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Self-Assembly of Polyamines as a Facile Approach to Fabricate Permeability Tunable Polymeric Shells for Biomolecular Encapsulation

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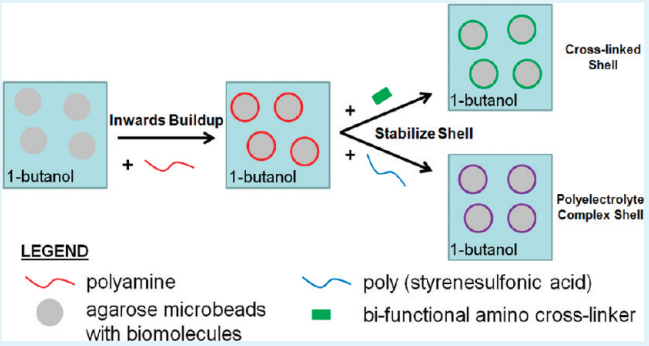
Supporting Information

ABSTRACT: In this article, the self-assembly of polyamines as a facile approach to fabricate permeability tunable polymeric shells for encapsulation of relatively low molecular weight (LM_w) hydrophilic biomacromolecules ($M_w \approx 4000$ Da) is presented. The entire process is performed in organic solvents within 2 to 4 h to allow for nearly 100% encapsulation yield. The polymeric shells are fabricated by a two-step process: 1) The self-assembly of polyamines (nonionized poly(allylamine) (niPA) or branched nonionized polyethyleneimine (niPEI)) within porous agarose microbeads via an inwards buildup self-assembly process. 2) Stabilization of assembled polyamines either via covalent (cross-linkers) or ionic bonding (complex with nonionized poly(styrene sulfonic acid) (niPSS)). Stable and distinct polymeric shells are formed in both cases. The shell thickness is demonstrated to be tunable within a range of 1 to 14 μm; and as the inwards buildup self-assembly technique is not a self-limiting process, shells with broader thicknesses can be achieved. Also, it is demonstrated that the polymer density of the shell can be tuned. Depending on the fabrication parameters, the resulting polymeric shells have been demonstrated to have different permeability characteristics for relatively LM_W dextran ($M_W \approx 4000$ Da). For example, niPEI shells are observed to have a higher permeability than niPA shells. Therefore, polymeric capsules can be fabricated via this facile approach for either retention of relatively LM_w hydrophilic biomacromolecules or designed to passively or responsively release the biomacromolecule payload. This two-step shell fabrication process represent an alternative and facile approach for the fabrication of self-assembled polymeric shells in the fields of capsule-based reactors/sensors and drugs/gene delivery where relatively LM_w macromolecules are concerned.



KEYWORDS: core−shell materials, self-assembly, inwards buildup, macromolecules, microencapsulation

INTRODUCTION

Encapsulation of various materials within polymeric capsules is of growing importance as exemplified by the many different applications. Polymeric micelles are frequently used as drug[1] or gene[2] delivery vehicles and polymersomes have been utilized as enzyme nanoreactors.[3] layer-by-layer (LbL) polymeric capsules have been widely applied in many fields such as bioreactors,[4] biosensors,[5] drug delivery,[6] tissue engineering,[7] and as immunization tools.[8]

To achieve successful encapsulation of hydrophilic macromolecules for biomedical applications such as biosensors and bioreactors, it is critical that leakage of the encapsulated biomacromolecules is minimized while ensuring that the bioactivity of these biomacromolecules are maintained; whereas for drug/gene delivery applications, the capsules must be able to passively or responsively release the encapsulated materials. Of the many methods available to fabricate polymeric capsules, the LbL polymer self-assembly technique is the most common method utilized for encapsulation of hydrophilic biomacromolecules because of the simplicity involved and stability of capsules produced.[9] Also, using microfluidics for the automation of the LbL technique and with potential high throughput has been reported.[10] Through the process of polymer self-assembly via the LbL technique, encapsulation of enzymes have been demonstrated as possible capsule-based bioreactors[4] or biosensors;[5] and DNA has been encapsulated and presented as potential gene delivery systems.[11] Various responsive release mechanisms for encapsulated materials have been realized with the conventional aqueous LbL technique. For example, LbL polymeric capsules that respond to either changes in pH,[12] salt[13] conditions or specifically to optical irradiation,[14] in a mimicked reductive cellular environment[15] or in the presence of a specific ligand[16] have been established.

Recently, we have demonstrated the encapsulation of hydrophilic molecules within polymeric capsules via the Reverse-Phase

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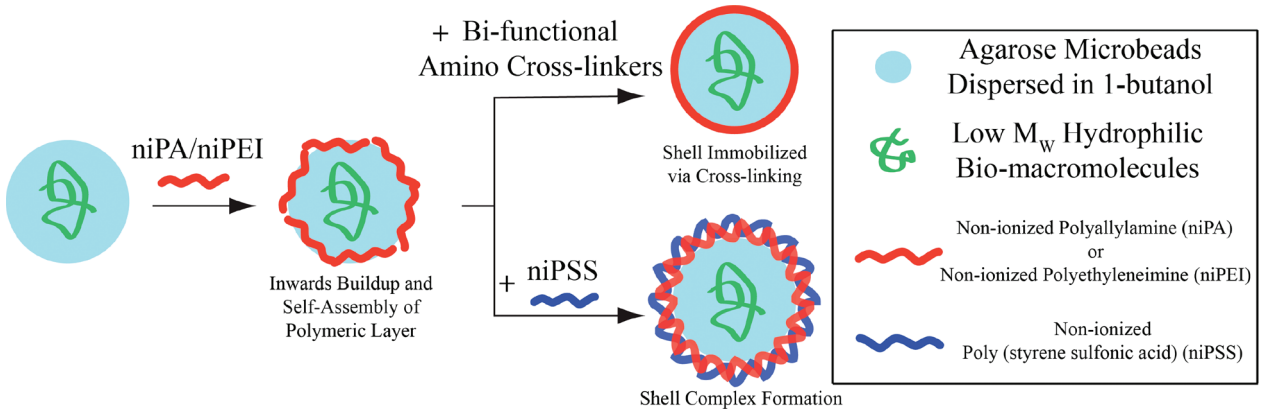

Figure 1. Schematic diagram illustrating the fabrication process of permeability tunable polymeric shells in two steps for biomolecular encapsulation.

LbL (RP-LbL) technique.[17] It was also demonstrated that high encapsulation efficiency and retention stability of encapsulated biomacromolecules can be achieved within RP-LbL fabricated capsules on agarose template cores.[18] We have also developed the inwards buildup self-assembly technique for the simultaneous encapsulation of biomacromolecules and encoding of the polymeric capsules.[19] In the inwards buildup self-assembly technique, nonionized polyallylamine (niPA) was demonstrated to spontaneously diffuse into the matrices of porous agarose microbeads and self-assemble to form well-defined concentric polymeric layers within the microbeads. Cross-linking these layers with noncleavable bifunctional amino cross-linkers conferred stability to these layers. In both techniques mentioned, the polymeric capsules fabrication process was performed using an organic solvent to minimize loss of encapsulated biomacromolecules and model enzymes that were encapsulated had retained bioactivity.

Although many works involving self-assembled polymeric shells have presented the encapsulation of hydrophilic macromolecules, encapsulation of relatively low molecular weight (LM_w) hydrophilic macromolecules with high retention stability remains a challenge. This has recently been demonstrated through the heat treatment of LbL multilayer shells[20] where dextran (M_w 10 000 Da)[20b] and SIINFEKL (standard amino acid abbreviations) peptides[8] were effectively encapsulated and retained within the LbL polymeric capsules. Hydrogen-bonded polymer capsules have the potential to encapsulate relatively LM_w hydrophilic macromolecules as well but the payload loading was performed at acidic conditions.[21] Fabrication of LbL multilayer shells requires a multistep process (repeated incubation and washing) and the necessity of heating or acidic conditions to encapsulate or retain relatively LM_w materials would not be suitable for temperature or pH sensitive biomacromolecules respectively. Therefore, we sought to develop a facile approach to fabricate self-assembled polymeric shells that requires fewer steps, does not require heating or acidic conditions, and that can encapsulate relatively LM_w hydrophilic biomacromolecules (NB: In the following sections of this article, relatively LM_w hydrophilic biomacromolecules is defined as macromolecules of approximately M_w 4,000 Da). The permeability of the resulting polymeric capsules should also be tunable to achieve high retention stability or to allow for release of encapsulated biomacromolecules.

Here we demonstrate the self-assembly of polyamines for the facile fabrication of permeability tunable polymeric shells and encapsulation of relatively LM_w hydrophilic macromolecules. First, porous agarose microbeads containing the biomacromolecule payload are formed through an emulsification process. Then, the shells are formed by a two-step process. Polyamines (nonionized poly(allylamine) (niPA) or branched nonionized polyethyleneimine (niPEI)) are first self-assembled and deposited within the peripheral matrices of porous agarose microbeads dispersed in 1-butanol (Figure 1) via an inwards buildup self-assembly technique. Next, the polyamine assembly is stabilized via either using a cross-linker or using nonionized poly(styrene sulfonic acid) (niPSS) to form covalent or ionic bonds respectively. In both cases, stable polymeric shells encapsulating the biomacromolecule payload is formed. If the last stabilization step is not performed, the deposited polyamines will disperse when the microbeads are transferred into an aqueous dispersant. Therefore in this work, stabilization of the self-assembled polyamines is absolute essential to form water stable polymeric shells. In contrast to the LbL polymer self-assembly technique, the inwards buildup polymer self-assembly technique is not self-limiting and the thickness of the polymeric shells is proportional to the polyamine concentration and incubation time. Therefore, polymeric shells of any micrometer thickness can be achieved in two-steps as described in this article; instead of performing numerous steps via the LbL technique to obtain polymeric shells of similar thicknesses. In addition, the density of the polymeric shells of this work can be tuned by varying the agarose concentration (%). We demonstrate that by changing the fabrication parameters (type of polyamine, agarose %, polymeric shell stabilizing approach, incubation time, and the type of cross-linker), polymeric shells of different permeability properties toward relatively LM_W biomacromolecules can be fabricated. These shells will be useful for either very high retention or passive/responsive release of encapsulated relatively LM_W biomacromolecules.

EXPERIMENTAL SECTION

Materials. Dextran-FITC M_w 4000 Da, dextran-TRITC M_w 65 000−76 000 Da, insulin-FITC from bovine pancreas, branched polyethyleneimine (PEI) M_w 25 000 Da, fluorescein isothiocyanate (FITC), 3,3′-dithiodipropionic acid di(N-hydroxysuccinimide ester) (DSP), DL-dithiothreitol (DTT), hydrochloric acid 1.0 N (HCl), 1-butanol anhydrous 99.8%, sodium nitrite and mineral oil were

purchased from Sigma. Span 80 was purchased from Fluka. Poly(allylamine) (PA) M_w 65 000 Da, 4-(2-aminoethyl)aniline 97% and ADOGEN 464 were purchased from Aldrich. Poly(styrenesulfonic acid) (PSS) 30% solution in water M_w 70 000 Da was purchased from Polysciences Inc. Low-melting agarose was purchased from Promega. Absolute ethanol was purchased from Fisher Scientific. PBS was purchased from BASE. Dimethyl sulfoxide (DMSO) was purchased from MP Biomedicals, Inc. Chloroform was purchased from BDH chemicals. All materials were used as received. Double distilled water (dd H_2O) used was distilled using a Fistreem Cyclone machine.

Preparation of Agarose Microbeads. An 8% w/v low-melting agarose in dd H_2O was prepared and kept molten at a temperature of 45 °C. The molten agarose was then mixed with the desired materials (fluorescence dextran or insulin) to prepare a mixture with the desired percentage of agarose containing the desired concentrations of materials. All reagents were prewarmed and kept at a temperature of 45 °C. The agarose/materials mixture was added to prewarmed mineral oil at 45 °C containing 0.1% Span 80 and stirred vigorously for 10 min to form water-in-oil emulsion droplets. Typical volume ratio of the agarose/materials mixture to mineral oil was 200 μL:5 mL. The droplets were then cooled in an ice water bath while stirring for another 10 min to allow solidification of the molten agarose droplets into agarose microbeads. The solidified agarose microbeads were further stabilized by placing at −20 °C for 5 min.

Preparation of Nonionized and Fluorescence Labeled Poly(allylamine) in 1-Butanol. Nonionized poly(allylamine) (niPA) in 1-butanol was prepared by placing the purchased PA solution at 65 °C overnight to remove any water content, followed by fully saturating 1-butanol with the dried PA. One mL of the saturated 1-butanol was dried, weighed and the solution was then diluted with 1-butanol to prepare a 1 mg mL^{-1} niPA solution. This was used as stock solution. Fluorescence labeled niPA was prepared by dissolving and reacting FITC with niPA in 1-butanol at a ratio of 1:100 (fluorescence monomer: PA monomer). Lower concentrations of niPA/niPA-FITC were prepared by diluting the stock solutions.

Preparation of Nonionized and Fluorescence Labeled Polyethyleneimine in 1-Butanol. Nonionized polyethyleneimine (niPEI) in 1-butanol was prepared by keeping the niPEI solution at 65 °C overnight to remove any water content. Next the niPEI was dissolved in 1-butanol at a concentration of 10 mg mL^{-1}. This was used as the stock solution. Fluorescence labeled niPEI was prepared by dissolving and reacting FITC with niPEI in 1-butanol at a ratio of 1:100 (fluorescence monomer: PA monomer). Lower concentrations of niPEI/niPEI-FITC were prepared by diluting the stock solutions.

Preparation of Nonionized and Fluorescence Labeled Poly(styrenesulfonic acid) in 1-Butanol. Nonionized poly (styrenesulfonic acid) (niPSS) in 1-butanol was prepared by keeping the niPSS solution at 65 °C overnight to remove any water content. Next the niPSS was dissolved in 1-butanol at a concentration of 5 mg mL^{-1}. This was used as the stock solution. Synthesis of fluorescence labeled nonionized polystyrenesulfonic acid (niPSS) was carried out by a photoinitiated condensation reaction between PSS moieties and a FITC-diazonium salt precursor. First, FITC and 4-(2-aminoethyl)aniline were dissolved and mixed in DMSO at a molar ratio of 1:1 to result in a FITC-aniline derivative as previously described.[22] This FITC-aniline derivative solution is then mixed with the as purchased PSS solution at a molar ratio of 1:50 (i.e., FITC derivative: PS monomer), followed by conversion of the FITC-aniline derivative into the diazonium salt by addition of excess sodium nitrite (∼100 times) in an ice bath. No additional acidification was necessary to generate the diazonium salt as PSS has sufficient acidic strength in an aqueous solution. The cooled mixture was exposed to UV light (λ = 365 nm, 7−8 mJ/cm^2 s) for 5 s in a UVC 500 Cross-linker (GE Healthcare, United Kingdom) which caused the condensation of sulfonic acid moieties with diazonium moieties and formed a aromatic sulfonic ester bond. The resulting PSS-FITC solution was acidified with HCl (to pH 2) to deionize the polymers and purified by repetitive washing and centrifugation with a Vivaspin 20 membrane (50,000 Da MWCO) centrifugation column (Sartorius Stedim Biotech S.A, France). The purified aqueous solution containing the PSS-FITC solution was filtered through a syringe filter with pore size of 0.22 μm and dried. niPSS-FITC solutions were obtained by dissolution of the dried PSS-FITC in 1-butanol at a concentration of 5 mg mL^{-1}. Lower concentrations of niPSS/niPSS-FITC were prepared by diluting the stock solutions.

Fabrication of Polymeric Capsules via the Inwards Build-up Technique. To transfer the agarose microbeads from oil to 1-butanol, 1 mL of ethanol containing 0.5% ADOGEN 464 was added to the agarose microbead-in-oil suspension and mixed vigorously, followed by centrifugation. The mineral oil and ethanol supernatant was then discarded and the pellet containing the agarose microbeads was washed with 1-butanol containing 0.5% ADOGEN 464. The resulting agarose microbeads were then incubated with the desired concentrations of niPA/niPA-FITC or niPEI/niPEI-FITC in 1-butanol containing 0.5% ADOGEN 464 for 30 min under gentle vortexing, followed by removal of excess polymer and washing with 1-butanol. Typically, 200 μL beads were incubated with 1.5 mL of the polymer solution. This forms the concentric polymeric layer. To stabilize this concentric polymeric layer (or to form the polymeric shell), we incubated the microbeads either with excess DSP (10 mg mL^{-1} in chloroform) for 2 h to cross-link the polyamines or with the desired concentration of niPSS in 1-butanol containing 0.5% ADOGEN 464 for 30 min to complex with the polyamines. To transfer the polymeric capsules from 1-butanol to PBS, we washed the polymeric capsules twice with 1-butanol, twice with ethanol and then with PBS/ethanol solutions of increasing PBS content (0.01×; 10% and 50%) before transferring to pure 0.01× PBS.

Tunable Shell Thickness and Density Studies. Polyamine concentration-dependent shell thickness studies of all polymeric capsules was performed using 2% agarose as template. Agarose % dependent shell thickness of self-assembled niPA-FITC and niPEI-FITC, was performed at incubation concentrations of 0.5 mg mL^{-1} and 0.25 mg mL^{-1} respectively. Shell thickness was measured from confocal images using ImageJ software (Scion Corp., USA) and average fluorescence intensity was obtained concomitantly from the linear measurement of shell thickness. (NB: measurements were made from confocal images of capsules ∼125 μm in diameter and dispersed in an aqueous phase). The average fluorescence intensity measurements correspond to the density of the polymeric shell. All confocal images were taken with the capsules dispersed in 0.01× PBS unless otherwise stated.

Determination of Polyamine Concentration Left in Supernatant. The microbeads were centrifuged after incubation with the desired niPA-FITC or niPEI-FITC solution and the supernatant was checked for fluorescence using a microplate reader, FLUOstar OPTIMA (BMG LABTECH, Germany). Bandpass filters with λ_{ex} 485 nm and λ_{emm} 520 nm were used for FITC detection. The concentration of polyamine left in the supernatant was estimated by comparing against the fluorescence of the stock niPA-FITC or niPEI-FITC solution used. The concentration of polyamine used was calculated by subtracting the concentration of polyamine left in the supernatant from the original concentration.

Relative Retention Efficiency Studies. Fluorescence dextran was first emulsified within agarose microbeads of desired percentage to a final concentration of 1 mg mL^{-1}. Next, the concentric polymeric layer within each microbead was fabricated using 1.5 mL of the desired concentrations of niPA or niPEI with an incubation time of 30 min. The polymeric layers were next stabilized with either DSP or with the desired concentration of niPSS. The retention efficiency study was performed by transferring these polymeric capsules containing the fluorescence labeled dextran into 0.01× PBS and analyzing the fluorescence intensity

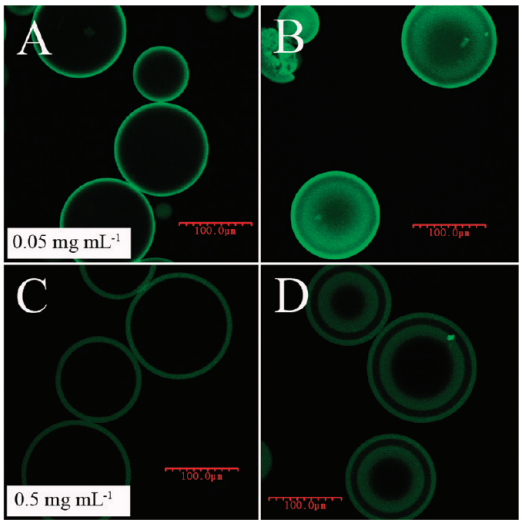

Figure 2. Confocal fluorescence images of agarose microbeads, dispersed in 1-butanol, after incubation with (A) niPSS-FITC, (B) niPSS-FITC/niPSS/niPSS-FITC, (C) niPEI-FITC, and (D) niPEI-FITC/niPEI/niPEI-FITC.

emitting from individual polymeric capsules (diameter ~100 μm); where the fluorescence intensity reflects the retention of fluorescence dextran. The zeroth day represents the fluorescence intensity measured immediately after transferring the samples from 1-butanol to 0.01× PBS and the fluorescence intensity of polymeric capsules obtained at the zeroth day were taken as 100% retention efficiency. The fluorescence intensities measured within individual polymeric capsules after dispersion in 0.01× PBS, at different days, compared to the fluorescence intensity of polymeric capsules at the zeroth day reflects the relative retention efficiency.

Encapsulation of Insulin-FITC within niPA/niPSS Polymeric Capsules. Insulin-FITC was first emulsified within 4% agarose microbeads to a final concentration of 1 mg mL^{-1}. Next, the polymeric shell within each microbead was fabricated using 1.5 mL of 0.5 mg mL-1 of niPA and stabilized with an equivalent amount of niPSS. An incubation time of 30 min was used for each polymer solution. The capsules were then transferred to 0.01× PBS and imaged immediately.

Optical and Fluorescence Microscopy. Phase contrast and fluorescence microscopic images were recorded using a CCD color digital camera, Retiga 4000R (QImaging, Canada) connected to a system microscope (Olympus BX41) with a mercury arc (Olympus HBO103W/2) excitation source. Bandpass filters with λ_{ex} 488 nm and λ_{emm} 520 nm were used for FITC detection; and λ_{ex} 540 nm and λ_{emm} 605 nm were used for TRITC detection. Images were captured with QCapture Pro software (Version 5.1.1.14, QImaging, Canada) and analyzed by ImageJ software (Scion Corp., USA). Confocal fluorescence microscopic images were captured using a laser scanning confocal microscope, FluoView FV300 (Olympus Corp., Japan).

RESULTS AND DISCUSSION

Inwards Buildup of Nonionized Polyethyleneimine (niPEI) and Nonionized Poly(styrenesulfonic acid) (niPSS) into the Matrices of Porous Agarose Microbeads. Previously, only nonionized poly(allylamine) (niPA) had been demonstrated for the inwards buildup self-assembly technique. Here, the use of niPEI and niPSS for the inwards buildup self-assembly technique is studied. One concentric layer of niPSS-FITC (0.05 mg mL^{-1}) and niPEI-FITC (0.5 mg mL^{-1}), fabricated via the inwards buildup technique, within the periphery of 4% agarose microbeads is depicted in images A and C in Figure 2, respectively. niPSS was selected as its polyelectrolyte form is well studied in the LbL field,[9,23] whereas branched niPEI was selected as it possesses primary amino side groups for cross-linking and is structurally different from niPA. It can be observed that, for the same incubation time, the concentric layers are of similar thickness for both sample sets. Analysis of the niPEI-FITC supernatant reveals that ~68% of incubated niPEI-FITC had been used to form the layer and this indicates that niPEI-FITC had formed a concentric layer of higher polymer density than niPSS-FITC. Repeated incubation with niPSS (Figure 2B) and niPEI (Figure 2D) for the purpose of encoding highlights that niPSS does not form well-defined concentric layers as compared to niPA and niPEI. niPSS and agarose are inherently "negatively charged" and could explain the resulting poor self-assembly phenomenon. The results also suggests that the self-assembly of polymers for the formation of well-defined concentric rings, via the inwards buildup self-assembly technique, is dependent on the availability of amino groups within the polymer chain for electrostatic interaction (see the Supporting Information S1) and preferential adsorption[19] with the agarose polymers; i.e., niPA and niPEI are suitable for the formation of dense and well-defined concentric polymer layers.

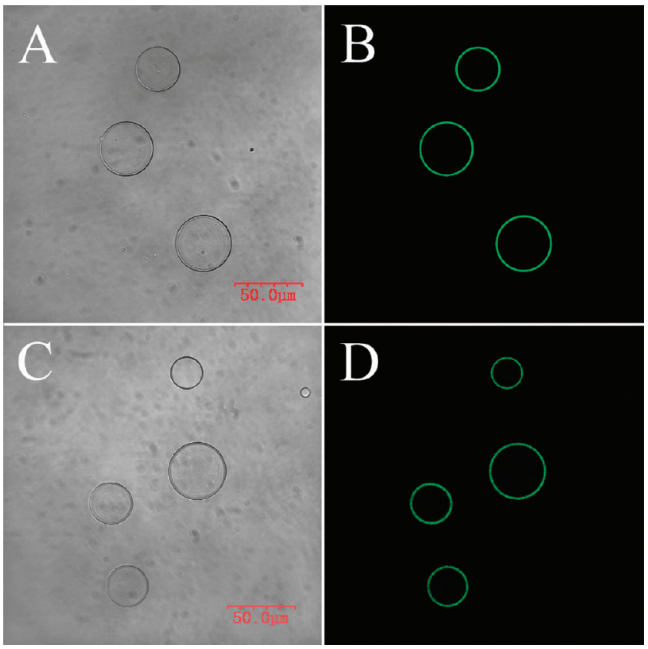

Figure 3. Optical transmission and corresponding confocal images of (A, B) cross-linked niPA-FITC polymeric capsules and (C, D) cross-linked niPEI-FITC polymeric capsules. The capsules are dispersed in aqueous phase.

Two-Step Fabrication Process of Cross-Linked Self-Assembled niPA and niPEI Polymeric Shells with Tunable Shell Thickness and Density. As highlighted in Figure 1, polymeric shells can be fabricated by a two-step approach within biomacromolecule loaded agarose beads: (1) self-assembly and deposition of polyamines via the inwards buildup self-assembly technique followed by, (2) "immobilization" of the polyamines with a bifunctional amino cross-linker. Figure 3 shows the optical and corresponding confocal images of cross-linked niPA-FITC

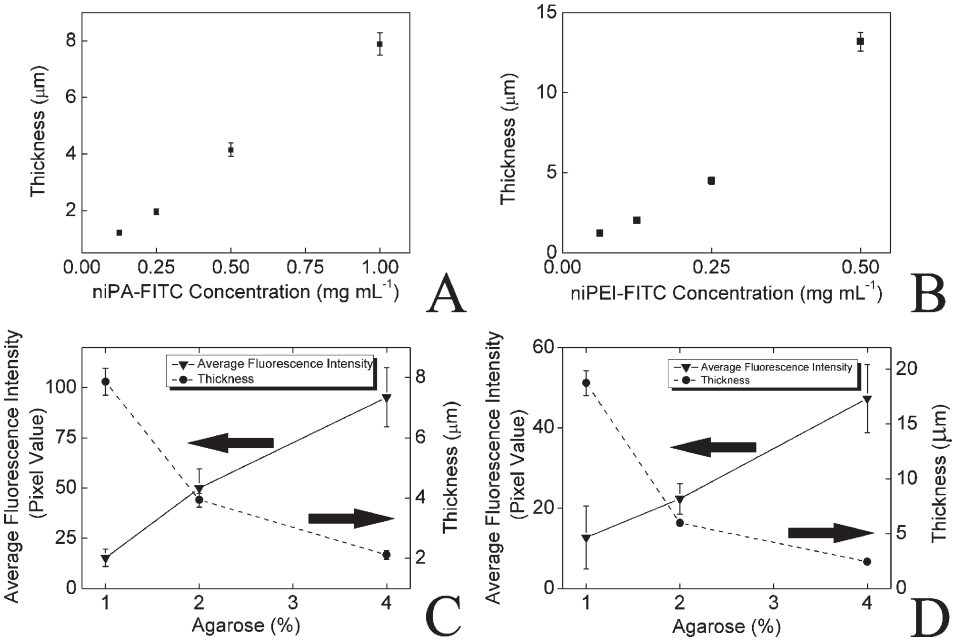

Figure 4. Shell thickness of cross-linked polymeric capsules as a function of incubated (A) niPA-FITC and (B) niPEI-FITC concentration. Average fluorescence intensity (▼) and shell thickness (●) as a function of agarose % for cross-linked polymeric capsules incubated with (C) niPA-FITC and (D) niPEI-FITC. The solid/dotted lines and arrows only serve to guide the eye.

(Figure 3A,B) and niPEI-FITC (Figure 3C,D) polymeric capsules dispersed in an aqueous phase. It is observed that the capsules are stably dispersed and do not collapse (contributed by having an agarose core). More interestingly, the polymeric shells of these cross-linked self-assembled niPA-FITC and niPEI-FITC capsules, fabricated via the two-step process, exhibit micrometer thickness that can be tuned by simply changing the concentrations of polyamines (Figure 4A,B, respectively). It can be clearly observed that the thickness of the shells is proportional to the concentration of polyamines. For example, incubation with 0.25 and 0.5 mg mL^{-1} of niPA will result in capsules with shell thickness of 2.0 and 4.2 μm, respectively.

Increasing the agarose concentration was observed to have resulted in a decrease of layer thickness and accompanied by an increase in average fluorescence intensity as depicted in panels C and D in Figure 4 for cross-linked niPA-FITC and niPEI-FITC polymeric capsules respectively. This intensity increment indicates that more polyamine is deposited per unit shell volume (or increment of polyamine density) when the template consist of more agarose polymers. It can thus be concluded that the polyamine density of the polymeric shells is a function of the agarose concentration (%) of the microbeads. It also further supports our conjecture that the concentric polyamine layers are formed by a self-assembly and deposition of the polyamines onto the agarose matrix and not by "filling up" the porosity within the agarose. Comparing the same unit concentration of niPA and niPEI used for the incubation period, it can be observed that niPEI always forms a thicker shell than niPA. Analysis and comparison of the polyamine supernatant after incubation reveals that more niPA (~15%) is consistently being packed into the shells. Combining these two observations, it highlights that self-assembled linear niPA always forms a denser polymeric shell than self-assembled branched niPEI. This difference in self-assembly and deposition mechanics is attributed to the different structures of niPA and niPEI. We believe that adsorbed branched niPEI creates a bigger steric hindrance for deposition of neighboring branched niPEI as compared to linear niPA that led to self-assembled niPEI shells of lower polyamine density.

In summary, the results here highlight a few points. First, polymeric capsules of cross-linked niPA or niPEI shells with micrometer thickness can be fabricated in two-steps. (1) Incubation of polyamines with agarose microbeads dispersed in 1-butanol and (2) addition of cross-linkers as "immobilizing" agents. Next, the shell thickness and polymer density of these cross-linked polymeric shells can be tuned by changing the polymer concentration and agarose % respectively; both of which are not characteristics manifested by the LbL technique. Lastly, the structure of the self-assembled polyamine will affect the properties of the polymeric shell; branched niPEI will result in a thicker but less dense polymeric shell than linear niPA of the same incubation concentration.

Two-Step Fabrication Process of niPA/niPSS and niPEI/niPSS Complex Polymeric Shells with Tunable Shell Thickness and Density. Alternatively, niPSS can be used to stabilize the self-assembled polyamines (Figure 1) through formation of polyelectrolyte complexes with the polyamines. Stable niPA-FITC/niPSS (Figure 5A,B) and niPEI-FITC/niPSS polymeric capsules (Figure 5C,D) could also be observed after dispersion in an aqueous phase, and the distinct peripheral fluorescence observed from both sets of polymeric capsules indicates the successful stabilization of self-assembled niPA-FITC and niPEI-FITC within the peripheral of agarose microbeads with niPSS. Figure 6A shows a series of confocal images, imaged with the same parameters, after incubation with decreasing amounts of niPSS-FITC (in 1-butanol and no washing) for a fixed concentration of incubated niPA (0.5 mg mL^{-1}). Using an excess of niPSS-FITC resulted in the diffusion of niPSS into the core of the microbeads and this is undesirable as niPSS might complex with encapsulated biomacromolecules and affect the bioactivity. Figure 6B shows a similar series of confocal images, imaged with

the same parameters, for incubated niPEI (0.5 mg mL^{-1}). For the niPEI system, insignificant niPSS-FITC was observed to have diffused into the core for all niPSS-FITC concentrations. This is presumably because adsorbed branched niPEI molecules have been reported to be able to associate with more interacting species.[24] Figure 6C and 6D shows the % thickness and average fluorescence intensity, as a function of niPSS-FITC concentration, analyzed from niPA/niPSS-FITC and niPEI/niPSS-FITC polymeric capsules, respectively, after dispersion in an aqueous phase.

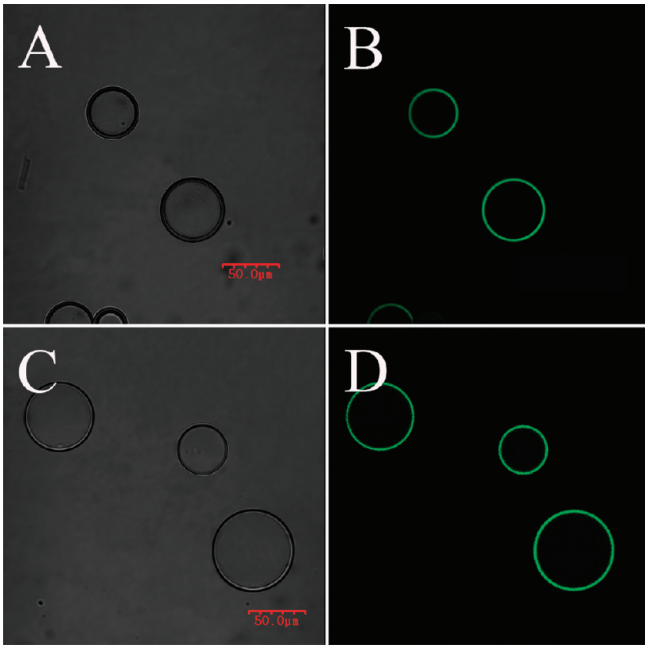

Figure 5. Optical transmission and corresponding confocal images of (A, B) niPA-FITC/niPSS polymeric capsules and (C, D) niPEI-FITC/niPSS polymeric capsules. The capsules are dispersed in aqueous phase.

The shell thickness was calculated as % thickness and is given by

$$\left(\text{shell thickness of capsules in aqueous phase}/\text{layer thickness (in 1-butanol) formed after incubation with 0.5 mg mL}^{-1}\text{niPA or niPEI}\right) \times 100\%$$

It clearly demonstrated that the thickness and polymer density of the shells are a function of the niPSS-FITC concentration. From the combined results as observed in Figure 6 and confocal line profile studies (see the Supporting Information, Figure S2), the optimum concentration of niPSS to be incubated with self-assembled layers formed with 0.5 mg mL^{-1} niPA and 0.5 mg mL^{-1} niPEI is 0.5 and 1.5 mg mL^{-1}, respectively, which translates to similar number of molecules to be incubated between the polyamines and niPSS. At these concentrations, almost 100% thickness of the polymeric shell is achieved and no niPSS is observed to have diffused into the capsules. Following these observations, further experiments involving the fabrication of niPA/niPSS and niPEI/niPSS polymeric capsules were carried out with equivalent mass ratios of niPSS to niPA but triple mass ratios of niPSS to niPEI. Given that the thickness and density of cross-linked polymeric capsules were tunable, the same experiments were performed for niPA/niPSS and niPEI/niPSS polymeric capsules. Not surprisingly, the thickness and density of niPA/niPSS and niPEI/niPSS polymeric shells were also found to be tunable by varying the concentration of polyamines and

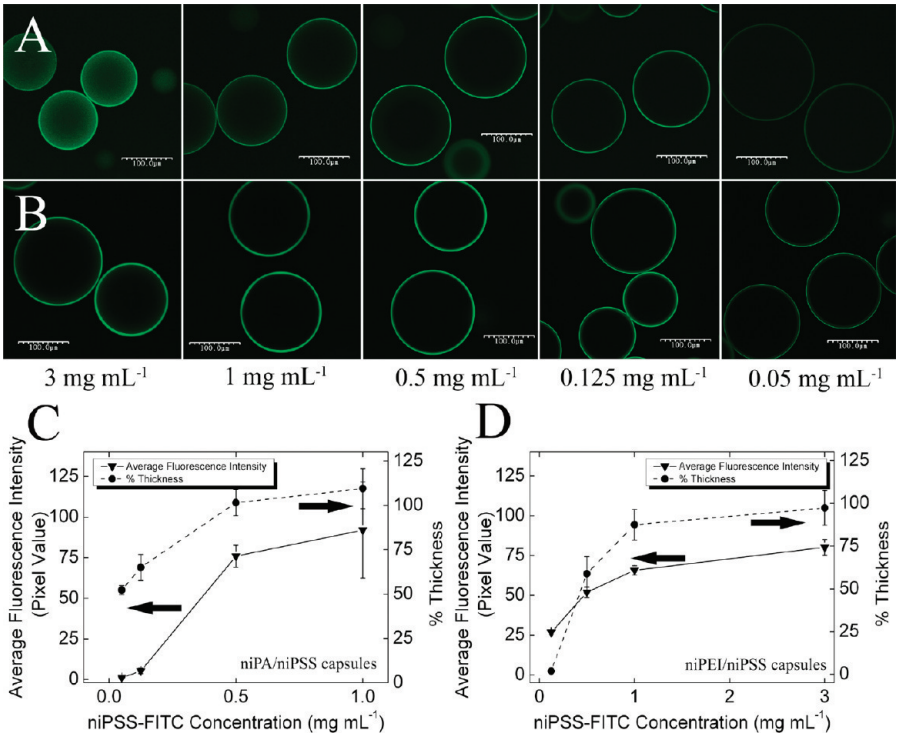

Figure 6. Confocal images of (A) niPA/niPSS-FITC and (B) niPEI/niPSS-FITC polymeric capsules, while dispersed in 1-butanol, after 30 min incubation with decreasing concentrations of niPSS-FITC. Average fluorescence intensity (▼) and % thickness (●) as a function of niPSS-FITC concentration for (C) niPA/niPSS-FITC and (D) niPEI/niPSS-FITC polymeric capsules that are dispersed in an aqueous phase.

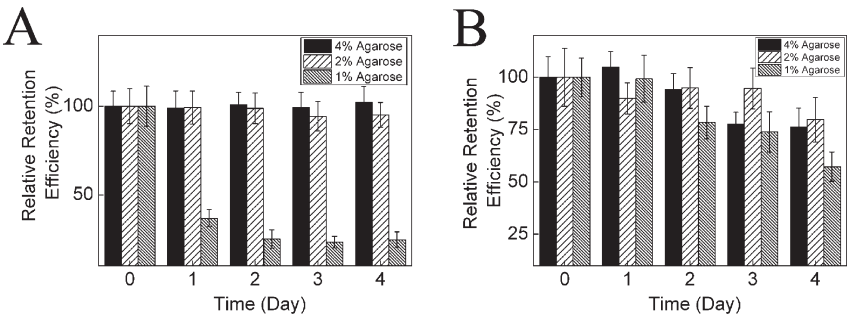

Figure 7. Relative retention efficiency of dextran-FITC (M_w 4,000 Da) within (A) cross-linked niPA and (B) cross-linked niPEI polymeric capsules fabricated using 4, 2, and 1% agarose as template. Incubation period was 4 days in an aqueous phase.

agarose % of microbeads, respectively (see Supporting Information S3). In addition, it should be noted that the polymeric shells of niPA/niPSS and niPEI/niPSS polymeric capsules also exhibit micrometer dimensions.

Tunability of Cross-Linked Polymeric Shells for High Retention or Release of Relatively LM_W Biomacromolecules. This next study encompasses the retention stability of relatively LM_W hydrophilic biomacromolecules within the cross-linked niPA and niPEI polymeric capsules (Figure 7); where dextran-FITC (M_w 4000 Da) was used as a model relatively LM_W biomacromolecule. Dextran-FITC cannot be cross-linked by amino cross-linkers used in this work and is a noncharged polymer. Performing retention stability studies is useful as it would reveal the permeability of the fabricated polymeric shells. Figure 7A shows the relative retention efficiency of the fluorescence dextran within cross-linked niPA (0.5 mg mL^{-1}) capsules fabricated with different agarose % and 4 days of incubation in an aqueous dispersant. High stability retention of ~100 and ~95% of the encapsulated dextran was observed within cross-linked niPA capsules fabricated with 4 and 2% agarose, respectively, whereas poor retention (~24%) and rapid passive release of encapsulated dextran was observed for cross-linked niPA capsules fabricated with 1% agarose and after 4 days of incubation. Using lower concentrations of niPA (0.25 and 0.125 mg mL^{-1}), i.e., formation of thinner polymeric shells, a rapid passive release of dextran (11% and 4.5% dextran-FITC remaining respectively) was also observed after two days of incubation in an aqueous phase (see the Supporting Information, Figure S4). This demonstrates that the capsule thickness (controlled by niPA concentration) and capsule density (controlled by the agarose %) of the cross-linked niPA shell strongly influences the permeability. With fewer polymers forming the shell, in terms of thickness or per unit shell volume, the shell gets more permeable and will allow encapsulated dextran to diffuse out more easily.

In contrast, polymeric capsules fabricated with cross-linked niPEI (0.25 mg mL^{-1}) did not exhibit high retention stability but demonstrated a continuous passive release of the encapsulated dextran; with 1% agarose polymeric capsules exhibiting the fastest release (43%) of encapsulated dextran after 4 days of incubation in an aqueous dispersant (Figure 7B). The increasing rate of passive release when the agarose % decreases further confirms that the agarose % is an important factor in controlling the permeability of the cross-linked polymeric shells fabricated by the two-step approach. niPEI with a concentration of 0.25 mg mL^{-1} was chosen to fabricate the shells as the resultant shells were of similar thicknesses with that of niPA (0.5 mg mL^{-1}) when 4% template agarose microbeads were used (Figure 4C,D). Given that the shell thickness was similar, the difference in retention stability observed for cross-linked niPA and cross-linked niPEI capsules indicates that niPEI forms polymeric shells of higher permeability and further supports our earlier observations that self-assembled branched niPEI forms less dense polymeric shells than linear niPA. The difference in poor retention efficiency as observed between cross-linked niPA and niPEI polymeric capsules fabricated with 1% agarose is probably due to the significantly thinner shell formed by cross-linked niPA (Figure 4C,D); thus highlighting that shell thickness also plays an important role in tuning the permeability of the polymeric shells.

Polymeric capsules have been designed for the responsive release of anticancer drugs in the presence of a reducing agent so as to mimic the responsive release of drugs within the reductive environment of cells.[15] To demonstrate the potential of these cross-linked capsules as responsive release vehicles, we have incorporated a cross-linker with a cleavable disulfide bond (3,3′-dithiodipropionic acid di(N-hydroxysuccinimide ester, (DSP)) into the fabrication process. A rapid disintegration of cross-linked niPEI shells was observed upon addition of 0.1 M DL-dithiothreitol (DTT) whereas the disintegration of cross-linked niPA shells was much slower (see the Supporting Informatin, Figure S5). This is presumably due to more disulfide bonds that had to be cleaved for disintegration as cross-linked niPA shells are denser. Disintegration of the cross-linked polymeric shells was initiated by cleavage of the disulfide bonds by DTT and which consequently causes the polyamines to dissolve and disperse into the surrounding aqueous phase. Without a stable polymeric shell, any encapsulated material can escape into the surrounding environment and this demonstrates the potential of using these cross-linked capsules as responsive release vehicles. Other cross-linkers of amino groups having different responsive release mechanisms include disuccinimidyl tartarate (central diol group, cleavable by sodium periodate) and bis-[2-(succinimidyloxycarbonyloxy)ethyl]sulfone) (central sulfone group, cleavable under alkaline conditions).

The results demonstrated in this section highlight that by using the two-step approach and selecting the appropriate parameters (i.e., linear or branched polyamines, incubation concentration of polyamines and agarose %) one can easily achieve polymeric shells of different permeability properties. This allows for the encapsulation of relatively LM_W biomacromolecules with high retention or for passive release; where the release rate may be tuned by changing the permeability of the cross-linked polymeric shells. Also, inclusion of a cleavable bifunctional amino cross-linker can allow for responsive release. Such capsule characteristics are especially useful for either

capsule-based biosensors/bioreactors or capsule-based macromolecular drug (such as dextran-doxurubicin[25] or poly(L-glutamic acid)-doxorubicin[26] conjugates)/gene delivery applications.

Biomacromolecule Retention Studies in niPA/niPSS and niPEI/niPSS Polymeric Capsules. niPEI/niPSS polymeric capsules (fabricated with 4% agarose and 0.5 mg mL^{-1} of niPEI) were preloaded with relatively LM_w dextran-FITC (M_w 4000 Da) but exhibited extremely poor retention stability. There was almost no fluorescence observed from individual capsules after transferring into an aqueous phase (data not shown); indicating immediate loss of encapsulated dextran-FITC. Therefore, no further retention studies concerning niPEI/niPSS capsules will be presented. However, these niPEI/niPSS capsules exhibited the potential for retention of higher M_w biomacromolecules (see the Supporting Information, Figure S6). In contrast, niPA/niPSS capsules (fabricated with 4% agarose and 0.5 mg mL^{-1} of niPA) exhibited high retention stability of encapsulated relatively LM_w dextran-FITC (M_w 4000 Da) during the initial two days of incubation in an aqueous dispersant and a subsequent slow passive release of encapsulated dextran over the next 2 days (see the Supporting Information, Figure S7). This result contrasts sharply with results obtained using niPEI/niPSS polymeric capsules and further emphasizes the fact that using niPEI results in polymeric shells of higher permeability than niPA. The slow passive release characteristic of niPA/niPSS polymeric capsules also differs from the high retention stability exhibited by cross-linked niPA polymeric capsules fabricated under the same parameters (Figure 7A) and can be attributed to the difference in stabilizing approach. Rearrangement of polymers within polymeric multilayer films have been induced either by changes in pH[27] or salt[28] conditions; and polymeric multilayer films assembled in salt conditions have been reported to become more porous after exposure to pure water.[29] Therefore, we believe that polymers assembled in an organic solvent will undergo polymer rearrangements after hydration and which may have resulted in the increase of permeability over time. Cross-linking of polymers, on the other hand, results in rigid and immobile polymers within the polymeric shell. We are in the process of understanding the structural properties of niPA/niPSS multilayers assembled from an organic phase and its changes after exposure to an aqueous environment, and these results will be published in future.

To summarize, these results have suggested that the stabilization approach of self-assembled polyamines also contributes to the permeability tuning of polymeric shells fabricated by the two-step approach. More notably, stabilization of self-assembled niPA layer with niPSS results in polymeric shells of unique permeability properties; where the shells are initially impermeable to encapsulated dextran (M_w 4000 Da) and gradually become permeable.

Encouraged by this results, we sought to encapsulate insulin (M_w of ~5800 Da), an important peptide hormone in the regulation of blood glucose, within niPA/niPSS polymeric capsules. Figure 8 shows the fluorescence image of niPA/niPSS polymeric capsules in an aqueous dispersant and with encapsulated insulin-FITC. High fluorescence intensity is observed from the interior of the capsules with little background noise from the surrounding dispersant; demonstrating the successful encapsulation of insulin-FITC within niPA/niPSS polymeric capsules fabricated by the two-step process. The low background signal indicates that immediate leaching of insulin-FITC from the capsules was not significant. Insulin has been demonstrated to retain its native-like secondary and tertiary structure in

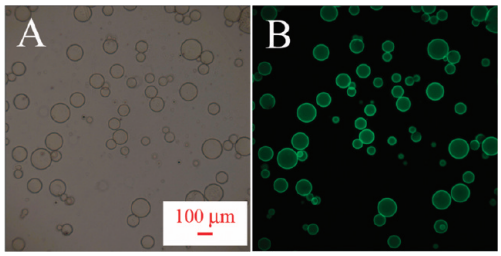

Figure 8. Optical (A) bright and corresponding (B) fluorescence images of niPA/niPSS polymeric capsules containing encapsulated insulin-FITC.

1-octanol[30] and was also shown to preserve its bioactivity after release from insulin-loaded microcapsules fabricated by a single phase oil-in-oil solvent evaporation method.[31] Given the stability of insulin, it is believed that the two-step complex formation approach introduced in this work will not significantly affect the biological properties of insulin. Also, coupling the observed successful encapsulation of insulin-FITC within niPA/niPSS polymeric capsules as highlighted in Figure 8 with capsule release characteristics as highlighted in the Supporting Information, Figure S7, it is reasonable to suggest that biocompatible insulin vessels with a timed release feature can be produced if biocompatible polymers (e.g., poly-L-lysine and poly-L-glutamic acid) are used with the two-step shell fabrication technique.

CONCLUSION

We have demonstrated the self-assembly of polyamines as a facile two-step approach for the fabrication of permeability tunable polymeric shells for biomolecular encapsulation. By using this approach, the shell permeability was tuned over a wide range to create capsules spanning from high retention to fast release of encapsulated biomacromolecules with relatively LM_w. The two-step approach involves an initial self-assembly of niPA or niPEI into the peripheral matrices of porous agarose microbeads via the inwards buildup self-assembly technique. Followed by stabilization of the self-assembled niPA or niPEI layer by covalent cross-linking or via electrostatic complex formation with niPSS. Self-assembled polymeric shells of micrometer thickness can be easily achieved by this two-step approach; and tunable shell thickness and polymer density were demonstrated by changing the concentration of polyamine and agarose % of the microbeads, respectively.

Retention studies using relatively LM_W dextran-FITC (M_w 4000 Da) demonstrates that depending on the fabrication parameters, the resulting polymeric shells will have different permeability properties. It was shown that polymeric shells fabricated from branched niPEI consistently resulted in shells of higher permeability than linear niPA. Using lower agarose % as template also resulted in shells of higher permeability and stabilization of polyamines with niPSS resulted in polymeric shells of different passive release characteristics. Inclusion of a cleavable cross-linker to stabilize the polyamine layer allows for responsive release of encapsulated materials. Therefore, as demonstrated in this work, self-assembled polymeric shells can be fabricated by a two-step approach to either retain encapsulated relatively LM_w hydrophilic biomacromolecules with high stability or can be designed to passively or responsively release the encapsulated biomacromolecules by simply manipulating the fabrication parameters. The two-step shell fabrication process

presented here represent an alternative, facile and exciting approach for the fabrication of polymeric capsules applied in the fields of capsule-based reactors/sensors and capsule-based delivery of polymeric drugs/genes where relatively LM_w hydrophilic macromolecules are concerned.

■ ASSOCIATED CONTENT

Supporting Information. FT-IR spectra of dried samples from agarose microbeads, agarose microbeads incubated with niPA and then transferred to d.d H_2O; and agarose microbeads incubated with niPA and retained in 1-butanol. Confocal fluorescence images and line plot profiles of niPA/niPSS-FITC and niPEI/niPSS-FITC capsules after 30 min incubation of niPSS-FITC (without washing) and also in aqueous phase. Shell thickness of niPA-FITC/niPSS and niPEI-FITC/niPSS polymeric capsules as a function of incubated niPA-FITC and niPEI-FITC concentration respectively. Average fluorescence intensity and shell thickness as a function of agarose % for niPA-FITC/niPSS and niPEI-FITC/niPSS polymeric capsules. Relative retention efficiency of dextran-FITC (M_w 4000 Da) within cross-linked niPA polymeric capsules fabricated using different niPA concentrations. Fluorescence images of niPEI-FITC and niPA-FITC polymeric capsules cross-linked with DSP before and 30 s after addition of 0.1 M DTT. Fluorescence intensity of supernatant as a function of time after addition of 0.1 M DTT to niPA-FITC polymeric capsules cross-linked with DSP and dispersed in an aqueous phase. Relative retention efficiency of dextran-TRITC (M_w 65 000−76 000 Da) within niPEI/niPSS polymeric capsules. Relative retention efficiency of dextran-FITC (M_w 4000 Da) within niPA/niPSS polymeric capsules. This material is available free of charge via the Internet at http://pubs.acs.org.

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