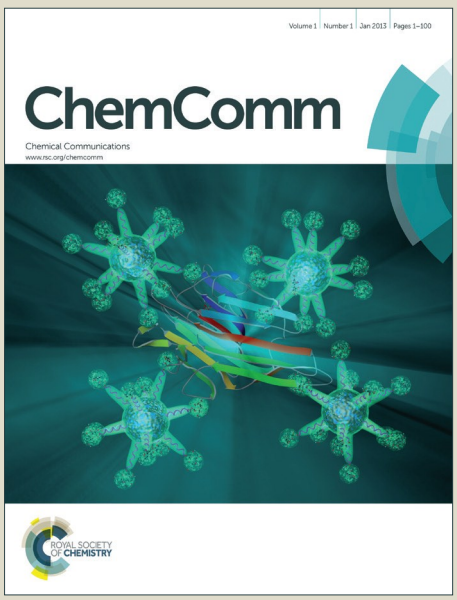
ChemComm

Accepted Manuscript



ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/chemcomm

Chemical Communications

COMMUNICATION

Enhanced ELISA with handheld pH meters and enzyme-coated microparticles for the portable, sensitive detection of proteins†

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

www.rsc.org/

Yun Zhang,* Jiani Yang, Jinfang Nie,* Juanhua Yang, Dong Gao, Lang Zhang and Jianping Li

This work describes a general methodology of enhanced enzyme-linked immunosorbent assay (ELISA) that integrates enzyme-coated microparticle probes for robust yet highly efficient signal amplification and a handheld pH meter for simple, portable, quantitative readout. Its utility is well demonstrated with detection of target protein with a 14-fold enhancement of sensitivity in comparison with conventional optical ELISA.

Robust, sensitive identification and quantification of protein targets of interest is a crucial need for research, forensic, and clinical applications. For instance, the sensitive detection of low abundance clinically relevant protein biomarkers in biological samples such as serum from patients could substantially improve disease diagnostics.[1-8] The enzyme-linked immunosorbent assay (ELISA), which has origins in 1970s,[9,10] is the most widely used and reliable clinical routine method for the detection of important protein markers.[11-13] This fundamental technique can produce satisfactory analytical specificity and sensitivity because it combines the specificity of antibodies with high-turnover catalysis by enzymes.[14,15]

Most ELISAs today are carried out in microtitter plates consisting of multiple, small-volume-capacity wells made of plastic such as polystyrene.[11,14-17] In a typical microtitter plate-based sandwich ELISA (as depicted in Fig. 1A), an antigen analyte is sandwiched between a primary antibody absorbed on the microtitter well and a biotinylated second antibody that further binds a streptavidin-conjugated enzyme via a biotin-streptavidin linkage. A substrate is then added to chemically amplify the colorimetric or fluorometric output signal of the enzyme reporter that can be detected by using a benchtop spectrophotometer as plate reader.[15] The use of microtitter plates is advantageous in that it not only allows users a facile separation of the antigen-antibody and biotin-streptavidin complexes from interferences in complex matric and unbound reagents, respectively, by washing the well surface, but also allows them to simultaneously process many samples.[10,11] As a consequence, the format of microtitter plate-based ELISAs has not changed significantly since its advent in the early 80's.[11,18]

Despite its popularity, however, the routine optical ELISA has rarely been applied to practical analyses outside of big laboratories,[19] as it requires an expensive, bulky plate reader (spectrophotometer) and well-trained users. In an attempt to meet a growing demand of affordable, user-friendly, portable ELISAs for use in resource-limited settings in both developed and developing countries (e.g., remote regions, small laboratories, or private clinics, etc.), a series of creative modified ELISAs have been reported in recent years, mainly including plasmonic ELISAs by the naked eye,[17,20] paper-based ELISAs with a digital camera or a colour densitometer for quantitative readout,[21,22] and plastic microchip-based ELISAs using a cell phone or a charge-coupled device as a portable quantitative reader.[23,24] Nevertheless, these methods still suffer from a few drawbacks. For example, the plasmonic colorimetric ELISAs only offer qualitative or semi-quantitative analysis. The paper- or plastic microchip-based ELISAs have lower detection sensitivity than the traditional optical ones. In this regard, developing a general ELISA that is sensitive, cost-

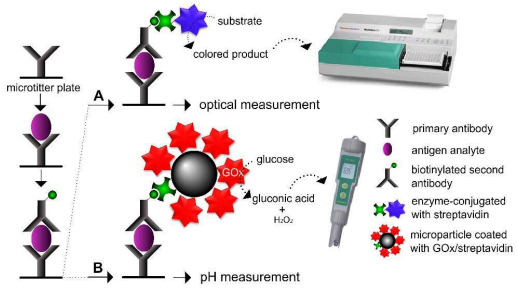


Fig. 1 Schematic illustration of (A) the conventional optical ELISA with a benchtop spectrophotometer and (B) the handheld pH meter-based portable ELISA that uses microparticles functionalized with glucose oxidase (GOx) and streptavidin as signal amplification probes.

Guangxi Key Laboratory of Electrochemical and Magnetochemical Function Materials, College of Chemistry and Bioengineering, Guilin University of Technology, Guilin 541004, P. R. China.
E-mail: zy@glut.edu.cn, Niejinfang@glut.edu.cn
† Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here]. See DOI: 10.1039/x0xx00000x

efficient, portable for use in these resource-poor settings, operable by minimally-trained users, and applicable to existing commercially-available ELISA setup, could be of high interest.

Herein, we describe a proof-of-concept of a complementary ELISA that has many of these merits, by using microparticles functionalized with glucose oxidase (GOx) and streptavidin for robust yet highly efficient signal amplification and a handheld pH meter as a cheap, portable quantitative reader (as depicted in Fig. 1B). A target antigen is captured with its specific primary antibody on the well surface. After a biotinylated second antibody binds the analyte, a microparticle probe coated with a large number of GOx and a streptavidin is subsequently used to rapidly recognize the biotin molecule via gravity effect. The GOx tags covalently conjugated on the microparticle maintain highly efficient catalytic activity for specific glucose oxidation to generate a great amount of gluconic acid and hydrogen peroxide (H_2O_2) (Fig. S1 in ESI†).[27] The enzymatic reaction-induced change in hydrogen ion (H^+) level is then determined using a handheld pH meter. The pH change positively depends on the analyte concentration in the sample. This concept was demonstrated by quantifying the level of a model analyte, human oncogenic protein (HOP, BCR-ABL p190), a biomarker associated with acute lymphoblastic leukaemia.[25,26] The detailed procedures of this new ELISA were shown in ESI†.

Battery-powered handheld pH meters are one family of portable quantitative devices among the most successful sensors in the world.[28,29] These devices have many attractive features that make them being routinely used for a wide range of applications such as laboratory measurements and field environmental analysis.[29] Key among these features includes low price, ease of use, portability, and accurate, sensitive pH detection.[29] Moreover, the advantage of using microparticles as detection probes is that each microparticle carries with it a large number of GOx tags per HOP binding event, therefore resulting in robust yet highly efficient signal amplification. The results show that the proposed handheld pH meter-based ELISA enables portable, rapid, sensitive, selective detection of the HOP target in buffer and human serum samples at fg mL^{-1} levels. Since such an ELISA design does not modify significantly the basic workflow and detection reagent kit of conventional ELISA (Fig. 1), it thus holds great potential to be applicable to most of existing commercially-available ELISA setup. To the best of our knowledge, this is the first report of using handheld pH meters as quantitative readers to develop affordable, user-friendly, and portable ELISAs for resource-poor settings.

The key concept of our method focuses on converting the HOP detection into the detection of pH of gluconic acid and H_2O_2 generated from the GOx-catalyzed oxidation of glucose using the handheld pH meter. Thus, in order to successfully carry out this new ELISA, the first challenge is to realize sensitive, accurate, precise detection of pH change that is positively associated with the level of H^+ ionized from the enzymatic products such as gluconic acid. In general, a buffer with a pH range of 5-7 is beneficial to the GOx-catalyzed oxidation of glucose. However, due to the buffering action of 10 mM phosphate-buffered saline (PBS, pH 5.8) solution, the addition of gluconic acid in a concentration rang of 0.62 μg

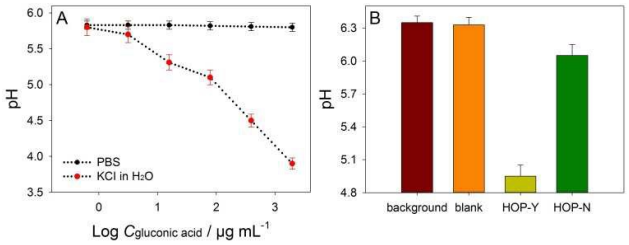


Fig. 2 (A) Comparison of the pHs of gluconic acid solutions of different levels in 10 mM PBS (pH 5.8) or 2.5 mM KCl in ultrapure water. (B) pHs obtained from the 2.5 mM KCl solution (background) and the assays of a blank sample (PBS without HOP) and 27 pg mL^{-1} HOP samples with (HOP-Y) or without (HOP-N) using the functionalized microparticle probes. The error bars reflect the standard deviations from three repetitive experiments of each solution or sample.

mL^{-1} – 1.96 mg mL^{-1} caused no obvious changes in the pHs of final mixture solutions (Fig. 2A, black dots). In this regard, to maximize the pH change originated from the generation of gluconic acid (and H_2O_2), the glucose solution was prepared with ultrapure water (with a 18.2 MΩ·cm resistivity) for enzymatic reaction. Nevertheless, the ultrapure water with low conductivity did not allow for rapid and robust pH measurement because of lack of adequate electrolytes (Fig. S2 in ESI†). Potassium chloride (KCl) is a commonly used neutral salt additive to improve solution conductivity. It was found that precise pH measurement could be achieved using a 2.5 mM KCl aqueous solution (Fig. S2 in ESI†). As a result, clearly distinguishing pH signals were observed from these gluconic acid solutions in this optimized electrolyte solution (i.e., 2.5 mM KCl in water) (Fig. 2A, red dots). The pH responses negatively relied on the gluconic acid levels. Moreover, the presence of glucose of different levels in a gluconic acid solution had no effect on its pH measurement (Fig. S3 in ESI†). Additionally, since the used pH meter has a built-in function of temperature compensation, it enabled accurate pH detection of low or high abundance gluconic acid solutions in a broad temperatures range of 4 – 45 °C (Fig. S4 in ESI†).

Under the optimized conditions for enzymatic reactions and pH measurement, the feasibility of pH meter-based ELISA was first demonstrated. Assays of a blank sample (PBS buffer without HOP) and a 27 pg mL^{-1} HOP sample were carried out according to the procedures schematically shown in Fig. 1B. After the generation of gluconic acid and H_2O_2, the pH of final reaction mixture for each sample was measured using the handheld pH meter, comparing with the background pH of a 2.5 mM KCl solution. Fig. 2B displays that no obvious pH changes are obtained between the blank sample and the background solution. In contrast, the pH recorded from the HOP sample is ca. 4.95, which is far lower than the background pH (ca. 6.35). The dramatically reduced pH value observed in the HOP assay reflects that after the target was captured with its primary antibody on the well, it was further traced by a microparticle probe coated with GOx tags that could still highly efficient catalyse the oxidation of glucose in ultrapure water to create a large amount of gluconic acid and H_2O_2. Moreover, a

pH of ca. 6.05 was obtained using GOx-streptavidin conjugates for the analysis of 27 pg mL^{-1} HOP. It is relatively lower than the background pH from the 2.5 mM KCl, but is far higher than that obtained from the microparticle probe-based assay of the HOP sample at the same level. The data suggests that as each microparticle probe carried with it a great number of GOx labels per HOP binding event, the amount of gluconic acid and H_2O_2 produced in this case was far higher than that created in the absence of such probes. In other words, in comparison with the single-enzyme method used in conventional ELISA (Fig. 1A), the microparticle-based multi-enzyme design in the new ELISA strategy (Fig. 1B) is able to significantly amplify the signal of each antigen-antibody binding event. Thus, a considerably improved assay sensitivity could be expected. The gravity action of the microparticle probes additionally facilitated their efficient mass transport from bulk solution to the well surface. Their sedimentation was fast (within 5 min), thus leading to a reduced total time (ca. 20 min) for the binding of biotinylated second antibody-HOP complex with the streptavidin on their surfaces. It typically takes a longer period of time (ca. 1 h) in traditional ELISA to finish such reactions due to low mass transport of streptavidin-conjugated enzyme tags via Brownian movement, although these processes themselves are rapid.[30]

Next, after demonstrating the feasibility of the pH meter-based ELISA and the signal amplification by means of GOx-coated microparticles, its detection specificity was studied by comparatively assaying a PBS sample with a final HOP level of 40 pg mL^{-1}, 40 ng mL^{-1} bovine serum albumin (BSA), 40 ng mL^{-1} human serum albumin (HSA), undiluted normal human serum (NHS), and a NHS sample with a final HOP level of 40 pg mL^{-1}. The corresponding ΔpH results were shown in Fig. 3A. One can clearly find from Fig. 3A that while a ΔpH as large as 1.5 is obtained from the HOP sample, the ΔpHs from both BSA and HSA are less than 0.1, although the levels of the two non-specific proteins are 3 orders of magnitude higher than the HOP level. The assay of the undiluted NHS also led to a small pH change (ca. 0.07) that could be negligible. Moreover, the interferences in the NHS caused no obvious effects on the ΔpH signals of the HOP-spiked NHS and the HOP sample in buffer that had the same analyte level. These results indicate that the new ELISA inherits from conventional ELSIAs good specificity. This is attributed to that the primary antibody and second antibody kept their molecular recognition capacity for the HOP.[14,15] In addition, the microtitter well-based washing steps in each assay could minimize the effects of unbound reagents or interferences in the complex serum matric.[10,11]

Then, a series of HOP samples in buffer with varying analyte levels were assayed to prove sensitivity and robustness of this new approach. The pH change signal for each sample was defined as $\Delta pH = pH_s - pH_b$, where pH_s and pH_b were the pH values recorded by the pH meter for the HOP sample and a PBS blank sample, respectively. The relationship between the resultant ΔpHs and logarithm values of HOP concentrations ($Log C_{HOP}$) tested was illustrated in Fig. 3B (black dots). It is found from Fig. 3B that as the HOP concentration increases, the ΔpH increases, clearly displaying HOP level-controlled ΔpH responses. This new ELISA is linearly sensitive to the analyte in

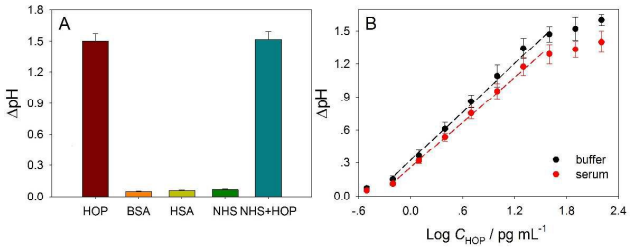


Fig. 3 (A) pH changes (ΔpHs) obtained from the assays of 40 pg mL^{-1} HOP, 40 ng mL^{-1} BSA and HSA, undiluted NHS, and 40 pg mL^{-1} HOP in NHS, respectively. (B) Exponential relationship between ΔpHs obtained from different HOP samples and logarithm values of concentrations of HOP ($Log C_{HOP}$). Both linear ranges of the final HOP levels in the buffer and serum are from 620 fg mL^{-1} to 40 pg mL^{-1} with regression equations of $y = 0.7593x + 0.3093$ ($R = 0.9978$) and $y = 0.6718x + 0.2630$ ($R = 0.9961$), respectively. The error bars reflect the standard deviations from three repetitive experiments of each sample.

the buffer samples in a level range of 620 fg mL^{-1} – 40 pg mL^{-1} (Fig. 3B, black dots). The limit of detection (LOD) for HOP was estimated to be ca. 570 fg mL^{-1} (3σ). The comparison results of the proposed ELISA and the conventional ELISA were shown in Table S1 in ESI†. While the traditional ELISA was conducted by well-trained users using an expensive (ca. $ 15700), benchtop spectrometer, this new method required only a quite cheap (ca. $ 30), easy to use, handheld pH meter to offer portable quantitative analysis of the HOP analyte. Furthermore, its LOD (ca. 570 fg mL^{-1}) is ca. 14 times lower than that of the former (ca. 8 pg mL^{-1}), presumably due to the use of microparticle amplification probes. The relative standard deviations (RSDs) in three repetitive assays of 0.6, 1.3, 2.5, 5, 10, 20 and 40 pg mL^{-1} HOP were 3, 5.2, 6.2, 6, 7.7, 7.3 and 6.8%, respectively, implying the acceptable reproducibility of the new ELISA.

Based on these good results in hand, in order to assess the reliability of the developed portable ELISA system, assays of HOP-spiked undiluted NHS samples was conducted according to the same procedures. The resulting linear concentration range and LOD for HOP was 620 fg mL^{-1} – 40 pg mL^{-1} (Fig. 3B, red dots) and 607 fg mL^{-1} (3σ), respectively. The average RSD of all the tests was ca. 7.2%. The same linear concentration ranges and similar LODs for the buffer and NHS samples therefore demonstrate the reliability of this new approach. Moreover, recovery experiments of HOP in both buffer and serum samples were also performed. The final HOP levels in the PBS or NHS were in the linear detection ranges above. One can find from Table S2 in ESI† that the obtained recovery results range from 94.2 to 102.9% and the RSDs are in the range of 1.1 – 8.5% ($n = 6$), thus validating the acceptable recovery, accuracy and practicability of the new method for quantifying the HOP in complex matrics such as body fluids.

In conclusion, we have developed successfully an enhanced portable ELISA that integrates functionalized microparticles and handheld pH meters. Each microparticle probe that carries itself with a great number of enzyme tags can allow for highly efficient signal amplification for each antigen-antibody specific binding event. The cheap, widely accessible, battery-powered,

handheld pH meter is well demonstrated to be easy-to-use and sensitive for portable quantitative readout. This new ELISA strategy inherits main advantages of conventional ELSIA such as excellent specificity. More importantly, it offers improved performance especially in terms of portability, sensitivity, assay cost, and technical requirement. Moreover, it addresses many shortcomings of these recently reported portable ELISAs, mainly including lack of quantitative ability and unsatisfactory detection sensitivity (Table S3 in ESI†).

In this initial proof-of-concept study, the HOP only served as a model analyte. Because the developed handheld pH meter-based portable ELISA system does not modify significantly the basic workflow and detection reagent kit of traditional ELISA (Fig. 1), it holds great potential to be generally applicable to most of existing commercially-available ELISA setup for routine quantification of analytes of interest in various resource-limited settings in either developed or developing countries.

It should be noted that another type of ubiquitous, portable, quantitative devices, namely personal glucose meters, have attracted considerable interests for developing various cost-efficient, user-friendly, and field bioassays in recent years.[31-40] Nevertheless, glucose meters are primarily designed to detect glucose in a concentration range of 198 μg mL^{-1} – 5.99 mg mL^{-1} (Table S4 in ESI†). Interestingly, the initial studies of a pH measurement-based glucose assay, in which the as-prepared GOx-coated microparticles were used to catalyse the oxidation of glucose to create gluconic acid and H_2O_2 that was further detected using a handheld pH meter, showed that it could provide a ca. 227-fold enhancement of sensitivity for glucose detection compared with the glucose meter technique (Fig. S5 and Table S4 in ESI†). Thus, the proposed handheld pH meter-based assay platform offers new opportunities for the design of simple, sensitive, and portable detection systems where the glucose acts as a secondary analyte.

This work was financially supported by National Natural Science Foundation of China (21105017, 21205021, 21365009 and 21565012), Natural Science Foundation of Guangxi (2014GXNSFAA118033 and 2015GXNSFFA139005), Projects of High Level Innovation Team/Outstanding Scholar and Key Laboratory of Food Safety and Detection in Guangxi Colleges and Universities, and Open Foundation of State Key Laboratory of Chemo/biosensing and Chemometrics at Hunan University.

Notes and references

1 L. Rodríguez-Lorenzo, R. de la Rica, R. A. Álvarez-Puebla, L. M. Liz-Marzán andM. M. Stevens, *Nat. Mater.,* 2012, **11**, 604.
2 N. A. W. Bell and U. F. Keyser,*J. Am. Chem. Soc.,* 2015, **137**,2035.
3 X. P. Yu, Y. N. Li, J. Wu and H. X. Ju,*Anal. Chem.,* 2014, **86**, 4501.
4 H.Xie, Q. Q. Zhang, Q. Wang, Y. Q. Chai, Y. L. Yuan and R. Yuan,*Chem. Commun.,* 2015,**51**, 7132.
5 H. Q. Zhang, F. Li, B. Dever, C. A. Wang, X. F. Li and X. Chris Le,*Angew. Chem. Int. Ed.,*2013, **52**, 10698.
6 C. Yin, M. Wang, C. Y. Lei, Z. Wang, P. Li, Y. Li, W. Li, Y. Huang, Z. Nie and S. Z. Yao,*Anal. Chem.,* 2015, **87**, 6311.
7 J. F.Nie, Y. Zhang, L. W. Lin, C. B. Zhou, S. H. Li, L. M. Zhang and J. P. Li. *Anal. Chem.,*2012, **84**, 6331.
8 H. Li, Q. Wei, G. L. Wang, M. H. Yang, F. L. Qu, Z. Y. Qian,*Biosens. Bioelectron.,*2011, **26**, 3044.
9 B. K. Van Weemen and A. H. W. M. Schuurs, *FEBS Lett.,* 1971, **15**, 232.
10 E. Engvall and P. Perlmann, *Immunohistochemistry,* 1971, **8**, 871.
11 J. H. Kai, A. Puntambekar, N. Santiago, S. H. Lee, D. W. Sehy, V. Moore, J. Han and C. H. Ahn, *Lab Chip,* 2012, **12**, 4257.
12 H. J. Lin,Y. F. Liu,J. R. Huo, A. H. Zhang, Y. T. Pan, H. H. Bai, Z. Jiao, T. Fang, X. Wang, Y. Cai, Q. M. Wang, Y. J. Zhangand X. H. Qian,*Anal. Chem.*, 2014, **86**, 10021.
13 A. Bhimji, A. A. Zaragoza, L. S. Live and S. O. Kelley,*Anal. Chem.,* 2013, **85**, 6813.
14 R. Edwards, Immunodiagnostics, Oxford University Press, Oxford, 1999.
15 A. W. Martinez, J. L. Gong, C. R. Mace, S. T. Phillips, E. Carrilho, K. A. Mirica and G. M. Whitesides, *Angew. Chem. Int. Ed.,* 2010, **49**, 4771.
16 N. Guarrotxena, B.Liu, L. Fabris and G. C.Bazan, *Adv. Mater.,* 2010, **22**, 4954.
17 R. de la Rica andM. M. Stevens, *Nat.Nanotechnol.,* 2012, **7**, 821.
18 D. Wild, The immunoassay handbook, 2005.
19 J. H. Cho,S. M. Han,E. H. Paek,H. Choand S. H. Paek, *Anal. Chem.,*2006, **78**, 793.
20 D. Cecchin, R. de la Rica, R. E. S. Bain, M. W. Finnis, M. M. Stevens and G. Battaglia,*Nanoscale,* 2014, **6**, 9559.
21 R. C. Murdock,L. Shen, D. K. Griffin, N. Kelley-Loughnane, I. Papautsky and J. A. Hagen, *Anal. Chem.,* 2013, **85**, 11634.
22 J. F. Tian, X. Li and W. Shen,*Lab Chip,* 2011, **11**, 2869.
23 S. Q. Wang, X. H. Zhao, I.Khimji, R. Akbas, W. L. Qiu, D. Edwards, D. W. Cramer, B. Ye and U. Demirci, *Lab Chip,* 2011, **11**, 3411.
24 S. Sun, M. H. Yang, Y. Kostov and A. Rasooly*Lab Chip,* 2010, **10**, 2093.
25 E.Tse, M. N. Lobato, A. Forster, T. Tanaka, G. T. Y. Chung and T. H. Rabbitts,*J. Mol. Biol.,* 2002,**317**, 85.
26 J. W.Voncken, V.Kaartinen, P. K. Pattengale, W. T.V. Germeraad, J.Groffenand N.Heisterkamp,*Blood,*1995, **86**, 4603.
27 D. Müller, *Biochem. Z.,* 1928, **199**, 136.
28 D. Kwon, J. Joo, S. Leeand S. Jeon,*Anal. Chem.,*2013, **85**, 12134.
29 S. B. Xie, Y. L. Yuan, Y. Song, Y. Zhuo, T. Li, Y. Q. Chai and R. Yuan,*Chem. Commun.,* 2014, **50**, 15932.
30 J. S. Rossier, G. Gokulrangan, H. H. Girault, S. Svojanovsky, G. S. Wilson, *Langmuir,* 2000, **16**, 8489.
31 L. Yan, Z. Zhu, Y. Zou, Y. S. Huang, D. W. Liu, S. S. Jia, D. M. Xu, M. Wu,Y. Zhou, S. Zhou and C. Y. James Yang,*J. Am. Chem. Soc.,*2013, **135**, 3748.
32 Y. Xiang and Y. Lu, *Anal. Chem.,* 2012, **84**, 4174.
33 X. T. Xu, K. Y. Liang andJ. Y. Zeng, *Biosens. Bioelectron.,*2015, **64**, 671.
34 L. B. Fu, J. Y. Zhuang, W. Q. Lai, X. H. Que,M. H. Luand D. P. Tang, *J. Mater. Chem. B,* 2013, **1**, 6123.
35 Q. Wang, H. Wang, X. H. Yang, K. M. Wang, F. Liu, Q. Zhao, P. Liuand R. J. Liu, *Chem. Commun.,* 2014, **50**, 3824.
36 J. Xu, B. Y. Jiang,J. Q. Xie, Y. Xiang,R. Yuan and Y. Q. Chai, *Chem. Commun.,* 2012, **48**, 10733.
37 Y. M. Wang, M. H. Lu, J. H. Zhu and S. F. Tian, *J. Mater. Chem. B,* 2014, **2**, 5847.
38 Y. Xiang and Y. Lu, *Chem. Commun.,* 2013, **49**, 585.
39 H. Mohapatra and S. T. Phillips, *Chem. Commun.,* 2013, **49**, 6134.
40 X. Zhu,H. F. Xu,R. L. Lin, G. D. Yang, Z. Y. Lin and G. N. Chen, *Chem. Commun*., 2014, **50**, 7897.