Focusing Review

Development of high-performance analytical methods using biomolecules encapsulated in silicate nanomaterials.

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Abstract

High-throughput assay system using immobilized biomolecules with high accuracy and sensitivity would make drug discovery more efficient. To utilize the biomolecules into analytical systems, we created immobilization techniques of biomolecules, especially proteins, using hydrogels derived from sol-gel reaction of alkoxysilane or silica nanoparticles. The silicate formed networks around proteins, and then gelled, effectively entrapping the proteins. We analyzed in detail the network patterns formed by the silica nanoparticles by means of the high-resolution transmission electron microscopy. Furthermore, FT-IR study indicated immobilized proteins maintained their structure even after encapsulation in the gel. This immobilization technique enabled the integration of biochemical reaction, separation, and detection into capillary electrophoresis, capillary electrochromatography, microchip electrophoresis, and biochip systems.

Key Words: Sol-gel, Alkoxysilane, Silica nanoparticle, Biomolecule

1. Introduction

Recently, genomic drug discovery technology, organic synthesis, and *in silico* drug design technology have been used to develop molecularly targeted medicines [1, 2]. Under these conditions, high-throughput assay system with high accuracy and sensitivity would make drug discovery more efficient. Biomaterials, as typified by proteins, serve various biological functions including molecular recognition, binding, catalysis, molecular transport, and signal transduction. These functions are extremely accurate and efficient owing to their conformational accuracy and diversity. Therefore, the assay system using biomolecules is very attractive for drug discovery.

The various bio-immobilization methods reported to date include covalent attachment to supports, adsorption onto solid supports, cross-linking with bifunctional reagents, entrapment in gels, and encapsulation in membranes such as microcapsules, liposomes, hollow fibers, or dialysis membranes [3]. Silica matrices produced by means of the sol-gel process have attracted much attention as ideal materials for bio-immobilization because this process can be used for many of the immobilization methods mentioned above and because it can generate various matrix forms. In addition, the physical and chemical properties of silica are quite attractive for joining inorganic materials with biomolecules.

We developed the methodology for bio-immobilization and the application of sol-gel technology to analytical devices, especially microfluidic flow-through systems: capillary electrophoresis, capillary electrochromatography, microchip electrophoresis, and biochip systems; to achieve rapid analysis, reduced sample consumption and cost [4, 5]. Furthermore, the specific and efficient biological reactions may benefit greatly with microfluidic devices that offer the means for handling small-volume samples (i.e., nL to μL) in confined reaction zones.

2. The Development of Sol-gel Technology using Alkoxysilanes

The basic sol-gel process consists of the following steps: (1) hydrolysis of an alkoxysilane, (2) dimerization of the resulting sila-

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nol to form siloxane bonds (≡Si−O−Si≡); and (3) polycondensation reactions that link additional silanol molecules to form cyclic oligomers (Fig. 1). The most common starting materials are tetramethoxysilane (TMOS) and tetraethoxysilane (TEOS) because they undergo hydrolysis and condensation readily under mild conditions.

Elleby *et al.* have developed a modified version of the process that removes the need for the addition of methanol. This version does not expose the biomolecules to the damaging effects of low pH [6]. These improvements enabled encapsulated proteins to retain their structure in nm-size porous structure [3, 6, 7] and biological activity for a prolonged period [8] and to enhance its utility as new type of matrix for biomolecules. However, the uses of the revised version had been limited to static formats, including silica-gel film [9] or monoliths in cuvettes for optical biosensors [6, 8, 10] and it had not been applied to the flow-through supports for proteins, except for immunoaffinity chromatography in which IgG-encapsulated sol-gel glass was ground and packed into a column [11].

Based on the Ellerby's method, we developed a novel sol-gel method for monolithic capillary columns that encapsulate proteins. The greatest challenge in the design of the continuous gels was to find controlled conditions showing the suitable characteristics as chromatographic packing material, that is, the proper mechanical strength and flow penetration. The properties of sol-gel matrices depend on the relative rates of hydrolysis and condensation, which can be altered by varying the pH, temperature, reagent concentrations, and reaction time [12, 13].

First of all, we searched for the optimal gelation condition with no use of proteins, by varying buffer concentration, pH, and volume. After that, other factors were examined by dissolving proteins in the buffer.

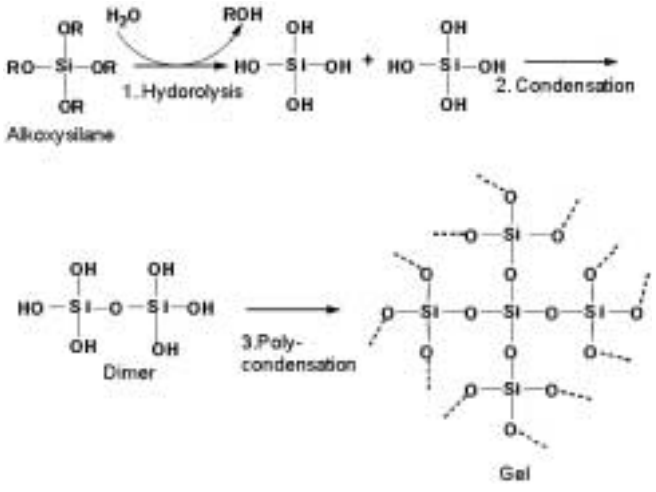

Fig. 1 Typical sol-gel reaction using alkoxysilane.

In CEC, the mobile phase contains an electrolyte and is transported through the capillary column by electroosmotic flow (EOF). The EOF is generated at the interface between the solution and a charged surface, such as the deprotonated silica [14]. The sol-gels also possess some unreacted silanols, which are believed to be responsible for the resultant EOF. Attenuated Total Reflection (ATR) -FT-IR was used to measure the silanol content during the time-course of gelation.

Fig. 2 a shows a typical IR spectrum of the gel in the region between 1300 cm^{-1} and 800 cm^{-1}. The band at 960 cm^{-1}, indicated by number 1, corresponds to silanol groups. The band at 1016 cm^{-1}, indicated by number 2, corresponds to MeOH, which was generated in the process of hydrolysis and condensation. The bands indicated by 3-6 correspond to SiO_2 structure with different vibration

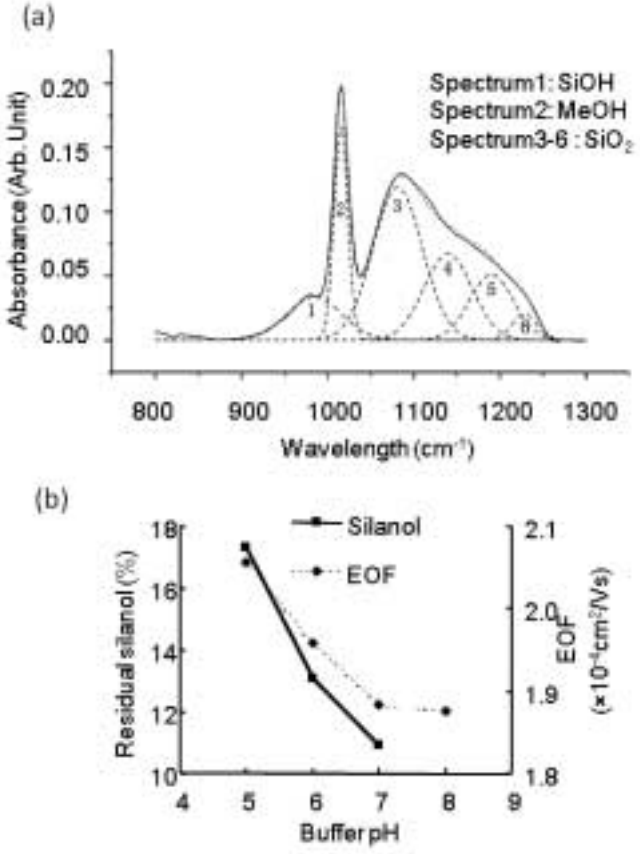

Fig. 2 Effect of residual silanol groups on EOF.
(a) Deconvolution of the IR absorption band at 800−1300 cm^{-1} of hydrogel. Solid line: experimental spectrum. Dashed line: decomposed spectrum. Dotted line: sum of the six decomposed spectra. Sample: hydrogel fabricated using 5 mM phosphate buffer.
(b) Relationship between the electroosmotic mobility and the relative contents of silanol groups. CEC conditions: Sample: 5 mM thiourea. Mobile phase: phosphate buffer. Applied voltage: 4 kV. Injection: 4 kV, 5 s. Column length: 36 cm. Gel length: 5 cm. Detection: 254 nm.

modes. The peak area of the silanol peak gradually decreased and the peak area of SiO_2 gradually increased, as the gelation process progressed.

As shown in Fig. 2 b, the residual silanol groups decreased as buffer pH of gel preparation increased. This change coincided with that of electroosmotic mobility. The residual silanol groups also decreased with an increase in the ionic strength, which is associated with the decrease in the electroosmotic mobility. These results indicate that electroosmotic mobility have close relationship with the content of residual silanol groups in gels [13]. Therefore, EOF can be controlled by changing the gelation conditions. Of course, other factors such as mobile phase conditions largely influence electroosmotic mobility [12] .

IR spectra also give a variety of information on the protein conformation [15]. The conformation of BSA in gel and in free solution was investigated using ATR-FT-IR. As illustrated in Fig. 3, two representative bands originated from BSA were observed in the region between 1800 cm^{-1} and 1400 cm^{-1}. The signal around 1657 cm^{-1} is the amide I band, which mainly consists of $\upsilon_{C=O}$ stretching vibrations. The signal at 1549 cm^{-1} is the amide II band, which mainly consists of υ_{N-H} bending vibrations. These two bands change its position depending on the structural changes as well as the degree of hydrogen bonding [16]. It is also reported that the amide I/ amide II intensity and area ratios may be associated with conformational changes of the protein [16]. The peak (aminde I and amide II) positions of each peak show no differences in both formats. Furthermore, the ratios of peak area are very close with each other. These strongly suggest that BSA maintain its conformation after encapsulation in the sol-gels [13].

The conformational stability of biomolecules is very important to maintain its biological activity. Silica hydrogels contain a sufficient amount of trapped interstitial water. Our hydrogel consist of about 80% water in their weight. Therefore the environment within hydrogel is essentially aqueous, allowing the retention of structure and reactivity of the encapsulated biomolecules. Our results support that this encapsulating technique is very effective to make use of biomolecules in biological assay system.

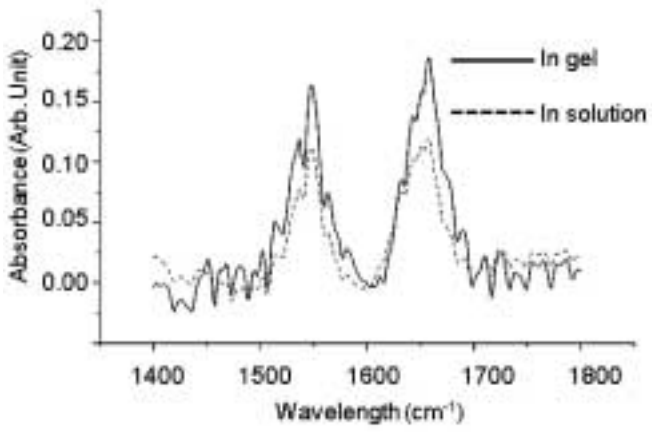

Fig. 3 IR spectra of BSA in gel or in free solution.

3. Application to analytical systems using CE or CEC

The analytes of our assay system are mainly small molecules and they can come in the silica networks and interact with the immobilized biomolecules. Two chiral compounds, bovine serum albumin (BSA) and ovomucoid (OVM) from chicken egg white, were encapsulated in tetramethoxysilane (TMOS)-based hydrogel and their chiral selectivity was evaluated for the separation of some selected enantiomers (tryptophan, benzoin, eperisone and chlorpheniramine). Two different proteins, BSA and OVM, were successfully encapsulated into the gel matrices by changing the alkoxysilane compositions of the gel and could separate the enantiomers (Fig. 4) [12]. Run-to-run repeatability was quite satisfactory. The consecutive analysis of the neutral compound, benzoin, by the OVM-encapsulated column showed good repeatability in the retention time (RSD=1.23% for the first peak, N=10). Under optimized conditions, the theoretical plate number for the first peak of benzoin reached 72,000 plates/m (Fig. 4).

We prepared a column containing encapsulated trypsin at the inlet of the capillary, and the column digested substrates (an amino acid derivative and peptides) and separated the substrates and products within a single capillary (Fig. 5) [17]. We also prepared a column with encapsulated microsomes containing drug-metabolizing enzymes [18, 19] . This column was used to produce metabolites and then separate them from the substrates. With this system, the sample size could be reduced by 3 orders of magnitude relative to

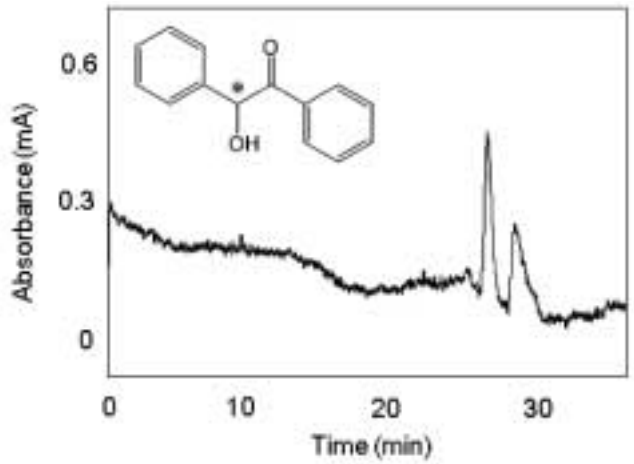

Fig. 4 Electrochromatogram of benzoin on OVM-encapsulated column.

Conditions: Sample: 0.1 mg/mL benzoin. Mobile phase: 20 mM phosphate buffer (pH 7.0)- 2-propanol (95:5, v/v). Applied voltage: 59 V/cm. Injection: 2 kV, 3 s. Column length: 34 cm. Detection: 254 nm.

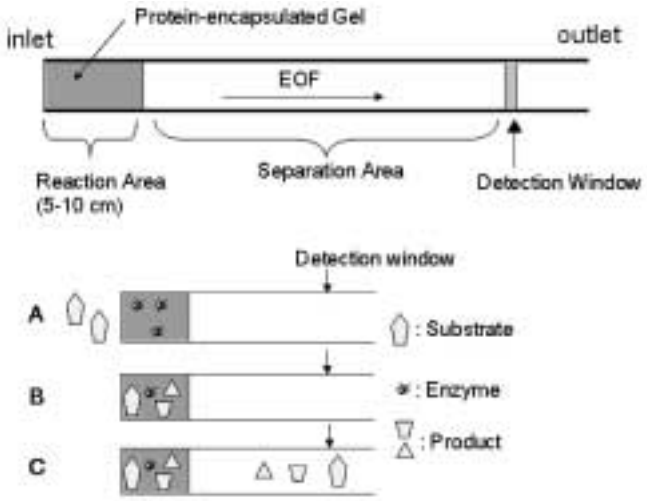

Fig. 5 The schematic illustration of an on-line enzyme reactor integrated into CE.

A. Substrates are introduced electrokinetically into the enzyme-encapsulated reactor.

B. Substrates are catalyzed into products while they flow through the enzyme-encapsulated gel by electrophoresis and EOF.

C. The products and unreacted substrates were separated at the separation section of the capillary by electrophoresis.

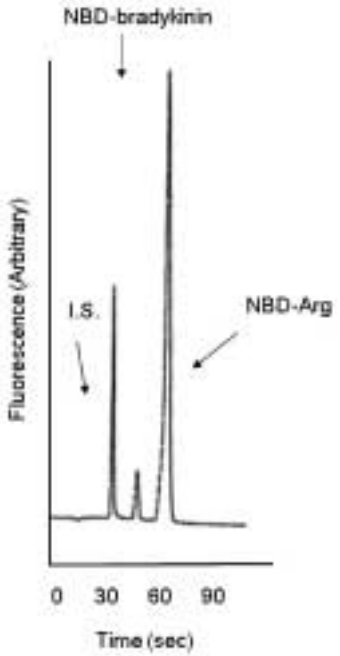

Fig. 6 Electropherogram of an on-chip tryptic reaction using NBD-bradykinin as a substrate.

Conditions: Sample: 0.1 mM I. S., 0.18 mM NBD-bradykinin; detection point, 16 mm; I. S. (internal standard), NBD-ethylenediamine. NBD-bradykinin was digested at the peptide bond of N-terminal Arg, and NBD−Arg was produced.

the same size required for conventional reaction schemes. This system could be also used as an electrophoretic enzyme-inhibitor assay just by injecting an inhibitor onto a column along with the substrate-coenzyme mixture [19]. Because the reaction, separation, and detection were integrated into a single system, the system could be automated, and thus analytical time and the amount of solvent required were reduced. Because CE and CEC allow high-speed analysis and efficient separation performance, these results are very encouraging; and monolithic capillary columns appear to open new options for the design and construction of high-throughput systems.

Integration of analytical and microfluidic systems has been used in many fields because the required sample volume is small, response is fast, highly parallel analyses can be carried out, and cross contamination is minimal [4, 5]. The technique is expected to be suitable for high-throughput screening. Not surprisingly, sol-gel technology was used to immobilize biomolecules on microfluidic chips. We used the previously mentioned trypsin-doped hydrogel for immobilization of trypsin on a microfluidic chip. Large molecules, such as peptides or proteins, were hydrolyzed, and substrate and product were separated on the microchip (Fig. 6) [20, 21].

4. The immobilization method of membrane proteins using silica nanoparticles

Although alkoxysilanes are widely used for the immobilization of biomolecules, alcohol are produced by hydrolysis of these alkoxysilanes. These alcohol byproducts will dissolve or destabilize encapsulated proteins or other biomolecules, such as biological lipid membranes. Therefore, another route using silica nanoparticles and aqueous sodium silicate as a starting material was developed to avoid the production of an alcohol [22]. In this route, silica nanoparticles behave as nucleation sites for condensation, and grow their particle diameter by condensation with sodium silicate. The grown silica nanoparticles form silica network and trap biomolecules. The microsomes containing expressed drug-metabolizing enzymes, P 450 s, and other electron transfer enzymes were immobilized on the microassay plate using sol-gel chemistry. A thin-film hydrogel containing microsomes was fabricated using aqueous silicate as a starting material. The transmission electron microscopy image clearly showed that the nanoclusters derived from the silica nanoparticles formed branched chains, and microsomes were entrapped in the silica network (Fig. 7). The different P 450 isozymes were immobilized on the microassay plate, and the metabolites by each isozyme were visualized as fluorescent images, which creates opportunity for the inhibitor assays. As shown in Fig. 8, the immobilized CYP 1 A 1 successfully metabolized ethoxyresorufin to resorufin, whose fluorescence was visualized. It was shown that the fluorescence intensity decreased as the concen-

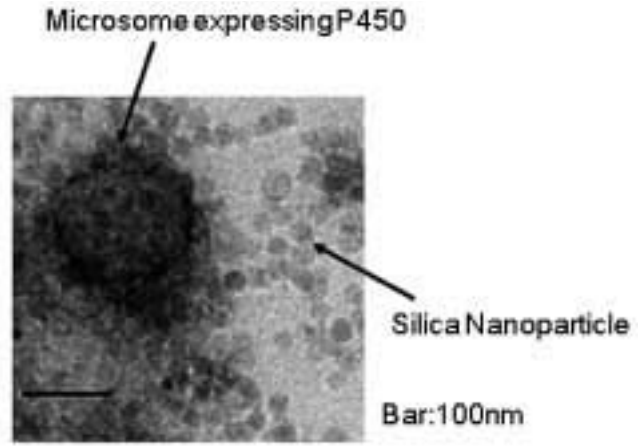

Fig. 7 Transmission electron microscopy image of microsomes immobilized in gels fabricated by using silica nanoparticles.

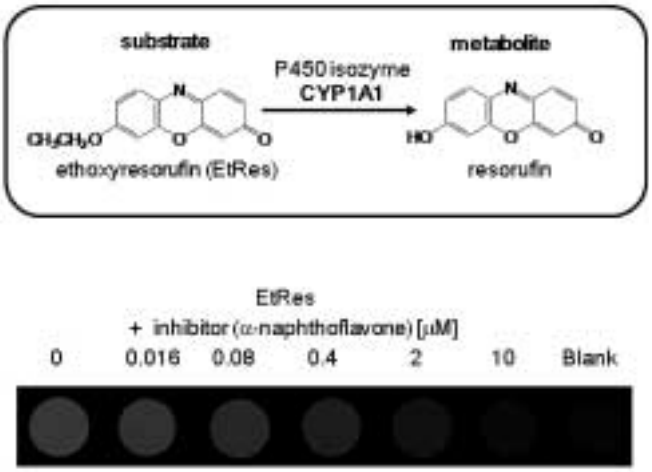

Fig. 8 Photograph of fluorescent metabolites on P 450 array containing CYP 1 A 1.

tration of α-naphthoflavone, an inhibitor of CYP 1 A 1, was increased [22]. Because this methodology enabled the development of assay system using P 450 that is unstable and involves other enzymes for its function, it can be applicable to various screening assays that require complicated reactions involving many biological components.

5. Conclusion

We created immobilization techniques of biomolecules, especially proteins, using hydrogels derived from sol-gel reaction of alkoxysilane or silica nanoparticles. The silicate formed networks around proteins, effectively entrapping the proteins. Because of the favorable properties of silicates (e.g., strength, transparency, permeability, and chemical inertness), the development of silica sol-gel processes has led to new applications in the field of analytical chemistry and materials science.

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References

[1] Hughes, B. *Nat. Rev. Drug Discov.*; **2009**, *8.* 181−181.

[2] Hopkins A. L.; Groom C. R. *Nat. Rev. Drug Discov.*; **2002**, *1.* 727−730.

[3] Sakai-Kato, K.; Kato, M.; Utsunomiya-Tate, N.; Toyo'oka, T. *Frontiers in Drug Design and Discovery*; **2006**, *2,* 273−294.

[4] Salimi-Moosavi, H.; Tang, T.; Harrison, D. J. *J. Am. Chem. Soc.*; **1997**, *119,* 8716−8717.

[5] Kopp, M. U.; de Mello, A. J.; Manz, A. *Science*; **1998**, *280,* 1046−1048.

[6] Ellerby, L. M.; Nishida, C. R.; Nishida, F.; Yamanaka, S. A.; Dunn, B.; Valentine, J. S.; Zink, J. I. *Science*; **1992**, *255,* 1113−1115.

[7] Edmiston, P. L.; Wambolt, C. L.; Smith, M. K.; Saavedra, S. S. *J. Colloid Interface Sci.*; **1994**, *163,* 395−406.

[8] Braun, S.; Rappoport, S.; Zusman, R.; Avnir, D.; Ottolenghi, M. *Mater. Lett.*; **1990**, *10,* 1−5.

[9] Yao, T.; Harada, I.; Nakahara, T. *Bunseki Kagaku*; **1995**, *44,* 927−932.

[10] Flora, K.; Brennan, J. D. *Anal. Chem.*; **1998**, *70,* 4505−4513.

[11] Cichna, M.; Knopp, D.; Niessner, R. *Anal. Chim. Acta.*; **1997**, *339,* 241−250.

[12] Kato, M.; Sakai-Kato, K.; Matsumoto, N.; Toyo'oka, T. *Anal. Chem.*; **2002**, *74,* 1915−1921.

[13] Sakai-Kato, K.; Kato, M.; Nakakuki, H.; Toyo'oka, T. *J. Pharm. Biomed. Anal.* **2003**, *31,* 299−309.

[14] Colón, L. A.; Guo, Y.; Fermier, A. M. *Anal. Chem.*; **1997**, *69,* 461 A−467 A.

[15] Grdadolnik, J.; Marechal, Y. *Biopolymers*; **2001**, *62,* 54−67.

[16] Giacomelli, C. E.; Bremer, M. G. E. G.; Norde, W. *J. Colloid Interface Sci.*; **1999**, *220,* 13−23.

[17] Sakai-Kato, K.; Kato, M.; Toyo'oka, T. *Anal. Chem.*; **2002**, *74,* 2943−2949.

[18] Sakai-Kato, K.; Kato, M.; Toyo'oka, T. *Anal. Biochem.*; **2002**, *308,* 278−284.

[19] Sakai-Kato, K.; Kato, M.; Toyo'oka, T. *J. Chromatogr. A.*; **2004**, *1051,* 261−266.

[20] Sakai-Kato, K.; Kato, M.; Toyo'oka, T. *Anal. Chem.*; **2003**, *75,* 388−393.

[21] Sakai-Kato, K.; Kato, M.; Ishihara, K.; Toyo'oka, T. *Lab Chip*; **2004**, *4,* 4−6.

[22] Sakai-Kato, K.; Kato,M.; Homma, H.; Toyo'oka, T.; Utsunomiya-Tate, N. *Anal. Chem.*; **2005**, *77,* 7080−7083.