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Fish collagen/alginate/chitooligosaccharides integrated scaffold for skin tissue regeneration application

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ABSTRACT

An emerging paradigm in wound healing techniques is that a tissue-engineered skin substitute offers an alternative approach to create functional skin tissue. Here we developed a fish collagen/alginate (FCA) sponge scaffold that was functionalized by different molecular weights of chitooligosaccharides (COSs) with the use of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride as a cross-linking agent. The effects of cross-linking were analyzed by Fourier transform infrared spectroscopy. The results indicate that the homogeneous materials blending and cross-linking intensity were dependent on the molecular weights of COSs. The highly interconnected porous architecture with 160–260 μm pore size and over 90% porosity and COS's MW driven swelling and retention capacity, tensile property and *in vitro* biodegradation behavior guaranteed the FCA/COS scaffolds for skin tissue engineering application. Further improvement of these properties enhanced the cytocompatibility of all the scaffolds, especially the scaffolds containing COSs with MW in the range of 1–3 kDa (FCA/COS1) showed the best cytocompatibility. These physicochemical, mechanical, and biological properties suggest that the FCA/COS1 scaffold is a superior candidate that can be used for skin tissue regeneration.

1. Introduction

Skin is frequently subjected to various injuries by physical and chemical means and is wounded, and initiated well-designed series of complicated events to reestablish its integrity and functions. Particularly in many acute and chronic conditions such as partial, full or deep-thickness wound conditions, the naturally initiated sequence of event fails to regenerate the skin tissue and reestablish the functions [1,2]. Skin grafting remains as a conventional gold standard method to treat those of severe wound conditions. Extensive limitations accompanied by grafting include donor site shortage, scarring at donor site, pain and risk of infection make the grafting complicated. Therefore, tissue engineering is a promising approach that received greater attention to restore wounded skin tissue [3]. However, synthesizing a biomimetic three-dimensional (3D) scaffold is still a challenging task in tissue engineering since it necessitates biomaterials that promise biological and mechanical properties which facilitate successful restoration of skin and its functions. Until today, numerous natural and synthetic biomaterials have been tested for the desired biological and mechanical

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properties for skin tissue regeneration. Consequently, marine-derived biomaterials have received a great attention due to their promising and exceptional characteristics in biomedical applications [4].

Collagen is a major extracellular matrix (ECM) structural protein in mammalian tissue, and due to its exceptional biological and functional properties, it has been used extensively in various tissue engineering applications [5–8]. However, the use of conventional collagens such as bovine and porcine collagen in biomedical application is problematic due to the risk of disease transmission. Therefore, fish collagen (FC) has become an excellent alternative for tissue engineering application as it exhibited more similar characteristics to those conventional collagens and no risk in disease transmission to human. *Paralichthys olivaceus* skin is also an excellent alternative source to extract collagen due to its high availability as a byproduct in the Korean seafood industry. However, characteristic low mechanical integrity and fast degradation of pure collagens in scaffolds had led them to be modified with chemical cross-links and combined with different bioactive polymers or compounds [9–12]. Therefore, we designed to fabricate tissue engineered skin substitutes *via* cross-linking FC, sodium alginate (SA) and chitooligosaccharides (COSs) with the use of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) as a cross-linking agent.

SA is a marine brown algae derived biopolymer that demonstrates a success in coupling with different biopolymers in order to obtain modified tissue engineered substitutes [13–15]. The addition of SA is a straightforward and effective means of fabricating mechanically upgraded porous scaffolds that emphasized it to be used as a matrix for the cellular growth in skin tissue regeneration [16–18]. The improvement in the mechanical properties of engineered substitutes modified by SA, such as toughness and stiffness of scaffolds, is made possible by the unique structure of the alginate molecules [19]. Moreover due to its ensuring biological induction in wound healing process, particularly in certain alginate containing wound dressing (eg-Kaltostat®) is able to promote the healing process in cellular level *via* stimulating the macrophages resulting the level of inflammatory cytokines including tumor necrosis factor and α-interleukin-6 elevation [18,20]. As a result of the promising aspects of collagen and SA in the application of tissue engineering, different combinations of collagen and alginate with different cross-linking treatments have been performed to fabricate biomimetic scaffolds [21–23].

EDC is a widely used zero-length, non-toxic cross-linker that conjugates carboxylates (COOH) to primary amines (NH_2) without remaining extra linking molecules. It is extensively applied to cross-link collagen, alginate or other free NH_2- and COOH-containing molecules [22,24–26]. COSs are also NH_2-containing promising biomaterials obtained from chitosan, that can be cross-linked with collagen and alginate by the use of carbodiimide reaction [27,28]. In addition, due to excellent characteristic biodegradability, biocompatibility, non-toxic, and non-allergenic properties, chitosan and COSs have been regarded as promising biomaterials for tissue engineering applications [29,30]. In skin tissue engineering, the application of chitosan has obtained a much prominent place [31] while the application of COSs is still under investigation. However, the anti-bacterial, anti-oxidant, anti-inflammatory, anti-tumor, anti-asthma properties of COSs [32] imply that COSs are a promising substitute for fabricating tissue engineered skin substitutes. Therefore, the intention of the present study is to elucidate the cooperative aspects of marine-derived FC, SA, and different molecular weight (MW) ranges of COSs (1–3 kDa, 3–5 kDa, 5–10 kDa, >10 kDa) in scaffolds, synthesized by freeze drying and EDC involved two step cross-linking treatment (Fig. 1) for skin tissue engineering application. The eligibility of the scaffolds for application of skin tissue regeneration was demonstrated through physicochemical, microstructural, mechanical and biological properties. The biological properties are described in means of normal human dermal fibroblasts-neonatal (NHDF-neo) cells behavior on the scaffolds. This study also aims to identify the effects of COSs in tissue engineered substitute and to identify the ideal COS fraction containing FCA scaffolds for the application of skin tissue regeneration.

2. Materials and methods

2.1. Materials

Collagen was extracted by the flatfish (*P. olivaceus*) collected from South Korean seas. Type I collagen from porcine skin was purchase from Matrixen™, Republic of Korea. High MW marker was obtained from Fermentas, (St. Leon-Rot, Germany). SA obtained from Sigma-Aldrich, USA was dissolved to obtain 3% (w/v) solution to be used in scaffolds fabrication. Four molecular range of COSs were purchased from Kittolife, Republic of Korea. EDC, *N*-hydroxysuccinimide (NHS), 2-morpholinoethane sulfonic acid (MES), fluorescein diacetate (FDA), and collagenase type I (125 CDU/mg), glutaraldehyde and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (MTT) were purchased from Sigma-Aldrich, USA. Dulbecco's minimum Eagle's medium (DMEM), Fetal bovine serum (FBS), trypsin (250 U/mg), penicillin/streptomycin and other materials used in cell culture experiment were purchased from GIBCO™, Invitrogen Corporation, USA. All other chemicals and solvents were of analytical grade, and water used in experiment was deionized.

2.2. Preparation of fish skin and isolation of collagen

P. olivaceus fish were collected from South Korean sea and the skins were mechanically detached form the fish. The skins were descaled by sharp knife and desalted by washing with cold water at 4 °C for one day and cut into small pieces. Acid-soluble collagen (ASC) was extracted from the prepared skin following the method described by Singh [33] with slight modifications. All steps of the procedure were carried out at 4 °C with gentle stirring. Non-collagenous proteins were removed with 0.1 M NaOH at a solid to solution ratio of 1:10 (w/v) for 3 days. The skins were then washed with dH_2O until the washed water become neutral or faintly basic pH. The skins were defatted with 10% butyl alcohol with a solid to solution ratio of 1:10 (w/v) for two days with every 12 h of changing new solution and thoroughly washed with dH_2O. Then skins were suspended in 0.5 M acetic acid with a solid to solution ratio of 1:10 (w/v) for 3 days and the suspension was centrifuged at 16,000 × *g* for 30 min at 4 °C. The supernatant was salted out by adding NaCl to obtain final concentration of 0.9 M. The resultant precipitate was collected by centrifugation at 16,000 × *g* for 1 h and then dissolved in 0.5 M acetic acid. The solution was then dialyzed against 0.1 M acetic acid for 3 days and dH_2O for 3 days. Dialysate was lyophilized and was referred as ASC.

2.3. Amino acid analysis

Amino acid compositions were analyzed using an automatic analyzer (Hitach Model 835-50, Tokyo, Japan) with a C18 column (5 μM, 4.6 × 250 nm, Watchers, MA, USA). The reaction was carried out at 38 °C, with the detection wave length at 254 nm and flow rate of 1.0 ml/min. All chemical analyses (from each tank) were carried out in triplicate.