Published in final edited form as:

Macromol Biosci. 2009 May 13; 9(5): 429–436. doi:10.1002/mabi.200800381.

Therapeutic Efficacy of 2-Methoxyestradiol Microcrystals Encapsulated within Polyelectrolyte Multilayers

Su He Wang,

Michigan Nanotechnology Institute for Medicine and Biological Sciences, University of Michigan,
Ann Arbor, MI48109, USA

Xiangyang Shi,

College of Chemistry, Chemical Engineering, and Biotechnology, Donghua University, Shanghai
201620, China

Xisui Chen, and

Michigan Nanotechnology Institute for Medicine and Biological Sciences, University of Michigan,
Ann Arbor, MI48109, USA

James R. Baker Jr.

Michigan Nanotechnology Institute for Medicine and Biological Sciences, University of Michigan,
Ann Arbor, MI48109, USA

Abstract

Development of a novel formulation of anticancer drugs to improve their water-solubility and bioavailability remains a great challenge. Herein, the potential anticancer agent 2-ME was selected as a model drug and was encapsulated within PE multilayers by layer-by-layer deposition of oppositely charged PEs onto the drug microcrystal surfaces. Cell viability and morphology observation of two cell lines reveal that the PE multilayer-encapsulated 2-ME microcrystals markedly decrease the cell viability, displaying similar inhibitory effect to that of the conventional formulation of 2-ME dissolved in ethanol. The current approach to encapsulate hydrophobic drug microparticles may be useful for formulating different drugs for a variety of biological applications.

Keywords

cancer therapy; drug delivery systems; 2-methoxyestradiol; polyelectrolyte multilayers; self-assembly

Introduction

The layer-by-layer (LbL) self-assembly process is primarily based on the sequential deposition of oppositely charged individual polymer layers.[1-3] This technique has been demonstrated as a powerful tool to construct various ordered functional thin films,[4] to design diversified functional capsules, core/shell particles, and hollow spheres,[5-11] and to encapsulate drug micro- or nanocrystals.[12-19] The driving force to achieve the successful LbL assembly can be electrostatic interaction,[4] hydrogen bonding,[20-25] covalent interaction,[26,27] and

hydrophobic interaction.[28-30] The physicochemical properties and stability of the multilayer films may be different when various driving forces are used to construct the multilayers.

Using the LbL self-assembly technique, there are generally three different approaches for drug encapsulation: (i) loading the drug into preformed LbL-assembled polymer multilayer capsules;[31-37] (ii) using the drug as a layer component to assemble it onto colloidal templates with a polyelectrolyte (PE) through hydrophobic interaction;[28] and (iii) sequentially depositing oppositely charged PEs onto drug microcrystal surfaces.[18,38] Approach (i) has been widely studied for *in vitro* and *in vivo* cancer therapeutic and antibiotic applications. [31-34,36,37] In comparison with approaches (i) and (ii), approach (iii) allows higher loading capacity of the drug since the drug micro- or nanocrystals are encapsulated within PE multilayers. However, there are only a few reports related to cell biological or *in vivo* studies of the drug efficacy when the drug crystals are encapsulated within PE multilayers.[17,18]

In a previous report, we have shown that 2-methoxyestradiol (2-ME) (Figure 1), a novel, potential anticancer drug, can be encapsulated within polymer multilayers through sequentially depositing dextran sulfate (DS) and dextran (DN) onto the drug microcrystal surfaces.[18] The encapsulated 2-ME displays effective drug activity. The driving force to assemble DS and DN multilayers is hydrogen bonding. We believe that the formed polymer capsules may be more stable when the polymer multilayers are formed through other stronger driving forces, e.g., electrostatic interaction.

In this present study, we selected two oppositely charged PE pairs that can form multilayers through electrostatic interaction. A biocompatible and biodegradable PE pair (DS and chitosan, CN) and a synthetic PE pair [poly(sodium 4-styrene sulfonate) (PSS) and poly(allylamine hydrochloride) (PAH)] were selected to form multilayers onto 2-ME microcrystal surfaces (Figure 1), respectively. The influence of the polymer biocompatibility and biodegradability on the drug efficacy was also investigated using the above PE pairs. The PE multilayer assembly onto 2-ME microcrystals and the hollow shell formation were confirmed by ζ -potential measurements, scanning electron microscopy (SEM), and transmission electron microscopy (TEM). The 2-ME drug efficacy was tested by an MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] cell viability assay and by monitoring the change of cellular morphology. The results will provide a basis for rational design of drug microcrystal-loaded polymer multilayer capsules for various biomedical applications.

Experimental Part

Materials

DS ($\bar{M}_w=500\ 000$), CN with C3646, and minimum 85% deacetylation, 2-ME, and dipalmitoyl-DL-R-phosphatidylcholine (DPPC) (Figure 1) were purchased from Sigma. PSS with $\bar{M}_w=70\ 000$, PAH with $\bar{M}_w=70\ 000$, and all other chemicals were obtained from Aldrich and used as received. The water used in all experiments was passed through a Millipore Milli-Q Plus 185 purification system and had a resistivity exceeding $18.2\ \text{M}\Omega \cdot \text{cm}$.

Assembly of DS/CN and PSS/PAH Multilayers onto 2-ME Microcrystals

The LbL assembly of DS/CN multilayers onto submicrometer-sized 2-ME crystals was carried out with minor modification from a published procedure.[18,19] 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 resultant product was purified by washing three times with water and finally resuspended in water. The precharged crystal particles by DPPC were then LbL-coated with DS and CN multilayers using a procedure described elsewhere with slight

modifications (In all cases, the PE concentration was sufficiently higher than that required for saturation coverage of the crystal particle surface).[19] A 10-mL DS solution ($2 \text{ mg} \cdot \text{mL}^{-1}$, 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 6 000 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, a 10-mL CN solution ($2 \text{ mg} \cdot \text{mL}^{-1}$, containing 0.5 M NaCl) was added into DS-modified 2-ME crystal suspension and purified in the same manner. Additional DS/CN multilayers were deposited onto the microcrystals in the identical fashion until the desired number of multilayers was achieved. Under similar conditions, PSS/PAH multilayers were assembled onto 2-ME microcrystal surfaces. The formed multilayer-encapsulated 2-ME microcrystals were lyophilized on a Labconco system for 2 d and dispersed into PBS buffer ($\text{pH} = 7.4$) before they were applied for cell biology studies.

Hollow Polymer Capsule Production

The 2-ME crystal core was removed by exposing 1 mg of the dried powder of 2-ME crystal capsules to 1 mL of ethanol for 30 min. The resultant hollow polymer capsules were then centrifuged at 13 000 rpm for 10 min, washed two times with water, and finally resuspended in water.

ζ -Potential Measurement

ζ -potential measurements were performed using a Malvern Zetasizer Nano ZS model ZEN3600 (Worcestershire, UK) equipped with a standard 633 nm laser. The 2-ME microcrystals modified with different layers of PE were dispersed in water before the measurement.

2-ME Loading Capacity Measurement

The loading percentages of 2-ME microcrystal within different PE multilayer capsules were calculated according to the following equation:

$$\text{Loading (\%)} = \frac{W_d}{W_c} \times 100\% \quad (1)$$

where W_c is the mass of the dried PE multilayer-encapsulated 2-ME microcrystals, W_d the mass of 2-ME drugs. The mass of 2-ME drug crystals in the PE multilayer capsules was determined by reverse-phase high-performance liquid chromatography (RP-HPLC). A given amount (0.1–0.5 mg) of dry PE multilayer-encapsulated 2-ME microcrystals was dissolved in ethanol according to the procedure described above for hollow polymer capsule production. After centrifugation, the supernatants were collected for RP-HPLC analysis based on a calibration curve of 2-ME drug. The RP-HPLC system used in this work consisted of a Waters Delta 600 separation module, a model 717 auto sampler equipped with a 100- μL loop, and a model 2996 PDA detector (Waters Corporation, Milford, MA). A Jupiter C5 silica-based RP-HPLC column ($250 \times 4.6 \text{ mm}^2$, 300Å) was purchased from Phenomenex (Torrance, CA). Two Phenomenex Widespore C5 safety guards ($4 \times 3 \text{ mm}^2$) were installed ahead of the Jupiter column. The mobile phase was a linear gradient beginning with 66:34 v/v water/acetonitrile (ACN) to 30:70 water/ACN within 20 min at a flow rate of $1 \text{ mL} \cdot \text{min}^{-1}$. The injection volume was 35 μL . The detection of eluted samples was performed at 205 nm.

Scanning Electron Microscopy

SEM was carried out with an AMRAY 1910 FE field emission microscope equipped with 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 three different SEM micrographs.

Transmission Electron Microscopy

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 sample suspension (5 μ L) onto a carboncoated copper grid and air-dried before the measurement.

Cell Biological Evaluation

FRTL-5 cells (a rat thyroid epithelial cell line, ATCC, Rockville, MD) were grown in Ham's F12 medium containing 10% fetal bovine serum (FBS) and 15 mIU $\cdot$ mL⁻¹ of bovine thyroid-stimulating hormone (TSH, Sigma). KB cells (a human epithelial carcinoma cell line, ATCC, CLL17, Rockville, MD) were continuously grown in RPMI 1640 medium supplemented with 10% heat-inactivated FBS, and 2.5 μ M FA. One day before experiments, cells (10 000 cells per well) were plated into a 96-well plate in a complete medium. The next day, 2-ME (10 μ M) in ethanol solution (1 μ L) and 2-ME microcrystals encapsulated within PE multilayers capsules with similar 2-ME concentration were added to cells and incubated for 48 h at 37 °C. An MTT assay was used to quantify the viability of the cells. After 48 h incubation with 2-ME in ethanol solution or 2-ME microcrystals within DS/CN or PSS/PAH multilayer capsules, the metabolically active cells were then detected by adding MTT to each well. Then, the plates were read at 570 nm. Mean and standard deviation for the triplicate wells were reported. After treatment with 2-ME in ethanol solution or 2-ME microcrystals within DS/CN or PSS/PAH multilayer capsules, the cell morphology was also observed by phase-contrast microscopy (Leica DMIRB fluorescent inverted microscope). The magnification is set at 200 $\times$ for all samples.

Results and Discussion

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.[39,40] 2-ME does not display considerable estrogenic activity at clinically efficacious doses and it does not seem to promote carcinogenesis. Moreover, it has been found to be active in inhibiting tumor growth in phase I/II clinical trials.[41,42] Using the LbL self-assembly technique to encapsulate the drug 2-ME microcrystals is expected to significantly improve the drug solubility in water and its bioavailability.

Similar to our previous studies, the 2-ME drug crystals were finely milled in the presence of 0.25 wt.-% DPPC, followed by intense sonication. This process affords the 2-ME drug microcrystals to be dispersible in water and also to be positively charged.[19] The positive charge of the 2-ME microcrystals allows subsequent electrostatic LbL assembly of PSS/PAH and DS/CN multilayers. Z-potential measurements were used to monitor each step of the assembly of 2-ME microcrystals (Figure 2) with PSS/PAH and DS/CN multilayers. The alternating charge reversal (from negative to positive) of 2-ME microcrystals in aqueous solution after each layer of coating with PSS/PAH and DS/CN pairs indicates the successful electrostatic assembly of PSS/PAH and DS/CN multilayers. PSS/PAH multilayer-coated 2-ME microcrystals appear to display higher negative and positive surface potentials than those coated with DS/CN multilayers, presumably due to the higher charge density of the two PEs in water.

SEM was used to monitor the surface morphology and particle distribution of the 2-ME microcrystals coated with PSS/PAH and DS/CN multilayers. Figure 3a and b show the SEM micrograph and size distribution histogram of DPPC-modified 2-ME particles coated with (DS/CN)₃ multilayers, respectively. Similar to our previous observation of DS/DN multilayer-assembled 2-ME microcrystals,[18] individual particles display quite different shapes (e.g., cubic, rectangular, spherical, etc.) with an average size of $3.2 \pm 1.4 \mu\text{m}$. (PSS/PAH)₃ multilayer-assembled 2-ME particles display similar morphology to those coated with (DS/CN)₃ multilayers (Figure 3c). However, the size of the (PSS/PAH)₃-coated 2-ME particles ($1.9 \pm 1.1 \mu\text{m}$) is much smaller when compared with those coated with (DS/CN)₃ multilayers (Figure 3d). For both cases, the 2-ME particle size distribution did not change significantly when more layers of DS/CN or PSS/PAH were deposited onto 2-ME particle surfaces. Individual bigger particles for both cases could be related to the aggregation of the 2-ME particles during the self-assembly process (Figure 3a and c). But this aggregation did not induce the precipitation of the 2-ME particles in aqueous solution. The smaller size of PSS/PAH-coated 2-ME particles may be due to the smaller molecular weight and the higher charge density of the two polymers when compared to DS and CN, thereby significantly prohibiting the possible aggregation of the 2-ME particles during the self-assembly process. It is interesting to note that both DS/CN- and PSS/PAH-coated 2-ME particles are significantly larger in size than those coated with DS/DN multilayers with a similar layer number through hydrogen bonding interaction in our previous study.[18] It is likely that the self-assembly through electrostatic interaction may introduce more aggregation of 2-ME particles than that formed through hydrogen bonding. Further detailed mechanistic studies are necessary to confirm this finding.

After exposure of 2-ME particles coated with both PSS/PAH and DS/CN multilayers to ethanol, hollow capsules were formed (Figure 4). This further confirms the successful assembly of PSS/PAH and DS/CN multilayers onto 2-ME microcrystals. For (PSS/PAH)₃ hollow capsules (Figure 4a), the surface of the capsules is distorted with abundant folds and creases produced by extraction of the solvent in the PSS/PAH layers prior to the TEM measurement.[3] The shells composed of PSS/PAH multilayers have a semi-transparent appearance. DS/CN multilayer capsules with similar layer number display similar morphology. With the increase in the layer number, the hollow capsules with 12 layers of DS/CN are fairly integrated (Figure 4b) with fewer folds and creases when compared to those with fewer layers.

The loading percentages of 2-ME microcrystals into different PE multilayer capsules were determined by exposing the PE multilayer-encapsulated 2-ME microcrystals to ethanol for 30 min. The dissolved 2-ME was analyzed using HPLC. It is clear that the loading percentage of 2-ME within all PE multilayer capsules with different composition and layer numbers is quite high within a range of 50–70% (Table 1). It is reasonable to see that for both DS/CN and PSS/PAH multilayer capsules, the loading percentage decreases with the increase in the layer number. In addition, PSS/PAH multilayer capsules load more 2-ME microcrystals than DS/CN multilayer capsules with similar layer number, which is presumably ascribed to the higher molecular weight and lower charge density of DS and CN polymers. Compared to a simple geometric estimation, the practical loading capacity of 2-ME (50–70%) is rather low. This might be largely due to the penetration of PE inside the capsules. As mentioned above, the bigger size of both DS/CN- and PSS/PAH-coated 2-ME particles (when compared with those coated with DS/DN multilayers with a similar layer number through hydrogen bonding interaction in our previous study[18]) is related to the aggregation of the 2-ME particles during the electrostatic LbL self-assembly process. Consequently, each micrometer-sized particle could be composed of many PE-coated nanometer-sized drug particles during the self-assembly process. The significant amount of PE penetration inside an entire capsule contributes to a lower loading efficiency in practice than that estimated theoretically. The formed 2-ME microcrystals encapsulated within either PSS/PAH or DS/CN multilayers are very stable in aqueous solution for at least 9 months. They can also be stored as a lyophilized form. The dry

powder of the 2-ME microcrystal-containing polymer multilayer capsules can be readily dispersed in water or PBS buffer for biological studies.

In order to test the drug efficacy of 2-ME microcrystals encapsulated within PE multilayers, we selected two different cell lines, FRTL-5 cells, and KB cells. 2-ME exerts its function through the induction of G2/M cycle arrest of the cells.[40] The G2/M cycle starts appearing around 48 h after cell incubation. Therefore, after incubation of the 2-ME microcrystal-loaded polymer capsules with cells for 48 h, an MTT assay was performed to evaluate the viability of FRTL-5 cells (Figure 5) and KB cells (Figure 6) treated with free 2-ME dissolved in an ethanol solution, and 2-ME coated with (DS/CN)₃; (DS/CN)₃DS; (DS/CN)₆; (DS/CN)₆DS; (PSS/PAH)₃; (PSS/PAH)₃PSS; (PSS/PAH)₆; and (PSS/PAH)₆PSS multilayers. It appears that both free 2-ME and 2-ME microcrystal coated with either DS/CN or PSS/PAH multilayers with different numbers of layers caused a significant loss of cell viability in FRTL-5 cells and KB cells when compared with the untreated corresponding cells. The ethanol (1 μ L) used to dissolve free 2-ME drug does not exert any favorable influence on the cell viability for both cells. The cell killing effect of 2-ME microcrystal capsules on KB cells is much more significant than that on FRTL-5 cells. This may be due to the fact that KB cell is a human epithelial cancer cell line, which may grow faster and have a cell cycle shorter than that of FRTL-5 cells, a normal rat thyroid cell line. As a cell cycle blocking agent, 2-ME drug could significantly inhibit the growth of KB cancer cells.

The outermost layer of 2-ME microcrystal capsules (DS vs. CN or PSS vs. PAH) did not influence the bioactivity of 2-ME. This finding is to some extent controversial to that reported by Wang et al.[37] In their study, the drug-loaded microcapsules were incubated with cells for 2 h, followed by a washing step to remove the non-adsorbed microcapsules. Therefore, microcapsules with positive charge can interact with negatively charged cell membrane and can be internalized within cells, while microcapsules with negative charge are washed away and do not exert any drug effect. In our case, 2-ME microcrystal capsules with both positive and negative surface charges were incubated with cells for 48 h. It is expected that the 2-ME microcrystal capsules with either positive or negative charge can be internalized into cell cytoplasm through lipid-raft-mediated uptake.[43,44] Both DS/CN and PSS/PAH multilayers with different number of layers could be dissociated in the cell plasma, and then 2-ME drugs are released from the capsules and display cell growth inhibition activity. For DS/CN multilayers, the polymers are able to degrade at the cellular environment upon interaction with cells.[45] The bioactivity of 2-ME microcrystal capsules appears to be approximately similarly independent of the number of corresponding polymer layers for each cell lines tested. Future release kinetics studies may be necessary to help the complete understanding of the detailed mechanism related to the therapeutic effect of 2-ME microcrystals encapsulated within polymer microcapsules. Although the therapeutic efficacy of 2-ME microcrystal capsules is different when different cell lines were treated, for a given cell line, the therapeutic efficacy is more or less similar, regardless of the biocompatibility and biodegradability of the coated polymer multilayers (biocompatible and biodegradable DS/CN multilayers vs. synthetic PSS/PAH multilayers). Using biocompatible and biodegradable DS/CN multilayers may be beneficial for future *in vivo* studies. In our ongoing studies, the additional toxicity experiments with different wall materials (including PAH, PSS, DS, and CN), as well as the toxicity of therapeutically non-effective 17- β -estradiol microcrystals (used as a negative control) encapsulated within PSS/PAH and DS/CN multilayers will be tested. It is likely that the 17- β -estradiol microcrystals encapsulated within PSS/PAH and DS/CN multilayers do not display bioactivity, which is a subject of our further confirmation.

The cytotoxic effect of 2-ME microcrystal capsules was further confirmed by phase contrast microscopic visualization of the cell morphology change after treatment with 2-ME with different formulations. Figure 7 shows the morphology of untreated KB cells, KB cells treated

with 2-ME ethanol solution, and 2-ME microcrystals coated with (DS/CN)₃; (DS/CN)₆; (PSS/PAH)₃PSS; and (PSS/PAH)₆PSS multilayers, respectively. Both 2-ME in ethanol solution (Figure 7d) and 2-ME microcrystals coated with different numbers of DS/CN and PSS/PAH multilayers (Figure 7b, c, e, and f) with similar 2-ME concentration (10 μ M) induced similar cell morphology changes. A significant portion of the cells became rounded and non-adherent, indicative of the fact that cells are approaching death (Figure 7). In contrast, no rounded and detached cells can be visualized in control cells without 2-ME treatment (Figure 7a). Similar cell morphology changes were observed for FRTL-5 cells (images not shown), which is consistent with our previous study.[18]

Using PE multilayer-encapsulated 2-ME microcrystals as a drug formulation could overcome the water-insolubility and improve the bioavailability of the drug, similar to our previous study. [18] 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 the drug is preserved.[46,47] The *in vitro* test of the drug activity did not show clear advantage of 2-ME microcrystal capsules formed through electrostatic interaction in comparison to those formed through hydrogen bonding reported in our previous study.[18] These differences in the stability of 2-ME microcrystal capsules formed through different mechanisms will be elucidated through future extensive *in vivo* studies.

Conclusion

In summary, a novel formulation of 2-ME drug has been developed by sequential deposition of DS/CN or PSS/PAH multilayers onto DPPC-modified 2-ME microparticles. The selection of PSS/PAH multilayers was used as a model for the understanding of the DS/CN multilayers for *in vitro* studies of 2-ME drug efficacy, since the pair of PSS/PAH polymers are not biocompatible for *in vivo* studies. The fabrication, morphology, hollow shell formation, and loading capacity of DS/CN and PSS/PAH multilayer-coated 2-ME particles were extensively investigated using ζ -potential measurements, SEM, TEM, and HPLC. The formed 2-ME microcrystal capsules with high loading capacity are bioactive and can inhibit the proliferation of both a rat thyroid cell line and a human epithelial carcinoma cell line. We show that 2-ME microcrystal capsules with different composition and number of polymer multilayers (DS/CN vs. PSS/PAH) display similar therapeutic effect to the corresponding cell lines. The construction of 2-ME microcrystal capsules provides a new approach to develop formulations of various hydrophobic anticancer drugs for therapeutic as well as other pharmaceutical applications for cancer.

Acknowledgments

This research is financially supported in part by the *National Cancer Institute* (NCI), *National Institutes of Health* (NIH), under Contract no. NOI-CO-97111, and in part by NIH R01 A137141 and P60DK20572. X. S. thanks the support from the Program for Professor of Special Appointment (Eastern Scholar) at *Shanghai Institutions of Higher Learning*.

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Figure 1. Molecular structures of 2-ME drug, DPPC, and the used polymers of CN, DS, PAH, and PSS.

Figure 2. ζ -potential of polymer multilayer-encapsulated 2-ME microcrystals as a function of PE layer number. Layer number 0 indicates the DPPC-modified 2-ME microcrystals.

Figure 3. SEM micrographs of DPPC-modified 2-ME particles coated with $(\text{DS/CN})_3$ (a) and $(\text{PSS/PAH})_3$ (c) multilayers. (b) and (d) show the size distribution histograms of the $(\text{DS/CN})_3$ and $(\text{PSS/PAH})_3$ multilayer-encapsulated 2-ME microcrystals, respectively.

Figure 4. TEM micrographs of (PSS/PAH)₃ (a) and (DS/CN)₆ (b) multilayer hollow capsules formed after dissolution of 2-ME microcrystals in ethanol.

Figure 5.

MTT assay of FRTL-5 cell viability after treatment with free 2-ME dissolved in 1 μ L ethanol, ethanol (1 μ L), 2-ME particles coated with (DS/CN)₃, (DS/CN)₃DS, (DS/CN)₆, (DS/CN)₆DS, (PSS/PAH)₃, (PSS/PAH)₃PSS, (PSS/PAH)₆, (PSS/PAH)₆PSS multilayers for 48 h. The data are expressed as mean $\pm$ S. D.

Figure 6.

MTT assay of KB cell viability after treatment with free 2-ME (10 μ M) dissolved in 1 μ L ethanol, 2-ME particles coated with (DS/CN)₃, (DS/CN)₃DS, (DS/CN)₆, (DS/CN)₆DS, (PSS/PAH)₃, (PSS/PAH)₃PSS, (PSS/PAH)₆, (PSS/PAH)₆PSS multilayers for 48 h. The data are expressed as mean $\pm$ S. D.

Figure 7.

Phase-contrast photomicrographs of control KB cells without treatment (a), the same cells treated with free 2-ME (10^{-5} M in ethanol solution) (d), and 2-ME particles coated with (DS/CN)₃ (b), (DS/CN)₆ (c), (PSS/PAH)₃PSS (e), and (PSS/PAH)₆PSS multilayers (f), respectively.

Table 1

Loading percentage of 2-ME microcrystals in DS/CN and PSS/PAH multilayer capsules

Coated multilayer	<u>Loading</u>
	%
(DS/CN) ₃	68.3
(DS/CN) ₃ DS	66.6
(DS/CN) ₆	50.2
(DS/CN) ₆ DS	47.6
(PSS/PAH) ₃	72.0
(PSS/PAH) ₃ PSS	68.8
(PSS/PAH) ₆	62.2
(PSS/PAH) ₆ PSS	57.1