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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.

Cover Letter

06-10-2017

Dear Prof. Boccaccini,

Thank you very much for giving us the opportunity to submit a revision of our manuscript “Phytase-mediated enzymatic mineralization of chitosan-enriched hydrogels” (MLBLUE-S-17-02521) for publication in Materials Letters.

We have made the changes requested by the reviewer. These are marked in the revised text using the “Track Changes” function. We have also prepared a point-by-point response to the reviewer’s comments.

Many thanks for your attention and we look forward to hearing from you.

Yours faithfully,

Timothy E.L. Douglas

Corresponding author

Response to Reviewer Comments

Reviewer #1: The authors describes the elaboration of hydrogels of anionic polysaccharide gellan gum (GG), enriched with phytase and different concentrations of chitosan oligomer, and mineralized in calcium glycerophosphate (CaGP) solution to create hydrogel-CaP composites. Sufficiently methodological details have been provided in terms of characterization, cell adhesion and proliferation, osteogenic differentiation, metabolic activity and citocompatibility. However, in the reviewer´s opinion, an indeep discussion of the novelty of the manuscript in the context of previous literature is needed.

AUTHORS:

We thank the reviewer for the positive appraisal of our manuscript.

We agree with the reviewer that a deeper discussion of the novelty is desirable. We have stated the novelty more explicitly in a new paragraph at the end of the introduction. We have added more discussion and cited more previous literature in an extra paragraph at the end of the discussion section. We have tried to add as much discussion as possible while respecting the word limit. The methodological details of the cell experiments are necessary and take up a lot of space. We are glad that the reviewer considers the description of the methods sufficient.

Highlights

- Gellan Gum (GG) hydrogels enzymatically mineralized using phytase
- Chitosan oligomer used to enrich GG hydrogels
- MG-63 osteoblast-like cells and human adipose-derived stem cells (ADSC) used
- Mineralization promotes MG63 and ADSC adhesion and proliferation
- Effect of mineralization on MG63 cells enhanced by chitosan

Phytase-mediated enzymatic mineralization of chitosan-enriched hydrogels

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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 (50043), 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 212-30011501) 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 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 72h 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 (6mm 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 18000 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 10x objective.

For osteogenic differentiation experiments, human adipose-derived stem cells (ADSC) were isolated from lipoaspirates following published protocols [7]. Hydrogels (10mm diameter) were incubated in a Mesenchymal Stem Cell Medium (MSCM, ScienCell, cat. no. 7501) for 24h then in 24-well plates in a suspension of 100000 cells in 1.5 ml MSCM. After 3d, osteogenic medium (alpha-MEM with 15% FBS, 2mM 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 4h 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 minutes 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 (10000/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 were performed by ANOVA. Multiple comparisons were counted by the Student–Newman–Keuls method; multiple comparisons versus control were counted by the Dunnett test. $p \leq 0.05$ was considered significant.

3. Results and Discussion

Hydrogel mineralization and mechanical properties were most strongly enhanced by 1.5% chitosan (Figure 1a,1b). The exact reason for these observations remains unclear. Possibly, extra ionic crosslinking occurs between GG and chitosan. FTIR bands at approximately 1650 (H–O–H vibration) 1070, 980 (P–O stretching mode) 875 (P–O(H) stretching) and 790 (P–O–H out-of-plane bending) cm^{-1} suggest the presence of phosphate and hydrogen phosphate (Figure 1c) [8].

On day 7, MG63 cells on samples without phytase formed islets but cells on samples with phytase covered larger areas (Figure 2a-h). Furthermore, the metabolic test on day 7 showed significantly higher cell growth on samples containing phytase (Figure 2i). Eluates of all samples decreased cell viability to approx. 80%, but with increasing dilution of eluate, viability increased again in most cases (Figure 2j), suggesting that samples are non-toxic but that compounds released from the samples do not promote cell growth.

Only four sample types were chosen for osteogenic differentiation experiments with ADSC: hydrogels with or without 1.5% chitosan and with or without phytase.

On day 1 after seeding, ADSC on the hydrogels without phytase had adhered individually or in small groups. Cells on the hydrogels with phytase had adhered in larger islets (Figure 3a-d). On day 3, islets of cells were visible on all sample types (Figure 3e-h).

Hydrogels without phytase supported ADSC growth very poorly after 13d (Figure 3i). Hence, the ALP activity test was performed after 14d only on hydrogels with phytase. There was no significant difference between the hydrogels with and without chitosan (Figure 3j). Cells cultured on hydrogels (both with and without chitosan) in non-osteogenic medium displayed ALP activity, howewer less so than cells cultured in osteogenic medium.

The difference between MG63 and ADSC cells in ability to adhere and grow on the hydrogels might be caused by the different size of these two cell types. Average area of MG63 cells on PS was 1441 μm^2, average area of ASC cells was 3076 μm^2, both on day 1 after seeding (our unpublished results). The smaller MG63 cells may produce lower traction forces. This may help them to adhere and grow on soft materials such as hydrogels. Very soft surfaces can hamper cell adhesion, spreading and proliferation due to their high deformability under cell traction forces [9]. Material stiffness is also positively correlated with stem cell osteogenic differentiation [10].

ALP-mediated GG hydrogel mineralization with CaP promoted adhesion and growth of MC3T3-E1 osteoblast-like cells [3, 4]. Here, a similar positive effect of enzymatic mineralization was demonstrated for MG63 cells and ADSC. Chitosan's promotion of MG63 adhesion and growth may result from MG63 cell adhesion to chitosan, or increased mineralization or stiffness resulting from the presence of 1.5% chitosan (Figure 1a,b). Osteogenic differentiation of ADSC on methacrylated GG has been promoted by collagen [11]. Hence, hydrogel loading with judiciously-chosed biomolecules like chitosan may be a promising strategy to promote stem cell differentiation.

4. Conclusions

Mineralization with phytase supported MG63 and ADSC adhesion and proliferation. For MG63 cells, phytase's effect was enhanced by chitosan. Phytase-mineralized hydrogels, with and without chitosan, were non-toxic. ADSC adhesion and proliferation was poor without phytase-mediated mineralization. Chitosan did not affect ADSC osteogenic differentiation regardless of media. These results demonstrate the importance of mineralization for ADSC growth.

5. Acknowledgement

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7. **Figure captions**

Figure 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.

Figure 2: a-i: MG63 cell morphology after 7 days. Ch 0, Ch0.75, Ch 1.5, Ch 3: chitosan concentration 0%, 0.75%, 1.5%, 3%, respectively. Ph: in the presence of phytase. a-d: without phytase, e-h: with phytase, i: polystyrene (PS) control. j: resaurin proliferation test. k: cytocompatibility test using eluates.

Figure 3: a-h: ADSC morphology. a-d: after 1 d. e-h: after 3 d. GG: gellan gum only (no chitosan or phytase). GG-Ch: with chitosan (1.5%). GG-Ph: with phytase: GG-Ph-Ch: with phytase and chitoaan.. i: resazurin metabolic test after 13d. j: ALP activity test after 14 d. *: $p<0.05$ with respect to sample cultured in osteogenic medium.

Figure 1

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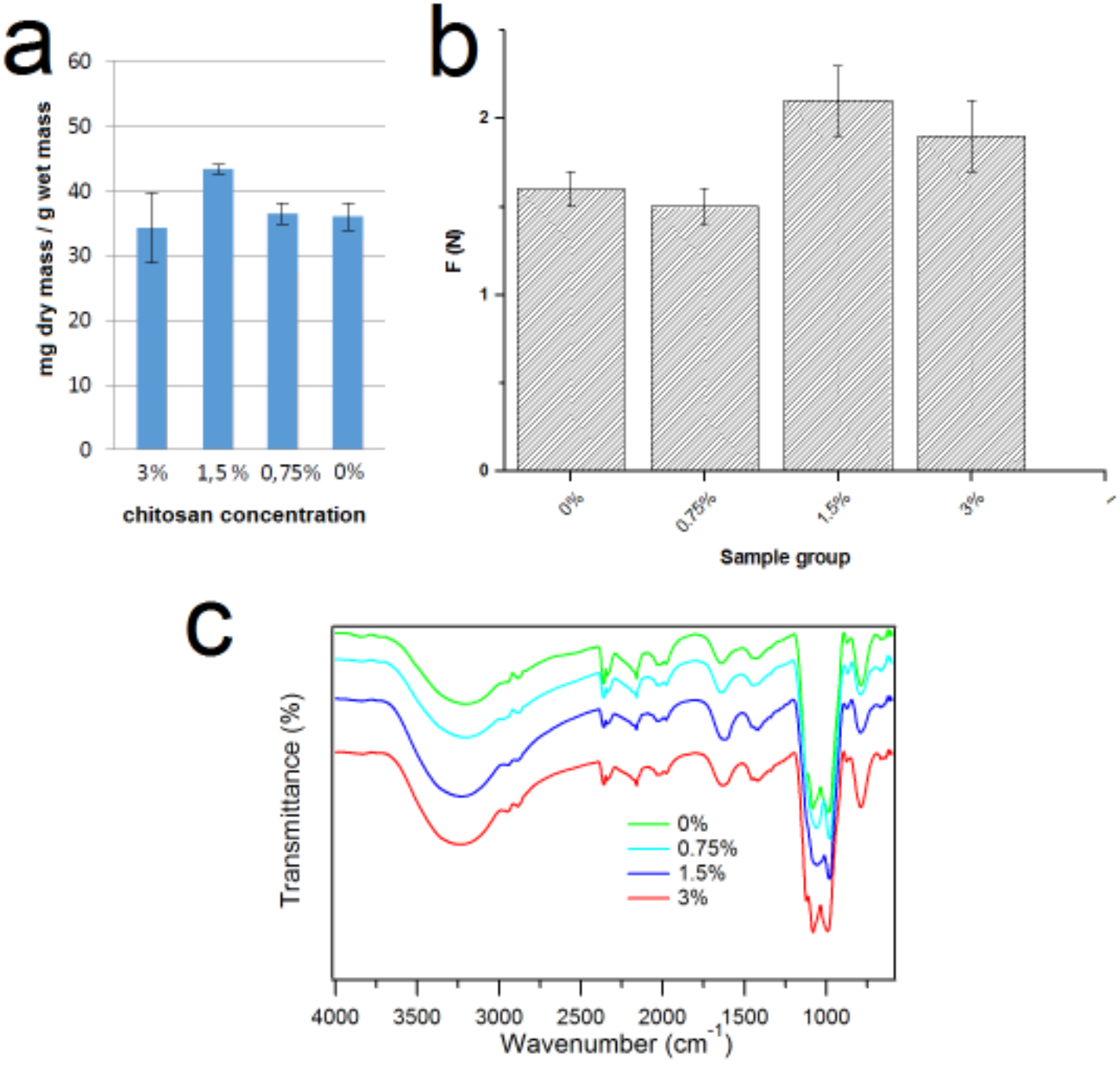

Figure 2

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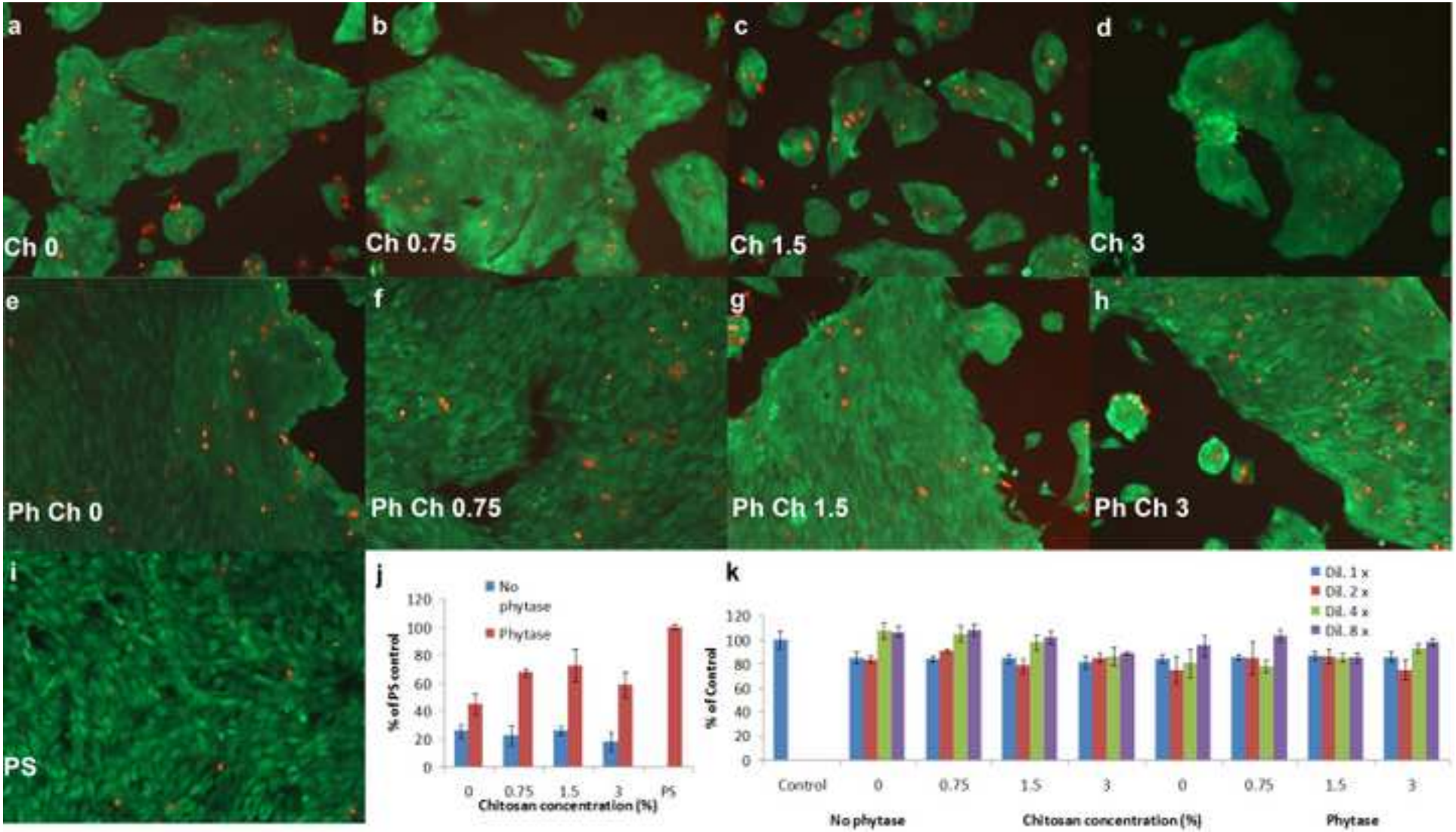

Figure 3

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