LSPR-based cholesterol biosensor using a tapered optical fiber structure

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Abstract: Accurate cholesterol level measurement plays an important role in the diagnosis of severe diseases such as cardiovascular diseases, hypertension, anemia, myxedema, hyperthyroidism, coronary artery illness. Traditionally, electrochemical sensors have been employed to detect the cholesterol level. However, these sensors have limitations in terms of sensitivity and selectivity. In this paper, a localized surface plasmon resonance (LSPR) -based biosensor is demonstrated that accurately detects and measures the concentration of cholesterol. In the present study, a tapered optical fiber-based sensor probe is developed using gold nanoparticles (AuNPs) and cholesterol oxidase (ChOx) to increase the sensitivity and selectivity of the sensor. Synthesized AuNPs were characterized by UV-visible spectrophotometer, transmission electron microscope (TEM), and energy dispersive X-ray spectroscopy (EDS). Further, coating of AuNPs over fiber was confirmed by scanning electron microscope (SEM). The developed sensor demonstrates for a clinically important cholesterol range of 0 to 10 mM, and the limit of detection is found to be 53.1 nM.

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1. Introduction

Cholesterol is an essential component of human body. It is produced by liver and is also a part of healthy diet because it is precursor of very essential biological materials such as bile acid, steroid hormones [1] and vitamin D [2]. An abnormal level (above 200 mg dL−1) of cholesterol in blood may cause several common diseases such as hypertension, heart disease, coronary artery disease, cerebral thrombosis, arteriosclerosis [3,4]. Nowadays, major and sudden deaths of human occur due to hypercholesterolemia [5]. A low level of cholesterol also causes various diseases such as anemia, hyperthyroidism, and malabsorption [6]. Various conventional techniques such as gas chromatography (GC) [7], high-performance liquid chromatography (HPLC) [8], electrochemical methods [9], voltammetric [10], colorimetric [11] and non-enzymatic technique [12] are used for detection of cholesterol. But these techniques are prone to several limitations such as low sensitivity, poor selectivity, complex and expensive development procedure. However, these techniques were replaced by enzymatic methods that are based on spectrophotometry [13]. For the fabrication of enzymatic cholesterol biosensor, cholesterol oxidase (ChOx) plays an important role [6,14]. ChOx converts the cholesterol into cholestenone in the presence of oxygen (O2) and produces the hydrogen peroxide (H2O2). There is a cofactor flavin adenine dinucleotide (FAD) in ChOx, which are responsible for oxidation process [6]. Budiyanto et al. [15] developed cholesterol biosensor using intensity modulation. They have shown that the sensed peak voltage decreases linearly with the increase in the concentration of cholesterol [15]. Dhawane et al. [14] developed colorimetric based biosensor using chitosan nanofiber mat for detection.
of cholesterol. The nanofiber is immobilized with ChOx and horseradish per-oxidase (HRP) which is used in detection of different cholesterol concentrations. Many researchers have also used nanoparticles (NPs) for sensing which demonstrates significant property of enzyme immobilization thereby increasing sensitivity [5,16,17]. A special characteristic of gold nanoparticles (AuNPs) shows strong absorption band [18] that triggers localized surface plasmon resonance (LSPR) when an incident photon frequency is resonant with collective oscillation of the conduction electron [19]. The local environment of AuNPs can change the resonance frequency of the LSPR. An absorbance and peak wavelength of AuNPs are sensitive towards changes in refractive index (RI) of analytes and binding phenomena of immobilized NPs. Recently, Nirala et al. [20] reported a colorimetric method based cholesterol biosensor using functionalized AuNPs with ChOx that shows limited sensitivity. A fluorescence-based fiber optic biosensor using hydrogel-immobilized enzyme for cholesterol and glucose sensing has been proposed by Lin et al. [21] but is temperature dependent.

In this paper, we have developed a sensor based on AuNPs-immobilized tapered fiber for accurate measurement of cholesterol levels. The fiber probe acting as a sensor node is functionalized with the ChOx to enhance the selectivity. The paper consists of six sections including this introductory section. Section 2 describes the process for fabricating tapered fiber. Material and methods form Section 3 wherein techniques for synthesis of AuNPs, immobilization of AuNPs over taper fiber structure, and characterization of AuNPs/AuNPs-immobilized taper fiber is described. Functionalization of ChOx over AuNPs coated taper fiber and preparation of different cholesterol solution is also discussed in this section. Section 4 consists the experimental setup to detect the cholesterol. Section 5 consists the results and analysis. Finally, section 6 comprises the conclusion of work.

2. Fabrication of taper fiber

The hydrogen-oxygen flame-brushing technique was used to manufacture the tapered fiber by tapering a matched cladding single mode fiber (model - A0Q0059SC0 LWPF), wherein the core and cladding diameters are 8.2 µm and 125 µm, respectively.

![Fig. 1. Schematic of fiber tapering process using hydrogen-oxygen flame-brushing technique.](image)

There are three regions on tapered fiber. First and most important region is tapered sensing region that actually works as a sensing region. Second region is transition-stretching region and third region is general fiber region. The lower waist diameter of taper fiber is more useful for the fabrication of sensor probe. Before tapering process, plastic coating of optical fiber was removed with fiber stripper from a small section of fiber and cleaned with ethanol. The hydrogen-oxygen flame-brushing technique was used to fabricate the tapered fiber sensor probe. To taper a fiber, both sides of fiber were pulled simultaneously while heating the fiber as shown in Fig. 1. It is easy to fabricate the smaller diameter (even less than 4 µm) through hydrogen-oxygen flame. We have kept constant temperature and pulling speed to avoid the
fragility of the tapered fiber. The pulling stages and the flame were controlled by the computer program. To get the waist diameter of taper fiber of ~4 µm, flow set was 40 and stretch dimension was 15000 µm. Fabricated tapered fibers exhibit very less stress and strain with optimum fabrication parameters.

Thus, there were no possibilities of fragility of the tapered fiber during fabrication. But, it was very difficult to handle the taper fiber structure of smaller waist diameter during cleaning, gold nanoparticles (AuNPs) coating and enzyme functionalization process. We had used an ultrasonic cleaner to clean the fiber before coating of AuNPs. Many taper fibers were broken during cleaning with an ultrasonic cleaner. Therefore, only half part of tapered fiber was obtained using cleaver. Reflection spectra was measured through the half-part of taper fiber. It eases the fabrication process and stabilizes the sensor probe. Taper fiber structures of waist diameter ~4 µm and waist length 10 mm were fabricated for the purpose of sensor probes.

3. Materials and methods

3.1 Materials

Gold(III) Chloride trihydrate (Hydrogen tetrachloroaurate - HAuCl₄), tri-sodium citrate and ultrapure water were used for the synthesis of AuNPs. Acetone, hydrogen peroxide solution (H₂O₂), sulfaric acid solution (H₂SO₄), ultrapure water, (3-mercaptopropyl)trimethoxysilane (MPTMS), ethanol and nitrogen gas were used for immobilization of AuNPs over tapered fiber. Cholesterol oxidase from Streptomyces, phosphate buffered saline (PBS), N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC) and N-Hydroxysuccinimide (NHS), 11-Mercaptoundecanoic acid (MUA) were used for functionalization of specific enzyme for cholesterol detection. A different concentration of cholesterol is prepared with 5% Triton X-100. Most of the reagents were purchased from Sigma-Aldrich, Shanghai. These reagents are of analytical grade and were used in the experiment without further purification. All aqueous solutions used in experiments were prepared with ultrapure water with a specific resistance of 18 MΩ cm.

3.2 Methods: synthesis of AuNPs

AuNPs was synthesized with the help of Turkevich method [22]. First, HAuCl₄ (150 µL, 100 mM) solution was added in 14.85 mL ultrapure water and was heated until getting the reflex action inside the bottle. Further, trisodium citrate (1.8 mL, 38.8 mM) was added, and solution was allowed to boil further for 5 minutes. It turns the mixture to red color solution (as shown in inset of Fig. 2(a)), which denotes the formation of AuNPs approx 10.5 ± 0.5 nm size. The prepared AuNPs solution can be stable up to three months at room temperature.

3.3 Characterization of AuNPs

The normalized absorption spectrum of AuNPs was obtained using a UV-visible spectrophotometer (HITACHI-U-3310) at room temperature in a quartz cuvette of 1 cm path length. Absorbance peak of AuNPs was obtained at 520 nm, as shown in Fig. 2(a). This spectral characterization was beneficial to select the wavelength of source to excite LSPR for subsequent sensing experiments. Transmission electron microscope (TEM) (Talos L120C, Thermo Fisher Scientific) was used to analyze the size and shape of the AuNPs particle. TEM image of AuNPs is shown in Fig. 2(b). The particle size distribution analysis of TEM image obtained using ImageJ software shows that mean diameter of synthesized AuNPs is 10.5 ± 0.5 nm (Fig. 2(c)). Figures 2(d)-2(f) display the energy dispersive X-ray spectroscopy (EDS) of AuNPs that confirms for synthesized material as gold.
3.4 Immobilization and characterization of AuNPs-immobilized taper fiber

The tapered fiber used for immobilization was cleaned by three-step process. The fiber needs careful handling during various processes to prevent from breaking since the waist diameter of taper fiber was very low (~4 µm). In first step, the tapered fiber was kept in acetone for ultrasonic bath for 20 min. Thereafter, the tapered fiber was kept in Piranha solution for another 30 min. Piranha solution was prepared using mixture of three part of H₂O₂ and seven part of sulfuric acid solution (H₂SO₄). Later on, the tapered fiber was cleaned using ultrapure water. Taper fiber was dried at a temperature of 70 °C under vacuum before further process of coating.

(3-mercaptopropyl)trimethoxysilane (MPTMS), which is an adhesive layer, used for coating of thin film of AuNPs over taper fiber structure. Taper fiber was kept in a freshly prepared solution of MPTMS (250 µL of MPTMS in 25 mL of ethanol) for soaking of MPTMS over taper fiber surface for 12 hr. As shown in Fig. 3, AuNPs is attached with SH group of MPTMS that helps in formation of a thiol-terminated self-assembled monolayer.
(SAM) of MPTMS over taper fiber structure. Thereafter, ethanol and nitrogen gas were used to remove the unbound monomers of MPTMS from taper fiber surface.

![Image](image_url)

Fig. 4. Scanning electron micrographs (SEM) of (a) AuNPs-immobilized taper fiber (b) AuNPs at the surface of taper fiber structure.

Thereafter, thiol-functionalized tapered fiber was immersed in AuNPs solution for 48 hrs to obtain a nanoparticle-immobilized tapered fiber. Again, ethanol and nitrogen gas were used to remove the unbound AuNPs from AuNPs-immobilized fiber [19]. Scanning electron microscope (SEM) (Gemini, Carl Zeiss Microscopy) was used to check the coating of AuNPs over taper fiber structure. For SEM, small taper section of fiber structure was placed on 3x3 mm silicon wafer. Figure 4(a) shows the tapered fiber after immobilization of AuNPs and 4b shows the presence of AuNPs on the surface of taper fiber structure. It is apparent from Fig. 4(b) that there is a uniform coating of AuNPs over taper fiber structure (sensor probe).

### 3.5 Functionalization of sensor probe with cholesterol oxidase

The AuNPs-immobilized taper fiber was cleaned with ultrapure water then it is kept for soaking in a freshly prepared aqueous solution of MUA (5 ml, 0.5 mM) for 5 hrs. MUA produces a layer of carboxylic group over the surface of AuNPs-immobilized taper fiber structure. The carboxylic group was activated by keeping the tapered fiber into a 2 ml solution of EDC (200 mM) and NHS (50 mM) for 10 min. Thereafter, the activated carboxylic fiber structure was dipped for 12 hrs in a freshly prepared solution of ChOx. The ChOx solution was prepared by mixing 0.32 mg of ChOx in 0.5 ml of cold PBS solution (pH 7.4). After the coating of MUA/EDC/NHS/ChOx, the functionalized taper fiber sensor probe was obtained. After enzyme functionalization, the sensor probe was kept in a refrigerator at 4 °C. Before using the probe for sensing purpose, it was dipped in cold PBS solution for 15 min.

### 3.6 Preparation of cholesterol solution

Different concentrations of cholesterol solutions were prepared for the characterization of sensing probe. Concentration of cholesterol in the range of 10 nM to 10 mM is clinically considered to be an effective range in a human being. First, the stock solution of 10 mM was prepared by dissolving 38.7 mg of cholesterol in 10 ml of 5% Triton X-100 solution. The cholesterol is waxy in nature so it was required to heat and stir to dissolve in a solution form. Different concentration (10 nM, 100 nM, 250 nM, 1 uM, 1 mM, 10 mM) of cholesterol were prepared by diluting the stock solution using ultrapure water. Similarly, solutions of glucose, urea, galactose, L-cysteine, and ascorbic acid each with 10 mM concentration were also prepared for selectivity experiment.
4. Experimental setup for detection of cholesterol

The absorbance spectra of AuNPs was obtained at 520 nm that lies in the visible range of optical source. So, tungsten halogen white light source (HL-2000, Ocean Optics Inc., USA) was used in the experimental setup.

![Experimental setup](image)

Fig. 5. Experimental setup for reflection spectra through AuNPs-immobilized and enzyme functionalized tapered fiber sensor probe using spectrometer.

A three-port circulator was used to measure the reflectance intensity through a biosensor probe.

As shown in Fig. 5 the optical source was connected to the first port of circulator. This optical source works in the visible and near-infrared region. The second port of circulator was connected to the AuNPs-immobilized taper fiber (sensor probe). The LSPR phenomena occur at the tapered fiber - AuNPs surface. The small size of AuNPs enhances the surface area for LSPR phenomena. ChOx enzyme is specific for cholesterol which oxidizes the cholesterol in the presence of oxygen and produces H₂O₂ and cholestenone. The level of H₂O₂ varies the RI at the surface of AuNPs and changes the reflectance intensity. The reflection spectra was measured through the USB2000 + spectrometer (Ocean Optics Inc., USA) that detection range was from 200 to 1100 nm.

5. Results and discussions

5.1 Reflection intensity before and after AuNPs-immobilization

Reflection spectra were measured through bare taper fiber and AuNPs-immobilized taper fiber using experimental setup shown in Fig. 5. 10 mM of cholesterol solution was used to check the sensor probes. It is clearly visible from Fig. 6 that several random peaks are generated in the case of without AuNPs-immobilized taper fiber structure. Interestingly, only a single major peak arises after coating of AuNPs over taper fiber structure which can be quite useful for the bio-sensing purpose.
5.2 Cholesterol sensing

The LSPR spectra of different cholesterol concentration (10 nM to 10 mM) was recorded by proposed sensor. Figure 7(a) shows the reflectance spectra of the tapered fiber sensor probe in different cholesterol concentration.

It can be observed from the result that reflection intensity increases with the increasing cholesterol concentration. This is due to the presence of ChOx at sensor probe. It oxidizes cholesterol and produces cholestenone and H$_2$O$_2$ as per concentration of cholesterol. The amount of H$_2$O$_2$ varies the RI surrounding the sensor probe and produces a shift in reflection peak. The variation of LSPR spectra occurs due to enzymatic reaction of ChOx with the cholesterol. Thereafter, LSPR spectra for 5% Triton X-100 solution were recorded in order to calculate the detection limit of proposed sensor. Figure 7(b) shows the linear variation of the reflection intensity with cholesterol concentration. It can be written as, reflection intensity = 517.77 + 125.47 C, where C is the cholesterol concentration in µM. The standard deviation of the blank sample was 2.223685 a.u. Based on the data, the detection limit of the proposed sensor is 53.1 nM and resonance wavelength is 607 nm. Three samples of different cholesterol concentration in the range of 10 nM – 10 mM were tested with developed biosensor with a sensitivity of 0.125%/mM, linearity of R$^2$ = 0.9952 and a resolution of 0.12.
5.3 Reproducibility and reusability of sensor probe

Reproducibility and reusability of sensor probe were tested with three consecutive results as shown in Fig. 8. For verifying the reproducibility, 1 µM of cholesterol were tested with three different sensor probes.

![Image](image_url)

Fig. 8. (a) Reproducibility test of proposed sensor probe at 1 µM cholesterol concentration, (b) reusability test of taper fiber sensor probe with different concentration of cholesterol solution.

The reflectance intensities of sensor probes, as observed in Fig. 8(a) are identical that confirms the reproducibility of sensor probes. The reusability of sensor probe was tested for 10 nM, 1 µM and 10 mM cholesterol concentration. The sensor probe was rinsed it in PBS solution (pH = 7.4) and dried after each measurement. It can be observed from Fig. 8(b), that reflection spectra in all cases were slightly decreased during second time measurement since ChOx is utilized away during the first testing. It is evident from result that the reflection intensity for 10 mM cholesterol solution is higher than the lower concentrations (1µM and 10 nM) even in second time measurement.

5.4 Selectivity of proposed sensor

Different biomolecules such as cholesterol, glucose, urea, L-cysteine, ascorbic acid and galactose which are normally present in serum were tested with the proposed sensor in order to check its selectivity. The selectivity plot for various biomolecules present in test cases is shown in Fig. 9. The reflection intensity peak of 10 mM concentration of cholesterol, glucose, urea, L-cysteine, ascorbic acid and galactose are 776.49 a.u., 491.48 a.u, 546.98 a.u, 513.11 a.u, 501.77 a.u, and 493.49 a.u, respectively. It can be observed from the result that sensor exhibit higher selectivity for cholesterol. This is due to the presence of a very specific enzyme (ChOx) at sensor probe, which was used to detect the cholesterol. The sensor probe is non-reactive in the case of another analytes.
5.5 Comparison of sensing performance

To date, few optical fiber sensors have been developed to detect the cholesterol. The fabrication processes of the previously developed sensors were complex and demonstrate limited detection limit.

Table 1. Comparison of sensing performance of proposed sensor with developed sensors

| Materials used                  | Mechanism used          | Linear range         | Detection Limit | Ref.   |
|---------------------------------|-------------------------|----------------------|-----------------|--------|
| Nickel oxide (NiO)              | Electrochemical technique| 0.12 mM - 10.23 mM   | 0.10 mM         | [9]    |
| AuNPs                           | Amperometric technique  | 0.04 mM - 0.22 mM    | 34.6 μM         | [23]   |
| poly(N-isopropylacrylamide)     | Fluorescence quenching  | 0.52 mM/L - 6.47 mM/L| n.r.*           | [21]   |
| (PNIPAAm)                       |                         |                      |                 |        |
| AgNPs                           | SPR technique           | 0 mM - 10 mM         | n.r.*           | [24]   |
| Ag/GO/AgNPs                     | SPR/LSPR technique      | 0 mM - 10 mM         | 1.131 mM        | [25]   |
| AuNPs                           | LSPR technique          | 10 nM - 1 μM         | 53.1 nM         | This work |

* not reported.

Table 1 shows the comparison of sensing performance of proposed sensor with respect to previously reported results. The comparison has been made in terms of the materials used for sensor fabrication and mechanism used. The results have been primarily compared in terms of linearity and detection limit. It can be clearly observed that the overall performance of the proposed sensor is better than the previously reported sensors.

6. Conclusion

In this paper, a biosensor is developed to detect and measure the cholesterol concentration. Taper fiber structure is fabricated using hydrogen-oxygen flame technique. 10.5 ± 0.5 nm of AuNPs is synthesized for AuNPs-immobilization process whose absorbance spectra is 520 nm. AuNPs with smaller size have a large light absorption and scattering cross-section in the LSPR wavelength region, thereby increased the sensitivity of the sensor. Further, the sensor probe is functionalized with ChOx that improves the selectivity of sensor. The cost of instruments required to characterize the sensor probe are low due to LSPR spectra lies in the visible region. Different cholesterol concentration in the range of 10 nM – 10 mM are tested with developed biosensor with a sensitivity of 0.125%/mM, linearity of $R^2 = 0.9952$ and a resolution of 0.12. The linearity range of sensor probe is 10 nM to 1 μM and its detection
limit is 53.1 nM. The fabrication process and performance of the proposed sensor probe is easy than existing sensors. The developed sensor probe is more stable. The reproducibility and reusability of sensor probe is also high.

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The authors declare that there are no conflicts of interest related to this article.

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