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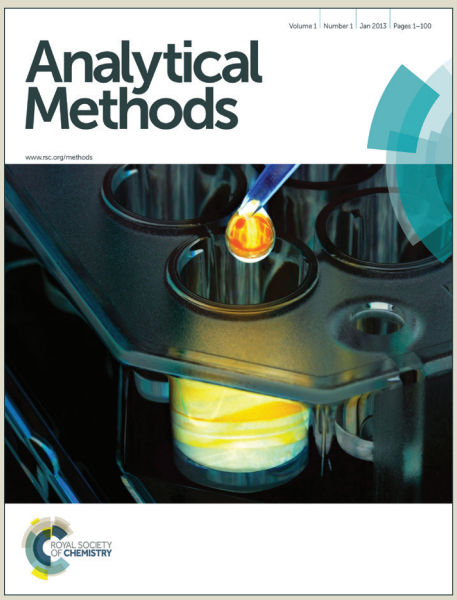

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ARTICLE

A fluorescent aptasensor for sensitive detection of human hepatocellular carcinoma SMMC-7721 cells based on graphene oxide

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In this work, we reported a simple, rapid and sensitive fluorescence method for the direct detection of cancer cells using graphene oxide (GO)-based aptasensor. This method took advantage of the exceptional quenching capability of GO for the dyes labelled aptamer, and aptamer was employed as the molecular recognition element. As proof of concept, an aptamer sequence was selected using a cell-based SELEX strategy in our laboratory for human liver cancer cell line SMMC-7721 with high specificity and high affinity, which applied in this method. Samples with the target cells present a high fluorescent intensity while nontarget samples still showed low fluorescent intensity. This GO-based aptasensor exhibited high sensitivity and specificity with the detection limits of 200 SMMC-7721 cells in 200 μL binding buffer. In addition, our strategy can directly detect target cells without washing and separation. Furthermore, future design of detectors for other cancer cells only requires changing the recognition element for the target cell. More importantly, flow cytometry was used for specific recognition of the target cells from mixture cells in fetal bovine serum to demonstrate the potential application of this method for medical diagnostics.

Introduction

Hepatocellular carcinoma is one of the most serious and highly malignant cancers in the world.[1, 2] Sensitive, selective, rapid, and cost-effective detection of cancer cell is important for the early cancer diagnosis and clinical treatments, since surgical resection or liver transplantation is effective treatment for early cancers.[3] Current diagnostic methods for liver cancer are mainly relying on measuring the serum level of Alpha-fetoprotein (AFP), as well as ultrasound imaging.[4-7] However, the sensitivity and specificity are very limited by interference of many other liver diseases based on AFP. In addition, imaging only gives limited information with morphology and it is hysteresis quality. In order to achieve these goals, many methods based on different levels have been developed, including ion, DNA, microRNA, protein, whole cell.[8-18] However, some of these methods are expensive, time-consuming, and dual fluorescence-labelled or need advanced instrumentation. Aptamer-based biosensor for detection of cancer cell has greatly expanded that owe to the development of cell-SELEX. Various aptamer-based methods, including colorimetric methods, fluorescence methods and electrochemical methods have been developed.[19-24] Although the results and prospect are attractive, they are still at the primary research stage.

With the development of nanotechnology in recent years, graphene has received much attention in recent years in materials science due to their unique optical properties, such as half-integer quantum hall effect and ballistic and extraordinary electron transport.[25, 26] Taking advantages of GO can server as a super quencher with the long-range nanoscale energy transfer property, which, in combination with the unique DNA/GO interactions, forms the basis of a GO-based strategy for analysis, such as DNA, metal ion, protein, and even pathogen analysis.[27-30] In addition, although some researchers have been already focused on detection of the intracellular molecules based on GO.[31] However, direct detection of cancer cells by GO-based aptasensor still need to be explored.

Herein, taking advantages of the high fluorescence quenching efficiency of GO, a GO-based aptasensor for cancer cell detection is developed. As proof-of-concept, Cy5-labelled aptamer zy1 is used as recognition element and the GO-based nanoprobe is used as signal element. By applying the whole cell-SELEX technology, we have developed panels of aptamers against SMMC-7721 human hepatocellular carcinoma cells.[32] Previous studies have demonstrated that these aptamers can recognize not only the human liver carcinoma cancer cell line SMMC-7721, but also the in vivo tumour and the tumour tissue section.[33, 34] GO can act as a good quencher with high fluorescence quenching efficiency and realize the sensitive cell detection. This work may contribute to introducing the new aptamer as a highly specific molecular probe for liver carcinoma diagnosis.

Experimental

Materials

Oligonucleotides designed in this study (aptamer zy1 probe: 5'-cy5-ACGCGCGCGCGCATAGCGCGCTGAGCTGAAGATC GTACCGTGAGCGCGT-3'; aptamer ls2n probe: 5'-cy5-ATGGAATGTGGGAGGGGGACTCAGGACAGTCACGGG A-3'; random sequence: N50) were synthesized by Sangon Biotech (Shanghai, China). 0.01M pH 7.4 PBS was purchased from Beijing Dingguo Changsheng Biotechnology CO. LTD. (Beijing, China). Other chemicals were of analytical grade and used without further purification. The solutions in the experiments were prepared with ultrapure water (Milli-Q 18.2 MΩ•cm, Millipore System Inc.).

Cell lines and buffers

The cell lines of human origin used in this study, including TCA, A549, Hela cells were purchased from American Type Culture Collection. Bel-7404, SMMC-7721 and L02 were purchased from the Shanghai Institute of Cell Biology of the Chinese Academy of Science. All of the cells were cultured in RPMI medium 1640 supplemented with 10% FBS (heat-inactivated, GIBCO) and 100 U/ml penicillin-streptomycin (Cellgro). All cells were maintained at 37 ℃ in a 5% CO2 atmosphere. Cells were washed before and after incubation with DNA library during selection with washing buffer (4.5 g/L glucose and 5 mM $MgCl_2$ in PBS, pH = 7.4). To reduce nonspecific binding, BSA (1mg/mL, Dingguo) and yeast tRNA (0.1 mg/mL, Sigma) were added to the washing buffer, which was used as the binding buffer for selection.

Apparatus and fluorescence measurements

Fluorescence spectra were measured using a multi-function plate reader Infinite® M1000 (Tecan, Switzerland) with a personal computer data processing unit. Excitation and emission slits were all set for 5.0 nm band-pass. The fluorophore of Cy5 was excited at 630 nm, and the emission spectra from 645 to 745 nm were collected. The fluorescence intensity at 665 nm was used to evaluate the performance of the proposed assay strategy. All measurements were carried out at room temperature unless stated otherwise.

Cell detection

Cultured cell treated with trypsin, and suspend cells in binding buffer, then prepared for different cell concentrations. Add aptamer probe into the cell suspension, mix thoroughly and incubate the mixture on ice for 30 min on a rotary shaker. Then the GO was added into the detection system, and reacted for 10 min, finally, the resulting solution was used for fluorescence measurements.

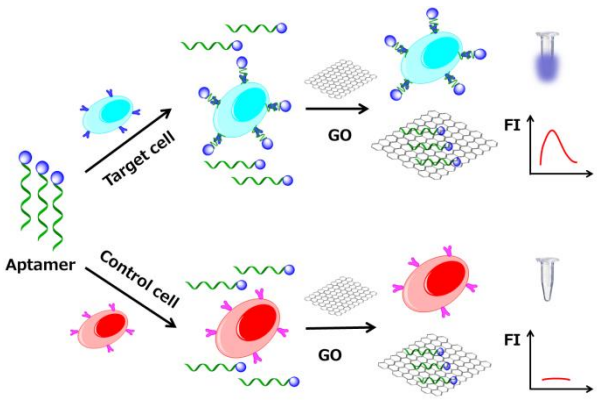
Flow cytometry analysis of mixture cells

To perform the ability of detection target cell in mix cell by this GO-based aptasensor, a detailed procedure of the binding experiments was performed by the following procedure. SMMC-7721 cells and Bel-7404 cells were mix under different rate, and then incubated with aptamer probes labelled at the 5' end with Cy5 (100 nM) in 200 μL of binding buffer on ice for 30 min. Then 22 ug GO was added into the detection system. After incubation for 10 min, the cells could be analyzed directly by flow cytometry with no required washing and separation steps. The fluorescence intensity of the cells was recorded with the FACScan flow cytometry (FACScalibur, BD Bioscience) by counting 10000 events. The Cy5-labeled random sequence was used as the negative control.

Results and discussion

The design and feasibility of GO-based aptasensor for cell detection

The detection principle of the aptamer and GO-based strategy for cancer cell detection was displayed in Scheme 1. Aptamer zy1 was used as recognition element, when in the present of the target cell, detectable fluorescent signals generated without influence by adding the GO, but the random sequences. While in absence of target cell or in presence of non-target cell, the fluorophore labelled aptamers were absorbed onto the surface of GO, and the fluorophores got closer to the GO surface, resulting in fluorescence quenching. All these data demonstrated that the aptamer recognized target cell with no influence on GO, but in system of the non-target cell or the non-aptamer, the aptamer will absorbed onto GO, and quenched the fluorescence of the probes.



Scheme 1. Schematic diagram of sensitive fluorescence detection of cancer cells using GO-based aptasensor.

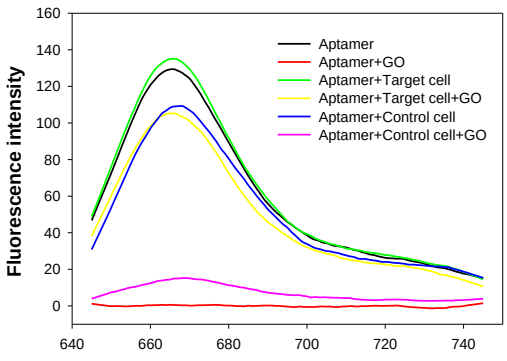


Figure 1. Feasibility test of GO-based aptasensor for cancer cell detection. Corresponding fluorescence intensity of target SMMC-7721 cells or control Bel-7404 cells incubated with different probes.

To confirm our assay method, the fluorescent experiments have been carried out. Keys to the success of our strategy are the free aptamer absorbed on the surface of GO and the aptamer bound with target cell. As is shown in Figure 1, the fluorescence of cy5-labeled aptamer was detected, it is the initial signal generated by the probe. After 10 min incubation with GO, nearly 100% fluorescence quenching with very fast kinetics was observed on aptamer-cy5/GO because of fluorescence resonance energy transfer (FRET) between cy5 and GO. While incubated with cells, the change of fluorescence

was not obvious, even added the GO into it, but compared to the non- target cell, the fluorescence intensity was stronger, and the S/B is more than 10.

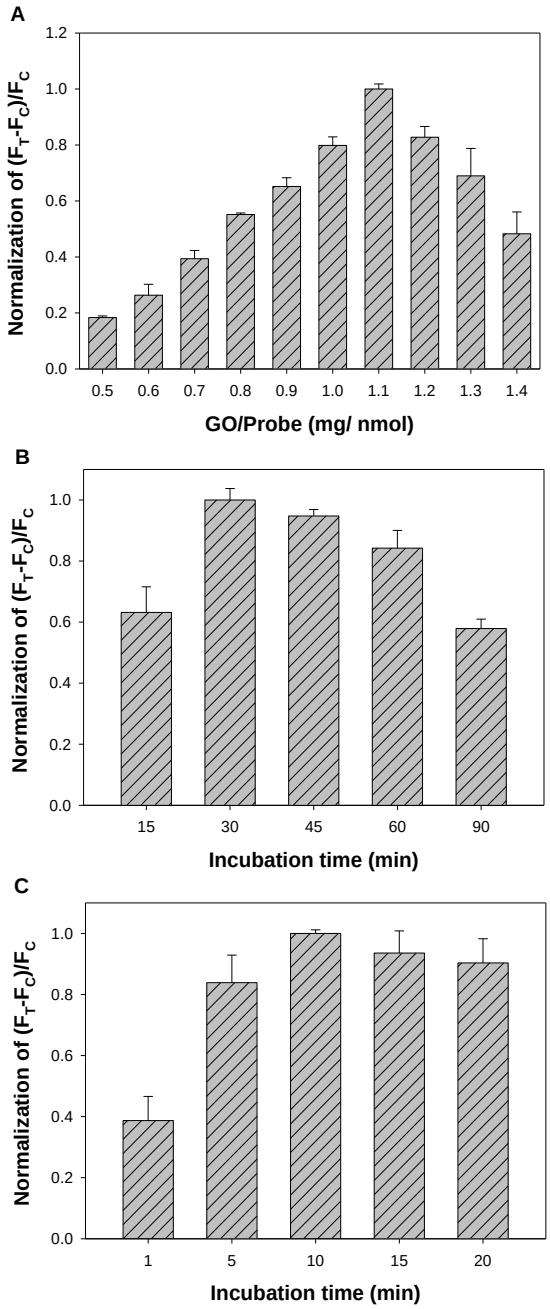


Figure 2. Optimization of experimental conditions for sensitive detection cancer cell. (A) Optimization of the ratios of GO to probes; (B) Optimization of incubation time, and the incubating time of SMMC-7721 cells with the aptamer probe is 15 min, 30 min, 45min, 60min, and 90 min, respectively; (C) Optimization of incubation time, and the incubating time of GO with the mixture of SMMC-7721 cells and the aptamer probe is 1 min, 5 min, 10 min, 15 min, and 20 min, respectively. (FT and FC mean the fluorescence response of target cell and control cell, respectively, and error bars were deduced from N = 3 experiments.)

Optimization of experimental conditions

In order to achieve a better limit of detection for the assay, several facts may be investigated to optimize the detection system. First of all, the concentration of the probe and GO, here using the ratio of aptamer/GO, is important to this detection system, since the incompletely quenched fluorescent will raise the background significantly. As is shown in Figure 2A, different ratio of the aptamer/GO were used in the cell detection solution, the highest S/N is achieved by the 1.1 mg/nmol. This suggested that when the ratio of the aptamer/GO reached 1.1 mg/nmol, the GO can quench the probes well. Other experimental conditions have been optimized, such as the incubation time of the cell and aptamer probes (Figure 2B), and the incubation time of GO and cell-aptamer probe mixture (Figure 2C). Finally, we perform the experience on ice, and the probes incubated with cell for 30 min, and the GO incubated with the cell-aptamer probe mixture for 10 min.

Cell detection

Under the optimized detection conditions, a series of SMMC-7721 cell samples were quantificational detected by this GO-based aptasensor. The sample was dispersed in 200 μL of binding buffer, followed by addition of the aptamer zy1 probe with 30 min of incubation, and then the 10 min of incubation with GO was performed. Then samples were analyzed by a plate reader spectrometer ranging from 2×10^4 to 0 in 200 μL of solution. As cell number increased, the fluorescent intensity increased accordingly (Figure 3A). Background signal was tested by the same method without any cells in the binding buffer. Background signal plus 3 times the standard deviation were considered to be the available value in this work. Cell detection calibration curve has been shown in Figure 3B. It should be noted that the minimum concentration of

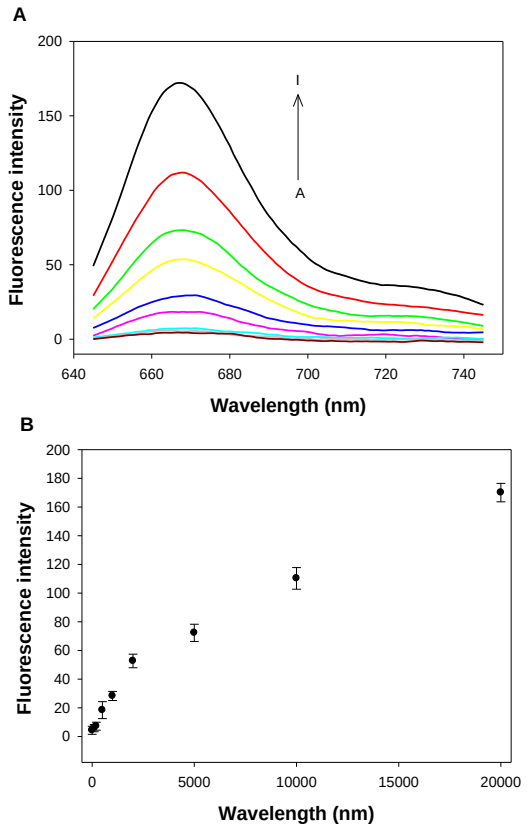


Figure 3. (A) Fluorescence response for the different concentrations of the SMMC-7721 cells: 0, 100, 200, 500, 1000, 2500, 5000, 10000, 20000 cells mL^{-1}; (B) Cell detection calibration curve of the GO-based aptasensor for SMMC-7721 cells detection, and dependence of fluorescence peak intensity at 665 nm on the SMMC-7721 cell concentration. (Error bars show the standard deviation of three experiments.)

200 cells could raise the signal to distinguish from the background signal, so the limit of detection is 200 cells in 200μL binding buffer. The sensitivity of this GO-based aptasensor was found to be comparable with some other cell detection methods, such as electrochemical methods and fluorescent methods.[20, 21, 35, 36]

Specificity of the GO-based aptasensor for cancer cell detection

To evaluate the selectivity of the GO-based aptasensor, normal cell and a serial of cancer cells have been chosen for performance, and fluorescent measurements were performed to monitor signal changes after aptamer probe binding with the cells. As is shown in Figure 4, due to the special aptamer specificity, only SMMC-7721 cells showed obvious fluorescence, neither L02 cells, Hela cells, Bel-7404 cells, TCA cells, nor A549 cells was observed with any vivid fluorescent. These results indicate that this method has good selectivity for the assay of target cells.

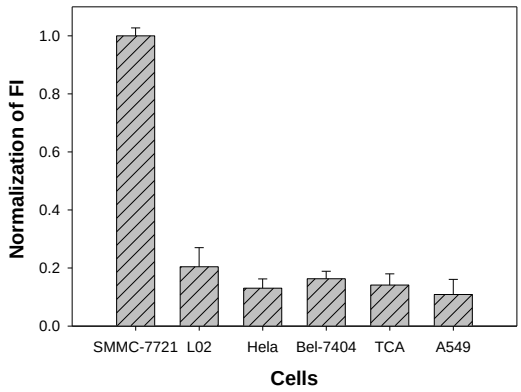


Figure 4. Specificity test, comparing signals from the target SMMC-7721 cells (1000 cells per 200 μL) and other nontarget cells (L02, Hala, Bel-7404, TCA, and A549 cell, 10000 cells per 200 μL).

Detection of target cell in mix cell

Moreover, we further evaluated the applicability of this strategy to detect target cancer cells from cell mixtures. Figure 5 presents the results from artificial complex samples by mixing Bel-7404 cells and SMMC-7721 cells with different concentration ratios (0:1, 1:4, 1:2, 1:1, 2:1, 4:1, 1:0). The total amount of cells was 1×10^5, and the final volume of each sample was 200 μL. From Figure 5, we found that with the population of target cells gradually increasing, two clear peaks could be observed with increasing fluorescence for target cells and decreasing fluorescence for the remainder. The above results confirmed the powerful capabilities of this strategy toward target cells in term of high selectivity.

General applicability of the GO-based aptasensor

In order to investigate the general applicability of our strategy, we first used the same principle to design a recognition probe to target Bel-7404 cells by changing the recognition element with aptamer ls 2n. Ls2n is an aptamer for specifically recognizing another human hepatocellular carcinoma cell line Bel-7404 with Kd of 44.4 ± 3.1 nM.[37] As expected, after target Bel-7404 cells incubated with probe, the fluorescence intensity raised with the concentration of target cell. (Figure 6A and B). It should be noted that the minimum concentration of 500 cells could induce distinguishable response from the background signal, finally the limit of detection is 500 in 200μL. It is a little lower than the limit of detection of SMMC-7721 cells, that because the Kd value of zy1 (2.18 nM) is lower than that of ls2n.

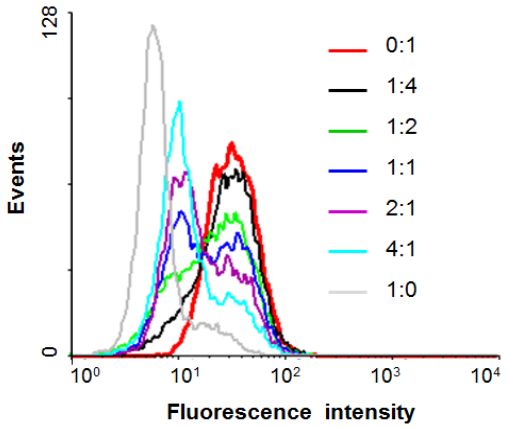


Figure 5. Flow cytometry analysis of mixture cells (Bel-7404 and SMMC-7721) in fetal bovine serum by using GO-based aptasensor. The ratio of Bel-7404 to SMMC-7721 is as follows: (A)0:1(red), (B) 1:4 (Black), (C) 1:2 (Green), (D)1:1 (Blue), (E) 2:1 (Purple), (F) 4:1 (Brillant blue) and (G)1:0 (Grey), respectively.

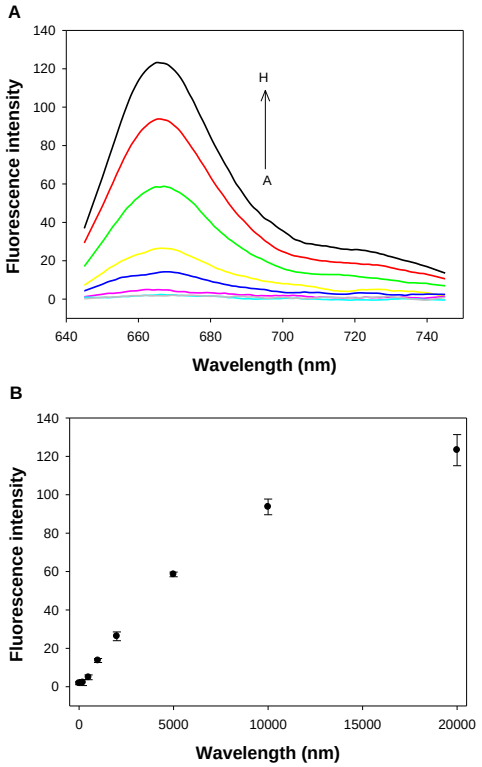


Figure 6. The fluorescence responses of GO-based aptasensor for the different concentrations of the Bel-7404 cells. A to H represent 0, 200, 500, 1000, 2500, 5000, 10000, 20000 cells mL^{-1}, respectively.

Conclusion

In summary, we have successfully developed a GO-based aptasensor for detecting cancer cells. This method possesses some remarkable features compared to the known assay methods for cancer cells. Firstly, this method is very simple and rapid, because only two steps are needed in cell detection. In addition, our strategy can directly detect target cells without washing and separation. Furthermore, this strategy is universal to multiple cancer cells through employing the relevant aptamers. More importantly, it demonstrates the potential

application of this method for medical diagnostics to detect the target cells in mixture cells containing fetal bovine serum. Therefore, this simple, rapid, sensitive, universal, and specific cancer cell detection strategy may provide a new insight into the detection of cancer cells. Given the unique and attractive characteristics, this study illustrates the potential of this GO-based aptasensor in bioanalytical applications for both fundamental and practical research.

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Notes and references

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1. Editorial, *Lancet*, 1987, **330**, 1183-1184.
2. M. Tanaka, F. Katayama, H. Kato, H. Tanaka, J. Wang, Y. L. Qiao and M. Inoue, *J Epidemiol.*, 2011, **21**, 401-416.
3. U. Cillo, A. Vitale, M. Bassanello, P. Boccagni, A. Brolese, G. Zanus, P. Burra, S. Fagiuoli, F. Farinati, M. Rugge and D. F. DAmico, *Ann. Surg.*, 2004, **239**, 150-159.
4. K. Hayashi, T. Kumada, S. Nakano, Takeda I, K. Sugiyama, S. Kiriyama, Y. Sone, A. Miyata, H. Shimizu and S. Satomura, *Am. J Gastroenterol.*, 1999, **94**, 3028-3033.
5. F. Farinati, D. Marino, M. De Giorgio, A. Baldan, M. Cantarini, C. Cursaro, G. Rapaccini, P. Del Poggio, M. A. Di Nolfo, L. Benvegnù, M. Zoli, F. Borzio, M. Bernardi and F. Trevisani, *Am. J Gastroenterol.*, 2006, **101**, 524-532.
6. G. T. Huang, J. C. Sheu, P. M. Yang, H. S. Lee, T. H. Wang and D. S. Chen, *J Hepatol.*, 1996, **25**, 334-338.
7. J. M. Lee, F. Trevisani, V. Vilgrain and C. Wald, *Liver Transpl.*, 2011, **17**, S34-S43.
8. G. Sivaraman, T. Ananda and D. Chellappa, *Analyst*, 2012, **137**, 5881-5884.
9. G. Sivaramana and D. Chellappa, *J. Mater. Chem. B.*, 2013, **1**, 5768-5772.
10. G. Sivaraman, T. Ananda and D. Chellappa, *RSC Adv.*, 2012, **2**, 10605-10609.
11. N. Ren, L. X. Qin, H. Tu, Y. K. Liu, B. H. Zhang and Z. Y. Tang, *J Cancer Res. Clin. Oncol.* 2006, **132**, 399-407.
12. K. Z. Qu, K. Zhang, H. Li, N. H. Afdhal and M. Albitar, *J Clin. Gastroenterol.*, 2011, **45**, 355-360.
13. M. Furuta, K. I. Kozaki, S. Tanaka, S. Arii, I. Imoto and J. Inazawa, *Carcinogenesis*, 2010, **31**, 766-776.
14. Y. Mao, H. Yang, H. Xu, X. Lu, X. Sang, S. Du, H. Zhao, W. Chen, Y. Xu, T. Chi, Z. Yang, J. Cai, H. Li, J. Chen, S. Zhong, S. R. Mohanti, R. Lopez-Soler, J. M. Millis, J. Huang and H. Zhang, *Gut.*, 2010, **59**, 1687-1693.
15. R. Masuzaki, S. J. Karp, M. *Omata, Semin. Oncol.*, 2012, **39**, 434-439.
16. L. N. Zhang, H. H. Deng, F. L. Lin, X. W. Xu, S. H. Weng, A. L. Liu, X. H. Lin, X. H. Xia and W. Chen, *Anal. Chem.*, 2014, **86**, 2711-2718.
17. C. D. Medley, S. Bamrungsap, W. H. Tan and J. E. Smith, *Anal. Chem.*, 2011, **83**, 727-734.
18. X. R. Sun, Y. P. Li, H. L. Huang, B. H. Yang and Y. Z. Wang, *Luminescence.*, 2014, doi: 10.1002/bio.2629.
19. Y. J. Guo, G. M. Sun, L. Zhang, Y. J. Tang, J. J. Luo and P. H. Yang, *Sens Actuators B Chem.*, 2014, **191**, 741-749.
20. X. L. Zhu, Y. Cao, Z. Q. Liang and G. X. Li, *Protein Cell*, 2010, **1**, 842-846.
21. J. J.Yin, X. X. He, K. M. Wang, F. Z. Xu, J. F. Shangguan, D. G. He and H. Shi, *Anal. Chem.*, 2013, **85**, 12011-12019.
22. L. Kashefi-Kheyrabadi, M. A. Mehrgardi, E. Wiechec, A. P. Turner and A. Tiwari, *Anal. Chem.*, DOI: 10.1021/ac500375p.
23. C. F. Ding, H. T. Liu, N. N. Wang and Z. F. Wang, *Chem. Commun.*, 2012, **48**, 5019-5021.
24. G. Jie, J. Zhang, G. X. Jie and L. Wang, *Biosens. Bioelectron.*, 2014, **52**, 69-75.
25. K. S. Novoselov, A. K. Geim, S. V. Morozov, D. Jiang, Y. Zhang, S. V. Dubonos, I. V. Grigorieva and A. A. Firsov, *Science*, 2004, **306**, 666-669.
26. A. K. Geim and K. S. Novoselov, *Nat. Mater.*, 2007, **6**, 183-191.
27. S. J. He, B. Song, D. Li, C. F. Zhu, W. P. Qi, Y. Q. Wen, L. H. Wang, S. P. Song, H. P. Fang and C. H. Fan, *Funct. Mater.*, 2010, **20**, 453-459.
28. E. Morales-Narváez and A. Merkoçi, *Adv. Mater.*, 2012, **24**, 3298-3308.
29. C. H. Lu, H. H. Yang, C. L. Zhu, X. Chen and G. N. Chen, *Angew. Chem. Int. Ed.*, 2009, **48**, 4785-4787.
30. X. Q. Liu, F. An. Wang, R. Aizen, O. Yehezkeli, and I. Willner, *J. Am. Chem. Soc.*, 2013, **135**, 11832-11839.
31. 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.
32. State Intellectual Property Office of the P.R.C, CN 102766693, 2013.
33. H. Shi, W.S. Cui, X.X He, Q. P. Guo, K. M. Wang, X. S. Ye and J. L. Tang, *PLoS ONE*, 2013, **8**, e70476.
34. X. H. Yang, X. Z. Zhang, K. M. Wang, Q. Wang, Y. Y. Tan, Q. P. Guo, M. Chen and Y. Zhou, *Anal. Methods*, 2014, **6**, 3506-3509.
35. C. F. Pan, M. L. Guo, Z. Nie, X. L. Xiao and S. Z. Yao, *Electroanalysis*, 2009, **21**, 1321-1326.
36. F. He, Q. Shen, H. Jiang, J. Zhou, J. Cheng, D. D. Guo, Q. N. Li, X. M. Wang, D. G. Fu and B. A. Chen, *Talanta*, 2009, **77**, 1009-1014.
37. Q. P. Guo, X. D. Liu, Y. Y. Tan, K. M. Wang, X. H. Yang, Y. Zhou, L. Ye and X. Y. Zhao, *Chin. Sci. Bull (Chin Ver)*, 2013, **58**, doi: 10.1360/972013-360.