



## Brief communication

## Exogenous mineralization of cell-seeded and unseeded collagen–chitosan hydrogels using modified culture medium

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## ABSTRACT

Induced biomineralization of materials has been employed as a strategy to increase integration with host tissue, and more recently as a method to control cell function in tissue engineering. However, mineralization is typically performed in the absence of cells, since hypertonic solutions that lack the nutrients and culture components required for the maintenance of cell viability are often used. In the present study, we exposed fibroblast-seeded three-dimensional collagen–chitosan hydrogels to a defined culture medium modified to have specific concentrations of ions involved in biomineralization. The modified medium caused a significant increase in calcium deposition in collagen–chitosan gels, relative to constructs incubated in a standard medium, though serum supplementation attenuated mineral deposition. Collagen–chitosan constructs became opaque over 3 days of mineralization in modified Dulbecco's modified Eagle medium (DMEM), in contrast to translucent control gels incubated in standard DMEM. Histological staining confirmed increased levels of mineral in the treated constructs. Rheological characterization showed that both the storage and loss moduli increased significantly in mineralized materials. Mineralization of fibroblast-seeded constructs resulted in decreased cell viability and proliferation rate over 3 days of incubation in modified medium, but the cell population remained over 75% viable and regained its proliferative potential after rescue in standard culture medium. The ability to mineralize protein matrices in the presence of cells could be useful in creating mechanically stable tissue constructs, as well as to study the effects of the tissue microenvironment on cell function.

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## 1. Introduction

There is a clear need for materials and methods to improve bone healing outcomes, particularly in cases of large defects and non-unions. The natural healing response is often not adequate to obtain full repair, and in such cases strategies to augment bone regeneration can be applied. Autografts and allografts are currently used clinically, but are hampered by issues of tissue availability and consistency [1]. The generic tissue engineering approach is to combine cells, biomaterials, and growth factors in a controlled fashion to create living materials that can replace damaged tissue and/or enhance regeneration. In the case of bone tissue, a wide range of strategies have been employed, using a variety of cell types, materials, and biochemical factors [2].

One strategy for potentiating the bone healing response is to use materials that have been exogenously mineralized using defined ionic solutions. Simulated body fluid (SBF) is a solution formulated with ion concentrations similar to blood plasma, which mineralizes the surfaces and pore walls of both natural [3–7] and

synthetic [8–10] scaffolds if thermodynamic conditions are appropriate. Kokubo et al. [11] first described how soaking a biomaterial in SBF leads to the *ex vivo* formation of a bone-like apatite coating, and later studies showed that such coatings can be both osteoconductive [10] and osteoinductive [13], and can facilitate the regeneration of bone [12,14]. SBF-induced mineralization has been further examined as a method for controlling osteoconductivity [5,9,15], as well as for protein [13,16] and gene delivery [17,18]. Taken together, this body of work has shown that SBF can be a useful tool to modify biomaterials for bone tissue engineering applications.

Previous studies using SBF to modify material scaffolds have been performed in the absence of cells, since the high ionic concentrations and lack of nutrients in SBF are not conducive to the maintenance of cell growth. However, cell-seeded materials have been proposed for a number of orthopedic applications. Natural biomaterial hydrogels are of interest in such cases due to their ability to mimic the natural extracellular matrix [19] and provide tissue-specific cues to enhance cell attachment and stem cell differentiation [20]. Direct encapsulation of cells during gel formation can be used to facilitate homogenous cell distribution in hydrogels. Numerous natural polymers including collagen [3], chitosan [21], and

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composite matrices [22–25] have been employed to engineer tissues and have shown promise in bone regeneration [26]. A drawback of natural hydrogel materials is that they often lack mechanical strength and represent only the protein component of the native bone tissue. Mineralization of such matrices has been pursued as a strategy to improve their mechanical properties and more closely mimic the native matrix [6]; however, the cellular component is typically not included during the mineralization process.

In the present study, we mineralized three-dimensional (3-D) hydrogels using a modified culture medium that combined the ionic constituents of SBF with the nutrients, vitamins, and amino acids needed to maintain cell viability. The model tissue constructs consisted of fibroblast cells embedded in collagen–chitosan hydrogel matrices developed previously in our laboratory [24,26]. Fibroblasts were used as a model cell type to examine the feasibility of mineralization in the presence of cells, since this non-mineralizing cell type allowed us to isolate the effects of mineralization to the medium alone. Both unseeded and fibroblast-seeded hydrogels were exposed to mineralizing solutions that were formulated to induce biomineralization while also supporting cell growth, and the effects of such treatment on mineral content, mechanical properties, and cellular viability were determined. Our primary goal was to demonstrate that mineralization of protein-based hydrogels is possible in the presence of cells. The ability to mineralize cell-seeded protein matrices could be useful in creating mechanically stable and osteogenic tissue constructs, as well as in studying the process of biomineralization.

## 2. Materials and methods

### 2.1. Media formulations

The composition of the mineralization medium was based on previously studied simulated body fluid (SBF) formulations, with modifications to enhance both mineralization and the ability to support cell growth. The base medium was Dulbecco's modified Eagle medium (DMEM; high glucose, Invitrogen, Carlsbad, CA), which was supplemented with ionic salts. Table 1 shows the ion concentrations of relevant biological fluids and mineralizing media. The main augmentation to the modified medium formulation was a fourfold increase in calcium ( $\text{Ca}^{2+}$ ) and phosphate ( $\text{PO}_4^{3-}$ ) in order to promote biomineralization, and an increased carbonate ( $\text{HCO}_3^-$ ) level to provide buffering capacity. These modifications are further discussed in Section 3.

The modified medium (mDMEM) formulation was prepared by adding salts directly to DMEM to achieve final concentrations of 141 mM NaCl, 5.3 mM KCl, 10 mM  $\text{CaCl}_2 \cdot \text{H}_2\text{O}$ , 1.0 mM  $\text{MgCl}_2$ , 8.4 mM  $\text{NaHCO}_3$ , 0.8 mM  $\text{MgSO}_4$ , and 4.0 mM  $\text{KH}_2\text{PO}_4$ . The medium was prepared at 25 °C and titrated to a pH of 7.4. In experiments examining the effect of serum, mDMEM and control DMEM were supplemented with varying concentrations of fetal bovine serum (FBS; Invitrogen) and 1% penicillin/streptomycin (P/S; Invitrogen). As per previous protocols, mDMEM was changed every 12 h to avoid precipitation in the culture solution and DMEM

was changed every 3 days. In subsequent mineralization experiments using cells, the mineralization medium (MM) used was mDMEM supplemented with 2% FBS. The control medium (DM) was DMEM supplemented with 10% FBS.

### 2.2. Collagen–chitosan gel fabrication

Collagen–chitosan gel composites were formed through a  $\beta$ -GP induced mechanism as previously described [24]. Briefly, 4.0 mg  $\text{ml}^{-1}$  bovine Type I collagen (MP Biomedicals, Solon, OH) was dissolved in 0.02 N acetic acid (Sigma) and was mixed with 2.0 wt.% chitosan (93% DDA; Biosyntech, Quebec, Canada) dissolved in 0.1 N acetic acid at a mass ratio of 50/50 collagen/chitosan. Beta-glycerophosphate ( $\beta$ -GP) and glyoxal were added as physical and chemical cross-linkers, respectively, at concentrations of 7.0 wt.%  $\beta$ -GP and 0.5 mM glyoxal. A 400  $\mu\text{l}$  aliquot of the pre-gelled mixture was injected into a well of a 24-well plate to create a disk-shaped construct with diameter of 1.5 cm. Gelation was then initiated by incubation of the mixture at 37 °C for 30 min. Gels were washed three times in phosphate buffered saline (PBS; Invitrogen) for 10 min to remove excess  $\beta$ -GP prior to use.

### 2.3. Calcium quantification

Calcium deposition on acellular gels after 3 days of incubation in either modified medium or DMEM containing 0%, 2%, 5%, and 10% FBS was quantified using an orthocresolphthalein complexone (OCPC) method as previously described [27]. Briefly, collagen–chitosan gels were washed three times in PBS for 10 min and frozen at days 0, 1, and 3. Samples were then digested in 0.5 ml of 1.0 N acetic acid overnight. 10  $\mu\text{l}$  of the dissolved solution was then incubated at 10 min at 25 °C with 300  $\mu\text{l}$  of a working solution consisting of 0.05 mg  $\text{ml}^{-1}$  of OCPC solution and ethanolamine/boric acid/8-hydroxyquinoline buffer (Sigma). Samples were read spectrophotometrically at 575 nm. Calcium values were quantified via a standard curve prepared from 0.0 to 100  $\mu\text{g ml}^{-1}$ .

### 2.4. Gel morphology and von Kossa staining

Acellular gel morphology was examined 3 days after incubation in mineralization medium (MM = mDMEM + 2% FBS) and in control medium (DM = DMEM + 10% FBS). Gels were washed three times in PBS for 10 min each and then transferred to a 12-well plate for imaging using a standard CCD camera in manual mode with a constant exposure setting.

For von Kossa staining, acellular gels were washed three times in PBS for 10 min and then placed in zinc-buffered formalin (Anatech LTD, Battle Creek, MI) for 30 min, followed by immersion in 70% ethanol (Fisher Scientific, Pittsburgh, PA). Samples were cryosectioned into the top, middle (300  $\mu\text{m}$  from the top face), and bottom face at the Histology Core Facility at the University of Michigan Dental School. Gels were stained with von Kossa reagent, embedded in paraffin and then mounted on slides. Images were taken at 4 $\times$  magnification using an Olympus IX15 Microscope system (Olympus America, Center Valley, PA) and stitched together

**Table 1**  
Ionic composition of biological fluids and media formulations.

	Na <sup>+</sup>	K <sup>+</sup>	Ca <sup>2+</sup>	Mg <sup>2+</sup>	HCO <sub>3</sub> <sup>-</sup>	SO <sub>4</sub> <sup>2+</sup>	HPO <sub>3</sub> <sup>2-</sup>	pH
<i>Media formulation ion concentrations (mM)</i>								
Blood plasma	142	3.6–5.5	2.1–2.6	1.0	27	1.0	0.65–1.45	7.2–7.4
Simulated body fluid (SBF)	141	4.0	2.5	1.0	4.2	0.5	1.0	7.4
Dulbecco's modified Eagle's medium (DMEM)	110	5.3	1.8	0	20	0.8	0.9	7.4
Modified DMEM (mDMEM)	141	5.3	10	1.0	8.4	0.8	4.0	7.4

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