Optimization of the mineral content in polymeric gels:
The effect of calcium to phosphate molar ratio

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Abstract

The influence of calcium to phosphate (Ca/P) molar ratio on the extent of mineralization in a model (poly)acrylamide gel was investigated under simulated physiological conditions. We hypothesized that the optimal growth of hydroxyapatite crystals will take place at the stoichiometric Ca/P molar ratio of 1.67. Phosphate ions were incorporated during the polymerization of the gel and mineralization was initiated by submersion of the gel in calcium acetate solution. Ca/P molar ratios were varied in the range of 0.5–5.0. The mineralized gel was characterized by Raman spectroscopy, scanning electron microscopy (SEM) and mineral weight fraction analysis via ashing. Raman spectra captured across the bulk of the gels indicated the presence of mineral at the core section. The phosphate symmetric stretching peak was observed in the range of 955–960 cm\textsuperscript{-1} which is characteristic of hydroxyapatite. SEM images showed that crystals formed at Ca/P = 2.0 were denser and larger in size than at other molar ratios. In agreement with SEM images, the dry weight fraction of mineral reached the maximum at the molar ratio of 2.0 and the extent of mineralization rapidly declined as the molar ratio diverged from 2.0. Also, the crystallinity of the mineral was optimum at the molar ratio of 2.0. Thus it appears that for effective mineralization, the molar ratio of the two ions needs to be in excess of the stoichiometric requirement, suggesting that ions are expended in processes other than the formation and growth of hydroxyapatite crystals. Therefore, the optimal level of mineralization in biomimetic-based growth of calcium phosphate crystals in sol–gel environment requires consideration of a range of molar ratios as opposed to using the molar ratios corresponding to that of the crystal species intended to grow.

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1. Introduction

The replacement of bone loss due to fractures, trauma, diseases and tumors is routine in
reconstructive orthopaedic surgery. According to the US Census Bureau, about 800,000 surgeries were performed in the US during the year 2000 to treat bone loss [1]. The primary choice for a bone substitute is the autologous bone taken from the iliac crest of the patient; however, autologous grafts have serious limitations such as donor site morbidity, increased operative time, blood loss and limited availability [2–4]. The secondary choice is allograft bone taken from cadavers, which introduces the risk of disease transmission [5–7]. Depending on the severity of the injury and the availability of autologous/allogeneic bone, man-made acellular biodegradable materials such as calcium salts [8,9], biodegradable polymers [10–13] and their composites [10–15] are utilized. Acellular biodegradable materials are used to fill voids and they are resorbed and replaced over time. However, they perform poorly under the action of physiological mechanical loads. In the light of the limitations of these existing natural and synthetic biomaterials, there is a real need for alternative bone substitutes.

Bone is a complex biocomposite of carbonated apatite mineral crystals and hydrated organic matrix that is predominantly composed of type I collagen. The combination of the compliant collagen and stiff mineral phases creates a unique nanocomposite with superior mechanical function. One approach to fabricate bone substitute material is to mimic the actual process by which bone is synthesized in the nature. The natural process involves the synthesis of a compliant collagen phase which is later mineralized through the growth of carbonated apatite crystals that provides strength and rigidity. Mimicking this process requires the synthesis of a polymer and the introduction of a secondary mineral phase.

The secondary mineral phase is generally introduced by growing calcium phosphate crystals within the polymeric gels. In one such approach, the gel is kept at the interface between anionic and the cationic solutions [16,17]. A second method involves the entrapment of phosphate ions to the gel during the polymerization [18]. The placement of the phosphate embedded gel within calcium solution results in the growth of calcium phosphate crystals. Imai et al. [18] reported the growth of hierarchically laminated calcium phosphate through the Liesegang phenomenon, which displays double periodicity at micron and submicron scales.

The purpose of this study was to improve the way of introducing the mineral phase by inducing the selective growth of only hydroxyapatite crystals and also to maximize the amount of mineral deposition. Hydroxyapatite is similar to that of the mineral found in the bone, is osteoconductive and can also be a good carrier of human bone morphogenetic protein [19–22]. The influence of Ca/P molar ratio on the amount and stoichiometry of hydroxyapatite crystals grown in (poly)acrylamide-gel substrate was investigated under simulated physiological conditions. It was hypothesized that the optimal growth of hydroxyapatite crystals will take place at a Ca/P molar ratio of 1.67: the Ca/P molar ratio present in hydroxyapatite crystals. The crystal growth was characterized using the Raman spectroscopy, scanning electron microscope (SEM) and weight fraction analysis.

2. Materials and methods

2.1. Preparation of (poly)acrylamide gel

The (poly)acrylamide gel was prepared from 30% acryl/bis (Bio-Rad Laboratories, Hercules, CA, USA). The pH of the solution was maintained at 7.0 using tris-HCl. Gelation was then initiated by using the catalysts N,N,N,N-tetramethylene diamine (Bio-Rad Laboratories, Hercules, CA, USA) and ammonium peroxodisulfate (PSF) (Fisher Biotech, Fair Lawn, NJ, USA) in a 4” diameter glass petri dish. A transparent gel, 0.1” thick was obtained.

2.2. Mineralization of (poly)acrylamide gel

After the addition of PSF, 10 ml of phosphate solution (diammonium hydrogen phosphate) of varying molarities (0.05, 0.1, 0.15, 0.167, 0.2, 0.225, 0.25, 0.3, 0.5) was added during the gelation so as to ensure uniform distribution of phosphate ions throughout the gel. This gel was then placed
in a special fixture with holes at the bottom. This fixture was submersed in a beaker containing 10 ml of calcium acetate solution, of varying molarities (0.05, 0.1, 0.15, 0.167, 0.2, 0.225, 0.25, 0.3, 0.5) at 37°C and pH 7.0 for 24 h with constant stirring (Fig. 1). The Ca/P molar ratio was thereby systematically varied from 0.5, 1.0, 1.5, 1.67, 2.0, 2.25, 2.5, 3.0 to 5.0 by adjusting the molarities of phosphate and calcium solution. The molar ratio of 1.67 corresponds to the theoretical stoichiometric ratio of Ca and P in hydroxyapatite. The mineralized gel was then separated equally into two halves. The nature of the crystals of the mineralized gel was studied by taking the Raman spectra across the thickness of each half of the gel. Following that, one half of the mineralized gel was investigated by SEM for the size, shape and density of the crystals and the other half for mineral weight fraction analysis via ashing.

2.3. Raman microspectroscopic assessment

In the laser Raman microspectroscopy, a continuous laser beam is microfocused on the sample through a microscope. The photons interact with the molecules of the sample by the phenomenon of Raman scattering, an inelastic light scattering process in which a small amount of energy is removed from the laser photon and is transferred to a molecular vibration. Crystallographic characteristics of the mineral crystals can be obtained from wavenumbers and bandwidths of peaks in the recorded spectrum. A confocal Raman microscope (LabRam, Jobin-Yvon, Edison, NJ, USA) consisting a point-focus 633 nm He–Ne laser source, a microscope (Olympus BX41) and a stigmatic spectrometer was used. The sample was placed on the stage of the microscope using a custom-made fixture such that the sample thickness was oriented perpendicularly to the laser beam incident from a 50× microscope objective. Four spectra in the range 400–1200 cm\(^{-1}\) were captured across the thickness of the gel to verify that the mineral growth was not limited to surface alone but also included the diffusion of calcium and phosphate ions into the bulk of the gel. Locations of interest were positioned with a motorized x–y stage and an optical camera. Each point scan lasted 180 s and the average of three consecutive scans was taken to obtain the final spectrum at a given location. An 1800 g/mm grating provided a spectral dispersion of about 1 cm\(^{-1}\)/pixel. The data were acquired using the Labspec software (Jobin Yvon Horiba, Edison, NJ, USA). The crystallinity of the mineral phase was calculated as the inverse of the bandwidth at half the maximum intensity of the symmetric phosphate stretch band. A greater crystallinity factor indicates larger crystals and/or stoichiometrically more ordered composition.

2.4. Scanning electron microscopy analysis

One half of the mineralized gel was lyophilized (Freeze Dryer 3, Labconco, Kansas City, MO, USA) for 24 h, coated with gold and the morphology of crystals was viewed using a JSM 6100 SEM (JEOL, Peabody, MA, USA).

2.5. Weight fraction analysis

The other half of the mineralized gel was cut into four portions and lyophilized for 24 h as above. Weights of the crucibles were measured using an analytical balance (Denver Instrument
Company, CO, USA) before \( (w_1) \) and after \( (w_2) \) placing the lyophilized mineralized gel. The mineralized gels were ashed in programmable muffle furnace (Isotemp, Winona, MT, USA) at 800 °C for 48 h and the ashed weight along with the crucible was determined \( (w_3) \). The weight of the mineral portion was calculated as \( (w_3 - w_1) \) and the percentage weight fraction of the mineral in the dried gel was calculated as \( \left(\frac{w_3 - w_1}{w_2 - w_1}\right) \times 100 \).

The average and standard deviations of mineral-weight fraction, wavenumber and crystallinity were calculated and the significance of the difference between any two molar ratios was tested by a Mann Whitney-\( U \) test \( (p<0.05) \).

3. Results

After submersion in the calcium solution for 24 h, the transparent (poly) acrylamide gels became opaque with a consistent white hue, indicating a homogenous deposition of mineral crystals across the bulk of the gel. Raman spectra captured at the mid-thickness of gels confirmed the fact that the process of mineralization was a bulk phenomenon rather than a mere surface effect. A typical Raman spectrum of a mineralized gel is shown in Fig. 2. The phosphate symmetric-stretch peak was observed in the range of 955–960 cm\(^{-1}\) (Fig. 3), which is characteristic for hydroxyapatite [23]. An increasing wavenumber for this peak indicates a more crystalline stoichiometry of hydroxyapatite particles. As the molar ratio increased from 0 to 2.25, the wavenumber and the crystallinity declined systematically with the exception of a spike at the molar ratio of 2.0. At Ca/P molar ratios >2.25, wave number and crystallinity increased.

SEM images shown in Fig. 4 indicate that crystals formed at Ca/P = 2.0 were denser and larger in size than at other molar ratios. The needle-shaped crystals, characteristic of hydroxyapatite, were arranged in clusters in the form of spheres. In agreement with SEM images, the dry weight fraction of mineral reached the maximum at Ca/P molar ratio of 2.0 (Fig. 5) and the extent of mineralization rapidly declined as the molar ratio diverged from 2.0 on either side of the compositional scale.

When the results are interpreted altogether the emerging picture is such that the maximum
amount of mineral and optimal stoichiometry is attained at a molar ratio of 2.0. While the crystallinity of mineral crystals were comparable at several molar ratios (0.5, 1 and 5) to that observed for the molar ratio of 2.0, the extent of mineralization at these molar ratios were nowhere near that attained by the molar ratio of 2.0.

4. Discussion

To develop novel biomaterials that mimic bone, it is essential to develop strategies for crystal growth in polymer matrices. Furthermore, it is desirable to grow crystals under physiologically friendly conditions as the polymer may include cells for tissue engineering purposes. While there are many factors which affect the mineralization process, molar ratios of the reacting species has primary importance. This study investigated the influence of Ca/P molar ratios on the extent of mineralization in a model (poly)acrylamide-gel under simulated physiological conditions. It was hypothesized that the optimal growth of hydroxyapatite crystals would take place at the stoichiometric Ca/P molar ratio of 1.67 which corresponds to the molar ratio of Ca and P in hydroxyapatite crystals. In testing our hypothesis, (poly)acrylamide gel was selected since it is a good model substrate to test the feasibility of hydroxyapatite crystal growth and to bracket the optimal values for different variables involved in the crystallization process. Although (poly)acrylamide-gel is non-biodegradable and therefore cannot be used for tissue-engineering purposes, the hindsight gained from the (poly)acrylamide-gels can be utilized for growing hydroxyapatite crystals in biodegradable and biocompatible polymers such as (poly)vinylalcohol, (poly)glycolic acid and (poly)lactic acid. The results refuted our hypothesis and a non-stoichiometric Ca/P ratio of 2.0 yielded the greatest mineral weight fraction, with larger and more crystalline morphology.

Different methods have been followed for growing crystals in polymeric gels. The method of keeping the gel at the interface of anionic and cationic solutions [16,17] results in the growth of mineral crystals by counter diffusion of ions. Following this method, octacalcium phosphate crystals were grown in polyacrylamide gel to understand the tooth enamel apatite formation...
But the size of the crystals that grew in the gel was not completely uniform. The crystallization of plate-like crystals of hydroxyapatite using a colloidal media of sodium silicate at the physiological temperature of 37 °C has been reported [24] where periodic white discs (Liesegang rings) of hydroxyapatite microcrystals were observed inside the gel from gel–solution interface. In another study, hydroxyapatite was grown in an agarose gel but in a highly alkaline medium at a temperature of 85 °C [25] and these crystals were free from brushite and $\alpha$ or $\beta$ tricalcium phosphate phases but the presence of calcium oxide was observed. The work presented in this study followed the method of Imai et al. [18] which incorporates phosphate ions during the gelation, and the subsequent diffusion of calcium cations into the gel results in bulk mineralization of the gel with the development of uniform size crystals. The existence of mineral crystals across the internal cross-section of the gel confirmed that the process of mineralization was not limited to the surface but involved the bulk of the gel.

At the onset of the study, the amount of mineralization was expected to be optimal at the molar ratio of 1.67, the theoretical Ca/P ratio for hydroxyapatite. However, as the results indicated, the amount and the purity of hydroxyapatite phase turned out to be optimal at a molar ratio of 2.0. Thus, it appears that the molar ratio of the two ions needs to be in excess of the stoichiometric requirement for optimum mineralization in a gel environment, suggesting that ions may be expended in processes other than crystal growth, such as entrapment in the structure of the gel itself. An increase in supersaturation of the reacting ions causes the driving force for crystallization to be greater [27]. The estimation of supersaturation effect with respect to hydroxyapatite is difficult in the current study due to numerous ionic interactions and also due to changes in supersaturation during precipitation of a given phase. In order to grow, the initially formed hydroxyapatite first dissolves, releasing its constituent calcium and phosphate ions in the immediate microenvironment. This release of ions causes an increase in the supersaturation, which subsequently leads to the precipitation of new apatite crystals through incorporation of other calcium and phosphate ions, located in the surrounding microenvironment. The growth of hydroxyapatite crystals consequently requires the presence of sufficient calcium and phosphate ions in the vicinity of the seed crystals and the current results suggest that Ca/P molar ratios <2.0 were unable to provide the required amount of calcium and phosphate ions in the poly(acrylamide) gel.

It was also observed that the amount and the purity of crystals were negatively affected as the Ca/P molar ratio gradually increased beyond 2.0. Marques et al. [28] have shown that supersaturation values for hydroxyapatite are greater than for other intermediate phases for all conditions and incubation times. Thus, with an increase in Ca/P ratio, the formation of hydroxyapatite is expected on thermodynamic grounds, but intermediate phases (e.g., octacalcium phosphate) are not excluded, due to kinetic factors [28]. Consequently, the selective growth of hydroxyapatite crystals will be hindered with an increase in Ca/P ratio because of the formation of other forms of calcium phosphates, as has been confirmed by our observations of declining crystallinity with increasing molar ratio beyond 2.0.

Besides the normal physicochemical parameters that influence crystal growth under in vitro conditions such as spatial constraints and ion fluxes, the process of biomineralization is further complicated by the role of different proteins. This makes it all the more difficult to compare the optimal Ca/P molar ratio for the in vitro growth of hydroxyapatite crystals, as deduced from our study, to the Ca/P molar ratio present in human body fluids. Physiologically, hydroxyapatite crystals are formed because they have a lower solubility product than the calcium phosphate ionic product of body fluids. Li et al. [26] have shown that the precipitation of hydroxyapatite resulted in an increase pH of the solution, which led to the formation of calcium phosphates with a low Ca/P molar ratio. The physical conditions of the current study were a rough approximation of that present in the human body fluids because of the high buffering capacity in vivo. Therefore, it will be premature at this point to relate the present result of the requirement of Ca/P ≈ 2.0 for in vitro
growth of hydroxyapatite crystals in (poly)acrylamide gel to the presence of Ca/P = 2.5 in human body fluids. Further studies have to be carried in order to corroborate this relationship.

In conclusion, the optimal level of mineralization in biomimetic-based growth of crystals in sol–gel environment requires consideration of a range of molar ratios. The lower range of this optimal molar ratio is determined by the supersaturation requirements for growth of hydroxyapatite crystals whereas the higher end is limited by the emergence of intermediate calcium phosphate based species other than hydroxyapatite. Furthermore, the actual concentration of the ions required for the growth of mineral in a gel may be affected by the structure of the gel being mineralized. In a nutshell, in vitro mineralization models in polymers require similar experiments to ascertain the maximum amount of mineralization and the appropriate stoichiometric ratio.

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