Original Article

An efficient electrochemical biosensor for Vitamin-D₃ detection based on aspartic acid functionalized gadolinium oxide nanorods

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ABSTRACT
In this study, an efficient electrochemical biosensor for Vitamin-D₃ detection using gadolinium oxide nanorods (Gd₂O₃NRs) has been reported. Gd₂O₃NRs were hydrothermally synthesized and functionalized with aspartic acid (Asp-Gd₂O₃NRs). Asp functionalization did not change the phase, shape and structure of Gd₂O₃NRs. The high-resolution transmission electron microscopy study revealed the diameter of Asp-Gd₂O₃NRs as 14.26 ± 0.13 nm with enhanced dispersivity. The Gd₂O₃NRs and Asp-Gd₂O₃NRs showed zeta potential of +29 and +24 mV, respectively. Asp-Gd₂O₃NRs exhibited enhanced hydrophilicity and electrochemical property than the bare Gd₂O₃NRs. A thin film of Asp-Gd₂O₃NRs was deposited on a glass substrate coated with indium-tin-oxide (ITO) by electrophoretic deposition. The immobilization of Vitamin-D₃ monoclonal antibody (Ab-VD) was done on the surface of Asp-Gd₂O₃NRs/ITO electrode for determination of Vitamin-D₃. The study of BSA/Ab-VD/Asp-Gd₂O₃NRs/ITO immunoelectrode response with different Vitamin-D₃ concentration was investigated using differential pulse voltammetry technique. The results of response study exhibited an improved sensitivity value of 0.38 μA ng⁻¹ mL⁻² with a linear range of 10–100 ng mL⁻¹ for Vitamin-D₃ detection while the detection limit of 0.10 ng mL⁻¹ was obtained. This immunosensor showed a satisfactory response to commercially available Vitamin-D₃ oral solution. Besides this, in vitro study of Gd₂O₃NRs and Asp-Gd₂O₃NRs was performed on RAW 264.7 and MCF-7 cells.

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1. Introduction

In recent years, Vitamin D (Vit-D) has captured the attention of the present world as it is an important and deciding factor for bone health and its deficiency is prevalent in all age groups [1]. Vit-D plays an essential job in bone strengthening
and a lower value of Vit-D causes rickets in children and osteomalacia in adults [2]. Various research reports and clinical studies showed that Vit-D deficiency might be associated with serious health problems including hypertension, Parkinson’s, Alzheimer’s, cardiovascular and cancer diseases [3–8]. Vit-D is a fat-soluble secosteroid and have two main forms ergocalciferol (Vit-D2) and cholecalciferol (Vit-D3) [9]. Among two metabolic form, Vit-D3 form is measured in serum samples and hence the preferable form over Vit-D2 [10]. The various reports on Vit-D suggest the required level of Vit-D is about 30 ng mL−1 and the value lower than 30 ng mL−1 is associated with Vit-D deficiency [11,12]. The conventional techniques used for detecting Vit-D concentration are enzyme-linked immunosorbent assay, mass spectrometry, chromatography, and radioimmunoassay etc. [13–15]. These techniques consume more time, require sophisticated instrumentation and are expensive so there is an increased demand for analytical techniques for Vit-D determination which should be reliable, selective, easy to operate, rapid and economical. Biosensor plays an important role in analytical sciences due to some exceptional capabilities like specificity, sensitivity, low cost, compact size and user friendly operation. Also, among the available transducer approaches electrochemical detection is fast, suitable, easy detection and cost-effective technique have been reported in the present study. The literature available regarding Vit-D detection is very limited and few research groups have reported sensors for determining Vit-D values. The biosensors for 25-OH Vit-D detection was developed by Carlucci et al., 2013 by utilizing 4-ferrocenylmethyl-1,2,4-triazoline-3,5-dione derivative. The electrochemical parameters obtained were a sensitivity value of 0.020 μA mL ng−1 and limit of detection (LOD) as 10 ng mL−1 with the electrochemical approach [16]. The Au electrodes used in SPR technique are costly and also experts are required for handling the instruments. The LOD obtained through electrochemical detection was 10 ng mL−1 that can be improved further to get a lower value. Other report was published by Ozbakir et al., 2015 which was based on an electrode which was modified with an enzyme and a detection range of 5–200 ng mL−1 was found for 25-OH Vitamin-D3 [17]. This method follow a time consuming fabrication process as there is expression and purification of proteins which is complex and lengthy process. Further, Canevari et al., 2014 developed electrochemical sensor for Vit-D3 determination using SiO2/GO/Ni(II)/GCE [18] and reported LOD of 3.26 × 10−9 mol dm−3 (1.25 μg of Vit-D3). The electrode SiO2/GO/Ni(II)/GCE used for Vit-D3 detection lacks in specificity as this detection method didn’t use antibodies specific to Vit-D3 but detected Vit-D directly through the catalytic activity of the material. In our lab, some preliminary work has been done for determination of Vit-D metabolites using electrochemical approach based on different nanomaterials. The nanomaterials were deposited on a glass substrate coated with indium tin oxide (ITO) and further used to immobilize antibodies and bovine serum albumin (BSA). Sarkar et al., 2017 had developed biosensing platform using carbon dots (CDs) embedded in chitosan (CH) matrix. The developed platform was used for detecting Vit-D2 and the BSA/Ab-VD2/CD-CH/ITO bioelectrode gave sensitivity value of 0.2 μA ng−1 mL cm−2 with LOD value of 1.35 ng mL−1 [19]. Also, electrospun polyacrylonitrile nanofibers with Fe3O4 NPs incorporated into it were utilized to fabricate an immunoelectrode (BSA/Anti-VD/Fe3O4-PANFs/ITO) for Vit-D3 detection as reported by Chauhan et al., 2018. The biosensing parameters obtained as sensitivity of 0.90 μA ng−1 mL cm−2 with LOD of 0.12 ng mL−1. The immunoelectrode responded in a linear range of 10–100 ng mL−1 [20].

Recently, applications of various rare earth metal oxide nanostructured (nRE-MO) materials in different fields, including materials science, bioscience and biotechnology have increased due to their interesting properties [21–24]. Gadolinium oxide (Gd2O3) is extensively explored because of its unique properties like high thermal conductivity, non-toxic, efficient charge transfer ability and interesting electrocatalytic properties [25–28]. Hence, Gd2O3 could be a suitable nanomaterial for electronics devices and it has a wide scope to explore its application in the electrochemical sensor [29]. Hydrothermally synthesized Gd2O3 nanorods (Gd2O3-NRs) have an advantage due to homogeneously grown anisotropic nanostructure with uniform shape [30]. Their well-controlled dimension and anisotropy of nanomaterials (1D) enable rapid charge transport along the axial direction that makes it suitable for electrochemical biosensor [20,31,32]. As the nanomaterials have a high tendency of agglomeration, the surface functionalization is required for potential biomedical applications [33]. Various reports were published on the surface functionalization of nRE-MO with an amine, carboxyl, and hydroxyl groups [34,35]. The surface functionalization enhances the dispersivity, biocompatibility, electrochemical properties and interactions with biomolecules for their potential use in biosensor applications [36–38]. Many researchers have used different chemicals like poly ethylene glycol, oleylamine, (3-aminopropyl) triethoxysilane (APTES) and oleic acid, etc. for surface functionalization [39–41]. Chaudhary et al., 2015 reported the surface coating of Gd2O3 NPs with different glycols having varied chain lengths ranging from ethylene glycol to tetramethylene glycol for p-nitrophenol sensing. However, proteins, amino acid and biopolymers were found to be more promising materials for surface functionalization of nanomaterials [41,42]. Amino acids act as capping agents for nanomaterials in numerous biological applications [42,43]. The various available amino acids along with aspartic acid (Asp) have desired functional groups (carboxyl and amine) which provide sufficient binding sites and hydrophilic environment for biomolecules attachment [44–46].

In this context, the present work describes the hydrothermal synthesis of Gd2O3-NRs and functionalization with Asp (Asp-Gd2O3-NRs). These Gd2O3-NRs and Asp-Gd2O3-NRs were characterized by different techniques. A thin film of these Asp-Gd2O3-NRs was deposited on ITO via electrophoretic deposition (EPD) technique and used for immobilization of monoclonal antibody of Vit-D3 (Ab-VD). This constructed BSA/Ab-VD3/Asp-Gd2O3-NRs/ITO immunoelectrode was used for Vit-D3 detection using differential pulse voltammetry (DPV) technique. DPV is considered as highly sensitive and extremely useful technique for detection of trace levels of analytes [47,48]. This electrochemical biosensor exhibited the improved sensitivity, specificity and detection range towards the Vit-D3 as compared to other reported biosensor in the literature (Table 1). This report based on Asp-Gd2O3-NRs for
electrochemical detection of Vit-D₃ is the first one as per our knowledge.

2. Experimental section

2.1. Materials

Gadolinium nitrate [Gd(NO₃)₃] and L-aspartic acid (Asp) were purchased from Central Drug House (P) Ltd., New Delhi, India. N-Hydroxysuccinimide (NHS), N-ethyl-N-[3-(dimethylaminopropyl)] carbodiimide hydrochloride (EDC), and bovine serum albumin (BSA) were purchased from Sigma-Aldrich, USA. Sodium hydroxide (NaOH), sodium chloride (NaCl), sodium phosphate monobasic anhydrous (NaH₂PO₄), and sodium phosphate dibasic dihydrate (Na₂HPO₄) were purchased from Thermo Scientific. ITO having a resistance of 25Ω sq⁻¹, thickness of 1.1 mm and transmittance of 90% were procured from Blazers, UK. Raw 264.7 cells were obtained from National Centre for Cell Science, Pune and MCF-7 cells were obtained from European Collection of Cell Culture (ECACC). Fetal calf serum (FCS) and Dulbecco’s Modified Eagle’s medium (DMEM) were procured from Gibco, while methylthiazoltetrazolium (MTT) and dimethyl sulfoxide (DMSO) were purchased from Sigma and phosphate buffer saline (PBS), pH 7.4, 1X was procured from Hi-Media for cell lines studies. Ascorbic acid, cholesteral, oxalic acid, urea and uric acid were purchased for SRL Pvt. Ltd, India and glucose was procured from SDFCL, India. Vit-D₃ monoclonal antibody (Ab-VD) was obtained from M/s My BioSource, USA and antigen Vitamin-D₃ (Vit-D₃) from Cayman Chemical Company, USA. The different concentrations of Vit-D₃ were prepared by diluting the stock solution (1 mg mL⁻¹) in series using absolute ethanol. PBS (0.1 M) having 5 mM of [Fe(CN)₆]³⁻/⁴⁻ with different pH were prepared using a salt solution of Na₂HPO₄ and NaH₂PO₄ in presence of 0.9% NaCl.

2.2. Synthesis and surface functionalization of Gd₂O₃ NRs

Gd₂O₃ NRs were prepared using a precipitation process at room temperature and aging of the precipitate under hydrothermal conditions [30,49]. During synthesis, 0.2 M of Gd(NO₃)₃ solution [4.11 g of Gd(NO₃)₃ in 60 mL DI] was continuous stirred at 40 °C. NaOH (1 M) solution was added drop wise into Gd(NO₃)₃ solution until pH reached up to 10 and white precipitates formed. The resulting solution was further stirred for another 3 h and then transferred into a hydrothermal vessel and kept at 180 °C for 24 h. The product was washed 3-4 times with DI water and ethanol (50%) via centrifugation at 5000 rpm for 30 min. The final precipitates of Gd₂O₃ NRs were obtained and dried by maintaining a temperature of 80 °C for 12 h.

$$2\text{Gd(NO}_3\text{)}_3 + 6\text{NaOH} \xrightarrow{\text{hydrothermal}} \text{Gd}_2\text{O}_3 + 6\text{NaNO}_3 + 3\text{H}_2\text{O} \quad (1)$$

For surface functionalization, Gd₂O₃ NRs (5.5 mM) were dispersed in 50 mL DI water under sonication condition [temperature (30 ± 5 °C), frequency (40 KHz), and power (100 W)] for 1 h. An aqueous solution of Asp (50 mL; 15 mM) was added drop wise almost 1 drop per 2 s in the uniformly dispersed Gd₂O₃ NRs (50 mL) and stirred for 5 h at room temperature. Asp-Gd₂O₃ NRs were washed thrice using DI water and ethanol repeatedly using centrifugation at 5000 rpm for 30 min and dried at 60 °C for 12 h. Asp was conjugated on the surface of Gd₂O₃ NRs via electrostatic interaction between the positively charged surface of Gd₂O₃ NRs (zeta potential, +29 mV) and negative terminal (COO⁻) of Asp.

2.3. In vitro cytotoxicity assay

The cytotoxicity (in vitro) of Gd₂O₃ NRs and Asp-Gd₂O₃ NRs with the varying concentration of both NRs from 62.5–500 μg mL⁻¹ was evaluated on Raw-264.7 and MCF-7 cells using MTT assay. For this study, the cells were maintained in a complete growth medium DMEM with 10% FCS at a temperature of 37 °C with humidified 5% CO₂ environment. The cells were plated at 10 × 10³ cell/well in 96-well tissue-culture plate having DMEM media for this assay and left to grow for 24 h. The well dispersed suspension of NRs in DI water was prepared by sonication. After the completion of 24 h, medium was changed and the well dispersed suspension of NRs was poured to the growth medium of the cells at a concentration of 62.5, 125.0, 250, 500 μg mL⁻¹ and incubated for 24 h under same condition. After 24 h the cells medium was replaced with MTT in DMEM without phenol red. The incubation of 3–4 h was further given to cells at 37 °C and allowed to reduce the MTT dye to the crystals of formazan. The formation of formazan was observed under inverted microscope after addition of MTT. The solubilisation of formazan was done in 100 μL of DMSO and absorbance was measured at 570 nm. The value of absorbance gives an indication of the cell proliferation. The calculation of relative cell viability was done with respect to cells that were not given treatment of NRs (control 0%). The NRs concentration was used in triplicate using three independent wells and each experiment was repeated three times, and then calculation of average cell viability was done.
2.4. **Electrophoretic deposition (EPD) of Asp-Gd$_2$O$_3$NRs onto ITO surface**

A colloidal suspension of Asp-Gd$_2$O$_3$NRs (5 mg) was prepared using acetonitrile and ethanol solution in 3:2 volume ratios by sonication for 1 h. EPD was done on hydrolyzed ITO substrate using two electrode systems in which ITO acted as anode and a platinum wire as a cathode. The two electrodes were placed parallel to each other at 1 cm apart and colloidal solution of Asp-Gd$_2$O$_3$NRs (4 mL) and 10 µL of magnesium nitrate solution (0.5 M) were added in the cell. The uniform deposition of a film on ITO was done at an optimized voltage (40 V) and time (3 min). These Asp-Gd$_2$O$_3$NRs/ITO electrodes were rinsed with DI water so that unbound material got remove and then dried overnight at 25 °C.

2.5. **Immobilization of biomolecules on Asp-Gd$_2$O$_3$NRs/ITO electrode**

A stock solution of Ab-VD (50 µg mL$^{-1}$) was prepared freshly in PBS (0.9% NaCl, pH 7.0) prior to experiments. For effective binding of available carboxylic groups (COO$^-$) on Ab-VD with amine (NH$_2^+$) groups of Asp molecules, well-known EDC-NHS chemistry was used. The solution of Ab-VD with NHS (0.1 M) and EDC (0.4 M) was prepared in a volumetric ratio of 2:1 (v/v) to activate the COO$^-$ groups available in fragment crystallizable (Fc) region of Ab-VD and kept for 30 min [50]. 20 µL of activated antibodies was uniformly dispersed onto the surface of Asp-Gd$_2$O$_3$NRs/ITO electrode and these electrodes were kept undisturbed in a humid chamber for 6 h. During this activation process, activated COO$^-$ of Ab-VD will bind through a covalent bond (OC-NH) with the NH$_2^+$ groups of Asp molecules present on Asp-Gd$_2$O$_3$NRs/ITO electrode [51,52]. The immunoelectrode (Ab-VD-Asp-Gd$_2$O$_3$NRs/ITO) was washed with PBS to remove the unbound Ab-VD from the surface. Finally, to block the nonspecific active sites of Ab-VD/Asp-Gd$_2$O$_3$NRs/ITO immunoelectrode, 10 µL of BSA (100 µg mL$^{-1}$) was drop cast on the top of electrode surface [53]. The immunoelectrode BSA/Ab-VD/Asp-Gd$_2$O$_3$NRs/ITO was washed thoroughly with PBS (pH 7.0) before use and then stored at 4 °C. Scheme 1 shows procedure followed during fabrication of Asp-Gd$_2$O$_3$NRs and BSA/Ab-VD/Asp-Gd$_2$O$_3$NRs/ITO immunoelectrode.

2.6. **Preparation of spiked samples**

The spiked samples were prepared using Vit-D$_3$ oral solution (Vit-D$_3$-OS) (De Purva), a product of Sanofi Pvt. Ltd. Different spiked samples were prepared by mixing 5 µL of the standard sample and 5 µL of Vit-D$_3$-OS. 10 µL of each concentration was used for measurement.

2.7. **Characterizations**

The crystalline properties and structure of synthesized Gd$_2$O$_3$NRs and Asp-Gd$_2$O$_3$NRs were analyzed using X-ray diffractometer (XRD, PANalytical X’pert PRO 2200 diffractometer) having a wavelength of X-ray $\lambda = 1.5406$ Å. XRD in diffraction patterns was recorded by varying the angle from 10° to 60° with a counting rate of 2/min. High-resolution transmission electron microscope (HR-TEM, JEOL JEM-2200 FS-Japan) instrument operating at a voltage of 200 kV was used to determine the shape and size of Gd$_2$O$_3$NRs and Asp-Gd$_2$O$_3$NRs. For analysis of TEM, firstly the colloidal solution of Gd$_2$O$_3$NRs and Asp-Gd$_2$O$_3$NRs in ethanol was prepared and then drop-casted on a TEM grid. For investigating the surface morphology of the electrodes field emission scanning electron microscope (FESEM with FIB and EBL) was carried out using Tescan LYRA3 XMU instrument. EPD technique was used for film formation of Gd$_2$O$_3$NRs and Asp-Gd$_2$O$_3$NRs on ITO substrate using a regulated DC power supply (Autonix). Fourier transforms infrared spectroscopy (FTIR-Varian FT-Raman and Varian 600 UMA) was used to investigate the different functional groups and various bonds present in Asp (powder), Asp-Gd$_2$O$_3$NRs, Ab-VD/Asp-Gd$_2$O$_3$NRs/ITO and BSA/Ab-VD/Asp-Gd$_2$O$_3$NRs/ITO immunoelectrode. The surface wettability was estimated by observing the contact angle (CA) measurement for which “SURFTENS universal” (OEG GmbH Germany) instrument was used.

To measure the surface charge, electrophoresis light scattering was done (model ZC-2000, Microtec, Japan). The surface potential ($\phi$) of sphere carrying charge in colloidal solution (in D.I.) was measured and theoretically the approximation of $\phi$ value expresses the zeta potential ($\zeta$) as given by the equation below:

$$\zeta = \phi = 4\pi(\sigma/\varepsilon\kappa)$$

In this equation, $\sigma$ represents the surface charge density of the particle, $\kappa$ and $\varepsilon$ give Debye-Huckel parameter and dielectric constant of the solution, respectively. PARSTAT (Princeton Applied Research; MODEL: PMC CHS08A - PARSTAT Multichannel (PMC) Chassis) instrument was applied during all electrochemical studies for BSA/Ab-VD/Asp-Gd$_2$O$_3$NRs/ITO using three electrode systems. Here, Ag/AgCl taken as a reference electrode and platinum as a counter electrode while the fabricated BSA/Ab-VD/Asp-Gd$_2$O$_3$NRs/ITO immunoelectrode used as working electrode. All electrochemical studies were carried out using electroanalytical techniques including differential pulse voltammetry (DPV) and cyclic voltammetry (CV) in PBS (0.1 M, pH 7.0, 0.9 % NaCl) with 5 mM of $[Fe(CN)_{6}]^{3-/-4}$.

3. **Results and discussion**

3.1. **X-ray diffraction studies**

The pattern of XRD for Gd$_2$O$_3$NRs & Asp-Gd$_2$O$_3$NRs is shown in Fig. 1(a) & (b). The diffraction peaks were observed at $2\theta = 20.08^\circ$, 28.56°, 33.09°, 35.16°, 39.02°, 42.57°, 47.50°, 52.07° and 56.37° corresponds to planes of body-centered cubic (BCC) phase of Gd$_2$O$_3$ as (211), (222), (400), (411), (332), (134), (440), (611), (622), respectively [54]. All the observed peaks were well matched and in agreement with JCPDS NO: 65-3181 for BCC phase of Gd$_2$O$_3$. There was no change in XRD peaks position but only intensity decreases for Asp-Gd$_2$O$_3$NRs. Hence, Asp functionalization did not alter the phase and nature of Gd$_2$O$_3$NRs which proves the successful synthesis of Asp-Gd$_2$O$_3$NRs. The average crystallite size “D” of as synthesized Gd$_2$O$_3$NRs and Asp-Gd$_2$O$_3$NRs was estimated to be 18.5 nm and 21.4 nm, respectively, corresponds to sharp peak.
at $2\theta = 28.56^\circ$ and the Scherrer formula was used for this calculation as given below:

$$D = \frac{0.9\lambda}{\beta \cos \theta}$$  \hspace{1cm} (3)

Where $\lambda = 1.540 \, \text{Å}$ is X-ray wavelength, $\theta$ is the Bragg’s diffraction angle and $\beta$ is the value of full width at half-maxima (angular width in radians).

3.2. Transmission electron microscopic studies

TEM studies were employed to confirm the morphologies of the Gd$_2$O$_3$NRs and Asp-Gd$_2$O$_3$NRs [Fig. 2 (a–f)]. The electron microscopy study of Gd$_2$O$_3$NRs reveals a cluster of NRs (image a). The alignment of these NRs was almost straight in length with an ultra-thin smooth surface. However, the degree of agglomeration of Asp-Gd$_2$O$_3$NRs was decreased (image d), increasing the dispersivity of NRs. Asp-Gd$_2$O$_3$NRs have enhanced dispersion due to the presence of functional groups (NH$_3^+$ and COO$^-$) as compared to Gd$_2$O$_3$NRs. The average diameter of Gd$_2$O$_3$NRs was found to be about 11.70 ± 1.93 nm [Image (a) inset]. Image (b) shows the corresponding atomic-scale image, comprising of organized lattice planes of Gd$_2$O$_3$NRs with BCC phase. The inter plane spacing (D-spacing) was 0.254 nm marked by red colour, which corresponds to the BCC phase with XRD plane (411) of Gd$_2$O$_3$NRs. Image (c) shows the selected area electron diffraction (SAED) pattern with planes (440) and (622). These results are well matched with the XRD data. Image (d) inset shows the diameter distribution of Asp-Gd$_2$O$_3$NRs and found slightly increased as 14.26 ± 0.13 nm. HR-TEM of Asp-Gd$_2$O$_3$NRs (image e) shows the noticeable thin layer with an approximate thickness of 2.03 nm as marked by a dark black line, indicating that the Gd$_2$O$_3$NRs successfully functionalized with Asp via edges of NRs. The inter plane spacing (D-spacing) obtained from HRTEM was 0.187 nm as marked by a yellow colour, which corresponds to the BCC phase with XRD plane (440) of Gd$_2$O$_3$NRs. While the D-spacing value calculated through Image-J software at some different grain was found to be 0.257 nm (white color) that corresponds to the XRD (411) plane. Image (f) shows the SAED pattern of Asp-Gd$_2$O$_3$NRs, showing clearly visible diffraction rings with planes (440), (400) and (611), which describe the crystallinity of Asp-Gd$_2$O$_3$NRs.

3.3. Zeta potential study

Zeta potential measurement study was done to find out the electrical potential of Gd$_2$O$_3$NRs and Asp-Gd$_2$O$_3$NRs. Gd$_2$O$_3$NRs and Asp-Gd$_2$O$_3$NRs showed the positive zeta potential of +29 mV and +24 mV, respectively. The aqueous solution of Gd$_2$O$_3$NRs and Asp-Gd$_2$O$_3$NRs has a pH value of 5.8 and 5.5, respectively. Beside this, there is an electrostatic interaction takes place between positively charged surface of Gd$_2$O$_3$NRs.
and negative terminal (COO\(^{-}\)) of Asp. Thus, positive group of Asp remain free, provided a positive charge of +24 mV of Asp-Gd\(_2\)O\(_3\)NRs. Similar results for zeta potential of Gd\(_2\)O\(_3\) NRs (+18 mV) and after capping with CTAB (+21.8 mV) are reported in literature [33].

### 3.4. In vitro studies of Gd\(_2\)O\(_3\)NRs and Asp-Gd\(_2\)O\(_3\)NRs

The cytotoxicity of Gd\(_2\)O\(_3\)NRs and Asp-Gd\(_2\)O\(_3\)NRs with the varying concentration of both NRs from 62.5 to 500 \(\mu\)g mL\(^{-1}\) was evaluated in vitro on Raw 264.7 and MCF-7 cells using MTT assay [Fig. 3A (a, b)]. The cells ability to reduce MTT to formazan crystal at all concentrations of NRs was monitored in comparison to control cells (0 % — without NRs). The MTT assay results revealed that the cells are viable up to 80% with both NRs even at higher concentration of 250 \(\mu\)g mL\(^{-1}\). Further, increase in the concentration (500 \(\mu\)g mL\(^{-1}\)) of both NRs, the percentage cell viability obtained was 73 % for Raw 264.7 cells and almost 60 % for MCF-7 cells. These results indicated that both types of NRs under this range can be used for any biomedical application.

### 3.5. Contact angle measurements of Gd\(_2\)O\(_3\)NRs/ITO and Asp-Gd\(_2\)O\(_3\)NRs/ITO electrodes

The Contact angle (CA) values were found to be 38.46° and 14.55° for Gd\(_2\)O\(_3\)NRs/ITO and Asp-Gd\(_2\)O\(_3\)NRs/ITO electrodes, respectively [Supplementary data; Fig. S1]. The decreased CA value for Asp-Gd\(_2\)O\(_3\)NRs/ITO electrode as compared to Gd\(_2\)O\(_3\)NRs/ITO electrode indicates the hydrophilic nature of Asp-Gd\(_2\)O\(_3\)NRs/ITO electrode.

### 3.6. Field emission scanning electron microscope studies

The morphological investigation of fabricated Asp-Gd\(_2\)O\(_3\)NRs/ITO and BSA/Ab-VD/Asp-Gd\(_2\)O\(_3\)NRs/ITO electrodes was done using FE-SEM [Fig. 3B]. Image (a) reveals the uniform dispersion of Asp-Gd\(_2\)O\(_3\)NRs and appears as a rough surface that provides the increased surface area for immobilization of biomolecules (Ab-VD and BSA). However, in FESEM nanorods are not clearly visible as compared to TEM images. Because, a thin film of Asp-Gd\(_2\)O\(_3\)NRs was deposited onto ITO surface via EPD technique and during film formation, NRs were deposited layer by layer onto ITO surface and visualized at micrometer scale (2 \(\mu\)m). After immobilization of biomolecules, the electrode surface completely changed into smooth morphology and was covered with biomolecules. Moreover, BSA/Ab-VD/Asp-Gd\(_2\)O\(_3\)NRs/ITO immuno electrode was formed as densely well-packed electrode surface [Image (b)]. The change in surface morphology indicates the successful immobilization of biomolecules onto Asp-Gd\(_2\)O\(_3\)NRs/ITO electrode surface.
Fig. 3 – A: Cell viability of RAW 264.7 and MCF-7 cells with varying concentrations (62.5, 125, 250, 500 μg mL⁻¹) of Gd₂O₃NRs and Asp-Gd₂O₃NRs for 24 h using MTT assay. B: FESEM images of (a) Asp-Gd₂O₃NRs/ITO electrode & (b) BSA/Ab-VD/Asp-Gd₂O₃NRs/ITO immunoelectrode. C: FT-IR spectra of (a) Asp (b) Asp-Gd₂O₃NRs/ITO (c) Ab-VD/Asp-Gd₂O₃NRs/ITO & (d) BSA/Ab-VD/Asp-Gd₂O₃NRs/ITO immunoelectrode.
3.7. Fourier transform infrared spectroscopic studies

Fig. 3C shows the FT-IR spectrum of (a) Asp, (b) Asp-Gd₂O₃NRs/ITO (c) Ab-VD/Asp-Gd₂O₃NRs/ITO (d) BSA/Ab-VD/Asp-Gd₂O₃NRs/ITO immunoelectrode. Curve (a) represents the FT-IR spectrum of Asp showing one peak at 1604 cm⁻¹ and another peak in 1400–1300 cm⁻¹ range due to NH₃⁺ and COO⁻ of amino acid [55]. The FT-IR spectrum of Asp-Gd₂O₃NRs/ITO electrode (curve b) exhibits a characteristic peak at 545 cm⁻¹ and reasons of this peak is the symmetric stretch of Gd−O bond. A signature peak at 1068 cm⁻¹ attributed to C=N stretch (C=NH₂) in primary amines. Moreover, IR peaks in the range of 1530–1490 cm⁻¹ were assigned to deformation of amino acid (NH₃⁺). These results indicated the presence of Asp on Gd₂O₃NRs surface and supported the functionalization process. Also, the decreased IR peak intensity in the range of 1400–1300 cm⁻¹ reveals the binding of COO⁻ of Asp with Gd³⁺ ions on the surface of Gd₂O₃ NRs. Besides this, IR peak at 850 cm⁻¹ was assigned to NH₂⁺ groups of Asp onto Gd₂O₃. However, Ab-VD/Asp-Gd₂O₃NRs/ITO immunoelectrode (curve c) exhibits the two sharp peaks appearing at 1590 and 1410 cm⁻¹ are assigned to NH₂⁺ of 1² alkyl amides (amide II bond between COO⁻ of Ab-VD and NH₂⁺ of Asp) and C−N stretch of 1³ amides (amide III bands), respectively [55,56]. Besides this, the broad IR peak centered at 3345 cm⁻¹ corresponds to OH stretching [57]. The peaks at 1590 and 1410 cm⁻¹ merged with reduced intensity (curve d) and at 850 cm⁻¹ (NH₂⁺ groups of Asp-Gd₂O₃) completely gone which confirms that the nonspecific sites on immunoelectrode get blocked by BSA. These results confirm the successful biomolecules (Ab-VD and BSA) immobilization and modification of electrode (Asp-Gd₂O₃NRs/ITO).

4. Electrochemical studies

4.1. Effect of pH and scan rate

The pH value of an electrolytic solution influences the electrochemical response of immunosensor. So, the effect of pH on the response of BSA/Ab-VD/Asp-Gd₂O₃NRs/ITO immunoelectrode was observed with different pH solution ranging from pH 6.0 to 8.0. DPV technique was used to observe the effect of pH in PBS solution containing [Fe(CN)₆]³⁻/⁴⁻. The potential range of −0.2 V to +0.6 V with a pulse width of 50 ms and a pulse height of 25 mV was applied for DPV study and maximum value of peak current was at pH 7.0 [Fig. S2]. So, whole electrochemical studies were done at pH 7.0 of electrolyte solution under similar conditions. Interface kinetics of Asp-Gd₂O₃NRs/ITO and BSA/Ab-VD/Asp-Gd₂O₃NRs/ITO immunoelectrode was observed at different scan rate (10–100 mV s⁻¹) [Fig. S3]. The value of anodic peak current (iₐ) and cathodic peak current (iₖ) linearly increased with the increase the scan rate for both electrodes suggesting a quasi-reversible or slow electron transfer kinetics.

4.2. Electrochemical comparison of modified electrodes using DPV technique

DPV response of each modified electrodes (i) ITO (ii) Gd₂O₃NRs/ITO (iii) Asp-Gd₂O₃NRs/ITO (iv) Ab-VD/Asp-Gd₂O₃NRs/ITO and (v) BSA/Ab-VD/Asp-Gd₂O₃NRs/ITO were analyzed to compare the electrochemical behavior in the potential range of −0.2 V to +0.6 V. During DPV measurements a pulse width of 50 ms and a pulse height of 25 mV were applied [Fig. 4]. A significant decrease in ΔI value for Gd₂O₃NRs/ITO electrode (37.46 μA; curve ii) was observed as compared to ITO (113.62 μA; curve i). These results indicate that the deposited layer of Gd₂O₃NRs onto ITO surface retards the transfer of electrons at the electrode/electrolyte interface due to less conductivity of Gd₂O₃ NRs as compared to bare ITO electrode [50]. However, ΔI value of Asp-Gd₂O₃NRs/ITO electrode increased (81.37 μA; curve iii) as compared with Gd₂O₃NRs/ITO electrode. This increase in ΔI value is due to sufficient functional groups available on Asp-Gd₂O₃NRs/ITO electrode surface which provides a continuous conduction path for electrons flow generated from oxidation/reduction of the [Fe(CN)₆]³⁻/⁴⁻ redox species [58]. After the immobilization of Ab-VD on Asp-Gd₂O₃NRs/ITO, an increase in ΔI was observed (89.37 μA; curve iv). This increase may be due to electrostatic interaction that is present on free sites of Ab-VD (NH₃⁺) and redox species present in the electrolyte which facilitates the electron diffusion by shortening the tunneling distance between Ab-VD and Asp-Gd₂O₃NRs/ITO electrode surface [59,60]. A similar increase in ΔI value after Ab immobilization has been previously observed in some reports [43,61]. The ΔI decreases significantly (50.13 μA; curve v) for BSA/Ab-VD/Asp-Gd₂O₃NRs/ITO electrode due to blocking of active sites (non-binding) onto the electrode surface. These results confirmed the successful immobilization of biomolecules (Ab-VD & BSA) onto Asp-Gd₂O₃NRs/ITO electrode.

![Fig. 4 – DPV curve of (i) ITO (ii) Gd₂O₃NRs/ITO (iii) Asp-Gd₂O₃NRs/ITO (iv) Ab-VD/Asp-Gd₂O₃NRs/ITO & (v) BSA/Ab-VD/Asp-Gd₂O₃NRs/ITO immunoelectrode in PBS containing [Fe(CN)₆]³⁻/⁴⁻ at potential range of −0.2 to +0.6 V with a pulse width of 50 ms and a pulse height of 25 mV.](image-url)
4.3. Response studies of BSA/Ab-VD/Asp-Gd$_2$O$_3$NRs/ITO immunoelectrode

Incubation time study was performed to check the response of BSA/Ab-VD/Asp-Gd$_2$O$_3$NRs/ITO immunoelectrode with a fixed Vit-D$_3$ concentration (10 ng mL$^{-1}$) and variation of time from 0 to 20 min (at an interval of 3 min). The immunocomplex formation at immunoelectrode depends on the time and specificity of interaction of Ab-VD with Vit-D$_3$. Fig. 5A (a) shows the magnitude of $\Delta$I increases with time up to 20 min after that it gets saturated. So, 20 min was used for immunochemical interaction of Ab-VD and Vit-D$_3$ for response study.

The electrochemical performance of BSA/Ab-VD/Asp-Gd$_2$O$_3$NRs/ITO immunoelectrode with different concentrations of Vit-D$_3$ (10–100 ng mL$^{-1}$) was recorded using DPV technique [Fig. 5A (b)] under similar conditions. The value of $\Delta$I increased linearly in each step with the increase in the concentration of Vit-D$_3$ at the interface of electrode/electrolyte. This can be assigned to the specific binding of antibody and antigen (Ab-VD and Vit-D$_3$) [62]. Due to this binding a rearrangement occurred at electrode surface which facilitate the transfer of charge via [Fe(CN)$_6$]$_{3^{-/-}}$ and resulted in the increase of current value [63,64]. Moreover, the differences of isoelectric point (IEP) values between immunoelectrode (net IEP will change upon antigen attachment to immunoelectrode) and available redox species in the buffer [65,66]. The calibration curve was plotted between Vit-D$_3$ concentrations and $\Delta$I values [Fig. 5A (c)] and results revealed that BSA/Ab-VD/Asp-Gd$_2$O$_3$NRs/ITO immunoelectrode respond linearly in the range of 10–100 ng mL$^{-1}$. Also, the other electrochemical parameters obtained as the sensitivity of 0.38 $\mu$A ng$^{-1}$ mL cm$^{-2}$ with LOD of 0.10 ng mL$^{-1}$ and regression coefficient ($R^2$) 0.99. To calculate the value of LOD, formula $3\sigma/m$ was used, $m$ indicates slope of the calibration curve and $\sigma$ indicates the standard deviation from the blank signal.

This fabricated immunoSENSOR shows linearity in the physiological range of Vit-D$_3$ (10–100 ng mL$^{-1}$) and better as compared to the reported Ab-25OHD/SPE/FMTAD electrode linearity (5–50 $\mu$g mL$^{-1}$) [16]. Also, the LOD value of fabricated immunoelectrode was 0.10 ng mL$^{-1}$ which is better than reported for previous 25OHD/SPE/FMTAD electrode (1000 ng mL$^{-1}$). The biosensing parameters of present immunoSENSOR were compared with the other reported biosensors [Table 1].

The estimation of binding affinity between Ab-VD and Vit-D$_3$ on the surface of the BSA/Ab-VD/Asp-Gd$_2$O$_3$NRs/ITO immunoelectrode, Hanes–Woolf plot was used [Fig. 5A (d)]. The Hanes–Woolf plot is a plot between [Vit-D$_3$ conc.] and [Vit-D$_3$ conc./$\Delta$I value] that gives the binding affinity regarding the dissociation constant ($K_d$) [67]. A smaller value of $K_d$ is attributed to the higher affinity of the immunoelectrode for Vit-D$_3$. The value of $K_d$ was found to be 0.0578 ng mL$^{-1}$, calculated by dividing the intercept by slope value of Hanes–Woolf plot. This low value of $K_d$ indicates the strong binding affinity of Vit-D$_3$ towards BSA/Ab-VD/Asp-Gd$_2$O$_3$NRs/ITO immunoelectrode [61,68].

The control experiment was performed using bare ITO to check the benefits of Asp-Gd$_2$O$_3$NRs. Ab-VD and BSA were immobilized onto ITO surface (without Asp-Gd$_2$O$_3$) under the same condition. The response was monitored for different concentrations of Vit-D$_3$ from 10 to 100 ng mL$^{-1}$. It was observed that the $\Delta$I was increased for 10–20 ng mL$^{-1}$ after that no change in $\Delta$I observed or even decreased from 30 to 100 ng mL$^{-1}$ [Fig. 5B]. These results suggested that the bare ITO not supported for immunosensor as sufficient antibodies were not immobilized onto the ITO surface due to lack of appropriate functional group. Hence, the Asp-Gd$_2$O$_3$NRs provide more specific surface area and abundant availability of functional group for the binding of higher amounts of Ab-VD. These Asp-Gd$_2$O$_3$NRs not only help in immobilizing Ab-VD, but also improves the biosensing parameters (detection range and LOD limit with better stability).

The response of BSA/Ab-VD/Asp-Gd$_2$O$_3$NRs/ITO immunoelectrode was also monitored with the commercially available Vit-D$_3$-OS, with varying concentration of 10–100 ng mL$^{-1}$ using DPV technique under similar conditions. During this measurement, 10 $\mu$L of each concentration of the spiked samples were used, which were prepared by mixing of 5 $\mu$L of Vit-D$_3$ and 5 $\mu$L of Vit-D$_3$-OS. Fig. 5A (a) shows the variation of DPV response BSA/Ab-VD/Asp-Gd$_2$O$_3$NRs/ITO immunoelectrode with different concentration of spiked samples. The magnitude of $\Delta$I increased slightly with the spiked samples as compared to Vit-D$_3$. The calibration curve was plotted between Vit-D$_3$-OS concentrations and $\Delta$I values [Fig. 6A (b)] and was found that BSA/Ab-VD/Asp-Gd$_2$O$_3$NRs/ITO immunoelectrode responded in a range of 10–100 ng mL$^{-1}$. The other parameters calculated were sensitivity of 0.85 $\mu$A ng$^{-1}$ mL cm$^{-2}$, LOD 0.781 ng mL$^{-1}$ and regression coefficient ($R^2$) 0.96. It has been used in 10–100 ng mL$^{-1}$ range of analyte and performed well up to 50 ng mL$^{-1}$. Although, it can detect up to 70 ng mL$^{-1}$ but the linearity was good for 50–50 ng mL$^{-1}$ range. Fig. 6A (c) shows a comparison of $\Delta$I obtained for the standard Vit-D$_3$ samples and spiked samples. The response was found satisfactory with the relative standard deviation (% RSD) varies from 0.44% to 4.58%. Thus, this electrode can be used for the detection of Vit-D$_3$ in the range of 10–100 ng mL$^{-1}$ in the presence of other biological components.

4.4. Immunoelectrode characterization

The specificity of BSA/Ab-VD/Asp-Gd$_2$O$_3$NRs/ITO immuno-electrode was observed with other metabolites present in serum sample like ascorbic acid (AA; 0.1 mM), cholesterol (CH; 4 mM), glucose (GLU; 4 mM), oxalic acid (OA; 1 mM), urea (2 mM) and uric acid (UA; 0.5 mM). During this study, each individual interferent (10 $\mu$L) was added in the electrolyte in presence of a fixed conc. of Vit-D$_3$ (10 ng mL$^{-1}$). The electrochemical response was observed in term of $\Delta$I values for each interferent. Fig. 6B (a) shows the bar graph of $\Delta$I values with respective interferents observed from DPV response. There was no significant change in the $\Delta$I value after the treatment of BSA/Ab-VD/Asp-Gd$_2$O$_3$NRs/ITO immunoelectrode with interfering compounds.

The shelf-life of immunoelectrode was also observed at a regular interval of 7 days up to 8 weeks using DPV technique under similar experimental conditions to check its stability. Fig. 6B (b) shows the graph between the value of $\Delta$I and time interval (weeks) and it was found that there was no significant...
change in $\Delta I$ value. Thus, this BSA/Ab-VD/Asp-Gd$_2$O$_3$NRs/ITO immunoelectrode was stable for almost 8 weeks.

Reproducibility and repeatability of immunoelectrode were also observed using DPV techniques under similar conditions. Relative standard deviation value (RSD) values for reproducibility and repeatability were 2.32% and 1.83% which was in the acceptable range [Supplementary data; Fig. S4].

**Fig. 5 -** A: (a) Incubation time study and (b) response study of BSA/Ab-VD/Asp-Gd$_2$O$_3$NRs/ITO immunoelectrode for different conc. of Vit-D$_3$ using DPV in PBS containing [Fe(CN)$_6$]$^{3-}/4-$ under similar conditions. (c) Calibration graph between value of $\Delta I$ and Vit-D$_3$ concentrations & (d) Hanes–Woolf plot between [Vit-D$_3$ conc.] and [Vit-D$_3$ conc./change in $\Delta I$]. B: The electrochemical response of BSA/Ab-VD/ITO immunoelectrode as function of Vit-D$_3$ using DPV technique in a potential range of $-0.2$ V to $+0.6$ V in PBS containing [Fe(CN)$_6$]$^{3-}/4-$.
Fig. 6 – A: Response study of BSA/Ab-VD/Asp-Gd$_2$O$_3$NRs/ITO immunoelectrode for different conc. of Vit-D$_3$ in the spiked samples (b) Calibration graph between value of $\Delta$I and spiked sample (Vit-D$_3$ and Vit-D$_3$-OS) concentrations & (c) Comparison of Vit-D$_3$ with spiked sample. B: (a) Effect of various Interferents & (b) Shelf-life of BSA/Anti-VD/Asp-Gd$_2$O$_3$NRs/ITO immunoelectrode monitored using DPV in PBS containing [Fe(CN)$_6$]$^{3-/4-}$ under similar conditions.
5. Conclusions

In summary, Gd$_2$O$_3$NRs were synthesized using hydrothermal method and successfully functionalized with the Asp without any change in the phase. However, the size of Asp-Gd$_2$O$_3$NRs was slightly increased as observed by XRD and TEM results. The Asp-Gd$_2$O$_3$NRs exhibited enhanced dispersivity and hydrophilicity. The electrochemical characterization proves that Asp-Gd$_2$O$_3$NRs electrode surface facilitates the transfer of electrons between the electrode/electrolyte interfaces. Asp-Gd$_2$O$_3$NRs exhibited ability as a linker towards the covalent immobilization of EDC–NHS activated Ab-VD. The response study of BSA/Ab-VD/Asp-Gd$_2$O$_3$NRs/ITO gives electrochemical performance such as sensitivity of 0.38 μA ng$^{-1}$ mL cm$^{-2}$ and detection limit of 0.10 ng mL$^{-1}$ toward Vit-D$_3$ detection, which is better than previously reported biosensor. Also, the range of detection was 10–100 ng mL$^{-1}$ which covers the physiological range of Vit-D$_3$. This immunosensor does not show any interference effect and shows a satisfactory response to commercially available Vit-D$_3$ oral solution. Besides this, in vitro study of Gd$_2$O$_3$NRs and Asp-Gd$_2$O$_3$NRs on RAW 264.7 and MCF-7 cells clearly demonstrated their biocompatible nature. These Asp-Gd$_2$O$_3$NRs facilitate a new path for designing a simple, sensitive, selective and biocompatible immunosensing platform for detection of Vit-D$_3$.

Conflicts of interest

The authors declare no conflicts of interest.

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Appendix A. Supplementary data

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