

Cardiovascular Marker Proteins Detection in the Blood Serum Using an LSPR Chip Based on Au Nanobipyramid

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Abstract. The localized surface plasmon resonance (LSPR) based technology allows the fabrication of inexpensive biosensors with very simple design for the detection of diseases. In the present work, we systematically fabricated an LSPR sensor chip using Au nanobipyramids (Au NBPs). Au NBPs with longitudinal LSPR band in the near-IR region (~900nm) exhibiting higher refractive index (RI) sensitivity are used for the sensor chip fabrication. The immobilized Au NBPs on a silanized glass coverslip were chemically modified using 11-mercaptopundecanoic acid (11-MUA) and Octanethiol monolayer, followed by activation using EDC-NHS chemistry for the immobilization of the protein molecules. For cardiovascular marker protein detection, monoclonal antibodies were immobilized on the sensor chip, and the marker proteins were detected from the blood serum obtained from the patients. Cardiovascular marker proteins N-terminal pro-B-type natriuretic peptide and cardiac troponin T (CTnT) were successfully detected on the fabricated LSPR sensor chip. © 2023 Journal of Biomedical Photonics & Engineering.

Keywords: localized surface plasmon resonance (LSPR); Au nanobipyramids; alkanethiol monolayer; NT-proBNP; cardiac troponin T (CTnT).

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1 Introduction

The localized surface plasmon resonance (LSPR) properties of metal nanoparticles have been utilized in different fields such as photocatalysis [1], biosensing [2–5], optical filters [6], nanophotonics [7] and surface-enhanced Raman scattering [8, 9]. These nanoparticles can be synthesized using physical and chemical methods. Among physical methods, electron beam lithography [10, 11], nanosphere lithography [12, 13], and the growth of nanostructure, particularly nanorods using oblique angle deposition techniques [14], have gained enormous interest. Although these physical methods can provide well-defined nanostructures on the substrates, these methods are expensive and need a skilled workforce to operate the equipment. On the other hand, chemical methods are inexpensive and are explored extensively. However, several byproducts appear in chemical synthesis along

with the main product [15]. Also, the dispersion in the size of nanoparticles is another major concern in the chemical synthesis method. The use of capping molecules to prevent aggregation also leads to their inferior performance [15]. In the past decade, advancement in the separation and purification of colloidal nanoparticles and their immobilization on substrates paves the way to fabricate nanoparticle-based sensor chips for various applications.

Gold (Au) nanobipyramids (NBPs) are a unique nanostructure being synthesized using chemical methods [16–20]. These nanoparticles, similar to nanorods, show transverse and longitudinal LSPR bands due to transverse and longitudinal oscillation of surface electrons. The longitudinal LSPR band of nanorods and NBPs can be easily tuned from visible to infrared spectral regions. However, compared to nanorods, NBPs show narrow plasmon resonance, which is a critical factor in sensing applications and provides better spectral

resolution when the nanoparticles are immobilized on a substrate.

Although most of the sensing applications involving nanoparticles synthesized using a chemical route have been performed in colloidal solution, scattered works show nanoparticle immobilization on a substrate and their sensing applications [16, 21–24]. The availability of selected conjugating molecules allows the immobilization of nanostructures on transparent substrates such as glass or quartz. It has been observed that the refractive index of coupling molecules between glass and nanostructure alters the plasmonic property significantly [24]. Any such modulation may lead to a change in the sensing capability. Therefore, the suitability of the substrate surface is also essential for immobilizing the nanostructure.

Cardiovascular diseases (CVD) are among the most pronounced cause of mortality in humans, and nearly 32% of deaths occur due to CVD [25, 26]. During myocardial infarction, the level of specific cardiac marker proteins gets elevated. Early detection of these proteins is of utmost importance for the on-time diagnosis of CVD. The noticeable CVD marker proteins include cardiac troponin I (cTnI), cardiac troponin T (cTnT), N-terminal pro-B-type natriuretic peptide (NT-proBNP), and myoglobin. Several methods have been used to detect CVD marker proteins [26–28]. Recently we have used Au thin film-based SPR to detect cTnI and cTnT [29]. There are only a few reports on the detection of cardiac marker proteins using the nanostructure-based LSPR technique. L. Tang et al. discussed the LSPR biosensing method using gold nanorods to detect cardiac biomarkers [30]. The group has developed a multiplexed gold nanorod biosensor using gold nanorods of different aspect ratios to detect multiple cardiac biomarkers. The gold nanorods synthesized using the seed-mediated growth method were terminated with carboxylic groups and immobilized with antibodies to detect cardiac troponin I (cTnI) and myoglobin (MG). Y. M. Bae et al. developed an LSPR substrate using gold nanoparticles for detecting two prominent cardiovascular biomarkers, namely low-density lipoprotein (LDL) and high-density lipoprotein (HDL) [31]. T. Liyanage et al. discussed using triangular gold nanoprisms (Au TNPs) in LSPR biosensors to detect cTnT biomarkers in human body fluids [32]. The chip-based LSPR sensors developed by the group were more sensitive compared to the commercial biosensor.

In the present work, the focus is on two objectives. First, we aim to develop an LSPR-based sensor chip utilizing Au NBPs systematically. The NBPs exhibiting longitudinal plasmon band in the near-IR spectral region were selected as they show higher RI sensitivity [33]. For efficient immobilization of Au NBPs, surfactant CTAB was removed, followed by the surface modification of NBPs for the CVD marker protein antibody immobilization. As the second objective, we show the successful detection of the cardiac marker proteins NT-proBNP and cTnT from the blood serum sample of cardiac patients on the prepared LSPR sensor chip.

2 Materials and Methods

2.1 Materials

For the synthesis and conjugation of gold nanobipyramids, tetrachloroauric(III) acid trihydrate ($\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$), cetyltrimethylammonium chloride (CTAC), cetyltrimethylammonium bromide (CTAB), 11-mercaptoundecanoic acid (11-MUA), 4-mercaptophenylboronic acid (MPBA), nitric acid (HNO_3), silver nitrate (AgNO_3), 8-hydroxyquinoline (HQL), (3-mercaptopropyl)trimethoxysilane (MPTMS), 1-ethyl-3-(3-Dimethylaminopropyl)carbodiimide hydrochloride (EDC), anti-Troponin T monoclonal antibody, bovine serum albumin (BSA), and human serum were procured from Sigma Aldrich. Anti-human NT-proBNP monoclonal antibody (clone 1312), cTnI, anti-Troponin I monoclonal antibody, hydrogen peroxide, sulfuric acid, were procured from Merck. 1-Octanethiol, ethanol was procured from Alfa Aesar. N-hydroxysuccinimide (NHS) and sodium borohydride (NaBH_4) were procured from Spectrochem. L-ascorbic acid (AA) was procured from Loba Chemie Pvt Ltd. Citric acid was procured from Qualigens fine chemicals.

2.2 Experimental Methods

2.2.1 Synthesis of Au NBPs

The seed-mediated approach reported earlier is used to synthesize the Au NBPs [33, 34]. Briefly, the seed nanoparticles were prepared by adding 72 μL of 0.25 M HNO_3 and 80 μL of 25 mM HAuCl_4 to 8 mL of 66 mM CTAC. The solution is mixed well, followed by adding 100 μL of freshly prepared 50 mM NaBH_4 in NaOH. The solution color was changed to brownish orange. After 1 min of stirring, 60 μL of 0.1 M citric acid was added, and the seed solution was heated at 85 $^\circ\text{C}$ for 1 h in a water bath. For the growth solution preparation, 20 mL of 46.5 mM CTAB, 200 μL of 25 mM HAuCl_4 , 165 μL of 5 mM AgNO_3 , and 255 μL of 0.4 M ethanolic HQL were mixed. In a beaker, 200 μL of seed solution was added, followed by 20 mL of growth solution and incubation of the mixture solution in a hot-air oven at 85 $^\circ\text{C}$ for 10 min.

The synthesized Au NBPs were purified by centrifuging the colloidal solution at 8000 rpm thrice. For the immobilization of Au NBPs on the MPTMS-modified glass surface, the excess of CTAB was removed, keeping ~ 70 μL supernatant after the second wash.

2.2.2 Fabrication of LSPR Sensor Chip

For the sensor chip fabrication, glass coverslips were cleaned glass coverslips in piranha solution (1:3 ratio of H_2O_2 and conc. H_2SO_4). The cleaned glass coverslips were incubated in 10% MPTMS in ethanol overnight. Excess of MPTMS was removed by mild sonication of the substrates in ethanol two times. To ascertain the MPTMS functionalization, the water contact angle of the substrates before and after the silanization process was

investigated using a lab-built contact angle setup [35]. Contact angle measurements were performed with a 10 μL water droplet and the analysis was carried out using the 'drop analysis' plugin in the ImageJ software [36, 37]. For the immobilization of Au NBPs, the MPTMS-coated glass coverslips were incubated in purified Au NBPs solutions for about 3 h.

The Au NBPs immobilized substrate was immersed in acetone for ~ 1 h to remove the remaining CTAB bilayer from the NBPs surface [38, 39], followed by immersion in an ethanolic solution of 1-Octanethiol, 11-MUA, and MPBA in 3:1:1 ratio. The carboxylic-COOH groups of 11-MUA were activated by a 1:1 mixture of 400 mM EDC and 100 mM NHS crosslinkers.

For the immobilization of the NT-proBNP antibody, the EDC-NHS modified sensor chip was incubated with 50 μL of 100 $\mu\text{g/mL}$ of NT-proBNP antibody in PBS buffer (pH:7.4) for 3 h. The sensor chip was rinsed with PBS buffer and DI water after incubation. CTnT monoclonal antibody was also immobilized on the sensor chip using a similar approach.

2.2.3 Blood Sample Collection and Serum Separation

The blood samples (3 mL) were collected from 3 CVD patients with informed consent from the intensive care unit, department of Cardiology, kasturba medical college, Manipal. The collected blood samples were centrifuged at 3000 rpm for 10 min. The supernatants containing serum were separated and stored at -20°C . Ethical approval was obtained from the institutional ethical committee (IEC number IEC551/2020), Kasturba Medical College & Hospital, for all the work related to human blood.

2.2.4 Bulk RI Sensing Using Au NBPs Substrate

The bulk refractive index sensitivity experiments were performed to ensure the successful removal of the CTAB bilayer from the Au NBPs surface. The experiments were performed with the substrate before and after the acetone treatment. For experiments, methanol (1.326 RI), water (1.33 RI), ethanol (1.36 RI), and toluene (1.496 RI) were used as sample mediums. Besides bulk RI sensitivity experiments, the absorption spectra of the sensor chip were also recorded after each step of the functionalization process. All these measurements were carried out using our lab-built setup described elsewhere [40].

3 Results and Discussion

3.1 Effect of Seed Age in Au NBPs Synthesis

The quality of seed nanoparticles plays a critical role in forming monodispersed and reproducible Au NBPs [41]. Factors affecting the quality of seed nanoparticles include the concentration of reducing agent, surfactant, temperature, and seed aging. As mentioned in the

experimental section, the prepared seeds were heated at 85°C before their use for the Au NBPs synthesis. The heat-treated seeds were further aged to improve the seed quality. Fig. 1(a) shows the effect of aging time on the seed nanoparticles. It is evident from the figure that the absorption spectrum of seed nanoparticles, immediately after synthesis, has a distinct but less pronounced LSPR band. The aging of nanoparticles for more than 15 min shows a strong increase in the intensity of the LSPR band. Fig. 1(b) shows the normalized absorption spectra of Au NBPs prepared using aged seed nanoparticles. It is evident from the figure that the longitudinal LSPR band shifts towards a higher wavelength with an increase in the seed aging time. Therefore, seed nanoparticles aged up to 60 min were used in the following work.

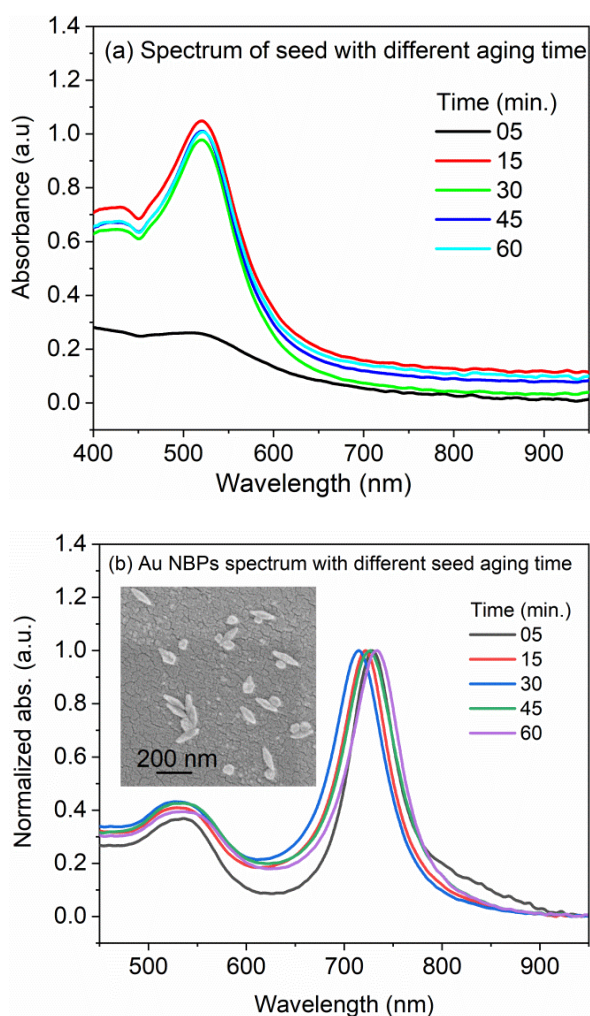


Fig. 1 Absorption spectra of (a) seed nanoparticles with different aging times and (b) Au NBPs synthesized using seed nanoparticles of different ages. Inset in (b) figure shows the FESEM image of the synthesized Au NBPs.

3.2 Purification and Immobilization of Au NBPs

Glass coverslips as an LSPR substrate were used in the following work. Before immobilization, the coverslips were cleaned with piranha solution (1:3 ratio of H_2O_2 and

conc. H_2SO_4). The cleaned glass coverslips were incubated in 10% MPTMS in ethanol overnight. The measurement of the water contact angle ascertained the silanization of coverslips. Figs. 2(a) and (b) show the contact angle images before and after the silanization process. As seen in Fig. 2, the water contact angle on the piranha-treated substrate was 21.8° indicating the hydrophilic nature of the substrate [42]. The water contact angle was increased to 64.6° after the silanization process with MPTMS due to the partial hydrophobic nature of the MPTMS-coated substrate [43].

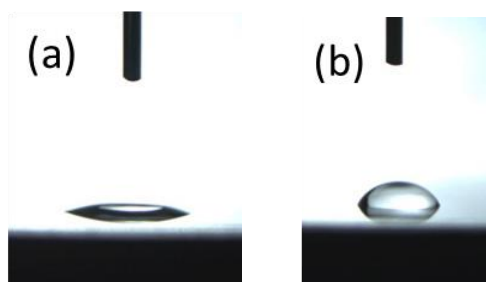


Fig. 2 Water contact angle (a) on piranha solution treated coverslip (contact angle: 21.8°), (b) on MPTMS coated coverslip (contact angle: 64.6°)

In the synthesis of Au NBPs, the CTAB forms a bilayer around NBPs, preventing them from aggregation. Due to the CTAB bilayer formation around nanoparticles, the NBPs acquire an overall positive surface charge. The immobilization of Au NBPs on the substrate is strongly hindered by the electrostatic repulsion between positively charged NBPs, resulting in lower NBPs density on the substrate [44]. Removal of CTAB from the NBPs surface can improve the Au NBPs immobilization and RI sensitivity. However, the complete removal of CTAB by several washing steps using centrifugation can cause shape deformation and aggregation of NBPs. For the successful immobilization of the Au NBPs on the glass substrates, optimization of the number of washing cycles and centrifugation time are performed. A constant centrifugation rate at 8000 rpm was maintained in all washing cycles.

For the removal of CTAB, 2 approaches were used. In the first approach, the Au NBPs colloidal solution was centrifuged at 8000 rpm for 5 min, and the supernatant was discarded. This washing process was repeated 3 times.

Figs. 3(a) and (b) show the LSPR spectra of Au NBPs as-synthesized and after 3 washing cycles, respectively. It was observed that the longitudinal LSPR band shifts towards a lower wavelength after 3 washing steps. Also, the intensity ratio of longitudinal to transverse LSPR bands changes from 2.61 to 0.97. The observed changes could be due to the complete removal of CTAB. It has been observed earlier that the complete removal of CTAB can cause the aggregation of nanoparticles along with the deformation of Au NBPs. The observed blue shift could be due to the aggregation of particles, whereas the decrease in the longitudinal to transverse intensity (L/T) ratio could be due to the deformation of Au NBPs. An effort was made to immobilize the nanostructures on the MPTMS-modified glass substrates. The corresponding LSPR spectrum of the substrate is shown in Fig. 3(c). The broad spectrum from the substrate clearly shows that the Au NBPs get aggregated on the substrate surface.

In the second approach, to avoid aggregation or deformation of Au NBPs, the presence of CTAB was ensured by leaving a small amount ($\sim 100 \mu\text{L}$) of supernatant after each centrifugation step of the colloidal solution. Figs. 4(a) and (b) show the LSPR spectra of Au NBPs as prepared and after 3 washing cycles, respectively. It is evident from the figure that the longitudinal band after 3 washing steps shows a slight blue shift of 5 nm. However, the L/T ratio improved from 2.61 to 3.6, indicating the suppression of aggregation and deformation of Au NBPs. The Au NBPs, obtained after centrifugation, were immobilized on the MPTMS-modified glass coverslips. Fig. 4(c) shows the spectrum of immobilized Au NBPs. It is evident that though the absolute absorbance decreases upon immobilization, the L/T ratio is maintained.

The immobilization of Au NBPs on silanized coverslips was further improved by optimizing the immobilization time. Fig. 5 shows the absorption spectra of immobilized Au NBPs after an immobilization time of 45 min and 3 h. A substantial increase in absorbance was observed with higher immobilization time. It is also observed that upon immobilization, the FWHM of the spectrum increases marginally, along with a small red shift of the longitudinal LSPR band. The observed increase in the FWHM and redshift could be due to substrate and MPTMS, which is used as a linker molecule between glass and Au NBPs.

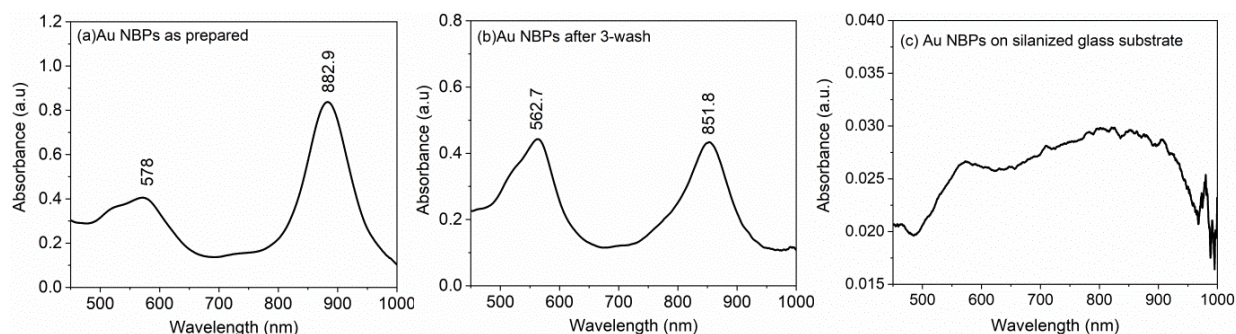


Fig. 3 Absorption spectra of Au NBPs (a) as-synthesized, (b) after 3 washes with complete removal of the supernatant, and (c) on MPTMS functionalized glass coverslip.

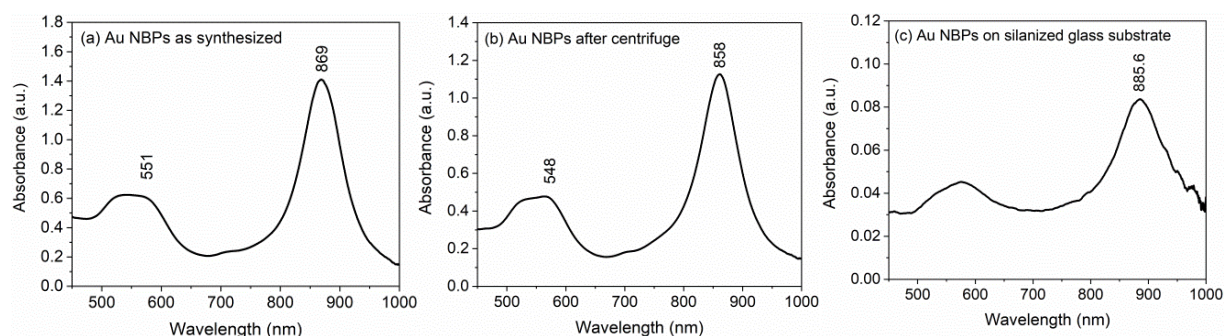


Fig. 4 Absorption spectra of Au NBPs (a) as-synthesized, (b) after 3 washes with partial removal of the supernatant, and (c) on the MPTMS functionalized glass coverslip.

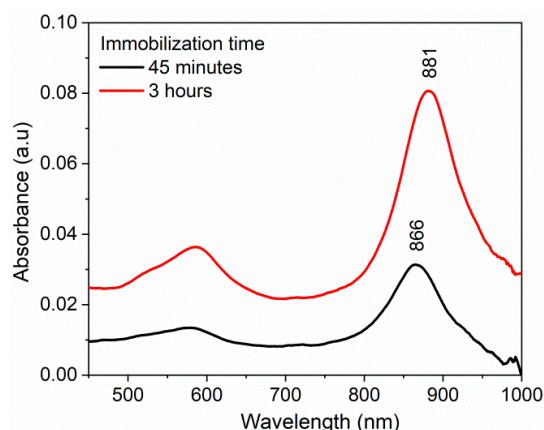


Fig. 5 Au NBPs immobilized on MPTMS-modified glass coverslip with an incubation time of 45 min and 3 h.

3.3 Surface Functionalization of Au NBPs

3.3.1 Non-Specific Binding (NSB) of BSA on the Sensor Chip

The Au NBPs immobilized on the MPTMS-treated glass coverslips were further modified for the immobilization of the protein molecules. It has been observed that acetone is helpful in the removal of the CTAB [38]. The immobilized Au NBPs were treated with acetone for 1 h to remove CTAB from the Au NBPs surface. The immobilized NBPs were treated with an ethanolic mixture of Octanethiol and 11-MUA (3:1 ratio), followed by the acetone treatment. The use of Octanethiol with 11-MUA ensures that the 11-MUA with -COOH terminal group is immobilized on the NBPs surface without any steric hindrance. It is well known that for protein conjugation, the terminal group -COOH of 11-MUA needs further modification, which is performed by incubating the substrate in the 1:1 mixture of EDC (0.4 M) and NHS (0.1 M) for 1 h.

The suitability of the EDC-NHS-modified substrate for protein conjugation was investigated using bovine serum albumin (BSA). The substrate is incubated in BSA solution in PBS buffer (10 $\mu\text{g/mL}$) for this. To prevent any temperature-dependent degradation of BSA, the incubation process was performed at 2–8 $^{\circ}\text{C}$. The LSPR spectra were recorded at each stage and are shown in

Fig. 6. The figure shows that after the removal of CTAB by acetone, the longitudinal band in the LSPR spectrum shifts towards a lower wavelength of ~ 8.5 nm. The immobilization of 11-MUA and Octanethiol leads to the shift of the LSPR wavelength to red by 1.8 nm. The EDC-NHS modified substrate shows a further increase in the LSPR band maximum wavelength by 12.3 nm. The BSA further shifts the LSPR band maximum by 9 nm. The observed wavelength shifts are expected and corroborate our earlier results [33].

The non-specific binding of protein appeared as a common issue in SPR and LSPR measurements. In several reported works, polyethylene glycol that imparts hydrophilicity to the substrate surface is utilized to prevent non-specific binding. However, due to the long polymeric chain and size of PEGs, degradation of sensing performance was observed. The present work uses a small thiolated molecule, 4-mercaptoboronic acid (MPBA), as a non-specific binding-preventing molecule.

MPBA has a -SH group in a phenyl ring group and boronic acid with 2 hydroxyl groups at the para position. After the EDC-NHS treatment of a self-assembled monolayer of alkanethiols, MPBA binds to the Au NBPs surface and prevents the non-specific binding of protein molecules on the NBPs surface. In the experiments reported here, 115 mM concentration of MPBA was used.

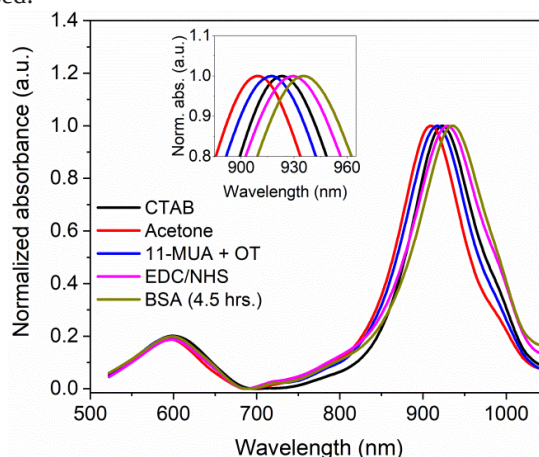


Fig. 6 Absorption spectra of the sensor chip at different functionalization stages. Inset shows the expanded spectral region of the longitudinal LSPR band.

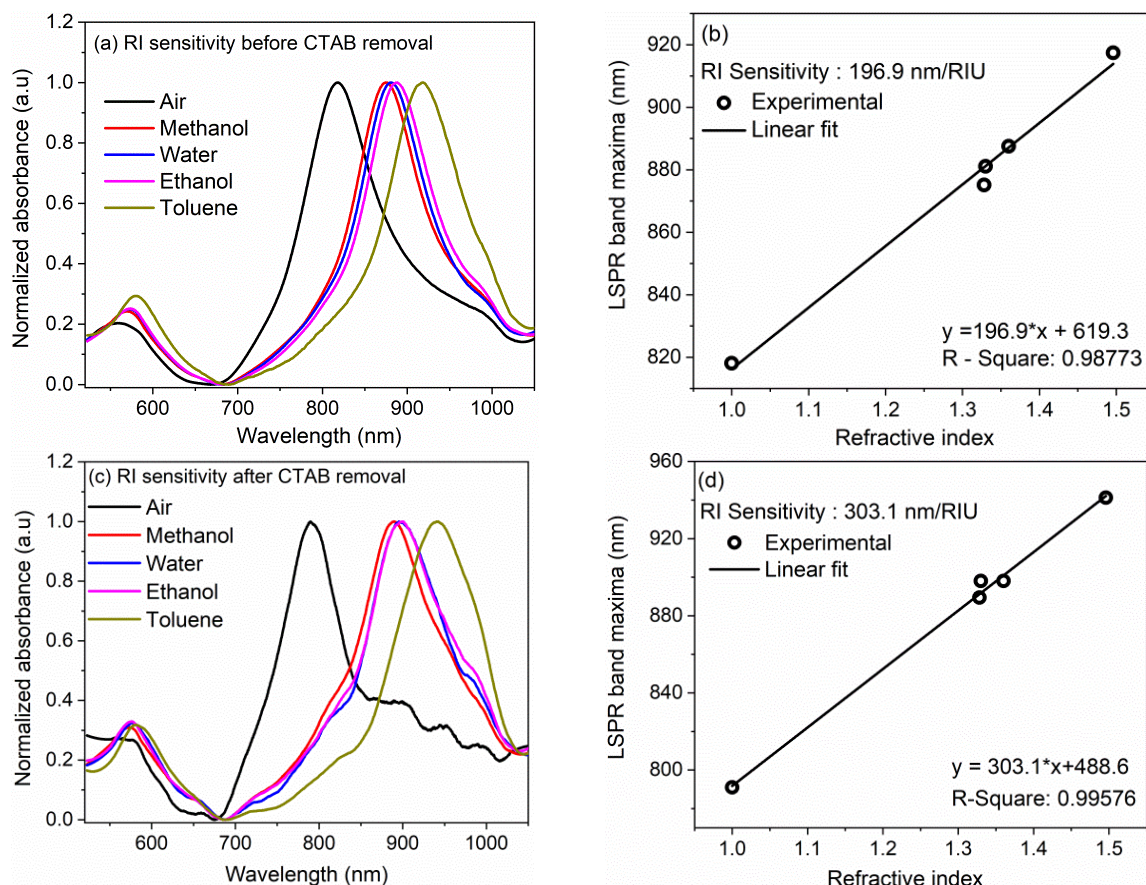


Fig. 7 (a) LSPR spectra of Au NBPs in different refractive index media before the removal of CTAB, (b) linear fit to the longitudinal band maxima position before CTAB removal, (c) LSPR spectra of Au NBPs in different refractive index media after the removal of CTAB, and (d) linear fit to the longitudinal band maxima position after CTAB removal.

3.3.2 Bulk RI Sensing of LSPR Sensor Chip

The replacement of CTAB with thiolated molecules was ascertained by examining the refractive index sensitivity of the sensor substrates. For the RI sensitivity measurement, methanol, water, ethanol, and toluene were standard samples. The RI sensitivity experiments were performed on immobilized NBPs with CTAB and immobilized NBPs after functionalization with thiolated molecules. Fig. 7(a) shows the LSPR spectra of the sensor substrate with immobilized CTAB-capped Au NBPs. The figure shows a clear wavelength shift in the sample medium from water to toluene. Fig. 7(b) shows the LSPR band maxima plotted against the RI of samples. The slope of the linear fit to the data represents the RI sensitivity. For the NBPs without acetone treatment immobilized on the sensor substrate, the RI sensitivity of 196.9 nm/RIU was observed. Figs. 7(c) and (d) show similar plots for the Au NBPs immobilized on sensor substrate, followed by acetone treatment and thiolation of immobilized NBPs. In this case, the RI sensitivity of 303.1 nm/RIU was obtained, 105 nm/RIU higher than the CTAB-capped immobilized NBPs. Our earlier work observed similar behavior where CTAB was replaced with 11-MUA [33]. The observed increase in the RI sensitivity is due to the replacement of the CTAB bilayer on the NBPs surface

by thiolated molecules, which makes the nanostructure more accessible and sensitive towards the sample medium.

3.4 CVD Biomarker Detection from Clinical Samples

3.4.1 Detection of NT-proBNP

NT-proBNP is a cardiac marker protein in the bloodstream. The B-type natriuretic peptide (BNP) plays a vital role in maintaining homeostasis in the cardiovascular system, serving as a counter-regulatory hormone for volume and pressure overload. The increase in the level of NT-proBNP in the bloodstream indicates insufficient blood pumping by the heart. Therefore, the level of NT-proBNP is widely measured in clinical applications for the diagnosis, risk stratification, and management of patients with heart failure. Early detection of elevated levels of NT-proBNP in the bloodstream is helpful in the timely treatment of the problems associated with heart failure.

The prepared sensor chips with immobilized Au NBPs were utilized to detect cardiac marker protein NT-proBNP. For the detection, the sensor chip was treated with acetone for 1 h, followed by incubation in the 3:1:1 mixture of Octanethiol, 11-MUA, and MPBA overnight.

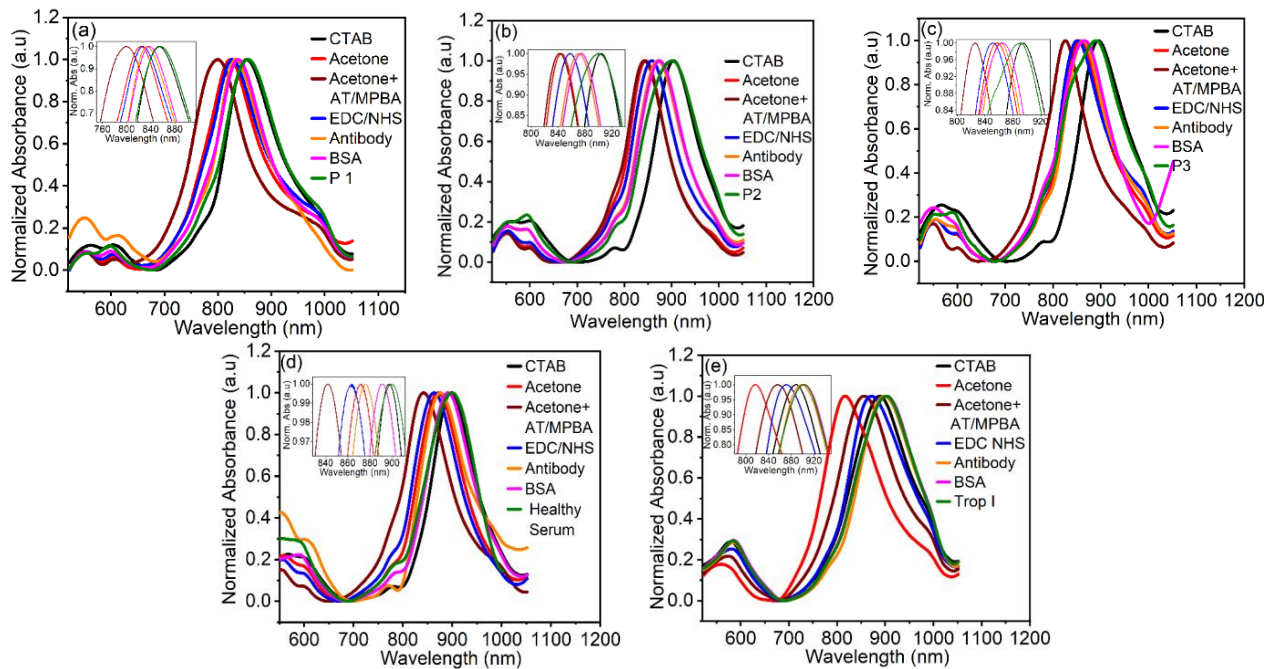


Fig. 8 LSPR spectra of the substrate at different functionalization and detection stages of NT-proBNP (a) for the blood serum of patient 1, (b) for the blood serum of patient 2, (c) for the blood serum of patient 3, (d) for commercial human serum, and (e) specificity determination using CTnI on NT-proBNP antibody immobilized sensor chip.

Table 1 LSPR band positions of sensor chip at different stages of functionalization and detection of NT-proBNP.

Coverslip (CS)	LSPR band position (nm) at different functionalization stages				
	a	b	c	d	e
before acetone	855.2	904.6	895.4	897.8	888
after acetone	823.6	845.3	857.7	871.7	817.5
after alkane thiols (AT)	798.6	842.7	825.9	843	856.4
after EDC/NHS	828.01	857.9	851.5	863.8	872.6
after antibody	835.2	871.1	862.4	876.7	898.4
after BSA	838.8	874.5	864.6	891.6	902.2
after cardiac patient serum	856.5	898.6	887.9	—	—
after commercial serum	—	—	—	898.6	—
after 100 ng/mL CTnI	—	—	—	—	901.6

After washing with ethanol, the terminal -COOH groups of 11-MUA were activated using EDC and NHS. After activation of the NBPs surface, the sensor chip was incubated in 100 $\mu\text{g/mL}$ NT-proBNP monoclonal antibody for 4 h. The antibody-immobilized sensor chip was treated with 1% (10 mg/mL) BSA for 30 min to lower the non-specific binding. The cardiac marker protein NT-proBNP was detected in the serum sample of cardiac patients. The sensor chip with immobilized monoclonal antibody blocked for non-specific binding was incubated in the blood serum and separated from cardiac patients' blood for 2 h. Subsequently, the LSPR spectra were recorded. Figs. 8(a–c) show the LSPR spectra of the sensor chip at different functionalization and detection of

NT-proBNP from the 3 patients marked as P1, P2, and P3. Fig. 8(d) corresponds to the response of the NT-proBNP antibody immobilized chip toward the commercially obtained blood serum of a healthy human. The detection specificity of NT-proBNP was ensured by using CTnI protein (100 ng/mL) diluted in PBS buffer. Fig. 8(e) shows the response of the CTnI on the NT-proBNP antibody immobilized sensor chip. The LSPR band maxima observed in these figures are listed in Table 1. It is also evident from the table that a substantial redshift in the LSPR band maxima position occurs when the sensor chip is treated with the serum of cardiac patients, whereas the presence of troponin I do not show a significant band position shift ensuring the specificity of the measurements.

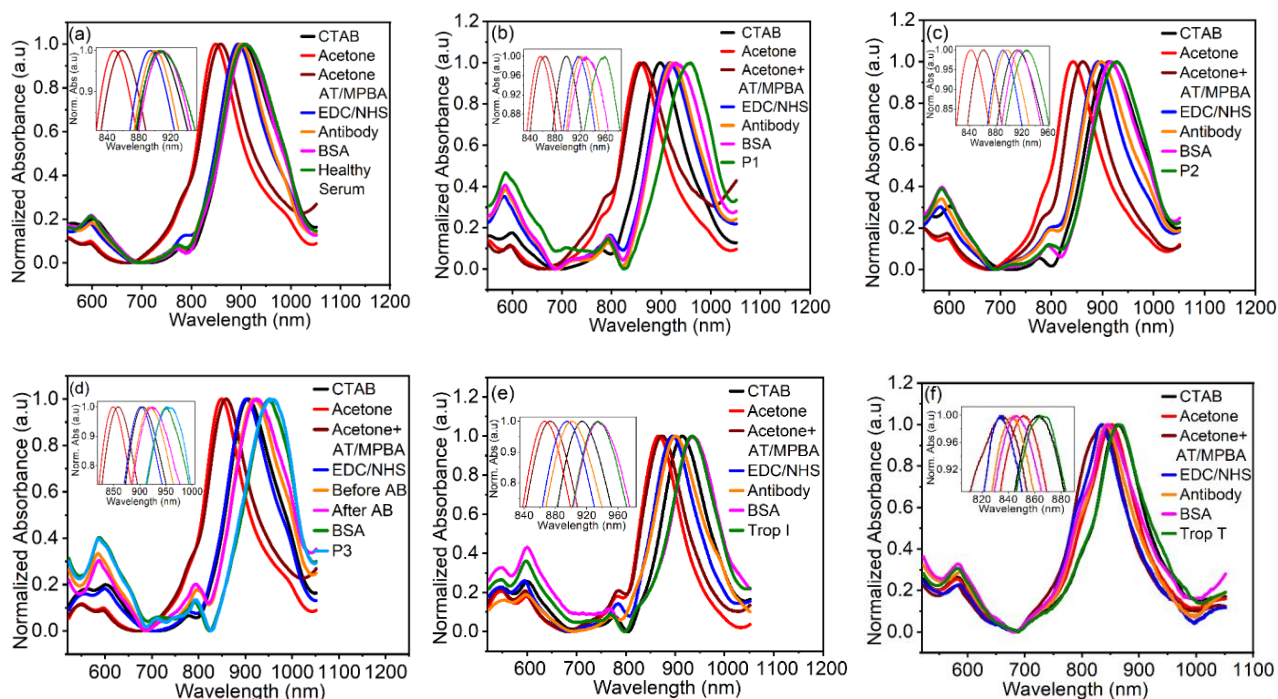


Fig. 9 LSPR spectra of the substrate at different functionalization and detection stages of NT-proBNP (a) for commercial human serum (b), for blood serum of patient 1, (c) for blood serum of patient 2, (d) for blood serum of patient 3, (e) specificity determination using CTnI, and (f) CTnT spiked in commercial human serum.

Table 2 LSPR band positions of sensor chip at different stages of functionalization and detection of CTnT.

Coverslip (CS)	LSPR band positions (nm) at different functionalization stages					
	a	b	c	d	e	f
before acetone	905.9	898.2	913.6	906.3	914.8	862.9
after acetone	849.2	857.4	843.1	849.4	867.3	851.3
after alkane thiols (AT)	858.8	864.2	862.2	859	873.7	834.8
after EDC/NHS	894.5	917.4	892	920.2	895	835.2
after antibody	900.3	923.2	898.1	925.2	901.4	842.7
after BSA	910.6	931.5	917	949.7	936.9	846.6
after patient serum	—	955.6	927.5	955	—	—
after commercial serum	908.6	—	—	—	—	—
after 100 ng/mL CTnI	—	—	—	—	934.9	—
after 100 ng/mL CTnT	—	—	—	—	—	864.7

3.4.2 Detection of Cardiac Troponin T (CTnT)

The prepared LSPR chip is used for the detection of another cardiac marker protein CTnT. Prior to the detection of CTnT, a monoclonal antibody specific to CTnT is immobilized on the EDC-NHS modified sensor chip by using a 50 µg/mL solution of CTnT. To avoid non-specific binding, the substrates were treated with 1% (10 mg/mL) BSA for 30 min. For the detection of CTnT, the antibody-immobilized sensor chip was incubated in the blood serum and separated from the cardiac patient blood samples for 2 h. As mentioned earlier, the LSPR spectra were recorded at each step of the functionalization of the

sensor chip and detection of CTnT. Figs. 9(a–f) show the absorption spectra at different stages of sensor chip functionalization for 6 sensor chips. Corresponding Table 2 lists the LSPR band maxima position at each stage of sensor functionalization. The insets in Fig. 9 show the expanded spectral region adjacent to the longitudinal band maxima for clarity purposes. It is clearly evident from Fig. 9(f) and Table 2 that the LSPR band maxima shift 18 nm towards red after CTnT incubation. Similarly, red shifts in the LSPR band maxima are also seen for the blood serum of patients 1, 2, and 3 by ~24, ~10, and ~5 nm, respectively. A blue shift of nearly 2 nm was seen for the healthy serum and CTnI (100 ng/mL). The clear absence

of red shift for CTnI confirms the specificity of the sensor chip. Also, it is very much evident that CTnT can be detected from the patient's blood serum easily using the developed approach.

4 Conclusions

The Au NBPs with longitudinal LSPR band in the near-IR spectral region were synthesized using a seed-mediated approach. After removing a significant amount of capping molecule CTAB, successful immobilization of the nanostructure is demonstrated on the MPTMS silanized glass coverslips. The immobilized Au NBPs were further functionalized using 11-MUA and Octanethiol after completely removing CTAB using acetone. The removal of CTAB was investigated using RI sensitivity measurements. It was found that the RI sensitivity before the removal of CTAB was 197 nm/RIU, which becomes 303 nm/RIU after the removal of CTAB. Successful fabrication of the sensor chip is demonstrated after activation of the terminal group of 11-MUA with EDC-NHS treatment, followed by immobilization of

antibodies of CTnT and NT-proBNP. Successful detection of the corresponding marker proteins was also shown with blood serum collected from cardiac patients. The specificity of the prepared sensor chip is also shown using another cardiac marker protein, CTnI. The approach demonstrated in the present work will be extremely helpful in the fabrication of LSPR sensor chips for detecting cardiac marker proteins in blood serum samples with very high specificity.

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Disclosures

The authors declare no conflict of interest.

References

1. S. Lv, Y. Du, F. Wu, Y. Cai, and T. Zhou, "Review on LSPR assisted photocatalysis: effects of physical fields and opportunities in multifield decoupling," *Nanoscale Advances* 4(12), 2608–2631 (2022).
2. H. Zhang, X. Zhou, X. Li, P. Gong, Y. Zhang, and Y. Zhao, "Recent Advancements of LSPR Fiber-Optic Biosensing: Combination Methods, Structure, and Prospects," *Biosensors* 13(3), 405 (2023).
3. T. Ghodselahi, T. Neishaboorynejad, and S. Arsalani, "Fabrication LSPR sensor chip of Ag NPs and their biosensor application based on interparticle coupling," *Applied Surface Science* 343, 194–201 (2015).
4. Y. Ziai, C. Rinoldi, P. Nakielski, L. D. Sio, and F. Pierini, "Smart plasmonic hydrogels based on gold and silver nanoparticles for biosensing application," *Current Opinion in Biomedical Engineering* 24, 100413 (2022).
5. B. Sepúlveda, P. C. Angelomé, L. M. Lechuga, and L. M. Liz-Marzán, "LSPR-based nanobiosensors," *Nano Today* 4(3), 244–251 (2009).
6. Y. Song, V. Tran, and J. Lee, "Tuning plasmon resonance in magnetoplasmonic nanochains by controlling polarization and interparticle distance for simple preparation of optical filters," *ACS Applied Materials & Interfaces* 9(29), 24433–24439 (2017).
7. N. C. Lindquist, P. Nagpal, K. M. McPeak, D. J. Norris, and S.-H. Oh, "Engineering metallic nanostructures for plasmonics and nanophotonics," *Reports on Progress in Physics* 75(3), 036501 (2012).
8. M. Fan, G. F. Andrade, and A. G. Brolo, "A review on recent advances in the applications of surface-enhanced Raman scattering in analytical chemistry," *Analytica Chimica Acta* 1097, 1–29 (2020).
9. R. Pilot, R. Signorini, C. Durante, L. Orian, M. Bhamidipati, and L. Fabris, "A review on surface-enhanced Raman scattering," *Biosensors* 9(2), 57 (2019).
10. G. Barbillon, J.-L. Bijeon, J. Plain, M. L. de la Chapelle, P.-M. Adam, and P. Royer, "Electron beam lithography designed chemical nanosensors based on localized surface plasmon resonance," *Surface Science* 601(21), 5057–5061 (2007).
11. Y. Lin, Y. Zou, Y. Mo, J. Guo, and R. G. Lindquist, "E-beam patterned gold nanodot arrays on optical fiber tips for localized surface plasmon resonance biochemical sensing," *Sensors* 10(10), 9397–9406 (2010).
12. T. R. Jensen, M. D. Malinsky, C. L. Haynes, and R. P. Van Duyne, "Nanosphere lithography: tunable localized surface plasmon resonance spectra of silver nanoparticles," *The Journal of Physical Chemistry B* 104(45), 10549–10556 (2000).
13. C. L. Haynes, R. P. Van Duyne, "Nanosphere lithography: a versatile nanofabrication tool for studies of size-dependent nanoparticle optics," *The Journal of Physical Chemistry B* 105(24), 5599–5611 (2001).
14. J. D. Driskell, S. Shanmukh, Y. Liu, S. B. Chaney, X.-J. Tang, Y.-P. Zhao, and R. A. Dluhy, "The use of aligned silver nanorod arrays prepared by oblique angle deposition as surface-enhanced Raman scattering substrates," *The Journal of Physical Chemistry C* 112(4), 895–901 (2008).
15. S. Kaabipour, S. Hemmati, "A review on the green and sustainable synthesis of silver nanoparticles and one-dimensional silver nanostructures," *Beilstein Journal of Nanotechnology* 12(1), 102–136 (2021).

16. H. Chen, X. Kou, Z. Yang, W. Ni, and J. Wang, "Shape-and size-dependent refractive index sensitivity of gold nanoparticles," *Langmuir* 24(10), 5233–5237 (2008).
17. N. R. Jana, L. Gearheart, and C. J. Murphy, "Seed-Mediated Growth Approach for Shape-Controlled Synthesis of Spheroidal and Rod-like Gold Nanoparticles Using a Surfactant Template," *Advanced Materials* 13(18), 1389–1393 (2001).
18. M. Liu, P. Guyot-Sionnest, "Mechanism of Silver(I)-Assisted Growth of Gold Nanorods and Bipyramids," *The Journal of Physical Chemistry B* 109(47), 22192–22200 (2005).
19. X. Zhang, M. Tsuji, S. Lim, N. Miyamae, M. Nishio, S. Hikino, and M. Umezu, "Synthesis and Growth Mechanism of Pentagonal Bipyramid-Shaped Gold-Rich Au/Ag Alloy Nanoparticles," *Langmuir* 23(11), 6372–6376 (2007).
20. Y. Xu, X. Wang, L. Cheng, Z. Liu, and Q. Zhang, "High-yield synthesis of gold bipyramids for in vivo CT imaging and photothermal cancer therapy with enhanced thermal stability," *Chemical Engineering Journal* 378, 122025 (2019).
21. S. Barbosa, A. Agrawal, L. Rodríguez-Lorenzo, I. Pastoriza-Santos, R. A. Alvarez-Puebla, A. Kornowski, H. Weller, and L. M. Liz-Marzán, "Tuning size and sensing properties in colloidal gold nanostars," *Langmuir* 26(18), 14943–14950 (2010).
22. S. M. Marinakos, S. Chen, and A. Chilkoti, "Plasmonic detection of a model analyte in serum by a gold nanorod sensor," *Analytical chemistry* 79(14), 5278–5283 (2007).
23. H. R. Hegde, S. Chidangil, and R. K. Sinha, "Refractive index sensitivity of Au nanostructures in solution and on the substrate," *Journal of Materials Science: Materials in Electronics* 33(7), 4011–4024 (2022).
24. H. R. Hegde, S. Chidangil, and R. K. Sinha, "Refractive index and formaldehyde sensing with silver nanocubes," *RSC advances* 11(14), 8042–8050 (2021).
25. G. A. Roth et al., "Global, regional, and national age-sex-specific mortality for 282 causes of death in 195 countries and territories, 1980–2017: a systematic analysis for the Global Burden of Disease Study 2017," *The Lancet* 392(10159), 1736–1788 (2018).
26. J. A. Reyes-Retana, L. C. Duque-Ossa, "Acute Myocardial Infarction Biosensor: A Review From Bottom Up," *Current Problems in Cardiology* 46(3), 100739 (2021).
27. S. Upasham, A. Tanak, and S. Prasad, "Cardiac troponin biosensors: where are we now," *Advanced Health Care Technologies* 4, 1–13 (2018).
28. A. Qureshi, Y. Gurbuz, and J. H. Niazi, "Biosensors for cardiac biomarkers detection: A review," *Sensors and Actuators B: Chemical* 171–172, 62–76 (2012).
29. R. K. Sinha, "Wavelength modulation based surface plasmon resonance sensor for detection of cardiac marker proteins troponin I and troponin T," *Sensors and Actuators A: Physical* 332, 113104 (2021).
30. L. Tang, J. Casas, "Quantification of cardiac biomarkers using label-free and multiplexed gold nanorod bioprobes for myocardial infarction diagnosis," *Biosensors and Bioelectronics* 61, 70–75 (2014).
31. Y. M. Bae, S. O. Jin, I. Kim, K. Y. Shin, and D. Heo, "Detection of biomarkers using LSPR substrate with gold nanoparticle array," *Journal of Nanomaterials* 2015, 302816 (2015).
32. T. Liyanage, A. Sangha, and R. Sardar, "Achieving biosensing at attomolar concentrations of cardiac troponin T in human biofluids by developing a label-free nanoplasmonic analytical assay," *Analyst* 142(13), 2442–2450 (2017).
33. P. N. Deviprasada, R. K. Sinha, "Highly Stable 11-MUA Capped Gold Nanobipyramid for Refractive Index Sensing," *Journal of Biomedical Photonics & Engineering* 9(1), 010308 (2023).
34. D. Chateau, A. Desert, F. Lerouge, G. Landaburu, S. Santucci, and S. Parola, "Beyond the concentration limitation in the synthesis of nanobipyramids and other pentatwinned gold nanostructures," *ACS Applied Materials & Interfaces* 11(42), 39068–39076 (2019).
35. R. K. Sinha, "A simple and inexpensive surface plasmon resonance setup for phase detection using rotating analyzer ellipsometric method," *Laser Physics* 30(2), 026202 (2019).
36. C. A. Schneider, W. S. Rasband, and K. W. Eliceiri, "NIH Image to ImageJ: 25 years of image analysis," *Nature Methods* 9(7), 671–675 (2012).
37. A. F. Stalder, T. Melchior, M. Müller, D. Sage, T. Blu, and M. Unser, "Low-bond axisymmetric drop shape analysis for surface tension and contact angle measurements of sessile drops," *Colloid Surfaces A* 364(1–3), 72–81 (2010).
38. J. Casas, M. Venkataramasubramani, Y. Wang, and L. Tang, "Replacement of cetyltrimethylammonium bromide bilayer on gold nanorod by alkanethiol crosslinker for enhanced plasmon resonance sensitivity," *Biosensors and Bioelectronics* 49, 525–530 (2013).
39. A. Wijaya, K. Hamad-Schifferli, "Ligand Customization and DNA Functionalization of Gold Nanorods via Round-Trip Phase Transfer Ligand Exchange," *Langmuir* 24(18), 9966–9969 (2008).
40. H. Hegde, C. Santhosh, and R.K. Sinha, "Seed mediated synthesis of highly stable CTAB capped triangular silver nanoplates for LSPR sensing," *Materials Research Express* 6(10), 105075 (2019).
41. A. Sánchez-Iglesias, N. Winckelmans, T. Altantzis, S. Bals, M. Grzelczak, and L. M. Liz-Marzán, "High-yield seeded growth of monodisperse pentatwinned gold nanoparticles through thermally induced seed twinning," *Journal of the American Chemical Society* 139(1), 107–110 (2017).

42. M. Ghorbanpour, C. Falamaki, “[A novel method for the fabrication of ATPES silanized SPR sensor chips: Exclusion of Cr or Ti intermediate layers and optimization of optical/adherence properties](#),” *Applied Surface Science* 301, 544–550 (2014).
43. H. Jung, C. K. Dalal, S. Kuntz, R. Shah, and C. P. Collier, “[Surfactant activated dip-pen nanolithography](#),” *Nano Letters* 4(11), 2171–2177 (2004).
44. A. R. Ferhan, L. Guo, and D. H. Kim, “[Influence of ionic strength and surfactant concentration on electrostatic surfacial assembly of cetyltrimethylammonium bromide-capped gold nanorods on fully immersed glass](#),” *Langmuir* 26(14), 12433–12442 (2010).