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Enrichment of thermosensitive chitosan hydrogels with glycerol and alkaline phosphatase for bone tissue engineering applications

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Thermosensitive injectable chitosan hydrogels can be formed by neutralization of acidic chitosan solutions with sodium beta-glycerophosphate (Na-β-GP) coupled with increasing temperature to body temperature. Such hydrogels have been considered for applications in bone regeneration. In this study, chitosan hydrogels were enriched with glycerol and the enzyme alkaline phosphatase (ALP) with a view to improving their suitability as materials for bone tissue engineering. Mineral formation was confirmed by infrared spectroscopy (FTIR) and increases in the mass fraction of the hydrogel not consisting of water. Incorporation of ALP in hydrogels followed by incubation in a solution containing calcium ions and glycerophosphate, a substrate for ALP, led to formation of calcium phosphate within the hydrogel. MG-63 osteoblast-like cells were cultivated in eluates from hydrogels containing ALP and without ALP at different dilutions and directly on the hydrogel samples. Hydrogels containing ALP exhibited superior cytocompatibility to ALP-free hydrogels. These results pave the way for the use of glycerol- and ALP-enriched hydrogels in bone regeneration.

Key words: *biomaterials, composites, mineralization, hydrogels*

1. Introduction

Chitosan, a polysaccaride produced by deaceylation of chitin, has been widely used in the pharmaceutical, cosmetic and biomaterial fields thanks to its excellent physicochemical and biological characteristics, namely biocompatibility, degradability, non-toxicity and non-immunogenicity [8]. The advantage of chitosan is that it can be processed into injectable forms which may be administered locally by minimally-invasive methods (i.e., injection), in order to enhance tissue regeneration and healing. Particularly interesting are chitosan formulations, which are able to form hydrogels when heated to body temperature [23].

Such thermosensitive chitosan hydrogels can be formed by neutralization of acidic chitosan solutions with sodium beta-glycerophosphate (Na-β-GP) [6], [23]. As a result of neutralization, electrostatic repulsion between positively charged chitosan chains is lowered. Na-β-GP consists of a glycerol and a phosphate part. Temperature increase causes chitosan chain dehydration, enhanced by Na-β-GP's glycerol part, as well as proton transfer from chitosan to Na-β-GP's phosphate part, which in turn enhance hydrophobic interactions and interchain hydrogen bonding [2], [5]. All the factors mentioned above promote chitosan hydrogel formation. Such hydrogels have supported osteogenic differentiation of rat muscle-derived stem cells in vivo [21]. Membranes created by

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freeze-drying of such hydrogels have been developed for guided bone regeneration and demonstrated biocompatibility in an in vivo rat model [7]. Chitosan has also been used as a component of injectable bone substitute materials [3].

In this study, chitosan hydrogels were enriched with glycerol and the enzyme alkaline phosphatase (ALP) with a view to improving their suitability as materials for bone regeneration. Incorporation of ALP in hydrogels followed by incubation in a solution containing calcium ions and glycerophosphate, a substrate for ALP, has led to formation of calcium phosphate (CaP) within the hydrogel [11], [13], [17], [18]. Hydrogel mineralization with CaP has been regarded as desirable for bone regeneration applications (for reviews, see [14], [19]). In previous work, incorporation of ALP in chitosan/Na-β-GP hydrogels caused mineralization with CaP and also reduced time required for hydrogel formation [16].

The second component of our system, glycerol, has also been used in medical applications. It has been added to pre-mixed calcium phosphate cements to improve handling [26] and was found to be biocompatible *in vivo* [1], [24]. Glycerol has also been used in composite hydrogels consisting of alginate and CaP, which also demonstrated good biocompatibility [4].

The hydrogel samples produced in this study were characterized physiochemically with respect to mineralizability and type of mineral formed and biologically using cells of the osteoblastic cell line MG-63. To our best knowledge, chitosan enrichment with both glycerol and ALP remains a relatively unexplored area of research, although it has a good potential in a development of a new generation of injectable biomaterials to enhance bone regeneration.

2. Materials and methods

2.1. Hydrogel production

Unless stated otherwise, all chemicals were obtained from Sigma-Aldrich, including chitosan powder (shrimp-derived, deacetylation degree (DD) 83.4%, molecular weight (Mw) 862 kDa), HCl, glycerol, ALP (bovine intestinal mucosa-derived product no. P7640) and Na-β-GP (product no. 50020). Chitosan was dissolved in 0.1 M HCl at a concentration of 25 mg/ml. Na-β-GP was mixed with an equal volume of MilliQ-water to yield a 1 g/ml solution-suspension. Chitosan solutions and NaGP solution-suspensions were mixed for 24 h under rotation. ALP was dissolved in MilliQ-water at a concentration of 25 mg/ml. A 2.75 ml chitosan solution, 0.35 ml Na-β-GP solution-suspension, 0.5 ml glycerol and 0.5 ml (or 0 ml ALP solution) and 0.9 ml (or 1.4 ml MilliQ-water) were mixed together using a pipette to yield 5 ml hydrogels, which are hereafter denoted as S1 (Chit-Gl-NaGP) and S2 (Chit-Gl-NaGP-ALP). The final ingredient concentrations are shown in Table 1. Gelation took place in wells of six-well plates at 37 °C overnight.

Table 1. Final composition of chitosan hydrogels investigated in this study

Sample	Chitosan [%]	Glycerol [%]	Na-β-GP [%]	ALP [mg/ml]
S1 (Chit-Gl-NaGP)	1.4	10	7	0
S2 (Chit-Gl-NaGP-ALP)	1.4	10	7	2.5

2.2. Mineralization studies

Cylindrical S1 and S2 hydrogel samples were cut out from 5 ml hydrogels using a 6 mm diameter hole punch and were subsequently incubated for 6 days in 0.1 M calcium glycerophosphate (Ca-GP), rinsed three times with Milli-Q water and incubated in Milli-Q water for 24 h. Ca-GP served as a source of calcium ions and substrate for ALP. The dry mass percentage, which served as a measure of extent of mineralization, was calculated as: (weight after incubation and subsequent freeze-drying for 24 h/weight before freeze-drying)*100. After mineralization studies and freeze-drying, the molecular structure of hydrogels was examined using attenuated total reflectance Fourier-transform infrared spectroscopy (ATR-FTIR). Transmittance spectra were acquired using a Cary 630 FTIR spectroscope from Agilent Technologies and MicroLab Software. Spectra were recorded at room temperature over the range 4000–600 cm^{-1} with a resolution of 4 cm^{-1} and averaging of 64 scans for each spectrum.

2.3. Cell culture studies

Prior to cell culture studies, chitosan and Na-β-GP were sterilized using ethylene oxide (EO) as described previously [15]. ALP solution was sterilized by filtration.

Cell biological characterization was evaluated by determining the viability of MG-63 cells (European Collection of Cell Cultures, Salisbury, UK) which

were cultured in Eagle's minimal essential medium (EMEM, PAN BIOTECH, Germany) supplemented with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin, 0.1% sodium pyruvate (PAA, Austria). Tissue culture polystyrene (TCPS, Nunclon, 24-well plates) served as a reference.

Cytocompatibility was evaluated by determining the viability of MG-63 cells after culture in eluate from hydrogel samples. Eluate was produced by incubating hydrogel samples, S1 or S2, of diameter 15 mm, height 10 mm and volume 2 ml in 3.5 ml EMEM for 24 h. The eluate was diluted in EMEM by factors of 1 (undiluted), 2, 4, 8 and 16. MG-63 cells (10 000/well of a 48-well plate) were cultured for 24 h and then subsequently incubated in the eluate (1 ml) at the aforementioned dilutions for the next 24 h.

Cell viability was then evaluated using Alamar Blue reagent (In Vitro Toxicology Assay Kit, Resazurin based). 0.1 ml of Alamar Blue reagent was added and the cells were incubated for 3 h at room temperature. Reduction of Alamar Blue was measured fluorescently (excitation wavelength 530 nm, emission wavelength 590 nm) (FLUOstar Omega, BMG labtech) and calculated according to the following formula

$$\%\ \text{Reduction of Alamar Blue} = \frac{S^{x} - S^{\text{control}}}{S^{100\%\ \text{reduced}} - S^{\text{control}}} \cdot 100\%$$

where:

S^{x} – fluorescence of samples,

S^{control} – fluorescence of medium without cells,

$S^{100\%\text{reduced}}$ – fluorescence of reagent reduced in 100% (reagent with medium was placed in autoclave for 15 min at 121 °C).

The result of this measurement is the reduction ratio of the reagent (the higher the reduction, the more cells). This test was conducted twice, after 24 h and 5 days. Measurements were performed in triplicate.

Cell attachment, distribution and viability were evaluated 24 h and 5 days post-seeding by fluorescence microscopy using an Axiovert 40 microscope (Carl Zeiss, Germany) after live/dead staining (Calcein AM/propidium iodide) as described previously [20].

Hydrogel samples of volume 0.5 ml were formed by pouring ingredient mixtures into wells of 24-well plates with subsequent gelation for 24 h. After gelation, 30 000 cells suspended in 1 ml EMEM were seeded onto each hydrogel sample and cultured for 24 h and 5 days. Subsequently 0.1 ml Alamar Blue reagent was added and viabitily measurements were carried out as described above. The viability was calculated as a percentage of control cultures. Cells grown on hydrogels were visualized after 24 h of culture by using live/dead staining as described above.

2.4. Statistical analysis

Data were presented as mean ±S.D. (Standard Deviation). Multiple comparison procedures were performed with ANOVA. A value of $P \leq 0.05$ was considered significant.

3. Results

Hydrogels containing 2.5 mg/ml ALP (S2) showed significantly higher dry mass percentage than ALP-free hydrogels (S1) (Fig. 1).

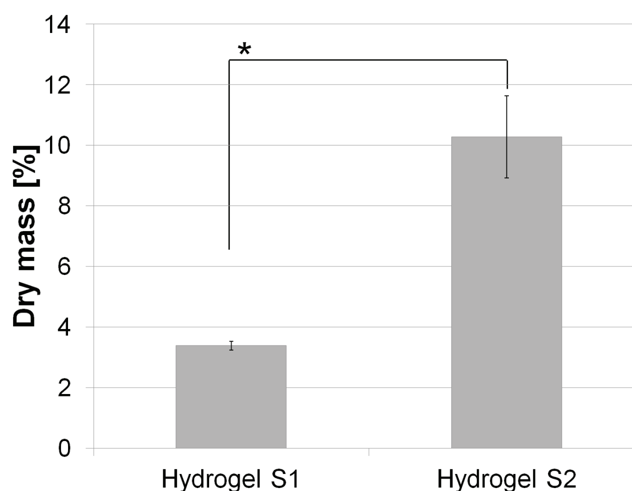

Fig. 1. Dry mass percentage of chitosan hydrogel sample groups S1(Chit-Gl-NaGP) and S2 (Chit-Gl-NaGP-ALP) incubated for 6 days in 0.1 M CaGP.
Error bars show standard deviation.
Statistical significance: $p^* < 0.001$ according to ANOVA

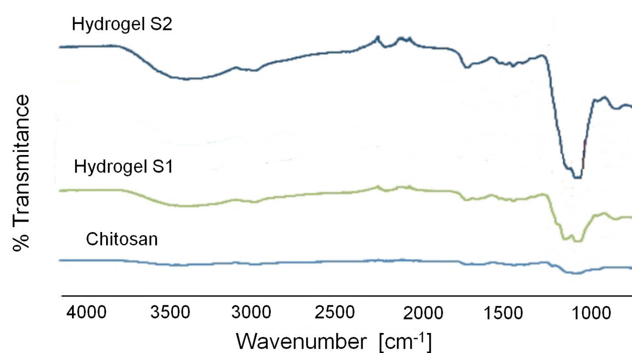

Fig. 2. FTIR spectra of chitosan hydrogel sample groups S1 (Chit-Gl-NaGP) and S2 (Chit-Gl-NaGP-ALP) incubated for 6 days in 0.1 M CaGP.
Pure chitosan powder is provided as a reference

ATR-FTIR measurements (Fig. 2) showed the appearance of a distinct band at 1030 cm^{-1} corresponding to stretching vibrations of phosphate groups [16], [22]. This band was much less pronounced in ALP-free hydrogels (S1) than in ALP-containing hydrogels (S2).

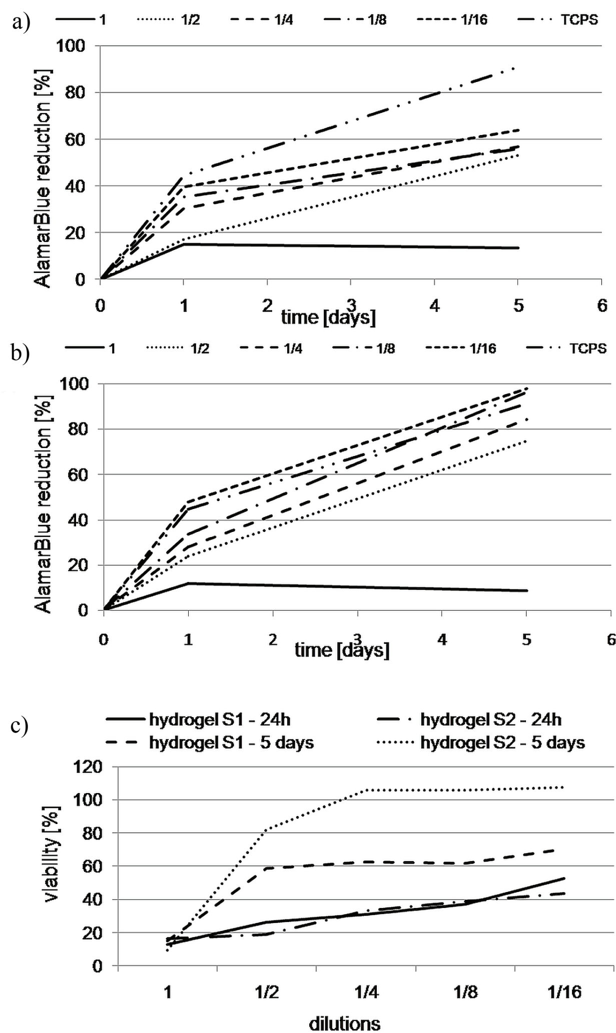

Fig. 3. Reduction of Alamar Blue in cells in contact with eluate (1) and its dilutions (1/2, 1/4, 1/8, 1/16) from hydrogel types S1 (Chit-Gl-NaGP) (a) and S2 (Chit-Gl-NaGP-ALP) (b) in comparison with cells cultured on control TCPS in pure EMEM, investigated after 24 h and 5 days. Cell viability in contact with extraction medium (1) and its dilutions (1/2, 1/4, 1/8, 1/16) as investigated by Alamar Blue reduction (c) on hydrogel types S1 (Chit-Gl-NaGP) and S2 (Chit-Gl-NaGP-ALP) after 24 h and 5 days. Viability of cells on control TCPS in pure EMEM was assumed to be 100%

In vitro results (Fig. 3b) showed that reduction of Alamar Blue in eluate of hydrogels containing ALP (S2) was lowest for dilution 1 (undiluted) and increased with the higher eluate dilutions (1/2, 1/4, 1/8, 1/16). In the case of hydrogels without ALP (S1) (Fig. 3a), reduction of Alamar Blue also increased with higher eluate dilution. Values of cell viability (Fig. 3c) were similar for both hydrogels after 24 h, but were markedly higher for hydrogels containing ALP (S2) after 5 days. Cell viability in eluate from hydrogel S2 with dilution 1/4 after 5 days was even higher than for cells on TCPS cultured in pure EMEM.

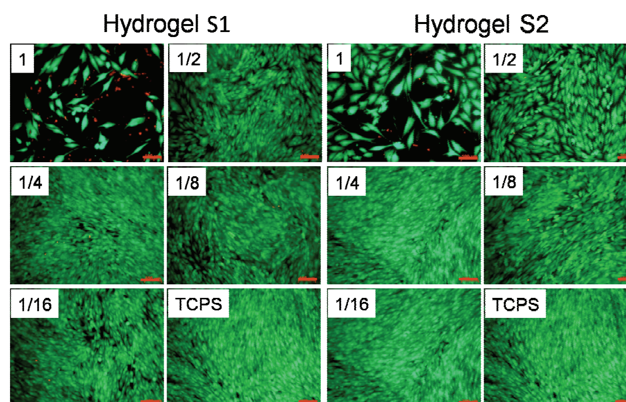

Fig. 4. Live/dead staining of MG-63 cells incubated for 5 days in contact with eluate (1) and its dilutions (1/2, 1/4, 1/8, 1/16) on hydrogel types S1 (Chit-Gl-NaGP) and S2 (Chit-Gl-NaGP-ALP) and cultured on TCPS in pure EMEM (served as a control). Green, viable cells; red, dead cells. Scale bar = 100 µm in all cases

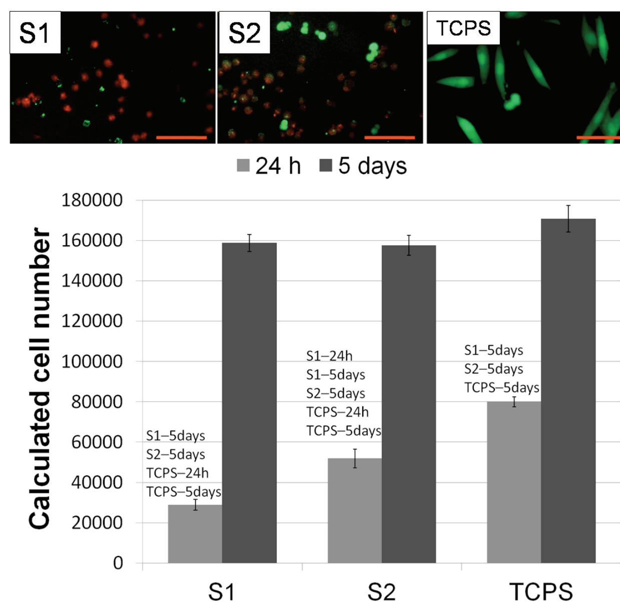

Fig. 5. Top: Live/dead staining of MG-63 cells cultured for 24 h on hydrogels S1 (Chit-Gl-NaGP) and S2 (Chit-Gl-NaGP-ALP) and TCPS (served as a control). Green, viable cells; red, dead cells. Scale bar = 100 µm in all cases. Bottom: Cell viability on hydrogel types S1 (Chit-Gl-NaGP) and S2 (Chit-Gl-NaGP-ALP) and TCPS (served as a control), investigated by Alamar Blue reduction after 24 h and 5 days. Error bars show standard deviation, $n = 3$ for all groups. Statistical significance $p < 0.05$ according to one-way ANOVA marked above each sample group

Results of live/dead staining of MG-63 cells after 5 days (Fig. 4) were consistent with results of Alamar

Blue reduction. Markedly fewer cells were observed for dilution 1 of ALP-containing hydrogels (S2) and ALP-free hydrogels (S1) and more cells on S1 were dead, i.e., stained red. However, no marked differences with controls were seen at higher dilutions.

Alamar Blue reduction test results indicated a higher number of MG-63 cells on ALP-containing hydrogels (S2) than on ALP-free hydrogels (S1) and the highest cell number on TCPS after 24 h (Fig. 5). After 5 days of culture, similar cell numbers were observed on ALP-containing hydrogels (S2) and ALP-free hydrogels (S1) and slightly higher cell numbers on TCPS. Results of live/dead staining after 24 h were consistent with Alamar Blue reduction test results. The highest number of cells was observed on TCPS, followed by ALP-containing hydrogels (S2) and ALP-free hydrogels (S1). Moreover, cells on TCPS appeared to be viable and spindle shaped, while on ALP-containing hydrogels (S2) round both live and dead cells were observed, while on ALP-free hydrogels almost only dead cells were seen (S1).

4. Discussion

The goals of this study were to produce chitosan-based hydrogels that would be i) more suitable for bone tissue regeneration thanks to their increased mineralizability and ii) cytocompatible for bone cells.

The results of dry mass percentage measurements (see Fig. 1) demonstrated indirectly that mineralization had taken place in hydrogels containing ALP. ATR-FTIR measurements (see Fig. 2) demonstrated directly that mineralization was much more pronounced in hydrogels containing ALP (S2). Hence, addition of ALP to chitosan-based hydrogels containing Na-β-GP and glycerol resulted in enhancement of the mineralization process and creation of calcium phosphate deposits within the samples.

The in vitro tests show that cell viability increased with a higher dilution of eluates for both hydrogels (S1 and S2). Interestingly, for eluates from hydrogels containing ALP with dilution 1/4, cell viability was even higher after 5 days than for cells cultured on TCPS in pure EMEM (see Fig. 3). The cell number, proliferation and viability were reduced on S1 hydrogels (not containing ALP) as compared to S2 (containing ALP) as shown by microscopic studies (see Figs. 4 and 5).

Thus, the results demonstrate that both hydrogels (containing ALP and ALP-free) are slightly toxic for cells. It can be speculated that this mild cytotoxicity is due to the use of hydrochloric acid and the high concentration of Na-β-GP (0.07 g/ml) in hydrogels. It has been reported that glycerol as a component of cell culture medium has inhibited proliferation of various non-osteoblastic cell types [10], [25], [27]. However, the results of this study show that the incorporation of 10% glycerol into hydrogels does not necessarily compromise cell proliferation. Indeed, the cell number on hydrogels after 5 days was comparable to that on TCPS, which is known to be a substrate which supports cell proliferation. The reasons for the higher cell numbers on hydrogels containing ALP (see Fig. 5) and in eluates from hydrogels (Fig. 3) remain unclear and discussion of the mechanism by which ALP promotes cell proliferation must remain highly speculative. The concentration of ALP used in this study, namely 2.5 mg/ml, has been used to enrich a wide range of hydrogels in previous studies [12], [17]. However, these studies focused on biological characterization of mineralized hydrogels and the influence of eluate containing ALP was not investigated in detail. The results in this study show that this ALP concentration may be used in further studies without worrying that bone cell survival and number may be compromised.

To our best knowledge, this is the first study demonstrating a direct positive effect of ALP addition to chitosan-based hydrogels on bone cell vitality and proliferation. In order to promote hydrogel mineralization, the ALP concentration should be maximized. Dash et al. incorporated ALP into porous crosslinked solid chitosan scaffolds by soaking in ALP solutions at concentrations up to 60 mg/ml [9]. Extent of mineralization was maximized by soaking in 20 mg/ml ALP; soaking at higher concentrations did not lead to increased mineralization. Furthermore, the authors found that formation of apatite was promoted at ALP concentrations up to 20 mg/ml, but brushite formation was favored at 40 and 60 mg/ml. Further studies are necessary to determine the optimal ALP concentration to stimulate cell proliferation. However, the optimal concentrations to maximize the amount of mineral formed and obtain the desired type of CaP should also be determined.

5. Conclusions

Thermosensitive chitosan-Na-β-GP hydrogels containing glycerol both enriched with 2.5 mg/ml ALP and ALP-free, supported growth of MG-63 osteoblast-like cells. Cytocompatibility of ALP-containing hydrogels

was superior to that of ALP-free hydrogels. These results pave the way for further investigations into the use of glycerol as a component of chitosan hydrogel biomaterials for bone tissue regeneration, and the enrichment of biomaterials with ALP to stimulate not only mineralization, but also cell proliferation.

Acknowledgement and conflict of interest statement

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