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pH-controlled drug loading and release from biodegradable microcapsules

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Abstract

Microcapsules made of biopolymers are of both scientific and technological interest and have many potential applications in medicine including their use as controlled drug delivery devices. The present study employs the electrostatic interaction between polycations and polyanions to form a multilayered microcapsule shell and also to control the loading and release of charged drug molecules inside the microcapsule. Micron-sized $CaCO_3$ particles were synthesized and integrated with chondroitin sulfate (CS) through a reaction between Na_2CO_3 and $Ca(NO_3)_2$ solutions suspended with CS macromolecules. Oppositely-charged biopolymers were alternately deposited onto the synthesized particles using electrostatic layer-by-layer self-assembly, and glutaraldehyde was introduced to crosslink the multilayered shell structure. Microcapsules integrated with CS inside the multilayered shells were obtained after decomposition of the $CaCO_3$ templates. The integration of a matrix, i.e. CS, enabled the subsequent selective control of drug loading and release. The CS integrated microcapsules were loaded with a model drug, i.e. bovine serum albumin labeled with fluorescein isothiocyanate (FITC-BSA), and it was shown that pH was an effective means of controlling the loading and release of FITC-BSA. Such CS integrated microcapsules may be used for controlled localized drug delivery as biodegradable devices, which have advantages in reducing systemic side effects and increasing drug efficacy.

Keywords

Microcapsule; controlled release; drug delivery; biodegradable

1. Introduction

One major focus of drug delivery is to develop suitable carriers for therapeutic molecules [1]. The recent availability of many protein therapeutics has challenged and thus stimulated research on developing advanced drug delivery systems. Polyelectrolyte microcapsules, introduced in 1998 [2, 3], have attracted great attention as potential drug carriers [4, 5] due to their ease of preparation, absence of hazardous procedures, use of simple building blocks,

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and the potential introduction of multifunctionality. So far, most of the effort has been dedicated to developing polyelectrolyte microcapsules using poly(styrene sulfonate) (PSS), poly(allylamine hydrochloride) (PAH), and poly(acrylic acid) as capsule shell components. For biomedical applications, especially for drug delivery, biodegradable polyelectrolyte microcapsules would be preferred to non-degradable ones, and there is increasing interest in microcapsules of biopolymers including polypeptides and polysaccharides. For instance, polysaccharides, proteins, polypeptides, and lipids [6–14], have been used to form multilayer shells onto colloids such as melamine formaldehyde (MF), polystyrene (PS), poly-DL-lactic acid, and $CaCO_3$ particles to fabricate polyelectrolyte microcapsules. Recently, De Geest *et al.* have reported a new type of microcapsules [15]. These microcapsules have self-rupturing properties and may be used as pulsed drug delivery systems. In addition, advances have been made in developing enzymatically-degradable polyelectrolyte microcapsules, which may lead to enzyme-responsive release of encapsulated drugs [16, 17]. For instance, chitosan/dextran sulfate microcapsules have been prepared and can degrade in the presence of chitosanase [16], and poly(L-arginine)/dextran sulfate microcapsules may degrade in a cell culture system and could have promising potential for intracellular drug delivery [17]. Meanwhile, there is increasing attention on *in vitro* and *in vivo* studies of the interactions between biodegradable polyelectrolyte microcapsules and cells and tissues [17–19].

Polyelectrolyte microcapsules, compared to traditional liposomes, have controllable stability and high permeability for polar molecules [20, 21], and have attracted great attention for encapsulation of various materials. Polyelectrolyte microcapsules have been used as carriers of drugs; the simplest method of encapsulation is to use the template itself as a substance to be encapsulated [8, 22]. However, such substances should be able to form colloidal particles and have low solubility under the assembly conditions. This substantially limits the application of such an encapsulation method since there are very few such materials. Also, drugs may be pre-loaded into the templates, usually porous and decomposable, before the formation of microcapsule shells [23, 24]; however, decomposition of the templates may affect the integrity of the capsules and may change the properties of the drugs.

Alternatively, drugs can be loaded and released by finely tuning the capsule interior or wall properties. Efforts have been, on one hand, devoted to controlling drug loading. Preformed hollow capsules have been loaded with a variety of materials by changing the permeability of the capsule wall, the swelling-shrinking behavior of capsules, and the solubility of small molecules [25–32]. More recently, a spontaneous encapsulation of charged molecules based on electrostatic interaction with a pre-cursor matrix was reported [31, 33–37]. Macromolecules such as PAH have been immobilized in capsules as a pre-loaded polymer by coprecipitating PAH with multivalent metal ions into decomposable templates, followed by multilayer shell formation and template decomposition [34, 35]. PSS has also been entrapped as a pre-cursor polymer within polyelectrolyte microcapsules through a polymerization synthesis process [33]. The polymerization approach has been applied to form several polymers within microcapsules. However, there are some concerns such as uncontrollable formation of polymers within the capsule shells and thereby modification of the properties and possible destruction of the capsule shells [33]. On the other hand, some efforts have been dedicated to controlling drug release, and light- [38–41], magnetic- [42], pH- (PSS/PAH based) [43–45], salt- [46, 47], glucose- [48, 49], and redox-responsive [10, 50] polyelectrolyte microcapsules have emerged recently. Such drug delivery systems, releasing their payload in response to internal or external triggers, offer great advantages. However, the development of stimuli-responsive polyelectrolyte microcapsules, especially those made of biopolymers, is still in the early stage.

Owing to the short development history of polyelectrolyte microcapsule technology, great potential for biomedical applications, and the almost infinite possibility of varying parameters in the preparation process, further studies are needed to advance the development of biodegradable drug delivery systems. The focus of this work was to develop biodegradable microcapsules with controllable loading and release capabilities for drug delivery applications. $CaCO_3$ particles, integrated with chondroitin sulfate (CS), were prepared, and CS, a polyanion, was used as a tool to control the subsequent loading and release of charged drugs. The $CaCO_3$ particles integrated with CS are referred to as $CaCO_3$(CS). Poly-L-lysine (PLL), a polycation, and CS were alternately deposited onto $CaCO_3$(CS) particles, and crosslinking of the multilayered shell was achieved using glutaraldehyde (GA), which has been extensively used on scaffolds and implants in animals and humans. No apparent histological or other evidence of toxicity related to the use of GA has been observed [51]. GA was also used to crosslink multilayered microcapsules of PAH and PSS [52], and of chitosan and alginate [53]. No cytotoxicity was detected in the *in vitro* studies of human fibroblasts cultured with GA crosslinked multilayered microcapsules [53]. Microcapsules with CS integrated inside the shells were obtained after decomposition of the templates (i.e. $CaCO_3$ particles) using disodium ethylenediaminetetraacetic acid (EDTA). A model drug, bovine serum albumin labeled with fluorescein isothiocyanate (FITC-BSA), was successfully incorporated into the microcapsules through its electrostatic interaction with the CS macromolecules integrated inside the multilayered shells. The control of loading and release of FITC-BSA by means of pH was studied.

2. Methods

2.1 Materials

Poly-L-lysine hydrobromide (PLL, MW=150–300 kD), chondroitin sulfate sodium salt (CS), glycine, sodium carbonate, calcium nitrate tetrahydrate, FITC-BSA, and GA were purchased from Sigma (St. Louis, MO, USA). EDTA was obtained from Fisher (Fair Lawn, NJ, USA) and quartz slides were purchased from Electron Microscopy Sciences (Hatfield, PA, USA). 0.05 M glycine solution of pH 5.5 was prepared and used throughout this study. PLL (1 mg/mL) and CS (1 mg/mL) solutions were prepared by dissolving PLL and CS, respectively, in the glycine solution. To quantify the amount of BSA loaded or released, its labeled product, i.e. FITC-BSA, was used and its solutions (1 mg/mL) of pH 3.4, 3.8, 4.2, 4.6, and 5.0 were prepared. Solutions of pH 7.4, 5.0, and 1.0 were also prepared for the FITC-BSA release study.

2.2 Preparation of $CaCO_3$ microparticles integrated with CS

Calcium carbonate ($CaCO_3$) microparticles integrated with CS were synthesized by mineralization of Na_2CO_3 and $Ca(NO_3)_2$ solutions suspended with CS (Figure 1 A→B). A 100 mL 0.33 M $Ca(NO_3)_2$ solution was mixed with 2 mL of 10% CS solution, to which 100 mL 0.33 M Na_2CO_3 solution was rapidly poured under agitation. $CaCO_3$ microparticles were formed immediately and collected through centrifugation. CS content in the $CaCO_3$(CS) microparticles was measured by inductively coupled plasma optical emission spectroscopy (ICP-OES). Briefly, 0.5 g $CaCO_3$(CS) microparticles were dissolved in 5 mL HCl and 10 mL HNO_3, then supplemented with deionized water to 50 mL. The solution was heated at 118 °C for 2.5 h then cooled to ambient temperature. Deionized water was added to bring the solution volume back to 50 mL, and the solution was analyzed by ICP-OES.

2.3 PLL/CS multilayer assembly and microcapsule preparation

Multilayered microcapsules were fabricated using electrostatic layer-by-layer (LBL) self-assembly nanotechnology. PLL and CS were alternately assembled onto $CaCO_3$(CS) microparticles. 200 mg $CaCO_3$(CS) colloidal particles were rinsed twice with the glycine

solution and then incubated in 1 mg/mL PLL solution for 10 min. The particles were then washed three times by immersing in glycine solution for 1 min followed by centrifugation at 5,000 rpm for 30 s. In the next step, the microparticles were incubated in 1 mg/mL CS solution for 10 min, followed by three washings. Repeating the deposition of PLL and CS led to the formation of PLL/CS multilayers with the desired number of layers on $CaCO_3$(CS) microparticles (Figure 1 B→C). After completion of the multilayer assembly, the template-shell particles were subjected to crosslinking using GA at ambient temperature (Figure 1 B→C), and the particles were treated with glycine solution overnight to eliminate any unreacted aldehyde groups. The template-shell particles were rinsed three times in deionized water and then transferred to 30 mL 0.1 M EDTA solution for 1 h to decompose the $CaCO_3$ templates (Figure 1 C→D); this process was repeated once and microcapsules integrated with CS were obtained. The prepared microcapsules were washed three times in deionized water before further use. The morphology of the CS integrated microcapsules was examined using scanning electron microscopy (SEM, Hitachi S-4000, Japan). Similar deposition and crosslinking procedures (with no centrifugation) were used to prepare PLL/CS multilayered films on quartz slides, which allowed monitoring of the multilayer formation using a UV-vis spectrophotometer (Biomate 3, Madison, WI, USA).

2.4 Loading of BSA into CS integrated microcapsules

FITC-BSA was used as a model drug and the effect of pH on drug loading was studied. The pH of FITC-BSA studied was in the range of 3.4–5.0. 2×10^8 microcapsules were mixed with 200 μL 1 mg/mL FITC-BSA and incubated for 12 h at ambient temperature, followed by centrifugation at 3,500 rpm for 5 min. FITC-BSA was quantified, using a UV-vis spectrophotometer, according to its absorbance peak at 458 nm. Raw data were converted to concentration (μg/mL) of BSA using the standard curves obtained at various pH values (data not shown). The loading of FITC-BSA was also confirmed using a Zeiss LSM 510 confocal laser scanning microscope (CLSM, Thornwood, NY, USA).

2.5 Release profiles of BSA from CS integrated microcapsules

2×10^8 microcapsules were mixed with 200 μL 1 mg/mL FITC-BSA solution at pH 3.8 and incubated at ambient temperature for 12 h. After centrifugation at 3,500 rpm for 5 min and removal of the supernatant, the FITC-BSA loaded microcapsules were mixed with 1 mL PBS (pH 7.4) or 1 mL HCl solution (pH 5.0 or 1.0) and incubated at ambient temperature under gentle shaking. A 200 μL supernatant was taken out at a given time and supplemented with 200 μL fresh PBS or HCl solution. Each 200 μL supernatant was diluted with PBS or HCl solution, the absorbance of FITC-BSA at 458 nm was recorded, and the cumulative release of FITC-BSA was calculated. Statistical analysis of the release at different pH values (i.e. pHs 7.4, 5.0 and 1.0) at certain time periods (i.e. 30 and 65 min) was carried out. One-way Anova and Duncan's multiple-range test for post hoc comparisons were conducted, and $\alpha = 0.05$ significant level was used.

3. Results

In this study, $CaCO_3$ microparticles integrated with CS were prepared, and LBL was applied to form multilayered shells on the particles. After dissociation of the $CaCO_3$ particles, polyelectrolyte microcapsules were obtained, and the integration of CS inside the capsules was intended to alter the physical properties of the capsule interior, which would allow control of subsequent drug loading and release via pH. A model drug, FITC-BSA, was loaded and its release was investigated. pH was explored as a means to control the loading and release of FITC-BSA. We would like to point out that our focus was to study the effect of pH on the loading and release of BSA and the impact of pH on structural change or bioactivity of BSA was not investigated.

3.1 Preparation of multilayered microcapsules

$CaCO_3$(CS) microparticles were prepared by mineralization of Na_2CO_3 and $Ca(NO_3)_2$ solutions suspended with CS (Figure 1 A→B). ICP-OES tests showed that there was approximately 1.8 wt.% CS inside the $CaCO_3$(CS) microparticles. The diameter of the $CaCO_3$(CS) microparticles was found in the range of 3–6 μm, which was obtained from SEM images of 50 randomly selected particles. To form microcapsules, oppositely-charged polyelectrolytes, i.e. PLL and CS, were deposited on $CaCO_3$(CS) microparticles using LBL technology (Figure 1 B→C). EDTA was used to decompose the $CaCO_3$ templates and to obtain microcapsules (Figure 1 C→D). Energy dispersive X-ray analysis showed no calcium (Ca) in the microcapsule samples (data not shown).

The stability of microcapsules was improved by crosslinking using GA. It was reported that the aldehyde and amine groups react and produce -C=N- bonds (Schiff base) that have a characteristic peak at a wavelength around 268 nm [52]. Conditions for crosslinking were optimized based on the study on quartz slides, which allowed us to monitor formation of PLL/CS multilayered coatings using a UV-vis spectrophotometer. Figure 2 shows the absorbance spectra of PLL/CS multilayers at 268 nm. Crosslinking was very effective in the first 2 h and continued to increase the absorbance of the coatings and thereby the mass deposited up to 24 h (Figure 2A). Absorbance of the multilayered coatings also increased with the increase of GA concentration, in the range of 0–5% (Figure 2B). Correspondingly, stable microcapsules (Figures 3C, D and E) were obtained after crosslinking for 24 h when GA in the range of 0.5–5% was used.

3.2 pH-controlled loading of BSA inside the CS integrated microcapsules

pH was applied to control the loading of FITC-BSA, a model protein drug, into the CS integrated microcapsules (Figure 1 D→E). Figure 4 shows that the loading of FITC-BSA varied remarkably with pH changes. The microcapsules had the lowest loading of FITC-BSA at pH 5.0. The loading of FITC-BSA increased substantially, from 1.0 mg/mL to 5.6 mg/mL, as the pH decreased from 5.0 to 3.8, and maximum loading of FITC-BSA was reached at pH 3.8. The loading of FITC-BSA decreased as pH decreased from 3.8 to 3.4. The loading of FITC-BSA inside CS integrated microcapsules was further confirmed by CLSM images (Figure 5). FITC-BSA was incorporated both in the capsule interior and also within the multilayered shells, and high fluorescent intensity was observed in the microcapsule cores (Figure 5B).

3.3 pH-controlled release of BSA from the CS integrated microcapsules

pH was used to trigger or tune the release of encapsulated FITC-BSA from the CS integrated microcapsules (Figure 1 E→F); the release profiles are shown in Figure 6 A. There was a burst release at the early stage (0–65 min), followed by a sustained release (Figure 6 A inset). The release rate of BSA was higher at a higher pH. 60% of BSA was released at 65 min at pH 7.4 and, by contrast, only 44% and 30% of BSA were released at pH 5.0 and 1.0, respectively. The release (e.g. at 30 and 65 min) was significantly different between pHs 7.4, 5.0, and 1.0 (Figure 6 B).

4. Discussion

Development of biodegradable drug delivery systems with controllable loading and release capabilities is of importance. Despite the progress made in the last couple of years in preparation of polyelectrolyte microcapsules, the control of drug loading and release from polyelectrolyte microcapsules is still a challenging task for researchers in biotechnology and medicine. The present study reports a new strategy to load charged drug molecules in polyelectrolyte microcapsules for triggered or sustained release. Electrostatic interaction is

employed to control the loading and release of a model drug, i.e. FITC-BSA, by integrating CS into the biopolymer microcapsules. As shown in Figure 1, we intended to incorporate CS macromolecules inside the templates, i.e. $CaCO_3$ particles, to control the subsequent capture and release of charged drug molecules after microcapsule formation and template removal. We prepared $CaCO_3$(CS) microparticles by mineralization of Na_2CO_3 and $Ca(NO_3)_2$ solutions suspended with CS (Figure 1 A→B). CS was incorporated in the $CaCO_3$ particles, as evidenced by ICP-OES tests: ~ 1.8 wt.% CS was found inside the $CaCO_3$(CS) microparticles. LBL was successfully used to build a multilayer of PLL and CS, i.e. (PLL/CS)$_5$, on the $CaCO_3$(CS) microparticles (Figure 1B→C). To enhance stability of the polyelectrolyte multilayers, GA was introduced and crosslinking was achieved through the reaction of aldehyde groups of GA with amino groups of PLL. As shown in Figures 2 and 3, the stability of microcapsules could be controlled by crosslinking time and concentration of GA, and stable microcapsules were prepared after crosslinking.

The $CaCO_3$ templates were then removed using EDTA and microcapsules were prepared (Figure 1C→D). Removal of $CaCO_3$ was confirmed by energy dispersive X-ray analysis as no calcium (Ca) was detected in the microcapsule samples. The use of $CaCO_3$ microparticles as templates is advantageous compared to the use of PS or MF templates. Polyelectrolyte microcapsules made of PSS/PAH, were initially obtained by dissolving PS or MF microparticles under harsh conditions (e.g. pH 1.0). However, the removal of PS or MF has created problems in maintaining the integrity of the capsules [37, 54], and drug molecules should be loaded after the dissociation of the templates. By contrast, the use of $CaCO_3$ microparticles as templates for polyelectrolyte microcapsules has shown great promise for biomedical applications, since $CaCO_3$ particles can easily be dissolved in mild conditions by EDTA, and can be loaded with drug molecules during or after their preparation. In addition, the developed PLL/CS multilayered microcapsules are advantageous, to some degree, to the recently reported CS microcapsules, which were continually produced in a multi-loop reactor and had an average diameter of 1.0 mm [55]. This is because the LBL technique (i) allows the formation of microcapsules of sizes from hundreds of nm to mm [2, 32], (ii) permits the control over the shell thickness (in the nm scale), composition, and density, and (iii) enables the subsequent selective control of drug loading and release (Figures 4 and 6) by introducing a matrix (i.e. CS) within PLL/CS microcapsules.

It is known that the interior of polyelectrolyte microcapsules can be used to encapsulate proteins, nucleotides, antibiotics, or other biologically relevant molecules, since polyelectrolyte multilayers developed by LBL are porous [56] and drug molecules can penetrate in and out of the capsules [57]. Meanwhile, the physicochemical differences between the capsule interior and exterior can be used to control the capture of drug molecules inside the capsules. Immobilization of a matrix, such as CS macromolecules, inside polyelectrolyte microcapsules may modify the physical properties (type of charge, charge density, hydrophobicity) of the capsule interior from those of its exterior medium. This may enable selective control of subsequent drug loading and release. In this study, CS macromolecules were integrated inside the polyelectrolyte microcapsules, and the influence of pH on subsequent drug loading and release were investigated. We showed that FITC-BSA, a model protein drug, was loaded inside the CS integrated microcapsules (Figure 5). Electrostatic interaction was the main driving force for BSA incorporation (Figure 1 D→E), while other forces such as hydrophobicity might also play a role in drug loading as a small amount of FITC-BSA was loaded at pH 5.0 (Figure 4). The CS macromolecules inside the microcapsules are negatively-charged, while BSA has no net charge at its isoelectric point (pI, i.e. pH 5.0 [58]) and has more net positive charges as the pH decreases. As a result, the microcapsules had the lowest loading of BSA at pH 5.0 (Figure 4) in the pH range 3.4–5.0. Therefore, by integrating CS macromolecules inside the capsules and by choosing the right

pH conditions, charged drug molecules, e.g. FITC-BSA, could be loaded into microcapsules, and more FITC-BSA was loaded into the CS integrated microcapsules at a lower pH. The decrease of BSA loading at pH 3.4 compared to that of pH 3.8 might be associated with a possible structural change of BSA.

pH can also be used to trigger or tune the release of encapsulated drug molecules from CS integrated microcapsules. Figure 6 shows that the encapsulated FITC-BSA could be released from the CS integrated microcapsules and its release was pH controlled. The concentration gradient between the microcapsule and its environment causes diffusion of FITC-BSA out of the microcapsule. It is believed that any release condition that weakens the interaction between BSA and the CS inside the microcapsule will lead to a faster releasing rate and higher releasing amount. The electrostatic attraction of BSA and CS decreases as pH increases from pH 1.0 to 7.4. This is because BSA has net negative charges above its pI (pH 5.0) and net positive charges below its pI, and CS is negatively-charged. As a result, BSA and CS repel each other at a high pH (e.g. pH 7.4) and attract each other at a low pH (e.g. pH 1.0). This leads to a faster release of BSA at a higher pH (Figures 6 A and B).

In summary, a biodegradable drug delivery system with controllable loading and release properties was developed. We integrated CS macromolecules, intended to facilitate the control of subsequent drug loading and release via pH, into $CaCO_3$ particles during the preparation of the particles. We formed a multilayered shell on the CS integrated $CaCO_3$ particle using LBL nanotechnology. After dissociation of the $CaCO_3$ templates, biopolymer multilayered microcapsules integrated with CS were obtained. A model drug, i.e. FITC-BSA, was loaded and its release was studied. pH-controlled loading and release behavior was observed. Substantially more BSA was loaded at pH 3.8 than at pH 5.0 (i.e. pI of BSA), and the release of BSA was faster at a higher pH. With further research still to be done, CS integrated biopolymer microcapsules appear promising for controlled drug delivery and other release applications.

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List of Abbreviations

CS	chondroitin sulfate
FITC-BSA	bovine serum albumin labeled with fluorescein isothiocyanate
PSS	poly(styrene sulfonate)
PAH	poly(allylamine)
MF	melamine formaldehyde
PS	polystyrene
$CaCO_3$(CS)	$CaCO_3$ particles integrated with CS
PLL	poly-L-lysine
GA	glutaraldehyde
EDTA	disodium ethylenediaminetetraacetic acid
ICP-OES	inductively coupled plasma optical emission spectroscopy

LBL	layer-by-layer
SEM	scanning electron microscopy
CLSM	confocal laser scanning microscope
pI	isoelectric point

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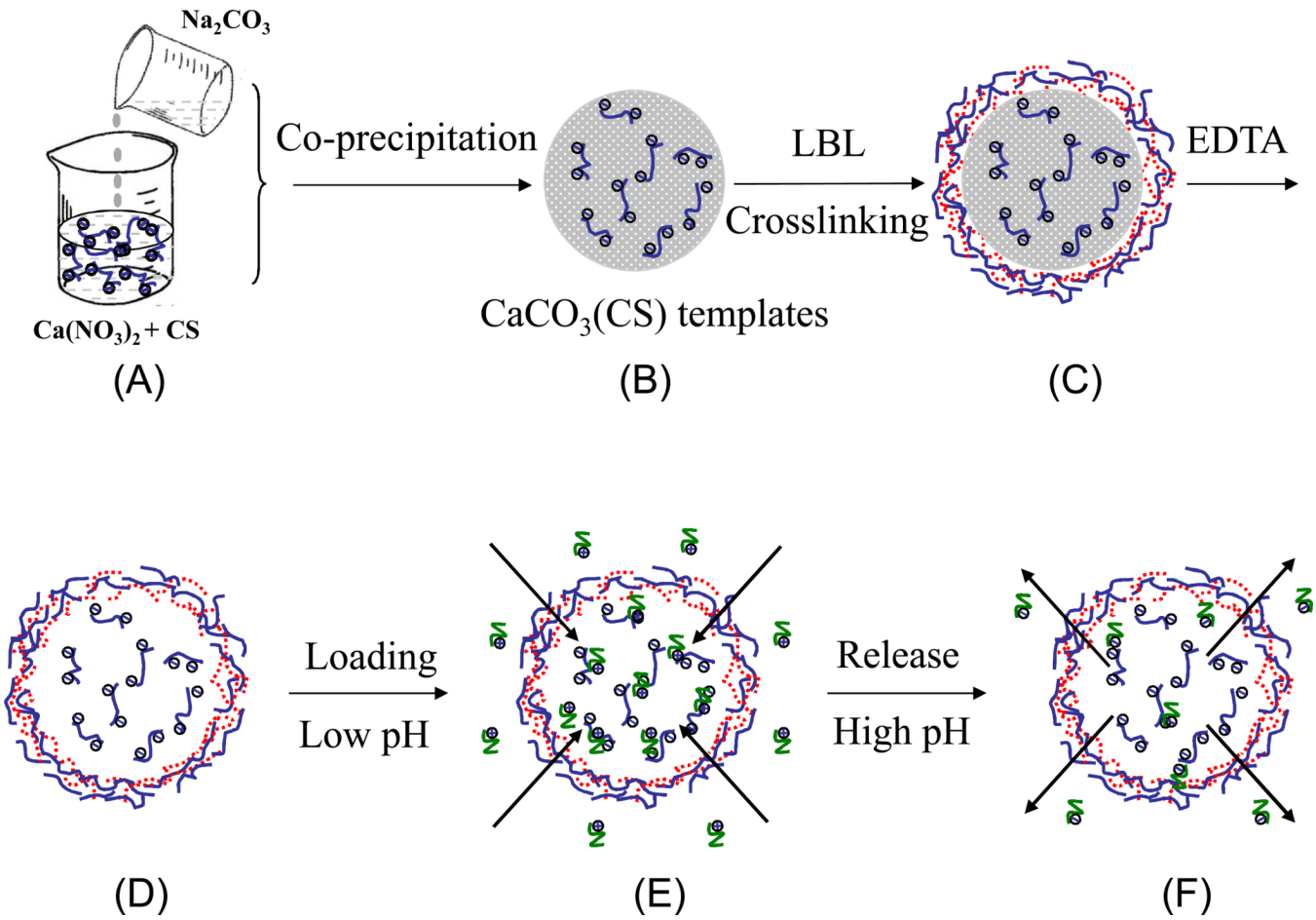


Figure 1.

Schematic diagram for the preparation of CS integrated multilayered microcapsules and related loading and release of FITC-BSA. (A→B): preparation of CS integrated $CaCO_3$ templates, (B→C): self-assembly and crosslinking of multilayered shell on $CaCO_3$(CS) templates, (C→D): decomposition of the $CaCO_3$ templates and formation of CS integrated multilayered microcapsules, (D→E): loading of BSA into CS integrated microcapsules at a pH at which BSA has net positive charges, and (E→F): release of BSA from CS integrated microcapsules at neutral pH where BSA has net negative charges. CS, crosslinked multilayered shell, BSA. The net charge of BSA is positive below its pI (pH 5.0) and negative above its pI.

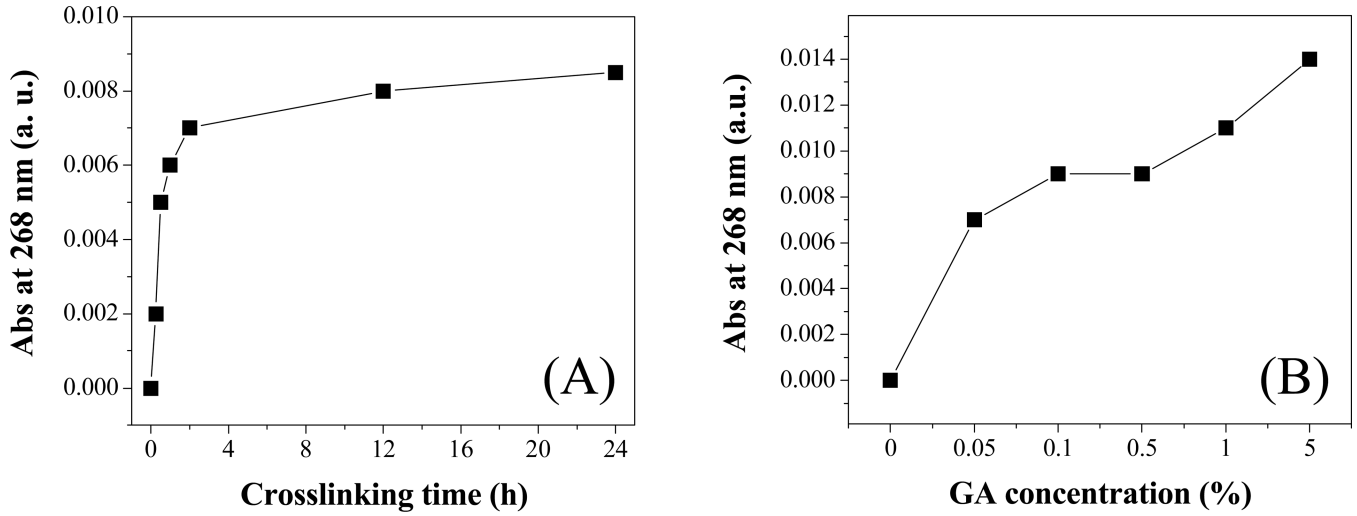


Figure 2.

Absorbance of $(PLL/CS)_5$ multilayers on quartz slides (A) after crosslinking using 0.5% GA as a function of time, and (B) after crosslinking with different concentrations of GA for 24 h.

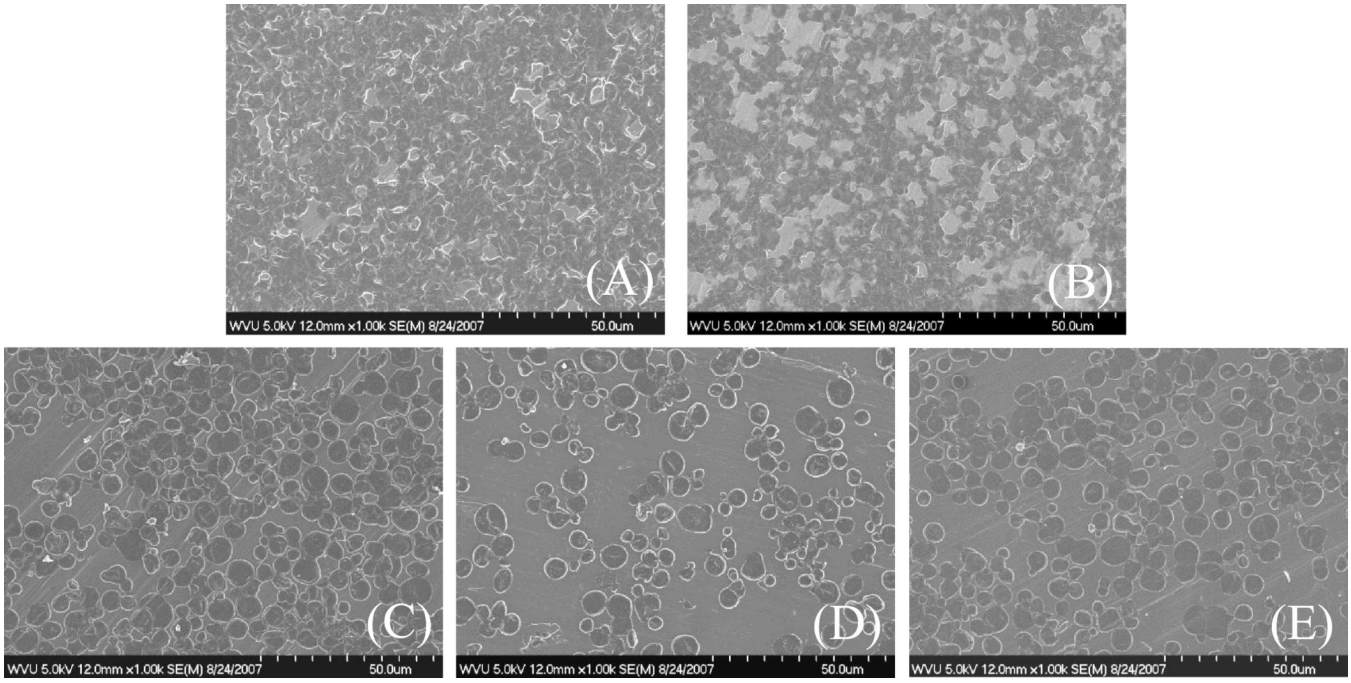


Figure 3.
SEM images of $(PLL/CS)_5$ microcapsules crosslinked for 24 h using GA at a concentration of (A) 0.05%, (B) 0.1%, (C) 0.5%, (D) 1%, and (E) 5%.

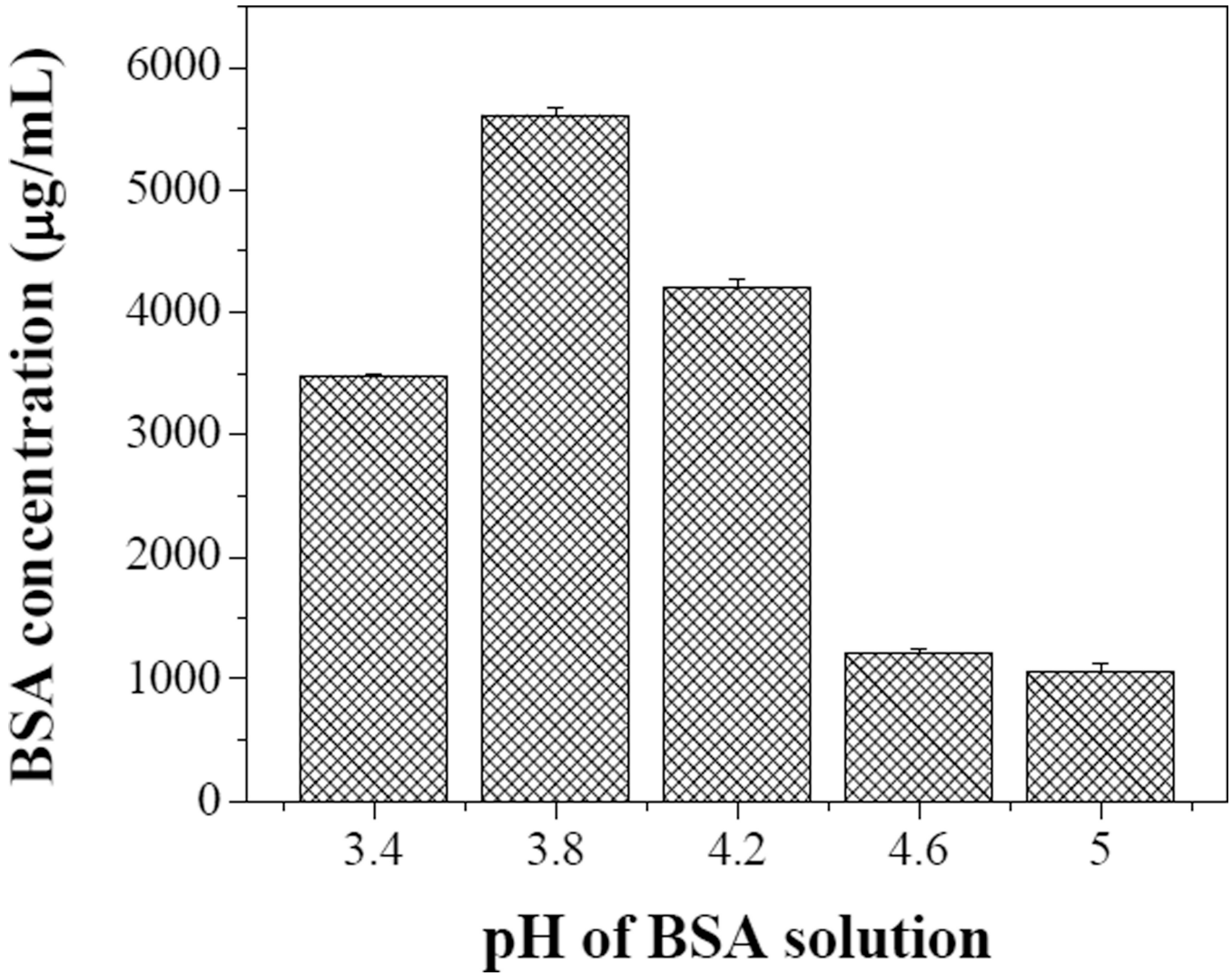


Figure 4.
FITC-BSA concentrations in the microcapsule interior as a function of pH of BSA solution.

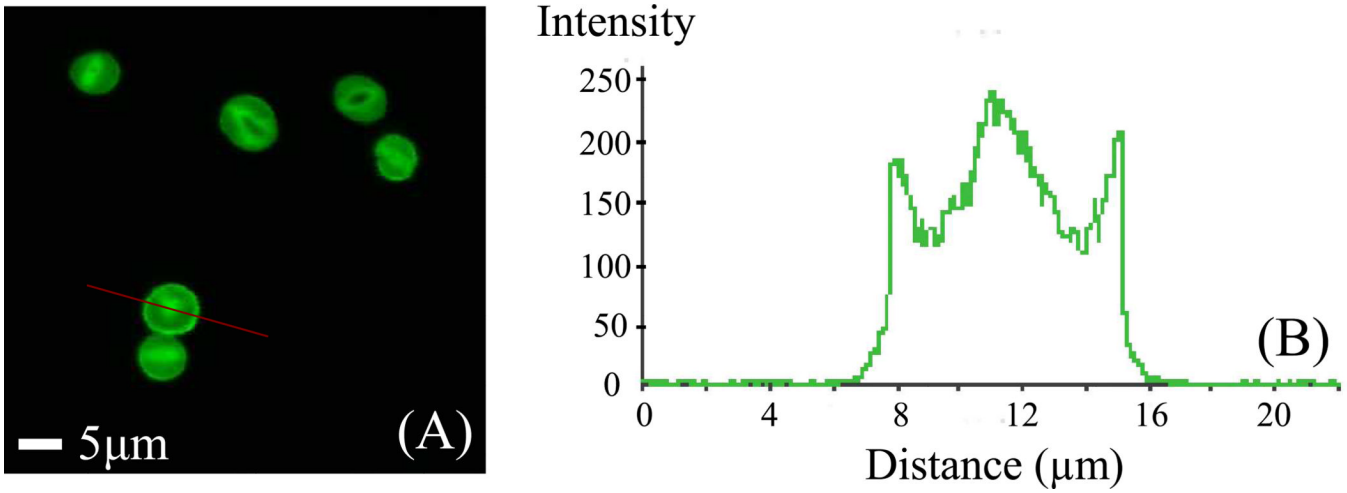
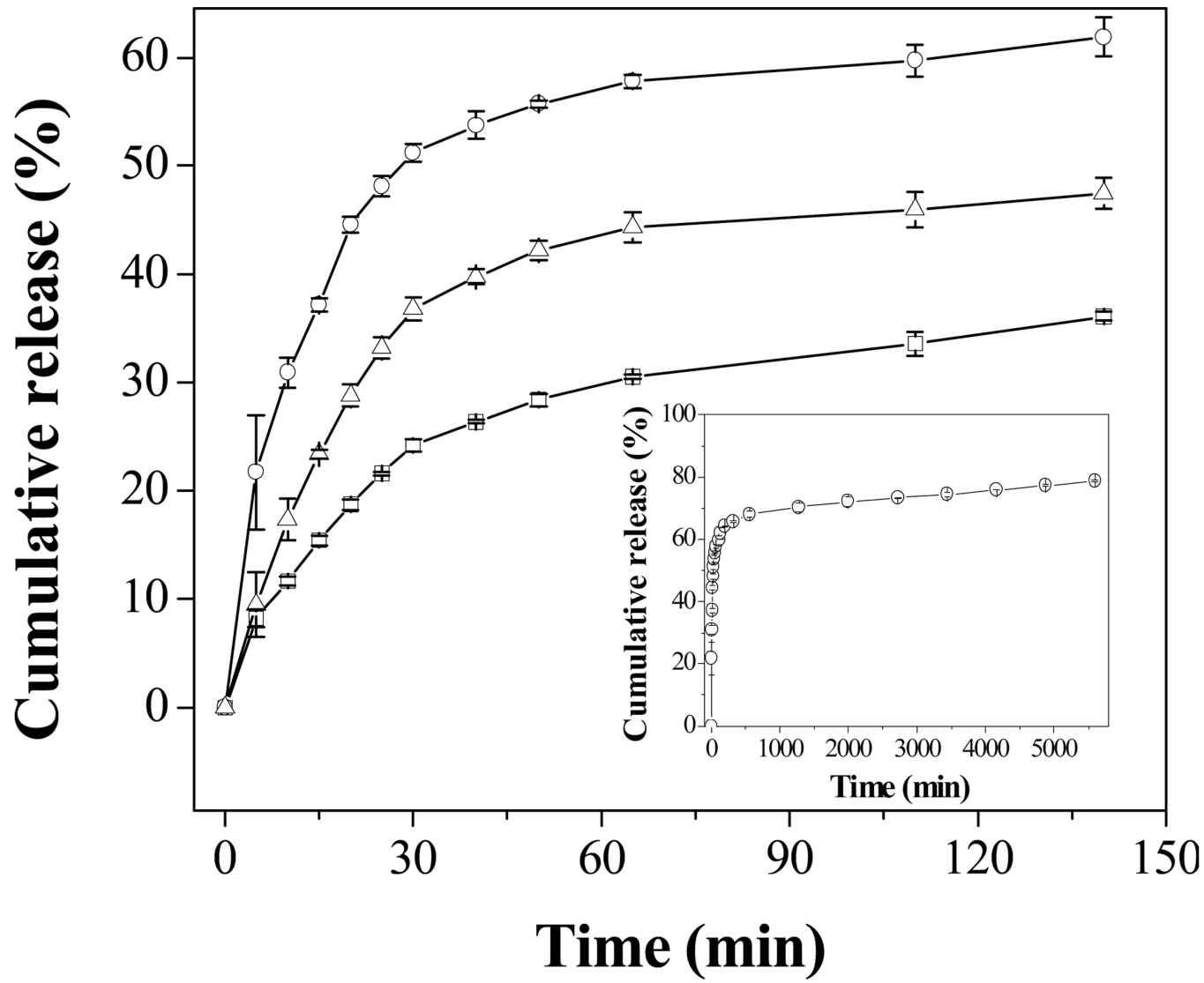


Figure 5.
Loading of FITC-BSA within CS integrated $(PLL/CS)_5$ microcapsules at pH 3.8. (A) CLSM image, and (B) fluorescence profile of the microcapsule shown in (A).



A.

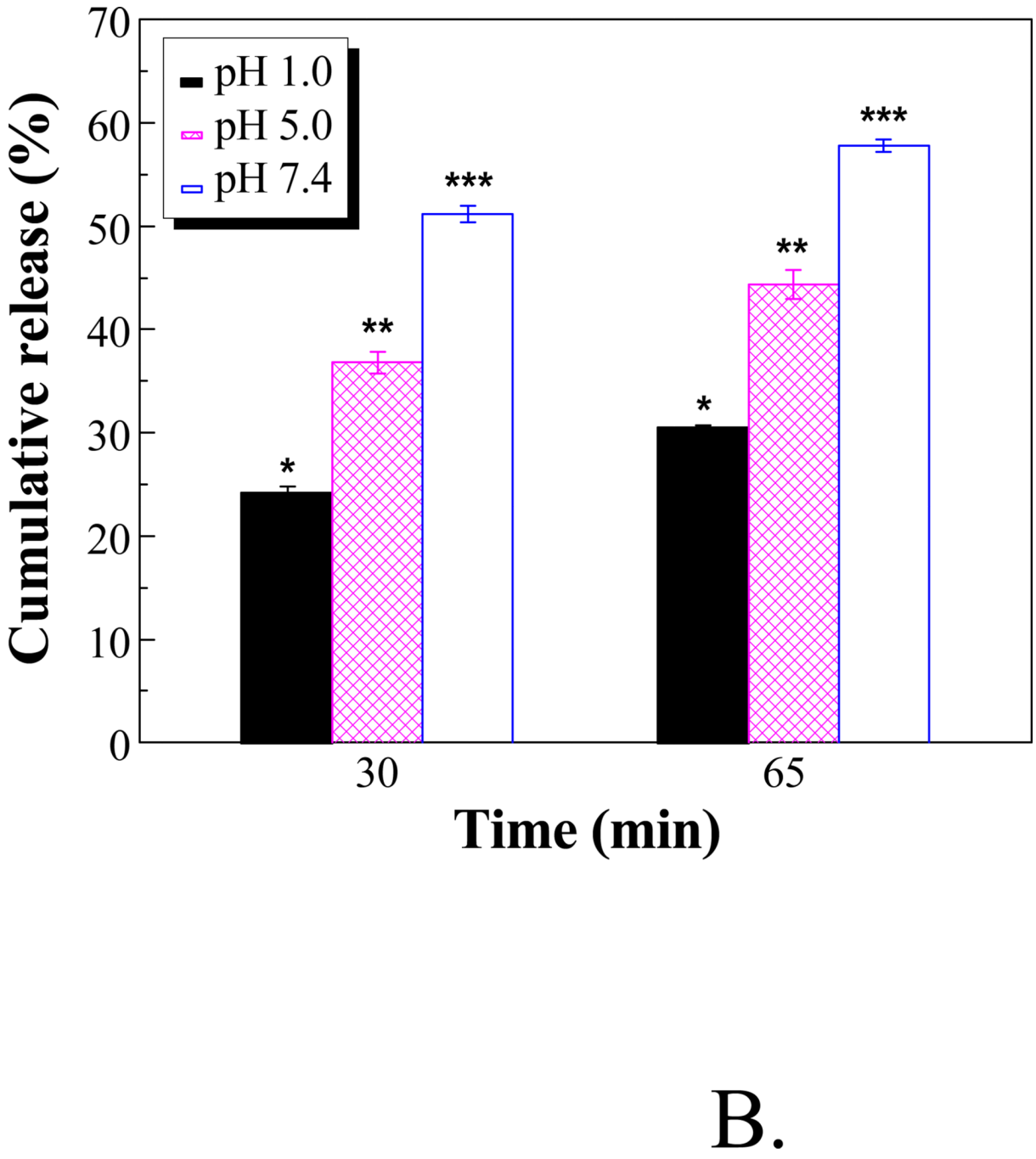


Figure 6.

FITC-BSA cumulative release from CS integrated microcapsules *vs.* incubation time. (A) Release profiles at pHs 7.4, 5.0, and 1.0. The inset is the release of FITC-BSA at pH 7.4 for up to 5,600 min. (B) Release of FITC-BSA at 30 and 65 min. *Release was significantly different between the pH values tested. Release was conducted at 37 °C at pH 7.4 (○), 5.0 (△), and 1.0 (□) solutions.