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**Prediction of key properties of molecular
(nano)systems for biological and industrial application
through HPC-based techniques and experimental
validation**

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Abstract

Understanding how molecular and nanoscale systems behave in complex biological environments is central to advancing modern biomedicine, drug design, and nanotechnology. This thesis integrates high-performance computing (HPC)-based molecular simulations with a comprehensive array of biophysical and spectroscopic techniques to elucidate the structural, energetic, and dynamic determinants of molecular recognition across multiple biological interfaces. The work investigates three interconnected areas: the binding of clinically relevant BRAF and MEK inhibitors to human serum albumin (HSA), with implications for pharmacokinetics, bioavailability, and therapeutic performance; the off-target interaction of small molecules with DNA, using vemurafenib as a model to assess binding modes, thermodynamic signatures, and potential genotoxic liabilities; and the multiscale behavior of amphiphilic dendrimers and their self-assembled nanomicelles, focusing on their interactions with serum proteins, protein corona formation, and suitability as drug-delivery platforms.

Across these case studies, synergistic computational-experimental cycles, including molecular dynamics simulations, free-energy calculations, UV-Vis and fluorescence spectroscopy, CD, ITC, DLS, TEM, and NMR, enabled quantitative characterization of binding affinities, conformational responses, and emergent cooperative effects. Representative quantitative outcomes include dissociation constants in the low micromolar range for BRAF/MEK inhibitor-HSA complexes, corresponding to binding free energies of approximately -7 to -9 kcal mol⁻¹, indicative of pharmacokinetically relevant serum association. In the DNA case study, binding affinities were significantly weaker, consistent with non-covalent groove or intercalative interactions driven primarily by entropic contributions. For amphiphilic dendrimers and their nanomicelles, self-assembly and surface charge modulation produced measurable shifts in hydrodynamic diameter and ζ -potential upon protein adsorption with a moderate binding affinity in the micromolar range, confirming that corona formation is strongly dependent on aggregation state and interfacial chemistry. The results reveal how structural and chemical variations propagate from the atomic scale to mesoscopic behavior, influencing drug transport, stability, biodistribution, and nanocarrier performance. Collectively, this thesis demonstrates the predictive power of integrating HPC with experimental validation, establishing a unified framework for probing molecular interactions and guiding the rational design of pharmaceuticals and nanomaterials with improved efficacy, selectivity, and safety.

Sommario (Italian Abstract)

Comprendere il comportamento di sistemi molecolari e nanoscopici in ambienti biologici complessi è fondamentale per il progresso della biomedicina moderna, della progettazione farmacologica e della nanotecnologia. Questa tesi integra simulazioni molecolari basate su high-performance computing (HPC) con un ampio insieme di tecniche biofisiche e spettroscopiche, al fine di chiarire i determinanti strutturali, energetici e dinamici del riconoscimento molecolare attraverso diverse interfacce biologiche. Il lavoro esplora tre ambiti interconnessi: il legame degli inibitori di BRAF e MEK clinicamente rilevanti con l'albumina sierica umana (HSA), con implicazioni per farmacocinetica, biodisponibilità e performance terapeutica; l'interazione off-target di piccole molecole con il DNA, utilizzando vemurafenib come modello per valutare modalità di legame, firme termodinamiche e potenziali rischi genotossici; e il comportamento multiscala di dendrimeri anfifilici e delle loro nanomicelle autoassemblate, focalizzandosi sulle interazioni con proteine sieriche, la formazione della corona proteica e l'idoneità di tali sistemi come piattaforme di drug delivery.

Attraverso questi casi di studio, in sinergia tra simulazione computazionale ed esperimenti, comprendenti dinamica molecolare, energia libera, spettroscopia UV-Vis e di fluorescenza, CD, ITC, DLS, TEM e NMR, hanno permesso una caratterizzazione quantitativa delle affinità di legame, delle risposte conformazionali e dei processi cooperativi derivanti dall'interazione collettiva delle componenti del sistema. Tra i risultati quantitativi rappresentativi si evidenziano costanti di dissociazione nell'ordine dei bassi micromolari per i complessi tra inibitori di BRAF/MEK e HSA, corrispondenti a energie libere di legame comprese approssimativamente tra -7 e -9 kcal mol⁻¹, indicative di un'associazione sierica farmacocineticamente rilevante. Nel caso dello studio sull'interazione con il DNA, le affinità di legame risultano significativamente più deboli, coerenti con interazioni non covalenti di tipo groove-binding o intercalativo guidate prevalentemente da contributi entropici. Per quanto riguarda i dendrimeri anfifilici e le loro nanomicelle autoassemblate, l'autoaggregazione e la modulazione della carica superficiale determinano variazioni misurabili del diametro idrodinamico e del potenziale ζ in seguito all'adsorbimento proteico con un'affinità di legame moderata nell'intervallo micromolare, confermando che la formazione della corona dipende in modo marcato dallo stato di aggregazione e dalla chimica dell'interfaccia. I risultati mostrano come variazioni strutturali e chimiche si propaghino dalla scala atomica a quella mesoscopica, influenzando trasporto dei farmaci, stabilità, biodistribuzione e performance dei nanovettori. Complessivamente, la tesi dimostra il potere predittivo derivante dall'integrazione tra HPC e validazione sperimentale, stabilendo un quadro unificato per lo studio delle interazioni molecolari e guidando la progettazione razionale di farmaci e nanomateriali con maggiore efficacia, selettività e sicurezza.

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1. Introduction

At the interface between chemistry, biology, and materials science lies the enduring challenge of understanding how molecular systems behave in complex biological environments. Whether a small drug molecule binds to its target protein, interacts nonspecifically with DNA, or becomes part of a nanoscale carrier coated by plasma proteins, the physicochemical principles governing these interactions are fundamentally the same: electrostatics, hydrogen bonding, hydrophobic forces, and dispersion interactions. Yet the balance and manifestation of these forces depend critically on molecular architecture, environmental context, and dynamic adaptability. Predicting and controlling this balance is central to modern biomedicine, where therapeutic efficacy and safety are inseparably linked to the microscopic details of molecular recognition.

This thesis explores these questions by integrating high-performance computing (HPC)-based molecular simulations with a comprehensive suite of biophysical and spectroscopic experiments. The goal is to predict and rationalize key physicochemical properties of molecular and nanoscale systems that are relevant for both biological and industrial applications. Through a synergistic “computational–experimental cycle,” the work aims to establish a quantitative and mechanistically grounded framework that links molecular structure and dynamics to measurable biophysical observables.

The methodological philosophy underpinning this research rests on the conviction that neither simulations nor experiments alone can fully capture the complexity of molecular interactions. Experimental techniques such as ultraviolet-visible (like UV-Vis) spectroscopy, fluorescence spectroscopy (FS), circular dichroism (CD), isothermal titration calorimetry (ITC), nuclear magnetic resonance (NMR), and transmission electron microscopy (TEM) provide direct access to thermodynamic, structural, and kinetic parameters. Molecular simulations, in turn, offer an atomistic window into the dynamic rearrangements and energetic landscapes underlying those measurements. Together, they form a cooperative workflow in which experiments validate, constrain, and challenge the models, while simulations interpret and extend experimental findings, ultimately yielding a unified, holistic and mechanistic picture of molecular behavior.

The first part of the thesis focuses on BRAF and MEK inhibitors, two classes of small-molecule anticancer drugs that have transformed the treatment of metastatic melanoma through selective inhibition of the MAPK pathway. Despite their clinical success, these inhibitors exhibit complex pharmacokinetic profiles and sometimes severe side effects, often linked to off-target binding or altered plasma protein interactions. Among the latter, human serum albumin (HSA) plays a central role as the primary carrier of hydrophobic and amphiphilic drugs in the circulation. By combining

fluorescence quenching, CD spectroscopy, and ITC with molecular docking and atomistic molecular dynamics (MD) simulations, this work dissects the binding of representative inhibitors, vemurafenib, cobimetinib, encorafenib, and binimetinib, to HSA. The integrated analysis provides not only binding affinities and thermodynamic signatures but also atomistic descriptions of the conformational adjustments and residue-level contacts that stabilize each complex. These results highlight how subtle structural variations among inhibitors translate into measurable differences in binding energetics and dynamic stabilization, ultimately influencing bioavailability, half-life, and therapeutic index.

The second research axis examines the interaction between small molecules and DNA, a subject that bridges therapeutic targeting and toxicological assessment. DNA represents both a legitimate pharmacological target in the case of anticancer intercalators and a potential site of off-target adverse effects for many drugs. Here, vemurafenib is used as a model compound to probe noncanonical drug–DNA interactions, combining UV-Vis absorption, melting curve analysis, and modulation of ionic strength with computational free-energy calculations and structural modeling. The spectroscopic results reveal characteristic hypochromic and hyperchromic shifts that indicate distinct binding modes, whereas simulations elucidate the geometry and dynamics of these interactions at the base-pair level. The combined interpretation of experimental and theoretical data enables the deconvolution of electrostatic, enthalpic, and entropic contributions to DNA binding, offering insight into the delicate balance between therapeutic activity and genotoxic liability. This section thus illustrates how the coupling of spectroscopy and simulation can transform empirical binding data into mechanistic models with predictive power.

Building upon these molecular-scale investigations, the third and most complex part of the thesis transitions into the nanoscale domain, exploring amphiphilic dendrimers (ADs) and their interactions with biological environments. These self-assembling systems bridge the gap between molecular design and supramolecular organization, forming nanomicelles or dendrimersomes capable of encapsulating hydrophobic drugs. Their promise as delivery vehicles lies in their tunable architecture and surface functionality, but in biological fluids, their identity is rapidly reshaped by the formation of the protein corona (PC), a dynamic shell of adsorbed biomolecules that governs nanoparticle fate. Through a combination of spectroscopic techniques (DLS, TEM, CD, ITC) and molecular simulations, this work characterizes the multiscale interactions between amphiphilic dendrimers and serum proteins, with particular emphasis on HSA. The computational component provides atomistic insight into the energetics and dynamics of PC formation, while experiments validate these models and quantify binding constants, aggregation behavior, and thermodynamic parameters. This integrated approach clarifies how dendrimer composition, surface charge distribution, and

supramolecular organization influence protein and how PC formation, in turn, modulates nanocarrier stability, biodistribution, and drug release behavior.

Across these case studies, a coherent “red thread” emerges: from small molecules binding to macromolecules, to nanosystems that involve entire proteomes, molecular recognition remains governed by the interplay of structure, dynamics and energetics. The biological consequences of these interactions, like pharmacokinetic modulation, genotoxic risk, or nanocarrier biocompatibility, are all manifestations of the same physical laws operating at different scales. The strength of the combined HPC–experimental duality lies in its ability to reveal this continuity, connecting atomistic interactions to mesoscopic behavior and macroscopic observables with predictive consistency. By quantifying how atomic-scale interactions propagate across scales, the cooperative approach adopted here provides a unified description of molecular and nanoscale systems, capable of guiding rational design across disciplines.

Finally, the thesis emphasizes the transformative role of high-performance computing as both a technological and epistemological tool in molecular science. Modern HPC infrastructures make it possible to simulate biomolecular systems of hundreds of thousands of atoms for microsecond timescales, to compute free energies with chemical accuracy, and to integrate simulation data with experimental observables in real time. In doing so, they elevate molecular modeling from a descriptive discipline to a predictive one. When tightly coupled with rigorous experimental validation, HPC-based molecular simulations can anticipate binding affinities, delineate preferred conformations, identify emergent cooperative effects, and predict system-level behavior in complex biological environments.

In summary, this thesis demonstrates that by uniting computation and experiment, one can construct a consistent and predictive picture of molecular interactions that transcends individual systems. From drug–protein to drug–DNA and nanoparticle–protein interfaces, the same mechanistic insights emerge, pointing toward a universal molecular language that connects structural chemistry, biology, and nanotechnology. This cooperative framework not only advances the fundamental understanding of biomolecular interactions but also provides a practical foundation for the rational design of more effective, selective, and biocompatible therapeutic and nanotechnological systems.

2. Background and State of the Art

2.1. Drug-Biomolecule Interactions: General Mechanistic Framework

At the molecular scale, therapeutic agents and nanocarriers seldom remain isolated in biological environments. Instead, they establish a complex network of interactions with essential biomolecules such as nucleic acids and proteins. These interactions govern both the intended pharmacological outcome and the unintended side effects that can compromise safety and efficacy. A mechanistic understanding of how small molecules and nanostructured carriers engage with DNA, plasma proteins, and other biomolecular partners is therefore indispensable for rational drug development and the design of advanced delivery systems.

The interaction of small molecules with DNA represents one of the oldest and most extensively studied paradigms. Classical anticancer drugs, such as anthracyclines, exert their therapeutic activity through intercalation, whereby planar aromatic groups insert between adjacent base pairs, stabilizing the double helix while distorting its structure. Other ligands exploit the minor groove, where their curved geometry allows selective recognition of AT- or CG-rich regions, often accompanied by hydrogen bonding and van der Waals contacts. Major groove binding, although less common for small molecules, underlies the recognition motifs of many proteins and synthetic ligands. Additional mechanisms include electrostatic association of cationic groups with the negatively charged phosphate backbone and, in the case of alkylating agents, covalent adduct formation that irreversibly compromises replication and transcription. Each of these binding modes impacts distinct thermodynamic and structural signatures: intercalators typically induce pronounced conformational changes and large enthalpic stabilization, while groove binders often rely more on entropy gains from solvent release, producing less disruptive structural effects. In the context of modern kinase inhibitors such as vemurafenib, the possibility of off-target groove binding highlights the need for predictive approaches capable of distinguishing subtle binding preferences and their biological consequences. Equally central are drug–protein interactions, with HSA serving as the prototypical model. HSA is the most abundant plasma protein and functions as a carrier of endogenous metabolites and a wide array of xenobiotics. Drugs typically engage HSA through noncovalent binding at Sudlow’s sites I and II, deep hydrophobic cavities whose physicochemical environment favors amphiphilic ligands. The thermodynamics of these interactions vary widely: some drugs are stabilized primarily by enthalpic contributions arising from hydrogen bonding and van der Waals contacts with key residues, while others exhibit entropy-driven binding dominated by the hydrophobic effect and solvent displacement. These interactions profoundly influence pharmacokinetics, modulating the free fraction of drug available for therapeutic action, extending or shortening circulation half-life, and

mediating drug–drug competition in polypharmacy contexts. Structural studies consistently reveal that HSA maintains its overall fold upon ligand binding, but subtle conformational adjustments fine-tune the interaction and can affect cooperative binding with other molecules. Computational simulations, when combined with spectroscopic and calorimetric validation, provide a detailed picture of these events, revealing not only the affinity but also the dynamic stability and specificity of drug-HSA complexes.

The introduction of self-assembling nanocarriers, such as dendrimers, adds an additional layer of complexity. In biological fluids, these nanosystems are rapidly coated by proteins, resulting in a protein corona that redefines their identity and biological fate. The PC is not static: tightly bound proteins form a persistent “hard” layer, while more loosely associated species constitute a “soft” corona in dynamic exchange with the environment. HSA is frequently a dominant component, reflecting its abundance and high affinity for amphiphilic surfaces. The formation of such complexes is governed by electrostatic interactions, hydrogen bonding, and hydrophobic contacts at the nanocarrier interface and is strongly modulated by the carrier’s architecture, charge distribution, and functional groups. Although corona formation can stabilize nanocarriers and modulate their circulation time, it can also trigger immune recognition, alter biodistribution, or interfere with controlled drug release. Understanding these phenomena is critical, as the therapeutic efficacy of nanocarriers depends not only on the encapsulation capacity and stability but also on how the biological milieu reshapes their surface properties. Computational and experimental studies converge to demonstrate that the thermodynamics of PC formation resemble those of drug–protein binding, although amplified by the multivalent and cooperative nature of nanosystem surfaces.

Taken together, these interactions reveal a unifying principle: whether a small molecule intercalates into DNA, binds reversibly to HSA, or assembles into a nanostructure subsequently coated by plasma proteins, the driving forces are fundamentally the same—electrostatics, hydrophobicity, hydrogen bonding, and van der Waals contacts. What distinguishes each system is the balance of these forces and the degree of structural adaptability of the biomolecular partner. DNA, with its relatively rigid double helix, exhibits distinct binding signatures depending on the ligand geometry. Proteins, with their dynamic tertiary structures, adjust conformationally to optimize binding. Nanocarriers, on the contrary, present multiscale surfaces that promote cooperative and competitive interactions. By employing high-performance computing techniques alongside spectroscopic and calorimetric experiments, it becomes possible to not only catalog these interactions but also to predict their affinities, specificities, and consequences for therapeutic efficacy and safety.

2.2. Melanoma skin cancer and BRAF/MEK-inhibitor drugs

Cutaneous melanoma (CM) is the most lethal form of skin cancer, originating from the malignant transformation of melanocytes, pigment-producing cells located primarily in the basal layer of the epidermis. Although it accounts for a minority of skin cancer cases, CM is responsible for the majority of skin cancer-related deaths due to its high metastatic potential and resistance to conventional therapies.

According to data from the American Cancer Society (ACS), approximately 106,200 new cases of melanoma were diagnosed in the United States in 2021, with an estimated death toll of 7,200 individuals (4,600 men and 2,600 women) [1]. By 2025, projections from the Surveillance, Epidemiology, and End Results (SEER) Program of the National Cancer Institute (NCI) estimated 104,960 new melanoma cases and 8,430 related fatalities in the United States, positioning melanoma as the fifth most commonly diagnosed cancer and accounting for 5.1% of all new cancer cases [2]. In Europe, the European Cancer Information System (ECIS) reported 106,369 new melanoma cases and 16,488 deaths in 2020 across the EU-27 nations (Figure 2.1). Projections indicate a continued upward trend, with expected increases in incidence and mortality of 13.7% and 29.4%, respectively, by 2040. Despite advancements in screening and public awareness, these figures underscore the persistent global burden of melanoma [3].

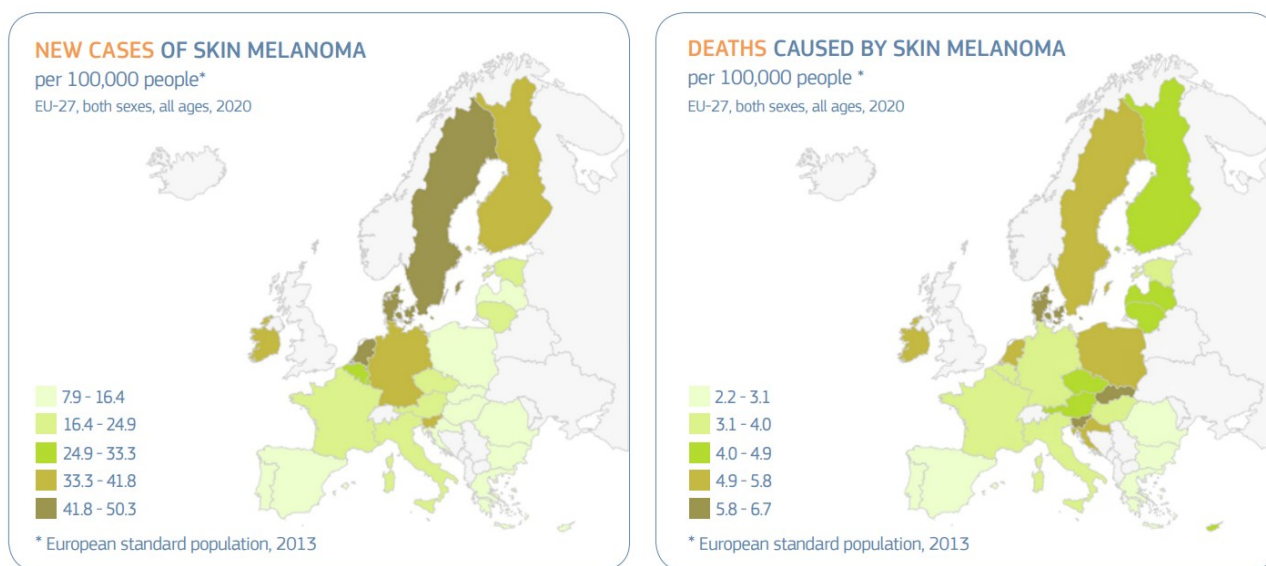


Figure 2.1. New cases (**left**) and deaths (**right**) caused by melanoma skin cancer in the EU-27 nations in 2020 [3].

The clinical outcome of melanoma is primarily determined by the stage at diagnosis. The American Joint Committee on Cancer (AJCC) TNM staging system stratifies melanoma into four stages based on tumor thickness and ulceration (T), regional lymph node involvement (N), and distant metastasis (M) [4]. The median age at diagnosis is approximately 59 years, with the main risk factors including

sun exposure, indoor tanning, immunosuppression conditions, moles (nevi), family history, and obesity. In this context, the most important measure of prevention is the use of sunscreen: evidence from randomized controlled trials indicates that consistent use of sunscreen significantly decreases rates of melanoma over time. The ACS recommends avoiding sun exposure between 10 a.m. and 4 p.m., or alternatively using protective clothing, hats, and broad-spectrum sunscreen with an SPF of 30 or higher, as well as complete avoidance of artificial UV sources. Additionally, research suggests that skin examinations directed to lesions can detect cancer at rates comparable to full-body exams while being more efficient in time [5].

Early-stage disease (stage I–II) is associated with an excellent prognosis, with five-year disease-specific survival rates exceeding 90% and reaching as high as 99.4% for certain subgroups. The majority (approximately 83%) of melanoma cases are diagnosed at these stages. However, survival declines sharply with advancing disease. Stage III melanoma, characterized by regional nodal involvement, shows five-year survival rates ranging from 36% to 68%, depending on the number and extent of nodal metastases. Stage IV disease, which is defined by distant metastases, is associated with a dismal prognosis—five-year survival is less than 15%, with a one-year survival rate of 26% and a median progression-free survival (PFS) of approximately 1.7 months. Only around 4% of patients present with stage IV disease at the time of diagnosis [5].

At the molecular level, the pathogenesis of melanoma is frequently driven by aberrant activation of the mitogen-activated protein kinase (MAPK) signaling pathway, which regulates key cellular processes such as proliferation, differentiation, and survival. The MAPK cascade involves the sequential activation of the RAS, RAF (including A-RAF, B-RAF, and C-RAF isoforms), MEK, and ERK kinases (Figure 2.2) [6].

Among these, mutations in the *BRAF* gene are the most common driver events in melanoma, found in approximately 50% of cases. The corresponding most frequent mutation in the B-RAF kinase is the V600E missense substitution (valine to glutamic acid at position 600), accounting for 74–90% of all BRAF mutations. Other clinically relevant BRAF V600 alterations include V600K (15–25%), V600R, and V600D, along with rarer variants such as V600A, V600M, and V600G. These mutations result in constitutive activation of BRAF kinase activity, thereby promoting sustained MAPK pathway signaling independent of upstream regulatory inputs, and contribute to uncontrolled cell growth and tumor progression [7].

The discovery of BRAF mutations as oncogenic drivers in melanoma has led to the development of targeted therapies that have revolutionized the treatment paradigm, particularly for patients with advanced or metastatic disease harboring BRAF V600 mutations.

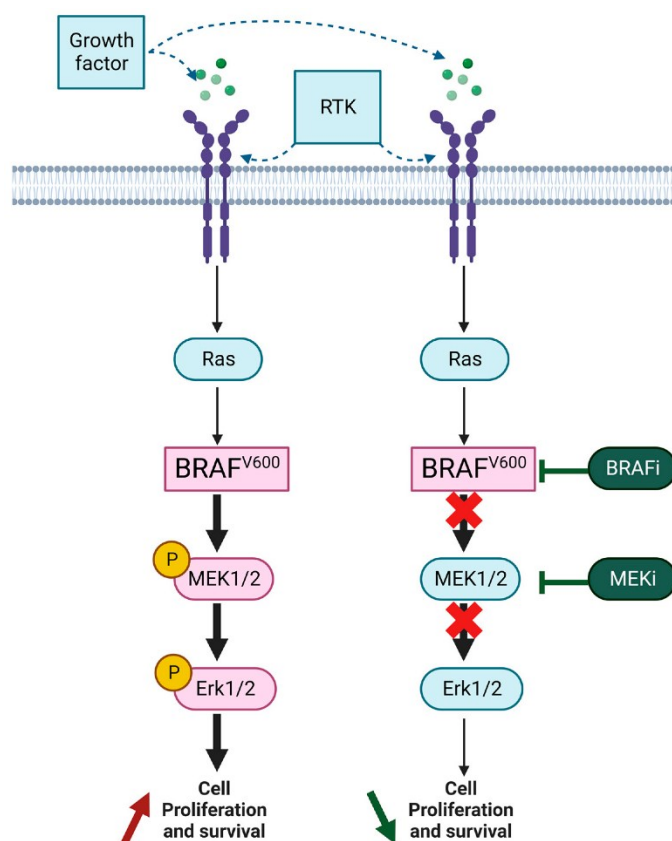


Figure 2.2. Schematic representation of alterations in the RAS-BRAF-MEK-ERK MAP kinase signaling pathway with V600-mutated BRAF and sites of therapeutic kinase inhibition (BRAFi: BRAF inhibitor; MEKi: MEK inhibitor) [8].

The first BRAF inhibitor (BRAFi) approved by the U.S. Food and Drug Administration (FDA) and European Medicines Agency (EMA) was vemurafenib (Zelboraf, Roche, Figure 2.3, left), based on pivotal clinical trials demonstrating a 63% reduction in the risk of death and a 74% reduction in disease progression compared to conventional chemotherapy. Dabrafenib (Tafinlar, GlaxoSmithKline, Figure 2.3, right) followed in 2013 as an orally bioavailable BRAFi approved for unresectable or metastatic melanoma with BRAF V600E mutations. Both agents have shown substantial clinical benefits but are associated with characteristic adverse effects, including dermatologic toxicities, photosensitivity, fatigue, and pyrexia [9].

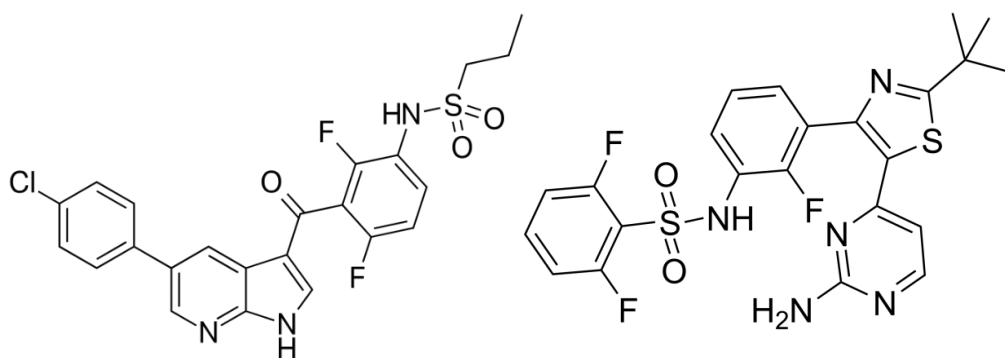


Figure 2.3. Chemical structure of vemurafenib (left) and dabrafenib (right).

A particularly concerning side effect of BRAFi monotherapy is the development of cutaneous squamous cell carcinomas (cuSCCs) and keratoacanthomas, which occur in approximately 14-26% of treated patients, often within the first 2-3 months of therapy. This paradoxical tumorigenesis is attributed to BRAFi-induced activation of the MAPK pathway in keratinocytes harboring pre-existing RAS mutations.

To mitigate BRAFi-associated toxicities and improve therapeutic efficacy, the combination of BRAF and MEK inhibitors has become the standard of care. MEK inhibitors (MEKi), such as cobimetinib (Cotellic, Genentech, Figure 2.4 left) and binimetinib (Mektovi, Array BioPharma, Figure 2.4 right), target downstream components of the MAPK pathway, offering a synergistic effect when used with BRAF inhibitors [10].

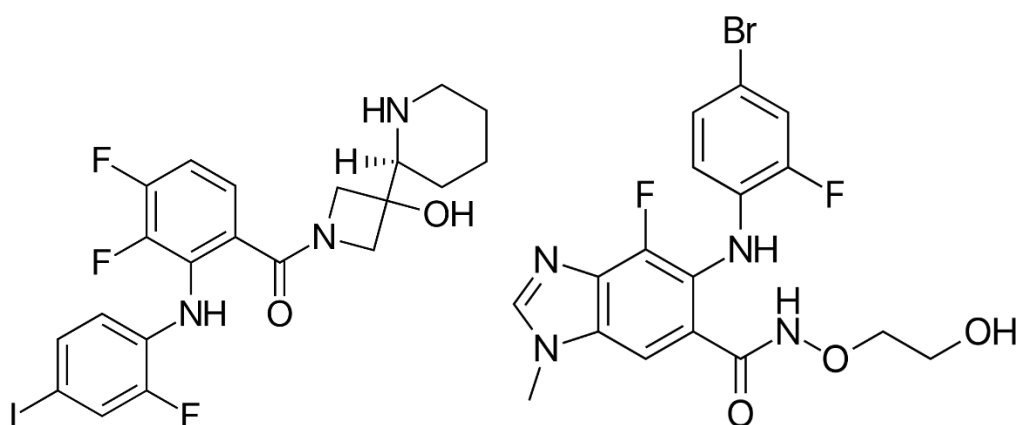


Figure 2.4. Chemical structure of cobimetinib (**left**) and binimetinib (**right**).

In preclinical and clinical studies, dual inhibition has shown superior outcomes in terms of tumor response, delayed resistance, and reduced incidence of cuSCCs. For example, the COLUMBUS trial evaluated the combination of encorafenib (Braftovi, Figure 2.5) and binimetinib in patients with BRAF V600-mutated metastatic melanoma. This trial demonstrated a median overall survival (OS) of 33.6 months for the combination therapy, compared to 23.5 months for encorafenib monotherapy and 16.9 months for vemurafenib [11].

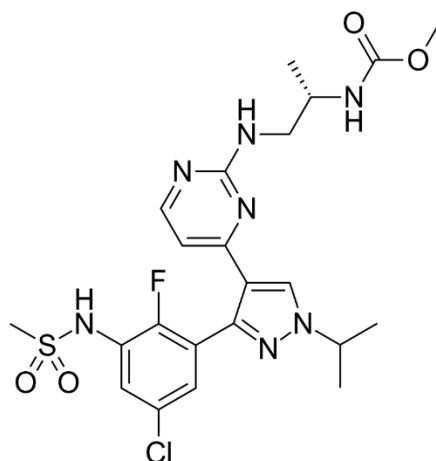


Figure 2.5. Chemical structure of encorafenib.

The rationale behind combination therapy is to achieve a more complete blockade of the MAPK pathway while preventing compensatory feedback activation. Additionally, MEKi co-administration reduces the risk of cutaneous adverse events by suppressing paradoxical pathway activation in non-tumor cells.

2.3. Amphiphilic Dendrimers (ADs)

The growing convergence of nanotechnology and medicine has led to a new generation of nanoscale carriers for therapeutic and diagnostic applications. Among these, dendrimers represent a class of synthetic macromolecules distinguished by their precisely branched three-dimensional architectures, monodispersed, and highly functionalizable surfaces. Their unique structural characteristics confer advantages that are unmatched by traditional polymeric systems. Initially conceptualized in the late 1970s and synthesized with increasing precision since the mid-1980s, dendrimers have since become central to research in drug delivery, gene therapy, imaging, and beyond.

Dendrimers are composed of three fundamental architectural elements (Figure 2.6): a central core, multiple layers of repetitive branching units (generations), and a multitude of surface terminal groups. This symmetrical and fractal-like structure allows for precise control over molecular size, shape, and surface chemistry. From a synthetic standpoint, this architecture is traditionally achieved through either divergent or convergent stepwise covalent approaches. The divergent method involves growth from the core outward, whereas the convergent approach constructs dendritic fragments from the periphery inward, later attaching them to a central core. Both methods offer precise control, but are hindered by synthetic complexity, long reaction sequences, and low yields at higher generations due to steric hindrance and purification challenges [12].

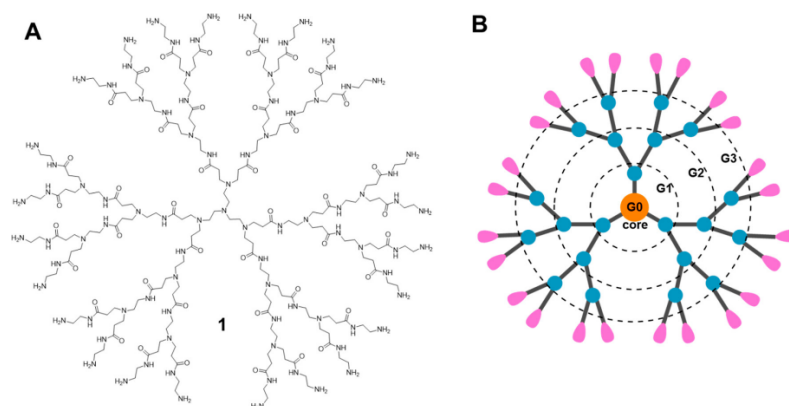


Figure 2.6. (A) Chemical structure of the G3 poly(amidoamine) (PAMAM) dendrimer (1) as an example. (B) Cartoon illustration of a dendrimer comprising a central core or generation 0 (G0), repeating branching units forming consecutive levels or generations (G1, G2, and G3), and the terminal groups on the dendrimer surface [12].

One of the most studied families of dendrimers is the poly(amidoamine) or PAMAM class [13]. These dendrimers are constructed from ethylenediamine cores and amidoamine branching units, resulting in water-soluble and biocompatible architectures. Their interiors contain tertiary amines and amide bonds that mimic peptide backbones, while their surfaces are densely functionalized with primary amines. This structural configuration makes PAMAM dendrimers particularly attractive for biomedical applications such as nucleic acid delivery and therapeutic encapsulation. Nevertheless, despite their synthetic success and commercial availability, the laborious production of high-generation PAMAM dendrimers remains a significant obstacle to large-scale or clinical deployment. To circumvent the intrinsic limitations of covalent dendrimer synthesis, Prof. Ling Peng and coworkers pioneered a novel strategy based on the spontaneous self-assembly of small amphiphilic dendritic molecules into supramolecular dendrimers (Figure 2.7A). These systems are constructed not through covalent bonding but via the accumulation of weak noncovalent interactions, including van der Waals forces, hydrogen bonding, and electrostatic interactions [14]. The fundamental concept involves the use of amphiphilic dendrons comprising a hydrophobic tail (typically long alkyl chains) and a hydrophilic dendritic head (such as PAMAM branches), which, upon dispersion in aqueous environments, spontaneously organize into nanostructures with dendrimer-like features. These supramolecular dendrimers retain the multivalent cooperativity, core-shell architecture, and functional versatility of their covalent counterparts, but are considerably easier to synthesize, modulate, and apply in biomedical settings (Figure 2.7B) [14].

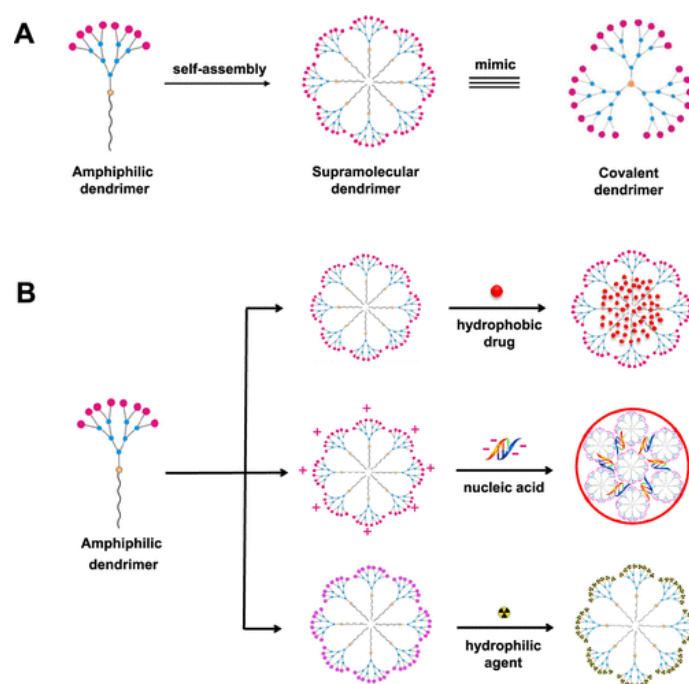


Figure 2.7. Cartoon representations of (A) the self-assembly of a small amphiphilic dendrimer into a supramolecular dendrimer that mimics a covalently constructed dendrimer. (B) Delivery of hydrophobic and hydrophilic pharmaceutical agents as well as negatively charged nucleic acid therapeutics [12].

A landmark application of supramolecular dendrimers lies in the field of nucleic acid delivery. The delivery of small interfering RNA (siRNA) poses significant barriers due to the molecule's inherent hydrophilicity, negative charge, enzymatic instability, and poor cellular uptake. Traditional delivery systems often fall short in providing efficient protection, targeting, and endosomal escape. This challenge was addressed by developing a special type of amphiphilic dendrimers, which features an 18-carbon alkyl tail linked to a low-generation PAMAM dendron with terminal primary amines (Figure 2.8A). These molecules self-assemble into nanomicellar structures that encapsulate siRNA via electrostatic interactions with the dendrimer's cationic surface, ensuring protection from nuclease degradation and promoting intracellular delivery. Upon internalization, the "proton sponge" effect of the amines facilitates endosomal escape, allowing the siRNA cytoplasmic release and the subsequent gene silencing activity (Figure 2.8B). The system's modularity is demonstrated by variations of this compound. For instance, replacing terminal primary amines with tertiary amines (Figure 2.8A), significantly reducing cytotoxicity due to lower charge density at physiological pH. Furthermore, incorporating guanidinium-rich arginine residues on the periphery (Figure 2.8A), mimicking cell-penetrating peptides and improving cellular uptake via hydrogen bonding and ionic interactions with anionic cell membrane components. These subtle modifications highlight how small changes to terminal groups can profoundly modulate the biological behavior and delivery performance of the supramolecular dendrimers [12].

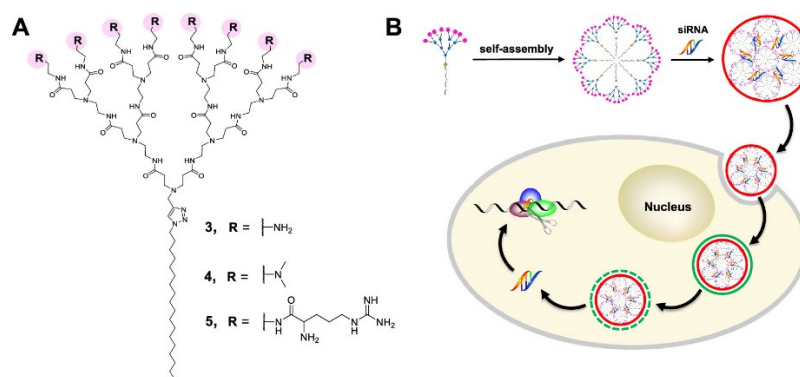


Figure 2.8. Amphiphilic dendrimers 3–5 and their self-assembly into supramolecular dendrimers for small interfering RNA (siRNA) delivery. (A) Chemical structures of 3–5 with primary amine, tertiary amine, and arginine residue terminals, respectively. (B) Cartoon illustration of the self-assembly of an amphiphilic dendrimer into a supramolecular dendrimer for siRNA binding and delivery, including cell uptake, endosome release, and subsequent gene silencing [12].

Further studies investigated the importance of the hydrophobic tail's length and the balance of amphiphilicity. Dendrimers with shorter alkyl chains (C12, C14) failed to form stable micelles, whereas chains of C16 and longer promoted robust self-assembly and effective siRNA delivery. These findings underscore the central role of hydrophobic interactions in stabilizing the supramolecular dendritic architecture [15].

One particularly innovative design is a dendrimer, which features two hydrophobic tails instead of one (Figure 2.9A). This amphiphile self-assembles into vesicle-like structures known as dendrimersomes. Upon binding to siRNA, these vesicles undergo a vesicle-to-micelle transition, exposing more cationic surfaces for stronger complexation with the siRNA. This adaptive behavior enhances the stability and cellular uptake of siRNA-loaded nanoparticles (Figure 2.9B), enabling siRNA delivery to notoriously difficult targets such as primary T cells, macrophages, and natural killer cells.

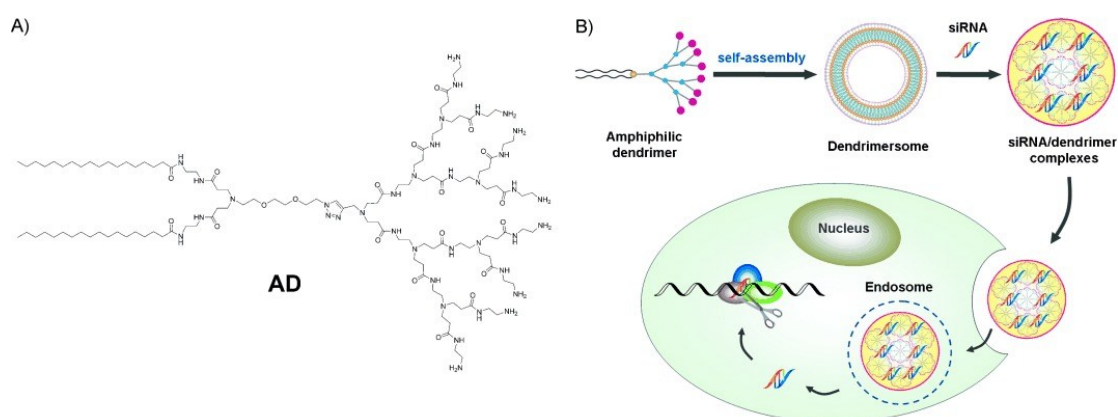


Figure 2.9. (A) The structure of the AD dendrimer and (B) representation of its adaptive self-assembly upon interaction with siRNA for siRNA delivery [16].

The targeting capabilities of supramolecular dendrimers can be further enhanced by attaching specific ligands. For example, incorporating a peptide containing the RGDK (arginine-glycine-aspartate-lysine) motif that binds both integrins and neuropilin-1 receptors, enables dual-targeting of tumor vasculature and tumor cells. Such ligand decoration significantly improves *in vivo* tumor localization and gene silencing outcomes compared to non-targeted counterparts [17].

Beyond gene delivery, supramolecular dendrimers also offer an effective platform for the encapsulation and controlled release of small-molecule drugs. The large internal cavities formed during self-assembly provide hydrophobic environments for drug loading. In a notable study, doxorubicin (DOX), a widely used anticancer drug, was encapsulated within micelles. The resulting DOX-loaded nanocarriers achieved a drug-loading capacity of up to 40%, a remarkably high figure for dendrimeric systems. These carriers exhibited enhanced tumor accumulation via the enhanced permeability and retention (EPR) effect, bypassed cellular drug-efflux mechanisms, and demonstrated increased anticancer efficacy *in vivo* compared to free DOX, all while reducing systemic toxicity [18].

The EPR effect (Figure 2.10) is a physiological phenomenon, fundamental in the delivery field, which explains the preferential accumulation of macromolecules and nanoparticles within tumor tissues. This effect arises from the unique pathophysiological characteristics of tumor vasculature, including irregular vessel morphology, abnormally wide endothelial junctions (100-800 nm), and poor lymphatic drainage, which collectively enable enhanced vascular permeability and impaired clearance of macromolecular agents. As solid tumors grow, hypoxic cancer cells secrete vascular endothelial growth factor (VEGF), which induces angiogenesis and leads to the formation of leaky and disorganized vessels that facilitate nanoparticle extravasation and retention. The EPR effect forms the cornerstone of passive tumor targeting, in which nanocarriers accumulate in the tumor interstitium without the need for specific ligand–receptor interactions. Furthermore, it serves as a prerequisite for active targeting, enhancing drug delivery efficiency when nanoparticles are functionalized with ligands such as antibodies, peptides, or carbohydrates to bind specific tumor-associated receptors [19].

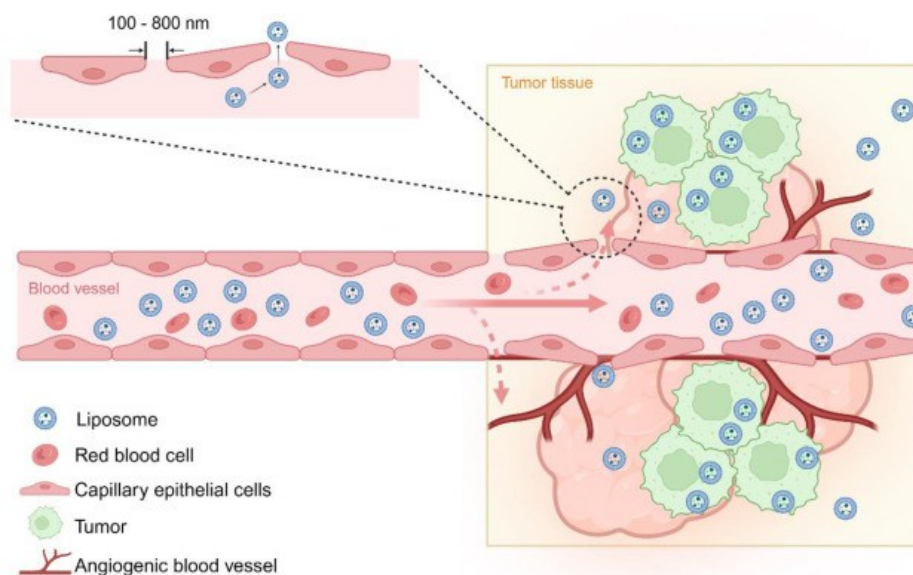


Figure 2.10. Schematic diagram of the EPR effect. Normal tissues have a relatively tight vascular system with tightly aligned vessel walls, which limits drug penetration. Tumor tissues have abnormal vascular structures with irregular gaps and fractures and looser vessel walls, resulting in easier penetration of intravascular drugs into tumor tissues and improved drug permeability. The poor lymphatic drainage function of tumor tissues makes drugs stay longer in the tumor environment, thereby increasing the accumulation of drugs in the lesion [20].

Based on these findings, the application of amphiphilic dendrimer nanomicelles was extended to the encapsulation of two of the clinically approved BRAF inhibitors used in melanoma therapy, namely dabrafenib (DAB) and vemurafenib (VEM). An amphiphilic dendrimer (AD) bearing two C18 aliphatic chains and a G2 PAMAM head self-assembled into uniform core-shell nanomicelles of approximately 10 nm diameter with excellent encapsulation efficiencies (~70% for DAB and ~60% for VEM) and high drug-loading capacities (~27% and ~24%, respectively). Spectroscopic, calorimetric, and scattering analyses confirmed stable encapsulation without significant alteration of micellar morphology, while molecular simulations revealed distinct localization patterns for the two drugs: DAB preferentially interacting with the hydrophilic shell and VEM residing near the hydrophobic core. *In vitro* release experiments demonstrated pH-dependent behavior governed by Fickian diffusion, with accelerated release under acidic conditions (pH 5.0) mimicking the tumor microenvironment. Notably, both DAB- and VEM-loaded nanomicelles exhibited enhanced antiproliferative effects compared with the free drugs across multiple melanoma cell lines, suggesting improved intracellular delivery and retention [21].

The application of supramolecular dendrimers extends to diagnostic imaging, where the capacity for multivalent surface modification and drug encapsulation can be leveraged to construct imaging probes (Figure 2.11). In the domain of nuclear medicine, dendrimers were used to chelate radionuclides using macrocyclic cages. The first compound incorporated the DOTA chelator with [^{111}In] for SPECT imaging. Despite good tumor uptake, high off-target accumulation was noted,

particularly in the liver. The second molecule, bearing the smaller NOTA chelator, provided improved biodistribution and higher imaging contrast. For PET imaging, a third compound chelated $[^{68}\text{Ga}]$ via NOTA, yielding stable and sensitive imaging probes that outperformed $[^{18}\text{F}]$ FDG in detecting certain tumors. The success of these systems stems from the ability of the supramolecular dendrimers to present multiple imaging moieties and accumulate in tumors via the EPR effect.

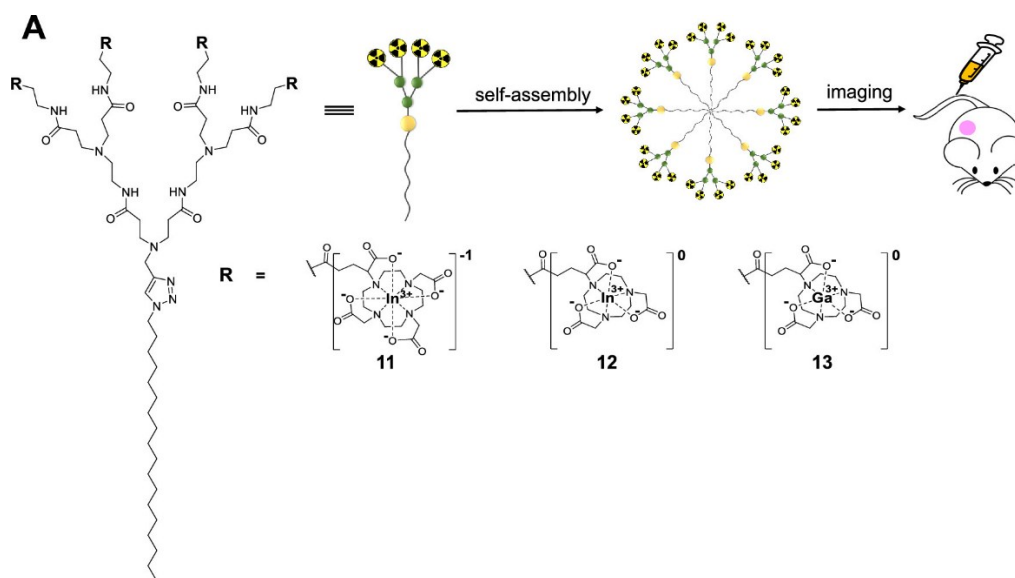


Figure 2.11. Amphiphilic dendrimers 11, 12, and 13 bearing radionuclide $[^{111}\text{In}]\text{In}^{3+}$ or $[^{68}\text{Ga}]\text{Ga}^{3+}$ chelated to macrocyclic cage 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA) or 1,4,7-triazacyclononane-1,4,7-triacetic acid (NOTA) at the dendrimer terminals for self-assembly into supramolecular dendrimer nanoprobes for tumor imaging based on single photon emission computed tomography (SPECT) and positron emission tomography (PET) [12].

Another imaging modality explored was near-infrared fluorescence (NIRF). Hydrophobic dyes like DiR are poorly soluble in aqueous media, but upon encapsulation within dendrimer micelles, the resulting DiR/dendrimer probes achieved aqueous solubility and maintained their optical properties. In vivo imaging demonstrated efficient tumor accumulation and prolonged retention compared to free dye, confirming the utility of supramolecular dendrimers as multimodal imaging platforms [12].

Despite their promise, several challenges remain. The noncovalent nature of supramolecular dendrimers raises concerns about their long-term stability in physiological environments, where dilution and protein interactions may lead to disassembly. Their pharmacokinetic profiling, immunogenicity assessments, and scalable and reproducible manufacturing techniques remain critical hurdles for clinical translation. Nevertheless, the lessons learned from lipid nanoparticles and other supramolecular nanomedicines can provide a roadmap to address these limitations for future therapeutic applications.

2.4. Human serum albumin: central role in pharmacokinetics, drug transport, and nanomaterial biodistribution

Human serum albumin (HSA, 66.5 kDa, 585 aa) is the most abundant protein in plasma ($\sim 43 \text{ g L}^{-1}$, 0.6 mM), constituting 60% of total protein [22]. Within its monomeric heart-shaped structure, albumin contains 67% of alpha helixes and no beta sheets. It is modular, with three homologous domains connected by random coil, named I, II and III, each containing two subdomains, A and B, that probably originated from gene duplication (Figure 2.12).

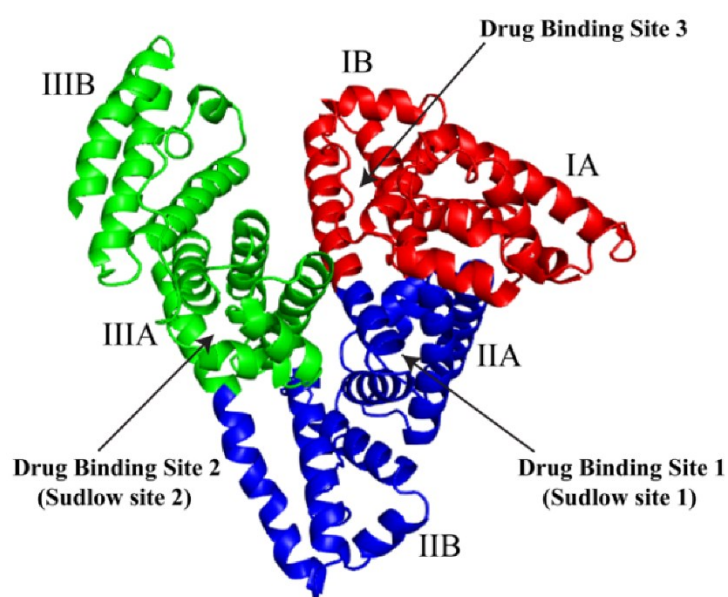


Figure 2.12. Domain organization of human serum albumin. Domain I (red), domain II (blue), and domain III (green) [23].

With an approximate half-lifetime of 19 days [24], this protein is found in both extravascular and intravascular spaces where it performs a number of fundamental physiological functions including the regulation of blood pH and oncotic pressure, and binding/transport of a wide range of endogenous and exogenous ligands of different nature (*e.g.*, fatty acids, hormones, amino acids, drugs, metabolites and nutrients, metal ions, and other proteins). This binding capacity plays a central role in pharmacokinetics, drug transport, and nanomaterial biodistribution, influencing their distribution, half-life, and clearance [22], [25]. Exogenous compounds bind predominantly at two multi-specific high-affinity sites located in subdomains IIA and IIIB, also known as Sudlow Site (Ss) I and II (Figure 2.12) that regulate free drug concentration and therapeutic efficacy [26].

HSA Ss I is a large, flexible binding pocket within subdomain IIA. It can accommodate multiple ligands, including bulky heterocyclic molecules and dicarboxylic acids, often with a central negative charge. Structural studies revealed overlapping and independent binding regions with Trp214 located in the warfarin-specific area. The pocket consists mainly of apolar clusters with key polar residues

(Tyr150, His242, Arg257, Lys195, Lys199, Arg218, and Arg222) that stabilize binding. Ligands such as warfarin and CMPF fit snugly within the cleft, forming hydrogen bonds, and the site exhibits low stereoselectivity, reflecting its structural flexibility. Ss II, located in subdomain IIIA of HSA, is a narrower and less flexible binding pocket. It is mainly hydrophobic with a polar patch at the entrance (Tyr411, Arg410), which stabilizes typical ligands such as aromatic carboxylic acids (*e.g.*, diazepam, ibuprofen, diflunisal). Interactions involve hydrogen bonds and salt bridges, with stereoselectivity often observed—for instance, (R)-ibuprofen binds more strongly than (S)-ibuprofen. Unlike site I, site II has not been divided into subsites and generally accommodates smaller molecules, though some structural adaptability is seen. Crystallographic studies show limited but significant side-chain rearrangements (*e.g.*, Leu387, Leu453) to fit larger ligands, and binding of fatty acids indicates that the site retains some flexibility despite being more restricted than site I [27].

The clinical importance of drug binding to HSA in this context is clear. Drug-albumin interactions can be altered by co-administered drugs, endogenous substances, or disease-related changes in albumin levels or structure, potentially affecting drug pharmacokinetics and pharmacodynamics. Drug–drug interactions often arise from competitive or allosteric displacement at albumin sites, although enhanced pharmacological effects (*e.g.*, warfarin with phenylbutazone) are now understood to result primarily from metabolic inhibition rather than binding displacement. However, in some cases (*e.g.*, diclofenac-nabumetone therapy), transient displacement and increased free drug fraction can influence clinical outcomes. Albumin displacement has also been exploited therapeutically, for example to restore the diuretic response in nephrotic syndrome or improving imaging in radiopharmaceutical diagnostics. Diseases like renal failure, uremia, and liver disease reduce drug binding due to hypoalbuminemia, structural modifications, or competition from endogenous ligands (*e.g.*, bilirubin, fatty acids, uremic toxins). This alters the free fraction (fp), which requires careful interpretation of drug levels and therapeutic windows. Clinical tools such as Albumin Binding Capacity (ABiC) help assess binding impairment, especially in liver disease. Finally, extracorporeal albumin dialysis systems (*e.g.*, MARS®, SPAD, Prometheus) use albumin's binding capacity to remove protein-bound toxins in liver failure or drug overdose. These techniques outperform conventional dialysis for insoluble or albumin-bound toxins and have shown effectiveness in cases such as phenytoin, theophylline, and diltiazem intoxication, although further optimization is needed [27].

HSA is also a highly versatile drug delivery vehicle because of its long half-life, ability to recycle via FcRn-mediated endosomal pathways, and natural accumulation in tumor tissues through the EPR effect. These properties allow albumin to transport anticancer drugs efficiently into tumors, exploiting the leaky and poorly organized vasculature characteristic of malignant tissues. Multiple strategies

have been developed to exploit HSA for drug delivery. Covalent conjugation to reactive residues such as Cys34, lysines, or histidines allows stable attachment of drugs without altering albumin's biological activity, while non-covalent binding to hydrophobic Sudlow sites I and II enables the transport of various small molecules. Albumin can also be engineered as nanoparticles or fused with peptides and proteins through recombinant techniques, further expanding its applications. A key element in conjugate design is the choice of linkers or spacers acid-sensitive, enzyme-cleavable, or self-immolative linkers can ensure controlled release of drugs specifically in pathological environments such as tumors. Clinically, these strategies have already produced successful examples, including albumin–fatty acid conjugates for diabetes drugs (liraglutide, semaglutide) and albumin-bound chemotherapeutics (aldoxorubicin, Abraxane). In general, HSA offers a flexible and biocompatible platform to improve pharmacokinetics, prolong circulation, and enhance targeted delivery of both small molecules and biologics [28].

HSA also displays diverse enzymatic functions, one of which is esterase-like activity. This refers to the ability of HSA to hydrolyze ester bonds or to form covalent intermediates with ester substrates (*e.g.* α -naphthyl acetate, *p*-nitrophenyl acetate (NPA), fatty acid esters and aspirin) via specific amino acid residues, ultimately generating alcohol and carboxylic acid. NPA, a classical substrate of carboxylesterase, is widely used to study the hydrolytic behavior of albumin because its hydrolysis yields *p*-nitrophenol, a yellow product easily detected spectrophotometrically at 400–412 nm. Acetylation of HSA by NPA occurs at up to 82 sites, primarily involving lysine, serine, threonine, and tyrosine residues, with Tyr411 (within Sudlow site II) identified as the primary and most reactive target. This residue is acetylated within minutes, reflecting pseudo-esterase activity that leads to covalent adduct formation [29]. This pseudo-esterase activity is thought to physiologically contribute to the metabolism of ester-containing drugs and xenobiotics, and may be altered under pathological conditions such as, for example, hepatic disorders.

This property has been exploited in applied settings; for instance, HSA-based biosensors have been designed to detect ester-bearing compounds or to monitor ester hydrolysis in biochemical processes. Ester bonds are also utilized in drug delivery systems as cleavable linkers, enabling controlled drug release. Furthermore, a wide range of drugs and environmental contaminants may interact with HSA at Sudlow's binding sites I and II, either specifically or nonspecifically, thereby influencing its esterase function. Such interactions can markedly affect the pharmacokinetic and toxicokinetic behavior of xenobiotics, with consequences for their distribution, metabolism, therapeutic action, safety, and toxicity.

Pollutants and drugs can inhibit HSA's esterase activity reversibly or irreversibly. Reversible inhibition may occur in several forms: competitive (binding to the catalytic site), uncompetitive

(binding to the enzyme–substrate complex), or noncompetitive (binding at an allosteric site, inducing conformational changes that reduce activity). In contrast, irreversible inhibition involves covalent modification of the protein. Understanding such interference is essential for toxicological risk assessment, and an elucidation of the mechanisms, whether reversible or irreversible, and their biological consequences requires detailed experimental investigation and kinetic analyses [30].

Protein corona

The emergence of nanotechnology in medicine has revolutionized diagnostics and therapeutics by enabling the precise delivery and modulation of drugs, genes, and imaging agents. However, as nanoparticles (NPs) are introduced into biological fluids, they acquire a new "biological identity" through the spontaneous adsorption of biomolecules, predominantly proteins, onto their surfaces. This complex and dynamic structure is known as the protein corona (PC). First formally conceptualized in 2007 by Dawson's group [31], though rooted in decades of earlier colloid science, the protein corona fundamentally alters the physicochemical properties of nanoparticles and thus governs their interactions with cells, tissues, and organs. When nanoparticles are introduced into a biological fluid such as plasma or interstitial fluid, they interact with a multitude of biomolecules (Figure 2.13). Among these, proteins rapidly and preferentially adsorb onto the NP surface because of their amphiphilic nature, conformational adaptability, and structural flexibility. This adsorption process is mediated by a combination of non-covalent forces — including van der Waals forces, electrostatic interactions, hydrogen bonding, and hydrophobic effects — and is strongly influenced by both nanoparticle properties (*e.g.*, size, charge, hydrophobicity, surface chemistry) and environmental parameters (*e.g.*, pH, ionic strength, temperature) [32].

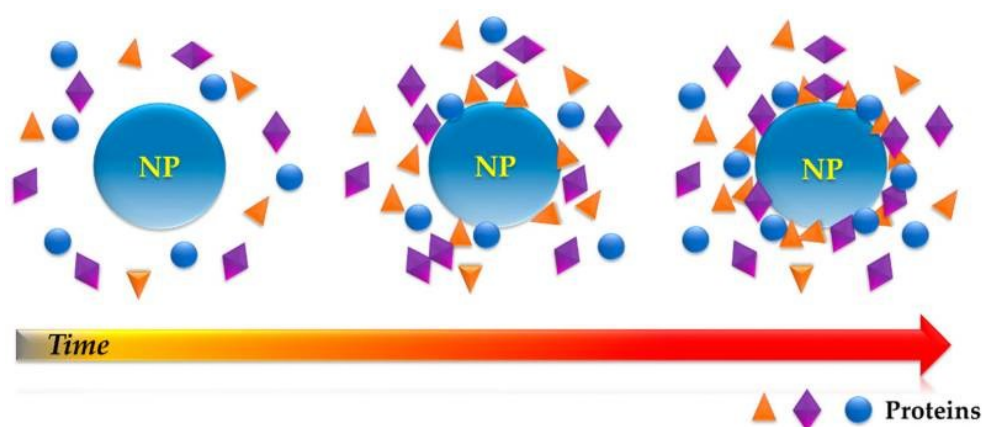


Figure 2.13. Protein adsorption onto a nanoparticle (NP) overtime leading to the formation of the so-called “protein corona” (cross-section view) [32].

The resulting PC is not static; it undergoes continuous remodeling in response to changes in the biological environment. Initially, proteins with high mobility but lower affinity form a transient "soft" corona. This outer layer includes proteins with weaker and more transient interactions with the NP surface. These proteins rapidly associate and dissociate depending on the local environment. Despite their dynamic nature, soft corona proteins can still participate in cellular recognition and influence short-term biodistribution. Over time, these proteins may be displaced by others with higher binding affinities, leading to the establishment of a more stable "hard" corona. This inner layer is composed of proteins with strong affinity to the nanoparticle surface, which forms the stable core of the corona. These proteins often undergo conformational changes upon binding, potentially altering their biological function. The hard corona persists over extended periods of time and is primarily responsible for dictating the long-term biological identity of the NP (Figure 2.14).

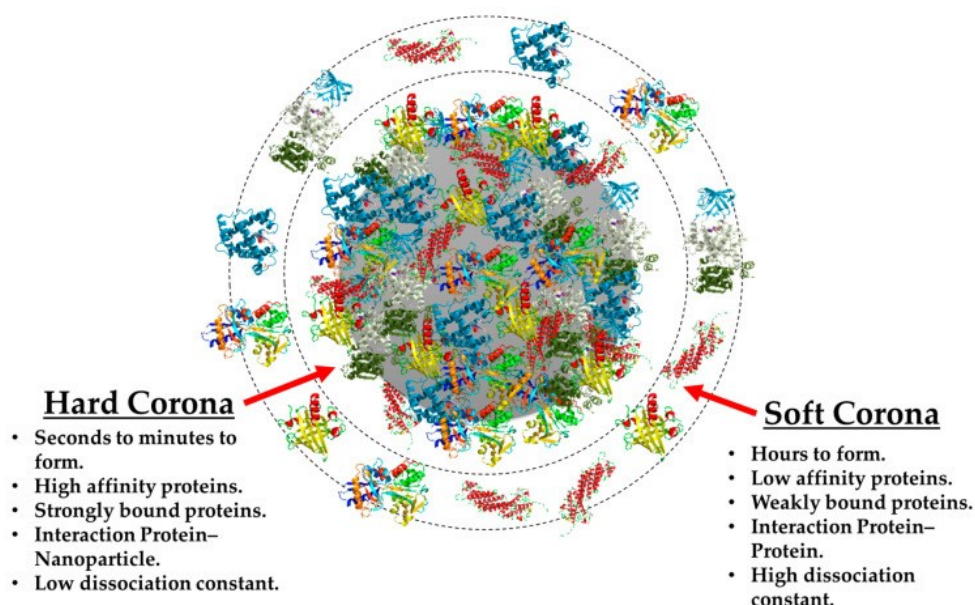


Figure 2.14. Schematic comparison of the main differences between the “hard” corona and the “soft” corona [32].

As an example, apolipoprotein A-I (ApoA-I) demonstrates high affinity for nanoparticle surfaces and has been consistently identified as a core component of hard coronas, even displacing more abundant proteins. Such findings underscore the importance of employing complementary analytical strategies: while techniques such as SDS-PAGE and protein quantitation assays are more suited to detecting stable, strongly bound components, ITC and DLS provide insights into weaker, transient interactions that characterize the soft corona [33].

Importantly, both layers can coexist, with the hard corona comprising tightly bound proteins that are less susceptible to exchange, and the soft corona containing loosely associated proteins that can

dynamically interact with the biological surroundings. The interplay between the soft and hard coronas significantly affects nanoparticle behavior *in vivo*, influencing parameters such as colloidal stability, immune clearance, opsonization, and cellular uptake [34]. Among the myriad proteins found in human plasma, two classes are most frequently and prominently involved in PC formation: HSA and immunoglobulin G (IgG). Their abundance, structural characteristics, and surface activity make them prototypical corona constituents. Yet, their roles differ significantly depending on the nanoparticle system. Detailed experimental analyses have revealed that HSA, although dominant in plasma, often exhibits weak binding affinities and is largely excluded from the hard corona despite its high concentration [33].

Another important parameter affecting the formation of a hard and soft PC is the NP's dimension: experimental evidence indicates that when nanoparticles and proteins are of comparable dimensions (around 7–9 nm, or < 20/40 nm) the adsorption model reaches its limits. Instead, weak and reversible interactions dominate, with nanoparticle-protein hetero-aggregation prevailing over the classical formation of a hard corona. The unique dimensional proximity induces pronounced surface curvature effects, which attenuate attractive forces and destabilize the corona, making aggregation reversible rather than permanent (Figure 2.15). This dimensionality shift alters both colloidal stability and the protein conformational response, resulting in nanoparticle-protein complexes that are easily dissociated, especially in environments simulating physiological conditions [35].

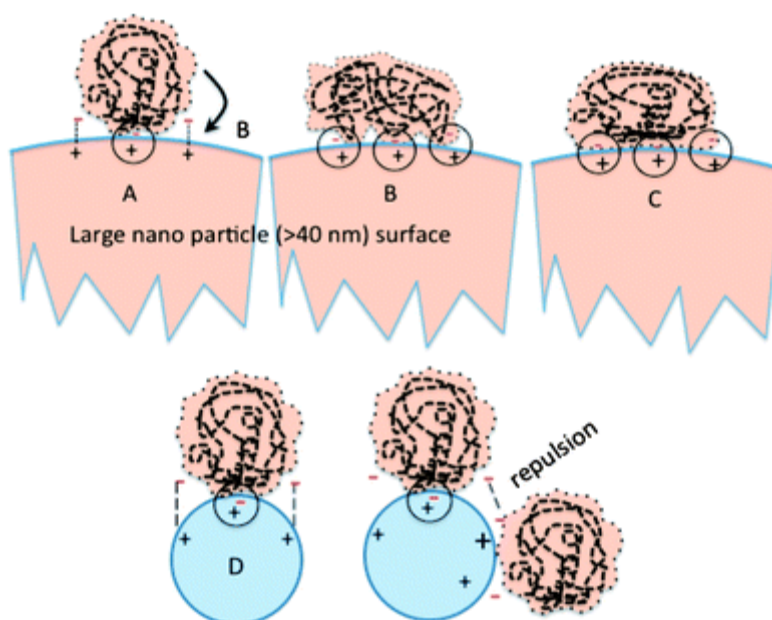


Figure 2.15. Evolution of the protein adsorption process with time on a large surface (A–C) and on small NPs (D) [35].

Despite progress, several unresolved challenges in the study of the protein corona continue to limit the safe and effective development of nanomedicines.

Although many studies have identified which proteins bind to NP surfaces, understanding how specific patterns of proteins, such as multifaceted recognition domains, affect NP recognition by biological systems is still limited. Interactions between protein coronas and cell membranes may involve multiple, transient receptor–ligand interactions, as shown in Figure 2.16. These can recruit intracellular signalling molecules, forming “bionanosynapses” that prepare cells for further biological responses. However, it remains unclear which protein arrangements influence processes like NP uptake, biodistribution, immune signaling, or cytokine secretion, making this one of the most complex and still largely unresolved open questions [36].

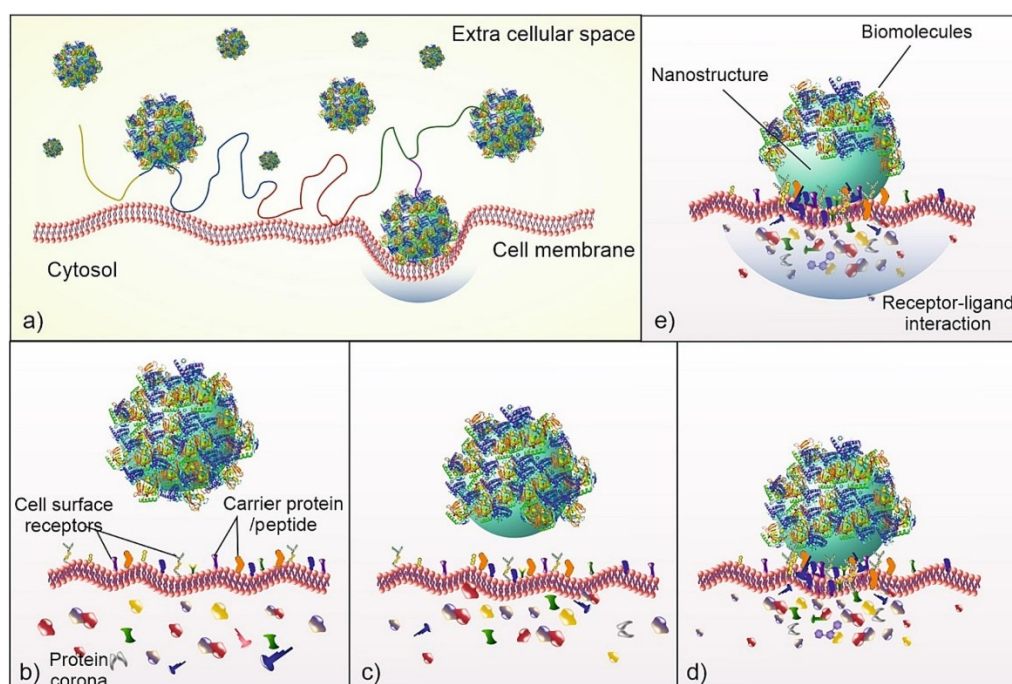


Figure 2.16. Sequence of events of NP-protein corona complex across cell membrane (a) Multiple collision of nanostructures with the membrane in a short period of time, forming ephemeral loose interface contacts with soft corona. Despite such short duration, these interactions induce surface rearrangements and follow a course that either leads in particle diffusion or to more intense and sustained interaction and biological interaction (b) Nanostructures surrounded by biomolecules before coming in contact with cell membrane (c) The progression of tight complex by releasing loosely bound particles (d) Recruitment of new biomolecules and membrane, further tightening and remodeling of interface followed by post-translational changes of proteins in signal transduction cluster (e) Transfer of this information across cell membrane completing recognition event [37].

Protein coronas can both mitigate and exacerbate NP toxicity. Common serum proteins (albumin, fibrinogen, apolipoproteins, immunoglobulins, etc.) are often found on NPs, but their presence can dramatically alter physiological responses. For instance, albumin or fibrinogen coatings on carbon nanotubes reduce platelet aggregation, while conformational changes in proteins like fibrinogen or IgG after adsorption can trigger immune signaling, cytokine release, or oxidative stress. Current studies remain limited in scope, preventing broad conclusions about how specific protein–NP

interactions drive toxicity or therapeutic effects and highlighting the need for more systematic, mechanistically grounded investigations [36].

The immune system poses an additional layer of complexity. Corona-coated nanoparticles are prone to opsonization and complement activation. Complement protein C3, for instance, has been shown to bind to iron oxide nanoworm coronas, promoting clearance through innate immune pathways. Because corona formation is inherently dynamic and exchangeable, nanoparticles may undergo repeated immune recognition, resulting in rapid clearance and diminished therapeutic efficacy over multiple dosing cycles [36].

Corona-bound immunoglobulins and complement factors cooperate to trigger all three complement activation routes (classical, lectin and alternative) leading to C3 deposition, generation of anaphylatoxins (e.g., C3a, C5a), and in some context formation of membrane attack complexes. Which pathway predominates depends on both corona composition and surface chemistry: for instance, adsorption of IgG or IgM promotes classical activation via C1q, whereas certain surface chemistries (including negatively charged or hydroxylated moieties) and nanoparticle types appear to favor alternative-pathway engagement through recruitment of factor B and factor D. Complement opsonization has two immediate consequences for nanoparticle fate: engagement of complement receptors on phagocytes that accelerates clearance, and local proinflammatory signaling mediated by anaphylatoxins. Immunoglobulins themselves exert nuanced effects that depend on isotype, density and orientation. Only a small number of bound Igs can suffice to trigger complement, yet the orientation of the antibody (exposure versus masking of the Fc region) critically influences its ability to recruit C1q and engage Fc γ receptors on phagocytes. Surface modifications and hydrophobic interactions alter Ig adsorption and structural rearrangement; consequently, two particles with similar nominal coatings can display markedly different opsonization profiles and aggregation behaviors depending on how antibodies settle within the corona and how stable those rearrangements remain under physiological shear and ionic conditions [36].

Attempts to mitigate these responses have focused on engineering “stealth” nanoparticles via surface functionalization, such as PEGylation or zwitterionic coatings, which reduce protein adsorption and prolong circulation (Figure 2.17). However, these approaches cannot fully prevent corona formation; rather, they modulate its composition and, in some cases, strategically enrich for proteins like clusterin that may contribute to stealth properties and *in vivo* persistence [32].

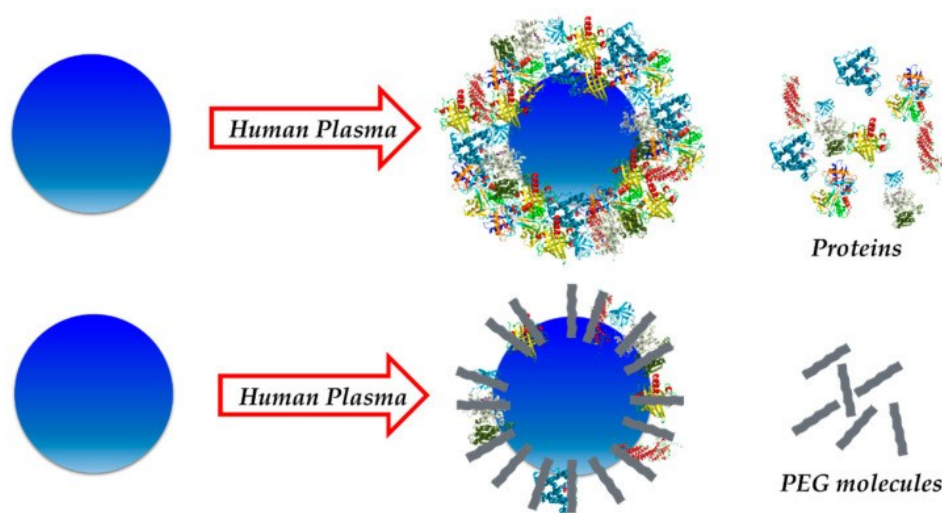


Figure 2.17. PEGylation of NPs minimizes interaction between proteins and NPs, leading to the formation of a smaller protein corona (PC) (cross-section view) [32].

Repeated exposure to particular surface chemistries (for example, PEG) elicits anti-PEG antibodies that accelerate subsequent clearance, illustrating how engineered “stealth” can paradoxically become immunogenic over time. Moreover, coronas enriched with unfolded or aggregated proteins are more likely to provoke antigen-specific responses, including T-cell activation, while complement-opsonized particles can modulate B-cell function through complement receptors. Consequently, particle design must anticipate not only the immediate innate interactions but the potential for adaptive memory that will alter pharmacokinetics and safety on repeated dosing and across different patient immunological backgrounds [36].

Two translational challenges emerge repeatedly. First, the corona formed *in vivo* is temporally and spatially heterogeneous: composition can change within minutes to hours after injection, and species-specific plasma proteomes produce distinct coronas that complicate extrapolation from animal models to humans. Second, much of current knowledge derives from ensemble measurements of corona composition; single-particle heterogeneity and the role of the soft corona remain poorly characterized despite their likely biological relevance. Complement proteins themselves undergo dynamic exchange on particle surfaces *in vivo*, further complicating static conceptions of opsonization and stealth. Together, these factors explain why materials that show promising behavior *in vitro* frequently encounter unexpected immune interactions *in vivo* and often display markedly reduced efficacy or altered biodistribution profiles upon translation [36].

2.5. DNA as a target/off-target: relevance of studying small-molecule interactions for genotoxicity and safety profiling

DNA is both an attractive therapeutic target and a major liability in safety assessment. Small molecules can engage DNA through a range of covalent and non-covalent modes, as shown in Figure

2.18 (intercalation, minor- or major-groove binding, electrostatic surface interactions) [38]. These interaction modes differ in their thermodynamic drivers, kinetics (on/off rates and residence times), structural consequences for the double helix, and biological outcomes, from useful modulation of gene expression to replication stress, mutagenesis, and cytotoxicity. Understanding how molecules bind DNA and what they do after binding is therefore central to both rational drug design and genotoxicity/safety profiling and remains a cornerstone of early-stage safety pharmacology.

Non-covalent interactions dominate many drug–DNA relationships and are typically classified as (Figure 2.18):

- Intercalation: planar aromatic moieties slide between base pairs, lengthening and locally unwinding the helix. Intercalators can alter duplex mechanics and obstruct polymerases. Single-molecule and bulk biophysical studies have shown that intercalation can produce measurable mechanical deformation and distinct kinetic fingerprints.
- Groove binding: heterocyclic or polyamide ligands bind in the minor or major groove, often with sequence preference (A-T versus G-C biases) and via hydrogen bonding and shape complementarity. Groove binders can be high affinity yet less disruptive to global helix geometry than intercalators, particularly when their binding does not induce significant backbone distortion.
- Electrostatic/backbone association: cationic molecules may associate broadly with the negatively charged phosphate backbone; these interactions are often lower specificity but can influence higher-order structure and aggregation and modulate DNA condensation under certain ionic conditions.
- Covalent adduct formation: certain electrophiles form covalent bonds with bases (*e.g.*, alkylators), producing lesions that are directly mutagenic and trigger DNA repair pathways such as base-excision or nucleotide-excision repair.

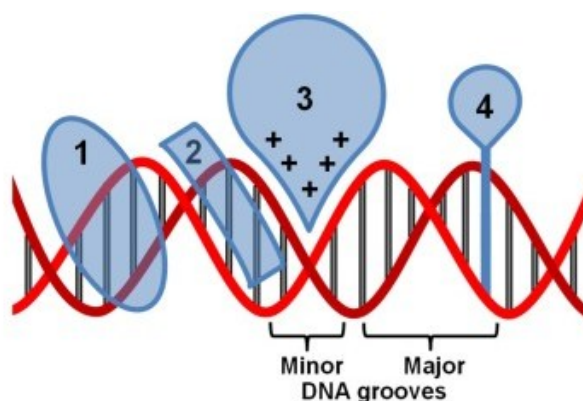


Figure 2.18. Schematic diagrams of different DNA binding modes: **1** is a major groove binder, **2** is a minor groove binder, **3** represents electrostatic/allosteric binding and **4** is an intercalator. The DNA backbone is in red and the base pairs in gray [38].

Affinity alone is an incomplete predictor of genotoxic risk. Kinetic parameters such as the association rate (k_{on}), dissociation rate (k_{off}), and residence time (t_{R}), often correlate more strongly with cellular outcomes. Long residence on DNA can prolong obstruction of polymerases and persistently engage repair systems, increasing the chance of replication fork collapse or error-prone repair. Single-molecule force spectroscopy (SMFS), surface plasmon resonance (SPR), ITC, NMR, fluorescence quenching, CD, UV-Vis and stopped-flow kinetics (SFK) are complementary tools that reveal these kinetic and mechanical signatures; combining techniques yields a mechanistic picture from thermodynamics to dynamics and helps disentangle mode-of-action-specific contributions [39].

Small-molecule–DNA interactions can cause genotoxicity through multiple paths:

- Direct lesions and miscoding: covalent adducts or chemically modified bases can mispair during replication, producing mutations and triggering error-prone repair pathways.
- Replication/transcription blockage: tightly bound or structurally disruptive ligands can stall polymerases, causing fork collapse, double-strand breaks, or transcriptional stress that may propagate into genome-wide instability.
- Interference with DNA-processing proteins: small molecules may occlude binding sites for essential factors (replicative helicases, topoisomerases, recombination proteins), leading to indirect genotoxicity and pathway-specific replication stress.
- Alteration of DNA secondary structure equilibrium: stabilizing non-canonical structures (e.g., G-quadruplexes, junctions) can dysregulate local genome function and increase the probability of error during DNA metabolism through misregulation of replication and repair factors.

These mechanisms explain why two compounds with similar equilibrium affinities may have markedly different genotoxic profiles, depending on residence time, structural alteration and repair-pathway engagement.

To distinguish on-target therapeutic DNA engagement from hazardous off-target interactions, a layered strategy is recommended:

- Biophysical profiling: use orthogonal assays (UV, ITC, NMR, SMFS, X-ray/cryo-EM where possible) to define binding mode, affinity, kinetics, and structural consequences. These data deconvolute intercalation vs groove vs junction binding and yield kinetic signatures predictive of biological persistence and mechanistic liability.

- In vitro genotoxicity assays: integrate standard genotoxicity tests (Ames, γ H2AX induction, COMET, micronucleus assays) with mechanistic readouts (replication fork progression assays, topoisomerase poisoning assays) to capture both mutagenic potential and DNA-processing interference in a pathway-specific manner.
- Cellular and genomic context: test across relevant cell types and consider genome-wide readouts (e.g., damage-seq, RPA/ γ H2AX ChIP-seq) to detect sequence/structure-biased damage and define tissue-specific vulnerabilities.
- ADME/metabolism profiling: many compounds are bioactivated or detoxified, and metabolites may have different DNA reactivity and residence times.
- Computational and structure-based filtering: *in silico* docking, predictive models of reactivity, and machine-learning classifiers trained on kinetic/structural features can prioritize candidates with lower DNA liability before costly experimental testing.

Not all DNA-binding is unacceptable: some therapeutics (oncology intercalators, alkylators) intentionally target DNA but must be dosed and monitored carefully. For non-oncology indications, evidence of covalent DNA modification, long residence times, polymerase stalling, or micronucleus formation raises major safety concerns and are generally unacceptable. Interpreting findings requires weighing mechanistic plausibility (does the binding mode plausibly produce the observed lesion?), dose/exposure margins, and therapeutic context to ensure an appropriate safety window [40].

2.6. Molecular Dynamics Simulations in Life Sciences

Molecular dynamics (MD) simulations constitute a cornerstone of modern computational biology, offering a unique means of interrogating the structural and dynamic properties of biomolecules at atomic resolution. Unlike experimental approaches that predominantly provide static structural information, such as X-ray crystallography or cryo-electron microscopy, MD simulations allow for the explicit observation of temporal evolution, thereby granting access to molecular motions and conformational fluctuations that are essential to biological function. Driven by advances in HPC, software development, and methodological innovation, MD has evolved from a niche computational technique into a mainstream tool across the life sciences and a key component of predictive molecular modeling workflows.

The importance of MD in life sciences is grounded in its capacity to capture the intrinsic dynamism of biomolecular systems. Proteins, nucleic acids, and lipid assemblies are not rigid entities; their functions depend critically on their ability to undergo conformational transitions, form transient

interactions, and respond to environmental perturbations. MD enables visualization and quantification of these dynamic processes, ranging from side-chain rearrangements to large-scale domain movements (some examples are illustrated in Figure 2.19), thereby linking structural fluctuations to functional outcomes.

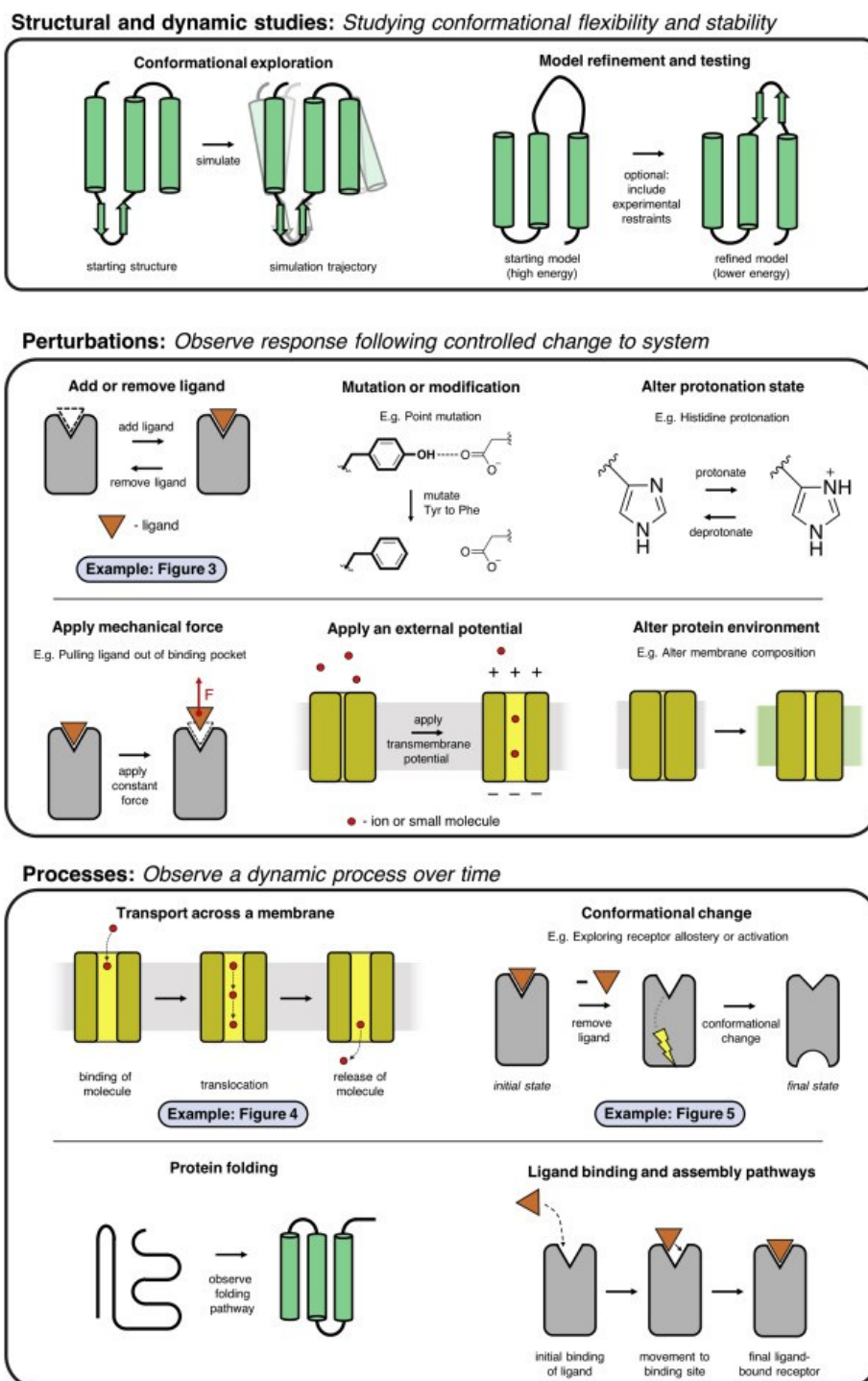


Figure 2.19. Some of the most common applications of Molecular Dynamics [41].

This capability has profound implications. First, MD simulations provide insights into mechanistic processes that are often experimentally inaccessible, such as the pathway and energetics of ligand binding, the propagation of allosteric signals, or the role of solvent molecules in stabilizing specific conformations. Second, they offer predictive power, enabling the estimation of thermodynamic quantities such as free energies, enthalpic and entropic contributions to binding, and kinetic barriers separating metastable states. Finally, in applied contexts such as structure-based drug design, MD has proven invaluable in elucidating how ligands interact with biomolecular targets, predicting the structural consequences of mutations, and revealing binding-site plasticity that may not be captured by static experimental structures or single-conformation docking approaches.

The methodological foundation of MD is the numerical integration of Newton's equations of motion for systems of interacting atoms. The force field is central to this process, as it defines the potential energy landscape governing atomic interactions. Classical biomolecular force fields (*e.g.*, AMBER, CHARMM, OPLS, GROMOS) parameterize bonded terms like bond lengths, bond angles, dihedrals as well as non-bonded interactions such as van der Waals forces and electrostatics. The accuracy of a simulation is intimately tied to the reliability of the chosen force field, which must be carefully validated against experimental benchmarks and assessed for its transferability across chemical environments [41].

The numerical integration of atomic trajectories is commonly performed using Verlet-based algorithms with femtosecond time steps, ensuring adequate resolution of high-frequency vibrational motions. To approximate bulk behavior, simulations typically employ periodic boundary conditions in combination with long-range electrostatics treatments, such as the Particle Mesh Ewald method. Furthermore, thermostats (*e.g.*, Nosé-Hoover, Langevin dynamics) and barostats (*e.g.*, Monte Carlo, Parrinello–Rahman, Berendsen) are introduced to maintain the desired thermodynamic ensemble and ensure correct sampling of phase-space distributions.

A critical consideration in biomolecular MD is the role of solvent. While implicit solvent models can reduce computational cost, explicit water models (*e.g.*, TIP3P, SPC/E, TIP4P) are generally required to accurately capture solvation effects, hydration dynamics, and the role of water molecules in mediating biomolecular interactions and stabilizing transient conformational states [41].

It is important to acknowledge that biologically relevant events frequently occur on timescales (microseconds to milliseconds or longer) that exceed the reach of conventional atomistic MD. To address this, a variety of enhanced sampling techniques have been developed, including metadynamics, replica exchange molecular dynamics (REMD), accelerated MD, and Markov state modeling. These approaches facilitate exploration of the conformational landscape by overcoming

kinetic barriers, thereby improving convergence and interpretability and enabling reconstruction of free-energy surfaces that would otherwise remain inaccessible.

The overarching objective of MD simulations is to establish a quantitative and mechanistic link between molecular structure, dynamics, and function. Specific applications include:

- Elucidation of conformational equilibria and allosteric transitions in proteins and nucleic acids and identification of intermediated metastable states.
- Quantification of ligand–receptor interactions, including binding free energies and binding pathways with atomistic details.
- Characterization of folding and misfolding pathways in proteins, with implications for understanding diseases associated with protein aggregation and conformational disorder.
- Investigation of membrane dynamics, ion channel gating, and lipid–protein interactions under physiologically relevant conditions.
- Integration with experimental methods, such as NMR spectroscopy or cryo-EM, to refine structural models and validate dynamic hypotheses through hybrid simulation-experimental workflows.

In pharmaceutical sciences, MD simulations serve as a computational complement to experimental drug discovery pipelines. By probing binding site flexibility, identifying cryptic pockets, and evaluating the effects of point mutations, MD contributes to the rational design of next-generation therapeutics and supports early-stage mechanism-of-action elucidation.

Despite its strength and success, MD is subject to important limitations. The timescale gap (ns-ms vs ms-s), characteristic of many biological processes, remains a challenge. While enhanced sampling strategies mitigate this limitation, they introduce additional methodological complexity and require careful validation to avoid biasing the underlying free-energy landscape.

Moreover, the accuracy of simulations is constrained by the imperfections of current force fields, which may inadequately capture weak interactions, protonation dynamics, metal coordination, or complex lipid environments. Computational cost remains a challenge as well: although advances in GPU acceleration and high-performance computing have dramatically extended the reach of MD, simulations of large systems (*e.g.*, virus capsids, organelle-scale assemblies) remain prohibitively expensive and require specialized parallelization strategies [41].

MD simulations are not a substitute for experiment; rather, their predictive value depends on rigorous integration and cross-validation with empirical data to ensure that MD trajectories provide biologically meaningful insights with physical realism and interpretable uncertainty bounds.

The role of High-Performance Computing in Drug Discovery and Molecular Simulation

High-performance computing (HPC) has become an indispensable driver of innovation in the life sciences, particularly in the fields of drug discovery and molecular simulation. Traditional drug development is an extraordinarily costly and time-consuming endeavor, typically requiring more than a decade of research and billions of dollars in investment. By contrast, computational drug discovery and design (CDDD), when supported by state-of-the-art HPC infrastructures, can drastically reduce both the financial and temporal burdens of this process. Estimates suggest that computational approaches save hundreds of millions of dollars per drug and shorten development timelines by approximately a year, making them an essential complement to experimental pharmacology and a cornerstone of modern preclinical decision-making [42].

The power of HPC lies in its ability to manage the vast complexity inherent in biological systems and drug discovery pipelines. Supercomputing resources enable researchers to perform large-scale virtual screening of millions of candidate compounds against biomolecular targets in a matter of hours or days, a task that would be practically impossible using conventional laboratory methods. These *in silico* screenings allow the rapid identification of promising lead compounds, which can then be validated experimentally. Furthermore, HPC facilitates drug repurposing efforts, where existing pharmaceuticals are computationally assessed against new targets, offering an efficient pathway to therapeutic innovation. Such strategies have proven particularly valuable in urgent scenarios, such as the outbreaks of SARS, avian influenza, and Ebola. In these cases, the capacity of supercomputers like Tianhe-2 to perform ultra-high-throughput virtual screening provided a means of identifying potential antivirals within days, thereby offering a timely response to global health emergencies and illustrating the real-world impact of HPC-enabled computational triage [42].

Beyond virtual screening, HPC has revolutionized MD simulations, which calculate the time-dependent behavior of biomolecules at atomic resolution. The complexity of biological macromolecules, often involving millions of atoms, makes their simulation computationally demanding, but with the resources of modern supercomputing centers, it has become feasible to investigate processes that were previously inaccessible. MD simulations have been instrumental in revealing conformational states and allosteric binding sites that are invisible to static structural methods such as crystallography. For instance, simulations of the glucagon receptor uncovered a closed conformation stabilized in the absence of ligand, while studies of potassium channels revealed novel druggable cavities within their voltage-sensor domains. Such insights not only advance fundamental biological understanding but also guide rational drug design strategies by exposing new

structural determinants of therapeutic relevance and dynamic features not captured by traditional experimental pipelines.

HPC has also enabled the development of novel algorithms specifically optimized for large-scale simulations. An example is the NUMD method, which combines normal mode analysis with umbrella sampling to efficiently explore protein conformational transitions and associated free energy landscapes. By distributing thousands of conformational states across thousands of CPU cores, NUMD provides a powerful approach to studying the large-scale motions of proteins such as kinases and calcium-binding proteins, which are critical to cellular regulation and drug targeting. Similarly, in neurodegeneration research, HPC-driven MD simulations have yielded unprecedented insights into the aggregation of amyloid β -peptides in Alzheimer's disease. Parallel simulations revealed helix-to-sheet transitions, identified aggregation-prone intermediate states, and guided the design of small-molecule inhibitors capable of stabilizing non-toxic conformations. These achievements underscore the role of HPC not merely as a facilitator of faster computation, but as a transformative force shaping the very questions that can be asked in biomedical research and expanding the mechanistic resolution with which these questions can be addressed [42].

The importance of HPC extends even further in the era of precision medicine. Personalized therapeutic strategies demand the integration of vast amounts of heterogeneous data, including genomic sequences, transcriptomic and proteomic profiles, metabolic networks, and clinical records. Only high-performance infrastructures possess the computational capacity to store, process, and analyze such biomedical "big data" in real time. Moreover, the predictive and integrative power of HPC is indispensable for translating these data into actionable medical insights, enabling individualized treatment regimens tailored to the molecular signatures of patients and supporting continuous model refinement as new clinical information becomes available. The advent of exascale computing is expected to be particularly transformative, as it will provide the computational throughput required to simulate complex systems at physiologically relevant timescales and to manage the ever-expanding biomedical datasets associated with personalized healthcare while allowing simultaneous coupling of multi-omic and clinical decision-support models [42].

Nowadays high-performance computing has transitioned from being a supportive technology to a central pillar of drug discovery and molecular simulation. By accelerating virtual screening, enabling long-timescale molecular dynamics, guiding the understanding of protein folding and misfolding, and managing the complexity of precision medicine, HPC has established itself as an essential infrastructure for modern biomedical research. The continuous growth of computational power, culminating in exascale systems, will not only further enhance the efficiency of traditional discovery pipelines but will also make possible entirely new paradigms of therapeutic design and healthcare

delivery. In the life sciences, the trajectory of progress is now inseparable from the trajectory of HPC itself and will increasingly depend on hybrid AI–HPC ecosystems capable of real-time inference and large-scale simulation.

The Cooperative Cycle of Molecular Simulation and Experiment

Molecular simulation and experiment form a reciprocal epistemic pair: simulations generate mechanistic, atomistic hypotheses that can rationalize averages and kinetics measured in the laboratory, while experiments provide the empirical constraints necessary to test, calibrate and, when necessary, correct the models that underlie those simulations. This reciprocity is valuable because each modality addresses different aspects of the same physical reality. Simulations access high-dimensional configurational distributions and femtosecond–nanosecond detail that are typically invisible to experiment, whereas experiments supply ensemble- and time-averaged observables that ultimately anchor theoretical claims to empirical fact and help delimit the physically permissible space of microscopic interpretations. The methodological challenge is therefore to translate between the two representational languages in a way that preserves uncertainty, avoids overinterpretation, and extracts the maximum information from their (dis)agreement. Conceptually, two broad paradigms for cooperation have emerged. The first uses simulation as a prior model that is adjusted a posteriori to fit experimental observables; the second alters the simulation protocol or force field itself so that generated ensembles better reflect measured data through iterative refinement cycles [43].

Validation requires awareness of six sources of uncertainty: the choice of degrees of freedom and physical model; the interaction function or force field; convergence and sampling; boundary and thermodynamic conditions; software reliability; and user practice. Even perfect agreement between computed and measured averages does not, by itself, prove that the underlying microscopic model is correct, because experimental observables are often indirect, averaged, and dependent on forward models that map configurations to measured signals. Hence, rigorous cooperation demands not only algorithmic methods for merging data and simulation, but also careful matching of what is averaged and how, explicit error quantification for both experimental observables and forward models, and repeated, independent validation efforts that test the robustness of conclusions under alternative modeling assumptions [44].

The study by Jiang et al. (2023) exemplifies this principle of cooperation in practice. Investigating the binding between ibrutinib and human alpha-1 acid glycoprotein (HAG), the authors combine multi-spectroscopic techniques (UV–vis, FS, CD) with molecular docking and molecular dynamics simulations. Experimentally, fluorescence quenching and Förster resonance energy transfer reveal that ibrutinib binds HAG with moderate affinity ($\sim 10^5 \text{ M}^{-1}$), driven by hydrogen bonds and

hydrophobic interactions. Conformational changes in the protein backbone are corroborated by CD spectroscopy. Theoretical simulations then validate and extend these findings: docking locates ibrutinib within the hydrophobic β -barrel pocket of HAG, while MD simulations confirm the stability of the complex, quantify the contributions of individual residues, and provide a dynamic picture of conformational adjustment during binding with atomistic temporal resolution. What is striking in this case is that experimental results and simulations not only converge but also compensate for each other's limitations. Spectroscopy identifies binding events and global conformational consequences but cannot by itself pinpoint residue-level interactions; simulation fills this gap. Conversely, docking predictions of binding pockets require empirical confirmation to avoid spurious conclusions, which spectroscopic quenching and CD spectra supply. The joint analysis therefore produces a more robust and fine-grained model of drug-protein interaction than either approach alone could achieve. This case study also highlights the translational value of the cooperative paradigm: understanding plasma protein binding is essential for predicting pharmacokinetics and drug efficacy, yet it depends on both empirical quantification and atomistic mechanistic insight. By situating simulations within experimental contexts, one achieves not only validation of computational models but also expansion of experimental interpretability and improved confidence in the mechanistic plausibility of proposed binding modes [45].

Finally, there are some community-scale needs. Robust, transferable force fields will increasingly be developed in a data-driven fashion, but progress depends on curated, high-quality experimental databases with well-characterized errors, on standards for forward models, and on practices that make simulations reproducible (clear reporting of boundary conditions, sampling protocols and uncertainties). Only in this ecosystem can discrepancies between simulation and experiment be interpreted reliably as opportunities to learn rather than as incurable contradictions and can methodological improvements propagate across the field in a transparent and reproducible manner [43].

3. Methodological Framework

3.1. Experimental techniques

3.1.1. UV–Vis Spectroscopy

Ultraviolet–visible (UV–Vis) spectroscopy is among the most widely employed optical techniques in both fundamental research and applied sciences, owing to its versatility, relative simplicity, and sensitivity. It is based on the fundamental interaction of electromagnetic radiation with matter, wherein photons in the ultraviolet (200–400 nm) and visible (400–800 nm) spectral ranges are absorbed by molecules or materials, resulting in characteristic electronic transitions. These transitions typically involve promotion of valence electrons from lower-energy ground states to higher-energy excited states. In organic and biological molecules, the most relevant processes correspond to $\pi \rightarrow \pi^*$ transitions of conjugated systems and $n \rightarrow \pi^*$ transitions involving lone pairs of heteroatoms. The energy separation between these states dictates the wavelength of absorption, thereby providing a molecular fingerprint that can be correlated with electronic structure and functional group composition and used as a diagnostic tool for monitoring local chemical environments [46]. The quantitative description of absorption processes is given by the Lambert-Beer law, expressed as:

$$A = \varepsilon c l \quad (1)$$

where A is the absorbance (a dimensionless quantity), ε is the molar extinction coefficient ($\text{L mol}^{-1} \text{cm}^{-1}$), c is the concentration of the absorbing species (mol L^{-1}), and l is the optical path length of the sample cuvette (cm). This linear relationship provides a direct means of determining solute concentrations, if the system adheres to ideal conditions where scattering and instrumental artifacts are negligible. The Beer–Lambert law thus underpins much of the quantitative analytical power of UV–Vis spectroscopy and remains foundational for validating concentration-dependent experimental designs [47].

When monochromatic light passes through a medium, part of the radiation is absorbed by electronic transitions, while the remainder is transmitted. The transmitted intensity decreases exponentially with path length and concentration, leading to the observed logarithmic dependence of absorbance on incident and transmitted intensities. Beyond simple absorbance, modern UV–Vis analysis often considers spectral band shapes, molar absorptivity values, and solvent-dependent shifts (bathochromic or hypsochromic effects), which provide insight into electronic structure, solvation, and intermolecular interactions as well as microenvironment-induced perturbations of chromophores [48].

Furthermore, in complex biological or material systems, the interpretation of UV–Vis spectra extends beyond mere absorption maxima. Phenomena such as charge-transfer bands, excitonic coupling, and surface plasmon resonance (in nanomaterials) can be probed to gain mechanistic and structural information. This makes UV–Vis spectroscopy not only a tool for quantification but also a method for structural elucidation and dynamic monitoring capable of capturing subtle electronic perturbations and real-time changes in molecular or nanoscale assemblies.

Instrumentation

Modern UV–Vis spectrophotometers (Figure 3.1) are typically configured as either single-beam or double-beam instruments. Single-beam designs measure the transmitted light through the sample against a baseline, while double-beam systems simultaneously compare the absorbance of a sample with that of a reference, thereby minimizing fluctuations in lamp intensity and detector sensitivity. The latter are generally preferred for precise and reproducible measurements and for long-term kinetic acquisitions.

The light sources used in UV–Vis instruments are selected to provide continuous emission across the desired spectral regions. A deuterium discharge lamp is employed for the ultraviolet range (approximately 190–350 nm), while a tungsten–halogen lamp covers the visible and near-infrared region (approximately 320–2500 nm). Optical elements such as diffraction gratings are used to disperse the radiation into narrow wavelength bands, which are then directed through the sample. Detection is commonly achieved with photodiodes, photomultiplier tubes, or charge-coupled devices (CCDs), depending on the sensitivity and wavelength range required and on the temporal resolution needed for fast spectral acquisitions.

Samples are typically contained in quartz cuvettes, which are transparent across both the UV and visible regions. The choice of solvent is critical, as many organic solvents exhibit strong absorbance in the UV region; thus, optically transparent solvents such as water, ethanol, or acetonitrile are preferred to avoid baseline distortion and secondary absorption features [46].

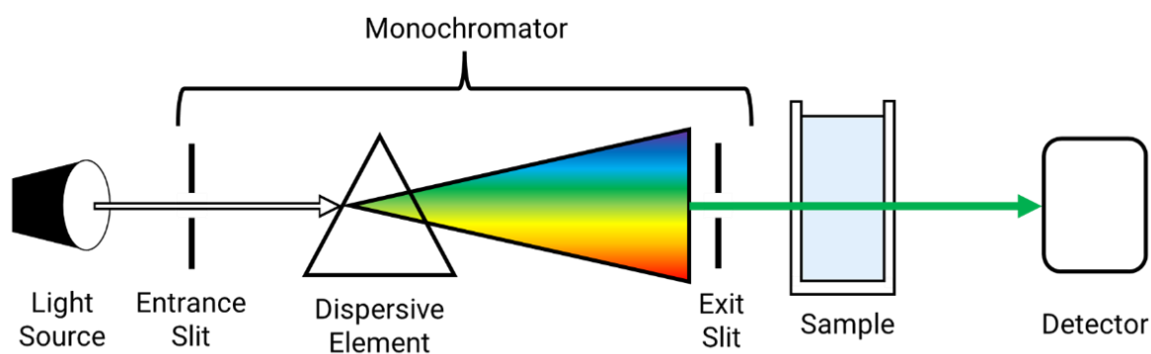


Figure 3.1. A basic block diagram of the elements in a single beam UV-Visible spectrometer.

Drug/nucleic acid interactions using UV

UV spectroscopy represents a powerful tool for studying the structural properties of nucleic acids and their interactions with small molecules such as drugs. A central concept in this context is the modulation of UV absorbance, known as hyperchromism and hypochromism, which arises from changes in the electronic environment of the DNA bases. In its native double-helical form, DNA exhibits reduced absorbance compared to its single-stranded state. This phenomenon, referred to as hypochromism, is the result of base stacking within the helical structure, where the electronic interactions between closely aligned bases limit their ability to absorb UV light individually and restrict the transition dipole moments of the bases. Conversely, when DNA undergoes denaturation through heating or the action of chemical agents, the hydrogen bonds stabilizing the double helix are disrupted, and the bases become unstacked and exposed. This structural transition leads to hyperchromism, characterized by a marked increase in absorbance due to the greater accessibility of the bases to UV radiation and the loss of excitonic coupling present in the stacked configuration.

These effects are not only diagnostic of DNA denaturation but also serve as sensitive indicators of interactions between DNA and potential ligands. For instance, when a small molecule binds to DNA, it can induce structural modifications ranging from partial unwinding of the helix to stabilization of the stacked conformation, depending on the binding mode. Intercalating agents, by inserting themselves between adjacent bases, often disrupt base stacking and give rise to hyperchromic shifts, whereas groove-binding ligands may stabilize the double helix and reinforce hypochromism by enhancing stacking cooperativity and reducing base mobility. Monitoring these spectral changes provides valuable insights into the nature and strength of binding interactions, allowing researchers to infer whether a ligand promotes DNA destabilization, stabilization, or subtler conformational rearrangements at local or global levels.

The study of hyperchromism and hypochromism through UV spectroscopy thus offers an accessible and informative approach to probing biomolecular interactions. It enables the characterization of the fundamental processes underlying drug–DNA recognition and contributes to the broader understanding of how small molecules influence the stability and function of nucleic acids. In this way, UV spectroscopic analysis remains an indispensable technique in biophysical chemistry and molecular pharmacology thanks to its sensitivity, simplicity, and ability to capture early structural perturbations that precede larger-scale conformational changes [49], [50].

DNA Melting Study

UV spectroscopy is widely employed to investigate DNA melting, a process that reveals fundamental aspects of nucleic acid stability. The principle underlying these studies is the hyperchromic effect, whereby the absorbance of DNA at 260 nm increases as the double helix denatures and the bases

become unstacked. Monitoring absorbance as a function of temperature generates a melting curve, from which the melting temperature (T_m), the point at which half of the DNA molecules are denatured, can be determined. This parameter serves as a quantitative indicator of DNA thermal stability and is sensitive to a range of physicochemical variables, including sequence composition, ionic environment, and buffer type as well as the presence of co-solutes that modulate hydration and electrostatic screening.

In practice, DNA melting studies rely on gradual heating of the sample while recording absorbance at 260 nm. The resulting sigmoidal curve reflects the cooperative disruption of base pairing and stacking interactions, with sharper transitions typically associated with more homogeneous DNA samples and with fewer sequence-dependent microdomains. The magnitude of the hyperchromic shift provides additional information about the extent of strand separation. Variations in T_m can be correlated with differences in guanine–cytosine content, since GC pairs, stabilized by three hydrogen bonds, require higher thermal energy to denature compared to AT pairs. Beyond sequence effects, melting profiles are profoundly influenced by the surrounding solution. For instance, salts and buffers alter the electrostatic environment of the DNA backbone, thereby modulating strand stability. As shown in systematic studies, DNA melting curves acquired in different buffers demonstrate that both buffer concentration and ionic strength strongly affect T_m , with stabilizing effects arising from enhanced screening of electrostatic repulsion between negatively charged strands and from altered water activity in the hydration shell.

The significance of UV-based melting studies extends beyond assessing DNA stability under controlled conditions. They are crucial for evaluating how environmental factors, chemical agents, or ligands alter DNA conformation. By comparing melting curves in the absence and presence of drugs or other interacting molecules, one can infer whether these agents stabilize the helix, destabilize it, or induce conformational changes such as partial unwinding, groove widening, or altered stacking cooperativity. Moreover, the methodology provides a relatively simple yet powerful experimental approach, combining thermodynamic insight with structural sensitivity. For these reasons, UV melting analysis remains a cornerstone in biophysical and biochemical research, offering an accessible window into the interplay between DNA structure, environment, and molecular interactions while requiring minimal sample manipulation and instrumentation [51].

Effect of the Ionic Strength

UV spectroscopy provides a simple yet powerful means to investigate the interactions between small molecules and DNA, particularly when exploring how ionic conditions modulate binding affinity and binding modes. The principle relies on monitoring changes in the absorption spectra of the interacting system, typically at characteristic wavelengths of the dye or drug, and at 260 nm where DNA bases

absorb strongly. When a molecule binds to DNA, its electronic environment is altered, leading to measurable spectral changes such as hypochromism, hyperchromism, or bathochromic and hypsochromic shifts. These effects are diagnostic of the type of binding interaction, whether intercalative, groove-binding, or primarily electrostatic and they provide early spectral fingerprints of ligand-induced structural perturbations.

Ionic strength plays a central role in determining the strength and nature of these interactions. DNA is a polyanion, with its negatively charged phosphate backbone strongly influencing how positively charged or planar aromatic ligands interact with it. At low ionic strength, the electrostatic attraction between cationic dyes and the DNA backbone is more pronounced, often enhancing binding constants and favouring both electrostatic interactions and intercalation. As the ionic strength of the surrounding medium increases, counterions such as sodium or phosphate anions compete with the ligand for binding sites, effectively screening the charges on DNA. This screening can weaken electrostatic attraction, leading to a reduction in binding affinity for some molecules. In other cases, however, higher ionic strength may stabilize DNA structure and facilitate intercalation by reducing repulsion between phosphate groups, thereby enhancing the apparent affinity of certain ligands by promoting tighter base stacking and narrowing of the inter-base spacing.

UV spectroscopic titrations of DNA with dyes or drugs under varying salt concentrations provide quantitative insight into these effects. Binding constants can be extracted by monitoring systematic changes in absorbance as a function of DNA concentration and comparing results across different ionic environments. Such studies reveal not only the strength of the interaction but also how environmental conditions modulate the binding equilibrium. For instance, methylene green has been shown to bind more strongly under low ionic strength, while Victoria blue B exhibits increased binding at higher ionic strength, illustrating how individual molecular structures dictate sensitivity to electrostatic screening and to salt-dependent conformational adjustments of the DNA helix.

The broader significance of these observations lies in both fundamental and applied contexts. On a mechanistic level, they illuminate the balance between intercalative stacking, groove binding, and electrostatic association in drug–DNA interactions. On a practical level, they help assess the biological impact of potentially carcinogenic dyes or the therapeutic potential of DNA-binding drugs. By correlating UV spectral changes with ionic conditions, researchers can map out how molecules interact with nucleic acids under conditions that mimic cellular environments and capture how subtle electrostatic fluctuations tune ligand affinity and DNA stability. Consequently, UV spectroscopy, coupled with controlled modulation of ionic strength, remains a cornerstone technique for dissecting the complex interplay between small molecules and DNA [52].

Esterase-like Enzymatic Activity Inhibition Assay

UV spectroscopy is also a valuable technique for monitoring enzymatic reactions and their inhibition, including assays designed to evaluate esterase-like activity. In the case of HSA, which exhibits pseudo-enzymatic properties resembling those of esterases, UV-based methods provide a straightforward and sensitive way to follow substrate hydrolysis and its modulation by ligands or inhibitors. The principle of the assay relies on the fact that ester-containing substrates undergo cleavage in the presence of HSA, leading to the release of chromophoric or UV-absorbing products whose spectral features can be quantified over time with minimal sample manipulation.

In practice, a model ester substrate, commonly *p*-nitrophenyl acetate (NPA) or analogous compounds, is incubated with HSA under controlled conditions. The enzymatic-like hydrolysis releases *p*-nitrophenol, a product with a characteristic absorption maximum in the UV-visible region, which can be continuously monitored by recording absorbance at around 400 nm. The rate of increase in absorbance directly reflects the catalytic efficiency of HSA and allows real-time tracking of reaction progress. When inhibitors or potential ligands are introduced, they may interact with the protein's active regions or alter its conformational state, leading to measurable changes in the reaction rate. A decrease in the slope of absorbance versus time plots indicates effective inhibition of HSA's esterase-like activity and provides a quantitative basis for comparing ligand effects.

The strength of UV-based esterase assays lies in their ability to provide kinetic parameters with high temporal resolution. By applying Michaelis–Menten analysis, values such as the apparent K_m and V_{max} can be derived, and their shifts in the presence of inhibitors yield mechanistic insights into the inhibition process, whether it is competitive, noncompetitive, or mixed and whether the ligand perturbs catalytic residues directly or exerts allosteric control. The following kinetic parameters were determined using enzyme kinetic calculations and the Michaelis–Menten equation:

$$v_0 = \frac{V_{max}[S]}{K_m + [S]} \quad (2)$$

where V_0 is the initial velocity of *p*-nitrophenol formation, V_{max} is the maximum velocity of *p*-nitrophenol formation, $[S]$ is the substrate concentration, and K_m is the Michaelis–Menten constant reflecting the substrate concentration at which the reaction rate reaches half of V_{max} .

To verify the parameters examined, a Lineweaver-Burk plot (aka double reciprocal plot) was also plotted between the inverse of velocity and the inverse of substrate concentration, in both the absence and presence of inhibitors [53]:

$$\frac{1}{v_0} = \frac{K_m}{V_{max}} \frac{1}{[S]} + \frac{1}{V_{max}} \quad (3)$$

This linearization enables straightforward extraction of kinetic parameters from the slope and intercept and, therefore, also allows qualitative discrimination among competitive, non-competitive, and mixed inhibition mechanisms.

3.1.2. Steady-State Fluorescence Spectroscopy

Fluorescence spectroscopy (FS) is a highly sensitive and versatile analytical technique that probes the photophysical properties of molecules through the detection of their spontaneous emission following excitation with electromagnetic radiation. In steady-state fluorescence spectroscopy, the sample is irradiated with light of a specific excitation wavelength, and the resulting emission spectrum is recorded under conditions where the excitation and emission intensities are temporally constant (i.e., time-averaged rather than time-resolved). This technique provides rich information about the local environment of fluorescent species, intermolecular interactions, and dynamic processes at the molecular scale by reporting subtle changes in polarity, quenching efficiency, or microviscosity.

A typical Jablonski diagram is shown in Figure 3.2. Upon absorption of a photon, a fluorophore is promoted from its ground electronic singlet state (S_0) to an excited singlet state (S_1 or higher). The molecule then undergoes a series of ultrafast relaxation processes, including internal conversion and vibrational relaxation, before returning to the lowest vibrational level of the first excited singlet state (S_1). From this state, the system can relax back to the ground state through multiple competing pathways. Among these, radiative relaxation results in the emission of a photon, perceived as fluorescence and characterized by a Stokes-shifted emission whose wavelength reflects the reorganization of the surrounding environment.

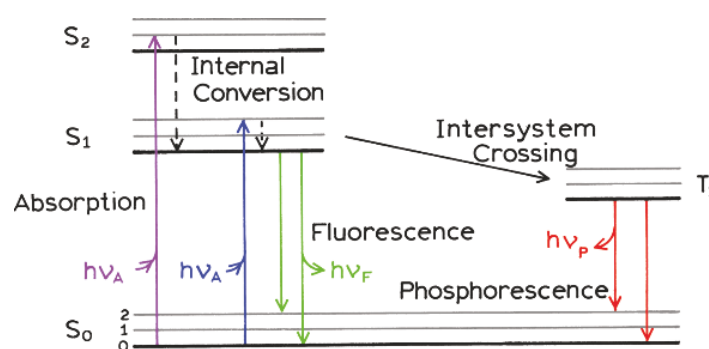


Figure 3.2. One form of a Jablonski diagram [54].

Since part of the excitation energy is dissipated non-radiatively (e.g., through collisions, vibrational relaxation, or solvent reorganization), the emitted photon typically exhibits a lower energy (longer wavelength) than the absorbed photon. This energy difference between the excitation and emission maxima is referred to as the Stokes shift, which is a fundamental characteristic of fluorescence

processes and provides a convenient spectral separation for detection and minimizes spectral overlap between excitation and emission pathways [54].

The observed fluorescence intensity I can be expressed as:

$$I = 2.303 * \Phi I_0 \epsilon c l \quad (4)$$

where Φ is the fluorescence quantum yield (i.e., the ratio of emitted photons to absorbed photons), I_0 is the incident excitation intensity, ϵ is the molar extinction coefficient of the fluorophore at the excitation wavelength, c is the concentration of the fluorophore, and l is the optical path length of the sample. This equation underscores the dependence of fluorescence signals on both intrinsic molecular properties (quantum yield, extinction coefficient) and experimental conditions (excitation intensity, sample concentration, highlighting the importance of maintaining linearity conditions to avoid inner-filter effects [55].

Importantly, fluorescence signals are highly sensitive to the microenvironment surrounding the fluorophore. Factors such as solvent polarity, pH, ionic strength, and the presence of quenchers or binding partners can induce spectral shifts, intensity changes, or alterations in emission anisotropy, thereby enabling fluorescence spectroscopy to serve as a powerful probe of molecular interactions and conformational dynamics with sensitivity to even subtle perturbations in the local chemical environment.

Instrumentation

A typical fluorescence measurement is performed using a spectrofluorometer, which is conceptually similar to a UV–Vis spectrophotometer but with additional optical elements to separately control excitation and emission (Figure 3.3). The main components include:

1. **Excitation Source.** Continuous-wave xenon arc lamps are commonly employed due to their broad spectral output (UV to near-infrared). In some cases, lasers are used when high spectral brightness, monochromatic excitation, or time-resolved studies are required, providing superior temporal precision and selectivity.
2. **Monochromators.** Two monochromators are generally incorporated: one to select the excitation wavelength and another to disperse the emitted light. This dual control allows precise tuning of both excitation and emission spectra, enabling excitation–emission matrix (EEM) measurements for complex systems and improving discrimination among overlapping fluorophores.

3. **Sample Compartment.** Samples are typically contained in quartz cuvettes transparent in the UV–Vis range. Temperature-controlled sample holders are often employed in biochemical studies to maintain physiological conditions or to investigate thermally induced conformational changes, ensuring reproducibility and minimizing thermal drift.
4. **Detectors.** Fluorescence emission is inherently weak compared to excitation light, necessitating highly sensitive detectors. Photomultiplier tubes (PMTs) are widely used owing to their high gain and low noise, while charge-coupled devices (CCDs) and avalanche photodiodes are increasingly adopted for rapid and multiplexed detection where spatial resolution or high throughput is required.

The geometry of the measurement is typically configured at 90° between excitation and emission paths to minimize interference from scattered excitation light. Additional optical filters (e.g., cutoff or band-pass filters) are frequently employed to suppress stray light and improve signal-to-noise ratios and to protect detectors from excessively strong excitation leakage [54][54].

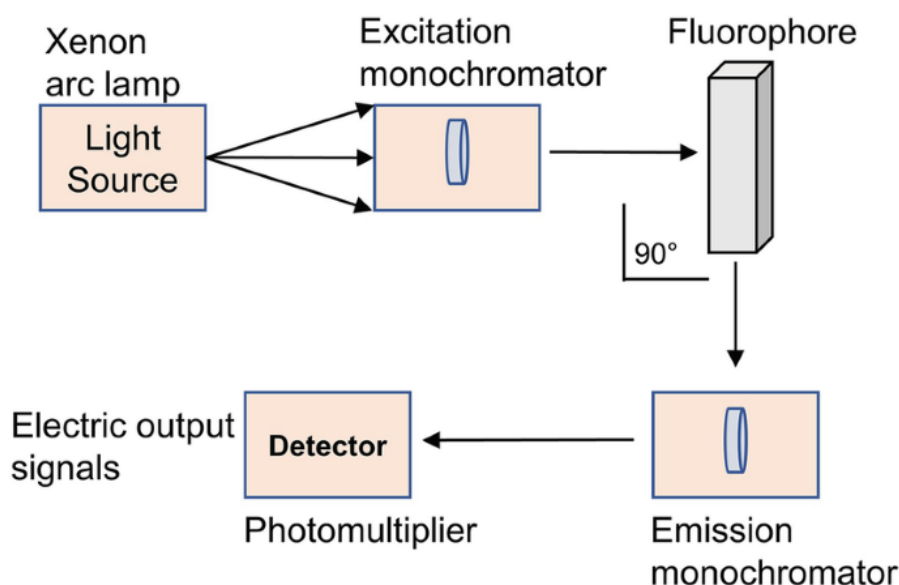


Figure 3.3. Schematic illustration of the instrumentation for fluorescence spectroscopy [56].

Fluorescence Quenching

Fluorescence spectroscopy has become an indispensable technique for investigating the interactions between drugs and proteins, primarily because the intrinsic fluorescence of aromatic amino acids such as tryptophan, tyrosine, and phenylalanine is exquisitely sensitive to the local environment. When a ligand binds to a protein and perturbs this environment, changes in fluorescence intensity, emission wavelength, or lifetime provide a window into the molecular mechanism of interaction. Among the most informative manifestations of these changes is fluorescence quenching, a process in which the

emission of the fluorophore is diminished by the presence of a quencher, in this case the drug molecule.

Two principal mechanisms underlie quenching: dynamic and static. In dynamic quenching, collisional encounters between the excited-state fluorophore and the quencher result in non-radiative deactivation before emission occurs. This process is time-dependent, leading to a measurable reduction in fluorescence lifetime as quencher concentration increases. In contrast, static quenching arises from the formation of a non-fluorescent ground-state complex between fluorophore and quencher. Here, the average lifetime of the fluorescent species is unaffected, since only the fraction of non-complexed fluorophores contributes to the signal. Deviations from linear behavior in quenching plots often reveal the coexistence of both static and dynamic contributions. A related phenomenon, the so-called sphere-of-action mechanism, occurs when quenchers situated within a defined volume surrounding the fluorophore can instantaneously suppress emission upon excitation. These subtleties underscore the need for careful analysis of both steady-state and time-resolved data to distinguish between quenching modes, especially when proteins contain multiple fluorophores with different solvent exposures.

The mathematical framework for quantifying quenching is typically described using the Stern–Volmer relationship, which provides a quantitative basis for distinguishing dynamic from static contributions and for extracting quenching constants under controlled conditions:

$$\frac{F_0}{F} = 1 + K_{SV}[Q] = 1 + k_q\tau_0 \quad (5)$$

where F_0 and F are the fluorescence intensities in the absence and presence of quencher, $[Q]$ is the quencher concentration, K_{SV} is the Stern–Volmer constant, k_q is the bimolecular quenching rate constant, and τ_0 is the fluorescence lifetime of the fluorophore in the absence of quencher. When k_q exceeds the diffusion-controlled limit in aqueous solution, static or other quenching mechanisms must be invoked. In systems where both dynamic and static quenching coexist, the observed Stern–Volmer plot typically exhibits upward curvature, which can be modelled using modified forms of the equation, including exponential terms or combined linear–quadratic dependencies that account for multiple quenching pathways.

From these quenching experiments, it is possible to extract information not only about the quenching process itself but also about binding equilibria between drugs and proteins. If one assumes that fluorescence quenching is directly proportional to the formation of a protein–ligand complex, binding constants can be derived using logarithmic linearization. A commonly applied modified Stern–Volmer equation, which is particularly useful in systems where fluorophores are heterogeneously exposed or where multiple binding sites contribute differently to the quenching response, is

$$\log\left(\frac{F_0 - F}{F}\right) = \log K_a + n \log [Q] \quad (6)$$

where K_a is the apparent association constant and n the number of binding sites. Or

$$\log \left(\frac{F_0 - F}{F} \right) = n \log K_a - n \log \left[\frac{1}{([L_T] - (F_0 - F)[P_T]/F_0)} \right] \quad (7)$$

where $[L_T]$ and $[P_T]$ are the total ligand and the total protein concentration, respectively. Once K_a is obtained, the dissociation constant follows straightforwardly as $K_d = 1/K_a$. This approach, while widely used, requires caution since not all binding events produce equivalent quenching responses, and partial or allosteric effects can complicate interpretation. Additionally, the derivation of this expression relies on strong and frequently violated assumptions:

- that all binding sites behave identically (equal individual K_a values)
- that ligand binding fully and exclusively abolishes the measured fluorescence (complete quenching of the fluorescent state)
- that intermediate partially-bound species (protein with 1 of n sites occupied) can be neglected.

These simplifications are inapplicable in most realistic protein systems (e.g., serum albumins) and render the fitted parameters ambiguous or misleading. Crucially, the parameter n derived from the double-log transform is not a reliable stoichiometric count but at best resembles a Hill coefficient, reflecting apparent cooperativity rather than true site multiplicity. Two orthogonal artefacts commonly bias quenching assays. First, the inner-filter effect (attenuation of excitation or emission by ligand absorption) reduces observed fluorescence independently of binding; routine but approximate corrections are not always applied or are inadequate at high absorbances, producing spurious apparent binding. Second, most derivations assume the bound complex is non-fluorescent and do not account for residual emission or microenvironment-dependent quantum-yield changes, which further distort fitted parameters [57]. Correcting for these effects, like the inner-filter effect, is essential to ensure that changes in fluorescence truly reflect binding, using the standard equation [54]:

$$F_{corr} = F_{obs} * 10^{\frac{A_{exc} + A_{em}}{2}} \quad (8)$$

where F_{corr} and F_{obs} represent the corrected and observed fluorescence intensities, respectively, and A_{exc} and A_{em} are the absorbances of the system at the excitation and emission wavelengths.

A further dimension of analysis is provided by the temperature dependence of binding constants. By determining K_a at different temperatures and applying the van't Hoff equation, the thermodynamic parameters that govern binding can be extracted.

$$\ln K_a = -\frac{\Delta H}{RT} + \frac{\Delta S}{R} \quad (9)$$

Where ΔH is the enthalpy, ΔS the entropy of the reaction, T the temperature and R the ideal gas constant. From these, the Gibbs free energy change (ΔG) is calculated as

$$\Delta G = \Delta H - T\Delta S \quad (10)$$

The sign and magnitude of these parameters are instructive: negative free energy indicates spontaneity, while the balance of enthalpic and entropic contributions provides insight into the forces stabilizing the complex. Exothermic, enthalpy-driven binding often reflects hydrogen bonding and electrostatic interactions, whereas entropy-driven binding, particularly with positive ΔS points toward hydrophobic effects and the release of ordered water molecules from the binding interface. However, there are critical caveats and limitations in the Van't Hoff analysis: if the K_a values are themselves affected by the methodological artefacts discussed above, such as inner-filter effects, incomplete quenching, or inappropriate linearization, the resulting ΔH and ΔS are physically meaningless. Even small systematic errors in K_a at each temperature, well within nominal experimental scatter, can propagate into large and inconsistent apparent enthalpy or entropy terms. Moreover, fluorescence intensities are inherently temperature-dependent due to factors unrelated to binding, including temperature-induced changes in quantum yield, solvent relaxation dynamics, and subtle perturbation of protein tertiary-structure. Highlighting these limitations on the calculation of K_a and thermodynamic parameters using fluorescence quenching, such data should never be interpreted without independent validation by isothermal titration calorimetry (ITC) or other calorimetric methods (see Paragraph 3.1.5) [57].

Likewise, complementing steady-state measurements with lifetime data is critical for distinguishing between dynamic and static mechanisms. When combined with binding isotherm analysis and thermodynamic evaluation, fluorescence quenching emerges as a powerful, multifaceted tool for elucidating the nature of drug-protein interactions, yielding insights that range from microscopic quenching kinetics to the fundamental thermodynamic forces underlying molecular recognition [58], [59].

Competitive Site Marker Displacement Experiments

Fluorescence spectroscopy is widely employed to investigate the binding characteristics of small molecules to serum albumins, particularly because the intrinsic fluorescence of tryptophan residues provides a sensitive probe of changes in the microenvironment surrounding the binding sites. For example, when a ligand interacts with bovine serum albumin (BSA), alterations in fluorescence intensity or wavelength shifts can be observed, which reflect quenching processes arising from either static complex formation or dynamic collisional encounters. However, intrinsic fluorescence quenching alone does not unequivocally reveal the precise location of the binding site. To address

this limitation, competitive site marker displacement experiments are commonly performed. This approach relies on the use of well-characterized ligands, such as warfarin and diazepam, which selectively bind to Sudlow's site I and site II, respectively, to probe the competitive behavior of the drug under study and to dissect site-specific affinities that cannot be resolved through intrinsic fluorescence alone.

In such experiments, the protein is incubated with a site marker and subsequently titrated with the drug of interest. If the marker's fluorescence signal is significantly perturbed, or if the quenching behavior of the drug is diminished in the presence of the marker, this indicates competition for the same binding pocket. Conversely, minimal changes suggest that the drug binds at an alternative site. For example, in the case of aspirin–BSA interactions, the presence of diazepam leads to a pronounced reduction in binding affinity compared to marker-free conditions, demonstrating that aspirin occupies site II as its principal binding location. The smaller effect of warfarin implies a weaker, secondary interaction at site I. These displacement patterns provide a powerful means of mapping the distribution of ligands across the available binding regions of albumin, thereby revealing not only the preferred site but also the potential for multi-site occupancy or overlapping allosteric effects that can influence co-administered drugs [60].

3.1.3. 3D and Synchronous Fluorescence Spectroscopy

Synchronous and three-dimensional (3D) fluorescence spectroscopy provide complementary approaches for probing the structural consequences of drug–protein interactions with high sensitivity. In synchronous fluorescence, excitation and emission wavelengths are scanned simultaneously with a constant interval ($\Delta\lambda$), allowing selective monitoring of specific chromophores. When $\Delta\lambda$ is set at 15 nm, the spectral response is dominated by tyrosine residues, while at $\Delta\lambda = 60$ nm the signal primarily reflects tryptophan. This spectral discrimination enables a detailed examination of microenvironmental changes around these residues upon drug binding. For instance, a decrease in fluorescence intensity without a notable shift in wavelength suggests that ligand association perturbs the internal packing of the protein rather than altering the polarity of the immediate environment of tyrosine or tryptophan side chains, indicating subtle tertiary-structure adjustments rather than large-scale polarity changes. Such findings highlight the subtlety with which small molecules can influence protein tertiary structure while leaving local residue polarity largely intact.

Three-dimensional fluorescence spectroscopy offers a broader mapping of protein conformational changes by simultaneously recording excitation–emission matrices. This approach provides multiple characteristic peaks, including those associated with Rayleigh scattering and with aromatic amino

acid transitions ($\pi \rightarrow \pi^*$) as well as polypeptide backbone transitions ($n \rightarrow \pi^*$). While the scattering peaks typically remain unaffected, variations in the intensities of peaks corresponding to aromatic residues and the peptide backbone yield insights into conformational rearrangements. A marked reduction in the intensities of these peaks upon drug binding indicates modifications in backbone polarity, hydrophobicity, or tertiary packing surrounding the tryptophan residues. In contrast, the absence of peak shifts may suggest that the overall spatial distribution of the chromophores remains unaltered, even as local environments undergo significant perturbation, providing a nuanced distinction between global structural stability and local microenvironmental reorganization.

Together, synchronous and 3D fluorescence techniques create a multidimensional picture of drug–protein interactions. Whereas synchronous fluorescence highlights residue-specific effects on tyrosine and tryptophan, 3D fluorescence captures global conformational alterations of the protein matrix, yielding a comprehensive spectroscopic fingerprint of ligand-induced structural changes [60].

Instrumentation

Both 3D and synchronous fluorescence measurements are conducted using advanced spectrofluorometers, which incorporate dual monochromators under precise software control. The hardware setup is similar to that used for steady-state fluorescence but with additional automation and data-processing capabilities:

- For 3D spectroscopy, the software coordinates sequential scanning of excitation and emission monochromators to generate the matrix dataset, often with correction algorithms applied to account for instrumental artifacts, lamp intensity fluctuations, and detector sensitivity, ensuring accurate reconstruction of excitation-emission landscapes.
- For synchronous fluorescence, the system enforces the constant wavelength difference ($\Delta\lambda$) between excitation and emission monochromators throughout the scan, allowing selective enhancement of residue-specific spectral features while suppressing overlapping background signals.

3.1.4. Circular Dichroism (CD) Spectroscopy

Circular dichroism (CD) spectroscopy is a rapid, sensitive optical technique that reports on molecular chirality and the chiral arrangement of chromophores. In biomolecular science it is used routinely to

- identify and quantify protein secondary structure,
- follow folding–unfolding equilibria and extract thermodynamic parameters,

- characterize nucleic-acid conformations and nucleic-acid–ligand binding modes
- monitor structural changes that occur upon adsorption to or interaction with nanoparticles or other supermolecular assemblies.

CD arises because chiral molecules absorb left- and right-circularly polarized light (Figure 3.4) to different extents. If $A_L(\lambda)$ and $A_R(\lambda)$ are the absorbances for left and right circular polarizations at wavelength λ , the CD signal is the difference:

$$\Delta A(\lambda) = A_L(\lambda) - A_R(\lambda) \quad (11)$$

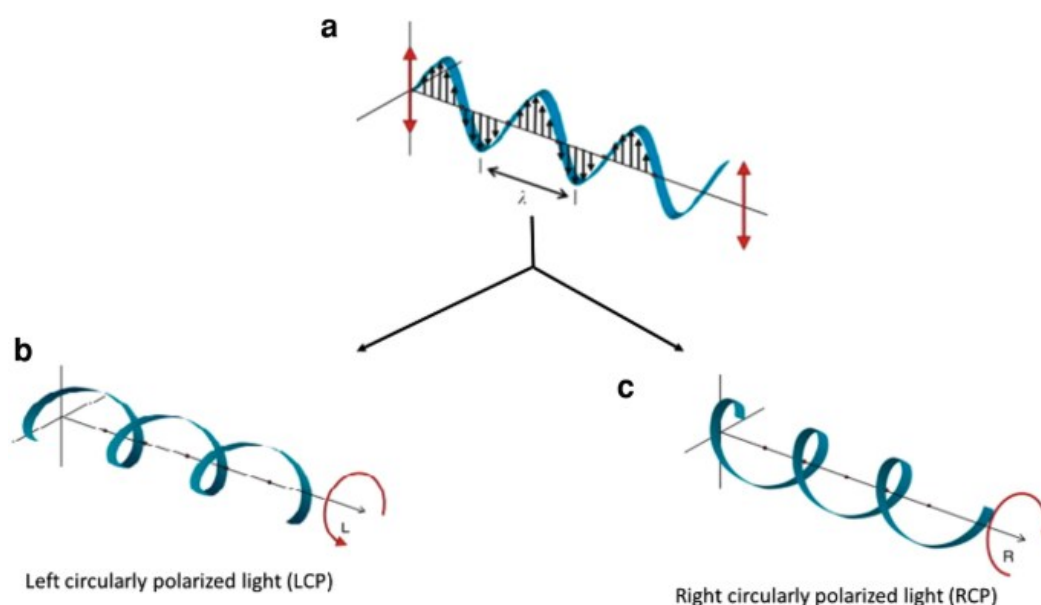


Figure 3.4. Representation of circularly polarized light where the helix represents the path followed by the component electric field of a light beam. **(b)** Left polarized light. **(c)** Right polarized light [61].

This differential absorption encodes how chromophores are arranged in 3D space, making CD exquisitely sensitive to light conformational rearrangements in global or local molecular conformation.

Most biomolecular chromophores (peptide bonds, aromatic side chains, nucleobases) are achiral in isolation but become optically active when placed in a chiral environment (for example when arranged into a helix or stacked). The difference ΔA is typically very small (parts in 10^{-3} to 10^{-5}) but modern instruments measure it with high precision. In practice instruments report ellipticity $\theta(\lambda)$ (usually in millidegrees, mdeg) calculated as follows:

$$\theta = 2\pi 180 * \Delta A \quad (12)$$

This representation is convenient because ellipticity directly reflects the amplitude of chiral absorption, making even minute variations in secondary structure readily detectable.

For comparative and quantitative analysis, particularly in protein structural studies, it is customary to convert ellipticity into molar ellipticity $[\theta]$, which normalizes the signal with respect to concentration and path length:

$$[\theta] = \frac{10\theta_{obs}}{clM} \quad (13)$$

where θ_{obs} is the observed ellipticity (in degrees), M is the molar mass of the solute (g mol^{-1}), c is the concentration (g mL^{-1}), l is the optical path length (cm).

In protein studies, a further refinement is the mean residue ellipticity (MRE), which accounts for the number of amino acid residues (N) in the polypeptide chain:

$$[\theta]_{MRE} = \frac{10\theta_{obs}}{clN} \quad (14)$$

where $[\theta]_{MRE}$ is expressed in $\text{deg}\cdot\text{cm}^2\cdot\text{dmol}^{-1}$ per residue. This normalization enables direct comparison of CD spectra across proteins of different sizes and concentrations, making it possible to derive quantitative estimates of secondary structure content by fitting spectra to reference databases [61], [62].

Instrumentation

CD spectroscopy requires specialized instruments known as spectropolarimeters (Figure 3.5), which combine elements of conventional absorption spectroscopy with optical components designed to generate and detect circular polarization. The core components include:

1. **Light Source.** A xenon arc lamp is typically employed due to its high intensity and broad spectral output spanning the UV and visible regions, providing sufficient photon flux for far-UV measurements where CD signals are weakest.
2. **Polarization Modulation System.** A photoelastic modulator or linear polarizer with a quarter-wave plate is used to convert linearly polarized light into alternating left- and right-circularly polarized components. This rapid modulation is essential for isolating true chiral absorption from background noise or instrumental drift.
3. **Sample Compartment.** Samples are placed in quartz cuvettes with path lengths typically between 0.1 and 1.0 mm, necessary due to the strong absorption of biological samples in the

far-UV range. For highly absorbing systems, shorter path lengths are essential to avoid saturation and ensure adequate signal detection while maintaining linearity of the CD response.

4. **Detector.** High-sensitivity photomultiplier tubes (PMTs) are most commonly used, as CD signals are often several orders of magnitude weaker than conventional absorbance. Their high gain and low noise make them ideal for detecting millidegree-level ellipticity changes.
5. **Temperature Control Systems.** Since CD is frequently applied to study conformational stability and unfolding transitions, precise temperature regulation (via Peltier devices or circulating water baths) is often integrated, ensuring reproducibility and enabling acquisition of thermal denaturation profiles under controlled conditions.

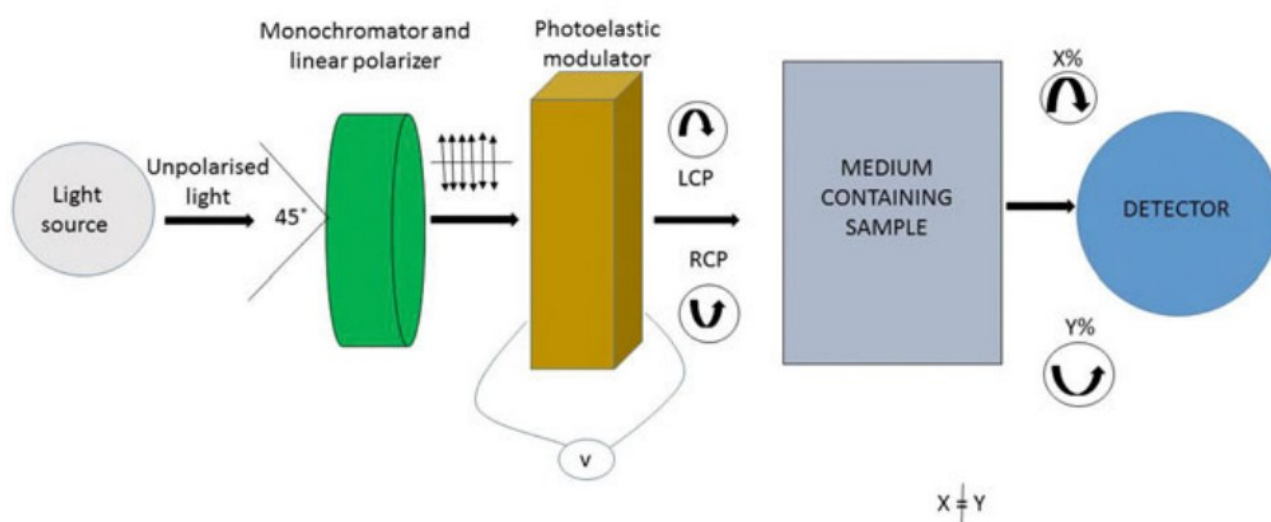


Figure 3.5. Representation of instrumentation of a CD spectroscope [61].

Modern instruments are coupled with advanced software for data acquisition, baseline correction, and deconvolution analysis, allowing quantitative estimation of secondary structural content and enabling reliable comparison of spectra across different experimental conditions or protein variants.

Protein secondary-structure determination by CD

Far-UV CD (≈ 190 – 250 nm) probes peptide-bond $\pi \rightarrow \pi^*$ and $n \rightarrow \pi^*$ transitions; regular secondary structures produce characteristic spectral shapes: the canonical α -helix has strong negative bands near 222 and 208 nm and a positive band near 190–195 nm, β -sheet gives a negative band near ≈ 218 nm and a positive band near ≈ 195 – 200 nm, and random coil shows a small negative feature around 195 nm (Figure 3.6). These spectral signatures provide a distinctive fingerprint for each secondary-structure motif and form the basis for quantitative deconvolution algorithms.

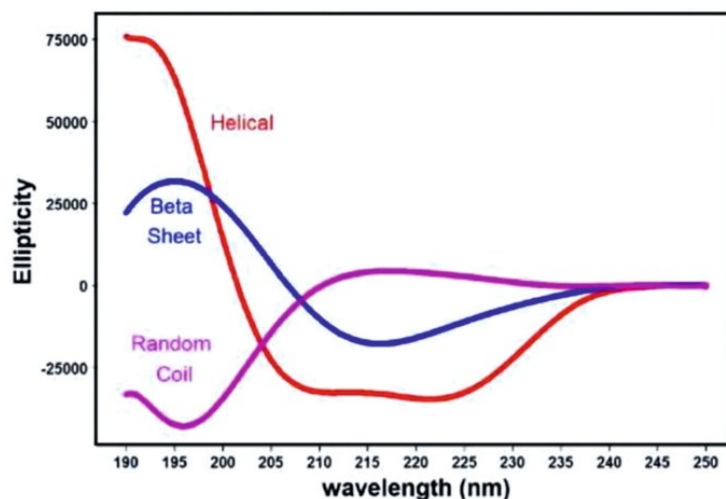


Figure 3.6. Far-UV CD spectra of proteins with α -helix (red) β -sheet (blue) and random (pink) structure in predominance [61].

Because CD reports an average over all chromophores, the spectrum provides the fractional composition of structural motifs rather than residue-level resolution. Nonetheless, even modest shifts in band intensity can reveal conformational changes induced by ligand or cofactor binding, offering insights into allosteric regulation and drug–protein interactions.

The secondary structural parameters of proteins were determined by analyzing the CD spectra through the CD Multivariate SEE program with standard calibration models, while in some cases, using the mean residue ellipticity ($[\theta]_{\text{MRE}}$). The α -helical content was further estimated from ($[\theta]_{\text{MRE}}$ values at 208 nm using the equation:

$$\% \alpha - \text{helix} = \frac{\text{MRE}_{208} - 4000}{33000 - 4000} * 100 \quad (15)$$

where 4000 is the MRE of β -form/random coil structures and 33000 corresponds to a pure α -helical conformation at 208 nm [62]. This empirical relation provides a convenient first-order estimate of helical content, particularly when full spectral deconvolution is impractical.

Thermal stability and denaturation from CD

Thermal stability is assessed using CD by following changes in the spectrum with increasing temperature. In some cases, the entire spectrum in the far- or near-UV CD region can be followed at several temperatures. Alternatively, a single wavelength can be chosen which monitors some specific feature of the protein structure, and the signal at that wavelength is then recorded continuously as the temperature is raised. CD is often used to assess the degree to which solution pH, buffers, and additives such as drugs, sugars, amino acids or salts alter the thermal stability and unfolding pathway. By following changes over the entire far-UV CD region or a specific wavelength (e.g. 222 nm or 208 nm for α -helix), it can be determined whether at high temperatures the protein is losing all its secondary structure, loses only a portion of its secondary structure, or simply undergoes

conformational change involving a change in secondary structure. In some cases, unfolding is not merely a loss of structure but involves transitions to well-defined alternative conformational states. Occasionally, the unfolded form of a protein will possess a defined, but totally different secondary structure than the native form [63].

Determination of nucleic acid conformations and nucleic acid–ligand interactions

CD spectroscopy is a powerful method for distinguishing and monitoring the conformations of nucleic acids, as well as their interactions with ligands. In the 200–320 nm region, the canonical double-helical forms of DNA and RNA display distinctive CD signatures that allow their identification. Both A-RNA and A-DNA share similar overall shapes, with A-RNA exhibiting a maximum near 260 nm, a minimum around 210 nm, and a small negative band near 290–300 nm. A-DNA, in contrast, displays its principal maximum at 270 nm, a minimum near 210 nm, and negligible signal beyond 300 nm. B-DNA is characterized by a conservative spectrum with nearly symmetric positive (≈ 275 nm) and negative (≈ 245 nm) components centered around 260 nm, although the magnitude of its maximum is less than half that of A-DNA. The left-handed Z-DNA exhibits a conservative couplet above 240 nm, consisting of a strong negative band near 290 nm and a positive band around 260 nm.

Vacuum-UV CD studies further discriminate right- and left-handed helices. Right-handed helices (A-DNA, B-DNA, A-RNA) show a pronounced positive band near 186 nm and a strong negative signal below 180 nm. Conversely, left-handed helices (Z-DNA, Z-RNA) exhibit an intense negative band at 190–195 nm, a crossover at 184 nm, and a positive peak below 180 nm. Computational and experimental analyses confirm that below 220 nm, right-handed duplexes display a positive CD couplet, whereas left-handed duplexes show the opposite sign. Consequently, CD measurements in the 170–220 nm range provide a reliable spectroscopic signature of the handedness of nucleic acid helices.

CD spectroscopy is also highly sensitive to environmental perturbations that alter nucleic acid structure. Changes in temperature, solvent composition (e.g., alcohols), or ionic strength often induce measurable decreases in specific spectral bands, such as the 275 nm feature characteristic of B-DNA. Notably, condensed DNA in biological assemblies such as bacteriophage, adenovirus, chromatin, and nucleosomes frequently exhibits a spectrum distinct from canonical B-form DNA, often referred to as the “C-form,” suggesting that CD can capture conformational states relevant to genome packaging and regulation and providing a window into higher-order structural organization.

Beyond conformational analysis, CD provides a direct means to investigate nucleic acid–ligand interactions. Small molecules typically bind DNA either by intercalation between base pairs or by insertion into the major or minor grooves. Many of these ligands are achiral but acquire optical

activity upon binding, giving rise to induced CD (ICD) signals. Acridine orange exemplifies this phenomenon. Free acridine orange is optically inactive; however, upon binding to DNA it exhibits strong ICD bands whose nature depends on the binding mode. At low dye-to-DNA ratios, intercalative binding aligns the aromatic chromophore parallel to base pairs, generating a negative CD signal with a minimum ΔA of approximately $-8 \text{ M}^{-1}\text{cm}^{-1}$. At higher binding ratios, groove binding dominates, leading to positive CD bands up to $+30 \text{ M}^{-1}\text{cm}^{-1}$. At still higher occupancies, exciton coupling between adjacent bound dyes produces more complex positive–negative patterns. These results underscore the capacity of CD to distinguish binding modes and to report on cooperative or aggregation effects arising from ligand–ligand interactions on the DNA template [62].

Circular Dichroism for Nanostructural Analyses and Biomolecule-Nanoparticle Interactions

CD spectroscopy provides valuable insights into the conformational behavior of biomolecules adsorbed on nanoparticle surfaces and encapsulated within nanocarriers. While CD alone cannot definitively determine whether a biomolecule retains full bioactivity, it allows quantitative assessment of structural changes such as partial denaturation by comparing the spectra of native and surface-bound species. For example, plasma proteins, including bovine serum albumin (BSA) and human fibrinogen (FNG), show reduced α -helical content upon adsorption onto nanopolymer surfaces such as poly(2-methoxyethylacrylate) (PMEA), PHEMA, and poly(2-ethylhexylacrylate) (PEHA). The α -helical content of BSA decreased from 51% in solution to 37%, 15%, and 8% on PMEA, PHEMA, and PEHA surfaces, respectively, demonstrating the sensitivity of CD to surface-induced conformational changes and to variations in polymer hydrophobicity [64].

Similarly, the interaction of DNA with functionalized nanoparticles can be probed by CD. Mixed monolayer-functionalized gold nanoparticles, widely studied as DNA-regulating molecules and transfection vectors, can significantly alter the CD signal of DNA. Cationic nanoparticles, such as amine-terminated NP_L-Phe, induce denaturation as evidenced by decreased ellipticity at 280 nm. Hydrophobic nanoparticles with aromatic side chains (e.g., NP_L-Trp, NP_L-Phe) promote more pronounced DNA unwinding through π – π stacking interactions with DNA bases, whereas hydrophilic nanoparticles with guanidinium groups (e.g., NP_L-Arg) perturb DNA more than lysine-terminated nanoparticles due to the formation of stable hydrogen bonds with guanine residues [65]. Together, these patterns reveal how side-chain chemistry and surface functionality govern the balance of electrostatic, hydrophobic, and hydrogen-bonding forces at the nano–bio interface, underscoring CD's capacity to differentiate the molecular mechanisms of nanoparticle-DNA interactions.

3.1.5. Isothermal Titration Calorimetry (ITC)

Isothermal titration calorimetry (ITC) is one of the most powerful and direct techniques for the quantitative characterization of molecular interactions. Unlike spectroscopic or fluorescence-based methods, which infer binding indirectly through changes in optical properties, ITC directly measures the heat exchanged during molecular association in real time. This thermodynamic approach provides a complete description of the energetics of binding, including the equilibrium dissociation constant (K_d), binding stoichiometry (n), enthalpy change (ΔH), entropy change (ΔS), and Gibbs free energy change (ΔG), offering a uniquely comprehensive energetic fingerprint of the interaction.

The fundamental principle of ITC is the detection of heat released or absorbed when a ligand binds to a macromolecule under isothermal conditions. Binding is inherently accompanied by changes in non-covalent interactions, such as hydrogen bonding, van der Waals forces, electrostatic interactions, and hydrophobic effects, as well as solvent reorganization. These processes are energetically manifested as measurable heat changes, which ITC detects without the need for labels or secondary probes, making it one of the few truly label-free and solution-phase techniques available.

The thermodynamic signature of binding is captured by the relation:

$$\Delta G = -RT\ln(K_d) = \Delta H - T\Delta S \quad (16)$$

where R is the gas constant, T is the absolute temperature, ΔG is the Gibbs free energy change, ΔH is the enthalpic contribution, ΔS is the entropic contribution and K_d is the dissociation constant. This equation illustrates how ITC not only provides the affinity of binding but also decomposes the free energy change into enthalpic and entropic contributions, thereby distinguishing between different modes of molecular recognition (enthalpy-driven vs. entropy-driven interactions) and offering mechanistic insight unavailable from purely spectroscopic methods.

The reliability of ITC measurements depends critically on experimental design. Protein and ligand solutions must be highly pure, accurately quantified, and dissolved in identical buffer conditions to minimize artefacts from dilution or buffer mismatch. It is generally recommended to dialyze the protein extensively and dissolve the ligand in the final dialysis buffer. Degassing of all solutions is essential to prevent bubble formation, which interferes with heat transfer and syringe performance. The choice of buffer requires particular attention. Binding processes often involve protonation or deprotonation events, meaning that the observed enthalpy (ΔH_{obs}) is the sum of intrinsic binding enthalpy and buffer ionization enthalpy. Performing the same experiment in buffers of different ionization enthalpies allows correction for this effect and, at the same time, provides valuable mechanistic information on proton transfer coupled to binding and on the role of proton-linked equilibria in modulating affinity. Another central parameter in ITC design is the so-called Wiseman

c-value ($c = n[\text{protein}]K_a$). The shape of the binding isotherm, and hence the ability to extract accurate binding constants, depends on choosing concentrations that yield c-values between approximately 10 and 100 for medium affinities, or between 5 and 500 more generally. For strong interactions (nanomolar affinity), protein concentrations must be reduced to avoid isotherms that resemble step functions, while weak interactions require higher concentrations to generate detectable heat signals. Typical protocols employ protein concentrations in the micromolar range in the calorimetric cell, with ligand concentrations 5–10 times higher in the injection syringe. Small, repeated injections ensure a gradual approach to the saturation of binding sites and improve the fidelity of the integrated heat profile, producing a sigmoidal binding curve from which K_a , ΔH , and n can be extracted. Control experiments are indispensable for accurate interpretation. Heat changes measured in ITC reflect not only binding but also ligand dilution, buffer mixing, and ionization effects. Therefore, titrations of ligand into buffer and buffer into protein are generally performed and subtracted from the binding data. Moreover, the first injection is often excluded from analysis, as mixing and diffusion during equilibration tend to produce anomalously low heat signals and do not reflect true binding thermodynamics [66], [67].

The scope of ITC spans virtually all fields of molecular and structural biology, chemistry, and pharmaceutical sciences, but in particular the characterization of protein–ligand interactions, self-assembly processes and nanoparticles-biomolecules will be highlighted given their complex energetic landscapes and the unique ability of ITC to quantify them directly.

Instrumentation

An ITC experiment is performed by titrating a ligand solution into a calorimetric cell containing the macromolecule of interest, while maintaining constant temperature. As shown in Figure 3.7, the instrumentation consists of:

- Two highly insulated cells: a sample cell containing the macromolecule and a reference cell filled with water.
- Precision thermal control system: keeps both cells at constant temperature, typically within $\pm 0.1 \mu\text{K}$, ensuring exceptional baseline stability.
- Automated microsyringe: delivers small aliquots (1–10 μL) of ligand solution into the sample cell with high volumetric accuracy and reproducibility.
- Feedback system: detects the differential power required to maintain thermal equilibrium between sample and reference cells, which constitutes the primary measured ITC signal.

The observed signal is a series of power peaks corresponding to heat changes after each ligand injection. These peaks are integrated to yield the molar heat change (ΔQ) per injection, producing a binding isotherm when plotted against the molar ratio of ligand to macromolecule. Non-linear regression analysis of the resulting isotherm allows extraction of the key thermodynamic parameters described above.

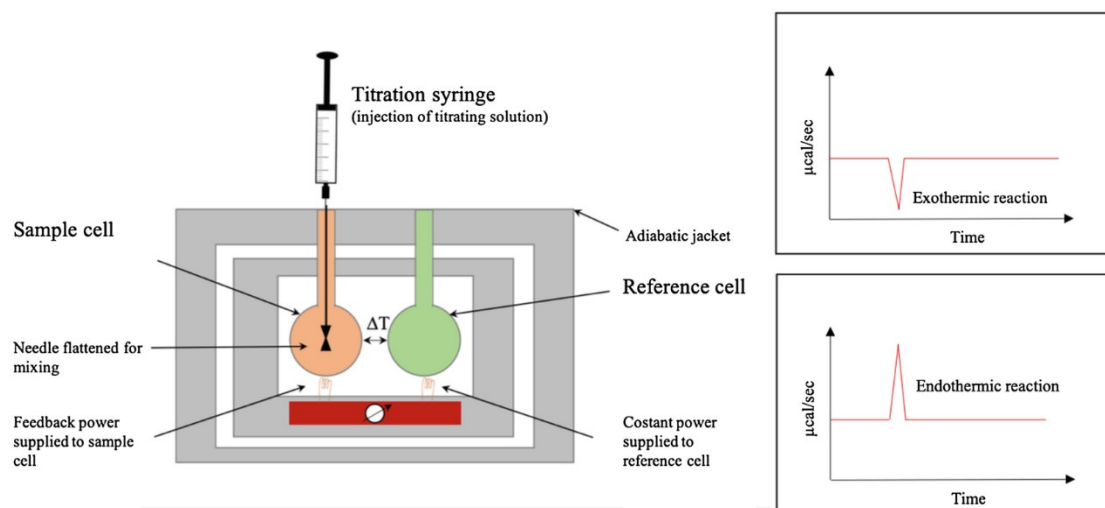


Figure 3.7. Schematic representation of an ITC instrument [68].

Protein/DNA–ligand interactions with ITC and complementarity with FS

In the warfarin–albumin study, ITC titrations were performed with HSA or BSA placed in the calorimetric cell and titrated with warfarin solutions prepared in the same HEPES buffer. Careful attention was given to sample preparation, degassing, and the inclusion of appropriate control titrations (ligand into buffer, buffer into protein) to correct for dilution and mixing heats. The resulting calorimetric curves revealed binding constants on the order of 10^4 – 10^5 M^{-1} , with binding stoichiometries close to unity for HSA and somewhat higher for BSA. Importantly, the enthalpy changes were consistently negative (≈ -4 to -6 kcal mol^{-1}), indicating that complex formation is predominantly enthalpy-driven, likely reflecting hydrogen bonding and electrostatic interactions within the Sudlow I site.

Fluorescence spectroscopy served as a complementary method in this context. While ITC measures the global energetics of the system, FS provides a local and residue-sensitive probe of changes in the environment of intrinsic tryptophan residues in albumins, especially Trp-214 in HSA and Trp-134/213 in BSA. By monitoring the quenching of protein fluorescence upon warfarin binding, FS confirmed the formation of a static complex and yielded binding constants in excellent agreement with those obtained calorimetrically. The use of synchronous fluorescence measurements minimized interference from warfarin's own fluorescence, allowing reliable estimation of binding affinities.

Furthermore, FS studies across different temperatures enabled van't Hoff analyses that produced enthalpy values consistent with ITC, thus reinforcing the robustness of the thermodynamic interpretation.

The combination of ITC and FS thus emerges as particularly powerful. ITC provides the complete thermodynamic signature of binding (affinity, stoichiometry, enthalpy and entropy) while FS offers high-sensitivity confirmation of complex formation and local structural perturbations around specific residues. When both approaches converge, as in the case of warfarin–albumin, confidence in the measured binding parameters is substantially increased. At the same time, discrepancies between global calorimetric signatures and local fluorescence responses can highlight the existence of multiple binding sites or higher-order interactions, which in this system were later confirmed by frontal analysis capillary electrophoresis [69].

ITC can play also a significant role in the quantification of the interaction between a drug and DNA: for example, the case of distamycin A, its carbamoyl derivatives, and DNA quadruplex structures. ITC provided not only a measure of the strength of the binding, with equilibrium constants in the range of 10^5 – 10^6 M⁻¹, but also insights into the driving forces underlying complex formation. The study revealed that distamycin A binds the model quadruplex [d(TGGGGT)]₄ with markedly different behavior depending on the ionic environment. In K⁺ buffer, ITC data indicated two binding events, with stoichiometries of 2:1 and 4:1 (drug/quadruplex) and Gibbs free energies of approximately –32 and –37 kJ/mol, respectively. By contrast, in Na⁺ buffer the interaction was weaker, with a single 1:1 binding event and ΔG° around –30 kJ/mol. These differences illustrate how the coordination of cations within the G-quadruplex core influences the accessibility and mode of ligand binding. Importantly, ITC also distinguished between enthalpic and entropic contributions to binding. For distamycin A, binding to [d(TGGGGT)]₄ in K⁺ was associated with a small favorable enthalpy and a large positive entropy term, indicating that the interaction is predominantly entropy driven, as expected for groove-binding ligands. ITC in this study proved indispensable for defining not only how strongly small molecules interact with quadruplex DNA but also how buffer composition, quadruplex sequence, and ligand structure reshape the thermodynamic landscape of binding [70].

Monitoring self-assembly processes with ITC

ITC provides a direct and quantitative means to monitor self-assembly phenomena of amphiphilic molecules, offering thermodynamic resolution that is difficult to obtain with other techniques. When applied to micellization, ITC measures the heat exchanged during a controlled dilution (or demicellization) experiment in which a concentrated suspension of preformed micelles is titrated into solvent. Because micelle formation is reversible, the calorimeter, by recording the differential power

required to maintain isothermal conditions after each injection, reports the energetics of the underlying processes: monomer dilution, micelle dissociation and re-formation, and micelle dilution once the system is driven well above the critical micelle concentration (CMC). Integration of the heat transients for each injection yields an integrated heat curve (Q versus concentration) that contains the complete thermodynamic signature of the self-assembly transition.

A typical demicellization thermogram exhibits three regions (Figure 3.8). At low cumulative concentration in the calorimetric cell, each injection predominantly causes micelle dissociation and monomer dilution, producing a characteristic heat signal. As the concentration approaches the CMC the heat per injection changes markedly and the integrated curve adopts a sigmoidal profile; the inflection point of this transition corresponds to the CMC. Beyond the CMC the heat per injection again levels off, reflecting only the dilution of micellar species. This characteristic progression initial dissociation, cooperative transition, and high-concentration dilution provides a clear thermodynamic hallmark of micellization that is difficult to access by other methods.

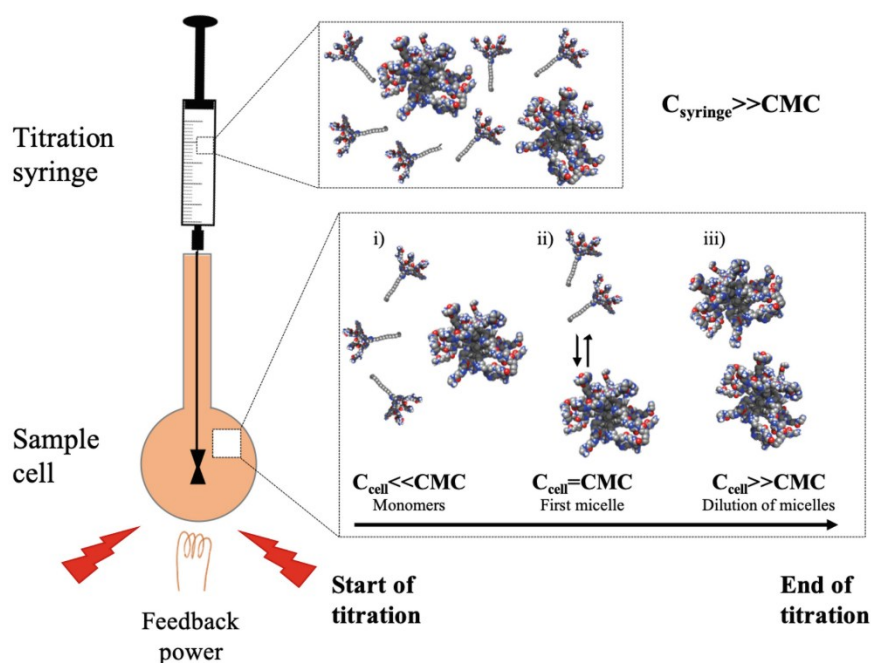


Figure 3.8. Schematic illustration of micellization events during an ITC experiment [68].

Practically, reliable determination of the CMC from ITC data is achieved by fitting the integrated Q versus amphiphile concentration profile (typically using a Boltzmann sigmoidal function) and locating the midpoint of the transition or the maximum of the first derivative dQ/dC (Figure 3.9). In the example reported in literature for the PAMAM-based amphiphile ACD1, fitting the integrated calorimetric data produced an ITC-derived CMC of $\approx 11.2 \mu\text{M}$; this value was independently confirmed by a pyrene fluorescent probe assay ($\text{CMC} \approx 9.96 \mu\text{M}$).

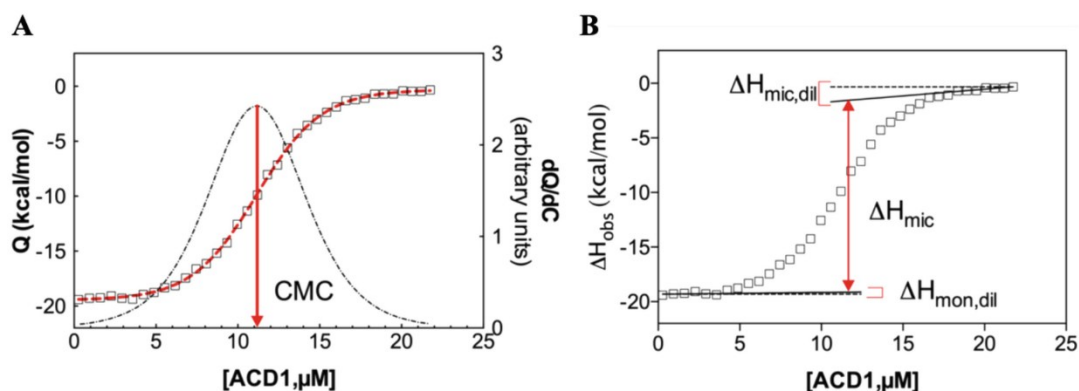


Figure 3.9. (a) Integrated isothermal calorimetry profile of ACD1 fitted with a Boltzmann sigmoidal function (red dotted line, $R^2 = 0.9987$) and the corresponding first derivative of the curve. The CMC is taken as the x-value at the maximum of dQ/dC curve (indicated by the red arrow). (b) Determination of the ΔH_{mic} obtained from the integrated ITC data [68].

Beyond locating the CMC, ITC also yields the micellization enthalpy (ΔH_{mic}). Because the experiment measures the reverse process (demicellization), the observed enthalpy components must be carefully separated: the total observed enthalpy (ΔH_{obs}) contains contributions from monomer dilution, micelle dilution and the micellization/demicellization process itself. In practice ΔH_{mic} is obtained by taking the difference between the extrapolated pre- and post-transition plateaus of the sigmoidal transition. For ACD1 the protocol reported an observed micellization enthalpy of $18.67 \text{ kcal mol}^{-1}$ (expressed here as the demicellization counterpart), with small monomer/micelle dilution contributions (reported in the protocol as ≈ -0.042 and $-0.035 \text{ kcal mol}^{-1}$). These values are then used as inputs for subsequent mass-action analysis and estimation of secondary thermodynamic parameters.

Interpretation of ITC self-assembly data requires an underlying thermodynamic model. Two common approaches are the pseudo-phase separation model (PPM), which treats micellization as a phase-like transition between monomers and a bulk micellar phase, and the mass-action model (MAM), which explicitly treats micelles as finite aggregates of defined aggregation number and accounts for counterion binding. While PPM can be adequate for large, classical micelles, the MAM is preferable for small aggregates or ionic amphiphiles because it explicitly incorporates aggregation number (N_{agg}) and counterion effects. Using the MAM and a regression approach that minimizes the sum of squared residuals between observed and predicted heats, one can estimate N_{agg} from the calorimetric data. For the ACD1 example the in-house analysis yielded an average aggregation number $N_{agg} \approx 10.1$, a value consistent with a small, well-defined micellar assembly.

Ionic amphiphiles also require explicit treatment of counterion condensation because the surface charge of the micelle alters the free energy of micellization. The degree of counterion binding (β) can

be determined experimentally by measuring the CMC as a function of added counterion concentration and applying the Corrin–Harkins relationship: a plot of $\ln(\text{CMC})$ versus $\ln([\text{counterion}])$ is linear, and the slope provides β . Applying this protocol to ACD1 in NaCl solutions produced $\beta \approx 0.65$, indicating substantial but incomplete counterion association at the micellar surface and a non-negligible electrostatic contribution to the self-assembly thermodynamics.

With CMC, N_{agg} and β in hand, the full thermodynamic characterization can be completed by calculating the standard free energy of micellization and its entropic component. Using a refined mass-action formalism for ionic micelles, ΔG_{mic} can be derived from the CMC, N_{agg} and β , and the entropy term obtained from the Gibbs relation $T\Delta S_{\text{mic}} = \Delta H_{\text{mic}} - \Delta G_{\text{mic}}$. For the ACD1 system these calculations led to a micellization process that is overall favorable and predominantly entropy-driven, a typical signature when hydrophobic desolvation and release of structured water and/or counterions dominate the free energy balance [68].

Protein Corona Characterization with ITC

The interaction of nanoparticles with biological systems is profoundly shaped by the formation of the protein corona, a dynamic layer of biomolecules adsorbed onto the nanoparticle surface upon exposure to physiological environments. This corona determines critical parameters such as circulation time, cellular uptake, biodistribution, and toxicity, thereby dictating the ultimate biological identity of the nanoparticle. While much research has concentrated on the effects of nanoparticle size, charge, and morphology, the influence of surface porosity and roughness has remained relatively underexplored. To address this gap, recent work has highlighted the potential of ITC as a key analytical tool to investigate nanoparticle–protein interactions in the context of mesoporous silica nanoparticles (MSNs).

In the case of MSNs, ITC experiments revealed that protein adsorption is not a single binding event but rather a sequential process. Initially, proteins loosely interact with the nanoparticle surface, driven primarily by entropic contributions arising from the release of structured interfacial water molecules. Subsequently, more stable interactions occur as proteins undergo conformational rearrangements that allow them to fit into the pores, a process accompanied by distinctly exothermic transitions. These observations suggest that pore size plays a decisive role in stabilizing adsorbed proteins and shaping the composition of the corona. The capacity of ITC to distinguish these thermodynamic phases provides valuable mechanistic insights. For example, nanoparticles lacking pores display only the initial endothermic interaction, confirming that the exothermic stabilization step is directly linked to protein accommodation within mesopores. This clear differentiation between surface adsorption and pore-mediated binding underscores the utility of ITC in disentangling the multiple contributions to protein corona architecture [71].

3.1.6. Transmission Electron Microscopy (TEM)

Transmission Electron Microscopy (TEM) represents one of the most transformative innovations in modern science, shaping fields from cell biology to materials chemistry. Since its invention by Ernst Ruska in 1931, TEM has provided unparalleled access to structural information at scales ranging from the micrometer down to the angstrom level. However, this advantage is counterbalanced by the strong interaction of electrons with matter, which produces both useful elastic scattering and damaging inelastic scattering. For soft, hydrated systems, this means that radiation sensitivity, vacuum requirements, and limited contrast are persistent challenges that must be addressed through both careful sample preparation and imaging strategies [72].

Sample preparation remains decisive. An important adjunct in many TEM workflows is staining with uranyl acetate, a heavy-metal salt that binds to a wide range of biomolecules, including proteins, lipids, and nucleic acids, increasing electron density and thus enhancing contrast in thin sections. It can be used in *en bloc* staining (before embedding and sectioning) or applied post-section to ultrathin slices. When used properly, uranyl acetate improves the visibility of fine ultrastructural detail, particularly in regions that would otherwise produce weak contrast under phase-contrast TEM. However, there are trade-offs: uranyl acetate is toxic, radioactive, light-sensitive, and prone to forming precipitates, which can obscure or distort structure if not carefully controlled. Protocols often include steps to avoid precipitation (freshly prepared solutions, protected from light, proper pH control), and to remove precipitates (for example, brief washes with diluted oxalic acid) without damaging sample detail. Moreover, legal and safety regulations may limit its use, and some labs explore non-radioactive alternatives or substitutes in staining [73], [74]. Furthermore, traditional drying or staining methods can introduce structural artifacts, while vitrification by cryo-fixation preserves samples in their near-native state and enables high-resolution imaging.

Instrumentation

A modern TEM consists of the following core components (Figure 3.10):

1. Electron source: typically, a thermionic tungsten filament or a field emission gun (FEG). The FEG provides a highly coherent and bright electron beam, which is essential for high-resolution imaging.
2. Electron acceleration system: electrons are accelerated through high voltages (80–300 kV) to shorten their wavelength and enhance penetration power.

3. Electromagnetic lenses: condenser, objective, and projector lenses guide, shape, and focus the electron beam. Unlike glass lenses in light microscopy, this consists of coils generating precisely controlled magnetic fields.
4. Sample holder and stage: the specimen, mounted on a thin copper or gold grid, is inserted into the electron path. Advanced stages allow tilting, rotation, and cryogenic operation (as in cryo-TEM).
5. Image formation system: transmitted electrons are detected using a fluorescent phosphor screen, CCD cameras, or direct electron detectors, the latter offering superior resolution and sensitivity for structural biology.

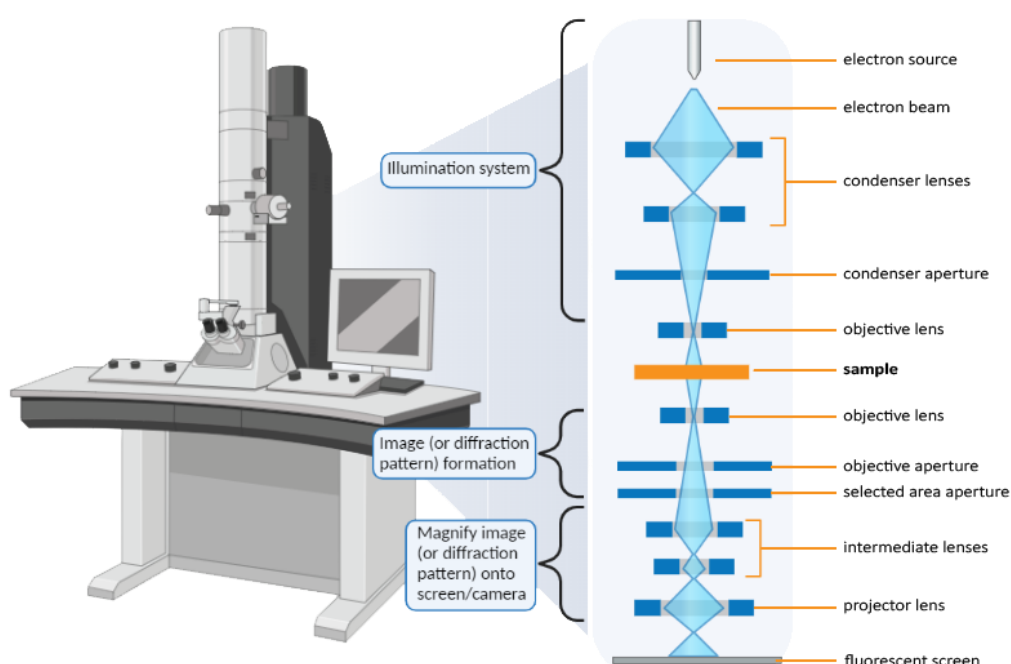


Figure 3.10. Simplified TEM diagram highlighting the major components throughout the column [75].

The TEM optical path includes condenser lenses that control illumination, the objective lens which forms the initial image of the specimen, and a series of intermediate and projector lenses that magnify the image onto the detector. Image contrast in soft matter arises primarily through phase contrast, which depends on defocus and is mathematically described by the contrast transfer function (CTF). Computational correction of the CTF, or the use of phase plates, makes it possible to extract structural information that would otherwise be invisible in weakly scattering samples such as vesicles, micelles, or polymers.

3.1.7. Dynamic Light Scattering and Zeta Potential Measurements

Dynamic Light Scattering

Dynamic Light Scattering (DLS), also referred to as photon correlation spectroscopy or quasi-elastic light scattering, has become one of the most widely used techniques for nanoparticle characterization, particularly in the field of drug delivery systems for the assessment of the hydrodynamic size distribution of colloidal particles and engineered nanoparticles. Its popularity stems from its simplicity, non-destructive nature, and the minimal amount of sample preparation required.

At its core, DLS exploits temporal fluctuations in the intensity of scattered laser light produced by particles undergoing Brownian motion. The intensity autocorrelation function (ACF) of the scattered light, commonly written as $G(\tau)$, is fitted to extract the translational diffusion coefficient (D_t). For an ideal monodisperse dispersion the ACF follows a mono-exponential decay of the form

$$G(\tau) = 1 + be^{-2D_t q^2 \tau} \quad (17)$$

where b is an instrument-dependent constant, τ the delay time and q the scattering vector. The scattering vector is determined by the optical geometry and solvent refractive index via

$$q = \frac{4\pi n_0}{\lambda_0} \sin\left(\frac{\theta}{2}\right) \quad (18)$$

where n_0 is the refractive index of the solvent, λ_0 is the wavelength in vacuum and θ is the scattering angle. Conversion of D_t into a hydrodynamic radius (R_h) relies on the Stokes–Einstein relationship

$$D_t = \frac{K_B T}{6\pi\eta R_h} \quad (19)$$

where T is the temperature, K_B the Boltzmann constant and the medium viscosity η . These mathematical relations are the determinative link between optical signals and the hydrodynamic descriptors reported by the instrument.

A note on the meaning of R_h : R_h represents the radius of an ideal hard sphere that diffuses at the same rate as the particles measured by DLS. It is a theoretical value since real colloidal particles are often non-spherical and surrounded by a dynamic solvation or hydration layer (corona) whose composition changes with the environment. Thus, DLS gives only an approximate particle size. On the other side, the other geometrical radius to describe the dimension of a particle is the radius of gyration (R_g), but it is the mass-weighted average distance of all atoms from the particle's center of mass. It is measured by small angle X-ray scattering (SAXS) for small nanoparticles, by static light scattering (SLS) for larger or computationally (see Paragraph 3.2.5).

Two interconnected sources of bias merit early emphasis. First, light scattering intensity scales strongly with particle size (approximately with the sixth power of the particle radius in the Rayleigh regime (particle size $< \lambda/10$)), so larger species dominate the intensity signal and can mask populations of smaller particles. Second, standard DLS outputs an intensity-weighted size distribution, and conversion to volume- or number-weighted distributions requires additional, model-dependent processing and can be misleading for polydisperse samples. For these reasons DLS is best understood as delivering a hydrodynamic, intensity-weighted descriptor that must be interpreted alongside orthogonal measurements when polydispersity or multimodality is suspected.

The sample must be optically clear, free of dust and macroscopic precipitates, and prepared in a dispersant chosen to control interparticle interactions (for example, including dilute inert electrolyte to screen long-range electrostatic forces where appropriate). Both overly concentrated and excessively diluted dispersions produce artefacts: high concentration promotes multiple scattering and apparent size underestimation, whereas too low a scattering signal reduces signal-to-noise and degrades autocorrelation fits.

The method has intrinsic resolution limits. In practice, DLS struggles to resolve two size populations unless they differ substantially (commonly by a factor of ~ 3 or greater), and populations present at small number fractions will be obscured by even modest amounts of larger material. This limitation explains why TEM/AFM (which provide number- and morphology-based information) and nanoparticle tracking analysis (NTA, which tracks individual particles and can better represent multimodality) are frequently used to complement DLS. For imaging NPs, many forms of electron microscopy, such as TEM, are widely used. It is now feasible to extract NP size distributions from TEM pictures together with details on mean size, standard deviation, and total PDI estimation using image analysis tools (such as ImageJ). However, because DLS is an intensity-based approach and TEM is a number-based technique, the information from TEM pictures frequently does not correlate well with DLS data. TEM operates on dry samples under ultrahigh vacuum conditions, whereas DLS samples are solvated. While TEM offers the projected surface area based on the amount of incident electrons that were transmitted, DLS measures the R_h of the scattered particles. Thus, DLS quantifies the solvated hydrodynamic radius, whereas TEM reports a dehydrated, projected morphology, making systematic discrepancies expected.

Conversely, techniques that separate by density or mobility (differential centrifugal sedimentation, SMPS, laser diffraction) offer different advantages and systematic biases. No single technique is universally authoritative: DLS excels for rapid, low-volume screening and for detecting changes in

hydrodynamic size (for instance, due to protein adsorption or agglomeration) but should be combined with microscopy or orthogonal sizing for rigorous characterization.

When DLS is applied to biologically relevant environments the interpretation becomes more complex. Adsorption of biomolecules (protein corona formation, Figure 3.11) alters both the particle surface and the effective hydrodynamic shell; the corona is dynamic and composition-dependent, so that the measured R_h may evolve with incubation time and with changes in medium composition. Cell-culture media, serum and other complex matrices contain small and large components that either contribute background scattering or form aggregates that confound measurement. Practical strategies include using serum-free formulations for primary DLS assessment, monitoring time-courses rather than single end-points, and corroborating corona formation and its functional consequences with orthogonal biochemical assays [76].

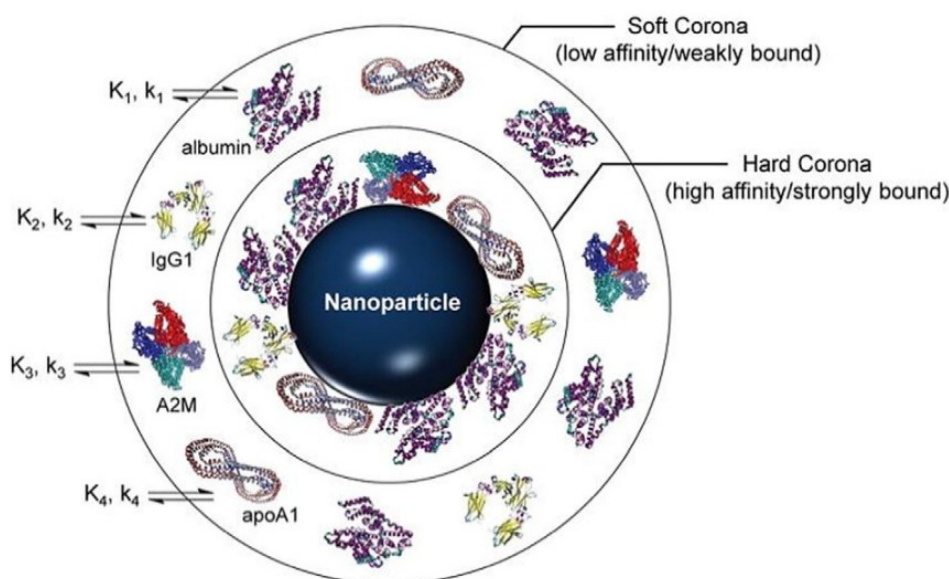


Figure 3.11. Schematic of soft and hard corona formed on the surface of a NP. Protein adsorption is a kinetic (k) and thermodynamic (K) function of both the individual proteins and NP properties such as surface modification, composition, and diameter. Initially, high-abundance and/or high-mobility proteins bind to the nanoparticle surface. Over time, these proteins are replaced by lower-mobility proteins with a higher binding affinity. Serum proteins commonly observed in NP coronas are shown as a representative corona: serum albumin, immunoglobulin G1 (IgG1), alpha-2 macroglobulin (A2M), and apolipoprotein A-1 (apoA1) [76].

Zeta Potential

Zeta potential (ZP), also termed electrokinetic potential, is the principal parameter used to quantify surface charge characteristics of colloidal particles in dispersion. Importantly, ZP is not a direct measurement of the true surface potential but rather the electrical potential at the so-called slipping or shear plane, i.e. the notional boundary between the moving particle–double-layer complex and the surrounding dispersant during electrophoresis. Its determination has become routine in nanomedicine

because surface charge profoundly influences colloidal stability, protein adsorption, cellular uptake, and ultimately the safety and efficacy of nanoparticulate drug delivery systems.

When a particle carrying surface charges is immersed in a liquid medium, an electrical double layer (EDL) develops (Figure 3.12). This consists of an inner Stern layer of counter-ions tightly adsorbed to the surface, and a more diffuse outer region of ions whose distribution decays exponentially with distance, as described by Debye-Hückel theory.

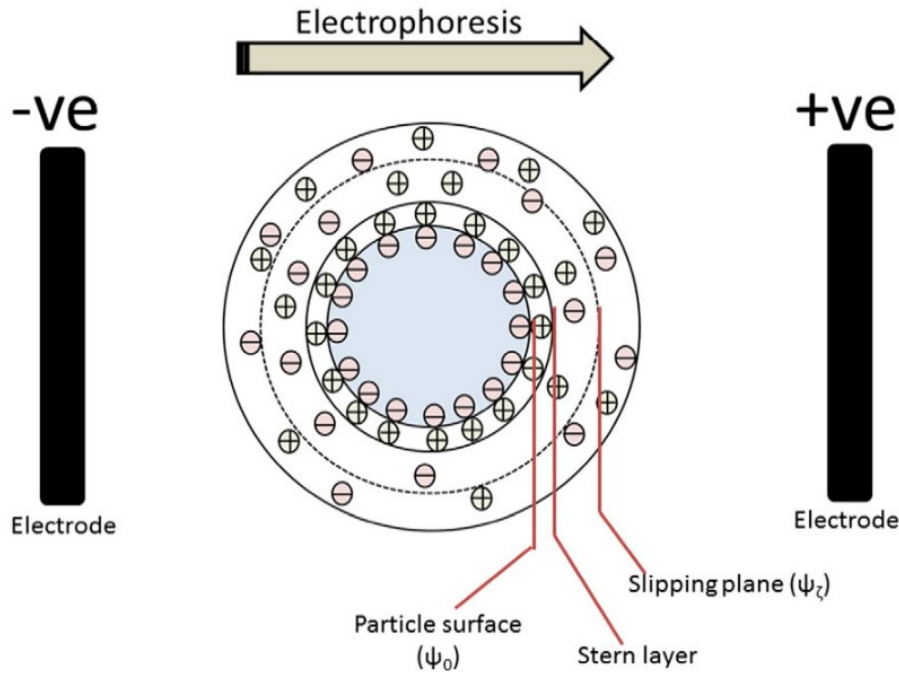


Figure 3.12. Cartoon showing the EDL on a negatively charged particle. Immediately on top of the particle surface there is a strongly adhered layer (Stern layer) comprising of ions of opposite charge i.e. positive ions in this case. Beyond Stern layer a diffuse layer develops consisting of both negative and positive charges. During electrophoresis the particle with adsorbed EDL moves towards the electrodes (positive electrode in this case) with the slipping plane becoming the interface between the mobile particles and dispersant. The ZP is the electrokinetic potential at this slipping plane [76].

The ZP corresponds to the potential at the slipping plane, where the mobile colloidal entity (particle plus attached layers) interacts hydrodynamically with the bulk solvent. The true surface potential (Nernst potential, ψ_0) is experimentally inaccessible, and the measured ζ is always a lower value reflecting electrostatic screening and the compression of the EDL. ZP is inferred from the electrophoretic mobility (μ_e), i.e. the velocity of the particles per unit electric field, through Henry's equation:

$$\mu_e = \frac{2\varepsilon_r\varepsilon_0\zeta^*f(K_a)}{3\eta} \quad (20)$$

where ε_r is the dielectric constant of the medium, ε_0 the permittivity of free space, η the viscosity, and $f(K_a)$ Henry's function, which depends on the ratio of particle size to EDL thickness. For common

aqueous dispersions of pharmaceutical nanoparticles, the Helmholtz–Smoluchowski (HS) approximation ($f(K_a) \approx 1.5$) applies, simplifying the relation to

$$\mu_e = \frac{\varepsilon_r \varepsilon_0 \zeta}{\eta} \quad (21)$$

By contrast, for very small particles in low ionic strength media, the Hückel approximation ($f(K_a) \approx 1$) is more appropriate. These relations highlight how solvent permittivity, ionic strength, viscosity, and particle-size regime condition the validity of calculated ζ values [76].

Instrumentations

Modern DLS instruments consist of four main components (Figure 3.13):

1. **Laser:** the laser provides a stable, coherent light source, and its power can be adjusted using an attenuator. Wavelength selection is typically optimized for aqueous samples to minimize absorption and heating artifacts.
2. **Sample:** measurements are made in clean, transparent cuvettes (glass or plastic, various sizes). Samples must be homogeneous and free of precipitates. The required sample volume ranges from a few microliters to a few milliliters, depending on the model, and proper filtration or mild centrifugation is recommended to remove dust and large aggregates.
3. **Detector:** modern detectors are typically placed at a 173° backscatter angle, which reduces noise from multiple scattering and improves detection of weak signals from small particles. This configuration also increases sensitivity for dilute or low-scattering systems. The setup also allows adjusting the illuminated area depending on sample concentration.
4. **Software Interface:** current software allows users to create custom standard operating protocols by entering parameters for solvents, materials, and experimental conditions. Results include z-average size, polydispersity index (PDI), and size distributions (by intensity, volume, or number). Advanced data visualization tools display results as plots or histograms and can be exported easily.

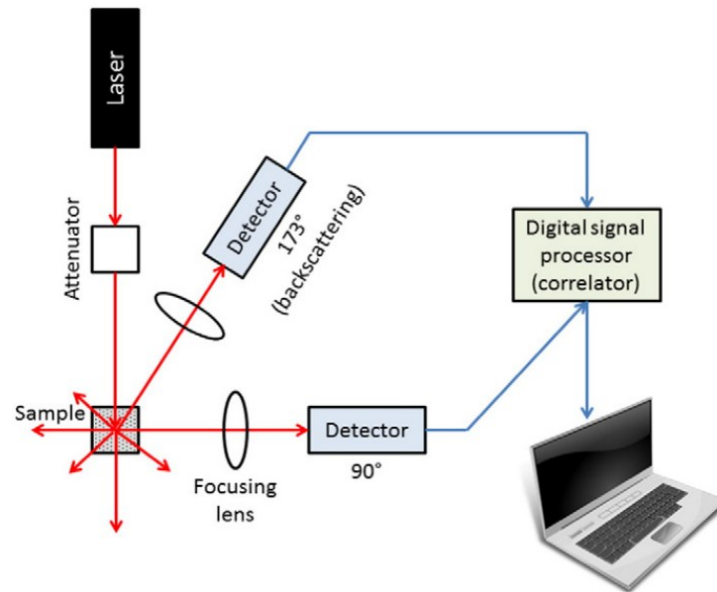


Figure 3.13. Schematic showing the instrumentation of DLS [76].

Zeta potential is determined by measuring the electrophoretic mobility of particles under an applied electric field, using the Electrophoretic Light Scattering (ELS) technique: it is based on the principle that moving particles scatter incident laser light at a slightly shifted frequency (Doppler shift), which is proportional to their velocity. In practice, the frequency shift is extremely small, requiring phase-analysis light scattering (PALS) algorithms for accurate detection. The instrument splits the laser into two beams, one passes through the sample, and the other serves as a reference. Interference between the scattered and reference beams enables precise reconstruction of the Doppler spectrum, allowing calculation of particle velocity, from which ZP is derived. The instrumentation used for this technique is shown in Figure 3.14.

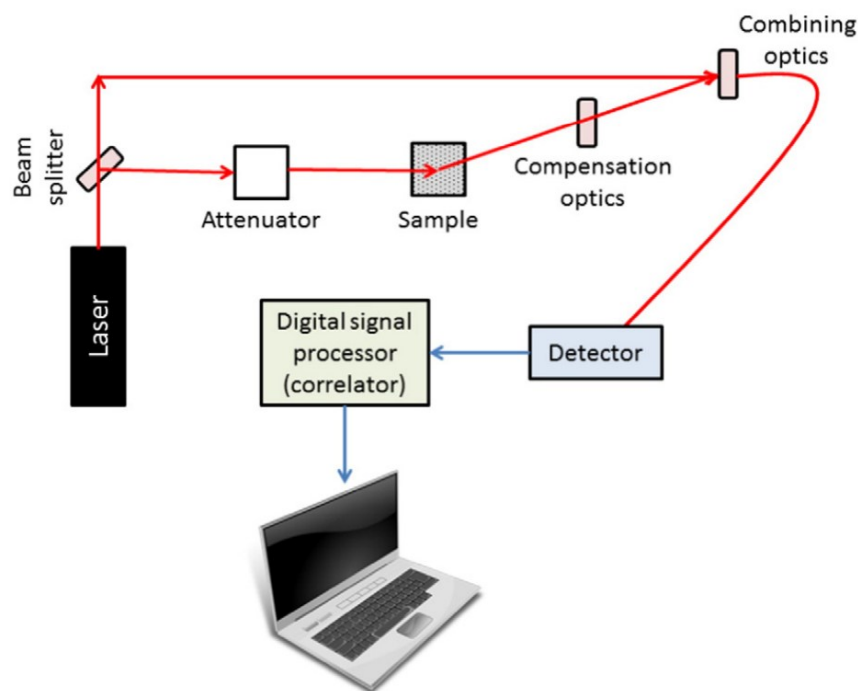


Figure 3.14. Schematic showing the instrumentation of ZP measurement by electrophoretic light scattering [76].

Instruments such as the Malvern Zetasizer series integrate DLS and ZP measurements in disposable cuvettes fitted with electrodes, enabling parallel characterization of size and surface charge. This combined setup minimizes sample handling, reduces contamination risks, and ensures that both parameters are measured under identical physicochemical conditions.

3.1.8. Nuclear Magnetic Resonance Spectroscopy

Nuclear Magnetic Resonance (NMR) spectroscopy is one of the most powerful analytical tools available for the elucidation of molecular structure and the study of molecular dynamics. It exploits the magnetic properties of certain nuclei, most notably ^1H and ^{13}C , to obtain direct, atom-level information about chemical environments and connectivities. Unlike other spectroscopic techniques, NMR is non-destructive and yields a wealth of complementary data (*e.g.*, chemical shifts, couplings, relaxation parameters, and integral intensities), collectively providing a comprehensive picture of molecular architecture.

At the core of NMR lies the quantum property of nuclear spin. A spin- $\frac{1}{2}$ nucleus, when placed in an external magnetic field B_0 , occupies one of two quantized orientations: parallel (low energy) or antiparallel (high energy) relative to the field. The small energy difference between these two states is given by:

$$\Delta E = \hbar\gamma B_0 \quad (22)$$

where $\hbar$ is the reduced Planck constant and γ the gyromagnetic ratio of the nucleus. The frequency at which resonance absorption occurs is the Larmor frequency:

$$\nu_0 = \gamma B_0 / 2\pi \quad (23)$$

Excitation by a radiofrequency (RF) pulse matching this energy gap perturbs the spin population, and the return to equilibrium produces the measurable NMR signal. The signal is recorded in the time domain as the free induction decay (FID), which is subsequently converted into a frequency-domain spectrum by Fourier transformation [77].

The Chemical Shift

The concept of the chemical shift is central to NMR interpretation. In a uniform magnetic field, each nucleus experiences not only the external field B_0 but also a local shielding effect generated by electron circulation. This shielding alters the effective field experienced by the nucleus and therefore shifts its resonance frequency relative to a reference standard. The chemical shift is expressed in parts per million (ppm):

$$\delta = \nu - \nu_{ref} \nu_0 * 10^6 \quad (24)$$

where ν is the observed resonance frequency, ν_0 is the operative frequency of the instrument and ν_{ref} the frequency of a reference compound, typically tetramethylsilane (TMS) in organic solvents.

Chemical shifts provide a sensitive probe of local chemical environment. Electronegative substituents deshield nuclei, shifting resonances downfield (higher δ). Conversely, electron-donating groups and magnetic anisotropy effects (as in aromatic rings) shift nuclei upfield (lower δ). This sensitivity makes chemical shift the first and most direct indicator of functional groups and electronic environment within a molecule [77].

Spin–Spin Splitting (J-Coupling)

In addition to chemical shift, NMR signals often exhibit splitting due to scalar (through-bond) spin–spin coupling between neighboring nuclei. The interaction of magnetic moments is transmitted via bonding electrons, producing a fine structure that encodes connectivity.

The multiplicity of a resonance follows the well-known $n+1$ rule: a nucleus coupled to n equivalent neighbors appears as a multiplet of $n+1$ peaks. The relative intensities of these components follow Pascal's triangle (*e.g.*, 1:2:1 for a triplet, 1:3:3:1 for a quartet, etc.). The separation between peaks is the coupling constant J , measured in Hz and independent of the magnetic field strength.

As an example, in ethanol the methyl group resonates as a triplet due to coupling with two vicinal methylene protons, while the methylene group appears as a quartet due to coupling with three methyl

protons. Splitting patterns thus provide a direct window into the number and arrangement of adjacent nuclei, supporting precise structural assignments.

It must be noted, however, that the simplicity of Pascal's triangle holds only in first-order systems, where coupled signals are well separated in frequency. In second-order cases (small chemical shift differences relative to J), signal distortion and complex multiplet patterns arise, requiring full spin simulations for accurate analysis [77].

Integration

The area under each NMR resonance is directly proportional to the number of nuclei contributing to that signal. Integration, usually displayed as stepped curves over the spectrum, thus yields the relative numbers of protons at different chemical shifts.

For instance, in ethanol the integral of the methyl triplet corresponds to three protons, while that of the methylene quartet corresponds to two. The ratio of integrals reflects the ratio of nuclei and allows rapid determination of stoichiometric and structural relationships.

Care must be taken to distinguish integral area from peak height: multiplets divide the signal intensity among several lines, but the integral remains proportional to the total number of contributing nuclei. Accurate integration requires proper baseline correction, adequate signal-to-noise ratio, and a relaxation delay long enough to ensure full recovery of longitudinal magnetization, preventing underestimation of slowly relaxing signals [77].

Saturation Transfer Difference NMR Spectroscopy

Saturation Transfer Difference (STD) NMR is a powerful ligand-based spectroscopic method that has become central in fragment-based drug discovery and in the study of biomolecular recognition. It exploits the transfer of saturation from a large biomolecular receptor, typically a protein, to small ligands upon binding. The experiment is conceptually elegant yet experimentally straightforward, and it allows rapid identification of binding events and mapping of ligand–receptor interaction epitopes. Since its introduction in the 1990s, STD NMR has been widely adopted both in academic structural biology and in pharmaceutical lead discovery, as it bridges the gap between biophysics and medicinal chemistry with remarkable sensitivity and speed.

The underlying mechanism of STD NMR rests on selective radiofrequency irradiation. In an STD experiment, the protein (receptor) is selectively saturated at frequencies corresponding to its resonances, typically aliphatic regions devoid of small-molecule signals. Through intramolecular spin diffusion within the protein, this saturation spreads throughout the macromolecule. When a ligand transiently binds to the receptor, magnetization is transferred from the saturated protein nuclei to the bound ligand by means of the nuclear Overhauser effect (NOE).

Upon dissociation, the ligand carries with it the saturation, which manifests as a reduction in signal intensity in the ligand's free-state spectrum. By subtracting spectra acquired with and without protein irradiation, one obtains the STD spectrum, which highlights only those ligand protons that have received saturation via binding. The technique therefore provides a direct signature of binding and simultaneously maps the interaction by ranking proton-specific STD responses, thereby revealing which parts of the ligand make the closest and most persistent contact with the receptor surface (Figure 3.15).

Mathematically, the STD amplification factor is defined as:

$$STD_{amp} = \frac{I_0 - I_{sat}}{I_0} \frac{1}{[L][P]} \quad (25)$$

where I_0 is the reference intensity without saturation, I_{sat} the intensity with saturation, $[L]$ the ligand concentration, and $[P]$ the protein concentration. This normalized parameter accounts for concentration effects and provides a quantitative measure of saturation-transfer efficiency, independent of sample stoichiometry.

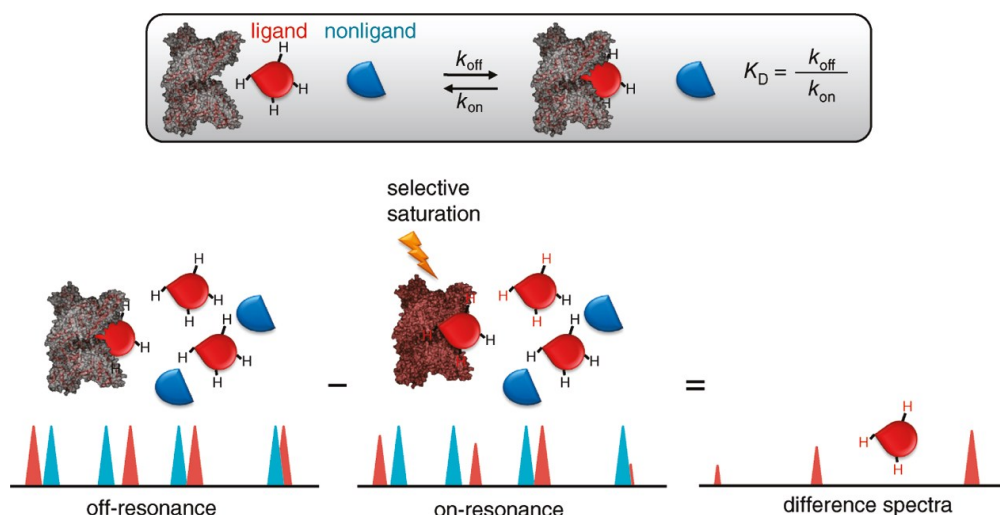


Figure 3.15. Scheme of the STD-NMR experiment. The exchange between free and bound ligand allows intermolecular transfer of magnetization from the receptor to the bound small molecule [78].

STD NMR benefits from several practical strengths. First, it requires no isotopic labelling of the protein, making it accessible for a wide range of biomolecular systems. Second, it tolerates high ligand concentrations relative to protein, which enhances sensitivity and allows screening of complex mixtures. Third, the experiment is fast: spectra can be acquired in minutes, enabling high-throughput ligand screening.

Importantly, STD NMR is not limited to affinity detection but also yields topological information. Ligand protons in close contact with the receptor surface exhibit stronger STD effects, while distal

groups yield weaker signals. This “epitope mapping” provides valuable insight into binding orientation and supports medicinal chemistry optimization.

STD NMR has been extensively applied in fragment-based lead discovery. It allows efficient screening of chemical libraries to identify weakly binding fragments, which can then be elaborated into higher affinity leads. Because binding events can be detected even for millimolar-affinity ligands, the method complements biophysical assays that are less sensitive in this regime.

STD NMR was also employed to characterize the interaction of a diverse set of natural and synthetic scaffolds with biologically relevant targets. The results highlight its utility in identifying novel ligands and mapping interaction epitopes not always accessible through crystallography or other high-resolution techniques. Moreover, STD NMR integrates seamlessly with complementary NMR experiments, molecular docking, and computational chemistry to refine models of ligand binding.

Despite its strengths, STD NMR has intrinsic limitations. It is primarily qualitative: while binding can be detected and mapped, precise affinity constants are not directly obtainable without complementary experiments. Sensitivity is constrained by protein solubility and stability; very large complexes or membrane proteins may pose experimental challenges. Furthermore, the technique requires the exchange of the ligand between bound and free states to be fast on the NMR timescale. Very tight binders may not yield strong STD signals because dissociation is too slow to accumulate saturation in the free ligand pool [79].

3.2. Computational techniques

A rigorous description of interatomic interactions would, in principle, require solving the Schrödinger equation [80] using quantum mechanical methods. However, the computational demands of such approaches make them impractical for systems of biological relevance, such as proteins or nucleic acids. By invoking the Born-Oppenheimer approximation [81], one can bypass the explicit treatment of electrons and instead represent each atom as a classical particle. In this framework, the dynamics of atoms are governed solely by the positions and velocities of their nuclei, according to Newton's second law, expressed as

$$-\frac{dE_{pot}(R)}{dR} = m \frac{d^2R}{dt^2} \quad (26)$$

At the atomistic scale, atoms are modelled as spheres centered on their nuclei, with defined masses and charges. Chemical bonds are approximated as springs, characterized by a force constant and an equilibrium length. Angular and torsional motions are similarly represented by harmonic or periodic potentials, capturing the essential features of molecular flexibility.

To achieve an accurate atomistic description, one must approximate the potential energy of the system. Otto Ermer defined a *force field* as “the analytical expression that describes the potential energy of the studied molecular complex and all the parameters needed to describe it.” Thus, the term *force field* encompasses both the mathematical form of the interatomic potential and the associated parameters. Once a system is described by an appropriate force field, its equilibrium configuration can be obtained by solving

$$\frac{dE_{pot}(R)}{dr_i} = 0 \quad (27)$$

where the potential energy depends on the atomic coordinates r_i . This minimization removes steric clashes and directs the system toward a mechanically stable geometry.

Molecular mechanics (MM) provides a practical means of eliminating unfavorable energetic conformations, which often arise in the early stages of simulations. However, MM alone represents only a preliminary step in the modelling of complex systems, since it neglects temperature effects and the contribution of kinetic energy,

$$E_{kin} = \frac{3}{2}k_bT \quad (28)$$

which requires consideration of the system's full-time evolution. For this reason, MM energy minimization is typically followed by molecular dynamics, where both potential and kinetic components are included to model realistic atomic motion.

3.2.1. Classical Molecular Dynamics

Molecular Dynamics (MD) can be understood as a time-dependent extension of Molecular Mechanics (MM): at each step of the simulation, the system evolves according to the forces acting on its atoms, gradually approaching equilibrium. The underlying principle is straightforward: starting from an initial molecular configuration, defined by atomic positions and their connectivity, the forces on each atom are calculated, and from these, the trajectories are obtained as the time-dependent evolution of the system. Unlike MM, MD requires the assignment of an initial set of random atomic velocities, sampled according to the desired starting temperature. During the simulation, both atomic positions and velocities are updated at each step. The system then fluctuates around equilibrium while conserving the total energy (E_{tot}). To ensure this conservation, velocities must be periodically adjusted, as excessive deviations must remain consistent with the imposed temperature and total energy. For this purpose, thermostats and velocity-scaling algorithms have been developed.

At the core of MD lies the repeated solution of Newton's second law:

$$F_i = m_i a_i(t) \quad (29)$$

where the force on atom i at time t is derived from the potential energy function $V(R)$, defined by the chosen force field:

$$F_i = -\nabla V_i(R_1, \dots, R_n) \quad (30).$$

Once the force is determined, the acceleration is obtained, and the atomic coordinates are updated using numerical integration schemes. A widely used method is the Verlet algorithm, expressed as:

$$R_i(t + \Delta t) = 2R_i(t) - R_i(t - \Delta t) + a_i(t)d\Delta t^2, \quad (31)$$

which computes new atomic positions from their previous coordinates and current accelerations. From the updated positions, the potential energy ($E_{\text{pot},i}$) of the system is recalculated, while velocities are estimated as:

$$v'_i(t) = \frac{R_i(t+\Delta t) - R_i(t-\Delta t)}{2\Delta t} \quad (32)$$

These velocities allow the calculation of kinetic energy for each atom,

$$E_{\text{kin},i} = \frac{1}{2} m_i [v'_i(t)]^2 \quad (33)$$

and the verification of the conservation of the total energy,

$$E_{\text{tot},i} = E_{\text{pot},i} + E_{\text{kin},i} \quad (34).$$

This cycle of force evaluation, integration, and energy assessment is iteratively repeated at each time step for the entire duration of the MD simulation, thereby generating a detailed trajectory of the molecular system. The resulting time series of coordinates forms the basis for all subsequent structural, dynamical, and thermodynamic analyses.

The choice and application of an appropriate force field is critical for ensuring the reliability of molecular modelling. For example, a force field developed for general materials science may yield entirely incorrect results when applied to proteins, and in many cases may not even allow a calculation to proceed. An essential feature of a suitable force field is transferability, that is the ability to apply a parameter set validated on a limited number of systems to a wide range of different contexts.

The studies reported in this work were conducted using AMBER (versions 12 and 14) [82], a well-established suite of molecular mechanics force fields for biomolecular simulations, originally developed by Peter Kollman's group at the University of California, San Francisco, together with the associated simulation package implementing them.

The functional form of the AMBER force field is:

$$V(R^N) = \sum_{bonds} \frac{1}{2} K_b (l - l_0)^2 + \sum_{angles} k_a (\theta - \theta_0)^2 + \sum_{torsions} \frac{1}{2} V_n [1 + \cos(n\omega - \gamma)] + \sum_{j=1}^{N-1} \sum_{i=j+1}^N \{ \epsilon_{i,j} [(\frac{\sigma_{i,j}}{R_{i,j}})^{12} - 2 (\frac{\sigma_{i,j}}{R_{i,j}})^6] + \frac{q_i q_j}{4\pi\epsilon_0 R_{i,j}} \} \quad (35).$$

This expression comprises four main contributions:

- Bond stretching: represented by a harmonic potential around the equilibrium bond length (l_0), with the force constant k_b depending on the atoms involved. This approximation is accurate near equilibrium but less so at large separations.
- Angle bending: describing deviations of bond angles from their ideal values, reflecting the geometry imposed by electron orbital hybridization, with force constant k_a .
- Torsional rotation: modelling the energy associated with twisting around bonds, dependent on bond order and the influence of neighboring atoms or electron lone pairs. These periodic terms capture conformational preferences such as staggered or eclipsed arrangements.
- Non-bonded interactions: consisting of van der Waals forces (modelled by a Lennard-Jones potential) and electrostatic interactions (Coulomb's law). This last term dominates long-range behavior and are crucial for reproducing hydrogen-bonding networks, packing, and solvation effects.

The parameters required to evaluate these terms (force constants, equilibrium lengths and angles, atomic charges, etc.) are not known *a priori*. AMBER therefore provides a library of parameter sets tailored to different molecular classes. These include:

- ffXX (e.g., ff95, ff12, ff14), OL15: optimized for peptides, proteins, and nucleic acids;
- GAFF (Generalized AMBER Force Field) [83]: designed for small organic molecules, enabling simulations of ligands and drug-like compounds in complex with biomolecules;

These parameter sets ensure internal consistency between bonded and non-bonded terms, which is essential for stable and physically meaningful simulations.

In this thesis, the ff14 and OL15 force fields were employed for proteins and nucleic acids, respectively, as they are among the most widely validated for these biomolecules. GAFF was applied to parameterize unconventional organic molecules such as dendrimers and drug compounds.

As previously mentioned, AMBER refers not only to a family of biomolecular force fields but also to the simulation package that implements them. In this work, several AMBER modules were employed:

- Antechamber [84]: identifies atom and bond types, and assigns partial charges to nonstandard molecules using the GAFF force field;
- parmchk: generates missing parameters required for small organic molecules parametrized with GAFF;
- leap: prepares all necessary input files for simulations, including solvation and ion addition, system parametrization, and the construction of simple nucleic acid fragments;
- parmed: allows modification and editing of parameter files;
- sander (*Simulated Annealing with NMR-Derived Energy Restraints*): the main simulation engine, providing tools for energy minimization and molecular dynamics under a wide variety of conditions;
 - pmemd (*Particle Mesh Ewald Molecular Dynamics*): an optimized version of *sander* designed for efficient parallel computations;
 - pmemd.cuda [85], [86]: a GPU-accelerated implementation of *pmemd*, which significantly enhances simulation performance;
- nmode: performs normal mode analysis to calculate system entropies;

- MMPBSA.py: evaluates free energies of binding from snapshots extracted from MD trajectories within the Molecular Mechanics/Poisson-Boltzmann Surface Area framework of theory; MMPBSA.py [87]: evaluates free energies of binding from snapshots extracted from MD trajectories;
- cpptraj [88]: a versatile analysis program for processing and interpreting simulation results.

In this thesis, missing GAFF parameters for drug-like molecules were computed using the semiempirical Austin Model 1 (AM1) method within *antechamber*, corrected by the bond charge correction (BCC) scheme for improved treatment of bond charges [89]. This workflow offers accuracy comparable to fully quantum-mechanical charge derivation while substantially reducing computational cost. For dendrimers, a more rigorous charge derivation method, RESP (Restrained Electrostatic Potential), was employed, using the capabilities of the R.E.D. server [90].

The Autodock Vina software was exploited for the docking studies of the protein/drug/dendrimer complexes.

Trajectory visualization and structural analysis were primarily conducted with UCSF Chimera [91] and VMD (Visual Molecular Dynamics) [92]. These programs enabled the inspection of MD trajectories, conformational changes, docking outcomes, and sequence alignments, providing an intuitive bridge between numerical data and structural interpretation.

Creation of the systems

The procedure outlined in this section is presented in the most general form, since the majority of steps are common across all systems investigated.

Because the objective of these simulations is to reproduce biological systems under conditions as close as possible to reality, it is essential to carefully mimic the physiological environment. The molecules studied in this work are naturally present under the physiological conditions of the human body; therefore, the simulated systems were prepared in aqueous solution with explicit solvent modelled using TIP3P [93] water molecules, and with a salt concentration corresponding to the typical human ionic strength (0.15 M NaCl). This setup ensures appropriate screening of electrostatic interactions and realistic solvation behavior.

AMBER supports several input file formats for starting structures (e.g., *.pdb*, *.xyz*). For proteins and nucleic acids, the most employed format is the Protein Data Bank (*.pdb*) file. This format, accessible from the Protein Data Bank repository (<https://www.rcsb.org>), provides coordinate information for experimentally resolved biomolecular structures, most often obtained by X-ray crystallography. A *.pdb* file contains essential information such as atom indices and names, the residue names and numbers to which each atom belongs, and the Cartesian coordinates of all atoms in the structure.

When needed, missing atoms or unresolved regions can later be reconstructed using specialized AMBER tools.

All simulations were conducted in explicit solvent and under periodic boundary conditions (PBCs), where the simulation box is replicated in three dimensions (Figure 3.16). In practice, this means that when atoms or molecules exit the box from one side, they re-enter from the opposite side, ensuring that the overall contents of the system remain constant. This approach eliminates boundary artifacts: although only one copy of the system is explicitly simulated, each particle interacts not only with other particles in the box but also with the periodic images of neighboring boxes. As a result, the system behaves as if embedded in an effectively infinite bulk environment, which is essential for realistic biomolecular simulations.

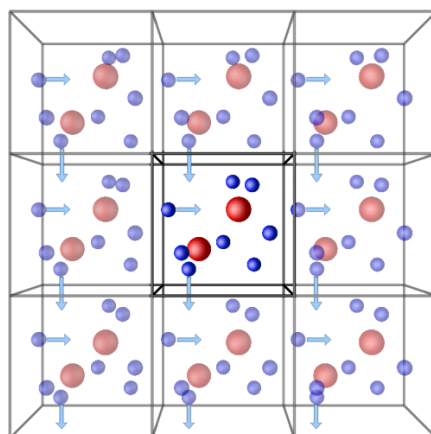


Figure 3.16. Schematic representation of the idea of periodic boundary conditions.

At first glance, this method might appear computationally prohibitive, as it would seem to require the evaluation of an infinite number of interactions. However, long-range electrostatics are efficiently computed using the Ewald summation method, or more commonly its optimized variant, the Particle Mesh Ewald (PME) algorithm [94]. PME separates the calculation into a real-space component and a reciprocal-space component, thereby allowing accurate evaluation of infinite electrostatic interactions. Even so, nonbonded interactions within PBCs require the application of a cutoff distance, typically in the range of 8-10 Å for production runs. This balance between accuracy and efficiency is essential for tractable biomolecular simulations.

The leap module in AMBER can handle input coordinate files that already include solvent and ions, such as structures for lipid membranes generated with the CHARMM-GUI interface [95]. Alternatively, leap can construct a solvated system starting from a “dry” solute by embedding it in a water box and adding counterions as needed to reproduce the desired physiological conditions. This flexibility allows consistent preparation of heterogeneous systems, from isolated proteins to complex assemblies.

In AMBER, two principal geometries are commonly employed for water boxes under periodic boundary conditions: truncated octahedral and rectangular (or cubic) boxes. The choice of box geometry depends on the shape of the solute, with the goal of minimizing the number of solvent molecules while maintaining proper solvation. For solutes with approximately spherical symmetry, an octahedral box is advantageous, as it requires fewer water molecules to fill the same effective volume, thereby reducing the number of “redundant” atoms in the system. Conversely, for elongated or highly anisotropic solutes, such as nucleic acid (DNA) strands, a rectangular/tetragonal box is generally preferred, as it accommodates their geometry more efficiently and provides greater flexibility. Selecting an appropriate box shape thus directly impacts computational cost and simulation stability.

The leap module can generate solvent boxes of specified dimensions and geometry, placing the solute at the center. A key requirement in molecular dynamics is that solute molecules should not interact directly with their own periodic images. For this reason, the box size is determined both by the cutoff distance applied to van der Waals interactions and by the expected mobility of the solute during the simulation. In the present work, a minimum buffer distance of 10-15 Å between the solute surface and the box boundary was employed to prevent spurious self-interactions. This criterion ensures physically meaningful trajectories and avoids artifacts related to artificial self-contact.

In explicit solvent simulations, water is by far the most abundant component of the system. Several water models are available, differing in their balance between physical accuracy and computational efficiency. Some models increase accuracy by including more than three interaction sites to better reproduce dipole effects, whereas others simplify the representation by fixing certain parameters, thereby reducing computational cost. The choice of model therefore reflects a trade-off between realism and performance.

In all simulations reported in this work, the TIP3P water model was employed. This model represents water with three interaction sites: partial positive charges located on the two hydrogen atoms and a compensating negative charge on the oxygen atom. Nonbonded van der Waals interactions are computed exclusively between oxygen atoms using a Lennard-Jones potential, while hydrogen atoms do not participate in van der Waals interactions. The intramolecular geometry is rigid, with a fixed H-O-H bond angle, which significantly reduces computational demand compared to flexible models. This simplicity has made TIP3P one of the most widely used water models in biomolecular MD.

To maintain this rigidity during molecular dynamics simulations, the SHAKE algorithm [96] is applied to constrain all bonds involving hydrogen atoms. This approximation allows the use of a larger integration time step. In unconstrained simulations, the time step must be shorter than the fastest vibrational motion in the system, typically the high-frequency hydrogen vibrations, which

limits the time step to ~ 1 fs. By constraining these motions, the time step can be safely increased to 2 fs, enabling more efficient sampling of conformational space within the same total simulation time. This improvement is crucial for simulations extending into the hundreds of nanoseconds or microseconds.

After solvating the molecular complex in water, it is necessary to introduce counterions into the system. Ions play a significant role in modulating solute dynamics and can influence the trajectories obtained during the simulation. The number of ions to be added is determined both by the net charge of the solute and by the solvent box volume, to reproduce the physiological ionic strength of 0.15 M NaCl. Maintaining the correct ionic environment is essential for realistic electrostatic screening.

In AMBER, counterions such as Na^+ and Cl^- can be added using the leap module. Placement of ions is guided by the electrostatic potential surface of the solute to ensure realistic distribution. When an ion is introduced at a position that overlaps with an existing water molecule, the water molecule is replaced by the ion, which is positioned at the water's center of mass. Given the large number of water molecules typically present in the system, most ions are inserted in this way.

This substitution, however, introduces a local non-uniformity in the solvent box, since a single ion replaces three water atoms. For this reason, during the initial equilibration stage under conditions of constant pressure and temperature (NPT), which allows the simulation box to adjust its dimensions, the system undergoes a reduction in box volume to restore the correct solvent density. This equilibration step ensures that both solute and solvent adopt physically meaningful densities before production dynamics begin.

3.2.2. Simulation Procedure

Pmemd is the AMBER module that exhibits the best scalability for parallel computations and now incorporates nearly all functionalities of the older sander module. In this work, all simulations were performed using pmemd, and whenever possible, the GPU-accelerated version, pmemd.cuda, was employed to further enhance computational efficiency. The standard simulation workflow is described in the following sections. This choice significantly reduces wall-clock time and enables longer, more statistically meaningful trajectories.

It is important to note that, in addition to monitoring numerical parameters throughout the simulation, visual inspection of both the minimized structures and the resulting trajectories remains essential. This manual review allows the rapid identification of potential issues or anomalies that may arise during molecular dynamics simulations, which might not be immediately apparent from automated checks alone. Such inspections help detect artifacts such as unfolding events, vacuum bubbles, or unrealistic motions.

Minimization

Prior to initiating molecular dynamics simulations, it is recommended to minimize the system geometry, as the initial structure obtained during system preparation may contain steric clashes or unfavorable overlaps between atoms in different residues. Energy minimization serves to remove these bad contacts and to relax the system according to the chosen force field. A well-minimized structure ensures numerical stability when dynamics begin.

In this work, the minimization protocol consisted of three steps. First, 500 steps of Steepest Descent were performed, followed by 500 steps of Conjugate Gradient minimization with a restraint of $5.0 \text{ kcal} \cdot \text{mol}^{-1} \text{ \AA}^{-2}$ applied to the solute, allowing the solvent to relax around the fixed solute. Finally, an additional 1000 steps of Conjugate Gradient minimization without restraints were executed to fully eliminate any remaining steric clashes and ensure a well-relaxed starting configuration for molecular dynamics. This staged approach progressively relieves strain while preventing excessive movement of the solute during early relaxation.

Heating and density relaxation

Following energy minimization, the molecular dynamics (MD) simulations were initiated. During the initial equilibration phase, a time step of 1.0 fs was employed to prevent excessive deviations arising from temperature increases and the rapid fluctuations of solvent molecules. The system was gradually heated to the target temperature of 300 K, with a restraint of $5.0 \text{ kcal mol}^{-1} \text{ \AA}^{-2}$ applied to the solute to maintain its structural integrity. This controlled heating avoids abrupt conformational changes and ensures a smooth transition from the minimized state to dynamic conditions.

The heating phase was conducted over 50 ps in the canonical ensemble (NVT), where volume and temperature are held constant, using the Langevin or Berendsen thermostat for temperature regulation (collision frequency = 2.0 ps^{-1}). The use of stochastic temperature control helps dampen high-frequency motions typical of early equilibration. Subsequently, a further 50 ps of simulation was performed in the isothermal-isobaric ensemble (NPT), while maintaining the same solute restraints, to allow the solvent box to equilibrate its density. Pressure control was achieved using a Berendsen or Monte Carlo barostat with a pressure relaxation time of 1.0 ps. This stage ensures that the system reaches a stable density before the restraints are released and production dynamics begin. The heating phase was conducted over 50 ps in the canonical ensemble (NVT), where volume and temperature are held constant, using the Langevin [97] or Berendsen [98] thermostat for temperature regulation (collision frequency = 2.0 ps^{-1}). Subsequently, a further 50 ps of simulation was performed in the isothermal-isobaric ensemble (NPT), while maintaining the same solute restraints, to allow the solvent box to equilibrate its density. Pressure control was achieved using a Berendsen or Monte Carlo barostat with a pressure relaxation time of 1.0 ps.

Equilibration

After the initial equilibration, all restraints were removed, gradually if necessary, and the systems were equilibrated in the NPT ensemble using a 2.0 fs time step. The duration of this equilibration phase varies depending on the solute's conformational flexibility, ranging from a few nanoseconds to several tens of nanoseconds. This stage allows the system to fully adapt to the target thermodynamic conditions without artificial constraints.

To monitor the progress of equilibration, several key properties are typically assessed, including temperature (T), pressure (P), volume (V), potential energy (E_{pot}), and the root-mean-square deviation (RMSD) of structural atoms. The thermodynamic parameters provide information on the energetic and volumetric stability of the system, whereas the RMSD reflects structural and vibrational equilibration.

In most cases, energetic equilibrium is achieved rapidly, often within the first tens of picoseconds, once the system reaches the target temperature. Vibrational equilibration, however, requires significantly longer, ranging from several nanoseconds up to tens of nanoseconds for a relatively stable drug–protein complex. For more flexible systems, such as nucleic acid or dendrimer complexes, at least 50 ns of equilibration was performed to ensure stability. Once the RMSD reached a clear plateau with no systematic drift, the system was considered fully equilibrated, and the production phase was initiated, during which the simulation data could be regarded as reliable for subsequent analysis.

Production

The production phase is the stage of the simulation during which data are collected for subsequent analysis. If large fluctuations in system density are not anticipated, the simulation can be performed in the NVT ensemble to improve computational efficiency. In such cases, the first 2 ns of the trajectory were discarded to account for any minor perturbations resulting from the ensemble change. This practice ensures that only steady-state dynamics contribute to the analysis.

The duration of the production phase was not fixed; simulations were continued until the convergence of the properties of interest was achieved. Typically, this required between 10 and 100 ns of simulation time, depending on the system and the specific observables being analyzed. Longer trajectories were used when necessary to capture slow conformational rearrangements or rare events.

3.2.3. Free Energy Methods

Molecular Mechanics/Poisson–Boltzmann Surface Area (MM/PBSA)

The Molecular Mechanics/Poisson–Boltzmann Surface Area (MM/PBSA) method [99] is widely employed to estimate the binding free energy of a molecular complex using snapshots extracted from equilibrated MD trajectories (in this work, typically ~200 snapshots were analyzed). This post-processing approach allows the evaluation of energetics without performing additional simulations. Both approaches are implemented in the MMPBSA.py module of AMBER and can be applied either to quantify the binding affinity between two interacting molecules (e.g., dendrimer/protein or drug/protein systems), or to compare the free energies of distinct solvated conformations of the same molecule.

These methods evaluate the free energy difference directly between two end states, without requiring the computation of intermediate configurations along a reaction coordinate. While this simplification reduces computational cost, it inherently introduces larger uncertainties compared to more rigorous free energy perturbation (FEP) or thermodynamic integration (TI) calculations. Nevertheless, despite their approximate nature, MM/PBSA and MM/GBSA (Molecular Mechanics Generalized Born Surface Area) typically provide binding free energy estimates (ΔG_{bind}) in reasonable agreement with experimental data. In particular, calculations of relative free energies ($\Delta\Delta G$) for macromolecular complexes and ligand–receptor interactions have been shown to achieve excellent consistency with experimental observations, while remaining computationally efficient. This balance of speed and accuracy is one of the major strengths of MM/PBSA.

The approach relies on the thermodynamic cycle (Figure 3.17) that relates the binding free energy in solution ($\Delta G_{\text{bind,solv}}$) to that in vacuum ($\Delta G_{\text{bind,vacuum}}$) through a solvation correction term (ΔG_{solv}):

$$\Delta G_{\text{bind,solv}} = \Delta G_{\text{bind,vacuum}} + \Delta G_{\text{solv}} \quad (36).$$

The solvation free energy term is decomposed into two contributions, a polar and a nonpolar component:

$$\Delta G_{\text{solv}} = \Delta G_{\text{POLAR}} + \Delta G_{\text{NP}} \quad (37).$$

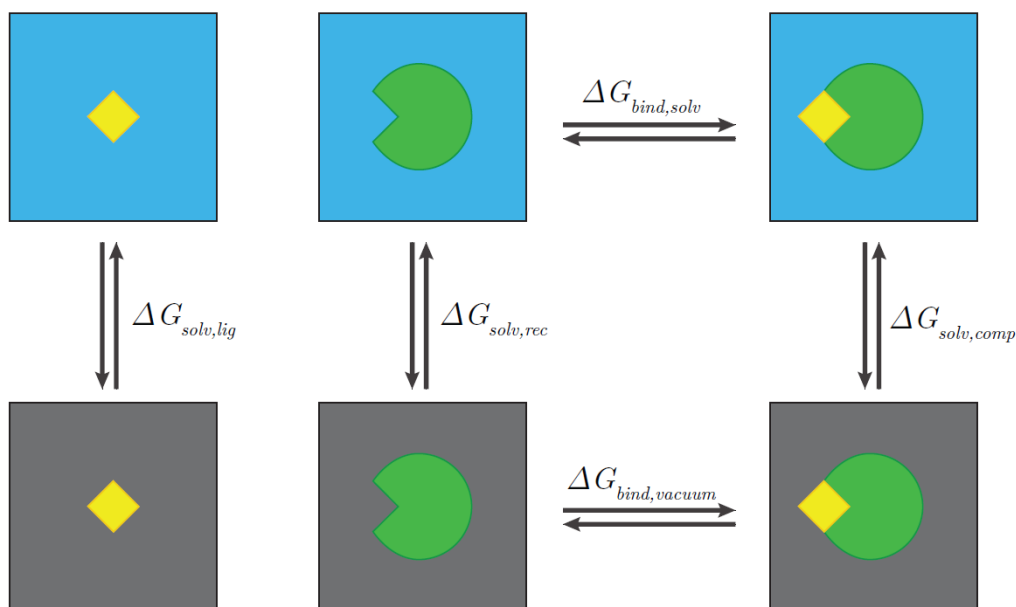


Figure 3.17. Thermodynamic cycle.

The polar term (ΔG_{POLAR}) accounts for electrostatic solvation effects, computed using either the Poisson–Boltzmann (PB) or Generalized Born (GB) models, whereas the nonpolar term (ΔG_{NP}) estimates hydrophobic contributions proportional to the solvent-accessible surface area.

ΔG_{POLAR} is obtained by solving the PB equation, as implemented in the *pbsa* module of AMBER (MM/PBSA), or by using the GB approximation available in *sander* (MM/GBSA). ΔG_{NP} accounts for hydrophobic effects, is estimated empirically as a linear function of the solvent-accessible surface area (SASA):

$$\Delta G_{\text{NP}} = \gamma \text{SASA} + \beta \quad (38)$$

where $\gamma = 0.00542 \text{ kcal mol}^{-1} \text{ \AA}^{-2}$ and $\beta = 0.92 \text{ kcal mol}^{-1}$. The SASA is calculated using either the LCPO algorithm implemented in *sander* or the *molsurf* method in *cpptraj*. This empirical term captures dispersion and cavity-formation effects associated with transferring a solute from vacuum to solvent. The SASA is calculated using either the LCPO algorithm [100] implemented in *sander* or the *molsurf* method in *cpptraj*.

The gas-phase molecular mechanics energy (E_{MM}) is calculated according to the force field parameters used to generate the system topologies, and it can be expressed as:

$$E_{\text{MM}} = E_{\text{int}} + E_{\text{vdW}} + E_{\text{ele}} \quad (39)$$

where E_{int} includes the bonded terms:

$$E_{\text{int}} = E_{\text{bond}} + E_{\text{angle}} + E_{\text{tors}} \quad (40).$$

The energy difference between the complex and its separate components gives:

$$\Delta E_{MM} = E_{MM,complex} - (E_{MM,receptor} + E_{MM,ligand}) \quad (41)$$

The binding free energy in vacuum is then given by:

$$\Delta G_{bind,vacuum} = \Delta E_{MM} - T\Delta S \quad (42)$$

where $T\Delta S$ represents the entropic contribution. The entropy term is usually estimated by normal mode analysis (NMA) in the gas phase, using the *nmode* module. Because NMA is computationally demanding, it is typically applied to a subset of representative snapshots from the molecular dynamic trajectory. This approach balances computational cost with a reasonable estimate of the entropic penalty associated with binding.

Finally, the binding free energy in solution is obtained as:

$$\Delta G_{bind,solv} = \Delta E_{MM} + \Delta G_{solv} - T\Delta S = \Delta H_{bind} - T\Delta S_{bind} \quad (43).$$

A more negative value of ΔH_{bind} indicates that the complex is energetically more stable than the separated species, and therefore that its formation is thermodynamically favored. This enthalpic term reflects the net balance of intermolecular interactions that stabilize the bound state.

In explicit solvent simulations, most of the energy contributions arise from solvent–solvent interactions, which are much larger in magnitude than the binding energy of interest. Consequently, the total energy exhibits fluctuations that hinder convergence, making the calculations extremely demanding in terms of CPU time. To circumvent this, the explicit solvent molecules and counterions are typically stripped from the trajectory files, allowing the calculations to converge more rapidly by preventing solvent–solvent interactions from overwhelming the relevant energetic contributions. This preprocessing step preserves all solute–solute and solute–solvent effects encoded in the MD snapshots while eliminating unnecessary computational overhead.

WHAM (Weighted Histogram Analysis Method)

The Weighted Histogram Analysis Method (WHAM) [101] constitutes a statistically rigorous extension of Ferrenberg and Swendsen’s multiple histograms reweighting technique, designed to improve the efficiency and accuracy of free-energy calculations in biomolecular systems. It is particularly useful for evaluating the potential of mean force (PMF) along reaction coordinates and for determining free-energy differences associated with conformational changes or molecular interactions. Because it optimally combines data from multiple simulations, WHAM has become a standard tool for enhanced-sampling analyses.

WHAM is conceptually related to the umbrella sampling method, in which biased simulations are performed by introducing restraining potentials to enhance sampling in otherwise rarely visited regions of phase space. Unlike conventional umbrella sampling, which requires “stitching” together

overlapping biased distributions in an approximate manner, WHAM systematically combines data from multiple biased simulations into a unified estimate of the unbiased probability distribution. This is achieved by minimizing statistical errors across overlapping histograms, thereby providing an optimal estimate of free energies [80]. In practice, this leads to smoother and more reliable PMF profiles.

Mathematically, WHAM determines the unbiased probability density by solving a set of self-consistent equations that couple the observed histograms from each biased simulation with the free-energy offsets of the respective biasing potentials. The method iteratively refines these free-energy offsets until convergence is reached, typically requiring only a modest number of iterations. Importantly, WHAM naturally incorporates contributions from all available simulations, regardless of overlap complexity, and allows for multiple overlaps to improve robustness [80]. This iterative refinement ensures that both well-sampled and poorly sampled regions contribute appropriately to the final free-energy surface.

The advantages of WHAM over earlier methods are threefold. First, it yields statistically optimal free-energy estimates by weighting simulation data according to sampling quality and overlap. Second, it provides a built-in estimation of statistical errors, enabling objective determination of where additional sampling is required to reduce uncertainty. Third, it generalizes readily to situations involving multiple restraining potentials, coupling parameters, or temperatures, making it broadly applicable to molecular dynamics and Monte Carlo studies of biomolecular systems [80]. These features make WHAM one of the most robust and versatile approaches for reconstructing free-energy landscapes.

COSMOmic

COSMOmic [102] is a mechanistic computational framework developed to model the partitioning and spatial distribution of molecules within micellar systems and biological membranes. It extends the COSMO-RS (Conductor-like Screening Model for Real Solvents) theory, which combines quantum-chemical calculations with statistical thermodynamics of molecular surface interactions, to anisotropic environments such as lipid bilayers and micelles. Unlike empirical approaches based on octanol-water partitioning, COSMOmic offers a physically grounded methodology that is largely free from system-specific fitting parameters [81]. This level of physical rigor enables predictions that remain robust across diverse chemical classes and membrane compositions.

The central concept of COSMOmic is to approximate membranes or micelles as layered liquids, where each layer possesses a distinct local composition of surfactant, solvent, and potential ions. This discretized, depth-dependent representation captures the intrinsic heterogeneity of interfacial systems. Quantum-chemical COSMO calculations provide surface polarization charge densities (σ -

profiles) of the constituent molecules, which are then translated into depth-dependent chemical potentials across the membrane. This enables the calculation of position- and orientation-dependent free energy landscapes for solutes. From these data, COSMOmic predicts both global membrane-water partition coefficients (K_{mw}) and internal concentration profiles of solutes within the membrane [81]. Such profiles offer mechanistic insight into preferential localization sites, enabling rational interpretation of permeability and accumulation trends.

Methodologically, COSMOmic requires as input the COSMO polarization surface data of the solute and relevant membrane components (e.g., phospholipids, water). The membrane structure itself is described using atomistic distributions derived from molecular dynamics simulations or experimental sources. By combining quantum-derived solvation energetics with realistic membrane density profiles, the method creates a seamless connection between quantum chemistry and mesoscale biophysics. COSMOmic then evaluates the partition function by systematically sampling all possible solute orientations and insertion depths, yielding not only equilibrium partitioning constants but also depth-resolved free-energy barriers associated with membrane permeation [81]. These depth-resolved barriers directly correspond to key kinetic bottlenecks in passive diffusion.

The technique has been validated against experimental membrane-water partition data and demonstrates predictive performance comparable to or surpassing traditional bulk solvent models. Importantly, it provides mechanistic insights into solute localization, orientation, and energetics within membranes: properties that are critical for understanding drug permeability, receptor access, and toxicological effects. Compared to molecular dynamics simulations, COSMOmic offers a significant reduction in computational cost while retaining the capacity to describe anisotropic solvation phenomena at the molecular level [81]. It therefore provides a powerful, computationally economical alternative for screening membrane-active compounds and rationalizing their biophysical behavior.

3.2.4. Enhanced Sampling Techniques

Umbrella Sampling MD

Umbrella sampling (US) [103] is a computational technique developed to address the sampling limitations of conventional molecular dynamics and Monte Carlo simulations when exploring free-energy landscapes. In many systems of chemical, biochemical, or materials interest, the relevant processes involve transitions across substantial free-energy barriers. Because such rare events are visited only infrequently under unbiased Boltzmann sampling, direct simulation often fails to provide statistically meaningful data on the regions of configurational space that dominate the

thermodynamics of transitions. Umbrella sampling circumvents this problem by artificially biasing the simulation, ensuring a more uniform exploration of the chosen reaction coordinate [82]. This approach allows rare events to be probed on timescales accessible to practical simulations.

The method begins with the definition of a reaction coordinate that captures the progress of the process under study, for example the distance between two atoms in a binding event or a torsional angle describing conformational change. Instead of allowing the system to evolve freely along this coordinate, umbrella sampling introduces bias potentials, typically harmonic restraints, which confine the system to specific regions of the coordinate. Because no single restraint can efficiently sample the entire range of interest, the coordinate is divided into partially overlapping segments, often called “windows.” Separate simulations are then performed within each window, and the bias potentials guide the system to explore regions that would otherwise be inaccessible on reasonable simulation timescales [82]. This window-based strategy ensures both continuity and controlled overlap across the sampled domain.

The biased sampling achieved in each window does not directly yield the true thermodynamic distribution. To reconstruct the unbiased free-energy profile, one must remove the influence of the bias potential. This is accomplished through post-processing methods such as the Weighted Histogram Analysis Method (WHAM) or umbrella integration. WHAM combines histograms from the different windows and iteratively determines the free-energy offsets that ensure consistency across overlapping regions, ultimately producing a global potential of mean force. Umbrella integration, by contrast, exploits the fact that harmonic bias potentials allow one to compute mean forces directly and integrate them into a free-energy profile, often with straightforward error estimation [82]. Both approaches yield continuous, smooth free-energy surfaces that can be interpreted mechanistically.

The effectiveness of US depends critically on several factors. The choice of reaction coordinate is central: if the coordinate fails to capture the essential physics of the process, the resulting free-energy profile may be misleading. Equally important are the placement and overlap of windows, which must balance computational efficiency against statistical accuracy, as well as the strength of the bias potentials, which must be sufficient to overcome free-energy barriers without unduly narrowing the accessible configurational space [82]. Properly chosen parameters ensure robust sampling, accurate reconstruction, and meaningful interpretation of the underlying free-energy landscape.

On-the-Fly Probability Enhanced Sampling Metadynamics

On-the-fly probability enhanced sampling (OPES) [104] represents a novel advancement in the field of enhanced sampling techniques, designed to overcome the limitations of traditional metadynamics (MetaD). Classical MetaD relies on the progressive construction of a bias potential through the deposition of Gaussian kernels in the space of selected collective variables (CVs). This bias gradually

flattens the free energy landscape, enabling the system to escape metastable states. Despite its widespread success, MetaD presents challenges, particularly when the chosen CVs are suboptimal or multidimensional, often leading to slow convergence and strong oscillations in the estimated free energy surface [83]. These drawbacks become especially pronounced in complex biomolecular systems, where identifying optimal CVs is inherently difficult.

The OPES framework, proposed by Invernizzi and Parrinello, rethinks this paradigm by shifting the focus from bias construction to probability distribution reconstruction. Instead of directly accumulating bias, OPES builds an on-the-fly estimate of the unbiased probability distribution through a weighted kernel density estimation scheme. By grounding the bias in a statistically optimized distribution rather than incremental Gaussian deposition, OPES avoids the uncontrolled growth of bias that characterizes classical MetaD and prevents sampling in physically irrelevant regions of phase space [83]. As a result, the dynamics remain smooth and stable, even in rugged or high-dimensional landscapes.

A crucial innovation in OPES lies in its adaptive management of the kernel bandwidth and normalization. The bandwidth is dynamically rescaled during the simulation according to Silverman's rule of thumb, which ensures an optimal balance between resolution and statistical robustness as sampling proceeds. In parallel, the normalization of the probability distribution is restricted to the region of the CV space actually explored at a given time, thereby mitigating the inefficiencies encountered in high-dimensional problems. To further preserve computational efficiency, OPES employs a kernel compression algorithm to limit computational costs by merging redundant kernels while preserving accuracy [83]. Together, these adaptive mechanisms allow OPES to maintain high statistical quality without incurring the typical overhead of KDE-based methods.

The resulting method offers several key advantages over standard MetaD. First, it exhibits significantly faster convergence, especially under suboptimal CV choices. Second, it maintains a quasi-static bias profile, which enhances the stability and accuracy of reweighting procedures, allowing for straightforward recovery of unbiased ensemble statistics. Third, OPES introduces an intrinsic upper bound on the applied bias, controlled by a barrier-related parameter, granting users fine control over the extent of exploration and preventing runaway diffusion. Importantly, OPES achieves these improvements while requiring only a minimal set of input parameters: the update frequency of the bias, the initial kernel bandwidth, and an approximate estimate of the free energy barriers of interest [83]. This combination of simplicity, stability, and efficiency makes OPES a powerful next-generation tool for free-energy calculations in complex molecular systems.

Frequency adaptive metadynamics

Molecular dynamics simulations are inherently constrained by the requirement of femtosecond integration time steps, making the direct observation of processes on millisecond or longer timescales exceedingly challenging. Many biologically relevant reactions, such as ligand binding and unbinding or large conformational transitions in proteins, fall into the category of “rare events,” where long waiting times in metastable states dominate over rapid barrier-crossing events. Enhanced sampling strategies have therefore become essential for accessing these time scales, with metadynamics being one of the most widely adopted approaches [84]. These methods enable accelerated exploration of configurational space while preserving the essential physics of activated processes.

In classical metadynamics, a history-dependent biasing potential is periodically deposited along a set of CVs to discourage revisits to already explored free-energy basins, thereby accelerating transitions and facilitating free-energy surface reconstruction. More recently, the development of infrequent metadynamics (InMetaD) has demonstrated that, under careful biasing conditions, it is possible not only to accelerate transitions but also to recover unbiased kinetic rates. The key principle of InMetaD is to ensure that bias deposition occurs rarely enough so that transition state (TS) regions remain effectively unbiased, preserving the integrity of transition pathways. However, this constraint necessarily slows the pace at which deep basins are filled, limiting the practical efficiency of the method.

Frequency-Adaptive Metadynamics (FaMetaD) [105] was introduced to address this trade-off between efficiency and accuracy. The central idea of FaMetaD is to modulate the bias deposition frequency dynamically during a simulation. At the beginning of the trajectory, when the system resides in the depths of a free-energy basin, bias is deposited at a relatively high frequency, enabling rapid filling of the well. As the simulation progresses and the system approaches barrier regions, the deposition frequency is gradually reduced, thereby minimizing the risk of perturbing TS configurations. This smooth transition from aggressive to conservative bias deposition provides rapid basin exploration while maintaining the fidelity of kinetic information. This adaptive strategy is achieved by linking the deposition stride to the instantaneous acceleration factor of the simulation and introducing a threshold parameter (θ) that governs the switchover from frequent to infrequent biasing.

Benchmark applications of FaMetaD on systems of increasing complexity, including peptide conformational transitions, protein unfolding, and ligand binding/unbinding, demonstrated several advantages over InMetaD. First, FaMetaD consistently improved the robustness of kinetic estimates, as evidenced by more reliable Poissonian statistics of transition times. Second, the method yielded more accurate rate constants across a range of parameter choices, showing a more gradual degradation

of accuracy under aggressive biasing conditions compared to InMetaD. Finally, FaMetaD frequently reduced the overall computational cost required to achieve converged kinetics, making it particularly advantageous for systems with high free-energy barriers.

3.2.5. Structural Analysis

Root-mean-square deviation

The root-mean-square deviation (RMSD) is defined as

$$RMSD = \sqrt{\frac{1}{N} \sum_{i=1}^N \delta_i^2} \quad (44)$$

where δ is the distance between N pairs of equivalent heavy atoms from a reference starting position. RMSD provides a straightforward measure of structural drift and global stability over time and is routinely used to assess equilibration and conformational changes during MD simulations.

Radius of gyration

A parameter that provides a quantitative characterization of the size of a molecule is the radius of gyration R_g . For a given molecule of N atoms, the mean-square radius of gyration is defined as

$$\langle R_g^2 \rangle = \frac{1}{M} \langle [\sum_{i=1}^N m_i [r_i - R]^2] \rangle \quad (45)$$

where R is the center of mass of the molecule, r_i and m_i are the position and mass of the i^{th} atom, and M is the total mass of the molecule. This metric is particularly useful for distinguishing compact from expanded conformations and for monitoring folding or unfolding events.

Radial Distribution Function

The radial distribution function ($g(r)$, RDF) describes how the atomic density varies as a function of distance from a chosen reference point. It is defined as the ratio between the local number density at distance r and the average bulk density, providing a probability-based measure of spatial organization. In practice, the analysis is performed by selecting a reference point - typically the center of mass (COM) of a macromolecule or a specific residue - and computing how atoms of another species are distributed as the distance from this reference increases. Peaks in $g(r)$ indicate preferred interaction distances, while plateaus correspond to bulk-like behavior. RDF analysis therefore reveals solvation structure, preferred contact distances, and local ordering around biomolecules, complementing other structural descriptors used to characterize molecular complexes.

4. Case studies

4.1. Biophysical and docking study on the interaction of BRAFi/MEKi anticancer drugs with HSA

Introduction

According to data from the Surveillance, Epidemiology, and End Results (SEER) database, the National Cancer Institute (NCI), and the American Cancer Society (ACS), approximately 97,610 new cases of melanoma and 7,990 associated deaths were projected in the United States for the year 2023. Melanoma of the skin ranks as the fifth most common cancer, accounting for 5.2% of all newly diagnosed malignancies. In Europe, the European Cancer Information System (ECIS) estimated 106,369 new melanoma cases and 16,488 related deaths in the EU-27 countries in 2020, with projections indicating a 13.7% and 29.4% increase in incidence and mortality, respectively, by 2040. Cutaneous melanoma (CM) is the most lethal form of skin cancer, with a median age at diagnosis of 59 years. Prognosis depends primarily on the stage at detection, as defined by the American Joint Committee on Cancer (AJCC) TNM system, which classifies melanoma into stages I–IV based on tumor thickness and ulceration (T), lymph node involvement (N), and distant metastasis (M). Between 2011 and 2017, 5-year survival rates reached 99.4% for stage I–II, 68.0% for stage III, and 29.8% for stage IV, with only 4% of diagnoses occurring in stage IV.

Molecular characterization has revealed that nearly 50% of metastatic cutaneous melanomas harbor activating mutations in the BRAF gene, most commonly the V600E variant, followed by V600K, V600R, and rarer mutations such as V600A, V600M, and V600G. These mutations lead to constitutive activation of the mitogen-activated protein kinase (MAPK) pathway, promoting uncontrolled cell proliferation and survival. The discovery of this driver alteration revolutionized melanoma management through the development of selective BRAF inhibitors (BRAFi), such as Vemurafenib (VEM) and Dabrafenib (DAB), which received regulatory approval following pivotal clinical trials demonstrating substantial improvements in overall survival and progression-free survival compared to conventional chemotherapy. These targeted therapies have reshaped first-line treatment strategies and significantly improved clinical outcomes.

Despite their efficacy, BRAF inhibitors are associated with distinct adverse effects, including photosensitivity, fatigue, arthralgia, and cutaneous squamous cell carcinoma (cuSCC), occurring in 14–26% of patients typically within the first 2–3 months of treatment. This phenomenon is attributed to paradoxical MAPK activation in keratinocytes harboring preexisting RAS mutations. To mitigate these effects and delay resistance, combined inhibition of BRAF and MEK emerged as a rational

therapeutic strategy. Clinical and preclinical data have shown that this approach enhances antitumor efficacy, prolongs disease control, and reduces cuSCC incidence. The dual-inhibition paradigm has therefore become the standard of care for BRAFV600-mutant melanoma.

Currently approved BRAF/MEK inhibitor combinations include Encorafenib (ENC, Braftovi) plus Binimetinib (BINI, Mektovi), developed by Array BioPharma and approved by the FDA and EMA in 2018 for BRAFV600E/K-mutant melanoma based on the phase III COLUMBUS trial, which demonstrated a median overall survival (OS) of 33.6 months for the combination versus 23.5 months and 16.9 months for Encorafenib and Vemurafenib monotherapies, respectively. Another clinically relevant combination is Vemurafenib with Cobimetinib (COB, Cotellic), which achieved an objective response rate of 87% in BRAF inhibitor-naïve patients in a phase 1b trial. These results underscore the synergistic clinical impact of coordinated MAPK pathway suppression.

The pharmacokinetic (PK) and pharmacodynamic (PD) properties of anticancer agents, governed by absorption, distribution, metabolism, and excretion (ADME), play a decisive role in therapeutic efficacy and toxicity. A critical factor influencing these processes is plasma protein binding (PPB), which determines the fraction of drug available to reach its biological target. Among plasma proteins, human serum albumin (HSA) is the most abundant, synthesized in the liver and circulating at concentrations of approximately 7×10^{-4} M. HSA serves as a versatile carrier for endogenous and exogenous ligands, including fatty acids, hormones, bilirubin, and numerous drugs, modulating their solubility, transport, and bioavailability. Alterations in HSA–drug interactions can therefore have immediate consequences on systemic exposure and therapeutic window.

Both ENC and BINI exhibit high plasma protein binding, 86% and 97.2%, respectively, as do VEM and COB, suggesting potential competitive or synergistic interactions at HSA binding sites when coadministered. Such interactions can alter the unbound drug fraction, thereby impacting distribution, metabolism, and therapeutic outcomes. Additionally, HSA possesses esterase-like catalytic activity, contributing to drug biotransformation; thus, interference by therapeutic agents may have toxicological relevance. Understanding the interplay between drug–drug and drug–protein interactions is therefore essential for optimizing combination regimens.

Given the growing clinical use of BRAF and MEK inhibitor combinations, elucidating their binding behavior with HSA is essential for understanding drug–drug interactions, pharmacokinetics, and safety profiles. In this work, we present a combined experimental and computational investigation of the interactions between HSA and the clinically approved inhibitors Encorafenib (ENC), Binimetinib (BINI), Vemurafenib (VEM), and Cobimetinib (COB). Steady-state fluorescence quenching, isothermal titration calorimetry, and molecular simulations were employed to characterize the binding thermodynamics, kinetics, and site specificity of these interactions, while circular dichroism and

synchronous fluorescence spectroscopy were used to assess conformational changes induced in HSA upon drug binding. Collectively, these multi-technique results provide an integrated view of the molecular determinants governing HSA–drug recognition in targeted melanoma therapies and their potential implications for pharmacokinetic optimization and clinical efficacy.

Material and Methods

Reagents and Chemicals

Globulin and fatty acid-free human serum albumin (HSA, A1887, PubChem CID = 72941834) were obtained from Sigma-Aldrich Inc. (Saint Louis, MO, USA). Encorafenib (ENC, CID = 71735876), Binimetinib (BINI, CID = 25227658), Vemurafenib (VEM, CID = 42611257), Cobimetinib (COB, CID = 16222096), indomethacin (IND, CID = 3715), ketoprofen (KET, CID = 3825), and hemin (HEM, CID = 455658) were purchased from MedChemExpress (Monmouth, NJ, USA). All other reagents and solvents were of analytical grade and supplied by Sigma-Aldrich.

Sample Preparation and Analytical Procedures

Stock solutions of HSA were prepared by dissolving the protein in phosphate-buffered saline (PBS, pH 7.4; 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄) at 25 °C. The protein concentration was determined by UV–vis spectroscopy at $\lambda = 280$ nm using a molar extinction coefficient (ϵ_{280}) of 35,700 M⁻¹ cm⁻¹. This approach follows standard procedures and the absorption theory outlined in Section 3.1.1.

Stock solutions of ENC, BINI, VEM, and COB were prepared in ethanol or DMSO, depending on solubility, while IND, KET, and HEM were prepared in DMSO. All solutions were stored in the dark at 4 °C until use. Control experiments confirmed that the solvent concentrations used (<1% v/v) did not alter the spectral properties of HSA.

Ultraviolet–Visible (UV-Vis) Spectroscopy

UV–Vis absorption spectra of HSA and the studied drugs were recorded on a Jasco V-730 spectrophotometer (Jasco, Japan) at 25 °C. Spectra were collected in the range of 200–500 nm to monitor potential complex formation and to detect any ligand-induced perturbations in HSA's electronic transitions, as previously described in Section 3.1.1. No additional mathematical treatment beyond baseline correction and standard normalization was required.

Steady-State Fluorescence Spectroscopy

Steady-state fluorescence (SSF) measurements were performed on a Jasco FP-8350 spectrofluorometer or a Jasco J-1500 spectropolarimeter equipped with a thermostatic cell holder and

a 1 cm quartz cuvette. The excitation wavelength (λ_{exc}) was set at 280 nm, and emission spectra were recorded in the 300–450 nm range using 5/10 nm slit widths at 25, 31, and 37 °C.

The HSA concentration was maintained at 2 μM , while the concentration of each drug (ENC, BINI, VEM, or COB) varied as follows: 1, 2, 3, 4, 5, 6, 10, 20, and 40 μM . Samples were incubated for 2 h in the dark prior to measurement. Because several of the selected drugs exhibit partial absorption at the excitation and/or emission wavelengths, fluorescence intensities were corrected for the inner filter effect following the procedure described in Section 3.1.2. In this correction, the absorbances used correspond to A_{exc} at 280 nm and A_{em} at 343 nm, which is the emission maximum of HSA under the chosen excitation conditions. Only the corrected fluorescence values were used for subsequent quenching and binding analyses.

Synchronous and Three-Dimensional Fluorescence Spectroscopy

Synchronous fluorescence spectroscopy (SFS) was performed with scanning intervals ($\Delta\lambda$) of 15 nm and 60 nm to selectively probe the microenvironment of tyrosine and tryptophan residues, respectively. Excitation and emission slit widths were set to 5 and 10 nm. Each HSA sample (2 μM) was titrated with increasing concentrations (0–40 μM) of the tested drugs.

Three-dimensional (3D) fluorescence spectra were acquired with emission wavelengths scanned between 220 and 500 nm, and excitation wavelengths increased from 220 to 350 nm in 5 nm steps at 25°C. Samples containing HSA (2 μM) with VEM (4 μM) or COB (40 μM) were incubated for 2 h prior to analysis.

Isothermal Titration Calorimetry

Isothermal titration calorimetry (ITC) experiments were performed using a MicroCal PEAQ-ITC microcalorimeter (Malvern, UK) at 25 °C (cell volume = 208 μL). The sample cell contained a 20 μM HSA solution in PBS, while the syringe was filled with a 200 μM solution of the corresponding inhibitor (ENC, BINI, VEM, or COB). All solutions and buffers were degassed for 30 min before titration to prevent bubble formation and baseline artifacts. Each titration consisted of 18–19 sequential injections (2 μL each) under stirring at 750 rpm.

Control experiments were performed to measure heats of dilution, and these values were subtracted from the raw thermograms before fitting. All titrations were performed in triplicate to ensure reproducibility. Data acquisition and analysis were conducted using the MicroCal PEAQ-ITC analysis software and nonlinear fitting of binding isotherms was performed using GraphPad Prism 8.0.0 (GraphPad Software, San Diego, CA, USA).

Far-UV Circular Dichroism (CD) Spectroscopy

Far-UV CD spectra were acquired on a Jasco J-1500 spectropolarimeter equipped with a thermostatic cell holder and PML-534 FDCD detector at 25 °C. Spectra were recorded from 200 to 260 nm (scan rate: 20 nm min⁻¹, step size: 0.5 nm, bandwidth: 1 nm) using a 0.2 cm quartz cell.

Baseline-corrected spectra were obtained by averaging three consecutive scans, and results were expressed as mean residue ellipticity (MRE) using the standard conversion described in Section 3.1.4. For these calculations, the following experimental parameters were used: protein concentration 2 µM, number of residues 585, and optical path length: 0.1 cm. The α -helical content was estimated from the MRE value at 208 nm, following the procedure outlined in Section 3.1.4, and further refined through secondary structure deconvolution using the CD Multivariate SSE program with standard calibration models.

Competitive Site Marker Displacement Experiments

Competitive binding assays were carried out using established HSA site markers: indomethacin (IND) for Sudlow's site I (subdomain IIA), ketoprofen (KET) for Sudlow's site II (subdomain IIIA), and hemin (HEM) for the heme binding site (subdomain IB). Each marker (2 µM) was preincubated with HSA (2 µM), followed by incremental addition of the test drug (ENC, BINI, VEM, or COB; 0–40 µM). Fluorescence spectra were collected following the same procedures described in Section *Steady-State Fluorescence Spectroscopy* above, and changes in fluorescence intensity were evaluated to assess site-specific displacement by each inhibitor.

Esterase-Like Enzymatic Activity Assay

The esterase-like activity of HSA and its inhibition by VEM and COB were assessed using p-nitrophenyl acetate (p-NPA, CID = 13243) as a substrate. The hydrolysis of p-NPA was monitored at 405 nm on a UV–vis spectrophotometer. The assay mixture contained 5 µM HSA and 0–0.8 mM substrate, in the absence or presence of 25 or 50 µM of the tested drug. Initial reaction velocities (v_0) were obtained from the linear portion of the absorbance–time traces and analysed according to the Michaelis–Menten formalism, as described in Section 3.1.1. Kinetic parameters ($V_{\max}$, K_m) were derived by nonlinear fitting to the Michaelis–Menten model and further checked using Lineweaver–Burk representations of the same data (Section 3.1.1).

Drug-induced changes in $V_{\max}$ and K_m were used to assess the inhibition of HSA's esterase-like activity and to infer the underlying mode of inhibition.

Molecular Simulations

Docking Studies

Docking simulations were conducted using AutoDock Vina. The crystal structure of HSA (PDB ID: 4K2C) was retrieved from the RCSB Protein Data Bank, and hydrogen atoms were added using the H++ web server. Ligand structures were generated and energy-minimized using Avogadro.

Docking was focused on subdomain IIA (Sudlow's site I) for ENC/BINI and subdomain IIIA (Sudlow's site II) for VEM/COB, as indicated by competition experiments. The exhaustiveness parameter was set to 25. The top-ranked binding conformations were used for subsequent molecular dynamics (MD) simulations. Visualization and figure preparation were performed using UCSF Chimera.

Molecular Dynamics (MD) Simulations

MD simulations were performed with AMBER22 using the ff14SB force field for HSA and gaff2 parameters for the ligands. Partial charges were derived via the RESP method using the RED server. The systems were solvated in TIP3P water boxes with a 0.15 M NaCl concentration.

After energy minimization (steepest descent and conjugate gradient, 6000 steps), equilibration was carried out under NVT and NPT conditions with gradual heating from 150 K to 298 K, followed by five 100 ps restrained simulations. Production runs (50–500 ns) were conducted using the pmemd.cuda module of AMBER on the CINECA Leonardo supercomputer and an in-house GPU/CPU cluster. The PME method was applied for long-range electrostatics, with SHAKE constraints and a 2 fs timestep.

Metadynamics Simulations

The frequency-adaptive metadynamics (FaMetaD) method implemented in PLUMED v2.9.1 was used to evaluate the unbinding kinetics and residence time of COB from HSA. Two reaction coordinates were defined:

1. the distance between the ligand center of mass and the residues forming the binding pocket,
2. the local hydration state (number of water molecules within 8 Å).

Gaussian widths were set to 0.05 Å (RC1) and 0.1 (RC2), with an initial hill height of 1 kcal/mol. Fifteen independent replicas were performed, and results were analyzed via bootstrap and Kolmogorov–Smirnov tests to validate Poisson-distributed unbinding times.

Data Analysis

All experiments were conducted in triplicate, and results are presented as mean $\pm$ standard deviation (SD). Statistical significance was assessed using the Student's *t*-test, with $p \leq 0.05$ considered

significant. Data processing and graph generation were performed using GraphPad Prism 8.0.0 (GraphPad Software, San Diego, CA, USA).

4.1.1. Biophysical and docking study on the interaction of anticancer drugs encorafenib and binimetinib with human serum albumin¹

Results and Discussion

Steady-state fluorescence quenching of HSA

The SSF quenching of aromatic amino acids is a proficient approach for investigating the binding affinity and mechanism underlying ligand-protein interactions. Typically, upon HSA excitation at a wavelength (λ) of 280 nm, the fluorescence spectrum consists of tryptophan and tyrosine residues. However, HSA displays a dominant emission maximum at $\lambda = 343$ nm, arising almost entirely from its single tryptophan residue (W214), located within Sudlow's site I in subdomain IIA. Upon gradual addition of ENC or BINI within the 1-40 μ M concentration range (in Figure 4.1.1), the fluorescence intensity of HSA decreased progressively at 343 nm, while neither the spectral profile nor the position of the emission maximum exhibited any significant shift. This behavior indicates that the microenvironment of W214 is perturbed upon drug binding, consistent with static quenching and local conformational rearrangements within Sudlow's site I. The observed decrease in fluorescence intensity therefore reflects energy transfer and direct interaction between HSA and the kinase inhibitors.

¹ The material presented in this chapter was published at: "Biophysical and docking study on the interaction of anticancer drugs encorafenib and binimetinib with human serum albumin". G. Cavalieri, G. Cilurzo, L. Pettorosso, A. Mansueto, E. Laurini, S. Priol, European Journal of Pharmaceutical Sciences 2023, 189, 106550.

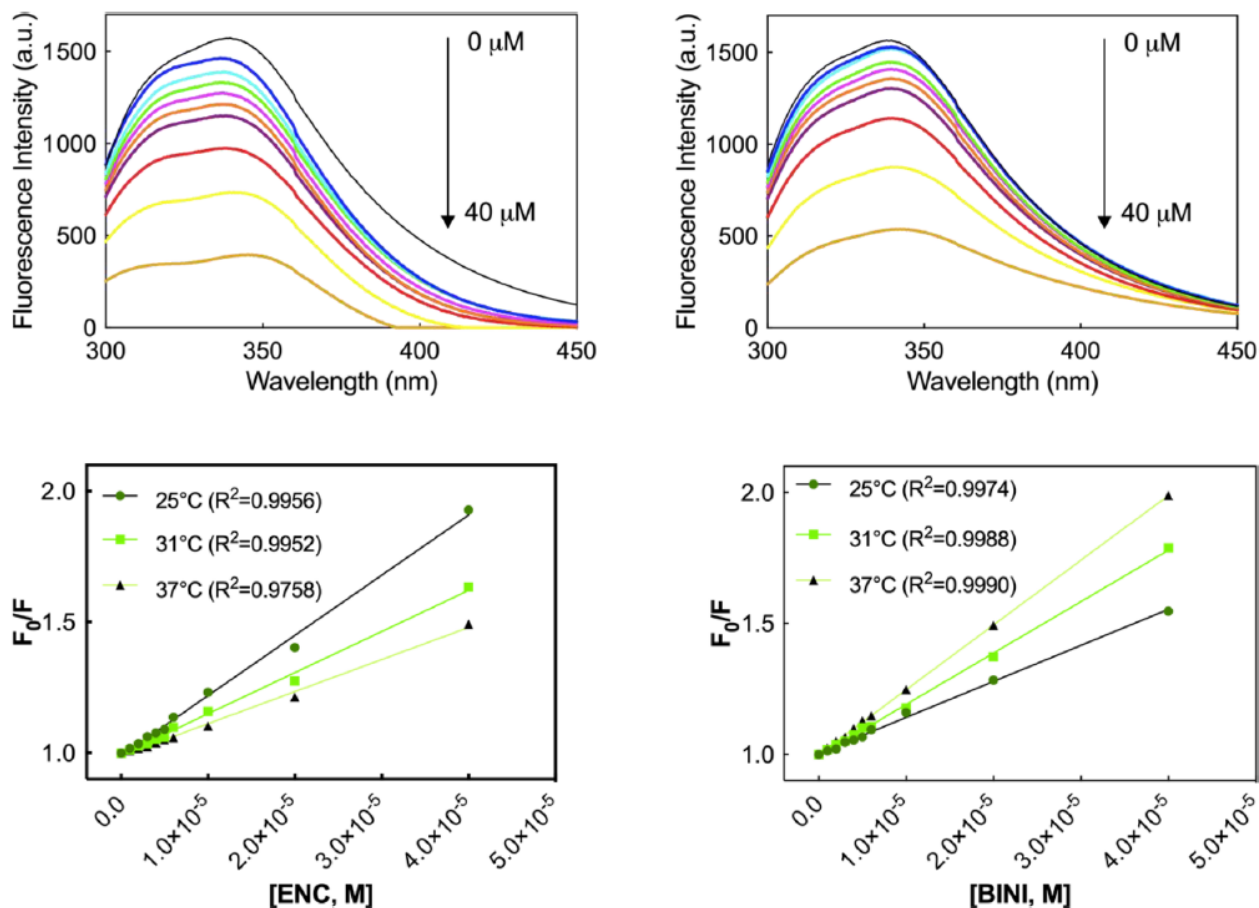


Figure 4.1.1. (Top panel) Steady-state fluorescence quenching of HSA (2 μM) in the presence of increasing concentrations (0–40 μM) of ENC (left) and BINI (right) (PBS buffer, pH = 7.4, $T = 25\text{ }^\circ\text{C}$, and $\lambda_{\text{exc}} = 280\text{ nm}$). **(Bottom panel)** Stern-Volmer plots for HSA fluorescence quenching induced by ENC (left) and BINI (right) at three different temperatures (25 $^\circ\text{C}$, 31 $^\circ\text{C}$, and 37 $^\circ\text{C}$).

To further investigate the quenching mechanism governing the interaction of ENC and BINI and HSA, the protein SSF quenching data recorded at 25 $^\circ\text{C}$, 31 $^\circ\text{C}$, and 37 $^\circ\text{C}$ were analyzed using the Stern-Volmer equation (Eq. (5) in Section 3.1.2).

As expected for static, binding-related quenching, k_q values exceeding $2.06 \times 10^{10}\text{ M}^{-1}\text{ s}^{-1}$ were observed for both inhibitors at all temperature considered (Table 4.1.1), confirming that the fluorescence attenuation arises from complex formation rather than collisional processes.

The Stern-Volmer plots for ENC and BINI are shown in Figure 4.1.1 (lower panels), while the corresponding calculated values of K_{SV} and k_q are listed in Table 4.1.1. For ENC/HSA, both K_{SV} and k_q decrease with increasing temperature, consistent with the classical behavior of static quenching. In contrast, the BINI/HSA system displays increasing K_{SV} and k_q values as temperature rises, suggesting that although quenching remains static in nature, entropy makes a substantial contribution to complex formation and stabilization (Figure 4.1.1 and Table 4.1.1).

ENC			BINI		
T (°C)	K_{sv} ($10^4 M^{-1}$)	K_q ($10^{12} M^{-1}s^{-1}$)	T (°C)	K_{sv} ($10^4 M^{-1}$)	k_q ($10^{12} M^{-1}s^{-1}$)
25	2.30 ± 0.07	3.89	25	1.38 ± 0.06	2.33
31	1.57 ± 0.04	2.66	31	1.96 ± 0.06	3.33
37	1.23 ± 0.08	2.08	37	2.47 ± 0.09	4.19

Table 4.1.1. Stern-Volmer constants (K_{sv}) and bimolecular quenching rate constants (k_q) for HSA tryptophan fluorescence quenching by ENC and BINI at different temperatures.

Binding parameters and thermodynamic parameters analysis

Given that static quenching was established for both inhibitors, the binding constant (K_a) and the corresponding stoichiometry of ligand binding n were evaluated using the double-logarithmic version of the Stern-Volmer equation described in Section 3.1.2.

Linear fitting of the double-logarithmic plots (Eqs. (6) and (7), Section 3.1.2) (Figure 4.1.2, upper panel) yielded the K_a and n values reported in Table 4.1.2. For both ENC and BINI, n values were consistently close to 1 at all temperatures, indicating the presence of a single dominant binding site on HSA for each inhibitor. The K_a values for ENC and BINI were of the order of $10^4 M^{-1}$ (Table 4.1.2), consistent with moderate affinity, a characteristic that facilitates reversible binding and efficient plasma transport. Interestingly, the temperature dependence of K_a differed between the two systems. Specifically, for ENC/HSA K_a decreased with increasing temperature, as expected for classical enthalpy-driven binding whereas for BINI/HSA, by contrast, K_a increased as temperature rose, suggesting that the association is entropy-favored. This opposite thermal behavior reinforces the view that BINI–HSA binding is stabilized predominantly by hydrophobic or disorder-enhancing contributions, whereas ENC–HSA binding follows the canonical enthalpy-driven profile.

Ligand	T (°C)	K_a ($10^4 M^{-1}$)	n (-)	ΔH (kcal/mol)	ΔS (cal/mol K)	ΔG (kcal/mol)
ENC	25	2.31 ± 0.07	1.08	-4.09	+6.19	-5.93
	31	1.85 ± 0.05	1.03			-5.97
	37	1.77 ± 0.06	1.05			-6.01
BINI	25	2.06 ± 0.04	1.09	+2.01	+26.6	-5.87
	31	2.15 ± 0.03	1.07			-6.03
	37	2.35 ± 0.05	1.04			-6.19

Table 4.1.2. Binding parameters of HSA with ENC and BINI derived from fluorescence quenching data using Eq. (7) and from the vant' Hoff plots (Figure 4.1.2, lower panels). The values of ΔG are derived from the ΔH and ΔS values obtained from the corresponding van't Hoff plots (Eq. (9)) using Eq. (10).

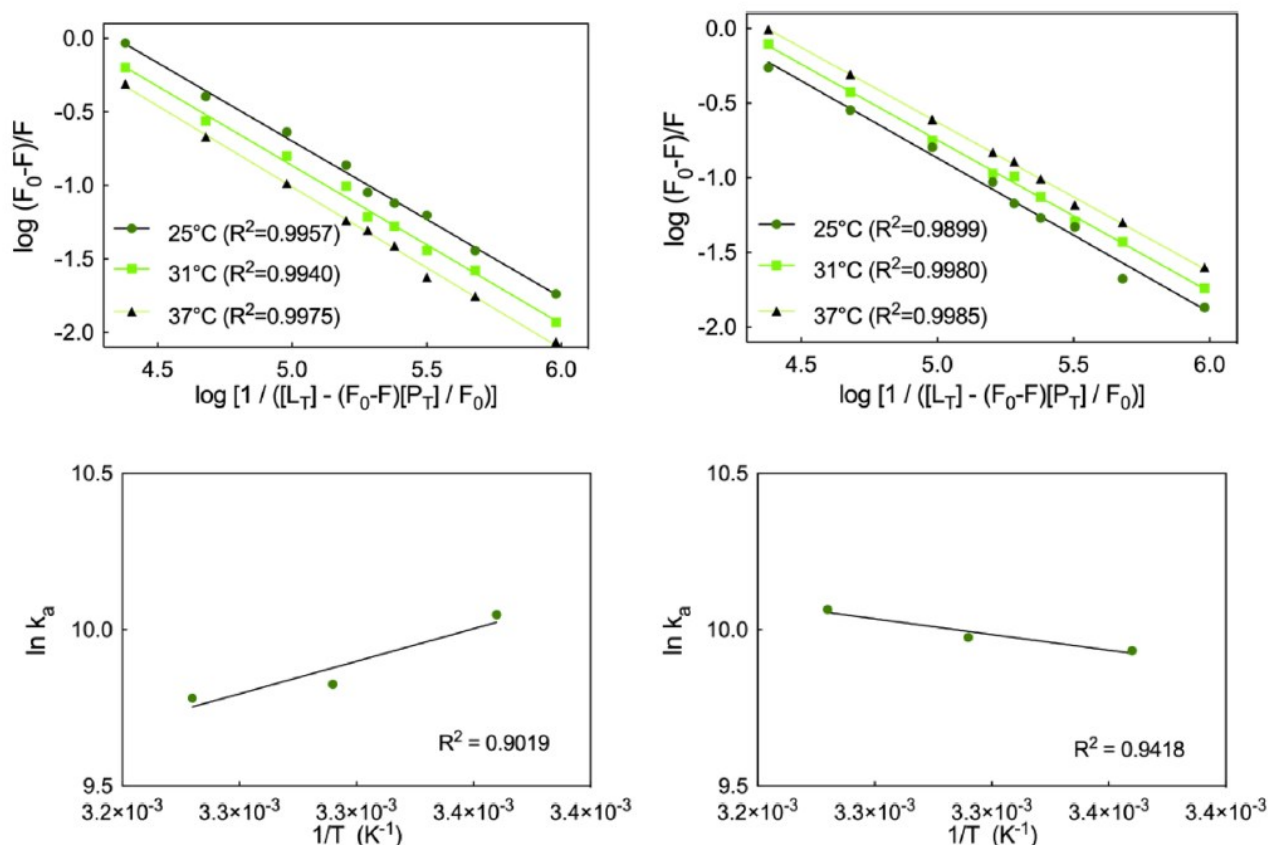


Figure 4.1.2. (Upper panel) Plots of $\log(F_0-F)$ vs. $\log[1/([L_T] - (F_0-F)[P_T]/F_0)]$ for HSA fluorescence quenching by ENC (left) and BINI (right) at three different temperatures (25 °C, 31 °C, and 37 °C). **(Bottom panel)** The van't Hoff plots ($\ln K_a$ vs $1/T$, see Eq. (9)) for ENC/HSA (left) and BINI/HSA (right) binding interactions.

Thermodynamic parameters offer essential insight into the intermolecular forces stabilizing ligand–protein complexes. Hydrophobic interactions, van der Waals forces, electrostatic interactions, and bonding are typically the dominant factors governing these associations. Consequently, to further elucidate the energetic nature of ENC/HSA and BINI/HSA binding, the variation in binding enthalpy (ΔH), entropy (ΔS) and free energy (ΔG) were obtained using the van't Hoff analysis described in Section 3.1.2 based on the linear relationship between $\ln K_a$ and $1/T$.

From the slopes and intercepts of the van't Hoff plots (Figure 4.1.2, lower panels). ΔH and ΔS were extracted, and the corresponding Gibbs free energy changes (ΔG) were computed as $\Delta G = \Delta H - T\Delta S$. The resulting parameters are summarized in Table 4.1.2.

The negative values of ΔG obtained for both systems confirm that binding of ENC and BINI to HSA is spontaneous and thermodynamically favorable at all investigated temperatures. For the BINI/ HSA complex, the positive ΔH value (+2.01 kcal/mol) denotes that the binding event is endothermic in agreement with the experimentally observed increase in K_a as temperature rises. (Table 4.1.2). The large positive ΔS value (+26.5 cal/(mol K)) suggests that hydrophobic interactions dominate the

stabilization of this complex, consistent with entropy-driven association strengthened at higher temperatures. In contrast, the ENC/HSA interaction exhibits a favorable enthalpic contribution ($\Delta H = -4.09$ kcal/mol, Table 4.1.2), pointing to stabilizing hydrogen bonding and/or polar interactions. At the same time, however, the positive entropy change ($\Delta S = +6.19$ cal/(mol K)) also suggests that hydrophobic forces contribute meaningfully, resulting in an overall stabilization mechanism that reflects a balanced interplay of enthalpic and entropic factors.

ITC binding analysis

ITC experiments were performed to quantify the binding thermodynamics of BINI and ENC to HSA at 25 °C, and the resulting isotherms are shown in Figure 4.1.3, with the fitted parameters summarized in Table 4.1.3. Both inhibitors displayed a clear 1:1 binding stoichiometry, in agreement with the SSF-derived values and consistent with occupation of a single dominant site on the protein. Specifically, for the ENC/HSA complex, the integrated heat profile yielded $K_a = 2.14 \times 10^4 M^{-1}$, $\Delta G = -5.91$ kcal/mol, and $n = 1.09$, whereas for the BINI/HSA ensemble the corresponding values were $K_a = 1.48 \times 10^4 M^{-1}$, $\Delta G = -5.69$ kcal/mol, and $n = 1.13$ (Table 4.1.3). These values show excellent agreement with those obtained from SSF quenching experiments (Table 4.1.2), thus reinforcing the reliability of the fluorescence-derived binding constants and stoichiometries. The ITC-derived enthalpy and entropy contributions further validate the mechanistic picture inferred from SSF. Thus, for ENC/HSA, the binding enthalpy is negative ($\Delta H = -4.24$ kcal mol⁻¹) and its magnitude exceeds the positive entropic contribution ($T\Delta S = +1.67$ kcal mol⁻¹). This indicates an enthalpy-driven association, consistent with stabilizing hydrogen bonding and polar interactions, complemented by a secondary but favorable hydrophobic component. In contrast, BINI/HSA binding exhibits ΔH and $T\Delta S$ positive values, indicating an endothermic yet entropy-driven process. In fact, the entropic term ($T\Delta S = +8.22$ kcal mol⁻¹) is substantially larger than the corresponding enthalpic penalty ($\Delta H = +2.53$ kcal mol⁻¹), confirming that hydrophobic interactions dominate the stabilization of this complex. Accordingly, the strongly favorable entropy term results in $\Delta G < 0$, rendering the overall binding process thermodynamically spontaneous.

Ligand	K_a ($10^4 M^{-1}$)	n (-)	ΔH (kcal/mol)	$T\Delta S$ (cal/mol)	ΔS (cal/mol K)	ΔG (kcal/mol)
ENC	2.14 ± 0.18	1.09 ± 0.11	-4.24 ± 0.08	+1.67	+5.60	-5.91
BINI	1.48 ± 0.21	1.13 ± 0.15	2.53 ± 0.16	+8.22	+27.6	-5.69

Table 4.1.3. Thermodynamic parameters (ΔH , $T\Delta S$, ΔG , n , K_a) involved in the formation of drug/HSA complexes as obtained from ITC experiments at 25 °C. All ITC experiments were in run in triplicate. Errors on ΔH are within 10%.

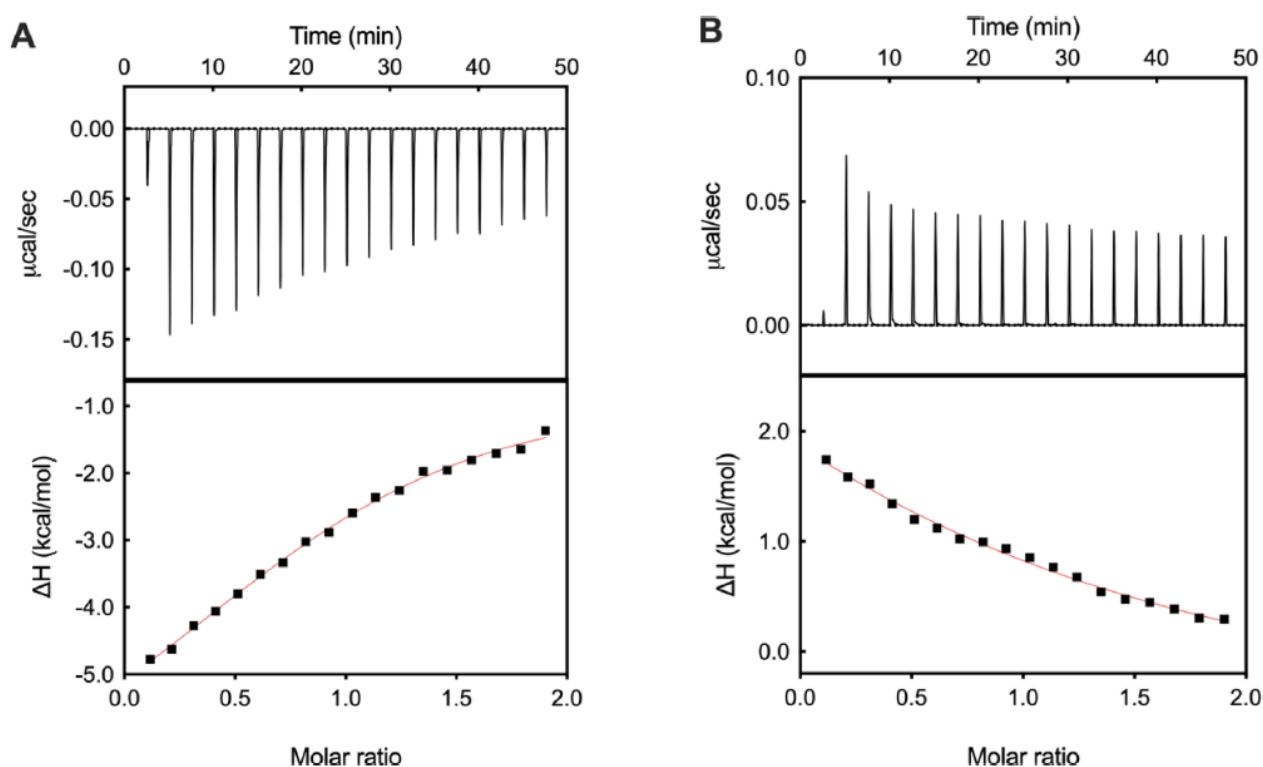


Figure 4.1.3. ITC raw data (**upper panels**) and plots of the integrated ITC data (**bottom panels**) for the interaction between ENC (A) and BINI (B) with HSA at 25 °C ([HSA] = 20 μM , PBS buffer, pH = 7.4). The solid red line corresponds to the data fitting with the one-site (1:1 binding stoichiometry) isothermal binding model.

Synchronous fluorescence spectra of HSA in presence of ENC and BINI

SFS can provide sensitive information on the on the local microenvironment of protein aromatic residues and shifts in the synchronous maximum wavelength (λ_{max}) are routinely used to probe polarity changes around tyrosine ($\Delta\lambda = 15$ nm) and tryptophane ($\Delta\lambda = 60$ nm). Figure 4.1.4 shows the SFS spectra of HSA recorded in the presence of increasing concentrations of ENC and BINI at both wavelength offsets. For both inhibitors, a progressive decrease in fluorescence intensity was observed, in agreement with the SSF quenching results. However, subtle but significant differences emerged between the two systems. In fact, In the ENC/HSA complex, a red shift of λ_{max} is evident at both $\Delta\lambda$ values, indicating that ENC increases the polarity of the microenvironment surrounding both Y and W residues (Figure 4.1.4, left panels). In contrast, for the BINI/HSA system, a slight red shift is detected in λ_{max} only at $\Delta\lambda = 60$ nm, with no appreciable change at $\Delta\lambda = 15$ nm (Figure 4.1.4, right panels). This suggests that BINI selectively perturbs the microenvironment of W214, with minimal influence on tyrosine residues.

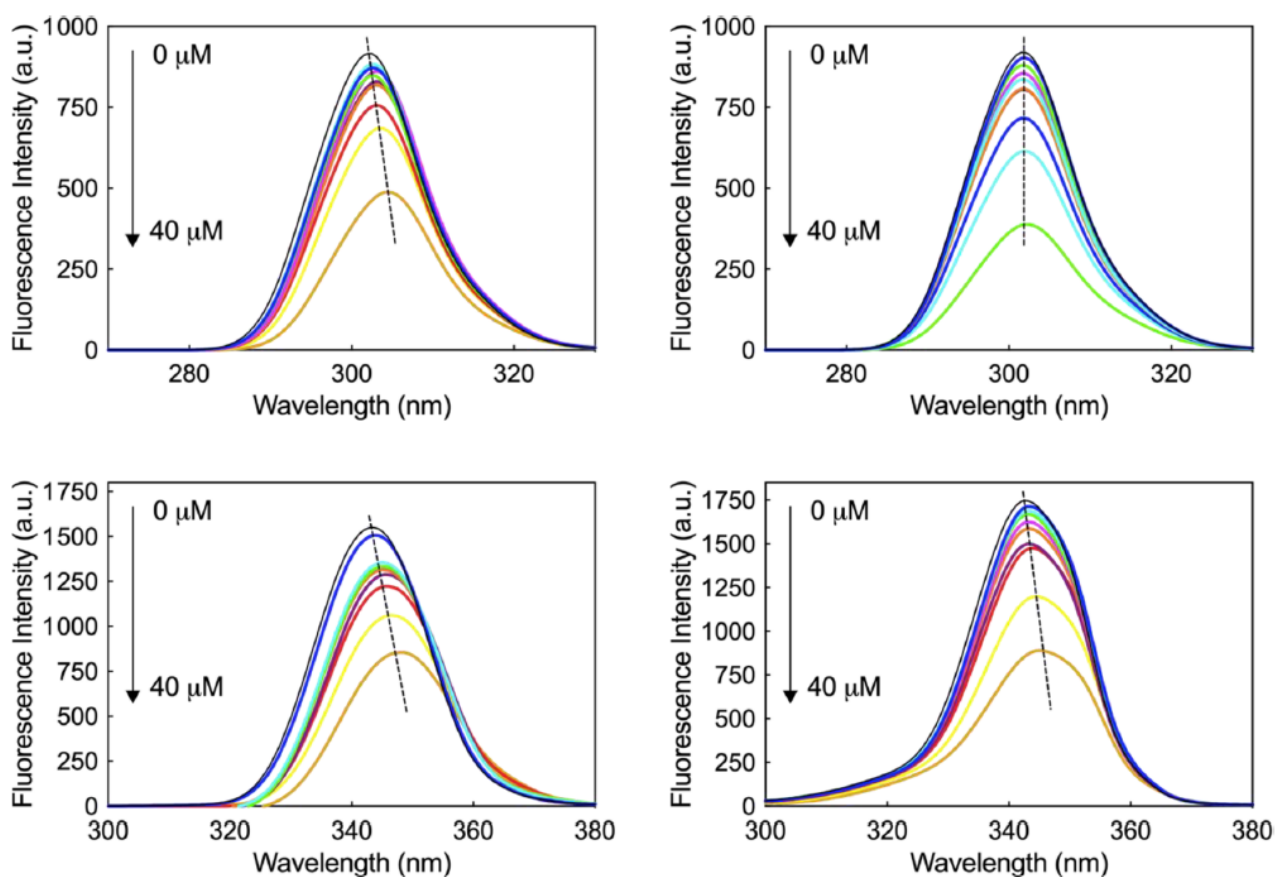


Figure 4.1.4. The synchronous fluorescence spectra of HSA (2 μM) at $\Delta\lambda = 15$ (**upper panels**) and 60 nm (**bottom panels**) in the presence of ENC (left) or BINI (right) at 25 $^{\circ}\text{C}$.

Structural analysis of the ENC/HSA and BINI/HSA complexes

Circular dichroism spectroscopy

Circular dichroism is a highly sensitive technique used to monitor conformational changes in proteins. The CD spectrum of pure HSA displays two negative bands ($\lambda = 208$ nm and 222, nm, respectively), which are indicative of the α -helical structures of the protein. The former band arises from the exciton splitting of electronic transitions from the peptide amide nonbonding orbital π_{nb} to the antibonding orbital π^* , while the latter results from the electronic transition from an oxygen lone pair orbital n to the π^* orbital. To investigate the potential impact of ENC and BINI binding on the secondary structure of this serum protein, far-UV CD measurements were performed on pristine HSA and in the presence of each inhibitor at 25 $^{\circ}\text{C}$ and at three different drug/protein molar ratios (0.5, 1, and 2), as shown in Figure 4.1.5. Intriguingly, for both the ENC/HSA and BINI/HSA systems the CD spectra did not exhibit any noteworthy alterations compared with HSA alone, except for the complex with the BRAFi at its maximum concentration (Figure 4.1.5). Even in this case, however, only a minor decrease in band intensity was observed, with no detectable shift in the band minimum upon addition of ENC. This indicates that, overall, the protein's secondary structure is only slightly affected. To corroborate

this observation, the estimated content of α -helical motifs (% α -helix) of free and drug-bound HSA were compared. The values reported in Table 4.1.4 are consistent with literature data and confirm that the α -helical composition of HSA remains substantially unchanged upon binding of either drug at all concentration tested. The only exception is the BRAFi at the highest drug/protein molar ratio, for which a slight decrease in α -helical content (56.1%) was estimated.

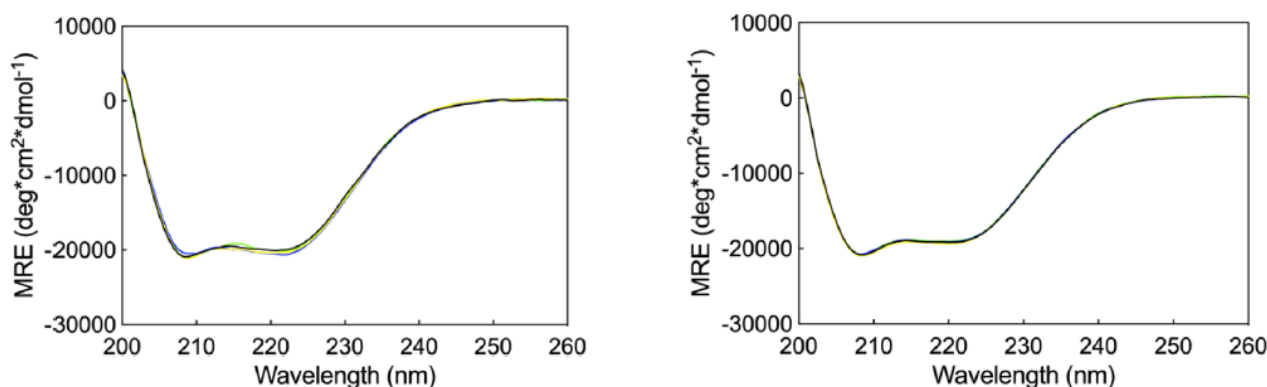


Figure 4.1.5. Far-UV CD spectra of HSA alone (black line) and in the presence of ENC (**left**) and BINI (**right**) in a molar ratio of 0.5 (yellow line), 1 (green line) and 2 (blue line). The CD experiments were carried out in PBS buffer solution, at 25 °C and at [HSA] = 2 μ M.

Ligand	Ligand/HSA Molar Ratio	α -helix (%)
-	-	58.0 ± 0.3
ENC	0.5	58.3 ± 0.2
	1	58.0 ± 0.1
	2	56.1 ± 0.4
BINI	0.5	58.4 ± 0.1
	1	57.8 ± 0.1
	2	57.7 ± 0.2

Table 4.1.4. α -helical content (% α -helix, $\pm$ standard errors) of HSA in the absence and in presence of ENC and BINI calculated from CD experiments at 25 °C using Eqs. (14) and (15).

Drug displacement experiments

Site-selective probes were used to conduct displacement experiments aimed at identifying the specific binding site for ENC and BINI in the structure of HSA. As already discussed in Section 3.1.2, HSA subdomains IIA and IIIA correspond to Sudlow's sites I and II, respectively, both of which are known to exhibit high affinity for a wide range of pharmaceutical compounds. In addition, previous studies have reported that subdomain IB contains an additional binding cavity, commonly referred to as drug binding site III. In this study, we purposely selected three active molecules, indomethacin (IND),

ketoprofen (KET), and hemin (HEM), which serve as established site markers for binding sites I, II, and III, respectively. We then recorded the fluorescence emission spectra obtained during the titration of each premixed HSA-site marker solutions with progressively increasing concentrations of ENC or BINI. Utilizing Eq. (7), the resulting data were fitted to assess the influence of each site markers on the binding of the BRAFi and MEKi to HSA as determined from the binding constants K_a listed in Table 4.1.5.

	ENC	BINI
<i>Site Marker</i>	$K_a (10^4 M^{-1})$	$K_a (10^4 M^{-1})$
none	2.31 ± 0.07	2.06 ± 0.04
IND	1.86 ± 0.04	1.57 ± 0.07
KET	2.03 ± 0.03	2.37 ± 0.11
HEM	2.01 ± 0.09	2.35 ± 0.12
<i>Competitor</i>	$K_a (10^4 M^{-1})$	$K_a (10^4 M^{-1})$
ENC	-	1.26 ± 0.10
BINI	1.35 ± 0.09	-

Table 4.1.5. Binding constants for ENC and BINI to HSA derived from fluorescence quenching data obtained at 25 °C in the absence and presence of IND, KET, HEM using Eq. (7).

As shown in Table 4.1.5, the K_a values of ENC and BINI in the presence of IND are significantly reduced compared to those obtained for the free protein. In contrast, their affinity remains largely unaffected in the presence of the other site markers KET and HEM. To further support these observations, additional experiments were conducted to examine the competitive interplay between ENC and BINI in their binding to HSA. Consistent with Table 4.1.5, a clear reduction in binding capacity is observed when each inhibitor is titrated in the presence of a fixed concentration of the alternative drug, compared with the corresponding isolated inhibitor/HSA system. These results indicate that the two antitumoral agents possess comparable ability to compete for occupancy of the same binding region within the HSA structure, where they mutually impair each other's binding. Overall, the data strongly suggests a displacement interaction between ENC and BINI during their association with HSA, and identify Sudlow's site I, located within subdomain IIA, as the most probable binding cavity for both compounds.

Molecular simulations studies of HSA binding with ENC and BINI

To obtain detailed molecular insights into the interactions between ENC and BINI with HSA, the results obtained from displacement experiments (see Section *Drug displacement experiments*) were used to guide molecular dynamics (MD) simulations of the BRAFi and the MEKi within the IIA

protein subdomain (Figure 4.1.6). Molecular docking was first employed to position ENC and BINI inside the experimentally identified binding pocket, and the most favorable docking pose for each compound was subsequently used as the starting point for MD simulations. This computational approach was applied to qualitatively describe the binding mechanism of the HSA/ ENC and HSA/BINI complexes and to evaluate any potential structural effects induced by the drugs on the protein. Beginning with the BRAF inhibitor, the ENC/HSA complex is characterized by the presence of a permanent hydrogen bond between the sp³ carbamate oxygen atom of ENC and the hydroxylic group of the side chain of Y150 of HSA (Figure 4.1.6, upper left). Additional stabilizing polar interactions are observed between the hydrophilic side chains of K195 and S287 and the pyrazole moiety of the drug and the carbamate group of the drug, respectively. The complex is further stabilized by the presence of stable π -cation interactions involving the chloro- and fluoro-substituted aromatic ring of ENC and the positively charged side chains of R218 and R222. Finally, throughout the entire timeframe of the MD simulation, a network of hydrophobic and van der Waals interactions are also consistently detected between the apolar functional groups of ENC and the lipophilic side chains of the albumin residues L198, F211, W214, A215, L238, and V455. These findings align with the binding thermodynamics obtained from fluorescence spectroscopy and ITC measurements (see Sections *Binding parameters and thermodynamic parameters analysis* and *ITC binding analysis*), which indicate a predominantly enthalpic contribution to the ENC–HSA interaction. Moreover, the direct contacts with Y150 and W214 identified in the simulations further corroborate the results of the SFS analysis (see Section *Synchronous fluorescence spectra of HSA in the presence of ENC and BINI*).

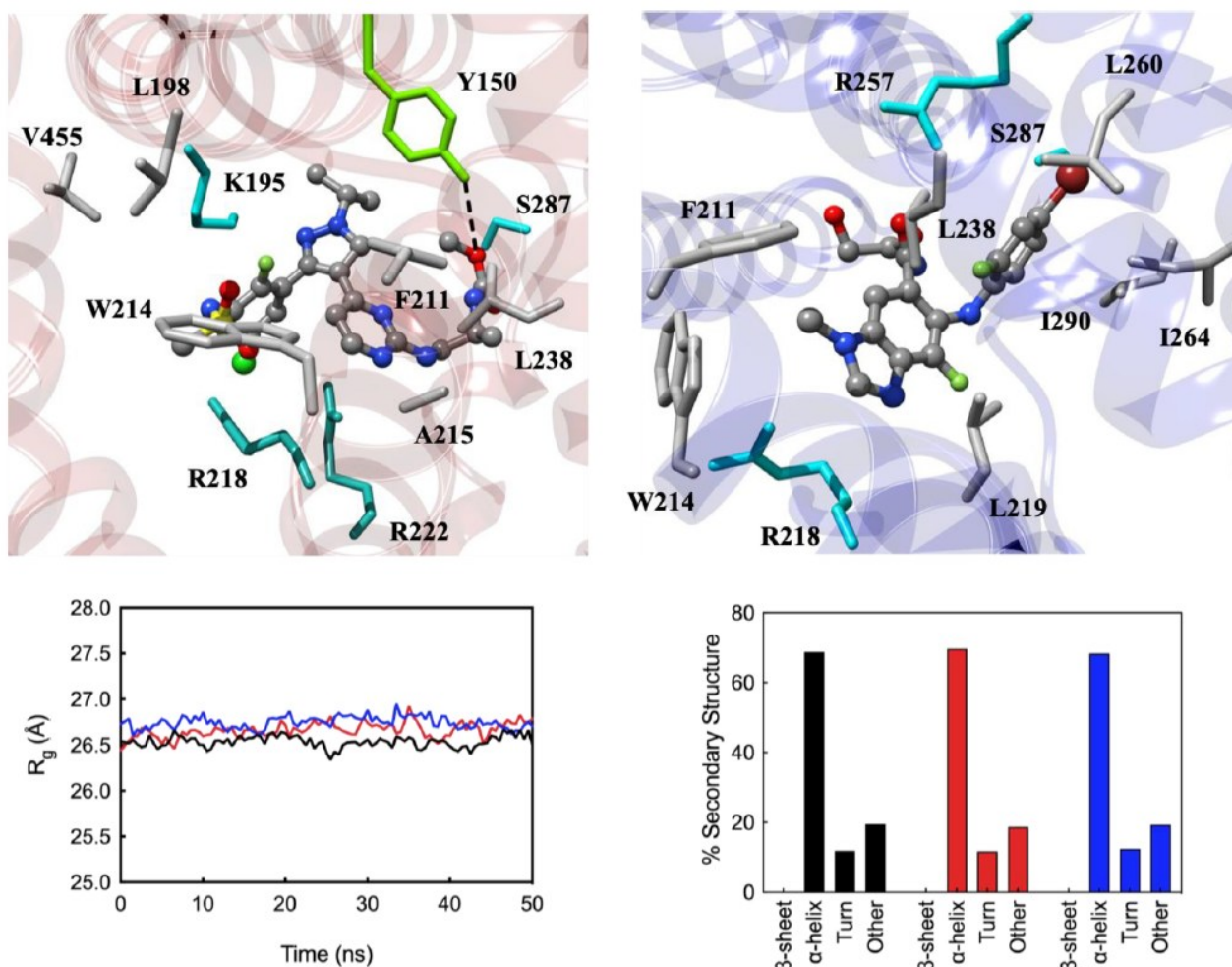


Figure 4.1.6. (Upper panels) Binding of ENC (left) and BINI (right) in the Sudlow's site I (subdomain IIA) of HSA. The two compounds are depicted as atom-colored sticks (C, grey; O, red; N, blue; S, yellow; F, light green; Cl, forest green), while the protein is shown in transparent ribbons. HSA residues mainly involved in drug binding are highlighted and labelled. Hydrogen bonds are indicated by broken black lines and labelled. Hydrogen atoms, water molecules, ions, and counterions are not shown for clarity. **(Lower panels)** Plot of the radius of gyration (left) as a function of the simulation time for free HSA (black) and in complex with ENC (red) and BINI (blue), respectively. Plot of the HSA secondary structure estimation (right) averaged during the 50 ns of MD simulation for free HSA (black) and in complex with ENC (red) and BINI (blue), respectively.

Moving to the complex between HSA and the MEK inhibitor, the upper right panel of Figure 4.1.6 shows that, although ENC and BINI occupy the same binding pocket, the network of stabilizing differs substantially. During the corresponding MD simulation, in fact, the 4-bromo-2-fluorobenzene ring of BINI remains nestled within a hydrophobic cavity formed by the side chains of HSA residues L238, L260, I264 and I290, while the benzimidazole moiety is strongly stabilized by hydrophobic contacts with the side chains of F211, W214, and L219. Only weak polar interactions are detected within the cavity, involving the hydroxy-ethoxy group of BINI with R257 and S287, and the imidazole nitrogen atoms of the drug with the charged side chain of R218. Consistent with the

experimental thermodynamics data derived from fluorescence spectroscopy and calorimetric analysis (see Sections *Binding parameters and thermodynamic parameters analysis* and *ITC binding analysis*), these contacts largely account for the BINI-HSA binding mode, supporting an interaction profile primarily driven by the entropic contribution. MD simulations were also carried out to evaluate potential structural changes in HSA induced by the binding of each drug. In particular, structural descriptors such as the radius of gyration (R_g) and the percentage of secondary structure were calculated (Figure 4.1.6, bottom panels). R_g reflects the overall compactness of the protein and highlights any dimensional perturbations caused by ligand binding, while the secondary-structure content enables direct comparison with the spectroscopic results (see Section *Circular dichroism spectroscopy*). The average value of R_g for the isolated serum protein was 26.54 ± 0.06 Å, in agreement previously reported data. For the ENC/HSA and BINI/HSA complexes, the R_g values were 26.67 ± 0.08 Å and 26.75 ± 0.07 Å, respectively (Figure 4.1.6, bottom left panel). These results confirmed that the compactness and the folding state of HSA remain essentially unchanged upon drug binding, in line with the CD experiments. Further confirmation was provided by the secondary-structure percentages calculated for both complexes and compared with the pristine protein (Figure 4.1.6, bottom right). Indeed, the secondary structure of HSA was essentially unaffected by the inclusion of either ENC or BINI in their respective binding pockets.

Conclusions

In this study, we employed a integrated strategy combining experimental methodologies and molecular simulation techniques to elucidate the binding process between human serum albumin and two anticancer drugs, namely encorafenib and binimetinib, which together represent one of the most effective combination treatments for melanoma. Overall, CD spectroscopy enabled the characterization of the protein's secondary structure in the presence and absence of each drug. The structure observed for ligand-free HSA agrees with literature data, and the interaction with ENC or BINI did not induce any significant structural modifications in the protein. Fluorescence studies and calorimetric analysis revealed that both ENC and BINI bind to Sudlow's site I, located within subdomain IIA of the protein, with moderate and comparable affinities, though through distinct mechanisms. Specifically, the ENC/HSA association is primarily enthalpy-driven, supported by hydrogen bonding and additional π and nonpolar interactions that collectively stabilize the complex. In contrast, the binding of BINI to HSA is mainly governed by entropic contributions, arising from an extensive network of hydrophobic contacts. In conclusion, this work provides valuable insights into the molecular basis of ENC and BINI binding to HSA, thereby improving our understanding of their transport and delivery in the bloodstream and, in turn, their pharmacokinetics profile. These

findings may also contribute to enhancing the therapeutic potential of the ENC/BINI combination in melanoma by supporting the design and optimization of dosage regimens required to achieve the desired anticancer efficacy.

4.1.2. Molecular Interactions of Cobimetinib and Vemurafenib with Human Serum Albumin: a Comparative Biophysical and Computational Analysis²

Results and Discussion

Fluorescence Quenching of HSA Induced by COB

As illustrated in Figure 4.1.7A, the fluorescence emission spectrum of HSA shows a strong emission peak at 343 nm upon excitation at 280 nm, attributable to its single tryptophan residue. Upon addition of increasing concentrations of COB (0-40 μM), a progressive decrease in fluorescence intensity is observed, without any significant shifts in the emission maximum. This quenching effect suggests the formation of an HSA/COB complex and indicates alterations in the local environment surrounding the tryptophan residue, likely as a consequence of ligand binding. The fluorescence quenching mechanism between a protein and a small molecule can generally be classified as either static or dynamic. In static quenching, a nonfluorescent ground-state complex forms between the fluorophore (e.g., the protein) and the quencher (drug or ligand). In contrast, dynamic quenching arises from collisional encounters between the fluorophore and the quencher that occur while the fluorophore is in the excited state, without formation of a stable complex. These quenching processes can be analyzed using the Stern–Volmer equation. A higher K_{SV} value indicates greater quenching efficiency, and a linear Stern–Volmer plot typically implies the prevalence of a single quenching mechanism. To distinguish between static and dynamic contributions, eq (5) can be rearranged by expressing K_{SV} as the product of the bimolecular quenching constant k_q and the average fluorescence lifetime of the unquenched fluorophore τ_0 (as shown in the right-hand side term of eq (5), which for HSA has the value of 5.6×10^{-9} s). If the calculated k_q exceeds the diffusion-controlled limit ($2.06 \times 10^{10} \text{ M}^{-1} \text{ s}^{-1}$), the quenching mechanism is considered static, indicating complex formation. Conversely, if k_q falls below this threshold, the process is classified as dynamic. Furthermore, the temperature dependence of K_{SV} provides additional insight into the quenching mechanism. An increase in K_{SV} with rising temperature supports dynamic quenching, as higher thermal energy enhances molecular motions and collision frequency. In contrast, a decrease in K_{SV} with temperature

² The material presented in this chapter was published at: “Molecular Interactions of Cobimetinib and Vemurafenib with Human Serum Albumin: a Comparative Biophysical and Computational Analysis” G. Cavalieri, D. Marson, A. Starz, E. Laurini, S. Priel, *Molecular Pharmaceutics* 2025 22 (8), 4969-4982.

suggests static quenching, because elevated temperatures destabilize the ground-state complex, with the notable exception of entropy-driven binding, in which static association may increase with temperature.

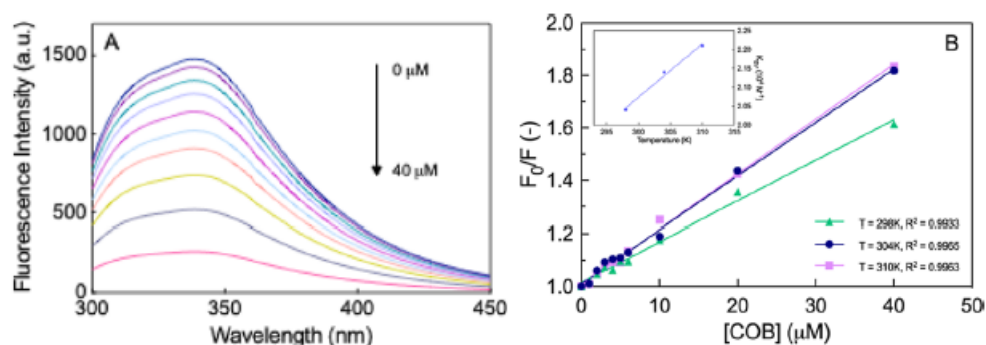


Figure 4.1.7. (A) Steady-state fluorescence quenching of HSA (2 μM) in the presence of increasing concentrations (0–40 μM) of COB (PBS buffer, pH = 7.4, $T = 25\text{ }^{\circ}\text{C}$, and $\lambda_{\text{exc}} = 280\text{ nm}$). (B) Stern–Volmer plots for COB-induced HSA fluorescence quenching at three different temperatures (25 $^{\circ}\text{C}$, 31 $^{\circ}\text{C}$ and 37 $^{\circ}\text{C}$).

To elucidate the nature of the HSA quenching mechanism induced by COB, fluorescence spectra of HSA in the presence of the drug were next recorded at three different temperatures. The corresponding Stern–Volmer plots are shown in Figure 4.1.7B, and the calculated values of the K_{SV} and k_{q} are summarized in Table 4.1.6.

T ($^{\circ}\text{C}$)	K_{sv} (10^4 M^{-1})	k_{q} ($10^{12}\text{ M}^{-1}\text{ s}^{-1}$)
25	2.04	3.64
31	2.14	3.82
37	2.21	3.95

Table 4.1.6. Stern–Volmer Constants (K_{SV}) and Bimolecular Quenching Rate Constants (k_{q}) for HSA Tryptophan Fluorescence Quenching by COB at Different Temperatures (the errors in the listed values are within 10%).

A clear temperature-dependent increase in K_{SV} is observed, indicating that the quenching efficiency becomes slightly higher at elevated temperatures. Moreover, the calculated k_{q} values are on the order of $10^{12}\text{ M}^{-1}\text{ s}^{-1}$, which far exceeds the maximum diffusion-controlled limit for dynamic quenching ($\sim 10^{10}\text{ M}^{-1}\text{ s}^{-1}$). Taken together, these results support a static quenching mechanism, in which complex formation between COB and HSA occurs in the ground state prior to excitation. Although an increase in K_{SV} with temperature is typically associated with dynamic quenching, such a trend can also arise in static quenching systems involving an entropy-driven binding mechanism, where thermal energy promotes ground-state complex formation due to favorable entropy gain. This finding therefore suggests that entropy may play a substantial role in promoting and stabilizing the formation of the COB/HSA complex. Furthermore, the observed thermal dependence of k_{q} is consistent with the

modest destabilization of the protein–ligand complex at higher temperatures, a common feature of static quenching behavior.

Thermodynamics and Kinetics of HSA-Drug Binding via ITC

Isothermal titration calorimetry is a highly sensitive, label-free technique widely used to characterize molecular interactions in solution. By directly measuring the heat exchanged during ligand binding, ITC provides comprehensive thermodynamic information, including the dissociation constant K_d , the change in enthalpy ΔH , and the binding stoichiometry n . From these parameters, the variation in Gibbs free energy ΔG can be calculated, describing the overall spontaneity of the binding process. The entropy contribution $T\Delta S$ is then obtained, offering insight into the fundamental driving forces of complex formation, whether enthalpy- or entropy-driven. In addition to this thermodynamic profiling, ITC can also be used to estimate kinetic parameters such as the association k_{on} and dissociation k_{off} rate constants, from which the residence time $\tau_r = 1/k_{off}$ can be derived. Together, these parameters provide a detailed, quantitative description of both the strength and duration of ligand binding under near-physiological conditions.

Accordingly, ITC experiments were first performed to determine the thermodynamics of COB binding to HSA at 25 °C, and the results are presented in Figure 4.1.8 and Table 4.1.7.

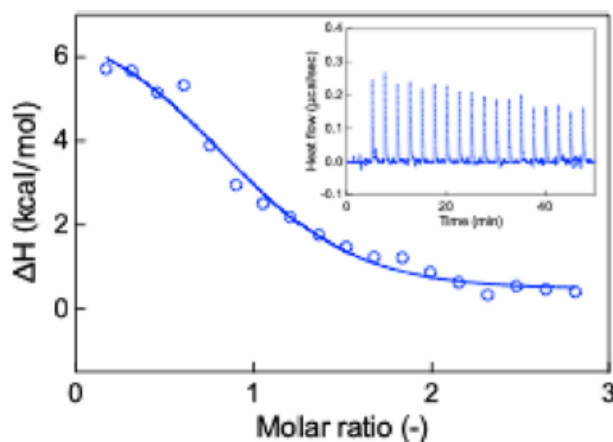


Figure 4.1.8. Integrated ITC binding isotherm for the interaction between COB and HSA at 25 °C in PBS buffer (pH 7.4 [HSA] = 20 μM). The blue line represents the best fit using a single-site binding model (1:1 stoichiometry). The inset displays the raw heat change data from successive COB injections into the HSA solution.

Thermodynamic data		Kinetic data	
n	1.14 ± 0.07	$k_{on} (M^{-1} s^{-1})$	1.19×10^4
$K_d (\mu M)$	58.2	$K_{off} (s^{-1})$	0.69
$\Delta G (kJ/mol)$	-24.19	$\tau_r (s)$	1.45

ΔH (kJ/mol)	5.88 ± 0.32	$\tau_{r,MD}$ (s)	0.87 ± 0.14
$T\Delta S$ (kJ/mol K)	$+30.1$		

Table 4.1.7. Summary of the Thermodynamic and Kinetic Data Extracted from ITC for the Binding of COB to HSA, including the Drug Residence Time $\tau_{r,MD}$ Estimated from Molecular Dynamics Simulations.

The ITC data confirms the spontaneous formation of a drug-protein complex with a 1:1 binding stoichiometry. Specifically, for the COB/HSA complex, integration of the heat curve yielded the following parameters: $K_d = 58.2 \mu\text{M}$, $\Delta G = -24.19 \text{ kJ/mol}$, and $n = 1.14$ (Table 4.1.7). These results clearly support the spontaneous nature of COB/HSA association. The ITC analysis also highlights the distinct contributions of different interaction forces governing the binding between the MEK inhibitor and HSA. The spontaneous nature of COB/HSA complexation is thus clearly supported. The positive enthalpy change ($\Delta H = +5.88 \text{ kJ/mol}$) indicates an endothermic process, which is energetically unfavorable from an enthalpic standpoint. However, the substantial positive entropy contribution ($T\Delta S = +30.1 \text{ kJ/mol}$) compensates for this enthalpic penalty, suggesting an entropy-driven binding mechanism. This thermodynamic signature is consistent with the burial of hydrophobic surfaces and the release of structured water molecules from the binding interface, features commonly associated with hydrophobic interactions. In addition, the lack of a favorable enthalpic component ($\Delta H > 0$) further suggests that strong polar interactions, such as hydrogen bonds or electrostatic contacts, are not the primary stabilizing forces in the COB/HSA complex.

To gain a comprehensive understanding of drug–target interactions, it is essential to evaluate both thermodynamic and kinetic parameters, as their combined analysis more accurately reflects the complexity of physiological conditions. In particular, estimating the lifetime of a drug–target complex provides crucial insights into pharmacological efficacy, since a drug remains active only while bound to its molecular target. Among the available kinetic descriptors, the drug residence time τ_r serves as a key nonequilibrium parameter for assessing the temporal stability of such complexes.

Given the critical role of kinetic properties in shaping drug–target lifetimes, a comparative analysis of VEM and COB, two agents frequently coadministered in the treatment of BRAF mutant melanoma, was therefore performed to explore possible differences in their pharmacokinetic behavior. As we previously reported, VEM exhibits a k_{on} value of $1.12 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$ and a k_{off} value of $5.42 \times 10^{-2} \text{ s}^{-1}$, respectively, yielding a τ_r of 18.5 s and a K_d value of $4.84 \mu\text{M}$ toward HSA. These parameters indicate a moderate residence time combined with a favorable kinetic profile, supporting a stable yet reversible interaction. In contrast, COB displays a markedly different kinetic signature. Based on ITC-derived thermodynamic data and kinetic extrapolation (Table 4.1.7), COB shows a comparable k_{on} ($1.19 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$) but a substantially higher k_{off} (0.69 s^{-1}), resulting in a significantly shorter

residence time of 1.45 s. This pronounced difference reveals a much more transient binding of COB to HSA, characterized by rapid dissociation and a more dynamic engagement under physiological conditions.

Overall, while VEM and COB share comparable association kinetics, their dissociation behaviors differ markedly, with VEM exhibiting a residence time roughly 13 times longer than that of COB. These findings highlight the importance of considering not only binding affinity but also kinetic stability when evaluating drug behavior, particularly in coadministration scenarios, where differences in HSA binding kinetics can influence drug distribution, free plasma concentrations, and overall pharmacokinetics. In this context, aligning drug-HSA interaction dynamics may represent a key factor in optimizing systemic availability and enhancing the combined therapeutic impact of these agents.

Structural Analysis of the HSA/COB Complex

Circular Dichroism Spectroscopy

CD spectroscopy is a widely used technique for analyzing protein secondary structure, offering a rapid and sensitive means of detecting conformational changes upon ligand binding. In the far-UV region (190-250 nm), CD spectra primarily reflect the content of α -helices, β -sheets, and random coil structures, with characteristic signals that enable the monitoring of potential structural perturbations. This technique is particularly valuable for assessing the stability of protein–ligand complexes and for estimating the melting temperature T_m through thermal denaturation experiments.

To evaluate the impact of COB binding on the secondary structure of HSA, far-UV CD spectra were recorded in the absence and presence of increasing concentrations of COB. As shown in Figure 4.1.9, the CD spectrum of native HSA (black curve) displays two characteristic negative bands near 208 and 220 nm, indicative of its predominant α -helical content. Upon titration of COB at concentrations of 1, 2, 4, and 20 μ M into a 2 μ M HSA solution, subtle changes in spectral intensity were observed, suggesting potential ligand-induced conformational perturbations. The corresponding spectra are presented in Figure 4.1.9, and a quantitative analysis of the α -helical content is provided in Table 4.1.8.

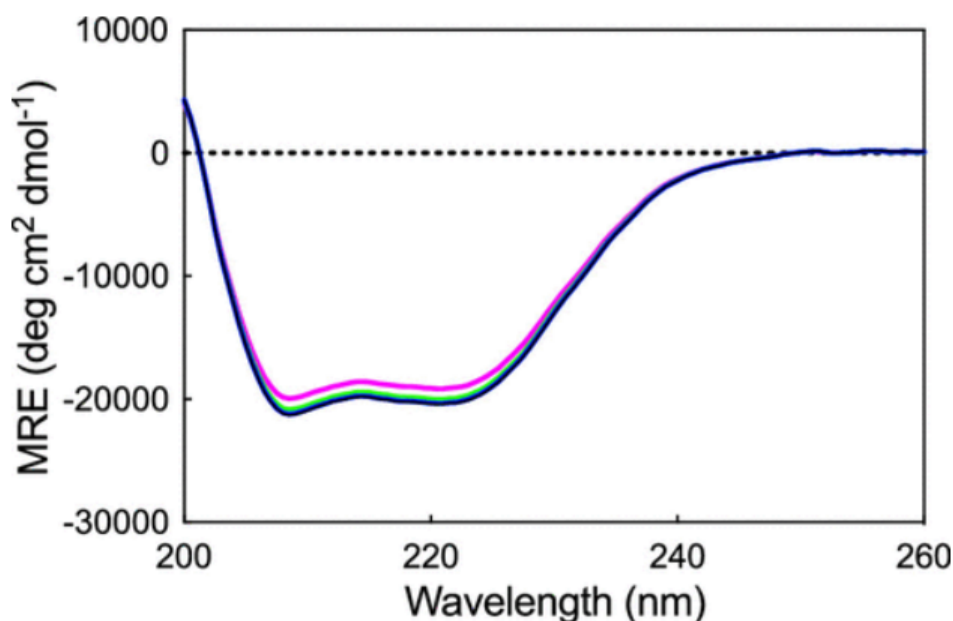


Figure 4.1.9. Far-UV CD spectra of HSA alone (2 μM) and in the presence of COB at increasing concentrations [1 (blue), 2 (cyan), 4 (green), and 20 (purple) μM].

Sample ratio	α -helix (%)	T_m ($^{\circ}\text{C}$)
Free HSA	59.2 ± 0.8	73.59 ± 0.68
1:0.5	59.0 ± 0.9	76.05 ± 0.93
1:1	58.9 ± 1.2	71.30 ± 0.89
1:2	57.8 ± 1.0	69.13 ± 2.26
1:10	54.7 ± 1.5	65.37 ± 0.88

Table 4.1.8. Percentage of α -Helix of the HSA Upon COB Binding, as Calculated from CD Spectra at 25 $^{\circ}\text{C}$, and Corresponding Melting Temperatures (T_m) of the Protein (Mean values are reported along with their standard deviations (SD). Statistical comparisons were performed using Student's t-tests between each condition and the free HSA control. Only the sample at a 1:10 HSA/COB molar ratio showed a statistically significant difference in α -helix content ($p \leq 0.05$)).

Collectively, these data indicate that COB does not induce significant alterations in the secondary structure of HSA at lower concentrations (molar ratios of 1:0.5, 1:1, and 1:2). VEM also exerts only a marginal effect on the secondary structure of HSA under the same concentration conditions. However, at a higher ligand-to-protein ratio (1:10), a $\sim 5\%$ decrease in α -helical content is observed, suggesting a perturbation of the protein structure, likely involving the binding site region.

To assess the thermal stability of HSA in the presence of COB, additional CD measurements were conducted between 25 and 95 $^{\circ}\text{C}$, monitoring ellipticity at $\lambda = 222$ nm to determine the protein melting temperature T_m . The T_m values are listed in Table 4.1.8. Consistent with the secondary structure data, the thermal stability of HSA remains largely unchanged at low COB concentrations, but decreases

substantially (by approximately 8 °C) at the 1:10 ratio. This significant reduction in T_m indicates a destabilizing effect of COB at higher concentrations, underscoring the impact of drug binding on the HSA secondary structure.

CD analysis thus shows that high ligand ratios lead to a slightly decrease in HSA's α -helix content and T_m , indicating structural destabilization. Since the biological function of HSA as a drug and ligand carrier relies on its stable, helix-rich architecture, such alterations could modestly affect binding pocket geometry, reduce affinity or selectivity, and impair transport capacity. Moreover, a lower T_m suggests increased susceptibility to thermal or conformational stress, potentially influencing drug pharmacokinetics by altering binding stability or increasing the fraction of free drug in solution. However, it is important to note that the 1:10 HSA/COB molar ratio used in these experiments is not physiologically relevant. The high drug concentrations used *in vitro* are intended to saturate binding sites and amplify structural effects for analytical purposes, rather than to reproduce the protein's behavior under physiological conditions.

Synchronous Fluorescence of COB and VEM Interacting with HSA

As shown in Figure 4.1.10, both COB/HSA and VEM/HSA complexes display spectral shifts at the selected wavelength intervals, reflecting changes in residue polarity upon ligand interaction. Specifically, the COB/HSA complex exhibits a slight red shift of 0.5 nm at $\Delta\lambda = 15$ nm (Figure 4.1.10, top left panel), suggesting an increase in the polarity surrounding tyrosine residues upon COB binding. In contrast, the VEM/HSA complex shows a blue shift only at $\Delta\lambda = 60$ nm (Figure 4.1.10, bottom right panel), indicating that the unique HSA tryptophan resides in a more hydrophobic environment once the drug is bound to the protein. These findings highlight the distinct binding-induced microenvironmental changes triggered by the two inhibitors, reflecting different modes of interaction with serum albumin.

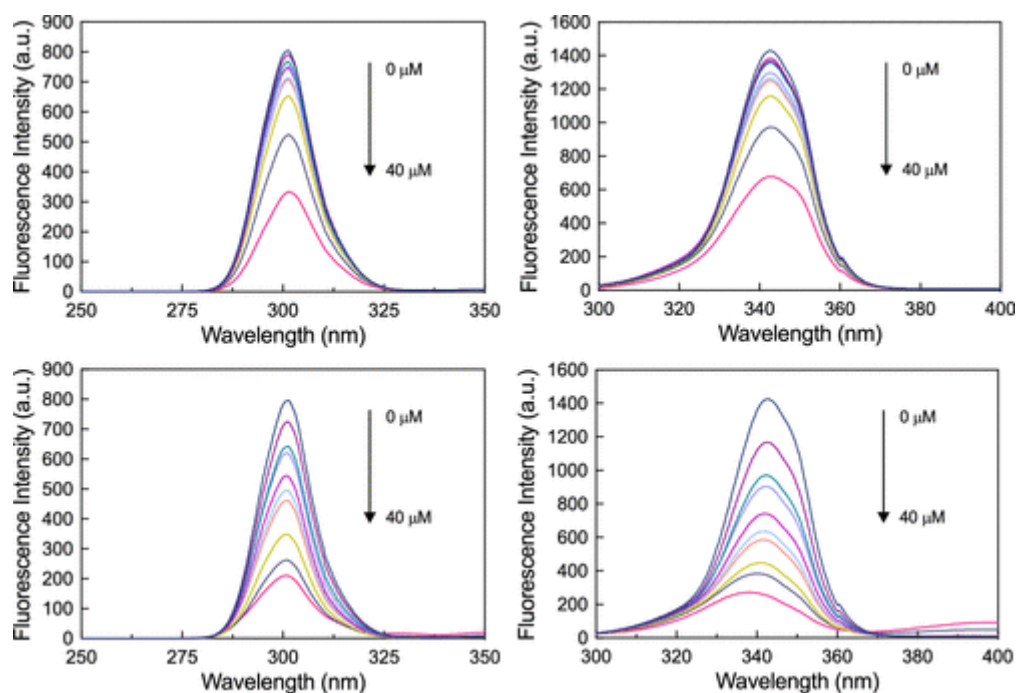


Figure 4.1.10. Synchronous fluorescence spectra of HSA (2 μM) recorded at $\Delta\lambda = 15$ nm (**left panels**) and $\Delta\lambda = 60$ nm (**right panels**) in the presence of increasing concentrations (0-40 μM) of COB (**top panels**) or VEM (**bottom panels**) at 25 $^{\circ}\text{C}$.

3D Fluorescence Study of COB and VEM Interacting with HSA

3D fluorescence spectroscopy is a highly sensitive technique for probing molecular interactions and conformational changes in proteins, offering detailed insight into the microenvironment surrounding intrinsic fluorophores such as tryptophan and tyrosine residues. Accordingly, this method was applied to investigate the effects of VEM and COB binding on the tertiary structure and local microenvironmental properties of HSA. The 3D fluorescence spectra and their corresponding contour plots provide a comprehensive overview of the conformational and microenvironmental properties of HSA and how these are modulated upon drug interaction. In the control condition (Figure 4.1.11, top panel), HSA displays a well-defined fluorescence peak centered at an excitation wavelength of ~ 280 nm and emission at ~ 340 nm, characteristic of the intrinsic fluorescence of Trp-214. The intensity and sharpness of this peak reflect the relatively hydrophobic and conformationally stable environment surrounding the fluorophore in the native protein. Upon addition of VEM (Figure 4.1.11, middle panel), a significant decrease in fluorescence intensity is observed, without a substantial shift in the maximum emission. This marked quenching, occurring without polarity changes, suggests a static quenching mechanism, consistent with the formation of a ground-state VEM/HSA complex. The altered shape of the intensity landscape, visible as a compressed and more localized contour pattern, may indicate reduced conformational flexibility or tighter packing around the fluorophore, likely reflecting drug-induced structural stabilization within specific subdomains. In the COB/HSA complex (Figure 4.1.11, bottom panel), a more moderate quenching effect is observed. While the

fluorescence peak remains well-defined, the intensity is slightly reduced compared to native HSA, and the emission profile appears marginally broader. This confirms that COB also binds to HSA and perturbs the local environment of Trp-214, albeit to a lesser extent than VEM. The absence of a spectral shift again suggests a predominantly nonpolar interaction environment, whereas the broader emission distribution may indicate partial exposure of the fluorophore or a redistribution of conformational dynamics upon drug binding.

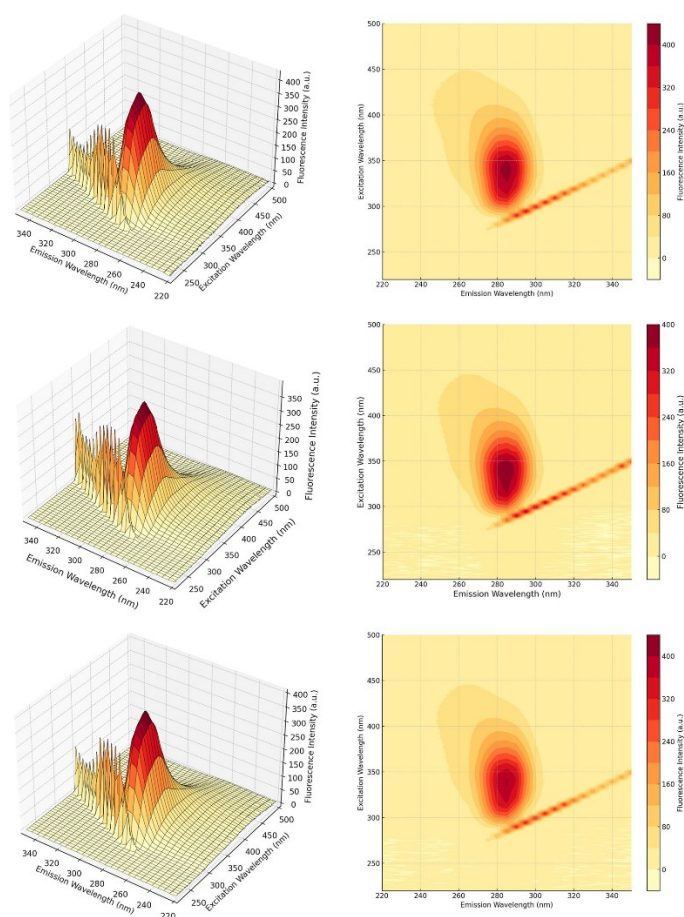


Figure 4.1.11. 3D fluorescence spectra (**left panels**) and corresponding two-dimensional contour maps (**right panels**) of 2 μ M HSA in the absence (top row) and presence of 2 μ M VEM (middle row) or 2 μ M COB (bottom row).

Determination of the COB Binding Site on HSA

To elucidate the preferential binding site of COB on HSA, site-displacement assays were conducted using established fluorescent markers selective for the three primary ligand-binding domains: indomethacin (IND) for Sudlow's site I, ketoprofen (KET) for Sudlow's site II, and hemin (HEM) for site III. Preformed HSA-marker complexes were titrated with increasing concentrations of COB, and the resulting fluorescence emission spectra were analyzed to assess competitive displacement. The corresponding Stern–Volmer quenching constants K_{SV} were calculated to quantify the extent of marker displacement and are reported in Table 4.1.9.

Site marker	K_{SV} (10^4 M^{-1})
none	2.02
IND	1.99
KET	1.21
HEM	2.07

Table 4.1.9. Stern–Volmer Constants (K_{SV}) for COB to HSA Derived from Fluorescence Quenching Data Obtained at 25 °C in the Absence and Presence of the Site-Selective Probes IND, KET, and HEM (the errors in the listed values are within 10%).

The data in Table 4.1.9 reveal that the value of K_{SV} for COB is markedly reduced in the presence of KET, indicating competitive binding at Sudlow’s site II. In contrast, the presence of IND or HEM has minimal impact on the K_{SV} value. These results suggest that COB preferentially binds to site II on HSA, with negligible interaction at the other two canonical binding regions. This hypothesis is further explored and substantiated through molecular dynamics simulations aimed at characterizing the binding mode and key stabilizing interactions within the protein pocket, as discussed below.

Molecular Simulations

To further investigate the structural basis of COB binding to HSA, classical molecular dynamics (cMD) simulations were performed using the binding site information inferred from the side-competition experiments discussed above. The MEK inhibitor was initially positioned within subdomain IIIA of HSA through molecular docking, targeting a well-characterized binding pocket. The top-ranked pose, selected based on docking score, was used as the starting conformation for the cMD simulations. The computed docking energy for this pose was -33.58 kJ/mol, which is in qualitative agreement with the experimental affinity data. However, it is important to note that docking scores are intrinsically approximate and are not designed for quantitative prediction of binding free energy. For this reason, no further decomposition of the docking energy was pursued, given the methodological limitations inherent to docking approaches. Instead, this study emphasizes the use of cMD simulations to qualitatively characterize the binding mechanism and stability of the complex, complemented by enhanced-sampling MD techniques aimed at describing the kinetics of the interaction.

Unrestrained cMD simulations revealed that COB remains stably bound (Figure S1) within two adjacent hydrophobic pockets of HSA (Figure 4.1.12). The piperidine moiety is accommodated within a cavity lined by residues F403, L407, L387, N391, Q390, L394, and L398. Simultaneously, the 2-fluoro-4-iodophenyl and 3,4-difluorophenyl groups are embedded in a second hydrophobic pocket formed by residues T409, N405, M548, and the nonpolar regions of K402, E542, and K545. A

stabilizing polar interaction between COB and the side chain of R410 further anchors the ligand within the binding site (Figure 4.1.12 left panel and Figure S2). This interaction cannot be described as a stable hydrogen bond, as the geometrical requirements (distance and angular orientation) for hydrogen bonding are not consistently satisfied throughout the trajectory. Rather, it is best described as a polar contact, with the mean distance between the relevant heavy atoms measured at 4.14 ± 0.84 Å over the equilibrated cMD trajectory. These structural and intermolecular observations are in good agreement with the thermodynamic profile obtained from ITC, which indicates an entropy-driven binding process. The absence of persistent hydrogen bonds or strong polar contacts in the COB/HSA complex, as revealed by MD simulations, suggests that the binding is not primarily stabilized by enthalpic contributions. Moreover, the predominance of hydrophobic contacts and the burial of nonpolar regions are consistent with a binding mechanism dominated by favorable entropic contributions. Moreover, analysis of the cMD trajectory also demonstrated that COB binding does not significantly perturb the secondary structure of serum albumin (Figure S3). In particular, the α -helix content in the presence of the drug remains closely comparable to that of free HSA, further supporting the results obtained from CD spectroscopy.

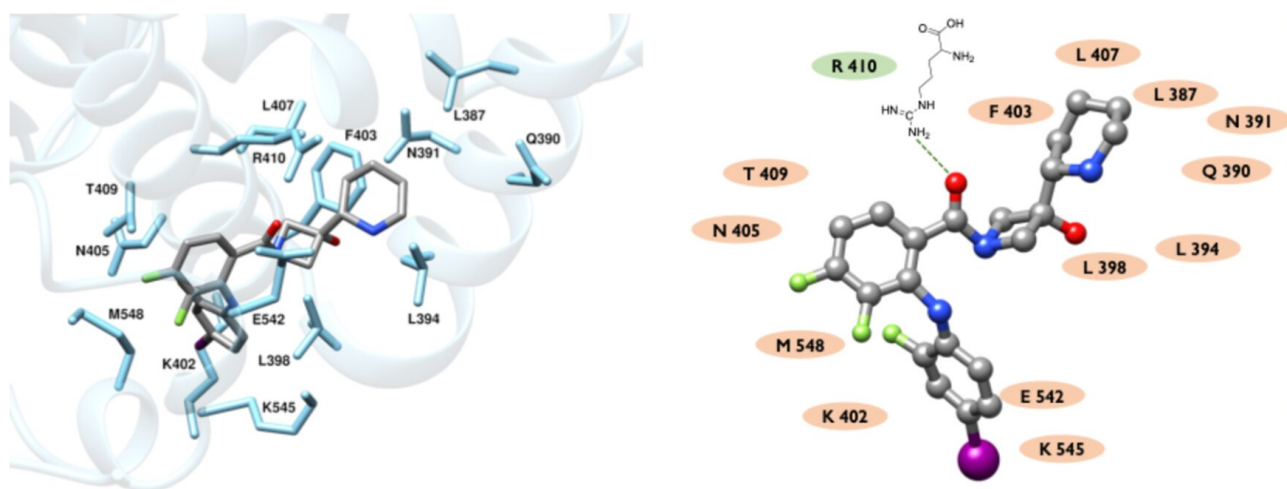


Figure 4.1.12. Binding mode of COB within Sudlow's site II of HSA. **(Left panel)** Representative snapshot from the cMD simulation showing COB nested in the binding pocket. Hydrogen atoms, water molecules, and ions are omitted for clarity. HSA is shown as transparent blue ribbons, with key interacting residues labeled and highlighted. COB is displayed as atom-colored sticks (C: gray, O: red, N: blue, F: light green, I: purple). **(Right panel)** Schematic representation of the binding site, highlighting HSA residues involved in ligand interaction as identified from the cMD trajectory. COB is shown as balls and sticks (hydrogens hidden), using the same color scheme. R410, forming a polar contact with the ligand (green dashed line), is colored light green, while residues contributing to hydrophobic interactions are shown in light pink. R410 is colored light green, and its polar contact with the ligand, characterized by a mean heavy-atom distance of 4.14 ± 0.84 Å and does not satisfy typical hydrogen bond geometry, is indicated with a green dashed line. Consistent with the experimentally derived thermodynamic signature suggesting an entropy-driven binding process, hydrophobic contacts (depicted as light pink ovals) appear to dominate the interaction network.

Molecular simulations were further employed to estimate the residence time $\tau_{r,MD}$ of COB within the HSA binding site. To capture rare events such as ligand unbinding within accessible simulation time scales, the FaMetaD technique was applied. This enhanced sampling method introduces a history-dependent biasing potential along selected reaction coordinates at controlled intervals, effectively flattening the free energy landscape and accelerating the occurrence of infrequent events (Figure 4.1.13, upper panel). Each simulation is terminated upon detection of a complete unbinding event, from which a biased residence time is obtained. An acceleration factor, calculated from the cumulative bias added during the FaMetaD trajectory, enables the recovery of unbiased unbinding times by appropriately scaling the biased simulation times collected from each trajectory. To ensure statistical robustness of the kinetic model, a total of 15 independent FaMetaD simulations were performed. The estimated $\tau_{r,MD}$ value was then extracted as the characteristic time constant (τ) of a homogeneous Poisson process governing ligand dissociation (Figure 4.1.13, lower panel).

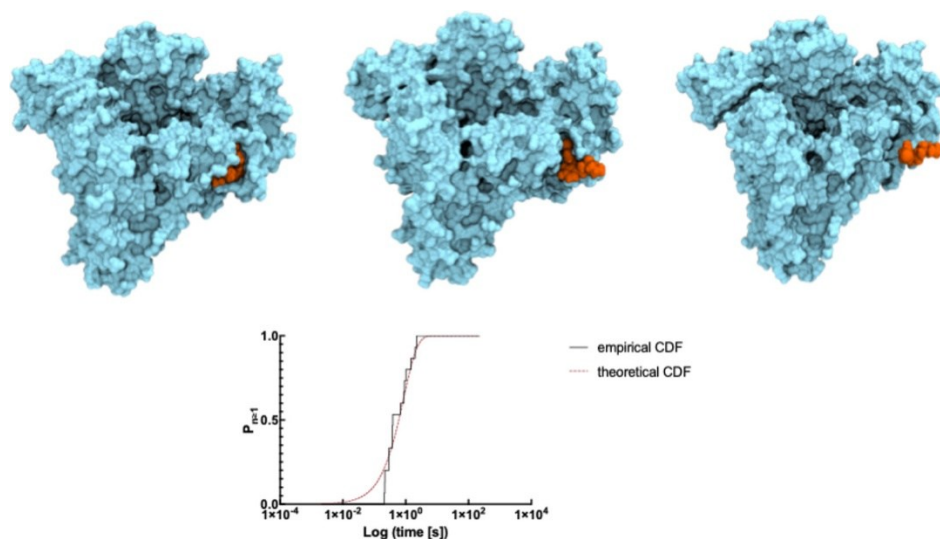


Figure 4.1.13. (Upper panel) Representative snapshots from a frequency-adaptive metadynamics (FaMetaD) simulation illustrating the unbinding of COB (shown as orange spheres) from its binding site on HSA, represented by its solvent-accessible surface in light cyan. These frames capture the progression of COB dissociation over time. **(Lower panel)** Superposition of the empirical cumulative distribution function (CDF_E) of unbiased release times derived from 15 independent FaMetaD simulations and the theoretical CDF (CDF_T) expected for a homogeneous Poisson process. The agreement between the two confirms the statistical reliability of the computed residence time.

Hence, the empirical cumulative distribution function (CDF_E) of the unbiased residence times was fitted via least-squares to the theoretical cumulative distribution function (CDF_T) of a homogeneous Poisson process, described by

$$CDF_T = 1 - e^{-t/\tau}. \quad (46)$$

To further validate the consistency between the simulated and theoretical dissociation kinetics, a Kolmogorov–Smirnov (KS) test was performed to compare CDF_E and CDF_T . The resulting p -value of 0.434 indicates no significant deviation between the two distributions, supporting the robustness of the residence time estimation. Based on the unbiased residence times obtained from 15 independent FaMetaD simulations, the mean residence time $\tau_{r,MD}$ of COB was determined by fitting the data to a Poisson cumulative distribution function, yielding a value of 0.87 ± 0.14 s (Table 4.1.7). These results provide valuable insight into the kinetic stability of COB within the HSA binding pocket and reflect the strength and specificity of its interaction network.

Importantly, the simulated mean value of $\tau_{r,MD}$ for COB (0.87 ± 0.14 s) is in good agreement with the experimentally derived residence time obtained from ITC kinetic analysis (1.45 s, Table 4.1.7), further supporting the consistency between computational predictions and biophysical measurements.

Effect on HSA Esterase Enzyme Activity by VEM and COB

Given that COB and VEM are frequently coadministered in clinical settings, understanding their combined influence on the physiological functions of HSA is of particular importance. As discussed above, both drugs are capable of forming stable complexes with HSA, which may interfere with its esterase-like activity, an enzymatic function crucial for the hydrolysis of various ester-containing drugs. A well-known example is the conversion of aspirin to salicylic acid, a reaction catalyzed in part by HSA. Inhibition of this activity through drug binding may reduce the metabolic turnover of aspirin and similar compounds, potentially impairing their therapeutic efficacy. This raises concerns about potential pharmacokinetic interactions during treatment with COB and VEM. To investigate this, the esterase activity of HSA was assessed in the absence and presence of each drug (Figure 4.1.14). The corresponding kinetic parameters, *i.e.*, the Michaelis–Menten constant K_m and the maximum reaction velocity V_{max} for the hydrolysis of *p*-nitrophenyl acetate (*p*-NPA), are reported in Table 4.1.10 for native HSA and for the protein in complex with either COB or VEM.

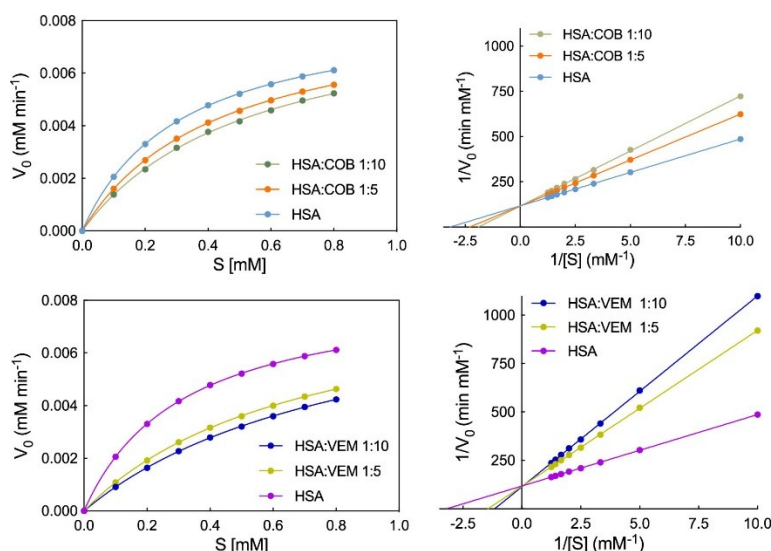


Figure 4.1.14. (Left panel) Michaelis–Menten plots and Lineweaver–Burk plots (right panel) of the esterase enzyme activity of HSA on binding with COB (top panel) and VEM (bottom panel). Lines represent data regression obtained at different substrate concentrations $[S]$.

Sample	Sample ratio	$V_{\max}$ (mM/min)	K_m (mM)
HSA/COB	free HSA	$8.520 \pm 0.501 \times 10^{-3}$	$3.146 \pm 0.373 \times 10^{-1}$
	1:5	$8.602 \pm 0.973 \times 10^{-3}$	$4.375 \pm 0.902 \times 10^{-1}$
	1:10	$8.684 \pm 0.431 \times 10^{-3}$	$5.307 \pm 0.450 \times 10^{-1}$
HSA/VEM	free HSA	$8.520 \pm 0.501 \times 10^{-3}$	$3.143 \pm 0.373 \times 10^{-1}$
	1:5	$8.672 \pm 0.242 \times 10^{-3}$	$7.000 \pm 0.305 \times 10^{-1}$
	1:10	$8.865 \pm 0.381 \times 10^{-3}$	$8.761 \pm 0.543 \times 10^{-1}$

Table 4.1.10. Michaelis–Menten Parameters (eq (2)) of HSA on Interaction with Increasing Concentration of COB and VEM.

Our findings demonstrate that the esterase-like activity of HSA diminishes progressively with increasing concentrations of either COB or VEM. This inhibition is primarily attributed to specific binding interactions within the HSA/drug complexes, particularly at Sudlow’s site II, which contains catalytically important residues essential for the esterase activity of the protein. Occupation of this site by COB or VEM may disrupt the local environment or directly interfere with substrate accommodation. Also, the kinetic analysis of *p*-NPA hydrolysis reveals a competitive inhibition mechanism for both drugs, evidenced by a marked increase in the apparent K_m without significant changes in $V_{\max}$. Specifically, in the presence of COB, K_m increased from 0.315 mM (free HSA) to 0.438 mM (COB 1:5) and 0.531 mM (COB 1:10), while $V_{\max}$ remained relatively stable (from 8.52×10^{-3} to 8.668×10^{-3} mM/min, Table 4.1.10). Similarly, VEM caused an increase in K_m from 0.314 mM (free HSA) to 0.700 mM (VEM 1:5) and 0.876 mM (VEM 1:10), with corresponding $V_{\max}$ values

ranging from 8.52×10^{-3} to 8.87×10^{-3} mM/min (Table 4.1.10). These findings align with previous reports indicating that VEM binds to Sudlow's site II, and now extend this behavior to COB, suggesting a shared binding mechanism. Overall, the results highlight the potential of both COB and VEM to modulate the enzymatic activity of HSA through site-specific interactions, which may have important implications for the metabolism and efficacy of ester-containing drugs coadministered with these agents.

Conclusions

The molecular characterization of drug–human serum albumin interactions offers critical insights into protein binding mechanisms and functional modulation. In this study, biophysical analyses and molecular simulations were integrated to delineate how cobimetinib and vemurafenib engage with HSA and influence its structural and enzymatic properties. Using a multidisciplinary approach combining fluorescence spectroscopy, isothermal titration calorimetry, circular dichroism, and advanced molecular simulations, we demonstrate that COB binds predominantly to Sudlow's site II on HSA through an entropy-driven mechanism dominated by hydrophobic interactions. CD spectroscopy effectively enabled evaluation of HSA's secondary structure in the presence and absence of the ligand. The structural profile of ligand-free HSA was consistent with previously reported data, and COB binding did not result in significant alterations to the protein's secondary structure. Moreover, COB exhibits a remarkably short residence time on HSA, suggesting dynamic and reversible engagement, in contrast to the longer binding lifetime observed for VEM. These kinetic differences underscore the importance of considering both binding strength and temporal stability when evaluating drug–carrier interactions. Additionally, we show that both COB and VEM can interfere with the esterase-like enzymatic activity of HSA, potentially affecting the metabolism of coadministered ester-containing drugs. Collectively, our findings provide valuable molecular insight into the distinct binding behaviors of COB and VEM and suggest that differential albumin-binding kinetics may contribute to variability in drug efficacy and toxicity. This study presents the first comprehensive biophysical and structural characterization of COB's interaction with HSA, offering important implications for pharmacokinetics, drug safety, and formulation development. These results may guide future optimization of combination regimens and support the development of improved therapeutic strategies in precision oncology.

4.2. Multitechnique Insight into the DNA-Vemurafenib Interaction³

Introduction

The interaction between small molecules and nucleic acids, particularly DNA, is fundamental to understanding gene regulation, molecular recognition, and drug development. Such interactions (occurring through intercalation, groove binding, electrostatic forces, or covalent linkage) play key roles not only in basic nucleic acid chemistry but also to the rational design of therapeutic agents, especially anticancer and antimicrobial drugs.

Vemurafenib (VEM), a selective BRAF inhibitor used in the treatment of metastatic melanoma harboring the BRAF^{V600E} mutation, exemplifies a clinically important small molecule. Although its primary target is the BRAF kinase rather than DNA, investigating its potential DNA-binding behavior is crucial for assessing possible off-target or genotoxic effects that may contribute to secondary malignancies or interfere with essential DNA processes such as replication, repair, and transcription. Understanding these interactions is therefore vital to refining its pharmacological and safety profile, particularly in long-term treatments.

To elucidate the molecular mechanisms of VEM–DNA binding, this study employed an integrated set of experimental and computational approaches. Biophysical techniques, including UV–visible absorption, steady-state fluorescence, and circular dichroism spectroscopy, were used to characterize both the mode and strength of interaction and to monitor conformational changes in DNA upon VEM binding. Viscosity measurements further contributed to clarifying the binding mechanism. Thermodynamic parameters such as binding enthalpy, entropy, and affinity constants were determined through isothermal titration calorimetry. Complementary computational analyses, including classical molecular dynamics and advanced enhanced-sampling simulations (OPES-METAD), provided atomic-level insight into binding sites and interaction dynamics.

Overall, this multidisciplinary investigation delivers a detailed and coherent picture of VEM–DNA interactions, enabling a more comprehensive evaluation of the drug’s molecular behavior and potential off-target effects, and thereby contributing to the optimization of its therapeutic safety and efficacy.

³ The material presented in this chapter was published at: “Into the Groove: A Multitechnique Insight into the DNA–Vemurafenib Interaction”. Cavalieri, G.; Pison, R.; Marson, D.; Laurini, E.; Pricl, S.. *Appl. Biosci.* 2024, 3, 468–483.

Material and Methods

Reagents and Chemicals

Rhodamine B (RhB), Acridine Orange (AO), calf thymus DNA (ctDNA), and all other analytical-grade reagents were obtained from Sigma Aldrich Inc. (Saint Louis, MO, USA), while VEM was purchased from MedChemExpress (Monmouth, NJ, USA).

Sample Preparation and Analytical Procedures

Stock solutions of ctDNA, RhB, and AO were prepared by dissolving the appropriate amounts in 1× phosphate-buffered saline (PBS, pH = 7.4). These stock solutions were stored at 4 °C in the dark. The purity of ctDNA was verified by UV absorbance measurements, ensuring an A₂₆₀/A₂₈₀ ratio greater than 1.8. The 10 mM stock solution of VEM was prepared in DMSO.

UV-Visible Absorption Spectroscopy

UV-vis absorption spectra were recorded at 25 °C using a V-730ST spectrophotometer (Jasco, Tokyo, Japan) equipped with a 1.0 cm quartz cuvette. To ensure complete interaction between DNA and the small molecules, each sample was allowed to equilibrate for 2 h. prior to measurement. For DNA-centered titrations, spectra of a 50 µM ctDNA solution were collected in the range of 225–350 nm before and after the addition of five incremental concentrations of VEM (5–100 µM). Corresponding VEM-only solutions were used as references. Similarly, the UV spectra of a 50 µM VEM solution was acquired in the range of 240–400 nm before and after sequential additions of incremental ctDNA concentrations (from 5 to 100 µM). All experiments were performed in triplicate.

DNA Melting Study

ctDNA melting studies were carried out by monitoring absorbance at $\lambda = 260$ nm for a 50 µM ctDNA solution over a temperature range of 25–100 °C, both in the absence and presence of equimolar concentrations of VEM, RhB, or AO. The temperature was increased at a rate of 1 °C/min. Data are presented as the fraction of single-stranded ctDNA (f_{ss}) as a function of temperature, where f_{ss} is expressed as $f_{ss} = (A - A_0)/(A_f - A_0)$. Here, A is the absorbance of the system at each temperature, A_f is the absorbance at the final temperature (100 °C), and A_0 is the absorbance at the initial temperature (25 °C). The melting temperature (T_m) was determined numerically as the point where $f_{ss} = 0.5$, using the first derivative of the melting curve.

Effect of the Ionic Strength

The influence of ionic strength on the ctDNA-VEM interaction was investigated by measuring the absorbance at 260 nm of solutions containing ctDNA (50 µM) and VEM (10 µM) at varying NaCl concentrations (0–0.1 M) at room temperature. Results are reported as mean values from three

independent measurements and expressed as the normalized absorbance ratio A_0/A , where A_0 is the absorbance of the ctDNA/VEM system without added salt and A is the absorbance of the system at each NaCl concentration.

Viscosity Measurements

The viscosity of ctDNA (100 μM) in the presence of VEM, RhB, and OA was measured at room temperature using an Ostwald viscometer. Temperature control was maintained with a thermostatically regulated water bath, and measurements were performed at different ligand-to-ctDNA molar ratios ([ligand/ctDNA]). To ensure equilibrium binding, each mixture was allowed to stand for 30 min prior to measurement. The relative viscosity (η/η_0) of each sample was determined by measuring the flow time of the solution through the viscometer (where η_0 represents the viscosity of ctDNA alone, and η is the viscosity of the ctDNA–ligand complex). The results are presented as $(\eta/\eta_0)^{1/3}$ as a function of [ligand/ctDNA]. For each solution, the flow time was recorded in triplicate using a digital timer, and the mean value was used to calculate the corresponding viscosity.

Circular Dichroism Spectroscopy

CD spectra were acquired between 220 and 320 nm at a scan speed of 20 nm/min and a temperature of 25 °C using a J-1500 spectropolarimeter (Jasco, Japan) equipped with a thermostatic cell holder and a PML-534 FDCCD detector. A 0.2 cm path length quartz cuvette was used, with spectral resolution parameters set to a step size of 0.5 nm and a bandwidth of 1 nm. In these experiments, the ctDNA concentration was fixed at 50 μM , while the effects of VEM addition were evaluated at drug concentrations of 50 and 100 μM . Spectra were corrected by subtracting the baseline obtained from the pure buffer sample, and final CD profiles represent the average of three accumulations. All spectra were processed and analyzed using the Jasco Spectra Manager software (version 2.15.01, Jasco Corporation, Japan, 2020).

Steady-State Fluorescence Spectroscopy

The FP-8350 spectrofluorometer (Jasco, Japan), equipped with a thermostatic cell holder and a 1 cm quartz cuvette, was employed for all SSF measurements. Fluorescence emission spectra were acquired for mixtures of ctDNA (50 μM) with the fluorescent probes RhB (5 μM) or AO (5 μM), both in the absence and presence of varying concentrations of VEM (0–100 μM). The excitation wavelength was set to 465 nm for RhB and 495 nm for AO, with emission spectra recorded at 576 nm and 525 nm, respectively. All measurements were performed in triplicate, and the final spectra are presented as the averaged results.

Isothermal Titration Calorimetry Studies

The thermodynamics of the ctDNA-VEM complex formation was investigated by ITC using a MicroCal PEAQ-ITC calorimeter (Malvern, UK) at 25, 30, 37, and 40 °C with a cell volume of 208 μ L. The sample cell contained a 40 μ M ctDNA solution in PBS, while the syringe was loaded with a 200 μ M VEM solution. All solutions and buffer were degassed for 30 min at each corresponding temperature prior to each experiment. Titrations were performed in 19 successive injections of 2 μ L at a constant stirring speed of 750 rpm. Control experiments were conducted to account for non-specific heats, and the resulting data were corrected by subtracting these values. Heat capacity changes (ΔC_p) were calculated from the slope of the enthalpy vs temperature plots. All experiments were performed in triplicate, and data acquisition and analysis were completed using GraphPad Prism 8.0.0 (GraphPad Software, San Diego, CA, USA).

Computer Simulations

Atomistic molecular dynamics (MD) simulations were performed to gain deeper insight into the interactions between VEM and DNA. As a representative model for ctDNA, a 14-mer with the sequence (5'-CAACGTTGGCCAAC-3'), previously used in similar studies, was selected. The initial DNA structure was created with the *nab* (Nucleic Acid Builder) tool in AMBER22 and parametrized with the OL15 forcefield. VEM parameters were taken from our earlier study, employing the GAFF2 force field with RESP-derived charges from the ESP Charge Derive server.

Because no experimental structural information exists regarding the interaction between VEM and DNA, an enhanced MD method was employed, namely the On-the-Fly Probability Enhanced Sampling with METADynamics-like target distribution (OPES-METAD) as implemented in the PLUMED library. The manually assembled DNA-VEM complexes were solvated with TIP3P water molecules in a periodic box, maintaining at least 24 Å from each solute atom to the box edge. Sodium and chloride ions were added to the system to achieve a physiological salt concentration of 0.15 M NaCl. Energy minimization consisted of 5000 steepest-descent steps followed by 5000 conjugate-gradient steps. The minimized structures were heated from 0 to 300 K over 100 ps in the NVT (constant number of particles, temperature, and volume) ensemble using a Langevin thermostat (collision frequency 2 ps⁻¹). This was followed by 10 ns of density equilibration in the NPT (constant number of particles, temperature, and pressure) ensemble using a Berendsen barostat ($P = 1$ atm). Snapshots extracted from these equilibrated simulations were then used as starting points for OPES-METAD. Within the framework of OPES-METAD, a bias was added along selected collective variables (CVs) during the simulation every 1 ps, assuming a maximum free energy barrier of 25 kcal/mol, enabling rapid exploration of low probability states. The ensemble of configurations obtained can then easily be reweighted to obtain the unbiased ensemble. Two CVs were selected to

drive the OPES-METAD simulations: a) the distance from the center of mass (COM) of VEM and the COM of the first base pair of the DNA molecule (dEDGE), and b) the number of contacts between DNA and VEM heavy atoms, calculated with the PLUMED COORDINATION function (parameters $r_0 = 6 \text{ \AA}$, a and b equal to 6 and 12, respectively). Additionally, a restraint potential ($k = 25 \text{ kcal/mol}$) was applied to the latter CV to prevent sampling unbound states by acting when the number of contacts fell below 150. The multiple-walkers variant of OPES-METAD was exploited, launching 32 parallel OPES-METAD trajectories, each 100 ns long (total simulation time: 3.2 μs). Reweighted frames were then analyzed to define two new CV sets as the minimum distance between the COM of VEM and the COM of any CG (dCG) or AT (dAT) base pair, and dEDGE combined with dCG. Analysis of the corresponding free-energy surfaces (FESs) revealed a well-defined binding site for VEM, extracted from the global minimum region. The minimum energy complex was then subjected to a 4 μs unbiased MD simulation in the NPT ensemble (pressure maintained by the Monte Carlo barostat). In all MD simulations, the SHAKE algorithm was applied to constrain all hydrogen atoms and the hydrogen mass repartitioning scheme was used to enable a 4 fs time step. Electrostatic interactions were calculated using the particle mesh Ewald (PME) method. Unbiased MD simulations were run with the GPU-accelerated pmemd module of AMBER22, while the OPES-METAD simulations were carried out with GROMACS 2022.4, patched with the PLUMED library (version 2.9.1). All simulations were conducted on our hybrid GPU–CPU cluster, as well as on the pre-exascale Leonardo supercomputer (CINECA high-performance computing (HPC) infrastructure, Bologna, Italy). Trajectories were analyzed with PYTRAJ (AMBER22) and in-house Python scripts.

Results and Discussion

UV–Visible Spectroscopy Assays

Absorption Spectra of the Interaction of Vemurafenib with ctDNA

The UV-vis absorption spectroscopy experiments performed in this study provided valuable insights into the interaction dynamics between VEM and ctDNA. By examining changes in absorbance as a function of varying drug or DNA concentrations, it was possible to elucidate the binding properties of VEM and evaluate its influence on the structural integrity of the nucleic acid. When the concentration of DNA was kept constant and the concentration of VEM gradually increased, the UV-vis absorption spectra displayed a moderate hyperchromic effect (Figure 4.2.1a). This increase in absorbance, particularly near the characteristic DNA peak at 260 nm, indicates alterations in the electronic environment of the nucleobases. Hyperchromicity in DNA spectra typically reflects perturbations in base-stacking interactions within the double helix. In the case of VEM, the moderate hyperchromic effect observed suggests that the drug interacts with DNA in a way that only slightly

disturbs base stacking, without causing substantial structural modifications to the overall helix. Such spectral behaviour is consistent with small molecules that bind within the minor groove, where they induce localized structural changes rather than the extensive helix unwinding elongation associated with intercalation. Importantly, this moderate hyperchromicity occurs without any bathochromic (red) shift in the absorbance maximum. A red shift would normally be expected for intercalating agents, as insertion between base pairs increases π – π stacking interactions between the drug and nucleobases. The absence of this shift in the UV spectra supports the conclusion that VEM does not intercalate into DNA but rather binds within the minor groove, modifying the electronic environment of the bases while leaving the helical pitch and base-pair spacing largely unchanged.

In a complementary set of experiments, the UV-vis absorbance of VEM was examined while varying the concentration of DNA and keeping the drug concentration constant. In this scenario, a hypochromic effect was observed, whereby the absorbance of VEM decreased progressively as more DNA was added to the solution (Figure 4.2.1b). Hypochromicity is a common indication of binding interactions in which the chromophore (in this case, VEM) experiences reduced electronic transition probabilities due to binding-induced conformational changes or shielding effects arising from the DNA environment. The absence of a bathochromic shift in this setup further supports the hypothesis of groove binding. If VEM were intercalated into the DNA, one would expect not only pronounced hyperchromicity but also a noticeable shift in the maximum absorption wavelength, reflecting enhanced π – π stacking interactions between the drug and the nucleobases. Instead, the observed hypochromicity, without any significant wavelength shift, is consistent with VEM external binding along the DNA helix, most likely within the minor groove. In this binding mode, VEM is expected to interact with the edges of the base pairs and the sugar–phosphate backbone, fitting snugly into the groove without disrupting the helical pitch or the base-pair stacking arrangement. Such an interaction profile is particularly relevant, as it suggests that VEM potentially does not induce significant genotoxic stress or compromise the integrity of the DNA double helix.

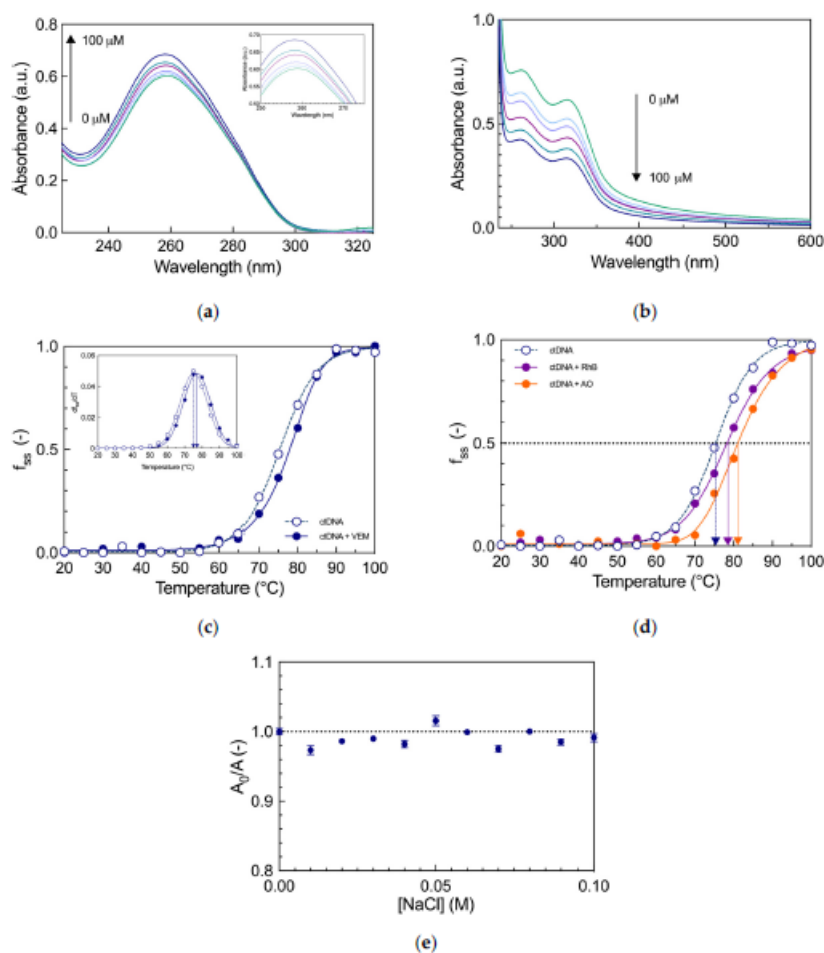


Figure 4.2.1. (a) UV-vis spectra of ctDNA (50 μM, green) before and after the addition of VEM (5 μM sky blue, 10 μM cornflower blue, 25 μM purple, 50 μM dark cyan and 100 μM navy blue) (PBS buffer, pH = 7.4, 25 °C). The inset shows a magnified view of the absorption region from 250 to 275 nm. (b) UV-vis spectra of VEM (50 μM, green) before and after the addition of ctDNA (5 μM sky blue, 10 μM cornflower blue, 25 μM purple, 50 μM dark cyan and 100 μM navy blue) under the same conditions. (c) Thermal denaturation profiles of ctDNA (50 μM) in the absence and presence of VEM (50 μM). The inset shows the first derivative of the thermal denaturation curves, used for the precise determination of the melting temperature T_m . (d) Thermal denaturation profiles of ctDNA in the absence and presence of RhB (50 μM) and AO (50 μM), with arrows indicating the T_m values for each system. In panels (c,d), errors are within 2%. (e) Normalized absorbance ratio as a function of NaCl concentration for the ctDNA–VEM complex (PBS, pH = 7.4, 25 °C).

DNA Melting Study

The melting temperature (T_m) of DNA is a fundamental parameter reflecting the thermal stability of the double-stranded helix. T_m is defined as the temperature at which 50% of the DNA in a sample transition from the double-stranded to the single-stranded state, a process known as DNA denaturation. This transition is typically monitored by UV-vis spectroscopy through the increase in absorbance at 260 nm as hydrogen bonds are disrupted. Because T_m is highly sensitive to interactions that stabilize or destabilize the helical structure, small molecules that bind to DNA often produce measurable changes in this parameter. Accordingly, the effect of VEM on the thermal stability of

ctDNA was next investigated by determining the T_m in the absence and presence of the drug. For comparison and validation purposes, analogous measurements were performed in the presence of Rhodamine B (RhB, a known DNA minor groove binder) or Acridine Orange (AO, a prototypical DNA intercalator). As shown in Figure 4.2.1c, the melting temperature of DNA alone was found to be 75.2 °C, consistent with its expected stability under physiological ionic strength conditions. This value reflects the inherent optimized balance of hydrogen bonding and base-stacking interactions that maintain the double helix. Upon VEM addition, the melting temperature of the ctDNA was only slightly affected, with a determined T_m value of 77.1 °C (Figure 4.2.1c). Contextually, the presence of RhB produced a similarly modest increase in T_m (78.2 °C), while the intercalator AO caused a pronounced stabilization of the helix, raising T_m to 81.5 °C (see Figure 4.2.1d). These findings are highly informative regarding the interaction mode of VEM with DNA. In fact, intercalating agents typically induce a substantial T_m increase (*e.g.*, 5 to 8 °C or more) as their insertion between base pairs enhances π - π stacking interactions and markedly stabilizes the double helix. This is precisely the behaviour observed for AO, which produced a ΔT_m of 6.3 °C (Figure 4.2.1d). Conversely, minor-groove binders generally cause little to no change in T_m , as they interact primarily with the edges of base pairs and the sugar-phosphate backbone, without significantly altering the global helical structure or base stacking. This behaviour is exemplified by RhB (for which ΔT_m of only 3 °C was measured) and is closely mirrored by VEM. The fact that VEM, like RhB, does not appreciably alter the T_m of ctDNA strongly supports a minor-groove binding mode, consistent with the UV-visible spectral data and the absence of intercalative signatures.

Effect of Ionic Strength

The nature of the interaction between small molecules and DNA can be significantly influenced by the ionic strength of the surrounding environment. Electrostatic binding, arising from the attraction between positively charged ligands and the negatively charged phosphate backbone of DNA, is particularly sensitive to changes in ionic strength. Typically, an increase in ionic strength screens these electrostatic interactions, reducing binding affinity and resulting in observable changes in UV-vis absorbance. On the contrary, groove binding, which primarily involves hydrophobic interactions and hydrogen bonding within the minor or major grooves of the DNA helix, is far less affected by variations in ionic strength. To investigate the possibility of electrostatic binding in the VEM-DNA assembly, Uv-vis spectroscopy measurements were performed at 260 nm for six DNA-VEM mixtures prepared in solutions of increasing ionic strength. These experiments aimed to detect any changes in absorbance that might indicate a weakening of electrostatic interactions between the drug and the nucleic acid. However, the results showed that the absorbance at 260 nm remained essentially unchanged at all ionic strength conditions (Figure 4.2.1e). This stability in absorbance strongly

suggests that the main interaction between VEM and DNA is not electrostatic in nature, further reinforcing the conclusion VEM likely binds within the DNA groove rather than through charge-based attraction to the DNA backbone.

Viscosity Studies

The viscosity of nucleic acid is a powerful indicator for distinguishing between intercalative binding and groove or electrostatic modes of interaction. Intercalative binding typically produces a pronounced increase in solution viscosity, as the insertion of planar aromatic ligands between adjacent base pairs forces local unwinding and elongation of the DNA helix. By contrast, groove binding and electrostatic interactions do not substantially alter DNA contour length and therefore exert only minimal effects on viscosity. To further elucidate the binding mechanism of VEM with ctDNA, the relative viscosity of the nucleic acid solution was measured at increasing concentrations of the drug (Figure 4.2.2a). For comparison, the relative viscosity profiles of ctDNA in the presence of RhB, a well-established minor groove binding, and AO, a prototypical DNA intercalator, were also evaluated and reported in the same Figure. The results show that both VEM and RhB similarly induced only a modest increase in the relative viscosity at all tested concentrations. In contrast, the viscosity of the ctDNA solution increases steadily in the presence of AO, as expected for a classical intercalator that lengthens the DNA helix upon insertion. Taken together, these observations support a groove-binding interaction between and ctDNA, consistent with the behaviour observed for RhB and clearly distinct from the helix-elongating effects induced by AO.

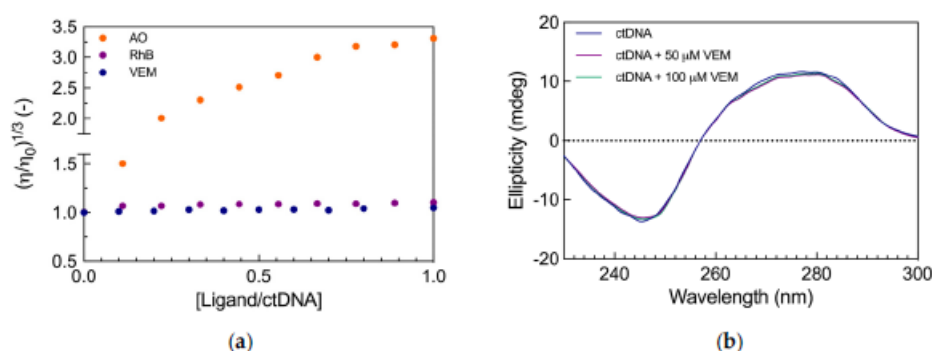


Figure 4.2.2. (a) Effect of increasing concentrations of VEM, RhB, and AO on the viscosity of ctDNA ($[ctDNA] = 50 \mu M$, $[ligand] = 0-50 \mu M$, PBS buffer, pH = 7.4, 25 °C). Each data point represents the mean of three independent measurements, with standard deviations smaller than the size of the data markers. (b) CD spectra of ctDNA recorded in the absence and presence of 50 μM and 100 μM of VEM in PBS buffer (pH 7.4, 25 °C).

CD Spectroscopy

Circular dichroism is a powerful and sensitive technique widely used to probe the conformational properties of nucleic acids, particularly DNA. This method is especially effective in detecting even

subtle changes in the secondary structure of DNA, such as transitions between different helical forms or distortions induced by ligand binding. The CD spectrum of free ctDNA displayed the expected two characteristic bands: a negative band at 243 nm, and a positive band at 277 nm (Figure 4.2.2b). These spectral features are well-established and reliable markers of the canonical B-form of DNA and are highly sensitive to structural perturbations. Upon addition of VEM at increasing concentrations, the CD spectra of ctDNA remained essentially unchanged, showing no significant variations in either the intensity or position of the two hallmark bands, even at the highest drug of the drug tested. The absence of spectral shifts or band perturbations in the presence of VEM indicates that the kinase inhibitor does not induce measurable changes in the secondary structure of DNA. This observation is fully consistent with a groove-binding interaction mode, which typically preserves the native conformation of DNA. These findings also reinforce the UV-visible spectroscopy results discussed earlier, collectively demonstrating that VEM interacts with ctDNA without disrupting its B-helical structure.

Fluorescence Spectroscopy

Fluorescence spectroscopy is a versatile tool for investigating interactions between small molecules and DNA; however, both DNA and VEM are inherently weakly fluorescent. This limitation complicates the direct monitoring of their binding behaviour using such technique. To circumvent this issue, competitive displacement assays were employed again using AO and RhB. When bound to DNA, both dyes display a pronounced increase in fluorescence intensity, making them excellent probes for monitoring binding displacement by other molecules. The principle underlying this method is straightforward: if VEM can displace one of the fluorescent dyes from DNA, a decrease in the dye's fluorescence signal will occur. This reduction provides indirect but reliable evidence of drug binding and, importantly, offers insight into the mode of interaction, distinguishing between groove binding and intercalation. As shown in Figure 4.2.3a, the fluorescence spectrum of the ctDNA–RhB complex, with its peak emission at 576 nm, exhibits a clear and progressive decrease in intensity upon VEM addition. This pronounced fluorescence reduction in fluorescence indicates that VEM competes with and effectively displaces RhB from the minor groove of the ctDNA. In contrast, the fluorescence spectrum of the DNA-AO complex, characterized by its peak emission at 525 nm, remains largely unchanged with increasing VEM concentrations (inset in Figure 4.2.3a). The minimal decrease in fluorescence suggests that VEM does not displace AO from its intercalative binding sites between the DNA base pairs and thus does not interact with ctDNA through an intercalative mechanism. Taken together with the complementary evidence from UV–visible spectroscopy, melting analysis, ionic strength dependence, viscosity measurements, and CD spectroscopy discussed above, these results

strongly support the conclusion that VEM interacts with ctDNA predominantly through a groove-binding mode, with no indication of classical intercalation.

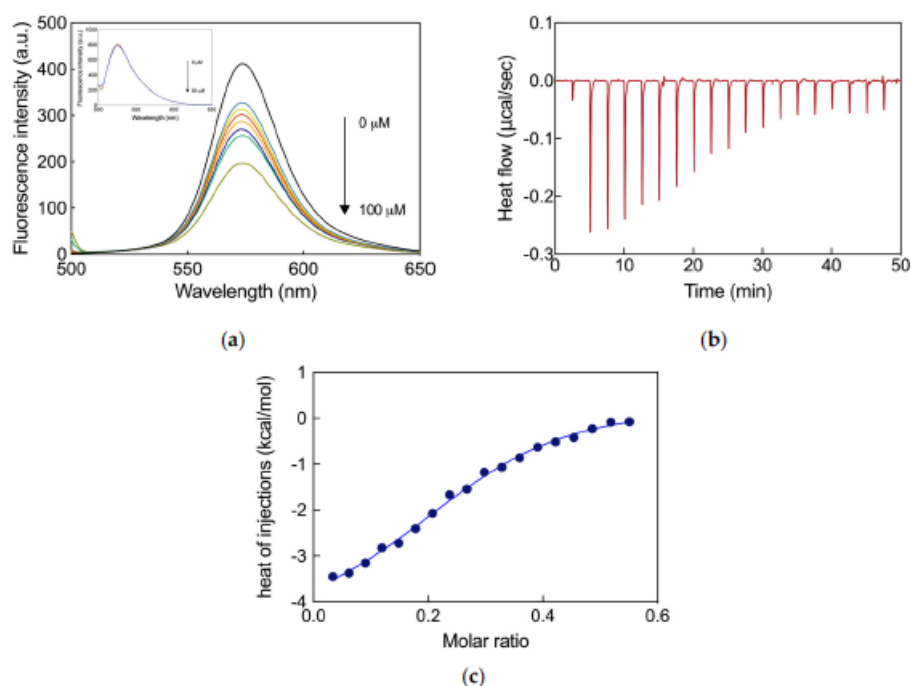


Figure 4.2.3. (a) Fluorescence spectra of the DNA–RhB complex in the presence of increasing concentrations of VEM (0 μM black, 2.5 μM cornflower blue, 5 μM dark khaki, 10 μM red, 15 μM orange, 20 μM blue, 50 μM green and 100 μM olive drab) (PBS buffer, pH = 7.4, 25 $^{\circ}\text{C}$). The inset shows the fluorescence spectra of the DNA-AO complex with increasing VEM concentrations (0 μM black, 2.5 μM blue, 5 μM cyan, 10 μM green, 20 μM orange, 50 μM red) under the same conditions. (b) ITC raw data and (c) the plot of the integrated ITC data for the interaction of ctDNA (40 μM , PBS buffer, pH = 7.4) and VEM at 25 $^{\circ}\text{C}$. The solid line corresponds to the data fitting with the one-site isothermal binding model.

Isothermal Titration Calorimetry

Accordingly, ITC was used to quantitatively assess the thermodynamic parameters underlying the interaction between VEM and ctDNA. In this experiment, a fixed concentration of ctDNA was titrated with incremental aliquots of VEM, enabling a detailed analysis of the binding affinity and the determination of the enthalpic and entropic contributions to the overall free energy change. The raw thermogram analysis (Figure 4.2.3b) clearly shows that each injection of VEM produces a negative heat flow peak, indicating that the interaction between the nucleic acid and the anticancer drug is exothermic. The sigmoidal profile of the integrated ITC data (Figure 4.2.3c), particularly the slope at the inflection point, allowed determination of the binding constant K_d , which reflects a moderate affinity between ctDNA and VEM with a average value of 11.2 μM (Table 4.2.1). The corresponding variation in the change in free energy for the binding interaction ΔG was calculated as -6.76 kcal/mol, consistent with a spontaneous and thermodynamically favorable binding process. Further

thermodynamic analysis provided comprehensive insight into the interaction mechanisms characterizing the VEM-ctDNA binding. The binding enthalpy change ΔH was determined to be -3.49 kcal/mol, confirming the exothermic nature of the interaction. This negative enthalpy variation suggests that favourable interactions, such as hydrogen bonding and van der Waals forces, contribute to stabilizing the VEM–DNA complex. The entropy term $T\Delta S$ contributed 3.27 kcal/mol to the overall change in free energy. The positive value of $T\Delta S$ indicates of the presence of stabilizing hydrophobic contacts between the two binding partners and an increase in system disorder upon binding, likely arising from the release of structured water molecules or ions from the DNA groove as VEM associates with the double helix. This entropy gain therefore favours the binding process. The estimated stoichiometry value $n = 0.239$ is consistent with expectations for a molecule of the size of VEM acting as a groove binder. Indeed, the reciprocal value $1/n \sim 4$ reasonably represents the number of base pairs interacting with the ligand. ITC experiments were performed at four temperatures (25 °C - 40 °C), to evaluate the molar heat capacity change ΔC_p associated with binding. As shown in Table 4.2.1, increasing temperature led to a more negative ΔH , while the entropic contribution revealed an opposite trend. Both ΔH and ΔS displayed linear temperature dependence and compensate each other, resulting in an almost temperature-independent ΔG . This enthalpy–entropy compensation is characteristic of systems dominated by hydrophobic interactions. The value of ΔC_p , obtained from the slope of the ΔH vs. temperature plot, was -172 cal/mol °C (Table 4.2.1). The negative sign of ΔC_p further supports the involvement of hydrophobic interactions, typically reflecting the burial of nonpolar surface areas upon complex formation. Finally, the binding stoichiometry remained essentially invariant across the temperature range, consistent with the values obtained at 25 °C.

T (°C)	K_d (μ M)	n (-)	ΔH (kcal/mol)	- $T\Delta S$ (kcal/mol)	ΔG (kcal/mol)	ΔC_p (cal/mol°C)
25	11.2	0.239	-3.49	-3.27	-6.76	-172
30	11.8	0.258	-4.08	-2.76	-6.84	
37	10.7	0.241	-5.32	-1.74	-7.06	
40	10.3	0.223	-6.08	-1.07	-7.15	

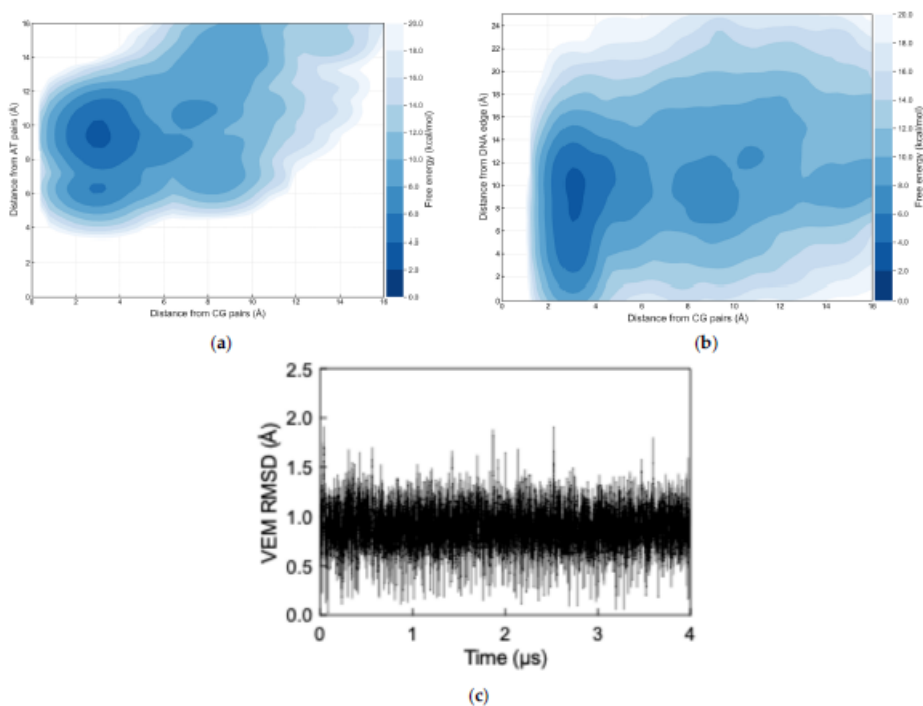
Table 4.2.1. ITC-derived thermodynamic parameters for the ctDNA-VEM complex at different temperatures. The errors in the listed values are within 10%.

Molecular Dynamics Simulations

Despite the extensive experimental validation of VEM binding to ctDNA reported above, the precise molecular details of this interaction remain elusive due to the absence of a crystal structure of the VEM-ctDNA complex. Under such circumstances, however, advanced computational methodologies

can be effectively employed to investigate and characterize the binding interactions at an atomic level. Traditional molecular dynamics simulations provide useful insights but are often limited in their ability to capture the full complexity of drug- ctDNA interactions, particularly in systems with multiple potential binding sites and significant conformational flexibility. To address these challenges, the On-the-Fly Probability Enhanced Sampling with METADynamics-like target distribution (OPES-METAD) technique was employed in this work. Indeed, OPES-METAD enables a comprehensive exploration of the free energy landscape (or free energy surface, FES) by applying a dynamic bias along selected collective variables (CVs), allowing the system to traverse energy barriers that would otherwise be inaccessible in conventional MD simulations. This enhanced sampling technique therefore facilitates the identification of low-energy states corresponding to potential binding sites. The first FES plot (Figure 4.2.4a) illustrates the free energy landscape of the interaction between VEM and ctDNA as a function of the minimum distance from CG base pairs (dCG, x-axis) and AT base pairs (dAT, y-axis). The contour levels depict free energy levels, with deeper-blue regions corresponding to lower free energy states and, hence, more favorable binding configurations for VEM. A distinct low-energy region appears between 2 and 4 Å from the CG base pairs and 8 and 12 Å from the AT base pairs. This finding indicates that VEM has a stronger affinity for regions close to CG base pairs, likely stabilized by direct interactions such as hydrogen bonding. The positioning of the energy minimum also implies that VEM binds within the minor groove of the DNA, where it can establish close contact with CG pairs. Although interactions with AT pairs are also present, these interactions are less pronounced, as reflected by the shallower energy minimum in AT-rich regions. Thus, while VEM engages with both CG and AT bases, its primary interactions are clearly associated with CG pairs, which play a dominant role in stabilizing the drug within the groove. The second FES plot (Figure 4.2.4b) maps the free energy landscape as a function of the distance from the CG base pairs (dCG, x-axis) and the distance from the ctDNA edge (dEDGE, y-axis). Again, a pronounced low-energy region appears between 2 and 4 Å from CG base pairs and 8 and 12 Å from the ctDNA edge. This confirms that VEM preferentially binds to the central CG-rich regions of the sequence, specifically, the CG pairs located at positions 4-5 and 10-11. Although these CG pairs are flanked by AT-rich regions, interactions of VEM with the CG bases are energetically more favorable. The location of the energy minimum suggests that VEM is positioned deep within the minor groove of the ctDNA (rather than at the edge), where it can form optimal contacts with the CG pairs. Overall, the FES analyses clearly identify the central CG-rich region of the ctDNA as the primary binding sites for VEM, supporting a stable, low-energy binding mode characteristic of minor-groove association. In order to characterize in greater detail the binding mode and the underlying interactions between VEM and ctDNA, a configuration corresponding to the lowest free energy state in the FESs

discussed above was extracted, and an additional 4 μ s of unbiased MD simulations were carried out. The stability of the VEM–ctDNA complex was monitored by evaluating the root mean square displacement (RMSD) of VEM within the binding site (Figure 4.2.4c). Figure 4.2.4d shows a representative snapshot isolated from the equilibrated portion of the 4 μ s unbiased MD simulation trajectory of the VEM–ctDNA complex, while panel (e) in the same figure provides a close-up view of the drug–ctDNA. Two permanent hydrogen bonds (HBs) were observed. The first involves the H atom on the pyrrole nitrogen of VEM and the N3 atom of G4, with an average dynamic length (ADL) of 2.76 ± 0.2 Å and a lifetime (LT) of 78.0% over the 5 μ s trajectory. The second HB involves the amino group (NH₂) of the same nucleotide and the N atom in the pyridine ring of the drug (ADL = 2.43 ± 0.3 Å, LT = 75.7%). Interestingly, two water molecules were found to bridge the drug and DNA through hydrogen bonds. As seen again in Figure 4.2.4d, the first water molecule donates two HBs (LT = 68.6%), one to the G4 phosphate group (ADL = 1.82 ± 0.2 Å) and one to the sulfonyl group of the drug (ADL = 1.85 ± 0.2 Å). The second water molecule accepts an HB (LT = 71.5%) from the sulfonamide HN- moiety (1.95 ± 0.1 Å) and donates two further HBs to the carbonyl group (-C=O) of C5 on one strand (1.94 ± 0.2 Å) and the same moiety of T6 in the opposite strand (1.73 ± 0.1 Å). These HBs, together with the network of favorable van der Waals, polar, and hydrophobic stabilizing VEM within the binding region, fully account for the affinity of VEM for the DNA minor groove and provide a molecular-based rationale for the experimental binding affinity measured by ITC.



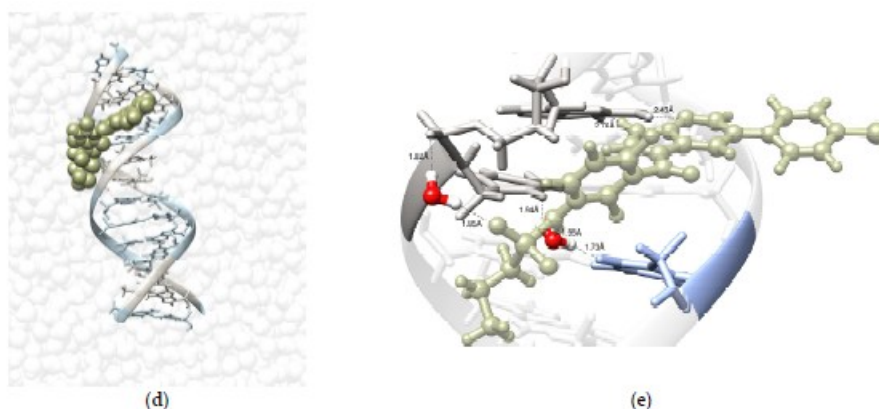


Figure 4.2.4. Free energy surface (FES) as obtained from OPES-METAD simulations for the interaction between VEM and DNA **(a)** as a function of the minimum distance from CG base pairs (dCG) and AT base pairs (dAT), and **(b)** as a function of the distance function of the distance dCG and the distance from the DNA edge (dEDGE). **(c)** Root mean square displacement (RMSD) of VEM over time in the unbiased simulation. **(d)** Snapshot extracted from the equilibrated portion of the 4 μ s unbiased MD simulation trajectory of the VEM–DNA complex. The DNA bases are shown in sticks and balls and colored as follows: adenines and thymines, winter sky; guanines and cytosines, moonstruck gray. VEM is portrayed in fern-colored spheres, while surrounding water molecules are depicted in transparent lucent white. Ions and counterions are not shown for clarity. **(e)** Main interactions involved in the DNA minor groove binding by VEM as obtained from the relevant equilibrated portion of the corresponding 4 μ s unbiased MD simulation trajectory. The water molecules involved in hydrogen bonds with the NA and the drug are shown in atom-colored sticks and balls (O, red; H, white). All other water molecules, ions, and counterions are not shown for clarity.

Conclusions

This work provides a comprehensive and multifaceted understanding of the interactions between Vemurafenib and calf thymus DNA, revealing critical insights that extend beyond its primary mechanism of action as a BRAF inhibitor. Using advanced biophysical techniques and molecular dynamics simulations, it has been demonstrated that VEM binds to DNA through a non-intercalative, groove-binding mechanism, specifically interacting with CG-rich regions in the DNA minor groove. This binding mode is essential as it ensures minimal disruption to the structural integrity of the DNA helix, which is a significant finding given the concerns around potential genotoxicity associated with small-molecule therapeutics like VEM. The experimental data, including UV-vis absorption spectroscopy, circular dichroism, and fluorescence spectroscopy, consistently support the conclusion that VEM does not induce significant structural changes in DNA. This is in sharp contrast with intercalative agents, which often cause substantial perturbations to the DNA double helix and can lead to genomic instability. The slight changes observed in the thermal denaturation studies further reinforce the notion that VEM interacts with DNA in a relatively benign manner, reducing the likelihood of inducing DNA damage or interfering with essential biological processes such as replication and transcription. The thermodynamic parameters obtained from isothermal titration

calorimetry add an additional layer of understanding. The exothermic nature of the interaction, characterized by favorable enthalpic and entropic contributions, highlights the role of hydrogen bonding, van der Waals interactions, and hydrophobic contacts in stabilizing the VEM–DNA complex. The positive entropy contribution, associated with the release of water molecules during binding, is consistent with groove-binding interactions in which the ligand displaces interfacial water without causing major conformational changes in the nucleic acid. Molecular dynamics simulations complement these findings by providing atomic-level detail the binding process. The simulations a strong preference of VEM for CG-rich regions in the minor groove, where it forms stable hydrogen bonds with the DNA bases. This binding configuration is further reinforced by bridging water molecules that help stabilize the VEM–DNA complex through a network of hydrogen bonds and favorable contacts. These computational insights closely mirror the experimental evidence and allow for a cohesive, detailed model of the interaction mechanism. These findings have important implications for the clinical use of VEM, particularly in long-term treatments where cumulative off-target effects could pose a risk to patients. The fact that VEM binds to DNA in a manner that avoids major structural disruptions suggests that its potential for genotoxic side effects may be lower than previously anticipated. Nonetheless, continued monitoring of long-term effects remains essential, especially given the extended treatment regimens typical in oncology. Contextually, understanding these interactions at a molecular level is critical for optimizing the therapeutic profile of VEM and ensuring that its benefits in treating melanoma and other cancers outweigh any potential risks. In conclusion, this study not only deepens the molecular understanding of VEM-DNA interactions but also underscores the value of integrating experimental and computational approaches to fully characterize drug-DNA interactions. The insights gained in this work will not only inform the clinical optimization of VEM but may also guide the rational design of future therapeutics that target, or have the potential to interact with, genomic material. By elucidating the precise nature of VEM-DNA binding, this study lays the groundwork for more informed decisions in drug design and therapy optimization, ultimately contributing to the development of safer and more effective treatment strategies in precision oncology.

4.3. Investigating the Complex Interaction between Self-Assembling Dendrimers and Serum Proteins via Computational and Experimental Methods

Introduction

Dendrimers represent a unique class of highly branched macromolecules characterized by a well-defined, tree-like architecture. Their structure, comprising a central core, successive layers of branching units, and terminal functional groups, confers exceptional uniformity, high surface-to-volume ratios, and versatile functionality. Unlike conventional linear polymers, dendrimers offer precise control over molecular size, shape, and surface chemistry, making them particularly suitable for advanced biomedical and nanotechnological applications. Owing to these properties, dendrimers have been extensively investigated for their potential in drug and gene delivery, diagnostics, and materials science.

Among the most widely studied dendrimer families are poly(amidoamine) (PAMAM) dendrimers, which are appreciated for their biocompatibility and peptide-mimetic properties. However, traditional PAMAM synthesis, based on iterative, multistep processes, poses significant challenges, including the formation of structural defects that complicate purification and scalability, thus limiting their clinical translation. To overcome these issues, recent research has focused on self-assembling amphiphilic dendrimers capable of forming stable supramolecular nanostructures through noncovalent interactions. These amphiphilic dendrimers (ADs), featuring hydrophobic alkyl chains and hydrophilic PAMAM dendrons, spontaneously organize into nanomicelles (NMs) (or nanovectors, NVs) in aqueous environments.

Our research group has designed and synthesized a new amphiphilic dendrimer AD comprising two C18 alkyl chains linked to a PAMAM dendron head (Figure 4.3.1). This molecule self-assembles into spherical nanomicelles (~10 nm in diameter) with well-defined physicochemical characteristics, including a low critical micellar concentration (3.2-5.7 μM) and an aggregation number of approximately 17 AD monomers per micelle. These AD NMs have demonstrated high encapsulation efficiencies and drug-loading capacities for poorly soluble anticancer drugs such as Vemurafenib (VEM) and Dabrafenib (DAB), enhancing their therapeutic efficacy in melanoma cell lines compared to the free drugs.

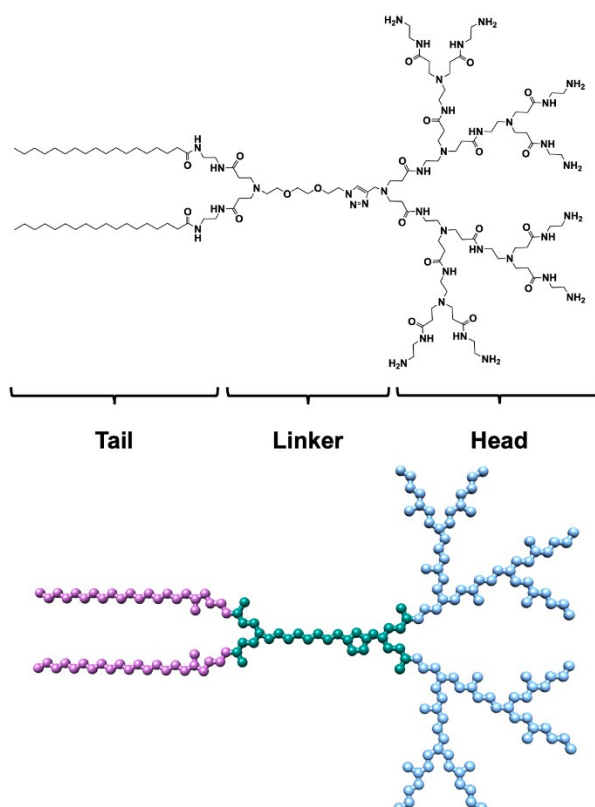


Figure 4.3.1. (Upper panel) 2D chemical structure of the AD monomer. **(Lower panel)** Atomistic representation of the AD monomer. The atoms are represented as spheres and colored by the corresponding region as follows: hydrophobic tails, purple; linker, teal; polar head, sky blue. For clarity, hydrogen atoms are missing.

A crucial aspect influencing the biological performance of nanovectors like AD NMs is their interaction with proteins in physiological environments, leading to the formation of a protein corona (PC). Upon exposure to biological fluids, nanomaterials rapidly adsorb a layer of biomolecules, predominantly proteins, which alter their physicochemical properties, biodistribution, cellular uptake, and toxicity. The PC is typically classified into a *soft corona*, composed of weakly and reversibly bound proteins, and a *hard corona*, consisting of proteins that are tightly and stably adsorbed on the nanocarrier surface. Understanding and controlling this dynamic layer is essential for predicting the in vivo fate of nanomaterials.

Among the various plasma proteins, human serum albumin plays a dominant role due to its high abundance and its impact on the pharmacokinetics, bioavailability, and toxicity of drugs. HSA adsorption onto nanocarrier surfaces can modulate their circulation time, immune recognition, and biological activity, either protecting the nanovector and prolonging its lifetime or hindering its interaction with target cells. Therefore, characterizing the molecular interactions between AD-based nanostructures and HSA is a fundamental step toward optimizing their biomedical performance.

In these studies, the interaction between HSA and the previously described AD as well as HSA and a set of structurally related amphiphilic PAMAM dendrimers bearing different terminal groups, namely

positively charged primary (1-A) and tertiary (1-TA) amines and negatively charged carboxylic acids (1-COOH), in both their monomeric and self-assembled nanomicellar forms (Figure 4.3.2) was systematically investigated.

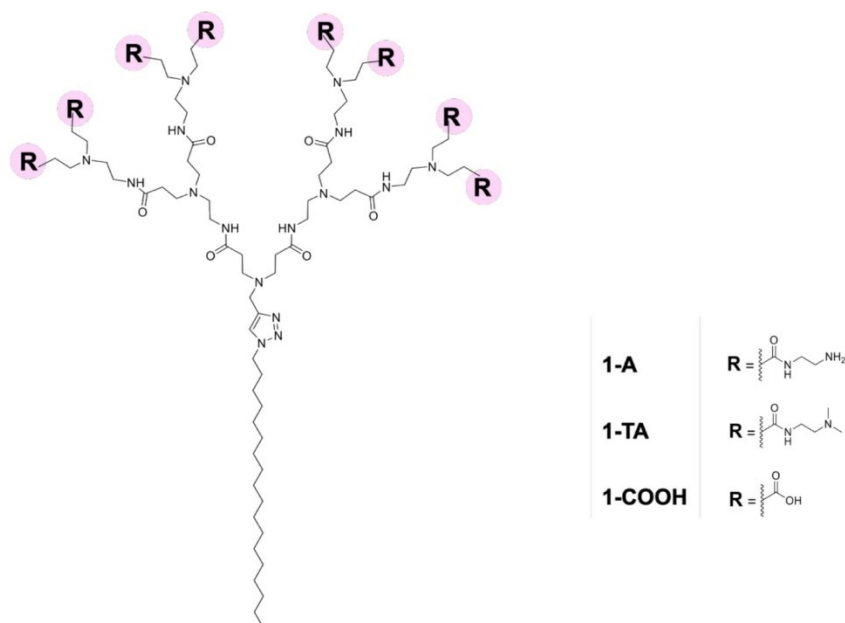


Figure 4.3.2. Chemical structures of AD-PAMAM dendrimers 1-A, 1-TA and 1-COOH bearing different terminal groups.

A comprehensive combination of experimental and computational methods, including fluorescence spectroscopy (FS), isothermal titration calorimetry (ITC), circular dichroism (CD), dynamic light scattering (DLS), and atomistic molecular dynamics (MD) simulations, was employed in order to:

1. quantify the binding affinities and corresponding thermodynamic parameters,
2. identify the interaction sites and the molecular determinants governing binding,
3. assess possible conformational changes induced upon complex formation.

This integrated approach allows to obtain a detailed understanding of dendrimer–protein recognition mechanisms, thus contributing to the rational design of next-generation amphiphilic dendrimers with optimized bio-interactions and enhanced therapeutic potential.

Materials and Methods

Reagents and Chemicals

Globulin- and fatty acid-free human serum albumin (product number A1887) was purchased from Sigma-Aldrich Inc. (Saint Louis, MO, USA). The amphiphilic dendrimer AD) used for the AD/HSA study, as well as the amphiphilic dendrimers 1-A, 1-TA, and 1-COOH used for the comparative series,

were synthesized and fully characterized according to procedures reported in our previous works. All other reagents and solvents were of analytical grade and were obtained from Sigma-Aldrich.

Sample Preparation and Analytical Procedures

HSA stock solutions were prepared by dissolving the appropriate amount of protein in phosphate-buffered saline 1× (PBS, pH = 7.4). Protein concentration was determined by UV spectroscopy at 280 nm using a molar extinction coefficient of $\epsilon = 35,700 \text{ M}^{-1} \text{ cm}^{-1}$.

For the AD/HSA system, AD nanocarrier stock solutions were prepared in PBS at a final concentration of 2.0 mM. For the 1-A, 1-TA, and 1-COOH systems, both monomeric dendrimers and their nanomicellar counterparts (1@-A, 1@-TA, 1@-COOH) were prepared at 2.0 mM in PBS and used at working concentrations either below or above their respective critical micellar concentrations (CMCs), depending on the experiment.

Spectroscopic Studies

Far-UV Circular Dichroism (CD) Spectroscopy

CD experiments were conducted to investigate the impact of dendrimer binding on the HSA secondary structure. Measurements were performed on a J-1500 spectropolarimeter (Jasco, Japan) equipped with a thermostated cell holder. For the AD/HSA system, spectra were acquired from 200 to 260 nm at 298 K, using a 0.2 cm pathlength cell, a 0.5 nm step size, and a 1 nm bandwidth. The concentration of HSA was fixed at 2 μM , while the AD concentration was varied between 1 and 25 μM . Each spectrum represents the average of three independent scans. For the HSA–1-A/1-TA /1-COOH systems, spectra were recorded under the same conditions with dendrimer concentrations either below the CMC (2 μM , monomeric form) or above the CMC (50 μM , micellar form). The secondary structure contents of HSA in all systems were calculated from mean residue ellipticity (MRE) values at 208 nm using validated standard calibration models.

Steady-State and 3D Fluorescence Spectroscopy

Fluorescence spectroscopy (FS) was employed to evaluate the binding affinity and quenching mechanisms of HSA in the presence of the amphiphilic dendrimers. All measurements were performed on an FP-8350 spectrofluorometer (Jasco, Japan) equipped with a thermostatic cell holder and a 1 cm quartz cuvette.

- For the AD/HSA system, the excitation wavelength (λ_{exc}) was set at 280 nm and the emission spectra were recorded from 300 to 450 nm at 25 °C (excitation and emission bandwidths were 5 and 10 nm, respectively). The HSA concentration was maintained at 2 μM , and the AD concentration was varied between 2 and 20 μM . Each mixture was incubated for 2 h prior to

spectral acquisition. 3D fluorescence spectra were obtained with λ_{exc} between 220–350 nm and λ_{em} between 220–500 nm.

- For the HSA–1-A /1-TA /1-COOH systems, the fluorescence spectra were recorded in the range of 300–400 nm at 25, 31, and 37 °C. The HSA concentration was fixed at 2 μ M, while the dendrimer or nanomicelle concentrations were adjusted according to their CMC values. Inner filter effects were corrected using standard equations, and competitive binding assays were performed using warfarin (Sudlow’s site I marker) and stearic acid (fatty-acid binding site marker) to validate the binding site predictions.

All fluorescence measurements were conducted in triplicate ($n = 3$), and the results are reported as mean $\pm$ standard deviation (SD).

Ultraviolet–Visible (UV–Vis) Spectroscopy

UV–vis absorption spectra were recorded on a Jasco V-730 spectrophotometer (Japan) using 1 cm quartz cuvettes. Spectra were collected over the 240–400 nm range at 25 °C, under the same buffer composition and concentration conditions adopted for the fluorescence assays ($n = 3$).

Isothermal Titration Calorimetry (ITC)

ITC experiments were performed with a MicroCal PEAQ-ITC calorimeter (Malvern Analytical, UK) at 25 °C (cell volume = 208 μ L).

- For the AD/HSA system, a solution of HSA (30 μ M, in the cell) was titrated with 19 injections (2 μ L each) of AD (900 μ M, in the syringe). AD concentrations were maintained above the CMC throughout the titration in order to selectively probe nanomicellar interactions. Corrected thermograms were analyzed using a “one set of sites” binding model.
- For the HSA–1-A/1-TA/1-COOH systems, two different titration protocols were performed: (i) *Monomeric form*: HSA (50 μ M, syringe) was titrated into dendrimer solutions (5 μ M, in the cell) under sub-CMC conditions and (ii) *Micellar form*: HSA (30 μ M, in the cell) was titrated with dendrimeric micelles (900 μ M, syringe) under above-CMC conditions.
- Integrated data were fitted using the same “one set of sites” model to determine the binding constants (K_d), enthalpy changes (ΔH), entropy contributions ($-T\Delta S$), and Gibbs free energy values (ΔG).

The molar ratio was expressed using the mass conservation relation:

$$[AD_{tot}] = [AD_{mon}] + N_{agg} * [AD_{mic}] \quad (47)$$

where N_{agg} represents the aggregation number (17 for AD nanomicelles).

All ITC measurements were performed in triplicate, and non-specific heats were subtracted from the raw thermograms.

Dynamic Light Scattering) and Zeta Potential Measurements

DLS and ζ -potential analyses were carried out using a Zetasizer Nano-ZS (Malvern, UK) at 25 °C (He-Ne laser, $\lambda = 633$ nm, scattering angle $\theta = 173^\circ$). For the AD/HSA, the technique was used to monitor micellar size and surface charge both before and after protein binding. For 1-A/1-TA /1-COOH, DLS was employed to evaluate micelle formation and hydrodynamic size variations upon interaction with HSA under different surface charge conditions.

Transmission Electron Microscopy (TEM)

For the AD/HSA system, TEM imaging was employed to visualize the morphology of the AD/HSA complexes. Samples (5 μ L) were deposited onto carbon-coated copper grids, air-dried for 1 h, stained with uranyl acetate, and imaged with a Philips EM 208 electron microscope (operating at 100 kV) equipped with a Quemesa camera (Olympus Soft Imaging Solutions, Germany). Images were acquired and processed using RADIUS 2.3 software.

Saturation Transfer Difference NMR Spectroscopy (STD-NMR)

STD-NMR samples ($[AD] = 300$ μ M, $[HSA] = 300$ μ M) were prepared in D₂O (99.98% D) provided by EurIsotop (Saint Aubin, France). STD-NMR experiments were recorded at 300 K on Bruker AVANCE spectrometer (Karlsruhe, Germany), operating at an ¹H Larmor frequency of 500 MHz, equipped with a double resonance broadband fluorine observe (BBFO) of 5 mm probe head, and z-gradient coil. The solutions were transferred into a 5 mm NMR tube. The on-resonance irradiation of the protein was performed at 1.5 ppm. Off-resonance irradiation was applied at -40 ppm.

Computational Details

Computational studies were performed for both the AD/HSA and HSA–1-A/1-TA /1-COOH systems in order to complement and support the experimental results.

System Setup and Parameterization

For all systems, GAFF2 atom types were assigned, and partial charges computed using the RESP method through the RED server. The HSA structure (PDB ID: 4K2C) was parameterized using the AMBER ff14SB force field.

Electrostatic potential distributions were calculated with Delphi 8.5, using an internal dielectric constant of 4, an ionic strength of 0.15 M, and a SURFPOT value of 4.0 Å, to assist in nanomicelle–protein orientation and ζ -potential estimation.

Molecular Docking and Dynamics

For the HSA-1-A/1-TA /1-COOH systems, initial binding sites were selected based on canonical fatty acid-binding pockets (FA5-FA7), and molecular docking was performed using AutoDock Vina. The best-scoring poses were subsequently refined by atomistic molecular dynamics simulations.

For the AD/HSA system, the AD nanomicelle model was constructed using parameters previously derived from SAXS, NMR, and ITC data. Electrostatic surface analyses were employed to the positioning of HSA molecules near oppositely charged micellar domains.

All complexes were solvated in TIP3P water boxes with a minimum padding of ≥ 15 Å padding, and Na⁺/Cl[−] ions were added to reach an ionic strength of 0.15M. The systems were energy-minimized, equilibrated, and subjected to 1 μ s NPT MD simulations at 298 K and 1 atm. Trajectory analyses were performed on the last 100 ns of stable simulation regime.

Energetic and Structural Analysis

Binding free energies were calculated using the MM/PBSA or MM/GBSA approaches as implemented in AMBER22 (MMPBSA.py), with an internal dielectric constant of 16 to account for the highly charged interface. Structural analyses were carried out using CPPTRAJ and custom Python scripts.

All simulations were performed on local GPU/CPU hybrid clusters and on high-performance computing facilities at CINECA (Bologna, Italy).

4.3.1. Investigating the Complex Interaction between Self-Assembling Dendrimers and Human Serum Albumin via Computational and Experimental Methods⁴

Results and Discussion

Experimental Binding Analysis of the HSA/AD Complex

Fluorescence Spectra and Binding Constants of the HSA/AD Complexes

Human serum albumin is composed of a single chain containing 585 amino acids, with a unique tryptophan residue (W214) located within subdomain II A. The emission from W214 primarily dictates the fluorescence spectra of HSA in the UV range. Upon interaction with other molecules, alterations in tryptophan fluorescence may arise, influenced either by conformational changes of the protein or direct quenching effects. Accordingly, changes in HSA fluorescence were monitored to investigate the binding of HSA with the AD nanomicelles (Figure 4.3.3A). The most notable change observed in the fluorescence spectrum upon addition of AD was the progressive quenching of fluorescence intensity (Figure 4.3.3A). Increasing concentrations of AD led to a linear decrease in tryptophan fluorescence emission. Therefore, the quenching behavior was analyzed using the Stern–Volmer (SV) equation (Eq. 5, see Section 3.1.2)

The SV equation assumes a linear relationship between F_0/F and $[Q]$ (see inset in Figure 4.3.3A), with the slope representing the value of K_{SV} . In the present case, the calculated value of K_{SV} for AD is $4.9 (\pm 0.2) \times 10^4 \text{ M}^{-1}$.

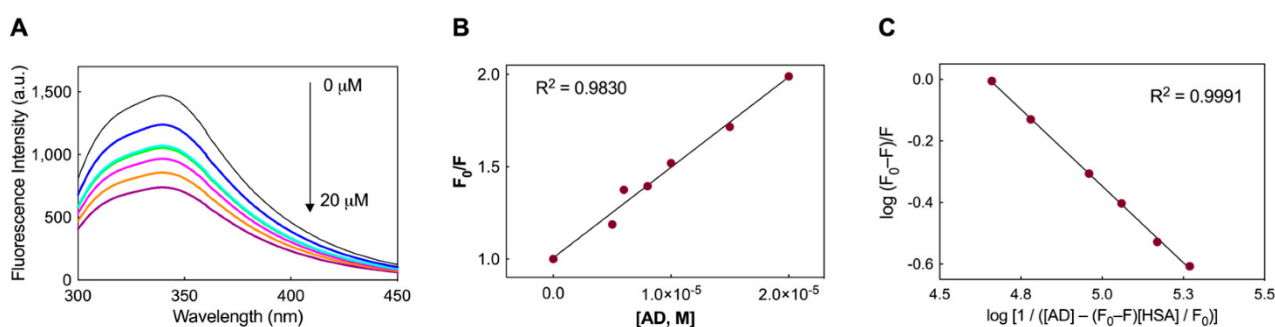


Figure 4.3.3. (A) Fluorescence emission spectra recorded for the HSA/AD NM systems. The spectra include measurements for free HSA (2 μM, black) and HSA at 2 μM with varying concentrations of AD NMs (5 μM in blue, 6 μM in cyan, 8 μM in magenta, 10 μM in orange, and 20 μM in purple). (B) Stern-Volmer plot illustrating the fluorescence quenching of HSA induced by the

⁴ The material presented in this chapter was published at: “Molecular Ballet: Investigating the Complex Interaction between Self-Assembling Dendrimers and Human Serum Albumin via Computational and Experimental Methods”. Cavalieri, G.; Marson, D.; Giurgevich, N.; Valeri, R.; Felluga, F.; Laurini, E.; Pricl, S. *Pharmaceutics* 2024, 16, 533.

AD NMs ($R^2 = 0.9830$). (C) Plots of $\log(F_0 - F)/F$ vs. $\log[1/([AD]) - (F_0 - F)[HSA]/(F_0)]$ for HSA fluorescence quenching by AD NMs ($R^2 = 0.9991$).

Under the assumption that HSA and AD interact via a single independent binding site within the protein structure, the binding constant K_d values can be estimated from the plot in Figure 4.3.3B by using the following modified version of the Stern-Volmer equation (Eq. 7). The K_d value was determined by evaluating the linear correlation between the two logarithmic terms in the modified Stern-Volmer equation. This analysis resulted in an affinity value of $22.9 \pm 4.2 \mu\text{M}$, indicating a moderate binding interaction between the nanomicelles and the serum protein.

Isothermal Titration Calorimetry Binding Analysis

An isothermal titration calorimetry study was conducted to quantitatively describe and characterize the interactions between the self-assembling AD nanomicelles and HSA in aqueous solutions at physiological pH (7.4). Different ITC conditions were systematically explored to accurately determine the thermodynamic binding parameters between HSA and AD, aiming to elucidate the interaction mechanisms between these two entities. This comprehensive analysis involved not only the assessment of binding affinity, expressed through the dissociation constant K_d and the relevant Gibbs free energy ΔG , but also the enthalpic ΔH and entropic $-T\Delta S$ components, which collectively characterize the interaction mechanism. Upon examination of the raw thermogram (Figure 4.3.4, upper panel insert), it is evident that each injection of AD, administered in discrete amounts, correlates with a negative heat flow peak, indicating an exothermic reaction between the protein and the nanocarrier. The determination of K_d values can be directly inferred from the sigmoidal profile of the integrated ITC data (Figure 4.3.4, upper panel), specifically from the slope at the inflection point. The binding strength obtained, represented by a mean affinity value of $13.4 \pm 3.1 \mu\text{M}$, indicates a moderate binding interaction between the serum macromolecule and the AD nanomicelles. Notably, the values obtained from the ITC experiments are fully consistent with the results obtained from the fluorescence assay for the HSA/AD complexes.

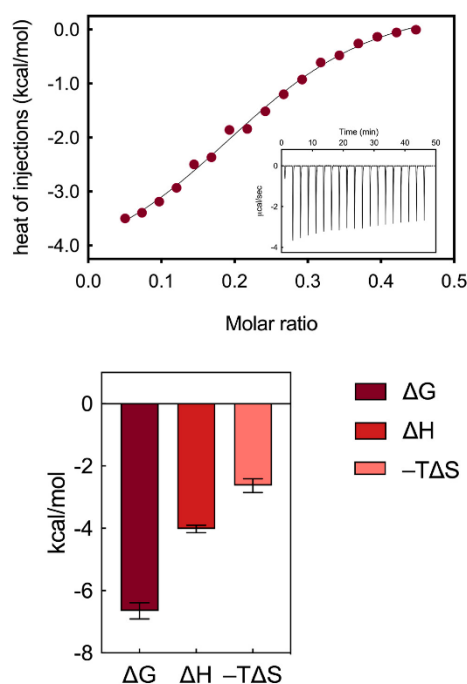


Figure 4.3.4. (Upper panel) ITC plot for the titration of HSA with AD nanomicelles (inset shows the measured heat power vs. time elapsed during HSA/AD binding). **(Bottom panel)** Summary of thermodynamic data extracted from ITC for the binding of AD nanosystems to HSA.

The analysis of the complete binding thermodynamics (Figure 4.3.4, bottom panel) enabled a detailed characterization of the interaction mechanism between the AD nanocarriers and HSA. Notably, the binding of the HSA with the AD nanomicelles was found to be thermodynamically spontaneous, as indicated by the negative value of ΔG (-6.65 ± 0.26 kcal/mol, Figure 4.3.4). By integrating these thermodynamic findings with the distinctive molecular features of the HSA/AD complex, the main interaction mechanisms between the AD nanovector and HSA could be delineated as follows:

1. robust electrostatic interactions are established between the positively charged terminal groups of the AD NMs and the side chains of selected aspartic and glutamic acid residues within HSA;
2. a limited network of hydrogen bonds (HBs) is formed between the internal groups of the AD molecules and specific amino acid residues of the serum protein, favoring the exposure of side chains that act as counterparts for proton bridge formation. Notably, within the AD dendron, the oxygens of the internal amide groups act as hydrogen bond acceptors, with the -NH moieties of the same groups function as HB donors;
3. these two classes of interactions are predominantly enthalpic in nature, as indicated by the favorable ΔH value obtained from the titrations (-4.02 ± 0.12 kcal/mol, Figure 4.3.4).

A couple of additional, non-negligible interactions must be considered to complete the thermodynamics scenario. In particular, the following contributions were identified:

1. weak hydrophobic interactions occur between the hydrocarbon/aliphatic components of the nanocarrier heads and the side chain of hydrophobic residues of HSA;
2. secondary hydrophobic reorganizations occur upon complex formation at the respective surfaces, leading to the release of structured water molecules, ions, and counterions. These latter contributions generate favorable entropic changes, enhancing the stability of the interaction between the protein and the NMs, as demonstrated by the value of $-T\Delta S = -2.63 \pm 0.22$ kcal/mol (Figure 4.3.4).

Structural and Thermal Perturbation of HSA upon AD Binding

Determination of the HSA Secondary Structure and Melting Temperature through Circular Dichroism Spectroscopy

Circular dichroism spectroscopy is a valuable technique for elucidating protein structures and examining conformational changes induced by ligand interactions. In the far-UV CD spectra of HSA, two negative bands are observed at 208 ($\pi\text{-}\pi^*$) and 220 nm ($n\text{-}\pi^*$), indicative of an α -helix-rich protein secondary structure. To ascertain the impact of AD nanovector binding on the secondary structure of HSA, CD spectra of free HSA and its AD complexes (Figure 4.3.5A) were recorded. These experiments were conducted to compare the percentage of α -helix content in free HSA and in the presence of the nanovector, at concentrations below (monomer form) or above (nanomicelles) its critical micelle concentration (CMC), with CD spectra values recorded between 200 and 260 nm at 25 °C. The percentage α -helicity of HSA was estimated from the Mean Residue Ellipticity (MRE) values at 208 nm using Eqs. (14) and (15) (see Section 3.1.4). The findings of this analysis are depicted in Figure 4.3.5B. The CD spectra indicate a percentage of α -helix of 59.3% for the unbound HSA, in good agreement with previous studies reported in literature.

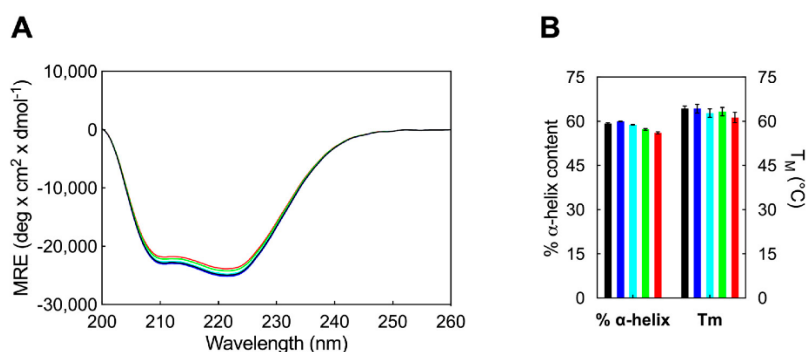


Figure 4.3.5. (A) Far-UV CD spectra of HSA alone (black line) and in the presence of AD in concentration of 1 μ M (blue line), 5 μ M (cyan line), 10 μ M (green line) and 25 μ M (red line). CD

experiments were carried out in PBS buffer solution at 25 °C, with [HSA] maintained at 2 μ M. **(B)** The α -helical content (left y-axis) and melting temperature (right y-axis) of HSA are depicted in the absence (black columns) and presence of AD at concentrations of 1 μ M (blue columns), 5 μ M (cyan columns), 10 μ M (green columns), and 25 μ M (red columns).

The results of the CD experiments indicate that the secondary structure of HSA remains essentially unaffected by the interaction with AD at concentrations below its CMC (e.g., 1 μ M), demonstrating that AD in its monomeric form does not induce significant conformational changes in the secondary structural motifs of the protein. Upon increasing AD concentration above its CMC, a slight decreasing trend is observed, resulting in a final α -helix percentage of 56.1% (Figure 4.3.5A), measured at the highest AD concentration of 25 μ M.

Furthermore, temperature-dependent CD spectroscopy was employed to investigate the potential impact of AD on the melting temperature (T_M) of HSA. T_M values were determined by recording CD spectra at 222 nm across a temperature range of 25 °C to 95 °C, with the addition of nanomicelles below and above their CMC. The results obtained are presented in Figure 4.3.5B. Consequently, the assessment of HSA thermal stability, based on T_M estimation, yielded results fully consistent with those derived from the standard CD spectra. In fact, no significant fluctuations were observed in the T_M value of HSA in the presence of AD monomers or at lower molar ratios of HSA/AD nanomicelles (Figure 4.3.5B). However, at higher concentrations, AD NMs were capable of reducing the albumin T_M by approximately 3 °C. Thus, only in the presence of high amounts of AD amphiphiles in their self-assembled form does HSA exhibit a slight decrease in thermal stability, in agreement with the modest reduction observed in α -helical content (Figure 4.3.5B).

In summary, the interaction between serum albumin and AD nanomicelles does not induce any substantial structural alterations in the protein. This observation is consistent with the moderate binding strength of the HSA/AD complexes, as previously determined by fluorescence spectroscopy and ITC analysis.

Three-Dimensional Fluorescence Spectroscopy

3D fluorescence spectroscopy represents as a further, valuable tool for probing changes in the conformation of HSA. The fluorescence intensity of the primary peak in the 3D FS spectrum is associated with both the polarity of the microenvironment and the secondary structure of the protein. Notably, the peak ($\lambda_{ex}/\lambda_{em}$ = 280/343 nm/nm) predominantly reflects the spectral characteristics of the W214 residue of HSA, while the other peaks correspond to the first-order Rayleigh scattering peak ($\lambda_{ex} = \lambda_{em}$). To corroborate the findings obtained from CD spectroscopy, 3D FS spectra were recorded, as shown in (Figure 4.3.6).

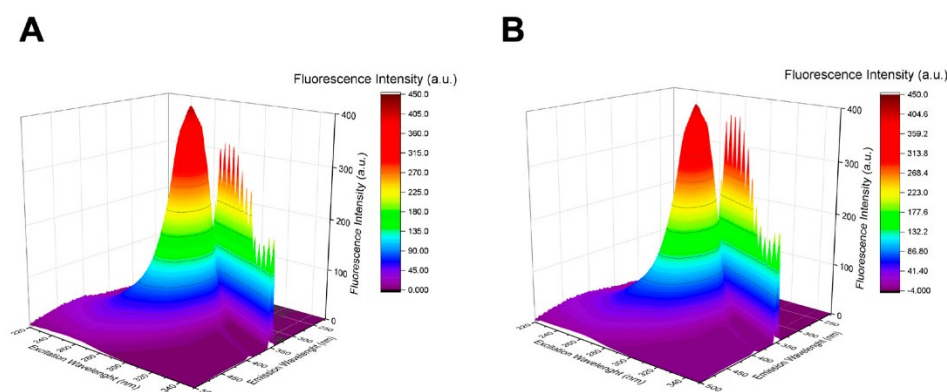


Figure 4.3.6. 3D fluorescence spectra of HSA in absence (A) and in presence (B) of AD nanomicelles (10 μ M).

As seen in Figure 4.3.6, no significant shifts in the characteristic peaks of HSA were observed in the presence of AD nanomicelles. These results indicate that no relevant alterations occur in the local environment surrounding the W214 residue upon interaction with AD. Consequently, it can be inferred that the binding between HSA and the AD nanomicelles likely occurs predominantly at the external protein surface, mainly involving the charged surface of the nanomicelles.

Transmission Electron Microscopy and Dynamic Light Scattering Analysis

Transmission electron microscopy (TEM) analysis of the HSA/AD NM systems revealed the formation of a spherical molecular complex with an average diameter of approximately 20 nm (Figure 4.3.7A). Dynamic light scattering) experiments performed in aqueous solution provided measurements of the hydrodynamic diameter (d) and the ζ -potential (ζ), yielding values of 17.6 ± 0.3 nm (Figure 4.3.7B) and +8.3 mV, respectively. In our previous work, the ζ -potential for pristine AD NMs was reported as +27 mV, while literature data suggests that pure HSA typically exhibits negative ζ values around -15 mV. This suggests that although the positively charged AD NMs primarily interacts with the negative residues of HSA, each HSA molecule within the complex adopts an orientation in which its more positively charged regions remain exposed to the solvent. This evidence, together with the calculated N_{agg} for the AD nanomicelles and the molar ratio detected from the ITC experiments, supports a plausible stoichiometry of six serum proteins per single AD nanoassembly, in agreement with experimental observations reported for similar systems.

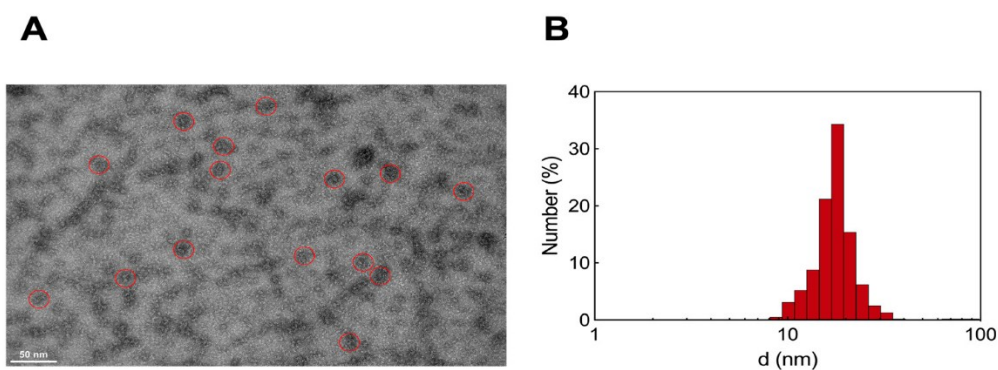


Figure 4.3.7. (A) TEM images of HSA/AD complexes. Red circles highlight three representative HSA/AD NM structures in each TEM ensemble. (B) Hydrodynamic diameter (d) of HSA/AD complexes at 25 °C as measured by DLS.

Epitope Mapping of Bound AD with the Use of STD-NMR

The binding mode of artemisinin with HSA was also investigated by employing STD NMR spectroscopy, which allows the identification of ligand moieties that interact with the binding site of the protein, as described in Section 3.1.8. The STD-NMR spectra of AD-HSA systems with a molar ratio of AD to HSA of 1:1 is displayed in Figure 4.3.8 (upper panel).

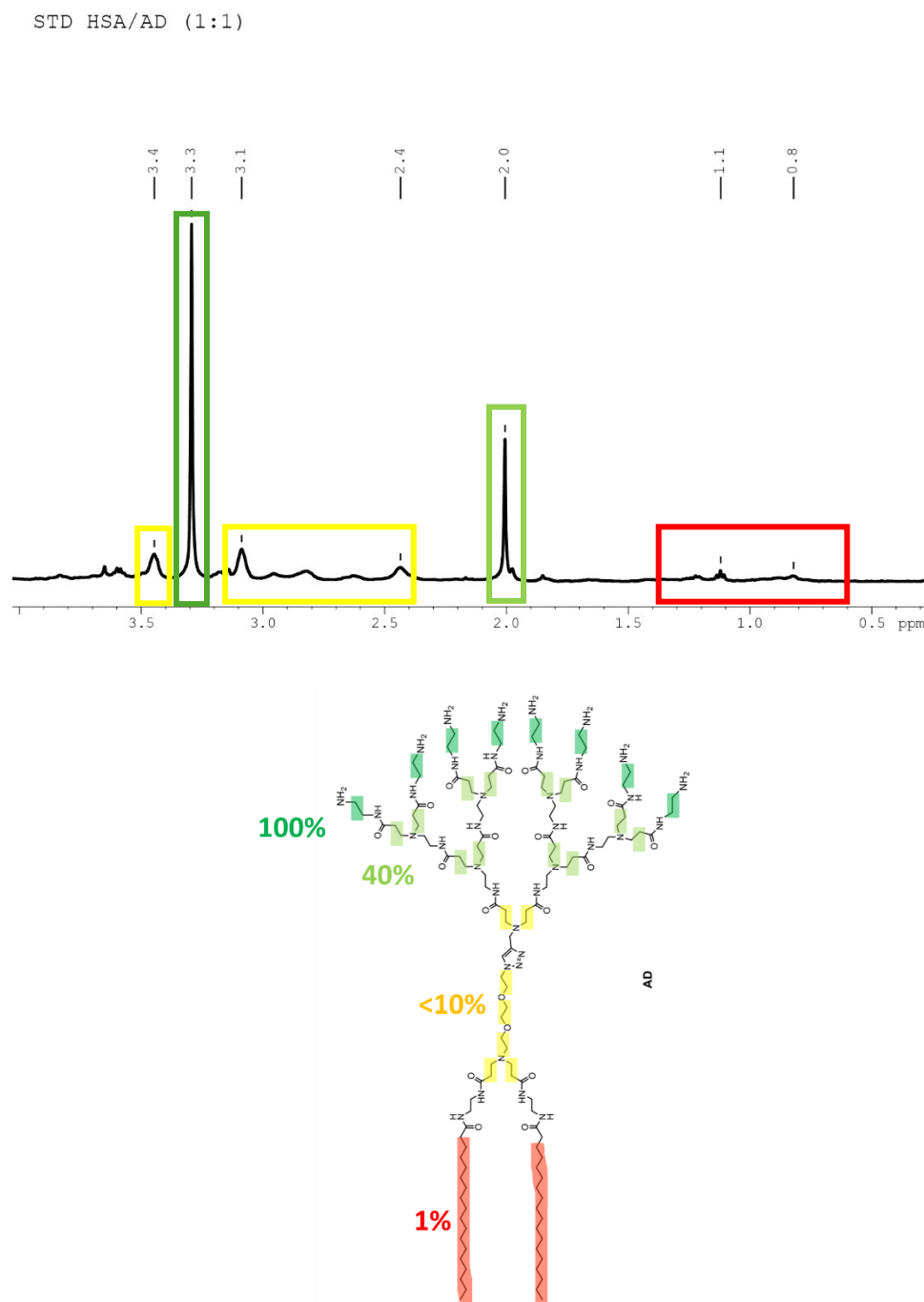


Figure 4.3.8. (Upper panel) STD-NMR spectrum of AD in the presence of HSA. [AD] = 300 μ M, [HSA] = 300 μ M, pH = 7.4, T = 300 K. (Lower panel) Epitope maps for the AD created from STD NMR. The percentage values for each epitope map is the R_{STD} . Green indicates the strongest interacting proton, light green the second, yellow the third, and red the weakest.

The ligand proton that lies closest to the protein experiences the strongest magnetization transfer and therefore shows the highest relative signal intensity. Binding epitopes were determined by calculating the relative STD (%) effect (R_{STD}), which indicates how much saturation is transferred from the protein through cross-relaxation and how near each proton is to the protein's binding site. To obtain

the R_{STD} values, the STD signal with the highest integral was assigned a value of 100%, and all remaining STD signals were scaled relative to this reference.

The STD-NMR analysis reveals a clear hierarchy of interactions between the dendrimer and human serum albumin (HSA) as shown in Figure 4.3.8, lower panel. The most intense STD response is observed for the methylene protons at δ 3.3 ppm, assigned to CH_2 groups adjacent to the terminal amines, indicating that these peripheral functionalities constitute the primary contact region with HSA (R_{STD} = 100%, highlighted in green in Figure 4.3.8, lower panel). A second significant contribution arises from the CH_2 units at δ 2.0 ppm, located between nitrogen and carbonyl groups within the dendrimer core or branching points, demonstrating that portions of the internal scaffold also participate in binding events (R_{STD} = 40%, highlighted in light green in Figure 4.3.8, lower panel). Signals in δ 3.4 ppm, corresponding to $O-CH_2-CH_2-O$ motifs of PEG-like linkers or spacer segments, exhibit medium STD intensities, suggesting that the central spacer domain plays an auxiliary but non-negligible role in the interaction, while the protons in the δ 2.4–3.1 ppm region, associated with CH_2 groups adjacent to amide linkages, display a certain degree of saturation transfer, consistent with their intermediate spatial position between the dendrimer core and its outer branches (R_{STD} = 10%, highlighted in yellow in Figure 4.3.8, lower panel). In contrast, the low-intensity responses at δ 1.1–0.8 ppm, attributed to hydrophobic aliphatic tails, indicate basically no contact with the protein surface (R_{STD} = 1%, highlighted in red in Figure 4.3.8, lower panel). Overall, the STD pattern highlights a binding mode dominated by the terminal amine region, with secondary contributions distributed across both the internal framework and the linker units.

Computational Studies

Binding Analysis of the HSA/AD Complex

Based on the experimental findings, it is likely that HSA and the AD NMs primarily interact through surface-mediated interactions involving the polar external amino acid residues of the protein and the hydrophilic, positively charged heads of the nanomicelles. To elucidate the interactions between these two molecular entities at the atomistic level, an investigation was initiated by examining the electrostatic potential distribution surrounding the serum protein. While the serum protein typically exhibits an abundance of negatively charged amino acids under physiological conditions, the analysis revealed that these residues are not homogeneously distributed on its surface. Nonetheless, a distinct surface region characterized by a marked overall negative potential was identified, particularly over the IA, IIA, and IIB domains of HSA (Figure S4), thus indicating a preferential site for binding with the positively charged AD nanomicelles. This electrostatic complementarity facilitates the formation of the initial complex between HSA molecules and AD nanomicelles. Consistent with our

experimental evidence and literature data reported for similar systems, the resulting complex is composed of six protein molecules making up the protein corona around one self-assembled AD nanomicelle. The determination of the surface electrostatic potential enabled further validation of the 6:1 stoichiometry of the HSA/AD complex. Specifically, the ratio between the solvent accessible surface area (SASA) of the AD nanomicelle ($35,618 \text{ \AA}^2$) and the SASA corresponding to the region of HSA exhibiting the most negative potential distribution (Figure S4, $5835 \text{ \AA}^2$) within a single protein yielded a value of 6.1, closely resembling the experimentally determined HSA/AD ratio. Consequently, after establishing the appropriate reciprocal orientation of HSA molecules relative to AD nanomicelle, six HSA molecules were manually positioned in proximity to the AD nanoassembly. Each HSA molecule was placed at a minimum distance of $10 \text{ \AA}$ from the AD nanomicelle, with the protein molecules evenly distributed over its surface (Figure 4.3.9).

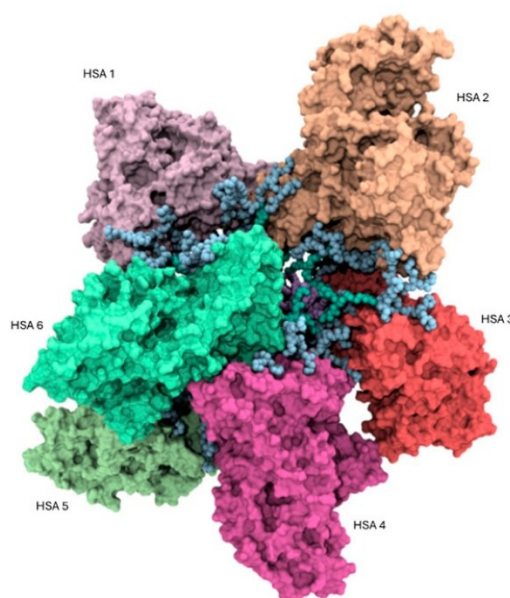


Figure 4.3.9. Atomistic representation of the HSA/AD (6/1) complex. Each protein structure is depicted as a colored surface, while the atoms of the AD nanomicelle are represented as spheres colored according to the scheme adopted in Figure 4.3.1.

Focusing on the primary interactions at the atomic level between HSA and the AD nanomicelle, the initial phase involved the assessment of the contacts between the micelle's atoms and the residues of the protein along the corresponding equilibrated MD trajectory. Specifically, this process entailed determining the closest distance between every amino acid of the HSA molecules and any atom within the micelle for each snapshot of the MD simulation (Figure S5). Initial observations revealed a consistent interaction pattern, where the HSAs making up the protein corona were found to contact the nanomicelle at very similar regions and amino acids. Subsequently, the frequency of protein-nanomicelle contacts was calculated by defining a contact as a scenario where the minimum distance between two interacting atoms (belonging to the protein and the nanoassembly, respectively), was

below 6.5 Å in any given MD frame. This approach resulted in the generation of a more detailed interaction fingerprint between each protein and the AD nanomicelle, as illustrated in Figure 4.3.10.

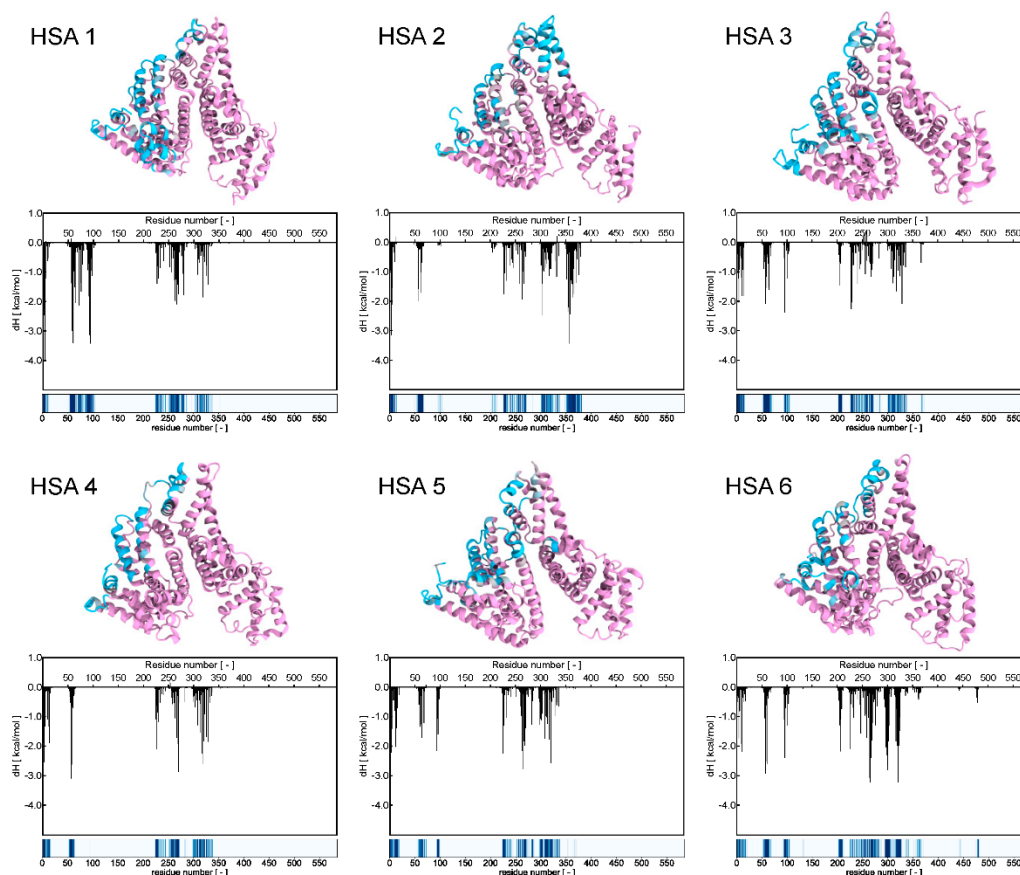


Figure 4.3.10. The six panels represent each HSA bound to the AD micelle in **Figure 4.3.9**. (**top panels**) HSA protein is represented by its secondary structure, and the aminoacid residues are colored in pink (no contact) or cyan (consistent contacts). (**middle panels**) the enthalpic free energy decomposition for each HSA residue as obtained from the MM/PBSA analysis. (**bottom panels**) The interaction fingerprint is based on the frequency of interaction of each HSA residue with the AD micelle. Fingerprints are colored based on the contact frequency with shades of blue: white representing 0 contact frequency while dark blue representing a constant contact throughout the MD trajectory.

Examination of these binding fingerprints revealed highly consistent interaction patterns across all HSA molecules (Figure 4.3.10, bottom panels). To quantitatively characterize these patterns in terms of energy, an MM/PBSA analysis was conducted. This analysis allowed for the decomposition of the enthalpic binding energy into individual contributions from each amino acid within the protein. Subsequently, comparison between the interaction fingerprints (Figure 4.3.10, bottom panels) and the energy contributions of each amino acid (Figure 4.3.10, middle panels and Table S1) revealed a strong correlation between the energy profiles and the structural data.

For a deeper understanding of the interaction between HSA and the AD nanomicelle, the number of charged AD groups involved in stable salt bridges (SBs) with the protein's residues was quantified.

These SBs were deemed significant if they persisted for at least 50% of the MD simulation duration. Results revealed that approximately 73% of the charged terminals on the AD NM formed stable SBs with negatively charged (*i.e.*, aspartic and glutamic acid) residues on HSA. While salt bridges play a pivotal role in binding and represent a significant portion of the interactions identified through contact and energy analysis, they are not the sole contributors. Hydrogen bonds and van der Waals forces also contribute to the stabilization of the macromolecular complexes, as represented in Figure S6. Although the interaction sites are widely distributed across the protein's surface (Figure 4.3.10, upper panels), two regions of HSA appear to be particularly crucial for binding with the positively charged branches of the AD nanomicelle. These regions include the helix containing residues E252, D255, D256, D259, E266, and D269, as well as the small loop containing residues E60 and D63. These regions form highly stable salt bridges with the nanomicelle terminal nitrogen in each HSA molecule comprising the protein corona (Figure 4.3.10).

Binding Analysis of the HSA/AD Complex

Further analysis of the atomistic MD data for the HSA/AD nanomicellecomplex revealed that the overall sizes of both the HSA chains and the AD nanoassembly remain essentially unchanged upon the formation of the protein corona, as evidenced by the corresponding value of the radius of gyrations (R_g). This parameter serves as an indicator of the compactness of a given molecular (super)structure and directly reflects its size in solution. As illustrated in Figure 4.3.11A, there is virtually no significant alteration in the micelle's R_g upon formation of the HSA protein corona, with R_g values of 30.82 ± 0.62 Å for the pristine AD nanomicelle and 30.98 ± 0.32 Å within the HSA/AD complex.

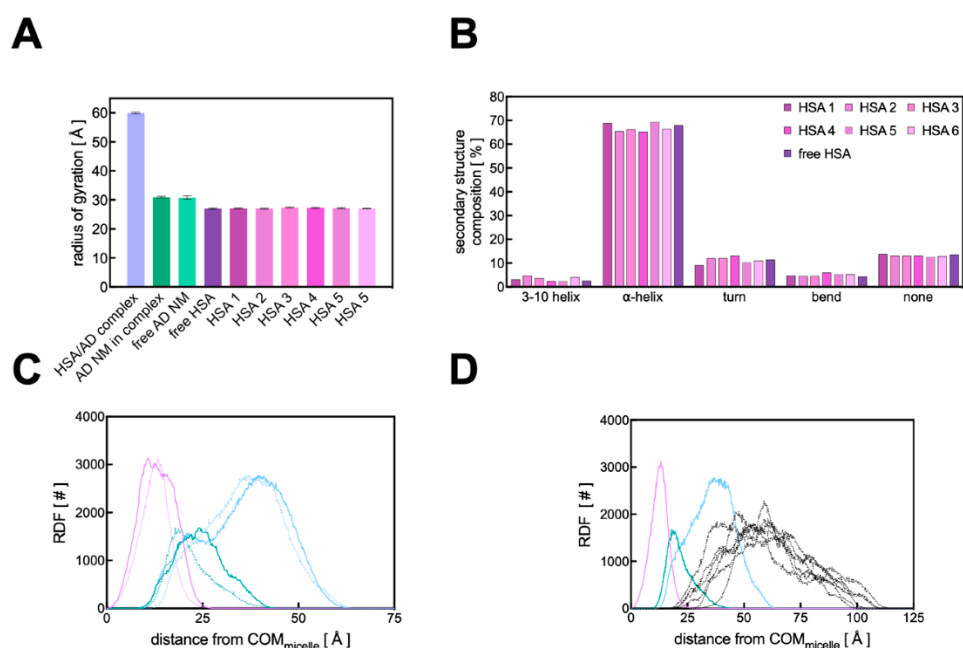


Figure 4.3.11. (A) Comparison of the radius of gyration (R_g) values for the HSA/AD complex, the AD NM within the complex, a free AD NM, a free HSA molecule, and each of the 6 HSA chains within the HSA/AD assembly. (B) Comparison of the secondary structure composition for the 6 HSA molecules in the AD complex and an isolated HSA chain. (C) Radial distribution functions of the AD NM atoms (tail in purple, linker in aquamarine, head in cyan), showing variations between unbound and complexed states. Dotted lines represent the RDF values for the unbound AD NM, while the solid lines represent the values for the AD NM in complex with HSA. (D) RDF of the complete HSA/AD complex relative to the center of mass of the AD NM. The RDF of the protein atoms are represented as black lines.

As previously documented by SAXS experiments, the AD NMs exhibit a triaxial ellipsoid shape in solution. Accordingly, the analysis of the gyration tensor's principal components performed in the present work provided further insights into the overall geometry of the HSA/AD complex. The square roots of the gyration tensor's eigenvalues represent the characteristic semi-axis lengths of the ellipsoid characterizing both the AD NM and its complex with HSA. Remarkably, upon complexation, the AD micelle maintains its triaxial ellipsoid configuration (with semi-axis lengths of 45.8 ± 0.4 Å, 39.78 ± 0.3 Å, and 37.18 ± 0.3 Å), indicating that its interaction with serum proteins does not induce measurable structural alterations in the AD nanostructure, despite protein distribution across its surface. Notably, when examining the overall 6:1 HSA/AD complex, its shape resembles that of an oblate spheroid, featuring a discernible minor axis (with semi-axis lengths of 81.8 ± 0.5 Å, 80.2 ± 0.3 Å, and 71.4 ± 0.7 Å).

From the perspective of the protein, its interaction with the micelles does not seem to significantly perturb its overall structure. As demonstrated in Figure 4.3.11A, the size of each protein when bound to the AD micelle (as indicated by the R_g value) remains largely unchanged upon complex formation. In fact, the calculated R_g values for the different HSA chains range from 26.98 to 27.35 Å, with a maximum standard deviation of 0.18 Å.

These values align with the R_g value computed for a single HSA under similar conditions (27.01 ± 0.16 Å) and are consistent with previously reported literature data on HSA. To delve deeper into the potential impacts of AD nanomicelle binding on the internal structure of HSA, the secondary structure content of the proteins, both free and bound to the AD nanomicelle, was analyzed using the DSSP algorithm, which assigns secondary structures to amino acids based on their 3D coordinates. The secondary structure of a single HSA simulated under similar conditions predominantly comprises α -helices (68.0%), along with some turns (11.5%), and no detectable β -sheets, consistent with existing literature. In complex with the AD nanomicelle, the α -helix percentages vary between 65.3 and 69.4%, and the turns between 9.2 and 13.2%, with again no β -sheet formation observed. These findings suggest that the interaction between the HSA chains and the AD nanomicelle does not significantly alter the arrangement of protein secondary structure, as evidenced in Figure 4.3.11B. Furthermore, considering again the 6:1 full HSA/AD nanomicelle complex, the corresponding

computed R_g is $60.01 \pm 0.27 \text{ \AA}$ (Figure 4.3.11A). This value implies that the proteins and the AD nanoassembly do not interact as rigid objects, for which a substantially higher R_g value would be expected.

The surface-averaged electrostatic potential (SAEP) was next calculated to estimate the ζ -potential of the HSA/AD NM complex. Initially, the ζ -potential of the pure HSA protein was determined to be -11.5 mV , while the value for the AD nanomicelle alone was calculated to be 30.48 mV . Upon complexation with HSA, the overall positive ζ -potential of the complex decreased to 10.2 mV . This suggests that although the positively charged AD nanomicelle is largely neutralized by the negative residues of HSA, each HSA molecule in the complex is oriented with its more positively charged regions (Figure S4, right panel) facing the solvent. This unique orientation of HSA thus contributes to the observed positive ζ -potential value of the resulting supramolecular assembly.

Finally, to complete the investigation of the structure of the HSA/AD complex and the relevant interactions, the radial distribution function (RDF) analysis was performed. This method involves selecting a reference point, typically the center of mass (COM) of a large molecule or a specific residue and then calculating the distribution of atoms from another molecular entity as the distance from this reference point increases. Initially, this technique was employed to examine the arrangement of each AD amphiphile within the nanomicelle itself and to determine whether the adsorption of the HSA proteins onto the nanomicelle altered its internal organization (see Figure 4.3.1 for details). As shown in Figure 4.3.11C, the internal distribution of the AD NM atoms remains largely unchanged upon complexation with HSA, consistent with the previously described results. By plotting the RDF of HSA atoms around the COM of the AD nanomicelle (Figure 4.3.11D), it becomes evident that there is a high degree of interpenetration between the proteins and the nanomicelle, which corroborates the previously described R_g value estimated for the complex, and in agreement with the STD-NMR experimental findings described in Section *Epitope Mapping of Bound AD with the Use of STD-NMR* above. Furthermore, upon analyzing the distribution of HSA atoms relative to the different components of the nanomicelle, a strong interpenetration of the proteins within the polar heads of the AD nano structure is observed, in agreement with the overall binding mechanism underlying the HSA/AD complex formation.

Conclusions

Dendrimers, which are complex compounds with highly branched nanostructures, offer precise control over their size, shape, and functionality, making them unique macro molecular systems. Their uniform architecture provides several advantages, such as high surface-to-volume ratios and monodispersity, which are crucial for their applications in drug delivery and diagnostics. However,

challenges arise due to structural anomalies during dendrimer synthesis, which can affect their large-scale production and clinical translation. Recent research efforts have focused on self-assembly strategies to address these limitations. One promising approach involves the use of amphiphilic dendrimers, which form stable nanomicelles suitable for drug delivery. However, understanding how these dendrimers interact with biological environments, particularly with human serum albumin, is essential for realizing their biomedical potential.

Accordingly, in the present study, the interaction between HSA and the recently designed self-assembling amphiphilic dendrimer AD was investigated using various experimental techniques. Fluorescence spectroscopy revealed a linear decrease in tryptophan fluorescence of HSA upon binding to AD nanomicelles, indicating a clear quenching behavior, which was analyzed using the Stern-Volmer equation. The calculated Stern-Volmer quenching constant K_{SV} for the AD nanomicelles was determined to be $4.9 \times 10^4 \text{ M}^{-1}$, while the affinity value obtained from the modified Stern–Volmer equation was equal to $22.9 \pm 4.2 \text{ }\mu\text{M}$, indicating a moderate affinity between the protein and the nanoassembly. Additionally, isothermal titration calorimetry confirmed the moderate strength of the interaction between HSA and the AD nanomicelles, with a mean affinity value of $13.4 \pm 3.1 \text{ }\mu\text{M}$. Thermodynamic analysis indicated thermodynamically spontaneous binding process, with favorable enthalpic contributions mainly arising from electrostatic interactions and hydrogen bonding. Circular dichroism spectroscopy showed no significant structural changes in HSA upon interaction with AD below its critical micellar concentration, with only slight decreases in α -helical content and thermal stability observed at higher AD concentrations. Three-dimensional fluorescence spectroscopy further supported these findings, indicating no significant alterations in the microenvironment surrounding the HSA tryptophan residue (W214) upon AD binding. Computational analysis of the HSA/AD complex binding revealed dominant surface interactions between polar amino acid residues of HSA and the hydrophilic, positively charged heads of the nanomicelle. Delving into atomistic details, electrostatic potential around HSA was analyzed, revealing a predominantly negative potential over specific domains, ideal for binding with AD nanomicelles. Moreover, molecular dynamics simulations unveiled highly consistent interaction patterns between HSA and the AD nanomicelle at the atomistic level. Further energetic analysis elucidated dominant enthalpic contributions, with stable salt bridges playing a pivotal role in complex stabilization. Finally, structural analysis indicated minimal alteration in the overall structure of HSA upon complexation with AD micelles, corroborated by radial distribution function analysis and zeta potential measurements. Overall, this study sheds light on the binding mechanisms and thermodynamic parameters governing the interaction between HSA and the self-assembled AD nanomicelles, offering valuable insights into their potential biomedical applications.

In a broader context, the observed moderate binding pattern between HSA and the AD nanomicelles holds considerable implications for pharmaceutical research, especially within the realm of drug delivery. Understanding the nature of the interactions between these entities is paramount for refining drug delivery systems and enhancing their efficacy. In the domain of drug delivery, a moderate binding pattern can be construed as advantageous, as it fosters three primary beneficial outcomes:

1. mitigated carrier-induced toxicity, since an excessive binding affinity between any drug vector and HSA can perturb normal protein functions. A moderate binding pattern serves to alleviate such risks, thus increasing the safety of the drug delivery system;
2. enhanced circulation stability, since moderate binding ensures a stable interaction between the drug carrier and HSA, which in turn can enhance the circulation time of the drug delivery system in the bloodstream. This prolonged circulation time improves drug bioavailability and increases the likelihood of reaching target tissues;
3. diminished immunogenicity since nanovector binding to HSA may mitigate the recognition of the drug delivery system as foreign by the immune system. This effect could potentially decrease immune responses, such as clearance by the reticuloendothelial system or the initiation of inflammatory reactions.

Finally, understanding the binding mechanisms and thermodynamic parameters enables the rational engineering of nanocarriers with tailored properties, optimizing drug loading, circulation stability, and targeted drug release, thereby advancing therapeutic outcomes in pharmaceutical applications.

4.3.2. Molecular Insights into the Binding of Human Serum Albumin to Charged Dendritic Nanomicelles: A Synergistic Experimental and In Silico Approach⁵

Results and Discussion

Analysis of Fluorescence Quenching

Intrinsic fluorescence spectroscopy of HSA, dominated by its single tryptophan residue (W214), is widely recognized as a well-established tool for investigating conformational changes and protein-ligand interactions [106]. In this study, fluorescence quenching experiments were employed to assess the binding interactions between HSA and both monomeric and nanomicellar forms of the ADs. However, the quantitative interpretation of quenching data, particularly in terms of binding constants

⁵ The material presented in this chapter was published at: “Molecular insights into the binding of human serum albumin to charged dendritic nanomicelles: A synergistic experimental and in silico approach”. G. Cavalieri, D. Marson, D. Dhumal, L. Peng, E. Laurini, S. Priol, *Journal of Molecular Liquids*, Volume 438, Part B, 2025, 128770, ISSN 0167-7322.

(*e.g.*, K_a or n) has increasingly come under scrutiny. In particular, van de Weert and colleagues [57] critically noted that the underlying assumptions of Stern–Volmer-based analysis are frequently violated in complex systems like HSA/drug ensembles. This is particularly relevant when ligands exhibit heterogeneous binding modes, induce local environmental perturbations near the fluorophore, or promote ligand-dependent protein conformation changes. As a result, affinity constants derived from fluorescence data may differ significantly from thermodynamic measurements and can even be misleading when interpreted mechanistically.

In light of these considerations, fluorescence data in this study were not used to extract thermodynamic binding parameters. Instead, fluorescence quenching was employed qualitatively and semi-quantitatively to confirm the occurrence of binding events and to compare the relative quenching efficiency among the various AD derivatives. The Stern-Volmer constant K_{SV} , obtained by linear fitting of the fluorescence intensity ratio (F_0/F) versus quencher concentration, is used here as an empirical indicator of interaction strength, enabling a comparative ranking of the systems. These findings are subsequently interpreted alongside calorimetric data, which offer a more rigorous thermodynamic characterization.

Accordingly, changes in the intrinsic fluorescence intensity of HSA were monitored upon addition of 1-A, 1-TA, and 1-COOH at concentrations both below and above their respective CMCs. The corresponding results are shown in Figure 4.3.12 and Figure S8. A gradual decrease in fluorescence intensity was observed with increasing concentrations of both the monomeric and nanomicellar forms of each amphiphilic system, consistent with binding interactions between the ADs and HSA.

Fluorescence quenching may arise from a variety of mechanisms, including dynamic quenching (via molecular collisions), static quenching (via complex formation), energy transfer, or protein conformational changes. As such, the observed quenching of HSA fluorescence by the different amphiphilic systems provides valuable insights into the nature of the binding events. The extent of quenching can be quantitatively described using the Stern-Volmer (SV) equation (Eq. 5. See Section 3.1.2):

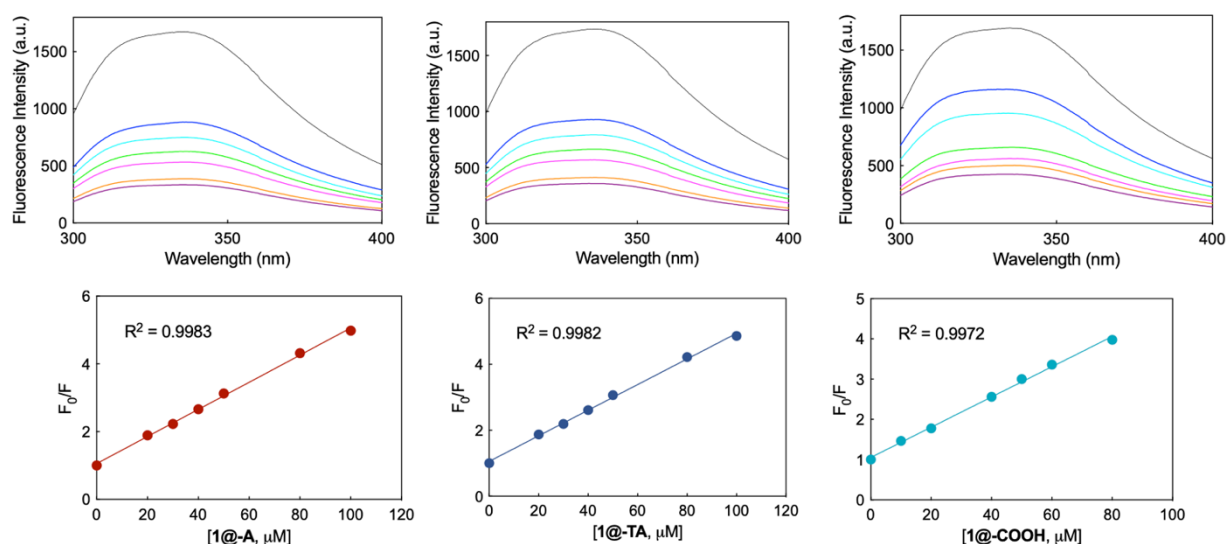


Figure 4.3.12. (Top panels) Fluorescence emission spectra of HSA (2.0 μM) in the presence of increasing concentrations of 1@-A (left), 1@-TA (center), and 1@-COOH (right) nanomicelles. Spectra include free HSA and HSA incubated with nanomicelles at the following concentrations: 20, 30, 40, 50, 80 and 100 μM for 1@-A and 1@-TA, and 10, 20, 40, 50, 60 and 80 μM for 1@-COOH. **(Bottom panels)** Stern-Volmer plot depicting the fluorescence quenching of HSA induced by the three AD nanomicelles. Data are shown as mean ± SD (n = 3).

Monomeric state	HSA/1-A	HSA/1-TA	HSA/1-COOH
$K_{SV} [10^4 M^{-1}]$	1.61 ± 0.23	1.82 ± 0.15	1.52 ± 0.31
Micellar state	HSA/1@-A	HSA/1@-TA	HSA/1@-COOH
$K_{SV} [10^4 M^{-1}]$	4.01 ± 0.27	3.88 ± 0.39	3.76 ± 0.33

Table 4.3.1. Stern-Volmer constants (K_{SV}) for HSA fluorescence quenching in the presence of different AD-PAMAM derivatives at 298 K. Reported values represent the mean ± standard deviation from three independent experiments.

For each amphiphilic derivative, two Stern–Volmer constants K_{SV} are reported in **Table 4.3.1**: one for the monomeric state (1-A/1-TA/1-COOH) and one for the nanomicellar state (1@-A/1@-TA/1@-COOH), reflecting the different physical organizations of the analytes. The calculated K_{SV} values indicate that the three monomeric amphiphilic dendrimers induce only modest quenching of HSA intrinsic fluorescence, with values in the range of $(1.5\text{--}1.8) \times 10^4 M^{-1}$. This relatively low quenching efficiency suggests that the monomeric species interact weakly with HSA, likely establishing limited physical contacts and inducing minimal perturbations to the protein structure. Under these conditions, only subtle conformational changes are expected, resulting in minor alterations to the local environment surrounding W214 and thus a comparatively small decrease in fluorescence intensity. In contrast, a marked increase in fluorescence quenching is observed for the nanomicellar systems

(Figure 4.3.12 and Table 4.3.1), with K_{SV} values reaching approximately $(3.7\text{-}4.0) \times 10^4 \text{ M}^{-1}$. This enhancement can be attributed to the multivalent nature of nanomicellar aggregates, which may offer a larger interaction surface and/or simultaneously engage multiple interaction regions on the HSA surface and/or induce more extensive structural rearrangements. Furthermore, the higher local density of terminal functional groups at the nanomicelle surface likely promotes more sustained interactions with regions in close proximity to W214, thereby amplifying the quenching effect.

UV-vis measurements were acquired under the same conditions as the fluorescence experiments to corroborate the quenching observations. For the monomeric systems, the UV-vis spectra closely resembled that of free HSA with no appreciable spectral changes in peak position or intensity, consistent with weak or negligible ground-state interactions. In contrast, the nanomicellar systems exhibited a reproducible increase in absorbance of the HSA band around 280 nm (Figure S9), indicating modest-to-moderate ground-state contacts at the protein-micelle interface. This qualitative trend mirrors the fluorescence quenching results, where K_{SV} values are significantly lower for monomeric dendrimers and substantially higher for micellar aggregates, supporting with a scenario in which ground-state interactions are minimal for monomers and appreciable for nanomicelles.

Moreover, temperature-dependent fluorescence quenching was evaluated at 31 °C and 37 °C (Table S2). For the monomeric forms, the K_{SV} values increased with temperature, a trend consistent with entropically favored association, in line with prior reports on protein-ligand and protein-nanomaterial systems where higher temperatures promote hydrophobic desolvation and enhance configurational sampling. Conversely, for the nanomicellar forms, the K_{SV} values decreased with increasing temperature, a behavior consistent with enthalpically driven association, in which complex formation becomes less favored at elevated temperatures, in agreement with previously reported protein binding to multivalent or clustered ligands and soft coronas.

Although, as previously discussed, fluorescence quenching cannot reliably provide quantitative binding constants in complex environments, the observed differences in K_{SV} among the tested systems still offer meaningful qualitative insight into their relative interaction strength. These findings support the notion that self-assembled aggregates interact more extensively with HSA than their monomeric counterparts - a hypothesis that will be further examined through ITC in the next section. Within its qualitative scope, the Stern-Volmer analysis thus provides valuable preliminary insight into the binding behavior of the amphiphilic AD-PAMAM derivatives toward HSA, confirming the occurrence of interactions and highlighting formulation-dependent differences in interaction propensity.

Binding of Monomeric and Nanomicellar ADs to HSA assessed using Isothermal Titration Calorimetry

Understanding protein–nanomaterial interactions is essential for predicting the behavior of nanoscale systems in biological environments, particularly with respect to protein corona formation. Isothermal titration calorimetry represents a powerful analytical technique for probing these interactions, providing direct insight into the molecular driving forces involved. As it directly measures the heat released or absorbed during binding events in solution, ITC provides key thermodynamic parameters, including the variation in enthalpy (ΔH) and entropy (ΔS), and the dissociation constant (K_d), thereby offering mechanistic insight into both the strength and nature of the interaction. To quantitatively characterize the interaction between HSA and the AD systems, ITC experiments were conducted under physiological conditions (pH 7.4). The resulting thermodynamic profiles revealed clear differences between the monomeric and nanomicellar forms, allowing assessment of how self-assembly modulates protein recognition. The corresponding data are summarized in Table 4.3.2, Figure 4.3.13 and Figure S10.

	HSA/1-A	HSA/1-TA	HSA/1-COOH
K_d [mM]	112 ± 11	89 ± 21	104 ± 18
ΔH [kcal/mol]	3.12 ± 0.10	2.88 ± 0.14	3.03 ± 0.07
$-T\Delta S$ [kcal/mol]	-8.51 ± 0.21	-8.41 ± 0.23	-8.46 ± 0.25
ΔS [kcal/mol K]	28.5 ± 0.7	28.2 ± 0.8	28.4 ± 0.8
ΔG [kcal/mol]	-5.39 ± 0.27	-5.52 ± 0.31	-5.43 ± 0.32
	HSA/1@-A	HSA/1@-TA	HSA/1@-COOH
K_d [mM]	1.15 ± 0.64	8.10 ± 0.11	4.29 ± 0.57
ΔH [kcal/mol]	-4.07 ± 0.05	-4.45 ± 0.07	-6.53 ± 0.04
$-T\Delta S$ [kcal/mol]	-4.09 ± 0.40	-2.50 ± 0.08	-0.80 ± 0.04
ΔS [kcal/mol K]	13.7 ± 1.3	8.4 ± 0.3	2.7 ± 0.1
ΔG [kcal/mol]	-8.16 ± 0.35	-6.95 ± 0.01	-7.33 ± 0.08

Table 4.3.2. Thermodynamic parameters of HSA interactions with monomeric and nanomicellar AD-PAMAM derivatives obtained by ITC at 25 °C.

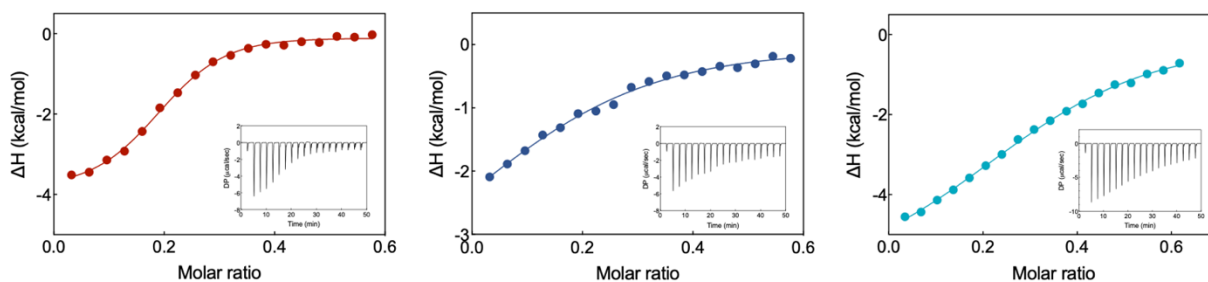


Figure 4.3.13. Integrated heat curves and corresponding raw thermogram (insets) from ITC experiments of HSA titrated with **1@-A**, **1@-TA**, and **1@-COOH**.

For the monomeric forms (1-A, 1-TA, and 1-COOH), the binding thermodynamics indicate spontaneous interaction with HSA, as reflected by negative Gibbs free energy values (ΔG). All three amphiphiles exhibit dissociation constants in the mid-micromolar range (89-112 mM), along with positive enthalpy changes ($\Delta H = +2.88 \pm 0.14$ to $+3.12 \pm 0.10$ kcal/mol). This endothermic signature, combined with strongly favorable entropy contributions ($-T\Delta S = -8.41 \pm 0.23$ to -8.51 ± 0.21 kcal/mol), suggests that binding is predominantly entropy-driven. Such thermodynamic behavior is characteristic of interactions mediated by hydrophobic effects, where apolar groups insert into hydrophobic pockets of the protein, displacing structured water molecules. In these systems, the gain in entropy from desolvation and increased conformational freedom offsets the enthalpic penalty, producing an overall favorable ΔG . In contrast, the nanomicellar systems (**1@-A**, **1@-TA**, and **1@-COOH**) display a markedly different thermodynamic profile. Titration curves for these self-assembled aggregates show clear exothermicity, with negative enthalpy values ranging from -4.07 to -6.53 kcal/mol. The magnitude of the enthalpic contribution increases from **1@-A** to **1@-COOH**, suggesting progressively stronger non-covalent interactions between the nanomicelle terminal groups and the protein surface. Concurrently, the entropy contributions ($-T\Delta S$) become less favorable (from -4.09 ± 0.40 kcal/mol for **1@-A** to -0.80 ± 0.04 kcal/mol for **1@-COOH**), likely reflecting entropic penalties associated with interfacial water ordering or conformational constraints upon complex formation. Nevertheless, the resulting free energy values ($\Delta G = -6.95 \pm 0.01$ to -8.16 ± 0.35 kcal/mol) confirm spontaneous binding, with significantly enhanced affinity compared to the monomeric forms ($K_d = 1.15$ -8.10 μ M). Overall, the ITC data reveal that the binding mechanism is strongly influenced by the aggregation state of the ADs. Monomeric interactions are entropy-driven and dominated by hydrophobic desolvation, whereas nanomicelle binding is primarily enthalpy-driven, involving electrostatic interactions, hydrogen bonding, and milder entropic contributions. This shift is consistent with the supramolecular organization of the nanomicelles, where peripheral charged groups are solvent-exposed and readily available for interaction with complementary sites on the HSA surface. Additional contributions likely arise from weak hydrophobic contacts and interfacial water

rearrangement. The clear transition from entropy- to enthalpy-driven binding upon micellization highlights the impact of nanoscale self-assembly on protein recognition, with important implications for the design of functional nanocarriers.

Structural and Thermal Perturbation of HSA Upon Nanomicelle Binding

Determination of the HSA Secondary Structure and Thermal Stability by Circular Dichroism Spectroscopy

Circular dichroism spectroscopy is a well-established technique for probing protein secondary structures and monitoring conformational changes upon interaction with small molecules [107]. In this study, far-UV CD measurements were performed to assess possible structural alterations in HSA upon interaction with the amphiphilic dendrimers. The CD spectrum of native HSA exhibited the characteristic negative ellipticity bands at 208 and 222 nm, consistent with a predominantly α -helical secondary structure (**Figure 4.3.14** and **Table 4.3.3**).

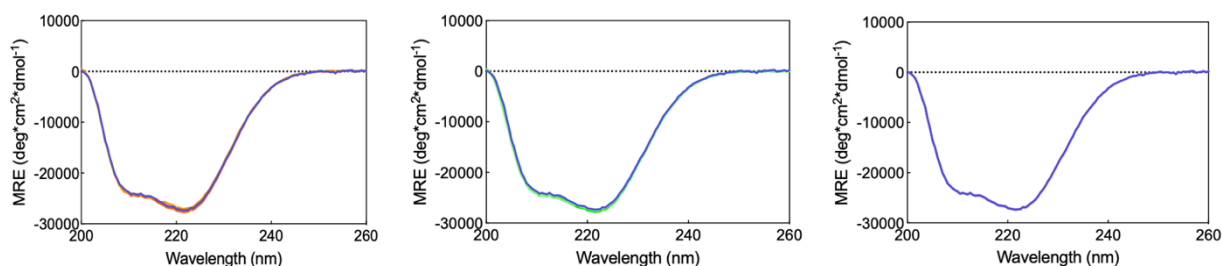


Figure 4.3.14. UV CD spectra of HSA in the absence (blue) and in the presence of: **1-A** (2 μ M, orange) and **1@-A** (50 μ M, dark orange) (left); **1-TA** (2 μ M, light green) and **1@-TA** (50 μ M, forest green) (middle); **1-COOH** (2 μ M, magenta) and **1@-COOH** (50 μ M, purple) (right). Measurements were performed at 298 K in PBS buffer (pH 7.4).

Sample	% α -helix	T_m [K]
HSA	62.6 ± 0.2	338.30 ± 0.49
HSA + 1-A	63.2 ± 0.3	338.87 ± 0.26
HSA + 1@-A	63.6 ± 0.6	337.31 ± 0.32
HSA + 1-TA	63.4 ± 0.3	336.52 ± 0.48
HSA + 1@-TA	63.9 ± 0.4	337.27 ± 0.28
HSA + 1-COOH	62.8 ± 0.7	337.48 ± 0.36
HSA + 1@-COOH	62.5 ± 0.1	338.46 ± 0.31

Table 4.3.3. Percentage of α -helical content and melting temperature (T_m) of HSA in the absence and presence of monomeric and nanomicellar AD-PAMAM derivatives, as determined by CD spectroscopy. Data are reported as mean $\pm$ SD from 3 independent experiments.

Deconvolution of the CD spectra confirmed an α -helical content of approximately 62.6% for native HSA, in good agreement with literature values. Upon addition of the monomeric forms of the dendrimers no significant changes in secondary structure were observed, with the α -helix content remaining essentially unchanged (62.8% to 63.4%,) indicating that these compounds do not appreciably perturb the native conformation of the protein. A comparable trend was observed for the nanomicellar forms. Slight increases in α -helix content were noted for **1@-A** (63.6%) and **1@-TA** (63.9%), while **1@-COOH** induced a negligible decrease (62.5%). Overall, these findings suggest that neither the monomeric nor the self-assembled forms of the three dendrimers promote meaningful structural destabilization or unfolding of HSA. Thermal denaturation experiments were performed by monitoring ellipticity at 222 nm over a temperature range of 298-393 K, in order to determine the melting temperature (T_m) of HSA in the presence of the various dendrimers. Native HSA exhibited a T_m of 338.30 K. Upon addition of both monomeric and nanomicellar AD derivatives, only minor shifts in T_m were detected, typically within ± 1 K. The most pronounced decrease was recorded for **1-TA**, lowering T_m to 336.52 K. Conversely, **1@-COOH** slightly increased protein stability, with a T_m of 338.46 ± 0.31 K. Collectively, these results indicate that both monomeric and self-assembled ADs exert minimal influence on the thermal stability of HSA, supporting the formation of stable protein–dendrimer complexes without significant perturbation of the native protein fold.

Conformation Stability Through Three-dimensional Fluorescence Spectroscopy

Three-dimensional fluorescence spectroscopy is a sensitive technique for detecting subtle changes in the local environment of aromatic residues, particularly tryptophan, providing insights into protein conformational stability. This approach enables simultaneous acquisition of excitation-emission matrices while distinguishing intrinsic fluorescence from scattering phenomena, and is widely used to study protein folding, ligand binding, and protein–nanostructure interactions. In this study, 3D-FS spectra were recorded for HSA alone and in the presence of all AD derivatives, both in their monomeric and nanomicellar forms. As a representative example, Figure 4.3.15 illustrates the fluorescence landscapes of HSA in the absence and in the presence of **1-A** and **1@-A**. The characteristic fluorescence peak ($\lambda_{ex}/\lambda_{em} = 280/343$ nm), attributed to W214 located in the hydrophobic core of the protein serves as a sensitive probe for tertiary structure and local polarity. As seen in Figure 4.3.15, no substantial shifts in peak position or changes in intensity were observed upon complexation with either **1-A** or **1@-A**. Similar results were obtained for the other ADs (see Figure S11), indicating that none of the derivatives induce significant perturbations in the microenvironment of W214. These findings indicate that AD derivatives do not elicit appreciable conformational rearrangements in HSA, in agreement with the CD spectroscopy results discussed above and the molecular dynamics analyses that will be presented.

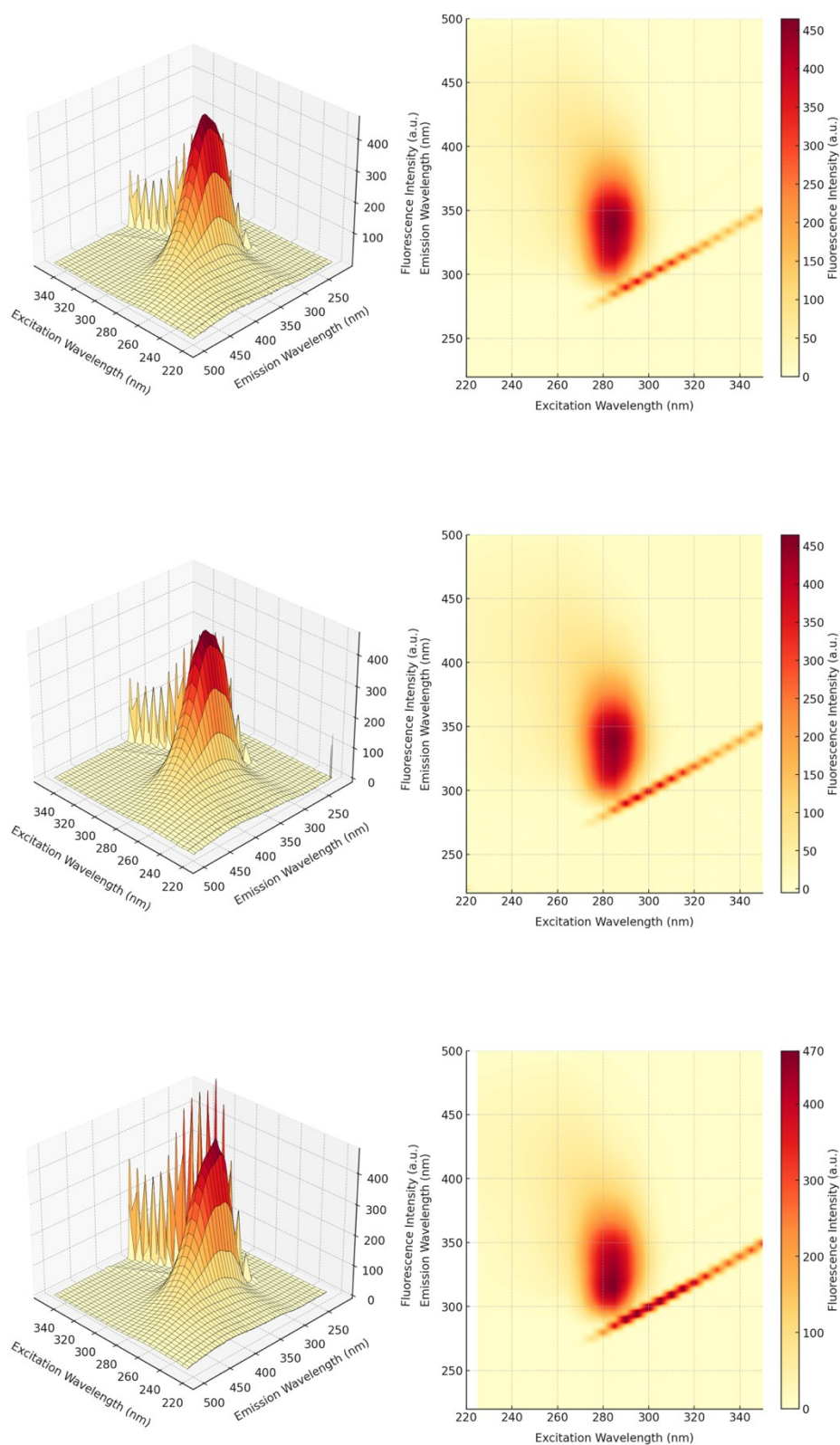


Figure 4.3.15. Three-dimensional fluorescence spectra (**left panels**) and corresponding two-dimensional contour maps (**right panels**) of 2 mM HSA in the absence (top row) and presence of the amphiphilic AD-PAMAM derivative, shown in its monomeric (**1-A**, 2 mM, middle row) and nanomicellar (**1@-A**, 50 mM, bottom row) forms.

Size and z-potential Analysis of HSA–Nanomicelle Complexes via Dynamic Light Scattering

Dynamic light scattering is a widely employed technique for characterizing the size and surface charge of nanoscale colloidal systems in solution. It provides key parameters such as hydrodynamic diameter and z-potential, offering valuable insights into colloidal stability, surface interactions, and complexation behavior of soft nanomaterials, including protein-nanoparticle assemblies [108]. In this study, the hydrodynamic size and surface charge of the HSA/nanomicelle complexes were assessed by DLS under aqueous buffered conditions. The recorded diameters were 15.9 ± 0.3 nm for HSA/1@-A, 15.6 ± 0.4 nm for HSA/1@-TA, and 14.2 ± 0.6 nm for HSA/1@-COOH (Figure 4.3.16), consistent with compact supramolecular architectures. These values closely match the estimated protein-to-nanomicelle stoichiometry derived from thermodynamics and computational analyses.

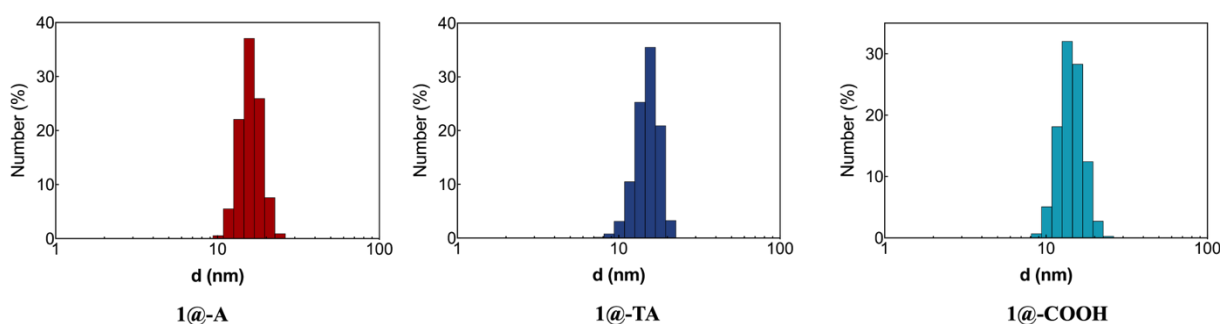


Figure 4.3.16. Hydrodynamic diameter (d) of HSA in complex with 1@-A (left), 1@-TA (middle), and 1@-COOH (right) at 25 °C as measured by DLS.

Z-potential measurements revealed distinct electrostatic profiles for the three dendrimer/HSA nanoassemblies. Specifically, the complexes formed by HSA with 1@-A and 1@-TA exhibited z-potential values of +6.1 and +4.3 mV, respectively, suggesting partial screening of the nanomicelle's positive charge by the negatively charged residues on the HSA surface. In contrast, the complex with 1@-COOH displayed a z-potential of −17.9 mV, consistent with a complete inversion of surface polarity and indicative of strong electrostatic interactions with basic regions of HSA. Taken together, these data support a stoichiometry of four HSA molecules per nanomicelle in the 1@-A and 1@-TA systems, and three HSA molecules per nanomicelle in the case of 1@-COOH. This composition is coherent with the previously determined aggregation numbers and is further supported by the molar ratios obtained from ITC, molecular dynamics simulations, and comparative experimental data from analogous protein–nanoparticle complexes.

Computational analysis

Computational characterization of HSA/monomer binding

Molecular dynamics simulations were firstly employed to investigate the binding interactions between the AD monomers and HSA, as well as their potential structural consequences. The results revealed distinct and well-defined binding features for the AD monomers toward specific fatty acid (FA) pockets in HSA. Specifically, the cationic derivatives **1-A** and **1-TA** preferentially occupied the FA7 pocket, which overlaps structurally with Sudlow's site I, while the anionic monomer **1-COOH** engaged the FA5 site, a region enriched in basic residues. Competitive fluorescence assays were carried out to validate the docking/MD-predicted binding sites. HSA quenching was monitored in the presence of canonical site markers. Warfarin (FA7) reduced the apparent K_{SV} for **1-A** and **1-TA**, whereas stearic acid (FA5) reduced K_{SV} for **1-COOH** and produced no appreciable effect on **1-A/1-TA**. This reciprocal pattern supports the site-selective engagement inferred from docking/MD (**1-A/1-TA** at FA7 and **1-COOH** at FA5). Detailed values of K_{SV} are provided in Table S3. All three binding modes were stable over the full 200 ns of MD simulation, as evidenced by backbone RMSD plateaus and the plot of the radius of gyration (R_g) over the production window (Figure S12 and Figure 4.3.17). As shown in Figure 4.3.17 (upper panels), the ligands remained consistently anchored within the selected pockets throughout the trajectory. In each case, the hydrophobic C_{18} tail was deeply embedded in the protein cleft, minimizing solvent exposure and contributing significantly to complex stabilization through persistent hydrophobic interactions. The polar head groups of **1-A** and **1-TA** remained exposed to the solvent and formed only transient contacts with the protein surface (Figure 4.3.17, upper left and center). In contrast, the negatively charged head group of **1-COOH** penetrated deeper into the binding cleft, consistent with the distribution of internal basic residues (Figure 4.3.17, upper right). Despite these localized interactions, none of the trajectories showed persistent hydrogen bonds or stable salt bridges, indicating that hydrophobic burial of the alkyl chain represents the primary stabilizing force. Critically, the binding modes observed *in silico* align with the thermodynamic signatures obtained by ITC, corroborating both the identity of the binding pockets and the relative affinities of the three monomers.

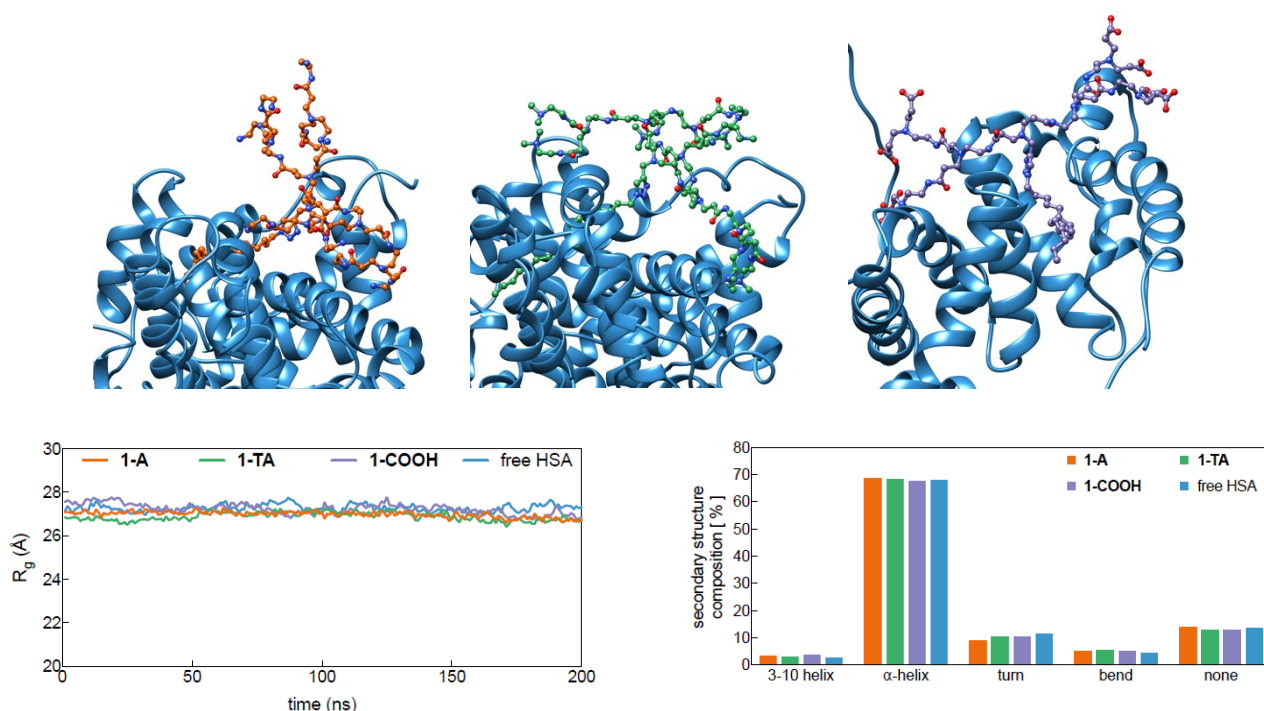


Figure 4.3.17. (Upper panels) Final MD snapshots of **1-A** (left), **1-TA** (center), and **1-COOH** (right) bound to HSA within the fatty acid binding region. The AD monomers are shown in ball-and-stick representation (O: red; N: blue; C: orange, green and purple, respectively). The HSA is rendered in blue. Hydrogen atoms, water molecules, and ions are omitted for clarity. (Lower left panel) Radius of gyration of HSA over time during the MD simulations in complex with each dendrimer monomer. (Lower right panel) Secondary structure content of HSA, averaged over the last 100 ns of simulation, for both free and bound protein systems.

To evaluate potential structural effects of monomer binding, global metrics such as R_g and secondary structure content were monitored throughout the simulations (Figure 4.3.17, lower panels). The R_g of unbound HSA remained stable at 27.27 ± 0.18 Å, consistent with reported values. Complexation with **1-A**, **1-TA**, and **1-COOH** resulted in comparable R_g values (26.99 ± 0.14 , 26.89 ± 0.19 , and 27.24 ± 0.24 Å, respectively), indicating no detectable loss of molecular compactness (Figure 4.3.17, lower left panel). Secondary structure profiles were likewise preserved in all systems (Figure 4.3.17, lower right panel), confirming that the overall protein fold remains structurally unperturbed upon binding. These observations align closely with the CD spectroscopy data, further reinforcing the conclusion that binding of the AD monomer does not compromise the structural integrity of HSA.

Computational Analysis of Structural Properties of HSA/AD Nanomicelle Complexes

The structural integrity of the nanomicelles in complex with HSA was also assessed through all-atom MD simulations. Analysis of R_g and asphericity indicated that both the overall size and supramolecular shape were largely preserved upon protein binding. As shown in Figure 4.3.18 (upper panel), R_g values increased only modestly (≈ 1 Å) following complexation, a trend that was consistent for all three systems. In detail, for the free nanomicelles average R_g values were 22.56 ± 0.40 Å (**1@-A**),

$24.06 \pm 0.55 \text{ \AA}$ (**1@-TA**), and $15.43 \pm 0.19 \text{ \AA}$ (**1@-COOH**). Upon binding to HSA, these values shifted to $23.47 \pm 0.33 \text{ \AA}$, $24.63 \pm 0.21 \text{ \AA}$, and $16.51 \pm 0.15 \text{ \AA}$, respectively. These minimal variations support the evidence that nanomicelle compactness is retained in the presence of the protein, confirming that the formation of an HSA-based protein corona does not significantly perturb the core structure of the AD aggregates.

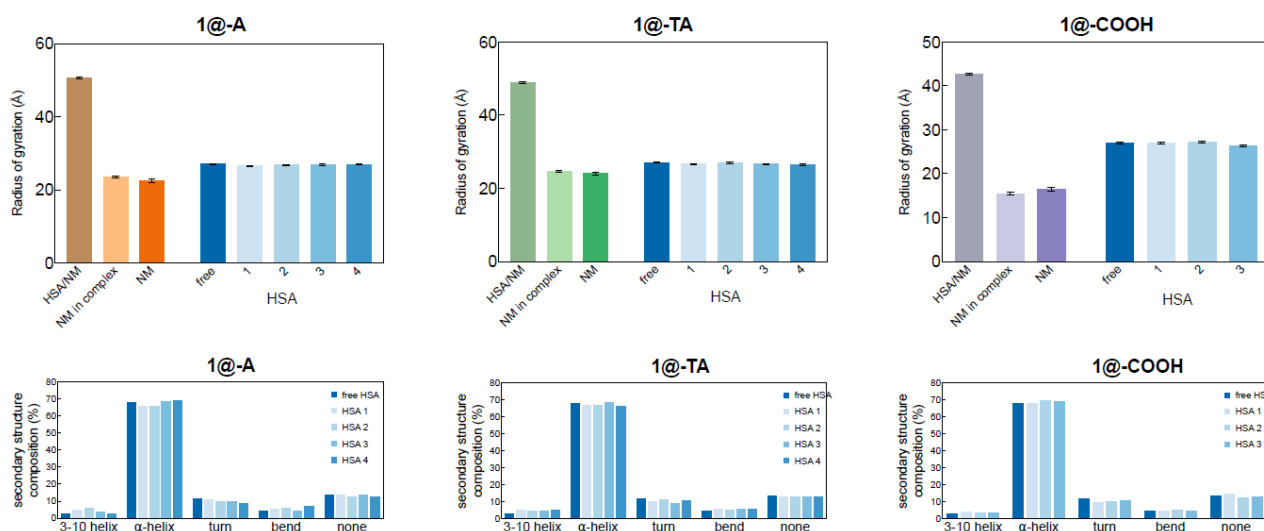


Figure 4.3.18. (Upper panels) Radius of gyration (R_g) for each system: free HSA, free nanomicelle, full complex, and individual HSA chains within the complex. Left to right: **1@-A** (four HSA chains), **1@-TA** (four HSA chains), and **1@-COOH** (three HSA chains). **(Lower panels)** Secondary structure content of free HSA and of the individual HSA chains in the corresponding nanomicellar complexes.

Asphericity values, calculated from the principal moments of the gyration tensor, remained low and comparable between free and HSA-bound nanomicelles, confirming that the overall spherical morphology is retained upon complexation. Average values of 0.11 ± 0.04 , 0.07 ± 0.04 , and 0.06 ± 0.03 for the free **1@-A**, **1@-TA**, and **1@-COOH** nanomicelles increased only slightly in the corresponding complexes (0.14 ± 0.03 , 0.09 ± 0.02 , and 0.06 ± 0.02 , respectively). These results indicate that nanomicelle architecture remains robust in the presence of the HSA protein corona, likely due to uniform surface coverage and largely isotropic interactions involving the dendrimer termini, which prevent the asymmetric deformations often observed in protein–nanoparticle systems. From the protein's perspective, nanomicelle binding has no measurable effect on overall size or compactness, as evidenced by stable backbone RMSD plateaus and an essentially invariant R_g over the equilibrated simulation window (Figure S13 and Figure 4.3.18). Indeed, the R_g of HSA in the complexes ranged from 26.53 to 27.20 Å (with standard deviation $\leq 0.16 \text{ \AA}$), closely matching that of the unbound protein ($27.01 \pm 0.18 \text{ \AA}$) and previously reported values under comparable conditions. To assess possible conformational changes, secondary structure profiles of HSA were analyzed using

the DSSP algorithm. In the unbound state, the protein exhibited 68.0% α -helix and 11.5% turns, with no detectable β -sheet content, consistent with established structural data. Upon nanomicelle binding, the α -helical content remained within 65.5-69.4%, and turn content varied between 8.8% and 10.6%, again without β -sheet formation. These results (Figure 4.3.18, lower panel) confirm that the native fold of HSA is preserved in all complexes, with no evidence of structural destabilization.

To characterize the structural and electrostatic properties of the HSA-nanomicelle complexes at atomistic resolution, their size, surface potential, and internal organization were systematically analyzed. The R_g of the complexes revealed similar values for the positively charged nanomicelles: 50.64 ± 0.62 Å for HSA/1@-A and 48.97 ± 0.70 Å for HSA/1@-TA, indicating comparable degrees of compaction. In contrast, the HSA/1@-COOH complex exhibited a smaller R_g value (42.64 ± 0.56 Å), consistent with the reduced number of HSA molecules forming its corona. To further assess electrostatic behavior, surface-averaged electrostatic potentials (SAEP) were computed and used to estimate effective z -potentials. HSA alone was predicted to carry a z -potential of approximately -11.5 mV, in line with experimental reports. Upon HSA complexation, 1@-A and 1@-TA retained net positive potentials (+5.94 mV and +6.00 mV, respectively), reflecting partial charge neutralization by HSA while preserving an overall cationic surface. This behavior is consistent with a binding mode in which negatively charged HSA regions face the dendrimer core, leaving cationic domains exposed to the solvent. In contrast, the 1@-COOH complex displayed a z -potential of -14.4 mV, suggesting preferential interaction of the dendrimer's terminal carboxylates with localized basic patches on HSA, while exposing anionic protein surfaces. The internal structure of the nanomicelles and their interfacial organization were also examined by radial distribution function (RDF) analysis, calculated relative to the nanomicelle center of mass. As shown in Figure S14 the micellar core remained stable in all systems, particularly for the cationic dendrimers. In the HSA/1@-COOH complex, a slight expansion of the nanomicelle surface was observed, attributed to outward extension of the flexible anionic branches toward nearby cationic domains on the protein.

Computational Characterization of HSA/nanomicelles Binding

Protein–nanomicelle interfaces were analyzed by quantifying atomic contacts between individual HSA residues and nanomicelle atoms along the MD trajectories. For each frame, the minimum interatomic distance between every residue and any nanomicelle atom was calculated (Figure S15). Contact analysis revealed highly consistent interaction profiles among the HSA molecules within a given corona, as well as across complexes formed with nanomicelles of similar charge. Contact frequency maps were generated by defining a contact as any interatomic distance ≤ 6.5 Å (Figure 4.3.19). These maps provided residue-level interaction fingerprints, which, although generally non-

specific, showed clear charge-dependent differences among the nanomicelles. Binding free energy decompositions using MM-GBSA were then performed to evaluate the energetic contributions of individual HSA residues (Figure S16). A strong correlation emerged between the contact frequency patterns and the binding energy profiles for all systems, reinforcing the internal consistency and reliability of the interaction analysis. In all complexes, electrostatic interactions were identified as the dominant driving force, in line with the complementary charge distribution at the protein-nanomicelle interface.

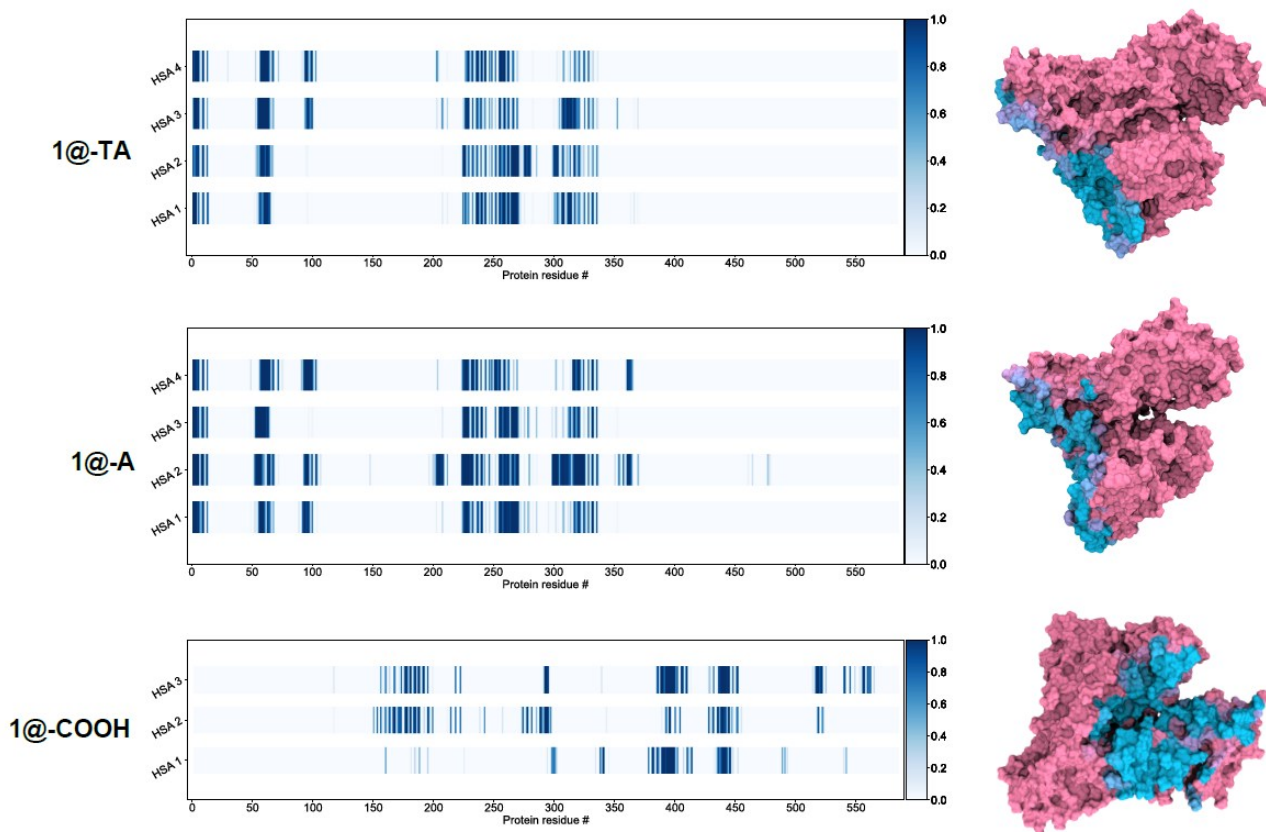


Figure 4.3.19. (Left panel) Residue-level contact frequency maps for the HSA–nanomicelle complexes, computed along the MD trajectories. Darker bars indicate higher contact persistence (defined as interatomic distance ≤ 6.5 Å). Rows correspond to individual HSA chains within each complex. **(Right panel)** Solvent-accessible surface representations of one representative HSA molecule per complex, colored by residue contact frequency with the nanomicelle. Residues not involved in interactions are shown in pink; residues with persistent contacts are highlighted in cyan.

Stable salt bridges and polar contacts between nanomicelle branches and HSA residues were analyzed to further characterize the nature of the interaction. Approximately 80% of the charged dendrimer terminals in 1@-A and 1@-TA formed salt bridges persisting for more than 50% of the trajectory, whereas only 40% of branches in 1@-COOH engaged in such interactions. This lower frequency reflects the limited availability of basic residues on the HSA surface. In contrast, polar contacts involving oxygen and nitrogen atoms were more frequent in 1@-COOH (25%) than in 1@-TA (10%)

and **1@-A** (6%). The broader charge distribution of **1@-TA** likely reduces salt bridge persistence, favoring alternative polar interactions. Notably, **1@-A** exhibited the most stable salt bridges, with ~50% persisting for over 95% of the simulation, compared to ~33% in **1@-TA**. These results indicate that both charge localization and dendrimer flexibility modulate the dynamic nature of AD nanomicelle–protein binding. In addition to electrostatic and polar interactions, extensive van der Waals contacts contributed to complex stabilization. Overall, binding appears to occur through a flexible, non-specific interface driven by a combination of electrostatic, polar, and hydrophobic forces, rather than by defined docking sites. At the residue level, interaction profiles of the cationic nanomicelles showed a preference for surface-exposed, negatively charged helices on HSA, particularly those spanning residues E252-D259 and E266-D269 (Figure 4.3.20).

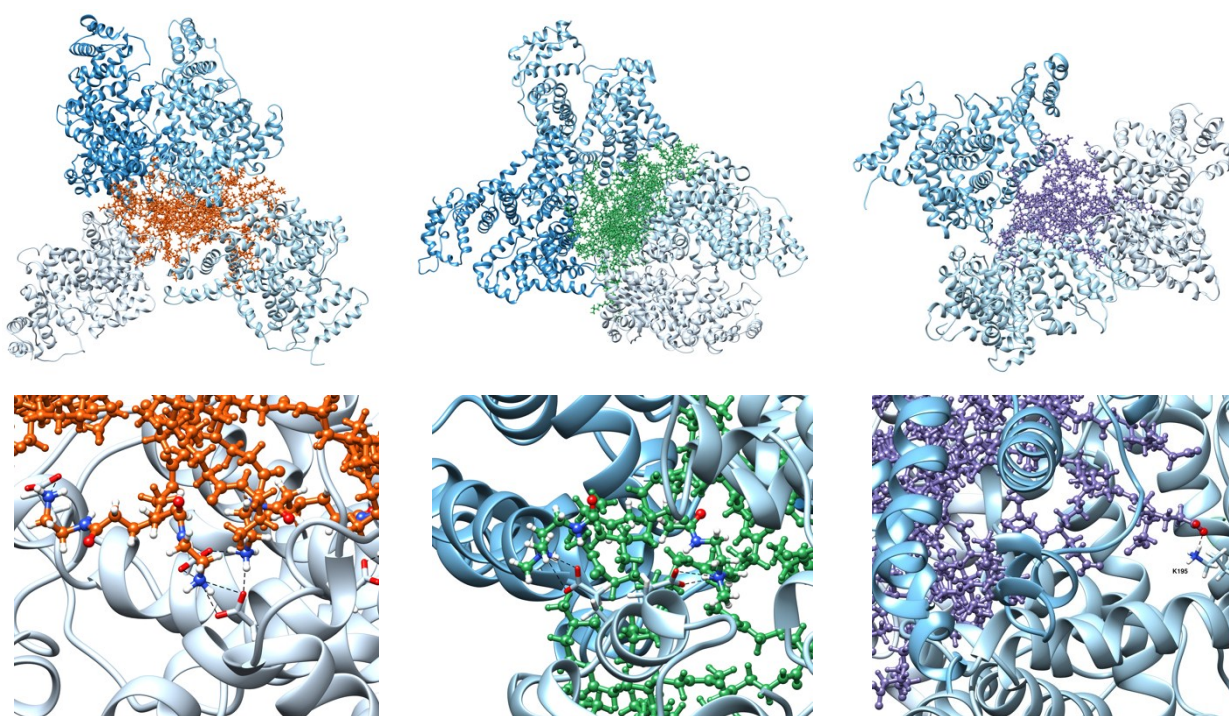


Figure 4.3.20. (Top panel) Representative MD snapshots of the HSA–nanomicelle complexes. HSA is shown in cartoon representation, with individual chains colored in different shades of blue. Nanomicelles are depicted as sticks and balls, with dendrimer branches colored in orange (**1@-A**, left), green (**1@-TA**, center), and purple (**1@-COOH**, right). (Bottom panel) Zoomed-in views highlighting representative polar and electrostatic interactions between nanomicelle branches and selected HSA residues. Key residues involved in persistent salt bridges or hydrogen bonding are shown as dotted lines. All images omit water molecules, ions and counterions for clarity.

In contrast, the negatively charged **1@-COOH** nanomicelle was able to penetrate more deeply into the protein, establishing stable contacts with internal residues such as K195. Radial distribution analyses (Figure S17) supported this observation, showing that charged atoms of **1@-COOH** approached the HSA center of mass to within ~11 Å, whereas those of **1@-A** and **1@-TA** remained

beyond 20 Å. Visualization of contact frequencies (Figure 4.3.19, left panels) confirmed this trend: while positively charged nanomicelles primarily engaged surface residues, **1@-COOH** accessed more buried regions of the protein. This deeper insertion likely accounts for the reduced number of HSA molecules in the **1@-COOH** corona. Such internal binding limits the availability of dendrimer branches for additional interactions, favoring the formation of more compact, three-protein complexes despite a comparable nanomicelle surface area.

Conclusions

This study provides a comprehensive molecular-level characterization of the interaction between human serum albumin and PAMAM-based amphiphilic dendrimers, examined in both monomeric and self-assembled nanomicellar forms. Through an integrated experimental and computational approach, binding affinity, thermodynamic signatures, structural effects, and interfacial organization were systematically evaluated. Micellization was found to markedly enhance HSA binding affinity, primarily via multivalent electrostatic interactions. Isothermal titration calorimetry revealed dissociation constants in the low micromolar range for nanomicelle–HSA complexes, in contrast to the significantly weaker interactions observed for monomeric dendrons. Thermodynamic profiling indicated distinct binding modes: entropy-driven in monomers, and enthalpically stabilized in nanomicelles, consistent with the formation of extended interfacial contacts. Spectroscopic data, including fluorescence quenching, circular dichroism, and 3D fluorescence spectroscopy, confirmed that binding occurs without significant perturbation of the secondary or tertiary structure of the serum protein, supporting a non-disruptive, surface-localized interaction. Atomistic molecular dynamics simulations further reinforced these observations, detailing the organization of protein coronas and identifying the residues most involved in complex stabilization. More specifically, converging evidence from spectroscopy, calorimetry, and MD indicates weak association for monomers and modest-to-moderate association for nanomicelles, consistent with a soft, reversible corona and limited structural perturbation of HSA. Under the conditions tested, no major impairment of HSA structural integrity, global folding or functional competence is anticipated. Future studies will implement dedicated functional assays (*e.g.*, ligand-transport/displacement and esterase-like readouts) to quantify any subtle effects.

Speaking about the plausible formation of the protein corona in this system, the protein–nanoparticle interface is best described as a soft rather than hard corona. The measured dissociation constants indicate moderate and reversible association, which is not compatible with the strong, quasi-irreversible adsorption typical of hard coronas but is consistent with transient, dynamic contacts. In addition, the nanoparticles display a small hydrodynamic diameter (~15 nm), comparable to that of

many serum proteins; as reported in the literature, when nanoparticle size approaches that of the protein, the resulting high surface curvature limits the number of simultaneous contact points and reduces adsorption energy, favoring weak, reversible interactions and heteroaggregation-like behavior rather than the formation of a stable, hard corona. Taken together, these elements support a soft, dynamic corona, with proteins exchanging rapidly with those in the surrounding medium.

Collectively, the results highlight the key role of supramolecular architecture and surface charge distribution in modulating protein recognition. Positively charged nanomicelles engaged up to four HSA molecules, while negatively charged systems favored three-HSA coronas, underscoring the impact of nanoscale presentation on binding stoichiometry and dynamics.

These insights provide a robust foundation for the rational design of dendritic nanocarriers with tunable interfacial properties. Future investigations should explore the behavior of such systems under biologically relevant conditions, including competitive protein adsorption and complex media. Extending this framework to *in vivo* studies will be essential to assess how protein corona formation influences circulation, immune recognition, and the overall therapeutic profile of dendritic nanostructures.

4.4 Free Energy Profiles and Partition Coefficients for Antimelanoma Drugs in Self-Assembled Nanomicelles via COSMOmic and Atomistic Molecular Dynamics Simulations⁶

Introduction

Melanoma is one of the most aggressive cancers of the skin. The discovery that mutant BRAF can be selectively targeted by small-molecule inhibitors transformed melanoma therapy. Vemurafenib (VEM) became the first FDA- and EMA-approved BRAF inhibitor (BRAFi) in 2011, followed by dabrafenib (DAB) and encorafenib (ENC). Other inhibitors such as AZ 628 (AZ), PLX8394 (PLX), and ponatinib (PON) are currently under investigation (Figure 4.4.1). Despite their clinical success, these BRAF inhibitors suffer from poor aqueous solubility and limited bioavailability, factors that complicate oral administration and contribute to systemic side effects. For this reason, nanocarrier-based intravenous delivery systems have been proposed as a promising strategy to enhance solubility, tumor targeting, and optimize pharmacokinetic profiles.

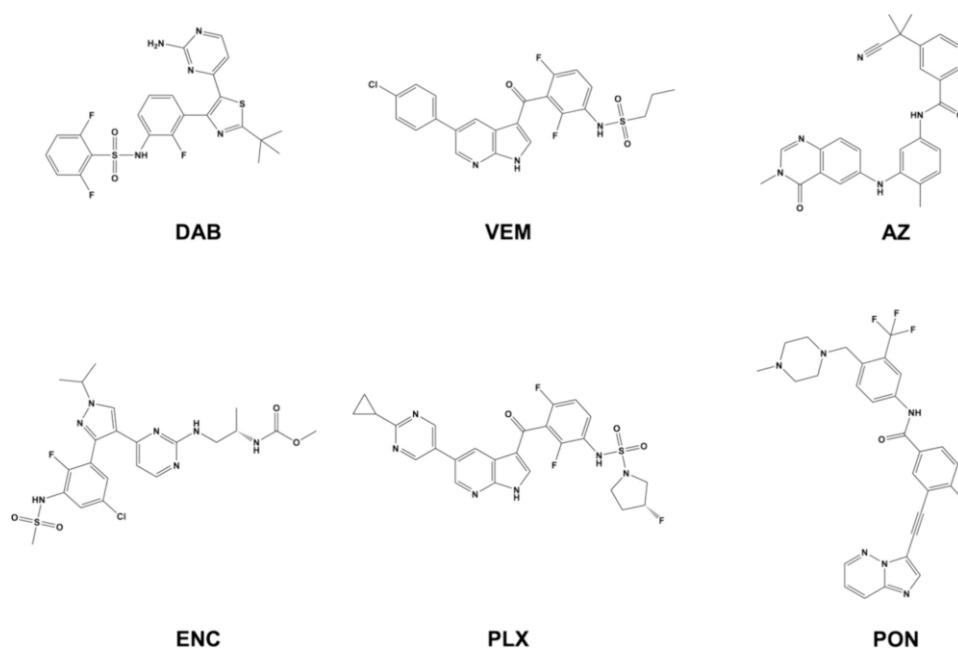


Figure 4.4.1. Chemical structure of the six BRAF inhibitors considered in this work.

In this context, the amphiphilic dendrimers ADs have emerged as promising nanocarriers. The AD molecule, composed of two C18 hydrophobic lipid-like tails linked to a PAMAM dendron bearing

⁶ The material presented in this chapter was published at: “Beyond the Veil: Free Energy Profiles and Partition Coefficients for Antimelanoma Drugs in Self-Assembled Nanomicelles via COSMOmic and Atomistic Molecular Dynamics Simulations”. Andrea Mio, Domenico Marson, Gabriele Cavalieri, Maurizio Fermeglia, Erik Laurini, and Sabrina Pricl Journal of Chemical & Engineering Data, 2024, Article ASAP.

cationic terminal groups, spontaneously self-assembles in water into spherical nanomicelles (NMs) of approximately 9 nm in diameter, containing about 17 monomers per micelle (Figure 4.4.2). These self-assembly yields a gradual polarity gradient from the hydrophobic core to the polar surface, creating a versatile microenvironment for hosting hydrophobic molecules.

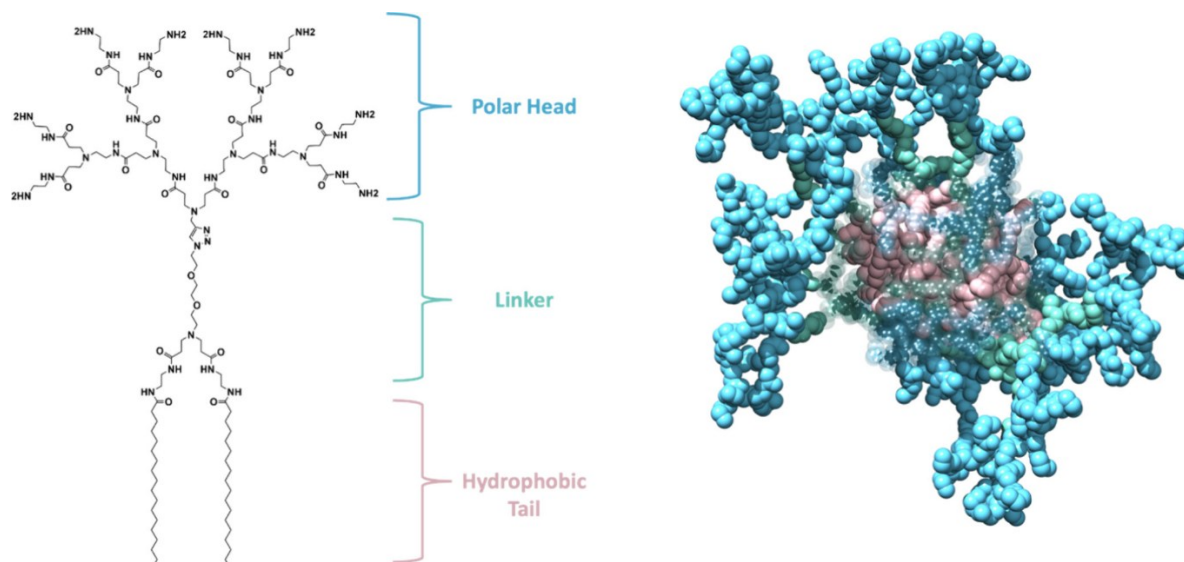


Figure 4.4.2. (Left) 2D chemical structure of the AD monomer. **(Right)** Atomistic model of the self-assembled AD nanomicelle. The atoms are represented as spheres and colored by the corresponding region as follows: hydrophobic tails, pink; linkers, aquamarine; polar heads, deep sky blue. For the sake of clarity, some linker or head atoms are represented as transparent spheres to show the hydrophobic core of the micelle.

Previous studies have shown that AD NMs exhibit minimal toxicity, both *in vitro* and *in vivo*, and effectively encapsulate BRAF inhibitors like DAB and VEM, enhancing their apparent bioactivity. Building upon these findings, the present study aimed to:

- 1.1 Investigate the molecular interactions between AD nanocarriers and six clinically relevant BRAF inhibitors (VEM, DAB, ENC, AZ, PLX, and PON);
- 2.1 Experimentally determine the encapsulation efficiency (EE) of AD nanomicelles toward each drug;
- 3.1 Compute the partition coefficients (K) and free energy profiles of the BRAF inhibitors within the nanocarriers to elucidate their preferred encapsulation mechanisms.

The partition coefficient (K) quantifies the distribution of a solute between the micellar and aqueous phases. Although experimental approaches (*e.g.*, solubility assays, micellar liquid chromatography, ultrafiltration) can determine K, they do not capture the intrinsically anisotropic structure of micelles, which significantly influences drug positioning. Therefore, molecular dynamics simulations and the COSMOmic model (an extension of COSMO-RS theory) were employed. MD provides atomic-level

free energy profiles, identifying energetically favorable regions for drug localization, while COSMOmic predicts partition equilibria based on quantum-chemical descriptors coupled with statistical thermodynamics.

By integrating experimental encapsulation data with computational modeling (MD + COSMOmic), this study offers a detailed characterization of the encapsulation modes of the selected BRAFis within AD nanomicelles, providing molecular-level insights essential for optimizing nanocarrier-based delivery of hydrophobic anticancer drugs.

Materials and Methods

Reagents and Chemicals

As reported in Table 4.4.1, ponatinib, AZ 628, PLX8394, and encorafenib were obtained from MedChemExpress (Monmouth, NJ, USA), while all other reagents and chemicals (all of analytical grade) were purchased from Sigma-Aldrich Inc. (Saint Louis, MO, USA). Dabrafenib and vemurafenib experimental data were retrieved from previous work conducted by our group.

Chemical name	CAS number	Supplier	Purity
AZ 628 (AZ)	878739-06-1	MedChemExpress	99.85%
Encorafenib (ENC)	1269440-17-6	MedChemExpress	99.63%
PLX8394 (PLX)	1393466-87-9	MedChemExpress	99.28%
Ponatinib (PON)	943319-70-8	MedChemExpress	99.43%
Phosphate-buffered saline (PBS)	7778-77-0	Sigma-Aldrich	≥99.9%
Dimethyl sulfoxide (DMSO)	67-68-5	Sigma-Aldrich	≥99.9%

Table 4.4.1. CAS Registry Number, Suppliers, and Mass Fraction Purity of Chemicals.

Drug Encapsulation Efficiency

For each drug/NM encapsulation, the amount of encapsulated drug was evaluated via absorbance measurements using the ultraviolet–visible (UV–VIS) spectrophotometer V-730ST (JASCO, Japan) at the appropriate wavelength λ for each compound ($\lambda_{\text{DAB}} = 340$ nm; $\lambda_{\text{VEM}} = 310$ nm; $\lambda_{\text{AZ}} = 315$ nm; $\lambda_{\text{PLX}} = 310$ nm; $\lambda_{\text{ENC}} = 325$ nm; $\lambda_{\text{PON}} = 320$ nm). The corresponding concentrations were then derived by interpolation using standard absorbance curves. The drug encapsulation efficiency (EE, %) was then calculated according to its standard definition as follows:

$$EE = \frac{\text{encapsulated mass of drug}}{\text{initial mass of drug used during encapsulation}} * 100. \quad (48)$$

Molecular Dynamics Simulations

The molecular models and parametrization procedures for the AD amphiphile, DAB, and VEM were obtained from our previous works. The same protocol was applied to parametrize all other drug molecules, as shown in Table 4.4.2. Briefly, molecular partial charges were determined using the RESP (Restrained Electrostatic Potential) approach through the RED (RESP ESP charge Derive) server, while the antechamber and parmchk2 utilities from the AMBER22 suite were employed to assign gaff2 force-field atom types. Pre-equilibrated self-assembled AD NMs were utilized in the current study. In this case, the drug molecules were manually positioned within approximately 10 Å from the micelle surface using the UCSF Chimera software. Conversely, each self-assembly experiment involved randomly placing a consistent number of AD monomers (17) and one drug molecule within the simulation box via the PACKMOL software.

Chemical name	Chemical or linear formula	Model
AZ 628 (AZ)	C ₂₇ H ₂₅ N ₅ O ₂	gaff2 with RESP chrges
Dabrafenib (DAB)	C ₂₃ H ₂₀ F ₃ N ₅ O ₂ S ₂	gaff2 with RESP chrges
Encorafenib (ENC)	C ₂₂ H ₂₇ ClFN ₇ O ₄ S	gaff2 with RESP chrges
PLX8394 (PLX)	C ₂₅ H ₂₁ F ₃ N ₆ O ₃ S	gaff2 with RESP chrges
Ponatinib (PON)	C ₂₉ H ₂₇ F ₃ N ₆ O	gaff2 with RESP chrges
Vemurafenib (VEM)	C ₂₃ H ₁₈ ClF ₂ N ₃ O ₃ S	gaff2 with RESP chrges
AD monomer	C ₁₂₅ H ₂₄₇ N ₃₇ O ₂₀	gaff2 with RESP chrges
Water	H ₂ O	TIP3P

Table 4.4.2. Chemical Compounds and Models.

The AMBER22 tleap program was used to generate the initial topology for each system. TIP3P water molecules were added, along with an appropriate number of Cl[−] counterions to maintain overall system neutrality. Unless otherwise stated, pressure was regulated at 1 atm using the Monte Carlo barostat, while the temperature was maintained at 300 K employing the Langevin thermostat with a collision frequency of 2 ps^{−1}. Electrostatic interactions were computed via the particle mesh Ewald (PME) method, and the SHAKE algorithm was applied to all hydrogen atoms to enable a time step of 2 fs for integration.

The energy minimization process, consisting of 5,000 steps each of steepest descent and conjugated gradient algorithms for all solvated system. Subsequently, the temperature was gradually increased to 150 K over 10 ps of canonical (NVT) ensemble MD simulations, during which solute positions were restrained with a force constant of 10 kcal/mol·Å² using a 1 fs time step. Upon release of the positional restraints, the system underwent further heating to 300 K over 10 ps in the isothermal–

isobaric (NPT) ensemble using a Berendsen barostat. Density equilibration was then achieved through 500 ps of repeated NPT MD runs, again using the Berendsen barostat. Production simulations were subsequently carried out (following an additional 5 ns of density adjustments) with a duration of 1 μ s for the equilibration of the preassembled AD nanomicelle-drug complexes and 5 μ s for the self-assembly simulations.

US-MD Simulations, MM/PBSA, and Structural Analysis

To initialize the starting configurations for each window in the umbrella-sampling-molecular dynamics (US-MD) simulations, a pulling procedure was implemented to guide each drug molecule toward and away from the center of mass (COM) of the micelle using a pull rate of 20 $\text{\AA}/\text{ns}$ and a force constant of 3,300 $\text{kJ}/\text{mol}\cdot\text{nm}^2$. Subsequently, a total of 68 US simulations were executed for each solute, systematically varying the distance between the COM of the micelle and the COM of the drug. Notably, the distance between consecutive windows was maintained at 1 $\text{\AA}$, increasing to 2 $\text{\AA}$ for windows located fully within the aqueous phase. Data collection within each window spanned 50 ns following an equilibration period of 10 ns, with data saved every 0.4 ps. The force constant for the umbrella potential was 3,300 $\text{kJ}/\text{mol}\cdot\text{nm}^2$, as this value ensured adequate histogram overlap across adjacent windows (see Figure S18). The weighted histogram analysis method (WHAM) was next applied to compute the unbiased free energy profiles, employing the software from the Grossfield Lab. Bootstrap analysis (500 subsamples) was performed to estimate statistical uncertainties in the free-energy profiles. The energetic contributions were further examined through the MM/PBSA methodology implemented in the MMPBSA.py module of AMBER22 (50 representative frames extracted from the US-MD trajectory were employed). Concurrently, the entropy contributions were computed utilizing the Interaction Entropy (IE) method. Additional simulation details are summarized in Table S4.

Structural analyses were performed using AMBER22 CPPTRAJ in combination with custom Python scripts. Hydrogen bonds were identified with the *hbond* command in CPPTRAJ, using default geometrical parameters. Polar contacts were investigated using the *distance* command in CPPTRAJ selecting the relevant atoms and defining a contact whenever the interatomic distance fell below 5 $\text{\AA}$. All simulations were carried out on an in-house GPU/CPU hybrid cluster, as well as on Tier-0 Marconi-100 and pre-exascale Leonardo supercomputers (CINECA high-performance computing (HPC) infrastructure, Bologna, Italy).

COSMOmic

The partition equilibria of solutes were estimated utilizing the COSMOmic model, an extension of the COSMO-RS framework specifically designed to describe anisotropic systems such as membranes

and micelles. Grounded in statistical thermodynamics and quantum chemical theory, COSMOmic subdivides the micelle structure into discrete radial shells (48 slices selected, with $r_{\text{max}} = 72 \text{ \AA}$). Within each layer, COSMO-RS computations are performed for each solute molecule in 162 orientations, allowing configurations that may span multiple layers. Each layer is assumed to be compositionally homogeneous, containing a defined distribution of ions, water, and surfactant atoms. Consequently, an accurate description of the atomic density profiles within the water/micelle system is essential. To achieve this, averaged atomic distributions derived from MD simulations of the pristine AD micelle, recognized as more realistic than single structural snapshots, were employed. COSMO-RS calculations were conducted at 300 K using the parameter file BP_TZVP_C30_1701.ctd, an encoded file that does not provide direct access to individual parameter values. Each molecular species requires at least one conformer for the quantum chemical evaluations. For amphiphilic molecules, whose conformations depend strongly on their microenvironment, representative surfactant monomers were extracted directly from MD simulations to ensure that their geometries reflected the micellar state. Following recommendations from the literature, conformers were selected based on solvent accessible surface criteria and subsequently subjected to density functional theory (DFT)/COSMO single-point calculations using Turbomole 6.6. For all investigated drug molecules, conformers were extracted from equilibrated MD simulations using a DBSCAN clustering procedure (RMSD of heavy atoms as the distance metric, $\epsilon = 0.5 \text{ \AA}$, $\text{minPoints} = 5$). These conformers were then used to perform the corresponding DFT/COSMO calculations in Turbomole 6.6, providing the required input for the COSMOmic analysis. All DFT calculations employed the Becke-Perdew (BP86) functional and the triple-z valence polarization (def-TZVP) basis set. The resolution-of-identity (RI) approximation was also applied throughout.

Correction of the Free Energy Profiles and Partition Coefficient Calculations

In contrast to the more straightforward bilayer configuration formed by simpler surfactants micellar systems exhibit structural features that require specific corrections in the calculation of their free energy profiles. In a micelle, the volume of each shell depends on its distance from the center of the micelle, resulting in a nonuniform volume distribution across the self-assembled structure. Thus, if the thickness of each layer is kept constant, layers located farther from the micellar core possess larger volumes than those close to the center. This spatial variation affects the shape of the computed free-energy profile because the profile directly reflects the probability of finding the solute within each region of the micelle. Hence, regions with larger volume correspond to lower apparent free-energy values, indicating a higher probability of solute occupancy. This effect becomes particularly evident in the aqueous phase, where the free energy curve shows a continuous decline rather than fluctuating around a constant value, as might otherwise be expected. To correct for this inherent geometric bias

and obtain physically meaningful free energy profiles, the profiles were adjusted by incorporating the appropriate shell-volume correction using:

$$\Delta G(z) = \Delta G_{nc}(z) + 2k_B T \ln(z) \quad (49)$$

where $\Delta G(z)$ is the corrected free energy, $\Delta G_{nc}(z)$ denotes the uncorrected profile as computed by either US-MD simulations or COSMO-RS calculations, k_B is the Boltzmann constant, T is the absolute temperature, and z denotes the radial coordinate relative to the micelle center.

The free energy profiles obtained from both US-MD simulations and COSMO-RS calculations were utilized to define the boundary between the micellar and aqueous phases, thereby enabling the computation of the partition coefficient between these two regions. The aqueous phase was defined as the region where the free energy returns to and fluctuates around zero, maintaining a stable baseline value. The partition coefficient K was then calculated using Equation 3, where the micellar phase encompasses all layers from the first layer to the surface layer n_{surf} , while the aqueous phase comprises all subsequent layers from $n_{surf} + 1$ to the final layer n :

$$K = \frac{V_W}{m_{mic}} \frac{\sum_{i=1}^{n_{surf}} V(z_i) e^{-\beta \Delta G(z_i)}}{\sum_{j=n_{surf}+1}^n V(z_j) e^{-\beta \Delta G(z_j)}} \quad (50)$$

Here, $V(z_{i,j})$ is the volume of the shell (i,j) , V_w denotes the volume of the water phase, and m_{mic} represents the mass of the micellar phase. The parameter $b = 1/(k_B T)$ corresponds to the inverse thermal energy, with k_B the Boltzmann constant and T the absolute temperature.

Results and Discussion

Drug Encapsulation Efficiency of the AD Nanomicelles.

For each drug/NM system, the amount of encapsulated drug was quantified by UV-vis absorbance measurements using a Jasco V-730ST spectrophotometer (JASCO, Japan) at the appropriate wavelength l for each compound ($l_{DAB} = 340$ nm; $l_{VEM} = 310$ nm; $l_{AZ} = 315$ nm; $l_{PLX} = 310$ nm; $l_{ENC} = 325$ nm; $l_{PON} = 320$ nm). The corresponding concentrations were determined by interpolation from drug-specific calibration curves. The drug encapsulation efficiency (EE, %) was then calculated according to its standard definition (Equation (48)). In our recent work, the EEs for AD NMs loaded with DAB and VEM were determined at three different AD/drug weight ratios (Table 4.4.3). For both nanosystems, increasing the drug concentration did not appreciably influence the EE values, and both nanocomplexes displayed good encapsulation performance with average EE values (EE_{AVG}) of 73.1% for DAB and 61.1% for VEM, respectively. Using the same methodology, AZ, ENC, PLX, and PON were encapsulated within the AD nanoassemblies to evaluate their corresponding EE values. As observed for DAB and VEM, variations in the drug amount within the explored experimental range

did not significantly alter the encapsulation capacity of the nanomicelles (Table 4.4.3). Interestingly, although all EE_{AVG} values fall within the typical range of good encapsulation performance, the dataset revealed two distinct clusters. DAB, ENC, and PLX exhibit higher EEs of approximately 75%, whereas the remaining three inhibitors (VEM, AZ, and PON) show lower EE values of around 60% (Table 4.4.3).

AD/DAB ratio (w/w)	EE (%)	EE_{avg} (%)	AD/VEM ratio (w/w)	EE (%)	EE_{avg} (%)
10:1	75.3 ± 0.2	73.1	10:1	61.6 ± 0.9	61.1
10:2	74.2 ± 0.4		10:2	61.9 ± 1.1	
10:5	69.8 ± 0.9		10:5	59.7 ± 0.4	
AD/AZ ratio (w/w)	EE (%)	EE_{avg} (%)	AD/ENC ratio (w/w)	EE (%)	EE_{avg} (%)
10:1	60.3 ± 0.6	59.3	10:1	77.4 ± 1.4	75.8
10:2	60.1 ± 1.0		10:2	78.6 ± 0.3	
10:5	57.5 ± 0.5		10:5	71.4 ± 0.7	
AD/PLX ratio (w/w)	EE (%)	EE_{avg} (%)	AD/PON ratio (w/w)	EE (%)	EE_{avg} (%)
10:1	79.5 ± 1.3	74.7	10:1	61.3 ± 0.8	59.7
10:2	73.3 ± 0.5		10:2	61.8 ± 0.7	
10:5	71.2 ± 0.6		10:5	56.1 ± 1.1	

Table 4.4.3. AD/Drug Ratios and Relative Drug Encapsulation Efficiency (EE) and Averaged Encapsulation Efficiency (EE_{avg}).

Calculation of Free Energy Profile and Partition Coefficient in AD Micelles through US-MD and COSMOmic Techniques.

To rationalize the encapsulation efficiency data, we adopted a dual computational strategy, combining COSMOmic and atomistic US-MD simulations to predict both the free energy profile (ΔG) and the partition coefficient K for the six BRAFis within the AD NMs. The upper panel of Figure 4.4.3 presents the comparison of the two ΔG profiles obtained from the two approaches, along with the preferred micellar location of each drug as determined from the energy minimum extracted from the US-MD simulations. As illustrated, the ΔG profiles obtained with COSMOmic and US-MD are highly comparable, demonstrating a strong complementarity between the two computational methodologies. Moreover, the energetic minima for all six inhibitors fall within a remarkable narrow range (-30 to -32 kJ/mol), suggesting a similar strength of interaction between each BRAFi and the AD nanocarriers. What substantially differentiates the energy profile of the various anticancer molecules within the AD NMs is the position of the free-energy minimum along the radial coordinate, a clear signature that each inhibitor adopts a specific preferential location within the nanomicelle

depending on its unique molecular structure and functional group distribution. Three characteristic sites were identified inside the micellar assembly (Figure 4.4.3A), as detailed below:

Position 1 (POS 1), the innermost and most hydrophobic location and situated between the alkyl tails and the central linker of the amphiphiles. AZ, PON, and VEM exhibit their lowest ΔG values in this region, indicating a stable energetic preference for POS 1.

Position 2 (POS 2), the region centered on the amidoaminoethoxytriazolic linker, which connects the hydrophobic tails to the polar headgroups. DAB and PLX preferentially occupy this site, as reflected in their ΔG minima. By contrast, the three BRAFi that prefer POS 1 (AZ, PON, and VEM) experience a notable energetic penalty (approximately 5 kJ/mol) when approaching this intermediate region of the micelles.

Position 3 (POS 3), the outermost, more polar zone of the micelles. The second-generation inhibitor ENC displays the most favourable ΔG values in this region, consistent with its structural polarity (bottom panel of Figure 4.4.3B). As shown in the top panel of Figure 4.4.3, DAB and PLX incur only a modest energetic cost when moving toward the more polar regions of the micelles, while AZ, PON, and VEM are substantially disfavored in this more polar region, with ΔG increase exceeding 10 kJ/mol compared to their preferred POS1 environment (Figure 4.4.3). Further validation of these findings was obtained through independent classical MD simulations of AD self-assembly in the presence of each BRAFi. Starting from a random distribution of 17 AD monomers and 1 drug molecule in a solvated box, 5 *ms* MD simulations consistently revealed that the final position of each inhibitor within the assembled micelles matched the location predicted by the minimum of its US-MD free-energy profile (Figure S19).

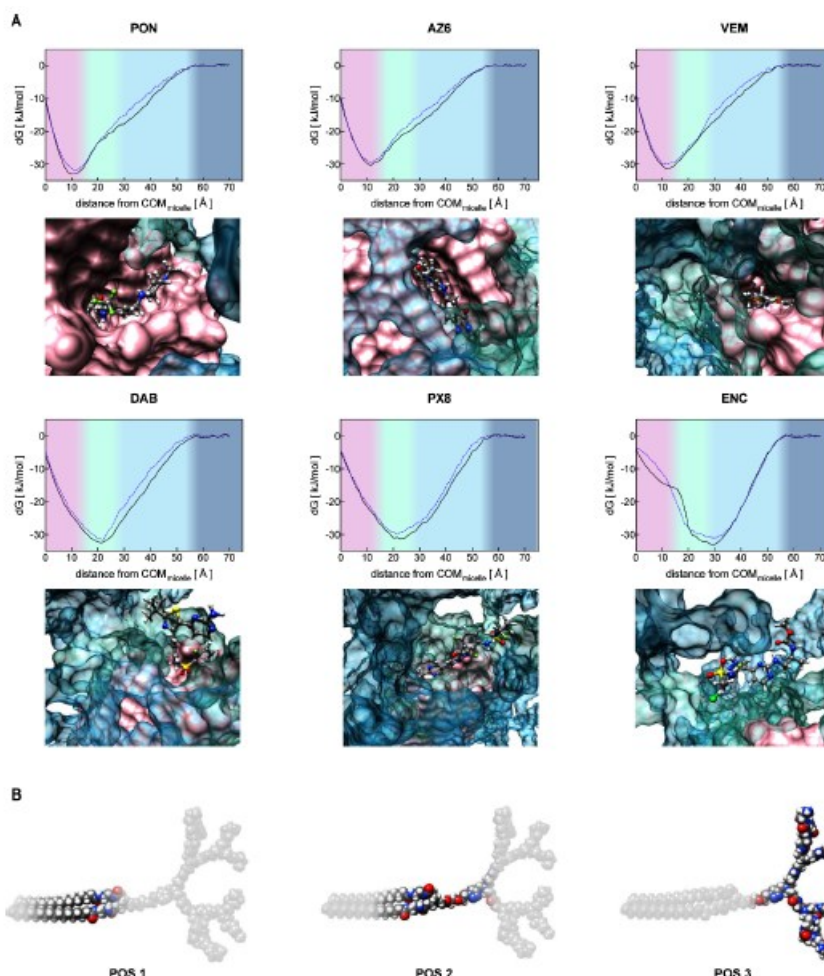


Figure 4.4.3. (A) (Upper panels) Free energy profiles of the six BRAF inhibitors in an AD micelle calculated from US-MD simulations (black line) and from the COSMOmic model (purple dotted line). (Bottom panels) Zoomed in view of the drug/micelle complex extracted from the energy minimum of each US-MD free energy profile. The drug is shown in a ball-and-stick style and colored by element. The atoms of the hydrophobic AD tails are represented as spheres, while the AD linkers and polar heads are shown as transparent van der Waals surfaces (see the caption of Figure 4.4.2 for the color code). Water molecules and counterions are not shown for the sake of clarity. (B) Schematic representation of the three preferred locations in the AD micelle found for the six antitumoral drugs (see text for more details). In each position, the atoms of the AD monomer most interacting with the inhibitor are shown as spheres colored by element while the other atoms are depicted as transparent spheres.

Next, using the same MD trajectories, the partition coefficient ($\log K$) between the micellar and aqueous phases was determined for each BRAF inhibitor (Table 4.4.4). As expected from the direct relationship between $\log K$ and the underlying ΔG profile, the values obtained from US-MD simulations and COSMOmic are comparable. Moreover, the combined dataset reveals two distinct clusters with equal population among the six inhibitors. Specifically, DAB, ENC and PLX exhibit higher $\log K$ values ($\log K_{\text{(US-MD)}} = 5.42\text{-}5.99$; $\log K_{\text{(COSMOmic)}} = 5.26\text{-}5.89$), whereas VEM, AZ, and PON display $\log K$ values below 5 ($\log K_{\text{(US-MD)}} = 4.61\text{-}4.94$; $\log K_{\text{(COSMOmic)}} = 4.37\text{-}4.80$). This behavior is consistent with the characteristics of the BRAFi free-energy profiles: the broader and

more extended ΔG minima of DAB, ENC and PLX support more favorable partitioning into the micellar phase, while the sharper, narrower minima of VEM, AZ and PON correspond to less favorable $\log K$ values (Figure 4.4.3).

System	$\log K_{(\text{US-MD})}$	$\log K_{(\text{COSMOmic})}$	System	$\log K_{(\text{US-MD})}$	$\log K_{(\text{COSMOmic})}$
AD/AZ	4.61 ± 0.11	4.37	AD/DAB	5.42 ± 0.12	5.26
AD/VEM	4.75 ± 0.11	4.54	AD/PLX	5.45 ± 0.14	5.36
AD/PON	4.94 ± 0.08	4.80	AD/ENC	5.99 ± 0.13	5.89

Table 4.4.4. Predicted Partition Coefficients ($\log K$) calculated *via* US-MD and COSMOmic Simulations. All values are expressed as kg(AD)/L(water).

Classic MD Simulations and Energetic Analysis via the Molecular Mechanics/Poisson–Boltzmann Surface Area (MM/PBSA) Approach

The analysis of the free energy profiles and partition coefficients values discussed above revealed that inhibitors interacting more efficiently with the hydrophobic core of the self-assembled AD micelles exhibit a slightly reduced affinity for the micellar phase, whereas those preferentially located in the more polar regions of the nanocarrier show comparatively higher affinity. To further elucidate the underlying binding mechanism and reinforce the molecular rationale, additional classical MD simulations within the MM/PBSA framework of theory were carried out. For each drug/AD NM system, a representative snapshot was first extracted from each of the three regions along the corresponding free energy landscape (POS 1, POS2, and POS3) and then subjected to 2 ns of classical MD simulations. This strategy enabled the calculation of the principal components of the binding enthalpy ΔH (namely, ΔE_{vdW} , ΔE_{ele} , and ΔE_{solv}) along with the corresponding entropic contribution ($-T\Delta S$) for each system (Figure 4, left panel). The acquired data provided a more mode detailed and quantitative characterization of the energetic differences among the six BRAFis, as discussed below.

Position 1. POS 1 is the region of the AD NMs with the highest lipophilic character and is located approximately 10 Å away from the nanomicelle center of mass ($\text{COM}_{\text{MICELLE}}$). In this deeply hydrophobic zone, VEM, AZ, and PON are optimally accommodated within the hydrocarbon tails of the aggregated amphiphiles, resulting in a markedly more favorable enthalpic contribution compared to the remaining inhibitors (Figure 4.4.4A, left and middle panels and Figure S20). The dominant stabilizing factor in this system is the strong network of van der Waals interactions, with highly favorable ΔE_{vdW} exceeding -75 kJ/mol ($\Delta E_{\text{vdW,VEM}} = -76.61 \pm 6.89$ kJ/mol; $\Delta E_{\text{vdW,AZ}} = -75.83 \pm 4.55$ kJ/mol; $\Delta E_{\text{vdW,PON}} = -78.07 \pm 4.68$ kJ/mol; Table S2). This trend is fully consistent with the large number of hydrophobic contacts detected during the simulations (Figure 4.4.4A, right panel), with average contact counts of 248 ± 16 (VEM), 252 ± 18 (AZ), and 239 ± 22 (PON) (Table S6). In

contrast, the other three inhibitors experience an enthalpic penalty of approximately 10 kJ/mol when located at POS1, as they establish just over half the number of hydrophobic contacts observed for VEM, AZ and PON (Figure 4.4.4A, right panel). The remaining enthalpic terms contribute only marginally and can be considered negligible in describing AD/drug interactions at this site. Entropy, on the other hand, provides a destabilizing (negative) contribution to micelle-drug binding at POS1. However, although VEM, AZ, and PON display the largest entropic penalties, this term does not alter the ranking determined by the enthalpic contributions (Figure 4.4.4A, right panel).

Position 2. This region is centered on the linker between the hydrophobic tails and the polar headgroups, located approximately 20 Å away from the COM_{MICELLE}. According to the free energy minima in their profiles (Figure 4.4.3, top panel), DAB and PLX exhibit a more favorable enthalpic contribution at this location compared to the other inhibitors, with values of $\Delta H_{\text{DAB}} = -79.27 \pm 5.51$ kJ/mol and $\Delta H_{\text{PLX}} = -75.73 \pm 5.22$ kJ/mol for PLX, respectively (Figure 4.4.4B, left panel and Table S5). The thermodynamic signature at POS2 resembles the pattern observed for POS 1: binding is predominantly enthalpic, with entropy contributing a destabilizing (negative) term that, once again does not alter inhibitor ranking. However, in contrast to POS1, DAB and PLX achieve a nearly perfect balance between van der Waals and electrostatic stabilizations ($\Delta E_{\text{vdW,DAB}} = -43.45 \pm 3.91$ kJ/mol, $\Delta E_{\text{ele,DAB}} = -47.16 \pm 3.77$ kJ/mol; $\Delta E_{\text{vdW,PLX}} = -42.18 \pm 3.80$ kJ/mol, $\Delta E_{\text{ele,PLX}} = -43.78 \pm 3.50$ kJ/mol; Table S5). These favorable interactions arise from a high number of polar contacts in the binding region (50 ± 6 (DAB); 49 ± 6 (PLX); Table S3), together with a network of four stable hydrogen bonds (HBs), present in $\geq 80\%$ of frames (Figure 4.4.4B, middle and right panels). ENC also interacts favorably with the nanomicelles at POS2, but its enthalpic contribution is approximately 10 kJ/mol less favorable. This reduced stabilization correlates with a slightly lower number of polar contacts (43 ± 8) and three HBs (Tables S5 and S6). In contrast, VEM, AZ, and PON experience significant energetic penalties at POS2, with marked reductions in all stabilizing interactions, as shown in Figure 4.4.4B (left and right panels). A particularly noteworthy observation is the distinct behavior of PLX and VEM, despite their structural similarity. The sulfonamide group in VEM is unable to establish the HB network that the same functional group in PLX successfully forms with the amide groups of the linker (Figure 4.4.4B, center panel). This difference arises because VEM is compelled to orient its sulfamide moiety toward the more polar region of the nanomicelle as its chlorophenyl group preferentially embeds into the hydrophobic tails of the amphiphiles. Conversely, PLX can efficiently position its piperidine substituent within the hydrophilic environment near the polar heads of AD, enabling stronger polar and H-bonding interactions.

Position 3. The last identified position (POS 3) is the outermost portion of the nanomicelle and is located approximately 30 Å from the $COM_{MICELLE}$. It is the most polar region of the self-assembled structure, owing to the presence of the amidoamine groups and the greater water penetration within this layer (Figure S19). ENC is the BRAFi that shows the stronger preference for POS 3, establishing robust enthalpic interactions with AD NMs (Figure 4.4.4C, left panel and Table S2; $\Delta H_{ENC} = -76.72 \pm 4.62$ kJ/mol). The principal stabilizing contribution arises from electrostatic interactions ($\Delta E_{ele,ENC} = -57.33 \pm 3.44$ kJ/mol), which result from a high number of polar contacts (110 ± 15 , Figure 4.4.4C, right panel and Table S2) and an average of 4 stable HBs maintained throughout the entire MD trajectory (Figure 4.4.4C, middle panel). In parallel, the hydrophobic portion of ENC contributes to a strong network of van der Waals interactions ($\Delta E_{vdW,ENC} = -39.25 \pm 2.75$ kJ/mol; Table S5), further enhancing its binding to the AD NMs. DAB and PLX, by contrast, are less efficient in forming stabilizing interactions with this highly polar region, experiencing an enthalpic loss exceeding 25 kJ/mol. This decrease directly linked to their reduced number of polar contacts, as shown in the left and right panels of Figure 4.4.4C. The remaining inhibitors (VEM, AZ, and PON) are also strongly energetically penalized at POS 3, as they fail to form an effective network of electrostatic interactions with the micelle headgroups. In addition, they experience a larger solvation-energy penalty compared than at either of the other two positions (Figure 4.4.4C, left and right panels, and Tables S5 and S6).

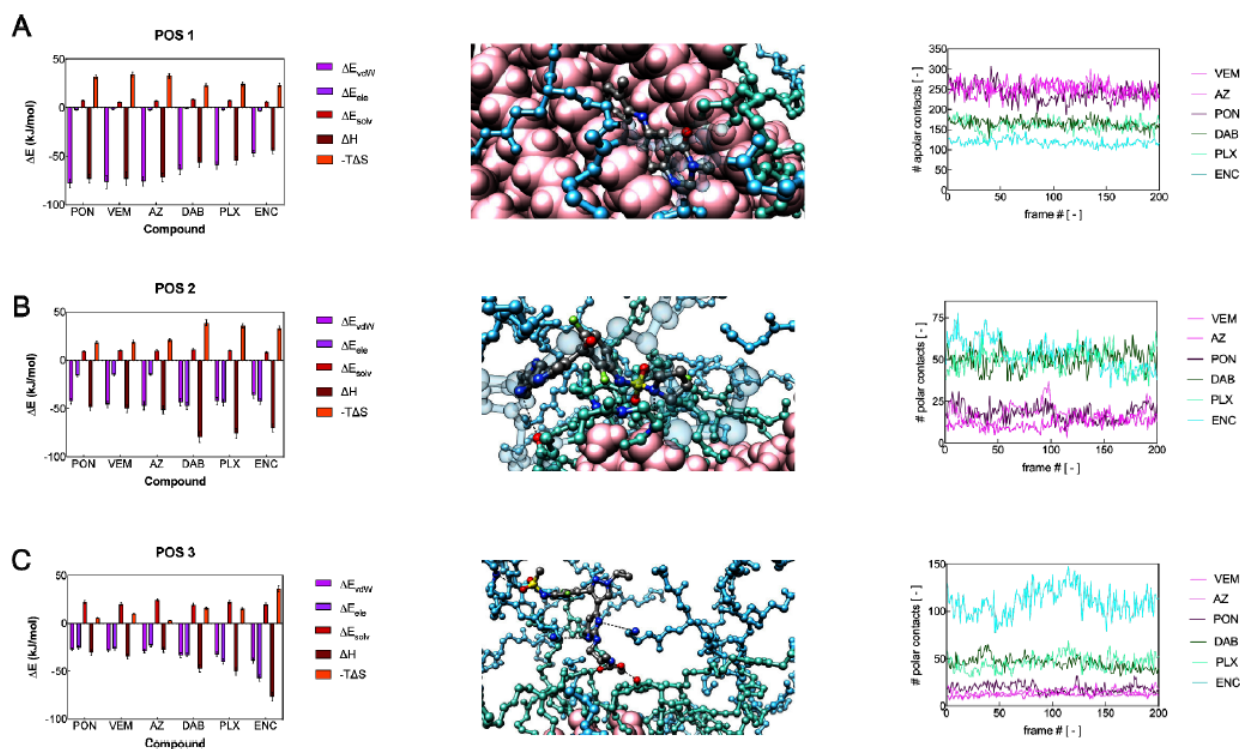


Figure 4.4.4. (Left panel) MM/PBSA energetic results of the six drug/NM complexes starting with the inhibitors in POS 1 (A), POS 2 (B), and POS 3 (C). (Middle panel) Atomistic snapshot extracted from the equilibrated MD simulations of AD micelle in complex with AZ (A), PLX (B), and ENC

(C). The drugs are depicted in a ball-and-stick style and colored by element. The atoms of the AD hydrophobic tails are represented as spheres while the AD linkers and polar heads are shown as balls and sticks (see the caption of Figure 4.4.2 for the color code). Water molecules and counterions are not shown for the sake of clarity. **(Right panel)** Number of apolar contacts for POS 1 (A) and of polar contacts for POS 2 (B) and POS 3 (C) detected during the entire MD simulation for each drug/NM complex.

Conclusions

This investigation elucidated the encapsulation efficiency and molecular interactions within NMs formed through the self-assembly of the amphiphilic dendrimer AD with six BRAFis inhibitors currently under clinical or translational evaluation for melanoma treatment, namely dabrafenib, vemurafenib, encorafenib, AZ 628, PLX8394, and ponatinib. Experimental determination of encapsulation efficiency was complemented with two computational methodologies, US-MD and COSMOmic simulations, which were used to predict free energy profiles and partition coefficients of the BRAFis within the AD NMs. Encapsulation experiments demonstrated that all six BRAFis can be efficiently incorporated into the AD NMs, albeit with varying EE values. DAB, ENC, and PLX showed higher EE values of approximately 75%, whereas VEM, AZ, and PON displayed slightly lower efficiencies, with EE values around 60%. Computational analyses from both US-MD and COSMOmic simulations provided convergent insights into the preferential location of each BRAFi into the AD NMs, identifying three distinct regions (POS 1, POS 2, and POS 3) based on the corresponding free energy minima. The hydrophobic inner region (POS 1) was favored by VEM, AZ, and PON, the intermediate linker region (POS 2) was preferred by DAB and PLX, while the outer polar region (POS 3) showed optimal interactions with ENC. These findings were consistent across both computational approaches, reinforcing the robustness of the predicted localization patterns. The computed partition coefficients ($\log K$) further reflected the affinity of each BRAFi for the micellar or aqueous phases, respectively. DAB, ENC, and PLX exhibited higher $\log K$ values, consistent with their stronger association with the nanocarrier, while VEM, AZ, and PON showed lower values and correspondingly reduced affinity. To refine the molecular picture further, additional classical MD simulations and MM/PBSA energetic analyses were carried out. These revealed distinct enthalpic signatures in the three positions, with hydrophobic interactions dominating in POS 1 and POS 2, and electrostatic interactions being critical for stabilization in POS 3. Overall, the integration of experimental encapsulation tests with two complementary computational approaches generated a comprehensive and coherent understanding of the encapsulation behavior and molecular interactions of the selected six BRAF inhibitors within the self-assembled AD NMs. Such insights are essential for optimizing drug delivery strategies and supporting the development of more effective therapeutic approaches for melanoma treatment.

4.5 Generalized Mechanistic Framework and Boundary Conditions

The methodological approach (Figure 4.5.1) developed throughout this thesis can be formalized as a reusable, scale-independent workflow for investigating molecular and nano-bio interactions. The process begins with physicochemical fingerprinting of the system, including structural features, charge distribution, hydrophobicity, and aggregation state, to identify the dominant interaction regime. An experimental screening phase then establishes the presence, qualitative strength, and structural consequences of binding using minimal spectroscopic or scattering readouts. When interaction is confirmed, thermodynamic profiling, primarily through calorimetric or entropy/enthalpy proxies, defines the global driving forces governing association. Targeted molecular modeling subsequently translates these macroscopic observables into atomistic mechanisms, resolving binding geometries, hydration effects, charge regulation, dynamic stability, and cooperative phenomena. The computational and experimental layers are iteratively cross-validated to ensure mechanistic consistency rather than mere numerical agreement. Finally, the results are extrapolated to biological implications, such as pharmacokinetic modulation, genotoxic liability, or corona-mediated biological identity. By relying on a minimal yet sufficient dataset and a decision-based progression from global observables to spatially resolved mechanisms, this workflow provides a transferable framework for predictive interpretation across drug-protein, drug-DNA, and nanoparticle-protein systems.

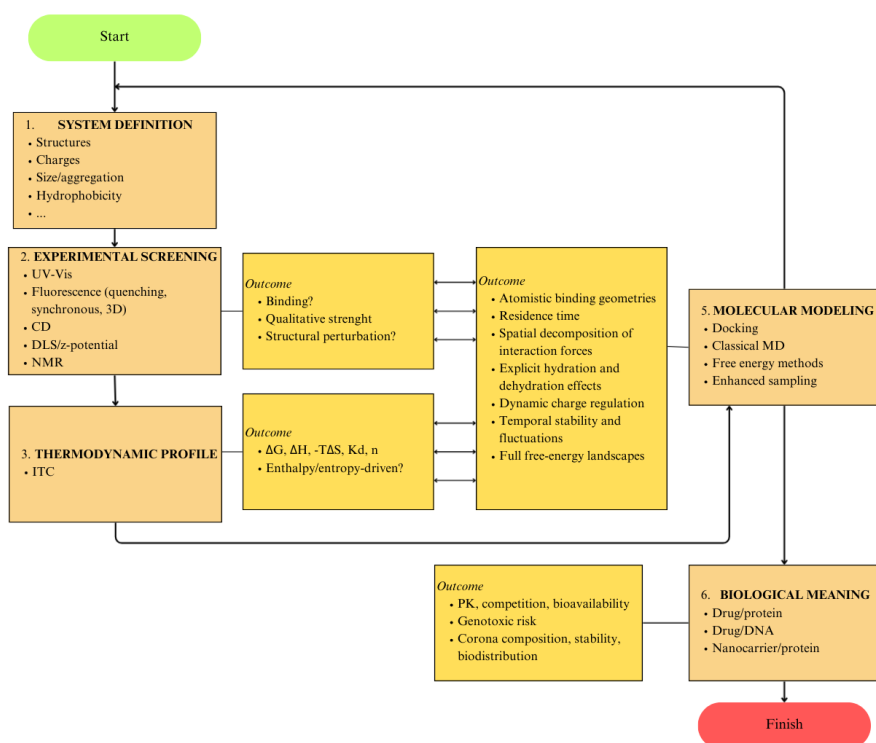


Figure 4.5.1. Overview of the reusable generalized physicochemical workflow integrating experimental observables and targeted molecular modeling.

Although the integrative computational-experimental workflow developed in this thesis provides mechanistic insight across molecular and nanoscale systems, its predictive scope is defined by several explicit limitations and boundary conditions. Classical molecular dynamics simulations rely on fixed-charge force fields, predefined protonation states, and specific water and ion models, rendering quantitative outcomes sensitive to parameterization choices and limiting explicit treatment of polarization and dynamic charge regulation. Simulated timescales, typically spanning the nanosecond-to-microsecond regime, capture metastable binding configurations but may not fully explore rare conformational transitions or long-term remodeling processes. Experimentally, measurements were conducted under controlled buffer conditions and simplified matrices; therefore, derived affinities and thermodynamic signatures reflect intrinsic interaction propensities rather than fully competitive physiological environments. In the drug-HSA studies, the single-protein model does not account for plasma-level competition or disease-dependent albumin modifications. In the DNA case study, interactions were evaluated on idealized B-form duplexes without chromatin context or genome-wide sequence variability, limiting direct extrapolation to cellular settings. For amphiphilic dendrimers and nanomicelles, simulations and experiments address pairwise or simplified protein systems, whereas *in vivo* protein corona formation is inherently matrix-dependent, species-specific, and dynamically remodeled under flow and immune surveillance. Consequently, corona composition should be interpreted as an interaction tendency rather than a universal biological identity. The framework is best suited for mechanistic rationalization and early-stage predictive screening under near-equilibrium conditions, rather than direct quantitative extrapolation to complex *in vivo* systems.

5. Conclusion

This thesis has demonstrated that the integration of high-performance computing with advanced biophysical experimentation constitutes a powerful and necessary paradigm for understanding the complex behavior of molecular and nanoscale systems in biologically relevant environments. Across the diverse case studies examined, ranging from the interaction of targeted anticancer inhibitors with human serum albumin, to the off-target association of small molecules with DNA, to the multiscale behavior of self-assembling amphiphilic dendrimers and their protein coronas, a coherent mechanistic picture emerges. The physicochemical forces that govern molecular recognition remain fundamentally consistent across systems of very different sizes and complexity; what differs is the way structure, dynamics, and environment modulate the manifestation of these forces.

The combined computational-experimental framework adopted in this thesis has proven capable of capturing this interplay with a degree of resolution and predictive power that neither simulations nor experiments could achieve independently. Spectroscopic and calorimetric techniques provided direct access to measurable thermodynamic and structural descriptors, while atomistic simulations revealed conformational landscapes, binding pathways, and energetic determinants underlying these observables. Through this iterative dialogue between measurement and modeling, the work elucidated not only binding affinities and structural perturbations, but also the dynamic stability, specificity, and emergent cooperative effects characteristic of complex biomolecular interactions.

In the context of anticancer therapeutics, the results deepen our understanding of how subtle chemical variations among BRAF and MEK inhibitors translate into distinct binding behaviors toward serum albumin, ultimately influencing pharmacokinetic profiles and clinical performance. The investigation of vemurafenib-DNA interactions highlights the importance of dissecting noncanonical binding modes for genotoxicity assessment, showing how hybrid computational-experimental approaches can transform spectroscopic signatures into atomistic interpretations with relevance for drug safety. Finally, the multiscale analyses of amphiphilic dendrimers provide mechanistic insight into nanocarrier design and behavior, demonstrating how self-assembly, surface charge, and corona formation collectively shape biodistribution, stability, and drug-release properties.

Together, these findings underline a central conclusion: predictive understanding in modern molecular science emerges most robustly when data and simulations are tightly interwoven, each constraining and enriching the other. As HPC capabilities continue to grow and simulation methodologies become increasingly accurate and scalable, such integrative strategies will assume an even more central role in guiding rational molecular and nanomaterial design. The approaches developed and applied here thus offer not only answers to specific biochemical questions, but also a

generalizable framework for future investigations at the interface of chemistry, biology, and nanotechnology.

To conclude, this thesis contributes to the advancement of a unified, mechanistic perspective on molecular recognition processes across scales. By bridging atomistic detail with mesoscopic behavior and connecting physical principles with biological consequences, it provides conceptual and methodological foundations that can support the development of safer, more effective therapeutic agents and next-generation nanomaterials. The broader implication is clear: the future of molecular and nanoscale design lies in the continued convergence of experiment and computation, enabled by high-performance computing and grounded in rigorous, mechanistic inquiry.

Supplementary Appendix

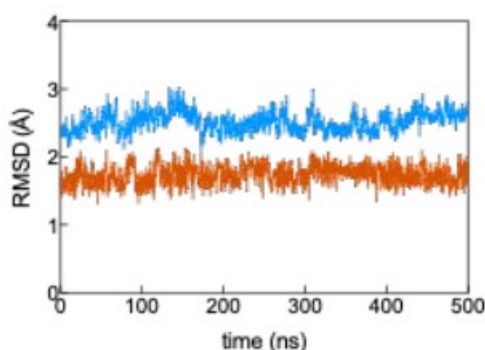


Figure S1. Root mean square deviation (RMSD) of COB heavy atoms (orange line) and HSA C α atoms (blue line), calculated over the equilibrated portion of the cMD simulations using the docked structure as the reference frame.

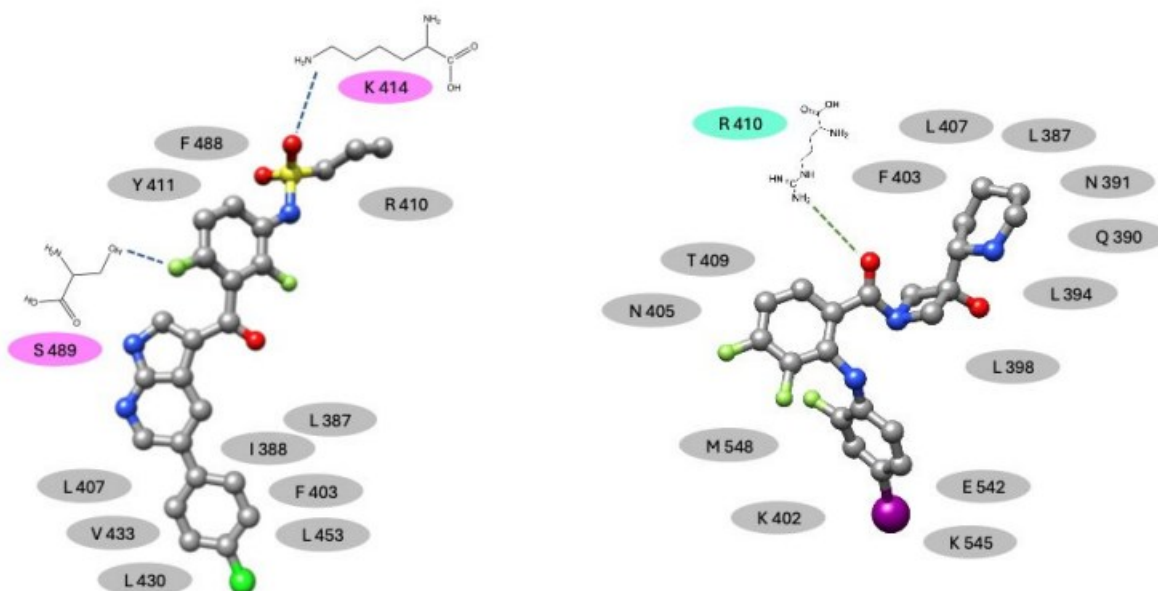


Figure S2. Schematic representation of the HSA binding pocket for VEM (**left panel**) and COB (**right panel**), highlighting residues involved in ligand interactions as identified from the cMD trajectories. Ligands are shown as balls and sticks (hydrogens omitted for clarity) with the following atom color scheme: carbon (gray), oxygen (red), nitrogen (blue), fluorine (light green), iodine (purple), chlorine (forest green). Residues forming key interactions are labeled: hydrogen bonds are indicated with pink ovals and blue dashed lines, while polar interactions are shown with cyan ovals and green dashed lines. Residues contributing to hydrophobic interactions are shown in light gray ovals.

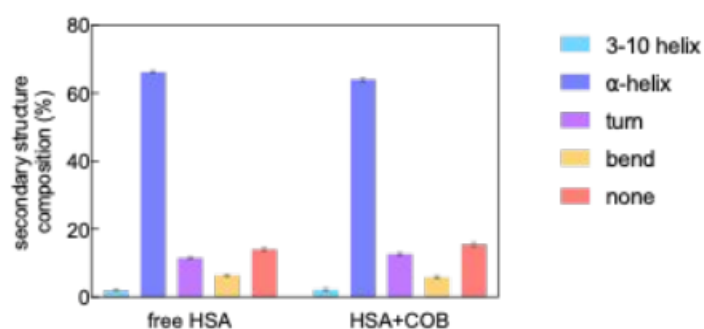


Figure S3. Secondary structure composition of HSA derived from the equilibrated cMD trajectory.

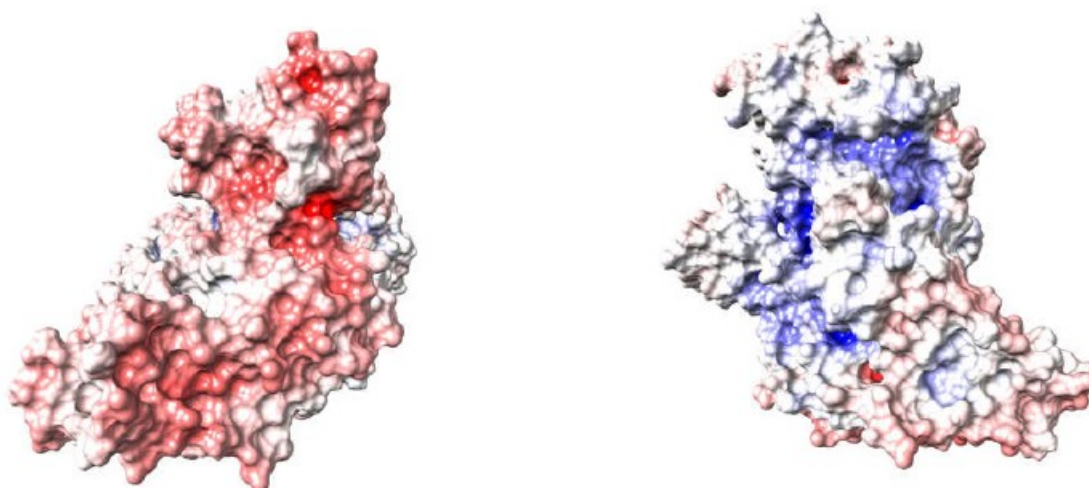


Figure S4. Solvent-accessible surface of HSA. HSA molecule is represented by its solvent-accessible surface, colored by the electrostatic potential. Areas predominantly negatively charged and positively charged (left and right panels, respectively) are shown.

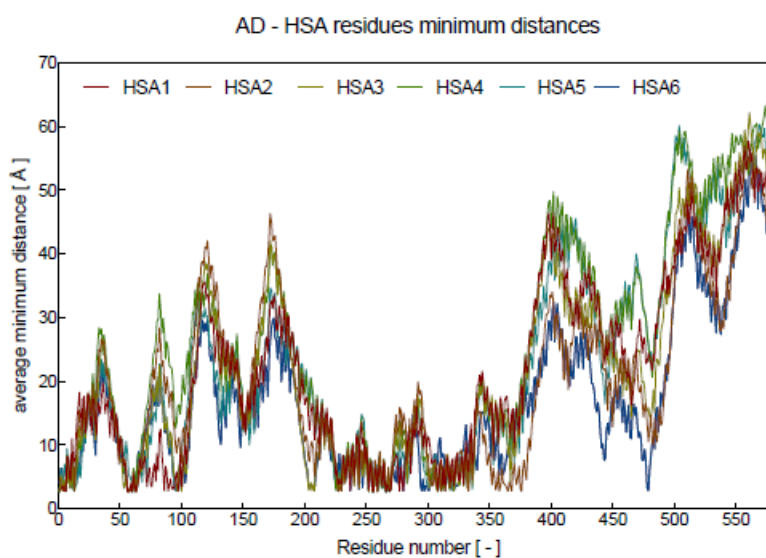


Figure S5. Average minimum distances. The average minimum distance between each aminoacid of each HSA and the micelle atoms.

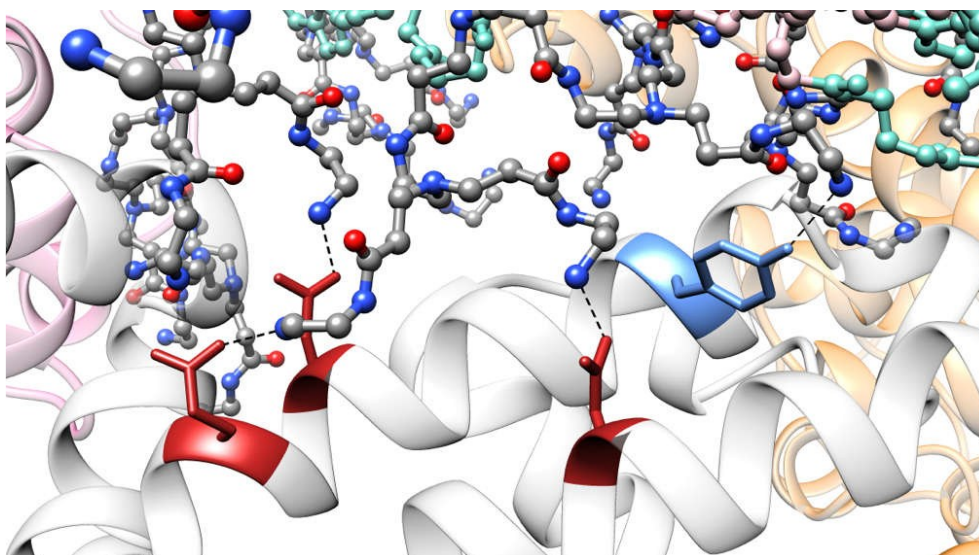


Figure S6. Representative interactions between AD and HSA. Salt bridges (SB) and H-bond (HB) are represented by black dotted line. The proteins are shown by colored ribbons while the AD NM is depicted in ball&stick style. The protein residues involved in SB or in HB are labelled and colored in firebrick or in cornflower blue, respectively. The AD head atoms are colored by element while the AD atoms belonging to linker and tail are colored according to the color scheme in Figure 4.3.1 of the Paragraph 4.3.1.

Table S1. The enthalpic free energy decomposition values, obtained from the MM/PBSA analysis, for each HSA residue. All data are expressed in kcal/mol.

RESIDUE NUMBER	HSA 1	HSA 2	HSA 3	HSA 4	HSA 5	HSA 6
1	-0.66	-0.85	-1.04	-1.57	-1.16	-0.51
2	-2.24	-0.95	-0.39	-1.89	-0.29	-0.31
3	-2.47	-3.14	-1.22	-2.56	-2.23	-0.36
4	-4	-2.12	-2.05	-1.33	-1.4	-1.74
5	-1.22	-1.54	-0.93	-0.61	-0.12	-0.82
6	0.04	-0.07	-0.44	-0.14	-0.45	-0.79
7	-0.11	-0.16	-0.13	-0.06	-0.05	-0.08
8	-0.1	-0.04	-0.53	-0.12	-0.06	-0.1
9	-0.63	-0.35	-1.19	-1.09	-0.43	-1.92
10	-0.52	-0.66	-1.83	-1.09	-0.8	-2.19
11	-0.04	-0.02	-0.1	-0.13	-0.22	-0.14
12	-0.14	-0.04	-0.93	-1.33	-2.03	-0.35
13	-0.09	0.2	-1.81	-1.88	-1.39	-0.52

14	-0.03	-0.06	-0.17	-0.26	-0.84	-0.12
15	-0.01	-0.01	-0.03	-0.2	-0.72	-0.09
16	-0.01	0	-0.02	-0.04	-0.25	-0.24
17	-0.01	0	-0.01	-0.01	-0.02	-0.47
18	-0.01	0	-0.02	-0.02	-0.28	-0.06
19	-0.02	-0.01	-0.03	-0.03	-0.05	-0.04
20	-0.01	0	-0.01	-0.01	-0.01	-0.06
21	0	0	0	0	-0.01	-0.01
22	-0.01	-0.01	-0.01	-0.01	-0.02	-0.02
23	-0.01	0	-0.01	0	-0.01	-0.01
24	0	0	0	0	0	-0.01
25	-0.01	0	0	0	0	-0.01
26	-0.01	0	-0.01	0	0	-0.01
27	-0.02	0	-0.01	0	-0.01	-0.01
28	0	0	0	0	0	0
29	-0.01	0	-0.01	0	0	-0.01
30	-0.02	-0.01	-0.02	0	0	-0.03
31	-0.01	0	0	0	0	0
32	0	0	0	0	0	0
33	0	0	0	0	0	0
34	0	0	0	0	0	0
35	0	0	0	0	0	0
36	0	0	0	0	0	0
37	0	0	0	0	0	0
38	0	0	0	0	0	0
39	-0.01	0	0	0	0	0
40	0	0	0	0	0	0
41	-0.01	0	0	0	0	0

42	-0.02	0	0	0	0	0
43	-0.01	0	0	0	0	-0.01
44	-0.01	0	0	0	0	-0.01
45	-0.03	0	-0.01	0	-0.01	-0.01
46	-0.02	-0.01	-0.01	-0.01	-0.01	-0.01
47	-0.01	0	-0.01	-0.01	-0.01	-0.02
48	-0.04	-0.01	-0.01	-0.01	-0.01	-0.01
49	-0.1	-0.07	-0.07	-0.02	-0.04	-0.04
50	-0.02	-0.01	-0.04	-0.02	-0.01	-0.02
51	-0.03	-0.01	-0.06	-0.05	-0.02	-0.09
52	-0.07	-0.03	-0.09	-0.05	-0.01	-0.04
53	-0.14	-0.12	-0.78	-0.13	-0.04	-0.18
54	-0.12	-0.04	-0.9	-0.32	-0.07	-0.19
55	-0.15	-0.05	-0.67	-0.52	-0.01	-0.35
56	0.14	-0.19	-0.43	-0.39	-0.04	-1.06
57	-1.38	-1.58	-2.09	-3.09	-0.13	-2.94
58	-3	-2.01	-1.55	-2.62	-0.59	-2.52
59	-1.24	-0.25	-1.16	-0.65	-0.43	-0.6
60	-3.4	-0.23	-0.5	-0.71	-1.34	-2.6
61	-0.5	-0.18	-0.39	-0.09	-0.26	-0.3
62	-0.97	-1.02	-0.73	-0.3	-0.3	-0.38
63	-1.51	-1.71	-1.53	-0.23	-1.26	-0.2
64	-2.05	-0.7	-1.6	-0.05	-1.72	-0.16
65	-0.32	-0.56	-0.6	-0.03	-0.48	-0.05
66	-0.1	-0.15	-0.11	-0.05	-0.07	-0.08
67	-0.17	-0.14	-0.47	-0.02	-0.12	-0.19
68	-0.32	-0.03	-0.2	-0.01	-1.21	-0.04
69	-0.16	-0.02	-0.06	-0.01	-0.15	-0.02

70	-0.06	-0.01	-0.03	-0.01	-0.03	-0.02
71	-0.14	0	-0.01	0	-0.03	-0.01
72	-2.12	0	-0.04	0	0.08	-0.01
73	-0.23	0	-0.01	0	-0.02	-0.01
74	-0.11	0	-0.01	0	-0.01	-0.01
75	-1.36	0	-0.01	0	-0.02	-0.01
76	-1.66	0	0	0	-0.01	0
77	-0.1	0	0	0	0	0
78	-0.1	0	0	0	0	0
79	-0.74	0	0	0	0	0
80	-0.04	0	0	0	0	0
81	-0.26	0	0	0	0	0
82	-0.01	0	0	0	0	0
83	-0.01	0	0	0	0	0
84	-0.04	0	0	0	0	0
85	-0.07	0	0	0	0	0
86	-0.04	0	-0.01	0	0	-0.01
87	-0.14	0	-0.01	0	0	-0.01
88	-0.25	0	0	0	0	0
89	-0.93	0	-0.01	0	-0.01	-0.01
90	-1.82	0	-0.02	0	-0.01	-0.03
91	-0.71	0	-0.01	0	-0.02	-0.01
92	-3.14	0	-0.01	0	-0.02	-0.02
93	-3.12	0	-0.09	0	-0.12	-0.24
94	-3.42	-0.01	-0.53	-0.01	-2.17	-1.1
95	-0.52	-0.02	-0.7	-0.01	-1.07	-0.54
96	-0.41	-0.11	-2.38	-0.01	-1.59	-2.41
97	-0.94	-0.02	-0.01	0	-0.04	-0.72

98	-1.12	-0.01	-0.13	0	-0.41	-0.13
99	-0.07	-0.09	-0.28	0	-0.04	-0.27
100	-0.03	-0.06	-1.23	0	-0.05	-1.31
101	-0.17	-0.01	-0.17	0	-0.01	-0.14
102	-0.08	-0.01	-0.06	0	-0.02	-0.04
103	-0.02	-0.14	-0.39	0	-0.01	-0.57
104	-0.09	-0.01	-0.29	0	-0.01	-0.25
105	-0.02	0	-0.02	0	0	-0.02
106	-0.01	-0.01	-0.02	0	0	-0.02
107	0	0	-0.01	0	0	0
108	0	0	0	0	0	0
109	0	0	0	0	0	0
110	0	0	0	0	0	0
111	0	0	0	0	0	0
112	0	0	0	0	0	0
113	0	0	0	0	0	0
114	0	0	0	0	0	0
115	0	0	0	0	0	0
116	0	0	0	0	0	0
117	0	0	0	0	0	0
118	0	0	0	0	0	0
119	0	0	0	0	0	0
120	0	0	0	0	0	0
121	0	0	0	0	0	0
122	0	0	0	0	0	0
123	0	0	0	0	0	0
124	0	0	0	0	0	0
125	0	0	0	0	0	0

126	0	0	0	0	0	0
127	0	0	0	0	0	0
128	0	0	0	0	0	0
129	0	0	0	0	0	0
130	0	0	0	0	0	-0.01
131	0	0	0	0	0	-0.03
132	0	0	0	0	0	-0.07
133	0	0	0	0	0	-0.01
134	0	0	0	0	0	0
135	0	0	0	0	0	-0.01
136	0	0	0	0	0	-0.01
137	0	0	0	0	0	0
138	0	0	0	0	0	0
139	0	0	0	0	0	0
140	0	0	0	0	0	0
141	0	0	0	0	0	0
142	0	0	0	0	0	0
143	0	0	0	0	0	0
144	0	0	0	0	0	0
145	0	0	0	0	0	0
146	0	0	0	0	0	0
147	0	0	0	0	0	-0.01
148	-0.01	-0.01	-0.01	0	0	-0.02
149	0	0	0	0	0	-0.01
150	-0.01	-0.01	-0.01	-0.01	-0.01	-0.02
151	0	0	0	0	0	-0.01
152	-0.01	-0.01	-0.01	-0.01	-0.01	-0.01
153	-0.01	0	-0.01	0	-0.01	-0.01

154	0	0	0	0	0	0
155	-0.01	0	-0.01	0	-0.01	-0.01
156	-0.01	0	0	0	-0.01	-0.01
157	0	0	0	0	0	0
158	0	0	0	0	0	0
159	0	0	0	0	-0.01	-0.02
160	0	0	0	0	0	0
161	0	0	0	0	0	0
162	0	0	0	0	0	-0.01
163	0	0	0	0	0	0
164	0	0	0	0	0	0
165	0	0	0	0	0	0
166	0	0	0	0	0	0
167	0	0	0	0	0	0
168	0	0	0	0	0	0
169	0	0	0	0	0	0
170	0	0	0	0	0	0
171	0	0	0	0	0	0
172	0	0	0	0	0	0
173	0	0	0	0	0	0
174	0	0	0	0	0	0
175	0	0	0	0	0	0
176	0	0	0	0	0	0
177	0	0	0	0	0	0
178	0	0	0	0	0	0
179	0	0	0	0	0	0
180	0	0	0	0	0	0
181	0	0	0	0	0	0

182	0	0	0	0	0	0
183	0	0	0	0	0	0
184	0	0	0	0	0	0
185	0	0	0	0	0	0
186	0	0	0	0	0	0
187	0	0	0	0	0	0
188	0	0	0	0	0	0
189	0	0	0	0	0	0
190	0	0	0	0	0	0
191	0	0	0	0	0	0
192	0	0	0	0	0	0
193	0	0	0	0	0	0
194	0	0	0	0	0	0
195	0	0	0	0	0	-0.01
196	0	-0.01	-0.01	0	0	-0.01
197	0	0	-0.01	0	0	-0.01
198	0	0	-0.01	0	0	-0.01
199	0	-0.01	-0.02	0	0	-0.02
200	0	-0.02	-0.04	0	0	-0.04
201	0	-0.01	-0.05	0	0	-0.06
202	0	-0.02	-0.08	0	0	-0.12
203	-0.01	-0.18	-0.43	-0.01	-0.01	-0.46
204	-0.01	-0.34	-1.45	0	0	-1.14
205	0	-0.03	-0.66	0	0	-1.29
206	0	-0.03	-0.37	0	0	-2.18
207	0	-0.05	-0.72	0	0	-1.09
208	-0.02	-0.21	-0.13	-0.01	-0.01	-0.19
209	-0.01	-0.17	-0.07	-0.01	-0.02	-0.75

210	0	-0.01	-0.02	0	0	-0.09
211	-0.01	-0.03	-0.04	-0.01	-0.01	-0.06
212	-0.02	-0.1	-0.09	-0.02	-0.02	-0.13
213	-0.01	-0.01	-0.01	-0.01	-0.01	-0.02
214	-0.01	-0.01	-0.01	-0.01	-0.01	-0.02
215	-0.01	-0.01	-0.01	-0.01	-0.01	-0.01
216	-0.02	-0.02	-0.04	-0.02	-0.02	-0.02
217	0	-0.01	-0.01	-0.01	-0.01	-0.01
218	-0.01	-0.01	-0.01	-0.01	-0.01	-0.02
219	-0.02	-0.02	-0.02	-0.02	-0.02	-0.03
220	-0.01	-0.02	-0.02	-0.02	-0.03	-0.02
221	-0.01	-0.01	-0.01	-0.01	-0.02	-0.04
222	-0.01	-0.01	-0.01	-0.01	-0.02	-0.05
223	-0.04	-0.02	-0.03	-0.06	-0.08	-0.08
224	-0.03	-0.03	-0.04	-0.07	-0.21	-0.18
225	-0.36	-0.09	-0.08	-1.08	-2.25	-2.09
226	-0.3	-0.17	-0.17	-0.62	-0.58	-0.13
227	-1.4	-1.77	-1.35	-2.11	-1.31	-0.58
228	-0.81	-1.1	-2.27	-0.52	-0.4	-0.12
229	-0.7	-1.05	-2.03	-0.86	-0.28	-0.24
230	-1.22	-0.25	-0.35	-1.18	-0.09	-0.7
231	-0.07	-0.11	-0.15	-0.09	-0.08	-0.07
232	-0.08	-0.13	-0.77	-0.08	-0.09	-0.13
233	-0.86	-1.31	-0.75	-0.52	-1.05	-1.03
234	-0.15	-0.09	-0.1	-0.11	-0.12	-0.15
235	-0.04	-0.04	-0.07	-0.03	-0.04	-0.06
236	-0.19	-0.19	-0.35	-0.05	-0.26	-0.24
237	0.01	-0.18	-0.37	-0.25	-0.14	-0.16

238	-0.04	-0.04	-0.06	-0.03	-0.03	-0.06
239	-0.03	-0.18	-0.09	-0.02	-0.02	-0.06
240	-0.62	-0.79	-1.4	-0.28	-0.32	-0.41
241	-0.06	-0.09	-0.15	-0.07	-0.04	-0.11
242	-0.02	-0.04	-0.05	-0.01	-0.01	-0.04
243	-0.03	-0.57	-0.3	-0.01	-0.01	-0.13
244	-0.16	-0.7	-0.91	-0.13	-0.07	-0.59
245	-0.01	-0.02	-0.03	-0.01	-0.01	-0.03
246	-0.01	-0.06	-0.04	0	0	-0.07
247	-0.03	-0.88	-0.73	-0.01	-0.01	-1.57
248	-0.01	-0.03	-0.02	0	0	-0.05
249	-0.08	-0.12	-0.26	-0.03	-0.02	-0.16
250	-0.02	-0.02	-0.03	-0.01	-0.01	-0.04
251	-0.07	-0.05	-0.07	-0.04	-0.05	-0.12
252	-0.82	-0.28	0.22	0.24	-0.33	-0.35
253	-0.06	-0.07	-0.09	-0.04	-0.04	-0.13
254	-0.05	-0.04	-0.08	-0.05	-0.05	-0.08
255	-0.99	-0.4	0.26	0.12	0.02	-0.97
256	-0.53	-0.44	-1.07	-0.36	0.06	-0.92
257	-0.1	-0.06	-0.12	-0.11	-0.08	-0.11
258	-0.15	-0.16	-0.62	-0.58	-0.16	-0.2
259	-0.53	-1.41	0.4	-0.35	-0.64	-1.72
260	-0.26	-0.1	-0.12	-0.19	-0.19	-0.3
261	-0.12	-0.03	-0.05	-0.05	-0.07	-0.09
262	-1.27	-0.26	-0.47	-0.54	-0.97	-1.04
263	-1.97	-1.92	-0.67	-1.06	-2.16	-3.08
264	-0.15	-0.07	-0.07	-0.1	-0.16	-0.2
265	-1.23	-0.05	-0.14	-0.14	-0.24	-0.55

266	-2.09	-0.98	-0.72	-0.93	-2.78	-3.24
267	-0.71	-1.41	-1.17	-1.71	-1.84	-2.41
268	-1.43	-0.09	-0.36	-0.31	-0.66	-1.8
269	-1.32	-0.3	-0.93	-1.92	-1.99	-1.81
270	-1.75	-0.49	-0.73	-2.88	-1.39	-1.21
271	-0.33	-0.11	-0.14	-0.52	-0.27	-0.25
272	-0.1	-0.02	-0.03	-0.07	-0.11	-0.17
273	-0.07	-0.01	-0.02	-0.03	-0.25	-0.51
274	-0.04	-0.01	-0.01	-0.02	-0.06	-0.42
275	-0.09	-0.01	-0.03	-0.03	-0.08	-0.17
276	-0.46	-0.01	-0.06	-0.02	-0.32	-1.29
277	-0.19	0	-0.01	0	-0.02	-0.1
278	-0.12	0	-0.01	-0.01	-0.02	-0.06
279	-1.05	-0.01	-0.04	-0.02	-0.08	-0.49
280	-1.78	0	-0.04	-0.01	-0.06	-0.64
281	-0.26	0	-0.01	-0.01	-0.05	-0.11
282	-0.07	0	-0.02	-0.01	-0.23	-0.07
283	-0.06	-0.07	-0.56	-0.5	-1.27	-0.17
284	-0.02	-0.01	-0.04	-0.03	-0.48	-0.04
285	-0.02	0	-0.01	-0.01	-0.05	-0.03
286	-0.15	-0.01	-0.04	-0.03	-0.12	-0.15
287	-0.02	-0.01	-0.02	-0.02	-0.03	-0.02
288	-0.01	0	-0.01	-0.01	-0.01	-0.01
289	-0.02	0	-0.01	0	-0.01	-0.02
290	-0.02	-0.01	-0.02	-0.02	-0.03	-0.04
291	0	0	0	-0.01	-0.01	-0.01
292	-0.01	0	0	0	-0.01	-0.01
293	-0.01	0	-0.01	-0.01	-0.01	-0.04

294	-0.01	0	0	0	-0.01	-0.4
295	0	0	0	-0.01	-0.02	-0.68
296	-0.02	-0.01	-0.01	-0.04	-0.1	-0.5
297	-0.01	-0.01	-0.01	-0.03	-0.38	-2.05
298	-0.01	-0.02	-0.02	-0.05	-0.5	-2.32
299	-0.03	-0.07	-0.05	-0.48	-1.28	-2
300	-0.01	-0.05	-0.15	-0.33	-1.07	-2.67
301	-0.01	-0.78	-0.28	-0.29	-1.1	-2.81
302	-0.04	-0.77	-0.13	-0.28	-1.12	-1.83
303	-0.22	-2.48	-0.23	-0.67	-0.9	-1.3
304	-0.05	-1.41	-0.12	-0.17	-0.12	-0.24
305	-0.06	-0.21	-0.1	-0.21	-0.11	-0.05
306	-0.03	-0.95	-0.09	-0.07	-0.03	-0.04
307	-0.18	-0.99	-0.89	-0.44	-0.1	-0.32
308	-1.08	-0.35	-0.29	-1.29	-0.23	-0.1
309	-0.55	-0.18	-0.3	-1.04	-1.76	-0.04
310	-0.04	-0.86	-0.19	-0.05	-0.16	-0.02
311	-0.04	-0.59	-1.38	-0.12	-0.98	-0.02
312	-0.67	-0.2	-1.18	-0.44	-1.02	-0.04
313	-0.1	-0.71	-1.17	-0.56	-0.78	-0.06
314	-0.44	-0.34	-0.6	-1.54	-1.24	-0.26
315	-0.33	-0.11	-0.14	-0.27	-0.17	-0.07
316	-0.04	-0.59	-0.05	-0.11	-0.1	-0.33
317	-0.3	-1.13	-0.49	-2.25	-1.78	-1.83
318	-1.85	-0.25	-1.66	-2.61	-1.2	-0.73
319	-0.09	-0.9	-0.15	-0.16	-0.17	-0.59
320	-0.05	-0.77	-0.24	-0.21	-0.42	-1.99
321	-0.47	-0.91	-0.95	-1.83	-2.58	-3.22

322	-0.66	-0.2	-0.46	-0.91	-0.49	-1.59
323	-0.07	-0.37	-0.13	-0.1	-0.18	-1.98
324	-0.04	-0.08	-0.09	-0.07	-0.04	-1.49
325	-0.77	-0.12	-1.15	-1.23	-0.12	-1.02
326	-1.12	-0.11	-0.78	-1.7	-0.36	-0.16
327	-0.06	-0.05	-0.06	-0.09	-0.03	-0.08
328	-0.04	-0.02	-0.07	-0.05	-0.02	-0.03
329	-1.43	-0.64	-2.09	-1.86	-0.98	-0.06
330	-0.06	-0.06	-0.08	-0.13	-0.1	-0.02
331	-0.02	-0.03	-0.03	-0.03	-0.03	-0.02
332	-0.13	-1.15	-0.78	-0.33	-0.61	-0.07
333	-0.05	0.2	-0.35	-0.52	-0.5	-0.05
334	-0.01	-0.04	-0.03	-0.03	-0.04	-0.02
335	-0.01	-0.01	-0.01	-0.01	-0.02	-0.02
336	-0.11	-0.48	-0.84	-0.3	-0.65	-0.29
337	-0.05	-0.1	-0.07	-0.17	-0.18	-0.17
338	0	-0.01	-0.01	-0.01	-0.02	-0.11
339	0	-0.01	-0.01	-0.01	-0.02	-0.14
340	0	0	0	0	-0.01	-0.2
341	0	-0.01	0	0	-0.01	-0.04
342	0	0	0	0	0	-0.01
343	0	0	0	0	0	-0.01
344	0	-0.01	0	0	0	-0.01
345	0	-0.01	0	0	0	-0.01
346	0	-0.01	-0.01	-0.01	-0.01	-0.01
347	0	-0.02	-0.01	0	0	-0.01
348	0	-0.08	0	0	0	-0.01
349	0	-0.06	0	0	-0.01	-0.01

350	0	-0.03	-0.01	-0.01	0	-0.01
351	0	-0.22	-0.01	0	0	-0.1
352	0	-1.26	0	0	0	-0.01
353	-0.03	-0.19	-0.03	-0.04	-0.05	-0.03
354	-0.01	-0.39	-0.01	-0.01	-0.01	-0.19
355	0	-2.36	0	0	0	-0.12
356	0	-2.2	-0.01	0	-0.01	-0.02
357	-0.02	-0.33	-0.02	-0.03	-0.02	-0.06
358	0	-3.43	-0.01	-0.01	-0.01	-0.16
359	0	-2.44	0	0	0	-0.07
360	0	-0.28	-0.01	-0.01	-0.01	-0.04
361	-0.01	-1.27	-0.01	-0.01	-0.02	-0.35
362	0	-1.41	0	0	0	-0.42
363	0	-0.58	0	0	-0.01	-0.16
364	0	-1.42	-0.01	0	-0.01	-0.2
365	0	-1.87	-0.04	0	-0.02	-0.05
366	0	-0.9	-0.02	-0.01	-0.02	-0.17
367	-0.01	-1.74	-0.69	-0.03	-0.08	-0.02
368	0	-0.28	-0.06	0	-0.01	-0.01
369	0	-0.26	-0.01	0	0	-0.01
370	-0.02	-0.22	-0.06	-0.03	-0.05	-0.03
371	0	-0.29	-0.07	0	-0.01	0
372	0	-1.37	-0.01	0	0	0
373	0	-0.58	-0.01	0	-0.01	-0.01
374	-0.01	-0.16	-0.02	-0.01	-0.03	-0.01
375	0	-0.03	0	0	0	0
376	0	-1.11	0	0	0	0
377	0	-0.18	-0.01	0	-0.01	-0.01

378	0	-0.06	0	0	0	0
379	0	-0.2	0	0	0	0
380	0	-0.48	0	0	0	0
381	0	-0.01	0	0	0	-0.01
382	0	-0.01	0	0	0	0
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474	0	0	0	0	0	-0.04
475	0	-0.01	0	0	0	-0.06
476	0	0	0	0	0	-0.01
477	0	0	0	0	0	-0.04
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479	0	-0.03	0	0	0	-0.52
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481	0	-0.01	0	0	0	-0.06
482	0	-0.02	0	0	0	-0.02
483	0	-0.02	0	0	0	-0.01
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583	0	0	0	0	0	0
584	0	0	0	0	0	0
585	0	0	0	0	0	0



Figure S7. HSA molecule is represented by its solvent-accessible surface, colored by the electrostatic potential as computed by Delphi. Areas predominantly negatively charged and positively charged (left and right panels, respectively) are shown.

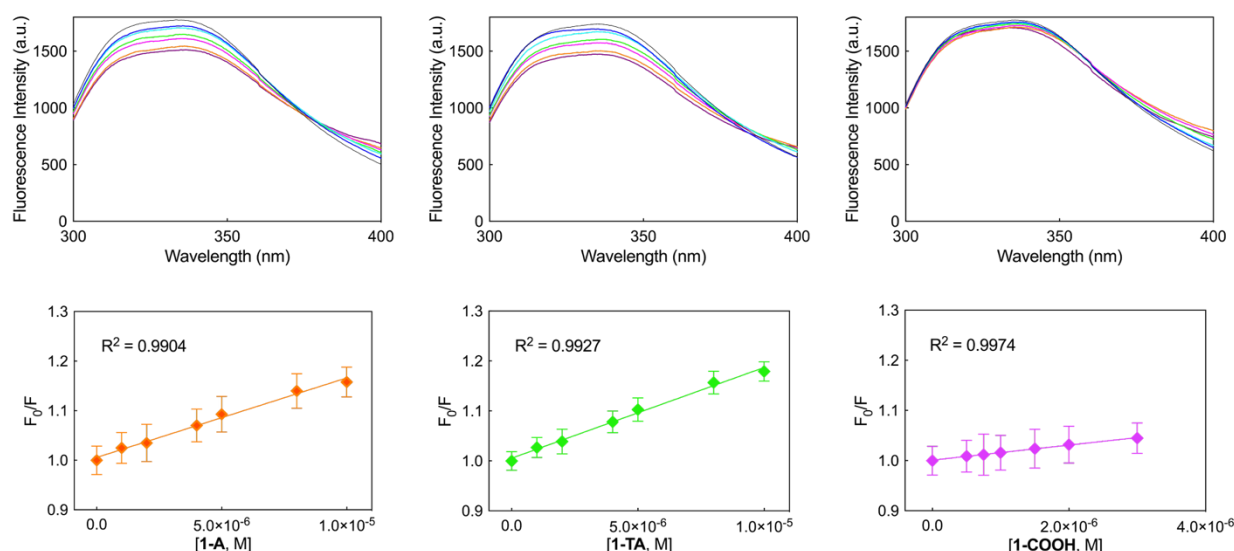


Figure S8. (Upper panels) Fluorescence emission spectra recorded for the HSA in complex with **1-A** (left), **1-TA** (middle), and **1-COOH** (right). The spectra include measurements for free HSA (2 μ M) and HSA at 2 μ M with varying concentrations of monomers, as follows: 1 μ M, 2 μ M, 4 μ M, 5 μ M, 8 μ M and 10 μ M for **1-A** and **1-TA**; 0.5 μ M, 0.75 μ M, 1 μ M, 1.5 μ M, 2 μ M and 3 μ M for **1-COOH**. (Lower panels) Stern-Volmer plot illustrating the fluorescence quenching of HSA induced by the different AD-PAMAM monomers.

Monomeric state	Temperature (K)	HSA/1-A	HSA/1-TA	HSA/1-COOH
K_{SV} [$10^4 M^{-1}$]	304	1.69 ± 0.26	1.99 ± 0.18	1.61 ± 0.34
	310	1.79 ± 0.28	2.12 ± 0.17	1.93 ± 0.38
Micellar state	Temperature (K)	HSA/1@-A	HSA/1@-TA	HSA/1@-COOH
K_{SV} [$10^4 M^{-1}$]	304	3.32 ± 0.24	3.49 ± 0.34	2.82 ± 0.27
	310	3.17 ± 0.21	3.15 ± 0.29	2.52 ± 0.22

Table S2. Stern-Volmer constants (K_{SV}) for HSA fluorescence quenching in the presence of different AD-PAMAM derivatives at 304 K and 310 K. Reported values represent the mean $\pm$ standard deviation from three independent experiments.

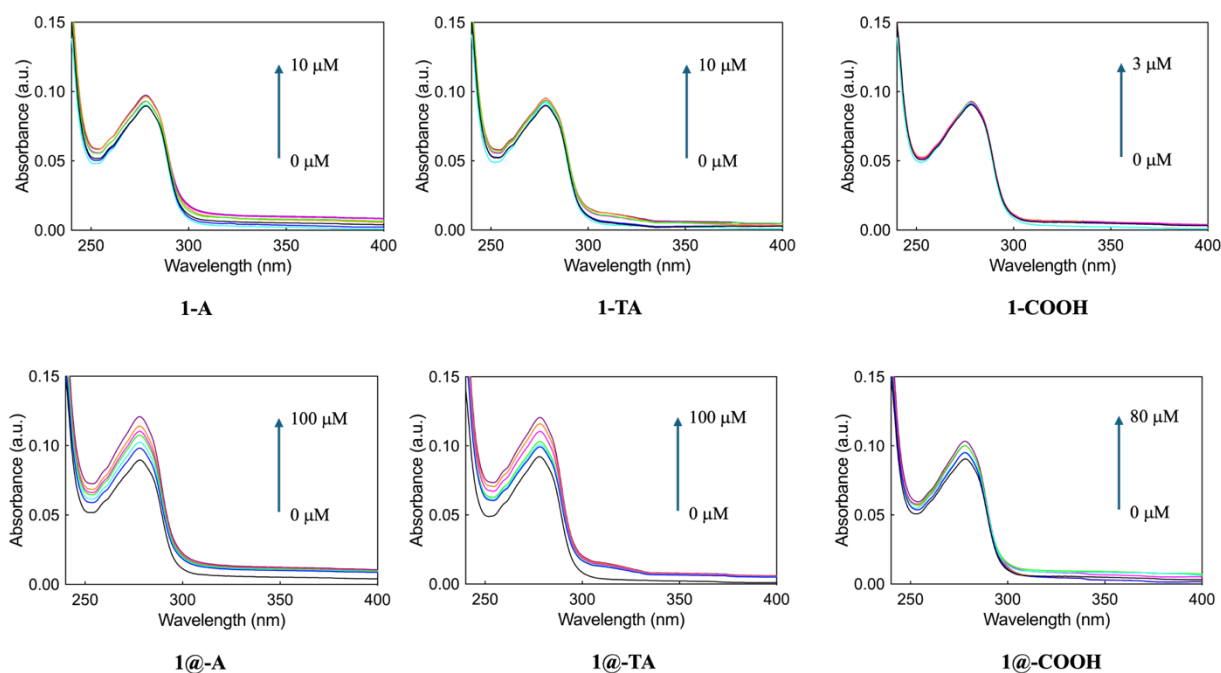


Figure S9. (Upper panels) UV absorbance spectra recorded for the HSA in complex with **1-A** (left), **1-TA** (middle), and **1-COOH** (right). The spectra include measurements for free HSA (2 μ M) and HSA at 2 μ M with varying concentrations of monomers, as follows: 1 μ M, 2 μ M, 4 μ M, 5 μ M, 8 μ M and 10 μ M for **1-A** and **1-TA**; 0.5 μ M, 0.75 μ M, 1 μ M, 1.5 μ M, 2 μ M and 3 μ M for **1-COOH**. **(Lower panels)** UV absorbance spectra recorded for the HSA in complex with **1@-A** (left), **1@-TA** (middle), and **1@-COOH** (right). The spectra include measurements for free HSA (2 μ M) and HSA at 2 μ M with varying concentrations of micelles, as follows: 20, 30, 40, 50, 80 and 100 μ M for **1@-A** and **1@-TA**, and 10, 20, 40, 50, 60 and 80 μ M for **1@-COOH**.

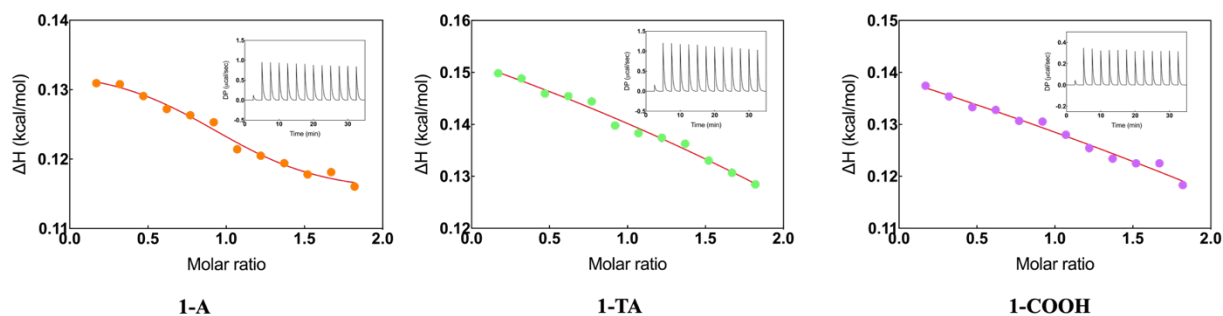


Figure S10. Integrated heat curves and corresponding raw thermogram (insets) from ITC experiments of HSA titrated with **1-A**, **1-TA**, and **1-COOH**.

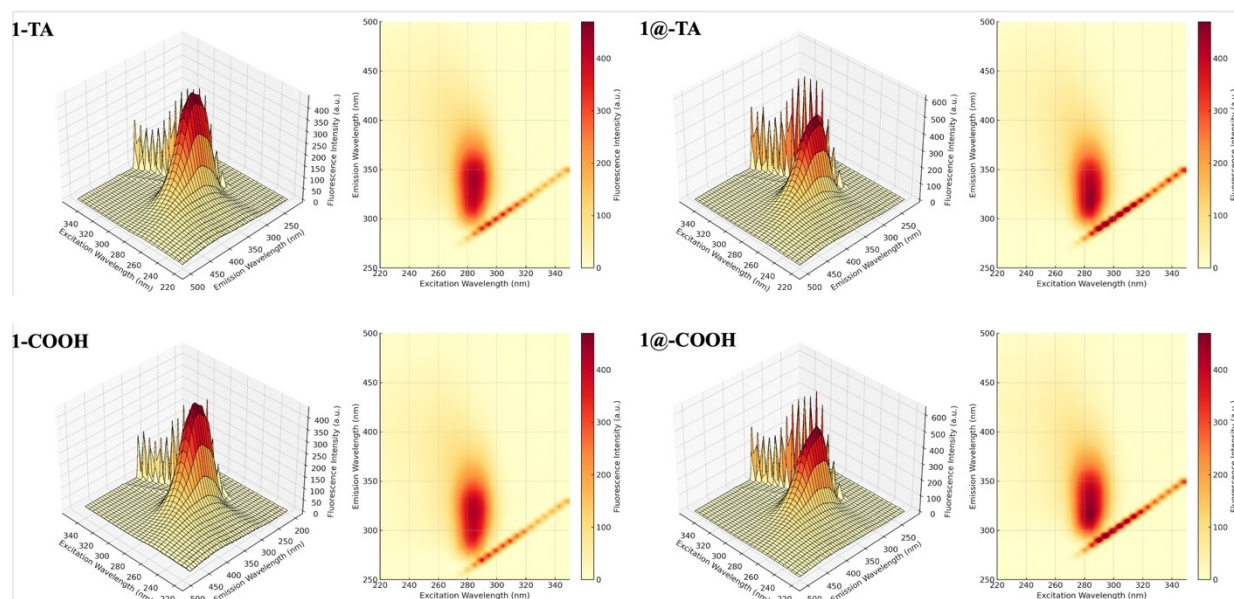


Figure S11. Three-dimensional fluorescence spectra (**left panels**) and corresponding two-dimensional contour maps (**right panels**) of 2 mM HSA in presence of the amphiphilic AD-PAMAM derivatives in its monomeric (**1-TA**, 2 mM, upper row; **1-COOH**, 2 mM, upper row) and nanomicellar (**1-TA@**, 50 mM, upper row; **1-COOH@**, 50 mM, lower row) forms.

Monomeric state	Site marker	HSA/1-A	HSA/1-TA	HSA/1-COOH
$K_{SV} [10^4 M^{-1}]$	none	1.61 ± 0.23	1.82 ± 0.15	1.52 ± 0.31
	Warfarin	1.23 ± 0.24	1.38 ± 0.19	1.55 ± 0.23
	Stearic Acid	1.58 ± 0.27	1.87 ± 0.20	0.99 ± 0.26

Table S3. Stern-Volmer constants (K_{SV}) for HSA fluorescence quenching of the different HSA/monomers systems obtained at 298 K in the absence and presence of the selective site markers Warfarin and Stearic Acid. Reported values represent the mean $\pm$ standard deviation from three independent experiments.

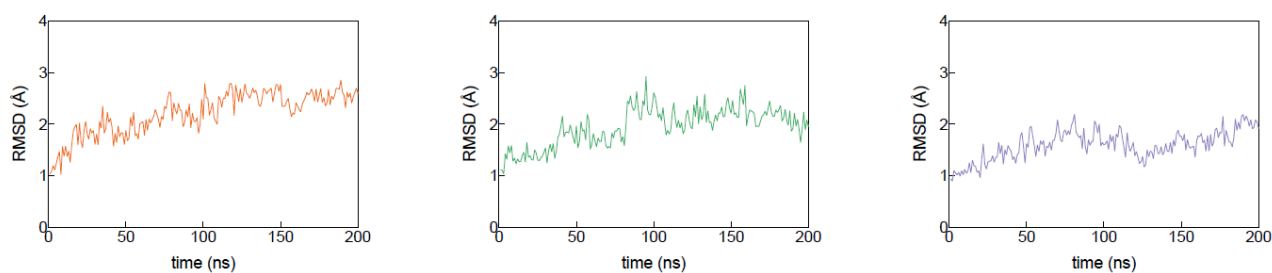


Figure S12. Root mean square deviation (RMSD) of HSA backbone atoms during unrestrained MD simulations (from left to right, HSA in complex with **1-A**, **1-TA**, and **1-COOH**).

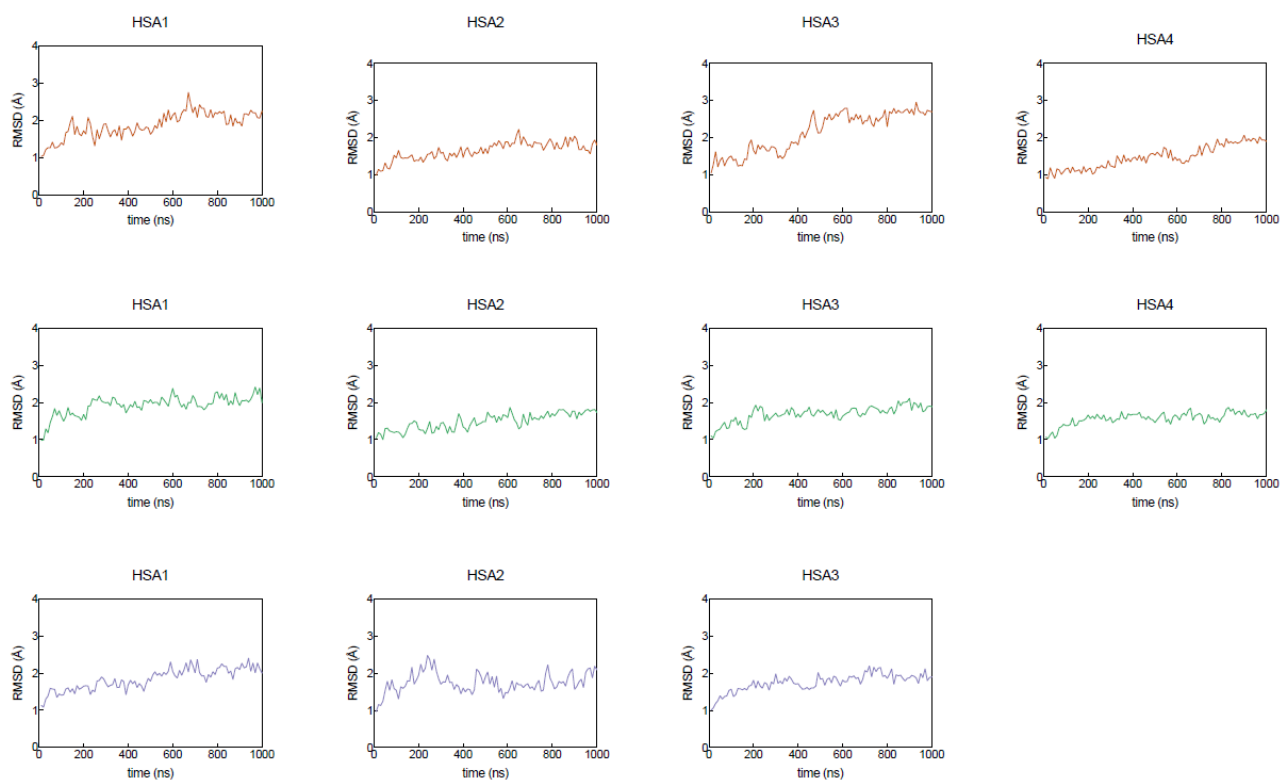


Figure S13. Root mean square deviation (RMSD) of HSA backbone atoms during unrestrained MD simulations (from top to bottom, HSAs in complex with **1@-A**, **1@-TA**, and **1@-COOH**).

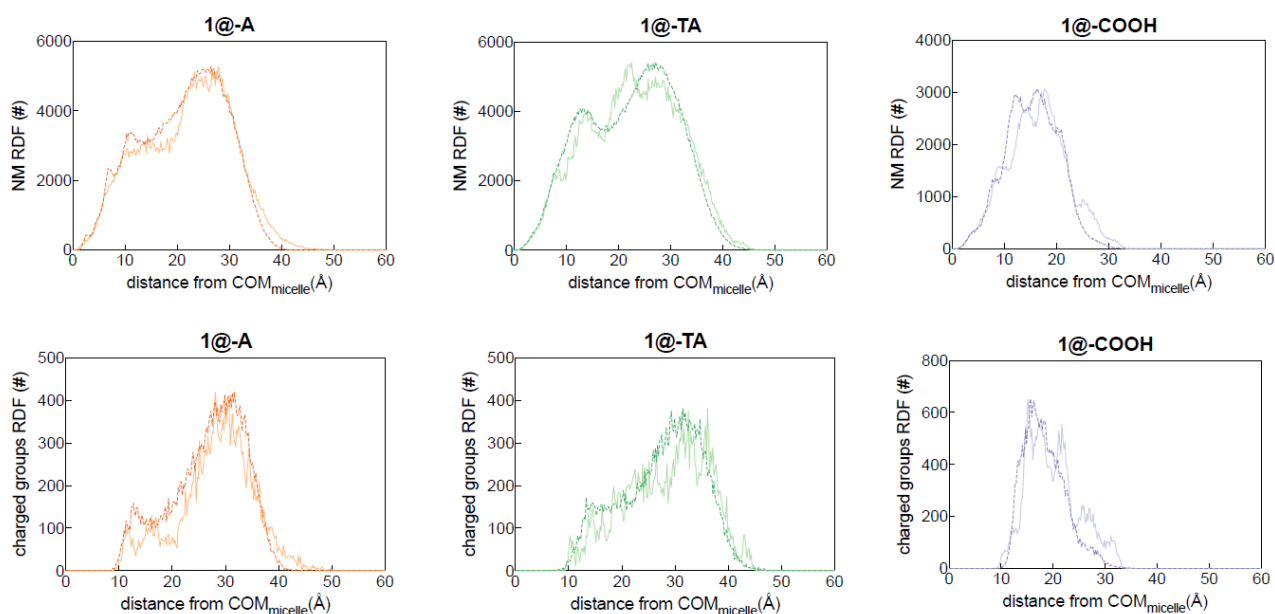


Figure S14. Top panels: radial distribution of the atoms of the dendrimer around the micelles' center of mass. Bottom panels: radial distribution of the atoms of the terminal branches of each dendrimer, around the dendrimers' center of mass. Color code: free **1@-A** orange continuous lines, **1@-A** in complex with HSA light orange dashed lines; free **1@-TA** green continuous lines, **1@-TA** in complex with HSA light green dashed lines; free **1@-COOH** purple continuous lines, **1@-COOH** in complex with HSA light purple dashed lines.

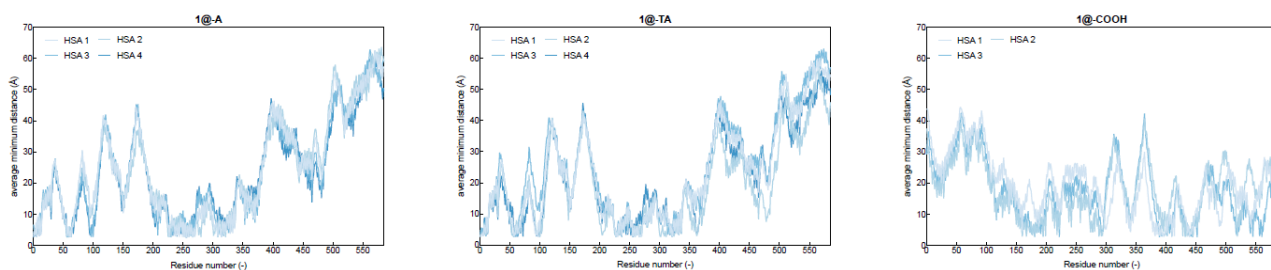


Figure S15. Average minimum distance between each amino acid of each HSA and the micelles' atoms (**1@-A** left panel, **1@-TA** central panel, and **1@-COOH** right panel).

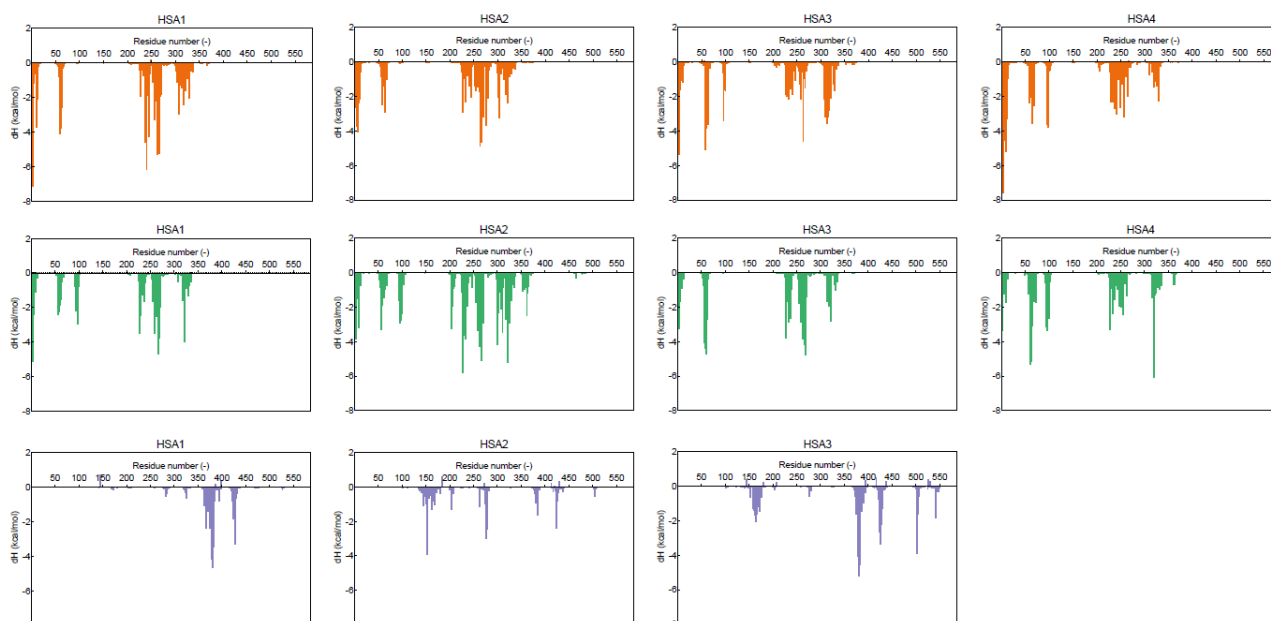


Figure S16. Per residue decomposition of the binding enthalpy between the micelle and each HSA molecule, as computed by MMGBSA. Top panels **1@-A**, middle panels **1@-TA**, and bottom panels **1@-COOH**.

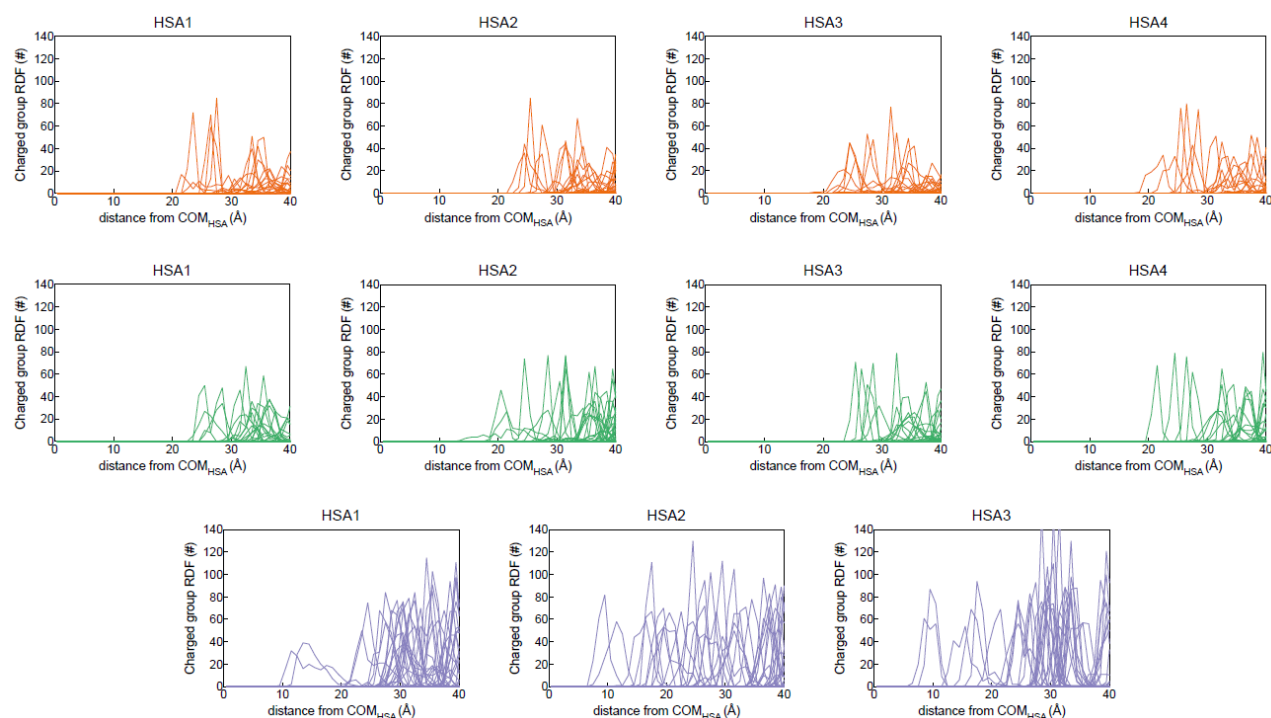


Figure S17. Radial distribution of the terminal charged atoms of each micelle around the center of mass of each HSA molecule. Top panels **1@-A**, middle panels **1@-TA**, and bottom panels **1@-COOH**.

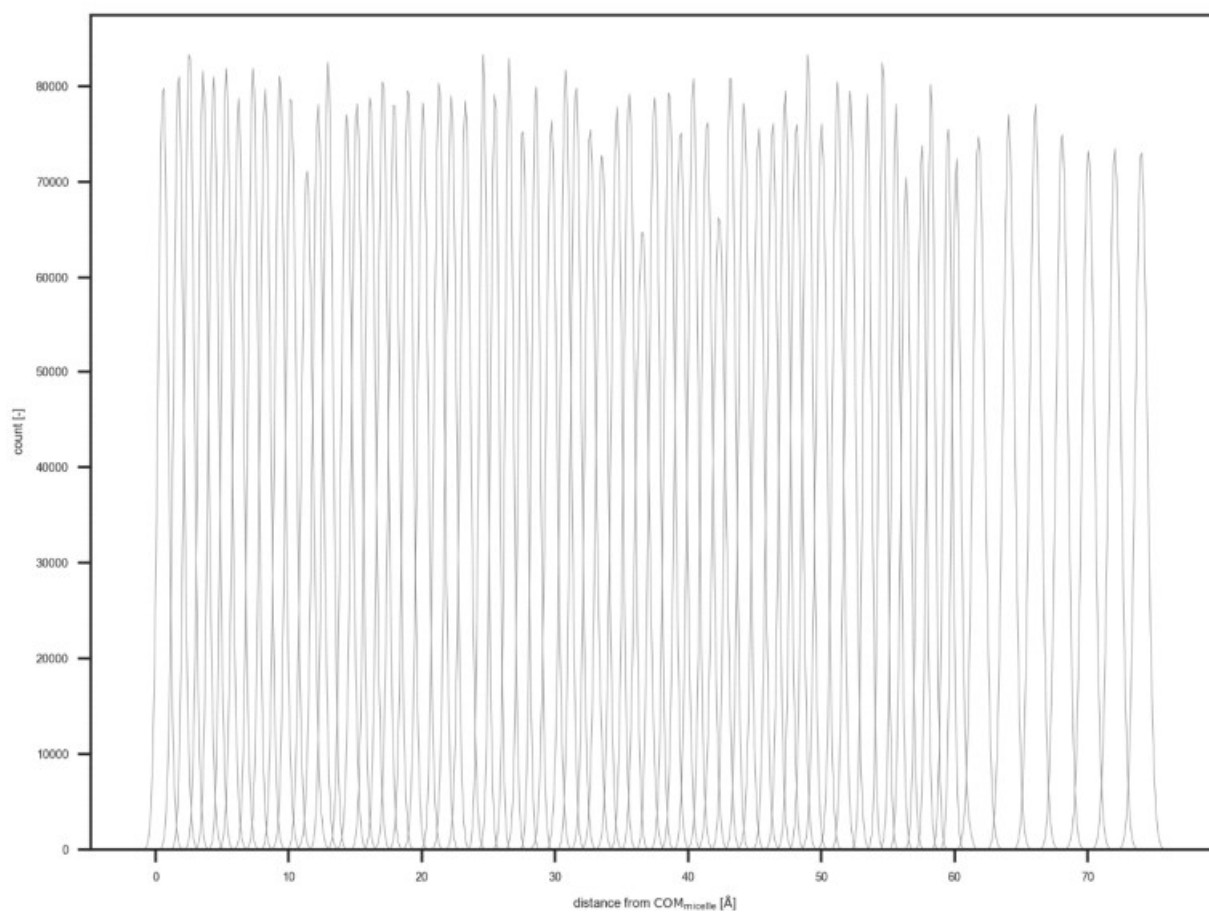


Figure S18. Histograms of the configurations within the umbrella sampling windows

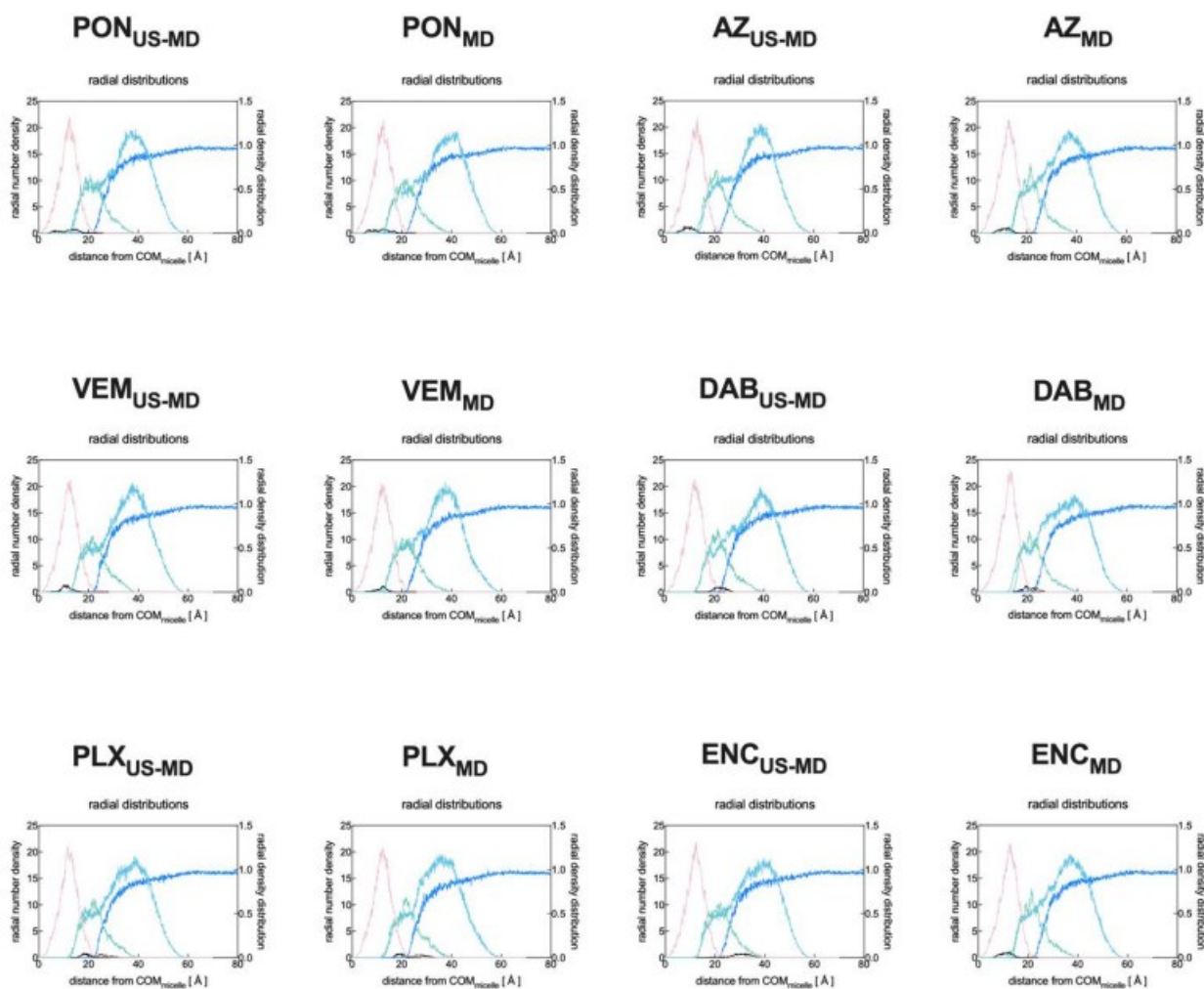


Figure S19. Radial distribution function of the hydrophobic tails (pink), linkers (aquamarine), polar heads (deep sky blue), drug (black), and water molecules (blue) of each drug/micelle complex from Umbrella Sampling Molecular Dynamics (US-MD) and classic Molecular Dynamics (MD) simulations.

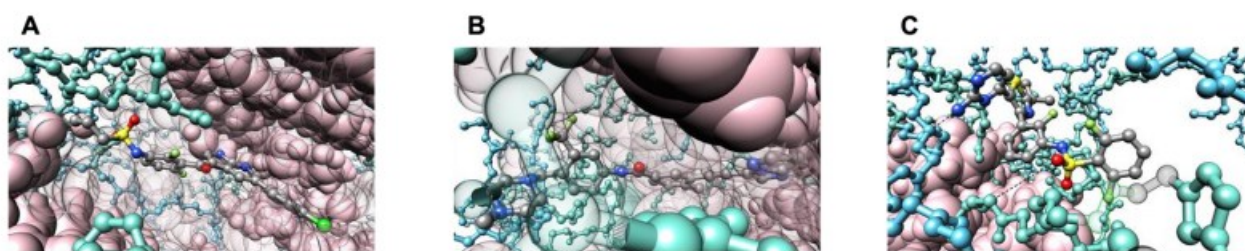


Figure S20. Atomistic snapshot extracted from the equilibrated MD simulations of AD micelle in complex with VEM (POS 1; A), PON (POS 1; B), and DAB (POS 2; C). The drugs are depicted in ball&stick style and colored by element. The atoms of the AD hydrophobic tails are represented as spheres while the AD linkers and polar heads are shown as ball&stick (see Figure 4.4.2 caption for the color code). Water molecules and counterions are not shown for clarity

Drug	Simulation	Water [#]	Cl- [#]	Atoms [#]	Equilibrated volume [$\text{\AA}^3$]	Data collection [μs]
AZ	random	78,613	136	243,465	2,482,408	5.0
	US	57,024	136	178,698	1,829,183	3.4
DAB	random	78,611	136	243,453	2,483,020	5.0
	US	56,666	136	177,618	1,820,434	3.4
ENC	random	78,761	136	243,911	2,485,844	5.0
	US	56,667	136	177,629	1,818,359	3.4
PLX	random	78,608	136	243,448	2,481,278	5.0
	US	56,667	136	177,625	1,818,469	3.4
PON	random	78,602	136	243,437	2,480,817	5.0
	US	56,663	136	177,620	1,816,291	3.4
VEM	random	78,607	136	243,437	2,479,421	5.0
	US	56,666	136	177,614	1,818,990	3.4

Table S4. Description of simulation details

POS 1					
System	ΔE_{vdW} (kJ/mol)	ΔE_{ele} (kJ/mol)	ΔE_{solv} (kJ/mol)	ΔH (kJ/mol)	-TAS (kJ/mol)
AD/PON	-78.07	-2.43	7.32	-73.18	31.86
AD/VEM	-76.61	-1.99	5.39	-73.21	34.18
AD/AZ	-75.83	-2.69	6.75	-71.77	32.98
AD/DAB	-63.81	-1.44	8.68	-56.57	22.94
AD/PLX	-59.48	-2.34	7.34	-54.48	24.28
AD/ENC	-46.90	-3.45	5.98	-44.37	23.30

POS 2					
System	ΔE_{vdW} (kJ/mol)	ΔE_{ele} (kJ/mol)	ΔE_{solv} (kJ/mol)	ΔH (kJ/mol)	-TAS (kJ/mol)
AD/PON	-42.15	-15.77	9.35	-48.57	18.46
AD/VEM	-45.49	-14.82	10.21	-50.10	19.09
AD/AZ	-46.91	-14.58	10.03	-51.46	20.92
AD/DAB	-43.45	-47.16	11.34	-79.27	38.86
AD/PLX	-42.18	-43.78	10.23	-75.73	35.74
AD/ENC	-36.21	-42.39	8.32	-70.28	33.30

POS 3					
System	ΔE_{vdW} (kJ/mol)	ΔE_{ele} (kJ/mol)	ΔE_{solv} (kJ/mol)	ΔH (kJ/mol)	-TAS (kJ/mol)
AD/PON	-27.27	-25.45	22.07	-30.65	5.74
AD/VEM	-28.38	-26.43	20.12	-34.69	9.82
AD/AZ	-29.02	-23.31	24.43	-27.9	2.87
AD/DAB	-33.45	-33.16	19.32	-47.29	15.72
AD/PLX	-32.37	-40.18	22.21	-50.34	15.26
AD/ENC	-39.25	-57.33	19.86	-76.72	36.16

Table S5. Thermodynamics parameters (see details in the main text) obtain from MM/PBSA analysis for each drug/NM system. All errors are less than 10% of the value.

System	POS 1	POS 2		POS 3	
	# apolar contacts	# polar contacts	# hydrogen bonds	# polar contacts	# hydrogen bonds
AD/PON	249 ± 16	12 ± 3	1 ± 1	12 ± 2	1 ± 1
AD/VEM	252 ± 18	17 ± 5	1 ± 1	15 ± 3	1 ± 1
AD/AZ	239 ± 22	18 ± 5	1 ± 1	19 ± 5	1 ± 1
AD/DAB	165 ± 12	50 ± 6	4 ± 1	46 ± 6	2 ± 2
AD/PLX	161 ± 14	49 ± 6	4 ± 1	46 ± 8	2 ± 1
AD/ENC	118 ± 7	43 ± 9	3 ± 2	110 ± 15	5 ± 2

Table S6. Averaged number of apolar contacts (POS 1), polar contacts and hydrogen bonds (POS 2 and POS 3) detected during the entire MD simulation for each drug/NM complex.

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