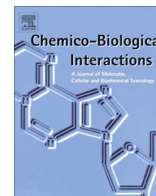




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Oxalic acid capped iron oxide nanorods as a sensing platform

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ABSTRACT

A label free impedimetric immunosensor has been fabricated using protein bovine serum albumin (BSA) and monoclonal antibodies against *Vibrio cholerae* (Ab) functionalized oxalic acid (OA) capped iron oxide (Fe_3O_4) nanorods for *V. cholerae* detection. The structural and morphological studies of Fe_3O_4 and OA- Fe_3O_4 /ITO, were characterized by X-ray diffraction (XRD), transmission electron microscopy (TEM), Fourier transform infrared (FTIR) spectroscopy and dynamic light scattering (DLS) techniques. The average crystalline size of Fe_3O_4 , OA- Fe_3O_4 nanorods were obtained as about 29 ± 1 and 39 ± 1 nm, respectively. The hydrodynamic radius of nanorods is found as 116 nm (OA- Fe_3O_4) and 77 nm (Fe_3O_4) by DLS measurement. Cytotoxicity of Fe_3O_4 and OA- Fe_3O_4 /ITO nanorods has been investigated in the presence of human epithelial kidney (HEK) cell line 293 using MTT assay. The cell viability and proliferation studies reveal that the OA- Fe_3O_4 nanorods facilitate cell growth. The results of electrochemical response studies of the fabricated BSA/Ab/OA- Fe_3O_4 /ITO immunosensor exhibits good linearity in the range of 12.5–500 ng mL^{-1} with low detection limit of 0.5 ng mL^{-1} , sensitivity 0.1 $\Omega/\text{ng mL}^{-1} \text{ cm}^{-2}$ and reproducibility more than 11 times.

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1. Introduction

Recently, investigators are being utilizing several types of nanostructured materials including iron oxides (mostly maghemite, $\gamma\text{-Fe}_2\text{O}_3$ and $\alpha\text{-Fe}_2\text{O}_3$ or magnetite, Fe_3O_4), among which magnetite is a very promising candidate. These iron oxide nanomaterials exhibits wide applications in various fields including electronic devices, drug delivery, ferrofluid technology, contrast agents hyperthermia treatment, cell separation, enzymatic assays biosensors, etc. [1–9] due to their peculiar physical and electronics properties such as small size, super-magnetic, low toxicity, biocompatibility and stability under physiological conditions. Although, these materials are more susceptibility towards oxidation and their tendency to agglomerate due to availability of strong dipole–dipole interactions between particles which can be prevented by functionalization with other materials [10]. The proper functionalization of nanomaterial with desire solvent is important to prevent aggregation and obtain thermodynamically stable nanoparticles. Thus, this nanomaterial can be functionalized with different materials including chitosan, carbon, polymers,

surfactants, bio-molecules, silica, metals, small polar molecules including citric acid (CA) etc. [2,11,12] which provide improved stability and biocompatibility nanoparticles. Moreover, the functionalized nanoparticles with carbon and silica capped $\alpha\text{-Fe}_3\text{O}_4$ and $\gamma\text{-Fe}_2\text{O}_3$ NPs control the phase transition of Fe_3O_4 nanorods at the time of electrophoretic film deposition and provide improved the electrochemical properties [2].

It has been observed that nanomaterial functionalized with carboxylic acid terminal group exhibit great importance because carboxyl group not only render the particles more water dispersible but also provides a site for further surface modification that can be utilized for wide applications including biomedical [13–17]. In this context, carboxylic group containing small molecules like oxalic acid (OA; $\text{C}_2\text{H}_2\text{O}_2$, OA) and citric acid (CA; $\text{C}_6\text{H}_8\text{O}_7$) found more suitable for functionalization of Fe_3O_4 nanorods. Because, OA and CA can be adsorbed onto the surface of the nanomaterial by coordinating via one or two of the carboxylate functionalities, leaving at least one carboxylic acid group exposed, making the nanomaterial surface hydrophilic, preventing particle agglomeration, and providing other functional groups to be used for further surface modification including oligonucleotides and antibodies or proteins via covalent bonding [18]. It has been observed that most of studies reported in literature are based on CA coated iron oxide nanomaterial for their biomedical applications including in contrast agent, drug delivery, targeted cellular imaging, hyperthermia and

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biodetection [10,11,19]. De Sousa has been reported that CA coated nanoparticles (NPs) stable in water and suitable for energy dissipation under an external ac magnetic field in the *rf* range which appropriated for magnetic hyperthermia therapy [20]. Cheraghipour et al., reported that CA capped NPs exhibit remarkable heating effect during the application of a magnetic field, which make them attractive for magnetic fluid hyperthermia applications [21]. NPs coated with both oleic acid and pluronic loaded with high doses of hydrophobic drugs has been used for drug delivery [22]. Oxalic acid (OA), has gained importance in green chemistry organic oxalic acid is essential for human body. The OA has been utilized as a reductant for silver nanoparticles synthesis and stabilizing by cetyltrimethyl-ammonium bromide (CTAB). Thus there is a wide scope to explore the studies on OA functionalized Fe₃O₄ for electrochemical biosensing applications.

Here, we have fabricated *in situ* capped OA-Fe₃O₄ nanorods for immunosensor application. A thin film of OA-Fe₃O₄ nanorods has been prepared using electrophoretic deposition technique onto Indium-tin-oxide (ITO) coated glass substrate and utilized for functionalization with monoclonal antibodies specific towards the *Vibrio cholerae* and bovine serum albumin (BSA) for *V. cholerae* detection using impedimetric spectroscopic technique. *V. cholerae* is known to be highly pathogenic which creates a serious health problem like diarrhea and acidosis in human. Thus, development of point of care devices has recently gained increasing attention in diverse fields including clinical diagnosis, environmental monitoring and food safety etc. [23,24]. Few immunosensors have been reported based on nanostructured materials for *V. cholerae* detection [25–28]. To the best of our knowledge, this is first time report on OA capped synthesized Fe₃O₄ nanorods and even for fabrication of immunosensor for pathogen (*V. cholerae*) detection. These 1D nanomaterials (OA-Fe₃O₄) nanorods provide direct attachment of antibodies via electrostatic interactions that may preserve the structural integrity of the antibodies resulted in higher sensitivity as compared to CA capped Fe₃O₄ nanoparticles [19]. Moreover, the cytotoxicity effect of Fe₃O₄ nanorods is still unexplored for application to point-of-care devices.

2. Experimental section

2.1. Material and methods

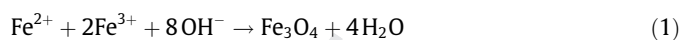
FeCl₃ (Fe³⁺); FeCl₂ (Fe²⁺) and NaOH was purchased from Fisher Scientific Ltd. Oxalic acid (OA; C₂H₂O₄) was purchased from SDFCL, Mumbai-30. All these chemicals were used to prepare solutions in deionized water without further purification. Indium-tin-oxide (ITO) coated glass plates were obtained from Balzers, UK, (Baltracom 247 ITO, 1.1 mm thick) with a sheet resistance and transmittance of 25 Ω sq⁻¹ and 90%, respectively. Monoclonal antibodies specific to *V. cholerae* (Ab-Vc) and bovine serum albumin (BSA) have been obtained from M/s Genetix Biotech Asia Pvt. Ltd, India. All other chemicals were of analytical grade and used without further purification. Deionized water from Organo Biotech Laboratories, India, was used to prepare the solutions.

2.2. Preparation of OA capped Iron oxide nanorods

The magnetite nanorods have been prepared using co-precipitation technique which is the simpler process followed by as reported in literatures [29]. We have used 0.2 M (25 mL) of FeCl₃ and 0.1 M (25 mL) of FeCl₂ and dissolved in de-ionized water with stirring, the proposed reaction is give below (1). The capping was done by maintaining the 1:1 ratio of resulting solution and the capping agent (OA) of 0.5 gm (10 mL⁻¹) was added drop wise

followed by continuous stirring at 80 °C. Then NaOH solution of 2 M (100 mL) was added drop wise at 90 °C with vigorously stirring for 30 min as reported by [30]. The precipitating agent is NaOH solution (basic medium). Thus obtained dark brown precipitation is taken for magnetic decantation and washed several times with de-ionized water until the pH 7 was noted. Here bare magnetite nanorods were prepared without the functionalization, means the capping agent (oxalic acid; OA) are not dissolved in the salt solution. The same procedure is followed to obtain the bare magnetite particle.

The principle reaction for the co-precipitation method is:



2.3. Cell proliferation study

The biocompatibility test of bare Fe₃O₄ and OA-Fe₃O₄ has been monitored on cells line survival/proliferation (human epithelial kidney cell line HEK 293) was studied using Methyl Thiazol Tetrazolium (MTT) assay. HEK 293 is used as test cell line. The HEK 293 cells are procured from NCC, Pune, India. The cells are maintained in completed growth medium [10% FBS containing Dulbecco's Modified Eagle's Medium (DMEM) medium] at 37 °C under humidified 5% CO₂ environment. For the assay, cells were plated at about 6000/well in 96-well tissue-culture plate and were allowed to attach for 48 h. Next day, the medium on cells was replaced with MTT containing DMEM medium. The cells were kept for 6 h at 37 °C to allow reduction of MTT dye to formazan crystals by living cells. Cells in each wells were solubilized in 200 μL of dimethyl sulfoxide (DMSO), followed by absorbance measurement at 570 nm. The % change in proliferation was calculated with respect to control cells that were not exposed to NPs (control). All experiments were done in triplicates.

2.4. Preparation of OA-Fe₃O₄/ITO electrodes by electrophoretic deposition (EPD)

The thin film of OA-Fe₃O₄/ITO was deposited on onto the ITO surface using electrophoretic deposition (EPD) technique using a DC battery (BioRad, model 200/2.0) as the power supply. 0.25 mg of OA capped Fe₃O₄ (OA-NPs) was dispersed in 10 mL acetonitrile solution and ultra-sonicated for 30 min. To increase the deposition rate of nanorods onto the ITO surface Mg ions enhance the positive charge on the material surface which help in transportation of OA-Fe₃O₄ towards the cathode surface [26,31]. A platinum foil (1 cm × 2 cm) was used as an anode and a hydrolyzed ITO-coated glass plate as a cathode. The two electrodes, placed parallel to each other with a separation of 1 cm, were dipped in capped Fe₃O₄ NPs colloidal suspension. We have optimized the film deposition onto an ITO plate (0.25 cm²) at different voltages ranging from 30 to 60 V for 40 s and then removed from the suspension followed by washing with de-ionized water to remove any unbound particles.

2.5. Biofunctionalization of OA-Fe₃O₄/ITO electrodes with antibodies (Ab) and BSA

The OA-Fe₃O₄/ITO electrodes were functionalized with monoclonal anti-*Vibrio cholera* antibodies (Ab). The stock solution of monoclonal antibodies specific towards *V. cholerae* (Ab; 1 ng mL⁻¹) was freshly prepared in phosphate buffer (50 mM, pH 7.0) and antigen (*V. cholerae*, Vc) aliquot in different working concentrations (1–500 ng mL⁻¹). 10 μL of Ab (1 ng mL⁻¹) solution was uniformly loaded onto OA-Fe₃O₄/ITO electrode surface via physical absorption and kept undisturbed overnight in a humid chamber.

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