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Delivery of doxorubicin and paclitaxel from double-layered microparticles: The effects of layer thickness and dual-drug vs. single-drug loading

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ABSTRACT

Double-layered microparticles composed of poly(D,L-lactic-co-glycolic acid, 50:50) (PLGA) and poly(L-lactic acid) (PLLA) were loaded with doxorubicin HCl (DOX) and paclitaxel (PCTX) through a solvent evaporation technique. DOX was localized in the PLGA shell, while PCTX was localized in the PLLA core. The aim of this study was to investigate how altering layer thickness of dual-drug, double-layered microparticles can influence drug release kinetics and their antitumor capabilities, and against single-drug microparticles. PCTX-loaded double-layered microparticles with denser shells retarded the initial release of PCTX, as compared with dual-drug-loaded microparticles. The DOX release from both DOX-loaded and dual-drug-loaded microparticles were observed to be similar with an initial burst. Through specific tailoring of layer thicknesses, a suppressed initial burst of DOX and a sustained co-delivery of two drugs can be achieved over 2 months. Viability studies using spheroids of MCF-7 cells showed that controlled co-delivery of PCTX and DOX from dual-drug-loaded double-layered microparticles were better in reducing spheroid growth rate. This study provides mechanistic insights into how by tuning the layer thickness of double-layered microparticles the release kinetics of two drugs can be controlled, and how co-delivery can potentially achieve better anticancer effects.

Statement of Significance

While the release of multiple drugs has been reported to achieve successful apoptosis and minimize drug resistance, most conventional particulate systems can only deliver a single drug at a time. Recently, although a number of formulations (e.g. micellar nanoparticles, liposomes) have been successful in delivering two or more anticancer agents, sustained co-delivery of these agents remains inadequate due to the complex agent loading processes and rapid release of hydrophilic agents. Therefore, the present work reports the multilayered particulate system that simultaneously hosts different drugs, while being able to tune their individual release over months. We believe that our findings would be of interest to the readers of Acta Biomaterialia because the proposed system could open a new avenue on how two drugs can be released, through rate-controlling carriers, for combination chemotherapy.

1. Introduction

Chemotherapy is an integral aspect of cancer treatment, both in the early as well as in the advanced stages. First-line chemotherapy necessitates the use of different drugs, either concurrently or

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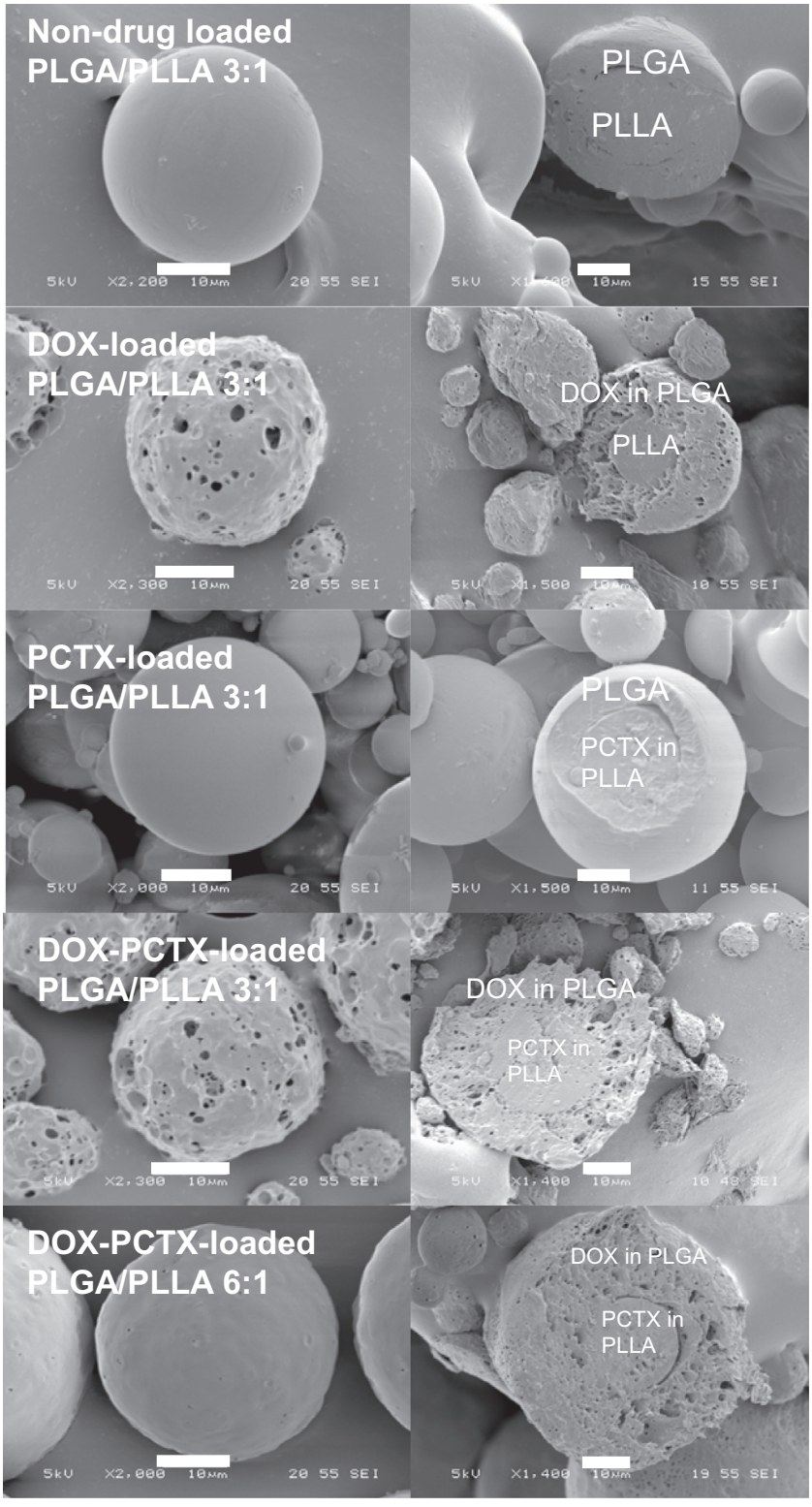

Fig. 1. Internal and external structure of double-layered PLGA/PLLA microparticles. (Row 1: non-drug loaded PLGA/PLLA 3:1, Row 2: DOX-loaded PLGA/PLLA 3:1, Row 3: PCTX-loaded PLGA/PLLA 3:1, Row 4: DOX–PCTX-loaded PLGA/PLLA 3:1, Row 5: DOX–PCTX-loaded PLGA/PLLA 6:1). Scale bar = 10 μm.

sequentially, with regimens lasting for as long as 3–6 months. However, such a treatment regimen would often demand high parenteral dosage and this is frequently associated with systemic toxicity [1,2].

Doxorubicin hydrochloride (DOX) and paclitaxel (PCTX) are two of the most widely used drugs for cancer chemotherapy [3,4]. At present, few drug delivery systems for cancer therapy using these drugs are commercially available. Those commercially available include Doxil®, Caelyx® and Myocet® – DOX-loaded liposomes. However, issues of drug leakage and aggregation, which can affect therapeutic efficacy, and difficulties with sterilization have not been resolved. The phospholipids are thermo-labile and thus

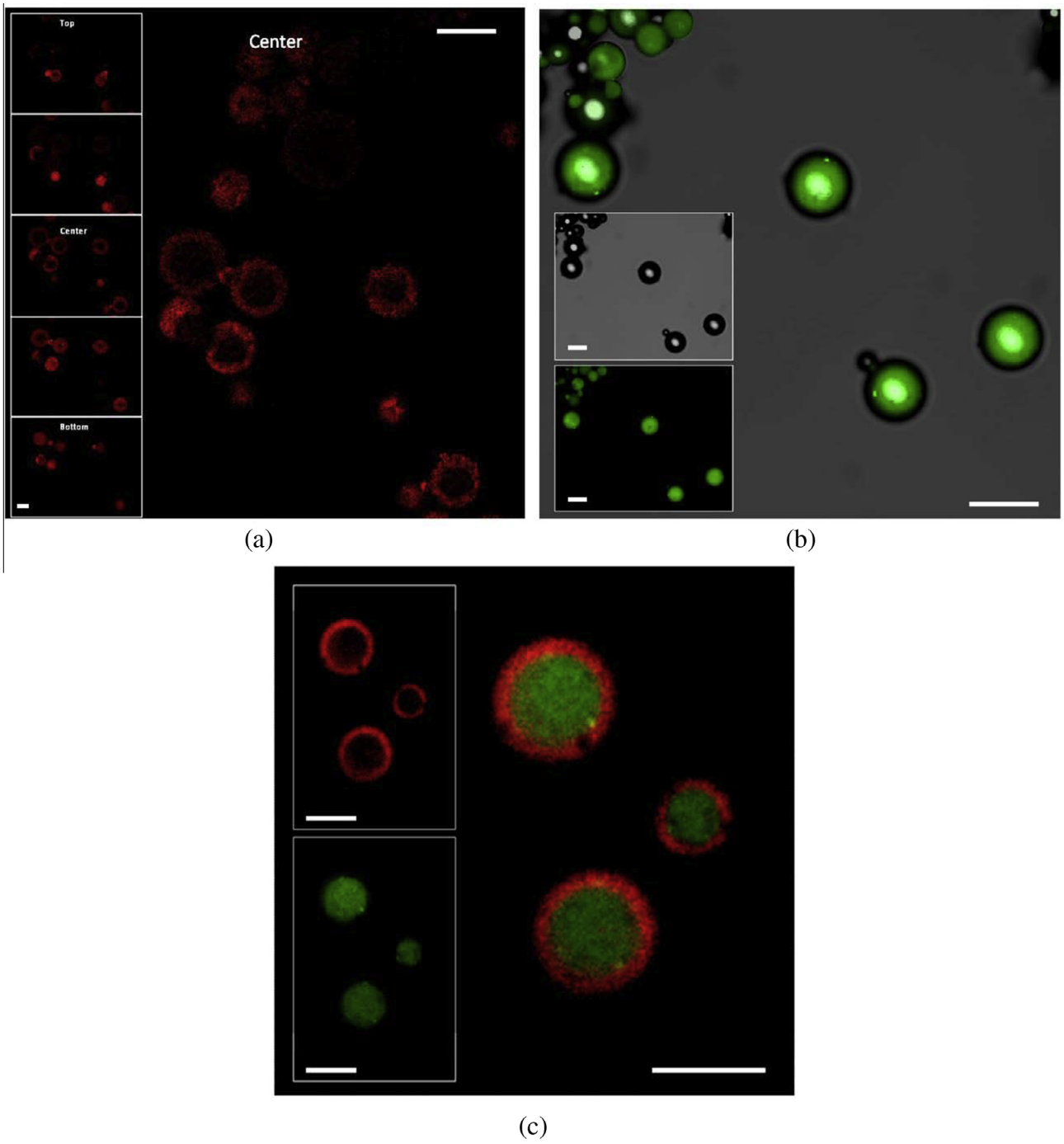

Fig. 2. CLSM images of double-layered PLGA/PLLA microparticles. (a) A composite z-stack comprising five confocal sections was obtained for DOX (red) with a z-interval of 5.5 μm between images measured below and above the center plane of the DOX-loaded microparticles. (b) PCTX (green)-loaded microparticles. The insets are the differential interference contrast image and the emission of dansyl chloride-tagged PCTX (green), respectively. (c) DOX-PCTX-loaded microparticles. The insets are the CLSM images of DOX (red) and dansyl chloride-tagged PCTX (green). Scale bar = 30 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

preclude heat sterilization, while on the other hand, gamma-irradiation and other chemical sterilizing agents can produce residual toxic contaminants [5]. Furthermore, constituents such as Cremophor EL and dehydrated ethanol, which are formulated with Taxol® to enhance the solubility of PCTX, lack controlled release capabilities and are associated with poor drug tolerability [6]. In addition, since most current delivery systems deliver only a single anticancer drug, they would have to be given in combination with other chemotherapeutics to achieve better anticancer effects [3,7]. Therefore, a single carrier that simultaneously entraps and releases more than one drug in a controlled and sustained fashion would clearly be advantageous for cancer therapy.

While the release of different drugs with specific release kinetics has been reported to achieve successful apoptosis and minimize drug resistance [4,7–11], most conventional particulate systems can only release one drug at a time. Recently, although some anticancer formulations (e.g. micellar nanoparticles, liposomes) have been successful in releasing more than one therapeutic agent, such as small molecule drugs, siRNA, plasmid DNA or peptide [12–16], sustained co-delivery of these agents remains inadequate due to

Table 1
Drug loading efficiencies (LE) (%) in the microparticles.

Samples	DOX	PCTX
DOX-loaded PLGA/PLLA 3:1	16.4 ± 3.3	–
PCTX-loaded PLGA/PLLA 3:1	–	90.5 ± 5.6
DOX–PCTX-loaded PLGA/PLLA 3:1	21.1 ± 1.5	87.9 ± 6.9
DOX–PCTX-loaded PLGA/PLLA 6:1	45.3 ± 3.3	90.1 ± 6.3
DOX-loaded PLGA	53.4 ± 3.9	–
PCTX-loaded PLLA	–	85.4 ± 8.3

the complex agent loading processes and rapid release of hydrophilic agents that are adsorbed onto the surface. The encapsulation and release of drugs with varying water solubilities generally require multiple carriers or solvents. Therefore, to formulate multiple drug-loaded particles, while controllably releasing each drug from this delivery system, is a challenge.

Biodegradable particulate systems are designed to mitigate drug degradation, control drug release and improve treatment outcomes. For example, mitoxantrone-loaded mono-layer microspheres showed that continuous release of mitoxantrone resulted in greater tumor growth suppression [17]. However, such monolithic particulate systems are limited by burst release, the difficulty in achieving controlled release, and the inability to encapsulate more than one drug due to possible drug-drug interactions. By virtue of the compartmentalized internal structure, multilayered polymeric microparticles, on the other hand, have multidrug loading capacity (through selective localization of drugs in specific layers), and can be structurally tailored to control drug release kinetics. For example, Matsumoto et al. [18] showed that multi-reservoir-type microspheres can not only retard burst release of cisplatin but also provide a sustained release. Shi et al. [19] showed that double-walled microspheres achieved a sustained and complete release of hydrophilic bovine serum albumin and hydrophobic cyclosporin A. Multilayered particles therefore have the potential to alter drug release kinetics by manipulating drug-layer localization and structural attributes, such as layer thickness [20–22].

In this paper, the aim was to investigate how DOX–PCTX-loaded, double-layered microparticles, and of differing layer thickness, can further inhibit tumor growth against a single drug. Here, the DOX-loaded shell was composed of poly(DL-lactic-co-glycolic acid, 50:50) (PLGA), and the poly(L-lactic acid) (PLLA)-core contained PCTX. It is hypothesized that two different drugs can be timed-released from double-layered microparticles, providing a controlled and sustained drug release, along with enhanced antitumor efficacy. In order to ensure that DOX would not exhibit a prolonged lag in its release caused by a hydrophobic PLLA shell [23,24], PLGA was chosen as the DOX-containing shell. Unlike more complex electrohydrodynamic atomization and electrospraying technique involving the use of syringe pumps, voltage generators and a series of concentric nozzles [24,25], the emulsion solvent evaporation method used in this study is a convenient one-step fabrication technique [26]. Polymer mass ratios can be manipulated to alter layer thickness, which in turn modulates drug release profiles. Subsequently, efficacy of these particulate systems against spheroids, composed of MCF-7 cells, was investigated.

2. Materials and methods

2.1. Materials

Poly(L-lactic acid) (PLLA, molecular weight (MW): 360 kDa, Bio Invigor), poly(DL-lactic-co-glycolic acid, 50:50) (PLGA, MW: 45 kDa, Bio Invigor) and poly(vinyl alcohol) (PVA, molecular weight (MW): 30–70 kDa, Sigma–Aldrich) were used, as obtained from the suppliers. Paclitaxel (PCTX) was bought from International Laboratory (USA), and doxorubicin hydrochloride salt (DOX) was bought from Xingcheng Chempharm Co., Ltd. (China). Solvents used were tetrahydrofuran (THF), dichloromethane (DCM), acetonitrile (ACN), and chloroform. These were obtained from Tedia Company Inc. and were of High-Performance Liquid Chromatography (HPLC) grade. For release studies, phosphate buffered saline (PBS) solution and Tween 80 were supplied by OHME Scientific and Sigma–Aldrich (both Singapore), respectively. More information on the consumables used for spheroid study can be obtained from Supplementary Information (SI).

2.2. Microparticle production

The microparticles comprising PLGA/PLLA were produced, and loaded with drugs, through a previously established method [27,28]. Firstly, PCTX was solubilized in PLLA (0.1 g)/DCM (7.5% w/v) solution, and DOX was added to PLGA (0.3 g)/DCM (7.5% w/v) solution, sonicated using an ultrasonic probe (Sonic Vibra-cell VC 130) to break down the drug crystals. The theoretical loading of PCTX and DOX were set at 1% w/w and 20% w/w, respectively. Both solutions were then mixed together using ultrasound for 1 min. This polymer solution was then added into 350 mL of PVA solution (5.0% w/v) to achieve an oil-in-water emulsion. An overhead stirrer (Calframo BDC1850-220) was used to stir this emulsion at 2000 rpm for 4 h (25 °C). Here, DCM evaporates to phase separate PLGA and PLLA, producing double-layered microparticles [27]. The product was then washed with deionized water, before freeze-drying and storing in a desiccator. Other double-layered and single-layered particles were prepared using similar method but the type of drugs loaded and the mass ratio of polymers used were changed accordingly to obtain the desired particle sample parameters.

2.3. Characterization

2.3.1. Particle size and morphology

The scanning electron microscope (SEM, JEOL JSM-6360A) was used to ascertain particle structure, morphology and size. SEM was operated at 5 kV. The particulate samples were cross-sectioned by putting into liquid nitrogen before sectioning using a razor blade, and coating with gold. Sizes of the particles were analyzed with ImageJ software. The diameter of at least 30 particles on 2 random fields in independent SEM micrographs was measured, and was expressed as mean ± standard deviation.

2.3.2. Drug distribution

The confocal laser scanning microscope (CLSM, LSM710) was used to determine drug distribution within the particles. Here, PCTX was tagged with dansyl chloride (green: excitation peak at 405 nm, emission wavelength of 500 nm), while DOX is fluorescent (red: excitation peak at 488 nm, emission wavelength of 580 nm). The aqueous suspension containing particles were drop-wise added to a glass slide before sealing with a glass cover slip. All images were obtained using 63×/1.40 oil objective lens, at a magnification of ×1, and the AxioCan MRm camera. Drug distribution was determined at the center of the microparticles. To ensure consistency, the same setting was used for all samples. Analysis of the images was done with the ZEN 2011 software.

2.3.3. Drug loading efficiency

Drug loading efficiency (LE) was determined by taking measured drug amount loaded divided with theoretical amount. For DOX, microparticles (3 mg) were first dissolved in DCM (1 mL). After which, deionized water (10 mL) was added, whereby doxorubicin preferentially partitioned into. This was vortexed, allowed to phase separate, before collecting the aqueous layer. This was done

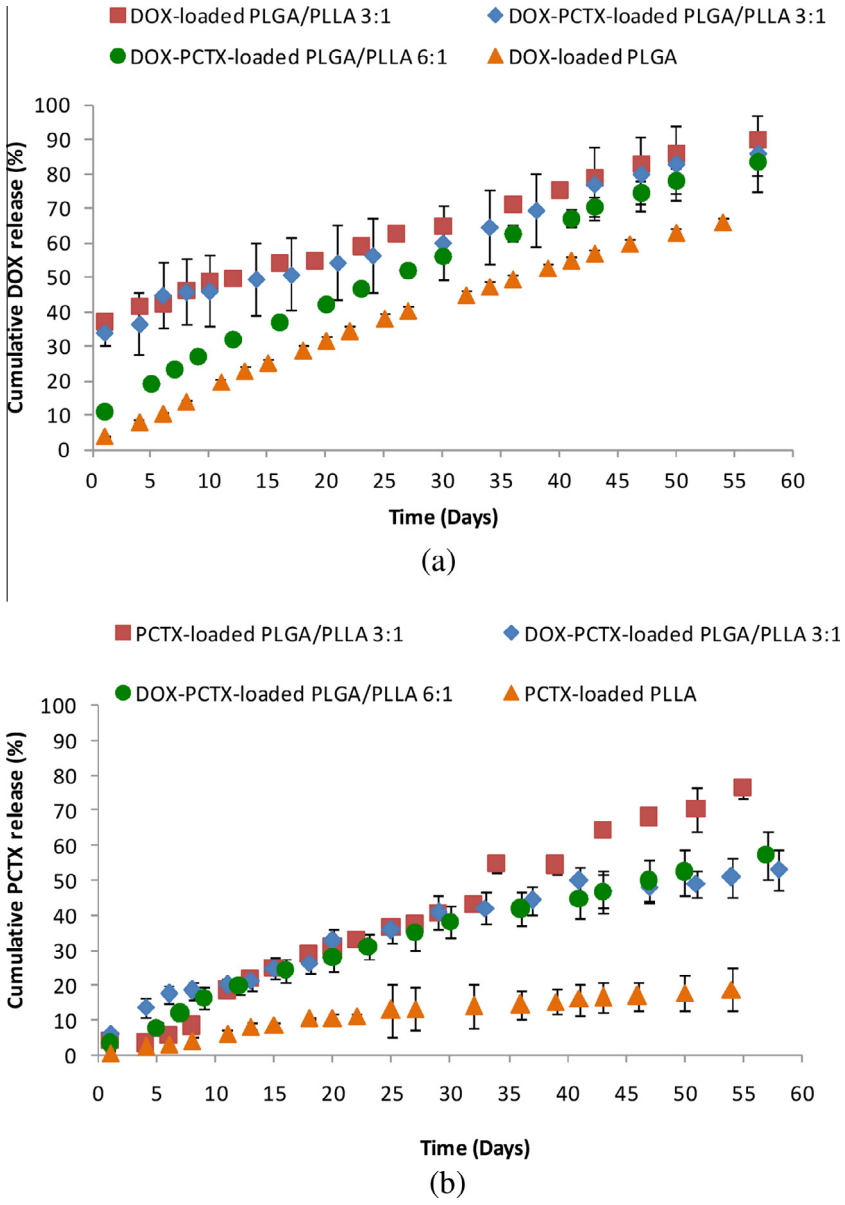

Fig. 3. Cumulative release of (a) DOX and (b) PCTX from single-layered and double-layered PLGA/PLLA microparticles.

thrice to maximize extraction of DOX (~90%). The concentration of DOX in water phase was compared against a calibration curve obtained with an ultraviolet–visible (UV–vis) spectrophotometer (Shimadzu UV-2501) at 480 nm. As for PCTX, similar method was conducted except that ethanol (10 mL) was added, instead of deionized water, to precipitate the polymers [29]. Centrifugation was then conducted, before drying of the supernatant. Dried PCTX was then dissolved in ACN for analysis with UV–Vis at 227 nm. The calculated loading efficiency was corrected accordingly to overcome extraction inefficiency. For this, the same above steps were conducted for blank PLGA/PLLA particles, and for the mixture of pure free drug (i.e. PCTX) with blank PLGA/PLLA particles, and extraction efficiency was measured to be ~90%. All measurements were done in triplicate.

2.4. Drug release study

Microparticles (5 mg) were introduced to 5 mL PBS (pH 7.4) with Tween 80 (0.05%) in vials, and agitated using a shaking incubator at 37 °C (50 rpm) ($n = 3$). For each sample, 4 mL of medium was withdrawn, at specific time points, before replenishing with new medium (4 mL) to achieve sink condition. DOX concentration was analyzed with UV–vis spectrophotometer at 480 nm. For PCTX, it first underwent extraction with 4 mL of DCM. Extracted PCTX after DCM evaporation was then introduced to a known amount of ACN. PCTX concentration was measured with UV–vis spectrophotometer at 227 nm, after subtracting for the background [30–33]. This was done with blank particles that were incubated in the medium, and using the same method above. Extraction efficiency was determined by using pure PCTX, of a known mass, and with the same extraction procedure, which was found to be ~70%. The amount of PCTX released was therefore corrected with this percentage figure.

2.5. Degradation of microparticles

Microparticles (50 mg) were introduced to vials that contain 50 mL of PBS/Tween 80 (0.05%) ($n = 3$). These were incubated in a shaking incubator at 37 °C. The particles were taken out at specific time points, and washed with deionized water before

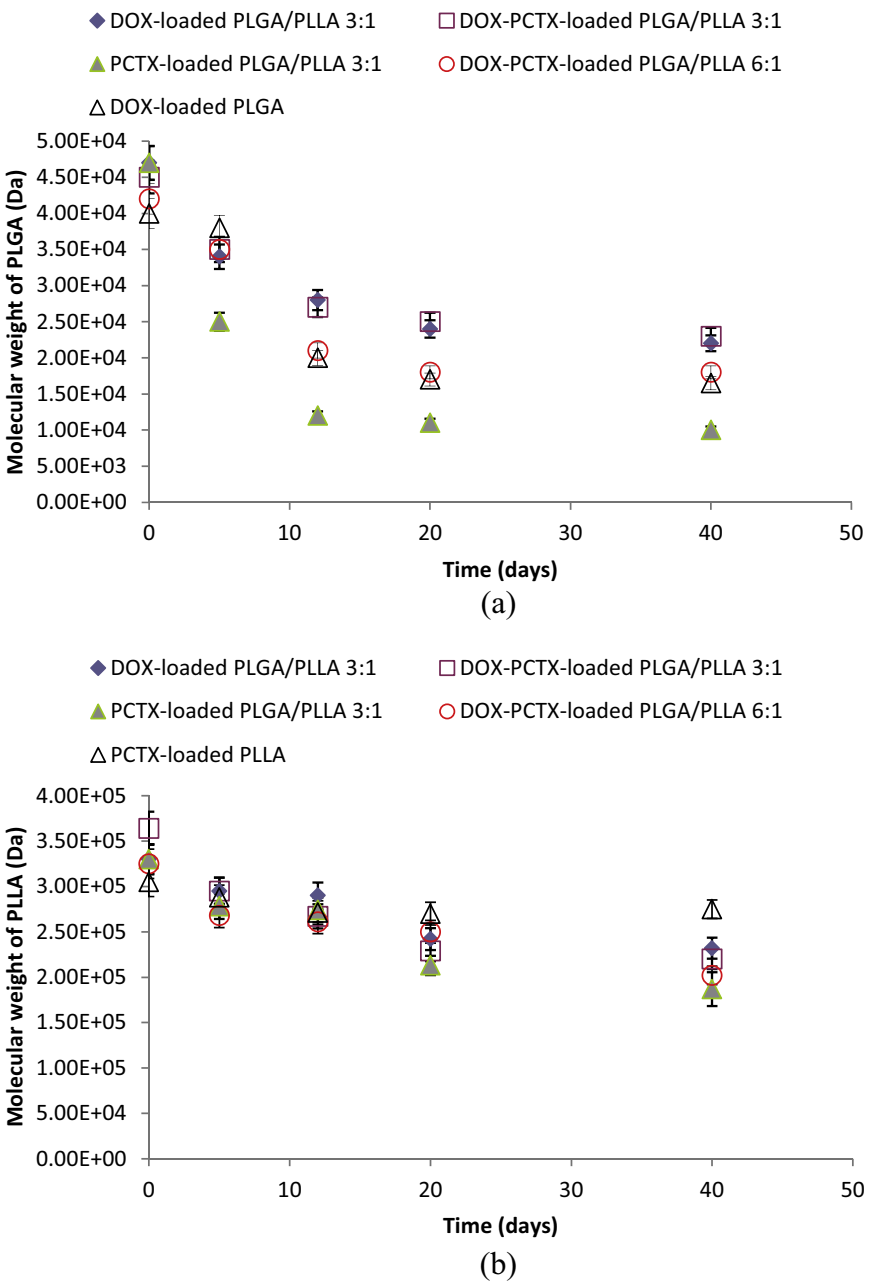

Fig. 4. Average molecular weights of (a) PLGA and (b) PLLA from single-layered and double-layered PLGA/PLLA microparticles against drug release time.

freeze-drying. Samples were imaged using SEM, CLSM and collected for size-exclusion chromatography (SEC) analysis. SEC analysis (Agilent 1100 Series LC System) was used to ascertain the average molecular weight of polymer (relative to polystyrene standards). Solvent used was chloroform, and analysis was performed at 30 °C, flow rate of 1 mL min^{-1} and using a reflective index detector (RID). For double-layered microparticles, the polymers were separated using the THF dissolution method [28,34], as only PLGA is solubilized in THF. PLLA and PLGA were then separated by centrifugation. PLGA and PLLA, after oven drying, were then dissolved individually in chloroform (1 mL), followed by SEC analysis.

2.6. Effects on 3D spheroids

The materials and methods to generate the MCF-7 spheroids are described in the SI. Magnetic spheroids (diameter size of ~400 μm) were introduced to either free drug (DOX or PCTX), or single-drug microparticles, or DOX–PCTX-loaded microparticles (at 0.4 μg mL^{-1} DOX and 0.08 μg mL^{-1} PCTX). For the free drug group, the amount of free drugs introduced to the spheroids was equivalent to the total cumulative drug released from the particles after 28 days at 37 °C (from drug release study). Microparticles were separated from the spheroids, to facilitate better imaging, by using a Transwell-96 Permeable Support with 3.0 μm pore polycarbonate membrane (Sigma CLS3385) [28]. The scheme of this setup is shown in SI. For each test condition, the spheroids ($n = 5$) were incubated for 28 days. The free drugs and the particles were added just once to the media at day 0. For the free drug group, drug-containing media were completely removed after 6 h before replenishing with new medium. This simulates acute free drug exposure and subsequent clearance during systemic administration, i.e. *in vivo* conditions. For the drug-loaded particles, they were maintained in the culture media, as this models the controlled release of drugs from the particles after they have been

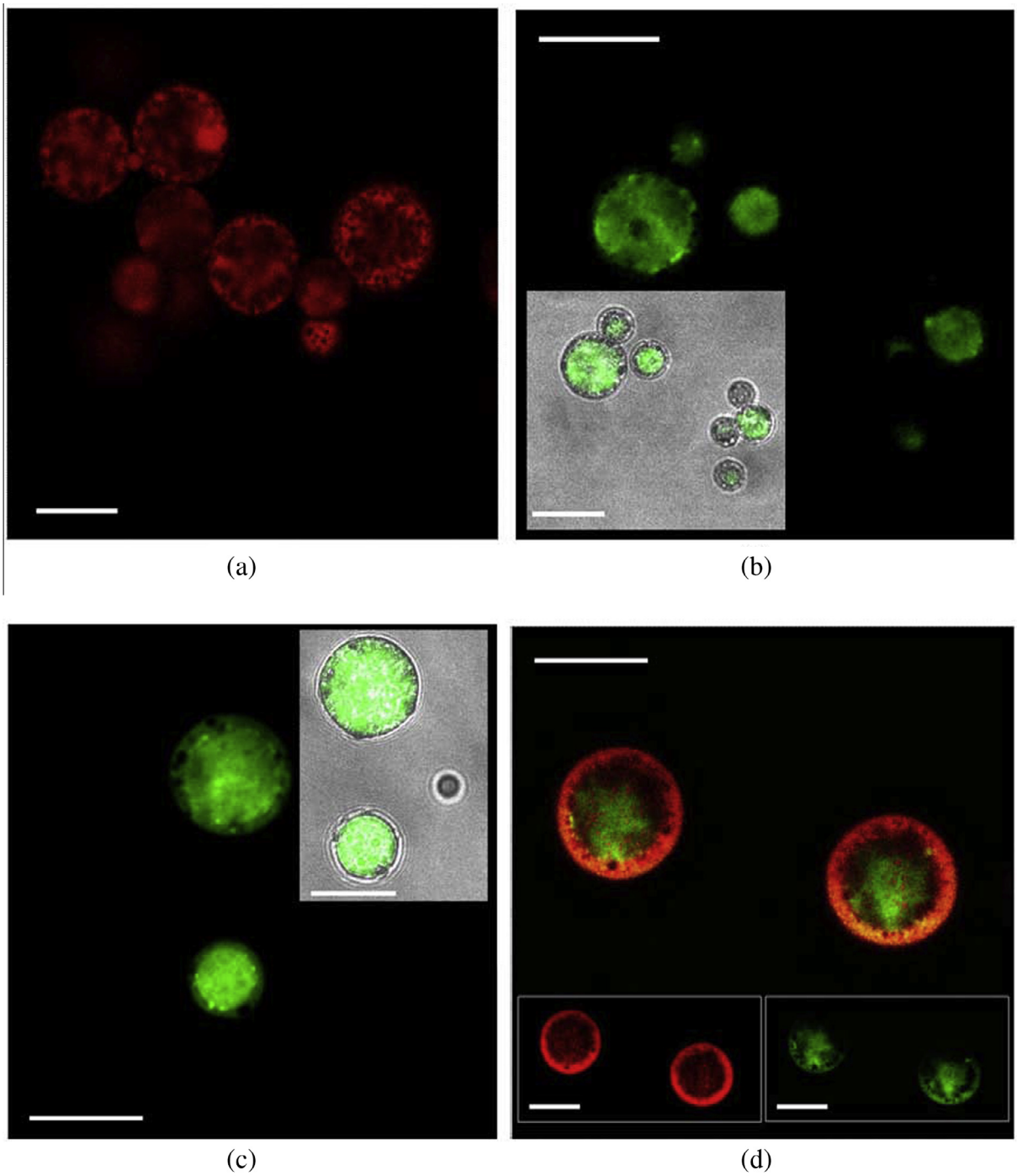

Fig. 5. CLSM images showing the distribution of drugs in double-layered PLGA/PLLA 3:1 microparticles during drug release. (a) DOX-loaded microparticles after 30 days of release. PCTX-loaded microparticles after (b) 5 days and (c) 12 days of release. The insets are overlays of differential interference contrast image and the emission of dansyl chloride-tagged PCTX (green). (d) DOX–PCTX-loaded microparticles after 20 days of release. The insets are the images of red DOX and green dansyl chloride-tagged PCTX, respectively. Scale bar = 30 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

administered *in vivo*. Over the entire experiment, cell culture media were refreshed with dilution every 2 days, so as to mimic systemic clearance of drugs released from the particles. Spheroid size was measured from the orthogonal diameters of each spheroid under bright field microscopy for volume calculation. Acid phosphatase assay was performed, at the end, to assess spheroid viability, as described in SI.

2.7. Statistical analysis

Comparisons of data obtained from different particle samples were done using unpaired Student's *t*-test and one-way ANOVA analysis coupled with Tukey's multiple comparison tests. Statistically differences were verified when $p \leqslant 0.05$.

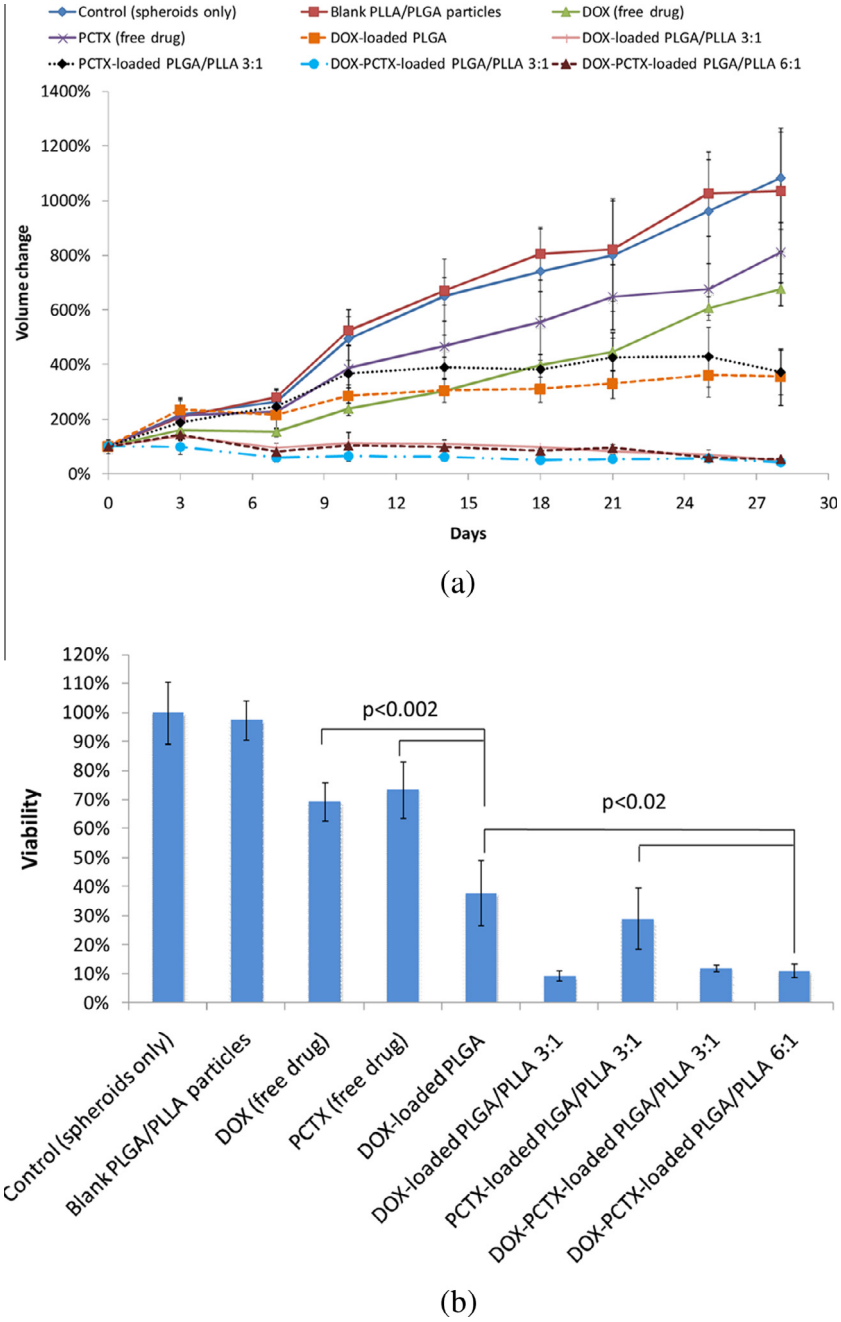

Fig. 6. (a) Growth of spheroids exposed to free drug (DOX or PCTX) or blank particles or single-drug microparticles or DOX–PCTX-loaded microparticles over 28 days. (b) Viability of spheroids as measured using acid phosphatase assay at the end of study (day 28).

3. Results and discussion

3.1. Drug-loaded double-layered microparticles

The SEM images of the drug-loaded microparticles produced are shown in Fig. 1 (Figs. S1 and S2 show numerous particles). Spherical microparticles were obtained, with a double-layered configuration. The particle sizes (i.e. diameter) of DOX–PCTX-loaded double-layered PLGA/PLLA 3:1 microparticles measured by SEM were found to be 34.7 ± 9.1 μm; all other samples had similar particles sizes. These particle size ranges are suitable as drug delivery depots [17,23,35], as they can be localized at the injection site while offering better syringeability [20]. For DOX–PCTX-loaded double-layered PLGA/PLLA 3:1 microparticles, the shell thickness and core diameter were 8.1 ± 1.3 μm and 18.3 ± 2.8 μm, respectively. Changing the polymer mass ratio was to PLGA/PLLA 6:1, there was an increase in the PLGA shell thickness to 11.9 ± 1.7 μm due to a higher PLGA content, whereas the core diameter of PLLA reduced to 13.3 ± 2.1 μm.

CLSM aided in determining drug localization within microparticles. CLSM images obtained for DOX-loaded only, PCTX-loaded only and DOX–PCTX-loaded double-layered microparticles are shown in Fig. 2. For DOX-loaded microparticles (Fig. 2a), a composite z-stack comprising five confocal sections was captured. At the center plane, red rings (DOX) were observed (z stack, center image). The red rings became smaller and eventually disappeared to form a solid circle when extended to the surface of the particle (z-stack, bottom image). As such, the composite z-stack images

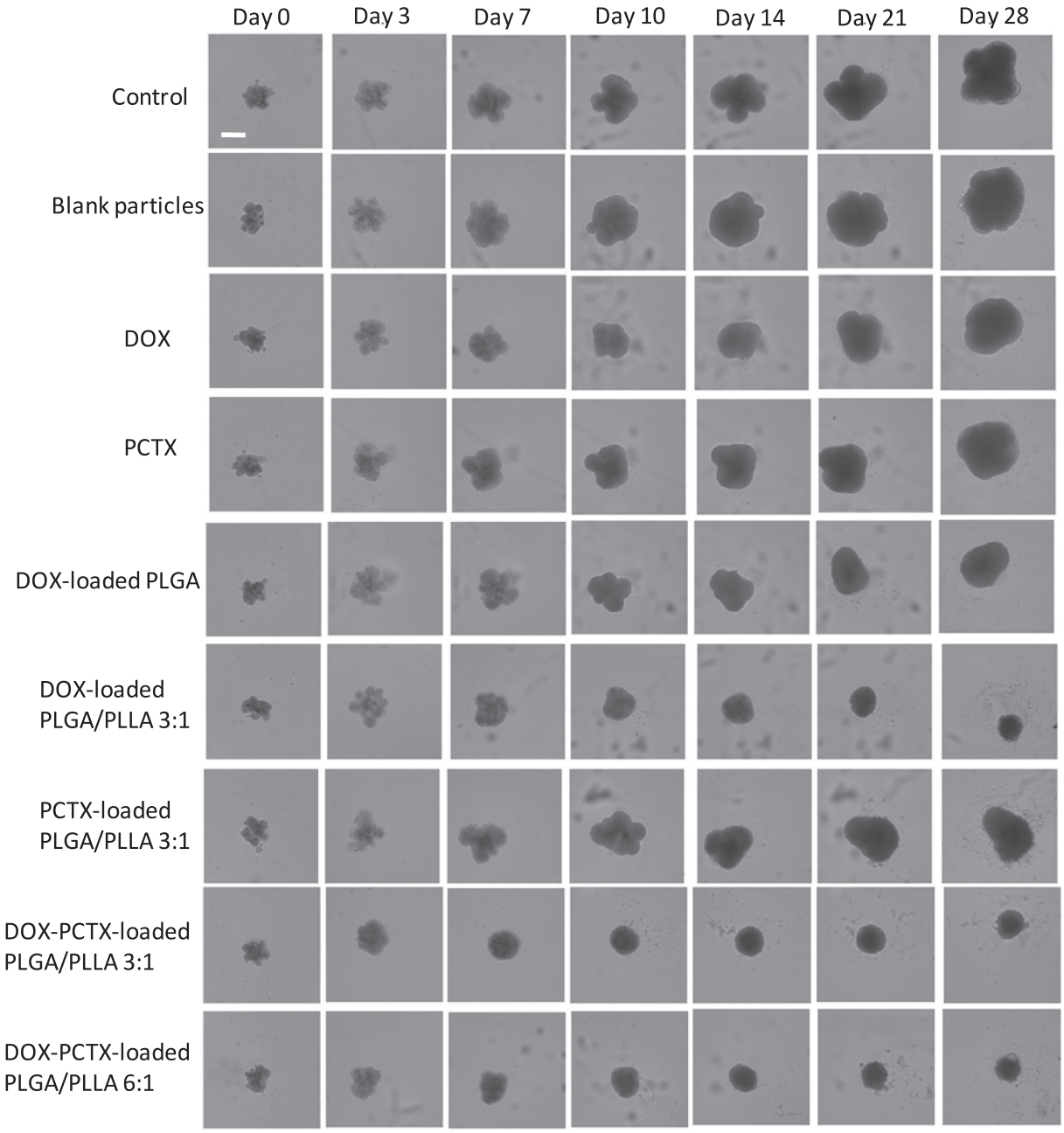

Fig. 7. Bright-field images of MCF-7 spheroids exposed to free drug (DOX or PCTX), blank PLGA/PLLA particles, single-drug microparticles, or DOX–PCTX-loaded microparticles. Scale bar = 400 μm.

depicted the localization of DOX in the PLGA shell. For PCTX-loaded microparticles (Fig. 2b), when the optical image of fluorophore-tagged PCTX (green) was overlaid with the differential interference contrast (DIC) image, the fluorescence from PCTX did not exceed the periphery of the PLLA core, suggesting PCTX localized within the PLLA core. For DOX–PCTX-loaded microparticles, the specific localization of two drugs in the layers can be seen from Fig. 2c, as revealed from the color distribution of PCTX and DOX. Such drug localization could be attributed to affinity between polymer and drug [23,26,34]. Highly hydrophobic PCTX was preferentially localized in hydrophobic PLLA, while hydrophilic DOX was localized within the relatively more hydrophilic PLGA.

From the SEM images (Fig. 1), the existence of drugs in the particles was observed to affect the morphology of these drug-loaded microparticles. Non-drug-loaded microparticles, as a reference, exhibited smooth, non-porous interior and exterior morphologies. PCTX-loaded microparticles yielded the same morphology as the non-drug-loaded microparticles. Microparticles (PLGA/PLLA 3:1) containing DOX, on the other hand, were observed to have porous PLGA shell layers (both exterior and interior). This is likely due to osmotic pressure whereby the dissolution of DOX salt drives water from the surrounding into the emulsion droplets, thus causing the leaching of DOX into the water phase. A much lower loading efficiency (LE) of DOX was therefore achieved for PLGA/PLLA 3:1 microparticles than PLGA/PLLA 6:1 microparticles (Table 1). It is believed that a higher content of PLGA (PLGA/PLLA 6:1) reduces the leaching of DOX out of the emulsion droplets due to longer mass transport pathway, thus forming a denser surface. This could

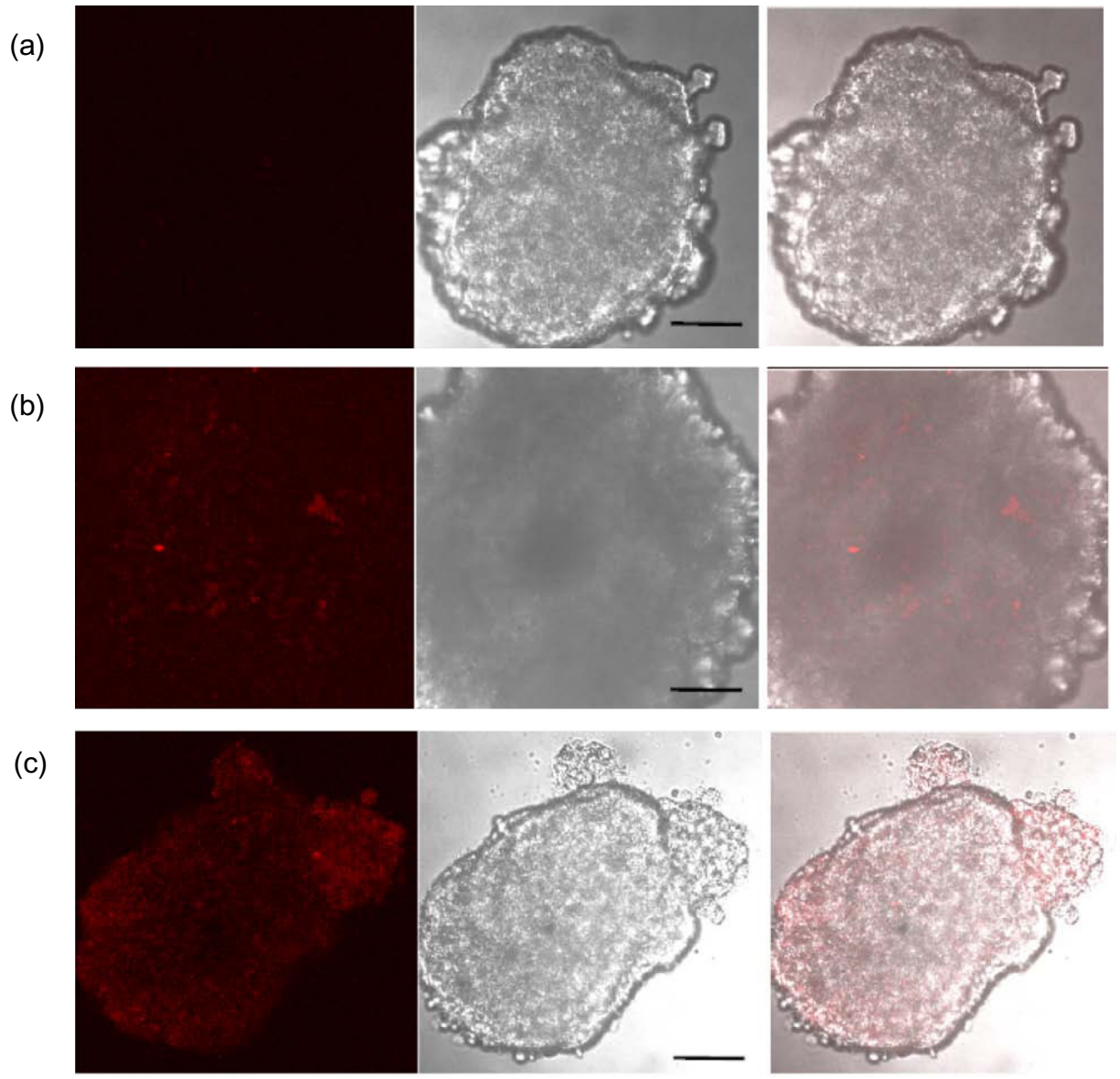

Fig. 8. CLSM images showing the penetration of DOX into the spheroids during drug release from double-layered DOX-loaded PLGA/PLLA 3:1 microparticles. (a) Represents the control group (spheroid not treated with particles). (b) and (c) represent spheroids treated with particles after 3 days and 10 days, respectively. Left: fluorescence images of red DOX; Center: differential interference contrast images; Right: overlays of differential interference contrast image and the emission of DOX (red). Scale bar = 100 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

be substantiated by the observation that DOX-loaded neat PLGA microparticles yielded non-porous surface (Fig. S3) and higher LE, in comparison to PLGA/PLLA 3:1 particles.

3.2. Drug release study

3.2.1. Single- vs. dual-drug-loading

Here, the effect of how one drug can influence the release of another, from the same microparticle, was investigated. The release of DOX from double-layered microparticles (DOX only) and microparticles loaded with dual drugs (i.e. DOX and PCTX) are plotted in Fig. 3a. DOX was initially localized within the PLGA shell. For PLGA/PLLA 3:1 microparticles, it was evident that the release profile of DOX was not influenced by the core-localized PCTX of dual-drug-loaded microparticles. Similar DOX release profiles were achieved with microparticles loaded with DOX only and DOX–PCTX-loaded microparticles (PLGA/PLLA 3:1) because of comparable polymer degradation rates, as represented in Fig. 4a and b. From Fig. 3a, it was also noted that both samples (PLGA/PLLA 3:1) displayed a burst release (~35%) of DOX on the first day, followed by a sustained release over 2 months. The burst release could be due to the highly porous particle surface [36]. Some DOX was also observed to have diffused into the PLLA core, as evidenced from the CLSM image of the double-layered microparticles (DOX only) after 30 days of incubation (Fig. 5a), which would further prolong the DOX release. The drug located in the inner region of the particles takes a longer time to diffuse before being released into the medium.

The PCTX release profiles of PCTX-loaded microparticles and DOX–PCTX-loaded microparticles are reflected in Fig. 3b. The graph shows that the slow degrading PLLA microparticles released PCTX at the slowest rate. In contrast, for double-layered microparticles, the relatively hydrophilic PLGA shells underwent more rapid degradation, accelerating the degradation of PLLA cores (Fig. 4b)

[37], and thus a faster release of PCTX over 2 months. During the early release period (up to day 10), a slower release of PCTX was observed for PCTX-loaded double-layered microparticles, as compared with DOX–PCTX-loaded double-layered PLGA/PLLA 3:1 microparticles ($p < 0.05$). As shown in Fig. 5b and a larger fraction of PCTX was still dispersed within the PLLA cores in PCTX-loaded microparticles after 5 days of incubation, affirming that the dense particle morphology (Fig. S4a) served as a rate-limiting barrier against PCTX release. After 12 days, an increase in porosity and degradation of PLGA (Figs. 4a and S4b) accelerated the release of PCTX. This is confirmed from a significant diffusion of green fluorescence to the outer circumference of the particles (Fig. 5c). Drastic PLGA degradation in the PCTX-only microparticles, as compared with the DOX-containing particles (Fig. 4a), could be due to acidic oligomers, as hydrolysis products, entrapped in the originally dense shell, leading to an autocatalytic degradation of PLGA. For the multiple drug release from DOX–PCTX-loaded microparticles (Fig. 5d), inward diffusion of some DOX from shell regions occurred over the course of the release and thus sustaining its release, while some PCTX diffused into the shell before being released into the medium.

3.2.2. Double-layered microparticles: effect of layer thickness

The effect of layer thickness on drug release from dual-drug-loaded microparticles was also investigated. For microparticles with thicker shell (PLGA/PLLA 6:1), the DOX release profile was more sustained, and with minimal burst release (Fig. 3a). This difference can be attributed to surface porosities and layer dimensions of PLGA/PLLA 3:1 and 6:1 microparticles. As a reference (i.e. neat PLGA microparticles), DOX release was also sustained, which could be due to the dense particle surface and uniform dispersion of DOX within monolithic matrix. PLGA/PLLA 6:1 microparticles of thicker shells showed significantly less porous surfaces relative to the thinner shell 3:1 microparticles before degradation (Fig. 1) and their relatively less porous surface was maintained even after 20 days of release period (Fig. S5). For PLGA/PLLA 6:1 microparticles, a denser surface together with thicker shell aided in minimizing any initial burst of DOX, while sustaining its release over two months. The existence of PLGA shell was observed after 40 days of incubation, as the degrading PLGA was still detectable on the SEC (Fig. 4a). PLGA is known to exhibit an exponential decrease in the molecular weight with degradation time (pseudo-first-order degradation kinetics) [38]. The degradation involves random chain scission, where the molecular weights of polymers decrease significantly with no appreciable mass loss in the initial stage. The degrading PLGA shell served as a diffusion barrier against its release from the inner region of the shell.

From Fig. 3b, increasing PLGA shell thickness (from PLGA/PLLA 3:1 to 6:1) gave a similar PCTX release rate for dual-drug-loaded particles ($p > 0.05$). Here, the degradation of PLGA proceeded more rapidly for the thicker shell with less porous surface (PLGA/PLLA 6:1) (Fig. 4a), likely due to the autocatalytic degradation caused by immobilized acidic oligomers [39]. This accelerated degradation would compensate for the longer diffusion pathways of PCTX through the thicker shell, resulting in similar release rates, as similarly reported by Klose et al. [40].

3.3. Efficacy against 3D multicellular tumor spheroids

Two-dimensional (2D) cell monolayers have been widely used to evaluate drug efficacy over 24–72 h [41]. However, the 2D monolayers poorly represent the complex 3D microenvironment in which cells are in close contact and interact with other types of cells and the extracellular matrix [42]. Three-dimensional cell culture, on the other hand, can better model the actual *in vivo* microenvironment [43]. Moreover, multicellular 3D spheroids provide a means for continuous and quantitative analysis in prolonged studies of which the durations are similar to animal studies [44]. Using a platform developed recently [45], magnetic MCF-7 spheroids were exploited to elucidate the therapeutic benefits of drug-loaded microparticles in a 28-day long study. These spheroids enable facile media exchange as they respond to magnetic field gradients [46,47]. For treatments with free drugs at 0.4 $\mu g\,mL^{-1}$ DOX and 0.08 $\mu g\,mL^{-1}$ PCTX concentrations, growth recovery of spheroid was observed 7 days after the introduction of free drug (Fig. 6a). This cellular recovery is likely due to acute free drug exposure (6 h) and a reduction in drug penetration into the spheroids at lower drug concentrations, which result in lower diffusion gradients within the spheroids [45,48]. Free drug-containing media were removed after 6 h, so as to mimic acute free drug exposure and subsequent systemic clearance after drug administration. It was found that MCF-7 spheroids responded to the drugs in a dose-dependent manner (Fig. S6), in which continuous growth inhibition of spheroids occurred with drug treatment at higher concentrations ($p < 0.01$ when comparing 4 $\mu g\,mL^{-1}$ and 0.4 $\mu g\,mL^{-1}$ for DOX and 0.4 $\mu g\,mL^{-1}$ and 0.04 $\mu g\,mL^{-1}$ for PCTX at day 21). The growth of spheroids incubated with drug-loaded particles was inhibited to a greater extent ($p < 0.01$ when compared with free drugs at day 28) and no recovery of growth was observed even after 28 days, implying continued exposure to therapeutic doses due to the prolonged release of the chemotherapeutics (Figs. 6a and 7). Bright-field images of MCF-7 spheroids (Fig. 7) exposed to free drug or various particle groups are shown to visualize the change in the spheroid sizes with time.

Simultaneous release of both DOX and PCTX from double-layered microparticles significantly decreased spheroid growth rate, as compared to PCTX-loaded PLGA/PLLA and DOX-loaded PLGA microparticles ($p < 0.01$, Tukey's multiple comparison tests) (Fig. 6a). Notably, the release kinetics of these two single-drug microparticle groups were similar to that of each drug from dual-drug-loaded PLGA/PLLA 6:1 microparticles (Fig. 3). The initial slow release of single drug from PCTX-PLGA/PLLA and DOX–PLGA microparticles was shown to produce an insignificant reduction in spheroid growth rate up to day 7. Better drug efficacy for dual-drug PLGA/PLLA 6:1 microparticles could arise from the drug combination of DOX and PCTX. The addition of relatively low amount of PCTX resulted in a complementary cytotoxic effect when both DOX and PCTX released from PLGA/PLLA 6:1 microparticles were delivered to the spheroids in a sustained manner. Early presence of DOX released from the dual-drug-loaded particles efficiently prevents the excess growth of tumor cells in the initial stage by intercalating with DNA strands and inhibiting further DNA and RNA biosynthesis [49]. A controlled and sustainable release of PCTX can induce the apoptosis of tumor cells continuously by stopping microtubules disassembly, thereby preventing cell division [16,50]. Therapeutic efficacy can be maximized and development of drug resistance can be minimized, when two antitumor drugs with different physicochemical properties and mechanisms are co-delivered to tumors [12,51]. Therefore, rate-controlling delivery systems that employ multiple antitumor drugs are advantageous to improve conventional approach through monotherapy that only delivers a single drug in a bolus dose. Clinical studies have also demonstrated that combination of DOX and PCTX increased tumor regression rates relative to just the individual drugs [3,7]. Results from the acid phosphatase assay also indicated that dual-drug-loaded microparticles could cause a significant decrease in spheroid viability (after 28 days) relative to free drugs and single-drug-loaded particles (i.e. PCTX–PLGA/PLLA and DOX–PLGA particles) (Fig. 6b).

Interestingly, a significant reduction in spheroid growth rate and spheroid viability similar to dual-drug-loaded particles was observed for DOX-loaded PLGA/PLLA 3:1 microparticles ($p > 0.05$

when comparing DOX-loaded PLGA/PLLA 3:1 and dual-drug-loaded PLGA/PLLA microparticles) (Fig. 6a and b). The initial burst of DOX released from DOX-loaded PLGA/PLLA 3:1 microparticles could impose significant toxicity to tumor spheroids, and slowed the initial growth phase because the culture media for particle groups were refreshed 2 days after drug treatment with subsequent dilution every 2 days. The released DOX from the initial burst would have 2 days to exert its cytotoxic effect. Cells at the outer circumference of the spheroids are more exposed to the released drugs and therefore undergo apoptosis. Substantial apoptosis, arising from initial 2-day DOX exposure at considerably high concentration, could expand the interstitial space along the circumference of the spheroids, thereby enhancing penetration of DOX into the primed tumors [52]. The penetration of DOX into the spheroids treated with DOX-loaded PLGA/PLLA 3:1 microparticles is shown in the CLSM images (Fig. 8). The subsequent continuous release of DOX could suppress the recovery of spheroid growth. Nevertheless, a burst release from these PLGA/PLLA 3:1 microparticles *in vivo* would produce systemic toxicity and adverse effects (e.g. toxicity to normal cells) [53]. Notably, tracking the volume of spheroids over the release period showed that those incubated with dual-drug-loaded PLGA/PLLA 6:1 microparticles displayed a similar reduction in growth rate (i.e. comparable efficacy) relative to DOX and dual-drug-loaded PLGA/PLLA 3:1 microparticles (Figs. 6 and 7). The increased shell thickness for PLGA/PLLA 6:1 was found to suppress the burst release of DOX (Fig. 3a), and this could potentially minimize toxicity to surrounding normal cells and side effects. Since sustained and controlled release of both DOX and PCTX is achievable with double-layered microparticles (PLGA/PLLA 6:1), this particulate drug delivery system would have great potential to inhibit tumor growth with minimal systemic toxicity.

4. Conclusions

Dual-drug-loaded, double-layered PLGA/PLLA microparticles were produced through a one-step fabrication process. DOX was localized in the PLGA shell, whereas PCTX was localized in the PLLA core. The drug release properties of dual-drug-loaded microparticles were compared with those of single-drug-loaded microparticles. Direct manipulation of polymer mass ratios produced microparticles with different layer morphologies and drug encapsulation efficiencies, which resulted in variable release kinetics of drugs. Controlled and sustained release of two drugs was observed for PLGA/PLLA 6:1 particles when the shell thickness was increased. Three-dimensional spheroid studies showed that release of DOX and PCTX from dual-drug-loaded double-layered microparticles (PLGA/PLLA 6:1) achieved a therapeutic advantage over single-drug microparticles, while having the potential to minimize toxicity to the surrounding normal cells as well as side effects. From this study, we presented a promising approach to tailor the release rates of drugs from multidrug-loaded particles by manipulating layer structures, thus allowing such "designer" release system to find use in combination chemotherapy.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.actbio.2015.08.051.

References

[1] H.L. Wong, R. Bendayan, A.M. Rauth, Y. Li, X.Y. Wu, Chemotherapy with anticancer drugs encapsulated in solid lipid nanoparticles, Adv. Drug Deliv. Rev. 59 (2007) 491–504.
[2] S. Kim, J.-H. Kim, O. Jeon, I.C. Kwon, K. Park, Engineered polymers for advanced drug delivery, Eur. J. Pharm. Biopharm. 71 (2009) 420–430.
[3] D.L. Gustafson, A.L. Merz, M.E. Long, Pharmacokinetics of combined doxorubicin and paclitaxel in mice, Cancer Lett. 220 (2005) 161–169.
[4] D. Mavroudis, C. Kouroussis, S. Kakolyris, S. Agelaki, K. Kalbakis, N. Androulakis, et al., Phase I study of paclitaxel (Taxol) and pegylated liposomal doxorubicin (Caelyx) administered every 2 weeks in patients with advanced solid tumors, Oncology 62 (2002) 216–222.
[5] Y. Barenholz, Liposome application: problems and prospects, Curr. Opin. Colloid Interface Sci. 6 (2001) 66–77.
[6] H. Gelderblom, J. Verweij, K. Nooter, A. Sparreboom, Cremophor EL: the drawbacks and advantages of vehicle selection for drug formulation, Eur. J. Cancer 37 (2001) 1590–1598.
[7] E. Briasoulis, V. Karavasilis, E. Tzamakou, D. Rammou, K. Soulti, C. Piperidou, et al., Interaction pharmacokinetics of pegylated liposomal doxorubicin (Caelyx) on coadministration with paclitaxel or docetaxel, Cancer Chemother. Pharmacol. 53 (2004) 452–457.
[8] S. Song, B. Yu, Y. Wei, M.G. Wientjes, J.L.-S. Au, Low-dose suramin enhanced paclitaxel activity in chemotherapy-naive and paclitaxel-pretreated human breast xenograft tumors, Clin. Cancer Res. 10 (2004) 6058–6065.
[9] J. Lehár, A.S. Krueger, W. Avery, A.M. Heilbut, L.M. Johansen, E.R. Price, et al., Synergistic drug combinations tend to improve therapeutically relevant selectivity, Nat. Biotechnol. 27 (2009) 659–666.
[10] H. Zhang, G. Wang, H. Yang, Drug delivery systems for differential release in combination therapy, Expert Opin. Drug Deliv. 8 (2011) 171–190.
[11] X. Xu, X. Chen, Z. Wang, X. Jing, Ultrafine PEG–PLA fibers loaded with both paclitaxel and doxorubicin hydrochloride and their in vitro cytotoxicity, Eur. J. Pharm. Biopharm. 72 (2009) 18–25.
[12] L. Fan, F. Li, H. Zhang, Y. Wang, C. Cheng, X. Li, et al., Co-delivery of PDTC and doxorubicin by multifunctional micellar nanoparticles to achieve active targeted drug delivery and overcome multidrug resistance, Biomaterials 31 (2010) 5634–5642.
[13] Y. Wang, S. Gao, W.-H. Ye, H.S. Yoon, Y.-Y. Yang, Co-delivery of drugs and DNA from cationic core–shell nanoparticles self-assembled from a biodegradable copolymer, Nat. Mater. 5 (2006) 791–796.
[14] H. Wang, P. Zhao, W. Su, S. Wang, Z. Liao, R. Niu, et al., PLGA/polymeric liposome for targeted drug and gene co-delivery, Biomaterials 31 (2010) 8741–8748.
[15] C. Zheng, M. Zheng, P. Gong, J. Deng, H. Yi, P. Zhang, et al., Polypeptide cationic micelles mediated co-delivery of docetaxel and siRNA for synergistic tumor therapy, Biomaterials 34 (2013) 3431–3438.
[16] H. Wang, Y. Zhao, Y. Wu, Hu. Y-l, K. Nan, G. Nie, et al., Enhanced anti-tumor efficacy by co-delivery of doxorubicin and paclitaxel with amphiphilic methoxy PEG–PLGA copolymer nanoparticles, Biomaterials 32 (2011) 8281–8290.
[17] B.A. Almond, A.R. Hadba, S.T. Freeman, B.J. Cuevas, A.M. York, C.J. Detrisac, et al., Efficacy of mitoxantrone-loaded albumin microspheres for intratumoral chemotherapy of breast cancer, J. Control. Release 91 (2003) 147–155.
[18] A. Matsumoto, Y. Matsukawa, T. Suzuki, H. Yoshino, Drug release characteristics of multi-reservoir type microspheres with poly(DL-lactide-co-glycolide) and poly(DL-lactide), J. Control. Release 106 (2005) 172–180.
[19] M. Shi, Y.Y. Yang, C.S. Chaw, S.H. Goh, S.M. Moochhala, S. Ng, et al., Double walled POE/PLGA microspheres: encapsulation of water-soluble and water-insoluble proteins and their release properties, J. Control. Release 89 (2003) 167–177.
[20] C. Berkland, A. Cox, K. Kim, D.W. Pack, Three-month, zero-order piroxicam release from monodispersed double-walled microspheres of controlled shell thickness, J. Biomed. Mater. Res. 70A (2004) 576–584.
[21] L.E. Kokai, H. Tan, S. Jhunjhunwala, S.R. Little, J.W. Frank, K.G. Marra, Protein bioactivity and polymer orientation is affected by stabilizer incorporation for double-walled microspheres, J. Control. Release 141 (2010) 168–176.
[22] C. Berkland, E. Pollauf, D.W. Pack, K. Kim, Uniform double-walled polymer microspheres of controllable shell thickness, J. Control. Release 96 (2004) 101–111.
[23] E.C. Tan, R.Y. Lin, C.H. Wang, Fabrication of double-walled microspheres for the sustained release of doxorubicin, J. Colloid Interface Sci. 291 (2005) 135–143.
[24] Q. Xu, S.E. Chin, C.-H. Wang, D.W. Pack, Mechanism of drug release from double-walled PDLLA (PLGA) microspheres, Biomaterials 34 (2013) 3902–3911.
[25] D.H. Choi, C.H. Park, I.H. Kim, H.J. Chun, K. Park, D.K. Han, Fabrication of core–shell microcapsules using PLGA and alginate for dual growth factor delivery system, J. Control. Release 147 (2010) 193–201.

[26] W.L. Lee, E. Widjaja, S.C.J. Loo, One-step fabrication of triple-layered polymeric microparticles with layer localization of drugs as a novel drug-delivery system, Small 6 (2010) 1003–1011.
[27] W.L. Lee, E. Widjaja, S.C.J. Loo, Designing drug-loaded multi-layered polymeric microparticles, J. Mater. Sci.: Mater. Med. 23 (2012) 81–88.
[28] W.L. Lee, W.M. Guo, V.H. Ho, A. Saha, H.C. Chong, N.S. Tan, et al., Inhibition of 3-D tumor spheroids by timed-released hydrophilic and hydrophobic drugs from multilayered polymeric microparticles, Small 10 (2014) 3986–3996.
[29] P. Perugini, I. Genta, B. Conti, T. Modena, D. Cocchi, D. Zaffe, et al., PLGA microspheres for oral osteopenia treatment: preliminary "in vitro"/"in vivo" evaluation, Int. J. Pharm. 256 (2003) 153–160.
[30] W. Peter Wuelfing, K. Kosuda, A.C. Templeton, A. Harman, M.D. Mowery, R.A. Reed, Polysorbate 80 UV/vis spectral and chromatographic characteristics – defining boundary conditions for use of the surfactant in dissolution analysis, J. Pharm. Biomed. Anal. 41 (2006) 774–782.
[31] M.J. Heslinga, E.M. Mastria, O. Eniola-Adefeso, Fabrication of biodegradable spheroidal microparticles for drug delivery applications, J. Control. Release 138 (2009) 235–242.
[32] X. Xu, P. Lu, M. Guo, M. Fang, Cross-linked gelatin/nanoparticles composite coating on micro-arc oxidation film for corrosion and drug release, Appl. Surf. Sci. 256 (2010) 2367–2371.
[33] Y.H. Yu, E. Kim, D.E. Park, G. Shim, S. Lee, Y.B. Kim, et al., Cationic solid lipid nanoparticles for co-delivery of paclitaxel and siRNA, Eur. J. Pharm. Biopharm. 80 (2012) 268–273.
[34] W.L. Lee, C. Loei, E. Widjaja, S.C.J. Loo, Altering the drug release profiles of double-layered ternary-phase microparticles, J. Control. Release 151 (2011) 229–238.
[35] R. Liggins, S. D'amours, J. Demetrick, L. Machan, H. Burt, Paclitaxel loaded poly (L-lactic acid) microspheres for the prevention of intraperitoneal carcinomatosis after a surgical repair and tumor cell spill, Biomaterials 21 (2000) 1959–1969.
[36] X. Huang, C.S. Brazel, On the importance and mechanisms of burst release in matrix-controlled drug delivery systems, J. Control. Release 73 (2001) 121–136.
[37] W.L. Lee, P. Yu, M. Hong, E. Widjaja, S.C.J. Loo, Designing multilayered particulate systems for tunable drug release profiles, Acta Biomater. 8 (2012) 2271–2278.
[38] W.L. Lee, W.X. Shi, Z.Y. Low, S. Li, S.C.J. Loo, Modeling of drug release from biodegradable triple-layered microparticles, J. Biomed. Mater. Res. 100 (2012) 3353–3362.
[39] D. Klose, F. Siepmann, K. Elkharraz, S. Krenzlin, J. Siepmann, How porosity and size affect the drug release mechanisms from PLGA-based microparticles, Int. J. Pharm. 314 (2006) 198–206.
[40] D. Klose, F. Siepmann, K. Elkharraz, J. Siepmann, PLGA-based drug delivery systems: importance of the type of drug and device geometry, Int. J. Pharm. 354 (2008) 95–103.
[41] T.L. Moore, S.W. Grimes, R.L. Lewis, F. Alexis, Multilayered polymer-coated carbon nanotubes to deliver dasatinib, Mol. Pharm. 11 (2013) 276–282.
[42] L.C. Kimlin, G. Casagrande, V.M. Virador, In vitro three-dimensional (3D) models in cancer research: an update, Mol. Carcinog. 52 (2013) 167–182.
[43] K.M. Yamada, E. Cukierman, Modeling tissue morphogenesis and cancer in 3D, Cell 130 (2007) 601–610.
[44] V.H. Ho, W.M. Guo, C.L. Huang, S.F. Ho, S.Y. Chaw, E.Y. Tan, et al., Manipulating magnetic 3D spheroids in hanging drops for applications in tissue engineering and drug screening, Adv. Healthcare Mater. 2 (2013) 1430–1434.
[45] W.M. Guo, X.J. Loh, E.Y. Tan, S.C.J. Loo, V.H. Ho, Development of a magnetic 3D spheroid platform with potential application for high-throughput drug screening, Mol. Pharm. 11 (2014) 2182–2189.
[46] V.H. Ho, K.H. Müller, A. Barcza, R. Chen, N.K. Slater, Generation and manipulation of magnetic multicellular spheroids, Biomaterials 31 (2010) 3095–3102.
[47] V.H. Ho, N.K. Slater, R. Chen, PH-responsive endosomolytic pseudo-peptides for drug delivery to multicellular spheroids tumour models, Biomaterials 32 (2011) 2953–2958.
[48] J. Friedrich, C. Seidel, R. Ebner, L.A. Kunz-Schughart, Spheroid-based drug screen: considerations and practical approach, Nat. Protoc. 4 (2009) 309–324.
[49] S. Lee, M. Baek, H.-Y. Kim, J.-H. Ha, D.-I. Jeoung, Mechanism of doxorubicin-induced cell death and expression profile analysis, Biotechnol. Lett. 24 (2002) 1147–1151.
[50] K. Obara, M. Ishihara, Y. Ozeki, T. Ishizuka, T. Hayashi, S. Nakamura, et al., Controlled release of paclitaxel from photocrosslinked chitosan hydrogels and its subsequent effect on subcutaneous tumor growth in mice, J. Control. Release 110 (2005) 79–89.
[51] F. Ahmed, R.I. Pakunlu, A. Brannan, F. Bates, T. Minko, D.E. Discher, Biodegradable polymersomes loaded with both paclitaxel and doxorubicin permeate and shrink tumors, inducing apoptosis in proportion to accumulated drug, J. Control. Release 116 (2006) 150–158.
[52] Z. Lu, M. Tsai, D. Lu, J. Wang, M.G. Wientjes, J.L.-S. Au, Tumor-penetrating microparticles for intraperitoneal therapy of ovarian cancer, J. Pharmacol. Exp. Ther. 327 (2008) 673–682.
[53] T.M. Allen, P.R. Cullis, Drug delivery systems: entering the mainstream, Science 303 (2004) 1818–1822.