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# A new size and shape controlling method for producing calcium alginate beads with immobilized proteins

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## ABSTRACT

**A method for producing size- and shape-controlled calcium alginate beads with immobilized proteins was developed. Unlike previous calcium alginate bead production methods, protein-immobilized alginate beads with uniform shape and sizes less then 20 micrometers in diameter could successfully be produced by using sonic vibration. BSA and FITC-conjugated anti-BSA antibodies were used to confirm protein immobilization in the alginate beads. Protein diffusion from the beads could be reduced to less than 10% by cross-linking the proteins to the alginate with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and *N*-hydroxysulfosuccinimide (NHSS). The calcium alginate beads could also be arranged freely on a slide glass by using a femtosecond laser.**

**Keywords:** Calcium Alginate Beads; Size Controllable Production Method; Protein Immobilized Beads; Femtosecond Laser; Laser Manipulation

## 1. INTRODUCTION

Calcium alginate beads have been widely used for immobilizing DNA [1,2,3,4], proteins [5,6], and cells [7] for applications in a variety of fields. In our laboratory, alginate beads have successfully been used for DNA transfection into microorganisms [1], plants [2, 3], and [4] animal cells. Another important application of calcium alginate beads is protein-immobilized alginate beads. Protein-immobilized alginate beads can be used for oral drug delivery [8], protein characterization [9], etc.

The size of the beads is an important factor for applications of calcium alginate beads, since it have been reported that smaller beads are more biocompatible than larger beads [10] and that lower shear forces due to reduced size may increase their long-time stability [11].

Several methods for producing protein-immobilized calcium alginate beads have been reported in previous studies, such as dropping an alginate solution into a gently stirred calcium chloride solution [12], adding an alginate solution and a calcium chloride solution into a gently stirred oil phase [13], and dropping an alginate solution into a calcium chloride solution containing a surfactant using a high voltage electrostatic generator [14]. However, while some of those methods produce calcium alginate beads less than 200 μm in diameter [14], it is difficult to produce beads under 50 μm with a uniform size. Moreover, protein-retention capacity seriously affects the future applications of protein-immobilized alginate beads.

In this study, we produced protein-immobilized calcium alginate beads with uniform shape smaller than 20 μm in size by using a vibration method. The small beads made by this method are easy to arrange by optical tweezers or laser manipulation. This should open the door to new applications of protein-immobilized calcium alginate beads, such as the development of protein arrays using such alginate particles. To enhance the protein-retention capacity of the bio-beads, the analyte proteins were cross-linked to the alginate carboxyl groups with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and *N*-hydroxysulfosuccinimide (NHSS). EDC is commonly used for the covalent linking of proteins to other molecules [15], and catalyzes the formation of amide bonds between the carboxylic groups of alginate and the amine groups of proteins. The cross-linking reaction is promoted by NHSS [16]. The beneficial effectiveness of cross-linking on protein retention is demonstrated. In addition, femtosecond laser irradiation of the target calcium alginate beads and laser arrangement of the calcium alginate beads into alphabetical patterns was performed.

## 2. MATERIALS AND METHODS

**Chemical materials** Sodium alginate with a viscosity of 100~150 cP, isoamyl alcohol, isopropyl alcohol, 1-ethyl -3-(3-dimethylaminopropyl)carbodiimide (EDC) and *N*-hydroxysulfosuccinimide (NHSS) were purchased from Wako Co. (Osaka, Japan). Bovine serum albumin (BSA) was purchased from Nakalai Tesque Co. (Kyoto, Japan). Bovine serum albumin labeled with fluorescein isothiocyanate (FITC) and anti-bovine serum albumin antibody were purchased from Sigma Co. (St. Louis, MO, USA). EZ-Label™ FITC Protein Labeling Kit was purchased from Takara Co. (Shiga, Japan).

**Calcium alginate beads production** A solution containing isoamyl alcohol, isopropyl alcohol, and aq. $CaCl_2$ (2:1:1) was added into a 1.5 ml test tube. Sodium alginate solution (alginate concentration: 1 % w/w) containing protein (25 μg/ml FITC-labeled BSA) was forced from a 100 μl syringe (1710RN 100 μl GL Sciences, Tokyo, Japan) by a syringe pump (MSP-RT As One, Osaka, Japan) through a fused silica capillary (30-75 μm) (GL Sciences) at a constant flow rate (0.1-2 μl/min) (Table 1), and dropped into the mixture while vibrating with a loudspeaker (FR-8, 4 Ω, Visaton, Germany) which was connected to a sine wave sound generator (AG-203D Kenwood, Tokyo, Japan) to produce calcium alginate beads (Figure 1). The frequency of the sine wave sound generator was set to 200 Hz. To harvest the calcium alginate beads produced, the test tube was centrifuged at 5,000 rpm for 3 min. The upper isoamyl alcohol phase was discarded, taking care not to remove the calcium alginate beads. After adding 100 mM $CaCl_2$, the suspension was mixed using a micro-tube mixer (CST-040; Asahi Technoglass, Tokyo, Japan) until the precipitated calcium alginate beads were completely re-suspended. Centrifugation was conducted at 5,000 rpm for 3 min. This washing step was repeated at least 3 times, and the final volume was adjusted to 50 μl.

**Calcium alginate beads size measurement** Calcium alginate beads were produced under 7 different conditions (Table 1). Adequate amounts of calcium alginate beads were re-suspended in a fresh 100 mM $CaCl_2$ solution on a glass slide and digital images of calcium alginate beads were captured through an inverted fluorescent

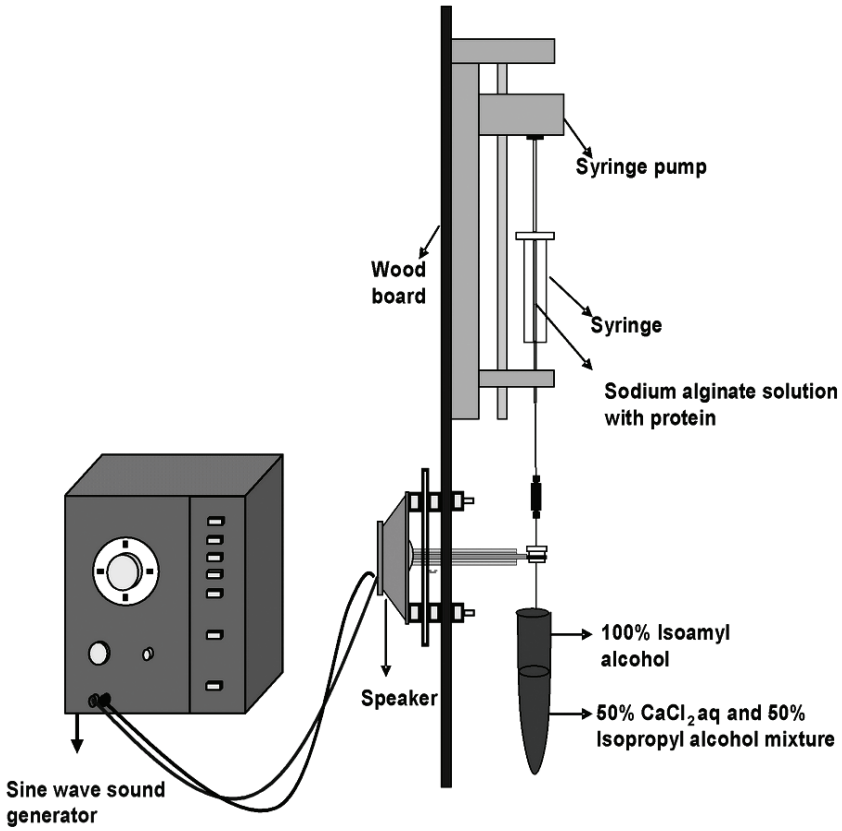


**Figure 1.** Apparatus for producing calcium alginate beads by the vibration method, comprising a syringe pump for forcing sodium alginate solution from a syringe, a loudspeaker, and a sine wave sound generator.

**Table 1.** Conditions for calcium alginate beads production.

| Conditions | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
|---|---|---|---|---|---|---|---|
| **Capillary ϕ (μm)** | 75 | 75 | 75 | 75 | 75 | 30 | 30 |
| **Flow rate (μl/min)** | 2 | 1 | 0.8 | 0.5 | 0.4 | 0.2 | 0.1 |
| **Diameter of beads** | 14.09±1.90 | 12.96±2.35 | 11.87±1.91 | 9.61±1.24 | 8.77±1.04 | 8.72±0.62 | 6.36±1.34 |
| **Number of beads measured** | 52 | 53 | 53 | 53 | 53 | 53 | 54 |

microscope (IX-70 Olympus, Tokyo, Japan) equipped with an RGB color CCD video camera. The original images of the calcium alginate beads were introduced into a personal computer and the area of each bead in the images was measured with ImageJ® image analysis software. The calcium alginate beads were assumed to be spherical, and their diameters were determined from the projection area. For each condition, at least 100 beads were collected, and of these, 371 isolated beads in total were measured.

**BSA and anti-BSA antibody reaction in calcium alginate beads** Anti-BSA antibody was labeled with FITC by an EZ-Label™ FITC Protein Labeling Kit according to manufacturer's instructions. BSA (50 μg/ml) protein was immobilized in calcium alginate beads. Calcium alginate beads without protein and calcium alginate beads with non-specific protein Glutathione S-transferase (GST 50 μg/ml) were used as negative controls. After washing 3 times, the beads were collected into three 1.5 ml tubes. Aqueous 5% skim milk was prepared as a blocking solution; since the skim milk was difficult to dissolve, it was centrifuged (4°C, 1,500 rpm, 10 min), and the supernatant was used.

The beads were incubated with 0.5 ml blocking solution for 1 hour. After blocking, the beads were washed 3 times with aq. $CaCl_2$ (100 mM). FITC-antiBSA antibody was diluted 5,000-fold with aq. $CaCl_2$ (100 mM). Into each of the 3 tubes was added 200 μl aq. FITC-antiBSA, followed by incubation for another hour. After washing 3 times, the beads were investigated by using the CCD video camera-equipped fluorescence microscope.

**Protein-retention capacity observation** EDC and NHSS were added to a sodium alginate solution (1% w/w) to give a final concentration of 2.5 μg/ml EDC and 0.8 μg/ml NHSS. The protein solution (FITC-BSA 25 g/ml) was mixed with this cross-linker-containing alginate solution (1:2 v/v), and stood at room temperature for 15 minutes.

The solution containing isoamyl alcohol, isopropyl alcohol, and aq. $CaCl_2$ (2:1:1) was added into the test tube to generate a $CaCl_2$ concentration gradient. The protein (25 μg/ml FITC-BSA) and aq. alginate (100 μl), with or without cross-linker, was forced from a syringe through a silica capillary by the bead-production instrument (capillary ϕ 75 μl, flow rate 2 μl/min), and dropped into the mixture solution.

The protein-retention capacity was evaluated by analyzing the intensity of fluorescence of each bead's surface. Adequate amounts of calcium alginate beads were re-suspended in a fresh 100 mM $CaCl_2$ solution and placed on a glass slide and digital images of the calcium alginate beads were captured through an inverted fluorescent microscope equipped with the CCD video camera. The original images of the calcium alginate beads were introduced into the personal computer and the intensity value of each bead was analyzed with MATLAB® software. Images of beads were taken at the 3rd day, the 6th day, and the 14th day after bead production. From each sample, the fluorescence intensities of 30~50 beads were measured.

**Calcium alginate beads arrangement** Sample calcium alginate beads produced by the vibration method were deposited on a 2% 3-aminopropyltrimethoxysilane (APS, Tokyo Chemical Industry Co. Tokyo, Japan)-coated cover glass by a Cytospin centrifuge (Shanpon Cytospin® 4, Thermo Scientific, Cheshire, UK) at 2,000 rpm for 5 min and placed above a target slide glass. A water layer of 100 μm was maintained between the two glasses by a silicone rubber spacer. The source and target substrates were set on an inverted microscope (Olympus), equipped with a 100× objective lens (PLN100XO, NA 1.25, WD 0.15, Olympus). The laser beam from a regeneratively amplified Ti:sapphire laser (Spectra Physics, Hurricane, 800 nm, 120 fs) was introduced to the inverted microscope. The beam diameter was adjusted with collimator lenses to be about 5 mm to match the size of the back aperture of the 100× objective lens, and the laser beam was focused on the image plane of the microscope. The protein-beads were patterned by scanning a motorized microscope stage (BIOS-102T, Sigma Koki, Tokyo, Japan) with a linear velocity of 90 μm/s, while irradiating a focused femtosecond laser pulse train with a repetition rate of 1 kHz. The laser pulse energy was 63 nJ/pulse (**Figure 2**).

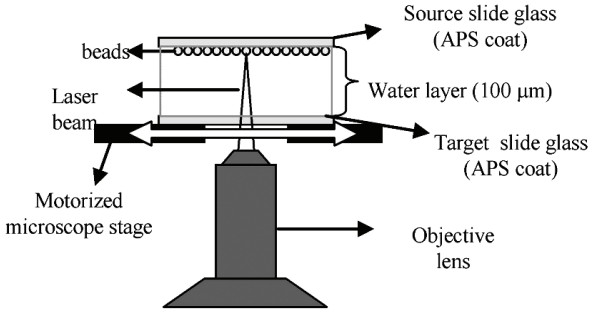


**Figure 2.** Experimental setup for micro-patterning calcium alginate beads by focused femtosecond laser.

## 3. RESULTS

**Calcium alginate beads production** Protein-immobilized calcium alginate beads with uniform size were successfully produced using the bead-production equipment (Figure 3). When the bead-production conditions were set as capillary ϕ , 75 μm, and flow rate, 2 μl/min, the average diameter of the calcium alginate beads was approximately 14 μm. At a flow rate of 0.8 μl/min, the size decreased to approximately 12 μm. When the flow rate was further reduced to 0.4 μl/min, the bead size did not change. To get smaller beads, the capillary was changed to 30 μm, and the diameter of most of the beads could be controlled to approximately 5 μm (Figure 4, Table 1).

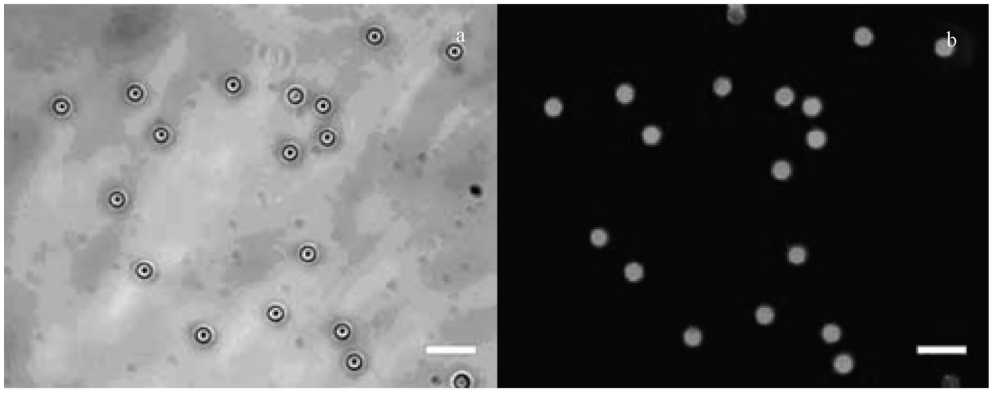


Figure 3. Images of protein-immobilized calcium alginate beads made by the vibration method. Images were photographed under a fluorescent microscope by cooled CCD camera. Bars: 20 μm. (a) microscope image of FITC-BSA-immobilized beads. (b) fluorescence image of the same beads.

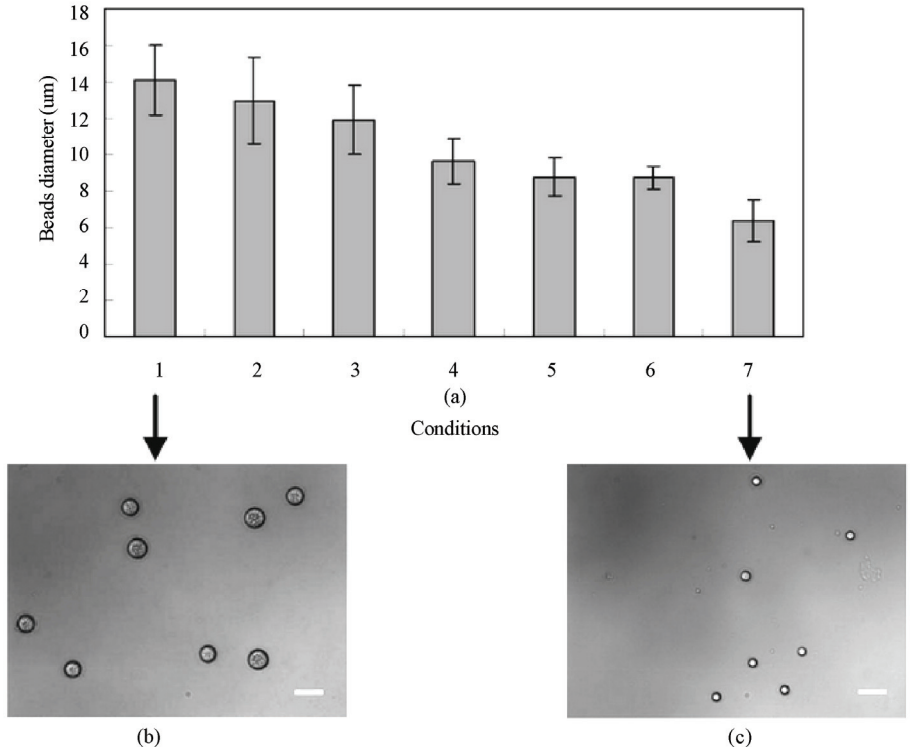


Figure 4. (a) Mean values of the sizes of at least 50 beads for each of 7 different conditions for calcium alginate beads production. Condition 1: capillary ϕ 75 μm, flow rate 2 μl/min. Condition 2: capillary ϕ 75 μm, flow rate 1 μl/min. Condition 3: capillary ϕ 75 μm, flow rate 0.8 μl/min. Condition 4: capillary ϕ 75 μm, flow rate 0.5 μl/min. Condition 5: capillary ϕ 75 μm, flow rate 0.4 μl/min. Condition 6: capillary ϕ 30 μm, flow rate 0.2 μl/min. Condition 7: capillary ϕ 30 μm, flow rate 0.1 μl/min. (b) Calcium alginate beads made under the 1st condition. (c) Calcium alginate beads made under the 7th condition. Bars: 20 μm.

 

**BSA and anti-BSA antibody reaction in calcium alginate beads** To confirm that the protein was immobilized in the alginate beads, antigen-antibody reaction in the alginate beads was performed by using BSA and FITC-labeled anti-BSA. Alginate beads without any encapsulated proteins and beads with encapsulated nonspecific protein (GST) were used as negative controls. BSA-encapsulated beads were clearly observed with FITC-labeled anti-BSA antibody under a fluorescence microscope. Almost no fluorescence was detected from GST protein-immobilized calcium alginate beads (**Figure 5(b)**). Weak signals were observed from non-protein calcium alginate beads (**Figure 5(a)**). However the intensity was barely more than a third that of BSA-immobilized calcium alginate beads (**Figure 5(c)**). These results suggest that the protein-immobilized calcium alginate beads would be useful for detecting antigen-antibody reactions.

**Protein-retention capacity observation** The protein-retention capacity was observed by using 2 types of calcium alginate beads: protein-immobilized alginate beads produced by the vibration method either with or without cross-linking. One group of calcium alginate beads had FITC-BSA cross-linked to the alginate carboxyl groups by EDC and NHSS, whereas the standard beads had no FITC-BSA cross-linking. After analyzing the captured images of the samples, the fluorescence data showed that the small alginate beads made by this vibration method showed a good protein-retention capacity. Two weeks after production of the beads, the image intensity of the standard beads had decreased only 22%, while the intensity reduction of the cross-linked beads was less then 10% (**Figure 6**) and the cross-linked beads could hold more protein than the standard beads. These results suggest that both of the standard beads and protein-cross-linked beads have excellent ability for protein-retention.

**Calcium alginate beads arrangement** Calcium alginate beads produced by using the vibration method were deposited on an APS (2%)-coated cover glass by centrifugation. The cover glass was placed above another glass slide where the calcium alginate beads would be arranged. A water layer of 100m was maintained between the two glasses by a silicone rubber spacer. The source and target slides were set on an inverted microscope equipped with a 100× objective lens. Laser scanning arranged the beads on the target slide into the pattern "F U K U I" (**Figure 7**). This result suggests that a

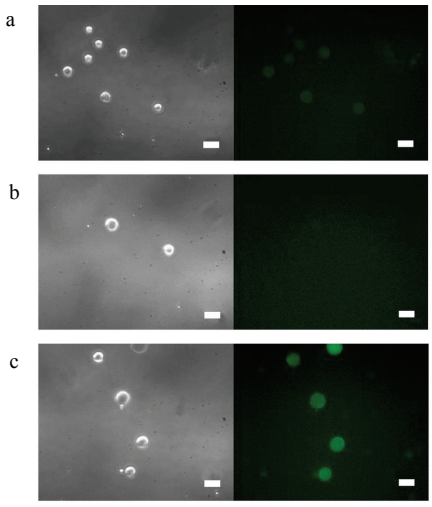


**Figure 5.** Calcium alginate beads produced by the vibration method. The images were taken under a fluorescence microscope by cooled CCD camera. Bars: 20m. (a) Negative control, calcium alginate beads without any immobilized protein. (b) Negative control, calcium alginate beads with nonspecific protein (GST). (c) calcium alginate beads with immobilized BSA.

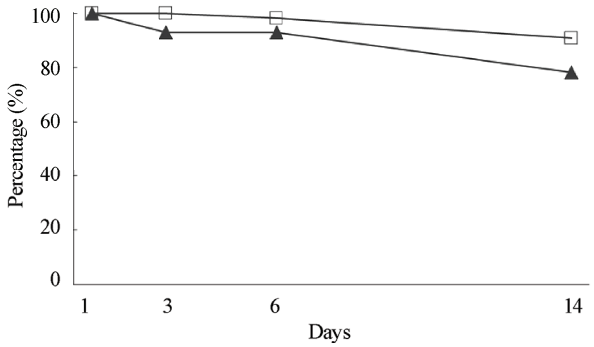


**Figure 6.** Intensity changes for cross-linked beads and standard beads. Squares, cross-linked beads. Triangles, standard beads.

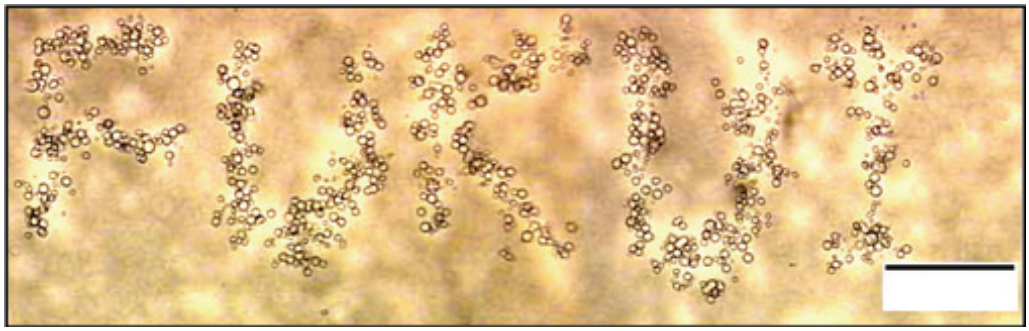

**Figure 7.** Microsopic image of target slide after laser irradiation with a 63 nJ/pulse energy. Bars: 200 μm.

femtosecond laser could serve as a useful manipulation tool for the arrangement of protein-immobilized calcium alginate beads on glass slides and for future applications of the small alginate beads.

## 4. DISCUSSION

In previous studies, for alginate beads size control, a droplet generator with a constant electrostatic potential [14,17] showed good potential for size control. The size of the capsules is mainly governed by voltage, flow, and needle diameter [17]. However, since the production of a micro-diameter needle is still difficult, the size adjustment is also limited. In this study, by connecting a flexible silica capillary to the syringe needle, reduction of the needle diameter was achieved. Furthermore, by changing from a droplet generator with constant electrostatic potential to a loudspeaker that was connected to a sine wave sound generator, continuous, smooth and fine vibrations could be generated. Consequently the size of the alginate beads could be controlled very accurately at the micro-scale. Calcium alginate beads in the range of 5 to 20m with a uniform size could be produced by using this new method. Moreover, by reducing the inner diameter of the silica capillary, and slower the flow rate of alginate solution from the syringe, the smaller alginate beads would be the produced.

Besides protein-immobilization, calcium alginate beads are also widely used for cell-immobilization. Reduction in capsule size has been emphasized to enhance mass transfer of both nutrients into encapsulated cells and products from the encapsulated cells out of the capsule. It has been shown that the response time of encapsulated islets to glucose increases with capsule size [18]. Thus the method developed by us might also be used for immobilizing cells. Furthermore, by adjusting the beads' size and the concentration of the cells-containing alginate solution, one cell per one bead should be possible.

Since BSA protein was successfully immobilized in the calcium alginate beads, and the reaction with FITC labeled anti-BSA was detected successfully by using alginate beads, this indicated that the protein- immobilized alginate beads have the potential to be used to detect antigen-antibody reactions.

Previously, a serum albumin-alginate membrane has been used for coating alginate beads to reduce protein diffusion [19]. However, in this report, even when the beads were coated, over 80% of the protein diffused within 8 days. However, by cross-linking the protein to the alginate, the protein diffusion could be reduced to less than 10% over 14 days. The data also showed that, even without cross-linking, the alginate beads produced by using the vibration method have a high ability for protein-retention.

In conclusion, we have succeeded in the development of a method for producing size- and shape-controlled calcium alginate beads with immobilized proteins. The protein-immobilized calcium alginate beads produced have a small and uniform size, can retain protein within the beads for long periods, are easy to manipulate, and are useful for the detection of antigen-antibody interactions. Therefore the alginate beads production method reported here should find wide application in many biotechnological fields.

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