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Rheological, biocompatibility and osteogenesis assessment of fish collagen scaffold for bone tissue engineering

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ABSTRACT

In the present investigation, an attempt was made to find an alternative to mammalian collagen with better osteogenesis ability. Three types of collagen scaffolds – collagen, collagen-chitosan (C—CH), and collagen-hydroxyapatite (C—HA) – were prepared from the cartilage of Blue shark and investigated for their physico-functional and mechanical properties in relation to biocompatibility and osteogenesis. C—CH scaffold was superior with pH 4.5–4.9 and viscosity 9.7–10.9 cP. Notably, addition of chitosan and HA (hydroxyapatite) improved the stiffness (11–23 MPa) and degradation rate but lowered the water binding capacity and porosity of the scaffold. Interestingly, C—CH scaffolds remained for 3 days before complete *in-vitro* biodegradation. The decreased amount of viable T-cells and higher level of FAS/APO-1 were substantiated the biocompatibility properties of prepared collagen scaffolds. Osteogenesis study revealed that the addition of CH and HA in both fish and mammalian collagen scaffolds could efficiently promote osteoblast cell formation. The ALP activity was significantly high in C—HA scaffold–treated osteoblast cells, which suggests an enhanced bone-healing process. Therefore, the present study concludes that the composite scaffolds prepared from fish collagen with higher stiffness, lower biodegradation rate, better biocompatible, and osteogenesis properties were suitable biomaterial for a bone tissue engineering application as an alternative to mammalian collagen scaffolds.

1. Introduction

Biomaterial in the form of scaffold plays a major role in bone tissue engineering, since it has a potential influence on the matrices of tissue formation. The selection of the most suitable material to prepare a better scaffold for bone tissue engineering applications is an important key step towards the creation of a tissue-engineered product [1,2]. In general, scaffolds must fulfill a few basic requirements such as high porosity, proper pore size, adequate cell adhesion properties, differentiation, and proliferation with desirable mechanical integrity to maintain the predesigned tissue structure, non-cytotoxicity, and osteoconductivity [3–5]. Therefore, the favorable attachment of target cells into the porous scaffold is the one of the major aspects in tissue engineering, which is also predetermined by the type of biomaterials used for scaffold preparation.

A growing body of research has applied scaffolds from biopolymer in tissue engineering due to its divergent cellular response towards the structural and biochemical properties [6–8]. Also, biomaterials used for scaffold preparation not only decide the physical properties such as biodegradability, mechanical stability, and biocompatibility but also control the right signals that directing the cellular response to tissue formation [8,9]. Recently, collagen has become one of the most preferred materials for artificial cellular membranes in tissue engineering applications [10]. Similarly, the biocompatibility of collagen scaffolds has also been considered as a proper candidate for tissue-engineered scaffolds. Conversely, a number of practical problems still exist, such as difficulty in controlling the *in-vivo* degradation, low efficiency of cell seeding, cytotoxicity of the breakdown products resulted from scaffold degradation, and incompatible poor mechanical properties with

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natural hard tissues [1,2,11]. To address these problems, composite collagen scaffold with other biomaterials such as chitosan, MMT (Montmorillonite), calcium, and hydroxyapatite were explored for the preparation of bone tissue engineering scaffolds.

Chitosan biomolecules and calcium acetate salts are expected to stabilize collagen-based scaffolds, as they possess unique properties to minimize biodegradation. Chitosan, being a linear polysaccharide, comprises β1- to β4-linked D-glucosamine residues. This primary monomer has a similar framework as that of glycosaminoglycans (GAGs) of the cartilage and has been found responsible for cartilage repair [12]. In addition, chitosan also activates cellular reconstruction and regeneration, stops hemorrhaging, and provides anesthetic properties. The composition and structure of HA is more or less similar to natural bone mineral, and therefore it has been deliberated as an ideal material in scaffold preparation for bone tissue engineering. Previous studies have shown that HA forms a chemical bond directly to bone when it was used as an implanted material for the treatment of bone regeneration [13]. Despite osteoconductivity and osteoinductivity activity of HA, its brittleness and poor mechanical stability limit its use for practical application. Several researchers have studied the osteogenetic effect of HA scaffolds with addition of other polymers [13,14]. It was reported that addition of chitosan and calcium acetate improved the mechanical properties of Ox skin collagen films [15].

Osteogenesis is categorized by a sequence of events induced by osteoblastic cells, which involve cell attachment and cell proliferation and followed by the expression of osteoblast phenotype. Chiu et al. [16] reported that healing of bone damage is regulated by the interaction between type II collagen and bone marrow-derived mesenchymal stem cells (BMDMSC). Further, they explained that type II collagen is critical to the BMDMSC differentiation during the early stage of embryonic bone development and fracture healing process. Subsequently, it is theorized that type II collagen is a chief regulator for osteogenesis of BMDMSC through an endochondral ossification-like process. Moreover, it has been demonstrated that type II collagen stimulates BMDMSC osteogenesis and prevents adipogenesis, stipulating a clue that type II collagen itself may play an important role in cell fate commitment during the early stage of bone marrow derived mesenchymal stem cell differentiation [17]. It was well defined that type I collagen aids osteogenic differentiation through activation of the ERK1/2 signaling pathway. Therefore, type I collagen has been widely used for bone regeneration and was combined with other biopolymers, hydroxyapatite, and/or calcium as a bone filling material [18,19]. On the other side, the effects of the above biopolymer with type-II collagen in osteogenic activity and bone regeneration have not been explicated. Therefore, the fish type-II collagen scaffold prepared with two important biomaterials chitosan and HA and their physico-mechanical, structural, biodegradation, biocompatibility, and osteogenesis properties were compared with a mammalian collagen scaffold.

2. Materials and methods

2.1. Extraction of collagen

Blue shark (*Prionace glauca*) cartilage was purchased from M/s. Yueqing Ocean Biological Health Care Product Co. Ltd. Zhejiang, China and brought to Shanghai Ocean University for the extraction of collagen. Type-II collagen (CII) from the blue shark cartilage was extracted with 0.5 M acetic acid containing 1% pepsin and was purified by gel filtration column chromatography as per the standard method [20]. Type II collagens from bovine (Xiamen Huaxuan Gelatin Co., Ltd., Fujian, China), and all other analytical grade reagents used in this study were acquired from local vendors.

2.2. Preparation of collagen scaffolds

Three types of collagen scaffolds were prepared: collagen, collagen-chitosan (C—CH) and collagen-hydroxyapatite (C—HA). Collagen scaffolds were prepared by dissolving 1% collagen (w/v) along with 0.2% glutaraldehyde (GTA) (v/v) in 10 ml-distilled water. For C—CH scaffolds, 1% chitosan (w/w) (95% degree of deacetylation) was dissolved in 0.3 M acetic acid and heated for 20 min at 45 °C to obtain a complete suspension, then added to the 1% collagen-0.2% GTA solution. For C—HA scaffolds, 1% hydroxyapatite (w/w) was added directly to the 1% collagen-0.2% GTA solution. The solution was stirred in a magnetic stirrer (2000 rpm) at 4 °C for 4 h to produce foam and was immediately injected into a plastic tube (2 cm in diameter and 5 cm long) and kept at −80 °C overnight. The tube was then lyophilized in a lyophilizer, and their physico-functional and mechanical properties were examined.

2.3. Viscosity, pH, and mechanical properties

The pH and viscosity of the collagen scaffold-forming solution was measured [21]. The compressive strength was tested for the collagen scaffold by standard ASTM D 882 methods [22] using a Universal Testing Machine (TA-XT Plus, Stable Micro Systems, SMS, UK). A collagen scaffold was cut into a cylindrical shape of size 15 mm × 10 mm and fixed on the grips of the device. The compression test was analyzed using a perpendicular plunger to the flat portion of the scaffold at a uniform stress speed of 1 mm s-1 with 5.0 g trigger force up to a maximum distance of 2 mm. The compression was determined by performing linear regression on the resulting equilibrium stress (σ)–strain (ε) plot.

$$E = \sigma/\varepsilon$$

σ denotes force per cross section area = F/A, where F is the Force, and A is the area. ε denotes the deformation of material under stress = 1-L/L0, where L0 and L represent the thickness before and after compression, respectively.

2.4. Porosity

Collagen scaffold porosity was measured by following the method of Wang et al. [23] with slight modifications. Briefly, the scaffold was immersed in the displacement liquid ethanol as it is expected to permeate the scaffolds without swelling or shrinking the matrix. Scaffolds porosity was calculated as follows:

$$\text{Porosity}(\%) = (Vb - Vl)/(Va - Vb),$$

where V*b* and Va represent the volume of ethanol before and after submersion of the scaffold, respectively. V*l* represents the volume of ethanol after the sample was removed from the liquid.

2.5. Water binding capacity

The water binding property of the scaffolds was studied according to the method of Pan et al. [24]. In brief, phosphate buffered saline (pH 7.4) was used as a suspension medium, and the scaffold was completely immersed in PBS solution for 1 h at room temperature. Then, the surface adsorbed water was removed by wiping with filter paper and was weighed (wet weight). The water binding capacity was determined using the following equation:

$$\text{Waterbindingcapacity}(\%) = (Ww - Wd)/Wd \times 100$$