1.3.6

Natural Materials

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Introduction to Natural Materials

The use of naturally occurring materials as matrices or scaffolds to support cell growth and proliferation significantly impacted the origin and progress of tissue engineering and regenerative medicine (TERM). However, the majority of these materials failed to provide adequate cues to guide cell differentiation toward the formation of new tissues. Over the past decade, a new generation of multifunctional and smart natural-based materials has been developed to provide biophysical and biochemical cues intended to specifically guide cell behavior.

Natural-based polymers originate from millions of years of nature's evolution in different environments, which resulted in an outstanding range of well adapted macromolecular designs to perform multiple structural and biological functions (Mano et al., 2007). In general, these can be categorized into three types of biopolymers: (1) proteins–chains of amino acids (e.g., collagen, elastin); (2) polysaccharides–chains of sugar (e.g., chitin, cellulose, glycosaminoglycans); and (3) nucleic acids–chains of nucleotides (DNA, RNA) (Table 1.3.6.1). From the point of view of origin, they might be derived from plants, animals (xenogenic), or humans (allogenic and autologous). Natural polymers offer several advantages with respect to synthetic polymers (Fig. 1.3.6.1): (1) they frequently avoid the immunogenic response and toxicity typical of synthetic polymers, thus presenting higher biocompatibility; (2) they contain bioactive motifs enabling local remodeling and cell spreading and a fibrillar architecture that can be deformed by cells, thus better mimicking the extracellular matrix (ECM); and (3) they can be recognized and metabolically processed by the body. However, natural polymers have historically been associated with some disadvantages, including batch-to-batch variability, lower modularity, and inadequate biomechanical properties. Recent developments in the field have led to a reduction in these drawbacks, and allowed exploration of the full potential of naturally occurring polymers to develop a number of biomaterials that mimic key aspects of the native ECM (Mano et al., 2007).

In this chapter, we first review the use of ECM proteins and blood-derivatives intrinsic capacity to mimic the biophysical and biological characteristics of native tissues. Furthermore, the design of a variety of nanostructures using the well-explored characteristics of nucleic acids is summarized. In the second section, the exploitation of supramolecular chemistry to create new dynamic functional hydrogels that mimic the ECM structure and/or composition is surveyed. Finally, we focus on the incorporation of nanoelements in polymeric networks for the design of smart nanocomposite materials with tailored functionalities to guide cell behavior.

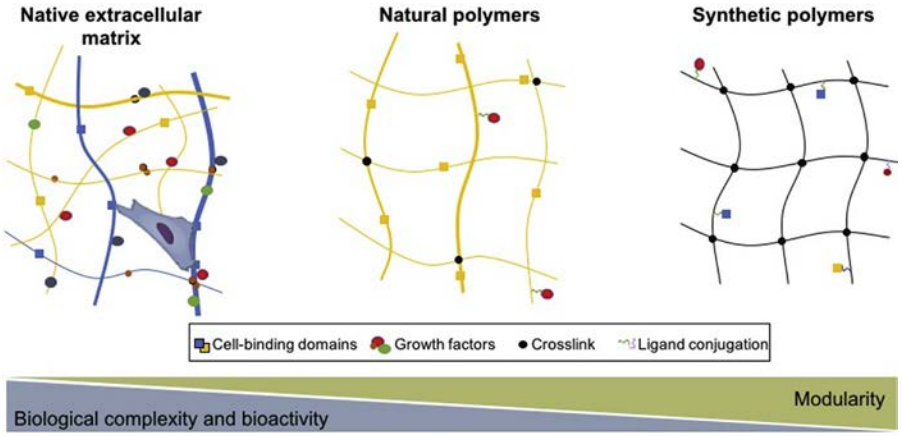
Natural Based-Biomaterials Exploring Structural Molecules

Extracellular Matrix-Based Biomaterials

The ECM comprises a complex milieu of proteins and glycosaminoglycans (GAGs) that provides a physical scaffold for maintaining the structural integrity of tissues, serves as an adhesive substrate for cell attachment and organization, and as a reservoir for biochemical cues to support cell survival and differentiation (Hussey et al., 2018; Mecham, 2001). These results in the establishment of specialized environments that contribute to the specific cell phenotypes and functions, making the ECM composition

TABLE 1.3.6.1 **Origin and Relevant Properties of Selected Natural Polymers**

Natural Material	Source	Relevant Properties
Proteins		
Collagen or denatured collagen (gelatin)	Animal tissues/cell culture	ECM protein. Provides cell-binding sites. Thermal gelation. Easy chemical modification
Decellularized ECM	Animal tissues/cell culture	ECM composition and structure mimetic. Tissue-specific. Provides cell-binding sites
Blood derivatives	Blood	ECM mimetic. Provides biochemical cues related to tissue healing/regeneration. Natural enzymatic gelation with thrombin (clot formation). Provides cell-binding sites
Polysaccharides		
Chondroitin sulfate	Animal tissues	Sulfated GAG present in connective tissues. Different sulfation patterns/growth factors affinity depending on source
Hyaluronic acid	Animal tissues/bacterial fermentation	ECM component. Nonsulfated GAG. Easy chemical modification
Cellulose nanocrystals	Plants, bacteria and tunicates (marine invertebrate animal)	High strength and aspect ratio. Used as nanofiller and reinforcement element
Nucleic acids		
DNA	Cells	Predictable biophysical and biochemical behavior. Different applications



• **Figure 1.3.6.1** Native extracellular matrix is a heterogeneous fibrillar network that provides biochemical and biophysical cues to cells. The use of natural polymers aims to mimic the native ECM complexity, which is very difficult to achieve using synthetic polymers. On the other hand, the modularity and chemical modification possibilities are wider on synthetic polymers.

tissue/organ-specific. Most ECMs are formed by a hydrogel-like porous network of fibrous proteins and linear GAGs. This fibrillar structure of the ECM influences its physical properties, signaling of growth factors (GFs), cell fate, and transport of nutrients and waste (Prince and Kumacheva, 2019). In order to recreate the biophysical and biochemical characteristics of the native tissues, the use of ECM-based biomaterials has gained interest in the last few years.

Proteins

Naturally occurring materials processed from purified or recombinant ECM proteins, such as collagen, laminin, fibrin, fibronectin, or elastin, have been widely used to create a myriad of biomimetic hydrogels and scaffolds for diverse TERM applications. The most abundant and well-studied protein of mammalian ECM is collagen, which accounts for nearly 90% of the dry weight of most tissues and organs. Other abundant ECM proteins include fibronectin and elastin. Collagen and its partially hydrolyzed form, gelatin, are among the most preferred ECM proteins used in TERM due to their wide availability in nature (bovine, porcine, and marine), and ease of processing and modification using various techniques and chemistries. For example, the modification of gelatin with methacryloyl residues (also known as GelMA) has been widely used to generate constructs with controlled architectures using micromolding, photomasking, bioprinting, self-assembly, and microfluidic techniques. The derived structures have been explored in a wide range of applications including bone, cartilage, cardiac, vascular tissues engineering, and drug and gene delivery (Yue et al., 2015).

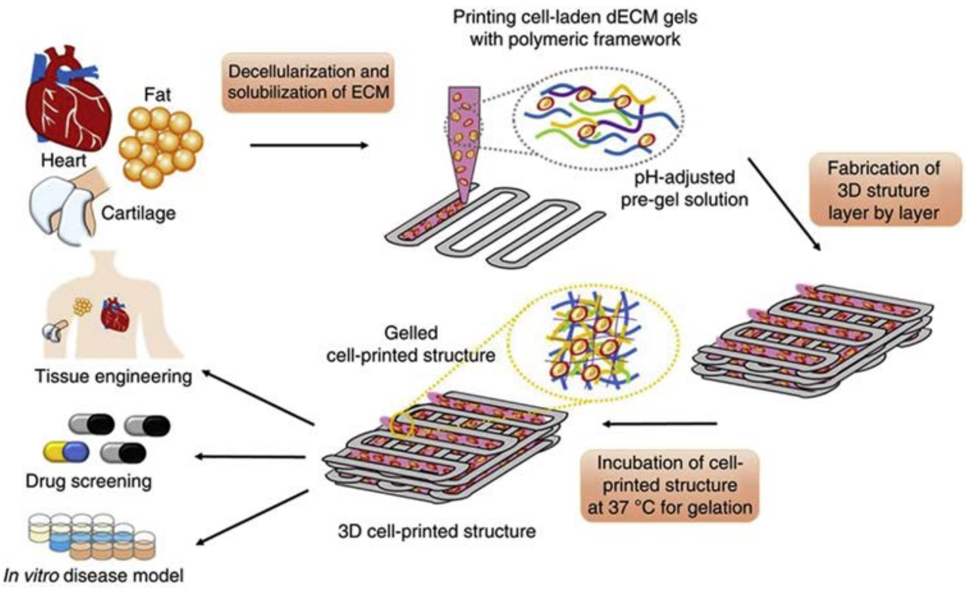
Elastin is an extremely durable ECM protein responsible for the repetitive and reversible elastic recoil of tissues. The insoluble nature of elastin makes its processing challenging and incompatible with some techniques, which restricts its use in TERM approaches. Tropoelastin, on the other hand, is the soluble precursor of elastin and combines the similarities to elastin with easy handling, making this monomer a versatile building block to engineer biomaterials (Mithieux et al., 2013; Wise et al., 2009). It has been incorporated as a bulk material to build highly elastic hydrogels (Annabi et al., 2017, 2010), films produced through casting (Hu et al., 2011), or electrospun fibrous scaffolds (Machula et al., 2014; Nivison-Smith et al., 2010) to enhance cell adhesion, proliferation and migration, neovascularization, and even direct stem cell commitment (Yeo and Weiss, 2019). It has also been used as surface coatings to enhance cell interaction with the interface and to potentiate integration within the body (Yeo et al., 2017, 2012). In a different approach, recombinant and synthetic elastin-like polypeptides [repetition of the pentapeptide valine-proline-glycine-valine-glycine (VPGVG) from elastin], which mimic the extensibility and thermal properties of natural elastin, have been used to produce a range of biomaterials, including nanoparticles, electrospun fibers, and hydrogels cross-linked used different chemical strategies (Rodríguez-Cabello et al., 2018). These polypeptides also have been modified to incorporate "clickable" sequences (azides and alkynes) that react orthogonally to form an irreversible covalent bond, which resulted in hydrogels with fully tunable viscoelastic properties (González de Torre et al., 2014; Testera et al., 2015).

In order to recapitulate the cell-binding properties of ECM proteins, different biomaterials/formats have been conjugated with the bioactive domains of these proteins. The majority of the published studies (89%, from 1979 to early 2018) used the tripeptide arginine-glycine-asparagine (RGD), which is the minimal binding domain of some ECM proteins such as fibronectin, vitronectin, and laminin to integrin receptors (Huettner et al., 2018). Beyond this, many binding domains derived from ECM proteins have been recently described, which has opened new avenues for designing complex strategies to target diverse cell receptors (Huettner et al., 2018). For example, PCL scaffolds coated with RGD and tyrosine-isoleucine-glycine-serine-arginine (YIGSR) promoted axonal regeneration, functional recovery, and vascularization within regenerating nerve tissues in vivo through the activation of independent signaling pathways (Zhu et al., 2017a). Nevertheless, although the short binding domains can support cell attachment, migration, and activation of specific pathways, they have severely reduced binding affinity and specificity compared with the same peptide presented within its associated full-length 3D protein structure (Li et al., 2017; Martino et al., 2009). Interestingly, the presentation of RGD in fibronectin sequences containing the major integrin-binding domain has been shown to improve binding affinity and modulate GF signaling (Martino et al., 2011). Therefore, the presentation of multiple cell-adhesion domains in their correct three-dimensional (3D) structural context can open new avenues for precisely controlling cell function and identifying synergistic effects that promote tissue regrowth and regeneration.

In addition, decellularized ECM (dECM)-based hydrogels have emerged as promising "game-changer" materials to develop biomimetic bioinks for 3D bioprinting (Choudhury et al., 2018). These bioinks are prepared from decellularized tissues and organs that are typically mixed and/or cross-linked using different natural and synthetic polymers or cross-linking agents to make them printable alone or in combination with biocompatible thermoplastics (e.g., PCL) to obtain mechanically reinforced scaffolds (Fig. 1.3.6.2). So far, different dECM bioinks have been derived from various organs such as heart, liver, fat, cartilage, skeletal muscle, skin, and vascular tissue (Choudhury et al., 2018). In a pioneering work, heart dECM bioink was developed, which allowed myoblasts to produce the cardiac-specific proteins actinin and fast myosin heavy chain-6, when compared with a collagen bioink (Pati et al., 2015, 2014). Thus, dECM bioinks have great potential to direct and mediate the differentiation of stem cells to create biomimetic and functional tissues, which would have applications in drug screening, disease modeling, and regenerative medicine. Nevertheless, since dECM is prepared either from human donors or animals, immunological issues need to be considered as well as the batch-to-batch high variability and limited availability of tissues. In order to overcome these issues, the use of cell-culture-derived matrix to produce dECM could offer a reliable alternative (Hussey et al., 2018). Using this approach, autologous cells can be cultured to obtain specific ECMs, avoiding concerns about the immune response. Furthermore, it could be obtained on a larger scale with higher reproducibility using cell expansion factories, although it remains very far from clinical translation.

Glycosaminoglycans

GAGs are negatively charged linear polysaccharides frequently found in the ECM conjugated with proteins, forming proteoglycans. They play important structural and regulatory roles in the ECM and are involved in many important cellular signaling processes governing tissue growth and development. Important types of GAGs that differ in chemical composition, structure, and function include the nonsulfated hyaluronan, the sulfated heparan sulfate and the closely related heparin, the sulfated chondroitin sulfate and the related dermatan sulfate, and keratan sulfate. Considering the diverse cell-instructive roles of GAGs in native tissues, GAG-based biomaterials have attracted huge attention in the field of TERM (Dinoro et al., 2019; Freudenberg et al., 2016).



• **Figure 1.3.6.2** Three-dimensional printing using specific cell-laden dECM bioinks. (Reproduced with permission from Pati, F., Jang, J., Ha, D.-H., Won Kim, S., Rhie, J.-W., Shim, J.-H., Kim, D.-H., Cho, D.-W., 2014. Printing three-dimensional tissue analogues with decellularized extracellular matrix bioink. Nat. Commun. 5, 3935. https://doi.org/10.1038/ncomms4935.)

Chondroitin sulfate (CS) is composed of repeating glucuronic acid and N-acetylgalactosamine units and has a high negative charge density. The main sources of CS for biomedical applications are cartilaginous tissues from bovine trachea and, in recent years, also from marine sources such as shark cartilage (Valcarcel et al., 2017). CS is a very heterogeneous family of polysaccharides with high variability in terms of sulfation pattern and chain length, which depend mainly on their sources. CS has traditionally been used for polyelectrolyte complexation strategies to build, e.g., membranes, fibers, and nanoparticles (Costa-Almeida et al., 2016; Santo et al., 2015; Silva et al., 2016).

Hyaluronic acid (HA) is the only nonsulfated GAG, and consists of repeating disaccharide units of N-acetylglucosamine and glucuronic acid. For biomedical and biotechnological use, it has been traditionally isolated from animal sources, mainly rooster combs, but in recent years microbial fermentation has emerged as the preferred alternative in industrial production of HA (Liu et al., 2011). Although HA is among the preferred polymers for hydrogel matrices in the biomedical field, it does not have any natural and effective physical mechanisms of gelling (e.g., thermal, ionic) and therefore, chemical modification is required for this type of application (Highley et al., 2016). Readers are addressed to the section "Dynamic Hydrogels Exploring Supramolecular Chemistry" of this chapter and to the excellent reviews from Burdick's group to gain a better overview of HA-based biomaterials for biomedical applications (Burdick and Prestwich, 2011; Highley et al., 2016). Among several strategies, recent works have developed an injectable hydrogel using a combination of dynamic covalent cross-linking with thermoresponsive engineered proteins provided by hydrazine-modified elastin-like protein and aldehyde-modified HA. This biomaterial allowed the encapsulated cells maintain their ability to differentiate into multiple lineages after injection (Wang et al., 2017) and the increase in cartilage-marker gene expression and GAG deposition while minimizing undesirable fibrocartilage phenotype (Zhu et al., 2017b).

Blood Derivatives as a Source of Bioinstructive Materials

Upon tissue injury, blood components, together with the provisional fibrillar matrix, are crucial to achieve hemostasis, to support cell infiltration, and to establish a spatial-temporal chemotactic gradient of proteins (e.g., GFs, cytokines, morphogens) that can tightly regulate the complex wound healing microenvironment (Anitua et al., 2019; Mendes et al., 2018a). During normal wound repair, the mimetic fibrillar network embedded with biologically active proteins is remodeled, being gradually degraded and replaced by mature ECM, until tissue repair is achieved. In a biomimetic approach, blood derivative formulations have been extensively studied to guide the wound healing process into regenerative pathways by tailoring the biophysical and biochemical cues present on the biomimetic ECM (Mendes et al., 2018a). In particular, platelet-rich blood derivative strategies led to the development of a panoply of novel human-based biomaterials that are making their way to clinical application.

Under physiological conditions, activated platelets release an enriched milieu of proteins, while circulating fibrinogen is polymerized, producing an insoluble fibrin matrix (clot) through multiple molecular mechanisms (Piechocka et al., 2010; Ryan et al., 1999). Briefly, fibrin polymerization is initiated by thrombin cleavage, which exposes the central domain of fibrinogen-binding sites to interact with complementary sites at the terminal domains of other fibrin molecules. These noncovalent interactions induce the self-assembly of fibrin monomers and the formation of half-staggered two-stranded protofibrils. When protofibrils achieve a threshold length, they start to associate laterally to form fibers that branch into a stable three-dimensional network due to plasma transglutaminase (factor XIIIa) covalent cross-linking and calcium ions presence. It is clear that the self-assembled fibrin matrix not only provides a provisional hierarchical ECM to facilitate cellular infiltration, but also contains numerous binding domains for the cells, ECM proteins, and secreted GFs (Brown and Barker, 2014; Laurens et al., 2006). Interestingly, fibrin contains binding sequences for integrins, which facilitate the adhesion of platelets, endothelial cells, smooth muscle cells, fibroblasts, and leukocytes (Lishko et al., 2004). Moreover, fibrin through its heparin-binding domain shows a promiscuous high affinity to bind platelet-derived GF (PDGF), vascular endothelial GF (VEGF), and fibroblast GF (FGF), which can be released from the activated/disrupted platelets (Martino et al., 2013). The complex and hierarchical fibrin structure, in combination with the unique molecules binding interactions, highlight the important and ubiquitous role of fibrin as a bioinstructive platform that provides the biophysical and biochemical cues to modulate a number of complex cellular responses.

In a biomimetic regenerative medicine approach, blood derivatives composed by a high content of structural proteins have been easily produced from autologous or heterologous whole blood samples (Mendes et al., 2018a). Thus, blood derivative formulations can be used directly as a liquid or a liquid-to-gel fibrin-based scaffold through the activation of collagen, calcium, or/and thrombin. The modulation of fibrinogen, thrombin, and calcium/salt concentrations has a marked impact over fibrin polymerization dynamics and the resulting fibrin scaffold properties, namely on fiber diameter, mechanical properties, and bioactive molecule sequestration (Brown and Barker, 2014; Dohan Ehrenfest et al., 2012; Piechocka et al., 2010). Throughout the last few decades, fibrin-based blood derivative formulations have been already successfully translated to the clinics (e.g., fibrin glue) due to their positive effects in the localized and sustained delivery of therapeutic GFs in the treatment, for instance, of periodontal and soft tissue wound healing (Anitua et al., 2019; Heher et al., 2018; Miron et al., 2017; Weisel and Litvinov, 2013). However, the lack of standardization in the formulations' preparation, poor characterization of formulations, and conflicting codelivery of multifunctional biomolecules led to conflicting results in the therapeutic outcomes, opening a debate on their real potential within the field (Marx, 2004; Wang and Avila, 2007). Moreover, the use of fibrillar gels produced from blood derivative precursors has attracted significant interest in the promotion of vascularization in regenerative strategies due to their natural reservoir in angiogenic GFs (e.g., VEGF) (Fortunato et al., 2016). Nevertheless, these strategies also exhibit limited ability to protect biomolecules from fast clearance and proteolytic degradation, have low mechanical properties, and show a high contractile effect upon cell encapsulation, which severely limits their potential as wound-healing modulators (Robinson et al., 2016; Sadeghi-Ataabadi et al., 2017).

In order to overcome the above-mentioned drawbacks, a wide range of natural and synthetic polymers, inorganic materials, or their blends have been recently combined with blood derivatives to tightly modulate spatiotemporal biomolecules delivery and to reinforce its inherent mechanical and structural properties (Mendes et al., 2018a). Recently, Faramarzi et al. embedded the inert alginate polymer matrix with the enriched bioactive milieu of platelet-rich plasma (platelets suspended in platelet-poor plasma at 1×10^6 platelet μL^{-1} concentration) to obtain a biofunctional alginate bioink for 3D bioprinting (Faramarzi et al., 2018). The engineered patient-specific bioink exhibited a gradual release of bioactive proteins and positively affected the function of mesenchymal stem cells and human umbilical vein endothelial cells, which could be explored to induce a healing response in cardiovascular and musculoskeletal bioprinted tissue constructs. Our research group has been focused on the modulation of biomolecule release kinetics from platelet lysate (platelet disruption by freeze–thaw cycles) hydrogels in a timely and a controlled space by solely using magnetic stimulation (Silva et al., 2018). The incorporation of magnetic nanoparticles in a methacrylated chondroitin sulfate hydrogel loaded with platelet lysate enabled the modulation of hydrogel physical properties (swelling, matrix stability, and degradation) and control over the GF profile release. This magnetic stimulation control led to a synergistic impact on cell morphology and synthesis of tendon- and bone-like matrix in an in vitro interfacial coculture model. In a different work, using polyelectrolyte complexation between two oppositely charged polysaccharides——chitosan and CS—resulted in the formation of nanoparticles that were loaded with PL and human adipose-derived stem cells. These particles showed an initial burst release of growth factors that correlated with higher cell proliferation, compared to cultures on unloaded particles and in the form of cell pellets (Santo et al., 2015). Along the modulation of bioactive molecules profile release, nanomaterials have also been explored to fine-tune hydrogel mechanical properties to trigger specific cellular responses (Memic et al., 2015). This concept was explored on the development of an injectable fibrillar nanocomposite hydrogel based on platelet lysate combined with cellulose nanocrystals (CNC) (Mendes et al., 2018b). Here, by increasing CNC loading it was possible to increase stiffness, improve biomolecule sequestering, and hinder the typical extensive retraction upon 3D cell culture, enabling therefore the modulation of encapsulated human adipose-derived stem cell behavior.

The development of blood derivatives-based biomaterials with biochemically, mechanically, and structurally tunable properties will clearly improve control over the proteins' milieu composition and kinetics release profile. These findings can be explored to fine-tune stem cell behavior toward specific lineages and, in combination with recent developments in the regenerative mechanisms, to engineer the wound-healing microenvironment toward tissue regeneration.

Multifunctional Biomaterials Based on DNA

Deoxyribonucleic acid (DNA) is a very old and well-known molecule due to its role as genetic information carrier. However, recently, it has also become a key player in materials science. Exploiting the biophysical and biochemical features of single- and double-stranded DNA, a variety of DNA-based materials showing programmable and multifunctional characteristics are used for biomedical applications (Ke et al., 2018; Shahbazi et al., 2018; Zhang et al., 2018). In this section, we focus on hydrogels, aptamers, and nanostructures based on DNA for tissue engineering applications.

The hydrated nature of DNA hydrogels, which can mimic the properties of natural tissues, has awakened the interest in the design of DNA-based tissue engineering scaffolds. DNA hydrogels are normally created using ligase cross-linking or self-assembly reactions, enabling the gelling to occur under physiological conditions and, thus, the encapsulation of cells. These gels have shown excellent biocompatibility and biodegradation (Stoll et al., 2017). In addition, they have been designed to respond to a variety of stimuli, such as temperature, pH, enzymes, light, or magnetic forces to alter their conformation (Shahbazi et al., 2018; Zhang et al., 2018). For example, Liu's group reported a DNA hydrogel that undergoes a pH-driven conformational transition (called the DNA motor), which influences the spatial distance between cross-linking points and subsequently the stiffness of the hydrogel. By changing the pH between 5.0 and 8.0, the storage modulus of the hydrogel could be tuned reversibly from 1000 to 250 Pa (Zhou et al., 2016).

The aptamers, also known as "nucleic acid antibodies," are designed to specifically bind with high affinity to a target molecule, have been classically used for disease diagnosis and therapy (Keefe et al., 2010; Zhang et al., 2018). DNA aptamers are generated using SELEX, an in vitro selection and evolution process, it being theoretically possible to create aptamers that target any molecule of interest (Gelinas et al., 2016). Unlike antibodies, the generation of aptamers is much cheaper and easier and reduces ethical and immunogenic constraints, offering new perspectives in biomedical applications. In a pioneering work using DNA aptamers to design GF mimetics for regenerative medicine, an aptamer for the hepatocyte GF (HGF) receptor (also known as c-Met), which plays a key role in cancer metastasis, inhibited HGF-induced c-Met activation, which suppressed cancer cell mobility in vitro (Ueki and Sando, 2014). More recently, aptamers were engineered to bind the PDGF-BB and VEGF. One end of the aptamer was linked to an RGD peptide and the other end to a functional group able to covalently link the aptamer to different material systems such as glass, polymers, or collagen scaffolds. In this new strategy the specific GFs are delivered using cell traction forces on the ECM, avoiding the need for exogenous triggers (Stejskalová et al., 2019).

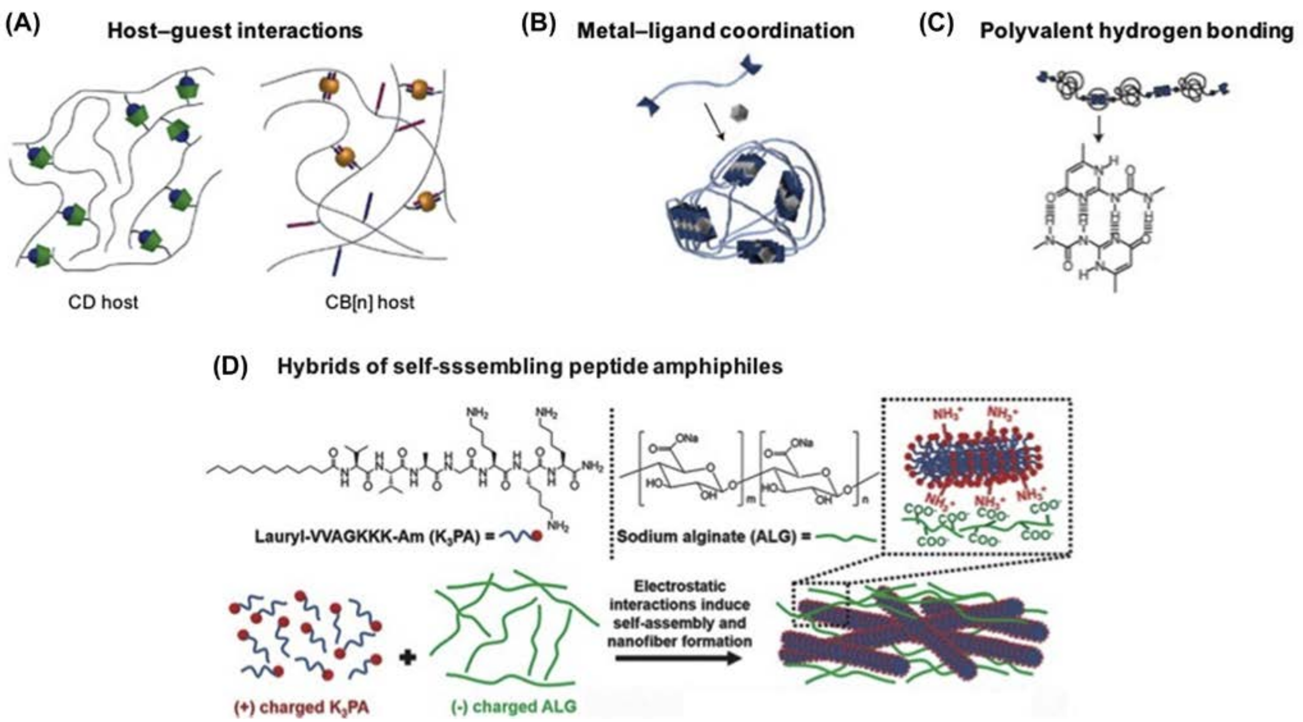
Exploiting nitrogenous bases complementarity and DNA predictable folding, the DNA origami technique provides a versatile platform to engineer nanoscale structures and devices that can sense, compute, and actuate (Hong et al., 2017; Zhang et al., 2018). This has opened a wide range of applications in chemistry, biology, physics, material science, and computer science (Hong et al., 2017). In the field of biomedicine, the most frequent application of these nanostructures is for targeted drug delivery of anticancer drugs, while its use in classical tissue engineering approaches is still limited to a conceptual stage, which is addressed in the last section of this chapter.

Dynamic Hydrogels Exploring Supramolecular Chemistry

A plethora of biocompatible hydrogel cross-linking mechanisms have been developed along the years to recreate hydrated mesh-like architectures of native tissue ECM. Nonetheless, conventional hydrogel systems typically rely on stable, permanent covalent cross-linking bonds that do not capture its highly dynamic character. The inability to relax and dissipate stress, a phenomenon that happens in native ECM by reorganization of physical cross-links or entanglements, or the restrictions that they pose to the spreading, migration, and proliferation of encapsulated cells, are limitations of most static hydrogels (Rosales and Anseth, 2016). In some cases, hydrogel degradation may proceed through hydrolytic or cell-mediated enzymatic degradation (Kharkar et al., 2013). However, the degradation of permanently cross-linked hydrogels presents several drawbacks in TERM applications, such as fast disappearance or deterioration and spatial inhomogeneity of mechanical properties over time. These features pose significant challenges in studying and controlling how cells respond to local biophysical cues, which is increasingly recognized as a key parameter in determining the fate of cells (McKinnon et al., 2014; Rodin et al., 2010).

Reversible Hydrogels Based on Supramolecular Cross-Linking of Polymeric Precursors

Hydrogels based on reversible (or adaptable) linkages that can be broken and reformed in a reversible manner (self-healing) under physiological conditions without external triggers (such as changes in pH or temperature) are becoming increasingly popular in the design of biomaterials (Fig. 1.3.6.3). Several comprehensive reviews covering this topic can be found in the literature (Mann et al., 2018; Saunders and Ma, 2019; Wang and Heilshorn, 2015). The dynamic nature of the linkages (built through physical associations or dynamic covalent bonds) applied to obtain reversible hydrogel systems has significant advantages over static hydrogels. Inherently, it enables the spatiotemporally controlled addition and removal of biochemical signals, repeated changes in matrix mechanics and, having shear-thinning (viscous flow under shear stress) and self-healing (time-dependent



• **Figure 1.3.6.3** Supramolecular biomaterials formed by cross-linking of polymeric precursors through (A) host–guest complexation using macrocyclic hosts, (B) coordination of metals with ligands (end-terminated or grafted on polymer chains), and (C) multiple hydrogen-bonding motifs. (D) Hybrid supramolecular combining high-molecular-weight biopolymers and oppositely charged low-molecular-weight peptide amphiphiles. (Figure adapted with permissions from: (A) Mann, J.L., Yu, A.C., Agmon, G., Appel, E.A., 2018. Supramolecular polymeric biomaterials. Biomater. Sci. 6, 10–37. https://doi.org/10.1039/C7BM00780A; (B) Burnworth, M., Tang, L., Kumpfer, J.R., Duncan, A.J., Beyer, F.L., Fiore, G.L., Rowan, S.J., Weder, C., 2011. Optically healable supramolecular polymers. Nature 472, 334–337. https://doi.org/10.1038/nature09963; (C) Dankers, P. Y. W., Harmsen, M. C., Brouwer, L. A., Van Luyn, M. J. A., Meijer, E. W., 2005. A modular and supramolecular approach to bioactive scaffolds for tissue engineering. Nat. Mater. 4, 568–574. https://doi.org/10.1038/nmat1418; and (D) Borges, J., Sousa, M.P., Cinar, G., Caridade, S.G., Guler, M.O., Mano, J.F., 2017. Nanoengineering hybrid supramolecular multilayered biomaterials using polysaccharides and self-assembling peptide amphiphiles. Adv. Funct. Mater. 27, 1605122. https://doi.org/10.1002/adfm.201605122.)

recovery upon relaxation) characteristics, they allow the encapsulation and delivery of cells though minimal invasive strategies (Guvendiren et al., 2012; Rosales and Anseth, 2016). This set of characteristics further makes them interesting materials to be applied as bioink for 3D bioprinting (Wang and Heilshorn, 2015). This biofabrication strategy is an emerging and rapidly expanding field of research in which additive manufacturing techniques in combination with cell printing are exploited to generate hierarchical tissue-like structures. In fact, the bioinks, materials that combine printability with cytocompatibility, are currently one of the biggest bottlenecks of 3D bioprinting technology. Thus, new polymeric biomaterials that can overcome these limitations are particular needed, making reversible hydrogel systems appealing alternatives in this field. Moreover, self-healing hydrogel with rapid self-integrating properties may provide a novel injectable solution for the regeneration of tissue interfaces needing different spatiotemporal biochemical and biophysical cues but that seamlessly integrate at their interfaces (e.g., bone–cartilage tissue complex).

Among the possible approaches to produce reversible hydrogels, systems based on transient polymeric networks formed through supramolecular cross-linking of polymer chains such as host–guest inclusion complexes (Fig. 1.3.6.3A), multiple hydrogen bonding, or metal–ligand coordination (Fig. 1.3.6.3B–C) are currently among the most promising strategies (Appel et al., 2012; Webber et al., 2016). Here, we highlight some representative studies exploring these reversible hydrogel design strategies with potential application in TERM.

Functionalization of polymers and macromeres with pendant host–guest motifs has been among the preferred strategies to produce building blocks of reversibly cross-linked hydrogels. Examples of these systems are the host–guest inclusion complexes using macrocyclic host molecules such as cyclodextrin (CD) and cucurbit[n]uril (CB[n], n = 5–8), showing high affinity for small hydrophobic guest molecules (Mann et al., 2018; Wang and Heilshorn, 2015; Webber et al., 2016). Although other macrocyclic hosts have been explored (e.g., crown ethers, catenanes, and cyclophanes), CD and CB[n] are the most commonly applied in TERM. This is mainly because of their compatibility with aqueous use since the interaction between host and guest occurs in a hydrophobic cavity that minimizes polar solvent interactions (Assaf and Nau, 2015; Mann et al., 2018). Both CD and CB[n] bind guest molecules through hydrophobic and van der Waals interactions in the hydrophobic cavity. However, CB[n] has a higher bind versatility. Whereas CB[6] tends to bind neutral and positively charged organic guests, CB[7] binds larger amphiphilic guests, and CB[8] binds positively charged and large organic guests (Assaf and Nau, 2015; Mann et al., 2018). These different affinities contribute to widening the number of possible hydrogel designs. Moreover, different binding affinities exist not only between CB[n], but also between the host and different guest molecules, a particular behavior that has been recently explored to develop hydrogels with a broad range of bulk dynamic properties without changing network topology (Zou et al., 2019). Both natural (e.g., HA) and synthetic polymeric (e.g., polyacrylamide or PEG) precursors have been cross-linked using these recognition motifs to produce reversible supramolecular hydrogels. The precursor polymers can be grafted with either a host or its complementary guest molecule to form hydrogels when the pairs are mixed. This cross-linking strategy has as an additional advantage the possibility of using the same grafted supramolecular motifs for the modular noncovalent functionalization of polymer hydrogel through biorthogonal synthesis pathways.

Numerous hydrogel systems based on these supramolecular cross-linking strategies have been developed and optimized in the past for 3D cell encapsulation and as injectable cell carries (Wang and Heilshorn, 2015). The β-CD–adamantane host–guest pair is perhaps one of most well-known cross-linking systems explored in the TERM field. For example, HA has been modified with these pendant moieties to produce a hydrogel that enables shear-thinning injection and high target site retention (>98%) (Rodell et al., 2015). Stabilization of the network through secondary Michael addition reaction (between thiol- and Michael-acceptor modified HA macromers) further improved the positive outcomes on the treatment of myocardial infarct in an in vivo mouse model. In a similar approach, the low stability and mechanical properties of β-CD–adamantane cross-linked HA has been improved by first promoting the host–guest complexation between monoacrylated β-CD host monomers and adamantane-functionalized HA guest polymers, to fabricate the supramolecular hydrogels by UV-induced polymerization of preassembled host–guest complexes (Wei et al., 2016). These hydrogels demonstrated self-healing behavior and showed promising results as injectable cell carriers, supporting chondrogenesis of human mesenchymal stem cells (hMSCs) and promoting cartilage regeneration in a rat model. Injectable and cytocompatible hydrogels have also been produced based on CB[6]-modified HA mixed with the respective complementary guest, diaminohexane conjugated HA (DAH-HA), that was applied for the controlled chondrogenesis of hMSCs (Jung et al., 2014). Interestingly, this host–guest pair was not only used to produce the hydrogel as a cell carrier, but was also leveraged to functionalize the polymer backbone with a prochondrogenic drug conjugated to CB[6], demonstrating the modular nature of these type of systems. The versatility of this strategy was later explored to support the long-term survival and prolonged transgene expression of bioengineered MSCs, a potential platform biomaterial that can be applied in the treatment of cancer and other intractable diseases (Yeom et al., 2015).

Functionalization of polymers or macromers with multiple hydrogen bonding motifs, such as the quadruple hydrogen bonding motif ureidopyrimidinone (UPy), is another widely reported strategy for the production of supramolecular hydrogels with viscoelasticity, shear-thinning, and self-healing behavior (Saunders and Ma, 2019; Webber et al., 2016). Several hydrogels based on UPy cross-linking have been used as biologic, and recently, also as cell delivery systems. A notable example of this strategy is a dextran-based hydrogel where the polymer backbone was functionalized with multiple pendant UPy units per chain (Hou et al., 2015). This supramolecular hydrogel was used as a cell carrier and drug-delivery system (chondrocytes, bone marrow stem cells, and bone morphogenetic protein 2). Moreover, due to its self-healing and shear-thinning nature, the system has the potential to be applied in the regeneration of complex tissue interfaces.

Metal–ligand interactions are an alternative cross-linking strategy that are gaining relevance in the biomaterials field. This class of supramolecular cross-linking, in which two or more ligands donate a nonbinding electron pair to empty orbitals in a transition metal cation, include the well-known Fe-catechol coordination complexes that have stability and strength approaching covalent bonds (Mann et al., 2018; Webber et al., 2016). Several mussel-inspired metal–ligand hydrogels based on Fe-catechol cross-linking have been developed in the past few years. For example, HA functionalized with pendent catechol can be cross-linked with Fe^{3+} ions, resulting in reversible, self-healable, and tissue-adhesive hydrogels (Lee et al., 2016; Park et al., 2016; Shin et al., 2015). These biocompatible injectable hydrogels were demonstrated to be effective for cell transplantation (Shin et al., 2015) and could potentiate the in vivo stem cell-mediated angiogenesis and osteogenesis potential in tissue defect models (Park et al., 2016), making them promising materials for TERM applications.

The unique rheology and gelation properties of the above-mentioned supramolecular hydrogels make them obvious candidates as bioinks materials for 3D bioprinting. Burdick's research group has published a representative example of such applications. Their approach explores an interesting printing strategy based on HA bioink that cross-links through supramolecular assembly of β-CD–adamantane host–guest complexes (Highley et al., 2015; Ouyang et al., 2016). Since the host–guest supramolecular bonds can be disrupted by the shearing stress applied during the extrusion process and rapidly reform after printing without any further trigger, this system allows reaching new levels of 3D printing complexity, such as the direct writing of shear-thinning bioinks at any position of a 3D space into supporting self-healing hydrogels made of similar materials. The printed structures can be further stabilized by secondary cross-linking by, e.g., introducing methacrylates into the HA and post-printing photopolymerization. The versatility of this platform has shown that supramolecular cross-linked hydrogels are biomaterials with new and existing properties to be explored in the field of 3D bioprinting.

Hydrogels Based on Natural Supramolecular Self-Assembly

Based on DNA self-assembly capacity, hybrid DNA hydrogels can be developed using short DNA sequences mixed or coupled onto the polymer backbones physically or chemically. Contrary to DNA-based hydrogels addressed in the section “Multifunctional Biomaterials Based on DNA,” hybrid hydrogels only require a small quantity of DNA, and self-assembled DNA acts as both the cross-linker and switchable element (Shahbazi et al., 2018; Shao et al., 2017). For example, Li and colleagues combined single-stranded (ss) DNA attached to a polymer backbone with a double-stranded DNA with ending sequences complementary to the ssDNA to develop a biodegradable bioink with encapsulated cells (Li et al., 2015). The hydrogel was biocompatible and could be used for rapid formation of 3D constructs for tissue engineering.

In another very interesting approach, a stimuli-responsive liposome–DNA hydrogel was created by the functionalization of polyacrylamide with cholesterol-modified DNA motifs (Lyu et al., 2018). The cholesterol interacts with the lipid bilayers of the liposomes and cross-links the polymer in a reversible manner (sol–gel–sol transitions) due to the thermosensitive nature of DNA motifs. Moreover, the system can serve as a release system upon the presence of a restriction endonuclease enzyme.

Peptides are versatile building blocks for supramolecular assembly of tissue engineering constructs. The use of peptides to create biomaterials provides a number of advantages such as natural biodegradation, and the possibility to mimic the structural and functional aspects of native ECM. Peptides can be used alone, or designed for conjugation or coassembly with polymers in order to improve bioactivity and structural complexity. In particular, the supramolecular assembly capacity of peptide amphiphiles (PAs), consisting of a charged hydrophilic head, a β-sheet forming domain, and a hydrophobic alkyl tail (Fig. 1.3.6.3D), has been used to create one-dimensional self-assembled fibrillar structures with many applications in biomedicine (Borges et al., 2017; Brito et al., 2019; Radvar and Azevedo, 2019; Webber et al., 2016). Although not covered in this chapter, for more information readers are directed to a recent review on the topic by the Stupp’s group, pioneers in the field (Hendricks et al., 2017).

PA also can be coassembled with hydrophobic polymer tails or polypeptides to construct self-assembling bioactive and biomimetic hydrogels (Borges et al., 2017; Radvar and Azevedo, 2019). In a recent work, a biodegradable self-healing polymer–peptide hydrogel consisting of a poly(γ-glutamic acid) polymer network was physically cross-linked via conjugated β-sheet peptide sequences (Clarke et al., 2017). The authors could tailor the mechanical properties of the hydrogels over an order of magnitude range of 10–200 kPa, which is in the region of many soft tissues, by altering the β-sheet peptide graft density and concentration. In a different approach, using hybrid PA–protein systems, Mata and colleagues produced complex hierarchical fibrillar membranes and 3D printed constructs for tissue engineering applications. On one hand, hybrid PA–elastin-like polypeptides were used as building blocks to fabricate foldable bioactive membranes that guide the growth of endothelial and adipose-derived stem cells into tubular structures (Inostroza-Brito et al., 2015). In a later work, using a range of ECM biomolecules as the protein component of the hybrid system, adipose-derived stem cells were bioprinted with high viability into complex hierarchical structures, demonstrating that the combination of self-assembly with 3D bioprinting has a huge potential in the field (Hedegaard et al., 2018).

Soft Nanocomposite Smart Materials

The nanoscale interactions between the cells and the tissue microenvironment control the function and fate of the cells. Hence, the rational design of biomimetic and functional biomaterials is subjective to these cues (Memic et al., 2015). The design of “smart materials,” stimuli-responsive materials that respond to external stimuli such as pH, temperature, magnetic, electric, or any other stimulus, is gaining increased relevance in the biomedical field (Merino et al., 2015). Among the numerous strategies to incorporate new and tunable functionalities in biomaterials, the incorporation of nanoparticles in hydrogels has become a popular option, not only as mere reinforcement nanofillers, but also as functional nanoelements to meet the specific needs of the different tissues (Alarçin et al., 2016). These nanoelements can be manipulated to interfere with cell processes like growth, differentiation, proliferation, and alignment in a controlled manner (Carrow and Gaharwar, 2015). In this section, we highlight some of the latest developments on soft nanocomposites developed using natural materials.

Stimuli-Responsive Soft Nanocomposites

The intrinsic properties of iron-based magnetic nanoparticles (MNPs) are highly attractive to produce responsive biomaterials providing the liberty to control the spatial distribution and orientation of MNPs through remote magnetic fields (Tibbitt et al., 2015). For example, a hybrid hydrogel composed of type II collagen, hyaluronic acid, and polyethylene glycol, incorporating MNPs was guided to defect tissue sites using a remote magnetic field (Zhang et al., 2015). This work paves a path for further investigation of tunable magneto-responsive nanocomposites for minimally invasive tissue engineering strategies.

Furthermore, the use of rod-shaped CNC, the nature's "carbon nanotubes," has been explored by our group and others to cross-link and reinforce soft HA hydrogel networks (Domingues et al., 2015) and to produce anisotropic domains within natural and synthetic 3D hydrogels (Araújo-Custódio et al., 2019; De France et al., 2017). These CNC could also be decorated with MNPs to create injectable gelatin hydrogels presenting an aligned microstructure, which was achieved by the alignment of the nanoparticles under the influence of low magnetic fields (106 mT) (Araújo-Custódio et al., 2019). At the same time, hydrogel 3D anisotropy induced the alignment of encapsulated and seeded adipose tissue-derived stem cells, when compared with isotropic hydrogels.

In a different approach, the incorporation of electroconductive nanoelements can play a key role in the improvement of regenerative strategies for electro-responsive organs/tissue, such as the heart, muscles, and neural tissue (Palza et al., 2019). For example, an electro-responsive nanogel was achieved via incorporation of gold nanoparticles in a chitosan hydrogel. The amount of nanoparticle in the hydrogel was directly proportional to electrical conductivity. Mesenchymal stem cells seeded on these hydrogels showed high viability and were able to migrate and proliferate, while showing evidence of accelerated cardiomyogenic differentiation in compassion with control hydrogels (Baei et al., 2016).

Future Perspectives

Tissue engineering was born with the promise of revolutionizing health care by providing artificially engineered functional tissue and organ substitutes (Langer and Vacanti, 1993). During the last few decades, extraordinary accomplishments have been achieved and significant scientific knowledge spanning from cell biology up to advanced biomaterials synthesis and processing technologies has been produced. However, the vast majority of strategies has been developed as "one-size-fits-all." Addressing the increasing demand for precision and personalized medicine treatments, in which health care is tailored on the basis of individual complexities (Hodson, 2016), the extension of this concept to the field of TERM will allow to produce biomaterials with precise and specific functions that will be integrated with technological advances such as microfabrication, 3D bioprinting, and stem cells, to select the most suitable approach to treat diseases or injuries in a specific patient or subset of patients (Aguado et al., 2018). Furthermore, using the same principles, personalized tissue constructs have the incomparable potential to be used as disease models that will change the future of drug-discovery pipelines in a manner that substantially deviates from traditional platforms, namely, 2D cell monolayer cultures and animal disease models (Skardal et al., 2016).

The combination of blood derivatives with biomaterials has emerged as a synergistic strategy to modulate the release of signaling molecules that orchestrate the swing between tissue regeneration, tissue repair, and scar formation (Mendes et al., 2018a). In this sense, the use of biomimetic biomaterials described in this chapter incorporating standardized blood derivatives might enable engineering the wound-healing environment toward tissue regeneration. In a translational perspective, we think that this strategy holds great potential to produce biomimetic and bioactive materials for different TERM applications due to the increasing trend on the production and use of standardized clinical-grade human PL as a xeno-free alternative to animal-derived serum in cell culture, which represents an advantage over similar materials such as dECM. This further strengthens the use of blood derivative formulations not only for research purposes, but also in terms of compliance with good manufacturing practices and clinical relevance.

DNA-based materials represent a forefront frontier for the biomaterials field due to their versatility. In particular, an underexplored area is the use of DNA origami for TERM applications. These nanostructures could be used to provide direct cell behavior on surfaces (2D) and hydrogel (3D) environments. For example, the creation of anisotropic DNA origami patterns, as shown in a chemically modified graphene surface (Yun et al., 2012), could be used to provide enough biophysical cues to guide the appropriate stem cells toward the regeneration of anisotropic tissues. Furthermore, the potential of DNA origami nanostructures to bind serum proteins could be explored for the selective/preferential binding of GFs and to control its delivery to target tissues.

In addition, so far mostly the methods to achieve anisotropy within hydrogel matrices depend on external stimuli such as magnetic or electrical, which is not universally practical for large-scale production (Chen et al., 2018). Hence, we believe that continued development in this area of soft nanocomposites is essential to acquire scalable orderly structures, with excellent mechanical properties, and novel responses to stimuli for use in innovative applications in the fields of biomedicine, sensors, actuators, and biomimetic materials.

Using an out-of-the-box concept, a new generation of living biomaterials that contain genetically modified bacteria have been proposed to modulate the microenvironment in a dynamic way. For this, bacteria are engineered to produce proteins and GFs triggered by external stimuli such as molecules and drugs, in a dose-dependent manner (Hay et al., 2018; Sankaran et al., 2018). Furthermore, the combination of additive manufacturing or other advanced technologies and bacteria (Liu et al., 2018; Schaffner et al., 2017) might bring a new generation of living responsive materials on demand, if serious bacterial safety concerns are addressed.

Despite the added functionalities that provide many new materials, the clinical translation of tissue-engineered products has been significantly slower than would be expected. The complexity of some strategies represents the main barrier for clinical translation, as practical, economic, and regulatory barriers favor simplicity (Abou-El-Enein et al., 2017; Darnell and Mooney, 2017). However, tissues and organs have multiscaled architectures, multiple cell types and ECM components, and possess a complex vascular, neural, and lymphatic network to support cell activity in a finely orchestrated dynamic microenvironment. Balancing the need for simplicity with this natural complexity creates the necessity for biomaterials researchers to identify the strategies, within the large design space now available, with the minimum necessary complexity to recreate native tissues.

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