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ORIGINAL PAPER

Enzyme sensor for simultaneous determination of cholesterol, triglyceride and HDL cholesterol in serum

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Abstract

A three-electrode lipid biosensor that simultaneously measures the total cholesterol (CHO), triglyceride (TG), and HDL cholesterol (HDL-C) in standard serum has been developed. The lipid biosensor is designed for clinical use where production cost, low sample requirement, portability, stability, and speed are high priorities. The device design filters out blood cells and lipoproteins from the serum, where the target molecules are catalyzed by enzymes encapsulated in carboxymethyl cellulose (CMC) to produce electron mediators, potassium ferrocyanide. These electron mediators were subsequently detected by amperometric determination. The sensor exhibit high selectivity towards the targets and can measure the target lipids in 4 min with 10 µL of serum. Lastly, the device can be stored up to 18 months with a minimal decrease in catalytic efficiency.

Keywords Enzyme sensor · Cholesterol · Triglyceride · HDL cholesterol

Introduction

Blood analysis using enzyme biosensors has been an essential technique for clinical tests [1–3]. In diabetes treatment, the quality of life of diabetic patients has been dramatically improved by the invention of self-monitoring blood glucose (SMBG) sensors. Since then, variations of enzyme-based glucose sensors have been proposed [4, 5], at times coupled with investigations into their adaptability to point-of-care use. Recently, the development of sensors using crystal oscillators [6] or waveguide spectroscopy [7] has been promising, but they have not yet been put into practical use because of the need for miniaturization and cost reduction. In that regard, electrochemical biosensors are great alternatives since they produce reliable and reproducible measurements on relatively small, lightweight devices. In particular, amperometric biosensors leveraging immobilized enzyme electrode offer added benefits of high sensitivity, high selectivity, and short response time without requiring substantial or complex instrumentation, making enzyme-coupled electrochemical sensors a promising method to quantify target substances [8–11].

This investigation focuses on adapting enzymatic sensors to measure blood lipid concentrations. In recent years, there has been an increased focus on on-site determining blood lipid concentration as an indicator predicting coronary atherosclerotic disease. Of the blood lipids to monitor, low-density lipoprotein (LDL) concentration is a valuable metric as it promotes arteriosclerosis, which increases the likelihood of myocardial and cerebral infarction. In addition, oxidized LDL damages the blood vessels walls and impairs the vasodilatory effects of healthy blood vessels. However, currently the most accurate way to determine the concentration of LDL cholesterol in blood is to use the Friedewald equation, where total cholesterol, HDL cholesterol, triglyceride concentration are used to measure the LDL cholesterol concentrations. High-density lipoprotein (HDL) plays an essential role as an antiatherogenic marker in the reverse cholesterol transport pathway [12] where it inhibits the low-density lipoprotein (LDL) oxidation [13]. Furthermore, HDL encourages anti-inflammatory activity by inhibiting the production of inflammatory

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cytokines. Those induce expression of vascular cells and intracellular adhesion molecules on the coronary vascular [14]. Therefore, decreased blood level of HDL is an indicator for increased risk for developing metabolic syndrome, atherosclerosis, and coronary heart disease [15, 16]. Current clinical methods approximate the serum concentration of actual HDL particles by indirectly measuring cholesterol bound to HDL (HDL-C) in the serum [17].

These tests are primarily used for clinical diagnosis in hospitals where large sample volumes can be processed. In recent years, the focus shifted to developing rapid tests aimed to support the treatment plan for dyslipidemia, test for the effectiveness of medication, and monitoring effects of lifestyle changes. For these purposes, a simplified device that samples blood from the finger tips instead of venous blood drawl is more suitable. However, there are many challenges to consider for the sensor:

1. Blood cells within the blood cells interfere with the cholesterol and triglyceride measurement system.
2. Other lipoproteins interfere with the measurement of the HDL cholesterol.
3. It is necessary to design a sensor that could complete these processes at the same time using a small amount of blood.

To meet the clinical demands, we have developed a blood cell and non-HDL lipoproteins separation system using an electrochemical sensor capable of detecting HDL, triglyceride and total cholesterol.

In this study, the measurement sample was preprocessed concurrently by two methods: For cholesterol and triglyceride measurements, blood cells were filtered out from the sample as it flows through the device flow path, leaving the serum containing the lipoproteins to be measured at the electrode. Other lipoproteins and interfering substances except HDL were removed for HDL detection and measurement on a parallel flow path. By selecting an electrochemical method, low signals generated can be detected immediately over a low sample volume, decreasing the wait time for overall results, as speed is an essential factor for point-of-care testing. The sensor outputs concentrations of total cholesterol, triglyceride, and HDL cholesterol in 3.5 min using only 0.01 mL of serum. This sensor setup provides new usefulness for blood lipid measurements in clinics where rapid and low-cost tests are prioritized.

Experimental

Materials

Polyethylene naphthalate film was purchased from Teijin Limited, Japan. Double-sided adhesive tape and adhesive tape were purchased from LINTEC, Japan. Polyethylene terephthalate film was purchased from Toray Industries, Inc., Japan. Glass microfiber filters and microporous membranes were purchased from Whatman, UK. Standard serum for lipid measurement JCCRM223 was purchased from Health Care Technology Foundation, Japan. The concentrations and deviations listed in its "certificate" were used in the experiments. Cholesterol: 191 ± 2.5 mg/dL 215 ± 2.5 mg/dL 260 ± 3 mg/dL, Triglycerides: $88 + 3/-2$ mg/dL $128 + 4/-3$ mg/dL 287 ± 6 mg/dL and HDL cholesterol: 44 ± 1 mg/dL 57 ± 1.5 mg/dL 60 ± 1.5 mg/dL. Lipoprotein lipase, cholesterol esterase, glycerol kinase, and glycerol-3-phosphate oxidase were purchased from TOYOBO, Japan. Cholesterol oxidase was purchased from Amano Enzyme, Japan. Microgranular cellulose was purchased from Whatman, UK. Carboxymethyl cellulose (CMC) (MW: ca.10,000) BSH-10 was purchased from DKS Co. Ltd., Japan. All other reagents were of analytical grade.

Sensor chip and apparatus

The lipid biosensor was designed to measure cholesterol, triglyceride, and HDL cholesterol concentrations simultaneously in a sample (Fig. 1a). The sensor was assembled by combining the flow path part of the sample solution and the flexible printed circuit supporting the reaction layer. The sensor layers are shown in Fig. 1b. Blood enters the sensor's tip by capillary action. Glass fiber is placed in the middle of the flow pathway to separate the red blood cells. The serum continues to pass through the flow pathway towards the electrodes, encouraged using a vacuum pump at the opposite end of the chip. The serum continues to pass through the flow pathway and reaches the electrodes.

The flow path part of the sample solution and lipoprotein aggregation membrane for removing lipoproteins other than HDL.

Figure 1b shows the flow path of the sample solution formed by stacking a 0.125 mm thick polyethylene terephthalate film, a 0.15 mm thick adhesive tape, a 0.3 mm thick double-sided adhesive tape, a 0.125 mm thick polyethylene terephthalate film, a 0.725 mm thick double-sided adhesive tape, a 0.06 mm thick double-sided adhesive tape, a 0.125 mm thick polyethylene terephthalate film and a 0.1 mm thick double-sided adhesive tape, in order. The size of the sensor strip is 10×40 mm and the diameter of the working electrodes is 0.6 mm. The total volume of the space for the working electrode is approximately 2μL.

The device features two channels: one for sending the sample solution to the cholesterol measuring electrode and the triglyceride measuring electrode, and the other is for sending the sample solution to the HDL cholesterol measuring electrode. Glass microfiber filters with the purpose of filtering out large particles from the serum sample were

placed in each channel, with one filter carrying a lipoprotein flocculating solution containing 0.01 mg sodium dextran sulfate, 0.1 mg magnesium chloride, and 0.02 mg sodium chloride. Microporous membranes were placed under the glass microfiber in each channel to filter the particles in the sample. In Fig. 1b, the vertical arrows indicate the sample flow direction. As the sample moves downwards, it reaches the electrode surface to start the enzymatic reaction.

Electrodes

Figure 2 shows the structure of the flexible printed circuit. The electrodes on the polyethylene naphthalate film consist of six working electrodes and two counter electrodes. The surface of the working electrode is formed with gold plating, and the counter electrode is covered with a mixture of silver and silver chloride. The electric circuit was covered with an insulation layer deposited by photolithography except for six working electrodes, two counter electrodes, and terminals. The functions of the six working electrodes are as follows (Fig. 2):

(i) Measurement of HDL cholesterol.
(ii) Measurement of interfering substances around the working electrode for HDL cholesterol measurement.
(iii) Measurement of triglyceride.
(iv) Measurement of interfering substances around the working electrode for triglyceride measurement.
(v) Measurement of cholesterol.
(vi) Measurement of interfering substances around the working electrode for cholesterol measurement.

Figure 3 shows a cyclic voltammogram of the working electrode in 0.5 M potassium chloride solution containing 10 mM potassium ferricyanide. The potential of the electrode is controlled using Hokuto Denko HA-501 potentiostat and Hokuto Denko HB-104 function generator, scanned at a rate of 100 mV/s. The cyclic voltammogram shows that the electrical properties of this working electrode are equivalent to the gold disk electrode.

The Ag / AgCl layer was screen-printed on the gold layer to form two reference electrodes. Ag / AgCl electrode being placed near the working electrode iii), iv), v) and vi) is used for cholesterol and triglyceride measurement, and Ag / AgCl electrode being placed near the working electrode i) and ii) is used for HDL cholesterol measurement. A terminal for connecting working or reference electrodes to the equipment was formed on the edge of the electric circuit. Two holes were made in the polyethylene naphthalate film to connect the channels to the air pump. Finally, photoresist ink was layered over the exposed gold electrodes, with the exception

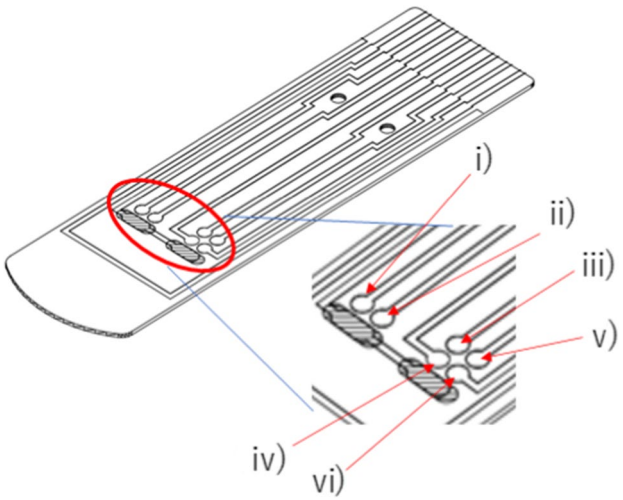
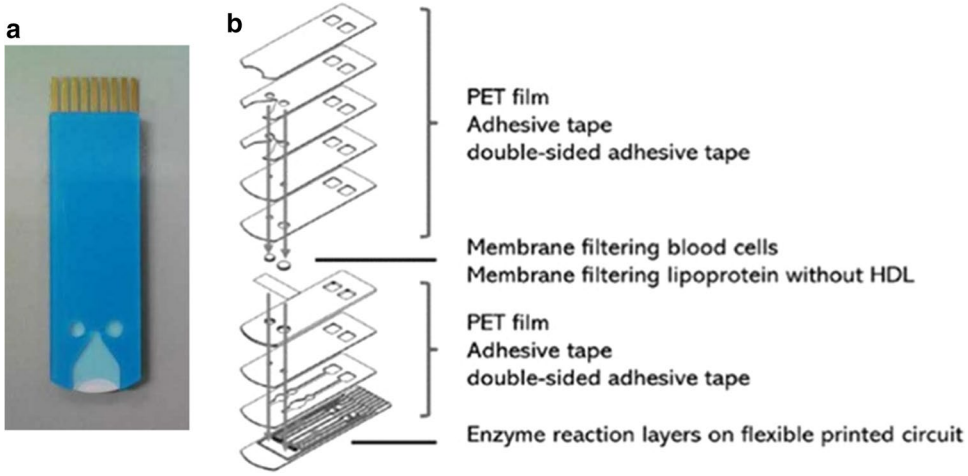


Fig. 2 Structure of flexible printed circuit i) Measurement for HDL cholesterol measurement, ii) Measurement of interfering substances around the working electrode for HDL cholesterol measurement, iii) Measurement of triglyceride, iv) Measurement of interfering substances around the working electrode for triglyceride measurement, v) Measurement of cholesterol, vi) Measurement of interfering substances around the working electrode for cholesterol measurement

Fig. 1 **a** Lipid biosensor, **b** Structure of the sensor



of the 6 working electrodes and reference electrodes to minimize noise signals.

Reaction layer

The enzyme mixture for each target was drop-casted on the working electrode to form an enzyme reaction layer. Mixtures for cholesterol measurement contained 0.5 units lipoprotein lipase, 0.5 units cholesterol esterase, 0.3 units cholesterol oxidase, 200 ng CMC, 250 ng Triton X-100, and 5 ng potassium ferricyanide. Mixtures for triglyceride measurements contained 1 unit lipoprotein lipase, 0.15 unit glycerol kinase, 0.15 unit glycerol 3-phosphate oxidase, 200 ng CMC, 250 ng Triton X-100, 5 ng potassium ferricyanide, 10 ng magnesium chloride, 100 ng adenosine triphosphate, and 1000 ng microgranular cellulose. Mixtures for HDL cholesterol measurement contain the same components as mixtures for cholesterol measurement. 10 mM sodium phosphate buffer pH 6.6 was used as the solvent for each mixture.

Amperometric determination and evaluation of lipid biosensor

0.01ml of standard serums containing supplier-certified cholesterol, triglyceride, and HDL cholesterol concentrations were used.

The lab-fabricated equipment consisting of an amperometric measurement system and a sample transfer system evaluated the lipid biosensor (Fig. 4). The sample transfer system incorporated an air pump, and this system can be programmed to control sample transfer and conditions for HDL separation and enzymatic reactions. The total measurement time was 4 min:2 min for HDL separation and 2 min for the enzymatic reaction. After the sample reaches the electrode and stop, 0.545 V is applied between each working electrode and reference electrode. The electric currents of the six electrodes are monitored 120 s after the start of the voltage application (60 s for HDL-C). To exclude the influence of interfering substances, the difference of the electric currents between electrodes i and ii, electrodes iii and iv and electrodes v and vi is calculated. The concentrations of cholesterol, triglyceride, and HDL cholesterol were calculated using calibration curves derived from the equipment's data processor from currents. To account variations potentially introduced by device production, a calibration curve is produced per manufacturing lot of the sensor before sample quantification. Each difference in electric currents are substituted into the calibration formula to calculate the lipid concentration. The calibration formula has a coefficient specific to the manufacturing lot of the sensor card. The measurements are repeated 12 times at each concentration and the regression equation is calculated by the least squares method. We consider that this protocol will cancel the errors in each measurement.

Storage stability

Storage stability was evaluated under the following conditions.

The sensor lots were divided into three groups and stored at 4 °C. The sensor lots were used to measure each standard serum 12 times every 0, 4, and 18 months.

Results and discussion

This enzyme sensor was accomplished by the flow path shown in Fig. 5.

The flow path is described below.

1. The sample is deposited at the sample inlet and pulled into the device by a vacuum pump.
2. The sample is divided into two flow paths, one towards the channel for CHO and TG measurement (CG flow

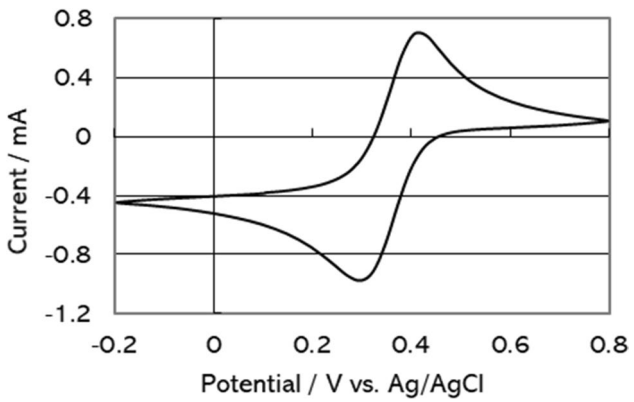


Fig. 3 Cyclic voltammogram of gold plating electrode in 0.5 M potassium chloride solution containing 10 mM sodium ferricyanide

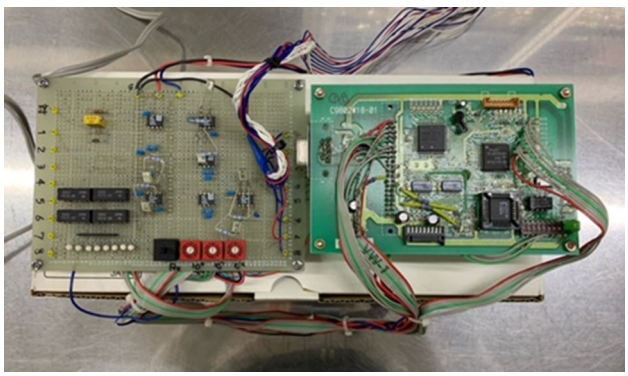


Fig. 4 Amperometric measurement system

path), while the other towards the channel for the HDL-C measurement (H flow path).

3. In the CG flow path, large particles such as the blood cells are captured by the filter, but smaller molecules such as CHO and TG continues to pass through until it reaches the terminals for CHO and TG enzymatic reaction layer. The enzymatic reaction is allowed to proceed for 120 s, the concentration of ferrocyan ions (reduced by the oxidation reaction of cholesterol or triglyceride) is quantified by measuring the applied current.
4. In the H flow path, a filter with precipitation reagent is placed to aggregate non-HDL lipoprotein. After aggregation, when the vacuum pump is applied again, non-HDL aggregates (low-density lipoprotein (LDL), very-low-density lipoprotein (VLDL), and chylomicrons) remain on the filter, allowing the HDL in the serum to travel towards the electrode. The enzyme reaction is allowed to proceed for 120 s, and then the voltage application current is measured (by the oxidation reaction of cholesterol) to quantify the ferrocyan ions. The sampling time of 120 s was decided by screening to minimize the effects of the variation in parameters after optimizing the concentration of components such as enzymes, the size of the flow path, and the thickness of the reaction layer.

Cholesterol

The reaction for cholesterol determination is described below:

$$\text{Cholesterol ester} + H_2O \xrightarrow{\text{Cholesterol esterase, Lipoprotein lipase}} \text{Cholesterol} + \text{Fatty acid} \quad (1)$$

$$\text{Cholesterol} + \text{Ferricyan ion} \xrightarrow{\text{Cholesterol oxidase}} \text{Choleste} - 4 - \text{en} - 3 - \text{on} + \text{Ferrocyan ion} \quad (2)$$

Cholesterol esters in lipoproteins are hydrolyzed by cholesterol esterase and lipoprotein lipase to produce cholesterol and fatty acids. Cholesterol and ferricyan ion are catalyzed by cholesterol oxidase to produce choleste-4-en-3-on and ferrocyan ions. The oxidative current of the ferrocyan ion is measured on the surface of the gold electrode. Fig. S1a shows the amperometric responses of biosensors to injections of different concentrations of cholesterol in Serum, ranging from 54 mg/dl to 215 mg/dl, respectively. Monitoring was initiated 15 s after sample injection and quantified after 105 s. The amperometric response initially increases and then decreases as it is consumed by the electrode due to the trace cholesterol concentration in the serum. Figure 6 shows the electric currents of working electrode v), vi) and the difference of them. The electric current of the working electrode vi) is the oxidation current of impurities in the sample and impurities in the enzyme reaction layer. (Electrode numbers are associated with Fig. 2). The difference indicates the oxidative current of the ferrocyan ion generated by the enzymatic reaction, which is increased by the enzymatic reaction and then decreased by the diffusion from the enzymatic reaction layer and the oxidation on the electrode.

Figure 7 shows the comparison results between the certified value of standard serum cholesterol concentration and the determination result by the sensor at 191, 215, 260 mg/dL concentrations. Each sample was measured 8 times. The coefficient of variation of each sample is 5.0–6.0%, which meets the clinical requirements for cholesterol measurement.

Triglyceride

The scheme of cholesterol determination is as follows:

$$\text{Triglyceride} + H_2O \xrightarrow{\text{Lipoprotein lipase}} \text{Glycerol} + \text{Fatty acid} \quad (3)$$

$$\text{Glycerol} + \text{ATP} \xrightarrow{\text{Glycerol kinase}} \text{Glycerol} - 3 - \text{phopsphate} \quad (4)$$

$$\text{Glycerol} - 3 - \text{phopsphate} + \text{Ferricyan ion} \xrightarrow{\text{Glycerol}-3-\text{phosphate oxidase}} \text{Dihydroxyacetone phosphate} + \text{Ferrocyan ion} \quad (5)$$

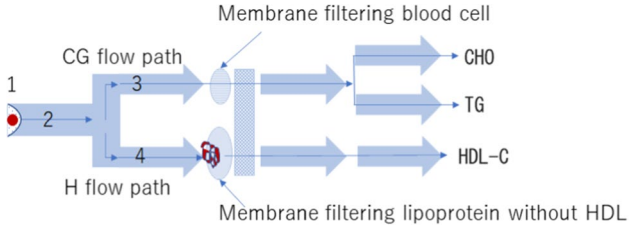


Fig. 5 Flow path of sensor chip

Triglycerides in lipoproteins are hydrolyzed by lipoprotein lipase to produce glycerol and fatty acids. Glycerol is phosphorylated by glycerol kinase to produce glycerol-3-phopsphate. Glycerol-3-phopsphate and ferricyan ion are catalyzed by glycerol-3-phosphate oxidase to produce dihydroxyacetone phosphate and ferrocyan ion. The oxidative current of the ferrocyan ion is measured on the surface of the gold electrode. Fig. S1b shows the amperometric response of the biosensor when the serum triglyceride concentration was varied from 72 mg/dl to 287 mg/dl. Monitoring began 15 s after sample injection and was quantified

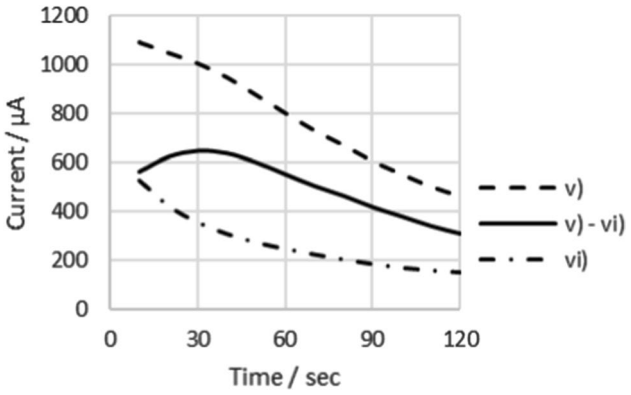


Fig. 6 Electric currents of working electrode v), vi) and the difference of them. (See Fig. 2 for electrode's number.)

after 105 s. The amperometric response increased with increasing TG. Figure 8 shows the comparison results between the certified value of standard serum triglyceride concentration and the determination result by the sensor across three samples 88, 128 and 287 mg/dL. Each sample was measured 8 times, and the coefficient of variation for each sample was 4.0–8.2%. The reaction for the triglyceride measurement is completed by a three-step reaction, and glycerol is an intermediate product produced by the first reaction, but it is also present in the blood. Therefore, both of the glycerol produced by the first reaction and the glycerol already present in the blood are the substrates for the second reaction. Generally, the glycerol in the blood will be pre-erased to solve this problem, however, this step complicates the measurement procedure. In this sensor, an electrode for measuring interfering substances was used to solve the problem. Both of interfering substances and glycerol in the blood were measured by the working electrodes for the interfering substances, and the signal only glycerol produced from triglycerides was calculated. This method is available for many other measurement items. The triglyceride sensor's coefficient of variation and reproducibility is good, indicating that the sensor meets the clinically required performance.

HDL cholesterol electrode

The scheme of HDL cholesterol determination is as follows:

$$\text{Lipoprotein (HDL, LDL, VDL, and Chylomicron)} \xrightarrow{\text{HDL selection filter}} \text{HDL},$$

$$\text{Cholesterol ester in HDL} + \text{H}_2\text{O} \xrightarrow{\text{Cholesterol esterase, Lipoprotein lipase}} \text{Cholesterol} + \text{Fatty acid},$$

$$\text{Cholesterol} + \text{Ferricyan ion} \xrightarrow{\text{Cholesterol oxidase}} \text{Choleste} - 4 - \text{en} - 3 - \text{on} + \text{Ferrocyan ion}$$

Before the blood reaches the enzyme layer, non-HDL lipoproteins are sieved by an HDL selection filter.

Cholesterol esters in HDLs are hydrolyzed by cholesterol esterase and lipoprotein lipase to produce cholesterol and fatty acids. Cholesterol and ferricyan ion are catalyzed by cholesterol oxidase to produce choleste-4-en-3-on and ferrocyan ions. The oxidative current of the ferrocyan ion is measured on the surface of the gold electrode. Fig. S1c shows

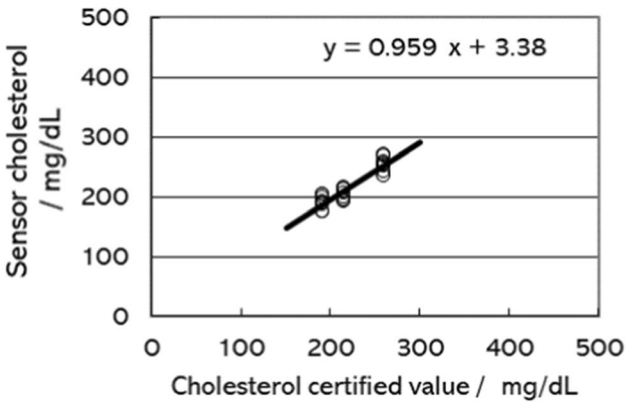


Fig. 7 Correlation between certified value of standard serum cholesterol and determination result by the sensor

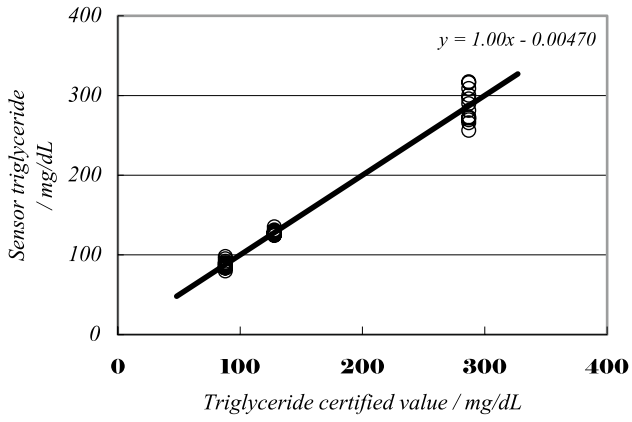


Fig. 8 Correlation between certified value of standard serum triglyceride and measurement result by the sensor

the amperometric response of the biosensor when the serum HDL cholesterol concentration was varied from 11 mg/dl to 44 mg/dl. Monitoring began 15 s after sample injection and was quantified after 105 s. The amperometric response, as well as the cholesterol concentration in serum, initially increased and then decreased as it was consumed by the electrode due to the trace cholesterol concentration in the serum. The reason for this may be that it is quickly consumed by the three enzymes. Figures 9, 10 shows the comparison results between the certified value of standard serum HDL cholesterol concentration and the determination result by the sensor across three samples 44, 57 and 60 mg/dL. The number of samples was 3, and each sample was measured 8 times. The coefficient of variation for each sample was 6.7–6.9%.

Selectivity of HDL-C

Figure 10. shows that there was no correlation between the CHO certified values and the calculated values by the HDL-C measuring unit. Figures 9 and 10 suggest that the HDL-C measuring unit measures the HDL-C concentration in the sample selectively.

The commercialization unit with the performance of the demo machine

We made a 9.5 × 17 cm size apparatus for the commercialization unit with the performance of the demo machine (ca. 30 × 30 cm including the pump) (Fig. 11). Next, the stability was evaluated using this sensor unit.

Stability of the sensor

The storage stability of the sensor chip was measured using a commercialization unit equipped with the performance of the demo machine. The long-term storage stability of the sensor was investigated by assessing its response to CHO, TG and HDL-C using a sensor chip stored at 4 degrees Celsius, sealed under desiccant. As shown in Fig. 12 and Table S1, in all items (CHO(a), TG(b) and HDL-C(c)), the sensor chip showed good stability and detection efficacy across low, medium and high target concentrations, and there was no noticeable change in response after storage for more than 18 months. In fact, it retained a near-initial current response during that period Fig. 12.

Conclusion

In this study, we have developed a system that can simultaneously determine total cholesterol, triglyceride, and HDL cholesterol in 4 min. The detections were achieved using the flow path and lipoprotein aggregation membrane to remove

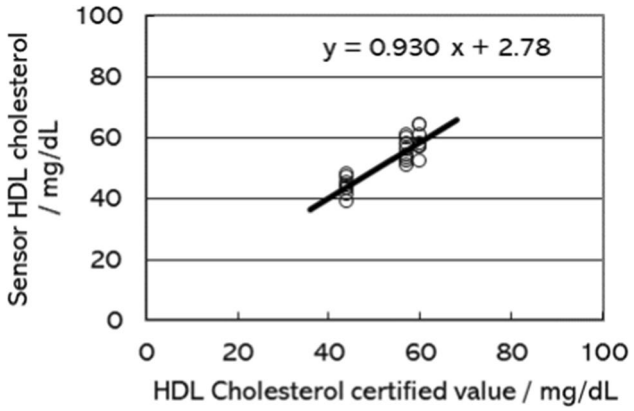


Fig. 9 Correlation between certified value of standard serum HDL cholesterol and measurement result by the sensor

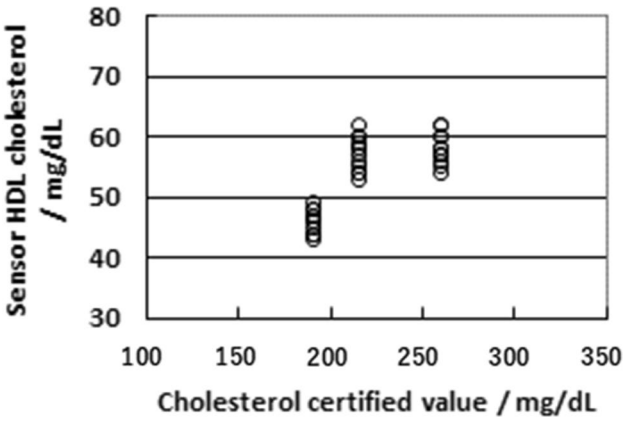


Fig. 10 Correlation between certified value of standard serum cholesterol and determination result by HDL-C measurement unit in the sensor

lipoproteins other than HDL, electrochemically treating interfering substances that passed through the membranes using a differential method. In this report, the device evaluated the concentrations of the target molecules compared to the known lipids in standard serum samples, where the detection deviation is < 12%. A follow-up evaluation will be performed on whole blood. The device retains initial detection efficiency after 18 months at room temperature. In this demonstration we have established a universal detection platform that can be modified to perform measurement of other molecules common in clinical practice, expanding its usability and effectiveness. In the future, we would like to develop new devices based on this platform for the clinical testing market, especially for the point-of-care testing market. Furthermore, by integrating with IT, we can use the advantages of electrochemical sensors for the health management of individuals, telemedicine, and home care for elderly patients.

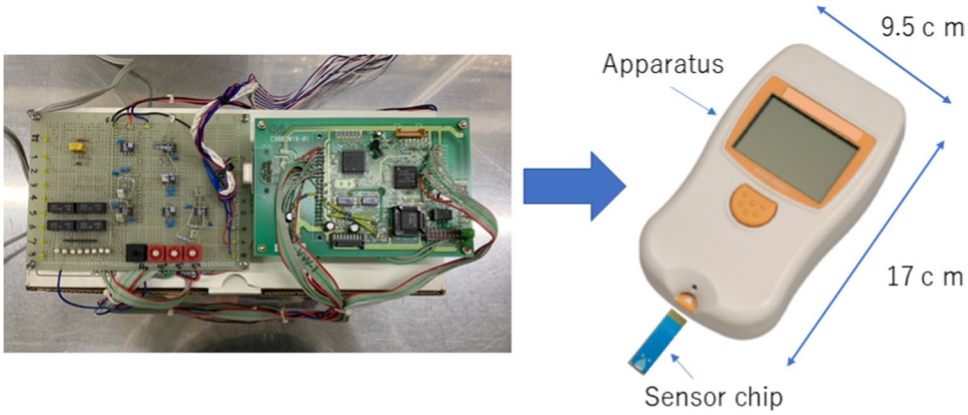


Fig. 11 The commercialization unit with the performance of the demo machine

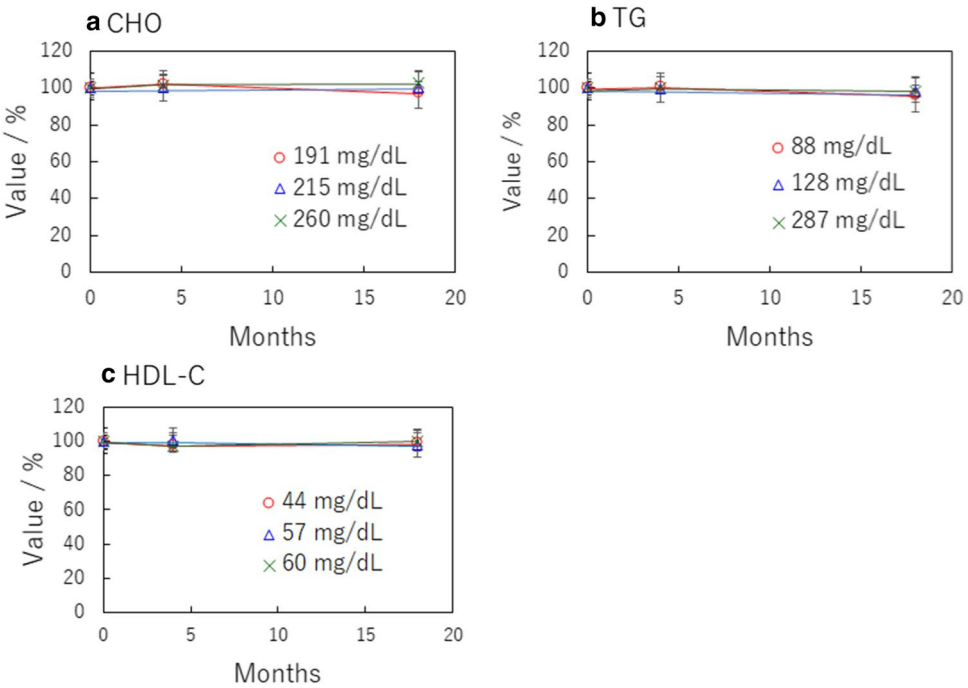


Fig. 12 The storage stability of the sensor chip was measured using a commercialization unit equipped with the performance of the demo machine. **a** CHO, **b** TG, **c** HDL-C. Each point shows the mean values of 12 measurements

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s44211-022-00148-w.

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Data availability statement The dataset generated during the current study are not publicly available but are available from the corresponding author on reasonable request.

Declarations

Conflict of interest On behalf of all authors, the corresponding author states that there is no conflict of interest.

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