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Design and characterization of 3D hybrid collagen matrixes as a dermal substitute in skin tissue engineering

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ABSTRACT

The highly interconnected porous dressing material was fabricated with the utilization of novel collagen (COL-SPG) for the efficient healing of the wound. Herein, we report the fabrication of 3D collagen impregnated with bioactive extract (COL-SPG-CPE) to get rid of infection at the wound site. The resultant 3D collagen matrix was characterized physiochemically using Fourier transform infrared spectroscopy (FTIR), scanning electron microscopy (SEM) and mechanical property. The dressing substrate possesses the high swelling ability, increase in the porosity, *in vitro* enzymatic degradability and antibacterial property. The *in vitro* biocompatibility and fluorescence activity of the collagen scaffold against both NIH 3T3 fibroblast and Human keratinocyte (HaCaT) cell lines assisted in excellent cell adhesion and proliferation over the collagen matrix. Furthermore, the *in vivo* evaluation of the COL-SPG-CPE 3D sponge exhibited with enhanced collagen synthesis and aids in faster reepithelialization. However, the rate of wound healing was influenced by the expression of vascular endothelial growth factor (VEGF), epidermal growth factor (EGF) and transforming growth factor (TGF-β) growth factors promotes the collagen synthesis, thereby increases the healing efficiency. Based on the results, COL-SPG-CPE has a potential ability in the remodeling of the wound with the 3D collagen as wound dressing material.

1. Introduction

After a physiological injury, the complex process of wound healing goes through a series of wound healing phases containing hemostasis, inflammation, proliferation and remodeling [1]. Once the skin is injured, the damaged blood vessels undergo the formation of fibrin network to stop bleeding. The inflammation process occurs simultaneously with vasodilation through the accumulation of individual chemicals, neutrophils, and macrophages [2]. The fibroblast infiltration and collagen synthesis activate the formation of new granulation tissue during the proliferative phase. Finally, remodeling phase involves the formation of cellular connective tissue resulting in contraction and healing of wounds. The reconstruct of skin tissue at the wound site has to be approached with the wound dressing material [3].

In the past few years, many wound dressing products have been approved commercially. However, the use of collagen from the marine origin showed advantageous with both physical and chemical with certain unique properties such as self-assembly, self-aggregation, triple helical structure, high glycine amino acid content low antigenicity and excellent biocompatibility [4,5]. The three-dimensional structure of collagen provides cell-cell and cell-matrix interactions with the fibroblast by the formation new granulation tissue and epithelium [6,7]. The ideal porous scaffold holds an important role in skin tissue regeneration, which provides a mechanical support that mimics the function and properties of the extracellular matrix [8].

Commercially various types of wound dressing material are available with both natural and synthetic materials in the different form, *i.e.*, gels, sheet or films and sponges, *etc.* [9]. The porous collagen material finds useful in various types of wounds such as leg ulcers, diabetic ulcers, peptic ulcer because it prevails more tissue exudates, maintain the moist environment and enhances the growth of new granulation tissue to make the wound healing efficient [10]. The wound dressing material made of collagen sponge with highly interconnected pores has to possess certain necessary property such as allowing oxygen permeability, maintaining antimicrobial activity, absorption of wound exudates and biocompatibility [11].

The proteinaceous nature of the collagen assists in high porosity and supports in absorbing of wound exudates, but raise the chance of invasion of the exogenous microorganism to cause infection around the wound site. The remedy for the cause mentioned above can be overwhelmed with the help of collagen dressing with antibiotics [12, 2]. The prevalent wound microbial floras are developing resistance towards the existing antibiotics due to the use of the new antimicrobial

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agents. Emphasis has been given to the use of plant extracts that possess bioactive ingredients that have to be incorporated into the collagen dressing to impart potential antimicrobial property [13]. The traditional significance and pharmacologically effective *Coccinia grandis* (CPE) (Family: Cucurbitaceae) is one of the commonly known plant widely available in Asia. Over the decades the villager's used crude leaf extracts as a traditional medicine for wound healing that possesses antioxidant and antibacterial properties to treat various skin diseases and wound healing [14,15]. Cheirmadurai et al.have done a hybrid collagen with *Delonix regia* seed as the wound dressing. However, some researchers have used different materials apart from other than collagen as wound dressing [16]. In this context, drug loaded bi-layered sponge consisting of hyaluronic acid and chitosan [17], alginate fibers with silver nanoparticles [18], mixed charge copolymers [19], silk sponge [20] and gellan gum hyaluronic acid sponge-like hydrogels [21] were used as the wound dressing material. The novel collagen originated from the marine fish source as a durable 3D scaffold. Moreover, the obtained collagen was unlikely to be free from certain diseases as bovine spongiform and foot mouth disease occurred in the use of collagen from land animals. Nevertheless, the bioactive extract from the *Coccinia grandis* (CPE) not only act against bacterial infection but also enhances the stability and porosity of the 3D scaffold for wound healing application [22–24].

The aim of the present study was to evaluate the feasibility of the *Arothron stellatus* 3D collagen sponge (COL-SPG), and COL-SPG impregnated separately with antibacterial drug ciprofloxacin, and bioactive CPE extract was prepared and assessed for their physio-chemical and biological properties. The *in vitro* biocompatibility and *in vivo* wound healing studies were performed for the developed dressing material.

2. Materials and methods

The *Arothron stellatus* fish was collected from the deep sea in the Bay of Bengal near Nagapattinam, Tamil Nadu, India. The leaves of *Coccinia grandis* (Cucurbitaceae) were collected from Thiruvarur District, Tamilnadu, India. Tris-HCl, Tris buffer, Glycine, Dulbecco's modified Eagle's medium (DMEM), Fetal bovine serum (FBS), and supplementary antibiotics for tissue culture were purchased from Sigma-Aldrich, India. 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 Coccinia grandis plant extracts (CPE)

Healthy and fresh leaves of *Coccinia grandis* were shade dried and finely powdered. Ten grams of the finely powdered leaf was subjected into a Soxhlet apparatus to get the aqueous extract. The aqueous extract was concentrated and stored at 4 °C until further use.

2.2. Fabrication of 3D collagen sponge incorporated with Coccinia grandis plant extracts (CPE)

The highly interconnected 3D scaffold from *Arothron stellatus* skin collagen was fabricated by the procedure reported in our previous study [22]. Briefly, 25 mL of 2% w/v collagen solution in 0.1 M acetic acid was prepared and uniformly mixed using the Homogenizer (Ultra Turrax T-50, IKA Werke, Germany) at 24,000 rpm for 10 min at 4 °C. Subsequently, the viscous solution was poured into a Teflon plate and step by step freezing from 4 °C, −20 °C and −80 °C at 1, 8 and 24 h respectively were carried out. Further, the frozen sample was lyophilized at to get the COL-SPG sponge. The COL-SPG-CPE scaffold was fabricated by adding 0.5 mg/mL of CPE in the 25 mL of collagen solution, and rest of the procedure was followed as mentioned earlier. Similarly, the Ciprofloxacin (D) loaded scaffold was fabricated (COL-SPG-D). All the preparative procedures were conducted at 4 °C in the cold room [23]. The fabricated COL-SPG, COL-SPG-D and COL-SPG-CPE spongy scaffolds were ethylene oxide sterilized and stored at room temperature until further use.

2.3. Characterization

2.3.1. Fourier transform infrared spectra (FTIR)

FTIR analysis was carried out to identify the formation and changes in the functional groups of prepared 3D sponge. The spectral measurements were measured at a resolution of 4 cm^{-1} in the frequency range of 4000–500 cm^{-1} using ABB 3000 FTIR spectrometer [25].

2.3.2. Scanning electron microscope (SEM)

The morphology of prepared samples (COL-SPG-D and COL-SPG-CPE) was analyzed by Scanning Electron Microscopy (SEM) (JEOL JSM-6460 LV and F E I Quanta FEG 200 - HRSEM). The samples were coated with gold to enhance the surface conductivity before scanning [11,25].

2.4. Swelling study

The composite scaffold was cut into a square piece (1 × 1 cm) and immersed into phosphate buffer solution (PBS, pH 7.4) at room temperature 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_o and W_e are the initial and the final weight of the film, respectively [24].

2.5. Porosity

The porosity of the prepared samples was achieved by the liquid displacement method. Ethanol was used as a displacement liquid because of its easy penetration through the pores of the samples [11]. A known weight (W) of the sample was immersed in known volume (V1) of ethanol. A series of brief evacuation and repressurization cycles were conducted and repeated until the air bubbles stopped forming. The total volume of ethanol and ethanol impregnated samples was then recorded as V2. The samples impregnated in ethanol were removed from the cylinder was recorded as V3. The percentage of porosity was obtained using:

$$\text{Porosity}\ (\%) = (V1 - V3/V2 - V3) \times 100 \quad (2)$$

2.6. In vitro enzymatic degradation

Biodegradation *in vitro* was analyzed by monitoring the weight loss of the samples when exposed to collagenase. A known weight of each sample (COL-SPG, COL-SPG-D, COL-SPG-CPE) in triplicate was taken and they were dried overnight. Breifly, 100 units per mL of collagenase was added to all the test samples and incubated for 24 h at 37 °C. The change of degradation in each sample was examined. The extent of biomaterial degradation was determined gravimetrically through the weight loss [26].