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Partially phosphonated polyethylenimine-coated nanoparticles as convenient support for enzyme immobilization in bioprocessing

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ABSTRACT

Partially phosphonated polyethylenimine (PEIP) has been developed as an easily functionalisable coating agent for iron oxide nanoparticles. Trypsin immobilization takes special advantage of the properties of this new material. Numerous enzymes can be loaded on the polymer by a covalent bounding with numerous amino groups. The PEIP contributes to the high stability of the material, through a strong covalent P—O—Fe bond. Resistance to hydrolysis and to temperature increasing ensure to obtain a highly recyclable magnetic nanomaterial designed for proteomic analysis.

1. Introduction

Trypsin (EC 3.4.21.4) is an essential key in mass spectroscopy-driven proteomics [1–3]. Its ability for specifically sequence C-terminal peptide bonds of arginine and lysine residues in a protein makes it a very useful tool for protein identification and analysis [4,5]. However, the conventional in-solution digestion for proteomic is limited by two principal factors [6]. Trypsin has a low thermostability at 37 °C, and undergoes a rapid autolysis at basic pH. Many processes have been developed to overcome these two major drawbacks [7,8]. Among them, the covalent immobilization of trypsin on a magnetic support is widely studied, and the benefits of this methodology are multiple [9–11]. First, the restriction of degrees of freedom enhances stability of enzyme and permit reduction of autolysis. In addition, the magnetic support leads to easy enzymes recovery with an electromagnetic field and the so grafted enzyme can be reused; it clearly helps the purification of products and elimination of trypsin mass-fingerprints.

Magnetic microbeads for enzyme support have been the subject of many investigations [12–19]. In these cases, in addition to a low specific surface area, the micro or millimetric scale of support can highly hamper the approach of the substrate [20,21]. Even if the affinity of trypsin for substrate is more or less preserved, proteolysis speeds can be extremely decreased. Considering this point of view, we try to focus on a material at the nanometric scale, with a size similar to an enzyme. The support would only slightly hinder the substrate docking conducting to better speed.

We have chosen to work on magnetic maghemite nanoparticles (NP) suitable for biofunctionalization. A poly (aminomethylenephosphonic) acid has been previously synthetized in our laboratory as a sorbent for metal ions in wastewaters [22,23]. Then, an only partially phosphonated version (PEIP) has been used for coating nanoparticles (NP-PEIP) [24]. The nanometric size and high number of free primary amine on PEIP permit the grafting of biomolecules and offers a well-adapted material for enzyme immobilization. The choice of the support has been carefully selected: compared to commonly used supports, like chitosan, alginate or silica, the stability is better toward acidic or basic hydrolysis and temperature increasing [25–28]. P—O—Fe covalent bonds between maghemite and PEIP achieve a strong stability of the material. The consequence is that NP-PEIPs properties are not deteriorated; and a large number of reuses can be obtained with a major stability toward increasing of temperature.

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2. Materials and methods

2.1. Materials

Hyperbranched polyethylenimine with a molecular weight of 25,000 Da was purchased from BASF. Dialysis tubing was obtained from Roth. Phosphorous acid, formaldehyde, hydrochloric acid, nitric acid, aqueous ammonia solution were purchased from VWR; ferrous, ferric and nitrous chloride from Alfa. Glutaraldehyde 50% in water, sodium cyanoborohydride, trypsin from pancreas porcine (EC 3.4.21.4) and albumin from bovine serum were bought from Sigma–Aldrich. Unless otherwise noted, all reagent-grade chemicals were used as received. Millipore water was used in the preparation of all aqueous solutions.

2.2. Preparation of polyethylenimine with 5% of amine phosphonated-PEIP

A mixture of PEI (0.186 mmol, 4.64 g), phosphorous acid (5.40 mmol, 0.443 g, 0.05 equiv. of the critical amount of PEI monomers MW = 43) in 10 mL of water was vigorously stirred, then irradiated at 150 W in a microwave oven (Prolabo Synthewave) for one minute. Then, pH was adjusted to 4 with hydrochloric acid 6 mol L^{-1}. A formaldehyde aqueous solution (35%, 10.8 mmol, 0.1 equiv.) was added and the mixture was irradiated for 5 min. The excess of formaldehyde was eliminated in vacuo. The polymer was dialyzed in water with a nitrocellulose membrane, yielding therefore 85% of PEIP (8.25 g).

2.3. Synthesis of maghemite nanoparticles (NPs) and coating on PEIP

First, maghemite nanoparticles were synthesized as previously described [24]. Ferrofluid was obtained with an iron concentration of 1.24 mol L^{-1}. Micrographs from TEM show a system where nanoparticles have a nearly spherical structure. According to parameters fit of the magnetization curve and crystalline size calculated from Debye Scherer formula, medium diameter of nanoparticles is estimated to 7 nm and polydispersity to 0.32.

0.5 mL of diluted precursor acid ferrofluid with a ferric concentration of 10^{-2} mol L^{-1} was slowly added to 2 mL of a solution of 50 g L^{-1} PEIP in water adjusted to pH 2 with concentrated nitric acid, under vigorous stirring. After 5 min, the mixture was basified by adding sodium hydroxide until a precipitation was observed. Supernatant containing excess of PEI was removed and the precipitate redispersed in a diluted nitric acid solution. In a second time, the solution was concentrated by evaporating water and acetone was added leading to the flocculation of PEIP-coated nanoparticles (NP-PEIP).

2.4. Trypsin immobilization

Glutaraldehyde was first mixed with active charcoal and filtered. This operation was repeated until the absorbance peak at 235 nm on UV-spectroscopy had disappeared. 2 mL of a solution of filtered glutaraldehyde (25%) were mixed to 2 mL of an aqueous solution of previously synthetized NP-PEIP ([Fe] = 10^{-2} mol L^{-1}) and 10 mL of acetate buffer (0.1 mol L^{-1}, pH 4.8), and the mixture was stirred during 2 h. Excess of glutaraldehyde was eliminated by ultracentrifugation (20,000 rpm, 30 min.). After removing of supernatant, the precipitate was redispersed in 25 mL of acetate buffer. Rinse cycles were effected two times. 10 mg of trypsin were introduced by small portions to the mixture, followed by addition of 100 mg of sodium cyanoborohydride previously dispersed in 5 mL of acetate buffer. After 5 h of incubation, immobilized trypsin was obtained by decantation of the mixture on magnetic plates. Supernatant was removed, and particles redispersed in water. Operation was repeated until no free trypsin was detected in wash water by spectrophotometry. The amount of immobilized trypsin was calculated by the difference between the introduced protein amount and that found in the supernatants. The UV absorption value of the supernatant solutions was measured at 280 nm and compared to the UV absorption value of the trypsin solution before immobilization.

2.5. Determination of activity

Concerning the unmodified trypsin, the substrate solution consists in 4.2 mmol of Nα-Benzoyl-L-arginine 4-nitroanilide (BApNA) dissolved in 150 mL of Tris–HCl buffer (pH = 7.8, 0.1 mol L^{-1}, containing 20% of DMSO) at 25 °C. Free enzyme solution was prepared by dissolving 5 mg of trypsin in 3 mL of a 2 mol L^{-1} HCl solution. At the start of the run, 0.1 mL of enzyme solution was then added, and the mixture maintained at 25 °C. Activity of immobilized enzyme was assayed by the introduction of 10 mg of NP-PEIP-T in 150 mL of the Tris–HCl buffer containing 4.2 mmol L^{-1} of BAPNA, under stirring at 25 °C. Stability tests are started after 30 min of incubation at the required temperature.

The released p-nitroaniline was estimated by spectrophotometry against a blank after 15 min at 405 nm using a molar extinction coefficient value of 8800 L mol^{-1} cm^{-1}. This measure was repeated three times and the mean and standard deviation were calculated. The enzymatic activity was defined as the amount of enzyme able to hydrolyze one mmol of BApNA per minute under the established conditions.

2.6. Mass spectrometry

A solution of bovine serum albumin (BSA) at 5 g L^{-1} was first prepared by dissolving BSA in Tris-buffer (20% DMSO, 0.1 mol L^{-1}, pH 7). The in-solution digestion was performed by adding free trypsin into 10 mL of the protein solution at a substrate-to-enzyme ratio of 50:1. Evaluation of supported trypsin consisted in adding 2 mL of NP-PEIP-T dispersed in the same Tris-buffer ([Fe] = 5×10^{-3} mol L^{-1}) into 10 mL of protein solution. Both mixtures were incubated at 37 °C for 12 h. After digestion, 1 μL of nitric acid was added into the solutions to stop the reaction, and in the second case magnetic particles were separated by using a permanent magnet.

MS experiments were carried out on an AB Sciex 5800 proteomics analyzer equipped with TOF TOF ion optics and an OptiBeam™ on-axis laser irradiation with 1000 Hz repetition rate. The system was calibrated immediately before analysis with a mixture of Angiotensin I, Angiotensin II, Neurotensin, ACTH clip (1–17), ACTH clip (18–39) and mass precision was better than 50 ppm. For MS analysis, a 1 μL volume of the peptide solution was mixed with 10 μL volumes of solutions of 5 g L^{-1} α-cyano-4-hydroxycinnamic acid matrix prepared in a diluent solution of 50% ACN with 0.1% trifluoroacetic acid. The mixture was spotted on a stainless steel Opti-TOF™ 384 targets; the droplet was allowed to evaporate before introducing the target into the mass spectrometer. A laser intensity of 3200 was typically employed for ionizing. MS spectra were acquired in the positive reflector mode by summarizing 1000 single spectra (5 × 200) in the mass range from 800 to 4000 Da.

3. Results and discussion

3.1. Immobilization of trypsin on coated nanoparticles

3.1.1. Synthesis of PEIP-coated nanoparticles (NP-PEIP)

Whole reaction pathway of the trypsin immobilization is resumed in Scheme 1. The first part concerns the preparation