

Xiaojun Liu
Roger C. Lo
Frank A. Gomez

Department of Chemistry and
Biochemistry, California State
University, Los Angeles, CA, USA

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Research Article

Fabrication of a microfluidic enzyme reactor utilizing magnetic beads

An enzyme-catalyzed microfluidic assay using magnetic micro-beads is described. Here, diaphorase (DI) (E.C. 1.6.99) is covalently attached to the magnetic micro-beads (2.7 μm) and integrated into a short section of a microchip fabricated from PDMS. DI converts non-fluorescent resazurin to fluorescent resorufin in the presence of nicotinamide adenine dinucleotide phosphate (NADH). In this work, an embedded magnet holds the micro-beads in place within the microchannel while a solution of resazurin and NADH in buffer is flowed through the beads. Incorporation of the micro-beads into the microchannel requires only a few minutes and offers well-defined spatial resolution and reproducibility. At a flow rate of 41.2 $\mu\text{L}/\text{h}$, a stable state for the enzyme reaction in the microfluidic format was achieved within 50 s. The maximum conversion of the reaction was obtained at a concentration of 1.25 mM NADH. The reaction yield is affected by ZnCl_2 and at concentrations in excess of 90.0 mM, the activity of DI was almost double without ZnCl_2 . At 5.2 mM potassium chloride, the activity of DI reached its maximum value. Overall, the conversion of resazurin in microfluidic format was more than twice than that in a batch assay.

Keywords:

Diaphorase / Enzyme / Magnetic micro-beads / Microfluidics / Microreactor

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1 Introduction

Miniaturization of enzyme reactors offers a number of advantages over benchtop instruments including smaller dead volumes, decreased reagent consumption, shorter analysis time, lower cost and greater sensitivity and flexibility in use [1–6]. Microfluidic reactors have been proven to be a powerful tool in chemistry and biotechnology [7–9] and are particularly useful when limited quantities of material (enzymes and/or substrates) are available. While enzymes can be studied in solution using a microfluidic reactor, the immobilization of enzymes has been preferred by many researchers since immobilization allows for the reuse of enzyme, lowers the rate of denaturation or inactivation, avoids secondary reactions like autoprolysis, simplifies product separation and improves the enzymatic reaction rate [10–21].

Enzyme immobilization can be classified into two categories according to the immobilization location. One, a wall or assembly membrane of channels (so-called open channel) [10–13] and, two, a solid support inside channels such as a monolith, sol-gel, controlled pore glass, silica and polystyrene beads [14–25]. The open channel type has limited immobili-

zation capacity due to a smaller surface-to-volume ratio. Enzyme immobilization onto monoliths or in sol-gels requires multi-step reactions. In addition, their preparation is time-consuming and they suffer from non-specific adsorption and poor reproducibility. The packing of controlled pore glass and polystyrene beads into channels needs weirs or frits microfabricated inside the channels to stop beads which makes design and fabrication of microfluidic reactors more complicated. Recently, Nomura *et al.* showed that magnetic particles could be packed into Teflon tubes trapped between two 6×5 mm permanent magnets [26]. In our previous work, we studied the affinity binding of peptides to teicoplanin (a glycopeptide antibiotic) using magnetic micro-beads (2.7 μm in diameter) derivatized with teicoplanin. Teicoplanin-modified micro-beads (used as the affinity column) and underivatized micro-beads (used as the control column) were simultaneously packed in a few minutes into given sections of a microchannel with the aid of a “holding magnetic” (2 mm diameter) imbedded into the PDMS [27]. The length and density of the two columns could be easily controlled and with good reproducibility. The extent of interaction between the peptide and the two types of beads in either microchannel resulted in differences in migration time of the peptide, and this difference was used to obtain a value for the binding constant of the peptide to teicoplanin.

Herein, we demonstrate a facile method, employing an embedded magnet, of fabricating an enzyme-catalyzed microfluidic reactor. In these studies, diaphorase (DI) (a flavin-bound enzyme) is used to convert resazurin (non-fluorescent) to resorufin (fluorescent) in the presence of nicotinamide adenine dinucleotide phosphate (NADH)

Correspondence: Dr. Frank A. Gomez, Department of chemistry and Biochemistry, California State University, Los Angeles 5151 State University Drive, Los Angeles, CA 90032-8202, USA
E-mail: fgomez2@calstatela.edu
Fax: +1-323-343-6437

Abbreviations: DI, diaphorase; NADH, nicotinamide adenine dinucleotide phosphate

(Fig. 1). DI was immobilized onto carboxylic-terminated magnetic micro-beads and then packed into a given section of a microchannel. Optimal conditions for the microreaction were examined and are detailed herein.

2 Materials and methods

2.1 Chemicals and reagents

Resazurin sodium salt, BSA, NADH, *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride and DI (E.C. 1.6.99) from *Clostridium kluyveri*, glycidyl methacrylate, dimethylacrylamide and TEMED were purchased from Sigma (St. Louis, MO). Zinc chloride, MES and potassium chloride were purchased from Fisher Scientific. Negative-type photoresist SU-8 2025 and its developer were obtained from Microchem (Newton, MA). PDMS oligomer and its cross-linking agent Sylgard 184 were purchased from Dow Corning (Midland, MI). Carboxylic group-terminated Dynal[®] magnetic beads (~2.7 μm diameter) were purchased from Invitrogen (Carlsbad, CA).

Zinc chloride was used as an activator. A stock solution of zinc chloride was prepared in 5 mM HCl. 0.20 M Tris solution was used as the running buffer. A stock solution of NADH and resazurin was dissolved in the running buffer before use. A stock solution of potassium chloride was prepared daily in the running buffer containing 0.02% BSA. The substrate solution was prepared by mixing the stock solutions at different ratios.

2.2 Instrumentation

A fluorescence microscope (Nikon Eclipse TE-2000-U, Nikon, Kyoto, Japan) with a 100 W mercury lamp and a charge-coupled device camera was used to monitor the DI-catalyzed reaction in real time. Red fluorescence was observed with Nikon filter Y-2E/C (ex/em 540–580 and 600–660 nm, respectively).

2.3 Fabrication of DI-catalyzed microfluidic reactor

The procedure for packing magnetic beads into the microfluidic channel was described in our previous study [27]. Briefly, a three-step procedure was used to fabricate the microfluidic enzyme reactor: (i) magnetic micro-bead

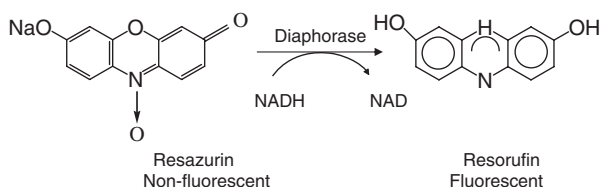


Figure 1. DI-catalyzed reaction between non-fluorescent resazurin and NADH to yield fluorescent resorufin.

derivatization with DI; (ii) fabrication of a hydrophilic microchip and; (iii) packing of DI-modified magnetic beads into the microchannels.

2.3.1 Immobilization of DI onto magnetic micro-beads

A solution (100 μL) of carboxylic acid-terminated magnetic beads from a stock solution (2×10^9 beads/mL) was washed ($5 \times$) with MES buffer (25 mM, pH 5). A solution (60 μL) of DI (1 mg/mL) in MES (25 mM, pH 5) was added to the beads and mixed. A solution (30 μL) of freshly prepared *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride solution (100 mg/mL in cold water) and MES buffer (10 μL) were added to the bead solution and the mixture was incubated for 6 h at room temperature with slight shaking. The solution was then discarded and beads were suspended in Tris (50 mM, pH 7.4) buffer for 15 min in order to quench non-reacted carboxylic acid groups. The beads were then washed with PBS (100 mM phosphate, pH 7.4, 150 mM NaCl, 0.1% BSA) ($5 \times$) and re-suspended in PBS (1 mL) (100 mM, pH 7.4, 150 mM NaCl) buffer.

2.3.2 Microchip fabrication

The microchip was prepared by conventional soft photolithography techniques [28]. The straight pattern was designed using AutoCAD software (San Rafael, CA) and printed as a high resolution (10 000 dip) photomask (CAD/Art Services, OR). Negative-type photoresist was spin-coated onto a 3 in. silicon wafer at 1200 rpm for 30 s. The channel is 150 and 25 μm wide and high, respectively. The mold was spin-coated with degassed PDMS pre-polymer solution at 1300 rpm for 150 s and then kept at 70°C for 15 min. A 2 mm diameter rare earth magnet used as holding magnet and was placed above the channel where the magnetic micro-beads were initially packed. Degassed PDMS pre-polymer solution was poured onto the assembly and baked for 2 h at 70°C. The assembly was peeled off from the mold. Holes (3 mm diameter) used as the sample and buffer reservoirs were punched. The PDMS assembly was irreversibly sealed to a glass slide

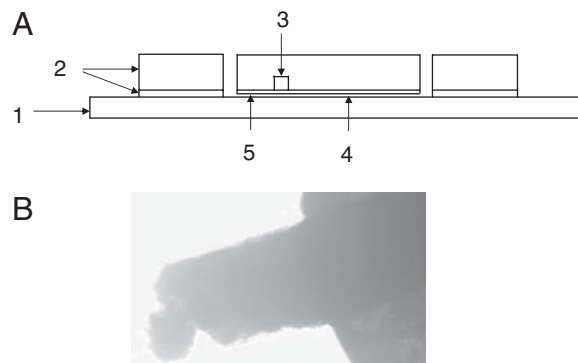


Figure 2. (A) Side view of microfluidic chip ((1) glass slide, (2) PDMS, (3) magnet in 2.0 mm diameter, (4) detection point, (5) microchannel) and (B) top view of the beads in the microchannel.

after treating with oxygen plasma (Fig. 2A). To suppress non-specific adsorption, the channel surface was modified to be permanently hydrophilic following the method described by Wu *et al.* [29]. In brief, 5.0% w/v dimethylacrylamide and 0.1% v/v glycidyl methacrylate were mixed in distilled water, and thoroughly degassed for 10 min. To initiate the polymerization reaction, 0.1% TEMED v/v and 0.05% potassium persulphate w/v were added into the mixture and the reaction was allowed for 30 min at room temperature. To remove unpolymerized monomers and small polymer molecules, the polymer solution was extensively dialyzed by 3500 molecular weight cut-off dialysis membranes for 2 days with distilled water changed every 12 h. The polymer solution was introduced into the just sealed microchannel, and incubated for 15 min at room temperature. The solution was completely pumped out and PDMS microchip was directly heated in 110°C for 10 min.

2.3.3 Incorporation of DI, the modified beads, into the microchannel

The channel was rinsed with redistilled water and the running buffer for 3 min using a peristaltic pump. Aliquots (5.0 μL) of the suspension of DI-modified beads were then injected into the channel at a flow speed of 2.1 $\mu\text{L}/\text{h}$. At this flow rate, the magnetic micro-beads were retained by the holding magnet imbedded into PDMS. The channel was rinsed with the running buffer in both directions at an increasing flow rate to finally form a 1.2 mm magnetic micro-bead column. The DI-catalyzed microfluidic reactor was ready for use.

2.4 Video acquisition and analysis

The substrate solution passed through the DI-catalyzed microfluidic reactor driven by a peristaltic pump. All the runs were repeated twice. Between two runs, the microfluidic reactor was washed with the running buffer, PBS buffer and the running buffer for 3, 5 and 5 min, respectively.

The progress of each enzymatic reaction was monitored in real-time by a fluorescence scope and saved as a MPEG video file. To obtain the intensity data of the fluorescent product, the MPEG video file was first exported to a sequence of 8-bit TIFF images with QuickTime Player (Version 7.5.5; Apple) and then the average intensity within a square area (10 \times 10 pixels) in the center of the microchannel using macros in ImageJ (Version 1.42g) was extracted. The fluorescence trace was then plotted *versus* the elapsed time for further calculations.

3 Results and discussion

3.1 Non-specific adsorption of enzyme

In our study, a 2 mm diameter rare earth magnet used as holding magnet was imbedded above the microchannel. At a flow speed of 2.1 $\mu\text{L}/\text{h}$, magnetic micro-beads were retained by the holding magnet when the bead suspension passed through the microchannel. Moreover, the hydrophilic surface of the magnetic micro-beads [30] and the channel [29] suppressed the non-specific adsorption of the magnetic beads onto the wall of the microchannel outside the holding magnet and facilitated the packing of the beads only under the holding magnet (Fig. 2B).

3.2 Amount of DI loaded into the channel

To estimate the total amount of enzyme loaded into a microchannel, DI was cleaved from the magnetic beads after incubating the beads with 100 mM NaOH solution for 2 h at room temperature. The concentration of DI was determined to be 0.40 $\mu\text{g}/\mu\text{L}$ by the absorption OD value at 280 nm using a UV/Vis spectrophotometer. According to the concentration and volume of beads suspension, the amount of DI loaded into the channel was calculated to be 2.0 μg .

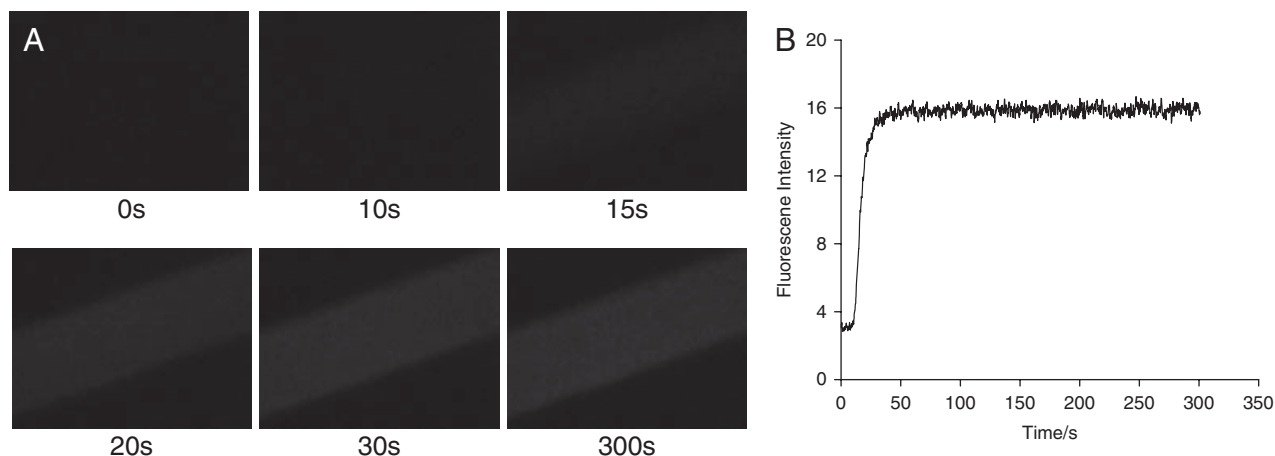


Figure 3. (A) Fluorescence microscopic graphs and (B) the corresponding curve from the video taken at a flow rate 41.2 $\mu\text{L}/\text{h}$.

3.3 Optimization of reaction yield

A mixture containing 8.0 μg DI, 1.5 mM NADH, 30 mM zinc chloride, 1.2 mM resazurin and 2.6 mM potassium chloride at pH 8.5 was allowed to react for 15 min and the corresponding conversion of resazurin to resorufin was used as a batch assay standard. In subsequent experiments, the

concentration of resazurin was kept at 1.2 mM and the conversion of resazurin in the microfluidic reactor was always compared with the batch assay standard. The ratio of the conversion of resazurin in the two reactors was defined as the normalized conversion of resazurin in the microfluidic reactor (Eq. (1)):

$$\text{Normalized Conversion}\% = Fr/Fs * 100 \quad (1)$$

Here, Fr and Fs were the fluorescence intensity from resorufin in microfluidic reactor and batch reactor, respectively.

The conversion of resazurin in the microfluidic reactor is dependent on the flow rate, pH of the solution, and the concentration of NADH, activator (ZnCl_2) and salt. Figure 3 shows the fluorescence microscopic graphs and the corresponding curve exported from the video taken at a flow rate of 41.2 $\mu\text{L}/\text{h}$. At a flow rate of 41.2 $\mu\text{L}/\text{h}$, a stable state for the enzyme reaction in a microfluidic format was achieved within 50 s. Figure 4A shows the effect of flow rate on the conversion of resazurin. The maximum conversion was obtained at a flow rate of 4.1 $\mu\text{L}/\text{h}$. The conversion of resazurin in a microfluidic reactor decreased with increasing flow rate. We attribute this to the difference in time the substrate is in contact with the DI-derivatized micro-beads. As the flow rate increases, contact time decreases. The conversion of resazurin in the microfluidic reactor exceeded its conversion in the batch reactor at a flow rate of less than 41.2 $\mu\text{L}/\text{h}$. This result could be explained by the effect of the ratio of enzyme to substrate. For a given plug of substrate at a flow rate of less than 41.2 $\mu\text{L}/\text{h}$, the enzyme-to-substrate ratio is much higher in the microfluidic reactor than in the batch reactor. A flow rate of 41.2 $\mu\text{L}/\text{h}$ was used in subsequent experiments.

The pH of the solution is an important parameter in most enzyme-catalyzed reactions. Figure 4B shows the influence of pH on the activity of DI. As can be seen, the activity of DI is very sensitive to pH. The maximum activity of DI was obtained at a pH 8.50. At pH 7.5 and 9.0, the activity of DI decreased by almost 50%. These observations agreed well with the behavior of DI in solution.

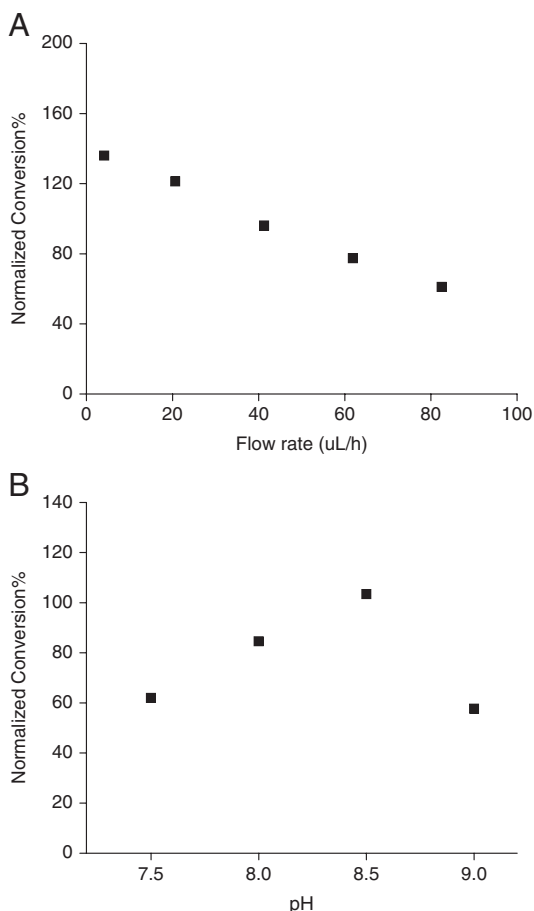


Figure 4. (A) Effect of flow rate and (B) pH on the conversion of resazurin in microfluidic reactor.

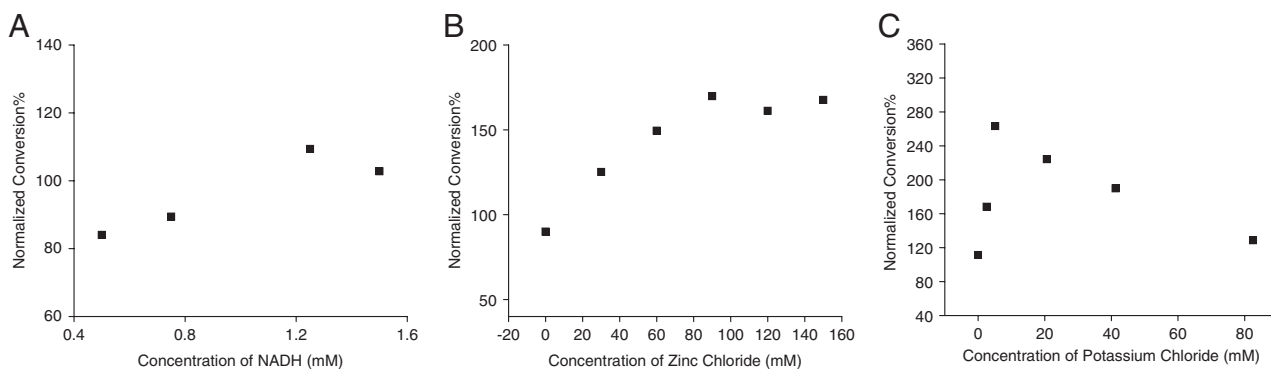


Figure 5. Measured conversion of resazurin at various concentrations of (A) NADH, (B) zinc chloride and (C) potassium chloride in the microfluidic reactor.

The yield of resorufin was strongly affected by the concentration of another substrate, NADH. Figure 5A shows the dependence of the conversion of resazurin with NADH. The yield of resorufin did not always increase with the concentration of NADH. The maximum conversion was obtained at a concentration of 1.25 mM NADH. A concentration of 1.25 mM NADH was used in subsequent experiments.

It is known that ZnCl₂ can activate DI [31]. Figure 5B illustrates the conversion of resazurin at various concentrations of ZnCl₂. The reaction yield is strongly affected by ZnCl₂ at low concentrations and relatively stable at concentrations of more than 90.0 mM. At ZnCl₂ concentration of more than 90.0 mM, the activity of DI was almost twice the activity of DI without ZnCl₂.

For the DI reaction, a suitable concentration of salt is needed. Figure 5C shows how the activity of DI in the microfluidic reactor changes when the concentration of potassium chloride is varied from 0 to 82.5 mM. The activity of DI was sensitive to the concentration of salt. At 5.2 mM potassium chloride, the activity of DI reached the maximum value and was more than twice of the batch assay. At 0.0 and 80.0 mM potassium chloride, the activity of DI was decreased by more than 50%.

4 Concluding remarks

In the present work, an enzyme-catalyzed microfluidic reactor was prepared using DI-modified magnetic microbeads. The enzyme-modified beads could be easily packed into the microchannels with well-defined spatial resolution and reproducibility under the help of a holding magnet imbedded into PDMS. Optimal conditions for the enzyme reaction were obtained and the conversion of resazurin was more than twice that when compared with a batch assay standard. This work has great potential in the development of microscale enzyme assays. The amounts of enzyme and substrate required are orders less than that used in typical enzyme assay protocols, which should benefit those working with expensive reagents. Future work is focused on examining other enzyme systems and in developing multi-step enzyme reactions.

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The authors have declared no conflict of interest.

5 References

- [1] Figeys, D., Pinto, D., *Anal. Chem.* 2000, 72, 330A–335A.
- [2] Jakeway, S. C., deMello, A. J., Russell, E. L., *Fresenius J. Anal. Chem.* 2000, 366, 525–539.
- [3] Jensen, K. F., *Chem. Eng. Sci.* 2001, 56, 293–303.
- [4] Koha, W.-G., Pishko, M., *Sens. Actuators B* 2005, 106, 335–342.
- [5] McCreedy, T., *Trends Anal. Chem.* 2000, 19, 396–401.
- [6] Xie, B., Ramanathan, K., Danielsson, B., *Trends Anal. Chem.* 2000, 19, 340–349.
- [7] Miyazaki, M., Maeda, H., *Trends Biotechnol.* 2006, 24, 463–470.
- [8] Heo, J., Thomas, K. J., Seong, G. H., Crooks, R. M., *Anal. Chem.* 2003, 75, 22–26.
- [9] Shiddiky, M. J. A., Rahman, M. A., Park, J.-S., Shim, Y.-B., *Electrophoresis* 2006, 27, 2951–2959.
- [10] Thomsen, M. S., Nidetzky, B., *Eng. Life Sci.* 2008, 8, 40–48.
- [11] Mao, H., Yang, T., Cremer, P. S., *Anal. Chem.* 2002, 74, 379–385.
- [12] Yakovleva, J., Davidsson, R., Lobanova, A., Bengtsson, M., Eremin, S., Laurell, T., Emneus, J., *Anal. Chem.* 2002, 74, 2994–3004.
- [13] Limbut, W., Loyprasert, S., Thammakhet, C., Thavarungkul, P. et al., *Biosens. Bioelectron.* 2007, 22, 3064–3071.
- [14] Kerby, M. B., Legge, R. S., Tripathi, A., *Anal. Chem.* 2006, 78, 8273–8380.
- [15] Koh, W.-G., Pishko, M. V., *Anal. Bioanal. Chem.* 2006, 385, 1389–1397.
- [16] Russom, A., Tooke, N., Andersson, H., Steme, G., *Anal. Chem.* 2005, 77, 7505–7511.
- [17] Wang, S.-C., Chen, H.-P., Lai, Y.-W., Chau, L.-K. et al., *Biomicrofluidics* 2007, 1, 034104.
- [18] Seong, G. H., Heo, J., Crooks, R. M., *Anal. Chem.* 2003, 75, 3161–3167.
- [19] Peterson, D. S., Rohr, T., Svec, F., Frechet, J. M. J., *Anal. Chem.* 2002, 74, 4081–4088.
- [20] Ma, J., Liang, Z., Qiao, X., Deng, Q. et al., *Anal. Chem.* 2008, 80, 2949–2956.
- [21] Luckarift, H. R., Ku, B. S., Dordick, J. S., Spain, J. C., *Biotechnol. Bioeng.* 2007, 98, 701–705.
- [22] Logan, T. C., Clark, D. S., Stachowiak, T. B., Svec, F., Frechet, J. M. J., *Anal. Chem.* 2007, 79, 6592–6598.
- [23] Tang, Z., Wang, T., Kang, J., *Electrophoresis* 2007, 28, 2981–2987.
- [24] Ma, J., Zhang, L., Liang, Z., Zhang, W., Zhang, Y., *J. Sep. Sci.* 2007, 30, 3050–3059.
- [25] Xu, X., Wang, X., Liu, Y., *Rapid Commun. Mass Spectrom.* 2008, 22, 1257–1264.
- [26] Nomura, A., Shin, S., Mehdi, O. O., Kauffmann, J.-M., *Anal. Chem.* 2004, 76, 5498–5502.
- [27] Liu, X., Gomez, F. A., *Electrophoresis* 2009, 30, 1194–1197.
- [28] Duffy, D. C., McDonald, J. C., Schueller, O. J. A., Whitesides, G. M., *Anal. Chem.* 1998, 70, 4974–4984.
- [29] Wu, D., Qin, J., Lin, B., *Lab chip* 2007, 7, 1490–1496.
- [30] Dynabeads® M-270 Immunoassays (Carboxyl) Manuals, Invitrogen.
- [31] Prasad, A. S., Oberleas, D., Wolf, P., Horwitz, J. P. et al., *J. Clin. Invest.* 1967, 46, 549–557.