Ultrasensitive Detection of Trace Protein by Western Blot Based on POLY-Quantum Dot Probes

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Ultrasensitive Detection of Trace Protein by Western Blot Based on POLY-Quantum Dot Probes

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In this study, we describe an ultrasensitive quantum dots (QDs)-based Western blot. With the high affinity of avidin-functionalized POLY-QDs and simplification of the detection process, this enabled the quantitative analysis of protein by Western blotting. To prepare the POLY-QDs, CdTe quantum dots were first coated with biotinylated denatured bovine serum albumin and then, via the effect of the biotin−avidin system, the biotinylated denatured bovine serum albumin-coated QDs, which had strong fluorescence, were linked together. With this series of modifications, the fluorescence intensity of CdTe QDs was significantly increased. Using the POLY-QDs as labels, the signal of Western blotting was more sensitive in tracing the protein than traditional dyeing. In the present study, trace protein A was applied to POLY-QDs-based Western blotting as a model. The linearity of this method was from 30 pg to 1.5 ng, and the sensitivity was up to low pictogram values. The final fluorescence signal on the polyvinylidenedifluoride (PVDF) membrane was retained for at least 40 min. The results of this study indicate that the POLY-QDs-based Western blot is an excellent quantitative analytical method for trace protein analysis.

Semiconductor nanoparticle quantum dots (QDs) have been applied widely in the biochemical field,[1,2] not only because of their size-dependent tunable photoluminescence (PL) with broad excitation spectra and narrow emission bandwidths but also due to convenience as they can be coupled to biochemical molecules. Owing to the surface properties of QDs, it is possible to use a similar approach to conjugate QDs of any color to biomolecules of interest.[3−8] These unique properties of QDs make them appealing as in vivo and in vitro fluorophores in a variety of biological investigations. Many researchers have used a number of methods to acquire coupling products, such as antigen−quantum dot complexes and antibody−quantum dot complexes which have been used in immunoassays or as immunofluorescent probes.[9−15] However, there are still many unsolved problems related to QDs as fluorescence probes, such as the stability of multifunctionalized QDs in different reaction systems, which obstruct the further use of QDs as fluorescence probes. Thus, many surface modifications of QDs have been studied by researchers. For example, QDs synthesized in Sol−Gel-derived composite silica spheres were coated with calix arene as luminescent probes for pesticides,[16] QDs capped with *S*-glutathione were used in cell imaging,[17] and QDs capped with 3-mercapto-1,2-propanediol achieved higher yield and better stability.[18] Therefore, when CdTe QDs were coated with dBSA, the chemical stability and photoluminescence of the QDs

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(1) Peng, C.; Li, Z.; Zhu, Y.; Chen, W.; Yuan, Y.; Liu, L.; Li, Q.; Xu, D.; Qiao, R.; Wang, L.; Zhu, S.; Jin, Z.; Xu, C. *Biosens. Bioelectron.* **2009**, *24*, 3657–3662.
(2) Wang, L. B.; Zhu, Y. Y.; Jiang, Y.; Qiao, R. R.; Zhu, S. F.; Chen, W.; Xu, C. L. *J. Phys. Chem. B* **2009**, *113*, 7637–7641.
(3) Chen, I.; Choi, Y.-A.; Ting, A. Y. *J. Am. Chem. Soc.* **2007**, *129*, 6619–6625.
(4) Chen, W.; Peng, C. F.; Jin, Z. Y.; Qiao, R. R.; Wang, W. Y.; Zhu, S. F.; Wang, L. B.; Jing, Q. H.; Xu, C. L. *Biosens. Bioelectron.* **2009**, *24*, 2051–2056.
(5) Jin, W. J.; Costa-Fernández, J. M.; Pereiro, R.; Sanz-Medel, A. *Anal. Chim. Acta* **2004**, *522*, 1–8.
(6) Lagerholm, B. C.; Wang, M.; Ernst, L. A.; Ly, D. H.; Liu, H.; Bruchez, M. P.; Waggoner, A. S. *Nano Lett.* **2004**, *4*, 2019–2022.
(7) Medintz, I. L.; Berti, L.; Pons, T.; Grimes, A. F.; English, D. S.; Alessandrini, A.; Facci, P.; Mattoussi, H. *Nano Lett.* **2007**, *7*, 1741–1748.
(8) Van Tilborg, G. A. F.; Mulder, W. J. M.; Chin, P. T. K.; Storm, G.; Reutelingsperger, C. P.; Nicolay, K.; Strijkers, G. J. *Bioconjugate Chem.* **2006**, *17*, 865–868.
(9) Goldman, E. R.; Clapp, A. R.; Anderson, G. P.; Uyeda, H. T.; Mauro, J. M.; Medintz, I. L.; Mattoussi, H. *Anal. Chem.* **2004**, *76*, 684–688.
(10) Hu, F. Q.; Ran, Y. L.; Zhou, Z. A.; Gao, M. Y. *Nanotechnology* **2006**, *17*, 2972–2977.
(11) Medintz, I. L.; Uyeda, H. T.; Goldman, E. R.; Mattoussi, H. *Nat. Mater.* **2005**, *4*, 435–446.
(12) Shen, J.; Xu, F.; Jiang, H.; Wang, Z.; Tong, J.; Guo, P.; Ding, S. *Anal. Bioanal. Chem.* **2007**, *389*, 2243–2250.
(13) Sundara, R. S.; Vu, T. Q. *Nano Lett.* **2006**, *6*, 2049–2059.
(14) Wang, S. P.; Mamedova, N.; Kotov, N. A.; Chen, W.; Studer, J. *Nano Lett.* **2002**, *2*, 817–822.
(15) Xing, Y.; Chaudry, Q.; Shen, C.; Kong, K. Y.; Zhau, H. E.; Wchung, L.; Petros, J. A.; O'Regan, R. M.; Yezhelyev, M. V.; Simons, J. W.; Wang, M. D.; Nie, S. *Nat. Protoc.* **2007**, *2*, 1152–1165.
(16) Li, H. B.; Qu, F. G. *Chem. Mater.* **2007**, *19*, 4148–4154.
(17) Zheng, Y.; Gao, S.; Ying, J. Y. *Adv. Mater.* **2007**, *19*, 376–380.
(18) Rajh, T.; Micic, O. I.; Nozik, A. J. *J. Phys. Chem.* **1993**, *97*, 11999–12003.

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significantly improved.[19,20] In our study, we coated the CdTe QDs with biotinylated denatured bovine serum albumin (B-dBSA) and then prepared the conjugation of the avidin and biotin-modified denature BSA coated QDs (POLY-QDs). In this way, based on the POLY-QDs, we not only solved the common agglomeration problem during the cross-linking reaction but also increased the stability and the photoluminescence intensity of the QDs. We then used the POLY-QDs system in Western blotting for the detection of trace amounts of protein.

Western blotting is a common method for protein analysis. Following the sections of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and semidry electrophoretic transfer, multiconstituent protein samples can be divided into several single bands, which facilitate their subsequent detection. Diaminobenzidine (DAB) and 3,3′,5,5′-tetramethylbenzidine (TMB) are commonly used for detection in Western blot analysis;[21] however, the detection limits of these compounds are too high and are not suitable for the analysis of micro amounts of protein samples. In our study, POLY-QDs were used in Western blot analysis, for the simple and ultrasensitive quantification of Western blot protein, due to high specificity of the antibody−antigen complex[22−24] and excellent fluorescent characteristic of QDs.

In this study, protein A was used as a model in the POLY-QDs-based Western blot. Protein A is a cell wall constituent which is characterized by its binding affinity to the Fc portion of some immunoglobulins, especially the IgG class, which affect the reaction of the antigen with IgG. In the presence of large amounts of IgG together with protein A, the detection limit of traditional ELISA is too high to detect trace protein. Base on the SDS-PAGE and semidry electrophoretic transfer Western blot which dissociates protein mixture samples into several separate bands, the interference effect was reduced or eliminated. Furthermore, using the POLY-QDs prepared, the sensitivity and stability of the detection were also enhanced. Both make the POLY-QDs a potential tool in the field of Western blot based protein analysis.

EXPERIMENTAL SECTION

Preparation of POLY-QDs. *Preparation of Denatured dBSA.* Denatured BSA was prepared by treating BSA with $NaBH_4$, as described in Gao's report.[25] Briefly, 264 mg of BSA was dissolved in 10 mL of ultrapure water; then, 6.7 mg of $NaBH_4$ was added to the solution as a reductant under stirring. The mixture was incubated for 1 h at room temperature and then incubated in a waterbath at 60−80 °C until no more gas (H_2) was generated. After these treatments, the excess $NaBH_4$ was hydrolyzed and BSA was denatured when the disulfide bonds were converted to sulfhydryl groups. The final concentration of dBSA aqueous solution was 40×10^{-5} mol/L.

Preparation of Biotinylated dBSA (B-dBSA). NHS−biotin was dissolved in dimethyl sulfoxide (DMSO) at 20 mg/mL. dBSA was diluted in phosphate buffered saline (PBS) (0.01 mol/L, pH 8.0) at 5×10^{-5} mol/L. Then, 25.6 μL of NHS−biotin solution was added to 1 mL of dBSA solution under stirring and incubated for 1 h at room temperature. The molar ratio of NHS−biotin to dBSA was 30:1. The solution was then centrifuged at 5000*g* for 15 min using a super filtering tube to remove excess NHS−biotin. Then, the solution above the super filtering membrane was collected and dissolved in an appropriate amount of PBS (0.01 mol/L, pH7.4) to obtain the desired concentration. The purified B-dBSA was identified by ultraviolet scanning (Figure S1 in the Supporting Information) and then stored at 4 °C until use.

Preparation of B-dBSA Coated CdTe Quantum Dots. The QDs stabilized by the thioglycolic acid (TGA) were dialyzed in PBS (0.01 mol/L, pH 7.4) for 2 days to remove free TGA. Then, the purified QDs were mixed with the B-dBSA solution and incubated at 60 °C for 15 min. The solution was then kept at room temperature for 2 days to ensure a sufficient reaction.

The B-dBSA-coated CdTe QDs were identified by SDS-PAGE with 8% polyacrylamide separation gel at a constant 100 V and 1 h total run time (Figure S2 in the Supporting Information).

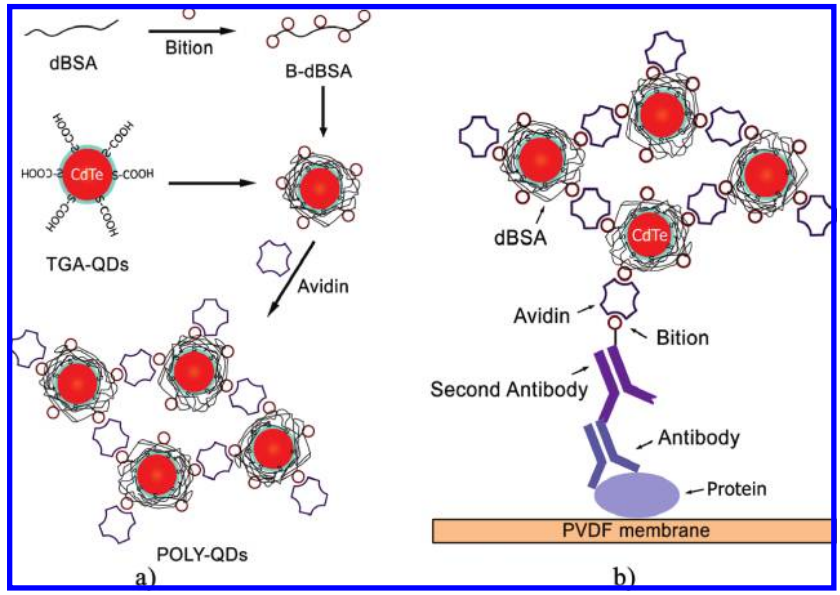
Preparation of Avidin-Functionalized POLY-QDs. PBS buffer was added to the B-dBSA-coated CdTe QDs to obtain the appropriate concentration. Avidin solution at 1 mg/mL was added to the measured B-dBSA-coated CdTe QDs under stirring, the molar ratio was 4:1. Approximately 30 min later, a flocculent precipitate could be seen at the bottom of the container. The mixture was then centrifuged at 3000*g* for 5 min at 4 °C; the supernatant was discarded, and the precipitate was redispersed in PBS.

Sample Preparation. The sample was an antibody solution treated with protein A column immunoadsorption apheresis. The concentration of total protein in the eluted samples was determined by a UV-spectrometer. All samples were diluted to 1 mg/mL. A 10 μL aliquot of each sample was placed in a microcentrifuge tube, and an equivalent volume of Laemmli buffer was added. The samples were heated in boiling water for 10 min and then cooled for 10 min at room temperature. The samples were centrifuged at 13 000 rpm for 5 min at room temperature, and the supernatants were used for detection.

Western Blot Analysis. Denatured protein samples were separated first by SDS-PAGE with 10% polyacrylamide separation gel. Then, the gel was transferred for 40 min at 2.5 mA/cm^2 to a polyvinylidenedifluoride (PVDF) membrane (Immobion-P, Millipore) in semidry transfer units. The principle of protein detection in Western blotting is described in Scheme 1. The PVDF membrane was immersed in blocking buffer (5% w/v BSA, 0.05% v/v Tween-20) for 1 h at room temperature, then incubated with primary antibody (rabbit antiprotein A) for 1 h at room temperature, incubated with a second biotinylated antibody (goat antirabbit) for 1 h at room temperature, and then incubated with the POLY-QDs solution for 2 h. After that, the membrane was washed three times with PBS. The fluorescence signal of POLY-QDs on the blot bands was detected immediately using the fully

(19) Kuo, Y. C.; Wang, Q.; Ruengruglikit, C.; Yu, H. L.; Huang, Q. R. *J. Phys. Chem. C* **2008**, *112*, 4818–4824.
(20) Wang, Q.; Kuo, Y. C.; Wang, Y. W.; Shin, G.; Ruengruglikit, C.; Huang, Q. R. *J. Phys. Chem. B* **2006**, *110*, 16860–16866.
(21) Hoeben, A.; Landuyt, B.; Botrus, G.; De Boeck, G.; Guetens, G.; Highly, M.; van Oosterom, A. T.; de Bruijn, E. A. *Anal. Chim. Acta* **2006**, *564*, 19–33.
(22) Xu, C. L.; Peng, C. F.; Hao, K.; Jin, Z. Y.; Wang, W. K. *Luminescence* **2006**, *21*, 126–128.
(23) Peng, C. F.; Chen, Y. W.; Chen, W.; Xu, C. L.; Kim, J. M.; Jin, Z. Y. *Food Chem.* **2008**, *109*, 647–653.
(24) Xie, H. L.; Ma, W.; Liu, L. Q.; Chen, W.; Peng, C. F.; Xu, C. L.; Wang, L. B. *Anal. Chim. Acta* **2009**, *634*, 129–133.
(25) Gao, X. H.; Chan, W. C. W.; Nie, S. M. *J. Biomed. Opt.* **2002**, *7*, 532–537.

Scheme 1. Sketch of the Preparation of POLY-QDs (a) and the Detection Protocol by Western Blot Based on the POLY-QDs (b)



automatic image acquisition and analysis system ZF-258 with emission UV-light at 365 nm (Jiapeng, Shanghai).

Real Sample Test. On the basis of the above-mentioned detection method, the result was compared with that of the commercial detection kit (Pierce Biotechnology, Rockford, USA) at the same detection conditions. Besides, the recovery test was performed by measuring the protein A added into the different protein sample (purified rat−antigentamicin monoclonal antibody). Different protein A solutions at different concentrations were spiked with the rat−antigentamicin monoclonal antibody solution and measured in triplicate. The recoveries were obtained from comparing the measured amounts with the added amounts of protein A. The mean and standard deviation values for recovery were obtained from three replicate determinations by the above-mentioned Western blot analysis method of the spiked samples.

RESULTS AND DISCUSSION

In this study, a new style of fluorescence probes, POLY-QDs, were applied to Western blot analysis to achieve an ultrasensitive detection limit. The POLY-QDs we prepared showed super optical properties and good stability. The results and discussion on the identification of POLY-QDs and the Western blot analysis of protein A are as follows:

Preparation of POLY-QDs. In previous studies, researchers modified QDs directly with biotin or avidin[26−29] or modified the QDs indirectly by means of lysylamine or ligase.[30,31] In the present study, we carried out a new method to biotinylate the QDs indirectly by coating with biotinylated dBSA and then producing POLY-QDs (Scheme 1).

Before BSA was biotinylated with NHS−biotin, it was first denatured by $NaBH_4$; thus, the BSA was denatured to peptide chains which have less steric hindrance than the native BSA for the reaction with NHS−biotin. In addition, the disulfide bonds of BSA were opened which made it possible for the native capping agent TGA on the surface of QDs to be substituted by the thiol-group of dBSA through ligand exchange.[20] As a result of the above reaction, QDs were biotinylated indirectly but more efficiently than the direct method. Then, the QDs were functionalized with avidin using a reasonable molar ratio. When the molar ratio reached 3:1, the QDs began to aggregate at the bottom of the PE tube, due to the formation of POLY-QDs. We chose a molar ratio 4:1 to ensure sufficient reaction (Figure S3 in the Supporting Information), and excess avidin was removed by centrifugation. Then, we rediluted the POLY-QDs aggregates with the same buffer solution to the certain concentration for the following research (Figure S4 in the Supporting Information). Finally, owing to the protective effect of B-dBSA and avidin on the surface of the CdTe QDs,[32] the stability and fluorescence intensity of QDs significantly increased (Figure S5 in the Supporting Information), which contributed to the quantitative analysis of proteins by Western blotting.

Western Blot Analysis of Protein. The critical issue in the application of QDs used as labels in such immunoassays as Western blotting is the stability of QDs. Because B-dBSA and avidin passivate the surface of QDs significantly, the stability of POLY-QDs was significantly increased. In addition, due to the effect of the biotin−avidin system, the optical signal of POLY-QDs was visibly amplified. Thus, POLY-QDs were very suitable for the analysis of trace multicomponent protein samples using Western blotting. Through the Western blot immune reaction, the quantity of proteins was expressed by the value of the integral optical density of POLY-QDs.

(26) Bakalova, R.; Zhelev, Z.; Ohba, H.; Baba, Y. *J. Am. Chem. Soc.* **2005**, *127*, 9328–9329.
(27) Goldman, E. R.; Balighian, E. D.; Mattoussi, H.; Kuno, M. K.; Mauro, J. M.; Tran, P. T.; Anderson, G. P. *J. Am. Chem. Soc.* **2002**, *124*, 6378–6382.
(28) Lingerfelt, B. M.; Mattoussi, H.; Goldman, E. R.; Mauro, J. M.; Anderson, G. P. *Anal. Chem.* **2003**, *75*, 4043–4049.
(29) Sapsford, K. E.; Medintz, I. L.; Golden, J. P.; Deschamps, J. R.; Uyeda, H. T.; Mattoussi, H. *Langmuir* **2004**, *20*, 7720–7728.
(30) Garske, A. L.; Denu, J. M. *Biochemistry* **2006**, *45*, 94–101.
(31) Joo, K.-I.; Lei, Y.; Lee, C.-L.; Lo, J.; Xie, J.; Hamm-Alvarez, S. F.; Wang, P. *ACS Nano* **2008**, *2*, 1553–1562.
(32) Wang, Q.; Pan, D. C.; Jiang, S. C.; Ji, X. L.; An, L. J.; Jiang, B. Z. *Chem.−Eur. J.* **2005**, *11*, 3843–3848.

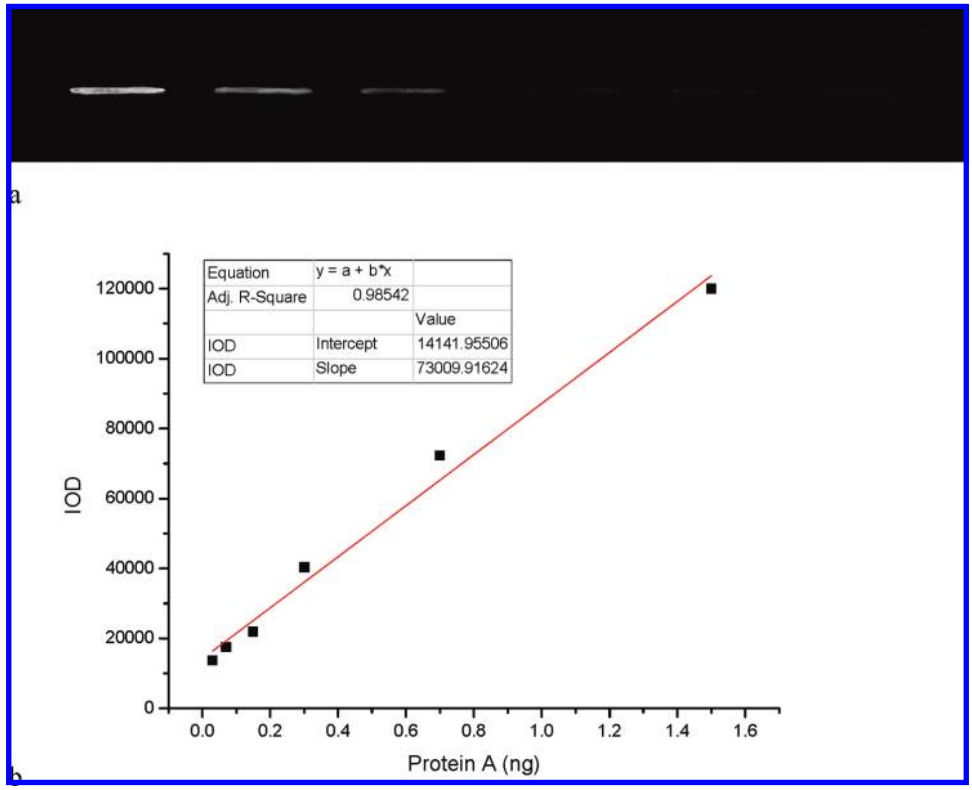


Figure 1. (a) Image of Western blot analysis with POLY-QDs, the quantity of protein A: 1.5, 0.7, 0.3, 0.15, 0.07, and 0.03 ng. (b) The standard curve for the absolute quantity of protein A vs integral optical density (IOD).

Table 1. Detection of Trace Protein A in Real Samples

samples	original quantity (pg)	spiked quantity (pg)	detected quantity (pg) mean ± RSD	recovery (%) mean ± SD
antibody solution	28.7	10	38.2 ± 0.16	95.0 ± 1.9
	53.2	50	104.9 ± 0.20	103.4 ± 2.0
	74.6	100	171.6 ± 1.31	97.0 ± 1.4
	107.5	200	305.5 ± 2.61	99.0 ± 1.2
	122.3	500	623.1 ± 3.13	100.2 ± 1.7

A standard curve for absolute quantity of the protein A samples vs the integral optical density (IOD) was established (Figure 1). The absolute quantity of protein A samples were 1.5, 0.7, 0.3, 0.15, 0.07, and 0.03 ng. We recommend that the absolute quantity of protein samples analyzed should be less than 10 μg, otherwise the fluorescence signal will reach a saturated state, and there would be no distinct differences in the overloaded protein samples. Meanwhile, the detection results of the developed method based on the POLY-QDs were also compared with that of the commercial detection kit at the same detection conditions. At the same absolute quantity of the protein A, the detection based on the commercial kit could only show the signal of the bands until the 150 pg although the ideal limit of detection of the commercial kit could achieve 20 pg. (The detailed procedure and results are in the Supporting Information.)

Besides, the final fluorescence signal on the PVDF membrane was retained for at least 40 min (Figure S5 in the Supporting Information), which was long enough for acquisition of image and image analysis. The PVDF membrane can be kept for a longer period in PBS buffer in dark conditions.

To estimate the stability of the POLY-QDs-based Western blot, purified rat−antigentamicin monoclonal antibody was used as a real matrix sample, and the recovery of protein A from the spiked rat monoclonal antibody was measured. Each sample was detected in triplicate. The recovery ranged from 95.0% to 103.4%. The results are shown in Table 1, which demonstrate that the POLY-QDs-based Western blot is a stable and reproducible method of detecting protein A.

CONCLUSIONS

In this study, we aimed to establish an ultrasensitive quantitative Western blot by introducing novel avidin-functionalized POLY-QDs as fluorescence labels. The B-dBSA- and avidin-modified QDs exhibited high stability and fluorescence intensity. The fluorescence signal on the PVDF membrane was retained for at least 40 min, and the sensitivity of this system was at the low pictogram level. The results of the Western blot analysis of protein A showed the superiority of POLY-QDs adequately, and these characteristics indicated that this system is an attractive analytical method. We believe that the application of POLY-QDs in the analysis of many other proteins will be distinctive.

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SUPPORTING INFORMATION AVAILABLE

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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