Simple and Cost-effective Enzymatic Detection of Cholesterol Using Flow Injection Analysis

Murugan THIRUPPATHI,*1 Ching-Ying TSAI,*1 Tzu-Wen WANG,*2 Yu TSAO,*1 Tsung-Hung WU,*1 and Ja-an Annie HO*1,*2,*3,*4

*1 BioAnalytical Chemistry and Nanobiomedicine Laboratory, Department of Biochemical Science and Technology, National Taiwan University, Taipei 10617, Taiwan
*2 Department of Chemistry, National Tsing Hua University, Hsinchu 30013, Taiwan
*3 Department of Chemistry, National Taiwan University, Taipei 10617, Taiwan
*4 Center for Biotechnology, National Taiwan University, Taipei 10617, Taiwan

A flow-injection analytical (FIA) system was developed for the determination of cholesterol concentrations based on enzymatic reactions that occurred in a cholesterol oxidase (CHOx)-immobilized, fused-silica capillary followed by electrochemical detection. The production of hydrogen peroxide from cholesterol in an enzymatic reaction catalyzed by CHOx was subsequently oxidized electrochemically at an electrode. Our FIA system demonstrated its cost-effectiveness and utility at an applied potential of 0.6 V (vs. Ag/AgCl), a flow rate of 100 μL/min and, under optimal conditions, the resulting signal demonstrated a linear dynamic range from 50 μM to 1.0 mM with a limit of detection (LOD) of 12.4 μM, limit of quantification (LOQ) of 44.9 μM, and the coefficient of variation of 5.17%. In addition, validation of our proposed system using a reference HDL-cholesterol kit used for clinical diagnosis suggested our FIA system was comparable to commercial kits for the determination of the cholesterol incorporation amount in various aqueous liposomal suspensions. These good analytical features achieved by FIA could make the implementation of this methodology possible for on-line monitoring of cholesterol in various types of samples.

Keywords Cholesterol, cholesterol oxidase, enzyme, oxidation, hydrogen peroxide, flow injection analysis, FIA

(Received March 5, 2020; Accepted May 6, 2020; Published September 10, 2020)
a rapid, sensitive, continuous flow, quantitative analytical system for determining target analytes in biological and environmental samples.27–29

In this study, we developed a FIA platform coupled with an electrochemical detector for cholesterol determination. A series of optimizations, such as surfactant effect, enzyme concentration, applied voltage, mobile phase flow rate, and cholesterol-containing sample injection volume, were investigated to determine the best conditions for cholesterol detection. The platform exhibited high sensitivity toward cholesterol detection under the optimized conditions. Finally, the biochemical relevance of this method was validated by using a reference HDL-cholesterol kit.

**Experimental**

**Reagents and chemicals**

Cholesterol oxidase (CHOx; EC 1.1.3.6., from *Cellulomonas* sp., 115 units/mg protein), cholesterol, (3-glycidyl-oxypropyl) trimethoxysilane (GTMS), hydrogen peroxide (H2O2, 35 wt.%), potassium carbonate (K2CO3), sodium metaperiodate (NaIO4), sodium dihydrogen phosphate (NaH2PO4), and disodium hydrogen phosphate (Na2HPO4) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Triton X-100 was obtained from Amersham Pharmacia Biotech (Amersham Place, UK). All other chemicals in this study were of analytical grade and were used as received. Na2HPO4 and NaH2PO4 were used to prepare 0.1 M, pH 7.4 phosphate buffer solution (PB). Deionized distilled water (18.2 MΩ cm) was obtained with a Milli-Q system (Milford, MA, USA) and used for all solution preparations. For the preparation of the stock solution, cholesterol was dissolved in an aliquot of 1% (v/v) Triton X-100 that was prepared in PB.

**Apparatus**

Electrochemical experiments were carried out with a BAS workstation (Model LC-4C, West Lafayette, IN, USA) and a BAS Model CC-5E electrochemical cell, in which a dual platinum-based electrochemical probe (Model 001012, ALS) was used as the working electrode (3 mm in diameter), Ag/AgCl as the reference electrode (Model RE-3VT, ALS), and a stainless steel auxiliary electrode block (012798, ALS) as the counter electrode. The dynamic amperometric experiments were performed using a FIA system, and a computer with a HW-2000 chromatography workstation (Great Tide Instrument Co., Taipei, Taiwan) was used for all data collection.

**Immobilization of CHOx enzyme on the inner surface of a capillary reactor**

Generally, high enzyme loading is required for sensitive detection. The immobilization of cholesterol oxidase enzyme on the surface of a silica capillary reactor was modified based on previous studies.27,30–32 The modification procedures for the cholesterol oxidase enzyme capillary were initiated by washing the fused-silica capillary (85-cm, 0.53 i.d.) with deionized distilled (DD) water, followed by treatment with 1 M NaOH overnight (Fig. 1). After carefully washing the capillary with 1 M HCl, followed by DD water, the silylation was subsequently performed by filling with 3-glycidoxypropyltrimethoxysilane (GPTMS) at 90°C for 2 h. We converted the epoxy groups to diols by treating with 10 mM H2SO4 at 90°C for 10 min, washing the capillary with DD water, and then we allowed this to react with 20 mM sodium metaperiodate (NaIO4) and 2 mM potassium carbonate (K2CO3) for 1 h at room temperature to convert diols to aldehydes. Then, 0.1 M, pH 7.4 phosphate buffered solution (PB) was passed slowly into the capillary and incubated overnight with cholesterol oxidase enzyme at 4°C to form a Schiff base. Finally, the capillary was rinsed, filled with PB (pH 7.4), and stored at 4°C prior to use.

**The setup for flow injection analysis**

Here, we developed FIA of cholesterol for practical purposes by automating the analyses, which resulted in decreased human error and analysis time that increased accuracy and precision. Our FIA system was constructed with (1) an isocratic pump (Waters 515 HPLC pump, MA, USA) at the inlet of the cholesterol biosensing system to control the mobile phase (0.1 M, pH 7.4 PB) and to maintain an appropriate flow rate, and (2) a Rheodyne (Model 7725) injector valve with a 20-μL sample loop (Rainin, Emeryville, CA) for injection of the samples. Commercially available polyether ether ketone (PEEK) tubing (0.020 i.d.) and standard finger tight fittings were purchased from Upchurch Scientific (Oak Harbour, WA). The water line of the pump was connected to the injector and then to the flow cell, and the mobile phase (buffer) and sample
were passed through the capillary reactor where they finally reached the working electrode (the schematic diagram of FIA is depicted in Fig. 2).

**Real sample analysis**

Cholesterol has been used in many liposomal formulations due to its effect on the physical properties of lipid bilayers. The ratio of cholesterol in the liposomal formulation plays a decisive role in regulating fluidity and permeability of bilayers. Therefore, the determination of actual cholesterol amount incorporated in the liposomal bilayer becomes essential in the characterization of liposomes. To validate the performance of our biosensing system, liposomal samples were prepared from a lipid mixture using the reversed-phase evaporation method as reported previously. A lipid mixture that consisted of phosphatidylcholine (PC), phosphatidylglycerol (PG), and cholesterol (7 mg:0.5 mg:0.8/2.2/4 mg) was dissolved in a 2-mL mixture of chloroform, isopropyl ether, and methanol (6:6:1 volume ratio). After sonication of the mixture under nitrogen for 1 min, 1 mL PBS (10 mM, pH 7.4) was added with subsequent sonication under nitrogen for another 3 min. The organic solvent was evaporated under reduced pressure (∼50 psi) at 45°C, which left a milky-white jelly of pro-liposome. Another aliquot of 1 mL PBS was added to this residue, and the mixture was sonicated again under nitrogen for 3 min. Finally, the liposome suspension was incubated for an additional 20 min at 45°C and then stored at 4°C until use. Immediately prior to use, the liposomal sample solutions were sonicated for 1 min before dilution with 1% Triton X-100 that was made in PBS, and we subsequently introduced this to the system.

**Results and Discussion**

**Optimization for cholesterol determination**

The FIA-based electrochemical cholesterol biosensor was developed using cholesterol oxidase that was immobilized on the capillary, and the cholesterol oxidase catalyzed the cholesterol to hydrogen peroxide and cholestenone in the presence of oxygen. The hydrogen peroxide that was produced was determined by the amperometric method (Fig. 2). To optimize the perfect condition for quantifying the concentration of cholesterol, the effect of Triton X-100 (surfactant), cholesterol oxidase concentration used for preparing enzyme capillary column, applied voltage, mobile phase flow rate, and sample injection volume were examined thoroughly. The non-ionic surfactant Triton X-100 was used for the preparation of cholesterol in aqueous solution due to its amphiphilic properties. As shown in Fig. 3A, the higher the hydrogen peroxide concentration, the greater the peak area, and the sample with 1% surfactant exhibited a reasonable peak area and acceptable deviation. The capillary bioreactor that was immobilized with 25 and 50 UN/mL of enzyme were examined for the determination of various cholesterol concentrations; 50 UN/mL enzyme provided the highest peak area (Fig. 3B). Similarly, the effects of the applied voltage, sample injection volume and mobile phase flow rate were also investigated by plotting each against peak area (Figs. 3C – 3E).

Thus, applied voltage of 0.6 V and the sample volume of 20 μL with a flow rate of 0.1 mL/min produced the highest peak area and, therefore, this was chosen as the operating condition in our experimental setting. Overall, we found that 1% Triton-X with sample, applied voltage of 0.6 V, 20 μL sample injection volume, 0.1 mL/min flow rate, and 50 UN/mL enzyme concentration were optimal for our FIA sensing platform. The following equation was used to determine the signal-to-noise ratio (S/N).

\[
\frac{S}{N} = \frac{\text{Signal of the sample}}{\text{Signal of the blank}}
\]

**FIA detection of cholesterol**

To further access the analytical application, detection of cholesterol was accomplished by our FIA-based electrochemical sensor, in which hydrogen peroxide was oxidized electrochemically at 0.6 V applied potential (vs. Ag/AgCl), which yielded quantitative current responses that corresponded to the concentration of cholesterol. From the calibration plot (Fig. 4), we obtained a wide linear range between 50 μM and 1 mM with a regression coefficient of 0.9953 and a low detection limit of...
Fig. 3 Optimization of system performance (A) Effect of Triton-X: without Triton-X, with 1% surfactant in sample, and with 1% surfactant in mobile buffer on detection of hydrogen peroxide, (B) the effect of enzyme (cholesterol oxidase) concentration on the inner face of the capillary, (C) effect of applied voltage, (D) effect of flow rate, and (E) effect of sample injection volume on the determination of cholesterol standards. All data represent average values of at least three replicates, and the error bars reflect standard deviation.

Fig. 4 (A) Calibration plot for determination of cholesterol using FIA. Linear dynamic range: 50 μM to 1 mM; limit of detection (LOD) was calculated as 12.4 μM, and the limit of quantification (LOQ) was calculated to be 44.9 μM. All data represent average values of at least three replicates, and the error bars reflect standard deviation. (B) Reproducibility of signal peaks (generated by analyzing 0.05, 0.1, 0.5, and 1 mM cholesterol standard solution, respectively).
12.4 μM. The sensing system was used for an average of 5 h a day, and the capillary enzymatic reactor remained stable for at least 80 uses at room temperature under operational conditions, which was stored at 4°C when not in use. The repeatability and reproducibility of the proposed system were examined by injection of 1 mM cholesterol standards, and the uniformity of the amperometric peaks that were generated by triplicate injections were investigated. The largest value for the coefficient of variation for triplicate measurements was 5.17%, which indicated that results could be obtained by this newly developed FIA system that were easily reproducible.

Validation of method by using a reference clinical-use cholesterol kit

The comparison between the proposed system and the commercial enzyme cholesterol assay kit (OSR6195, Beckman Coulter, Lismeehan, Ireland) to measure cholesterol concentrations in samples was conducted to validate the application of our methodology. In this study, the proposed system and ELISA kit were tested with three samples that contained various cholesterol levels. For both methods, sample preparation was done by diluting with 1% Triton-X that was prepared in PB prior to analysis. The suspensions were either injected into our system or analyzed by a commercial cholesterol kit. The best-fit regression line of analysis of the proposed system when compared to that of a reference cholesterol kit indicated a strong correlation between these two data sets ($R^2 = 0.9510$) (Fig. 5), which suggested that the proposed system was comparable to commercial kits for the detection of cholesterol in various samples.

Determination of the cholesterol incorporation rate for various aqueous liposome samples

In addition to the biochemical function of cholesterol, it plays a strategic role in liposome composition as well. The appropriate quantity of cholesterol incorporated in the liposome bilayer guaranteed a controlled and reproducible liposomal drug delivery and release. The feasibility of using our biosensing system to determine the incorporated cholesterol amount for 3 liposome preparations was evaluated herein. As indicated in Fig. 6, the amount of cholesterol incorporated in 3 liposomal sample solutions, analyzed both in the proposed system and commercial kit, agreed with each other ($R^2 = 0.9435$). The more cholesterol was used for preparing liposomes, the higher cholesterol incorporation was achieved. The results suggest our proposed system is a suitable tool for determining the level of cholesterol in the presence of other phospholipids.

![Fig. 5](image1.png) Correlation plot obtained by analyzing various concentrations of cholesterol sample solutions between the results obtained by the proposed capillary cholesterol biosensing system (FIA) and a reference cholesterol kit.

![Fig. 6](image2.png) Cholesterol incorporation rates, determined by both the proposed biosensing system and a commercial cholesterol kit, for three liposomal sample solutions that contained various levels of cholesterol.

| Component | Reproducibility | Technique | Linear range | LOD | Sensitivity | Stability | Ref. |
|-----------|-----------------|-----------|--------------|-----|-------------|----------|------|
| CHOx, CE, POD | <4% | Electrochemistry | 1.2 μM - 1 mM | 12 nM | — | 25 days | 25 |
| CHOx, CE, POD | <10% | Electrochemistry | 10 - 250 mg/dL | — | — | 26 |
| CHOx, CNT, microfluidic chip | — | Electrochemistry | 50 - 400 mg/dL | 10 mg/dL | 0.0512 nA/mg/dL | >30 days | 36 |
| CD, Hb | — | Optical | 0 - 800 μM | 56 μM | — | 14 |
| CHOx, GNP | — | Optical | 0.5 - 8 mg/dL | 19 mg/dL | 2470 μA/mM/cm² | >10 days | 37 |
| Fe₃O₄, CHOx, CE | — | Electrochemistry | 0.1 - 1 mM | 80 μM | 73.88 μA/mM/cm² | >25 days | 38 |
| CdTe/CdSe/ZnSe QD, CHOx | — | Electrochemistry | 0.1 - 10 mM | 75 μM | 111.16 μA/mM/cm² | 30 days | 39 |
| Pt/rGO/P3ABA nanocomposite film, CHOx | 3.5% | Electrochemistry | 0.25 - 4 nM | 40.5 μM | 15.94 μA/mM/cm² | 7 days | 40 |
| ChOX | 5.17% | Electrochemistry | 0.05 - 1 mM | 12.4 μM | 510.28 a.u./mM/cm² | 30 days | Present work |
| | | | | | | | |
| CHOx, Cholesterol oxidase; CE, cholesterol esterase; POD, peroxidase; CNT, carbon nanotube; CD, carbon dot; Hb, hemoglobin; GNP, gold nanoparticle; Fe₃O₄, magnetite nanoparticle; QD, quantum dot; Pt, platinum; rGO, reduced graphene oxide; P3ABA, poly(3-aminobenzoic acid). |
Conclusions

We developed a flow-injection enzymatic system with amperometric detection for determining cholesterol. The concentration of cholesterol was directly proportional to the concentration of hydrogen peroxide produced from an enzymatic reaction catalyzed by cholesterol oxidase. The performance of our FIA system was compared with other cholesterol sensors and the findings are shown in Table 1. Reproducibility, linear dynamic range, stability and detection limit are comparable to other designs described previously, \(^{14,25,26,36-40}\) and the good cost effectiveness of our FIA system was clearly realized considering its simple design, the use of a single enzyme and re-useability of enzymatic capillary reactor. Our enzymatic capillary reactor had operational and storage stability >30 days, which permitted direct detection of samples that contained cholesterol without complicated sample preparation and high cost. The successful demonstration of delivery capability offers new opportunities for construction of a rapid, sensitive, and multi-functional bioanalytical system for sensing different target analytes.

Conflicts of Interest

The authors declare no conflict of interest.

Acknowledgements

The authors gratefully acknowledge financial support provided by the Taiwan Ministry of Science and Technology (MOST) under grant nos. 98-2113-M-002-025-MY3, 101-2113-M-002-026-MY3, 102-2628-M-002-004-MY4, 106-2113-M-002-014-MY3 and 107-2811-M-002-026-102-2113-M-002-025-MY3, 101-2113-M-002-003-MY3, 1003-MY3, 102-2628-M-002-004-MY4, and 107-2811-M-002-026-102-2113-M-002-025-MY3, 101-2113-M-002-025-MY3,101-2113-M-002-014-MY3 under grant nos. 98-2113-M-002-025-MY3, 101-2113-M-002-026-102-2113-M-002-025-MY3, and 107-2811-M-002-026.

The authors gratefully acknowledge financial support provided by the Taiwan Ministry of Science and Technology (MOST) under grant nos. 98-2113-M-002-025-MY3, 101-2113-M-002-026-MY3, 102-2628-M-002-004-MY4, 106-2113-M-002-014-MY3 and 107-2811-M-002-026.

References

1. J. Xu, D. Jiang, Y. Qin, J. Xia, D. Jiang, and H.-Y. Chen, Anal. Chem., 2017, 89, 2216.
2. Y. Zhang, Y.-N. Wang, X.-T. Sun, L. Chen, and Z.-R. Xu, Sens. Actuators, B, 2017, 246, 118.
3. A. N. Sekretaryova, V. Beni, M. Eriksson, A. A. Karyakin, A. P. F. Turner, and M. Y. Vagin, Anal. Chem., 2014, 86, 9540.
4. H.-C. Chang and J.-A. a Ho, Anal. Chem., 2015, 87, 10362.
5. A. Devadoss and J. D. Burgess, Langmuir, 2002, 18, 9617.
6. H. Iwai and S. Akihama, Chem. Pharm. Bull., 1986, 34, 3471.
7. A. Kaushik, P. R. Solanki, K. Kaneto, C. G. Kim, S. Ahmad, and B. D. Malhotra, Electroanalysis, 2010, 22, 1045.
8. T. Nagaoka, S. Tokonami, H. Shigi, H. Matsumoto, Y. Takagi, and Y. Takahashi, Anal. Sci., 2012, 28, 187.
9. C. Zhao, L. Wan, L. Jiang, Q. Wang, and K. Jiao, Anal. Biochem., 2008, 383, 25.
10. G. A. Tığ, D. K. Zeybek, and Ş. Pekyarmdmc, Chem. Papers, 2016, 70, 695.