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Role of non-mulberry silk fibroin in deposition and regulation of extracellular matrix towards accelerated wound healing

Dimple Chouhan [a], Bijayshree Chakraborty [b], Samit K. Nandi [b], Biman B. Mandal [a,*]

[a] Biomaterial and Tissue Engineering Laboratory, Department of Biosciences and Bioengineering, Indian Institute of Technology Guwahati, Guwahati – 781039, Assam, India
[b] Department of Veterinary Surgery and Radiology, West Bengal University of Animal and Fishery Sciences, Kolkata – 700037, West Bengal, India

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ABSTRACT

Bombyx mori silk fibroin (BMSF) as biopolymer has been extensively explored in wound healing applications. However, limited study is available on the potential of silk fibroin (SF) from non-mulberry (*Antheraea assama* and *Philosamia ricini*) silk variety. Herein, we have developed non-mulberry SF (NMSF) based electrospun mats functionalized with epidermal growth factor (EGF) and ciprofloxacin HCl as potential wound dressing. The NMSF based mats exhibited essential properties of wound dressing like biocompatibility, high water retention capacity (440%), water vapor transmission rate (~2330 g m^{-2} day^{-1}), high elasticity (~2.6 MPa), sustained drug release and antibacterial activity. Functionalized NMSF mats enhanced the proliferation of human dermal fibroblasts and HaCaT cells *in vitro* as compared to non-functionalized mats ($p \leqslant 0.01$) showing effective delivery of EGF. Extensive *in vivo* wound healing assesment demonstrated accelerated wound healing, enhanced re-epithelialization, highly vascularized granulation tissue and higher wound maturity as compared to BMSF based mats. NMSF mats treated wounds showed regulated deposition of mature elastin, collagen and reticulin fibers in the extracellular matrix of skin. Presence of skin appendages and isotropic collagen fibers in the regenerated skin also demonstrated scar-less healing and aesthetic wound repair.

Statement of Significance

A facile fabrication of a ready-to-use bioactive wound dressing capable of concomitantly accelerating the healing process as well as deposition of the extracellular matrix (ECM) to circumvent further scarring complicacies has become a focal point of research. In this backdrop, our present work is based on non-mulberry silk fibroin (NMSF) electrospun antibiotic loaded semi-occlusive mats, mimicking the ECM of skin in terms of morphology, topology, microporous structure and mechanical stiffness. Regulation of ECM deposition and isotropic orientation evinced the potential of the mat as an instructive platform for skin regeneration. The unique peptide motifs of NMSF assisted the augmented recruitment of fibroblast, keratinocytes and endothelial cells leading to accelerated wound healing. Early progression of mature granulation, faster re-epithelialization and angiogenesis in the wounds in *in vivo* rabbit model forwarded the blended nanofibrous mats of NMSF and PVA ferrying EGF, apt for scarless healing.

1. Introduction

Skin protects the internal organs from various foreign elements, pathogens and mechanical stress. Breaching of skin may lead to physiological imbalance of the body often resulting in fatal consequences. More than 6.5 million people are suffering from chronic cutaneous wounds such as diabetic foot ulcers, venous ulcers, pressure sores and traumatic injuries [1]. Wound healing is a complex process comprising four sequential phases – hemostasis, inflammation, cell proliferation and tissue remodeling [2,3]. Obstruction in any phase of the healing pathway may lead to complications like chronicity or fibrosis [2,3]. There is a necessity of bioactive wound dressing which not only accelerates the healing process but also regulates the extracellular matrix (ECM) deposition to avoid further scarring complicacies. Hence, efficient wound dressing which aims to improvise all the four phases of wound healing is the ultimate need for aesthetic wound repair.

* Corresponding author.
E-mail addresses: biman.mandal@iitg.ernet.in, mandal.biman@gmail.com (B.B. Mandal).

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Electrospinning technique holds immense potential in the wound healing applications as it not only facilitates the easy incorporation of bioactive molecules but also closely mimics the nanofibrous structure of natural ECM, thereby providing a 3D environment for better cell recruitment [4–6]. Over the past decades, extensive research endeavors have been directed towards the development of nanofibrous mats using various synthetic and natural polymers. The most explored biopolymers in wound dressing applications are collagen, chitosan, silk fibroin, hyaluronic acid, cellulose, alginate, dextran, elastin, poly(vinyl alcohol) (PVA), poly (ethylene glycol) (PEG), poly(lactic co-glycolic acid) (PLGA), polylactic acid (PLA), poly(caprolactone) (PCL) and silicone [7–9]. Current strategies to develop dressing materials focus only on the accelerating healing events to quickly seal the wound. However, scar-less skin regeneration is not addressed by most of the products [10]. Achieving regulated ECM deposition by tuning the fibroblast response using functional wound healing substrate needs to be explored more [11]. The current study focuses on fabricating bioactive wound dressings and their role in the regulated deposition of ECM towards wound repair.

Among the various biomaterials reported till date, silk fibroin (SF) from mulberry silkworm *Bombyx mori* (BM) has been proved to be an accepted material for biomedical applications due to its biocompatibility, biodegradability, low immunogenicity, material versatility, cost-effectiveness, easy processing and tensile properties [12]. In the present study, we explored the potential of SF protein from non-mulberry silk varieties *Antheraea assama* (AA) and *Philosamia ricini* (PR). Non-mulberry silk fibroin (NMSF) possesses inherent Arginine, Glycine and Aspartate (RGD) motifs in the protein sequence, facilitating binding to the integrin receptors of cells [13]. This provides functional advantage of mediating cell-material interactions for wound repair. To fabricate NMSF based electrospun mats, PVA (a non-protein polymer) was used as the blending material as the proteolytic rich environment of chronic wounds demand stable wound dressing [2]. Blend of PVA and BMSF has been previously used in the format of membranes and electrospun mats manifesting the efficacy of the blend for wound dressing applications [14,15]. In the present study, blends of PVA with three different types of SF were electropsun using aqeous solvent which were also functionalized with epidermal growth factor (EGF) and ciprofloxacin (CIP) to re-establish growth factor pool in wound microenvironment and prevent infection respectively [16,17]. The present study focuses on the assessment of essential physical properties and functional advantage of the novel material for wound healing applications. Four different functionalized mats were prepared: one pristine mat of only PVA and three hybrid mats from the blends of PVA with three varieites of SF for comparative study. We have looked into the substrate's ability to accelerate the *in vivo* wound healing process and potential to regulate the deposition of collagen, elastin and reticulin fibers which majorly constitute the ECM of skin.

2. Materials and methods

2.1. Preparation of silk fibroin solutions

Solution of *Bombyx mori* silk fibroin (BMSF) was isolated from the silk cocoons as described in the standard protocol [18]. Briefly, *B.mori* cocoons were degummed twice for 15 min in boiling water using 0.02 M sodium carbonate (Merck, India). The dried silk fibers were dissolved in 9.3 M lithium bromide solution (Sigma Aldrich, USA) at 60 °C for 4 h and subsequently dialyzed against Mili-Q water using a 12 kDa cellulose dialysis membrane (Sigma Aldrich, USA) for 3 days at room temperature (RT) to obtain aqueous BMSF solution. Silk fibroin, AASF and PRSF were extracted from the silk glands of 5th instar matured larvae of *Antheraea assama* and *Philosamia ricini* silkworms respectively according to previously described protocol [19]. Briefly, the fibroin protein was extruded out from the glands using forceps and was subsequently dissolved in 1% (w/v) aqueous sodium dodecyl sulfate (SDS) (Himedia, India) solution. The protein solution was further dialyzed against Mili-Q water using a 12 kDa cellulose dialysis membrane for 4 h at 4 °C to obtain pure AASF and PRSF aqueous solutions. Isolation procedure of SF from the three silk varieties was completely aqueous and further fabrication steps also involved all-aqueous procedures bringing green synthesis of the nanofibrous mats.

2.2. Fabrication of functionalized electrospun nanofibrous mats

PVA (1,700–1,800 degree of polymerization and 98–99 mol% hydrolysis) was procured from LobaChemie Pvt. Ltd., India. PVA stock solution (13% w/v) was prepared by dissolving PVA granules in lukewarm de-ionized water at 50 °C for 6 h with constant stirring. PVA aqueous solution was then blended separately with the three different aqueous solutions of SF (3% w/v) (BMSF, AASF and PRSF) in the ratio of 4:1 (PVASF) (w/w) to make hybrid mats. Pristine nanofibrous mat of PVA was fabricated using 8% PVA (w/v) solution. Viscosities of pristine PVA solutions and blend solutions were measured using a viscometer (Fungi Lab, Visco Basic Plus) with the spindle calibrated at 0 cP for water, rotating with a speed of 100 rpm. The four types of nanofibrous mats were electrospun using electrospinning machine (E-spin nanotech, India). Briefly, the solution was filled in a syringe and spilled with a flow rate of 0.800 ± 0.100 mL/h via a 21 gauge blunt needle. Rotating drum collector (at a rotating speed of 500 rpm) was placed at a distance of 15 cm from the needle while the voltage was kept at 25 ± 3 kV. Four different mats were prepared under the similar electrospinning parameters: one pristine mat of only PVA solution (PVA) and three hybrid mats from the blends of PVA and SF– PVA + *B. mori* (PVABM), PVA + *A. assama* (PVAAA) and PVA + *P. ricini* (PVAPR). To fabricate functionalized nanofibrous mats, human recombinant EGF (expressed in *E. coli*) (Sigma Aldrich, USA) was mixed in the spinning solution prior to electrospinning. The final concentration of EGF in all the solutions was 1 μg/mL. β-sheet in the hybrid electrospun mats were induced by slightly modifying the solvent vapor method [20]. For this, mats were kept in a vacuum desiccator saturated with 70% ethanol vapors for 6 h. The average thickness (300 μm) was kept constant for all the mats by spinning 12 mL solution. For the evaluation of preliminary antibacterial assessment, the nanofibrous mats were coated with ciprofloxacin hydrochloride monohydrate (Himedia, India, MW 385.82 Da). Drug incorporation was done by coating onto the processed mats instead of co-spinning to achieve higher burst release. The mats (6 mm diameter) were coated with 50 μL of CIP solution (30 mg/mL) by which a total amount of 1.5 mg CIP was adsorbed on each sample. Coating of CIP was done in 5 cycles by adding 10 μL of drug solution and drying after every one hour.

2.3. Physico-chemical characterization of nanofibrous mats

Morphology and size of nanofibers were investigated using field emission scanning electron microscopy (FESEM; Zeiss, sigma). Image J software (Wayne Rasband, National Institute of Health, USA) was used to determine the diameter of nanofibers by measuring 100 randomly selected nanofibers from the magnified images. The surface topography and roughness of the nanofibers were assessed using atomic force microscopy (AFM Agilent, Model 5500 series, non-contact mode) with silicon cantilever having a spring constant of 42 N/m at a resonance frequency of 320 kHz. AFM images were further analyzed by WSxM software. The functional groups and chemical bonds of SF and PVA in the nanofibrous