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A novel microfluidic chip electrophoresis strategy for simultaneous, label-free, multi-protein detection based on a graphene energy transfer biosensor†

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A new type of high-throughput and parallel optical sensing platform with a single-color probe based on microfluidic chip electrophoresis combined with aptamer–carboxyfluorescein/graphene oxide energy transfer is reported here. Label-free protein multi-targets were detected, even in challenging complex samples without any pre-treatment.

Introduction

When the emission spectrum of a donor fluorescent molecule and the absorption spectrum of a receptor overlap, and the distance between two molecules is within 10 nm, Förster resonance energy transfer (FRET) occurs as a phenomenon of non-radioactive energy transfer *via* long-range dipole–dipole interactions. This effect forms the basis of the wide application of elaborately-designed FRET with pronounced selectivity and sensitivity.[1] The simultaneous detection of multiple analytes is appealing for industrial, environmental and biosecurity applications. Sensitive and multiplex protein detection is vitally important in numerous areas because it can provide necessary information for deeper understanding of the genomic expression, molecular diagnostics and pharmacoproteomics. Nevertheless, it is noteworthy that simultaneously parallel multiple-target sensing remains challenging. In order to increase the efficiency in this kind of mix-and-detect assay, multi-color DNA probes have been synthesized.[2,3] But the advance towards high-throughput sensing is hampered by the limited number of colored probes available. In the same way as an electrochemical sensor array, one kind of sensor array with ensemble aptamers for a wider range of targets has been reported.[4] Capillary electrophoresis (CE) has also been employed to assist the simultaneous detection of dual single-base mutations in a given DNA oligonucleotide using two probes,[5] in addition to being used as an effective separation of donor–acceptor complexes from free donor and acceptor to avoid the interference of any unhybridized molecular beacon (MB) signal.[6,7] However, as far as we know, a separation technique based, label-free, multiple detection with individual color probes has never been reported. The striking emphasis of no sample pre-treatment in traditional mix-and-detect methods actually increases the difficulty to realize multi-target analysis. Being the most efficient separation technique, chromatography could be combined with RET, instead of being incompatible, to innovate multi-target sensing. Actually, if the separation ability is high enough, the interaction between probe and target could also be more conveniently demonstrated, avoiding the trouble of probe array immobilization. In this sense, chromatography can also be regarded as a kind of array technology without the difficulty of probe array immobilization.

What is the cause of no reports on this until now? It was reasonable to infer that the dedicated interaction balance between nanomaterial, energy donor and bioconjugate pair makes it difficult for the biosensor system to adapt to the interaction with the stationary phase and harsh elution conditions in traditional liquid chromatography separation. CE has been employed[5–7] due to its free solution environment and mild separation conditions. However, in order to further increase the detection number of targets in CE, all strategies to improve the separation resolution should be considered.[8] Microfluidics has been greatly advanced since μ-TAS (miniaturized total analysis systems) was coined by Manz *et al.*[9] Common analytical assays, including polymerase chain reactions, protein analysis, DNA separation, and cellular studies, have been performed in microfluidic devices.[10] Among the many characteristics of multidimensional chip electrophoresis, including speed, low-volume sample consumption and portability *etc.*, the most striking and advantageous characteristic, especially compared with CE, is the higher peak capacity which is expected easily to allow the high-throughput and parallel analysis of targets.

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Thanks to these advantages, chip-based chromatography might bring us a new result.

There are many kinds of functional nanoparticles, such as quantum dots, metal nanoparticles, and carbon nanomaterials used in optical biosensing. Graphene has received great interest in various areas because of its extraordinary electronic, thermal, and mechanical properties.[11] As a result of p-stacking interactions, graphene oxide (GO) sheets can strongly adsorb single-stranded nucleic acids, and then quench the fluorescence of an organic dye, which is labelled on ssDNA.[12–14] Very recently, the biological applications of GO, have been a very active research field in the development of biosensors because of the ultrahigh quenching efficiency of GO.[2,4,15–19] And then, in order to investigate the possibility of multi-detection based on chip-CE, in the same way as the literature, in this paper GO was anticipated to serve as an energy acceptor of the fluorescein FRET system to construct a fluorescent sensor for the sensitive detection of the biorecognition event between an aptamer and protein. Three kinds of proteins were chosen as target proteins considering that their aptamers had been utilized by others demonstrating their stronger binding affinity.[20–22] Furthermore, since most sensing work using oligonucleotide probes has been performed on model systems, and few clinically relevant targets have actually been tested,[23] the ability of this proposed multiplex detection in a complex matrix was also demonstrated here.

Experimental

Reagents

Three kinds of DNA aptamers, targeting thrombin (5′-FAM-AGTCCGTGGTAGGGCAGGTTGGGGTGACT-3′),[21] cytochrome c (5′-FAM-AGTGTGAAATATCTAAACTAAATGTGGAGGGTGGGACG GGAAGAAGTTTATTTTTCACACT-3′)[22] and lysozyme (5′-FAM-ATCTACGAATTCATCAGGGCTAAAGAGTGCAGAGTTACTTAG-3′),[23] respectively, were synthesized by Sangon Biotech (Shanghai, China), with a 6-FAM label attached to the 5′-end of each aptamer. Thrombin, cytochrome c, lysozyme and carrier ampholytes (pH 3–10) were all purchased from Sigma-Aldrich (St. Louis, MO). Other reagents were purchased from Beijing Chemical Works (Beijing, China). Graphene oxide (GO) was synthesized from graphite powder using Hummer's method.[24] The electrophoresis chip utilized a spin-coated chromium plate (SG2506, UM grade, chromium layer thickness 145 nm; Az-1805 photoresist, 570 nm thickness; Hunan Changsha Shaoguang Microelectronics Co.) as the substrate, using the same type of sheet glass as a cover slip. All aqueous solutions were prepared with purified water using a Millipore Purification System (Billerica, MA).

Microchip fabrication

Glass chips were fabricated according to standard procedures involving photolithography, wet chemical etching, hole drilling, and high-temperature bonding.[25] A photomask with a multi-dimensional microchannel structure designed using Illustrator software was placed onto a glass substrate with a chromium and photo-resistant coating *via* UV exposure using a model JKG-2A lithographic device (Photomechanical, Shanghai, China). Then, the glass plate was immersed into 0.15% NaOH for 2 min, followed by water flushing, drying at 110 °C for 15 min and chromium removal. Then, the microchannels were etched in HF/HNO_3. After etching, the etched substrate and cover plate were washed sequentially with acetone and water and chromium was removed again. The plate was dried by heating, and then drilled. The plate was activated in concentrated sulfuric acid for 8 h, followed by washing with water, bonding and heating at 60 °C. Finally, the chip was placed in an oven at 110 °C for high-temperature bonding.

The experimental device and the microfluidic chip with its double-'T' structure are shown in Fig. 1. One kind of two-dimensional chip with a double-'T' structure is shown, including a 263 μm-wide and 1.4 cm-long straight horizontal IEF channel intersected by a secondary dimensional CZE separation channel 130 μm wide and 3.5 cm long. Another kind of two-dimensional chip, in which the IEF channels (263 μm wide and 1.4 cm long) were joined by four narrower channels (130 μm wide and 3.5 cm long) for secondary CZE separation, is also given.

Microchip electrophoresis for protein analysis

Sample preparation. Firstly, 2 μM aptamer of thrombin, 10 μM aptamer of cytochrome c and 10 μM aptamer of lysozyme were added into 0.3 mg mL^{-1} GO solution, followed by incubating in an oven at 90 °C for 5 min. The mixture was then cooled to room temperature. After that, thrombin, cytochrome c and lysozyme (0.25 mg mL^{-1}) were added to the solution with the volume ratio of GO, the three fluorescently-labelled aptamers and three proteins of 1 : 2 : 2 : 2 :2 : 2 : 2. Finally, the ampholyte was mixed into the solution with the volume ratio of 10% v/v.

The complex protein samples were prepared by extraction from normal bacteria cells. The cultured *Escherichia coli* (DH5α) cells were centrifuged at 4 °C, 10 000*g* for 15 min to precipitate

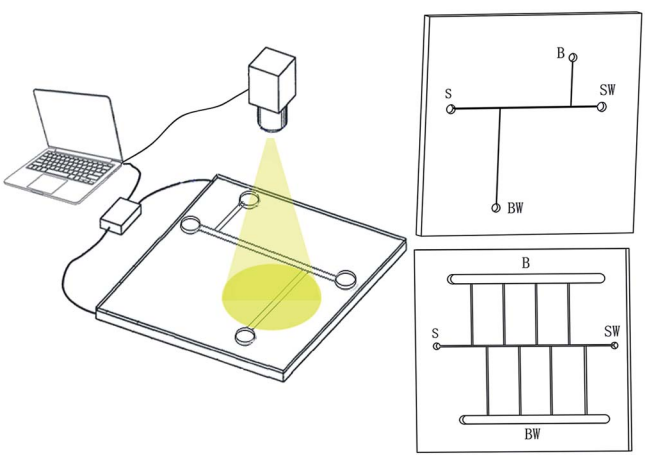

Fig. 1 (a) The scheme of the experimental device; (b) the chip for electrophoresis with a simple double-T structure (upper) and another kind of chip structure with multichannels (below).

the thallus. The precipitate was collected and re-suspended in PBS buffer (pH 7.5) followed by the addition of cell lysis buffer. The whole proteins of *E. coli* (0.25 mg mL^{-1}) were introduced into the incubation solution with GO, thrombin, cytochrome c and lysozyme, and their specific FAM-labelled aptamer in the volume ratio of 2 : 1 : 2 : 2 : 2 : 2 : 2 : 2 respectively. Finally, ampholyte (10% v/v) was mixed into the solution. After that, the sample could be injected into the chip microchannel for further analysis.

Two-dimensional microfluidic chip electrophoresis

As indicated in Fig. 1, isoelectric focusing (IEF) was performed between reservoirs S and SW, and secondary capillary zone electrophoresis (CZE) was performed between reservoirs B and BW. Microfluidic chip electrophoresis was carried out as follows: (a) before each electrophoresis measurement, the microchannels were washed sequentially with 1 mol L^{-1} NaOH for 20 min, water for 10 min and then 0.5% methyl cellulose (MC) dissolved in Tris–HCl for 10 min; (b) the 0.5% MC solution in the first-dimension channel was pumped out and changed with sample prepared above; (c) an electric potential was then applied to the microchannels by means of platinum electrodes placed in the reservoirs, using a computer-controlled high-voltage power supply (XCDY microfluidic chip electrical source purchased from Shandong Normal University), between the acid reservoir (reservoir S, anode) and the alkaline reservoir (reservoir SW, ground), for 30 s to initiate protein IEF; (d) after IEF, the electrical voltage was switched to reservoir B (ground) and reservoir BW (anode) for second-dimensional CZE separation. The typical separation medium in the secondary channels was 0.5% MC solution. The progress of the applied voltage and time for two-dimensional electrophoresis is listed in Table 1 (in ESI†).

Data collection and image analysis

The whole process was monitored and recorded using an IX70 inverted fluorescence stereomicroscope equipped with a CCD camera (Olympus, Japan). The field of view of the inverted microscope was aimed at the downstream of the secondary dimensional channel as demonstrated in Fig. 2. The spacing from the first channel to the fourth channel was in the diameter size of the microscope field. And then the separation behaviours in all channels could be captured and recorded by the microscope. ImageJ software (http://rsb.info.nih.gov/ij) was used to convert the photographs captured from the videos of electrophoresis into 3D electropherograms based on the fluorescence intensities in the channels.

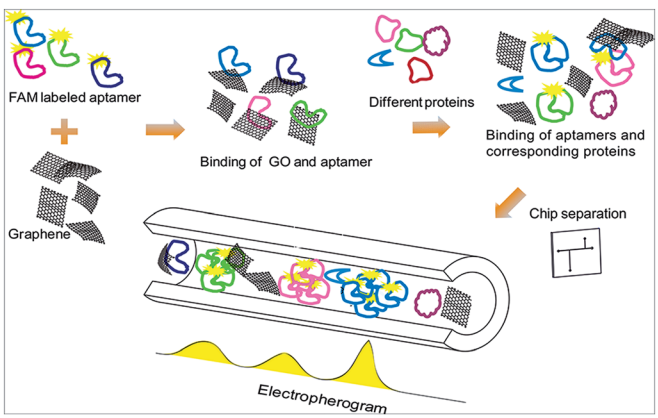

Fig. 2 The schematic diagram of chip-CE-assisted biosensor for the simultaneous detection of protein.

Results and discussion

As we assumed that the well-established FRET process involved two basic steps in this proposed system. First, fluorescein-based dye (FAM)-labelled ssDNA of aptamers assembled non-covalently onto GO. Due to the strong interaction between the aptamers and GO, the fluorescence of the labelled FAM was quenched by FRET between GO and FAM. Second, upon the introduction of proteins, the specific binding between the aptamer and its corresponding protein target resulted in a change of the interaction between the aptamer and GO, and then the release of the ssDNA probe from the surface of GO. As a result, the fluorescence of the FAM-labelled aptamer was restored. Otherwise, there was no recovery of the fluorescence without the target protein. The schematic diagram of chip-CE-assisted biosensor for the simultaneous detection of protein is given in Fig. 2.

Initially, experiments were carried out to optimize the ratio of GO and FAM-labelled aptamer. On the one hand, the interference of the fluorescent background brought about by free FAM-labelled aptamers was thus avoided. On the other hand, the proper ratio between GO and FAM-labelled aptamer made it efficient for the aptamer–protein complex to release from the surface of the GO, so that an obvious FRET signal change could be observed in the presence of the target analyte. A value of 0.3 mg mL^{-1} was finally chosen the as optimized GO concentration because the fluorescence intensity was only 0.4% of the original one. In addition, the ratio of aptamer to GO conjugates added into the mixture was not the same among different aptamers because it was found that the extent of the fluorescence recovery after binding of the aptamer and protein was different. According to literature,[20–22] it may be ascribed to the diverse affinity between these three pairs of protein–aptamer whose sequence structure was designed. With only one kind of fluorescent probe being used, the existence of multi-targets could not be determined by the current fluorescence spectral-based ensemble measurement. Chip-CE played a crucial role in achieving multiplex detection. One of the first issues confronting the experiment was achieving enough separation ability. In this paper, one kind of isoelectric focusing (IEF) and capillary zone electrophoresis IEF-CZE coupled two-dimensional electrophoresis chip designed with a double-T structure (Fig. 1, upper right) was used. It was found through the fluorescent electrophoresis images collected after first dimensional IEF, that the mixture failed to be separated by only the IEF separation mode between S and SW (data in ESI†), whereas it was not the case with multi-dimensional separation (Fig. 3).

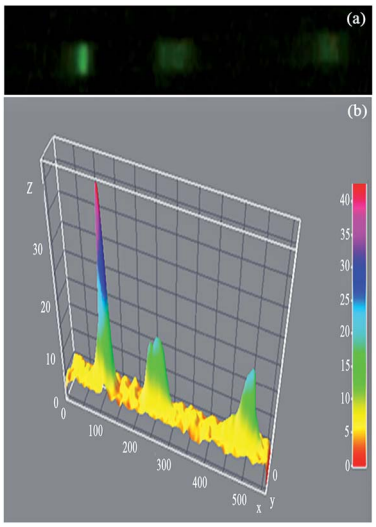

Fig. 3 (a) Fluorescent separation images using IEF and CZE coupled two-dimensional microchip electrophoresis (thrombin, cytochrome c and lysozyme from left to right); (b) the 3D plot spectrum of (a).

Fortunately, taking advantage of the high-performance IEF/CZE coupled 2D chip, electrophoresis with the double-T structure chip, as shown in Fig. 1, three protein–aptamer complexes were baseline separated after being further separated by the second dimension of CZE. The existence of the target protein could be determined according to the occurrence of the electrophoresis peak which corresponded to the aptamer and protein complex. In this way, three proteins can be detected simultaneously and in parallel. As for other chromatography-based strategies, such as western-blotting and CE[5–7] in which all the components are detected without selectivity, the successful separation of all components is a prerequisite for target analysis. In our system, only the targets specifically binding with the aptamer could create the fluorescent component which could then be observed through the next electrophoresis separation. The limited amount of fluorescent components that need to be detected certainly greatly reduces the burden on the subsequent separation.

The sizes of the GO particles existing in the buffer background during electrophoresis should be small enough to avoid any influence on protein separation and the subsequent fluorescent detection through light-scattering. At the same time, it was assumed by us that the main difference in our experiment among the various sizes of GO smaller than the one-size limit was the adsorption ability. Therefore, the ratios of GO with different sizes and fluorescent-labelled aptamer should be optimized respectively. In the study described in this paper, GO was synthesized from graphite powder using the Hummers' method.[24] The clear GO aqueous solution was also prepared in the same way as in the literature for FRET-based optical sensors. Through atomic force microscopy (AFM) analysis, the size of graphene used for this experiment was about 2 μm (data in the ESI†). From our experiments, satisfactory electrophoresis separation and fluorescent detection were obtained when this kind of GO was used, thus demonstrating that this size of GO was appropriate; accordingly, this size of GO was chosen for investigation. Based on that, we could further optimize the ratio of GO and fluorescently-labelled aptamer to realize multi-target detection. Another thing worth mentioning is how, after IEF, to transfer the fluorescent protein–aptamer complexes of focus from the IEF channel into the secondary dimension. Some components might be focused outside the two junctions of the first and secondary channels, for example the pI value of proteins was outside the pI range of ampholine used for IEF (thrombin (pI = 6.6, MW = 37 000), cytochrome c (pI = 10.7, MW = 12 000) and lysozyme (pI = 11, MW = 14 300)). In this experiment, the positions of the fluorescent protein–aptamer complex of interest were within the two junctions of the first and secondary channels. There might be a degree of variation of pI value of the proteins after binding with the aptamer. In the case of transferring a focused band that is outside the junctions, the adjustment of the position of the focused band with TEMED[26] could be applied.

The next goal was to confirm the selectivity and the robustness of the assay for multiplex protein sensing in a real complex mixture. This method was then used to detect three target proteins within all the proteins of the *E. coli* cell. There is the possibility of the existence of non-specific interaction interference by *E. coli* proteins. However, a minimal fluorescence increase was observed in the presence of interference proteins, while a significant fluorescence enhancement was observed for the target proteins. It was illustrated that the non-specific interaction between the aptamers and *E. coli* was not strong enough; that is, it could not compete with the specific interaction between the aptamers and their corresponding protein targets to release ssDNA from GO. The interference could also be corrected from the background through control experiments. In addition, for those blank GO substrates without adsorbed aptamer, the non-specific interaction between GO and other proteins did not influence the binding between the aptamers and their corresponding protein targets, and thus, the analysis of the target protein. Therefore, our system was not influenced by non-specific interactions. The selectivity and specificity of the aptamer–GO-based FRET biosensor in complex samples has also been described in other reports.

All that remains now is the chip electrophoresis separation in which the achievement of three target-pair separation within a complex protein matrix was the key challenge. In the case of extremely complicated samples, chip electrophoresis with the simple double-T structure (Fig. 1, upper right) could not realize adequate separation of the target complexes, suggesting a limited separation capacity (Fig. 4(a)). Still, the consistent position of the electrophoresis peak with the analysis in a simple sample matrix as in Fig. 3 displayed the separation reproducibility of our proposed assay. To further increase the resolving power of this sensor system, another kind of chip structure for multichannels in the secondary CZE dimension (Fig. 1, lower right) with greatly increased separation capacity was employed. Significantly, three fluorescence bands corresponding to three aptamer target protein could be observed in the microchannels (Fig. 4(b)). The peak identifications were confirmed by separating a cell homogenate sample spiked with

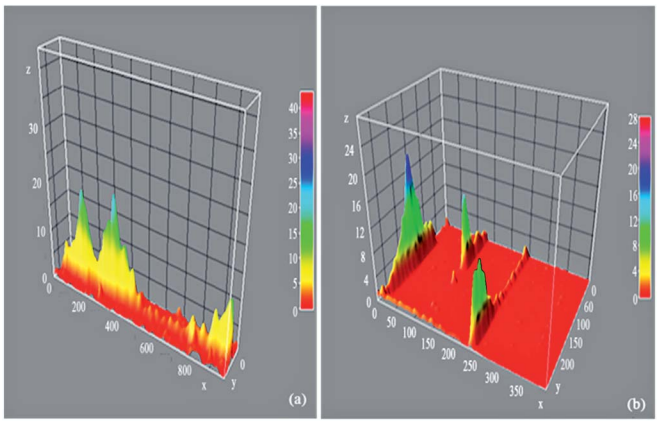

Fig. 4 3D plot of the fluorescent separation images of IEF–CZE coupled two-dimensional microchip with (a) double-T structure and (b) with multi-channels in the secondary dimension.

the three respective proteins. Since the peaks in the electropherogram were ascribed to protein and FAM-labelled aptamer complexes, therefore the migration behaviour of cytochrome c and lysozyme with similar pI values and molecular weights were not the same. Their migration behaviours (especially the intervals between the peaks) were not only influenced by the pI value and molecular weight of the protein alone, but also by non-selectively labelled FAM, especially for the longer-bound ssDNA chains with 61 and 42 bases.

The necessity of the high performance separation ability was then indicated again. The simultaneous detection of multi-targets depends not only on the selective recognition of aptamers for the targets but also on the separation ability of chip electrophoresis. The proposed method was not affected by the other thousands of proteins. Also, strikingly, the raw complex sample could be analysed directly without any pre-treatment and pre-labelling. For this experiment, due to the existence of the high fluorescence background in blood samples, the whole protein of *E. coli*, instead of an extra blood sample, was employed here to prepare a simulated real complex sample. Its great advance toward a real clinical assay could be envisioned without a doubt. Considering that a certain separation capacity corresponds to a certain channel structure chip, simply increasing the GO and aptamer concentration[4] which might influence separation was not a proper solution. There are many reasonable choices, for example, a fluorescent acceptor[27] or chemiluminescence resonance energy transfer[28] can be employed to overcome the background interference brought by the matrix.

Conclusions

A new chromatography based probe array technology was proposed here. The novel combination of a resonance energy transfer (RET) based sensor method with high performance chip electrophoresis offers several advantages. First, the investigation here provides a new technique to achieve high throughput and parallel sensoring by the combination of chromatography with a RET based optical sensor assay. There are three striking characteristics: (i) there was no immobilization involved, unlike in the strategy of sensor array biochips; (ii) there was no sample pre-treatment or pre-labeling involved; (iii) no multicolor DNA probes are required. This methodology should in theory have wide applicability because, with sufficient specific sensing elements, the number of targets would be considerable as long as the separation resolution allows. Secondly, a high detection sensitivity with low background noise was ensured by the super-quenching ability of GO. Furthermore, ratiometric imaging is also expected to increase the signal-to-background ratio, and thus afford a greater sensitivity in fluorescent digital imaging. Meanwhile, the irreplaceable advantage of integration makes the microfluidic chip a good candidate to realize high-resolution separation with further improved sensitivity detection. The lowest detection limit of our method and how to increase the number of targets detected are now under way with further experiment optimization. Third, one emphasis in bioassays nowadays is the move to methods that are quick, simple, use a small sample size and portable equipment. Micro- and nanofluidic devices are becoming the focus of investigations. The unique characteristics of speed, high separation performance, low-volume sample consumption and simple operation bestowed by the chip, combined with the design flexibility of recognition biosensors, promise great potential capacity for any high-throughput recognition in real complex-sample assays.

Acknowledgements

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References

1 S. Song, Y. Qin, Y. He, Q. Huang, C. Fan and H. Chen, *Chem. Soc. Rev.*, 2010, **39**, 4234–4243.
2 W. Yang, K. R. Ratinac, S. P. Ringer, P. Thordarson, J. J. Gooding and F. Braet, *Angew. Chem., Int. Ed.*, 2010, **49**, 2114–2138.
3 C. H. Lu, H. H. Yang, C. L. Zhu, X. Chen and G. N. Chen, *Angew. Chem., Int. Ed.*, 2009, **48**, 4785–4787.
4 N. Varghese, U. Mogera, A. Govindaraj, A. Das, P. K. Maiti, A. K. Sood and C. N. R. Rao, *ChemPhysChem*, 2009, **10**, 206–210.
5 N. Mohanty and V. Berry, *Nano Lett.*, 2008, **8**, 4469–4476.
6 S. He, B. Song, D. Li, C. Zhu, W. Qi, Y. Wen, L. Wang, S. Song, H. Fang and C. Fan, *Adv. Funct. Mater.*, 2010, **20**, 453–459.
7 H. Pei, J. Li, M. Lv, J. Wang, J. Gao, J. Lu, Y. Li, Q. Huang, J. Hu and C. Fan, *J. Am. Chem. Soc.*, 2012, **134**, 13843–13849.
8 Y. Wen, F. Xing, S. He, S. Song, L. Wang, Y. Long, D. Li and C. Fan, *Chem. Commun.*, 2010, **46**, 2596–2598.

9 Y. Wang, Z. H. Li, D. H. Hu, C.-T. Lin, J. H. Li and Y. H. Lin, *J. Am. Chem. Soc.*, 2010, **132**, 9274–9276.
10 S. Guo and S. Dong, *Chem. Soc. Rev.*, 2011, **40**, 2644–2672.
11 S. Myung, A. Solanki, C. Kim, J. Park, K. S. Kim and K. B. Lee, *Adv. Mater.*, 2011, **23**, 2221–2225.
12 S. Bi, T. Zhao and B. Luo, *Chem. Commun.*, 2012, **48**, 106–108.
13 J. H. Kim, S. Chaudhary and M. Ozkan, *Nanotechnology*, 2007, **18**, 195105.
14 Y. Q. Li, J. H. Wang, H. L. Zhang, J. Yang, L. Y. Guan., H. Chen, Q. M. Luo and Y. D. Zhao, *Biosens. Bioelectron.*, 2010, **25**, 1283–1289.
15 A. Ramachandran, M. Q. Zhang, D. Goad, G. Olah, J. R. Malayer and Z. El Rassi, *Electrophoresis*, 2003, **24**, 70–77.
16 J. H. Wang, W. Wang, Y. H. Liu, L. B. Duo, L. J. Huang and X. F. Jiang, *Mol. Biol. Rep.*, 2009, **36**, 1903–1908.
17 Y. Q. Li, L. Y. Guan, J. H. Wang, H. L. Zhang, J. Chen, S. Lin, W. Chen and Y. D. Zhao, *Biosens. Bioelectron.*, 2011, **26**, 2317–2322.
18 A. Manz, N. Graber and H. M. Widmer, *Sens. Actuators, B*, 1990, **1**, 244–248.
19 H. Chen and Z. H. Fan, *Electrophoresis*, 2009, **30**, 758–765.
20 B. Juskowiak, *Anal. Bioanal. Chem.*, 2011, **399**, 3157–3176.
21 D. M. Tasset, M. F. Kublik and W. Steiner, *J. Mol. Biol.*, 1997, **272**, 688–698.
22 D. J. F. Chinnapen and D. Sen, *Biochemistry*, 2002, **41**, 5202–5212.
23 J. ColinCox and A. D. Ellington, *Bioorg. Med. Chem.*, 2001, **9**(10), 2525–2531.
24 W. S. Hummers and R. E. Offeman, *J. Am. Chem. Soc.*, 1958, **80**, 1339.
25 C. A. Emrich, I. L. Medintz, W. K. Chu and R. A. Mathies, *Anal. Chem.*, 2007, **79**, 7360–7366.
26 T. Manabe, H. Miyamoto, K. Inoue, M. Nakatsu and M. Arai, *Electrophoresis*, 1999, **20**, 2677.
27 B. Juskowiak, *Anal. Bioanal. Chem.*, 2011, **399**, 3157–3176.
28 S. Bi, T. T. Zhao and B. Y. Luo, *Chem. Commun.*, 2012, **48**, 106–108.