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Matching Together Living Cells and Prototissues: Will There Be Chemistry?

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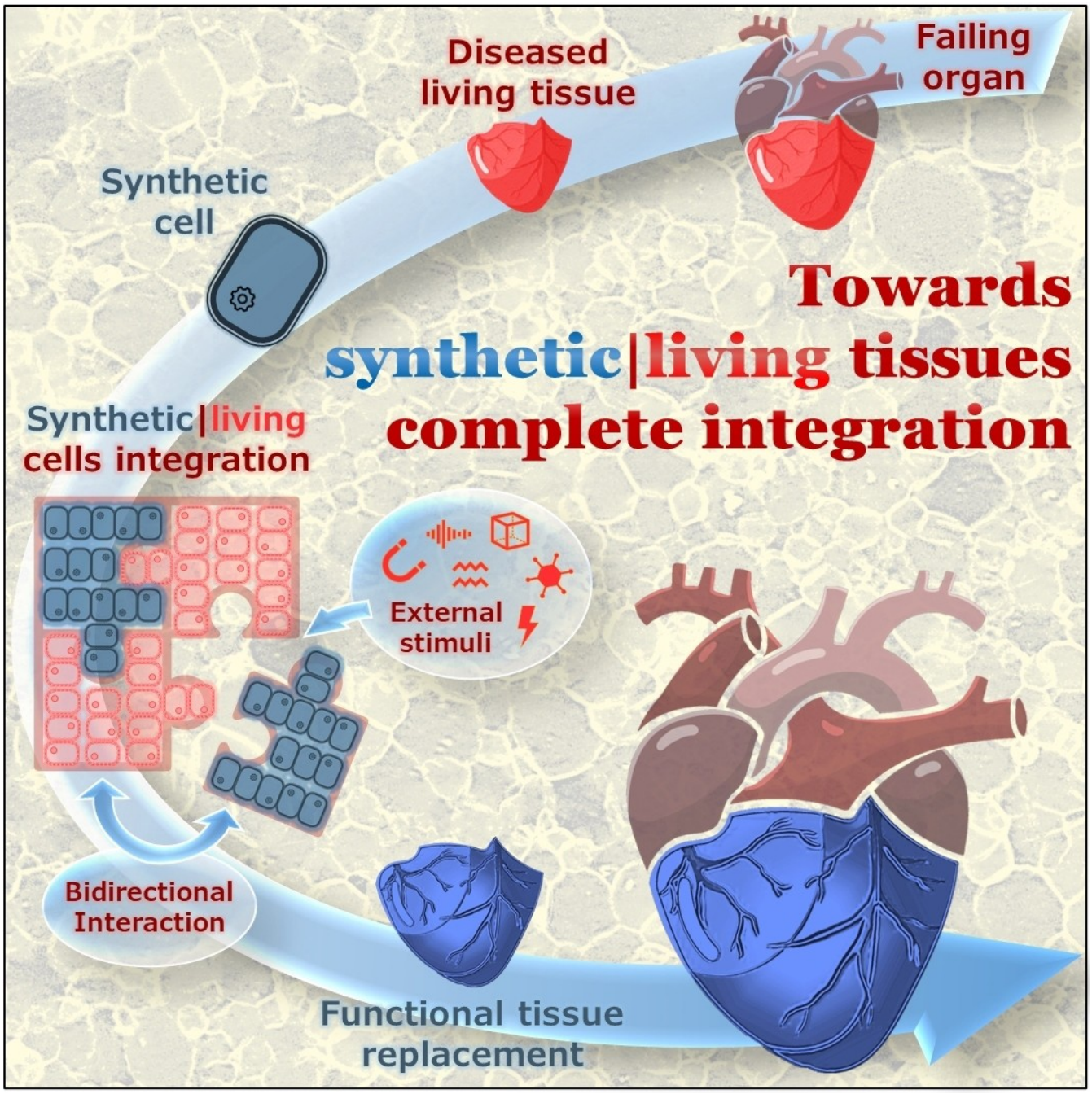

Scientific advancements in bottom-up synthetic biology have led to the development of numerous models of synthetic cells, or protocells. To date, research has mainly focused on increasing the (bio)chemical complexity of these bioinspired micro-compartmentalized systems, yet the successful integration of protocells with living cells remains one of the major challenges in bottom-up synthetic biology. In this review, we aim to summarize the current state of the art in hybrid protocell/living cell and prototissue/living cell systems. Inspired by recent breakthroughs in tissue engineering, we review the chemical, bio-chemical, and mechano-chemical aspects that hold promise for achieving an effective integration of non-living and living matter. The future production of fully integrated protocell/living cell systems and increasingly complex prototissue/living tissue systems not only has the potential to revolutionize the field of tissue engineering, but also paves the way for new technologies in (bio)sensing, personalized therapy, and drug delivery.

1. Introduction

Unravelling the intricate molecular mechanisms governing the microscopic world of living cells stands as one of the paramount challenges of our time.[1] In recent years, a synergistic combination of chemical sciences and synthetic biology has given rise to the field of "bottom-up synthetic biology".[2] This cutting-edge, high-impact field of research seeks not only to redesign and build from scratch systems analogous to biological cells using molecules, materials and chemical reactions,[3] but also to innovate systems that can outperform their living counterparts for a wide range of applications in medicine, computing[4] and industry.[5] Through this cross-disciplinary background, bottom-up synthetic biology has transitioned from studying the basics of how Nature works to the construction of simple molecular and genetic devices. It is now venturing into the integration of chemical building blocks into systems of greater complexity and functionality, capable of executing more sophisticated tasks.[6] Amidst this evolution, novel trends emerge in physically and chemically combining synthetic life-like systems with natural living systems, posing a whole new set of scientific challenges, especially for chemists. In this review, we aim to elucidate and discuss the advances in the field of bottom-up synthetic biology with a specific emphasis on chemical strategies that facilitate the interface and integration of non-living and living matter.

At the forefront of bottom-up synthetic biology lies the endeavor to reimagine and fabricate living cells mimics from scratch. Research efforts in this direction gave rise to the concept of synthetic cells or "protocells". Over the past decade, protocell engineering has equipped researchers with a very powerful toolkit to craft a variety of protocell models. While existing reviews delve into the plethora of protocell types and models developed thus far,[7] the focus of the first section of this work is to highlight the interactions between synthetic and living cells. We underscore key factors such as preparation methods, surface properties promoting adhesion and interaction, protocell membrane composition, spatial arrangement of synthetic/living cell populations, and the spectrum of protocell-cell signaling (chemical, mechanical, thermal, luminous *etc.*).

Following how complex hierarchical architectures naturally occur in multicellular living systems, the second section of this review explores the next step in protocell engineering, that is the assembly of synthetic tissue-like materials termed "prototissues" from protocell units. While prototissue engineering is still in its infancy, it has already yielded remarkable outcomes, including complex materials displaying light-activated electric communication[8] and thermally-induced mechano-chemical inhibition of enzyme cascade reactions.[9] In this section we review the major contributions of prototissue engineering to the bottom-up synthetic biology field, highlighting key features related to building block composition, prototissue synthetic construction and their resulting tissue-like properties.

At present, one of the primary challenges in prototissue engineering is integrating prototissues with living cells and tissues. Successful integration holds promise for the application of prototissues in tissue engineering, regenerative medicine, and personalized therapy.[10] For example, we can envision using prototissues as advanced substrates for tissue engineering capable of providing cells with both chemical and mechanical cues to guide their spreading, growth, and differentiation. Moreover, we can also imagine the prospect of conjugating fully autonomous prototissues to failing living tissues to repair them. This challenge is clearly cross-disciplinary and demands expertise in synthetic chemistry, bottom-up synthetic biology, and tissue engineering. Therefore, in the third section, we briefly introduce the field of tissue engineering to the reader and summarize its most recent developments in scaffold design and fabrication, especially with the consolidation of bioprinting techniques.[11] Emphasis is placed on methods to enhance substrate biochemical compatibility and integration through materials that can convey specific stimuli to cells. Additionally, we dedicate a subsection to some current examples of substrates delivering mechanical cues to living cells using light stimulation, because we believe that light can provide invaluable opportunities for cell manipulation and signaling in tissue engineering.

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Finally, in the fifth section, we present the first examples of prototissues capable of interacting with living cells and discuss what we believe are the forthcoming challenges in this pivotal research area. These challenges include achieving precise spatiotemporal arrangements of protocell units, scaling-up prototissue fabrication methods, and enhancing the biochemical complexity of protocells and prototissues.[12]

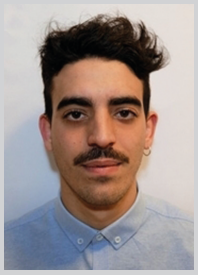
Considering how rapidly the fields of living tissue and prototissue engineering are advancing, we believe that it is only a matter of time before these fields are effectively "matched" together. While preliminary examples of prototissues chemically interacting with living cells and tissues exist in the literature,[13] the perfect "chemistry" has not been achieved yet. The take-home message of this review is that this complex challenge at the interface of non-living and living matter can be addressed and overcome only through a synergistic interplay between synthetic chemistry, bottom-up synthetic biology, and tissue engineering. We believe that the combination of the knowledge and methodologies of these different research fields will be the winning approach toward realizing the next generation of protocell and prototissues capable of seamless integration with their living counterparts.

2. Synthetic Cell/Living Cell Interactions

The progress in integrating living and synthetic tissues is inextricably linked with studying the fundamental physical and biochemical aspects ensuring their compatibility and successful interactions. Living cells/organisms are extremely complex yet fragile systems to handle, therefore bioengineers and material scientists must collaborate synergistically to find the right chemistry. In order to do so, attention must be focused on a smaller level, questioning the optimal characteristics and

Stefano Valente is a synthetic and materials chemist. In 2021 at the University of Trieste (Italy) he received his PhD in nanotechnology for his work on hybrid nanoparticle systems for theranostics. After one year of post-doc at the Joint Research Centre of the European Commission in Ispra (Italy), in 2022 he joined the group of Prof. Pierangelo Gobbo where he is currently working on the ERC-funded "PROTOMAT" project, focusing on the design and development of synthetic protocellular materials and their integration with living cells.

Agostino Galanti obtained his PhD at the Institut de Science et d'Ingénierie Supramoléculaires of the University of Strasbourg (France) in 2018, under the supervision of Prof. P. Samorì. He joined the group of Prof. Pierangelo Gobbo in 2020 at the University of Bristol (UK), where he started working on the synthesis of bioinspired materials using protocells as building blocks. The group then moved to the University of Trieste (Italy), where he is currently working on the development of free-standing protocellular materials capable of transducing external stimuli to mechanical and chemical responses.



Edoardo Maghin is a biotechnologist with a PhD in Developmental Medicine and Health Planning Sciences. He specializes in skeletal muscle tissue engineering and regeneration, focusing on the extracellular matrix in healthy and pathological conditions. He has experience with decellularization methods, bioreactor design and development, 3D bioprinting, and establishing innovative 3D in vitro models. Currently, Edoardo is working on developing a hybrid living/non-living material composed of synthetic protocellular material and living cells.

Nahid Najdi was born in Shiraz, Iran and graduated in Applied Chemistry at Islamic Azad University of Iran in BSc. She continued her studies in Medicinal Chemistry in Islamic Azad University, Kazeroon branch under the supervision of Dr. Maryam Rajabzadeh with the focus on heterogeneous metal hollow sphere catalysts in oxidation of benzyl alcohols and magnetic catalysts in Ullmann reactions. She Joined the Gobbo group as a PhD student in November 2023 and is working on the synthesis of different crosslinking polymers for the development of advanced protocellular materials.

Martina Piccoli has a background in biological sciences, with specific training and expertise acquired in pediatric and developmental research. Her research includes the characterization of stem cells and the development of biomaterials for tissue engineering purposes. During her postgraduate training, she developed technical skills in the field of tissue engineering and in particular on decellularization methods to produce different formulations of natural biomaterials. Since 2017 she has been the principal investigator of the Tissue Engineering laboratory of the Istituto di Ricerca Pediatrica Città della Speranza. Her lab is focused on the production of 3D constructs with different approaches and techniques.

Pierangelo Gobbo received his PhD in 2016 at the University of Western Ontario (Canada). In 2016 he joined the research group of Prof. Stephen Mann, FRS at the University of Bristol (UK) as an NSERC of Canada Postdoctoral Fellow first, and then as an EU Marie Curie Postdoctoral Fellow. In 2019 he established his independent research group at the University of Bristol and started his work on biomimetic tissue-like materials. In 2021 he moved his research group to the University of Trieste (Italy), where he is currently working as an Associate Professor of Organic Chemistry.

features that the single protocellular unit must embody to interact successfully with living cells. In the first part of this section, we categorize and summarize the key features in protocell design, fabrication, and composition that play a role in the interaction with cells. We also emphasize spatial arrangements and the type of communication pathways between protocells and living cells.

In the final part of this section, we report some noteworthy examples where synthetic and living cells interact, elucidating key biochemical details (protocell model, type of signal) and their interaction mechanism, aligning each example with the categorization provided in the previous section.

2.1. Features that Influence Synthetic/Living Cell Interaction

The key factors that determine and influence the interactions between micro-compartments encompass the morphological, mechanical, and chemical properties of the membrane, the molecules and the nanometric structures constituting their external surface or residing in the lumen. Although the construction of micro-compartments and their interaction occur spontaneously in the living world, replicating these factors through a bottom-up approach poses a non-trivial challenge. Unidirectional interaction, especially from synthetic to living cells, is relatively easy to achieve. The literature is rich with examples of synthetic cells functioning as "smart capsules" for *in-situ* drug production and delivery for therapeutic purposes.[14] This is one of the simplest forms of synthetic/living cells unidirectional interaction researchers are seeking, while others involve more complex signaling and transduction. However, protocell and prototissue technology aims to reach a deeper level of integration, where synthetic and living cells or tissues interact and influence each other in a chemical communication system characterized by continuous feedback. Achieving such synergy between living and non-living entities is currently one of the major challenges of protocell and prototissue engineering.

A fundamental aspect impacting synthetic/living cell integration is the chemical nature of the synthetic components. With the idea of identifying molecular building blocks that were first of all biocompatible, researchers turned their attention towards what in Nature is already used to build cellular or subcellular structures. Amphiphilic molecules, proteins, natural polymers, as well as inorganic nanoparticles and synthetic polymers, have all been exploited to craft microcompartments such as polymersomes,[15] DNAsomes,[16] coacervates,[17] proteinosomes,[18] emulsion droplets[19] and other types of colloidosomes.[3f] However, in order to mimic the natural cell membrane, lipid or polymer-lipid biomolecules are the building blocks of choice to form the bilayer constituting the artificial cell membrane, with giant-unilamellar vesicles (GUV) being the most common type of synthetic cell exploited so far in literature for synthetic biology applications.[20] All these systems possess structures and shapes that are determined by the particular technique employed for their preparation (*e.g.* microfluidics,[21] emulsion,[22] 3D-bioprinting[11a]) and the type of physical-chemical interactions holding the membrane components together (covalent or non-covalent – *e.g.* H-bonding, electrostatic or hydrophobic/hydrophilic interactions). In particular, droplet microfluidics relies on the manipulation of very small volumes of oil and aqueous phases using laminar flow within micrometer channels, enabling complex chemical and biological experiments.[23] In synthetic biology, this technique ensures precise molecular and geometrical composition of the synthetic cells with high throughput and automation and allows manipulation of living cells.[24] Consequently, this methodology has been successfully used to prepare droplets as cell-like reactors,[25] coacervate organelles in liposomes[26] as well as 2D or 3D interface bilayer networks.[27] Typically, the resulting vesicle prepared with any technique mentioned above is a spherical structure that remains stable in physiological environment or in the specific conditions in which the preparation has been made. Nonetheless, several groups have reported the possibility of transitioning from static structures to dynamic vesicles capable of reprogramming membrane domains or their shape.[28] These advancements represent a step forward in designing and constructing life-like systems, which are crucial to make the synthetic micro-compartments interact with living cells.

Finally, a distinguishing feature between an isolated synthetic cell functioning as a mere compartment and one that can interact with the environment is the ability to communicate with other synthetic cells or with living cells through the exchange of diffusible chemical signals or other physical stimuli. This feature is becoming increasingly vital with the goal of conjugating synthetic cells and tissues, as evidenced by studies in tissue engineering. These demonstrated how cells require not only a suitable substrate for growth and differentiation, but also specific chemical or mechanical stimulation to direct their maturation into a living tissue.

Despite significant attention has been dedicated to the topic of synthetic/living cell communication,[29] successful attempts of harnessing biochemical reactions and signaling that constitute the language of living cells and applying them to real problems still remain exiguous, albeit promising.

Reviewing the available literature on interactions between artificial and living cells[30] allows few different categorizations based on the distance between the two communicating entities, the direct or indirect nature of the communication, or the type of the signal. On the one hand, in terms of distance, we can distinguish between contact, short-range, or long-range communication. Contact communication requires the sender and receiver membranes to be connected through interfacial recognition biochemical elements[31] and therefore this communication is usually assisted by membrane proteins[32] or substrate/receptor systems.[1c] Short-range communication usually takes place when sender and receiver elements are enclosed within the same biological structure[33] or matrix;[34] Finally, long-range communication arises when these elements are spatially separated.[35] On the other hand, communication can also be categorized as direct or indirect. Direct communication involves that the signal from the sender element arrives and is elaborated straightforwardly by the receiver element.[36] Con-

versely, indirect communication involves the transduction (*e.g.* physical to chemical[37] or *vice versa*[38]) or transformation (*e.g.* chemical-chemical[39]) of an incoming signal by the receiving element.

Another important categorization involves the geometrical arrangement of synthetic and living elements participating in the interaction phenomenon.[30a] Possible arrangements include discrete populations within a physical substrate or an appropriate medium ensuring signal transmission,[35] or the encapsulation of the synthetic cell inside a living cell (or *vice versa*) resulting in the fabrication of a hybrid system where signals are elaborated within the host structure.[40] Finally, synthetic and living elements can be arranged in an extended network in which they are interconnected directly to one another by chemical or physical links.[41]

A further categorization is based on the chemical or physical element on which the synthetic cell/living cell interaction relies. In Nature, the type of communication employed for the most part depends on a small molecule chemical signal. This is also the case when interaction between synthetic and living elements are considered. However, a few literature examples also demonstrated the involvement of macromolecules such as DNA strands[4] or even mechanical,[42] thermal,[35] or luminous stimuli[8] for signal transmission between synthetic and living systems.

Finally, considering the integration of synthetic cells with natural substrates for tissue engineering, another distinction concerns the effect that the aforementioned interactions have on living cells, which may either lead to upregulation (*e.g.* promoting cellular differentiation[43] or giving growth support[44]) or downregulation (*e.g.* inhibiting protein formation[45] or tumor cell killing with therapeutic proteins synthesized *in situ*[46]) of specific biological mechanisms and reactions.

2.2. Examples of Synthetic Cell/Living Cell Interactions

Early endeavors in bottom-up synthetic biology focused on developing methodologies to recreate rudimental chemical communication pathways between protocells. Drawing inspiration from the natural world, scientists began to use biological machineries such as enzymes,[47] riboswitches,[39] membrane proteins,[48] and transcription factors[49] to trigger and control protocell-protocell signaling, primarily using chemical inputs, but also exploring the potential of using physical inputs such as light and temperature. Several of these methods have also proven effective in establishing communication between synthetic and living cells, with chemical signals being the predominant mode of interaction.

One of the first successful attempts of synthetic/living cell interaction was demonstrated by the group of B. G. Davis in 2009. They managed to compartmentalize a complex system performing the formose reaction[50] to mimic a carbohydrate protometabolism within a lipid vesicle made of a synthetic phospholipid. The carbohydrate-borate complex produced from this cycle diffused out of the membrane and was detected by the bacterium *Vibrio harveyi*, triggering its quorum sensing mechanism, a natural bacterial communication process.[51] The harsh conditions required for the protometabolism, including high formaldehyde concentration, high pH, and metal cations as catalysts, were successfully created and contained inside the vesicle to preserve bacteria viability.

A different type of mechanism to trigger a quorum sensing response was proposed by R. Lentini and coworkers.[39] They engineered synthetic cells in the form of phospholipid vesicles containing DNA, a transcription-translation machinery, and isopropyl b-D-1-thiogalactopyranoside (IPTG), a molecule sensed by the bacterium *Escherichia coli* to trigger its quorum sensing system. Externally added theophylline molecules could enter the lipid membrane, activate the riboswitch present in the DNA filament, and allow the expression of the gene that translates for the protein α-hemolysin. This protein would migrate to the lipid membrane, creating a pore through which the chemical signal (IPTG) could be excreted and reach the bacterium. In this way they have used the synthetic cell to translate a chemical message (theophylline to IPTG), enhancing the sensory ability of the bacterium without altering its genetic content.

A recent study on synthetic/living cell interaction employing a different quorum sensing system involved GUVs (sender) endowed with a DNA template and a cell-free protein synthesis (CFPS) machinery, along with engineered *E. coli* (receiver) containing complementary components of the quorum sensing system based on the acyl-homoserine lactone synthase from the bacterium *Bradyrhizobium japonicum* (BjaI/BjaR quorum sensing system).[52] Here, the UV light served as the trigger: upon excitation, a photocleavable block on the gene that hampers the expression of the BjaI synthase is removed and the protein can be synthesized within the GUV. With the precursors previously inserted inside the lumen and the newly formed BjaI protein, the synthetic cell could synthesize a chemical signal (*N*-isovaleryl-L-homoserine lactone), which passed the lipid membrane, was collected by the bacterium, and triggered a fluorescent response after interacting with the BjaR protein.

Advancements from bacteria to mammal cells interacting with synthetic cells under physiological conditions were successfully demonstrated by D. Toparlak and coworkers (Figure 1.1).[43] In this example, the protocell model comprised a vesicle made of phospholipids and cholesterol containing two DNA templates. Translation of these templates produced a brain-derived neurotrophic factor (BDNF) and perfringolysin O (PFO) proteins. BDNF regulates the development and function of the nervous system, while PFO can assemble in oligomers that form pores in the protocell membrane, from where BDNF can be released. PFO monomers synthesis required both LuxR protein and N-3-oxohexanoyl homoserine lactone (3OC6 HSL) molecules, which can be used as external trigger to prompt the artificial cell release the BDNF protein signal, thereby stimulating neuronal differentiation.

A promising form of protocell-cell communication only recently explored is the one mediated by light signals. This was achieved by combining together the fascinating phenomenon of bioluminescence[53] with light-sensitive biomolecules. A

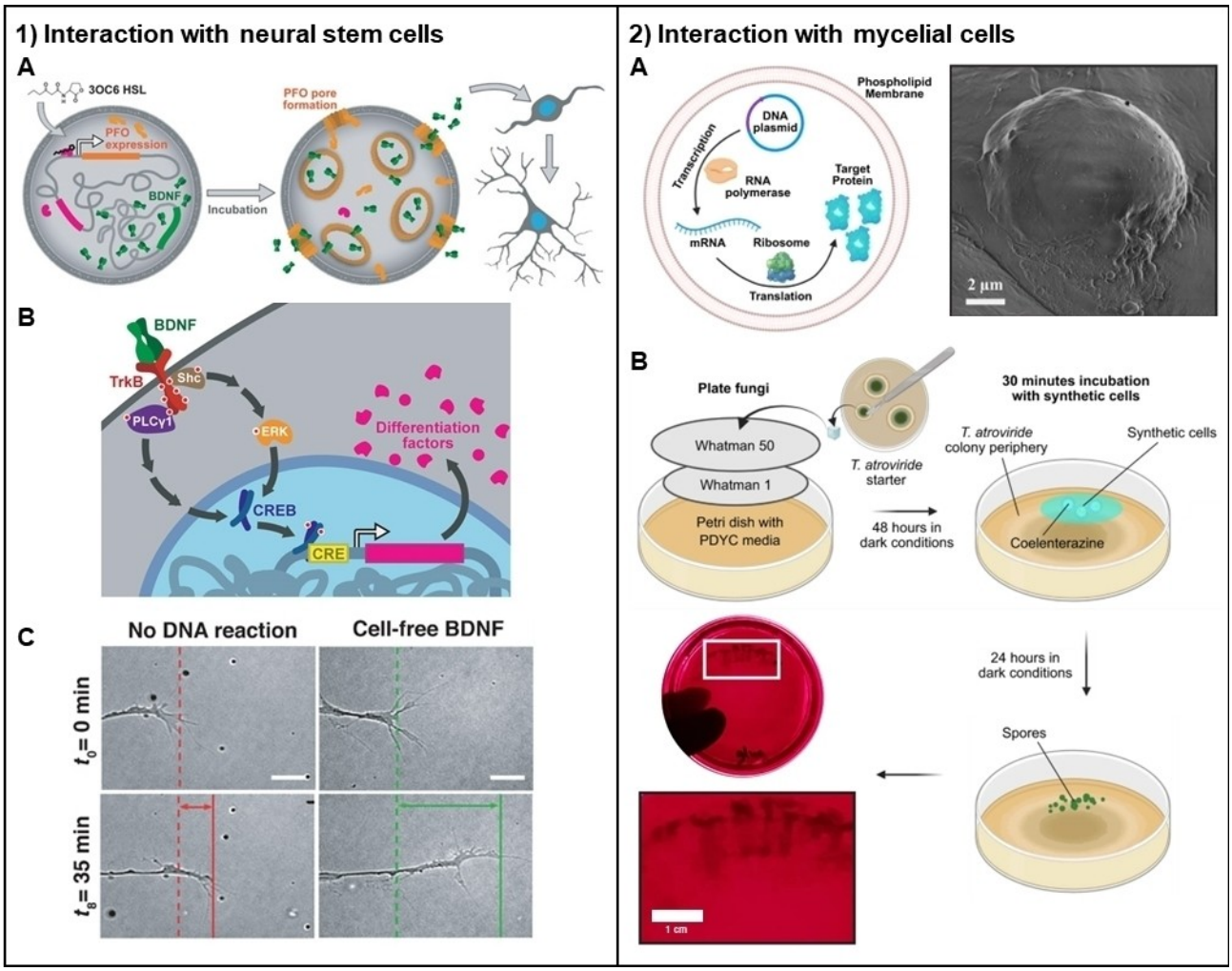


Figure 1. Examples of synthetic/living cells interaction (part 1). **(1)** Interaction of lipid-based protocells containing DNA and transcription-translation machinery with neural stem cells. A) Scheme illustrating the complete communication mechanism. B) Close-up of the transmembrane signaling pathway in the neural stem cells. C) Axons growth displayed in single axon live imaging indicating an increase in outgrowth velocity upon exposure to the growing factor. Scale bars, 10 μm. Adapted with permission from reference.[43] Copyright (2020) AAAS. **(2)** Interaction of light-generating lipid-based protocells with mycelial cells. A) Scheme of the liposomal synthetic cell and the internalized protein synthesis system (left) and the related cryo-SEM image (right). B) Illustration of the experimental setup with fungal cells incubated for 30 minutes in a Petri dish with light-generating synthetic cells (top and right), with images of the plate showing the spores generated after exposure to the light generated by the synthetic cells localized in the white rectangle (bottom left). Adapted with permission from reference.[36] Copyright (2022) Springer Nature.

recent study demonstrated for the first time the possibility of preparing lipid-based synthetic cells capable of sending bioluminescent intercellular and intracellular signals (Figure 1.2).[36] At the heart of this communication pathway lies the encapsulation of the bioderived machinery for bioluminescence within synthetic cells. Here, the activity of the luciferase enzyme converts the coelenterazine into its oxidized (luminescent) form. Intercellular signals triggered bioprocesses in natural cells of the fungus *Trichoderma atroviride*, where the blue light emitted by the synthetic cells was sufficient to induce the conidiation in its mycelial cells, thus initiating asexual sporulation. Instead, intracellular signaling employed luminous signals to self-activate the gene transcription of a DNA plasmid for protein expression inside the synthetic cell or to recruit tagged proteins on the membrane. This study is particularly important, as it demonstrated for the first time that protocells can be programmable light sources for controlling light-triggered intercellular and intracellular processes.

Interactions between synthetic and living cells can also occur when the two entities are in direct contact. This is a very common phenomenon in nature, as seen in virus infection, immune system response, or simply between neighboring cells within a living tissue.

For instance, in a recent study X. Xu and colleagues employed a particular self-assembly technique to create synthetic cells used to transfect 293 T cells, delivering mRNA sequences produced inside the protocell to express proteins inside the living receiver.[54] The protocell structure comprised phospholipids, polyoxyethylated triglycerides (Cremophor EL), and Matrigel, a gel mainly composed of extracellular matrix proteins.[55] These components created a droplet with a corona of lipid chains on its membrane. This self-assembled structure

was able to protect the encapsulated mRNAs and create a contact with the 293 T cell membrane, after which it was assimilated, degraded, and the cargo was released.

A more complex interaction system involving an invasion-defense loop was proposed by Y. Zhang and coworkers (Figure 2.1).[33] By using liquid coacervate microdroplets containing glucose oxidase (GOx), researchers induced hydrogen peroxide production inside human liver cancer cells (HepG2), which in turn led to internal damage following the production of reactive oxygen species (ROS). The destruction of the plasma membrane by ROS was connected to the leakage of cytosolic lactate dehydrogenase (LDH). Catalase enzyme (CAT) artificially introduced inside the cell acted as an internal defense mechanism, protecting cells from H_2O_2 by catalyzing its decomposition into water and dioxygen. In this work, protocell internalization (phagocytosis) inside living cells was initiated by electrostatic binding, being the coacervate formed with an excess of the polycation diethylaminoethyl-dextran hydrochloride (DEAE-dextran) with respect to the polyanion (DNA). Specifically, the presence of an excess of DEAE-dextran induced a positive charge on the surface of the coacervate micro-droplet facilitating the interaction with the negatively charged living cell membrane.

Synthetic and living cells can also be arranged in a hybrid system, where one is embedded inside the other. This interesting concept finds its roots in the endosymbiosis theory.[56] Depending on whether the protocell is enclosed within the living cell or *vice versa*, it is possible to create artificial systems supporting and augmenting the metabolism, or repairing living cells from the inside,[57] or also to have synthetic cells powered and sustained by internal bioreactors.

O. Staufer and colleagues investigated various synthetic compartments able to perform simple tasks in support of cell metabolism, akin to organelles within a living cell.[58] They prepared synthetic peroxisomes (oxidative organelles containing enzymes for H_2O_2 generation and scavenging), endoplasmic reticulum, or magnetosomes as GUVs that were subsequently taken up by cells through passive endocytosis, as the membrane of these vesicles results very similar to the one of natural organelles.[59] For instance, synthetic peroxisomes were prepared by encapsulating bovine catalase within phosphatidylcholine-structured vesicles and taken up via endocytosis by keratinocytes. Analogously as described in the last example, catalase enables the decomposition of H_2O_2, hence this synthetic organelle helped reducing ROS level, maintaining the redox homeostasis, and mitigating the oxidative stress. Furthermore, a synthetic calcium storage organelle containing a UV-sensitive Ca^{2+}-chelating molecule was prepared to mimic the natural functionality of calcium buffer of the endoplasmic reticulum or mitochondria inside a cell. This synthetic organelle released Ca^{2+} ions upon irradiation, regulating the cell calcium homeostasis. Finally, similarly as it happens in magnetotactic bacteria,[60] rat kidney fibroblasts were hybridized with magnetosome-like synthetic organelles bearing 50 nm diameter Fe_2O_3 nanoparticles which were able to confer the exotic feature of magnetic field sensing and cell migration to cells that do not naturally display this ability.

Increasing the GUV size allows for the reverse configuration, entrapping living cells inside vesicles. Y. Elani and coworkers[40] achieved this using a microfluidic system to incorporate various cell types inside a lipid-based compartment made from a bilayer of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine lipids (Figure 2.2). In particular, they studied how a single colon carcinoma cell within the GUV acted as an organelle-like reactor, transforming the internal feedstock of lactose molecules into glucose and working in tandem with a synthetic enzymatic cascade (GOx and horseradish peroxidase, HRP) contained in the same structure. The integration was successful thanks to the lumen inside the vesicles which created a viable and controlled environment and the structure itself that protected the cell from the external environment.

Another notable example involves the inclusion of living cells within synthetic cell architectures as proposed by K. Jahnke and coworkers.[61] In this work, they genetically engineered *E. coli* to overexpress xenorhodopsin (which is a light-driven inward-directed proton pump) and encapsulated these bacteria inside phospholipid-based GUVs. The researchers then used this construct to regulate the pH of the protocell lumen upon light exposure, activating an internal DNA-based nanomechanical switch capable of triggering the attachment of DNA origami on the outside of the GUV. This research showcased an efficient method to regulate some physical-chemical conditions within synthetic cells lumen and modify their membrane post-production.

To increase the number of interconnected elements and achieve both more complex pathways of communication and an advanced level of spatial organization of the communicating elements, some groups tried to establish chemical communication between synthetic and living cells arranged in 2D or 3D hybrid networks.

One pioneering example was presented by M. Schwarz-Schilling and colleagues. They employed water-in-oil droplets, stabilized by a surfactant and prepared by microfluidics, which were then inserted into a capillary to form a linear network (Figure 2.3).[62] Within this network, certain droplets housed bacteria while others contained CFPS systems. Aligned inside the capillary, each droplet interacted solely with its two neighboring droplets. This setup was used to demonstrate droplet-droplet interaction based on the diffusion of genetic inducers. To demonstrate this, the researchers engineered two distinct genetic circuits based on the LuxI/LuxR quorum sensing system: a "sender" circuit producing N-(3-oxo-hexanoyl)-L-homoserine lactone (AHL) with IPTG as the genetic inducer and a "receiver" circuit acting as an AND gate requiring both IPTG and AHL to express a fluorescent protein. Both IPTG and AHL could permeate the droplet membrane, diffusing into neighboring droplets. Additionally, they also inserted one circuit in the bacteria and the other one in the protocell, showcasing both "bacterial-to-cell-free" and "cell-free-to-bacteria" droplet communication.

A more sophisticated network of interacting cells and protocells within a 2D platform was proposed in 2020 by S. Mann's group (Figure 2.4).[41] They successfully fabricated arrays of GUVs and red blood cells using ultrasound standing waves

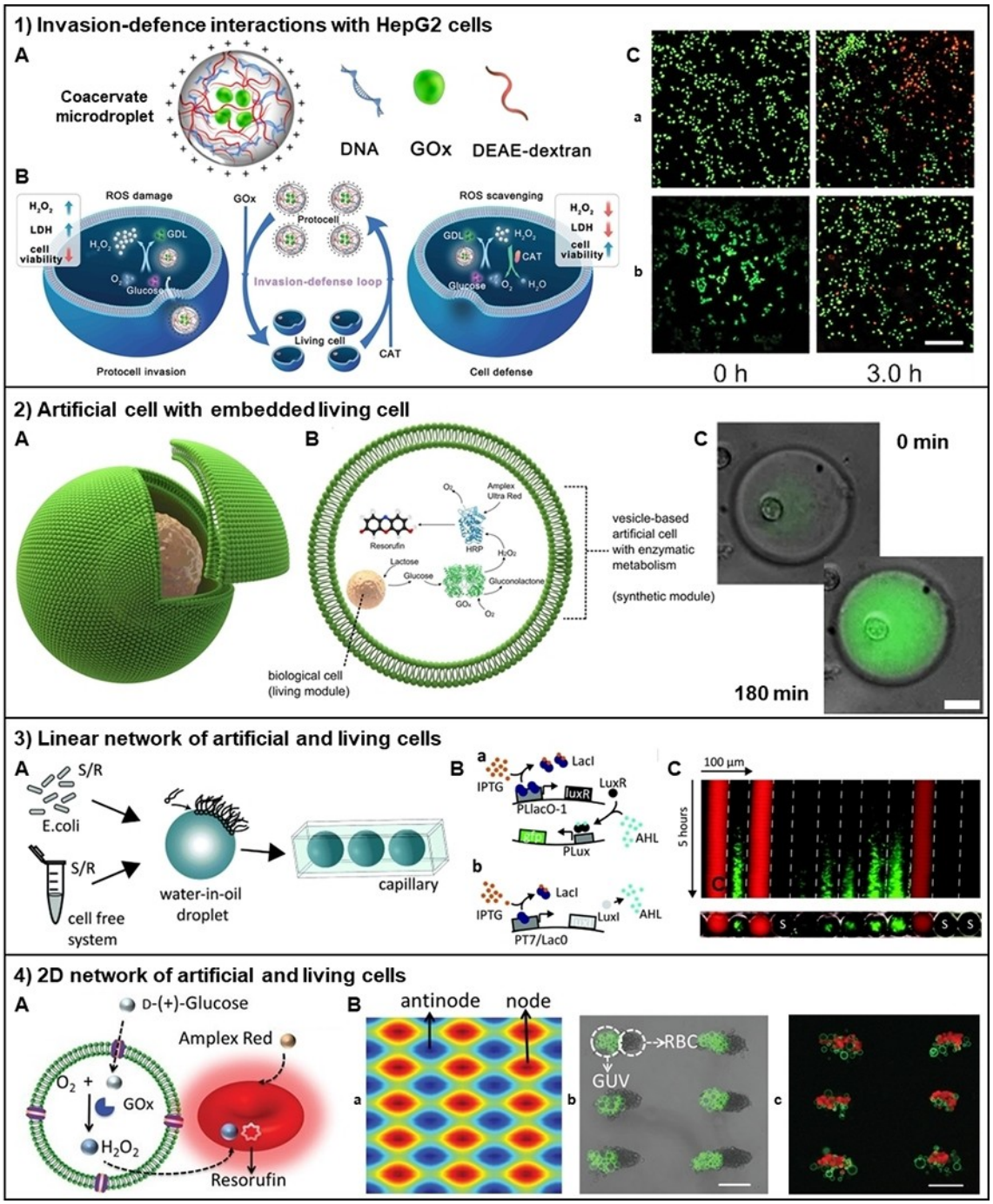


Figure 2. Examples of synthetic/living cells interaction (part 2). **(1)** Invasion-defense interactions between coacervate microdroplets and HepG2 cells. A) Scheme and composition of the coacervate microdroplet encapsulating GOx. B) Illustration of the invasion (left) and defense (right) mechanism between synthetic and living cells involving protocell internalization and intracellular H_2O_2 production, subsequently scavenged and neutralized by internal catalase (CAT). C) Fluorescence microscopy images of staining assays for live/dead (green/red) cells after exposure to protocells without (row a) and with (row b) defense mechanism enabled. Scale bar, 5 µm. Adapted with permission from reference.[33] Copyright (2020) Wiley-VCH GmbH. **(2)** GUV hybrid protocells with embedded BE colon carcinoma cells. A) Scheme of the living/synthetic hybrid cell. B) Illustration of the organelle-like function of the encapsulated cell performing the lactose to glucose hydrolysis as part of a multi-step enzymatic pathway inside the GUVs. C) Brightfield/fluorescence microscopy images showing the successful synthesis of the fluorescent resorufin product. Scale bar, 25 µm. Adapted with permission from reference.[40] Copyright (2018) Springer Nature. **(3)** Linear network of emulsion droplets containing bacterial cells or gene expression systems. A) Encapsulation of *E. coli* or cell-free protein synthesis (CFPS) system constituted by transcription/translation machinery inside droplets and preparation of the linear network. B) Overview of the genetic circuit of the receiver droplet containing the living cell (a) and the sender droplet containing the synthetic system (b). C) Fluorescence microscopy image kymograph showing the activation of receiver droplets (green) by means of sender (S) and inducer (red) droplets. Adapted with permission from reference.[62] Copyright (2016) The Royal Society of Chemistry. **(4)** 2D network of GUV and red blood cells. A) Scheme of the synthetic cell and the signal transduction mechanism. B,a) Simulation of the acoustic pressure distribution of high (blue) and low (red) pressure antinodes used in the acoustic trapping device to group the cell colonies, B,b) Brightfield/fluorescence microscopy image of the final network, and B,c) Fluorescence microscopy image of the 2D network of synthetic (green) and red blood cells (red) indicating the resorufin production. Scale bars, 50 µm. Adapted with permission from reference.[41] Copyright (2020) Wiley-VCH GmbH.

and making them interact within a two-step enzymatic cascade reaction. The sender vesicles were equipped with the GOx enzyme and the pore-forming cationic peptide melittin for the harvesting of glucose molecules from the external environment. Meanwhile, the receiver red blood cells naturally acted as peroxidase agents[63] thanks to the internal hemoglobin, intaking and processing the H_2O_2 produced and Amplex Red molecules to generate the fluorescent product resorufin as output.

These examples collectively underscore that the technology to "match" synthetic and living cells already exists and that the biochemistry of natural metabolisms is sufficiently well-known for us to build simplified models replicating natural functions. With the aim of developing applications in tissue engineering and regenerative medicine, the focus now needs to shift towards the design and fabrication of synthetic tissues that serve as advanced substrates for living cells and tissue manipulation and growth.

3. Increasing Complexity: Prototissue Engineering

Just as living cells aggregate to form living tissues, protocells can be organized into prototissues or synthetic tissues. Within a prototissue, protocells can be envisioned as volumetric pixels (voxels) of the material, where each protocell functions as an independent chemical reactor, capable of emitting chemical signals with precise spatiotemporal control. While individually addressing and manipulating each voxel remains nontrivial, what is even more important is that the overall functionality of a prototissue should transcend that of its constituent protocells. In fact, prototissues developed thus far often exhibit collective emergent properties through inter-unit interactions,[64] for example long-range communication, macroscopic deformation, signal propagation and enhanced chemical gradients sensing.[65] This significance is particularly notable in the field of tissue engineering, where innovative biocompatible materials featuring chemically programmable microcompartments hold the promise to revolutionize the field. Prototissues could significantly elevate the complexity of the material used as a substrate for cell and tissue culture, potentially facilitating the growth of complex tissues (such as liver and brain), currently beyond the capabilities of existing technologies.[66] However, before achieving complete integration with living tissues, several major challenges must be addressed, including constructing larger prototissues, developing different types of communication and interaction pathways within the prototissue and living cells, and modulating the mechanical properties of prototissues.[3d] Nevertheless, a few pioneering examples of prototissues have recently emerged in the literature. The following section aims to gather those examples and examine their distinctive features, starting with the nature of the protocell building blocks and the way in which these units are interconnected to form a self-standing material and progressing to the assembly methods and the life-like functions they are capable of replicating. Our objective is to highlight strengths and weaknesses of these innovative constructs and analyze the essential steps needed to apply prototissues to advanced tissue engineering and guide their future development.

Arguably, the most remarkable feature of synthetic tissues as opposed to bulk hydrogels, materials or aggregates is their modularity, that is, they are assembled from populations of protocells with different phenotypes (compositions and functions). This concept is best understood by drawing an analogy with biological tissues. These chemically programmable microcompartments, like living cells in biological tissues, serve as "building blocks". Over the past few years, several research groups have tackled the challenges of identifying suitable protocell building blocks and interconnecting them through (bio)chemistry to create freestanding and functional tissues, employing both wet chemistry and more engineering-oriented approaches.

For example, H. Bayley's group has demonstrated the possibility of programming the composition of synthetic tissues made of thousands of interconnected lipid-based protocells with single protocell resolution using a custom-made 3D-printing setup (Figure 3.1).[67] These protocells are essentially water droplets in oil encapsulated in lipid monolayers which are kinetically stabilized by the formation of lipid bilayers at the interface between two droplets (droplet-interface bilayer, DIB) once they come in contact.[19,68] This enables the creation of a cohesive 2D or 3D material as a droplet network directly on a glass surface or in an oil drop suspended in a bulk aqueous solution. This network comprises numerous microcompartments capable of communicating through membrane proteins to transmit an electric signal via an ionically conductive pathway. Furthermore, by setting different osmolarity between adjacent layers of droplets, it is possible to induce macroscopic deformation of the synthetic tissue – the layers shrink or swell complementarily until reaching equilibrium through an osmotic flow of water, while the structure folds.

Subsequently, the same research group took an important step towards developing a functional mimic of neuronal transmission.[8] In this case, DIB-type 3D-printed tissue-like materials were composed of synthetic cells as aqueous compartments separated by lipid bilayers endowed with an internal light-activated transcription-translation system. The researchers prepared a gene with the promoter segment blocked by photocleavable biotin-streptavidin complexes, removable upon UV irradiation. Upon light exposure, the gene could be expressed, leading to the synthesis of α-hemolysin pore-protein which created junctions between neighboring liposomes, allowing the passage of an electric current through the synthetic tissue.

Spheroidal prototissues were assembled by P. Gobbo and coworkers using a double emulsion technique for the preparation of protocells and their interconnection into a robust structure.[9] They prepared colloidosome-type protocells exploiting a peculiar amphiphilic protein-polymer nanoconjugate and employing the Pickering emulsion technique.[69] The protein-polymer nanoconjugates comprised a poly(*N*-isopropylacrylamide)-based thermoresponsive polymer conjugated to a cation-

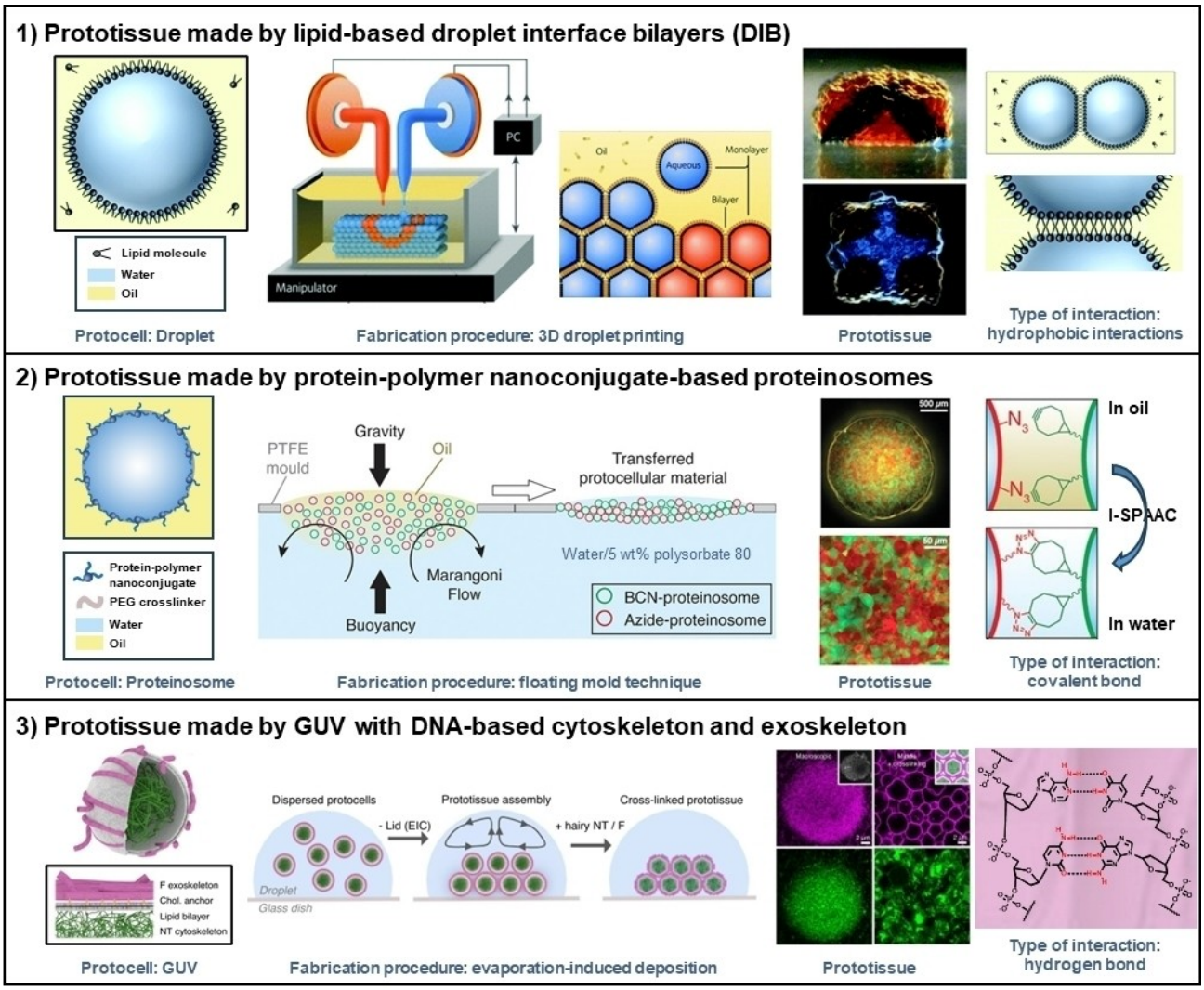


Figure 3. Relevant examples of synthetic tissues. **(1)** Prototissue prepared from lipid-based droplets using an aqueous 3D droplet printer and exploiting hydrophobic interactions between the lipid molecules to form droplet interface bilayers (DIBs). Adapted with permission from reference.[19] Copyright (2017) The Royal Society of Chemistry. **(2)** Prototissue prepared from protein-polymer colloidosomes using the floating mold technique and exploiting covalent protocell-protocell linking via the interfacial strain-promoted alkyne–azide cycloaddition (I-SPAAC) reaction. Adapted with permission from reference.[71] Copyright (2021) Wiley-VCH GmbH. **(3)** Prototissue prepared from giant unilamellar vesicles (GUV) using an evaporation-induced convection (EIC) technique and exploiting base-pairing interactions between the DNA strands forming protocell cytoskeleton and exoskeleton. Adapted with permission from reference.[73] Copyright (2023) Springer Nature.

ized bovine serum albumin, and were further functionalized with either azides or strained alkynes. The spheroidal prototissues were then prepared as water-in-oil-in-water double Pickering emulsions from a 1:1 mixture of azide- and strained alkyne-functionalized colloidosomes as water-in-oil droplets. Subsequent removal of the inner oil phase through dialysis against ethanol/water triggered the bio-orthogonal interfacial strain-promoted alkyne-azide cycloaddition (I-SPAAC)[70] between the complementary reactive colloidosome membranes. Notably, their study showed that the thermoresponsive properties of the interlinked colloidosomes could be collectively harnessed to generate prototissue spheroids capable of reversible contractions. These contractions could be enzymatically modulated and exploited for mechano-chemical transduction, defined as the conversion of a mechanical stimulus (contraction) into the down/up-regulation of an enzyme cascade reaction hosted within the colloidosome that composed the prototissue.

In a subsequent endeavor, A. Galanti and colleagues developed a floating mold technique to assemble millions of azide- and strained alkyne-functionalized colloidosomes into free-standing millimeter-size prototissues, exploiting always the I-SPAAC reaction (Figure 3.2). In brief, they injected an emulsion containing a 1:1 population of azide- and strained alkyne-functionalized colloidosomes inside a PTFE mold floating on an aqueous solution of a surfactant. The formation of a surface tension gradient between the bulk oil phase of the emulsion and the bulk aqueous phase generated a Marangoni flow. Assisted by the surfactant, this flow progressively removed the oil phase, bringing the reactive colloidosomes in contact, and

allowing the formation of the protocellular material (PCM), which concurrently was transferred to water. Using the floating mold technique, they could generate prototissues with complex 3D architectures. Remarkably, this technique also enabled the creation of 2D periodic arrays of prototissues displaying an emergent non-equilibrium spatiotemporal sensing behavior. These arrays were capable of collectively translating the chemical information provided by the external environment and encoded in the form of propagating reaction-diffusion fronts into an optically readable dynamic signal output.[71]

J. R. Burns' group introduced one of the latest examples in the literature concerning prototissues. Starting from the preparation of classic GUV protocells, the researchers integrated oligonucleotides that spontaneously assemble into DNA nanotubes (NT) or fibers. The oligonucleotides endowed the protocells with biomimetic inner and outer skeletal frameworks, improving a DNA-based self-assembly technology recently applied to protocells to mimic the complexity of the natural cytoskeleton (Figure 3.3).[72] The prototissue was formed via evaporation-induced convection (EIC) of a suspension of protocells, where adherence was reached via weak electrostatic attractive forces exerted by the exoskeleton. Crosslinking of the units was ensured upon the addition of DNA fibers featuring a single-strand region complementary to those present in the DNA fibers constituting the exoskeleton. Anticipating potential biomedical applications, this variety of prototissue possesses several compelling attributes: a) individual protocells demonstrate non-toxicity to human blood cells under serum-free conditions, b) the location and alignment of the cytoskeleton they contain can be manipulated via lipophilic interactions or magnetism, and c) the same cytoskeleton can be used to immobilize smaller vesicles, which, in turn, are stabilized against human serum by the cytoskeleton and the protocell membrane, effectively acting as an exoskeleton.[73]

Another recent example of prototissues has been proposed by Y. Ji and colleagues. They developed a functional assembly of membranized coacervate protocells enclosed by a layer of rigid polysaccharides to enhance stability and permeability for chemical signal exchange. These protocells could be further structured into tissue-like assemblies exploiting hydrogen bonding.[74] Moreover, the group of X. Han has focused on synthetic tissues made of GUVs, demonstrating that these building blocks can be assembled using either electric[75] or magnetic stimuli.[13,64]

While the breakthroughs reported above trace a promising trajectory for prototissues development, each system presents drawbacks. Specifically, (i) efforts are still required to further advance the integration of prototissue with living organisms; (ii) research should continue to focus on developing new building blocks and expand their chemical versatility; (iii) methods to generate more detailed and complex 3D architectures should be developed, possibly with the help of microfluidics and/or 3D printing. Nonetheless, prototissues should also show sufficient robustness and shelf-life to ensure ease of manipulation and guarantee their employment even after prolonged storage. The prototissue models developed thus far have their advantages and disadvantages. For example, prototissues based on DIBs require a specific and custom-made 3D-bioprinter for their formation and, although stable, they may be somewhat fragile. To address this, researchers are currently experimenting to find the optimal conditions of lipid types and concentration, oil phase, droplet size, inter-droplet contact angle, and temperature to prevent issues such as water loss, shrinkage, and collapse, while maintaining the stability of the bilayer, a sufficient molecular exchange rate between DIBs, and the outstanding single-protocell resolution achieved through the 3D-printing technique. Conversely, spheroids and protocellular materials made of bio-orthogonally reactive colloidosomes exhibit significant chemical and mechanical stability due to the covalent I-SPAAC reaction employed for bonding protocells together. Moreover, they offer the possibility to further expand the range of physical-chemical stimuli that can be used to trigger advanced behaviors using stimuli responsive materials, such as thermoresponsive polymers. Moreover, they can be fabricated with various shapes using custom-made molds. However, since these prototissues are formed through random mixing of their building blocks, it is still very difficult to control the spatial organization of protocells with high precision. Finally, prototissues made of protocells endowed with DNA-based nanofibers for synthetic cytoskeleton and exoskeleton display considerable stability in osmotic stress conditions and advanced complexity of structural internal organization. Furthermore, the fiber assembly can be easily tuned requiring only a minimum set of building blocks. However, interactions between the protocells composing this prototissue and living cells or the external environment have not yet been demonstrated, which we believe is a crucial requirement for complete integration and communication between synthetic/living tissues.

In conclusion, with the goal of achieving future applications in tissue engineering and regenerative medicine and in contrast with bulk substrates or amorphous vesicle aggregates that fail to show complex functionalities, we think that a prototissue, to be defined as such, should:

1) Present a multi-micro-compartmentalized structure;
2) Be capable of promoting adhesions with both protocells and living cells;
3) Present communication both between its constituent units and the external environment;
4) Display higher-order behaviors;[65]
5) Be chemically and mechanically designed to provide specific spatiotemporal cues to living cells.

4. Past, Present, and Future of Tissue Engineering

Tissue engineering is an innovative field whose aim is the preparation of artificial (living) tissues able to function as native tissues through the manipulation of cells, small molecules, and materials (biological or synthetic).[10] For this reason, tissue engineering is a multidisciplinary field that bridges chemistry, biology, medicine, and engineering. It intervenes when native

tissues or organs suffer severe disease or damage from conditions like cancer, congenital anomaly, or trauma when conventional pharmaceutical treatments are no longer applicable. In such cases, organ transplantation or artificial tissues and organs growth become the sole options for replacing or reconstructing the unusable ones.[76] To assemble artificial tissues or organs, the approach employed in this field is to provide a suitable substrate or scaffold to substitute or help the growth of living cells from the patient, forming tissues recognized by the receiving organism. In this context, the need for materials and structures specifically designed to be biocompatible and able to be successfully integrated with living cells has made scaffold design and fabrication the major area of biomedical research in this field. As a matter of fact, over the past two decades, a huge deal of work has been dedicated to the development of scaffold materials for tissue engineering.[77]

Synthetic tissues or prototissues hold great potential as substrates for tissue engineering considering that, ideally, they could be specifically designed to mimic functions, structures, and properties of living tissues. However, the technology for preparing and modifying prototissues is still in its infancy and there are still many challenges for researchers aspiring to employ prototissue engineering in tissue growth.[78] For example, important challenges that need to be tackled include cell adhesion and integration, biocompatibility, and immunogenicity, the small size (ranging from few μm^2 to few mm^2) of current synthetic tissues, and the lack of complex natural structure and functions, such as vascularization or repairing systems.

We believe that reviewing the main concepts behind the development of efficient scaffolds for tissue engineering will offer valuable insight into designing the next generation of protocells and prototissues. In the following section, we collect a selection of biocompatible scaffolds based on the most common materials used in the literature. We explore the strategies for their preparation and the characteristics contributing to their successful integration within tissue engineering. Additionally, we delve into a particular conceptual advancement in this field regarding the use of stimuli provided by the synthetic substrate for cell growth, manipulation, and differentiation. Here we highlight some promising examples demonstrating how scaffolds and biomaterials can interact with living cells and tissues through chemical and physical stimuli. Finally, we introduce some methods to stimulate cells mechanically using light, which holds particular promise for manipulating cell orientation and promoting their differentiation, thanks to the spatial resolution, speed, and repeatability of light as an external trigger.

4.1. Biocompatible Scaffolds

Tissue engineering relies heavily on scaffolds, 3D porous solid biomaterials pivotal for tissue regeneration and repair. These scaffolds are designed to perform some or all of the following functions: a) promote cell-biomaterial interactions, cell adhesion, and extracellular matrix (ECM) deposition, b) permit sufficient transport of gases, nutrients, and regulatory factors essential for cell survival, proliferation, and differentiation, c) biodegrade at a controllable rate that matches tissue regeneration under the culture conditions of interest, and d) provoke a minimal degree of inflammation or toxicity *in vivo*. As such, an ideal scaffold should possess very specific characteristics in terms of size, shape, strength, degradation rate, porosity, and microstructure.[79]

In tissue engineering, substrate preparation can be performed following two primary approaches, akin to those in synthetic biology. The top-down approach involves obtaining scaffolds from living tissue, removing cells and preserving native ECM architecture, organization and composition through decellularization-based methods.[80] The final tissue-derived scaffold retains specific features like the spatial organization, 3D microarchitecture, unique ECM protein composition, trapping of chemokines and soluble factors, *etc*.[81] Conversely, the bottom-up strategy takes advantage of minimal hybrid systems based on synthetic biomaterials functionalized with biomolecules that can pattern and organize the interacting cells and deform their shape and conformation under controlled exogenous stimulations, opening the door for the real spatiotemporal control of bio-constructs. These scaffold-like structures are designed to mimic the *in vivo* environment by presenting specific ligands/proteins, integrin-like or cadherin-like motifs, and precise tension and strain distribution in a time-dependent manner.[82]

In this scenario, the materials predominantly adopted in scaffold fabrication include polymers, bioceramics (*e.g.* calcium phosphate, aluminosilicates, zirconia ceramics), and other hybrid materials. Within polymers, both natural (*e.g.* chitosan, collagen, fibrin) and synthetic (*e.g.* polyglycolic acid, polycaprolactone, and polylactic acid) can be used, offering distinct advantages. Natural polymers are usually more biocompatible and less immunogenic, while synthetic polymers present higher mechanical stability, improved cell attachment (depending on the choice of functional side-groups), and can be shaped even in complex forms comprising small details and features that require high precision like channels and undercuts.[83]

Tissue-specific hydrogels, derived directly from the native tissue of interest, are another popular choice as supportive materials and ECM-like scaffolds.[84] Although hydrogel formulations often lead to the loss of structural complexity and organization of the decellularized ECM-derived (dECM) scaffolds, this type of biomaterial is considered a good compromise between functionality and biocompatibility. This is because dECM retains tissue-specific features, such as specific proteins like collagen and laminin. The compositional complexity of the dECM hydrogel is challenging, if not impossible, to replicate synthetically by combining proteins, chemicals, and different soluble factors with bottom-up approaches. Despite this, the stability and versatility of these hydrogels are not yet fully optimized and require refinement to meet clinical grade and standardization criteria. Consequently, researchers are starting to explore new ways to functionalize and control tissue-derived hydrogels, improving their biological and biomechanical properties. This includes addressing common issues like inadequate stiffness and resistance, as well as achieving fully integrated *in vitro* post-processing cultures.[85]

4.2. Stimuli-Driven Tissue Engineering

In recent years, with the advent of affordable 3D bioprinting techniques, researchers have been developing and refining novel approaches that move beyond the traditional view of tissue engineering. Rather than seeing the scaffold solely as a substrate for cell growth and differentiation, it is now recognized as an active player capable of controlled interaction with cells over time. Scientists are currently exploring innovative smart hybrid biomaterials designed to mimic the native ECM, aiming to deliver various stimuli to the growing tissue. These advanced scaffolds are expected to dynamically adjust their properties and features in defined and organized spatiotemporal arrangements influencing cell interactions, maturation, and organization. The evolution of this field has started to be called stimuli-driven tissue engineering (Figure 4).[86]

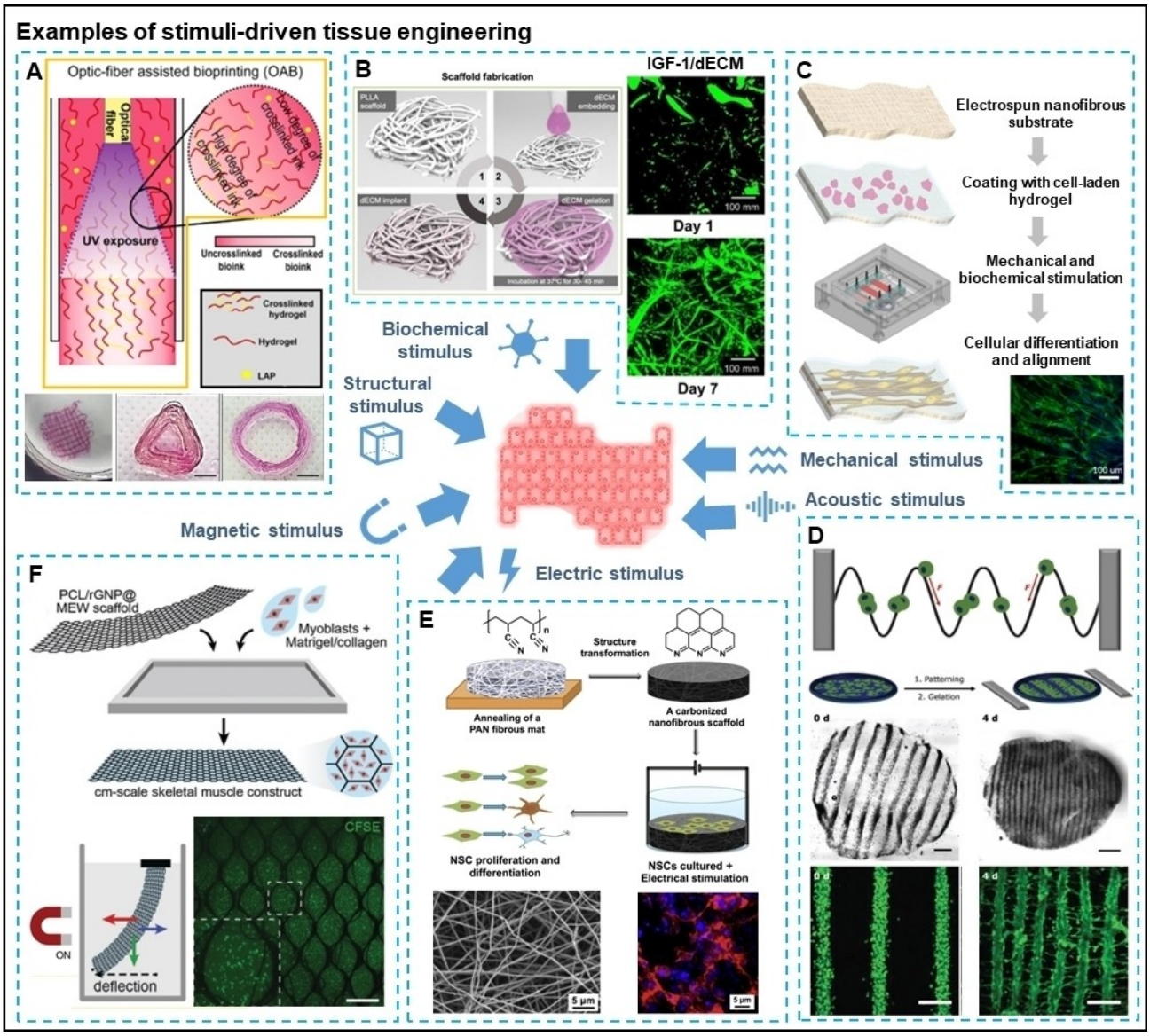


Figure 4. Examples of stimuli-driven tissue engineering. **(A)** Bioprinted photo-crosslinked hydrogels for muscle tissue restoration under structural alignment and topographical cues. The nozzle is designed with a grooved surface to induce uniaxial cell alignment upon extrusion, while optical-fiber-assisted crosslinking allows the preparation of asymmetric crosslinked portions of the hydrogel and different shapes. Scale bars, 1 mm (left) and 5 mm (center, right). Adapted with permission from reference.[92] Copyright (2023) Springer Nature. **(B)** Muscle-specific scaffold for tissue regeneration infused with a myogenic factor (IGF-1) which gives the biochemical stimulus to induce myoblast proliferation and differentiation (right). Adapted with permission from reference.[93] Copyright (2020) Elsevier Inc. **(C)** Mechanical stimulation of mesenchymal stem cells on 3D multilayered composite scaffold. Adapted with permission from reference.[98] Copyright (2019) ACS. **(D)** Acoustic myoblasts patterning with ultrasound standing waves in collagen hydrogels, showing retaining of the patterned configuration even upon matrix contraction after 4 days (bottom). Scale bars, 500 μm (brightfield) and 200 μm (fluorescence). Adapted with permission from reference.[99] Copyright (2018) Wiley-VCH GmbH. **(E)** Neural stem cell proliferation and differentiation upon electrical stimulation on carbon nanofibrous scaffold. SEM micrograph of the annealed scaffold (bottom left) and immunofluorescence micrograph of differentiated NSCs after electrical stimulation (bottom right). Adapted with permission from reference.[100] Copyright (2017) Elsevier Inc. **(F)** Magneto-active microfiber scaffold for remote stimulation of myoblasts. Epifluorescence microscopy image of labeled myoblasts encapsulated in the magnetic scaffold (bottom right). Scale bar 1 mm. Adapted with permission from reference.[103] Copyright (2023) Wiley-VCH GmbH.

These strategies can be applied to all tissues and organs, with particular relevance in skeletal muscle tissue engineering. In this field, high levels of spatiotemporal organization and communication between different biological components – including myoblasts, fibroblasts, myofibers, ECM, and others – are crucial. These components work together to develop, maintain, and repair muscle tissues. During *in vivo* muscle homeostasis, myoblasts must coordinate with each other and with the surrounding environment to respond to mechanical stimuli. Muscle cells perceive local tension, strain direction, soluble factors, chemical cues, assessing the state of the surrounding ECM. Consequently, they selectively activate specific signaling pathways when there has been tissue damage or simple cell turnover.[87]

Historically, the top-down approach has been the predominant methodology used globally to obtain starting biological material for general tissue engineering applications, including skeletal muscle repair. Scaffolds produced through decellularization were believed to provide sufficient stimulation to prompt cells to engraft and exhibit behaviors akin to that in the physiological environment. However, since the groundbreaking work of H. Vandenburgh and S. Kaufman in 1979,[88] it has become evident that, in addition to scaffold guidance, structure, and biochemical properties, exogenous mechanical and/or electrical stimuli that mimic physiological functions of muscular tissue are essential for achieving mature and organized tissue-like structures.[89] These stimuli are sensed by cells, leading to higher levels of organization, alignment, maturation, and ultimately contractile activity. Typically, such cells are cultured *in vitro* using specific bioreactor systems.[90]

Innovatively, W. Kim and coworkers combined skeletal muscle-specific dECM hydrogels with methacrylate modifications to enhance scaffold stability via photo-crosslinking.[91] They used 3D printing of cell-laden structures to control myotube and muscle formation. Their study showed that the combination of biochemical cues from the ECM and mechanical stability from methacrylate crosslinking synergistically increased myoblast coordination and maturation, outperforming single hydrogel formulations.

Moreover, in a recent publication, J. Y. Lee and colleagues optimized the printing method using optical fiber-assisted 3D bioprinting with *in situ* photo-crosslinking of gelatin-methacrylate (GelMA) or skeletal muscle dECM-methacrylate bio-ink to produce a shape-morphing hydrogel that boosts skeletal muscle maturation over time (Figure 4A).[92] Additionally, the group of S. J. Lee developed a muscle-specific dECM hydrogel doped with insulin growth factor-1 (IGF-1), an essential myogenic factor for cell migration and proliferation (Figure 4B). They incorporated the hydrogel solution within a supportive poly(L-lactic acid) (PLLA) scaffold network, showcasing how this strategy enhances cell recruitment and support both *in vitro* and *in vivo*.[93] Interestingly, this approach has already been exploited with synthetic cells capable of synthesizing recombinant growth factors on demand in order to trigger tissue angiogenesis and regeneration.[94]

Tissue ECM-derived hydrogels are extensively studied and employed in other types of organ regeneration. B. Kang and coworkers reported a bioprinting process based on dECM-based hydrogels from different porcine tissues (heart, liver, colon), mixed and loaded with ruthenium/sodium persulfate (Ru/SPS) photo-initiator fragments to obtain a dual crosslinkable bioink by either photo- or thermal-activation.[95] By using the same photo-initiator system, J. Jang's group obtained photoactivatable ECM-derived hydrogels from corneal and cardiac tissues.[96] Other photo-initiators, such as Eosin-based crosslinkers in ECM-derived hydrogel from smooth muscle mucosa, are also under investigation for similar purposes, as reported by S. Yeleswarapu and coworkers.[97]

Following the bottom-up approach, researchers are designing from scratch scaffold-like hybrid systems to mimic the *in vivo* environment, presenting biomolecules and precise patterns of strain and tension cues. For instance, C. Rinoldi and coworkers fabricated a polymeric multilayered nanofibrous scaffold by electrospinning that was capable of mimicking the native ECM.[98] On top of that, they deposited a thin layer of cell-laden hydrogel containing mesenchymal stem cells. The objective was to induce tenogenic differentiation, and this was accomplished by incubating the hybrid scaffold in a bioreactor designed to provide both a biochemical stimulus in the form of the growth factor bone morphogenetic protein 12 and a mechanical stimulus in the form of periodic stretching to mimic the natural function of tendons and promote cell alignment and differentiation (Figure 4C).

Remarkably, the group of M. M. Stevens showed how skeletal muscle cells can be organized and patterned upon external stimulation, eliminating the need for scaffold patterning and guidance, by using simple ultrasound standing waves to dispose and arrange cells inside supportive hydrogels (Figure 4D).[99] More specifically, they arranged myoblast populations within different formulations of Collagen 1 or GelMA hydrogel to obtain aligned muscle fibers based solely on the initial pattern disposition of the myoblast cell population. This occurs without material cues or guidance during engraftment and culture. This patterning approach can be very useful to induce and coordinate the cells' location, quantity, and distribution.

Another form of external stimulus capable of promoting cell differentiation is electric stimulation (Figure 4E). W. Zhu and colleagues created an electrospun substrate composed of polyacrylonitrile (PAN), which, after annealing, transformed into a conductive carbon nanofibrous scaffold suitable for culturing neural stem cells (NSCs). Through electrical stimulation, they induced cell proliferation and neuronal differentiation and maturation, resulting in various phenotypes depending on the electrical stimulus.[100]

S. Miao and coworkers further explored the concept of stimulation and crosstalk between synthetic smart materials and cells. They proposed shape-memory 3D-extruded smart biomaterials made of polyvinyl alcohol filaments that form a scaffold for seeding mesenchymal stem cells.[101] The anisotropic features and the tunable shape-memory ability ultimately generated topographical signals that directed cell alignment and enhanced myogenic differentiation. In a different way, the group of S. R. Shin adopted a co-axial bioprinting/wet-spinning

technique and photolithography to locally control myogenesis and/or fibroblast differentiation.[102] By encapsulating stem cells within GelMA hydrogel and modulating its stiffness and concentration of growth factors, they achieved spatial differentiation. In this way, they obtained a multilayered organized tissue-like structure composed of myofiber bundles surrounded by connective tissue, closely resembling native tissue.

Another technique, melt electro-writing (MEW), was employed to produce a synthetic scaffold for magneto-mechanical stimulation and architecture-guided organization of skeletal muscle cells, as reported by G. Cedillo-Servin and coworkers.[103] The meshes comprised polycaprolactone- and magnetic reduced graphene nanoplatelets-based (rGNP@) composite fibers that can be filled with myoblast in a hydrogel formulation (Matrigel/collagen) to create a centimeter-scalable skeletal muscle construct organized, reinforced, and stimulated by the magneto-responsive supportive scaffold (Figure 4F).

Overall, these examples illustrate that the literature is already rich in various biocompatible scaffolds for cell culture, ranging from those made directly from extracted ECM to hybrid ECM-based substrate and synthetic ECM-like scaffolds. The turning point here, which will propel the field of tissue engineering forward, is the capability of these substrates to provide specific stimuli or combinations thereof to precisely guide the growth and differentiation of cultured cells. It has already been demonstrated that stimuli are tissue-specific, and different cell lines may respond more favorably to certain cues than others. Therefore, in the future, it will be of paramount importance to select the appropriate scaffold to ensure both proper compatibility and stimulation.

4.3. Shining Light on Mechanical Interactions

Administrating static and dynamic mechanical cues is an important method for stimulating living cells and directing their growth and differentiation toward specialized tissue formation. It is now widely recognized that the mechanical properties of the ECM regulate essential cell behaviors, through a cascade of signaling events known as mechanotransduction.[104] Mechanotransduction has garnered considerable attention in recent years due to its established role in tumor development and progression,[105] as well as its potential applications in regenerative medicine. For instance, studies have shown that mechanical stimulation can drive mesenchymal stem cell differentiation[106] or that cardiac fibroblasts are capable of using internal mechanosensors to detect substrate stiffness variations.[107] Consequently, efforts are currently underway to develop methods for providing cells with dynamic mechanical stimulation in controlled *in vitro* experiments, ranging from single-cell micro-manipulation[108] to 2D cell sheet culture –[109] the latter being the most realistic model of mechanical strain to which cells are subjected *in vivo*. However, substrates for cell sheets that enable dynamic mechanical stimulation of living cells still lack precise spatiotemporal control over cell stimulation. Light emerges as an ideal candidate for mechanical cell stimulation. Indeed, light can provide a remote stimulus with high spatiotemporal resolution to induce the movement of soft micro-actuators that, in turn, deliver localized mechanical stimuli to living cells.

Recently, several intriguing examples of smart, light-responsive materials and arrays of micro-actuators have been reported to be successful for administering dynamic mechanical signals to living cells. Among the earliest examples is a study by J. Aizenberg's group, where they developed an array of elastomeric micropillars that could be remotely actuated by a light-triggered poly(*N*-isopropylacrylamide)-gold nanorods (p-(NIPAM)-AuNR) nanocomposite hydrogel (Figure 5.1). This array was then used as a cell culture platform.[110] The actuation of the hydrogel stems from the synergistic effect of the thermoresponsive nature of the p(NIPAM) polymer network and the photothermal effect exhibited by gold colloids. The gold colloids generate heat locally upon absorbing light at an energy corresponding to their surface plasmon resonance peak, inducing a volume phase transition in the p(NIPAM) hydrogel, resulting in its reversible localized contraction. Using such arrays, the authors applied uniaxial tension to murine mesenchymal stem cells and successfully demonstrated cell viability following the mechanical stimuli. Notably, this stimulation was administered reversibly for the first time using a highly localized light trigger.

Y. Chandorkar and colleagues subsequently introduced photothermally actuated hydrogels for cyclic mechanical cell stimulation, demonstrating the fabrication of patterned p-(NIPAM)-AuNR substrates capable of exerting mechanical forces on living cells upon beating at frequencies up to 10 Hz.[111] In their study, murine fibroblasts underwent repeated cyclical actuation using photoresponsive hydrogels as culture substrates. Comparison between stimulated and unstimulated cells confirmed that living cells sensed the mechanical strain resulting from substrate deformation. Stimulated cells exhibited changes in the formation of focal adhesions and migration rates compared to those grown on unactuated samples. In another study, the same photo-actuating hydrogel substrate was coupled with a NIR micro-projection system to provide mechanical stimulation to C2 C12 myoblast cells in localized areas demonstrating high spatial and temporal resolution.[112] The study revealed that cells respond differently to short-term (5 hours) and long-term (17 hours) actuations. Short-term actuation led to increased cell proliferation and migration, after which this effect is reversed, and cells show an increase in area linked with the lower ECM secretion. The authors hypothesized a link to cell differentiation, indicating that the continuous actuation has a "training" effect on cells. In a subsequent study, the same authors employed the photo-actuating p(NIPAM) hydrogel system with human mesenchymal stem cells, demonstrating that mechanical stimulation effectively modulated their fate towards osteogenesis without the use of biochemical differentiation factors.[113]

The p(NIPAM)-AuNR nanocomposite hydrogel represent just one example of light-triggered actuating system used in cell mechanical stimulation. For instance, stimuli-responsive elastomeric films based on poly(dimethylsiloxane) containing AuNRs have been employed as actuators displaying a reversible

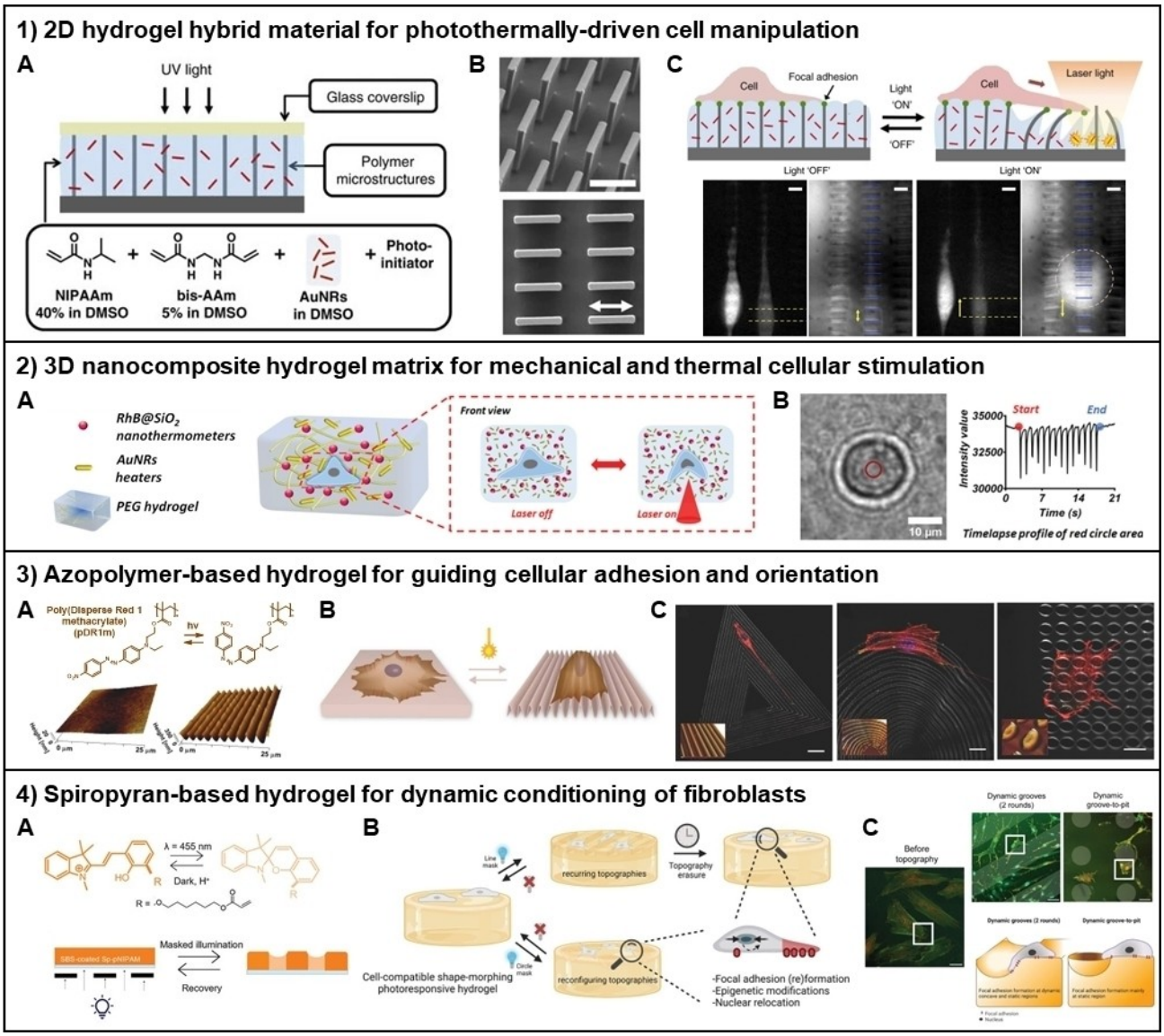


Figure 5. Examples of light-responsive biocompatible actuators employed to mechanically stimulate living cells. **(1)** 2D hydrogel micropillar array hybrid material for photothermally-driven cell manipulation. A) Illustration of the fabrication procedure and structure of the hydrogel system with the related constituents. B) SEM images of the polymer micropillars, side (top) and top views (bottom). Scale bar, 10 μm. C) Scheme illustrating the mechanism of light-triggered cell manipulation (top) and related epifluorescence microscopy images showing successful cell elongation (yellow dashed lines) due to the movement of the microstructures (blue dashes) (bottom). Scale bars, 10 μm. Adapted with permission from reference.[110] Copyright (2017) Springer Nature. **(2)** 3D nanocomposite hydrogel matrix for mechanical and thermal cellular stimulation. A) Scheme illustrating the mechanism of cell actuation in one layer of the hydrogel. B) Holographic microscopy image of the actuated cell (left) with the related excitation profile showing a "beating" effect (right). Adapted with permission from reference.[115] Copyright (2022) Wiley-VCH. **(3)** Azopolymer-based hydrogel for guiding cellular adhesion and orientation. A) Chemical structures of the azopolymer photoisomers (top) used for the preparation of the hydrogel substrate patterned with light (bottom). B) Illustration of cell response upon light excitation and substrate patterning. C) Confocal microscopy images showing cell orientations on different pDR1 m patterns. Scale bars, 10 μm. Adapted with permission from references[116a] and.[116b] Copyright (2015) ACS and (2016) Wiley-VCH GmbH. **(4)** Spiropyran photoacid-based hydrogel for dynamic conditioning of fibroblasts. A) Chemical structures of the spiropyran photoacid photoisomers (top) used for the preparation of the hydrogel substrate patterned with light (bottom). B) Scheme of the experimental procedure for dynamic topographical cell conditioning. C) Confocal microscopy images of cells before (left) and after the stimulation with different physical cues (top right) with related illustrations (bottom right). Scale bar, 150 μm. Adapted with permission from reference.[117] Copyright (2023) Wiley-VCH GmbH.

photoinduced expansion and causing a 2.4-fold stiffening effect in the expanded state. These collagen-functionalized substrates were employed to mechanically stimulate NIH-3T3 fibroblasts, and the actuation was shown to promote faster cell proliferation and increased focal adhesions at actin filaments periphery.[114]

Photoactuators based on plasmonic nanoparticles embedded in soft materials have also been used with poly(ethylene glycol) hydrogels, where the material acted as a 3D cell culture medium and irradiation with NIR light caused a local thermally induced deformation of the gel embedding cells (Figure 5.2).[115] Although information regarding cell behavior modifications post-actuation is limited, this example is note-

worthy as it represents the first photo-actuating 3D cell culture platform, whereas previous examples were confined to 2D substrates. Moreover, in this system, individual cells could be addressed with localized light stimuli, while simultaneously measuring temperature.

Plasmonic heaters are not the sole light-absorbing systems used to induce reversible deformations in soft materials; photochromic moieties such as azobenzene (Figure 5.3) or spiropyran are also harnessed for this purpose.[116] In a recent study, M. Bril and colleagues employed a spiropyran-containing p(NIPAM) hydrogel coated with a styrene-butadiene-styrene (SBS) elastomer thin film as a cell culture platform (Figure 5.4).[117] The stimulation of the spiropyran photoacid embedded in the hydrogel with blue light (455 nm) triggered the shrinkage of the p(NIPAM) hydrogel, resulting in a localized change in morphology of the system without substantial modification of its mechanical properties. Here it should be highlighted that this light-induced transition is reversible upon removal of the light stimulus, albeit at a significantly slower rate compared to plasmonic nanoparticle-based systems (minutes to hours versus seconds). In this investigation, the authors used the system to mechanically stimulate human dermal fibroblasts by reversibly altering the morphology of the substrate, thus showing that plasmonic heaters are not the only chemical species that can be employed as light absorbers to actuate cell culture substrates.

All these examples underline once again the rapidly growing interest in mechanical stimulation of living cells. Unfortunately, we cannot detail yet any examples in the literature on the use of prototissues to provide mechanical stimulation to living cells. However, even if the examples of this are just starting to appear in literature, considering that the efforts are clearly directed towards the use of light-triggered artificial materials, we can say that the future of this research field is surely bright.

5. Predicting Synthetic/Living Tissues Interactions

Despite significant efforts in tissue engineering research, several challenges persist in the development and successful implantation of complex living tissues, such as brain or kidney tissue. Nowadays, the creation of living tissues from isolated cells hinges heavily on the use of a 3D scaffold onto which stem cells are seeded and cultured.[118] This scaffold must not only ensure successful cell attachment and delivery of nutrients and gases but also provide spatially and temporally controlled biochemical signals, such as growth/differentiation factors and DNA or interference RNA, to stimulate cell growth and differentiation.[119] Additionally, the morphological and mechanical properties of the scaffold used exert a significant influence on the success of cell growth and differentiation toward tissue formation. Although 3D printing enables precise control of scaffold morphology, its structural and chemical complexity remains limited with current technologies. Indeed, the level of complexity achievable for regenerated tissues is constrained by two key issues. Firstly, our capacity to administer chemical and/or mechanical cues to growing cells with a high degree of spatial and temporal control is restricted. Secondly, during *in vitro* cellularization of the tissue (the most common technique for tissue regeneration), cells face high levels of stress before implantation due to non-ideal culturing conditions and post-implantation due to the immunological responses of the recipient organism.

The driving force that pushes forward the advancement of this field from laboratory applications to clinical tests is the need to address the challenges mentioned above. Benefitting from its life-like design and mimicry, prototissue engineering has the potential to tackle and solve these open challenges. Indeed, some preliminary examples of synthetic tissues integrated with living cells and tissues have already been documented. In the following sections, we aim to underline the advantages that synthetic cell-mediated tissue engineering can provide to improve the current natural cell-mediated approach and examine the biochemistry underlying these interactions.

5.1. Natural Cell-Mediated *vs.* Synthetic Cell-Mediated Tissue Engineering

The complete recreation of the intricate biological features of living tissues and organs represents a major scientific challenge. This complexity arises from the dynamic interactions of structural components and environmental stimuli with cells, as well as intercellular and intracellular dynamics. All these interactions modulate cell behavior and are hard to replicate with a sufficient level of accuracy. For this reason, current tissue engineering approaches typically use either a scaffold for *in vitro* cell culturing before implanting the newly formed tissue into the patient, or use an implanted scaffold for *in-situ* cell recruitment and tissue formation.[120] These scaffold-mediated and natural cell-mediated approaches can also be combined. In any case, interactions between living cells and biomaterials are crucial to their effectiveness. Naturally derived substrates are usually recognized as "natural" by the cells and are highly effective in triggering the same molecular pathways that the cell would spontaneously activate *in vivo*. Therefore, from a biological point of view, the use of naturally derived materials, such as collagen, hyaluronic acid, general ECM components, or even paper-based[121] or textile-based[122] substrates offer significant advantages.

The majority of scaffolds available for tissue engineering are currently composed of bioderived bulk materials, such as dECM. These materials are used in order to minimize the issue of foreign body response typically displayed by receiving organisms if synthetic scaffolds are employed.[123] Despite the widespread use of dECM-based scaffolds in tissue engineering, there remains a limited understanding of how the production and modification steps of dECM affect the immunogenicity of the final tissues. Unfortunately, immunological responses are still prominent when dECM is used as the scaffold for tissue construction,[124] so this bioscaffold is still far from ideal. However, as highlighted previously, the material is not the only

important factor in bioscaffold design; morphology, structure, and stiffness also play critical roles in the success of tissue growth and implantation. Scaffold morphology can now be controlled using a variety of 3D bioprinting methods that have been recently developed.[125]

Nevertheless, any natural cell-mediated approach shows some drawbacks, and *in vitro* cell manipulation and growth are not straightforward. Specifically, it is challenging to control the delivery of molecular oxygen, nutrients, and molecular cues, and different cell types require different nutrients and in different amounts, and sometimes multiple cell populations are needed to recreate complex tissues. Moreover, naturally derived scaffolds are difficult to manipulate and customize because they degrade easily and lose their biocompatibility. For instance, it is still difficult to keep functional biomolecules (such as DNA, RNA, growth factors, enzymes, and other proteins) incorporated within scaffolds for long periods of time, and the structure itself has short shelf life, especially after cells have been seeded. Another important issue with the use of naturally derived substrates is the lack of reproducibility and tunability of standardized methodologies. In addition, a common problem with both *in vitro* and *in-situ* approaches is the lack of spatiotemporal control of growth factors delivery at different stages of tissue regeneration.

On the other hand, the use of prototissues as scaffolds may hold promise for overcoming the aforementioned obstacles. Synthetic materials can be manipulated and modified according to specific needs or requirements. Moreover, the fabrication of synthetic cell-based scaffolds may be performed on a large scale and easily achieve high batch-to-batch consistency, allowing high reproducibility, avoidance of ethical concerns regarding natural cell manipulation, and standardization of clinically approved processes, especially when microfluidic and printing techniques are exploited. The aforementioned building blocks for synthetic cells production are under constant development to achieve an increasingly high level of resistance to chemical (pH, salt, organic solvents) or physical (strain, compression, electrical fields) conditions, ideally surpassing what living cells could ever withstand, thus expanding the range of applications of synthetic cell-mediated tissue engineering.

As illustrated in the previous sections, the use of synthetic cells and their assemblies offers a great opportunity to build synthetic scaffolds that could greatly improve the current tissue engineering approaches. Features such as chemical communication and interaction with cells (either synthetic or living) and substrates, cargo loading and release, production of active molecules, gene expression, and autonomous motility are all powerful tools that increase the level of complexity, versatility, and functionality of the synthetic cell-based scaffolds.[78]

5.2. Prototissues Interacting with Living Matter

As stated before, of greatest interest to the advancement of synthetic/living tissue interactions is the mechanical and biochemical signaling that would enable greater control over the fate of living cells interfacing with the artificial tissue. Recent studies have highlighted that (bio)chemical communication between synthetic tissues and living cells is indeed possible. In an intriguing study by A. Alcinesio and coworkers, the authors successfully introduced live bacteria to a synthetic tissue structure and transmitted a nucleic acid signal from a population of protocells within the synthetic tissue. This resulted in the production of a fluorescent output from the living cells.[126] In another fascinating study by S. Mann's group, modular prototissues were generated by suspending phospholipid-enveloped poly(diallyldimethylammonium chloride)/double-stranded DNA coacervate droplets in agarose hydrogels (Figure 6.1). Different populations of lipophilically modified enzyme-containing coacervates were patterned in a three-layer concentric arrangement to form a tubular prototissue using a sequential procedure involving a tube and three rods with different diameters as a template. The prototissue contained three layers of different protocells, displaying GOx, HRP, and CAT respectively from the outer to the inner layers of the tube. The three enzymes were employed to produce an H_2O_2-free flux of NO in the central channel of the prototissue-like vessel. Given the anti-coagulant properties of NO, this output was used to control and inhibit coagulation of rabbit plasma and whole blood.[45]

The examples above demonstrate how synthetic tissues offer a reliable strategy for concentrating (bio)chemical species locally within soft materials that display increased levels of internal organization. This allows for the spatial coupling of different chemical reactions within the synthetic tissue to produce signaling and network systems that display a spatial distribution in a way that would not be achievable within a bulk soft material, such as a hydrogel.

In addition to biochemical signaling, electrical signals have also been used to stimulate living cells. In a recent example reported by H. Bayley's group, they showed the modulation of neuronal network activity in neural progenitor cells and mouse brain tissues using an ionic current produced by micro-scale hydrogel droplet networks (Figure 6.2).[127] Even though this material may not strictly be termed a synthetic tissue, as the protocell membranes are disrupted upon the formation of the hydrogel, it nonetheless showcased the potential of sophisticated micro-compartmentalized systems to deliver complex signals to living cells and tissues.

To the best of our knowledge, only one example of interfacing synthetic and living tissues has been reported. In the fascinating study by X. Zhang and coworkers, prototissues were composed of GUVs assembled using the magneto-Archimedes effect (Figure 6.3). The authors successfully patterned different populations of GUVs as well as C6 glioma cells using non-homogeneous magnetic fields. Initially, two populations of GUVs (containing GOx and L-arginine respectively) produced nitric oxide (NO) in the presence of glucose as GOx substrate and the glioma cells then sensed the NO produced. More importantly, in the same study, the authors reported that a prototissue made of a binary population of GOx- and HRP-containing GUVs was capable of producing NO in the presence of glucose and hydroxyurea. The NO produced, which possesses

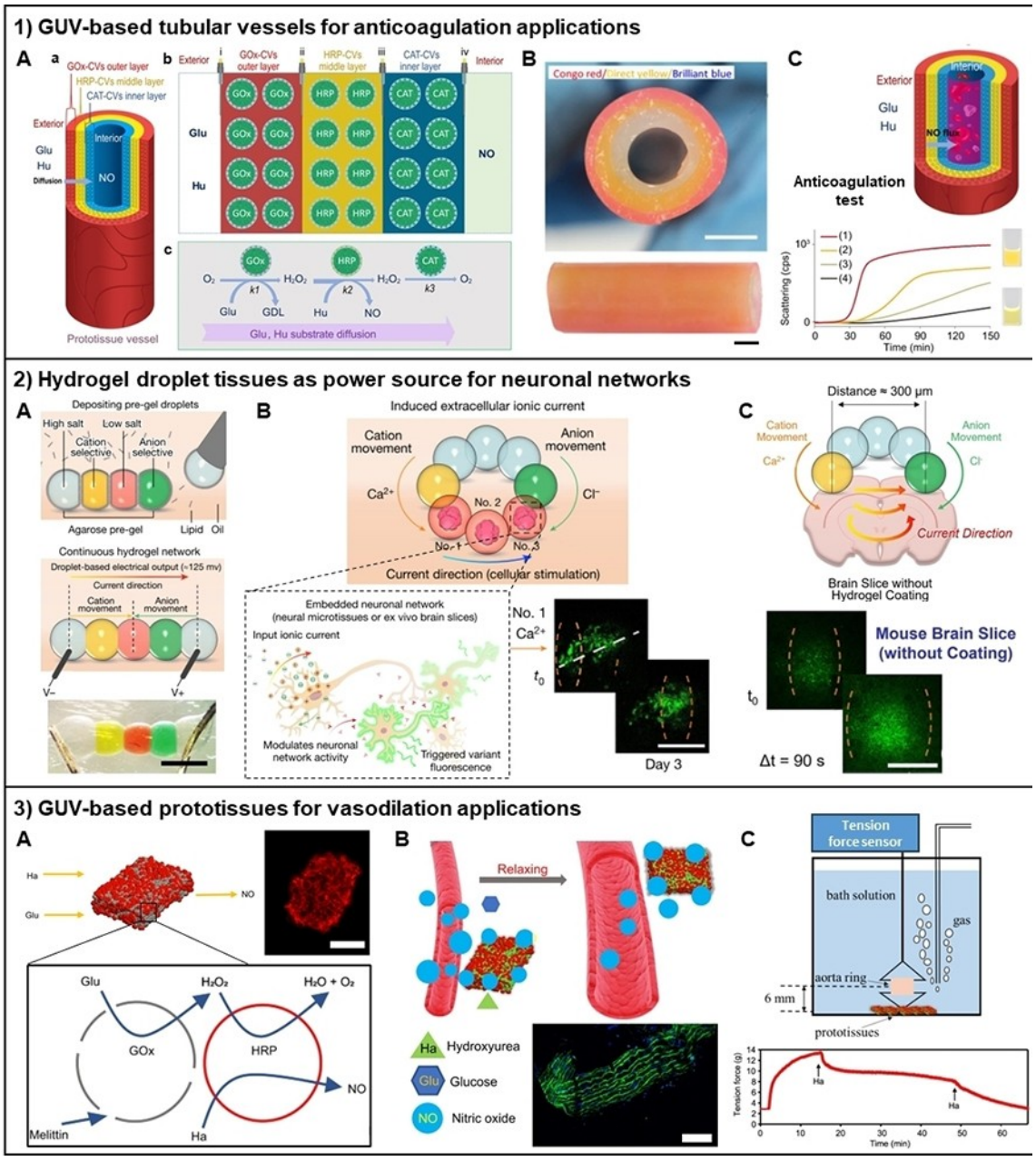


Figure 6. Examples of interactions between synthetic and living tissues. **(1)** GUV-based tubular vessels for anticoagulation applications. A) Illustration of the tubular prototissue vessel (a) with different vesicles enclosing various enzymes (b) performing a cascade reaction that produces NO on the inside (c). B) Picture showing the section (top) and the side-view (bottom) of the tubular prototissue. Scale bars, 5 mm. C) Illustration of the tubular prototissue exerting its NO-flux mediated anticoagulation function on blood sample (left) and the related scattering plot showing increasing anti-coagulation after increasing exposure to NO (bottom). Adapted with permission from reference.[45] Copyright (2022) Springer Nature. **(2)** Hydrogel droplet tissues as a power source for neuronal networks. A) Scheme of the fabrication process for the hydrogel droplet tissue (top), the final network obtained after gelation able to produce an electric current in scheme (center) and in brightfield microscopy after the insertion of the electrodes (bottom). Scale bar, 500 μm. B) Example of tissue interaction with droplets containing neural microtissues (top, bottom left) and related fluorescence microscopy images showing the neuronal activity in droplet 1 upon changing the modulated area (orange dashed lines) (bottom right). Scale bar, 150 μm. C) Scheme illustrating the interaction between the hydrogel network and a mouse brain slice (top) with related fluorescence microscopy images showing the neuronal activity upon changing the modulated area (orange dashed lines) (bottom). Scale bar, 150 μm. Adapted with permission from reference.[127] Copyright (2023) Springer Nature. **(3)** GUV-based prototissues for vasodilation applications. A) Illustration of the nitric oxide-producing prototissue with the scheme illustrating the signal cascade between the two protocell types containing glucose oxidase (GOx) and horseradish peroxidase (HRP) (bottom) and the fluorescence microscopy image of the tissue (red: HRP-containing protocells) (top right). Scale bar, 100 μm. B) Illustration of vasodilation induced by NO-prototissues upon hydroxyurea and glucose feeding (top) and fluorescence image of vascular sections in contact with NO-prototissues showing NO-responding parts (green) and nuclei (blue) (bottom left). Scale bar, 100 μm. C) Setup for the assessment of prototissue-induced vasodilation *in vitro* (top) and the relative tension curve showing the relaxation after hydroxyurea feeding (bottom). Adapted with permission from reference.[13] Copyright (2022) Springer Nature.

anti-coagulant properties, induced relaxation of living blood vessel tissue obtained from rats.[13]

In conclusion, while experiments aimed at interfacing living cells with synthetic tissues are still at an early stage, they exhibit promising potential for the field of tissue engineering and regeneration. To achieve this significant objective, besides improving the biocompatibility and stability of synthetic tissues in biologically relevant conditions, it is vital to investigate the role of the interface between the synthetic tissue and living cells to optimize cell attachment to these materials. Currently, coating cell culture substrates and materials with ECM proteins[128] or cell adhesion peptides are the most widely employed strategies for achieving successful cell attachment.[129] The preliminary work conducted so far demonstrates that synthetic tissues can effectively administer physical and chemical stimuli to living cells with a high spatiotemporal resolution – a capability superior to that achievable with traditional unstructured materials. We envision that further development in prototissues design and fabrication could pave the way for their use as advanced 2D and 3D artificial scaffolds for tissue engineering purposes, offering potential applications in regenerative medicine.

6. Summary and Outlook

In synthetic biology, the prospect of fabricating prototissues is drawing significant interest among researchers engaged with regenerative medicine and tissue engineering. This interest stems from the potential application of prototissues in supporting and directing the growth of living cells and tissues using both chemical and physical stimuli.

However, despite the plethora of biomaterials and models proposed for their construction, so far very few works in the literature have effectively integrated synthetic tissues with living cells and sustained cell survival and interaction. Rather than being a matter of substrates, we suggest that this lack of scientific advancement stems from biochemical matching issues: we are still uncertain about how to implement the fundamental mechanisms of cellular interaction within synthetic micro-compartments to sustain two-way reciprocal communication and symbiosis with living systems.

We believe that there could be (bio- or physical−) chemistry between synthetic and living systems if we find the correct way to match them. In this quest, signaling chemistry – meaning the use of ions, molecules, or macromolecules to convey a signal or to trigger a biochemical or structural response[130] – is certainly an approach that must be studied in the context of living cells and their functioning. Researchers have already employed bio-derived chemical communication systems such as enzyme cascades, DNA strand displacement, and gene-mediated communication between protocells,[3g] and in this review we have described several examples in which the same systems have started to be used between synthetic and living cells, mostly unidirectionally. Important steps forward in the advancement of chemical communication in synthetic biology would be the consolidation of bidirectional and feedback-controlled communication systems and the implementation of fully synthetic signaling and transduction machinery, like synthetic enzymes or *synzymes* (molecular[131] or nanomaterial-based[132]). In addition, exploiting the correct interface chemistry – meaning how chemical and physical stimuli can be transduced and propagated to living tissues upon contact or adhesion[133] – is another fundamental step towards full synthetic/living matter integration. Living cells use substrate/receptor systems for recognition and triggering, membrane proteins for adhesion, channel formation, molecules trafficking, and signal transduction and we are successfully starting to implement these systems in synthetic cells. For further progress, supramolecular chemistry could help to develop novel synthetic supramolecular systems for the regulation of these mechanisms,[134] which have so far only been employed as proof-of-concept. If we are able to face these critical challenges, as is the case with synthetic cell populations,[135] we can envision the application of protocell/prototissue technology in tissue engineering as, for example, a patch of synthetic tissue attached to the pancreas of diabetic patients could be able to sense the glucose level and release insulin when needed through the proper interface chemistry and feedback loop control. Another possible future application could be related to regenerative medicine, where prototissue materials could be implanted in the patient's body and fully integrated with the living tissue. This full integration could make it possible not only to prevent or cure a disease, but also to monitor the course of the patient's recovery if the prototissue is programmed to release specific molecular markers that report on the state of the living tissue or organ. Importantly, the prototissue material should also be progressively degraded and metabolized by the patient's body after its function is completed.

With this review, we aim to take stock of the current state of the art and to delineate the recent developments in a young and cross-disciplinary field that we believe could have a profound impact on our lives. Initially, we have scrutinized the most recent articles pertaining to the progress in synthetic/living cell communication. We have highlighted the types of interactions that have been demonstrated so far, focusing on the biological machinery and structures used in synthetic protocells up to now. We claim that exploiting molecular building blocks other than lipids can lead to other forms of integration between non-living and living matter, and that harnessing the power of biological structures that already exist is currently sufficient to demonstrate how living cells can be artificially influenced.

Subsequently, we have outlined the types of artificial tissues that are predominantly adopted in tissue engineering, with a particular emphasis on prototissues, which are synthetic tissues composed of synthetic protocell units. The examples we have cited are diverse, employing various types of molecular elements and structural designs along with their respective advantages and disadvantages in terms of stability, 3D architecture, and permeability. In the realm of tissue engineering research, we have sought to underscore the current trend of stimuli-driven tissue engineering, emphasizing the latest results that rely on synthetic tissues. We also believe that it is

imperative to endow synthetic tissues with the capacity to exert different types of cues in order to foster deeper interactions with living tissues. This is particularly crucial in the context of creating cell substrates that can respond in different ways during cell growth and maturation, and potentially adapt based on cellular feedback.

Finally, we have attempted to anticipate how this research area could advance further by reporting current successful examples of synthetic tissues that have influenced the behaviors of living cells or tissues, as well as more unconventional types of interactions, such as photo-mechanical stimuli that can only be exerted by the culture substrate thus far. We view these examples of applied prototissues as significant benchmarks for achieving synthetic/living tissue full interaction, from which future research should draw inspiration in terms of biocompatibility, structure design, and advanced functionalities. Furthermore, we anticipate that photo-mechanical transduction applied to enzymatic reactions, cell manipulation, and small molecule administration is poised to play a major role in the future within the tissue engineering field, owing to the ease and precision with which light stimuli can be delivered.

The array of approaches and techniques utilized by researchers to successfully prepare synthetic cells and tissues and to enable their interaction thus far provides an excellent starting point for developing the next paradigm of bottom-up synthetic biology. Although many challenges remain, we have begun to comprehend how to combine and to promote interactions between simple life-like structures and living systems. It is evident that the next revolution in tissue engineering will depend on the full integration and communication between synthetic and living tissues, and we hope to have shed light on how these two realms can finally be matched and harmonized.

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Conflict of Interests

The authors declare no conflict of interest.

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REVIEW

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In the field of tissue engineering, the combination of synthetic and living tissues could be the turning point. In this work we review examples of hybrid protocell/living cell and prototissue/living cell systems, focusing on the chemistry that makes the integration between non-living and living matter efficient. We also highlight key breakthroughs in stimulus-driven tissue engineering and predict the advent of photo-mechanical interactions.