



Short communication

CdSe/ZnS quantum dots based electrochemical immunoassay for the detection of phosphorylated bovine serum albumin

Kulwadee Pinwattana^a, Jun Wang^b, Chiann-Tso Lin^b, Hong Wu^b, Dan Du^b, Yuehe Lin^{b,*}, Orawon Chailapakul^{a,c,**}^a Department of Chemistry, Faculty of Science, Chulalongkorn University, Patumwan, Bangkok 10330, Thailand^b Pacific Northwest National Laboratory, 902 Batteley Boulevard Richland, WA 99352, United States^c Center of Excellence for Petroleum, Petrochemicals and Advanced Materials, Chulalongkorn University, Patumwan, Bangkok 10330, Thailand

ARTICLE INFO

Article history:

Received 27 May 2010

Received in revised form 31 July 2010

Accepted 12 August 2010

Available online 20 August 2010

Keywords:

Electrochemical immunoassay

Quantum dots

Biomarker

Phosphorylated bovine serum albumin

ABSTRACT

A CdSe/ZnS quantum dot (QD) based electrochemical immunoassay of phosphorylated bovine serum albumin (BSA-OP) as a protein biomarker is presented. The QDs were used as labels for amplifying electrochemical signals and were conjugated with a secondary anti-phosphoserine antibody in a heterogeneous sandwich immunoassay. In this assay, the model phosphorylated protein BSA-OP was added to the primary BSA antibody coated polystyrene microwells, and then the QD labeled anti-phosphoserine antibody was added for completing immunorecognition. Finally, the bound QD was dissolved in an acid-dissolution step and was detected by electrochemical stripping analysis. The measured current responses were proportional to the concentration of BSA-OP. Under optimal conditions, the voltammetric response was linear over the range of 0.5–500 ng mL⁻¹ of BSA-OP, with a detection limit of 0.5 ng mL⁻¹. It also shows good reproducibility with a relative standard deviation of 8.6%. This QD-based electrochemical immunoassay offers great promise for simple and cost-effective analysis of protein biomarkers.

© 2010 Elsevier B.V. All rights reserved.

1. Introduction

Protein phosphorylation is one of the most important and most thoroughly investigated post-translational modifications in living organisms (D'Ambrosio et al., 2007; Hu et al., 2008; Paradela and Albar, 2008). Because of its fundamental role in the regulation of cellular functions, such as cell division, signal transduction, and cell differentiation, much effort has concentrated on the development of detection methodologies for the different substrates (D'Ambrosio et al., 2007). The phosphorylation process has been demonstrated to involve some 518 protein kinases and 100 phosphoprotein phosphatases in the human genome (Kerman and Kraatz, 2009; Mann et al., 2002). Approximately 30% of all proteins in eukaryotic cells are supposed to be phosphorylated at any given time (Mann et al., 2002). Recently, many human diseases have been recognized to be associated with the abnormal phosphorylation of cellular protein and protein phosphorylation is also associated with the pathways of cancer (Hu et al., 2008).

Therefore, the analysis of protein phosphorylation has increasingly become the focus of attention in the field of proteomics due to its highly dynamic nature and low stoichiometry (Paradela and Albar, 2008). In general, the detection method of protein phosphorylation includes mass spectrometry (MS), radiolabeling, high-performance liquid chromatography (HPLC), electrophoresis, enzyme-linked immunosorbent assays (ELISA), and fluorescence-based immunoassay (D'Ambrosio et al., 2007; Kerman et al., 2007; Mann et al., 2002; McLachlin and Chait, 2001; Paradela and Albar, 2008; Yan et al., 1998). Unfortunately, the above-mentioned methods often involve radiation hazards, are time-consuming and labor-intensive, or requiring highly qualified personnel and sophisticated instrumentation.

Currently, electrochemical immunoassays combining the recognition events of specific antibodies with electrochemical detection techniques have been successfully developed (Pastor-Navarro et al., 2007; Roucaïrol et al., 2007; Tramontina et al., 2007). Electrochemical techniques have numerous advantages for use in biosensor development: for example, they are relatively inexpensive, inherently simple and applicable to optically opaque media, and suitable for miniaturizable instrumentation with low power requirements (Kuramitz et al., 2006; Tang et al., 2007, 2008). Thus, the combination of the specificity of immunological recognition and electrochemical detection can achieve the detection of analytes with high sensitivity and specificity (Chen et al., 2007; Dungchai et al., 2008; Preechaworapun et al., 2008). Furthermore, a larger

* Corresponding author. Tel.: +1 509 376 0529; fax: +1 509 376 5106.

** Corresponding author at: Department of Chemistry, Faculty of Science, Chulalongkorn University, 254 Phayathai Road, Patumwan, Bangkok 10330, Thailand. Tel.: +66 2 218 7615; fax: +66 2 218 7615.

E-mail addresses: yuehe.lin@pnl.gov (Y. Lin), corawon@chula.ac.th (O. Chailapakul).

number of labels, such as various nanoparticles (gold and silica), QDs (CdS, PbS, and ZnS), and apoferritin nanoparticles, have been successfully developed for labeling (Kerman et al., 2007; Liu and Lin, 2007; Song et al., 2008; Wang et al., 2008a,b; Wu et al., 2007). In particular, to enhance the sensitivities, biosensors and bioassays using electrochemical stripping analysis based on QDs have shown great potential for the detection of trace biomolecules (Wang et al., 2008b; Wu et al., 2007). Especially, QDs have been widely used for the labeling and biosensing of electrochemical immunoassays because of their unique advantages such as nanometer size similar to proteins and versatility in surface modification with various biomolecules (peptides, oligonucleotides, and proteins) (Wang et al., 2008b; Wu et al., 2007; Yang et al., 2009).

In this research, we report on an electrochemical immunoassay approach for the quantification of BSA-OP using QDs as electrochemical labels. The serine site of BSA was phosphorylated and BSA-OP was chosen as a model phosphoprotein in this work. The results demonstrated that this approach is simple and sensitive for the detection of phosphorylated proteins.

2. Materials and methods

2.1. Reagents

Phosphoserine-bovine serum albumin (BSA-OP), bovine serum albumin (BSA), phosphate buffer saline (PBS 0.01 M), Tween20, γ -globulins, cadmium chloride (CdCl_2), and hydrochloric acid (HCl) were purchased from Sigma–Aldrich (St. Louis, MO). Anti-phosphoserine antibody was purchased from Abcam, Inc. (Cambridge, MA). Monoclonal anti-bovine serum albumin (anti-BSA) was purchased from Rockland Immunochemicals, Inc. (Gilbertsville, PA). Polyethylene glycol-6000 (PEG) was obtained from Fluka. The Qdot®655 antibody conjugation kit was purchased from Molecular Probes, Inc. (Eugene, OR), which includes QD nanocrystals (CdSe/ZnS), succinimidyl-4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (SMCC) stock solution, dithiothreitol (DTT) stock solution, dye-labeled markers for antibody elution, β -mercaptoethanol, separation media, and exchange buffer. All reagents were of analytical grade and were prepared using deionized or autoclaved water.

2.2. Apparatus

A C24 Incubator Shaker was purchased from New Brunswick Scientific (Edison, NJ). A benchtop Eppendorf centrifuge 5415D (Eppendorf, Germany) was used in the conjugation purification step. Square-wave voltammetric (SWV) measurements were performed with a μ Autolab type III Potentiostat with a GPES 4.9 software package (Eco Chemie, Netherlands) connected to a personal computer. Disposable screen-printed electrodes (SPEs) were purchased from Alderon Biosciences, Inc. (Durham, NC). The 96-well microplates for immunoassay were purchased from Costar (Corning, NY).

2.3. Preparation of QD-anti-phosphoserine conjugates

The QD-anti-phosphoserine conjugates were prepared with the Qdot®655 antibody conjugation kit following the manufacturer's protocol. The QDs were coated with a layer of polymer by the manufacturer. This process involved six steps: (A) QD nanocrystals in dimethylsulfoxide solution were activated with hetero-bifunctional crosslinker (10 mM of SMCC) at room temperature for 1 h, and then excess SMCC was removed via a NAP-5 desalting column with the exchange buffer provided in the kit as the elution solvent. Approximately 500 μL of the QDs with maleimide-nanocrystal surfaces were collected. (B) 300 μL

of 0.5 mg mL^{-1} anti-phosphoserine antibody was reduced with 20 mM DTT at room temperature for 0.5 h to expose free sulfhydryl groups and then (C) labeled with the dye-labeled marker and purified with the NAP-5 desalting column, collecting approximately 500–600 μL of the colored fraction. (D) The activated QD nanocrystals and reduced antibodies were then mixed and allowed to conjugate (sulfur–maleimide bond formation) at room temperature for 1 h. (E) The reaction was quenched by the addition of β -mercaptoethanol and concentrated to a final volume of 20 μL via ultrafiltration (7000 rpm for approximately 10–15 min on a benchtop Eppendorf centrifuge) using the 0.5 mL concentrator supplied in the kit. (F) Finally, the QD-anti-phosphoserine conjugates were purified by Superdex 200 gel chromatography with PBS as the eluent buffer and collecting the initial dark brown-colored eluent in the dead space of the column ($\sim 200 \mu\text{L}$), which was stored at 4°C before use. The QD-anti-phosphoserine conjugates were further characterized with transmission electron microscopy (TEM). The sample was prepared by dispersing 5 μL of 200-fold diluted QD-anti-phosphoserine solution on the surface of the carbonate support film of a TEM grid, and allowing it to dry at room temperature.

2.4. Immunoassay procedure

This procedure is divided into six steps: (A) 50 μL of a 200-fold dilution of the anti-BSA antibody stock solution was added into a 96-well microplate and incubated at 4°C overnight with gentle shaking. (B) After the solution was removed, the wells were washed four times with washing buffer (0.05% (v/v) Tween20 in 0.01 M PBS) followed by the addition of 250 μL of blocking buffer (2% (w/v) PEG and 3% (w/v) globulin in 0.01 M PBS) and incubating at 37°C for 2 h with gentle shaking to block the remaining active sites. (C) The plates were then washed, followed by the addition of 50 μL of BSA-OP of the desired concentration into the wells and incubation at 37°C for 1 h with gentle shaking. (D) The wells were then washed four times, as mentioned above, prior to the addition of 50 μL of a 200-fold dilution of the QD-anti-phosphoserine conjugates and incubation at 37°C for 1 h with gentle shaking in order to obtain the sandwich immunoreactions. (E) The completed microplate was washed four more times. Finally, to release cadmium ions from the captured QDs, 20 μL of 1 M HCl was added into each well, and then the solution was mixed with 50 μL of acetate buffer with 10 ppm Hg. (F) The resulting solution was quantified by square wave voltammetry, as described in the following section.

2.5. Electrochemical measurements

SWV measurements were performed with a SPE consisting of a carbon working electrode, a carbon counter electrode, and an Ag/AgCl reference electrode. Each SPE was first pretreated electrochemically by cyclic voltammetric scanning 10 times at a potential range of 0–1.5 V in 0.05 M PBS. After washing and drying with an airstream, 50 μL of sample solution was dropped onto the sensing area of the SPE. The cadmium solution was measured with SWV using an *in situ* plated mercury film on the SPE. The SWV parameters used were a conditioning potential of 0.6 V for 60 s, deposition potential of -1.2 V for 120 s, equilibration time of 10 s, amplitude of 25 mV, step potential of 4 mV, and a frequency of 15 Hz. A baseline correction of the resulting voltammogram was performed using the GPES 4.9 software.

3. Results and discussion

In this study, the synthesis of QD-anti-phosphoserine conjugates was schematically summarized in Fig. 1A. To facilitate the conjugation with biological molecules, the QD coated with a

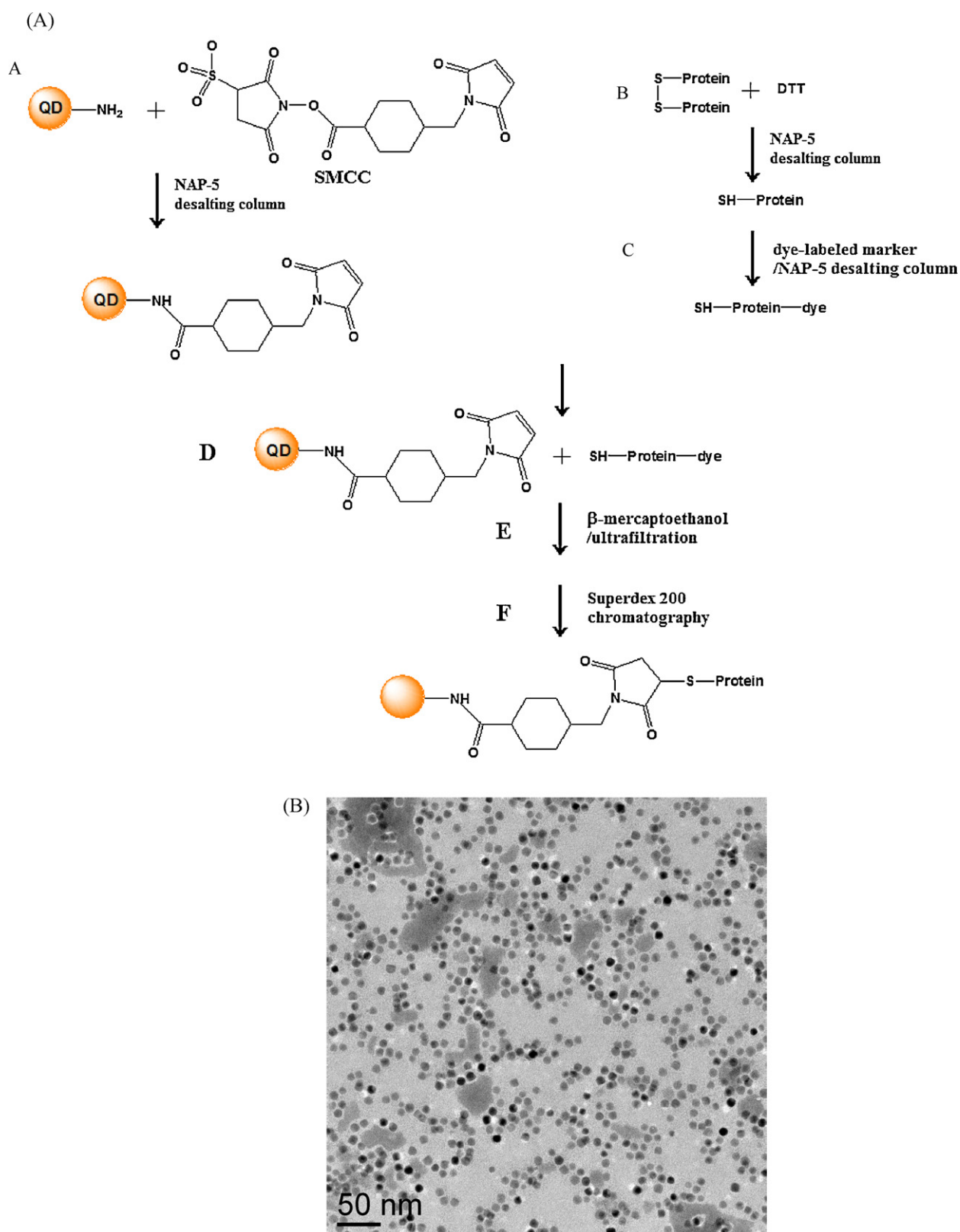


Fig. 1. (A) Schematic illustration of the synthetic procedure for the preparation of the QD-anti-phosphoserine conjugates. (B) Transmission electron microscopy (TEM) image of the QD-anti-phosphoserine conjugates.

polymer shell. The QD-anti-phosphoserine conjugates were characterized by measuring the protein concentration (bicinchoninic acid assay (BCA) method) and QD concentration (using a molar extinction coefficient of $650,000 \text{ M}^{-1} \text{ cm}^{-1}$ and a measurement

wavelength of 638 nm). The average number of anti-phosphoserine antibodies per QD was estimated to be four. As shown in Fig. 1B, TEM image shows that the QD-anti-phosphoserine conjugates are uniform in size, without aggregation, and have an average diameter

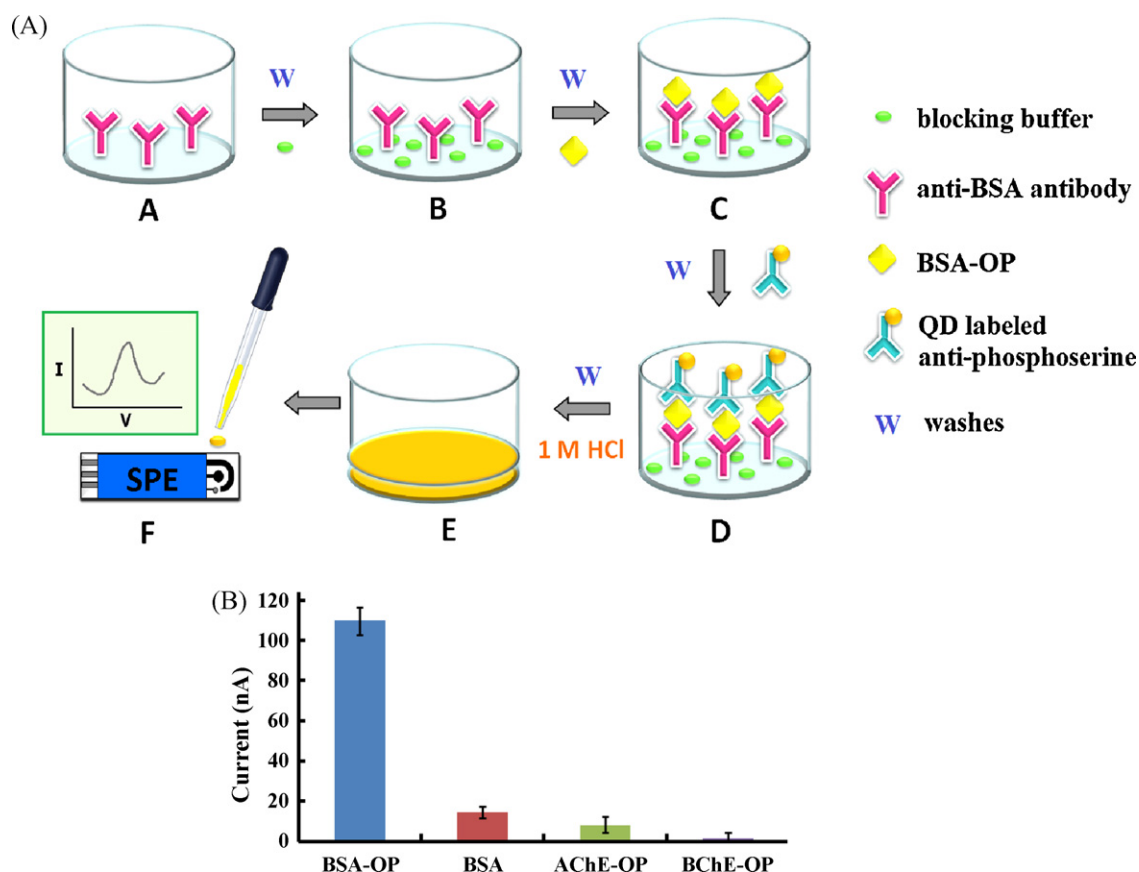


Fig. 2. A schematic illustration of the determination of BSA-OP based on a sandwich electrochemical immunoassay using quantum dots as labels. (A) The anti-BSA antibody was immobilized onto the microplate wells; (B) spare sites were blocked with blocking buffer; (C) BSA-OP was captured by the immobilized antibodies; (D) QD-anti-phosphoserine conjugates recognized the OP moiety of BSA-OP; (E) QD labels were dissolved by acid solution; (F) released cadmium ions were determined by SWV using a disposable SPE. B: The electrochemical response of the assays in different protein solutions including BSA-OP, BSA, AChE-OP, and BChE-OP. The concentration of the four different proteins is 100 ng mL^{-1} .

of approximately 10 nm which was similar to other reports (Wang et al., 2008b; Wu et al., 2007).

Fig. 2A displays the schematic illustration for the determination of BSA-OP based on the QD labels and the electrochemical immunoassay. Firstly, BSA-OP was captured with the primary anti-BSA on the well of the microplate, and then the QD-anti-phosphoserine conjugates were added to form a sandwich immunoassay. By adding HCl, the QDs from the complex were dissolved and cadmium ions were released. These cadmium ions were measured with SWV using an *in situ* plated mercury film on the SPE. The concentration of cadmium ions was directly proportional to the amount of BSA-OP. It was found that both the stripping peak potential both of standard cadmium ions and of those obtained from the dissolved QDs were -0.75 V .

It was found that there is non-specific binding of the QD-anti-phosphoserine conjugates for this assay. Optimization of experiments conditions to minimize the non-specific binding was further performed. The QD-anti-phosphoserine conjugates were diluted with 3% (w/v) globulin in 0.01 M PBS to make a series of concentrations from the stock solution for testing. A 200-fold dilution was found to provide the lowest background, so this ratio was chosen for the assay (data not shown). To further reduce the non-specific adsorption of the conjugates, the effect of blocking buffers on signal to noise ratio between the electrochemical responses of 10 ng mL^{-1} BSA-OP and 0 ng mL^{-1} BSA-OP was investigated (see Supporting information Fig. S1). It was found that the blocking buffer containing 3% (w/v) globulin and 1% PEG could mostly lower background and achieve higher signal to noise ratio. Changing the concentration of PEG from 0.5% to 3% (w/v) (all in the co-presence

of 3% (w/v) globulin in PBS) was studied, and it was found that 2% (w/v) PEG works best in term of the blocking efficiency of the blocking buffer for this assay. In addition, the high concentration of the primary anti-BSA antibody used for immobilization in the microplate well was found to generate a high background signal. We found that 200-fold dilution of the primary anti-BSA antibody stock solution was optimal for the assay.

The selectivity of the assay to BSA-OP is examined in the presence and absence of BSA-OP (Fig. 2B). The results showed that the SWV response from BSA-OP is highest than that from BSA, AChE-OP, and BChE-OP, indicating the assay is specific to BSA-OP. The results demonstrated the good selectivity of this immunoassay.

Under these optimal conditions, the proposed method was used to detect BSA-OP based on the QD labels. As shown in Fig. 3, the SWV response increased with the increase of the BSA-OP concentration from 0 to 500 ng mL^{-1} . It can be seen that the SWV response in the absence of BSA-OP (curve a) was lower than that in the presence of 0.5 ng mL^{-1} BSA-OP (curve b), attributing to excellent blocking and washing of the assay. As shown in the inset of Fig. 3, the SWV responses linearly increase with the increase of the logarithm of BSA-OP concentration in the range of $0.5\text{--}500 \text{ ng mL}^{-1}$, with a correlation coefficient of 0.9961 ($n=3$). The detection limit of this method was estimated to be 0.5 ng mL^{-1} (about 7.8 pM). To test the reproducibility of the method, a series of six wells repetitive measurements of 25 ng mL^{-1} BSA-OP was investigated and yielded reproducible electrochemical signals with a relative standard deviation of 8.6%.

We further compared this method with the conventional enzyme-linked immunosorbent assay (ELISA) using horseradish

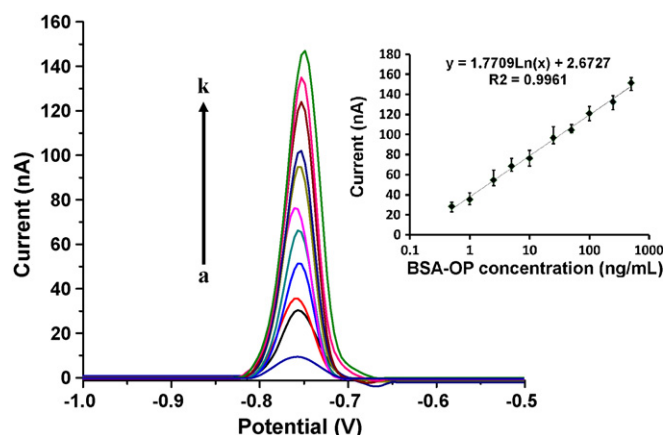


Fig. 3. Square wave voltammograms acquired in 0 ng mL⁻¹, 0.5 ng mL⁻¹, 1 ng mL⁻¹, 2.5 ng mL⁻¹, 5 ng mL⁻¹, 10 ng mL⁻¹, 25 ng mL⁻¹, 50 ng mL⁻¹, 100 ng mL⁻¹, 250 ng mL⁻¹, and 500 ng mL⁻¹ of BSA-OP (a–k, respectively) using the assay method under the optimal conditions. The inset: a plot of the peak current versus the logarithm of 0.5 ng mL⁻¹, 1 ng mL⁻¹, 2.5 ng mL⁻¹, 5 ng mL⁻¹, 10 ng mL⁻¹, 25 ng mL⁻¹, 50 ng mL⁻¹, 100 ng mL⁻¹, 250 ng mL⁻¹, and 500 ng mL⁻¹ BSA-OP.

peroxidase (HRP)-anti-phosphoserine conjugates for analysis of BSA-OP (Supporting information, Fig. S2). The results demonstrated that the electrochemical immunoassay showed a lower detection limit compared with ELISA.

4. Conclusions

This work demonstrated good performance of QD labels-based electrochemical immunoassay for the detection of BSA-OP, as a model analyte (serine phosphoprotein). To increase the analytical sensitivity of the assay, QD labels were conjugated to the secondary anti-phosphoserine antibody in a sandwich immunoassay format. Under optimal conditions, the obtained detection limit of the assay was 7.8 pM using a 60-min immunoreaction time. The proposed method provides a simple, sensitive, and reproducible assay for phosphorylated proteins. This work may be extended to the simultaneous detection of multiple biological phosphorylated protein biomarkers using different QD labels, such as CdS, ZnS, and PbS.

Acknowledgments

K.P. would like to thank the Office of the Higher Education Commission, Thailand for supporting by grant fund under the program Strategic Scholarships for Frontier Research Network for the Join PhD Program Thai Doctoral Degree for this research. O.C. would also like to thank the Thai Government Stimulus Package 2 (TKK2555), under the Project for Establishment of Comprehensive Center for

Innovative Food, Health Products and Agriculture, Chulalongkorn University, and PCU028.2010. This work was performed at Pacific Northwest National Laboratory (PNNL) and partially supported by Grant U01 NS058161-01 from the National Institutes of Health CounterACT Program through the National Institute of Neurological Disorders and Stroke, National Institute of Health. The contents of this publication are solely the responsibility of the authors and do not necessarily represent the official views of the federal government. The TEM characterization work was performed at the Environmental Molecular Sciences Laboratory (EMSL), a national scientific user facility sponsored by the Department of Energy's Office of Biological and Environmental Research located at Pacific Northwest National Laboratory. PNNL is operated for DOE by Battelle under Contract DE-AC05-76L01830.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2010.08.021.

References

- Chen, Z.-P., Jiang, J.-H., Zhang, X.-B., Shen, G.-L., Yu, R.-Q., 2007. *Talanta* 71 (5), 2029–2033.
- D'Ambrosio, C., Salzano, A.M., Arena, S., Renzone, G., Scaloni, A., 2007. *J. Chromatogr. B* 849 (1–2), 163–180.
- Dungchai, W., Siangproh, W., Chaicumpa, W., Tongtawe, P., Chailapakul, O., 2008. *Talanta* 77 (2), 727–732.
- Hu, L., Zhou, H., Li, Y., Sun, S., Guo, L., Ye, M., Tian, X., Gu, J., Yang, S., Zou, H., 2008. *Anal. Chem.* 81 (1), 94–104.
- Kerman, K., Chikae, M., Yamamura, S., Tamiya, E., 2007. *Anal. Chim. Acta* 588 (1), 26–33.
- Kerman, K., Kraatz, H.B., 2009. *Biosens. Bioelectron.* 24 (5), 1484–1489.
- Kuramitz, H., Dziewatkoski, M., Barnett, B., Halsall, H.B., Heineman, W.R., 2006. *Anal. Chim. Acta* 561 (1–2), 69–77.
- Liu, G., Lin, Y., 2007. *Talanta* 74 (3), 308–317.
- Mann, M., Ong, S.-E., Grønborg, M., Steen, H., Jensen, O.N., Pandey, A., 2002. *Trends Biotechnol.* 20 (6), 261–268.
- McLachlin, D.T., Chait, B.T., 2001. *Curr. Opin. Chem. Biol.* 5 (5), 591–602.
- Paradela, A., Albar, J.P., 2008. *J. Proteome Res.* 7 (5), 1809–1818.
- Pastor-Navarro, N., Gallego-Iglesias, E., Maquieira, Á., Puchades, R., 2007. *Talanta* 71 (2), 923–933.
- Preechaworapun, A., Ivandini, T.A., Suzuki, A., Fujishima, A., Chailapakul, O., Einaga, Y., 2008. *Anal. Chem.* 80 (6), 2077–2083.
- Roucaïrol, C., Azoulay, S., Nevers, M.-C., Créminon, C., Grassi, J., Burger, A., Duval, D., 2007. *Anal. Chim. Acta* 589 (1), 142–149.
- Song, H.F., Kerman, K., Kraatz, H.B., 2008. *Chem. Commun.* 4, 502–504.
- Tang, D., Yuan, R., Chai, Y., 2008. *Anal. Chem.* 80 (5), 1582–1588.
- Tang, H., Chen, J., Nie, L., Kuang, Y., Yao, S., 2007. *Biosens. Bioelectron.* 22 (6), 1061–1067.
- Tramontina, F., Leite, M.C., Cereser, K., de Souza, D.F., Tramontina, A.C., Nardin, P., Andreazza, A.C., Gottfried, C., Kapczinski, F., Gonçalves, C.-A., 2007. *J. Neurosci. Methods* 162 (1–2), 282–286.
- Wang, H., Wang, J., Timchalk, C., Lin, Y.H., 2008a. *Anal. Chem.* 80 (22), 8477–8484.
- Wang, J., Liu, G.D., Wu, H., Lin, Y.H., 2008b. *Small* 4 (1), 82–86.
- Wu, H., Liu, G.D., Wang, J., Lin, Y.H., 2007. *Electrochem. Commun.* 9 (7), 1573–1577.
- Yan, J.X., Packer, N.H., Gooley, A.A., Williams, K.L., 1998. *J. Chromatogr. A* 808 (1–2), 23–41.
- Yang, H., Ji, J., Liu, Y., Kong, J., Liu, B., 2009. *Electrochem. Commun.* 11 (1), 38–40.