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Preparation, functionalization and characterization of engineered carbon nanodots

Luka Đorđević[1,4*], Francesca Arcudi[1,5*] and Maurizio Prato[1,2,3*]

Carbon-based dots (CDs) and their functionalized (nano)composites have recently attracted attention due to their seemingly easy preparation and numerous potential applications, ranging from those in the biomedical field (i.e., imaging and drug delivery) to those in (opto)electronics (i.e., solar cells and LEDs). This protocol details step-by-step procedures for synthesis, purification, functionalization and characterization of nitrogen-doped carbon nanodots (NCNDs), which we have been preparing for the past few years. First, we describe the bottom-up synthesis of NCNDs, starting with the use of molecular precursors (arginine (Arg) and ethylenediamine (EDA)) and making use of microwave-assisted hydrothermal heating. We also provide guidelines for the purification of these materials, through either dialysis or low-pressure size-exclusion chromatography (SEC). Second, we outline post-functionalization procedures for the surface modification of NCNDs, such as alkylation and amidation reactions. Third, we provide instructions for the preparation of NCNDs with different properties, such as color emission, electrochemistry and chirality. Given the fast evolution of preparations and applications of CDs, issues that might arise from artifacts, errors and impurities should be avoided. In this context, the present protocol aims to provide details and guidelines for the synthesis of high-quality nanomaterials with high reproducibility, for various applications. Furthermore, specific needs might require the CDs to be prepared by different synthetic procedures and/or from different molecular precursors, but such CDs can still benefit from the purification and characterization procedures outlined in this protocol. The sample preparation takes various time frames, ranging from 4 to 18 d, depending on the adopted synthesis and purification steps.

Introduction

Nanotechnology, the manipulation of matter with at least 1D between 1 and 100 nm, has produced a multitude of nanomaterials and multiple applications[1–4]. The specific physical and chemical properties allow nanomaterials to be exploited in a variety of research areas and commercial products, ranging from electronics to medicine. Carbon, an element of many faces[5], is an example of how size, shape, composition, surface topology, chemistry and morphology are pivotal for the development and application of nanomaterials and nano-based devices[6,7]. In recent years, CDs have emerged because of interest in their properties, which are different but equally appealing with respect to other carbon nanomaterials (such as carbon nanotubes and graphene)[8–14].

CDs are considered quasi-spherical nanoparticles, with size <10 nm, comprising carbon, oxygen and hydrogen atoms. In addition to their apparently harmless, abundant and inexpensive nature, they have shown intriguing fluorescence properties, which have brought them the label of 'carbon nanolights'[8] and have sparked numerous applications[14–23]. CDs can be prepared via two main routes: top-down and bottom-up approaches[24,25]. Whereas the former consists mainly of cutting down larger carbon nanostructures (such as carbon nanotubes and graphene), the latter route has shown the real potential of CDs by including numerous synthetic methods, as well as a number of small molecules as starting materials. For example, cheap precursors and accessible protocols can be used in a straightforward way, as well as a way to teach nanotechnology and fluorescence techniques at the high-school and undergraduate levels[26,27].

It is, however, this widespread access to starting materials and procedures, as well as the apparently easy preparation, that may have caused inconsistencies, such as those in their structural classification and fluorescence properties. These need to be overcome for the CDs to truly shine as next-generation nanomaterials and to compete with inorganic-based quantum dots. In addition, the literature

[1]Department of Chemical and Pharmaceutical Sciences, INSTM UdR Trieste, University of Trieste, Trieste, Italy. [2]Carbon Nanobiotechnology Laboratory, CIC biomaGUNE, Donostia-San Sebastián, Spain. [3]Basque Foundation for Science, Ikerbasque, Bilbao, Spain. [4]Present address: Simpson Querrey Institute, Northwestern University, Chicago, IL, USA. [5]Present address: Department of Chemistry, Northwestern University, Evanston, IL, USA. *e-mail: dordevic.luka@gmail.com; francescarcudi@gmail.com; prato@units.it

presents critical discrepancies in defining CDs, because they should be classified depending on the presence of amorphous or crystalline structures, as well as their fluorescence origin[28]. The family of CDs embraces three main types, namely carbon nanodots (CNDs), carbon quantum dots (CQDs) and graphene quantum dots (GQDs). Amorphous carbon-based nanoparticles without quantum confinement are called CNDs, whereas crystalline structures and quantum effects are present in spherical CQDs or in single-layer GQDs. Categorizing CDs into one of these types can still pose a challenge, especially in cases where fluorescence has contributions from both the structure and molecular-like excited states. Therefore, it might be easier to refer to all quasi-spherical carbon nanoparticles (which include those with quantum effects or molecular-like behavior and all those that have varying contributions from both) as CDs and categorize them on the basis of characteristic structural elements: graphitic (*g*-CDs) and amorphous (*a*-CDs)[9,11]. In addition, as in the case of the heteroatom-doped nanoparticles reported herein, this information can be included by an additional prefix, such as in *a*-N-CDs, which stands for nitrogen-doped amorphous carbon nanoparticles.

Inconsistencies in the literature on CDs may also be due to vague (and/or sometimes inadequate) synthetic protocols, as well as unsatisfactory (or lacking) purification and characterization procedures[29]. Finally, aggregation of the carbonaceous materials and lack of size control and uniformity are common issues for both the top-down and bottom-up procedures and ultimately affect the quality of the prepared materials.

In recent years, we have worked on the synthesis and purification of amorphous nitrogen-doped carbon nanoparticles (*a*-N-CDs, also referred to as NCNDs)[30,31], the modification of their (chiro) optical[32,33] and electrochemical properties[34], and the preparation of (non)covalent hybrids[35–37] and (nano)composite materials[38,39]. This work is based on our original procedure, which uses a two-component approach, together with a fast microwave-assisted hydrothermal synthesis[30]. More specifically, by using Arg and EDA, it is possible to prepare NCNDs that feature an amorphous core and an amino-rich surface. The two-component approach, given the different reactivity of the components, results in nanoparticles with the core originating from Arg, whereas the surface emerges from EDA. The prepared CNDs are small, with a rather homogeneous size distribution (2.47 ± 0.84 nm), and show blue fluorescence (fluorescence quantum yield, $\phi_f = 17\%$) after proper purification. In addition to dialysis, NCNDs can be purified by using SEC, which yields three distinct fractions of NCNDs that feature different sizes, surface functional groups and fluorescence properties (e.g., the smallest fraction has the highest ϕ_f at 46%). We have managed to expand this procedure in order to not only modulate and tailor the properties of NCNDs to exhibit different fluorescence[33], confer chirality[32], tune their band gap[34] and prepare donor–acceptor (supra)molecular assemblies[32,37], but also exploit the amino-rich surface to prepare covalent hybrids[35,36] and composites with clay[38] or ionogels[39].

Overview of the procedure

Part 1 of the Procedure covers the synthesis (Steps 1–9) and purification of NCNDs using either dialysis (Step 10A) or SEC (Step 10B). Beyond purification, the characterization procedures (Step 11) of CDs are in need of uniformity, and therefore we propose proper UV–visible (UV-VIS) absorption (Step 11A) and fluorescence emission (Step 11B) protocols as characterization procedures. Quantification of reactive groups on the surface is also pivotal for (post)functionalization reactions. In this protocol, given the abundant presence of amines, we have used the colorimetric ninhydrin-based Kaiser test (Step 11C). For other types of CDs, such as those rich in carboxylic acids, which can be prepared from precursors such as citric acid, conductometric titrations can be used. Therefore, quantification measurements should be tailored to the specific need and CD surface. In addition, atomic force microscopy (AFM) characterization (Step 11D) can provide useful information about the CD size (height) and the uniformity of the sample, which cannot be always easily determined with transmission electron microscopy (TEM) experiments. In cases in which the determination of energy levels for NCNDs is important and when the CDs are tailored to specific tasks, as in energy-related applications, electrochemical characterization is a critical characterization technique (Step 11E). Another tunable property of NCNDs that we describe in this protocol is chirality; consequently, we detail chiroptical characterization with circular dichroism (Step 11F).

We further describe, in part 2 (Steps 12 and 13), the post-synthetic modification/functionalization reactions that utilize the reactive amino-rich surface and then discuss the quality control tools of the resulting modified NCND materials. Finally, in Box 1, we illustrate how our synthetic reaction can also be used for the preparation of NCNDs with tunable properties (optical, chiroptical and electrochemical).

Box 1 | Preparation and purification of NCND derivatives

1 Various NCND derivatives can be prepared and purified by following Steps 1–10 of the main procedure and varying the precursors. As an example, we outline how to tune the electrochemical (option A), emission (option B) or chiroptical (option C) properties of NCNDs. These properties can be modified by using quinone, chromophore or chiral precursors in the reaction mixture.

(A) **Tuning the electrochemical properties of NCNDs ● Timing 3–4 d**

(i) Weigh 87.0 mg of powdered Arg into a microwave vessel.

(ii) Add 45.5 mg of powdered 2,3-dimethoxy-5-methyl-*p*-benzoquinone to the microwave vessel.

(iii) Add a stir bar to the reaction vessel.

(iv) Add 100.0 μL of Milli-Q water with a micropipette.

▲ **CRITICAL STEP** Water should be added to the bottom of the vessel. Avoid touching the walls of the vessel with the pipette tip. If water is placed incorrectly, the microwave heating will not result in the preparation of NCNDs with the anticipated properties.

(v) Add 33.0 μL of EDA with a micropipette.

! **CAUTION** This step should be carried out in a fume hood while wearing gloves, goggles and a lab coat. Dispose of the micropipette tip in the appropriate waste container.

▲ **CRITICAL STEP** EDA should be added to the bottom of the vessel. Avoid touching the walls of the vessel with the pipette tip. If EDA is placed incorrectly, the microwave heating will not result in the preparation of NCNDs with the anticipated properties.

(vi) Close the vessel with a microwave cap.

! **CAUTION** Vessels and caps are designed for single use and should not be used more than once.

(vii) Stir briefly (30 s) on a magnetic stirrer (300 r.p.m.) to mix the reagents.

(viii) Place the capped vessel inside the attenuator of the microwave reactor and use the following method: power cycling (12 cycles total; total time 180 s) of 200 W, 15-s power intervals, 5-s cooling intervals, with 250 °C as maximum temperature and 240 °C as minimum temperature (Supplementary Fig. 3).

? **TROUBLESHOOTING**

(ix) Wet the filter membrane with Milli-Q water (using a wash bottle).

(x) Remove the cap from the vessel and add ~2 mL of Milli-Q water to the dark amber reaction mixture inside the microwave vessel, shake gently and filter the mixture. Rinse the vessel (~2 mL Milli-Q water) and filter again (twice or until all the materials are collected).

! **CAUTION** The cap should be removed from the vessel in a fume hood, because gases are formed during the reaction.

? **TROUBLESHOOTING**

■ **PAUSE POINT** The obtained mixture can be stored at −20 °C for 1 week without obvious changes in properties.

(xi) Dialyze against ultrapure water as outlined in Step 10A and finally lyophilize to obtain NCND derivative as powdered material (63.4 mg).

(B) **Tuning the emission properties of NCNDs ● Timing 3–4 d**

(i) Weigh 20.0 mg of powdered Arg into a microwave vessel.

(ii) Add 43.0 mg of powdered Br_2NDA to the microwave vessel.

(iii) Add a stir bar to the reaction vessel.

(iv) Add 260.0 μL of Milli-Q water with a micropipette.

▲ **CRITICAL STEP** Water should be added to the bottom of the vessel. Avoid touching the walls of the vessel with the pipette tip. If water is placed incorrectly, the microwave heating will not result in the preparation of NCNDs with the anticipated properties.

(v) Add 4.0 μL of EDA with a micropipette.

! **CAUTION** This step should be carried out in a fume hood, while wearing gloves, goggles and a lab coat. Dispose of the tip in the appropriate waste container.

▲ **CRITICAL STEP** EDA should be added to the bottom of the vessel. Avoid touching the walls of the vessel with the pipette tip. If EDA is placed incorrectly, the microwave heating will not result in the preparation of NCNDs with the anticipated properties.

(vi) Close the vessel with a microwave cap.

! **CAUTION** Vessels and caps are designed for single use and should not be used more than once.

(vii) Stir briefly (30 s) on a magnetic stirrer (300 r.p.m.) to mix the reagents.

(viii) Place the capped vessel inside into the attenuator of the microwave reactor and use the following method: power cycling (18 cycles, total time 200 s) of 300 W, 15-s power intervals, 5-s cooling intervals, with 230 °C as maximum temperature and 220 °C as minimum temperature (Supplementary Fig. 4).

(ix) Wet the filter membrane with Milli-Q water (using a wash bottle).

(x) Remove the cap from the vessel and add ~2 mL of Milli-Q water to the dark-red reaction mixture inside the microwave vessel, shake gently and filter the mixture. Rinse the vessel (~2 mL Milli-Q water) and filter again.

! **CAUTION** The cap should be removed from the vessel under a fume hood, because gases are formed during the reaction.

(xi) The pH of the filtrate is then adjusted to 7.2 (by using a 0.2 M aqueous HCl solution) and the solution is filtered again.

■ **PAUSE POINT** The obtained mixture can be stored at −20 °C for 1 week without obvious changes in properties.

(xii) Dialyze against ultrapure water as outlined in Step 10A and finally lyophilize to obtain the NCND derivative as a powdered material (16.5 mg).

(C) **Tuning the chiroptical properties of NCNDs ● Timing 3–4 d**

(i) Weigh 87.0 mg of powdered Arg into a microwave vessel.

(ii) Add 57.0 mg of powdered (*R,R*)- or (*S,S*)-CHDA to the microwave vessel.

▲ **CRITICAL STEP** CHDAs are light-yellow solids that are hygroscopic. The weighing should be done quickly and under moisture-free conditions.

(iii) Add a stir bar to the reaction vessel.

(iv) Add 100.0 μL of Milli-Q water with a micropipette.

▲ **CRITICAL STEP** Water should be added to the bottom of the vessel. Avoid touching the walls of the vessel with the pipette tip. If water is placed incorrectly, the microwave heating will not result in the preparation of NCNDs with the anticipated properties.

(v) Close the vessel with a microwave cap.

! **CAUTION** Vessels and caps are designed for single use and should not be used more than once.

(vi) Stir briefly (30 s) on a magnetic stirrer (300 r.p.m.) to mix the reagents.

(vii) Place the capped vessel inside the attenuator of the microwave reactor and use the following method: power cycling (12 cycles, total time 180 s) of 200 W, 15-s power intervals, 5-s cooling intervals, with 250 °C as maximum temperature and 240 °C as minimum temperature (Supplementary Fig. 5).

Box 1 | Preparation and purification of NCND derivatives (Continued)

(viii) Wet the filter membrane with Milli-Q water (using a wash bottle).

(ix) Remove the cap from the vessel and add ~2 mL of Milli-Q water to the dark amber reaction mixture inside the microwave vessel, shake gently and filter the mixture. Rinse the vessel (~2 mL Milli-Q water) and filter again.

! CAUTION The cap should be removed from the vessel under a fume hood, because gases are formed during the reaction.

■ PAUSE POINT The obtained mixture can be stored at −20 °C for 1 week without obvious changes in properties.

(x) Dialyze against ultrapure water as outlined in Step 10A and finally lyophilize to obtain the NCND derivative as a powdered material (NCND-*R*: 20.5 mg; NCND-*S*: 22.0 mg).

Advantages and limitations

The hydrothermal microwave-assisted synthesis of NCNDs detailed in this protocol has several advantages. The synthesis uses starting materials that are commercially available, cheap and abundant precursors, and the solvent used is water. By using a laboratory microwave reactor, the conditions (temperature and pressure) can be easily controlled (or fine-tuned), and the synthesis is highly reproducible. The reaction time is short (only 3 min), and therefore the power consumption for the synthesis of NCNDs is limited. The synthesized NCNDs possess high solubility in water and apparently lack toxicity[40]. The multi-component approach allows the use of various starting materials and tuning of the properties of the NCNDs. This approach can also be extended to different core (and surface) precursors, for example, citric acid instead of Arg[32]. Solvents other than water can be used for the reaction, and therefore solvothermal syntheses can also be performed.

The purification involves either dialysis or SEC. Dialysis is performed against Milli-Q water over a period of 48 h, and the obtained NCND solution is lyophilized for at least 24 h. The solid obtained after lyophilization is highly hygroscopic and requires storage under inert gas or in a desiccator. The dialysis procedure reported herein is optimized specifically for the purification of NCNDs obtained by this protocol. The molecular weight cut-off (MWCO) of the dialysis membrane and the step-by-step process (in terms of time and water replacement), however, could require some optimization for the specific nanomaterial, as has been reported and suggested recently[29]. SEC is performed using Sephadex LH-20 resin, which can be costly and requires the use of methanol (MeOH; instead of water, in order to speed up the procedure), which is a toxic and flammable liquid. SEC purification, however, can be quite versatile in terms of medium type, particle and pore sizes, column size and solvent, and therefore can be used for various types of CDs, not only the NCNDs reported here[41].

The synthesis is fairly scalable. Our usual scale-up procedure includes a total of eight syntheses, the products of which are then filtered (over the same filtration membrane), collected and divided into four dialysis membranes containing 10 mL each (a higher amount of mixture in one dialysis membrane results in incomplete purification), which therefore require four stirrers and easy access to Milli-Q water. Obtaining gram-scale quantities could therefore be time consuming or require further optimization.

Alternative methods

The synthesis described here is one of several routes toward CDs. As mentioned, the two main routes are top-down and bottom-up. The top-down route consists mainly of using arc-discharge, laser ablation and (electro)chemical oxidation to cut up larger carbon nanostructures, such as soot, graphite and carbon nanotubes[42–44]. Chemical oxidation of nanocarbons is a common procedure that involves the use of oxidizing acids[45]. Even coal can be used as a starting material, but the procedure requires fuming sulfuric or nitric acid and heat treatment. Electrochemistry offers milder oxidation conditions and easy manipulation of synthetic parameters. In particular, water oxidation can be exploited for the formation of reactive intermediates that can then oxidize graphite[46].

Bottom-up routes utilize monomeric (or polymeric precursor) starting materials, which can undergo dehydration and dehydrogenation reactions under hydro- or solvothermal conditions or thermal carbonization. The most commonly used starting materials are amino acids, citric acid or carbohydrates[47–50], which are usually thermally treated above their decomposition temperatures. Generally, using temperatures <300 °C will result in amorphous CDs, whereas heating to >300 °C will produce graphitized CDs, unless π-conjugated precursors are used[11]. Solvothermal and hydrothermal syntheses are usually carried out in (polytetrafluoroethylene-lined) autoclaves or microwave reactors, which are then heated in ovens or microwaves, respectively[51,52]. The starting material(s), solvent, temperature and reaction time can be optimized to obtain CDs with different surface composition/

properties, such as fluorescence, size and crystallinity[53–57]. In addition, adding a second precursor is a common route to improve the photophysical properties of CDs, because it allows surface passivation and/or heteroatom doping during the synthesis[58]. In addition, the electrochemical procedure in the bottom-up route has been successfully applied for the carbonization of starting materials (such as alcohols)[59].

Alternative purification methods include centrifugation and/or ultrafiltration, but these were usually found to be insufficient to obtain purified CDs[29]. In addition to gel-filtration chromatography, CDs—depending on their size and surface chemistry—can be separated by gel electrophoresis, ion-exchange chromatography or normal- or reversed-phase chromatography[11,60].

In addition to the characterization methods described in this protocol, several alternative approaches can be used. For complete characterization, high-resolution TEM can yield useful structural information, including morphology, size and crystallinity, but CNDs are usually small nanoparticles that tend to aggregate on the grid and provide (too) low contrast; therefore, TEM is not the best technique for morphological characterization[11]. Structural characterization techniques should also include X-ray photoelectron spectroscopy and Fourier transform infrared spectroscopy[61]. Further structural information can be gathered from NMR spectroscopy, especially ^{13}C-NMR, but such techniques usually require high concentrations, long acquisition times and/or ^{13}C-enriched precursors[30]. Fluorescence quantum yields, instead of relative quantum yields, can also be reported as absolute ϕ_f, but this requires use of an integrating sphere. Another useful experimental technique is zeta potential measurement, which yields information about the electrostatic potential at the electrical double layer surrounding the CDs in solution and supramolecular complexation events. An alternative to electrochemical characterization and determination of conduction band and valence bands of CDs and their (nano)composites, can be performed through a combination of UV photo-electron spectroscopy and optical band gap measurement[62].

Applications

The procedure we describe could be particularly useful for researchers interested in or working in the field of CDs, or nanotechnology in general. The main goal of this article is to guide researchers with a reliable and standard set of protocols for the hydrothermal synthesis of tailored NCNDs, as well as the purification procedures and characterization steps needed for the preparation of high-quality nanomaterials. We hope that our protocols will aid in the utilization and understanding of NCND properties, as well as help to eliminate errors and artifacts that have occurred during the fast and intense research of recent years[29,63–66]. We expect our purification and quality control instructions to also be used by researchers who are preparing other types of CDs, including CQDs and GQDs, as well as their hybrids or composite materials. For example, NCNDs prepared through this route can be purified using SEC, which can be performed in a variety of solvents and therefore can be adapted to the solubility of CD-based nanoparticles[67].

The multitude of starting materials and synthetic procedures yielded a variety of CDs that differ in, for example, size, crystallinity and surface chemistry. As an emerging and diverse class of fluorescent nanomaterials, CDs have found applications in the biomedical and energy-related fields, which we have also targeted[33–35,40]. The fluorescence emissions and tunable surfaces of CDs have been exploited in both in vitro and in vivo bio-applications, which include cell/tissue imaging, photodynamic therapy, photoacoustic imaging and drug delivery[68–76]. The most promising applications related to the energy field include the use of CDs as phosphors for LEDs[77], photocatalytic energy conversion[78–80], solar photovoltaics[81], and other energy-conversion and storage-oriented applications[82].

Experimental design

This protocol presents a straightforward synthetic strategy to prepare NCNDs through a bottom-up approach, starting from commercial and low-cost precursors. Arg and EDA are used as both carbon and nitrogen sources, allowing simultaneous nitrogen doping and surface passivation during the synthetic step. NCNDs result from processes of condensation, polymerization and aromatization from the thermal carbonization of the precursors. We reported that, whereas Arg is mainly responsible for the NCND core, EDA leads to the formation of the outer shell of the CNDs[30,32,35]. Our procedure involves the use of water (as reaction medium) and microwave-assisted conditions. We propose a useful synthetic approach that avoids long reaction times and has been successfully applied as an effective and user-friendly method for the preparation of various materials[52], not only NCNDs. It has been already reported that the heating parameters, such as the heating time, can affect the size of the

nanoparticles and their photophysical properties[83,84]. However, many of the reported microwave syntheses are carried out using domestic microwave ovens[66], in which the reaction conditions are difficult to control, in terms of temperature or power, and therefore may suffer in terms of reproducibility. We use a microwave reactor, applying a constant power and a narrow temperature interval during the course of the reaction (3 min)[30]. The microwave parameters are optimized to obtain nanoparticles with an average size of ~2.5 nm, and the uniform carbonization process limits the formation of aggregates, which can be removed through a simple filtration step, followed by appropriate purification procedures, such as dialysis and SEC, to remove small-size by-products. The produced NCNDs possess high solubility in water and in common polar organic solvents, as well as fluorescence emission in the blue region of the visible spectrum. Moreover, the surface, rich in primary (or secondary) amino groups, can be used for further functionalization reactions and makes the CNDs water-soluble carriers for hydrophobic molecules[36,40]. Alternatively, the amino groups can be easily modified to tertiary amines or other functional groups for specific purposes[35].

Finally, our synthetic strategy can be extended in order to modify the properties of the NCNDs. By using Arg, EDA and naphthalene dianhydride derivatives, in appropriate ratios, as precursors it is possible to modulate the fluorescence emission, affording CNDs that emit light across the entire visible spectrum[33]. Alternatively, by using Arg and EDA, together with quinones as starting molecules, a redox library of NCNDs can be prepared[34]. In addition, we describe the preparation of chiral NCNDs by using a chiral diamine as a precursor that is able to transfer its chirality to the nanoparticle under the relatively harsh conditions used[32].

Part 1: preparation and purification of NCNDs

CNDs are synthesized in this protocol with a multi-component approach that includes hydrothermal microwave-assisted heating of Arg and EDA. NCNDs can be synthesized at a relatively large scale with fairly easy and inexpensive methods. Removing large carbon aggregates or particles, as well as small by-products, from the synthesis is pivotal for obtaining high-quality nanomaterials. We describe how dialysis or SEC can be used for the purification of NCNDs. Finally, the nanomaterial is obtained as a powder after lyophilization.

It is important to check the quality of the prepared and purified NCNDs on the basis of various characterization approaches, such as measurement of UV-VIS (Step 11A) and fluorescence (Step 11B) spectra, the Kaiser test (Step 11C) and AFM (Step 11D). UV-VIS spectra of NCNDs in water should be recorded as a quality check, given that the UV peak absorbance (286 nm, with the absorption onset starting at 388 nm) and the mass absorption coefficient (~3.7 (g^{-1} L) cm^{-1} at 286 nm) indicates whether the synthesis and purification have been performed correctly. Broad fluorescence emission and excitation dependence are common phenomena observed for NCNDs. The fluorescence peaks of NCNDs in water shift from 356 to 474 nm when the excitation wavelength changes from 300 to 420 nm, and the fluorescence intensity decreases as the peak red-shifts. The Kaiser test is used to estimate the amount of (primary and secondary) amino groups present on the surface of the NCNDs; a value of ~1,350 μmol g^{-1} is expected when the nanoparticles have been synthesized, purified and lyophilized correctly. Finally, microscopy (AFM) is used to confirm the quasi-spherical shape, the small size (2.47 nm), and the uniform size distribution (±0.84 nm).

Part 2: post-functionalization of NCNDs

The amino groups present on the surface of the NCNDs can be further modified to target specific applications. In part 2 of this protocol, we outline how to transform primary (and secondary) amino groups into tertiary ones, which we have exploited for electrochemiluminescence purposes.[35] We also describe an amidation reaction between NCND amino groups and molecules bearing carboxylic acid moieties. We use 5,10,15-tri(4-*tert*-butylphenyl)-20-(4-carboxyphenyl)porphyrin as an example of a dye for the preparation of donor–acceptor hybrids.[36] However, this procedure can also be applied for the preparation of various nanohybrids, including covalent attachment of luminophores[35] or biologically active molecules[40].

The successful modification or covalent attachment of molecules is confirmed by performing the same quality control steps as for the pristine materials, which include measurement of UV-VIS (Step 11A) and fluorescence (Step 11B) spectra, the Kaiser test (Step 11C) and AFM (Step 11D).

Preparation of NCNDs' derivatives and purification

By using the multi-component approach, it is possible to endow CNDs with different properties, if appropriate precursors are used. In Box 1, we outline how to tune the emission properties of NCNDs

by using chromophore precursors (such as naphthalene dianhydride derivatives) that can react in the microwave-assisted hydrothermal conditions, together with Arg and EDA (step 1A). Then we show how to confer chirality upon the NCNDs by using a chiral diamine (instead of EDA) as surface precursor (step 1B). In addition, the energy levels of the NCNDs can also be tuned to produce nanomaterials with desired energy levels for specific applications—thus improving their photocatalytic performance—by incorporating quinones into their structures (step 1C). Purification is performed through filtration and dialysis.

The quality of the NCND derivatives is checked by using the aforementioned characterization approaches, such as measurement of UV-VIS (Step 11A) and fluorescence (Step 11B) spectra, the Kaiser test (Step 11C) and AFM (Step 11D). Furthermore, depending on the targeted property, additional characterization techniques can be used. When energy levels of NCNDs are modified, it is appropriate to record cyclic voltammograms (Step 11E). On the other hand, when chiral NCNDs are prepared, they can be characterized by recording their circular dichroism spectra (Step 11F).

Materials

Reagents

! CAUTION Use all reagents and solvents in a well-ventilated area away from heat sources, dust, fumes, gases, vapors and sprays. All the flammable, corrosive, toxic, carcinogenic, irritant and harmful reagents and solvents listed below should be handled in a fume hood. Wear protective clothes, protective gloves, eye protection and face protection **▲ CRITICAL** Reagents and solvents are used as purchased, without degassing or drying. All reagents are stored per manufacturer's instructions and used without further purification.

Preparation of NCNDs (part 1)

- Arginine (Arg; 98%; Fluorochem, cat. no. M03558). Store between 0 and 8 °C.
- Ethylenediamine (EDA; ≥99%; Sigma-Aldrich, cat. no. E26266). Store tightly closed in a ventilated place. **! CAUTION** EDA is flammable, toxic and harmful upon ingestion, inhalation or skin contact.
- Milli-Q water. We use bi-distilled water that is fed to the Milli-Q ultra-pure water system, producing Milli-Q water with 18.2 MΩ × cm resistivity.
- Methanol (MeOH; ≥99%; Sigma-Aldrich, cat. no. 34860). Store tightly closed in a cool and ventilated place. **! CAUTION** Methanol is highly flammable in liquid and vapor form. It is toxic if inhaled or swallowed and if it comes into contact with the skin. It causes damage to the organs through prolonged or repeated exposure.
- Ethanol (EtOH; ≥99.8%; Sigma-Aldrich, cat. no. 32205). Store tightly closed in a cool and ventilated place. **! CAUTION** Ethanol is highly flammable in liquid and vapor form. It causes severe eye irritation.

Post-functionalization of NCNDs (part 2)

- *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC·HCl, ≥99.0%; Sigma-Aldrich, cat. no. 03449). Store at −20 °C and under an inert atmosphere. **! CAUTION** This compound is an irritant.
- 4-(Dimethylamino)pyridine (DMAP; ≥99%; Sigma-Aldrich, cat. no. 107700). Store tightly closed in a ventilated place. **! CAUTION** DMAP is irritant and harmful.
- *N,N*-dimethylformamide (DMF; anhydrous, 99.8%; Sigma-Aldrich, cat. no. 227056). Store under an inert atmosphere in a ventilated place. **! CAUTION** DMF is flammable, irritant and toxic.
- Paraformaldehyde (95%; Sigma-Aldrich, cat. no. 158127). Store between 0 and 8 °C. **! CAUTION** Paraformaldehyde is carcinogenic, flammable, irritant and harmful.
- Formic acid (≥95%; Sigma-Aldrich, cat. no. F0507). Store in a cool and ventilated place. **! CAUTION** Formic acid is flammable, corrosive and toxic.
- 4-*tert*-Butylbenzaldehyde (97%; Sigma-Aldrich, cat. no. 384038). Store tightly closed in a ventilated place. **! CAUTION** 4-*tert*-Butylbenzaldehyde is toxic, harmful and may cause an allergic reaction.
- Methyl 4-formylbenzoate (99%; Sigma-Aldrich, cat. no. 244740). Store tightly closed in a ventilated place.
- Pyrrole (98%; Sigma-Aldrich, cat. no. 131709). Store under inert gas, between 0 and 8 °C. **! CAUTION** Pyrrole is flammable, toxic, harmful and irritant.
- Boron trifluoride diethyl etherate (BF_3·OEt_2; ≥46.5% BF_3 basis; Sigma-Aldrich, cat. no. 216607). Store under inert gas, between 0 and 8 °C. **! CAUTION** BF_3·OEt_2 is flammable, toxic and corrosive.

- *p*-Chloranil (tetrachloro-1,4-benzoquinone; 99%; Sigma-Aldrich, cat. no. 232017). Store tightly closed in a ventilated place. **! CAUTION** *p*-Chloranil is irritant.
- Triethylamine (Et_3N; ≥99%; Sigma-Aldrich, cat. no. T0886). Store tightly closed in a ventilated place. **! CAUTION** Triethylamine is flammable, harmful and corrosive.
- Potassium hydroxide (KOH; ≥85%; Sigma-Aldrich, cat. no. 221473). Store tightly closed in a ventilated place. **! CAUTION** KOH is corrosive and harmful.
- Chloroform ($CHCl_3$; ≥99%, contains EtOH as a stabilizer, Sigma-Aldrich, cat. no. 288306). Store tightly closed in a ventilated place. **! CAUTION** Chloroform is harmful, toxic, irritant and a suspected carcinogen.
- Sodium hydrogen carbonate ($NaHCO_3$; ≥99.7%; Sigma-Aldrich, cat. no. 31437). Store tightly closed. **! CAUTION** $NaHCO_3$ is irritant.
- Dichloromethane (CH_2Cl_2; ≥99.9%; Sigma-Aldrich, cat. no. 32222). Store tightly closed in a ventilated place. **! CAUTION** Dichloromethane is irritant and may cause cancer.
- Cyclohexane (CHX; ≥99.5%; Sigma-Aldrich, cat. no. 179191). Store tightly closed in a ventilated place. **! CAUTION** Cyclohexane is flammable, irritant and may be harmful.
- Ethyl acetate (AcOEt; ≥99.5%; Sigma-Aldrich, cat. no. 319902). Store tightly closed in a ventilated place. **! CAUTION** Ethyl acetate is flammable and irritant.
- Pentane (≥99%; Sigma-Aldrich, cat. no. 60489). Store tightly closed in a ventilated place. **! CAUTION** Pentane is flammable and harmful.
- Hydrochloric acid (HCl; 37%; Sigma-Aldrich, cat. no. 435570). Store tightly closed in a ventilated place. **! CAUTION** Hydrochloric acid is corrosive.
- Sodium chloride (NaCl; Sigma-Aldrich, cat. no. S9888). Store tightly closed. **! CAUTION** Sodium chloride is irritant.
- Sodium sulfate (Na_2SO_4; Sigma-Aldrich, cat. no. 239313)
- Toluene (≥99.5%, Sigma-Aldrich, cat. no. 179418). Store tightly closed in a ventilated place. **! CAUTION** Toluene is flammable, irritant and harmful.

Preparation of NCND derivatives

- 2,6-Dibromonaphthalene-1,4,5,8-tetracarboxylic dianhydride (Br_2NDA; >98.0%; TCI Europe, cat. no. D4339). Store at room temperature (RT; 20–30 °C) and under an inert atmosphere. **! CAUTION** Br_2NDA is an irritant.
- 2,3-Dimethoxy-5-methyl-*p*-benzoquinone (99%; Sigma-Aldrich, cat. no. 299561). Store tightly closed in a ventilated place. **! CAUTION** 2,3-Dimethoxy-5-methyl-*p*-benzoquinone is harmful and an irritant.
- (1*R*,2*R*)-(−)-1,2-Diaminocyclohexane ((*R*,*R*)-CHDA; 98%; Sigma-Aldrich, cat. no. 346721). Store under an inert atmosphere. **! CAUTION** (1*R*,2*R*)-(−)-1,2-Diaminocyclohexane is corrosive and harmful.
- (1*S*,2*S*)-(+)-1,2-Diaminocyclohexane ((*S*,*S*)-CHDA; 98%; Sigma-Aldrich, cat. no. 346713). Store under an inert atmosphere. **! CAUTION** (1*S*,2*S*)-(+)-1,2-Diaminocyclohexane is corrosive and harmful.

Quality checking

- Quinine hemisulfate salt monohydrate (suitable for fluorescence measurement; 99.0–101.0%; Sigma-Aldrich, cat. no. 22640). Store under an inert atmosphere and in dark. **! CAUTION** Quinine hemisulfate salt monohydrate is an irritant.
- Kaiser test kit (Ninhydrin test kit; Sigma-Aldrich, cat. no. 60017). Store tightly closed in a ventilated place. **! CAUTION** The components of the kit are flammable, harmful and toxic. They may cause respiratory irritation. The kit contains an ethanolic solution of ninhydrin (flammable liquid), potassium cyanate (flammable liquid with acute toxicity) and phenol (a flammable liquid with acute toxicity; it causes skin corrosion and is a suspected mutagen).
- Ferrocene (98%; Sigma-Aldrich, cat. no. F408). Store tightly closed in a ventilated place. **! CAUTION** Ferrocene is flammable, toxic, corrosive and harmful.
- Tetrabutylammonium hexafluorophosphate ($TBAPF_6$, for electrochemical analysis; ≥99.0%; Sigma-Aldrich, cat. no. 86879). Store under an inert atmosphere.

Equipment

- Spatula (double-headed micro-spoon/spatula; VWR International, cat. no. RSGA025.150)
- Analytical balance (Mettler-Toledo, model no. AB135-S/FACT)
- Pressure vessels (10-mL microwave vessel, Pyrex; CEM, cat. no. 908535)
- Vessel cap (silicone/polytetrafluoroethylene, 10 mL; CEM, cat. no. 909210)

- Stirring bar (micro stir bar; CEM, cat. no. 162810)
- Pipette (100 μL; PIPETMAN P100, Gilson, cat. no. F123615)
- Pipette tips (200-μL tip volume; Diamond towerpack D200; Gilson, cat. no. F161330)
- Microwave reactor (CEM, Discover-SP model) running Synergy application software and connected to a compressed air source
- Milli-Q ultrapure water system (Millipore, model no. Milli-Q Plus 185) equipped with a purification cartridge (QPAK 1; Millipore, cat. no. CPMQ004R1)
- Vacuum filtration system (25-mm, funnel 15 mL, spring clamp and fritted glass base with stopper; Millipore, cat. no. XX1002500), filtration flask (Pyrex micro-filtering flask, 125 mL; Sigma-Aldrich, cat. no. CLS5360125) and pump (diaphragm pump; Vacuubrand, model no. MD 1C)
- Filter membrane (0.1 μm, 25 mm, Millipore; Merck, cat. no. JVWP02500)
- Dialysis membrane (Float-A-Lyzer, MWCO 0.5–1 kDa; Spectrum Labs, cat. no. G235063)
- Stirring bars (cylindrical, 60-mm length, 9-mm diameter, BRAND; Sigma-Aldrich, cat. no. Z328871)
- Magnetic stirrer (RCT basic; IKA, model no. 2581001)
- Freeze-drier (CoolSafe 9L; Labogene, model no. CoolSafe-9)
- Sephadex LH−20 (Sigma-Aldrich, cat. no. LH20100)
- UV-VIS spectrophotometer (Agilent, model no. Cary 5000)
- Fluorescence spectrophotometer (Agilent, Cary Eclipse model)
- UV-VIS/fluorescence cuvettes (quartz, four polished windows, 10 × 10-mm light path; Hellma Analytics, model no. 111-070-40-QS)
- AFM instrument (Veeco, model no. Multi-Mode V)
- Mica substrate (highest grade; V1 Mica; 50 × 75 mm, 2 × 3 inches; Ted Pella, cat. no. 56-75)
- AFM probe (HQ:NSC19/Al BS probe, 65 kHz, 0.5 N m^{-1}; MikroMasch, cat. no. HQ:NSC19/Al BS-15)
- pH meter (with electrode, PH 25+; Crison Instruments, cat. no. 25 50)
- Electrochemical workstation (Autolab, model no. PGSTAT302N; or CH Instruments, model no. CHI 750C)
- Electrochemical glass cell (CH Instruments, cat. no. CH222) equipped with a polytetrafluoroethylene cap (CH Instruments, cat. no. CHI223)
- Glassy carbon (GC) working electrode (3-mm diameter; Cypress Systems, cat. no. 66-EE047)
- Platinum (Pt) wire counter electrode (CH Instruments, cat. no. CHI115)
- Quasi-reference electrode (QRE) consisting of either a silver wire (Sigma-Aldrich, cat. no. GF06769200) or a non-aqueous Ag/Ag^{+} reference electrode w/ porous polytetrafluoroethylene tip (CH Instruments, cat. no. CHI112)
- Electrode polishing kit (CH Instruments, cat. no. CH120)
- Circular dichroism spectrometer (scanning rate: 50 nm min^{-1}, data pitch of 0.2 nm, data integration time of 2 s; each circular dichroism spectrum is an average of at least five scans; Jasco, model no. J-810)
- Two-necked round-bottom flasks (25 mL; Sigma-Aldrich, cat. no. Z334499; and 2000 mL; VWR International, cat. no. 76012-794)
- Single-neck round-bottom flask (50 mL; Sigma-Aldrich, cat. no. Z548863)
- Rubber septa (Precision Seal; cat. nos. Z553964 and Z553999)
- Beaker (2,000 mL, DWK Life Sciences Kimble; Fisher Scientific, cat. no. 11736333)
- Glass rod (8 mm × 200 mm, Sigma-Aldrich, cat. no. Z549649)
- Handheld UV lamp (254/365 nm, Consort; VWR International, cat. no. CONSVL6-LC)
- Wash bottle (500 mL, Scienceware; Sigma-Aldrich cat. no. Z423106)
- Graduated cylinder (10 mL, BRAND; Sigma-Aldrich, cat. no. Z327263)
- Centrifuge tubes (Falcon 15 mL and 50 mL; VWR International, cat. nos. 734-0452 and 734-0453)
- Parafilm tape (roll size 4 in. × 250 ft; Sigma-Aldrich, cat. no. P7668)
- Glass vial (20 mL liquid scintillation vials, with cap; Sigma-Aldrich, cat. no. V8255)
- Syringe needle (disposable; Sigma-Aldrich, cat. no. Z192473)
- Rotary evaporator (Büchi Rotavapor RII evaporator with jack and water bath; Sigma-Aldrich, cat. no. Z563994)
- Desiccator (Scienceware Secador desiccator cabinet model 4.0, vertical profile; Sigma-Aldrich, cat. no. Z553220)
- Diamond Suspension (MetaDi Supreme; Buehler, cat. no. 40-6627)
- Support stand with rod (Sigma-Aldrich, cat. no. Z509442), double bosshead (VWR International, cat. no. LENZ06250000) and universal clamp (VWR International, cat. no. 241-7311)
- Columns for flash chromatography (glass columns with threaded joints and fritted disc; Sigma-Aldrich, cat. nos. Z202983, Z416746, Z416754 and Z416762)
- Reflux condenser (Sigma-Aldrich, cat. no. Z517410)

- Inert gas (Ar or N_2)
- Liquid N_2
- Silica gel (SiO_2, 60 Å, 230–400 mesh particle size, 40–63-μm particle size; Sigma-Aldrich, cat. no. 717185). Store tightly closed.
- Weighing paper (Whatman; Sigma-Aldrich, cat. no. WHA10347671)
- Polypropylene syringes (Sigma-Aldrich; cat. nos. Z230723 and Z248029)

Software
- OriginPro 8.1 (commercial; https://www.originlab.com/demodownload.aspx)
- Gwyddion SPM 2.50 (free and open-source software: http://gwyddion.net)

Reagent setup

Porphyrin derivative synthesis

Prepare the porphyrin derivative according to a previously reported protocol[36]. In brief, charge a dried two-necked round-bottom flask (2.0 L) with 4-*tert*-butylbenzaldehyde (0.37 mL, 2.19 mmol), methyl 4-formylbenzoate (0.33 g, 2.19 mmol), pyrrole (0.30 mL, 4.38 mmol) and $CHCl_3$ (1.0 L, containing 0.5–1.0% (vol/vol) EtOH as stabilizer), under argon (Ar). Add $BF_3{\cdot}OEt_2$ (0.36 mL, 2.91 mmol) and allow to stir for 1 h in the dark, then add *p*-chloranil (0.40 g, 0.33 mmol) and allow to stir for another 1 h. Quench the reaction with Et_3N (0.28 mL, 3.81 mmol). Concentrate the reaction mixture under reduced pressure, and then pass it over a silica plug (SiO_2, Ø = 5.7 cm, l = 7 cm, CHX/CH_2Cl_2, 1:1 (vol/vol)). The pure porphyrin is separated from the mixture by column chromatography (SiO_2, 40–63 μm, Ø = 7.6 cm, l = 22 cm, dry load from CH_2Cl_2, CHX and then CHX/AcOEt 95:5 (vol/vol)), followed by crystallization with CH_2Cl_2/pentane (294 mg, 16% yield). The porphyrin ester is converted into its acid by refluxing overnight a mixture of the abovementioned porphyrin (0.10 g, 0.12 mmol) in EtOH (0.05 L) and 2 M aqueous KOH (0.05 L). After cooling, the mixture is acidified with 2 M aqueous HCl and then concentrated under reduced pressure. The porphyrin product is extracted with $CHCl_3$; the organic phase is washed with 1 M aqueous $NaHCO_3$ and brine, dried (with Na_2SO_4) and removed under reduced pressure. Purification by chromatography (SiO_2, 40–63 μm, Ø = 4.4 cm, l = 15 cm, CH_2Cl_2 followed by CH_2Cl_2/MeOH 90:10 (vol/vol)) affords pure product (86 mg, 88% yield). The obtained purple solid can be stored in the dark and at RT before the coupling reaction (Step 12A) for up to 2 years. We expect that the solid can be stored for longer times, but we have not tested this.

Equipment setup

Microwave synthesis

Use the power cycling control option to irradiate at a defined power and bring the reaction to a maximum temperature (power interval); then cool the sample until the minimum temperature (cooling interval) is reached. Program the power cycling method, inserting the maximum amount of microwave power to be applied, the maximum and minimum temperatures, the power and cooling intervals (microwave maximum amount of time allowed to reach the maximum and minimum temperatures) and the number of cycles (power intervals). The automated IntelliVent moves into proper position to seal the vessel. The system begins heating and cooling through the number of cycles programmed and, once the cycles are completed, begins to cool the vessel to permit its removal from the instrument. The microwave power, maximum and minimum temperatures, power and cooling intervals, power intervals and consequent total time of the reaction are specified for each synthesis.

Filtration system

Filtration of the reaction mixture from microwave is performed on a 0.1-μm membrane mounted between a glass funnel (25 mm) and base with an aluminum clamp. The filtration device, with the silicon stopper, is fitted on top of a vacuum flask and vacuum is applied with a pump.

Dialysis system

Float-A-Lyzer dialysis membranes must be pre-wet before use and the reader should refer to the instructions (Spectra/Por Float-A-Lyzer G2 Ready-to-Use Dialysis Devices, spectrumlab.com). Briefly, unscrew the cap, fill the device with 10% (vol/vol) ethanol in water, cap and immerse the device in the same alcohol solution (2.0 L) for 10 min. Replace the alcohol solution with only water by

removing the device, unscrewing the cap, removing the alcohol solution inside, flushing and then filling the device with water. Screw the cap on and then place the device in only water (2.0 L) for 20 more min. Repeat the last step. Then slowly load the sample solution inside the device, place the floating ring and finally leave the device to dialyze, under slow stirring.

SEC

Sephadex LH-20, supplied as dry powder, has to be swollen before use. The reader is encouraged to follow the instructions from GE Healthcare (56-1190-97 AD). Briefly, 32.0 g of Sephadex is weighed in a beaker and suspended in 128.0 mL of methanol (approximate bed volume: 3.9–4.1 mL g^{-1}); then it is allowed to swell for at least 3 h, without stirring (as it may break the beads). The slurry is resuspended and poured into the column (Ø = 2.5 cm, l = 58 cm) with one continuous motion, with the aid of a glass rod held against the column wall (to prevent introduction of air bubbles). The column is left to pack/equilibrate overnight, making sure that it does not run dry, until the medium bed is stable. The column outlet is then opened until the solvent reaches the medium bed, and then the sample is loaded as a solution in methanol (~2 mL). The elution is carried out by gravity at RT (the lower the flow rate, the better the resolution; lower flow rate is especially recommended during the initial stages) and can be followed by using a handheld UV lamp (365 nm). The various fractions are checked by measuring UV-VIS and fluorescence spectra and then collected.

Electrochemistry

Electrochemical characterization is carried out in a typical three-electrode glass cell (10 mL), composed of a GC working electrode (Ø = 3 mm), a platinum wire as counter electrode and a silver wire as QRE. The potential of the reference electrode, in the case of a silver QRE, is calibrated after each set of measurements using a ferrocene/ferrocenium (Fc/Fc^+) redox couple as the internal standard (~1.0 mM). The GC electrode is stored in ethanol and is polished before use with a 0.05-μm diamond suspension (MetaDi Supreme Diamond Suspension) and ultrasonically rinsed with deionized water for 10 min and with ethanol for 10 min. The electrode is electrochemically activated in the background solution by means of several voltammetric cycles at 0.5 V s^{-1} between the anodic and cathodic solvent/electrolyte discharges. In the meantime, NCNDs are dissolved in a solution of dry DMF/$TBAPF_6$ (0.1 M) by aid of ultrasonication (or periodical heating) (concentration of ~1 mg mL^{-1}). The cyclic voltammetries (CVs) are performed at different scan rates, ranging from 0.05 to 0.1 V s^{-1}.

AFM

An aqueous solution of NCNDs (concentration of ~0.1 mg mL^{-1}) is drop-cast on a mica substrate and left to dry overnight (and covered to avoid deposition of dust). Acquire images using tapping mode and a HQ:NSC19/AlBs probe (65 kHz; 0.5 N m^{-1}). Save the acquired AFM images and then analyze them in Gwyddion 2.50. The statistical analysis is performed on ~100 nanoparticles, and the average size is calculated from the size histogram with a curve fit to the data using a Gaussian model.

Procedure

Part 1: preparation of NCNDs ● Timing 30 min

▲ **CRITICAL** Steps 1–10 describe how to prepare and purify NCDCs. All the necessary reagents for the synthesis of NCNDs are photographed in Fig. 1a. For examples of how to tune the electrochemical, fluorescence emission or chiroptical properties of NCNDs, see Box 1.

1 Weigh 87.0 mg of powdered Arg into a microwave vessel (Fig. 1b).

2 Add a stir bar to the microwave vessel.

3 Add 100.0 μL of Milli-Q H_2O with a micropipette (Fig. 1c).
▲ **CRITICAL STEP** Water should be added to the bottom of the vessel. Avoid touching the walls of the vessel with the pipette tip. If water is placed incorrectly, the microwave heating will not result in the preparation of NCNDs with the anticipated properties.

4 Add 33.0 μL of EDA with a micropipette.
! **CAUTION** This step should be carried out in a fume hood, while wearing gloves, goggles and a lab coat. Dispose of the tip in the appropriate waste container.
▲ **CRITICAL STEP** EDA should be added to the bottom of the vessel. Avoid touching the walls of the vessel with the pipette tip. If EDA is placed incorrectly, the microwave heating will not result in the preparation of NCNDs with the anticipated properties.

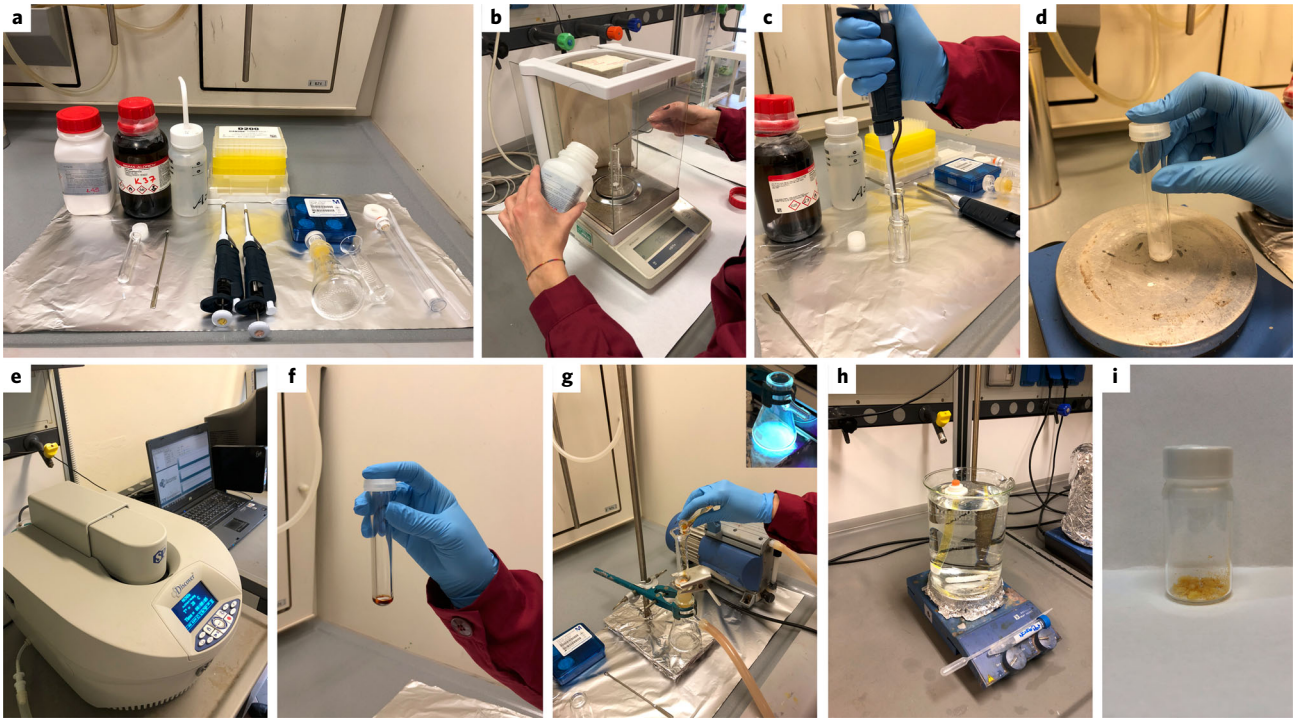

Fig. 1 | Time-course illustration of key steps in the preparation of NCNDs. A more detailed time course is presented in Supplementary Fig. 1. **a**, Reagents and reaction setup for the synthesis of NCNDs. **b**, Weighing of the Arg powder in a microwave vessel (Step 1). **c**, Adding of liquids (Milli-Q water, followed by EDA; Steps 3 and 4). **d**, Capping of the reaction vessel and mixing of the components (Steps 5 and 6). **e**, Microwave-assisted synthesis of NCNDs (Step 7). **f**, Reaction vessel after microwave irradiation (after Step 7). **g**, Filtering of the crude reaction through a micro-filter (inset shows the filtered solution under 365-nm light) (Step 9). **h**, Dialysis of the filtered solution against pure water of (Step 10A(iv and v)). **i**, Powdered NCNDs after freeze-drying of the dialyzed solution (Step 10A(viii)).

5 Close the vessel with a microwave cap.

! CAUTION Vessels and caps are designed for single use and should not be used more than once.

6 Stir briefly (30 s) on a magnetic stirrer (300 r.p.m.) to mix the reagents (Fig. 1d).

7 Place the capped vessel inside the attenuator of the microwave reactor (see 'Equipment setup' section, Fig. 1e) and use the following method: power cycling (12 cycles total, total time 180 s) of 200 W, 15-s power intervals, 5-s cooling intervals with 250 °C as maximum temperature and 240 °C as minimum temperature (Supplementary Fig. 2).

? TROUBLESHOOTING

8 Wet the filter membrane with Milli-Q H_2O (using a wash bottle).

9 Remove the cap from the vessel and add ~2 mL of Milli-Q H_2O to the dark amber reaction mixture inside the microwave vessel (Fig. 1f), shake the vessel gently and filter (see 'Equipment setup' section) the mixture. Rinse the vessel (~2 mL Milli-Q H_2O) and filter again (Fig. 1g).

! CAUTION The cap should be removed from the vessel under a fume hood, as gases are formed during the reaction.

■ PAUSE POINT The obtained mixture can be stored at −20 °C before purification for up to 1 week. We expect that the frozen solution can be stored for longer times, but we have not tested this.

Part 1: purification of NCNDs ● Timing 2–3 d

10 Purify the NCNDs either by dialysis (option A) or SEC (option B). Dialysis may provide higher yields than the fractions recovered by chromatography because multiple reactions can be purified at once, but doing this results in less control over size distribution and emission properties. In addition, dialysis is usually performed in water, whereas other solvents are used for chromatographic separation.

(A) **Dialysis**

(i) Transfer the combined filtrate (from Step 9) from the filtration flask to a graduated cylinder (or a 15-mL centrifuge or Falcon tube) and dilute with Milli-Q H_2O up to a total volume of 10 mL. This solution is ready to be transferred to a dialysis membrane.

▲ **CRITICAL STEP** For effective dialysis purification, one dialysis membrane can contain up to two filtered reaction mixtures from part 1. The volume of the two filtrates combined should be maximally 10 mL. Loading more material from more reaction mixtures into a single dialysis membrane will result in ineffective purification.

(ii) Before transferring the mixture, the dialysis device must be prepared (pre-wet). First prepare 2.0 L of 10% (vol/vol) EtOH in Milli-Q H_2O solution in a 2-L beaker. Transfer 10 mL of this solution into a dialysis membrane, close the membrane and place it inside the 2-L beaker. Place a stir bar and stir (50–100 r.p.m.) for 10 min.

(iii) Remove, uncap and flush the device with 10 mL of Milli-Q H_2O and repeat Step 10A(ii) with 2.0 L of Milli-Q H_2O.

(iv) Remove, uncap and transfer the 10 mL of light-yellow filtrate solution from Step 10A(i) to the emptied dialysis device and dialyze against 2.0 L of pure Milli-Q H_2O for 48 h (Fig. 1h).

(v) Replace the Milli-Q H_2O five times: the next morning (after 12 h), then twice during the day (every 4–6 h) and then twice the next day (every 4–6 h).

? TROUBLESHOOTING

(vi) Transfer the solution from the dialysis membrane to a plastic Falcon tube (50 mL) and freeze it at −20 °C.

! CAUTION Liquid N_2 should be handled with insulating gloves, safety goggles and a lab coat with long sleeves because it can cause severe frostbite (or eye damage) upon contact. Work in an open room, because the release of N_2 can displace oxygen.

PAUSE POINT The tube can be capped and left at −20 °C for months without obvious changes in properties. Alternatively, freeze the solution by immersing the Falcon tube in liquid N_2.

(vii) Remove the plastic cap, cover the opening with Parafilm tape and produce a series of small holes with a steel needle. Freeze-dry the solution for 24 h.

(viii) Remove the sample from the freeze-drier, transfer (with the aid of a spatula) the yellow solid to a glass vial (previously weighed), then weigh the solid (~23.0 mg), pass a stream of Ar (or N_2) over the mouth of the vial, close the plastic cap tightly and cover it with Parafilm (Fig. 1i).

? TROUBLESHOOTING

PAUSE POINT The solid can be stored inside a desiccator (that blocks UV light) at RT for at least 12 months without obvious changes in properties.

(B) **SEC**

(i) Transfer the combined filtrate from Step 9 from the filtration flask to a single-neck 50-mL round-bottom flask and remove water under reduced pressure. Recover the yellow oily residue with MeOH (~2 mL). The solution is ready to be transferred to an SEC column.

(ii) Open the column outlet until the solvent reaches the medium bed and the sample is loaded. The elution can be carried out by gravity and can be followed by using a handheld UV lamp. The slower the flow rate, the better the resolution, and slower flow rates are especially recommended during the initial stages.

? TROUBLESHOOTING

(iii) Check the various fractions by collecting UV-VIS and fluorescence spectra and group them into three main fractions.

(iv) Separately transfer the three grouped fractions to single-neck 50-mL round-bottom flasks and remove the MeOH under reduced pressure with the temperature of the water bath at 40 °C.

(v) Take up separately the three residues with H_2O (between 5 and 10 mL) and freeze-dry, as described in Step 10A(vi–viii). Weigh the three solids (expected recovery = 16.5, 8.7 and 9.6 mg) and store them tightly closed and under inert gas.

PAUSE POINT The solids can be stored inside a desiccator (that blocks UV light) at RT for at least 3 months without obvious changes in properties.

Part 1: quality control

11 Before using the NCNDs or before proceeding with post-functionalization reactions, ensure the quality of the prepared materials by using one or several of the recommended control tests (see 'Experimental design' section for details). Possible control tests include recording UV-VIS (option A) and fluorescence (option B) spectra, the Kaiser test (option C) and AFM (option D). In addition,

the electrochemical properties of the NCNDs can be probed with cyclic voltammetry (option E) in the case that the electrochemical properties are being tuned in the NCNDs (Box 1, step 1A), and the chiroptical properties can be investigated by circular dichroism (option F) if the NCNDs are endowed with chirality (Box 1, step 1C).

(A) **UV-VIS absorbance spectra** ● **Timing 1–2 h**

(i) Record the UV-VIS absorption spectra (240–700 nm) for NCND aqueous solution samples. The dialyzed NCNDs should present a characteristic band at ~285 nm. Prepare at least three different samples at different concentrations of NCNDs (recommended concentrations are between 1 and 10 g L^{-1}) and record the UV-VIS spectra. Then plot the absorbance (at 285 nm versus concentration). Make sure that the mass absorption coefficient is ~3.7 (g^{-1} L) cm^{-1} (at 285 nm).

? TROUBLESHOOTING

(B) **Fluorescence emission spectra** ● **Timing 1–2 h**

(i) Record the emission spectra (310–650 nm) for the NCND aqueous solution samples, with increments of 10 nm in the excitation wavelength. For dialyzed NCNDs, a broad emission centered at 356 nm is observed when the sample is excited at the optimal excitation wavelength (i.e., 300 nm). When the excitation wavelength changes from 300 to 420 nm, the emission intensity decreases and red-shifts from 356 to 474 nm.

(ii) Determine the quantum yield (ϕ_f) of the NCNDs according to the previously published protocol[85]. Briefly, the relative fluorescence quantum yield relies on the comparison of the integral emission spectra between the NCND sample and the standard (quinine hemisulfate salt monohydrate), measured under identical conditions with the standard's absorbance matching that of the sample at the chosen excitation wavelength.

(C) **Kaiser test** ● **Timing 1–2 h**

(i) Run a Kaiser test according to a published procedure[86]. In a typical test, weigh 1.00 mg of NCNDs. Add 75 μL of the kit solution of phenol (80% (vol/vol) in EtOH) and 100 μL of the kit solution of KCN (in H_2O/pyridine) and sonicate the solution for 2 min. Subsequently, add 75 μL of the kit solution of ninhydrin (6% (vol/vol) in EtOH) and heat the mixture at 120 °C for 10 min. Cool the solution to RT, dilute it at a known concentration with EtOH (60% (vol/vol) in H_2O) to record the absorbance spectrum. A control solution is prepared in the same way, but without the NCNDs, for background correction of the absorbance. Finally, calculate the amine loading (in micromoles per gram) by using the absorbance at 570 nm (ε = 15,000 M^{-1} cm^{-1}) as an average of at least two different tests.

? TROUBLESHOOTING

(D) **AFM** ● **Timing 1 d**

(i) Prepare the substrate by cutting a small piece (~5 × 5 mm) of mica.

(ii) Cleave the mica by inserting a sharp edge into a corner of the mica sheet and gently separating the layers.

(iii) Drop-cast an aqueous solution of NCNDs (~0.1 mg mL^{-1}) and allow the substrate to dry overnight.

(iv) Use AFM to image the quasi-spherical shape of the NCNDs deposited on the mica substrate (see 'Equipment setup' section). MultiMode AFM standard operating procedures can be followed[87].

(E) **Cyclic voltammetry** ● **Timing 1–4 h**

▲ **CRITICAL** Cyclic voltammetry measurements can be set up and performed according to published procedures[88,89]. A shorter version of this procedure (see 'Equipment setup' section) is described here.

(i) Measure a cyclic voltammogram of the NCND solution (an ~1 mg mL^{-1} NCND concentration, dissolved by heating and sonication cycles) in dry and degassed 0.1 M $TBAPF_6$ in DMF solution (8.0 mL), at 100 mV s^{-1}, with a GC electrode (Ø = 3 mm), a silver wire QRE and a platinum wire counter electrode.

(ii) When using a QRE, add a redox couple as the internal standard, such as ferrocene/ferrocenium (Fc/Fc^+), (to obtain a concentration of ~1.0 mM) as the last measurement, at 100 mV s^{-1}.

(iii) Calculate the energy levels of the NCNDs from the onset oxidation potential ($E_{onset,ox}$) and the onset reduction potential ($E_{onset,red}$), using published procedures[90,91]. The onset potentials are defined as the potentials at which there is an evident rise in anodic or cathodic currents, which represent the initial injection of holes or electrons into the highest occupied molecular orbital (HOMO) and lowest unoccupied molecular orbital

(LUMO), respectively. The HOMO and LUMO levels are then compared to the internal standard (Fc/Fc^+) and calculated as $E_{HOMO} = -(5.1\ eV + E_{[onset,ox\ vs.\ Fc/Fc+]})$ and $E_{LUMO} = -(5.1\ eV + E_{[onset,red\ vs.\ Fc/Fc+]})$. Finally, assuming an Fc/Fc^+ value of 0.45 V versus saturated calomel electrode (SCE), in 0.1 M $TBAPF_6$ in DMF, the energy levels can be reported as $E_{HOMO} = -(4.65\ eV + E_{[onset,ox\ vs.\ SCE]})$ and $E_{LUMO} = -(4.65\ eV + E_{[onset,red\ vs.\ SCE]})$.

(F) **Circular dichroism** ● **Timing 30 min–2 h**

▲ **CRITICAL** Equipment preparation and data collection can be performed by following the relevant parts of previously published procedures[92,93]. A shorter version of this procedure is described here.

(i) In a quartz cuvette (10 mm light path), prepare an ~0.65 mg mL^{-1} solution of chiral NCNDs in ultrapure H_2O.

(ii) Record the circular dichroism spectra as an average of at least five scans, with a scanning rate of 50 nm min^{-1}, data pitch of 0.2 nm, and data integration time of 2 s.

Part 2: post-functionalization and purification of NCNDs

12 According to specific needs and applications, the NCNDs can be modified by post-functionalization reactions. Herein we report two different reactions that exemplify the surface amine modifications of the NCNDs. Follow option A for carbodiimide coupling with molecules bearing carboxylic groups and follow option B for reductive methylation (Eschweiler–Clarke reaction)[94,95].

(A) **Amidation reaction** ● **Timing 1–2 d**

(i) Charge a 25-mL dry two-necked flask with 5,10,15-tri(4-*tert*-butylphenyl)-20-(4-carboxyphenyl)porphyrin (44.8 mg, 0.05 mmol) (see 'Reagent setup' section), DMAP (12.2 mg, 0.10 mmol) and dry DMF (5.0 mL), under Ar.

! CAUTION This reaction should be carried out in a fume hood while wearing gloves, goggles and a lab coat.

(ii) Cool the solution to 0 °C using a water–ice bath and then add EDC·HCl (19.2 mg, 0.10 mmol).

(iii) Allow the mixture to stir for 30 min and, during this time, prepare a dispersion using the NCNDs (25.0 mg) purified in Step 10A in dry DMF (5.0 mL) by sonication (and periodic heating), under Ar.

? TROUBLESHOOTING

(iv) Add the NCND solution to the activated acid–EDC porphyrin solution with a syringe, under Ar.

(v) Leave the solution to warm up gradually to RT and allow it to stir overnight under Ar.

(vi) Transfer the solution to a single-neck 50-mL round-bottom flask and remove the DMF under reduced pressure (toluene can be added to accelerate the evaporation).

(vii) Recover the residue with MeOH and purify the materials by SEC (as detailed in Step 10B (ii–v)). An NCND–porphyrin covalent conjugate is obtained as a purple solid after lyophilization (18.1 mg).

■ **PAUSE POINT** The solid can be stored inside a desiccator (that blocks UV light) at RT for at least 12 months without obvious changes in properties.

(B) **Reductive alkylation reaction** ● **Timing 2–4 d**

(i) Prepare a formalin solution[96] (37% (wt/vol), 3.0 mL, 0.01 mol) and transfer it to a 25-mL single-neck flask equipped with a reflux condenser.

! CAUTION This step should be carried out in a fume hood, while wearing gloves, goggles and a lab coat.

(ii) Add formic acid (4.0 mL, 106.0 mmol), followed by NCNDs (80.0 mg) purified in Step 10A to the formalin solution, warm to and keep the reaction mixture at 101 °C for 48 h.

(iii) Cool the solution to RT and concentrate the mixture under reduced pressure.

! CAUTION The excess formaldehyde should be removed with a rotary evaporator in a fume hood.

(iv) Take up the residues with 10 mL of H_2O and dialyze against pure water as outlined in Step 10A(ii–viii). Methylated NCNDs (mNCNDs) are obtained as a brownish solid after lyophilization (47.5 mg)

■ **PAUSE POINT** The solid can be stored inside a desiccator (that blocks UV light) at RT for at least 12 months without obvious changes in properties.

Part 2: quality control ● Timing 1–2 d

13 Characterize the post-functionalized NCNDs to confirm the successful outcome of the reactions. Control steps include recording UV-VIS (Step 11A) and fluorescence (Step 11B) spectra, the Kaiser test (Step 11C) and AFM (Step 11D).

Troubleshooting

Troubleshooting advice can be found in Table 1.

Table 1 | Troubleshooting table

Step	Problem	Possible reason	Solution
7	Reaction profiles do not match those in Supplementary Fig. 2	Incorrect amount of reagents inside the vessel	Add reagents to the bottom of the microwave vessel and be sure they are not placed on the walls
		Incorrect microwave heating	Turn on the microwave at least 30 min before running the reaction and be sure that the compressed air source is open and well connected. Alternatively, run a blank reaction (only water), before running the actual reaction
10A(v)	Loss of material through the dialysis membrane or incomplete dialysis	The dialysis membrane was punctured with a glass pipette	Use plastic pipettes when transferring the solution from/to dialysis membranes
		The dialysis membrane was not conditioned properly	Condition the dialysis membrane according to the specification: soaking the membrane less or more than the specified time can result in an unsatisfactory dialysis
10A(viii)	The NCNDs are not in powder form	Starting materials were incompletely removed	Check and make sure that synthesis/purification has been done correctly
		The lyophilization was not efficient	Freeze-dry again for longer time and make sure that it is well frozen
	The NCNDs become oily upon weighing	The NCNDs are highly hygroscopic	Weigh the sample quickly Weigh the sample under an N_2 stream Add a small amount of water and freeze-dry again
10B(ii)	NCND fractions are not separated	Too much sample was loaded	The sample volume should be ~1–2% of the total bed volume
		Not enough bed volume or fast flow rates	Prepare a longer column and use recommended flow rates of 1–10 cm h^{-1}, in accordance with the specification of the medium
		The column, left equilibrating overnight, ran dry	Make sure to tightly seal the column with enough solvent and check that the column is not leaking
11A(i)	The mass absorption coefficient is not ~3.7 (g^{-1} L) cm^{-1} (at 286 nm) or the absorption profile is different	NCNDs are hygroscopic and the weight is incorrect	Quickly weigh the solid under an N_2 stream (water adsorption results in higher weights)
		Incorrect synthesis, dialysis and/or lyophilization	Redo Steps 1–10, taking into account the troubleshooting advice
11C(i)	The amount of amines (for NCNDs) is not ~1,350 μmol g^{-1}	NCNDs are hygroscopic and the weight is incorrect	Quickly weigh the solid under an N_2 stream (water adsorption results in higher weights)
		Incorrect synthesis, dialysis and/or lyophilization	Redo Steps 1–10, taking into account the troubleshooting advice
12A(iii)	The mixture is a suspension instead of a solution	NCNDs are not solubilized in DMF	Heat the NCND suspension (it is stable at 120 °C) for up to 30 min Add few drops (~4 drops) of MeOH to improve the solubility of the NCNDs
Box 1, step 1A(viii)	The total reaction time is not 180 s	The total reaction time is affected by the reactivity of the quinone used: if the power/cooling interval is insufficient to reach the minimum/maximum temperature, the instrument will skip the cooling/power cycle and continue the next heating/cooling cycle until the minimum/maximum temperature is reached	Use the same quinone or complete the 12 cycles in less or more than 180 s, depending on the amount of quinone used
Box 1, step 1A(x)	The reaction mixture is stuck to the walls of the microwave vessel	Stronger carbonization could be occurring if you are using quinones as starting materials	Adding water and sonicating (for at least 15 min) help to recover most of the material from the vessel

Timing

Part 1: preparation, purification and quality control of NCNDs
Steps 1–9, preparation of NCNDs: 30 min
Step 10, purification of NCNDs: 2–3 d
Step 11, quality control: 1–2 d
Part 2: post-functionalization and purification of NCNDs
Step 12A, amidation reaction: 1–2 d
Step 12B, reductive alkylation reaction: 2–4 d
Step 13, quality control: 1–2 d
Box 1, step 1A, tuning the electrochemical properties of NCNDs: 3–4 d
Box 1, step 1B, tuning the emission properties of NCNDs: 3–4 d
Box 1, step 1C, tuning the chiroptical properties of NCNDs: 3–4 d

Anticipated results

Part 1: preparation, purification and quality control of NCNDs

The NCNDs prepared by the procedure in Steps 1–10 are obtained as a yellow solid (23.0 mg). These carbon nanoparticles are highly soluble in water (up to 80 mg mL^{-1}). The aqueous solution appears yellow in daylight at a concentration of 0.5 mg mL^{-1}, emits blue luminescence upon excitation under a 365-nm UV lamp and has an absorption band at 286 nm, with the absorption onset starting from 388 nm (Fig. 2a). They are fluorescent in the blue region of the UV-VIS spectrum, with the fluorescence peaks shifting from 356 to 474 nm when the excitation wavelength changes from 300 to 420 nm, and the fluorescence intensity decreases as the peak red-shifts with $\phi_f = 17\%$ (Fig. 2b).

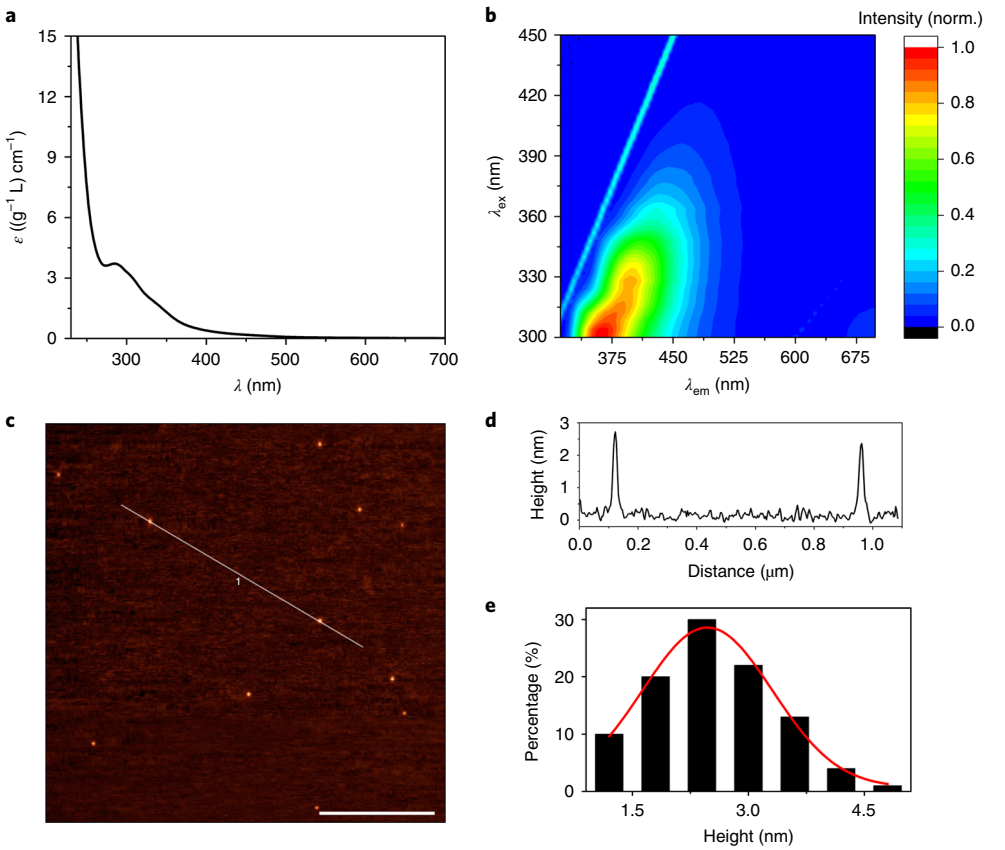

Fig. 2 | Characterization of NCNDs (Step 11). a, UV-VIS spectrum in water (298 K) (Step 11A(i)). **b**, Fluorescence matrix scan in water (298 K) (Step 11B(i)). **c**, Tapping-mode AFM image (1.7 × 1.7 μm) from a drop-cast aqueous solution on a mica substrate (scale bar, 500 nm) (Step 11D(iv)). **d**, Height profile (Step 11D(iv)). **e**, Size histogram with curve fit to the data using a Gaussian model (Step 11D(iv)).

Their size, as determined by AFM, is 2.47 nm, with a homogeneous size distribution (±0.84 nm) that obeys a Gaussian distribution with a full-width at half-maximum (FWHM) of 1.98 (Fig. 2c–e) Furthermore, the surface of the NCNDs is rich in primary (and secondary) amino groups, as determined by the Kaiser test (1,350 μmol g^{-1}).

In the case of our NCNDs, a dialysis membrane with a MWCO of 0.5–1 kDa is used, which was found to be efficient in removing low-MW and polymeric by-products from the carbon nanoparticles (Supplementary Fig. 6a,b). The dialysis procedure (in terms of time and water exchange) was optimized for our NCND system. To be confident of the purification, the absorption and emission properties should become stationary throughout the dialysis. As shown before, during the dialysis, small organic by-products are removed and these are usually more fluorescent than the CDs[29] (Supplementary Fig. 6c–e). Therefore, the ϕ_f decreases during the dialysis, until a stationary phase is reached. For NCNDs, characterization of the leftover filter material shows fluorescence excitation dependence ($\phi_f < 1\%$, Supplementary Fig. 6d), whereas the dialysate is characterized by $\lambda_{em} = 421$ nm ($\phi_f = 35\%$, Supplementary Fig. 6e), which agrees with the characteristics of low-MW material of small molecular-like fluorophores. Because the dialysate and NCNDs show markedly different fluorescence behavior, we conclude that dialysis (0.5–1 kDa cut-off) is adequate for the separation of NCNDs from small by-products. This is also confirmed by diffusion-ordered NMR spectroscopy of the NCND sample, which does not show low-MW by-products[30].

Part 2: post-functionalization, purification and characterization of NCNDs

The surface amino groups of the NCNDs are modified through chemical reactions; we use amidation and alkylation reactions as examples. In the first case, the amino groups from the NCNDs and carboxylic acids from the porphyrin dye are combined through a carbodiimide condensation reaction (Step 12A). In the second case, the primary amino groups are converted to tertiary ones through reductive alkylation (Step 12B). In both cases, the post-functionalized NCNDs are purified and, after lyophilization, are obtained as solids.

The covalent attachment of the porphyrin is confirmed by recording UV-VIS and fluorescence spectra of the hybrid material; the typical Soret and Q bands of the porphyrin are visible in absorption[30], whereas electronic interaction is confirmed in the emission properties[36]. The number of amino groups on the surface is decreased as compared to that of the pre-functionalized NCNDs, whereas AFM shows the size of the hybrid to be ~5 nm (ref. [36]).

The reaction to afford NCNDs with tertiary amino groups on the surface can be monitored with the Kaiser test (~30 μmol g^{-1} after completion of the reaction)[35]. The changes observed in their photophysical properties are consistent with the NCND surface modification. Although the UV-VIS absorption and fluorescence emission spectra did not show substantial differences compared to those of the NCNDs, the CV of the mNCNDs exhibits an easier to oxidize peak (anodic peak of an irreversible process) at +0.80 V versus SCE (compared to $E^{ox}_{p,a} = +1.18$ V versus the SCE of NCNDs), which can be attributed to the presence of tertiary amines[35].

Preparation, purification and characterization of NCND derivatives

The electrochemical, emission and chiral properties of NCNDs are tailored by following the procedures described in Box 1. Tuning of these properties includes using the multi-component approach and the hydrothermal microwave-assisted synthesis already described in part 1. Modifying the electrochemistry is accomplished by adding quinones to the Arg and EDA starting materials in the synthesis[34], whereas emission is tuned by adding core-substituted naphthalene dianhydrides as chromophores along Arg and EDA[33]; finally, chirality is conferred by using chiral CHDAs instead of EDA[32].

The NCND derivatives are purified and finally obtained as solids. In addition to standard characterization techniques, supplemental quality control steps are performed on the basis of the tailored property. For example, addition of quinones to the synthesis results in NCNDs that are more easily reduced (as compared to the cathodic peaks of irreversible processes, $E^{red}_{p,c} = -1.52$ V versus SCE compared to $E^{red}_{p,c} = -2.52$ V versus SCE of NCNDs of part 1), as evidenced by the cyclic voltammograms (Fig. 3a). In addition, from the reduction onset potentials in Fig. 3a, the energy levels of the LUMOs can be calculated ($E_{LUMO} = -1.8$ V versus SCE for NCNDs from part 1 and $E_{LUMO} = -0.9$ V versus SCE for the NCNDs from Box 1, Step 1A). Furthermore, the quinone-based NCNDs have a higher absorptivity and a red-shifted absorption onset (Fig. 3b). Tuning the emission

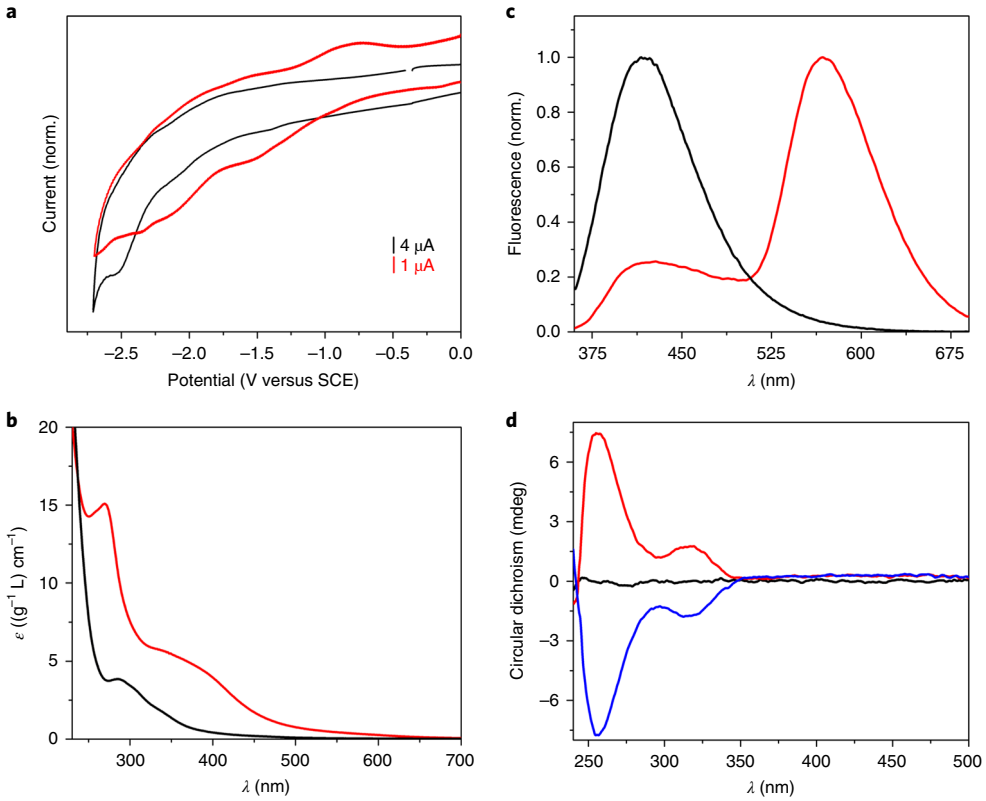

Fig. 3 | Characterization of NCND derivatives (Box 1). a,**b**, Cathodic CVs (in 0.1 M $TBAPF_6$ in DMF solution, GC electrode, scan rate = 100 mV s^{-1}, Pt wire used as counter electrode and the potential is referenced to SCE, at RT) (measured in Step 11E(i)) (**a**) and UV-VIS spectra in water (298 K) of NCNDs from part 1, Step 10A(viii), measured in Step 11A(i) (black line) and NCNDs prepared from 2,3-dimethoxy-5-methyl-*p*-benzoquinone (red line) from Box 1, step 1A(xi), measured in Step 11A(i) (**b**). **c**, Normalized emission spectra (norm.; 350 nm as excitation wavelength) of NCNDs from part 1, Step 10A(viii), measured in Step 11A(i) (black line) and NCNDs prepared from Br_2NDA (red line) from Box 1, step 1B(xii), measured in Step 11B(i). **d**, Electronic circular dichroism spectra of NCNDs from part 1 (black line) and NCND-*S* and NCND-*R* (red and blue line, respectively) from Box 1, step 1C(x), measured in Step 11F(ii).

properties of NCNDs by using core-substituted naphthalene dianhydrides results in a red-shifted emission spectrum (Fig. 3c). Finally, preparation of chiral NCNDs is confirmed by recording circular dichroism spectra (Fig. 3d).

Reporting Summary

Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

The main data supporting the findings of this study are available within the article and its Supplementary Information files. Additional data are available from the corresponding authors upon request.

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Author contributions

L.Đ. and F.A. designed and performed the experiments and wrote the manuscript. M.P. planned the research, co-wrote the manuscript and secured the funding.

Competing interests

The authors declare no competing interests.

Additional information

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Correspondence and requests for materials should be addressed to L.Đ., F.A. or M.P.

Related links

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