



Dual growth factor releasing multi-functional nanofibers for wound healing



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ABSTRACT

The objective of this research is to develop a dual growth factor-releasing nanoparticle-in-nanofiber system for wound healing applications. In order to mimic and promote the natural healing procedure, chitosan and poly(ethylene oxide) were electrospun into nanofibrous meshes as mimics of extracellular matrix. Vascular endothelial growth factor (VEGF) was loaded within nanofibers to promote angiogenesis in the short term. In addition, platelet-derived growth factor-BB (PDGF-BB) encapsulated poly(lactic-co-glycolic acid) nanoparticles were embedded inside nanofibers to generate a sustained release of PDGF-BB for accelerated tissue regeneration and remodeling. In vitro studies revealed that our nanofibrous composites delivered VEGF quickly and PDGF-BB in a relayed manner, supported fibroblast growth and exhibited anti-bacterial activities. A preliminary in vivo study performed on normal full thickness rat skin wound models demonstrated that nanofiber/nanoparticle scaffolds significantly accelerated the wound healing process by promoting angiogenesis, increasing re-epithelialization and controlling granulation tissue formation. For later stages of healing, evidence also showed quicker collagen deposition and earlier remodeling of the injured site to achieve a faster full regeneration of skin compared to the commercial Hydrofera Blue[®] wound dressing. These results suggest that our nanoparticle-in-nanofiber system could provide a promising treatment for normal and chronic wound healing.

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1. Introduction

Wound healing is a dynamic, complex, multicellular process involving extracellular matrix (ECM), cytokines, blood cells and many other factors [1]. Due to the pathological and physiological complexity of the wound healing process, perfect tissue regeneration is difficult to achieve [2]. This is especially true for chronic wounds, which affect millions of patients and have the associated cost of ~US\$20 billion annually in the USA; hence progress of new treatment strategies or methodologies is greatly needed [3]. Traditional wound dressing normally acts as a temporary barrier for hemostasis and infection prevention purposes [4]. In recent decades, natural and synthetic skin grafts have been developed for replacement applications. However, most of them are expensive, require extensive care and do not regain full skin functionalities [5]. Hydrogel is another candidate for the wound treatment with benefits of maintaining moisture at the wound site and capability of drug delivery [6]. However, most hydrogels are non-degradable

and difficult to use for large wounds [7]. To date, current treatment methods failed to achieve satisfaction of regaining barrier functionality and the cosmetic appearance of natural skin [8].

Using growth factors and their combinations in vivo has been suggested as a promising treatment to promote active healing [9,10]. However, until now, only platelet-derived growth factor-BB (PDGF-BB) has successfully completed clinical trials [11]. One of the major obstacles is that growth factors are either easily degraded by proteinases [12] or removed by exudate before reaching the wound bed [13]. Recent effort has been made on delivering growth factors via electrospun fibers for diabetic wound healing applications [14–16]. Compared to other wound dressing formations, electrospun nanofibers provide an ECM-like scaffold to support skin regeneration [17,18]. However, without a fundamental understanding of the biological process and better administration techniques to release biological molecules at essential points of the healing process, optimal wound healing having a fast and finely orchestrated nature still remains a challenge [19,20].

Normally, skin wound healing shows three overlapping phases: initial inflammation, tissue regeneration to fill the wound bed and tissue remodeling to regain skin functionalities. Recent developments discovered that during each phase of the dynamic process,

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many cytokines and growth factors are critical to modulate various cellular processes, including hemostasis, cell migration, differentiation, ECM formation, angiogenesis, and so on [1,2]. Some of the most important factors, like epidermal growth factors (EGFs), platelet-derived growth factor (PDGF), transforming growth factor (TGF- β), vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF-2), present at different healing stages with certain functionalities [21]. For example, VEGF is a key mediator for angiogenesis and granulation tissue formation in the early stage of healing [21,22]. On the other hand, PDGF is crucial for inflammation, granulation, re-epithelialization and remodeling throughout the three phases of wound healing [1,21].

In this study, we intended to create a biomimetic system that could serve as scaffold to support wound healing while releasing VEGF and PDGF-BB in a relayed manner to simulate the angiogenesis and cell proliferation at various phases, thereby facilitating the wound healing process. The optimal goal will be creating an integration of biochemical stimulation, cell growth support and bacteria inhibition that promotes favorable cell behaviors in skin wounds. Electrospinning is used to fabricate nanofiber composites containing chitosan, poly(ethylene oxide), VEGF and nanoparticles, which are loaded with PDGF-BB. Unlike existing research that uses either electrospun fibers with antibacterial nanoparticles [23] or antibiotics [24,25], the anti-infection issue is addressed on the fiber itself in this work. It is well-known that chitosan is an antibacterial polymer [26]. We hypothesize that introducing VEGF in the early stage could promote new blood vessel formation and sequentially bring more nutrition and oxygen to the wound site, and providing PDGF-BB throughout the whole process will facilitate the wound healing. Thus, a relatively fast release is required for VEGF, which will be released from electrospun fibers; and a sustained release is critical for PDGF-BB, which will be released from polymeric nanoparticles within the fibers. Our unique device could provide a structural mimic of ECM and anti-infection behavior, and supply specific growth factors when they are necessary to synergistically improve the complex wound healing treatment.

2. Materials and methods

2.1. Materials

Chitosan (CS, medium molecular weight, 75–85% deacetylated), poly(ethylene oxide) (PEO, Mn: 600,000 Dalton), acetic acid, chloroform and other chemicals were purchased from Sigma Aldrich (St Louis, MO). Poly-lactic-co-glycolic acid (PLGA) (50:50) was purchased from Lakeshore Biomaterials (Birmingham, AL). PDGF-BB and vascular endothelial growth factor (VEGF) were purchased from Prospec (East Brunswick, NJ). Adult human dermal fibroblast (HDF) cells were purchased from ATCC (Manassas, VA). Gram-negative *Escherichia coli* (*E. coli* 25922) and gram-positive *Staphylococcus aureus* (*S. aureus* 25923) were also obtained from ATCC. All other chemicals if not specified were purchased from Sigma Aldrich (St Louis, MO).

2.2. Fabrication of PLGA nanoparticles

PLGA nanoparticles fabrication was carried out using the double-emulsion technique as previously described [27]. Briefly, 200 μ l 2% w/v PDGF-BB solution was added to 3.33 ml 3% w/v PLGA solution and sonicated at 30 W for 2 min. This o/w solution was then added dropwise to 12 ml 2% PVA solution and sonicated at 20 W for 2 min. Final w/o/w solution was de-solvated overnight using a magnetic stirrer. Centrifugation was then performed for 4,000 rpm for 5 min to get rid of particle aggregates. The PDGF-BB loaded PLGA nanoparticles were washed and

collected using ultracentrifugation, and then further obtained via freeze-drying. In addition, the supernatant from the nanoparticle formation process was also collected to determine the loading efficiency as previously reported [27].

2.3. Fabrication nanofibers via electrospinning

The CS stock solution was prepared at a concentration of 2.5% w/v CS in 90% acetic acid. PEO solution at 8% w/v was prepared in DI water at room temperature. Two CS/PEO blend solutions were prepared by mixing the two stock solutions at 1:1 and 2:1 chitosan to PEO volume ratios (in this study, nanofibers without nanoparticles/growth factors were named as 1:1 CS/PEO and 2:1 CS/PEO). In another experiment, 20 wt.% of PLGA nanoparticles to PEO weight was sonicated into the mixture CS/PEO solution for 10–15 min at 20 W for complete dispersion of the nanoparticles (nanofibers with nanoparticles loaded with VEGF and PDGF-BB were named as 2:1 CS/PEO-NPs).

For electrospinning, the blended solutions were loaded into 5 ml syringe and fitted with an 18-gauge blunt needle tip. The solution feed was driven using a syringe pump at a flow rate of 1.5 μ l min^{-1} , and a 15 cm distance and DC voltage of 18 kV were applied between the collector (aluminum mesh) and needle. All experiments were carried out at ambient temperature and relative humidity of 15–20%.

2.4. Characterization of fiber meshes

The surface morphology of the electrospun nanofiber mesh was characterized using a scanning electron microscope (SEM; Hitachi, S-3000N). All samples were first sputter-coated by silver. Fiber diameters were also determined using Image-J software. For each mesh, 100 fibers were considered from three different images to calculate the average diameter. To visualize the nanoparticles within the nanofibers, indocyanine green (ICG) loaded PLGA nanoparticles were prepared and electrospun. Fluorescent images were taken using fluorescent microscopy with a TRITC filter.

2.5. In vitro growth factor release from electrospun fiber meshes

To assess the growth factor release kinetics, VEGF was loaded in CS/PEO nanofibers, while PDGF-BB was encapsulated in the PLGA nanoparticle that embedded in the scaffolds. The scaffolds weighing 10.0–11.0 mg were loaded into 100 kDa dialysis membranes and placed in the 0.1 M PBS solution with pH 7.4. Samples were then placed on an orbital shaker at 37 °C. At predetermined time points, 1 ml of PBS solution was collected, stored at –20 °C for later analysis, and replenished with 1 ml fresh PBS. The release profiles of VEGF and PDGF-BB were analyzed using ELISA following the manufacturer's instructions (Invitrogen, Carlsbad, CA). Loading efficiency of VEGF was determined by dissolving the nanofibers in PBS and following with ELISA. Loading efficiency of PDGF-BB was determined indirectly by measuring the PDGF-BB concentration in washing solutions collected in the nanoparticle formation process by ELISA. Cumulative release over a period of 3 days was performed on all samples.

2.6. In vitro cell proliferation

Adult human dermal fibroblasts (HDFs) (passage numbers up to 10) were cultured in complete Dulbecco's modified Eagle's medium (DMEM) with supplements of 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Cells were sub-cultured until ~80% confluency and maintained at a humidified atmosphere of 95% air and 5% CO₂. For in vitro cell proliferation on nanofiber meshes, scaffold samples (3 mm diameter) were vacuum dried

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