Materials Letters 214 (2018) 186–189

Contents lists available at ScienceDirect

Materials Letters

journal homepage: www.elsevier.com/locate/mlblue

# Phytase-mediated enzymatic mineralization of chitosan-enriched hydrogels

Jana Lišková [a], Timothy E.L. Douglas [b,c,g,*], Robbe Wijnants [b], Sangram Keshari Samal [d,e], Ana C. Mendes [f], Ioannis Chronakis [f], Lucie Bačáková [a], Andre G. Skirtach [b,e]

[a] Dept. Biomaterials and Tissue Engineering, Institute of Physiology of the Czech Academy of Sciences, Czech Republic
[b] Dept. Molecular Biotechology, Ghent University, Belgium
[c] Engineering Dept., Lancaster University, UK
[d] Laboratory of General Biochemistry and Physical Pharmacy, Ghent University, Belgium
[e] Centre for Nano- and Biophotonics, Ghent University, Belgium
[f] Nano-BioScience Research Group, Technical University of Denmark (DTU), Kgs. Lyngby, Denmark
[g] Materials Science Institute (MSI), Lancaster University, UK

ARTICLE INFO

*Article history:*
Received 6 April 2017
Received in revised form 10 October 2017
Accepted 3 December 2017
Available online 5 December 2017

*Keywords:*
Biomaterials
Biomimetic
Composite materials

ABSTRACT

Hydrogels mineralized with calcium phosphate (CaP) are increasingly popular bone regeneration biomaterials. Mineralization can be achieved by phosphatase enzyme incorporation and incubation in calcium glycerophosphate (CaGP). Gellan gum (GG) hydrogels containing the enzyme phytase and chitosan oligomer were mineralized in CaGP solution and characterized with human osteoblast-like MG63 cells and adipose tissue-derived stem cells (ADSC). Phytase induced CaP formation. Chitosan concentration determined mineralization extent and hydrogel mechanical reinforcement. Phytase-induced mineralization promoted MG63 adhesion and proliferation, especially in the presence of chitosan, and was non-toxic to MG63 cells (with and without chitosan). ADSC adhesion and proliferation were poor without mineralization. Chitosan did not affect ADSC osteogenic differentiation.



## 1. Introduction

Mineralization of hydrogels with calcium phosphate (CaP) is desirable for applications in bone regeneration. One strategy is incorporation of phosphatase enzymes like alkaline phosphatase (ALP) followed by hydrogel incubation in a solution containing $Ca^{2+}$ and glycerophosphate (enzyme substrate), whereupon CaP forms inside the hydrogel [1].

Other macromolecules with biological activity may be incorporated in the hydrogel [1]. Chitosans are a family of cationic polysaccharides widely used as scaffold biomaterials [2]. Here, hydrogels of the anionic polysaccharide gellan gum (GG) were enriched with the plant-derived phosphatase enzyme phytase and different concentrations of chitosan oligomer and mineralized in calcium glycerophosphate (CaGP) solution. Adhesion and proliferation of osteoblast-like MG63 cells and human adipose-tissue derived stem cells (ADSC) on the mineralized hydrogels was investigated. ADSC expression of the early osteogenic differentiation marker ALP was also studied. Cytotoxicity of mineralized hydrogels was tested by preparing eluates in cell culture medium (CCM) and evaluating viability of MG63 cells grown in these eluates and their serial dilutions.

ALP-mediated hydrogel mineralization has been studied previously [1,3,4]. However, phytase-mediated mineralization and the effect of chitosan oligomer on hydrogel mineralization and cell behavior on mineralized hydrogels are novel.

## 2. Materials and methods

All materials, including GG (G1910, "Low-Acyl", 200-300 kD), CaGP (50,043), phytase (from wheat, P1259), Dulbecco's modified Eagle's Minimum Essential Medium (DMEM, D5648), DMEM with low glucose, without phenol red (D2902) were acquired from Sigma-Aldrich, unless stated otherwise. Chitosan oligomer (Chitoceuticals, batch 212-3,00,11,501) was obtained from Heppe Medical Chitosan (HMC+, Halle, Germany).

GG hydrogel discs (0.7% GG, 0.03% $CaCl_2$ (w/v), diameter 6 or 10 mm, height 2.5 mm) were prepared as described previously [5]. Hydrogels were incubated in solutions containing 0, 0.75, 1.5 or 3% (w/v) chitosan and 9 mg/ml phytase for 2d, transferred to 0.1 M CaGP solution for mineralization for 4d and autoclaved in

* Corresponding author at: Engineering Department, Lancaster University, Gillow Avenue, Lancaster, LA1 4YW, UK.
*E-mail address:* t.douglas@lancaster.ac.uk (T.E.L. Douglas).

https://doi.org/10.1016/j.matlet.2017.12.004
0167-577X/

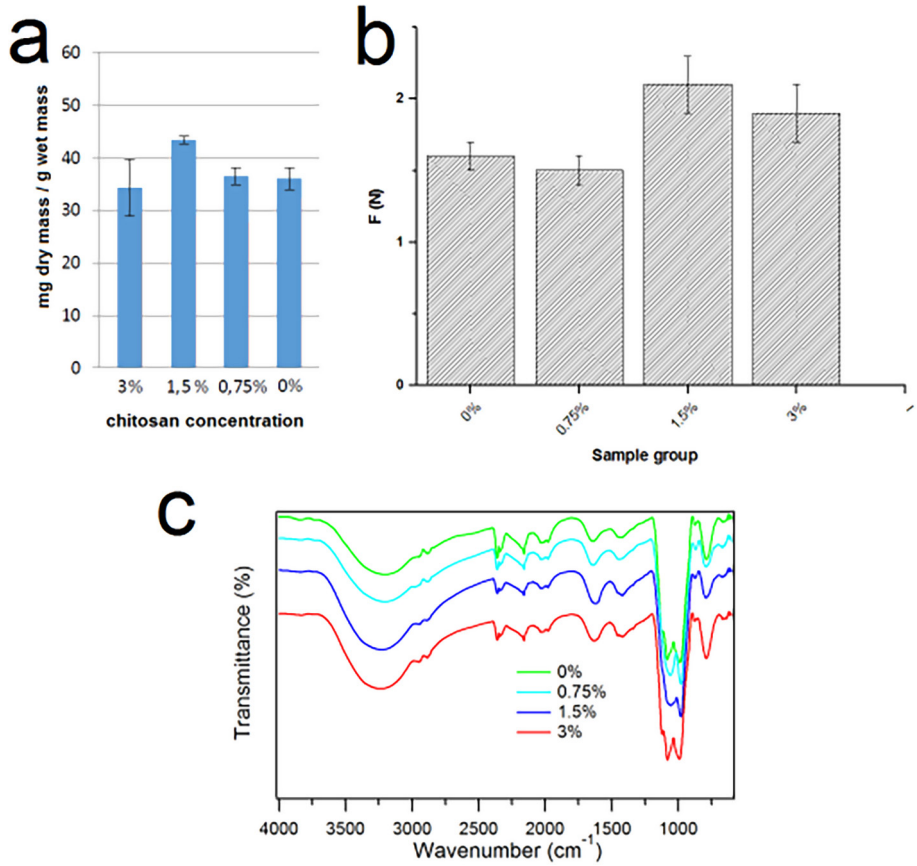


**Fig. 1.** Physicochemical characterization of gellan gum hydrogels preincubated in 9 mg/ml urease and different chitosan concentrations between 0 and 3% (w/v) and subsequently incubated in mineralization medium (0.1 M calcium glycerophosphate) for 4d and autoclaved. a a: Dry mass percentage as a measure of mineralization. b: Mechanical properties The y-axis shows the force required to compress samples by 80%. c: FTIR analysis. Error bars show standard deviation.

Milli-Q (121 °C, 15 min). Resistance to mechanical loading was performed as described before [5]. For physicochemical analysis, hydrogels were weighed, dried at 60 °C for 72 h and reweighed. Calculation of dry mass percentage, a measure of mineral formation, and FTIR were performed as described previously [6].

For initial cell adhesion and proliferation studies, hydrogels (6 mm diameter) were soaked in CCM containing DMEM, 10% fetal bovine serum (FBS, Gibco, Life Technologies) and gentamicin (40 μg/ml; LEK Pharmaceutical D. D.). for 24 h, placed in 96-well plates containing 18,000 MG63 cells (European Collection of Cell Cultures, Salisbury, UK, 86051601) in 0.2 ml CCM. Cell attachment and distribution on hydrogels were evaluated on day 7 post-seeding using fluorescence microscopy after LIVE/DEAD staining (Calcein AM/propidium iodide, Life Technologies) following the manufacturer's instructions. Fluorescence microscopy images were obtained using the inverted IX 51 epifluorescence microscope equipped with a DP 70 digital camera (all Olympus, Japan), under 10× objective.

For osteogenic differentiation experiments, human adipose-derived stem cells (ADSC) were isolated from lipoaspirates following published protocols [7]. Hydrogels (10 mm diameter) were incubated in a Mesenchymal Stem Cell Medium (MSCM, ScienCell, cat. no. 7501) for 24 h then in 24-well plates in a suspension of 1,00,000 cells in 1.5 ml MSCM. After 3d, osteogenic medium (alpha-MEM with 15% FBS, 2 mM L-glutamine, 10 mM dexamethasone, 20 mM β-glycerolphophate, 50 μM L-ascorbic acid phosphate and 40 μg/ml gentamicin) or control non-osteogenic medium (alpha-MEM medium supplemented with 15% FBS, 2 mM L-glutamine and 40 μg/ml gentamycin) was used and exchanged every 2d or 3d. Cell attachment and distribution were evaluated on day 1 and 3 using LIVE/DEAD staining and fluorescence microscopy. Metabolic and ALP activity tests were performed after 13d and 14d, respectively.

For metabolic testing, hydrogels were washed with Phosphate Buffered Saline (PBS), incubated in resazurin solution (Alamar blue, 40 μM work solution diluted in CCM without phenol red) in clean wells for 4 h in a cell culture incubator, and returned to original CCM. Resazurin fluorescence was measured at 590 nm with excitation at 530 nm. Results were normalized by the area of hydrogels and cell culture wells (polystyrene controls). Hydrogels were retained to measure ALP activity the next day. Hydrogels were washed twice with PBS in clean wells and substrate solution (Paranitrophenyl phosphate, 0.1 mg/ml in substrate buffer: 50 mM glycine, 1 mM $MgCl_2$, pH 10.5) was added for 15 mins at room temperature. Control hydrogels without cells were incubated simultaneously. Substrate solution was removed and mixed with an equal volume of 1 M NaOH solution. Absorbance (405 nm) was measured and ALP concentration calculated using a standard curve. Values produced by hydrogels without cells were subtracted. For all groups, n = 3.

For cytocompatibility testing, eluates were produced by incubating four hydrogels in 2 ml CCM at 37 °C for 48 h and diluted by factors of 1 (undiluted), 2, 4, and 8. MG63 cells (10,000/well, 96-well plate) were subsequently incubated in eluates/dilutions for 72 h. Eluate was replaced by 0.2 ml resazurin work solution and the metabolic test was followed (see above, n = 3). Viability was calculated as a percentage of control cultures incubated with CCM without eluate.

Quantitative results were presented as mean ± standard deviation. Statistical analyses were performed using SigmaStat (Jandel Corporation, San Jose, CA USA). Multiple comparison procedures