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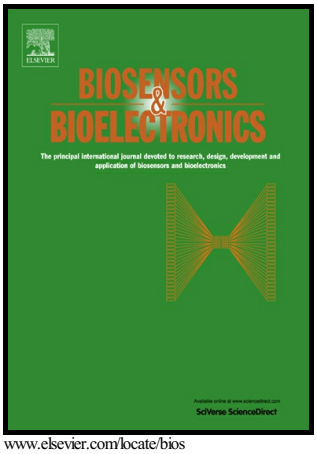

www.elsevier.com/locate/bios

PII: S0956-5663(18)30532-3
DOI: https://doi.org/10.1016/j.bios.2018.07.030
Reference: BIOS10619

To appear in: *Biosensors and Bioelectronic*

Received date: 3 May 2018
Revised date: 1 July 2018
Accepted date: 16 July 2018

Cite this article as: Gang Tian, Min Ding, Biao Xu, Yifan He, Wenjing Lyu, Mingchao Jin and Xiaoqing Zhang, A novel electrochemical biosensor for ultrasensitive detection of serum total bile acids based on enzymatic reaction combined with the double oxidation circular amplification strategy, *Biosensors and Bioelectronic,* https://doi.org/10.1016/j.bios.2018.07.030

A novel electrochemical biosensor for ultrasensitive detection of serum total bile acids based on enzymatic reaction combined with the double oxidation circular amplification strategy

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ABSTRACT

Serum total bile acids (TBA) level is used as a sensitive and reliable index for hepatobiliary diseases in clinics. Herein, a novel electrochemical biosensor was fabricated using enzymatic reaction coupling with the double oxidation circular amplification strategy for the detection of human serum TBA. With the catalysis of 3α-hydroxysteroid dehydrogenase (3α-HSD), 3α-bile acids reacted specifically with nicotinamide adenine dinucleotide (NAD^+). And then, the reduced nicotinamide adenine dinucleotide (NADH) was produced. After that, the NADH reacted with the electron mediator of tris(2,2'-bipyridine) ruthenium(III) ($Ru(bpy)_3^{3+}$), which was then transformed to $Ru(bpy)_3^{2+}$. Ultimately, $Ru(bpy)_3^{2+}$ was further oxidized to $Ru(bpy)_3^{3+}$ under a certain voltage, which was detected by the chronoamperometry assay. The detection was performed using a disposable unmodified screen-printed carbon electrode (SPCE) without sample preparation. The proposed biosensor showed high sensitivity and accuracy with the linear range from 5.0 to 150.0 pmol/L in 10^6-fold dilution serum. The established method had a good correlation with the enzymatic cycling method (r = 0.9372, P < 0.001, n = 72) commonly used in clinic. The electrochemical biosensor is simple, ultrasensitive and without sample pretreatment, showing great potential for point-of-care testing (POCT) of serum TBA in clinical

samples. In addition, the biosensor is cost-effective with a small volume of samples, especially suitable for those who have difficulties in blood collection, such as infants, children and some small animals.

Keywords: Bile acid; Reduced nicotinamide adenine dinucleotide; Tris(bipyridine)-ruthenium (II); Screen printed carbon electrode; Serum.

1. Introduction

Bile acids are synthesized from cholesterol in the liver and secret through the biliary tract into the small intestine, where they help lipid and fat-soluble vitamins absorption (Fiorucci and Distrutti 2015; Setchell et al. 2013). In humans, bile acids mainly include cholic acid (CA), chenodexycholic acid (CDCA), deoxycholic acid (DCA) and lithocholic acid (LCA), existing in free or glycine-/taurine- conjugated forms (Chiang 2009). As more than 95% of bile acids are reabsorbed in the enterohepatic circulation, the concentration is very low in the peripheral blood (Danielsson and Sjovall 1975; Goto et al. 2007; Slijepcevic et al. 2017). However, elevated serum bile acids level was found in patients with hepatobiliary (Belgaumkar et al. 2016; Chen et al. 2013; Sugita et al. 2015; Ye et al. 2007) and other diseases, such as colon cancer (Costarelli et al. 2002; Farhana et al. 2016) and diabetes (Gao et al. 2016; Sonne et al. 2016). Compared with other laboratory parameters, serum total bile acids (TBA) level is a more sensitive and reliable indicator for the diagnosis of primary biliary cirrhosis and nonalcoholic steatohepatitis (Puri et al. 2017; Tang et al. 2015). Currently, serum TBA level is used as an index for the diagnosis of intrahepatic cholestasis of pregnancy (ICP) by excluding other causes of cholestasis (Reyes 2016; Walker et al. 2002).

The following methods are usually used for the detection of serum bile acids, including radioimmunoassay (Wildgrube et al. 1983), high-performance liquid chromatography (HPLC) (Okuda et al. 1984a, 1984b), and enzymatic methods (Zhang et al. 2004). The radioimmunoassay is sensitive and specific. Whereas, it has radioactive contamination both for the environment and operators. The HPLC method with ultravoilet detector is not sensitive enough for the detection. Derivatization and hydrolysis is usually required for the HPLC method with fluorescence detector, which makes the operation complicated and time consuming that limits its widespread. HPLC with mass spectrum was also used for the detection of serum bile acids, but the cost is too expensive (Ye et al. 2007). The enzymatic cycling colorimetric assay is the most commonly used method for the determination of serum TBA in clinic (Ducroq et

al. 2010). Whereas, it needs a large volume of reagents and non-portable equipment. Some new strategies were also used for the TBA. In our group, an electrochemiluminescent assay based on enzymatic reaction method was established for the quantification of serum TBA (Zhang et al. 2013). The method was sensitive, but the electrode polishing cost so much time, which limits its application in clinical samples. In recent years, the research of electrochemical biosensors has attracted broad interest, because of their inherent advantages such as high sensitivity, rapid detection and potential miniaturization (Altintas et al. 2018; Zhao et al. 2018). The electrochemical detection strategies were also used for the measurement of TBA concentration in the phosphate buffer or the bovine serum (Bartling et al. 2009). Nevertheless, few studies based on electrochemical methods were reported for the TBA concentrations in clinical samples. The modification of the working electrode by enzymes, electro-mediators or nanomaterials was the conventional way for the electrochemical detection, which also made the operation complicated (Rani et al. 2004). A simple electrochemical sensor was developed for serum TBA level based on enzymatic reaction with the use of unmodified screen-printed carbon electrode (SPCE) as the working electrode in our group (Zhang et al. 2016). The sensor is reagent-saving and cost-effective. Whereas, the preparation steps for the serum samples (i.e., deproteinization, nitrogen-blow, redissolution) are tedious and time-consuming.

A novel electrochemical biosensor was fabricated using enzymatic reaction coupling with the double oxidation circular amplification strategy for the detection of human serum TBA. The strategy of enzymatic reaction combined with the double oxidation circular amplification greatly increases the sensitivity of the detection. Because of low cost and easy to miniaturization, the SPCE is widely used in the electrochemical measurement (Tian et al. 2016). Here, we used an unmodified SPCE without sample preparation for the electrochemical detection. Thus, the complicated modification process was avoided under the acceptable sensitivity. Meanwhile, as the pretreatment of the clinical samples is just to dilute with ultrapure water, this could eliminate the matrix effect in serum samples. The principle of the enzymatic reaction was elucidated in our previous work (Zhang et al. 2016). NADH, as the production of the enzymatic reaction, reacted with the electron mediator of tris(2,2'-bipyridine) ruthenium(III) ($Ru(bpy)_3^{3+}$), which was then transformed to $Ru(bpy)_3^{2+}$. Ultimately, $Ru(bpy)_3^{2+}$ was further oxidized to $Ru(bpy)_3^{3+}$ under a certain voltage, which was detected by the chronoamperometry assay. The electrochemical detection method

based on enzymatic reaction combined with the double oxidation circular amplification we established is sensitive, simple, cost-effective and suitable for clinical detection of TBA.

2. Material and methods

2.1 Materials and reagents

Deoxycholic acid (DCA), taurodeoxycholic acid (TDCA), glycoursodeoxycholic acid (GUDCA) and $Ru(bpy)_3Cl_2 \cdot 6H_2O$ (TBR) were purchased from Sigma Chemical (St. Louis, MO, USA). NAD^+ and NADH were purchased from Roche Co. (Switzerland). TBA test kit and 3α-HSD (enzymatic activity of 6.09 U/mg) were bought from Sichuan Maker Biotechnology Co., Ltd. (Chengdu, China). Other chemicals were of analytical grade and obtained from the Chongqing Company of Chemical Reagent (Chongqing, China). Ultrapure water was obtained from a Milli-Q system (Milford, MA, USA). Electrochemical measurements were performed on a CHI852C electrochemical analyzer (Chenhua Instruments Co. Ltd., Shanghai, China). Fourier transform infrared spectroscopy (FTIR) was recorded on a TENSOR 27 spectrometer (Bruker, Germany) from 3500 to 1500 cm^{-1} at a resolution of 4 cm^{-1}. The SPCE consists of a working electrode with the diameter of 2.5 mm, a Ag/AgCl reference electrode and a platinum auxiliary electrode, was fabricated in the laboratory as reported by Wu and co-workers (Wu et al. 2008; Jin et al. 2017).

2.2 Samples and electrochemical measurement

All serum samples were obtained from the First Affiliated Hospital of Chongqing Medical University (Chongqing, China) and stored at -20℃ until analysis. This study was approved by the ethical committees of the First Affiliated Hospital of Chongqing Medical University. The concentrations of TBA in serum samples were also detected using enzymatic cycling method by an automatic biochemical analyzer (Hitachi 7600, Hitachi Co., Tokyo, Japan) in the First Affiliated Hospital of Chongqing Medical University. TBA test kit was used strictly for the detection according to the kit instructions. 40 clinical serum samples with low concentrations (≤ 0.20 μmol/L) of TBA were mixed and used as a blank serum to establish and evaluate the method. To further investigate the correlation between the enzymatic cycling method and the established electrochemical method, 72 clinical serum samples with different concentrations of TBA were detected by both methods.

Serum samples were diluted with ultrapure water to eliminate the matrix effect of serum. 50 μL of the mixed solution containing 35 μL of 100 mmol/L PBS (70 mmol/L,

pH 9.50), 5 μL of 50 mmol/L NAD^+ (5.0 mmol/L), 5 μL of 10 kU/L 3α-HSD (1.0 kU/L) and 5 μL of 70 mmol/L $Ru(bpy)_3^{2+}$ (7.0 mmol/L) was added to the surface of the SPCE. And then, a constant potential of 1.15 V was applied to the SPCE. Subsequently, the diluted serum was added into the mixed solution and detected by a chronoamperometry method.

3. Results and discussion

3.1 Principle of the enzymatic reaction coupling double oxidization electrochemical detection of TBA

The mixed solution containing of 3α-HSD, NAD^+, $Ru(bpy)_3^{2+}$ and PBS was added to the SPCE surface. With a constant potential of 1.15 V was applied to the SPCE, tris(2,2'-bipyridine) ruthenium (Ⅱ) ($Ru(bpy)_3^{2+}$) was oxidized to the form of tris(2,2'-bipyridine) ruthenium (Ⅲ) ($Ru(bpy)_3^{3+}$). With the catalysis of 3α-hydroxysteroid dehydrogenase (3α-HSD), 3α-bile acids reacted with nicotinamide adenine dinucleotide (NAD^+) specifically. And then, the reduced nicotinamide adenine dinucleotide (NADH) was produced, which was illustrated in our previous work (Zhang et al. 2013). FTIR spectroscopy was used to characterize the structure of the bile acid before and after the enzymatic reaction. And the results were shown in Fig. S1 in the Supporting Information. After that, the NADH reacted with the electron mediator of $Ru(bpy)_3^{3+}$. The reaction results in the $Ru(bpy)_3^{3+}$ being converted to $Ru(bpy)_3^{2+}$, which was further oxidized to $Ru(bpy)_3^{3+}$ at the electrode (Downey and Nieman 1992). As a result, an oxidation current could be observed by electrochemical detection. It was confirmed by the cyclic voltametry assay, as shown in Fig. S2 in the Supporting Information. The principle of the reaction was illustrated in scheme 1.

< Scheme 1>

3.2 Enzymatic reaction combined with double oxidization electrochemical measurements of bile acids

To further testify bile acids could be detected by the enzymatic reaction combined with double oxidization method on an unmodified SPCE, the amperometric *I-t* curves of different reagents were collected (Fig. 1). In Fig. 1A, low current signals were observed when adding a free bile acid of DCA into PBS solution containing of NAD^+ and 3α-HSD. These results indicated that the electrochemical signal of the oxidation of NADH on SPCE is low. Whereas, elevated current responses were observed obviously when adding NADH into PBS solution containing of $Ru(bpy)_3^{3+}$. It indicated that NADH reacted with $Ru(bpy)_3^{3+}$ to form $Ru(bpy)_3^{2+}$, which was further oxidized to $Ru(bpy)_3^{3+}$ at the surface of the SPCE and produced increased electrochemical signals (Fig.1C). Additionally, bile acids were added into PBS

solution containing of NAD^+ and $Ru(bpy)_3^{2+}$, no obviously electrochemical signal was found because no production of NADH was produced in the enzymatic reaction process (Fig. 1B). When bile acids were added into PBS solution containing of NAD^+, 3α-HSD and $Ru(bpy)_3^{2+}$, the oxidization peak was also detected. It indicated that with the catalysis of 3α-HSD, NAD^+ and bile acids could react with each other, producing NADH which could react with $Ru(bpy)_3^{3+}$ to produce NAD^+ and $Ru(bpy)_3^{2+}$. Moreover, $Ru(bpy)_3^{2+}$ could be further oxidized to $Ru(bpy)_3^{3+}$ on the surface of the electrode and produced an electrochemical signal (Fig.1D). The result of the experiment matches well with the speculated enzymatic reaction combined with double oxidation electrochemical reaction of bile acids.

<Figure 1>

3.3 The effect of individual bile acids on the electrochemical measurement of TBA

Bile acids are a kind of 3α-hydroxysteroid compounds, existing either in the free form or conjugated form with taurine or glycine in human serum, which could react with NAD^+ at the catalysis of 3α-HSD. Therefore, a free bile acid of DCA, a taurine conjugated bile acid of TDCA and a glycine conjugated bile acid of GUDCA were chosen randomly to evaluate the effect of individual bile acids on the measurement of TBA. The electrochemical signals of 9.06±0.28 μA DCA, 9.20±0.33 μA TDCA and 9.16±0.19 μA GUDCA were found insignificantly different at the same condition with the *P* value of 0.312.

3.4 Preparation of serum samples

Matrix effect could suppress or enhance the responses of analysts, which influences the accuracy of the results (Barling et al. 2008). Dilution with solvent is a common approach to reduce or eliminate matrix effect (Ferrer et al. 2011). To investigate the matrix effect of serum, mixed blank serum and PBS solution were spiked with DCA, and then diluted with ultrapure water from 10 to 10^8-fold. At 10-fold dilution, the peak current in the mixed blank serum was higher than that in the PBS solution. A significantly decreased peak current was observed in the mixed blank serum when diluted from 10^2 to 10^3-fold. Whereas, the peak current increased in PBS solution and almost equivalent at 10^3-fold dilution in both the serum and PBS solution, indicating 10^3-fold dilution was effective to eliminate matrix effects in serum. However, increasing the dilution times from 10^3 to 10^6, the peak current increased significantly in both the serum and PBS solution, and reached the maximum value at

10^6-fold dilution. On the contrary, the peak current decreased sharply in both serum and PBS solution with the increased dilution times from 10^7 to 10^8, shown in Fig.2. The results illustrated that the matrix effect in serum interfered with the enzymatic reaction combined with the double oxidation reaction process. As 10^3-fold dilution was effective to eliminate the matrix effect in serum, the sensitivity is insufficient. As a result, 10^6-fold dilution was adopted in the subsequent work for the effective elimination of matrix effect in serum with satisfactory sensitivity.

<Figure 2>

3.5 Optimization of the experimental conditions

The detailed information about the optimization of the experimental conditions, including pH values of PBS solution, the activity of the 3α-HSD and the concentrations of NAD^+ and $Ru(bpy)_3^{2+}$ was illustrated in the Supplementary Material.

3.6 Calibration curve and linearity

The calibration solutions were prepared by adding 100 μL of DCA standard solution into 900 μL of the mixed blank human serum to generate a series of concentrations from 1.0 to 150.0 μmol/L. At the optimal experiment conditions, the spiked serum was diluted 10^6 times with ultrapure water and measured by the chronoamperometry assay. The linearity of the calibration curve was assessed based on seven sets independently prepared calibration standard solutions using linear regression. Calibration curve of DCA in the 10^6-fold diluted serum shows a linearity of the current I (μA) and concentration of DCA from 5.0 to 150.0 pmol/L. The calibration curve in serum was $I = 1.060 \ln c + 6.572$ ($r = 0.9983$, $n = 5$), as shown in Fig. 3. The limit of detection (LOD) was calculated as three times of the signal-to-noise ratio (S/N = 3). And the limit of quantification (LOQ) was acquired by diluting the lowest concentration of DCA gradually in the calibration curve till the last detectable concentration. The LOD and LOQ were 0.40 pmol/L and 2.0 pmol/L, respectively.

<Figure 3>

3.7 Precision and recovery

The precision and recovery of the electrochemical method were examined by measuring the concentration of DCA in 900 μL blank plasma before and after adding 100 μL of DCA standard solutions at high (100.0 μmol/L), medium (20.0 μmol/L) and low concentrations (5.00 μmol/L). The inter-day variation was assessed by measuring

the concentration of DCA in the working solutions on 5 consecutive days, while the intra-day variation was assessed by consecutive determinations of DCA in 5 samples with the same procedure in the same day. Precisions were expressed by the relative standard deviation (RSD). All of the variations were less than 11.8%, and the recoveries of different concentrations of DCA were from 91.4% to 108.0%, as listed in Table S2 in the Supplementary Material.

3.8 Interference test

Hemoglobin and bilirubin are usually defined as endogenous interferences in serum samples. The interference test was performed using the evaluation program by the Clinical and Laboratory Standards Institute (CLSI) of America. The concentrations of TBA in serum before and after adding standards of bilirubin were defined as X_C and X_T, respectively. The interference value (expressed as X_T - X_C) less than 1.96*S* showed no significant interferences which was expressed by N, while the interference value more than 1.96*S* indicated significant interferences which was expressed by I.

To assess the selectivity of the electrochemical assay, the concentrations of hemoglobin ranging from 0 to 40.0 mg/mL and bilirubin ranging from 0 to 171.0 μmol/L were added into solutions including 7.20 μmol/L of the serum TBA. The results showed that there were significant interferences when the concentration of hemoglobin was over 30.0 mg/mL and bilirubin was over 34.2 μmol/L, shown in Table S3 in the Supplementary Material.

3.9 Clinical application

72 clinical serum samples containing different concentrations of TBA were determined by both enzymatic cycling method in clinics and the established electrochemical method, and the results were shown in Fig. 4. Pearson's correlation analysis revealed a significant relationship between the established electrochemical method and the enzymatic cycling method (r = 0.9372, P < 0.001) for the serum TBA concentrations measurement. The equivalent test was used to further evaluate the deviations between the two methods. The cut-off value of the equivalent (δ) was 1.106 μmol/L (±10% RSD) to ensure less than 10% of RSD in the enzymatic cycling method. The data indicated that the established assay and the enzymatic cycling method were equivalent (t_1 = -0.073, p_1 = 0.47; t_2 = 0.035, p_2 = 0.49).

<Figure 4>

4. Conclusions

An ultrasensitive electrochemical detection assay was established by the enzymatic reaction combined with the double oxidation circular amplification strategy. The method is simple, ultrasensitive, cost-effective and without sample preparation except dilution for serum TBA. It correlated well with the enzymatic cycling method used in clinic and is quite suitable for the subjects with difficult blood collection, such as infants, children and some small animals. It has the potential to be used in point-of-care testing for the clinical samples. Our next work is to combine the sensing with micro-fluidic chips to separate bile acids components.

Acknowledgements

The authors thank the First Affiliated Hospital of Chongqing Medical University for collecting the serum specimens. The project was supported by the National Natural Science Foundation of China (81401228) and the Natural Science Foundation Project of CQ CSTC (cstc2014jcyjA10082).

Declarations of interest: None.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version.

References

Altintas, Z., Akgun, M., Kokturk, G., Uludag, Y., 2018. Biosens. Bioelectron. 100, 541-548.

Barling, J., Shiel, A., Weis, D., 2008. J. Anal. At. Spectrom. 23, 511-516.

Bartling, B., Li, L., Liu, C.C., 2009. Analyst 134, 973-979.

Belgaumkar, A.P., Vincent, R.P., Carswell, K.A., Hughes, R.D., Alaghband-Zadeh, J., Mitry, R.R., Le, R.C., Patel, A.G., 2016. Obes. Surg. 26, 1195-1202.

Chen, J.B., Deng, W.P., Wang, J.W., Shao, Y., Ou M.L., Ding, M., 2013. Int. J. Gynaecol. Obstet. 122, 5-8.

Chiang, J.Y.L., 2009. J. Lipid Res. 50, 1955-1966.

Costarelli, V., Key, T.J., Appleby, P.N., Allen, D.S., Fentiman, I.S., Sanders, T.A., 2002. Br. J. Cancer 86, 1741-1744.

Danielsson, H., Sjovall, J., 1975. Annu. Rev. Biochem. 44, 233-253.

Downey, T.M., Nieman, T.A., 1992. Anal. Chem. 64, 261-268.

Ducroq, D.H., Morton, M.S., Shadi, N., Fraser, H.L., Strevens, C., Morris, J., Thomas, M.A., 2010. Ann. Clin. Biochem. 47, 535-540.

Farhana, L., Nangia-Makker, P., Arbit, E., Shango, K., Sarkar, S., Mahmud, H., Hadden, T., Yu, Y., Majumdar, A.P.N., 2016. Stem Cell Res. Ther. 7, 181.

Ferrer C., Lozano A., Agüera A., Girón, A.J., Fernández-Alba, A.R., 2011. J. Chromatogr. A 1218, 7634-7639.

Fiorucci, S., Distrutti, E., 2015. Trends Mol. Med. 21, 702-714.

Gao, J.Y., Xu, B., Zhang, X.Q., Cui, Y., Deng L.L., Shi Z., Shao Y., Ding M., 2016. Clin. Chim. Acta. 459, 63-72.

Goto, T., Myint, K.T., Sato, K., Wada, O., Kakiyama, G., Iida, T., Hishinuma, T., Mano, N., Goto, J., 2007. J. Chromatogr. B Analyt Technol. Biomed. Life Sci. 846, 69-77.

Jin, M.C., Zhang, X.Q., Zhen, Q.N, He, Y.F., Chen, X., Lyu, W.J., Han, R.C., Ding, M., 2017. Biosens. Bioelectron. 98, 392-397.

Okuda, H., Obata, H., Nakanishi, T., Hisamitsu, T., Matsubara, K., Watanabe, H., 1984a, Hepatology 4, 3S-6S.

Okuda, H., Obata, H., Nakanishi, T., Hisamitsu T., Matsubara K., Watanabe H., 1984b, Hepatogastroenterology 31, 168-171.

Puri, P., Daita, K., Joyce, A., Mirshahi, F., Santhekadur, P.K., Cazanave, S., Luketic, V.A., Siddiqui, M.S., Boyett, S., Min, H.K., Kumar, D.P., Kohli, R., Zhou, H., Hylemon, P.B., Contos, M.J., Idowu, M., Sanyal, A.J., 2017. Hepatology 67,

29359.

Rani, K., Garg, P., Pundir, C.S., 2004. Anal. Biochem. 332, 32-37.

Reyes, H., 2016. Hepatology 63, 4-8.

Setchell, K.D.R., Heubi, J.E., Shah, S., Lavine, J.E., Suskind, D., Al-Edreesi, M., Potter, C., Russell, D.W., O'Connell, N.C., Wolfe, B., Jha, P., Zhang, W., Bove, K.E., Knisely, A.S., Hofmann, A.F., Rosenthal, P., Bull, L.N., 2013. Gastroenterology 144, 945-955.

Slijepcevic, D., Roscam Abbing, R.L.P., Katafuchi, T., Blank, A., Donkers, J.M., van Hoppe, S., de Waart, D.R., Tolenaars, D., van der Meer, J.H.M., Wildenberg, M., Beuers, U., Oude Elferink. R.P.J., Schinkel, A.H., van de Graaf, S.F.J., 2017. Hepatology 66, 1631-1643.

Sonne, D.P., Samuel, v.N.F., Kulik, W., Soeters, M.R., Vilsbøll, T., Knop, F.K., 2016. J. Clin. Endocrinol. Metab. 101, 3002-3009.

Sugita, T., Amano, K., Nakano, M., Masubuchi, N., Sugihara, M., Matsuura, T., 2015. Gastroenterol. Res. Pract. 2015, 717431.

Tang, Y.M., Wang, J.P., Bao, W.M., Yang, J.H., Lin-Kun, M.A., Jing, Y., Hui, C., Ying, X.U., Yang, L.H., Wen, L.I., 2015. Int. J. Mol. Med. 36, 377-385.

Tian, G., Zhang, X.Q., Zhu, M.S., Zhang, Z., Shi, Z.H., Ding, M., 2016. Sci. Rep. 6, 23569.

Walker, I.A., Nelson-Piercy, C., Williamson, C., 2002. Ann. Clin. Biochem. 39, 105-113.

Wildgrube, H., Stockhausen, H., Metz, P., Mauritz, G., Mahdawi, R., 1983. Clin. Chem. 29, 494-498.

Wu, J., Yan, F., Zhang, X., Yan, Y., Tang, J., Ju, H., 2008. Clin. Chem. 54, 1481-1488.

Ye, L., Liu, S.Y., Wang, M., Shao, Y., Ding, M., 2007. J. Chromatogr. B 860, 10-17.

Zhang, G.H., Cong, A.R., Xu, G.B., Li, C.B., Yang R.F., Xia, T.A., 2004. Biochem. Biophys. Res. Commun. 326, 87-92.

Zhang, X., Deng, W., Ban, Y., Gao, J., Ding, M., 2013. Analyst 138, 5074-5080.

Zhang, X., Zhu, M., Xu, B., Cui, Y., Tian, G., Shi, Z., Ding, M., 2016. Biosens. Bioelectron. 85, 563-567.

Zhao, M., Zhang, S., Chen, Z., Zhao. C., Wang, L., Liu, S., 2018. Biosens. Bioelectron.105, 42-48.

Figure captions:

Scheme 1 Principle of enzymatic coupling double oxidization electrochemical detection of TBA. Different types of bile acids have different groups for R, R_1, R_2 and

R_3, as listed in Table 1 in the Supporting Information (Table S1). In the enzymatic reaction, the carbon-oxygen bond at the 3α-OH group in bile acids is oxidized to carbon-oxygen double bond.

Fig. 1 Amperometric *I-t* curves. (A) 5.0 μL of 10.0 pmol/L DCA added into 50 μL of 70 mmol/L PBS (pH 9.50) containing 5.0 mmol/L NAD^+ and 1.0 kU/L 3α-HSD. (B) 5.0 μL of 10.0 pmol/L DCA added into 50 μL of 70 mmol/L PBS (pH 9.50) containing 5.0 mmol/L NAD^+ and 7.0 mmol/L $Ru(bpy)_3^{2+}$. (C) 5.0 μL of 10.0 pmol/L NADH added into 50 μL of 70 mmol/L PBS (pH 9.50) containing 7.0 mmol/L $Ru(bpy)_3^{2+}$. (D) 5.0 μL of 10.0 pmol/L DCA added into 50 μL of 70 mmol/L PBS (pH 9.50) containing 5.0 mmol/L NAD^+, 1.0 kU/L 3α-HSD and 7.0 mmol/L $Ru(bpy)_3^{2+}$.

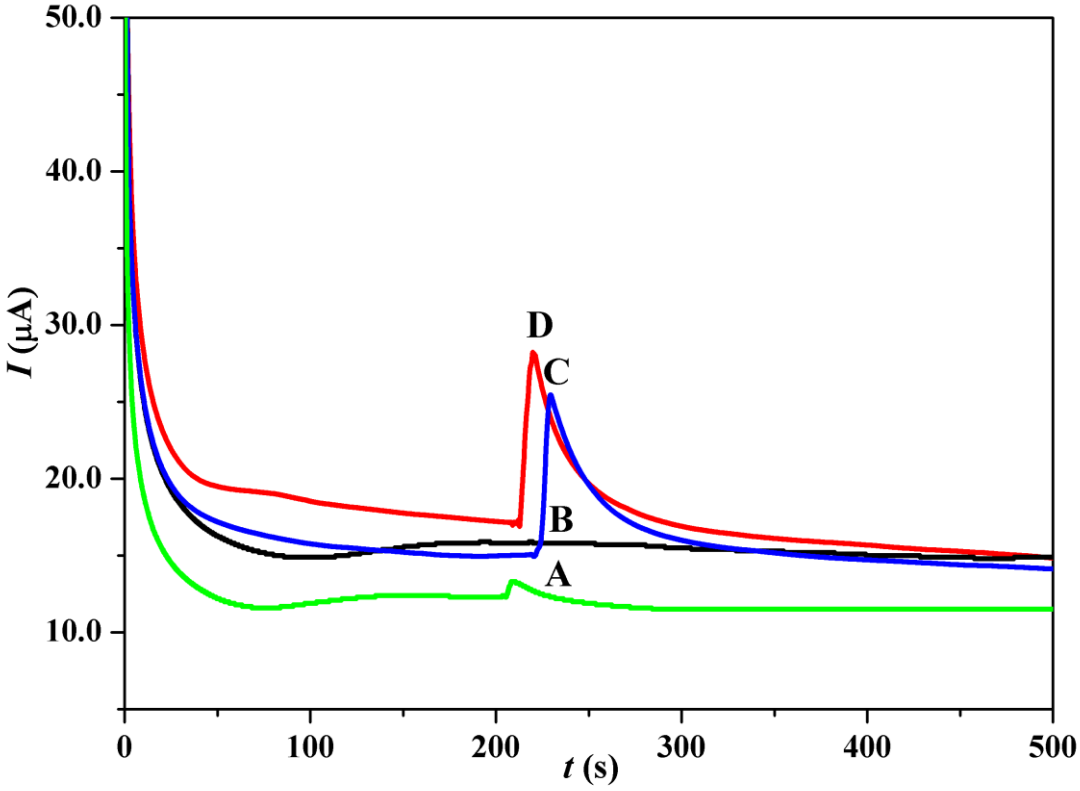
Fig. 2 The matrix effect of serum at different dilution times.

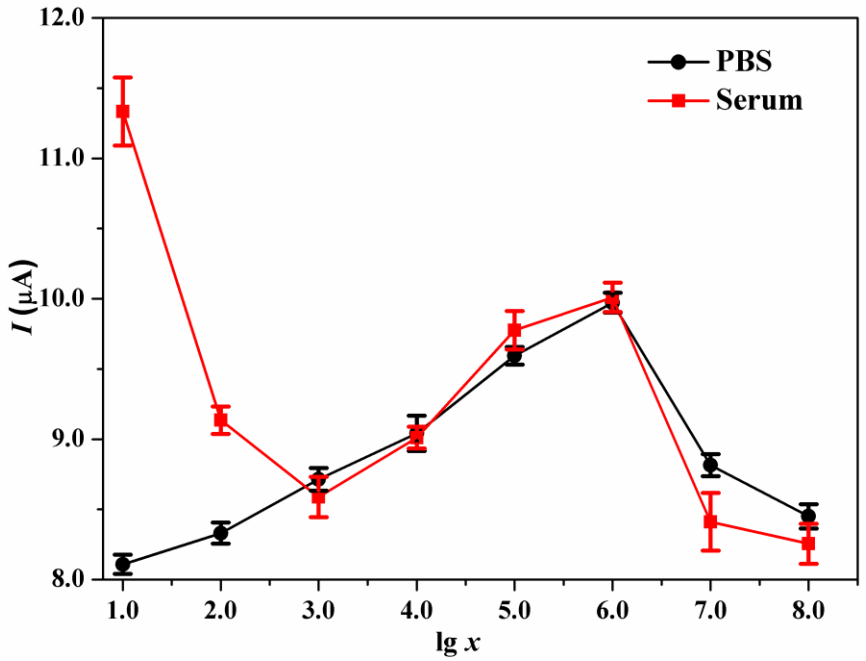
Fig. 3 The calibration curve of DCA by the established electrochemical method.

Fig. 4 The correlation of the two methods in the detection of serum TBA in clinical samples.

Highlights

- The sensitivity of the electrochemical biosensor for serum TBA was greatly improved by the use of a novel enzymatic reaction combined with the double oxidation circular amplification strategy.
- An ultrasensitive electrochemical biosensor for serum TBA was developed with disposable unmodified SPCE without any other sample preparation.
- The electrochemical biosensor was applied to detect serum TBA from clinical samples and the results were well correlated with the data from the routine enzymatic cycling method.
- As only a small amount of volume was required for the determination, the method is quite suitable for the serum TBA of individuals with difficult blood collection, such as infants, children and some small animals for scientific researches.

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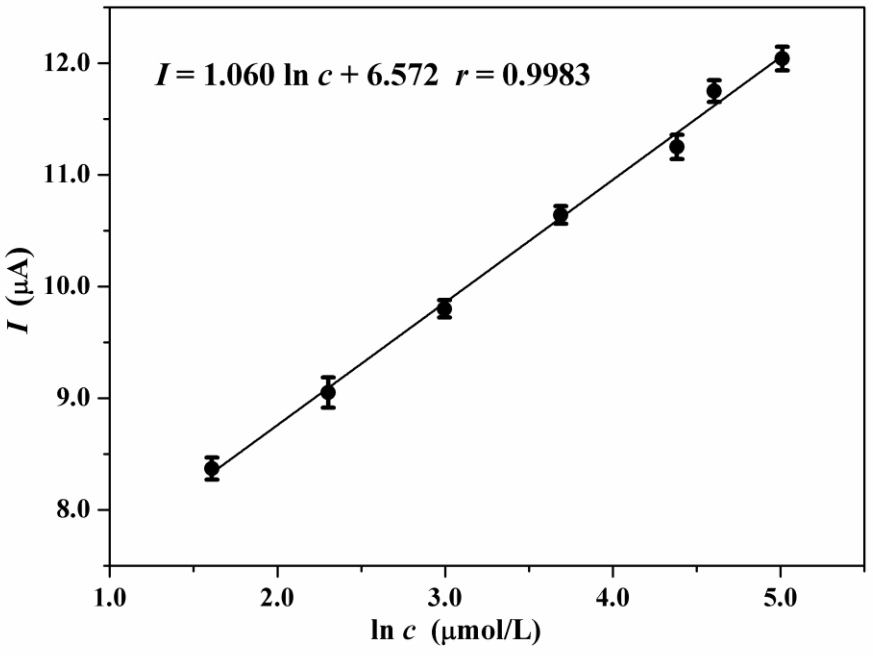
I (μA)
lg x


I = 1.060 ln c + 6.572 r = 0.9983
I (μA)
ln c (μmol/L)