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Channel-specific coatings on microfabricated chips

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Abstract

This paper reports channel-specific immobilization of fluorescein-5-isothiocyanate (FITC)-labeled bovine serum albumin and β-galactosidase on microchips with a central channel and two crossing channels; referred to as a double cross channel configuration. Solvent wells at the termini of all channels were used to store reagents. Coatings were applied in multiple steps using electroosmotically driven flow to deliver reagents to specific channels in the chip. The first step in all coating reactions was derivatization of the capillary walls with an organosilane having a reactive pendant functional group. As the silylating reagent was transported from the reagent storage well to a specific waste well, capillary walls in the route of transport were silylated. Flow was maintained throughout a reaction. The route of transport, and thus the specificity of channel coating, were controlled by the well to which negative potential was applied. Flow in a multichannel network takes the shortest route between the electrodes delivering the motive potential. The second reagent in the reaction was delivered from a different well and took a different path through the channel network, as did other reagents. Only the channel being coated was in the flow path of all the reagents used in the coating process. The zone of immobilization in the case of FITC-labeled albumin was determined with confocal fluorescence microscopy. Enzyme activity of immobilized β-galactosidase (β-Gal) was monitored by following the hydrolysis of fluorescein mono-β-D-galactopyranoside to fluorescein with laser-induced fluorescence.

Keywords: Chip technology; Immobilized proteins; Galactosidase; Albumin; Proteins

1. Introduction

It is clear from the scientific and commercial literature that the trend is toward integrating the components of analytical systems. The possibility that this is most easily achieved in microfabricated devices is one of the reasons for the interest in performing chemical reactions, separations, and detection on a single microchip [1,2]. Microfabricated systems have now been demonstrated for capillary electrophoresis [3,4], open channel electrochromatography [5], capillary gel electrophoresis [6], micellar electrokinetic capillary chromatography [7] and continuous bed liquid chromatography [8]. In addition to ease of fabrication and integration, miniaturized devices are often faster, cost less, are more portable, and consume less reagents than conventional instruments [9]. Potential areas of application might be in life science research, clinical analyses, high-throughput screening, biotechnology, quality control, and environmental monitoring.

Although analytical operations are being integrated in chip devices, it is desirable or even necessary in many cases that they are compartmentalized. Ideally this would be true of stationary organic phases as well. That has generally not been the case to date. Immobilized organic phases are generally found distributed throughout the channel

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network of the device. This could be problematic. Having stationary phase in the inlet channel of a liquid chromatography system would cause a separation of analytes to occur during loading unless the channel is exhaustively saturated. Similar types of problems could occur with immobilized enzymes.

The problem is how to coat a single channel in a network of channels on a chip. This paper addresses this problem by using electroosmotic flow to direct reagents to specific channels in a channel network. Immobilized proteins were chosen as models because the coating process required multiple steps and coated zones of protein are easily detected by confocal microscopy. The influence of the coating on the electroosmotic flow, directional control of the flow by voltages, and enzymatic assays with channel-specifically immobilized β-galactosidase are described.

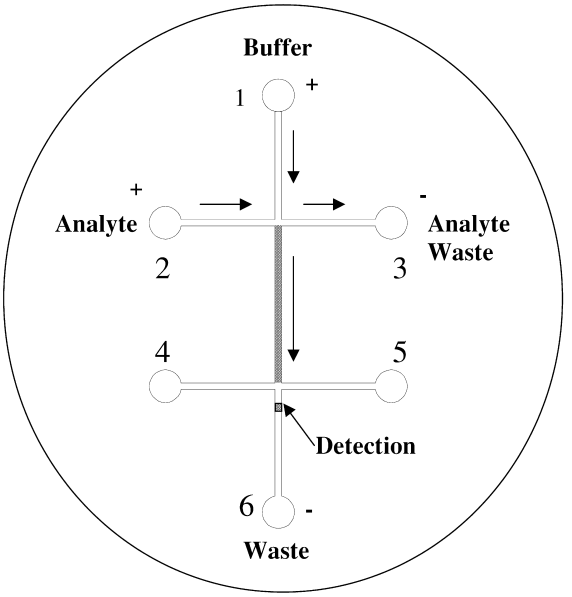

Fig. 1. Schematic of the chip used. The channels terminate at reservoirs containing the indicated solutions.

2. Experimental

2.1. Chemicals

γ-Aminopropyltriethoxysilane and glutaric dialdehyde were purchased from Aldrich (Milwaukee, WI, USA). Sodium cyanoboronhydride, β-galactosidase (β-Gal, ≠G6008, Grade VI, from *Escherichia coli*), fluorescein-5-isothiocyanate (FITC)-labeled bovine serum albumin (BSA), fluorescein mono-β-D-galactopyranoside (FMG), and sodium phosphate were obtained from Sigma (St. Louis, MO, USA). All the reagents and buffers were filtered through 0.2 μm filters to eliminate undissolved particles.

2.2. Microchips

Microchips were produced at the Stanford Nanofabrication Facility using 100 mm Borofloat borosilicate glass rounds of 0.7 mm thickness (Precision Glass and Optics, Santa Ana, CA, USA) coated with 1500 Å of amorphous silicon (AmSi) using a low-temperature CVD process (Strataglas, Mountain View, CA, USA). The wafers were then coated with one micron thick AZ3612 photoresist (Shipley Microelectronics), exposed on an Electronics Visions aligner (Electronics Visions Group, Phoenix, AZ, USA), covered with a mask of the design in Fig. 1, and developed with LDD26W developer (Shipley Microelectronics). The AmSi film in the exposed areas of the photoresist was removed using standard SF_6/Freon 115 chemistry in an MRC plasma etcher (Materials Research, Organgeburg, NY, USA). Thus defined, the channels were etched to a depth of 10±0.5 μm in 49% unbuffered microelectronic-grade hydrofluoric acid. The photoresist was removed in piranha cleaning solution (sulfuric acid–hydrogen peroxide, 70:30) and the remaining AmSi was removed using the same MRC plasma etching process. Because the ability of photoresist to protect and adhere to the bare glass in 49% HF is low, the AmSi film is used as a sacrificial etch-defining layer. Access holes were drilled (Cameron Microdrill Press, Treat Industries, Sonora, CA, USA) into the etched wafers using diamond bits 1 mm in diameter (Abrasive Technology, Westerville, OH, USA). The etched and drilled wafer was then thermally bonded to another Borofloat round at 625°C in a programmable vacuum furnace (Cole-Parmer Instruments, Vernon Hills, IL, USA). Pyrex glass tubing (1/4 in. I.D.×3/8 in. O.D.; 1 in.=2.54 cm) that had been cut into 1 in. lengths was bonded over the access holes with epoxy adhesive (Ciba, MI, USA) to form buffer

wells. Platinum wires were inserted into the wells to act as electrodes.

2.3. Chip treatment

Internal surfaces of the chip were activated with 1 *M* sodium hydroxide for 30 min, and then rinsed with water. The column was dried at 110°C overnight. A 10% (v/v) solution of γ-aminopropyltriethoxysilane (APS) in sodium-dried toluene with traces of methylene dichloride was loaded into all buffer wells with one outlet connected to a Barnant vacuum pump. A 20 mmHg vacuum was applied to fill channels with the silylation reagent (1 mmHg= 133.322 Pa). The chip was placed in a 90°C oven and baked for 2 h. Then the microchip was rinsed with toluene and (ACN)–water (1:1). Finally, the column was treated with 100 m*M*, pH 7.0 sodium phosphate buffer.

2.4. Microfluidic control

Liquid flow in channels was achieved by electroosmosis. The direction and velocity of flow in the channel system during each chemical reaction was controlled by applying an appropriate voltage to reservoirs at the terminus of each channel in such a way that the movement of liquid was directed to a single reservoir. A National Instruments (Austin, TX, USA) AT-A0-10 analog power output card was used to generate an analog voltage signal (0–10 V, 0–20 mA) for up to 10 channels. This analog voltage was then amplified 100-fold using a Gamma (Ormond Beach, FL, USA) PMT10-0.1 P/M power supplies to provide a voltage output of 0–10 000 V, and current output up to 100 μA for each channel. The power supplies were individually controlled by a personal computer running the Labview 5.0 software.

2.5. Channel-specific protein immobilization

Five percent (v/v) glutaric dialdehyde in 100 m*M* sodium phosphate, pH 7.0 buffer was loaded into the analyte well (2). Voltages were applied as shown in Fig. 3 to achieve the zig-zag flow pattern, $V(1) = 80$ V, $V(2) = 0$ V (ground), $V(3) = 250$ V, $V(4) = 200$ V, $V(5) = 500$ V, $V(6) = 350$ V. Trace amounts of 10^{-4} *M* fluorescein solution were added to well 2 to indicate the direction of flow. After a 2 h reaction, excess reagent was rinsed out of channels with 100 m*M* sodium phosphate, 1 m*M* $MgCl_2$, pH 7.5 buffer. Then 2 mg/ml of FITC-labeled BSA in the above buffer or β-galactosidase was added to well 3. Voltages in all the reservoirs were manipulated as in Fig. 4 to follow the opposite zig-zag flow pattern, $V(1) = 80$ V, $V(2) = 250$ V, $V(3) =$ ground, $V(4) = 500$ V, $V(5) = 200$ V, $V(6) = 350$ V. The reaction went on for 20 h at room temperature. The chip was then rinsed with cleaning buffer. By this means, only the middle separation channel was treated with all the chemical reagents and had the designated coating of proteins.

2.6. Confocal fluorescence microscopy

A Nikon Inverted Eclipse TE-300 optical microscope equipped with a TE-FM epi-fluorescence attachment was used for fluorescence imaging. A Nikon EF-4 B-2E/C FITC filter block equipped with a 465–495 nm excitation filter, a dichroic mirror of 505 nm, and a barrier filter of 515–545 nm was used to select an excitation beam of roughly 488 nm while blocking it at the detection window. A DVC-1310C digital color charge-coupled device (CCD) camera was attached to the side port of the microscope. Real time images were sent to a Windows NT workstation through the PIXCI image interface card (Vision 1, Bozeman, MT, USA). XCAP software (EPIX, Buffalo Grove, IL, USA) was used to carry out the data analysis.

2.7. Laser induced fluorescence detection

All enzymatic assays were monitored by a laser-induced fluorescence detection system. An air-cooled Uniphase (San Jose, CA, USA) 2011-10SL argon-ion laser with 4 mW output at 488 nm was used as the laser excitation source. Neutral Density Filters (Melles Griot, Carlsbad, CA, USA) were used to adjust the intensity of the excitation light. Plasma lines were removed with a dichroic mirror (Newport, Irvine, CA, USA). The laser was focused onto a single point on the chip after the separation channel. Fluorescence was collected with a 10× Melles Griot achromatic microscope objective. The holding device was connected to a Newport 460 XYZ positioner,

which provided sub-micrometer positioning precision for easy focusing of the laser beam. Two optical filters were used to eliminate the scattered laser beam. A Schott (Duryea, NY, USA) bandpass filter (0G515) reduced the passage of less than 490 nm light while a HSNF-488-1.5 Kaiser (Ann Arbor, MI, USA) super holographic notch filter selectively reduced the transmission of light at 488±5 nm. A side-on Hamamatsu (Bridgewater, NJ, USA) photomultiplier (PMT; #R928) was used to collect fluorescence. The multiplier had high sensitivity and broad spectral response from the ultraviolet to near-infrared region (185–930 nm, 400 nm peak); it also has high quantum efficiency (5–10% in the visible region), low dark current (3.0 nA after 30 min), and high linear current (0.1 mA). A Keithley 485 autorange picoammeter (2 nA to 2 mA) was used to collect signals from the PMT which were converted into voltages by employing an in-house *I* to *V* converter. A National Instrument AT-GPIB/TNT (plug and play ISA) was used to interface to the computer for data acquisition and storage.

3. Results and discussion

Current chip fabrication protocols generally do not allow or will destroy organic coatings when the two halves of the system are bonded. This means that organic coatings must be applied to the fully fabricated channel system, commonly by flowing reactants into the chip. The problem with this approach is that reagents are dispersed throughout the channel system and all channels are coated. This is undesirable in the case of chromatography systems and some types of immobilized enzyme reactors.

3.1. A channel-specific coating strategy

The objective in this work was to develop strategies for the application of a specific organic coating to the main separation channel (Fig. 1) of a ''double cross'' geometry chip. This was achieved by exploiting the fact that the channel walls of a glass chip are rich in silanol groups that cause extensive electroosmosis when an electric field is applied across water-filled (and acetonitrile-filled) capillaries, as in the case of fused-silica capillaries. Electroosmosis in capillary electrophoresis causes bulk electrolyte flow, known as electroosmotic flow (EOF), as a result of the formation of a counter-ion layer at the charged capillary surface [10]. The fact that EOF occurs along the shortest path between electrodes was used in these studies to direct the transport of reactants to specific channels within channel networks for the purpose of modifying channel walls.

Because silanol groups are necessary for EOF and they can be sequestered or eliminated by surface modification, there is the issue of maintaining electroosmosis during the coating process. It is well known that, at neutral pH or slightly higher, the number of silanol groups at the surface of fused-silica capillaries is so large that only a portion are eliminated or sequestered during most surface modification procedures. This means that the net zeta potential at modified surfaces will still be negative after coating and EOF will continue from the anode to cathode, albeit at a slower rate. Only in the case of amine-containing coatings, such as γ-aminopropyl silane, cationic polymers, and enzymes of high isoelectric point, is it possible for all the surface silanols to be sequestered. In fact, the number of amine groups in the coating can exceed the number of surface silanols and electroosmosis will be reversed.

Coating processes can require multiple steps. In these cases, reagents for each of the steps were brought into the coating zone from different wells on the chip in such a manner that only the channel being coated was exposed sequentially to all the reactants.

3.2. Protein immobilization

The reaction scheme for protein immobilization is outlined in Fig. 2. After silylation of all the channel walls in the chip with γ-aminopropyl silane (step 1 in Fig. 2), well 2 in the chip was loaded with glutaric dialdehyde and this reagent was caused to flow continuously to well 5 by electroosmosis. Channel walls were dynamically derivatized with dialdehyde by Schiff base formation along this path during the course of 2 h. Application of a positive potential at wells 1, 3, 4, and 6 of 80, 250, 200 and 350 V, respectively, prevented this reagent from entering and reacting in the channels leading from cross 1 to

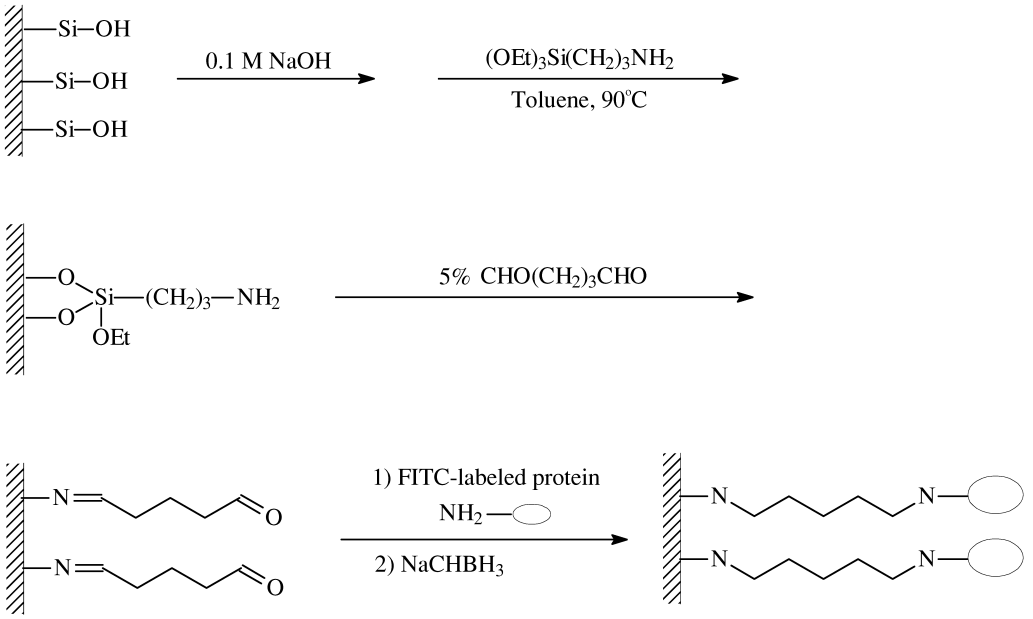

Fig. 2. Procedures for step-wise protein immobilization.

wells 1 and 3, and cross 2 to wells 4 and 6, as shown in Fig. 3.

In step 3, the fluorescein-labeled protein was introduced from well 3 to 4, as in Fig. 4b, with the following voltages applied to the wells: $V(1) = 80$ V, $V(2) = 250$ V, $V(3) =$ ground, $V(4) = 500$ V, $V(5) = 200$ V, $V(6) = 350$ V. The biggest voltage gradient between wells 3 and 4 transports FITC-BSA in the path directly between wells 3 and 4, driving in the direction from cross 1 to cross 2. As a result, the main separation channel is treated with all the derivatizing reagents and had channel-specific immobilization of the protein. Immobilization of the protein also made little contribution to EOF in the channel, since the number of charged groups in the protein seems to be much smaller than the number in the surface coating.

Using multiple chemical modification steps with controlled flow, FITC-labeled BSA was immobilized in the main channel of the chip between the two crosses. It is clear from Fig. 5 that only the specific channel is coated with the fluorescein-labeled protein. There is, however, some non-specific absorption of small fluorescent particles in the other channels. Also, at the beginning of the junction, a section of about 150 μm in length was not completely covered by the fluorescent-labeled protein. This is thought to be due to the fact that when the reagents used in the second and third steps of modification entered the channel they had not completely diffused across the width of the channel and the opposite side of the channel was not completely derivatized. This can probably be avoided by further adjusting the voltages applied to the side channel.

3.3. Control of the flow direction

Manipulation of the voltage at each reservoir allows the flow of reagents to be controlled in the desired direction. CCD images shown in Figs. 3 and 4 are examples of such control. In Fig. 3, reagent is flowing from well 2 to well 5, while pinched at the two intersections by the opposing voltages. Opposing voltage is applied to prevent reagent bleed. As shown in Fig. 6, if a side channel is left floating while potential is applied to cause flow of 10^{-4} *M* fluorescein into the main channel, there will be leakage of solution into the side channel. Reagent bleed into channels is related to both diffusion and convective flow. Experiments showed that the higher the applied potential, i.e. the higher the flow-rate, the more serious the reagent bleed problem can be.

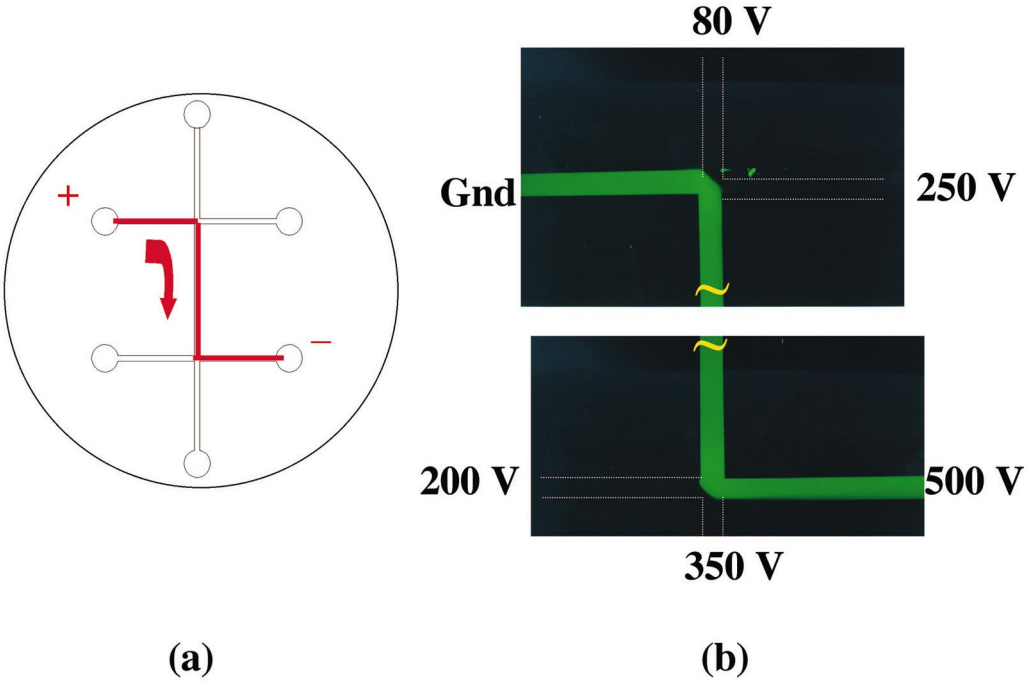

Fig. 3. (a) The flow direction of the second step chemical modification. The arrow indicates the direction of electroosmotic flow. (b) CCD image of the electrokinetic flow of 5% glutaric dialdehyde in 100 m*M*, pH 7.0 sodium phosphate buffer into the main channel.

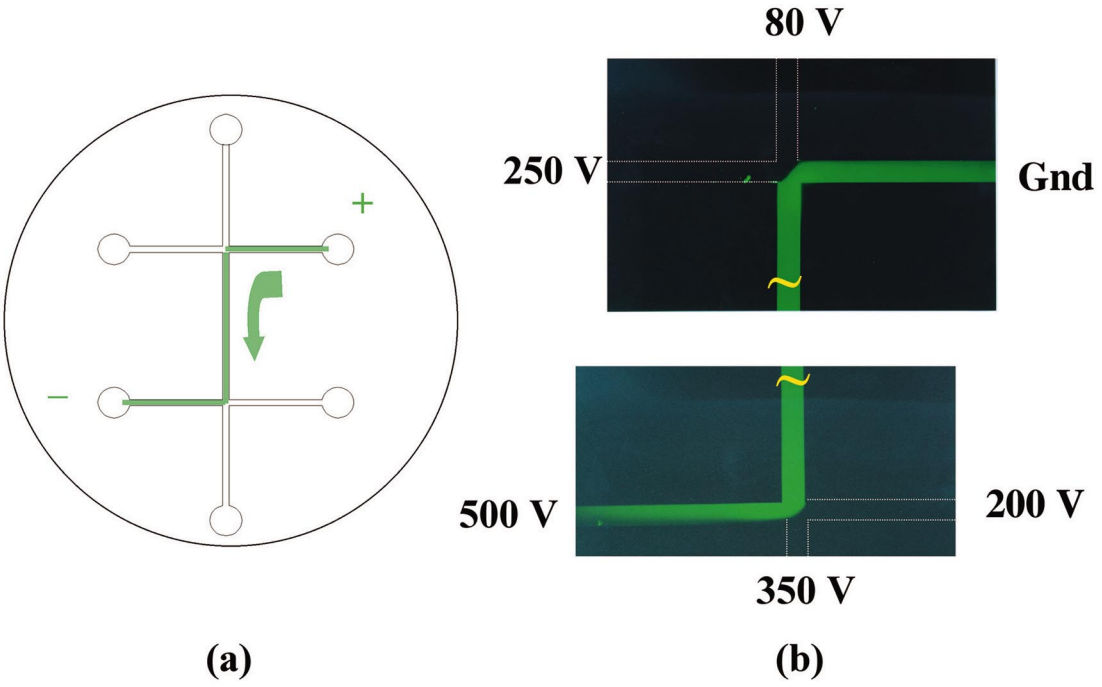

Fig. 4. (a) Flow direction of the third step chemical modification. (b) CCD image of the electrokinetic flow of 2 mg/mL FITC-BSA in 100 m*M* sodium phosphate buffer, pH 7.5, 1 m*M* $MgCl_2$ inside the channel network.

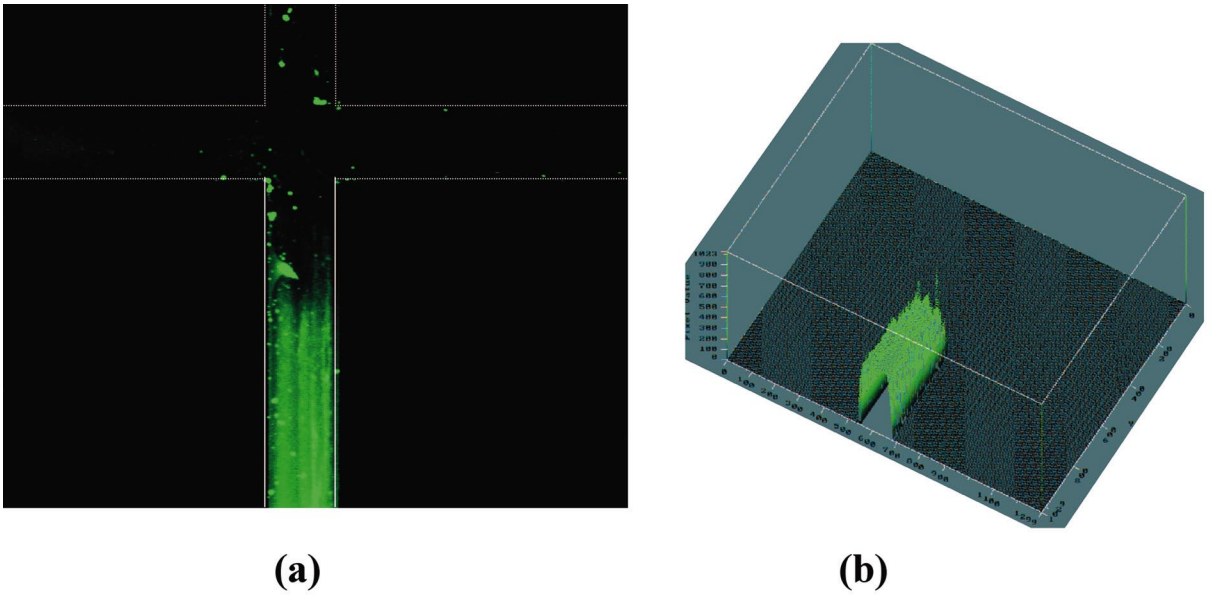

Fig. 5. (a) CCD image of the four-way reaction cross with channel-specific immobilization of fluorescein-labeled protein; only the main channel was coated with FITC-BSA, and not the other channels. (b) Three-dimensional image of the channel cross.

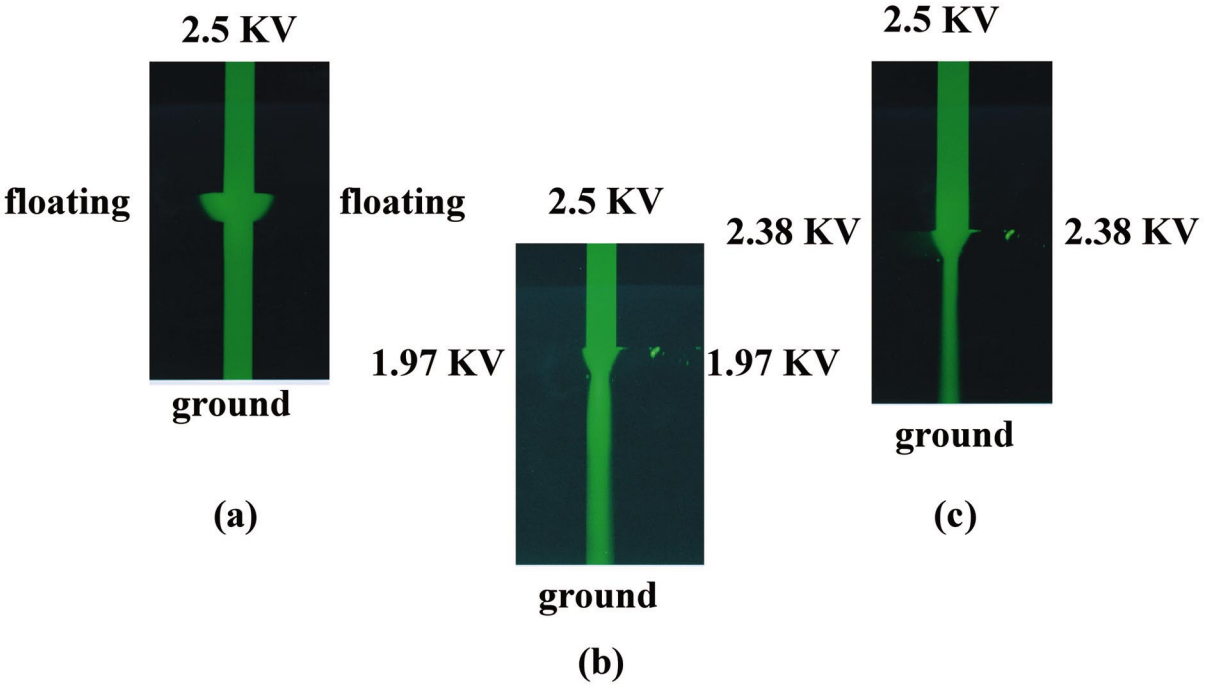

Fig. 6. (a) Reagent bleeding occurred when the side channels were left floating. 2.5 kV was applied to force the 10^{-4} *M* fluorescein solution to flow in the main channel. (b) Opposing voltages of 1.97 kV were applied to the side channels to prevent reagent leakage. (c) Opposing voltages of 2.38 kV were applied to the side channels.

Usually, the concentration of sample leaking into side channels was about 3% of that in the sample channel [11]. These effects can be avoided by applying voltages to several channels simultaneously. In Fig. 6b, 1.97 kV was applied to the side channel. Leakage stopped and the sample stream was focused. With an even higher applied opposing voltage, 2.38 kV (Fig. 6c), sample focusing is more significant.

3.4. Channel-specific enzymatic reactor

Using the same method, β-galactosidase from *E. coli* was covalently immobilized in the main channel. Channel-specific hydrolysis of 10^{-4} *M* fluorescein mono-β-D-galactopyranoside (FMG) was achieved by electrokinetically pumping this substrate into the main channel, where the enzyme is located. Enzymatic cleavage of FMG by β-Gal released fluorescein according to Fig. 7. Fluorescence intensity increased during passage through the zone of immobilized β-galactosidase as seen be observing fluorescence intensity at various points in the channel (Fig. 8a and b). The three-dimensional image in Fig. 8c shows the increase in fluorescence intensity beyond the intersection along the *Z*-axis.

A flow-through enzyme assay, monitored with single-point laser-induced fluorescence at the end of the immobilized enzyme zone, was used to observe the product of the on-chip reaction between β-Gal and FMG. Enzymatic assays on chips have been previously accomplished [12] by continuously mixing enzymes in solution and substrates at channel junctions on chips. In this experiment, the enzyme β-Gal was specifically immobilized on the surface of the chip channel. Immobilization of the enzyme can both stabilize the enzyme and allow reuse. A sample plug of the substrate FMG was electrokinetically injected into the enzyme column. Voltages applied are programmable through a voltage control program developed by VI engineering (Indianapolis, IN, USA). First, electroosmotic flow was directed from analyte well 2 to analyte waste well 3, as in Fig. 9a. When the channel connecting these two wells was uniformly fluorescent, the electroosmotic flow was redirected from well 1 to well 6. The substrate at the intersection of the cross was thereby injected into the column, as in Fig. 9b. The typical injection volume is approximately 2 nL. The results are shown in Fig. 10. The peak is relatively broad. This is thought to be due to the fact that the substrate (FMG) and product (fluorescein) differ in electrophoretic mobility. As they migrate along the column at different

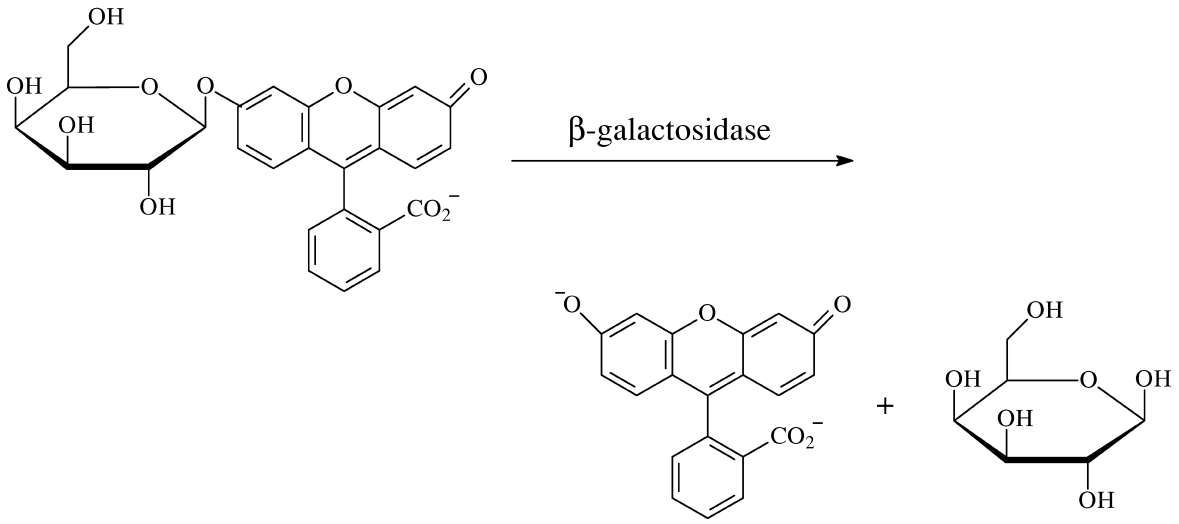

Fig. 7. Enzymatic cleavage of FMG by β-galactosidase releases fluorescein.

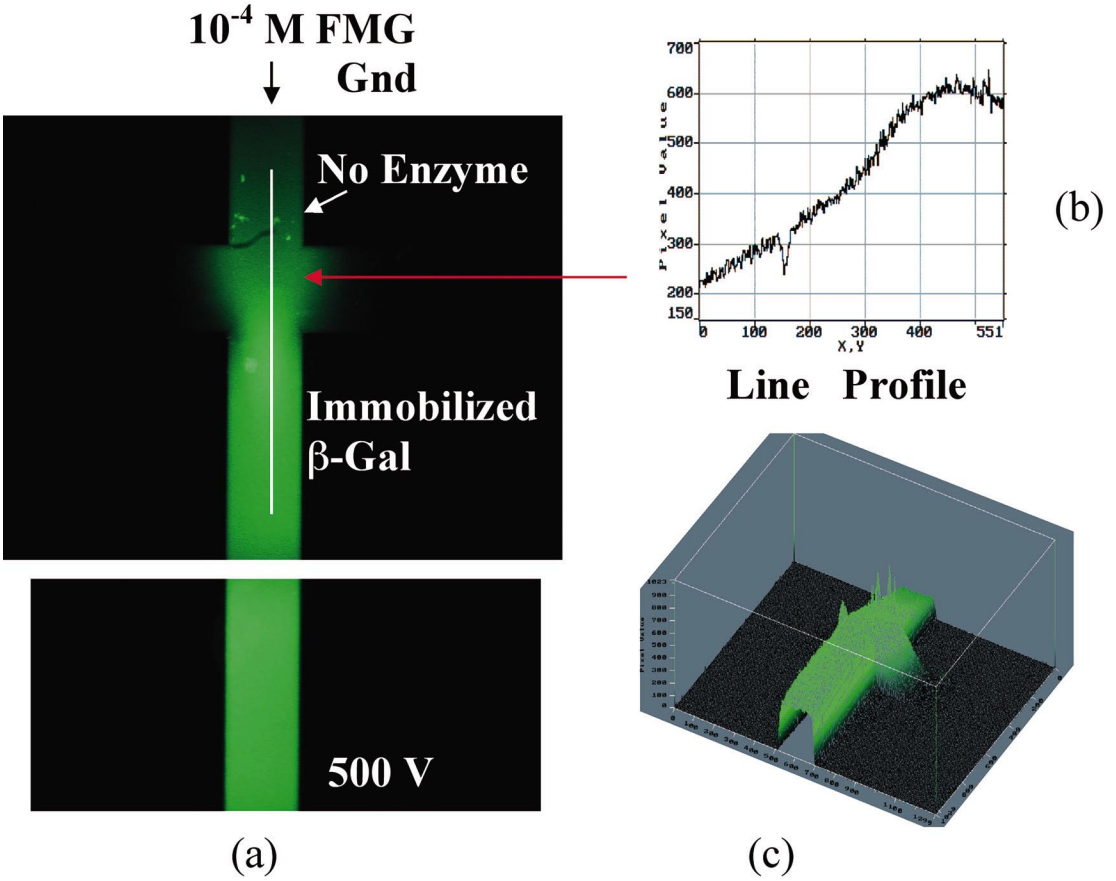

Fig. 8. (a) CCD image of the cross with channel-specific immobilization of β-galactosidase. The arrow indicates the flow direction of the injected substrate, 10^{-5} *M* FMG. (b) Quantitative line profile of the line in (a) drawn downstream of the main channel. (c) Three-dimensional image of the channel cross with the enzymatic reaction inside.

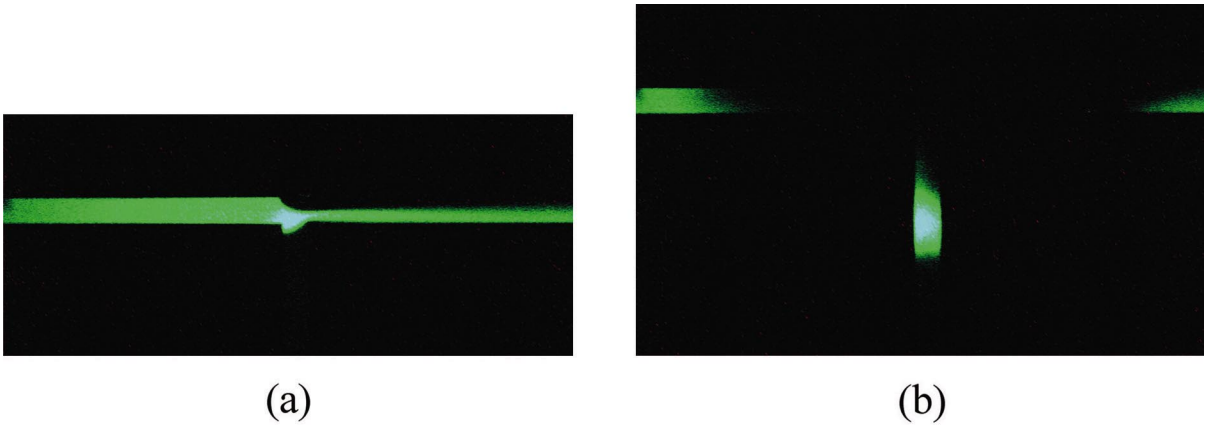

Fig. 9. (a) Immediately before the injection, 10^{-5} *M* of FMG was flowing from well 2 to well 3. (b) Injection of a FMG sample plug; the voltage field applied between well 1 and the waste was 700 V, 140 V/cm.

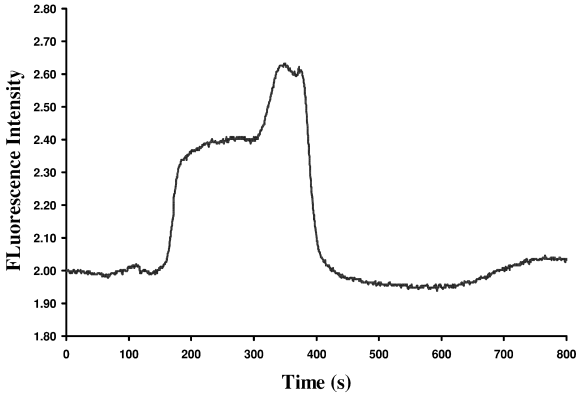

Fig. 10. Laser-induced fluorescence monitoring of product formed at the second cross. Conditions: 10^{-5} *M* FMG in 0.1 *M* sodium phosphate buffer, pH 7.5, 1 m*M* $MgCl_2$, electric field 140 V/cm.

velocities, the product plug keeps moving away from the substrate plug throughout the migration process and causes the product peak to be broad.

To avoid this problem, the substrate plug was incubated in the enzyme column for some time by switching the voltage off (Fig. 11a). After a 120 s injection and 100 s of migration, the operating potential was dropped to zero and FMG allowed to incubate in the enzyme channel for 100 s. Voltage was then applied again to elute the sample. Two discrete peaks were observed separated to the baseline. Incubation times of 200, 300, and 500 s were used in subsequent experiments (Fig. 11b–d). The intensity of peak 1 kept decreasing, while that of peak 2 kept increasing. It is quite obvious that peak 1 is the substrate FMG, and peak 2 is the fluorescein

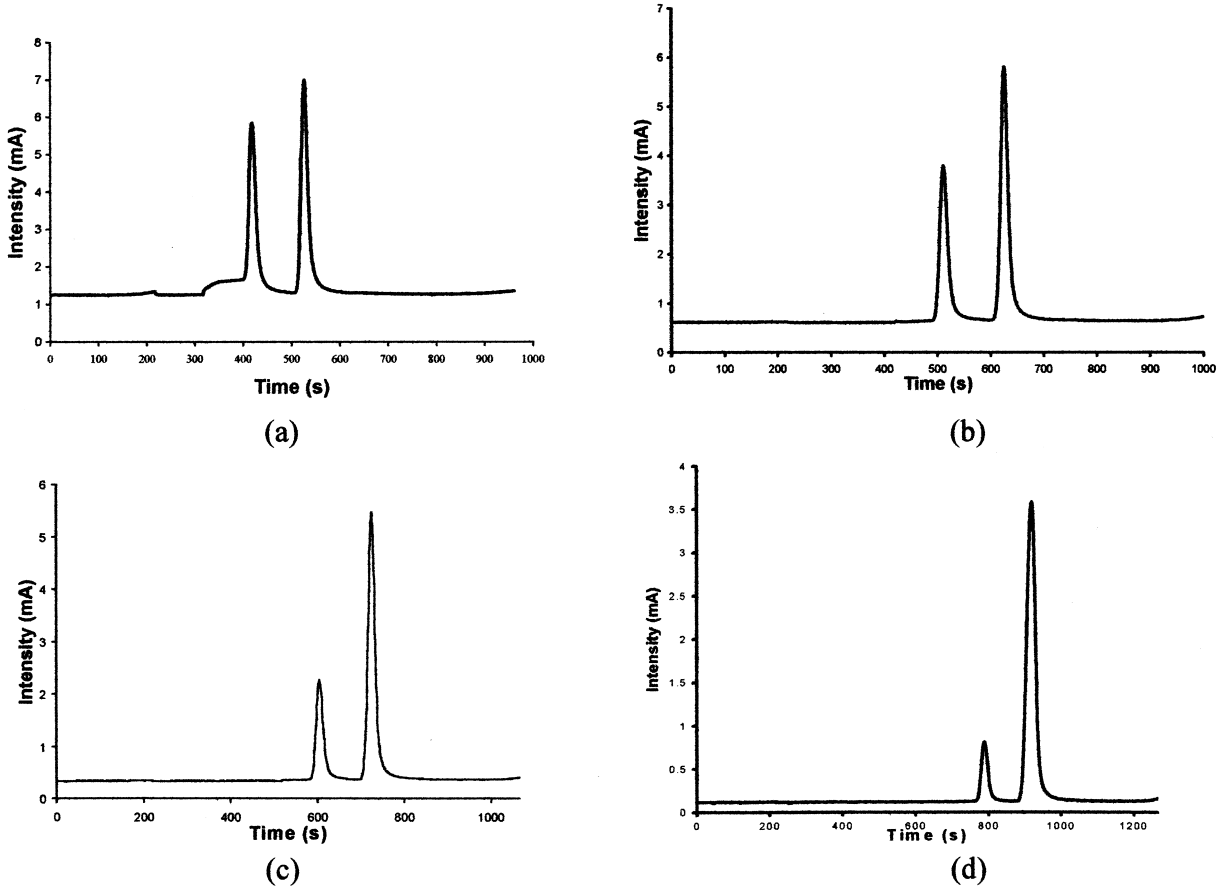

Fig. 11. Time course of the β-Gal and FMG reaction with various incubation times (a) 100 s, (b) 200 s, (c) 300 s, (d) 500 s. The injection was carried out at 120 s, the sample plug was allowed to migrate for 100 s, and then the incubation was started. The same eluting conditions were used as in Fig. 10.

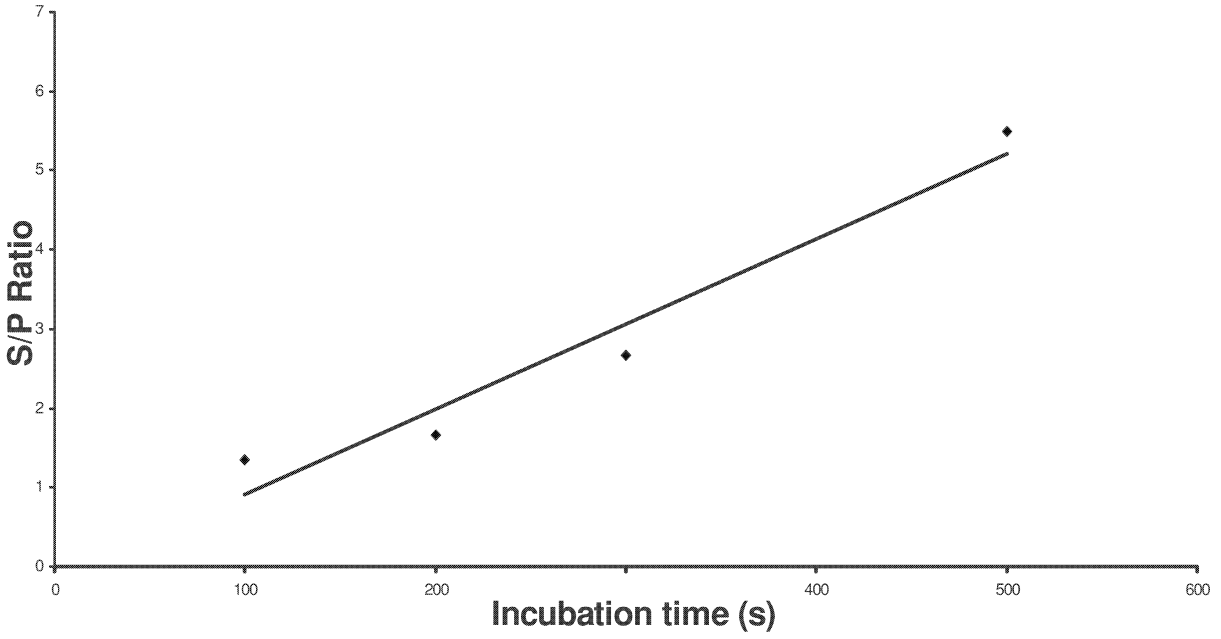

Fig. 12. Substrate-to-product conversion vs. incubation time.

product. The plot of the substrate-to-product conversion ratio is proportional to the incubation time (Fig. 12). The migration times of the two species are reproducible, as shown in Table 1. For a separation length of 2 cm, t_{fmg}(avg.) = 312 s, $t_{fluorescein}$(avg.) = 426 s, and the difference in electrophoretic mobility is 175 μm/s.

3.5. Other systems

Although not examined, it is likely that the reactions described above can be extended to many other coating procedures. The process described for bovine serum albumin and β-galactosidase immobilization is not unique. Both the reactions and reagents used are found in a wide variety of coating procedures ranging from application of stationary phases in liquid chromatography and capillary electrophoresis to the immobilization of a wide variety of proteins. Reactions times of up to a day, aqueous solvents, and reactions in acetonitrile can be accommodated.

Table 1
Elution time and substrate conversion

Incubation time (s)	S/P	Elution time (s)	
		Peak 1	Peak 2
100	1:1.35	319	426
200	1:1.66	312	425
300	1:2.67	302	426
500	1:5.49	293	422

4. Conclusions

Electroosmosis can be used to precisely control reagent transport through interconnected channel networks in terms of both direction and the channels involved. It has been demonstrated for the first time that this process can be used to achieve channel-specific immobilization of proteins on microchips. The use of immobilized enzymes on chips to execute enzyme assays has the advantage that system may be used for a long period of time without replacing the enzyme. In the specific case of β-galactosidase, a post-incubation separation of the fluorescein product from residual fluorescein mono-β-D-galactopyranoside substrate was demonstrated to enhance detection sensitivity.

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