Exploiting the Fundamentals of Biological Organization for the Advancement of Biofabrication

Jordan Hill[1,2], Ricky Wildman[1], Alvaro Mata[1,2,3*]

[1]Department of Chemical & Environmental Engineering, University of Nottingham, NG7 2RD Nottingham, UK.
[2]School of Pharmacy, University of Nottingham, NG7 2RD Nottingham, UK.
[3]Biodiscovery Institute, University of Nottingham, NG7 2RD Nottingham, UK.

* Corresponding author: a.mata@nottingham.ac.uk

Abstract

The field of biofabrication continues to progress, offering higher levels of spatial control, reproducibility, and functionality. However, we remain far from recapitulating what nature has achieved. Biological systems such as tissues and organs are assembled from the bottom-up through coordinated supramolecular and cellular processes that result in their remarkable structures and functionalities. In this perspective, we propose that incorporating such biological assembling mechanisms within fabrication techniques, offers an opportunity to push the boundaries of biofabrication. We dissect these mechanisms into distinct biological organization principles (BOPs) including self-assembly, compartmentalization, diffusion-reaction, disorder-to-order transitions, and out-of-equilibrium processes. We highlight recent work demonstrating the viability and potential of these approaches to enhance scalability, reproducibility, vascularization, and biomimicry; as well as current challenges to overcome.

1. Introduction

Biological development and regeneration

Biology possesses an unparalleled capacity to fabricate sophisticated structures such as cells, tissues, and organs with remarkable functionality. These biological systems are formed through the assembly and organization of a library of molecular and cellular building blocks that interact and signal cooperatively through controlled mechanisms. These biological organization processes are particularly heightened during the development of an organism or tissue regeneration (Fig. 1a). In the case of development, coordinated biochemical and mechanical cues emerge and lead to the development of anisotropic environments that activate different cell signalling pathways and alter gene expression. These concerted molecular milieu regulate self-assembly (SA) and self-organization, resulting in morphogenesis and the development of the embryo [1]. For example, reaction-diffusion processes guide the morphogenesis of the branched structure of the lungs and the folded topology of the brain. Out-of-equilibrium processes permit growth and self-replication of tissues, cells, and molecules, while molecular self-assembly facilitates the creation of macromolecules and new cells. Compartmentalization optimizes biological processes by grouping together biological constructs which perform similar tasks, such as organelles within cells. Order-to-disorder transitions provide function to proteins and can even govern catabolic processes [2]. During regeneration, mechanical or biochemical cues resulting from a wound trigger a multitude of biochemical and cellular responses that sequentially activate and regulate recruitment of cells, expression of factors, and a multitude of processes leading to healing of the tissue [3]. Supramolecular interactions regulate cell signalling and communications, controlling specific cellular responses to an adverse incident. Out-of-equilibrium processes facilitate the growth of new tissue in dissipative regeneration processes. In both of these events, the complex processes defined organize a plethora of molecular and cellular interactions (Fig. 1a), enabling biology to grow, self-replicate, self-heal, sense, and adapt to an ever-changing environment.

Organization principles

The mechanisms by which biology “biofabricates” these complex, hierarchical, and functional structures can be dissected into different molecular and supramolecular events. These “biological organization principles” (BOPs) emerge from cooperative interactions and chemical networks between multiple components, not observed in isolated molecules or reactions [4], which allow biology to diversify, respond, and ultimately optimize (Fig. 1a). Processes such as self-assembly, compartmentalization, diffusion-reaction, disorder-to-order transitions, and out-of-equilibrium processes enable the organization of multiple types of building-blocks across scales and within different thermodynamic landscapes, providing biological structures with their unique structural hierarchy, physical properties, and responsive nature. From the precise assembly of a DNA molecule to the active and dynamic behaviour of the actin cytoskeleton; from the selective and specific functions of the cell membrane to the rhythmic contractions which beat the heart, living systems rely on these organization principles.

Synthetic analogues of biological organization

As our understanding of biological systems has increased, so has the pursuit for recreating their structures and properties. The field of supramolecular chemistry, initially pioneered by Jean-Marie Lehn [5], has been instrumental in this effort, triggering ground-breaking work on self-assembling peptides resembling the nanofibrous architecture of the extracellular matrix (ECM) [6, 7], self-assembling surfaces [8], active supramolecular polymers [9], or immunomodulatory materials [10]. This work has inspired many and led to a plethora of remarkable advances including, for example, enzymatically-driven self-assembling materials [11], self-assembling molecular vaccines [12], self-replicating nanostructures [13, 14], and supramolecular motors [15]. However, despite these remarkable advances, we remain far from engineering practical and useful structures that can emulate the complex functions exhibited by living systems [16].

Biofabrication

Biofabrication, being the automated generation of biologically functional products through bioprinting or bioassembly [17], has opened opportunities to tackle this challenge from the top-down, enabling high resolution and reproducible engineering of micro and macrostructures from computer-assisted designs.

From the first inkjet bioprinter [18], the field has exploded in popularity [19]. The work of Dietmar Hutmacher [20] on electrospun fibres has facilitated the incorporation of electrospinning within biofabrication [21]. Extrusion printing too has been adapted for use with biomaterials [22]. There have also been great efforts developing more functional inks through multi-material approaches [23], with uniquely controllable architectures such as core-shell dual-polymer fibres [24], or using ECM-derived materials [25]. However, biofabrication in its current state is not without its limitations. For example, UV-crosslinking, chemical crosslinking, and the high stress imposed during bioprinting all may have adverse effects on cells and tissues, limiting the functionality of fabricated structures [26]. Other common limitations include difficulties incorporating a suitable vasculature, limited molecular diversity and tailored display, and a restricted capacity to control structure-function relationships.

As the need for more functional and life-like properties increases, it is essential to find new ways to biofabricate that can take us closer to resembling the remarkable complexity and functionality of biological systems. In this review, we propose that the integration of biological organization principles (BOPs) is the next natural step for the advancement of biofabrication, allowing the development of specifically tailored molecular and cellular structures in a manner analogous to how nature fabricates. We will explore these principles and discuss the state of understanding of biological processes, before presenting how the use of BOPs is beginning to be incorporated within fabrication techniques to overcome current limitations of biofabrication and tackle pressing biomedical challenges.

2. Principles of biological organization

Biology builds functional ensembles by assembling molecular building-blocks across scales through supramolecular mechanisms. These biological organization principles tend to be coordinated and, on many occasions, integrated with each other [27, 28]. In this section, we dissect and introduce specific BOPs that are being implemented within biofabrication strategies.

Self-assembly

Self-assembly is ubiquitous in nature, being the mechanism behind the formation of, for example, virus capsids from individual proteins [29], the bundling of collagen to form functional muscle fibres [30], or the neural network formed in the development of the brain [31]. In biological systems, self-assembly occurs primarily within and between molecules *via* non-covalent interactions such as π-π stacking, hydrogen bonding, or hydrophobic interactions, which heavily depend on both the structure of the assembling unit and the surrounding environmental conditions [32]. These processes occur near thermodynamic equilibrium, where the free energy of the system is minimized. At the cellular level, cells too self-assemble to form tissues exhibiting well-defined and functional structures [33].

Self-organization / non-equilibrium systems

While self-assembly bestows biology with an unparalleled capacity to reproducibly grow complex structures, the capacity of these structures to adapt and grow depends on dynamic self-assembling processes, also referred to as self-organization. These processes require the input of energy, operate away from thermodynamic equilibrium, and are guided by adaptive negative feedback loops. Growth rates in these systems may be modulated by the chemical potential (μ), with excess chemical potential (δμ) from the equilibrium state being the non-equilibrium driving force. This, however, is a simplification, and an in-depth discussion and review of statistical mechanics and physics behind non-equilibrium systems, including how they may modulate morphology in biological systems, has recently been published by Nguyen *et al*. [34]. These systems are present in cellular processes such as the assembly and disassembly of the actin cytoskeleton [35] (Fig. 1a), the unidirectional motion of kinesin [36], the organic-inorganic interactions that direct dental enamel formation [37], or the activation of amino acids by enzymes [38]. Non-equilibrium systems are vital in development and regeneration, fuelling all the metabolic processes which are essential to the emergence life.

Compartmentalization

The capacity of biological systems to spontaneously assemble different kinds of building-blocks into well-defined structures can lead to the formation of discrete environments in which isolated events may occur. These compartmentalized environments offer distinct milieus that perform specialized functions and can trigger further processes fuelled by diffusion of molecules or movement of cells. These compartmentalization processes may occur through concerted molecular pathways or through liquid-liquid phase separation in the case of biomolecular condensates [39]. Examples can be found at multiple scales expanding from intracellular organelles, to cell membranes, to larger membranous tissues across which diffusion of nutrients and waste are essential to ensure the function of organisms [40].

Reaction-diffusion / pattern formation

Patterns are commonly found in biology. Some patterns, such as *Turing Patterns,* named after Alan Turing's [41] mathematical description, are formed through reaction-diffusion mechanisms. Typically, this mathematical description is a semi-linear parabolic partial differential equation, the solution to which can describe the spatiotemporal changes in the concentration of a solitary chemical or a mixture of substances and subsequent pattern formation. The effect of reaction-diffusion mechanisms may be observed in the branching patterns of the lungs and kidneys and in the development of the brain. These processes are critical to give organs their structure and function [42, 43] and exhibit the capacity to be modulated and controlled [44].

Disorder-order transitions and synergies

It is increasingly evident that biology has evolved to display a unique ability to regulate biomolecular functionality by harnessing order and disorder. There is growing consensus that intrinsically disordered proteins (IDPs) and intrinsically disordered regions (IDRs) play a critical structural and functional role in the functionality of proteins, and consequently of tissues and organs [45]. Protein order is displayed as rigid α-helices, β-turns, or β-sheets whereas disorder can be seen in flexible and dynamic random coil structures. In tissues and organs, proteins operate as networks and their functions are regulated by interactions between then defining conformations, transitions, and synergies [46]. Understanding the fundamental mechanisms by which biological systems build with proteins is critical to fabricate structures with great molecular diversity, structure, and function [47, 48].

3. Integrating BOPs within biofabrication

The possibility to integrate BOPs within reproducible engineering processes offers an opportunity to push boundaries of biofabrication to develop systems with molecular diversity, nanoscale control, structural hierarchy, responsive and dynamic properties, and the capacity to better communicate with cells. In this section, we highlight recent examples demonstrating the feasibility and opportunities behind this multidisciplinary approach.

Molecular self-assembly within biofabrication

Incorporation of self-assembly within biofabrication offers the possibility to incorporate molecular programmability and nanoscale control without dependence on sophisticated equipment [49]. Furthermore, there is an unparalleled capacity to display bioactive signals [50, 51] (Table 1) and multiple functions [52], and the opportunity to systematically tailor cellular responses [53]. Additionally, the use of non-covalent interactions facilitates reversibility, enabling the generation of injectable materials [54, 55] as well as dynamic [56] and self-healing [57] structures with controlled vascularization. These materials can be designed to respond to environmental cues such as enzymatic activity [11] or be further modified for example with host-guest moieties to provide additional levels of assembling control to modulate degradation [57], bioactivity [58], and structural hierarchy [59]. These properties have enabled the generation of SA bioinks for bioprinting techniques such as extrusion (Table 1) [60, 61], inkjet [62], and electrospinning (Table 1) [63]. Pioneering work by Domingos *et al*. [64] incorporated extrusion bioprinting with self-assembling peptide-based materials to fabricate structures with adjustable stiffness and the potential to modulate cell response. Hauser and co-workers [65]* have utilized peptide-based bioinks to fabricate instantly-solidifying materials under

physiological conditions [66], overcoming limitations associated to scale and shape fidelity (Fig. 2Aii). Here, mechanically robust, self-supporting structures were 3D printed up to 4.0 cm in height and could maintain this structure for 30 days. This study represents a significant development in SA biofabrication, as it reports SA structures being bioprinted on a biologically relevant scale with high biocompatibility, shape fidelity, and mechanical strength without the requirement of additional crosslinking methods. SA can also be exploited to develop new biofabrication methods. For example, Hedegaard *et al.* [67] exploited hydrodynamic forces being generated during bioprinting to guide co-assembly of peptides and proteins hierarchically into geometrically complex scaffolds (Fig. 2Aiii). Furthermore, biofabricating with SA can lead to stimuli-responsiveness (Table 1) [68]. For example, Zhang and co-workers [69-73]* have developed vascularized 'smart' printed cardiac patches up to 8 mm in diameter with responsive curvatures (Fig. 2Ai), advanced neural tissue engineering in millimetre scale organoids, and biofabricated skeletal muscle tissue with millimetre-scale myofiber alignment. This printing of structures which can change with time is referred to as 4D-printing. These responsive materials offer advantages for clinical applications of biofabricated constructs, given their ability to adapt to the dynamic *in vivo* environment. BOPs can facilitate 4D-printing, which would greatly advance biomimicry and clinical applicability of biofabricated structures [74, 75]. Despite this potential, there remain general limitations in biofabricating with SA such as low stiffness, difficulty to control SA beyond the microscale, poor control over surface topology, and difficulty controllably integrating complex molecules such as proteins. However, new studies continue to emerge aiming to overcome these limitations such as enabling interpenetrating protein networks [76], biofabricating hierarchically, or rational design of self-assembling materials [65].

Cellular self-assembly and self-organization within biofabrication

The dynamic nature of cells and their capacity to communicate and spontaneously assemble into complex structures (*i.e.* tissues) makes them uniquely attractive to be viewed as self-assembling building-blocks [77]. In this area, much of the work has been focused on spheroids and organoids. Organoids are clusters of different cell types grown from stem cells while spheroids are defined as bodies of cell aggregates which have been driven towards specific phenotypes [78]. The increasing interest in organoids evidences this potential to develop biology-driven fabrication methods [79]. For example, Lutolf and co-workers [80]** have recently combined organoid self-assembly with extrusion bioprinting to enhance spatial control of organoid growth towards centimetre-scale tissue constructs (Table 1). Burdick and co-workers [81] used 3D bioprinting to control deposition of spheroids in a support consisting of a precursor for a self-healing hydrogel for high-cell density tissue growth (Fig. 2Bi). Together, these studies tackle previous limitations of organoid/spheroid engineering of scalability and issues with cell density. Organoid culture has also been integrated with inkjet printing for modelling of tumour growth [82] and with microfluidics to recapitulate embryonic development at the micrometre scale [83]. Beyond bioprinting, other biofabrication approaches are being developed using extrinsic forces to guide bioassembly. For example, Onbas and Yildiz [84] used magnetic levitation to assemble spheroids and modulate their characteristics (e.g. size, area, circularity), facilitating more reproducible, biomimetic modelling of biological systems. Here, 200 µm diameter tumour spheroids were intentionally engineered with necrotic cores, accurately reflecting *in vivo* tumours. This level of control is not possible through other methods, such as non-adherent microwells or spinner flasks, and there is the unique advantage over the hanging drop method in that spheroid size is not restricted by the size of a droplet. Additionally, the magnetic levitational assembly permits a move away from Matrigel as a culture medium, which would facilitate human applications. Currently, Matrigel poses important limitations to the clinical applicability of organoids due to its undefined animal components. Mironov and co-workers [85]* exploited the diamagnetic properties of organoids for magnetic levitational bioassembly aboard the International Space Station. Chondrospheres of 300 µm in diameter were achieved in this study under microgravity conditions (Fig. 2Bii & Table 1). The introduction of a microgravity environment permits a reduction in concentration of the cytotoxic paramagnetic Gd^{3+}. As there is a correlation between [Gd^{3+}] and cell death [84], this microgravity bioassembly holds promise for improving the overall cell viability. This biofabrication method also facilitates integration of inorganic materials, such as calcium phosphate, potentially allowing the bioassembly of bone tissue constructs. This is not possible under the Earth's gravity due to significant differences in densities between organic and inorganic materials. Acoustofluidic assembly has also grown in popularity to assemble and fuse brain organoids (Fig. 2Biii & Table 1) [86, 87] and acoustic levitational assembly to fabricate mesenchymal stem cell organoids [88]. However, these techniques tend to exhibit large

variations in size and shape, which is a general feature of organoids. The introduction of microfluidics to organoid culture, however, is advancing reproducibility through the controlled delivery of morphogen gradients from signalling centres located within a microfluidic device [89]. Furthermore, it has also been demonstrated that the use of bioprinting is facilitating spatial control over larger-scale cellular structures and manipulation of cellular self-assembly [80].

Compartmentalization and reaction-diffusion within biofabrication

Biofabrication processes that can control the diffusion of components offer an opportunity to enhance structural hierarchy, tailor compositional anisotropy, and modulate the nanoscale structure (Table 1) [90]. By incorporating reaction-diffusion mechanisms within biofabrication techniques, it may be possible to create a wide range of complex geometries, which can facilitate investigation of structure-function relationships. Exploiting liquid-liquid interfaces, Stupp and colleagues generated diffusion barriers leading to the directional co-assembly of peptide amphiphiles (PAs) and hyaluronic acid into millimetre-scale sacs and membranes [91]. Inspired by this work, we showed how PAs co-assembled with disordered elastin-like proteins (ELPs) can be manipulated on demand to generate compartments and trigger a diffusion-reaction process that can be controlled to fabricate tubular networks with the capacity to grow and self-heal millimetre-scale ruptures [56]. Reaction-diffusion models have been used to predict cell migration and proliferation in 3D printed scaffolds [92] and assembly of DNA strands within hydrogels [93]*. Here, the reaction-diffusion behaviour of DNA complexes and the resulting patterns within a scaffold are computationally modelled and predicted, facilitating the fabrication of centimetre-scale rings and patterns (Fig. 3Ai). Similar results have recently been achieved with the diffusion of a pH-responsive low molecular weight gelator (LMWG) and an acid to form self-supporting patterns within hydrogels [94]. In this case, the acidic protons induce a sol-gel transition of the LMWG to form the self-assembled hydrogel. This approach could be used for the patterning of hydrogels, but care must be taken given the toxicity of the required acid. Computational reaction-diffusion models have also has been used to design 3D printable architectures recapitulating the structure of human trabecular bone using a polylactic acid/sodium alginate/hydroxyapatite composite. (Fig. 3Aii & Table 1) [95]. This combination of spatial control afforded by 3D printing and BOPs resulted in better control of pore sizes in the printed structure compared to other fabrication methods such as solvent casting, gas foaming, or freeze drying. Pore size and interconnectivity are critical in scaffold design, with pore sizes between 325 and 420 μm improving vascularization and collagen type I network formation, while pore sizes of 275 μm inhibit the formation of functional bone [96]. Another study imposed spatiotemporal control on morphogen gradients to control cell fate patterning [89]. Here, artificial signalling centres – groups of morphogen-secreting cells – were used to manipulate the self-organization of cells to reproducibly align in a microfluidic environment at a micrometre scale. Such theory has been incorporated with 3D printing for tissue engineering, inducing neuronal differentiation with 3D-printed morphogen gradients for centimetre-scale tissue growth (Table 1) [97]. This degree of control over both the initial and final structure of a material, which is afforded by the combination of the BOP and bioprinting, facilitates greater control over the porosity and shape of a printed construct, resulting in the fabrication of a mechanically robust tubular structure for spinal cord repair. The precise spatial patterning of physiochemical cues, too, remains a limitation in current biofabrication techniques. Accuracy here is required for control over selective signalling and cellular organisation [98]. Recently, 2-photon patterning (2PP) has been applied to accurately place bioactive cues upon a hydrogel support with micrometre-millimetre precision, facilitating the accurate guidance of axons using 3D patterned nerve growth factor (Fig. 3Aiii) [99]. However, some limitations exist when trying to integrate these BOPs with biofabrication. For example, reaction-diffusion processes require precisely controlled environments which can be difficult to control in traditional bioprinting methods such as inkjet or extrusion. Scalability is also an issue, and some reaction-diffusion models cannot be easily scaled to biologically relevant sizes, although there is some development on this front [97], and solutions to this may facilitate the fabrication of controllable, macroscale patterns and designs. Compartmentalization processes require rapid assembly kinetics and tuneable diffusion profiles, both of which require adaptation to be integrated with additive manufacturing techniques.

Disorder-order transitions and synergies within biofabrication

There is an increasing interest in materials science to exploit the interplay between molecular order and disorder and investigate how the protein structure affects its function [47, 100, 101]. Proteins and peptides are rich and versatile building blocks with biofunctionalities that far surpass materials normally used in biofabrication [47]. Pioneering work demonstrated how molecular conformational transitions triggered thermally [102] or by guided intermolecular interactions [103] can generate microscopic structures while avoiding fabrication processes such as multiple-emulsion microfluidics (Table 1). Here, control of the molecular-scale structure influences size and geometry at the micrometre and millimetre scale, facilitating hierarchical control beyond those of current biofabrication techniques while also eliminating the need for cytotoxic crosslinking methods. This approach can also lead to intracellular material manipulation and potential engineering of cellular behaviour, such as modulating the expression of a desired protein [104]. However, before this level of cellular engineering can lead to a rational design of tissue growth, it is critical to advance our understanding of fundamental mechanisms of protein function, particularly with respect to IDPs and IDRs. Disorder-order transitions can also be exploited using small molecules, such as melanin-inspired materials [105] capable of controlled polymerization and resulting material properties (e.g. UV-absorbance, colouration, morphology, and electrochemical properties) depending on the level of assembling order. Our group has developed supramolecular biofabrication processes that exploit the interplay between protein order and disorder. In one approach, we take advantage of ELP disorder-to-order transitions to conform to and penetrate within graphene oxide (GO) stacks at liquid-liquid interfaces, resulting in highly stable ELP-GO complexes (Table 1) [106]**. This ELP-GO platform can be bioprinted to fabricate perfusable self-assembling fluidic structures of up to 12 cm in length and 2 mm in diameter, exhibiting rapid endothelialisation, the capacity to pulsate, and physiological permeability (Fig. 3Cii & Table 1) [107]. In another system, we tune ELP order:disorder ratios to generate supramolecular frameworks capable of nucleating and growing organized apatite nanocrystals into hierarchical structures (Fig. 3B & Table 1) [108, 109]. These examples are beginning to illustrate the unique opportunities of biofabricating while harnessing the interplay between order and disorder, opening opportunities to a greater diversity of biomaterials and assembling principles.

Out-of-equilibrium processes within biofabrication

Out-of-equilibrium processes offer an opportunity to move beyond a focus on structure and into more complex life-like properties such as the capacity to actuate, grow, and self-replicate [16, 110]. Incorporation with biofabrication makes it possible to create initial states or define boundary conditions from which non-equilibrium processes can emerge. For example, programmed bioassembly has been used to fabricate spatially controlled protocellular materials (PCMs) through the interfacial adhesion of two protein-polymer protocells, which are then capable of emergent non-equilibrium chemical sensing [111]. Printable biomolecular motor systems, principally constituted by 0.1 – 10 μm contractile units of the proteins kinesin-1 and light meromyosin, have been developed which are capable of actuation upon UV irradiation. These units can generate micronewton forces leading to millimetre-scale contractions (Fig. 3Ciii) [112]. This approach can lead to the fabrication of soft robotics and the design of dynamic biomaterials [113]. This actuation has also been used to effect cell differentiation by applying tuneable forces, exploiting both the action of molecular motors and the mechanosensing capabilities of cells to direct cell fate (Table 1) [114-116]. Materials capable of accessing non-equilibrium states on-demand can be used to biofabricate structures with emergent properties. Our group has exploited the possibility to access non-equilibrium by enabling and controlling gradients in chemical potential and mechanical perturbations to generate guided self-assembling systems (Fig. 3Ci) [56, 106]. This capacity to access non-equilibrium states enables temporal and spatial control of self-assembly as well as emergent properties such as growing and self-healing. These kinds of properties are difficult to achieve with traditional biofabrication approaches. Here, it can be seen that precise spatial control may aid in investigating structure-function relationships. However, there are challenges accessing and controlling non-equilibrium states, and thermodynamically dissipative systems are difficult to incorporate within traditional biofabrication techniques. A loss of function may be observed over time in a non-equilibrium system, such as an actuator, due to the requirement of an energy input and gradual degradation and deformation of actuating units. Nonetheless, recent developments in understanding of

statistical mechanics, such as thermodynamic uncertainty relationships [117] may make it easier to develop systems which facilitate access and stabilization of non-equilibrium states for novel biomaterials.

4. Conclusion and future outlook

Biological organization principles (BOPs) represent an opportunity to biofabricate with mechanisms that nature has evolved to optimize structure and functionality. This approach offers an opportunity to implement "bioinspired fabrication steps" that can complement biofabrication techniques to overcome major limitations such as the capacity to have molecular programmability and diversity, selectively communicate with cells, recreate hierarchical structures from the low nanoscale, reproducibly assemble complex cellular ensembles, and the ability to fabricate dynamic and responsive constructs with life-like properties. Furthermore, as our capacity to integrate these bottom-up mechanisms within biofabrication increases, the number of combinatorial approaches that take advantage of multiple BOPs simultaneously [93, 94, 106, 107] are likely to increase, offering higher levels of hierarchy, anisotropy, selectivity, and functionality. These are exciting possibilities but there remain important challenges to tackle including difficulties to implement BOPs within traditional fabrication methods, reproducibility of bottom-up assembly up to the macroscale, and the ability to guide cellular assemblies into complex structures reproducibly. Nonetheless, the studies discussed in this article evidence the viability of biofabricating with BOPs and highlight the opportunities behind innovative biofabrication concepts that unify bottom-up and top-down fabrication. From the BOPs presented here, we believe that molecular SA holds the most immediate promise for the advancement of biofabrication. For example, SA inks are already demonstrating a unique capacity to fabricate structures capable of selectively communicating with cells and guiding their behaviour. These SA inks tend to be modular, which additionally enable systematic modifications and enhance tuneability of both structure (e.g. hydrophobicity, charge, nanostructure shape, stiffness) and function (e.g. bioactivity, selectivity, degradability). Furthermore, SA can be incorporated with other BOPs to develop combinatorial approaches and can be modulated through top-down fabrication techniques to direct assembly across multiple size scales, enhancing structural hierarchy and tailoring physiochemical properties.

Acknowledgments

The work was supported by the ERC Starting Grant (STROFUNSCAF), the ERC Proof-of-Concept Grant (MINGRAFT), and the Medical Research Council (UK Regenerative Medicine Platform Hub Acellular Smart Materials 3D Architecture, MR/R015651/1).

References

1. Hannezo E, Heisenberg C-P, **Mechanochemical Feedback Loops in Development and Disease.** *Cell* 2019, **178**: 12-25.
2. Larion M, Salinas RK, Bruschweiler-Li L, Miller BG, Brüschweiler R, **Order–Disorder Transitions Govern Kinetic Cooperativity and Allostery of Monomeric Human Glucokinase.** *PLoS Biol.* 2012, **10**: e1001452.
3. Eming SA, Martin P, Tomic-Canic M, **Wound repair and regeneration: Mechanisms, signaling, and translation.** *Sci. Transl. Med.* 2014, **6**: 265sr6.
4. Azevedo HS, Perry SL, Korevaar PA, Das D, **Complexity emerges from chemistry.** *Nat. Chem.* 2020, **12**: 793-4.
5. Lehn JM, **Supramolecular chemistry.** *Science* 1993, **260**: 1762.
6. Hartgerink JD, Beniash E, Stupp SI, **Peptide-amphiphile nanofibers: A versatile scaffold for the preparation of self-assembling materials.** *Proc. Natl. Acad. Sci. U.S.A* 2002, **99**: 5133.
7. Zhang S, **Fabrication of novel biomaterials through molecular self-assembly.** *Nat. Biotechnol.* 2003, **21**: 1171-8.
8. Whitesides GM, Kriebel JK, Love JC, **Molecular engineering of Surfaces Using Self-Assembled Monolayers.** *Sci. Prog.* 2005, **88**: 17-48.

9. De Greef TFA, Smulders MMJ, Wolffs M, Schenning APHJ, Sijbesma RP, Meijer EW, **Supramolecular Polymerization.** *Chem. Rev.* 2009, **109**: 5687-754.
10. Cerritelli S, Velluto D, Hubbell JA, **PEG-SS-PPS: Reduction-Sensitive Disulfide Block Copolymer Vesicles for Intracellular Drug Delivery.** *Biomacromolecules* 2007, **8**: 1966-72.
11. Toledano S, Williams RJ, Jayawarna V, Ulijn RV, **Enzyme-Triggered Self-Assembly of Peptide Hydrogels via Reversed Hydrolysis.** *J. Am. Chem. Soc.* 2006, **128**: 1070-1.
12. Liu H, Moynihan KD, Zheng Y, Szeto GL, Li AV, Huang B, Van Egeren DS, Park C, Irvine DJ, **Structure-based programming of lymph-node targeting in molecular vaccines.** *Nature* 2014, **507**: 519-22.
13. Carnall JMA, Waudby CA, Belenguer AM, Stuart MCA, Peyralans JJP, Otto S, **Mechanosensitive Self-Replication Driven by Self-Organization.** *Science* 2010, **327**: 1502.
14. Boekhoven J, Hendriksen WE, Koper GJM, Eelkema R, van Esch JH, **Transient assembly of active materials fueled by a chemical reaction.** *Science* 2015, **349**: 1075.
15. von Delius M, Geertsema EM, Leigh DA, **A synthetic small molecule that can walk down a track.** *Nat. Chem.* 2010, **2**: 96-101.
16. Grzybowski BA, Huck WTS, **The nanotechnology of life-inspired systems.** *Nat. Nanotechnol.* 2016, **11**: 585-92.
17. Groll J, Boland T, Blunk T, Burdick JA, Cho D-W, Dalton PD, Derby B, Forgacs G, Li Q, Mironov VA, et al., **Biofabrication: reappraising the definition of an evolving field.** *Biofabrication* 2016, **8**: 013001.
18. Mironov V, Boland T, Trusk T, Forgacs G, Markwald RR, **Organ printing: computer-aided jet-based 3D tissue engineering.** *Trends Biotechnol.* 2003, **21**: 157-61.
19. Castilho M, de Ruijter M, Beirne S, Villette CC, Ito K, Wallace GG, Malda J, **Multitechnology Biofabrication: A New Approach for the Manufacturing of Functional Tissue Structures?** *Trends Biotechnol.* 2020, **38**: 1316-28.
20. Brown TD, Dalton PD, Hutmacher DW, **Direct Writing By Way of Melt Electrospinning.** *Adv. Mater.* 2011, **23**: 5651-7.
21. Mulholland EJ, **Electrospun Biomaterials in the Treatment and Prevention of Scars in Skin Wound Healing.** *Front. Bioeng. Biotechnol.* 2020, **8**.
22. Wang F, Shor L, Darling A, Khalil S, Sun W, Güçeri S, Lau A, **Precision extruding deposition and characterization of cellular poly-ε-caprolactone tissue scaffolds.** *Rapid Prototyp. J.* 2004, **10**: 42-9.
23. Bertassoni LE, Cardoso JC, Manoharan V, Cristino AL, Bhise NS, Araujo WA, Zorlutuna P, Vrana NE, Ghaemmaghami AM, Dokmeci MR, et al., **Direct-write bioprinting of cell-laden methacrylated gelatin hydrogels.** *Biofabrication* 2014, **6**: 024105.
24. Moroni L, Schotel R, Sohier J, de Wijn JR, van Blitterswijk CA, **Polymer hollow fiber three-dimensional matrices with controllable cavity and shell thickness.** *Biomaterials* 2006, **27**: 5918-26.
25. Cunniffe GM, Díaz-Payno PJ, Sheehy EJ, Critchley SE, Almeida HV, Pitacco P, Carroll SF, Mahon OR, Dunne A, Levingstone TJ, et al., **Tissue-specific extracellular matrix scaffolds for the regeneration of spatially complex musculoskeletal tissues.** *Biomaterials* 2019, **188**: 63-73.
26. Gibbs DM, Vaezi M, Yang S, Oreffo RO, **Hope versus hype: what can additive manufacturing realistically offer trauma and orthopedic surgery?** *Regen. Med.* 2014, **9**: 535-49.
27. Manalo MN, Pérez LM, LiWang A, **Hydrogen-Bonding and π–π Base-Stacking Interactions Are Coupled in DNA, As Suggested by Calculated and Experimental Trans-Hbond Deuterium Isotope Shifts.** *J. Am. Chem. Soc.* 2007, **129**: 11298-9.
28. Hildebrand PW, Günther S, Goede A, Forrest L, Frömmel C, Preissner R, **Hydrogen-bonding and packing features of membrane proteins: functional implications.** *Biophys. J.* 2008, **94**: 1945-53.
29. Buzón P, Maity S, Roos WH, **Physical virology: From virus self-assembly to particle mechanics.** *Wiley Interdiscip. Rev. Nanomed. Nanobiotechnol.* 2020, **12**: e1613.
30. Silver FH, Freeman JW, Seehra GP, **Collagen self-assembly and the development of tendon mechanical properties.** *J. Biomech.* 2003, **36**: 1529-53.
31. Hasan MF, Ghiasvand S, Wang H, Miwa JM, Berdichevsky Y, **Neural layer self-assembly in geometrically confined rat and human 3D cultures.** *Biofabrication* 2019, **11**: 045011.
32. Smith KH, Tejeda-Montes E, Poch M, Mata A, **Integrating top-down and self-assembly in the fabrication of peptide and protein-based biomedical materials.** *Chem. Soc. Rev.* 2011, **40**: 4563-77.
33. Napolitano AP, Chai P, Dean DM, Morgan JR, **Dynamics of the self-assembly of complex cellular aggregates on micromolded nonadhesive hydrogels.** *Tissue Eng.* 2007, **13**: 2087-94.

34. Nguyen M, Qiu Y, Vaikuntanathan S, **Organization and Self-Assembly Away from Equilibrium: Toward Thermodynamic Design Principles.** *Annu. Rev. Condens. Matter Phys.* 2021, **12**: 273-90.
35. Foffano G, Levernier N, Lenz M, **The dynamics of filament assembly define cytoskeletal network morphology.** *Nat. Commun.* 2016, **7**: 13827.
36. Yildiz A, Tomishige M, Vale RD, Selvin PR, **Kinesin Walks Hand-Over-Hand.** *Science* 2004, **303**: 676.
37. Moradian-Oldak J, George A, **Biomineralization of Enamel and Dentin Mediated by Matrix Proteins.** *J. Dent. Res.* 2021: 00220345211018405.
38. Beuning PJ, Musier-Forsyth K, **Hydrolytic editing by a class II aminoacyl-tRNA synthetase.** *Proc. Natl. Acad. Sci. U.S.A* 2000, **97**: 8916.
39. Banani SF, Lee HO, Hyman AA, Rosen MK, **Biomolecular condensates: organizers of cellular biochemistry.** *Nat. Rev. Mol. Cell Biol.* 2017, **18**: 285-98.
40. Misteli T, **The concept of self-organization in cellular architecture.** *J. Cell Biol.* 2001, **155**: 181-5.
41. Turing AM, **The chemical basis of morphogenesis.** *Philos. Trans. R. Soc. Lond., B, Biol. Sci.* 1952, **237**: 37-72.
42. Menshykau D, Michos O, Lang C, Conrad L, McMahon AP, Iber D, **Image-based modeling of kidney branching morphogenesis reveals GDNF-RET based Turing-type mechanism and pattern-modulating WNT11 feedback.** *Nat. Commun.* 2019, **10**: 239.
43. Lefèvre J, Mangin JF, **A reaction-diffusion model of human brain development.** *PLoS Comput. Biol.* 2010, **6**: e1000749.
44. Karig D, Martini KM, Lu T, DeLateur NA, Goldenfeld N, Weiss R, **Stochastic Turing patterns in a synthetic bacterial population.** *Proc. Natl. Acad. Sci. U.S.A* 2018, **115**: 6572.
45. van der Lee R, Buljan M, Lang B, Weatheritt RJ, Daughdrill GW, Dunker AK, Fuxreiter M, Gough J, Gsponer J, Jones DT, et al., **Classification of Intrinsically Disordered Regions and Proteins.** *Chem. Rev.* 2014, **114**: 6589-631.
46. Nevola L, Giralt E, **Modulating protein–protein interactions: the potential of peptides.** *Chem. Commun.* 2015, **51**: 3302-15.
47. Ulijn RV, Lampel A, **Order/Disorder in Protein and Peptide-Based Biomaterials.** *Isr. J. Chem.* 2020, **60**: 1129-40.
48. Schmid S, Hugel T, **Controlling protein function by fine-tuning conformational flexibility.** *eLife* 2020, **9**: e57180.
49. Hedegaard CL, Mata A, **Integrating self-assembly and biofabrication for the development of structures with enhanced complexity and hierarchical control.** *Biofabrication* 2020, **12**: 032002.
50. Dankers PYW, Harmsen MC, Brouwer LA, Van Luyn MJA, Meijer EW, **A modular and supramolecular approach to bioactive scaffolds for tissue engineering.** *Nat. Mater.* 2005, **4**: 568-74.
51. Sousa CFV, Saraiva CA, Correia TR, Pesqueira T, Patrício SG, Rial-Hermida MI, Borges J, Mano JF, **Bioinstructive Layer-by-Layer-Coated Customizable 3D Printed Perfusable Microchannels Embedded in Photocrosslinkable Hydrogels for Vascular Tissue Engineering.** *Biomolecules* 2021, **11**: 863.
52. Derkus B, Okesola BO, Barrett DW, D'Este M, Chowdhury TT, Eglin D, Mata A, **Multicomponent hydrogels for the formation of vascularized bone-like constructs in vitro.** *Acta Biomater.* 2020, **109**: 82-94.
53. Shi J, Du X, Yuan D, Zhou J, Zhou N, Huang Y, Xu B, **d-Amino Acids Modulate the Cellular Response of Enzymatic-Instructed Supramolecular Nanofibers of Small Peptides.** *Biomacromolecules* 2014, **15**: 3559-68.
54. Liu Y, Wong C-W, Chang S-W, Hsu S-h, **An injectable, self-healing phenol-functionalized chitosan hydrogel with fast gelling property and visible light-crosslinking capability for 3D printing.** *Acta Biomater.* 2021, **122**: 211-9.
55. Wang W, Zeng Z, Xiang L, Liu C, Diaz-Dussan D, Du Z, Asha AB, Yang W, Peng Y-Y, Pan M, et al., **Injectable Self-Healing Hydrogel via Biological Environment-Adaptive Supramolecular Assembly for Gastric Perforation Healing.** *ACS Nano* 2021, **15**: 9913-23.
56. Inostroza-Brito KE, Collin E, Siton-Mendelson O, Smith KH, Monge-Marcet A, Ferreira DS, Rodríguez RP, Alonso M, Rodríguez-Cabello JC, Reis RL, et al., **Co-assembly, spatiotemporal control and morphogenesis of a hybrid protein–peptide system.** *Nat. Chem.* 2015, **7**: 897-904.
57. Redondo-Gómez C, Abdouni Y, Becer CR, Mata A, **Self-Assembling Hydrogels Based on a Complementary Host–Guest Peptide Amphiphile Pair.** *Biomacromolecules* 2019, **20**: 2276-85.

58. Redondo-Gómez C, Padilla-Lopategui S, Azevedo HS, Mata A, **Host–Guest-Mediated Epitope Presentation on Self-Assembled Peptide Amphiphile Hydrogels.** *ACS Biomater. Sci. Eng.* 2020, **6**: 4870-80.
59. Edelbrock AN, Clemons TD, Chin SM, Roan JJW, Bruckner EP, Álvarez Z, Edelbrock JF, Wek KS, Stupp SI, **Superstructured Biomaterials Formed by Exchange Dynamics and Host–Guest Interactions in Supramolecular Polymers.** *Adv. Sci.* 2021, **8**: 2004042.
60. Gao F, Xu Z, Liang Q, Liu B, Li H, Wu Y, Zhang Y, Lin Z, Wu M, Ruan C, et al., **Direct 3D Printing of High Strength Biohybrid Gradient Hydrogel Scaffolds for Efficient Repair of Osteochondral Defect.** *Adv. Funct. Mater.* 2018, **28**: 1706644.
61. Sather NA, Sai H, Sasselli IR, Sato K, Ji W, Synatschke CV, Zambrotta RT, Edelbrock JF, Kohlmeyer RR, Hardin JO, et al., **3D Printing of Supramolecular Polymer Hydrogels with Hierarchical Structure.** *Small* 2021, **17**: 2005743.
62. Hart LR, Harries JL, Greenland BW, Colquhoun HM, Hayes W, **Molecular design of a discrete chain-folding polyimide for controlled inkjet deposition of supramolecular polymers.** *Polym. Chem.* 2015, **6**: 7342-52.
63. Mori da Cunha MGMC, Arts B, Hympanova L, Rynkevic R, Mackova K, Bosman AW, Dankers PYW, Deprest J, **Functional supramolecular bioactivated electrospun mesh improves tissue ingrowth in experimental abdominal wall reconstruction in rats.** *Acta Biomater.* 2020, **106**: 82-91.
64. Raphael B, Khalil T, Workman VL, Smith A, Brown CP, Streuli C, Saiani A, Domingos M, **3D cell bioprinting of self-assembling peptide-based hydrogels.** *Mater. Lett.* 2017, **190**: 103-6.
65. Susapto HH, Alhattab D, Abdelrahman S, Khan Z, Alshehri S, Kahin K, Ge R, Moretti M, Emwas A-H, Hauser CAE, **Ultrashort Peptide Bioinks Support Automated Printing of Large-Scale Constructs Assuring Long-Term Survival of Printed Tissue Constructs.** *Nano Lett.* 2021, **21**: 2719-29.

The authors report the use of ultrashort peptide bioinks for the fabrication of large-scale structures, a significant development in the use of self-assembling bioinks for biofabrication and overcoming a severe limitation of these inks.

66. Rauf S, Susapto HH, Kahin K, Alshehri S, Abdelrahman S, Lam JH, Asad S, Jadhav S, Sundaramurthi D, Gao X, et al., **Self-assembling tetrameric peptides allow in situ 3D bioprinting under physiological conditions.** *J. Mater. Chem. B* 2021, **9**: 1069-81.
67. Hedegaard CL, Collin EC, Redondo-Gómez C, Nguyen LTH, Ng KW, Castrejón-Pita AA, Castrejón-Pita JR, Mata A, **Hydrodynamically Guided Hierarchical Self-Assembly of Peptide–Protein Bioinks.** *Adv. Funct. Mater.* 2018, **28**: 1703716.
68. Zhou Y, Zhou D, Cao P, Zhang X, Wang Q, Wang T, Li Z, He W, Ju J, Zhang Y, **4D Printing of Shape Memory Vascular Stent Based on βCD-g-Polycaprolactone.** *Macromol. Rapid Commun.* 2021: e2100176.
69. Wang Y, Cui H, Wang Y, Xu C, Esworthy TJ, Hann SY, Boehm M, Shen Y-L, Mei D, Zhang LG, **4D Printed Cardiac Construct with Aligned Myofibers and Adjustable Curvature for Myocardial Regeneration.** *ACS Appl. Mater. Interfaces* 2021, **13**: 12746-58.
70. Miao S, Nowicki M, Cui H, Lee SJ, Zhou X, Mills DK, Zhang LG, **4D anisotropic skeletal muscle tissue constructs fabricated by staircase effect strategy.** *Biofabrication* 2019, **11**: 035030.
71. Miao S, Cui H, Esworthy T, Mahadik B, Lee S-j, Zhou X, Hann SY, Fisher JP, Zhang LG, **4D Self-Morphing Culture Substrate for Modulating Cell Differentiation.** *Adv. Sci.* 2020, **7**: 1902403.
72. Cui H, Liu C, Esworthy T, Huang Y, Yu Z-x, Zhou X, San H, Lee S-j, Hann SY, Boehm M, et al., **4D physiologically adaptable cardiac patch: A 4-month in vivo study for the treatment of myocardial infarction.** *Sci. Adv.* 2020, **6**: eabb5067.

Utilizing molecular self-assembly, the authors have constructed a 4D printed cardiac patch which is adaptive and responsive to the dynamic curvature of a beating heart. The patch demonstrated vascularization and cardiomyocyte growth at a greater level.

73. Esworthy TJ, Miao S, Lee S-J, Zhou X, Cui H, Zuo YY, Zhang LG, **Advanced 4D-bioprinting technologies for brain tissue modeling and study.** *Int. J. Smart Nano Mater* 2019, **10**: 177-204.
74. Yang Q, Gao B, Xu F, **Recent Advances in 4D Bioprinting.** *Biotechnol. J.* 2020, **15**: 1900086.
75. Morouço P, Lattanzi W, Alves N, **Four-Dimensional Bioprinting As a New Era for Tissue Engineering and Regenerative Medicine.** *Front. Bioeng. Biotechnol.* 2017, **5**: 61.
76. Da Silva K, Kumar P, van Vuuren SF, Pillay V, Choonara YE, **Three-Dimensional Printability of an ECM-Based Gelatin Methacryloyl (GelMA) Biomaterial for Potential Neuroregeneration.** *ACS Omega* 2021.

77. Laurent J, Blin G, Chatelain F, Vanneaux V, Fuchs A, Larghero J, Théry M, **Convergence of microengineering and cellular self-organization towards functional tissue manufacturing.** *Nat. Biomed. Eng.* 2017, **1**: 939-56.
78. Burdis R, Kelly DJ, **Biofabrication and bioprinting using cellular aggregates, microtissues and organoids for the engineering of musculoskeletal tissues.** *Acta Biomater.* 2021, **126**: 1-14.
79. Kim J, Koo B-K, Knoblich JA, **Human organoids: model systems for human biology and medicine.** *Nat. Rev. Mol. Cell Biol.* 2020, **21**: 571-84.
80. Brassard JA, Nikolaev M, Hübscher T, Hofer M, Lutolf MP, **Recapitulating macro-scale tissue self-organization through organoid bioprinting.** *Nat. Mater.* 2021, **20**: 22-9.

The authors have combined extrusion bioprinting with cellular self-assembly to deposit organoid-forming building blocks into a hydrogel precursor capable of forming a self-healing scaffold. This method facilitated the fabrication of centimetre-scale tissues with biomimetic structures such as vascularization and tubular intestinal epithelia.
81. Daly AC, Davidson MD, Burdick JA, **3D bioprinting of high cell-density heterogeneous tissue models through spheroid fusion within self-healing hydrogels.** *Nat. Commun.* 2021, **12**: 753.
82. Yoon WH, Lee H-R, Kim S, Kim E, Ku JH, Shin K, Jung S, **Use of inkjet-printed single cells to quantify intratumoral heterogeneity.** *Biofabrication* 2020, **12**: 035030.
83. Salmon I, Grebenyuk S, Fattah ARA, Rustandi G, Pilkington T, Verfaillie C, Ranga A, **Engineering neurovascular organoids with 3D printed microfluidic chips.** *bioRxiv* 2021: 2021.01.09.425975.
84. Onbas R, Arslan Yildiz A, **Fabrication of Tunable 3D Cellular Structures in High Volume Using Magnetic Levitation Guided Assembly.** *ACS Appl. Bio Mater.* 2021, **4**: 1794-802.
85. Parfenov VA, Khesuani YD, Petrov SV, Karalkin PA, Koudan EV, Nezhurina EK, Pereira FDAS, Krokhmal AA, Gryadunova AA, Bulanova EA, et al., **Magnetic levitational bioassembly of 3D tissue construct in space.** *Sci. Adv.* 2020, **6**: eaba4174.

By accessing microgravity conditions, the authors were capable of scaffold-free magnetic levitational bioassembly with reduced concentration of Gd^{3+} paramagnetic agent. This is the first example of scaffold-free bioassembly in space, revealing the potential for the advancement of space tissue engineering.
86. Ao Z, Cai H, Wu Z, Ott J, Wang H, Mackie K, Guo F, **Controllable fusion of human brain organoids using acoustofluidics.** *Lab Chip* 2021, **21**: 688-99.
87. Cai H, Ao Z, Wu Z, Song S, Mackie K, Guo F, **Intelligent acoustofluidics enabled mini-bioreactors for human brain organoids.** *Lab Chip* 2021, **21**: 2194-205.
88. Jeger-Madiot N, Arakelian L, Setterblad N, Bruneval P, Hoyos M, Larghero J, Aider J-L, **Self-organization and culture of Mesenchymal Stem Cell spheroids in acoustic levitation.** *Sci. Rep.* 2021, **11**: 8355.
89. Manfrin A, Tabata Y, Paquet ER, Vuaridel AR, Rivest FR, Naef F, Lutolf MP, **Engineered signaling centers for the spatially controlled patterning of human pluripotent stem cells.** *Nat. Methods* 2019, **16**: 640-8.
90. Dong Z, Cui H, Zhang H, Wang F, Zhan X, Mayer F, Nestler B, Wegener M, Levkin PA, **3D printing of inherently nanoporous polymers via polymerization-induced phase separation.** *Nat. Commun.* 2021, **12**: 247.
91. Capito RM, Azevedo HS, Velichko YS, Mata A, Stupp SI, **Self-Assembly of Large and Small Molecules into Hierarchically Ordered Sacs and Membranes.** *Science* 2008, **319**: 1812.
92. Buenzli PR, Lanaro M, Wong CS, McLaughlin MP, Allenby MC, Woodruff MA, Simpson MJ, **Cell proliferation and migration explain pore bridging dynamics in 3D printed scaffolds of different pore size.** *Acta Biomater.* 2020, **114**: 285-95.
93. Chen S, Seelig G, **Programmable patterns in a DNA-based reaction–diffusion system.** *Soft Matter* 2020, **16**: 3555-63.

Using computational modelling, the authors were able to precisely programme the diffusion of DNA embedded within a hydrogel to produce centimeter-scale patterns, showcasing the potential to fabricate macroscopic materials by considering the reaction-diffusion behaviour of biomolecules.
94. Cooke HS, Schlichter L, Piras CC, Smith DK, **Double diffusion for the programmable spatiotemporal patterning of multi-domain supramolecular gels.** *Chem. Sci.* 2021.

95. Fernández-Cervantes I, Morales MA, Agustín-Serrano R, Cardenas-García M, Pérez-Luna PV, Arroyo-Reyes BL, Maldonado-García A, **Polylactic acid/sodium alginate/hydroxyapatite composite scaffolds with trabecular tissue morphology designed by a bone remodeling model using 3D printing.** *J. Mater. Sci.* 2019, **54**: 9478-96.
96. Stoppato M, Carletti E, Sidarovich V, Quattrone A, Unger RE, Kirkpatrick CJ, Migliaresi C, Motta A, **Influence of scaffold pore size on collagen I development: A new in vitro evaluation perspective.** *J. Bioact. Compat. Polym.* 2013, **28**: 16-32.
97. Hamid OA, Eltaher HM, Sottile V, Yang J, **3D bioprinting of a stem cell-laden, multi-material tubular composite: An approach for spinal cord repair.** *Mater. Sci. Eng. C* 2021, **120**: 111707.
98. Straley KS, Foo CWP, Heilshorn SC, **Biomaterial Design Strategies for the Treatment of Spinal Cord Injuries.** *J. Neurotrauma* 2009, **27**: 1-19.
99. Broguiere N, Lüchtefeld I, Trachsel L, Mazunin D, Rizzo R, Bode JW, Lutolf MP, Zenobi-Wong M, **Morphogenesis Guided by 3D Patterning of Growth Factors in Biological Matrices.** *Adv. Mater.* 2020, **32**: 1908299.
100. Zhu H, Gomez M, Xiao J, Perale G, Betge F, Lyngstadaas SP, Haugen HJ, **Xenohybrid Bone Graft Containing Intrinsically Disordered Proteins Shows Enhanced In Vitro Bone Formation.** *ACS Applied Bio Materials* 2020, **3**: 2263-74.
101. Mohammadi B, Glatzel M, Altmeppen H, **Disordered structure and flexible roles: using the prion protein N1 fragment for neuroprotective and regenerative therapy.** *Neural Regen. Res.* 2021, **16**: 1431-2.
102. Mozhdehi D, Luginbuhl KM, Simon JR, Dzuricky M, Berger R, Varol HS, Huang FC, Buehne KL, Mayne NR, Weitzhandler I, et al., **Genetically encoded lipid–polypeptide hybrid biomaterials that exhibit temperature-triggered hierarchical self-assembly.** *Nat. Chem.* 2018, **10**: 496-505.
103. Roberts S, Miao V, Costa S, Simon J, Kelly G, Shah T, Zauscher S, Chilkoti A, **Complex microparticle architectures from stimuli-responsive intrinsically disordered proteins.** *Nat. Commun.* 2020, **11**: 1342.
104. Dzuricky M, Rogers BA, Shahid A, Cremer PS, Chilkoti A, **De novo engineering of intracellular condensates using artificial disordered proteins.** *Nat. Chem.* 2020, **12**: 814-25.
105. Lampel A, McPhee SA, Park H-A, Scott GG, Humagain S, Hekstra DR, Yoo B, Frederix PWJM, Li T-D, Abzalimov RR, et al., **Polymeric peptide pigments with sequence-encoded properties.** *Science* 2017, **356**: 1064.
106. Wu Y, Okesola BO, Xu J, Korotkin I, Berardo A, Corridori I, di Brocchetti FLP, Kanczler J, Feng J, Li W, et al., **Disordered protein-graphene oxide co-assembly and supramolecular biofabrication of functional fluidic devices.** *Nat. Commun.* 2020, **11**: 1182.

The authors report the fabrication of a robust, tubular structure of biological relevance by applying multiple BOPs. Self-assembling materials access a non-equilibrium state on-demand to co-assemble through a reaction-diffusion mechanism, while disorder-to-order transitions generate hierarchical constructs. This is the first instance of such an interplay of BOPs being demonstrated and manipulated for biofabrication.

107. Wu Y, Fortunato GM, Okesola BO, Brocchetti FLPD, Suntornnond R, Connelly J, De Maria C, Rodriguez-Cabello JC, Vozzi G, Wang W, et al., **An interfacial self-assembling bioink for the manufacturing of capillary-like structures with tuneable and anisotropic permeability.** *Biofabrication* 2021, **13**: 035027.
108. Elsharkawy S, Al-Jawad M, Pantano MF, Tejeda-Montes E, Mehta K, Jamal H, Agarwal S, Shuturminska K, Rice A, Tarakina NV, et al., **Protein disorder–order interplay to guide the growth of hierarchical mineralized structures.** *Nat. Commun.* 2018, **9**: 2145.
109. Deng X, Hasan A, Elsharkawy S, Tejeda-Montes E, Tarakina NV, Greco G, Nikulina E, Stormonth-Darling JM, Convery N, Rodriguez-Cabello JC, et al., **Topographically guided hierarchical mineralization.** *Mater. Today Bio* 2021, **11**: 100119.
110. Adamski P, Eleveld M, Sood A, Kun Á, Szilágyi A, Czárán T, Szathmáry E, Otto S, **From self-replication to replicator systems en route to de novo life.** *Nat. Rev. Chem.* 2020, **4**: 386-403.
111. Galanti A, Moreno-Tortolero RO, Azad R, Cross S, Davis S, Gobbo P, **A Floating Mold Technique for the Programmed Assembly of Protocells into Protocellular Materials Capable of Non-Equilibrium Biochemical Sensing.** *Adv. Mater.* 2021, **33**: 2100340.
112. Nitta T, Wang Y, Du Z, Morishima K, Hiratsuka Y, **A printable active network actuator built from an engineered biomolecular motor.** *Nat. Mater.* 2021.

113. Santos LJ, Reis RL, Gomes ME, **Harnessing magnetic-mechano actuation in regenerative medicine and tissue engineering.** *Trends Biotechnol.* 2015, **33**: 471-9.
114. Zheng Y, Han MKL, Zhao R, Blass J, Zhang J, Zhou DW, Colard-Itté J-R, Dattler D, Çolak A, Hoth M, et al., **Optoregulated force application to cellular receptors using molecular motors.** *Nat. Commun.* 2021, **12**: 3580.
115. Serzanti M, Baù M, Demori M, Calamaio S, Cominelli M, Poliani PL, Dell'Era P, Ferrari M, Ferrari V, **Arrangement of Live Human Cells through Acoustic Waves Generated by Piezoelectric Actuators for Tissue Engineering Applications.** *Appl. Sci.* 2020, **10**: 3477.
116. Zhou Q, Chen J, Luan Y, Vainikka PA, Thallmair S, Marrink SJ, Feringa BL, van Rijn P, **Unidirectional rotating molecular motors dynamically interact with adsorbed proteins to direct the fate of mesenchymal stem cells.** *Sci. Adv.* 2020, **6**: eaay2756.
117. Barato AC, Seifert U, **Thermodynamic uncertainty relation for biomolecular processes.** *Phys. Rev. Lett.* 2015, **114**: 158101.

BOP	Key Findings	Biofabrication Technique	Limitations	Ref.
Molecular self-assembly	Hierarchical interactions such as van der Waals forces improve printability and isotropy, and trigger alignment of cells.	Extrusion bioprinting.	Intrinsic low stiffness means a supporting gel is required for complex geometries.	[61]
	Specific bioactive epitopes can improve tissue growth and reduce scar tissue.	Electrospinning.	Complex geometries and structures are not provided.	[63]
	Shape-memory materials for controlled macroscale conformational transitions.	Extrusion bioprinting.	Shape-recovery *in vivo* requires an elevated temperature. 10 minutes at 40 °C provides a shape recovery ratio of just 66%.	[68]
	Scaffolds with controlled vasculature and bioactivities.	Extrusion bioprinting.	Difficult to construct complex 3D structures with macroscale x, y, and z dimensions.	[51]
Cellular self-assembly	Controllable organoid assembly can lead to reproducible and robust tissue models.	Acoustic levitational bioassembly.	Only a single organoid can be constructed at once, which may be time-consuming given the need to account for heterogeneity between samples.	[86]
	Levitational organoid assembly offers a scaffold-free route for tissue engineering.	Magnetic levitational bioassembly in microgravity.	Accessing microgravity conditions is incredibly high cost. The alternative is to use higher concentrations of cytotoxic Gd^{3+}.	[85]
	Controlled spatial arrangement of organoids can coerce tissue growth into a desired structure.	Extrusion bioprinting.	Cell viability dropped from 95% to 85% in the space of 24 hours. No longer-term viability data was provided.	[80]
Disorder-to-order transitions	Controlling disorder/order ratios can guide crystallographic alignment and mineralisation.	Guided mineralization.	Difficult to integrate this technique with traditional biofabrication techniques for spatial control.	[108]
	On-demand disorder:order transitions can fabricate mechanically-robust scaffolds for cell culture.	Extrusion bioprinting.	Cell viability is lower than a tissue culture plastic control.	[106]
	Harnessing molecular interactions of IDPs facilitates fabrication of complex architectures.	Microfluidics.	Stability augmentation requires use of UV-crosslinking, which may damage cells.	[103]
	Solutions to reaction-diffusion equations can	Extrusion bioprinting.	Suitable for designing stiff structures for bone tissue	[95]

Reaction-diffusion and compartment-alization	generate more accurately biomimetic architectures. 3D-printing with phase-separated peptides allows control of nanoscale geometries and porosity. Consideration and spatial control of morphogen gradients can direct cell differentiation.	Digital light processing. Extrusion bioprinting	engineering, but less applicable to softer tissues. Increased porosity may negatively impact mechanical stiffness. This technique required the use of UV curing, which may damage cells.	[90] [97]
Out-of-equilibrium processes	Piezoelectric actuators generate acoustic waves which can align cells for controlled growth. Molecular motors can direct mesenchymal stem cell differentiation. Accessing the non-equilibrium state on-demand facilitates the fabrication of novel biomaterials	Screen-printing. Molecular motors. Extrusion bioprinting	Useful for aligned tissues/cells, less applicable to other cell types. Difficult to integrate with traditional biofabrication techniques for spatial control. There are concerns over the cytotoxicity of graphene oxide.	[115] [116] [107]

Table