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Polyelectrolyte Multilayer Microcapsules Containing Fluorescein Isothiocyanate-Concanavalin A/Glycogen Conjugates for Fluorometric Determination of Sugars

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Polyelectrolyte multilayer microcapsules were prepared by a layer-by-layer deposition of poly(ethyleneimine) (PEI) or poly(allylamine hydrochloride) (PAH) and poly(styrene sulfonate) (PSS) on a calcium carbonate particle containing fluorescein isothiocyanate-concanavalin A (FITC-Con A)/glycogen conjugates. Fluorescence microscope and scanning electron microscope observations revealed that the microcapsules thus prepared are spherical with a diameter of 3–5 μm. The fluorescence intensity of the microcapsules was enhanced upon addition of sugars, depending on the type of sugar and its concentration. The enhanced fluorescence intensity was rationalized by sugar-induced decomposition of the FITC-Con A/glycogen conjugates and resulting dequenching of the fluorescence of FITC-Con A. The capsule can be used repeatedly a few times after washing in buffer solution, although FITC-Con A leaked out of the capsules gradually.

1. Introduction

Recently, polyelectrolyte multilayer microcapsules that are constructed using a layer-by-layer (LbL) deposition technique have been attracting much attention. The microcapsules can be prepared by LbL deposition of cationic and anionic polyelectrolytes on the surface of a core particle, followed by dissolution of the core substance. The procedure for constructing hollow capsules is simple and materials for the capsule membrane can be chosen arbitrarily. Due to these advantages, many different types

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of functional capsules have been reported, including enzyme-[1,2] and drug-containing capsules.[3,4] Some of the capsules are sensitive to such external signals as pH changes[5, 6] and sugars.[7]

We report here the fluorometric detection of sugars by functional microcapsules containing fluorescein isothiocyanate-concanavalin A (FITC-Con A)/glycogen conjugates. Con A is a lectin protein (molecular weight: 104,000) found in Jack beans and is known to contain four identical binding sites to sugars such as D-mannose and D-glucose.[8] On the other hand, glycogen is a polysaccharide composed of D-glucose units. Thus, it is possible to construct a multilayer thin film through biological affinity by depositing Con A and glycogen alternately on the surface of a solid support.[9–11] It has been known that the fluorescence of FITC derivatives is quite sensitive to the physical and chemical parameters of the environment where the dye is located. In fact, we have found that the fluorescence of FITC-Con A is quenched when FITC-Con A is conjugated with glycogen and dequenched upon the addition of sugars to the solution of the conjugate due to the decomposition of the conjugate (Fig. 1).[12] Thus, it was possible to detect sugars on the basis of changes in the fluorescence intensity of FITC-Con A/glycogen conjugates.[12] In the present study, we try to encapsulate the FITC-Con A/glycogen conjugates in polyelectrolyte multilayer microcapsules to develop sugar-sensitive systems that can be used repeatedly.

In relation to this, sugar-sensitive microcapsules have already been reported, in which the principle of detection was based on a fluorescence resonance energy transfer (FRET) system using tetramethylrhodamine isothiocyanate (TRITC)-labeled concavalin A (TRITC-Con A) and FITC-labeled dextran.[13] The capsule was prepared by coating LbL films on a melamine formaldehyde (MF) particle. This system, however, suffers from the fact that a large number of TRITC-Con A and FITC-dextran molecules cannot be encapsulated. Furthermore, use of MF particles may not be suitable for the encapsulation of biological materials because the MF core is usually dissolved in a strongly acidic medium (1 M HCl) to prepare hollow capsules.

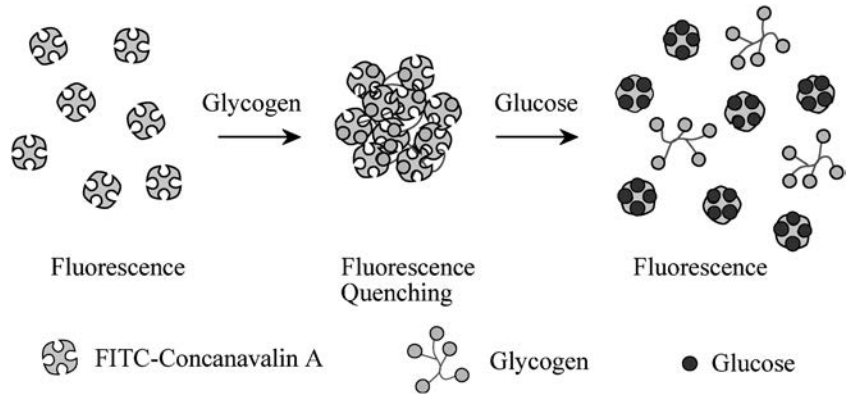

Fig. 1. Schematic representation of the formation of FITC-Con A/glycogen conjugate and its decomposition by D-glucose.

For these reasons, in this study, we used calcium carbonate ($CaCO_3$) as a core particle. The $CaCO_3$ particle was easily prepared by mixing a calcium chloride and ammonium carbonate solution, and, after being coated by LbL films, $CaCO_3$ was dissolved under a mild condition such as 100 mM EDTA (pH 7.4).[14] In addition, $CaCO_3$ is known to form a spherical particle of micrometer size during particle formation, other materials can easily be incorporated inside the particle.[15] In this way, it may be possible to encapsulate large amounts of FITC-Con A/glycogen in a $CaCO_3$ core without loss of its biological activity. We used here poly(styrenesulfonate sodium) (PSS) as polyanion and poly(allylamine hydrochloride) (PAH) and poly(ethyleneimine) (PEI) as polycationic counterparts. In the present study, we examined the sugar response and reusability of the microcapsules on the basis of the fluorescence response of the capsules. Note here that our system uses only one fluorescence probe (i.e., FITC-Con A) in contrast to the reported system where TRITC-Con A and FITC-dextran were needed for obtaining FRET response.[13]

2. Experimental Methods

2.1 *Materials*

FITC-Con A (molecular weight: 104,000) and TRITC were purchased from Funakoshi Co. (Kyoto, Japan). Glycogen was obtained from Tokyo Kasei Co. (Tokyo, Japan). Poly(styrenesulfonate) sodium salt (PSS, molecular weight: 500,000) was obtained from Scientific Polymer Products, Inc. Poly(allylamine hydrochloride) (PAH, molecular weight: 10,000) and poly(ethyleneimine) (PEI, molecular weight: 60,000–80,000) were purchased from Nittobo Co. (Tokyo, Japan) and Nacalai Tesque Co. (Tokyo, Japan), respectively. TRITC-labeled PAH (TRITC-PAH) and PEI (TRITC-PEI) were synthesized by the coupling reactions of TRITC and PAH or PEI. Other reagents used were of the highest grade available and used without further purification.

2.2 *Apparatus*

The shape and size of microcapsules were evaluated using a fluorescence microscope (IX70, Olympus, Japan) and a S-3200N scanning electron microscope (Hitachi, Japan). Fluorescence spectra were measured using a Shimadzu RF-5300PC spectrofluorophotometer (Kyoto, Japan).

2.3 *Preparation of microcapsules containing FITC-Con A/glycogen conjugates*

An FITC-Con A (20 μg mL^{-1}) solution was mixed with a glycogen solution (20 μg mL^{-1}) to form a complex (0.1 M Tris-HCl buffer containing 1 mM $MnCl_2$ and 1 mM $CaCl_2$, pH 7.4). The mixed solution was left standing for 60 min to complete complexation. A $CaCO_3$ core was prepared according to the reported procedure.[13] 222 mg of $CaCO_3$ was added to the FITC-Con A/glycogen conjugate solution (10 mL) (solution A). Ammonium carbonate (192 mg) and PSS (40 mg) were dissolved in 10 mL of distilled water (solution B). Solution A was poured into solution B quickly and stirred for 30 min. The precipitated $CaCO_3$ particles that included FITC-Con A/glycogen conjugates were collected by centrifugation after being washed in the Tris-HCl buffer solution.

Polyelectrolyte multilayer films were prepared on the surface of the $CaCO_3$ particles (Fig. 2). The $CaCO_3$ particles were dispersed in a PEI (or PAH) solution (2 mg mL^{-1}, in 0.1 M Tris-HCl buffer containing 1 mM $MnCl_2$ and 1 mM $CaCl_2$, pH 7.4) for 15 min to deposit the first PEI (or PAH) layer. After being washed in the working buffer for 1 min to remove any weakly adsorbed PEI (or PAH), the $CaCO_3$ particles were dispersed in a PSS solution (2 mg mL^{-1}, in 0.1 M Tris-HCl buffer containing 1 mM $MnCl_2$ and 1 mM $CaCl_2$, pH 7.4) for 15 min to deposit PSS through an electrostatic interaction between PEI (or PAH) and PSS. The second PEI (or PAH) layer was deposited in a similar manner on the surface of the $CaCO_3$ particles modified with the PEI/PSS (or PAH/PSS) bilayer membrane. The deposition was repeated to build up multilayer membranes. The multilayer-membrane-coated $CaCO_3$ particles were suspended in 0.1 M EDTA solution (pH 7.4) for ca. 5 min to dissolve the $CaCO_3$ core and washed twice with 0.1 M Tris-HCl buffer. For the observation of the microcapsules with a fluorescence microscope, the capsules were prepared using TRITC-PEI (or TRITC-PAH) instead of PEI (or PAH).

2.4 *Fluorescence measurements of microcapsules*

Microcapsules containing FITC-Con A/glycogen conjugates were diluted with 0.1 M Tris-HCl buffer (containing 1 mM $MnCl_2$ and 1 mM $CaCl_2$, pH 7.4) to O.D. 0.15 at 600 nm. The diluted dispersion was used for all fluorescence measurements in the present study. The fluorescence emission spectra of the dispersion were measured before and after adding sugars. The wavelength of excitation light was 488 nm because the absorption maximum of FITC-Con A was observed around this wavelength. The fluorometric response of the capsules to sugars was evaluated in terms of I/I_0, where I_0 and I denote the fluorescence intensities at 515 nm before and after the addition of sugar, respectively. The reusability of the capsules was examined by the following protocol. The capsules were dispersed in a disposable cuvette and the fluorescence intensity was measured before and after the addition of 100 mM D-glucose. Then, the microcapsules were centrifuged and dispersed again in 0.1 M Tris-HCl buffer (containing 1 mM $MnCl_2$ and 1 mM $CaCl_2$, pH 7.4) for 30 min. This rinsing process was repeated twice and the capsules were used for further measurements. The amount of FITC-Con A that leaked out of the capsules was evaluated by measuring the fluorescence intensity in the supernatant obtained by centrifugation. All experiments were carried out at room temperature (~ 20°C).

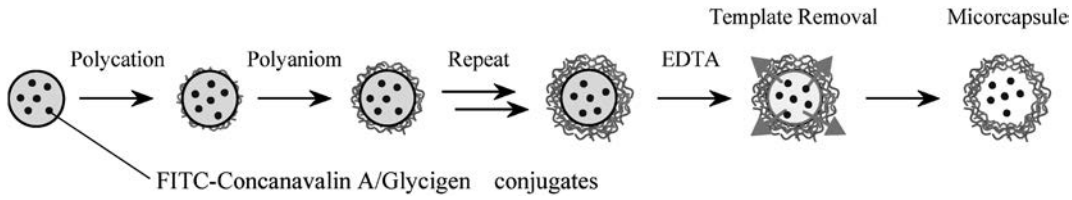

Fig. 2. Protocol for the preparation of microcapsule containing FITC-Con A/glycogen aggregate.

3. Results and Discussion

3.1 *Preparation of microcapsules containing FITC-Con A/glycogen conjugates*

The $CaCO_3$ particles prepared were observed using fluorescence and scanning electron microscopes. Figure 3(a) shows a fluorescence microscope image of the $CaCO_3$ particles. A fluorescence originating from FITC-Con A/glycogen conjugates in the particles was clearly observed, suggesting that FITC-Con A/glycogen conjugates were successfully included in the $CaCO_3$ particles. In addition, a scanning electron microscope image (Fig. 3(b)) shows that the particles were spherical in shape and the average size was 3–5 μm.

The polyelectrolyte membrane was prepared on the $CaCO_3$ particles as a core (template) using a PSS and TRITC-PAH or TRITC-PEI multilayer for the fluorescence microscope analysis. The formation of the multilayer capsule was monitored on the basis of TRITC fluorescence. It was found that microcapsules cannot be constructed when the (TRITC-PAH/PSS)$_n$ and (TRITC-PEI/PSS)$_n$ layers are thinner than 3 bilayers (n = 3 or smaller), probably due to the lack of mechanical strength. On the other hand, nearly spherical microcapsules were constructed when the (TRITC-PAH/PSS)$_n$ and (TRITC-PEI/PSS)$_n$ membranes with more than 4 bilayers were coated on the $CaCO_3$ core. Figure 4 shows (a) fluorescence microscope and (b) scanning electron microscope images of (TRITC-PEI/PSS)$_4$ microcapsules containing FITC-Con A/glycogen conjugates. We ascertain that the intensity of fluorescence from the surface of the microcapsules is higher than that from the inside upon excitation of TRITC-PEI (Fig. 4(a)). Figure 4(b) shows the

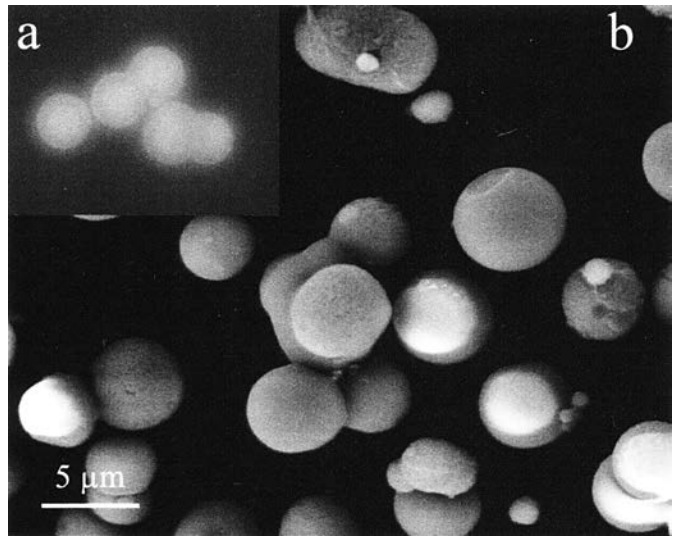

Fig. 3. Fluorescence microscope (a) and scanning electron microscope images (b) of $CaCO_3$ particles containing FITC-Con A/glycogen aggregate.

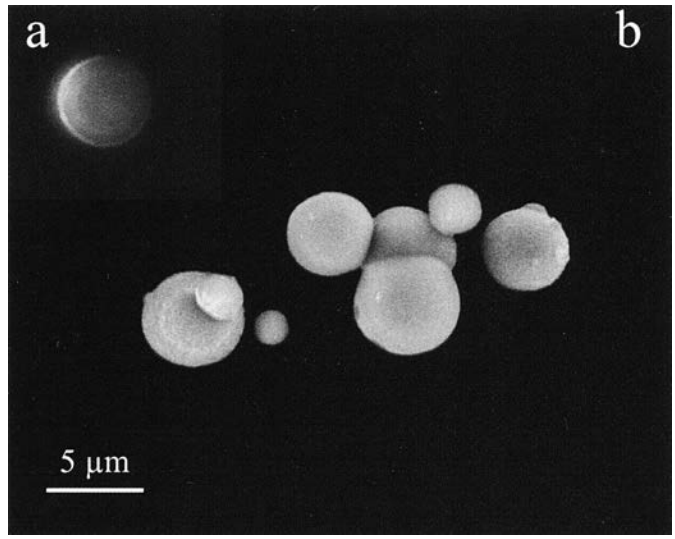

Fig. 4. Fluorescence microscope (a) and scanning electron microscope images (b) of hollow microcapsules with $(PEI/PSS)_4$ membrane.

successful construction of microcapsules, although the microcapsules are partly broken. These observations show that the polyelectrolyte multilayer capsules containing FITC-Con A/glycogen conjugates can be constructed by the present protocol.

3.2 *Sugar response of the microcapsules*

Figure 5 shows the fluorescence spectra of the FITC-Con A/glycogen conjugate-containing capsule with a $(PEI/PSS)_4$ membrane in the absence and presence of D-glucose. The intensity of the fluorescence was increased by the addition of increasing amounts of D-glucose. This shows that added D-glucose passed through the capsule membrane to bind with FITC-Con A competing with glycogen, which induced the decomposition of the FITC-Con A/glycogen conjugates in the capsules, as observed for Con A/glycogen LbL films.[10,11] As a result of the decomposition of the conjugates, the fluorescence of FITC-Con A was dequenched in a similar manner as in the case of the sugar-enhanced fluorescence of the conjugate in solution.[12] A time course of the change in fluorescence intensity of this capsule was monitored in the presence of different concentrations of D-glucose (data not shown). The rate of increase in the fluorescence intensity significantly depended on the concentration of D-glucose. The fluorescence intensity increased rapidly to reach a maximum value in 60 s upon the addition of 100 mM D-glucose, while more than 300 s was required to reach the equilibrium state in the presence of 10 and 20 mM D-glucose. These results suggest that D-glucose can be

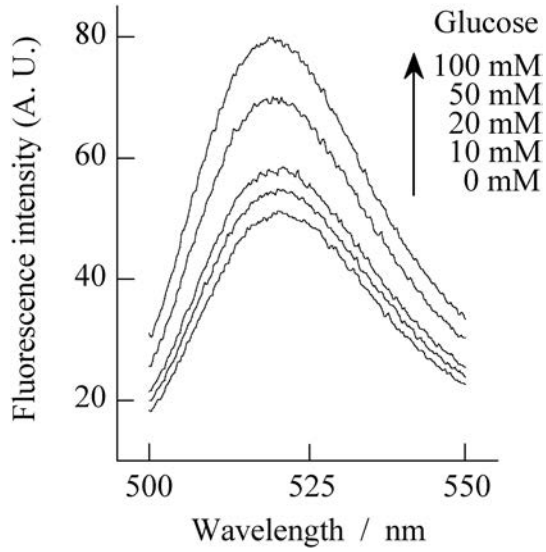

Fig. 5. Effects of D-glucose addition on the fluorescence spectra of FITC-Con A/glycogen microcapsule with $(PEI/PSS)_4$ membrane. Each fluorescence spectrum was recorded 5 min after the addition of glucose.

detected by measuring the fluorescence intensity of the capsule. The response times for other sugars were almost of the same as that for D-glucose.

The effects of the type of polycations and thickness of the capsule membrane were evaluated. Table 1 shows changes in fluorescence intensity at 515 nm (I/I_0) upon the addition of 100 mM glucose to the microcapsules prepared with $(PEI/PSS)_n$ and $(PAH/PSS)_n$ (n = 4–6) membranes. The fluorescence intensities of $(PEI/PSS)_4$ membrane capsules increased to I/I_0 = 1.55 in the presence of 100 mM D-glucose. On the other hand, the I/I_0 values decreased for the capsules with thicker $(PEI/PSS)_5$ and $(PEI/PSS)_6$ membranes. In addition, the capsules with $(PAH/PSS)_n$ membranes exhibited low response for D-glucose probably due to the lower permeability of the $(PSS/PAH)_n$ membranes to D-glucose. In relation to this, we previously reported that LbL thin films composed of PAH show a lower permeability than PEI-based films to small organic compounds such as ascorbic acid, uric acid, and acetaminophen due to a dense packing of PAH chains in the film, which in turn results from a different molecular geometry of the linear chain for PAH and a highly branched configuration for PEI.[16] On the basis of the above observations, the microcapsules with a $(PEI/PSS)_4$ membrane are used for further evaluation of sugar response.

Figure 6 shows the effects of the type of sugar on the response of the $(PEI/PSS)_4$ microcapsule containing FITC-Con A/glycogen conjugate. D-glucose, D-mannose,

Table 1
Fluorometric response of microcapsules containing FITC-Con A/glycogen conjugates: Effects of type of polycation and thickness of capsule membrane.

Capsule Membrane	I/I_0		
	$n = 4$	$n = 5$	$n = 6$
$(PEI/PSS)_n$	1.55	1.42	0.94
$(PAH/PSS)_n$	1.08	1.06	0.96

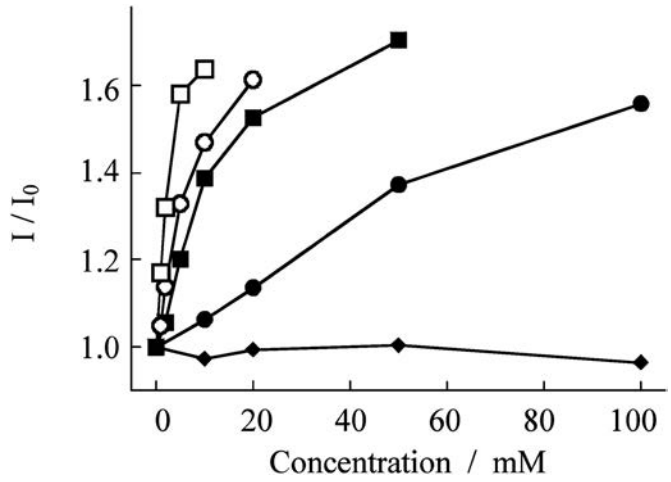

Fig. 6. Fluorometric response of FITC-Con A/glycogen microcapsule to D-galactose (filled diamonds), D-glucose (filled circles), D-mannose (filled squares), methyl-α-glucose (open circles), and methyl-α -mannose (open squares).

D-galactose, methyl α-D-glucopyranoside (Me-α-Glu) and methyl α-D-mannopyranoside (Me-α-Man) were tested. The calibration graph showed a wide detection range for D-glucose over the concentration range of 10–100 mM. The fluorescence was fully dequenched by 10 mM Me-α-Man and 20 mM Me-α-Glu because of the high affinity of the methylated sugars to Con A (the binding constants of Me-α-Man and Me-α-Glu are reported to be 2.1×10^4 and 4.9×10^3 M^{-1}, respectively).[17] D-mannose was also more effective than D-glucose (the binding constants of D-mannose and D-glucose are reported to be 2.2×10^3 and 0.8×10^3 M^{-1}, respectively).[18] In contrast, D-galactose did not induce any change in fluorescence intensity because of its negligibly low affinity to Con A. Thus, the increase in the fluorescence intensity of FITC-Con A depends on the

binding affinity of the sugars to Con A. In other words, FITC-Con A enclosed in the capsule retains its biological affinity. Note here that the dynamic range for D-glucose of the present system is comparable to or wider than those of electrochemical glucose sensors reported, although the lower detection limit is slightly higher.[19,20]

The reusability of the $(PEI/PSS)_4$ microcapsule was investigated (Fig. 7). The response of the microcapsule to 100 mM D-glucose was monitored four times repeatedly. When D-glucose was removed from the capsule by washing, FITC-Con A and glycogen formed a conjugate again in the capsule and exhibited a response to D-glucose again. However, the fluorescence intensity of the capsule decreased with increasing number of measurements, suggesting a leakage of FITC-Con A from the microcapsule upon the addition of D-glucose. Therefore, we have verified the leakage of FITC-Con A from the capsules in the presence and absence of D-glucose. Figure 8 shows the fluorescence intensity of the supernatant of the solution in which the capsule was dispersed. The fluorescence intensity scarcely increased before adding 100 mM D-glucose, showing the leakage of FITC-Con A/glycogen conjugate is negligibly small because of its large size. The slower leakage may originate from the dissociation of FITC-Con A in part from the conjugate. However, FITC-Con A leaked out of the capsule upon the addition of D-glucose as a result of the decomposition of the FITC-Con A/glycogen conjugate into its components. Therefore, a repeated use of the present microcapsule is limited at the present stage.

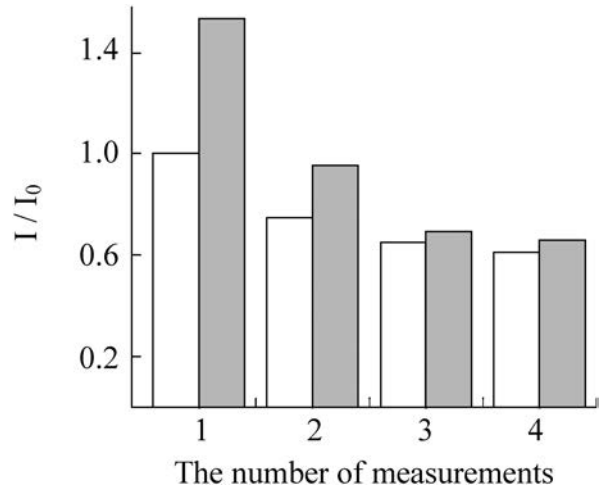

Fig. 7. Reusability of FITC-Con A/glycogen microcapsule. Fluorescence intensities of FITC-Con A/glycogen microcapsules in the absence (open pillar) and presence (filled pillar) of 100 mM D-glucose are shown.

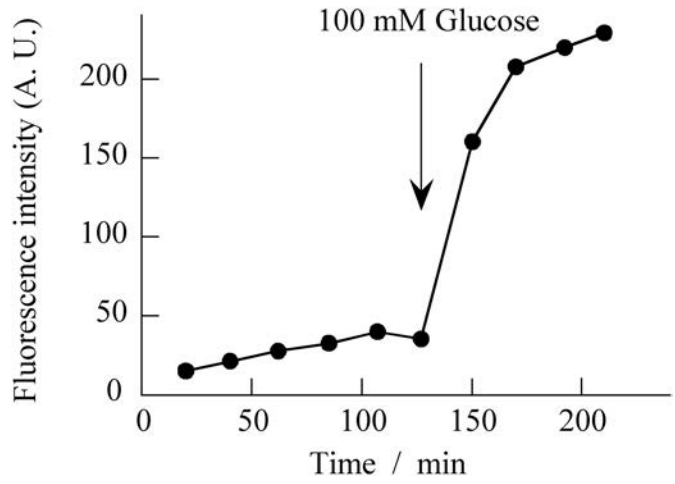

Fig. 8. Leakage of FITC-Con A from the microcapsule containing FITC-Con A/glycogen conjugates upon addition of D-glucose.

4. Conclusions

Polyelectrolyte multilayer microcapsules containing FITC-Con A/glycogen conjugates were prepared using a $CaCO_3$ particle as the core. The fluorescence intensity of the $(PEI/PSS)_4$ capsules increased in the presence of D-glucose and other sugars, depending on the concentration of the sugars. The fluorescence response is due to the dequenching of the fluorescence of FITC-Con A induced by the decomposition of the FITC-Con A/glycogen conjugate into components by sugars. The reusability of the capsules is still limited due to a leakage of FITC-Con A out of the capsules. It may be possible to improve the permeability of the LbL membranes by tuning the thickness and employing suitable materials. It is clear that the present technique is useful for developing functional microcapsules because the encapsulation of biological materials can be carried out in a simple operation under mild conditions.

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