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Comparative study of label-free electrochemical immunoassay on various gold nanostructures

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Electrochemical methods such as amperometry and impedance spectroscopy provide the feasibility of label-free immunoassay. However, the performance of electrochemical interfaces varies with the shape of gold nanostructures. In the present work three types of gold nanostructures including pyramid, spherical, and rod-like nanostructures were electrochemically synthesized on the gold electrode and were further transformed into immunosensor by covalent binding of antibodies. As a model protein, a cancer biomarker, Carcinoembryonic Antigen (CEA) was detected using amperometric and impedimetric techniques on three nanostructured electrodes, which enabled to evaluate and compare the immunoassay's performance. It was found that all three immunosensors showed improved linear electrochemical response to the concentration of CEA compared to bare Au electrode. Among all the spherical gold nanostructure based immunosensors displayed superior performance. Under optimal condition, the immunosensors exhibited a limit of detection of 4.1 pg ml^{-1} over a concentration range of five orders of magnitude. This paper emphasizes that fine control over the geometry of nanostructures is essentially important for high-performance electrochemical immunoassay. © 2013 AIP Publishing LLC. [<http://dx.doi.org/10.1063/1.4827381>]

I. INTRODUCTION

Nanostructures (NS) with different shapes and sizes are very attractive due to their unique physical and chemical properties, which have potential applications in different fields like sensors, electronic devices, and catalysis.^{1,2} A wide variety of different Au NS shapes such as spherical, rod, cube, triangular, and hexagonal have been studied.^{3,4} For example, Radha *et al.*⁵ used spherical and cube shaped Au nanoparticles (NPs) as extrinsic Raman label in surface enhanced Raman scattering (SERs) based immunoassay and showed that the cube shaped NPs improved the sensitivity of immunoassay. Similarly, Jianqi *et al.*⁶ used hexagon and boot shaped Au NPs and demonstrated that the boot shaped NPs induced 100 fold SER enhanced sensitivity as compared with hexagon shaped NPs, whereas Jiang *et al.*⁷ studied the electrochemical sensitivity of the coral shaped Au NPs for human IgG as a model analyte. The immunosensor so prepared exhibited excellent performance, e.g., showed the lower detection limit of 5 pmol l^{-1} .

In protein immunoassays, signals can be detected either by label specific or label free techniques as both techniques have their own merits and demerits. Labeled detection is widely used due to its ease of use, common availability of reagents, and simple instruments. In addition to conventional labeling strategy such as radioisotopes and fluorescent dyes,

many novel labels such as carbon nanotubes have recently been employed by Naimish *et al.*,⁸ where a novel immunosensor array was fabricated using carbon nanotubes microwell and obtained the limit of detection of 1.0 pg ml^{-1} . Extending it further, single wall carbon nanotubes (SWCNTs) forest sensor with RuBPY – silica nanoparticles as labels has further improved the detection limit to 100 fg ml^{-1} of the prostate specific antigen (PSA).⁹

On the other hand, the label free technique avoids interference due to tagging and is based on the real time bimolecular interactions. However the expensive fabrication, morphological variation in the sample and inadequate knowledge of biosensors often limits its sensitivity and thus its use. The advanced nanomaterials coupled with the electrochemical detection have provided some new opportunities for the development of label free immunosensor. Recently, Jaan *et al.*¹⁰ reported the detection of human lung cancer (EN01) by fabricating a Polyethylene glycol (PEG) layer and subsequently using anti EN01 tagged spherical Au NPs bioprobes and showed its response in a linear dynamic range from 10^{-8} to $10^{-12} \text{ g ml}^{-1}$ with an improved detection limit of 2.3 pg ml^{-1} . Similarly, Reda *et al.*¹¹ used spherical Au NPs for the detection of cancer biomarker, epidermal, the growth factor receptor in human plasma and brain tissue. The developed electrochemical immunosensor showed the detection limit of 0.34 pg ml^{-1} . Thus, the electrochemical approach enhanced by nanostructures offers high sensitivity, selectivity, and instrumental simplicity.

Efficient immobilization of protein plays a critical role for sensitive heterogeneous immunosensor.^{12,13} The enhancement

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in the sensitivity can be achieved by the use of various types of nanoparticles like gold^{14–18} and silver.¹⁹ The gold nanoparticles offer number of advantages such as its ease for functionalization and acceptable biocompatibility. Bonel *et al.*²⁰ has developed an immunosensor for detection of ochratoxin A (OTA) based on physical adsorption of OTA conjugated to albumin from brovine serum (OTA-BSA) and OTA-BSA bound to the gold nanoparticles (OTA-BSA-AUNPs). However, Liu *et al.*²¹ used gold nanoparticles microcomb electrode for detection of latoxin. Schafieid *et al.*²² present a colometric bioassays based on aggregation of carbohydrate stabilized gold nanoparticles for detection of *Ricinus communis* Agglutinin. Idegani *et al.*²³ prepared a sensitive immunosensor for human chorionic gonadotropin hormone (HCG) using direct electrical detection of gold nanoparticles. Tang *et al.*²⁴ had developed a novel transparent immune-affinity reactor by binding of functionalized gold nanoparticles to quartz crystal.

For the electrochemical detection of gold nanoparticles various techniques such as pioneering work based on anodic stripping voltammetry (ASV) were performed by Deguaire *et al.*²⁵ However, Gonzalez *et al.*²⁶ investigated an electrochemical method to monitor the biotin streptavidin interactions based on the use of colloids gold as an electrochemical label. In previous studies, overpotential deposition was employed for shape controlled electrodeposition of gold nanostructures.²⁷ The shape dependent electrochemistry of Cytochrome c (cyt c) and its electrocatalytical activity towards hydrogen peroxide was investigated for different types of nanostructures by Liu *et al.*²⁸

In the present work, carcinoembryonic antigen (CEA) was used as it is an important cancer biomarker for a number of cancers developed in the digestive system, especially for the colon cancer.^{29,30} Different types of nanostructures were used to compare the performance of the immunosensor. The electrochemical immunosensors were developed by the deposition of morphologically three different Au nanostructures with varied surface area on the Au electrode to improve the sensitivity as well as the selectivity. The modified electrodes were then used for the response of antibody–antigen interactions. The conductivity and the surface area were used to determine the electrochemical active area of each electrode. The effect of nanostructure decoration on the limit of detection (LOD) of the immunosensor was also determined for each sensor. The performance of nanostructured electrodes was also compared with bare Au surface. Another aspect of the present work was on the determination of the sensitivity and stability of the modified immunosensor.

II. EXPERIMENT

A. Chemicals and reagents

Hydrogen tetrachloroaurate (III) (HAuCl₄) trihydrate, perchloric acid (HClO₄), monoclonal CEA, N-hydroxy-succinimide (NHS), and N-ethyl-N'-(3-dimethylaminopropyl) carbodiimide (EDC) of analytical grade by Sigma Aldrich (USA) were used to modify and decorate the electrode surface. The phosphate buffer solution was prepared with de-ionized water. The potassium ferricyanide and potassium chloride

were used in cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS) measurements.

B. Electrode modification

The gold electrodes with 3 mm in diameter (CH Instruments, USA) were washed with 0.3 and 0.05 μm alumina powder followed by thorough rinsing with and sonication in de-ionized (DI) water. The Au electrodes were then dried with nitrogen. Subsequently, electrochemical technique (electrochemical overpotential deposition (OPD)) was employed to decorate electrodes surfaces with morphologically different kinds of nanostructures, i.e., pyramidal, spherical, and rod-like nanostructures. The pyramidal, spherical, and rod-like gold nanostructures were electrodeposited from the aqueous solution of 0.1 M HClO₄ containing 40, 40 and 4 mM HAuClO₄ at -0.8 , -0.2 , and -0.08 V with respect to the reference Ag/AgCl electrode, respectively, for 2 min.³¹

Different types of nanostructures in the present experiment were obtained by electrochemical process. The electrolytic conductivity in the process causes an Ohmic drop, which depends on the geometry of the electrochemical cell. In order to reduce or minimize the Ohmic drop, electrodes were kept in very close proximity during all deposition experiments and at a constant height from the container base.

The pyramidal, spherical, and rod-like decorated gold electrodes were then modified with the cysteamine self assembled monolayer by immersing the nanostructured electrode in cysteamine mixed in ethanol solution (5 mM) for 24 h to form the self assembled monolayer (SAM). Later, the electrodes were washed with ethanol and dried with nitrogen. The monoclonal CEA antibody (ACEA) ($1\ \mu\text{g ml}^{-1}$) was applied to the SAM modified electrode by means of micropipette so that whole electrode is covered with antibody. The cysteamine functionalized electrode were immersed in phosphate buffer solution (PBS) of pH 7 ACEA ($1\ \mu\text{g ml}^{-1}$) followed by addition of 0.2 M EDC and 0.05 M NHS for the activation of carboxylic group present in the ACEA as shown in Figure 1. The electrodes were incubated overnight and then carefully washed with PBS to remove excess antibodies. The schematic of the process sequence is shown in Figure 1. Afterwards electrodes were exposed to cysteamine-SAM activated with EDC/NHS and then were incubated for 2 h. The CEA concentration used ranged from 0.001 to 1000 ng ml⁻¹. Finally, electrodes were washed and dried to remove the physically adsorbed antibodies.

C. Characterization techniques

The morphologies of the prepared nanostructures were examined using high resolution field-emission scanning electron microscopy (FESEM, JEOLJEM-2100F) and the atomic force microscopy (AFM, SPM 3100, Veeco Instrument, Inc., USA). Cyclic voltammetry and impedance measurements were performed using a three cell electrode system at a scan rate of $50\ \text{mV s}^{-1}$. The platinum, saturated calomel (SCE) electrode, and modified electrode were used as the counter, reference, and working electrode, respectively. All the measurements were performed at room temperature.

FIG. 1. Schematic representation of the steps for the preparation of nanostructured electrodes. The nanostructures of three types, i.e., pyramids, spherical, and rod-like, were electrodeposited in step 1 and then functionalized with cysteamine self assembled monolayers in step 2. The electrodes were then immersed in antibody solution in the presence of EDC and NHS in step 3, and finally the attachment of antigen in step 4.

III. RESULTS AND DISCUSSIONS

A. Morphology of Au nanostructures

The SEM images shown in Figures 2(a)–2(c) demonstrate that the pyramids, spherical, and rod-like nanostructures were successfully synthesized on the gold surface and were dispersed uniformly on the entire surface. This was consistent with the AFM images shown in Figures 2(d)–2(f). In the case of pyramid nanostructures, the edge length of the base varied from 60 to 350 nm with an average aspect ratio of 1.82. The spherical nanostructures and nanorods had an average diameter of 15 nm and 120 nm, respectively. The roughness of the surface as determined from the AFM images was 1.65 ± 0.05 , 1.60 ± 0.20 , and 1.45 ± 0.05 nm, for pyramid, spherical, and rod-like nanostructured electrodes, respectively. The deposition of nanostructures effectively enhanced the surface area of the electrode, which was determined using the relation;³² $[S_o + n * (\text{average surface area of the nanostructure})]$, where S_o is the region of interest (ROI), i.e., area of the AFM/SEM scan and was $25 \mu\text{m}^2$ in the present case. n is the average number of nanostructures in the ROI. The determined surface area of the three nanostructured electrodes was 63, 70, and $60 \mu\text{m}^2$ for the pyramid, spherical, and rod-like nanostructured electrodes, respectively, as given in Table I. The surface area of the nanostructured electrodes was thus increased by about 2.5, 2.9, and 2.4 times for pyramid, spherical, and rod-like, respectively, to that of the plane Au electrode. It was interesting to observe the response to CEA and establish the relation between the surface coverage and surface area of each electrode.

B. Electrochemical behavior of Au nanostructures

For the comparative study of the three types of nanostructured electrodes, the electrochemical activity was determined by performing EIS and CV in 10 mM $\text{K}_3\text{Fe}(\text{CN})_6$ with 1 M KCl as supporting electrolyte and is shown in Figure 3, displaying the Bode plot of the bare Au and nanostructured electrodes. The measurements were performed at a scan rate

of 50 mV s^{-1} . The equivalent circuit used to evaluate different parameters is also shown in inset of Figure 3(a). The charge transfer resistance (R_{ct}) was evaluated using the software z-view. The values of resistance obtained for the bare Au electrode, pyramid, spherical, and rod-like nanostructured electrodes were $20.5 \text{ K}\Omega$, $12.3 \text{ K}\Omega$, $6.5 \text{ K}\Omega$, and $9.8 \text{ K}\Omega$, respectively. As observed, the spherical nanostructures decorated electrode showed the smallest value of charge-transfer resistance and thus displayed the highest conductivity among the three types of electrodes. Figure 3(b) shows the CV response of the bare Au and nanostructures decorated electrodes, which also confirmed the results observed in the EIS measurements. The potential scan was started from 0.4 V and brought down to 0 V and then back to 0.4 V (as the direction is shown in Figure 3(b)) to complete one cycle. Both the oxidation and reduction peaks could be observed. The scans shown in the Figure 3 were the third cycle of the cyclic voltammetry. The third cycle was chosen because current became stable in this cycle. The first scan started from 0.4 V is shown in the inset of the Figure 3, with zero current initially; however, it saturated at the positive current value when the first cycle was completed. It then became independent of the number of cycles. The separation between the oxidation and reduction peaks was measured for the bare and nanostructured Au electrodes. For bare Au electrode, the peak to peak separation (ΔE_p) was 90.5 mV. However, for the nanostructured Au electrodes, the difference between the two peaks (ΔE_p) was ~ 70.9 , 66.4, and 70 mV for pyramid, spherical, and rod-like nanostructured electrodes, respectively. The CV results suggested that the amperometric response of the modified electrodes strongly depended on the surface area of Au nanostructures and the surface coverage of the electrode. The current response was found to increase with the density and with the size of the Au nanostructures. EIS and CV measurements demonstrated that the response obtained for the electrode decorated with the spherical nanostructures was the best, which was due to the large surface area of the electrode surface.

The performance of nanostructured electrodes was also evaluated for the value of exchange current density (i_0) and

FIG. 2. (a)–(c) SEM images with the scale of 100 nm, (d)–(f) AFM images of the pyramid, spherical, and rod-like nanostructured electrodes, respectively. The side bar represents the height scale of the nanostructures.

the standard rate constant (k^0) as it was believed that for a better performance, the value of i_0 and k^0 should be higher. The value of i_0 and k^0 calculated using Butler–Volmer relationship as given by^{33,34}

$$i_o = \frac{RT}{FR_{ct}}, \tag{1}$$

$$k^o = \frac{i_o}{FC}, \tag{2}$$

$$i_p = 2.99 \times 10^5 n (n_a \alpha)^{1/2} A C D^{1/2} v^{1/2}, \tag{3}$$

where R , T , F , and C are the gas constant, absolute temperature, Faradic constant, and concentration of the reactant, respectively. n , A , D , and v are the number of electrons involved in the reaction, the electrochemically active surface area, the diffusion coefficient of the reactant species in solution, and the scan rate of the potential perturbation, respectively.

The values obtained are shown in Table I. The observed values of i_0 and k^0 were high for the electrode decorated with spherical nanostructures, which indicated its much faster charge-transfer rate compared to pyramids and rods decorated electrodes. As demonstrated above, the chemical

TABLE I. Summary of calculated parameters, i.e., surface area, exchange current density (i_o), standard rate constant (k_o), electrochemical active surface area (A), and surface coverage for pyramid, spherical, and rod-like nanostructure. The surface area and surface coverage were evaluated from AFM images of nanostructures.

Nanostructures	Surface area (μm^2)	i_o ($\mu\text{A cm}^{-2}$)	$k_o \times 10^{-8}$ (cm^{-1})	$A \times 10^{-6}$ (cm^2)	Surface coverage (%)
Pyramid	63	1.45	2.94	61	52.9
Spherical	70	2.92	5.94	68	63.4
Rod-like	60	0.91	1.78	55.9	40.7

FIG. 3. (a) Impedance response and equivalent circuit for the electrochemical cell. It consists of a charge transfer resistance R_{ct} connected parallel to double layer capacitance C_{dl} , and both are in series to the solution resistance R_s . (b) CV response of (black) bare Au, (red) pyramid, (blue) spherical, (green) rod-like nanostructures using 10 mM $\text{Fe}(\text{CN})_6^{3-}$ in 1 M KCl. The measurements were performed at a scan rate of 50 mV s^{-1} .

activity of the spherical nanostructured electrode was 1.56 and 2.25 times better than the pyramid and rod-like nanostructured electrodes. It is believed that this was due to the uniform field profile offered by the spherical nanostructured electrode which might not be the case with the other two modified electrodes. In addition to this, improved electrochemical activity may also be attributed to improved surface area of spherical nanostructured electrode.

The activity at the electrode surface also depended on the reaction rate and the electrochemically active surface area. The values of electrochemically active surface area were calculated using Eq. (3) and is given in Table I, which showed that the electrochemical active area was 61, 68, and $56 \mu\text{m}^2$ for pyramid, spherical, and rod-like nanostructured electrodes, respectively. Interestingly; the value of electrochemically active surface area was close to the actually determined surface area of the nanostructured electrodes. The incorporation of nanostructures on the surface of electrode enhanced the active

reaction area of the electrode which was responsible for the enhanced electrochemical signal and even allowed for lower detection limit and higher sensitivity for the analyte detection.

C. Optimization of experimental conditions

1. pH response of buffer

It is well known that acidic or basic environment can destroy the protein microstructure and can affect the biocatalytic performance of immunosensor. Thus, the effect of the solution pH on the performance of spherical nanostructured immunosensor (i.e., electrode with attached CEA antibody-antigen) was investigated by performing the electrochemical measurements in the range from acid to basic environment. The concentration of the antigen used was 100 ng ml^{-1} . The reported value of pH in most of the studied immunosensors has been found in the range of 6–7.³⁵ Figure 4 summarizes the electrochemical response obtained at different pH values, which showed the maximum current of the immunosensor appeared at $\text{pH} = 7.0$, and this was taken as the optimized value for all later experiment for a better biochemical activity.

2. Effect of the incubation time

The effect of incubation time on the performance of the nanostructured immunosensors was also studied, and results are shown in Figure 5. The attachment of the antigen (CEA) to the antibody (ACEA) was monitored up to 7 h, which showed exponential behavior. Electrodes modified with pyramids and rods almost showed identical behavior, while electrode modified with spheres showed higher response current and reached the saturation value quickly. The plot also demonstrated that electrodes decorated with rods and pyramids would take longer time to saturate. This was due to much shorter time for attachment with spheres compared to the other two nanostructures. As a reasonable amount of current was detected within first few hours of the incubation time, all subsequent measurements were done for 2 h of incubation. This was to ensure the prevention of saturation and for the better performance of immunosensor.

FIG. 4. Effect on response of peak current as a function of solution pH for spherical nanostructured immunosensor.

FIG. 5. Effect of incubation time on the detection of antigen for the three nanostructured electrodes; circle (red), square (black), and triangle (blue) represent spherical, pyramid, and rod-like nanostructured electrodes, respectively.

D. Analytical performance of immunosensor

1. Electrochemical response

CV was conducted to study the response of each electrode following every step of modification. Figures 6(a)–6(c) show the sequence of CV response curves of the bare electrodes (top), nanostructured electrodes (second from the top), modified with Cysteamine SAM (third from the top),

and finally with antibodies (bottom) for (a) pyramid, (b) spherical, and (c) rod-like nanostructures, respectively. The value of the peak current for the bare Au was $8.69 \mu\text{A}$, and potential separation between oxidation and reduction peak was 0.0716 V . It can be seen that in spherical nanostructured electrode, the peak current increased 1.40 times, and the potential separation decreased to 0.0583 V as compared to 0.0716 V for the bare Au electrode. Similarly the peak separation of 0.0616 V and 0.0591 V was observed for the pyramid and the rod-like nanostructured electrodes, respectively, as seen in Figures 6(b) and 6(c). This was attributed to the increase in the surface area of the conducting nanostructures, which provided a much faster and quicker exchange of charge carriers. This indicated an increase in the electrochemical reversibility of $\text{K}_3\text{Fe}(\text{CN})_6$ reactions at the nanostructured electrodes. The electrodes, when further modified with CEA antibody, showed 0.83, 0.76, and 0.77 times drop in the peak current for pyramid, spherical, and rod-like nanostructures, respectively. However, there was an increase in potential separation of 0.0627 V , 0.0707 V , and 0.0753 V between the oxidation and reduction peaks for the pyramid, spherical, and rod-like nanostructured electrodes, respectively. This showed an improved adhesion of antibodies to the nanostructured electrodes and excellent reversibility of the nanostructured electrodes.

Proper comparisons of nanostructured electrodes were made by defining two parameters, one was the $\Delta I = (I - I_0)/I_0$, i.e., the normalized current response, where I_0 was the current of bare gold surface and I was the current after antibody

FIG. 6. CV response of electrochemical immunosensor. (i) Bare Au surface, (ii) nanostructured electrode, (iii) development of cysteamine self assembled monolayer, (iv) immobilization of CEA antibody on (a) pyramid, (b) spherical, (c) rod-like nanostructured electrodes. (d) Variation of the normalized current response with normalized surface area for the (i) spherical, (ii) pyramid, and (iii) rod-like nanostructured electrodes.

attachment and the values of ΔI obtained were 0.62, 0.68, and 0.21 for pyramid, spherical, and rod-like nanostructured electrodes, respectively. The second parameter was the normalized surface area defined as the ratio of the average surface area to the average number density of nanostructures (A/ρ) for each nanostructured electrode. This has made the comparison of the response of different types of nanostructured electrodes independent of the morphology of the nanostructure and simple. The normalized surface area came out to be 0.23, 0.09, and 0.42 for pyramid, spherical, and rod-like nanostructured electrodes, respectively. The normalized current response for the three nanostructured electrodes as a function of normalized surface area is plotted in Figure 6(d), which clearly showed highest response with smallest surface area for the spherical nanostructured electrode. The Figure 6(d) demonstrated that the normalized current response was the highest for the small normalized surface area, and as the normalized surface area increased, the current response dropped drastically. The increase in the normalized surface areas was 2.5 times and 4.5 times in the case of pyramid and rod-like nanostructured electrodes, respectively, while the drop in normalized current was 20% and 75%, respectively, compared to spherical nanostructured electrode. The analytical response was correlated to the particle size and density. The pyramid nanostructures have a base length of about 120 nm and density of $197 \mu\text{m}^{-2}$ compared to 15 nm and $578 \mu\text{m}^{-2}$, 197 and $99 \mu\text{m}^{-2}$ for spherical and rod-like nanostructure, respectively. It was quite consistent with CV finding that best response current was obtained for spherical nanostructures.

2. Detection of CEA

The lower limit of the CEA antigen detection by the nanostructured immunosensors in optimized conditions was determined in the range from 0.001 to 1000 ng ml⁻¹. The results are plotted in Figure 7, which showed a drop in the peak response current with increase in the concentration of antigen, i.e., 9.9 to 4.7 μA , 10.1 to 5 μA , and 8.6 to 5 μA as the concentration increased from 0.001 to 1000 ng ml⁻¹ for pyramid, spherical, and rod-like nanostructures, respectively.

FIG. 7. The variation in the peak current for (a) bare Au, (b) pyramid, (c) spherical, and (d) rod-like nanostructured electrodes as a function of log of concentration. The concentration of PSA varied from 1 pg ml⁻¹ to 1000 ng ml⁻¹.

TABLE II. Comparison of sensitivity and association constant for bare Au, pyramid, spherical, and rod-like nanostructures.

Nanostructures	Bare Au	Pyramid	Spherical	Rod
Sensitivity ($\mu\text{A ng}^{-1} \text{ ml}$)	0.2107	0.339	0.457	0.312
Association constant $\times 10^9 \text{ M}^{-1}$	0.0653	0.0407	0.0821	0.0441

The behavior was identical for all three electrodes. This decay in current value was used to determine the theoretical value of the LOD using the equation $\text{LOD} = 3 \times \text{S.D.}/\text{sensitivity}$.³⁶ However, S.D. is the standard deviation of the blank solution measurements. The value of limit of detection determined was 0.07, 0.0039, 0.0036, and 0.0045 ng ml⁻¹ for the bare Au, pyramid, spherical, and rod-like nanostructured electrodes, respectively. This showed detection limit improved drastically when electrodes were decorated with nanostructures. The sensitivity obtained for three electrodes was different and higher than the bare Au. The detailed comparison is given in Table II. The sensitivity was high for small values of the normalized surface area and then dropped as the normalized surface area increased as can be seen in Figure 8. On the other hand, the surface coverage varied linearly with the normalized surface area, and the rate of the change of surface coverage was 68 ± 3 as obtained from the linear fit. It was interesting to note that in the case of spherical nanostructures, the value of sensitivity was higher. This was probably due to its higher surface area of about $52 \mu\text{m}^2$ compared to 46 and $42 \mu\text{m}^2$ for pyramid and rod-like nanostructures. This showed that sensitivity of an immunosensor could be drastically improved by increasing the surface area and the density of the nanostructures at the electrode surface.

3. Association constant

The interfacial interaction between the antibody-antigen³⁷ and the association constant for the three modified electrodes was determined by using Langmuir isotherm relation given by^{38,39}

$$K_a C = \frac{R_{\text{ct}(i)} - R_{\text{ct}(o)}}{R_{\text{ct}(o)}}, \quad (4)$$

FIG. 8. Plot of sensitivity and surface coverage as a function of the normalized surface area.

where K_a , C , $R_{ct(i)}$, and $R_{ct(o)}$ is the association constant, concentration, impedance of antigen and antibody, respectively. Figure 9 shows the electrochemical resistance response for varied concentrations of antigen. The value of charge transfer resistance was obtained by fitting the bode plot in z-view software. The charge transfer resistance after attachment of the antibody came out to be 6.2, 9.01, and 8.04 K Ω for the pyramid, spherical, and rod-like nanostructured electrodes, respectively. However the value of resistance increased to 15.02, 21.9, and 11.6 K Ω for the three electrodes when the antigen concentration reached to 100 ng ml $^{-1}$. The association constant was determined by taking the slope of the linear region in the concentration range from 10 to 1000 ng ml $^{-1}$. The affinity of protein towards the nanostructured electrode came out to be 0.065×10^9 , 0.0407×10^9 , 0.0821×10^9 , and 0.04407×10^9 M $^{-1}$ for bare Au, pyramid, spherical, and rod-like nanostructured electrodes, respectively. The inset in Figure 9 shows the calibration curve obtained for the normalized resistance as a logarithmic function of antigen concentration, which was linear with a regression coefficient of 0.9632 in the complete range, which was not close to 1 due to variation in the concentration (However, the regression coefficient was 0.98 when determined in the range of interest, i.e., 0.001 to 1 ng ml $^{-1}$. It was 0.99 when determined in the higher range from 1 to 1000 ng ml $^{-1}$. Thus, the mean regression coefficient came out to be 0.986, which was quite reasonable. It was inferred that difference with the value of goodness (=1) in the range of interest was more than in the higher concentration range. This was due to amplification of error in smaller concentration range occurred during the dilution of antigen at lower concentrations. The improved association constant was an indication of improved interaction between the antibody and nanostructured electrode.

4. Selectivity of immunosensor

For clinical purposes, the immunosensor must demonstrate high selectivity to a specific analyte in the presence of

FIG. 9. Impedance response of the immunosensor with the concentration of antigen. The inset shows the plot of normalized resistance as a function of the log of concentration.

FIG. 10. Selectivity of immunosensor with (i) CEA antibody, (ii) CEA antibody with CEA antigen, (iii) CEA antibody with HBsAg antigen, (iv) CEA antibody with AFP antigens, (v) CEA antibody with PSA antigens.

non-specific species. The selectivity of spherical nanostructured based immunosensor was determined by using some promising non-specific analyte; hepatitis B antigen (HBsAg), alfa fetoprotein antigen (AFP), and prostate specific antigen (PSA). The immunosensor was incubated in solution containing HBsAg, AFP, and PSA for 2 h of fixed concentration of 100 ng ml $^{-1}$. The electrochemical signal was measured with and without interfering analyte to determine the interference between antibody and the non specific analyte, and the results are shown in Figure 10. It can be seen from Figure 10 that when CEA antigen was used, the current dropped to 6.62 μ A, i.e., 1.3 times the electrode with PSA antibody. However, HBsAg, AFP, and PSA, each did not interfere significantly with the immunosensor, and no drop in the response current was observed. Thus no significant cross reactivity was detected for HBsAg, AFP, and PSA antigen. It suggests that the nanostructured immunosensor exhibits good selectivity for CEA assays.

5. Stability of immunosensor

The stability of the immunosensor decorated with spherical nanostructures was determined from the voltammetry current response. The modified electrodes were kept at 4 $^{\circ}$ C

FIG. 11. Cyclic voltammetric responses of the spherical nanostructured immunosensor as a function of the storage days.

in 0.1 M PBS (pH = 7.0), and measurements were repeated every 3 days for a month as shown in Figure 11. The response current remained stable during the first 25 days and then dropped but still retained almost 83% of the original value even after 30 days. Thus the protein conjugation with nanostructures was quite efficient as compared to the values quoted in the literature.⁴⁰ The immunosensor prepared by An *et al.* exhibited a storage stability of about three weeks, whereas present immunosensors showed improved stability and enhanced response, which was due to better biocompatibility of Au nanostructures with Cysteamine, and further cysteamine with protein. This suggested that the developed immunosensor provides a better and reliable tool for the detection of CEA.

IV. CONCLUSION

In this work, three different types of nanostructures, namely, spherical, pyramid, and rod-like nanostructures were electrodeposited onto the gold electrodes. The comparative study for antibody-antigen detection was performed on bare and nanostructured Au surface. It was found from the FESEM and AFM images that the spherical nanostructures were smaller in size and has larger value of surface area as compared to the pyramid and rod-like nanostructures. Due to the higher surface area, the spherical nanostructure showed better electrochemical performance than the other types of nanostructures. The normalized surface area of nanostructures was also evaluated, and it was observed that the spherical nanostructures having smaller value of aspect ratio gave an enhanced response current and in improved sensitivity for CEA antigen. It was thus proved that the higher the surface area of the gold nanostructures the better the performance of immunosensor would be. The limit of detection of the nanostructured electrodes was 4 pg ml^{-1} , and its stability was almost for a month. The present work demonstrates that simplicity, specificity, LOD, and stability of an immunosensor can be significantly enhanced by use of Au nanostructures.

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