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bFGF-grafted electrospun fibrous scaffolds via Poly (dopamine)-assisted for skin wound healing

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Abstract:

Electrospun fibrous membranes coated with basic fibroblast growth factor (bFGF) are effective medical devices to promote wound healing. However, current strategies of adding bFGF generally cause degradation of electrospun materials or damage to the bioactivity of the biomolecules. Here, we have developed a simple strategy for surface bFGF-functionalization of electrospun fibers in an aqueous solution, which maintained original fiber properties and growth factor bioactivity. Poly-dopamine (PDA) forming the mussel foot protein, was chosen as adhesive polymeric bridge-layer between substrate Poly (lactide-co-glycolide) (PLGA) fibers and bFGF. The bFGF-grafted PDA was analyzed using scanning electron microscopy, water contact angle, and X-ray photoelectron spectroscopy. Improved hydrophilicity together with a stable fibrous structure and biodegradable fibrous matrix suggested that the PLGA/PDA-bFGF electrospun fibrous scaffolds have great potential for promoting wound healing. *In vitro* experiments showed that the bFGF-grafted PLGA electrospun fibrous scaffolds highly enhanced adhesion, viability, and proliferation of human dermal fibroblasts. *In vivo* results showed that such scaffolds shortened wound healing time, accelerated epithelialization and promoted skin remodeling. Therefore, this PDA modification method can be a useful tool to graft biomolecules onto polymeric electrospun fibrous scaffolds which are potential scaffold candidates for repairing skin tissue.

Keywords: Electrospinning; Poly (lactide-co-glycolide); poly (dopamine); wound healing; basic fibroblast growth factor.

Introduction

Skin, the largest organ of the human being, covers the surface of the human body like armor and forms the first line of defense of the body against pathogens.[1] Wounds caused by serious trauma leading to partial or complete injury to the dermis and/or the sub-dermal tissues always require prompt closure. This is especially important for burn victims and patients with diabetes.[2-4] Skin wound healing is started by a series of interactions between extracellular matrix (ECM), cells and cytokines, a process that might last for years.[5, 6] This process can be divided into four overlapping phases involving hemostasis, inflammation, proliferation in the early stage and remodeling in the late.[7, 8] Therefore, to hasten the repair of skin damage, promoting wound healing in an early stage and skin remodeling in a late stage should be carried out. The traditional ways to repair skin damage includes allografts, autografts, xenografts and so on.[9, 10] However, these options have serious disadvantages such as high cost, limited skin graft availability and rejection reactions.[11, 12]

Biomaterial scaffolds such as poly (ε-caprolactone) (PCL), poly (L-lactic acid) (PLLA) and poly (lactide-*co*-glycolide) (PLGA) can provide a substrate for cell growth allowing for repairing of the damaged tissue and promoting new tissue regeneration after functionalized.[13-16] In addition, electrospun scaffolds with a high surface area can mimic ECM and thus be the substrate for tissue repair.[17, 18] Nowadays, electrospun scaffolds are used for a very wide range of applications in tissue engineering. Nevertheless，hydrophobic electrospun polyester materials are not conducive to cells adhesion and growth compared to the hydrophilic electrospun fibrous materials, so it is difficult to use them to promote wound healing in an early stage. For instance, Wu et al. found that hybrid poly (lactide-co-glycolide)/chitosan fibers by co-electrospinning and core/shell structured PLGA/chitosan fibers by coaxial electrospinning showed better cytocompatibility of fibroblasts than the electrospun PLGA membrane in adhesion, viability assays.[13] Therefore, to solve the problem of using electrospun fibrous membranes to repair skin damage in an early stage is an urgent need.

Promoting skin repair in an early stage mainly relies on the compatibility between the fibrous membrane and host tissue as well as on the ability of the membrane to promote wound healing.[15]

Accordingly, surface modification to improve the surface hydrophilicity of electrospun fibrous could heighten the biocompatibility between the fibrous membrane and tissue.[19] For instance, Lee et al. enhanced the hydrophilicity and biocompatibility of PCL nanofibrous membranes by dissolving collagen and PCL in a co-solvent and then fabricating mixed fibers.[15] Croll et al. modified the surface of PLGA through hydrolysis and aminolysis.[20] Park et al. chemically modified PLGA using oxygen plasma treatment and in situ grafting of hydrophilic acrylic acid to improve the hydrophilicity of this material.[21] All methods mentioned above were able to improve the hydrophilicity and biocompatibility of the polyester materials to some extent. However, co-solvent electrospinning changed the properties of the fibrous scaffolds and reduced the mechanical strength of the materials. Conventional chemical surface grafting in acid or alkaline solutions could cause degradation of the fibrous material, introduce toxic chemicals and inactivate active factors. Accordingly, these complex surface-modified scaffolds were of only limited benefit for tissue repair. Therefore, a better choice would be to improve the material surface hydrophilicity and biocompatibility while maintaining the original properties of the material. The electrospun scaffolds presented here display this dual functionality in promoting wound healing in an early stage and allowing skin remolding in a late stage.

Basic fibroblast growth factor (bFGF), a single-chain polypeptide, shows the potential to accelerate acute and chronic wound healing.[22] However, since bFGF is a protein, and proteins got physical and chemical instabilities, excessive processing would accelerate bFGF inactivation and reduce its effectiveness.[23-25] Previously, various delivery systems of bFGF have been studied.[26, 27] Demirdögen et al. firstly produced heparin-entrapped hyaluronic acid-gelatin microspheres in a w/o emulsion system using the adipic dihydrazide-mediated crosslinking method, and then incorporated bFGF into the microspheres;[28] while Guan et al. fabricated poly (ester urethane) urea (PEUU) scaffolds loaded with bFGF by thermally induced phase separation.[29] However, these methods were time-consuming, multi-step procedures and had a negative impact on the biological activity of the growth factor.

Poly-dopamine (PDA), a biomolecule, is found abundantly in the mussel adhesion protein and can

adhere to various substrates including metallic, inorganic, and organic materials.[30] In an aqueous solution, PDA can spontaneously polymerize onto the surface of substrates, and thus improve the wettability of the material.[31] This simple one-step method has been applied by many researchers to modify the surface properties of biomaterials by many researchers.[32, 33] Furthermore, PDA can also function as a coupling agent allowing the conjugation of other biomolecules onto the polymer scaffolds without surface pretreatment.[34] Lee et al. successfully conjugated trypsin onto the poly-dopamine-modified cellulose paper and the immobilized trypsin remained active.[35] Therefore, biologically active factors can be grafted onto the surface of the materials in a one-step process in an aqueous solution through a reaction with the poly-dopamine surface. Moreover, the grafted factors maintain high activity, and the grafting process will not change the structure of the material. The diameters, the water contact angles, chemical compositions of the fiber surface remained the same as before grafting the biologically active factors.

Here, bFGF was grafted onto the surface of PLGA electrospun fibers by immersing the fibers in solutions of dopamine and bFGF. The scaffolds were subsequently tested for their ability to promote wound healing and skin remolding. PLGA electrospun fibrous scaffolds were coated with PDA by immersion in a dopamine solution. The poly-dopamine-modified scaffolds were subsequently immerged in a bFGF solution for grafting of this growth factor, thereby improving the wettability and biocompatibility of the scaffold surface and accelerating wound healing. This study characterized the morphology, surface wettability, and degradation of the scaffolds and their influence on HDFs *in vitro*, and investigated the promotion of wound healing in an early stage and skin remodeling in a late stage *in vivo* in the rabbit ear model.

Materials and methods

Materials

Poly (lactide-co-glycolide) (PLGA, Mw=50 kDa, 90/10, Mw/Mn=1.83) was purchased from Jinan Daigang Co. (Jinan, China). Recombinant murine bFGF was obtained from PeproTech Inc. (Rocky Hill, NJ). Dopamine hydrochloride (98%) purchased from Aldrich. Ultrapure water (18.3 MΩ cm)

produced by a Human Ultrapure System (Human Corp., Korea) was used. All chemicals and solvents were of reagent grade, which were supplied by Guoyao Reagents Company (Shanghai, China).

Electrospinning

The electrospinning solutions were prepared by dissolving 1g PLGA in 3 g dichloromethane and 1 g N, N-dimethylformamide. The electrospinning process was performed as described elsewhere.[36] Briefly, electrospinning was performed using the following parameters: applied voltage, 12 kV; solution feed rate, 0.6 ml/min; distance between needle and collector, 15 cm; syringe needle diameter, 0.8mm. The fibers were vacuum dried at room temperature for 2 d to completely remove any solvent residue prior to further use.

PDA coating and bFGF grafting onto the electrospun fibers

PLGA electrospun fibrous scaffolds were sterilized in 75% ethanol at 25 ℃ for 5h. All other solutions were sterilized by filtration. PLGA electrospun fibrous scaffolds were immersed in a dopamine solution (2 mg/ml in 10mM Tris, pH 8.5) at 25 ℃ for 12 h and rinsed 5 times with deionized water for further use. The PDA-coated PLGA electrospun fibers were subsequently transferred into a 10 μg/ml bFGF solution (10mM Tris, pH 8.5) for 12 h. The modified electrospun fibrous scaffolds were rinsed 5 times with deionized water and dried under a stream of argon.[35, 37]

Characterization of electrospun fibrous scaffolds

Electron microscopy (SEM, FEI Quanta 200, Netherlands) was used to examine the morphology of the fibrous scaffolds. For the surface wettability test , electrospun fibrous scaffolds with 100 μm thickness were chosed and punched into small strips (70.0 mm ×10.0 mm), and the strips was stably posted on the glass slides using solid double-sided adhesive. Water contact angles (WCA) were measured to evaluate the surface wettability of fibrous scaffolds using a Kruss GmbH DSA 100 Mk 2 goniometer (Hamburg, Germany), followed by image processing of sessile drops with DSA 1.8 software at room temperature (n=8). Chemical compositions of the fiber surface were determined by X-ray photoelectron spectroscopy (XPS, XSAM800, Kratos Ltd, Britain), and data was processed

using Kratos VISION 2000 software.[38]

Detection of bFGF grafting onto the electrospun fiborous scaffolds

The graft pattern of bFGF on the PDA-coated PLGA film was analyzed by immunofluorescence staining. The bFGF grafted electrospun fibrous scaffolds were treated with a rabbit polyclonal anti-human FGF-2 antibody (N-19) (sc-1390; Santa Cruz, USA), in a 1:50 dilution overnight at 4 °C, incubated with Alexa488-conjugated goat anti-rabbit IgG, 1:500 (Invitrogen, CA, USA) for 30min at room temperature, and then washed three times with 1 × phosphate-buffered saline (PBS) solution. Blank PLGA electrospun fibrous scaffolds, the PLGA electrospun fiber coated PDA scaffolds and the blank PLGA electrospun fibrous scaffolds immersed in a 10 μg/ml bFGF solution for 12 h were used as controls and treated in the same way.

bFGF loading onto the electrospun fiborous scaffolds

PLGA/PDA electrospun fibrous scaffolds were incubated in a 10 μg/ml bFGF solution for 12 h. Fibers were subsequently washed in 5 ml PBS 3 times to remove unbound bFGF. The amount of unbound bFGF in the wash buffer was determined by ELISA (Uscn Life Science Inc., Wuhan, China) according to manufacturer's instructions. Measurements were performed as described elsewhere.[23, 39]

Degradation of electrospun fibers

The degree of degradation was estimated from changes in molecular weight before and after modification. Molecular weight was determined by gel permeation chromatography (Waters 2695 and 2414, Milford, MA) using polystyrene as a standard. The column used was a Styragel HT 4 (7.8×300 mm). The mobile phase consisted of tetrahydrofuran using regularity elution at a flow rate of 1.0 ml/min.[40]

***In vitro* cell culturing on scaffolds**

Human dermal fibroblasts (HDFs), used to detect the cell behavior on electrospun fibrous scaffolds, were isolated from human hypertrophic scars provided by the Department of Plastic and Reconstructive Surgery, Ninth People's Hospital Affiliated to Medical School of Shanghai Jiao Tong

University (Shanghai, China). The cells were cultured in DMEM supplemented with 10% FBS, penicillin (100 units/ml) and streptomycin (100μg/ml) (Sigma) in a humidified incubator at 37°C with 5% CO_2. Culture media was replaced every three days. Once the HDFs reached 70% confluence, they were passaged at a ratio of 1:3. The second to fourth passages of HDFs were used in this study.

Sterilized electrospun fibrous scaffolds cut into disks with a diameter of 5 mm were pre-wet with cell culture medium for 2 h. 50 μl of a HDFs suspension (3×10^4 cells/ml) was seeded on the surfaces of membranes and placed in 96-well culture plates (Costar, Corning, NY, USA). The plates were incubated at 37°C with 5% CO_2 for 4 h before adding 100 μl of culture medium into each well. The culture medium was changed every 3 days.

Observation of cell morphology

HDFs cultured on PLGA, PLGA/PDA, and PLGA/PDA-bFGF scaffolds were photographed by SEM 3 and 9 days after seeding. Since the connection between bFGF and PLGA in bFGF/PLGA scarffolds is weak without PDA, bFGF could easily be washed out of the bFGF/PLGA scarffolds after several washes, and make the bFGF/PLGA scaffold become into PLGA scaffold. Therefore, the control group of bFGF/PLGA group was not used as a control in this experiment.

Scaffolds were harvested, washed with phosphate-buffered saline (PBS) solution twice and fixed with 4% glutaraldehyde for 2 hours at 4°C. The samples were dehydrated using graded ethanol series followed by three rinses with distilled water. Dry specimens were sputter coated with gold and examined by SEM.

Detection of cell proliferation rate

HDF proliferation was investigated using a Cell Counting Kit-8 (CCK-8, Dojindo Laboratories, Kumamoto, Japan) after incubation for 3,6 and 9 days, respectively. Briefly, the culture medium was removed and cell-scaffold samples were washed twice with the PBS. Subsequently, 100 μl culture medium and 10μl CCK-8 reagent was added to each sample and incubated at 37°C for 2.5 h in a humidified incubator at 37°C with 5% CO_2 according to the reagent instruction. 100 μl of incubated medium was transferred to a 96 -well culture plate and the absorbance was measured at 450 nm

using a microplate reader (Thermo labsystems, USA). All experiments were performed in triplicate.

Rabbit ear model of wound healing and ethics

Sixteen healthy New Zealand white rabbits weighting 2.5-3 kg, male or female, were obtained from Animal Center of the Shanghai Ninth People's Hospital Affiliated Shanghai Jiao Tong University School of Medicine (Shanghai, China) and used in this study. The study was carried out with the approval of the Committee of Experimental Animal Administration of Shanghai Ninth People's Hospital Affiliated Shanghai Jiao Tong University School of Medicine, and in accordance with international ethics guidelines and the National Institutes of Health Guide concerning the Care and Use of Laboratory Animals. Animals were kept in a regulated environment. Food and water were given ad libitum throughout the experiments.

The rabbit model of wound healing was used as previously described.[41, 42] Briefly, the animals were anesthetized and operated under sterile conditions. Four to six wounds of 10 mm diameter were created on the ventral surface of each ear avoiding the central ear artery and marginal ear veins. In order to prevent the shedding of the scaffolds, 2mm normal skin was peeled around the wound, and the distance between each of the wounds was greater than 12mm. The epidermis, dermis and perichondrium in each wound were thoroughly removed.

Rabbits were randomly divided into four groups (n=4): group A (control group); group B (blank PLGA scaffolds group, PLGA); group C (PDA-coated PLGA scaffolds group, PLGA/PDA); group D (bFGF- conjugated PDA-coated PLGA scaffolds group, PLGA/PDA-bFGF). The wounds in group B-D were implanted with the corresponding scaffolds, respectively. The wounds in group A were left without any treatment after operation. After recovery from anaesthesia, the rabbits were returned to their cages.

Macroscopic evaluation and the wound healing rate

The healing of each wound was observed and recorded carefully. Rabbits in each group were separately sacrificed on postoperative day 7 and day 14. The wound healing rate was calculated as the number of healed wounds in each group/the number of all wounds in each group. Healed wounds were defined as wounds that were healed by more than 90%.

Morphologic Analysis of the wound tissue

The samples in each group were excised with a 5 mm margin of surrounding unwounded tissue and bisected across the middle of the wound. These specimens were fixed in 4% neutral formalin for 24 h, embedded in paraffin, cross-sectioned along the tissue, and stained using hematoxylin–eosin (H&E) for determination of re-epithelialization and modified Masson's trichrome for analysis of collagen deposition. Re-epithelialization was calculated as the ratio between the distance covered by the epithelium and the distance between wound ends using Image-ProPlus 6.0.

Statistical analysis

The data was analyzed by ANOVA with a post hoc Dunn or Bonferroni examination. All the data were processed by IBM SPSS Statistics 19 for Windows. A value of $p < 0.05$ was considered significant.

Results

Electrospun fibers characterization

As were shown in Fig. 1, the surface of the PLGA, PLGA/PDA and PLGA/PDA-bFGF fibers was uniform in shape. Even though there were some local adhesion points between fibers, a stable 3D fiber structure was maintained. So the grafting process did not cause a change of the structure of the fibrous scaffolds. The diameter of blank PLGA fibers was 1.56±0.36 μm (Fig. 1 a), while the diameter of modified electrospun fibers was 1.71±0.41 μm and 1.76±0.38 μm for PLGA/PDA and PLGA/PDA-bFGF, respectively (Fig. 1 b and c). The surface of the fibers became rougher and the diameter of the fibers increased after PDA-coating and again after grafting with bFGF. However, these differences between the electrospun fibers were not statistically significant ($p > 0.05$). The static water contact angle on PLGA, PLGA/PDA and PLGA/PDA-bFGF electrospun fibrous scaffolds were determined as a measure of wettability and were 116.7±3.5°, 0° and 0°, respectively. Since the optimum range of water contact angles for cell culture substrates is between 5° and 40°, and 0° actually is totally hydrophic, and the cell proliferation rate can improve if they grow on the materials with such water contact angle,[43-47] these results show that PLGA electrospun fibers were

hydrophobic, while electrospun fibers coated with PDA and grafted with bFGF were extremely hydrophilic. The difference was significant (Fig. 1 insert pictures).

XPS spectra of PLGA fibrous scaffolds revealed the presence of carbon (C 1s, 284.5 eV) and oxygen (O1s, 531.0 eV) (Fig. 2). A significant difference was observed between PLGA/PDA and PLGA/PDA-bFGF with respect to the high resolution nitrogen peak (N 1s, 399.8 eV), consistent with previous reports.[33, 48] This additional peak is attributed to the amine groups and amide bonds of PDA and bFGF on the electrospun PLGA fibers. In addition, the area of the N1s peaks is increased after grafting bFGF, which can be seen from the N1s peaks of line b and c in Fig. 2. The increase of the N1s peak area indicated that PDA and bFGF were successfully coated and grafted onto the PLGA scaffolds.

As was shown in Fig. 3d, bFGF grafted on the PLGA/PDA electrospun fibrous scaffolds could be clearly detected by immunofluorescence staining. However, there was no fluorescence staining in the PLGA, PLGA/PDA and PLGA fibers immersed in bFGF (Fig. 3 a, b and c), indicating that bFGF was successfully grafted only onto the surface of PLGA/PDA electrospun fibers. The amount of bFGF grafted onto PLGA/PDA electrospun fibrous scaffolds was measured, which was 143.8±4.7 ng/cm^2 for PLGA/PDA fibers after conversion calculation. The bFGF grafted, PDA-coated PLGA electrospun fibers were then transferred into a 10 μg/ml bFGF solution again(10mM Tris, pH 8.5) for 24 h and 36 h to see whether the extension of the grafting time could induced increasing of the amount of bFGF loading. The results showed that there was no significantly increasing of the amount of bFGF loading. These data showed that the amount of bFGF grafted onto PLGA/PDA electrospun fibrous scaffolds was balance for 12 h grafting time.

The degradation of PLGA fibers was determined by measuring changes in the molecular weight and total mass of electrospun fibrous scaffolds. Less than 3% of Mw reduction was found for PLGA/PDA (residual Mw=49.38±0.21 KDa) and PLGA/PDA-bFGF (residual Mw=49.05±0.28 KDa) fibrous scaffolds, and similar results were obtained for mass reduction. As indicated above, we found that there was no significant difference in the morphology of PLGA, PLGA/PDA and

PLGA/PDA-bFGF fibrous scaffolds. These results indicate that this kind of grafting in aqueous solutions enables the fibers to maintain a stable structure without induction of PLGA degradation.

Cell growth and proliferation on modified electrospun PLGA fibrous scaffolds

The cell morphology of HDFs grown on electrospun PLGA, PLGA/PDA and PLGA/PDA-bFGF scaffolds was studied by SEM at days 3 and 9 (Fig. 4). Compared to the PDA modified PLGA scaffolds, , HDFs were not easy to attach on the unmodified PLGA scaffolds at day 3 due to the hydrophobicity of the electrospun PLGA fibrous scaffolds. In contrast, HDFs were able to grow very well on the electrospun PLGA/PDA and PLGA/PDA-bFGF scaffolds, and the cells were characteristically spindle-shaped. However, the overall number of HDFs at day 3 was small due to a low HDF seeding density to allow for observation of cell proliferation over a 9 day span. Subsequently, at day 9 of culture, there were a number of HDFs on electrospun PLGA fibrous scaffolds. In comparison, on electrospun PLGA/PDA and PLGA/PDA-bFGF scaffolds, a large number of HDFs were found reaching an estimated confluence of about 50% and 90%, respectively. The PLGA/PDA-bFGF scaffolds were almost completely covered with a continuous HDF monolayer stretching across the fibrous substrate.

The HDFs proliferation on electrospun PLGA/PDA and PLGA/PDA-bFGF scaffolds was measured using a colorimetric CCK-8 assay at days 3, 6 and 9 (Fig. 5). PLGA scaffolds were used as control. It can be observed that the absorbance index of all groups increased with increasing culture time. The absorbance in cultures on electrospun PGLA fibrous scaffolds increased slowly after 9 days of culture, indicating slow HDF proliferation on the eletrospun PLGA scaffolds. There was a statistically significant difference ($p < 0.05$) between PLGA/PDA scaffolds and PLGA/PDA-bFGF scaffolds after only three days in culture. Moreover, this difference continued to increase until the day 9 of culture because of the bioactivity of bFGF.

Wound healing

All the wounds healed gradually and none of the wounds became infected. Photographs of the

wound healing status in each group are shown in Fig. 6A. On day 4 after operation, it was observed that electrospun PLGA/PDA and PLGA/PDA-bFGF fibrous scaffolds stick more closely with the wounds and surrounding tissue than unmodified PLGA scaffolds. From day 7, on the edge of the PLGA scaffolds was gradually set off from the surrounding tissue, and the PLGA scaffolds were gradually shed with the blood scab on day 10 to 14, which implied that the unmodified PLGA scaffolds were of poor biocompatibility with the surrounding tissue compared to the PLGA scaffolds modified by PDA. On day 14 post-operation, the rate of healed wound of the control and PLGA groups was 68% and 74% respectively. More wounds were healed in the PLGA/PDA and PLGA/PDA-bFGF on day 14 with wound healing rates in these groups being 86% and 92%, respectively.

Taken together, the wound healing rate is the lowest in the control group, gradually became higher in the PLGA and the PLGA/PDA group. The wound healing rate is the highest in the PLGA/PDA/bFGF group. (Fig. 6 B)

Re-epithelialization

The re-epithelialization results on day 7 and 14 are shown in Fig. 7. There were no significant differences among all groups on day 7. On day 14, the percentage of re-epithelialization in the PLGA/PDA (75.753±5.127%) and PLGA/PDA-bFGF (97.430±2.373%) groups was significantly increased when compared to the control (64.430±3.815%) and PLGA groups (56.520±4.142%) ($p< 0.05$). On day 14, the wounds in the PLGA/PDA-bFGF group were almost completely re-epithelialized, which implied that bFGF played a role in promoting re-epithelialization.

Collagen deposition

The result of collagen deposition in each group by modified Masson's trichrome staining on day 14 is shown in Fig. 8. Collagen fibers in the dermal layer of the Control and PLGA groups were arranged irregularly and sparser compared to the PLGA/PDA and PLGA/PDA-bFGF groups. In addition, the collagen fibers of the PLGA/PDA and PLGA/PDA-bFGF groups were in bundles with

regular arrangement, and the dermal layer of PLGA/PDA-bFGF group was much closer in appearance to that of normal skin. At day 14, in the PLGA/PDA and PLGA/PDA-bFGF groups, gaps where noted that were occupied by the scaffolds which were absent in the PLGA group. This might be due to the rejection of the unmodified PLGA scaffolds by the surrounding tissue in the early stage and its subsequent shedding with the scab.

Discussion

An ideal tissue engineering scaffold should be able to mimic the elements of the ECM, which could promote cell attachment and proliferation, as well as having the characteristics of non-toxicity, hydrophilicity and chemical stability.[49] In addition to the aforementioned, in recent years, numerous research groups added drugs or bioactive molecules to tissue engineering scaffolds for topical administration purposes.[50] Collectively, tissue engineering scaffolds that promote wound healing should not only act as substrates for cell growth, but also carry a drug that promotes regeneration.[51] In the present study, we fabricated bFGF grafted PLGA electrospun fibers via simple immersion of the fibers in dopamine and bFGF solutions, followed by an investigation in into their wound healing properties. We found that poly-dopamine could successfully be coated on the surface of the fibers and enhance the biocompatibility as shown by HDF growth and skin repair.

In this study, the growth factor, bFGF, was easily grafted onto the surface of electrospun fibers, and the approach only used aqueous solutions via simple dip-coating. Electrospun fibers were first reported to be coated with PDA, followed by conjugation of bFGF to the fibers. Dopamine contains catechol and amine functional groups and is found in high concentration in mussel adhesive proteins.[35, 42] At weak alkaline pHs, dopamine can form thin adherent poly-dopamine films that can react with amine and thiol groups (Scheme 1A).[37] Accordingly, polymerization of dopamine is consistent with the process of formation of melanin, which involves oxidation of catechol to quinone, further reacting with amines and other catechols/quinones to attach to the electrospun fibers. Next, bFGF was immobilized onto the poly-dopamine films by a reaction with the catechol and quinone functional groups (Scheme 1A).

PLGA electrospun fibrous scaffolds are considered to be potential ideal tissue engineering materials due to the fact that they are particular porous. Nevertheless, the water contact angle of PLGA electrospun fiber scaffolds is over 110°, which is a drawback for cell attachment and proliferation on these matrices.[52, 53] Previous studies have demonstrated that different surface modification strategies were able to improve the hydrophilicity of various no-wetting surfaces, for example, layer-by-layer assembly, self-assembly and photoinitiated surface-grafting polymerization.[54-56] Though these methods were able to change the surface properties of these materials, there were many limitations to their wide application such as the need of special instruments, complex processes or chemical specificity between interfacial modifiers and surfaces. Here, we employed mussel-inspired surface coating with poly-dopamine as a one-step surface modification, which has been shown to adhere to the surfaces of a wide variety of materials, including organic and inorganic substances.[34] Furthermore, this method of surface modification has been shown to be time-saving and non-toxic, which was consistent with the results of our study. As shown in Fig. 1, though the diameter of the fibers increased after modification with PDA compared to blank PLGA fibers, the difference was not statistically significant. Moreover, testing of the degree of degradation before and after modifying also indicated that the mussel-inspired surface modification did not change morphology of the fibers and maintained 3D fiber structure. On the other hand, unmodified PLGA fibers showed a higher static water contact angle (116.7±3.5°), which changed to zero after coating with PDA, indicating that the PDA-coating changed the PLGA properties from hydrophobic to hydrophilic. Many previous studies have shown that various cells (e.g., human umbilical vein endothelial cells and mouse osteoblast) were able to adhere to the PDA modified surface of the various materials, grow and proliferate well. As shown in Fig. 4, the HDFs were able to adhere, spread, and survive better on PDA-modified electrospun PLGA. A few of HDFs were detectable on the eletrospun PLGA fibers. Additionally, the results of CCK-8 assay (Fig. 5) suggested that more HDFs grew on PDA-modified electrospun PLGA fibers.

Another purpose of surface modification is to obtain a functional molecular layer. The methods of

biomolecule immobilization onto the surfaces of substances include physical adsorption, affinity immobilization, and conjugation reactions, each of which, for instance, the standard carbodimide techniques,[57] necessitate a variety of solvents, complex processes and specific chemical reactions.[35] Moreover, these approaches generally cause different degrees of degradation of the material and affect the biological activity and the release rate of the biomolecules.[58,59] After surface modification with PDA, various biomolecules can easily conjugate onto the surface of the PDA film, depending on catechols reacting with thiols and amines.[38] This strategy of creating a functional organic ad-layer only needs two steps, simple ingredients and mild reaction conditions.[39] To date, a number of biomolecules have been successfully grafted onto the PDA modified surfaces of various substances, and exhibited good biological activity, such as trypsin, hyaluronic acid and DNA.[38, 39, 41] To our knowledge, this study is the first that reports on grafting of bFGF onto the surface of an electrospun PLGA fibrous scaffold through a catecholamine polymer. The result of XPS spectra (Fig. 2) showed that the nitrogen peak increased following the bFGF grafting (N 1s, 399.8 eV) compared to the PDA only coated scaffold, which was attributed to the successful grafting of bFGF onto the PLGA scaffolds. As shown in Fig. 1, the diameter of PLGA/PDA-bFGF fibers (1.76±0.38 μm) was similar to that of PLGA/PDA fibers, and the difference compared to the unmodified PLGA fibers was not statistically significant, which indicated that the two-step surface modification did not change the morphology of the electrospun PLGA fibrous scaffolds. In addition, HDFs that were cultured on the PLGA/PDA-bFGF scaffolds almost completely covered the surface of the scaffold after day 9 in culture (Fig. 4) and absorbance index was almost twofold compared to that of PLGA/PDA fibrous scaffolds (Fig. 5). These results indicated that the biological activity of bFGF was maintained well after being grafted onto the surface of the PLGA fibers.

The bFGF is a multipotential glycoprotein that promotes many cells proliferation, such as dermal fibriblasts, keratinocytes, and endothelial cells. Due to its mitogenic and angiogenic characteristics, the bFGF can induce tissue remodeling, wound healing and neovascularization.[60] In order to test the improving effect of the PDA modified electrospun fibrous scaffolds and the electrospun fibrous

scaffolds grafted with bFGF *in vivo*, we used the scaffolds in the rabbit ear wound model. PLGA/PDA and PLGA/PDA-bFGF scaffolds were more compatible with the surrounding tissue than the unmodified PLGA scaffolds in an early stage of wound healing. All wounds healed eventually, but at day14, the rate of healed wound with implanted PLGA/PDA scaffolds was higher than that in the control and unmodified PLGA groups, while the wound healing rate of the PLGA/PDA/bFGF group was 6% higher than that of the PLGA/PDA group. These results demonstrated that the electrospun PLGA fibrous scaffolds that were grafted with bFGF via poly-dopamine could not only act as substrate for cell growth, but also could effectively promote wound healing.

Skin wound healing is a critical and complex biological process after trauma. This process is activated by signaling pathways of both epithelial and nonepithelial cells, which release a myriad of different cytokines and growth factors.[61] bFGF is a cytokine known to play multiple roles during the various stages of wound healing. Studies have showed that bFGF could significantly accelerates epithelial healing.[62] Re-epithelialization is crucial for wound healing, and defect of wound re-epithelialization will always result into delayed wound healing process. Thus promote re-epithelialization is very important for wound healing,[63] In our study, we found that the stimulating effect of PLGA/PDA-bFGF scaffolds leads to a shorter re-epithelization time, and the re-epithelization time is the shortest in the PLGA/PDA-bFGF group, while longest in the control group. In addition, at day 14, the collagen fibers in the PLGA/PDA and PLGA/PDA-bFGF groups were arranged more regularly and were closer in appearance to the normal skin than in the other groups, especially in the latter group (Fig. 8). These results demonstrated that one of the mechanisms of promoting wound healing effect of bFGF might be promoting re-epithelia process.

In addition, at day 14, the collagen fibers in the PLGA/PDA and PLGA/PDA-bFGF groups were arranged more regularly and were closer in appearance to the normal skin than in the other groups, especially in the latter group (Fig. 8). These results demonstrated that one of the mechanisms of promoting wound healing effect of bFGF might be promoting re-epithelia process. The result of collagen deposition analysis using Masson's staining suggested that the bFGF-grafted electrospun

PLGA/PDA fibrous scaffolds enabled skin remodeling to result in a tissue that was closer in appearance to normal tissue while also promoting wound healing.

Conclusion

We successfully fabricated bFGF-loaded hydrophilic electrospun PLGA scaffolds using a PDA ad-layer which improved the hydrophilicity of the fibrous scaffold and could as act as a bridge to bind bFGF to the polymeric fibers. We found that the bFGF-loaded hydrophilic electrospun PLGA scaffolds were able to maintain a stable fibrous structure and not induce degradation of the fibers, which was better in simulating the ECM to promote wound healing and skin regeneration. These results indicated that the mussel-inspired poly (dopamine) surface modification was a simple strategy to improve the hydrophilicity and to effectively immobilize functional compounds onto the surface of the PLGA scaffold. Furthermore, the creation of a bFGF-loaded PDA ad-layer on electrospun PLGA scaffolds is a promising and effective strategy for skin regeneration tissue engineering.

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Figure legends

Scheme 1. Illustration of PDA-coated and growth factor immobilization on PLGA electrospun fibers (A), and cell growth on modified PLGA electrospun fibers (B).

Fig. 1 SEM images and water contact angles (insert picture) of PLGA (a), PLGA/PDA (b) and PLGA/PDA-bFGF (c) electrospun fibers.

Fig. 2 XPS spectra of PLGA (a), PLGA/PDA (b) and PLGA/PDA-bFGF (c) electrospun fibers.

Fig. 3 Immunofluorescence staining of bFGF on the PLGA/PDA electrospun fibrous scaffolds. PLGA (a), the PLGA/PDA (b), PLGA immersed bFGF (c) and PLGA/PDA-bFGF (d).

Fig. 4 SEM images of HDFs growth on electrospun PLGA, PLGA/PDA and PLGA/PDA-bFGF scaffolds on day 3 and 9 after seeding.

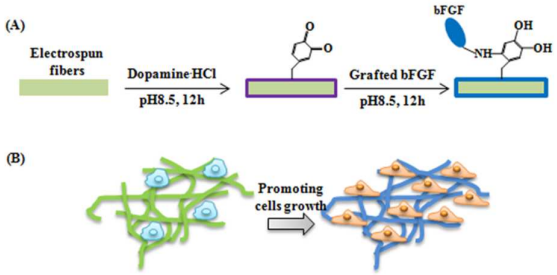
Fig. 5 CCK-8 assay of HDFs proliferation on electrospun PLGA, PLGA/PDA and PLGA/PDA-bFGF scaffolds fibers after 3, 6 and 9 days in culture. * represents $p < 0.05$ compared to the corresponding controls.

Fig. 6 Electrospun fibrous scaffolds in wounds and the rate of healed wound, Photographic imaging of the status of wound healing (A) and the rate of healed wounds of each group (B). Control (a), PLGA (b), PLGA/PDA (c) and PLGA/PDA-bFGF (d). Rate of healed wound was calculated as the number of healed wound in each group/the total number of wounds in this group.

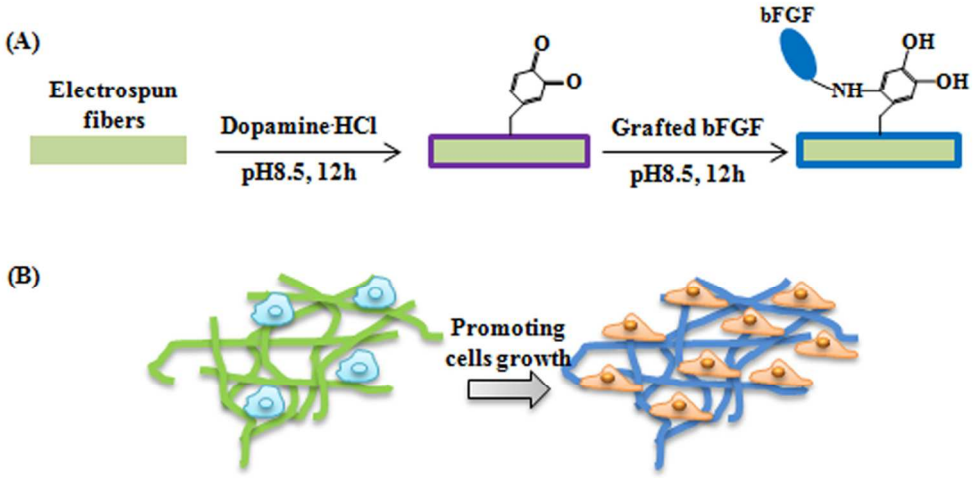
Fig. 7 Histological appearance of wounds harvested on day 7 and 14 of each group and re-epithelialization of each group on day 14 after operation, Hematoxylin–eosin (H&E) staining of wounds (A) and re-epithelialization on day 14 after operation of each group (B). The red arrows indicate the edges of epithelial regeneration. Control (a), PLGA (b), PLGA/PDA (c) and PLGA/PDA-bFGF (d). * represents $p < 0.05$ compared to with the corresponding controls.

Fig. 8 Masson staining of wounds of each group harvested on day 14. The red arrow indicates the gap, the scaffolds occupied

Colour Graphic



bFGF was facilely grafted on PLGA fibers surface in aqueous solution via poly-dopamine, which maintained fiber properties and bFGF bioactivity.



Scheme 1. Illustration of PDA-coated and growth factor immobilization on PLGA electrospun fibers (A), and cell growth on modified PLGA electrospun fibers (B).
48x24mm (600 x 600 DPI)

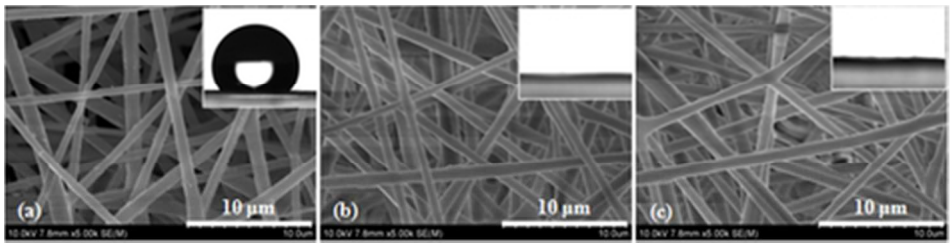


Fig. 1 SEM images and water contact angles (insert picture) of PLGA (a), PLGA/PDA (b) and PLGA/PDA-bFGF (c) electrospun fibers.
19x4mm (600 x 600 DPI)

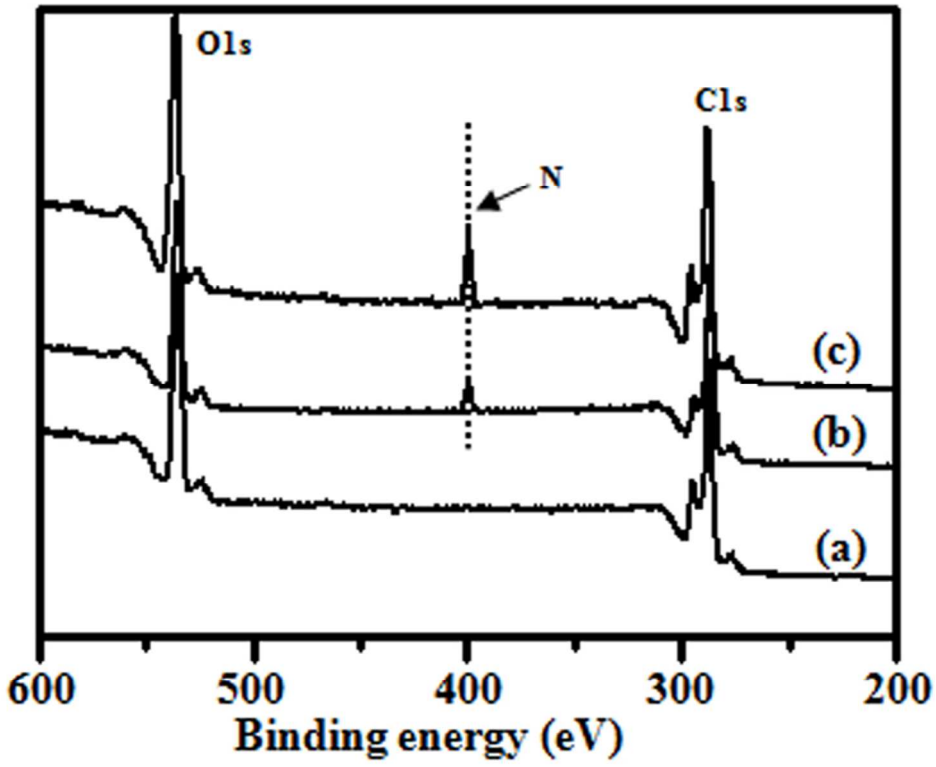


Fig. 2 XPS spectra of PLGA (a), PLGA/PDA (b) and PLGA/PDA-bFGF (c) electrospun fibers.
39x31mm (600 x 600 DPI)

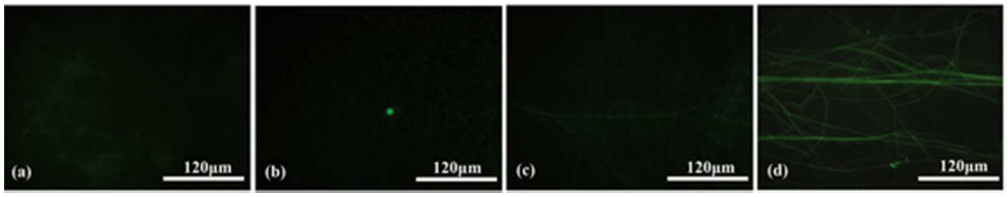


Fig. 3 Immunofluorescence staining of bFGF on the PLGA/PDA electrospun fibrous scaffolds. PLGA (a), the PLGA/PDA (b), PLGA immersed bFGF (c) and PLGA/PDA-bFGF (d).
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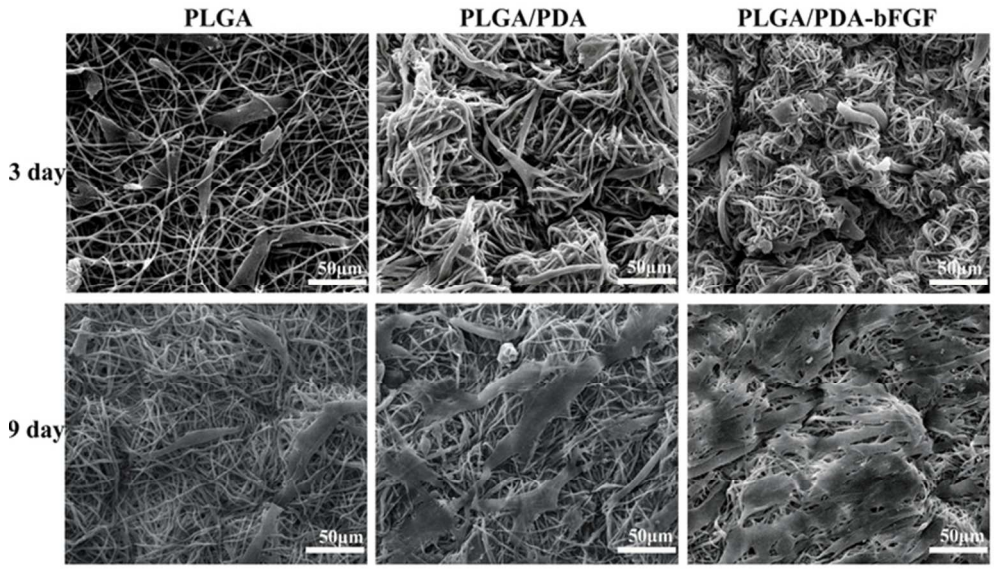


Fig. 4 SEM images of HDFs growth on electrospun PLGA, PLGA/PDA and PLGA/PDA-bFGF scaffolds on day 3 and 9 after seeding.
65x38mm (300 x 300 DPI)

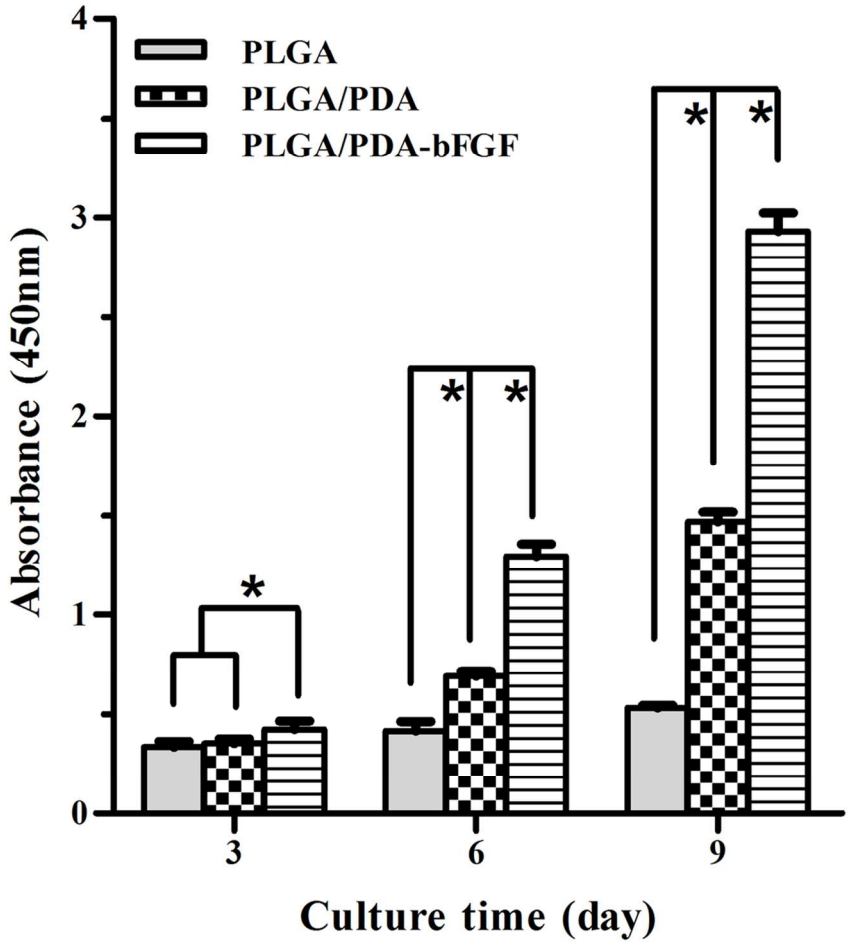
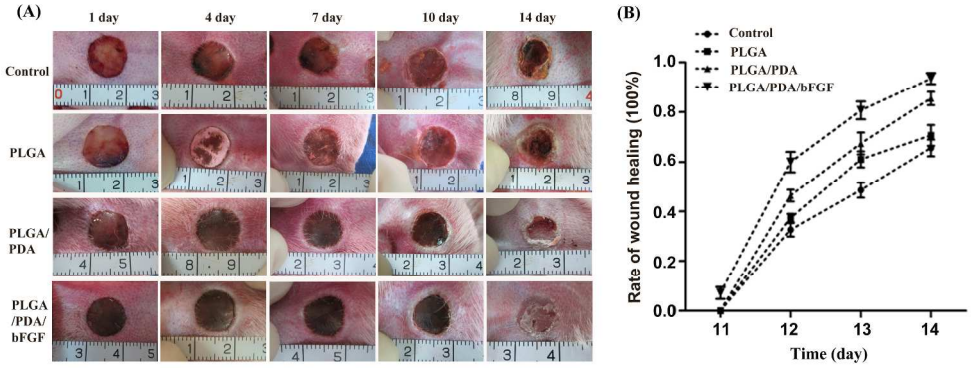


Fig. 5 CCK-8 assay of HDFs proliferation on electrospun PLGA, PLGA/PDA and PLGA/PDA-bFGF scaffolds fibers after 3, 6 and 9 days in culture. * represents $p < 0.05$ compared to the corresponding controls.
62x64mm (600 x 600 DPI)



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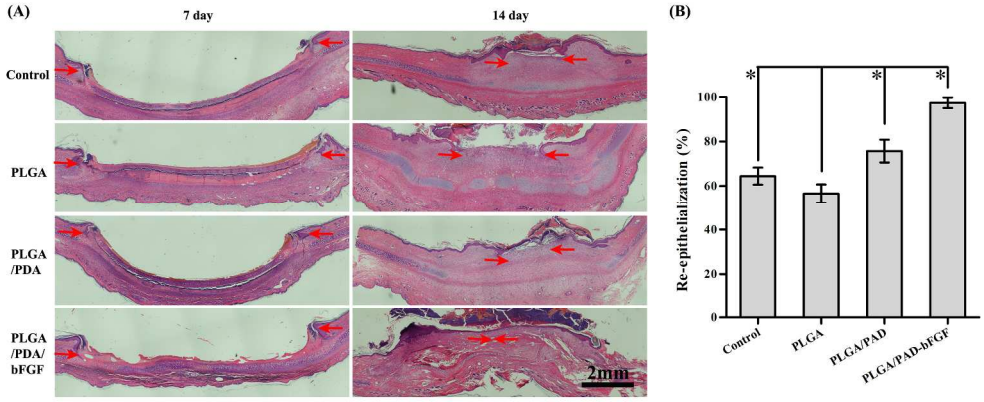

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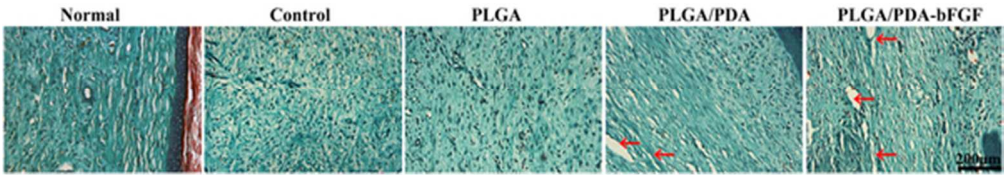

Fig. 8 Masson staining of wounds of each group harvested on day 14. The red arrow indicates the gap, the scaffolds occupied
25x4mm (600 x 600 DPI)