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Encapsulation of Submicrometer-Sized 2-Methoxyestradiol Crystals into Polymer Multilayer Capsules for Biological Applications

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Abstract: A facile approach has been developed to encapsulate submicrometer-sized drug crystals into polymer multilayer capsules produced by sequential deposition of polymers onto the drug particle surfaces. 2-Methoxyestradiol (2-ME) is a hydrophobic metabolite of 17-β estradiol, which has been demonstrated as a potential anticancer agent. It was selected as a model drug and was formulated into submicrometer-sized particles through fine milling followed by intense sonication in the presence of dipalmitoyl-DL-(*R*)-phosphatidylcholine (DPPC). The reserved positive charges on the 2-ME crystal surface by DPPC enhanced the water solubility of the particles and subsequent self-assembly of dextran sulfate (DS) and dextran (DN) multilayers through hydrogen bonding and physical adsorption. Upon the exposure of the drug capsules to ethanol, hollow DS/DN multilayer polymer shells can be formed. The encapsulation process and hollow polymer multilayer shell formation were confirmed by confocal laser scanning microscopy (CLSM) and transmission electron microscopy (TEM), while the surface morphology of the formed drug capsules was investigated using scanning electron microscopy (SEM). In vitro studies show that the inhibitory effect of the formed 2-ME capsules is the same as that of the conventional formulation of 2-ME in a concentrated ethanol solution, as demonstrated by dramatic changes in cell morphology and significantly decreased viability of target cells. We also demonstrate that the change of the outermost layer of the drug capsules does not significantly influence its bioactivity. The presented strategy to encapsulate submicrometer-sized hydrophobic drug particles is expected to provide a general pathway to fabricate drug capsules for various biological applications.

Keywords: Encapsulation; 2-methoxyestradiol; polymer multilayers; capsules; bioactivity

Introduction

Many compounds are encapsulated to provide their controlled release under specific conditions.[1−5] An ideal encapsulation system should allow the adjustment of membrane shell thickness, pore size, mechanical strength, and

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surface potential, as well as render sufficient stability of the carrier system to achieve delivery. The commonly used encapsulation systems such as liposomes, microparticles, and microemulsions do not readily permit simultaneous manipulation of permeability, mechanical and chemical stability, surface charge, and biocompatibility, thus limiting their applications. An attractive and flexible construction method that meets all these requirements for encapsulation is the layer-by-layer (LbL) self-assembly technique, which is essentially based on the sequential deposition of oppositely charged individual polyelectrolyte (PE) layers.

The LbL self-assembly technique was first developed by Decher and Hong in the early 1990s to construct PE multilayers as well as composite PE/clay multilayers onto planar surfaces.[6−8] In late 1990s, the LbL technique was extended by Donath and Caruso to assemble PE multilayers onto 3-dimensional spherical colloids.[9,10] Upon extraction of the colloid core particles by chemical or physical treatments, uniform and stable hollow capsules can be readily formed. The major advantage of the LbL technique is that the thickness and composition of the multilayer films can be controlled by varying the number of PE deposition cycles and the charged species, respectively. This technique has been demonstrated as a powerful tool to construct various ordered functional thin films,[11] as well as diversified functional capsules,[12] core−shell particles,[13] and hollow spheres.[14]

By utilizing the LbL approach, a variety of materials can be encapsulated either as layer components or as colloidal templates within the multilayers. The primary requirement for a material to be encapsulated is that the material should be charged prior to the LbL process. If the material is encapsulated as a layer component, it is often dissolved in aqueous solution and assembled with oppositely charged PEs onto colloidal templates. For example, dyes,[15] proteins,[16] enzymes,[17] DNA,[18] and inorganic nanoparticles[13] have been encapsulated onto colloidal templates using oppositely charged PEs. On the other hand, if the material is encapsulated as a colloidal template, surface charges should be rendered before LbL construction. Various polymeric,[10,14] inorganic colloid particles,[19] and inorganic[20] and organic microcrystals[21−23] can be encapsulated within semipermeable PE multilayers. This technique is also amenable for encapsulation of delicate solid biological materials[24,25] and drugs:[26−30] e.g., protein crystals of catalase have been coated by the sequential adsorption of polyanions and polycations for biocatalysis applications and immunoassays.[24,31]

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The LbL strategy has also been extended to uncharged microparticles in order to achieve the encapsulation of hydrophobic, poorly water-soluble, low molecular weight compounds.[32] The hydrophobic microcrystals should be precharged by the self-assembly of amphiphilic compounds or PEs onto their surface, thereby rendering them water-dispersible, followed by LbL deposition of oppositely charged PEs. Upon dissolution of microcrystals using organic solvents, hollow intact PE multilayer capsules can be formed. The formed PE multilayer capsules are semipermeable, i.e., permeable for low molecular weight dyes,[33] ions,[34] and oligomers,[10] but not for enzymes (e.g., catalase[25,31]) or polymers with a molecular weight of about 4000 or greater.[35] This provides an ideal carrier system to encapsulate small molecular drugs and release the drug at specified conditions. Additionally, the thickness of the PE multilayer shell can be controlled at the nanometer level (with a single PE layer typically being 1−2 nm thick),[33] which allows controlled release of substances straightforwardly through control of the multilayer shell thickness. The driving force for the LbL assembly is essentially electrostatic interaction, although other forces such as hydrogen bonding,[36−40] covalent interaction,[41] and hydrophobic interaction[15,42,43] have also been demonstrated to be highly useful. The physicochemical properties and stability of multilayer films may vary when different driving forces are used to construct the multilayers. For biology and medical applications, the inclusion of nanoparticles,[44,45] biomacromolecules,[16] and/or lipids[46,47] in the capsule walls may be an alternative means of controlling the permeability, surface potential, biocompatibility, cell affinity, and targeting specificity. This provides an extremely useful strategy when the capsules are used for cancer therapeutics.

Although PE multilayer capsule systems have been well developed, few studies related to PE multilayer capsules are applied to biological and medical applications.[48−50] There is currently no reported literature related to in vitro cell biological studies using polymer multilayer-encapsulated drug crystals. In this present study, a novel, potential anticancer drug, 2-methoxyestradiol (2-ME), was selected as a model drug and was encapsulated into PE multilayer capsules for in vitro cellular bioactivity studies. The drug 2-ME is present in the serum of women during the ovulatory and luteal phases of the menstrual cycle and during pregnancy. As a metabolite of 17-β estradiol, 2-ME has been demonstrated to be a potential anticancer agent.[51,52] The conventional formulation of 2-ME remains concentrated

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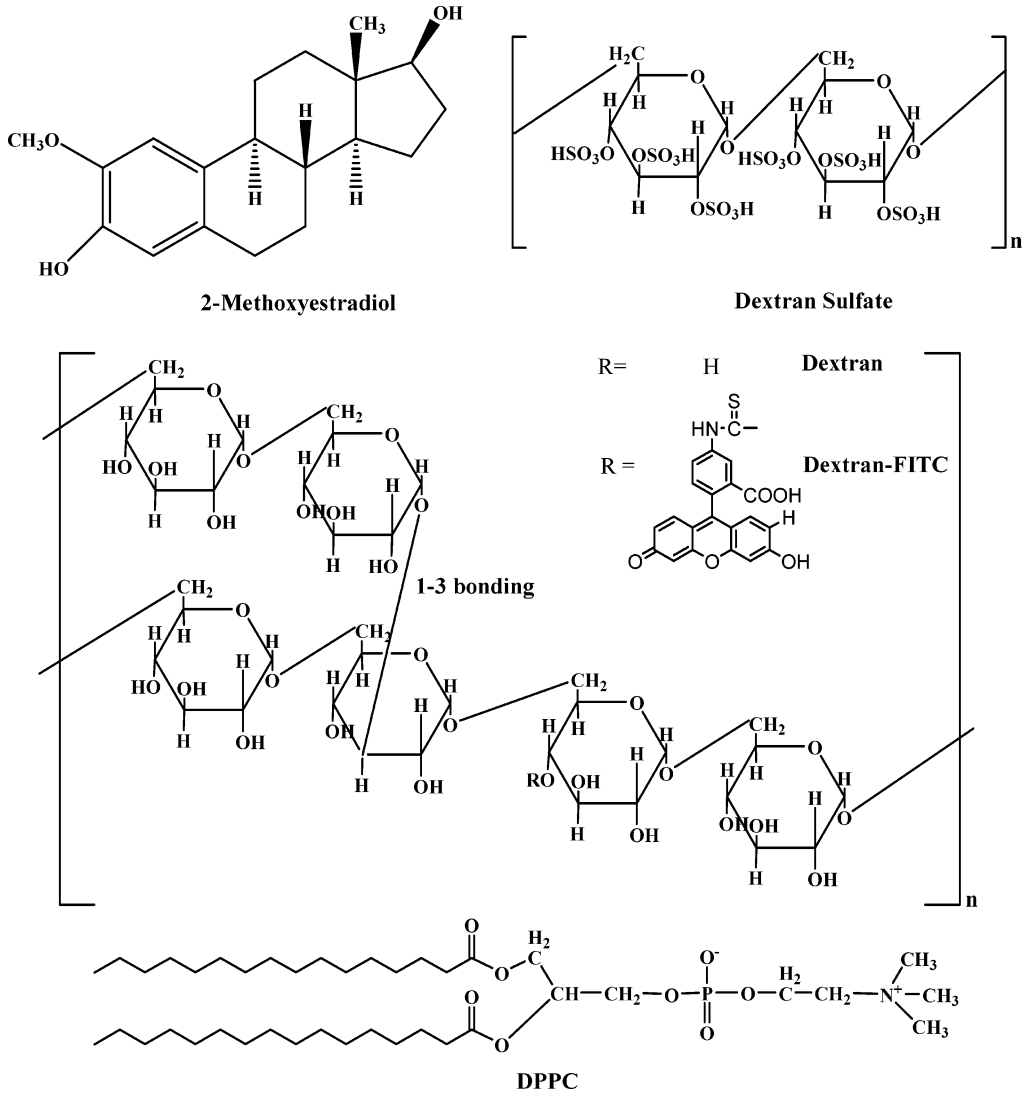
Scheme 1. Molecular Structures of 2-ME Drug, DPPC, and the Used Polymers of DN, DS, and DN-FI



ethanolic solutions because of its insolubility in water. Using the conventional formulation, 2-ME can be quickly metabolized, thus losing its inhibitory effect on tumor cells.[52] Though 2-ME is a relatively safe drug, high doses of 2-ME or frequent administration of it may cause hot flashes, fatigue, diarrhea, and reversible liver enzyme elevations. Therefore, continuous exposure of tumor cells to 2-ME at a low and stable level is necessary to maintain the inhibitory effect. We hypothesize that the encapsulation of 2-ME into PE multilayer capsules could maintain prolonged delivery of a therapeutic level of the drug, thereby providing a novel formulation of 2-ME for cancer therapeutics. Thus, the goal of this study is to demonstrate a new strategy to encapsulate 2-ME into PE multilayer capsules for cancer therapeutics. Various microscopy techniques including confocal laser scanning microscopy (CLSM), transmission electron microscopy (TEM), and scanning electron microscopy (SEM) were utilized to confirm the formation of 2-ME capsules and the hollow polymer multilayer shells. The bioactivity of the formed 2-ME capsules was investigated by monitoring the change of cellular morphology and an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. To our best knowledge, this is the first in vitro cell biological study to evaluate the pharmaceutical performance of polymer multilayer-encapsulated cancer drug microcrystals.

Experimental Section

Materials. Dextran (DN, MW = 500 000), dextran sulfate (DS, MW = 500 000), 2-ME, and dipalmitoyl-DL-(*R*)-phosphatidylcholine (DPPC) were purchased from Sigma. Dextran-FITC (fluorescein-5-isothiocyanate) (DN-FI, MW = 500 000) was purchased from Biochemika. The molecular structures of 2-ME, DPPC, DN, DS, and DN-FI are shown in Scheme 1. All other chemicals were obtained from Aldrich and used as received. The water used in all experiments was

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passed through a Millipore Milli-Q Plus 185 purification system and had a resistivity exceeding 18.2 MΩ·cm.

Assembly of DS/DN Multilayers onto Submicrometer-Sized 2-ME Crystals. The LbL assembly of DS/DN multilayers onto submicrometer-sized 2-ME crystals was carried out with minor modification from a published procedure.[23] The 2-ME crystals (50 mg) were finely milled in the presence of 0.25 wt % DPPC (2 mL in water), followed by intense sonication. Then the mixture was diluted with 0.25 wt % DPPC to 12 mL and kept for 1 h with occasional shaking. The resulting product was purified by washing 3 times with water and finally resuspended in water. The precharged crystal particles by DPPC were then LbL-coated with DS and DN with slight modifications of the procedure described elsewhere (in all cases, the polymer concentration was sufficiently higher than that required for saturation coverage of the crystal particle surface).[23] Ten milliliters of a DS solution (2 mg/mL, containing 0.5 M NaCl) was added to a 15-mL centrifuge tube containing 2 mL of the DPPC-coated, dispersed 2-ME crystals. After adsorption of DS for 15 min, the suspension was centrifuged at 6000 rpm for 10 min. The supernatant was then removed, and the coated 2-ME crystals were washed by three alternate cycles of centrifuging and resuspending the particles in pure water. Then 10 mL of DN solution (2 mg/mL, containing 0.5 M NaCl) was added into the DS-modified 2-ME crystal suspension and purified in the same manner. Additional DS/DN multilayers were deposited onto the microcrystals in an identical fashion until the desired number of multilayers was achieved. For CLSM measurement, DN-FI was deposited onto 2-ME crystal surfaces as the outermost layer. The formed 2-ME capsules were lyophilized on a Labconco system for 2 days and dissolved into PBS buffer (pH 7.4) before it was applied for cell biology studies.

Hollow Polymer Capsule Production. The 2-ME crystal core was removed by exposing 0.5 mL of the coated particle suspension to 1 mL of ethanol, allowing 30 min for core dissolution. The resulting hollow polymer capsules were then centrifuged at 13 000 rpm for 10 min, washed two times with water, and finally resuspended in water.

Confocal Laser Scanning Microscopy (CLSM). CLSM images were taken on an Olympus FV-500 confocal microscope. Twenty microliters of an aqueous sample solution was deposited onto the microscope cover slip, followed by covering the liquid with another cover slip.

Scanning Electron Microscopy (SEM). SEM was carried out with an AMRAY 1910 FE field emission microscope equipped with a backscattered electron detector at 15 kV. SEM samples (on silicon substrates) were sputter-coated with about 20 nm Au using a Polaron sputter coater system. The particle size distribution histogram was obtained by measuring 200 individual particles in 3 SEM micrographs.

Transmission Electron Microscopy (TEM). TEM measurements were performed at 60 kV on a Philips CM-100 microscope equipped with a Hamamatsu digital camera ORCA-HR operated using AMT software (Advanced Microscopy Techniques Corp, Danver, MA). TEM samples were prepared by deposition of a diluted particle suspension (5 μL) onto a carbon-coated copper grid and air-dried before the measurement.

Thyroid Cell Culture. FRTL-5 cells were obtained from the American Type Culture Collection (Rockville, MD). The cells were grown in Ham's F12 medium containing 10% fetal bovine serum (FBS) and 15 mIU/mL of bovine thyroid-stimulating hormone (TSH, Sigma). Before experiments, FRTL-5 cells were plated overnight in media supplemented with 5% charcoal-stripped FBS and TSH. The next day, 2-ME (5 μM) in ethanol solution and 2-ME capsules with the same concentration were added and cultured with the cells for an additional 2, 4, and 6 days. The concentration of 2-ME in DS/DN multilayer capsules was calculated based on the dry weight of the materials (assuming the weight of polymer multilayers is small enough to be neglected).

Quantitation of Cell Viability and Morphologic Observation. An MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was used to quantify the viability of cells. FRTL-5 cells were incubated with 5 μM 2-ME in ethanol solution and with 2-ME particles coated with DS/DN multilayers in aqueous solution for 48 h before the addition of MTT. After treatment with 5 μM of 2-ME in both ethanol solution and aqueous capsule formulation for 48 h, the cell morphology was observed by phase-contrast microscopy.

Results and Discussion

Previous literature shows that amphiphilic compound (e.g., surfactant, polymer, and lipid) can be used to render water solubility of uncharged organic crystals.[23,32] 2-ME is a water-insoluble uncharged organic compound (Scheme 1). In order to achieve its water solubility, the submicrometer-sized 2-ME particles were dispersed in water in the presence of DPPC, which is an amphiphilic compound. This process not only rendered the water solubility of 2-ME particles but also precharged the drug particle surfaces with positive charges.[23] The positive charge of organic crystals after DPPC modification has been reported in a previous report.[32] Then the subsequent deposition of a DS layer can be achieved through electrostatic interaction. Upon sequential deposition of DN and DS, multilayers were formed onto 2-ME particle surfaces. The formation of DS/DN multilayers was confirmed by CLSM imaging. Figure 1a shows a CLSM image of DPPC-modified 2-ME particles coated with $(DS/DN)_3DS$/DN-FI multilayers. DN-FI was selected as the outermost layer in order to image the entire particles of 2-ME. The fluorescence signal uniformly distributed onto each 2-ME particle, indicating that the outermost DN-FI layer coating was uniform. Upon dissolution of the 2-ME particles by ethanol, hollow capsules composed of $(DS/DN)_3DS/DN$-FI multilayers were formed (Figure 1b). In Figure 1b, green fluorescent rings indicate the intact morphology of hollow capsules, suggesting the successful assembly of DS/DN multilayers onto 2-ME particles. Without the previous multilayer coating, a single layer of DN-FI adsorption could not provide robust capsule formation. The hollow capsule

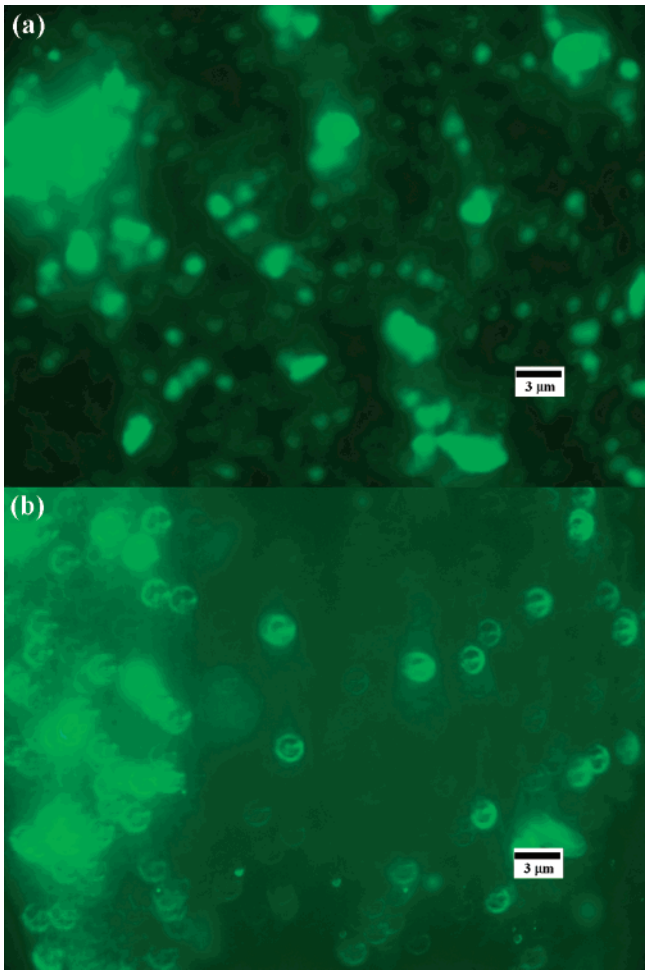

Figure 1. (a) CLSM images of DPPC-modified 2-ME particles coated with $(DS/DN)_3DS/DN$-FI multilayers and (b) the same particles treated with ethanol.

formation was also confirmed by TEM observation (vide infra). Please note that the spherical ring structures of the hollow capsules are different from those obtained by in situ dissolution of the drug particles coated with PE multilayers.[26] In the latter case, the shape of the formed capsules is similar to that of the drug particles, i.e., rectangular in shape. It is believed that the rectangular shape of the capsules gradually changes to spherical after osmotic pressure equilibrium.

SEM was utilized to observe the surface morphology and particle distribution of 2-ME particles coated with DS/DN multilayers. Figure 2a shows the SEM micrograph of DPPC-modified 2-ME particles coated with $(DS/DN)_3$ multilayers. The inset of Figure 2a shows a magnified SEM image. Individual particles display quite different shapes (e.g., cubic, rectangular, spherical, etc.); however, the particle size distribution is relatively homogeneous with an average diameter of 276 ± 76 nm (Figure 2b). The size distribution did not change significantly when more layers of DS/DN were deposited onto 2-ME particle surfaces. To further confirm the coating of DS/DN multilayers onto 2-ME particle surfaces, TEM was performed. Figure 3a shows a TEM micrograph of 2-ME particles coated with 9 layers of $(DS/DN)_4DS$. The core−shell structures of the particles due to

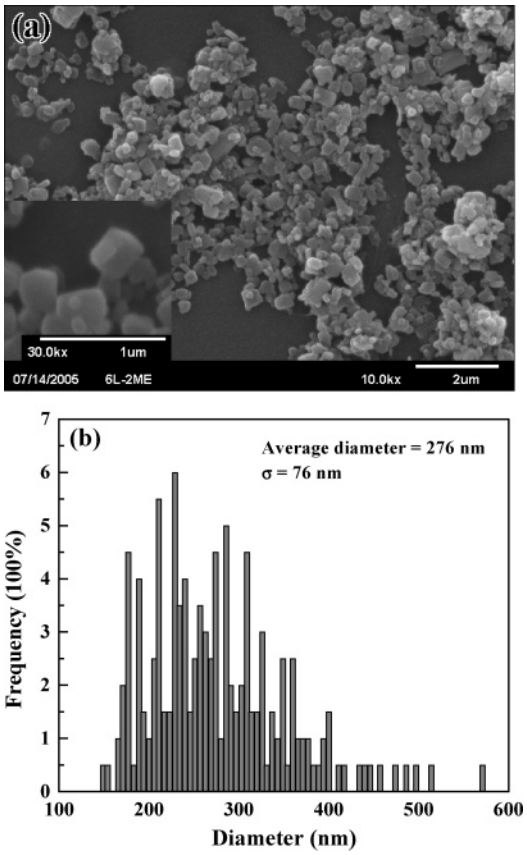

Figure 2. (a) SEM micrograph of DPPC-modified 2-ME particles coated with $(DS/DN)_3$ multilayers and (b) the size distribution histogram of the same particles. The inset of panel a shows a magnified SEM image of individual 2-ME particles coated with $(DS/DN)_3$ multilayers.

polymer multilayer coating can be clearly observed. Compared with the 2-ME drug particles, the polymer coating is much more transparent. The thickness of 9 layers' coating is approximately 45 nm. After exposure of 2-ME particles coated with DS/DN multilayers to ethanol, hollow capsules were formed even if 6 layers of DS/DN were coated onto 2-ME particles (Figure 3b). The surface of the capsules is distorted with abundant folds and creases produced by extraction of the solvent in the DS/DN layers prior to the TEM measurement.[10] The shells composed of DS/DN multilayers have a semitransparent appearance. It is interesting to note that the TEM image of the hollow DS/DN shells is not as sharp as that of hollow shells composed of polyallylamine hydrochloride (PAH)/poly(sodium sulfonate) (PSS) multilayers.[32] This is because the driving force to assemble DS/DN multilayers is through hydrogen bonding and physical adsorption (vide infra), which is much weaker than the electrostatic attraction between oppositely charge PEs that drives multilayer assembly. Consequently, the formed shell structures of DS/DN multilayers are more flexible than multilayers composed of oppositely charged PEs.

The driving force for LbL self-assembly is commonly electrostatic interaction. However, in this work, DN is a neutral polymer which contains many hydroxyl groups, while

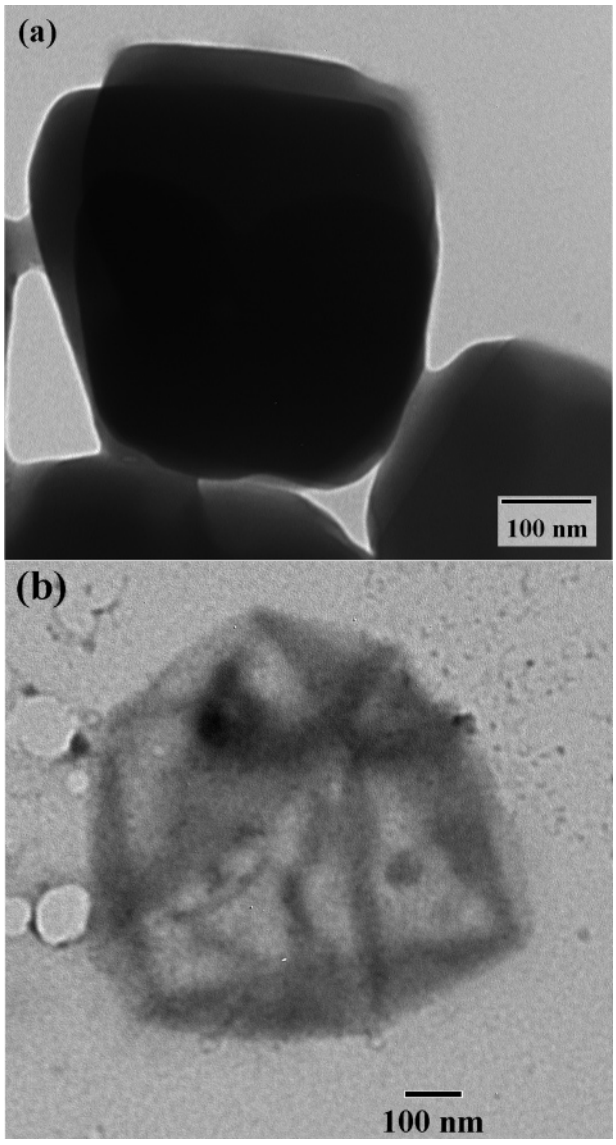

Figure 3. (a) TEM micrographs of DPPC-modified 2-ME particles coated with $(DS/DN)_4DS$ multilayers and (b) a hollow capsule composed of $(DS/DN)_3$ multilayers after dissolution of 2-ME in ethanol.

DS is a negatively charged derivative of DN. It is believed that there is no electrostatic interaction between DS and DN. We think that the formation of DS/DN multilayers is primarily based on hydrogen bonding related to the many hydroxyl groups possessed by both DS and DN molecules. Also, physical adsorption could be another driving force contributing to the buildup of DS/DN multilayers since DS and DN have similar backbone structures.[42] The structural similarity and difference between DS and DN is essential to assemble DS/DN multilayers. Exactly the same polymer structures are not likely to be used to form multilayers in an LbL manner. This is due to the fact that each polymer layer achieves saturated adsorption at each deposition cycle that limits the following similar polymer layer adsorption. In our case, we proved that DN/DN and DS/DS multilayers did not form by monitoring the UV−vis absorbance of the corresponding multilayers assembled onto quartz slides. In contrast, we confirmed the stepwise multilayer growth by assembling DS/DN multilayers onto quartz slides. The absorption intensity at the aliphatic region (220 nm) is increased regularly with the number of DS/DN assembled, although the total absorption intensity is small. There is no UV absorption difference between 15 min and 60 min adsorption, indicating that there are not several layers of DS/DN adsorbing each time.

The fabricated 2-ME particles coated with DS/DN multilayers are biologically active. It has been previously demonstrated that 2-ME can induce thyroid cell apoptosis.[51] In this study, FRTL-5 cell was selected as a model cell that can be killed when treated with 2-ME. Phase contrast microscopy was used to visualize the cell morphology change after treatment with 2-ME with different formulations (Figure 4). Both 2-ME in ethanol solution and 2-ME particles coated with $(DS/DN)_3$ multilayers with approximately the same molar concentration (5 μM) induced similar cell morphology changes. These changes included cell flattening and spreading, and a significant portion of the cells became rounded and nonadherent, indicative of the fact that cells undergo apoptosis (Figure 4b,c). In contrast, no rounded and detached cells can be visualized in control cells without 2-ME treatment (Figure 4a).

The viability of cells treated with free 2-ME, 2-ME coated with $(DS/DN)_3$ multilayers, and 2-ME coated with $(DS/DN)_3DS$ multilayers was also quantified by an MTT assay (Figure 5). It appears that both free 2-ME and 2-ME capsules coated with DS/DN multilayers caused a significant loss of cell viability in FRTL-5 cells compared with untreated cells. The outermost layer of 2-ME capsules (DN versus DS) did not influence the bioactivity of 2-ME. Regarding the possible interaction mechanism of 2-ME capsules with the cells, we think that DS/DN multilayers can be taken up and internalized into cell cytoplasma[50] and degrade at the cellular environment upon interaction with cells.[53] Then 2-ME drugs are released from the capsules and display cell apoptotic activity. It is interesting to note that 2-ME exerts its function through the induction of G2/M cycle arrest of the FRTL-5 cells.[51] The G2/M cycle starts to appear around 48 h after cell incubation. Literature data show that various drugs encapsulated within PE multilayers of different thickness can be completely released within several hours.[26−28,30] For this reason, it is not meaningful to investigate the influence of the capsule layer thickness on the drug release. However, the water solubility of 2-ME drug is overcome through its encapsulation within polymer multilayers. In other words, the goal of our study is to develop a polymer multilayer-encapsulated 2-ME drug formulation for our future in vivo studies. It is anticipated that, in in vivo studies, the side effect should be significantly decreased because of the protection of polymer multilayers, and the drug efficacy should be significantly improved because the crystalline structure of

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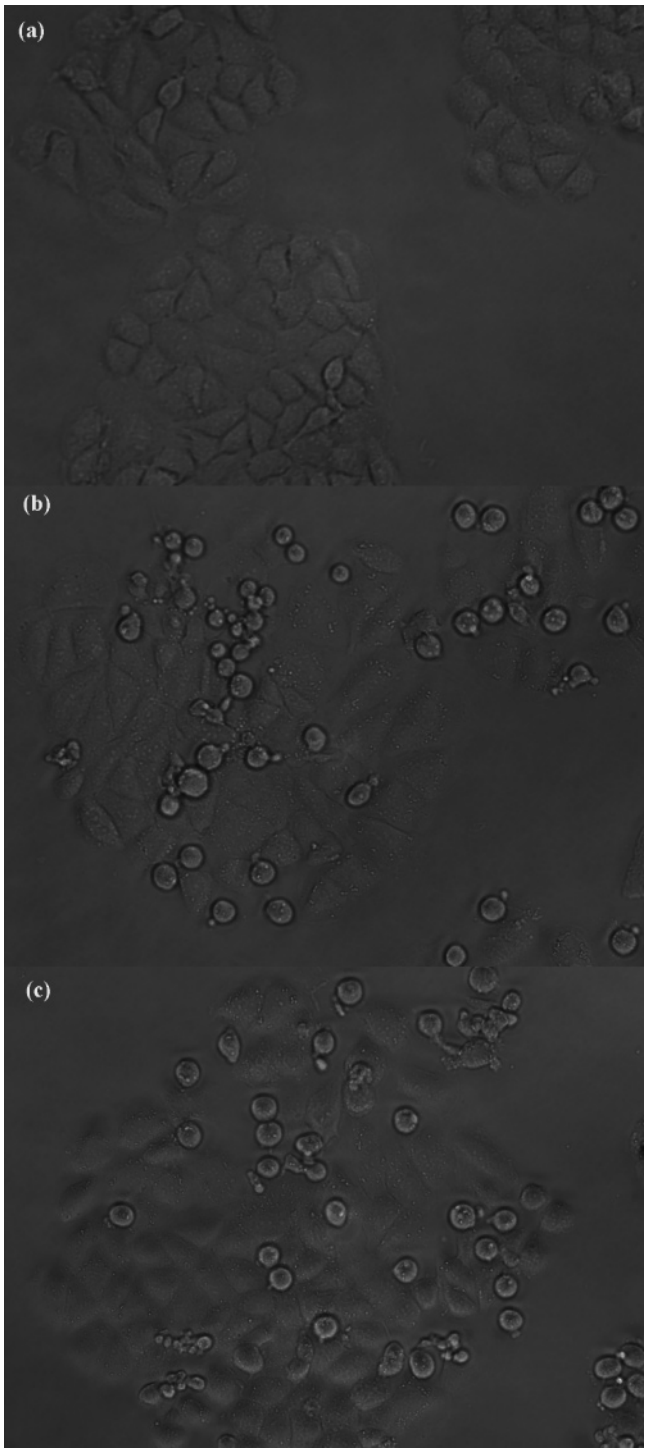

Figure 4. Phase-contrast photomicrographs of (a) control FRTL-5 cells without treatment, (b) the same cells treated with free 2-ME (5 μM in ethanol solution), and (c) 2-ME particles coated with $(DS/DN)_3$ multilayers.

the drug is preserved.[54,55] A detailed mechanism related to the release of 2-ME is still not clear yet. We also think that the DS/DN multilayers formed through hydrogen bonding and physical adsorption may not be strong enough compared with those formed through electrostatic interaction or covalent bonding. In order to verify the drug capsule/cell interaction mechanism, both synthetic and biodegradable polymers with opposite charges will be assembled onto 2-ME particle surfaces through electrostatic interaction for in vitro and in vivo studies. Dye-labeled polymer systems will also be employed to investigate the interaction between drug capsules and cells. The work is currently ongoing in our group, and the results will be reported in due course.

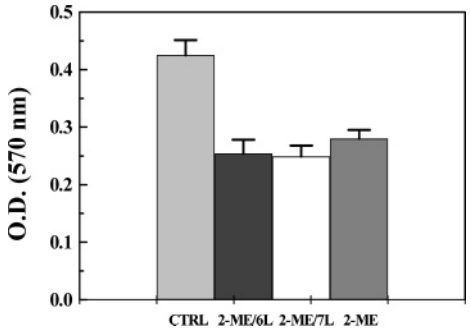

Figure 5. MTT assay of FRTL-5 cell viability after treatment with free 2-ME, 2-ME particles coated with $(DS/DN)_3$ multilayers, and 2-ME particles coated with $(DS/DN)_3DS$ multilayers for 48 h. The data are expressed as mean $\pm$ SD.

Conclusion

In summary, a novel formulation of 2-ME drug has been developed by sequential deposition of DS/DN multilayers onto DPPC-modified submicrometer-sized 2-ME particles. The formation and morphology of DS/DN multilayer-coated 2-ME particles were extensively investigated using CLSM, SEM, and TEM. Upon dissolution of 2-ME core particles with ethanol, hollow capsules composed of DS/DN multilayers were formed. The formed 2-ME capsules are bioactive and can induce thyroid cell apoptosis as verified by both phase contrast microscopy morphology studies and MTT assay. This approach of fabrication of 2-ME drug capsules opens a new pathway to encapsulate various drugs into polymeric multilayer capsules for a range of therapeutics applications.

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