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Lab-on-a-Chip for Monitoring the Quality of Raw Milk

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Abstract A lab-on-a-chip (LoC) was designed for simultaneous monitoring of microorganisms, antibiotic residues, somatic cells, and pH in raw milk. The LoC was fabricated from polydimethylsiloxane (PDMS) using microelectromechanical system (MEMS) technology, which consisted of two parts; a protein array and microchannel. The protein array was fabricated by immobilizing five types of antibodies corresponding to two microorganisms, two antibiotic residues, and somatic cells. A sol-gel film was deposited on a glass substrate to immobilize the antibodies. The target analytes in raw milk could be bound with the corresponding antibody by an immunoreaction, and the antigen-antibody complex was detected using fluorescence microscopy. SNARF-dextran was used as a pH indicator, and the SNARF-entrapped hydrogel was attached to the microchannel in the chip. After injecting the milk sample into the channel, the pH was measured by monitoring the change in fluorescence intensity by fluorescence microscopy. The on-chip simultaneous assay of two microorganisms (*E. coli* O157:H7 and *Streptococcus agalactiae*), two antibiotic residues (penicillin G and dihydrostreptomycin), and neutrophils was successfully accomplished using the proposed LoC system.

Key words: Lab-on-a-chip, raw milk, microorganism, antibiotics, somatic cell, pH, protein array

Lab-on-a-chip (LoC) technology was developed to integrate various analytical processes, including pretreatment, mixing, reaction, and separation. This technology can also be scaled down from conventional glassware to microfluidics, involving micron-sized channels in glass or a polymer chip [2, 6, 16, 18]. In recent years, the miniaturized analysis system has attracted considerable interest in chemical and biological applications [8, 29]. LoC technology offers several distinctive advantages over macroscopic systems: It can reduce the level of sample and reagent consumption, and provide fast analysis, high throughput, system integrating, and automation. Microfluidic devices are often described as miniature versions of their macro-scale counterparts. Although this analogy is true for some aspects of microfluidic devices, many phenomena cannot be scaled down linearly from large to small implementations because of the increased surface area-to-volume ratio and the omnipresence of laminar flow. Currently, several commercial microfluidic-based products have been reported [8]. In particular, several companies have developed microfluidic devices that work for highly predictable and homogeneous samples in the drug discovery process, compound screening, genomic analysis, and proteomics [4, 29].

Protein chips are an attractive method for measuring a wide range of proteins that are not possible using traditional DNA chips [4, 17, 24]. The use of proteins in microarray format is relatively new, and its application has increased. The most common use of protein microarrays is the immunoassay, in which arrays of different capture antibodies are immobilized and exposed to a biological sample. The analytical proteins bound to the immobilized capture antibodies are detected by fluorescence, luminescence, surface plasmon resonance (SPR), *etc.* [20–22, 27]. The potential application of protein chips has expanded to the study of the mechanism of drug action, environmental monitoring, as well as in the food industry for the detection of small quantities of substances, such as microorganisms, specific chemicals, and toxic compounds [5].

Milk and dairy products are an essential food in many countries. Therefore, quality control of raw milk is becoming increasingly important. Various analytical methods have been used to detect microorganisms, toxic substances, and somatic cells in raw milk [7, 15, 26, 27]. In recent years,

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methods for measuring toxic materials (antibiotics) at extremely low levels have been reported [7, 23, 27]. Separation techniques such as GC, HPLC, and capillary zone electrophoresis, and detection methods such as UV-vis, diode-array, fluorescence, and MS have been developed to detect substances at extremely low levels [1, 10, 11]. However, these methods have some disadvantages. These include high capital cost and long analysis time. Therefore, the immunoassay, receptor assays, and enzyme-linked immunosorbent assays have been developed to overcome these shortcomings [14, 19, 25, 28]. These methods cannot overcome the requirement of professional skill, the restriction of analytes, and the difficulties associated with field testing. Recently, immunobiosensors based on SPR have been developed to detect aminoglycosides and penicillin [3, 9, 12, 13], but simultaneous multidetection using this method is quite difficult. Therefore, protein chip technology has emerged as an attractive alternative for overcoming these disadvantages.

The aim of this study was to fabricate an integrated analytical system for the detection of the multicomponents in raw milk using the protein chip technology. The immunochemical analysis method was developed to simultaneously detect multicomponents using monoclonal antibodies (Mabs) against *E. coli* O157:H7, *Streptococcus agalactiae*, dihydrostreptomycin, penicillin G, and neutrophils. The monitoring of microbials, antibiotic residues, somatic cells, and pH is essential for the quality control of milk and dairy products. However, this analysis is time-consuming, often has a low sensitivity, and is difficult to use on the farm. The protein microarray can provide simultaneous analysis and is easy and rapid in comparison with traditional analysis methods. *E. coli* O157:H7 and *S. agalactiae* are representative microorganisms that are found in food poisoning and mastitis, and penicillin G and dihydrostreptomycin are representative antibiotic residues in raw milk among approximately 30 antibiotic species. Finally, neutrophils are leukocytes included in somatic cells. If the cow contracts mastitis, the number of leukocytes (*i.e.*, somatic cells) will increase. Therefore, an analysis of neutrophils will be an effective method of monitoring the progress of a cow's mastitis.

MATERIALS AND METHODS

Materials

Tetraethoxysilane (TEOS, M.W. 152.22, Tokyo Kasei Kogyo Co., Ltd., Tokyo, Japan) was used as a starting material to form the sol-gel film. The mercaptopropyl triethoxysilane (MPTS, M.W. 238.42) and bovine serum albumin (BSA) were purchased from Sigma Aldrich Co. (St. Louis, MO, U.S.A.). The glass slide (75 mm×25 mm) was purchased from Paul Marienfeld GmbH & Co. (Lauda-Koenigshofen, Germany). The monoclonal antibodies (Mabs) against *E. coli* O157:H7, *S. agalactiae,* and neutrophil were obtained from Biogenesis Ltd. (Poole, U.K.). The Mabs against dihydrostreptomycin and penicillin G were purchased from Biodesign International (Saco, ME, U.S.A.). The positive photoresist (AZP4620) and developer (AZ400K) solutions were obtained from Clariant Co. (Somerville, NJ, U.S.A.). Polydimethylsiloxane (PDMS) microfluidic devices were fabricated by curing the pre-polymer components of Sylgard 184 (Dow Corning, Midland, MI, U.S.A.). The dextran-seminaphtharhodafluore-1 (SNARF-1) was purchased from Molecular Probes (Eugene, OR, U.S.A.), and polyethylene glycol (PEG400) was from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Preparation of Sol-Gel Film

The sol-gel solution was prepared by mixing TEOS and MPTS in ethanol at 120 rpm and 25°C for 30 min. The alkoxy-group (-OR) was hydrolyzed by adding water and hydrochloric acid (HCl) to this solution. The solution was agitated continuously for 1 h to form the siloxane (Si-O-Si) structure. The sol-gel film on the glass slide was prepared by dipping the dry slides into the prepared sol-gel solution.

Fabrication of Protein Array

Before the immobilization process, 10% glycerol was added to the Mab solution to prevent dehydration of Mabs during the adsorption process. Various antibodies (Mabs against *E. coli* O157:H7, *S. agalactiae*, dihydrostreptomycin, penicillin G, and neutrophil) were spotted onto the sol-gel film surface. The protein array was constructed on the micro-scale using a microarrayer (Micro Pipetting System Nano-Plotter™, GeSIM, Germany). The amount per spot was 0.4 nl, and the size was 100 μm in diameter. The spotted substrate was incubated in a humid chamber at 4°C for at least 24 h. After incubation, a protein array was immersed in a PBS (pH 7.4) buffer solution containing 3% BSA as a blocking material. After 12 h, the protein array was washed with a PBS buffer solution for 20–30 min.

Fabrication of Lab-on-a-Chip

High-resolution transparencies containing the design of the channels and weirs were created using a computer-aided drawing package and patterned onto the slide glass to produce the photomasks. The positive photoresist compound was spin-coated onto a glass substrate at 15,000 rpm for 1 min, followed by baking at 92°C for 5 min. The photoresist-coated glass substrate was exposed to UV light for 3 min and developed in a developer solution to create the master. For polymer molding on the patterned glass master, a 10:1 mixture of PDMS pre-polymer and the curing agent was mixed thoroughly and degassed under vacuum. The polymer mixture was then poured onto the

master and cured for 1 h at 65°C. After curing, the PDMS replica was peeled from the master. The slide glass was cleaned and rinsed with a mixture of deionized water and ethanol. The PDMS and slide glass were placed in a low-energy plasma cleaner (Tesla Coil) and oxidized at medium power for 1 min. After removing the substrates from the plasma cleaner, they were immediately brought into conformal contact, and an irreversible seal was spontaneously formed.

Synthesis of Hydrogel

The hydrogel precursor solutions were prepared by mixing PEG, MPTS (20%), TEOS (H_2O:Si=4:1), and 10 mM SNARF-1 solution with a different volumetric ratio. A syringe pump was used to deliver the precursor solution and all subsequent fluids. After removing the PDMS mold first, the glass slide and a second PDMS mold with a channel wider than the micropatches were exposed to oxygen plasma. Upon contact, the two pieces formed an irreversible bond.

Analytical Methods

A secondary antibody labeled with a fluorescent dye (FITC) was used to analyze the protein chip. The fluorescence produced by the FITC-labeled secondary antibody was observed using fluorescence microscopy (Leica DML, Leica Microsystems AG, Wetzlar, Germany) under ambient conditions.

RESULTS AND DISCUSSION

Protein Immobilization on the Sol-Gel Film

Inorganic silica-based gels were used as substrates for creating the chemically patterned surfaces. The gels were produced by the hydrolysis of TEOS. The physical interactions between the proteins and synthetic surfaces were increased by adding the silane agents (MPTS). The optimized ratio of alkoxysilane:alcohol:water:catalyst of the sol-gel solution was 1:7:11:0.07 for a pore size of 5–100 nm. The pores increase the surface area and the amount of proteins (antibodies) immobilized on a substrate. Antibodies can be immobilized by the following mechanisms: entrapment in pores, sticking on pores, and adsorption on the surface of the pores. Using the physical coherence between the proteins and sol-gel film, a protein array can be made more durable and stable.

The protein array consisting of the FITC-labeled anti-*E. coli* O157:H7 antibody on a sol-gel surface was fabricated using a microarrayer. The spots were arrayed by delivering 0.38 nl of an antibody solution to an Au surface. After incubation for 6 h at 4°C, an image of the protein array was obtained using fluorescence microscopy (Fig. 1). Successful immobilization of the protein on the sol-gel surface was confirmed.

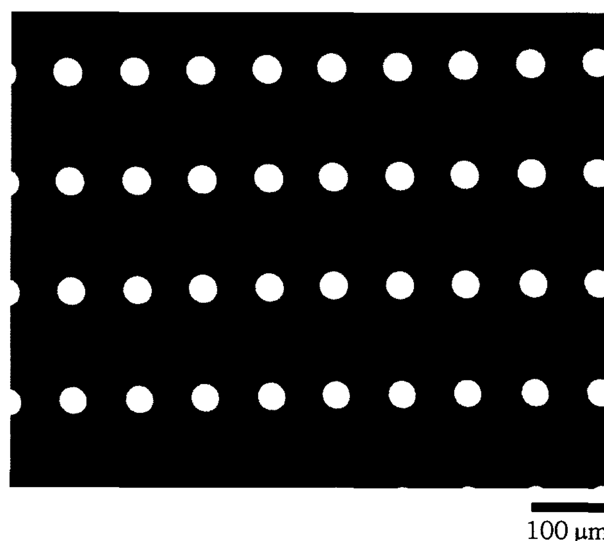

Fig. 1. The fluorescence image of the protein array consisting of FITC-labeled anti-*E.coli* O157:H7 antibodies on the sol-gel surface.

Immunoreaction of Immobilized Antibody

The reactivity of a single immobilized antibody was confirmed before fabricating the multicomponent chip. The detection of *E. coli* O157:H7, *S. agalactiae*, and Mabs against the corresponding pathogens were confirmed by their immobilization on the sol-gel surface. After immunoreaction for 2 h first, the FITC-labeled secondary Mab such as the ELISA structure were applied for 2 h. Figure 2A shows the fluorescence image of the immunoreaction for *E. coli* O157:H7 (cell density was 10^9 CFU/ml) and 2B that for *S. agalactiae* (cell density was 10^9 CFU/ml).

In order to verify the detection of the antibiotic residues, penicillin G and dihydrostreptomycin were detected by using the Mabs-immobilized array. The antibody-immobilized layer was immersed into a PBS buffer solution containing 3% BSA in order to avoid nonspecific binding. The antibody-immobilized layer was submerged into the sample solutions containing penicillin G and dihydrostreptomycin. After the immunoreaction between Mab and the

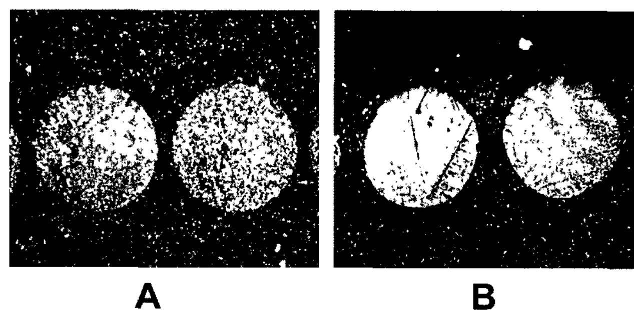

Fig. 2. The fluorescence images of the immunoreaction of Mabs for *E. coli* O157:H7 (10^9 CFU/ml) (**A**), and *Streptococcus agalactiae* (10^9 CFU/ml) (**B**).

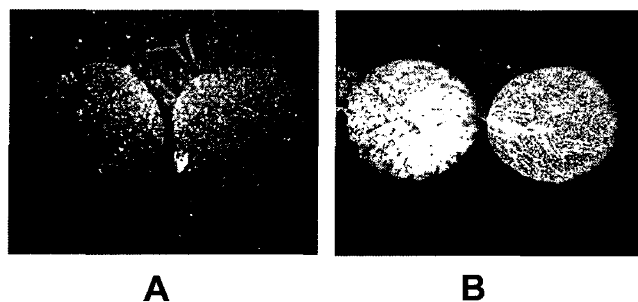

Fig. 3. The fluorescence images of the immunoreaction of Mab for antibiotics; **A.** penicillin G; **B.** dihydrostreptomycin.

corresponding analytes, the antibody layer was washed with a PBS buffer solution. The antibody-immobilized substrate was then submersed into the solution containing the FITC-labeled secondary antibody. Figure 3 shows the fluorescence images of this protein array. From these images, it was confirmed that the antibiotics were successfully detected by an antigen-antibody interaction using the fabricated antibody array.

The neutrophil obtained from bovine blood was detected using the antibody-immobilized array. The fabricated protein array was submerged into a sample neutrophil sample (200,000 cells/ml) and then re-reacted with the FITC-labeled secondary Mab solution. The image of the protein array was obtained by fluorescence microscopy, as shown in Fig. 4. This result verified the immunoreaction between immobilized antibody and neutrophil.

Simultaneous Multidetection Using Protein Chip

Various antibodies were immobilized on a chip in order to simultaneously detect multicomponents. Mabs against *E. coli* O157:H7, *S. agalactiae*, dihydrostreptomycin, penicillin G, and neutrophil were arrayed by the microarrayer in a row with a dot size of 100 μm. The raw milk sample, which was obtained from the dairy research farm at Konkuk University in Korea, was applied to the antibody-arrayed chip for 2 h. After the solution (containing five types of FITC-labeled antibody with respect to the analyte) had been applied, the fluorescence response of the antibody-arrayed chip was measured by fluorescence microscopy, as shown in Fig. 5. These experiments confirmed that various components in raw milk could be detected simultaneously by using the single protein chip with different kinds of immobilized antibodies.

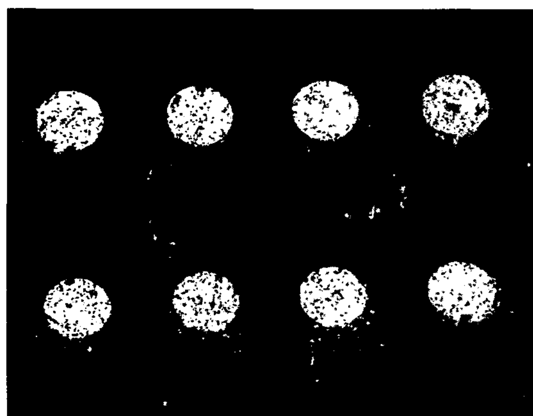

Fig. 4. A fluorescence image of immunoreaction between the antibody array and neutrophils.

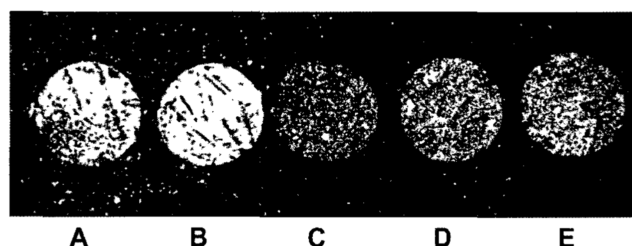

Fig. 5. The fluorescence response of the protein chip as a result of an immunoreaction between the protein array and corresponding analytes: **A.** *E. coli* O157:H7; **B.** *Streptococcus agalactiae*; **C.** penicillin G; **D.** dihydrostreptomycin; **E.** neutrophils.

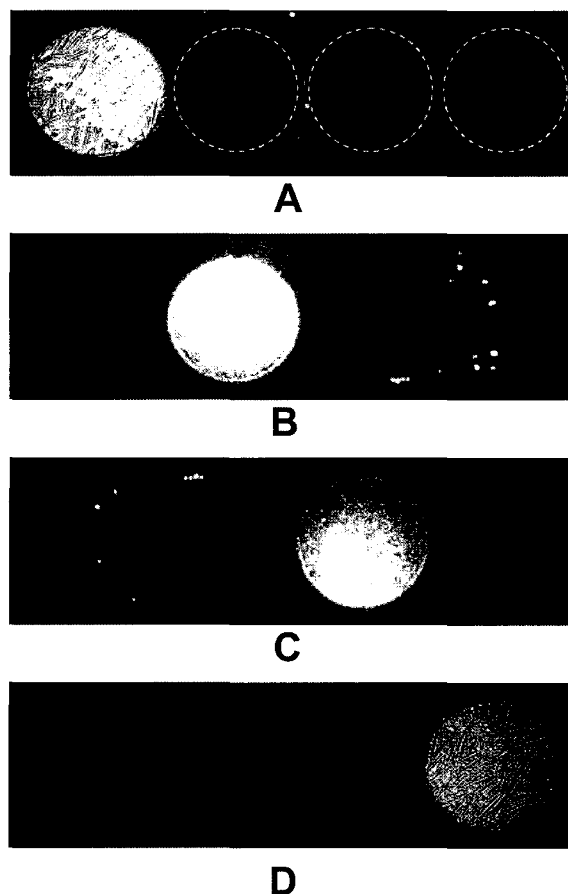

Fig. 6. The specificity of the immunoreaction between a fabricated protein array and analytes: **A.** *E. coli* O157:H7; **B.** *Streptococcus agalactiae*; **C.** dihydrostreptomycin; **D.** penicillin G.

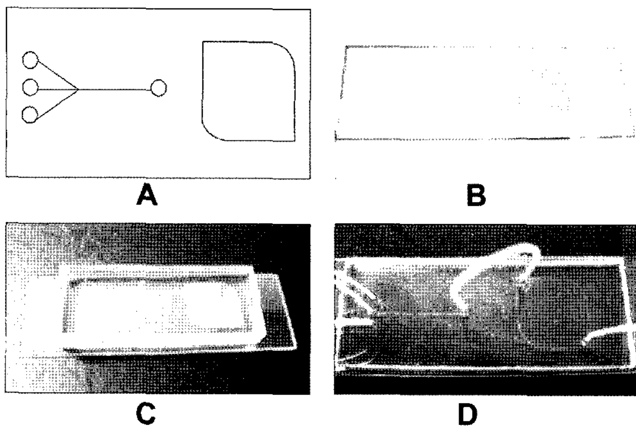

Fig. 7. Fabrication procedure for the lab-on-a-chip.
A. Making mask film; **B.** Patterning on glass; **C.** PDMS molding; **D.** Connecting microtube.

Specificity is a critical factor for a biosensor. In a proposed protein chip system containing an array of different types of antibodies, selectivity, in that one analyte should not react with the antibody dots specific to other analytes, needs to be confirmed. In order to verify the selectivity of a proposed protein chip system, a protein array was fabricated using four kinds of Mabs (against *E. coli* O157:H7, *S. agalactiae*, dihydrostreptomycin, and penicillin G). Figure 6A shows the fluorescence response for when a sample solution containing only *E. coli* O157:H7 was applied to the protein array. *E. coli* O157:H7 reacted only with the Mab dot against *E. coli* O157:H7, but not with the other Mab dots. This result was also applicable to the other analytes. Figures 6B–6E show that there was no nonspecific binding to the other antibodies, suggesting that it is possible to analyze multicomponents (microorganisms, antibiotics, and somatic cells) in a raw milk sample using one protein chip.

Multidetection using Fabricated Lab-on-a-Chip

The LoC was fabricated with PDMS, as shown in Fig. 7. The procedure used was as follows: **A.** Making mask

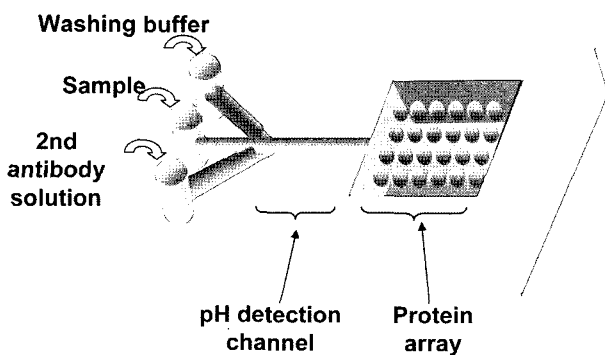

Fig. 8. Schematic diagram of fabricated sensor system.

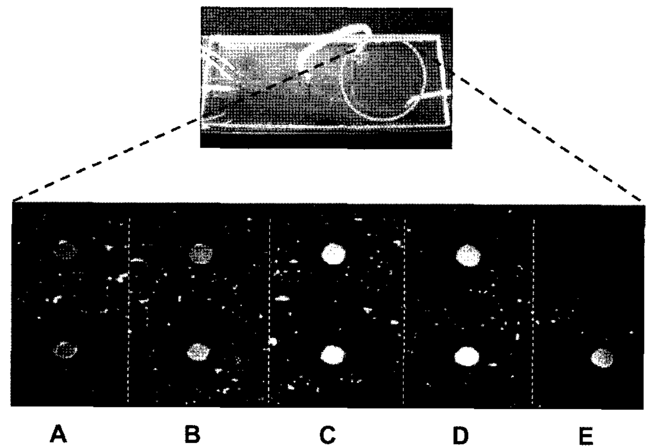

Fig. 9. The fluorescence image of a protein array on the lab-on-a-chip.
A. Penicillin G; **B.** Dihydrostreptomycin; **C.** *E. coli* O157:H7; **D.** *Streptococcus agalactiae*; **E.** Neutrophil.

film; **B.** Patterning on glass; **C.** PDMS molding; and **D.** Connecting microtube. The fabricated PDMS mold was attached to a glass slide, which had previously been immobilized with antibodies. The schematic diagram of the fabricated sensor system is shown in Fig. 8. By introducing the sample into the microchannel, the analytes in the sample should react with the corresponding antibody dots. The size of the microchannel was 0.20×4.4×0.15 mm (W×L×D). After washing with PBS buffer solution, the FITC-labeled antibody solution was injected in order to obtain the fluorescence response. Figure 9 shows the fluorescence images obtained.

The emission intensity of the pH indicator (SNARF-1-dextran) changed with pH. The SNARF-1-dextran entrapped

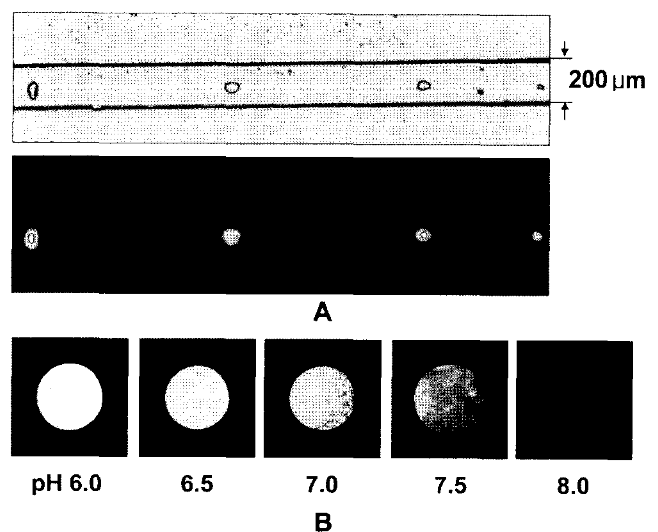

Fig. 10. A. Hydrogel in a microchannel; **B.** pH-dependent emission intensity of the hydrogel dot.

hydrogel was immobilized into the microchannel and was confirmed using fluorescence microscopy, as shown in Fig. 10A. As the buffer with different pHs was flowed into the channel, the emission intensity of the immobilized hydrogel was changed, as shown in Fig. 10B. This shows that a change in pH could be detected by a change in the emission intensity of SNARF-1-dextran.

A LoC was fabricated to test raw milk by employing a Micro Total Analysis System (μTAS) using microelectromechanical system (MEMS) technology. In this chip, several microorganisms, antibiotic residues, and neutrophils in raw milk were detected by an antibody microarray. The pH of the milk was measured using a pH indicator (SNARF-1-dextran) hydrogel entrapped in the micro channel. The antibodies were successfully immobilized onto the sol-gel surface by physical adsorption. In addition, the immunoreaction due to antigen-antibody interaction on the sol-gel surface was detected by fluorescence microscopy. This study highlights the possibilities of protein chip technology for the simultaneous detection of several components in raw milk. It was also shown that raw milk testing using LoC technology can be made easier, less time-consuming, and more economical. The proposed system can be applied to food, medical, and environmental monitoring systems.

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