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Spatiotemporal Control of Growth Factors in Three-Dimensional Printed Scaffolds

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Abstract

Three-dimensional printing (3DP) has enabled the fabrication of tissue engineering scaffolds that recapitulate the physical, architectural, and biochemical cues of native tissue matrix more effectively than ever before. One key component of biomimetic scaffold fabrication is the patterning of growth factors, whose spatial distribution and temporal release profile should ideally match that seen in native tissue development. Tissue engineers have made significant progress in improving the degree of spatiotemporal control over which growth factors are presented within 3DP scaffolds. However, significant limitations remain in terms in pattern resolution, the fabrication of true gradients, temporal control of growth factor release, the maintenance of growth factor distributions against diffusion, and more. This review summarizes several key areas for advancement of the field in terms of improving spatiotemporal control over growth factor presentation, and additionally highlights several major tissues of interest that have been targeted by 3DP growth factor patterning strategies.

Keywords

spatiotemporal; growth factor; pattern; gradient; bioprinting; printing; fabrication

1. Introduction

Three-dimensional printing (3DP) has emerged as a powerful collection of techniques for scaffold fabrication. These methods are particularly attractive for tissue engineering and regenerative medicine, as they can create biologically-inspired constructs of a variety of clinically-relevant sizes, generate complex external and internal architectures, deposit material in highly specific locations within a construct, and incorporate a growing number of synthetic and natural materials either independently or as composite mixtures. With these capabilities in mind, tissue engineers seek to leverage 3D printing for the fabrication of

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templates that a) mirror complex tissue architectures found in native tissue, b) deposit spatially and temporally specific biochemical cues for different tissue types, and c) ultimately promote new tissue formation, to solve current clinical challenges. A variety of 3DP techniques have already been adapted for biomedical research, and these strategies have been extensively reviewed.(1–8)

3D bioprinting stands as the next progression of these techniques for medical applications. In bioprinting, biological and composite materials are similarly deposited layer-by-layer to create three-dimensional structures. However, in contrast to acellular and/or biologically inert scaffolds, these techniques specifically focus on the incorporation of living cells and bioactive molecules directly into the printed solutions without loss of function.(1,9) The precise spatiotemporal control offered by 3DP allows tissue engineers and clinicians to create replacement tissues and tissue templates that more effective mimic the biochemical and physical microenvironment of the native tissue.(9)

Of particular interest in bioprinting is the ability to spatially pattern different growth factors (GFs) and other bioactive proteins, and furthermore to create concentration gradients of these molecules, in order to mimic the complex developmental profiles of different native tissues. Proper incorporation of these chemical cues within tissue scaffolds directly impacts new tissue formation, and it has been shown extensively that several different GFs often operate synergistically to facilitate or inhibit tissue growth.(10–14) Additionally, correctly controlling the release and temporal presentation of growth factors is critical to ensure that functional tissues are developed with the desired phenotype. In order to progress from simple, thin tissues to highly heterogeneous tissues, interfacial regions, and whole organs, both spatial and temporal concentration profiles and gradients must be well understood.(4) In particular, musculoskeletal tissues, nervous tissue, and vasculature are comprised of a heterogeneous collection of tissues with coordinated functions.(1) For example, the osteochondral unit contains bone, cartilage, and transitional layers with gradated mechanical and biological properties, and it has been demonstrated that synergistic interactions of bone morphogenetic proteins and transforming growth factor-β (TGF-β) directly result in the proper or improper formation of all three sections. (1,15–17) Similarly, proper formation of nervous tissue is highly dependent upon accurate directional gradients of several different growth factors, including nerve growth factor (NGF) and glial cell line-derived neurotrophic factor (GDNF).(18)

Despite the potential offered by 3D bioprinting, there remains a number of significant limitations with these techniques. Techniques for growth factor deposition are largely limited to inkjet printing, laser-assisted printing, and low-temperature extrusion bioprinting. Other techniques such as selective laser sintering and soft/stereolithography are often excluded due to their use of high temperatures, harsh solvents, or extended use of UV light, which may inhibit the activity of the bioactive molecules unless they are encapsulated within a protective shell or delivery vehicle.(9,19–21) Proper selection of a shear-thinning polymer solution must also be considered, as the shear stresses applied at the nozzle tip may impact this bioactivity. Additionally, achieving the proper resolution necessary for truly biomimetic tissue templates continues to pose a challenge. As Murphy and Atala note, the primary challenge is adequately reproducing the complexity of the ECM microenvironment so as to

restore biological function.(9) Creating accurate true biochemical gradients within scaffolds also remains out of reach, as most approaches in the literature have developed bi/tri phasic scaffolds with discrete layers rather than true gradients. Finally, improvements must be made in the temporal control of growth factor presentation after deposition, as the current standard for controlled release in these scaffolds is largely passive diffusion from hydrolyzed microparticles and hydrogels.(20,21) Ultimately, it is clear that functional tissue regeneration is dependent on the accurate coordination of multiple bioactive factors, both in terms of spatial localization as well as release kinetics.(20)

In this review, we discuss the current state of growth factor patterning within 3D bioprinted scaffolds, with a specific focus on achieving spatiotemporal control of these cues and its importance to the creation of functional tissue templates. Specifically, we describe recent advances in growth factor patterning and recurrent challenges associated with these methods, including limitations in printing resolution, difficulties in maintaining controlled release profiles of growth factors, and more. Finally, we highlight studies from recent literature describing bioprinting approaches towards several target tissues and provide a summary of future work that remains in order to progress these techniques towards the end goal of generating fully functional tissues and organs.

2. Fabricating Spatiotemporal Growth Factor Patterns

Spatiotemporal growth factor patterns and gradients are critical for tissue development, especially for heterogeneous tissues such as the osteochondral unit and directionally oriented tissues such as nerves and blood vessels.(1) By fabricating scaffolds with directional gradients of one or more growth factors, one can mimic the guided migration of cells and tissue morphogenesis seen in native tissue development – e.g., during embryonic tissue development.(22) In extrusion and inkjet printing techniques, the generation of patterns and gradients is often achieved by the printing and blending of multiple “inks” containing different growth factor formulations.(1,23) In light-mediated techniques such as stereolithography (SLA), multilayer scaffolds with gradient-like presentation of growth factors can be generated by using multiple resin solutions, which must be washed out or interchanged between the processing of successive layers.(23,24) Extrusion and inkjet methods present a more facile process for growth factor gradient printing by simply switching and/or blending bioinks between subsequent layers, provided that the printing system is equipped to handle multiple printheads or cartridges.(25) The level of granularity that can be achieved in growth factor patterning is thus highly dependent on both the number of bioinks that a printing system can handle as well as the ability of the print system to blend or rapidly switch between these bioinks during printing. Furthermore, the resolution at which 3DP systems can deposit or crosslink their inks/resins of choice will, accordingly, determine the resolution at which encapsulated growth factors can be patterned. The temporal control of spatial gradients after printing is another critical issue that is dependent on the kinetics of both growth factor release and subsequent diffusion.(26) Ideally, the rate of release for a particular growth factor included in a bioprinting approach should match that of the native tissue during embryonic development.(26) Additionally, growth factor release should be tuned to produce spatial patterns that are maintained in the presence of aqueous diffusion.(27) Finally, the printing of ceramic materials and other biochemical cues in

gradients can be used to complement growth factor gradients, creating more highly biomimetic environments for tissue development.(23,28)

The following discussion highlights key advances, challenges, and areas for improvement related to the spatiotemporal control of growth factor patterning – namely, printing of multiple bioinks, improving resolution of patterning, achieving temporal control of growth factor release, maintaining growth factor patterns against diffusion, co-printing biochemical gradients, and directly conjugating growth factors to scaffolds.

2.1. Printing of Multiple Bioinks

The ability to handle and print multiple bioinks is a critical prerequisite for growth factor patterning. While 3DP systems have traditionally allowed for the deposition of just a single ink or material, recent developments in extrusion and inkjet based systems have now enabled the printing of multiple inks in tandem using both custom and commercially available printers.(29) If multiple bioinks differ only in their growth factor composition, then a single printhead/nozzle may be used in combination with multiple cartridges.(25) If the bioinks differ in material composition, however – e.g., to generate physical or mechanical gradients accompanying the growth factor gradient – then multiple printheads/nozzles may be necessary to generate appropriate conditions such as temperature, pressure, etc. for each material.(1) One primary advantage of a single printhead/nozzle setup is, of course, the ability to switch rapidly between bioinks rather than having to pause in the range of 4–20 seconds for printhead/nozzle switching.(25,30) In addition to reducing the duration of printing, this allows for deposition to more closely resemble that of single, continuous flow, which helps reduce defect formation and furthermore, helps prevent errors traditionally caused by poor start/stoppage of flow or inconsistent nozzle alignment that are often seen with multi-printhead systems.(29,31) Liu et al. created one exemplary single printhead system which afforded near instantaneous switching of up to seven bioink cartridges, and furthermore allowed for coaxial fiber deposition of multiple bioink formulations.(25) In their setup, seven discrete channels, each connected to its own bioink cartridge, were fed at programmable proportions of pneumatic flow into a single dispensing printhead channel to generate fibers with coaxial distributions of several materials.(25) Excitingly, they patterned both multiple cell types and inks of varying hydroxyapatite concentration, which in the future can be used in tandem with growth factor patterning to generate complex, heterogeneous constructs for multi-unit tissues such as the osteochondral unit, which presents a gradient of both mineral composition and cell phenotype.(25) Hardin et al. developed another microfluidic printing system that enabled direct mixing of two extruded bioinks during the well-defined and tunable transition period between each ink.(31) However, their mixing of inks was achieved using polydimethylsiloxane inks with similar composition and rheological properties, and the mixing of inks of dissimilar polymer composition or behavior under flow could likely be more challenging.(31) The ability to seamlessly mix bioinks during printing is powerful because it enables the creation of true horizontal and vertical gradients by continuously adjusting the proportion of multiple bioinks, rather than simply creating pseudogradients composed of discrete layers. Tarafder et al. showcased another multi-cartridge printing approach towards temporomandibular joint (TMJ) disc regeneration in which alternating strands of poly(ε-caprolactone) (PCL) were

printed with poly(lactic-co-glycolic acid) (PLGA) microspheres containing either connective tissue growth factor (CTGF) or TGF-β3.(32) Other extrusion and inkjet techniques have utilized multi-printhead systems to great success for printing bioinks with distinct temperature, pneumatic pressure, and nozzle size requirements, such as the integrated tissue-organ printer system developed by Kang et al.(33) Ultimately, single-printhead systems may be most suitable for the printing of bioinks with highly similar material compositions, while multi-printhead systems on the other hand will benefit from improvements to speed of printhead switching and the ability to maintain continuous printing when switching between bioinks with print conditions.

2.2. Improving Resolution of Growth Factor Patterning

High resolution printing is critical to the development of functional growth factor templates for tissue development – as Zhu et al., among others, have noted, printing resolution "must still be developed down to the submicron scale for effective tissue fabrication".(34) Furthermore, distributing growth factors at the precise spatial locations where they are needed can help reduce the required concentration of growth factor loading, reducing the risks of off-site diffusion and ectopic tissue growth associated with delivering supraphysiological growth factor dosages.(35,36) As the resolution of 3DP techniques increases, so does the ability to print highly granular growth factor patterns and gradients. In general, SLA and other light-based techniques allow for the highest resolution of printing,(1) though for the aforementioned reasons, extrusion and inkjet printing are still generally preferred for their ease of patterning multiple bioink formulations. In extrusion based techniques, the resolution is limited to around 200 μm and is generally improved by using smaller diameter needles/nozzles.(25,37,38) Smaller diameter needles, in turn, require lower bioink viscosity for effective extrusion, and the viscosity of an ink formulation varies based on parameters related to the print material itself – such as molecular weight and chemical composition – to printing conditions such as temperature and pneumatic pressure.(37) Improving the resolution of printing and by extension, of growth factor patterning in extrusion-based systems, thus requires experimental determination of the optimal print conditions for one's bioink of choice. For inkjet systems, the resolution of printing is instead determined by droplet size instead, which is presently limited to about 1 picoliter or approximately 12 μm in diameter due to physical factors of droplet generation.(39) Compared to extrusion based systems, inkjet systems typically present an improved resolution of around 20–100 μm, though the precision of ink patterning can be compromised by the inherent heterogeneity of droplet size.(38)

Advancements in the field of additive manufacturing may pave the way for higher resolution bioprinting systems. For instance, Gunduz et al. discovered that the application of ultrasonic vibrations to the nozzle of an extrusion printer could significantly reduce wall friction and flow stress, enabling the printing of high viscosity materials.(40) One could imagine this approach being used to print bioinks of a given viscosity through increasingly small-diameter needles, thereby improving the resolution with which the bioinks can be deposited. Thus, fine spatial control over growth factor patterning will benefit from novel techniques for higher resolution 3D printing.

2.3. Temporal Control of Growth Factor Release Patterns

As discussed previously, the rate of growth factor release in 3DP scaffolds should match that seen in native tissue development, meaning that each area of a growth factor gradient or pattern should have differential release kinetics. Given that growth factors are typically delivered by encapsulation in intermediate vessels such as polymeric microparticles or secondary hydrogels, the release kinetics can be tuned by utilizing different vessels for each factor or by adjusting physical and chemical parameters of the vessel materials such as their surface area and hydrolytic degradability.(26) Lee et al. demonstrated one approach in which CTGF and TGF-β3 were encapsulated in microspheres of 50:50 and 75:25 poly(lactic acid):poly(glycolic acid), respectively, and delivered in separate locations of a construct to produce a spatially and temporally distinct growth factor release profile.(41) This approach took advantage of the greater hydrolytic degradability of poly(glycolic acid) to effect more rapid release of CTGF.(41) This approach is exemplary of many similar growth factor delivery strategies that utilize carriers with differential release kinetics, and many existing reviews have summarized the advances and ongoing pitfalls associated with such strategies.(26,42,43) Ultimately, combining the multibioink systems discussed previously with differentially degradable growth factor vessels can effect a combination of spatial and temporal control over growth factor release.

However the degradation of these secondary vessels is highly dependent on interactions with the *in vivo* microenvironment, and *in vitro* experiments cannot fully model the complex biochemical and physical factors present at the site of a tissue defect.(26) It can thus be beneficial to modulate growth factor release post-implantation using external stimuli. The usage of external physical stimuli to modulate behavior of tissue engineering scaffolds has gained popularity in recent years,(44–46) though few approaches have applied this methodology so far to 3DP scaffolds. One very interesting approach by Gupta et al. utilized 3D-printed PLGA shells doped with plasmonic gold nanorods (AuNRs) to achieve photothermally mediated rupture of the shells and subsequent release of a payload from the aqueous core (Figure 1).(47) By utilizing AuNRs of different length, they could print shells with different wavelength-specificity,(47) which, in a tissue engineering context, could be utilized for the selective spatiotemporal release of multiple growth factors.

The fine spatial control afforded by using a laser stimulus of specific wavelength and the high degree of temporal control afforded by external control shows the promise of this method to create user-defined spatiotemporal growth factor gradients *in situ*, with exciting clinical implications. Drawbacks, however, include the low penetration depth of lasers and other external physical stimuli, as well as the potential cytotoxicity of reactions such as the photothermal response.(47) While some studies have been performed on the 3D printing of light-responsive additives such as AuNRs and graphene oxide or magnetically responsive additives such as iron oxide nanoparticles, further research is needed to elucidate how the patterning of these materials can be used to for external spatiotemporal control of growth factor release.(47–49)

2.4. Controlling for the Effects of Growth Factor Diffusion

One major concern in growth factor patterning is the maintenance of fabricated growth factor patterns against diffusion. Growth factor gradients are particularly susceptible to losing granularity as released growth factors diffuse and disrupt any finely tuned gradient that may have been present upon scaffold fabrication.(27) A number of factors affect the aqueous diffusion of growth factors, including the molecular weight of the growth factor, the porous architecture of the scaffold, the degradation of scaffold materials, and more. As growth factor molecular weight decreases or scaffold pore size increases, for instance, growth factors will diffuse more readily.(26) Thinner constructs, especially those below about 100–200 μm in overall thickness as a rule of thumb, experience relatively greater diffusion of growth factors and other biomolecules, which can produce ectopic effects in the surrounding tissue.(1,33,50) Furthermore, sharper gradients are likely to at least retain some bioactive effects compared to shallower growth factor gradients, which may completely lose their gradient distribution as a result of diffusion.(23) Given that a multitude of biomolecule and scaffold-specific factors affect the survival/loss of printed growth factor gradients, it is important to optimize these parameters experimentally for any scaffold by measuring the effective growth factor concentrations produced *in vitro*.

Some approaches, however, have utilized diffusion as a means of actually producing growth factor gradients in scaffolds that target axially oriented tissues such as nerves and blood vessels.(18,51) Johnson et al. printed two hydrogels – one loaded with nerve growth factor and the other with glial cell line-derived neurotrophic factor – at different spatial distributions along the sensory and motor pathways of a bifurcated silicone conduit and allowed diffusion to form continuous gradients of these two growth factors along the construct.(18) Interestingly, they used a drug release model for thin film hydrogel systems to predict the formation of diffusive gradients in their printed constructs.(18) Akar et al. similarly modeled platelet-derived growth factor BB (PDGF-BB) diffusion and encapsulated PDGF-BB in PLGA microspheres at one end of a poly(ethylene glycol) (PEG) construct, enabling its subsequent burst release to gradually create a PDGF-BB gradient along the entire construct when implanted *in vivo* (Figure 2).(51)

Mathematical models of protein diffusion can thus be utilized to produce post-fabrication growth factor gradients in 3DP scaffolds, though one must be mindful that theoretical and even *in vitro* models cannot fully account for the complex physical factors that may be influence aqueous diffusion *in vivo*. Methods that utilize diffusion to generate spatial gradients thus present a higher degree of uncertainty and potentially less applicability to *in vivo* scenarios compared to more conventional growth factor patterning methods that utilize spatially defined printing to directly generate the patterns.

2.5. Co-Printing of Biochemical Gradients

Growth factor gradients can be printed in tandem with other biochemical gradients to generate microenvironments that more closely resemble the composition of native tissue.(52) Calcium phosphates such as hydroxyapatite, for instance, can be printed alongside osteogenic growth factor patterns to mimic the biochemical distribution of these cues in native bone matrix.(53,54) Several examples exist in the literature where calcium phosphates

have been printed in a vertical gradient with camphene,(55) poly(propylene fumarate),(56) and other scaffold materials(23) to generate biochemical distributions and physical architectures that mimic features of natural bone tissue – e.g., the transition from cortical to cancellous bone.(57) Literature is much sparser, however, on the creation of calcium phosphate gradients in combination with growth factor gradients. Ahlfeld et al. showed one case in which a two-channel plotting system was used to fabricate biphasic scaffolds containing a calcium phosphate cement (CPC) component and a vascular endothelial growth factor (VEGF)-loaded hydrogel component.(28) A gradient-like distribution was created in their scaffold by varying the proportion of CPC strands and VEGF-loaded hydrogel strands printed throughout the scaffold (Figure 3).(28) The co-printing of biochemical gradients in coordination with growth factors patterning is thus a highly unexplored approach towards biomimetic scaffold fabrication, and future studies could investigate the co-patterning of growth factors with other highly bioactive extracellular components such as glycosaminoglycans.(58)

2.6. Printing of Covalently Conjugated Growth Factor Patterns

An alternative approach to physical encapsulation is to covalently conjugate growth factors or bioactive peptide sequences directly to the scaffold materials, avoiding the aforementioned issues related to diffusion of soluble growth factors and effecting more constant presentation of patterned growth factors. Existing reviews have summarized the considerations related to bioconjugation of biomolecules to tissue engineering scaffolds,(59,60) and these strategies have occasionally been applied to 3DP scaffolds.(59,61,62) Gao et al., for instance, conjugated acrylated peptides to acrylated PEG during inkjet printing to generate crosslinked hydrogels with bioactive peptide presentation through the scaffold.(61) Lee et al., on the other hand, performed post-fabrication conjugation of biomolecules by using mussel-inspired adhesive chemistry to adhere bone morphogenetic protein-2 (BMP-2) to the surface of a 3D printed PCL scaffold.(62) While relatively few examples exist of biomolecule conjugation to 3DP scaffolds, the authors of this review could not find literature in which biomolecule patterns or gradients were generated on 3DP scaffolds by bioconjugation strategies. Future work in bioprinting could thus apply bioconjugation strategies such as the usage of “click” and activated ester chemistry to produce biofunctionalized inks for subsequent gradient and pattern printing.(60,63) One notable drawback of direct bioconjugation strategies, however, is the inability to temporally control growth factor presentation after scaffold fabrication, as biomolecules will be bound to the matrix until the scaffold itself degrades.(26) The relative importance of controlling growth factor release kinetics vs. maintaining constant growth factor presentation via bioconjugation should thus be considered for any tissue engineering application.

3. Target Tissues and Applications

Spatiotemporal growth factor patterning and gradient design can be used to create physiologically-inspired template scaffolds that present complex and/or sequential biochemical stimuli for the development of heterogeneous tissues.(1,10,64–67) Effective growth factor patterning requires an understanding of not only what growth factors are relevant to tissue formation and ingrowth and in what concentrations for each tissue, but also

how factors interact to synergistically enhance or inhibit tissue formation. For example, it is well-understood that bone morphogenetic proteins (BMP-2, BMP-3, BMP-7) play a critical role in bone formation and remodeling. However, it is also important to note that, depending on the type of bone and location relative to other tissues, a variety of additional factors may need to be present for tissue growth, including transforming growth factors (TGF-β1, TGF-β2, TGF-β3), VEGF, and insulin-like growth factor (IGF-1) among others.(68) The specific BMP molecules involved in bone formation also vary temporally; BMP-2 is often the osteogenic signal molecule early in bone formation, while other BMPs may drive bone development at later stages.(69) Similarly, Miller et al. presented a study in which BMP-2 and IGF-2 were printed in various amounts to determine the effects of relative concentration between the two factors on cell phenotype, and the authors observed that BMP-2-induced osteogenesis was increasingly inhibited by IGF-2. (70) Fibroblast growth factor 2 (FGF-2) can also induce partially differentiated cells to upregulate other factors such as BMP-2 and VEGF, but the level of induced upregulation is highly dependent on the extent of differentiation.(71)

This section thus serves to highlight recent literature in which growth factor patterning has been applied to different target tissues. Specifically, highly heterogeneous tissues such as cartilaginous and osteochondral tissues and highly directional tissues such as nerves are considered here.

3.1 Cartilaginous and Osteochondral Tissues

As a major recurrent clinical challenge, cartilage and the osteochondral unit are target tissues of importance in bioprinting, and these tissues have been studied extensively using a number of different growth factor-based approaches over the last several years. A variety of previous reports using non-3DP hydrogels have demonstrated that bilayered composites with growth factor gradients can significantly improve cartilage and bone healing and tissue formation.(16,17,27,64,67,72–77) These types of experiments, which created primarily bi- and triphasic constructs with discrete layers, can be considered precursors to the growth factor patterning techniques discussed here, and the incorporation of 3DP techniques to target these tissues is compelling when considering the ability to much more specifically design growth factor gradients. For example, Kundu et al. used a multi-head extrusion-based printing system to pattern TGF-β within chondrocyte-encapsulated polycaprolactone/alginate.(78) After 4 weeks *in vivo* in a nude mouse model, it was shown that chondrocyte-containing constructs and chondrocytes+TGF-β-containing constructs had improved collagen II formation compared to positive controls, and in particular the patterning of TGF-β led to more mature cartilage formation.(78) In a different experiment, Castro et al. attempted to mimic the organization of bone and cartilage layers within the osteochondral unit by delivering chondrogenic TGF-β1 in a gradient with osteogenic hydroxyapatite.(79) In their experiment, a PEG-diacrylate solution was used as the bulk phase, with 20, 10, and 0 wt% HA in the subchondral, interface, and chondral layers respectively and TGF-β1 deposited only in the chondral layer, and it was demonstrated that the triphasic gradient scaffolds yielded improved adhesion, proliferation, and chondrogenic and osteogenic differentiation of seeded hMSCs as observed by significantly increased GAG production, type II collagen synthesis, and extracellular calcium deposition compared to controls.(79)

Focusing on the TMJ, Tarafder et al. in several studies demonstrated the ability to pattern CTGF and TGF-β3 into distinct regions within PCL scaffolds after encapsulation within PLGA microspheres.(80,81) The authors observed that despite printing at high temperatures, PLGA microsphere-encapsulated growth factors maintained their bioactivity and a consistent release over 42 days, and that rabbit TMJ scaffolds created with CTGF and TGF-β3 microspheres led to heterogeneous fibrocartilage formation after 6 weeks in culture. When applied *in vivo*, 4-week TMJ disc explant samples displayed recovery of the defect site, being mechanically sound with a highly-organized fibrocartilage structure mirroring native tissue.(80) In a separate study, the authors also demonstrated that in addition to heterogeneous tissue formation after CTGF and TGF-β3 release, gene upregulation was highly dependent on the dose of growth factor used, with the 100mg microsphere/g scaffold dose yielded significantly increased collagen I and II and aggrecan expression than the 50mg/g dose.(81)

3.2 Bone and Bone-Interfacial Tissues

Bone tissues are of great interest from a bioprinting perspective, as there is still a high demand for treatment of critical size bone defects in the clinic. Akkineni et al. improved a cold-plotting 3DP technique for the fabrication of biphasic, VEGF-patterned CPC scaffolds, such that the minimum feature size for printed fibers was under 200μm in diameter.(28,54) 3D plotting remains a relatively new technique. Like high temperature extrusion 3DP, plotting deposits fibers through a print nozzle corresponding to xyz instruction sets; however, plotting can be conducted at mild conditions, as the CPC paste bulk phase is a biocompatible oil and hardens upon contact with water. For this reason, bioactive molecules and cells can be incorporated without loss of activity. The authors demonstrated that the activity of incorporated VEGF was not impacted by plotting within CPC pastes, leading to increased cell density of dermal microvascular endothelial cells on printed scaffolds, and that plotted scaffolds could be set in a humid environment and maintain structural stability while avoiding microfracture defects occasionally observed when immersing in water due to swelling.(54)

In a follow-experiment, Ahlfeld et al. achieving the mentioned reduced feature size as well as printing a variety of layer-by-layer patterns, and selectively designed a biphasic scaffold with a gradient of VEGF concentration radially from the center of the construct.(28) Fahimipour et al. similarly used a room temperature printing technique to generate gelatin/alginate/β-tricalcium phosphate scaffolds with PLGA microparticle-encapsulated VEGF for craniofacial defect applications.(82) Their technique was also demonstrated to maintain growth factor activity, as osteoblast proliferation was significantly increased between 7 and 14 days for VEGF-containing scaffolds compared to positive and negative controls, and ALP activity for seeded cells was higher than either control at both timepoints.(82) Despite a hydrogel bulk phase, these scaffolds also demonstrated desired mechanical properties; determined by uniaxial compression, the composite scaffolds had a compressive modulus of 98 MPa, which is in the range of cancellous bone.

Several other studies have focused on the coordination of multiple growth factors for bone bioprinting. Ker et al., for example, selectively oriented polystyrene fibers and patterned

BMP-2 and FGF-2 using an inkjet printer with desired use for tendon-bone interface engineering.(83) The authors observed that seeded populations of C2C12 and C3H10T1/2 cells each demonstrated directed differentiation based on their location; scaffold regions with no printed growth factor yielded myocyte formation, while cells seeded on BMP-2 and FGF-2 regions differentiated down osteoblast and tenocyte pathways respectively.(83) Finally, Gurkan et al. and Park et al. each investigated the effects of BMP-2/TGF-β1 and BMP-2/VEGF gradient delivery on cell fate.(84,85) In the latter study, the authors developed a biphasic scaffold using a PCL backbone with a 2% collagen/BMP-2 exterior and 10% alginate/10% gelatin/VEGF interior, with encapsulated dental pulp stem cells (DPSCs) throughout, as shown in Figure 4.(85) Four weeks after *in vivo* implantation in mice, it was observed that compared to the DPSC/collagen and DPSC/collagen/BMP-2 constructs, new vasculature had formed in the center of the biphasic scaffold in addition to the periphery, and that vessel formation at the periphery was significantly increased in this group as well. Finally, VE-Cadherin, VEGFR-2, ALP, and Runx2 were also significantly upregulated in the biphasic group, indicating synergistic osteogenic and vasculogenic effects.(85) .

3.3. Nervous Tissues

Nervous tissues are a unique target tissue in that a primary challenge is achieving directional tissue development. This also makes them prime targets for spatiotemporal growth factor patterning. In addition to an accurate coordination of chemical factors, these tissues require higher printing resolution to ensure accurate macro- and micro-/submicroscopic architecture. An early study by Ilkhanizadeh et al. demonstrated that seeded neural stem cells could be controllably differentiated *via* growth factor delivery.(86) The authors seeded NSCs onto HydroGel™ scaffolds with inkjet bioprinted patterns of FGF-2, ciliary neurotrophic factor (CTNF), and fetal bovine serum (FBS), and it was observed that cells grown on CTNF and FBS patterned areas developed into glial and smooth muscle cells respectively, while those seeded on FGF-2 patterned areas displayed no obvious differentiation.(86) In a related study, Johnson et al. designed dual growth factor gradient scaffolds for nerve cell regeneration, using 3DP silicon casts of the sciatic nerve bifurcation, as shown in Figure 5.(18) The authors designed the bifurcation with both spatial and temporal gradients of NGF and GDNF, such that both factors were concentrated at the inlet and then deposited in progressively further locations up to the bifurcation, after which the two factors were separately printed in the proximal and distal ends to mimic sensory and motor neuron branching.(18) Prolonged release of both factors was observed out to three weeks, and *in vitro*, it was observed that NGF and GDNF provided directional cues to axons and Schwann cells respectively. Finally, after three months implantation *in vivo* in a 10mm nerve gap injury in rats, it was observed that the 3DP scaffold facilitated nerve regeneration as well as Schwann cell population in the regenerated tissue, and that rats implanted with the dual-gradient scaffolds demonstrated significantly restored function *via* gait analysis compared to those with control treatments.(18)

Finally, Lee et al. demonstrated the combination of coaxial electrospraying with stereolithography printing, developing bovine serum albumin (BSA) or NGF-encapsulating core-shell PLGA nanoparticles *via* electrospraying and then mixed into PEG/PEG-diacrylate hydrogel print solutions.(87) The authors observed that, in addition to the effects of varying

the scaffold geometry, the encapsulation and delivery of NGF significantly improved neurite outgrowth compared to the blank and BSA controls.(87) Additionally, the directed extension of PC-12 cells was increased in NGF-patterned samples compared to blank scaffolds and those sprayed with non-localized NGF.(87)

Conclusions

The field of bioprinting has thus far led to the creation of highly biomimetic scaffolds that replicate the physical, biochemical, and architectural features of native tissue with a higher degree of fidelity than previously achievable. To recapitulate the dynamic array of biochemical cues seen during native tissue development, however, tissue engineers will need to achieve greater spatiotemporal control over growth factor presentation in these printed scaffolds. While significant progress has been made in the fabrication of spatially defined growth factor patterns and even growth factor gradients, notable challenges remain in several areas. The practice of growth factor patterning will greatly benefit, for instance, from improvements to the ability of print systems to handle and mix multiple bioink formulations and furthermore, to print and spatially pattern these inks at higher resolutions. Additionally, achieving true temporal control over growth factor presentation will require the development of controlled release mechanisms for multiple growth factors that can maintain these spatiotemporal distributions against the force of aqueous diffusion. Guiding growth factor release with stimuli-responsive materials may thus provide a means for greater spatiotemporal control, by using external physical cues such as light or magnetism. Other avenues which have yet to be fully explored include the co-printing of growth factor patterns with other biochemical cues such as calcium phosphates, as well as the covalent conjugation of growth factors to scaffold materials to generate biofunctionalized inks for patterned printing. By optimizing spatiotemporal control of growth factor patterning in these areas, more highly tissue-specific scaffolds can be created for heterogeneous and directionally oriented tissues such as the osteochondral unit, blood vessels, and nerve tissue. Thus, complex tissues which may have been out of reach with conventional scaffold fabrication techniques are now prime targets for repair by 3D printed scaffolds with spatiotemporally patterned growth factors.

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Abbreviations:

3DP	Three-dimensional printing
AuNRs	Plasmonic gold nanorods
BMP	Bone morphogenetic protein
BSA	Bovine serum albumin
CPC	Calcium phosphate cement

CTGF	Connective tissue growth factor
CTNF	Ciliary neurotrophic factor
DPSC	Dental pulp stem cell
FBS	Fetal bovine serum
FGF-2	Fibroblast growth factor 2
GDNF	Glial cell line-derived neurotrophic factor
GF	Growth factor
IGF-1	Insulin-like growth factor 1
NGF	Nerve growth factor
PCL	Poly(ε-caprolactone)
PDGF-BB	Platelet-derived growth factor BB
PEG	Poly(ethylene glycol)
PLGA	Poly(lactic-co-glycolic acid)
SLA	Stereolithography
TGF-β	Transforming growth factor β
TMJ	Temporomandibular joint
VEGF	Vascular endothelial growth factor

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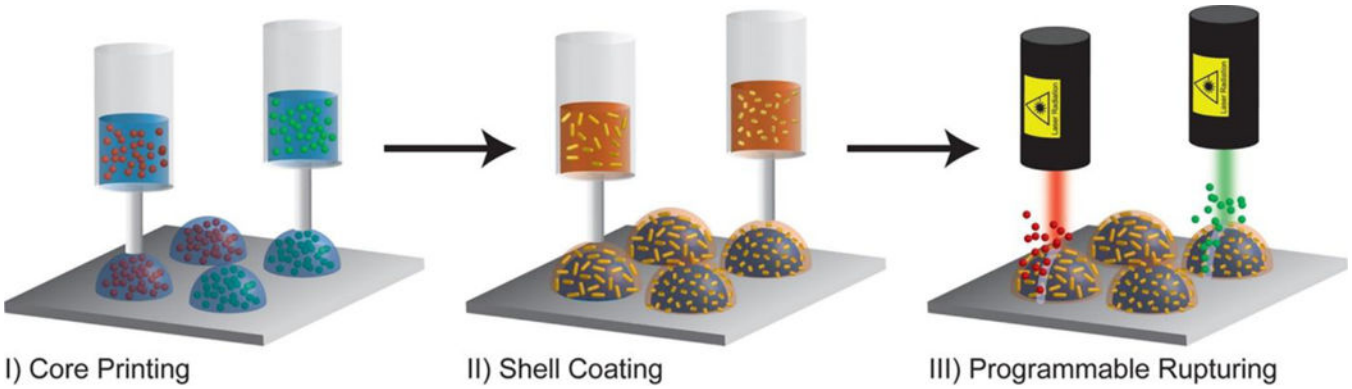

Fig 1:

Printing of drug-loaded aqueous cores, followed by deposition of AuNR-doped PLGA shells, and then wavelength-specific rupturing of shells to effect selective release of payloads. Reproduced with permission from (47).

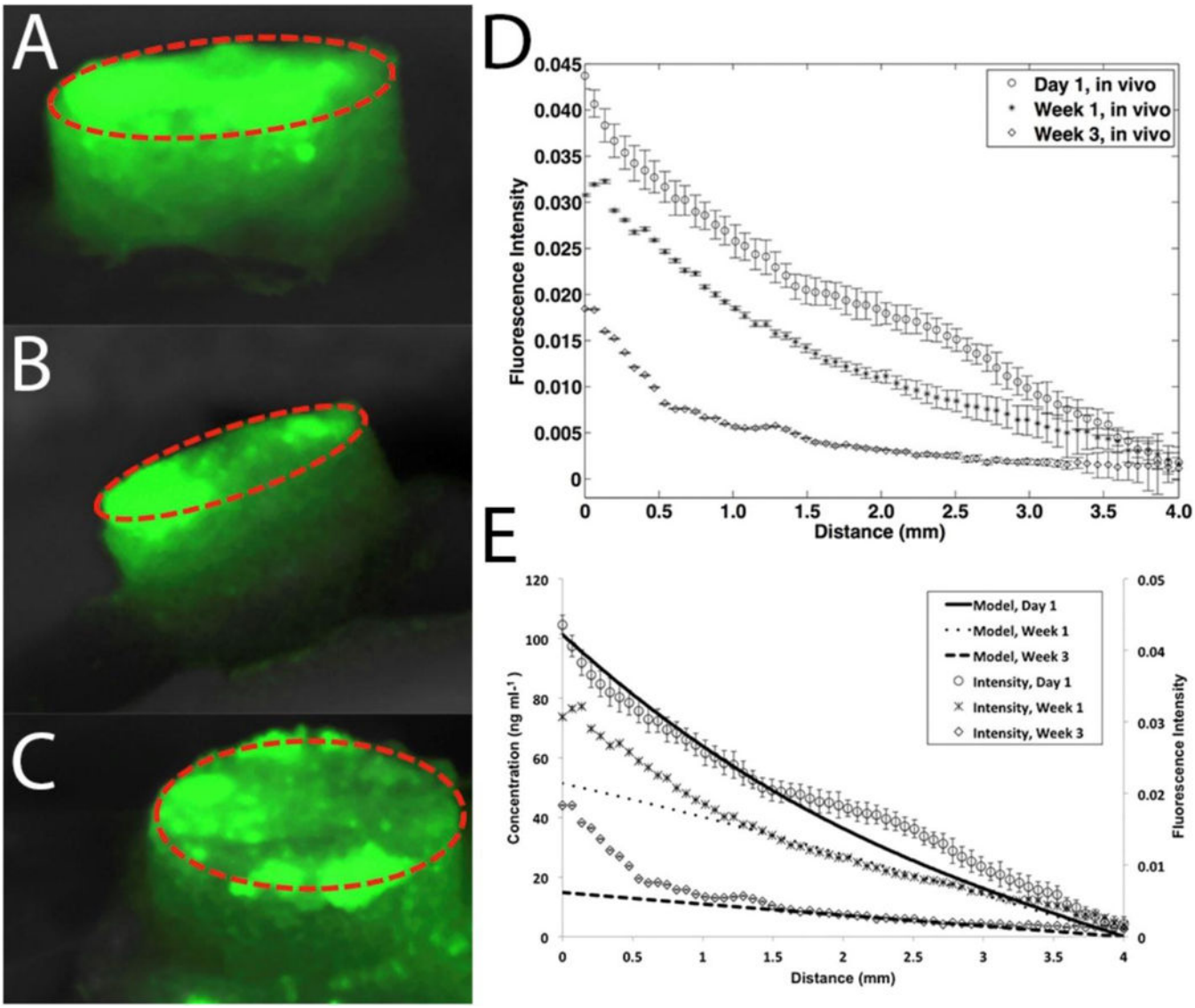

Fig 2:

In vivo gradient of fluorescently-labeled PDGF-BB created by diffusion of the growth factor from its original area of burst release (red circle). Fluorescent images were taken at day 1 (A), day 7 (B), and day 21 (C). Also shown are the spatial distribution of growth factor fluorescence (D) as well as a comparison of the experimental growth factor distribution to the predicted distribution from mathematical modeling (E). Reproduced with permission from (51).

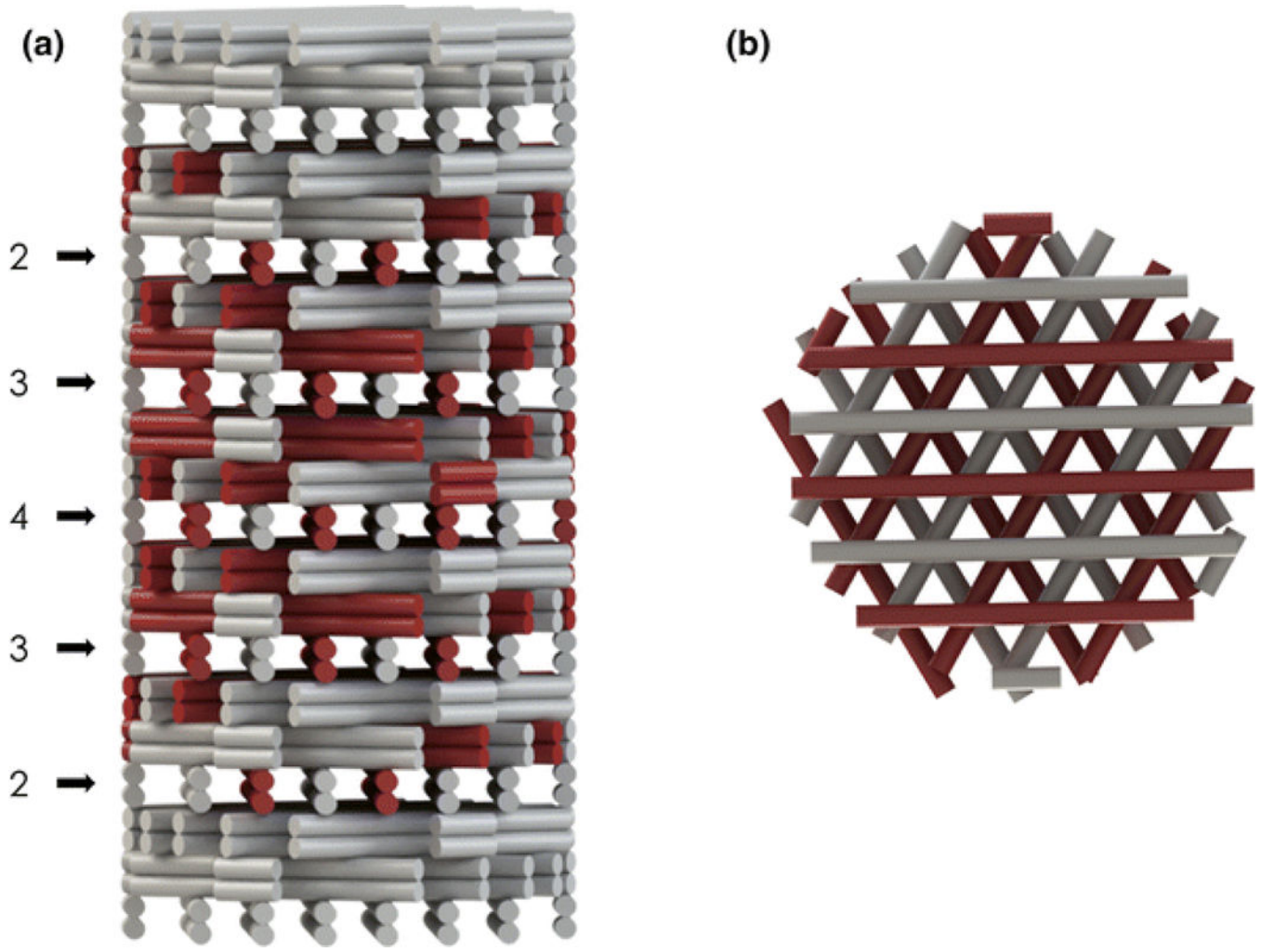

Fig 3:
Gradient-like distribution of CPC strands (white) and VEGF-loaded alginate-gellan hydrogel strands (red) generated by a two-channel plotting system. The 3D printed scaffold is shown from side (a) and top (b) views. Reproduced with permission from (28).

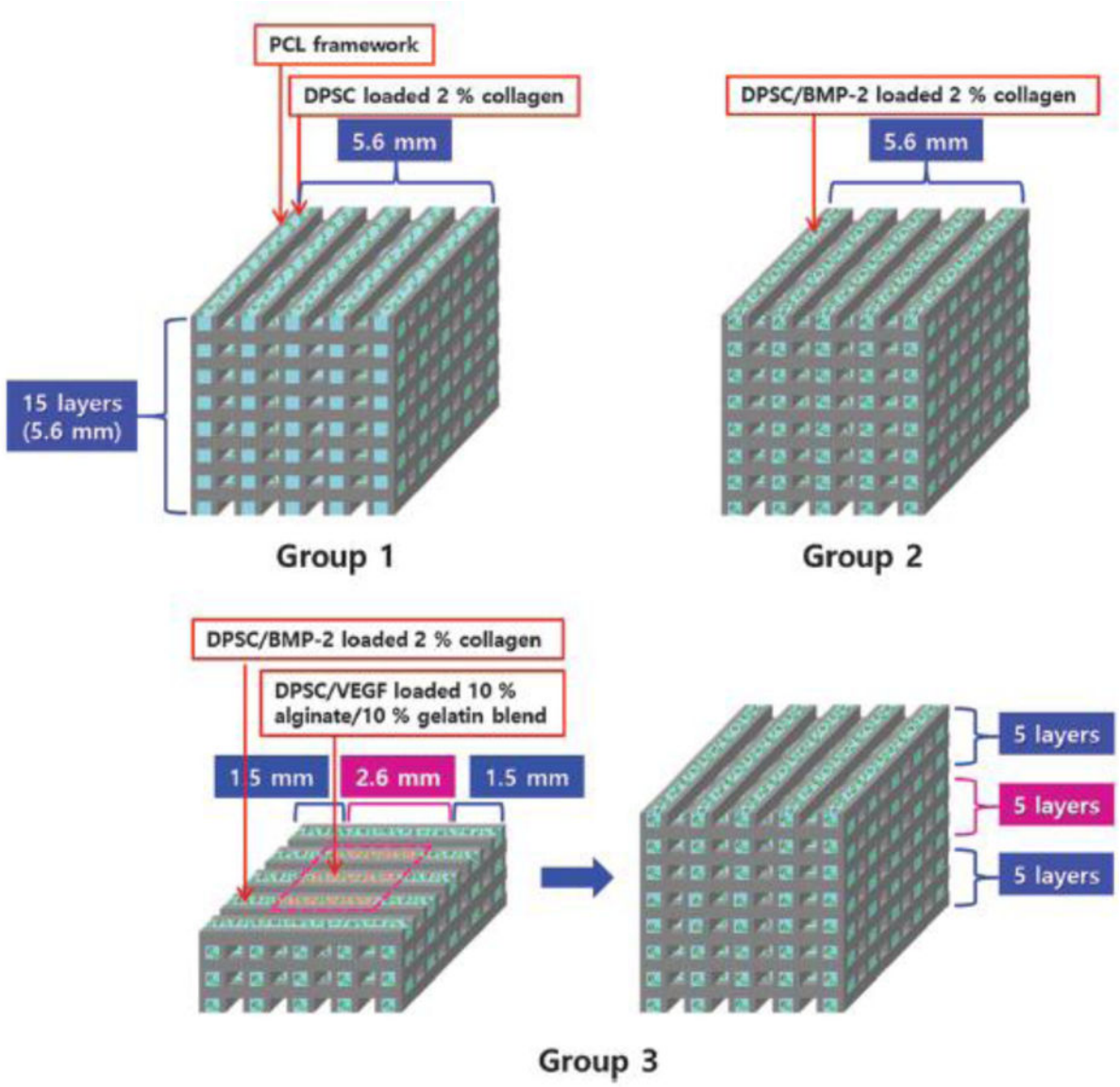

Fig 4:

3D printed scaffold scheme, displaying uniform PCL/DPSC/collagen (Group 1), PCL/DPSC/BMP-2/collagen (Group 2), and biphasic PCL/DPSC/collagen/BMP-2 exterior PCL/DPSC/gelatin/alginate/VEGF interior (Group 3) scaffolds used by Park et al. Reproduced with permission from (85).

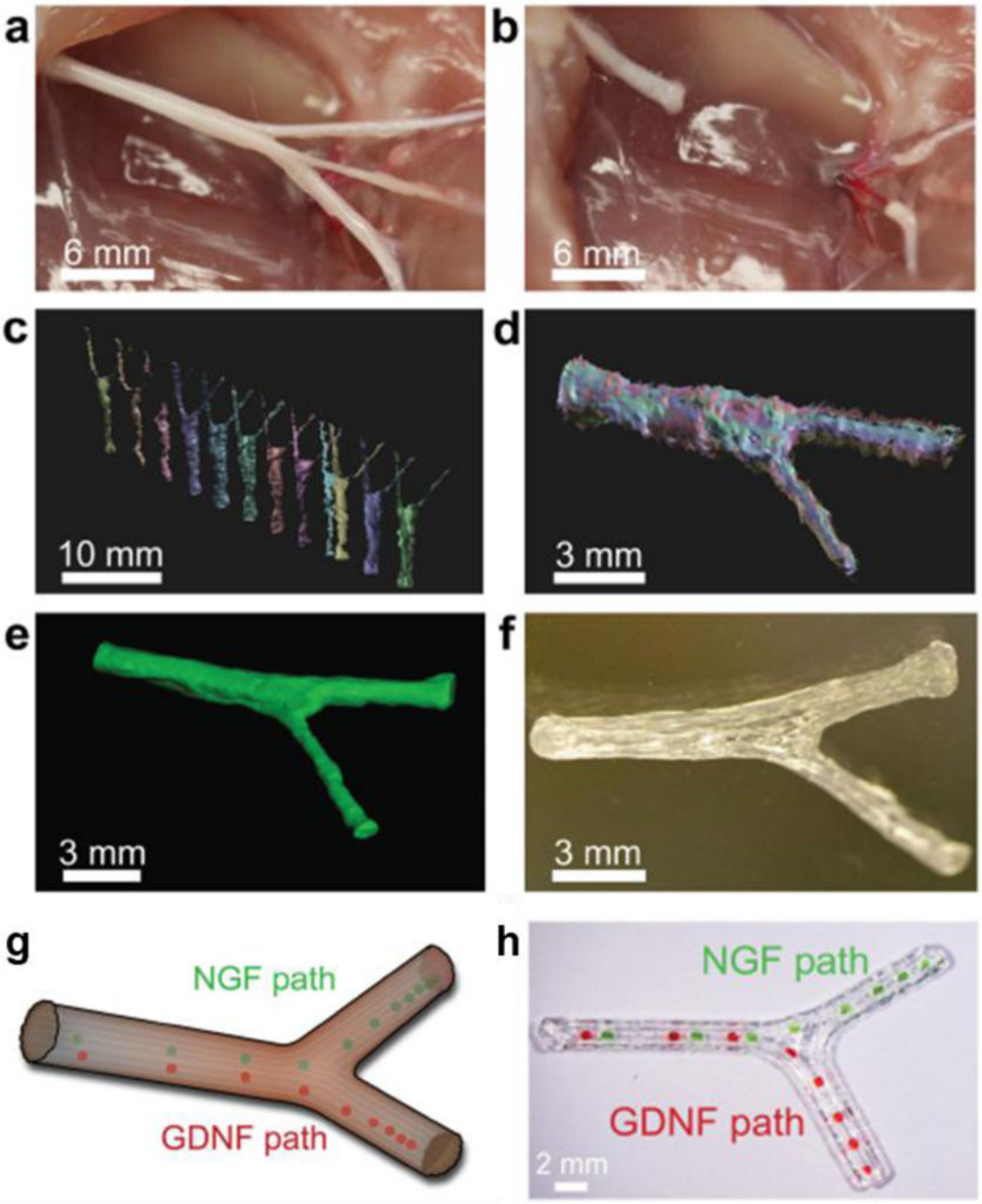

Fig 5:

3D printed bifurcating sciatic nerve developed from computed tomography scanning used by Johnson et al. a) Sciatic nerve with branching sensory and motor nerves. b) Sciatic nerve pathway was transected for modelling. c,d) Several scans of the geometry are taken and aligned into a 3D model of the nerve pathway. e) The aligned scans are reconstructed into a full 3D template. f) Using the reconstructed images, the scaffold is printed into a model mirroring the original sciatic nerve pathway. g) Schematic of designed model with GF placement, and h) 3D bioprinted scaffolds with NGF and GDNF spatiotemporal patterns

(green and red dyes used to indicate hydrogel droplet locations). Reproduced with permission from (18).