Reactive and Functional Polymers 93 (2015) 77–83

Contents lists available at ScienceDirect

Reactive and Functional Polymers

journal homepage: www.elsevier.com/locate/react

Tyramine modified alginates via periodate oxidation for peroxidase induced hydrogel formation and immobilization

Olivera Prodanovic [a], Dragica Spasojevic [a], Milos Prokopijevic [a], Ksenija Radotic [a], Nevena Markovic [b], Marija Blazic [b], Radivoje Prodanovic [c,*]

[a] Institute for Multidisciplinary Research, University of Belgrade, Kneza Višeslava 1, 11030 Belgrade, Serbia
[b] Institute of Chemistry, Technology and Metallurgy, University of Belgrade, Njegoševa 12, 11001 Belgrade, Serbia
[c] Department of Biochemistry, Faculty of Chemistry, University of Belgrade, Studentski trg 12-16, 11000 Belgrade, Serbia

ARTICLE INFO

Article history:
Received 10 March 2015
Received in revised form 4 June 2015
Accepted 7 June 2015
Available online xxxx

Keywords:
Hydrogel
Tyramine
Polymerization
Emulsion
Peroxide

ABSTRACT

Phenol and amino groups were introduced into alginate to different degrees via oxidation with 2.5, 5, 10, 15 and 20 mol% of periodate and reductive amination by tyramine. Modification of alginate with tyramine was confirmed by FTIR spectroscopy and UV–VIS spectroscopy, while concentration of phenol and ionizable groups was determined using absorbance at 275 nm and acid–base titration. All tyramine-alginates were able to form hydrogels after cross-linking with horse radish peroxidase (HRP) and hydrogen peroxide. Tyramine-alginates oxidized with up to 10 mol% of periodate were also capable of forming hydrogels with calcium ions. Tyramine-alginates were tested for HRP immobilization within micro-beads obtained by peroxidase catalyzed droplet polymerization using internal delivery of hydrogen peroxide via glucose oxidase and glucose. Highest activity of immobilized peroxidase was obtained with 20% (w/v) tyramine-alginate obtained via 20 mol% periodate oxidation. Immobilized enzyme was not leaking from the micro-beads and was further kinetically characterized for pyrogallol oxidation. K_m for pyrogallol was increased after immobilization from 1.93 mM for soluble HRP to 7.34 mM for immobilized HRP. The optimum pH was also increased from pH 7.0 to 8.0. Temperature and organic solvent stability improved significantly after immobilization, so that half-life at 70 °C increased around four times, while half-life in 80% (v/v) dioxane increased 22 times. After repeated use of 6 times in batch reactor for pyrogallol oxidation immobilized HRP retained 45% of original activity.

1. Introduction

Alginate is a polysaccharide obtained from brown seaweed algae that consist of regions rich in D-mannuronic acid (M block) and L-guluronic acid (G block). In the presence of calcium ions cross-linking and hydrogel formation occur. During cross-linking in the presence of Ca^{2+} and hydrogel formation small molecules, proteins and cells could be easily entrapped. Because of its hydrophilicity and easy of gelation alginate has been used for a long time as a material for immobilization of small molecules [1], enzymes [2], proteins [3] and cells [4]. Recently, due to its biocompatibility, low toxicity and structural similarity to extracellular matrices of living tissues [5], it has been receiving much attention also as a material for wound healing and tissue engineering [6]. One possible drawback of alginate could be its large pore size of polysaccharide network within alginate hydrogel that could lead to fast release of encapsulated small molecules and proteins. Therefore, chemical modification of native alginate structure and different cross-linking chemistries is used to precisely control bioactive substance release in drug delivery applications [7] and enzyme immobilization [8,9].

Alginate could be chemically modified by esterification and amidation of its carboxyl groups or oxidation and reductive amination of its vicinal hydroxyl groups [10]. Using these reactions various functional groups can be introduced and give alginate hydrogels novel properties. For example, alginate was previously oxidized with periodate and cross-linked by adipic acid dihydrazide to form hydrogels for tissue engineering applications [11,12], or modified with a linear alkyl groups (C8, C12, C16) by reductive amination for use as alginate derived polymeric surfactants [13]. During the oxidation reaction with periodate molecular weight of alginate rapidly decreases until an oxidation degree of 10 mol%. Alginates with a degree of oxidation higher than 10 mol% are not capable of forming hydrogels with calcium ions [14]. Recently alginate carboxyl groups were modified with tyramine by the use of carbodiimide coupling reaction and forming amide bond. Synthesized tyramide-alginates were both ionically and enzymatically

* Corresponding author.
E-mail addresses: olivearp@imsi.rs (O. Prodanovic), dragicaspasojevic@yahoo.com (D. Spasojevic), milos.prokopijevic@gmail.com (M. Prokopijevic), xenia@imsi.rs (K. Radotic), markovicnevena6@gmail.com (N. Markovic), marijabbl@gmail.com (M. Blazic), rprodano@chem.bg.ac.rs (R. Prodanovic).

http://dx.doi.org/10.1016/j.reactfunctpolym.2015.06.004
1381-5148/

cross-linkable and could form hydrogels both in the presence of calcium ions and in the presence of hydrogen peroxide and peroxidase [15]. Obtained hydrogels were used for cell encapsulation and showed potential for bioproduction and biomedical applications [16].

Horse radish peroxidase (HRP) was previously immobilized in alginate macro-beads for azo dye degradation [17] and phenol removal from waste waters [18]. In all of these studies because of large pore size of polysaccharide gel network and in order to keep enzyme entrapped, alginate macro-beads with several millimeters in diameter were used for immobilization and that lead to high diffusional limitations and decreased enzyme efficiency. In order to avoid diffusional limitations use of alginate micro-beads with diameters in micrometers range would be beneficial, but to prevent leakage of enzyme additional chemical steps are necessary [19,20]. One way to decrease horse radish peroxidase leakage from alginate beads is to use chemically modified alginate that could form covalent link with the enzyme molecule [21].

Since there is a growing need for novel biomaterials and hydrogels that could be used in tissue engineering, drug delivery and cell and enzyme immobilization studies, we have synthesized a new type of modified alginate that could form hydrogels both in the reaction with calcium ions and in the reaction with hydrogen peroxide and peroxidase. Obtained alginates were characterized and tested as hydrogels for horse radish peroxidase encapsulation within micro-beads obtained in a coupled enzymatic emulsion polymerization reaction.

2. Experimental

2.1. Materials

Alginic acid sodium salt from brown algae with medium viscosity (A2033-100G Sigma), horseradish peroxidase (lyophilized powder, 250 pyrogallol units/mg, P6782 Sigma), glucose oxidase (lyophilized powder, 160 U/mg, G2133 Sigma), H_2O_2, pyrogallol, sodium dihydrogen phosphate anhydrous, and sodium periodate were purchased from Sigma-Aldrich, while dioxane was obtained from Merck.

2.2. Synthesis of tyramine alginates

Sodium alginate was dissolved in water at a final concentration of 1% (w/v). Sodium metaperiodate was added to final concentrations of 1.25 mM, 2.5 mM, 5.0 mM, 7.5 mM and 10 mM so that molarity ratio of periodate to C6 glycoside units in alginate was set to 2.5 mol%, 5 mol%, 10 mol%, 15 mol% and 20 mol%. Reaction mixture was left in the dark for 24 h at +4 °C. Reaction was stopped by adding glycerol at 500 mM concentration and further incubation in the dark for 30 min at +4 °C. Oxidized alginate was precipitated from reaction mixture by adding NaCl at 1% (w/v) and 2 volumes of 96% (v/v) ethanol. Precipitation was repeated two times using the same procedure after dissolving oxidized alginate at 1% (w/v) concentration in water. At the end the precipitate was separated, dried and dissolved in 0.1 M sodium-phosphate buffer pH 6. When dissolved at 1% (w/v) concentration, solid tyramine hydrochloride was added at 1.5% (w/v) final concentration (86 mM) and the solution was stirred for half an hour. Afterwards, solid sodium cyanoborohydride was added at 0.5% (w/v) final concentration and the reaction mixture was left in the dark for 24 h at +4 °C. Modified alginate was precipitated by adding NaCl to 1 M final concentration and two volumes of 96% ethanol. Precipitation was repeated two times using the same procedure after dissolving modified alginate at 1% (w/v) concentration in water. Modified alginate was dried over the night at 40 °C in the dark and stored at −20 °C till use.

2.3. Spectral characterization of alginates

Alginates were dissolved in distilled water at 0.1% (w/v) final concentration. UV–VIS spectra for alginates were recorded in the range of wavelengths from 200 to 380 nm on UV–VIS spectrophotometer (Shimadzu Corporation UV-2501PC, Japan).

FT-IR spectra of alginates were obtained on Nicolet 6700 Thermoscientific spectrometer using ATR method.

2.4. Acid–base titration of alginates

Alginates were dissolved at final concentration of 1% (w/v) in 4 mL of 50 mM potassium chloride (KCl). pH level was reduced to pH 2 by adding HCl. pH was monitored while gradually adding 0.1 M NaOH until pH 10 is reached. From the volumes of spent hydroxide subtracted by the volume of hydroxide spent for KCl solution titration, we calculated the amount of mmols of NaOH used and concentration of ionisable groups per gram of alginate.

2.5. Calcium induced hydrogel formation

Alginates were dissolved in distilled water at 2% (w/v) concentration. Bead formation was tested by dropping the alginate solution using 1 mL pipet tips in the 5% (w/v) calcium chloride. Shape of the precipitated alginate was inspected visually.

2.6. Peroxidase induced hydrogel formation

Tyramine-alginates were dissolved in 0.1 M Tris HCl buffer pH 7.0 at 10% (w/v) final concentration. In the alginate solution horse radish peroxidase at a final concentration of 1 U/mL and hydrogen peroxide at a final concentration of 1 mM were added. After mixing, hydrogel formation was detected by touching the solution surface with pipette tips.

2.7. Peroxidase immobilization

Tyramine-alginates were dissolved in 0.1 M Tris HCl buffer pH 7.0 at final concentration of up to 20% (w/v). In 0.3 mL of tyramine-alginate solution were added 0.1 U of glucose oxidase, suitable amount of horse radish peroxidase (0.5 U, 2 U or 5 U) and glucose to final concentration of 10 mM. 300 μL of this mixture was immediately poured into 600 μL of light mineral oil containing 3% (v/v) Span 80 detergent. For micro-beads formation emulsion was stirred for 15 min at 1000 rpm using magnetic stirrer. Reaction was stopped by adding 1 mL of 0.5% (v/v) Triton X-100 and left stirring for 5 min. Obtained micro-beads with immobilized HRP were washed first three times with 1 mL of 0.5% (v/v) Triton X-100 and afterwards three times with 1 mL of 10 mM Tris HCl buffer pH 7.0 and left in the buffer till use.

2.8. Measurement of enzyme activity

Peroxidase activity was measured using pyrogallol and H_2O_2 as substrates. Typical tests were carried out by adding 10 μL of enzyme dilution into 1 mL of 13 mM solution of pyrogallol and 9.7 mM H_2O_2 in 0.1 M Tris HCl buffer, pH 7.0 and 20 °C. Absorbance was followed for the first 3 min at 420 nm and amount of enzyme activity was calculated by using absorbance coefficient of purpurogallin (12 mg^{-1} cm^2). For immobilized enzyme 100 μL of 50% (v/v) suspension was resuspended in 3 mL of 13 mM solution of pyrogallol in 0.1 M Tris HCl buffer, pH 7.0 and 20 °C. Reaction was started by adding 30 μL of 0.97 M H_2O_2 and performed under constant stirring for 3 min. Every 60 s aliquots were taken and filtrated and absorbance at 420 nm was measured. 1 U of enzyme activity was defined as the amount of enzyme that produces 1 mg of purpurogallin in 20 s at 20 °C. Specific activity of immobilized enzyme was calculated per volume of hydrogel.