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Halloysite-alkaline phosphatase system—A potential bioactive component of scaffold for bone tissue engineering

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ABSTRACT

Alkaline phosphatase (ALP) was sucessfully incorporated into the halloysite (HAL) nanotubes, as confirmed by FTIR-ATR and XRD analyses. The loading efficiency (LE) of ALP was found to be 13.5%, while its encapsulation efficiency (EE) was estimated to be around 27%, as determined using the Bradford test. The influence of the immobilization in HAL on the enzyme activity was measured using standard ALP activity assay. Immobilized ALP effectively induced the bomineralization process, as showed by SEM, EDS, and XRD studies. As a result, calcium phosphate was produced in the form of hydroxyapatite cauliflower-like structures, with a slight content of calcium hydroxide. Interestingly, the encapsulation of ALP guest molecules in the HAL nanotubes considerably increased its thermal stability, most probably due to the heat sink effect. The activity of HAL-bound ALP was also found to be pH-independent in the wide range of pH values (3–10) due to the amphoteric nature of the aluminum oxide lining the HAL nanotube internal surface. Due to an increased resistance to the unfavorable conditions, which are often encountered during scaffold preparation or sterilization, ALP-HAL nanocomposite material may constitute an attractive bioactive component of the scaffolds for bone regeneration.

1. Introduction

Halloysite (HAL) is a nanoclay mineral belonging to the kaolin subgroup, which is relatively abundant in nature. It is a two-layered (1:1) aluminosilicate, exhibiting a range of morphologies, among which the predominant form is a hollow tubular structure. The size of HAL nanotubes varies from 0.2 to 1 μm in length and from 15 to 100 nm in the inner diameter, depending on the deposit. HAL nanotubes can be used to encapsulate low molecular weight drugs for their subsequent delivery and controlled release [1]. Halloysite exhibits a high level of biocompatibility and very low cytotoxicity in vitro [2] and was already proposed as a component of the scaffolds [3]. Very recently it was also shown that, due to the relatively large diameter of the lumen of the nanotubes, also larger molecules, such as proteins, can be entrapped in HAL [4]. HAL nanotubes may not only serve as a delivery device for proteins, but they may also provide them with a necessary protection against unfavourable conditions (elevated temperature, unsuitable pH, proteolytic enzymes). As an additional advantage, the opposite charges of HAL interior (positive) and outer surface (negative) allow using the electrostatic interactions to increase the efficiency and selectivity of the protein entrapment.

The immobilization of several different proteins inside HAL was described very recently in the literature. Duce et al. [5] have shown that bovine serum albumin, α-lactalbumin and β -lactoglobulin loaded into HAL exhibit increased thermal stability. The authors have also observed changes in the secondary structure of the proteins entrapped inside HAL nanotubes. Lvov and Tully [6] have loaded several different enzymes in HAL, showing that in the case of negatively charged proteins more than half of the load was permanently immobilized inside nanotubes. They have also performed enzymatic activity studies on the HAL-immobilized enzymes [4] and found that the biocatalytic activity of the immobilized proteins exposed to the elevated temperatures was maintained for longer times. It was also shown that HAL-entrapped enzymes showed increased activity outside of the optimal pH range. However, the possible application of the obtained enzyme-HAL systems has not been proposed so far.

HAL is biocompatible [7] and thus suitable for biomedical applications. Due to its inorganic nature and lack of biodegradability, the applications of HAL as a component of the topically applied medications, wound-healing materials, and tissue engineering scaffolds seem

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to be preferable. One of more interesting applications of HAL could be in bone regeneration. HAL nanotubes improve the mechanical properties of hydrogel-based materials [8] and introduce additional mesoporosity into the scaffold. To increase the effectiveness of the bone repair process the scaffold can be made bioactive by incorporating the biologically active agent and HAL nanotubes seem suitable for that purpose.

Alkaline phosphatase (ALP) has been used in tissue engineering. It is a cell membrane-associated enzyme that hydrolyses inorganic pyrophosphate (biomineralization inhibitor), yielding inorganic phosphate, a substrate for hydroxyapatite formation [9]. Hydroxyapatite mineral is a main constituent of the osseous tissue, while ALP is one of the key proteins involved in the reconstruction of the bone. So far there were various attempts to introduce ALP into the scaffolds [10,11], but their outcomes, although positive, were not fully satisfactory. Here we describe, for the first time, the immobilization of ALP in HAL. The detailed studies on the physicochemical properties of the obtained, novel system are reported and the influence of the entrapment on the enzyme activity and stability is addressed. Although immobilization of several enzymes in HAL was already described, to the best of our knowledge, there is no literature reports dealing with application of such systems so far. Here, for the first time, we propose the HAL-protein system as a component of the bone scaffold. The preliminary tests on the biomineralization process promoted by ALP entrapped in HAL (ALP-HAL) confirmed the potential usefulness of the obtained ALP-HAL system as a bioactive component of the scaffold for bone regeneration.

2. Experimental

2.1. Materials

Alkaline phosphatase (ALP) from bovine intestinal mucosa (≥10 DEA units/mg solid), halloysite nanoclay (HAL), 2-amino-2-methyl-1-propanol (BioUltra, ≥99.0% (GC)) (AMP), 4-nitrophenol (spectrophotometric grade) (4-NP), 4-nitrophenyl phosphate bis(tris) salt (≥97%) (PNP), fluorescein isothiocyanate isomer I (for protein labeling, ≥ 90%) (FITC), calcium glycerophosphate (≥98%), Sephadex® G-50, PBS (tablets), and DMSO (for HPLC, ≥99.7%) were purchased from Sigma-Aldrich, Poland, and used as received.

2.2. Immobilization of ALP in halloysite

100 mg of ALP was dissolved in 2 ml of PBS buffer (pH = 7.4) In this solution 200 mg HAL was dispersed and thoroughly mixed. The resulting suspension was then placed in the vacuum oven (1400E-2 Sheldon Laboratory) and the pressure was reduced to 200 mmHg at 25 °C. After 15 min the pressure was allowed to return to ambient conditions, the sample was vortexed and the procedure was repeated two more times, allowing the substitution of the air inside the nanotubes of HAL with the enzyme solution. The number of cycles was established based on the preliminary studies. Finally, the suspension of ALP immobilized in HAL (ALP-HAL) wa centrifuged at 12 000 rpm for 5 min, washed twice with water and lyophilized.

2.3. The Bradford method for protein quantitation

The amount of ALP immobilized in 1 mg of HAL was determined using the Bradford method. Bradford assay is based on binding of the Coomassie Blue G250 dye to the analyzed protein. The amount of the protein in the sample is estimated based on the absorbance of the strong noncovalent complex formed between protein and dye in acidic conditions. As a result of the complex formation the brown solution turns blue (λ_{max} = 595 nm). The assay reagent was prepared by dissolving 100 mg of Coomassie Blue G-250 in 50 ml of 95% ethanol. The solution was then mixed with 100 ml of 85% phosphoric acid and made up to 1 l with distilled water. A calibration curve for the free ALP was obtained in PBS (Supplementary materials, Fig. S1A). To each of the ALP standard 5 ml of Bradford reagent was added and the resulting solution was mixed thoroughly. The samples were left for equilibration and after 35 min the absorbance of each solution was measured at 595 nm.

To estimate the amount of ALP entrapped in 1 mg of the HAL nanotubes, the concentration of the ALP in the initial solution and in the supernatant after the immobilization process was measured. The measurements were done according to the same procedure as the one described above for the standard solutions. To 100 µl of the ALP-containing sample 5 ml of Bradford reagent was added and the resulting solution was thoroughly mixed. After 35 min the absorption spectrum of the sample was measured and the absorbance value at 595 nm was used to calculate the amount of the protein in solution, based on the calibration curve. The weight of the encapsulated protein was calculated as the difference in the weight of ALP in the solution before and after immobilization.

The loading efficiency (LE) and encapsulation efficiency (EE) were then determined according to the equations shown below:

$$LE(\%) = \frac{The\ weight\ of\ ALP\ immobilized\ in\ HAL}{The\ weight\ of\ HAL\ sample} \quad (1)$$

$$EE(\%) = \frac{The\ weight\ of\ ALP\ immobilized\ in\ HAL}{The\ weight\ of\ ALP\ used\ for\ immobilization} \quad (2)$$

As the encapsulation efficiency of ALP depends on its concentration, we have studied various ALP:HAL weight ratios (1:4, 1:3, 1:2, 1:1), which corresponded to the ALP concentrations ranging from 25 mg/ml to 100 mg/ml. Based on the Bradford method described above we have selected 1:2 ratio as optimal (providing the highest loading and encapsulation efficiency values). Under these conditions the concentration of ALP solution used to encapsulate the protein in 200 mg of halloysite was 50 mg/ml. Both LE and EE were calculated for the optimized ALP:HAL weight ratio (1:2).

2.4. Enzymatic activity of immobilized ALP

The enzymatic activity of ALP was studied using Bessey and Lowry colorimetric method [12]. ALP enzyme cleaves the phosphate group from 4-nitrophenyl phosphate (4-NPP) to generate 4-nitrophenol (4-NP), which is then deprotonated in the alkaline environment to form 4-nitrophenolate. The resulting compound is characterized by an intense absorption band with a maximum at 405 nm, the intensity of which is directly proportional to the ALP activity. The calculations of ALP enzymatic activity were done based on the calibration curve obtained for the reaction product, 4-nitrophenol (4-NP) in 0.5 M AMP buffer.

To determine the activity of free ALP or entrapped in the nanoclay, 2 mg of ALP or ALP-HAL was mixed with 0.4 ml of 0.5 M AMP buffer and 0.4 ml of 5 mM 4-NPP substrate. The sample was then incubated for 1 h at 37 °C, while being constantly stirred. Then, 0.4 ml of 0.3 M NaOH was added to stop the enzymatic reaction. Finally, the sample was centrifuged (12 000 rpm, 5 min) and supernatant collected. The sample was diluted to keep the absorbance values within the linear range and the absorbance of the supernatant was measured at 405 nm. Based on the calibration curve the activity of the immobilized protein was calculated. Enzymatic activity (ACT) of 1 mg of ALP or ALP-HAL ($\frac{U}{mg}$) was determined as the amount of 4-NP (µmol) formed in enzymatic reaction per 1 min at pH = 9.8 and at 37 °C according to the formula:

$$ACT\left(\frac{U}{mg}\right) = \frac{c \bullet V \bullet 10^{-3}}{t \bullet m_s} \bullet D \quad (3)$$

where: c was the concentration of 4-NP determined in the supernatant, V was the total volume of the obtained supernatant, t - the incubation time, m_s - the weight of the ALP-HAL sample, and D was the dilution factor. The measurement was done in triplicate and the average value of ACT was calculated.