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3-Dimensional cell-laden nano-hydroxyapatite/protein hydrogels for bone regeneration applications

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ABSTRACT

The ability to encapsulate cells in three-dimensional (3D) protein-based hydrogels is potentially of benefit for tissue engineering and regenerative medicine. However, as a result of their poor mechanical strength, protein-based hydrogels have traditionally been considered for soft tissue engineering only. Hence, in this study we tried to render these hydrogels suitable for hard tissue regeneration, simply by incorporation of bioactive nano-hydroxyapatite (HAp) into a photocrosslinkable gelatin hydrogel. Different cell types were also encapsulated in three dimensions in the resulting composites to prepare cell-laden constructs. According to the results, HAp significantly improves the stiffness of gelatin hydrogels, while it maintains their structural integrity and swelling ratio. It was also found that while the bare hydrogel (control) was completely inert in terms of bioactivity, a homogeneous 3D mineralization occurs throughout the nanocomposites after incubation in simulated body fluid. Moreover, encapsulated cells readily elongated, proliferated, and formed a 3D interconnected network with neighboring cells in the nanocomposite, showing the suitability of the nano-HAp/protein hydrogels for cellular growth in 3D. Therefore, the hydrogel nanocomposites developed in this study may be promising candidates for preparing cell-laden tissue-like structures with enhanced stiffness and increased osteoconductivity to induce bone formation *in vivo*.

1. Introduction

Extracellular matrix (ECM), a complex mixture of proteins and sugars beyond the cell, provides mechanical support and signaling cues to the cells *in vivo* [1–3]. The success of tissue engineering as a feasible approach in regenerative medicine relies largely on our ability to generate three-dimensional (3D) scaffolds resembling that of the natural ECM [3,4].

Protein-based hydrogels with their beneficial characteristics such as high water retention ability and controllable biodegradability have been widely used for generating 3D ECM-like scaffolds [3,5]. In addition, their high permeability for transport of nutrients and metabolites make protein hydrogels as appealing candidates for cell delivery [6,7]. Among protein-based hydrogels, collagen which is the most abundant ECM protein has great potential to be used as the structural scaffolds for treatment of connective tissues including bone [1,5]. The less expensive and the most widely utilized form of collagen is gelatin which is obtained by thermal or chemical denaturation of collagen. In addition to the high biocompatibility and high swelling ratio, gelatin hydrogels also provide natural cell binding motifs, making them amenable for 3D cell encapsulation [3,4,6]. Gelatin molecules can also be treated by addition of methacrylate groups to their amine-containing side groups, allowing them to be photocrosslinkable [6,8,9]. The resulting cross-linked hydrogel is demonstrated to have superior mechanical properties with adequate biocompatibility, allowing for easy fabrication of 3D cell-laden microstructures [4,6]. However, methacrylated gelatin has not yet been adapted for bone tissue engineering, making its suitability for this purpose uncertain.

Many of the currently available hydrogels, including methacrylated gelatin, suffer from poor mechanical properties and inability to induce mineralization [5,10]. This is why hydrogels are traditionally served for soft tissue repair only. Current artificial materials used in the fabrication of orthopedic implants involve a range of various materials based on ceramics [11], metals [12], conventional polymers [13], and their combinations [14]. In contrast to the hydrogels, none of these materials can be adapted neither for fabrication of highly hydrated structure nor for the call-laden constructs. Moreover, most of these materials typically do not bear any functionality that encourages communication with their cellular environment. Therefore, it will be of great interest to develop a bioactive protein-based hydrogel with superior mechanical strength providing a stable structure for cell adhesion and bone tissue formation.

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The mechanical properties of hydrogels can be increased by a number of parameters such as density and chemistry of covalent cross-links [6,8] and chemistry, concentration, and molecular weight of the hydrogel precursors [15]. As mentioned earlier, molecules of gelatin can be cross-linked through addition of methacrylate groups followed by a simple photopolymerization; although methacrylation is an effective route to make gelatin more suitable for tissue engineering, but mechanical strength of the resulting hydrogel still remains inferior compared to the conventional polymers. In addition, it must be remembered that the use of high cross-linkers may lead to some toxic side-effects [6,16]. Therefore a method that further enhances the mechanical properties of gelatin without affecting their beneficial properties is needed. Gelatin composites based on carbon nanotubes have been shown to possess increased elastic modulus [3]; however their inability to induce mineralization makes them unsuitable for bone regeneration.

Hydroxyapatite (HAp, $Ca_{10}(PO_4)_6(OH)_2$) nanoparticles, as the major mineral constituents of vertebrate bone and tooth, are widely used in biomaterials owing to their excellent biocompatibility, affinity to biopolymers and high osteogenic potential [17,18]. It has been well documented that synthetic HAp can promote new bone ingrowth through osteoconduction mechanism without causing any local or systemic toxicity, inflammation or foreign body response [19,20]. To date, hybrids or composites of HAp with some traditional hydrogels have been developed [7,10,21,22]; however, to our knowledge, the combination of the cell-containing methacrylated gelatin with the nanosized HAp has not yet been reported. Given the increasing use of gelatin as tissue engineered scaffold, development of novel protein-based composites with the potential ability of cellular encapsulation and, at the same time, bone tissue formation may be of great benefit in bone tissue engineering. In this regard, the combination of bioactive HAp and methacrylated gelatin hydrogel leads to three distinctive advantages: (1) presence of HAp improves the mechanical properties of hydrogel and also makes the hydrogel bioactive, (2) calcification of hydrogel is expected to further enhance the mechanical strength of scaffold during implantation, and (3) gelatin hydrogel can act as a delivery vehicle of bioactive molecules and bone cells.

Here, we report a simple and cost-effective approach that uses HAp nanoparticles in order to reinforce protein-based hydrogels and make them bioactive; we then fabricate a bioactive 3D cell-laden gelatin scaffold with enhanced stiffness which may be useful for treatment of cancellous bone defects, or low load-bearing orthopedic applications. The correlation between the HAp concentration and the extent of mineralization and 3D cell spreading is also investigated. This work may be an important step toward the development of new generation of cell-laden hydrogel composites for application in bone scaffolds.

2. Experimental

For medical reasons, simple preparation techniques were used that did not involve the introduction of potentially harmful substances into the composites. The experimental steps involved in the current study, including preparation of cell suspension, preparation of HAp nanoparticles and methacrylated gelatin, and fabrication of 3D cell-laden bioactive nanocomposites are schematically illustrated in Fig. 1.

2.1. HAp preparation

The conditions for preparation of HAp nanoparticles were optimized in our earlier studies [23,24]. In brief, Ca^{2+} and HPO_4^{2-} solutions of 0.15 M concentration were prepared by dissolving appropriate amounts of $Ca(NO_3)_2 \cdot 4H_2O$ (Merck) and $(NH_4)_2HPO_4$ (Merck) in deionized water, respectively. Phosphate solution was then added into calcium solution at the rate of 15 mL/h under continuous and gentle stirring, while molar ratio of Ca/P was kept at stoichiometric amount (i.e. 1.67). During the precipitation, pH of the reaction was adjusted at 10 using NH_4OH solution (Merck). The resulting suspension was subsequently transferred to a 100 ml Teflon-lined stainless steel autoclave and aged at 90 °C for 60 h. Finally, the obtained powder was separated by centrifuging and washed by a mixture of deionized water and ethanol (volume ratio of 1:1).

The as-synthesized HAp powder was characterized using X-ray diffractometer (XRD, Model D5000, Siemens, Germany) with CuKα ($\lambda = 1.5418$ Å) radiation over the 2θ range of 10–60°. Phase composition, crystallite size, and crystallinity degree of HAp nanoparticles were then determined according to the methods described in the literature [23,25]. The morphology of particles was investigated by scanning electron microscopy (SEM, Model VEGAII, XMU, Tescan, Czech Republic). Mean particle size was then estimated using image analysis of three SEM micrographs. For this, average of particle size was calculated from measurement of 50 randomly selected individual particles. Moreover, elemental composition of nanoparticles was determined by an energy dispersive X-ray analyzer (EDX, Model QX2, Rontec) which was coupled with the SEM microscope. Simulated body fluid (SBF) which has the ionic concentrations similar to that of the human blood plasma was used to survey the bioactive behavior of powder. For this, a commonly used SBF solution of pH 7.4 was prepared according to the procedure recently described by Kokubo and Takadama [20]. The *in vitro* bioactivity of HAp sample was subsequently assessed by soaking the powder in the SBF solution for 30 days at 37 °C. The SBF solution was refreshed every three days. Finally, the powder was filtered and thoroughly washed with deionized water before analysis with SEM and EDX.

2.2. Hydrogel/HAp nanocomposites

2.2.1. Preparation of methacrylated gelatin

Methacrylated gelatin was synthesized according to an established method described in literature [6,9]. Briefly, type-A porcine skin gelatin (Sigma-Aldrich) was dissolved (10% (w/v)) in phosphate buffered saline (PBS) at 50 °C. Methacrylic anhydride (MA) (Sigma-Aldrich) was added dropwise to the gelatin solution to obtain a gelatin/MA solution of 0.8 mL MA per gram of gelatin. The reaction mixture was then left under continuous stirring at 60 °C for 2 h. Following a 5-time dilution with warm PBS (40–50 °C), the resulting solution was dialyzed against deionized water using 12–14 kDa cut-off dialysis membrane at 40–50 °C for one week while the deionized water was refreshed 2 times a day. Finally, the dialyzed solution was frozen and then lyophilized.

2.2.2. Preparation of gelatin/HAp hydrogels

First, a 0.5% (w/v) photoinitiator (PI) solution was prepared by dissolving 2-hydroxy-1-(4-(hydroxyethoxy) phenyl)-2-methyl-1-propanone (Irgacure 2959; CIBA chemical) in PBS at 70 °C. Certain amount of HAp nanoparticles was subsequently dispersed in the PI solution by 15-min sonication in a water bath sonicator. Methacrylated gelatin was then dissolved at a concentration of 5% (w/v) in the HAp dispersion at 70 °C. The resulting mixture was again sonicated in the water bath for at least 5 min. To produce hydrogel nanocomposites, 30 μL of the well-mixed solution/dispersion was pipetted onto a glass spacer consisting of two glass slides (500 μm thickness) placed on a glass coverslip; another glass slide was then quickly placed on top of the solution droplet resulting in an evenly distributed cylindrical mixture between the two glass slides. Subsequently, hydrogel nanocomposites were formed by exposing the solution/dispersion to 7.9 mW/cm^2 UV light (360–480 nm) for 10 s. Samples were incubated in PBS at room temperature for 2 h and then detached from the slide using a scalpel and kept free floating in PBS. In this study, the filler concentrations in the hydrogel nanocomposites were taken to be 0, 0.1, 0.5, and 2.5 mg/mL.

2.3. Structural characterization

Samples were investigated by optical microscopy using a Leica DM microscope (Germany) to roughly determine distribution of HAp filler