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Gelatin/Carboxymethyl chitosan based scaffolds for dermal tissue engineering applications

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ABSTRACT

The present study delineates the preparation, characterization and application of gelatin-carboxymethyl chitosan scaffolds for dermal tissue engineering. The effect of carboxymethyl chitosan and gelatin ratio was evaluated for variations in their physico-chemical-biological characteristics and drug release kinetics. The scaffolds were prepared by freeze drying method and characterized by SEM and FTIR. The study revealed that the scaffolds were highly porous with pore size ranging between 90 and 170 μm, had high water uptake (400–1100%) and water retention capacity (>300%). The collagenase mediated degradation of the scaffolds was dependent on the amount of gelatin present in the formulation. A slight yet significant variation in their biological characteristics was also observed. All the formulations supported adhesion, spreading, growth and proliferation of 3T3 mouse fibroblasts. The cells seeded on the scaffolds also demonstrated expression of collagen type I, HIF1α and VEGF, providing a clue regarding their growth and proliferation along with potential to support angiogenesis during wound healing. In addition, the scaffolds showed sustained ampicillin and bovine serum albumin release, confirming their suitability as a therapeutic delivery vehicle during wound healing. All together, the results suggest that gelatin-carboxymethyl chitosan based scaffolds could be a suitable matrix for dermal tissue engineering applications.

1. Introduction

Wound healing is a multi-factorial physiological event that involves the interaction and synchronization among different cells and tissues [1,2]. A number of solutions have already been recommended for the cure of wounds, but still there exist an urge for the development of more effective treatment strategies due to various limitations posed by the existing methodologies. Traditional approaches including allografts, autografts, and xenografts are still considered better due to their potential to support efficient and faster healing [3–5]. However, these grafting procedures are associated with a number of limitations including immune rejection of grafts, probability of transfer of infectious agents to the host and laborious surgical procedures [4,5]. In this regard, aspects of tissue engineering had provided an upper hand. The prime goal for the researchers is to regenerate skin with restoration of complete structural and functional properties of the wounded area. The current engineered skin substitutes rely on creating three dimensional scaffolds to mimic their native extracellular matrix [5]. This matrix allows them to guide the dermal fibroblasts and keratinocytes for adhesion, growth, proliferation and differentiation to form structurally and functionally defined skin tissue [6]. These scaffolds also provide a physical barrier against the external environment and prevent any chances of infection.

In recent years, a number of natural biopolymers including alginate, collagen and chitosan have been studied extensively for their potential to support wound healing process [7–9]. These biopolymers are preferred due to their biocompatibility, biodegradability and few structural similarities with the human tissues [10]. Gelatin and chitosan have also been used extensively for vari-

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ous tissue engineering applications. Gelatin is a denatured protein derived from the triple helix of collagen. It is a biodegradable and non-antigenic polymer, which provide hemostasis and facilitates cell adhesion and proliferation during healing process. However, poor mechanical properties, low elasticity, low shape stability, low thermal stability limit its use [11]. These disadvantages can be overcome either by crosslinking or combining it with other biopolymers [12]. In this regard, chitosan has been considered as a better choice by number of investigators due to its versatility. Chitosan (poly-1,4-D-glucosamine) is a polysaccharide biopolymer derived from chitin by alkaline deacetylation [13,14]. Although, it is a functionally versatile polymer; yet has various limitations including insolubility at neutral pH, slower and uncontrollable rate of degradation [12,15]. Thus, various derivatives of chitosan have been introduced in the market such as carboxymethyl chitosan, chitosan esters, N-trimethylene chloride chitosan *etc*., which have better solubility at neutral pH and improved degradability [15,16].

In previous studies, Mishra et al. had shown application of carboxymethyl chitosan, gelatin and nano-hydroxyapatite based injectable gel for bone tissue engineering application [12]. Zhou et al. have demonstrated the synthesis and characterization of silver nanoparticles, gelatin and carboxymethyl chitosan hydrogel based antibacterial hydrogels [17]. In addition, Huang et al. demonstrated the influence of carboxymethyl-chitosan and gelatin based hydrogel on cutaneous wound healing [18]. The report by Huang et al. was majorly concentrated on the biological characterization of carboxymethyl-chitosan and gelatin based hydrogels. However, physico-chemical characterization and pharmaceutical evaluation of this formulation were found missing.

Keeping the above perspective in mind, we report the preparation, characterization and application of gelatin (G)—carboxymethyl chitosan (C) based freeze-dried scaffolds for dermal tissue engineering. The scaffolds were subjected to physico-chemical characterization (SEM and FTIR). Suitability of the prepared scaffolds for dermal tissue engineering was analyzed using 3T3 mouse fibroblast cells. In addition, the scaffolds were also analyzed for their potential as a drug delivery vehicle.

2. Materials and methods

2.1. Materials

Gelatin (bloom number: ~300, average molecular weight: 50000–100000 Da), chitosan (medium molecular weight, 200–800cP viscosity and 75–85% degree of deacetylation) and glutaraldehyde (25% aqueous solution) were bought from Sigma Aldrich, Mumbai, India. Dulbecco's Modified Eagle's Media (DMEM), fetal bovine serum (FBS) and Calcien-AM were brought from Invitrogen, Mumbai, India. Trypsin-EDTA, antibiotic-antimycotic solution and ampicillin were purchased from Himedia, Mumbai, India. NIH 3T3 mouse embryonic fibroblast cell line was procured from NCCS, Pune, India.

2.2. Methods

2.2.1. Preparation of GC scaffolds

The *N*,*O*-carboxymethyl chitosan was prepared following the protocol previously described by Mishra et al.[12]. The GC scaffolds were prepared by freeze drying technique as per protocol described by Banerjee et al.[19]. In brief, gelatin and carboxymethyl chitosan were dissolved in milliQ water at a concentration of 10% (w/v) and 2% (w/v) respectively. Thereafter, both the solutions were mixed in a definite ratio (w/w) that include 3:1 (GC31), 1:1 (GC11) and 1:3 (GC13). The solutions were crosslinked by glutaraldehyde with a final concentration of 0.2% in the formulation (40 μl glutaraldehyde stock (25%) per 5 ml of the formulation), mixed uniformly and casted into a 20 mm diameter dish. The gels were allowed to crosslink for 1 h and thereafter, placed in −20 °C overnight prior to lyophilization for 12 h (Lyodel Freeze Dryer).

2.2.2. Physico-chemical characterization of the GC scaffolds

Morphological characterization of the lyophilized GC scaffolds (GC11, GC13 and GC31) was done by scanning electron microscopy (JOEL JSM-5800) at 20 kV post gold sputter coating. The porosity of the scaffolds was measured by liquid displacement method following the protocol described by Han et al. [6]. In brief, the scaffold was immersed in a known volume (V1) of absolute ethanol for 15 min. The total volume of absolute ethanol and the scaffold was recorded (V2). Thereafter, ethanol impregnated scaffold was removed and the residual volume of ethanol was recorded (V3). The total volume of the scaffold was calculated by equation:

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

The FTIR spectra of the GC scaffolds in the scanning range of 4000–400 cm^{-1} were obtained using FTIR spectrophotometer (NEXUS-870) by KBr pellet method.

The tensile strength of the GC membranes was tested using a Stable Microsystems (TA-HD plus, U.K.) tensile testing machine at the tension test mode. The pre- and post-test speed was kept at 1 mm/min and break sensitivity was adjusted to 10 g.

2.2.3. Equilibrium swelling and water retention analysis

The equilibrium swelling of the GC scaffolds was determined as per the protocol described by Pasparakis et al. [20]. In brief, accurately weighed, GC scaffolds (W1, 100 mg) were immersed in phosphate buffer saline (PBS; pH 7.4). At defined time intervals, the scaffolds were withdrawn, blotted to remove excess fluid and weighed (W2). The increase in the scaffold weight was measured as a function of time. Equilibrium swelling degree (ESD) was expressed as:

$$\text{ESD} = (W2 - W1)/W1$$

For water retention analysis, the accurately weighed samples (W3, 100 mg) were immersed in PBS (pH 7.4) at 37 °C for 24 h. The wet samples were taken and placed in an eppendorf tube containing a small piece of filter paper at the bottom. After centrifugation (Eppendorf Mini Spin Centrifuge, 500 rpm, 3 min), the weight of the samples was recorded (W4). The percentage water retention (%WR) was calculated as:

$$\%\text{WR} = (W4 - W3) - 100/W3$$

2.2.4. Biodegradation analysis

The biodegradation analysis of the GC scaffolds was carried out in collagenase type I, lysozyme and bacterial enzyme cocktail [6]. In brief, 100 mg of the dried scaffold was placed in PBS (pH 7.4) and allowed to reach swelling equilibrium (W5). Thereafter, the three enzymes were added to the solution at a concentration of 0.1% (v/v) in individual sets. At defined time intervals, the scaffolds were withdrawn, blotted and weighed (W6). The change in their weight was calculated as a function of time. The percentage degradation of scaffolds was expressed as:

$$\text{Degradation}\% = (W5 - W6) \times 100/W5$$

2.2.5. Biological characterization of the GC scaffolds

The biological compatibility of the GC scaffolds was evaluated using NIH 3T3 mouse embryonic fibroblasts. The cell line was maintained in DMEM supplemented with 10% heat inactivated FBS in a