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Novel diazoresin/carboxymethyl chitosan capillary coating for the analysis of proteins by capillary electrophoresis

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ABSTRACT

Capillary electrophoresis has become an effective tool for the separation and analysis of biomolecule macromolecules. In this work, a new method to prepare covalent connection capillary electrophoretic coatings is proposed. Photosensitive diazoresin (DR) as coupling agents and carboxymethyl chitosan as coating was used. Through layer by layer (LBL) self-assembly and unique photochemistry reaction of DR, a stable and efficient capillary coating can be obtained. The covalently bonded coatings have the ability of suppressing protein adsorption on the inner surface of silica capillary, and thus a baseline separation of Lysozyme (Lys), myoglobin (Mb), bovine serum albumin (BSA) and ribonuclease A (RNase A) can be achieved. The covalently linked DR/carboxymethyl chitosan capillary coatings presented good stability and repeatability. This method seems to be an environmental and simple way to prepare the covalently coated capillaries for CE.

KEYWORDS

Capillary electrophoresis; covalently coated capillary; diazoresin; carboxymethyl chitosan; protein

1. Introduction

Capillary electrophoresis (CE) with advantages of high efficiency, high sensitivity, speediness and economy is a powerful separation tool for biomacromolecule analysis [1,2]. However, one of the major difficulties of CE is the undesirable adsorption of protein onto fused-silica capillary walls when analyzing proteinaceous samples [3–5]. This phenomenon may lead to sample loss, poor resolution, peak broadening, long migration times, and unstable electroosmotic flow (EOF) [6]. In order to suppress the protein adsorption onto the capillary surface, the common used approach is surface modification with capillary coatings [7–8].

Generally, capillary coatings are classified into non-covalently and covalently bonded coatings [9]. The non-covalent coating can be produced by flushing the capillary with coating solutions, and the coating molecules absorb on capillary surface by weak interactions such as electrostatic, van der Waals, and hydrogen bonding. The preparation process of covalently bonded capillary coatings is usually complicated which includes multi-steps such as capillary pretreatment, introducing coupling agents,
and inserting target coating reagents [10,11]. But highly toxic and moisture sensitive silane coupling agents are traditionally used in the covalent coatings, which often cause environmental and quality problems during the manufacture and application [12,13].

In the preparation process of high-performance capillary electrophoresis coatings, how to combine the advantages of non-covalent bonds and covalent bonding coatings to avoid their shortcomings is one of the main directions. In this paper, we prepared a series of covalent linked capillary coatings, which is a new material using layer by layer self-assembly technology combined with photochemical reaction. The preparation, structure and properties of the coating are preliminarily studied and discussed.

2. Experiments

2.1. Reagents and solutions preparation

DR (Mn = 2500) was synthesized according to the method described elsewhere, respectively [14]. Carboxymethyl chitosan was bought from Shanghai Institute of Fine Chemical Industry (Tianjin, China). Lysozyme (Lys), cytochrome c (Cyt-c), bovine serum albumin (BSA), amylglucosidase (AMG), myoglobin (Mb) and ribonuclease A (RNase A) were purchased from Sigma (St. Louis, USA). N,N-Dimethyl formamide (DMF) was purchased from Yongda Chemical Reagent Company (Tianjin, China). Phosphate acid (H₃PO₄) was purchased from Fuyu Fine Chemical Company (Tianjin, China). Monosodium orthophosphate (NaH₂PO₄·2H₂O) and dibasic sodium phosphate (Na₂H₂PO₄·12H₂O) were bought from Shunqiang Chemical Reagent Company (Shanghai, China). Acetone was obtained from Sanhe Chemical Reagent Company (Tianjin, China). Sodium hydroxide (NaOH) and hydrochloric acid (HCl) were purchased from Hongyan Reagent Company (Tianjin, China). Phosphate buffer was used as separation medium, and the pH value was adjusted by NaOH (0.1 M) and H₃PO₄ (40 mM). The concentrations of Lys, Cyt-c, BSA, AMG, Mb, and RNase A in the testing samples were all 0.5 mg/mL. All solutions were filtered through a 0.45 μm membrane before use.

2.2. Preparation of the carboxymethyl chitosan-coated capillary

The preparation of the carboxymethyl chitosan coated capillary was as follows: a new bare fused silica capillary was rinsed with 0.1 M NaOH for 30 min and deionized (DI) water for 10 min. Then coating was performed by flushing the capillary with aqueous solution of DR (2 mg/ml) for 5 min, and then flushed with DI water for 1 min and air dried for 5 min. Subsequently, the capillary was flushed with aqueous solution of carboxymethyl chitosan (4 mg/ml) for 5 min, and then flushed with DI water for 1 min and air dried for 5 min. A self-assembled DR/carboxymethyl chitosan bilayer coating was completed. The coating cycle was repeated for several times to obtain multilayer DR/carboxymethyl chitosan coated capillary. Afterwards, the coated capillary was exposed to 365 nm UV light with an intensity of 350 μW/cm² for 15 min in order to form the covalently linked carboxymethyl chitosan capillary coatings. The schematic illustration of these preparation steps above is shown in Fig. 1.
2.3. Instrumentation and characterization

UV-vis spectrometer (TU-1810, China) was used for monitoring the LBL self-assembly coating process. The photo-crosslinking of the DR/carboxymethyl chitosan coating on the capillary was carried out using a 365 nm UV curing system (EXFO Omnicure S1000) with a lamp power of 100 W. Atomic force microscope (afm, CSPM 5500, China) was used for surface characterization of the coatings. The CE experiments were performed on a CL1020 high performance capillary electrophoresis instrument (Huayang liming instrument Co., China). Fused-silica capillaries of 75 μm ID and 375 μm OD were provided by Yongnian Optic Fiber (Hebei, China). The EOF measurements were carried out using a method reported elsewhere [15]. Phosphate buffers (40 mM) of pH 3.0–9.0 were prepared to determine EOF at different pH values. DMF with a concentration of 0.5 vol % was used as the EOF marker.

3. Results and discussion

3.1. Formation of DR/carboxymethyl chitosan coatings on the inner wall of capillary

3.1.1. LBL self-assembly

UV-vis spectroscopy is used to monitor the assembly process. The absorbance of the DR/carboxymethyl chitosan film is at 380 nm, which derives from the characteristic π−π* transition absorption of the diazo group of DR, increases linearly with the number of assembly cycles (Fig. 2). This indicates that the LBL assembly is carried out successfully and uniformly. The driving force of the assembly comes from the hydrogen bond between the diazo group of DR and hydroxyl group of carboxymethyl chitosan.
Since the 4-layer capillary coating is well formed and facile to fabrication, it is adopted for the following performance studies.

3.1.2. UV crosslinking

DR is a non-toxic photoactive component often used as cell culture supports [16,17], and the diazo groups involved in the DR/carboxymethyl chitosan multilayer films will be decomposed under UV irradiation, which results in a gradual decrease in the absorbance of the film at 380 nm (Fig. 3). The photoreaction that takes place in the multilayer films, which originates from the diazo decomposition, is a first-order reaction: $\ln[(A_0-A_e)/(A_t-A_e)]$ changes linearly with irradiation time, where $A_0$, $A_t$ and $A_e$ represent the absorbance of the

Figure 2. The UV-vis spectra of the assembly from the DR and carboxymethyl chitosan. Number of assembly cycles (bottom to top): 1, 2, 3, 4, 5 and 6.

Figure 3. UV-vis spectra of DR/carboxymethyl chitosan multilayer coatings at different irradiation times. Irradiation time (s) (top to bottom): 0, 5, 10, 15, 25 and 35 s; irradiation intensity (at 365 nm): 350 $\mu$W/cm$^2$. 
film before irradiation, after irradiating for time $t$, and at the end of irradiation (35 s), respectively.

### 3.1.3. Stability

As its covalently cross linked structure, the spectrum of the irradiated coating did not change after immersion in DMF for 30 min (Fig. 4a). However, the spectrum of the non-irradiated film changed dramatically by the etching of the DMF (Fig. 4b), because the force between them is hydrogen bonding which is so weak that can be easily eluted.

### 3.1.4. Morphology

AFM images (Fig. 5) exhibit the surface morphology of the bare and DR/carboxymethyl chitosan covalently coated capillaries. The inner surface of bare capillary is smooth which has an average surface roughness (Ra) of 0.22 nm, and after modified with DR/carboxymethyl chitosan covalent coatings, the Ra increases to 1.808 nm. The average thickness for the 4 layers of DR/carboxymethyl chitosan covalent coatings is about 7.306 nm. The increased thickness indicates that the coating process is carried out successfully.

### 3.2. Performance of the DR/carboxymethyl chitosan covalently coatings for CE analysis of proteins

#### 3.2.1. EOF

Figure 6 compares the EOF at different buffer pH in bare, DR/carboxymethyl chitosan non-covalently and covalently coated capillary columns. The magnitude of EOF in a capillary is dependent on the net surface charge density of chargeable groups. For uncoated capillaries, the dissociation of silanol groups is responsible for the generation of EOF, which increases with the increase of buffer pH, due to the strong dissociation of silanol groups in a high pH environment. By contrast, when the capillary is coated with DR/carboxymethyl chitosan, especially the covalently coated DR/carboxymethyl chitosan, the EOF is extremely low. For example, the EOF of the DR/carboxymethyl chitosan covalently coated column at pH 4.0 is $5.3975 \times 10^{-9}$ m$^2$V$^{-1}$S$^{-1}$ that is much less than that of the uncoated one ($1.963 \times 10^{-8}$ m$^2$V$^{-1}$S$^{-1}$), because the silanol...
groups on capillary surfaces which are responsible for the generation of EOF are mainly reacted and shielded by the DR/carboxymethyl chitosan coating.

3.2.2. Effect of coating types

Figure 7A–7C shows CE separation results of four proteins by using bare capillary, DR/carboxymethyl chitosan non-covalent, and DR/carboxymethyl chitosan covalent capillary coatings in the optimized conditions, respectively. The bare capillary performs a strong adsorption to the proteins, and thus a bad separation result with only two characteristic peaks is obtained and last for a long time. Although the separation performance of DR/
carboxymethyl chitosan non-covalent capillary coating is better than that of bare capillary, but the separate time is also long and the peak is wide, effective separation of the proteins cannot be achieved, and the stability of the coating is very poor due to lack of strong bondings to the capillary. Compared with them, the carboxymethyl chitosan covalent capillary coating has the best separation performance, take short time, and a stable and baseline separation of the Cyt-c, Lys, BSA and RNase A is achieved within 20 minutes.

4. Concluding Remarks

In this work, we present a new approach to the preparation of covalently connected capillary coatings and the introduction of a new material (carboxymethyl chitosan). As the coating, through the unique photochemical reaction between DR and carboxymethyl chitosan, the hydrogen bond is converted into covalently bound after treated with UV light. Compared with bare carboxymethyl chitosan coating, the covalent linked carboxymethyl chitosan capillary coating improves the CE separation performance of protein, and has good stability and reproducibility. Covalently bonded coatings inhibit protein adsorption on the inner surface of quartz capillary, so that

![Figure 7. Separation of four proteins using the bare capillary (a), 4-layer carboxymethyl chitosan noncovalent coated capillary (b) and 4-layer carboxymethyl chitosan covalently coated capillary (c). Separation conditions: buffer, 40 mM phosphate (pH = 4.0); injection, 20 s with a height difference of 20 cm; applied voltage, +15 kV; UV detection, 214 nm; sample, 0.5 mg/mL for each protein; capillary, 75 μm ID × 50 cm (41 cm effective); capillary temperature, 25°C. Peak identification: 1, Lys; 2, BSA; 3, Mb; 4, RNase A.](image-url)
lysozyme (Lys) is baseline separated. Myoglobin (Mb), bovine serum albumin (BSA) and ribonuclease (RNase A) are used in capillary electrophoresis (CE) in 20 min. In addition, this method is simpler than the traditional method, DR, with highly toxic and water sensitive silane coupling agents.

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