Published in final edited form as:

J Mater Chem B Mater Biol Med. 2013 ; 1(8): 1109–1118. doi:10.1039/C2TB00223J.

Multifunctional hybrid silica nanoparticles for controlled doxorubicin loading and release with thermal and pH dually response

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Abstract

Controlled drug loading and release into tumor cells to increase the intracellular drug concentration is a major challenge for cancer therapy due to resistance and inefficient cellular uptake. Here a temperature and pH dually responsive PNiPAM/AA@SiO₂ core-shell particles with internal controlled release were designed and fabricated for efficient cancer treatment, which could recognize the intrinsic pH differences between cancers and normal tissues. Upon lowering the temperature, doxorubicin was loaded into the PNiPAM/AA@SiO₂ nanoparticles, whereas by increasing the acidity, previously loaded doxorubicin was quickly released. Comparing with common mesoporous silica particles (MSNs), this core-shell particle has more uniform size and better dispersity. In addition, dried PNiPAM/AA@SiO₂ nanoparticles could be easily redispersed in distilled water. The in vitro cell culture experiments showed that not only PNiPAM/AA@SiO₂ particles were more biocompatible and lower cytotoxic than MSN, but also DOX@PNiPAM/AA@SiO₂ had higher drug releasing efficiency in the lysosomes and stronger inhibitory effect on tumor cell growth than DOX@MSN. All these features indicated that PNiPAM/AA@SiO₂ particles have great potential in therapy applications.

1. Introduction

Recently, various nanostructured materials have been developed for biomedical imaging, diagnostics and therapy, because they show improved pharmacokinetics and biodistribution and exciting efficacy for cancer treatments. However, the poor cellular internalization of nanoparticles and insufficient intracellular drug release always limits the amount of anticancer drugs that actually reach cancer cells, which hampers the efficacy of cancer chemotherapy. To conquer the challenges, stimuli-responsive nanoparticles have been regarded as one of the most promising carriers for drug delivery, which are sensitive to

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environmental stimuli such as temperature,¹⁻⁴ ionic strength,⁵ ultraviolet light,⁶ or magnetic field.⁷⁻⁹

It is well documented that pH values in different tissues and cellular compartments vary significantly. For example, the tumor extracellular environment is more acidic (pH 6.8)¹⁰ than blood and normal tissues (pH 7.4), and the pH values of late endosome and lysosome are even lower, at 5.0-5.5.¹¹ So pH sensitive delivery system is of special interest for controlled drug delivery.¹²⁻¹⁴

Mesoporous silica nanoparticles have been extensively explored as drug delivery systems due to their superior features such as high pore volume, large surface area, prominent biocompatibility, accessible surface functionalization, and effective protection for the payloads.¹⁵⁻¹⁸ With the aim to administer drug molecules specifically toward target tissues, pH sensitive molecules have been introduced to prepare hybrid nanoparticles with MSN.^{19, 20} The pore surface and opening of MSNs have been functionalized with stimuli-responsive groups,²¹⁻²⁴ inorganic nanoparticles,²⁵ and peptide²⁶ that worked as caps and gatekeepers.^{15, 27} Controlled release of encapsulated drugs can be triggered in responding to internal or external stimuli such as pH, temperature, redox potential, light, and enzymatic reactions.

For example, The folate was linked by disulfide bonds to construct the gate-like structure on the outlet of the pores of MSNPs, the controlled release can be triggered in the presence of reductant dithiothreitol or glutathione (GSH).²⁸ MSNP coated by PEG-DA-peptide macromer possessing MMP substrate polypeptides can be responsive to endogenous proteases triggered, localized drug release in vitro and in vivo.²⁹ Mesoporous silica nanocomposite nanoparticles immobilized pH responsive hydrazone bonds indicates pH-sensitive drug release.³⁰ Polyvalent mesoporous silica nanocarriers-aptamer bioconjugates were fabricated as controlled release drug delivery systems and were able to effectively target cancer cells.³¹

Despite the success of these approaches, they are needed to be improved because of tedious multiple-step syntheses, necessity of suitable surfactants, very low surface grafting efficiency or encapsulation efficiency, etc., more importantly, slow release of the encapsulated drug and low releasing efficiency caused by strong adsorption of MSNP. Therefore, it is of our interest to explore a simple and facile method to prepare drug carriers that are capable of recognizing the intrinsic pH differences between tumor and normal tissues and possessing higher releasing efficiency and faster release behavior at low pH.

Herein, we propose a facile and efficient strategy to introduce the pH/thermo-responsive nanocarriers with dually responsive poly(N-isopropylacrylamide) (PNiPAM)-co-acrylic acid (AA) hydrogel core enclosed in silica shell via self-assembly approach. The PNiPAM/AA@silica particles not only possess pH/thermal responsive feature, high dispersity and the unique features derived from silica shell, but also have improved drug release efficiency in cells. The physicochemical and pH/thermo- sensitive properties of PNiPAM/AA@silica composite microspheres were tested. Doxorubicin hydrochloride (DOX), a classic anticancer drug, was chosen as a model drug to assess the drug loading and releasing behaviors of the carriers. The cytotoxicity of PNiPAM/AA@silica and DOX@PNiPAM/AA@silica to MCF-7 cells was measured. The drug release efficiency of DOX@PNiPAM/AA@SiO₂ in cells had been compared with that of DOX@MSN.

2. Materials and methods

2.1. Materials

Cetyl trimethylammonium bromide (CTAB), N-isopropylacrylamide (NiPAM), N,N'-methylene bis(arylamide) (MBA), 3-(trimethoxysilyl) propylmethacrylate (MPS), Tetraethoxysilane (TEOS), 3-(4,5)-dimethylthiaziazol(-z-yl)-3,5-diphenyltetrazolium bromide (MTT) and trypsin were purchased from Sigma-Aldrich (St. Louis, USA). Lysotracker Green was purchased from Invitrogen. Acrylic acid (AA), sodium dodecyl sulfate (SDS) and ammonium persulfate (APS) was obtained from Shanghai Chemical Reagents Company (Shanghai, China). NiPAM was recrystallized from n-hexanes and dried in vacuum prior to use.

2.2. Synthesis of mesoporous silica nanoparticles

Mesoporous silica nanoparticles (MSNs) were synthesized as previously reported.³²⁻³⁴ Briefly, 200 mg of CTAB was dissolved in 96 mL of water, followed by the addition of 7 mL of 0.2M NaOH aqueous solution. The solution was heated to 80 °C and kept at the temperature for 30 minutes before 1 mL of TEOS was added. The solution went from clear to opaque, indicative of a hydrolysis process. The reaction was kept at 80 °C for 2 h. The resulting nanoparticles were centrifuged and washed with methanol. In order to remove the CTAB, the as-synthesized particles were suspended in 50 mL solution of methanol and 2.0 mL of 12 M hydrochloride acid. The solution was refluxed for 10 h and the MSNs were collected by centrifugation and washed with methanol.

2.3. Synthesis of PNiPAM/AA hydrogel nanoparticles

The PNiPAM/AA hydrogel particles were prepared by the precipitation polymerization of NiPAM, MBA and AA using APS as an initiator. More specifically, an appropriate amount of NiPAM, AA and MBA was dissolved in 120 mL of doubly distilled water containing 0.042 g of SDS. The dispersion was purged with nitrogen for 30 min under continuous mechanical stirring of 500 rpm (revolutions per minute) at room temperature. Then the solution was heated to 70 °C, and APS (0.053 g) dissolved in 3 mL of water was quickly injected to initiate the polymerization. The reaction mixture was stirred for 12 h at 70 °C under the nitrogen atmosphere. The obtained PNiPAM/AA nanoparticles were centrifuged and thoroughly washed with water and methanol to remove SDS and unreacted monomers.³⁵⁻³⁷ The purified PNiPAM/AA particles were redispersed in distilled water at a solid content of 0.5 wt% for subsequent use.

2.4. Synthesis of PNiPAM/AA @SiO₂ nanoparticles

The synthesis procedure was described as following: 10 mg of CTAB was dissolved in 5 mL of water, then a predetermined amount of above PNiPAM/AA particles and TEOS were added, and the mixture was ultrasonically treated for 30 minutes. Then the mixture was stirred at 37 °C for 24 h. The obtained PNiPAM/AA@SiO₂ nanoparticles were centrifuged and thoroughly washed with distilled water and then re-dispersed in water at a solid content of 1.0 wt% for further use.

2.5. Synthesis of FITC-PNiPAM/AA @SiO₂ nanoparticles

FITC-grafting PNiPAM/AA@SiO₂ nanoparticles were prepared with modified Stöber method.³⁸ A typical synthesis procedure was depicted as following: 5 mg of fluorescein isothiocyanate (FITC) was reacted with 25 mg 3-aminopropyl trimethoxysilane (APS) in 2 g absolute ethanol by stirring for 48 h at room temperature. Then 200 µL of the resulting solution was mixed with 200 mg PNiPAM/AA@SiO₂ and injected into the mixed solution of water (0.5 g) and ethanol (1.5 g). The mixed solution was magnetically stirred at room

temperature for 48 h. The obtained particles were centrifuged and washed with ethanol and distilled water three times, respectively. The FITC- grafting PNiPAM/AA@SiO₂ nanoparticles were finally redispersed in water.

2.6. Drug loading into SiO₂ and PNiPAM/AA@SiO₂ nanoparicles

Doxorubicin was dissolved in distilled water to prepare 3 mg/mL solutions. 1.5 mL of the doxorubicin solution was pipetted into test tubes containing 3 mL of 10 mg/mL SiO₂ or 3 mL of 10 mg/mL PNiPAM/AA@SiO₂ solution. The mixed solution was kept at 4 °C for 24 h, to reach maximum doxorubicin loading. Then the dispersion was centrifuged at 6000 rpm for 10 min to separate the loaded nanoparticles and carefully washed with distilled water twice. The amount of drugs loaded in the nanoparticles was determined by subtracting the amount of drug in the supernatant from that in the loading solution using a UV spectrophotometer with the detection wavelength of 485 nm.

2.7. In vitro drug release from the DOX@SiO₂ and DOX@PNiPAM/AA@SiO₂ nanoparicles

Nanoparticles loaded with doxorubicin were re-dispersed in 5 mL of PBS (pH 7.4 and 5.0) immediately after loading. The dispersion was then transferred into a dialysis bag (molecular weight cut off 7000 Da) and the bag was subsequently placed in a 50 mL centrifuge tube containing 25 mL of PBS. 5 mL of solution was sampled from the tube every half an hour during the first three hours, then sampled every hour in the following four hours. Finally, 5 mL of solution was taken out every day and the released drug was determined spectrophotometrically. The volume of the release medium in the flask was kept constant by adding equal volume of fresh medium back after each sampling. All drug release data were averaged with three measurements.

2.8. Cell culture

Human breast cancer cells (MCF-7) and human embryonic kidney (HEK293, normal cell) cells were maintained in Dulbecco's modified Eagle's medium (DMEM) and Minimum Essential Medium (MEM) supplemented with 10% (v/v) fetal bovine serum (FBS) in a humidified atmosphere containing 5% CO₂ at 37 °C.

2.9. The cytotoxicity assay

The cytotoxicity of DOX@PNiPAM/AA@SiO₂ against MCF-7 cells was determined by standard MTT assay. Briefly, the cells were seeded onto 96-well plates at a density of 5000 viable cells per well and incubated for 24 h to allow cell attachment. Then the cells were incubated with free DOX, DOX@SiO₂, DOX@PNiPAM/AA@SiO₂ with the doxorubicin concentrations ranging from 0.08~20 µg/mL, and blank PNiPAM/AA@SiO₂ from 0.4~200 µg/mL respectively. After 48 h, fresh medium containing MTT (0.5 mg/mL) were replaced and the cells were incubated for additional 3.5 h. Upon removing MTT solution, the purple formazan crystals were dissolved with 100 µL DMSO, and the absorbance was recorded at 570 nm with a microplate reader (TECAN Znfinite M200, Austria). Untreated cells in medium were used as control. Corresponding groups without cells were used as blanks. All experiments were carried out with four replicates.

2.10. Confocal Microscopy assay

MCF-7 cells were incubated with DOX@ PNiPAM/AA@SiO₂ or DOX@MSN for 1 or 4 h in petri dishes, washed with PBS three times and subsequently labeled with fluoroprobe Lysoracker Green in the culture medium at 37 °C for 30 min. After labeling, cells were washed with PBS buffer to remove the residual DOX or nanoparticles. The intracellular localizations of free DOX and released DOX from DOX@PNiPAM/AA@SiO₂ or DOX@MSN were directly visualized via a confocal laser scanning microscope (Carl Zeiss,

Germany). Lysotracker Green was excited at 488 nm and their emission was recorded at 505–525 nm. Doxorubicin was excited at 488 nm and its emission was recorded at 560–600 nm. In the assay, all experiments were carried out under a light-sealed condition to avoid photo-bleaching.

2.11. Cellular uptake by flow cytometry

Flow cytometry (FCM) was used to determine the drug transfer capability of the PNiPAM/AA@SiO₂ nanoparticles into cells. MCF-7 cells were seeded onto a 6-well plate (5×10⁵ cells per well), and cultured for 24 h, then treated with DOX, DOX@PNiPAM/AA@SiO₂ or DOX@MSN at the same final concentration of 5 µg/mL of equivalent DOX. The untreated cells were used as a blank control. After incubating for 1h, 4 h or 12 h, the media were removed, and the cells were washed twice with PBS buffer to remove residual nanoparticles. Then the cells were harvested after being treated with 0.25% trypsin solution, washed with PBS buffer three times, and finally suspended in PBS. The signals of DOX fluorescence were recorded by FCM (Attune® acoustic focusing cytometer, Applied Biosystems, Life Technologies, Carlsbad, CA).

2.12. General Analysis

The average hydrodynamic radius of MSN, PNiPAM/AA and PNiPAM/AA@SiO₂ nanoparticles at different temperatures and pH were determined by ZetaSizer Nano ZS (Malvern Instruments Ltd., Worcestershire, UK). All the measurements were performed with the nanoparticles suspended in distilled water at the concentration of 100 µg/mL.

TEM images were obtained using a Tecnai G2 20 S-TWIN transmission electron microscope (FEI Company) operating at 200 kV. Samples were deposited onto carbon coated copper grids, dried at room temperature and stained with uranyl acetate when necessary.

Infrared spectroscopy was recorded on a Spectrum One FT-IR spectrometer. The freeze-dried nanoparticles were grounded with KBr and pressed into a thin wafer. For each sample, 32 scans were recorded from 4000 to 400 cm⁻¹ with a resolution of 2 cm⁻¹.

Nitrogen adsorption-desorption isotherms were measured at -196°C by ASAP 2020 (M+C) (Micromeritics, America). Before the samples were analyzed, they were degassed in a vacuum at 150°C for 12 hours. Specific surface area was calculated using the multiple-point Brunauer–Emmett–Teller method. Pore volume was determined from the adsorption branch of nitrogen adsorption-desorption isotherm curve at a relative nitrogen pressure $P/P_0 = 0.992$ signal point. Pore diameter was calculated from the adsorption branch of the isotherms using the Barrett–Joyner–Halenda method.

3. Results and Discussion

3.1. Synthesis physicochemical characterization of nanoparticles

In order to achieve an optimal controllable delivery system for doxorubicin, three types of particles (Mesoporous silica particles, PNiPAM/AA hydrogel particles and PNiPAM/AA@SiO₂ particles) were prepared. Mesoporous silica nanoparticles (MSNs) with highly ordered mesostructures and spherical morphology were synthesized, using CTAB as template. As shown in Fig. 1a, spherical particles of MSNs with regular morphology and diameter of approximately 100 nm were obtained. The mean particle size of MSNs in H₂O determined by dynamic light scattering was about 525.8 nm, and the zeta potential was -29.1 mV (Table 1). The 2D cylindrical pores with the diameter of 2-3 nm were arranged in parallel. Transmission electron microscopy (TEM) images of PNiPAM/AA hydrogel

nanoparticles and PNiPAM/AA@SiO₂ nanoparticles are shown in Fig. 1b and c. The images show that the particles are homogeneous and well-dispersed. The hydrodynamic size of the NiPAM/AA@SiO₂ particles (247.3 nm) is smaller than that of NiPAM/AA hydrogel particles (306.2 nm), the reason for which might be that the size of PNiPAM/AA hydrogel cores decreased when NiPAM/AA@SiO₂ particles were fabricated at 37 °C. The zeta potential of the PNiPAM/AA@SiO₂ is similar to that of SiO₂, increasing from -14.5 mV of PNiPAM/AA particles to -22.9 mV, which further verifies that the PNiPAM/AA hydrogel particles are covered by SiO₂. As illustrated in Fig. 1c, many small SiO₂ fragments aggregated on the surface of PNiPAM/AA hydrogel particles. There are many visible gaps among the fragments, probably the channels connecting PNiPAM/AA cores and the outside facilitate drug loading and release. The result of nitrogen adsorption-desorption measurement indicates that PNiPAM/AA@SiO₂ and MSN have a specific surface area of 27.37 and 751.05 m²/g. Total pore volume and average pore diameter of PNiPAM/AA@SiO₂ and MSN are 0.12 cm³/g and 17.97 nm for PNiPAM/AA@SiO₂ and 0.42 cm³/g and 2.23 nm for MSN, respectively. It is well known that the morphology of mesoporous silica nanoparticles synthesized by the conventional methods may hamper its practical applications, because it was unfavorable for large scale productions. Most often, irregular or agglomerated particles are obtained, which limited therapeutic efficacy of the particles. Alternately, we used the PNiPAM/AA particles as templates, the size of the obtained PNiPAM/AA@SiO₂ nanoparticles was controlled by the size of cores and the dispersity was good, as shown in Table 1. This type of particles not only possesses the environmental responsive property of PNiPAM/AA hydrogel, but can also be modified like SiO₂ particles. These features in combination with its unique structural advantages render the PNiPAM/AA@SiO₂ nanoparticles an excellent candidate as drug carrier.

3.2. Fourier transforms infrared spectra (FTIR) characterization of particles

Fig. 2 provides the FTIR spectra of MSN, NiPAM/AA, and NiPAM/AA@SiO₂. In the framework region MSN FTIR spectrum shows strong absorption peaks at about 464 cm⁻¹, 808 cm⁻¹ and 1,091 cm⁻¹ due to the vibrations of Si-O-Si and Si-O linkages, the hydroxyl absorption peak at 3,700-2,900 cm⁻¹ resulted from hydrolyzed ethyl orthosilicate.^{39, 40} The FT-IR spectra of the NiPAM/AA hydrogels demonstrates broad bands of N-H stretch and vibration at 3299.0 cm⁻¹ and 1540.5 cm⁻¹, respectively. The strong peak at 1648.5 cm⁻¹ originates from the C=O carbonyl stretching vibration. Two typical bands of C-H vibration at 1387.5 and 1367.5 cm⁻¹ belong to the divided bands of the symmetric -CH(CH₃)₂ group. In the spectra of the NiPAM/AA@SiO₂, the typical peaks are similar with those of MSN and NiPAM/AA. These findings indicate that NiPAM/AA@SiO₂ shell-core particles were successfully obtained.

3.3. Determination of dispersity and modification of PNiPAM/AA@SiO₂ nanoparticles powder

One of problems in various nanotherapeutic candidates is that particles tend to agglomerate. Good dispersity of therapeutic nanoparticles is essential.⁴¹ Here, the dispersity of PNiPAM/AA@SiO₂ nanoparticles was evaluated and compared with that of MSN. Nanoparticles of PNiPAM/AA and MSN in ethanolic suspensions were dried using rotary evaporation and redispersed in distilled water (Fig. 3a). Well-suspended, optically transparent colloidal solution could be clearly seen after simply redispersing the powder in distilled water by ultrasonication for 5 min. The hydrodynamic size of the redispersed PNiPAM/AA@SiO₂ nanoparticles is ~274 nm in distilled water and slightly larger than as-synthesized PNiPAM/AA@SiO₂ nanoparticles (~234 nm). As shown in Fig. 3b, PNiPAM/AA@SiO₂ nanoparticles have much better dispersity than MSN.

In order to determine whether the prepared PNiPAM/AA@SiO₂ particles can be chemically modified similarly as MSN, FITC was grafted to the SiO₂ shell of the particles and their hydrodynamic size was measured using DLS. The hydrodynamic size distribution of synthesized FITC-PNiPAM/AA@SiO₂ particles was almost identical to the unmodified PNiPAM/AA@SiO₂ particles, as shown in Fig. 4a. Yellowish and transparent colloidal solutions showing Tyndall light scattering behavior further confirmed the dispersity of the FITC-PNiPAM/AA@SiO₂ particles (Fig. 4b). The fluorescence of FITC-PNiPAM/AA@SiO₂ particles under UV illumination was homogeneously distributed in water (Fig. 4c).

The above results indicate that PNiPAM/AA@SiO₂ nanoparticles obtained by the simple self-assemble method have a relatively better dispersity than MSN, nevertheless their surface could be modified just like MSN.

3.4. Thermal sensitive characterization of PNiPAM/AA@SiO₂ particles

Here, we further study the influence of temperature on the size of PNiPAM/AA@SiO₂ core-shell nanoparticles. As shown in Fig. 5, the particles, prepared at 37 °C, slightly swelled with temperature decreasing from 47 °C to 21 °C. It is interesting that after the particles was stored for 24 hours at room temperature, the size of the particles increased about 10 nm, and the size of the nanoparticles went back to the original size when the medium temperature was raised again above 37 °C. The reason for the size increment of the nanoparticles might be that the sustained expanding force of PNiPAM/AA cores at lower temperature for a long time acted on SiO₂ shells and the shells were broken open along the gap. When the temperature was raised, contraction force of the cores would draw the open shells back to original topography.

According to the results, PNiPAM/AA@SiO₂ nanoparticles will expand and the drug could be loaded into the nanoparticles when the medium temperature decreases. Then the medium temperature is recovered to its preparation temperature and the drug is capsulated into the nanoparticles, as shown in Fig. 6. In turn, the release of drug molecules from DOX@PNiPAM/AA@SiO₂ was accelerated when the temperature was decreased (Fig. S1).

3.5. Effect of pH values on drug release

In order to determine whether lower pH can trigger drug release at body temperature, PNiPAM/AA@SiO₂ nanoparticles were fabricated at 37 °C and doxorubicin was loaded for 48 h at 4 °C and neutral pH. Drug loading contents and encapsulating efficiency of MSN and PNiPAM/AA@SiO₂ nanoparticles are 9.98%, 71.59% and 5.98%, 51.89%, respectively. In vitro release profiles of DOX from MSN, PNiPAM/AA and PNiPAM/AA@SiO₂ nanoparticles were examined in phosphate buffer at pH 7.4 and pH 5.0, respectively. As shown in Fig. 7, the drug release rate of both particles was faster at low pH (pH 5.0) than at high pH (pH 7.4). There was slightly difference between the cumulative drug release from MSNs at pH 7.4 and pH 5.0, which was respectively about 50% and 67%. However, at pH 7.4 the amount of cumulative drug released from PNiPAM/AA@SiO₂ (about 20%) is significantly lower than that of MSN and PNiPAM/AA particles (about 60%, Fig. 7b). When the medium pH value was reduced to 5.0, the accumulated released drug from PNiPAM/AA@SiO₂ quickly improved to 80%. The results clearly show that PNiPAM/AA@SiO₂ improved drug release efficiency in acidic medium and decreased the amount of cumulative drug released from PNiPAM/AA cores in neutral medium. The reason for the result is that the protons in the acidic buffer solution can easily get into the core through the gap of shell to protonate the amino group of DOX, which accelerate the drug release. This release behavior is desirable for cancer treatment, i.e. most of the drug remains encapsulated

in the nano carrier during circulation, but when it reaches the acidic tumor tissue, the low pH triggers drug release.

3.6. Cell viability assay

To evaluate the potential of PNiPAM@SiO₂ nanoparticles as effective drug carrier for cancer therapy, *in vitro* cytotoxicity of DOX@MSN and DOX@PNiPAM/AA on MCF-7 cells was investigated. MCF-7 cells were incubated with DOX@MSN and DOX@PNiPAM/AA@SiO₂ at equivalent doxorubicin doses for 48 h, respectively. As shown in Fig. 8a, significant dose-dependent inhibition of MCF-7 cells' proliferation was observed when the cells were treated with DOX@PNiPAM/AA@SiO₂, but DOX@MSN showed mild toxicity to MCF-7 cells. On the other hand, the blank carrier of PNiPAM/AA@SiO₂ nanoparticles at the same concentration as drug carrier showed no cytotoxicity toward MCF-7 cells (Fig. S2), which differs from MSN, indicative of its better biocompatibility than MSN. Higher releasing efficiency of DOX from DOX@PNiPAM@SiO₂ particles taken up by MCF-7 cells might have contributed to the enhanced cytotoxicity of DOX@PNiPAM/AA@SiO₂.

The endocytosis of DOX loaded MSN and PNiPAM/AA@SiO₂ was evaluated by flow cytometry analysis after incubation for 1 h, 4 h and 12 h, respectively. As DOX is a fluorophore, the fluorescence intensity is proportional to the amount of DOX in MCF-7 cells. It could be seen from Fig. 8b that the cells without any treatment showed only autofluorescence. The fluorescence intensity of DOX@MSN and DOX@PNiPAM/AA@SiO₂ increased with the prolonging cultivation time. Although the DOX concentration and cell incubation time were the same, the fluorescence intensity of DOX loaded by PNiPAM/AA@SiO₂ was higher than that of DOX loaded by MSN at each time point, respectively. The flow cytometry results indicated that the DOX concentration loaded into the cells by PNiPAM/AA@SiO₂ was higher than that of DOX loaded by MSN, which was in agreement with the result of cellular toxicity. To verify pH responsive release behavior of DOX@PNiPAM/AA@SiO₂ in cells, the intracellular DOX distribution was analyzed by using DOX auto-fluorescence after incubation for 1 or 4 h. Lysotracker Green, a specific probe for acidic compartments, was used to confirm the lysosome colocalization of DOX@PNiPAM/AA@SiO₂. In the group of cells treated with DOX@PNiPAM/AA@SiO₂, partial DOX gathered in the lysosomes, of which the fluorescence overlapped with that of the Lysotracker, and partial DOX dispersed in cytoplasm, surrounding the lysosomes, as shown in Fig. 9a and b. In distinct contrast, in DOX@MSN treatment the red fluorescence of DOX was much weaker and mainly colocalized with that of the Lysotracker, and DOX fluorescence in cytoplasm was barely visible (Fig. 9c and d). This experiment further proved that PNiPAM/AA@SiO₂ outperformed MSN in drug release at cellular level. The difference in cytotoxicity of DOX@PNiPAM/AA@SiO₂ and DOX@MSN might be related to the following two factors. Superior dispersity of PNiPAM/AA@SiO₂ results that size of the nanoparticles in solution is smaller than that of MSN, which makes the former easier to enter cells;⁴²⁻⁴⁴ Furthermore, DOX@PNiPAM/AA@SiO₂ particles have higher drug releasing efficiency in the acidic intracellular microenvironment than DOX@MSN.

4. Conclusions

In summary, PNiPAM/AA@SiO₂ nanoparticles with good dispersity and the ability of being modified were fabricated through self-assembly of PNiPAM/AA particles and TEOS in water. Many gaps were observed in the aggregated silica shell by TEM. The drug of doxorubicin not only could be loaded into the DOX@PNiPAM/AA@SiO₂ nanoparticles at lower temperature and encapsulated inside PNiPAM@SiO₂ particles by silica shell at 37 °C and neutral pH, but also could be released quickly from the nanoparticles at pH 5.0. The drug release results indicate that PNiPAM/AA@SiO₂ particles have pH responsive characteristics and higher releasing efficiency than MSN particles, which is very attractive

for cancer treatment. In *in vitro* cell assays, significant growth inhibition of MCF-7 cells was observed when the cells were treated with DOX@PNiPAM/AA@SiO₂ particles, which was higher than that of DOX@MSN. The result of lysosome location and flow cytometry analysis demonstrated PNiPAM/AA@SiO₂ loaded with DOX was efficiently taken up by MCF-7 cells and had higher drug releasing efficiency in the acidic intracellular microenvironment than DOX@MSN. The results demonstrated that thermo/pH-sensitive PNiPAM/AA@SiO₂ particles could have great potential of selective release in tumor tissue.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported in part by Chinese Natural Science Foundation project (No.30970784, 81171455, 31200717, 21271059 and 20971034), National Key Basic Research Program of China (2009CB930200), Chinese Academy of Sciences (CAS) "Hundred Talents Program" (07165111ZX), Research Fund for the Doctoral Program of Higher Education of China (No.20111301110004) and CAS Knowledge Innovation Program. This work was supported in part by NIH/NCRR 3 G12 RR003048, NIH/NIMHD 8 G12 MD007597, and USAMRMC W81XWH-10-1-0767 grants. It was supported with the joint lab of nanotechnology for bioapplication, which was established with Life Technologies Corp. in the National Center for Nanoscience and Technology of China.

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Fig. 1. TEM images of mesoporous SiO₂ (a), PNiPAM/AA nanoparticles (b) and PNiPAM/AA@SiO₂ nanoparticles (c).

Fig. 2.
FT-IR spectra of MSN, PNiPAM/AA and PNiPAM/AA@SiO₂ nanoparticles.

(b)

Particles Type	PNiPAM/AA@SiO ₂ nanoparticles		MSN nanoparticles	
	before drying	after drying	before drying	after drying
Size in H ₂ O (nm)	233.8	273.9	526.0	1486
PdI in H ₂ O	0.045	0.081	0.253	0.434

Fig. 3.

(a) The photograph of colloidal solution of PNiPAM/AA@SiO₂ particles before and after drying; (b) Change of size and polydispersity of PNiPAM/AA@SiO₂ and MSN in water before and after drying.

Fig. 4. (a) hydrodynamic diameter distributions; (b) colloidal solutions of FITC-PNIPAM/AA@SiO₂ particles; (c) a photograph of aqueous solution of FITC-PNIPAM/AA@SiO₂ particles under illumination.

Fig. 5.
The effect of temperature changing on hydrodynamic diameter of PNiPAM/AA@SiO₂ nanoparticles.

Fig. 6.
The schematic diagram for the mechanism of drug loading of DOX@PNiPAM/AA@SiO₂ nanoparticles.

Fig. 7. DOX release profiles from DOX@MSN, DOX@PNiPAM/AA@SiO₂ (a) and PNiPAM/AA nanoparticles (b) under different pH.

Fig. 8.

The cytotoxicity and cellular uptake of different nanoparticles to MCF-7 cells. (a) Viability of cells cultured *in vitro* with DOX loaded PNiPAM@SiO₂, MSN and blank carriers; (b) Flow cytometric analyses of DOX@PNiPAM/AA@SiO₂ (red line) and DOX@MSN (green line) at different time.

Fig. 9. Intracellular location of free DOX, DOX@ PNiPAM/AA@SiO₂ and DOX@MSN. MCF-7 cells incubated with DOX@ PNiPAM/AA@SiO₂ after 1 h (a) and 4 h (b) and MCF-7 cells incubated with DOX@MSN after 1 h (c) and 4 h (d).

Table 1

Physicochemical characterization of the different nanoparticles.

Particles type	Size in H ₂ O (nm)	Zeta Potential in H ₂ O(mV)	PdI in H ₂ O
MSN	525.8	−29.1	0.253
PNiPAM/AA hydrogel particles	306.2	−14.5	0.006
PNiPAM/AA@SiO ₂ nanoparticles	247.3	−22.9	0.04