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Evaluation of protein immobilization capacity on various carbon nanotube embedded hydrogel biomaterials

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ABSTRACT

This study investigates effective immobilization of proteins, an important procedure in many fields of bioengineering and medicine, using various biomaterials. Gelatin, alginate and chitosan were chosen as polymeric carriers, and applied in both their composites and nanocomposite forms in combination with carbon nanotubes (CNTs). The prepared nano/composite structures were characterized using scanning electron microscopy (SEM), Fourier-transform infrared spectroscopy (FTIR), thermal gravimetric analysis (TG) and contact angle analysis (CA). Electrochemical impedance spectroscopy analysis revealed gelatin composites in general to exhibit better immobilization performance relative to the native gelatin which can be attributed to enhanced film morphologies of the composite structures. Moreover, superior immobilization efficiencies were obtained with the addition of carbon nanotubes, due to their conducting and surface enhancement features, especially in the gelatin–chitosan structures due to the presence of structural active groups.

1. Introduction

Hydrogels are three-dimensional networks constructed from hydrophilic homopolymers or copolymers crosslinked to form insoluble polymer structures. Thanks to their advanced biocompatibility, flexible methods of synthesis, and satisfactory physical characteristics, hydrogels have been one of the most frequently used materials for many applications specifically in sensing technology, tissue engineering, stem cell biotechnology and drug delivery [1]. During the last two decades, natural hydrogels like gelatin, alginate and chitosan have been gradually replaced with synthetic hydrogels having high water absorption capacity, high gel tenacity, and most importantly tremendous biocompatibility.

Hydrogels are also attractive materials in the fabrication of various biosensors as the hydrated gel structure provides a superior supporting base for encapsulation and immobilization of functional biological molecules like cells, antibodies, antigens, and oligonucleotides.

Gelatin, one of the hydrogels used in this study, is a biopolymer derived from collagen by hydrolytic degradation. Because of its unique functionality, gelatin is used in a wide variety of applications in biomedical engineering, ranging from sensors [2,3] to regenerative medicine [4] and micro/nano patterning [5]. Due to its sol–gel transition temperature of 30 °C, gelatin should be cross-linked chemically to avoid dissolution at body temperature like many other hydrogels. Gelatin is composed of a variety of chains enabling it to be modified according to demand using crosslinkers. The amphoteric behavior of gelatin is due to the presence of the amino and carboxyl groups of amino acids. In acidic environment, gelatin is positively charged whereas in an alkaline media, gelatin is negatively charged. At the isoelectric point (between 4.6–5.0), positive charges from ammonium radicals equal negative charges from the carboxyl radicals [6]. Chitosan, another hydrogel used in this study, is a partially deacetylated derivative of chitin, obtained from the shell crustaceans such as crab and shrimp. This biocompatible polymer dissolves in weak acidic media. Further increase of pH towards alkalinity results in gel-like precipitates of the hydrated polymer by neutralization of the amine groups [7]. Alginate, the last hydrogelic biopolymer used in this study, is a straight polysaccharide produced by some algae and microorganisms. Commercially available alginate is typically extracted from brown algae by treatment with aqueous alkali solutions. It is a linear binary copolymer consisting of linked mannuronic (M) and guluronic acid (G) residues. In the presence of divalent ions, such as calcium, alginate tends to form a gel which can typically be used in the form of a hydrogel in biomedicinal applications, including tissue engineering [8] and biosensors [9]. The advantage of using alginate for entrapment or encapsulation is due to the environmental conditions provided by the gel for the entrapped material as well as the high porosity provided by the open lattice structure.

The incorporation of nanomaterials for desired specifications makes hydrogels even more attractive in biomedical applications. In sensor technology, the addition of nanomaterials to the supporting systems increases biomolecule immobilization and electron transfer rate, thus decreasing sensing concentration and sensitivity. Since their discovery in 1991, carbon nanotubes (CNTs) have attracted a great deal of attention by scientists worldwide and played an initial role in many innovative investigations due to their unique structural, morphological and

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electronic properties. Although extensive efforts have been made since the 90's, many potential applications of this fantastic material still have not been discovered because of the processing and manipulation difficulties. Coupling CNTs with hydrogels as the sensing material is thought to improve the electron transfer rate, enhance the detection sensitivity of electrochemical sensors and provide both conductive and biocompatible matrices for biomedical applications. There are few papers in sensor applications of CNTs in combination with hydrogels. To the best of our knowledge, there is only one study based upon gelatin–CNTs nanocomposite material [10]. In this study, the direct electrochemistry of glucose oxidase (GOx) at gelatin-multiwalled carbon nanotube modified glassy carbon electrode (GCE) has been investigated. The surface coverage concentration of GOx in gelatin–CNTs composite film modified electrodes has been found at the levels of 10^{-9} mol·cm^{-2} indicating high enzyme loading. The electron transfer rate constant of GOx also reflects the enormous conducting properties of CNTs. Tang et al. [11] have also shown the direct electrochemistry of GOx using a chitosan matrix in combination with CNTs. It has been emphasized in the paper that the MWCNTs (multi walled carbon nanotubes) embedded in flavin adenine dinucleotide (FAD) were like "conductive wires" connecting FAD with the electrode, thus reducing the distance between them and being propitious to fast direct electron transfer. In another study, Zhao et al. [12] prepared an alginate–CNTs nanocomposite material with the aim of fabricating a hemoglobin immunosensor for the detection of peroxides. This study demonstrated that the direct electron transfer of hemoglobin immobilized alginate–MWCNTs composite film was largely facilitated and showed the excellent bioelectrocatalytic activity towards hydrogen peroxide reduction.

In this work, combinations of various hydrogel nano/composite materials were prepared and a comparative study on effective immobilization of myelin basic protein (MBP), selected as model protein on which our group carried out various studies [2,9], onto these composite materials was carried out. Subsequently, gelatin, gelatin–CNT, gelatin–alginate, gelatin–alginate–CNT, gelatin–chitosan, and gelatin–chitosan–CNT nano/composite biomaterials were prepared and characterized using FTIR, TG, SEM, and CA, before being modified on to screen-printed carbon electrodes. The immobilization efficiency of MBP to those materials was studied using electrochemical impedance spectroscopy (EIS). Finally, effective immobilization supporting systems which is an important issue for sensing systems was described.

2. Materials and methods

2.1. Materials

Myelin basic protein, chitosan, alginate, gelatin from porcine skin (Type A), N-hydroxysuccinimide (NHS), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), glutaraldehyde cross-linking agents, single-walled carbon nanotube, potassium chloride (KCl), potassium ferrocyanide ($K_4[Fe(CN)_6]$), and potassium ferricyanide ($K_3[Fe(CN)_6]$) were purchased from Sigma (St Louis, MO, USA). A Sunred Human MBP Enyzme-Linked Immunosorbant Assay (ELISA) Kit was used for Ultraviolet Spectroscopy (UV). UV measurements were carried out using a LambdaScan Model UV spectrometer. De-ionized water was purified using a MilliPore Simplicity unit to a resistivity ≥ 18.2 MΩ cm.

Electrochemical measurements were carried out on a Gamry Instrument using the Framework Version 5.50 software. All impedance data were calculated using Zview 2. Quanta 400 F Field Emission Scanning Electron Microscope was used for the investigation of structures after being sputter-coated with gold. TGA termograms were obtained using Shimadzu DTA60 and FTIR measurements were carried out with Shimadzu IRAffinity-1 model Infrared Spectrometer. Contact angles were imaged using Attention Theta Contact Angle instrument.

2.2. Preparation of nano/composite electrodes

Gelatin was prepared by dissolving in phosphate buffer (0.05 M, pH 7.4) whereas acetate buffer (0.05 M, pH 5.5) was used for chitosan to obtain a desired ratio of 2% (w/v) in the final solution. The gelatin electrode without CNTs and MBP was prepared by direct modification of 2% (w/v) gelatin and 0.003 M glutaraldehyde as cross-linker. Gelatin–chitosan and gelatin–alginate electrodes were prepared by mixing both polymer solutions to obtain a final concentration of 2% (w/v). Although glutaraldehyde (0.003 M) was used as cross-linker for the preparation of gelatin–chitosan composite, NHS/EDC (0.0035 M) was used as cross-linker for the gelatin–alginate composite due to the presence of carboxyl groups rather than amine groups on alginate. In order to prepare the electrodes containing CNTs, nanotubes were added (CNTs to polymer ratio is 1:10) to the microtubes in which the related polymers (gelatin, gelatin–chitosan or gelatin–alginate) were situated. Homogeneity was established by vortexing after the addition of CNTs for a period of 30 s. The nanocomposites were prepared for the modification procedure after the addition of the cross-linkers. Subsequently, 10 μL of the nano/composite solution was added drop wise to the screen-printed carbon electrode surface. The electrodes were left at room temperature for 2 h to ensure a stable dry surface. Finally, 10 μL of MBP (10 μg·mL^{-1}) and cross-linker solution (0.003 M of glutaraldehyde for gelatin and gelatin–chitosan; 0.0035 M of NHS/EDC for gelatin–alginate) was added to the electrode surfaces to obtain the MBP immobilized electrodes. After 20 min of incubation, the MBP immobilized electrodes were washed with de-ionized water and prepared for electrochemical measurements which were carried out in a 1 mL screen-printed electrochemical cell system. An example of schematic presentation of nanocomposite structure is given for the gelatin–CNTs–MBP system in Scheme 1.

2.3. Electrochemical study

All-in-one screen printed carbon electrodes (SPCE) with a 20 mm^2 working surface area, counter and reference electrodes were used for the electrode design. The reasons making SPE electrodes superior are their high stability, durability and conductivity. A 1 mL volume electrochemical cell for screen-printed electrode was used in all experiments. CV (cyclic voltammetry) and EIS were performed in PB buffer containing 0.1 M KCl and 0.5 mM $Fe(CN)_6^{3-/4-}$. Cyclic voltammograms were obtained by cycling the potential between −0.4 to 0.6 V with a scan rate of 100 m Vs^{-1}. EIS measurements were recorded within the frequency range of 0.01 Hz to 100 kHz at open circuit potential. All experimental studies were performed at least three times.

The effective surface areas were determined by CV in 0.5 mM $Fe(CN)_6^{3-/4-}$/0.1 M KCl solution in the potential range of −0.4 to +0.6 mV. Scan rates of 10, 50, 100, 250, 500, 750 and 1000 m Vs^{-1} were employed. The reduction peak current was determined and effective surface areas of electrodes were calculated by the Randles–Sevcik equation (Eq. (1)) [13] as described below.

$$i_p = \left(2.69 \times 10^5\right) n^{3/2} D^{1/2} C A v^{1/2} \quad (1)$$

where n is the number of transferred electrons for the redox reaction, D is the diffusion coefficient (6.70 × 10^{-6} cm^2 s^{-1}), C is the molar concentration of ferricyanide (0.5 mM), A is the effective surface area (cm^2), and v is the scan rate (Vs^{-1}). The value of n is equal to one for cyclic voltammograms obtained in $Fe(CN)_6^{3-/4-}$, due to the following half reaction taking place at the electrode:

$$Fe(CN)_6{}^{3-} + e^- \rightarrow Fe(CN)_6{}^{4-}.$$