Biotechnol Lett
DOI 10.1007/s10529-014-1547-9

ORIGINAL RESEARCH PAPER

Highly sensitive electrochemiluminescence biosensors for cholesterol detection based on mesoporous magnetic core–shell microspheres

Juanjuan Zhang · Shihong Chen · Xingrong Tan · Xia Zhong · Dehua Yuan · Yinfeng Cheng

Received: 21 March 2014 / Accepted: 1 May 2014

Abstract A sensitive electrochemiluminescence (ECL) biosensor for cholesterol detection based on multifunctional core–shell structured microspheres (Fe_3O_4@SiO_2–Au@mpSiO_2) is reported. This microsphere consisted of a core of silica-coated magnetite nanoparticle, an active transition layer of gold nanoparticles and a mesoporous silica shell. Scanning electron microscopy was employed to observe the morphology of the nanomaterials and transmission electron microscopy was used to further confirm the subtle structure of Fe_3O_4@SiO_2–Au@mpSiO_2. The microspheres possessed a large surface area that increased enzyme loading, and an active transition layer gold nanoparticles enhanced the ECL signal. They were used to immobilize cholesterol oxidase for cholesterol detection with a high sensitivity, low detection limit and wide linear range. The linear range was from 0.83 to 2.62 mM with a detection limit of 0.28 μM (S/N = 3). Moreover, the reproducibility, stability and selectivity of the biosensor were established.

Keywords Biosensor · Cholesterol oxidase · Electrochemiluminescence · Luminol · Magnetic core–shell microspheres

Introduction

Cholesterol and its esterified form are of vital importance for human beings since they build a block for body tissue and are the precursors of other biological materials, such as bile acid and steroid hormones. The level of total cholesterol is associated with numerous clinical disorders, such as arteriosclerosis, coronary heart diseases, hyperthyroidism, anaemia, etc. (Shih et al. 2009). Therefore, development of a biosensor that allows convenient and rapid determination of cholesterol is necessary for its regular estimation in clinical analysis/diagnosis.

Electrochemiluminescence (ECL) is a method of converting electrical energy into chemical energy. Compared with conventional chemiluminescence, it represents advantages such as high controllability, reproducibility, and sensitivity (Richter et al. 2012). Among various ECL systems, the luminol/H_2O_2-ECL

Electronic supplementary material The online version of this article (doi:10.1007/s10529-014-1547-9) contains supplementary material, which is available to authorized users.

J. Zhang · S. Chen (✉) · X. Zhong · D. Yuan · Y. Cheng
Key Laboratory of Luminescent and Real-Time Analytical Chemistry (Southwest University), Ministry of Education, College of Chemistry and Chemical Engineering, Southwest University, Chongqing 400715, People's Republic of China
e-mail: cshong@swu.edu.cn

X. Tan (✉)
Department of Endocrinology, 9th People's Hospital of Chongqing, Chongqing 400700, People's Republic of China
e-mail: Tangxr2832@sina.com

Published online: 27 May 2014

system was applied in constructing enzyme biosensors because luminol's co-reactant, H_2O_2, is the product of enzymatic reactions based on oxidase type enzymes (Ballesta-Claver et al. 2012). In the presence of H_2O_2, luminol(5-amino-2, 3-dihydro-1,4-phthalazinedione) can generate intense ECL emission. Therefore, some effective luminol-ECL systems were reported for the measurement of glucose, lactate, cholesterol, and choline (Zhang et al. 2012; Xu et al. 2010). Thus, luminol-based ECL biosensors greatly enlarged the application of enzyme biosensors.

Currently, magnetic core–shell nanomaterials have gained attention in nanoscience and nanotechnology due to their excellent optical, electrical, thermal, mechanical, magnetic and catalytic properties (Gulfam et al. 2011; Chen et al. 2009, 2010). Mesoporous materials have great potential in enzyme immobilization, adsorption, drug delivery, molecular sieves and separation (Ma et al. 2010). Several studies using mesoporous materials to immobilize enzymes for biosensors construction have been reported (Jiang et al. 2011; Zhao et al. 2013). Thus, mesoporous-magnetic core–shell nanomaterials endow further alternative properties for enzyme biosensors' construction.

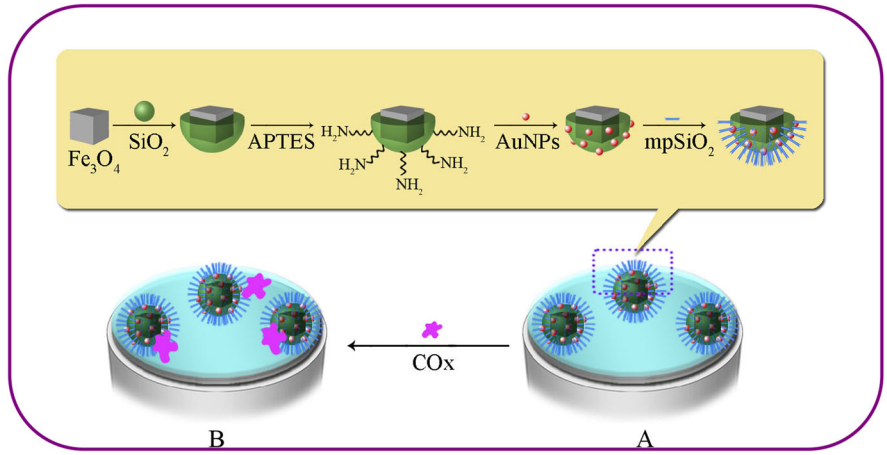
Enlightened by above observation, in this work, we have synthesized multifunctional nanospheres with a core of silica-coated magnetic nanoparticles, an active translation layer of uniform gold nanoparicles (AuNPs) and an outer shell of mesoporous silica ($mpSiO_2$) with ordered nanopore channels using such techniques as sol–gel technique, interfacial deposition and surfactant-templated synthesis. This multifunctional nanomaterial is designated as $Fe_3O_4@SiO_2$–$Au@mpSiO_2$. In this nanoarchitecture, Fe_3O_4 not only serves as the core to help the growth of shell structure SiO_2–$Au@mpSiO_2$ but also simplifies the synthesis process of $Fe_3O_4@SiO_2$–$Au@mpSiO_2$ microspheres due to its magnetically separable property. The mesoporous silica shell could well confine the AuNPs and make the AuNPs accessible to the reactant because of the ordered pore channels outside (Deng et al. 2008). Furthermore, mesoporous silica would be an optimal choice to immobilize cholesterol oxidase since the silica-based mesoporous structure provides many positions suitable for enzyme loading and retains the enzyme's activity to a large extent. The resultant microspheres demonstrated high magnetization, large surface area, highly open mesopores and high loading of AuNPs. They were applied to immobilize cholesterol oxidase to construct a cholesterol biosensor in luminol-ECL system for the first time. Due to the following reasons: (1) AuNPs with good conductivity, large surface area, and excellent electro-activity to luminol oxidization obviously enhanced the ECL intensity, (2) the mesoporous structure could increase enzyme loading and maintain the activity of the enzyme to a large extent at the same time, and (3) the nanoarchitecture with a unique structure effectively combined good properties of each component, this material would provide a new and promising platform for enzyme biosensors in luminol/H_2O_2 ECL system.

Reagents

Cholesterol oxidase (COx, EC 1.1.3.6, $\geq$50 U/mg) obtained from *Brevibacterium* sp., cholesterol, Triton X-100, chloroauric acid ($HAuCl_4$), trisodium citrate, 3-aminopropyltriethoxysilane (APTES) and tetraethyl orthosilicate (TEOS) were from Sigma. A stock 0.01 M cholesterol solution was prepared in PBS containing 10 % (w/w) of Triton X-100 at 65 °C, then stored at 4 °C. Cholesterol oxidase solution was prepared in 0.05 M PBS (pH 7.0), stored at 4 °C when not used. Other chemicals used were of analytical grade and were used as received. Deionized water was used throughout this study.

Apparatus

ECL emission was performed using a model MPI-A electrochemiluminescence analyzer (Xi'an Remax Electronic Science & Technology Co. Ltd., China), equipped with a photomultiplier tube (PMT) and set at 600 V for detection. Cyclic voltammetry (CV) was carried out with a CHI 600D electrochemical work station (Shanghai CH Instruments Co., China). The conventional three-electrode system, including a modified glassy carbon electrode (GCE) as working electrode, a saturated calomel electrode (SCE) or Ag/AgCl as reference electrode and a platinum wire as counter electrode was used in the detection process. Scanning electron microscopy (SEM) was conducted with a Hitachi scanning electron microscope (SEM, Hitachi, S-4800, Japan). Transmission electron microscopy (TEM) was performed on a TECNAI 10 (Philips Fei Co., Hillsboro, OR). All the experiments were carried out at room temperature.



Scheme 1 Illustration of the synthesis process of Fe_3O_4@SiO_2–Au@mpSiO_2 (the *inset*), and the biosensor preparation process. *Step A*: a 10 µL Fe_3O_4@SiO_2–Au@mpSiO_2 suspension was cast onto a pretreated GCE surface and dried at room temperature. *Step B*: a 3 µL COx solution was dropped onto the electrode

Synthesis of multicomponent Fe_3O_4@SiO_2–Au@mpSiO_2 microspheres

Fe_3O_4@SiO_2–Au@mpSiO_2 microspheres were synthesized according to Deng et al. (2008) and Schwertmann and Cornell (1991) with slight modifications. Firstly, Fe_3O_4 magnetite nanoparticles were synthesized as follows. $FeCl_2{\cdot}4H_2O$ (5.55 g) was dissolved in 100 ml deionized water previously saturated with N_2 and heated to 90 °C under N_2 bubbling. Then 50 ml water with 1.3 g KNO_3 and 9.35 g KOH was dropwise added under continuous stirring and N_2 bubbling and stirring was maintained at 90 °C for 2 h. The mixture was left overnight and separated with a magnet to recover the black powder (Fe_3O_4).

Secondly, silica-coated Fe_3O_4 (Fe_3O_4@SiO_2) and APTES-modified Fe_3O_4@SiO_2 nanoparticles were synthesized. Briefly, 0.2 g Fe_3O_4 was dispersed into 150 ml ethanol under sonication for 15 min in an ice bath. Then 400 µl TEOS and 12 ml 28 % (w/v) NH_4OH were added into the solution and reacted in an ice bath for 2 h under sonication. The resultant (Fe_3O_4@SiO_2) was separated with a magnet and thoroughly washed with ethanol. To obtain APTES-modified Fe_3O_4@SiO_2 nanoparticles, 0.15 g Fe_3O_4@SiO_2 was dispersed into 50 ml 2-propanol containing 1 ml APTES and then the dispersions were heated to 40 °C under stirring for 3 h. Subsequently, the black resultant APTES-modified Fe_3O_4@SiO_2 was thoroughly washed with ethanol and deionized water using a magnet.

Thirdly, Fe_3O_4@SiO_2–Au and Fe_3O_4@SiO_2–Au@mpSiO_2 microspheres were synthesized. Briefly, 12.5 ml APTES-modified Fe_3O_4@SiO_2 (0.8 % w/w in water) was mixed with 7.5 ml gold colloid solution in quick sonication in a water bath, then subjected to mechanical stirring for 8 h to obtain Fe_3O_4@SiO_2–Au nanoparticles. Gold colloid solution was prepared according to Deng et al. (2008). The resultant Fe_3O_4@SiO_2–Au nanoparticles were separated and washed with deionized water. To grow a mesoporous silica shell on the Fe_3O_4@SiO_2–Au nanospheres, 0.06 g Fe_3O_4@SiO_2–Au, 0.225 g CTAB, 1 ml 28 % (w/v) NH_4OH, 50 ml deionized water and 75 ml ethanol were mixed under sonication for 5 min and mechanically stirred for 30 min to form a uniform dispersion. Then, 1.5 ml TEOS was added dropwise to the dispersion under continuous stirring for 6 h at 30 °C. The resultant precipitate was collected and washed consecutively with ethanol and deionized water with the help of magnet and then was redispersed in 100 ml acetone and refluxed at 80 °C for 48 h to remove the template CTAB. The product (Fe_3O_4@SiO_2–Au@mpSiO_2) was washed with deionized water and dispersed in water for further use. The typical synthetic process is shown in Scheme 1. This nanomaterial was used to immobilize COx for achieving a cholesterol biosensor. The sensor preparation is also schematically shown in Scheme 1.

Result and discussion

Characterization of the synthesized nanomaterials

The morphological characteristics of the as-prepared nanomatericals were characterized by SEM. The Fe_3O_4 magnetic nanoparticles (Fig. 1a) showed

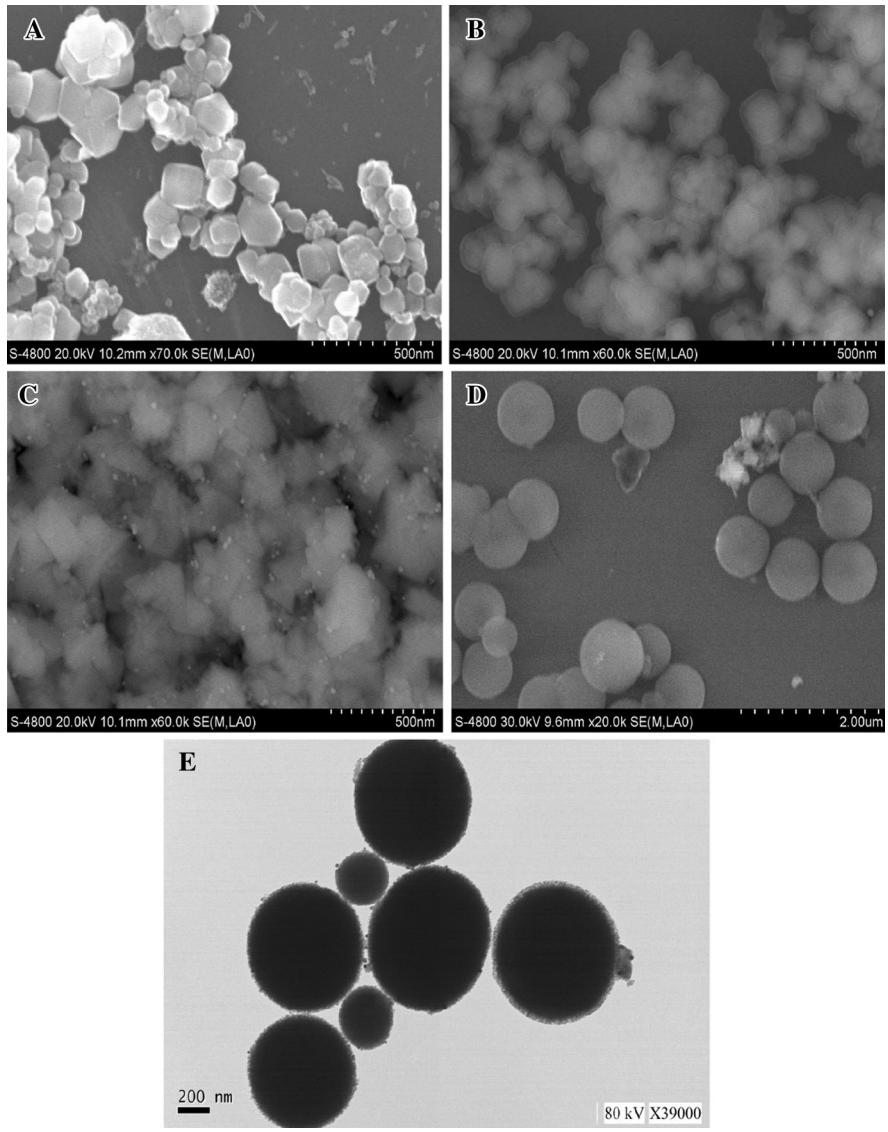


Fig. 1 SEM images of **a** Fe_3O_4, **b** $Fe_3O_4@SiO_2$, **c** $Fe_3O_4@SiO_2$–Au and **d** $Fe_3O_4@SiO_2$–Au@mpSiO_2, and **e** TEM image of $Fe_3O_4@SiO_2$–Au@mpSiO_2

irregular hexagons and quadrangles with aggregates of each other, due to the magnetic interactions between nanoparticles themselves. When a silica layer was modified onto the surface of Fe_3O_4, the resultant core–shell $Fe_3O_4@SiO_2$ microspheres (Fig. 1b) displayed more regular spherical shapes with smooth surface. The image of $Fe_3O_4@SiO_2$–Au microsphere is presented in Fig. 1c and AuNPs were obviously observed. After $Fe_3O_4@SiO_2$–Au nanoparticle was coated with a mesoporous silica layer, the resulting $Fe_3O_4@SiO_2$–Au@mpSiO_2 microsphere displayed a well-defined and uniform sphere structure with the diameter about 500 nm, as shown in Fig. 1d. To more clearly visualize the morphology of $Fe_3O_4@SiO_2$–Au@mp-SiO_2 nanocomposites, TEM technique was employed. As expected, Fig. 1e displayed a loose surface of the $Fe_3O_4@SiO_2$–Au@mpSiO_2 nanospheres.

Characterization of the biosensor fabrication process

SEM was employed to characterize the biosensor fabrication process. A typical SEM image of the resulting modified film of COx on the $Fe_3O_4@SiO_2$–

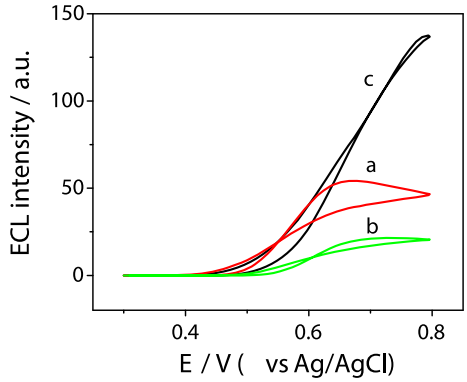


Fig. 2 ECL characterization of differently modified electrode: **a** bare GCE, **b** Fe_3O_4@SiO_2–Au@mpSiO_2/GCE, and **c** COx/Fe_3O_4@SiO_2–Au@mpSiO_2/GCE in 0.050 M PBS (pH 7.4) under 0.0067 mM cholesterol. Scan rate: 100 mV/s

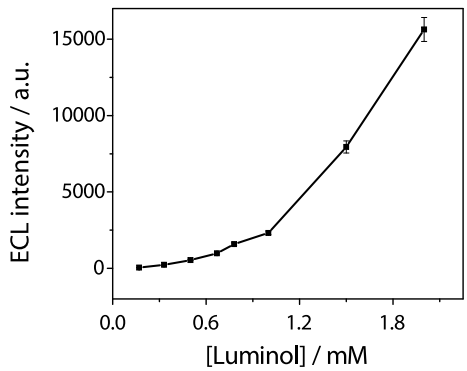


Fig. 3 The impact of luminol concentration on the ECL signal at the biosensor in 0.05 M PBS (pH 7.4)

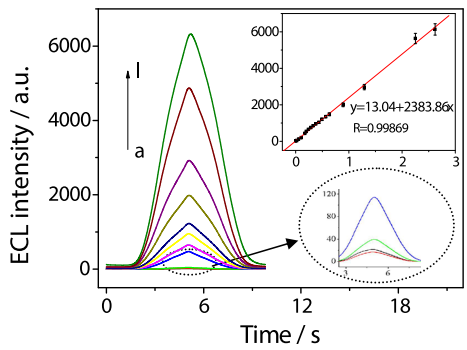


Fig. 4 ECL responses of the biosensor COx/Fe_3O_4@SiO_2–Au@mpSiO_2/GCE to 0 (**a**), 0.00083 (**b**), 0.022 (**c**), 0.055 (**d**), 0.17 (**e**), 0.24 (**f**), 0.67(**g**), 0.49 (**h**), 0.89 (**i**),1.3 (**j**), 1.5 (**k**) and 2.6 (**l**) mM cholesterol in the presence of luminol (0.67 mM). Inset shows linear relationship between the ECL signal intensity and the cholesterol concentration

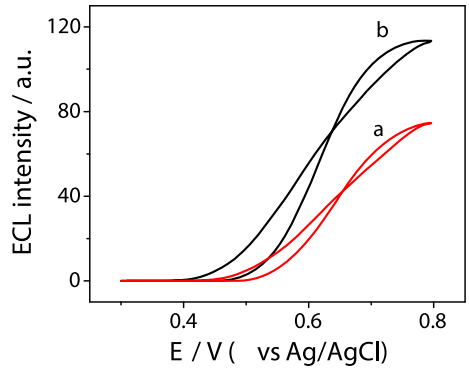


Fig. 5 The ECL responses of **a** COx/Fe_3O_4@SiO_2–Au and **b** COx/Fe_3O_4@SiO_2–Au@mpSiO_2 modified electrode in 0.05 M PBS (pH 7.4) in the presence of 0.017 mM cholesterol

Au@mpSiO_2 microspheres is shown in Supplementary Fig. 1. The sphere structure of Fe_3O_4@SiO_2–Au@mpSiO_2 was also visible but it became relatively blurry compared with Fe_3O_4@SiO_2–Au@mpSiO_2 modified film (Fig. 1d), indicating that COx was successfully immobilized on the surface of Fe_3O_4@SiO_2–Au@mpSiO_2 microspheres modified film.

Cyclic voltammetry (CV) is also a convenient and valuable technology to probe the interface features of the modified electrodes. Supplementary Fig. 2 shows the cyclic voltammograms (CVs) of different modified electrodes in 5 mM $[Fe(CN)_6]^{4-/3-}$ solution. A pair of well-defined oxidation and reduction peaks of $[Fe(CN)_6]^{4-/3-}$ was observed at the bare GCE (curve a). After the Fe_3O_4@SiO_2–Au@mpSiO_2 nanocomposites were modified on the surface of GCE (curve b), the peak current of the modified electrode decreased, indicating that the Fe_3O_4@SiO_2–Au@mpSiO_2 nanocomposites have been immobilized successfully. When COx was adsorbed on the electrode (curve c), the peak current further decreased, due to the hindrance caused by non-conductive COx.

The ECL characterization was performed and the results are shown in Fig. 2. Compared to the bare GCE (curve a), the ECL intensity of the Fe_3O_4@SiO_2–Au@mpSiO_2/GCE decreased (curve b), which may be attributed to the insulated property of silica. After COx was immobilized on the electrode (curve c), the ECL intensity was enhanced due to the H_2O_2 produced by the enzymatic reaction on the biosensor surface. The mechanism is as follows.

Table 1 Recoveries of cholesterol by the biosensor

Sample	$C^{a}_{Original}$(mM)	C_{Added} (mM)	$C_{Detected}$ (mM)	RSD (%)	Recovery (%)
1	0.020	0.020	0.038	2.3	95
2	0.30	0.20	0.49	4.6	98
3	0.50	0.20	0.77	4.1	110

[a] concentration of cholesterol

$$\text{Cholesterol} + O_2 \xrightarrow{\text{COX}} \text{Cholest - 4 - 3 - one} + H_2O_2 \quad (1)$$

Under positive potential, the resulting H_2O_2 in Eq. (1) reacted with luminol to generate an ECL signal (shown in Eq. 2). The intensity in ECL signal was directly proportional to the cholesterol concentration.

$$H_2O_2 + \text{luminol} \xrightarrow{\text{AuNPs}} \text{aminophthalate} + \text{hI} \quad (2)$$

Performance of the biosensor

To get an optimal performance of the proposed biosensor, the effect of luminol concentration in tested solutions on ECL signal was studied and the results are shown in Fig. 3. As seen, the ECL intensity increased with the increase in luminol concentration. If the concentration of luminol was further increased, the ECL intensity would continue to increase and even would exceed the maximum intensity accessible to the apparatus (the maximum value of ECL intensity that can be detected by the apparatus is about 17400 a.u.). A high concentration of luminol will therefore lead to a high background signal and will result in a narrow detectable range of cholesterol concentrations. Considering the sensitivity, detection limit and detectable range, 0.67 mM luminol was chosen for further experiments.

The performance of the proposed biosensor was evaluated by detecting standard cholesterol solutions using ECL technique in 0.05 M phosphate buffered saline (PBS) containing 0.67 mM luminol. As shown in Fig. 4, the ECL signal increased with the concentration of cholesterol. The inset of Fig. 4 describes the linear relationship between ECL intensity and the concentration of cholesterol. The linear range covered from 0.83 to 2.62 mM with a detection limit of 0.28 μM (signal to noise = 3). Compared with other cholesterol sensors (Supplementary Table 1) with different detection methods, this ECL biosensor established in this work had low detection limit and high sensitivity. The reasons may be ascribed to the synergistic effect of each component in the Fe_3O_4@-SiO_2–Au@mSiO_2 microspheres. The mesoporous nanostructure endowed high enzyme loading and the gold transition layer presented a catalytic property to enhance ECL signal in luminal-H_2O_2 system.

To demonstrate the effect of mesoporous silica (mSiO_2) shell with ordered nanopore channels on the ECL response to cholesterol, a control experiment between COx/Fe_3O_4@SiO_2–Au and COx/Fe_3O_4@-SiO_2–Au@mSiO_2 modified electrode was investigated in 0.05 M PBS (pH 7.4) in the presence of 0.017 mM cholesterol and the results are shown in Fig. 5. As expected, the ECL response of the biosensor with mSiO_2 (curve b) was greater than that of without mSiO_2 (curve a). This was due to the mSiO_2 shell endowing more enzyme loading, thus resulting in an improvement in ECL response to cholesterol.

The stability of the proposed biosensor was also investigated. It was measured at intervals of 2–3 days by ECL, and stored at 4 °C when not in use. The response of ECL signal decreased gradually and maintained about 90.3 % of the initial value in 1 week.

Specificity is an important factor for evaluating the performance of a biosensor. The change in ECL signal generated from glucose or tryptophan were negligible after 20-fold concentration of glucose or tryptophan were added into 0.05 M PBS (pH 7.4) containing 0.0067 mM cholesterol, which indicated the biosensor had a good anti-interference ability.

Recovery experiment

The analytical applicability of the biosensor was evaluated by standard addition method. Table 1 lists the recovery of three samples of different cholesterol concentrations. The recovery rate was between 95 and

110 %. The satisfying results demonstrated that the biosensor had potential for practical application.

Conclusions

A cholesterol ECL biosensor based on the synergistic effect of each component in the magnetic core–shell microsphere (Fe_3O_4@SiO_2–Au@m$pSiO_2$) is reported. This multifuntional nanosphere consists of a core of silica-coated magnetite nanoparticle, an active gold transition layer and an outer shell of mesoporous silica shell with nanopore channels. The nanocomposite with a mesoporous surface abled high COx loading. The nanoporous channels made the gold nanopartiles accessible which perform high catalytic ability to the luminol/H_2O_2 ECL system. Therefore, the proposed biosensor gave a satisfactory performance for cholesterol detection. The multifuntional nanospheres would offer a good substrate for enzyme immobilization for constructing an enzyme biosensor in luminol/H_2O_2 ECL system.

Acknowledgments This work is supported by National Natural Science Foundation of China (21075100, 21275119), Ministry of Education of China (708073), Specialized Research Fund for the Doctoral Program of Higher Education (20100182110015), State Key Laboratory of Electroanalytical Chemistry (SKLEAC 2010009), Natural Science Foundation Project of Chongqing City (CSTC-2011BA7003) and Fundamental Research Funds for the Central Universities (XDJK2012A004).

References

Ballesta-Claver J, Ametis-Cabello J, Morales-Sanfrutos J, Megía-Fernández A, Valencia-Mirón MC, Santoyo-González F, Capitán-Vallvey LF (2012) Electrochemiluminescent disposable cholesterol biosensor based on avidin–biotin assembling with the electroformed luminescent conducting polymer poly(luminol-biotinylated pyrrole). Anal Chim Acta 754:91–98

Batra N, Tomar M, Gupta V (2012) Realization of an efficient cholesterol biosensor using ZnO nanostructured thin film. Analyst 137:5854–5859

Chen YJ, Gao P, Wang RX, Zhu CL, Wang LJ, Cao MS, Jin HB (2009) Porous Fe_3O_4/SnO_2 core/shell nanorods, synthesis and electromagnetic properties. J Phys Chem C 113: 10061–10064

Chen YJ, Zhang F, Zhao GG, Fang XY, Jin HB, Gao P, Zhu CL, Cao MS, Xiao G (2010) Synthesis, multi-nonlinear dielectric resonance, and excellent electromagnetic absorption characteristics of Fe_3O_4/ZnO core/shell nanorods. J Phys Chem C 114:9239–9244

Deng YH, Qi DW, Deng CH, Zhang XM, Zhao DY (2008) Superparamagnetic high-magnetization microspheres with an Fe_3O_4@SiO_2 core and perpendicularly aligned mesoporous SiO_2 shell for removal of microcystins. J Am Chem Soc 130:28–29

Dhand C, Singh SP, Arya SK, Datta M, Malhotra BD (2007) Cholesterol biosensor based on electrophoretically deposited conducting polymer film derived from nano-structured polyaniline colloidal suspension. Anal Chim Acta 602: 244–251

Gulfam M, Lee JM, Kim J, Lim DW, Lee EK, Chung BG (2011) Highly porous core-shell polymeric fiber network. Langmuir 27:10993–10999

Jiang XY, Wu YH, Mao XY, Cui XJ, Zhu LD (2011) Amperometric glucose biosensor based on integration of glucose oxidase with platinum nanoparticles/ordered mesoporous carbon nanocomposite. Sens Actuators B 153:158–163

Karjalainen S, Sewon L, Soderling E, Larsson B, Johansson I, Simell O, Lapinleimu H, Seppanen R (1997) Salivary cholesterol of healthy adults in relation to serum cholesterol concentration and oral health. J Dent Res 76: 1637–1643

Ma GC, Yan XQ, Li YL, Xiao LP, Huang ZJ, Lu YP, Fan J (2010) Ordered nanoporous silica with periodic 30-60 nm pores as an effective support for gold nanoparticle catalysts with enhanced lifetime. J Am Chem Soc 132:9596–9597

Matharu Z, Arya SK, Singh SP, Gupta V, Malhotra BD (2009) Langmuir-Blodgett film based on MEH-PPV for cholesterol biosensor. Anal Chim Acta 634:243–249

Richter MM (2004) Electrochemiluminescence (ECL). Chem Rev 104:3003–3036

Safavi A, Farjami F (2011) Electrodeposition of gold–platinum alloy nanoparticles on ionic liquid–chitosan composite film and its application in fabricating an amperometric cholesterol biosensor. Biosens Bioelectron 26:2547–2552

Schwertmann U, Cornell RM (1991) Iron Oxides in the Laboratory. Preparation and characterization, 1st edn. VCH, Weinheim

Shih W-C, Yang M-C, Lin MS (2009) Development of disposable lipid biosensor for the determination of total cholesterol. Biosens Bioelectron 24:1679–1684

Xu SJ, Liu Y, Wang TH, Li JH (2010) Highly sensitive electrogenerated chemiluminescence biosensor in profiling protein kinase activity and inhibition using gold nanoparticle as signal transduction probes. Anal Chem 82: 9566–9572

Zhang MH, Yuan R, Chai YQ, Chen SH, Zhong HA, Wang C, Cheng YF (2012) A biosensor for cholesterol based on gold nanoparticles-catalyzed luminol electrogenerated chemiluminescence. Biosens Bioelectron 32:288–292

Zhao MG, Huan JY, Zhou Y, Chen Q, Pan XH, He HP, Ye ZZ (2013) A single mesoporous ZnO/Chitosan hybrid nanostructure for a novel free nanoprobe type biosensor. Biosens Bioelectron 43:226–230