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Acceleration of gelation and promotion of mineralization of chitosan hydrogels by alkaline phosphatase

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ABSTRACT

Thermosensitive chitosan hydrogels containing sodium beta-glycerophosphate (β-GP), whose gelation is induced by increasing temperature to body temperature, were functionalized by incorporation of alkaline phosphatase (ALP), an enzyme involved in mineralization of bone. ALP incorporation led to acceleration of gelation upon increase of temperature for four different chitosan preparations of differing molecular weight, as demonstrated by rheometric time sweeps at 37 °C. Hydrogels containing ALP were subsequently incubated in calcium glycerophosphate (Ca-GP) solution to induce their mineralization with calcium phosphate (CaP) in order to improve their suitability as materials for bone replacement. Incorporated ALP retained its bioactivity and induced formation of CaP mineral, as confirmed by SEM, FTIR, Raman spectroscopy, XRD, ICP-OES, and increases in dry mass percentage, which rose with increasing ALP concentration and incubation time in Ca-GP solution. The results demonstrate that ALP accelerates formation of thermosensitive chitosan/β-GP hydrogels and induces their mineralization with CaP, which paves the way for applications as injectable bone replacement materials.

1. Introduction

Chitosan, a polysaccharide derived by deacetylation of chitin, a major component of the exoskeletons of crustaceans, has been widely applied as a biomaterial. Chitosan and its derivatives represent a particularly attractive group of biocompatible and degradable polymers. Chitosan is biocompatible, degradable, non-toxic, and non-immunogenic, allowing its use in the medical, pharmaceutical, cosmetic and tissue construction fields. It also possesses antibacterial properties, which decrease with increasing degree of deacetylation (DD) [1,2]. and can therefore be considered as biodegradable. Biodegradability is increased by increasing DD [3], as is solubility, allowing formation of acidic chitosan solutions.

Hydrogels are hydrophilic polymers associated physically or chemically to form three-dimensional water-binding networks. Advantages of using hydrogels as biomaterials for tissue regeneration include injectability, exact filling of defects, and ease of integration of cells and water-soluble bioactive substances, including enzymes. Chitosan hydrogels can be formed by neutralization of an acidic chitosan solution by addition of sodium beta-glycerophosphate (β-GP) [4]. These hydrogels have the added advantage of thermosensitivity, i.e. gelation can be induced endothermically by increasing temperature to the range 30–60 °C, within which body temperature lies. The mechanism of temperature–induced gelation is believed to include reduction of mutual electrostatic repulsion between positively charged chitosan chains by neutralization with the weak base β-GP, as well as thermally induced transfer of protons from chitosan to the phosphate part of β-GP and thermally induced dehydration of chitosan chains, enhanced by the water structuring effect of β-GP's glycerol part,

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leading to increased hydrophobic interactions and hydrogen bonding between chains, in turn leading to sol-gel transition [5,6]

Chitosan/β-GP hydrogels have been shown to form in vivo [7]. Such chitosan gels have been applied as drug delivery systems [6] and scaffolds for tissue engineering of cartilage [4,8] and nucleus pulposus [9].

Advantages of using chitosan hydrogels for bone substituting applications include chitosan's ability to preferentially support adhesion of osteoblasts over fibroblasts [10] and the ease of incorporation of growth factors such as the osteoinductive bone morphorgenetic protein-2 (BMP-2) as well as mesenchymal stem cells [11,12].

With respect to bone substitution, the presence of a ceramic phase based on calcium phosphate (CaP) leads to a number of advantages, including increased bioactivity (formation of chemical bonds with surrounding bone after implantation) and affinity for biologically active proteins such as growth factors, which stimulate the natural healing processes of surrounding bone [13]. Since stiffer [14,15] and rougher [16] surfaces are known to promote differentiation of cells toward the osteoblastic phenotype, mineralization is expected to make chitosan more compatible to bone tissue.

The simplest strategy to introduce a CaP phase into gels is the incorporation of CaP particles [17]. However, CaP particles tend to aggregate, leading to uneven dispersion and poor reproducibility. Although CaP particles have been added to solid chitosan scaffolds (for reviews see [12,18,19]) and hydroxyapatite formation on chitosan membranes has been achieved by alternate soaking in solutions containing calcium and phosphate ions [20], these mineralized scaffolds or membranes are not injectable.

A more promising mineralization strategy for calcification of hydrogels is the incorporation of the enzyme alkaline phosphatase (ALP), the enzyme responsible for mineralization of bone. ALP is known to be involved in the process of bone mineralization [21]. As a biomaterial component, ALP has been applied as an enzymatic coating onto metallic bone implants [22–24] and covalently linked to fibrin and collagen scaffolds to increase mineral deposition in vivo [25–29]. The strategy of ALP incorporation followed by incubation in a solution containing calcium ions and glycerophosphate (GP) appears to be universally applicable to all hydrogels [30–34]. Both calcium ions and glycerophosphate diffuse into the gels containing ALP. ALP cleaves phosphate ions from glycerophosphate, which are then free to react with calcium ions to form insoluble CaP which precipitates and remains trapped within the gel, while by-products such as glycerol are free to diffuse out. Moreover, as one of the components of the thermogelling system, namely β-GP, is known to be a substrate for ALP, it was conceivable that the presence of ALP would cause changes in the gelation behavior by cleaving β-GP to glycerol and phosphate. β-GP is a small molecule able to diffuse through the chitosan gel network and thus reach entrapped ALP.

The current study aimed to use rheometry to investigate the effect of ALP incorporation on gelation properties such as gelation speed. The amount and nature of mineral formed due to ALP incorporation followed by incubation in a solution containing calcium glycerophosphate (Ca-GP), which served as a source of organic phosphate and calcium ions, was also investigated. Release of ALP from hydrogels was also monitored.

2. Materials and methods

2.1. Origin of chitosan preparations

In order to test the reproducibility of the studied mineralization process, four chitosan preparations of differing molecular weight were investigated. Three were obtained from Sigma–Aldrich: chitosan from shrimp (Product no. 50494), from crab (Product no. 50494) and Fluka chitosan, hereafter referred to as shrimp, crab and Fluka, respectively. One preparation was produced at Morski Instytut Rybacki (Marine Fisheries Institute) in Gdynia, Poland, and is hereafter referred to as 83% chitosan.

2.2. Determination of molecular weight (MW) of chitosan preparations

The molecular weight (MW) of chitosan preparations was determined by gel permeation chromatography (GPC/SEC) with the application of a high-performance liquid chromatography (HPLC) Knauer Smartline apparatus, equipped with an analytical isocratic Pump 1000 and differential refractive index (DRI) detector (S-2300/2400, Knauer). Determination took place in the connected columns Tessek HEMA 1000 BIO 10 μm as well as HEMA BIO 40 μm at a temperature of 30 °C and eluent flow rate of 1 ml/min (0.1 M NaCl, 0.3 M CH_3COOH). 20 μl of a standard dextran solution (PSS, Germany) or a solution of the sample at a concentration of 0.2% was fed to the column. Solutions were filtered beforehand through an injection filter with a membrane consisting of regenerated cellulose with a pore size of 0.2 μm. To obtain calibration curves, molecules of defined molecular weights ranging from 180 to 277000 g/mol were used, whose retention times ranged from 10.5 to 16.8 min. On the basis of calibration curves, the MW of chitosan preparations was determined by applying a system for the calculation of GPC integrated into "Chromgate" software (Knauer) to verify and analyze HPLC data.

The deacetylation degree (DD) (defined as the ratio of the number of–NH_2 groups formed in chitosan to the initial number of -NH-CO-CH_3 groups present in chitin) was determined by a titration method [35]. Chitosan (0.13–0.3 g) was dissolved in 43% orthophosphoric acid (100 ml). Next, a distillation process was carried out until the solution reached a temperature of 160 °C. The distillate was titrated with a 0.1 M aqueous solution of sodium hydroxide against phenolphthalein. A blank test was performed by distilling a 43% solution of orthophosphoric acid that was titrated with 0.1 M sodium hydroxide solution.

The acetylation degree (AD) [%] was calculated from the formula:

$$AD = \frac{2.03 \times V}{m}$$

where: V – is the number of ml of 0.1 M sodium hydroxide used for distillate titration, m – is the weighed portions of chitosan samples after taking into account moisture and ash content [g] while the deacetylation degree (DD) was calculated from the formula:

$$DD = 100 - AD$$

2.3. Production of hydrogels and ALP incorporation

Chitosan hydrogels were produced according to a protocol based on that of Chenite et al. [4]. Briefly, 0.4 g chitosan was dissolved in 16 ml 0.1 M HCl. 10 g sodium glycerophosphate were dissolved in 10 ml water. ALP was dissolved in water at concentrations of 0, 1.25 and 2.5 mg/ml.

3.6 ml chitosan solution was mixed with 0.4 ml Na-GP solution and 0.4 ml ALP solution, to yield a chitosan concentration of 20.5 mg/ml, a Na-β-GP concentration of 90.9 mg/ml and ALP concentrations of 0, 0.11 and 0.23 mg/ml.