Engineering Fluorescent Poly(dopamine) Capsules

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KEYWORDS: mussel-inspired, templating, fluorescence, cell imaging, cancer cells

ABSTRACT: The recent development of poly(dopamine) (PDA) capsules provides new opportunities for their application in biology and medicine. To advance the biomedical application of PDA capsules, strategies that enable the preparation of fluorescently labeled PDA (F-PDA) capsules are required, as this will allow evaluation of their cellular interactions using a range of fluorescence-based techniques. Herein, we report a facile approach for the fabrication of F-PDA capsules via the polymerization of dopamine (DA) on sacrificial templates in the presence of hydrogen peroxide (H_2O_2). F-PDA capsules with well-defined sizes are prepared by templating different organic and inorganic particles. The resulting F-PDA capsules show negligible cytotoxicity in HeLa cells after incubation for 48 h. We also demonstrate visualization of the F-PDA capsules following internalization by HeLa cells using conventional fluorescence microscopy, en route toward detailed investigations on their biological interactions.

INTRODUCTION

Poly(dopamine) (PDA), formed by the oxidative polymerization of dopamine (DA), is a well-known melanin-like biopolymer in many parts of living organisms.[1] By mimicking mussel protein adhesion, Messersmith and co-workers pioneered a simple but versatile surface modification approach based on DA polymerization in alkaline solution onto various organic and inorganic substrates, including noble metals, oxides, polymers, semiconductors, and ceramics.[2] The obtained PDA surface coating is reactive to amine and thiol groups via Schiff base formation or Michael addition, which provides a facile approach to modify surfaces with various functional molecules.[3] Alternatively, functional molecules can be incorporated into the PDA structure during the polymerization process, allowing the fabrication of hybrid coatings with diverse reactivity and physicochemical properties.[4]

The advantage of PDA coatings reside in their versatility, such as control over their thickness, surface chemistry, and excellent biocompatibility,[5] PDA-based polymer capsules have recently been prepared by templated assembly which represents an attractive approach for engineering nanostructures.[6] A number of particulate templates have been used, including porous[7] and nonporous[8] silica particles, and emulsion droplets.[9] Recently, biological studies of these PDA capsules have been reported; for example, submicrometer-sized PDA capsules have been used to immobilize doxorubicin via a pH-liable linker, and these drug-loaded PDA capsules show effective intracellular drug release in cancer cells in vitro.[10] The ability to track the mobility of PDA capsules in biological systems will provide important insights into their safety and efficacy as drug carriers. However, PDA is known to act as a fluorescence quencher, which complicates the fluorescent labeling of PDA capsules.[11]

To investigate the interactions of PDA capsules with biological systems, it is important to overcome such labeling challenges. Recently, we used a thiol-containing poly(methacrylic acid) polymer conjugated with Alexa Fluor 488 dye as a linker to label PDA capsules.[10] Although the polymer-dye conjugates reduce the influence of PDA on fluorescence quenching, the cross-linking and conjugation labeling approach requires multiple steps, often limiting the labeling efficiency. A recent study has reported that the oxidative treatment of DA in the presence of hydrogen peroxide (H_2O_2) yields fluorescent precipitates.[12] This finding inspired us to seek an alternative approach to prepare fluorescent PDA (F-PDA) capsules by combining the aforementioned oxidative treatment process with PDA templated assembly.

Herein, we report an approach to produce F-PDA capsules via template assembly, eliminating the need for conjugation with dye molecules. PDA capsules are synthesized by polymerization of DA on templates and subsequent core removal. The resulting PDA capsules serve as templates for additional treatment with DA in the presence of H_2O_2, leading to the formation of F-PDA capsules (Scheme 1). The size of the F-PDA capsules can be controlled by using different sized templates. We show that the fluorescence of the F-PDA capsules is pH-dependent, with the highest fluorescence observed at pH 3. Moreover, these F-PDA capsules exhibit negligible cytotoxicity in HeLa cells after incubation for 48 h.

EXPERIMENTAL SECTION

Materials. Silica (SiO_2) particles (1.1 or 2.5 μm in diameter (D)) and polystyrene (PS) particles (D = 3.55 μm) were purchased from Microparticles GmbH. Calcium carbonate ($CaCO_3$) particles (D = 5.5 μm) were synthesized according to the protocol reported by Imai et al.[13] Hydroxytyramine hydrochloride (dopamine), hydrofluoric acid (HF), ammonium fluoride

(NH_4F), tetrahydrofuran (THF), ethylenediaminetetraacetic acid (EDTA), phosphate-buffered saline (PBS), and 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma-Aldrich. Tris(hydroxymethyl)-aminomethane (Tris), ethanol, sodium phosphate (Na_2HPO_4), and citric acid monohydrate were purchased from Chem-Supply (Australia). The universal buffer was prepared from a mixture of Na_2HPO_4 and citric acid solution. By adjusting the volume ratio, buffers between pH 3 and 8 were prepared.[14] H_2O_2 aqueous solution (30% (w/w) in H_2O) was used for all oxidation experiments in the synthesis of F-PDA capsules. High-purity (Milli-Q) water used in all experiments, and was prepared by a Millipore Milli-Q water purification system with a resistivity greater than 18 MΩ·cm.

Synthesis of F-PDA Capsules. 10 mg of template particles (3.55 μm PS, 5.5 μm $CaCO_3$, 1.1 μm SiO_2, or 2.5 μm SiO_2) and 20 mg of DA were added to 50 mL of 10 mM Tris buffer (pH = 8.5), respectively. The suspensions were constantly agitated overnight at room temperature to form PDA core-shell particles, followed by washing with fresh Tris buffer by three centrifugation/re-dispersion cycles. PDA capsules were obtained after removing the template particles using: THF for PS particles; 0.2 M EDTA solution for $CaCO_3$ particles; and 2 M HF /8 M NH_4F solution (HF) at pH 5 for SiO_2 particles. ***[****Caution! HF solution is highly toxic. Extreme care should be taken when handling HF solution, and only small quantities should be prepared.****]*** The resulting PDA capsules were combined and resuspended in 10 mL Tris buffer (pH 10.5) with a DA concentration of 5 mg mL^{-1} and allowed to react for 60 min. Subsequently, 2.5 mL of H_2O_2 was added to the mixture and allowed to react for another 15 h. The obtained F-PDA capsules were washed with Milli-Q water three times through centrifugation/re-dispersion cycles.

Cell Culture. HeLa cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) GlutaMAX supplement (Gibco) containing 10% fetal bovine serum at 37 °C in a 5% CO_2 humidified atmosphere and subcultured prior to confluence using trypsin.

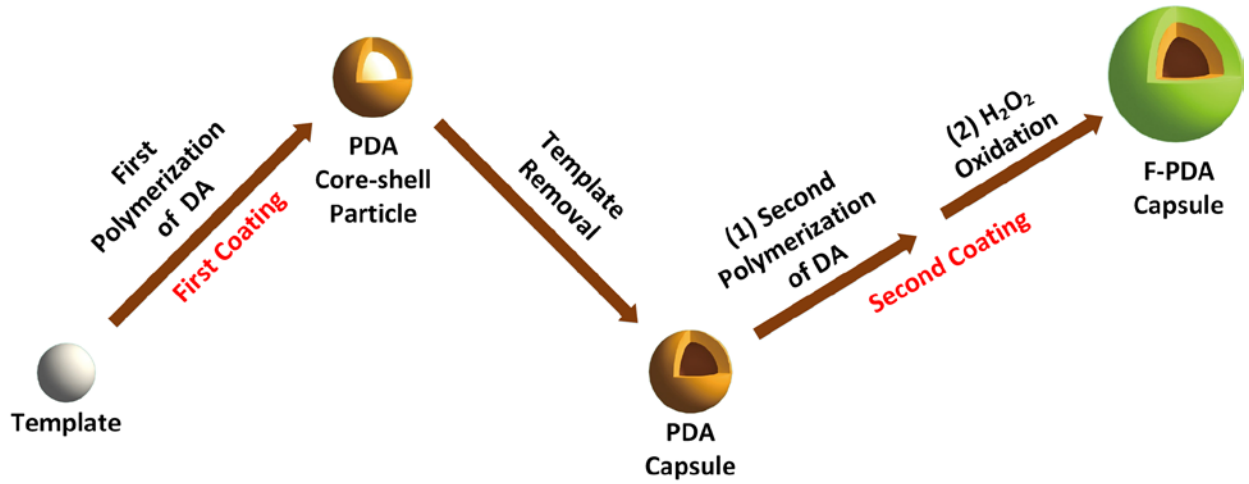
MTT Assay. HeLa cells were seeded at a density of 2 x 10^4 cells per well in 96-well plates. Cells were incubated for 48 h at 37 °C in a 5% CO_2 atmosphere after the addition of 200 μL of a suspension containing 1.1 μm F-PDA capsules (derived from SiO_2 templates) at various cell-to-capsule ratios: 1:0, 1:10, 1:25, 1:50, and 1:100. Plates were further incubated at 37 °C for 2 h after the medium was replaced with 200 μL of a MTT-containing medium (final concentration of 0.5 mg mL^{-1}). The absorbance of blue Formazan at 560 nm was measured with a plate reader (Multiskan Ascent, Thermo Scientific) after the addition of 150 μL of solubilization mixture (0.04 N HCl in isopropanol). The absorbance values of untreated cells were set as 100%, and cell proliferation was expressed as a percentage of untreated cells.

Cell Fixation and Staining. F-PDA capsules were incubated with HeLa cells. HeLa cells were plated at 2 × 10^4 cells per well into 8-well Lab-Tek I chambered coverglass slides (Thermo Fisher Scientific, Rochester) and allowed to adhere overnight. Cells were then incubated with F-PDA capsules at a cell-to-capsule ratio of 1:100 for 24 h at 37 °C in a 5% CO_2 atmosphere. Following this, the cells were gently washed with PBS three times and fixed with 4% paraformaldehyde for 30 min at room temperature. Nuclei and membrane were stained with Hoechst 33342 (2 μg mL^{-1}) and Alexa Fluor 488 Wheat Germ Agglutinin (5 μg mL^{-1}) after incubating cells with the labeling reagents (25 °C) for 30 min, individually.

Characterization Methods. Scanning electron microscopy (SEM, FEI Quanta 200) and transmission electron microscopy (TEM, Philips CM120 BioTWIN, operated at 120 kV) were

used to observe the capsule morphologies. Capsules were imaged on an Olympus IX71 inverted fluorescence microscope equipped with a fluorescein isothiocyanate (FITC) filter cube. The fluorescence intensity was determined using a fluorolog spectrofluorometer (Horiba) comprising a 450 W xenon arc lamp. Cell images were obtained using a deconvolution fluorescence microscope (DeltaVision, Applied Precision) equipped with a 60× 1.52 NA oil objective and a standard FITC/TRITC (excitation of 555 ± 14 nm, emission of 617 ± 37 nm) filter setting. The deconvolution fluorescence microscopy images were processed with Imaris (Bitplane). The UV-Vis absorption spectra of F-PDA capsules suspended in Milli-Q water were quantified using a NanoDrop 1000 spectrophotometer (Thermo Scientific). Capsule counting was performed on a CyFlow Space flow cytometer (Partec GmbH). The pH values of solutions were measured with a Mettler-Toledo MP220 pH meter.

Scheme 1. Schematic illustration for the synthesis of F-PDA capsules. Various template particles were coated with a PDA film through the first polymerization of DA. PDA capsules were obtained after template removal. A second PDA coating, which includes the second polymerization of DA and subsequent reaction with H_2O_2, yielded F-PDA capsules.



RESULTS AND DISCUSSION

Preparation of F-PDA Capsules. Organic (PS) and inorganic (SiO_2 and $CaCO_3$) template particles were used to synthesize PDA capsules (Scheme 1). The TEM, SEM, and fluorescence microscopy images of the F-PDA capsules prepared using the various templates are shown in Figure 1 (for SiO_2), Figure S1A and B (for PS), and Figure S1C and D (for $CaCO_3$). Different-sized monodisperse F-PDA capsules were obtained by varying the template size (D = 1.1 μm and 2.5 μm, respectively), and their fluorescence was confirmed by visualization using standard optical fluorescence microscopy with a FITC filter (excitation 555 ± 14 nm) (Figure 1C and D). All methods indicated that the individual capsules retain their size and spherical shape after removal of the template. The shell thickness of the F-PDA capsules (derived from 1.1 μm SiO_2) was determined by TEM to be 60 ± 10 nm (inset, Figure 2B). It is noted that an F-PDA layer can be formed directly on the pristine template particles without the first PDA coating; however, without the first PDA coating, the resulting capsules exhibited poor mechanical strength, and did not yield stable free-standing capsules after template removal. In addition, in the case of SiO_2 templates, treatment with HF for template removal resulted in a significant decrease in the fluorescence intensity of the F-PDA capsules compared with the F-PDA core-shell particles (Figure S2). Based on these two findings, we prepared F-PDA capsules by forming the fluorescent coating on preformed PDA capsules, as illustrated in Scheme 1.

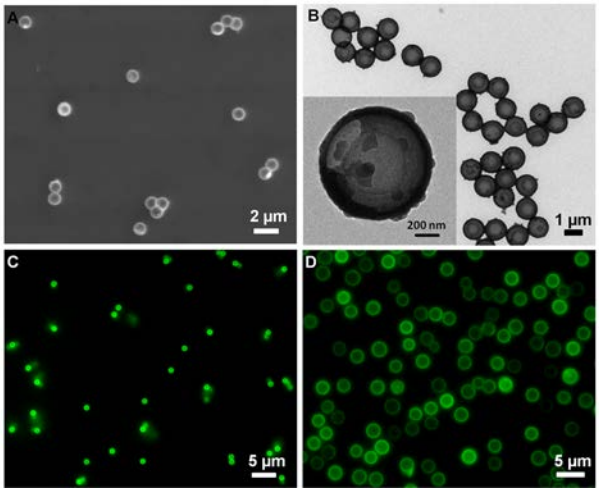


Figure 1. (A) SEM, (B) TEM, and (C) fluorescence microscopy images of F-PDA capsules prepared using 1.1 µm-diameter SiO_2 particles. The inset in (B) shows a single F-PDA capsule at higher magnification. (D) Fluorescence microscopy image of F-PDA capsules synthesized using 2.5 µm-diameter SiO_2 particles.

Fluorescence Properties of F-PDA Capsules. To identify the optimum excitation wavelength of the F-PDA capsules, UV-Vis absorption spectra of the F-PDA capsules were measured. F-PDA capsules exhibited a broad absorption, from 350-600 nm (Figure S3). The underlying broad absorption (350-600 nm) is expected due to the presence of PDA in the capsule walls. A broad monotonic absorption spectra is characteristic of PDA[15] and synthetic melanins,[16] although the exact reason for this unique absorption behavior is debated in literature.[16-17] Additionally, the UV-vis absorption spectra also exhibited a shoulder at 350-400 nm, which can be attributed to the presence of fluorescent species formed in solution, which are incorporated (either covalently or non-covalently) into the PDA films during the oxidative treatment process. Excitation of a suspension of F-PDA capsules at 400 nm results in fluorescence emission at ca. 480 nm. Moreover, the supernatant of the F-PDA coating process is also found to exhibit strong fluorescence at 480 nm when excited at 400 nm (Figure S4). In a control experiment, PDA

capsules were subject to H_2O_2 treatment in the absence of added DA. In contrast to PDA capsules treated with DA and H_2O_2, capsules reacted with only H_2O_2 do not display fluorescence (Figure S5). These results support the hypothesis that the fluorescence of F-PDA capsules arises due to incorporation of small fluorescent molecules formed in solution by a reaction between DA and H_2O_2, rather than a modification of the existing PDA film.

Next, we sought to optimize the fluorescence of the F-PDA capsules by varying the reaction time for the second DA polymerization step and the subsequent oxidation with H_2O_2. Different reaction times for both the DA polymerization (15 min, 30 min, 60 min, 180 min and 240 min) and H_2O_2 oxidation (5 h, 10 h, 15 h, 20 h and 30 h) steps were chosen. The second DA polymerization for 60 min (Figure 2A) and the subsequent oxidation with H_2O_2 for 15h (Figure 2B) yielded F-PDA capsules with the maximum fluorescence intensity. A decrease in fluorescence intensity of F-PDA capsules was observed at longer reaction times (>15 h). Significantly, strong oxidizing agents such as periodate[15b] and alkaline H_2O_2[18] have been reported to chemically degrade PDA and melanins respectively. Consequently, excessive treatment of F-PDA capsules with DA/H_2O_2 may lead to oxidative degradation and destabilization of the F-PDA structure, leading to a loss in fluorescence intensity.

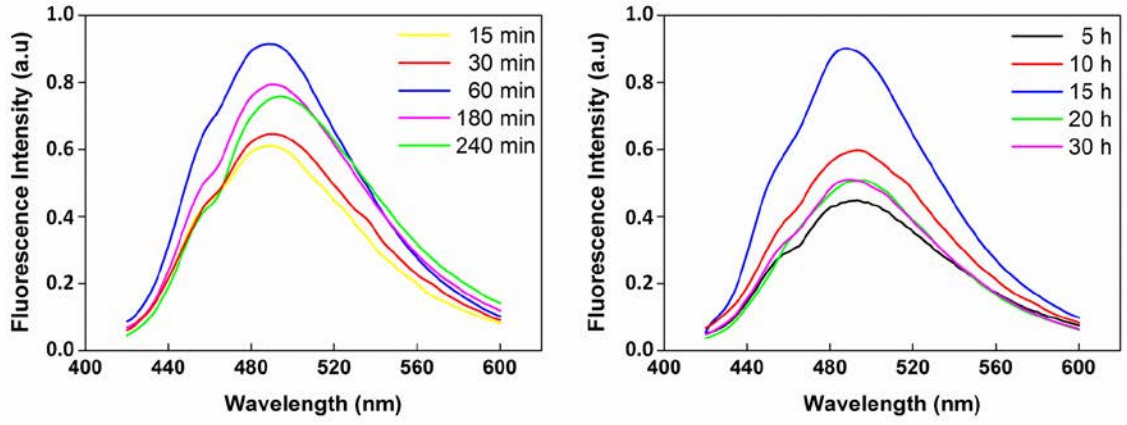


Figure 2. Normalized emission spectra of F-PDA capsules prepared from 1.1 μm-diameter SiO_2 particles using different reaction times: (A) varying the second DA polymerization time, keeping the H_2O_2 reaction time with constant at 15h; and (B) varying the H_2O_2 reaction time, keeping the second DA polymerization time constant at 60 min. All F-PDA capsules were excited at 400 nm.

The pH-dependence of the F-PDA capsules was also investigated. The F-PDA capsules were suspended in buffer solutions of different pH, and the fluorescence intensity was measured. The fluorescence intensity, from high to low, was pH 3 > pH 8 > pH 4 > pH 6> pH 7 > pH 5 (Figure 3A). The F-PDA capsules in solutions of pH 3, 6 or 8 were also imaged by fluorescence microscopy with identical exposure times and lamp power for direct comparison. The brightest F-PDA capsules were observed in pH 3 solution, with a slightly higher fluorescence intensity at pH 8 compared with pH 6 (Figure 3B, 3C and 3D). Interestingly, a different pH-dependence (from pH 3-8) of the supernatant from the DA/H_2O_2 treatment process was observed. The fluorescence intensity increased with an increase in pH (Figure S6). This suggests that the fluorescent compounds in the supernatant are further reacted with the PDA surface during the polymerization, resulting in different fluorescent moieties. However, due to the complexity of DA polymerization, elucidation of the exact chemical reactions and pH-dependence of the resulting fluorescence remain unknown.

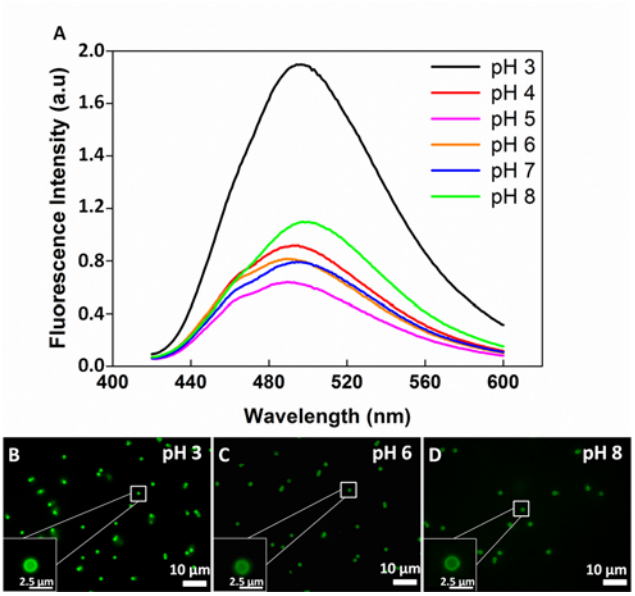


Figure 3. (A) Normalized emission spectra of F-PDA capsules dispersed in solutions of different pH. (B - D) Fluorescence microscopy images of F-PDA capsules dispersed in buffer solutions of different pH: (B) pH 3, (C) pH 6, and (D) pH 8. The capsules were prepared using 1.1 µm-diameter SiO_2 particle templates.

Cytotoxicity Studies and Cellular Internalization of F-PDA Capsules. MTT assays were conducted to examine the cytotoxicity of the F-PDA capsules. HeLa cells were treated with F-PDA capsules at various cell-to-capsule ratios: T1 (1:0), T2 (1:10), T3 (1:25), T4 (1:50), and T5 (1:100). No significant changes in cell viability were observed for all treatments (Figure 4), indicating negligible cytotoxicity of the F-PDA capsules.

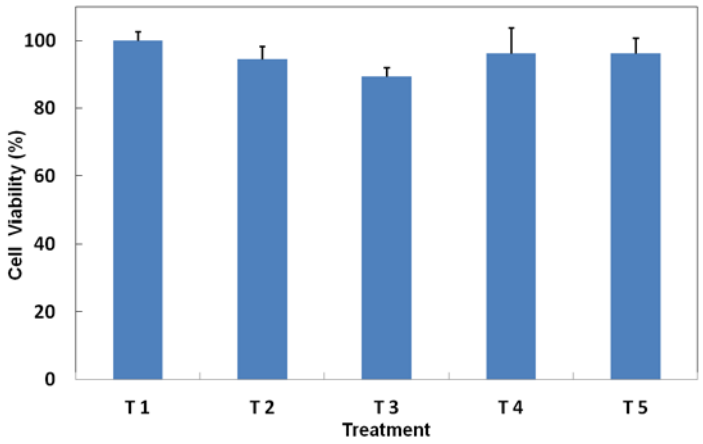


Figure 4. Cell viability of HeLa cells measured by MTT assays. The cells were incubated with F-PDA capsules at various doses (cell-to-capsule ratio): T1 (1:0), T2 (1:10), T3 (1:25), T4 (1:50), and T5 (1:100). The viability of untreated cells is set as 100%. Data are shown as the mean of the standard deviation from three independent experiments. Triplicates were performed for each experiment.

The F-PDA capsules were incubated with HeLa cells for 24 h, and the cells were fixed after the treatment. Subsequently, the cell membranes and nuclei were stained with fluorescent probes. By using a fluorescence deconvolution microscopy with a standard TRITC filter (excitation of 555 ± 14 nm, emission of 617 ± 37 nm), the F-PDA capsules were imaged, showing as spherical structures (Figure 5). Importantly, based on the cell membrane staining, the F-PDA capsules were found to be effectively internalized by HeLa cells (Figure 5B). It is noted that in contrast to many multilayered polymer capsules, these internalized F-PDA capsules retained the spherical morphology, showing little deformation. This is presumably due to the thicker F-PDA capsule shell (~60 nm) observed by TEM, leading to increased mechanical strength. The effective internalization of F-PDA capsules is also consistent with our previous report on the pH-triggered endocytic drug release using PDA capsules, although the two capsules possess some differences

in their physicochemical properties.[10] In addition, there has been considerable interest to engineer degradable PDA drug carriers based on biological cues, such as enzymes.[19] Therefore, the intrinsic fluorescence of PDA capsules reported in this study provides the possibility to track PDA materials in biological systems using fluorescence-based technologies. Further, when such PDA carriers are combined with responsive (degradable) moieties, or incorporated into structures assembled by existing stepwise assembly techniques[20] to form hybrid structures, new opportunities are likely to arise for the use of such systems in drug/gene delivery.

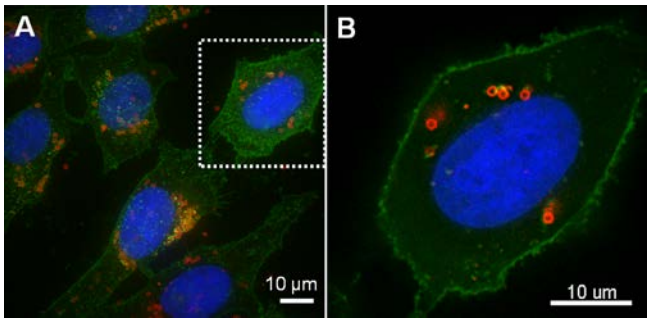


Figure 5. Fluorescence deconvolution microscopy images of F-PDA capsules in HeLa cells. (A) Image presented at the maximum intensity projection. (B) Image of single z-plane of the frame area in A. The internalized F-PDA capsules were visualized using a standard TRITC filter (red). The cell membrane was stained with AF488-labeled wheat germ agglutinin (green), and the nuclei were stained with Hoechst 33342 (blue).

CONCLUSION

We have reported the synthesis of F-PDA capsules with well-defined sizes via the polymerization of DA in the presence of H_2O_2. The resulting fluorescence of F-PDA capsules were shown to be pH-dependent, with the highest fluorescence intensity observed at pH 3. In addition, the F-PDA capsules (1.1 μm diameter) showed negligible cytotoxicity in HeLa cells

after incubation for 48 h at various doses. The internalized F-PDA capsules in HeLa cells were found to be readily visualized using conventional fluorescence microscopy, which allows further quantitative and qualitative analysis of the cellular interactions of PDA materials. This information will be valuable for the developments of PDA in various biological applications. Given the diversity of templates, it is expected that F-PDA capsules with different morphologies can also be fabricated through this versatile approach.

ASSOCIATED CONTENT

Supporting Information

Preparation of F-PDA capsules using PS and $CaCO_3$ templates, UV-Vis absorption of F-PDA capsules and decrease of fluorescence intensity of F-PDA capsules upon HF treatment. This material is available free of charge via the Internet at http://pubs.acs.org.

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ACKNOWLEDGMENT

This work was supported by the Australian Research Council under the Australian Laureate Fellowship (F.C., FL120100030) and Discovery Early Career Researcher Award (Y.Y., DE130100488). M. M. acknowledges The University of Melbourne for a McKenzie fellowship. X. C. acknowledges The University of Melbourne for a Melbourne Research Scholarship and a Melbourne International Research Scholarship. We acknowledge Dr. Nicholas Williamson and

Paul O'Donnell (Bio21 Mass Spectrometry and Proteomics Facility) for assistance with chromatography and mass spectroscopy analysis.

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