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Sensitive and non-invasive cholesterol determination in saliva *via* optimization of enzyme loading and platinum nano-cluster composition†

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An excessive cholesterol level can lead to cardiovascular diseases, such as stroke, hypertension, and myocardial infarction. A non-invasive, painless method of determining the cholesterol level in blood would improve the user's convenience. To provide rapid and accurate determination of cholesterol, we have developed a simple, disposable, enzyme-based electrochemical biosensor that can detect salivary cholesterol. It is possible to detect low concentrations of cholesterol in saliva using the optimized vertical structure of the platinum nano-cluster (Pt-NC) and the immobilization of a proper volume of an enzyme. The biosensor exhibited a linear range from 2 to 486 μM, the limit of detection was about 2 μM, and the sensitivity of the sensor was calculated to be 132 μA mM^{-1} cm^{-2}. It also showed good specificity for ascorbic acid, uric acid, dopamine, glucose, and lactate. In a test with an actual sample, the performance of the biosensor was confirmed by measuring total cholesterol in the saliva of a patient with hyperlipidemia. The cholesterol levels measured in the saliva of three patients with hyperlipidemia were 520, 460, and 290 μM. Therefore, the Pt-NC based enzyme sensor is a promising candidate for the detection of cholesterol in human saliva.

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1. Introduction

Cholesterol is an essential lipid and is crucial for normal body functioning. However, an abnormal level of cholesterol in the blood can significantly increase the risk of some diseases such as arterial diseases, coronary heart disease, arteriosclerosis, hypertension, and diabetes.[1] According to a report published by the World Health Organization (WHO) in 2008, globally, excessive serum cholesterol is estimated to cause 2.6 million deaths and 29.7 million disability-adjusted life years.[2] Therefore, determining the concentration of serum cholesterol is very important from a healthcare perspective on a worldwide basis.

Conventionally, high performance liquid chromatography (HPLC) has been used to determine the concentration of cholesterol in the patient's blood.[3] However, obtaining samples of patients' blood has some drawbacks, including pain, the risk of infection, and the stress caused by pricking a finger. Because of these problems, recent researchers have tried to measure cholesterol using other body fluids, such as sweat, tears, and saliva. The methods used to collect saliva are simple, non-invasive, and cost-effective compared to the collection of blood, and the samples are easy to store.[4] As a result, many researchers have focused on the detection of glucose in saliva,[5] but the detection of cholesterol in saliva is rarely used due to the poor limit of detection (LOD) in the previous studies.

Generally, the total concentration of cholesterol in the serum of a normal person's blood should be less than 200 mg dl^{-1} (<5.17 mM).[6] However, there is only a small amount of cholesterol in saliva, *i.e.*, the concentration ranges from 10 to 320 μM.[7] Therefore, it is very challenging in medical diagnostics to determine accurately the low levels of cholesterol in saliva. To date, several methods have been developed for conducting cholesterol assays, including colorimetric,[8] spectrophotometric,[9] and electrochemical methods.[1,10] Although these three methods conventionally are used by most researchers to detect cholesterol, they are severely limited or even unsuited for such use because they cannot detect accurately the low concentrations of cholesterol in saliva. However, the electrochemical technique for such detection offers several advantages, including rapid detection, low cost, and the ability

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to improve the sensitivity and selectivity by modifying either the sensor platform or the materials used extensively to measure the low cholesterol in saliva.

Generally, electrochemical cholesterol biosensors have been fabricated through the immobilization of an enzyme, such as cholesterol esterase or cholesterol oxidase, and the former converts esterified cholesterol into free cholesterol. In the presence of oxygen, cholesterol oxidase catalyzes the oxidation of cholesterol to form H_2O_2 and cholest-4-en-3-one.[11] The hydrogen peroxide that is generated can be measured through an electrocatalytic reaction as follows (1 and 2).

$$\text{Cholesterol esters} + H_2O + \text{ChEx} \rightarrow \text{cholesterol} + \text{fatty acids} \quad (1)$$

$$\text{Cholesterol} + O_2 + \text{ChOx} \rightarrow \text{cholestene-3-one} + H_2O_2 \quad (2)$$

Conventionally, a Pt or glassy carbon electrode (GCE) has been used for the electrochemical assay.[12] However, these electrodes have low sensitivity, and many researchers have tried to deal with this issue by exploring potential new materials for the electrodes, including two-dimensional materials,[1] conducting polymers,[13] and nanomaterials.[14]

The nanomaterials have unique chemical, physical, and electronic properties that are different from the properties of their bulk counterparts. They have been used in diverse ways, and they have been shown to improve the performance of biosensors.[15]

In this work, we fabricated a high performance sensor using Pt nano-clusters (Pt-NCs) that can measure the low concentrations of cholesterol in saliva. The optimization of the Pt-NCs and the optimization of the volume of enzyme that were loaded were critical factors for enhancing the sensor's detection limit. Thus, in order to obtain an accurate determination of the concentration of cholesterol in saliva, we investigated and controlled the structures of the Pt-NCs simply by controlling the electroplating time and by loading the optimal volume of the enzyme onto the Pt-NCs as shown in Fig. 1. The simply fabricated using electroplating and properly loaded enzyme will provide properties of low detection limit and stability by enhancing the electrocatalytic area for non-invasive detection. Scanning electron microscopy (SEM) and atomic force microscopy (AFM) were used to investigate the surface morphology and topology of the Pt-NC structure and to compare the enzyme loadings. In addition, cyclic voltammetry and amperometry were used to observe the properties of the biosensor with the Pt-NC which has optimized geometry for loading a sufficient amount of enzyme. Subsequently, a feasibility test of the sensor was performed by measuring the cholesterol concentration in a hyperlipidemic patient's saliva.

2. Materials and methods

2.1 Reagents

We used the polyimide, VTEC PI-1388, from Richard Blaine International, USA. The polyimide was used as a substrate of the sensor. Cholesterol oxidase, cholesterol esterase, and peroxidase were purchased from TOYOBO, Japan. Cholesterol, Nafion, Triton TM X-100, ascorbic acid (AA), dopamine (DA), uric acid (UA), glucose, and lactate were obtained from Sigma-Aldrich, S. Korea. The Pt-NC electroplating stock solution consists of 600 mg of chloroplatinic acid hydrate and 5 mg of lead(II) acetate trihydrate, 2 ml of HCl (DaeJung, Korea), and 20 ml of DI water. We also purchased a phosphate buffer solution (PBS) (1 M, pH 7.4) (Corning). Fifty milliliters of a cholesterol stock solution were prepared with 44 ml of 0.1 M PBS, 5 ml of Triton X-100, and 1 ml of isopropyl alcohol, and this solution was stored at 4 °C.

2.2 Preparation of the sensor with a Pt nano-cluster electrode

ESI Fig. S1† shows simple schematic illustration for the fabrication process of a cholesterol sensor with a Pt-NC electrode and a photograph image of the fabricated sensor. First, the liquid PI was spin-coated onto a silicon wafer to a thickness of 20 μm, and the wafer was placed inside a convection oven at 210 °C for 1 h to thermally cure the PI. After conventional photolithography processing with a photoresist (DNR-L300), Ti and Pt were deposited on the polyimide substrates using an RF sputter. A 20 nm thick layer of Ti was deposited as an adhesion layer. Then, a 150 nm thick layer of Pt was deposited.

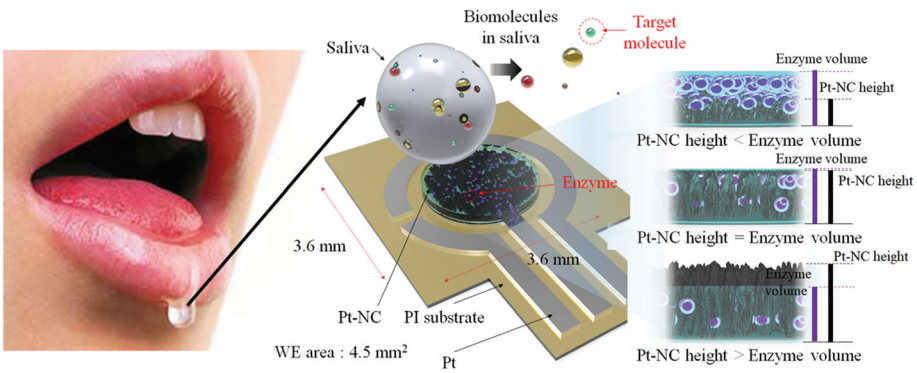

Fig. 1 A schematic for an electrochemical biosensor with a Pt nano-cluster and the strategic approach for improving sensor's performance by optimizing between the effective surface area and enzyme volume.

After the lift-off process using acetone, a second PI layer was spun to a thickness of 5 μm for insulation. In order to open the sites of Pt, the photoresist was patterned, and exposed PI was dry etched using O_2 plasma treatment by reactive ion etching (RIE) (Plasmatherm, USA). The diameter of the etched opening area of the conducting material was 2.2 mm. After the etching process, the flexible electrode was diced using a femtosecond laser micro-machining workstation (M-2000 laser). Then, the flexible electrode was easily detached from the silicon substrate,[16] after which the Pt-NC was electroplated selectively on the sputtered Pt working electrode using a potentiostatic mode (−200 mV, 100–500 s). The electroplating was performed separately from 1 to 5 times at 100 s intervals.

2.3 Preparation of the enzyme based biosensor

To fabricate a sensor with a Pt-NC/enzyme, the sensor that had been prepared was assembled in a customized jig. For the determination of cholesterol, we mixed 20 units per mg of cholesterol oxidase, 120 units per mg of cholesterol esterase, and 135 units per mg of peroxidase (powder type, TOYOBO Co., LTD). Then, 3 and 15 μl of the enzyme mixture were coated on the prepared Pt-NC working electrode (100, 200, 300, 400, and 500 s electroplated), respectively. Subsequently, 1.5 μl of Nafion were dropped on the working electrode and dried at room temperature for 25 min. Then, the Pt-NC/enzyme/Nafion sensor was fabricated.

2.4 Electrochemical measurements

In order to characterize the properties of the Pt-NC electrode based on the electroplating conditions, the working electrodes were electroplated for 100 to 500 s, at the interval of every 100 s. Then, they were characterized and compared in 0.1 M PBS and 1 M H_2SO_4 solutions, respectively, by using cyclic voltammetry with AUTOLAB (Metrohm, PGSTAT302N). The potential window and scan rate were from −0.65 to +0.8 V and 100 mV s^{-1}, respectively.

Chronoamperometry was used to determine the cholesterol level by using CHI (CH-Instruments, CHI 750B). A three-electrode-cell configuration was used for the measurements, which used Pt-NC/enzyme/Nafion as the working electrode, Pt as the counter electrode, and Ag/AgCl (CH-Instruments, Inc.) as a reference electrode (ESI Fig. S2†). In order to optimize the specific applied potential, the prepared sensor was investigated and its current response with respect to 100 μM cholesterol was compared to various applied potentials and the current response to various interfering species.

2.5 SEM and AFM information

Scanning electron microscopy (SEM) images were obtained using a Teneo Volume Scope (FEI, Teneo). The Pt-NCs were electroplated onto the sputtered Pt electrode. The power level was 15 kV. The surface topography of Pt-NC was observed by using an atomic force microscope (AFM, XE-100 Park System, Korea).

2.6 Preparation of human saliva samples containing cholesterol

The human saliva samples were obtained from three hyperlipidemia patients who took medicine for the condition. All the experiments based on human samples were carried out in accordance with the ethical principles for medical research involving human subjects, as described by the World Medical Association and approved by the Korea Institute of Science and Technology (KIST) Review Board (approval number: 2017-031). Informed consents were obtained from human participants of this study. The saliva sampling was prepared by the following steps: (a) rinse the mouth with water, and wait for 2 min, (b) hold the saliva in the mouth with minimal swallowing, and (c) saliva in the mouth is placed in sterilized tubes of about 100 μl. Then, the samples were stored at 4 °C. Before conducting the experiment, 50 μl of the original saliva samples were diluted with 500 μl of 0.1 M PBS to adjust their concentrations, and this was followed by vortexing to mix the samples properly. In the amperometric test, the 1/10 diluted saliva sample was diluted further 10 times by injecting a buffer solution.

3. Results and discussion

3.1 Comparison of morphological, topographical, and electrochemical analysis for roughened Pt nano-clusters

According to the different electroplating times, morphological and topographical changes in the Pt-NC as the working electrode were observed by SEM and AFM analyses as shown in Fig. 2. And also, the cyclic voltammetry technique is used in a 0.1 M PBS and 1 M H_2SO_4 solution to check the properties of the sensor with Pt-NC. The changes in the roughness of the electrochemical surface were compared for each condition based on the charge delivery capacity (CDC) and the roughness factor (RF).

Fig. 2(A) shows that the grain size of the Pt-NC that was formed increased gradually and formed larger clusters based on the increment of the electroplating time. Fig. 2(B) shows the roughness of the Pt-NC electrodes that were fabricated under different electroplating conditions, and the roughness values associated with the five different conditions were 0.7 ± 0.05, 1.3 ± 0.05, 2.1 ± 0.04, 3.5 ± 0.25, and 4.8 ± 0.83 μm, respectively. This means that the effective surface area tended to increase, which indicated that the efficiency of the electrode's performance was getting better.

Fig. 2(C) confirms that the CDC of the Pt-NC electrodes that had different electroplating conditions increased gradually.

The following equation was used to calculate the charge delivery capacity (CDC):

$$\mathrm{CDC} = \frac{1}{\nu A}\int_{E_c}^{E_a} |i| \mathrm{d}E \quad (\mathrm{C\ cm^{-2}})$$

where ν is the scan rate (V s^{-1}), A is the geometrical surface area of the electrode (cm^2), E_a/E_c is the anodic/cathodic potential limit (V), i is the measured current, and E is the electrode potential (V *vs.* SCE). The CDC values for Pt-NC electroplating

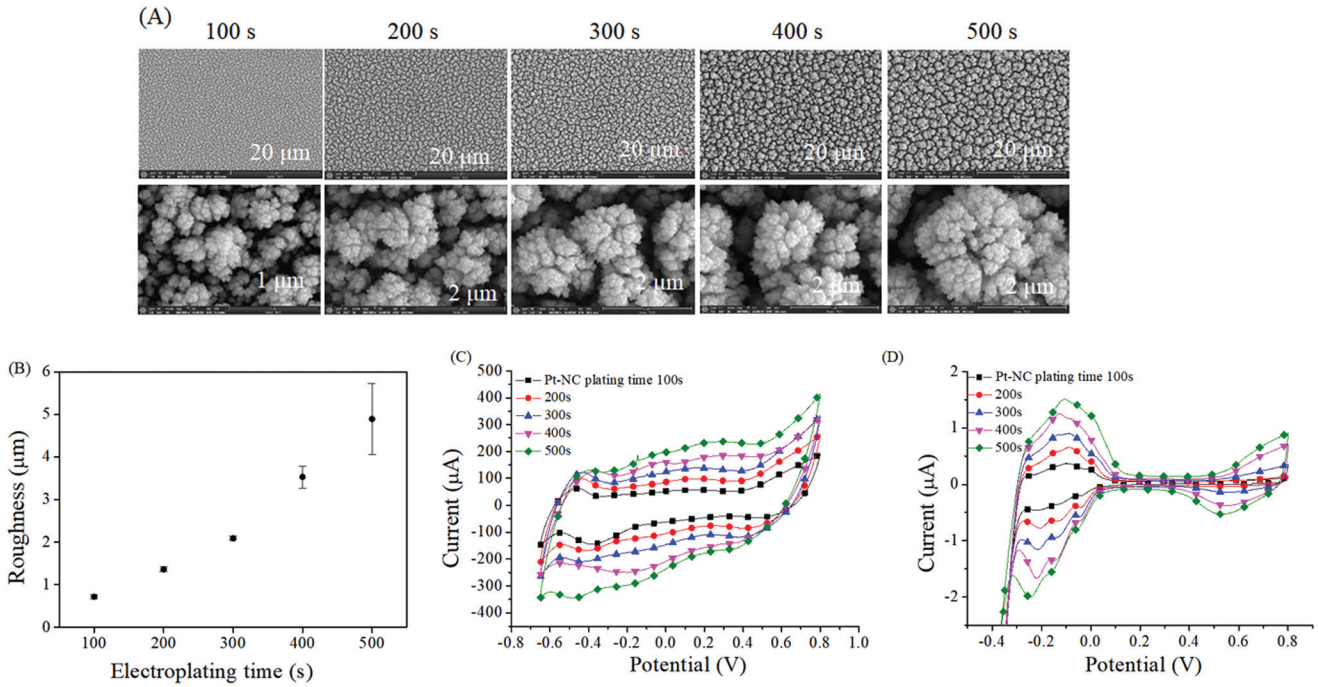

Fig. 2 (A) SEM images of a Pt nano-cluster for different electroplating times (100, 200, 300, 400, and 500 s); (B) the relationship between surface roughness and electroplating time by topographical analysis with AFM; (C) cyclic voltammograms in 0.1 M PBS and (D) 1 M H_2SO_4 for different electroplating times of 100, 200, 300, 400, and 500 s. The scan rate of 100 mV s^{-1} was applied.

times of 100, 200, 300, 400, and 500 s were 4.65, 6.97, 9.29, 11.02, and 16.6 mC cm^{-2}, respectively.[16]

Fig. 2(D) shows that the electrochemical RF of the Pt-NC was calculated using cyclic voltammetry in H_2SO_4.

The RF can be calculated as follows:[17]

$$f_r = A_r/A_g$$

where f_r is the calculated RF value, A_r is the real (true, actual) surface (interface) area, and A_g is the geometric surface (interface) area. The determination of the RF of the Pt-NC electrode was based on the formation of a hydrogen monolayer that was adsorbed electrochemically.

The respective RF values were 6.3, 13.04, 18.04, 23.09, and 32.3, which means that the actual surface area of the electrode increased proportionally with the increase of the electroplating time. The longer the electroplating time for forming Pt-NC, the larger the real activation area of the sensor.

The physical gap of 30 μm between the working electrode and the counter electrode could be electrically shorted by the over-plating of Pt-NC (ESI Fig. S3†), but this did not occur when the electroplating time was 500 s.

Since the surface area of Pt-NC is controlled by electroplating, the immobilized volume of the enzyme should be optimized to improve the performance of the sensor. Thus, we used SEM and amperometry to observe the surface of the electrode for different volumes for the enzyme.

Fig. 3 shows the SEM results of the Pt-NC cross-section under different electroplating conditions (100–500 s). Fig. 3(A) shows that the height of the Pt-NC increased as the electroplating time increased. The height at the electroplating time at 100 s was approximately 680 nm. When electroplating was performed for 200, 300, 400, and 500 s, the heights of the Pt-NCs were approximately 1400, 2100, 2800, and 3300 nm, respectively. Fig. 3(B) shows the immobilized enzyme, which had a volume of 3 μl on the surface of the Pt-NC. The Pt-NCs that were electroplated at 100, 200, 300, and 400 s were covered entirely by the enzyme. However, the Pt-NC that was electroplated for 500 s was not completely covered by the enzyme. Fig. 3(C) shows the immobilized enzyme, which had a volume of 15 μl on the surface of the Pt-NC. The Pt-NCs that were electroplated at 100, 200, and 300 s were covered completely with excess enzyme, and, when electroplating was done under 400 s conditions, the enzyme was well distributed on the Pt-NC. Also, the Pt-NC that had an electroplating time of 500 s was still not completely covered by the enzyme. We observed that the distribution of the enzyme depended on the structure of the Pt-NC and the volume of the enzyme.

3.2 Comparison of the morphological and electrochemical analyses of the enzyme/Pt nano-cluster

The electrochemical properties of the sensors with Pt-NC are expected to be suppressed if the enzyme is insufficiently distributed on the surface of the 500 s electroplated electrodes and to be overly distributed on the surfaces of the 100, 200, and 300 s electroplated electrodes. Thus, we concluded that 400 s of electroplating and 15 μl of enzyme immobilization were the optimal conditions.

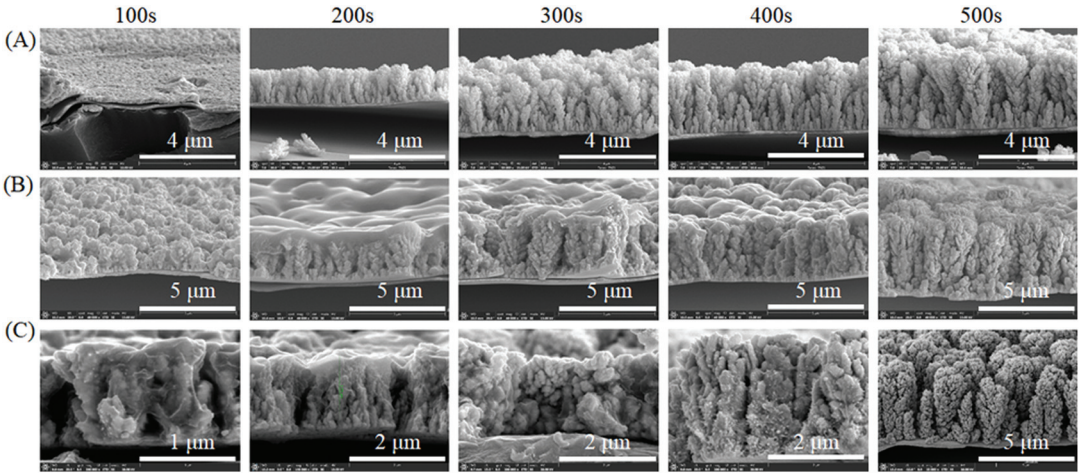

Fig. 3 (A) The cross sectional views for the Pt nano-clusters with different electroplating times; the cross sectional view for the enzyme immobilized Pt nano-cluster with an enzyme volume of 3 μl (B) and 15 μl (C), respectively.

Fig. 4(A) shows the current response to 100 μM cholesterol after immobilizing the enzyme volume of 3 μl on electroplated Pt-NC under different conditions. It was observed that the current response was less than 1 μA except for the Pt-NC that was electroplated for 200 s. The Pt-NC that was electroplated for 200 s had a current response of 1.25 μA. The SEM images in Fig. 3(B) show that the enzyme volume of 3 μl in the vertically formed Pt-NC seemed to be coated on the top surface of the Pt-NC, which reflected on a random current response irrespective of the expanded surface activation area by the Pt-NC structure.

Fig. 4(B) shows the amperometry response when the volume of the enzyme was 15 μl. The current responses for each electroplating condition were 1, 1.4, 2.1, 2.9, and 2.3 μA, respectively. Therefore, we chose the Pt-NC that was electroplated for 400 s as the optimal condition due to its high current response even though it had a relatively slow response time. The high current response originated from the well distributed, high volume enzyme in the vertically-formed Pt-NC, as shown in the SEM image of the 400 s condition in Fig. 3(C). However, the current response of the 500 s condition Pt-NC was not inferior to that of the 400 s condition Pt-NC. As shown in the 500 s conditions SEM image of Fig. 3(C), this was caused by the deficient immobilization of the enzyme under 500 s conditions of Pt-NC. Both an excess volume and a deficient volume of the enzyme may cause the degradation of the performance of the enzyme sensor. Also, a change in the structure of the surface of the electrode due to electroplating also may affect the immobilization of the enzyme. Thus, we used conditions in which 15 μl of the enzyme were immobilized on a 400 s electroplated Pt-NC surface.

3.3 Interference test

We investigated the influence of AA, UA, DA, glucose, and lactate, which commonly exist in salivary samples.[18] The sensor we fabricated was coated with a Nafion membrane, a negatively-charged polymer that prevents negatively-charged interference around the surface of the sensor. In addition, the optimal potentials for the sensor that was prepared with the Pt-NC/enzyme/Nafion were investigated because, if the applied potential is too high, it can induce a redox current due to other electroactive species in the samples.[19]

Fig. 5(A) shows the current response for various potentials applied to saliva that contained various biomolecules, such as cholesterol, AA, UA, DA, glucose, and lactate. We measured the

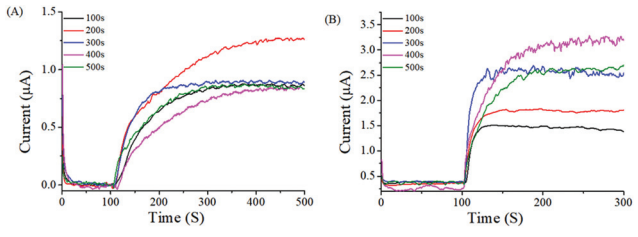

Fig. 4 (A) Amperometry response for the Pt nano-cluster (different electroplating times 100–500 s) with 3 μl (A) and 15 μl (B) of enzyme to the 100 μM total cholesterol under the conditions of continuous stirring in 0.1 M PBS.

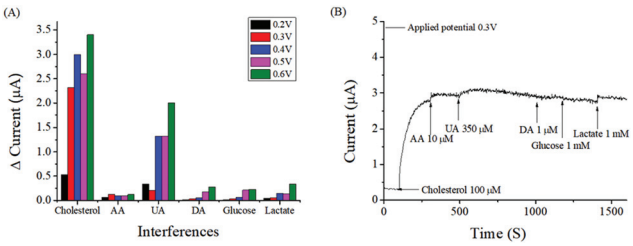

Fig. 5 (A) Effect of applied potential on the sensor response with Pt nano-cluster/enzyme/Nafion determined by amperometric experiment with 100 μM total cholesterol, 10 μM AA, 350 μM UA, 1 μM DA, 1 mM glucose, and 1 mM lactate in 0.1 M PBS. (B) Current response of the sensor with Pt nano-cluster/enzyme/Nafion at 0.3 V.

change in current that occurred for 100 μM cholesterol, 10 μM AA, 350 μM UA, 1 μM DA, 1 mM glucose, and 1 mM lactate by increasing the applied potential from 0.2 to 0.6 V.[20] In general, the average concentration of cholesterol in saliva is known to be about 120 μM,[7] and the concentration of AA in saliva is about 4 μM.[21] The AA measurement was performed with the higher concentration. The concentration of UA in saliva is about 350 μM,[22] which is the normal existing level in saliva. The concentration of glucose in saliva is about 80 μM.[23] The glucose measurement was performed at much higher levels than salivary levels. The concentration of lactate in saliva is about 350 μM.[24] The lactate measurement was performed at concentrations of lactate that were higher than the concentrations in saliva. We confirmed that the current response of cholesterol and interfering species increases when the applied potential increases. When the applied potential was increased from 0.2 V to 0.6 V at intervals of 0.1 V, the current responses for cholesterol were 0.53, 2.32, 3, 2.6, and 3.4 μA, respectively, as shown in Fig. 5(A). The largest current response among the interference was UA. Although the current response of UA was 58% of the current response of the cholesterol at 0.6 V, the current response of UA at 0.3 V was decreased. These results indicated that a high applied potential is not appropriate for the detection of cholesterol because the redox current of interference could influence the current response of cholesterol. Since the redox currents for AA, DA, glucose, and lactate at higher applied potential would be increased, lower applied potential can help to suppress the current caused by the interfering species. However, if a lower potential is applied, it can cause poor sensitivity to the detection of cholesterol. Thus, we selected 0.3 V as an optimal potential for the Pt-NC sensor.

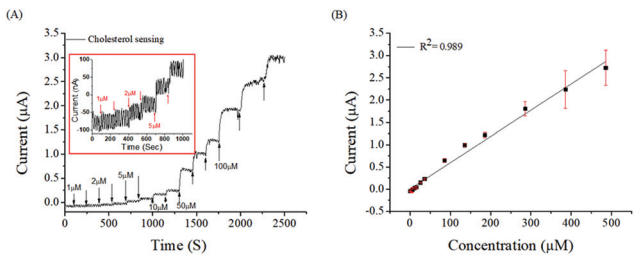

Fig. 6 (A) Amperometric response for sensors with Pt nano-cluster/enzyme/Nafion to the consecutive addition of total cholesterol under the conditions of continuous stirring in 0.1 M PBS buffer. (B) Calibration curve fitting.

Fig. 5(B) shows the amperogram that resulted from the interference test using an applied potential of 0.3 V. It was confirmed that the current responses of other interference species, including UA, are suppressed effectively. The relatively large current response of the UA (350 μM) was less than 8.6% of the current response of cholesterol, *i.e.*, 100 μM. The current responses of AA, DA, glucose, and lactate were only 5, 1.2, 1.2, and 2.1%, respectively, of the current response of cholesterol.

3.4 Amperometric measurements of total cholesterol

For the determination of cholesterol in saliva, the sensor must have high sensitivity and a linear response in the dynamic range, because the amount of cholesterol in saliva is known to be in the range of 0.1% to 1% of the amount in blood.

Fig. 6 shows the performance of the sensor in view of the dynamic range and linearity. Fig. 6(B) shows that the sensor with Pt-NC/enzyme/Nafion had a sensitivity of 132 μA mM^{-1} cm^{-2}, and an LOD of 2 μM in the range of 2–486 μM.

Table 1 compares our results with the results of previous researchers. A variety of materials were used for the detection of low levels of cholesterol. The GCE/MoS_2-AuNP/ChOx sensor had a very high sensitivity of 4460 μA mM^{-1} cm^{-2} and an LOD of 0.26 ± 0.015 μM. However, it had a narrow linear range of 0.54–48 μM, which does not cover the range of salivary cholesterol. The sensor with GR/TI(GR)/3DNS/CS/ChOx had a wide linear range of 50–8000 μM.[25] However, this linear range did not match with the clinical range of salivary cholesterol, and this sensor also had low sensitivity. Although the sensor with GR/CBNP/ChOx/GA had an LOD of 0.4063 μM,[13] it had low sensitivity and a very narrow linear range to measure the cholesterol in saliva. The sensor with GCE/AgNPs had a wide linear range of 100–2000 μM, but it could not detect the low levels of cholesterol in saliva.

As shown in previous studies, GCE is an electrode that is commonly used for measuring cholesterol. It has a rather low oxidation rate and a rather high chemical inertness.[12] Although it is appropriate for electrochemical measurement, there are still huge problems for commercializing this electrode due to its excessive cost and the difficulty of modifying it. In addition, the GCE causes an overpotential in the reaction with the biomolecule to be detected, which leads to problems with the selectivity and reproducibility of the sensor, and, as a result, it is difficult to measure biomolecules with good sensitivity.[26] In order to overcome these problems, 2d material or

Table 1 Comparison of modified electrodes with different materials for cholesterol detection

Electrode	LOD (μM)	Linear range (μM)	Sensitivity (μA mM^{-1} cm^{-2})	Ref.
GCE/MoS_2-AuNP/ChOx	0.26 ± 0.015	0.54–48	4460	1
GR/Ti(GR)/-3DNS/CS/ChOx	6	50–8000	3.82	25
GR/CBNP/ChOx/GA	0.4063	2.5–27.5	1490	13
GCE/AgNP	25.8	100–2000		26
Pt-NC/enzyme/Nafion	2	2–486	132	This work

GCE: glassy carbon electrode, GR: graphene.

metal nanoparticles have been used to modify the surface of the electrode.[15] The 2d materials, such as MoS_2, have been used extensively in recent sensor applications. MoS_2 has a flake-like structure, and it has a large surface area when processed on the surface of the electrode, which has the advantage of increasing the sensitivity of the sensor. However, a single layer of MoS_2 has poor conductivity and an inferior electron transfer rate. Therefore, there is the disadvantage that MoS_2 must be mixed with other materials or used in a reduced form.[25] Furthermore, although the high sensitive non-enzymatic cholesterol sensors, such as the sensor using Pt nanoparticles assembled on CNT[27] or Au–Pt hybrid on ZnO nanorods,[28] showed good properties for practical use, they are still not free from relatively complex electrode fabrication processes and selectivity issues for real samples. Conversely, the disposable sensor with the modified Pt-NC that we used in this work is easy to fabricate simply by controlling the electroplating time and the volume of the enzyme, which will provide reliable and stable detection properties.

3.5 Salivary cholesterol measurements of hyperlipidemia patients

The fabricated and optimized sensor with Pt-NC/enzyme/Nafion was investigated to determine its feasibility for analyzing human saliva. Saliva samples were collected from 3 hyperlipidemia patients.

Fig. 7(A) represents the result of the current response based on the patients' saliva samples. The current response obtained from each patient's sample had a similar slope between the 1/100 dilution and 1/10 dilution intervals due to the viscosity control of the saliva and the buffer-based amperometric measurement. The values of the slopes of patients 1, 2, and 3 were 1×10^{-6}, 1.16×10^{-6}, and 1×10^{-6}, respectively. Since the slopes of the measurements were similar, the performance of the sensor was considered to be similar. Fig. 7(B) shows that the estimated cholesterol concentration was obtained by substituting the current response obtained with the 1/10 dilution into the calibration curve (Fig. 6B) made by using standard cholesterol. The estimated cholesterol levels for patients 1, 2, and 3 were 520, 290, and 460, respectively.

The commercially-used blood-based cholesterol kit can measure cholesterol in the mM range. In order to detect salivary cholesterol at the 10 μM level and to conduct diluted sample measurements due to viscosity control, a sensor is required that is simple and easy to fabricate and has high sensitivity.

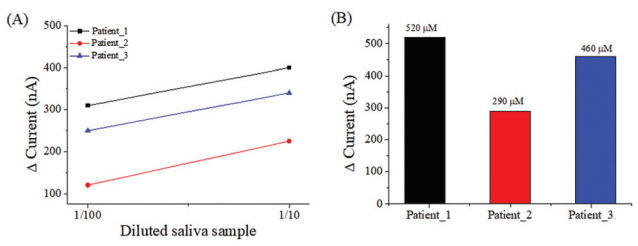

Fig. 7 (A) Current responses for sensors with Pt nano-cluster/enzyme/Nafion in a patients' saliva sample by an amperometric technique in 0.1 M PBS. (B) Comparison of the standard curve to the measured data in saliva cholesterol.

Table 2 Comparison of the pre-determined cholesterol level in a sample of human blood and saliva and the measured cholesterol level by using our sensor in human saliva samples

Sample No.	Blood (μM)	Saliva (μM)	Saliva (μM)
Patient 1	3950	129	520
Patient 2	2920	<103	290
Patient 3	4800	206	460
Assay	Commercial kit	Commercial kit (NA)	Sensor of this work

NA: Not available.

The results presented above indicate that this approach can be used to determine the cholesterol in saliva immediately without having to pretreat the saliva. To analyze the amount of cholesterol in saliva more precisely, an additional process was required, *i.e.*, the use of a calibration curve that shows the detection of different cholesterol concentrations. We used a commercially-available assay kit at Green Cross in Korea (Roche cobas 8000 C702 modular analyzer) to determine the concentration of total cholesterol in samples of human serum and saliva. We were unable to measure the concentration of human saliva in this analyzer due to the detection limit of the assay kit. Table 2 shows the pre-determined cholesterol level in a sample of human blood and saliva and the level of cholesterol measured by using our sensor in human saliva. The cholesterol level in saliva was about 10^{-1} less than the level in the human blood.

The stability of the sensor is one of the most important issues for minimizing the degradation of the original signal. This degradation occurs continuously with time for the lifetime of the enzyme, and the molecules are progressively escaping from the interface of the electrode. Thus, we conducted a sensor stability test, as shown in ESI Fig. S4.† The current responses to 120 μM cholesterol were recorded periodically (once a week) and compared. The sensors we prepared were stored in 0.1 M PBS (pH 7.4) at 4 °C, and, after three weeks, the current response of these sensors was 79% compared with the initial results (day 1). These results showed that the rough surface of the coated Pt-NC enhanced the stability by enhancing the active sites on the electrode for trapping the enzyme.

4. Conclusions

In this study, we fabricated, optimized, and tested a sensor with Pt nano-cluster/enzyme/Nafion for the high sensitive determination of cholesterol in saliva. The cholesterol sensor, fabricated with Pt nano-cluster/enzyme/Nafion as the working electrode, had a large surface RF value and a vertical structure that allowed high-volume loading of the enzyme. However, excessive loading of the enzyme decreased the sensing pro-

perties of the device. Therefore, the optimal volume loading of enzyme onto the vertical structure of a Pt nano-cluster resulted in the detection of low concentrations of cholesterol. In addition, the cholesterol sensor with Pt nano-cluster/enzyme/Nafion could detect cholesterol specifically without interfering toward AA, UA, DA, glucose, and lactate. The sensor exhibited a sensitivity of 132 μA mM^{-1} cm^{-2} and an LOD of 2 μM in the linear range of 2–486 μM. The sensor could provide meaningful information concerning the low concentration of cholesterol in saliva.

In the near future, the sensor will be investigated with more samples of saliva and the measured results will be compared with the serum cholesterol level. The correlation between serum cholesterol and saliva cholesterol levels will be demonstrated more clearly, and a non-invasive screening tool will provide a meaningful database for hyperlipidemia patients.

Conflicts of interest

There are no conflicts to declare.

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