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Improved stability of the carbon nanotubes–enzyme bioconjugates by biomimetic silicification

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ABSTRACT

Nano-materials have been applied in many fields due to their excellent characteristics, such as the high surface area-to-volume ratio, excellent physicochemical properties and biological compatibility. In this study, multi-walled carbon nanotubes (MWCNTs) were utilized to prepare MWCNTs–papain bioconjugates and then realized the immobilization of papain. MWCNTs functionalized with carboxyl- and amine- groups on their surface were used as immobilization carriers. The immobilization of papain on the functionalized MWCNTs through physical absorption was examined. The conjugates were denoted as MWCNTs–papain bioconjugates. To improve the stability, the bioconjugates were further coated by silica through the biomimetic silicification process that induced by papain (denoted as silica-coated bioconjugates). The as-prepared MWCNTs–papain bioconjugates and the silica-coated bioconjugates were characterized by scanning electron microscopy (SEM) and Fourier transform infrared (FTIR) spectroscopy. The preliminary results showed that the bioconjugates could retain most of the initial activity of papain. Compared to free papain and MWCNTs–papain bioconjugates, the silica-coated bioconjugates exhibited significantly improved thermal, pH and recycling stability. Comparisons of the kinetic parameters between MWCNTs–papain bioconjugates and the silica-coated bioconjugates revealed that the K_m value of the immobilized papain experienced a slight increase after silica coating, which suggested that the silica coating did not significantly hinder papain's access to substrate or release of product.

1. Introduction

Enzyme engineering is a fast-growing field in the pharmaceutical, bioanalytical applications and food markets. Proteases are one of the most important classes of industrial enzymes [1,2]. Many proteases from various sources are being studied extensively with respect to activity and structure. Papain (EC 3.4.22.2), a well-characterized enzyme, has a variety of industrial applications in food, pharmaceutical, leather, and cosmetic fields [3–8]. Given the aforementioned features, papain thus serves as an ideal model enzyme to study [9–11]. So in the present study, papain was chosen as the model enzyme to investigate.

In addition to the unquestionable advantages, there exist a number of problems for free enzymes in practical applications. For example, free enzymes are highly sensitive to pH and temperature [12]. Moreover, unlike conventional heterogeneous catalysts, most enzymes operate dissolved in water in homogeneous catalysis systems and cannot be recovered in the active form from reaction mixtures for reuse, which may also contaminate the product [13]. Several methods have been proposed to overcome these limitations; one of the most successful approaches is enzyme immobilization. As can be seen from the previous reports, many supports such as alginate bead, dye ligands [14–18], have been utilized to immobilize enzymes.

Recently, the nanomaterials–enzymes conjugates, which combine the advantageous features of the nanomaterials and the functionality of biocatalysts, show great potential for applications in bioconversion, biosensors and biomedical fields [19]. In particular, the high stability of the nanobiocatalyst has provided a promising future in enzymatic practical applications. Thus, many nanomaterials have been developed for enzyme immobilization. Among these nanomaterials, carbon nanotubes have attracted widespread attention due to their highly specific surface, extraordinary mechanical properties, high electrical conductive ability, high thermal conductivity, and good biocompatibility [20]. Apart from offering numerous opportunities in materials and electronics studies, carbon nanotubes also hold great potential for applications in biosensing, functional surfaces and coatings, biorecognition and drug delivery [21–23]. Karajanagi et al. [24] investigated the enzyme structure and function on carbon nanotubes, which was critical in designing optimal carbon nanotube–enzyme conjugates. Asuri et al. [25] studied the structure, activity and stability

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of three functionally unrelated enzymes covalently attached to SWCNTs and found out that the SWCNTs–enzyme conjugates possessed high activity, high stability and water soluble property. Wang et al. [26] assembled several types of enzymes on carbon nanotubes through layer-by-layer method and demonstrated that the resulting sandwich-like layer structure could retain the bioactivity of the enzyme. Pangule et al. [27] had designed antimicrobial coatings incorporating carbon nanotube–lysostaphin conjugates, which showed highly effective against antibiotic-resistant pathogens. A nanocomposite matrix made up of silylated chitosan and MWCNTs (CHIT–SiO_2–MWCNTs) was also fabricated to immobilize urease [28]. Dordick et al. have reported that the MWCNTs–enzyme conjugates are more stable under some harsh conditions [20], and protease can resist nonselective protein binding [29]. So MWCNTs–papain bioconjugates may be useful as antifouling materials. However, leaking and poor stability of the enzymes are still the bottlenecks for the applications of the bioconjugates. In order to solve the problems, silica coating may be a promising approach.

Generally, silica coating is realized by using the traditional sol–gel method. However, for enzymes immobilization, the relatively harsh reaction conditions of the traditional sol–gel method may induce the deactivation of the enzyme [30]. In contrast, the biomimetic silicification process can serve as an alternative method [30,31], which can be conducted at near neutral pH and ambient temperature [32–34]. Thus, the biomimetic silicification process, which combines the excellent properties of the silica matrix with a benign immobilization process, may become an efficient strategy for enzyme immobilization.

Therefore, in this study, papain was first immobilized onto the functionalized MWCNTs and then the MWCNTs–papain bioconjugates were obtained. To improve the stability of the bioconjugates, the possibility of using silica coating through the biomimetic silicification process was further explored. Additionally, the pH stability, thermostability, reusability and kinetic properties of the bioconjugates were also investigated.

2. Materials and methods

2.1. Materials

Multi-walled carbon nanotubes were purchased from Shenzhen Nanotech Port Co. Ltd. (NTP), China. Papain was obtained from Genview, America. Tetramethoxysilane (TMOS) was purchased from Sigma–Aldrich, America. All other chemicals, unless specified, were of analytical grade and used as received.

2.2. The functionalization of the carbon nanotubes

Pristine MWCNTs were suspended in a concentrated H_2SO_4/HNO_3 mixture (3:1 v/v) and sonicated for 3 h [35]. The suspension was centrifuged, washed with distilled water and dried at 50 °C in vacuum overnight (denoted as MWCNTs–COOH). The MWCNTs–COOH was suspended in a 20:1 mixture of thionylchloride and N,N-dimethyl formamide and refluxed at 70 °C for 24 h. After the acyl-chlorination, the MWCNTs derivatives were centrifuged, washed with anhydrous toluene and dried under vacuum at 50 °C overnight. The acyl-chlorinated MWCNTs were reacted with hexamethylenediamine (at 50% excess) using chloroform as solvent, at 80 °C for 3 days. The excess amine was removed first with chloroform, followed by anhydrous tetrahydrofuran and finally the poly-amine terminated MWCNTs were dried in vacuum at room temperature (denoted as MWCNTs–NH_2).

2.3. Preparation of MWCNTs–papain bioconjugates and the silica-coated bioconjugates

A quantity (5 mg) of the functionalized MWCNTs were sonicated and suspended in 9 mL of a pH 7.5 phosphate buffer saline (PBS) at room temperature for 1 h. Then the solution was mixed with 1 mL of a concentrated (6 mg/mL) papain solution and incubated at 30 °C for 4 h. The mixture was centrifuged, washed with PBS and MWCNTs–papain bioconjugates were obtained.

For the preparation of the silica-coated bioconjugates, hydrolyzed TMOS was used as the silicic acid precursor. TMOS was hydrolyzed in 1 M hydrochloric acid and diluted to be 0.1 M with pH 7.0 of PBS. The MWCNTs–papain bioconjugates were added to 0.1 M hydrolyzed TMOS solution, reacted for 15 min at room temperature. After centrifuged and washed, the silica-coated bioconjugates were obtained. The silica-coated bioconjugates were collected, washed several times with PBS.

The morphologies of the MWCNTs and bioconjugates were characterized by a JSM-6700F (JEOL, Japan) scanning electron microscope (SEM). Energy-dispersive X-ray spectrometry (EDX) equipment that was attached to the SEM system was used to analyze the elements that comprise the conjugates. The Fourier transform infrared (FTIR) analyses were collected on a Brukervector/22 (Bruker Corporation, Germany).

2.4. Determination of papain activity

The activity of papain was determined by using casein as substrate. 1 mL PBS and 2 mL of 0.5% casein solution were added to 1 mL of free papain (6 mg/mL) solution. After enzymatic reaction at 30 °C for 0.5 h with gently stirring, 5 mL of 10% trichloroacetic acid (TCA) was added immediately to terminate reaction. The precipitation was centrifuged and the filtrate was used to determine the papain activity at 275 nm by using UV–vis spectra. One unit of enzyme activity was defined as the tyrosine content formed by per enzyme in 1 min at 30 °C and pH 7.0. The bioconjugates were incubated in 1 mL of PBS (pH 7.0), and 2 mL of 0.5% casein solution was added, reacted at 30 °C for 0.5 h. Finally, the mixture was centrifuged and the filtrate was used to determine the papain activity by using UV–vis spectra. The bioconjugates were obtained as described in Section 2.3. Relative activity was the ratio of the immobilized enzymes to the highest activity in a series of experiments.

2.5. The stability and kinetic parameters

For pH stability, free papain, MWCNTs–papain bioconjugates and the silica-coated bioconjugates were separately suspended in various pH (3, 5, 6, 7, 8, 10, 12) PBS for 2 h at 30 °C. The activity of free papain was measured directly and the others were centrifuged and then measured (as described in Section 2.4).

To determinate the thermal stability, the free papain solution, MWCNTs–papain bioconjugates and the silica-coated bioconjugates were suspended in pH 7.0 PBS for various durations separately (0, 0.5, 1, 1.5, 2, 3, 4 h) at 60 °C. Then the activity was measured following the method described in Section 2.4.

The recycling of the bioconjugates was also studied. After the first cycle of the catalytic reaction with casein as substrate, the reaction mixture was centrifuged to remove the supernatant and washed with PBS. The bioconjugates were used in the next batch. They were reused seven times and evaluated the activity each time.

Kinetic constants were determined by measuring the activity of papain in the presence of various casein (substrate) concentrations (0.1%, 0.2%, 0.3%, 0.4%, and 0.5%). Michaelis–Menten constant (K_m) values and the maximum velocities (V_{max}) were determined by using the Lineweaver–Burk double-reciprocal plot, in which the reciprocals of the initial velocities of the papain activity were plotted against the reciprocals of the concentrations of casein used. MWCNTs–papain bioconjugates and the silica-coated bioconjugates were added to 1 mL of pH 7.0 PBS for 0.5 h and catalyzed various concentrations of casein for 0.5 h at 30 °C. The mixture was centrifuged and the supernatant was measured by using UV–vis spectra at 275 nm. Free papain catalyzed various concentrations of casein directly and the tyrosine content was measured. All the experiments were carried out in triplicate and average values were reported.

3. Results and discussion

3.1. Preparation of MWCNTs–papain bioconjugates and the silica-coated bioconjugates

3.1.1. The functionalization of MWCNTs

MWCNTs were firstly acid-oxidized to form carboxylic acid groups on the surface of the nanotubes in a concentrated H_2SO_4/HNO_3 mixture, and the carboxyl-modified carbon nanotubes were denoted as MWCNTs–COOH. Then, the carboxylic acid groups were acyl-chlorinated in the mixture of thionylchloride and N,N-dimethyl formamide, and the acyl-chlorinated carbon nanotubes were denoted as MWCNTs–COCl. Finally, the MWCNTs–COCl reacted with hexamethylenediamine and formed amine-modified carbon nanotubes, which were denoted as MWCNTs–NH_2 (as shown in Scheme 1). Through the acid–base titration method [36], it was found that the content of carboxyl was about 2.29 mM/g. The content of amino groups on functional MWCNTs was about 0.801 mM/g, which was determined through the De-Fmoc group method [37,38].

FTIR spectra of the pristine and modified MWCNTs were shown in Fig. 1. Compared with the FTIR spectrum of pristine MWCNT (Fig. 1(a)), the FTIR spectra of MWCNTs–COOH (Fig. 1(b)) and MWCNTs–NH_2 (Fig. 1(c)) clearly showed the peaks at 1572 cm^{-1}