Influence of Different Polyelectrolytes on Layer-by-Layer Microcapsule Properties: Encapsulation Efficiency and Colloidal and Temperature Stability

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The fabrication of colloidal and temperature stable microcapsules for encapsulation of biomolecules based on matrix-assisted layer-by-layer (LbL) encapsulation by polyelectrolyte self-assembly has been demonstrated. In brief, the process is based on the emulsification of a hydrogel in warm oil for microdroplet formation. The hydrogel acts as a matrix for the later encapsulation process and can be loaded with biomolecules. After microdroplets of, for example, protein loaded hydrogel are formed by emulsification, cooling leads to solidification of the droplets to form microbeads, followed by encapsulation of the hydrogel microbeads with polyelectrolyte multilayers through an LbL self-assembly process to form polymeric capsules. Colloidal stability, encapsulation efficiency, and temperature stability of the LbL hydrogel microcapsules composed from different polyelectrolytes with various ionic strengths and charge densities have been studied. Microcapsules fabricated with strong polyelectrolytes showed better colloidal stability, while microcapsules fabricated with weak polyelectrolytes showed better encapsulation efficiency and temperature stability. After temperature treatment, microcapsules fabricated with different polyelectrolytes exhibited different morphological changes from complete rupturing over broken microcapsules with deformed hollow shells to intact microcapsules. Among all the studied polyelectrolyte pairs, the PAH/PSS polyelectrolyte system was found to be the best material to fabricate microcapsules with good colloidal and temperature stability and high encapsulation efficiency. Microcapsules with PSS as the outermost layer remained similar in size after temperature treatment, while microcapsules with PAH as the outermost layer shrunk by 76% in capsule volume. The present study provides a detailed overview on properties and design of LbL microcapsules as a function of polyelectrolyte materials and layer number. As a result of the versatility of loading LbL hydrogel microcapsules with various biomolecules or mixtures, potential applications are in the fields of diagnostics, drug delivery, and life sciences.

Introduction

Over the past decades, most biochemical reactions were performed in reaction flasks or reaction tubes. With the demands on performing a high number of parallel biochemical reactions for high throughput screening or diagnostics, microreaction tubes, microtiter plates, or microcavities were designed to achieve higher integration density. The use of small volumes for biochemical reactions is desirable because it reduces reaction time and reagent consumption and thereby cost and the amount of chemical waste produced. Microreaction tubes and microtiter plates have reaction compartments in a two-dimensional arrangement. Only by staging microtiter plates and using robotics are more reactions possible per given laboratory bench space, but the limitation of this technology is already reached. With the technology drive to further miniaturized biochemical reaction systems, microcompartments such as microcapsules suspended in a three-dimensional solution allow performing a very high number of individual reactions. The use of microcapsule suspensions is an alternative approach, and microcapsules are a promising "reaction tube" of the future. Interestingly, high concentrations of biomolecules/polymers leading to a "crowded environment" enhance biochemical reactions[1] and may in the same way improve performance of microcapsule based biochemical reactions.

The layer-by-layer (LbL) technology based on self-assembly of alternating cationic and anionic polyelectrolytes onto a template to form thin films is a proven method to design polymeric microcapsules. Microcapsules can be tailored to meet different requirements such as controlled capsule permeability,[2−4] biofunctionality,[5−7] biocompatibility,[8,9] and optical[10−12] and

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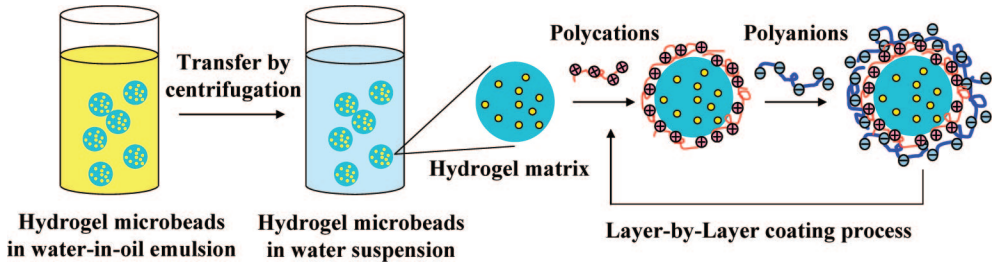

Figure 1. Schematic diagram illustrating matrix-assisted LbL encapsulation by polyelectrolyte self-assembly.

magnetic[13−15] properties. Various strategies for encapsulating biomolecules, drugs, reagents, and cells within LbL microcapsules have been reported. Tiourina et al. demonstrated the encapsulation of α-chymotrypsin into a hollow LbL microcapsule by using a pH induced pore opening/closing system.[16] The hollow microcapsules contain pores that were opened for α-chymotrypsin penetration at pH 6 and closed above pH 8. A similar strategy for encapsulation of urease into hollow LbL microcapsules based on dielectric induced pore opening/closing has been reported by Lvov et al.[17] The hollow microcapsule pores opened upon exposure to a water/ethanol mixture while closed in water. However, loading the protein into hollow microcapsules based on pH or dielectric switching may influence the activity of the proteins due to pH or hydrophobicity changes. Because such microcapsule filling systems are based on diffusion, only a very small percentage of the initial protein can be loaded and the loading concentration is limited to the concentration of the dissolved protein. Hollow LbL microcapsules have the tendency to collapse and are fragile, making their handling and manipulation difficult. An alternative approach for encapsulation of biomolecules by utilizing crystalline proteins as template for LbL coating, followed by dissolution of the encapsulated crystalline protein in buffer, was introduced by Caruso et al.[18] Although this method leads to very high concentration of protein within the microcapsule, a high osmotic pressure will develop after dissolution of the protein crystal, and therefore microcapsules with high mechanical stability are necessary.

Coating of polyelectrolyte multilayers on the flat hydrogel surface to control human blood coagulation was demonstrated by Sakaguchi et al.[19] Recently, LbL coating of hydrogel beads to develop drug delivery systems have been introduced.[20,21] However, the studies on influence of different polyelectrolytes on the encapsulation efficiency and the temperature stability of LbL microcapsules are limited.

The encapsulation of biomolecules based on matrix-assisted LbL encapsulation has the advantage of using a hydrogel matrix which serves as template for the LbL polyelectrolyte self-assembly encapsulation. This hydrogel matrix can be loaded with virtually any water soluble macromolecule, mixtures of macromolecules, or cells. The process is depicted in Figure 1. First, emulsification of hydrogel loaded with protein and/or DNA in warm oil for microdroplet formation is performed. Then cooling of the emulsion leads to solidification of the droplets to form microbeads, followed by encapsulation of the hydrogel microbeads with polyelectrolyte multilayers through an LbL self-assembly process to form polymeric capsules. Such microcapsules are of micrometer diameter with a nanometer thick LbL membrane and nanoliter volume. The thickness and permeability of the resulting capsules can be controlled depending on the number of LbL polyelectrolyte layers. The permeable capsules can retain encapsulated large protein molecules within the inner hydrogel matrix environment and allow small molecules to diffuse through the capsule wall.[22,23] The hydrogel core of our LbL hydrogel microcapsules contain almost 98% water and provide a favorable physiological environment for biochemical reactions to take place. Also, the hydrogel matrix acts as a scaffold and provides mechanical support to maintain the spherical shape of the capsules and facilitate the manipulation of the LbL hydrogel microcapsules as stable compartments for biochemical reactions. Some biochemical reactions such as the polymerase chain reaction (PCR) require higher temperature or temperature cycling of up to 95 °C; therefore, temperature stable microcapsules are desirable. In the following, the matrix-assisted LbL encapsulation process is described in detail, and the encapsulation efficiency of the LbL hydrogel microcapsules composed of polyelectrolytes pairs with various ionic strengths and charge densities is studied. Temperature stability and retention of biomolecules within the microcapsules as a function of LbL layer number and polyelectrolyte material is studied, and theories to explain microcapsule behavior and stability are discussed.

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Experimental Section

Materials. Polyethylenimine (PEI, linear average MW 250 000 g mol^{-1}) was purchased from Polysciences, Inc., U.S.A. Polyacrylic acid (PAA, average MW 450 000 g mol^{-1}), poly(diallyldimethylammonium, chloride) (PDA, average MW 100 000−200 000 g mol^{-1}), poly(sodium 4-styrenesulfonate) (PSS, average MW 200 000 g mol^{-1}), and poly(allylamine hydrochloride) (PAH, average MW 70 000 g mol^{-1}) were purchased from Aldrich, U.S.A. Dextran sulfate (DS, average MW 500 000 g mol^{-1}) and Span 80 (sorbitan monooleate) were purchased from Fluka, U.S.A. Bovine serum albumin fluorescein isothiocyanate conjugate (BSA-FITC) and mineral oil were purchased from Sigma, U.S.A. Agarose (low-melting point) was purchased from Promega, U.S.A.

Matrix-Assisted LbL Encapsulation by Polyelectrolyte Self-Assembly. Preparation of agarose microbeads: A solution of 4% low-melting agarose (0.1 M Tris buffer, pH 7) as matrix material was prepared and kept molten at a temperature of 40 °C. A solution of the biomolecule (protein) to be encapsulated, typically 0.1 to 10 mg mL^{-1}, was prepared with Tris buffer, pH 7; in the further experiments 1 mg mL^{-1} BSA-FITC was used as a model substance. The molten agarose solution was then mixed with the BSA-FITC solution at a volume ratio of 1:1 to achieve a mixture with a final concentration of 0.5 mg mL^{-1} BSA-FITC in 2% agarose. An aliquot of 200 μL of mixture was added to 1.8 mL of prewarmed mineral oil which contains 0.5% Span 80 at 40 °C. The oil/BSA-FITC agarose mixture was shaken vigorously until a milky emulsion was formed. The resulted emulsion was poured into 5 mL ice-cooled mineral oil containing 0.5% Span 80 under stirring to allow solidification leading to the formation of microbeads. A number of 10^3 to 10^6 microbeads depending on the volume and emulsification conditions can be formed by this process in parallel.

Encapsulation of agarose microbeads by polyelectrolyte multilayers was accomplished as follows: The resulting BSA-FITC agarose microbeads were coated with the first polyelectrolyte layer by transferring into an aqueous phase of 0.1 M Tris buffer containing 5 mg mL^{-1} positively charged polyelectrolyte (pH 7), occasionally mixed and incubated for 10 min. After incubation, the mineral oil and the excess polyelectrolyte was removed from the cationic polyelectrolyte coated microbeads by three washing and redispersion cycles with 0.1 M Tris buffer (washing with buffer; centrifugation at 1200 rpm (150 c.f.g.) for 5 min; removal of supernatant; redispersion in buffer). The resulting cationic polyelectrolyte coated agarose microbeads were overcoated with a second layer of opposite-charged polyelectrolyte by addition of 2 mL of anionic polyelectrolyte solution (5 mg mL^{-1}, 0.1 M Tris buffer, pH 7), occasionally mixed and incubated for 10 min. After the adsorption process, excess polyelectrolyte was removed by similar centrifugation and redispersion cycles as described above. Alternative deposition of cationic and anionic polyelectrolytes onto the agarose microbeads was performed until the desired number of layers was achieved.

Encapsulation Efficiency Studies. Fluorescence labeled bovine serum albumin (BSA-FITC) with a molecular weight of ~65 kD was used as a model protein to study the encapsulation efficiency of the process as a function of the polyelectrolyte material. BSA-FITC loaded microcapsules fabricated with various polyelectrolyte pairs were prepared with the same procedure as described above. The amount of BSA-FITC retained within the microcapsules after eight layers of LbL coating were examined by a fluorescence microscope and analyzed.

Temperature Stability Studies. An aliquot of 50 μL of BSA-FITC loaded microcapsule suspension was transferred into a microreaction tube, and 35 rounds of temperature cycling using a Peltier Thermal Cycler (PTC-200, MJ Research, U.S.A.) with a cycling profile of 95 °C for 0.5 min, 65 °C for 1 min, and 72 °C for 2 min was performed. After temperature cycling, the microcapsules were examined by fluorescence microscopy and analyzed.

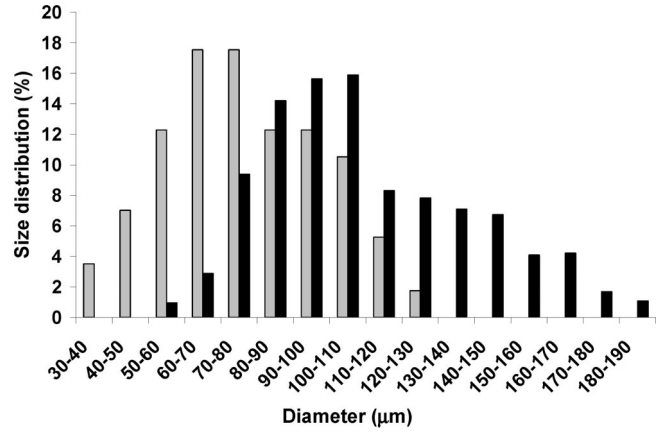


Figure 2. Size distribution curve of microcapsules with eight polyelectrolyte multilayers and PSS as the outermost layer (black). Size distribution curve of microcapsules with nine polyelectrolyte multilayers and PAH as the outermost layer after heating (gray).

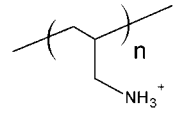
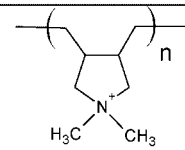
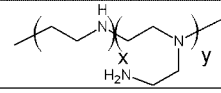
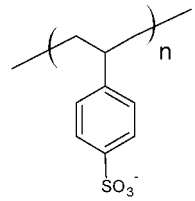
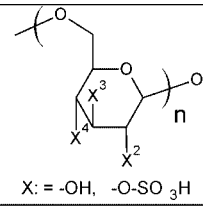
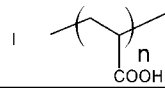
Optical Microscopy. Phase contrast and fluorescence microscopic images were recorded using a CCD color digital camera (Retiga 4000R, QImaging, Canada) connected to a system microscope (Olympus BX41) with a mercury arc (Olympus HBO103W/2) excitation source. Bandpass filters with excitation wavelength of 488 nm and emission wavelength of 520 nm were used for FITC detection. Images were captured with QCapture Pro software (Version 5.1.1.14, QImaging, Canada) and analyzed by QCapture Pro software or Scion Image software (Scion Corp., MY, U.S.A.).

Results and Discussion

Morphology and Colloidal Stability of Polyelectrolyte Coated Agarose Microbeads. Microcapsules were fabricated using a water-in-oil emulsification technique to produce microbeads followed by LbL encapsulation with polyelectrolyte multilayers to form microcapusles. The morphology and size distribution of the microcapsules were examined with phase contrast microscopy. The produced microcapsules have a perfect spherical shape with 90% of the capsule diameter falling in the range from 70 to 170 μm. The size distribution follows a Gaussian distribution with an average diameter of 111 μm ± 30 μm (Figure 2). The volume of an individual microcapsule ranged from about 0.17 to 2.6 nL with an average volume of 0.7 nL; about 100 000 times smaller than a 96-well microtiter plate reaction volume. The size of the microcapsules can be adjusted by varying the concentration of the surfactant, the ratio between the aqueous and the oil phase, and the homogenizing force during the emulsification process. Most desirable are capsules in the micrometer regime: They can be loaded with sufficient amount of biomolecules for biochemical reactions; a large number of capsules can be present per volume element, and they can be easily observed and analyzed.

Various cationic and anionic polyelectrolyte pairs with different pK_a values were tested for the LbL deposition process (Table 1). To fabricate individual microcapsule compartments to perform a high number of parallel biochemical reactions, it is important to maintain the colloid stability of the microcapsules during the fabrication process.

Table 1. Chemical Structures and Properties of Polyelectrolytes

Name	Structure	pKa	M.W. (g mol^{-1})
Poly(allylamine hydrochloride) (PAH)		~8.5	70,000
Poly(diallyldimethylammonium, chloride) (PDA)		—	150,000
Polyethylenimine (PEI)		~9.7	250,000
Poly(styrenesulfonate) (PSS)		~3.5	200,000
Dextran sulfate (DS)		—	500,000
Polyacrylic acid (PAA)		~4.5	450,000

Any aggregation of capsules may lead to flocculation and sedimentation and destroy the colloid. From phase contrast microscopy observations, most of the microcapsules fabricated from different polyelectrolyte pairs exhibited a good colloidal stability. An example of microcapsules fabricated from the PAH/PSS polyelectrolyte pair is shown in Figure 3A. In contrast, microcapsules fabricated from the PAH/PAA polyelectrolyte pairs are unstable and aggregate instantaneously after the second layer of polyelectrolyte coating, and severe flocculation occurred as the number of coating layers increased (Figure 3B). It was reported that the charge density and pK_a value of the deposited polyelectrolyte influence the colloidal stability of the resulting microcapsules.[24] PAH is a weak polyelectrolyte with a pK_a value of about 8.5 and

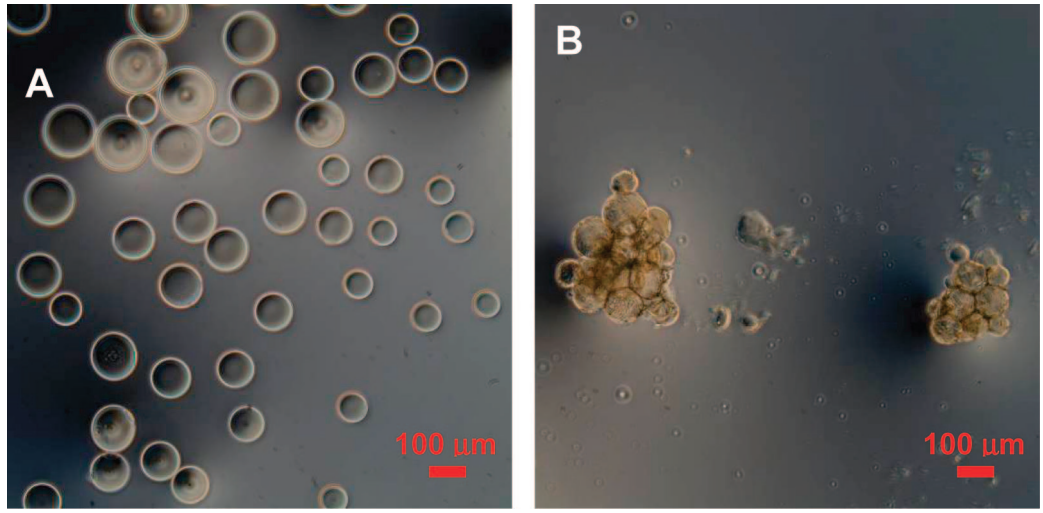


Figure 3. (A) Phase contrast micrograph of microcapsules fabricated with PAH/PSS polyelectrolyte pairs showing good colloidal stability and (B) microcapsules fabricated with PAH/PAA polyelectrolyte pairs showing aggregated.

the charge density of PAH in the coating solution with pH 7.0 is relatively low compared to other cationic polyelectrolytes such as PEI ($pK_a \sim 9.7$) and PDA (side groups are charged at almost all pH). The low charge density of the polyelectrolyte resulted in a lower colloidal stability. However, the colloidal stability can be reinforced if the second anionic polyelectrolyte layer is a strong polyelectrolyte with a high charge density such as PSS and DS. Aggregation only occurred if both cationic (PAH) and anionic (PAA) polyelectrolytes are of low charge density (weak polyelectrolyte). The proposed aggregation hypothesis is only observed for LbL encapsulation of hydrogel matrix beads while no aggregation was observed when using polystyrene and other solid particles such as calcium carbonate particles as the core materials with similar coating condition and pH (0.5 M NaCl, pH 7.0).[25]

Encapsulation Efficiency. To perform biochemical reactions within microcapsules, it is important to determine the amount or activity of biomolecules encapsulated within the microcapsules. The amount of biomolecules retained within the microcapsule compared to the initial amount reflects the encapsulation efficiency of the microcapsule fabrication process. The encapsulation efficiency is mainly influenced by the molecular weight of the biomolecules, the pore size of the matrix material for the formation of microbeads and the polyelectrolytes applied for the capsule fabrication process as well as handling procedures. High molecular weight biomolecules and higher concentration of the matrix material result in higher encapsulation efficiency. There is almost no loss of biomolecules during the first emulsification step because biomolecules are insoluble in oil; the LbL capsule fabrication is the critical step to achieve high encapsulation efficiency. The LbL polyelectrolyte film coated onto the surface of the microbeads reduces or seals the pores of the microbeads and prevents further leakage of biomolecules from microcapsules. The porous nature of the hydrogel may result that some polyelectrolyte penetrates into the hydrogel template during the coating process. Serizawa et al. state "During the first assembly step, the polyelectrolytes should entangle with the polymer segments on the hydrogel surfaces, instead of conventional electrostatic or solvophobic adsorption of the corresponding polymers, and this results in the penetration of the polyelectrolytes near the hydrogel surfaces".[26] The fluorescence ring observed in Supporting Information Figure 1 represents the penetration of the polyelectrolyte during the LbL coating procedure. The fluorescence intensity profile of the capsule formed from eight LbL layers was plotted. The green line represents the largest slope of the fluorescence intensity increase, and the capsule thickness was calculated as $2d$. A number of 10 capsules were examined with a mean capsule wall thickness of ~ 7 μm. This value is different from that in the literature stating that each polyelectrolyte layer is about 2 nm. Our results indicate that polyelectrolytes probably diffuse a certain distance (about 7 μm) into the hydrogel during the LbL coating process.

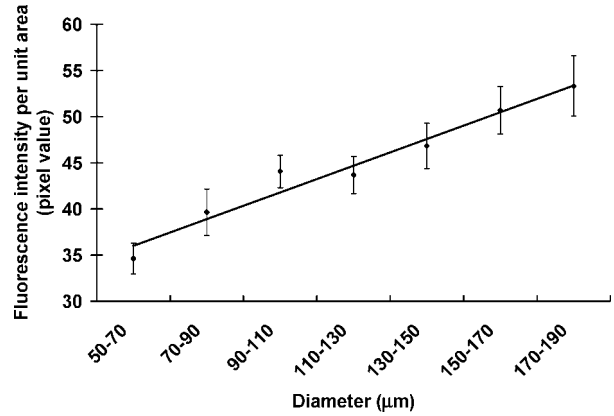


Figure 4. Fluorescence intensity as a function of capsule diameter for microcapsules loaded with BSA-FITC.

Fluorescence labeled bovine serum albumin (BSA-FITC) with a molecular weight of about 65 kDa was used as a model material to study the encapsulation efficiency. Various combinations of anionic and cationic polyelectrolytes pairs were employed to fabricate eight-layer microcapsules loaded with BSA-FITC. The retained BSA-FITC fluorescence intensity within individual microcapsules is a function of the encapsulation efficiency of the microcapsule fabrication process. The size of microcapsules influences the amount of BSA-FITC within different microcapsules and the resulting fluorescence intensity. It was observed that there is a linear correlation between the fluorescence intensity and the microcapsule diameter with a regression equation of $y = 3.26x + 32.1$ ($R^2 = 0.96$), where y is the fluorescence intensity (pixel value) and x is the microcapsule diameter (μm) (Figure 4). Therefore, the relative encapsulation efficiency was defined as the fluorescence intensity per unit diameter of microcapsules. The initial concentration of BSA-FITC (0.5 mg mL^{-1}) entrapped within the microbead, before the LbL capsule fabrication process, was normalized as 100% efficiency. Fluorescence intensities of microcapsules after coating of eight LbL polyelectrolyte layers were measured. Microcapsules with more polyelectrolyte layers are possible but not necessary and practical for this study; a detailed study on the influence of polyelectrolyte layer number on microcapsule stability is presented later.

Figure 5 shows that the relative encapsulation efficiency of microcapsules fabricated with PAH as the cationic polyelectrolyte has the highest encapsulation efficiency followed by PEI and PDA. The best encapsulation efficiency of 45% was achieved with the pair PAH/PSS. An average capsule of about 110 μm diameter with a 0.7 nL volume formed by this experiment contains about 10^9 protein molecules. Moreover, an inverse relationship between the pK_a value of the cationic polyelectrolyte (PDA > PEI > PAH) and the encapsulation efficiency (PAH > PEI > PDA) was observed (i.e., cationic polyelectrolyte with a lower pK_a value resulting in higher encapsulation efficiency). Cationic polyelectrolytes with a lower pK_a value (weak polyelectrolyte) are more coiled, while the cationic polyelectrolytes with a higher pK_a value (strong polyelectrolyte) are more rigid

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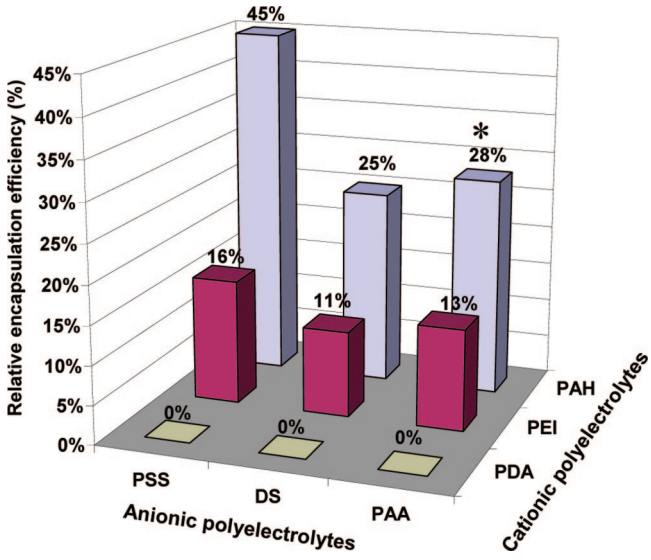

Figure 5. Relative encapsulation efficiency for microcapsules formed by eight polyelectrolyte layers as a function of the polyelectrolyte pairs. (*) indicates the result taken from aggregated microcapsules.

and elongated.[27] It was proposed that the rigid structure of the polyelectrolyte may create a topological restriction for the formation of electrostatic ion pairs between oppositely charged polyelectrolytes resulting in less electrostatic bonds and larger pore size. A similar observation has been reported by Krasemann et al.; the bond density of a PSS/PDA complex was as low as 0.0625 ion pairs per carbon atom, while the bond density of a PSS/PAH complex was 0.11 ion pairs per carbon atom.[28] Therefore, the encapsulation efficiency is mainly influenced by the pK_a value of the cationic polyelectrolyte that affects the pore size of the resulting microcapsule membrane.

In addition to the encapsulation efficiency, the activity of the biomolecules after the encapsulation process was studied. Microcapsules encapsulated with *E. coli* or enzyme (glucose oxidase and peroxidase mixture) were tested for bioactivity after the encapsulation process. Results showed the observation of a viable cell colony inside the microcapsule after cell culture (Supporting Information Figure 2A,B) and the formation a blue color product after addition of glucose and TMB substrate (Supporting Information Figure 2C) indicated the activity of the biomolecules were preserved after the encapsulation process.

Temperature Stability of the Hydrogel Microcapsules. Certain biochemical reactions require high temperature cycling, therefore studying the temperature stability of microcapsules is necessary. Microcapsules prepared from polyelectrolytes pairs including PAH/PSS, PAH/DS, PEI/PSS, PEI/DS, and PEI/PAA with good colloidal stability and encapsulation efficiency were employed for temperature stability studies. Microcapsules formed by eight polyelectrolyte layers encapsulating BSA-FITC were subjected to 35 rounds of temperature cycling (65 to 95 °C). The morphology and fluorescence content of the microcapsules was examined after the temperature cycling. Microcapsules fabricated with PEI as the cationic polyelectrolyte were unstable at high temperature; neither microcapsules nor hollow polyelectrolyte shells were observed (Figure 6A), while microcapsule fabricated with PAH/DS left behind broken and deformed hollow polyelectrolyte shells without fluorescence content after the temperature cycling (Figure 6B). Capsule rupturing may have occurred because of development of osmotic pressure created from the molten agarose at high temperature combined with the weakening of electrostatic interactions between polyelectrolytes of the capsule. Fluorescence intensity was observed from hollow shells resulting from some nonspecific deposition of BSA-FITC onto the polyelectrolyte capsule. In contrast, microcapsules fabricated with PSS/PAH remained stable with perfect spherical shape and with their fluorescence content retained inside the microcapsules (Figure 6C). Although some broken hollow capsules were observed, it was found that 75% of the microcapsules were intact. Polyelectrolyte capsules appeared deep yellowish and with increased surface roughness after temperature cycling. Ibarz et al. reported the effect of temperature on LbL multilayers and suggested that at high temperature the polyelectrolyte layers within the capsule walls rearrange and the capsule wall density increases.[29] As the polyelectrolyte layers rearrange, optical property of the polyelectrolyte layer may change causing the observation of yellowish colored microcapsule; however, the color change is not fully understood. Some studies have reported a significant shrinking behavior of hollow $[PDA/PSS]_4$ microcapsule at high temperature.[30] However, shrinking of microcapsules was not observed in our hydrogel filled microcapsule system with PSS as the outermost layer. After the temperature cycling microcapsules revealed an average diameter of 105 μm $\pm$ 30 which does not significantly differ from the initial diameter before temperature cycling (111 μm $\pm$ 30). At high temperature the agarose core of our microcapsules is molten, but due to its high molecular weight the agarose (and proteins) cannot diffuse out and will create an osmotic pressure. We believe that the generated osmotic pressure is counteracting the shrinking force and the capsule diameter is almost constant. This hypothesis is further supported by the observation from broken hollow capsules without BSA-FITC agarose content (Figure 7L); those broken capsules appeared shrunk and deformed in shape. Besides the physical stability of the microcapsules, the retention of biological materials within microcapsules after temperature cycling was determined. The study is based on the measurements of fluorescence intensity of individual microcapsules resulting from the BSA-FITC encapsulated within the microcapsules (hollow capsules without the BSA-FITC agarose content were omitted from the study). The fluorescence intensity of the microcapsules before and after temperature cycling was compared. The difference in the fluorescence intensity is related to the retention efficiency for biomolecules within the microcapsule at high temperature. The retention of BSA-FITC within the microcapsules after temperature cycling was 102.5% which is similar to the microcapsule before temperature cycling. The retention study demonstrates that

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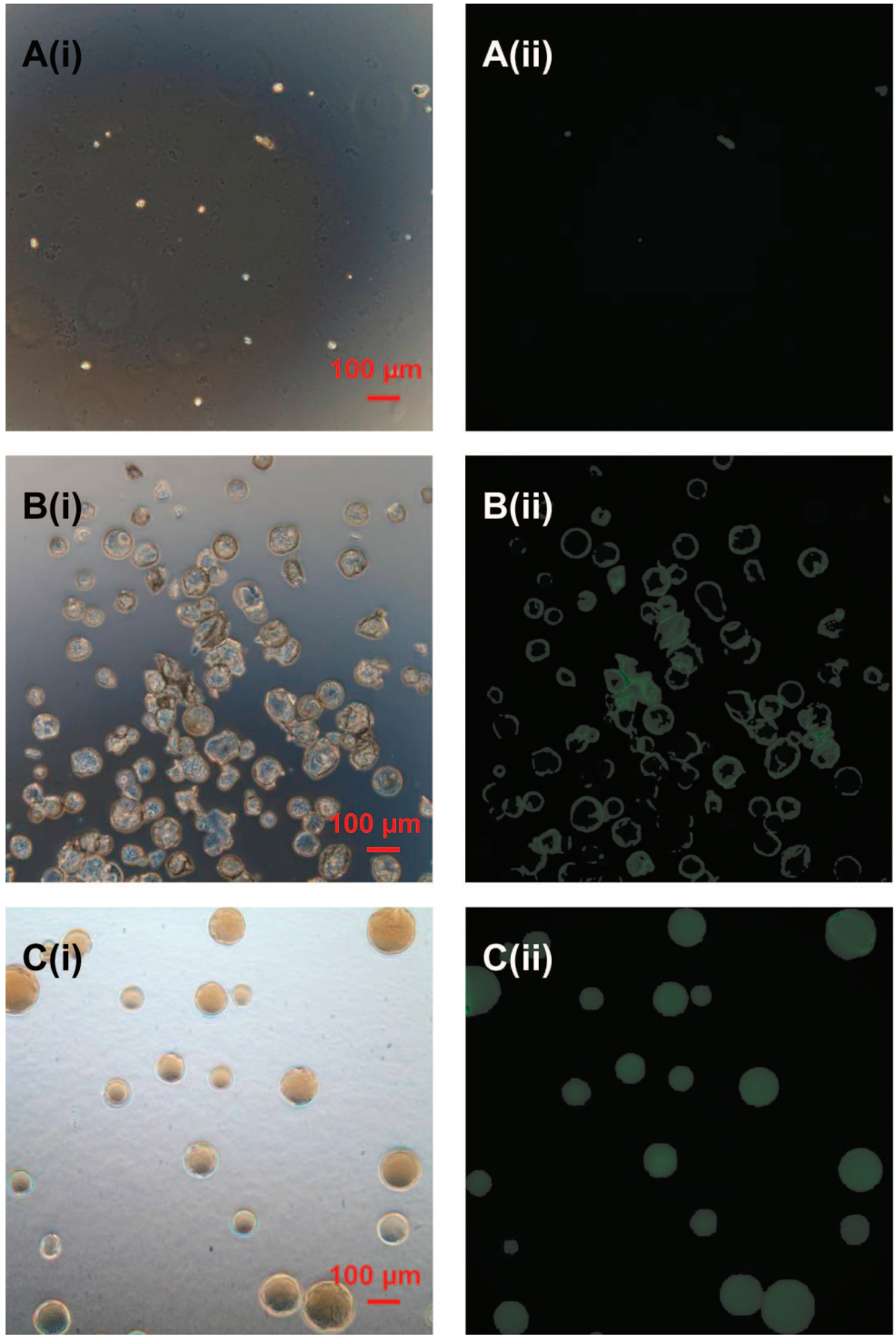

Figure 6. Temperature stability of microcapsules. Phase contrast micrographs (i) and the corresponding fluorescence micrographs (ii) of microcapsules after 35 rounds of temperature cycling at temperature between 65 and 95 °C. (A) Microcapsules fabricated with $[PEI/PSS]_4$ were destroyed, (B) microcapsules fabricated with $[PAH/DS]_4$ leaving behind a deformed hollow capsules, and (C) microcapsules fabricated with $[PAH/PSS]_4$ remain intact.

microcapsules fabricated with PSS/PAH are heat stable up to 95 °C and able to encapsulate and retain biomolecules without leakage.

Thermal Behavior of Hydrogel Microcapsules with Different Layer Numbers. Further detailed studies on the thermal behavior of the PAH/PSS hydrogel microcapsules and the critical number of polyelectrolyte layers required to fabricate heat stable microcapsules were performed. PAH/PSS hydrogel microcapsules with layer numbers from 1 to 11 encapsulating BSA-FITC were fabricated and subjected to temperature cycling and analysis by microscopy. No intact microcapsule was observed with microcapsules fabricated with one to five layers of polyelectrolyte (Figure 7A−E). Hollow polyelectrolyte shells were observed with microcapsules fabricated with six layers $[PAH/PSS]_3$ of polyelectrolyte

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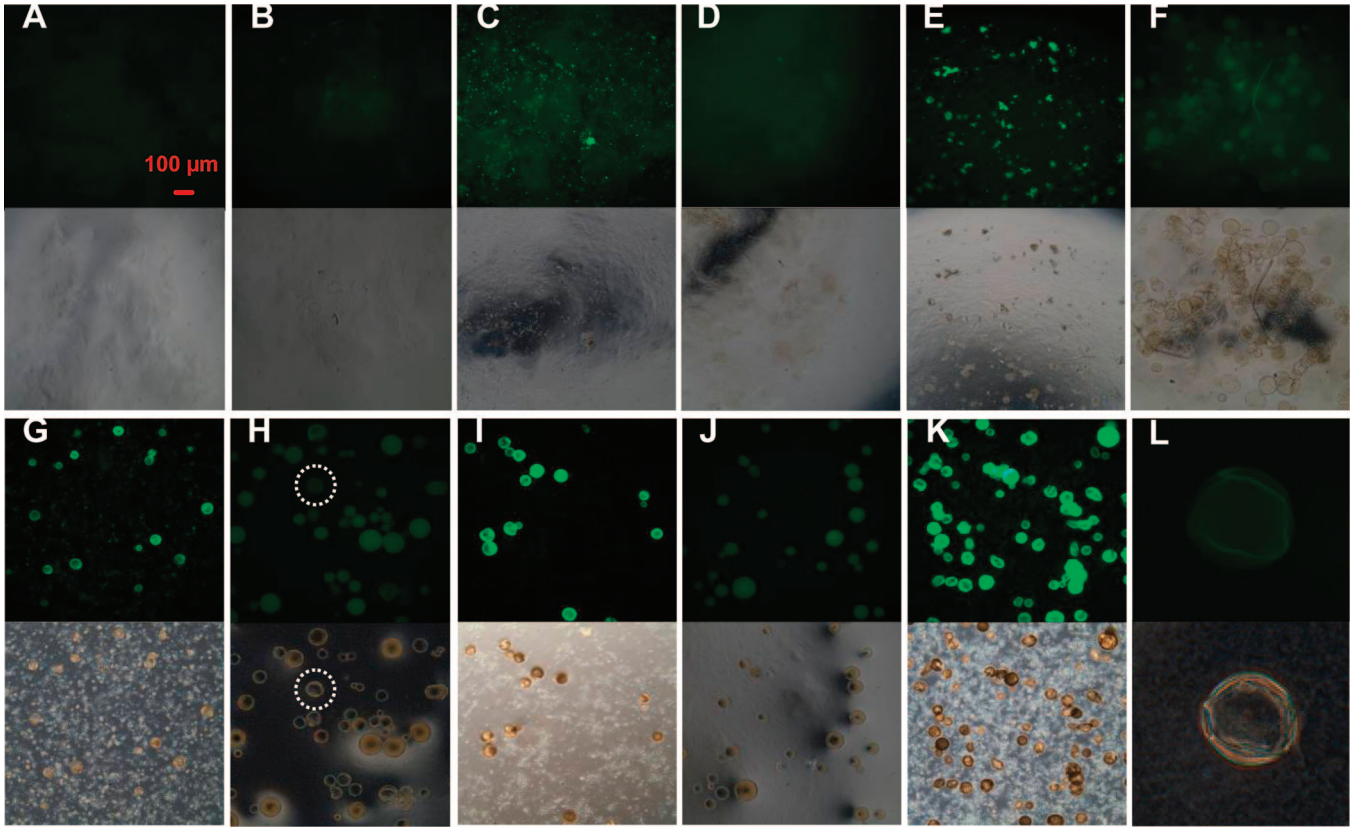

Figure 7. Temperature stability of microcapsules fabricated with the PAH/PSS polyelectrolyte pair as a function of layer number(s). Micrographs A−K corresponding to microcapsules formed from 1 to 11 polyelectrolyte layer(s). Micrograph L shows the enlarged hollow microcapsule circled in H. The top shows the fluorescence, and the bottom shows the phase contrast micrographs.

(Figure 7F, phase contrast micrograph). Intact microcapsules with the encapsulated fluorescence content retained were observed with microcapsules fabricated with eight and more polyelectrolyte layers (Figure 7H−K). These results clearly demonstrated that eight polyelectrolyte layers are the minimum number required to fabricate heat stable microcapsules. Microcapsules with a cationic polyelectrolyte (PAH) as the outermost layer exhibited shrinking and increase in the fluorescence intensity (Figure 7G,I,K), while microcapsules with an anionic polyelectrolyte (PSS) as the outermost layer remained similar in size and fluorescence intensity (Figure 7H,J). Microcapsules with PAH as the outermost layer shrunk from an average diameter of 111 to 77 μm, resulting in a 76% decrease of the microcapsule volume and 2.5-fold increase in fluorescent intensity. Johnston et al. reported that when fluorescently labeled PSS was used for LbL coating, there was a clear increase in the fluorescence intensity after the adsorption of nonfluorescence PAH as the outermost layer due to a change in the local environment of the fluorophore.[31] In our study, the BSA-FITC was loaded into the hydrogel core and the interaction between the BSA-FITC and PAH is limited. Therefore, the increase in fluorescence intensity of the shrunken microcapsule likely results from the decrease in microcapsule volume causing an increase in the concentration of encapsulated BSA-FITC.

Leporatti et al. reported the shrinking behavior of hollow PAH/PSS polyelectrolyte multilayer capsules upon heating.[32] A small shrinkage was observed with capsules having an outermost layer of PSS, and a severe shrinkage was observed with capsules having an outermost layer of PAH. Leporatti suggested rearrangement and annealing of the polyelectrolytes to a low entropy state as the driving force. In contrast, Köhler et al. reported that the hollow capsules consisting of PSS as the outermost layer shrunk, while capsules consisting of PDA as the outermost layer swelled upon heating.[33,34] Köhler suggested whether the capsules undergo shrinking or swelling is determined by the overall uncompensated charge of the polyelectrolyte multilayers (i.e., capsules with larger uncompensated charge undergo swelling, while with balanced charge ones undergo shrinking). The detailed mechanisms behind the thermal behavior of hollow polyelectrolyte multilayer capsules are still unclear and debatable. The thermal behaviors of polyelectrolyte multilayer capsules discussed above relates to hollow capsules without a core material. The temperature behavior and stability of microcapsule fabricated with a hydrogel core materials may differ if the hydrogel matrix acts like a cytoskeleton which maintains the size and shape of the microcapsule. From our experiments we found that hydrogel filled microcapsule

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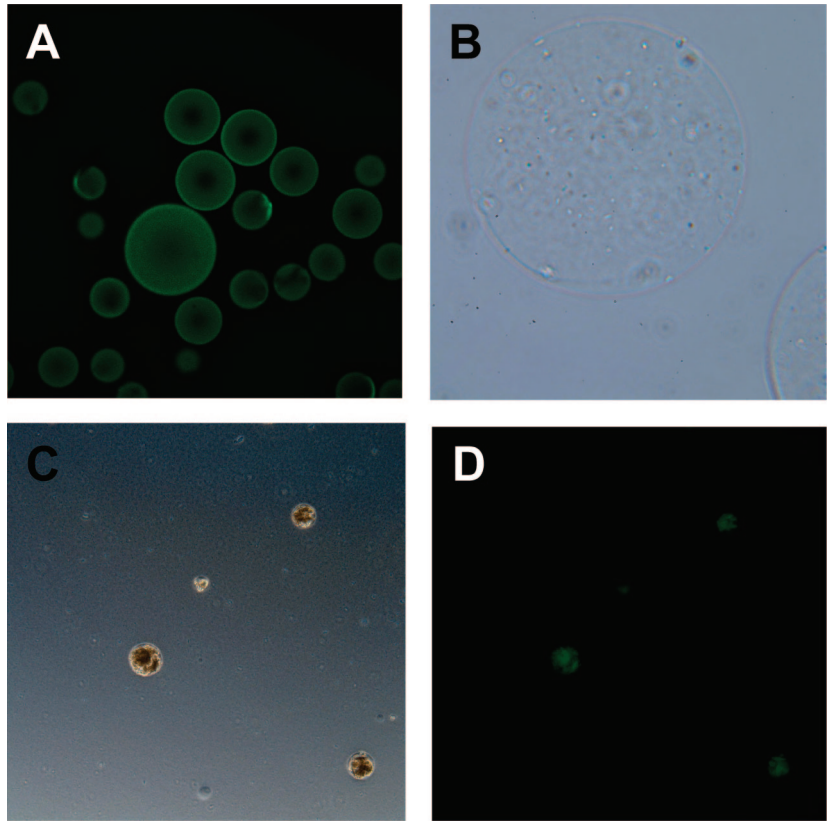

Figure 8. Encapsulation of biological materials into LbL hydrogel microcapsules. (A) DNA (fluorescence-labeled oligonucleotides with 22 bases) and (B) individual *E. coli* cells are seen as small rods in a capsule of about 100 μm diameter. (C) Phase contrast micrograph of the *E. coli* loaded microcapsules after incubation in a culture medium and (D) the corresponding fluorescence micrograph of the microcapsules after fluorescein diacetate stain indicating the cell viability.

shrinkage was only observed when the capsule outermost layer consisted of PAH, while no significant shrinkage was observed when the capsule outermost layer consisted of PSS. High molecular weight hydrogel molecules (and proteins) encapsulated within the inner core of the microcapsule are unable to penetrate through the capsule layers and would induce an osmotic pressure in the capsule. If the osmotic pressure is strong enough to balance the shrinkage force, the capsule size remains similar. By the fact that the shrinkage force with PAH as the outermost layer is much stronger than with PSS as the outermost layer;[26] we conclude that hydrogel filled microcapsules with PSS as the outermost layer would maintain a balance between the shrinkage force and the internal osmotic pressure resulting in heat stable microcapsules with constant diameter upon heating. However, for hydrogel microcapsules with PAH as the outermost layer, the shrinkage force is larger than the internal osmotic pressure resulting in shrunken microcapsules.

Encapsulation of Various Biological Materials and Biological Activity. The matrix-assisted LbL encapsulation provides a versatile method for the encapsulation of various biological materials. The feasibility of the LbL hydrogel microcapsule for the encapsulation of DNA and *E. coli* cells has been demonstrated (Figure 8A,B). In addition, the biological activity of the encapsulated *E. coli* cells was investigated. LbL hydrogel microcapsules loaded with *E. coli* cells were incubated in a culture medium (1% Laboratory-Lemco powder and 1% Bacto peptone) for 12 h at 37 °C, and the growth of colonies from a single cell within the capsule was observed. The resulted microcapsules were stained with fluorescein diacetate (FDA) and observed with a fluorescence microscope. Figure 8C shows *E. coli* colonies were observed inside the microcapsules, and the corresponding fluorescence micrograph (Figure 8D) demonstrated the cell viability.

Conclusion

The matrix-assisted LbL encapsulation process has the ability to fabricate temperature stable microcapsules loaded with biomolecules. Polyelectrolytes with various ionic strengths and charge densities showed significant influence on the properties of microcapsules, such as encapsulation efficiency and colloidal and temperature stability. Microcapsules fabricated with most polyelectrolyte pairs exhibited good colloidal stability, and aggregation only occurred for microcapsules fabricated with the weak cationic and anionic polyelectrolytes pair PAH/PAA. The PAH/PSS polyelectrolyte pair achieved the highest encapsulation efficiency of 45%. The temperature behavior of microcapsules fabricated with different polyelectrolyte pairs include PEI/X (where X are PSS, DS, or PAA), PAH/DS, and PAH/PSS resulted in completely ruptured microcapsules, broken microcapsules with deformed hollow shells, and intact microcapsules, respectively. We found that strong polyelectrolytes with high charge density can form microcapsules with better colloidal stability, while weak polyelectrolytes are more coiled and can form microcapsules with better encapsulation efficiency and temperature stability. At least eight polyelectrolyte layers

of $[PAH/PSS]_4$ are required to fabricate temperature stable microcapsules. Microcapsule shrinkage was only observed in microcapsules with PAH as the outermost coating layer, while no significant shrinkage was observed in microcapsules with PSS as the outermost layer. Among all the studies, the PAH/PSS polyelectrolyte pair was found to be the best material to fabricate microcapsules with good colloidal stability, encapsulation efficiency, and temperature stability. Results from our study give clear design parameters on polyelectrolyte pairs and layer number to create a microcapsule with the desired properties. We have also demonstrated the encapsulation of biomolecules (DNA) and microorganisms (*E. coli*) by using matrix-assisted LbL encapsulation. The method provides a "mild" way to encapsulate biomolecules and cells demonstrated by the ability of *E. coli* to grow and form colonies within capsules. A better understanding of biomolecule filled LbL microcapsule properties as a function of polyelectrolyte pairs and layer number may open future applications in the area of diagnostics, drug delivery, and life science.

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Supporting Information Available: Confocal fluorescence micrograph of a microcapsule after fluorescent staining formed from eight LbL layers and the corresponding fluorescence intensity profile (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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