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Encapsulation and Stability Properties of Nanoengineered Polyelectrolyte Capsules for Use as Fluorescent Sensors

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This is the first report about a novel fluorescence sensor technology based on hollow micro- and nanoscale polyelectrolyte capsules. The nanostructured shells were constructed using the electrostatic layer-by-layer assembly process to deposit multilayer polyion films onto microtemplates (melamine formaldehyde microspheres). The latex cores were subsequently dissolved and removed, leaving hollow shells. The capsules were then loaded with a model fluorescent assay consisting of a sodium-sensitive dye and a reference fluorophore. Fluorescence spectroscopy was used to analyze properties of the capsules with respect to their potential application as biosensors. The results show that multiple dye molecules can be introduced into the interior of the capsules with excellent control over relative levels, and the capsules retain >99% of fluorescence during 30 days of storage in a buffer. The findings also demonstrate that the capsules are mechanically robust, and only extremes in solvent pH cause significant leaching of fluorophores from the interior of the shells. Finally, results from sodium sensitivity experiments suggest that capsules have excellent potential for use as sensors, with a highly linear response over a broad range (0–100 mM).

1. Introduction

Chemical sensors are important for monitoring of chemical processes and biological systems.[1] In recent years there has been an increased interest among researchers in

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developing novel, improved approaches for determining biochemical concentrations.[2] While modern biosensors provide a basic ability to quantitatively measure chemical substances, many suffer from an inability to provide adequate specificity, spatial resolution, and real-time sensing capability. The use of many such sensors is further hindered by associated damage to biological tissues due to implantation of large sensors, analyte consumption, performance degradation over time, or repeated invasive use.[2–34]

Optical sensing techniques have gained popularity as alternatives to the more common electrochemical methods. Advantages of these sensors include minimal consumption of analytes, potential for real-time monitoring, insensitivity of the signal to electrical interference, electrical isolation, and high sensitivity to analytes.[5,6] Specifically, fluorescence spectroscopy and microscopy have been utilized for the real-time, specific and extremely sensitive monitoring of biochemicals, pH, and voltage.

The most common use of fluorescence in biological measurements is the injection of a fluorescent indicator dye directly into a cell or tissue sample. In principle, these fluorescent indicators operate based on the change in excitation or emission properties (intensity, spectral shape, and/or lifetime) due to binding with analyte molecules, quenching of fluorescence by the analyte molecules, or fluorescent resonance energy transfer (FRET).[7,8] Disadvantages of these methods include cytotoxicity of the assay chemistry, intracellular sequestration, protein binding, and diffusion of dye from the area of interest, all of which affect the surrounding tissues as well as the experimental results.[8] Thus, efforts to immobilize the sensing chemistry into a biocompatible probe configuration have been pursued.

The majority of researchers in the field of fluorescence sensing have studied various configurations for fiber-optic probe-based sensors.[1,6,9] A pH sensor was the first fiber-optic-based device designed specifically for physiological use.[10] Since then, a number of novel fiber-optic, integrated probe systems have been developed to detect important analytes such as glucose, oxygen, and carbon dioxide.[2,11,12] While these fiber-optic probe-based sensors offer high strength and stability, they still require the physical connection of fibers to the sensing elements and have not found widespread application.[13]

Recently there have been attempts to decouple the sensing chemistry from optical fibers. This capability is important for intracellular measurements and chronically implanted sensors that can be interrogated transdermally. It can also enable fluorescence imaging, with the physical entrapment of assay molecules to avoid leaching, and the added potential for protecting the cells from toxic chemicals. In addition, encapsulation of indicator dyes by biocompatible materials enables sensors to remain unaffected by protein adsorption.[8]

Potential solutions to these problems that are currently under investigation involve the use of fluorescent indicators embedded in polymer or silica matrices,[8,14–16] and encapsulated by dialysis membranes or liposomes.[17,18] Advances in polymer and sol-gel systems, the most intensely investigated, have largely overcome a number of problems in terms of sensor sensitivity, response time, indicator leaching, and reversibility[8] with recent improvements in fabrication processes.[15] However, the fluorescence intensity and homogeneity of particles produced using polymerization is still dependent on the solubility of assay chemistry in precursor solutions, which often results in poor homogeneity, as well as

particle-to-particle and batch-to-batch repeatability. In addition, the use of solid polymer or hydrogel systems alone complicates the use of competitive-binding reactions, which require free movement of molecules within a restricted range such as is obtained with dialysis membranes. This option, however, while suitable for miniaturization, is not amenable to efficient fabrication, will have poor mechanical stability and durability, and requires relatively large end-products that cannot be deployed intracellularly.[17]

An interesting possibility for improving the current methods is the use of tiny fluid-filled shells: in the past few years, a new technique for the fabrication of hollow capsules has been developed to produce tiny shells that can potentially be employed as carriers for fluorescent sensors. The electrostatic layer-by-layer (LBL) assembly technique has been elaborated to deposit thin layers of charged molecules onto micro- and nanoscale templates, with subsequent removal of the core material to achieve hollow shells.[19–27] The method is very versatile, and shell properties can be controlled: inner diameters are dependent upon the size of templates used for assembly, wall thicknesses can be controlled precisely to within a few nanometers, and walls may be constructed using a diverse selection of charged molecules (polyelectrolytes, proteins, dyes, inorganic particles, co-polymers, etc.).[24] The versatility in the construction of these tiny capsules and their control over properties makes them attractive for use in sensor applications.

The aim of this work was to study the potential for real-time sensors based on hollow polyelectrolyte capsules with specific transport properties. This novel concept was studied to determine the potential of dye-loaded shells for measurement of chemical concentrations. A model two-component sodium assay was chosen, which included a sodium-sensitive dye and a reference fluorophore, the latter of which is used for an internal intensity reference. Sodium was chosen as an initial analyte because of its importance to cellular function, as well as the availability of sodium-specific fluorescent probes.[28] Using the assay-loaded capsules, three sets of tests were performed. The loading properties of shells were studied to determine the possibility of simultaneous encapsulation of multiple molecules and the controllability of relative amounts of the multiple internalized species. The fluorescence intensity of loaded shells in suspension was compared as a measure of signal levels. The stability and robustness of the capsules were tested under mechanical insult (vortexation, sonication, smashing) and pH modulation. Finally, simple assay response experiments were conducted to confirm that the indicator maintains its function after encapsulation as well as to determine the potential range and sensitivity achievable with such sensors.

2. Materials and Methods

2.1 *Reagents*

Polyelectrolytes used for the formation of the shells were sodium poly(styrenesulfonate) (PSS, MW~70 k, polyanion) and poly(diallyldimethylammonium) chloride (PDDA, MW~100 k, polycation) (Aldrich Chemicals, Milwaukee, WI). The substrate particles were positively charged, 2.3-μm-diameter melamine formaldehyde (MF) in 10% V/V solution of KCl (Microparticles GmBH, Germany). The sodium-sensitive dye used was sodium-binding benzofuran isophthalate (SBFI) and the reference fluorophore was 7-

methoxycoumarin-3-carboxylic acid (MCA) (Molecular Probes, Eugene, Oregon). The pH 7.6 (0.02 M) buffer was made with Trizma acid and base (Sigma Chemical, St. Louis, MO).

2.2 *Instrumentation*

A 1510 Sonicator (Branson, Danbury, CT) and a Genie 2 Vortex (Daigger, Bohemia, NY) were used for dispersion and suspension of particles in microcentrifuge tubes. A 5404R Centrifuge (Eppendorf, Westbury, NY) was used to separate the particles from ambient solution. A Nitrogen laser (LSI, Franklin, MA) was used as the excitation source for fluorescence experiments. A fiber-optic 2048-element PDA spectrometer (StellarNet, Inc, Oldsmar, FL) was connected to a custom cuvette holder using an SMA-terminated probe comprising seven (six around one) 400 μm fibers. The data were collected using vendor-supplied software (SpectraWiz), then imported and analyzed using Microsoft Excel.

2.3 *Spectroscopy*

Samples were placed in 1 cm cuvettes and were stirred prior to data collection to maintain homogeneity. Three replicate scans were taken for each sample so instrumental variations could be assessed. Spectra were collected using 2000 ms integration time, and five consecutive scans averaged to produce a single spectrum.

2.4 *Shell fabrication*

The shells were fabricated using layer-by-layer assembly techniques that have been described in detail elsewhere.[29–33] Briefly, polyelectrolytes were mixed with 0.5 M KCl to obtain stock solutions (2 mg/ml PSS, 3 mg/ml PDDA). It is known that ionic strength is a determining factor in the structure of assembled layers and, for this study, this variable was held constant by using the same stock solutions of PSS and PDDA for all deposition reactions. Approximately 100 μL of the MF particle suspension (~10^9 particles) was placed in each of four micro-centrifuge tubes; then 1 mL of PSS stock solution was added to each tube. The particles were vortexed and sonicated for one minute, suspended in the PSS stock for ten minutes to allow polyelectrolyte adsorption, then centrifuged at 10,000 rpm for ten minutes to separate particles from solution. The rinse cycle involved removing the supernatant, adding 1 mL of Trizma buffer, sonicating, vortexing and centrifuging. Each batch was rinsed three times for each step. After the third cycle, the supernatant was removed, and 1 mL of PDDA stock solution was added to the particles. The particles were vortexed, sonicated and suspended for ten minutes. The three-cycle rinse process was repeated, leaving the particles with one complete bilayer. This procedure was repeated until there was a total of five bilayers on the MF particles.

Once the layering process was complete, the cores were dissolved by placing the particles in a 0.1 M NaCl solution.[24] The particles were suspended in the NaCl for 24 h. During this time the MF cores dissolved and diffused out of the shell. The microshells were centrifuged and rinsed ten times to remove residual NaCl. This process resulted in hollow capsules surrounded by five bilayers of polyelectrolytes.[24]

2.5 *Dye loading experiments*

For the capsules to be used as carriers for fluorescence sensor chemistry, a means of introducing the molecules into the shells is necessary. Initial tests studied diffusion due to the simplicity of the approach. Individual stock solutions of SBFI (0.05 mg/mL or 55 μM) and MCA (0.1 mg/mL or 454 μM) were prepared, then a mixture of 3:2:5 (SBFI:MCA:buffer) was prepared from stock solutions, resulting in a mixture of 0.015 mg/mL (16.5 μM) SBFI and 0.02 mg/mL (91 μM) MCA. Hollow capsules were suspended in 1 mL of the SBFI/MCA solution for a period of 24 h to allow diffusion of dyes through the bilayer membranes and into the interior of the capsules. The shells were rinsed ten times to remove the free dye. Figure 1 depicts the steps of capsules formation, from the nanoassembly of polyion layers on the particle surface to the loading of dyes.

Control experiments exposing only MF particles (no polyions), MF particles with a five-bilayer polyion coating (MF core intact), and five-bilayer hollow capsules (core dissolved) to the same dye mixture were used to determine whether dyes adsorbed onto MF, whether they stuck to or became entrapped within polyion films, or whether they diffused into the interior of polyion shells.

Another essential aspect of carriers for fluorescent indicators is a way to control the amount of material entering the vehicle interior. Because of the need for two-dye systems in quantitative measurements, some understanding of how to tailor the relative amounts of the two molecules loaded into the shells was also important. A series of experiments to

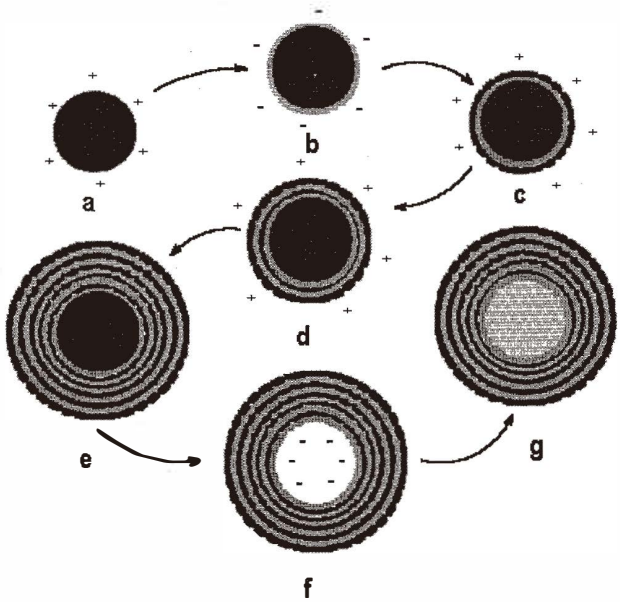

Fig. 1. Steps of capsules fabrication. Layering of polyelectrolytes (a–e) until the desired number of bilayers are deposited. Once the layering process is complete, the core is dissolved, leaving behind an empty shell (f). The hollow shells are then loaded with sensor chemistry (g).

follow the simultaneous diffusion of both indicator and reference molecules over time was used. After the template particles had been dissolved and capsules isolated, the shells were loaded with the same 3:2:5 volume ratio (SBFI:MCA:buffer) solution as noted above. To achieve this, the capsules were suspended in 1 mL of the dye mixture at room temperature. After the shells had been in the solution for one hour, 100 μL of the suspension was removed and capsules were separated and rinsed using five cycles of centrifugation at 10,000 rpm for five minutes. They were then suspended in 0.5 mL of Trizma buffer and fluorescence spectra of the suspension were recorded. After allowing some time (~1 h) for the capsules to settle to the bottom of the cuvette, the solution was scanned using the fiber probe with the laser coupled into the central fiber for excitation and the surrounding six fibers for collection. This allowed excitation of the sample only where the probe was placed. The solution was scanned at the top, in the middle, and at the bottom, where the particles had aggregated. This process was repeated after 2, 3, 4, 5, 6, 9, 12, 18 and 24 h had elapsed, so loaded shells could be observed after different periods of immersion in the dye mixture. Spectra of the 3:2:5 SBFI:MCA:buffer mixture used for loading the capsules were also collected for purposes of comparison.

2.6 *Stability testing*

Longevity and stability are both desirable qualities of sensors, particularly those for biomedical applications. Containment of assay chemistry is a critical property that affects the shelf-life of sensor materials as well as longevity in continuous use. For the hollow capsules described here, it was important to demonstrate that assay chemistry, once loaded, would be retained inside the shells for a sufficient time period under normal storage conditions. Furthermore, it is important to know whether the capsule walls lose structure (rupture) when mechanically stressed or in a hostile chemical environment. In this work, dye-loaded capsules were studied under exposure to different conditions to determine the integrity of capsule walls and the potential duration of encapsulation. These conditions included vortexing and sonicating, long-term storage, and suspension in acidic and basic solutions.

Stability during storage. Long-term storage tests were performed to determine how much dye leached out of the capsules over a period of one month. Loaded capsules were suspended in a fresh buffer solution, and baseline fluorescence spectra of the shell suspension and supernatant (after centrifugation) were collected. The shells were re-suspended in a microcentrifuge tube and placed in the refrigerator for storage. Every other day, the suspension was centrifuged, supernatant was drawn off and placed in a cuvette, and fluorescence spectra were collected. The supernatant was then placed back into the centrifuge tube with the capsules and they were re-suspended. The fluorescence intensity of the capsules was measured only on the first and last days of the testing. These steps were taken to gain some insight into the leaching of dye molecules from the interior of the shells into the ambient solution.

Stability during Vortexation and Sonication. The purpose of this experiment was to determine if fluorescence molecules moved out of the capsules during vortexing or sonication of the capsules, due to either destruction of walls or increased permeability. It

is noteworthy that, as described above, both of these procedures were used throughout the polyion shell fabrication process as a means of suspending the particles in solution. After the capsules were loaded with dyes, one vial was marked for vortex only; the other for sonicate only. Throughout the rinsing cycles, each vial was either vortexed or sonicated for one minute in accordance with its notation. After ten rinses, the capsules were left suspended in the pH 7.6 buffer and fluorescence spectra were collected. Then the capsules were centrifuged, and only the supernatant was scanned.

Stability in Acidic and Basic Environment. Polyion capsules are proposed as a versatile technology for encapsulation of fluorescence chemistry. It is also important, then, to understand how they respond when placed in a basic or acidic environment. This is because many fluorescent indicators are not soluble at neutral pH and thus loading into capsules would likely require suspension of the capsules in an acidic or basic solution. In addition, it is possible that capsules might eventually be used for applications that require operation in a nonneutral medium, for example, as pH sensors. It has already been shown that pH does affect the wall permeability of capsules fabricated using multilayer polyelectrolyte films.[21] For this work, simple tests were performed to determine the relative amount of the fluorophores released from the loaded capsules in both acidic and basic solutions. After the capsules were loaded and rinsed ten times, a pH 9.0 buffer was added to one vial, and a pH 4.11 buffer was added to another vial. Fluorescence scans of both the particles and supernatant were performed. The capsules were suspended overnight in their respective buffers. After 24 h, fluorescence spectra of shells and supernatant were again collected.

2.7 *Sodium response*

A final concern regarding the suitability of the shells as carriers for fluorescence assays is the ability of small molecules to equilibrate across the capsule walls, and interact with the indicator molecules. Using the sodium assay described above, initial experiments were performed to demonstrate the feasibility of capsules loaded with SBFI to respond to changing sodium levels. A cuvette containing 0.5 mL of loaded capsules suspended in pH 7.6 tris-buffer was placed into the sample holder. A NaCl solution was prepared by dissolving 2.925 g of NaCl in 100 mL of tris-buffer. This NaCl solution was prepared such that adding 10 μL of NaCl solution to the 0.5 mL capsules/buffer solution gave a 10 mM increase in NaCl concentration. The NaCl concentration in the capsules/buffer solution was increased in 10 mM increments from 0 mM to 100 mM. At each concentration, the suspended capsules were excited with the LSI laser, and three scans from the spectrometer were recorded.

3. Results and Discussion

3.1 *Dye loading experiment*

The dye loading experiment was performed in an attempt to understand the properties of diffusion of individual dyes into the capsules. Figure 2 shows fluorescence spectra of capsules after immersion for 1, 12, and 24 h in a dye mixture. The spectra in Fig. 2 have

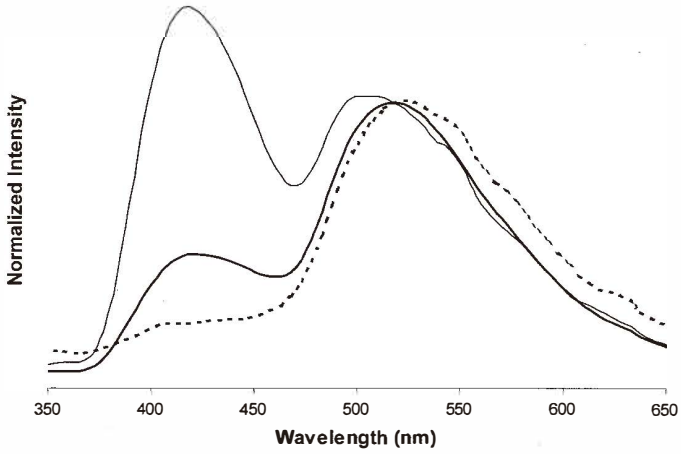

Fig. 2. Intensity (normalized to 518 nm) of capsules suspensions at 1 (thick dashed), 12 (thick solid), and 24 h (thin solid) during the loading experiment.

rising relative fluorescence at the shorter-wavelength peak, which indicates increasing encapsulation of MCA in the shells for longer loading times. The data seem to show that SBFI enters the shells at a faster rate initially, but the capsules continue to take up MCA until the fluorescence peak intensity surpasses that from SBFI as the system approaches equilibrium. This trend is surprising, because the SBFI molecule is almost three times larger than the MCA molecule. Furthermore, there were many more MCA molecules in the loading mixture. The observations suggest that neither wall permeability nor concentration gradients determine the transport behavior of the dye molecules to the interior of the capsules. Further experiments are needed to fully understand the processes involved in dye uptake, but the intention here was to determine whether the process is truly encapsulation and whether the results are repeatable.

Control experiments showed no appreciable fluorescence of solid MF particles or MF particles with five outer polyions bilayers when compared to hollow capsules after suspension in the dye mixture. These results indicate that the fluorophores are not simply adsorbing to the surface of particles or becoming entrapped within the polyion films. The explanation for dye retention within the capsules after diffusion is still unclear, though several hypotheses have been developed. These include: 1) dye aggregation in the capsule interior, 2) dye molecules complex with remaining fragments MF in the capsule, and 3) a layer of charged dye molecules coats the inner surface after diffusion, resisting outward diffusion of other molecules by electrostatic repulsion. Polarization experiments showed anisotropy values of 0.6–0.7 for encapsulated dyes, which suggests some binding/immobilization of molecules or high viscosity in the capsule interiors. Future work using confocal fluorescence microscopy, absorption spectroscopy, and polarization experiments is needed to address this question in more depth. From an engineering standpoint, however, it is sufficient to know that the process is repeatable and controllable.

Figure 3 shows the fluorescence peak ratio (MCA:SBFI) as a function of time of removal from the dye solution for the shells during loading. During the first twelve hours of loading, there was little change in the fluorescence peak ratio of MCA:SBFI. However, there was a significant and steady increase in peak ratio during hours 12–24. The importance of this behavior is that one can "tune" the relative amount of loading of two molecules by carefully controlling the immersion time. The curve fit to the data points suggests that there is an almost exponential increase in peak ratio with immersion time. Further experiments showed that this curve can be used to select a time that will result in a peak ratio within 10% of that estimated by the equation, if the same dye mixture, shell size, wall thickness, and wall composition are used.

3.2 *Liquid phase vs. encapsulated dyes*

It is well known that fluorescent molecules are sensitive to the environment in which they are placed. It was important to compare emission spectra of capsules to spectra of dyes in solution, as that is an indication of whether the fluorophores truly migrate to the interior, adsorb to the surface, or get entrapped in the polyion walls of the shells. In theory, the interior of the polyion shell is similar to the surrounding solution. If this is true, the location of emission peaks in spectra collected from capsules should be very similar to those seen in the loading mixture, unless there is a significant change (*e.g.*, pH shift) encountered in the capsule interior.

Figure 4 contains plots of emission spectra for capsules and a dye mixture used to load the shells. The data show that the emission peaks of the respective dyes occur at the approximately the same wavelengths for both liquid phase and encapsulated dyes. The observed wavelength of MCA emission is approximately 420 nm, and the observed SBFI emission peak occurs at approximately 550 nm. The results show that, other than differences in overall intensity and a slight peak shift, the spectra are essentially the same. The SBFI and MCA emission peaks at approximately the same spectral position in both the

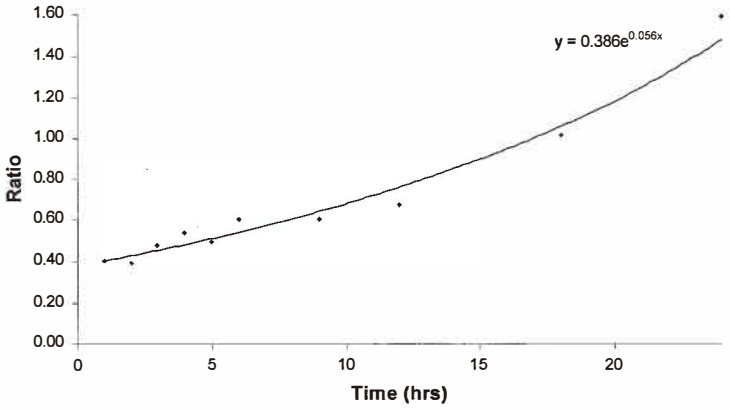

Fig. 3. Ratio of fluorescence intensity at peaks (418 nm: 518 nm) over the 24 h period during immersion in dye solution. Markers show computed ratio values, while the line is an exponential fit.

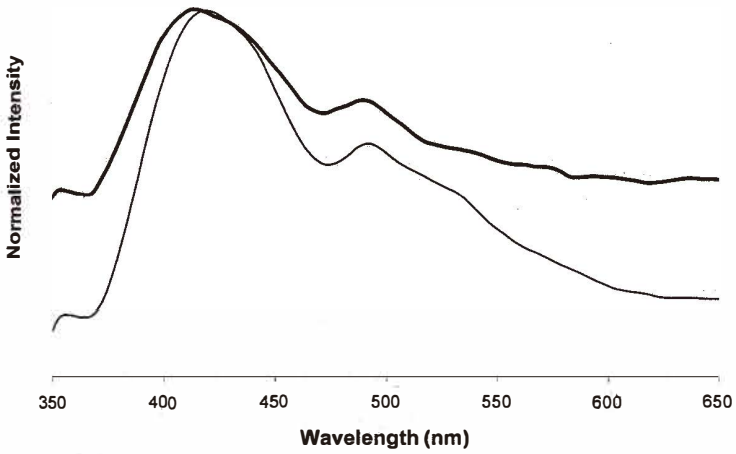

Fig. 4. Comparison of normalized emission spectra of dyes in solution (thick solid) to loaded capsules (thin solid).

capsules and solution phase. These findings indicate that the dyes are most likely diffusing into the capsules instead of adsorbing to the surfaces, which experience shows would give significantly different spectra. It should also be noted that different batches of SBFI had to be used throughout the experiments, and significant batch-to-batch variability in properties of this particular dye has been previously reported,[34] so shifts in emission properties were expected even between solution spectra.

Another important characteristic of carriers for optical sensors is the ability to take up enough of the indicator molecules to produce detectable signals. A desirable property is an emission intensity that exceeds that from dye in solution. The fluorescence obtained from capsules was compared to the dye mixture used to load the sensors (0.015 mg/mL SBFI and 0.02 mg/mL MCA). Figure 5 contains spectra from an experiment that used the same instrumental setup to record data from both samples. The fluorescence of the loaded capsules suspension is two orders of magnitude more intense than that of the dyes in solution. This result far exceeded expectations, and bodes well for applications of the fluorescent capsules to low-light situation. An interesting point is that during the loading process, the concentration of dyes does not equilibrate between the interior and exterior of the shells. If equilibrium were reached, the suspension of capsules could never even approach the intensity of solution, because the volume of dye and, hence, the number of fluorophores would always be smaller.

3.3 *Stability tests*

Long-Term Stability. The long-term stability experiment was performed in order to test the ability of the capsules to retain the encapsulated dyes over a period of one month. Figure 6 shows spectra of capsules on the last day of the study together with the fluorescence of supernatant on selected days throughout the period. It is noted that the capsules used in this experiment were loaded over a short time and, therefore, exhibit a

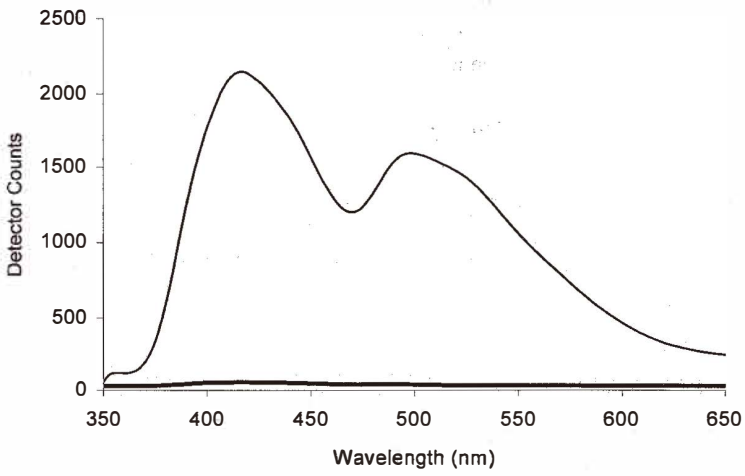

Fig. 5. Comparison of relative fluorescence intensity of dye solution (thick solid, 0.015 mg/mL SBFI and 0.02 mg/mL MCA) to dye-loaded capsules (thin solid).

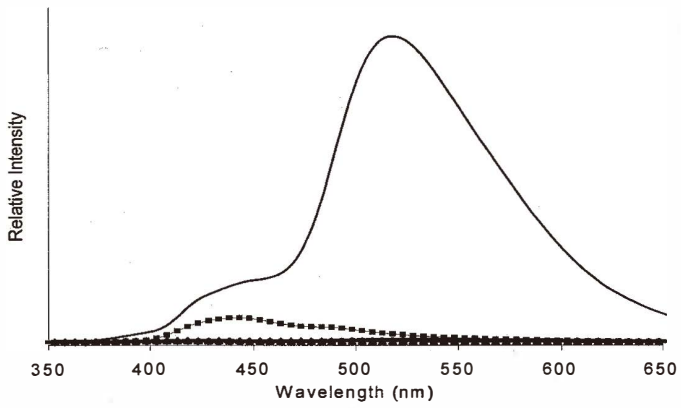

Fig. 6. Spectra of capsules and supernatant during the one-month storage period. The more intense spectra were collected from a capsule suspension at the end of the 30-day experiment. Weaker spectra that are barely visible were collected from supernatant on days 1 (triangle), 9 (diamond), 17 (square), and 29 (circle).

larger emission at 510 nm (SBFI) compared with that at 400 nm (MCA); this explains the relationship between Figs. 2 and 6. The supernatant spectra in Fig. 6 are one order of magnitude weaker in fluorescence in all cases, and can barely be seen on the graph. It is clear from these data that the fluorescence of the capsules was much more intense than each

of the supernatant scans taken throughout the month, even at the end of the period. This demonstrates that the amount of dye leaving the interior of the capsules is at the noise level when compared with the fluorescence from the capsules.

The relative loss of dye to solution over the one-month storage period was placed on a quantitative scale by computing the change in fluorescence intensity of the supernatant as a percentage of the initial capsule fluorescence at 510 nm. Calculations showed that the loss of dye to the ambient solution results in a fluorescence signal less than 0.1% of the intensity of the fluorescent capsule suspension. Thus, leaching did not occur significantly over the testing period. Thus, the contribution of nonencapsulated dye to fluorescence measurements will not be appreciable, reducing the likelihood of spurious signals or widely varying nonspecific background levels of fluorescence.

Mechanical and Chemical Stability. Initial mechanical tests were conducted to discover whether processes such as vortexing and sonicating had any adverse effects on the containment of the dyes inside the hollow cores. Figure 7 shows spectra from experiments where loaded capsules were subjected to vortexation and sonication. The spectra are similar to those obtained from the storage experiments. Again, the fluorescence intensity from the supernatant is extremely small compared to that obtained from the capsules, to the point that most spectra cannot even be observed when placed on the same scale as the capsules. This indicates that very little dye is lost through the shell during these procedures. A small increase in leaching is noted when comparing fluorescence from vortexed capsules versus that from sonicated capsules. Overall, however, the supernatant spectra exhibit extremely low levels of fluorescence with respect to the capsules; for these

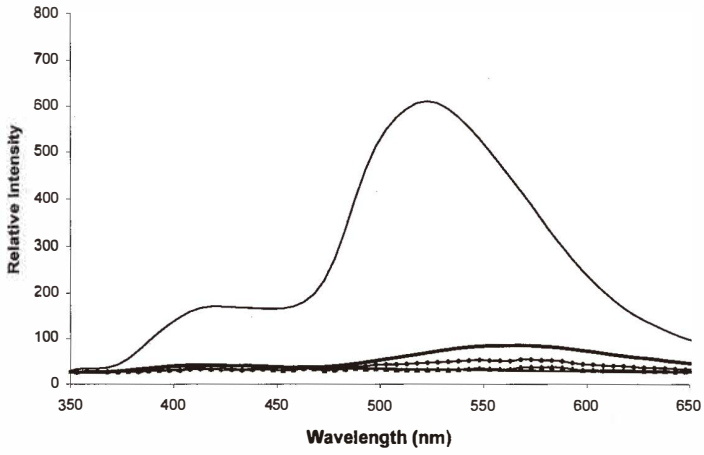

Fig. 7. Fluorescence spectra of capsules (thin solid) and supernatant subjected to mechanical insult (post-sonication: triangle, post-vortexation supernatant: diamond) and pH changes (pH 9: plus-sign, pH 4: bold).

experiments, a maximum of about 6% of fluorescence comes from dye free in supernatant relative to capsules. These results indicate that, in qualitative terms, the polyelectrolyte shells are stable under mechanical stress.

The capsules were also suspended in varying pH, and Fig. 7 contains supernatant spectra collected from shells immersed in acidic and basic solutions. In comparison with the mechanical tests, the changes in pH resulted in a much higher level of fluorescence in the supernatant. In particular, shells suspended in pH 4.11 showed greater leaching of dyes, showing approximately 15% when compared with capsule fluorescence. This result was expected, as others have observed similar behavior.[21] A possible explanation of this occurrence is that at high or low pH, the polyelectrolytes in the shell wall rearrange, causing the pores on the shells to expand, and enabling diffusion of dyes out of the capsules. Fortunately, in most potential physiological applications, the particles should be in a suitable pH environment. This may, however, be a limiting factor for other potential uses of the capsules.

3.4 *Sodium response tests*

The response of loaded capsules to increasing sodium levels was studied as an initial feasibility test. Figure 8 shows three representative spectra for different sodium concentrations after normalization (418 nm) to correct for source drift. The sodium concentration was increased in 10 mM increments, and it can be easily seen that the SBFI peak intensity increases significantly with increasing sodium, though the plot only includes spectra for 0, 50, and 100 mM. These results are very promising, as the data indicate that the encapsulated analyte-sensitive dye retains its indicator functionality.

Another critical point is the necessity of including an internal reference when making fluorescence intensity-based measurements. Although it is not obvious from the normal-

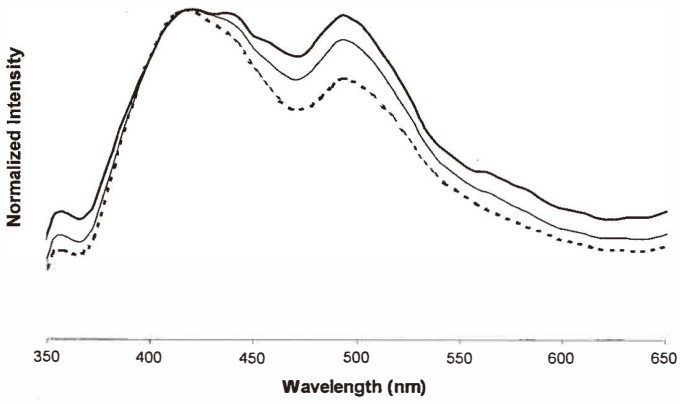

Fig. 8. Representative fluorescence spectra (normalized to 418 nm) of capsules suspended in buffer of increasing sodium levels. Spectra for concentrations of 0 mM (thick dashed), 50 mM (thin solid), and 100 mM (thick solid) are shown.

ized plots, the importance of the reference dye is clear when observing raw spectra. Due to variations in the intensity of the laser source over the integration period, even replicate spectra collected from the same sample vary significantly in overall intensity. Thus, without a reference point, quantitative measurements would be extremely difficult because any instrumental variations would lead to drift. For the system described here, replicate scans overlap completely after normalization to the reference peak.

Figure 9 places the peak ratio changes with increasing sodium concentration on a quantitative scale. The first observation is that the peak ratios exhibit an approximately linear relationship with increasing sodium concentrations. Secondly, this effect is observed over the entire range of concentrations tested, and may extend beyond this point. Another important point is that, according to the standard deviations described by error bars, 20 mM accuracy can be achieved. The error bars decrease in size as the concentration increases, most likely due to the development of a strong peak from SBFI from the less well-defined band superimposed on the strong MCA peak that is evident at low concentrations. Thus, the response is clearly seen from the data, and it is realistic to believe that the accuracy and range can be improved over that obtained for the simple example presented here.

4. Conclusion

The paper describes a novel, extremely general technology for encapsulating fluorescence assays using nanoengineered liquid-core shells. The results of initial experiments performed toward the understanding of loaded polyelectrolyte capsule properties are extremely promising when considering potential sensor applications. It has been shown that it is possible to encapsulate fluorescent probes in stable, polyelectrolyte microshells with excellent controllability, and sodium-sensitivity experiments suggest that encapsu-

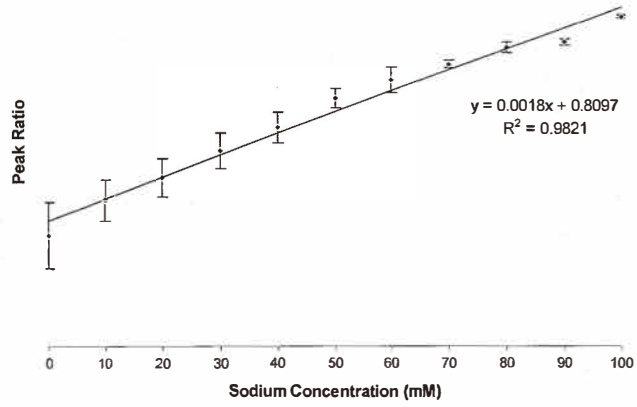

Fig. 9. Fluorescence intensity ratio (493 nm: 418 nm) of capsule spectra with increasing sodium concentration. Markers indicate the average peak ratio and error bars show one standard deviation; all values were computed from three replicate scans at each concentration.

lated fluorescent indicators can be useful as sensors. The long-term robustness of capsules has been shown, and it is likely that these shells will prove flexible in the detection of other analytes.

The potential implications of the findings are that tremendous improvements may be made over current methods being investigated for implantable biosensors. Because the interior of capsules is similar to the ambient solution, they can accommodate a much larger number of fluorophores per unit volume than any polymer-based approach. This results in increased signal intensity as well as sensing range. Furthermore, the ability to control the wall thickness with a precision of a few nanometers hints at the possibility of tuning the permeability of the capsules, thereby controlling the response time of encapsulated sensors. Transport will likely be much more rapid for capsules than for polymer carriers.

While these potential improvements are attractive, much work is needed to better grasp the capabilities of capsules for use as biosensors. Future investigations will seek to determine whether the above suggestions are accurate, and must consider a number of biosensor-related issues including calibration for quantitative measurements, reversibility, biocompatibility, and interfaces with instrumentation. In addition, while the versatility of the capsules as carriers for many fluorescent assays seems obvious, the limitations in loading different molecules must be identified. It is anticipated that future work will prove capsules to be extremely general and useful in producing novel sensors for both intracellular and extracellular measurements.

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