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Dual-layered 3D nanofibrous matrix incorporated with dual drugs and their synergetic effect on accelerating wound healing through growth factor regulation

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ABSTRACT

The by-product of the slaughter house was utilized for the development of promising regenerative wound dressing material. Currently, dual-layered nanofibrous spongy scaffold was fabricated for tissue engineering applications. Herein, Keratin (K)-Fibrin (F)-Gelatin (G) 3D sponge loaded with Mupirocin (M) was fabricated with the naturally derived materials from bovine origin using freeze drying method. Moreover, poly(3-hydroxybutyric acid) (P) and Gelatin(G) solution loaded with Curcumin (C) were electrospun to get the dual drug loaded dual-layered nanofibrous spongy 3D scaffold (KFG:M-PG:C). The fabricated biomaterial was assisted with physical, biological and mechanical property. The *in vitro* cell viability and fluorescence staining of NIH 3T3 and HaCaT cells assist in cell adhesion and proliferation of the dual-layered scaffold. Moreover, *in vivo* assessment using silicone splint animal model was employed. The nanofibrous surface aids in the migration of fibroblast for the increased the collagen deposition and granulation tissue formation. Nonetheless, the 3D spongy surface promotes the gaseous exchange and absorption of exudates. The fabricated KFG:M-PG:C scaffold has the ability to produce perusable material that can integrate with the host tissue. Overall, the three dimensional (3D) dual-layered nanofibrous spongy scaffold with synergistic effect of dual drugs prevents from infection and facilitates as highly durable substrate in tissue engineering application.

1. Introduction

In recent year, several proteins have played important role in the wound healing with the naturally derived biomaterials. The development of biomaterial from collagen, gelatin, fibrin, silk and cellulose have been reported widely [1]. However, only few reports were being given on the utilization of keratin from the bovine horn as the major slaughter house waste. The increase in the food industry has rendered millions of tons of keratin biomass for the meat and slaughter house market. The forecast of worldwide meat production during 2015 was estimated with 58.3 million tons per year from leading exporters in United States, India, Brazil and China [2–4]. Accordingly, keratin based material have exhibited a promising biocompatibility, biodegradability, and mechanical stability [5].

Keratin (K) was insoluble protein associated with intermediate filaments and epidermal appendgeal structures such as hairs, nails, horns, hoofs, and feathers. Most notably, the higher cysteine content residues and the cell binding motifs play significant role in the physical and biological property of the biomaterial [6–8]. The physiologically clotted Fibrin (F) from the bovine blood is one of the good haemostatic agent aids in tissue rebuilding, absorption of exudates and act as the one of the key component in biomaterial development [9,10]. Sastry et al., has reported that physiologically clotted fibrin from the slaughter house waste has been utilized for developing various wound dressing and biomedical products [11]. Gelatin (G), denatured collagen from the bovine origin supports the formation of highly porous spongy matrix for cell adhesion and proliferation in tissue engineering application [12]. The hydrophobic polymer poly(3-hydroxybutyric acid) (P) is highly biocompatible nontoxic since it is common metabolite of the higher living organism. Moreover, it exhibits to have potential durability to support the cell proliferation and adhesion of both fibroblast and keratinocytes with better biodegradability [13,14].

The electrospinning is simple and versatile process for the fabrication a nanofiber matrix with high surface to volume ratio and unique morphological feature. The nanofiber matrix enhances the cell adhesion, differentiation and proliferation. The use individual polymer will not facilitate in smart biomaterial development [15]. However, the system with both natural and synthetic polymers blended into a spongy porous and nanofiber matrix with use of phase separation and electrospinning technique respectively [16]. The antibiotic mupirocin acts against pathogens, thereby reducing the infection at the wound site [17]. The Curcumin is herbal medicine with antibiotic property, which supports cutaneous wound healing by formation of granulation

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tissue and deposition of collagen initiates the fibroblast and epithelial cell formation, tissue remodelling, granulation tissue formation and collagen deposition. The antibiotic and biological property of the drugs responsible for prevention of infection at wound site and faster wound healing process [18,19].

The skin wound healing, needs a durable substrate for the tissue repair and damage with use of the smart biomaterial for the reconstruction of skin tissue formation. Biomaterial act as a matrix or scaffold emerges as the predominant in restore and repair of the damaged tissue with tissue engineering approach [20]. The tissue engineering is an interdisciplinary scientific area which improves in the development of dual-layered scaffold for the tissue damage and function for enhanced wound healing [21]. The function of the extracellular matrix was enhanced by the development of the nanofibers based biomaterial to assist in cell-cell and cell-matrix interactions in the wound healing process [22]. Such material with well-defined three dimensional (3D) spongy matrix and the nanofibrous matrix supports the cellular adhesion and proliferation, deposition of collagen, gaseous exchange, and exudates absorption during wound healing process [23]. The tissue repair or wound enters into a series of overlapping events, such as haemostasis, inflammation, proliferation and remodelling [24]. The skin injury with damaged tissue was suddenly sealed by the platelets and enters into the haemostasis phase. Here, the clotting of blood with fibrin network formation cause to stop bleeding of blood [25]. Simultaneously, macrophages arrive at the wound, which enters inflammation phase with multiple processes such as digestion of cell debris, release of various growth factors like endothelial growth factor (VEGF), epidermal growth factor (EGF), transforming growth factor (TGF-β), and cytokine supports the stimulation of angiogenesis, cell signalling, cell migration, cell differentiation and endothelial cells [26]. During proliferation phase, the formation of granulation tissue and deposition of collagen takes place with the migration of fibroblast. Finally, strengthening of connective tissue and reepithelialization involves during the remodelling phase [27]. Moreover, the skin substitute with highly active antimicrobial substance has to be loaded in the scaffold for preventing infection at the wound site [21]. Further, the spongy matrix with stable nanofiber matrix to support the new tissue formation was development as the dual-layers scaffolds with dual drugs.

The aim of the present work is to develop a dual drug loaded, dual-layered nanofibrous spongy 3D matrix by electrospinning. The polymer blends of poly(3-hydroxybutyric acid) (P) and Gelatin (G) loaded with Curcumin (C) over a 3D spongy, Keratin (K)-Fibrin (F)-Gelatin (G) substrate loaded with Mupirocin (M) as a smart biomaterial in tissue engineering application. The fabricated scaffolds were evaluated *in vitro* for their physiochemical, biological and mechanical properties. Further, *in vivo* wound healing properties of the developed 3D matrix with the expression levels of VEGF, EFG and TGF-β were examined using male albino Wister rats.

2. Material and methods

Bovine horn and bovine blood was collected from the slaughter house at Perambur, Chennai, India. Gelatin, Tannic acid, Mupirocin, Curcumin, poly(3-hydroxybutyric acid), 1,1,1,3,3,3 hexafluoro-2-propanol, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), 4,6-diamino-2-phenylindol (DAPI), 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. All other chemicals were purchased from Sigma Aldrich unless specified otherwise. The NIH 3T3 fibroblast and Human (HaCaT) keratinocytes cell lines was obtained from the National Centre for Cell Science (NCCS), Pune, India. All experiments were performed in compliance with Committee for the Purpose of Control and Supervision of Experiments on Animals guidelines (CPCSEA), and performed after the approval from the Institutional Animal Care and Use Committee (IACUC) [466/01a/CPCSEA].

2.1. Fabrication of keratin-fibrin-gelatin 3D sponge

The bovine horn keratin (K) and fibrin (F) was extracted using the methods as reported in our previous work [28]. The gelatin form the base material for the fabrication of highly porous 3D spongy matrix Thus KFG sponge was fabricated with, 2 wt% concentration of horn Keratin (K), Fibrin (F), and Gelatin (G) were blended at 1:1:3 ratios by dissolving them uniformly in water using Homogenizer (Ultra Turrax T-50, IKA Werke, Germany) at 24,000 rpm for 20 min at 4 °C. An appropriate volume of 10 wt% aqueous solution of tannic acid was added to the mixture and stirred for a given time at room temperature. Further, the well homogenized solution was poured into a Teflon template (measuring 5 cm × 10 cm) and pre-frozen step by step from −20 °C to −80 °C for 24 h. Subsequently, the frozen samples were lyophilized to get KFG Sponge. By adding 50 mg of Mupirocin (M) drug to 100 mL of KFG solution and the rest of the procedure was followed as mentioned above to fabricate the KFG:M scaffold [28].

2.2. Electrospinning of dual-layered nanofibrous spongy 3D matrix scaffold (KFG-PG)

The schematic representation of the fabrication of the dual-layered scaffold was depicted in Fig. 1 To fabricate dual-layered nanofibrous spongy KFG-PG 3D matrix scaffold, 4% (w/v) concentration of polymer solution were prepared by dissolving 0.4 g of P and G in 10 mL of 1, 1, 1,3,3,3 hexafluoro-2-propanaol for 12 h with constant stirring. The blended solution of PG in 1:1 ratio was allowed at constant stirring for 8 h to obtain uniform blending. The well blended solution was electrospun over the prepared KFG sponge placed over the grounded rectangle aluminium substrate, placed at a distance of 10 cm perpendicular to the 24 G needle connected to positive terminal of the high voltage DC power supply (ZEONICS, Bangalore, India). The polymer solution was extruded with 1.5 mL/h using a controlled syringe pump subjected to an electric potential of 1.5 kV/cm. Thus KFG-PG dual-layered scaffold was fabricated by electrospinning. Curcumim (C) was loaded into the nanofibrous scaffold by adding 50 mg of drug 10 mL of PG solution and stirred for 4 h to get a uniform mixing of both polymer and the drug and rest of the procedure was followed as mentioned above. The sterilization of the dual layered nanofibrous matrix scaffolds was performed using ethylene oxide. Further the prepared scaffolds were stored at room temperature until further use [29].

2.3. Characterization techniques

The surface morphology of the scaffolds were visualized using FE-SEM, (AURIGA, Carl Zeiss AG, Jena, Germany) operating at an accelerating voltage of 5–20 KV after being coated with gold. Fifty different fibres were measured using the UTHSCA Image tool software to determine the average diameter of the fibres for different concentrations of the polymer solution [29,30]. The chemical and conformational changes of the dual-layered nanofibrous scaffolds were investigated using Fourier transform infrared (FTIR) spectroscopy measurements, to determine the functional groups present in the prepared KFG-PG and KFG:M-PG:C nanofibrous scaffolds. The spectra were measured at a resolution of 4 cm^{-1} in the frequency range of 4000–600 cm^{-1} using ABB 3000 spectrometer with Grams as the operating software [29,30].

2.4. Tensile strength measurement of the nanofibrous scaffolds

All the scaffolds were cut into dumb-bell shaped specimens (100 × 16 mm), prepared, and load-elongation measurement was measured using a universal testing machine (INSTRON model 1405) according to Vogel at an extension rate of 5 mm/min [29].