Reactive & Functional Polymers 83 (2014) 62–69

Contents lists available at ScienceDirect

Reactive & Functional Polymers

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

Enzyme immobilization on smart polymers: *Catalysis on demand*

Giuseppe Cirillo [a], Fiore Pasquale Nicoletta [b], Manuela Curcio [a], Umile Gianfranco Spizzirri [a,*], Nevio Picci [a], Francesca Iemma [a]

[a] Department of Pharmacy, Health and Nutritional Sciences, University of Calabria, I-87036 Rende (CS), Italy
[b] Department of Chemistry and Chemical Technologies, University of Calabria, I-87036 Rende (CS), Italy

ARTICLE INFO

Article history:
Received 19 March 2014
Received in revised form 27 May 2014
Accepted 5 July 2014
Available online 12 July 2014

Keywords:
Thermo-responsive hydrogels
On demand catalysis
Enzyme immobilization
Radical polymerization
Pepsin

ABSTRACT

A new approach for the synthesis of hydrogel films with thermo-sensitive enzymatic activity is reported. Pepsin (PEP) was covalently immobilized on thermo-responsive hydrogels by radical polymerization in the presence of N-isopropylacrylamide and poly-(ethylene glycol) dimethacrylate 750, acting as functional monomer and crosslinking agent, respectively. Hydrogels showing lower critical solution temperatures between 32.9 and 36.1 °C were synthesized by UV-irradiation of reaction batches differing in the PEP/monomers ratio. The derivatization degree of the hydrogels was expressed as mg of PEP per gram of matrix and found to be in the range of 6 to 11% as assessed by Lowry method. Scanning electron microscopy analysis and water affinity evaluation allowed to highlight the porous morphology and thermo-responsivity of hydrogels as a function of temperature. Using bovine serum albumin as a substrate, kinetics parameters were determined by Lineweaver–Burk plots and the catalyst efficiency evaluated. The influence of temperature on enzyme activity, as well as the thermal stability and reusability of devices, were also investigated.

1. Introduction

Enzymes are biocompatible and biodegradable catalysts from renewable resources, operating under mild conditions (room temperature, atmospheric pressure and physiological pH) in water, with high rates and selectivity [1–3]. Consequently, they are sustainable, environmentally friendly and cost-effective [4,5]. The application of enzymes in different industries (food, pharmaceutical, chemical, textile) is continuously increasing, especially during the last two decades, in order to match the growing demand for green and sustainable chemicals manufacture [6–9]. Furthermore, immobilization of enzymes onto organic or inorganic polymer matrices has been developed to overcome some drawbacks associated to their routine use, such as the lack of long-term stability and the difficulty in their recovery and reuse [10–15]. Immobilization allows the enhancement of the mechanical properties of enzymes and their stability to environmental changes [16,17]. In addition, the products can be easily purified without any contamination, and reused in continuous operational cycles [18,19].

Due to the high chemical versatility of the employed supports, a great variety of bioreactors for immobilization were fabricated [20,21]. Traditionally, three methods are used for enzyme immobilization, namely binding to a support (carrier), entrapment (or encapsulation), and cross-linking [16,17]. The binding to a polymer carrier can be reached by either physical (hydrophobic and van der Waals forces) or covalent interactions [22]. In the first case, the weakness of the involved interactions results in the formation of materials with low-term resistance to the hard reaction conditions of industrial processes, such as high ionic strengths and reactant/product concentrations, leading to a desorption of the catalyst from supports [23]. Alternatively, a stronger linkage is obtained by the covalent coupling of the enzyme to a matrix, but the possibility to irreversibly deactivate the catalyst could occur, carrying out to un-effective materials [24].

The covalent bonds are generally formed through reactions involving the functional groups of unmodified enzyme side chains, such as lysine, cysteine, or aspartic and glutamic acid residues [16]. As an example, the exposed functional groups of these residues can react with supports bearing active esters, the most common are N-hydroxysuccinimide or epoxide-functionalized materials such as diglycidyl ethers. The most general method for the immobilization on aspartic and glutamic acid residues is their conversion into the corresponding active esters *in situ* with a carbodiimide coupling agent and an auxiliary nucleophile. As an alternative, the coupling

* Corresponding author. Address: Department of Pharmacy, Health and Nutritional Sciences, University of Calabria, Edificio Polifunzionale, I-87036 Rende (CS), Italy. Tel./fax: +39 0984493011.
E-mail address: g.spizzirri@unical.it (U.G. Spizzirri).

http://dx.doi.org/10.1016/j.reactfunctpolym.2014.07.010
1381-5148/

of aldehyde groups with exposed amino groups is followed by the transformation to stable secondary amines using suitable reducing agents. Finally, stable thioether bonds can be formed by reaction of cysteine thiol groups with unsaturated carbonyls (e.g., maleimides) [17].

Another interesting approach for enzyme immobilization is the introduction of polymerizable groups in the side chains in order to produce reactive macromers [25].

This study report on the possibility to introduce pepsin (PEP) in a polymeric network in order to synthesize a solid support owing catalytic activity.

In literature, PEP was immobilized by different routes. It was entrapment in a conducting polymer matrix based on polyaniline to fabricate haemoglobin sensor [26]. A bioconjugate retaining the biocatalytic activity and showing an improved stability compared to the free enzyme, was obtained by reaction of PEP with gold colloidal particles surface, via thiolate linkages through the cysteine residues. [27]. Hybrid alumina nanoparticles were employed as support to covalent immobilized phosphorylated PEP molecules by interaction between the phosphoserine on pepsin and the alumina surface in an orientation-specific manner [28]. To synthesize material for biological and chromatographic application, chemically modified silica and polymethylmethacrylate particles were used to covalently link PEP molecules by the introduction of aldehyde functional groups [29–31]. Alternatively, radical copolymerization of 2-vinyl-4,4-dimethylazlactone and ethylene dimethacrylate allows the introduction of azlactone groups able to readily react with the reactive amine functionalities of PEP, leading the attachment of the enzyme to the monolithic support through a dipeptide spacer [32]. In addition, employing a condensating agent, such as carbonyl diimidazole and N-hydroxysuccinimide, PEP was successfully immobilized on dextran-modified fused-silica capillary and poly-methylmethacrylate microchips, respectively [33,34]. Finally, PEP was immobilized in silicone elastomers utilizing condensation-cure room temperature vulcanization of silanol-terminated poly(dimethylsiloxane) [35].

Stimuli-responsive or 'smart' devices can be obtained when the support is a polymer matrix exhibiting a volume phase transition in response to environmental changes such as temperature, pH and ionic strength [17,36]. A well-known example is the thermo-responsive polymer poly-N-isopropylacrylamide (pNIPAAm) characterized by a lower critical solution temperature (LCST) around 32 °C, below which it readily dissolves in water, while it is insoluble above LCST [37]. Hence, a homogeneous catalysis occurs in enzyme-pNIPAAm bioconjugates below LCST, while the catalyst precipitates when temperature rises allowing an easy recovery and reuse. As a consequence, it is possible to reversibly control the reaction by varying the temperature, gaining a *catalysis on demand* (inhibited above the LCST and re-activated when temperature decreases) [38].

In this paper, a new approach is proposed for the manufacture of a device for *catalysis on demand* based on innovative thermo-sensitive hydrogel films with enzymatic activity. In particular, PEP was copolymerized by UV-initiated radical polymerization with N-isopropylacrylamide (NIPAAm) and poly-(ethylene glycol) dimethacrylate 750 (PEGDMA750), acting as functional monomer and crosslinker, respectively. The main goal of this work was to avoid any preliminary derivatization of the employed enzyme, exploiting the reactivity of the heteroatoms towards radical processes.

Our challenge was the PEP immobilization in a polymer network without damaging its biological peculiarities and, at the same time, the modulation of its enzymatic activity by a temperature-depending swelling/shrinking mechanism of the hydrogel support. In this way, smart devices able to control *on demand* the substrate access to catalytic sites by temperature changes, were obtained. The physico-chemical and biological properties of the hydrogels were extensively investigated by scanning electron microscopy, differential scanning calorimetry, and determination of the catalysis kinetic. The catalytic activity was studied as a function of sample composition and temperature.

2. Materials and methods

2.1. Materials

Pepsin (PEP), N-isopropylacrylamide (NIPAAm), poly-(ethylene glycol) dimethacrylate 750 (PEGDMA750), ninhydrin, KBr, Na_2CO_3, NaOH, $KNaC_4H_4O_6{\cdot}4H_2O$, $CuSO_4{\cdot}5H_2O$, Folin–Ciocalteu reagent, bovine serum albumin (BSA, 66 kD), tyrosine and trichloroacetic acid were purchased by Sigma–Aldrich (Milan, Italy). Irgacure 2959 (1-[4-(2-hydroxyethoxy)-phenyl]-2-hydroxy-2-methyl-1-propane-1-one, with a maximum absorption at around 275 nm, from Ciba) was used as photoinitiator.

2.2. Synthesis of catalytic hydrogels

An aqueous solution of PEP at the desired concentration, as shown in Table 1, was heated to 30 °C and continuously stirred for 15 min. Then, NIPAAm and PEGDMA750 were added and the solution was purged with gaseous nitrogen for 20 min. Irgacure 2959 was finally added and the solution was transferred into curing cells, made by two $10 \times 10\ cm^2$ glass plates brought together by clips and separated by 1.0 mm thick Teflon spacers. Polymerization was initiated by a high pressure mercury lamp (Philips HPK 125, 500 mW cm^{-2}, wavelength 253 nm, irradiation time 10 min), which provided the film formation. The obtained polymer films (code # P100, P200, and P300) were extensively washed with water for 72 h to remove unreacted species and, then, dried under vacuum.

2.3. FT-IR spectroscopy

Fourier-Transmission IR (FT-IR) spectra of monomers and hydrogels were measured as pellets in KBr with a FT-IR spectrophotometer (Jasco, model FT-IR 4200) in the wavelength range of 4000–400 cm^{-1}. Signal averages were obtained from 100 scans at a resolution of 1 cm^{-1}.

2.4. Scanning electron microscopy

Morphology analysis (top views and cross sections) was performed by a Leica LEO 420 scanning electron microscope (SEM). Samples were placed on appropriate stubs and, then, sputtered with gold (thickness $\sim$ 100 Å) under argon atmosphere to achieve the necessary conductivity. To analyze film cross sections, samples were cryo-fractured with liquid nitrogen. The accelerating voltage was 15 kV under high vacuum conditions.

2.5. Thermal behavior

Thermal analysis was performed by a differential scanning calorimeter (Netzsch, DSC200 PC) to determine sample LCSTs. Films were soaked in distilled water at room temperature for at least 2 days in order to reach the swollen state. About 10 mg of swollen film were placed in an aluminium pan and, then, hermetically sealed by an aluminium lid. The thermal range was from 25 °C to 55 °C, under dry nitrogen atmosphere with a flow rate of 25 ml min^{-1} and a heating rate of 2 °C min^{-1}.