Pharm Res (2015) 32:1249–1263
DOI 10.1007/s11095-014-1530-0

RESEARCH PAPER

pH-Responsive Nano Carriers for Doxorubicin Delivery

Shahla Bagherifam • Frode Miltzow Skjeldal • Gareth Griffiths • Gunhild M. Mælandsmo • Olav Engebråten • Bo Nyström • Vasif Hasirci • Nesrin Hasirci

Received: 4 February 2014 / Accepted: 22 September 2014 / Published online: 7 October 2014

ABSTRACT

Purpose The aim of this study was to design stimuli-responsive nanocarriers for anti-cancer drug delivery. For this purpose, doxorubicin (DOX)-loaded, polysebacic anhydride (PSA) based nanocapsules (NC) were combined with pH-sensitive poly (L-histidine) (PLH).

Method PSA nano-carriers were first loaded with DOX and were coated with poly L-histidine to introduce pH sensitivity. The PLH-coated NCs were then covered with polyethylene glycol (PEG) to reduce macrophage uptake. The drug release profile from this system was examined in two different buffer solutions prepared as acidic (pH5) and physiological (pH 7.4) media. The physical and chemical properties of the nanocapsules were characterized by Fourier transform infrared spectroscopy (FTIR), dynamic light scattering (DLS), ultraviolet and visible absorption spectroscopy (UV–VIS), and scanning electron microscopy (SEM). *In vitro* studies of the prepared nanocapsules were conducted in MDA-MB-231 breast cancer cells.

Results The results obtained by SEM and DLS revealed that nanocapsules have spherical morphology with an average size of 230 nm. Prepared pH sensitive nanocapsules exhibited pH-dependent drug release profile and promising intracellular release of drug. PEGylation of nanoparticles significantly prevented macrophage uptake compared to non-PEGylated particles.

KEY WORDS doxorubicin · nanocapsule · pH-responsive · poly L-histidine · polysebacic anhydride

ABBREVIATIONS

ATCC	American type culture collection
DAPI	4′,6-diamidino-2-phenylindole
DCM	Dichloromethane
DLS	Dynamic light scattering
DNA	Deoxyribonucleic acid
DOX	Doxorubicin
EE	Encapsulation efficiency
EPR	Enhanced permeability and retention
FDA	Food and drug administration
FTIR	Fourier transform infrared spectroscopy
GPC	Gel permeation chromatography
H-NMR	Proton nuclear magnetic resonance
LC	Loading capacity
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
MW	Molecular weight
NCs	Nanocapsules
PBS	Phosphate buffered saline
PDI	Poly dispersity index

S. Bagherifam · V. Hasirci · N. Hasirci
Graduate Department of Polymer Science and Technology, Middle East Technical University, Ankara, Turkey

S. Bagherifam · F. M. Skjeldal · G. Griffiths
Institute of Molecular Biosciences, University of Oslo, Oslo, Norway

G. M. Mælandsmo · O. Engebråten
Institute for Cancer Research, Oslo University Hospital, University of Oslo, Oslo, Norway

B. Nyström
Department of Chemistry, University of Oslo, Oslo, Norway

V. Hasirci · N. Hasirci
Graduate Department of Biotechnology, Middle East Technical University, Ankara, Turkey

V. Hasirci
Department of Biological Sciences, Middle East Technical University Ankara, Turkey

N. Hasirci
Department of Chemistry, Middle East Technical University Ankara, Turkey

V. Hasirci · N. Hasirci (✉)
BIOMATEN - Center of Excellence in Biomaterials and Tissue Engineering, Middle East Technical University, Ankara 06800, Turkey
e-mail: nhasirci@metu.edu.tr

PEG	Polyethylene glycol
PFA	Paraformaldehyde
PLH	Poly (L-histidine)
PLL	Poly L-lysine
PSA	Polysebacic anhydride
PVA	Polyvinyl alcohol
RPMI	Roswell park memorial institute
SEM	Scanning electron microscopy
TPA	12-O-tetradecanoyl-phorbol-13-acetate
UV–VIS	Ultraviolet and visible absorption spectroscopy

INTRODUCTION

Chemotherapy is widely used for cancer treatment *via* the use of cytotoxic anti-cancer agents and is often applied in combination of other cancer treatments. This type of therapy is mainly used to reduce the size of the tumor. However, a significant undesirable side-effect of chemotherapeutic agents is their toxicity for normal cells, especially cells with a high turnover, such as those in hair, skin, and blood cells [1, 2]. Anti-cancer drugs also affect cells of vital organs such as liver, heart, and kidney. Because of these side-effects, alternative and more selective therapies have been sought. Targeting of chemotherapeutic agent to the cancer cells is the main strategy used to try to efficiently internalize the delivery vehicle and intracellular release of cancer treatment agent. In the literature different targeting vehicles, either active or passive, have been utilized and reported for the delivery of anti-cancer agents. When an active targeting drug delivery system is modified with some 'smart' molecules, such as antibodies, proteins and ligands that are able to selectively bind ligands enriched on the surface of cancer cells, they will detect tumor-specific or tumor-associated antigens and bind to the related receptors. In contrast, in passive targeting, stimuli-responsive delivery systems are developed to show their bioactivity against properties or physiological changes occurring at the targeted/activated site [3].

Based upon the measured pH value of the most solid tumors in different patients, it was observed that pH of tumor sites, dependent on tumor growth rate, is shifted toward the acidic region with pH values in the range 5–7, which is related to extreme amounts of metabolites like lactic acid and CO_2, while the normal blood pH remains constant at 7.4 [4]. In most of solid tumors, the rapid development of tumor vasculature by poorly controlled abnormal angiogenesis leads to formation of vessels with porous-walls. Macromolecules, lipids or nanoparticles with sizes in the range of 200–400 nm can easily pass these pores and remain in the tumor area for longer time [3]. This phenomenon has been characterized and termed as enhanced permeability and retention (EPR) effect. This pathophysiology of tumors has been considered as an ideal trigger for the delivery anti-cancer agents *via* pH sensitive delivery systems. The strategy to construct this type of system is to find components associated with the delivery vehicle that is susceptible to changes in pH.

Poly (L-histidine) (PLH), a polyamino acid, exhibits high potential to cell membrane fusion after protonation of the imidazole groups in acid medium (pH below 6). The imidazole side chain of histidine has a pK_a of approximately 6.0, and overall the amino acid has a pK_a of 6.5. This means that below a pH of 6, the imidazole ring is mostly protonated leading to hydrolysis of the polymer. PLH is known to have an endosomal membrane disruption activity [5]. In this case, all nanoparticles (NPs) are internalized by cells *via* endocytic mechanisms. This means that the NP is surrounded by an endosomal or lysosomal membrane. If the NP or its contents need to have access to the cytoplasm it is essential that this membrane be disrupted or lysed. For this purpose, cationic peptides or lipids are often used. Previously a number of studies have focused on the application of PLH for either DNA delivery [6] or drug delivery [7], especially for the delivery of anticancer agents such as doxorubicin (DOX) [8]. Although poly (L-histidine) is a promising pH-sensitive compound, some limitations, such as difficulty in blocking the imidazole group, the control of the molecular weight of the polymer during the synthesis, and its low solubility in organic solvents have limited the production and investigation of this pH sensitive polymer. Nevertheless, there are many studies being attempted to overcome these limitations by either making copolymers or combining the polymer with other molecules to design pH responsive drug carrier systems [9].

In this study a nano-sized pH-responsive delivery system has been designed for targeting the anti-cancer agent DOX by using PLH as a smart molecule covering the delivery system. For this purpose, the main part of the delivery system was prepared from polysebacic anhydride (PSA). PSA is a promising material for drug delivery since it exhibits biocompatibility, controlled erosion that starts from the surface, degrade to non-toxic metabolites, can easily be obtained from natural sources, and it is a low cost product [10–12]. PSA can be easily synthesized *via* condensation polymerization of sebacic acid, leading to conversion of carboxylic acid groups to anhydride linkages [13]. Therefore, synthesized PSA was used to produce nanocapsules and then they were modified with PLH to add pH responsiveness to particles. To prevent macrophage uptakes of the delivery system during future intravenous application, the prepared pH-responsive nanocapsules were then modified by covering the entities with polyethylene glycol (PEG). A schematic illustration of the prepared system and the chemical structures of the ingredients used are shown in Fig. 1.

There are several studies on the application of PLH for either drug or gene delivery. Bello *et al.* have designed a complex system, based on polylysine substituted with L-histidine for DNA delivery, and showed that protonation of

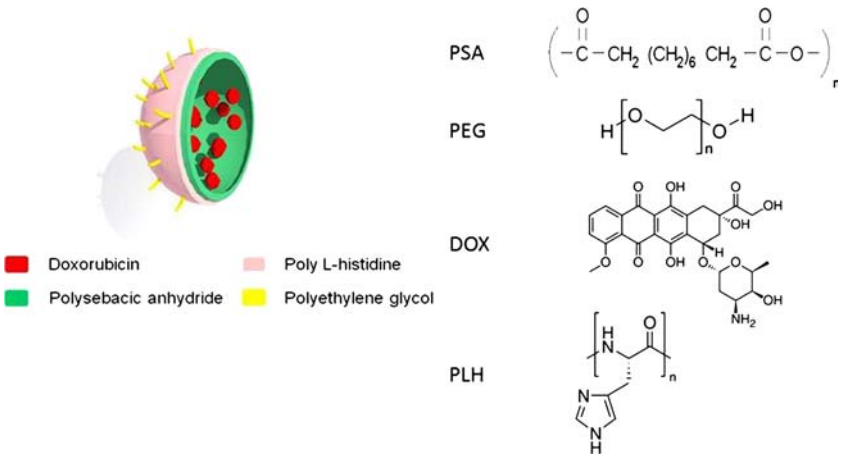

Fig. 1 Schematic illustration of pH responsive DOX-loaded nanocapsules.

the imidazole group in PLH significantly improves the transfection efficiency of the formulated system [6]. In another DNA delivery study, nanoparticles prepared from a grafted copolymer of poly L-histidine and poly L-lysine (PLH-co-PLL) were investigated for DNA transfection and were found to exhibit higher transfection efficacy compared to poly L-lysine particles [14]. Lee *et al.* investigated pH-dependency of micelles prepared from PLH and they reported that the micelles produced at basic media (pH: 8.0) were mostly destabilized in lower pH media (pH: 7.4) [7]. Further studies with PLH focused on delivery of DOX showed higher drug release when the pH was reduced from 8 to 5 [8]. The same group also observed that accumulation of DOX in solid tumors was almost 20-fold higher when folate conjugated nanoparticles of poly L-histidine (containing PEG and poly L-lactic acid block copolymers) were compared to non-folate conjugated particles [8].

The purpose of this study was to prepare an effective nanoparticle carrier system to target DOX to the tumor environment. For this goal, polysebacic anhydride was synthesized and DOX-loaded nanocapsules were prepared from the synthesized polymer. pH-sensitive nanocapsules were obtained by using poly L-histidine as an outer shell of the drug loaded nanocapsules. To reduce macrophage uptake of nanocapsules, the surfaces of the pH responsive nanocapsules were modified once more with polyethylene glycol (PEG). The anti-tumor activity, intracellular release and cell initialization of the nanocapsules were then examined by using MDA-MB-231 human breast cancer cells.

EXPERIMENTAL

Materials

Ethyl ether, petroleum ether, dichloromethane, chloroform, polyvinyl alcohol (MW 13000–23000), acetic anhydride, poly L-histidine (MW:5000–25000), polyethylene glycol, and monomethyl ether (MW:5000) were all purchased from Aldrich Chemicals (Gillinham, UK). Sebacic acid was obtained from Fluka (Gillinham, UK). Doxorubicin was acquired from Sandoz (Istanbul, Turkey). Coumarin-6 was purchased from Sigma Aldrich (St. Louis, MO-USA).

Polysebacic Anhydride Synthesis

Polysebacic anhydride (PSA) was prepared by a method similar to that described by Shen with some modifications [15]. Briefly, 6 g sebacic acid was put in 58 mL acetic anhydride and then the mixture was refluxed at 140°C under dry nitrogen gas sweep for 30 min. The access amounts of acetic anhydride and acetic acid that were produced during the reaction were removed by rotary evaporation at 40°C. The prepared sebacic anhydride was dissolved in 50 mL chloroform. Non-reacted sebacic acid was removed *via* precipitation of sebacic anhydride from chloroform solution in 200 mL of 1:1 mixture of anhydrous petroleum ether and diethyl ether. Polysebacic anhydride was prepared *via* condensation polymerization by curing of 5 g of pure sebacic anhydride prepolymer at 180°C for 1.5 h in vacuum oven. The prepared polymer was dissolved in dichloromethane, precipitated in anhydrous diethyl ether, filtered, dried, and kept in vials under argon atmosphere till used.

Polysebacic Anhydride Characterization

The chemical structure of the polymer was characterized by ^{1}H-NMR (950 US2, Bruker, Bremen, Germany) by dissolving the polymer in deuterated chloroform. The structure of the synthesized polysebacic anhydride was analyzed and compared with sebacic acid by using FTIR (Spotlight 65, Perkin Elmer, MO, USA). The weight-average molecular weight of the synthesized polymer was determined by gel permeation chromatography (GPC) (PL-GPC 220, CA, USA). The analysis was performed at 30°C in tetrahydrofuran with a flow rate

of 1 mL/min. In the GPC experiments polystyrene was used as standard.

Preparation of DOX Loaded Nanocapsules

DOX loaded nanocapsules (PSA-DOX-NCs) were prepared by utilizing a modified double emulsion protocol that has been described in the literature [16]. For this purpose, 50 mg polysebacic anhydride was dissolved in 5 mL dichloromethane (DCM). To get w_1/o emulsion, 1.75 mL aqueous solution of DOX (2 mg/mL) was added into the polymer solution and sonicated for 20 s by a probe sonicator operating at 60 W power output. The homogeneous emulsion that was obtained was subsequently added to 15 mL of a 4% polyvinyl alcohol solution and sonicated again for 20 s. To the prepared $w_1/o/w_2$ emulsion, it was added an extra amount of polyvinyl alcohol solution (75 mL) with a concentration of 1%. The prepared emulsion was stirred at room temperature to evaporate DCM and to precipitate DOX loaded NCs. Following the complete evaporation of DCM *via* rotary evaporator, solidified particles were collected by centrifugation (14,000 rpm at 4°C), and the product was washed twice with 1 mL double distilled water. Blank nanocapsules, without DOX, were also prepared *via* the same procedure. The only difference was that the same amount of distilled water was employed instead of the DOX solution to obtain the w_1/o emulsion. Fluorescence-labeled nanoparticles were made in a similar way by adding coumarin-6 dye to the organic phase of polysebacic anhydride. For this purpose, 20 μL of coumarin-6 stock solution (20 μg coumarin-6 in 1 mL DCM) was added to the polymer solution. The prepared NCs were dried by lyophilization in a freeze-drier for further studies. Table I shows the types and the codes of the prepared nanocapsules.

Characterization of the DOX Loaded Nanocapsules

The yield of the prepared nanocapsules (NCs) containing doxorubicin (DOX) was calculated by using the mass of NCs obtained per mass of the polymer used initially. The morphology of NCs was analyzed by employing scanning electron microscopy (SEM, QUANTA 400, Oregon, USA).

Table I Types of the prepared nanocapsules

Codes	Samples
PSA-NCs	Polysebacic anhydride (PSA) nanocapsules
PSA-DOX-NCs	Doxorubicin (DOX) loaded PSA nanocapsules
PLH-PSA-DOX-NCs	Poly (L-histidine) (PLH) coated and DOX loaded PSA nanocapsules
PEG-PLH-PSA-DOX-NCs	PEGylated, PLH coated and DOX loaded PSA nanocapsules

Size and size distributions were obtained by using a particle size analyzer (Malvern Mastersizer, Worcestershire, UK).

Preparation of pH-Responsive Nanocapsules

Both the control NCs (PSA-NCs) and the DOX-loaded NCs (PSA-DOX-NCs) were made pH-responsive by coating them with PLH and this also made them electrostatically stabile. Before the coating process, the zeta potentials of both DOX loaded NCs and pure PLH were measured by the zeta sizer in dilute acidic medium at pH ~ 6. To check the calibration of the instrument, zeta potential of a standard sample (−68 ± 6.8 mV) was first measured. To perform the coating, PLH was dissolved in acidic aqueous medium. For this purpose, 0.01 M HCl was added drop-wise to 10 mL double-distilled water containing PLH until the pH was adjusted to approximately 6. DOX-loaded nanocapsules were put into the solution. To cover the surface of NCs with PLH, NaOH (0.01 M) was added drop-wise to the solution to increase the pH up to ca. 8. PLH coated nanocapsules are named as PLH-PSA-DOX-NCs. They were collected by using a centrifuge at a speed of 10,000 rpm at 4°C and afterwards the samples were freeze-dried. The level of PLH-coating on these NCs was analyzed by FTIR. In this analysis, FTIR spectra of NCs coated with poly (L-histidine) (PLH-PSA-DOX-NCs) were compared with the FTIR spectra of DOX loaded NCs (PSA-DOX-NCs) and pure PLH as control groups.

PEGylation of pH-Responsive Nanocapsules

To improve the transport performance of the prepared pH-responsive carriers (PLH-PSA-DOX-NCs) and to prevent them from being taken up by the macrophages, the surface of the pH responsive NCs was covered with PEG (molecular mass of 5,000 Da) to produce PEGylated particles and these NCs are named as PEG-PLH-PSA-DOX-NCs. For this purpose, NCs were stirred in 5 mL cold (~4°C) and 3% aqueous solution of polyethylene glycol monomethyl ether for 10 min. The particles were then collected by centrifugation at 10,000 rpm for 15 min, freeze-dried and characterized by FTIR. The effects of PEG coating on size and morphology of NCs were examined by DLS and SEM analysis.

Drug Loading Capacity and Encapsulation Efficiency

To assess the drug concentration in the nanocapsules, UV absorption measurements were performed. The DOX concentration in the supernatant was analyzed by a UV spectrophotometer (1420 Wallac Victor, Turku, Finland) and calculated *via* a calibration curve with $R^2 = 0.992$ prepared previously with different concentrations of DOX in phosphate

buffered saline (PBS, 0.01 M, pH = 7.4). Percentage of encapsulation efficiency of DOX was obtained using the equation given below.

$$EE\% = \left[W_{(1)} - W_{(2)}/W_{(1)}\right] \times 100$$

where; EE% is encapsulation efficiency, $W_{(1)}$ is weight of initial DOX, $W_{(2)}$ is weight of DOX in supernatant at the end of the loading process, and $(W_{(1)} - W_{(2)})$ is the weight of DOX entrapped in the nanocapsules. Percentage of loading capacity of NCs was calculated by using the equation given below.

$$LC\% = \left[W_{(DOX)}/W_{(NC)}\right] \times 100$$

In this equation, $W_{(DOX)}$ is the mass of DOX in nanocapsules and $W_{(NC)}$ is the mass of the nanocapsules.

DOX Release Profile of pH-Responsive NCs (PEG-PLH-PSA-DOX-NCs)

The release behavior of the PEGylated pH responsive nanocapsules was studied in PBS buffered solution (0.01 M) at two different pH values; where one is acidic with a pH of 5.0, and the other is the physiological pH of 7.4. For this purpose, 5 mg of nanocapsules (PEG-PLH-PSA-DOX-NCs) was resuspended in 0.5 mL double distilled water and transferred into dialysis bags. The release studies were conducted in 4.5 mL of the corresponding buffer under shaking in an incubator at 37°C. To provide sink condition, 0.5 mL aliquots were removed from the release medium at certain times for UV absorbance measurements, while equal amounts of fresh PBS solutions were added into the media. The amount of released DOX was calculated by using the calibration curve prepared previously, that was based on the absorbance intensity of DOX at 490 nm. In the assessment of drug release behavior, the cumulative amount of the released drug was calculated, and the percentages of released drug from the nanocapsules were plotted *versus* time. All experiments were carried out in triplicates.

Efficiency of pH-Responsive Nanocapsules (PEG-PLH-PSA-DOX-NCs)

The anti-tumor activity of the prepared pH responsive NCs was examined on the MDA-MB-231 breast cancer cells purchased from the American Type Culture Collection (ATCC). Cell viability assay included blank wells containing medium only, untreated control cells and cells treated with the empty NCs, free DOX and DOX-loaded nanoparticles. Briefly, the breast cancer cells were seeded in 96-well plates, incubated for 24 h and treated by 100 μL sterile PBS (blank groups) as well as PBS solution containing unloaded nanocapsules (blank NCs), free DOX (0.6 μg/mL) and DOX containing nanocapsules (PEG-PLH-PSA-DOX-NCs) (0.6 μg/mL and 1.2 μg/mL). After 3 days of incubation at 37°C and 5% CO_2, cell viability was estimated by adding 10 μL of WST Kit-8 that was added to each well and incubated for 2 h. The absorbance values of the wells were obtained by UV spectroscopy (Wallac Victor plate reader, Turku, Finland) at 450 nm.

Intracellular Uptake of DOX Containing Nanocapsules into the Cancer Cells

To visualize the intracellular uptake of DOX containing nanocapsules by the cells, confocal laser scanning microscopy was employed. To accomplish this, MDA-MB-231 cells (15,000 cells per well) were seeded in 8-well glass slide (Lab-Tek II, NY, USA), incubated for 24 h and then treated with sterile PBS (0.01 M), PBS containing free DOX (0.6 μg/mL) and nanocapsules containing the same amount of DOX (PEG-PLH-PSA-DOX-NCs). After 3-days incubation, the medium was removed and the cells were rinsed twice with sterile PBS (0.067 M, pH 7.4). Fixing was accomplished by adding 400 μL of cold paraformaldehyde (PFA, 4%) and by incubating for 15 min at room temperature. Excess paraformaldehyde was removed by rinsing with sterile PBS (0.067 M, pH 7.4). After fixing, the cell nucleus was labeled with DAPI (4′,6-diamidino-2-phenylindole) by adding one droplet of prolong gold antifade reagent containing DAPI (Invitrogen, Oregon-USA) to each well. Fixed cells were covered by cover slips, kept at 2–8°C for 24 h and they were inspected by a Zeiss LSM 710 confocal microscope, equipped with Plan-Apochromatic 63×1.4 NA oil immersion objective (Zeiss-Germany). The image processing and visualization were performed by using the ZEN 2011 software (Carl Zeiss, Germany). Cell uptake of nanocapsules by cancerous cells was also examined by fluorescence microscopy. In this experiment, MDA-MB-231 breast cancer cells were seeded in 8-well glass slide (15,000 cells per well) and treated with sterile PBS as a control group, free DOX and Coumarin-labeled PEG-PLH-PSA-DOX-NCs. Treated cells were incubated for 1 day and fixed by PFA (4%) as described above.

Macrophage Uptake of pH-Responsive Nanocapsules

The influence of PEGylation of nanocapsules on macrophage uptake was analyzed on human acute monocytic leukemia cells (THP-1) that were obtained as a kind gift from Dr. Lina Prasmickaite and grown in RPMI 1640 cell culture medium (Lonza, Verviers, Belgium) containing 10% fetal bovine serum, 1% penicillin-streptomycin and 2 mM Glutamine. THP-1 cells were seeded (15,000 cells per well) in 8-well glass slide and induced to differentiate into macrophages by adding 200 μL culture medium containing 10 μM TPA (12-O-

tetradecanoyl-phorbol-13-acetate) and incubated at 37°C in the presence of 5% CO_2. After 48 h, the macrophages were treated by replacing TPA containing medium with sterile PBS, PEGylated NCs (PEG-PLH-PSA-DOX-NCs) and non-PEGylated NCs (PLH-PSA-DOX-NCs) with DOX concentration of 0.6 μg/mL. Treated macrophages were incubated for 6 h and fixed by paraformaldehyde (4%) as described above for the microscopy study. From three representative experiments, we analysed 10 cells with the uptake of non-PEGylated beads and 17 cells with the uptake of PEGylated beads. The level of total Doxorubicin fluorescence in the individual cells was measured as a function of relative difference in integrated intensity to the nucleus (DAPI) labeling of the cells. No saturated pixels were present and no background subtraction was performed. The individual nucleus was manually masked by freehand selection to avoid any discrepancies due to size, fluorescence intensity or shape. The total fluorescence intensity of the DOX loaded into PEGylated and non-PEGylated particles was measured with ImageJ [17]. Data is presented as mean and standard deviation, and the statistical significance was tested with a two-tailed Mann–Whitney test. The values having $p \leq 0.0014$ was considered significant (GraphPad, Prism).

RESULTS AND DISCUSSION

Polysebacic Anhydride Characterization

The ^{1}H-NMR spectra of both sebacic acid and polysebacic anhydride are compared in Fig. 2. It was found that the peak related to the OH groups located at 10.49 ppm, representing the monomer structure [3, 14] has disappeared in the spectrum of the synthesized polysebacic anhydride. This phenomenon is related to the conversion of carboxylic acid groups to anhydride. In addition, FTIR analysis confirmed the synthesis of the polysebacic anhydride. The FTIR spectra of both sebacic acid and polysebacic anhydride are shown in Fig. 3. The spectrum of sebacic acid showed characteristic absorption bands at 1,697, 1,300 and 930 cm^{-1}, representing carboxylic acid groups (Fig. 3a). The broad band presented at 3,335–2,500 cm^{-1} was ascribed to the strong hydrogen bonding of the -OH groups of the free acid. In the case of the spectrum for the polymer (Fig. 3b), these bands disappeared and the polysebacic anhydride characteristic absorption band was observed at 1,816–1,740 cm^{-1}. A sharp peak in the range of 1,090–1,030 illustrates stretching of the anhydride groups (−CO-O-CO-) [12]. Peaks observed in the domain of 2,920 and 2,870 cm^{-1} are related to stretching of C-H bonds in CH_2 groups.

Results obtained from GPC demonstrated that the molecular weight of the prepared PSA is about 2,500 Da with a polydispersity index (PDI) of 1.30.

Characterization of pH-Responsive Nanocapsules

Morphology, Size and Size Distribution

Although the main strategy of this study was to target DOX *via* pH-responsive nanocapsules, the enhanced permeability and retention (EPR) effects of nano-size particles were also expected to contribute to the entrapping of nanocapsules to the solid tumor [18]. To exploit the EPR effect in tumor

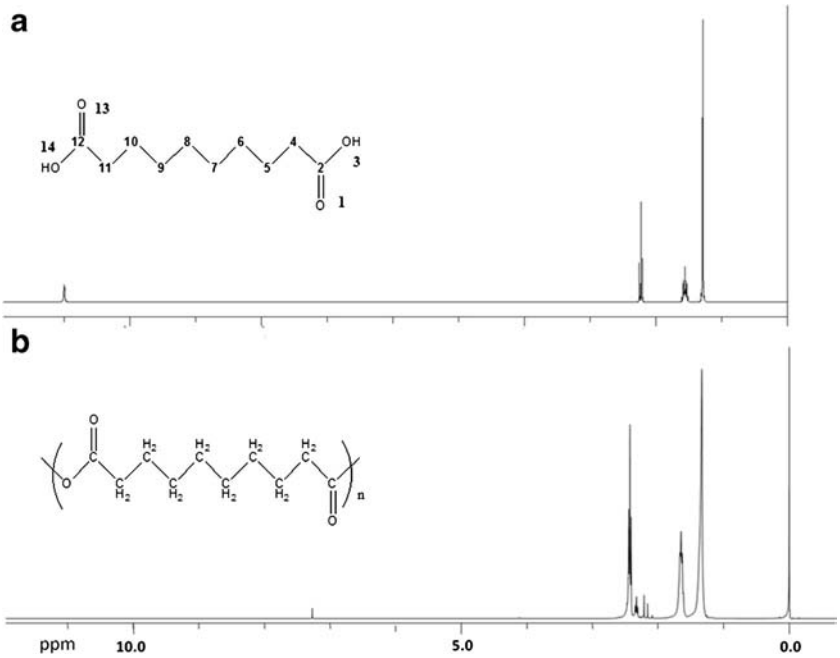

Fig. 2 ^{1}H-NMR spectrum. (**a**) sebacic acid (**b**) polysebacic anhydride.

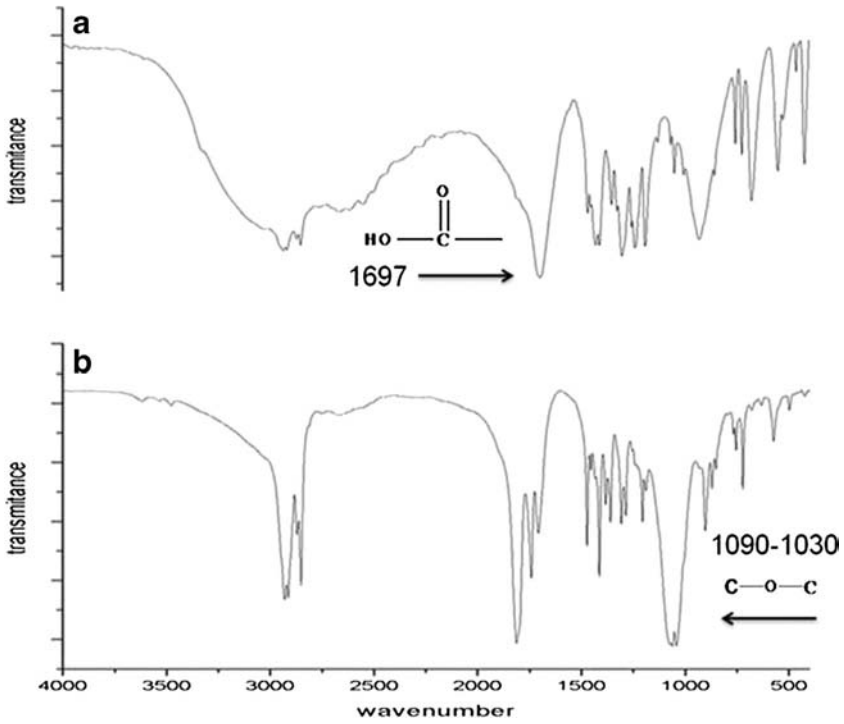

Fig. 3 FT-IR spectrum. (**a**) sebacic acid (**b**) polysebacic anhydride.

targeting, relatively small nanocapsules are required [3]. The results from dynamic light scattering (DLS) for DOX loaded NCs, before coating with PLH and PEG (PSA-DOX-NCs), are shown in Fig. 4. The size distribution of particles was rather broad, but most of the particles were located in the range of 150–350 nm. SEM micrographs exhibited a fine spherical morphology for PSA-DOX-NCs (Fig. 5).

Drug Encapsulation and Loading Capacity

The drug delivery systems are usually defined as a delivery technique to carry at least two-fold dosage of pharmaceutical agents compared to conventional drug administration [19]. Hence, encapsulation efficiency and loading capacity of NPs are important for sustained delivery. The challenge for polymer-based drug delivery systems is to obtain a sufficiently high drug loading capacity. The water-soluble DOX exhibits weak amphipathic properties (pK_a 8.3) and it is generally difficult to encapsulate the drug from an aqueous medium. There are also various challenges related to improving the DOX loading efficiency by preventing its migration from the organic to the aqueous phase, which is the main cause of the high amount of non-loaded drug in the supernatant. In some studies, cooperation of DOX with anionic polymers [20, 21] and interaction of an anionic surfactant [22] was used to develop DOX encapsulation efficiency up to 42.5 and 49.3%, respectively. In our study, encapsulation efficiency and loading capacity of DOX in polysebacic anhydride based nanocapsules were estimated to be 48 and 5.3%, respectively. These values are close to the reported [20–22] values for encapsulation of DOX in nanocapsules. In the literature there are plenty of studies aimed to improve encapsulation efficiency of highly hydrophilic DOX. It was shown that encapsulation efficiency of DOX can be significantly improved by either using negatively charged polymers [23] or co-encapsulation of DOX with other gradients such as ammonium sulfate [24]. Consequently, NCs fabricated in this study *via* the double emulsion method exhibited acceptable drug encapsulation for DOX delivery, without any modification of the polymer to entrap more DOX.

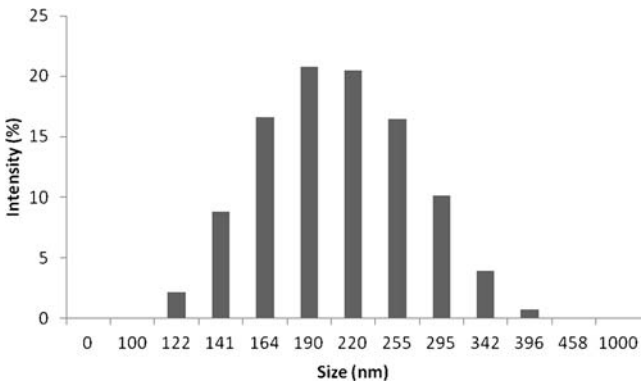

Fig. 4 Size distribution of DOX loaded NCs before coating (PSA-DOX-NCs) from DLS.

pH Sensitive System

Poly (L-histidine) (PLH) is a biodegradable polyamino acid. The polymer backbone has many imidazole functional groups

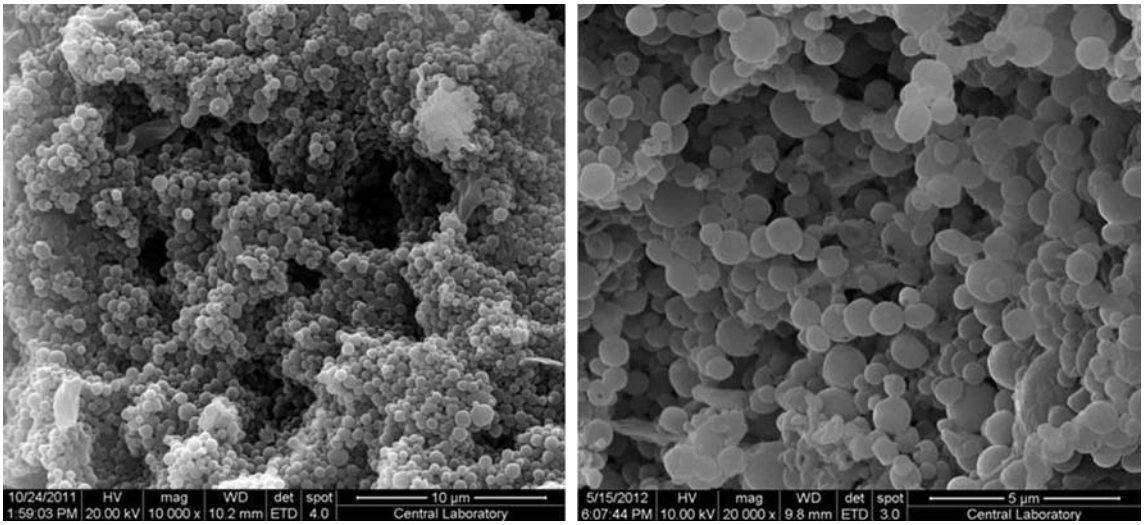

Fig. 5 SEM micrographs of DOX loaded NCs before coating (PSA-DOX-NCs).

with pKa around 6.0 and PLH exhibits buffering properties in the physiological pH range. Imidazole groups can be protonated at pH below 5.8, and as a consequence PLH can be dissolved in dilute acids [25]. This feature has been used to design different PLH based complexes as pH-sensitive delivery systems. For instance, PLH has been applied either as an outer shell or in cooperation with alginate to prepare microcapsules used for protein delivery [26]. In these studies, PLH was defined as a promising compound for protein delivery. In order to test the pH-response of poly (L-histidine), DOX loaded NCs (PSA-DOX-NCs) were coated with PLH by adsorbing PLH molecules on the NCs surfaces through electrostatic interactions. For this purpose, zeta potential of both DOX loaded NCs and PLH were measured in dilute acid medium with the intension to create the same condition needed for coating. The results showed zeta potential values of +29.4 and −35 mV for PLH and PSA-DOX-NCs, respectively. The results favor the conjecture that positively charged PLH can be adsorbed onto negatively charged NCs. To provide more evidence for this hypothesis, PLH-coated NCs (PLH- PSA-DOX-NCs) were characterized by FTIR to establish whether the absorbance peaks coming from PLH functional groups can be detected in the FTIR spectrum of the NCs. Figure 6 shows FTIR spectra for DOX loaded nanocapsules (PSA-DOX-NCs) (Fig. 6a), DOX loaded nanocapsules coated with PLH (PLH-PSA-DOX-NCs) (Fig. 6b), and pure PLH used as blank (Fig. 6c) to compare the success of the coating.

A comparison of FTIR-spectra reveals that the spectrum of PLH-PSA-DOX-NCs (Fig. 6b) not only displayed peaks originating from DOX loaded nanocapsules (Fig. 6a), but the spectra also reveal absorbance peaks representing imidazole groups located in the PLH polymer chain (Fig. 6c). These results demonstrate the presence of PLH on the surface of NCs.

PEGylation of pH Responsive NCs

Through conjugation of PEG on to the nanocapsules created PEG-PLH-PSA-DOX-NCs. PEGylation affected the uptake of these carriers by the macrophages and their uptake was significantly reduced (see the discussion below). The modified nanocapsules were characterized by FTIR to observe whether there are peaks originating from PEG functional groups in the FTIR spectrum of the pH-responsive nanocapsules. FTIR spectra of the nanocapsules are presented in Fig. 7. The spectrum of PEG was used as a blank to establish the success of the modification.

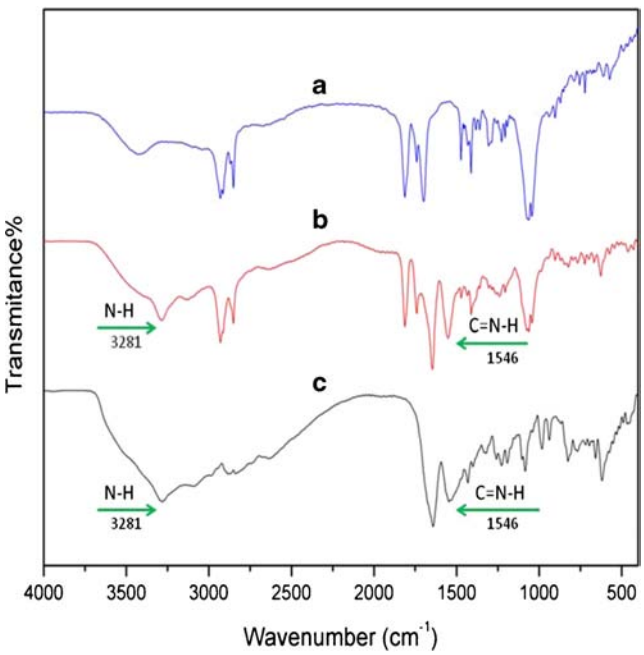

Fig. 6 FTIR spectra. (**a**) DOX loaded NCs (PSA-DOX-NCs), (**b**) PLH coated and DOX loaded NCs (PLH-PSA-DOX-NCs), (**c**) pure PLH.

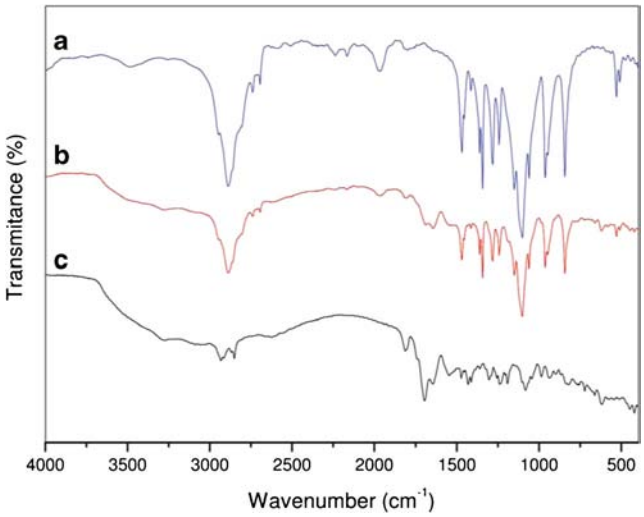

Fig. 7 FTIR spectra. (**a**) PEG, (**b**) PEGylated, PLH coated and DOX loaded NCs (PEG-PLH-PSA-DOX-NCs), (**c**) PLH coated and DOX loaded NCs (PLH-PSA-DOX-NCs).

By comparing these spectra, it was concluded that the spectrum of PEGylated NCs (Fig. 7b) displays peaks representing pH responsive NCs, (Fig. 7c), with the absorbance peak appearing at 2,820 cm^{-1}, originating from the PEG functional groups (Fig. 7a). As mentioned above, the size and size distribution of the NCs are important for the evaluation of the EPR effect. Thus, the final size of NCs coated with PLH and modified with PEG is an important parameter to establish. To monitor the morphology and size of the modified nanocapsules, we employed SEM and DLS. The results from DLS (see Fig. 8) of the modified NCs show that the size of the capsules is not significantly affected by coating and PEG-modification (see Fig. 4).

SEM micrographs shown in Fig. 9 illustrate the final morphology of PEGylated and pH-responsive NCs. It seems that the shape of the modified NCs has been changed slightly during the coating and PEG-modification process. The spherical shape of nanoparticles was changed after coating with poly L-histidine. The coating took place by acid–base titration with vigorous stirring, which causes heterogeneous adsorption of precipitated poly L-histidine on the surface of nanoparticles, which are suspended in water. This phenomenon may be a result from the heterogeneous coating of NCs with PLH during the precipitation of PLH on the surface of NCs.

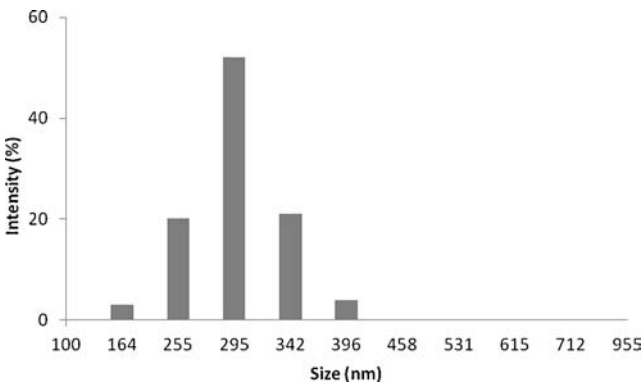

Fig. 8 Size distribution of NCs after coating with PLH and modification with PEG (PEG-PLH-PSA-DOX-NCs).

In situ DOX Release Study from PEG-PLH-PSA-DOX-NCs

The *in situ* release profiles of DOX from the pH responsive nanocapsules (PEG-PLH-PSA-DOX-NCs) in PBS at two different pH values (pH 5 and pH 7.4) are given in Fig. 10. The release profiles of DOX over several days in PBS are depicted at pH 5 and 7.4 and demonstrated significantly different results. It is evident that poly (L-histidine) (PLH) coated nanocapsules release a higher amount of drug at acid pH, both in the burst and sustained release steps, because PLH dissolves at acid pH and this facilitates the release of the drug. In this process, the drug concentration can reach an effective dose in a relatively short period of time (approximately 15–20 h). Subsequently, because of slow degradation of the polysebacic anhydride, the drug will be released in a sustained manner. In the case of pH 7.4, it took significantly longer time to achieve an effective dose. For instance, after 46 h it can be seen that the percentage of release is about 19% at pH 7.4, while at pH 5 the released amount is ca. 40%. A more efficient decrease of the release rate at pH 7.4 can probably be accomplished in future by using high molecular weight polymers and/or double coating the nanocapsules by PLH.

Cell Culture Study of pH Responsive Nanocapsules

Anti-Tumor Efficiency

Anti-tumor activity of free DOX and PEG-PLH-PSA-DOX-NCs was next analyzed by using WST cell counting kit-8. In these experiments, cells were seeded on 96-well plates and treated with 0.6 μg/mL free DOX, drug loaded NCs containing 0.6 or 1.2 μg/mL of DOX, or blank NCs containing no DOX (control group). The pH for cell culture medium (RPMI 1640) was 7.0–7.4. Cell viability experiments were done after 3-days of incubation. Relative cell viability was calculated by assuming the control sample had 100% viable cells. The results obtained from these experiments show that drug free NCs (control group) did not exhibit any detectable cytotoxicity on the MDA-MB-231 cells (Fig. 11).

The mechanism by which DOX kills cancer cells is still not unequivocally established [27]. The main proposed effect of DOX is to prevent DNA duplication and cause inhibition of topoisomerase II [28]. More recently an alternative, cytoplasmically-initiated mechanism has been proposed involving intra-membrane proteolysis of an endoplasmic reticulum protein (CREB3L1), thereby releasing its cytoplasmic domain that contains a transcription factor. The pleiotropic

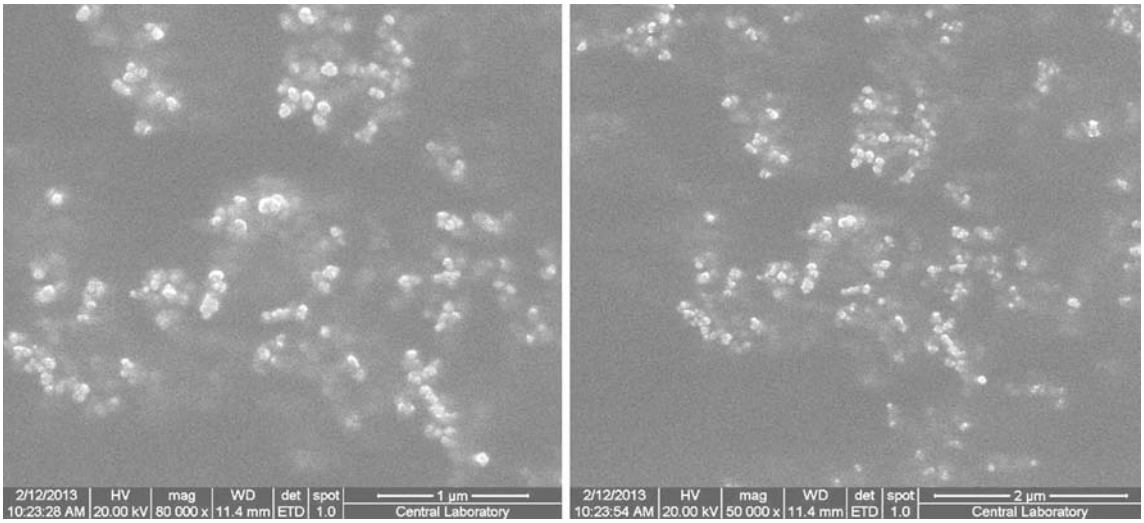

Fig. 9 SEM micrograph of NCs after coating with PLH and modification by PEG (PEG-PLH-PSA-DOX-NCs).

downstream effects of this transcription factor include effects on lipid metabolism and apoptosis [29].

Figure 11 shows the relative cell viability of MDA-MB-231 cells exposed to DOX concentrations of 0.6 and 1.2 µg/mL, in free and encapsulated state. The results reveal that higher cytotoxicity levels are observed in cells treated with higher concentrations of DOX, as expected. By comparing the behavior of free and loaded DOX, it is evident that although the same amount of DOX has been used in both cases, more antitumor activity was observed for DOX-loaded nanocapsules. This effect can presumably be linked to the cellular uptake and intracellular effect that results from high endosomal-lysis capacity of the outer shell of the NCs consisting of the pH responsive poly (L-histidine) [30]. In other words, the capacity of the NPs or their cargo to enter the cytoplasm is high.

Internalization of DOX and Cell Uptake

Cell internalization of drug *via* nanoparticles is a promising strategy to develop the therapeutic efficacy of anticancer drugs. There are various challenges to improve the properties of DOX-loaded polymeric carriers and to enhance the intracellular concentration of DOX, especially in drug-resistant cells, an important phenomenon in cancer therapy [31]. It has been reported that PLH can be protonated in acidic medium, and it may form phospholipid bi-layers by performing 16 interactions between the protonated molecules and the negatively charged phospholipids [32].

The cell internalization of DOX-loaded NCs was probed by confocal microscopy. For this, non-treated cells and cells treated with the same concentration of free DOX and nanocapsules loaded with DOX were analyzed. To determine the location of drug, the nucleus was stained using the DNA-binding dye DAPI (blue) after the cell fixation process. A TRITC filter responding to red fluorescence signals was employed to recognize the presence of DOX. Figure 12 shows confocal microscopy images of the internalization of DOX *via* pH responsive NCs. In the control cells, only treated by PBS, there was no indication of any red fluorescence signal (Fig. 12a). In the case of free DOX-treated cells, DOX

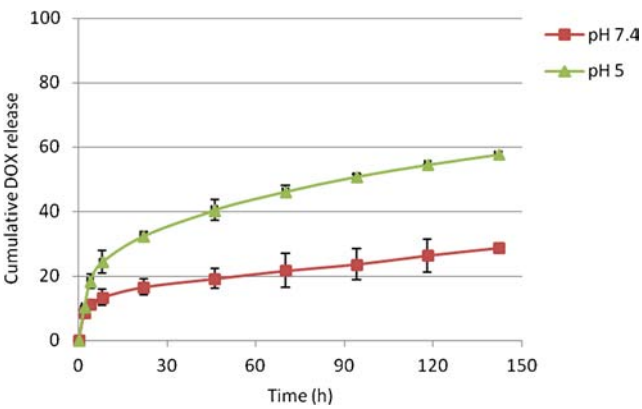

Fig. 10 DOX release profiles of PEG-PLH-PSA-DOX-NCs at pH 5.0 and pH 7.4.

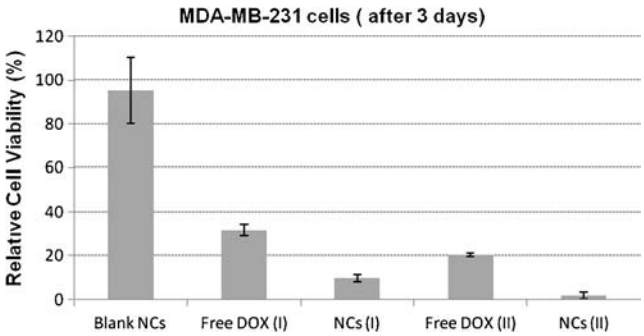

Fig. 11 WST kit-8 cell viability assay of blank NCs (PEG-PLH-PSA-NCs), free DOX 0.6 µg/mL (I) and 1.2 µg/mL (II), PLH coated NCs (PEG-PLH-PSA-DOX-NCs) containing 0.6 µg/mL (I) and 1.2 µg/mL (II) DOX. Data are shown as mean ± s.d. of biological replicates ($n = 4$). The *error bars* indicate the standard deviation with p value < 0.05.

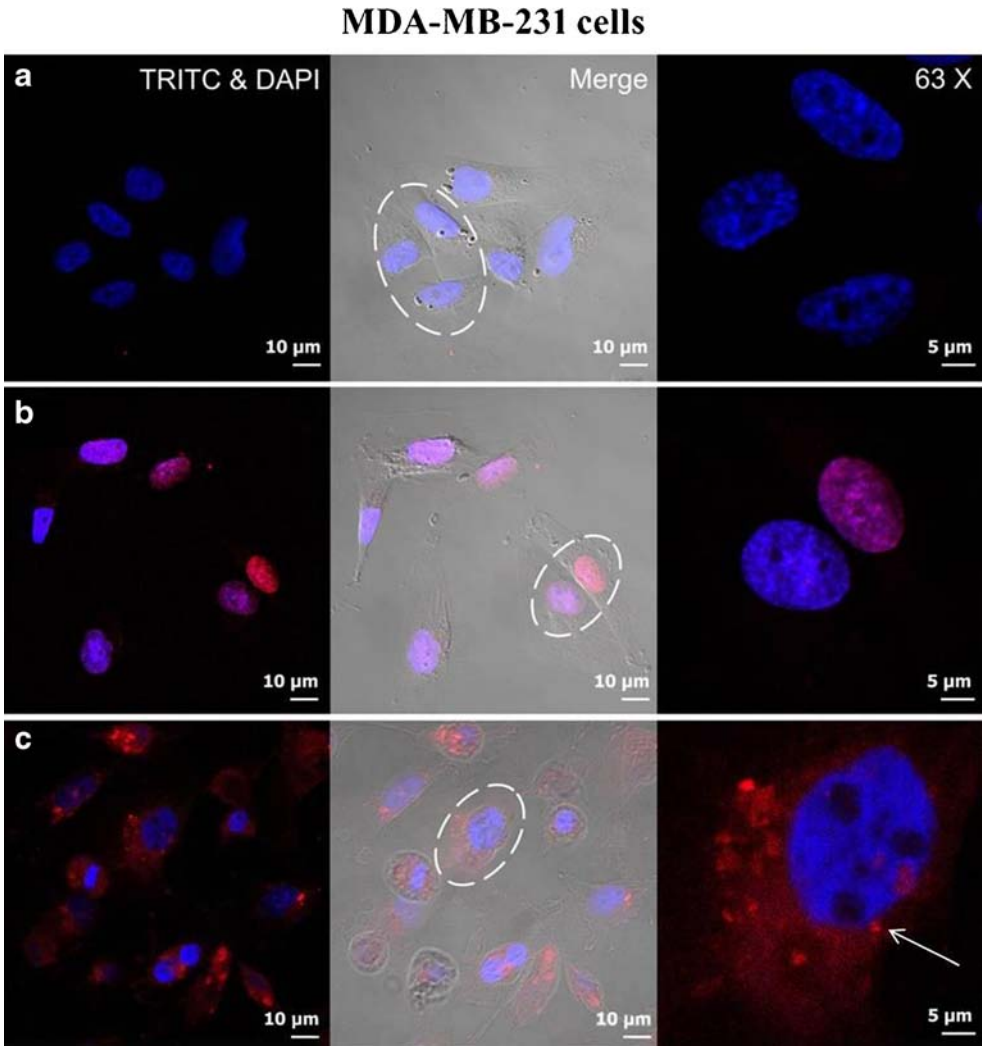

Fig. 12 Confocal microscopy images to show internalization of DOX in MDA-MB-231 cells. The nucleus was stained by using the DNA-binding dye DAPI (*blue*) after the cell fixation process. A TRITC filter responding to red fluorescence signals was employed to recognize the location of DOX with red fluorescence. (**a**) Untreated cells, (**b**) cells treated by free DOX, (**c**) cells treated with PEGylated, PLH coated and DOX carrying NCs (PEG-PLH-PSA-DOX-NCs). The *arrow* in 12-c shows concentrated red fluorescence points, which most likely represent encapsulated DOX.

primarily enters into the MDA-MB-231cells and the drug was easily detected inside the nuclei (Fig. 12b), in agreement with previous studies [11]. For cells exposed to PLH coated NCs (PEG-PLH-PSA-DOX-NCs), a significant increase in DOX fluorescence intensity was evident in the cancer cells (Fig. 12c). The concentrated foci of red fluorescence is considered most likely to represent DOX still encapsulated within cell internalized NPs, probably within endocytic organelles (as evident in Fig. 13). In addition the entire cytoplasm was labeled uniformly red, arguing for a general release of DOX into the cytoplasm. However, in contrast to the free DOX treatment, there was little evidence of DOX from NPs accumulating in the nucleus. We can offer two possible explanations for this unexpected observation, in light of the fact that the NP-DOX is able to kill the cells. First, sufficient toxic concentrations of DOX may bind to DNA to kill the cells without giving an obvious co-localization by light microscopy. Alternatively, the DOX released into the cytoplasm from NPs may kill the cells by a cytoplasmically-initiated mechanism [27] for example related to lysosome lysis [33, 34]. Further studies are needed to address this issue.

As a consequence of the high water-solubility of DOX, an alternative scenario to explain the high cytoplasmic concentration of DOX is that the drug may be released from NPs in the culture medium (before NP internalization) and then be taken up by the cells as free drug (this would be the scenario for the extracellular release of drug inside tumors). To confirm whether or not NCs are taken up into the cells, cellular internalization of NCs was visualized by staining them with fluorescent reagent coumarin-6 (green fluorescence reagent). In order to achieve this, polysebacic anhydride was labeled by the poorly water-soluble coumarin-6 before the preparation of the nanocapsules. DOX loaded NCs were prepared from coumarin-6-labeled polymer and then coated by PLH and modified by PEG as was done earlier. The cellular uptake of labeled NCs was examined on MDA-MB-231 cells. The cells were treated with PBS and PBS containing DOX loaded and coumarin-6-labeled NCs.

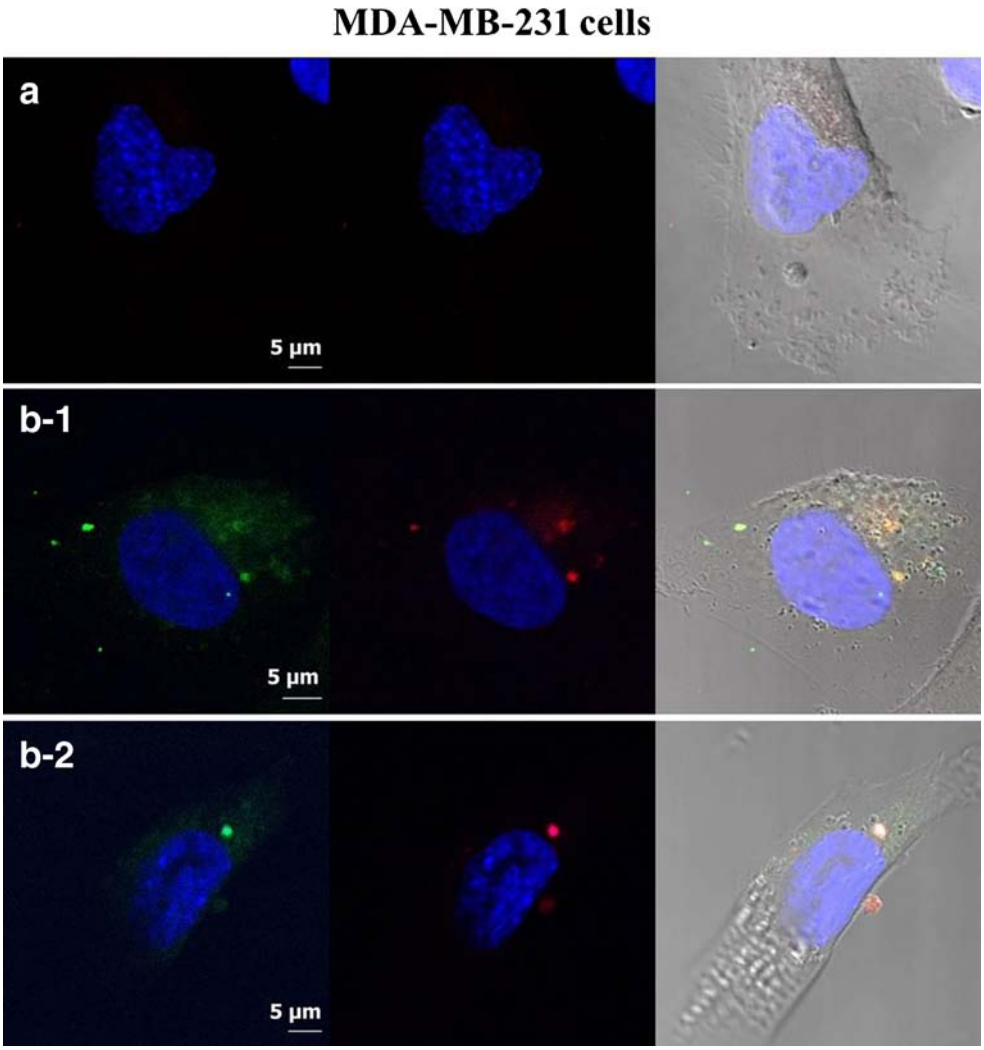

Fig. 13 Confocal microscopy images to show cellular uptake of nanoparticles by MDA-MB-231 cells. The nucleus was stained by using the DNA-binding dye DAPI (*blue*) after cell fixation. A TRITC filter responding to red fluorescence signals was employed to recognize the location of DOX with red fluorescence. Nanocapsules were labeled by coumarin-6 (*green*). (**a**) Cells treated with PBS, (**b-1** and **ba2**) cells treated with NCs PEGylated, PLH coated, DOX carrying and coumarin-6-labeled.

To prevent release of the poorly water-soluble coumarin-6 in cell culture medium, the cells were fixed one day after treatment. Figure 13 shows microscope images related to MDA-MB-231 cells treated by PBS (Fig.13a) and coumarin-6-labeled NCs (Fig. 13b-1 and b-2).

The images in the left column illustrate pictures obtained by the green fluorescence filter that is selective for coumarin-6. The middle column depicts images obtained by the TRITC red fluorescence filter that responds to DOX. Images in the right column show merged images of green, red and phase contrast with DAPI filters. As shown in Fig. 13b-1 and b-2, the green fluorescent particles can be observed after 6 h of incubation with coumarin-6 labeled nanocapsules. This result shows that the prepared nanocapsules (PEG-PLH-PSA-DOX-NCs) can be internalized into MDA-MB-231 cells. In this figure, the nanoparticles are observed to be distributed throughout the cytoplasm of the cells indicating that after internalization the NCs do not localize only in lysosomes but rather get distributed in the cytoplasm.

Macrophage Uptake

In the last decades, the *in vivo* applications of polymeric drug delivery systems have changed from macro-size (≥1 mm) to micro (5–20 µm) and finally to nano (100–1,000 nm) [35, 36]. These nano-sized particles exhibit significant advantages for nanomedical applications. For instance they can be investigated without any surgery [37], applied for multi-drug delivery [38], and targeted to tumor site *via* EPR effect [39]. Some negative aspect of these carriers that may limit their clinical application is their uptake by phagocytic cells, leading to their clearance from the blood circulation [40]. PEGylation (using poly ethylene glycol) has been known to reduce uptake of nanoparticles by macrophages. PEG molecules either bonded or adsorbed to the surface of nanoparticles inhibit blood protein absorption by steric hindrance and repulsion effects of PEG chains [41].

In this study, the influence of PEGylation on the macrophage uptake was determined by using THP-1 human monocyte cells differentiated to macrophages by using 10 µM TPA

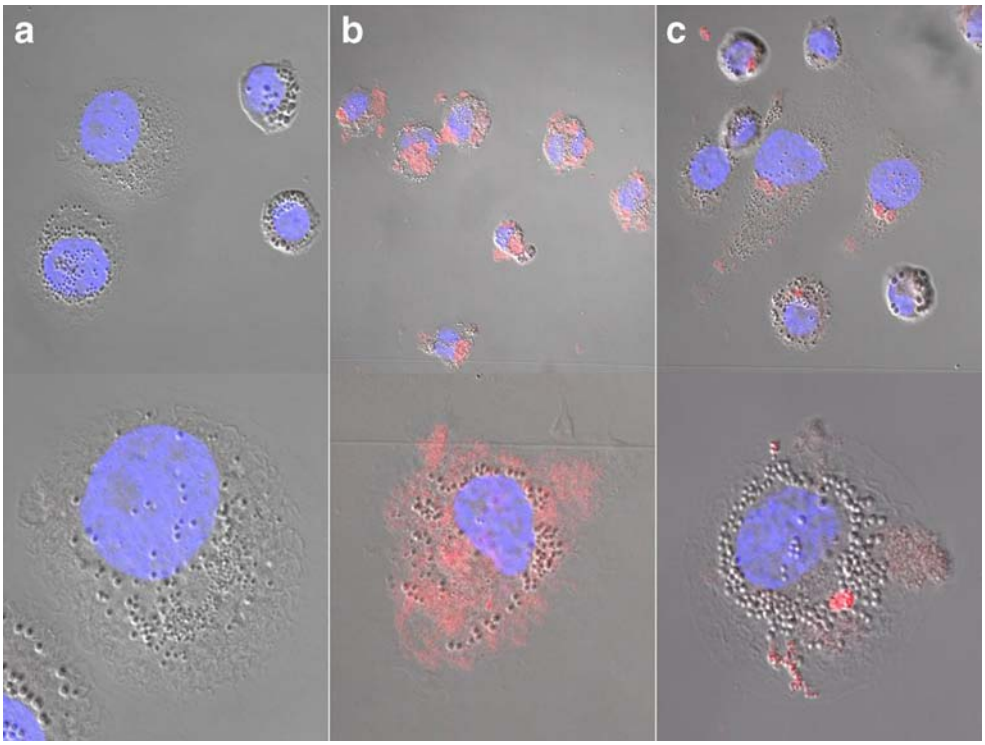

Fig. 14 Confocal microscopy images for HTP-1 macrophage uptake in (**a**) control cells, (**b**) non-PEGylated NCs (PLH- PSA-DOX- NCs), (**c**) PEGylated and PLH coated NCs (PEG-PLH- PSA-DOX- NCs).

(12-O-tetradecanoyl-phorbol-13-acetate). The macrophage uptake was analyzed "indirectly" by relating the fluorescence intensity coming from encapsulated DOX to the amount of NPs taken up by macrophages. In order to quantify this difference, macrophages were treated with PEGylated and non-PEGylated beads. Confocal micrographs were acquired and the total DOX fluorescence was analyzed as shown in Fig. 15 (see materials and methods). In this experiment, PBS treated macrophages were used as the control group. Confocal micrographs of THP-1 monocytes are presented in Fig. 14. The bubbles surrounding all the cells are an artifact happened during the embedding process. Figure 14b represents non-PEGylated NCs. The more concentrated red parts seen in non-PEGylated sample are attributed to more internalization of NPs into macrophages. This effect arises from loaded DOX inside the NCs. By comparing the pictures of PEGylated NCs (Fig. 14c) with non-PEGylated (Fig. 14b) particles, it is seen that more DOX fluorescence intensity is observed in macrophages treated by non-PEGylated NCs relative to PEGylated ones. This result was linked to more internalization of non-PEGylated NCs by phagocytic pathways.

DOX release from non-PEGylated particles in macrophages was significantly higher than PEGylated nanocapsules. The data are shown as the total fluorescence of DOX relative to the individual nuclear DAPI staining. Error bars represent the standard deviation and the significance $p \leq 0.0014$ by a Mann–Whitney test.

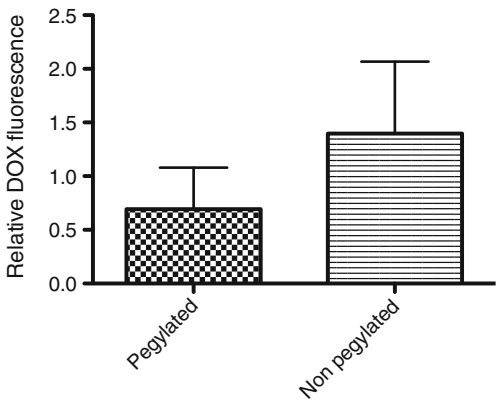

Fig. 15 Image J analysis of macrophages treated by PEGylated and Non PEGylated NCs.

CONCLUSION

Nanocapsules prepared in this work exhibited high loading capacity without any modification to allow efficient encapsulation of water-soluble DOX. Nevertheless, we have already high loading capacity in comparison to some previous studies [20–22]. The size of the prepared nanocapsules is hypothesized to be suitable for further exploitation of the EPR effect for passive targeting into tumors. Another important property of our nanocapsules is the application of polysebacic anhydride as a base matrix. This study showed that polysebacic anhydride exhibits promising properties for internalization of DOX, which is useful for cancer cells that exhibit drug

resistance. This biodegradable polymer displays surface erosion, leading to the release of encapsulated drug *via* a controlled pattern of behavior. Collectively, our results demonstrate that the pH responsive poly (L-histidine) coated nanocapsules based on PSA are promising vehicles for the intracellular delivery of an anticancer agent that are worthy of further studies towards eventual clinical testing.

ACKNOWLEDGMENTS AND DISCLOSURES

This research was supported by The Scientific and Technological Research Council of Turkey (TUBITAK) with grant No: 111M385. GG and BN were supported by funding from the Norwegian Cancer Society.

REFERENCES

1. Muvaffak A, Gürhan I, Hasirci N. Prolong cytotoxic effect colchicines released from biodegradable microspheres. J Biomed Mater Res B Appl Biomater. 2004;71B(2):295–304.
2. Chen Y, Wan Y, Wang Y, Zhang H, Zhijun J. Anticancer efficacy enhancement and attenuation of side effects of doxorubicin with titanium dioxide nanoparticles. Int J Nanomedicine. 2011;6:2321–6.
3. Torchilin VP. Passive and active drug targeting: drug delivery to tumors as an example. Handb Exp Pharmacol. 2010;197:3–53.
4. Tian L, Bae YH. Cancer nanomedicines targeting tumor extracellular pH. Colloids Surf B: Biointerfaces. 2012;99:116–26.
5. Johnson RP, Chung CW, Jeong Y, Kang DH, Suh H, Kim I. Poly(L-histidine)-tagged 5-aminolevulinic acid prodrugs: new photosensitizing precursors of protoporphyrin IX for photodynamic colon cancer therapy. Int J Nanomedicine. 2012;7:2497–512.
6. Bello RM, Midoux P. Histidylated polylysine as DNA vector: elevation of the imidazole protonation and reduced cellular uptake without change in the polyfection efficiency of serum stabilized negative polyplexes. Bioconjug Chem. 2001;12(1):92–109.
7. Lee ES, Shin HJ, Na K, Bae YH. Poly(L-histidine)–PEG block copolymer micelles and pH-induced destabilization. J Control Release. 2003;90(3):363–74.
8. Lee ES, Na K, Bae YH. Doxorubicin loaded pH-sensitive polymeric micelles for reversal of resistant MCF-7 tumor. J Control Release. 2005;103(2):405–18.
9. Kim D, Lee ES, Oh KT, Gao ZG, Bae YH. Doxorubicin-loaded polymeric micelle overcomes multidrug resistance of cancer by double-targeting folate receptor and early endosomal pH. Small. 2008;4(11):2043–50.
10. Leong KW, Brott BC, Langer R. Bioerodible polyanhydrides as drug-carrier matrices. I: characterization, degradation, and release characteristics. J Biomed Mater Res. 1985;19(8):941–55.
11. Zhang C, Wang W, Liu T, Wu Y, Guo H. Doxorubicin-loaded glycyrrhetinic acid-modified alginate nanoparticles for liver tumor chemotherapy. Biomaterials. 2012;33(7):2187–96.
12. Liang Y, Xiao L, Zhai Y, Xie C, Deng L, Dong A. Preparation and characterization of biodegradable poly(sebacic anhydride) chain extended by glycol as drug carrier. J Appl Polym Sci. 2013;127(5):3948–53.
13. Hasirci V, Yilgor P, Endogan T, Eke G, Hasirci N. Polymer fundamentals: polymer synthesis. In: Ducheyne P, Healy K, Hutmacher DE, Grainger DW, Kirkpatrick CJ, editors. Comprehensive biomaterials. New York: Elsevier Science; 2011. p. 349–71.
14. Benns J, Choi JS, Mahato RI, Park JS, Kim SW. pH-sensitive cationic polymer gene delivery vehicle. Bioconjug Chem. 2000;11(5):637–45.
15. Shen E, Pizsczek R, Dziadul B, Narasimhan B. Microphase separation in bioerodible copolymers for drug delivery. Biomaterials. 2001;22(3):201–10.
16. Liu J, Qju Z, Wang S, Zhou L, Zhang S. A modified double-emulsion method for the preparation of daunorubicin-loaded polymeric nanoparticle with enhanced in vitro anti-tumor activity. Biomed Mater. 2010;5(6):065002.
17. ImageJ. 2013 December 15. Available from: http://rsb.info.nih.gov/ij [Website].
18. Huynh NT, Morille M, Bejaud J, Legras P, Vessieres A, Jaouen G, *et al.* Treatment of 9L gliosarcoma in rats by ferrociphenol-loaded lipid nanocapsules based on a passive targeting strategy via the EPR effect. Pharm Res. 2011;28(12):3189–98.
19. Bankar VH, Gaikwad PD, Pawar SP. Novel sustained release drug delivery systems: review. IJPRD. 2011;3(12):1–14.
20. Khandekar SV, Kulkarni MG, Devarajan PV. Polyaspartic acid functionalized gold nanoparticles for tumor targeted doxorubicin delivery. J Biomed Nanotechnol. 2014;10:143–53.
21. Wong H, Bendayan R, Rauth AM, Wu XY. Development of solid lipid nanoparticles containing ionically complexed chemotherapeutic drugs and chemosensitizers. J Pharm Sci. 2004;93(8):1993–2008.
22. Chavanpatil MD, Khdair A, Patil Y, Handa H, Mao G, Panyam J. Polymer-surfactant nanoparticles for sustained release of water-soluble drugs. J Pharm Sci. 2007;96(12):3379–89.
23. Yousefpour P, Atyabi F, Farahani EV, Sakhtianchi R, Dinarvand R. Polyanionic carbohydrate doxorubicin-dextran nanocomplex as a delivery system for anticancer drugs: in vitro analysis and evaluations. Int J Nanomedicine. 2011;6:1487–96.
24. Bajelan E, Haeri A, Vali AM, Ostad SN, Dadashzadeh S. Co-delivery of doxorubicin and PSC 833 (Valspodar) by stealth nanoliposomes for efficient overcoming of multidrug resistance. J Pharm Pharm Sci. 2012;15(4):568–82.
25. Patchornik A, Berger A, Katchalski E. Poly (L-histidine). J Am Chem Soc. 1957;79:5227–30.
26. Chen AZ, Chen MY, Wang SB, Huang XN, Liu YG, Chen ZX. Poly(L-histidine)-chitosan/alginate complex microcapsule as a novel drug delivery agent. J Appl Polym Sci. 2012;124(5):3728–36.
27. Patel AG, Kaufmann SH. How does doxorubicin work? eLife. 2012;1:e00387.
28. Hande RK. Topoisomerase II, inhibitors. Updat Cancer Ther. 2008;3(1):13–26.
29. Denard B, Lee C, Ye J. Doxorubicin blocks proliferation of cancer cells through proteolytic activation of CREB3L1. elife Camb. 2012;18(1):e00090.
30. Varkouhi AK, Scholte M, Storm G, Haisma HJ. Endosomal escape pathways for delivery of biologicals. J Control Release. 2011;151(3):220–8.
31. Gao Y, Chen Y, Ji X, He X, Yin Q. Controlled intracellular release of doxorubicin in multidrug-resistant cancer cells by tuning the shell-pore sizes of mesoporous silica nanoparticles. ACS Nano. 2011;5(12):9788–98.
32. Uster PS, Deamer DW. pH-dependent fusion of liposomes using titratable polycations. Biochemistry. 1985;24(1):1–8.
33. Thorn CF, Oshiro C, Marsh S, Hernandez-Boussard T, McLeod H, Klein TE, *et al.* Doxorubicin pathways: pharmacodynamics and adverse effects. Pharmacogenet Genomics. 2011;21(7):440–6.
34. Mizutani H, Oikawa S, Hiraku Y, Murata M, Kojima M, Kawanishi S, *et al.* Distinct mechanisms of site-specific oxidative DNA damage by doxorubicin in the presence of copper(II) and NADPH-cytochrome P450 reductase. Cancer Sci. 2003;94:686–91.
35. Kipper MJ, Shen E, Determan A, Narasimhan B. Design of an injectable system based on bioerodible polyanhydride microspheres for sustained drug delivery. Biomaterials. 2002;23(22):4405–12.

36. Muvaffak A, Gürhan I, Hasirci N. Cytotoxicity of 5-fluorouracil entrapped in gelatin microspheres. J Microencapsul. 2004;21(3): 293–306.
37. Berkland C, Kipper MJ, Narasimhan B, Kim KK, Pack DW. Microsphere size, precipitation kinetics and drug distribution control drug release from biodegradable polyanhydride microspheres. J Control Release. 2004;94(1):129–41.
38. Hsu W, Lesniak MS, Tyler B, Brem H. Local delivery of interleukin-2 and adriamycin is synergistic in the treatment of experimental malignant glioma. J Neurooncol. 2005;74(2):135–40.
39. Maeda H, Wu J, Sawa T, Matsumura Y, Hori K. Tumor vascular permeability and the EPR effect in macromolecular therapeutics: a review. J Control Release. 2000;65(1–2):271–84.
40. Soma CE, Dubernet C, Barratt G, Benita S, Couvreur P. Investigation of the role of macrophages on the cytotoxicity of doxorubicin and doxorubicin-loaded nanoparticles on M5076 cells in vitro. J Control Release. 2000;68(2):283–9.
41. Owens DE, Peppas NA. Opsonization, biodistribution, and pharmacokinetics of polymeric nanoparticles. Int J Pharm. 2006;307(1):93–102.