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# Emerging Trends in Information-Driven Engineering of Complex Biological Systems

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Synthetic biological systems are used for a myriad of applications, including tissue engineered constructs for in vivo use and micro-engineered devices for in vitro testing. Recent advances in engineering complex biological systems are fueled by opportunities arising from the combination of bioinspired materials with biological and computational tools. Driven by the availability of large datasets in the "omics" era of biology, the design of the next generation of tissue equivalents will have to integrate information from single-cell behavior to whole organ architecture. This review discusses recent trends in combining multi-scale processes to enable the design of the next

generation of biomaterials. Any successful microprocessing pipeline must be able to integrate hierarchical sets of information to capture key aspects of functional tissue equivalents. Micro- and biofabrication techniques that facilitate hierarchical control as well as emerging polymer candidates used in these technologies are also reviewed.

## 1. Introduction

Recent advances in micro- and biofabrication are fueled by opportunities arising from the combination of bioinspired materials with biological and computational tools ("biomateriomics") to pioneer a new frontier in engineering of complex biological systems[1]. In regenerative medicine, this approach requires integration of complex biological systems with synthetic polymer materials to achieve appropriate physical, mechanical and biological properties[2,3]. Recent advancements in additive manufacturing techniques has resulted in increased functionality and complexity of engineered tissue structures[4]. However, broader breakthroughs have been hampered by technological tradeoffs posed by a limited understanding of the structural complexity of biological tissues. To be able to mimic native tissue, the complex interplay between cells of different phenotypes, their local microenvironment and their time-dependent interactions need to be defined[1]. Beside a clear set of biospecifications, individual anatomical architectures are key elements required for mimicking natural tissue. In this context, medical imaging enables visualization of the anatomical structure, leading to information-guided engineering of 3D models in computer aided design (CAD) of the target tissue[5]. With the help of advanced computer-aided manufacturing (CAM) techniques, polymer scaffolds with high resolution can be produced to support stability and flow transport as part of the extracellular microenvironment. Considering the often orthogonal sets

of material requirements ranging from mechanical properties to biodegradability, the choice of materials remains an important consideration during the design process[6–8]. Additionally, a cell-instructive protein matrix, which resembles the native extracellular matrix (ECM) as closely as possible in guiding cell attachment, orientation, proliferation and differentiation, is required[9]. This review will focus on the various steps and challenges from computer-assisted manufacturing of scaffolds to cell seeding techniques with the goal to produce functional tissues (**Figure 1**).

## 2. Hallmarks of functional tissue

In designing complex biological systems, engineers must consider the most basic properties of tissues that enable function. Classical tissue engineering relies on mimicking the extracellular matrix through the use of natural or synthetic materials, typically referred to as "scaffolds" that support cells[10]. Scaffold composition, architecture, mechanical properties, and biologically active additives should be precisely designed for the target tissue or question of interest[11] (**Figure 2**). In considering this vast design space, it should be appreciated that native matrix molecules assemble hierarchically to form complex and diverse suprastructures that enable diverse functionality ranging from the transmission of light in the cornea to the sustained contraction of cardiac muscle over entire lifetimes[12]. Nature's capacity for precise hierarchical organization enables vast functionality that tissue engineers and materials scientists struggle to compete with. In this section, we briefly focus on defining aspects of the material-biological interface that are critical to designing structures that retain biological relevance (**Table 1**).

### 2.1 Structure and Organization

#### *2.1.1 Organizational hierarchy*

From the assembly of amino acids into proteins to the arrangement of single cells into complex organisms, life is distinct in its remarkable ability to achieve complexity from simpler building blocks. During human embryogenesis, for example, the single-celled zygote undergoes many rounds of mitosis to transition from the single-layered blastula to a tri-layered gastrula composed of the ectoderm, mesoderm, and endoderm[11,13]. From these three germ layers, sheets of connected cells (epithelium) or individual meshes of cells (mesenchyme) give rise to the four types of tissue (connective, epithelial, muscular, and nervous) that constitute all organs. Complex tissues are arranged in a hierarchical fashion that enable specific function. Skeletal muscle, for instance, is a tissue comprised of muscle cells that fuse to form myofibers composed of sarcomeres made of actin and myosin filaments that facilitate muscle contraction. Such hierarchical organization is evident across all tissue types and allows for the diversity of functions found in the human body[14].

The major participants that are critical to tissue structure and function are (i) cells, (ii) the extracellular matrix, and (iii) and signaling molecules[15]. These components work in concert to achieve a targeted function. Establishing the precise balance of these critical elements to either recapitulate tissue or facilitate its repair is the goal of tissue engineering[16]. While nature drives these processes through self-assembly, most engineers use guided or direct assembly to achieve spatial control over these critical elements. Strategies for harnessing multiscale control over biological systems have recently been reviewed[14,17]. Here, we limit the discussion to key findings of the physical and chemical aspects of the microenvironment.

*2.1.2 Physical properties*

Increased attention has been given to the role of substrate physical properties, such as roughness, topography, and mechanical properties (*e.g.*, stiffness and elasticity), in governing cell behavior[18]. Control of these physical properties is recognized as an essential prerequisite for successful tissue-engineered constructs[7,15,19]. Though cells are micron-sized, they contain sensory machinery that are below 100 nm. Underlying substrate architecture with features below 50 nm has enabled specific cell patterning[20]. Topographical features at the nanometer scale can influence cell orientation and motility[21,22]. Contact guidance, a phenomenon whereby a cell's interaction with its external environment influences its morphology and movement, is typically governed by micro- (1-50 μm) and nanometer (<1 μm) architectures[23,24]. Again, nanotopography can mediate the substrate features with which cells interact[25]. For example, surface roughness can alter wettability and affect protein binding and exchange at the surface[26]. Additionally, the geometric packing configurations of adsorbed proteins is affected by surface nanotopography[27].

*2.1.3 Extracellular matrix composition*

The extracellular matrix (ECM) is a complex milieu of biomacromolecules that plays both a structural and functional role in supporting cell morphology and behavior[28,29]. ECM composition varies significantly depending on the tissue type and disease state, but is generally composed of water, proteins, polysaccharides, proteoglycans and a host of ECM regulators and secreted factors[28]. A large-scale survey of the ECM atlas found 1027 genes associated with the human matrisome[30]. Still, elucidating the exact distributions and roles of each extracellular component remains elusive for many tissue types. Given the expansive and complex roles and interactions of these components, recent efforts have aimed at organizing and mining existing datasets to uncover or quantify cellular function[31,32]. This and other biomedical data should be used to inform the decision-making process

when designing complex biological systems. Tissue engineers, for instance, recognize the importance of core proteins and glycoproteins (*e.g.*, collagens, fibronectin, laminin), proteoglycans (*e.g.*, chondroitin sulfate, heparan) and glycosaminoglycans (*e.g.*, hyaluronic acid) in directing cell behavior[33]. These critical elements work in concert to mediate cell adhesion and movement, morphology, differentiation, and overall gene expression. ECM suprastructures modulate cell behavior through ECM/ECM receptor interactions *via* the presence of binding domains such as RGD in the case of fibronectin, GFOGER for collagen, and YIGSR for laminin[34]. Modification of materials with peptides mimicking these cell binding domains is a classic approach for promoting cell guidance and has been used to achieve cell patterning. A major challenge lies in reducing the complexity of the ECM to a combination of materials that is scalable, cost-effective, and most importantly, retains biological relevance for the task at hand. Several strategies for mimicking the ECM are later discussed or have been reviewed elsewhere[35,36].

**2.2 Biological activity**

The function of healthy tissue can be summarized as to support regular cell behavior while preventing irregular behavior. These functions, though clearly distinguished by tissue type, are generally regulated through balancing critical elements including cell population, ECM composition, or transmission of signaling molecules. Furthermore, all tissues require transport of nutrients, waste, and information. Transport, which may occur passively through diffusion, or actively through energy-driven processes, is often facilitated *in vivo* by sophisticated networks of vasculature and lymphatic systems. Recreating these biological networks is essential for the reconstruction of any tissue construct that supersedes the passive diffusive limit of oxygen (~200 μm)[37].

ECM-affiliated proteins (*e.g.*, syndecans), regulators (*e.g.*, transglutaminases) and secreted factors (*e.g.*, TGF-β, VEGF) play a significant role in regulating cell and tissue behavior. Increasingly, these factors are recognized as critical elements in designing biomaterials that retain functionality, particularly in promoting wound healing, repairing diseased tissues, and in stimulating vasculogenesis. Key considerations for designing materials include natural or synthetic affinity between the factors and the biomaterial surface or matrix, overall stability and spatiotemporal control, mode of delivery, and stimuli-responsiveness.

Based on the above concepts, key aspects of biological complexity that must be considered in designing biomaterials include the chemical composition, physical and mechanical properties, and the overall hierarchical organization that enables precise assembly of desired inert or biological components.

**3. Information-driven materials design**

Traditional techniques for fabricating substrates in regenerative medicine, such as solvent casting and particle leaching, rely on processes that lack precise control over architecture and cellular composition[38]. Emerging micromanufacturing techniques aim to integrate computer-aided design or manufacturing (CAD/CAM) to allow for the information that must be obtained using advanced imaging techniques to guide the materials design process. Beyond this approach, the design of next-generation biomaterials is predicted to further incorporate biological information transfer at the single-cell level[39]. In combination with computational and statistical models, efficient designs that consider aspects of anatomical hierarchy can be pursued.

**3.1 Imaging**

While standard biomaterials, for example, in the form of implants, are widely available today, personalized biomaterial development has not yet been fully embraced[40]. To reconstruct individually-designed tissue replacements, morphological information of the original tissue is necessary. Computed tomography (CT) and magnetic resonance imaging (MRI) provide access to series of 2D images[41], which allow for the characterization of the micro-structures of hard tissue scaffolds due to the difference in density[42].

Hollister e*t al.,* for example, used these two medical imagining techniques for designing a human mandibular condyle bone tissue [43] (**Figure 3** C). Computational topology design (CTD) and Boolean image techniques led to a 3D geometric model of the tissue harnessing information about the global anatomic and integrated architecture[43]. The calculated CAD data of the tissue geometry can be translated into a vector script and sent for microfabrication, which rebuilds individual scaffolds for the target tissue (Figure 3 A). Alternatively, iCAT-CT (Xoran Technologies® Inc.) data of a porcine periodontal defect have been used to inform the design of tissue equivalents[44]. To mimic the surface morphology, interfacial micro-channels were added. After manufacturing the hybrid scaffold via STL (Surface Tessellation Language) files, contrast agent and micro-CT was used to evaluate host adaption[44].

An ultrathin tubular free-form structure was recently fabricated which provides sufficient mechanical flexibility. The modeling of the 3D structure in STL format of a bile duct is based on medical image data using MRI. 2D and 3D Magnetic resonance cholangiopancreatography (MRCP) images were therefore taken of the tested rabbits for modeling as well as investigating the interconnection between the artificial scaffold and the native bile duct[45] (Figure 3 D). Creating 3D models of living organs based on imaging can also be used in medical research[40] (Figure 3 B). Markl

*et al.* for example combined flow-sensitive 4D MRI with rapid prototyping technology and computational fluid dynamics (CFD) to investigate the gas flow in the human tracheas and bronchial tree[46].

To date, MRI and CT are the most frequently used techniques for image-guided design of scaffolds in tissue engineering. However, combining different medical imaging techniques will provide even more detailed information. For example, with the help of endoscopy in combination with fluorescence imaging or confocal laser scanning systems, the tissue and its properties can be locally assessed with high precision. Positron-emission tomography (PET) or single-photon emission computed tomography (SPECT) makes it possible to analyze metabolic processes of tissues[47,48]. The images of the region of interest can be computationally transformed into a 3D triangle mesh using CAD software and mathematical modeling based on a set of theoretical rules to spatial organization[49][50].

**3.2 Omics-inspired materials design**

Classical approaches to biomaterial design rely heavily on a low-throughput trial and error methodology that has inevitably led to a substantial number of scientific articles in the field, with minimal clinical successes. While a number of factors contribute to this disproportionate scientific output, some key technical challenges that have hindered progress in the field can now be addressed with the latest tools available to researchers[39]. These include cutting-edge advances in biology such as single-cell omics, data-reduction tools to aid in experimental design and data analysis, and high throughput polymer libraries that facilitate rapid materials screening.

"Omics" is a neologism generally referring to the fields of biology focused on studying the totality of a major aspect of the cell (*e.g.*, genome, proteome, metabolome). More generally, the suffix "-omics" can be considered as "all constituents considered collectively" and has, with somewhat intemperance, made its way into the vernacular of many other disciplines (*e.g.*, radiomics, video-omics). Understandably, the major success of the Human Genome Project has sparked interest in applying this approach to other disciplines. For instance, The Materials Genome Project was launched with the intent to create new materials-innovation infrastructure from discovery through deployment[51]. One outcome of this initiative is The Materials Project, which has the mission of combining informatics and materials science with recent advances in scientific computing to accelerate the discovery of new inorganic materials[52]. As a result, hundreds of thousands of materials now exist in the database to aid in designing electronics, batteries, and other inorganic compound-based structures. Importantly, workflows for computational materials science have been generated that may serve as a template for other tangential disciplines[53].

The emerging materiomics approach in biomaterials design and development proposes using iterative materials synthesis and biological characterization cycles to unwind the complexity of material property effects on biological systems[39,54,55]. Such an approach relies on the convergence of materials science and engineering, chemistry, data science, and biology to leverage the advances listed above. The term "biomateriomics," originally defined by Cranford and Buehler, may be considered a materiomics approach to studying biological systems[1]. Several examples where such approaches may be leveraged to discover and/or engineer new biomaterial properties are discussed elsewhere in more detail[1,39,55]. We summarize a vision for how these approaches may be enacted through discussing proposed experimental design schemes.

Complex synthetic biological systems have two major components: (i) the biological, or "living", and (ii) the non-biological, or "non-living". As discussed, biological components have hierarchies that enables function, while non-biological components have an almost limitless design space that can be explored through materials selection and design. At the interface of these two components, *i.e.*, "biointerface", is a combinatorial library of possible interactions that achieve a coupled function ("synergy")[55]. Achieving a desired function requires great understanding about both the biological (*e.g.*, cell behavior) and non-biological (*e.g.*, material properties) components, but also about how their interactions drive responses in the other. The pursuit of a holistic understanding of the interactome of these components is an emerging goal among researchers that is expected to drive advances in nanomedicine, medical device development, tissue engineering, and material science[55].

Analogous to the way combinatorial chemistry has led to the discovery of new drugs and accelerated clinical outcomes, biomaterials development will benefit from rapid property discovery and biological assessment[56]. Key to the success of this approach is defining a concise parameter design space, targeted biomarker profiling, and limiting experimental scope (*e.g.*, through implementing design of experiments (DOE)) to reduce the number of experiments that result in datasets that take years and highly sophisticated techniques to analyze. In combination with computational modelling, information-driven materials design offers a powerful approach that has the potential to produce materials with improved clinical function[57].

A standard workflow for the next-generation of biomaterials design may involve three major stages: screening, surface response, and optimization[54]. In the screening stage, partial- or full- factorial design is implemented for high-throughput production of materials with varying parameters of interest. Typically, a base material (*e.g.*, polyethylene glycol (PEG)) is selected that can easily be

modified for high-throughput production and iteration. This library is then assessed for specific outputs of interest, such as driving a cellular response (*e.g.*, live/dead assessment, specific biomarker production, cell adhesion, etc.). At this stage, a subset of the original parameter selection will guide the second stage, referred to as "surface response," where finer tuning of the material parameters can be explored with more complex output assessment. The final "optimization" stage involves a small number of designs that can be assessed for their functionality in the most advanced assays (*e.g.*, *in vivo* animal testing).

Full characterization of the final design may involve high resolution genomics, transcriptomics, and proteomics. At each of these stage iterations, computational and statistical modelling can be implemented to maximize efficiency. This and similar approaches have recently been implemented to identify polymers that are resistant to bacterial attachment[58], optimize delivery of proteins for cardiac repair[59], design zwitterionic polymer brushes for stem cell growth[60], optimize seeding efficiencies for dermal scaffolds[61], and predict cardiac reprogramming outcomes on biomaterials[62].

While these approaches are promising for demystifying the relationship between biological components and synthetic substrates, a number of challenges plague this area of research. These include lack of standards in characterizing biomaterials, parameterization of cell and material responses, validating the biomarkers used for assessing biological outcome, and managing the large datasets these types of experiments produce[39,54,63]. Standardizing reporting in bio-nano literature was recently covered, though establishing rigorous standards for biomaterials reporting is still warranted[64]. Material-biological property parameterization was also recently addressed[63]. Cellular parameterization includes characterizing cells through gene expression analysis and high-content imaging, while important material parameters include chemical composition and spatial

organization[63]. Implementing data-dimensionality reduction strategies, improved visualization tools, and more efficient machine-learning algorithms will be required to address the critical issue of managing and analyzing large datasets[65–69]. An additional challenge remains in curating datasets that provide information in a robust, reproducible manner. A number of databases exist separately for materials scientists and biologists. Unifying the two has remained a significant challenge, however. Hebels *et al.* recently released the Compendium for Biomaterial Transcriptomics (cBiT), a first-of-its-kind repository designed for researchers to search biomaterial-based transcriptomics data[70]. Efforts such as these are necessary for progress in this field, and the paucity of resources for understanding material-biological interactions will continue to impede progress. Developing mathematical models to understand these interactions is critical, especially as the biotechnology sector enters "Industry 4.0", which relies on the development of digital representations of products and processes to optimize their design[63,71]. This cannot be achieved without reliable, consistently annotated data repositories. Together, these efforts are aimed at providing resources and integrating a holistic approach toward understanding how materials and the biological components they interact with may be controlled for desired function.

## 4. Characterization and validation of biomaterials

### 4.1 Biomaterial candidates for micromanufacturing

There is a plethora of materials used in the micromanufacturing of bio-integrative systems with various *in vitro* or *in vivo* applications. These materials are comprised of metals, ceramics, macromolecules, or composites thereof. Apart from polymers and naturally derived materials, ceramic and metal materials have a long and successful history in dental and orthopedic applications

that have been reviewed elsewhere[72–74]. Furthermore, there are many applications of biomaterials as medical devices that will not be discussed here, because they have been discussed elsewhere[75,76]. In contrast, this review will focus on emerging trends in the employment of macromolecular materials in tissue engineering with a specific focus on challenges associated with their validation and clinical translation (**Table 2**). Naturally occurring biomacromolecules are employed as biomaterials and are primarily comprised of polysaccharides and proteins. They generally feature physiologically relevant compositions, biocompatibility, abundant availability or bio-inductive properties[77,78]. Synthetic macromolecules used in biomaterials applications are generally comprised of synthetic polymers, such a polyesters, polyurethanes, hydrogels or acrylate functionalized polymers. Synthetic materials often allow for more precisely controlled physical properties such as chemical composition, stiffness, degradability and architecture, as well as the potential to elude the immune system[77,79].

*4.1.1 Synthetic polymers*

Polyesters are biodegradable, tend to be biocompatible, and have a long history of use in various *in vivo* applications, such as sutures. Common polyester biomaterials are poly(lactic acid) (PLA), poly(lactic-*co*-glycolic acid) (PLGA), poly(ε-caprolactone) (PCL), and poly(lactide-*co*-caprolactone) (PLCL). These polymers are frequently electrospun to create fibrous mats used for tissue engineering[80–82]. While electrospun mats are common in tissue engineering, there are subtler biological implications, namely the potential for protein fouling on implanted scaffolds to initiate an adverse immune response that need to be addressed. Kostina *et al*. are addressing this issue by modifying the surface of PCL fibers with non-fouling coatings[83]. Our lab has leveraged the chemical functionality of polyesters to create electrospun bi-phasic fibers of PLGA derivatives to direct the

attachment of cells on microfibers[84]. Polyesters are also favored for their inherent degradability, which occurs through acid or base catalyzed hydrolysis of the ester backbone. For PLGA this results in the release of metabolites, *i.e.*, glycolic acid and lactic acid, which can be cleared by the host. The degradation rate can be controlled by the ratio of lactic acid and glycolic acid blocks, as well blending PLGA with other polymer derivatives[85,86].

Given that polyesters are thermoplastic, they can easily be incorporated into melt extrusion or filament based 3D printing systems[87–89]. Generally, using these techniques larger fibers (>100 μm) are produced, which may not be desired for certain tissue engineering applications since features would ideally be subcellular (<20 μm). Recently, Wunner *et al*. developed a melt-electrospinning technique to create porous scaffolds comprised of 20 μm diameter fibers[90]. Our lab has recently reported an electrospinning-based jet writing technique that allows for 3D printing of scaffolds comprised of very fine PLGA fibers (≤10 μm diameter) that were highly successful in repairing a cranial defect in a mouse model[91]. Furthermore, polyesters are amenable to other manufacturing techniques such as microsphere sintering, solvent casting, and phase separation[92]. Other efforts involved similar techniques in combination with a sacrificial template technique to create porous PLGA scaffolds with multi-length scale features for spinal cord injury repair[93]. Beyond polyester materials, high resolution 3D printing of polyelectrolyte solutions can be used to create tissue scaffold structures. These inks are combinations of polyanions like poly(acrylic acid) (PAA) and polycations like poly(ethylenimine) (PEI), or poly(allylamine hydrochloride) (PAH) that can be written into structures with filament sizes as small as 1μm[94,95].

Outside of the aforementioned processing techniques, light-based polymerization of synthetic polymers is very attractive for the manufacturing of complex materials systems because of its

potential for ultra-fine resolution and spatiotemporal control. Various acrylates or acrylate-modified polymers are used for the advantageous photopolymerization which allows for precisely tunable properties. Commercially available photoresist like OrmoComp® has been used in conjunction with multi-photon polymerization to create ≤1 μm sized features which can be selectively functionalized to guide cell attachment[96]. This material is a hybrid organic/inorganic molecule comprised of a silicon based component and photopolymerizable component[97].

Photopolymerization based strategies are widely used in additive manufacturing techniques because of their practical compatibility with many printing strategies. Generally, these chemistries rely on a photoinitiator that forms radicals upon illumination, which polymerizes a monomer that possesses multifunctional crosslinkers[98]. Various additives, including other polymers, can be added to tune solution properties critical for 3D printing[98]. Photopolymerization techniques are applied heavily to hydrogels and other polymers for tissue engineering applications and have been reviewed in details elsewhere[98,99]. Hydrogels make up a large class of water-laden polymer networks that are typically biocompatible and have physiological stiffnesses similar to many soft tissues. Hydrogels can be crosslinked *via* covalent bonds (chemical hydrogels) or non-covalent (physical hydrogels) molecular interactions[99]. PEG is a ubiquitous hydrogel in tissue engineering that is highly bio-inert, yet amenable to dramatic chemical modifications to create a diverse array of functional PEG derivatives[100]. PEG can be functionalized to be photo-reactive, with PEG di-acrylates (PEGDA) and PEG methacrylates (PEGMA) being the most common candidates[99]. When 3D printing hydrogels, there are many considerations ranging from fluid properties, nozzle design and the choice of crosslinking method (physical vs. chemical) to solution properties, such as shear thinning, thickening, viscosity, and time to gelation. Gaining deeper control over these solution properties, especially

those occurring dynamically during gelation/crosslinking is key for the future of 3D printing hydrogels[99]. High-resolution hydrogel structures have been demonstrated by Richter *et al.*, where 1 μm sized PEGDA structures were created to engineer protein repellant portions of the aforementioned microstructures[96].

Outside of photopolymerization and 3D printing, PEG can be formed into monolithic gels using other crosslinking methods such as enzymatic crosslinking of functionalized PEGs. These gels can contain relevant cell binding motifs and biodegradabable linkages to create biochemically relevant material surfaces that have been shown to be dramatically influence cell behavior[101–103]. Recently, advances have been made to improve encapsulation and spatial localization of single cells in functionalized biodegradable PEG microspheres with the potential to study single-cell behavior in controlled 3D niches[104]. Additionally, PEG has been demonstrated to be incredibly versatile and amenable to modification with various glycosaminoglycans (GAGs) to produce GAG composites with tunable properties, which offers a potential route to the critical role of these polysaccharides in ECM biology (reviewed elsewhere)[105]. Other bio-inert hydrogels used in tissue engineering applications include poly(2-hydroxy ethyl methacrylate) (PHEMA), poly(acrylamide) (PA), and poly(N-isopropyl acrylamide) (PNIPAAm) and have been discussed elsewhere[38]. While hydrogels may give rise to precise control over physical parameters like stiffness and degradability, many synthetic hydrogels lack physiologically relevant architectural motifs, such as fibril structures, which in part gives rise to interest in utilizing naturally-derived materials[79].

*4.1.2 Naturally-derived biomacromolecules*

Protein-based biomaterials include, for example, collagen, fibrin (fibrinogen and thrombin), laminin, fibronectin and elastin. Examples of polysaccharide-based biomaterials include alginate, chondroitin sulfate, heparin sulfate, chitosan, and hyaluronic acid. Virtually all of these materials, either alone or in combination with another natural/synthetic material, have been processed into tissue scaffolds using electrospinning[106–109]. While traditional electrospun tissue scaffolds recapitulate the fibril structure of the ECM, they tend to be dense, relatively thin, difficult to handle, and are difficult to produce with higher order, organized architecture. Other traditional manufacturing techniques such as freeze drying, phase separation and gas foaming techniques have been used with proteinaceous materials like gelatin and collagen to create porous scaffolds[110–113]. Some of these scaffolds may display ideal levels of porosity, but still lack precise control over microscale features and their hierarchal organization.

Furthermore, many of these biomaterials like alginate and collagen naturally form hydrogels that can be incorporated into 3D printing techniques. These systems tend to be more cell-compatible than synthetic 3D printing solutions; however, high resolution 3D printed structures using naturally derived materials can be challenging. Nevertheless, recent advances have been made in 3D printing of collagen scaffolds; however, these scaffolds have relatively large printed features (>100 μm) comprised of smaller collagen fibrils[114]. Collagen is widely used because of its innate propensity to auto-polymerize *in vitro* and form hydrogels comprised of physiological relevant fibril architecture. This simultaneously poses a drawback, because subtle changes in solution properties like temperature or concentration can alter the structural properties of the resultant collagen hydrogels. Beyond proteins, an exciting technique for producing DNA-based materials with high-precision uses DNA origami assembled from short complementary oligonucleotides[115].

The potential benefits of naturally derived materials in tissue engineering may include their relative abundance, their biochemical relevance, their biocompatibility, their inherent degradability, and their bio-inductive capacity. Many of these materials are not mechanically robust and require secondary crosslinking to stabilize them prior to cellularization. Furthermore, as a result of various phenomenological assembly processes of different naturally derived materials, they generally lack orthogonal control over physical properties such as stiffness, ligand density, and architecture[116].

*4.1.3 Composites and materials' complexity*

Fundamentally, biological organs may be considered to be composite materials comprised of complex tissues and interfaces where the ECM acts to direct cell fate with multi-faceted cues that hinge on their material properties. To engineer tissue at the organ level, multiphasic materials systems are necessary. Major challenges associated with tissue engineering includes overcoming poor mechanical stability of natural materials/hydrogels, recapitulating complex tissue characteristics like mechanical gradients, engineering the multi-phase architecture of tissues, and producing tissue scaffolds that recapitulate functional processes like nutrient transport or toxin filtration. Recent developments have been made to 3D print composite materials such as a mechanically robust PEG/alginate hydrogel, of which both systems are historically weak[117]. A mechanically complex tissue interface to recapitulate is that of the enthesis (where tendons and ligaments anchor to bone). Mechanical integrity at this interface is critical for orthopedic implants; however, the transition from soft tissue to stiffer bone (or bone replacement) makes this a very difficult problem to solve[118]. The skin is another organ comprised of distinct layers with unique biological roles and architectures; hence, researchers are leveraging innovative approaches to creating multi-phasic systems for the treatment of full thickness wounds, as well as the creation of

highly relevant *in vitro* skin equivalents that allow for long term culture (6 weeks) with immune and neuronal fractions for investigative studies[119,120].

Furthermore, creating functional and integrable vasculature networks remains a huge challenge for tissue engineering. Novel approaches have been taken to 3D print complex vascular networks out of combinations of a synthetic material like Pluronic® F127 and methacrylated gelatin (GelMA) with and without cells[121]. Considering broader applications, recently Gou *et al*. demonstrated an innovative approach to incorporate functional nanoparticles into a highly ordered 3D printed PEGDA hydrogel systems with to create a cell-free detoxification scaffold[122]. These examples underscore the necessity of creating composite biomaterial constructs, as the research field intends to engineer more complex tissue scaffolds.

**4.2 Characterization and validation of bio-instructive materials systems**

Biomaterials systems that aim to recapitulate the hierarchal biology found *in vivo* become increasingly difficult to characterize. Some characteristics such as biocompatibility are, in part, defined by regulatory agencies. For instance, the U.S. Food and Drug Administration (FDA) utilizes the International Organization for Standardization (ISO) standards to assess risk and biocompatibility (ISO 10993 standards), which is subcategorized into various toxicities, hemocompatability, degradation, sensitization, and implantation[123]. It is critical to note that the FDA regulates devices, not materials; hence, for regulatory agencies and researchers, all considerations of the appropriateness of a material is application dependent. Additionally, the comprehensive approach necessary for assessing safety and biocompatibility of a medical device seeking regulatory approval is challenging to be achieved by academic researchers; however, some of the subcategorized tests

laid out in the FDA guidelines may be useful in directing academic studies and help to solidify good research practices. Hence, it is recommended to assess the translational potential of a biomaterial during early technological development and with the regulatory proceedings in mind. Beyond biocompatibility, characterizing material properties like topology and stiffness in a translationally validated context becomes very difficult as well. There are some standard characterization methods such as those put forth by the American Society for Testing and Materials (ASTM) for biomaterials systems (F2150-13 and STP1173); however, complex composite materials, especially macromolecular systems, may not strictly adhere to the requisites of those tests[124,125]. Yet, as previously discussed, it is well accepted that cell behavior largely hinges on these inherent material properties (physical and biochemical). This underscores the need for deeper investigation into cells in 3D systems and approaching the characterization of biomaterials with standards and good practices in mind.

*4.2.1 Characterization of physical properties*

The stiffness or compliance of a material is thought to direct cell fate, which is well accepted using 2D models but becomes increasingly complex to assess in 3D systems. Depending on the inherent properties and size of a biomaterial, the characterization of stiffness may involve rheological techniques, unconstrained compression or tensile testing, contact model guided indentation (nanoscale to macroscale) or via ultrasound elastography[126–130]. In all cases, the underlying assumptions and limitations of the model and method chosen should be carefully considered, which may highlight the need for new models to be adapted for particular biomaterial systems. This is especially important when biomaterials *in vitro* are compared to the native, *in vivo*, tissue which often cannot be done directly considering different methodologies needed for each setting.

Stringent adherence to good practices, as well as differences in methodologies and test conditions are critical to address when interpreting and comparing the results of mechanical testing. Furthermore, any bulk material properties offer little information as to the cell-scale heterogeneity of mechanical properties, especially as the cells engage in a dynamic modulation of their 3D biomaterial environment through physical manipulation and chemical degradation. Using an elegant approach, Juliar *et al.* assessed angiogenic sprouting events in fibrin gels, *in situ* and correlated them to the mechanical properties at both the cell scale and bulk length scale using laser tweezer microrheology and bulk rheology, respectively[131]. This study revealed a significant amount of microscale stiffness heterogeneity surrounding sprouting events that changed over time. There was a general trend toward increased bulk stiffness over time likely associated with remodeling by the stromal cells. Ultimately, this work underscores the value in taking a more rigorous approach to assessing mechanical properties associated with biological phenomena, *in situ* and at various length scales.

Surface topography is known to influence cell behavior, as has been shown with various well-defined engineered 2D surfaces[132]. However, nano-scale topography under physiological conditions in 3D is very difficult to assess *in situ* considering the hydrated state of many biomaterials. Liquid phase atomic force microscopy (AFM) can be applied on relatively flat surfaces; however, many biomaterials have higher order, microscale topography that precludes the use of AFM-based assessment of nano-topography. Future advancements in environmental scanning electron microscopy (ESEM) or cryo-SEM techniques will likely address some of these shortcomings, and recent advancements in preserving aqueous, bio-based surfaces for imaging in ultra-high vacuum can be employed to better assess the aqueous phase topology of biomaterials[133]. Additionally,

advancements in ultra-high resolution fluorescent imaging technologies is helping to shed light on focal adhesion dynamics in 3D systems[134]. Advances in fluorescent microscopy will help to bolster our understanding of how cells interact with material topology in 3D.

Characterizing and engineering the biochemical composition of a tissue is also a non-trivial pursuit, in large part because tissues are compositionally diverse owning to the hundreds of different proteins and polysaccharides that make up a single tissue[135]. Often, there is a gap in knowledge about the complete composition of a target tissue and most importantly, which of the proteins are critical to facilitate the tissue's primary function at the cellular level. This gap in knowledge gave rise to a significant effort from a Swedish-based program in 2003 known as The Human Protein Atlas (HPA)[136]. The HPA has set out to map every protein from the cellular to the organ level with a multi-omics approach using transcriptomics, antibody-imaging, and mass spectrometry proteomics[137,138]. Efforts like these will give engineers a target to aim for, so that scaffolds and materials can be more intelligently designed.

*4.2.2 Validation and challenges*

Establishing functional benchmarks of *in vitro* systems against native tissues is yet another non-trivial endeavor. However, defining translationally relevant functional readouts is key to the success of any *in vitro* technology to ensure that different approaches can be benchmarked against one another. For instance, a bio-assembly method of producing primary hepatocyte spheroids has led to the ability to maintain viable, metabolically active, functional hepatocytes and translationally relevant cultures for up 5 weeks which is not possible to do using conventional 2D culture methods [139]. In 2017, AstraZeneca and Genentech Inc. used primary hepatocyte spheroids to demonstrate an

improvement in hepatotoxicity prediction power of this 3D model compared to 2D methods of culturing hepatocytes [140]. In addition to potential strides in preclinical drug safety assessment, the knowledge gained from understanding how the 3D microenvironment of a liver spheroid improves primary hepatocyte viability and function could potentially inform the next steps to recreating larger scale functional liver mimics for tissue engineering applications.

In cardiac engineering, readouts for tissue maturation include conduction velocity, force generation, and calcium handling[141,142]. Recent advances by Ronaldson-Bouchard *et al*. represent the state of the art in maturing iPSC derived cardiomyoctes *in vitro*[143]. While these and similar constructs will likely be first used to improve preclinical toxicity assessment, it stands to reason that the deeper understanding of growing cardiomyoctes in engineered 3D *in vitro* systems will glean critical details for how to better mimic and produce full scale tissues for implantation.

To date, there is a vast range of potential materials systems for any given biological question. As discussed previously, omics approaches are beginning to be applied to biomaterials development. One could imagine computational models for the design and implementation of biomaterials systems. As previously discussed, computational models can be employed to assess the critical functions of biomaterials systems and thereby more intelligently guide their design and implementation[39,54]. Hence, an omics approach to rationale biomaterials design should be employed. Additionally, to begin to weigh one material against another, head-to-head comparisons of various 3D biomaterials systems needs substantial investment. Further benchmarking of biomaterials systems against one another with more clearly defined characterization methods will help lead better design of biomaterials systems.

For a tissue scaffold to be clinically translatable it has to (i) demonstrate efficacy and validation in a tissue application, (ii) meet rigorous standards for safety, (iii) be commercially manufactured according to Current Good Manufacturing Practices (CGMP), and ultimately (iv) be economically viable. Many acellular scaffolds have been successful in the clinic; however, these challenges pose significant hurdles for cell-based therapies leading to fewer successes [144]. Cell-related challenges in tissue engineering have been outlined extensively[145]. Regulatory pathways may change for acellular compared to cell-based scaffolds, depending on the country, market of interest and intended medical application. In the United States, cell free scaffolds are typically treated as medical devices and regulated by the FDA's Center for Devices and Radiological Health (CDRH). If a tissue scaffold is cell-laden, then it may be characterized as a biologic and regulated by the FDA's Center for Biologics Development and Research (CBER). Cell based systems are subject to additional scrutiny for various reasons, including increased safety concerns and the need to translate varying academic research practices into strictly controlled manufacturing processes that adhere to CGMP, which stresses the importance of raw materials and reagents all the way up to reliably generating a consistent, characterized product at a commercial scale with excellent quality control[144–146]. Human pluripotent stem cells (hPSCs) have been an exciting cell source for cell-laden tissue scaffolds; however, their clinical translation is hindered by the choice of source (allogenic or autologous), the need to produce commercial scale quantities of cells, the need for strict control over differentiation to create pure cell populations with the desired phenotype/function, and the ability to do this in a cost-efficient manner (extensively reviewed elsewhere)[146]. Considering all of the hurdles associated with the clinical translation of tissue scaffolds (efficacy, validation, safety, commercialization, CGMP, and cost-efficiency), it becomes clear that a data driven approach to the rationale design and

implementation of new biomaterials systems is required through close collaboration between engineers, health professionals, bioinformaticians, fundamental scientists and commercially-minded people alike.

## 5. Scaffold micromanufacturing

### 5.1 Micromanufacturing techniques

In engineering complex biological systems, material properties may be considered input parameters and biological outcomes the output parameters. An aspect of information-driven design that is necessary for reliable experimental interpretation, then, is precise knowledge of input parameters. In other words, the material properties must be precisely defined. Control over these properties has advanced in recent years through the advent of micro- and bio-fabrication techniques that enable the precise placement of materials and biological components. These are promising as tools for advanced *in vitro* models for regenerative medicine applications[147]. Multiple approaches have been explored for the combination of materials and cells. Classic tissue engineering involves fabricating a scaffold cells grow and proliferate throughout. Modern biofabrication enables controlled deposition of cell-laden bioinks of synthetic or natural matrices. Recently, Moroni *et al.* introduced the spatial resolution/time for manufacturing (RTM) ratio as a quantitative metric for assessing fabrication efficiency, which we use here to compare techniques[148]. In this section, we discuss advanced fabrication approaches that are being developed for acellular scaffold production (**Table 3**).

In general, 3D printing has been utilized across multiple industries for rapid prototyping for multiple decades[149–151]. Classic 3D printing refers to the process whereby a jet of binder is directed at a powder bed to create pre-defined patterns. With the rapid and widespread adoption of 3D

manufacturing, dozens of other "3D printing" techniques have emerged. These include light-based approaches such as stereolithography (SLA), digital projection lithography (DLP), continuous liquid interface production (CLIP) and direct laser writing (DLW), ink or filament-based printing approaches such as fused deposition modelling (FDM), extrusion printing, direct ink writing (DIW), and inkjet printing, and electrospinning techniques[147,152–154].

Extrusion printing, sometimes referred to as "ink-based printing" encompasses additive manufacturing approaches that result in the 3D deposition of materials such as filaments and droplets using computer-aided design that allows for arbitrary structure design[95,153]. These materials may be subject to thermal, pneumatic, light-based, or mechanical treatment during deposition or in post-processing. Fused deposition modelling (FDM) was the earliest implementation of filament printing, whereby thermoplastic filaments are passed through a heated nozzle onto a build platform and structures are assembled layer-by-layer as they cool below their glass transition temperature. FDM has been applied for creating microfluidic devices, tablets, and implants with a wide range of materials, including ABS, PLA, PCL, PMMA, and PVA[155,156]. FDM dominates the desktop 3D printer market space due to the available materials, ease of use, and relatively efficient printing (RTM ratio ~1)[147]. In creating biomedical devices or other biomaterials, there are however many limitations to FDM such as a relatively large feature minimum feature width (~200 µm), and an overall limitation in materials that can be printed, many of which are not biocompatible or suitable for most tissue engineering applications.

In the context of producing acellular scaffolds, printing typically relies on soft materials such as polymeric or particulate matter that exhibit steady flow during the deposition process but achieve stability (*e.g.*, through gelation or cooling below the glass transition temperature) upon delivery.

Emphasis is placed on identifying conditions in which starting materials are printable, through optimizing parameters such as viscosity, thermal conductivity, and shear-thinning properties. Printing of cell-laden "bioinks" is often referred to as "bioprinting" and enables the precise placement of cells either alone or within a support structure. An added layer of complexity may be explored with dynamic materials (*i.e.*, materials that change over time or in response to a stimulus), often referred to as "4D printing"[157]. Several excellent reviews have discussed ink-based printing for tissue engineering[4,38,153]. Overall, biomanufacturing is trending toward faster printing speeds, improved resolution, and use of sophisticated materials. These advancements emerge through the development of novel materials and manufacturing approaches that enable complex designs that may recapitulate native *in vivo* tissues.

**5.2 Light-based 3D printing**

The major light-based printing methods include stereolithography (SLA), continuous liquid interface production (CLIP) and direct laser writing (DLW)[158]. These methods are based on the principle of bathing a photo-polymerizable resin with light at a specific location to generate a CAD structure[153].

While SLA, DLP, and CLIP allow for relatively efficient printing (RTM ratios ~0.5-2) and enable large build volumes, direct laser writing at the nanoscale is achievable using-two photon polymerization (2PP) [148,158]. 2PP relies on a photoinitiator that simultaneously absorbs two near-infrared photons to generate free radicals for initiating polymerization within a monomer reservoir, enabling unprecedented lateral resolutions of ~100 nm[159]. Most structures generated using this method therefore require photosensitive polymers and initiators, which have the potential to be toxic[160]. Commercially available cytocompatible photoinitators include certain Irgacure formulations and dye-

amine combinations (*e.g.*, Rose Bengal dye with amine as a co-initiator)[98]. One major challenge within light-based 3D printing methods has been exploring multimaterial printing, as it is typically difficult to alter the composition of a polymer reservoir during printing.

Another challenge lies in patterning multiple materials or ECM components at very small length scales[161]. As 3D printing techniques have emerged, a greater emphasis on spatially patterning more advanced structures with equal resolution to 2D approaches has evolved.

The first example of patterned ECM deposition in 3D using 2PP was achieved by Klein *et al*.[161,162]. Here, a protein-repellent PEG-DA PETA polymer framework was subsequently decorated with blocks of Ormocomp®, an inorganic-organic polymer containing siloxane linkages, that facilitated fibronectin binding. This sequential building of protein-binding structures on a protein-repellent background enabled selective cell attachment that has since been applied for cell elasticity measurements[163]. A potential limitation to this approach is the binary nature of the protein attachment that limits control over types or amounts of proteins attached. Spatial control over the scaffold surface chemistry was introduced via a multi-step process involving a two-photon-triggered cycloaddition whereby an Ormocomp® scaffold first undergoes silanization to generate photoactivatable dienes, is then irradiated in the presence of protein-ligand dieonophiles with a femtosecond pulsed laser, and then bioconjugated with fluorescently labeled proteins[162,164] (**Figure 4 a-c**). This strategy enables more selective attachment of specific moieties to the surface but is laborious and requires extensive processing. Another innovation relates to specific protein placement through introducing photoresists that are either protein adhesive, repellant, or selective[96] (Figure 4 d). In a step-wise process, a protein can non-specifically adhere to the first resist and then a second protein can be conjugated to the selective resist following an activation

strategy similar to the previously described approach (Figure 4 e). This strategy results in the selective 3D patterning of multiple ECM components and was subsequently used to explore cell-ECM interactions on 3D structures (Figure 4 f).

**5.3 The 3D electrojetting**

Using electrostatic forces to produce fibers is a well-known process for over 100 years. A high voltage source feeds a direct current with a certain polarity into a polymer solution or melt placed in a syringe with an applied constant flowrate. Grounding the collector leads to an electric field which stretches the polymer solution toward the collector. At a certain critical voltage, the electric stress increases sufficiently to distort the droplet on the needle of the syringe into a conical shape called a Taylor cone. When the electric field strength exceeds the surface tension of the solution, the liquid is accelerated to the collector as a fluid jet. By traveling through the surrounding gas phase, mostly air, the solvent of the jet evaporates and leads to the deposition of a solid polymer fiber on the grounded collector. During jet propulsion toward the ground electrode, a bending or whipping instability develops where the lower end of the jet undergoes a growing oscillatory circular deflection[165,166]. The whipping results in thinning of the jet to submicron scales which increases surface area and decreases the time needed for solvent evaporation. The remaining polymer fiber is deposited at the ground electrode as a non-woven mat of interconnected fibers. Nowadays a wide range of natural materials, biodegradable and non-degradable synthetic polymers can be used to produce fibers in high-throughput via this process termed “electrojetting”.

In the last 25 to 30 years, the interests of using fibers for tissue engineering applications has increased. Due to high porosity and the microtopography, non-woven fiber mats are often

considered to be structurally similar to the native extracellular matrix (ECM)[167]. Several research groups use electrojetted fiber mats to mimic the ECM for bone, skin, nerve and vascular tissue engineering.

Nevertheless, the two-dimensional electrospun scaffolds with randomly oriented fibers are limited in their application. Much research is therefore focused on orienting the fibers by applying appropriate collectors. Chang's group used a cylindrical collector with equally spaced circular protrusions to yield a fibrous tube with patterned architectures (**Figure 5** A). They demonstrated the production of 3D fibrous tubes with different diameters, lengths, and various cross-section shapes made of polycaprolactone (PCL) and poly(lactic acid) (PLA)[168]. However, the deposition of the fibers was still random with pore sizes less than 20 µm in spite of their directionality. This is due to the well recorded bending and ink instability of the jet using the electric fields as a driver for physical mass flow[169].

Pursuing a different approach, bicompartmental and biodegradable PLGA fibers were produced by using electrohydrodynamic co-jetting[84]. Each fiber was comprised of two distinguishable compartments, which can be selectively surface-modified[84,170]. By using aligned multicompartimental microfiber scaffolds as templates for spatioselective azide-peptide immobilization, a two dimensional cell culture substrate for guided cell adhesion of fibroblast was designed (Figure 5 B)[84]. The challenge is now to bring these microstructures into the third dimension, requiring stabilization of the migration path of the jet. Additive patterning of materials for applications in biotechnology, sensors[171] or printed electronics has stimulated the development of different techniques related to high-resolution e-jet printing, such as pyro-electrodynamic printing or other electrodynamic processes[172]. One example is near-field-electrospinning (NFES)[173]. By

setting the working distance between the spinneret and fiber collector to a position before the onset of the whipping instability, a predictable location control for the deposition of fibers is possible[174]. As the needle to collector distance is fixed to 500 µm - 1 mm, the solvent in the polymer jet may not have sufficient time to fully evaporate and may remain liquid after deposition[175]. By adding a computer assisted x-y translation stage the fiber gets additionally mechanically stretched, leading to thinner fibers with oriented deposition[176]. Parker *et al.* investigated the effect of various parameters, such as working distance, flow rate and stage speed on the morphology of PCL fibers and sugar-PCL core-shell fibers with similar microstructures to neuronal and muscle tissues[174]. He *et al.* fabricated high resolution PCL scaffolds with controlled micron scale patterns and multi-layer scaffolds with varied coiled pattern as shown in **Figure 6** A[176]. Nevertheless, the height of the scaffolds produced with NFES is limited by the short working distance.

Compared to most other electrospinning techniques, melt electrospinning is a solvent-free but heat-intensive technique. Melting the polymer in the supply zone to 80-300 °C and allowing sufficient cooling of the filament over a relatively long travel distance inhibits spin bonding that would create nonwoven fiber mats[177]. Dalton and Hutmacher *et al.* reported an elegant melt electrospinning-based direct writing approach. By combining a computer-controlled translating stage collector with the melt electrospinning setup allows the fabrication of orientated PCL structures over large areas[177,178]. Detailed analysis of process parameters such as electrical field strength, flow rate and spinneret geometry resulted in highly controlled filament deposition. Layering sub-micron fibers over each other resulted in structures with different grid sizes (Figure 6 B) up to a height of one millimeter. Through a computer based simulation to keep the electrostatic force at a constant level while varying the working distance, a height of 7 mm was achieved[90]. *In vitro* cell culture studies

showed good adhesion, growth and differentiation of primary human mesenchymal stromal cells (hMSCs), human periodontal ligament (hPDL) and mesenchymal precursor cells (Figure 6 C)[90,178].

Even though melt electrospinning is a solvent-free process, the high temperature limits the use of many biodegradable polymers and biological materials, such as proteins, used in tissue engineering and regenerative medicine[179,180]. Additionally, cells cannot directly be processed using melt electrospinning[181].

*5.3.1 The 3D jet writing process*

An alternative strategy to control the deposition of electro spun fibers is based on manipulating the electric field. This often involves designing a grounded collector in form of drums[182], rings[183] or poles[184]. Our lab recently presented a new method, termed 3D jet writing, to control the bending and whipping instability during jet propagation by applying a secondary electric field (**Figure 7** A)[91]. The outward directed jet movement was suppressed by a ring electrode which created an electric potential well and reversed the direction of the electric field toward the center of the circular ring. Combining the stable polymer jet with a computer assisted x-y-stage allowed precise patterning of biodegradable PLGA fibers into open-pore structures in different shape and sizes as shown in Figure 7 B. Human mesenchymal stem cells (hMSCs) were cultured on fibronectin coated honeycomb scaffolds and filled the entire free volume of the pores with 500 µm in length after three days of culture (Figure 7 C)[91]. The cell density was $1.4 \times 10^6$ cells per $mm^3$ PLGA, seven times higher than reported elsewhere[185]. Additionally, maximum cell-cell contact and differentiation toward an osteogenic lineage were determined. *In vivo* studies of attaching a cellularized scaffold on user-

defined defect areas affected bone tissue regeneration while maintaining cell-cell interaction (Figure 7 D).

Due to the precise deposition of the fibers at room temperature, 3D jet writing is a promising technique as a physiologically relevant 3D culture platform. Even though the system is still solvent based, it is open for further materials as well as water-based jetting. This might lead to direct cell-electrospinning with very high resolution in the future.

**6. Cell-instructive matrix design**

The ECM provides a three dimensional microenvironment for cells of structural and functional proteins, proteoglycans and glycoproteins[186]. Various tissues have unique compositions, conformations and architectures in their normal state, as well as unique signatures when diseased[187,188]. Yet, there are numerous proteins (*e.g.*, fibronectin, collagen, laminin, fibrinogen, vitronectin, thrombospondin, elastin, tenascin, and osteopontin) which are found in the ECM[188–191]. For example, laminin is predominantly found in the basement membrane of breast tissue which possess a more sheet like structure, whereas interstitial matrices of mesenchymal tissues are chiefly made of fibrillar proteins like collagens I, III and fibronectin[192,193]. In the case of pathogenic breast cancer, the soft mammary tissue is remodeled to a dense, fibrous, collagen-I rich matrix with aligned fibers[194–196].

The ECM macromolecules provides structural support and mechanical integrity of the local microenvironment, have attachment sites for cell surface receptors, and regulate the growth factors[197]. It can act as a reservoir for latent signaling factors that can be released via degradation and can influence cell processes such as migration and proliferation[186].

Additionally, cells actively remodel their local microenvironment by exerting forces on the matrix, secreting new proteins or degrading proteins through matrix metalloproteases (MMPs), which in turn leads to changes in the proliferation, migration and adhesion and creates a complex dynamic reciprocity between cells and the ECM[198].

Given that cells respond sensitively to their microenvironment, it becomes paramount to precisely control proteins used *in vitro* in order to mimic the target tissue as close as possible while keeping in mind how complex is complex enough, which is often times difficult to know. Parameters to consider when designing an artificial matrix are protein composition, morphology, relative amount, fibril density, matrix compliance and the orientation of the protein structures[199–201] (**Table 4**).

A common technique for creating an attachment surface for cells on synthetic material scaffolds involves physisorption of proteins. Thereby, the protein needs to undergo a change in conformation to solidify on the surface. Solution conditions such as concentration, solvent and substrate properties dominate the morphology characteristics of the adsorbed protein layer[199–201]. This stochastic adhesion may lead to denaturation or inaccessibility of binding sides[200]. *In vivo*, cells form protein matrices, especially fibrillar fibronectin under mechanical tension by stretching the protein leading to exposed self-association sites. To mimic this process *in vitro,* various approaches ranging from stirring to mechanically pulling over electrical forces, to the use of active denaturants, have been investigated[202–205].

Another technique is the production of decellularized matrices via cell secretions. The disadvantage is the time-consuming cell growth and the multiple processing step of complete decellularization before adding the target tissue cells or stem cells[206]. Even though the

matrix is close to nature, the chemical composition is undefined and makes it difficult to control the ECM properties.

Another possibility is to use the property that fibrillogenesis occurs at the interface of protein solution[207], air and the scaffold. Forcing the interface through the microporous scaffold resulted in fibronectin fibrils in the interpillar space[208]. These engineered fibronectin networks are fibrillar in nature and stable in cell culture conditions. This process can also be extended to other proteins. The shear-driven hydrodynamically deposited ECM forms remarkably stable fibrillar protein networks, which are similar to the protein matrix secreted by human mammary fibroblasts. Engineered ECMs will enable investigation into the bidirectional relationship between cells and their protein microenvironments [91,208]. Beside the use of natural proteins such as collagen or fibronectin, there are synthetic polymers available as well. Due to its durability and cell compatibility, hydrogels are used as a protein replacement, or addition, to provide a supportive cell environment[209]. Through its coordinated control, physical properties such as density and structure can be tuned to investigate cell behavior. Chemical modification of the bioactivity as well as the cell behavior can be influenced and makes it a promising and diverse material to investigate[210]. Hydrogel-based biomaterials can be spatially controlled by bioprinting or photo-patterning[161][211].

Synthetic polymers and naturally-derived proteins are being explored for their potential in 2PP. Some naturally derived materials are more biologically active than others. For instance, bovine serum albumin (BSA) is a common natural material used in 2PP, but it lacks relevance as a biomaterial for studying cell-ECM interactions. Ovsianikov *et al.* generated scaffolds composed of a methacrylate-modified gelatin (GelMod) for the expansion of

adipose-derived stem cells (**Figure 8** A) [212]. Su *et al.* reported on a series of 2PP printed structures composed of a mixture of laminin/BSA in the presence of Rose Bengal dye for studying stem cell migration (Figure 8 B)[213]. Collagen-I was also implemented in 2PP with high spatial resolution[214]. A critical outstanding question for all of these materials is whether they retain any of the biologically relevant protein configurations following the multiphoton crosslinking process. Subsequent studies should be focused on this aspect of their design.

**7. Cellularization of Scaffolds**

Once a material system has been developed for a given tissue engineering application, it can be categorized as an acellular or cellular scaffold. Cellularization of a scaffold can be done post-production of the scaffold prior to implantation, during production prior to implantation or by the host in which it is implanted.

**7.1 Acellular Scaffolds**

Examples of acellular scaffolds include many of the previously synthetic or naturally derived 3D printed or traditionally manufactured porous scaffolds. Acellular scaffolds can further be derived from decellularized tissues that have been reviewed in more detail elsewhere[215]. Though not a micromanufacturing strategy, these represent both biochemically and structurally complex tissue scaffolds. Ott *et al.* successfully decellularized a rat heart and gave rise to perfusion-decellularization of whole organs[216]. Since then, companies like Miromatrix Medical Inc. have scaled this to larger, human-relevant sized organs[217]. However, given their non-autologous source, these scaffolds have the potential to elucidate unwanted immune responses, and they can also be challenging to handle *in vitro* in an aseptic manner. While some organs can be efficiently decellularized, re-seeding those

decellularized tissues with autologous cells of the proper type, and spatial arrangement remains a great challenge.

Generally, acellular scaffolds can be directly implanted into the host and rely solely on integration of cells from the wound site into the scaffold. Alternatively, they can be cellularized *in vitro*. If cellularized *in vitro*, this can be done by either static or dynamic processes. Any post-production cell seeding inherently requires a porous scaffold. A static seeding process would imply one in which a cell suspension is exposed to an acellular scaffold without mixing, where cells would settle into the scaffold via gravitational force. If the scaffold is extremely porous this may be possible but likely will not lead to a homogenous distribution of cells. A dynamic process may be necessitated by a desire for homogeneity of seeding or if the scaffold is not as porous. Given that most mammalian cells are ~10-20 μm in diameter, porous features closer to size of cells 20-50 μm may require additional force be imparted on the system to seed cells. This could be in the form of a fluidic flow (mixing) or by a light centrifugation.

Once cells are seeded in the tissue scaffold, a major challenge is to direct them to arrange and behave in a way that is advantageous for the intended application. Early on, this gave way to substantial efforts to pattern 3D surfaces with adhesive ligands to orient cells spatially on microfibers, to utilize different peptide sequences to elicit variable cell binding, as well as to immobilize growth factors on surfaces to drive cell behavior[84,218,219]. Many of these instances have been exhaustively outlined elsewhere[78].

**7.2 Cellular scaffolds**

7.2.2 *Bioprinting*

Bulk encapsulation of cells into a hydrogel is a common strategy for both synthetic and natural scaffolds that may be too dense for a post-production seeding strategy. Modern approaches allow for selective deposition of bioinks containing cells or cell aggregates in a process referred to as "bioprinting"[148]. This is typically achieved via droplet- or extrusion- based printing. In both cases, either the printhead or stage are controlled and translate over xy and z directions. Droplet printing requires that the polymer or prepolymer solution have gelation kinetics that match the deposition speed, which can limit the library of materials available for this technique. Extrusion-based printing passes polymer or pre-polymer material through a nozzle in a continuous ejection method to maintain contact with the stage and is typically slower than droplet printing. In either case, the solutions may be subject to additional thermal, mechanical, or light treatment.

Cell-hydrogel printing of defined 3D structures can have advantages over classical seeding on acellular scaffolds, such as controlled cell placement, high seeding efficiencies, and control over cell-matrix properties. However, many limitations plague current systems, such as low printing resolutions, lengthy solution optimization procedures, and creating large 3D structures that do not collapse from their own weight. Several strategies for overcoming these limitations have been discussed, such as including sacrificial support structures, and rapid crosslinking to facilitate larger build volumes[220]. The underlying biofabrication techniques that enable these processes, as well as their advantages and disadvantages, have recently been reviewed[38,147,148,221–223]. Exciting emerging techniques focus on incorporating aspects of tissue heterogeneity that are found in native tissue, via deposition of multiple materials or compartments sequentially or simultaneously. Layer-by-layer deposition of scaffold support materials and cell-laden bioinks was achieved using a multi-head 3D printing system to print large-scale proof-of-concept architectures resembling tooth, kidney, ear,

and skin[220,224]. This system, known as the integrated composite tissues/organs building system (ICBS) and the integrated tissue and organ printer (ITOP) system are two recent examples of integrated systems for printing heterogeneous solutions (**Figure 9**). ITOP demonstrated a proof-of-concept printing of an anatomical defect of large tissue structures by incorporating micro-channels to facilitate nutrient diffusion and combining hydrogels and synthetic polymers for imparting mechanical strength[225].

Printing of vascularized constructs is another area in which precise deposition of cells has enabled significant progress (**Figure 10**)[226]. For example, vascularized perfusable scaffolds comprised of multiple cell types were generated using 3D bioprinting[121,227]. In this approach, vascular inks comprised of Pluronic F-127 and thrombin were printed on a perfusion chip along with cell-laden ECM bioinks of gelatin and fibrinogen. Casting of gelatin, fibrinogen, cells, thrombin and transglutaminase eventually induces polymerization into fibrin and crosslinking of the gelatin matrix[227]. After cooling, the vascular inks liquefy and are evacuated to create a hollow vascular network which is then seeded with endothelial cells and connected to an external pump. Using this approach, 1 cm thick osteogenic tissues were supported in long-term culture and provide the potential for studying *ex vivo* cell interactions in the future.

**8. Conclusion and further perspective**

Information-assisted manufacturing of complex functional tissues with various cell types is now achievable. When mimicking natural tissue, it is necessary to understand the cell's native environment, especially cell-cell-interactions as well as cell-ECM-interactions. The challenge is to implement vast amounts of available information about cells, tissue structure, and biological

interactions into an artificial product without dramatically increasing its complexity. High-throughput techniques based on experimental design and data analysis in material design and biological characterization will play an important role in building an intelligent architecture for imitating native tissue. Due to the emerging trend of using information-driven design and CAD-based micromanufacturing techniques, different structures and scaffold sizes can be produced. However, material choice, protein matrix design and cellularization will always depend on the target organ as they affect each other permanently. For example, fabrication of thick artificial tissue is limited due to passive transport of nutrients and metabolic waste. Further progress in integrating vascular tissue and combining different types of tissues will lead to more enhanced architectures and biological functions.

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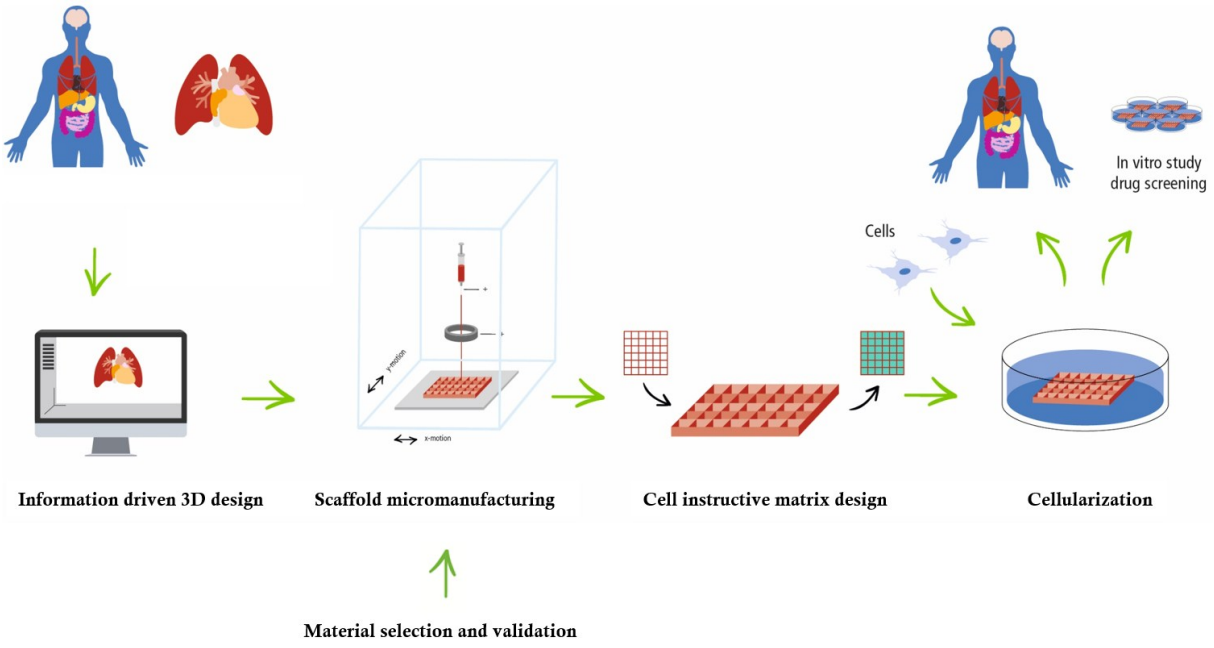


**Figure 1**. Workflow of engineering artificial tissue using information driven design for tissue engineering and regenerative medicine.

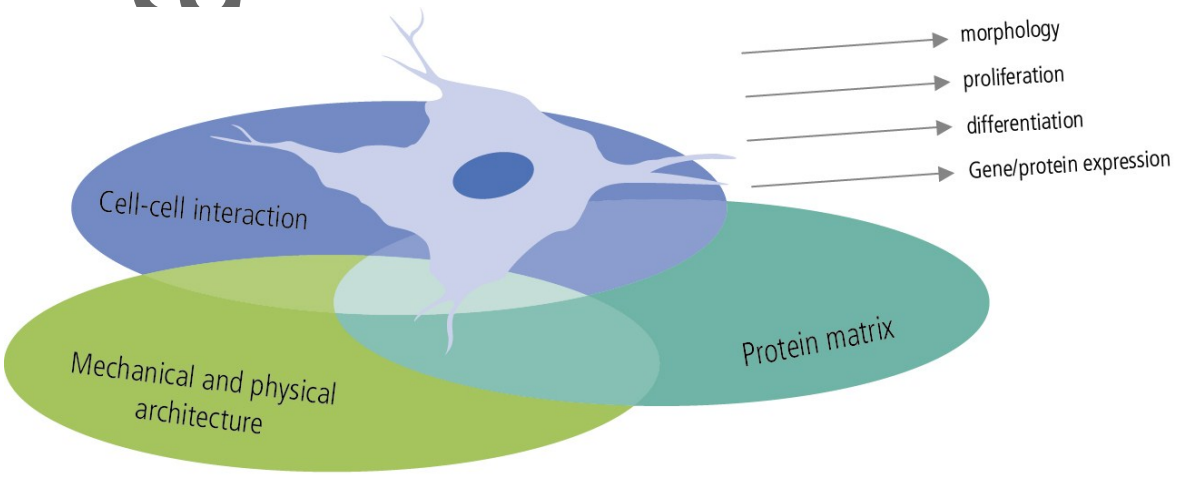


**Figure 2**. Cell behavior is influenced by microenvironmental cues provided by cell-cell interactions, the ECM, mechanical properties and physical architecture.

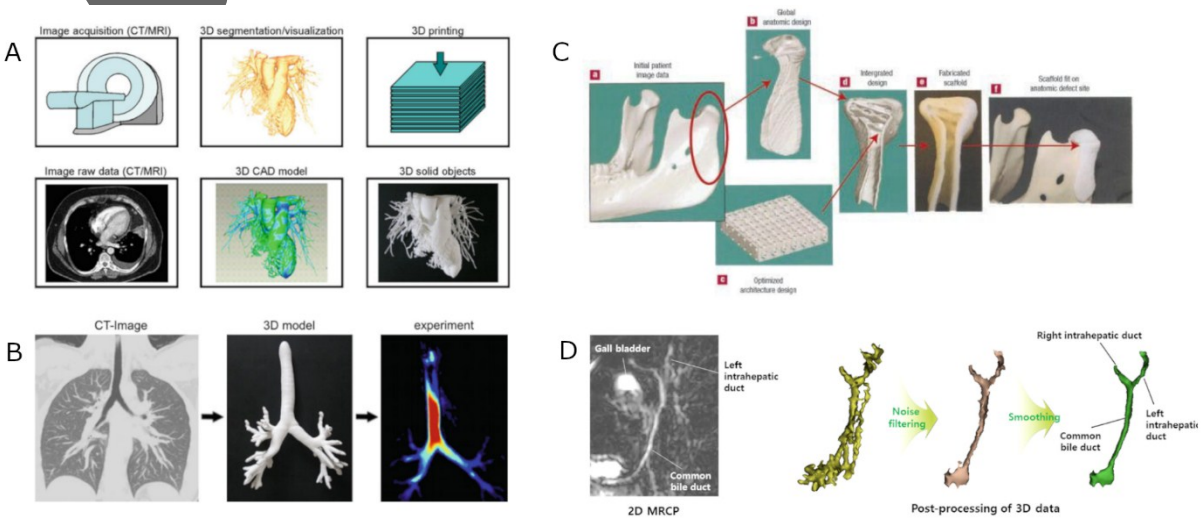


**Figure 3**. Imaging-based modeling used in the design of bioengineered scaffolds. (A) Schematic workflow of imaging-based modeling. Adapted with permission.[40] Copyright 2010, Springer Nature. (B) Using flow-sensitive 4D MRI to investigate the gas flow in the human traches and bronchial tree. Adapted with permission.[40] Copyright 2010, Springer Nature. (C) CT and MRI were used for designing a human mandibular condyle bone tissue. Reproduced with permission.[43] Copyright 2005, Springer Nature. (D) Modeling of the 3D structure in STL format of a bile duct based on medical image data using MRI. Adapted with permission.[45] Copyright 2017, American Chemical Society.

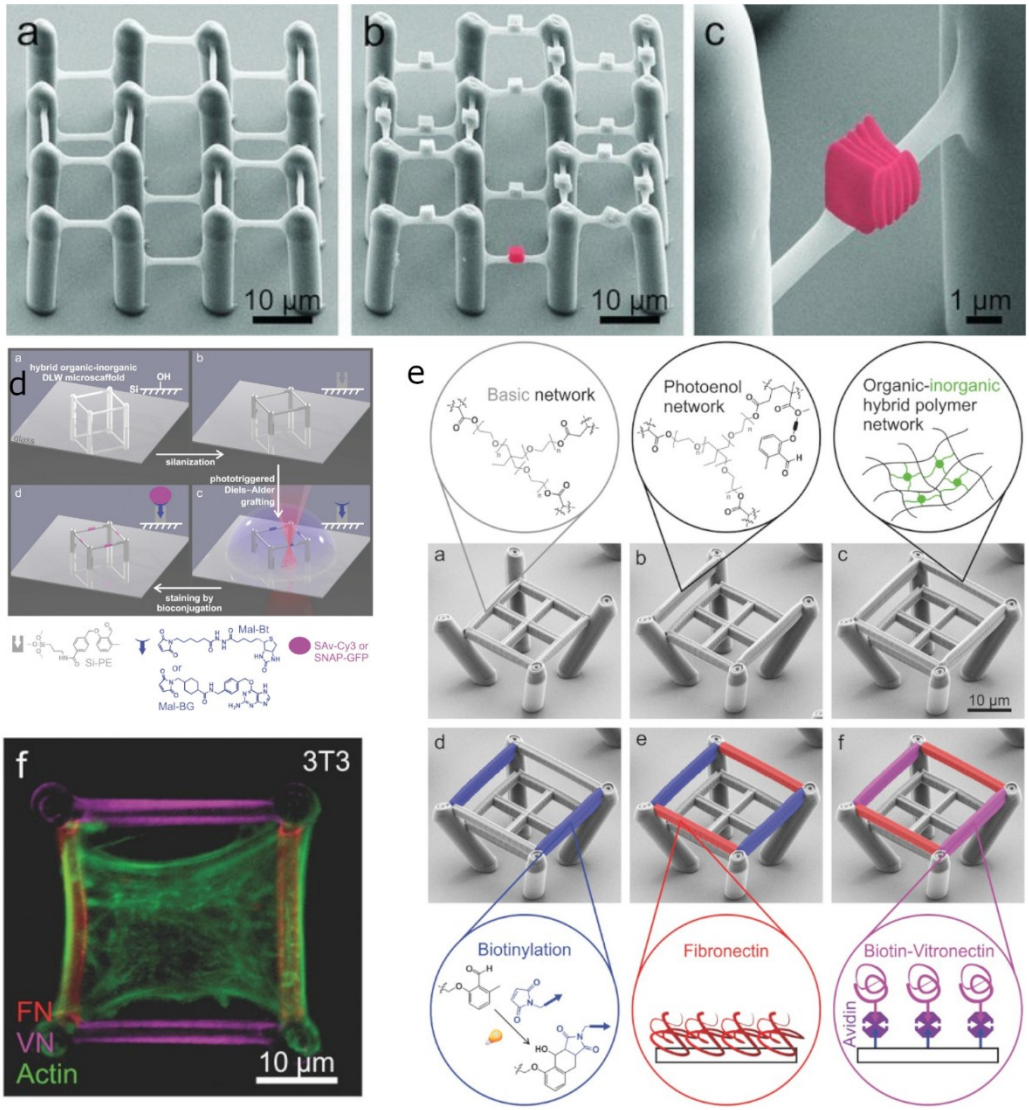


**Figure 4**. Multiphoton polymerization for production of spatially-patterned materials. (A-C) PEG-DA/PETA scaffolds are decorated with Ormocomp®. Reproduced with permission.[162] Copyright 2011, Wiley-VCH. (D,E) Strategy for selective patterning of proteins using selective patterning chemistries or sequential deposition of protein repellent, selective, and adhesive polymers. Reproduced with permission.[164] Copyright 2013, Wiley-VCH. (F) Mouse fibroblast (NIH-3T3) cell shows preferential binding to Fibronectin over Vitronectin on scaffolds made using approach shown in (E). Reproduced with permission.[96] Copyright 2016, Wiley-VCH.

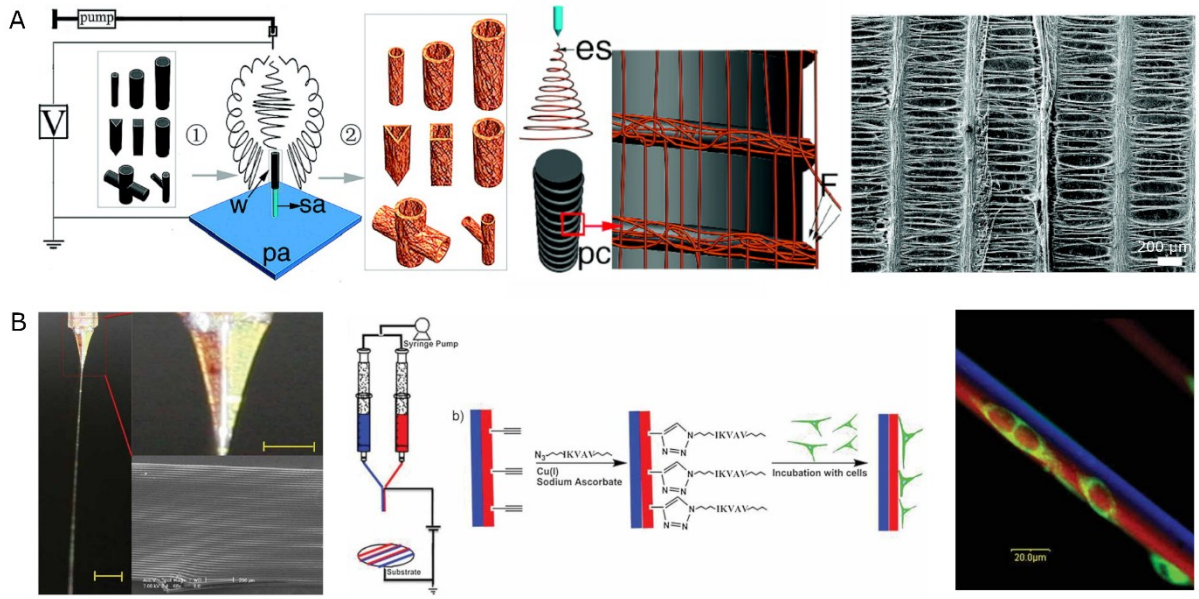


**Figure 5**. Various scaffold productions using electrospinning. (A) Illustration processing fibrous tubes using 3D columnar collectors with patterned architectures. Adapted with permission.[168] Copyright 2008, American Chemical Society. (B) Spatial controlled peptide immobilization onto PLGA fiber scaffolds for selective cell guidance. Reproduced with permission.[84] Copyright 2009, Wiley-VCH.

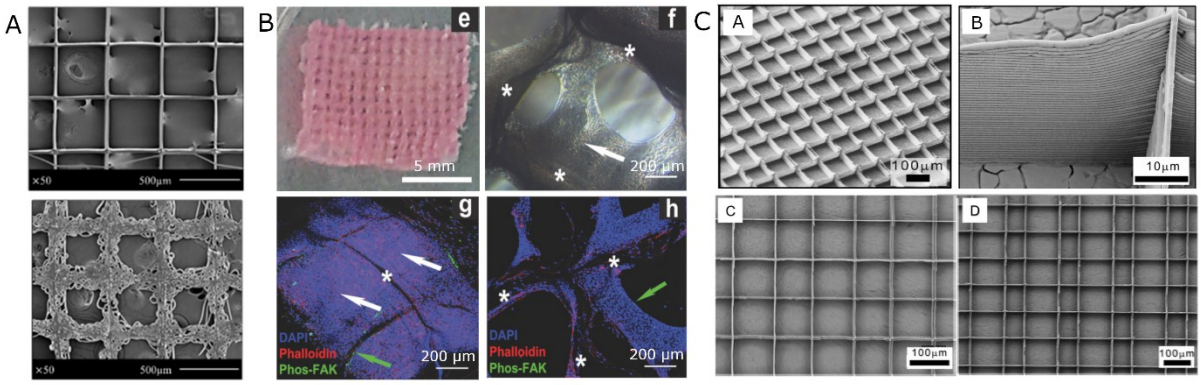


**Figure 6. Various oriented scaffolds fabricated with different 3D electrojetting techniques in combination with a computer assisted x-y translation stage. (A) PCL scaffolds fabricated via near-field-electrospinning (NFES). Reproduced under the terms of CC BY-NC 4.0.[176] Copyright 2017, the authors. (B) Highly ordered fiber architectures produced via electrospinning writing (MEW). Adapted with**

**permission.[90] Copyright 2018, Wiley-VCH. (C) Large volume scaffolds after seeding with hPDL cells and incubation in vitro. Adapted under the terms of CC BY 3.0.[178] Copyright 2015, the authors, published by IOP Publishing Ltd.**

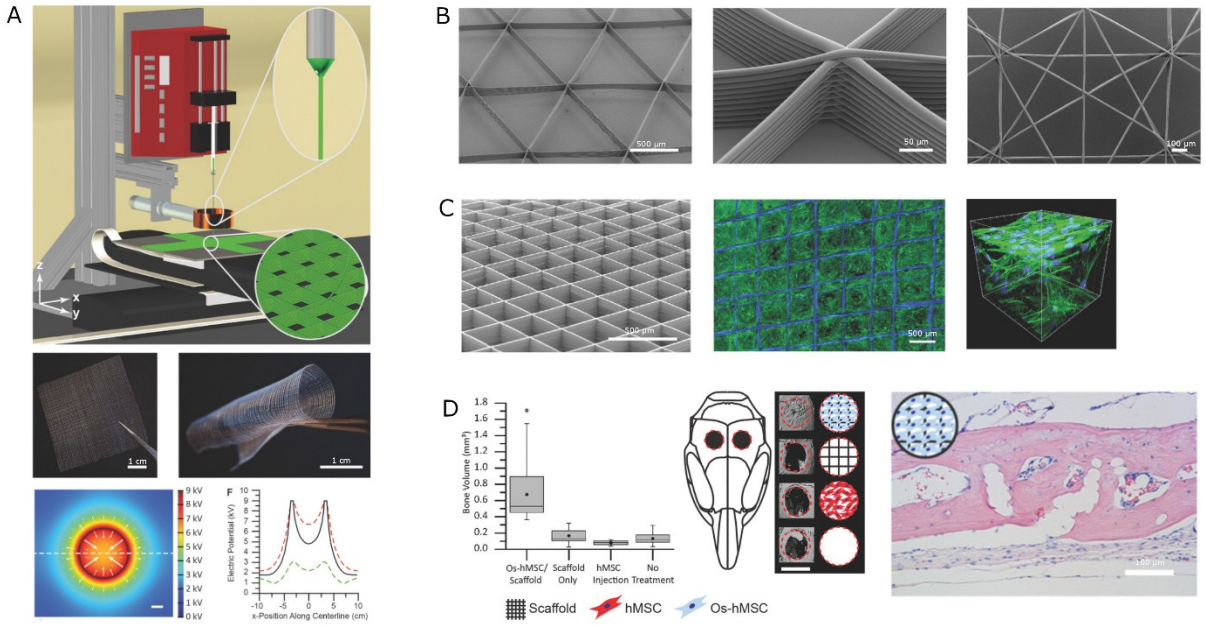


**Figure 7. Scaffolds fabricated using 3D jet writing. (A) 3D jet writing setup with computer simulations of the electric potential. (B) Tessellated scaffolds structures of different geometries manufactured by 3D jet writing. (C) hMSC culture on PLGA scaffolds in vitro after incubation with fibronectin. (D) 3D scaffolds regenerated bone tissue on a defect mouse skull in vitro. All panels adapted with permission.[91] Copyright 2018, Wiley-VCH.**

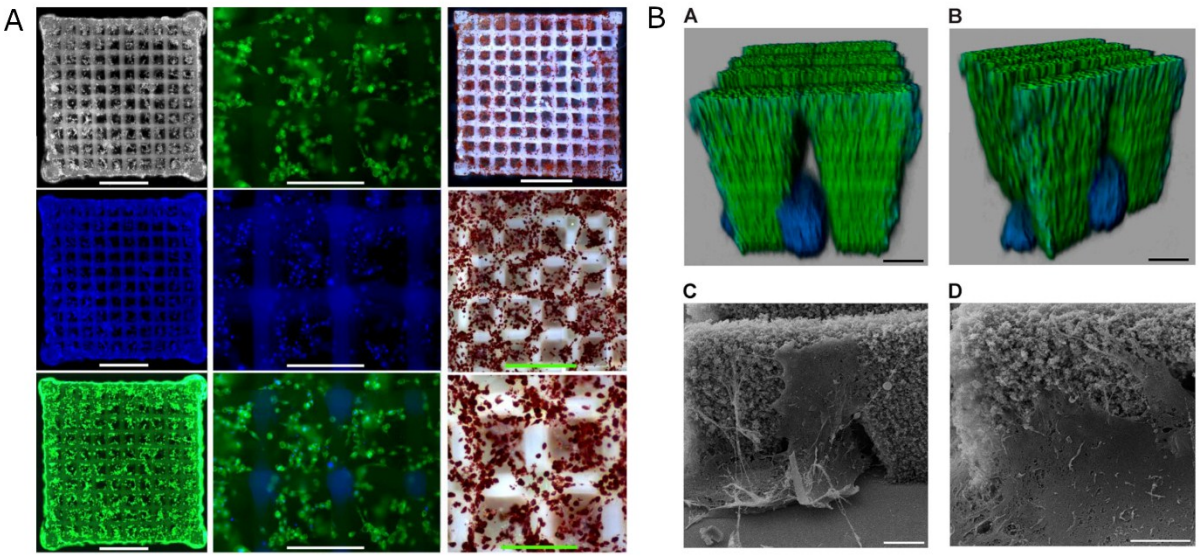


**Figure 8**. Scaffolds fabricated using multiphoton polymerization of biomacromolecules. (A) Gelatin scaffolds support the expansion of adipose-derived stem cells. Adapted under the terms of CC BY 3.0.[212] Copyright 2011, the authors. (B) Laminin and BSA modules are used to support mesenchymal stem cell growth. Reproduced with permission.[213] Copyright 2012, American Chemical Society.

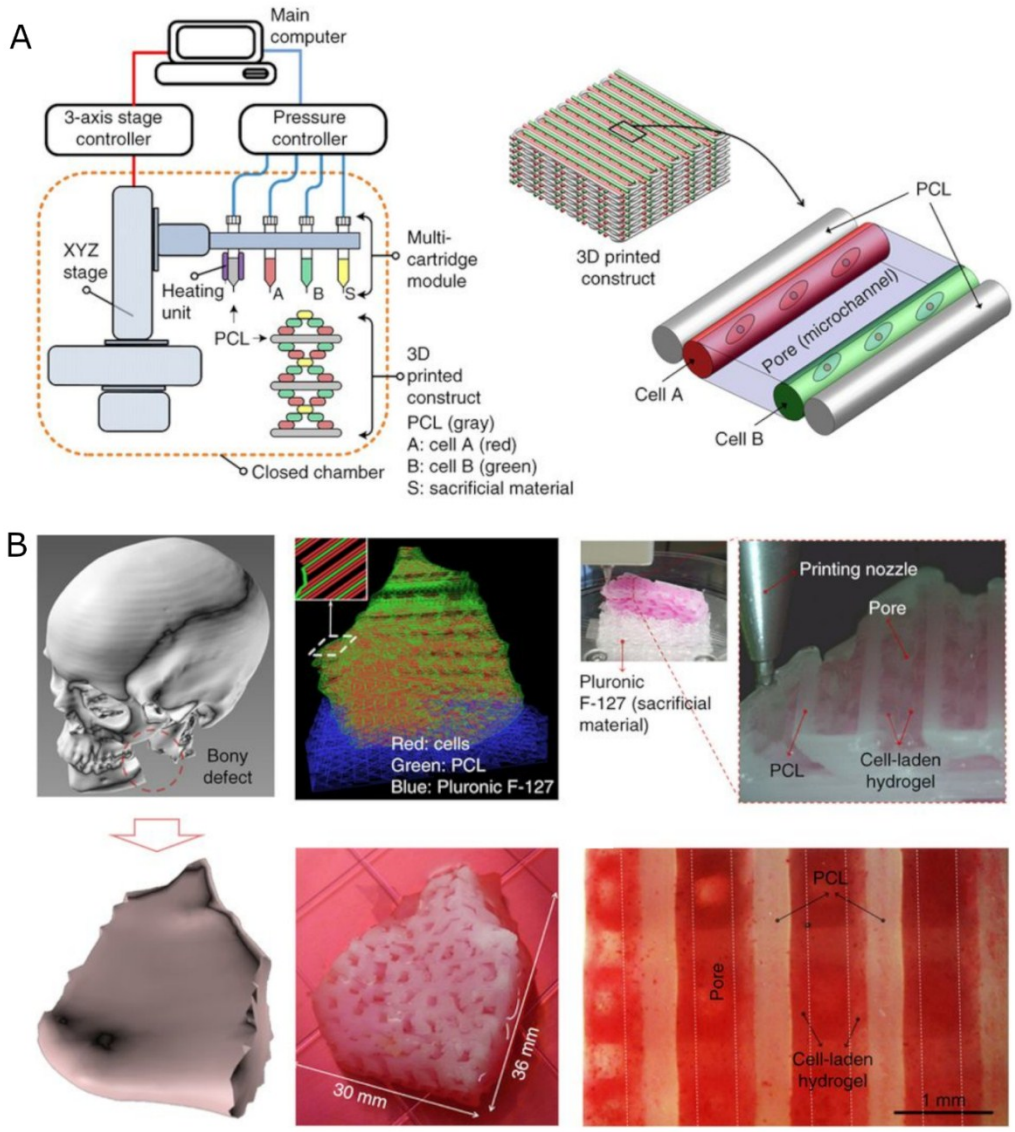


**Figure 9. System for 3D printing of heterogenous polymers, hydrogels, and cell solutions. (A) Schematic for the integrated tissue and organ printer (ITOP) unit and patterning architecture. (B) System is applied for the reconstruction of a calvarial defect. All panels reproduced with permission.[225] Copyright 2016, Springer Nature.**

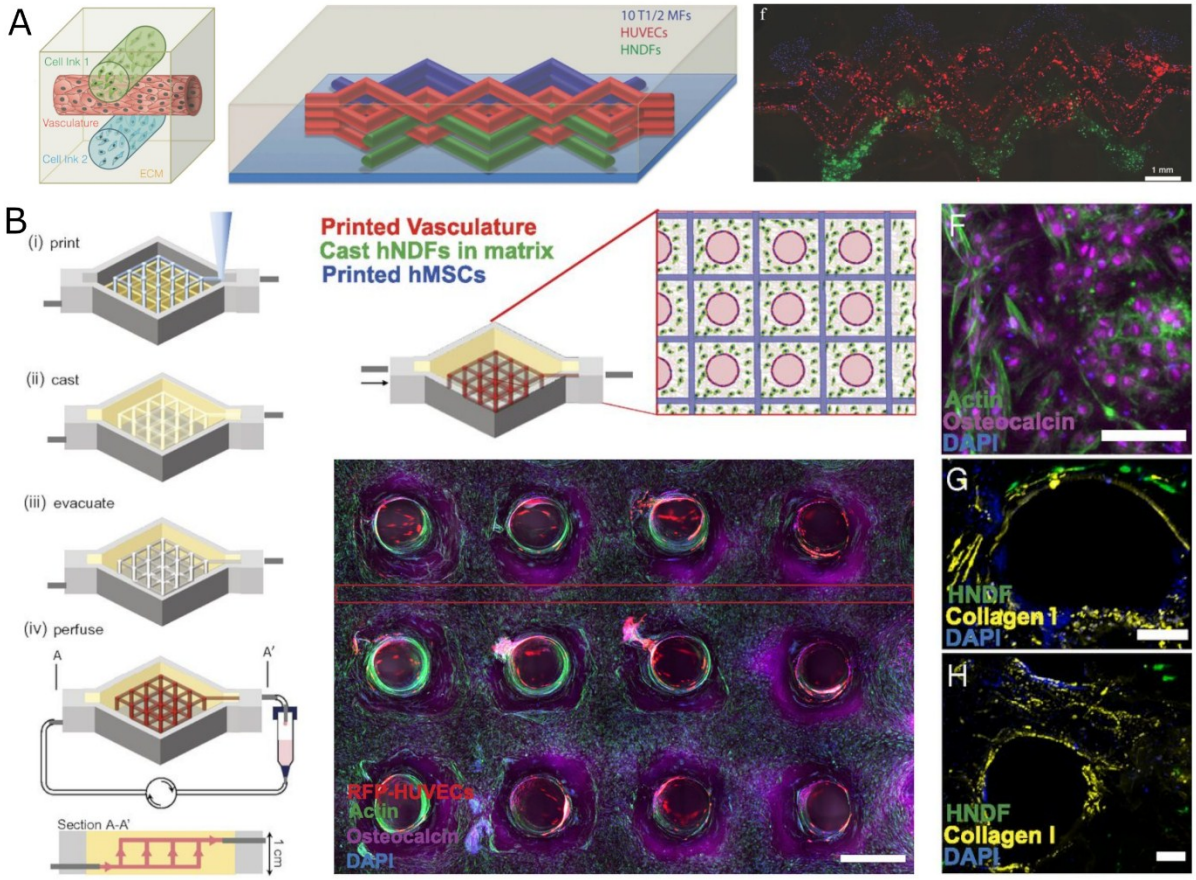


**Figure 10. 3D printing of vasculature. (A) Heterogeneous printing of three different inks (GelMA containing different cell types and a sacrificial Pluronic F127 ink). Adapted with permission.[121] Copyright 2014, Wiley-VCH (B) Perfusable vasculature enables thick (1 cm) osteogenic tissues to grow. Adapted with permission.[227] Copyright 2016, the authors.**

**Table 1**. Key elements of functional tissues

| **Key elements of functional tissues** |
| --- |
| • **Structure**<br>Provide hierarchical organization, lend particular physical and chemical properties to guide proper cell behavior.<br>• **Function**<br>Promote regular cell phenotype specific to the tissue of interest, and facilitate transport of nutrients, waste, and information. |

**Table 2.** Key elements of biomaterials

| **Key elements of biomaterials** |
| --- |
| • **Precise control over material properties**<br>An ideal biomaterial would allow for orthogonal control over physical characteristics and biochemical composition with tissue appropriate properties<br>• **Tunable bioactivity**<br>An idea biomaterial would have user prescribed bio-inertness or bio-inductive capacity depending on the intended application.<br>• **Biodegradation**<br>Tunable bio-associated degradability with definable kinetics and bio-compatible |

**Table 3.** Key elements of micromanufacturing techniques

**Key elements of micromanufacturing techniques**

- **Resolution and scalability**
  Achieving hierarchical design of tissue requires patterning of molecules at sub-micron scale while simultaneously being able to fabricate structures over large areas and build volumes.
- **Speed**
  Iterative processing for material design requires rapid prototyping.
- **Ease-of-use**
  Ideal techniques would allow for use by non-experts, decrease user-error, and diminish time required for optimizing fabrication parameters.
- **Cost**
  Cost for both the micromanufacturing apparatus and consumable materials should be minimized.
- **Materials compatibility**
  A wide range of synthetic and natural materials and biological components such as live cells would be able to be processed either simultaneously or sequentially.
- **Translational potential**
  Ideal micromanufacturing techniques will facilitate commercial and clinical translation.

**Table 4.** Key elements of ECM design

**Key elements of ECM design**

- **Tunable architecture and topology**

  The ECM provides structural support and mechanical integrity as well as orientation to the cells.

- **Tunable mechanical stiffness**

  The mechanical properties should fit to the target tissue to guide cell differentiation and integration of the artificial tissue into the surrounding environment inside the body

- **Tunable biochemical compositions**

Each kind of tissue has his own composition of proteins and growth factors. By tuning the material composition, the resulting tissue can be influenced.

- **Spatial control**

  Microfabrication techniques as 3D printing or photo-patterning provide special control of the biomaterials mimicking the ECM



**Anke Steier** studied Bioengineering at the Karlsruhe Institute of Technology (KIT) in Germany. She specialized in process engineering of bioproducts and separation techniques with focus on interdisciplinary interfaces during her bachelor and master studies. Beginning of 2017 she joined the department of Advanced Polymers and Biomaterials at the Institute of Functional Interfaces at KIT as a doctoral candidate. After a research stay at the Biointerface Institute at the University of Michigan, she is now concentrating on designing multicompartimental polymeric fiber architectures for controlling chemical and mechanical properties for biointerfaces.

**Ayse J. Muñiz** received her B.S. in Biochemistry from the University of New Mexico. She is currently pursuing her PhD in Macromolecular Science and Engineering from the University of Michigan. Her research focuses on engineering extracellular matrices for studying the influences of the microenvironment on cell fate.

**Dylan Neale** received his B.S. in Materials Science and Engineering from the University of Florida. He received his M.S.E. in Chemical Engineering and is currently pursuing his Ph.D. in Chemical Engineering at the University of Michigan. His research is focused on developing and utilizing 3D cell culture technologies for various biological applications.

**Table of contents**

**The engineering of complex biological systems** relies on pioneering new tissue designed constructs and micro-engineered devices to advance regenerative medicine and disease modelling. Modern advances in micro- and biofabrication are fueled by the combination of bioinspired materials with biological and computational tools. In this review, we discuss the integration of multi-scale approaches to enable next generation biomaterial design.

**Toc keyword: Tissue Engineering**

Anke Steier[+], Ayse Muñiz[+], Dylan Neale[+], and Joerg Lahann*

**Emerging Trends in Information-Driven Engineering of Complex Biological Systems**