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Bioactive polymeric scaffolds for tissue engineering

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ABSTRACT

A variety of engineered scaffolds have been created for tissue engineering using polymers, ceramics and their composites. Biomimicry has been adopted for majority of the three-dimensional (3D) scaffold design both in terms of physicochemical properties, as well as bioactivity for superior tissue regeneration. Scaffolds fabricated via salt leaching, particle sintering, hydrogels and lithography have been successful in promoting cell growth *in vitro* and tissue regeneration *in vivo*. Scaffold systems derived from decellularization of whole organs or tissues has been popular due to their assured biocompatibility and bioactivity. Traditional scaffold fabrication techniques often failed to create intricate structures with greater resolution, not reproducible and involved multiple steps. The 3D printing technology overcome several limitations of the traditional techniques and made it easier to adopt several thermoplastics and hydrogels to create micro-nanostructured scaffolds and devices for tissue engineering and drug delivery. This review highlights scaffold fabrication methodologies with a focus on optimizing scaffold performance through the matrix pores, bioactivity and degradation rate to enable tissue regeneration. Review highlights few examples of bioactive scaffold mediated nerve, muscle, tendon/ligament and bone regeneration. Regardless of the efforts required for optimization, a shift in 3D scaffold uses from the laboratory into everyday life is expected in the near future as some of the methods discussed in this review become more streamlined.
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1. Introduction

Bioactivity refers to the capability of a material to affect its biological surroundings. Langer and Vacanti first defined the term “tissue engineering” in the 1990s. Since then, three-dimensional (3D) structured, biomaterial-based scaffolds have traditionally been used to provide a bioactive environment in which cells adhere and proliferate [1]. This opened up avenues for tissue regeneration; with researchers hypothesizing that scaffolds could potentially provide structural stability and environment for cellular regeneration thus mimicking native tissue in functionality. Since then, 3D scaffolds have been evaluated for a wide variety of applications ranging from bone regeneration, nerve regeneration, muscle regeneration, tendon/ligament regeneration, and much more [2–4]. To accomplish these scaffolds, synthetic and natural polymers have been popular biomaterials due in large part to their vast diversity of properties and bioactivity [5–7]. Natural polymers were among the first biodegradable scaffold materials to be used clinically, due to their better overall interactions with various cell types, and lack of an immune response. However, synthetic polymers were later realized to be cheaper and allow for better functionality than natural polymers, despite the potential for an immune response or toxicity especially with the use of certain polymer combinations [8]. Among the synthetic polymers, poly(L-lactic acid) (PLLA), poly(glycolic acid) (PGA), poly(caprolactone) (PCL) and poly(lactic-co-glycolic) acid (PLGA) are currently the

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most popular for the creation of 3D structures in the form of scaffolds [9–11]. These polymers are also used in combination with natural polymers to improve untoward issues associated with hydrophilicity, cell attachment, and biodegradability. Moreover, the scaffold surfaces are functionalized using specific ligands such as protein molecules that help enhance cellular responses.

Using synthetic and natural biomaterials, 3D scaffolds such as nanofibers, hydrogels, and sintered microparticles have been explored widely [8,12]. These 3D, highly porous scaffolds are used to generate a local bioactive environment upon implantation to regenerate the damaged or lost tissue. Within a 3D scaffold, porosity has been shown to be an important determining factor for second-generation tissue engineering, which places a stronger emphasis on the need for vascularization and cellular ingress into the pores within the scaffold [13]. Previous studies have shown that cellular growth and attachment are largely dependent on both the size and density of the pores within a scaffold, which must be carefully manipulated to particular parameters depending on the material and application [14,15]. One of the main reasons why porosity is important is because cellular networks rely on interconnected pathways for nutrient transportation, cell signaling, and proliferation, mimicking the native extracellular matrix (ECM) environment in structure. However, the porosity and in turn surface to volume ratio of a scaffold should be not so large that it weakens its mechanical strength [16]. The 3D scaffolds for tissue engineering applications face two major constraints - a scaffold can neither be too porous (due to compromised mechanical strength) nor lack porosity significantly (due to lack of cellular ingress, vascularization, and signaling). This tradeoff is highlighted in Fig. 1, which demonstrates that greater cellular infiltration correlates with greater porosity and the porosity is generally inversely proportional to the mechanical strength of a given scaffold. This tradeoff is in general one of the fundamental concepts of tissue engineering and must always be taken into consideration during biomaterial fabrication.

From very small beginnings of their use, 3D scaffolds have exploded in popularity, as researchers worldwide have attempted to maximize their potential. Over time, a number of different guidelines have been established for the creation of 3D scaffolds. Currently, a 3D scaffold is expected to demonstrate a minimum of the following three characteristics for functionality room for cell and nutrient transportation and adhesion, mechanical properties suitable for the intended application, and biocompatibility in order to prevent an unwanted immune response [17].

A major advantage of the use of 3D scaffolds for tissue engineering is the ability to functionalize for mechanical strength, degradation rate, and cellular adhesion. There are a variety of different techniques to functionalize a scaffold, most of which depend on the material in question. Surface modification to add necessary molecules for cell proliferation has been used with many different polymeric materials. For instance, copolymerization using the same monomers has been used to functionalize polymeric scaffolds to promote cell adhesion to overcome the limitations of parent polymer [18]. Another advantage in terms of functionality lies in the fabrication technique, as 3D printing, electrospinning, microparticles, and hydrogels can all bring about very different mechanical properties, degradation rates, and cellular adhesion.

3D structures created via electrospinning in the form of nanofibers are one of the most widely used scaffold types and have been shown to be particularly useful in mimicking the extracellular environment, due to their high surface-to-volume ratios, excellent mechanical properties, high porosity, and pore size distribution [19]. Also, the diameter and orientation of the fibers in these scaffolds can be manipulated to obtain different cellular responses depending on the application [20]. 3D hydrogel based scaffolds are also used widely due to their unique, stimuli-responsive properties and their unique ability to maintain their original structure well. Moreover, by using hydrogel scaffolds, active agents such as growth factors can be released at a particular required rate, due to their bioactive agent encapsulation abilities [21,22]. In addition to these approaches, scaffolds fabricated using microparticles of PLLA, PLGA, and their blends with natural polymers have been popular due to their ability to reduce the degradation of various encapsulated biological molecules and also the ability to release for extended period of time [23–26].

Relatively new techniques to fabricate 3D scaffolds are decellularization and 3D printing. Decellularization is a process of creation and functionalization of natural 3D scaffolds, which involves obtaining an organ from an animal (of another species), removing all the cells using detergents, and then re-implanting the stem cells from a potential desired host. In order to accelerate differentiation, growth factors may be added to the decellularized scaffold [27]. Recently, several whole organs have been revitalized and cellularized using a donor's stem cells. One popular example is a recellularized heart, which begins to function again, demonstrating success in restoring functionality [28]. In addition to heart several other organs, such as the lungs and bladder, have also been recellularized *in vitro* [29,30]. However, decellularization has been shown to have a number of significant disadvantages, ranging from non-homogeneous distribution of cells, difficulty in retaining the full extracellular matrix, and immunogenicity if there is any trace material left over prior to de-cellularization. Excessive or aberrant immune response needs an immunosuppressant treatment for longer period of time which is risky [16].

Of these techniques, 3D printing has an advantage of having nanoscale precision in the dimensions of the scaffold, over the other traditionally used fabrication methods. Countless bioactive materials have been 3D printed into scaffolds over the years. Even hydrogels have been 3D printed in order to fabricate particular 3D scaffolds [31]. It is theorized that this technique will outperform previous techniques for 3D scaffold fabrication, such as porogen leaching. Later in this article, various aspects of 3D scaffold

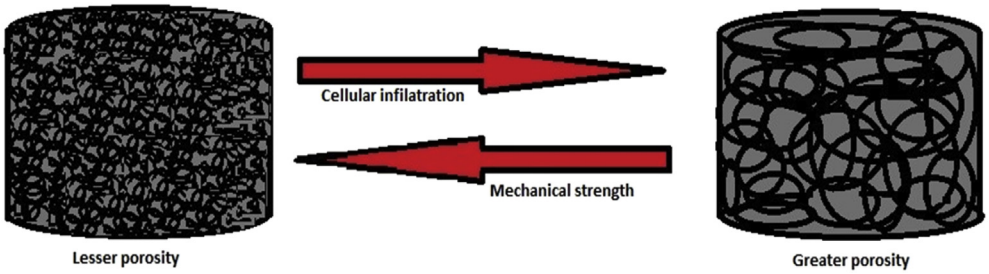

Fig. 1. There is always a tradeoff between mechanical strength and porosity, which must be fine-tuned depending on the tissue in question and the specific application.

fabrication using both synthetic and natural biomaterials are discussed.

2. Synthetic and natural biomaterials for tissue engineering

2.1. Polycaprolactone (PCL)

Polycaprolactone, a well-known polyester material first synthesized in the 1930s, is widely used for the fabrication of 3D scaffolds for tissue engineering applications. PCL is elastic in nature and consists of nonpolar methylene groups and one semi-polar ester group. PCL is used in various forms such as films, fibers, and microparticles. To improve its bioactive properties, it is used in the form of composite materials with other polymers, such as gelatin and chitosan, for various tissue regeneration applications [32–38]. In addition to its applications in tissue engineering, PCL is also used widely for various drug delivery applications, and has obtained FDA approval for a number of different products [39,40]. Many of these uses will be further elaborated upon in the applications section. Due to the fact that PCL is relatively elastic when compared to other polyesters, its mechanical use has been popularized [41]. Its advantages include very high drug permeability, a relatively slow degradation rate, and less acidic byproducts when compared to other polyesters, making it useful for applications within the fields of drug delivery and in recent years tissue engineering [39,42].

Polycaprolactone has been shown to have strong solubility and blend compatibility with other biomaterials [43–45]. However, its relatively slow degradation rate (2–4 years) acts as a disadvantage, making it an unideal scaffold for short term drug and growth factor delivery applications [46]. Furthermore, PCL has poor cellular adhesion properties on its own, without some form of functionalization [47]. Numerous approaches to improve its bioactivity, including copolymerization, surface functionalization, and blend formations have been used to overcome this disadvantage [48,49]. For instance, Chang et al. demonstrated that poly(epsilon-caprolactone)-graft-type II collagen-graft-chondroitin sulfate (PCL-g-COL-g-CS) scaffolds fabricated via particulate leaching and surface modification of PCL can be loaded with type II collagen and chondroitin sulfate to allow for proliferation of chondrocytes *in vitro* [50]. During the 4-week culture period, significant cellular proliferation was observed. Histological staining revealed a significant amount of secreted collagen, an important cellular viability marker. It has been demonstrated that, within a porous PCL scaffold, cells can maintain the exact same phenotype as chondrocytes within native cartilage tissue, demonstrating the viability of porous PCL scaffolds for tissue engineering applications and their ability to mimic the native tissue environment [51]. This finding is important for demonstrating the enormous capacity for functionalization of PCL scaffolds, and overcoming the lack of bioactivity of unmodified PCL.

2.2. Poly (L-lactic acid)

Poly (L-lactic acid) is a synthetic biodegradable polyester formed from the polymerization of l-lactide, obtained from renewable sources such as starch, and has a wide variety of applications such as sutures, drug delivery vehicles, prosthetics, vascular grafts, bone screws, skin regeneration scaffolds, and pins for fixation [52]. One example of an already FDA approved PLLA product is Sculptra™, which is an injectable that is used currently to treat facial atrophy [53]. Poly (L-lactic acid) degrades mostly into nontoxic byproducts and can be easily blended with other materials as well, popularizing its use [54]. While PLLA does have a faster degradation rate compared to PCL via bulk degradation, it is still considered relatively slow in comparison to other polymeric biomaterials used for tissue engineering scaffolds [55]. Furthermore, PLLA has high crystallinity in its degradable fragments, which can lead to inflammation in the body, and therefore is sometimes blended with other polymers to form a 3D scaffold [56]. To resolve this issue and allow for greater bioactivity, it has been demonstrated by Fukushima and Kimura that PLLA can be fabricated as a combination of L-lactic acid and D, L-lactic acid since D, L-lactic acid is degraded more rapidly and lacks the high crystallinity and the associated inflammation [57].

One example of a PLLA composite scaffold for inflammation reduction applications is shown in Fig. 2, which demonstrates the surface morphology of PLLA/Rg3 nanofibrous matrices using SEM microscopy. The fibers represented indicate pure PLLA (a), PLLA with 2%Rg3 (b), PLLA with 6% Rg3 (c), and PLLA with 10% Rg3 (d). The purpose of Rg3, a well-known scar reduction compound, is to both counteract the inflammation associated with PLLA and allow for skin regeneration at a more rapid rate [58]. It can be seen that the fibers are relatively uniform in nature, allowing for cellular infiltration into the pores of the scaffolds. In addition to electrospinning, various other techniques such as 3D printing and solvent casting may be used to obtain blended PLLA scaffolds for enhanced nontoxic bioactivity.

2.3. Poly(lactic-co-glycolic) acid

PLGA is a combination of the polyester polymers PLLA and PGA and is among the most commonly used biodegradable synthetic polymers for tissue engineering applications [59]. The higher the ratio of PGA within a PLGA scaffold, the faster PLGA is expected to degrade. The byproducts of its degradation, lactic acid and glycolic acid, are nontoxic [60]. PLGA has been popularized for a variety of reasons, such as biodegradability, adaptability and customization for different types of formulations, and surface modification for targeted drug delivery [59,61]. One example of an FDA approved PLGA scaffold is Osteofoam™ for bone regeneration applications, which has demonstrated a 3D morphology similar to that of human trabecular bone, and has been shown to allow for cell colonization

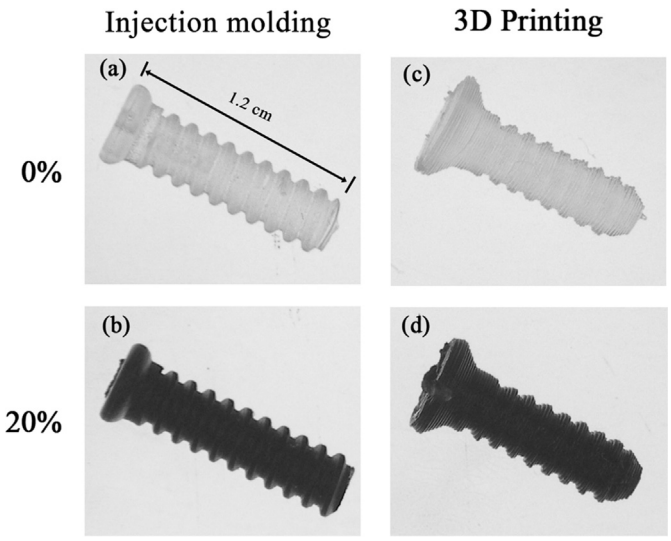

Fig. 2. The surface morphology of typical PLLA nanofibrous scaffolds can be seen using SEM microscopy [58]. The fibers represented indicate pure PLLA (a), PLLA with 2%Rg3 (b), PLLA with 6% Rg3 (c), and PLLA with 10% Rg3 (d). Rg3 is used to enhance the scaffold bioactivity as the compound plays a critical role in scar reduction, making such a material more useful for skin regeneration applications. It can be seen that the fibers are relatively uniform as well in structure, allowing for cellular infiltration of the scaffolds.

[62–64].

Unfortunately, PLGA has one major drawback limiting its bioactivity. Its degradation byproducts are highly acidic and, in large quantities, can be very difficult for the human body to metabolize rapidly [65]. This can particularly be an issue in drug delivery applications, in the presence of acid sensitive drugs. Because of this central disadvantage, some researchers have attempted to negate the effects of the degradation byproducts of PLGA. One traditionally popular method is to simply vary the ratio of PGA: PLLA such that there is greater amount of PGA, leading to a slower degradation rate, and less acidic byproducts all released at once. However, it has been shown recently that in the presence of particular salts, the pH of PLGA byproducts can be increased, thus leading to overall greater bioactivity, particularly in terms of delivery applications [66].

PLGA has been used to fabricate nanoparticles, microparticles, 3D scaffolds for drug delivery and tissue engineering applications, which will be discussed in greater depth in further sections in this article [67–71].

2.4. Silk

Unlike other polymers covered so far, silks are naturally occurring polymeric proteins, extruded from insects and worms. Also, referred to as silk fibroin, the protein component of silk that gives it its biocompatible properties, this biomaterial has made its way from solely textile applications into the tissue engineering field particularly scaffolding. It is particularly of use within the field of tissue engineering due to its notable cellular adhesion properties. While silk must first be cleansed of its secondary toxic protein component in a relatively lengthy procedure, namely sericin, the remaining fibroin component has been shown to possess relatively high tensile strength in addition to biocompatibility. For these reasons, it has been widely used in the form of gels, sponges, and films for cartilage, bone, tendon, nerve and ligament regeneration [72–78].

To obtain properties such as elasticity, fixed degradation rates, and porosity, silk composites have been used commonly. For instance, chitosan and silk fibroin nanofibers for wound dressing applications are quite common and are fabricated via electrospinning [79]. Other composites, such as silk and hydroxyapatite for wound dressing applications, have been created by adding hydroxyapatite powder to silk fibroin and gelatinizing the compound [80]. To fabricate 3D scaffolds of silk biomaterials, freeze drying, electrospinning, and 3D printing has been shown to be an effective technique [73,81,82]. Fabrication techniques and applications using silk based biomaterials will be discussed in detail in the later sections.

2.5. Collagen

There are approximately twenty-nine known types of collagen, which all possess different characteristics, and can be extracted from almost every species on the planet including humans. Collagens are present in extracellular matrix and in bones in the form of fibers or gels to support the tissues [83]. Because of its abundant nature and its role in growth and support of organs, they have been used as 3D scaffolds for a wide variety of tissue engineering application: applications ranging from hard tissue such as bone, to soft tissue regeneration such as cartilage, vasculature, and nerves [84–89]. Various types such as collagen I, II, III, V, and XI have been tested for tissue engineering applications. Out of these, type I collagen has been described by many scholars as the “gold standard”, because of the lesser immune reactivity that is associated with it [83]. In addition, there is a very small difference in collagen characteristics across different host species, that could lead to a potential non-intended differences within certain fabricated scaffolds [90]. Furthermore, collagen scaffolds can be relatively difficult to synthesize without significant alterations to the integrity of their intended structure, as is unfortunately sometimes the case with proteins [83]. Collagen scaffolds usually display a relatively rough surface morphology, contributing to its fibrous nature and structural porosity of the sample. While Fig. 3A shows the gross view of a typical collagen scaffold and its dimensions, Fig. 3B shows the surface and cross sectional view. The porosity with an average pore size of about 80 μm is viewed using SEM microscopy techniques. The figure also highlights the rough surface morphology often associated with collagen. The scaffolds were fabricated by freeze drying technique in order to create the porous structure shown.

As far as fabrication techniques go, aligned fibrous collagen scaffolds are created via standard electrospinning technique and it has been shown that rabbit conjunctiva fibroblast cells proliferate more rapidly on aligned collagen fibrous scaffolds than on random collagen fibrous scaffolds, emphasizing the role of fabrication technique used [92,93]. Porous collagen-based scaffolds are also created via solvent casting-particulate leaching, phase separation, gas foaming, emulsion freeze drying, and fiber meshes. Many of these methods don't allow for cellular adhesion properties due to change in surface morphology. Thus solid freeform fabrication has been used and is shown to be more effective in terms of cellular adhesion [94]. Because cellular attachment on collagen scaffolds is highly dependent upon the surface morphology of the biomaterial, it is important to carefully consider the fabrication method used and its optimization.

2.6. Hyaluronic acid (HA)

Hyaluronic acid is a type of non-adhesive glycosaminoglycan, natural biodegradable material found mostly in connective, epithelial, and neural tissue [95]. Scaffolds of HA are used for both hard and soft tissue regenerations, which will be elaborated upon in future sections. One frequent use of HA for tissue engineering applications, however, is in the form of hydrogel material. This is due to its swelling capabilities, and ability to encapsulate cell and other materials for delivery applications.

Cellular viability on HA hydrogel structures is shown in Fig. 4. This figure demonstrates the cell viability on HA scaffolds indicated by the secretion of specific cellular viability markers important for proliferation, namely collagen and aggrecan. Staining for collagen and aggrecan increased and the increase is dependent upon the concentration of crosslinking agent used for the hydrogel material. Towards the edge of the constructs, interestingly, both collagen and aggrecan were highly concentrated than towards the center. Even though the mechanical properties needs adjustment via chemical modification to resemble the native tissue, HA has similar physical and biological functions [96–98].

In addition to their role as hydrogels, there are also less common methods to fabricate HA scaffolds. HA scaffolds may be fabricated via electrospinning to form a fibrous scaffold [99] or blended with other biomaterials in order to create porous scaffolds via leaching of salt particles [100]. More recently “wet spinning”, a novel spray-assisted, layer-by-layer type of assembly technique has been used to deposit various polyelectrolyte films onto porous HA scaffolds, allowing for greater cellular adhesion and proliferation of human keratinocytes *in vitro* [101]. Lastly, a promising technique for the fabrication of HA scaffolds appears to be 3D printing, which allows for microscale precision of the scaffold parameters, potentially leading to greater cellular performance in animal models [102]. The popularity of HA is most likely

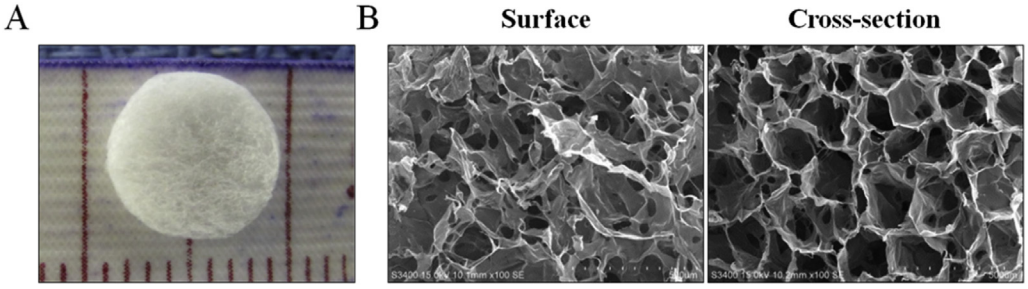

Fig. 3. A typical collagen scaffold is shown above, where (A) is the scaffold with 8 mm diameter and 2 mm thickness and (B) is a surface view (left) via SEM technology and a cross sectional view (right) [91]. Scaffolds have an average pore size of about 80 μm and were fabricated with the use of a lyophilizer in order to create the porous structures shown. The rough surface morphology associated with many collagen type II scaffolds can be seen.

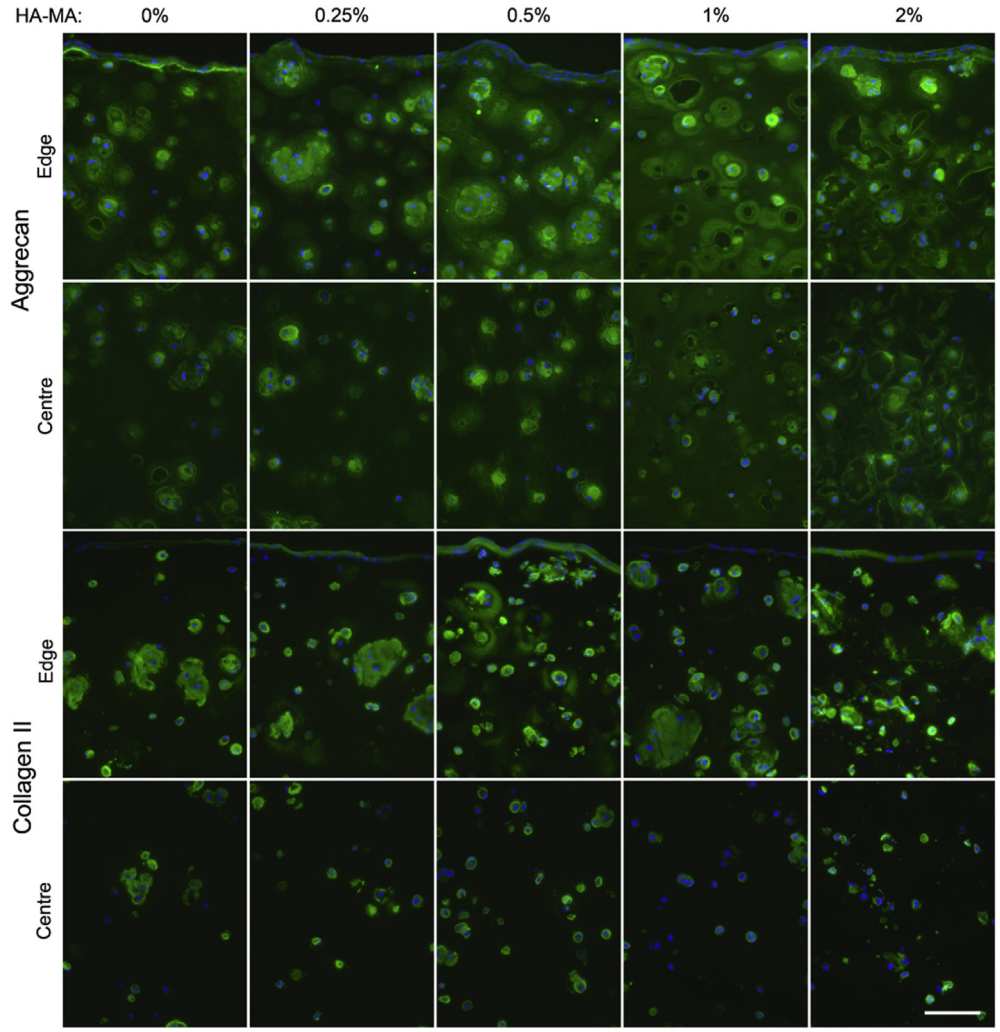

Fig. 4. Cellular staining for collagen type II and aggrecan markers demonstrates cellular viability on photo-crosslinked HA scaffolds *in vitro* for cartilage tissue engineering applications [103]. Collagen type II and aggrecan are important byproducts for demonstrating cellular viability in many applications. One can observe the secretion of collagen and aggrecan markers as the amount of crosslinking is increased throughout the scaffold. It can also be observed that collagen type II and aggrecan markers are more concentrated at the edge of the constructs rather than towards the center.

in part due to the wide variety of fabrication techniques, paving the way for the biomaterial to be used for nearly any tissue regeneration application.

2.7. Chitosan

Chitosan is a linear biodegradable polysaccharide derived from the partial deacetylation of chitin via chemical hydrolysis. 3D chitosan scaffolds have demonstrated functional properties as well as structural characteristics similar to that of glycosaminoglycans, naturally occurring in the human body as a lubricant. This holds testimony to its bioactivity – as well as that it naturally promotes cellular adhesion without any further functionalization unlike a few biomaterials already discussed [104–106]. Hence, 3D scaffolds of

chitosan alone or in combination with other natural polymers are used in the gel, sponge, or fiber forms for numerous tissue engineering applications [7,107]. Chitosan as a biomaterial has limited solubility at physiological pH which is advantageous for its use for an extended period of time [108,109]. Thus, chitosan is believed to be a viable biomaterial for tissue engineering applications in the form of 3D scaffolds. Moreover, because of its multifunctional structure and ability to crosslink, chitosan is often blended with other biomaterials to modify properties of the scaffolds. An SEM image of composite 3D scaffold of fabricated chitosan-collagen microparticles is indicated in Fig. 5. It can be noticed that these scaffolds exist in a uniform porous structure, with suitable mechanical stability. These structures also show that blending of chitosan with other well accepted biomaterials will result in scaffolds with desired bioactive properties. For instance, chitosan with HA has been shown to have exceptional structural performance, with a positive cartilage staining of collagen type II and GAG [110]. Also, chitosan/PCL scaffolds used for tissue engineering applications, show that bovine articular chondrocytes attach and proliferate after twenty-one days *in vitro* on such 3D scaffolds [111]. For nerve regeneration applications, it has been demonstrated that collagen-chitosan scaffolds carrying RGD, stimulated linear axonal growth in rats with 15 mm long nerve defects after four months [112]. These applications will be further discussed in greater detail in future sections.

3. Types of bioactive 3D structures

3.1. 3D printed structures

The recent advancement of 3D bio-printing has revolutionized the field of tissue engineering. Using different complex algorithms,

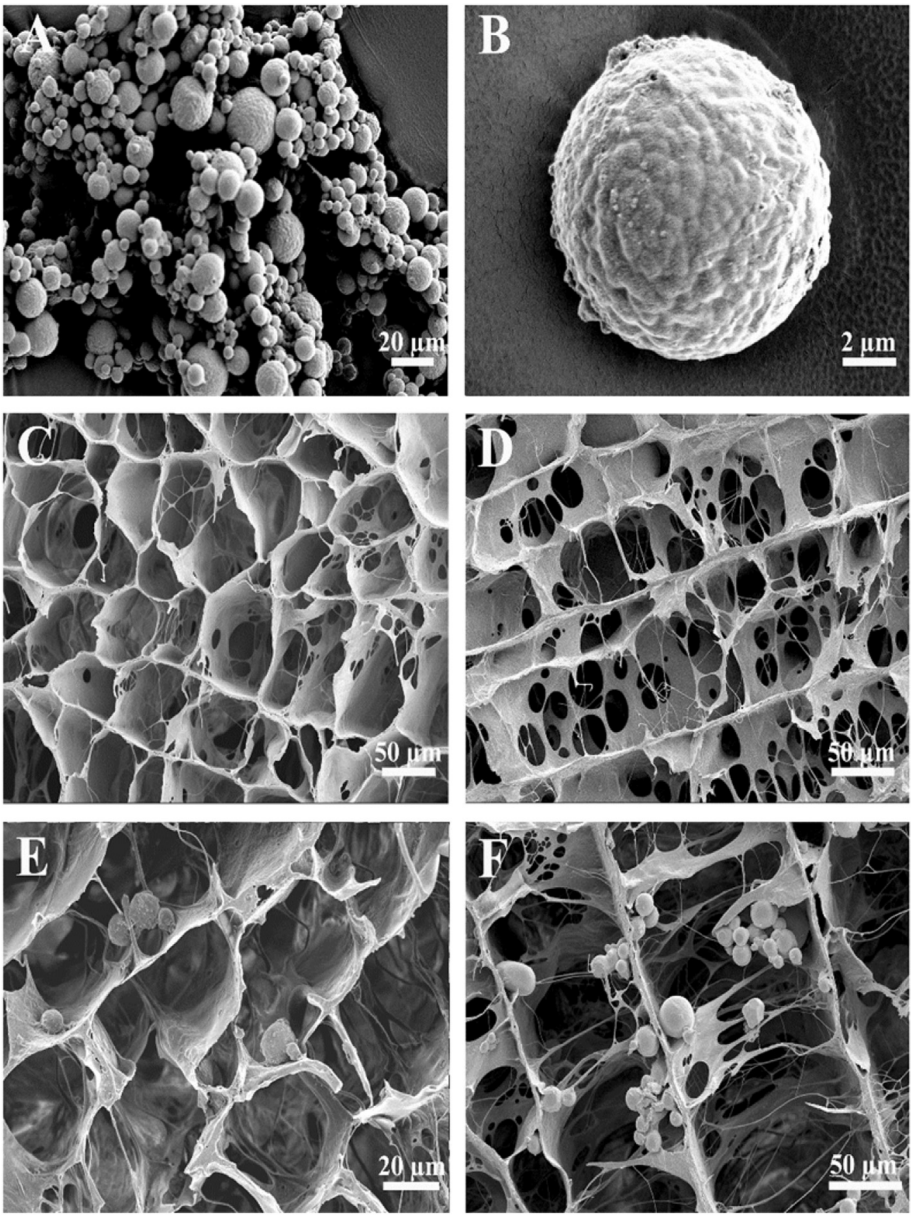

Fig. 5. A typical chitosan-collagen composite microstructure is shown above using SEM technology [113]. Part (A) is a zoomed out image of the structure, while (B) demonstrates the spherical nature of the microparticles. Part (C) displays the honey-bomb structure, while parts (D), (E), and (F) display the unique orientation of the micro-channels throughout the structure, which are formed by interconnected microparticles.

a two-dimensional image on a computer screen can be used to fabricate a 3D equivalent in real-time. When a biocompatible, 3D scaffold has to be printed, there is a series of different steps involved [114]. Precision ranges from millimeter to nanoscale, and the greater the precision the higher is the cost. Fig. 6 shows the precise similarities between an original scan and a 3D printed model. Using only an MRI scan in Part (A), a digitally rendered image is constructed in Part (B) using specialized software. The final version is shown in Part (C). The finely interconnected lobes are demonstrative of the excellent precision that 3D printing gives way to.

Similarly, several different tissue types have already been fabricated and transplanted via 3D printing, ranging from multi-layered skin, bone, vascular grafts, tracheal splints, heart tissue, and cartilaginous structures [116]. More complex geometrical parameters can now be fabricated using 3D bio-printing– a major advantage over traditional techniques such as porogen leaching. Furthermore, it offers a much more streamlined approach for enhanced productivity at a potentially more cost-effective rate [117]. Three dimensional printed scaffolds have been fabricated and applied for both hard and soft tissue engineering applications such as bone, cartilage, nerve, and muscle to name a few [118–121]. It has been demonstrated, for example, that human chondrocytes which are bio-printed onto a nano-cellulose alginate bioink, show a viability of 73% after 1 day and 86% after 7 days of culturing cells on the scaffold *in vitro*, demonstrating the viability of 3D printed scaffolds for tissue engineering applications [119]. Similarly, collagen scaffolds can also be 3D printed, and it was demonstrated that hMSCs display attachment and proliferation *in vitro* over a four week period on 3D bio-printed collagen scaffolds with predefined capillary networks [122]. While it is worth pointing out that 3D printing technology has fabricated such scaffolds with cell viabilities similar to those obtained by traditional methods, there are drawbacks associated with 3D printing technique. For example, the biological material must be transformed into liquid form in order to print the droplets and the difficulty in achieving biologically relevant cellular densities [116]. As stated earlier, one of the major advantages of 3D printing for tissue engineering applications is that it allows for extreme precision in the spatial parameters of networks.

3.2. Nanofibers

Nanofibers have been popularized primarily due to their ability to mimic the native properties of the extracellular matrix on such a small precision scale, as shown in Fig. 7 [123,124]. Fig. 7 shows a uniform nanofibrous matrices. Part (a) of the figure demonstrates the very fine diameter from a close-up view, showing the uniformity of individual fibers. Parts (b) and (c) show a zoomed-out view, where the uniform nature of fibers can still be visualized. Usually fabricated via electrospinning, such fibers are frequently used as strong reinforcements in nanocomposites [125,126]. In addition to their ability to mimic the native extracellular matrix, nanofibrous scaffolds also display a higher surface-to-volume ratio, leading to greater cellular attachment than larger fibers [127]. Moreover, nanofiber reinforced composites have been shown to have greater mechanical strength than traditional unfilled or carbon/glass fiber filled composites, adding a major advantage to scaffolds which require high mechanical strength [128].

To fabricate nanofibers variety of materials, such as PCL, and chitosan, to name a few have been used [38,130–133]. Such fibers can either be in an aligned or random orientation. Aligned nanofibers have very specific applications, and can be used to specify the direction of tissue growth. It has been reported that neurites obtained from the dorsal root ganglia explants, when seeded onto electrospun PLA nanofibers in a uniform alignment, have been shown to grow outward from the ganglia in the direction of the fibers *in vitro* [134]. Also, it has been shown that poly(vinyl alcohol) random fibrous scaffolds with chondroitin sulfate enhanced mesenchymal stem cell chondrogenic differentiation *in vitro*, and

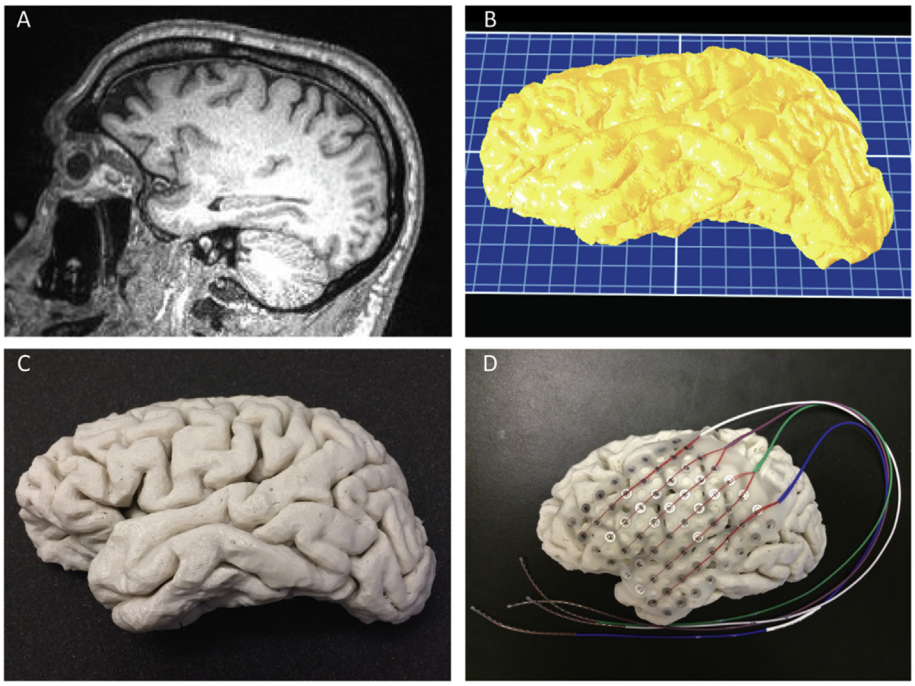

Fig. 6. A 3D printed brain model is shown [115]. Part (A) shows a sagittal view of the brain from an MRI scan, (B) shows the rendered digital image, (C) shows the 3D printed result, and (D) shows electrical stimulation of the model. The model can be electrically stimulated *in vitro* for cell studies, albeit being able to replicate the functionality of the human brain in its entirety is still a very long way.

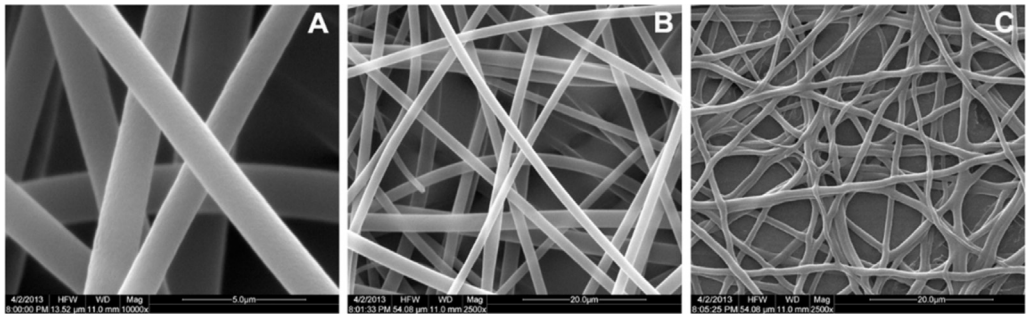

Fig. 7. Nanofiber morphology via SEM is shown [129]. Part (A) shows the superfine diameter of the fibers, while (B) and (C) demonstrate the morphology before and after dipping in aqueous solution respectively. Polystyrene (PS) is used as a material. Through inspection, it can be visually noted that the fibers are of a uniform diameter, which is important for cellular compatibility.

enhanced proliferation in osteogenic defects *in vivo* in rats, demonstrating that nanofibers may be useful for cartilage tissue engineering applications [135]. One major advantage of random fibers, however, is that mechanical properties can be altered for improved stiffness and resistance in all directions when subject to mechanical testing [136–139]. This is a major advantage over aligned nanofibers, which only exhibit strong mechanical properties in the direction of the fibers. Thus, the fine tuning of mechanical properties is of utmost importance when fabricating nanofibrous scaffolds, creating a general rule of thumb: while aligned, fibers exhibit greater strength in one direction and therefore are more useful for tendon and ligament regeneration applications, random nanofibers exhibit similar mechanical properties in all directions and therefore are more useful for skin and cartilage regeneration applications.

3.3. Microparticles

Microparticles, initially developed as carriers for anti-cancer drugs, have now made their way to the field of tissue engineering. What is so unique about microparticles is that they are able to deliver growth factors or soluble drugs in a slow, controlled fashion, and can be manipulated to provide for some degree site-specific targeting [140]. Due to these specific properties, when embedded within 3D scaffolds, it is has been shown that there can be a steady, cumulative release of growth factors or drugs [141].

In addition to their role as delivery vehicles when embedded within a 3D scaffold, microparticles also have a number of other applications and have been especially popularized as injectable scaffolds, demonstrating overlap with drug delivery applications. Chitosan microparticles crosslinked with genipin and seeded with goat marrow stromal cells (GBMCs) in a 3D construct were shown to provide feasibility as injectable scaffolds after evaluation of cellular viability at 7 and 14 days *in vitro*, paving the way for injectable applications [25]. More recently, injectable microspheres have been demonstrated *in vivo* in rats. Lovastatin microparticles in combination with polyurethane (PUR) scaffolds resulted in a sustained release of lovastatin over a period of 14 days, and stimulated BMP-2 growth factor expression in osteoblast cells in defects [142]. Porous PLLA microparticle scaffolds containing PVA and treated with serum demonstrated better cellular adhesion properties than other types of 3D microparticle scaffolds *in vitro* [24]. Such findings indicate that scaffolds based upon microparticles provide a unique overlap between tissue engineering and drug delivery applications.

Atomizing, spray drying, and sintering have all been traditionally used to fabricate and functionalize microparticles [143]. Oil-in-water dispersion techniques have been relatively common for fabrication of microparticles, as well [144]. A very common functionalization method today is perhaps laser sintering, which allows for further bioactivity on the surface of the microparticles, paving the way for the attachment of various molecules as well as cellular adhesion properties [145]. *In vitro* tests demonstrate that sintered 3D chitosan/PLGA microspheres are useful for bone tissue engineering applications as MC3T3-E1 osteoblast-like cells adhere and proliferate on the surface of the material [146].

3.4. Hydrogels

Hydrogels, fabricated using either synthetic or natural polymers, are water-absorbing polymeric materials that are largely hydrophilic and highly flexible. Hydrogels have a number of advantages that make them popular for 3D scaffolds for tissue engineering applications, such as structural similarities to native ECM and potential for drug or growth factor delivery in a non-invasive manner [147]. However, hydrogels are usually quite mechanically weak due to their massive water content (up to 90% sometimes) and are inefficient with loading cells, therefore requiring further modification to make it more bioactive [148].

A number of different synthetic and natural materials can be used in the form of hydrogels, with HA and PEG being two examples already discussed in previous sections [98]. Other examples of natural polymers include collagen, gelatin, fibrin, alginate, and agarose [22,149,150]. Synthetic polymers that can be used in the form of hydrogels include poly(acrylic acid) (PAA), poly(ethylene oxide) (PEO), PVA, polyphosphazene, and various polypeptides [151]. Hydrogels have been fabricated using both physical and chemical techniques. Warming/cooling a polymeric solution to form a gel, crosslinking in aqueous solution, lowering pH in aqueous solution, mixing solution to form a coacervate gel, gelling a polyelectrolyte solution with a multivalent ion of opposite charge, and crosslinking certain polymers in a solid state with different kinds of radiation and/or chemical crosslinkers are among the most common techniques for hydrogel fabrication [148].

One relatively recent application of these hydrogels is cell encapsulation, which provides a hydrated environment for cells to proliferate. Degradation of such an encapsulating hydrogel has been shown to be dependent upon segments on the hydrogel itself, with the use of natural biopolymers which can be degraded by enzymes [152]. Hydrogels can also be functionalized with peptides like RGDS for additional bioactivity, which have been shown to allow for cellular attachment capabilities far beyond that of a typical non-functionalized hydrogel scaffold [153]. Similarly, hydrogels have also been used to encapsulate growth factors for drug delivery applications, which can be slowly released over time [150].

Thus, hydrogels are similar to microspheres discussed in the

previous section in that they are commonly used as encapsulation materials thus having tremendous overlap with drug delivery. However, they are more commonly used for loading cells rather than being embedded within a scaffold for the release of growth factors or other secondary materials.

4. Applications

4.1. Nerve regeneration

Nervous system is divided into central nervous system (CNS) and the peripheral nervous system (PNS). It is important to differentiate between the two, in that CNS consists of the brain and spinal cord, and the PNS consists of the ganglia and nervous tissue outside of the CNS. Nerve regeneration applications in tissue engineering have traditionally dealt with both systems [154–156]. There are a number of different challenges that have yet to be addressed, however, such that *in vivo* models don't hold up well because the nerve axons in mice are smaller and shorter than those in humans, and atrophy in target tissues makes functional recovery difficult to achieve [156]. Because of this, further *in vivo* testing on large animal models similar to humans is necessary in order to bridge the gap between what is theorized and what is demonstrated.

Schwann cells are at the forefront of examination in PNS regeneration applications, as they provide structural support and contribute to myelination of axons. Thus, 3D scaffolds used for nerve regeneration applications must be bioactive in that they promote myelination, outgrowth, and structural support for axons via Schwann cells. Of the 3D structures mentioned earlier, nanofibers appear to play an important role in nerve regeneration, particularly when dealing with nerve guidance conduits (NGCs), designed to guide the axonal outgrowth of neurites. NGCs have been reported to have multiple uses, such as the ability to present multifunctional properties aiming to direct the growth of axons from one proximal nerve end, to secrete growth factors aiding in regeneration of tissue, and in the reduction of inhibitory scar tissue at the site of injury [157,158]. Fig. 8 demonstrates a digital image of a typical NGC. Part (A) shows a digitally constructed image of the NGC, while part (B) shows the two-severed nerve ends and part (C) demonstrates the typical placement of the NGC, joining the severed nerve from both ends. From visual inspection, it can be seen that the NGC is intended to bridge the nerve gap between the two broken ends and allow for outgrowth of the nerve itself. This has made recovery from nerve injuries much more likely in recent years.

In the laboratory setting, NGCs are frequently subject to novel customization. Xie et al. reported that a novel combination of both aligned and randomly orientated electrospun PCL nanofibers in a double layer is useful in guiding the outgrowth of neurites *in vitro* when pre-seeded with Schwann cells, and has allowed for moderate functional recovery in a 14 mm rat sciatic nerve injury model *in vivo* [160]. This novel combination of bilayer NGCs, composed of randomly orientated nanofibers on the outside layer and aligned nanofibers in the inside layer, were also observed during surgical procedures to be much more tear-resistant when compared to traditional NGCs composed solely of traditional, aligned nanofibers. Such scaffolds that are much more robust and tear resistant *in vivo* demonstrate their real-world capability. Interestingly enough, while nanofibers still remain highly popular for NGCs, it was discovered recently that protein films comprised of blended silk fibroin and coated human tropo-elastin protein demonstrate a significant amount of neurite extension, as well as Schwann cell area growth *in vitro* [161]. This novel biodegradable scaffold obtained both a robust-like biomaterial component from the silk and an improved 2.4-fold increase in neurite extension capabilities from the tropo-elastin protein in comparison to standard poly-d-lysine film coating. NGCs are often also functionalized with proteins such as collagen or laminin, which further stimulate the outgrowth of nerve axons and improve nerve functional recovery as well [162].

Recently hydrogels have also been employed as scaffolds for nervous system regeneration, as findings suggest that scaffolds of this nature also may play a role in aiding Schwann cell based axonal recovery. Tseng et al. recently demonstrated that chitosan-based hydrogels (1.5 kPa stiffness) containing proliferating and differentiating neuro-progenitors can be injected *in vivo* for the regeneration of the central nervous system using a zebrafish injury model [163]. The novelty of this study comes from the fact that this is one of the few instances in which a self-healing hydrogel, which is expected to gain major popularity in coming years, has been shown to be effective *in vivo* for nerve regeneration applications. Silk fibroin-based hydrogels are unfortunately still rarely used for nerve regeneration applications, despite the fact that they have shown little to no cytotoxicity and allow for significant nerve regeneration when used with Schwann cell cultures *in vitro* [164]. Much of the success of the hydrogel scaffold has to do with stiffness. It has been reported that culturing neural stem cells on softer hydrogels allows for differentiation into astrocytes and neurons, while stiffer hydrogels tends to allow for differentiation into oligodendrocytes [165]. For these reasons stiffness is usually emphasized, as was the case in the research done by Tseng et al. mentioned earlier.

4.2. Bone regeneration

Synthetic polymeric scaffolds for tissue engineering applications are starting to eliminate the need for bone grafts, which have traditionally been used to treat osteogenic defects [166]. Recently, there has also been a move towards nanostructured materials for bone regeneration, due to their ability to create reactions at the cellular level [167]. Among the most popular scaffold materials for bone regeneration are hydroxyapatite, beta tricalcium phosphate (β-TCP), and bioactive glass, due to structural similarities [168]. The advantages of inorganic materials like these appear to be a great deal of compressive strength and osteoconductivity potential [169].

These materials are often blended with other biodegradable polymers for greater bioactivity. Recently a new type of blended scaffold was presented as Zhang et al. demonstrated that porous nano-hydroxyapatite/PCL spiral-structured scaffolds (1:4 ratio), fabricated using a modified salt leaching technique and seeded with human fetal osteoblasts (hFOBs) over a 14-day period, significantly increased the amount of mineralized extracellular matrix material. Bone mineralization markers ranging from bone sialoprotein (BSP), osteonectin (ON), osteocalcin (OC), and type I collagen were measured using reverse transcriptase polymerase chain reaction analysis [170].

Grain size is one of the most important aspects of scaffold in tissue engineering for bone regeneration. Grain size refers to the size of each individual fragment of material or “grain”. Smaller grain sizes are shown to be more favorable with respect to cellular attachment and proliferation, and differentiation of most osteogenic lineages [171]. The causes of this phenomenon are disputed and unclear, since studies have failed to demonstrate the mechanisms involved. Nonetheless, due to the observed cell attachment profile, fabrication processes are often performed to produce smaller grain sizes. Grain size has also been demonstrated to be dependent upon sintering temperature using hydroxyapatite scaffolds. A temperature of 1325 °C was found to be an ideal sintering temperature in order to achieve an ideal, smaller grain size without compromised porosity [172]. Such findings are a significant

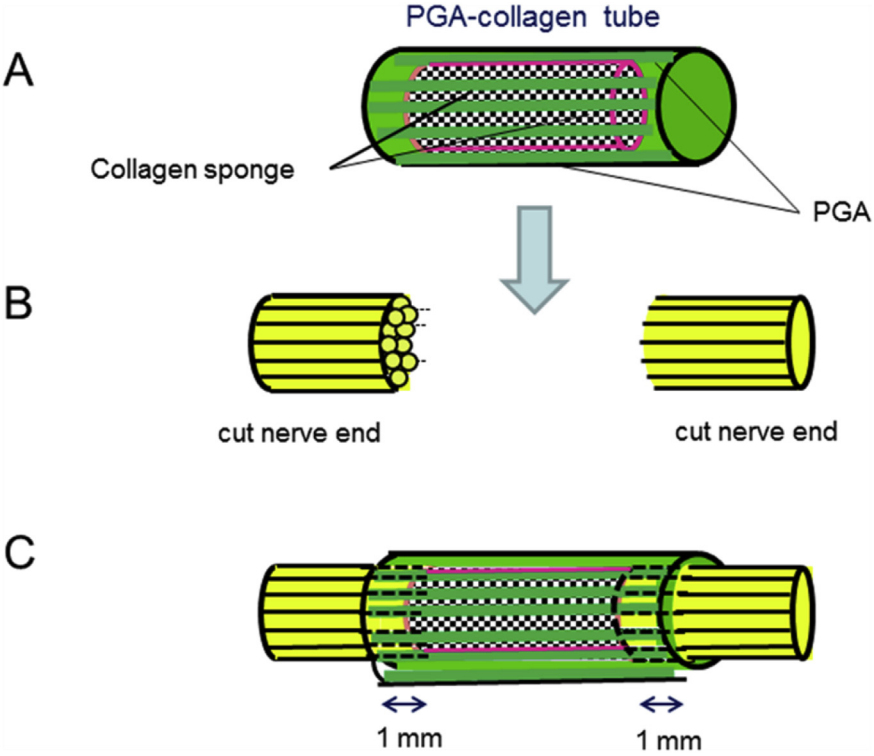

Fig. 8. A typical PGA/Collagen NGC is shown above [159]. Part (A) shows a digital image of the conduit, with (B) and (C) demonstrating how it fits into a nerve gap. The broken nerve has two cut ends and the tube fits directly between them, allowing for further outgrowth toward the gap itself.

breakthrough for researchers who desire a more standardized procedure for sintering these biomaterials.

Apart from grain size, another critical factor for the regeneration of bone is mechanical strength that mimics native tissue. For this reason, polymeric materials with high mechanical strength are often used as scaffolds for bone tissue engineering applications, such as silk protein, PLLA, chitosan nanofibers, and bioactive glass materials to name a few [139,173–175]. Metallic scaffolds, such as those of titanium, have been largely popularized due to their high compressive strength, porosity, and fatigue resistance [176]. Recently, Guneta et al. attempted a novel breakthrough, demonstrating that 3D printed titanium scaffolds are viable for bone tissue engineering applications [177]. By varying the sintering temperature of these titanium scaffolds from 1250 °C to 1370 °C, pore diameters ranging from 17 μm to 24 μm were achieved, and in turn it was theorized that mechanical properties can be optimized from a combination of both 3D printing specifications and variable sintering temperatures. This is consistent with other findings of pore size being dependent upon sintering temperature mentioned earlier. Unfortunately, the mechanical strength of scaffolds currently available for bone tissue engineering applications, as well as vascularization potential, are still lacking despite such a wide variety of scaffolds to choose from Ref. [178]. However, research into 3D printed scaffolds by Guneta et al. and others does perhaps offer a viable future alternative.

Vascularization remains one of the key challenges for bone tissue engineering, albeit various potential solutions have been demonstrated in recent years. It is known that insufficient vascularization can lead to a strong deficiency of the critical nutrients for cell survival within a scaffold, and can lead to unexpected and dangerous irregularities in differentiation [179]. One technique to stimulate vascularization is to introduce certain growth factors into a scaffold. Growth factors like vascular endothelial growth factor (VEGF) have been demonstrated by Wernike et al. to greatly increase blood vessel density, and to bring osteoprogenitor cells to defect sites *in vivo* in mice models [180]. Using intravital microscopy, vascularization was reported over a 28-day period. Interestingly the concentration of VEGF was shown to be a determining factor for vascularization, but only in limited concentrations, as high local concentrations of the growth factor produced unfortunate malformed vasculature. Growth factors may also be combined in a scaffold to serve multiple functions. A combination of BMP-2 (loaded in PLGA microspheres for bone regeneration) and VEGF (loaded in gelatin hydrogel for vascularization) growth factors embedded onto a polypropylene (PP) scaffold are shown by Kempen et al. to allow for both enhanced bone formation and enhanced vascularization in rat bone defect models *in vivo* [181]. Interestingly enough, a combination of VEGF and BMP-2 was found to increase bone formation over the 56-day period more than BMP-2 by itself, despite the fact that the traditional role of VEGF involves mostly vascularization. This demonstrates that a combination of growth factors is desired in most instances. Fig. 9 further demonstrates this phenomenon shown by Kempen et al. and discussed earlier, as it can be seen that with the incorporation of VEGF and BMP-2, both vascularization and further bone growth are simultaneously induced. In comparison to part (a) and (b) of the figure which contain no growth factor, the growth factor in parts (c) and (d) leads to greater cell proliferation and differentiation associated with vascularization and osteogenesis.

In order to fulfill both the mechanical strength and provide environment conducive for vascularization, PLGA scaffolds have especially been deemed viable candidates for bone tissue engineering, particularly when blended with other bioactive materials for greater cell attachment [64,182]. Sheik et al. recently presented a novel PLGA/silk hybrid scaffold for bone tissue engineering applications in which the degradation rate of PLGA was combined

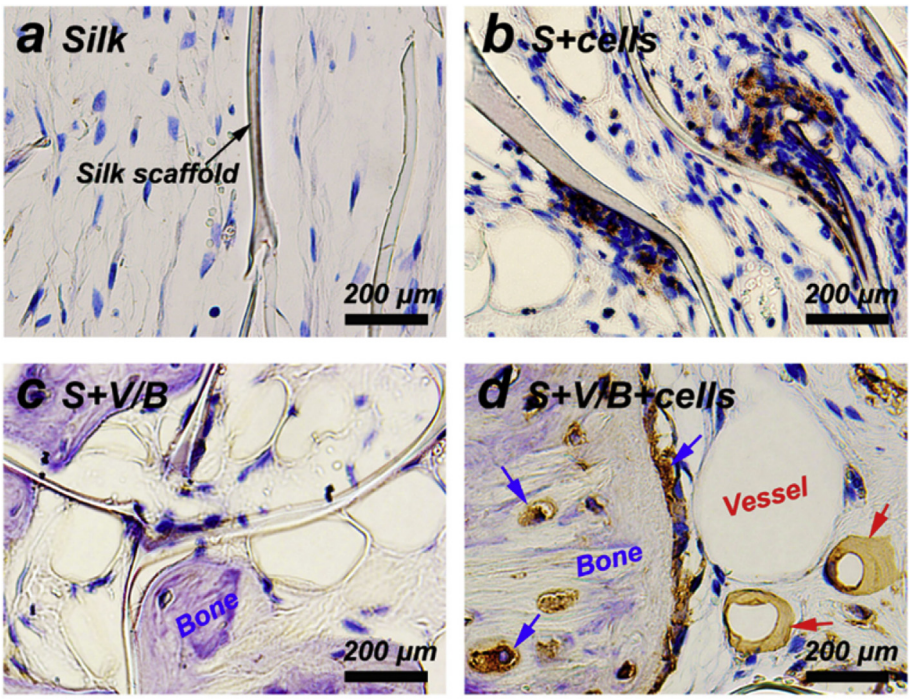

Fig. 9. Silk fibroin scaffolds were stained with GFP at 8 weeks post *in vivo* operation [170]. Part (a) represents pure silk, (b) is cell proliferation on the scaffold, (c) shows silk with VEGF and BMP-2 growth factors for vascularization, and (d) demonstrates cellular proliferation once again. New vasculature can be seen forming via VEGF stimulation.

with the hydrophilic silk polymer, as well as hydroxyapatite nanoparticles to further improve biocompatibility, and effectiveness was evaluated both *in vitro* and *in vivo* [183]. Variable-pressure field emission scanning electron microscopy (VP-FE-SEM) was used to demonstrate the porous nature of the scaffold, and contact angle measurements demonstrated that the silk component added further hydrophilicity to the scaffold. These findings are an important step in demonstrating how PLGA scaffolds can be modified or blended for greater cell infiltration and overall bioactivity. Osteoblasts were cultured for 14 days *in vitro* on the scaffold and an MTT assay following, showed cell attachment and proliferation during the culture period. Moreover, when implanted into rat calvarial defect for a 4-week period, Haemotoxylin and Eosin (H&E) staining revealed bone formation. Thus, concepts such as grain size, mechanical strength, and vascularization continue to remain dilemmas within the bone regeneration field, with a continuous research to optimize these parameters.

4.3. Muscle regeneration

Muscle tissue regeneration, perhaps more so than both nerve and bone tissue regeneration, has presented some very difficult challenges. These challenges are largely due to the fact that scaffolds must have both structural integrity while at the same time be able to induce both strong contraction and force regeneration [184]. It should be noted that there are three types of muscle – cardiac, smooth, and skeletal. Cardiac muscle tissue is predominantly located at the walls of the heart, smooth muscle tissue is located mostly in the walls of several other organs, and skeletal muscle fibers share a special attachment to the skeleton. Of these, cardiac muscle regeneration is of particular importance, because cardiac muscle tissue generally has extremely limited natural regenerative capacity in most mammals [184].

At the forefront of cardiac muscle regeneration are cardiomyocytes, which are capable of differentiating to form the most basic structures for cardiac tissues. Cardiomyocytes are frequently seeded onto PGA, gelatin, alginate, or collagen scaffolds, although it has been proposed that layering cell sheets in a 3D structure without an artificial scaffold may lead to greater bioactivity [185]. Peptides like RGD attachment to the scaffold enhance cellular functionality. Previously it has been shown that neonatal rat cardiac cells seeded onto RGD-immobilized macroporous alginate scaffolds promoted greater cellular adhesion and accelerated cardiac tissue regeneration *in vitro* on heart patches than unmodified alginate scaffolds [186]. Western blotting demonstrated the expression of proteins essential to cellular vitality, such as α-actinin, *N*-cadherin and connexin-43. Additionally, cellular apoptosis decreased significantly in the RGD-immobilized macroporous alginate scaffolds in comparison to the control group. Also aiding in the overall functionality of a scaffold, particularly when it comes to cellular adhesion, are cellular adhesion molecules such as vitronectin and fibronectin, which serve similar roles as RGD [187].

Smooth muscle cells are unique in that they can shift reversibly on a continuum from quiescent, contractile phenotypes to synthetic phenotypes, presenting a challenge for tissue engineering applications, particularly in reversing them to a contractile phenotype [188]. Nanofibers, such as those of PCL and collagen, have been shown to be particularly useful for smooth muscle tissue engineering scaffolds, as growth is guided by the nanofiber orientation and the cells are able to maintain a typical phenotype shape [189]. However, crosslinking can also be utilized for advantageous mechanical properties and proliferation in numerous directions. Recently it has been demonstrated that crosslinked, multilayer, electrospun gelatin nanofibers (±45° orientation) have been useful biomaterials for culturing human umbilical vein smooth muscle cells (HUVSMCs) *in vitro*, as cell viability reached between 80% and 92% after 9 days and cellular growth occurred along the various fibers [190].

Skeletal muscle has a massive, already built-in capacity for regeneration without external intervention, as such tissue is

constantly being destroyed, repaired, and remodeled. However, in the case of very severe injury, this regenerative capacity may either be limited or completely lost. During the repair and regeneration phase, satellite cells are known to play a very important role in migrating to the defect site, proliferating, and differentiating to restore functional properties [191]. Collins et al. demonstrated the importance of satellite cells for skeletal muscle tissue engineering applications, showing that seeding only seven of these cells onto irradiated mice muscle tissue was able to create over one-hundred myofibers *in vivo* [192]. Thousands of myonuclei were reported. Popular biomaterials as scaffolds for the seeding of stem cells to regenerate skeletal muscle tissue are hydrogels, fibrous meshes, and patterned substrates [193].

Furthermore, electrical stimulation is shown to be a novel catalyst with potential for the expansion of many myogenic progenitor cells on 3D scaffolds *in vitro*, which may be an important future method for the expansion of other cell types such as satellite cells [158]. While the reasons for this are unclear, papers like those by Serena et al. have shown that routine electrophysiological stimulation improves the differentiation potential of muscle precursor cells (MPCs) both *in vivo* and *in vitro* [194]. MPCs were seeded onto 3D collagen scaffolds and routinely electrically stimulated. While electrical stimulation did not impact cellular viability, it was noted that NO(x), a satellite cell activator, increased with a 65% greater release rate. Other myogenic markers, such as desmin, also increased compared to the control group. Serena et al. also implanted their novel electrically-stimulated scaffolds into the tibialis anterior muscles of mice *in vivo*, and after 10 days noted new myofiber formation.

From this section, it is important to conclude that muscle regeneration is unique in that it covers a wider variety of tissue – from cardiac muscle to smooth muscle, to skeletal muscle. Each tissue type has its own key players and there is most likely no one scaffold that would be useful for the regeneration of all tissue type.

4.4. Tendon/ligament regeneration

Compared to skeletal muscle tissue, previously discussed, tendons do not naturally regenerate very well after injury, and even minor injuries may present challenges in the overall healing process. For these reasons, 3D scaffolds are of critical importance for tendon regeneration. Traditional methods, such as grafts, fail to bring back the mechanical and structural properties of the original tendon as well as stimulate cellular proliferation, and a number of solutions have been proposed over the years to this dilemma. The ECM of tendon is mostly composed of collagen type I and presents itself in a complex, interwoven structure, making a 3D, biomimetic environment relatively difficult to replicate.

One major hurdle appears to be in the regeneration of the Achilles tendon (AT), due in large part to constant mechanical load being placed onto it [195]. For this regeneration application, collagen has been popularized as a scaffold. Juncosa-Melvin et al. reported that Rabbit AT defects 2 cm long decreased to 85% of their original maximum stress and modulus simply by the implantation of a collagen type I gel loaded with MSCs (0.08 M/mg cell-to-collagen ratio) *in vivo* after 12 weeks [196]. Juncosa-Melvin et al. concluded that even lower cellular densities should be tested with higher stiffness, so as to not allow excessive contraction that causes tearing in culture and weaker mechanical properties.

For all tendon regeneration applications, polymeric materials that demonstrate exceptionally strong mechanical properties have also been utilized as scaffolds, such as silk and PLGA. It is reported that PLGA/silk fibrous scaffolds are used as devices to release bFGF, stimulating mesenchymal progenitor cell (MPC) differentiation and attachment *in vitro* [197]. PLGA fibers were used to encapsulate and release the bFGF, while microfibrous silk was used as a reinforcement. Results showed an increase in gene expression of common ligament and tendon ECM proteins and increased collagen production. Other past studies like those of Ouyang et al. reported that knitted PLGA loaded with bone marrow stromal cells (bMSCs) stimulated the production of collagen type I on 10 mm long rabbit AT defects *in vivo*, helping to bring back the native tendon environment [198]. Compared to the control groups of knitted PLGA by itself and the defects left alone, there was no lymphocyte infiltration reported on the PLGA scaffold loaded with bMSCs. Immunohistochemical analysis revealed that collagen type I and collagen type III were strongly expressed, indicating a reconstruction of the native ECM environment.

Ligaments, composed mostly of fibroblasts surrounded by collagen types I and III in a dense matrix, have gained attention within the field of tissue engineering due to the critical role that the anterior cruciate ligament (ACL) plays in knee stabilization. Due to the poor healing capabilities of ACL and requires exceptional mechanical stability, polymeric scaffolds such as PLLA and silk fibers have been used to deliver growth factors and mimic the mechanical properties of the native tissue environment [199]. Recently a novel scaffold for ACL regeneration has been reported, composed of mechanically strong extruded PLLA nanofibers combined with a flexible shell of electrospun PCL nanofibers, allowing for incorporation of bFGF and platelet-derived growth factor (PDGF) in a controlled release manner, as well as proliferation of hMSCs *in vitro* [200]. Both the PLLA and PCL fibers were fabricated via electrospinning. Gene expression showed an upregulation of critical ligament markers necessary for cellular viability, such as collagen type I, collagen type III, tenascin-C, and scleraxis over a 21-day period. The AT and ACL appear to be the two largest hurdles to overcome for tendon and ligament regeneration due to both their complexity and frequency of injury cases – thus, it will likely be a combination of bioactive materials that is ultimately able to mimic the complexity of the native tissue.

5. Conclusions and perspective

While there are still a number of challenges, 3D structured biomaterial based scaffolds hold a promising future for tissue engineering applications. Since the emergence of tissue engineering in the 1990s, what was once deemed science fiction is now becoming a reality. Scaffolds have been fabricated using a variety of biomimetic materials, both synthetic and natural. Some of the major challenges of current techniques involve inducing sufficient vascularization (particularly for bone tissue engineering), finding a way to streamline the manufacturing of such scaffolds outside of the laboratory environment, fine tuning the degradation rate for a specific application, and fabricating a scaffold with biomimetic mechanical and structural properties. Of particular importance in this review article is the fabrication technique known as 3D printing, which offers hope for future mass production capabilities of 3D scaffolds, perhaps even more so than other methods mentioned. Growth factors are also still at the forefront of scaffolding, as differentiation and proliferation of cells can be brought about at a faster rate, particularly when it comes to the vascularization hurdle. It is clear that there are a variety of different methods to choose from. In the end, it will most likely be a combination of different fabrication methods, different growth factors, and different biomaterials that will make the superior scaffold for a very specific application. While NGCs for nerve regeneration are certainly among the most complex scaffold structures mentioned in this review, the movement toward even greater complexity will likely continue, as more and more studies are combining different materials and methods. With new discoveries being made

constantly, the future generation of scaffolding for tissue regeneration applications will likely utilize more functionalization techniques for enhanced bioactivity as opposed to the previous generation of tissue engineering which relied mostly on the properties of the unmodified biomaterials themselves.

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