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Protein imprinting and recognition via forming nanofilms on microbeads surfaces in aqueous media

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ABSTRACT

In this paler, we present a technique of forming nanofilms of poly-3-aminophenylboronic acid (pAPBA) on the surfaces of polystyrene (PS) microbeads for proteins (papain and trypsin) in aqueous. Papain was chosen as a model to study the feasibility of the technique and trypsin as an extension. Obtained core–shell microbeads were characterized using scanning electron microscopy (SEM), Raman spectroscopy, X-ray photoelectron spectroscopy (XPS) and BET methods. The results show that pAPBA formed nanofilms (60–100 nm in thickness) on the surfaces of PS microbeads. The specific surface area of the papain-imprinted beads was about 180 $m^2 g^{-1}$ and its pore size was 31 nm. These imprinted microbeads exhibit high recognition specificity and fast mass transfer kinetics. The specificity of these imprinted beads mainly originates from the spatial effect of imprinted sites. Because the protein-imprinted sites were located at, or close to, the surface, the imprinted beads have good site accessibility toward the template molecules. The facility of the imprinting protocol and the high recognition properties of imprinted microbeads make the approach an attractive solution to problems in the field of biotechnology.

1. Introduction

Molecular imprinting technology is an attractive synthetic approach to mimic natural molecular recognition [1–5]. The technology is now well-established in the field of synthetic molecular recognition, offering a generic, robust, and cost-effective alternative to existing techniques such as monoclonal antibodies [6]. During the last few years, a number of applications of molecular imprinting materials have been used in liquid chromatography, biosensors, catalysts, bio imprinting, binding assays and food chemistry [7–10].

The nature of molecular imprinting lends itself to the generation of 3D imprint systems where binding sits are formed throughout a bulk material. However, molecular imprinting on surfaces (2D imprinting) is gaining momentum especially when forming imprints against large macromolecules such as proteins and in sensor design [11]. Papain is a food grade, highly active endolytic cysteine protease and is one of the widely used industrial and medicinal enzymes. It is used for cell isolation, in breweries, food and pharmaceutical as digestive enzyme, leather, cosmetic and textile industries [12–14]. In recent years, many proteins have been investigated as templates for molecular imprinting [15–26].

To date, there have been no reports concerning the polymers of papain molecularly imprinting, whereas the work has a great interest for the separation, recovery and detection of food proteins. In our previous works [27,28], a surface molecularly imprinting technique has been used for proteins (Lysozyme and bovine hemoglobin). In this paper, similar technique was applied to papain molecular imprinting for the first time. We designed and fabricated the nanofilms of papain imprinting on the surfaces of PS microbeads with the technique, conducted characterizations of various samples and evaluated adsorption characteristics of imprinting beads. As an extension, the microbeads of trypsin imprinting were also prepared and characterized. The characterization results verified that nanofilms of pAPBA were able to self-assemble onto the PS microbeads through aromatic ring electron-pairing interaction and to form complex with template proteins. Also, by evaluating the adsorption performance of these imprinting microbeads, we verified that these beads displayed high template binding capacity and fast mass transfer.

2. Materials and methods

2.1. Materials

Styrene (analytical reagent), 2,2′-azobisisobutyronitrile (AIBN, analytical reagent), and Tween-20 (chemical reagent) were from

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Tianjin Guangfu Fine Chemical Research Institute (Tianjin, China). 3-aminophenylboronic acid (APBA) was obtained from Beijing Element Chem-Tech Co. (Beijing, China). Papain (molecular weight 23,000; p*I* = 9.5), Lysozyme (molecular weight 14,400; p*I* 11.1) and hemoglobin (Hb, molecular weight 65,000; p*I* 6.7; from bovine blood) were purchased from Sigma. Trypsin (molecular weight 23,300; p*I* 10.5) was purchased from Gibco. Bovine Serum Albumin (BSA, molecular weight 68,000; p*I* 4.6) was purchased from Roche. All other chemicals and solvents were obtained from commercial sources. Styrene was distilled to remove inhibitor before use. All other materials were used without further purification. Deionized and distilled water was used throughout all experiments.

2.2. Preparation of PS microbeads

Suspension polymerization was used for preparing PS beads. A 500-mL, spherical three-necked vessel was fitted with an anchor-type mechanical stirrer, a condenser and a thermometer. The vessel was put into a water bath and charged with 40 mL of 3% poly(vinyl alcohol) aqueous solution and 250 mL of water. Then 10 mL of styrene containing 0.3 g of AIBN was added dropwise into the vessel under stirring (400 rpm). This mixture was heated to 82–85 °C and the polymerization was conducted for about 2 h. The reaction mixture was then allowed to cool to room temperature before it was transferred to a beaker. After the beaker was stood undisturbed, the supernatant was abandoned. The left beads were rinsed repeatedly with methanol and dried at room temperature.

2.3. Preparation of the imprinted and blank core–shell microbeads

Protein-imprinted core–shell beads were prepared as follows: 10 mL of 100 mM APBA water solution containing 2 mg mL^{-1} protein and 1 g of PS micrbeads were added orderly in a 25 mL open vessel. After gentle stirring for 30 min, 10 mL of 50 mM ammonium persulfate water solution was added in order to start the polymerization reaction. The synthesis was carried out at 22 °C for 40 min, after which the resultant particles were separated through filtration and washed repeatedly with water. The particles were then cured at room temperature for 6 h. Following this, they were washed first with 3% acetic acid containing 0.1% Tween-20 to remove template until no free protein was detectable in the eluate using a UV–vis spectrophotometer at 280 nm and then five times with water. The final products were dried at room temperature. Blank core–shell microbeads were formed under the same conditions in the absence of the template.

2.4. Characterization of samples

The PS and core–shell microbeads were mounted onto metal stages, coated with gold by sputtering, and then observed using a SEM (JSM-6390LV, Japan). The surface area measurements and pore size of papain-imprinted microbeads (removed template) were carried out at liquid-nitrogen temperature (77.4 K) with a nitrogen adsorption/desorption analyzer (NOVA 1000, U.S.A.). The samples were degassed at 80 °C for 6 h before measurements. Specific surface areas were calculated using the Brunauer–Emmett–Teller (BET) model, and pore size was evaluated from the desorption branches of the nitrogen isotherms using the Barrett–Joyner–Halenda model. XPS of PS, non-imprinted and protein-imprinted microbeads were recorded on an X-ray photoelectron spectrometer (Escalab MKII, U.S.A.), using non-monochromatized Al Kα X-ray radiation as the excitation source. The pass energy was at 50 eV. Raman scattering spectra of PS, blank and protein-imprinted microbeads were recorded at room temperature under ambient conditions on a FT-IR/Raman spectrometer (Nicolet Nexus U. S.A.).

2.5. Batch rebinding tests

The protein solutions were prepared with 50 mM sodium phosphate buffer (PB, containing 0.1% Tween-20) and the initial protein concentrations were 0.1 mg mL^{-1}. 10 mL of the solution and 0.1 g of imprinted/blank microbeads were placed in a 25-mL vessel. The vessel was then sealed and shaken at room temperature. After incubation, the microbeads were filtered through a porous poly(vinylidene fluoride) membrane (hydrophilic membrane, millipore pore size = 0.45 μm). The amount of protein adsorbed by the microbeads at the end of each run was determined by the following formula:

$$Q = \frac{(C_i - C_f)V}{m}$$

where Q (μg/mg) is the mass of protein adsorbed per gram of microbeads, C_i (0.1 mg mL^{-1}) is the initial protein concentration, C_f (mg mL^{-1}) is the final protein concentration, V (10 mL) is the total volume of the adsorption mixture, and m (100 mg) is the mass of microbeads in each rebinding mixture. The final concentration, C_f, was determined by using a UV–vis spectrophotometer at 280 nm. All tests were conducted in triplicates. Moreover, control experiments also carried out in which PB did not contain protein. The control experiments were used to eliminate the influence of the pAPBA on detection of the protein because pAPBA also absorbs at 280 nm.

3. Results and discussion

3.1. Morphology of the PS and core–shell microbeads

Suspension polymerization has some advantages, including facile separation of products, easy removal of dispersant, high purity and uniform sized particles. Here suspension polymerization was used to prepare homopolymerized styrene microbeads and the beads were as the cores of core–shell microspheres. A scanning electron micrograph of the beads in the dry state is shown in Fig. 1a. The beads are of sizes around 50 μm and take on good uniformity, regular shape and clean surfaces. This avails subsequent molecular imprinting and potential application as packing materials.

In order to verify the success of grafting pAPBA on the surfaces of PS microbeads, blank core–shell microbeads were also characterized by SEM. From the image (Fig. 1b), pAPBA has been grafted on the surface of PS microbeads and formed nanofilm. The nanofilm was 60–100 nm in thickness.

3.2. Surface Area and pore size of papain-imprinted microbeads

The nitrogen sorption/desorption results showed that the specific surface areas and pore size of the imprinted microbeads (removed templates) were about 180 $m^2 g^{-1}$ and 31 nm, respectively. This proves the nanofilms are porous. Their larger surface and middle-pores are advantageous for loading target molecules and rebinding kinetics.*XPS spectra of the PS, blank and imprinted microbeads*

XPS has become an established technique for the characterization of polymer surface [26,29,30]. Here XPS was used for determining the elemental surface compositions of the samples. Fig. 2 depicts XPS spectra of the samples. For the PS microbeads, an external signal (O 1s) was observed (see Fig. 2a), which is due to the water adsorbed onto the surfaces of the sample. For the blank microbeads and imprinted microbeads (un-removed template), the appearance of the signals (B 1s) (see Fig. 2b, c and d) indicates that pAPBA had to be grafted onto surfaces of PS microbeads. Most importantly, imprinted samples displayed a significant increase in N 1s/C 1s intensity ratio compared to blank samples. This illustrated the formation of the complex between pAPBA and