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Fabrication of multiwalled carbon nanotubes–magnetite nanocomposite as an effective ultra-sensing platform for the early screening of nasopharyngeal carcinoma by luminescence immunoassay

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ABSTRACT

The hybrid nanocomposite that consists of multiwalled carbon nanotubes (MWCNTs) and magnetite (Fe_3O_4) was fabricated by chemical co-precipitation method. Briefly, CNTs were oxidized with acids to form carboxylic group and then co-precipitated with Fe_3O_4 to form CNT–Fe_3O_4 nanocomposites. The nanocomposites were characterized by SEM, HRTEM, XRD, FTIR X-ray photoelectron spectrometry (XPS) and SQUID. The XRD results indicated the high crystallinity of Fe_3O_4 nanoparticles with spinel structure and the transmission electron microscope images depicted the intercalated iron oxide magnetic particles on the surface of CNTs. The MWCNTs–Fe_3O_4 was applied as a sensing interface to perform luminescence enzyme immunoassays. Firstly, EBNA-1 antigen was immobilized onto the carboxyl group functionalized MWCNTs–Fe_3O_4, followed by binding with anti-EBNA-1 IgA antibodies. The diluted secondary antibodies (anti-human IgA-HRP) were then added to the CNTs/Fe3O4–PEG–EBNA-1–anti-EBV IgA ab complex and act as a catalyst to produce a visible light upon reaction with the substrate luminol. The formed RLU is proportional to the amount of IgA anti-EBV antiobodies on the MWCNTs. The detection limit of proposed CNTs/Fe_3O_4 based luminescence enzyme immunoassay was in the order of 0.00128 EU/mL (1:100,000 fold dilution) for the detection of anti-EBV IgA antibodies, whereas the commercial ELISA and magnetic beads' assay was accounted for up to the dilution fold of 1000 (i.e., 0.128 EU/mL). The initial findings showed that CNTs/Fe_3O_4 nanocomposites have a great potential in luminescent enzyme immunoassays and could be used as a sensing platform for the early screening of nasopharyngeal carcinoma.

1. Introduction

Carbon nanotubes (CNTs) are remarkably a new material and are defined as allotropes of carbon with a cylindrical nanostructure with diameter ~1–10 s of nm. MWCNTs are usually made from several cylindrical carbon layers with diameters in the range of 1–3 nm for the inner tubes and 2–100 nm for the outer tubes [1]. CNTs are considered as a promising nanomaterial for various biomedical applications, delivery of therapeutics [2], cancer diagnostics [3], tissue engineering, etc. The potential applications of CNTs in bio/immuno sensing are mainly due to its exceptional thermal, electrical and mechanical properties. Despite the advantages of CNTs, several limitations like toxicity and biocompatibility of CNTs still need to be evidently addressed. Non-specific binding of biomolecules on the hydrophobic nanotube surface was considered as potential disadvantage [4], however, it could be resolved by different functionalization methods. A number of different CNTs functionalization have been proposed either by covalent or non-covalent approaches. Oxidation of CNTs using strong acids is a method commonly used for generating covalent functionalization [2]. The chemical functionalization of CNTs not only offers the compatibility with the host, but also prevents agglomeration and improves the solubility in various solvents, which is an essential criterion for biosensing applications.

The carbon nanotube (CNT)/magnetic nanoparticle hybrids have gained more and more attention, due to their good stability, unique structural and excellent magnetic properties. The enormous attention behind the fabrication of Fe_3O_4 nanocomposites is mainly explained by the superparamagnetic property of iron oxides. Further, the

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superparamagnetic property of Fe_3O_4 facilitates the recovery of functionalized CNTs using an NdFeB permanent magnet. A range of CNT–magnetite inorganic hybrid materials have been developed and used for variety of applications, like, magnetic property for microscopy, in biosensors and drug delivery [5,6]. The magnetite (Fe_3O_4) nanoparticles were loaded onto the surface of CNTs by different in situ methods like chemical precipitation and solvothermal method [7,8]. The carboxylic derivative of pyrene was used as a crosslinker for the attachment of capped magnetic nanoparticles on the surface of CNT to increase the solubility of CNTs in organic solvents [9]. It was reported that hybrid materials that contain at least two nanocrystals have received considerable interest for the rapid and sensitive detection of tumor markers [10].

The concept of using nanomaterials for biosensing is, it offers great functional surface area for biomolecule loading. In addition, the unique properties of nanometerials, such as optical, electronic and mechanical, offer the fabrication of sensors with ultrasensitive detection limit. Epstein–Barr virus (EBV) infection is causatively associated with a variety of human cancers, including nasopharyngeal carcinoma (NPC) and Epstein–Barr nuclear antigen (EBNA-1), and is the only viral nuclear protein expressed in NPC able to promote oncogenesis by altering cellular properties [11]. Nasopharyngeal carcinoma (NPC) is a highly prevalent malignancy in southern China and most in Southeast Asia and North Africa [12]. Hence, it was suggested to screen anti-EBA antibody level for the early diagnosis of nasopharyngeal carcinoma. Indirect Immunofluorescence Assay (IFA) is still widely used for EBV serodiagnosis in NPC [13], but this method is time consuming and not suitable for large-scale analysis [14]. Enzyme-linked immunosorbent assay (ELISA) provides a promising alternative with potential for automation and mass screening but it lacks standardization [15]. Sensitive and simple techniques for the precise detection of clinical markers are incredibly sought in disease monitoring especially in cancers. Recently, novel immunoassay formats using carbon nanotubes based analytical techniques offering better sensitivity and practicality than other bioanalytical techniques for the ultrasensitive detection of cancer markers [16]. Fabrication of quantum dots (QD), nanocomposite and Fe_3O_4 nanoparticle combined with electrochemiluminescence (ECL) immunoassay were proposed for the ultrasensitive detection of carcinoembryonic antigen 19-9 (CA 19-9) [17]. Here, we report the synthesis of CNTs/Fe_3O_4 hybrid nanocomposites by in situ chemical co-precipitation method. The as-prepared CNT–magnetite composites were extensively characterized and applied for an effective detection of anti-EBA IgA antibodies by luminescent immunoassay. The sensitivity of CNTs/Fe_3O_4 based luminescent immunoassay was compared with the commercial ELISA and magnetic beads assay. The system capable of ultrasensitive detection of anti-EBV IgA antibodies is greatly beneficial in the primary screening of nasopharyngeal carcinoma.

2. Experimental

2.1. Materials

Multiwall carbon nanotubes (MWCNT) were purchased from Sigma Aldrich, USA, with 7–15 nm in diameter, and 0.5–200 μm in length. Ferrous chloride 4-hydrate, ferric chloride, and poly(ethylene glycol) bis(amine) (PEG) were also purchased from Sigma Aldrich, USA. Epstein–Barr nuclear antigen 1 (EBNA-1) was purchased from Development Center for Biotechnology, Taiwan. MediPro anti-EBV IgA ELISA was purchased from Formosa Biomedical Tech Corp., Taiwan. SuperSignal ELISA Pico chemiluminescent substrate was purchased from Thermo Scientific (USA). The commercial magnetic beads (Dynabeads® M-270 Carboxylic Acid) were purchased were purchased from Life Technologies (USA).

2.2. Oxidation of carbon nanotubes

400 μg of CNTs was stirred with the mixture of concentrated sulfuric acid/nitric acid (3:1) solutions and refluxed at 80 °C for 4 h to graft a carboxylic acid groups on the surface of CNTs. Followed by, the solution was diluted with 1 L of distilled water and the excessive acid was removed through a polycarbonate filter paper with 0.1 μm pore size. The prepared samples were then dried in an oven at 60 °C overnight.

2.3. Preparation of CNTs/Fe_3O_4 nanocomposites

The CNTs/Fe_3O_4 hybrid nanocomposites were prepared by in situ chemical co-precipitation of Fe^{2+} and Fe^{3+} ions (molar ratio 2:1) in alkaline solution to the MWCNTs. Briefly, the oxidized CNTs (400 μg) were first added to a 100 mL solution containing $FeCl_2 \cdot 4H_2O$ (0.10 g), $FeCl_3$ (0.16 g) and heated to 80 °C for 30 min with stirring under N_2 atmosphere. The solution was further heated at 65 °C for 30 min and the pH of the solution was adjusted to 12.0 with 2 N NaOH (3 mL) to form iron oxide precipitation. CNTs/Fe_3O_4 impurities were removed by washing several times with distilled water and the synthesized nanocomposites were collected using an NdFeB permanent magnet. The as-prepared nanocomposites were subsequently dried under vacuum and stored at room temperature. The equation of concomitant precipitation of Fe_3O_4 occurred in the above procedure was described below:

$$2Fe^{3+} + Fe^{2+} + 8NH_3\ H_2O \rightarrow Fe_3O_4 + 4H_2O + 8NH_4^+$$

2.4. Characterization

The morphologies of CNTs and CNTs/Fe_3O_4 nanocomposites were observed by scanning electron microscope (SEM, JEOL JSM-6300) at an accelerating voltage of 10 kV. Fourier transform infrared (FTIR) spectra of the samples were measured with a JASCO 4100 series spectrometer in the range of 4000–400 cm^{-1}; resolution 0.9 cm^{-1}. The X-ray diffraction patterns were collected on a Rigaku Rint-2200 diffractometer with Cu-kα ($\lambda = 1.5430$, 30 kV, 20 mA) radiation. The selected area electron diffraction (SAED) of CNT/Fe_3O_4 was performed by transmission electron microscopy (TEM) (Philips TECNA1 F20 at 220 kV). The qualitative analysis of surface elements was performed by X-ray photoelectron spectroscopy (XPS) equipped with PHI5000 Versa Probe (ULVAC-PHI, Chigasaki, Japan). A magnetic characterization was performed using an MPMS-7 magnetometer system (Quantum Design Company, San Diego, CA, USA).

2.5. Functionalization of CNTs/Fe_3O_4 nanocomposites

The as-prepared CNTs/Fe_3O_4 nanocomposites (100 μg) were conjugated with poly(ethylene glycol) bis(amine) (PEG) through EDC coupling. Briefly, CNTs/Fe_3O_4 was immersed in MES buffer with EDC (1%) for 30 min at 4 °C and the unreacted or the residual EDC in CNTs/Fe_3O_4 solution was removed by washing with distilled water. The activated CNTs/Fe_3O_4 was treated with PEG bis-amine (2%) for 1 h at room temperature. The activated carboxyl groups of CNTs/Fe_3O_4 will effectively bind to the amine groups of PEG through the formation of amide bond.

2.6. Immobilization of EBNA-1 onto CNTs/Fe_3O_4–PEG nanocomposites

The CNTs/Fe_3O_4–PEG nanocomposites were conjugated with EBNA-1 antigen via EDC coupling. In order to activate the carboxylic acid group, 20 μg of CNTs/Fe_3O_4–PEG was mixed with MES buffer containing 1% EDC. The solutions were transferred to a 96-wells