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Formulation and cytotoxicity of doxorubicin loaded in self-assembled bio-polyelectrolyte microshells

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Abstract

A bio-polyelectrolyte microshell composed of alginate sodium (ALG) and chitosan (CHI) was fabricated by electrostatic layer-by-layer (LbL) self-assembly technique. The resulting ALG-CHI microshells were found to be able to effectively load anti-cancer drug doxorubicin (DOX) in the interior of the shells under modest conditions without addition of other reagents, as demonstrated by confocal laser scanning microscopy (CLSM). The mass of DOX loaded in one capsule of four alginate/chitosan layers (i.e. the volume $V = 2.5 \times 10^{-10}$ cm^3) is calculated as ca. 1.4×10^{-13} g, which corresponds to 1.5×10^8 DOX molecules. Also, the release of DOX in the shells is dependent on the number of assembled layers of the shells. Colorimetric XTT cell viability assay results showed that the DOX-loaded microshells at high concentrations tested could kill cancer cells more efficiently than free-DOX alone.

Keywords: Bio-polyelectrolyte; Cytotoxicity; Doxorubicin; Microshell; Self-assembly

1. Introduction

Encapsulation technology for the formulation of anti-cancer drugs offers numerous advantages compared to conventional dosage forms, which include improved efficacy, minimized systemic toxicity, and improved patient compliance and convenience (Tardi et al., 2000; Boisdron-Celle et al., 1995; Morgan et al., 2005). In recent years several conventional encapsulating systems such as liposomes, nano and microparticles have been reported in anti-cancer drug delivery (Harivardhan et al., 2005; Feng et al., 2004; Mu and Feng, 2003; Azarmi et al., 2006). Notwithstanding, it is still needed to develop new systems that allow the manipulation of surface charge, shape and size, permeability, mechanical stability and biocompatibility adjustable to practical applications in cancer patients.

Layer-by-layer (LbL) self-assembly (Decher, 1997; Ruths et al., 2000) of oppositely charged polyelectrolytes onto dissolvable colloidal particles has recently been utilized to build ultrathin nano- and microshells with customized structure. The constructed hollow shells have been shown to be efficient in nanoscale encapsulation of drugs, proteins, dyes, and nanomaterials (Gao et al., 2002; Peyratout and Dähne, 2004). In consideration of drug formulation development, the encapsulation of anti-cancer drugs in the microshells can a provide a means of concentrating and protecting the drug molecules in a defined volume as well as decreasing toxic side effects to normal organics. Further, particular advantages of the polyelectrolyte microshells e.g. the tailored wall thickness on a nanometer-scale range, the modulated wall composition, together with the controlled size and shape render the shells ideal drug delivery vehicles suitable to various cancer patients. However, very few examples to date have shown that the self-assembled microshells totally composed of natural bio-polyelectrolytes are employed to encapsulate anti-cancer drugs under modest conditions without addition of other reagents or without compromise on outmost immobility layers (Liu et al., 2005; Khopade and Caruso, 2002).

Natural polysaccharides such as chitosan (CHI) and alginate sodium (ALG) have been widely investigated for applications in coating membranes, controlled-release drug delivery, food

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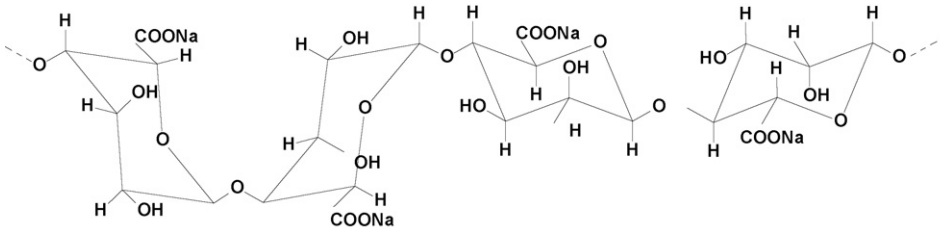
industry and biomaterials (Ye et al., 2005; George and Abraham, 2006; Yan et al., 2001; Gaserod et al., 1999; Baruch and Machluf, 2006). CHI is a natural cationic polymer derived from chitin *N*-deacetylation. ALG is an anionic polymer constituted of a naturally occurring block copolymer of two monosaccharide units obtained from marine brown algae. The bio-polyelectrolyte microshells constructed by using ALG and CHI as building blocks with the LBL self-assembly technique have been recently studied (Ye et al., 2006; Tao et al., 2006), in which emphasis is mainly placed on the protein encapsulation and peculiar property of the AlG-CHI shells themselves. Tong et al. investigated the loading and cumulative release of insulin in the AlG-CHI microshells and found that increasing the loading temperature not only increased the insulin loading capability, but slowed down its release rate as well (Ye et al., 2006). In very recent study, we found that compared with the shells composed of synthetic poly(styrenesulfonate sodium) (PSS) and poly(allylamine hydrochloride) (PAH) that collapse or deform at a 4 wt.% or more than 4 wt.% PSS concentration, the contructed ALG-CHI microshells displayed unexpected higher stability and mechanical property (Tao et al., 2006). Considering these proper characteristics, a new formulation by loading anti-cancer drugs in the interior of the preformed bio-polyelectrolyte ALG-CHI shells is expected.

Here, we, utilizing the colloidal templating LbL technique, construct multilayered bio-polyelectrolyte microshells based on ALG and CHI. Doxorubicin hydrochloride (DOX), a water-soluble fluorescent drug, is the most widely used anticancer drug, and in this work is chosen as model drug. The loading of DOX in the shells was traced using confocal laser scanning microscopy (CLSM). The cytotoxic effects of blank microshells, free DOX or DOX-loaded microshells on H460 and A549 lung cancer cell lines were also assessed using colorimetric XTT cell viability assays for the first time.

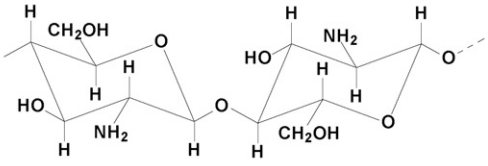
2. Materials and methods

2.1. Materials

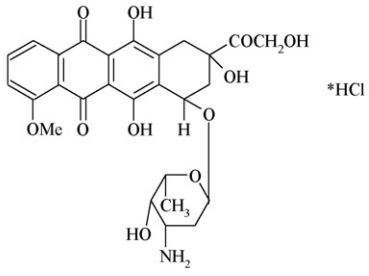
ALG (M_w = 12,000–80,000) was obtained from Sigma, Canada. CHI (M_w = 30,000) was obtained from Primex Biochemicals, Norway. Melamine formaldehyde (MF) particles (4.94 ± 0.14 μm) were purchased from Microparticles GmbH, Germany. Rhodamine isothiocyanate (RITC) was purchased from Aldrich. DOX was purchased from Pharmacia Canada Inc. All chemicals were used as received. Millipore water was used throughout the study. For reference, the chemical structures of ALG, CHI, and DOX are shown below:



Alginate sodium (ALG)



Chitosan (CHI)



Doxorubicin (DOX)

2.2. Preparation of bio-polyelectrolyte microshells

1.5 ml of alginate solution (1 mg/ml in 0.5 M NaCl) or 1.5 ml of chitosan solution (1 mg/ml in 0.5 M NaCl at pH 3.8), with a charge opposite to that of MF templates or the last layer deposited, was added to a template colloidal solution (0.3 ml), and left to absorb for 1 h. The excess of added species was removed after each layer was deposited by three repeated centrifugation (2500 g, 3 min)/washing/redispersion cycles with dilute aqueous NaCl. Subsequent layers were deposited until the desired number of multi-layers was achieved. Generally, CHI was chosen as outmost layer of shell walls unless otherwise noted. Hollow microshells were obtained by dissolving the MF cores with HCl (0.1 M), centrifuging (2500 g, 5 min) and washing three times with water. The as-prepared shells are centrifuged, and then re-dispersed in 0.01 M phosphate buffer solution (PBS) of pH 7.4 in stock.

2.3. Loading and release of DOX

Equal amount of microshell suspension (aged for appropriate 2 days) and DOX (2 mg/ml) were mixed together overnight. The filled shells were washed three times with water and used in

release experiments immediately. The release experiments were carried out by dipping the DOX-loaded microshells with different layers into physiological NaCl solution (0.154 M). After immersed for 24 h, the shells are separated by centrifuge, and then the DOX contents in the supernatants were determined by UV/vis spectrophotometer. The loading and release of DOX in the shells was quantified by calculating the difference between the input amount and the superfluous amount from the supernatant liquids, combined with a standard curve of DOX solutions ranging in concentrations from 5 to 40 μg/ml.

2.4. Activity testing

A colorimetric XTT assay system was utilized to determine the cytotoxicity of the microshells alone, free DOX alone or DOX-loaded microshells on the H460 and A549 cancer cell lines. Cells were grown in a humidified 5% carbon dioxide atmosphere at 37 °C on a 96-well microplate, with each well containing 5000 cells immersed in 100 μl of 10% fetal bovine serum, and 1% penicilin/streptomycin. The cells were allowed to adhere for 14 h. The cells were then incubated with blank microshells, free DOX or DOX-loaded microshells at various concentrations for 48 h. Following incubation, 50 μl XTT labeling mixture was added to each well. The microplate was incubated for a further 4 h. A Benchmark microplate reader (BIO Rad Laboratory, Mississauga, Ontario, Candana) with a 492 nm optical filter and a 650 nm reference wavelength was utilized to measure the absorbance of each well. The fraction of viable cells was calculated unity asbtract the opital density fraction of treated cells to untreated cells. Each arrangement had a minimum of two independent measurements.

2.5. Characterization

Scanning electron microscope (SEM) images were recorded by Hitachi S-2500 equipped with a backscatter detector and a PCI digital imaging system. The sample for SEM was prepared as below: the shell suspension was immersed in sequence with 2.5% glutaraldrehyde and 1% OsO4 buffer solution, and then deposited on a coverslip pre-coated with poly-L-lysine for 30 min, followed by washing, drying, and coating with a thin gold layer.

Confocal micrographs were taken with a LSM 510 confocal laser scanning microscopy (CLSM, Carl Zeiss Inc.) equipped with multiple laser lines from UV to infrared for excitation of fluorophores. The microshells were visualized either by RITC-PAH or DOX with the excitation wavelength of 543 nm. The PAH labeling was prepared according to a method reported in the literature (Kaschak and Mallouk, 1996).

Beckman DU 7400 spectrophotometer was employed for DOX quantification.

3. Results and discussion

The bio-polyelectrolyte microshells were prepared by alternate adsorption of ALG and CHI onto weakly cross-linked MF colloidal particles, followed by the sacrifice of the template cores. Fig. 1 shows a scanning electron microscopy (SEM) image of hollow microshells with four alginate/chitosan layers templated onto MF latex particles. Almost all the fabricated hollow microspheres maintain intact spherical shape after removal of the template. In order to gain further insight into the interior architecture of the microshells, the microshell-suspended solution was treated with liquid nitrogen freezing, followed by externally mechanical pressure. A SEM image of the fractured microshells (inset in Fig. 1) provides direct visualization of the empty shells formed with alginate and chitosan. The wall thickness of hollow microshells of four alginate/chitosan layers in dried state is ca. 20 ± 5 nm, which is slightly larger than that of the PAH-PSS shells (Peyratout and Dähne, 2004).

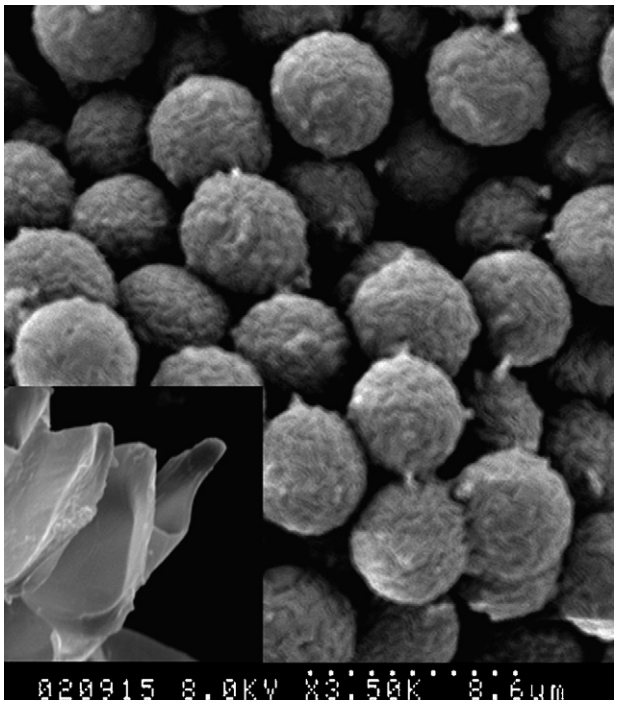


Fig. 1. Scanning electron microscopy (SEM) image of biocompatible hollow microshells composed of (alginate/chitosan)$_4$ with a diameter of 4.9 (m. The inset shows a SEM image of the fractured shells after the treatment with liquid nitrogen freezing and externally mechanical pressure.

Confocal laser scanning microscopy (CLSM) images provide further visualization that the composite microshells of ALG-CHI were successfully fabricated by the LbL self-assembly technique. A typical CLSM image of the ALG-CHI shells (labelled with RITC-PAH) is shown in Fig. 2. Evidently, the constructed microshells display intact spherical shape with inner hollow structure. It is worth mentioning that the (ALG/CHI)$_4$ shell size in the PBS solution is ca. 7.8 μm, which is remarkably larger than the template size e.g. 4.9 μm. The highly expanded shells are more likely to be arisen from the occurrence media of the microshells according to Baijpai et al. in an investigation of water uptake behavior of ALG-CHI particles (Bajpai and Tankhiwale, 2006), that is, the wall composite architecture might become more loosely in PBS solution, and hence leading to takeup of more water in shell walls. Besides, the formation of the hydrogen bonds between the two polysaccharides as well as the hydrogen bonds between the polysaccharides and water could also be

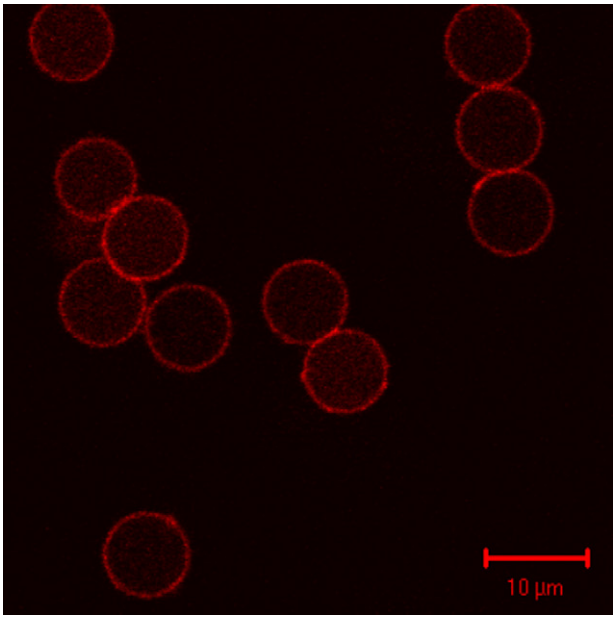


Fig. 2. A CLSM image of hollow microshells of $(ALG/CHI)_4$ templated on MF particles, where RITC-PAH was used to label the shells.

considered as a reasonable explanation for the expanded shells (Lee and Mooney, 2001). Furthermore, the expanded shells are stable and remain spherical shape intact during the period of at least 1 month, indicating the degree of cross-linking of the shells between ALG and CHI (i.e. the interactions between $–NH_2$ groups of ALG and $–COO^-$ groups of CHI) in PBS solution of pH 7.4 might not have decreased to a larger extent. Combined with our recent experimental results, the constructed ALG-CHI shells themselve display higher stability and mechanical property compared to the conventional PSS-PAH shells. All these characteristics of the self-assembled shells i.e. good stability and strong mechanical properties and bio-compatibility are also indispensable to the bio-polyelectrolyte shells as ideal drug vehicles in vivo.

Loading of anti-cancer drugs inside the assembled hollow shells was performed under moderate conditions by the mixture of a suspension of the shells and a solution of doxorubicin hydrochloride (DOX). DOX is a fluorescent anti-cancer drug. CLSM images directly verified the efficient loading of DOX in the interior of the ALG/CHI shells with structural integrity, yielding a higher fluorescence intensity (proportional to the concentration of DOX) than in the bulk, as shown in Fig. 3. The floccular morphology of the encapsulated DOX in the interior of the shells (see Fig. 3a) suggests that the loaded DOX may not exist in the free DOX form. The driving force of DOX loading inside the shells probably is due to the spontaneous diffusion of DOX from low- to high-concentration regions, and the deposited DOX could exist in an aggregated or complex form so that the real concentration of the loaded substances within the interior of the microshells is lower than in the bulk solution, and hence leading to the efficient loading of DOX (Gao et al., 2002).

A magnified DOX-loaded shell is shown in Fig. 3b. From the corresponding fluorescence intensity profile, one can see the intensity change along the line at the DOX-loaded shell cross-section. As expected, the main fluorescence emission originates from the interior of the shells. The $(ALG/CHI)_4$ shell size loaded with DOX is ca. 7.8 ± 0.3 μm, which is similar to those of the hollow shells with four layers (see Fig. 2). Thus it can be seen that the shell size is predominantly dependent on the num-

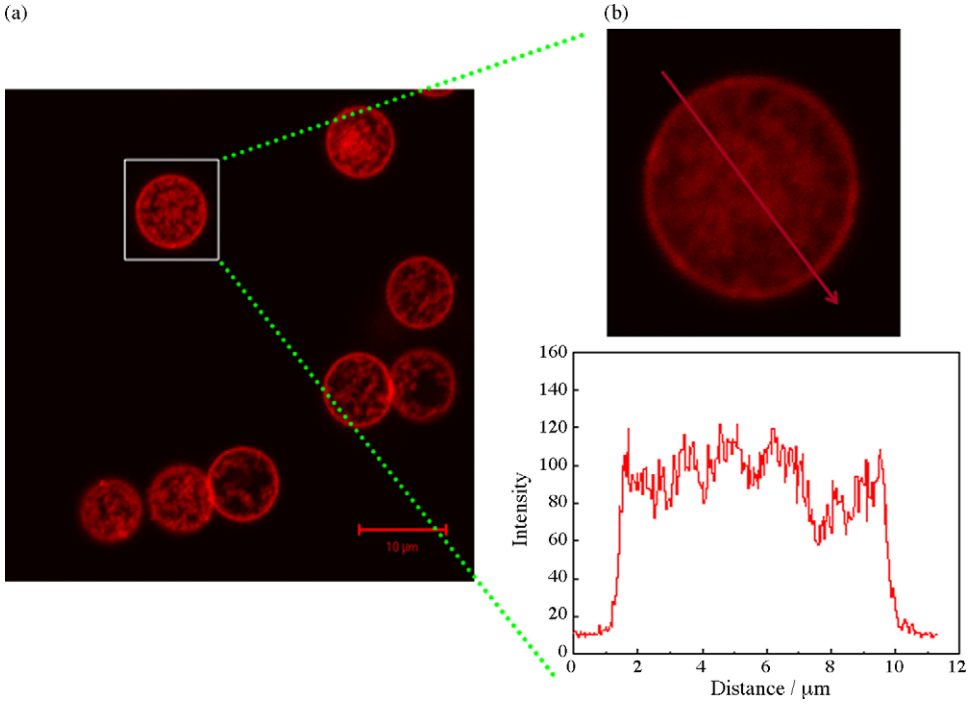


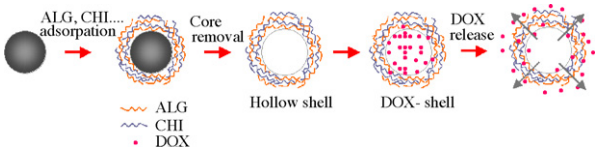
Fig. 3. (a) CLSM image of capsules of $(alginate/chitosan)_4$ loaded with DOX; (b) a magnified CLSM image of a selected individual shell as indicated in (a) and fluorescence intensity profile along the line as drawn above.

ber of assembled layer of the shells templated onto fixed MF particles.

The quantity of DOX in the shells of $(ALG/CHI)_4$ can be determined by the spectrophotometric absorbance difference between the initial DOX concentration and the superfluous DOX from the supernatant liquids, where a DOX standard curve is combined. As a result, the mass of DOX loaded in one capsule of four alginate/chitosan layers (i.e. the volume $V = 2.5 \times 10^{-10}$ cm^3) is calculated as ca. 1.4×10^{-13} g, which corresponds to 1.5×10^8 DOX molecules. This loading value is comparable to the 1.4×10^7 to 1.0×10^9 molecules/shell in the literature (Ye et al., 2006).

Loading and release of DOX for the (ALG-CHI) shells with different layers were investigated. From Table 1, one can see that the shell size gradually increases from 6.9 to 8.9 μm with increasing the number of assembled layer of the (ALG-CHI) shells i.e. from 3 dilayer to 7 dilayer. Accordingly, the loading amount of DOX in one shell composed of $(ALG-CHI)_n$ ($n = 3$, 5, 7) is 1.20×10^{-13}, 1.53×10^{-13}, and 1.80×10^{-13} g, respectively. The enhancement of loading amount with the increase of layer numbers is understandable owing to the increase of volumes of the shells themselves. The release experiments were carried out by dipping the DOX-loaded microshells with different layers into physiological NaCl solution (0.154 M) for 24 h. The release ratio for the shells of $(ALG-CHI)_n$ ($n = 3, 5, 7$) under current experimental conditions is calcucated as 97%, 93% and 88%, respectively, that is, the greater release ratio is obtained if the fewer layer (at least three dilayer assembled for the stable shells here) are used as shell walls. This suggests that the release ratio of drugs can be controlled by the number of assembled layers of the shells.

In this study, we attempt to provide a new formulation by loading anti-cancer drug DOX in the interior of the LbL self-assembled bio-polyelectrolyte shells based on ALG and CHI (see Scheme 1). As a matter of fact, the constructed ALG-CHI shells can also be considered as drug delivery vehicles for other active drugs by the fact that another anti-cancer drug peptide BH_3 has also been successfully loaded inside the ALG-CHI shell in more amounts. Thus, the proposed formulation can be extended by loading one or more kinds of drugs inside the shells adjusted to the specific application in patients.



Scheme 1. Schematic illustration of the procedure for the loading and release of DOX in the LbL self-assembled ALG-CHI shells.

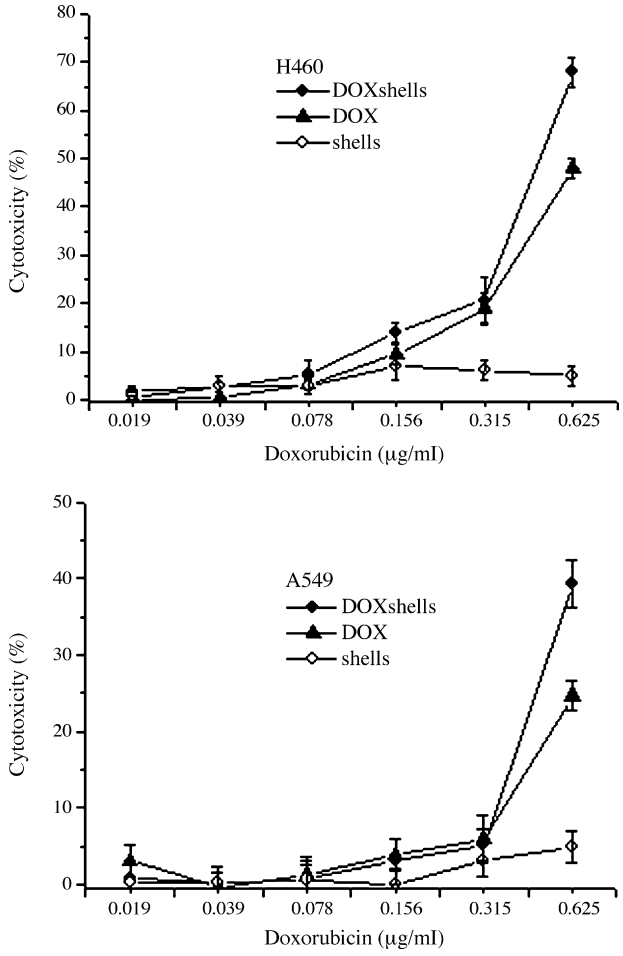


Fig. 4. Comparison of cell toxicity of DOX-loaded $(ALG/CHI)_5$ microshells (●), free DOX (▲) and control $(ALG/CHI)_5$ microshells (◯) in H460 (a) and A549 lung cancer cells (b). Each data point represents the average result of three wells in two independent experiments. Note: Blank microshells had the same concentration based on weight as DOX-loaded microshells examined.

In order to further verify the biological activity of the loaded DOX, the cytotoxic effects of control microshells, DOX-loaded capsules and free-drug on H460 and A549 lung cancer cell lines were assessed using colormetric XTT cell viability assays (Fig. 4). From the Fig. 4, we can see that under the same concentration of DOX percent cell kill here was consistently higher in the chemo-sensitive lung cancer (H460 cell line) than the

Table 1
Loading and release amount of DOX for hollow microshells with different layers[a]

Shell composition	Shell diameter (μm)	Loading amount[b] ($\times 10^{-13}$ g capsule^{-1})	Release amount[c] ($\times 10^{-13}$ g capsule^{-1})	Release ratio (%)
(ALG/CHI)3	6.9 ± 0.3	1.20	1.16	97
(ALG/CHI)5	8.2 ± 0.3	1.51	1.40	93
(ALG/CHI)7	8.9 ± 0.3	1.83	1.61	88

[a] The loading and release of DOX (2 mg/ml) in shells is kept for 24 h, respectively.
[b] The loading amount per micro shell was calculated based on the pipette volume and the physical parameters of particles (diameter $d = 4.9$ μm, the density $p = 1.51$ g/cm and solids content of wt. 10% (i.e. l00 mg/ml)).
[c] The release of DOX in shells was performed in physiological NaCl (0.154 M).

chemo-resistant one (A549 cell line). At concentrations higher than 0.4 μg/mL, DOX-loaded microshells in both cell lines were shown to be more cytotoxic than free-DOX alone. No significant cytotoxic activity for the control microshells on their own was observed. This synergy can be explained by the known interaction between the microshell's chitosan outer wall and plasma membranes (Erbacher et al., 1998; Dodame et al., 1999). The binding of the microshells to the H460/A549 human lung cancer cells would effectively result in a higher local concentration of DOX in the direct vicinity of the cells and likely accounts for the greater cytotoxicity observed.

4. Conclusion

A novel formulation by loading DOX in the interior of biopolyelectrolyte microshells is presented, in which multilayered microshells based on ALG and CHI are constructed utilizing the colloidal templating LbL technique, followed by the DOX loading by the simple mixture of microshell suspension and DOX under modest condition. The loading of DOX in the shells has been confirmed using confocal laser scanning microscopy. The mass of DOX loaded in one capsule of four ALG/CHI layers is ca. 1.4×10^{-13} g, which corresponds to 1.5×10^{8} DOX molecules. Also, the release ratio of DOX can be controlled by the number of assembled layers of the shells. Colorimetric XTT cell viability assay results show that DOX-loaded microshells possess more cytotoxic than free-DOX alone.

Acknowledgements

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