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Single-enzyme nanoparticles based urea biosensor

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ABSTRACT

Single-enzyme nanoparticles (SENs) with excellent activity and stability were successfully fabricated via the surface modification and in situ aqueous polymerization of separate urease molecule. A novel piezoelectric biosensor was developed for urea determination based on SENs immobilization onto nanoporous alumina membranes prepared by electrical anodization. The process of SENs immobilization was optimized and the performance of the developed urea biosensor was evaluated. The high selectivity, shorter response time (12 s), wider linear range (0.08 μM–1 mM) and lower limit of detection (LOD, 0.05 μM) were observed for the present biosensor. Moreover, a stability study showed a very high stability over time for the frequency response of the biosensor with separated porous alumina/SENs electrode, testifying for the protective nature of the nanoporous alumina membranes and the interest of SENs. The clinical analysis of the urea biosensor confirmed the feasibility of urea determination in urine sample.

1. Introduction

Enzyme biosensors have attracted much attention in recent years due to their potential applications in clinical diagnostics, pharmaceutical, environmental analysis and food industry [1–3]. The crucial step in the development of an enzyme based biosensor is the effective immobilization of an enzyme on the electrode surface [4]. In the past years, various organic and inorganic materials have been developed to immobilize enzyme, such as polyacrylonitrile-glycopolymer [5], poly(ethylene glycol) diglycidyl ether [6], polyvinylferrocenium [7], fiber [8], NiO nanoparticles [9], nanoporous alumina membrane [10], titania [11] and silica [12]. Among them, nanoporous alumina membrane which can be easily fabricated via electrical anodization of high purity aluminum is of current interest in enzyme immobilization owing to its relatively high surface area, high porosity, and high chemical, biological and mechanical stability [13]. The enzymes have been successfully immobilized onto nano-structured anodic alumina membranes for the biosensing applications [14,15]. However, the expected high stability and activity of enzymes cannot be usually met due to the loss of enzymatic functions. The encapsulation of single-enzyme molecules, a novel way to modify and stabilize enzymes, provided an efficient route to inhibit enzyme inactivity. SENs have been fabricated via a two-step or multistep procedures [16,17]. Our group reported the protocol of the synthesis of SENs with better magnetism and conductivity, the obtained SENs showed high activity and long-term stability [18].

The electrode-separated piezoelectric sensor (ESPS) is sensitive sensor and can monitor the frequency shift caused by conductive change of solution [19,20]. Compared to the conventional conductometry, the electrical double layer in the ESPS sensing system is eliminated owing to its high working frequency (9 MHz). Meanwhile, it does not produce thermal effects for the biological materials because the detector cell is set in the feedback network of the oscillator and no obvious current flows through it. Moreover, the ESPS can detect a slight change in solution conductivity in the presence of an excess of foreign electrolyte, and the sensitivity and accuracy are better than those obtained in the absence of the foreign electrolyte [21]. In the present work, urease was selected as a model enzyme and the ESPS/FIA system was employed to monitor the enzymatic reaction. A novel piezoelectric biosensor has been developed based on SENs immobilization onto nanoporous alumina membranes. The constructed ESPS biosensor showed excellent performance for urea determination.

2. Experimental

2.1. Materials and apparatus

High purity aluminum foils (99.99%, from VWR) were used to prepare nanoporous alumina membranes. Pyrrole

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monomer was obtained from Merck and distilled before use. N-hydroxysuccinimide (NHS) and 1-ethyl-3-(3-dimethyl amino propyl) carbodiimide hydrochloride (EDC) were purchased from Sigma Chem. Co. and used as received. Crylic acid and acryloyl chloride were purchased from Aldrich. N-(3-aminopropyl)-pyrrole and pyrrole-N-propylsulfonic sodium were prepared as described previously [22]. Urease (EC 3.5.1.5, from jack bean, 50 U/mg) was purchased from Sigma. Chitosan (degree of deacetylation: 88%, molecular weight: 210,000) was supplied by Hengsheng Biochemical Co., Qingdao, China. All other chemicals were of analytical grade and used without further purification. Deionized (DI) water (resistivity of 18 MΩ cm) was obtained from a Millipore Milli-Q Water System (Millipore Inc.), and was used for rinsing and for makeup of all aqueous solutions.

Piezoelectric measurements were performed in an ESPS/FIA sensing system which was described in our earlier report [15]. A nanoporous alumina membrane (4 cm^2) with SENs was used as the separated electrode. The distance between the separated electrode and the quartz crystal disc was set as 3 mm. The separated electrode and gold electrode were connected to a TTL-IC oscillator. The solution was introduced into flow cell by FIA setup. The oscillating frequency of the ESPS was monitored using a universal frequency counter (Iwatsu, Model SC-7201) attached to personal computer, and the data was acquired and stored using a computer-based data acquisition system. Transmission electron microscope (TEM) image was obtained at 120 kV on a Hitachi model H-800 transmission electron microscope. Infrared (IR) spectra were recorded on Nicolet 200SXV Fourier transform infrared (FTIR) spectrometer using a KBr wafer.

2.2. Fabrication of the urea biosensor

The nanoporous alumina membranes were prepared by the two-step anodization method described elsewhere [23], the alumina electrodes with pore diameter (200 nm) and pore length (105 μm) were employed in our experiments unless otherwise stated. The SENs were synthesized according to the procedure described in our previous study except for no addition of $FeCl_3$ and $FeCl_2$ [18]. The SENs immobilization onto nanoporous alumina membranes was performed by the following processes. The nanoporous alumina membranes were firstly immersed in a pH 6.5 phosphate buffer solution containing 1 mg mL^{-1} SENs for 1.5 h at 25 °C, and then were washed with water. Subsequently, 10 μl of 1% chitosan in 1% acetic acid was spread on the above alumina membranes and kept overnight for coating. Finally, the as-prepared urea biosensor was washed with water and stored at 4 °C before use.

2.3. Piezoelectric measurements

The piezoelectric measurements of the obtained urea biosensor were carried out in pH 7.4 aqueous solution at 35 °C [15]. After mounting nanoporous alumina electrode with immobilized SENs and quartz crystal disc into the flow cell, a small amount of aqueous solution was introduced into the flow cell. When the frequency became stable, urea solution was injected into the detection system. Time-dependent change in the frequency was recorded by a frequency counter and stored in a microcomputer.

3. Results and discussion

3.1. Characterization of the SENs

The morphology of the resultant encapsulated enzyme molecule was characterized by TEM. As shown in Fig. 1A, the as-prepared SENs were quite polydisperse and ellipsoidal in shape with a size of about 45 nm in diameter. The particle size distribution of the

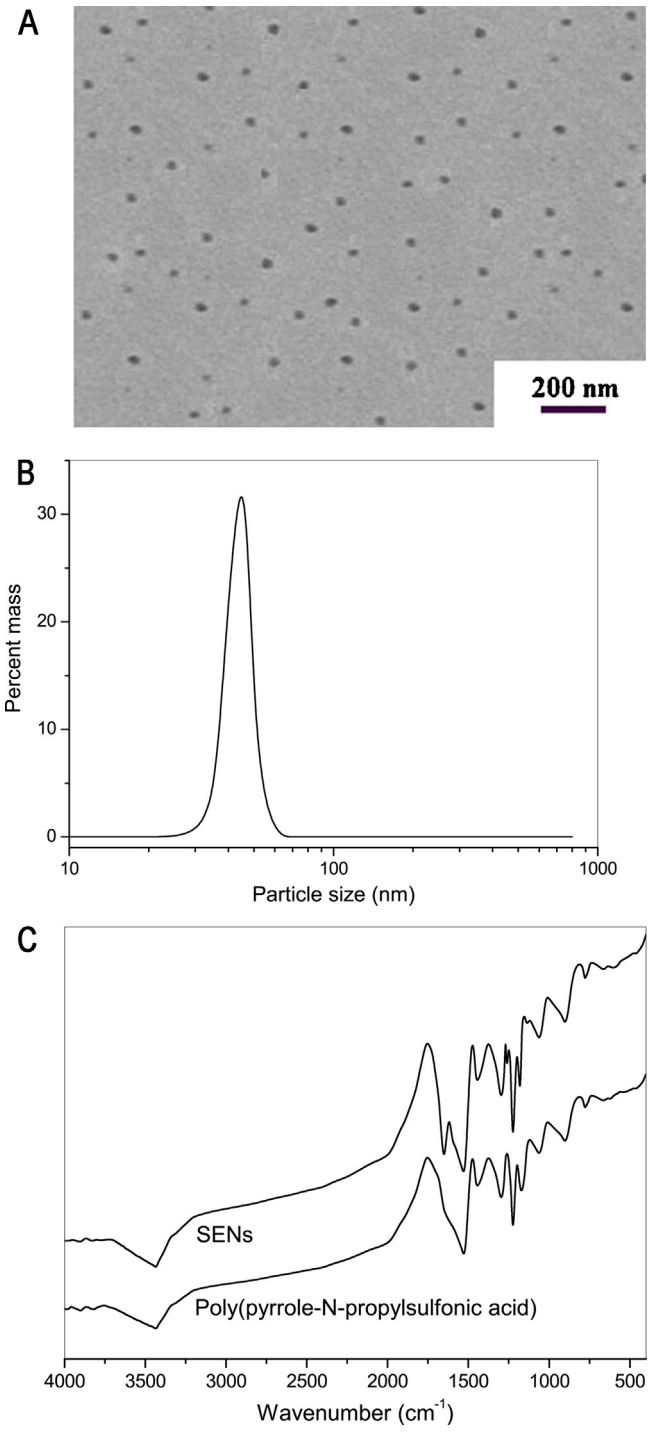

Fig. 1. TEM image (A), particle size distribution (B) and FTIR spectra (C) of synthesized SENs.

SENs measured by laser particle analyzer is shown in Fig. 1B, a narrow size distribution was observed and the average particle size of the SENs was around 44.2 nm, which was consistent with TEM observation. The obtained results indicated that this technique was available to realize the encapsulation of single enzyme molecule by polymer layer. In order to further verify the formation of a polymer on enzyme molecule, FTIR was employed to gain evidence for the SENs. Fig. 1C shows the FTIR spectrum of poly(pyrrole-N-propylsulfonic acid) and SENs. The main bands at 1540, 1456, 1300, 1066, 902 and 780 cm^{-1}, which were in good agreement with the characteristic bands of poly(pyrrole-N-propylsulfonic acid),