Nanoscale

RSC Publishing

COMMUNICATION

Patterned polymer nanowire arrays as an effective protein immobilizer for biosensing and HIV detection

Cite this: *Nanoscale*, 2013, **5**, 527

Received 7th October 2012
Accepted 21st November 2012

DOI: 10.1039/c2nr33069e

www.rsc.org/nanoscale

Yue Shen,[ab] Yingyi Liu,[c] Guang Zhu,[a] Hao Fang,[a] Yunhui Huang,[b] Xingyu Jiang*[c] and Zhong L. Wang*[a]

We report an array of polymeric nanowires for effectively immobilizing biomolecules on biochips owing to the large surface area. The nanowires were fabricated in predesigned patterns using an inductively coupled plasma (ICP) etching process. Microfluidic biochips integrated using the substrates with arrays of nanowires and polydimethylsiloxane channels have been demonstrated to be effective for detecting antigens, and a detection limit of antigens at 0.2 μg mL^{-1} has been achieved, which is improved by a factor of 50 compared to that based on flat substrates without the nanowires. In addition, the high sensitivity for clinical detection of human immunodeficiency virus (HIV) antibody has also been demonstrated, showing a 20 times enhancement in fluorescent signal intensity between the samples with positive and negative HIV.

The development of biochips is a major thrust of the rapidly growing biotechnology owing to their diverse applications in genomics,[1,2] proteomics,[3–6] diagnosis[7,8] and pharmaceuticals.[9] Often, biochips need solid substrates to immobilize biomolecules such as DNA, antibodies, antigens, enzymes, polysaccharides, *etc.*[10,11] The capacity of the solid substrate to adsorb biomolecules determines the sensitivity of the assays.[12,13] Enlarging the specific surface areas of the solid substrate can lead to improved sensitivity, thus the diagnosis of diseases such as AIDS[14] and flu can be more effective. A lot of work has been done to fabricate 3-D or quasi 3-D films to increase the specific surface area of the solid substrate such as electrospun nanofibrous membranes,[14–17] track-etched polymeric membranes,[18] *in situ* polymerized porous structures,[19,20] polydimethylsiloxane (PDMS) microwells,[21] polyvinylidene fluoride[22] and nitrocellulose[23] membranes, polyacrylamide gel pads,[24] polystyrene microbeads,[25,26] *etc.* Among these methods, electrospun nanofibrous membranes showed superior capability of immobilizing biomolecules. However, electrospinning does not apply to all kinds of polymers, especially for thermal setting polymers, and it is hard to make micron scale patterns, so that the liquid diffusion is always a problem.[27]

In this paper, we report a novel method for fabricating arrays of polymer nanowire structure[28,29] for effectively immobilizing biomolecules on biochips owing to the increased surface area. The polymer nanowire arrays are fabricated in the patterned trenches on a biochip with inductively coupled plasma (ICP). The detection limit of the biochips based on polymer nanowire arrays is lowered to 1/50 of that based on flat substrates. The nanowires can be made with any polymer material or even polymers mixed with other organic compounds.

We invented a new method for fabricating patterned and vertically aligned polymer nanowire arrays of any polymer.[28,29] The polymer nanowires are formed by inductively coupled plasma (ICP) etching a polymeric film that has a roughened surface. The local etching rate is higher at a local dip than at a protrusion. This leads to an amplification of initial surface modulations and deepened surface roughness. The formation mechanism is suggested to be due to a dependence of the cone-shaped interaction volume between the ion and the polymer on its local incident-angle at the modulated surface.

The density, length and position of the nanowires can be well controlled. For the polyethylene terephthalate (PET) nanowire arrays with highest density (diameter = 100 nm, length = 15 μm, density = 5×10^7 mm^{-2}), the surface area can be two hundred times larger than that of the flat substrates.[28] The average diameter of these nanowires is around 100 nm and the average distance between the nanowires is around 300 nm. Considering the size of the protein or DNA molecules commonly immobilized on biochips is in the range from 1 nm to 10 nm, the size of the polymer nanowires is suitable for biomolecule adsorption. And unlike nano-porous materials, in which it is hard for the biomolecule to diffuse deeply into the pores, the whole surface of the nanowires has larger size of the in-between area, which can be easily reached by the biomolecules. Furthermore, because the nanowires can be confined to pre-designed micro-scale patterns so that the other areas remain flat, it solves the liquid diffusion problem.[14,27] The majority of the adsorbed molecules are

[a]*School of Material Science and Engineering, Georgia Institute of Technology, Atlanta, Georgia 30332, USA. E-mail: zlwang@gatech.edu*

[b]*College of Material Science and Engineering, Huazhong University of Science and Technology, Hubei, 430074, China*

[c]*National Center for Nanoscience and Technology, Chinese Academy of Sciences, Beijing, 100190, China. E-mail: xingyujiang@nanoctr.cn*

accumulated in the area covered by nanowire arrays. Flat areas can be used to assemble with other complex structures by van der Waals forces, such as microchannels, electronic or optical devices, to make high output/multi-functional biochips.

Our method can be applied on any polymeric material or even on polymers mixed with other organic compounds. As examples, we have tried this method on polymethyl methacrylate (PMMA, cross-linked and uncross-linked), polystyrene (PS, cross-linked and uncross-linked), poly(dimethylsiloxane) (PDMS, cross-linked), polyethylene naphthalate (PEN), polyethylene terephthalate (PET, cross-linked), Kapton film, a thin layer of fullerenes, polytetrafluoroethylene (PTFE), and so forth. Although the highest density of the final nanowires varies from 5×10^6 mm^{-2} to 5×10^7 mm^{-2} with different materials, we have not found any polymeric sample that cannot be etched into nanowires applying this method. So it is possible for us to choose the substrate materials with proper functional groups for further surface modifications to enhance or prevent the adsorption of specific biomolecules.

These unique characteristics of this kind of polymer nanowires perfectly meet the requirements of solid substrates of biochips. We demonstrate the microfluidic immunoassay chip fabrication with patterned PET nanowire arrays, as a substrate. We chose PET because it is cheap, transparent, insoluble in water, mechanically strong and can form high density nanowires.

The fabrication process of the microfluidic chip is shown in Fig. 1. A silicon shadow mask with the designed pattern was used to confine the ICP etching area on the PET substrates. As a demo, we exposed areas of 200 μm in width and 5 mm in length. Then, Au sputtering and ICP treatment were consequently carried out. As a result, the exposed area was etched into high density nanowire arrays using the method we have developed (Fig. 1b). The morphology of the PET nanowires was reproducible as long as the fabrication process remained the same.

To integrate the polymer nanowire arrays in a microfluidic chip, a polydimethylsiloxane (PDMS) slab with designed trenches was carefully coated on the fabricated PET slide to form enclosed microchannels for carrying out immunoassays. Because both the area without nanowires on the PET slide and the area on the slab of PDMS were completely flat, the adhesion between the PET slide and the PDMS was strong enough to prevent leakage during the

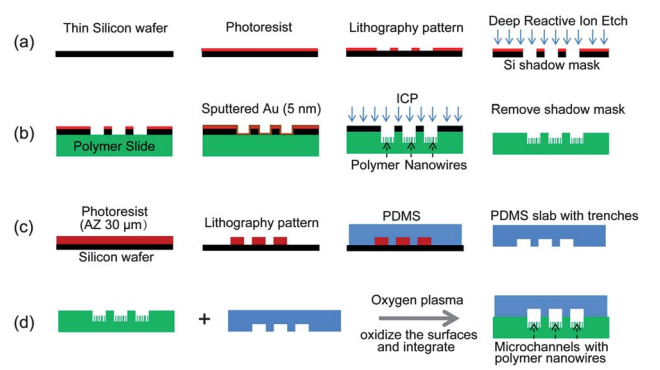

Fig. 1 (a) Fabrication of the Si shadow mask. (b) Fabrication of the substrate with patterned polymer nanowire arrays. (c) Fabrication of the PDMS slab with designed trenches. (d) Integration of the microfluidic chip.

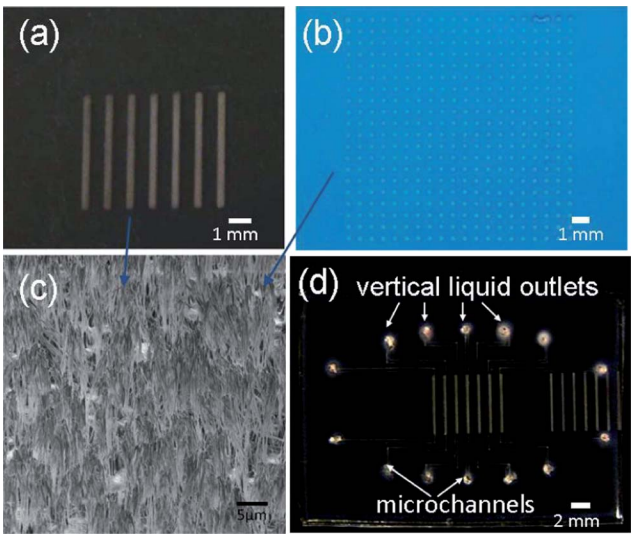

Fig. 2 (a and b) Images of the substrate with patterned polymer nanowires in trenches. (c) SEM image of the polymer nanowire array. (d) Image of the microfluidic chip.

microfluidic operations in the immunoassay in the current report. The fabricated microfluidic chip is shown in Fig. 2.

Then an immunoassay to detect a certain antigen was carried out on the chip (Fig. 3). The antigen (Rabbit IgG, from Jackson-Immuno research) solutions with different concentrations were introduced into the microchannels and adsorbed on the polymer nanowires to form protein patterns. Then 5% casein solution was introduced to saturate the surface and reduce the nonspecific adsorption. Next the unadsorbed biomolecules were washed away with 0. 1 mL of phosphate buffer saline and the channels were incubated with fluorescent dye-labeled antibody (Goat anti-rabbit IgG-FITC) for 1 h, when the antibody would specifically bind to the immobilized antigen. At last, the channels were purged with 0.1 mL of PBS. The fluorescent dye-labeled antibody would only stay in the channels with

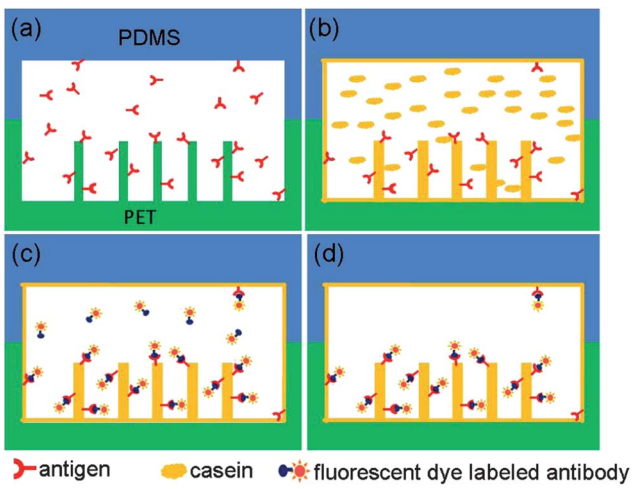

Fig. 3 The process of the immunoassay: (a) immobilizing antigen on the surface of the substrate. (b) Introducing 5% casein solution to prevent non-specific adsorption. (c) Introducing fluorescently labeled antibodies to bind with the antigens. (d) Purging the channel with PBS buffer to wash away free antibodies.

immobilized antigens because of the specific adsorption. Without specific adsorption, the antibody would be washed away. The chip was observed under an inverted fluorescence microscope. The fluorescent intensity in the areas with nanowire arrays has been much stronger than that in the flat areas because of the larger amount of antigen adsorption on the surface (Fig. 4). If the limit of detection (LOD) is defined as signal/noise = 2 : 1, the LOD with the nanowire arrays was 0.2 μg mL^{-1}. This number was 1/50 of that using the flat polymer substrate (10 μg mL^{-1}) and was even lower than that using electrospun nanofibers (0.625 μg mL^{-1}).[17] It should be noticed that the concentration of the fluorescent dye-labeled antibody was fixed, so at a high antigen concentration range, the increase of the fluorescent signal was not linear.

A cross-adsorption experiment has been carried out to show the good selectivity of the antigen-functionalized nanowires. Polymer nanowires were localized in 300 nm diameter spots as shown in Fig. 2b. The principle was the same with the experiment to detect a single antigen. But 3 different antigen solutions (mouse IgG, Human IgG, and Rabbit IgG) were injected in different rows and 3 fluorescently labeled antibodies (Cy3 labeled Goat anti-rabbit IgG, red fluorescence; FITC labeled Goat anti-Human IgG, green fluorescence; and AF488 labeled Goat anti-mouse IgG, green fluorescence) were introduced in different columns. The results are shown in Fig. 5. Only spots with specific adsorption were bright under the fluorescence scanner.

The biochips with polymer nanowires can be applied in immunoassay based clinical diagnosis. Here we demonstrate the performance improvement of such biochips with HIV-positive serum antibody detection. Such diagnostic assays typically attempt to detect primary antibodies (IgGs) in patient's serum which is specific for HIV ENV antigens. The biochemical principle of our protocol follows that in enzyme-linked immunosorbant assay (ELISA) but only requires 1/100 to 1/10 000 of the amount of serum sample and biochemical reagents commonly consumed in multi-well plate ELISA. Firstly gp120, which was a glycoprotein exposed on the surface of the HIV envelope, was adsorbed on the polymer nanowires. Then a 2% BSA block solution was introduced to reduce non-specific adsorption. Next, HIV positive and HIV negative

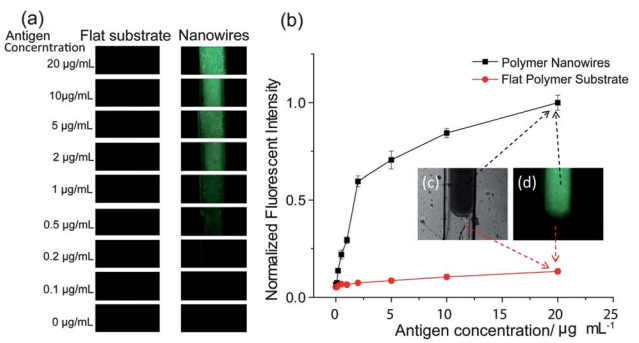

Fig. 4 The immunoassay results: (a) the fluorescent image of the microfluidic channels with different antigen concentrations. The left column shows the images taken from the flat part of the substrate and the right column shows the images taken from the part with nanowire arrays. (b) The fluorescent intensity at different antigen concentrations. (c and d) Bright-field optical image and fluorescent image of the nanowire array/flat substrate junction area, the upper part of the channel was with polymer nanowires.

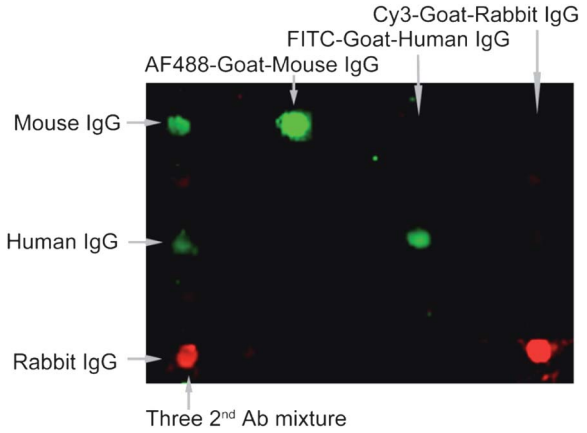

Fig. 5 Cross-analysis of different antigen–antibody interactions.

serums were introduced and incubated for 30 min. The anti-gp120 antibody would specifically bind to the gp120 if the serum is HIV positive. Finally, the fluorescent dye labeled secondary antibody that was specific for anti-gp120 was injected and incubated for 30 min to determine whether there was anti-gp120 antibody in the serum. After each step, 0.01 M PBS buffer with 0.2% Tween 20 was used to wash away the free molecules. In Fig. 6 we can see that the biochip with polymer nanowires provide a much brighter fluorescence signal with HIV positive samples than the HIV negative samples. Without the nanowires, both the HIV positive and negative samples remained dark. It should be noticed that for HIV negative samples, there was also a weak fluorescence emission from non-specific absorption. But the fluorescent signal intensity difference between the HIV positive and negative samples was amplified 20 times by using polymer nanowires, which is a significant improvement in sensitivity potentially for clinical diagnosis. For future development, it is also possible to use special gases in the ICP treatment step to modify the surface of the nanowires with certain functional groups (*e.g.* ammonia gas for amino-groups), which may be further chemically modified with desired anchor biomolecules or non-specific adsorption inhibitors to increase the signal to noise ratio.

In conclusion, we have fabricated a patterned polymer nanowire structure for enhancing the sensitivity of biochips. The output fluorescent signal was magnified and the detection limit of biomolecules was lowered. It has potential application in a lot of clinical diagnosis based on specific adsorption of biomolecules

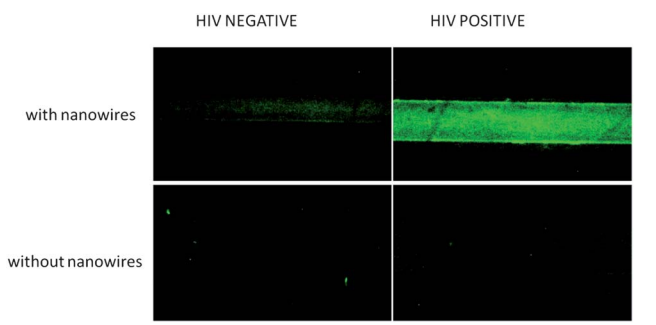

Fig. 6 The HIV positive serum detection result using biochips with and without polymer nanowires. All the four pictures were taken from one experiment on one chip.

such as HIV antibody immunoassay. Besides making protein or DNA micro arrays, it is also possible to use such substrates for cell culture to study the influence of surface morphology on the cells' adhesion, growth, migration and differentiation.

Experimental section

Fabrication of the patterned polymer nanowire arrays

First, a silicon shadow mask was made by lithography and deep reactive ion etching (Bosch process) on a thin silicon wafer (thickness = 50 μm). The areas to be covered by polymer nanowire arrays were etched through (Fig. 1a). Then, the shadow mask was put on a clean and flat PET slide (thickness = 0.3 mm). After that, 10 nm Au was sputtered on the slide with shadow mask to roughen the exposed surface. Next, the ICP etching was carried out with the following conditions: Ar, O_2, and CF_4 gases were introduced in the ICP chamber with flow rates of 15.0, 10.0, and 30.0 sccm (standard cubic centimeter per minute), respectively. The operation temperature was 55.0 °C with a pressure of 15 mTorr. One power source of 400 W was used to generate a large density of plasma while another power source of 100 W was used to accelerate plasma ions toward the polymer surface. The etching process lasted for 0.5 hour. The silicon shadow mask was reusable.

Integration of the microfluidic chip

A polydimethylsiloxane (PDMS) slab with designed trenches was made *via* a soft lithography technique (Fig. 1c). It was carefully coated on the fabricated PET slide to form enclosed microchannels for carrying out immunoassays. To further enhance the attachment between the PDMS slab and the polymer substrate with nanowires (not just for PET, but also for other polymer substrates), oxygen plasma (40 W, 2 min, with an oxygen plasma cleaner) was used to generate high energy surfaces before the sealing of the microchannels (Fig. 1d).

Antigen detecting experiment

Antigen (Rabbit IgG, from Jackson-Immuno research) solutions with different concentrations were introduced into the microchannels and were incubated for 2 hours. Then 5% casein solution was introduced, and the antigen microarray was incubated for 2 hours to saturate the surface and reduce the nonspecific adsorption. Next, 0. 1 mL of phosphate buffer saline (PBS, pH = 7.4, from Sigma-Aldrich) was used to purge each channel. After that, the channels were incubated with fluorescent dye-labeled antibody (Goat anti-rabbit IgG-FITC, 5 μg mL^{-1}, from Beyotime Institute of Biotechnology) for 1 h, when the antibody would specifically bind to the immobilized antigen. At last, the channels were purged with 0.1 mL of PBS. The chip was observed under an inverted fluorescence microscope.

Cross-adsorption experiment of 3 different antigen/antibody pairs

Polymer nanowires were localized in 300 nm diameter spots as shown in Fig. 5. Different antigen solutions (mouse IgG, Human IgG, Rabbit IgG, 5 μg mL^{-1} in carbonate buffer (CB, pH = 9.6), from Jackson-Immuno research) were injected into horizontal aligned PDMS microchannels and incubated for 15 min to functionalize different rows of nanowire spots. PBS was used to purge the channels after incubation. Then the PDMS slab was peeled off from the substrate and the whole substrate was incubated in the blocking solution (5% BSA in PBS, pH = 7.2) for 15 min to decrease nonspecific adsorption. After incubation, the substrate was washed with PBS. Next, another piece of PDMS slab with designed channels perpendicular to the first one was coated on the spots and fluorescently labeled antibodies (Cy3 labeled Goat anti-rabbit IgG, red fluorescence; FITC labeled Goat anti-Human IgG, green fluorescence; AF488 labeled Goat anti-mouse IgG, green fluorescence; 5 μg mL^{-1} in PBS, pH = 7.2, from Beyotime Institute of Biotechnology) were introduced in different columns and incubated for 15 min. Finally, each channel was purged with PBS once to wash away the free molecules. The chip was observed under a Typhoon Trio fluorescence scanner (from GE, blue-excited fluorescence was 488 nm; green-excited fluorescence was 532 nm. The multicolor fluorescence images were overlaid and exported by the custom software of the instrument).

Detection of HIV antibody in human serum

A solution of gp120 (50 μg mL^{-1} with carbonate buffer, pH = 9.6, from ProSpec-Tany TechnoGene) was introduced in the microchannels and incubated for 30 min. Then a 2% BSA block solution was introduced and incubated for 30 min. Next, HIV positive and HIV negative serums were introduced and incubated for 30 min. Finally, the fluorescent dye labeled secondary antibody (specific for anti-gp120, 20 μg mL^{-1}, from Beyotime Institute of Biotechnology) was injected and incubated for 30 min. After each step, 0.01 M PBS buffer with 0.2% Tween 20 was used to purge the channel.

Abbreviations

ICP	Inductively coupled plasma
PDMS	Polydimethylsiloxane
HIV	Human immunodeficiency virus
PET	Polyethylene terephthalate
PMMA	Polymethyl methacrylate
PS	Polystyrene
PEN	Polyethylene naphthalate
PTFE	Polytetrafluoroethylene
PBS	Phosphate buffer saline
LOD	Limit of detection
Cy3	Cyanine dye 3
FITC	Fluorescein isothiocyanate
AF488	Alexa Fluor 488
IgG	Immunoglobulin
ELISA	Enzyme-linked immunosorbant assay
BSA	Bovine serum albumin.

Acknowledgements

The research was supported by NSF, BES DOE, NSFC (51202076), MOST, and the Knowledge Innovation Program of the Chinese Academy of Sciences (Grant no. KJCX2-YW-M13).

References

1 O. P. Kallioniemi, *Ann. Med.*, 2001, **33**, 142–147.
2 R. B. Stoughton, *Annu. Rev. Biochem.*, 2005, **74**, 53–82.
3 K. R. Kondabagil and B. S. Kwon, *J. Microbiol. Biotechnol.*, 2001, **11**, 907–914.
4 P. R. Srinivas, S. Srivastava, S. Hanash and G. L. Wright, *Clin. Chem.*, 2001, **47**, 1901–1911.
5 F. von Eggeling, K. Junker, W. Fiedler, V. Wollscheid, M. Durst, U. Claussen and G. Ernst, *Electrophoresis*, 2001, **22**, 2898–2902.
6 M. F. Templin, D. Stoll, J. M. Schwenk, O. Potz, S. Kramer and T. O. Joos, *Proteomics*, 2003, **3**, 2155–2166.
7 A. H. C. Ng, U. Uddayasankar and A. R. Wheeler, *Anal. Bioanal. Chem.*, 2010, **397**, 991–1007.
8 V. Mikhailovich, D. Gryadunov, A. Kolchinsky, A. A. Makarov and A. Zasedatelev, *BioEssays*, 2008, **30**, 673–682.
9 B. H. Weigl, R. L. Bardell and C. R. Cabrera, *Adv. Drug Delivery Rev.*, 2003, **55**, 349–377.
10 F. Rusmini, Z. Y. Zhong and J. Feijen, *Biomacromolecules*, 2007, **8**, 1775–1789.
11 I. Barbulovic-Nad, M. Lucente, Y. Sun, M. J. Zhang, A. R. Wheeler and M. Bussmann, *Crit. Rev. Biotechnol.*, 2006, **26**, 237–259.
12 J. de Jong, R. G. H. Lammertink and M. Wessling, *Lab Chip*, 2006, **6**, 1125–1139.
13 J. de Jong, B. Ankone, R. G. H. Lammertink and M. Wessling, *Lab Chip*, 2005, **5**, 1240–1247.
14 D. Y. Yang, X. Niu, Y. Y. Liu, Y. Wang, X. Gu, L. S. Song, R. Zhao, L. Y. Ma, Y. M. Shao and X. Y. Jiang, *Adv. Mater.*, 2008, **20**, 4770–4775.
15 Y. Lee, H. J. Lee, K. J. Son and W. G. Koh, *J. Mater. Chem.*, 2011, **21**, 4476–4483.
16 E. Jo, M. C. Lim, H. N. Kim, H. J. Paik, Y. R. Kim and U. Jeong, *J. Polym. Sci., Part B: Polym. Phys.*, 2011, **49**, 89–95.
17 D. Y. Yang, X. Liu, Y. Jin, Y. Zhu, D. D. Zeng, X. Y. Jiang and H. W. Ma, *Biomacromolecules*, 2009, **10**, 3335–3340.
18 X. Y. Jiang, J. M. K. Ng, A. D. Stroock, S. K. W. Dertinger and G. M. Whitesides, *J. Am. Chem. Soc.*, 2003, **125**, 5294–5295.
19 T. Rohr, C. Yu, M. H. Davey, F. Svec and J. M. J. Frechet, *Electrophoresis*, 2001, **22**, 3959–3967.
20 C. Yu, F. Svec and J. M. J. Frechet, *Electrophoresis*, 2000, **21**, 120–127.
21 H. Zhu, J. F. Klemic, S. Chang, P. Bertone, A. Casamayor, K. G. Klemic, D. Smith, M. Gerstein, M. A. Reed and M. Snyder, *Nat. Genet.*, 2000, **26**, 283–289.
22 G. Walter, K. Bussow, D. Cahill, A. Lueking and H. Lehrach, *Curr. Opin. Microbiol.*, 2000, **3**, 298–302.
23 T. O. Joos, M. Schrenk, P. Hopfl, K. Kroger, U. Chowdhury, D. Stoll, D. Schorner, M. Durr, K. Herick, S. Rupp, K. Sohn and H. Hammerle, *Electrophoresis*, 2000, **21**, 2641–2650.
24 D. Guschin, G. Yershov, A. Zaslavsky, A. Gemmell, V. Shick, D. Proudnikov, P. Arenkov and A. Mirzabekov, *Anal. Biochem.*, 1997, **250**, 203–211.
25 T. Lilliehorn, M. Nilsson, U. Simu, S. Johansson, M. Almqvist, J. Nilsson and T. Laurell, *Sens. Actuators, B*, 2005, **106**, 851–858.
26 Q. Zhang, J. J. Xu and H. Y. Chen, *Electrophoresis*, 2006, **27**, 4943–4951.
27 Y. Y. Liu, D. Y. Yang, T. Yu and X. Y. Jiang, *Electrophoresis*, 2009, **30**, 3269–3275.
28 H. Fang, W. Z. Wu, J. H. Song and Z. L. Wang, *J. Phys. Chem. C*, 2009, **113**, 16571–16574.
29 J. R. Morber, X. Wang, J. Liu, R. L. Snyder and Z. L. Wang, *Adv. Mater.*, 2009, **21**, 2072–2076.