

Fibrinogen and fibrin based micro and nano scaffolds incorporated with drugs, proteins, cells and genes for therapeutic biomedical applications

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Abstract: Over the past two decades, many types of natural and synthetic polymer-based micro- and nanocarriers, with exciting properties and applications, have been developed for application in various types of tissue regeneration, including bone, cartilage, nerve, blood vessels, and skin. The development of suitable polymers scaffold designs to aid the repair of specific cell types have created diverse and important potentials in tissue restoration. Fibrinogen (Fbg)- and fibrin (Fbn)-based micro- and nanostructures can provide suitable natural matrix environments. Since these primary materials are abundantly available in blood as the main coagulation proteins, they can easily interact with damaged tissues and cells through native biochemical interactions. Fbg- and Fbn-based micro and nanostructures can also be consecutively furnished/ or encapsulated and specifically delivered, with multiple growth factors, proteins, and stem cells, in structures designed to aid in specific phases of the tissue regeneration process. The present review has been carried out to demonstrate the progress made with micro and nanoscaffold applications and features a number of applications of Fbg- and Fbn-based carriers in the field of biomaterials, including the delivery of drugs, active biomolecules, cells, and genes, that have been effectively used in tissue engineering and regenerative medicine.

Keywords: biomaterial, polymer composite, cross-linking, growth factor, drug delivery, controlled release, tissue regeneration

Introduction

Tissue engineering is a multidisciplinary area that encompasses biology, chemical engineering, material science, and chemistry. Existing techniques have the potential to create specific tissues and organs in vitro and in vivo. For fabrication of biomimetic scaffolds, a range of synthetic and protein-based polymer scaffolds, such as collagen, gelatin, elastin, proteoglycan, hyaluronan, laminin, silk fibroin, soybean, fibrinogen (Fbg), and fibrin (Fbn) have been widely used. Among these polymers, Fbg- and Fbn-based scaffolds have been widely used to construct functional substitutes (tissues and organs), which may provide a permanent solution to damaged tissue and organs without need for additional therapies, due to their nonimmunogenicity (autograft) and their ability to mimic the organization of native tissue structures. Fbg is a 340 kDa blood protein involved in blood coagulation and platelet aggregation (thrombogenesis). When Fbg and thrombin solutions are mixed together in the presence of Ca^{2+} forms Fbn through polymerization, it can be digested by the proteolytic enzyme (plasmin) through fibrinolysis.¹ Similar to collagen, Fbg and Fbn scaffolds can attain a high cell seeding efficiency and uniform cell distribution and can then proliferate, migrate, and differentiate into specific tissues/organs by secreting extracellular matrix (ECM)

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(which is involved in the generation of tissue).² Fbg has major healing advantages, as it provides a favorable surface for cellular attachment and proliferation – a three-dimensional (3-D) fibrous structural support and nanotextured surfaces, which consist of a fibrous network for cell signaling, and cell–matrix and cell–cell interactions.³ Especially in tissue engineering, Fbg- and Fbn-based scaffolds induce ECM production that renders support to connective tissues, such as cartilage, ligament, bones, tendons, nerves, blood vessels, and skin.^{4–8}

Fbg and Fbn can be easily formulated into diverse structural scaffolds because of their micro- and nanocharacteristic; micro/nanoporous scaffolds, such as microspheres,⁹ microfibers,¹⁰ microtubes,¹¹ nanoparticles,¹² nanofibers,¹³ and hydrogels,¹⁴ have been formulated for diverse tissue engineering applications. In a vast range of severe pathological conditions, such as chronic wounds, diabetic ulcer, and ischemic heart disease, the rate of reepithelialization and neovascularization can predict the therapeutic approach towards perfusion and healing of damaged tissue.^{15–17} Since Fbg and Fbn scaffolds are considered to be natural provisional ECM, they also play a significant role in the early stages of hemostasis and wound repair, in addition to blood clotting. Furthermore, Fbg contains two Arginine–Glycine–Aspartate (RGD) integrin-binding sites, which usually bind endothelial and fibroblast cells.¹⁸ Fbg also has a high affinity towards biomolecules, such as vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), and many other tissue-inductive cytokines.^{5,19}

Current 3-D scaffolds may incorporate different structural features, such as micro/nanoporous matrices or composites, micro/nanofibers with porous characteristics, micro/nanospheres or particles, and micro/nanofiber composites within porous scaffold. Micro/nanospheres or particles within a porous scaffold (shown in Figure 1) have attracted special interest for potential tissue engineering and regenerative applications. In many cases, growth factors/cells/deoxyribonucleic acid (DNA) can be integrated into Fbg and Fbn scaffolds by bulk encapsulation, specific/nonspecific physical adsorption, surface fictionalization, or by the use of protein or cell adhesive binders, as illustrated in Figure 2. Micro/nanocarriers or composites encapsulated with growth factors and/or drugs can be delivered to a targeted site to induce/mimic ECM productions, where tissues need to be regenerated. For specific tissue engineering applications, cell proliferation and differentiation towards specific phenotype is very favorable. To attain this successfully, in situ and in vivo sustained delivery with various drug and growth factors within Fbg and Fbn micro/nano scaffolds have

been studied.^{20,21} Further, these scaffolds loaded with growth factors, such as VEGF,²² epidermal growth factor (EGF),²³ nerve growth factor (NGF),²⁴ and bone morphogenic protein (BMP)²⁵ were shown to stimulate blood vessel, skin, nerve and bone growth, respectively. Such scaffolds have also been extensively studied with various tissue inductive growth factors, such as transforming growth factor (TGF),²⁶ FGF,²⁷ fibronectin (FN),²⁸ keratinocytes,²⁹ etc, for the growth of various tissues. Furthermore, the controlled release of these growth factors from Fbg and Fbn scaffolds provides a number of vital advantages, as growth factors transported into the target environment may locally govern multiple processes of the cell, such as attachment, proliferation, differentiation, and morphogenesis.

The development of techniques for the regeneration of different tissues (skin, nerve, vascular, and cartilage) is not only based on the delivery of corresponding growth factors, but is also dependent upon the polymer carrier and types of cell interactions. For example, alginate,³⁰ dextran,³¹ hyaluronan,³² heparin,³² polyvinyl alcohol,³³ and poly lactic-co-glycolic acid (PLGA)³⁴ scaffolds have all been used for vascular repair/regeneration, wound healing, and other tissue engineering applications. However, these polymeric materials have their own disadvantages, such as rapid degradation, denaturation of growth factors due to irreversible complexation, lack of cell proliferation, and production of acidic environments in the ischemic wound area, which could reduce protein moiety functions, as well as cause potential immune responses and acute inflammation.³⁵

The material used for fabricating an ideal scaffold must meet a number of basic criteria, such as providing an appropriate environment for enabling cellular function, cell migration, proliferation, and differentiation, and also must allow for tissue regeneration/development. Most importantly, the scaffold should not evoke any immune reactions, while providing a feasible model for cellular interaction/proliferation. Moreover, scaffolds should mimic both the fibrillar form and complex biochemical role of native ECM.³ In this review, we highlight the methods for fabricating Fbg- and Fbn-based micro/nanocarriers, including the use of hydrogel and Fbg/Fbn blended natural and synthetic matrices that are used for biomedical applications like drug release, wound dressing, and in tissue engineering and cancer therapy.

Herein we present a detailed discussion of the theory behind Fbg and Fbn use and of their use as micro/nanoscaffolds, integrated with biomolecules and cells, in tissue engineering applications; their applications for

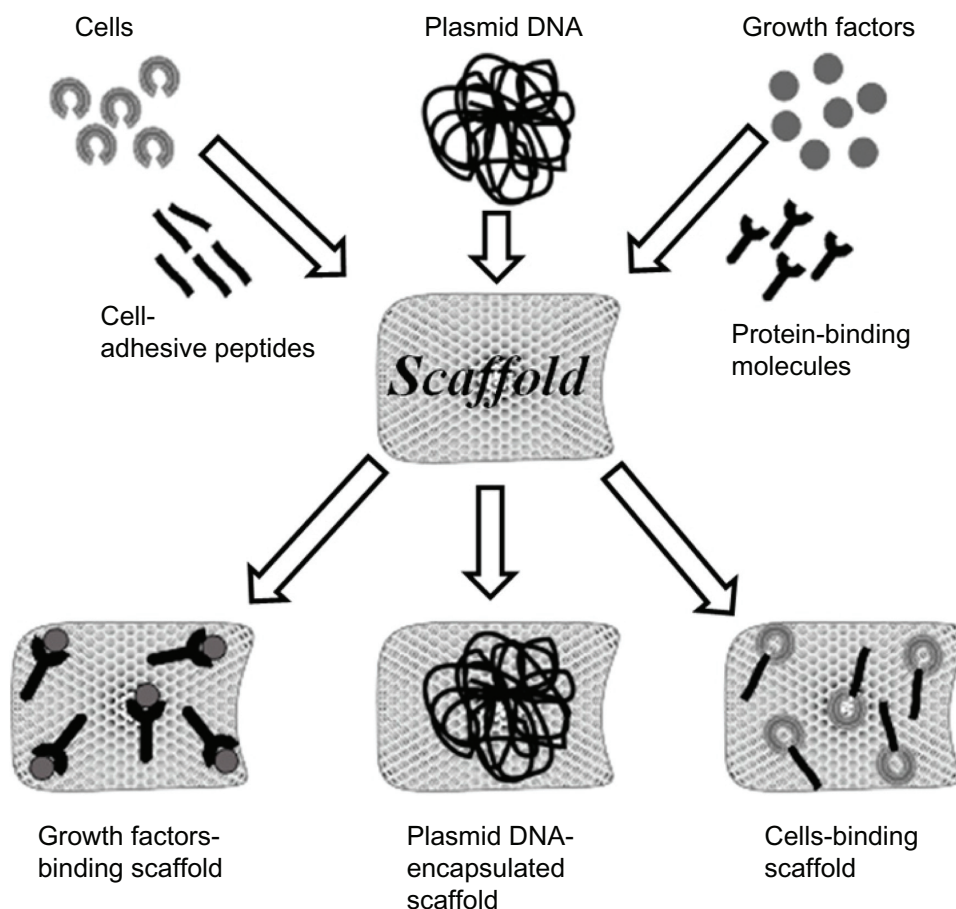


Figure 2 Incorporation of cells/DNA/growth factors into the scaffold matrix.
Abbreviation: DNA, deoxyribonucleic acid.

Fbg and Fbn microspheres/beads and nanoparticles as scaffolds

Fbg- and Fbn-based microspheres/beads and nanoparticles are widely used in a diverse range of pharmaceutical and biomedical applications, such as drug delivery, protein/peptide delivery, chemotherapy, and wound dressings and hemostatic bandages. Miyazaki et al were the first to report the fabrication of Fbg microspheres using a water and oil (W/O) emulsification method.³⁶ The prepared microspheres were then applied as a novel drug carrier for anticancer drug delivery. A few years later, Fbn microbeads were prepared by mixing both Fbg and coagulation factors (thrombin and calcium ions); additionally, glutaraldehyde was added to this system to minimize the coagulation of Fbn beads. The beads were then successfully used for various macromolecular delivery systems (insulin, lysozyme, ovalbumin, and bovine serum albumin).³⁷ In another study, Fbg-coated albumin microcapsules were administered via intravenous administration, to reduce bleeding in animals and to reduce bleeding time in surgical wounds in muscles and abdominal skin.³⁸

Generally, microspheres contain pore structures with interconnected networks, which assist in cell infiltration and in cell–matrix interaction. Recently, our group prepared Fbg microspheres (100–150 μm) with pore structures using a W/O emulsification solvent extraction method, for use in various biomedical applications. These are shown in Figure 3. Using this method, the spheres size and uniformity can be controlled with the Fbg solution concentrations (wt%), flow rate, and the stirrer rotating speed.⁹ Using the same method, Fbg nanospheres were also developed by further optimization of the above parameters, as shown in Figure 4. However, since the data is not yet published, the preparation method is not described here. Table 1 lists methods for fabricating Fbg and Fbn micro- and nanoscale scaffolds, which have been used for various pharmaceutical applications.

Fbg and Fbn micro/nanofiber as scaffolds

In tissue engineering, 3-D fibrous scaffolds with a large surface area can generally support high cell density and long-term cultures, and provide a better link between single

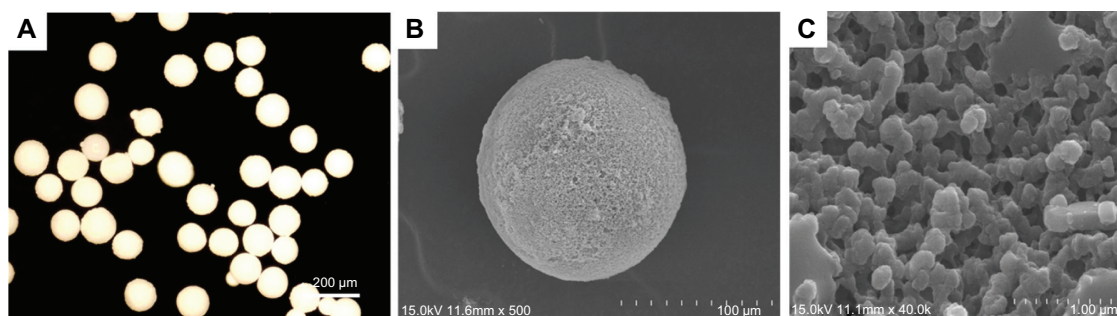


Figure 3 Microscopic images of Fbg microspheres.

Notes: (A) Light microscope image shows the microsphere sphericity and approximately uniform sizes, between 100–150 μm ; (B) SEM image further confirmed sphericity; (C) Magnified image of (B), reveals the porous structures. Copyright © 2011, Springer Science+Business Media. Reproduced with permission from Rajangam T, Paik HJ, An SSA. Development of fibrinogen microspheres as a biodegradable carrier for tissue engineering. *BioChip J.* 2011;5(2):175–183.⁹

Abbreviations: Fbg, fibrinogen; SEM, scanning electron microscope.

cells and organs than do conventional 2-D cultures. There is a list of methods available for the fabrication of polymer micro and nanofibrous scaffolds with 3-D structures: electrospinning, self-assembly, phase separation, 3-D printing, and fiber bonding. Each method has its own advantages and disadvantages, although the choice of method is also based on the polymer characteristics.

To fabricate 3-D Fbg fibers, electrospinning methods have been preferred due to their practicality and flexibility. Wnek et al were the first to report the fabrication of Fbg nanofiber using an electrospinning method: First, an Fbg suspension was prepared with 1,1,1,3,3,3-hexafluoro-2-propanol (HFP) and minimal essential medium (9:1); the mixture was left overnight and then, the homogenous solution was electrospun with a high-voltage supply (22 kV).¹³ The electrospun nanofiber showed an average size of 80–700 nm diameter, and the 3-D fiber was effectively used as a wound dressing and hemostatic bandage. McManus et al also prepared a Fbg nanofiber, using the same method and suspending agents, and in order to further evaluate the fiber surface porosity, nanofibers with two different Fbg

concentrations were fabricated.⁴³ The nanofiber produced with the lower Fbg concentration (110 mg/mL) showed a higher average porosity value (59%) than did the high-concentration Fbg (130 mg/mL) nanofiber (54%), due to the greater degree of solvent evaporation. However, both spun fibrous mats could act as a biomimetic scaffold and were deemed to be especially appropriate for use as wound dressings. The same research group, in other studies, assessed the cellular interaction and scaffold remodeling with Fbg nanofibers, using neonatal rat cardiac fibroblast cells; in these, the postculture mat histology evidenced that the Fbg scaffold was easily remodeled by the deposition of native collagen.⁴⁴ Electrospinning-prepared Fbg nanofiber was also used to evaluate in vitro cell migration on the scaffold surface.⁴⁵ Carlisle et al analyzed the mechanical properties of a single electrospun Fbg nanofiber, using a combined atomic force/fluorescence microscopic technique, and the result indicated that Fbg nanofiber had a high extensibility.⁴⁶ High-extensibility fiber scaffolds have been found to be more widely applicable for in vitro and in vivo tissue engineering due to their flexibility. Electrospun Fbg nanofibers that

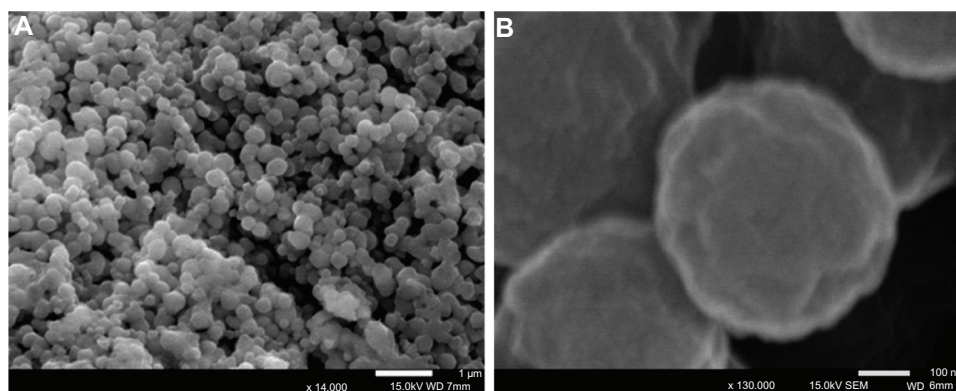


Figure 4 SEM images of Fbg nanospheres shown at different magnifications (image “A $\times 14,000$ ” and image “B $\times 130,000$ ” magnification).

Abbreviations: Fbg, fibrinogen; SEM, scanning electron microscope.

Table 1 Microcarrier fabrication methods using Fbg and Fbn and their biomedical applications

Matrix structures	Preparation methods	Applications	Ref
Fbg microspheres	Emulsification solvent extraction	Carrier for injectable (5-fluorouracil) anticancer therapy	36
Fbg microspheres	Emulsification solvent extraction	Carrier for injectable (Adriamycin) anticancer therapy	39
Fbn microbeads	Preheated oil emulsion	Wound healing	40
Fbn microbeads	Moderate heat condensation	Stem cell carriers for wound tissue regeneration	8
Fbn microbeads	Emulsion	Deliver macromolecules and protein drugs	41
Fbg nanoparticles	Two-step coacervation technique	Cancer drug delivery	42

Abbreviations: Fbg, fibrinogen; Fbn, fibrin.

interacted with human bladder smooth muscle cells showed rapid proliferation and demodulation as well as relatively slow rates of degradation.⁴⁷ In other studies, McManus et al reported the fabrication of Fbg-polydioxanone composite nanofibers with high mechanical strength for potential in situ urologic tissue development.⁴⁸ In electrospinning, the fiber size can be controlled by controlling the voltage and the distance between the needle and the collecting plate.

Usually, 3-D fiber scaffolds of the micron size range have greater mechanical strength, a property that could enhance cell adhesion, proliferation, and ease of handling during in vitro postmodification or implantation over the nanofiber scaffolds. On the other hand, they lack nanotextured topographical features, which are needed for the scaffold to mimic like native ECM.⁴⁹ Further, successes in directing cells towards certain cellular morphologies, functions, and differentiations have been studied with diverse types of nanofibers.^{49,50} Recent studies of tissue engineering have paid great attention to combining these two scaffold features towards the development of ideal scaffold fabrication. Recently, we developed nanofiber-aligned Fbg microfibers, which showed a higher rate of biomolecule encapsulation and proliferation than other highly porous and less pores Fbg microfibers.⁵¹ The structurally different Fbg microfibers were prepared by a simple tubular gel-solvent extraction method without high-power voltage or high pressure (Figure 5). Here, the Fbg suspension was prepared using aqueous phosphate-buffered saline. In our laboratory, we also prepared a highly porous Fbg sheet composed of micro- and nanostructures, using a freeze-drying method (Figure 6). However, since the data

has not yet been published, the preparation method has not been described.

Fbg and Fbn hydrogel as scaffolds

Fbn hydrogels have been most widely used as scaffolds for the reconstruction of different tissue and have been used as a biological adhesive for various procedures, such as abdominal, vascular oral, thoracic, and endoscopic surgeries.^{52–54} Mostly, Fbn hydrogels have been prepared by mixing Fbg with thrombin solution in the presence of Ca²⁺. Thrombin can cleave peptide fragments from soluble Fbg, thereby yielding insoluble Fbn peptides that aggregate to form fibril hydrogel networks.¹⁴ The characteristics of the Fbn fibril network formation were mainly based on the thrombin concentrations. For example, an increased thrombin concentration was found to be associated with shorter gelation time, the formation of more fiber bundles with thinner fibers size, and with low porosity. On other hand, lower thrombin concentrations were found to have longer gelation time, fewer fiber bundles with thicker fibers size, and more porous structures.^{55–57} In many cases, the constructed hydrogels have lacked mechanical strength, and to overcome this problem, the hydrogels were blended with biocompatible, biodegradable synthetic polymers⁵⁸ or were seeded with cells either by static or dynamic methods.⁵⁹

Rowe et al proposed a tubular, 3-D smooth muscle cell-seeded Fbn hydrogel construct for vascular tissue engineering. Here, the cells were directly mixed with the Fbg solution, were injected into an annular mold, and were then polymerized by the addition of thrombin. Further evaluation in this study concluded that the concentration of thrombin plays major roles in the outcome, influencing mechanical properties, fibril morphology, and cellular behavior.¹⁴ In another study, Almany and Seliktar developed unique Fbg-based biosynthetic hybrid hydrogels. The Fbg fragments were cross-linked with a dysfunctional polyethylene glycol (PEG) side chain to make a PEG-Fbg hybrid hydrogel. The developed hydrogel had both good cell adhesion and mechanical properties because Fbg offers cellular adhesive domains, while PEG provides mechanical and structural properties.⁶⁰ PLGA microsphere-immobilized Fbn hydrogel provides an improved elastic modulus for chondrocyte proliferation and ECM production.⁶¹ Fbn hydrogel containing BMP-2 loaded poly(L-lysine) nanoparticles and human mesenchymal stem cells (MSCs) evidenced that the Fbn constructs significantly improved the proliferation of human MSCs in bone regeneration. The study also suggested that this might be one of the most promising methods for enhancing bone

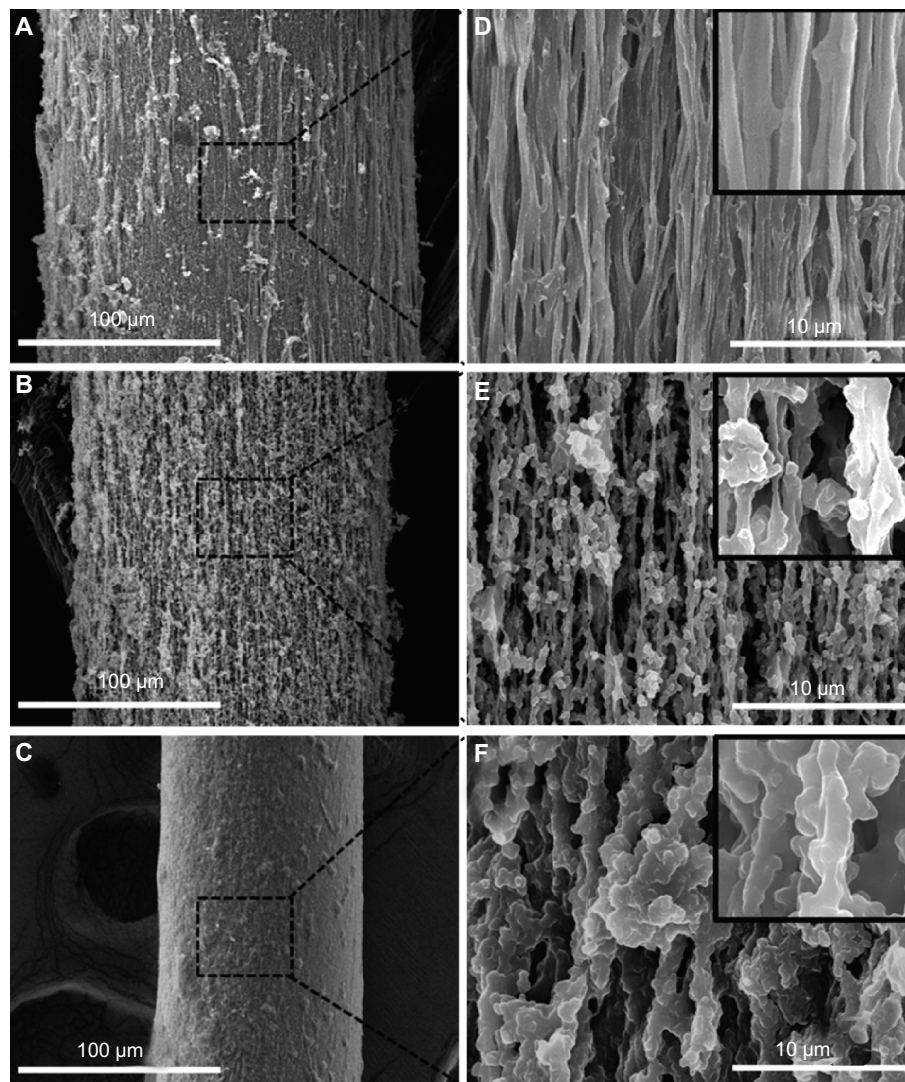


Figure 5 SEM images of Fbg microfibers fabricated from three different concentrations of Fbg.

Notes: Decreasing fiber diameter was observed with decreasing Fbg concentration. Images (A–C) show 200 μm , 150 μm , and 75 μm fibers, respectively, which were made from 15, 10, and 5 wt% Fbg, respectively. Images (D–F) represent highly aligned nanostructures, highly interporous fibers with aggregated structures, and less porous fibers with unaligned structure, respectively, and are the same as the lower magnification images in (A–C), respectively (inlet images scale bar is 1 μm). Copyright © 2012, Dove Medical Press Ltd. Reproduced with permission from Rajangam T, An SS. Improved fibronectin-immobilized fibrinogen microthreads for the attachment and proliferation of fibroblasts. *Int J Nanomedicine*.2013;8:1037–1049.⁵¹

Abbreviations: Fbg, fibrinogen; SEM, scanning electron microscope.

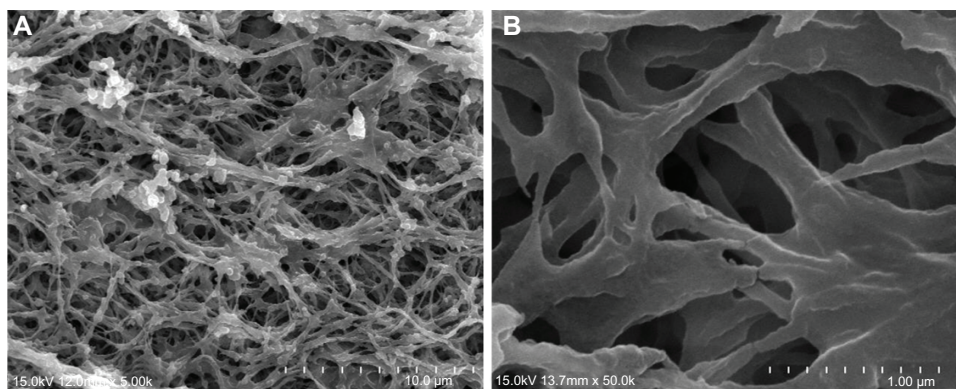


Figure 6 SEM images of an Fbg sheet reveal highly porous micro- and nanostructured networks (A and B shown at different magnifications).

Abbreviations: Fbg, fibrinogen; SEM, scanning electron microscope.

regeneration.⁶ Recently, Lei et al developed a novel Fbn-based hydrogel to deliver concentrated and nonaggregated nonviral genes, demonstrated by in vivo study.⁶² The hybrid hydrogel, made with Pluronic F127-Fbg, showed superior physical properties for in vitro cell culture. Moreover, the hybrid hydrogel retained Fbg biocompatibility with added mechanical strength, and at the same time, Pluronic F127 did not lose its unique self-assembly properties.⁶³ Fbg- and Fbn-based hydrogels have remarkable merit over other types of natural polymer hydrogels (gelatin and collagen) in that they can be made available autologously, thus avoiding viral infection transmission and the potential risks of foreign body reaction.⁶¹ Fbn hydrogels seeded with smooth muscle cells,⁶⁴ skeletal muscle cells,⁶⁵ and chondrocyte cells⁶⁶ have been used to produce engineered tissues with a high degree of mechanical strength.

Fbg and Fbn tubes as vascular graft

Our laboratory constructed small-diameter Fbg grafts as biodegradable and biocompatible scaffolds for use in microvascular tissue engineering, using a tubular gel-solvent extraction method. The tube showed a rough outer surface and smooth inner surface. The measured inner and outer diameters of the Fbg microtube were approximately

750–850 μm and 950–1050 μm , respectively (Figure 7) (data unpublished).

Fbg/Fbn–synthetic polymer composites as micro/nano scaffolds

Synthetic polymers⁶⁷ and copolymers⁶⁸ have been frequently used in the field of tissue engineering, as they have ability to precisely control the mechanical strength of the matrices, but often, these polymers provide inadequate cell regulation for specific cellular events. Fbg and Fbn have been widely mixed/blended with other natural/synthetic polymers to fabricate different composite biomaterials for vascular regeneration, bone regeneration, chronic wound healing, osteogenesis, and reepithelialization.^{69–71} In addition, biocomposite materials acquire adequate mechanical strength, enhanced cell adhesion, proliferation, and ECM production as well as the potential regulation of other cellular process activities for specific cellular events.

PEG-Fbg composites furnish attachment motifs for smooth muscle and endothelial cells adhesion; these cells have demonstrated their capability to pass through scaffolds to carry out their respective functions.⁶⁰ The PLGA microspheres immobilized with Fbg matrix have shown enhanced chondrocyte proliferation and ECM

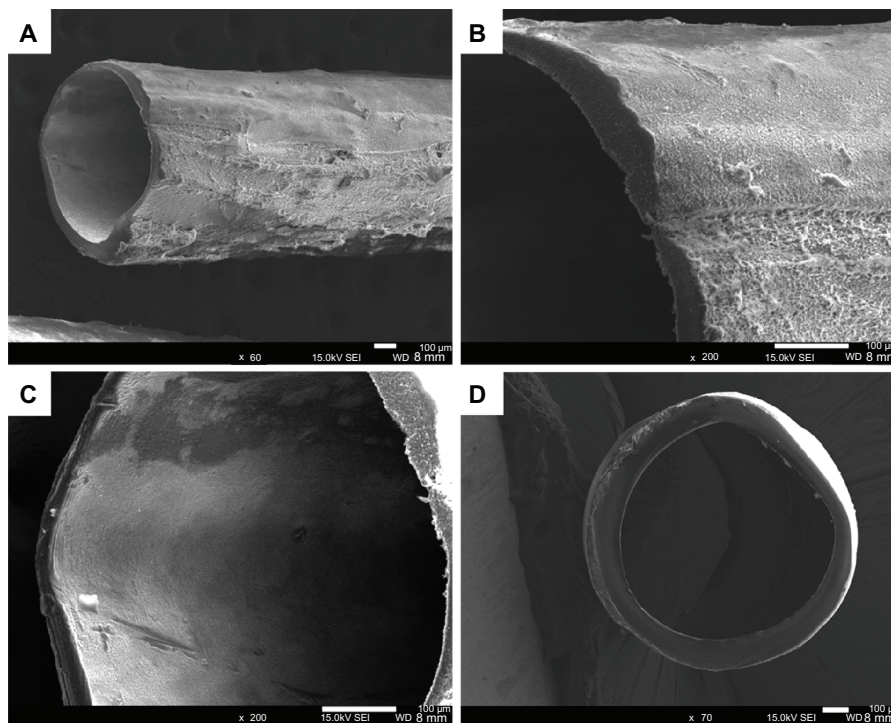


Figure 7 SEM images of a Fbg microtube. (A) The outer morphology of the tube shows the rough surfaces; (B) Magnified image of microtube shown in (A); (C) The tube's inner surface was revealed to be much smoother than the outer surface; (D) The cross-sectional SEM image reveals the round shape, with approximately uniform wall thickness. (data unpublished).

Abbreviations: Fbg, fibrinogen; SEM, scanning electron microscope.

production.⁶¹ PEGylated albumin combined with PEGylated Fbg hydrogel was shown to reveal comprehensive drug release and degradation properties.⁷² Macro/microporous biphasic calcium phosphate granules combined with Fbn glue were shown to provide a moldable and self-hardening biocomposite, which has been used in types of orthopedic and maxillofacial applications, due to their osteoconductive properties.⁷³ Fbn-PLGA composites were shown to encourage early chondrogenesis in rabbit articular cartilage, and this indicates that Fbn-PLGA might act as a possible cell delivery vehicle to promote articular cartilage development.⁵⁸ Frisman et al reported a method for fabricating nanostructured biocompatible and biosynthetic polymeric hydrogel by cross-linking PEG and Fbg. The constructed 3-D hydrogel provided enhanced cellular behavior and superior mechanical strength.⁷⁴ Polycaprolactone valves coated with Fbn demonstrated proper opening and closing dynamics in cardiac tissue engineering, with good biocompatibility,⁷⁵ and in a further study, Fbn-polyurethane microporous hydrogel cultured with osteogenic cells exhibited promising results in articular tissue development in vivo.⁷⁶

The delivery of genetic material and DNA by polymer-based hydrogels would add to the applications in the field of gene therapy and cancer therapy.⁷⁷ However, the delivery of

DNA through a synthetic cationic polymer (“polyplexes”) hydrogel has not previously succeeded because of high aggregation and inactivation of the cationic polymer inside the hydrogel matrix.⁷⁸ But, the same polymer hydrogel when functionalized with Fbg, could be effectively used to deliver concentrated DNA without aggregation.⁶² Osathanon et al reported a novel method of constructing a calcium phosphate–Fbn hydroxyapatite-based composite scaffold, which revealed a tightly interconnected network and a more porous structure, and the biodegradable composite supported primary osteoblast-like cell adhesion, proliferation, and differentiation in bone tissue engineering, in vitro and in vivo.⁷⁹ Fbg and Fbn also have been combined with different types of synthetic polymers, such as polydioxanone,⁴⁸ poly(ϵ -caprolactone),⁸⁰ etc. Other composite materials that have been used in similar applications are listed in Table 2.

Fbg/Fbn–natural polymer composites as micro/nano scaffolds

These combination natural polymer matrix–based cell delivery systems have become a promising approach in the development of many current cell therapies, as such systems exhibit synergistic effects on cellular interaction, promoting integration into host tissue, as well as offering variable material

Table 2 Delivery systems for different biomolecules and cell types using Fbg/Fbn composites, and their tissue engineering applications

Micro/nano scaffolds/composites	Types of growth factor/cell delivery	Targeted tissue	Respective function	Ref
Fbn-calcium phosphate	rhBMP-2	Bone	The highly interconnected porous composite promoted bone formation in mouse calvarial defect model	79
Fbg-polydioxanone	Bladder smooth muscle cells	Urologic tissue	Promoted cellular ingrowth and in situ urologic tissue regeneration	48
Alginate-Fbn-hyaluronan	TGF- β 1	Cartilage	Promoted chondrogenic differentiation of MSCs and sulfated glycosaminoglycans synthesis, inducing cartilage tissue formation	93
Silk-Fbn-hyaluronic acid	Human chondrocytes	Spinal disc	Provided both mechanical strength and biochemical support for nucleus pulposus cartilage formation	91
Fbn-poly(ϵ -caprolactone)	Human umbilical vein endothelial cells	Cardiovascular tissue	The composite scaffold provided integrated physicochemical character with biomimetic property for cardiovascular tissue regeneration	80
Fbn-poly(L-lactide)	Auricular chondrocyte	Cartilage	The porous scaffolds furnished significantly higher level of rabbit auricular chondrocyte viability, with better mechanical strength and cytocompatibility for cartilage tissue engineering	61
PEtU-PDMS/fibrin	VEGF and bFGF	Blood vessels	The composite stimulated new blood vessels formation in ischemic tissues in general and in particular, in ischemic cardiac tissues	94
Collagen-hyaluronan-Fbn	Chondrocytes	Cartilage	The implanted scaffold enhanced cartilage regeneration of osteochondral defects by the supporting of the hyaline cartilage formation	95
Fbg-alginate	Chondrocytes	Cartilage	Human articular chondrocytes mixed with Fbn-alginate composite promoted sufficient chondrocyte proliferation and differentiation in the formation of specific cartilage matrix	92
Fbn-collagen-Fbn	Fibroblast, keratinocyte and epidermal	Skin	The constructed composite scaffolds containing fibroblasts, keratinocytes, and epidermal cells promoted rapid skin regeneration	86

Abbreviations: bFGF, basic fibroblast growth factor; Fbg, fibrinogen; Fbn, fibrin; MSCs, mesenchymal stem cells; PEtU-PDMS, poly(ether)urethane-polydimethylsiloxane; rhBMP, recombinant human bone morphogenetic protein; TGF, transforming growth factor; VEGF, vascular endothelial growth factor.

properties with good degradation kinetics.⁸¹ In this section, we discuss Fbg/Fbn–natural polymer composites and their application in tissue regeneration therapies. In one study, Fbn-gelatin composite film was shown to exhibit a high equilibrium content of water, which could effectively improve the efficacy and properties of the matrix when applied as a wound dressing.⁸² In other studies, a physiologically coated Fbn-chitosan gelatin composite film was shown to provide a fibrous and porous structure with a high water absorption capacity, which could improve the encapsulation efficacy of hydrophilic protein and peptides.⁸³ Hepatic cell-seeded Fbg-gelatin matrix stabilized by thrombin exhibited a very high compressive strength. The study also suggested that Fbn could be a favorable biological component for cell assembly/delivery compared with other natural protein-based scaffolds.⁸⁴ Fbg- and thrombin-coated collagen patches were used as a topical hemostatic agent and were applied for rapid hemostasis and wound closure after procedures such as liver, thoracic, neuro-, kidney, spleen, plastic, and minimally invasive surgery, *in vivo*⁸⁵. By combining collagen, chitosan, and Fbn, Han et al prepared novel asymmetric porous scaffolds.⁸⁶ In other studies, Lorusso et al reported that Fbg/thrombin-coated collagen patches were effectively used for the control of severe intraoperative pulmonary hemorrhage and air leakage after correction of a ruptured thoracic aortic aneurysm.⁸⁷ Recently, a Fbn-chitosan composite loaded with human fibroblasts showed good cells spreading effect and ECM interaction.⁸⁸ Both Fbn- and collagen-based vascular grafts could be used as substitutes for bypass grafts or as models for vascular studies.⁸⁹ Very recently, Zhou et al successfully demonstrated the controlled delivery of recombinant human epidermal growth factor (rhEGF) using a Fbn-chitosan composite – the composite achieved a high protein loading efficacy due to the presence of chitosan and a lower initial burst release due to the Fbn.⁹⁰ Fbg and Fbn have also been blended or coated with different natural polymers, such as poly(L-lactide),⁶¹ silk fibroin,⁹¹ alginate,⁹² etc. Other such composites are listed in Table 2.

Cross-linking of Fbg and Fbn micro/nanoscaffolds

In tissue engineering and regenerative medicine, shape stability is a significant factor for specific medical applications, such as those relating to bone, blood vessel, and nerve regeneration.^{11,96} Fbg micro/nanoscaffolds can lose their structure within a few days through degradation by various protease enzymes, such as plasmin and trypsin, *in vitro* and *in vivo*.^{97,98} For complete regeneration of various body tissues, including cartilage and bone, scaffolds should maintain their size and

shape in the long term in order to deliver comprehensive mechanical strength. In order to achieve such strength, Fbg and Fbn scaffolds have been cross-linked with different chemical and biological agents.

In 1963, Mihalyi was the first to describe a cross-linking procedure for bovine Fbg, using formaldehyde.⁹⁹ A few years later, in 1975, human Fbg cross-linking was introduced by Furlan and Beck, using glutaraldehyde and tetranitromethane. They further confirmed the participation of different chains (α , β , and γ) in intermolecular cross-linking, by performing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), the results of which evidenced that enhanced intermolecular cross-linking occurred when high concentrations of glutaraldehyde were used.¹⁰⁰ Fbn clots are naturally cross-linked through the inclusion of activated transglutaminase (factor XIII), which forms covalent cross-linking between glutamyl and lysyl residues of adjacent Fbn molecules,⁴⁵ but factor XIII is comparatively expensive. On the other hand, glutaraldehyde is commonly used as a cross-linking agent for natural proteins and is currently used to hasten tissue regeneration by fixing collagenous tissue.^{101,102} Glutaraldehyde is also reasonably inexpensive and has the ability to be cross-linked rapidly over large distances, with a broad range of amino groups.¹⁰³ However, aldehydes are non-zero-length cross-linkers that can induce poly- or bifunctional cross-links into the protein structure during cross-linking. Moreover, aldehydes could leach toxic substance after carrier degradation, thereby presenting an increased risk of cytotoxicity and material calcification.^{101,102} To compare cellular toxicity and other physical properties, Sell et al cross-linked bovine Fbg scaffolds using two different cross-linkers, namely glutaraldehyde vapor and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC).⁴⁵ The EDC cross-linked scaffolds showed considerably increased peak stress modulus values and less toxicity with significant sustained degradation, when compared with glutaraldehyde cross-linked scaffolds, *in vitro*. Dare et al cross-linked Fbn hydrogels incorporated with human articular chondrocytes using naturally-occurring genipin, and the result demonstrated a superior mechanical strength, with excellent gene expression, ECM production, and a slower rate of degradation. Moreover, genipin cross-linked-Fbn hydrogel did not noticeably affect cell viability and importantly, increased the shear moduli and dynamic compression of the hydrogel.¹⁰⁴ Recently, Fbg nanoparticles were cross-linked with calcium chloride and were shown to have potential as a promising therapeutic carrier for sustained anticancer drug release *in vitro*.¹²

EDC is a water soluble and zero-length cross-linker. Most of all, EDC cannot be incorporated into protein molecules, as it can be simply washed away as a water soluble urea derivative, thereby decreasing the potential for cytotoxic effects.^{101,102} Fbg microspheres that were cross-linked with various concentrations of EDC (1, 3, 5 and 10 mM) revealed a slower rate of degradation with increasing EDC concentrations.⁹ Compared with this, nanostructure aligned and highly porous Fbg microfibers that were cross-linked with EDC showed slower fibrinolytic degradation. Thus, increased Fbg cross-linking is not only based on the concentration of cross-linkers, but also depends on scaffold structural characteristics. For example, Fbg scaffolds made with nanostructures attained higher cross-linking compared with microstructures because of their higher exposure of Arginine and Lysine residues, which are abundantly present on the Fbg surface. To minimize or reduce the proteolytic degradation of Fbn, researchers have commonly used Fbn degradation inhibitors and their stabilizers, such as factor XIII, aprotinin, and ϵ -amino-n-caproic acids,^{59,65,93,105} along with sufficient amounts of thrombin and calcium, which all prevent excessive degradation of Fbn.¹⁰⁶

Tissue plasminogen activator can activate plasminogen to active plasmin (serine protease), which in turn, degrades Fbg and Fbn by cleaving the carboxyl terminal peptide bond of the Arginine or Lysine residues; therefore, for long-term degradation there needs to be an increase in the number of peptide bonds with suitable cross-linking agents.^{99,101,102} Various studies have disclosed the potential of Fbg and Fbn scaffolds for cell viability, gene expression, and ECM production. However, to obtain better tissue engineering results, scaffolds should maintain their size and shape for prolonged periods, and to achieve that, it is necessary that they be stabilized with an appropriate cross-linking agent. Furthermore, the rate of degradation can be controlled by either increasing the concentration of cross-linking agents or by producing a nanostructured scaffold.

Cancer therapy

Cancer remains an incurable disease, and there are more than 10 million new patients identified every year. On the other hand, due to the development of new technology and surgical intervention (radiation and chemotherapy), the death rate related to cancer has been declining for the past few years. However, chemotherapeutics kill normal cells and cause a variety of toxicities to patients. To minimize this, many research groups have focused their research towards biodegradable polymer carrier (micro/nano) drug delivery systems,

and many have attained satisfactory results.¹⁰⁷ Among the biodegradable polymer carriers, Fbg-based micro/nano carriers are widely used in cancer treatment. Fbg and Fbn can inhibit the progression of cancer cell growth by countering excessive coagulation in and around tumor tissues.¹⁰⁸ Because of this, Fbg-based micro- or nanocarriers are widely used in cancer treatment.

The anticancer drug-loaded Fbg micro/nano carriers have shown promising effects on various cancer cells. In one study, water soluble, 5-fluorouracil-loaded Fbg microspheres were effectively used as a novel injectable drug delivery system for anticancer treatment and expressed sustained release for more than 5 days, as demonstrated by in vitro experiment.³⁹ The same research group demonstrated doxorubicin release in mice, and positive results were observed on the basis of animal survival data. The doxorubicin solution-injected mice showed decreased lifespan, with 50% dead at 10 days and no survivors after 28 days, whereas doxorubicin-loaded-Fbg-microsphere-injected mice showed significantly increased lifespan, and 50% mortality occurred after 60 days.³⁶ Similarly, micronized droplets of olive oil containing docetaxel coated with Fbg markedly improved the therapeutic efficacy of docetaxel for the treatment of mammary tumor growth.¹⁰⁹ Recently, Fbg nanoparticles were effectively used as a carrier for 5-fluorouracil⁴² and in curcumin delivery.¹¹⁰

Fbg and Fbn micro/nano scaffolds integrated with biomolecules and cells for tissue engineering applications

An engineered scaffold should be biocompatible, provide sufficient mechanical strength for the specific application, and give the necessary functional support for cell growth and survival. In 3-D matrices, cell growth would be different from that on a 2-D/flat scaffold surface. When cells are growing in a 3-D scaffold, the third dimension provides another direction for cell-cell interactions, cell migration, and cell morphogenesis, all of which are essential in regulating cell cycles and tissue functions. For enhanced tissue regeneration the scaffold also needs to be grafted/immobilized with growth factors, other biomolecules, or different cells that can accelerate tissue regeneration and improve the function of the regenerated tissue through either direct release of biomolecules or via the secretion of proteins from immobilized cells. Growth factors are tissue-promoting agents that show therapeutic potential for various types of tissues regeneration (skin, nerve, vascular, dental, cartilage, bone, oral, and craniofacial) as well as for

“therapeutic regeneration” processes.^{111–113} For example, in the treatment of chronic wounds, FGF and VEGF were successfully used in a bioartificial dermal substitute to improve angiogenesis through soft-tissue formation and collagen synthesis, in preclinical trials.^{114,115} Similarly, VEGF has been used in the treatment of new blood vessel formation and cardiac ischemia, in different clinical trials and animal models.^{112,116,117} Furthermore, local administration of FGF-basic can stimulate wound healing, as has been detailed by clinical trials.^{118,119} However, the direct administration of growth factors have drawbacks, such as rapid clearance from the injected or implanted area (due to the administration of a more soluble form), faster rates of degradation, and short half-lives (usually less than 1 hour).¹²⁰ To overcome this problem, the natural polymer-based delivery system has emerged, as biopolymers can stabilize growth factors, allowing for their release in a controlled manner.¹²¹ The following section describes growth factor- and cell-loaded Fbg and Fbn scaffolds which have been used in a diverse range of tissue regeneration applications. The schematic diagram in Figure 8 illustrates the general representation of growth factors and cell delivery from designed scaffolds, for a number of tissue engineering applications.

Nerve tissue regeneration

Peripheral nerve damage/injury often requires neural reconstruction, and the ability of the spinal cord to regenerate after injury is limited. From a clinical viewpoint, a number of scientific articles suggested that tissue engineering is a favorable option to facilitate the process of nerve repair/regeneration.^{7,24,121,122} The neurotrophins are a family of growth factors that maintain neuron survival and axonal elongation,¹²³ and include NGF, neurotrophin-4/5, neurotrophin-3 (NT-3), and brain-derived neurotrophic factor (BDNF).¹²⁴ NGF can be used to treat various neurodegenerative disorders, such as Alzheimer’s disease, Huntington’s disease, and peripheral nerve injuries; NGF also plays an essential role in sensory and sympathetic neuron endurance and preservation.¹²⁵ NGF has been explored as a significant drug for the treatment of spinal cord injury, to stimulate neural sensory fiber growth around chronic wounds.¹²⁶ NT-3 fulfills a central role in neurogenesis (by stimulating the differentiation of new neurons) and promotes corticospinal axonal growth.¹²⁷ NT-3 has been used as a potential therapeutic agent for spinal cord injury because it encourages neural cell growth and survival in the development of the corticospinal tract.¹²³

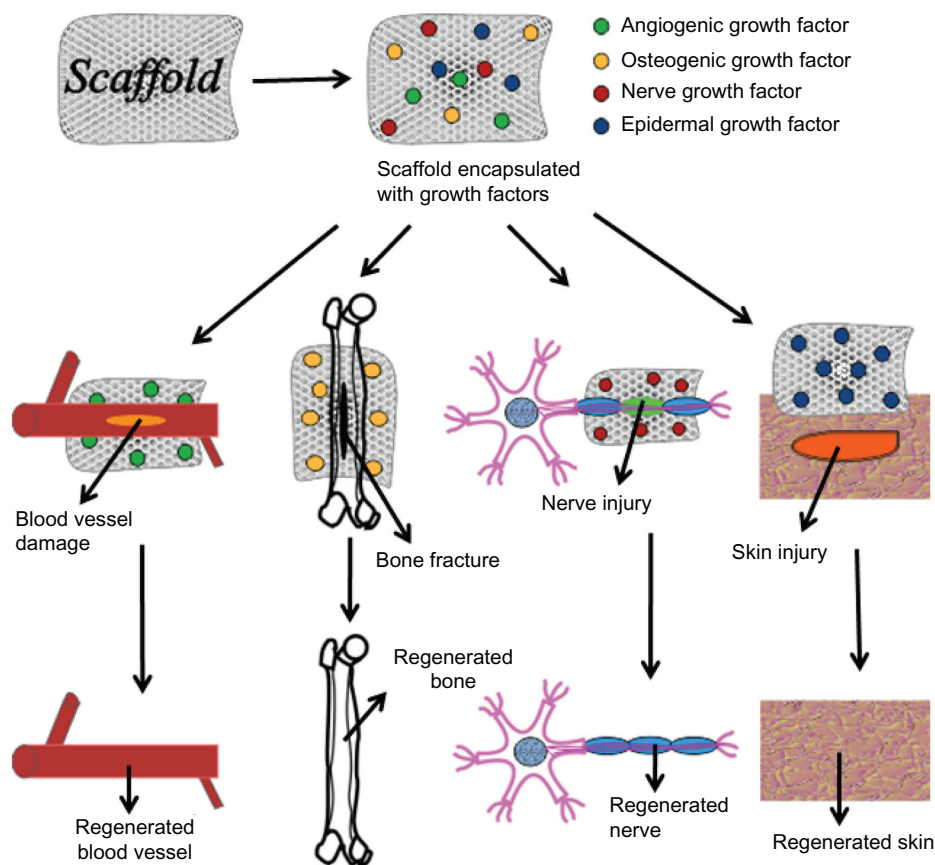


Figure 8 Specific growth factor-loaded scaffolds used for regeneration of various tissues.

The direct injection of neurotrophins at the site of spinal cord injury has been shown to promote neuronal growth; however, the regenerative efficacy was reduced by various factors, such as loss of neurotrophins into the cerebrospinal fluid, aggregation, and proteolysis, and so this may not be applicable for continuous functional restoration and neuron regeneration.^{122,128} To achieve long-term protein release as well as neuronal fiber sprouting, a suitable polymer scaffold will release NGF at the specific site and at a specific rate for a specific duration.^{121,129,130} The specific cell- and protein-binding characteristics of Fbg and Fbn scaffolds could be suitable for application to nerve conduit, providing an optimal microenvironment as well as molecular and electrical cues for efficient neural cell regeneration.^{7,131}

NT-3-loaded Fbn scaffolds initiate regenerative responses and promote axonal growth by increasing neuronal fiber sprouting and cell migration into the injury site; moreover, the scaffold can dramatically reduce glial scar formation at the white matter border of the lesion, as demonstrated in one *in vivo* animal experiment.¹²² In another study, a heparin-functionalized Fbn scaffold immobilized with NT-3, via noncovalent interactions, delivered protein in a controlled fashion to an injured spinal cord, as studied in an adult rat model. Furthermore, the heparin-Fbn-affinity-based system released the drug through cell-mediated processes and restricted drug loss by diffusion. Therefore, the controlled delivery of NT-3 from Fbn scaffolds indicated improved neuronal fiber development/growth.¹³² The β -NGF-incorporated Fbn matrix provided enhanced nerve regeneration by effectively releasing NGF via the proteolytic activity of plasmin, and this kind of delivery system represents an improvement in nerve regeneration.¹³³ Further, it was shown that Fbn glue soaked with NT-3 expressed better dorsal root regeneration in the spinal cord and offered a strategy for injured spinal reflex arc stimulation. This study also suggested that Fbn glue is an effective exogenous vehicle for the intraspinal delivery of neurotrophic growth factors.¹³⁴ Fbn implantation with NT-3 induced neuronal fiber formation in rat spinal cord lesions.¹²⁴

In artificial nerve regeneration, other than nerve stimulating factors, stem cells, such as Schwann cells (SCs) and MSCs, also could enhance nerve growth by, themselves, secreting nerve stimulating factors.¹³⁵ MSCs can be induced to differentiate into SCs and to promote the regeneration of injured sciatic nerves.¹³⁶ SCs have a prominent specific role in nerve tissue regeneration because they can secrete NGF and other nerve regenerating proteins, which can enhance the survival of cholinergic neurons.¹³⁷ Because of their protein secretion advantage, these cells have a

potential use in the treatment of various neurodegenerative disorders, such as Alzheimer's and Huntington's disease.¹²⁶ Both MSC- and SC-seeded polyhydroxybutyrate-fibrin (PBH-Fbn) nerve conduits have demonstrated enhanced continuous proliferation and differentiation throughout the conduit over conduits fabricated using only PBH, *in vivo*.⁷ Fbn scaffolds cultured with embryonic stem cells stimulate neurons and oligodendrocytes and have been used as a platform for neural tissue engineering.¹²⁵ It has been suggested that both Fbg and Fbn can be an excellent scaffold for nerve regeneration, and in particular, Fbn gel has been demonstrated to encourage axonal regeneration and cell migration in short-gap nerve injury.¹³⁸

Bone and cartilage tissue regeneration

The poor healing potential of cartilage and bone has been a well-known concern in orthopedic surgery.¹³⁹ On the other hand, studies of bone tissue engineering have looked at regeneration of osteoblasts to accelerate fracture healing, correction of major defects in bones that cannot usually be rebuilt, spinal cord strengthening, and bone mineral density loss by aging.^{6,25,79,140} The treatment of bone loss has been conventionally managed using direct bolus injection of cells, open implantation of bone-forming cells, and scaffold-based drug delivery systems.¹⁴¹ The skeletal system holds numerous growth factors, including TGF- β , FGF, VEGF, insulin-like growth factors (IGF-I and IGF-II), platelet-derived growth factor (PDGF) and BMPs, that contribute to cartilage and bone repairs.¹⁴¹ Among these peptide growth factors, BMPs play a significant role in mediating the growth, remodeling, and regeneration of the skeleton system, due to their better osteoinductive properties,¹⁴² and also in stimulating osteoblast proliferation and skeletal muscle component synthesis (collagen).¹⁴³ bFGF has mitogenic and angiogenic properties for cartilage regeneration that have been consistently confirmed.¹⁴⁴ TGF can increase bone thickness,¹⁴⁵ and the anti-inflammatory properties of TGF- β 1 have been shown to stimulate cartilage regeneration via chondrocyte differentiation.¹⁴⁶ The IGFs are also viewed as one of the most significant groups of skeletal growth factors, due to their important actions on bone and cartilage cell function, repair, and regeneration.¹⁴⁷

Since 1980, Fbn has been extensively used for bone repair/regeneration; its various characteristics, including slow drug release and enhanced cellular growth, have made it a suitable carrier for different orthopedic surgery applications.¹⁴⁸ Fbn provides a natural environment for bone-forming cells,¹⁴⁹ and Fbn glue was effectively used because

of its chemotactic, hemostatic, and mitogenic properties.¹⁴⁸ Recently, in bone tissue engineering, Fbn scaffolds loaded with different osteogenic growth factors and bone forming cells have been found to promote cartilage structure and viability.⁶⁸ In one study, BMP-2-immobilized Fbn gel, via cell-activated plasmin, resulted in a significant improvement in bone-defect healing.¹⁵⁰ Elsewhere, a heparin-incorporated Fbn-FN matrix model was reported to provide an optimal delivery system for BMPs, where the Fbn matrix acted as the platform to accommodate the infiltrating tissue, while FN established a connection between Fbn and heparin and also provided an adhesion site for tissue-repair cells.¹⁵¹ Kang et al reported that Fbn scaffold itself has good bone cell inductive properties, and when it's loaded with peptide growth factors, such as with BMP-2 shows enhanced bone regeneration.¹⁵²

Recently, bone marrow cells and MSCs were incorporated into Fbn scaffolds to encourage meniscal healing in an animal model.^{153–156} MSCs are multipotent and are capable of producing mesenchymal tissues like bone, cartilage, and tendon.⁵⁰ The MSC-loaded Fbn matrix was used as an autogenous bone graft because MSCs can easily undergo many cycles of cell division without losing their osteogenic capacity/properties, a feature that holds strong implications for orthopedic surgery and oral and maxillofacial reconstructions.¹⁵⁷ MSCs-Fbn microbeads secrete mineralized ECM around the implantation area, and bone formation is promoted by secretion of osteogenesis (ascorbic acid, β -glycerophosphate, and dexamethasone) and osteoblast growth factors.¹⁵⁸ Isogai et al reported that periosteal cell-cultured Fbn glue stimulated new bone formation at heterotopic sites, in an in vivo animal study.¹⁵⁹ In another study, Fbn hydrogel mixed with freshly isolated bovine chondrocytes showed coherent cartilaginous ECM production and distribution.¹⁰⁶ Currently, studies concerned with the fabrication of Fbn-based scaffolds for bone and cartilage regeneration have observed the advantages of this material when used to deliver multiple osteogenic growth factors and cells.^{6,92,104} These advantages include the stimulation of osteoblasts, osteocytes, and osteoid cell proliferation, extending the application of such procedures beyond the treatment of isolated defects and fractures. However, effective bone and cartilage regeneration depend on the associated drug/cell delivery systems, the duration of biological cues release, and biochemical interactions between cells and the polymer carrier.

In bone tissue engineering and orthopedic surgery, Fbn composites have been widely used because of their good adhesive properties and their reduction of blood loss intra- and

postoperatively as well as for their ease of manipulation.^{160,161} Fbn-coated calcium phosphate granules implanted in male Swiss albino mice depicted new bone formation and good healing responses at the extraskelatal site. Furthermore, bone formation was found to be conspicuous on Fbn-coated granules, whereas the uncoated granules did not show any de nova bone formation.¹⁶² The Fbn-based biocomposite scaffolds seemed to stimulate mineralization on the Fbn network that was present within the ceramic grains; moreover, it was suggested Fbn glue could stabilize the granules at the implantation site, to mold the bone defects without an empty space and also provide favorable microenvironmental conditions for the osteogenic cells, leading to rapid bone healing effects.⁷³ Fbn, calcium phosphate, and hydroxyapatite-based composites could effectively induce bone repair. However, for better bone tissue regeneration, scaffolds should have well-controlled micro/nanopore interconnected networks, so that they can provide ease of cell attachment and proliferation.⁷³ In one study, the porous scaffold developed by a Fbn-calcium phosphate composite showed tightly interconnected networks and porous structure. The biodegradable composite supported primary osteoblast-like cell adhesion, proliferation, and differentiation in bone tissue engineering, in vitro and in vivo experiments.⁷⁹ In another study, the combination of Fbn sealant and ceramic depicted a synergic effect in bone and cartilage tissue engineering; also the bioceramics showed enhanced physical properties compared with ceramic alone.¹⁶³

Vascular tissue regeneration

The selection of suitable biomaterials that are capable of inducing endothelialization and blood vessel formation is significant in therapeutic regeneration, especially for vascular tissue engineering. Angiogenesis is another eminent concern for vascular tissue engineering; angiogenesis is the process by which the preexisting vasculature gives form to new blood vessels. The stimulation of neovascularization is based on the various angiogenic factors, and each angiogenic factor would function respectively, to make perfect functional blood vessels. Several growth factors have been applied to therapeutic angiogenesis, including the angiopoietins, hepatocyte growth factor (HGF), FGF (acidic and basic), and VEGF. Among these stimulators, VEGF is one of the major groups of proteins involved in blood vessel formation, by directly stimulating endothelial cells migration and proliferation; the VEGFs include VEGF-A, -B, -C, -D, -E and -F.¹⁶⁴ After VEGF, the FGFs are the most widely applied proteins for restoration of the blood supply in ischemic tissues, and these stimulate reendothelialization of wounded arteries;^{165–167} in particular,

bFGF binds to endothelial cells and enhances their migration and proliferation for new blood vessel formation.^{168,169} HGF, neurotrophin, erythropoietin, and IGF are all also used for targeted vascular repair.⁶⁸ In addition to other growth factors, such as PDGF, ephrin B2, and angiopoietin 1, these help to stabilize the newly shaped blood vessels, by recruiting smooth muscle cells to the nascent endothelial capillary.¹⁷⁰ Angiopoietins 1 and 2 function through the Tie2 receptor, which controls signal survival, migration of endothelial cells, regulation of blood vessel remodeling, and maintenance of vascular integrity.¹⁷¹ Generally, for new blood vessel formation around ischemic tissues or associated with implants, it is presumed that efficient neovascularization requires the sustained delivery of angiogenic proteins to settled tissue over a period of weeks.^{172,173}

Fbg/Fbn is a blood protein which has the ability to augment the biochemical and mechanical integrity of the scaffold for vascular regeneration.¹¹ The enzymatic fragmentation of fibrin yields a variety of degradation products that are capable of inducing angiogenesis; in particular, Fbn fragment E is a potent angiogenic factor, which has been studied alongside chicken chorioallantoic membrane (CAM).^{70,164} In one study, the heparin-functionalized Fbn matrix immobilized with VEGF was shown to be highly effective in delivering the growth factor over a period of months. Moreover, the matrix maintained VEGF bioefficacy for long term better than the other polymeric systems.³⁵ VEGF-grafted Fbn gel also showed enhanced therapeutic angiogenic outcomes, in a rabbit ischemic hind limb model.³⁵ Elsewhere, covalently immobilized VEGF in a Fbn hydrogel revealed dose-dependent protein release with excellent growth of endothelial cells, which further stimulated an angiogenic response in an ischemic region.¹⁷⁴ The VEGF-modified Fbn gel was shown to induce new blood vessel formation in CAM.¹⁷⁴ Similarly, bFGF-incorporated Fbn scaffolds have shown a comparatively slow release, for continuous angiogenesis.⁵ Fbn scaffolds derived from blood clots were used as a local injectable carrier for VEGF, as this approach prevents the initial burst-release issue and maintains a controlled release.¹⁷⁵ Both VEGF- and bFGF-immobilized Fbn-based scaffold systems have shown increased neovascularization, with the establishment of more mature blood vessels compared with the delivery of single growth factor.¹⁷⁶ Arkudas et al investigated whether VEGF- and bFGF-immobilized Fbn gels significantly induce higher absolute blood vessels formation in an arteriovenous loop mode.¹⁷⁷ Composite scaffolds made with poly(ether)urethane-polydimethylsiloxane/Fbn can also be effectively used as a sustained delivery system for angiogenetic growth factors.⁹⁴

Both Fbg and Fbn contain binding sites for various angiogenic factors, including adipose-derived stem cells and bone marrow stem cells.^{178,179} Both Fbg and Fbn provide a high seeding efficacy with uniform cell distribution for certain stem cells, such as hematopoietic stem cells, endothelial progenitor cells, smooth muscle cells, and MSCs, which are potentially differentiated into vascular-promoting cells in vitro.¹⁸⁰ Endothelial cell-seeded-Fbn-hydrogel-induced blood vessels were shown to achieve good mechanical strength, with significant production of collagen and elastin, and showed considerable vasoreactivity within 2 weeks when implanted into 12-week-old lambs.¹¹ Similarly, a 3-D Fbn gel containing human aortic myofibroblast cells also resulted in good vascular development, with controlled degradation in vitro.⁶⁵ Smooth muscle cells can easily bind with Fbn via integrin $\alpha_v\beta_3$, evoking a number of biochemical responses, such as secretion of proteolytic enzyme and production of the extracellular matrices collagen and elastin, which lead to Fbn degradation and increased mechanical strength, respectively, aiding in the formation of blood vessels.¹⁸¹ Moreover, Fbn-based carriers immobilized with angiogenic cells and vascular-stimulating proteins can be effectively used in vascular repair and in the induction of angiogenesis.¹⁸²

Skin tissue regeneration

Skin injury requires a coordinated communication of hemostatic and inflammatory systems, influenced by cytokines and other growth factors, which locally regulate cellular proliferation and skin restoration.¹⁸³ A number of growth factors are involved in skin regeneration, such as EGF, FGF, keratinocyte growth factor (KGF), PDGF, and TGF- β , each of which has been studied for its promotion of wound healing. EGF is a powerful growth factor for epidermal cell growth and is widely used as a therapeutic agent for epidermal cell migration, proliferation, and for stimulation of skin reepithelialization.¹⁸⁴ EGF can also promote the synthesis of collagen and other cell-adhesive materials through epidermal cell proliferation, leading to increased rates of wound healing.^{184,68} FGF has therapeutic potential for skin tissue regeneration; specifically, bFGF stimulates collagen synthesis, soft-tissue formation, and reepithelialization, as shown by in vivo study of animal models.^{118,185} FN is another potent mitogen that plays a crucial role in wound healing; upon injury it is bound to Fbn and is deposited at the site of injury, coagulating blood and providing a provisional matrix for epidermal cell migration and differentiation.²⁸ KGF is also a member of the FGF family (FGF-7) and plays a significant role in epithelial

development and the promotion of wound healing.^{186,187} The TGF- β members includes three isoforms namely, TGF- β 1, TGF- β 2, and TGF- β 3, which are involved in either scar-free healing or scarring repair.

Fbg/Fbn is a natural wound healing matrix that provides 3-D cell-infiltration scaffolds and facilitates cell adhesion and migration within wound areas.¹⁸ During wound healing, Fbg could improve the verity of ECM proteins (hyaluronan and laminin) secretion which may additionally encourage the properties and function of engineered skin tissues.²⁰ bFGF can be easily bound to Fbg and Fbn due to the specific interactions; the bFGF-grafted Fbg and Fbn play a role in fibroblast adhesion and spreading, as well as in proliferation, ECM synthesis, and reepithelialization.¹⁹ Fbn scaffold supplemented with TGF- β 2 was shown to improve the wound healing of cutaneous wounds compared with Fbn alone in an *in vivo* animal study.²⁶ In another, recent study it was shown that PDGF-embedded Fbn gel enhanced wound healing in excisional wounds.¹⁸⁸ Elsewhere, the application of an Fbn hydrogel cross-linked with KGF showed long-term, controlled release and accelerated healing, which are difficult to achieve through the normal healing process, *in vitro* and *in vivo*.¹⁸⁹ Gwak et al reported that the combined delivery of EGF and KGF accelerated rapid epidermal regeneration compared with therapy using either EGF or KGF alone.¹⁹⁰ The combined therapy also resulted in significantly improved epidermal thickness compared with each separate therapy alone. EGF-immobilized Fbn scaffolds resulted in effective regeneration of keratinocytes.¹⁹¹ Zhou et al developed a novel Fbn-based delivery system for rhEGF delivery, which may have great potential in topical wound healing applications, such as burns, surface destructions, ulcer fissures, and blisters.⁹⁰ Finally, Fbg matrix covalently cross-linked to FN with factor XIII was shown to stimulate macrophage, fibroblast, and endothelial cell migration at the site of tissue injury and is involved in full expression of macrophage immobilization.^{192–195}

Fibroblast cells enrolled to the site of tissue injury are considered to be essential for successful skin regeneration.¹⁹⁶ Human keratinocyte cells and melanocytes have also been evaluated for potential application in wound healing.¹⁹⁷ Fibrocytes are believed to have diverse biochemical functions in wound healing processes and also are believed to induce angiogenesis *in vitro* and *in vivo*.¹⁹⁸ Fibroblast-coated Fbn microbeads have been found to stimulate granulation in tissue formation,⁸ and keratinocytes suspended in Fbn matrix were successful in reconstituting full-thickness wounds, in both mouse and human.¹⁹⁹ Keratinocyte cell-seeded Fbn gel secretes a major cytokine (TGF- α) that promotes

keratinocyte proliferation and initiates reepithelialization via the EGF-receptor/TGF- α pathway.²⁰⁰ A recent study revealed that transplantation of an autologous keratinocyte-suspended Fbn scaffold could be efficiently used in the treatment of chronic venous leg ulcers.²⁹ Similarly, Fbn scaffolds incorporated with adenovirus encoding endothelial nitric oxide synthase (eNOS) were shown to enhance NO production at the site of diabetic wounds.²⁰¹ The enhanced NO production stimulated angiogenesis, endothelial and epithelial cell proliferation, and cell migration.²⁰²

Conclusion and future perspectives

In the last three or four decades, a number of outstanding reviews and research articles have been printed on natural and synthetic polymers and their combination, which have significant relevance toward the development of drug delivery systems, cancer treatment, and tissue engineering applications. Specifically, protein-based polymer scaffolds containing micro/nanoporous structures have been the focus of this review, where Fbg- and Fbn-based micro/nanoscaffold systems with micro/nanoporous structures, exclusively used for tissue engineering applications, have been discussed. The advantages of these materials include their biocompatibility, biodegradability, and nonimmunogenicity, and together with their various cell-adhesive/binding properties have resulted in their widespread and successful application as a biomaterial. Fbg/Fbn-immobilized growth factors have been shown to be continuously released for several days in a controlled manner, resulting in a significant improvement in cartilage/bone, nerve, vascular, and skin regeneration/repair. The release of growth factors and other tissue-inductive proteins is only sufficient when there are enough cells at the site of injury to stimulate or respond to respective signals or regeneration. Once the severity of a wound is deep/thick, the local cell population may be inadequate to regenerate the wound, and so, extra cells must be introduced in order for complete repair to take place. This is one of the major indications for tissue engineering applications, and Fbg and Fbn scaffolds have proven to be useful candidates in vascular, cartilage, bone, skin and nerve regeneration. Various scaffold developments in our laboratory, such as Fbg microspheres, Fbg nanospheres, Fbg microfibers, highly porous Fbg sheets, and Fbg microtubes, could serve in further innovations in tissue engineering applications. The developed scaffolds showed good results in terms of biomolecule grafting, cell attachment, and cell proliferation in tissue engineering, and could play a significant role in artificial tissue constructs, especially in wound dressing preparation and in artificial vascular development. Fbg/Fbn scaffolds

have some limitations, such as weak mechanical strength and quicker degradation rate; however, their mechanical strength can be improved through either mixing with other natural/synthetic polymers or incorporation of micro/nanospheres and cells, while their quicker degradation can be controlled by various cross-linking methods. Next-generation scaffolds should be immobilized with growth factors, other biomolecules, and suitable cell lines so as to accelerate, augment, and support cell proliferation and differentiation, enhancing the regeneration of fully functional tissue. In conclusion, Fbg and Fbn as drug delivery carriers and tissue engineering scaffolds can be used to deliver the drug and growth factors in a controlled manner, and also can be used to deliver the variety of cells by local injection, which may accelerate tissue regeneration. However, the overall processes of wound healing and vascular repair are complex processes, and there are a plethora of factors that may be considered in the continuous advancement of tissue engineering.

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Disclosure

The authors report no conflicts of interest in this work.

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