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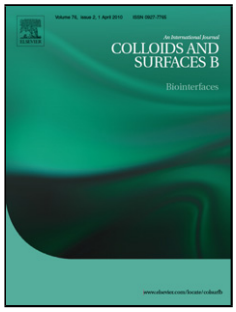

Title: Doxorubicin-loaded biodegradable self-assembly zein nanoparticle and its anti-cancer effect: preparation, *in vitro* evaluation, and cellular uptake

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PII: XXXXXXXXXXX
DOI: http://dx.doi.org/doi:10.1016/j.colsurfb.2015.12.048
Reference: XXXXXXXX

To appear in: *Colloids and Surfaces B: Biointerfaces*

Received date: 9-9-2015
Revised date: 24-11-2015
Accepted date: 24-12-2015

Please cite this article as: Fangyuan Dong, Xiaoli Dong, Liping Zhou, Huihui Xiao, Pui-Yu Ho, Man-Sau Wong, Yi Wang, Doxorubicin-loaded biodegradable self-assembly zein nanoparticle and its anti-cancer effect: preparation, in vitro evaluation, and cellular uptake, Colloids and Surfaces B: Biointerfaces http://dx.doi.org/10.1016/j.colsurfb.2015.12.048

Doxorubicin-loaded biodegradable self-assembly zein nanoparticle and its anti-cancer effect: preparation, *in vitro* evaluation, and cellular uptake

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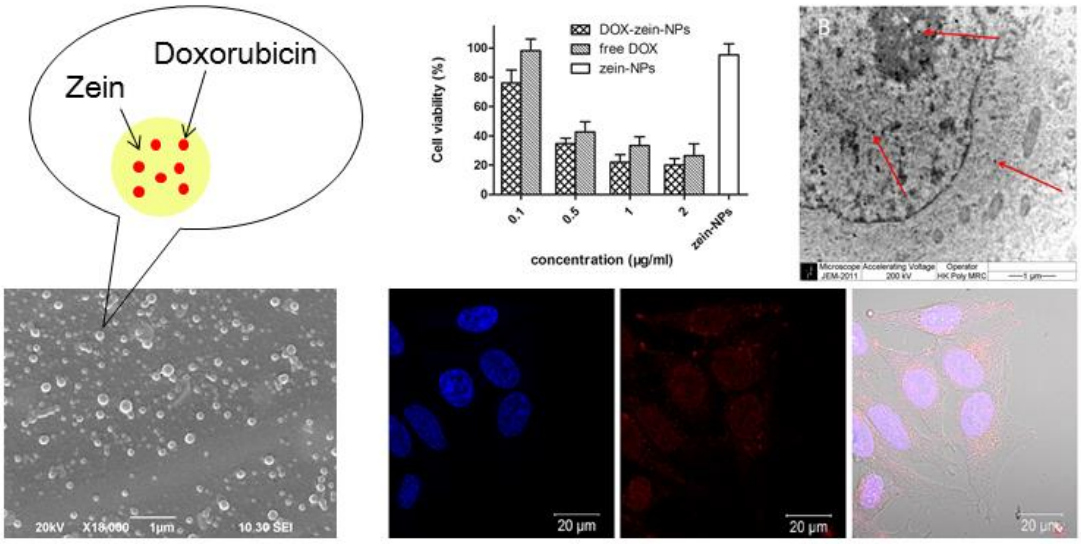
Graphical Abstract



Highlights

1. The formation of Doxorubicin-loaded zein nanoparticles (DOX-zein-NPs) is facile;
2. The shell material zein is biodegradable and biocompatible;
3. DOX-zein-NPs have a uniformly spherical shape with a particle size around 200 nm;
4. DOX-zein-NPs have better anti-cancer effect compared to free DOX;
5. DOX-zein-NPs can enter into both cell cytoplasm and nucleus.

Abstract

Cancer is one top leading cause of the deaths worldwide. Various anticancer drugs, which can effectively kill cancer cells, have been developed in the last decade. However, the problem is still about the low therapeutic index of the drugs, which means that the effective dose of drugs will cause cytotoxicity to normal cells. A strategy based on drug nano-encapsulation is applied to achieve an effective anti-cancer therapy. In this study, we use zein, which is an amphiphilic protein, to make the anti-cancer drug nano-encapsulation. Doxorubicin (DOX), a popular anti-cancer drug, is selected as the core drug. The results show that DOX could be successfully encapsulated into zein to form spherical nanoparticles. The encapsulation efficiency and loading efficiency could reach as high as 90.06% and 15.01 mg/g, respectively. The cumulative release result showed a desired pH-responsible release behavior: DOX could be released faster in acidic buffer solutions (pH 5.0 and 6.5) than neutral one (pH 7.4). The effects of the nano-encapsulation on the anti-proliferation of HeLa cells were also examined. It indicated that, compared with free DOX, the DOX-loaded zein nanoparticles (DOX-zein-NPs) had a better effect on cancer cell killing at low DOX concentrations. We also investigated the cellular uptake of DOX-zein-NPs using confocal laser scanning microscopy (CLSM), flow cytometry, and transmission electron microscopy (TEM). And the endocytosis mechanism of DOX-zein-NPs entering into HeLa cells was studied using various endocytosis pathway inhibitors.

Keywords: nano-encapsulation; zein; doxorubicin; drug delivery; cellular uptake.

1. Introduction

Cancer counts for one in four deaths in the 21th century [1]. Currently, tons of anti-cancer drugs have been developed. However, the general problem of these drugs is that they lack efficient selectivity toward tumor cells. They kill both normal and cancer cells and result in toxicity to normal tissues. Because of the weak selectivity, drugs reach the target site in concentrations much less than effective [2]. In this case, the drug dose has to be further increased to make it effective, and thus, more toxicity will be given to the normal tissues. Severe side effects decrease the life quality of the patients and can be fatal at times. Site-specific delivery, which protects drugs from release until the target site is reached, is applied to increase the drug concentration at the target site, and thus lower the dose used and reduces incidence of the side effects. The site-specific delivery can be achieved using a drug delivery system. A drug delivery system is often associated with particulate carriers, such as polymeric micelles, emulsions, liposomes [3], nanotubes, dendrimers [4], and nanoparticles (NPs) [4-8], which are designed to localize drugs at the target sites [9].

Nano-encapsulation is a superior method that nano-carriers are designed for drug delivery systems [9, 10]. Drugs are wrapped and protected by ultrafine vehicles or capsules to form nano-sized core-shell structures. In nano-encapsulation, there is one kind of NP carrier used for encapsulating drugs. This kind of NP-mediated delivery provides numerous advantages. Most drugs formulated with organic solvents have poor solubility and low bioavailability. The use of polymeric NPs allows for the preparation of hydrophobic cancer medications which have improved bioavailability [11]. Because of their small particle size and large surface, NPs can improve the drug solubility and stability, and enter the tumor cells through the enhanced permeability and retention (EPR) effect [12]. Besides, as a protection to the drugs, NP-mediated delivery can achieve a controlled slow release over a required time frame [13]. It also allows increased intracellular accumulation of the therapeutic agent and limits the uptake by healthy tissues. NP-mediated delivery could also circumvent the multidrug resistance (MDR) problem in cancer therapies. For example, paclitaxel-loaded NPs showed 10-fold lower IC_{50} values than free paclitaxel in MDR cells [14].

NPs that are used in NP-mediated deliveries are produced from a wide variety of materials including carbon, heavy metals, semiconductors, and polymers, which have their own advantages and disadvantages. The material selection for the drug delivery agents is important and it will determine the selectivity and specificity as well as the toxicity of the agent. It is hard to ignore that many potential candidates of the polymeric micelles fail in preclinical studies because of high toxicity. To surmount the dilemma, biodegradable polymeric materials with low immunogenicity, controlled degradation, and good mechanical properties are becoming one of the most powerful candidates. The biocompatibility, toxicity, and biodegradability are always the first concerns about the materials used for drug delivery. So far, site-specific delivery is still a problem because of the selection of the carrier materials, and a balance between the biocompatibility and functionality of the carrier materials is difficult to achieve. The best choices could be the natural materials derived from plant based materials,

which are non-toxic, and even edible. However, the interiors of general plant derived natural materials are their hydrophilicity, easy water absorption, and high digestibility [15, 16].

Zein, a major corn protein, unlike most natural materials, is amphiphilic, having both hydrophobic and hydrophilic compounds in its molecule. Zein is insoluble in water but readily disperses in ethanol-water mixture [17]. It is inexpensive, abundant, biodegradable, and biocompatible [18]. Zein is well applied in food and pharmaceutical industries because of its good biocompatibility and bioavailability. Zein has an amphiphilic molecular structure where over 50% of the amino acid in zein is hydrophobic. An amphiphile, such as zein, can self-assemble into various structures, including spheres, sponges and films [6, 17, 19-21]. Zein can self-assemble to form biobased films, hydrophobic surfaces, and encapsulations, and have potential applications in food packaging, electronic devices, and for oral administration enhancement [22]. Because of its self-assembly ability, zein has been extensively studied to encapsulate bioactive compounds, such as essential oil [23], flax oil [24], vitamin D3 [25], α-tocopherol [18], and citral and lime [19]. Nano-scaled drug carriers prepared by amphiphilic molecules are of great interest to scientists because of their unique core–shell structures, and the core provides an ideal compartment for drug loading, especially hydrophobic drugs [26, 27]. In addition, amphiphilic particles have the ability to transfer through cell membranes. They have better stability than hydrophobic or hydrophilic particles because of the unique surface properties.

There are many FDA approved drugs for cancer treatments. Doxorubicin (DOX), a water-soluble anti-cancer drug, is one of them and has been proved effective in a series of cancers [28]. However, the clinical use of DOX is limited because it is harmful to healthy tissues and has significant side effects, such as cardio toxicity, myelosuppression, weight loss, and alopecia. The objective of this work is to apply zein NPs to encapsulate DOX for anti-cancer therapies. The formation of the DOX-loaded zein NPs (DOX-zein-NPs) and their *in vitro* release profiles were studied. The anti-proliferative effects on HeLa cells and the cellular uptake were also investigated.

2. Materials and methods

2.1. Materials.

Zein was purchased from Wako Pure Chemical Industries, Ltd. (Tokyo, Japan). Ethanol (96% v/v) was obtained from Guangdong Guanghua Sci-Ttch Co., Ltd (Guangzhou,China).
[3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetr azolium] (MTS) was purchased from Promega Corporation (Madison, USA). Doxorubicin hydrochloride, sodium caseinate (CAS), phenazine methosulfate (PMS), 4',6-Diamidino-2-phenylindole dihydrochloride (DAPI), nystatin, chlorpromazine, nocodazole, and cytochalasin D were purchased from Sigma-Aldrich Chemical Co. Ltd. (St. Louis, MO). Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS), penicillin and streptomycin, trypsin, and phosphate buffer saline (PBS)

were all purchased from Life Technologies (Thermo Fisher Scientific Inc., NY, USA). All other reagents were of analytical grade. All chemicals were used as received.

2.2. Nanoparticle preparation

DOX-zein-NPs were prepared using phase separation method. Different formulations were listed in Table 1. In brief, 10 mg/ml zein and 0.5 mg/ml DOX were first dissolved in 1 ml of 80% ethanol-water. The mixture was then sonicated for 15 min to form a transparent stock solution. Sodium caseinate (CAS), used as stabilizer, was dissolved in deionized water to form aqueous solutions of different concentrations [29]. Then 2.0 ml of CAS solution was rapidly poured to the stock solution under vigorously vortex for 10 min, and the NPs were spontaneously formed by self-assembly. To prepare control blank zein-NPs, the corresponding amount of zein was dissolved in 1 ml of 80% ethanol-water. 2.0 ml of CAS solution was rapidly poured into the pure zein stock solution under the same condition. The ethanol in the formed NP suspensions was removed under nitrogen stream, and the suspensions were lyophilized in the dark to obtain dry NP powders. The prepared powders were stored at 4℃ with light protection before further analyses.

2.3. Particle characterization

The particle size and the corresponding zeta potential were obtained using Malvern ZETASIZER 3000HSA (London, England) at room temperature (25℃). All measurements were conducted in triplicate. The prepared NP powders were re-dispersed in deionized water and then transferred into the cuvette for size measurement and the particle size was obtained as an average. As for zeta potential measurement, the pH of the re-dispersed NP suspensions was adjusted to 7.4 before measurement. The morphology of the NPs was observed using a JEOL JSM-6490 scanning electron microscope (SEM, Tokyo, Japan). The samples were gold coated (300Å) with an Edwards S150B sputter coater to help improve electrical conductivity and observed under the SEM.

2.4. Drug encapsulation and loading efficiency

The encapsulation efficiency (EE) and loading efficiency (LE) of DOX in DOX-zein-NPs were determined using UV-vis spectrometer (GBC-Cintra 40, Australia). The absorbance of DOX was obtained at 480 nm. The calibration curve was obtained from the UV absorbance of several standard DOX aqueous solutions. The freshly prepared NP suspensions were centrifuged at 14,000 rpm for 55 min. The supernatants were then removed for the determination of free DOX in the suspensions. All measurements were in triplicate. The EE and LE were calculated according to the following equations:

$$\mathrm{EE} = \frac{total\ DOX\ -\ free\ DOX}{total\ DOX} \times 100\%$$

$$\mathrm{LE} = \frac{mass\ of\ DOX\ in\ particles\ (mg)}{mass\ of\ nanoparticles\ (g)}$$

2.5. In vitro *drug release*

The *in vitro* release profiles of DOX from DOX-zein-NPs were studied in PBS buffer solutions with various pH values (5.0, 6.5, and 7.4) at 37℃. 10 mg DOX-zein-NPs were re-dispersed in 2 ml of PBS, and the suspension was then put into a membrane dialysis bag with molecular weight cut off at 10 kDa. The membrane dialysis bag was suspended in 50 ml of PBS. The free DOX was used as control group. The entire system was kept in a vibrator at 250 rpm and 37℃. At predetermined time intervals, 2 ml of solution was collected from the release medium and replaced with 2 ml of fresh PBS. The concentration of DOX in the collected sample was determined by UV-vis spectrometer, as described in section 2.4.

2.6. *Cell culture*

HeLa cells were incubated in Dulbecco's Modified Eagle Medium (DMEM), which contained 100 mg/ml penicillin and 100 mg/ml streptomycin. The cells were supplemented with 10% FBS, and maintained in a humidified incubator at 37℃ in an atmosphere of 5% CO_2. Then, the cells were trypsinized using a 0.25% trypsin solution in PBS buffer for 5 min and re-suspended in the complete culture medium.

2.7. *MTS assay*

The anti-proliferative effects of DOX-zein-NPs on HeLa cells were evaluated using MTS assay. HeLa cells were cultured at a density of 5,000 cells per well in the growth medium for 24 h in the 96-well plates, with 8 wells in each group. Two control samples are set as the control without any treatment and the control with the treatment of zein-NPs. The cells were incubated with free DOX or DOX-zein-NPs at a DOX concentration of 0.1, 0.5, 1.0, and 2.0 μg/ml at 37℃ for 48 h, respectively. 20 μl of MTS/PMS solution (333μg/ml MTS and 25μM PMS dissolved in PBS) was added to each well and incubated at 37 ℃ for 2 h. The tetrazolium compound MTS, with the presence of electron coupling reagent PMS, was reduced by NADPH or NADH in metabolically active cells to form a colored formazan product that has a maximum absorbance at 490 nm [30]. The absorbance, which is directly proportional to cell viability, was recorded at 490 nm with a 96-well microplate spectrophotometer (Bio-Rad, Hercules, CA, USA). Results were expressed as the percentage MTS reduction assuming the cell viability of control cells was 100% [31].

2.8. *Confocal laser scanning microscopy (CLSM)*

The cellular uptake of DOX-zein-NPs was investigated using CLSM. HeLa cells (4×10^4 cells/well) were seeded onto sterile microscope slides in a 6-well plate and allowed to attach for 24 h after incubation, the cells were then incubated with free DOX and DOX-zein-NPs at a DOX concentration of 50 μg/ml at 37℃ for 5, 10, and 30 min, respectively. Then, the treated cells were washed three times with PBS and fixed with 4% paraformaldehyde for 20 min. Then, the fixed cells were stained by 1 μg/ml DAPI for 20 min and washed three times with PBS. The prepared cell samples

were examined using a confocal microscope (Leica, Germany). The cellular uptake of DOX was visualized at the excitation and emission wavelength of 488 and 590 nm, respectively. The cell nucleus was visualized at the excitation and emission wavelength of 340 nm and 488 nm, respectively.

2.9. Flow cytometry

Sterile microscope slides were put into a 6-well plate. HeLa cells were then seeded at 4×10^5 cells/well onto the slides and allowed to attach for 24 h. Then the original medium was replaced by fresh medium containing free DOX and DOX-zein-NPs, respectively, at a DOX concentration of 200 μg/ml. And the cells were incubated for 1 h and 4 h at 37℃, respectively. Then, the medium was removed and the treated cells were washed three times with PBS (pH7.4), and the cells were harvested by trypsin and centrifugation (1,200 rpm, 5 min). And the pelleted cells were re-suspended in 0.5 ml of 70% ethanol for fixing. Cells were washed three times with PBS and re-suspended into 0.5 ml PBS before flow cytometry analysis. The flow cytometry analysis was conducted using a BD FACS Aria II flow cytometer (BD Biosciences).

2.10. Transmission electron microscopy (TEM)

HeLa cells were seeded in a 6 well-plate at the density of 4×10^4 cells/well. After 24 h, cells were treated with DOX-zein-NPs at a DOX concentration of 50 μg/ml at 37℃ for 30 min. The treated cells were then washed 3 times and fixed by 4% glutaraldehyde and osmium tetroxide, followed by dehydration in graded ethanol and embedding. The prepared samples were cut into 70 nm slices and were examined under TEM (JEOL Model JEM-2011, Japan).

2.11. Cellular uptake inhibition study

The cellular uptake mechanism of DOX-zein-NPs was investigated. HeLa cells were seeded into a 6-well plate at a density of 4×10^5 cells/well and allowed for attachment for 24 h. Then, the cells were pretreated with various inhibitors for 30 min at the following concentrations: nystatin 25 μg/ml, chlorpromazine 10 μg/ml, nocodazole 10 μg/ml, and cytochalasin D 5 μg/ml [32-34]. Then, cells were incubated with 200 μg/ml DOX-zein-NPs for 2 h in the presence of the same inhibitor. Two control samples were set as the untreated cell samples exposed or not exposed to DOX-zein-NPs, respectively. The treated cells were collected and fixed for flow cytometry analysis. The experiments were conducted in triplicates.

3. Results and discussion

3.1. Formulation of DOX-zein-NPs

3.1.1. Nano-encapsulation of DOX by zein

The morphology of prepared DOX-zein-NPs was characterized using SEM (Fig.

1). The SEM images show that the zein concentration and the mass ratio of zein to DOX ($R_{Z/D}$) have little effect on the shapes of the formed DOX-zein-NPs. The spherical particles had uniform particle sizes ranging from 200 to 250 nm. For drug delivery, the size of the NPs is important, since it affects the drug release kinetics, the drug bio-distribution, and the drug clearance from the organism [35]. It is reported that NPs smaller than 250 nm actively accumulate in tumor tissue due to the EPR effect [12]. The particle sizes and zeta potentials (ZP) of DOX-zein-NPs prepared from various zein concentrations were also measured using DLS and shown in Table 1. The average hydrodynamic size of DOX-zein-NP was around 200-240 nm. The PDI value, around 0.15-0.20, indicated that the distribution of the particle sizes was narrow, which was in good agreement with the observation by SEM. The ZP had a high absolute value around 50, indicating that the formed DOX-zein-NPs were clear and stayed still from aggregation. The size distribution of the DOX-zein-NPs were also measured after being dispersed in the deionized water for 24 h (Supplementary material 1). As seen from the result, the particle size did not change much and still had a narrow size distribution after 24 h, indicating the good stability of the formed DOX-zein-NPs.

3.1.2. DOX encapsulation and loading efficiency

The EE and LE of the DOX-zein-NPs were measured and shown in Table 2. As shown, the highest EE and LE were 90.06±0.26 and 15.01±0.14 mg/g, respectively, when DOX-zein-NPs were prepared at the zein concentration of 10 mg/ml and $R_{Z/D}$ of 20. The effects of $R_{Z/D}$ on the EE and LE were investigated by comparing the results of the DOX-zein-NPs prepared with the same zein concentration. When comparing sample 1, 2, and 3, which were formed from the same zein concentration of 10 mg/ml, the EE and LE decreased when the $R_{Z/D}$ value increased from 20, 30, to 50. When comparing sample 5, 6, and 7, which were prepared from the same zein concentration of 30 mg/ml, the EE and LE decreased when the $R_{Z/D}$ value increased from 30, 60, to 120. It is believed that, when the zein concentration was 10 mg/ml and the $R_{Z/D}$ value increased from 20, or the zein concentration was 30 mg/ml and the $R_{Z/D}$ value increased from 30, $R_{Z/D}$ was moving away from its optimal for DOX-zein-NP formation so zein was not likely to make encapsulation structure with DOX but self-assemble into zein nanoparticles. This can be attributed to the interactions of zein and DOX in the solution. In this way, both EE and LE were decreased. However, when we prepared the DOX-zein-NPs at the conditions of zein concentration of 10 mg/ml and $R_{Z/D}$ less than 20, or zein concentration of 30 mg/ml and $R_{Z/D}$ less than 30, it was very difficult to form the DOX-zein-NPs, which meant the DOX-zein-NPs formation was very sensitive when the $R_{Z/D}$ value was below the optimal. The effects of the zein concentration on the EE and LE were investigated by comparing the results of the DOX-zein-NPs prepared with the same $R_{Z/D}$. For example $R_{Z/D}$ of 30, the EE of sample 2, 4, and 5, which were prepared from different zein concentrations, 10, 20, and 30 mg/ml, were almost the same in a narrow range of 83-86%. The LE of sample 2, 4, and 5 were also similar ranging from 9.2 to 9.6 mg/g. Therefore, the zein concentration had no significant effect on the EE and LE when $R_{Z/D}$ was the same.

Based on the discussion above, we conclude that there is an optimal $R_{Z/D}$ ($R_{Z/D\text{-}OPT}$) to each zein concentration, under which the zein nano-encapsulation of DOX has the highest EE and LE. Above or below $R_{Z/D\text{-}OPT}$, zein is more likely to self-assemble rather than interact with the DOX to form the encapsulation, and lead to the decreased EE and LE. And, since both zein and DOX were at low concentrations in this study, the effect of the change of zein concentration on the interaction among zein molecules, among DOX molecules, and between zein and DOX molecules was low. So, at the same $R_{Z/D}$, the EE and LE were almost the same when changing the zein concentration.

3.2. The in vitro *release of DOX from DOX-zein-NPs*

The *in vitro* release profiles of free DOX and DOX-zein-NPs in different buffer solutions, respectively, were shown in Fig. 2. As seen, the release behaviors of free DOX were similar at different pH values. The free DOX showed an initial burst release, and within 8 h almost all of the free DOX was released out regardless of the pH variation. The release of free DOX was mainly by diffusion. On the other hand, after the initial 6 h of rapid release, the release of DOX from DOX-zein-NPs reached a sustained release. It is believed that the release of DOX was much longer delayed because of the zein nano-encapsulation structure. The slow hydrolysis process of zein NPs in PBS allowed a sustained release of the encapsulated drugs for a few days [18, 36, 37]. Moreover, the release of DOX from DOX-zein-NPs also showed a pH-responsible feature. At acidic pH of 5.0 and 6.5, the cumulative DOX release from DOX-zein-NPs reached 69% and 83% after 100 h, respectively. In comparison, only 60 % of DOX was released out at pH 7.4 after 100 h. It has been reported that tumor extracellular matrix has a lower pH value (pH 6.5) than the blood and normal tissues (pH 7.4), and the organelles of tumor cells like endosomes and lysosomes have a much lower pH value (pH 5.0) [38]. The slow release of DOX from DOX-zein-NPs at normal extracellular pH and fast release at lower tumor intracellular pH can maintain drug molecules within the NPs during blood circulation, reduce the cytotoxicity to the normal cells, and enhance the targeted cytotoxic effects on the specific tumor cells [39].

3.3. In vitro *cytotoxicity of DOX-zein-NPs*

The *in vitro* cytotoxicity of zein-NPs, free DOX and DOX-zein-NPs, respectively, on HeLa cells were showed in Fig. 3. It shows that zein-NPs had no cytotoxicity since the cell viability was not changed after the addition of zein-NPs. When the concentration of free DOX was increased from 0.1, 0.5, 1.0, to 2.0 μg/ml, 98.1%, 42.6%, 33.4%, and 26.5% of HeLa cells was killed by free DOX while 76.0%, 34.7%, 22.0%, 20.1% was killed by the DOX-zein-NPs (with equivalent DOX concentration), respectively. The results indicated that, at the same concentration of DOX, zein nano-encapsulation could inhibit the proliferation of HeLa cells more effectively. In addition, at higher concentrations, compared to low DOX concentration of 0.1 μg/ml, the difference on the cytotoxicity between free DOX and DOX-zein-NPs was much

smaller. So, at low DOX concentrations, the NP-based anti-cancer drug delivery has a better effect on cancer cell killing than the free drug application.

3.4. Cellular Uptake of DOX-zein-NPs

3.4.1. CLSM

The mechanisms of cellular uptake and intracellular trafficking of DOX-zein-NPs in HeLa cells was investigated using CLSM. HeLa cells were seeded in culture dishes and treated with free DOX and DOX-zein-NPs (the DOX concentration: 200 μg/L) for 5, 10, and 30 min, respectively. As shown in Fig. 4, 5 min after the administration of DOX-zein-NPs, weak red fluorescence signals were observed in the HeLa cell cytoplasm. The intensity of the red fluorescence in the cytoplasm increased as the time increased to 10 min and 30 min. While, when using free DOX, there was no red fluorescence observed in the cytoplasm after 5 min, and there was no red fluorescence in the cytoplasm after 10 min or 30 min, either. It indicated that DOX-zein-NPs entered the HeLa cells and stayed in the cytoplasm. If we focus on the cell nucleus instead, 5 min after the administration of free DOX, weak red fluorescence was observed in the cell nucleus. And the red fluorescence enhanced when the time increased to 10 min and 30 min. Since the cytotoxicity of DOX on cancer cells was through its effect on the DNA in the cell nucleus, it is believed that the DOX entered the HeLa cells and further penetrated the cell nucleus membrane and accumulated in the cell nucleus. So, for free DOX, there was no accumulation of DOX in the cytoplasm so there was no red fluorescence observed in the cytoplasm. When we compare the fluorescence observed from the nucleus of the HeLa cells after the administration of free DOX and DOX-zein-NPs, respectively, at all the time points, 5, 10, and 30 min, the intensities of the red fluorescence in the cell nucleus after the DOX-zein-NPs administration were weaker than that after the free DOX application. Because the DOX-zein-NPs controlled the release of the DOX in a much longer time frame compared to the free DOX administration, which is beneficial to enable a prolonged curing effect against the cancer cells.

3.4.2. Flow cytometry

To further confirm the intracellular uptake of DOX-zein-NPs, flow cytometry was used to measure the fluorescence intensity of DOX-zein-NPs and free DOX, respectively, in HeLa cells during cell uptake (Fig. 5A). The cells with no treatment were used as control, and its fluorescence was shown as the red line in Fig. 5A.The fluorescence intensities of cells incubated with both free DOX and DOX-zein-NPs, respectively, increased with time, which indicated both the free DOX and DOX-zein-NPs continuously entered the HeLa cells during the incubation. And the fluorescence intensity of cells incubated with free DOX was higher than that of DOX-zein-NPs at the same time point, which was consistent with the CLSM results. The reason is that DOX-zein-NPs usually entered cells via endocytosis pathways while free DOX via faster passive diffusion [40, 41], and the DOX was controlled released from DOX-zein-NPs in a much longer time frame.

3.4.3. TEM

The endocytosis of DOX-zein-NPs was also studied using TEM (Fig. 5B). The TEM image shows that the DOX-zein-NPs had the ability to enter not only the cytoplasm but also the cell nucleus. It was also measured that the size of the DOX-zein-NPs in the cell nucleus was about 50 nm. Compared to the average size of the DOX-zein-NPs, around 200 nm as shown in Table 2, the NPs that could enter cell nucleus had a smaller particle size. By controlling the zein nano-encapsulation to a size as small as 50 nm, the nucleus-targeted drug delivery can be achieved using zein based nano-carriers.

3.4.4. The endocytosis pathway of DOX-zein-NPs

It is reported that endocytosis is the mechanism most relative to the cellular uptake of NPs [42]. NPs can interact with the plasma membrane and enter into cells via different endocytosis pathways. There are primarily two types of endocytosis: phagocytosis and pinocytosis. Phagocytosis is reported to be strict to particular mammalian cells, such as neutrophils, monocytes, and macrophages, while pinocytosis is commonly present in most cells [32]. Pinocytosis can be further classified into caveolae-mediated endocytosis, clathrin-mediated endocytosis, and macropinocytosis [43-46]. To investigate the endocytosis pathway of DOX-zein-NPs entering HeLa cells, various pathway inhibitors were selected: nystatin was used for the inhibition of caveolin-mediated endocytosis; chlorpromazine had the inhibition of clathrin-mediated endocytosis [47]; nocodazole and cytochalasin D, can disrupt microtubule and actin, respectively, which are fundamental parts of micropinocytosis [48, 49]. The effect of different inhibitors in HeLa cells was assessed quantitatively using flow cytometry and the result was shown in Fig. 6. The 0% uptake and 100% uptake was set according to the mean fluorescent intensities of two samples . The 0% uptake was determined from cell samples with no DOX-zein-NPs incubation, while the 100% uptake (control group) from cell samples with DOX-zein-NPs incubation. As shown in Fig. 6, neither nystatin, nor chlorpromazine had effect on the uptake of DOX-zein-NPs in HeLa cells. While, both nocodazole and cytochalasin D resulted in a decrease of cellular uptake of DOX-zein-NPs. The effect of nocodazole was stronger, with a 33% reduction of uptake relative to the control group. And cytochalasin D had less inhibition effect, where the uptake of DOX-zein-NPs reduced by 22 %. The result indicated that the endocytosis pathway of DOX-zein-NPs is not caveolin-mediated or clathrin-mediated pathways, but macropinocytosis.

4. Conclusion

In this study, zein was used to make nano-encapsulation of an anti-cancer drug DOX. DOX-zein-NPs were found to be uniformly spherical in shape with a particle size around 200 nm. The drug EE and LE ranged from 40-90% and 1-15 mg/g, respectively. The release profiles of DOX-zein-NPs was studied. Results indicated that DOX-zein-NPs exhibited a sustained pH-responsible release. DOX was released

faster at acidic pH values (pH 5.0 and 6.5) than at neutral pH value (pH 7.4). Compared to the rapid release of free DOX, DOX-zein-NPs showed a sustainable release behavior. It took 8 h for free DOX to reach 100% release, while, after 100 h, the cumulative release of DOX from DOX-zein-NPs in the environment of pH 5.0, 6.5 and 7.4 were 68%, 83%, and 61%, respectively. The anti-proliferative effect of DOX-zein-NPs on HeLa cells was studied. At the same concentration of DOX, DOX-zein-NPs killed more HeLa cells than free DOX. The CLSM result showed that DOX-zein-NPs entered the cytoplasm of HeLa cells and the DOX release was delayed and controlled by the zein encapsulation structure, which was confirmed by later flow cytometry result. TEM result shows that the small sized DOX-zein-NPs (around 50 nm), could enter both cell cytoplasm and cell nucleus. The endocytosis of nano-zein-DOX was inhibited by nocodazole and cytochalasin D, which indicated that macropinocytosis is the endocytosis pathway of DOX-zein-NPs. Zein nano-encapsulation could be considered as a prospective drug delivery system for cancer chemotherapy.

Acknowledgements

This work is financially supported by the National Natural Science Foundation of China (project number: 51303153), The Hong Kong Polytechnic University (project number: 1-ZVA9, 5-ZDAJ, G-UC07, and G-YK99), and Shenzhen Key Laboratory Fund (Project code: ZDSYS20140509142430241). We appreciate the help from the Material Research Center of the Hong Kong Polytechnic University.

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Figure Captions

Fig. 1. Selected SEM images of DOX-zein-NPs: (a) DOX-zein-NPs prepared at zein concentration of 10 mg/ml and $R_{Z/D}$ value of 20; (b) DOX-zein-NPs prepared at zein concentration of 20 mg/ml and $R_{Z/D}$ value of 30; (c) DOX-zein-NPs prepared at zein concentration of 30 mg/ml and $R_{Z/D}$ value of 30.

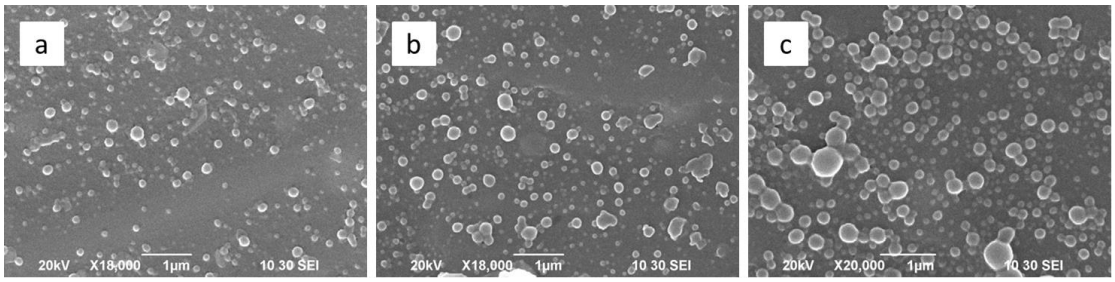


Fig. 2. The release profiles of DOX from DOX-zein-NPs and free DOX in different PBS buffer solutions (pH 5.0, 6.5, and 7.4).

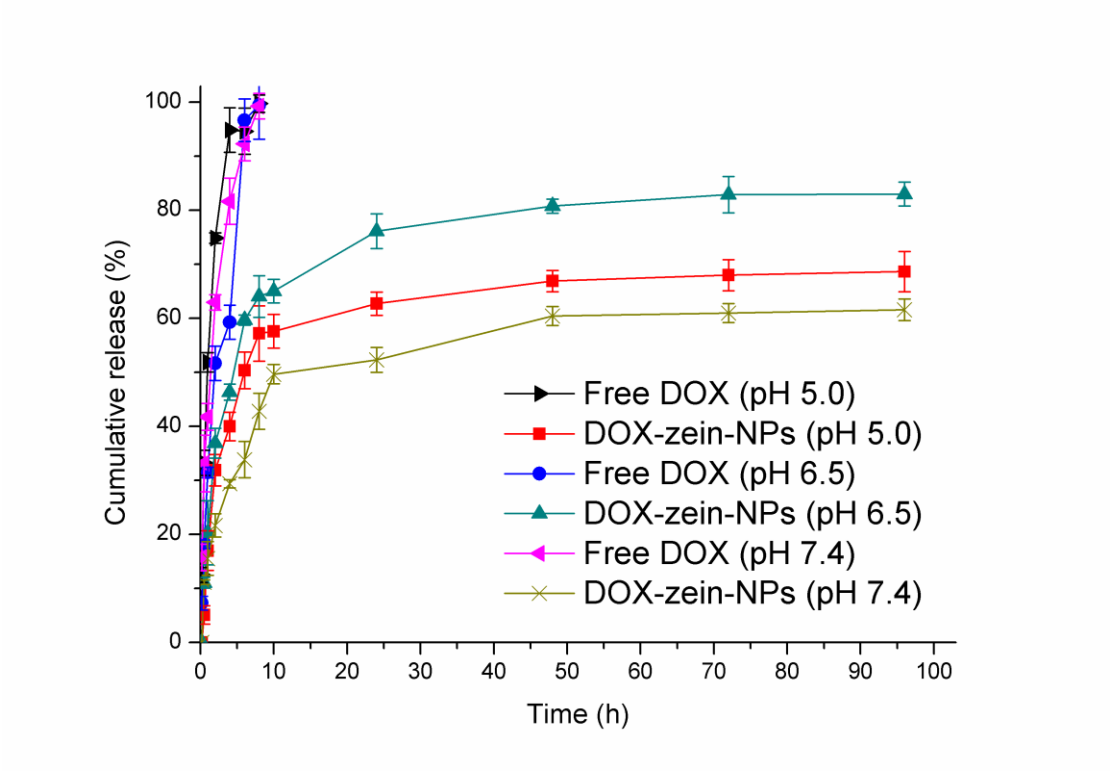


Fig. 3. MTT assay of HeLa cell viability after treatment with zein-NPs, free DOX, and DOX-zein-NPs at 37℃ for 48 h. The data are present as mean ± S.D (n=6).

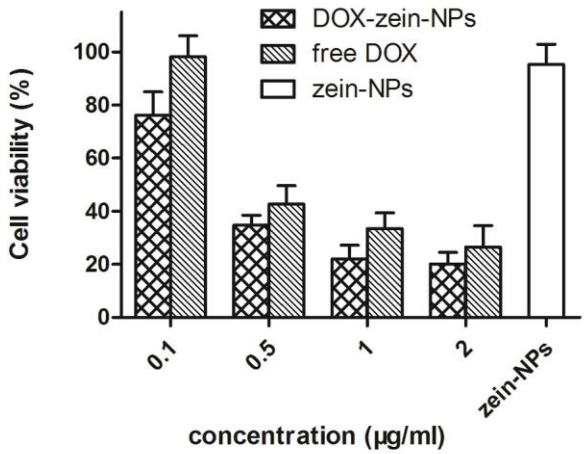


Fig. 4. CLSM images of HeLa cells after treatment with (a) free DOX and (b) DOX-zein-NPs for 5, 10, 30 min, respectively. *Red* is DOX fluorescence, *blue* is cell nucleus treated with DAPI, and *Merged*. Scale bar: 20 μm.

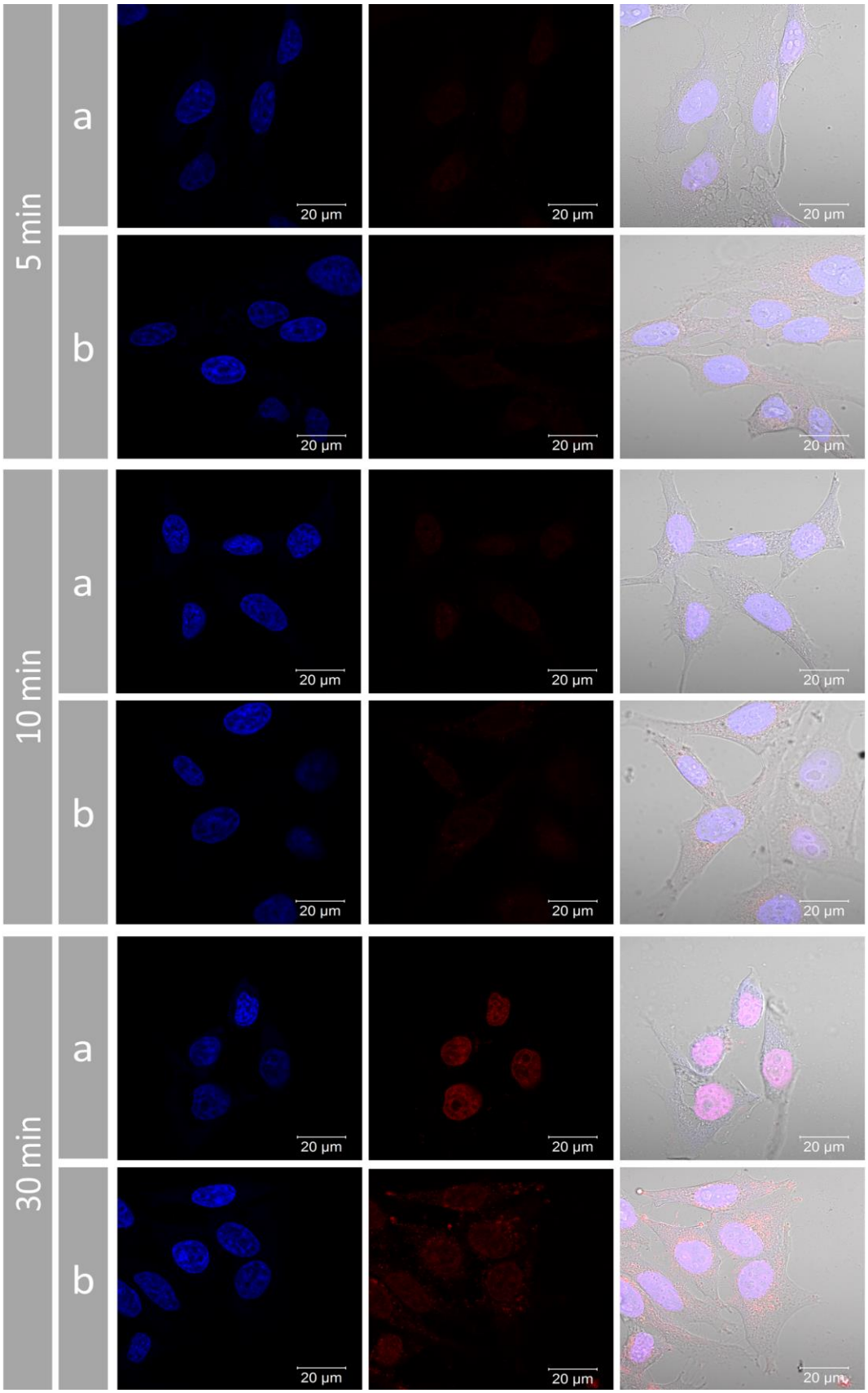


Fig. 5. (A) Flow cytometry histogram profiles of HeLa cells incubated with free DOX and DOX-zein-NPs for 1 h and 3 h, respectively. (B) TEM image of HeLa cells incubated with DOX-zein-NPs for 30 min.

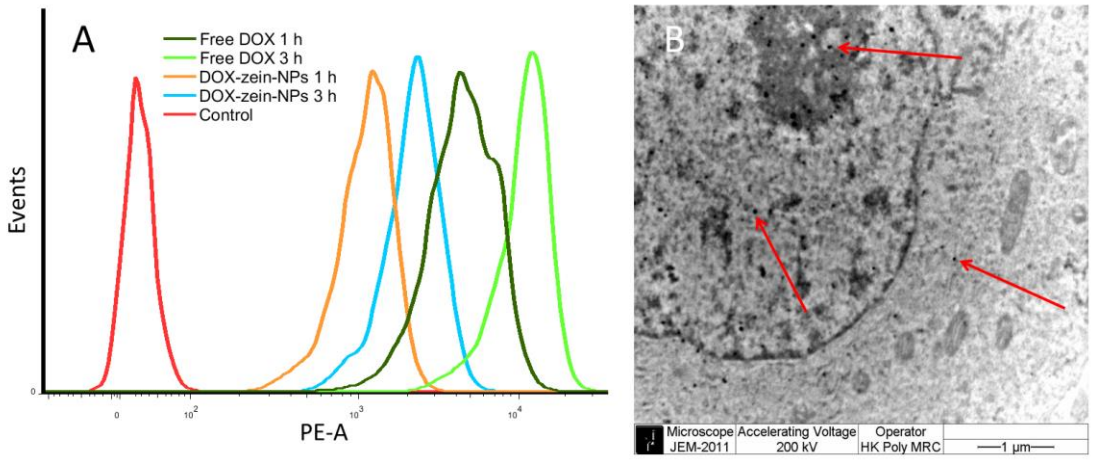
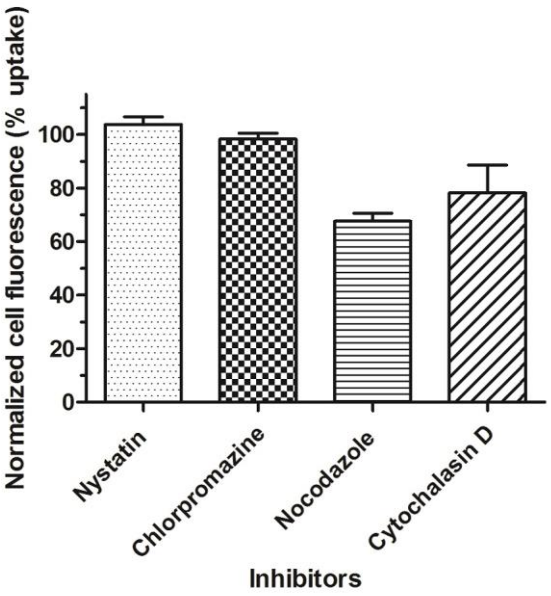


Fig. 6. Effects of pharmacological inhibitors on the uptake of DOX-zein-NPs into the HeLa cells. Mean values and standard deviations are given.



Tables

Table 1 The different formulations for preparation of DOX-zein-NPs.

Sample	Zein concentration (mg/ml)	zein to DOX mass ratio ($R_{Z/D}$)	CAS concentration (mg/ml)
1	10	20	10
2	10	30	10
3	10	50	10
4	20	30	20
5	30	30	30
6	30	60	30
7	30	20	30

Table 2 Characterization of different DOX-zein-NPs.

sample	ZC (mg/ml)	$R_{Z/D}$	Size (nm)	PDI	ZP (mV)	EE (%)	LE (mg/g)
1	10	20	204.53±1.53	0.17±0.02	-45.20±0.21	90.06±0.26	15.01±0.14
2	10	30	202.83±0.90	0.19±0.01	-44.33±0.74	84.42±1.36	9.38±0.15
3	10	50	198.73±1.67	0.16±0.01	-41.93±0.33	74.24±1.59	4.82±0.11
4	20	30	215.77±3.35	0.23±0.01	-52.97±8.58	86.26±2.71	9.58±0.30
5	30	30	244.03±28.60	0.19±0.04	-50.25±4.55	83.15±2.00	9.24±0.22
6	30	60	202.27±2.91	0.18±0.02	-41.95±0.45	65.92±2.35	3.66±0.13
7	30	120	247.00±21.31	0.16±0.05	-45.92±2.59	43.46±1.20	1.21±0.03