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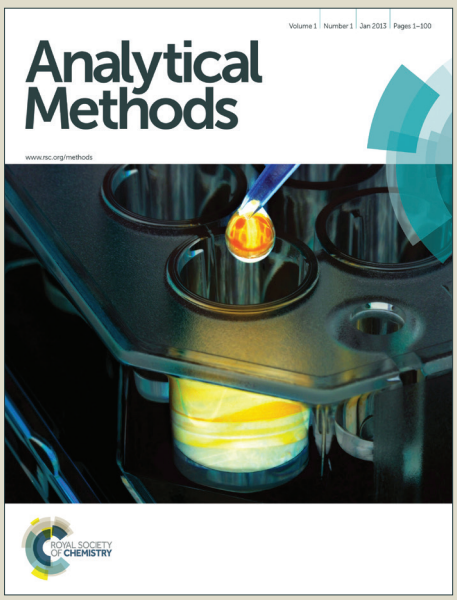

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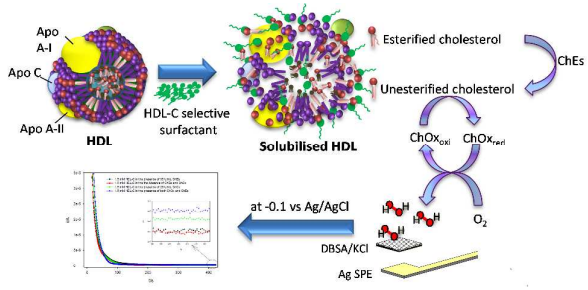

An electrochemical biosensor for selective measurement of HDL-C in serum

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ARTICLE TYPE

A biosensor for the determination of high density lipoprotein cholesterol employing combined surfactant-derived selectivity and sensitivity enhancements

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High density lipoprotein cholesterol (HDL-C) is a modifiable risk factor in cardiovascular disease and devices suitable for its determination at the point of care are critical to the future management of hypercholesterolaemia. An electrochemical biosensor for measuring HDL-C was developed. The biosensor was based on a homogeneous assay methodology for selective determination of HDL-C in combination with a printed electrochemical sensor for measuring the reduction of hydrogen peroxide at a silver paste electrode. The polyoxyethylene alkylene tribenzylphenyl ether surfactant (Emulgen B-66) was found to be capable of both the selective dissolution of HDL particles, as well as the enhanced electrocatalytic reduction of hydrogen peroxide. The resulting biosensor was shown to have a linear response to HDL-C from 0.5 to 4 mM (r^2=0.998) with an average r.s.d. of 7%. The biosensor was also used to analyse HDL-C in thirteen serum samples and had good agreement with a commercial spectrophotometric precipitation-based assay (r=0.72; $p < 0.058$).

Introduction

Cholesterol levels, including high density lipoprotein cholesterol (HDL-C) are modifiable risk factors for cardiovascular disease (CVD), a condition which remains the number one global cause of death.[1-3] Levels of HDL-C above 60 mg/dL (1.55 mM) are considered to have a positive protective role in heart disease, while low HDL-C levels (less than 40 mg/dL or about 1 mM) are linked to an increase in heart attack risk. For this reason, the importance of measurement of HDL-C has been emphasized by the National Cholesterol Education Programme (NCEP) since the late 1980s. [4, 5] The measurement of HDL-C is also important for two other purposes: [1, 6, 7]

1. The calculation of (low density lipoprotein cholesterol) LDL-C using the Friedewald formula: LDL-C = Total cholesterol (TC) - (HDL-C + TG/5)
2. The calculation of non-HDL cholesterol, determined by subtracting the HDL cholesterol concentration from the TC content. Non-HDL-C has been recommended as a target for preliminary CVD prevention.

The gold standard for measurement of HDL-C and other cholesterols is the method developed by the Centres for Disease Control and Prevention (CDC).[7] This method is highly complex and requires 5.0 mL of sample which is subjected to ultracentrifugation, precipitation and measurement using the Abell-Kendall method. Since there are only a few laboratories capable of performing the ultracentrifugation steps necessary in the CDC method, the Cholesterol Reference Method Laboratory Network (CRMLN) developed a simpler method based on a modified dextran sulphate procedure.[8] However, this technique, like the CDC method, also required large sample volumes and also required multiple manual processing steps including the removal of triglycerides which still renders this method unsuitable in most clinical laboratories and in automated analysers.[9, 10]

In the past three decades, chemical precipitation methods, and more recently, homogeneous assays have been used to measure serum HDL-C in clinical laboratories. Homogeneous assays were a major step forward in improving the precision of earlier precipitation methods. Full automation eliminated manual pipetting, off-line pre-treatment, centrifugation and separation steps and improved assay precision, in line with recommended NCEP criteria. To date, there are several commercial colorimetric assays available for the quantitative measurement of HDL-C in serum.[3, 11] All of these determine the amount of H_2O_2 produced from the enzymatic reaction of cholesterol present in HDL, to cholest-4-en-3-one, which can then be measured

spectrophotometrically.

Homogeneous assay methodologies have been developed which allow the direct and selective analysis of HDL-C in a single step.[3] In one method, a polyoxyethylene alkylene tribenzylphenyl ether surfactant (Emulgen B-66) was found to be capable of the selective solubilisation of HDL-C, allowing the enzymatic reaction of HDL-C to H_2O_2 as follows:

$$\text{HDL-C} + H_2O \xrightarrow{\text{ChEs}} \text{cholesterol} + \text{fatty acids} \quad (1)$$

$$\text{Cholesterol} + O_2 \xrightarrow{\text{ChOx}} \text{cholest-4-en-3-one} + H_2O_2 \quad (2)$$

where ChEs is cholesterol esterase and ChOx is cholesterol oxidase. However, such assays are only appropriate for laboratory analyses. The movement of many routine blood tests away from the central laboratory to the point-of-care is a major trend in healthcare provision. A point of care device that measures HDL-C directly is very attractive in biomedical diagnostics and would be highly advantageous in the self-management of hypercholesterolemia.[12-14] Electrochemical techniques lend themselves well to the fabrication of low cost, point of care and disposable diagnostic devices. Thus, it would seem a common sense approach to develop electrochemical biosensor methodologies that are capable of measuring HDL-C using a similar principle, with the measurement of H_2O_2 performed electrochemically.[15, 16] While a number of electrochemical biosensors for cholesterol have been developed,[17-20, 20-30] to date, there are just a few published examples of electrochemical biosensors for HDL-C.[31, 32] Kinoshita *et al.*, developed an amperometric sensor based on a homogeneous assay in which, a peroxidase-entrapped and ferrocene-embeded carbon paste electrode was used to measure the H_2O_2 produced after enzymatic reaction of HDL-C. PEG-modified enzymes in the presence of α-cyclodextrin sulphate and $MgCl_2$ were employed to impart selectivity to the measurement of HDL-C. This method was performed at 37°C and was only linear up to 0.04 mM. However, since it is important to measure HDL-C directly up to at least 2 mM preferentially at room temperature, their method would be unsuitable for the development of a point of care device. Foster *et al,* developed an electrochemical device for HDL-C based on a precipitation methodology in which phosphotungstic acid (PTA) and $MgCl_2$ were employed as the precipitation reagents.[32] However, incorporation of this method on a disposable platform has also proved challenging.

In the present work, an electrochemical sensor capable of the reduction of H_2O_2 at a modified screen-printed silver electrode was employed as the basis of a biosensor to perform the selective measurement of HDL-C. It has been shown previously that these electrodes, modified with lyotropic layers composed of surfactant and salt, exhibit the significantly enhanced electrocatalytic reduction of H_2O_2.[15, 16] This behaviour was exhibited for a broad range of surfactants including anionic, cationic and neutral types. Here, we demonstrate that the polyoxyethylene alkylene tribenzylphenyl ether surfactant Emulgen B-66, facilitates both the selective measurement of HDL-C in serum, as well as enhancing the electrocatalytic reduction of the H_2O_2 at the electrode when formed following the enzymatic catalysis of cholesterol esters and cholesterol with ChEs and ChOx, respectively. This resulted in a biosensor capable of the direct, room temperature measurement of HDL-C in the diagnostically relevant range of 0 to 4 mM.

Experimental

Materials

Dodecylbenzenesulphonic acid (DBSA-D0989) was purchased from TCI Europe. Polyoxyethylene octyl phenyl ether (Triton X-100), sodium chloride (NaCl), potassium chloride (KCl), potassium dihydrogen phosphate (KH_2PO_4), disodium hydrogen phosphate (Na_2HPO), *N*,*N*-dimethyl-*m*-toluidine and 4-aminoantipyrine (4-AAP) were purchased from Sigma-Aldrich (UK). 4-Aminoantipyrine HCl was from BDH (Dorset, UK). Cholesterol oxidase (O5F; 19.4 U/mg), cholesterol esterase (CE4F; 144 U/mg) and horseradish peroxidise (HRP, HRP4C; 295 U/mg) were purchased from BBI Enzymes (Gwent, UK). HDL-C and LDL-C isolated from human sera and delipidated difibrinated serum (S139) were purchased from Scipac Ltd. (Kent, UK). 30% (v/v) hydrogen peroxide solution was purchased from Merck (Nottingham, UK). Polyoxyethylene alkylene tribenzylphenyl ether (Emulgen B-66) was kindly donated by Kao Corporation (Japan). The HDL and LDL/VLDL cholesterol assay kit was purchased from Abcam (Cambridge, UK). Silver (PF-410), carbon (Electrodag 6017SS), and Ag/AgCl (Electrodag 6038SS) screen printing inks were from Henkel (Netherlands). Serum samples were kindly donated by Alere (San Diego).

Methods

Assay optimisation

The optimisation of the concentration of Emulgen B-66 required for the selective determination of HDL-C over LDL-C was performed spectrophotometrically. Solutions of either 0.5 mM HDL-C or LDL-C in PBS were mixed with Triton X-100 or Emulgen B-66 in a microtitre plate with an assay mixture containing 39 U/mL ChEs, 23 U/mL ChOx, 0.075 % (w/v) 4-AAP, 14 U/mL HRP and 0.06 % (v/v) *N*,*N*-dimethyl-*m*-toluidine. The resulting absorbance was measured at 545 nm on a FLUOstar Optima plate reader (BMG Labtech, UK) with Optima software (version: 2.1) after incubation for three min. at room temperature.

Biosensor development

All electrochemical experiments were performed on a 3 x 3 mm screen printed silver paste electrode (SPE) with a Ag/AgCl screen printed reference electrode and carbon counter electrode. Electrodes were used in either an open stirred batch system in 4 mL 0.1 M PBS pH 6.8 or in a low volume thin layer cell of 8 µL formed from a lid and 25 µm spacer layer and referred to in the text as 'encapsulated' electrodes. All electrochemical measurements were carried out using a PGSTAT128N potentiostat with NOVA 1.6 software (Metrohm, UK). Electrodes were either used without further modification or modified with an inkjet-printed layer of DBSA/KCl as previously reported.[15] Inkjet printing was performed using a Dimatix Materials Printer DMP-2831 with Dimatix Drop manager DMP-2800 series software (Fujifilm Dimatix, Inc., US). The effect of a number of reagents

on the electrocatalytic reduction of H_2O_2 at the electrodes was assessed in the presence of a range of assay reagents including 6% (v/v) Emulgen B-66, ChEs (39 U/mL), ChOx (23 U/mL) and HDL-C (1.5 mM) in serum, either individually or in combination. Measurement of HDL-C in delipidated serum was performed via the chronoamperometric measurement of the H_2O_2 produced after reaction of the HDL-C with ChEs (39 U/mL) and ChOx (23 U/mL) in 6% Emulgen B-66 at -0.1 V vs Ag/AgCl at 420 s following 180 s incubation at room temperature.

Analysis of clinical samples

Each of 13 serum samples was analysed once using the cholesterol assay kit (Abcam) and two or three times using the developed biosensor due to limitations in sample volume.

Results and discussion

Optimisation of sensor selectivity using Emulgen B-66

The polyoxyethylene alkylene tribenzylphenyl ether, Emulgen B-66 possesses a hydrophile-lipophile balance of 13.2 which is believed to result in the selective break down of HDL particles – as opposed to other lipoproteins such as LDL, very low density cholesterol (VLDL) and chylomicrons – thus allowing the selective enzymatic catalysis of HDL-bound cholesterols.[33-35] The exact mechanism of HDL solubilisation remains unclear. However, since apolipoprotein A-I is the major apolipoprotein in HDL, this surfactant may solubilize the polar lipids via a specific interaction with this apolipoprotein.[35] To assess the ability of Emulgen B-66 to achieve the selective break down of HDL, the recovery of cholesterol from serum samples containing HDL-C or LDL-C was measured spectrophotometrically.

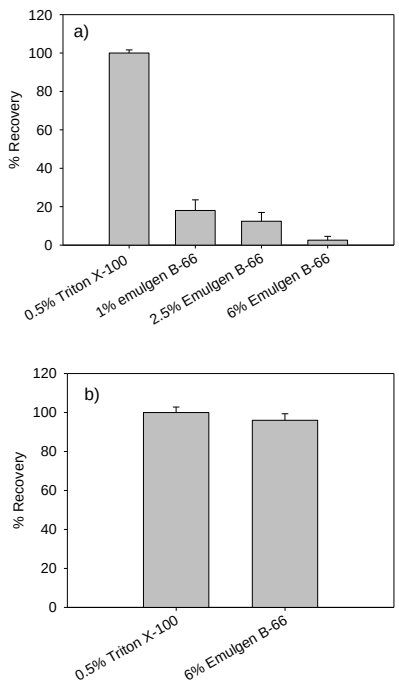

Fig. 1. Recovery efficiencies of (a) 0.5 mM LDL-C and (b) 0.5 mM HDL-C in serum to Emulgen B-66, relative to 0.5% (v/v) Triton X-100. Recovery in Triton X-100 was taken as 100%.

Fig. 1(a) shows the percentage recovery of 0.5 mM LDL-C in serum in the presence of different concentrations of Emulgen B-66 relative to 0.5% (v/v) Triton X-100, which is a non-specific surfactant and results in the release of total cholesterol (TC).[36, 37]

In this case, recovery of LDL-C was 18±6%, 12±5% and 2.5±2% (v/v) for Emulgen B-66 concentrations of 1%, 2.5% and 6%, respectively. Conversely, the percentage recovery of 0.5 mM HDL-C in the presence of 6% Emulgen B-66 was found to be 96±3 %, relative to 0.5% Triton X-100 (Fig. 1b).[35]

Effect of assay reagents on the reduction of hydrogen peroxide

In order to evaluate the electrochemical response of the electrode towards H_2O_2 in the presence of the assay components necessary to selectively measure HDL-C, time-based amperometric measurement of H_2O_2 was performed in the presence of individual or combined assay reagents. In the case of Emulgen B-66, enhanced reduction currents were observed in its presence (Fig. 2a). In the absence of surfactant, electrodes had a response of 1.33×10^{-7} A (curve 1). However, in the presence of 6% (v/v) Emulgen B-66, a response of 5.98×10^{-6} A was obtained for 3 mM H_2O_2 (curve 3), which was a 39% current increase over that achieved for the previously reported[16] combination of DBSA and KCl of 3.64×10^{-6} A (curve 2). This enhanced response might assist in achieving a lower limit of detection of H_2O_2 with the sensor.

As previously demonstrated, the electrocatalytic reduction of H_2O_2 has been shown to be significantly enhanced at a screen printed, silver paste electrode modified with a lytropic layer formed by surfactant and electrolyte.[16] The mechanism for this is not fully understood, but may be due to several effects including a change in silver paste morphology by creating a high surface area nanostructure, or the formation of micellar, hexagonal or lamellar structures by surfactant in the solution which become deposited onto the silver paste and creates an enhanced surface for the catalytic process.[16, 38] Emulgen B-66 is a nonionic surfactant which was prepared in an electrolyte solution of 0.1 M PBS pH 6.8. It is believed that an equivalent effect is achieved by this combination as has previously been demonstrated with other surfactant/salt combinations. However, further enhancement appears to result from operation of the sensor in a solution of this surfactant and electrolyte, as opposed to the modification of the surface alone with DBSA and KCl.[16] Fig. 2(b) shows the cyclic voltammograms for unmodified electrodes and those in 6% Emulgen B-66 in PBS. These again show significant modification of the surface with a capacitive double layer formed by Emulgen B-66 and NaCl, as has been previously observed.

The effect of the serum sample and the enzymes on the amperometric response of the sensor was also investigated using amperometry in 4 mL stirred solution. Fig. 3 shows the response of the modified sensor to H_2O_2 in the presence of 6% Emulgen B-66 before, during and following exposure of the sensor to HDL-C, ChEs and ChOx.

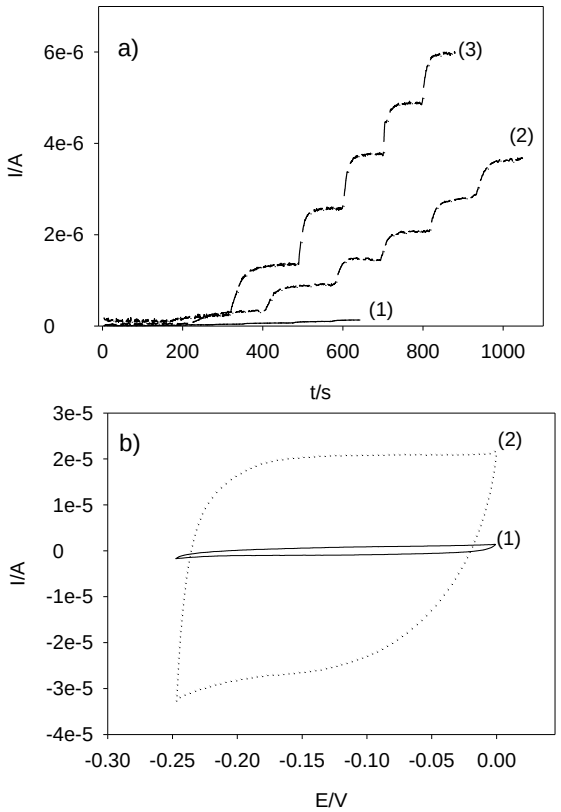

Fig. 2. (a) Amperometric measurement of 0.5 to 3 mM H_2O_2 at -0.1 V vs. Ag/AgCl in 4 mL stirred batch solution using: curve 1) unmodified Ag SPEs in 0.1 M PBS pH 6.8 solution; curve 2) DBSA/KCl modified Ag SPEs in 0.1 M PBS 6.8 solution; curve 3) SPEs in 6% (v/v) Emulgen B-66 solution. (b) Cyclic voltammograms (scan rate of 0.1 V/s vs Ag/AgCl in 0.1 M PBS, pH 6.8) of: curve 1) unmodified Ag SPEs and; curve 2) electrodes measured after 3 h in 6% (v/v) Emulgen B-66.

The response of the sensor was similar before (a) and after (b) the combined exposure of the electrode to these species. However, the response was reduced significantly in the presence of HDL-C (d) and ChOx (e) alone. The presence of HDL or ChOx appears to significantly disrupt formation of the lyotropic layer on the electrode surface. In the case of HDL, it has already been clearly demonstrated that it interacts selectively with Emulgen B-66 and may disrupt the lyotropic layers formed at the electrode surface.

In the case of ChOx, it has also been found to be capable of disrupting phospholipid membranes via the "active site lid" mechanism.[39]Phospholipid membranes are also formed from the organisation of amphiphilic molecules and are analogous to the lyotropic phases formed by the interaction of Emulgen B-66 and electrolyte at the electrode surface. Disruption may also relate to the highly hydrophobic nature of the ChOx active site and the presence of additional hydrophobic domains on its surface.[40] The sensor response was not significantly affected by the presence of ChEs. These results also demonstrated the reversible nature of the effect of both lipoprotein and ChOx on the electrocatalytic response of the sensor, further suggesting that only the formed lyotropic phase was affected and not the underlying electrode structure.

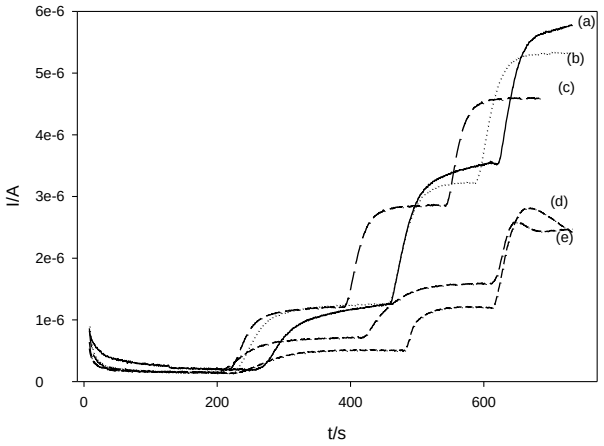

Fig. 3. Amperometric response of sensors to H_2O_2 (0.5 to 1.5 mM) in stirred solution containing 6% (v/v) Emulgen B-66: a) before exposure and; b) after exposure to a solution of ChEs (39 U/mL), ChOx (23 U/mL) and serum containing 1.5 mM HDL-C; c) in the presence of ChEs (39 U/mL); d) in the presence of serum containing 1.5 mM HDL-C; e) in the presence of ChOx (23 U/mL).

The final response of the biosensor system to 8 µL H_2O_2 in a thin layer cell is shown in Fig. 4. This response takes into account all processes which either enhance the electrocatalysis such as the presence of Emulgen B-66, or which interferes with it, such as HDL and ChOx. Measurement was again performed at -0.1 V vs Ag/AgCl and the current response recorded at 420 s. The sensor had excellent linearity from 0 to 10 mM H_2O_2 (r^2=0.996, n=3), which makes it suitable for the direct determination of HDL-C in serum, based on a resulting one to one stoichiometric relationship between the concentration of cholesterol and the concentration of H_2O_2 generated, assuming full enzymatic conversion.

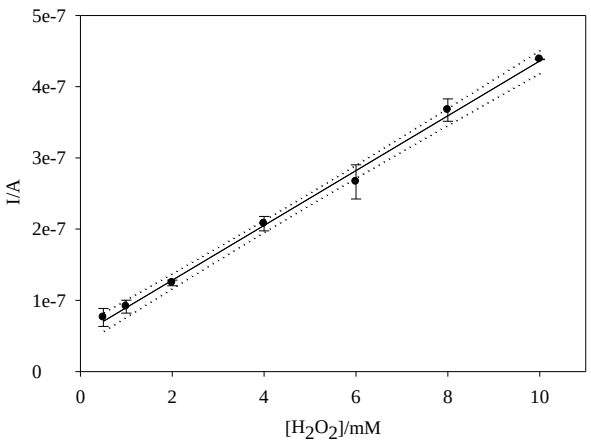

Fig. 4. Amperometric response to 8 µL of H_2O_2 (-0.1 V vs Ag/AgCl at 420 s) in the presence of 6% (v/v) Emulgen B-66, 39 U/mL ChEs and 23 U/mL ChOx at, (slope=3.85×10^{-8} A/mM, r^2=0.996, dotted line=95% confidence interval, n=3).

Measurement of HDL-C

The encapsulated biosensor was first applied to the measurement of HDL-C in delipidated serum and sensitivity and reproducibility studies were performed. The chronoamperometric responses of the biosensor in HDL-C from 0.5 to 4 mM are

shown in Fig. 5. Of note is the potential step chronoamperometric response occurring as the generated H_2O_2 is reduced at the electrode. In classical potential-step chronoamperometry, the signal decays to zero due to the total consumption of the reactant.[41] Theoretically, the amperometric response would fall to 93% after $t = L^2/D$, where L is the diffusion layer thickness (25 µm) and D is the diffusion coefficient of H_2O_2. Based on a value of D of 1.71 x 10^{-5} cm^2 s^{-1},[42] this would occur after 365 ms. However, in this work, a pseudo steady-state response was evident after several hundred seconds. This is most likely due to a combination of barriers to diffusion slowing the process of complete reduction of the available H_2O_2[32] and the continued production of some H_2O_2 via the enzymatic catalysis of cholesterol. Coulometry can also be employed as an alternative to amperometry. The amperometric response taken at 420 s was found to be proportional to the HDL-C concentration (Fig. 6). The biosensor had a linear response of 4.49×10^{-8} A/mM (r^2 = 0.998, n=3) between 0.5 and 4 mM HDL-C with an average r.s.d. of 7.0 %.

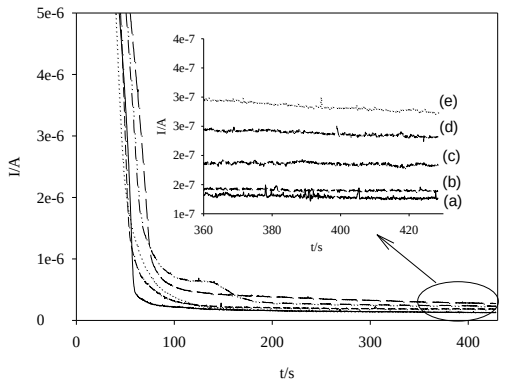

Fig. 5. Amperometric responses of the developed biosensor to HDL-C in 0.1 M PBS, pH 6.8 at -0.1V (vs Ag/AgCl). Inset: Detail of response between 360 and 420 s. HDL-C concentrations: a) 0.5 mM, b) 1 mM, c) 2 mM, d) 3 mM, e) 4 mM.

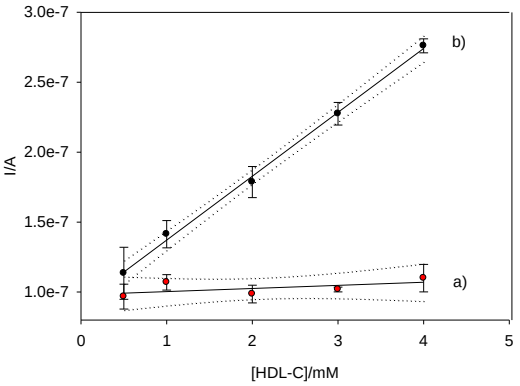

Fig. 6. Biosensor response to HDL-C in delipidated serum at -0.1 V (vs Ag/AgCl). a) Assay controls using delipidated serum in the presence of assay components for 0 mM concentration and in the absence of ChEs and ChOx for 0.5 to 4 mM concentrations ; b) Biosensor containing 6% (v/v) Emulgen B-66, 39 U/mL ChEs and 23 U/mL ChOx; (slope=4.49×10^{-8}, r^2=0.998, n=3)

The measurement of HDL-C in clinical serum samples was then studied using the developed biosensor. The HDL-C concentration was also measured using a spectrophotometric precipitation assay methodology (Abcam, UK) and correlated against the developed biosensor (Fig. 7). The correlation had a slope of 0.85 (r=0.72, p <0.058) for 13 clinical serum samples. The Bland–Altman plot which calculates the mean difference between the two methods of measurement demonstrated no bias between the two methods across the measured range with most of the measurements within two standard deviations of the mean (Fig. 8).[43] The biosensor indicated slightly higher HDL-C concentrations in some measurements compared to the assay kit. Although the exact reason for that is not known, it may be due to varying levels of free cholesterol present in the samples, as this has been shown to affect the response of this type of assay.[44] It has also been reported that most homogeneous assay methodologies give positive predictive values due to the presence of intermediate density lipoproteins (IDL) in the serum, or in the presence of high levels of Lp(a).[45] While the biosensor was developed based on the homogeneous assay methodology, the Abcam assay kit is based on the precipitation principle.[9, 10, 46, 47] Therefore, discrepancies between the two methods are very likely to be due to the differences in the methods and how they process and respond to the complex mixtures of lipids and lipoproteins in the sample.

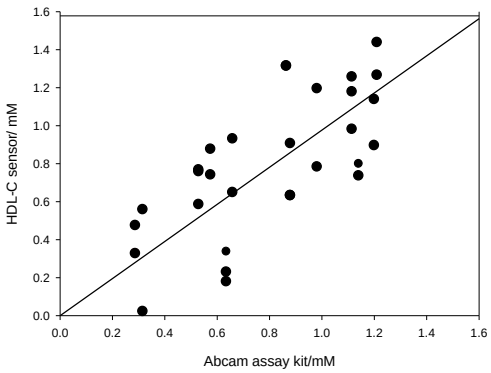

Fig. 7. Correlation of HDL-C in clinical serum samples measured by the biosensor and the Abcam assay kit (slope= 0.85, r=0.72).

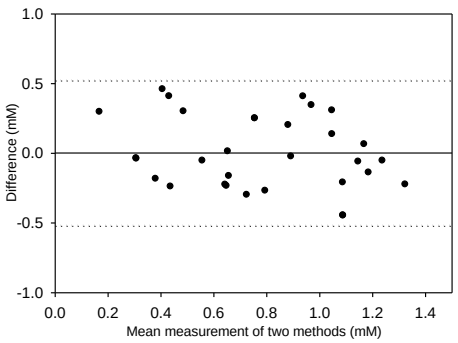

Fig. 8. Bland–Altman plot of the difference between the developed biosensor and Abcam assay kit against the mean of the two methods in 31 measurements. (dashed lines are mean difference ±2 sd).

Conclusions

An electrochemical biosensor was developed for the selective measurement of HDL-C. The sensor was able to achieve both selectivity and sensitivity enhancements using the surfactant Emulgen B-66. The presence of this surfactant was shown to selectively dissolve HDL over LDL. In addition, it also resulted in the enhanced electrocatalysis of H_2O_2 which is produced following the release of cholesterol ester and cholesterol from HDL and its catalysis by ChEs and ChOx, respectively. The biosensor was shown to have good linearity across the diagnostically relevant range. The biosensor was successfully applied to the measurement of HDL-C in real samples, although deviation from other methods was observed in some of the samples which might be due to the presence of variable quantities of other lipids.

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Notes and references

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† Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here]. See DOI: 10.1039/b000000x/

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