Materials Science & Engineering C 93 (2018) 455–464

Contents lists available at ScienceDirect

Materials Science & Engineering C

journal homepage: www.elsevier.com/locate/msec

Accelerated wound healing and its promoting effects of biomimetic collagen matrices with siderophore loaded gelatin microspheres in tissue engineering

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ARTICLE INFO

Keywords:
Collagen
Siderophore
Microspheres
Wound healing
Biocompatibility

ABSTRACT

The prolonged inflammation and elevation of Matrix Metalloproteniases (MMPs) at the wound site causes significant degradation of Extracellular matrix (ECM) which cause delays the process of wound healing. Hence the development of therapeutic dressing matrices to control and to positively regulate MMPs balance was considered important in achieving faster healing. The design of biomaterial matrices of collagen scaffold has the challenge to mimic the function of ECM and emulate to the attraction of fibroblast migration at wound site. Herein, we report the fabricated Collagen (COL) matrices impregnated with Siderophore loaded Gelatin Microspheres (SGM) as a delivery system to control both infection and protease levels in the wound site for accelerated healing. The fabricated collagen scaffold impregnated with siderophore loaded gelatin microspheres (COL-SGM) was characterized physiochemically using Fourier transform infrared spectroscopy (FTIR), Scanning electron microscopy (SEM) and swelling behaviour. The COL-SGM scaffold possesses good swelling ability and also exhibited better morphology for the cell adhesion and proliferation. The *in vitro* biocompatibility and *in vitro* fluorescence activity of the developed scaffold revealed to possess good cell proliferation and migration against NIH 3T3 fibroblast and Human keratinocytes (HaCaT) cell lines. Furthermore, the *in vivo* evaluation offered the advantage of neutralizing the excessive proteases and delivered the siderophore in controlled fashion depending on the level of wound exudates with modulated MMPs. Moreover, the COL-SGM scaffold exhibited with increase in the collagen synthesis and faster reepitheliazation of wounds. Thus the developed COL-SGM scaffold achieved improvements in biocompatibility and act as a potent MMP inhibitor to improve wound healing efficiency in tissue engineering application.

1. Introduction

The skin is one of the largest organs of human anatomy, composed of mainly epidermis and dermis and act as a functional protective barrier from the environment [1]. Upon occurrence of the skin injury, a complex dynamic wound healing mechanism, involving blood vessels, connective tissues, extracellular matrix (ECM) and a variety of cell mediators from fibroblast and keratinocytes to hasten the wound closure [2,3]. Basically a normal wound healing involves three phases, namely: inflammation, granulation tissue formation and tissue remodeling [4].

During tissue formation, growth factors and chemical mediators were synthesized by local and migratory cells to stimulate the fibroblast to migrate into the wound where they proliferate and construct an ECM [5,6] In some cases, the normal wound healing process is disturbed and prolonged; which can lead to chronic, non-healing wounds due to the elevation of the structurally related proteolytic enzymes called Matrix Metalloproteinases (MMPs) which leads to delay of wound closure. MMPs are zinc (Zn^{2+}) dependent endopeptidases produced in latent forms [7]. Once activated, they participate in both physiological and pathological processes. MMPs are involved in the degradation of extracellular matrix (ECM) components, remodeling of tissues, shedding of cell surface receptors and processing of various signalling molecules [8,9]. Among them gelatinase-A (MMP-2) and gelatinase-B (MMP-9) are capable to degrade extracellular matrix protein, including type IV collagen. These are linked to cell invasion and metastasis [10]. Hence, the regulation of MMPs at the wound sites needs more attention. Siderophore a secondary metabolite isolated from *Pseudomonas aeruginosa* Strain S1 capable of binding with divalent ions and arresting the metabolic function which in turn act as an inhibitor for MMPs and improves healing of wounds [11].

Availability of a wide range of wound dressings till date is probably

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https://doi.org/10.1016/j.msec.2018.08.026
Received 23 March 2018; Received in revised form 16 July 2018; Accepted 8 August 2018
Available online 09 August 2018

matched by the diversity in wound types. In addition a wide variety of wound dressings with targeted therapy and controlled drug release for specific problems have emerged in recent days. The application of inert or bio-active synthetic dressing materials remain largely limited to the healing process and minimization of scarring. Similarly moist wound dressings, which possess clinical problems by providing a favourable environment for microbial proliferation [12]. On the other hand, film dressings, by virtue of their semi permeable nature were claimed to provide a barrier to bacteria as well complying with most of the ideal characteristics needed for an effective wound dressing [13].

Among recently available dressings, collagen-based dressings have shown to actively influence the healing process by intervening with various tissue components [14]. Though many natural biomaterials are available, collagen stands apart from them, mainly due to its mode of interaction with the host tissue [15]. Collagen films, by virtue of their capability to regulate release profiles of incorporated drugs, have been used widely as carrier constructs [16,17]. Moreover, they possess all the characteristics, of ideal material for scaffolding. Hence, a controlled delivery system of siderophore at the wound sites was developed by preparing collagen scaffold impregnated with siderophore loaded gelatin microspheres to maintain the therapeutic level of MMPs [18], was used to evaluate both *in vitro* and *in vivo* in our current study.

The present work focused on the design and development of collagen based biomaterial for wound dressing application. In this study, siderophore an ion chelating agent, isolated from *Pseudomonas aeruginosa* S1, were used to inhibit MMPs at the wound sites. Furthermore, we have utilized the inherent nature of collagen to form thin scaffolds impregnated with siderophore loaded microspheres. Apart from its basic physio-chemical properties, the scaffold was assessed for its biocompatibility through *in vitro* fibroblast and keratinocyte proliferation. The *in vivo* potential of siderophore loaded gelatin microsphere impregnated collagen scaffold (COL-SGM) as well as collagen scaffold impregnated with DBHA loaded gelatin microspheres (COL-DGM) as an effective wound dressing material were evaluated using rat as the animal model. The influence of siderophore on MMPs as an inhibitor was analyzed through various biochemical parameters. Hence this work may be a probable solution to effectively manage wound infection, with active participation of wound dressing during the healing process and cause lesser concern towards the frequency of dressing.

2. Experimental methods

All glassware used in this study was soaked in 5% v/v of RBS concentrate-20 and washed with deionized water and dried. Dihydroxybenzoic acids (DBHA) a model compound of siderophore type was used as a standard. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), Calcein AM, Dulbecco's Modified Eagle's Medium (DMEM), Fetal Calf Serum (FCS), and supplementary antibiotics for tissue culture were purchased from Sigma Aldrich, India. The isolated catechol-type siderophore from *Pseudomonas aeruginosa* S1 (Accession No. KM881475) was used in this study [11]. The mouse NIH 3T3 fibroblast and Human keratinocytes cell lines (HaCaT) were obtained from the National Centre for Cell Science (NCCS), Pune, India. The rest of the chemicals and culture wares were purchased from Sigma-Aldrich unless specified otherwise. All experiments were performed in compliance with Committee for the Purpose of Control and Supervision of Experiments on Animals guidelines (CPCSEA) and completed after the approval from the Institutional Animal Care and Use Committee (IACUC) [466/01a/CPCSEA].

2.1. Extraction of collagen (COL) and preparation of siderophore loaded gelatin microspheres (SGM) and DBHA loaded gelatin microspheres (DGM)

The collagen was extracted through the salt precipitation method was adopted from the previous work [19]. The Siderophore (S) loaded Gelatin Microspheres (GM) were prepared by water in oil emulsion method adopted from our previous study. [18]. Briefly, 2% of the siderophore in 10 mL of 6 wt% concentration of gelatin was added to at constant stirring of liquid paraffin at 60 °C. Simultaneously, 4 wt% Sodium Tetra Meta Phosphate (STMP) was added and kept at continuous stirring for 1 h for cross linking. After the 1 h the traces oil phase was removed by frequent washing with acetone. Finally, the siderophore loaded gelatin microspheres (SGM) were lyophilized and stored at 4 °C. Similarly, Dihydroxybenzoic acids (D)-loaded gelatin microspheres (DGM) were also prepared and stored at 4 °C.

2.2. Fabrication of collagen scaffold impregnated with siderophore loaded gelatin microspheres (COL-SGM) and DBHA loaded gelatin microspheres (COL-DGM)

The scaffolds were prepared by solvent casting method. Here, 4 wt% collagen solution in 0.1 mL acetic acid was prepared. Further, 50 mL of the collagen solution was mixed uniformly. To this with 100 mg of prepared SGM were added and gently stirred to distribute the spheres homogeneously throughout the solution for 15 min. Finally, the viscous solution was uniformly cast over horizontally placed polypropylene platforms (measuring $10 \times 4\ cm^2$) and allowed to dry at room temperature (30 °C) until a thin COL-SGM scaffold was obtained. Similarly, the DGM loaded scaffold was fabricated (COL-DGM). The fabricated scaffolds were ethylene oxide sterilized and stored in light proof desiccators for further experimental purpose [20,21].

2.3. Physiochemical characterization

2.3.1. Morphological assessment of microspheres impregnated collagen scaffolds

The morphology of the fabricated scaffolds (COL-SGM and COL-DGM) was assessed by Scanning Electron Microscope (SEM) (JEOL-JSM-5200). The sample was mounted on aluminium stubs using a double side adhesive tape and coated with gold to enhance the surface conductivity before scanning [18,22].

2.3.2. Fourier Transform Infrared Spectra (FTIR)

Fourier Transform Infrared Spectroscopy (Perkin Elmer, USA) spectral measurements were carried out for the determination of their functional groups of the fabricated scaffolds. The spectral measurements were measured at resolution of $4\ cm^{-1}$ in the frequency range of 4000–400 cm^{-1} [18,21].

2.3.3. Swelling behavior

The fabricated scaffolds was cut into a square piece (1×1 cm) and immersed into phosphate buffer solution (PBS, pH 7.4) at room temperature (37 °C) until the film reached the equilibrium. The samples were removed and the absorbed buffer was gently removed with filter paper in each immersion intervals. The equilibrium E_{sw}-swelling ratio was calculated from the Flory-Huggins formulae.

$$E_{sw}(\%) = \frac{W_e - W_0}{W_0} \times 100 \quad (1)$$

where, W_0 and W_e are the initial and the final weight of the film, respectively [23].

2.3.4. Evaluation of functional integrity of collagen scaffolds

The functional integrity of siderophore after the formation of microspheres was analyzed by immersing the scaffolds (surface area of $4\ cm^2$) in 1 mM aqueous solution of ferrous sulphate ($FeSO_4 \cdot 7H_2O$) for 2 h. The iron complexed samples were further subjected to Arnow's assay to determine the catecholate type siderophore [24].

2.3.5. In vitro biocompatibility, cell adhesion and proliferation studies

The collagen scaffolds impregnated with siderophore (COL-SGM) and DBHA conjugated gelatin microspheres (COL-DGM) were washed