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# Detection of Skin Cholesterol by a Molecularly Imprinted Electrode

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## Abstract

Our research goal is based on the acquisition of bio-information from the analysis of skin components by using a simple test. Controlling the total cholesterol level is important for preventing lifestyle-related diseases. Approximately 11% of the body's cholesterol is present in the skin and is equal to the percentage found in the blood. Therefore, we focused on a simple and non-invasive method of measuring cholesterol by using a solvent to extract the skin component. The extracted solution was analyzed using high-performance liquid chromatography (HPLC) and a molecularly imprinted self-assembled monolayer (SAM) sensor. The molecularly imprinted SAM electrode had high responsiveness and sensitivity, which were attributed to its strong affinity toward hydrophobic cholesterol due to the presence of a long side chain and the complementary structure of the cavity. The subject with the highest skin cholesterol level showed higher blood cholesterol concentration, while the subject with the lowest skin cholesterol level showed the lowest blood cholesterol concentration. Since the coefficient of correlation of the non-invasive method using the SAM electrode and the invasive conventional method was 0.9408, it was concluded that the former method had great potential for practical use.

**Keywords:** skin cholesterol, electrochemical detector, HPLC

## 1. Introduction

Hypercholesterolemia, well known as a risk factor of arteriosclerosis and angina, is a lifestyle-related disease. The end products of cholesterol utilization are bile acids, which are synthesized in the liver. While the synthesis of bile acids is one of the main mechanisms underlying the excretion of excess cholesterol, excretion in this form is nonetheless insufficient to compensate for excess dietary intake of cholesterol.[1–3]

For centuries, physicians have employed many simple non-invasive methods for evaluating physical parameters in order to assess body function in health and disease. A non-invasive medical procedure does not penetrate or break the skin, for example, examinations for temperature, pulse, and blood pressure; auscultation of heart sounds; palpation; and changes in body weight. In contrast, biochemical examinations of blood and serum chemistry provide more beneficial information, such as blood sugar, blood erythrocyte, leucocyte, and total cholesterol levels. While collecting blood is important, the conventional method is inconvenient due to the necessity of going to a hospital for blood collection via invasive means and of using enzyme reactions for many procedures. A recently developed minimally invasive technique of blood glucose self-measurement that has reduced the frequency of medical examinations has become very popular internationally.

Control of the total cholesterol level in the body plays an important role in preventing lifestyle-related diseases. Approximately 11% of the body's cholesterol is present in the skin and is equal to the amount in the blood. Therefore, we focused on the measurement of skin cholesterol in order to develop a simple and non-invasive measurement technique involving high-performance liquid chromatography (HPLC) and a molecularly imprinted self-assembled monolayer (SAM) electrode.

A number of molecularly imprinted polymeric materials have been developed during the past decade.[4–7] These polymeric materials can recognize target molecules by using cavities with shapes that are complementary to those of the target molecules and have many applications in the recognition of biological compounds, drugs, and agrochemicals.[8–12] In particular, this method is useful for the detection and separation of inactive substances, i.e., hormones; bile acids, which have steroid backbones; and endocrine disrupters. The method is based on the complementary shape of a cavity, which relies heavily on the specific shape of a target, without recourse to chemical reactions. Recently, we reported that overoxidation of polypyrrole (PPy) can create a cavity that has a shape complementary to that of a dopant. Further, overoxidized PPy (OPPy) has an important advantage over previously reported polymers since a complementary cavity can be easily created by the extraction of an anionic template molecule via overoxidation (dedoping). OPPy films and colloids show high enantioselectivity toward amino acids.[13–16] This excellent enantioselectivity can be explained by the cavities, which are created by the dedoping process (extraction of a dopant) that occurs concomitantly with the irreversible overoxidation of PPy films and colloids. However, the cholesterol molecule has no potential as a dopant for the synthesis of an electric polymer and is an electroinactive species in a range of the potential window of the commonplace electrodes, for example, electrodes made of noble metals (gold and platinum) and metaloxides, excluding glassy carbon and diamond electrodes.[17] Therefore, we created a cavity with a shape complementary to that of cholesterol by using SAM instead of PPy matrix. Furthermore, we explored the molecular recognition property of molecularly imprinted SAM in order to evaluate its applicability as a sensing material for determining skin cholesterol by using ferrocyanide [$K_4Fe(CN)_6$] as a redox marker.

## 2. Experimental

### 2.1. Reagents

Cholesterol, squalene, oleic acid, and triolein were purchased from Wako (Japan). Cholesterol oxides, such as cholesterol 5α, 6 α-epoxide, and 7-ketocholesterol were purchased from Sigma. Stearylmercaptan; cholesterol esters such as acetate, n-caprylate, and linoleate; and bile acids such as cholate, chenodeoxycholate, deoxycholate, and dehydrocholate used were of reagent grade and purchased from Tokyo Chemical Industry (Japan). Milli-Q (Millipore) water was used throughout (>18.3 M ohm cm).

2.2. Non-invasive extraction of cholesterol from skin

Sampling for analysis was carried out only after obtaining consent from the test subjects. Informed consent, mentioned here, is the subject's right to be presented with sufficient information, by either the researchers or their representatives, to allow the subject to make an informed decision regarding whether or not to consent to a treatment or procedure. Express consent is what is normally referred to by consent, or when the subject directly consents via words, either written or verbal. First, a plastic cup (bore diameter: 25 mm, capacity: 8.0 mL) was filled with 0.5 mL ethanol. The lid-capped cup was held in the palm, keeping the ethanol on the palm for 60 s. The molecularly imprinted SAM electrode was immersed in the extracted solution and then used for electrochemical measurement.[18]

2.3. HPLC methods

Chromatographic analysis was carried out on an Agilent 1100 Series HPLC system, equipped with a diode array ultraviolet detector. Samples for HPLC (30 μL) were injected onto a Luna 5μ $C_{18}$ column (150 mm × 4.6 mm; Phenomenex) equilibrated with a mixture solvent (50% acetonitrile and 50% propanol) at a flow rate of 1.2 mL $min^{-1}$. Absorbance was monitored at 212 nm.

2.4. Preparation of molecularly imprinted SAM electrode

A molecularly imprinted SAM electrode consisting of stearylmercaptan was designed using cholesterol as a template molecule on a gold electrode as follows. A gold disk electrode (diameter: 1.0 mm) was mechanically polished and then electrochemically washed in 0.1 M $H_2SO_4$ aqueous solution by cycling the potential between –0.20 and +1.3 V (vs. Ag|AgCl) at 20 $mVs^{-1}$ up to be given a typical voltammogram. The electrode was rinsed well with water and then immersed in an ethanol solution containing 20 mM cholesterol and 0.1 mM stearylmercaptan for 30 min. The resulting electrode was immersed in ethanol for 3 h in order to wash and extract cholesterol as a template molecule. The extraction of cholesterol molecules created complementarily shaped cavities on the SAM electrode. Finally, we were able to obtain a molecularly imprinted SAM electrode that had cavities with a shape complementary to that of cholesterol, as shown in Fig. 1.

**3. Results and discussion**

The extracted ethanol solution included components such as cholesterol, cholesterol oxides, and squalene, which is a well-known precursor of cholesterol, while other cholesterol derivatives were not observed (Fig. 2). Therefore, we focused on cholesterol in the extracted solution and attempted to develop an electrochemical sensing method by using a molecularly imprinted SAM electrode.

We used ferrocyanide as a redox marker due to its electrochemical activity. Negligible response was obtained with modified gold electrode due to the fact that their surface is completely enclosed by electroinactive species, namely, stearylmercaptan and cholesterol as shown in Fig. 1. When the cavity recognizes the cholesterol molecule, the redox marker does not diffuse to the electrode surface, and the electro electrochemical signal then decreases. In contrast, when the cavity extracts cholesterol, the redox marker diffuses to the electrode, and the signal then recovers. Voltammograms at each step of the preparation and sensing are represented in the inset of Fig. 3. The redox peak recovered, as represented by curve c, after washing with ethanol in order to extract cholesterol. A decrease in oxidation peak current was observed in curve d, indicating cholesterol recognition. Therefore, the response ($\Delta I$) was normalized using the following formula:

$$\Delta I = I_0 - I_r \qquad [1]$$

$I_0$ and $I_r$ are the oxidation peak currents of ferrocyanide at the molecularly imprinted SAM electrode before and after the recognition of cholesterol, respectively. The current is related to the cavity concentration for the mass-transport of the redox marker on the molecularly imprinted SAM. When the SAM electrode recognized cholesterol, the current decreased due to the absence of marker diffusion to the electrode surface. In contrast, when the SAM electrode extracted cholesterol, the marker diffused to the electrode surface, and the current increased.

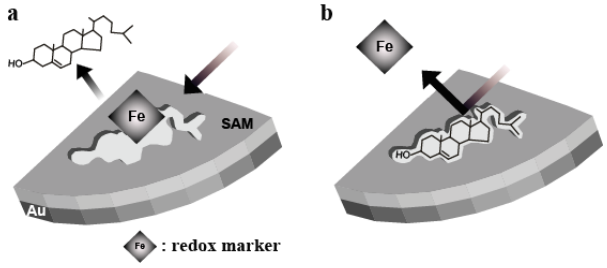


**Fig. 1** Illustration showing the cholesterol-recognition mechanism of the molecularly imprinted SAM electrode during the extraction (a) and uptake (b) processes.

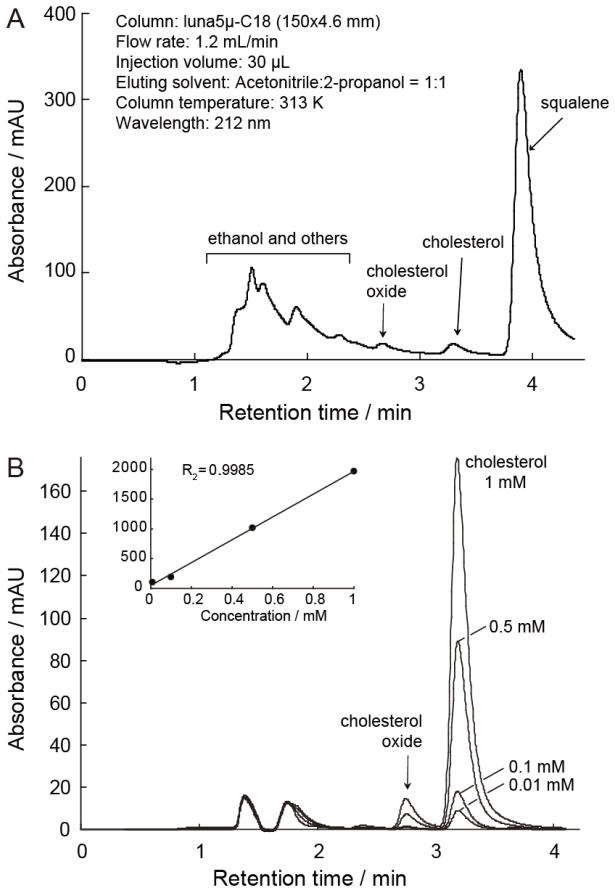


**Fig. 2** HPLC of extracted components (A) and cholesterol (B).

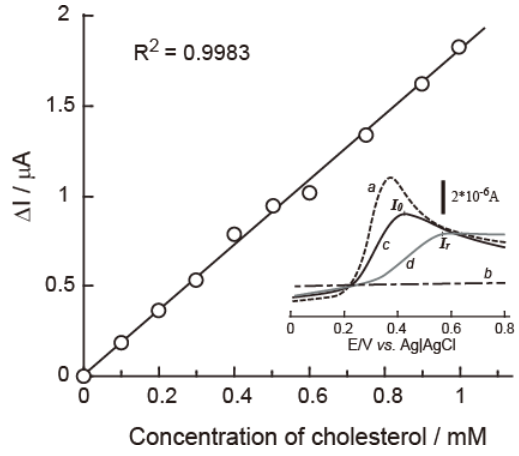


**Fig. 3** Dependence of the Δ*I* on the cholesterol concentration. The inset shows cyclic voltammograms for a bare gold electrode (a), a modified electrode (b), and a molecularly imprinted SAM electrode before (c) and after immersion in ethanolic 3 mM cholesterol solution (d). The electrolysis solution contained 5 mM $K_4Fe(CN)_6$ and 50 mM $KClO_4$ as background electrolytes.

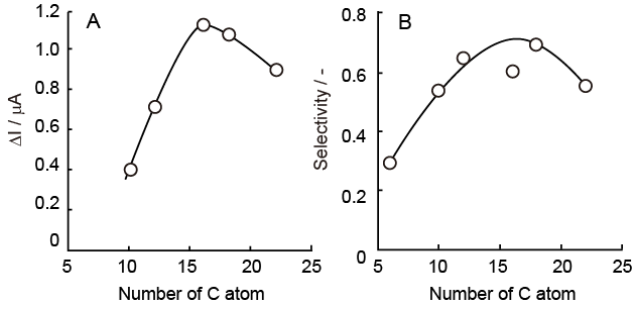


**Fig. 4** Dependences of the Δ*I* (sensitivity) (A) and the selectivity of the SAM, Δ*I*(cholesterol)/[Δ*I*(cholesterol) + Δ*I*(cholesterol acetate)] (B) on the number of carbon atom in the alkylthiol.

Fig. 3 shows the plot of Δ*I* versus the concentration of cholesterol, which exhibits a linear relationship even at low concentrations. The coefficient of correlation was excellent (0.9983). Moreover, this sensor had a rapid response, and the complete response time was under 30 s.

In the view points of sensitivity and selectivity, the length of the used alkylthiol for the SAM formation were optimized, as shown in Fig. 4. Although an increase in the number of carbon atom increases the response (Δ*I*), the highest response obtained around 16 of the number of carbon, and the Δ*I* decreases with an increase of the number of carbon over 18. On the contrary, the SAM modified electrode, which was prepared by using shorter alkylthiols less than 10 of the number of carbon, represented distinct oxidation peak currents of ferrocyanide without extracting cholesterol molecules. This means that the formation of the SAM by shorter thiol allows the diffuse of a redox marker to the gold electrode surface due to the thickness and the low dense of thiol molecules on the electrode. Also the selectivity increases with an increase in the number of carbon, the high selectivity was obtained around 18. This is attributable to the intermolecular interaction (van der Waals interaction), the longer alkylchain is stronger than those of the shorter one. On the contrary, the longer chain which is more than the molecular length of cholesterol may have control over the extraction of cholesterol and keep it in the SAM due to the larger size and stronger hydrophobic interaction, therefore, the Δ*I* and selectivity have decreased in Fig.4.

Fig. 5 demonstrates the procedure for measuring cholesterol, using the electrochemical SAM and HPLC methods. First, a plastic cup was filled with ethanol. The lid-capped cup was held in the palm, keeping the ethanol on the palm for 60 s. The SAM electrode was then immersed in the extracted solution and subsequently used for electrochemical measurement

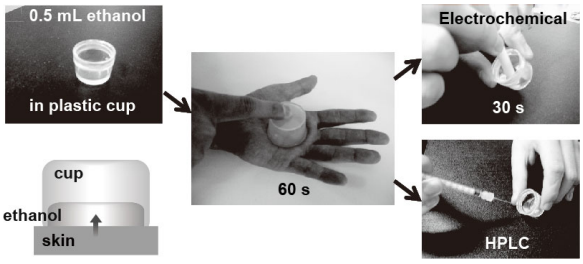


**Fig. 5** Procedure of extracting cholesterol from the skin for the HPLC and electrochemical measurements.

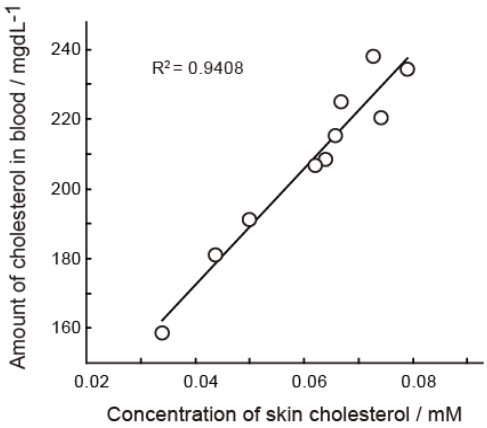


**Fig. 6** Coefficient of correlation of actual examinations of skin and blood cholesterols.
and HPLC analysis.

We attempted to use the SAM electrode for measuring skin cholesterol levels and compared the results to those of a clinical test; these results are shown in Fig. 6. Samples were collected from 5 persons for determining skin cholesterol levels, using SAM. The same subjects then underwent invasive catheterization—the test currently used to measure blood cholesterol. The subject with the highest skin cholesterol level (0.079 mM) showed higher blood cholesterol concentration (234 mg $dL^{-1}$). Moreover, the subject with the lowest skin cholesterol level (0.034 mM) showed the lowest blood cholesterol concentration (159 mg $dL^{-1}$). Since the coefficient of correlation of the non-invasive method using the SAM electrode and the invasive conventional method was 0.9408, it can be concluded that the former method has great potential for practical use.

**4. Conclusion**

The extracted solution included cholesterol, cholesterol oxides, and squalene; it did not contain other cholesterol derivatives. The high response and sensitivity of the SAM electrode can be attributed to its strong affinity toward hydrophobic cholesterol due to the long side chain and complementary structure of the cavity. The subject with the highest skin cholesterol level showed higher blood cholesterol

concentration. Further, the subject with the lowest skin cholesterol level showed the lowest blood cholesterol concentration. Since the coefficient of correlation of the non-invasive method using the SAM electrode and the invasive conventional method was 0.9408, it was concluded that the former method had great potential for practical use. It is expected that the molecularly imprinted SAM electrode built onto an electrochemical detector will be effective analytical procedure by combination with the flow injection system.

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