Biosensors and Bioelectronics 80 (2016) 79–83

Contents lists available at ScienceDirect

Biosensors and Bioelectronics

journal homepage: www.elsevier.com/locate/bios

Paper-based fluorescence resonance energy transfer assay for directly detecting nucleic acids and proteins

Hua Li [a], Xueen Fang [a,b,*], Hongmei Cao [a], Jilie Kong [a,**]

[a] Department of Chemistry and Institutes of Biomedical Sciences, Fudan University, Shanghai 200433, PR China
[b] Department of Chemistry and Institutes of Biomedical Sciences, Shanghai Suxin Co. Ltd., PR China

ARTICLE INFO

Article history:
Received 13 October 2015
Received in revised form
10 December 2015
Accepted 20 December 2015
Available online 21 December 2015

Keywords:
Paper-based microfluidic
FRET
Nucleic acid and protein detection

ABSTRACT

Paper-based fluorescence resonance energy transfer assay (FRET) is gaining great interest in detecting macro-biological molecule. It is difficult to achieve conveniently and fast detection for macro-biological molecule. Herein, a graphene oxide (GO)-based paper chip (glass fiber) integrated with fluorescence labeled single-stranded DNA (ssDNA) for fast, inexpensive and direct detection of biological macromolecules (proteins and nucleic acids) has been developed. In this paper, we employed the Cy3/FAM-labeled ssDNA as the reporter and the GO as quencher and the original glass fiber paper as data acquisition substrates. The chip which was designed and fabricated by a cutting machine is a miniature biosensor that monitors fluorescence recovery from resonance energy transfer. The hybridization assays and fluorescence detection were all simplified, and the surface of the chip did not require immobilization or washing. A Nikon Eclipse was employed as excited resource and a commercial digital camera was employed for capturing digital images. This paper-based microfluidics chip has been applied in the detection of proteins and nucleic acids. The biosensing capability meets many potential requirements for disease diagnosis and biological analysis.

1. Introduction

There is a growing need for low-cost detection of nucleic acids and protein. In particular, biological macromolecule detection is very important for clinical diagnostics, food safety testing, and forensics and so forth (Yetisen et al., 2013). Microfluidic devices fabricated with glass, PDMS, and other polymers have attracted considerable attention because of their potential for miniaturization, integration, and automation (Cao et al., 2012). However, their design and fabrication of these types of microfluidics would be complex and time consuming. Thus, simpler, more cost-effective methods must be investigated. As expected, some paper like filter paper, chromatography paper or glass fiber paper which are used as the carrier for various bioassays has gained great interest among biological analysis of industry due to intrinsic filtering property, biocompatibility and safe disposal. Biosensors based on paper chips have proven to be of particular utility and important because of their super-hydrophobicity, wide surface area, low cost, easy fabrication, and suitability for remote areas (Chen et al., 2012; Connelly et al., 2015). Especially, like conventional chips, they can be 96-well or 384-well plates, and be subjected to chromatographic or enzyme-linked immune-sorbent assays (ELISA) (Cheng et al., 2010). It is worth mentioning the glass fiber is more suitable than other paper as a chip due to its super compressive strength, therefore they can be fabricated as small as nanometer size. Advanced detection technologies such as gene microarrays, nucleic acid sequencing (Tamura et al., 2011; Tusher et al., 2001), and ELISA for protein analysis are commonly available in developed countries. However, there is a shortage of technical and financial support in developing countries, and while these advanced techniques cannot always help people in need. Therefore the Smartphone, digital camera and other instruments that can take a picture received new development in scientific research and expanded opportunities for the diagnostic assays of remote and resource-limited settings, they can be brought anywhere due to their microminiaturization and high resolution. (Ozcan, 2014; Vashist et al., 2015)

Fluorescence resonance energy transfer (FRET)-based hybridization assays are often used for detecting biological macromolecules because of their operational convenience and robustness (Li et al., 2015; Noor and Krull, 2013). A paper platform for nucleic acid hybridization with Hg^{2+} was recently reported (Li et al., 2013; Zhang et al., 2015a, 2015b). Fluorescence quenchers include nanoparticles (Tang et al., 2008), single-walled carbon nanotubes (Chen et al., 2012), and MoS_2 (Zhu et al., 2013). Here, we

* Corresponding author at: Department of Chemistry and Institutes of Biomedical Sciences, Fudan University, Shanghai 200433, PR China.
** Corresponding author.
E-mail addresses: fxech@fudan.edu.cn (X. Fang), jlkong@fudan.edu.cn (J. Kong).

http://dx.doi.org/10.1016/j.bios.2015.12.065
0956-5663/

selected graphene oxide (GO) as quencher, which is a two-dimensional material with high adsorption and quenching ability. It also has good solubility because of surface hydroxyl and carboxylic groups (Lu et al., 2009). In addition, GO could differentiate single-stranded DNA (ssDNA) from double-stranded DNA, and specific proteins from nonspecific proteins after contacting with glass fiber chip. When a fluorescence-labeled ssDNA was tightly bound to GO, it was immediately quenched (Yang et al., 2013). However, after adding a complementary strand or a specific protein, the DNA probe was released from the GO; the fluorescence was recovered.

Here, we described an integrated FRET assay on a GO-based paper chip for the detection of nucleic acids and proteins (Tang et al., 2008; Wei et al., 2014). This assay presented a novel direction of integrating fluorescence labeled single-stranded DNA (ssDNA) functionalized graphene oxide with digital imaging for the detection of proteins and nucleic acids. Especially, the selectivity of nucleic acid hybridization assay was demonstrated by single nucleotide polymorphism (SNP) detection. The selectivity of protein hybridization was assayed with four nonspecific proteins. The biosensing capability meets many potential requirements for disease diagnosis and biological analysis.

2. Materials and methods

2.1. Materials and reagents

Mucin 1 and other proteins were bought from Sigma Co., Ltd (Shanghai, China). GO was purchased from Sinopharm Chemical Reagent Co., Ltd (China). Glass fiber paper was purchased from Shanghai Kinbio Tec Co., Ltd. Superhyb solution for nucleic acid hybridization was obtained from Tianenze Tech Co. Ltd (Beijing, China). Phosphate buffer solution (PBS: 0.3 M NaCl, 10 mM Na_2HPO_4/NaH_2PO_4, pH = 7.0) for aptamer/protein hybridization was prepared in our lab. Oligonucleotides were synthesized, modified, and purified by Sangon Biotech Co., Ltd. (Shanghai, China). Nucleic acid sequences are given: nucleic acid specific probe (P1): 5′-Cy3-CTTCACCTTCTATGCCCCTGACAC; fully complementary for P1(FC): GTGTCAGGGGCATAGAAGGTGAAG; non-complementary(NC):AATTCCCCCC- CCCCCCAATT; single base mismatch (1BPM): GTGTCAGGGGCATAC- AAGGTGAAG; three base mismatch (3 BPM): GTGTCAGGGGCATACAACCTG- AAG; five base mismatch (5 BPM): GTGTCAGGGCGATACAACCTGAAG; mucin 1 specific probe (P2):5′-FAM- GCAGTTGATCCTTTGGATACCCTGG. All chemical reagents were analytical grade, and used without further purification, and all solutions were prepared with ultrapure water.

2.2. Paper-based microfluidic chip fabrication

In this study, we applied the glass fiber membrane instead of the common cellulose paper to fabricate the paper-based microfluidics system. A common home-use cutting machine was applied to create the microfluidic pattern on the glass fiber membrane. Many chips could be produced at one time by computer-directed cutting. The hydrophilic fiberglass chip was immobilized on a hydrophobic glass plate for patterning microwell plate (Cheng et al., 2010; Fang et al., 2010); every well had a width of 3 mm and a depth less than 1 mm. This paper-based microfluidic chip proves advantageous because sample could be easily introduced from center by capillary action through the hydrophobic channel to detection zones. This procedure was much more convenient and had a lower cost than fabrication of PDMS or PMMA chip. All reaction liquids were added by micropipette (Eppendoff), which was much more convenient than pumping liquids into polymer microfluidic chips (Li et al., 2012).

2.3. Microfluidic assays

We used two different protocols for the GO paper-based FRET assay. Every paper microwell was modified with 4 μL GO before hybridization. Previously, nucleic acid “post-mixing” was used; however, the nucleic acid hybridization was slow if we first mixed GO with Cy3-labeled ssDNA (He et al., 2010). Adding 2 μL of the probe and 3 μl of the target to 3 μl of the hybridization buffer solved this problem and decreased nonspecific binding (Umemura, 2015). We incubated the probe and ssDNA for 30 min in the dark at room temperature, and then mixed the above solution with the GO-functional paper for another 30 min before data collection.

The glycoprotein mucin 1 bound the specific aptamer with the sequence was listed before. The binding between the aptamer and specific protein was weak, the “post-mixing” did not have the expected results. Thus, the opposite approach (“pre-mixing”) was adopted, which had better compatibility for the detection of proteins (Bianying et al., 2013). We first immobilized GO and mucin 1 specific aptamer on a paper chip (He et al., 2012), and then added protein samples in PBS buffer to the paper microwells.

2.4. Data collection and data processing

We scanned the paper by a camera and a Nikon Eclipse Ti (Nikon, Japan), with laser (520 nm, 40 w) as excitation source for nucleic acid detection, and laser (480 nm, 40 w) for protein detection. Photos were processed with Photoshop software, and ImageJ software was used for analysis of the fluorescence intensities. Each sample was repeated in triplicate, and the final data were averaged (Zhou and Krull 2014).

3. Results and discussion

3.1. Glass fiber membrane microfluidic chip

A glass fiber-based microfluidic chip with multi-microwells for nucleic acid and protein detection was shown in Fig. 1. It was fabricated without precise valves or pumps (Hu et al., 2014a, 2014b), and target detection and readout could be simultaneously performed. The plate was divided into two parts according to the order of sample addition; *i.e.*, when one part was “ON”, the other was kept “OFF” (Davaji and Lee, 2014). The upper part (Part I of the Fig. 1) was prepared for nucleic acid detection and the bottom part (Part II of the Fig. 1) for protein detection. The plate was scanned with an excitation source and images were recorded with a camera.

The microfluidic device is low cost because of its small volume and inexpensive apparatus, and it takes less time because of its wide surface area. Furthermore, it does not need heat processing; room temperature and dark surroundings are adequate. FRET has an inherently robust sensitivity for nucleic acid and protein hybridization *in vitro*, and is compatible with paper-based devices. For the FRET assay, we designed a multi-microwell paper plate for high throughput analysis. It had a series of concentration gradients that were detected simultaneously, and every well had 12 μL of solution, which was far less than that required in other assays. The paper chip was freed from immobilization and washing steps, only required mixing paper-based GO with pre-hybridized Cy3-probe/targets. It took very little time, including the laser illumination and image capture (Noor and Krull, 2014). Both the camera and chip device are convenient to carry and easy to operate, and the data are easy to store and analyze. According to comparing the performance of this assay with others', we have reached a conclusion that the paper-based fluorescence resonance energy transfer assay for directly detecting nucleic acids and proteins was more rapid,

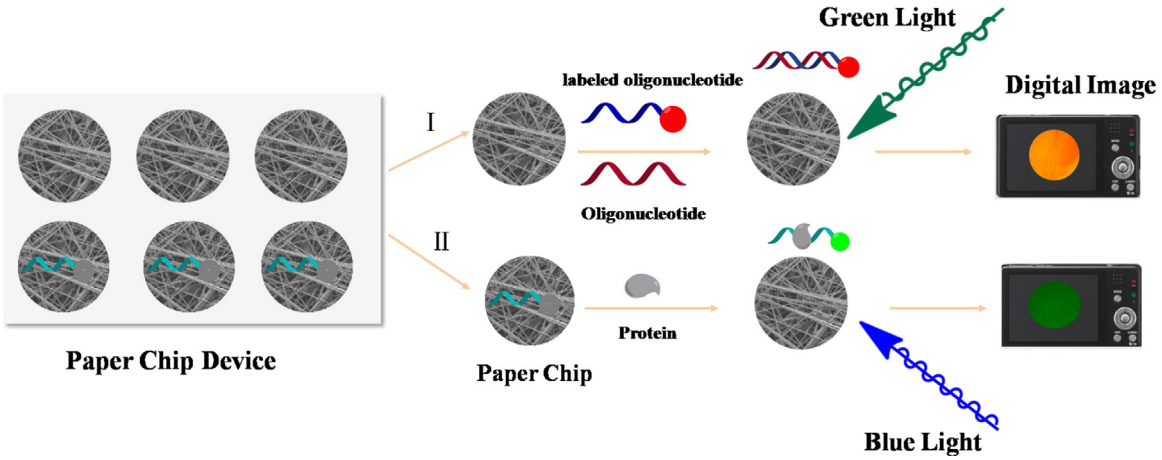

Fig. 1. Scheme of the paper-based FRET for the analysis of proteins and nucleic acids (I). The "post-mixing" processing for the detection of nucleic acids. (II). The "pre-mixing" processing for the detection of mucin 1.

simpler, and lower-cost.

In the FRET assay, we investigated different hybridization methods to establish fast and direct detection assays for proteins and nucleic acids. They were divided according to the order of addition of the labeled oligonucleotide. Pre-mixing with ssDNA can increase the hybridization speed because there is no competition between P1/GO adsorption and P1/FC, however, mucin 1/P2 adsorption is not specific enough. Therefore, the post-mixing strategy is not used for this step. Fortunately, we found that the pre-mixing strategy was better; it not only shortened the time required but also increased the specific binding. Thus, we implemented the both strategies.

3.2. *High-throughput detection of nucleic acids via "post-mixing" FRET*

To demonstrate the high-throughput paper-based FRET for the detection of nucleic acids, we used a laser (520 nm) and a camera for capturing the fluorescence images. We used a 12-channel paper-based device with 4 μL GO (1 mg/mL). The GO could rapidly diffuse over the large surface area, creating serial dilutions of target (0 nM, 1 nM, 10 nM, 40 nM, 80 nM, 500 nM, 1 μM, 2 μM, 5 μM, and 10 μM) mixed with fluorescent probe (10 μM) and hybridization buffer. These were incubated at room temperature before hybridization. The specific ssDNA was then added to the Cy3-labeled probe, and the interaction between them was much stronger than the GO adsorption *via* π-π stacking, restoring the fluorescence. In contrast, nonspecific ssDNA could not bind the fluorescent probe, or the interaction was too weak, and the fluorescence remained quenched by the GO glass fiber paper.

With 520 nm excitation source there was intense red fluorescence from the fully complementary ssDNA, while there was no change from the control assay. Fig. 2a shows red spots corresponding to fluorescence restoration in the paper chips. The variation in brightness corresponds to increasing concentrations of ssDNA. The whole process was conducted in the dark.

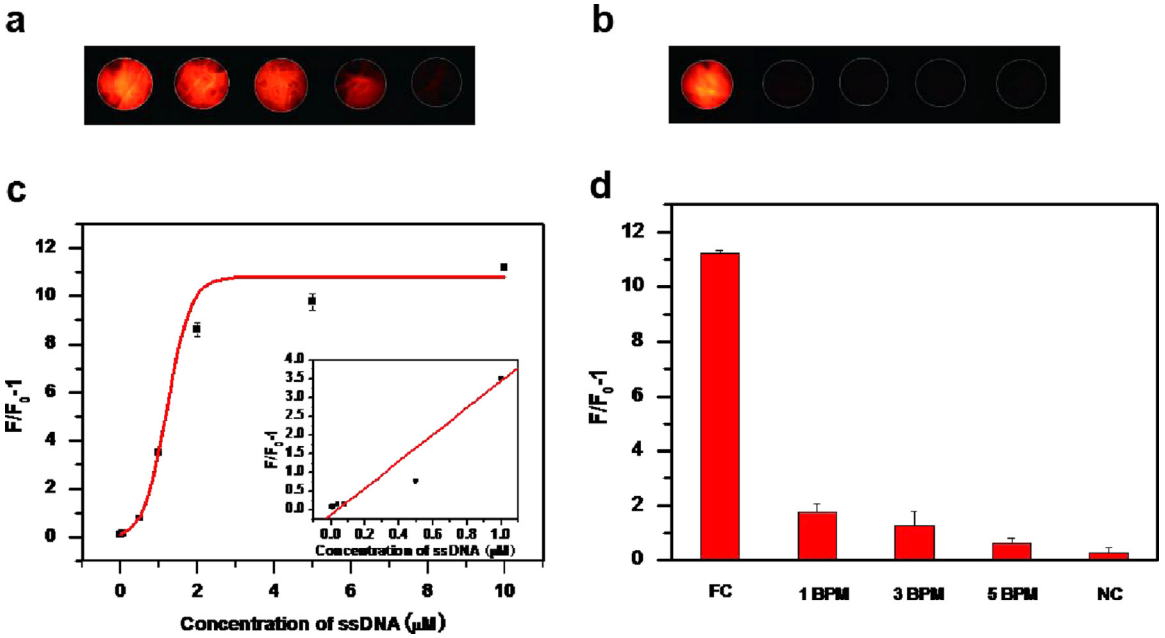

Fig. 2. The sensitivity and specificity of GO paper-based FRET for the nucleic acids. (a) Colored digital image of the different concentration of target, the white circles indicate the location of fluorescence restoration. (b) "FC" represents the fully complementary, and the "1 BPM" "3 BPM" "5 BPM" "NC" represents respectively the one base mismatch, three base mismatch, five base mismatch and noncomplementary, the image shows the contrast between the both in two parallel tests. (c) The relationship between the fluorescence intensity and the concentration of target. (d) The results of nonspecific discrimination of nucleic acid hybridization.

To obtain more ratio-metric information, we analyzed the fluorescence intensity of each channel using ImageJ, as shown in Fig. 2c, for increasing concentrations of the target. The data were analyzed in terms of (F/F_0-1), where F is the final fluorescence intensity and F_0 is the background intensity. The intensity of the red fluorescence in every microwell was proportional to the amount of target. The detection limit of 1 nM indicates that the assay is adequate, although less sensitive than other methods, of course, it may be possible to further decrease the limit of detection of this assay *via* nucleic acid amplification before the paper FRET assay.

We tested the selectivity of the assay using a single-base mismatch, a three-base mismatch, a five-base mismatch, and random oligonucleotides, which were hybridized with specific probes (10 μM) (Zhang et al., 2015a, 2015b). The assay was conducted in a commercial hybridization buffer with no other reagents; this was essential to increase specific binding and decrease nonspecific binding. We observed mismatch discrimination at the single-base-pair level shown in Fig. 2b. Thus the assay could reveal single nucleotide polymorphisms (SNPs), which is important for the clinical disease diagnosis and basic life science research. (Allen et al., 2012). As shown in Fig. 2d, the fluorescence intensity of the SNP (1 BPM) assay was extremely weak relative to that of the fully complementary target (6:1), while the intensity produced by the three-base mismatch, the five-base mismatch, and the random oligonucleotides were progressively lower. Hence, we concluded that nonspecific fluorescence was negligible. More importantly, this paper-based sensor was of sufficient selectivity to easily differentiate single mismatches, which offered the opportunity to allow single-nucleotide polymorphism (SNPs) analysis. It indicates that remote and resource-limited settings have the chance to achieve the detection of some critical diseases handily.

3.3. Detection of MUCIN 1 via "pre-mixing processing"

To test the paper chip for the detection of proteins, we used mucin 1 as a target. Mucin 1 is one of mucoprotein and a tumor marker, and its abnormal expression is relative to some cancers, such as, mammary cancer, oophoroma, pancreatic cancer and so on. After optimization of the GO and aptamer concentrations (1 mg/mL, 10 μM respectively), we determined that pre-mixing was the most effective method for the detection of protein. Over 97% of the fluorescence was quenched, which provided a reliable background for the assay (Hu et al., 2014a, 2014b). PBS buffer was used in this procedure for this assay (0.3 M NaCl, 10 mM Na_2HPO_4/NaH_2PO_4, pH=7.0). The NaCl increased the ionic strength, which did not interfere with the detection. As before, we immobilized the GO on the paper chip first, and then added 2 μL of aptamer that was specific for mucin 1. The fluorescence was quenched by GO paper immediately; in the meantime, we incubated 3 μL of mucin 1 solution and 3 μL of PBS *in vitro*. After the paper chip had dried, we mixed both, and kept them in a humid chamber for 10 min (Coskun et al., 2013). For simultaneous detection, a laser (480 nm) and a camera for capturing the fluorescence images, a protein concentration gradient (0, 0.1, 1, 10, and 100 μM) was prepared. The results were shown in Fig. 3a and c, which indicated a mucin 1 concentration dependent change of fluorescence intensity.

BSA, histone, and myoglobin were used to test the specificity of the assay. The FAM-labeled aptamer sensor exhibited good specificity against mucin 1 under the optimized condition discussed above. In this experiment, 2 μL of FAM-labeled aptamer was added to the paper chip, followed by mucin 1, BSA, histone, and myoglobin incubation for 10 min in separate microwells. As shown in Fig. 3b and d, the fluorescence from the nonspecific proteins changed little, while there was a large change for the mucin 1 (Nilghaz et al., 2012). The fluorescence introduced by BSA, histone and myoglobin could be neglected. These results indicated that the assay could be used to detect mucin 1 effectively, and have the potential use for the rapid detection of cancer biomarker.

4. Conclusions

We have demonstrated a GO paper-based mircofluidic chip for the simultaneous, multichannel detection of nucleic acids and proteins. The method has three main advantages: 1) its ease of use

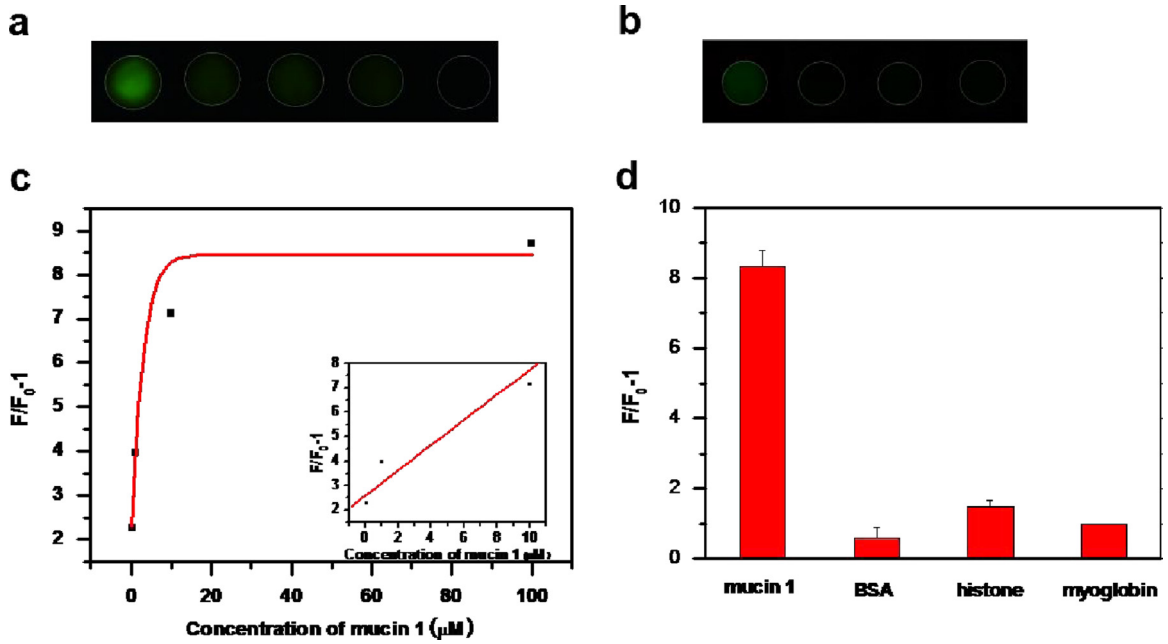

Fig. 3. GO-Paper chip based mircofluidic for the sensitive and selective detection of mucin 1. (a) Colored digital image of the different concentration of protein, the green area indicate the location of fluorescence restoration. (b) Colored digital image of the different protein. (c) The relationship between the fluorescence intensity and the concentration of proteins. (d) Selectivity tests for mucin 1 detection using paper-based microfluidic device. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

and low cost make it feasible for applications in developing countries; 2) it simplifies hybridization steps and decreases incubation times because of its high surface area and super-hydrophobicity; and 3) it could be applied for the single-nucleotide polymorphism (SNPs) analysis, therefore extending to disease diagnosis and biological analysis. With an improved sensitivity by background suppression, and more optimized experiment conditions, this GO paper-based mircofluidic chip is expected to have many widespread applications.

Acknowledgments

We are grateful for the kind help from the colleagues in our groups. We acknowledge the National Natural Science Foundation of China (21505024, 21427806, 21175029, 21335002, 21375027), the Introduce talents of Fudan University Research funding (IDH1615001), and the Shanghai Leading Academic Discipline Project (B109).

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2015.12.065.

References

Allen, P.B., Arshad, S.A., Li, B., Chen, X., Ellington, A.D., 2012. Lab Chip 12 (16), 2951–2958.
Bianying, F., Linjie, G., Lihua, W., Fan, L., Jianxin, L., Jimin, G., Chunhai, F., Qing, H., 2013. Anal. Chem. 85 (16), 7732–7737.
Cao, L., Cheng, L., Zhang, Z., Wang, Y., Zhang, X., Chen, H., Liu, B., Zhang, S., Kong, J., 2012. Lab Chip 12 (22), 4864–4869.
Chen, H., Wang, J., Liang, G., Zhang, P., Kong, J., 2012. Chem. Commun. 48 (2), 269–271.
Cheng, C., Martinez, A.W., Gong, J., Mace, C.R., Phillips, S.T., Carrilho, E., Mirica, K.A., Whitesides, G.M., 2010. Angew. Chem. Int. Ed. Engl. 49 (28), 4771–4774.
Connelly, J.T., Rolland, J.P., Whitesides, G.M., 2015. Anal. Chem. 87 (15), 7595–7601.
Coskun, A.F., Nagi, R., Sadeghi, K., Phillips, S., Ozcan, A., 2013. Lab Chip 13 (21), 4231–4238.
Davaji, B., Lee, C.H., 2014. Biosens. Bioelectron. 59, 120–126.
Fang, X., Liu, Y., Kong, J., Jiang, X., 2010. Anal. Chem. 82 (7), 3002–3006.
He, S., Song, B., Li, D., Zhu, C., Qi, W., Wen, Y., Wang, L., Song, S., Fang, H., Fan, C., 2010. Adv. Funct. Mater. 20 (3), 453–459.
He, Y., Lin, Y., Tang, H., Pang, D., 2012. Nanoscale 4 (6), 2054–2059.
Hu, J., Wang, S., Wang, L., Li, F., Pingguan-Murphy, B., Lu, T.J., Xu, F., 2014a. Biosens. Bioelectron. 54, 585–597.
Hu, R., Wen, W., Wang, Q., Xiong, H., Zhang, X., Gu, H., Wang, S., 2014b. Biosens. Bioelectron. 53, 384–389.
Li, F., Chao, J., Li, Z., Xing, S., Su, S., Li, X., Song, S., Zuo, X., Fan, C., Liu, B., Huang, W., Wang, L., Wang, L., 2015. Anal. Chem. 87 (7), 3877–3883.
Li, M., Zhou, X., Ding, W., Guo, S., Wu, N., 2013. Biosens. Bioelectron. 41, 889–893.
Li, X., Ballerini, D.R., Shen, W., 2012. Biomicrofluidics 6 (1), 11301.
Lu, C., Yang, H., Zhu, C., Chen, X., Chen, G., 2009. Angew. Chem. Int. Ed. 48 (26), 4785–4787.
Nilghaz, A., Wicaksono, D.H.B., Gustiono, D., Abdul Majid, F.A., Supriyanto, E., Abdul Kadir, M.R., 2012. Lab Chip 12 (1), 209–218.
Noor, M.O., Krull, U.J., 2013. Anal. Chem. 85 (15), 7502–7511.
Noor, M.O., Krull, U.J., 2014. Anal. Chem. 86 (20), 10331–10339.
Ozcan, A., 2014. Lab Chip 14 (17), 3187–3194.
Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., Kumar, S., 2011. Mol. Biol. Evol. 28 (10), 2731–2739.
Tang, B., Cao, L., Xu, K., Zhuo, L., Ge, J., Li, Q., Yu, L., 2008. Chem.–Eur. J. 14 (12), 3637–3644.
Tusher, V.G., Tibshirani, R., Chu, G., 2001. Proc. Natl. Acad. Sci. USA 98 (9), 5116–5121.
Umemura, K., 2015. Nanomaterials 5 (1), 321–350.
Vashist, S.K., van Oordt, T., Schneider, E.M., Zengerle, R., von Stetten, F., Luong, J.H.T., 2015. Biosens. Bioelectron. 67, 248–255.
Wei, Q., Luo, W., Chiang, S., Kappel, T., Mejia, C., Tseng, D., Chan, R.Y.L., Yan, E., Qi, H., Shabbir, F., Ozkan, H., Feng, S., Ozcan, A., 2014. ACS Nano 8 (12), 12725–12733.
Yang, K., Feng, L., Hong, H., Cai, W., Liu, Z., 2013. Nat. Protoc. 8 (12), 2392–2403.
Yetisen, A.K., Akram, M.S., Lowe, C.R., 2013. Lab Chip 13 (12), 2210–2251.
Zhang, Y., Zheng, B., Zhu, C., Zhang, X., Tan, C., Li, H., Chen, B., Yang, J., Chen, J., Huang, Y., Wang, L., Zhang, H., 2015a. Adv. Mater. 27 (5), 935–939.
Zhang, Y., Zuo, P., Ye, B., 2015b. Biosens. Bioelectron. 68, 14–19.
Zhou, F., Krull, U.J., 2014. Anal. Chem. 86 (21), 10932–10939.
Zhu, C., Zeng, Z., Li, H., Li, F., Fan, C., Zhang, H., 2013. J. Am. Chem. Soc. 135 (16), 5998–6001.