THE INFLUENCE OF MINERALIZATION CONDITIONS ON THE EFFECTIVENESS OF ENZYMATIC MINERALIZATION OF HYDROGELS

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Abstract

Polysaccharide hydrogels are widely used in food industry and medicine. Gellan gum (GG) recently gained a lot of attention as a promising material for tissue regeneration proposes due to its excellent biocompatibility and similarity to natural extracellular matrix. However, in unmineralized form it is not suitable for bone tissue engineering because of weak mechanical properties. Enzymatic mineralization (e.g. using alkaline phosphatase – ALP) is one of the methods of calcifying of hydrogels and it resembles natural processes occurring during bone healing.

The aim of this research was to investigate mineralization of hydrogels and to improve properties of gellan gum scaffolds by adjusting processing conditions. Since ALP does not form with GG covalent bonds, during incubation in mineralization medium (solution of calcium glycerophosphate - CaGP) it is diffusing from the samples. Therefore, mineralization effectiveness depends on the interplay between incoming CaGP and outgoing ALP molecules. We hypothesize that better CaGP availability, especially in the first hours of incubation, can result in more effective and homogenous precipitation of calcium phosphates (CaP) in GG samples.

To this end, samples with different GG and ALP concentration were subjected to two different mineralization regimes (more and less frequent CaGP exchanges). We proved that better CaGP availability (more frequent CaGP exchange) resulted in better mechanical properties (Young's modulus) and more effective mineral formation (higher dry mass percentage) of the samples compared to the same samples mineralized with lower accessibility of CaGP. This may be related to the fact, that in presence of fresh organic substrates, more CaP are formed in the outer parts of the samples at the beginning of the process, that limit ALP diffusion and allow more uniform mineralization.

Keywords: *enzymatic mineralization, hydrogels, bone tissue engineering*

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Introduction

Hydrogels are highly hydrated polymers that recently gained increasing interest for potential use in tissue engineering. Advantages of hydrogels include injectability, exact fitting to the defect site and easiness of incorporation of bioactive molecules and cells. Hydrogels have been mainly considered for applications in soft tissue engineering, but the versatility of hydrogels has resulted in increased interest in potential application of those materials in regeneration of bone and cartilage. However, hydrogels lack the capacity to calcify which limits their suitability for hard tissue regeneration [1-3].

A recent trend involves the development of hydrogels that possess the capacity to mineralize. Thanks to CaP presence within hydrogels, materials gain affinity for biologically active proteins. Mechanical reinforcement resulting from mineralization may help to overcome one of the main disadvantages of hydrogel materials, namely weak mechanical properties [4-5].

Hydrogels can be mineralized with several different ways: physically (by incorporation of inorganic calcium phosphate particles) [6], chemically (by incubation of hydrogels in solutions containing calcium and/or phosphate ions) [7] and enzymatically (by incorporation of enzymes) [8-10].

Alkaline phosphatase (ALP) is an enzyme involved in mineralization of bone by cleavage of phosphate from organic phosphate (e.g. calcium glycerophosphate, CaGP). In previous studies ALP was used to induce mineralization of hydrogels to improve their mechanical strength and render them more suitable for bone replacement applications [11].

Physical and biological properties of enzymatically mineralized gellan gum (GG) hydrogels with a focus on bone regeneration applications were studied by Douglas et al [11-12]. In these studies ALP was incorporated into hydrogel without any covalent bonding therefore it could easily diffuse outside. This method of ALP incorporation does not involve reactive and potentially harmful chemicals. However its main disadvantage is poor retention of ALP in material during the process of mineralization. It can be a limiting factor for achieving higher degree of mineralization. In all previous studies maximal content of CaP precipitates inside hydrogels did not exceed 10-12%. Moreover as it was shown in our previous study, fast diffusion of ALP from outer parts of hydrogels lead to inhomogeneous mineralization – the samples were less mineralized on the surface due to ALP loss. It was also noted that ALP released to the surrounding solution consumed CaGP and as a result CaP precipitated outside hydrogels. We assume that this phenomenon might reduce efficiency of enzymatic mineralization. It is particularly present in the first hours of incubation, because by that time considerable amount of the enzyme is released from the samples [12-13].

The aim of this study was to find out how to prevent outside mineralization and sustain steady influx of CaGP into the hydrogel in the first hours of mineralization, while it possess the highest mineralization capability due to high ALP concentration within the material.

We also wanted to measure a rate of ALP release from hydrogel during mineralization and compare it to diffusion of ALP from unmineralized hydrogel. We believe that CaP precipitation inside hydrogel may partially prevent enzyme loss and mineralization can sustain longer.

In this experiment we evaluated the influence of mineralization conditions on its effectiveness in the case of the samples with different GG and ALP concentrations. Two CaGP exchange regimes were applied. The main hypothesis was that more frequent CaGP exchange in the first day of incubation results in more efficient CaP formation within GG samples.

Materials and Methods

Materials

Aqueous solutions of GG (Gelzan™ CM, Sigma-Aldrich), alkaline phosphatase from bovine intestinal mucosa, lyophilized powder (ALP, P7640, Sigma-Aldrich) and calcium chloride ($CaCl_2$, POCH) were used. GG was mixed with UHQ-water, heated up to 90°C until complete dissolution and then cooled down to 50°C. $CaCl_2$ and ALP were dissolved in UHQ-water and heated up to 50°C. GG solution was first mixed with ALP and then $CaCl_2$ solution was added. After short mixing gel was purred into test tubes and left to gellify (4°C, 5 min).

Samples for ALP release measurement

To measure release rate of ALP from 0.7% GG during mineralization as well as from non-mineralized gel, two sets consisting of three types of samples were prepared. In the first set ALP was released to water, while in the second one to CaGP solution and at the same time gel underwent mineralization (FIG. 1.1). All samples were prepared in 10 ml test tubes. 2 ml 0.7% GG gel containing 2.5 mg/ml ALP and 0.03% $CaCl_2$ was poured into test tube.

Samples for physicochemical examination

Solutions were prepared in the same way as described before. Final concentrations of GG, ALP and $CaCl_2$ in samples are given in TABLE 1. For each sample group 8.8 ml GG, 0.2 ml of ALP and 1 ml $CaCl_2$ solutions were vigorously mixed, casted into Petri dish (10 cm diameter) and cooled down to 4°C. Samples (12 mm diameter and 4 mm height) were cut using a hole punch. For mineralization samples were then immersed in 0.1 M CaGP solution (Sigma-Aldrich). For each sample group two CaGP exchange regimes were used: A (2 h, 4 h, 6 h, 1 day, 3 days and 5 days) and B (1 day, 3 days and 5 days). After 7 days the samples were washed in UHQ-water and subjected to physicochemical examination. Preparation procedure is shown in FIG. 1.2.

Methods

ALP release measurement

Samples were prepared in 10 ml test tubes. Each 2 ml sample of GG gel after gellation was covered with 1 ml of UHQ-water or 0.1 M CaGP. Solution was exchanged after: 1 h, 2 h, 4 h, 8 h, 1 day and 3 days. Solution collected from each changing period was centrifuged at 3000 rpm for 4 min to separate CaP precipitating outside hydrogels. 600 µl of supernatant was collected and dried (37°C for 3 days), remaining precipitate was dissolved in 200 µl UHQ-water. 60 µl of this solution was transferred to 96-well plate in triplicate for each sample and mixed with 60 µl of BCA reagent (5 ml bicinchoninic acid solution, 20 mg of copper II sulfate anhydrous pure – both from Sigma-Aldrich and 500 µl H_2O). To determine a real concentration standard solutions of ALP were also prepared. After 30 min incubation absorbance at 562 nm was measured. Real concentration was calculated on the basis of calibration curve and known dilutions.

Mechanical testing

Mineralized hydrogels were subjected to compression testing using ZWICK 1435 (Zwick Roell, Germany). Hydrogels were placed between piston heads and displacement was applied at a rate of 1 mm/min until the samples were compressed to 10% of their original height. Young's modulus was determined based on the stress-strain curve. For each sample group 4 samples were tested.

TABLE 1. Final concentrations of GG, ALP and $CaCl_2$ in samples.

GG [% (w/v)]	ALP [mg/ml]	$CaCl_2$ [% (w/v)]
0.40	0.5	0.03
0.55		
0.70		
0.70	0.25	
	0.50	
	2.50	

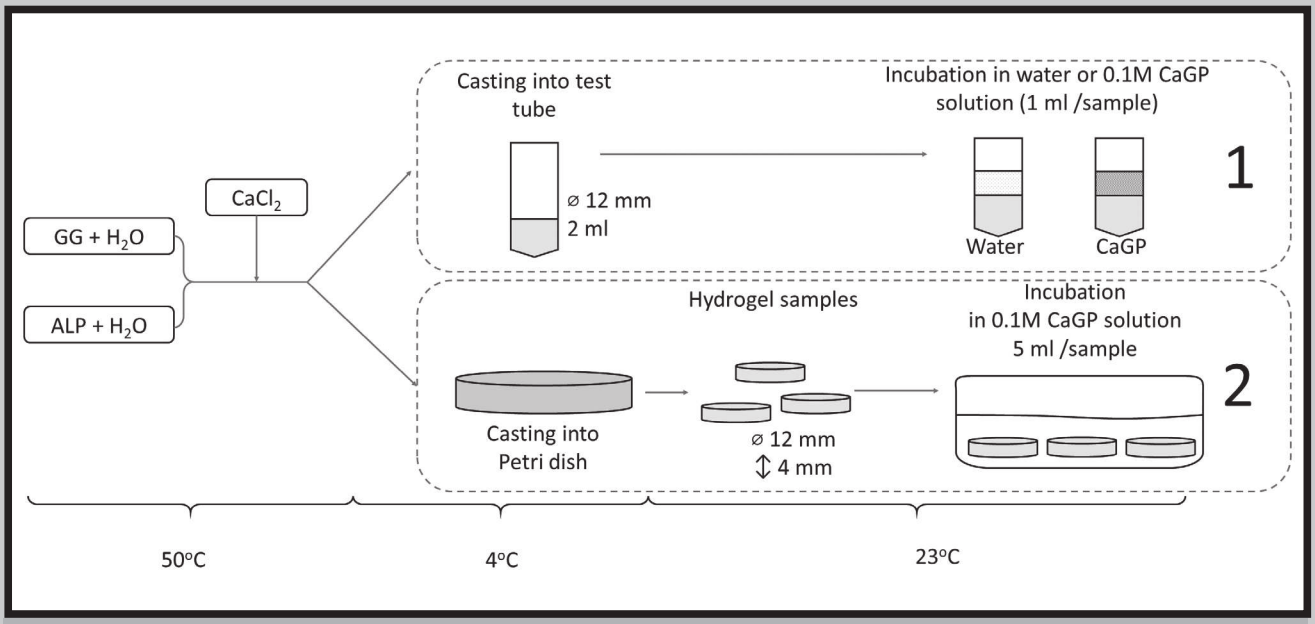

FIG. 1. Manufacturing of GG samples with incorporated ALP for ALP release measurements (1) and physicochemical testing (2).

Measurement of dry mass percentage

Prior to compressive strength testing wet samples were weighted, dried for 48 h and then weighted again. Dry mass percentage was calculated as follows: (weight after incubation and subsequent drying)/(weight after incubation before drying) * 100. For each sample group 4 samples were tested.

Scanning Electron Microscopy and Energy Dispersive X-ray Spectroscopy

Scanning electron microscopy (SEM) and energy dispersive X-ray spectroscopy (EDX) were performed using Nova NanoSEM 2000 (FEI, USA). Prior to analysis, samples were lyophilized and coated with a thin carbon layer. Micrographs were obtained with 5 kV accelerating voltage.

Results

ALP release measurement

The aim of ALP release measurements using BCA method was to evaluate the influence of mineralization process on a rate of ALP diffusion from hydrogel and to find out how mineral phase deposition interfere with this process. Release kinetics of ALP from hydrogel containing 0.7% GG and 2.5 mg/ml ALP is shown in FIG. 2. Regardless of the surrounding medium (water or CaGP) enzyme release was the most intensive in the first hours of incubation. The amount of ALP released to water was much pronounced as compared to the hydrogel soaked in CaGP and mineralized. The differences in release rate between those two sample groups were most noticeable in the first hours of the experiment, i.e. after the first hour the amount of ALP released from soaked in UHQ-water hydrogel was 3 times higher than from that soaked in CaGP one, but between 8 h and 20 h it was only about 1.5 times higher.

Mechanical testing

FIG. 3 shows Young's modulus (E) of mineralized hydrogels with different GG (a) and ALP (b) concentrations. For all sample groups E was higher when samples were mineralized using regime A (more frequent CaGP exchange). Those differences were most significant in samples with lower GG concentration (0.4% and 0.55% GG). In the case of 0.7% GG with 0.25 and 2.5 mg/ml ALP the influence of CaGP exchange regime was not significant. Regarding the influence of GG concentration, when regime B was applied, the stiffness of mineralized hydrogels increased with growing GG concentration. However, when regime A was used, there was no difference between samples containing 0.55% and 0.7% GG. Surprisingly, the increase in ALP concentration did not result in improvement in mechanical properties of the samples. Young's modulus was the highest in the samples containing 0.5 mg/ml ALP, whereas those with 0.25 and 2.5 mg/ml ALP had almost two times lower stiffness.

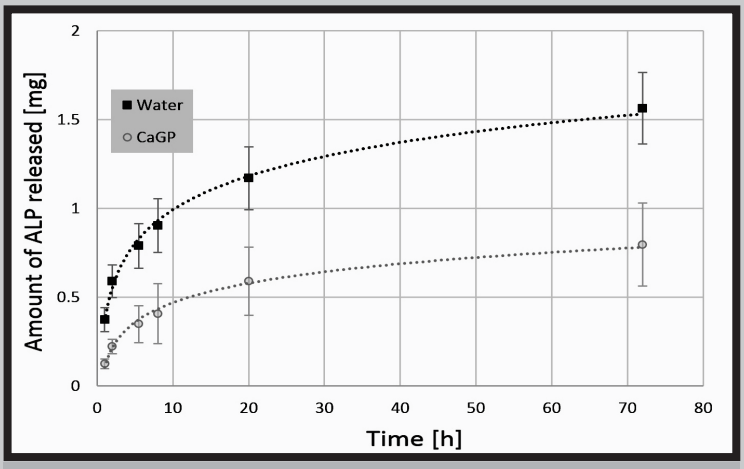

FIG. 2. Amount of ALP released from hydrogel to water and to CaGP during mineralization.

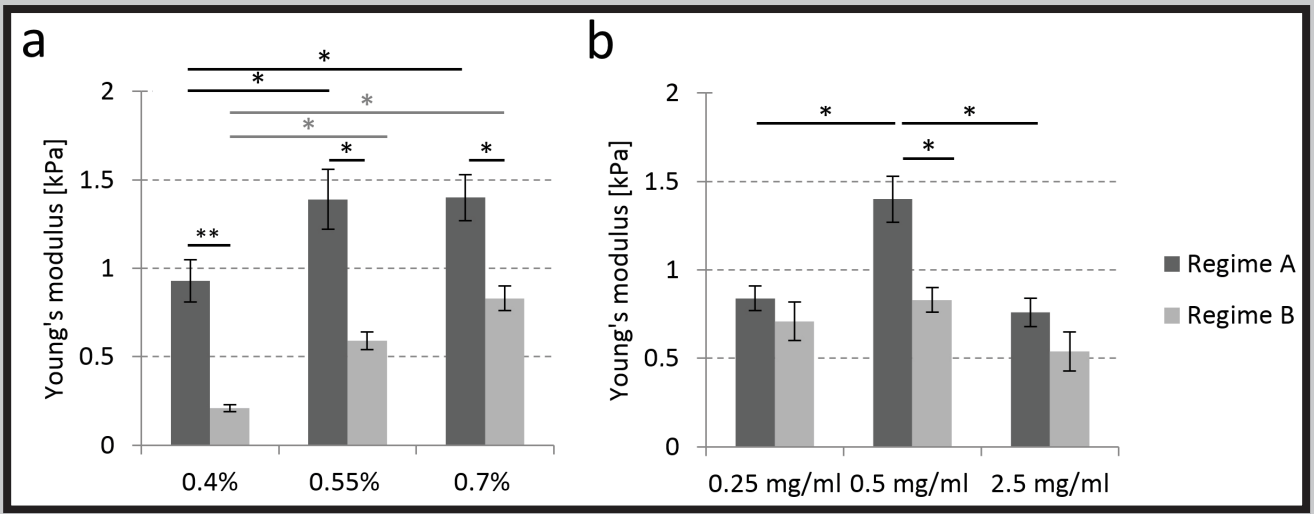

FIG. 3. Young's modulus of mineralized hydrogels with different GG (a) and ALP (b) concentrations; statistically significant difference in comparison to other groups with *P <0.05 or **P<0.01.

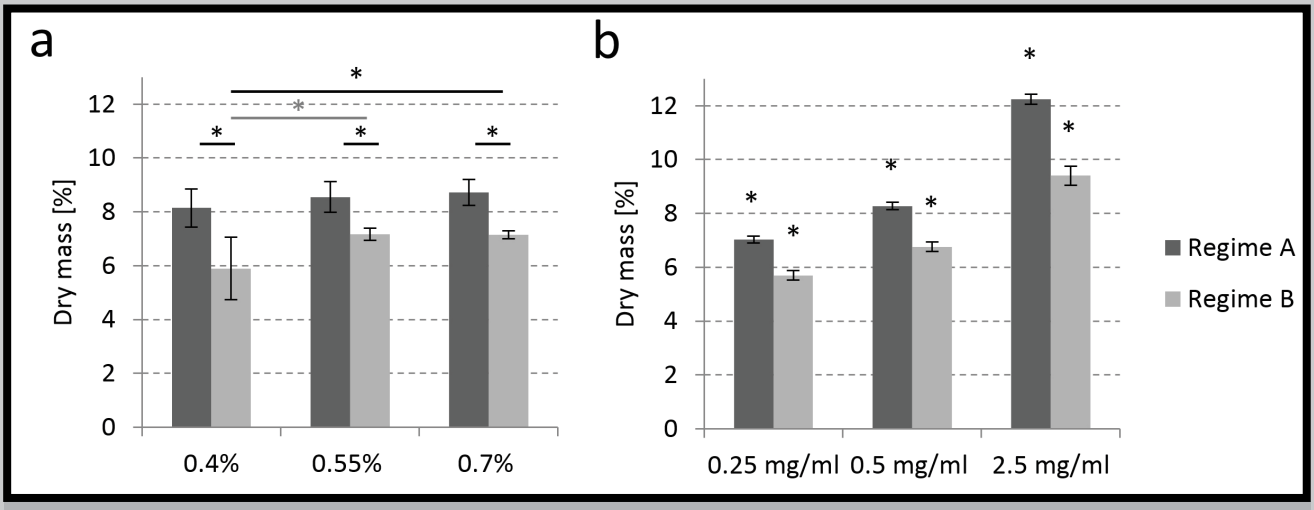

FIG. 4. Dry mass percentage of mineralized hydrogels with different GG (a) and ALP (b) concentrations (statistically significant difference in comparison to other groups with *P <0.05).

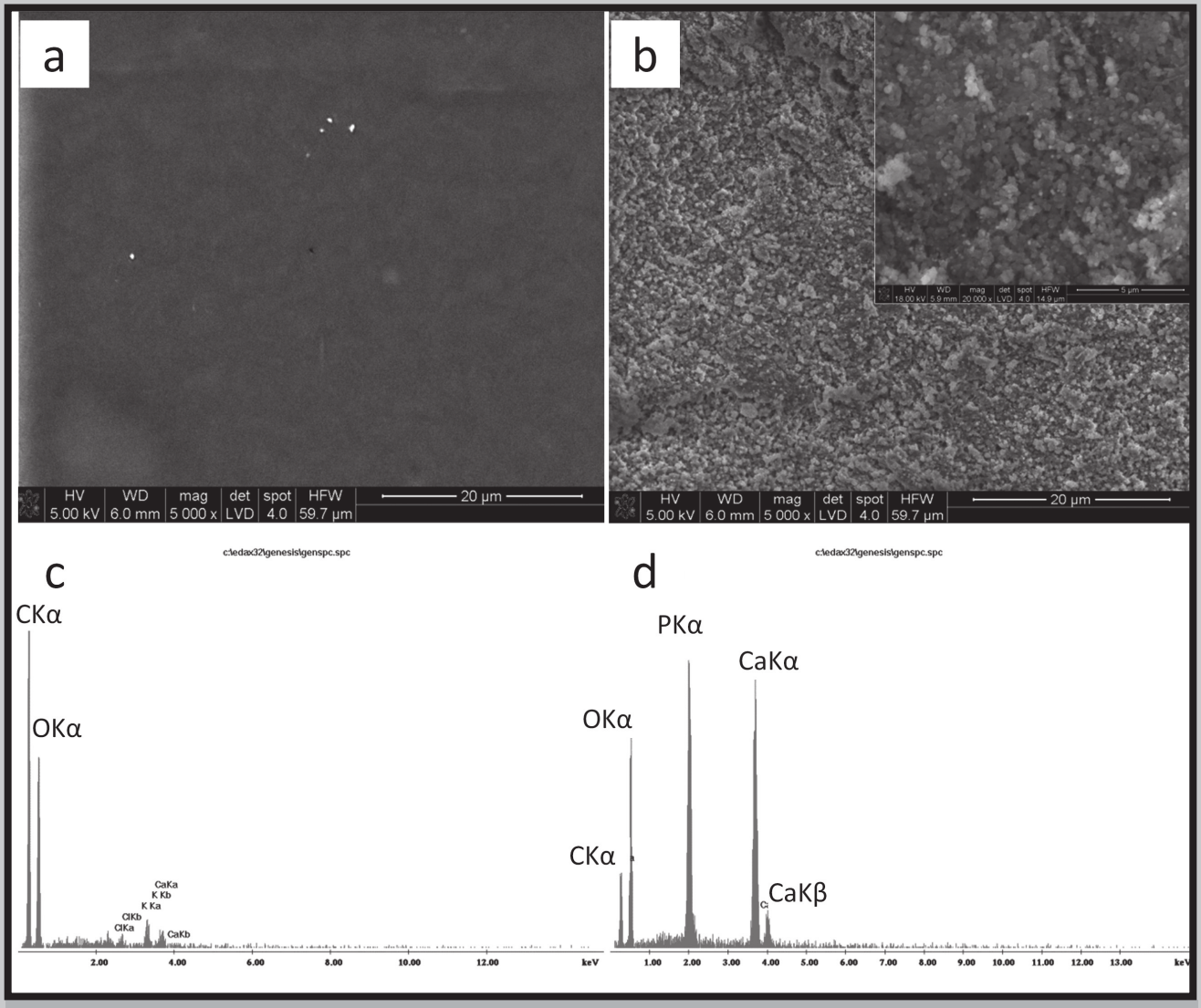

FIG. 5. SEM images (a, b) and EDX analysis (c, d) of non-mineralized (a, c) and mineralized (b, d) hydrogels.

Dry mass percentage

The amount of mineral formed during incubation in CaGP can be assessed by dry mass percentage measurements. The more effective mineralization, the more CaP precipitated within hydrogel and the higher dry mass percentage was observed. FIG. 4 shows dry mass percentage of mineralized hydrogels with different GG (a) and ALP (b) concentrations. Regime A resulted in more effective mineral precipitation than regime B, however those differences were not as distinct as in the case of Young's modulus. What can be expected, the increase in GG concentration did not cause significant increase in dry mass percentage, since the contribution of GG mass was negligible compared to the mass of CaP formed within the samples. Statistically significant differences were found only between samples containing 0.4% GG and samples with 0.55% GG and 0.7% GG mineralized using regime B. With increasing ALP concentration, dry mass percentage was also growing for both mineralization regimes.

Scanning Electron Microscopy and Energy Dispersive X-ray Spectroscopy

FIG. 5 shows SEM images of hydrogel containing 0.7% GG and 0.5 mg/ml ALP before (FIG. 5a) and after 7-day mineralization (FIG. 5b) according to regime A. Before mineralization hydrogel had smooth and uniform surface whereas after mineralization numerous precipitates can be seen on the surface. Size of majority of these precipitates is below 1 μm. EDX analysis (FIG. 5d) confirmed that precipitates consisted of calcium and phosphorus.

Discussion

Enzymatic mineralization of hydrogel is based on the reaction of disconnection of orthophosphate group from CaGP and formation of insoluble CaP. The reaction is catalyzed by enzyme, e.g. alkaline phosphatase (ALP), which is incorporated in hydrogel, but is not covalently bond to the polymer matrix. Therefore ALP diffusion from hydrogel is an important factor that can influence the effectiveness of mineralization. Other variables that should be also considered in term of mineralization process are: enzyme concentration and its activity, rate of CaGP diffusion into the hydrogel and hydrogel concentration. The assumption is that mineralization should be more effective in the case of higher ALP concentration and/or better substrate (CaGP) availability. Higher gellan gum concentration can also affect mineralization by obstructing reagents diffusion and limiting space for CaP precipitation. Furthermore, the influence of growing mineral deposits should be also considered as a diffusion barrier.

Previous experiments [11] showed nonuniform mineralization of hydrogels – less CaP was formed on the surface than in the middle of the samples. This phenomenon was related to high rate of ALP release and in consequence less effective mineral formation, especially in the outer parts of the samples. The aim of our study was to evaluate the influence of different parameters of both mineralization conditions and compositions of the samples on the effectiveness of the mineralization process.

The results of ALP release studies show that enzyme diffusion is the most intensive in the first hours of incubation, which makes that time crucial for the outcome of mineralization process. Just after immersing the sample in CaGP solution or water, gradient of ALP concentration is the highest and enzyme diffusion is fast and unhindered. With time passage, precipitation of CaP inside hydrogel creates a diffusion barrier and hampers ALP release – this is shown by the difference in ALP release between mineralized and non-mineralized sample (FIG. 2). However, it is not possible to completely impede ALP diffusion by rapid mineralization as it was shown for sample mineralized in the test tube. In this case there was still significant ALP release in the first hours of the process. Two gel samples – one incubated in water and one in CaGP – are two extreme conditions of mineralization: in the first case there is no substrate influx – only ALP release, in the second – mineralization rate is maximal and unaffected by enzyme loss (ALP is replenished by enzyme diffusing from deeper parts of cylindrical samples). Real mineralization conditions are always somehow between these two extreme cases. If mineral formation is slow at the beginning of the process (less CaGP available), the situation resembles more the unmineralized system (incubated in water), but when CaGP is exchanged very often, fast rate of precipitation of CaP can limit enzyme release. Therefore, it is very important to prevent irreversible ALP loss in the first hours of the process in order to obtain uniformly mineralized hydrogels. Constant influx of CaGP might be provided by a perfusion device, however in that case ALP would be leached out more extensively during this process.

Considering abovementioned observations, mineralization of gellan gum with different GG and ALP concentration was conducted using two regimes – A (more frequent CaGP exchange) and B (less frequent CaGP exchange). In all cases, regime A resulted in more effective mineralization compared to regime B for the same samples, as evidenced by higher Young's modulus and dry mass percentage. Surprisingly, in the samples with variable GG concentration, there was a significant difference in stiffness between samples mineralized with different regimes, but they did not correspond to dry mass of those samples (FIG. 3a and 4a). To explain this phenomenon, the CaP distribution within the hydrogel should be considered. Hydrogels mineralized with regime B had less mineral formed on the surface. Outer parts of the samples are softer and during compression test they can strongly affect the results, however after drying as the volume of this part is relatively small, it does not contribute significantly to total mass of the sample.

Regarding samples with different ALP content, discrepancies in Young's modulus and dry mass percentage are also noticeable. Noteworthy, mechanical properties of hydrogels containing 2.5 mg/ml ALP are much lower than those with 0.5 mg/ml ALP (regardless of mineralization regime). The cause of that drop in Young's modulus might be due to disturbance of hydrogel structure by too many ALP molecules available. In the case of hydrogels with 0.5 mg/ml ALP, for 1 mg of the enzyme there are 14 mg of GG, while in the case of samples with 2.5 mg/ml ALP, there are only 2.8 mg of GG. On the other hand, in samples with lower ALP concentration (0.25 mg/ml) mineralization rate was slower and therefore not limited by substrate influx. Mineralization regime did not affect properties of those samples as it was noticed in other groups.

Conclusion

Based on the conducted experiments, we can confirm that our hypothesis was correct and that better substrate availability can result in more effective and uniform mineralization of gellan gum. Properties of mineralized hydrogels can be also altered by adjusting ALP and GG concentrations. The most effective mineralization was observed in hydrogels containing 0.55% GG and 0.5 mg/ml ALP mineralized by regime A (more frequent CaGP exchange).

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References

[1] Douglas T.E., Pamula E., Leeuwenburgh S.C.: Biomimetic Mineralization of Hydrogel Biomaterials for Bone Tissue Engineering. Biomimetics: Advancing Nanobiomaterials and Tissue Engineering (2013) 51-67.

[2] Stevens M.M.: Biomaterials for bone tissue engineering. Materials today 11 (2008) 18-25.

[3] Gkioni K., Leeuwenburgh S.C., Douglas T.E., Mikos A.G., Jansen J.A.: Mineralization of hydrogels for bone regeneration. Tissue Engineering Part B: Reviews 16 (2010) 577-585.

[4] Morris E.R., Nishinari K., Rinaudo M.: Gelation of gellan - A review. Food Hydrocolloids 28 (2012) 373-411.

[5] Tang J., Tung M.A., Zeng Y.: Compression strength and deformation of gellan gels formed with mono-and divalent cations. Carbohydrate Polymers 29 (1996) 11-16.

[6] Pereira D.R., Canadas R.F., Silva-Correia J., Marques A.P., Reis R.L., Oliveira J.M.: Gellan gum-based hydrogel bilayered scaffolds for osteochondral tissue engineering. In: Key Engineering Materials (2014) 255-260.

[7] Taguchi T., Sawabe Y., Kobayashi H., Moriyoshi Y., Kataoka K., Tanaka J.: Preparation and characterization of osteochondral scaffold. Materials Science and Engineering: C 24 (2004) 881-885.

[8] Filmon R., Basle M., Barbier A., Chappard D.: Poly (2-hydroxy ethyl methacrylate)-alkaline phosphatase: A composite biomaterial allowing in vitro studies of bisphosphonates on the mineralization process. Journal of Biomaterials Science, Polymer Edition 11 (2000) 849-868.

[9] Filmon R., Grizon F., Basle M., Chappard D.: Effects of negatively charged groups (carboxymethyl) on the calcification of poly (2-hydroxyethyl methacrylate). Biomaterials 23 (2002) 3053-3059.

[10] Beertsen W., Van den Bos T.: Alkaline phosphatase induces the mineralization of sheets of collagen implanted subcutaneously in the rat. Journal of Clinical Investigation 89 (1992) 1974.

[11] Douglas T.E., Messersmith P.B., Chasan S., Mikos A.G., de Mulder E.L., Dickson G., et al. Enzymatic mineralization of hydrogels for bone tissue engineering by incorporation of alkaline phosphatase. Macromolecular bioscience 12 (2012) 1077-1089.

[12] Douglas T., Wlodarczyk M., Pamula E., Declercq H., Mulder E., Bucko M.M., et al.: Enzymatic mineralization of gellan gum hydrogel for bone tissue-engineering applications and its enhancement by polydopamine. Journal of tissue engineering and regenerative medicine 8(11) (2014) 906-918.

[13] Pietryga K., Costa J., Pereira P., Douglas T.E.L., Pamuła E.: Promotion of bone cells growth on gellan gum hydrogels by enzymatic mineralization. Engineering of Biomaterials 125 (2014) 6-12.