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Multifunctional silica nanoparticles for targeted delivery of hydrophobic imaging and therapeutic agents

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ABSTRACT

This article reports the development of a multifunctional silica nanoparticle system for targeted delivery of hydrophobic imaging and therapeutic agents. Normally, silica nanoparticles have been widely used to deliver hydrophilic drugs such as doxorubicin while difficult to carry hydrophobic drugs. A strategy for loading hydrophobic drugs onto silica nanoparticles via covalent attachment was developed in this study as a universal strategy to solve this problem. Docetaxel, one of the most potent therapeutics for cancer treatment is selected as a model hydrophobic drug and quantum dots (QDs) are used as a model imaging agent. Such a multifunctional delivery system possesses high drug loading capacity, controlled drug release behavior and stable drug reservation. A mixed layer of polyethylene glycol conjugated phospholipids is formed on the nanoparticle surface to further enhance the biocompatibility and cell fusion capability of the delivery system. Folic acid as ligand is then conjugated onto the surface layer for targeting. Such a multifunctional system for targeting, imaging and therapy is characterized and evaluated *in vitro*. Fluorescent confocal microscopy is used to monitor the cellular uptake by specific cancer cells. Cytotoxicity studies are conducted by using MTT assay.

1. Introduction

Silica nanoparticles (SNPs) have attracted extensive attention for biomedical applications (Vivero-Escoto et al., 2010). Delivery of anticancer drugs, as reported by a lot of studies, is one of the important fields where silica nanoparticles show outstanding performance (Barbé et al., 2004; Slowing et al., 2008; Zhao et al., 2010; He and Shi, 2011). Their hydrophilic surface, inert property and biocompatibility makes the nanoparticles ideal candidate to deliver therapeutics. However, the drugs that could be delivered by SNPs are hydrophilic in most cases so far in the literature (Chen et al., 2010; Knežević et al., 2011). Since the majority of potent anticancer drugs are in hydrophobic forms, applications of SNPs for anticancer drugs delivery have been largely restricted. It is thus highly essential to investigate the possibility of carrying hydrophobic drugs by SNPs for drug delivery purpose.

To date, the convenient and direct method developed by other research groups to load hydrophobic drugs in SNPs is to immerse mesoporous silica nanoparticles in highly concentrated or saturated solution of hydrophobic drugs, thus certain quantity of the drugs would be expected to be loaded into the silica matrix. This method takes advantage of large surface area and porous interior of the silica nanoparticles that could be used as reservoirs to store the hydrophobic drugs (Lu et al., 2007; Chang et al., 2010; Lay et al., 2010; Vivero-Escoto et al., 2009). The hydrophobic drugs would thus be loaded into the particles via physical adsorption. However, it is obvious that such method is not in high reproducibility due to the batch-to-batch variance of the amount of the loaded drugs that could not be adequately and precisely controlled. Moreover, such method might lead to insufficient drug loading and instable drug encapsulation (or say, dissociation of the drugs from the matrix). The waste of drug is also a problem: high concentration solutions of the drugs have to be prepared while the loading efficiency is unknown.

Therefore, alternative solution should be designed to overcome this problem. We developed a novel and universal strategy to realize the loading and delivery of hydrophobic anticancer drugs by SNPs. The drug was associated with SNPs by covalent attachment on the surface of the particles. Docetaxel (DTX) was used as a model hydrophobic anticancer drug here, which has been widely employed as a potent agent to be effective to a wide spectrum of cancers (Engels et al., 2007).

In order to protect the attached drugs from the exposure from biological fluids in human bodies, the surface of the drug attached

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SNPs should be properly modified first. A mixed layer of phospholipids was applied to coat on the particles. Phospholipids are one of the ideal candidates to decorate nanoparticles (Feng and Huang, 2001; Feng et al., 2002; Feng, 2004). Recently, some research groups including our group have developed a series of phospholipid modified polymeric or silica nanoparticles, showing the prosperous application of phospholipids in drug delivery area (Salvador-Morales et al., 2009; Liu et al., 2010; Koole et al., 2008). Moreover, phospholipids are essential biomolecules in the structure and function of living matter, e.g. the membrane of human cells. Hence, effective interaction of the phospholipid coated SNP with cancer cells would be expected.

Active targeting provides promising approaches for the drug attached SNPs to reach and penetrate into the malignant cells with overexpression of the corresponding receptors on their surface, and then release the loaded DTX in a controlled and sustained manner (Cho et al., 2008; Wang et al., 2008). The targeted delivery can be realized by conjugating molecular ligand onto the phospholipid coated SNP surface, where phospholipid molecules provide the site of conjugation on their end functional groups. For instance, the carboxylic group on the ligand molecule can be conjugated with the reactive primary amine group of phospholipids (Torchilin, 2005; Allen and Moase, 1996; Yang et al., 2007). Folic acid was selected as the model molecular ligand for targeted delivery of the drugs to a large number of cancer cells of folate receptor overexpression such as certain breast cancer and ovarian cancer cells. DTX loaded SNPs would be wrapped by mixed phospholipids: the PEGylated lipids to facilitate stealth NP formulation to escape from recognition by the reticuloendothelial system (RES) and thus increase the systemic circulation time of the SNPs (Duncan, 2003; Yamamoto et al., 2001); and the folic acid conjugated, PEGylated lipids for targeted delivery purpose.

Herein, a novel silica based system was developed for delivery of hydrophobic anticancer drugs with DTX as a model drug, which can be loaded to the silica matrix by covalent attachment. Such a drug delivery system possesses high drug loading efficiency, controlled release behavior and stable drug reservation. The complex nanoparticles were entrapped in a layer of polyethylene glycol (PEG) conjugated phospholipids to keep the activity of DTX, further enhance the biocompatibility of the system and increase the cell fusion capability of the SNPs. Folic acid was conjugated as targeting ligand to achieve targeted drug delivery. Such a multifunctional silica-based hydrophobic drug delivery system was then characterized and evaluated *in vitro*. Quantum dots (QDs) were encapsulated into silica matrix as imaging agents (Gao et al., 2004) in this study to monitor the cellular uptake behavior of the SNPs in cancer cells.

2. Materials and methods

2.1. Materials

Docetaxel (anhydrous, 99.56%) was purchased from Shanghai Jinhe Bio-Technology Co. Ltd, China. Taxotere® was provided by National Cancer Center, Singapore. 4-Nitrophenylformamate (NPC), cethltrimethylammonium bromide (CTAB), tetraethyl orthosilicate (TEOS), (3-aminopropyl) triethoxysilane (APTES), sodium hydroxide, ethanol, dichloromethane (DCM), triethylamine (TEA), acetonitrile (ACN), dimethyl sulfoxide (DMSO), pyridine, phosphate buffered saline (PBS), folic acid, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay, trypsin–ethylenediaminetetraacetic acid (EDTA) solution and 4′,6-diamidino-2-phenylindole (DAPI) were all purchased from Sigma–Aldrich (St. Louis, MO, USA). 1,2-Distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (DSPE–PEG_{2k}) was provided by Lipoid GmbH (Ludwigshafen, Germany). Poly[ethylene glycol]-5000 bis-amine (PEG_{5k} bis-amine) was offered by Laysan Bio, Arab, AL, USA. 1,2-Distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[folate(polyethylene glycol)-5000] (DSPE–PEG_{5k}–FOL) was synthesized by carbodiimide chemistry as previously reported (Wu et al., 2006). Tween-80 was from ICN Biomedicals, Inc., OH, USA. Qdot 655 ITK organic quantum dots, fetal bovine serum (FBS) and penicillin–streptomycin solution was purchased from Invitrogen. Dulbecco's Modified Eagle's Medium (DMEM) was from Sigma. All solvents used in this study were HPLC grade. MCF7 breast cancer cells were provided by American Type Culture Collection (ATCC). The water used was pretreated with the Milli-Q® Plus System (Millipore Corporation, Bedford, USA).

2.2. Preparation of silica nanoparticles

For preparation of the silica nanoparticles, 5 ml aqueous CTAB solution (0.5 mM) was added into the mixture of 10 ml anhydrous ethanol and 40 μl of 2 M NaOH. 20 μl TEOS was added into the mixed solution. The final solution was stirred overnight under room temperature. The product was washed with ethanol and centrifuged for three times to remove the CTAB surfactant. For preparation of QD loaded silica nanoparticles, 200 μl of 1 μM QDs were firstly dissolved in 5 ml aqueous CTAB solution (0.5 mM) and stirred vigorously for 30 min. The resulting solution was added into 10 ml anhydrous ethanol and 40 μl of 2 M NaOH was added into the mixture. After the solution was mixed well, 20 μl TEOS was added. The final solution was stirred overnight under room temperature. The product was washed with ethanol and centrifuged for three times.

2.3. Surface modification of silica nanoparticles

For surface modification, the silica nanoparticles were dried and redispersed into 5 ml ethanol, 1 ml APTES was added into the solution. The mixture was heated to 60 °C under reflux for 3 h. After that, the solution was cooled down to room temperature and washed with ethanol for three times. The final nanoparticles were dissolved in ethanol for further use.

2.4. Conjugation of docetaxel onto the silica matrix

The conjugation was achieved by using widely applied NPC method as reported previously with slight modification (Yoo and Park, 2001; Zhang et al., 2007; Sánchez-Chaves et al., 2008). Docetaxel was activated first by 4-nitrophenylformamate (NPC) at the hydroxyl positions. Weighed amount of DTX and NPC with 1:4 as molar ratio were dissolved in DCM separately. The solution was mixed under ice bath. The mixture was stirred for 3 h under room temperature in nitrogen atmosphere with pyridine as catalyst. Solvent was removed by rotary-evaporator. The residue was then dissolved in anhydrous ethanol and mixed with the amine modified silica particles in room temperature under nitrogen protection with TEA for 24 h. After the reaction was complete, the drug conjugated particles were collected by centrifugation and further washed thrice by ethanol. All of the supernatant was collected. The pellets were re-suspended in ultrapure water for further use.

2.5. Coating of phospholipid layers

DSPE–PEG_{2k} and DSPE–PEG_{5k}–FOL in molar ratio of 85:15 (concentration as of 1 mg/ml) were mixed and dispersed in water. The above prepared particles were added into the dispersion and the mixture was vigorously stirred overnight. Lipid coated particles were collected by centrifugation followed by three times wash.

The pellets were re-suspended in ultrapure water for characterization and further use. For the particles without targeting effect, only DSPE–PEG_{2k} was used.

2.6. Characterization of the nanoparticles

The particle size and size distribution of the samples were measured by dynamic light scattering (DLS, 90Plus, Brookhaven Instruments Co., TX, USA) in aqueous medium. The surface charge was determined by Zetasizer (Malvern Nano ZS, Malvern Instruments Ltd., Worcestershire, UK) at room temperature in ultrapure water. The pH value and concentration of the sample dispersion were determined before measurement. The amount of docetaxel attached on the silica nanoparticles was measured by high performance liquid chromatography (HPLC, Agilent LC1100) with thrice repeat. A reversed phase Inertsil® ODS-3 column (250 mm × 4.6 mm, particle size 5 μm, GL Science Inc., Tokyo, Japan) was used. The quantity of free drug dissolved in the supernatant mentioned in Section 2.4 was firstly determined using 50% acetonitrile in water solution (volume ratio) as mobile phase. The column effluent was detected at 230 nm with a UV/VIS detector. The drug loading in the particles is thus calculated by subtraction of the drug amount in supernatant from that added for conjugation. The unit of drug load is mg drug per mg NPs. The emission spectra of the QD loaded silica particles in suspension were investigated using a photoluminescence spectrometer (PTI Quantamaster) at excitation of 480 nm.

2.7. Particle morphology

The shape and surface morphology of the particles were investigated by field emission scanning electron microscope (FESEM, JSM-6700F, JEOL, Japan) and field emission transmission electron microscope (FETEM, JEM-2200FS, JEOL, Japan). The layer of the NP powder was obtained on copper tape for FESEM and copper grid for FETEM by evaporating water under reduced pressure from the particle dispersion. For FESEM, the dried particles were then coated by platinum carried out by the Auto Fine Coater (JEOL, Tokyo, Japan).

2.8. Surface chemistry analysis

The existence of various elements of interest on the particle surface was confirmed by X-ray photoelectron spectroscopy (XPS, AXIS His-165 Ultra, Kratos Analytical, Shimadzu Corporation, Kyoto, Japan). The elements were identified according to the specific binding energy (eV), which was recorded from 0 to 1200 eV with pass energy of 80 eV under the fixed transmission mode. The data were processed by specific XPS software.

2.9. In vitro drug release

The drug loaded particles (silica–DTX–DSPE–PEG–FOL NPs) were dispersed in PBS (0.1 M, pH 7.4 or 5.0) containing 0.1% v/v Tween-80, which improves the solubility of docetaxel in water to simulate the sink condition. The dispersion in tubes was then put in an orbital shaker shaking at 120 rpm in a water bath at 37 °C. At designated time intervals, the tube of the suspension was centrifuged and the pellet was drained and re-suspended in fresh medium to continue the drug release process. The drug released in the supernatant was extracted by DCM and transferred in the same mobile phase as used in measuring drug load. After the evaporation of DCM, docetaxel quantity was determined by the same HPLC procedure as mentioned above. The error bars were obtained from triplicate samples.

2.10. Cell culture

MCF7 breast cancer cells, which are of high folate receptor overexpression, were employed in this study. DMEM supplemented with 10% FBS and 1% penicillin–streptomycin solution was utilized as the cell culture medium. Cells were cultivated in humidified environment at 37 °C with 5% CO_2 (Sanyo incubator). Before experiment, the cells were pre-cultured until confluence was reached to 75%.

2.11. In vitro cellular uptake: confocal microscopy study

Cells were cultivated in the 4-well coverglass chamber (LAB-TEK®, Nagle Nunc., IL, USA) for 1 day. The particles dispersed in the cell culture medium at concentration of 0.250 mg/ml were added into the wells. Cells were washed three times after incubation for 2 h and then fixed by 70% ethanol for 20 min. The cells were further washed thrice by PBS (pH 7.4) and the nuclei were then stained by DAPI for 45 min. The fixed cell monolayer was finally washed thrice by PBS and observed by confocal laser scanning microscope (CLSM, Olympus Fluoview FV1000).

2.12. In vitro cytotoxicity

For cytotoxicity measurement, cells were incubated in 96-well transparent plates (Costar, IL, USA) at 5×10^3 cells/well (0.1 ml) and after 12 h, the old medium was removed and the cells were incubated with prepared doses of docetaxel for 24, 48 and 72 h incubation. The particles were sterilized with UV irradiation for 30 min prior to using. MTT assay was used to measure the cell viability at given time intervals. The absorbance of the wells was measured by the microplate reader with wavelength at 570 nm and reference wavelength at 620 nm. Cell viability is defined as the percentage of the absorbance of the wells containing the cells incubated with the samples over that of the cells only.

2.13. Statistical analysis

Data were expressed as the means with 95% confidence intervals. Statistical tests were performed with the Student's *t* test. For all tests, *P* values less than 0.05 were considered to be statistically significant. All statistical tests were two-tailed.

3. Results and discussion

3.1. Preparation of the DTX loaded, phospholipid coated SNPs

The illustrative structure of the complex SNPs was shown in Fig. 1. The particles were firstly prepared by modified Stöber's method with CTAB as surfactant. The method can also be used to produce the silica nanoparticles encapsulating QDs that would be utilized to visualize the interaction of the SNPs with cancer cells (Hu et al., 2009). After removing CTAB surfactants, mesoporous SNPs can be produced as the matrix for hydrophobic drug loading. Yet what is worth to be pointed out is that both the surface and interior of the SNPs are hydrophilic, therefore alternative strategy should be designed to realize hydrophobic drug loading on SNPs. We used the covalent conjugation strategy to attach DTX on the SNPs. DTX would be activated first and further coupled with the surface functionalized SNPs. The free hydroxyl groups of DTX on C2′-, C7- or/and C10-positions could be made reactive by NPC and subsequently conjugated on the silica particles via the linkage with the free amine groups attached on silica surface. The design could be understood more easily in a simpler way: DTX was converted to DTX–PEG conjugation as a prodrug (Du et al., 2007; Liu et al., 2008). In this case, we conjugated SNPs which are similar to PEG on DTX molecules

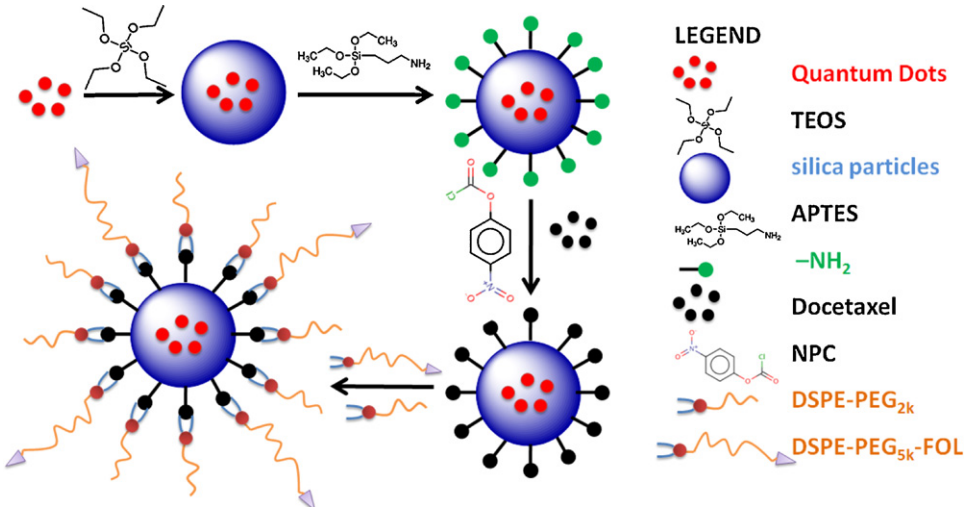

Fig. 1. Schematic representation of the production of the silica drug delivery system composed of QDs encapsulated in the silica matrix, DTX conjugated on the silica surface and a PEG functionalized phospholipid shell.

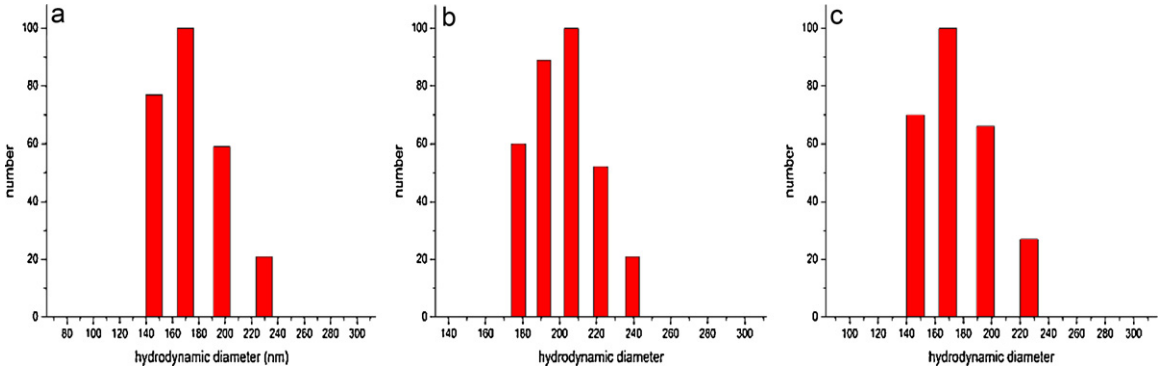

Fig. 2. Hydrodynamic diameter of (a) silica particles, (b) silica–DTX–DSPE–PEG NPs, and (c) silica–DTX–DSPE–PEG–FOL NPs.

to produce the DTX–SNPs prodrug and in turn, DTX loaded SNPs. Furthermore, in order to protect the attached drugs from the exposure from physiological fluids and keep the activity of the drugs, the PEG conjugated phospholipid (DSPE–PEG_{2k}) layer was, afterwards, coated on the drug loaded particles (silica–DTX–DSPE–PEG NPs). The interaction of the hydrophobic carbon rings on DTX molecules with the hydrophobic carbon chains on phospholipids formed the adsorption of the lipids on the attached DTX, and meanwhile, resulted in the coating of lipid layers on the particles. PEG attached phospholipids were recently used to formulate polymeric or silica nanoparticles to achieve enhanced biocompatibility, circulation lifetime and cellular interaction (Salvador-Morales et al., 2009; Liu et al., 2010; Koole et al., 2008). In addition, the coating of lipid layer provides the feasibility to introduce molecular probes for targeting effect. Folic acid as the targeting ligand was conjugated on PEG attached lipids to form DSPE–PEG_{5k}–FOL. Two types of lipids were mixed to coat on the drug loaded silica particles (silica–DTX–DSPE–PEG–FOL NPs), resulting in the combination of merits offered by PEG and phospholipids, as well as protection or shielding on the drug molecules.

3.2. Characterization of the nanoparticles

The sizes measured by laser light scattering (LLS) of those particles were all in the range of 140–240 nm (Fig. 2). Compared the size distribution of the particles before and after lipid coating, the number average size became slightly larger with the presence of the lipids. The zeta potential values are important indication of the stable dispersion of colloidal particles in aqueous medium. Initially, from Table 1, the surface charge of SNPs was measured to be positive, mainly due to the exposure of hydroxyl groups on silica surface in pH 5.5 environments. After surface functionalization by APTES, which is an amine contained silane coupling agent, the surface charge was even more positive. Subsequently, the coating of lipid layers on DTX loaded particles distinctively decreased the surface charge to be negative, which is partially due to the consumption of amine groups by activated DTX as well as the coating of the surface by negatively charged lipid molecules. The values of the surface charges of the lipid coated SNPs with DTX loaded demonstrated the stability in aqueous medium. Meanwhile, the drug load was measured as 815.8 ± 7.4 μg DTX per mg particles. This means that the drug loading of such nanoparticles was as high as 81.5%, which is much higher than that given in the literature for the mesoporous systems (Chang et al., 2010; He and Shi, 2011; Knežević et al., 2011). The high drug load value could be attributed to the covalent attachment of DTX with silica matrix rather than physical adsorption. Desired quantity of DTX could be loaded as long as corresponding

Table 1
Zeta potential of various particles. Data represent mean ± SD, $n = 3$.

Sample	Zeta potential (mV)
SNPs	5.34 ± 2.72
APTES decorated SNPs	41.50 ± 6.03
Silica–DTX–DSPE–PEG NPs	−21.40 ± 4.21
Silica–DTX–DSPE–PEG–FOL NPs	−26.20 ± 4.36

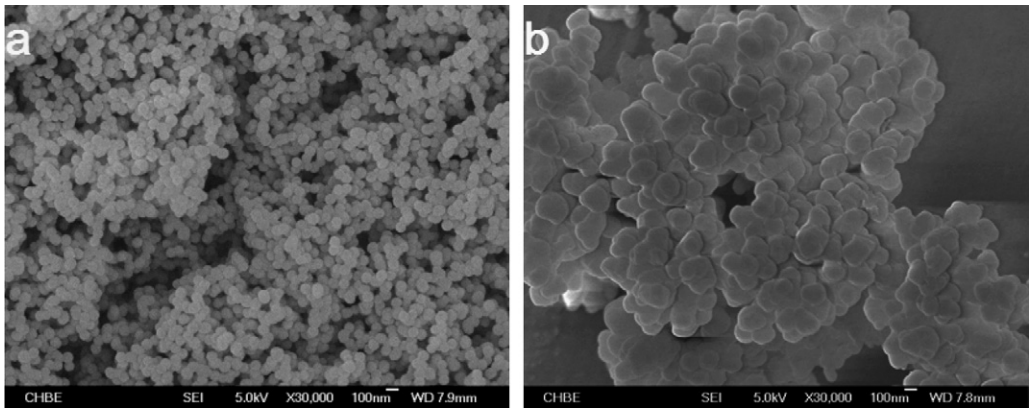

Fig. 3. Representative FESEM image of silica NPs (a) and silica–DTX–DSPE–PEG–FOL NPs (b).

amount of free amine groups could be provided. Since the surface density of amine groups from APTES on silica surface is able to be as high value, high drug load could also be realized. The conjugation method produced drug loaded SNPs via convenient synthesis strategy and could be widely applied to easily link other hydrophobic drugs with free hydroxyl or carboxyl groups onto amine decorated silica particles, showing huge potential application of this strategy.

3.3. Particle morphology

The spherical morphology of the silica particles with uniform size was shown in the field-emission scanning electron microscopy (FESEM) image (Fig. 3a). The size revealed under FESEM was in agreement with the results obtained from LLS test. Such SNPs were subsequently decorated by amine groups through coupling of APTES. DTX was followed to be activated by NPC chemistry and anchored on the silica matrix. The FESEM image in Fig. 3b displayed the morphology of the mixed phospholipid coated docetaxel loaded silica nanoparticles (silica–DTX–DSPE–PEG–FOL NPs). The surface of the particles can be seen as more sticky due to the presence of the lipids. The adsorption of lipids on the surface could be briefly hypothesized as the hydrophobic interaction of the carbon chains on lipid molecules with the hydrophobic backbone of DTX.

The transmission electron microscopy (TEM) was also employed to visualize the presence of QDs in the QDs loaded silica particles. The small dots as QDs of higher contrast can be seen inside the silica shell of lower contrast (Fig. 4a). Multiple QDs were encapsulated inside the silica shell. The photoluminescence spectra reveal the integrity of QDs after encapsulation into silica matrix since virtually no shift of maximum emission wavelength occurred (Fig. 4b). Such QDs loaded silica NPs were also subsequently attached with DTX and coated by phospholipids as imaging agents to monitor the cellular uptake behavior of the drug delivery system.

3.4. Surface chemistry analysis

The surface chemistry of the prepared particles was investigated afterwards to confirm the attachment of DTX on silica as well as the coating of phospholipids. Fig. 5a illustrated the wide scan of the DTX loaded silica particle surface. This spectrum showed the presence of nitrogen on the particles (around 396.5 eV), owning to the coupling of amine containing agent (APTES) with silica core. From Fig. 5b, it was shown that the presence of carbon in C–O and C=O bonds was identified on the sample surface (around 285.8 eV). Since only DTX contains such bonds (Fig. 5c) (neither silica nor APTES contains), it can be concluded that DTX was conjugated on the silica matrix. The PEG conjugated phospholipid layers were, afterwards, coated on the drug loaded particles, as proven by the presence of phosphorous signal peak in XPS spectra, detected from silica–DTX–DSPE–PEG–FOL NPs (Fig. 5d). The XPS spectra confirmed the successful coating of lipid layers on the particles.

3.5. In vitro drug release

In this study, the conjugation of DTX on silica matrix was achieved by an acid labile ester bond, which has been extensively used to produce prodrug of DTX. The linking bond could be easily cleaved in acidic environment, providing the possiblity of controlled drug release (Du et al., 2007). Such design could prevent drug from exposure of biological materials in bloodstream and

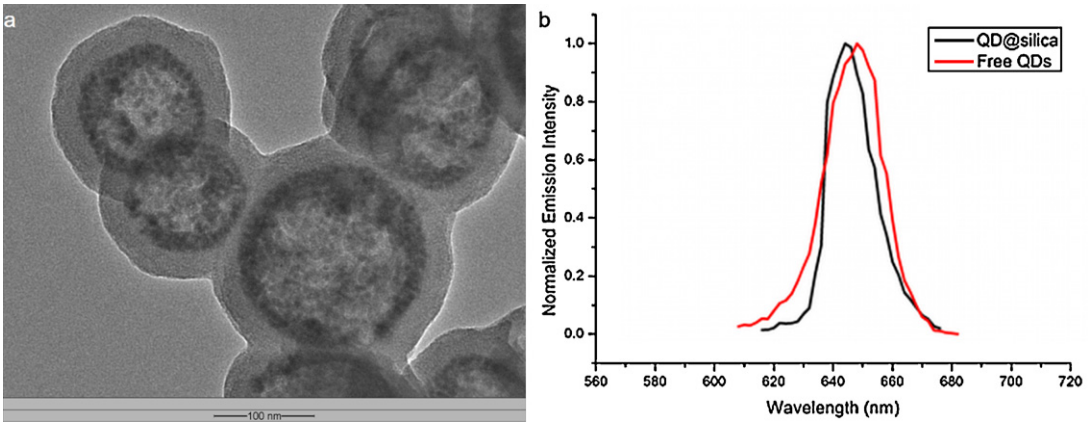

Fig. 4. (a) FETEM image of QDs loaded silica particles and (b) measurement of maximum emission wavelength of QDs encapsulated in silica matrix.

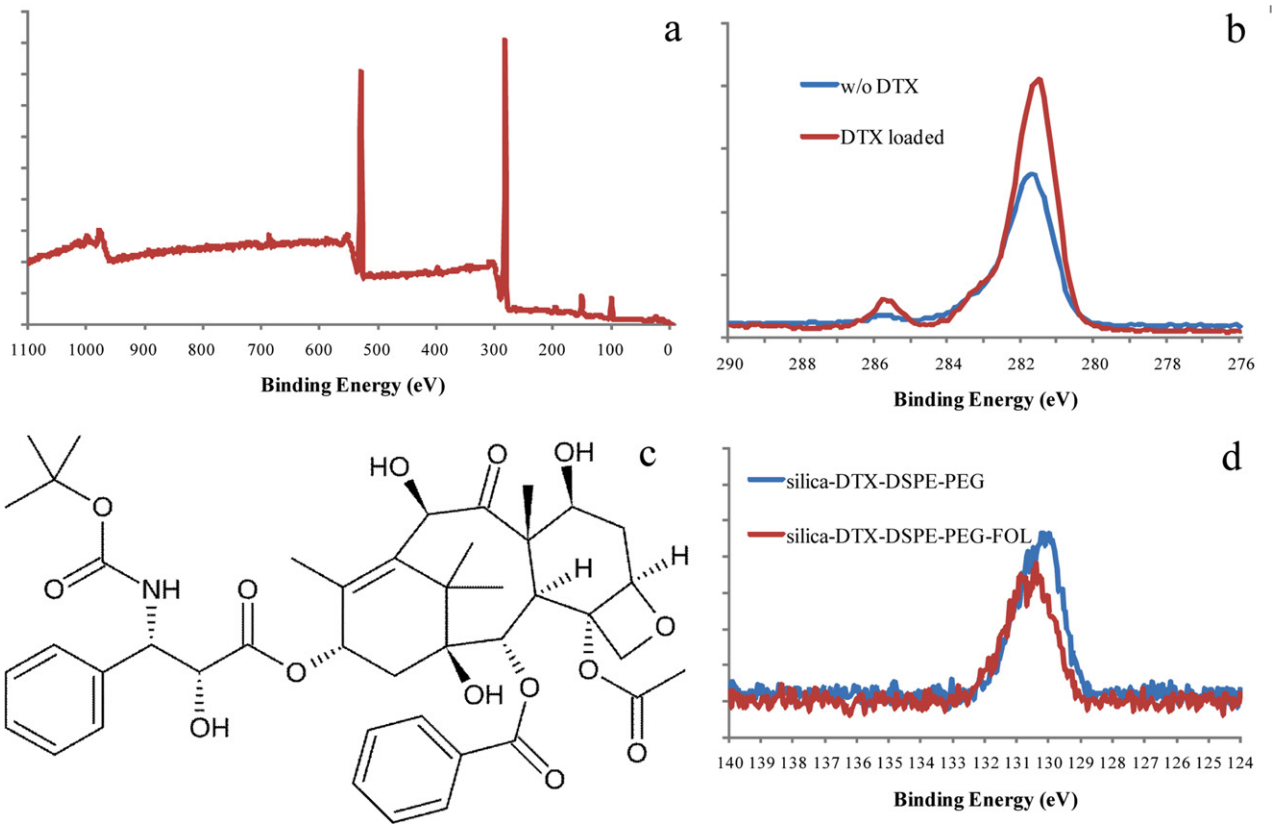

Fig. 5. Representative XPS spectrum of DTX loaded silica particles (a) and C 1s peaks from the APTES decorated SNPs and DTX loaded silica particles (b). Figure (c) shows the molecular structure of DTX which contains C–O and C=O bonds. Figure (d) is the representative XPS spectrum of P 2p peaks from the silica–DTX–DSPE–PEG NPs and silica–DTX–DSPE–PEG–FOL NPs.

release at undesirable sites but a rapid release somewhere with acidic environment such as endosomes or lysosomes in cytoplasm. Therefore drug release can be promoted after the particles are taken up by cancer cells. Fig. 6 lists the controlled DTX release from silica–DTX–DSPE–PEG–FOL NPs at pH = 5.0 or pH = 7.4 after 1, 3, and 7 days, respectively, which shows the pH triggered drug release manner, i.e. drug release at lower pH, which is close to that of late endosome and lyzosome, is significantly greater than that at higher pH. We hypothesized that the quantity of the released drugs per unit time would be almost constant due to the similar concentration of protons in the release medium, i.e. thus the same possibility of the protons to react on the labile linker leading similar quantity of dissociation drugs. After the drug molecules were cleaved, they were not reactive anymore with the hydrogen ions, which were soon collected for quantification of the drug release.

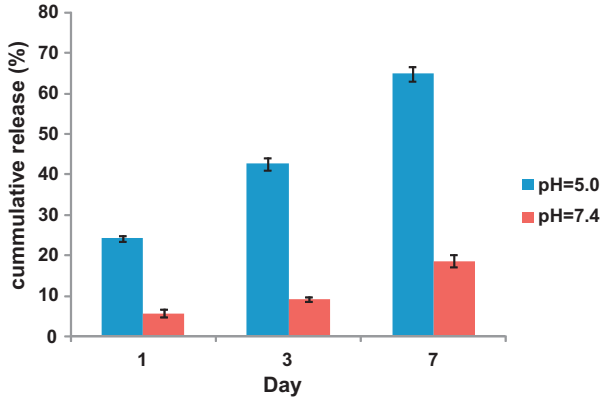

Fig. 6. Controlled release profile of DTX from silica–DTX–DSPE–PEG–FOL NPs at pH = 5.0 or pH = 7.4 environment. Data represent mean ± SD, $n = 3$.

Fig. 6 shows the different responses of drug release to the pH values. At lower pH value, which is close to that of late endosome and lyzosome, the quantity of released drug was significantly greater than that in higher pH medium. The result presented the controlled and sustained release behavior provided by the drug carriers, as well as proved the cleavable bond in acidic environment between DTX and silica matrix, as like the commonly used linkage of producing DTX prodrug. Through this strategy, most of the hydrophobic anticancer drugs could be covalently attached on SNPs via sensitive linkage. Silica based particles thus can be used as drug delivery carriers of hydrophobic molecules, which greatly expands the value of SNPs in biomedical and nanomedicine applications.

3.6. *In vitro cellular uptake: confocal microscopy study*

The lipid coated particles (silica–DTX–DSPE–PEG NPs and silica–DTX–DSPE–PEG–FOL NPs) can penetrate into and be taken up by MCF7 cancer cells through an endocytosis process. We used the particles with QDs loaded as the imaging agents to evidence the results from the visualized images of confocal laser scanning microscopy (CLSM) showing the internalization of the particles in the cells (Fig. 7). In this figure, row A showed the images of MCF7 cells incubated with QDs loaded silica NPs without lipid decoration, while row B showed the images using QDs loaded silica–DTX–DSPE–PEG NPs and row C presented the images of the cells incubated with QDs loaded silica–DTX–DSPE–PEG–FOL NPs. The red fluorescence emitted from QDs loaded in the silica matrix was utilized to represent the appearance of the complex particles. The images obtained from PI channel which showed the red

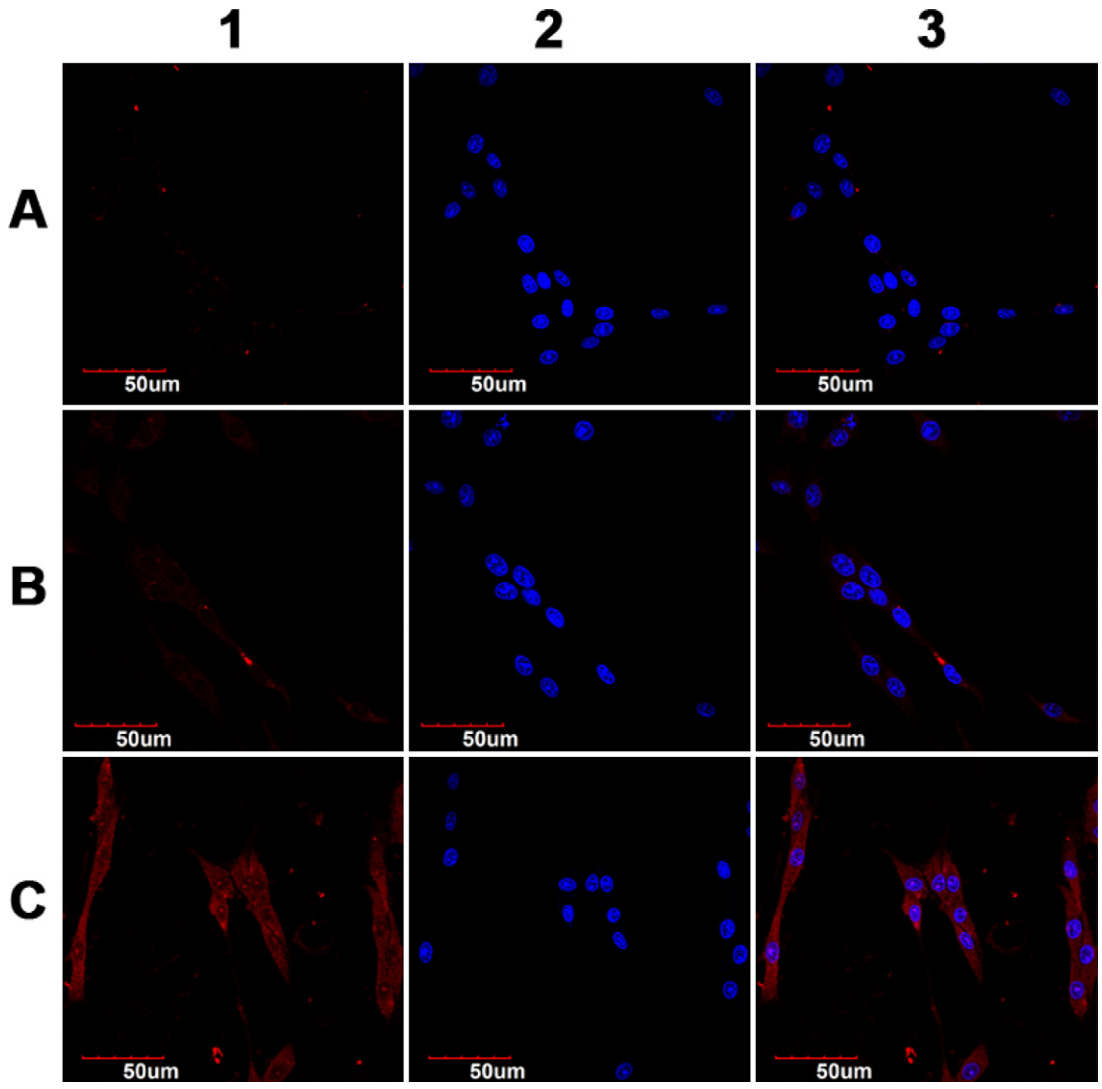

Fig. 7. Confocal laser scanning microscopy (CLSM) images showed the internalization of the NPs (0.250 mg/ml) in MCF7 cells with 2 h incubation.

fluorescence from the QDs capped in silica matrix were shown in column 1; column 2 listed the images obtained from the DAPI channel which showed the nuclei in blue fluorescence stained by DAPI; and column 3 listed the images obtained from the merged channels of PI and DAPI, from which it can be seen that, the blue fluorescence in the nucleus is surrounded by red fluorescence internalized in the cytoplasm. In addition, compared the red fluorescence intensity in row A with those in rows B and C, it is clearly to see that the intensity in rows B and C is higher than that in row A. The difference could be attributed to the favorable surface property of the lipid coated particles brought by phospholipids, which could enhance the interaction with the cell membranes by lipid–cell fusion (Ito et al., 1991). The targeting effect was also verified by comparison of the red fluorescence distribution and intensity in cytoplasm as shown in row B and C. Under the same exciting laser intensity from the same confocal microscope, it can be seen that the fluorescence from the particles with folic acid conjugated (row C) in the cytoplasm is much brighter than that from the particles without folic acid attached (row B). It can thus be concluded that the receptor-mediated endocytosis did facilitate and promote the entry of folic acid conjugated NPs into cells when the NPs contact with the folate receptor overexpressed cancer cells (Lee et al., 2003). Therefore, the silica particles coated by lipid layers can be used to treat cancerous cells by a targeting manner.

3.7. *In vitro cytotoxicity*

The efficacy of anticancer activity was reflected by the *in vitro* cytotoxicity upon the cancer cells. Fig. 8 illustrates the

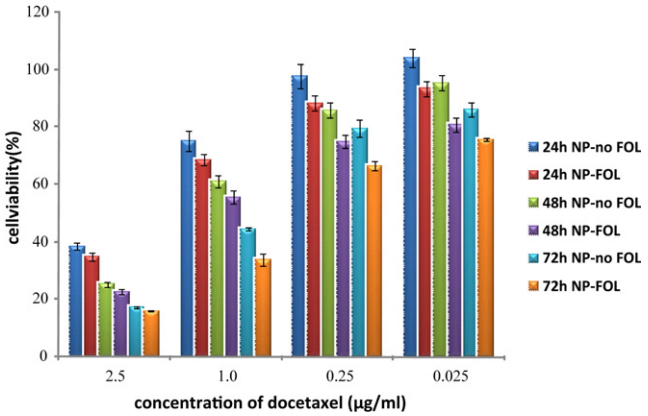

Fig. 8. Cell viability at various concentrations of the drug in silica–DTX–DSPE–PEG and silica–DTX–DSPE–PEG–FOL NPs under three treatment times. Data represent mean ± SE, $n = 6$.

Table 2
IC_{50} values of MCF7 cells treated by various formulations after 24, 48 and 72 h (unit as μg/ml).

	24 h	48 h	72 h
Taxotere®[a]	14.05	1.26	0.46
Silica–DTX–DSPE–PEG NPs	2.39	1.19	0.58
Silica–DTX–DSPE–PEG–FOL NPs	2.12	0.71	0.28

[a] Data as calculated from previous trials in our group and reported firstly on Pan et al. (2010).

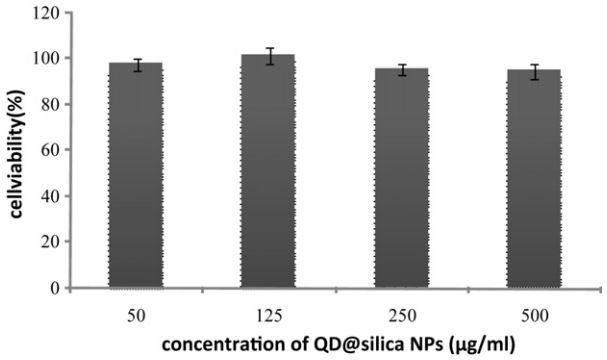

Fig. 9. Cell viability of MCF7 cells incubated with various concentrations of the QDs loaded silica particles for 72 h. Data represent mean ± SE, $n = 6$.

quantitative analysis on the cytotoxicity of DTX formulated in the NPs up to 72 h with various levels of drug concentration, as of 2.5, 1.0, 0.25, and 0.025 μg/ml ($P < 0.05$ under the two-tailed Student's *t* test in all cases). The lowest cell viability, i.e. the highest cell mortality, appeared at the highest concentration of the drugs after treatment for the longest time, which proved the controlled and sustained cell inhibition efficacy. Noteworthy, under the same drug concentration and exposure time, the silica–DTX–DSPE–PEG–FOL NPs would result in lower cell viability, or equivalently, higher cell cytotoxicity than silica–DTX–DSPE–PEG NPs. The explanation is straightforward since the particles with folic acid as targeting ligand on their surface can be more efficiently taken up by the folate receptor overexpressed cancer cells. This finding demonstrated the targeted delivery behavior of hydrophobic therapeutics by silica particles and meant that for the same therapeutic effect, the drug needed for the targeted formulation could be less than that for the non-targeted opponent. The drug delivery process by the NPs could be summarized as, following the uptake by targeted cells, the NPs were trafficked into the endosomes. The endosomes then fuse with lysosomes where the acidic labile ester bond between DTX and the particle would be cleaved, allowing the drug to be released from the silica surface and then to diffuse out of the lipid layers into cytoplasm, resulting in the anticancer activity intracellularly (Chen et al., 2010).

A quantitative evaluation of the *in vitro* therapeutic effect of a dosage form is IC_{50}, which is defined as the drug concentration required to kill 50% of the incubated cells in a designated time period. It can be calculated from the above *in vitro* cellular viability data that, after 72 h treatment, the targeted formulation is 51.7% and 39.1% more effective than the opponent and commercial formulation of DTX (Taxotere®), respectively, as judged by the IC_{50} values (Table 2).

Fig. 9 shows the cytotoxicity of MCF7 cells treated by non-drug loaded particles (QDs loaded silica NPs). It is noted that the particles without drug loading have virtually no cytotoxicity in a wide range of particle concentrations (50–500 μg/ml). The results suggested that the cytotoxicity effect shown in Fig. 8 was due to the loaded drugs but not the encapsulated QDs, silica matrix or phospholipids.

4. Conclusion and discussion

We have developed a silica based drug delivery system for hydrophobic anticancer drugs. The hydrophobic drugs could be simply and efficiently loaded onto silica matrix. Mixed phospholipid layers were coated on the docetaxel-conjugated silica nanoparticles. The drug delivery system with high drug loading provides a feasible, efficient and convenient solution for delivering hydrophobic anticancer drugs by silica nanoparticles, which enormously broadens the application of silica particles in nanomedicine realm. The system integrates cancer cell treatment, drug delivery and targeting effect in one complex nanostructure, as well as combines the merits of silica, PEG and phospholipids, such as non-toxicity, simple surface functionalization, effective cellular interaction, and controlled drug release. The cleavable drug conjugated bond could also decrease the amount of drug dissociated from the carrier prior to reaching the desired sites when circulating in bloodstream and, meanwhile, targeted release at specific areas of interest. The novel silica based drug delivery system for hydrophobic drugs possesses the potential of further *in vivo* investigation for practical applications in cancer diagnosis and treatment.

It should be pointed out that the linker in this study is essential to attach the docetaxel onto the silica carrier. Although it is possible to develop a non-linking strategy by co-encapsulating quantum dots and docetaxel in the silica nanoparticles, some of the advantages of the current system would be lost, e.g. the drug loading would not have been high and the drug release would not have been zero-order.

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