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Advancing Tissue Engineering: a Tale of Nano, Micro and Macro Scale Integration

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Abstract

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Tissue engineering has the potential to revolutionize the health care industry. Delivering on this promise requires the generation of efficient, controllable and predictable implants. The integration of nano- and microtechnologies into macroscale regenerative biomaterials is playing an essential role in the generation of such implants, by enabling spatiotemporal control of the cellular microenvironment. Here we review the role, function and progress of a wide range of nano- and microtechnologies that are driving the advancements in the field of tissue engineering.

Keywords

Tissue Engineering; Microfluidic; Micromaterial; Biomimetic; Microenvironment

1. Introduction

Tissue engineering has the potential to revolutionize our health care system. Conceptually, the lost or malfunctioning tissues will be replaced by man-made biological substitutes to restore, maintain, or improve their function. Tissue engineering has already shown great promise to contribute to treatments of a myriad of diseases including osteoarthritis, cancer, diabetes, skin burns, cardiovascular conditions and various traumatic injuries [1]. Tissue engineering is a highly multidisciplinary discipline that demands integration of knowledge, tools and skills from biology, chemistry, engineering and medicine. Conventionally it is based on combining cells, biomaterials, and bioactive molecules to form an implant. However, the lack of biologically relevant tissue complexity in the current tissue engineered implants is one of the main challenges that need to be overcome to further advance this breakthrough technology into clinically applicable therapies [2]. In particular, spatiotemporal control of the cellular microenvironment is desired to steer cell fate, function, and behavior. Integrating nano- and microtechnologies into clinically sized implants represents a major opportunity to gain control over cellular microenvironments. Such breakthroughs are expected to revolutionize our healthcare system by effectively generating next-generation regenerative implants. Furthermore, these innovations also provide a unique, designable and controllable artificial tissues/organs platform for the ex-vivo screening of (diseased) cells and tissue, bioactive molecules, pharmaceuticals, and (nano- and microstructured) biomaterials in an unprecedented high throughput manner. Here we aim to provide a comprehensive overview of the nano- and microtechnologies that have been explored to realize the next generation of tissue engineered implants.

2. Assembling Nano- and microfibers to form Biomaterials

Biomaterials are an essential component of tissue engineering that provides structural support and acts as a reservoir for biomolecule sequestration. In essence, biomaterials are instrumental in generating a controlled biomimetic microenvironment to elicit desired cell/tissue responses [3–6]. Over the years, many distinct material types have been explored including metals, ceramics, glasses, and polymers. Polymers are most commonly explored for nano- and microtechnology based tissue engineering approaches. Furthermore, polymers can be classified into two general categories, namely natural polymers and synthetic polymers. Polymers derived from natural sources include collagen, gelatin, elastin, fibrin,

hyaluronic acid, and alginate. While many natural polymers possess cell-adhesion ligands that promote cell-biomaterial interactions, they typically lack well-defined chemical composition and therefore present large batch-to-batch variations.

On the other hand, a wide variety of synthetic biomaterials such as poly(ethylene glycol) (PEG), poly(lactic acid) (PLA), poly(glycolic acid) (PGA), polycaprolactone (PCL) have been designed to possess well-defined chemical composition and properties. These natural or synthetic biomaterials can be easily processed into three-dimensional (3D) scaffolds of well-defined shapes, structures, and architectures at micro- and nanoscales with a variety of techniques that have been developed over the past three decades [7–10].

More recently, self-assembling molecules of short sequences have been explored, which are based on for example, the supra molecular chemistry [11–14] and self-assembling peptides [15–17]. These nanoscale self-assembly approaches can also be used to fabricate scaffolds in a bottom-up manner, in contrast to the conventional top-down techniques based on processing of bulk biomaterials.

3. Biofunctionalization of materials

Basic biomaterials often function as mere scaffolds. In order to create stimulating microenvironments most biomaterials require conjugation of bioactive molecules or distinct biomimetic elements [18–20]. This can be achieved amongst others via the incorporation of biofunctional elements, passively controlled release of bioactive molecules, and cell-mediated growth factor release.

3.1 Incorporating biofunctional elements

Numerous micro- and nano-scale elements have been developed to enhance the functionality of materials. These include aptamers, nanobodies, layer-by-layer (LBL) molecular assembly for conjugation of bioactive molecules usually on the surface of the biomaterials [21–23], and biomimetic peptides and polymer brushes to alter the intrinsic properties of the biomaterials [24–26].

Incorporating biofunctional elements can be achieved using a wide variety of distinct crosslinking chemistries [27, 28]. Of these, the most widely used approach for conjugation is based on carbodiimide chemistry, which crosslinks carboxyl group with primary amine group to form an amide bond (Figure 1A) [29]. In a typical process, carboxyl group is first activated by 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) to the O-acylisourea intermediate form, which can easily be displaced by nucleophilic attack from primary amines. The primary amine group then reacts with the active O-acylisourea intermediate to form a stable amide bond with the original carboxyl group, and an EDC by-product (a soluble urea derivative) is released. As the O-acylisourea intermediate form lacks stability in aqueous solution and easily degrades, a catalyst N-hydroxysuccinimide (NHS) is often added as an additive to the mixture to improve the coupling efficiency. Avidin-biotin interaction is another widely used method for biomaterial functionalization (Figure 1B). Avidin and its derivatives such as streptavidin binds to four biotin molecules with the strongest known non-covalent interactions between a protein and a ligand at a dissociation

constant in the range of 10^{-15} M [30]. Advantageously, the avidin-biotin complex is extremely stable and cannot be disrupted by harsh temperature, pH, and even detergents [30].

In addition to these approaches that homogeneously modify the entire bulk of a biomaterial, much research has been dedicated to modifying the surface of biomaterials. LBL molecular assembly through electrostatic interaction is a classical example, where thin films of polymers with alternating charges can be sequentially deposited onto a surface. Controlling the surface charge of a biomaterial can prevent or augment subsequent protein coating, while minimally distorting biomaterial structures (Figure 1C) [23, 31, 32]. For example, deposition of positively charged poly(diallyldimethylammonium chloride) (PDDA) and negatively charged clay platelets onto the surface of poly(acrylamide) (PAAm) hydrogel scaffold yields a nanoclay coating that facilitates cell adhesion [33, 34]. Similarly, such LBL assembly approach was also used to immobilize the negatively charged DL-1 notch ligands when a PDDA was used as the outermost nanolayer [33]. Besides protein ligands, the electrostatic interactions could be further adopted to immobilize DNA/viral particles. Dipping methods have been designed to generate a gradient of positively charged poly-L-lysine molecules on a collagen scaffold. The gradient could be conveniently controlled by the rate at which the scaffold was dipped into the poly-L-lysine solution. Subsequently negatively charged retrovirus particles could form the same gradient via interactions with poly-L-lysine (Figure 1D) [35]. The retrovirus gradient could then induce graded gene transfection of cells seeded onto the scaffold and eventual formation of a tissue interface. Moreover, LBL can be used to release molecules of interest in a staged manner when spatiotemporal release profiles are desired [36]. Incorporation of recombinant proteins into such layers yields immobilized and locally high concentrates of these molecules, which contributes to the stability and longevity of their biological activity [37]. Indeed, multilayered coated systems have already shown clinical prowess e.g. in improved integration of bone-based implants [38].

With regards to hard tissue engineering, it is more critical to allow modification of inorganic substances to promote osteogenesis. In this sense, an over-saturated simulated body fluid (SBF) is often used to deposit mineral nanostructures on the surface of polymer scaffolds for enhanced osteoinductivity and osteoconductivity [39, 40]. Interestingly, Xia and co-workers also utilized a dipping method to generate a gradient on a piece of nanofiber scaffold (Figure 1E) [41, 42]. The resulting scaffold exhibited a deposited calcium mineral gradient, which was controlled by the time the biomaterial was soaked in SBF (Figure 1, F and G) [42]. The gradient-wise fabrication of such nanolayer coatings can steer e.g. differentiation of stem cells and thus the control the generation of multi-tissue interfaces [42, 43].

3.2. Controlled release of bioactive molecules

Spatiotemporal control over the release of bioactive molecules is instrumental in our ability to intelligently steer implant function. Moreover, controlled release offers additional benefits such as protection of the molecules from physiological degradation in body fluids. Since the past decades, tremendous advancements have been made in the design and development of controlled release systems. In general, controlled release can be broadly classified into two

major categories according to their mode of operation: sustained release and stimuli-responsive release.

Sustained release aims to deliver biomolecules over an extended period of time, which maintains the concentrations of the molecules at reasonable levels of interest. This can be achieved by two major mechanisms: diffusion through an insoluble polymer shell or solid matrix, and erosion of a polymer matrix (Figure 2A) [44–46]. In diffusion-mediated release, the bioactive molecules can be either trapped inside the reservoir of a polymer shell or within a solid polymer matrix. The hollow formulation controls the release rate of biomolecules by simply modulating membrane properties. However, it is often associated with capsule rupture, which results in burst release at largely uncontrolled time points [47]. In contrast, solid matrix release systems almost always give a burst release at the beginning due to desorption or release of the biomolecules on or close to the surface, followed by more sustained release over time through diffusion [44]. In erosion-mediated mechanisms the biomolecules are encapsulated in a biodegradable polymer matrix. The release pattern of the molecules within can be easily controlled by tuning the erosion parameters of the matrix [48–50].

It is of an important note that a variety of these approaches can be multiplexed into a single scaffold to achieve simultaneous release of several biomolecular species. For example, one growth factor can be encapsulated directly in the matrix of a porous scaffold while another is first laden into nanoparticles prior to embedment in the same scaffold (Figure 2B) [51]. The growth factor directly loaded into the matrix of the scaffold showed rapid release profile mediated by diffusion through the polymer (Figure 2C), whereas those endured double protections exhibited different degrees of slower release depending on the nanoparticles' polymer viscosity (Figure 2D). The differential release of multiple growth factors is particularly useful when engineering tissues that require staged presentation of bioactive molecules such as the neovascularization process where earlier stages require vascular endothelial growth factor (VEGF) to promote angiogenesis and later stages require platelet-derived growth factor (PDGF) to mature vessels by recruiting and committing pericytes.

Stimuli-responsive release of biomolecules has attracted more and more attention in recent years due to its higher degree of on-demand control as compared to sustained release [52, 53]. Commonly used stimuli include pH, light, temperature, and magnetism, where corresponding mechanisms can be incorporated into the scaffolds to initiate the responsiveness [44, 54–56]. For example, thermal-sensitive hydrogels based on colloidal self-assembly and covalent gelation of submicrometer-sized microgels made from poly(N-isopropylacrylamide) (PNIPAm) have been fabricated (Figure 3A) [57]. The bulk scaffold exhibited similar responsiveness to temperature change as the isolated microgel particles (Figure 3B), and demonstrated significant shrinkage at higher temperature and swelling when the temperature was reduced (Figure 3C). Moreover, porous hydrogel scaffolds encapsulating magnetic nanoparticles termed ferrogel have been explored. These constructs can be remotely activated by a magnetic field to achieve microscale and macroscale compression (Figure 3, D–G) [56, 58–61]. Such delivery mechanism was discovered against the fact that the fibrin clot formed during the blood coagulation actually functions as a provisional extracellular matrix (ECM) and reservoir for secreted growth factors by the

infiltrating cells [62]. Fibrin(ogen) has thus been shown to exhibit strong affinity to many growth factors in the VEGF, PDGF, placenta growth factor (PIGF), fibroblast growth factor (FGF), bone morphogenetic protein (BMP), transforming growth factor (TGF) families as well as a few other growth factors (Figure 4A) [63]. The bonded growth factors all showed sustained release profiles dependent on the affinity between the molecules and the fibrin matrix (Figure 4, B and C). In addition, the heparin-binding domain of the fibrin(ogen) (Figure 4D) was shown to possess growth factor-binding capacity by itself (Figure 4E). Incorporation of such nanoscale bioactive ECM domain into a fibrin-mimetic matrix successfully reproduced growth factor-ECM interactions and promoted tissue repair [63].

3.3. Cell-mediated growth factor release

Cells embedded in a scaffold can be used as a direct source or as a trigger for the release of immobilized growth factors [64–66]. To achieve such functionality, a protein/enzyme sensitive linker moiety is typically co-polymerized into a hydrogel matrix to allow for local cleavage of the network by protease activity in the presence of cells (Figure 5A) [61, 67, 68]. This cell-based trigger can even be taken a step further by implementing a smart negative feedback system by co-immobilizing an enzyme labile moiety with an inhibitor of the enzyme into the hydrogel scaffold [69]. For example, matrix metalloproteinase (MMP)-sensitive peptide was used as the linker of the hydrogel network, which could be degraded upon MMP activation; at the same time the polysaccharide-bound MMP-inhibitor rTIMP-3 was liberated by the cleaving event, inducing local inhibition of the MMP activity that attenuated further hydrogel degradation (Figure 5B). This feedback system demonstrated a constrained release profile in comparison to the implant without the MMP inhibitor rTIMP-3 (Figure 5C).

Although successful, it is of important note that natural tissues have a higher dynamical complexity than provided via tissue engineered strategies, which mostly rely on the incorporation of a single type of responsive unit (e.g. MMPs-cleavable peptides). Natural tissues typically contain a multitude of cell types and express a complex and spatiotemporally defined panel of factors when required to heal. It is therefore expected that the development of novel moieties that are cell type-responsive and allow for timed release would greatly promote the advancement of tissue engineering. In particular, this is likely of great value where control over inflammation, differentiation and tissue maturation is desired for clinical success.

4. Bottom-up tissue engineering

Besides their contribution to the development of novel biomaterials and modifications for biomaterials, nano- and microtechnologies have made a significant impact on the processing of biomaterials [70]. In particular, it enabled reproducible and controllable fabrication of biomaterial entities at the nano- and microscale named nano- and micromaterials, respectively [71]. Some of these, based for instance on hydrogels, can be used for the production of objects containing a single, or multiple cells, by simply suspending the cells in the prepolymer solution.

The ability to produce micromaterials opens up a unique set of opportunities. The conventional manner to produce tissue engineered implants relies on providing a desired element homogeneously throughout a biomaterial, which is referred to as top-down tissue engineering [72–74]. Although elegant due to its simplicity, the resulting homogeneity impedes the creation of the exquisite structures as found in natural tissues and organs. Such structures not only define a tissues or organs shape, but are also essential for their function [75]. The lack of this spatial control diminishes our capability to accurately steer multiple functions as they occur within our body. Unfortunately, the clinical success of engineered implants heavily depends on the construct's ability to steer multiple in vivo processes [66, 76, 77]. In particular, an implant's efficacy is determined by its ability to provide protection from the implantation site's often harsh environment, facilitating host-implant integration by e.g. attracting blood vessels and offer continued stimulation to guarantee long-term tissue function [78]. It is therefore often argued that a high multifunctionality with spatial control is demanded of implants. The advent of micromaterials enabled the creation of designable and replicable building blocks that can be assembled into larger macro-sized structures that are reminiscent of organs. This approach is referred to as bottom-up tissue engineering [79]. In recent years a multitude of microtechnologies have been explored to advance bottom-up tissue engineering. These methods include soft lithography, photolithography, microfluidic, electrospinning, and micro-assembly techniques.

4.1. Soft lithographic micromaterials

Soft lithography refers to a set of microfabrication techniques where elastomeric stamps or molds are used to generate objects or features [80]. The use of micro-molds is a robust and reproducible method to generate 3D objects that are suitable for tissue engineering. Shape and size of objects can be precisely controlled in the micrometer to millimeter range [81]. Due to the use of a physical mold, to impose the shape of the object, the method is compatible with a wide range of materials including thermal-, photo-, enzymatic-, and ionic crosslinking materials. This also makes this method highly compatible for the encapsulation of cells since it does not inherently rely on conditions that are incompatible with cell survival and function.

A critical factor in the use of soft lithography for the creation of objects is the release of these objects from the mold. This is especially the case with shapes where the mold and object form interlocking structures. Several methods have been developed to mitigate this problem, including the use of soluble molds [82], or the use of thermoresponsive molds that change their shape based on a change in temperature in order to release the particles (Figure 6A) [83]. In this way, the objects can be easily harvested and subsequently used as building blocks for bottom up tissue engineering.

By using molds with smaller features, soft lithography can also be used to create nano-features on material surfaces, e.g. a two-step approach can be used to generate a PDMS mold that combines micro-features with a nano-topography [84]. After replicating these features into PMMA, it was shown that the cell orientation depended on the combination of both the micro- and nano-topography (Figure 6B) [85]. The application of nanostructures is also not limited to flat surfaces. The use of a mold containing micro- or nano-features has

been combined with thermal imprinting to generate 3D curved polylactic acid microwells with surface features. Cells seeded in these wells adapted their shape and alignment based on the surface features [86].

To generate features at an even smaller scale, soft lithography can also be used to pattern molecules such as proteins [80]. This approach is often used to pattern cell adhesive protein islands on a surface that otherwise prevents cell attachment [87]. In addition, protein stamping can also be used to create nanofabrics that make use of the natural organizational capacity of extracellular matrix proteins into nano-scale fibrils. For instance a study has been reported where fibronectin was patterned on a poly(N-isopropylacrylamide) (PIPAAm) surface with a resolution up to 10 μm . Lowering the temperature from 37 to 32 $^{\circ}\text{C}$ resulted in the release of the fibronectin from the surface, forming a flexible, free-standing nanofabric. Within the fabric, the fibronectin remodeled into nanoscale nodules with a diameter of approximately 50 nm, which is consistent with refolded fibronectin dimers (Figure 6C) [88].

It has become clear that soft lithography offers great flexibility in the fabrication of structures. However, indirect fabrication processes such as mold based approaches limit the variety of micromaterial shapes that can be produced, and also hampers the creation of libraries of individual slightly varying micromaterials. As such, this method is well positioned for the production or modification of multiple copies of micromaterials with relatively simple shapes, but is currently less suited for approaches that require variations between individual objects.

4.2. Photolithographic micromaterials

Photolithography employs light-induced polymerization of materials for the generation of 3D structures. Pre-polymer solutions are shaped into solid structures by inducing radical-based photosensitive reactions. The polymerization can be spatially controlled by using a photomask that blocks light in certain areas, while letting light through in other areas. The areas of the photosensitive material accessible to light are cross linked or solubilized depending on whether a negative or positive photoresist is used. The two-dimensional pattern of the photomask is thus replicated into a 3D pattern of the polymer. This method can be used to make non-cellular objects, but also for production of cell-laden micromaterials (Figure 7A) [89].

Even though this method results in 3D objects, the objects are in essence planar since they arise from a two-dimensional photomask. However, a modification of the standard photolithography principle in the form of dynamic optical projection stereo lithography has allowed for more flexibility in making truly 3D objects. This method uses a digital micro-mirror device (DMD) found in conventional computer projectors, to solidify a photosensitive liquid prepolymer using a light source. The dynamically interchangeable pattern is created by an array of micro-mirrors that can be switched on or off [90, 91]. Therefore, the digital mask allows for the use of controllable and interchangeable reflected light patterns rather than the static, more expensive physical masks used in conventional photolithography. In order to fabricate 3D structures, a 3D computer design of the object is deconstructed into a series of layers that are sequentially illuminated (Figure 7B) [91].

Two-photon lithography is another refined version of photolithography that generates both micro- and nanometer features [92–94]. This technique is based on controlled localization of the laser focal point and subsequent selective polymerization within the focal volume of the laser beam. This allows for the fabrication of true 3D micromaterials, while enhancing the printing resolution [95, 96]. An elegant example of the technical prowess of two-photon polymerization is represented by the ‘micro-bull’ sculpture, which was fabricated at a resolution close to the subdiffraction-limit of 120 nm (Figure 7C) [95]. Since then, the technology has also been applied in cell biology and tissue engineering in for instance the fabrication of a two-component scaffold. These scaffolds are composed of spatially defined areas that are either protein-repelling or protein-binding. Since cells would only attach to the protein-binding regions, the placement of these regions controlled cell adhesion and consequently cell shape in 3D (Figure 7C) [97].

Even though two-photon lithography is capable of producing objects with a large degree of freedom concerning shape and size, it is an inherently slow process due to its point-by-point writing. The process has been optimized allowing for writing speeds of 1 cm s^{-1} while maintaining micrometer resolution [98]. This allows for ground breaking academic exploration, but challenges industrial applications that require mass production. Moreover, only a few photo-initiators are compatible with two-photon lithography. Due to toxicity of these compounds, the process is less suitable for the production of cell encapsulated micromaterials [96].

Even though great advances have been made regarding resolution, freedom to prepare complex 3D shapes, and fabrication speed in photolithography, it remains challenging to fabricate objects over multiple length scales using this method. Processes are generally optimized for either a high resolution or a high fabrication volume. Therefore, gaining the ability to produce a macro-scale implants while not having to sacrifice control of the nano- and microscale remains as a major challenge.

4.3. Microfluidic micromaterials

Microfluidic technology has been used extensively for the generation of biomaterial droplets, mainly in the micrometer range [99]. However, microfluidic droplet generators have recently also been explored to synthesize libraries of nanoparticles in a well-controlled, reproducible and high-throughput manner [100]. Conceptually, a stream of a pre-polymer solution is periodically broken up by a second flow of an immiscible carrier fluid. When the two fluid streams meet, droplets form due to a balance between the interfacial tension and the shear of the continuous phase (the carrier fluid) acting on the dispersed phase (the pre-polymer solution) [99]. Subsequent gelation of the pre-polymer in the droplets results in 3D objects. Multiple methods of gelation can be integrated, including photo-, enzymatic-, thermal and ionic crosslinking. Many of these approaches are biocompatible and allow for the fabrication of cell-laden micromaterials [101].

The technology is especially fit for the fabrication of well-controlled spherical or rounded objects, which are hard to form with soft- and photolithography. Furthermore, microfluidics offers the ability to continuously alter the particle shape and size simply by changing the flow rates [102]. Adaptations of this concept allow for the creation of multi-zonal particles.

By including different flow channels containing different materials, either core-shell [103] or Janus particles, containing a surface with distinct regions of different physical or chemical properties, can be prepared (Figure 8A) [104].

In addition to producing spheres, flow focusing can also be used to generate microfibers. In this scenario, the flow conditions are designed to prevent the inner phase from breaking up and thus form a continuous stream instead of droplets. As with droplet microfluidics, different fluid flows can be combined in order to make more complex structures such as hollow fibers [105], or fibers in which different cell types are restricted to specific regions of the fiber [106]. Moreover, by incorporating valves into the microfluidic system, it is not only possible to make fibers with parallel variation (different regions in the same section of the fiber), but also serial variation (different regions in different sections of the fiber), and combinations of serial and parallel variation (Figure 8B) [106]. In addition, microfibers can be decorated with micro and even nanotopographic features by patterning the nozzle of the prepolymer channel [106].

As indicated, the range of shapes of particles that can be produced using flow focusing is limited. However, this range can be greatly extended by combining microfluidics and photolithography. For example, a photopolymerizable pre-polymer can be flown through a PDMS channel and illuminated with UV through a photomask, resulting in the formation of shaped objects in the flowing monomer. By using the diffusion of oxygen through PDMS, polymerization near the PDMS surface is inhibited, so that the objects do not stick to the surface and remain mobile in the flowing pre-polymer. Using this methodology, a wide range of shaped particles can be produced with in high throughput using relatively simple of device designs (Figure 8C) [107].

This method was later optimized by including a valve in the prepolymer's channel that stops the flow exactly when the pre-polymer is illuminated, which is referred to as stop-flow lithography [108]. This process allows for an increased resolution and a higher flexibility in the shapes of objects that can be prepared. Stop-flow lithography can be used to fabricate micromaterials with elaborate 3D features through the use of gradients of UV filtering nanoparticles in the prepolymer, as well as discontinuous photomask patterns (Figure 8C) [109].

Microfluidic micromaterials have been uniquely suited for the creation of well controlled 3D environments. This technique is expected to enable the study of the behavior and development of single cells or microtissues with unprecedented precision, control and detail. Encapsulating cells within microfluidically generated microgels provides unique opportunities for both direct and indirect co-cultures, high throughput screening of cell-biomaterial interactions, and decreasing the required amount of material allowing for more cost-effective investigations. Moreover, microgels can be laden into distinct biomaterials thereby allowing for decoupled tuning of the cell's adjacent and distant microenvironment. Several groups have started to pioneer these promising avenues for a variety of applications ranging from studying the behavior of cancer cell to improving tissue engineered constructs [110, 111].

4.4. Electrospun micromaterials

The above described technologies allow for the creation of well-defined objects at – mostly – the micrometer scale. However, their ability to fabricate biomaterials at the nanometer scale has remained limited. In contrast, electrospinning is a method that can produce long biomaterial fibers with a nanometer diameter. Conceptually, fibers are drawn by the flow of a viscoelastic polymer that is subjected to an applied electric field between an injecting needle and a collector plate (Figure 9A) [112]. Electrospinning provides a simple and versatile method for the generation of micro- and nanoscale fibers from a variety of materials, including polymers, ceramics, and composites. Using this technology, fibers can be fabricated with a constant and tunable diameter in the range of 100 nm to several micrometers. Multiple process parameters can be used to control the diameter of the fibers, including the polymer's physical properties, the polymer flow rate, the applied electric field, and the distance between the needle tip and the collector [113]. Apart from tuning the diameter of the individual fibers, process parameters can also be adapted in order to create micro- or nanoscale surface topographies on the fibers [114]. By controlling the solvent evaporation during electrospinning a phase separation process can be initiated, which can controllably yield surface features such as nanogrooves or nanoporosity (Figure 9B) [115, 116].

Coaxial needles enable the fabrication of fibers that contain an inner and an outer region. This approach greatly enhances the versatility of the method and can be used to fabricate hollow fibers [117], fibers containing spatially defined cells [118] and drug delivery agents such as liposomes [119] or nanoparticles [120]. Regardless, electrospinning is mainly used as a technology to create non-woven mesh scaffolds on which cells can be seeded. Meshes with randomly oriented or aligned fibers can be formed, by either using a stationary or a rotating collector. Furthermore, the shape and surface features of the collector can be adapted to further control the orientation of individual fibers. By using, for instance, a cylindrical collector with equally spaced protrusions, tubular scaffolds with a patterned architecture of fibers can be prepared (Figure 9C) [121].

Even though electrospinning is a method that is well suited for the production of nano- and microscale fibers, the practical applications of these fibers are currently still limited. Woven or non-woven mats can be prepared as scaffolds for cell seeding, but in order to reach satisfactory mechanical properties or clinically relevant thicknesses, the packing of the fibers is often so dense that it limits the cellular migration into the mats. Although of high scientific value, the amount of clinical procedures for which nano- or microscale electrospun fibers would prove the superior solution has remained limited.

4.5. Assembling micromaterials into macrostructures

Over the years many strides have been made in the development of distinct nano- and micromaterials using a wide variety of techniques. However, a key determinant of success is grounded in the efficient, elegant and cost-effective integration into clinically relevant therapies. A major application is the recapitulation of natural tissue hierarchy in man-made implants as this has remained a true challenge. Indeed, the integration of nano- and

microtechnologies in bottom-up tissue engineering approaches represent a unique opportunity to make these elusive man-made hierarchical tissues a reality.

A well-established method to create a multi zonal tissue is the layer-by-layer assembly of micrometer thick cell layers. Thermoresponsive polymer brushes are proving an ideal platform to control these sheets as they allow and improve the production and harvest of cell sheets by controlling cell adhesion strength and cell detachment (Figure 10A) [122]. Moreover, by layering oriented cell sheets in alternating manners, natural orientation can be created in the compiled implant. Cell sheet technology has already found its way to clinical translation and has shown promising clinical outcomes. As an example, stacks of micrometer thin cell sheets allowed for the reconstruction of severely damaged corneas [123].

Other more conventional bottom-up approaches have aimed to chemically or physically assemble cell laden micromaterials into macroscale implants. This is most commonly achieved via geometrical recognition between micromaterials gels or by the usage of biological glue. Geometrical recognition can be achieved via amongst others lock and key structures, which organizes micromaterials in larger predetermined geometric configurations by controlling external energy input, surface tension, and microgel dimensions [89]. Microgels can also be assembled via acoustic waves a non-invasive manner [124]. In addition, by including magnetic nanoparticles in the micromaterials complex, organized and 3D constructs could be created via the application of an external magnetic field (Figure 10B) [125]. Furthermore, oppositely charged microgels allow for the formation of a macrostructures containing a checkerboard pattern [126]. The creation of such unique microstructures could augment our control over the communication between cell types and as well recreate niche environments ideal for pharmacological or tissue engineering applications. However, these techniques only provide limited control and versatility in the placement of microgels.

DNA was recently explored as a programmable and sequence-specific biological glue to provide exquisite control and versatility over the assembly of micromaterials (Figure 10C). By modifying one or a few sides of the biomaterial with distinct DNA sequences, the self-assembly can be steered with unprecedented versatility [127]. This approach allowed the upscaling of nano- and micromaterials from the nano- to the multi millimeter scale. Besides conventional nanoscale hybridization bonds, DNA can assemble into a 2D brickwork lattice, 3D rhombohedral lattice and 3D origami structures [128]. The programmable nature of these approaches is expected to contribute to high fidelity and tightly orchestrated bottom-up tissue engineered implants.

All the mentioned techniques have shown promise, but are limited either by precision, throughput or scalability. In contrast, 3D biomaterial printing represents a unique tool that fulfills all these prerequisites for clinical translation [129, 130]. This technique is ideal for the accurate, rapid and reproducible placement of nano- and micromaterial to form macroscale implants [131]. 3D biomaterial printing can be achieved using multiple methods, but is ultimately based on the repeated and continuous deposition of droplet, fibers or sheets with micrometer sizes and nanometer features [132]. Moreover, by predetermining the

physical characteristics of the placed droplets the macroscale implant's shape can be controlled in a temporal manner (Figure 10D) [133]. However, one of the main challenges for this technology lies in the on-demand production and placement of many different nano- and micromaterials with a high resolution to form a clinically sized implant while maintaining a fast paced production process.

5. Conclusion and perspective

Nano- and microtechnologies have found their way into tissue engineering. This integrative movement has had a significant impact on our ability to create controllable, intelligent and versatile macroscale implants. Currently, nano- and microtechnologies are driving many of the most exciting developments in the creation of biomimetic microenvironments. Moreover, the continuing integration of these technologies in the field of tissue engineering is expected to revolutionize our ability to scale the production of tissue engineered implants.

Although tremendously successful, many challenges in nano- and microtechnologies for tissue engineering solutions yet persist. In particular, great strides have been made in creating stimulatory spatially defined microenvironments, but many of these approaches are relatively short lived. Current delivery mechanisms based on using nano-/microscale formulations can typically only achieve one-time loading and a limited release time. Similarly, using advanced bottom-up approaches e.g. using 3D printers to precisely place certain cells within certain regions. However, without additional stimulatory cues, the advantage of this technological feat will be limited. Therefore there remains a strong need in devising novel ways to control tissue engineered implants for over longer time scales to allow the newly formed tissues inside the scaffolds to fully mature and become self-sufficient.

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Figure 1.

A) Principle of the carbodiimide reaction. B) Schematics of the biotin-(strept)avidin binding. C) Layer-by-layer (LBL) assembly of alternatively charged polymers. D) Electrostatic immobilization of DNA gradient on scaffolds. E) Formation of a mineral gradient on the surface of electrospun nanofibers using a pulling method. F) Calcium gradient formed along the long axis of the nanofiber mat. G) SEM images showing the differential mineral contents on the nanofiber scaffold. Adapted with permission [30, 35, 41, 42].

Figure 2.

A) Illustration of three major mechanisms for achieving sustained drug release: diffusion through an insoluble polymer (I) shell or (II) matrix, and (III) erosion of a polymer matrix. B) Growth factors were incorporated into polymer scaffolds by either mixing with polymer particles before processing into scaffolds (VEGF), or pre-encapsulating the factor (PDGF) into polymer microspheres used to form scaffolds. The two growth factors were incorporated together into the same scaffolds by mixing polymer microspheres containing pre-encapsulated PDGF with lyophilized VEGF before processing into scaffolds. C) In vitro release kinetics of VEGF from scaffolds fabricated from PLG (85:15, lactide:glycolide). D) In vitro release kinetics of PDGF pre-encapsulated in PLG microspheres (filled triangle 75:25, intrinsic viscosity = 0.69 dl/g; filled square 75:25, intrinsic viscosity = 0.2 dl/g), before scaffold fabrication. Reproduced with permission [44, 51].

Figure 3.
A) Schematic for the controlled aggregation and colloidal gelation of the microgel particles.
B) Deswelling behavior of the single microgel particle and the hydrogel scaffold, plotted with the diameter, D, normalized by the initial diameter as a function of the temperature. C) A hydrogel scaffold before and after the transition temperature. D) Stress vs. strain curves for nanoporous ferrogel and macroporous ferrogel subjected to compression tests. E, F) Cylinders of a nanoporous ferrogel and a macroporous ferrogel reduced its height ~5% and ~70% when subjected to a vertical magnetic-field gradient of ~38 A/m². G) SEM images of a freeze-dried macroporous ferrogel in the undeformed and deformed states. Scale bar: 500 μm. H) On-demand release profiles of mitoxantrone from macroporous ferrogels. I) On-demand release profiles of plasmid DNA from macroporous ferrogels. J) On-demand release profiles of SDF-1α from macroporous ferrogels. Reproduced with permission [56, 57].

Figure 4.

A) Ability of growth factors to bind to fibrinogen. A signal significantly greater than 0.1 (gray box) was considered representative of a specific binding. B) Cumulative release of growth factors over 7 days. C) Fractions of FGF-2 and PDGF-2 remaining in the matrices after 7 days. D) Ribbon diagram representation of human fibrinogen and its central region E as well as relevant fibrinogen fragments in various colors. E, F, G) Growth factors binding to fibrinogen fragments. A signal significantly greater than 0.1 (gray box) was considered representative of a relevant binding. Reproduced with permission [63].

Figure 5.

A) Schematic Michael-type addition reaction between vinyl sulfone-functionalized multiarm PEGs and mono-cysteine adhesion peptides (step 1, in high stoichiometric deficit) or bis-cysteine MMP substrate peptides (step 2, to come up to stoichiometric equivalence) to form cell-laden gels that locally respond to local protease activity (step 3). B) MMPs degrade the hydrogel crosslinks (1), liberating polysaccharide-bound rTIMP-3, which inhibits local MMP activity (2), and attenuates further hydrogel degradation (3). C) Hydrogels with (filled symbols) and without (open symbols) encapsulated rTIMP-3 (10 μ g per 50 μ l gel) were incubated with (squares) or without (triangles) 20 nM rMMP-2. rMMP-2 was refreshed every two days to maintain enzyme activity (indicated by green arrows). Encapsulated rTIMP-3 attenuated rMMP-2-mediated hydrogel degradation, confirming activity of rTIMP-3 across the 14-day study. Adapted with permission [69, 134].

Figure 6.

A) Schematic depiction of the fabrication of multicellular microtissues using a thermoresponsive mold. B) Two-step approach to generate a PDMS mold that combines micro-features with a nano-topography on which cells orient themselves in the direction of the nanopatterns. C) Fabrication of free-standing fibronectin nanofabric. Using a PDMS stamp, fibronectin is patterned on PIPAAm coated glass. After dissolving of the PIPAAm layer, an assembled fibronectin matrix is released. By using multiple PDMS stamps, different micro-architectures can be prepared. Within the fabric, the fibronectin remodeled into nanoscale nodules with a diameter of approximately 50 nanometer, resulting in an additional nanotopography. Scale bars 40 μ m (top right), 1 μ m (bottom middle), 100 nm (bottom right). Adapted with permission [83, 85, 88].

Figure 7.

A) Fabrication of patternable hydrogel micro-units using a photomask to spatially control the photopolymerization of a prepolymer solution. Micromaterials could be assembled using a lock-and-key principle (scale bars 200 μm). B) Schematic depiction of dynamic optical projection stereolithography using a digital micro-mirror device (DMD) to solidify a photosensitive liquid prepolymer using a patternable light source. This approach allows for the fabrication of more complex 3D objects containing cells, which were visualized with phalloidin. C) Two-photon lithography further increases the resolution of photolithography in two dimensions. Objects can be fabricated at a resolution close to the subdiffraction-limit of 120 nanometer (middle, scale bar 2 μm). By combining this high degree of architectural flexibility with different materials that are either protein repelling or protein binding, this technology can be used to precisely control cell adhesion and consequently cell shape in 3D (right). Adapted with permission [89, 91, 95, 97].

Figure 8.

A) Microfluidic flow focusing for the fabrication of droplets. This is a highly flexible method that can be used for the mass production of particles mainly in the micrometer range. By tuning the processing parameters, droplets containing multiple internal phases (left, scale bar 100 μm) or droplets having two distinct surface phases can be prepared (right). B) Microfluidic flow focusing for the production of fibers. By incorporating multiple inlets and valves, the system is both capable of serial and parallel variation (middle, scale bar 1 mm). Using this variation, multicellular structures can be prepared (right, scale bar 100 μm). C) Combination of microfluidics with photolithography to extend the variety of shapes of particles that can be produced (left). This method was later optimized by including a valve in the prepolymer's channel that stops the flow exactly when the pre-polymer is illuminated. This combination enables the fabrication of micromaterials with elaborate 3D features (right, scale bar 50 μm) Adapted with permission [103, 104, 106, 107, 109].

Figure 9.

A) Schematic representation of the electrospinning process. Fibers are drawn by the flow of a viscoelastic polymer that is subjected to an applied electric field between an injecting needle and a collector plate. B) Nano- or microscale surface topographies can be created by adapting process parameters to create e.g. nanogrooves (left) or nanoporosity (right) on the fibers. C) Shaped collectors can be used to prepare amongst others branched tubular scaffolds (top right, scale bars 5 mm). By also including protrusions on the collector surface, an additional micro-architecture can be added to the meshes (bottom right, scale bars 100 μm) Adapted with permission [115, 116, 121].

Figure 10.

A) Schematic representation of thermo-responsive polymer brush mediated creation and release of cell sheets by adjusting polymer density system. B) Microgels can be assembled by employing electromagnetism to form macrosized hydrogel constructs. C) DNA can act as a biological glue to assembled hydrogels are an orthogonal chemical system for generation of complex 3D systems. D) 3D printed micro-droplets can form autonomous systems that change morphologies over time. Adapted with permission [122, 125, 127].