Synthesis of composite gelatin-hyaluronic acid-alginate porous scaffold and evaluation for *in vitro* stem cell growth and *in vivo* tissue integration

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ARTICLE INFO

ABSTRACT

Engineering three-dimensional (3-D) porous scaffolds with precise bio-functional properties is one of the most important issues in tissue engineering. In the present study, a three-dimensional gelatin-hyaluronic acid-alginate (GHA) polymeric composite was synthesized by freeze-drying, which was followed by ionic crosslinking using $CaCl_2$, and evaluated for its suitability in bone tissue engineering applications. The obtained matrix showed high porosity (85%), an interconnected pore morphology and a rapid swelling behavior. The rheological analysis of GHA showed a viscoelastic characteristic, which suggested a high load bearing capacity without fractural deformation. The influence of the GHA matrix on cell growth and on modulating the differentiation ability of mesenchymal stem cells was evaluated by different biochemical and immunostaining assays. The monitoring of cells over a period of four weeks showed increased cellular proliferation and osteogenic differentiation without external growth factors, compared with control (supplemented with osteogenic differentiation medium). The *in vivo* matrix implantation showed higher matrix-tissue integration and cell infiltration as the duration of the implant increased. These results suggest that a porous GHA matrix with suitable mechanical integrity and tissue compatibility is a promising substrate for the osteogenic differentiation of stem cells for bone tissue engineering applications.

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1. Introduction

Bone tissue engineering has a tremendous potential with respect to transforming the clinical practice of skeletal reconstruction. It provides an opportunity to engineer bones that have been damaged by disease or trauma and to aid neo-bone generation. However, several barriers have restricted the application of bone tissue engineering in clinical practice. In particular, large bone tissue defects caused by non-healing trauma fractures, revisions of orthopedic prostheses, osteonecrosis, spine fusion or maxillofacial reconstruction do not have high success rates [1]. To manage these clinical defects, several major improvements must be incorporated in engineered bone because current therapies, such as bone grafts, have many limitations [2,3]. The repair of bone loss is a complex process that requires sequential cellular and molecular events to generate new bone tissue. Using biomaterials could also be one of the ways to aid in repairing bone loss. However, developing an ideal biomimetic scaffold is challenging task because these matrices must act as a substrate for osteoid deposition and provide temporary mechanical support, which presents an important tissue engineering challenge. Particularly for bone engineering, the successful modulation of the adherence, proliferation and differentiation of cultured cells, specifically, tailor stem cell differentiation for osteogenesis in a three-dimensional (3-D) microenvironment, is required [4]. Scaffolds play a facilitator role in the creation of new bone, and the success of scaffold implantation depends upon the 3-D environment that is provided by the scaffold for cells.

Biodegradable polymeric scaffolds offer promising opportunities for the *in vitro* engineering of bioartificial tissues, and the use of 3-D porous scaffolds fabricated from biopolymers has several advantages, such as biocompatibility, biodegradability and similarities with native tissue and its microenvironment at macroscopic and cellular levels. Due to their versatilities, several biopolymers, such as chitosan, alginate, agarose, hyaluronic acid, collagen and

gelatin (its hydrolyzed form of collagen), are used by researchers in bone tissue engineering [5–8]. Gelation at cryotropic conditions is considered a straightforward, effective means for synthesizing porous scaffolds, and several cryotropic fabrication approaches, such as freeze-thawing and cryogelation, have been extensively used for various tissue engineering applications [9–15].

Ideally, a scaffold should provide a porous architecture for cell migration, growth and proliferation, and those scaffolds used for bone tissue engineering should be able to support and promote osteo-induction (osteogenic differentiation), osteo-integration (scaffold-host tissue integration) and the vascularization of regenerated bone tissues [1,16]. Furthermore, scaffold degradation should occur in a controlled manner because controlling this process reduces the risk of toxic end product generation and facilitates load transfer to the developing bone. Accordingly, the selection of suitable components for scaffold production is critical step because the scaffold plays a vital role in cellular growth. Hyaluronic acid (HA, a glycosaminoglycan) is one of the prominent polysaccharides and is found in most vertebratal tissue. HA is considered immuno-neutral and promotes the migration and differentiation of mesenchymal and other cells [17]. HA also increases collagen deposition and angiogenesis by providing mechanical integrity [18]. Alginate is another biocompatible polysaccharide with high functionality that is being explored in several biological applications. Alginate has been shown to have a potential as a carrier for cell transplantation and as a tissue engineering scaffold. Its slow degradation rate could be used for bone tissue engineering because *in vivo* bone regeneration is a slow process. However, alginate does not provide suitable mechanical integrity for cellular growth when used as a single component in scaffold fabrication [18]. In contrast, gelatin is a protein with inherent cell adhesion properties due to the presence of Arg-Gly-Asp (RGD) sequences and has been successfully used for making porous tissue engineering scaffolds [9–12,19].

In the present study, we attempted to synthesize a novel, biocompatible, degradable 3-D polymeric composite scaffold with interconnected macroporous architecture for stem cell growth. The biopolymers alginate, hyaluronic acid and gelatin were used in different concentrations to optimize scaffold characteristics for bone tissue engineering applications. The scaffolds were characterized physico-chemically and examined *in vitro* with respect to the cellular response of the stem cell culture. Finally, an *in vivo* experiment was performed to evaluate the ability of the scaffold to integrate with host tissue for bone tissue engineering applications.

2. Materials and methods

2.1. Materials

Alginic acid (from brown algae; low viscosity ~250 cP at 25 °C), hyaluronic acid (from human umbilical cord), gelatin (from cold water fish skin, MW ~60,000), Dulbecco's modified Eagle's medium (DMEM), penicillin-streptomycin antibiotic and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Chemical (St. Louis, MO, USA). Bone marrow-derived human mesenchymal stem cells (ATCC, Manassas, VA, USA) and an alkaline phosphatase assay (colorimetric) kit were purchased from Abcam (USA). Alkaline phosphatase live stain (Molecular Probes by Life Technologies, USA) and fetal bovine serum (FBS) were purchased from Hyclone (UT, USA). StemPro® Osteogenesis Differentiation Kits, DMEM (low glucose), MSC-qualified FBS, Glutamax-I and gentamicin were purchased from Gibco (Life Technologies, USA). All other chemicals were of analytical grade and used without further purification.

Table 1
Different ratios of polymers for the synthesis of a GHA scaffold.

Gelatin	Alginate	Gel property
0.5%	0.5%	Gel not formed
1%	0.5%	Gel not formed
1.5%	0.5%	Gel formed but unstable
2%	0.5%	Stable but very weak
2.25%	1%	Soft stable
2.25%	1.5%	Soft with increased strength
2.25%	2%	Mechanically stable
2.25%	2.25%	Mechanically stable

Hyaluronic acid used for fabrication of GHA was 10%.

2.2. Methods

2.2.1. Synthesis of porous gelatin-hyaluronic acid-alginate (GHA) scaffolds

Composite scaffolds were synthesized using gelatin, hyaluronic acid and alginate. Polymer ratios and synthesis conditions were optimized (Table 1). First, different ratios of gelatin (w/v) and alginate (w/v) were added to 10 ml degassed water, dissolved by heating at 60 °C in an air-tight tube and allowed to cool at room temperature (27 ± 2 °C) before mixing hyaluronic acid (10%, w/v) with vortexing to produce a homogenous mixture of the polymers. Polymer solutions were then transferred to a mold (plastic syringe) and cooled at -20 °C for 12 h. The gels obtained were then transferred to $CaCl_2$ solution (0.5%, w/v) and allowed to crosslink for 30 min. The resulting matrices were vacuum-dried at −45 °C using a lyophilizer (Freeze dryer FD8508, Ilshin, Korea) and stored at room temperature until required.

2.3. Microstructure analysis

2.3.1. Scanning electron microscopy

Physical morphologies of GHA scaffolds were studied using scanning electron microscopy (SEM, FEI Quanta 200). Scaffolds were vacuum-dried overnight to remove moisture and then platinum-coated using a sputter coater (Hitachi E-1030). The microscope was operated under high vacuum at 15 kV with a sample spot size of 4.5 mm. Pore size ranges, distribution, pore diameters, and interconnectivity were determined using SEM-associated image analysis software.

2.3.2. Micro-computed tomography (μ-CT) analysis

GHA scaffolds were sectioned at ~12 mm and mounted on a sample holder using sample glue. The samples were analyzed under an X-ray CT scanner (SKYSCAN 1172, German) at a magnification of 50× at 30 kV using an X-ray tube current of 173 mA. Images were recorded every 0.3° of sample rotation. μCT files were further processed to choose a threshold line that avoids any background noise that might be added during the scanning. Once the threshold was chosen, the CTvol software further processed the scan files to obtain a three-dimensional image along with the calculation of the pore size, average pore diameter, pore volume, porosity, surface area, open pores and closed pores. The pore size, porosity, and pore volume were all obtained using the CTan software provided by the manufacturer. Three-dimensional reconstruction of the internal pore morphology was performed using these axial images and analyzed using these two software programs.

2.3.3. Swelling behavior

The swelling equilibrium and the uptake kinetics of GHA matrices were calculated using a conventional gravimetric method. The sample weights in the initial dry condition were recorded, and then samples were immersed in phosphate buffer saline (PBS, pH 7.4) for pre-defined time intervals (30 s). Increases in wet weight were