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FOCUS REVIEW

Design of gene-activated matrix for the repair of skin and cartilage

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Recently, tissue engineering has advanced markedly in the development of the regeneration of injured or diseased tissues and organs. Gene-activated matrix combines gene therapy and tissue engineering to create a promising solution with great potential for the restoration of the structure and function of damaged or dysfunctional tissues. The present review provides a comprehensive overview of the developments, applications and future prospects of gene-activated matrix as a substitute for tissue repair and regeneration. Our current research on skin and cartilage regeneration with gene-activated matrix is presented, and the key issues for future studies are also proposed.
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INTRODUCTION

The loss of organs or tissues because of trauma and disease is always a serious problem in clinics. Traditional treatments use either autologous or allogeneic grafts. Over the past few decades, tissue engineering and regenerative medicine, which overcome the shortage of donated organs and the adverse reactions caused by allograft immunity, have advanced markedly in the regeneration of damaged tissues.[1–3] Normally, tissue engineering comprises three parts: cells, a scaffold and bioactive signals such as proteins and genes.[4]

Among these parts, the scaffold essentially acts as a three-dimensional template for tissue formation by supporting cell attachment, proliferation, differentiation, migration and extracellular matrix genesis. Ideally, a scaffold should meet the following criteria:[5–7] (i) biocompatible to ensure cell attachment and proliferation and to prevent inflammation and rejection response after implantation; (ii) biodegradable and bioresorbable with a controllable degradation and resorption rate to coincide with tissue regeneration; (iii) three-dimensional and highly porous with an interconnected pore network for cell infiltration and diffusion of both nutrients and metabolic waste; and (iv) mechanical properties consistent with the anatomical site into which it is to be implanted.

Although scaffolds can supply the essential mechanical support and attachment sites for cells, they often lack the biologic cues to regulate effectively cellular behaviors during tissue formation.[8] Growth factors, secreted by a wide range of cell types, are critical signaling molecules that transmit signals to modulate cellular processes in the biologic environment.[9] To regenerate functional tissue of the same quality as natural tissue, growth factors have been widely used to construct bioactive scaffolds. Although this approach could induce specific cellular responses and direct new tissue formation,[10,11] key challenges associated with growth factors such as a short half-life, denaturation during storage, immunogenicity and a high cost[12,13] limit their application in practice. With the development of gene therapy, it is recognized that cells can be transfected by genes to synthesize specific growth factors in a time-regulated and locally restricted manner. Thus, researchers have turned to replacing growth factors with DNA to allow seeded or infiltrating cells to produce the desired growth factors through transcription and translation of the delivered DNA.[14–16]

GENE-ACTIVATED MATRIX

Gene-activated matrix (GAM) combines gene therapy and tissue engineering to create a novel solution with a great potential for the restoration of structure and function of damaged or dysfunctional tissues. In general, GAM systems operate at four general levels as shown in Figure 1 and outlined as follows: DNA condensation with cationic transfection reagents such as polycations and liposome; DNA polyplex encapsulation into or coating on the scaffolds; release of DNA from the matrix after being implanted into defective tissue; and transfection, and thereby expression of the desired growth factors.[17,18] Owing to its better stability compared with growth factors, DNA can be easily incorporated into a scaffold, avoiding systemic distribution and reducing the immune response of the vector. Acting as a localized depot of genes, GAM systems can maintain an elevated concentration of DNA within the cellular microenvironment and achieve localized, sustained transgene expression, which promotes the expression of growth factors directly within the local environment and eventually tissue formation.

In GAM systems, nucleic acids, that is, DNA, RNA and small interfering RNA (siRNA), can be introduced alone or packaged using viral or non-viral vectors to increase expression of a therapeutic gene or knockdown expression of a specific gene to promote the desired

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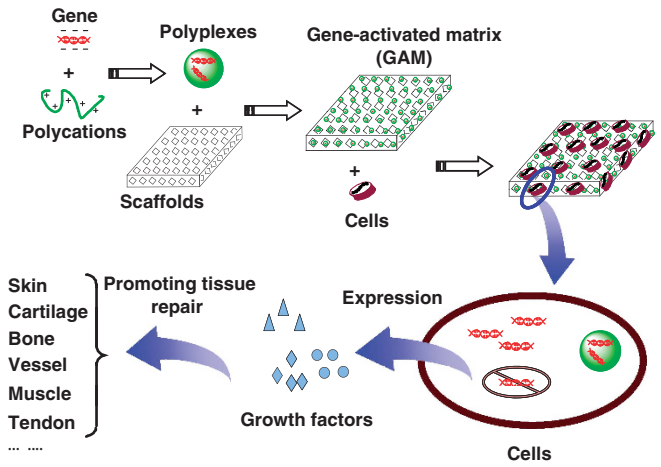

Figure 1 Schematic illustration of gene-activated matrix (GAM) construction and the mechanism by which it promotes tissue repair.

responses, leading to functional tissue formation. Polymer constructs, fabricated from naturally derived and synthetic polymers, have been formulated into hydrogels, sponges and fibers, as well as films capable of delivering DNA and supporting cell growth. In addition to the types of genes and matrices, the method of incorporating genes into the matrix is considered a key factor. Combining a broad range of different matrices with numerous non-viral and viral gene vectors, a wide range of methods have been developed to load genes into or onto the matrix. Naked DNA, as well as DNA complexes, have been encapsulated into a matrix through the formation of hydrogels,[19] sponges[20] or fibers scaffolds.[21] These GAM systems make it possible for the sustained release of DNA to transfect local cells *in vitro* and/or *in vivo*. However, the DNA may be damaged either during scaffold formation caused by interactions with organic solvents or degradation caused by the acidic environment of the degrading scaffolds.[22,23] Compared with encapsulation, the physical adsorption by dipping or spraying does less damage to DNA.[24,25] However, genes attached by such means are easily eluted from the scaffolds and spread to distal tissues, which results in low transduction efficiency and a high occurrence of side effects. When tethered to a matrix via a chemical bond,[26] the covalently coupled DNA has to be released by the degradation of either the tether linkages or the biomaterial, which may provide long-term release. However, this method faces some challenges owing to their harsh reaction conditions, which may render the DNA inactive. In addition, strong chemical bonds may limit the release of DNA from the matrix. Characterized by responsiveness, reversibility and competition, molecular recognition could lead to non-covalent linkages differing from those of covalent bonds.[27] The feasibility of incorporating plasmid DNA into scaffolds through supramolecular interactions to achieve long-lasting and site-specific DNA delivery has been the focus of many studies. In addition, specific interactions such as antigen–antibody interactions, biotin–avidin interactions, metal chelating systems and oligonucleotide interactions have also been applied to immobilize DNA onto the scaffolds.[28]

Recently, GAM has been applied to engineer various types of tissues. Shea *et al.*[29] proposed engineering tissues through the incorporation and sustained release of plasmids encoding tissue-inductive proteins from polymer matrices. It was observed that plasmid DNA (pDNA) was released over a period ranging from days to a month after being loaded into a poly (lactide-*co*-glycolide) (PLGA) matrix. *In vivo*, the quantity of platelet-derived growth factor secreted as a result of sustained gene delivery was sufficient to enhance matrix deposition and blood vessel formation.[29] The pioneering work by Klugherz *et al.*[30] reported the successful *in vivo* transfection by using the controlled release of DNA from a polymer coating on an expandable stent. Zhang *et al.*[31] designed a novel GAM with embedded chitosan/plasmid nanoparticles encoding human transforming growth factor-β1 (TGF-β1) based on a porous chitosan/collagen composite scaffold. After being implanted *in vivo*, it was found that enhanced green fluorescence protein-transfected human periodontal ligament cells can not only proliferate but can also recruit surrounding tissue to grow into the scaffold. Rujitanaroj *et al.*[32] investigated nanofiber scaffold-mediated RNA interference by controlling fibrous capsule formation *in vitro* and *in vivo*. By encapsulating complexes of siRNA with a transfection reagent within the poly (caprolactone-*co*-ethylene) nanofibers, a sustained release of siRNA was obtained for at least 28 days. A significant decrease in fibrous capsule thickness was observed after *in vivo* subcutaneous implantation of siRNA scaffolds for 2 and 4 weeks compared with plain nanofibers. Krebs *et al.*[33] reported an injectable alginate hydrogel containing preosteoblastic cells and calcium phosphate–DNA nanoparticles, and sustained release of DNA was observed when the nanoparticles were incorporated into these hydrogels. Furthermore, preosteoblast cells exhibited the capacity to form bony tissue in as little as two-and-a-half weeks when injected subcutaneously into the backs of mice.

Based on the concept of GAM, the strategy of gene activation has been successfully applied in our current studies, especially to skin and cartilage repair.

APPLICATIONS OF GAM FOR SKIN TISSUE REPAIR

Skin, the largest organ in the body, provides a protective barrier against physical, chemical and biologic pathogens, supports and maintains human health. However, owing to burn, trauma or chronic diseases, every year millions of patients suffer from skin loss, which is one of the most severe problems in clinics. Thus, skin repair has become a major focus in the field of wound healing.

Because of biocompatibility and biodegradability, in our studies, collagen porous scaffolds have been applied as a dermal equivalent to induce fibroblast infiltration and dermal regeneration. However, the rapid biodegradation rate and low mechanical strength of untreated collagen, which cannot match the demand of *in vitro* and *in vivo* applications, have limited further use of this material. Chitosan, which has a large number of amino groups in the molecular chain, was introduced into the glutaraldehyde crosslinked collagen-based scaffolds.[34,35] Under the glutaraldehyde treatment, the existence of chitosan can considerably improve the biostability of the collagen/chitosan scaffold. It was also shown that the GA-treated scaffold could retain the original good cytocompatibility of collagen to accelerate effectively infiltration and proliferation of dermal fibroblasts. More importantly, no obvious contraction was found in the glutaraldehyde crosslinked scaffolds.

The collagen-based three-dimensional scaffold could function as a 'regenerating template' to guide the differentiation and proliferation of cells into the targeted functional dermis. While the most important function of skin is to control water loss and inhibit bacterial entry, a silicone membrane was further used in our collagen/chitosan scaffolds to prepare a bilayer dermal equivalent (BDE) (Figure 2).[36] It was found that the water vapor permeability of the BDE was close to that of normal adult skin. The BDE had a tensile stress of 0.1 MPa and a strain of 30% in its wet state. It was demonstrated that the BDE had

no cytotoxicity and no remarkable sensitization and irritability. Fibroblasts could grow well and proliferate rapidly in the BDE. Animal tests showed that plenty of fibroblasts and extracellular matrix could be observed in the regenerative dermis after transplantation of the BDE for 4 weeks.[36,37]

It is essential to supply necessary nutrients and oxygen and to remove waste products during skin repair. Angiogenesis of BDE is one of the most important issues in the treatment of full-thickness skin defects. The use of angiogenetic factors such as vascular endothelial growth factor (VEGF),[38] angiogenin,[39] basic fibroblast growth factor, platelet-derived growth factor and TGF show positive effects on enhancing angiogenesis *in vivo*. To overcome the fragile disadvantage of growth factors, the DNA encoding VEGF was applied to enhance the angiogenesis of BDE.[40] The cationic gene delivery vector *N*,*N*,*N*-trimethyl chitosan chloride (TMC) was used to form complexes with the plasmid DNA encoding VEGF-165. The gene-activated BDE was developed by loading TMC/pDNA into BDE, and the pDNA released from BDE retained its intact structure and possessed good transfection efficiency. A significant improvement in the VEGF expression level from the TMC/DNA complexes containing BDE was demonstrated in a cell culture experiment. *In vivo* tests showed that the gene-activated BDE had the strongest VEGF expression in mRNA and protein levels and the highest densities of newly formed and mature vessels. The healing skin had a similar structure and ~80% tensile strength of the normal skin after the transplantation of an ultrathin graft for 112 days.

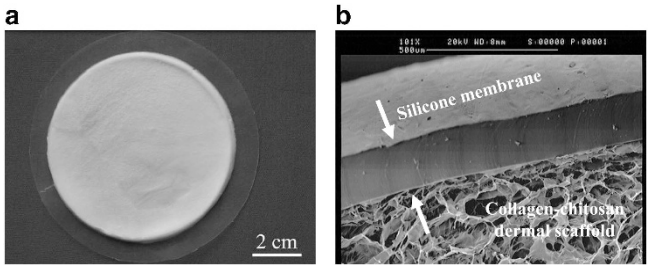

Figure 2 Macroscopic appearance (**a**) and microstructure (**b**) of the bilayer dermal equivalent (BDE).[36] A full color version of this figure is available at *Polymer Journal* online.

A burn wounds pig model, in which healing time from full-thickness burns is considerable, was used to test the gene-activated BDE treatment.[41] A significantly higher number of newly formed and mature blood vessels were found in the experimental group, which also showed a rapid regeneration rate of burned dermis. At 105 days grafting, the healing skin had ~70% tensile strength of that of normal skin (Figure 3).

For current skin grafts and bioengineered skins, scarring is a considerable problem. The scars can result in loss of dermal functions and can also cause issues such as disfiguration, itching and local ulceration. A scarless repair is not only an esthetic requirement but also a functional one. Therefore, prevention of scarring is a major challenge for the repair and regeneration of skin defects.[42] In a recent study by our group, TMC/siRNA complexes, which could induce suppression of the TGF-β1 pathway, were combined with BDE (RNAi-BDE) to inhibit scar formation.[43] A prolonged siRNA release was observed in the RNAi-BDE. Application to full-thickness skin defects of pigs confirmed that TGF-β1 expression was downregulated by the loaded siRNA in the BDE. This downregulation could inhibit the expression of other scar-related factors such as collagen type I, collagen type III and α-smooth muscle actin. Seventy-three days after transplantation, the skin regenerated by the RNAi-BDE was extremely similar in structure to that of normal skin (Figure 4).

Skin appendages such as hair follicles, sweat glands and sebaceous glands have an important role in thermoregulation, touch and excretion. Although most commercialized skin substitutes are

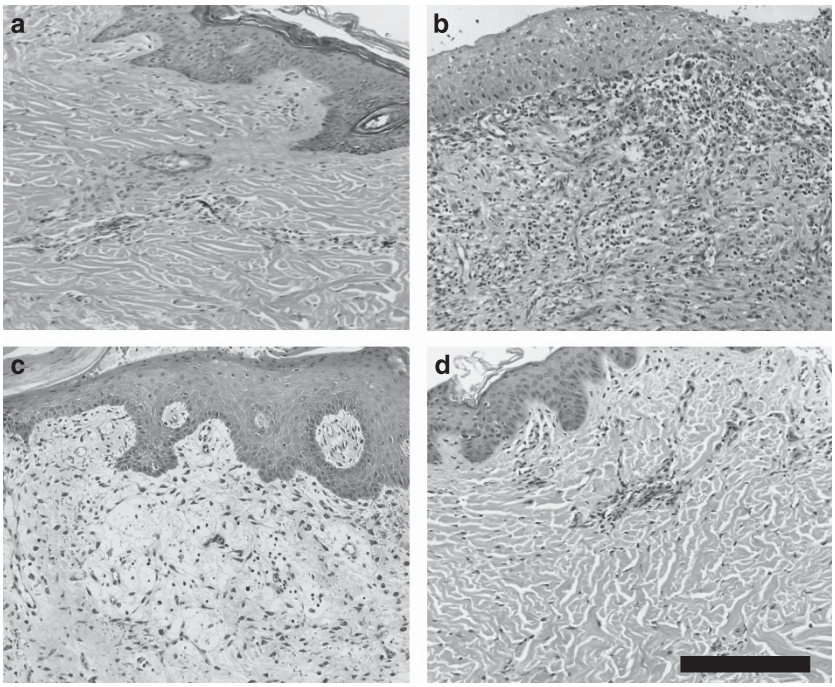

Figure 3 Hematoxylin and eosin staining of normal skin tissue (**a**) and tissue sections of burn wounds after being treated with gene-activated bilayer dermal equivalents (BDEs) for 14 days and then transplanted with ultrathin skin grafts for 28 days (**b**), 56 days (**c**) and 105 days (**d**). Bar indicates 200 mm.[41] A full color version of this figure is available at *Polymer Journal* online.

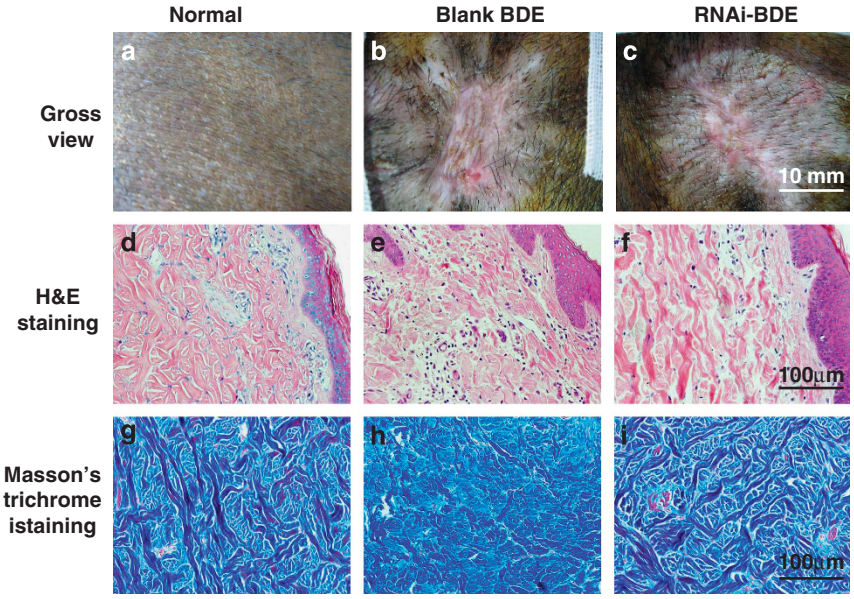

Figure 4 Gross views (**a**–**c**), hematoxylin and eosin (H&E) staining of sections (**d**–**f**) and Masson's trichrome staining (**g**–**i**) of normal skin (**a**, **d** and **g**) and wounds treated by blank bilayer dermal equivalents (BDEs) (**b**, **e** and **h**) and RNA interference (RNAi)-BDEs (**c**, **f** and **i**) for 73 days. Ultrathin autografts were transplanted at day 14.[43]

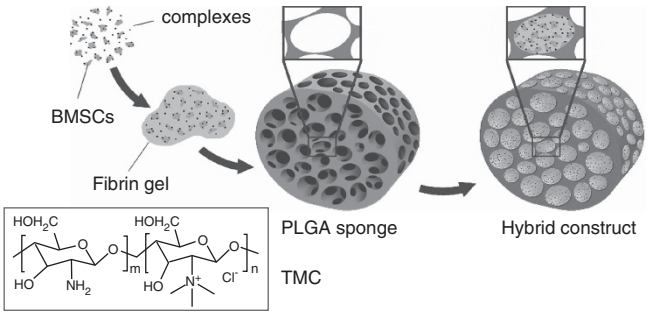

Figure 5 Schematic showing fabrication procedure of the composite construct by filling bone marrow mesenchymal stem cells (BMSCs), *N*,*N*,*N*-trimethyl chitosan chloride (TMC)/DNA complexes and fibrin gel into a poly (lactide-*co*-glycolide) (PLGA) sponge and the chemical structure of TMC.[45] A full color version of this figure is available at *Polymer Journal* online.

adequate at promoting wound healing, it remains a challenge to regenerate skin with complete appendages. Thus, regenerated skin cannot fully replace normal skin in terms of its function. By the same strategy, GAM may be a promising material to promote appendage regeneration as well as to fulfill the criteria of 'regenerated skin' with the complete structural, functional and esthetic properties of natural skin.

APPLICATIONS OF GAM FOR CARTILAGE TISSUE REPAIR

It is known that cartilage damage occurs frequently owing to sports and progressive aging. However, owing to its intrinsic biology such as limited blood supply and lack of self-repair capacity, cartilage is hard to repair once destroyed. Therefore, a substantial number of studies have been conducted on articular cartilage repair. In recent years, a hybrid of PLGA sponge filled with fibrin gel, bone marrow mesenchymal stem cells (BMSCs) and pDNA-encoding transforming growth factor-β1 was developed for cartilage repair in our group and was shown to be good at this function.[44–46]

With good degradability and biocompatibility, as well as good processibility and mechanical properties, PLGA is the most widely used synthetic biodegradable polymer for tissue engineering scaffolds, a d porous PLGA scaffolds have been extensively used in guiding the regeneration of cartilage. Various methods such as porogen leaching, phase separation, foaming, fiber processing and three-dimensional microprinting have been introduced to produce porous polymer scaffolds. Owing to its advantages of easy operation, accurately controlled pore size and porosity, the porogen leaching method was adopted in our research. Different types of particles such as paraffin spheres and gelatin particles have been used as porogens. Paraffin spheres were first used in our studies to prepare porous scaffolds.[47] With paraffin spheres as porogen, three-dimensional poly(L-lactic acid) scaffolds with high porosity and interconnected pores were conveniently prepared. However, there is a potential side effect because the paraffin spheres are rarely removed completely. To overcome this shortcoming, gelatin particles were used in the preparation of the porous scaffolds.[48,49] Containing bioactive sequences such as RGD (arginine–glycine–aspartic acid), gelatin can enhance cell–material interaction, and therefore, it is not necessary to be completely leached from the scaffolds. It was observed that the experimental scaffold had better biologic performance in comparison with the control scaffold fabricated with NaCl particles as porogen.[50,51]

However, chondrocytes tend to adhere and spread only on the surfaces of the pore walls when they are seeded directly onto a porous scaffold.[47] In normal cartilage, chondrocytes live in a gel-like matrix formed by positively charged type II collagen and negatively charged proteoglycans.[52] Therefore, a hybrid scaffold obtained by filling the fibrin gel into PLGA sponge was prepared by our group.[53,54] We found that the fibrin gel evenly distributed in the hybrid scaffold. Furthermore, we found that the chondrocytes distributed more evenly and kept a more rounded morphology than normal cartilage in the hybrid scaffold. Moreover, after *in vitro* culture of chondrocytes for 4 weeks, a greater amount of glycosaminoglycans was secreted into the hybrid scaffolds than into the PLGA sponges. Thus, the

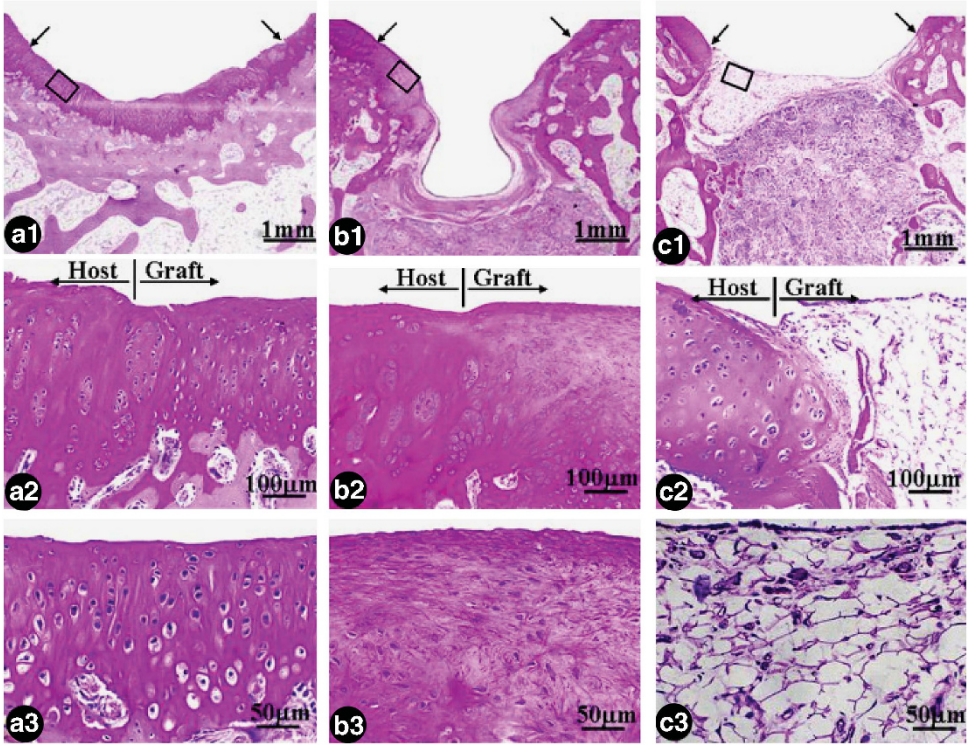

Figure 6 Glycosaminoglycan (GAG) staining by periodic acid Schiff in the neotissues. (**a**) Poly (lactide-*co*-glycolide) (PLGA)/fibrin gel/bone marrow mesenchymal stem cells (BMSCs)/*N*,*N*,*N*-trimethyl chitosan chloride (TMC)/pDNA-encoding transforming growth factor-β1 (pDNA-TGF-β1) complexes), (**b**) PLGA/fibrin gel/BMSCs and (**c**) PLGA/fibrin gel/(TMC/pDNA-TGF-β1 complexes). a3–c3 are higher magnification of the box zones in a1–c1, respectively.[45]

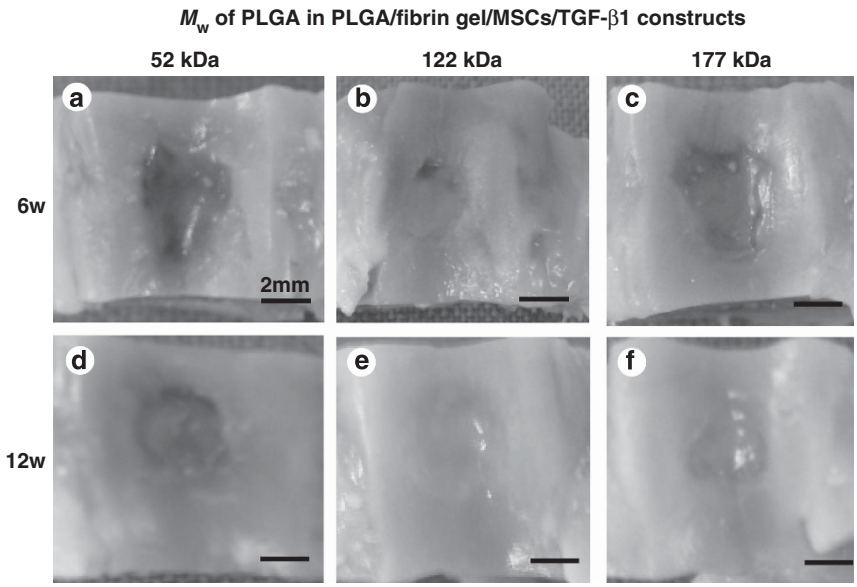

Figure 7 Photographs of full-thickness cartilage defects after being repaired by poly (lactide-*co*-glycolide) (PLGA)/fibrin gel/mesenchymal stem cells (MSCs)/transforming growth factor-β1 (TGF-β1) constructs with the PLGA molecular weight (M_w) of 52 kDa (**a** and **d**), 122 kDa (**b** and **e**) and 177 kDa (**c** and **f**) for 6 (**a**–**c**) and 12 weeks (**d**–**f**), respectively.[62] A full color version of this figure is available at *Polymer Journal* online.

fibrin/PLGA hybrid scaffolds were favorable in cartilage tissue engineering.

Presently, a cell-based therapy to repair articular cartilage defects has been suggested as a promising means to restore cartilage function.[55,56] Chondrocytes and mesenchymal stem cells (MSCs) are commonly used for cartilage regeneration. However, some disadvantages of chondrocytes such as morbidity at donor sites and loss of the chondrocyte phenotype with expansion limit its wide application.[57,58] To improve the quantity of regenerated cartilage, MSCs have increasingly become a promising alternative cell source.

When induced by the appropriate biologic cues, multipotential MSCs can differentiate into bone, cartilage, fat, muscle, marrow, stroma and other tissue types.[59,60]

To induce chondrogenic differentiation, TGF-β1, one of the most powerful growth factors, has been used in our previous research.[61] Herein, TGF-β1 was mixed with fibrinogen and MSCs to form a hydrogel, which was then filled into a PLGA porous scaffold to build the PLGA/fibrin gel/BMSCs/TGF-β1 constructs. Full restoration of osteochondral tissue was observed after implantation of PLGA/fibrin gel/BMSCs/TGF-β1 constructs into full-thickness cartilage defects for 12 weeks.

In consideration of the short half-life of TGF-β1, TMC/pDNA-encoding transforming growth factor-β1 complexes further displaced the growth factors to build the construct (Figure 5).[45] Application of the composite constructs on full-thickness cartilage defects in New Zealand rabbit joints showed that heterogeneous TGF-β1 was expressed in the experimental group, lasting for 4 weeks as observed by western blotting and qRT–PCR, and cartilage defects were successfully repaired after 12 weeks. More importantly, the neocartilage integrated well with its surrounding tissue and subchondral bone (Figure 6). According to the Wakitani's standard, an overall score of 2.83 was obtained.

Gene vectors have a vital role in gene transfection. Although TMC showed good transfection results as a carrier for pDNA encoding TGF-β1 in the above study, the potential cytotoxicity may limit its further application. Owing to its low cytotoxicity, the block copolymer poly(ethylene oxide)-*b*-poly(l-lysine) was further used as the gene carrier in our research.[46] The poly(ethylene oxide)-*b*-poly(l-lysine) complexes could transfect MSCs *in vitro* to produce TGF-β1 and upregulate the expression of chondrogenesis-related genes in the construct. After being implanted in the osteochondral defects of rabbits for 12 weeks, neocartilage and subchondral bone with abundant deposition of glycosaminoglycans and type II collagen were observed, and the regenerated cartilage had good integration with the host tissues.

Another criterion for designing scaffolds for tissue engineering is coherence between the degradation rate of the scaffold and the reconstruction rate of the extracellular matrix. On the one hand, when a scaffold degrades too slowly, it will block the infiltration of neotissue. On the other hand, if it degrades too rapidly, the scaffold will fail to support tissue formation. With limited migration ability of chondrocytes, this effect is particularly significant in cartilage regeneration. To address this, we further examined the influence of the degradation profiles of PLGA on the tissue repair outcome.[62] PLGA with different weighted-average molecular weights (52, 122 and 177 kDa) were used to fabricate the sponges (S52, S122, S177). Then, the sponges were built into the constructs of a PLGA/fibrin gel/MSCs/TGF-β1 (C52, C122 and C177). After being implanted into full-thickness defects of rabbit knees, the PLGA in C177 had the fastest degradation rate at the initial stage, whereas the PLGA 122 had a moderate degradation rate and the smallest mass loss. It was observed that full-thickness defects (both cartilage and subchondral bone were destroyed with a diameter and depth of 4 mm) repaired by the PLGA 122 group had formed a hyaline cartilage-like tissue with abundant glycosaminoglycans in the top layer and subchondral bone in the bottom layer after implantation for 12 weeks (Figure 7). The group also achieved the best macroscopic (11.3 ± 0.8) and histological scoring (Wakitani, 0.5 ± 0.6). Comparatively, the cartilage and subchondral bone defects remained unregenerated in the C52 and C177 groups. Meanwhile, the regenerated tissues by the PLGA 122 group expressed the highest SOX9 and collagen type II, but the smallest interleukin-1β and tissue necrosis factor α. Thus, the one with a moderate molecular weight (122 kDa) showed the weakest inflammatory response and the best cartilage regeneration.

CONCLUSIONS AND FUTURE PERSPECTIVES

By combining the concept of gene therapy and tissue engineering, GAM with higher bioactivities for tissue repair was developed. With the GAM system, better results were obtained for the repair of skin and cartilage. Additionally, with the GAM system, the incorporated genes activated cells to express the desired cytokines for reconstructing the niches needed for tissue regeneration. However, formation of the delicate structure and composition of the damaged tissues and organs are determined by various factors. The expression of several factors involved in the regenerating process is critical. Thus, another area of critical importance is to incorporate different types of genes into a GAM system to meet the demands of tissue repair in different phases.

In particular, simultaneous or sequential delivery of multiple genes is particularly important, as the cellular processes involved in directing tissue repair are complex. A recent study noted the feasibility of the sequential delivery of DNA from the substrate, layer by layer.[63] This study provides evidence that tissue regeneration through the combination of scaffold design and a spatially controlled, localized gene delivery system represents a promising strategy for facilitating the development of complex tissue or organ systems.

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