Preparation of a Molecularly Imprinted Polymeric Nanocapsule with Potential Use in Delivery Applications

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ABSTRACT: A molecularly imprinted nanocapsule was prepared, which showed excellent site accessibility and potential in delivery applications. The monomer−template complex (Est−Vinyl) was synthesized by the reaction of 3-isopropenyl-α,α-dimethylbenzyl isocyanate with estrone, where the template was linked to a polymerizable vinyl group via a thermally reversible urethane bond. Microemulsion polymerization of Est−Vinyl, styrene, and divinylbenzene was carried out in water and octanol by using potassium persulfate as an initiator to produce a nanocapsule having a cross-linked polymeric wall. The hollow structure of the nanocapsule was confirmed by transmission electron microscopy. The template was removed from the polymeric wall by means of a simple thermal reaction. In aqueous media, the imprinted nanocapsule solubilized hydrophobic pyrene. When the imprinted nanocapsules were previously incubated in a template (estrone) solution, pyrene could not transfer effectively into the interior of the nanocapsules, suggesting that the imprinted sites were blocked by the template molecules.

Introduction

Artificial receptors fabricated by molecular imprinting have attracted a great deal of interest due to their potential use in such applications as chemical sensors, catalysts, and the separation of toxic chemicals.[1] The advantages of molecularly imprinted materials, as compared to biological receptors, include their mechanical and chemical stability, low cost of preparation and wide range of operating conditions. However, they suffer from some drawbacks in certain applications, such as the heterogeneous distribution of the binding sites, low capacity and selectivity, and poor site accessibility. The development of suitable methods for overcoming these problems will open the door to considerably more diverse application opportunities than are available at present. In this context, many recent studies have focused on the development of new methodologies for producing imprinted materials exhibiting uniform structures and high affinity to the target molecules.[2]

We hypothesized that polymeric nanocapsules would make a good candidate imprinting material having high capacity and excellent site accessibility. Nanocapsules have a structure consisting of a hollow interior surrounded by a thin wall. Given that the size and wall thickness can be controlled, we postulated that a definite number of binding sites could be generated at the wall of the nanocapsule by molecular imprinting. In addition, we surmised that if the wall were sufficiently thin, a specific compound of interest could be encapsulated inside an imprinted nanocapsule, in which the imprinted site would act as the gate and the template as the stopper.[3]

In this work, we employed the method of microemulsion polymerization to prepare nanocapsules. Microemulsion polymerization has been actively studied for the preparation of nanoparticles[4] and more recently for the preparation of nanocapsules.[5] The polymerization of styrene and divinylbenzene in the presence of a monomer−template complex was carried out in oil-in-water microemulsion droplets. After polymerization, the template molecules were removed to generate template-shaped pores in the cross-linked polymeric wall. This approach was expected to endow the imprinted nanocapsules with excellent site accessibility, since all of the binding sites are located at the wall of the nanocapsules.

We previously reported the use of a thermally reversible bond in the process of template-monomer complexation for use in molecular imprinting. This method has certain advantages in that it is possible to easily remove the template molecule from the matrix and to introduce various functional groups into the cavities.[6] We extended this approach to the generation of the binding sites in the nanocapsule wall. Estrone, which was used as the template in this study, is a naturally occurring estrogen, which influences the normal development and maturation of the female. Estrogens have been suspected of having carcinogenic properties and adverse environmental effects, and have been subjected to extensive quantitative analysis.[7]

Experimental Section

Materials. Dibutyltin dilaurate (DBTDL), 3-isopropenyl-α,α-dimethylbenzyl isocyanate (IPDMBI), divinylbenzene, styrene, dodecyltrimethylammonium bromide (DTAB), pyrene, potassium persulfate, and isooctane were purchased from Aldrich Chemical Co. Estrone, testosterone, and testosterone propionate were obtained from TCI. THF was used after purification by standard methods. Other chemicals were used as received without further purification.

Synthesis of Est—Vinyl. Estrone (0.5 g, 1.85 mmol) and IPDMBI (0.37 g, 1.85 mmol) were dissolved in THF (30 mL). To the solution, DBTDL (1 mL) was added at 25 °C. The reaction mixture was stirred for 24 h at 75 °C. The solvent was evaporated and the product was isolated by column chromatography on silica gel (EA: hexane = 1:3, $CHCl_3$: MeOH = 9:1). Yield: 43%.

Anal. Calcd for $C_{31}H_{37}NO_3$: C; 78.95, H; 7.91, N; 2.97. Found: C; 78.69, H; 8.15, N; 2.60. ^{1}H NMR (300 MHz, DMSO-d_6): δ 8.16 (s, NH), δ 7.51 (s, 1H), δ 7.32 (s, 3H), δ 7.23 (d, 1H), δ 6.75 (d, 1H), δ 6.73 (s, 1H), δ 5.39 (s, 1H), δ 5.11 (s, 1H), δ 2.80 (bs, 2H), δ 2.50−1.95 (bm, 6H), δ 2.13 (s, 3H), δ 1.77 (bd, 1H), δ 1.60 (s, 6H), δ 1.50−1.36 (bm, 6H), δ 0.82 (s, 3H). ^{13}C NMR (75 MHz, DMSO-d_6): 219.6, 152.8, 148.7, 147.7, 142.9, 140.1, 137.4, 136.2, 128.1, 126.0, 124.1, 123.1, 121.6, 121.5, 118.9, 112.4, 69.8, 54.8, 49.5, 47.3, 43.5, 37.6, 35.4, 31.3, 29.4, 28.8, 25.8, 25.4, 21.6, 21.1, 13.5.

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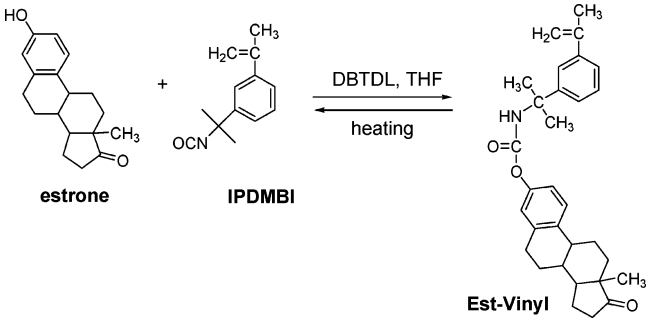
Scheme 1. Synthesis of the Monomer−Template Complex (Est−Vinyl)



Microemulsion Polymerization of Est—Vinyl, Styrene, and Divinylbenzene. DTAB (2.0 g, 10.3 wt %) was dissolved in MeOH (2.3 mL) and H_2O (20 mL). The solution was stirred for 1 h at 39 °C in order to form micelles in the water. Isooctane (4.2 mL) was added dropwise into the solution for 2 h and a mixture of Est−Vinyl (0.15 g, 0.32 mmol), styrene (1 g, 9.6 mmol) and divinylbenzene (1 g, 7.7 mmol) was added dropwise into the micelle formed solution for 2 h at 39 °C and the reaction mixture was heated to 70 °C. The initiator (potassium persulfate, 0.04 g) was added into the solution and the polymerization proceeded for 3 h. The residual surfactants were removed by excess methanol, and the nanocapsules were dried at room temperature for 3 days (yield: 57%).

Anal. Calcd: C; 91.33, H; 7.75, N; 0.21. Found: C; 90.91, H; 8.10, N; 0.14.

Preparation of Control Nanocapsule. Control nanocapsule was also prepared in a similar manner from styrene, divinylbenzene, and IPDMBI instead of Est−Vinyl (Yield: 49%).

Anal. Calcd: C; 91.98, H; 7.80, N; 0.22. Found: C; 91.42, H; 8.21, N; 0.15.

Measurements. The nanocapsules were investigated with a transmission electron microscope using JEM-3000F (JEOL, Japan). The sample was deposited on a carbon coated Cu grid after dispersed in methylene chloride. Fluorescence image of pyrene-loaded nanocapsules was obtained by using MRC-1024 confocal laser scanning microscope (Bio-Rad, U.K.). Reverse phase HPLC analysis was carried out using a M930 solvent delivery system, M720 absorbance detector and a software package Autochro-2000 (YOUNG LIN Instrument Co., Ltd., Korea). Analyses were performed using a MetaSil 5u ODS column from Metachem (Torrance, Canada) equipped with a UV−vis detector (set at 254 nm for all compounds) with methanol as eluent at a rate of 1.0 mL/min. For each analysis 20 μL of sample was injected. Excitation spectra of pyrene were investigated with a spectrofluorophotometer using RF-5301PC (Shimadzu, Japan).

Results and Discussion

The monomer−template complex (Est−Vinyl) was prepared by the reaction of 3-isopropenyl-α,α-dimethylbenzyl isocyanate (IPDMBI) with estrone in the presence of dibutyltin dilaurate (DBTDL) (Scheme 1). The reaction occurred between an isocyanato group of the monomer and a phenol moiety of estrone, forming a thermally cleavable urethane bond. It is known that the urethane bond formed between an isocyanate and a phenol is stable at room temperature, but that the reversible cleavage occurs at elevated temperatures.[8]

Figure 1 shows the fabrication process for the estrone-imprinted nanocapsule. Microemulsion polymerization of Est−Vinyl, styrene, and divinylbenzene was carried out according to Jang's procedure.[5] Micelles were first formed in water by using dodecyltrimethylammonium bromide (DTAB) as a surfactant. Isooctane and a mixture of Est−Vinyl, styrene, and divinylbenzene were added to the aqueous solution. Once isooctane and the monomers had penetrated into the micelles,

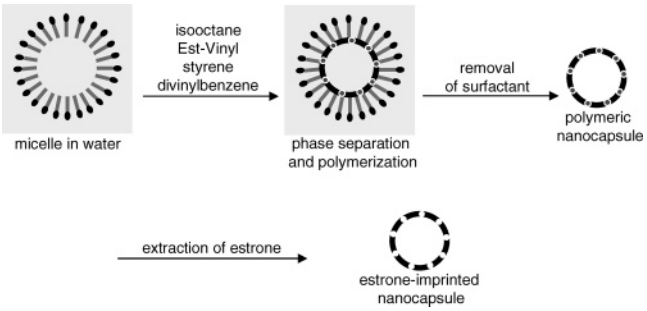


Figure 1. Fabrication of the estrone-imprinted nanocapsule.

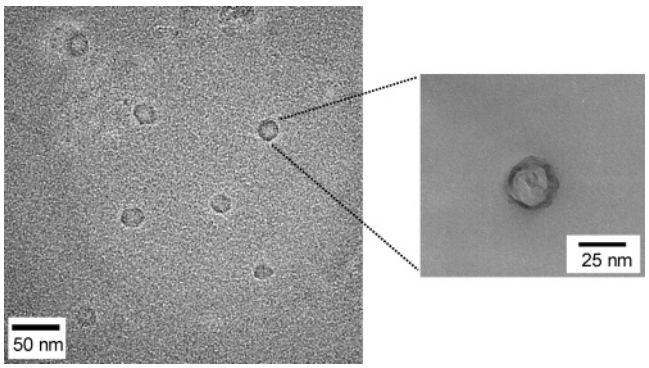
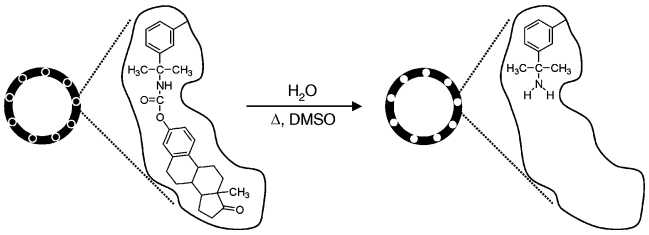


Figure 2. TEM images of estrone-containing nanocapsules.

Scheme 2. Extraction of the Template and Generation of the Recognition Site



the reaction mixture was heated to 70 °C and then the initiator (potassium persulfate) was added to the mixture. After polymerization for 3 h, the surfactants were removed by washing with excess methanol. It was reported that polymerization occurs primarily at the interface between isooctane and water, yielding the polymeric nanocapsules, due to the low interfacial energy in this system.[5]

The hollow structure of the nanocapsule was confirmed by transmission electron microscopy (TEM) (Figure 2). The diameters of the nanocapsules were in the range 20−25 nm, as estimated by TEM. The wall thickness was about 2.5 nm. A control nanocapsule, which had the pendant amine functionalities, was prepared in a similar manner from styrene, divinylbenzene, and IPDMBI instead of Est−Vinyl. Under the reaction conditions, the isocyanato group of IPDMBI was converted to the amino group by the reaction with water.

To extract the template molecule from the nanocapsule, estrone-containing nanocapsules were heated in DMSO at 180 °C in the presence of a small amount of water. During this process, isocyanato groups were generated by dissociation of the urethane bonds, which were subsequently converted to amino groups through their reaction with H_2O (Scheme 2).

The extraction of the template was confirmed by FT-IR spectroscopy (Figure 3). The stretching vibrations of the carbonyl groups of the urethane bond and estrone appeared at 1736 and 1722 cm^{-1}, respectively. These peaks disappeared after the extraction of the template molecules, while a new peak

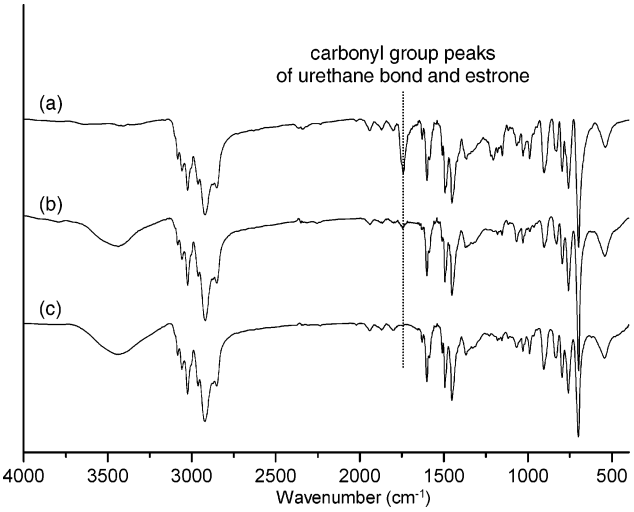


Figure 3. FT-IR spectra of the nanocapsule (a) before and (b) after extraction of template. (c) shows the spectrum of the control nanocapsule.

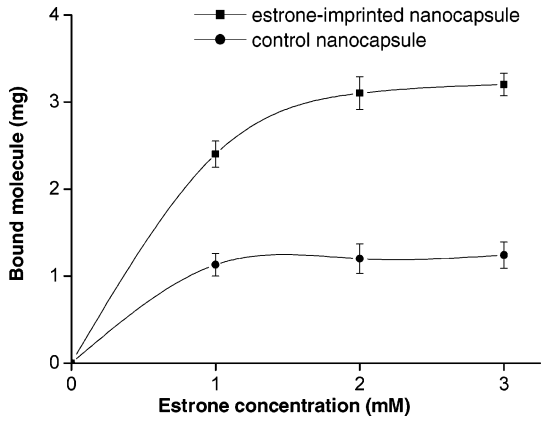


Figure 4. Amount of estrone bound by the estrone-imprinted nanocapsules and the control nanocapsules. All experiments were repeated three times.

corresponding to a primary amino group showed up at 3420 cm^{-1}.

The estrone recognition ability of the imprinted nanocapsules was investigated (Figure 4). An estrone-imprinted nanocapsule (50 mg) was added to solutions of estrone in methylene chloride (10 mL) at various concentrations (1, 2, and 3 mM). After incubating for 12 h, the nanocapsules were isolated by dialysis in methanol through a cellulose membrane tube with a molecular weight cutoff of 3500. The amount of estrone bound to the nanocapsules was determined by measuring the amount of residual estrone in the methanol by HPLC. The recognition ability of the control nanocapsule was measured in the same manner. As shown in Figure 4, the estrone-imprinted nanocapsules had much higher recognition ability than the control nanocapsules at all concentration ranges. It is noteworthy that the amount of estrone adsorbed on the recognition sites of the estrone-imprinted nanocapsules in 3 mM estrone solution was greater than 90% of the expected capacity.[9]

The kinetic uptake of estrone by the nanocapsules was investigated, to evaluate the site accessibility. A dialysis tube (molecular weight cutoff: 3500) containing a solution of the nanocapsule (50 mg) in methylene chloride (3 mL) was placed in a solution of estrone (10 mg) in methanol (100 mL), and then the concentration of estrone in methanol outside of the membrane tube was measured by UV spectroscopy every 20 min for 3 h. Under these conditions, the nanocapsules remained

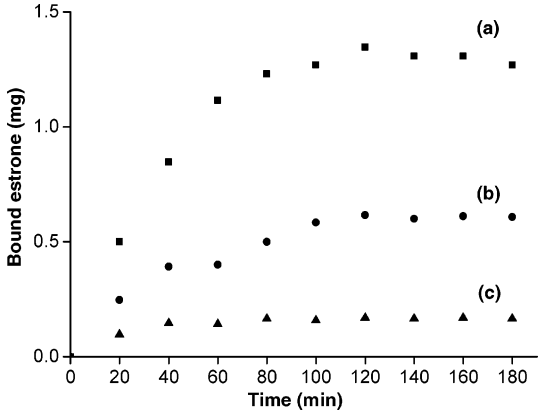


Figure 5. Kinetic uptake of estrone (a) by the estrone-imprinted nanocapsules, (b) by the control nanocapsules, and (c) by the dialysis membrane only.

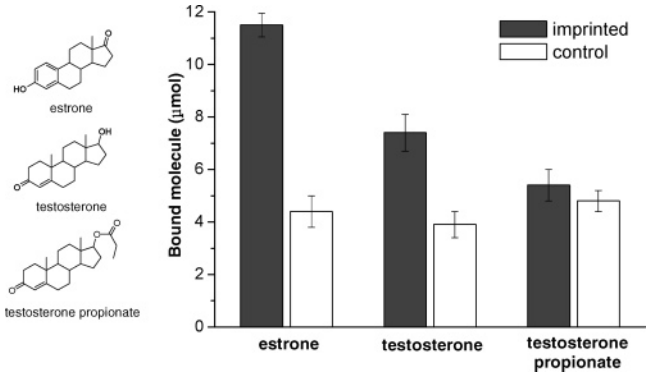


Figure 6. Amount of molecules bound by the estrone-imprinted nanocapsules and control nanocapsules. All experiments were repeated three times.

in the membrane tube, while estrone could freely pass through the membrane to access the nanocapsules. We also measured the amount of estrone adsorbed on the membrane in the absence of the nanocapsules. As shown in Figure 5, the estrone-imprinted nanocapsule possessed high site accessibility, exhibiting very fast uptake with saturation being reached in 2 h. The amount of estrone adsorbed on the membrane was not significant.

We investigated the specific recognition ability of the nanocapsules for testosterone and testosterone propionate, which are structural analogues of estrone, under the same conditions as those described above. The estrone-imprinted nanocapsule showed higher specific recognition ability for estrone than for either of the two chosen structural analogues (Figure 6).

Hydrophobic drugs can be delivered by nanocapsules in an aqueous environment.[10] We studied the encapsulation of a hydrophobic compound in the nanocapsules by using pyrene, which has a smaller size than estrone (template). The fluorescence of pyrene is known to be sensitive to changes in the microenvironment.[11] Figure 7 shows the fluorescence excitation spectra (λ_{em} = 393 nm) of pyrene in water in the presence or absence of the nanocapsules. Sample solutions were prepared as follows. A solution of pyrene (0.012 mg) in THF (0.12 mL) was added to distilled water (100 mL) and vigorously stirred for 30 min to evaporate THF, resulting in an aqueous pyrene solution (6×10^{-7} M) (sample a). Samples b and c were prepared by the addition of the estrone-imprinted nanocapsule and control nanocapsule (0.2 mg) dissolved in THF (0.2 mL) to the aqueous pyrene solution (6×10^{-7} M), respectively. The fluorescence excitation spectrum (Figure 7b) recorded in the presence of the estrone-imprinted nanocapsule (sample b) was red-shifted and the fluorescence intensity values near 323 and

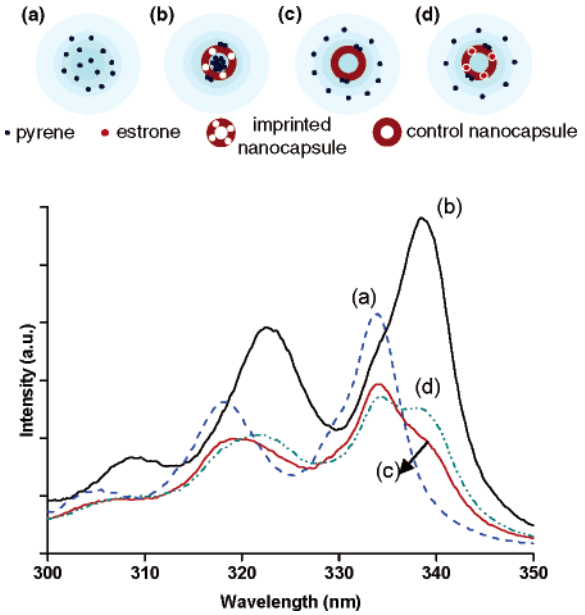


Figure 7. Fluorescence excitation spectra of pyrene [6×10^{-7} M in water (100 mL)] (a) in the absence of the nanocapsules (sample a), (b) in the presence of the imprinted nanocapsules (sample b), (c) in the presence of the control nanocapsules (sample c), and (d) in the presence of the imprinted nanocapsules incubated in an estrone solution in THF (3 mM, 2 mL) for 3 h (sample d).

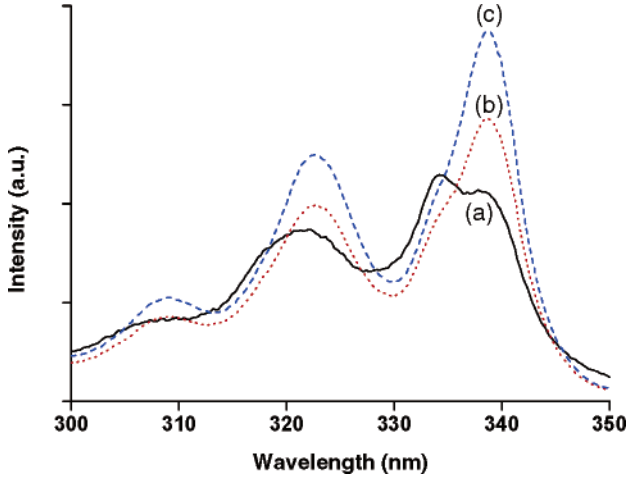


Figure 8. Fluorescence excitation spectra of pyrene [6×10^{-7} M in water (100 mL)] in the presence of the imprinted nanocapsules incubated in (a) an estrone solution, (b) a testosterone solution, and (c) testosterone propionate solution in THF (3 mM, 2 mL) for 3 h.

339 nm increased significantly, compared with the spectrum of pyrene only (sample a) (Figure 7a) or pyrene in the presence of the control nanocapsule (sample c) (Figure 7c). In sample d, estrone-imprinted nanocapsules (0.2 mg) incubated in an estrone solution in THF (3 mM, 2 mL) for 3 h were added to the aqueous pyrene solution (6×10^{-7} M). To be very interesting, the fluorescence spectrum of sample d (Figure 7d) showed much weaker peaks near 323 and 339 nm than that of sample b, and it was similar to that taken in the presence of the control nanocapsule (Figure 7c). These results indicate that pyrene molecules transferred into the hydrophobic interior of the nanocapsule mainly through the imprinted sites. In sample d, we presume that estrone blocked the gate to the hollow interior of the nanocapsules through the molecular recognition process, and thus, only a small amount of pyrene could enter into the interior of the nanocapsules.

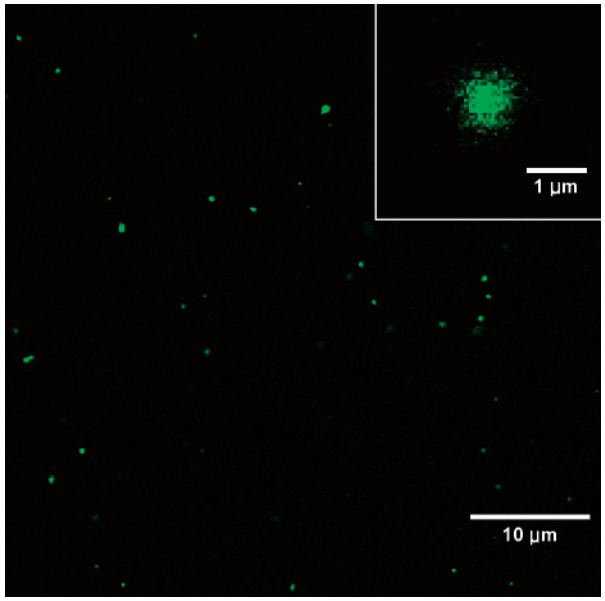


Figure 9. CLSM fluorescence images of pyrene-loaded nanocapsules.

Very interestingly, when the imprinted nanocapules were incubated in the solution of structural analogues of estrone, the fluorescence spectra were similar to that taken in the presence of nanocapsule without any incubation process, showing that testosterone and testosterone propionate cannot block the imprinted sites (Figure 8). The results also confirm that the imprinted site acted as a gate to the hollow interior of the nanocapsule and the template as the stopper.

The encapsulation of pyrene in the estrone-imprinted nanocapsules was also confirmed by confocal laser scanning microscopy (CLSM). The fluorescence image obtained from the imprinted nanocapsules (1 mg) in an aqueous pyrene solution (6×10^{-7} M, 10 mL) is shown in Figure 9. This image exhibits a green emission resulting from the encapsulated pyrene. As shown in the inset of this figure, the pyrene-loaded nanocapsules aggregated to form micrometer-sized green dots at this concentration.

Conclusions

We prepared estrone-imprinted nanocapsules. Recognition sites were generated at the cross-linked polymeric wall of the nanocapsule, resulting in high capacity and excellent site accessibility. We utilized a thermally reversible bond in the process of template-monomer complexation, which allowed us to readily remove the template from the polymeric wall by means of a simple thermal reaction. In aqueous media, the imprinted nanocapsules solubilized the hydrophobic pyrene. Previously when the imprinted nanocapsules were incubated in a template solution, the pyrene molecules could not transfer effectively into the interior of the nanocapsules. These results suggest that pyrene normally enters into the interior of the nanocapsules through the imprinted sites, which can be blocked by the template molecules. Future studies will be undertaken, to extend this discovery to the controlled release of hydrophobic drugs.

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(9) The expected capacity of estrone-imprinted nanocapsule was calculated on the assumption that all monomer−template complexes produced the imprinted sites; capacity of estrone-imprinted nanocapsule (mol/g) = 1 ÷ (mol wt of 3-isopropenyl-α,α-dimethylbenzylamine + mol wt of styrene × 30 + mol wt of divinylbenzene × 24). The amount of estrone adsorbed on the recognition sites was calculated by the difference between bound amount of imprinted nanocapusle and that of control nanocapsule.

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