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Pharmaceutical electrospinning and 3D printing scaffold design for bone regeneration

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ABSTRACT

Bone regenerative engineering provides a great platform for bone tissue regeneration covering cells, growth factors and other dynamic forces for fabricating scaffolds. Diversified biomaterials and their fabrication methods have emerged for fabricating patient specific bioactive scaffolds with controlled microstructures for bridging complex bone defects. The goal of this review is to summarize the points of scaffold design as well as applications for bone regeneration based on both electrospinning and 3D bioprinting. It first briefly introduces biological characteristics of bone regeneration and summarizes the applications of different types of material and the considerations for bone regeneration including polymers, ceramics, metals and composites. We then discuss electrospinning nanofibrous scaffold applied for the bone regenerative engineering with various properties, components and structures. Meanwhile, diverse design in the 3D bioprinting scaffolds for osteogenesis especially in the role of drug and bioactive factors delivery are assembled. Finally, we discuss challenges and future prospects in the development of electrospinning and 3D bioprinting for osteogenesis and prominent strategies and directions in future.

Contents

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1. Introduction

Bone tissue has a natural regenerative capacity, which is enough to heal small injury sites including cracks, bone defects and some types of fractures. However, the critical size of bone defect caused by pathological fracture or high-energy injury is still a great challenge in clinic. Therefore, bone grafting is always needed. Among all available grafts, although autograft has numerous limitations, involving high incidence rate of donor site, long operation time and poor usability, it is still regarded as the gold standard for bone replacement[1,2]. Hence, successful regenerative scaffolds design for osteogenesis is sorely needed to mimic the structures and components of natural bone tissue, by selecting suitable tunable synthetic or biomimetic natural materials including metals and composites, ceramics and polymers. Electrospinning and 3D bioprinting as pioneering technologies enable preparation of multi-scale, multicellular tissue and bionic structures with complex cell structure, tissue heterogeneity, structural and functional diversity as well as highly complex microenvironment[3]. Although the regeneration of tissue and organ is still a long way to go, great progress has been made in the design of bone regeneration scaffolds as grafting tissues in regenerative medicine.

The embryonic development, morphological structure and fracture healing process of bone provide inspiration for the innovation of bone regeneration engineering[4]. Natural bone is a kind of nanocomposite material, which is heterogeneous and anisotropic. Its main components have several structural levels from macro to nano scale. The structure from the outer dense/cortical bone to the inner sponge/trabecula represents the macro and micro structure levels respectively[5]. Nanocomposites, mainly composed by mineralized collagens and minerals are the characteristics of bone nanostructures. Therefore, in order to translate novel uncovers into new devices for clinical uses, it is urged to imitate natural bone functionality via some advanced technologies, including 3D bioprinting and electrospinning[6]. Concerning bone tissue as a natural nanocomposite, it is needed to feature organic collagens, multiple cell types and inorganic minerals into the scaffold rather than only fabricate a mechanically stable structure.

Fibrous scaffold is considered as a promising alternative for regenerative medicine simulating the structure of natural extracellular matrix (ECM) [7]. Among the various technologies that can be used to fabricate scaffolds, electrospinning is a simple method, which can produce cell attached scaffolds with large surface area, high distance between fibers for cell gas exchange, infiltration and nutrition as well as adjustable support according to tailor the needs. The polymer solution jet is accelerated and towed in the electric field in this technique. Relying on the equipment, preparation conditions of the solution and electric field superiority, the stretch jet is able to break and generate micro/nano droplets, or retain as filaments and produce nano / micro diameter fibers after drying. Significantly, the orientation of electrospinning fibers can provide guidance for attached cells by regulating their differentiation status and affecting their morphology, thereby promoting osteogenesis.

Moreover, bone possesses a complex structure and osteocytes are distributed in the ECM of bone. Thus, a suitable three-dimensional structure is required for mimicking the ideal repair process. Calcium phosphate scaffolds can be 3D-printed into scaffolds with controllable nanopores and customized macropore structures[8]. By promoting the interaction between growth factors and osteoblasts, nanopores structures can trigger the process of bone regeneration with the differentiation of progenitor cells into osteoblasts. By 3D bioprinting, traditional materials can be involved into advanced transplantation but without previous disadvantages including immune-rejection, invariable density and insufficient biochemical functionality[9-11].

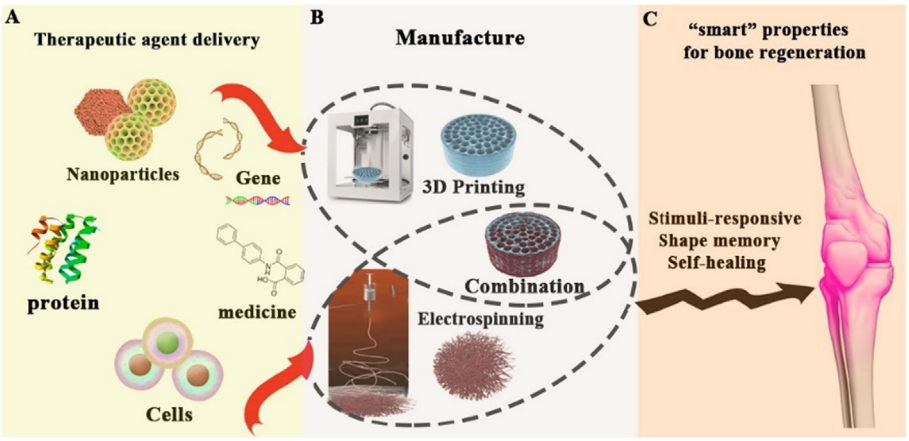
Several recent reviews have summarized the manufacturing technique or mechanical stability of these technologies. However, very few reviews have discussed the combination of electrospinning and 3D bioprinting in one set, based on the structure and functionality of natural bone. Herein, we summarize the pharmaceutical electrospun and 3D printing scaffold designs for bone regeneration including the material selection and structure designing for bone regeneration, drug delivery and bone organoid as shown in Scheme 1. We also discussed the combination of electrospinning and 3D bioprinting for bone regeneration. Initially, we give a brief introduction to illustrate bone biology and pathology as well as materials for bone regeneration. After that, electrospinning design for bone regeneration will be summarized, alongside its application in drug delivery. Correspondingly, we then sum up 3D bioprinting design for bone regeneration and drug delivery likewise. Finally, we briefly discuss the electrospinning and 3D bioprinting design for bone organoid.

2. Bone reparative and remodeling phases

To design effective scaffolds for bone regeneration, we need to understand the nature bone regeneration process. This process includes reparative phase and remodeling phase.

2.1. Reparative phase

Hematoma formation: In the steady-state, adjacent blood cells accumulate in the fracture site, preventing further bleeding. Blood vessel constricting also suppressed bleeding. After a few hours, a hematoma is formed at the site of fracture with the help of blood cells and plasma fibrinogen, contributing to the initiation of a template callus. FXIII can be detected as a crosslinked tool in the formation of hematoma and this tool provides relatively reliable strength and stability for the hematoma[12]. The hematoma equips the fracture site with various growth factors to initiate later



Scheme 1. Therapeutic agents delivery based on 3D printing and electrospinning technologies for responsive bone regeneration.

regeneration. Although this promoting function of the hematoma is useful in the case of a fracture, in the context of granulation tissue formation, the hematoma occupies the only space and has a bad impact on blood circulation, leading to the prolonged procedure of regeneration. Indeed, the clot contracts and undergoes proteolysis before epithelium penetrating it (Fig. 1A).

Granulation tissue and scar tissue formation: Initially, neutrophils instruct and drive an acute inflammation liquefying the necrotic tissue in order to create a proper microenvironment for further repair. Removing necrotic tissue depends on several types of immune cells, including macrophages and fibroblasts. They are recruited to the injury site by fibronectin derived from plasma and cellular debris. Macrophages are capable of ingesting liquefaction tissue while fibroblasts excel at secreting collagens. They have been viewed as crucial inducers in proliferation and neovascularization through various growth factor production. But macrophages can also serve as a collagenase producer, liquefying necrotic tissue as before[12]. Owing to the contribution of macrophages and neutrophils, a glistening and pebbled tissue first appears, called granulation tissue. Provisional matrix and newborn capillaries develop in the surroundings composed of inflammatory cells involving macrophages and fibroblasts. Monocytes and plasma cells are also conspicuous in the granulation tissue recruited from circulation.

Cells and cell cooperation seem like automatic movements in rapidly changing events concerning necrotic tissue movement and functional tissue regeneration. However, growth factors are really incorporated in the cell crosstalk. Growth factors appear at the beginning of damage and maintain their roles as instructors in the whole regeneration process such as proliferation, recruitment, and migration. Increase and decrease of growth factor amount stabilize the matrix: hypoxia activates vascular endothelial growth factor (VEGF) and its abundant expression induces generation of quiescent capillary endothelial cells; As shown in Fig. 1B, angiogenesis leads to the relatively sufficient oxygen and nutrients, mediating downregulating VEGF. VEGF relates to endothelial cell survival, migration, maturation, and proliferation[13]. Moreover, heparan sulfate-containing glycosaminoglycan chains affect the function of VEGF and integrins usually associate with modulating cellular sensitivity to VEGF. Unlike VEGF, integrins serve as locomotion helpers for cells exposed to the disorganized basement membrane, influencing migration and provisional matrix proteins. Without integrins and proper growth factor signaling, the capillary cannot sprout and survive.

Soft callus formation: The periosteum cells in the site of injury replicate and develop into chondroblasts forming hyaline cartilage 7–9 days after fracture. The cells proximal to the gap usually act as different players compared to distal ones. The periosteal cells distal to the fracture gap differentiate into osteoblasts, resorbing calcified cartilage, and recruiting bone cells. The so-called fracture callus consists of woven bone and granulation tissue. Osteoblasts produce woven bone while fibroblasts generate hyaline cartilage, composing into soft heterogeneous callus preparing for hard callus formation later. Generally, soft callus culminates on the fourteenth day after a fracture.

Hard callus formation: Hard callus often refers to replacing hyaline cartilage as well as woven bone. This procedure usually starts with the substitution of woven bone. When the mineral deposits at the collagen matrix, woven bone is transformed into lamellar bone. Although replacement happens later, the hyaline cartilage transformation mechanism is identical to the woven bone. Then interleukin-1 and tumor necrosis factor-α drive micro vessels and plenty of osteoblasts to manufacture lamellar bone exposing to those mineralized matrixes, also in the formation of cancellous bone. Actually, original strength of majority bone is derived from this hard callus formation.

2.2. Remodeling phase

Remodeling occurs three to four weeks after fracture and often keep functioning for 3–5 years (Fig. 1A). Osteoclasts resorb the trabecular bone and create compact bone. The original morphology and power of bone rely on remodeling, thus enhancing this process can bring functional and precise devices for regeneration. This improvement is able to be achieved through the formation of polarity in long bone loading, in which the positive convex surface activates osteoclasts and the negative concave surface activates osteoblasts.

3. Materials for bone regeneration

A widely range of biocompatible materials have been investigated comprehensively as latent organic and inorganic composition of natural bone tissue. These materials can be grouped broadly into, metals, bio-glass, ceramics, and composites that act as sustained substitution for bone defects and osteoporosis. Bone regeneration materials (BRM) are commonly employed in maxillofacial surgery, implant dentistry, neurosurgical cranial repair and orthopedic surgery [14-17]. BRMs were applied in the scaffold (i.e., cementless fixation prosthesis coating, screws, fixation plates), intra-osseous augmentation (i.e., cementoplasty, allograft), GBR membrane and other supplies for bone regeneration. BRM scaffold has many important parameters, including biocompatibil-

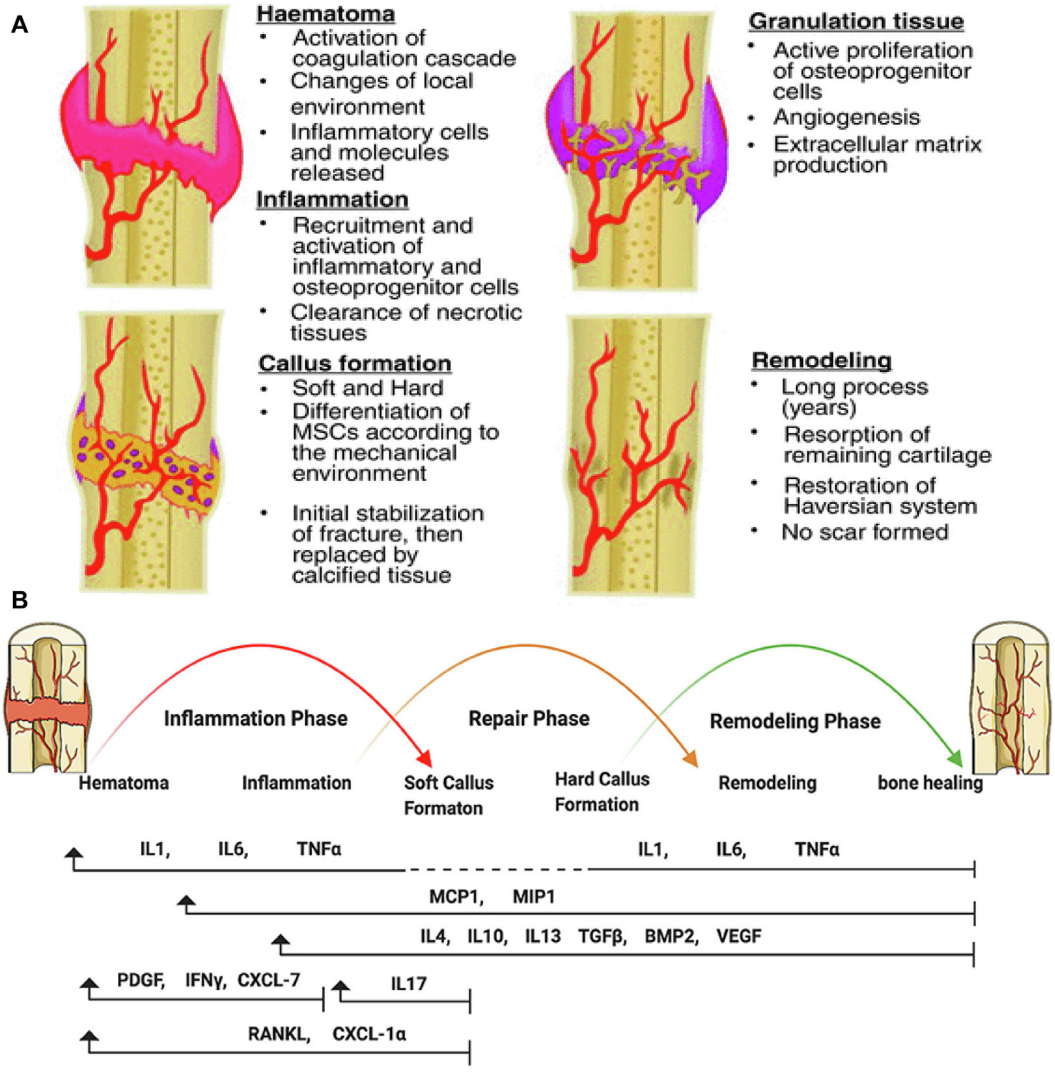


Fig. 1. (A) The hematoma formation, granulation tissue, soft callus formation and remolding stages of bone regeneration[12]. Adapted with permission. Copyright 2018, Springer Nature. (B) Schematic illustration of cytokines, growth factors and chemokines involved in different bone healing stages[13]. Adapted under the terms of the CC-BY license. Copyright 2020, Frontiers Media S.A.

ity, mechanical strength, osteo-conductivity, osteo-inductivity and osteointegration [18,19].

3.1. Metal materials

Metal 3D printing technology, as the most potential technology of bone regeneration, was the most important advanced manufacturing technology of bone scaffold products. At present, rapid prototyping methods to manufacture metal functional components were: powder bed fusion (PBF), direct energy deposition (DED), and binder jetting. DED method includes Wire Arc Additive Manufacturing (WAAM), Laser Engineered Net Shaping (LENS™), and Direct Metal Powder Laser Sintering (DMLS). PBF technology includes Selective Laser Sintering (SLS), Selective Laser Melting (SLM) technology and Electron Beam Selective Melting Technology (EBSMT). Below, we list few different production processes to maximize the bone regeneration properties of different metal materials, most importantly, to overcome the disadvantages.

Titanium metal trabecular bone reconstruction system: Titanium (Ti) and Co–Cr–Mo alloy alloys are extremely suitable and economical materials for load-bearing implant design since they have good mechanical strength and biocompatibility with corrosion resistant. However, due to the absence of trabecular structure and stress shielding resulting from the mismatch in modulus between the implant and nature bone (110–120 GPa for Ti alloys and only 10–30 GPa for human cortical bone), bone resorption may cause alveolar bone remodeling due to insufficient stimulation of bone tissue by stress, leading to implant loosening or loss. Due to this situation, the recommended strategy is to avoid bone resorbing by reducing the elastic modulus: adding alloying elements such as niobium, zirconium, and tin. However, the addition of these materials will affect the biological inertia; thus, titanium metal trabecular bone reconstruction system (TMTBRS) has emerged in porous coating or bone implant scaffolds.

TMTBRS by new generation DED not only were used to design porous structures mimicking cancellous bone, but also have the capability to form an implant using multiple materials by spraying the metal powder onto the base formed with different types of metal. The study of Dong et al. shows the application of 3D-DED metal printing technology to CoCr alloy with porous titanium coating does not affect the viability of osteoblasts and does not impair in-vitro osseointegration. However, for bone regeneration, there were no statistically significant between two groups (smooth and D.E.D. Ti-coated). Laser Engineered Net Shaping (LENS™) is a Laser

based solid free form (SFF) techniques that do not use a powder bed to fabricate near net shaped metallic parts with complex geometries. By adjusting processing conditions porosity rate can be modified.

EBMT, is an important branch of PBF technology that can construct the interface needed to support inward bone growth and widely used in clinical practice, a product like 3D Titanium Alloy Trabecular Filling Block (by AKMEDICAL Inc. Beijing, China) was developed and produced to repair bone defects for procedures like revision after arthroplasty. TMTBRS were often present with mesh and foam structures, characterized by α′-martensite with residual-α to induce bone regeneration. Electron beam melting (EBM) system is composed of an electron gun, which generates an electron beam to focus electromagnetically scanned onto powder layers gravity fed from cassettes and mechanically raked into layers for printing. Compared with the DED process, the prototype manufactured by electron beam machining has more exquisite directional microstructure, and the rigidity is stabilized, while the yield stress and elongation are also preserved. As for result, the EBM implant prototypes are fabricated for compatible bone stiffness in contrast to requisite density.

Customized porous tantalum augment: Tantalum (Ta), an extremely inert metal with biomimetic trabecular microstructure, is the most common bone defect repair metal material at present due to its outstanding biological activity and excellent corrosion resistance[20-22]. Research report that TA can induce bone regeneration by activating the BMP2/Smad4/Runx2 signaling pathway, which in terms causes BMSCs (Bone marrow derived mesenchymal stem cells) to undergo osteogenic differentiation[23]. However, Ta implant is often processed by deposition of extravagant pure Ta powder (cost about 600USD/kg) on carbon and mold by chemical vapor deposition (by Zimmer Inc. Warsaw, IN, USA). Due to the lack of customization, fixed Ta scaffold products often forced surgeons to compromise on the critical-sized bone defects, which may result in the procedure being less smooth and the stability of implantation weakened[24,25]. Selective laser melting (SLM) is a type of laser powder bed fusion (L-PBF) assisted additive manufacturing (AM). It utilizes a laser to melt and fuse prepositioned powder materials directly form parts which could be completely functionalized. Tantalum powder is deposited layer-by-layer through laser melting in an argon environment. Customized porous tantalum augment obtained through cleaning and disinfection.

Absorbable metal scaffold: In scaffold fabrication, biodegradable materials are preferred to reduce residual artificial matrix at the regenerated section. Moreover, the risks associated with permanent implants, such as chronic local inflammation, constant physical irritation, and implant-associated infections, can be avoided after the biodegradation of the implants. The surface and internal patterns of the scaffold are the key parameters to determine the initial success of cell attachment and subsequent endogenous growth of the scaffold. The porosity of the scaffold directly affects the rate of cell attachment, degradation and carriers release because it determines the surface area of cell-scaffold interactions

Magnesium (Mg) is the second most abundant intracellular cation in human, and more than half of Mg is stored in bone, which makes Mg alloy to be an appropriate material for absorbable scaffold[26]. Mg has superior advantages in bone regeneration. Firstly, the density and mechanical modulus of Mg are similar to natural bone; Secondly, Mg can be degraded in body and mainly metabolize into non-toxic magnesium oxide and Mg^{2+} during degradation, which can be completely passed out of our body [27-29]. However, the uncontrollable degradation rate of magnesium alloys impedes their applications in practice[30].

For the particularity of Mg powder, due to the high flammability of excessive total surface area, there are worrisome safety risks during production. Due to the rapid increase of vapor pressure during melting and boiling temperature, fine Mg powder particles have a high affinity for oxygen and will be excessively oxidized during the fabrication of the scaffold. These properties severely limit the availability of pre-alloyed magnesium powders with the required composition that can be used to regulate the biodegradation rate and mechanical strength of porous scaffolds. Therefore, high energy AM like PBF, the most commonly used bone scaffold AM technology, is not suitable for this type of metal material, despite structural fineness and integrity is more controllable.

Binder Jetting (BJT) is an additive manufacturing process which has a great potential for overcoming the limitations of structures, technologies, materials as well as other Am technologies. It offers several advantages, including (1) 3D printing in environmental conditions, (2) easy adjustment of the ink composition, (3) the possibility of providing load drugs or other biological agents, and (4) the potential to manufacture complex structures with graded holes and required alloy composition. In addition, Binder Jetting precise matching of solvent evaporation rates and printing parameters allows printing of stacked lattice structures with overhanging sections and the ability to produce helical free structures without any synthetic support. However, BJT method manufacture magnesium based materials still have limitations, including (1) due to the high reactivity of magnesium powder, the choice of adhesive components, resulting in printing and interaction in the process of degreasing, and ruled out the use applies to other metal adhesive system, (2) in the absence of external pressure magnesium powder sintering can be poor, because in the magnesium powder particles inevitably exist on the surface of a stable layer of oxide film, as a diffusion barrier. (3) The final step in the manufacturing process includes de-binding and sintering to remove the binder in ink, the thoroughly adhesive elute and stability of the whole product be maintained after elution were considered as key outcomes measurement. Therefore, the core research direction of BJT is to find more applicable adhesive system and low-temperature deposition manufacturing (LDM) technic for the corresponding product.

Doped metal materials for additive manufacturing: Zinc is necessary for skeletal development, and extensive researches have demonstrated that zinc has the ability to promote osteoblast proliferation and differentiation. Zinc can also increase alkaline phosphatase (ALP) activity[31,32]. Also, zinc ions cause damage to the bacterial membrane in achieving the purpose of antibacterial features. This feature is usually used to prevent surgical site infections (SSIS) and is realized as a chemical antibacterial method (CAM). CAM could be applied to biocompatible/biodegradable polymeric scaffold via dip coating (direct mineral addition method, electrophoretic deposition, metal-particle composite, or sputtering technique.

Zn alloy coatings that promote bone regeneration are often deposited onto the fabricated scaffold using chemical vapor depositions like RF (radio frequency) magnetron sputter system (MSS) and other plasma spraying technology. Zn alloy was most commonly doped into other metal、ceramics, and polymers to form composites material fabricating scaffold which promotes bone regeneration. 3D printing technology (direct inkjet printing) or electrospinning technology to ceramic-polymer composite solutions. High levels of free radicals can destroy osteoblast through the oxidation of proteins and lipids[33]. In this regard, cerium oxide (nanoceria) has garnered the interest of biomaterials engineer due to its exceptional antioxidant property by inhibiting the oxidation of other molecules from reactive oxygen species (ROS) due to its free radical scavenging[34].

There are other metals and their corresponding ions which have been demonstrated for in situ mineralization techniques for organic scaffolds to achieve the goal of more natural bone regeneration. The most common method is to immerse the organic scaffold in a solution containing calcium and phosphorus ions.

Mineral ions are deposited on the organic fibers and gradually crystallize and grow in the scaffold. Other alternative metal ion options include the following. Webster et al. found that the adsorption of calcium, vitronectin and collagen increased when yttrium was doped on Hap. In addition, zirconium and molybdenum can also be applied to other metal alloys, and used in plastic surgery and dental applications[35]. It was previously suggested that vanadium can promote bone regeneration [36]. Early studies found that vanadium derivatives can promote the proliferation and even differentiation of osteoblast-like UMR106 cells [37]. Strontium can treat osteoporosis through a dual mechanism. Strontium also promotes osteoblast differentiation and reduces the viability of osteoclasts [38].

In summary, the application of any metal alone as BRMs scaffold has its limitations and contradicting characteristics. Fortunately, the recently discovered additive manufacturing (AM) porous metal biomaterial has many advantages and can be used as an excellent bone substitute [39,40]. AM porous material can precisely control its topology design [41], and then can be used to simulate the mechanical properties of bone[42], and ultimately promote the proliferation and differentiation of bone cells [43].

3.2. Polymers

Natural polymers used for co-precipitating bone regeneration scaffold include specific proteins (collagen, gelatin, keratin, silk sericin, fibrin, fibroin, xanthan gum, carrageenan), polysaccharide (alginate, chitosan, hyaluronic acid) and polyhydroxyalkanoate (PHA). The synthetic polymer includes polylactic acid, PLGA, polycaprolactone, polyethylene glycol (PEG) and poly (propylene fumarate) (PPF) have been used as fabricating AM materials for 3d porous scaffolds[44-49].

Polymer scaffold fabrication methods can be roughly divided into powder and extrusion-based strategy. Polymers AM often co-precipitated with metal, bio-glass and ceramic or ions for in situ mineralization. Porous structures were produced by electrospinning, gas foaming, cryotropic gelation and porogen (NaCl particles) leaching method. And number of crosslinking techniques (chemical crosslinking, ion crosslinking and adhesive crosslinking) have been used to reinforce the material and controlling decomposition rate. The porous structure of polymers scaffold provides a shell for the newly formed osteoblast and sufficient sites for cell attachment, also can impregnate drugs, growth factors while providing temporary bone mechanical support. However, high temperature modeling (FDM), for example DED, PBF and fused deposition extrusion could thermally degrade these additives, and are not best suited for such applications.

Proteins: Collagen I (Col-I), the most significant component in the bone, is a force sensitive protein with a diameter of 80–100 nm. Col-I monomers are composed of two α1 chains and one α2 chain. They can form fibrils by self-assembly and present horizontal stripes at 67 nm, which is called the D cycle. At present, the most common preparation method for col-1 based AM scaffold is the direct mineral addition method. By directly adding hardness materials such as metal, ceramic, and bio-glass powder, the mechanical strength is enhanced and the biological properties of the additive materials are obtained. However, the composition and proportion of natural bones cannot be completely simulated by this technique, while in-situ mineralization method can obtain a more natural bone structure and achieve the goal of autologous mineral deposition by adding specific ions. Currently, the most popular research direction is the use of electrospinning technology creating nanoscale Col-I cell scaffolds to promote bone regeneration. By applying a high voltage to the needle between the solution and the fiber collector with opposite charges, the solution with the same electrode charges repels apart, causing the solution to stretch in the form of a fiber jet deviation filament.

Gelatin, keratin, silk sericin, fibrin, fibroin, xanthan gum were natural polymers that are very similar to col-I chemical composition. Gelatin has a peptide sequence, namely l-arginine glycine - aspartic acid (RGD) [50]. It helps to cell adhesion, proliferation and differentiation[51-54]. These advantages of gelatin have attracted many researchers to apply it to bone regeneration materials (Fig. 2A, B) [55-57]. Silk fibroin protein is a biomaterial for bone regeneration, not only because it stimulates extracellular matrix and has cellular compatibility, but also because it can template the growth of hydroxyapatite crystals, leading to bone integration. As an osteogenic biomaterial, silk fibroin protein has the potential to induce stem cell differentiation by inhibiting Notch pathway.

GelMA as a nature polymer material is made of gelatin and methacrylate anhydride, has attracted more and more attention for the regenerative medical investigation and clinical application. Due to the presence of methacrylate anhydride, GelMA is capable of producing thermally stable crosslinked hydrogels by applying photoinitiators and under UV irradiation, controllable degradation rate enables it to complete the bone regeneration process. Using this property, a solution of gelatin sponges could be carried into varied shaped bone defects.

Polysaccharide: Natural polysaccharides are often used in addition to making bio-hydroxyapatite products (Bio-HAp) to increase their toughness and cell adhesion. Chitosan is a biodegradable natural basic polysaccharide, and the by-product of degradation is non-cytotoxic. The combination of hydroxyapatite and chitosan showed good rigidity and good biocompatibility. The rough micromorphology of the AM coating can increase the contact area of the metal implant-bone interface and show good osteoattractivity.

Alginate is a biodegradable and biocompatible material that has been widely used for bone regeneration [58] due to its similar structure to natural extracellular matrix and easy to be crosslinked by Ca^{2+} ions. However, an obvious drawback of alginate scaffolds is their poor preference to adhere to cells, as the availability of cell adhesion sites is limited[59]. In order to overcome this weakness, alginate was modified by adding cell adhesion peptide [60] or mixed with other biological materials with a large number of cell attachment sites [61,62].

Polyhydroxyalkanoate: Poly (3-hydroxybutyrate-co-3-hydroxy hexanoate) (PHBHHx) is a naturally occurring biodegradable polymer synthesized by bacteria, a member of polyhydroxyalkanoate, PHBHHx is thermal processing making it possible for various high-temperature environments. Researches report that PHBHHx incorporated particulate hydroxyapatite (HA) composites can achieve mechanical strength in compression about the same magnitude of human bones. In vivo experiments also showed a strong tendency to reconstruct bone structure at the bone defect interface after implantation of the composited scaffold. Cell culture experiments have also shown that PHBHHx has the potential to support cell scaffolds.

Synthetic polymer: The main advantages of natural polymers are the biological suitability and cell adhesion. However, due to its uncontrollable degradation rate, none crosslinking capability, synthetic compounds have become the main reference for AM materials. Unfortunately, though most synthetic polymers have good mechanical properties, inflammation caused by acid and crystallinity degraded by-products remain a problem [63].

At present, some synthetic bio-hydroxyapatite bone scaffolds (such as PEG-HA bone scaffolds) have already been used in clinical practice. For the prediction of long-term effects, we should pay attention to the sensitization of the toxicity and elution of crosslinkers, as well as by-products after degradation. Such as reported that Poly-L-lactic acid (PLLA) crystallinity and Poly

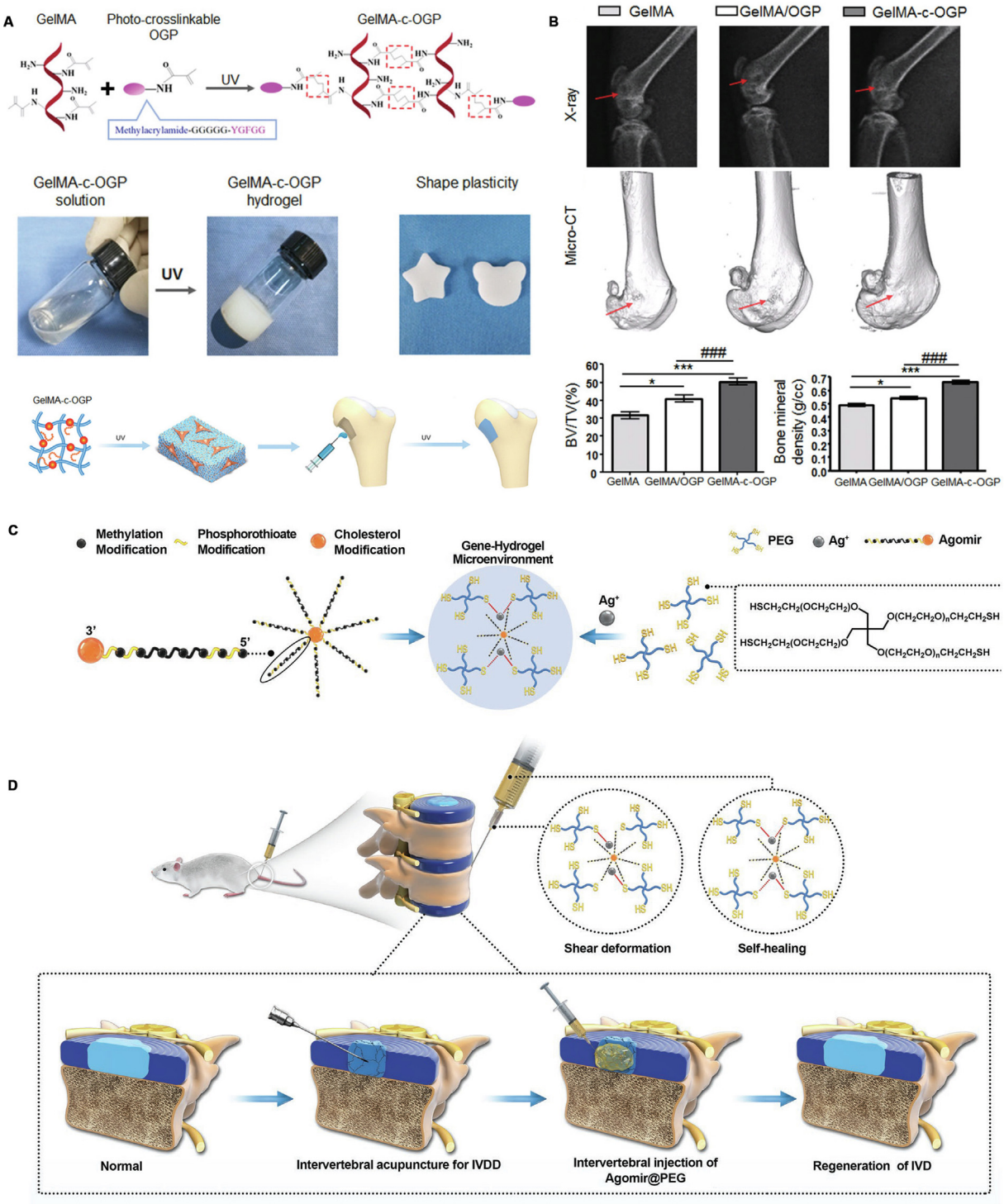


Fig. 2. (A) GelMA hydrogel structure conjugated with methacrylate modified OGP polypeptide and photo-crosslinked photo [56]. (B) The protein compound GelMA hydrogel showed a higher therapeutic effect under CT imaging [56]. Adapted with permission. Copyright 2019, Wiley. (C) Gene-Hydrogel construction [91]. (D) Agomir@PEG was administrated into the intervertebral space to establish the regeneration of intervertebral[91]. Reused with the authorization. Copyright 2020, Wiley.

(lactide-co-glycolic acid) (PLGA) acidic byproduct may cause inflammation.

3.3. Ceramic and bio-glass

A variety of ceramic bone graft substitutes have been investigated for the past decades [64-68]. Bio-ceramics are manufactured mostly by powder bed process (i.e. SLS/SLM, binder jetting). Cause in powder bed process, the ceramic powder is consolidated into the desired shape of the ceramic product using a colloidal ceramic process.

Hydroxyapatite: HA is composed of calcium ions and phosphates present in the body, so its biocompatibility is extremely high [69,70]. The HA surface supports the adsorption, growth and even differentiation of osteoblasts. At the same time, adding growth factors required for bone regeneration into HA can significantly

improve bone regeneration efficiency [71]. However, HA has a stable structure and cannot be degraded in the body, so the cells loaded inside are difficult to move and have poor adhesion [72-74]. With the continuous development of nanotechnology in recent years, the function of HA has become more powerful [75,76], not only can it achieve antibacterial function, the spatial rotation of HA particles makes HA even at heterotrophic sites also osteoinductive [77,78].

β-tricalcium phosphate: Beta-tricalcium phosphate belongs to the third generation of biomaterials, namely, those having appropriate micro- and macroporosities, good mechanical properties and promoting not only bone substitution but also bone regeneration [78,79] with different formulations as granules, blocks, injectable form [80,81] in many surgical sites of orthopedic surgery as spine, hip, knee, fractures.

Additively manufactured bio-ceramic: In view of the increasing demand of intraosseous augmentation surgery, it is urgent to find suitable materials to promote bone formation. However, compared with scaffold, intra-osseous augmentation needs to be fluid or granule and fixed into a supporting structure over time, synthetic bone graft substitutes currently see limited use clinically because of their inferior in vivo performance when compared to autogenous bone grafts[82]. Most synthetic bone grafts conventional materials like PMMA (polymethyl methacrylate), CSC (calcium sulfate cement) and hydroxyapatite cement have a limited capacity to reconstitute bone cause the lack of biodegradability, osteoinductivity or osteogenicity of autologous bone grafts[83,84]. The poly (L-lactic acid) (PLLA) composites containing calcium carbonate have much higher hydroxycarbonate apatite (HCA)-forming ability in simulated body fluid (SBF) [85,86] than conventional composites containing calcium phosphates[87,88].

Bioactive glass: Bioactive glass and glass-ceramics are two other preferred materials for bone regeneration because they enhance osteoblastic adhesion, differentiation of mesenchymal stem cells and progenitor cells, and angiogenesis. Bioactive glass is a material composed of crystalline phases embedded in a matrix of amorphous glass and eventually presents an amorphous structure. Therefore, glass-ceramics belong to the material category between the glass and polycrystalline ceramics. The mechanical and biological properties of glass-ceramics are significantly different from those of their parent glass phases.

Increasing the content of network modifier will decrease the connectivity and degree of polymerization of glass. Although vitreous silica is chemically very stable, the addition of silica network modifiers reduces its stability, thereby increasing its solubility and biological activity. The biological activity mechanism of these glasses is based on solution-mediated dissolution. A layer of hydroxyapatite is formed on the bioactive glass surface by ion exchange between this dissolved material and the material originally present in solution (humoral or simulated humoral). In general, researchers need to determine whether the bone defect they are repairing is in the weight-bearing area (lower extremity joints, lumbar vertebra) functional areas (teeth, upper extremity joints) or purely bone-covered areas (e.g., skull) before selecting the response material for manufacturing scaffold. The compromise between biodegradable materials for natural bone healing and mechanical support should be the main research direction in the future.

3.4. Potential applications

Two-dimensional (2D) materials have emerged as a new promising research topic. In particular, graphene and black phosphorus (BP) were emerging 2D crystal material with unique layered structure and excellent physicochemical properties, such as adjustable band gap, good biocompatibility and high photothermal conversion efficiency. It has been widely used in the biomedical field [89,90]. BP may be coated in negatively charged graphene oxide nanosheets and then adsorbed on a positively charged polymer three-dimensional scaffold. The increased surface area provided by GO nanosheets will enhance cell attachment in the initial phase. Also, the slow oxidation of the BP nanosheets coated in the bone tissue layer results in the continuous release of phosphate, an important promoter of osteoblastic differentiation designed to stimulate osteogenesis and new bone formation.

In addition, a report of an interpenetration network hydrogel is developed utilizing graphene oxide (GO) can significantly promote M2 type differentiation of macrophages and osteogenic differentiation of BMSCs. At the same time, sufficient mechanical stiffness, strength and stability of the hydrogel scaffold are ensured. At present, there are still many limitations in the common additive methods, such as the tool resolution, the need of support structure, ceramic shrinkage during high temperature processing and so on. As an alternative to the additive method, the molding technique of simultaneous assembly in the entire manufacturing chamber has recently emerged.

In the magnetic levitational assembly method, the shape and microstructure of the scaffold are determined by magnetic fields and electric fields. The desired geometry of the 3D scaffold based on the preliminary 3D model is achieved by calculating and simulating the specific distribution of the physical field in the 3D space. In order to provide conditions for the suspension of diamagnetic objects for magnetic assembly, a paramagnetic medium, such as gadolinium-based (Gd^{3+} -based) are commonly used by magnetic levitation methods

Another potential additive for bone regeneration is liposome nanoparticles, which regulates gene expression by delivering synthetic miRNA, provides an ideal potential tool for many application of bone defect repair (Fig. 2C, D) [91]. Lipid nanoparticles like Agomir are a fragment of miRNA modified by cholesterol, methylation and thiophosphate, which can simulate the function of miRNAs to regulate the expression of target genes[92]. As a non-viral vector, it has the characteristics of easy synthesis, low immune response, high biocompatibility and high safety[93].

4. Electrospinning design for bone regeneration

4.1. Morphology, arrangement and pattern control of bone regeneration

Nanofibers with diverse morphologies such as hollow, core sleeve as well as beaded structure and nanofibers of different fiber diameter or other unique structures, can be manufactured through spinnerets and collectors of other configurations, as well as by adjusting the parameters. Due to the special layer and structure of bone, in order to imitate the natural growth state of bone, electrospinning fiber membrane influences the proliferation and differentiation of osteoblasts by biomimetic roughness, dynamic compressive strains and layer. In essence, diverse surface texture augments the total available surface area, which makes cells attach and proliferate better on rough surface [94,95]. Previous researches have illustrated that electrospinning nanofibers with HA nanoparticles create appropriate rough surface for cell attachment and growth and also show better in mechanical properties. In another work, via crystallization, automatic phase separation and electrospinning, researchers successfully attained core-sheath structures in scaffold including chitosan (CS) as well as polylactic acid (PLA), which supported a more ideal adhesive interface among cells and promoted the MC3T3-E1 cell proliferation on the fibrous surface by increasing the alkaline phosphatase activity [95].

By a mass of techniques such as emulsion electrospinning, coaxial electrospinning, a sacrificial template, and the use of controllable heating of preformed nanofibers researchers make hollow nanofibers possible. Hollow nanofibers play a unique role in the osteoblastogenesis. The current study found that chondrogenic differentiation of the cells was able to be induced by a specific range of dynamic compressive strains which showed a stronger induction rather than osteogenic biochemical factors. While maintaining other morphological and chemical elements, researchers designed an electrospun scaffold with local strain gradients which was able to be adjusted by diverse mechanical properties varying with dynamic hollow core dimensions in the core-shell microfibrous scaffolds, thereby finding low localized compressive strains regions more differentiated hMSCs toward an osteoblastic-like phenotype while high strains led to chondrogenic differentiation[96]. Beyond that, to mimic the Haversian canal which facilitates the transport of nutrients and the discharge of metabolic wastes by small blood vessel in it, tubular nanofibers were used to create functional vessel-like structure (Fig. 3A). In a study, so as to create osteon-like structures with similar function like native osteons, osteon-like structures were designed to mimic the Haversian canal by creating a functional vessel-like tubular core with porous shell surrounded for bone tissue adhesion, which makes it possible to provide nutrients and oxygen to osteoblasts[97].

In addition to the mechanical stimulation mentioned above, electrical stimulation also affects cell differentiation. The piezoelectric properties of fibrous scaffolds made by poly (-vinylidene fluoride-trifluoroethylene) (PVDF-TrFE) were activated by applying cyclic compression at a physiological frequency. When electrical stimulation continued to generate, findings demonstrated that lower levels promoted chondrogenic differentiation and higher levels promoted an osteoblastic-like phenotype, which indicated the level of piezoelectric activity of the scaffold adjusted MSC differentiation (Fig. 3B)[98]. The diameter of electrospinning fibers also influences the differentiation and proliferation of cells. A study demonstrated that cell morphology and cell proliferation varied with surface topography by electrospun fibers diameter ranging of 0.14–2.1 μm [99].

Patterning of Nanofibers: High total available surface areas and roughness of patterned micro- and/or nanostructures of Nanofiber-based mats make it a research hotspot. In a study, using the technologies of UV-initiated photolithography, chemical

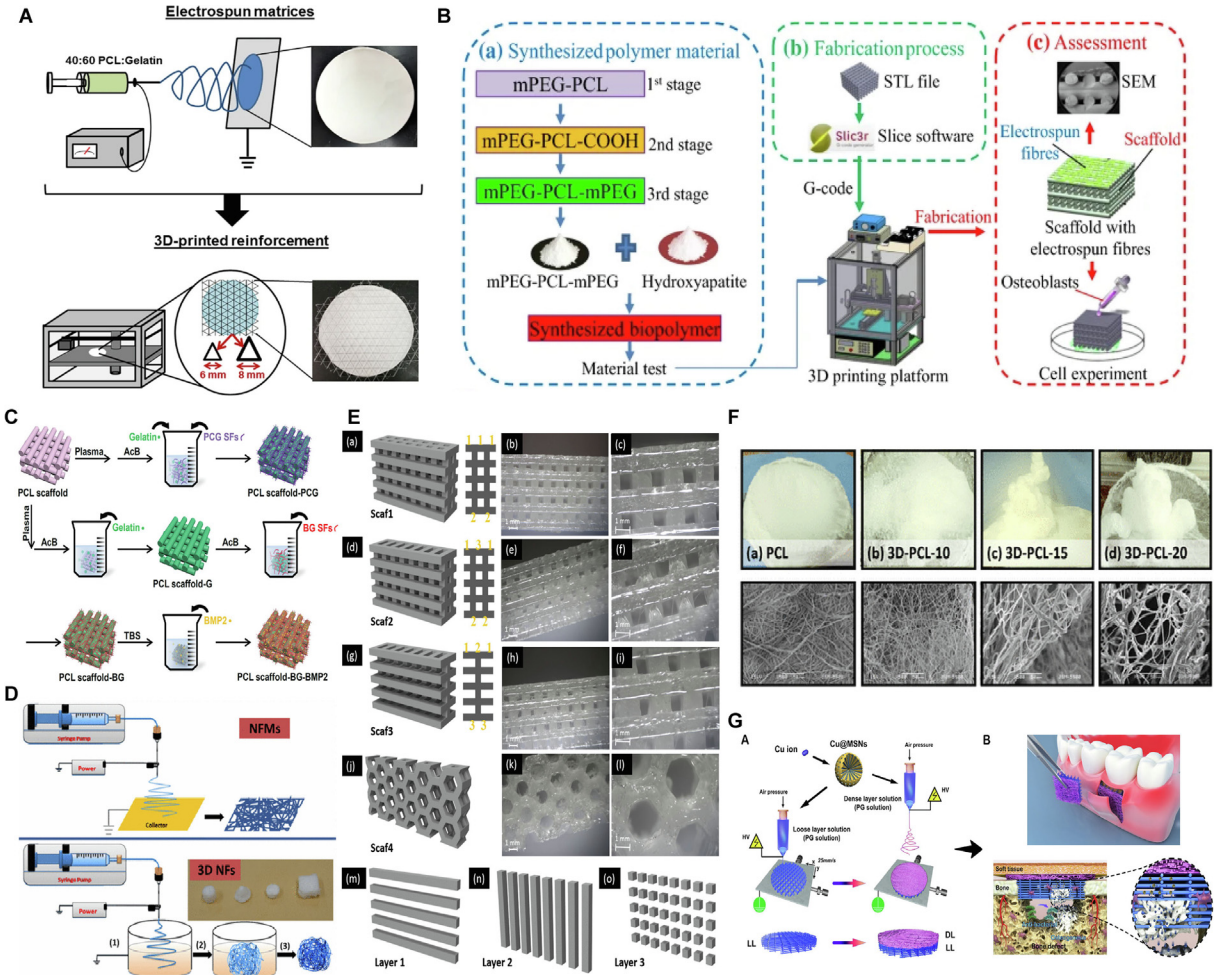


Fig. 3. (A) The methylene blue staining and SEM morphology of pre-osteoblasts cultured on shell–core PCL35 or PCL/BCP35 coil scaffolds[97]. Adapted with permission. Copyright 2013, Elsevier. (B) Representative photographs and histological results of as-spun PVDF-TrFE, annealed PVDF-TrFE and PCL scaffolds processing chondrogenesis dynamically[98]. Adapted with permission. Copyright 2017, Elsevier. (C) Immunofluorescence staining for focal adhesion protein of bone marrow mesenchymal stem cells on different nanofibrous micropatterned (NF-MP) matrix[100]. Adapted with permission. Copyright 2018, American Chemical Society. (D) Reconstruction molds of bone defect areas implanted with various patterned scaffolds. Adapted with permission[102]. Copyright 2020, Elsevier. (E) The SEM images of randomly oriented and aligned scaffolds, as well as the morphology of mesenchymal stem cells[8]. Adapted with permission. Copyright 2018, Elsevier. (F) Focal adhesion and cytoskeleton morphology of mesenchymal stem cells seeded on different scaffolds[103]. Adapted with permission. Copyright 2012, Elsevier. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

crosslinking and electrospinning researchers mimicked natural extracellular matrix in both chemical composition and architecture in which a single cell was confined to eliminate potential communications between other cells. Using the nanofibrous micropatterned matrix as a platform, they examined the relation between adhesion, proliferation and differentiation of a single bone marrow -derived stem cells (BMSCs) and the nanofibrous architecture (Fig. 3C). Compared to it on a non-nanofibrous surface, the BMSCs on the nanofibrous micro island obtained smaller spreading area, higher ALP activity and less focal adhesion, which exhibited a more in vivo-like morphology [100]. However, as the pore sizes in the electrospun scaffolds matrices are not big enough, cells are not able to efficiently infiltrate, which has been a known limitation. Additionally, without vascularization, the nutrient transport, waste discharge and the amount of tissue-ingrowth are limited by the small pore sizes. To solve this problem, electrospinning PEO sacrificial fibers was created to make the adjustable diameter of electrospinning pore, thus enhancing infiltration among cells[101]. In addition to micropattern, layered functional structure also has a unique function. Due to hierarchical complexities of bones, porosity and composition distributions in biodegradable constructs need to be designed to be functionally graded. For example, using the collagen self-assembly technologies as well as electrospinning, researchers designed hierarchical micro/nanostructure by the self-assembly of collagen liquor in the pores of electrospun fibers, which create an appropriate microenvironment to promote cell adhesion, differentiation and proliferation, thereby constructing similar structure to native proliferation (Fig. 3D)[102].

Control of Alignment: As we all know, smooth surface, circular cross-section and uniform diameter are typical morphological features of electrospun nanofibers. In addition, alignment is also one of its characteristics of great concern. In numerous studies, accessing random and aligned electrospun nanofibers is desired. It has been demonstrated that electrospun nanofibers as scaffolds provide attachment sites for cell adhesion, proliferation, and calcification. Likewise, mineralized collagen fibrous structure in natural bone could be mimicked better by combination of electrospinning techniques for bone tissue engineering[103]. Whereas, in the complex architecture of bone, cells are distributed throughout the bone tissue[104]. Hence, in order to promote cell growth and tissue regeneration, thereby accurately mimicking native structure of ECM, a well-defined structure is essential (Fig. 3F). Therefore, with numerous alignments, electrospun nanofiber scaffolds which show better in guiding cell adhesion, cell proliferation and cell differentiation, which has been proved both in vivo and in vitro should be a better choice[105]. Although not only random but also directional scaffold promoted cell proliferation and improved osteogenic differentiation of adipose derived mesenchymal stem cells, aligned scaffolds showed better osteogenesis (Fig. 3E)[8]. This might be the result of which the attached cells were able to be guided by the orientation of electrospun fibers in cell morphology and even cell differentiation[8,106]. In a present study, findings indicated that cultured on aligned scaffolds adipose mesenchymal stem cells preferred to the osteogenic differentiation.

In another study, distinct cytoskeleton regulation signaling appeared and PPAR signaling down-regulated in MSCs on the aligned fibers, which inhibited adipogenesis and initiated the osteogenic switch. To be specific, it has been showed that adhesion of cells is observed in a sole fiber whose diameter is greater than 10 μm while some fibers whose diameter was less than 10 μm. Therefore, morphology of the scaffolds is able to be controlled to adjust cell alignment especially in a single fiber. Hence, applicable alignment and diameter of electrospun nanofibers control the directional distribution of osteoblasts, thereby determining the apatite/collagen matrix alignment in bone tissues. Moreover, the alignment also is able to improve mechanical strength and decrease the diameter due to its additional drawing and better fiber-packing, which provides additional benefits[7,107].

In addition to the electrospinning as the most common way to product nanofibers for bone scaffolds, airbrushing which means that we can use compressed gas to blow polymer solutions into fibers for making tissue scaffolds. nanofibers yielded by air brushing showed more loosely packed bundles of aligned nanofibers than that made by electrospinning which seemingly appeared more to promote cell adhesion and cell proliferation. However, the advantages in osteogenesis need to be studied in more experiments.

Some challenges are also accompanied by the progress in electrospinning for bone tissue regeneration. The functions of biomimetic scaffolds based on nanofibers, including mechanical properties, release curves of bioactive substances, and interactions with cells, are highly dependent on equipment parameters and culture conditions. Although important progress has been made in the application of electrospinning in bone regeneration, the understanding of its mechanism is still insufficient, which limits the further development of this field. Meanwhile, more systematic studies are needed to establish the relationship between scaffold structural characteristics, biological delivery, cellular function, and bone regeneration. Rapid advances in genetic engineering and synthetic biology have brought more therapeutic potential to the field of bone regeneration, increasing complexity but also bringing new opportunities. This adds to the need for an interdisciplinary approach and collaboration in order to respond more effectively to challenges and to advance the development of the field.

4.2. Electrospinning with “smart” properties for bone regeneration

Electrospun materials have been bestowed with “smart” attributes over the past years, expanding their application spectrum to diverse fields, including bone regeneration. For instance, stimuli-responsive electrospun materials can experience volume and/or wettability alteration when encountering external stimuli including the change in temperature, magnetic field, and infection, thus enabling diverse applications, including controlled or on-demand agent release, minimally invasive surgical implantation, guided cellular response, among others.

Shape memory materials, as a subgroup of the stimuli-responsive materials, are qualified of transforming from an original state to a less invasive deformed state when encountering external stimuli such as temperature, magnetic or light trigger, thus providing an alternative instructional strategy for smart implants and medical devices[108,109]. Along this line, nanofibers with memorized morphology are frequently constructed by electrospun polymers which is able to take diverse structures as temperature varying. For example, researchers performed dual-electrospinning to electronspin the miscible nanofibers composed of poly (vinyl acetate) (PVAc) and poly (lactic acid) (PLA). The dynamic mechanical analysis demonstrated that the interwoven structure for PLA/PVAc composite exhibited two well-separated phase transitions, among which temperature serves as the switch[110]. With the incorporation of graphene nanoplatelets, which can improve properties of the fabricated PLA/PVAc polymer composite, further in vitro study revealed superior spreading and adherence of osteoblast-like G292 cells onto these PLA/PVAc polymer composite. Both the shape of recovery rate (Rr) and fixity rate (Rf), describing the capability of a pad to continue the mechanical distortion as well as to memorize its permanent shape, respectively, represent two essential quantities in assessing the shape-memory effect (Fig. 4A).

In the context of bone regeneration, an electrospun nanofiber-based mat, which exhibits high porosity, provides remarkably

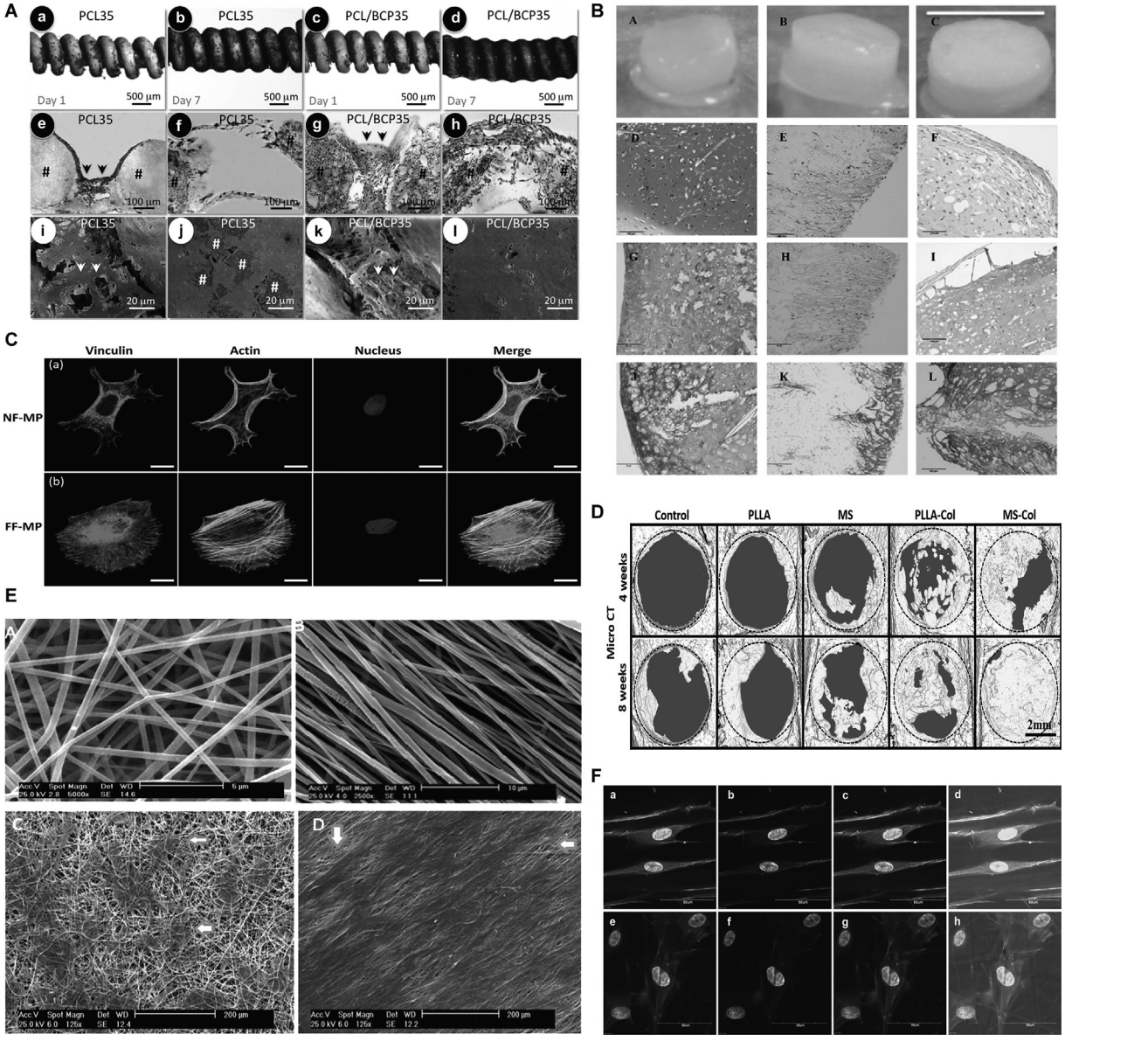


Fig. 4. (A) Sequential images illustrating the shape recovery process of PLA-G/PVAc-G nano-scaffolds from temporary structures to permanent shape[110]. Copyright 2019, Wiley. (B) Depiction and 3D printing molds of the thermal-responsive smart HA-PELGA nanocomposites. Adapted with permission[112]. Copyright 2019, The American Association for the Advancement of Science. (C) Photograph illustrations of the shape recovery behavior for electrospun nanofibrous PLMC scaffold[113]. Adapted with permission. Copyright 2014, American Chemical Society. (D) Digital photograph of the shape memory effect for the screw mimicked HAp/PLMC scaffold. Adapted with permission[116]. Copyright 2016, Royal Society of Chemistry. (E) Temperature responsive shape memory property of electrospun HA-PELGA and self-wrapping behavior of the osteoinductive membrane[117]. Adapted with permission. Copyright 2016, Wiley.

better shape recovery compared with a blockbuster. Additionally, the rapid recovery as well as continuing of morphology enable declined the slippage of polymer chain under the action of pressure, thereof debilitating the pressure relaxation. In this case, electrospinning nanofibers are ideal for circumstances demanding prompt control of the shape transition and excellent strain recovery, thus fulfilling the demand to reconstruct complex bone defects utilizing minimally invasive implant procedure. In one study, researchers developed an electrospun scaffold composed of poly (ε-caprolactone) (PCL) - polydimethylsiloxane (PDMS) copolymers that endow the composites with shape memory capacity. Through tailoring the ratio of these two segments, (9: 1, 8: 2, and 7: 3), PCL-PDMS fibers exhibited diverse fiber diameters, thermal behavior, and mechanical properties, while 7 thermo-mechanical cycles later, all fibers showed superior Rr ratios of > 90% and Rf ratios of > 92%, even after being engineered into the fibrous scaffold. Further, the PCL-PDMS scaffolds show great biocompatibility in fabricating osteoblast proliferation, enhancing the expression of biomineralization associated alkaline phosphatase and the deposition of mineral, as evidenced by biological assay[111].

In another study, a shape-memory graft made of HA-PELGA electrospun nanofibers were utilized to space-fill bone defects (Fig. 4B). Upon warm saline rise, quick deployment of the graft from their pre-compressed shape, to stiffen, swell, and end up in a 100% stable fixation state was observed. After that, those grafts extended to fill and match to the 5 mm critical flaw as early as 4 weeks. A single dose of 400 ng recombinant human bone morphogenetic A single dose of 400 ng recombinant human bone morphogenetic protein-2/7 heterodimer was used, and these osteoconductive macroporous grafts further support the entire repairing of torsional integrity, and full graft resorption by 12–16 weeks[112]. Similar studies also warrant the morphology

memory properties of fibrous poly (D, L-lactide-co-trimethylene carbonate) (PLMC) scaffolds in the application of bone regeneration (Fig. 4C)[113].

By exploring polymers with better application to the clinic feasible, for example, shape-memory composites encapsulating with drugs were arranged. Scientists have generated dexamethasone (Dex)/ PLMC composite nanofibers by incorporating the kind of synthetic bone-formation inducing factor Dex into a shape memory copolymer PLMC via co-electrospinning, which showed a uniform and smooth morphology with a diameter of 564 nm. Besides equipped with good mechanical performance and shape memory effect, researchers manipulated the release kinetics of the encapsulated Dex in Dex/PLMC composite by tailoring the acoustic power and insonation duration of ultrasound[114].

Additionally, by altering the chemical composition as well as thermomechanical characters of shape memory polymers is able to be manipulated for adapting to pressure among the grafts and nearing tissues, and thereof tailoring cellular activities by changing substrate topography. Researcher have conducted modification of the morphology memory polymers by having the biodegradable poly (3-hydroxybutyrate-co-3-hydroxy valerate) (PHBV) incorporated to form ultrafine composite fibers through electrospinning. The PLLA-PHBV (7:3) formulation is identified to offer superior shape memory properties with high Rf ratio (>98%) and Rr proportion (>96%) when benchmarked against PLLA fiber counterpart. In addition, the PLLA-PHBV (7:3) fibers also exhibit improved osteogenesis-inducing ability in the mouse bone mesenchymal stem cells, even under nonosteoinductive conditions[115]. Similar studies also reported on the generation of composite nanofibers of HAp/PLMC with diverse nanohydroxyapatite (HAp) proportions by incorporating HAp into a formation memory co-polymer PLMC via co-electrospinning. As shown in Fig. 4D, following in vitro findings verified that the combination of PLMC nanofibers and HAp remarkably promoted the secretion of alkaline phosphatase together with mineral deposition osteogenesis [116].

Interestingly, the capacity of shape-memory electrospun nanofibers can be further expanded to fabricate multifunctional scaffolds augmenting allograft healing/facilitating allograft tissue integration. When the electrospun nanofibers fabricated with shape-memory materials are incorporated within a polymer matrix, it endows the resultant composite with shape translation ability to enable close autogenous wrapping of allografts at body temperature while maintaining satisfactory mechanical force in the aqueous environment. The electrospinning meshes thus serve as a rubber phase and switching phase provides the permanent morphology. For example, the amphiphilic composed comprised of poly(lactide-co-glycolide)-b-poly (ethylene glycol)-b-poly(lacti deco-glycolide) (PELGA) matrix reinforced with HA showed an obvious shape-memory effect. HA-PELGA composite membranes were spread to 100% in the stain at 25 °C, after that cooled to 4 °C to fixed in a temporary shape, exhibiting rapid deployment within the 20 s in situ around the bone graft upon irrigation of 37 °C saline (Fig. 4E). Furthermore, the thermal behavior of HA-PELGA composites exhibit the desirable ability for cell seeding/cell sheet transform, when the temperature was declined from 37 to 20 °C, the polymers of HA-PELGA membrane undergo hydrophobic-to-hydrophilic transition thereby enabling readily release of the implanted cell onto allograft surfaces in vivo, thus facilitating allograft healing[117].

Apart from shape memory electrospun materials, there are a few other noteworthy examples for smart electrospun materials for bone regeneration that will be highlighted. For instance, inspired by the effects of the stimulation on osteoblasts, scientists particularly fabricated magnetic scaffolds and inspected their synergetic effect on osteoblast cells and bone regeneration. The magnetic biodegradable polyvinyl alcohol (PVA)/ chitosan (CS)/Fe_3O_4 nanofiber membranes were prepared via electrospinning by Yan Wei et al. The average fiber diameter was 230–380 nm and the porosity was 83.9–85.1%. Then, subsequent experiment figured out the appropriate data of applied voltage, polymer concentration as well as Fe_3O_4 nanoparticles loading with 4.5 wt%, 20 kV, and lower than 5 wt%, respectively, for the production of smooth, continuous as well as uniform Fe_3O_4/CS/PVA electrospun nanofibers, as verified by XRD, FI-TR and TEM data. Regarding the magnetic responsive property of these nanofibrous membranes, the author further explored their weak ferrimagnetic behaviors by vibrating sample magnetometer test. Cell growth dynamics in cell adhesion and proliferation revealed good bone biocompatibility of these Fe_3O_4/CS/PVA magnetic membrane, which can be further explored for the facilitation of osteogenesis. While the electrospun biomaterials offer a promising application for bone repair, the consequent soft tissue infection that occurs after orthopedic surgeries for biomaterials implantation may lead to serious problems, compromising their regeneration effect. In light of the utilization of stimuli-responsive electrospun nanofibers, a 2019 study postulated that grafting metronidazole, a kind of antimicrobial agents, on the surface of the PCL nanofibrous scaffold can generate an infection-responsive guided bone regeneration/ \guided tissue regeneration (GBR/GTR) membrane. These ester bonds broken up rapidly in response to infections, which was attributed, at least in part, to the cholesterol esterase releasing by the aggregated macrophages, whereas exhibited relatively stable in healthy tissue. These ester bonds are broken up rapidly in response to infections, which was attributed, at least in part, to the cholesterol esterase releasing by the aggregated macrophages, whereas exhibited relatively stability in normal tissue. The design of infective-response release avoids burst initial release while enabling selective drug release and effective drug concentration for the protection of infection attack. Further antibacterial test indicated that the result of PCL-MNA mat increased when blending with cholesterol esterase solutions with the dosage of 5 μ/mol as well as 10 μ/mol, as opposed with the decrease in bacteria activity from 36.9% to 34.0%, respectively.

4.3. Expansion of electrospinning in 3D for bone regeneration

A common restriction of electrospun scaffolds for bone regeneration and repair is the inherently weak mechanical properties, limiting these materials be used for clinical applications. Nicholas et al. creatively raised a technique that printed a mesh intensified electrospun scaffolds by three-dimensional (3D) print to improve their mechanical stiffness and strength (Fig. 5A)[118]. The mesh composed poly lactic acid (PLA) was directly 3D-printed on electrospun scaffolds made by a 60:40 ratio of gelatin to poly ε-caprolactone (PCL). And between the struts, PLA grids were printed on the scaffolds with a 0.6 or 0.8 cm distance. The intensify of electrospun scaffolds by 3D-printed reinforcements improved the mechanical properties and presented acceptable biocompatibility in rat cranial defects. Also, based on PCL, Nafisa et al. fabricated highly aligned mats of PCL using near-field electrospinning (NFE) with characteristics of collagen fibril architecture and resulted in cellular attachment and oriented growth. NFE allowed for the fabrication of geometrically controlled features for increased complex load-bearing architectures important for additive manufacturing of tissue engineered constructs, with the potential to synthesis heterogeneous structures of highly aligned fibers like bone-ligament interface, which offers promising applications for the development of tissue engineered gradients. Similar 3D scaffold involves a solution of PCL was reported by Sandy et al. and evaluated for their potential interest for bone regeneration[119].

In another study using 3D-printing, Dong et al. showed a platform with the able to fabricate elaborate shapes and controllable

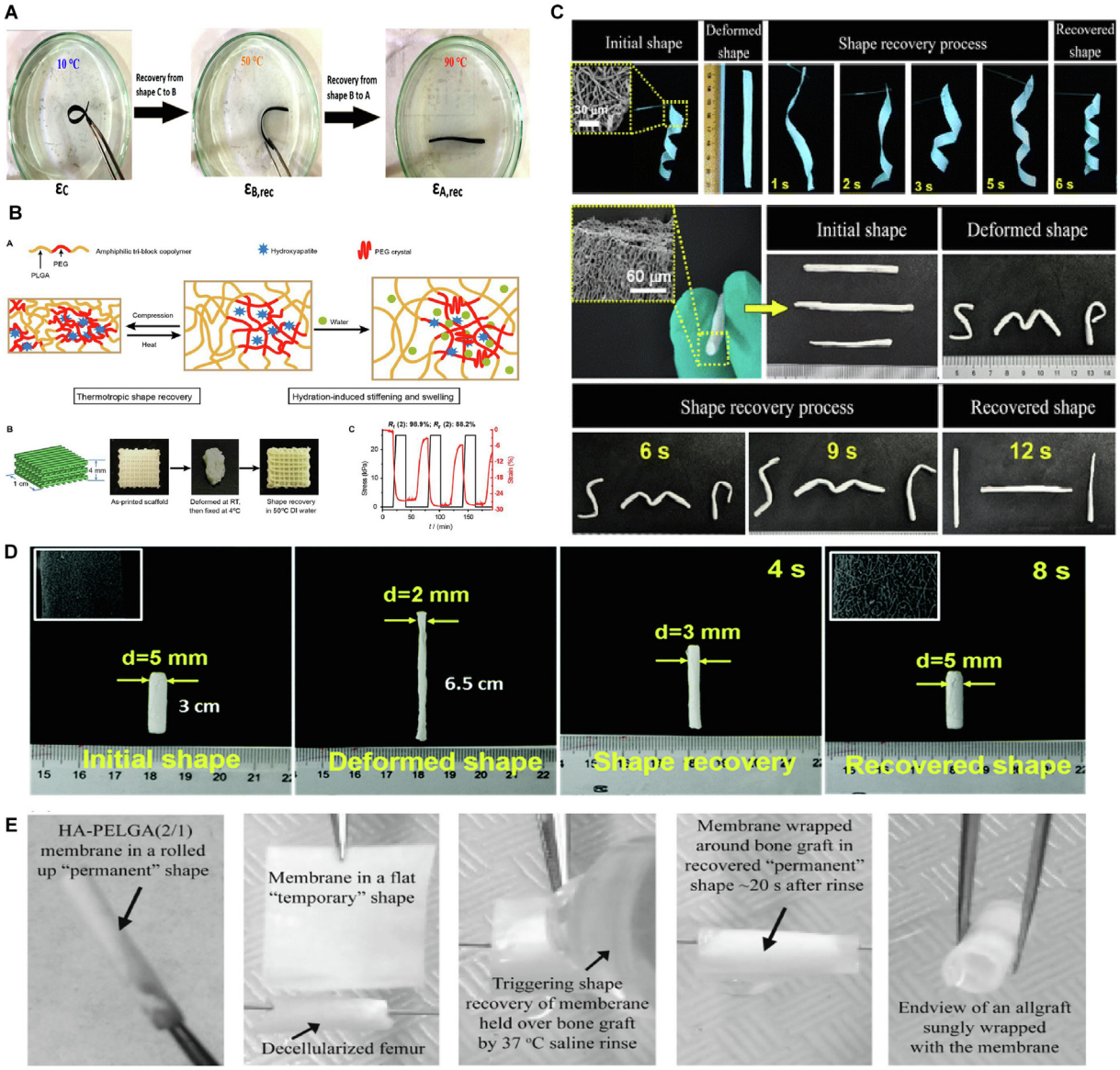


Fig. 5. (A) Schematic diagram showing the reinforced scaffolds by placing electrospun membranes in a 3D printed mesh [118]. Adapted with permission. Copyright 2019, Springer Nature. (B) Flow chart of the printed material synthesis as well as the fabrication of 3D scaffolds using homemade printing platform [120]. Adapted with permission. Copyright 2019, Springer Nature. (C) Illustration of the procedures for processing 3D printed PCL scaffold and BMP2 loaded PCL scaffold [121]. Adapted with permission. Copyright 2019, Wiley. (D) Schematic illustration of designing, developing and the structure of 2D nanofibers and 3D nanofibers [122]. Adapted with permission. Copyright 2017, American Chemical Society. (E) 3D models and their digital photograph of freeze dried scaffolds [123]. Adapted with permission. Copyright 2018, Elsevier. (F) The optical and SEM images of different 3D bioactive scaffolds [127]. Adapted with permission. Copyright 2020, American Chemical Society. (G)Schematic of fabrication processes and application backgrounds of the 3D electrowritten scaffolds [129]. Adapted with permission. Copyright 2020, Elsevier.

bone scaffolds by altering the electrospinning equipment or thermal-extrusion. Besides, alteration to pore size, porosity as well as pore interconnectivity can also be achieved by using various materials and different approaches. The scaffolds are applicable to improve the proliferation of MC3T3-E1 osteoblastic cells as well as support pretty mechanical stiffness (Fig. 5B)[120]. Apart from the complicated control on porosity, geometric shape and pore size, the alteration of biomolecule presenting capacity and biomimetic surface also are significant topics for 3D printed scaffolds, which decide their efficacious in regulating cell responses. As shown by Li et al., the short nanofibers-decorated 3D printed scaffolds display a biomimetic nanotopography without weakening the bulk mechanical strength, porosity, and pore size. Furthermore, the scaffolds were demonstrated to strongly improve the adhesion and proliferation of BMSCs and pre-osteoblasts, and enhances mRNA expressions of osteogenic markers (Fig. 5C)[121]. Data from Jin et al. also emphasized certain protein expression and cellular behavior. They reported a creative method to fabricate 3D-nanofibers by in situ polymerization and solution-assisted electrospinning technology, which showed enhanced biocompatibility to satisfy cell growth requirements in a dynamic environment (Fig. 5D). Besides, the 3D nanofibers facilitated the adhesion and collagen expression of human BMSCs under biomechanical regulation[122].

In a more coherent work, Izabella et al. Combined gelatin and PLLA, using electrospinning and 3D printing-fused deposition modeling to demonstrate a novel layered scaffold with multiple function for subchondral bone and nasal cartilages repair. 3D-implant materials with different geometry and structure were designed and constructed to solve the problem that otolaryngologists now

have with planting the nasal cartilage scaffolds with needle and threads. Besides, they tested the mineralization capacity of the implant by using the simulated body fluid (Fig. 5E). Also, they determined the morphology, cytotoxicity, and proliferation of Murine fibroblasts L929 cultured on obtained scaffolds[123]. Additional usage of stem cells was reported by Marie et al. They used 3D-electrospinning and printing approaches to offer a suitable environment for cells to accelerate bone reconstruction. In a rat calvarial defects model, the scaffolds cultured with BMSCs received better mineralized reconstruction and bone size during a 2-month experiment, suggesting a potential capacity for clinical use, especially for maxillofacial surgery[124].

Generating 3D constructs by aerogel or hydrogel was also reported by some researchers. As an example, by using ball milling and freeze-drying techniques, Zhang et al. incorporated the chitosan (CS) aerogels, cellulose acetate (CA) and PCL nanofibers fabricated. This study synthesized the nanofiber-strengthened aerogels mainly to maximize the interaction between cells and material interaction in the pure CS scaffold, which showed enhanced cell adhesion as well as osteogenic differentiation, which showed promising potential for clinical bone reconstruction[125]. Another approach was raised by Jinga et al., the porous 3D-scaffolds with synthetic fibers were manufactured by electrospinning, beginning with inorganic powders and polycaprolactone generated by the sol-gel method. The powders used were nano-scale with a glass-ceramic character, which lead to the material of a promising adhesion to living tissue under a physiological environment, with controllable properties of bioresorbability and bioactivity[126].

Hwang et al. raised a new in situ technique of cyclic utilizing the lactic acid (LA) to fabricate the osteoinductive biomolecules, calcium lactate (CaL), thereby synthesizing a bioactive PCL/CaL 3D-scaffold (3D-SCaL) for bone repair and reconstruction. The morphology of this fibrous scaffold and its packing degree could be precisely altered by changing the collector design and the component of spinning solution. Within 2 cell lines, MC3T3-e1 and BMSCs, 3D-SCaL presented impressive translation of CaL from LA and demonstrated greatly improved cell proliferation as well as growth, biomineralization capacity, and osteogenic differentiation (Fig. 5F)[127]. Still, the insufficient mechanical strength of hydrogel material should also be emphasized. To solve this problem, Maharjan et al. addressed this limitation by combining recycled cellulose nanofibers with chitosan (CS) hydrogel. This scaffold showed unique porous morphology and exhibited more stiffness compared to pure CS. Notably, the reinforced material also intensive pre-osteoblast cell (MC3T3-E1) attachment, viability, proliferation as well as increased biomineralization[128].

Data from other studies explored more delicate structures. As reported by Lian et al., they put forward and synthesis a novel bi-layered "GBR scaffold" with multiple function, which incorporating solution electrospinning (SES) and solution electrospinning writing (SEW) methods by a specific printer. Furthermore, copper-carried mesoporous silica nanoparticles (Cu@MSNs) were loaded into the fibrous matrix to fabricate a complex fiber scaffold. The obtained material composed a porous and loose SEW layer to promote cell growth. Also, a compact and dense SES layer to keep away from non-physiological obstruction (Fig. 5G)[129]. In another study did by Awasthia et al., they loaded the nanosheets into the PCL/zein composed polymerized network by using electrospinning. Their scaffold presented enhanced Young's modulus, improved cell adhesion, viability and differentiation[130]. Interestingly, Song et al. fabricated porous PCL nanofiber nets with different content of nano hydroxyapatite prepared by electrospinning, and the 3D nanofiber scaffolds were fabricated by special adhesive.

The 3D nano-fiber scaffolds demonstrated layered composition with connected pores with different sizes, and cells could migrate in different layers, even between the scaffolds, suggesting a potential for bone repair[131].

5. Drug delivery in electrospinning design for bone regeneration

5.1. Bioactive factors carrying

BMP: Bone morphogenetic proteins (BMPs), composed of twenty members, is a group with highly conserved functional proteins with similar structure. Originally, there were seven such proteins found because of their ability to guide bone and cartilage formation, and now, BMPs are deemed to orchestrate tissue architecture throughout the body, constituting a group of morphogenetic signals. The active form of BMPs composes of two disulfide-linked poly-peptide subunits, ranging in size from 30 to 38 kDa. According to the homology of amino acid sequence, osteogenic induced BMP was divided into OP-1 group (bmp-5-8) or BMP-2/-4 group and osteogenic protein-1.

The effect of BMPs on bone formation is the most widely studied. Particularly, it is BMP-2 that has been widely studied for its inducing MSCs to differentiate into chondrocytes, promoting osteoblast precursor cells differentiation into mature osteoblasts and other significant roles in bone formation and remodeling. However, complications follow, such as hematoma and soft-tissue edema, because it is only to promote the release of BMP without control, so the key to make good use of BMP-2 is to control its release. Meanwhile the emergence of electrospinning technology enables this idea to be realized. To achieve ossification and controlled release, we can crosslink BMP-2 onto electrospun fiber scaffolds, which can control the morphology and porosity of polymer bioproduction. In 2006, this proposal is supported by an experiment of external bone formation from hMSCs through silk fibroin scaffolds containing BMP-2. Li C et al. showed that compared with the control group, the nanofiber electrospun scaffolds holding BMP-2 possessed higher calcium deposition as well as enrich the expression of bone specific markers, indicating that these nanofibrous scaffolds were effective delivery systems of BMP-2[132]. It has been confirmed that electrospinning silk-fibroin based scaffolds were promising candidate materials for bone regeneration through the measurement of gene transcription related to mineralization and bone formation.

Similarly, Kim BR et al. fabricated BMP-2-loaded PCL–Gel–biphasic calcium phosphate scaffolds and they demonstrated the release of BMP-2 contributed to early bone formation [133]. They proved markable bone regeneration implanted in rat skull defects. The histological analyses and Micro-CT showed that BMP-2 can significantly improve the new bone formation efficiency, especially in the initial stage, and ultimately achieve rapid and early bone regeneration.

Actually, BMP2 was stably expressed from cartilage callus stage to callus stage (all phases of bone healing), so it is vital to design a slow and lasting release of BMP2 to guarantee its steadiness over the entire bone healing. As demonstrated by Cheng G et al, the bone formation was enhanced via the controlled release of BMP2 in their dual-delivery release system, fabricated by LBL technology and core-shell nanofiber assembly[134]. Wu R et al. demonstrated BMP-2 cross-linking can be used as an excellent sustained-release carrier on polydopamine coated PELA electrospun scaffolds to repair the acetabulum defects[135]. Besides, BMP-2 can also improve early accumulation of osteoblast precursor cells in bone injury site, and recently this proposal is supported by Cheng L et al. that BMP2-modified black phosphorus loaded electrospun fibrous scaffold recruited these cells and accelerating biomineralization supported by both in vivo and in vitro data[136]. Of the

BMPs, there are relatively few studies on evaluating the effectiveness of BMP6. However, in a present study, it was revealed that dual delivery of BMP6 on titanium surface had a critical effect on the proliferation of bone implant cells to enhance the early period of implant osseointegration[137].

FGF: The fibroblast growth factor (FGF) is a kind of cell signal protein, which is considered to be an effective regulator of cell growth and wound healing, and a key factor of normal development. In humans, there are 25 members of FGF family, among which 22 members have been firmly identified as structure related signal molecules, ranging in size from 17 to 34 kDa. In addition, FGF-2, also known as basic fibroblast growth factor, is the best family member for bone regeneration, synthesized primarily as a 155 amino acid polypeptide. Rubert M et al. successfully encapsulated FGF-2 in Coaxial electrospun PCL/PEO fibers, observed a long-term release as well as demonstrated its ability to enhance fibroblast cell viability and proliferation[138]. Moreover, Chi Zhang et al. demonstrated that the FGF-2 loaded by fiber scaffold has the ability of bone regeneration membrane and promote the formation of extra bone blood vessels and this might be the result of cell vascularization because of FGF-2 loaded in fibers[139].

In some studies, FGF-2 combined with BMPs enhanced bone regeneration timely as well as dose-dependent manner. By controlling the delivery of FGF-2 and BMP-2 and the synergistic effect of hydroxyapatite nanofiber coating, bone regeneration was enhanced, and the increased expression of osteogenic gene markers was confirmed by quantitative polymerase chain reaction analysis[140].

VEGF: Vascular endothelial growth factor (VEGF) was originally named as vascular permeability factor. it is a signal protein released by cells as well as the process of angiogenesis during bone regeneration. Farokhi M et al. shown the new bone tissue formation by the histology analysis after 10 weeks of implanting the biological nanocomposite scaffold as a VEGF delivery system[141]. Marta R et al. developed an engineered biological functional system which combines endogenous VEGF and BMP-2, with the ability to induce increased angiogenesis, and this was proved by the high expression of angiogenic markers on the constructs having VEGF[142]. Rosa AR et al. proved the PLGA/BSA/VEGF scaffolds improved cell adhesion and the scaffolds showed non-poisonous for cells[143]. Likewise, the gelatin/PLGA nanofiber scaffold was successfully constructed by An G et al. to release VEGF and BMP-2 in turn, promoting the proliferation, adhesion and differentiation of BMSCs[144].

PDGF: Platelet-derived growth factor (PDGF) modulates cell growth as well as tissue repair, and particularly participates in the process of angiogenesis. In both human and mouse, PDGF is a dimeric glycoprotein about 30 kDa and its family consists of five ligands, four homodimers including PDGF-AA, -BB, -CC and -DD. Among them, one heterodimer is PDGF-AB. It should be noticed that apart from its positive effect on promoting the proliferation of u undifferentiated stroma and some progenitor cells, PDGF also participates in the induction of pattern and morphogenesis in bone remodeling with the therapeutic potential in skeletal reconstruction.

Actually, to promote rapid angiogenesis within tissue-engineered constructs is necessary. However, insufficient blood supply is provided at the site of implantation is the major barrier. For this purpose, Farokhi M et al. in 2013 have reported that the osteoblasts proliferation, ALP production and osteoblast attachment were significantly up-regulated concerning interaction of osteoblasts with silk fibroin/calcium phosphate/polylactic-co-gly colic acid nanocomposite as a delivery vehicle for VEGF and PDGF[145]. As demonstrated by Briggs T et al. the incorporation of PDGF-BB into polymer scaffolds by emulsion electrospinning can enrich osteogenic markers[146]. Similarly, it was proved that the addition of PDGF-BB to nanofiber scaffolds increased the osteogenic differentiation potential, which may be the result of the synergistic effect of PDGF-BB and scaffolds[147].

5.2. Drug carrying

The need for injured tissue regeneration has stimulated the development of regenerative medicine as well as tissue engineering. Also, it has been realized that the combination of biomaterials, biological cells and molecules can powerfully promote the regeneration process. In this case, a variety of drugs have been encapsulated in scaffolds to achieve better drug function, including anti-inflammatory drugs, bone morphogenetic proteins, polyphosphate, deferoxamine and alendronate. Even though not all drugs target bone tissue, the huge benefits of this approach have been confirmed in the guided bone regeneration (GBR). AS a local drug delivery system, these biomaterials scaffolds could release drugs on demand and regulate the activity of the cells which are in contact with them, including their coordination, proliferation and differentiation. In one study, the adenosine was incorporated into polycaprolactone (PCL)/ polyvinyl alcohol scaffolds and showed an ordered release of adenosine and helped the osteogenesis from bone mesenchymal progenitor cells.

In 2014, Akhilesh K. Gaharwar and his coworkers reported that with the application of poly scaffold, they achieved continuous release of dexamethasone (Dex) for 28 days. What's worthy to note is that they used the amphiphilic beads as a drug carrier, where the hydrophobic Dex was well embedded and sustained released. Subsequently, Nelson Monteiro, et al. proposed a concept that considering the lipid solubility of many drugs, applying the liposomes as depots to carry drug molecules may lead to more efficient strategies. Afterwards, they immobilized the Dex-loaded liposomes on the surface of electrospun PCL and found that this system not only performed high levels of biocompatibility, but also entrapped drugs to guarantee sustained release.

The innovation of carrier device permits the greater possibility of sustained drug release. What's more, with the applying of electrospinning fiber technique, more breakthroughs were made in the properties of scaffold materials, thus researchers attempted to carry more different drugs to explore the potential of drug delivery. Polyphosphate (poly-P), functioning as osteogenic growth factors, was to be incorporated into the mesh of PCL/ poly (L lactic acid) electrospun nanofibrous, which displayed a high rate of osteogenic differentiation. A recent study reported that metformin was incorporated into PCL/chitosan nanofibrous membranes by electrospinning, and the improved cell adhesion, cell proliferation, and osteogenic differentiation were then investigated. During another study, Yaojie Wei et al. loaded the aspirin in poly lactic-co-glycolic acid and tried to create a nanofiber coating on titanium. Consequently, they observed that such drug carrier system could promote the performance of nanofiber in both anti-inflammation and osseointegration.

Besides, researchers also tried to regulate different concentration of drugs to achieve the greatest effects. Aiming at preventing infection, Jiajia Xue et al. fabricated GBR membranes of PCL and gelatin blended with metronidazole (MNA) by electrospinning. They revealed that the GTR/GBR membranes might behave differently depending on the content of the drug. For instance, drug crystals could only form when the MNA content increased to 20%, while only with the MNA content increased to 30%, nearby cells could attach to membranes and gradually proliferate without cytotoxicity. In the study of Ranjith Ramanujam's team, naringin loaded PCL fibers were fabricated with different concentrations of PCL and naringin and they also found an increased cumulative naringin release with increasing fiber diameters. Present evidence indicated that varying drug and polymer concentrations might act

as a tool to alter cumulative drug release within advantageous ranges for bone regeneration.

Furthermore, focusing on balancing the formation as well as bone resorption during the process of bone reshaping, researches even created multiple delivery systems. Yi Wang and his colleagues fabricated a mesoporous silicate PCL/gelatin scaffolds by electrospinning aiming to realize the multiple delivery of alendronate and silicate. As expected, they observed the synergetic effect of these two drugs in bone remodeling: alendronate prevented bone resorption by inhibiting the expression of guanosine triphosphate-related protein, while silicate facilitated bone formation by regulating the process of vascularization and calcification.

Notably, by layer-by-layer assembly technique, Yufei Yan and his coworkers developed a 3D-printed biodegradable scaffold to achieve the controlled release of deferoxamine. This 3D printed scaffold exerted excellent biocompatibility and sustained-release performance, which significantly improved the vascularity regeneration and bone regeneration. In some senses, this study further revealed a promising perspective of biomaterial scaffolds and drug delivery in bone tissue engineering.

5.3. Gene delivery

MicroRNAs (miRNAs) is a kind of non-coding as well as small RNA expressed in endogenous (around 21–22 nucleotides), which exists in organisms including plants animals as well as viruses, mediating transcriptional regulation and RNA silencing. They usually interact with complementary regions of target mRNA in 30 untranslated regions, leading to mRNA instability, decomposition and / or translation inhibition, so as to achieve their functions. Because miRNA plays a post transcriptional regulatory role in cytoplasm, miRNA-based therapy can regulate gene expression without entering the nucleus. Hence, it is one of the most attractive in recent years being the use of genetic materials. For example, isolate them from plasma and blood serum as diagnostic tools to indicate the health status of individuals. In addition, miRNAs can also play a role in determining the fate of cells, through their synthesis and delivery to target tissues to induce specific regulation, inhibit or enhance cell growth and proliferation. Recently, it has been systematically verified that miRNAs regard as post-transcriptional regulators of gene to promote tissue regeneration in vivo and in vitro.

There are various ways to deliver drugs to the target cells and tissues and the most common one is chitosan particles due to their which are delivered because of the high transfection efficiency. Unfortunately, however, this causes a intense cellular immune response. To maintain the flash point of particles as well as lower the immune response, in 2007, Nie H et al. developed a new DNA release system based on composite scaffolds prepared by electrospinning technology and their observations shown that PLGA/HAp composite scaffold coated with DNA/chitosan nanoparticles has prosperous application in the field of bone regeneration[148]. Similarly, recent method for miRNAs delivery method is nanoencapsulation using nanoparticles or nanofibers. The delivery of miRNAs into cells through nanofibers can accelerate its function and improve its efficiency.

MiRNAs are also involved in the process of gene expression, regulating bone formation and remodeling by controlling various signaling pathways and growth factors related to secretory molecules and transcription. MiRNAs also have variable profiles in the process of tissue formation and osteogenic differentiation and it has been demonstrated that during the osteogenic differentiation, the expression of miRNA-29b, miRNA-22 (miR-22), miRNA-196a, miRNA-2861 (miR-2861), miRNA-335-5p and miRNA-3960 will be increased, and others will be decreased, including miRNA-135, miRNA-141, miRNA-26a (miR-26a), miRNA-200a, and miRNA-133. Among them, miR-26a has become the promoter of osteogenic differentiation of bone marrow-derived MSC and has been proved to increase vascularization. Li R et al. used a comb-shaped polycation as an effective carrier to transfer miR-26a into bone marrow mesenchymal stem cells. The miR-26a is able to coordinate the coupling between osteogenesis and angiogenesis, promote the secretion of VEGF, and improved significantly the healing of rat skull defects[149].

MiR-126 is regard as the main regulator of physiological angiogenesis, which regulates vascular integrity and angiogenesis by regulating the signal of angiogenic growth factors like FGF as well as VEGF. Zhou F et al. incorporated miRNA-126 in the dual-functional electrospinning membrane and they planted vascular endothelial cells directly on electrospun membranes and the results showed that cell proliferation and adhesion were improved[150]. In another study, Tahmasebi A et al. filled both miR-126 and miR-22 in the PCL nanofibers and demonstrated the osteogenic differentiation potential of iPSCs in vitro level[151]. Likewise, Abazari MF et al. observed that iPSCs transduced with miR-2861 had a great positive impact in improving iPSCs osteogenic differentiation potential[152]. Whereas, miRNAs down-regulation also plays a direct role in osteogenic differentiation of MSCs. According to Sadeghi M et al. the co-application of HA and anti-miR-221 transfected cells could promote bone healing [153]. In a variety of tissue types, mir-29 family is not only an important positive regulator of osteoblast differentiation, but also an effective negative regulator of ECM synthesis. Previously, Eric N et al. seeded re-osteoblastic murine cell line on miR-29a inhibitor-loaded nanofibers and proved to synthesize more osteonectin and increase ECM production[154].

5.4. Cells carrying

As a dynamic organ, bone protects tissue homeostasis and repair through continuous reconstruction. However, the dynamic balance of bone reconstruction is disturbed when it comes to severe trauma, malignant tumors, infections, and some other reasons [155]. Therefore, bone grafts and other materials are used for surgical treatments under such circumstances. As a new method of bone engineering, electrospinning has been extensively used to remodel a suitable microenvironment for osteanagenesis from stem cells in vitro. Characterized excellent self-renewal and differentiation ability, stem cells are regarded as the ideal cells used for bone regeneration. Up to now, researchers have successfully used mesenchymal stem cells (MSCs), induced pluripotent stem cells (iPSCs), embryonic stem cells (ESCs) and others to load in electrospinning for bone regeneration[156-158].

iPSCs: In recent years, iPSCs have become a befitting cell for bone regeneration because of the autologous application progress and better proliferation and differentiation ability. D'Angelo et al. applied a support scaffold for carrying iPSCs which is PLLA electrospun mesh[159]. As shown in Fig. 6A, they found that the osteogenic markers including bone matrix molecular deposition as well as ALP activity, was higher expression in the cells differentiated on PLLA nanofibers. In this study, they evaluated the osteogenesis capacity of iPSCs in basic medium, and the results showed that it was promoted. Ardeshirylajimi et al. reported that they used polyethersulfone (PES) to induce bone formation and to observe the expression of upregulation mRNA, including osteocalcin, osteonectin, collagen (Fig. 6B)[160]. The differentiated cells of 21 days were compared with those of two-dimensional tissue culture polystyrene (TCPS) group, while there was no obvious difference on the 14th day. Worth to mention that, another study found that extraordinary low frequency pulsed electromagnetic field (PEMF) can promote bone forming ability of electrospinning[161]. Soleimanifar et al. used conditioned medium to culture iPSCs and

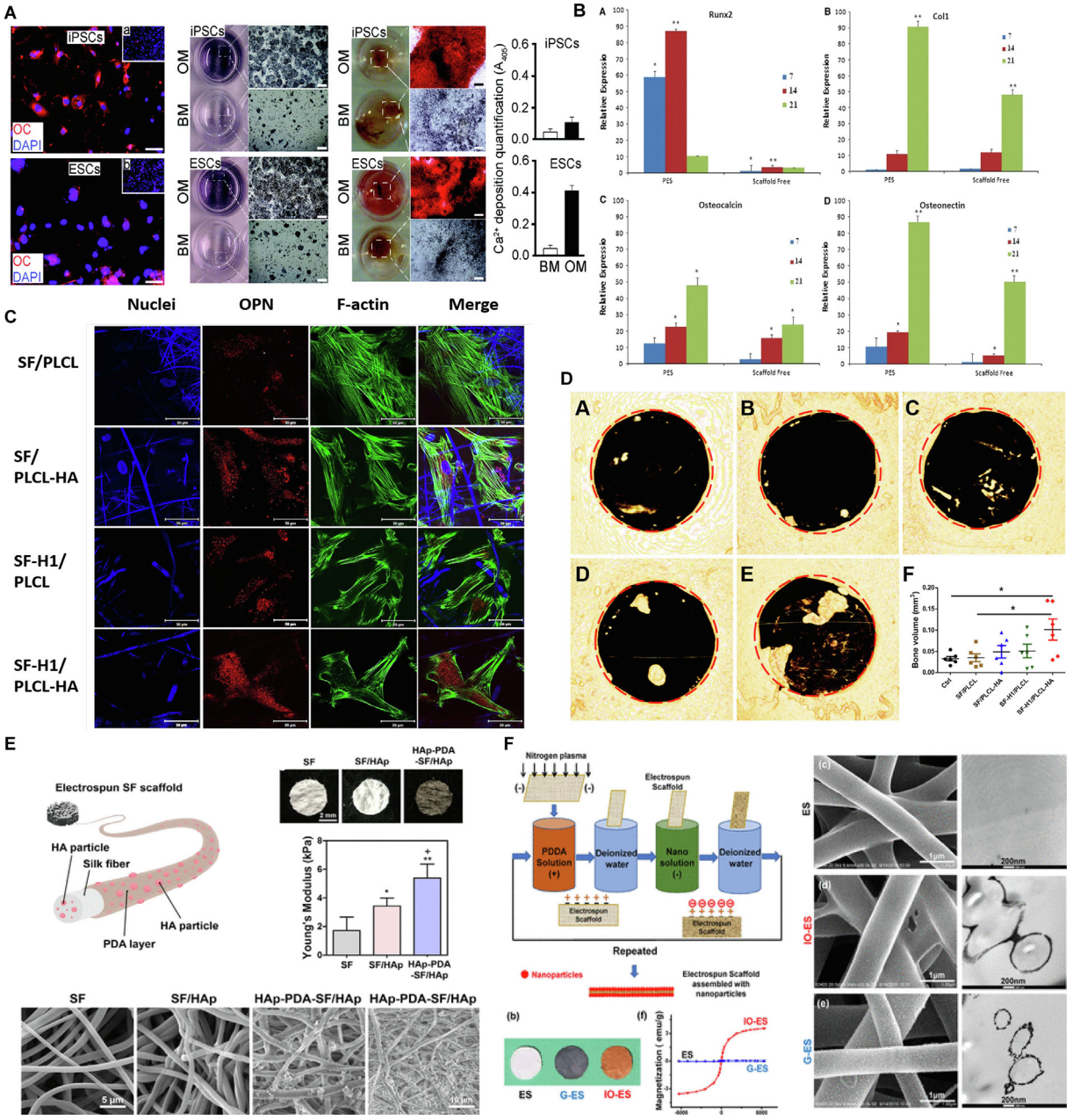


Fig. 6. (A) Representative results of iPSCs and ESCs cultured in osteogenic medium with expressing osteogenic proteins and Ca^{2+} deposition [159]. Adapted with permission. Copyright 2013, American Chemical Society. (B) Results of the gene expression levels of Runx2, Col1, Osteocalcin and Osteonectin by Real time PCR[160]. Adapted with permission. Copyright 2013, Springer Nature. (C) Immunofluorescence analysis of osteogenic differentiation for human iPS and MSCs on various core-shell scaffolds[164]. Adapted with permission. Copyright 2019, Elsevier. (D) Results of the repairing calvarial bone in different groups at 8 weeks after implantation[164]. Adapted with permission. Copyright 2019, Elsevier. (E) Illustration and SEM images of electrospun scaffolds functionalized with hydroxyapatite[165]. Adapted with permission. Copyright 2018, American Chemical Society. (F) The optical and SEM images of different 3D bioactive scaffolds[169]. Adapted with permission. Copyright 2018, American Chemical Society.

evaluated the osteoinductive potential[162]. The results showed that MSCs derived limited osteogenic medium and conditioned medium had the same differentiation capacity for iPSCs cultured on PCL.

Some studies found modified scaffolds and introducing biomaterials into the fibers before electrospinning could exert more potential in osteogenic differentiation of iPSCs. One study by Tahmasebi et al. surprisingly used aloe vera gel to coat the scaffold surface. It was found that ALP activity, calcium content and tissue-specific gene expression increased significantly on the 7th and 14th day of differentiation. Another study by Tahmasebi et al. demonstrated that on the 14th day of differentiation, calcium content as well as ALP activity of iPSCs could significantly improve by adding miR-126 and miR-22 to PCL electrospinning fibers[163]. In addition, compared with PCL without adding miRNA, the mRNA levels of runx-2, osteonectin and osteocalcin were added on the 7th and 14th day of differentiation[151]. Xu et al. found that the electrospun scaffolds loaded with H1 peptide and HA into mice showed higher bone volume in the cranial crest bone (Fig. 6C) [164]. Therefore, the incorporation of HA and H1 peptide increased the iPSCs differentiation to osteogenesis on SF/PLCL compound fiber. As shown in Fig. 6D, to conclude, electrospun scaffolds with iPSCs cultured and differentiated have presented promising results for bone regeneration. But more researches need to be done to

explore higher mechanochemical properties of electrospinning scaffolds.

MSCs: Like all kinds of stem cells, MSCs have the same potential to be multi-differentiation, and new studies in recent years have found that human MSCs can differentiate into tissue or cells of every germ layer including bone. Therefore, researchers considered it applicating in electrospun scaffolds for inducing bone regeneration. The PLA scaffold rich with MSCs have the ability to regenerate femoral segments and critical size skull defects, whereas bare scaffold had no effects. There was a novel nanofibrous scaffold developed by Ko et al. which was modified with hydroxyapatite (HAp) particles[165]. In vitro, human adipose-derived MSCs have been observed to differentiate well into bone, and in vivo showed better capacity in repair bone defect (Fig. 6E). These results showed two-stage HAp-modified silk fibroin (SF) scaffolds greatly reinforced the osteogenesis and mineralization, and also provided promising method of materials science for critical-sized calvarial bone defect. Xue et al. obtained MSCs from bone marrow, umbilical cord and adipose tissue that was investigated the potential of osteogenesis in electrospun scaffolds in vitro[166]. This study showed that PCL nanofiber scaffold was able to promote adhesion and proliferation of these MSCs. The researchers also revealed that it was Wnt/β-catenin as well as Smad3 signaling pathways consistently activation by PCL nanofiber scaffold that promoted osteogenesis of MSCs. Notably, it had been found that bone marrow-derived MSCs had higher differentiation ability through the comparison of these three proliferation and differentiation capacity. Moreover, Shin et al. reported that bone marrow-derived rat MSCs were transplanted into better vascularized areas in vivo using electrospun nanofiber scaffolds, and evaluated the situation of bone formation[167]. Characterized by immunohistochemistry and histology, mineralization and type I collagen were detected and extracellular matrix (ECM) started to form and cells differentiated all over the visible field.

Except for common stem cells like iPSCs and MSCs, scientist have exploited several other stem cells involving adipose-derived stem cells as well as human mesenchymal stromal cells, attempting to explore more effective ways aiming for bone regeneration through applicating in electrospun scaffolds. Gazquez et al. developed new flexible nanofibrous yttrium-stabilized zirconia (YSZ) scaffolds which present impressive multiscale mechanical properties[168]. Their results revealed that seeded human mesenchymal stromal cells showed osteogenesis and differentiation potential. Also, compared to the characteristic of bioinert behavior, nanofibrous structure of YSZ scaffolds could detected mineralization. In fact, adipose-derived stem cells take the advantages of clinical availability and safety therefore have been popular for bone repair in clinics. PLGA/ polycaprolactone (PCL) electrospinning scaffolds were modified by layered combination technique through using Iron oxide nanoparticles (IONPs) which were employed by Chen et al[169]. On adipose-derived stem cells, they then detected the cellular effect of this electrospun scaffold. The results demonstrated that the surface modification of the nanoparticle assembled membrane greatly enhanced the properties of osteogenic differentiation from adipose stem cells and promoted the mechanical force of cells (Fig. 6F). Generally, more and more potential stem cells as well as bioactive factors were discovered to apply bone repair and regeneration, as summarized in Table 1.

6. 3D bioprinting design for bone regeneration

The techniques of scaffolds transplantation have opened up new alternatives for bone defects due to the development of bone tissue engineering. An ideal scaffold should be able to provide appropriate biocompatibility, biodegradability and mechanical

Table 1
Drug delivery in electrospinning design for bone regeneration.

Name	Types	Mechanisms and Applications
BMP	Protein	Inducing MSCs to differentiate into chondrocytes; promoting osteoblast precursor cells differentiation into mature osteoblasts and early enrichment; contributing to early bone formation
FGF	Protein	Promoting extraosseous blood vessels formation; stimulating the proliferation of mesenchymal stem cells
VEGF	Protein	Promoting blood vessel formation; enhancing osteoblast maturation, ossification, and bone turnover; synergistic effects with BMP-2; increasing the bioactivity of scaffold and cellular adhesion
PDGF	Protein	Promoting hypertrophic cartilage remodeling, ossification and angiogenesis; stimulating chemotactic migration of osteoblasts, bone fill and matrix mineralization
Simvastatin	Small Molecular Drugs	Down-regulating the osteoblasts apoptosis; reducing the osteoclast activity; increasing the expression of the bone collagen I, non-collagen bone proteins and VEGF; promoting the mineralization
Bisphosphonates	Small molecule	Increasing the expression of osteoprotegerin; inhibiting the osteoblasts apoptosis; inducing the apoptosis of the osteoclast; activating the BMP signaling pathway
DEX	Small molecule	Osteoinductive and mineralization activity
Adenosine	Small molecule	Inhibiting osteoclastic differentiation; promoting the proliferation of osteoclast precursors; inducing osteogenesis of BMSCs
Metformin	Small molecule	Improving cell adhesion, cell proliferation, and osteogenic differentiation
Aspirin	Small molecule	Promoting proliferation and osteogenic differentiation of BMSCs; inhibiting osteoclast differentiation of macrophages
Ascorbic acid	Small molecule	Promoting the collagen biosynthesis and stabilizing the helical structure of the collagen; inducing the specific genes related to the osteoblast phenotype; eliminating the oxidative stress conditions and reducing the bone resorption
Bioceramics	Small molecule	Osteoconductive properties; romoting bone differentiation and regulating protein synthesis and mineralization; activating the transcription of the BMP-2 gene
MiRNA-26a	MicroRNAs	Promoting osteogenic differentiation of bone marrow-derived MSC, vascularization, the secretion of VEGF and maturation of new bone
MiRNA-126	MicroRNAs	Regulating vascular integrity and angiogenesis; promoting osteogenic differentiation and the expression of FGF and VEGF
MiRNA-2861	MicroRNAs	Enhancing bone differentiation, matrix creation and mineralization
MiRNA-29a inhibitor	MicroRNAs	Promoting ECM synthesis and osteogenic differentiation; increasing synthesis of osteonectin and type I collagen
iPSCs	Stem cells	Promoting osteogenic differentiation, strong mineral deposition and the production of MSCs
MSCs	Stem cells	Promoting osteogenic differentiation and mineralization
ESCs	Stem cells	Promoting osteogenic differentiation and mineralization
hADMSCs	Stem cells	Inducing bone collagen regeneration; promoting osteogenesis and differentiation

BMP, Bone morphogenetic protein; MSCs, mesenchymal stem cells; FGF, fibroblast growth factor; VEGF, Vascular endothelial growth factor; PDGF, Platelet-derived growth factor; miRNA, microRNA; DEX, Dexamethasone; BMSCs, bone mesenchymal progenitor cells; iPSCs, induced pluripotent stem cells; ESCs, embryonic stem cells; hADMSCs, human adipose-derived mesenchymal stem cells

properties. The 3D printing technology can accurately produce personalized tissue-engineered bone scaffolds based on CT/MRI imaging of bone defects and lesions to achieve perfect matching between the scaffold and the focus, imitating the microstructure of normal bone tissues. 3D bioprinting has been applied to construct scaffold models for bone defects of the maxillary, mandible, facial cranium, long bone, vertebra and joints.

6.1. Functionality

An optimal scaffold should be biocompatible, with the potency to integrate with the natural bone without triggering immunoreactions. As for non-absorbable materials such as metal or ceram, bone cell ingrowth should be considered as the key mechanism. Porous structures can construct a large surface area for the cell growth[170,171]. In certain metal such as titanium or tantalum, porosity between 60% and 80% and pore size ranging between 300 and 500 μm favor vascularization and osseointegration[172-175]. The pore size of porous hydroxyapatite bioceramics should be greater than 100–150 μm, while the ideal range is 300–400 μm[176,177].

The metabolism of bone repair can be affected by 3D bioprinted materials for bone regeneration by variety of ways, thereby promoting the bone remodeling process. Some grafts consist of or are made of materials containing calcium and/or phosphorus, thus can provide local targeted nutritional support for the bone defect through slow or controlled degradation. Calcium pyrophosphate, hydroxylapatite and biphasic calcium phosphate (BCP) are commonly seen in these materials. Calcium phosphate may facilitate bone regeneration because of the calcium and phosphorus elements and regulating the activation of osteoblasts, showing osteoconductivity and osteoinductivity[178]. Microspheres containing alginate and undoped carbonate hydroxyapatite or nanocrystalline 3.2% by weight of zinc-doped structure can release large amounts of calcium and phosphorus in bone defects[179]. Modification of these materials may promote the properties of the printed scaffold. The combination of natural calcium phosphate and biocompatible alloys, such as BCP-niobium pentoxide (Nb2O5) nanocomposite material, showed better physical, mechanical and biological properties compared with pure BCP[180].

There are some materials aiming at simulating similar mechanical properties, microstructure or other physical/chemical properties as human bone tissue[181]. When the nano-sized hydroxyapatite is alloyed with other materials to construct a composite scaffold, the composition and microstructure characteristics of natural bone such as trabecular bone can be simulated[182,183]. Several studies reported materials that can directly affect the osteogenesis-osteoclastic balance[184-187]. It is not a novel idea since it had been reported in 2002 that polyurethane scaffolds were able to induce the deposition of calcium phosphate crystals by Gogolewski et al[188]. Similar effects were observed in polyphosphodiesters (PPDEs) with strong affinity for minerals, which is similar to the bisphosphonates applied as anti-osteoporosis medicines, as well as enhancing the differentiation of osteoblasts and weakening the function of osteoclasts[189-191]. Poly D, L-lactide-glycolic acid copolymer (PLGA) had also been proved to increase the local calcium and phosphorus content in animal models[192].

6.2. Application

In addition to the universal properties, such as biocompatibility, that general 3D printing medical scaffolds share, the 3D printing scaffold used for bone regeneration should also satisfy different requirements unique to bone tissue. Thus 3D printed scaffolds for bone regeneration are required to have certain physical and chemical properties. Due to different functions of the bones, defects in maxillary, mandible, teeth, cranium, long bones, etc. require different properties of the scaffolds.

Calvarium: The key aims of calvarial reconstruction are cosmesis, to protect the brain, to restore the contours of mental health, and to normalize the neurological dysfunction that is common in patients with brain defects[193]. The average elastic modulus of calvarial cortex is reported to be about 12 GPa (between 1.1 and 1.3 GPa for one- to two-year-old children) with frontal calvarium having a greater stiffness than the parietal bone[194,195]. Unlike long bones that are modeled through the endochondral ossification mechanism, the calvarium and facial cranium are ossified through the intramembranous pathway[193,196-200].

Maxillary, mandible and tooth: Currently, implant placement is widely used for the remodeling of missing teeth[201-205]. In one study that the process of repairing the alveolar with additively manufactured bone tissue scaffold is exhibited [206-208] as shown by the finite element analysis during the chewing process[209]. Polylactic-polyglycolic acid (PLGA), Polycaprolactone (PCL), and heterogenous bone mineral have been reported to be applicable for the purpose[210-212]. In addition to periodontal tissue engineering, there are even studies attempting to regenerate the entire missing tooth through dental bioengineering[213,214].

Vertebra and pelvis: A typical vertebra mainly includes four parts: (1) The vertebral body as the central part to support the body weight; (2) The vertebral arch with the function of forming the spinal bony canal and protecting the spinal cord; (3) The spinous and transverse processes attaching muscles and ligaments; (4) The articular processes forming the facet or zygapophyseal joints. The vertebral body is the part most frequently requiring 3D printing scaffolds for repairing of the bone defects. [215-217]. For vertebral bone defects caused by various reasons (including iatrogenic causes such as corpectomy)[218]. Artificial vertebral body fabricated by 3D printing technology which possessed great mechanical properties have been reported by several studies [219,220]. 3D-printed AVB can significantly prevent subsidence comparing with traditional titanium cage, though its long-term effectiveness and security in humans still need confirmation of long-term follow-up[218]. Recent researches mainly focus on degradable materials. There have been several studies reported fascinating artificial intervertebral fusion cages with optimizing biodegradability[221-223].

Appendicular skeleton: Bone defect and fracture nonunion are the most common application scenarios of 3D bioprinting for bone regeneration of limbs and joints. Currently, autologous bone grafts are still be seen as the gold standard by surgeons. However, autografting is an additional operation with many of cases are reported to suffer complications related to collection of autologous bone [224,225].

Calcium phosphate (CaP) ceramic is a synthetic material composed of hydroxyapatite (HA), with compositions, property and osteoconductivity similar to that of natural bone matrix and is commonly used in long bone reconstruction[18,226,227]. Other degradable materials include bioglass, mostly synthetic silicate-based ceramics, which can be absorbed within two weeks after implantation, allowing rapid vascularized and formation of new bone[228,229]. Mostly, the strength of synthetic scaffold is insufficient for reconstruction of large defects, so there are many efforts to reinforce the scaffolds functionality by doping with growth factors or stem cell[230,231]. In other cases, HA can also be applied in the coating of metal substitute or prostheses to facilitate bone ingrowth[232]. In recent years, the role of hydrogel in the treatment of fracture nonunion has attracted increasing attentions. An optimal hydrogel for bone regeneration should be easy to produce, injectable, biocompatible, degradable, release appropriate active growth factors within 2–4 weeks, with properties varying

according to the required graft function[233]. Gelatin, hyaluronic acid and alginate are the most commonly used naturally derived polymer hydrogels assessed for bone regeneration, while polyethylene glycol (PEG) as the typical synthetic polymer hydrogel.

6.3. Printing technique

Stereolithography Apparatus (SLA): SLA is one of the earliest and most widely used 3D printing technologies today[234-236]. The SLA based on layer-by-layer printing technology with the liquid materials scanned by the light source undergoes photopolymerization and solidifies to form a thin layer[237,238].

Selective Laser Sintering (SLS): SLS usually uses a CO_2 laser with a wavelength of 10.6 μm as the heat source. At present, SLS has been widely used in medical research and clinical practice. Materials applied in SLS include a non-metallic inorganic material, metal powder and synthetic biopolymers such as polylactic acid (PLLA), polycaprolactone (PCL), polyether ether ketone (PEEK), polyvinyl alcohol (PVA), etc. The powder materials are softened or melted by the high-energy laser beam, of which the computer controls the path, and bonded to form a thin layer, and then the layers are superimposed to form a three-dimensional solid structure.

PLLA can be obtained by polymerizing pure L-lactic acid and lactide. Compared with general polylactic acid materials (amorphous polyracemic lactic acid), semi-crystalline PLLA has a longer degradation time, higher mechanical strength, larger stretch ratio and lower shrinkage rate. The glass transition temperature (Tg) is 60.5 °C, melting point (Tm) range is 172. 2–186.8 °C [239,240]. With good biocompatibility, biodegradability and high crystallinity, PCL is a chemically synthesized biodegradable high-molecular material that can be completely degraded after 24 months, and it can maintain satisfactory mechanical properties after being made into a film[241]. PVA is a non-biodegradable polymer material, a semi-crystalline copolymer of vinyl alcohol and vinyl acetate. The Tg and Tm depend on the degree of crosslinking of the polymer, and the range is 58–85 °C and 220–240 °C respectively[239]. PEEK is a semi-crystalline thermoplastic polymer with Tg of 143 °C, possessed excellent thermal stability, chemical resistance and abrasion resistance. PEEK has good biocompatibility and elastic modulus close to cortical bone. It is an ideal material for making bone tissue scaffolds and artificial joints[242]. The properties of these mentioned materials are quite suitable for SLS processing.

There are several novel scaffolds proved to be effective and bioactive, such as the biphasic calcium phosphate,[243] tetracalcium phosphate (TTCP),[244] solvent-free polylactide/calcium carbonate composite,[245] poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV),[246-248] polyamide/hydroxyapatite composites,[249] poly(D,L-lactide)/β-tricalcium phosphate composite,[250] etc.

Fused Deposition Modelling (FDM): FDM techniques for 3D printing have been developed in 1980s. Commonly used materials are thermoplastic polymers such as polyamide, polyester and polyethylene, in the form of powders or filaments, which can be fed into the extruder of molten plastics and deposited on the surface at a lower temperature at the outlet for a quick solidify. The advantages of this technology are high molding accuracy and high strength of the stent. As solvents or other additives are not needed, the molding does not require subsequent impurity removal. Yet the disadvantage is that the printing temperature is quite high, which could lead to degradation of polymer materials or bioactive factors.

Porous Design: In addition to focusing on the biocompatible properties, well studied bone substitute should also provide mechanical strength similar to the natural bone and avoiding stress shielding,[251] as well as facilitating bone ingrowth, [252,253] better to be biodegradable at an appropriate rate with the regeneration process of bones[254]. A fully-interconnected porous structure can benefit the properties mentioned above and should be considered in the bioprinting for bone regeneration. Tremendous materials have been applied in the porous design of bone substitutes. However, the mechanical properties of polymer-based biomaterials are commonly low, while ceramic-based biomaterials are inherently brittle[255,256].

Support bath: support bath refers to printing low viscosity bone tissue bioinks in a suspension medium, which is a method to maintain the activity of biological factors or cells in bioinks as much as possible. Generally speaking, the higher the viscosity of the bioink containing bone cells in 3D bioprinting is, the better it is to print precise bone details, but high viscosity means the loss of biological activity of bone tissue. Support bath technology allows low viscosity bioink to produce acute structure patterns, which solves the contradiction between viscosity and biological activity in bone tissue printing to a certain extent. At present, the most common use of microgels is to provide physical strength support for individual cells or cell spheres that are close to the liquid, and do not destroy the transport of bone cells. In the existing support bath model, human bone stem cells can differentiate into muscle and bone in high fidelity personalized organ structure without being affected.

Sacrificial manufacturing: different from support bath, sacrificial manufacturing aims to remove non-cellular components and retain cells and biological factors after printing, so as to solve the problem that highly active bone tissue bioink of insufficient viscosity cannot be printed. Sacrificial manufacturing can bring cells into the printing of anatomical structure details, which can more faithfully simulate the structure of human bone tissue. In the reported cases, high stability materials (such as alginate and polycaprolactone) were combined with femur cells to achieve accurate printing of femur. In addition, sacrificial manufacturing may be conducive to the production of vascular tissue, because when the template is removed, intercellular spaces will be left, which enhance the degree of vascularization.

Internal reinforcement: the internal reinforcement strategy also aims at the contradiction between printing requirements and bioactivity requirements by permanently embedding the scaffold to provide additional rigidity. This method may be suitable for bone structure printing, but it may not be suitable for soft tissue moldings such as muscle and joint. Previous studies have fully demonstrated that adding additional scaffolds can enhance the strength of the model without affecting cell viability: for example, fibroblasts encapsulated in hydrogels can proliferate, and even stronger matrix formation ability within 3 days in the presence of poly (ethylene glycol) silicate scaffolds. At the same time, the existence of an internal stent also destroys the potential channel formation, which is not conducive to the diffusion of oxygen and nutrition.

In general, 3D bioprinting methods are very rich, but none of them can meet all the requirements of bone regeneration. In fact, many new printing methods are committed to a basic contradiction: the requirement of a printer for the rigidity of printing materials and the requirement of bone tissue-related cells for the gap. The harder the material is, the better it seems to be for printing, but bone tissue-related cells are often difficult to survive on these hard materials; the thinner the material is, the better it seems to be for cell survival, but the existing printing equipment seems to be difficult to print these thin materials. From the technical point of view, all kinds of printing methods are the improvement of mature basic printing methods, so we should also show the advantages and disadvantages of several basic bone regeneration printing methods (inkjet, laser, extrusion) in order to design better printing strategies.

Inkjet bioprinting has the advantages of fast manufacturing speed, low cost, and a very accurate printing effect in bone tissue printing. A large number of studies have explored the use of this technology for bone regeneration, even in vascularization. However, the density of bone tissue cells produced by ink-jet bioprinting is low (10^6 cells/ml), and the viscosity it can support is limited (3.5–12 MPA / s), so the bone tissue produced often needs additional cross-linking structure to meet the requirements of bionics. Laser-assisted bioprinting can produce bone tissue with strong cell viability, and the printed pattern resolution is comparable to that of ink-jet bioprinting, so it can not only print living cells but also print DNA and other precise cell content. However, the cost of laser-assisted bioprinting is high, the printing speed is slower than that of inkjet, the viscosity range is also insufficient (1–300 MPa/s), and the cell density is limited (10^8 cells/ml). Therefore, there are few studies using laser-assisted bioprinting to directly produce blood vessels, which seems to be unable to meet the requirements of vascularization. Extrusion bioprinting can print spheres with extremely high cell concentration, and the viscosity of bioink is also low (30 to 60 $\times$ 10^7 MPA / s). Therefore, extrusion bioprinting can produce bone tissue structures that require more dense cells, such as blood vessels. However, the survival ability of cells extruded from bioprinting is insufficient, and it is often difficult to maintain vitality in the long-term model.

7. Drug delivery in 3D bioprinting design

A significant proportion of bone defects are due to brittle fractures of osteoporosis, and even other types of bone defects are associated with localized osteoporosis. Compactness and hardness of the surrounding bone are also key factors for a successful bone repair during the implantation of bone regeneration scaffold [257]. Unfortunately, the vast majority of oral medications currently in clinical practice are ineffective in treating osteoporosis. Often the drug causes nonspecific bone formation in areas where it is not needed. Also, the drug's onset time is too slow, lagging significantly behind the critical stage when bone tissue is embedded or attached to the scaffold[258].

Therefore, the application of drugs, bioactive substances, and even mesenchymal stem cells on bone scaffolds has been widely considered as an effective strategy, as shown in Table 2 [259].

Table 2
Drug delivery in 3D bioprinting design for bone regeneration.

Name	Types	Mechanisms and Applications
BMPs	Protein	Inducing osteogenesis and harmonizing osteoclast genesis; promoting bone regeneration
VEGF	Protein	Improving angiogenesis to promote bone regeneration
TGF-β1	Protein	Up-regulating the levels of RUNX2, osteocalcin, ALP and calcium deposition
abalone	Protein	As adjuvant of BMP-2; promoting osteoblast differentiation
deferoxamine	Iron chelator	Activating activate HIF-1α; inducing osteoinduction and vascularization
MSCs	Cells	Promoting osteogenic differentiation and promoting new bone formation
osteoblast	Cells	Cell proliferation and migration; enhancing new bone formation
HYSA	Chinese medicine	Up-regulating the expression of ALP, HIF-1α and BMP-2; stimulating osteogenesis
DMOG	Inhibitor of HIF-PH	Stabilizing HIF-1α expression, inducing bone-related gene expression of hBMSCs
DEX	Corticosteroid drug	Anti-inflammation; anti-bacterial; promoting osteoinduction
Gentamicin	Antibiotics	Prevent orthopedic infections; promoting bone regeneration
Heparan sulfate	Glycan	Stimulating osteoblast maturation and promoting bone repair.
Calcitonin	Hormone	Inhibiting osteoclast activity; temporarily reversing bone resorption
PTH	Hormone	Regulating bone anabolism; inhibiting osteoblastic apoptosis
Ag2+/AgNPs	Inorganic ion	Promoting cell proliferation, and enhancing higher alkaline phosphatase activity
Mg2+	Inorganic ion	Supporting bone growth and boosting local blood perfusion
strontium	Inorganic ion	Enhancing cell viability, proliferation, adhesion, and alkaline phosphatase activity
n-HA	Nanoparticle	Serving as a nano mechanical reinforcer and an osteoconductive factor
Ca-P	Nanoparticle	Improving cell activity; promoting osteogenic differentiation and bone growth
Silica/Silicate	Nanoparticle	Supporting the scaffold strength, drug release, cell adsorption; promoting osteogenic differentiation of hMSCs.
PLA	Biodegradable polyester	Reducing cell cytotoxicity; enhancing drug sustained release and osteogenesis
PCL	Biodegradable polyester	Serving as a porogen; enhancing cell attachment and biocompatibility

BMPs, bone morphogenetic proteins; VEGF, vascular endothelial growth factor; TGF-β1, transforming growth factor-β1; ALP, alkaline phosphatase; HIF-1α, hypoxia-inducible factor 1α; MSCs, mesenchymal stem cells; HYSA, hydroxy-safflower yellow A; DMOG, dimethyloxallyl glycine; HIF-PH, HIF prolyl hydroxylase enzyme inhibitors; hBMSCs, human bone marrow mesenchymal stem cells; DEX, dexamethasone; PTH, parathyroid hormone; AgNPs, silver nanoparticles; n-HA, nanocrystalline hydroxyapatite; PLA, polylactic acid; PCL, polycaprolactone.

7.1. Biomacromolecule

Whether loading drugs, biomacromolecules packaging, targeted markers bonding or cells culturing, none of its 3D printing strategies allow one-step high temperature molding, as most of these substances are extremely sensitive to temperature degradation. Thus, low temperature electrospinning, 3D printing or dip-coating, surface modification, packaging with hydrogel, and spray-coating on the finished scaffold surface were the common strategies[260,261]. For example, The mesoporous calcium silicate (MesoCS) 3D printed scaffold has excellent biological activity and can enhance the formation of bony apatite. Lin et al. loaded the biomacromolecule bone morphogenetic protein 2 (BMP-2) into the mesoporous calcium silicate (MesoCS) and prepared composite scaffolds by 3D printing technology. The results show that the 3D MesoCS / polycaprolactone scaffold exhibits excellent biocompatibility and physical properties. After being immersed in a simulated body fluid, a bony apatite layer can be formed. In addition, BMP-2 can be released continuously [262]. Also, for the macromolecule like acetylated nanocellulose has been proven to be used for cell culture due to its similarity with extracellular matrix [263]. Rojas et al. proved low degree of substitution of acetylated nanocellulose can be used for 3D printing to prepare scaffolds that can support cell proliferation [264]. On the other hand, Some polymers have inherent ability to adhere to large molecules, such as 2-methacryloyloxyethyl phosphorylcholine (MPC), by layer-by-layer printing strategy that concentrates the polymers on the outermost layer of the scaffold is the most direct method of fabrication[265]. Electrochemical oxidation strategies using nitric acid solution to corrode different scaffolds to surface functionalized hydroxyl groups, resulting in only a small amount of immobilized silane coupling agent is needed to carry the macromolecular protein to promote bone regeneration[266]. Also, N reacts with hydrogen atoms, where the scaffold oxidized is incubated with gaseous trifluoroacetic anhydride[267].

Calcitonin: Calcitonin can temporary and reversible effect on bone resorption through osteoclast activity inhibition. Long chains of hydrogels are connected together to form a network; Drugs are physically or chemically attached to hydrogel chains and networks

[268]. Hydrogels can be loaded with drugs by hydrogen bonding, electrostatic interactions, and other physical means. Hydrogel can also form chemical bonds with drug groups, leading to drug release[269]. With the degradation of hydrogel, the drug gradually diffuses from the net. Thus, the drug loading capacity of implant is increased and the duration of drug release is prolonged.

Bone Morphogenetic Proteins (BMPs): BMPs has been widely studied due to their unreplaceable ability to facilitate the bone regeneration. The mesoporous bioactive glass (MBG) was utilized for the surface coating of silicate 1393 bioactive glass (abbreviated as 1393@MBG) can enhance the alkaline phosphatase (ALP) activity[270]. It is reported a design of 3D printed osteochondral nanocomposite scaffolds, which bearing functional water-in-oil emulsions can greatly stimuli the cell growth in the surrounding bone sites[271]. Another 3D porous hollow cage adapted with rhBMP-2 and mesenchyme stem cells was reported to realize bone grafting [272].

Parathyroid hormone: Drugs like parathyroid hormone are utilized in the treatment of osteoporosis because they are more effective than bisphosphonates. The drug is also used for bone anabolism and its ability to inhibit osteoblastic apoptosis[273]. However, multiple injections are required for this class of drugs, and procedures can be used to generate solutions through a PTH-targeted delivery system. At the same time, the use of CS-PTH NPs was modified with poly-ethylene glycol as a delivery agent instead of daily injection can also improve the delivery pattern [274].

Drugs delivery: At present, the most commonly used method for drug loading of bone stent is dip coating. The method begins by dissolving the drug in phosphate buffered saline or diluted water and immersing the implant in the solution. During immersion, small molecules of the drug are physically delivered as an electrolytic coating, and deposited in the pores or surface of the implant. However, despite the advantages of convenience, this approach also has obvious disadvantages, such as limited drug loading doses, short drug release cycles and, most importantly, lipid-soluble drugs that are not permitted in implanted drug delivery systems due to sensitization by their solvents. At the same time, if the post-dissolution eluent is forcibly used, the drug will recrystallize and precipitate, leading to adverse reactions of by-products. Porous materials are widely used in drug delivery research due to their porous structure and adjustable surface functional modifiability [275-277]. It was reported that the deferoxamine (DFO) loaded 3D-printed scaffold has excellent vascularization and osteogenic activity, and can quickly promote the repair of huge bone defects in the distal femur of rats. The use of DFO to activate the HIF-1α signaling pathway not only has an important regulatory effect on the coupling of vascularization and osteogenesis and development, but also confirmed that it has an effect on the differentiation and maturation of osteoblast precursor cells into osteoblasts (Fig. 7A) [278].

7.2. Nanoparticle delivery

Experimental evidence has been shown to reflect how influencing the shape of a particle changes its ultimate functionality. It has been reported that using 3D-printing technology, nanoparticles can arranged tightly and form a macroscopic super crystalline structure (Fig. 7B) [279]. In one study, after graphene sheets were modified with magnetite nanoparticles, its application in the biomedical field was greatly improved. (Fig. 7C) [280].

Modification of the shape of micro- or nano-particles will also affect the particle's ability to target specific cells[281,282]. Due to the increased surface area, rod-like nanoparticles have been shown to increase interactions with cell receptors, resulting in higher uptake than that of spherical particles[283,284]. Altering a particle's shape from the traditional spherical particle also increases its duration in the blood stream due to a reduced likelihood of rejection by immune cells[282,285]. These should be noted in printing technologies to fabricate drug particles & devices with complex 3D shapes & structures at nano- & micro-scales[286]. It is important to note that the release profile of a 3D-printed hydrogel-based drug carrier is highly dependent on the resolution of the particle[287].

7.3. Cell delivery

Cultivating cells like mesenchymal stem cells (MSCs) for bone regeneration therapy is extremely expensive and time-consuming. Research should focus on how to promote the retention and proliferation of MSCs at the bone regenerative sites. The key is to maximize cell viability and minimize cell damage and rupture to promote the paracrine function. In recent years, the preparation of bone regeneration scaffolds by 3D printing technology for cell delivery has received increasing attention. Cells can be wrapped inside the material as bio-ink for 3D printing bone regeneration scaffolds. Lee et al. chemically introduced sulfate groups into alginate and formulated several bio-inks. These inks consist of alginate and various amounts of alginate sulfate. The results show that the alginate/alginate sulfate bio-ink can significantly promote bone regeneration, because by adding sulfate groups, the bio-scaffold can extend the activity of bone morphogenetic protein, thus obtaining good 3D cell printing ability and bone regeneration [288].

Traditional 3D printed scaffolds have a series of shortcomings for cell delivery, including low porosity and non-channel structure, which hinder the process of bone formation and vascularization. Inspired by the structure of the root of lotus, Wu et al. successfully prepared different materials (including ceramics, metals, and polymers) into biomimetic materials with a lotus root-like structure through 3D printing technology. This type of scaffold breaking the limitations of traditional 3D printing methods. Compared with the traditional 3D printed scaffold, the 3D printed scaffold with root structure significantly improves the proliferation of bone mesenchymal stem cells in vitro and the construction of blood vessel network [289]. Similarly, the operation of using cells as bio-ink for 3D printing also has certain disadvantages, including a large number of pre-culture periods and external stimuli (e.g. UV). Therefore, Whitely et al. developed a biodegradable hydrogel as an injectable stem cell delivery system and seeded it in situ into a 3D printed scaffold. The hydrogel system can achieve different curing rates by changing the content ratio of the internal oxidant and reducing agent, which is beneficial to control the efficiency of cell encapsulation inside the scaffold [290]. In short, the development of 3D printed bone regeneration scaffolds for cell delivery is very rapid, and how to obtain functional materials for the construction of the scaffold system is very important.

8. Electrospinning and 3D bioprinting design for bone organoid

Organoid, as an in vitro personalized specific 3D cell culture system which possessed fundamental characteristics of the presented organs, can not only be used for drug evaluation, but also accelerate the recovery of damaged organs [5]. However, cell-based bioproducts often suffering the disadvantages of unpredictable in vivo behavior, which severely hampering the clinical translation. Fracture healing, one of the bone repairing process, is depend on the intermediate products (“soft callus”) fabrication, which composed by skeletal stem cells from periosteum and can subsequently transform into bone. Therefore, the use of bone stem cell-based microsphere organoids, which can provide a homoge-

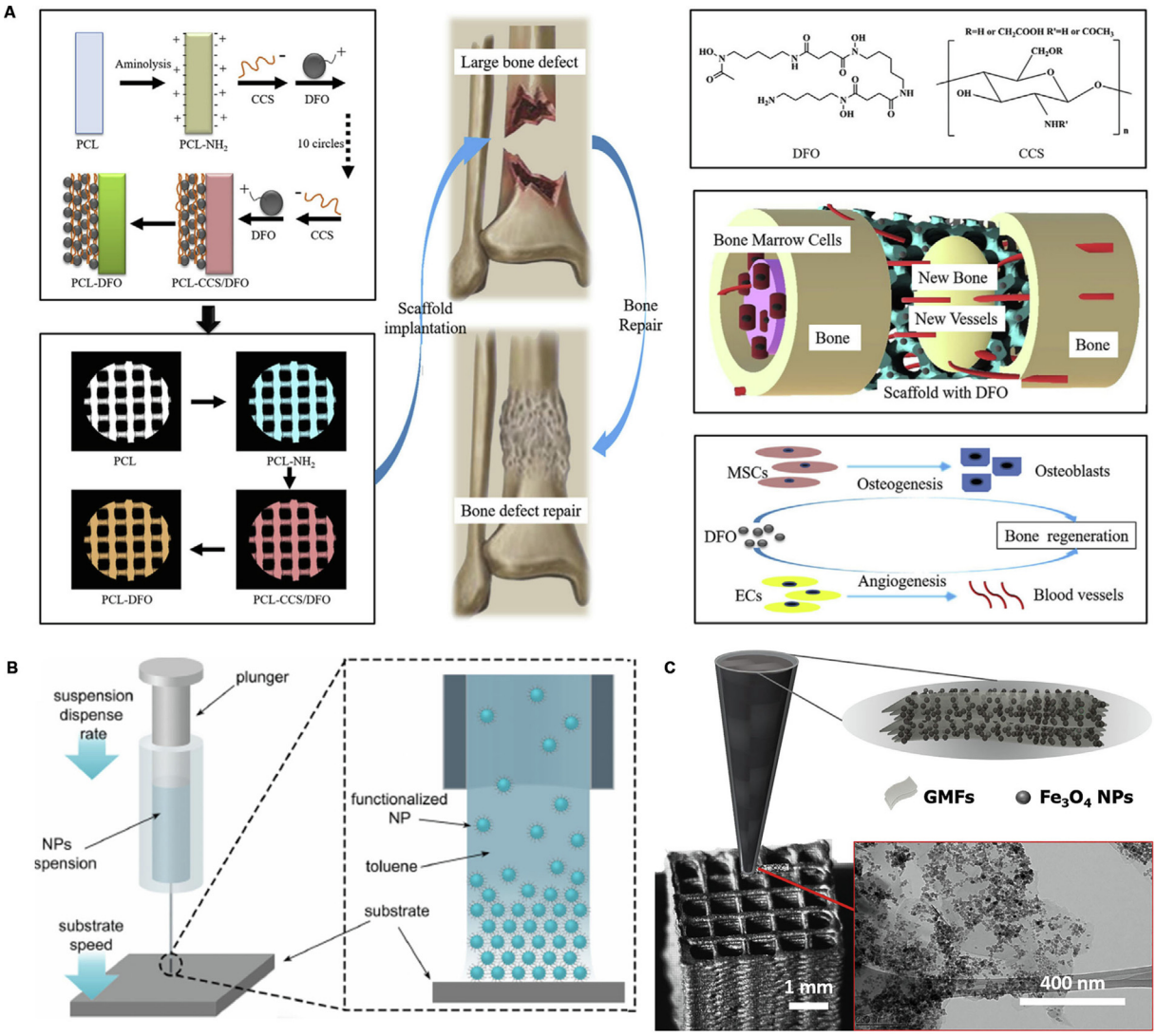


Fig. 7. (A) Deferoxamine (DFO) decorated 3D printed polycaprolactone scaffold and its microstructure as well as the mechanism for bone regeneration.[278]. Reused with permission. Copyright 2019 Elsevier. (B) 3D printing cooperated with the ceramic functionalized nanoparticles to form a powerful macroscopic super crystalline structure [279]. Adopted with the permission. Copyright 2020 Wiley. (C) Schematic diagram of functionalized graphene-polymer 3D mesh printing based on the principle of extrusion 3D printing[282]. Reused with permission. Copyright 2020 Elsevier.

neous three-dimensional construction for callus-like organoids fabrication had been extensively studied [291-294].

Typically, periosteum derived cells can spontaneously assemble together which allows the scalable production of semi-autonomous callus organs, which form bone micro-organs upon implantation [292] (Fig. 8A). At the same time, organoids can also be assembled into custom-made constructs according to different bio-applications [295] (Fig. 8B). Although microsphere organoids which fabricated by putting cells into different containers have made tremendous progress, it's still hard to precisely adjust the self-renewal and differentiation capabilities of the cells, as well as achieve the simultaneous construction of multiple components (e.g., bone stem cells or vascular endothelial cells) in the bone organoid.

Over the past decade, the rise of electrospinning and 3D bio-printing technology has deepened people's understanding of tissue engineering and organ reconstruction which can be assembled in vitro at the cell level. Recently, biological 3D printing based on microfluidic devices has shown great potential. By controlling the flow rate of each channel of the microfluidic control, microspheres with multiple complex structures can be printed [296,297]. For the manufacture of bone organs, through the air-assisted 3D bioprinting method, human multicellular bone organoids can be reconstructed with high-resolution and precise spatial structure in a limited size [298]. Experimentally, a 3D printer based on a multi-channel microfluidic chip was used to precisely inject hydrogels which containing different cell components (Human vein endothelial cells and human bone marrow mesenchymal stem cells) to produce partitioned gel droplets with adjustable proportions. At the same time, the airflow produced by a controllable jet nozzle caused the droplet to rotate, so that the cells were arranged in a spiral structure inside the droplet (Fig. 8C). The adopted endothelial cells were distributed around the mesenchymal stem cells in a spherical spiral structure, which vividly simulated the physiological characteristics of the human bone tissue and realized the controllable molding of the three-dimensional structure and improved the molding accuracy to single-cell resolution.

Alongside to the above-mentioned combination between microfluidics and 3D printing, enhanced mechanical and flexible structure can also be obtained when utilizing the electrospinning and 3D printing technology together[299,300], through a layer-by-layer method [301]. Therefore, under the premise that

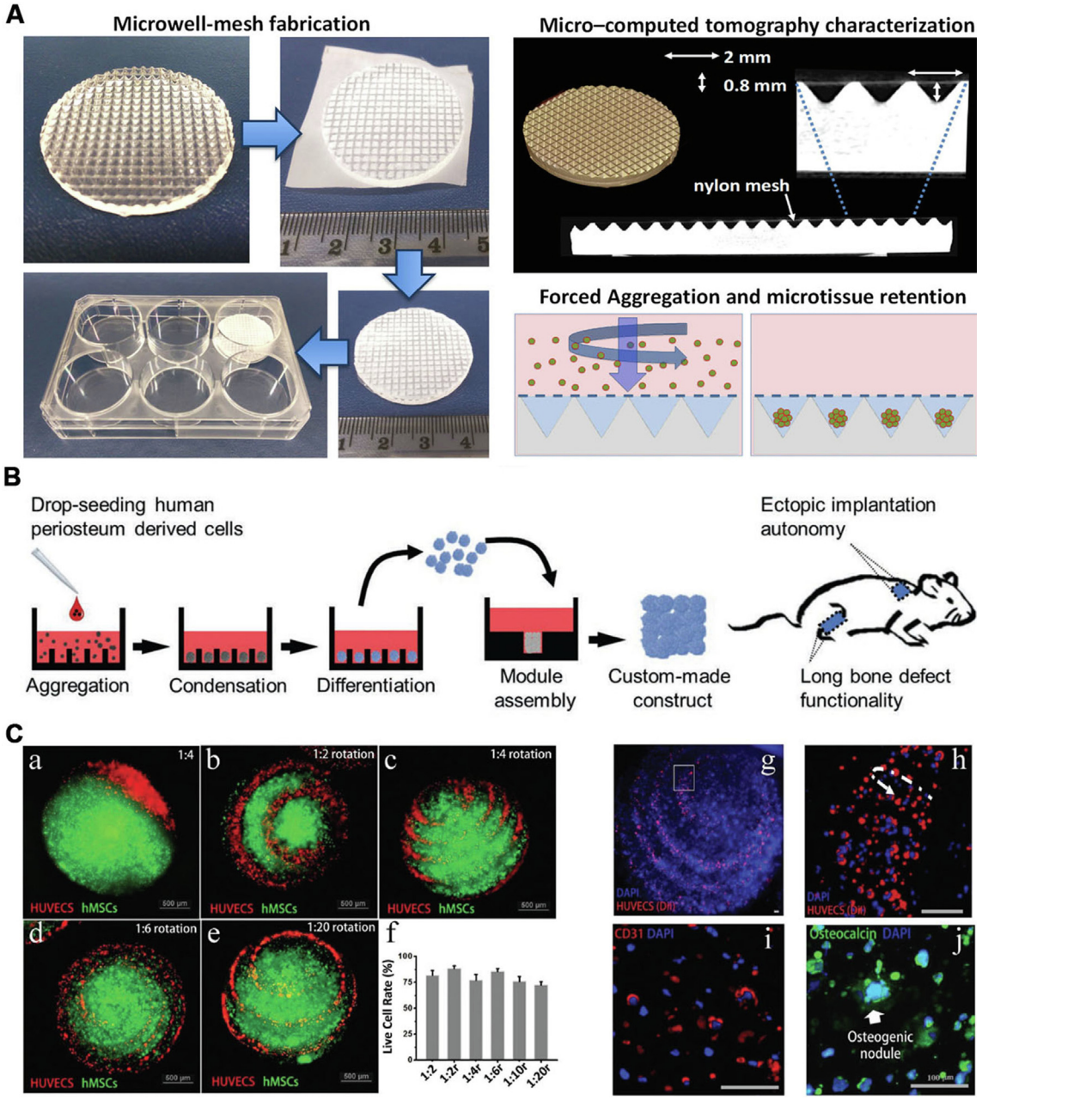


Fig. 8. Preparation and design of different bone organoids. (A) Preparation of degradable grids for self-assembly of cells into bone organoids through polystyrene mold. Reproduced with permission of Elsevier from [292], Copyright 2015. (B) Custom-made construction fabricated through Module assembly. Adopted with permission of Wiley from [295], Copyright 2020. (C) Microsphere organoids with different cell arrangements prepared by 3D printing. (a) Fluorescence pictures of traditional spacer microspheres. (b-e) HUVEC cells were spirally distributed in bone mesenchymal stem cell microspheres. (F) The survival rate of the cells after 10 days of culture in vitro. (g, h) The geometric characteristics of blood vessels in microsphere organoids after 10 days of culture. (i, j) Secretion of osteocalcin in organoids. Reused with permission of Wiley from [298], Copyright 2018.

mesenchyme stem cell (MSC) spheres organoids have higher bone regeneration potential in vitro and in vivo compared with monolayer cultured MSC, combining MSC organoids and electrospinning technology will lead to a greater acceleration to the spheres organoids based clinical translation.

9. Conclusions and future perspective

Nowadays, both electrospinning and 3D bioprinting exhibit great potential in the production of complex structures, such as bone, cartilage and osteochondral tissue for tissue engineering. Electrospinning nanofibers have been evaluated and studied as scaffolds for regenerative medicine and show high potential in the field of biomedicine and clinical treatment. Different kinds of electrospinning nanofibers are also being fabricated into scaffolds for bone regeneration with its powerful superiority in multifarious properties involving large surface areas, easy functionalization, excellent mechanical characters as well as available access to obtain. To perform the bio-functions of osteogenesis, meticulous design is necessary for electrospinning fibrous scaffolds to select the suitable material and engineer, modify or functionalize the morphology.

Moreover, the orientation of electrospinning fibers can provide guidance for attached cells by regulating their differentiation

status and affecting their morphology. Some agents including metal, antibiotic, antiphlogistic, anticarcinogen or other natural bio-activators are alternative to be loaded into electrospinning fibrous scaffolds for reinforcing the biological effectiveness as well. In general, electrospinning fibrous scaffolds are capable to accomplish their missions of promoting osteogenesis with above-mentioned elements. Likewise, the fabrication of 3D bioprinting scaffolds have been shown to be an encouraging solution with plenty of advantages including controlled porosity and design, enhanced biological activity as well as improved mechanical properties. Additionally, traditional materials can be utilized into advanced transplantation but without previous defects like immune-rejection, invariable density, and insufficient biochemical functionality through the application of 3D bioprinting. Considerable progress in electrospinning and 3D bioprinting has been realized, and it also provides a promising clinical platform for bone repair and regeneration. However, this technology is still in its infancy and some aspects need to be further investigated. Here, we put forward some of the major research and technical challenges need to be addressed for better development of electrospinning and 3D bioprinting scaffolds in biomedicine:

(I) The effect of material load levels on mechanical and biological properties is needed to study in depth, as a balance needs to be struck in both electrospinning and 3D bioprinting scaffolds to ensure optimal performance.
(II) Optimizing the addition and dispersion methods of electrospinning and 3D bioprinting for bone tissue-engineering scaffolds reinforced with nanomaterials, as materials need specific features to allow the fabrication way functioning efficiently as well as effectively.
(III) Utilize molecular biological approaches further explore the detailed mechanism under the induced biological phenomena. Only with proved molecular mechanism can we enhance properties of electrospinning and 3D bioprinting scaffolds engineering preferable products.
(IV) Adequately take advantage of existing electrospinning and 3D bioprinting scaffolds for recreating multifunctional ones. Currently, scaffolds possess accessibility as well as high clinical potential for manufacture, with which design cost is able to be saved.
(V) The success and safety of clinical translation need to be confirmed in clinical practice for coming decades. Attempt to introduce it into clinical treatment but with mandatory assessment of potential risk and safety evaluation for ensuring its specific biomedical uses.

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