Electronic Supplementary Material (ESI) for RSC Advances.

Supplementary Information

Portable and Quantitative Evaluation of Stem Cell Therapy towards Damaged Hepatocytes

Lifang Zhao[a] *, Qin Wei*[a]*, Hua Wu*[a]*, He Li*[a,b*]*, Dong Li*[c*]*, Shyam S. Mohapatra*[d*]

([a] Key Laboratory of Chemical Sensing & Analysis in Universities of Shandong, School of Chemistry and Chemical Engineering/ [b] School of Biological Science and Technology, University of Jinan, Jinan 250022, China

[c] Cryomedicine Lab, Qilu Hospital of Shandong University, Jinan 250012, China

[d] Department of Internal Medicine, Division of Translational Medicine-Nanomedicine Research Center, Tampa, FL 33612, US)

Experimental

1. 1 Apparatus and reagents

Low-glucose Dulbecco-modified Eagle medium (L-DMEM), RPMI-1640 medium, fetal bovine serum (FBS), penicillin, streptomycin, and trypsin were purchased from Gibco-BRL (Grand Island, NY). Vybrant Apoptosis Assay Kit #3 is from Invitrogen. The antibodies used in flow cytometry were against human antigens CD29, CD31, CD34, CD44, CD45, CD73, CD90, CD105, CD133, and CD271 (SeroTec, Raleigh, NC). Anti-albumin antibodys (ab135575 and ab106784) were purchased from Abcam, MA, USA. Albumin, insulin, invertase and bovine serum albumin (BSA) were purchased from Sigma-Aldrich (USA). Albumin ELISA kit was a kind gift from Dr. Wang. Graphite was obtained from Shanghai Carbon Co., Ltd (China). Chloroauric acid ($HAuCl_4 \cdot 4H_2O$) and trisodium citrate were obtained from Shanghai Chemical Reagent Co. (Shanghai, China). Iron trichloride hexahydrate ($FeCl_3 \cdot 6H_2O$), sodium acetate anhydrous, poly (acrylic acid), poly (diallyldimethylammonium chloride) (PDDA, 20%, w/w in water, MW = 200000−350000), and all other chemicals, obtained from Sinopharm Chemical Reagent Co., Ltd (Beijing, China), with analytical reagent grade or better quality. All other reagents were analytical grade without further purification. Double distilled water was used throughout the experiments.

The brand name of the personal glucose meter (PGM) and test trips used for the test in this work was *ACCU-CHEK Performa*. All electrochemical measurements were performed on a CHI 760E electrochemical workstation (Chenhua Instrument

Shanghai Co. Ltd, China). Electrochemical impedance spectroscopy (EIS) was obtained from the IM6e impedance measurement unit (ZAHNER elektrik, Germany). Transmission electron microscope (TEM) images were obtained from a JEOL-1200EX microscope (Japan). UV-vis spectra were performed on a Lambda 35 UV/vis spectrophotometer (Perkin Elmer, USA).

Umbilical cords (UCs, clinically normal pregnancies) were obtained from Qilu Hospital of Shandong University after normal full-term deliveries. Informed consent was obtained from the mothers. Tissue collection for research was approved by the Ethics Committee of Qilu Hospital. Human hepatic L02 cells were was obtained from Cell Bank of Chinese Academy of Sciences, Shanghai, China.

1.2 Mesenchymal stem cell culture and conditioned medium collection

UCs were excised and washed in 0.1 mol/L phosphate buffer (pH 7.4) to remove excess blood. The cords were dissected and the blood vessels were removed. The remaining tissues were cut into small pieces (1-2 mm^3) and placed in plates with L-DMEM, supplemented with 10 % FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin. Cultures were maintained at 37°C in a humidified atmosphere with 5% CO_2. The medium was changed every 3-4 days. Adherent cells proliferated from individual explanted tissues 7-12 days after initiating incubation. At this time, the small tissue pieces were removed from the culture and the adherent fibroblast-like cells were cultured to confluence, which took 2-3 weeks. The cells were then trypsinized using 0.25 % trypsin and passaged at 1×10^4 cells/cm^2 in the medium

described above. The cells were used after five or more passages.

Fifth-to seventh-passage cells were collected and treated with 0.25 % trypsin. The cells were stained with either fluorescein isothiocyanate-conjugated or phycoerythrin-conjugated monoclonal antibodies in 100 μL phosphate buffers for 15 minutes at room temperature, as suggested by the manufacturer. The antibodies used were against human antigens CD29, CD31, CD34, CD44, CD45, CD73, CD90, CD105, CD133, and CD271. Cells were analyzed using flow cytometry system (Guava easyCyte6HT, EMD Millipore, Billerica, MA) and the data were analyzed with Guava Incyte (EMD Millipore). Positive cells were counted and compared to the signal of corresponding immunoglobulin isotypes.

To obtain the conditioned medium (CM), cells were seeded at 2×10^6 cells/75 cm^2 and cultivated until 80% confluence. Thereafter, the medium was replaced and the supernatants were harvested to centrifuged (1000 g for 15 minutes), filtered using 0.22 μm Millipore filter and stored at -80 ˚C until further use.

1.3 Preparation of Au NPs

Au NPs with an average diameter of 13 nm were prepared according to the previous protocol [1]. Briefly, 100 mL of 0.01% $HAuCl_4$ was boiled with vigorous stirring and 2.5 mL 1% trisodium citrate solution was added to the boiling solution quickly. When the solution turned wine-red, indicating the formation of Au NPs, the solution was removed from heat and left to cool with stirring for 5 minutes.

1.4 Preparation of Fe_3O_4 functionalized by PDDA

First, Fe_3O_4 beads terminated by carboxyl groups were prepared following a previously reported protocol with minor modification [2]. Briefly, 1.35 g of iron trichloride hexahydrate, 3.2 g of anhydrous sodium acetate, and 0.5 mL of poly (acrylic acid) were dissolved in ethylene glycol (38 mL) with the help of ultrasound to form a clear solution. Then, the mixture was sealed in three poly tetrafluoroethylene reaction kettles (20 mL capacity), maintained at 200˚C for 6 hours and allowed to cool to room temperature. The black product was separated by the application of a magnetic field, washed alternately with deionized water and ethanol several times, and dried at 70˚C under vacuum.

A colloidal suspension of Fe_3O_4 (10.0 mg in 1 mL of water) was mixed with 1 mL of PDDA solution (1% w). The mixture was achieved after sonication for 30 minutes, and the product of PDDA-Fe_3O_4 was collected using an external magnetic field and washed three times with deionized water.

1.5 Preparation of graphene oxide

Graphene oxide (GO) was produced from graphite by a modification of Hummer's method [3]. In a typical experiment, 5 g of graphite was oxidized by reacting with 100 mL of concentrated H_2SO_4 with stirring for 12 hours. While immersing the reaction vessel in an ice bath, 30 g of $KMnO_4$ was added slowly. After the addition of $KMnO_4$, the solution was stirred at 100˚C for another 12 hours to oxidize the graphite completely to GO. The GO was then thoroughly washed three times with 30% HCl, ethanol and ether respectively. Finally, the brown yellow solid was vacuum-dried at room temperature.

The PDDA functionalized GO (PDDA-GO) was prepared by the method as reported by Lai [4]. 5 mL GO dispersion (0.5 mg/mL) and 10 mL 1% PDDA aqueous solution were mixed and sonicated for 2 hours. The mixed solution was centrifuged at 10000 rpm for 15 minutes to remove the remaining PDDA polymer. After washing with water, PDDA-GO was obtained. The PDDA-GO was suspended in 2.5 mL water and sonicated for 10 minute to obtain a homogeneous solution.

1.6 Experimental design and hydrogen peroxide-induced hepatocytes injury

Briefly, L02 cells were cultured in RPMI-1640 medium containing 20% FBS and 0.01 mg/ml insulin and randomly divided into the following four groups: (1) normal group (L02), (2) H_2O_2-damaged group (L02+H_2O_2), (3) H_2O_2-damaged and normal medium treatment group (L02+H_2O_2+RPMI-1640), and (4) H_2O_2-damaged and MSC-CM treatment group (L02+H_2O_2+CM).

L02 cells in the normal group were incubated in FBS-free RPMI-1640 medium for 2 hours. L02+H_2O_2 group were incubated in 1mM H_2O_2 for 2 hours. L02+H_2O_2+RPMI-1640 group were incubated for 2 hours with 1mM H_2O_2, and then in RPMI-1640 medium containing 20% FBS for 24 hours. In the L02+H_2O_2+CM groups, RPMI-1640 medium containing 20% FBS was replaced by CM. All cell experiments were replicated at least five times. The concentration of H_2O_2 (1mM) and the treatment time applied were determined by our preliminary experiments. The morphologic changes of the cells were observed using an inverted microscope (Olympus, IX71, Japan).

In order to detect the hydrogen peroxide-induced apoptosis, annexin V/propidium iodide (PI) labelling was performed using the Vybrant Apoptosis Assay Kit #3 (Invitrogen) according to the manufacturer's instructions, followed by flow cytometry. L02 cells were scanned in FL1-H (fluorescein isothiocyanate) versus FL2-H (PI) channels using flow cytometry system (Guava) and the data were analyzed with Guava Incyte (EMD Millipore).

1.7 Preparation of Fe_3O_4-Au-Ab_1 bioconjugate

Scheme 1A shows the procedure for the preparation of the Fe_3O_4-Au-Ab_1 bio-conjugate. The preparation process of Fe_3O_4-Au was described in supplementary material. The previously prepared Au NPs were added to the PDDA-Fe_3O_4 nanoparticles with stirring. Au NPs were attached to the surface of the PDDA-Fe_3O_4 nanoparticles through electrostatic interactions. The wine-red colloidal suspension of Au NPs turned colorless after all the Au NPs were attached to the surface of PDDA-Fe_3O_4 nanoparticles. Then, the product was removed from the solution by sedimentation with the help of an external magnetic field. This process was repeated multiple times until the color no longer changed with the addition of Au NPs. The final Fe_3O_4-Au nanocomposites were separated using an external magnetic field and dispersed in 5 mL of deionized water. 1 mL of 100 μg/mL primary anti-albumin antibody (Ab_1) was added to 1 mL of the Fe_3O_4-Au dispersion, and the mixture was shaken overnight at 4˚C. The reaction mixture was magnetically separated and washed three times with phosphate-buffered saline (PBS, 7.4). After discarding the supernatant, the product Fe_3O_4-Au-Ab_1 bioconjugate was resuspended in 1 mL of PBS

containing 1% BSA.

1.8 Preparation of GO-Au-Ab_2@invertase bioconjugates

Scheme 1B shows the procedure for the preparation of the GO-Au-Ab_2@invertase bio-conjugate. The GO-Au-Ab_2@invertase bioconjugates were prepared via a layer-by-layer assembly approach [5-7]. 1.0 mL of the above-synthesized PDDA-GO colloidal suspension and 1 mL of Au NPs solution were mixed and sonicated for 30 minutes to accomplish the conjugation of PDDA-GO and Au NPs. After centrifugation (6000 r/min, 10 minutes), the colorless supernatant was discarded and the purplish-red GO-Au was washed with water and dispersed in 1 mL of 0.1 M PBS. 0.01 mg of antibody solution (Ab_2) and 0.5 ml of invertase (various concentrations) were mixed with 0.5 mL of GO-Au and shaken for 12 hours at 4 ˚C. The resulting composites were centrifuged and washed with PBS. The GO-Au-Ab_2@invertase was suspended in 0.5 mL of PBS and stored at 4˚C before use. To optimize the responses obtained with the label, different ratios of invertase to GO-Au were used for preparation of the label.

1.9 Fabrication of the immunosensor

Scheme 1C indicates the fabrication procedure of the immunosensor. The 96 well polystyrene microplate (Axygen, USA) was first coated with 100 μL of Fe_3O_4-Au-Ab_1, followed by addition of 100 μL of albumin at different concentrations and incubated at 37˚C for 1 hour. Then the wells were washed with PBS (pH 7.4, containing 1 % BSA) three times. Subsequently, the GO-Au-Ab_2@invertase solution

(100 μL) was incubated for 1 hour at 37 ˚C, followed by washing with PBS. Finally, the sucrose solution (in PBS 7.0, 0.5 M, 100 μL) was added to the wells and allowed to react at 45˚C for 30 minutes. The glucose produced by invertase was then monitored by the PGM.

1.10 Statistical Analysis

All experiments were replicated a minimum of three times. Data were expressed as mean ± S.D. The differences between the values were determined by the independent samples test. A value of less than 0.05 was considered statistically significant.

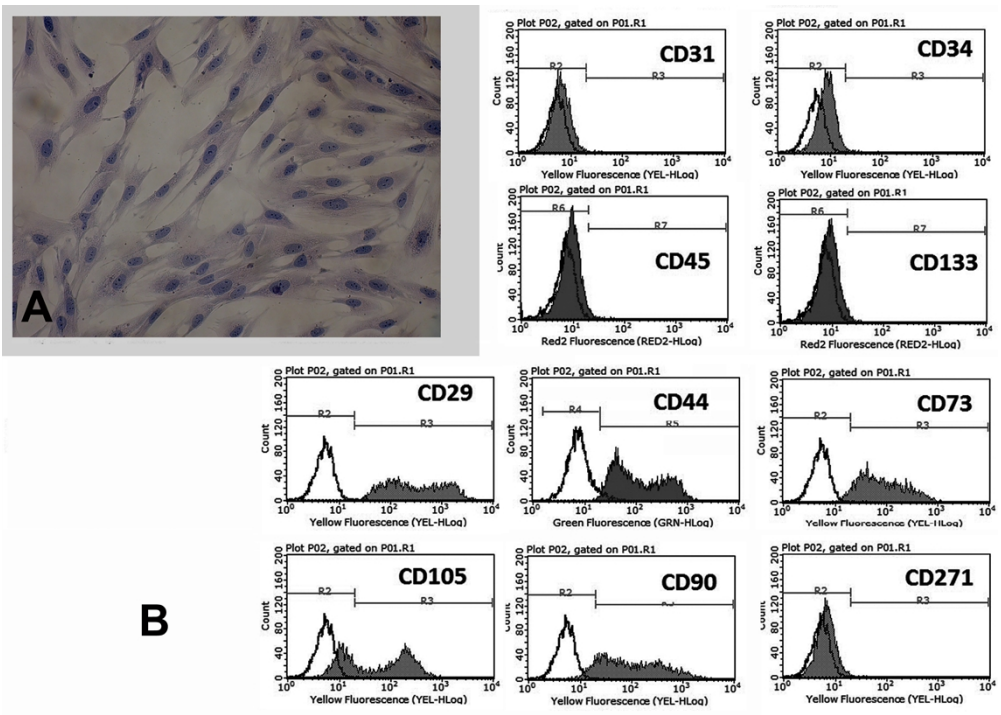
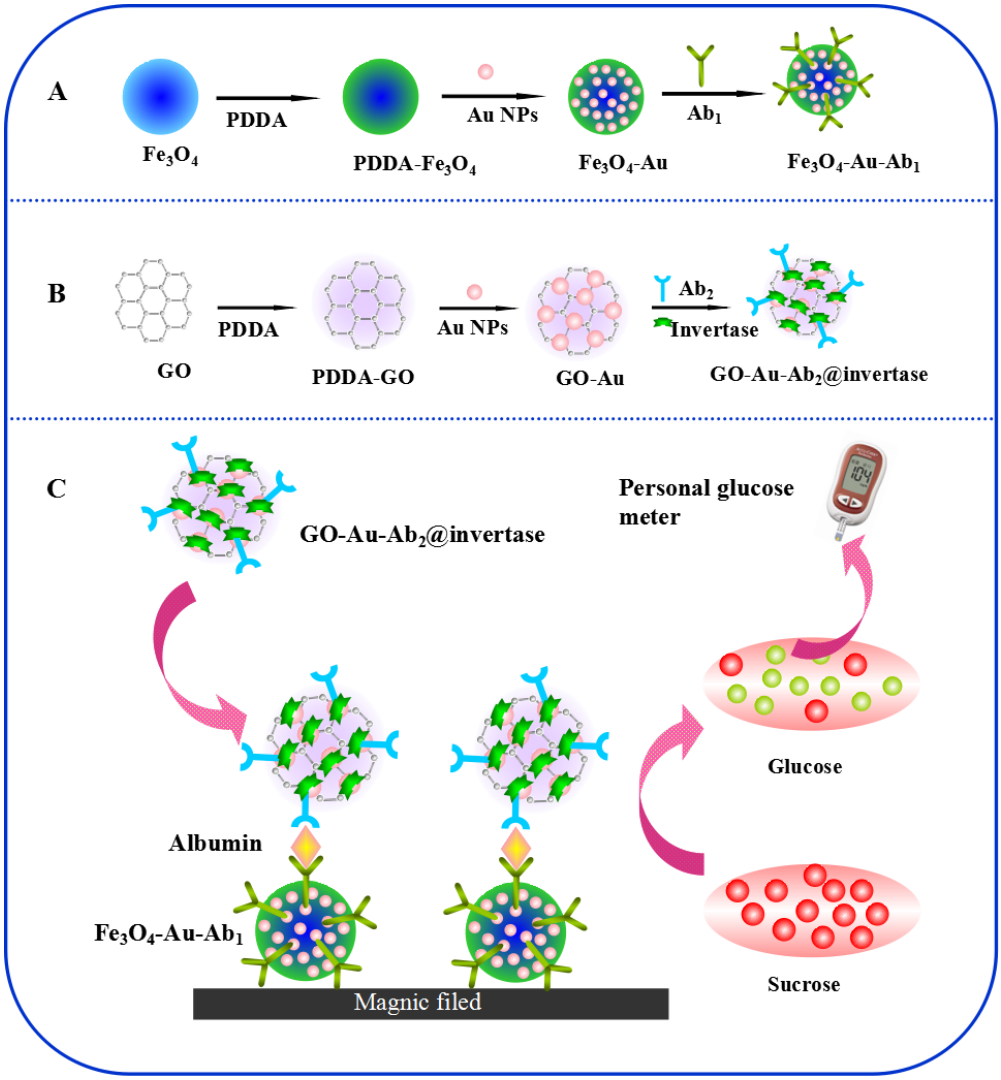


Figure S1 UC-derived MSCs in passaged cultures. (A) H&E staining of UC-derived MSCs. (B) Immunophenotype of UC-derived MSCs.



Scheme 1. Schematic representation of the preparation of the Fe_3O_4-Au-Ab_1 bioconjugate (A), GO-Au-Ab_2@invertase bioconjugate (B) and immunosensor (C).

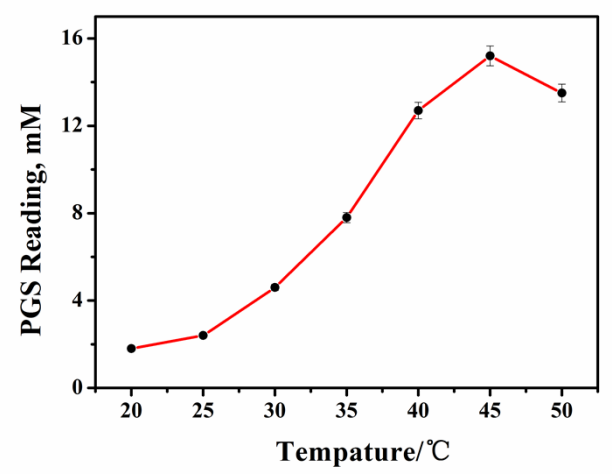


Figure S2. Detection of glucose using a PGM at different temperatures (100 μL 1 M sucrose dissolving in 7.4 PBS + 20 μL 1mg/mL invertase dissolving in 7.4 PBS, 40 minutes).

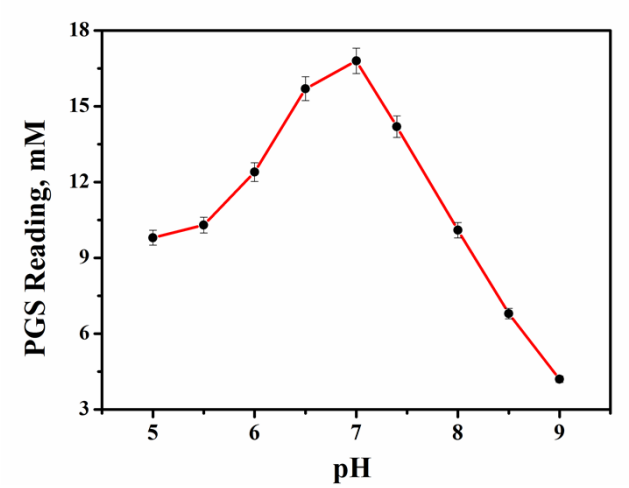


Figure S3. Detection of glucose using a PGM at different pH (100 µL 1 M sucrose + 20 µL 1mg/mL invertase, 45 ˚C, 40 minutes).

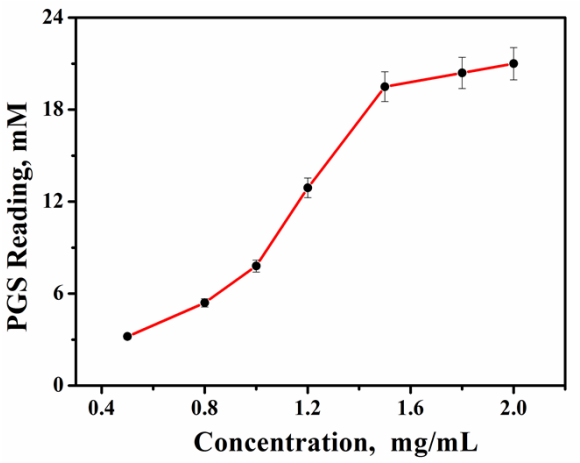


Figure S4. Detection of glucose using a PGM with various concentration invertase immobilized on GO-Au NPs (100 µL 1 M sucrose dissolving in PBS 7.0 + 20 µL GO-Au-Ab_2@invertase, 45 ˚C, 40 minutes).

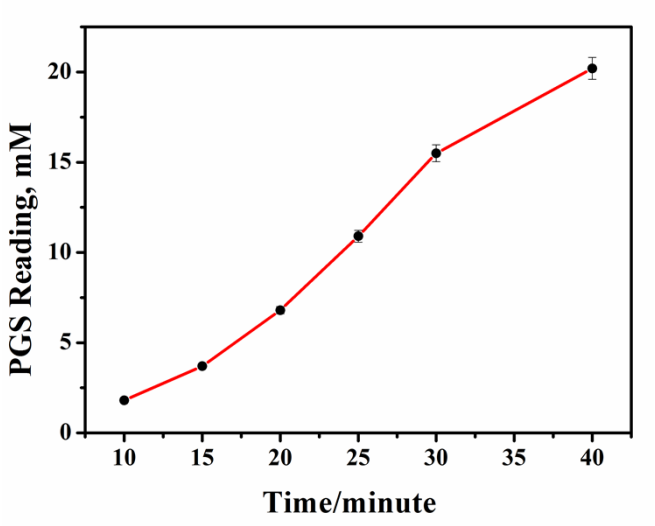


Figure S5. The response of immunosensor (albumin, 1 μg/mL) using a PGM with different reaction time for the hydrolyzing of sucrose (45 ˚C, 100 μL 1 M source dissolving in PBS 7.0).

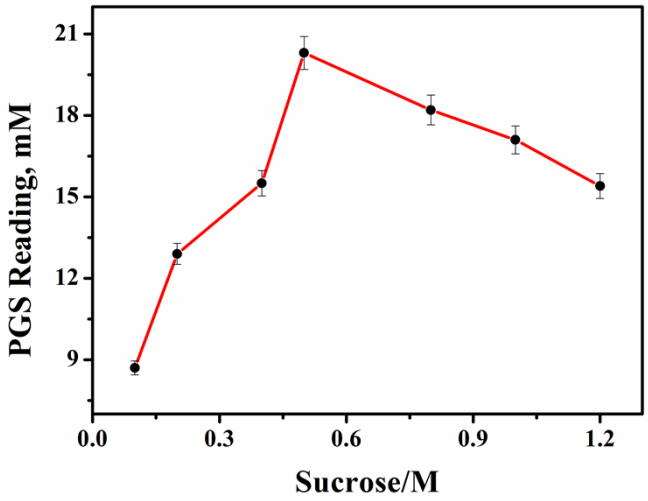


Figure S6. The response of immunosensor (albumin, 1μg/mL) using a PGM with different concentrations of sucrose (45 ˚C, 30 mintues).

Reference

[1] G. Lai, F. Yan and H. Ju, Dual signal amplification of glucose oxidase-functionalized nanocomposites as a trace label for ultrasensitive simultaneous multiplexed electrochemical detection of tumor markers, Analytical chemistry, 81 (2009) 9730-9736.

[2] T. Zheng, J.J. Fu, L. Hu, F. Qiu, M. Hu, J.J. Zhu, Z.C. Hua and H. Wang, Nanoarchitectured Electrochemical Cytosensors for Selective Detection of Leukemia Cells and Quantitative Evaluation of Death Receptor Expression on Cell Surfaces, Analytical chemistry, 85 (2013) 5609-5616.

[3] Q. Wei, T. Li, G. Wang, H. Li, Z. Qian and M. Yang, Fe_3O_4nanoparticles-loaded PEG–PLA polymeric vesicles as labels for ultrasensitive immunosensors, Biomaterials, 31 (2010) 7332-7339.

[4] Y. Lai, J. Bai, X. Shi, Y. Zeng, Y. Xian, J. Hou and L. Jin, Graphene oxide as nanocarrier for sensitive electrochemical immunoassay of clenbuterol based on labeling amplification strategy, Talanta, 107 (2013) 176-182.

[5] J.F. Quinn, A.P. Johnston, G.K. Such, A.N. Zelikin and F. Caruso, Next generation, sequentially assembled ultrathin films: beyond electrostatics, Chemical Society reviews, 36 (2007) 707-718.

[6] X. Hong, J. Li, M. Wang, J. Xu, W. Guo, J. Li, Y. Bai and T. Li, Fabrication of

magnetic luminescent nanocomposites by a layer-by-layer self-assembly approach, Chemistry of materials, 16 (2004) 4022-4027.

[7] J. Li, X.W. Zhao, Y.J. Zhao and Z.Z. Gu, Quantum-dot-coated encoded silica colloidal crystals beads for multiplex coding, Chem. Commun., (2009) 2329-2331.