Analyst

Cite this: *Analyst*, 2012, **137**, 4262

www.rsc.org/analyst

PAPER

A new strategy for the detection of adenosine triphosphate by aptamer/quantum dot biosensor based on chemiluminescence resonance energy transfer

Zi-Ming Zhou, Yong Yu and Yuan-Di Zhao*

Received 20th April 2012, Accepted 26th June 2012
DOI: 10.1039/c2an35520e

We designed an aptasensor for the detection of adenosine triphosphate (ATP) based on chemiluminescence resonance energy transfer (CRET). An adenosine aptamer was cut into two pieces of ssDNA, which were attached to quantum dots (QDs) and horse radish peroxidase (HRP), respectively. They could reassemble into specific structures in the presence of ATP and then decrease the distance of HRP and QDs. ATP detection can be easily realized according to the fluorescent intensity of QDs, which is excited by CRET between luminol and QDs. Results show that the concentration of ATP is linear relation with the fluorescent intensity of the peak of QDs emission and the linear range for the linear equation is from 50 μM to 231 μM and the detection limit was 185 nM. When the concentration of ATP was 2 mM, the efficiency of CRET is 13.6%. Good specificity for ATP had been demonstrated compared to thymidine triphosphate (TTP), cytidine triphosphate (CTP) and guanosine triphosphate (GTP), when 1 mM of each was added, respectively. This method needs no external light source and can avoid autofluorescence and photobleaching, and ATP can be detected selectively, specifically, and sensitively in a low micromolar range, which means that the strategy reported here can be applicable to the detection of several other target molecules.

Introduction

ATP is the basic biological energy source in life, which sustains every biological process. Existing research shows that the content of ATP *in vivo* has great influence on cell metabolism and death.[1] Moreover, many diseases demonstrate an abnormal content of ATP, such as cardiovascular disease,[2] parkinsonism,[3] and Alzheimer's.[4] Therefore, it's important to detect ATP in life science and clinical medical research.

At present, there is lots of methods for detection of ATP, like chromatography, bioluminescence assay, electrochemical, and fluorescent biosensor.[5] Chromatography is restricted to the tedious separation of sample and low accuracy detection. A broad method for detection of ATP at present is based on luciferase, which has a good sensitivity but its accuracy sometimes could be affected by quenching in matrix. Low specificity and sensitivity are always the challenges in electrochemical sensors. In a word, how to detect ATP with high specificity and sensitivity and more simply is a hot issue.

Aptamers, first reported by three groups independently in 1990,[6] are the artificial single-stranded DNA or RNA sequences, which are screened using the systematic evolution of ligands by the exponential enrichment (SELEX) approach. They could fold into special structures, and bind to certain targets with extremely high specificity, such as metal ions,[7] small molecules[8] and proteins.[9] Owing to its high specificity, thermostability, easy synthesis, and modification, aptamers have been used in many biosensors for the detection of thrombin,[10] cocaine[11] or ATP,[12] *etc.*, which are known as aptasensors. Fan's group has developed a target-responsive electrochemical aptamer switch technology (TREAS). In their research, ferrocene was used as a signal molecule to observe the change of current before and after aptamer bound to ATP to achieve electrochemical detection of ATP.[13] Plaxco and coworkers cut aptamers into two pieces.[14] When targets, like ATP or cocaine, were added, they worked as molecular linkers forming sandwich structures with the two pieces of aptamer, making signal molecules close to the electrode, which then induced the change of current.

Compared with other methods, the traditional fluorescence method needs small samples and has high sensitivity, high selectivity, and is easy to operate. Many works have been reported using this method. Brennan's group fixed aptamers in sol–gel-derived silica material,[15] using fluorescein as a signal molecule. The detection of ATP was realized after 4,4-dimethylamino-azobenzene-4′-carboxylic acid (DABCYL), the quencher molecule, was separated by adding ATP, recovering the fluorescence. Yamana's group reported the use of pyrene fluorophore labelled anti-ATP aptamer to detect ATP.[16] The light intensity of pyrene fluorophores is sensitive to conformational change of aptamer by ATP.

Britton Chance Center for Biomedical Photonics, Wuhan National Laboratory for Optoelectronics, Key Laboratory of Biomedical Photonics of Ministry of Education, Department of Biomedical Engineering, Huazhong University of Science and Technology, Wuhan 430074, People's Republic of China. E-mail: zydi@mail.hust.edu.cn; Fax: +86 27-8779-2202

In contrast to organic dye, quantum dots (QDs) have wide excitation, narrow emission, size-dependent character and resistance to light bleaching. As a new fluorescent probe, QDs have been used in aptamer biosensors to detect biomolecules and metal ions. Deng's group reported a biosensor based on aptamer/QDs, where QDs were used as donor and Cy5 as acceptor.[17] In their system, the hybridization of ATP aptamer and its complementary strand produced fluorescence resonance energy transfer (FRET) from QDs to Cy5. When ATP was added, the conformational change of aptamer led to weakening of FRET to achieve the detection of ATP. Aldissi and Bogomolova also used the specific affinity of ATP with its aptamer to replace the complementary strand, which induced fluorescence change to detect ATP.[18] Recently, Willner and coworkers designed an aptamer biosensor based on CRET.[19] When ATP or Hg^{2+} were present, the fragments of aptamer self-assembled into the active hemin-G-quadruplex DNAzyme structure, which could catalyze luminol by hydrogen peroxide inducing chemiluminescence. Moreover, the energy of chemiluminescence could be transferred to QDs labeled on aptamers, inducing the CRET signal which achieved the detection of ATP or Hg^{2+}. Compared with FRET, CRET needs no external light source, thus can avoid autofluorescence and photobleaching. In this paper, we propose a new strategy for the detection for ATP based on CRET of aptamer/quantum dots. Adenosine aptamer was cut into two pieces of ssDNA, which were attached to QDs and horse radish peroxidase (HRP), respectively. When ATP was added, the two fragments were reassembled by ATP that decrease the distance of QDs and HRP, which induced CRET. Therefore, the quantity of ATP can be estimated by the fluorescence intensity of QDs. This method needs no external light source and can avoid autofluorescence and photobleaching, and ATP can be detected selectively, specifically and sensitively in a low micromolar range.

Experimental

Materials

Chromium chloride (99%), tellurium powder (200 mesh, 99.99%), sodium borate and hydrogen peroxide were all purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Sulfo-SMCC, EDC·HCl, luminol, *para*-iodophenol, and reduced glutathione (GSH) were all obtained from Sigma-Aldrich Fine Chemicals (St. Louis, MO, USA). Sodium thioglycollate and HRP were purchased from Shanghai Sangon Biological Engineering Technology and Services Co., Ltd. (Shanghai, China). Ultrafiltration membrane was purchased from Micom (Millipore, Bedford, MA, USA). Zeba desalination columns were supplied by Thenno (USA). All other materials and reagents were of analytical grade. All oligonucleotides used in the present study were synthesized and purified by Shanghai Sangon Biological Engineering Technology and Services Co., Ltd. (Shanghai, China). Their base sequences are as follows: fragment A: 5′-NH_2–$(CH_2)_6$–ACCTGGGGGAGTAT-3′; fragment B: 5′-TGCGGAGGAAGGT–$(CH_2)_3$–SH-3′.

Characterization

Capillary electrophoresis analysis with fluorescence detection was carried out on a home-built system, consisting of a high-voltage supply (0–30 kV) (Shanghai Nucleus Research Institute, China), and an inverted fluorescence microscope (IX71, Olympus, Japan) equipped with a 100 W mercury lamp, and a fiber optic spectrometer (QE65000, Ocean Optics, USA). The absorption spectrum was measured by UV-vis spectrophotometer (UV-2550, Shimadzu, Japan). The fluorescence spectrum was measured by a luminescence spectrometer (LS-55, Perkin-Elmer, USA).

Preparation of CdTe quantum dots

CdTe quantum dots capped with GSH were synthesized according to previous reports.[20] Briefly, a chromium precursor solution was made by mixing a solution of chromium chloride and GSH in ultrapure water, and then adjusting the pH to 11.2 by NaOH. NaHTe solution was prepared by the reaction of Te powder with $NaBH_4$ in an oxygen-free condition immediately and then was added to the nitrogen-saturated chromium precursor solution under vigorous stirring. The resulting mixture solution with a typical molar ratio of 5 : 6 : 1 (Cd^{2+} : GSH : Te^{2+}) was heated to 100 °C. Different size of water-soluble CdTe QDs were obtained by controlled reaction time. The GSH-coated CdTe QDs were precipitated with isopropanol and then isolated by centrifugation and decantation to remove free GSH molecules. Finally, CdTe Qds were dissolved in 0.01 M phosphate buffered saline (PBS) (10 mM, pH 7.4). The obtained concentration of QDs and quantum yield was estimated followed ref. 21 and 22, respectively.

Preparation of aptamer-ATP biosensor

Water-soluble QDs were conjugated to fragment A using EDC as a coupling reagent.[23] Firstly, a reaction mixture containing QDs, DNA and EDC with a ratio of 1 : 5 : 200 in PBS (10 mM, pH 7.4) was prepared and left to stand for 8–10 h (in fact, 2 h is enough for the conjugation; we chose 8–10 h overnight simply to be compatible with working hours) at room temperature. Then, the mixture was purified using an ultra-filtration membrane. The conjugates were obtained and dissolved in 500 μL PBS. HRP were conjugated to fragment B using sulfo-SMCC as a coupling reagent.[24] First of all, a reaction mixture containing 3 mg HRP and 2 mg sulfo-SMCC in PBS (10 mM, pH 7.2; containing 0.15 M NaCl) was prepared, stirred for 30 min at room temperature, then filtered by Sephadex G-100 column (35 cm long and 1 cm diameter). The filtrate was diluted to 3 mg mL^{-1} and fragment B was added with a ratio of 1 : 3 (HRP : DNA), shook for 3 h, then purified using an ultra-filtration membrane.

ATP detection

Chemiluminescence (CL) spectra were measured with a fluorescence microscope with a closed mercury lamp using a 3 mL quartz cuvette (1 cm optical path) in a darkroom. ATP with different concentrations was added to a solution containing QD–fragment A (1 mM) and HRP–fragment B (10 mM). After incubation for 1 h, 0.5 mL CL reaction buffer (containing $Na_2B_4O_7$ 50 mM, 1×10^{-4} M luminol, 5×10^{-4} M H_2O_2, 5×10^{-4} M *p*-IP) was added to the quartz cuvette. CL reactions were initiated, and CL spectra were recorded immediately.

Results and discussion

ATP detection strategy

An illustration of aptamer/quantum dots biosensor based on CRET to detect ATP is shown in Fig. 1. Firstly, the ATP aptamer is cut into two pieces of ssDNA: fragment A has an amino group at the 5′ end and fragment B has a thiol group at the 3′ end. QD is conjugated to the former using EDC as a coupling reagent (Fig. 1(1)), and HRP is conjugated to the latter using sulfo-SMCC (Fig. 1(2)). The ATP, working as a molecular linker, reassembles the two pieces of ssDNA into the intact aptamer tertiary structure, and then the distance between QDs and HRP is decreased (Fig. 1(3)). If CL buffer is added, the oxidation of luminol can be catalyzed by HRP and induce chemiluminescence. The CL spectrum (the peak at 470 nm, Fig. 2, curve b) of luminol overlaps well with the absorption of QDs (Fig. 2, curve a). Therefore, CRET happens between luminol and QDs.

Characterization of conjugation of QD–fragment A

In our study, water-soluble CdTe QDs with an emission wavelength of 650 nm (Fig. 2, curve c) and quantum yield of 53% was adopted. Capillary electrophoresis was used to inspect the conjugation of QDs and fragment A (Fig. 3A). It was found that the QDs have a strong fluorescent peak at about 380 ± 0.6 s (Fig. 3A, curve b). After coupling with fragment A, the peak shifted to about 338 ± 4.5 s (Fig. 3A, curve a). This might be caused by the decrease of the charge–mass ratio after the conjugation of QDs and fragment A. Meanwhile, it could be seen that the QD–fragment A had a smaller peak width than QDs (Fig. 3A), indicating that the uniformity of charge distribution of QDs was optimized to some extent during the process of conjugation. UV-vis spectrophotometer was used to confirm whether fragment A was conjugated completely to QDs (the ratio of QDs to DNA is 1 : 5). It was found that free fragment A could get through the ultrafiltration membrane (Fig. 3B), but free QDs could not pass through it (Fig. 3D). When QDs were incubated with fragment A at that ratio, no peak was found in the filtrate (Fig. 3C), suggesting that there is no free DNA in the filtrate, and fragment A was completely conjugated to QDs.

Characterization of conjugation of HRP–fragment B

UV-vis spectrophotometer was also used to confirm whether fragment B was conjugated completely to HRP (the ratio of HRP to DNA is 1 : 3, figures were not given out). When there

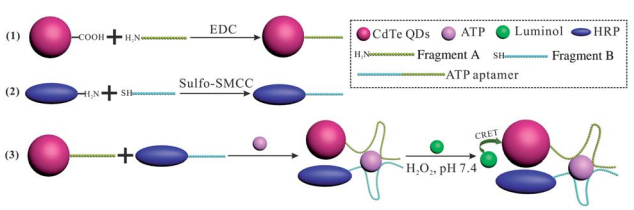

Fig. 1 Schematic depiction of the aptamer/QD biosensor based on CRET for detection of ATP.

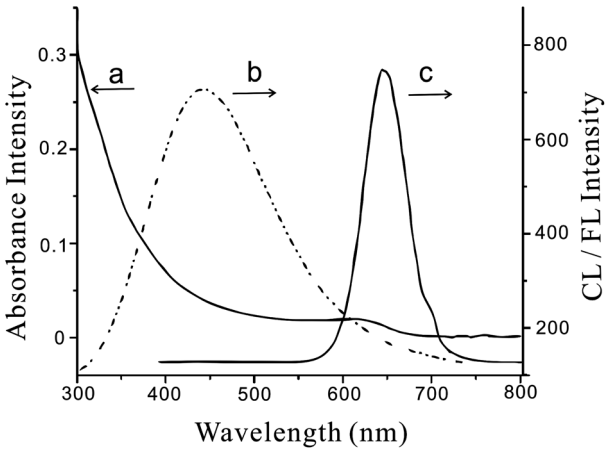

Fig. 2 UV-vis absorption of QDs (a), luminol CL spectrum (b), emission spectrum of QDs (c).

was no fragment B in solution, little absorption of free HRP in filtrate was found, but one peak at about 400 nm was found for the filtration residue on the ultrafiltration membrane, which was the same as the absorption of HRP,[25] indicating free HRP cannot get through the membrane. When HRP was incubated with fragment B at that ratio, no absorption of free HRP in the filtrate was found, but for the filtration residue on the ultrafiltration membrane, in addition to the peak at 400 nm due to HRP, there was another peak at 260 nm. According to the intensity of peak at 260 nm, it was calculated that almost all of the added fragment B was found on the ultrafiltration membrane, indicating fragment B was conjugated completely to HRP.

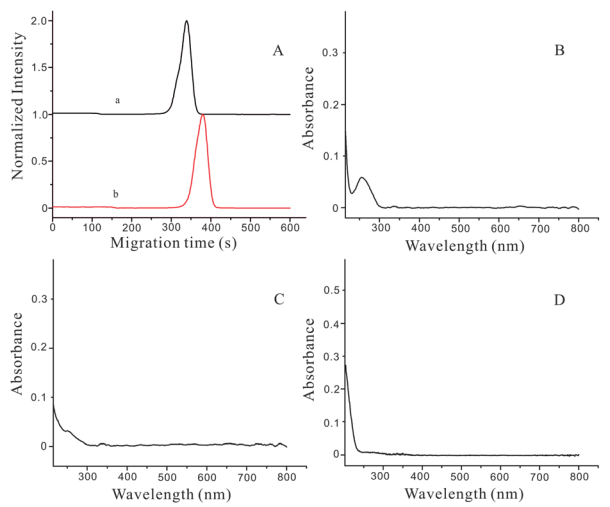

Fig. 3 Capillary electrophoresis for QDs incubated with (a) and without (b) fragment A (A); UV-vis spectrophotometer for the filtrate of fragment A (B); UV-vis spectrophotometer for the filtrate of QDs incubated with (C), or without (D) fragment A. A reaction mixture containing QDs, DNA and EDC with a ratio of 1 : 5 : 200 in PBS (10 mM, pH 7.4) was prepared and left to stand for 8–10 h at room temperature, then filtered using an ultrafiltration membrane. The intensities of electrophoresis peaks shown in A were normalized.

Characterization of conjugation of QD–fragment A with HRP–fragment B by ATP

Capillary electrophoresis was used to inspect the conjugation of QD–fragment A with HRP–fragment B in the absence/presence of ATP (Fig. 4). It was found that there was a peak at about 323 ± 2.7 s (Fig. 4, curve b) for QD–fragment A conjugated with HRP–fragment B in absence of ATP, which was closed to the peak of QD-fragment A. When ATP was added to the mixture of QD–fragment A and HRP–fragment B, the peak appears at about 301 ± 3.8 s (Fig. 4, curve a), which meant ATP linked HRP–fragment B to QD–fragment A.

Aptamer/QDs biosensor based CRET ATP detection

In our system, the CL spectrum of luminol/hydrogen peroxide (about 470 nm) overlaps well with the absorption of QDs used in this manuscript. This can induce resonance energy transfer under certain conditions if QDs are close to HRP. Then, when ATP was added, one peak was observed at 650 nm, which is emission peak of the QDs (Fig. 5). With the increase of concentration of ATP, the CL intensity of luminol decreased and emission intensity of QDs increased, suggesting the efficiency of CRET was increasing (inset in Fig. 5). The efficiency of CRET was determined according to the literature method[26] by the formula, $E = S/(S_0 + S)$, where E is the CRET efficiency, S is the area of QD emission spectrum, and S_0 is the area of luminol chemiluminescence spectrum. When the concentration of ATP was 2 mM, the efficiency was *ca.* 13.6%. It was found that when the concentration of ATP was at a low level, the emission intensity of QDs at the peak increased with increasing concentration of ATP. The relationship of ATP concentration and the fluorescence intensity of QDs at the peak was studied (Fig. 6). The concentration of ATP can be estimated by the formula, $F = kC + 7.53$, where C is the concentration of ATP, F is the fluorescence intensity of QD at the peak, and k is a constant with a value of 7.0. The linear range for the linear equation is from 50 μM to 231 μM and the detection limit was 185 nM based on a signal-to-noise ratio of 3.

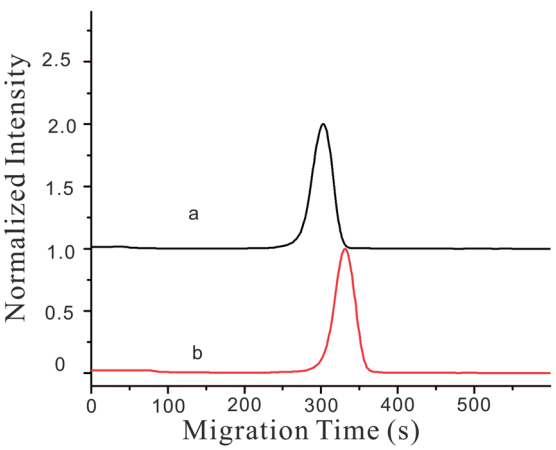

Fig. 4 Capillary electrophoresis for QD–fragment A with HRP–fragment B in the presence (a) and absence (b) of ATP. The intensity of electrophoresis was normalized.

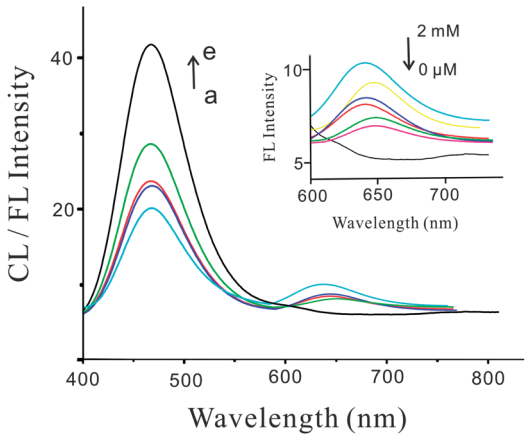

Fig. 5 CRET spectrum of QD–fragment A and HRP–fragment B in different concentrations of ATP: (a) 2 mM, (b) 400 μM, (c) 200 μM, (d) 100 μM, and (e) 0 M. The inset shows fluorescence intensity of QDs in different concentrations of ATP: 2 mM, 1 mM, 400 μM, 200 μM, 100 μM, 50 μM and 0 M from top to bottom.

ATP detection specificity

In this study, under optimized conditions, TTP, CTP and GTP were selected to study the specificity of aptamer/QDs biosensor based on CRET. It was found that ATP analogues such as TTP, CTP or GTP did not induce similar structural switching. Thus they did not lead to CRET. As shown in Fig. 7, the fluorescence intensity of QDs showed little change after the addition of TTP, CTP, or GTP at a concentration of 1 mM, but ATP caused a dramatic fluorescence of QDs with the same concentrations. The good selectivity of aptamer/QDs biosensor based on CRET is attributed to the high specificity of aptamer. Therefore, the biosensor can be applied to highly sensitive detection of ATP with high specificity.

ATP detection sensitivity

The biosensor for the detection of adenosine triphosphate based on chemiluminescence resonance energy transfer is sensitive in a low micromolar range, and the sensitivity is influenced by several factors, such as the inspection instrument, the physical property

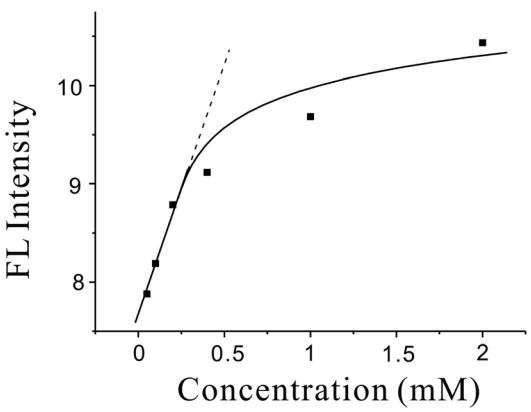

Fig. 6 The relationship of ATP concentration and the fluorescence intensity of QDs.

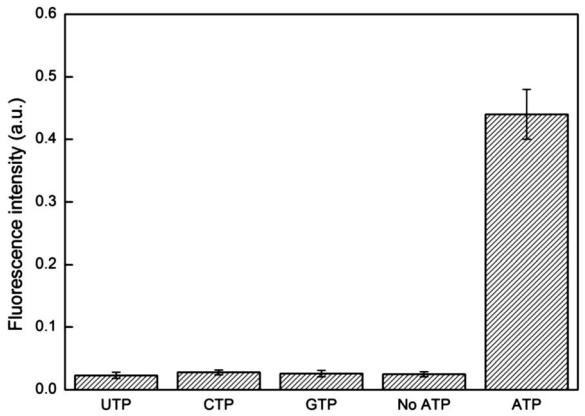

Fig. 7 Fluorescence intensity of QDs in the presence of TTP (1 mM), CTP (1 mM), GTP (1 mM), and ATP (1 mM), respectively. Other conditions are the same as in Fig. 5. The fluorescence intensity of each solution was normalized.

of QD and the concentration of materials used in the paper. The inspection instrument to detect the CRET was a home-built system, which is simple and crude. The sensitivity of the biosensor will be better if the property of the system is improved. The physical property of QD is an important factor to the biosensor's sensitivity based on CRET. The QDs used in the paper are home-synthesized. The sensitivity can be improved if QDs have better properties. In addition, the sensitivity will be improved by optimizing the test conditions, such as pH and the buffer solution.

Conclusion

In conclusion, a new strategy for detection of ATP using aptamer/QDs biosensor based on CRET was developed. The luminol/hydrogen peroxide CL reaction catalyzed by horseradish peroxidase (HRP) was chosen as the light source, and QDs as acceptor. Conformational change of aptamer with ATP specifically induced chemiluminescence resonance energy transfer. In contrast to FRET, CRET occurs by the oxidation of luminol without an excitation source. In addition, the new luminescent semiconductor nanocrystals have been adopted as acceptor, reducing greatly the interference of autofluorescence and photobleaching. It can be predicted that biosensors based on CRET has good potential in detection of ATP. Our future work is to optimize the system, enhance the efficiency of CRET, improve sensitivity, and develop detection at the cell level.

Acknowledgements

This work was supported by the National Key Technology R&D Program of China (2012BAI23B02), National Natural Science Foundation of China (Grant no. 81071229), Specialized Research Fund for the Doctoral Program of Higher Education of China (no. 20100142110002), the Fundamental Research Funds for the Central Universities (Hust, 2012TS016), and the Open Research Fund of State Key Laboratory of Bioelectronics, Southeast University. We also thank Analytical and Testing Center (HUST) for help with measurement.

Notes and references

1 Y. Eguchi, S. Shimizu and Y. Tsujimoto, *Cancer Res.*, 1997, **57**, 1835–1840.
2 H. Yokoshiki, M. Sunagawa, T. Seki and N. Sperelakis, *Am. J. Physiol.: Cell Physiol.*, 1998, **274**, C25–C37.
3 S. Przedborski and M. Vila, *Clin. Neurosci. Res.*, 2001, **1**, 407–418.
4 L. Annunziato, G. Pignataro and G. F. Di Renzo, *Pharmacol. Rev.*, 2004, **56**, 633–654.
5 (*a*) M. R. L. Stratford and M. F. Dennis, *J. Chromatogr., Biomed. Appl.*, 1994, **662**, 15–20; (*b*) B. U. Sevin, J. P. Perras, H. E. Averette, D. M. Donato and M. Penalver, *Cancer*, 1993, **71**, 1613–1620; (*c*) W. Li, Z. Nie, X. H. Xu, Q. P. Shen, C. Y. Deng, J. H. Chen and S. Z. Yao, *Talanta*, 2009, **78**, 954–958; (*d*) A. J. Moro, J. Schmidt, T. Doussineau, A. Lapresta-Fernandez, J. Wegener and G. J. Mohr, *Chem. Commun.*, 2011, **47**, 6066–6068.
6 (*a*) C. Tuerk and L. Gold, *Science*, 1990, **249**, 505–510; (*b*) A. D. Ellington and J. W. Szostak, *Nature*, 1990, **346**, 818–822; (*c*) D. L. Robertson and G. F. Joyce, *Nature*, 1990, **344**, 467–468.
7 T. Li, S. J. Dong and E. Wang, *Anal. Chem.*, 2009, **81**, 2144–2149.
8 H. X. Zhang, B. Y. Jiang, Y. Xiang, Y. Y. Zhang, Y. Q. Chai and R. Yuan, *Anal. Chim. Acta*, 2011, **688**, 99–103.
9 N. Hamaguchi, A. Ellington and M. Stanton, *Anal. Biochem.*, 2001, **294**, 126–131.
10 V. Pavlov, Y. Xiao, B. Shlyahovsky and I. Willner, *J. Am. Chem. Soc.*, 2004, **126**, 11768–11769.
11 B. R. Baker, R. Y. Lai, M. S. Wood, E. H. Doctor, A. J. Heeger and K. W. Plaxco, *J. Am. Chem. Soc.*, 2006, **128**, 3138–3139.
12 J. Wang, L. H. Wang, X. F. Liu, Z. Q. Liang, S. P. Song, W. X. Li, G. X. Li and C. H. Fan, *Adv. Mater.*, 2007, **19**, 3943–3946.
13 X. L. Zuo, S. P. Song, J. Zhang, D. Pan, L. H. Wang and C. H. Fan, *J. Am. Chem. Soc.*, 2007, **129**, 1042–1043.
14 X. L. Zuo, Y. Xiao and K. W. Plaxco, *J. Am. Chem. Soc.*, 2009, **131**, 6944–6945.
15 N. Rupcich, R. Nutiu, Y. F. Li and J. D. Brennan, *Anal. Chem.*, 2005, **77**, 4300–4307.
16 N. Kamekawa, Y. Shimomura, M. Nakamura and K. Yamana, *Chem. Lett.*, 2006, 660–661.
17 Z. Chen, G. Li, L. Zhang, J. F. Jiang, Z. Li, Z. H. Peng and L. Deng, *Anal. Bioanal. Chem.*, 2008, **392**, 1185–1188.
18 A. Bogomolova and M. Aldissi, *Biosens. Bioelectron.*, 2011, **26**, 4099–4103.
19 R. Freeman, X. Q. Liu and I. Willner, *J. Am. Chem. Soc.*, 2011, **133**, 11597–11604.
20 H. Zhang, Z. Zhou, B. Yang and M. Y. Gao, *J. Phys. Chem. B*, 2003, **107**, 8–13.
21 W. W. Yu, L. H. Qu, W. Z. Guo and X. G. Peng, *Chem. Mater.*, 2003, **15**, 2854–2860.
22 H. Q. Wang, Y. Q. Li, J. H. Wang, Q. Xu, X. Q. Li and Y. D. Zhao, *Anal. Chim. Acta*, 2008, **610**, 68–73.
23 S. Dhar, N. Kolishetti, S. J. Lippard and O. C. Farokhzad, *Proc. Natl. Acad. Sci. U. S. A.*, 2011, **108**, 1850–1855.
24 D. K. Tiwari, S. I. Tanaka, Y. Inouye, K. Yoshizawa, T. M. Watanabe and T. Jin, *Sensors*, 2009, **9**, 9332–9354.
25 N. Q. Jia, Q. Zhou, L. Liu, M. M. Yan and Z. Y. Jiang, *J. Electroanal. Chem.*, 2005, **580**, 213–221.
26 A. R. Clapp, Z. Li, Y. Wang, G. Zhang, W. Xu and Y. Han, *J. Lumin.*, 2010, **30**, 995–999.