[Frontiers in Bioscience 10, 3058-3067, September 1, 2005]

TEMPERATURE- AND PH-SENSITIVE CORE-SHELL NANOPARTICLES SELF-ASSEMBLED FROM POLY(*N*-ISOPROPYLACRYLAMIDE-*CO*-ACRYLIC ACID-*CO*-CHOLESTERYL ACRYLATE) FOR INTRACELLULAR DELIVERY OF ANTICANCER DRUGS

Jia-Shen Wei [1,2], Hong-Bo Zeng [3], Shao-Qiong Liu [1], Xiao-Gong Wang [3], Eng Hock Tay [1,2], and Yi-Yan Yang [1]

[1] Institute of Bioengineering and Nanotechnology, 31 Biopolis Way, The Nanos, Singapore 138669, [2] Department of Mechanical Engineering, National University of Singapore, Singapore, [3] Department of Chemical Engineering, Tsinghua University, Beijing 100084, P. R. China

TABLE OF CONTENTS

1. Abstract
2. Introduction
3. Materials and Methods
3.1. Materials
3.2. Synthesis and characterization of poly(N-isopropylacrylamide-co-acrylic acid-co-cholesteryl acrylate) (P(NIPAAm-co-AA-co-CHA))
3.3. Preparation of paclitaxel-loaded core-shell nanoparticles
3.4. Characterization of core-shell nanoparticles
3.4.1. Determination of LCST
3.4.2. Determination of critical association concentration (CAC)
3.4.3. Transmission electron microscopy (TEM)
3.4.4. Size and size distribution
3.4.5. Encapsulation efficiency of paclitaxel
3.5. In vitro drug release
3.6. Confocal laser scanning microscopy (CLSM)
3.7. Cytotoxicity test
4. Results and Discussion
4.1. Synthesis of P(NIPAAm-co-AA-co-CHA)
4.2. Critical association concentration (CAC) determination
4.3. LCST of the nanoparticles
4.4. Size, morphology and stability of the nanoparticles
4.5. Encapsulation efficiency of paclitaxel
4.6. In vitro drug release
4.7. Cellular uptake and in vitro cytotoxicity
5. Conclusion
6. References

1. ABSTRACT

Temperature- and pH-sensitive amphiphilic polymer poly(*N*-isopropylacrylamide-*co*-acrylic acid-*co*-cholesteryl acrylate) (P(NIPAAm-*co*-AA-*co*-CHA)) has been synthesized and employed to encapsulate paclitaxel, a highly hydrophobic anticancer drug, in core-shell nanoparticles fabricated by a membrane dialysis method. The nanoparticles are spherical in shape, and their size can be made below 200 nm by varying fabrication parameters. The lower critical solution temperature (LCST) of the nanoparticles is pH-dependent. Under the normal physiological condition (pH 7.4), the LCST is well above the normal body temperature (37ºC) but it is below 37ºC in an acidic environment (e.g. inside the endosome or lysosome). The critical association concentration of the polymer is determined to be 7 mg/L. Paclitaxel can be easily encapsulated into the nanoparticles. Its encapsulation efficiency is affected by fabrication temperature, initial drug loading and polymer concentration. *In vitro* release of paclitaxel from the nanoparticles is responsive to external pH changes, which is faster in a lower pH environment. Cytotoxicity of paclitaxel-loaded nanoparticles against MDA-MB-435S human breast carcinoma cells is slightly higher than that of free paclitaxel. In addition, doxorubicin is used as a probe to study cellular uptake using a confocal laser scanning microscope (CLSM). Doxorubicin molecules are able to enter the cytoplasm after escaping from the endosome and/or the lysosome. The temperature- and pH-sensitive nanoparticles would make a promising carrier for intracellular delivery of anticancer drugs.

2. INTRODUCTION

Polymeric core-shell nanoparticles (i.e. micelles) self-assembled from amphiphilic copolymers have recently been employed to target poorly water-soluble drugs to tumor sites (1,2). The core-shell nanoparticles having a small size (<200 nm) can solubilize hydrophobic drugs in their inner cores through hydrophobic interactions, while exposing their hydrophilic shells to the external environment. This enables them to exhibit prolonged activity in the systemic circulation by avoiding the scavenging of the reticuloendothelial systems (RES), and to protect the enclosed drug molecules from degradation. Through the enhanced permeability and retention (EPR) effect, the enclosed drug can be passively targeted to tumor sites (3). In addition, the conjugation of recognition signals onto the shell of the nanoparticles (4) and the use of pH- or temperature-sensitive polymers made active targeting of anticancer drugs possible (5,6). For example, the nanoparticles having the shells made from poly(*N*-isopropylacrylamide) (PNIPAAm) or its copolymers have a lower critical solution temperature of about 32ºC, above which the nanoparticles are well dispersed in an aqueous solution but below which their core-shell structure deforms and precipitates, releasing the enclosed drug molecules. Therefore, using the nanoaprticles having an LCST slightly higher than the normal body temperature, drug delivery can be controlled by superficially heating the local environment of the nanoparticles. However, such a heating treatment is not easily accessible to deep organs or tumors. To circumvent this issue, we have recently developed pH-triggered temperature-sensitive core-shell nanoparticles self-assembled from poly(*N*-isopropylacrylamide-*co*-*N,N*-dimethylacrylamide-*co*-10-undecenoic acid) (7). These nanoparticles had a pH-dependent LCST. Under the normal physiological condition (pH 7.4), the LCST is above the normal body temperature (37ºC) but it is below 37ºC at low pH, leading to the deformation and precipitation of the nanoparticles and the release of the enclosed doxorubicin molecules. Using these nanoparticles, moderately hydrophobic drugs can be delivered selectively to acidic tumor tissues or escape from biological barriers such as endosomes or lysosomes. Since the hydrophobicity of core-forming segment 10-undecenoic acid is relatively weak, it is difficult to encapsulate highly hydrophobic drugs such as paclitaxel into the nanoparticles.

Paclitaxel is a widely used anticancer drug in clinic, exhibiting high activity against ovarian cancer, breast cancer, lung cancer, head and neck malignancies (8). However, paclitaxel has poor solubility in conventional aqueous vehicles. Taxol® is the only formulation of paclitaxel currently available for clinical use, which is a mixture of Cremophor EL (polyxoyl 35 castor oil) and dehydrated alcohol (1:1, v/v) containing paclitaxel of 6 mg/mL (9). However, Cremophor EL is highly toxic, causing a number of serious side effects such as hypersensitivity reactions, neurotoxicity and cardiotoxicity (10). It was reported that it induced severe anaphylactoid hypersensitivity reactions and abnormal lipoprotein pattern (11). In addition, paclitaxel is an antimicrotubule agent, it is essential for paclitaxel-loaded nanoparticles to escape from the endosomes or the lysosomes and enter the cytoplasm for the effective functioning of paclitaxel. In this study, we will synthesize pH-triggered temperature-sensitive nanopartilces having a hydrophobic core of cholesteryl moieties and a shell of temperature- and pH-sensitive poly(*N*-isopropylacrylamide-*co*-acrylic acid) moieties. We will demonstrate that the nanoparticles provide a high loading capacity for paclitaxel and paclitaxel release is responsive to external pH changes. Meanwhile, it will be illustrated that these nanoparticles can deliver the enclosed drug molecules into the cytoplasm.

3. MATERIALS AND METHODS

3.1. Materials

N-isopropylacrylamide (NIPAAm, purchased from Sigma-Aldrich) was purified by re-crystallization from n-hexane. Cholesterol, 2,2'-azobisisobutyronitrile (AIBN), acrylic acid, acryloyl chloride, diethyl ether, benzene, 1,4-dioxane, paclitaxel, doxorubicin hydrochloride, pyrene, triethylamine (TEA), Dulbecco's Modified Eagles' Medium (DMEM), 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyl tetrazolium bromide (MTT), acetonitrile of HPLC grade, methanol of HPLC grade, dichloromethane (DCM), *N*, *N*-dimethylacetamide (DMAc), and dimethylformamide (DMF) were purchased from Sigma-Aldrich, and used as received. Fetal serum albumin (FBS) was supplied by Invitrogen. MDA-MB-435S human breast carcinoma cells were purchased from Interlab Cell Line Collection (Italy). All other chemicals were of analytical grade, and used as received.

3.2. Synthesis and characterization of poly(N-isopropylacrylamide-co-acrylic acid-co-cholesteryl acrylate) (P(NIPAAm-co-AA-co-CHA))

The monomer cholesteryl acrylate (CHA) was first prepared by refluxing cholesterol and an excess amount of acryloyl chloride in benzene for 8 hours (Scheme 1). The resulting ester was purified by crystallization from ether/ethanol for a few times. P(NIPAAm-*co*-AA-*co*-CHA) was then synthesized by radical copolymerization using AIBN as an initiator (Scheme 1). Briefly, *N*-isopropylacrylamide (11.3 g, 100 mmol), acrylic acid (0.36 g, 5 mmol), cholesteryl acrylate (0.441g, 1 mmol) and AIBN (0.054 g, 0.33 mmol) were dissolved in distilled 1,4-dioxane. The solution was degassed by bubbling with nitrogen for 15 minutes. The reaction mixture was refluxed for 24 hours under nitrogen at 60°C. Upon completion, the product was precipitated out by the addition of diethyl ether. The products were purified by re-precipitation twice from dichloromethane-diethyl ether and then vacuum-dried for use. The molecular weight (Mw) of the polymer was determined by a gel permeation chromatography (GPC) (Waters 2690, MA, USA) with a differential refractometer detector (Waters 410, MA, USA). The weight average molecular weight and polydispersity index were calculated from a calibration curve using a series of polystyrene standards (Polymer Laboratories Inc., USA). The ^{1}H-NMR spectra of the polymers were studied using a Bruker Avance 400 spectrometer (400 MHz), and chloroform-*d* ($CDCl_3$) was used as the solvent. Infrared spectra were measured using a Nicolet 560-IR spectrometer by incorporating the sample in a KBr disk.

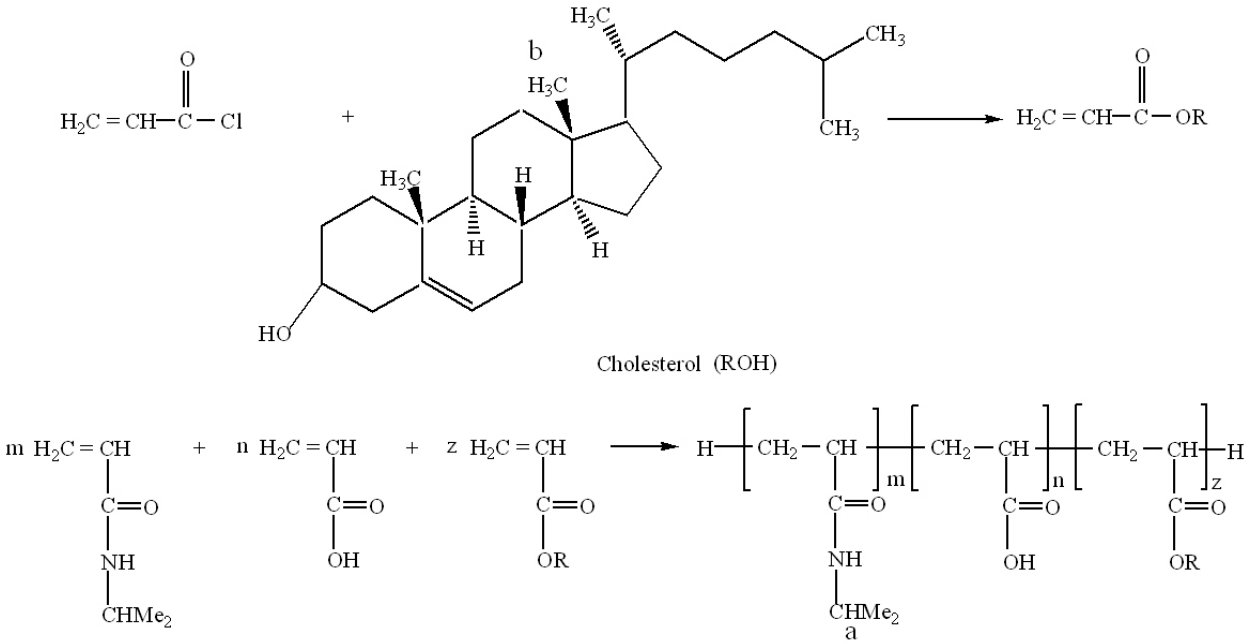

Figure 1. Synthesis of P(NIPAAM-*co*-AA-*co*-CHA)

3.3. Preparation of paclitaxel-loaded core-shell nanoparticles

Paclitaxel was loaded into the core-shell nanoparticles by a membrane dialysis method. Briefly, a fixed amount of P(NIPAAm-*co*-AA-*co*-CHA) (3-12 mg) and paclitaxel (1-3 mg) were dissolved in 5 mL of DMF. The solution was dialyzed against de-ionized (DI) water at different temperatures for 24 hours using a dialysis membrane with a molecular weight cut-off of 2,000 (Spectra/Por 7, Spectrum Laboratories Inc.). The water was replaced every two hours for the first eight hours. After dialysis, the solution in the dialysis bag was collected and filtered with 0.45 μm syringe filter and freeze-dried for two days before further examinations.

3.4. Characterization of core-shell nanoparticles

3.4.1. Determination of LCST

The core-shell nanoparticles were prepared in PBS buffers (pH 7.4 and pH 6.6) and phthalate buffers (pH 5.0 and pH 4.0) respectively. The optical transmittance of these nanoparticle solutions was measured at 500 nm at various temperatures using an UV/VIS/NIR spectrophotometer (Jasco, Japan). The temperature of the sample cell was thermostatically controlled using a temperature-controller (Jasco, Japan). Heating rate was 0.1°C/min. The LCST values of the nanoparticles were determined at the temperatures showing an optical transmittance of 50%.

3.4.2. Determination of critical association concentration (CAC)

The CAC value of the polymer in PBS was analyzed by fluorescence spectroscopy using pyrene as a hydrophobic fluorescence probe that preferentially partitions into the hydrophobic core of the nanoparticles (12). Fluorescence spectra were recorded by an LS50B luminescence spectrometer (Perkin Elmer, USA) at room temperature (20ºC). Aliquots of pyrene solutions in acetone (1.54×10^{-5} M, 400 μL) were added to empty containers, and acetone was allowed to evaporate overnight. Then, 10 mL of aqueous polymer solutions at a concentration ranging from 0.1 to 500 mg/L were added to the above containers. The solutions were equilibrated for 24 hours at room temperature. The emission spectra were recorded from 360 to 450 nm with an excitation wavelength of 340 nm. Both excitation and emission bandwidths were set at 2 nm. CAC of the polymer was determined by plotting the intensity (peak height) ratio (I_3/I_1) of the third band (393 nm, I_3) to the first band (372 nm, I_1) from the emission spectra against logarithm of polymer concentration. The CAC value was taken from the intersection of the tangent to the curve at the inflection with the horizontal tangent through the points at low concentrations.

3.4.3. Transmission electron microscopy (TEM)

A drop of the resulting nanoparticle solution containing 0.01% (w/v) phosphotungstic acid was placed on a copper grid coated with carbon film, and air-dried at room temperature overnight. The TEM observations were carried out on a JEM-2010 microscope (JEOL, Japan) with an electron kinetic energy of 300 k eV.

3.4.4. Size and size distribution

The size and size distribution of the nanoparticles were measured by dynamic light scattering (ZetaPALS, Brookhaven Instrument Corporation, USA) at a scatting angle of 90°. Multimodel software was employed for analysis. Each measurement was repeated three times. An average value was obtained from the three measurements.

3.4.5. Encapsulation efficiency of paclitaxel

To determine the encapsulation efficiency of

paclitaxel, the paclitaxel-loaded nanoparticles (8.5 mg) was first dissolved in 1 mL of chloroform, and 2 mL of ether was then added to precipitate the polymer. The suspension was centrifuged at 10,000 rpm for 10 minutes, and the supernatant was removed and dried. The dried sample was dissolved in 2 mL of the mixture of ammonium acetate solution (20 mM), methanol and acetonitrile (volume ratio=35:20:45). The solution was filtered using 0.22 μm PTFE syringe filter and analyzed for paclitaxel concentration by high performance liquid chromatography (HPLC) using the mixture as the mobile phase. The HPLC system consisted of a Waters 2690 separation module and a Waters 996 PDA detector. A Waters Symmetryshield^{TM} C_8 4.6×15.0 cm column fitted with C_8 guard column was used. The temperatures of column and samples were set at 28°C and 20°C, respectively. The detection wavelength was set at 229 nm. A calibration curve was constructed to determine paclitaxel concentration in the range of 5 to 50 ppm and the r^2 value was 0.999. The measurements were performed in triplicate. The encapsulation efficiency of paclitaxel was calculated as the following formula: Encapsulation efficiency (wt%) = (mass of paclitaxel in nanoparticles/mass of paclitaxel initially loaded) × 100 %.

3.5. *In vitro* drug release

In vitro release of paclitaxel from P(NIPAAm-*co*-AA-*co*-CHA) nanoparticles was studied as a function of pH. The freshly prepared nanoparticle solution was filtered using a 0.45 μm filter and put in a dialysis bag with a molecular weight cut-off of 10,000 (Spectra/Por 7, Spectrum Laboratories Inc.). The dialysis bag was then submerged into PBS buffer (pH 7.4) or phthalate buffers (pH 4.0 and pH 5.0), and maintained at 37ºC. At fixed time intervals, the entire medium was removed and replaced with fresh buffer. Paclitaxel was extracted from the release medium using 5 mL of DCM. The mixture was vigorously vortexed for 3 minutes. After phase separation, the organic phase was taken out carefully and evaporated overnight. The extraction efficiency was determined to be 92%. The solid samples were dissolved in 2 mL of the mobile phase and analyzed using HPLC as described in Section 2.4.5.

3.6. Confocal laser scanning microscopy (CLSM)

Doxorubicin (DOX), as a model compound, was encapsulated into the nanoparticles to study cellular uptake of the nanoparticles. Although the functioning sites of doxorubicin and paclitaxel are different (the nucleus and the cytoplasm respectively), doxorubicin gives strong fluorescence, allowing for the observations of cellular uptake of the nanoparticles and intracellular drug release. The encapsulation of DOX was based on the method reported in our previous study (7). MDA-MB-435S cells were incubated with DMEM (supplemented with 10% FBS, 5% penicillin and 2 mM L-glutamine) containing free DOX (5 mg/L) and DOX-loaded nanoparticles (DOX content: 5 mg/L) at 37°C for one hour. Samples were visualized by CLSM (Olympus FV300, Japan) at excitation wavelength of 532 nm and emission wavelength of 617 nm. All the observations were conducted under the same conditions.

3.7. Cytotoxicity test

Cytotoxicity of free paclitaxel and paclitaxel-loaded nanoparticles was tested against MDA-MB-435S cells. The cells were seeded onto 96-well plates at a density of 10, 000 cells per well and incubated for one day. Free paclitaxel and paclitaxel-loaded nanoparticles in DMEM were filtered with 0.22 μm syringe filter and diluted with the growth medium to give final paclitaxel concentrations of 0.0001, 0.001, 0.01, 0.1, 1.0, 5.0 and 10.0 mg/L. The blank nanoparticles in DMEM were filtered and diluted to 1, 10, 50, 100, 200 mg/L. The media in the wells were replaced with 100 μL of the pre-prepared samples. The plates were then returned to the incubator and maintained in 5% CO_2, at 37°C, for 48 hours. Fresh growth media and aliquots of MTT solution (10 μL) were used to replace the mixture in each well after 48 hours of exposure. The plates were then returned to the incubator and maintained in 5% CO_2, at 37 °C, for 3 hours. The growth medium and excess MTT in each well were then removed. 150 μL of DMSO was added to each well to dissolve the internalised purple formazan crystals. An aliquot of 100 μL was taken from each well and transferred to a fresh 96-well plate. Each sample was tested in eight replicates per plate. The plates were then assayed at 550 nm and 690 nm using Microplate Reader (PowerWave X, Bio-Tek Instruments). The absorbance readings of the formazan crystals were taken to be that at 550 nm subtracted by that at 690 nm. The results were expressed as a percentage of the absorbance of the blank control.

4. RESULTS AND DISCUSSION

4.1. Synthesis of P(NIPAAm-co-AA-co-CHA)

P(NIPAAm-co-AA-co-CHA) is synthesized in a two-step reaction. Firstly, monomer of cholesteryl acrylate (CHA) is prepared by an esterification reaction between cholesterol and acryloyl chloride with a yield of 78%. P(NIPAAm-*co*-AA-*co*-CHA) is then synthesized by radical copolymerization of *N*-isopropylacrylamide, acrylic acid and CHA in 1,4-dioxane. The chemical structure and composition of P(NIPAAm-*co*-AA-*co*-CHA) are analyzed by ^{1}H-NMR (Figure 1). The resonance peak at δ 4.0 (Signal a) is attributed to the protons of -NHC*H*Me$_2$ moieties. The resonance peak at δ 0.7 (Signal b) is from –C*H*$_3$ groups in the CHA moieties. The broad resonance peaks at δ 0.8-1.0 are from the protons of other methyl groups (C*H*$_3$) in cholesterol. The molar ratio of NIPAAm and CHA is calculated from the integration values of the peak at δ 4.0 and the peak at δ 0.7. Acrylic acid content of the polymer is measured by titration. The molar ratio of NIPAAm to acrylic acid to CHA is determined to be 84:4.4:1. The FT-IR spectrum of the polymer is shown in Figure 2. It exhibits strong absorptions at about 1650 cm^{-1} ($\nu_{C=O}$) and 1544 cm^{-1} (ν_{C-N}) from NIPAAm segments. The absorption of $\nu_{C=O}$ in CHA is also found at about 1720 cm^{-1}. The weight average molecular weight of the polymer is about 54 kDa with a polydispersity index of 2.0.

4.2. Critical association concentration (CAC) determination

Amphiphilic polymers can self-assemble into core-shell structure in aqueous media above a certain concentration that is known as the critical association concentration (CAC). CAC is an important parameter to characterize the stability of core-shell nanoparticles. The

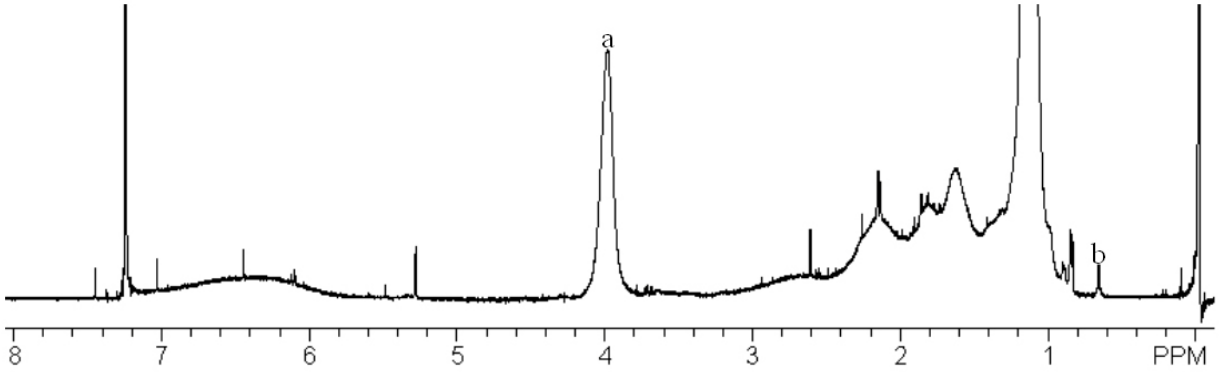

Figure 2. ^{1}H-NMR spectrum of P(NIPAAM-*co*-AA-*co*-CHA).

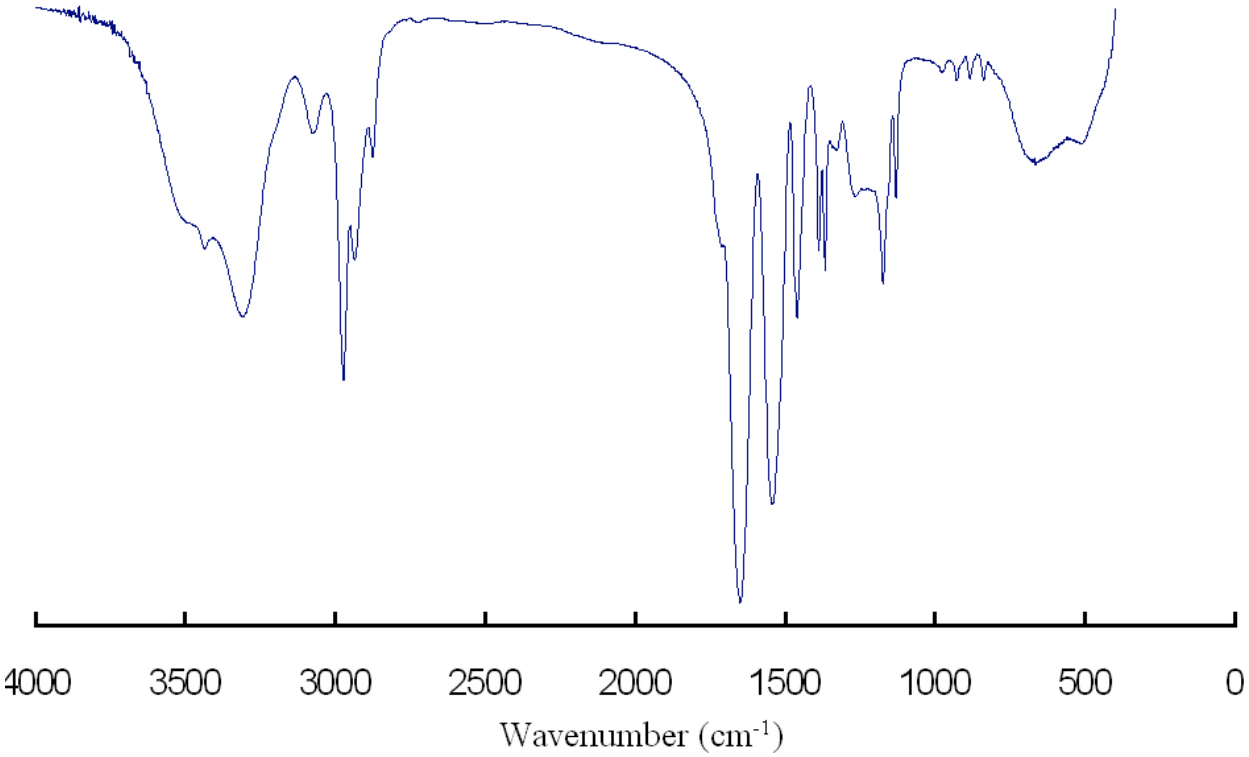

Figure 3. FT-IR spectrum of P(NIPAAM-*co*-AA-*co*-A).

CAC value of P(NIPAAm-*co*-AA-*co*-CHA) is determined using a fluorescence technique, where pyrene was chosen as a fluorescent probe. Typical emission spectra of pyrene at various concentrations are shown in Figure 3. The third peak in the spectra shifts gradually from 393 nm to 394 nm, indicating the change in vibration structure of pyrene emission (13). From the emission spectra, the ratio of I_3 (393nm) to I_1 (372nm) against polymer concentration ranging from 0.1 mg/L to 500 mg/L is plotted (Figure 3). At low concentrations, the intensity ratio changes slightly. However, as polymer concentration increases, the intensity ratio increases sharply, indicating the partitioning of pyrene into the hydrophobic core of the nanoparticles. The CAC value of the polymer in DI water is determined to be approximately 7.0 mg/L. Furthermore, because of the salt-out effect (6), it is anticipated that the CAC value will be lower in the physiological environment and thus the core-shell structure will be more stable than in water. The low CAC value indicates that such nanoparticles would remain stable under conditions of extreme dilution, as would be the case in the physiological environment, after administration.

4.3. LCST of the nanoparticles

PNIPAAm exhibits an LCST of 32ºC in water. The LCST can be modulated *via* introducing hydrophobic or hydrophilic monomers (14). In this study, a hydrophilic comonomer acrylic acid is employed to adjust the LCST of the copolymer and to introduce pH sensitivity to the

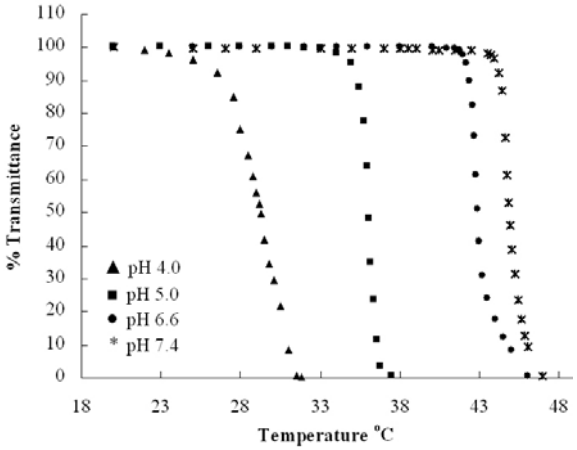

Figure 4. Plot of transmittance of P(NIPAAM-*co*-AA-*co*-CHA) nanoparticle solutions as a function of temperature at varying pH at 500 nm.

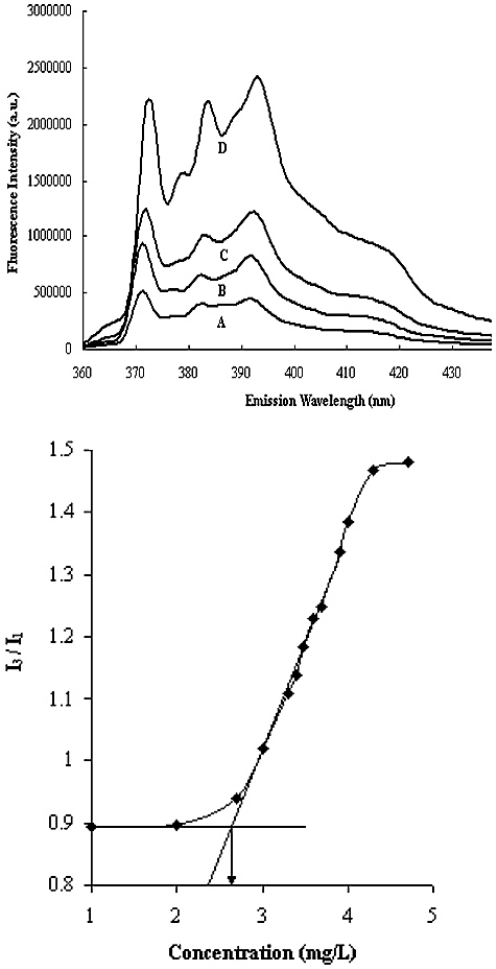

Figure 5. Fluorescent emission spectra of pyrene against polymer concentration (right, A: 0.1 ppm B: 1 ppm C: 10 ppm D: 100 ppm) and plot of the ratio of intensities (I_3/I_1) from the pyrene emission spectra as a function of logarithm of polymer concentration (left).

copolymer. The copolymer self-assembles into core-shell nanoparticles with well-isolated hydrophobic inner cores from an aqueous phase. Therefore, the composition of the shell determines the LCST of the nanoparticles regardless of the presence of cholesteryl moieties (15). The LCST values of nanoparticles made from P(NIPAAm-*co*-AA-*co*-CHA) are measured in buffers of different pH values. All the buffer solutions are prepared with an ionic strength of 154 mM. Figure 4 shows the optical transmittance of the nanoparticle solutions as a function of temperature. The LCSTs of the nanoparticles are pH-dependent. For instance, at pH 7.4, the LCST of the nanoparticles is 44.5ºC, well above the normal body temperature (37ºC). However, at pH 5.0 and 4.0, the LCSTs of the nanoparticles are 36.1ºC and 29.3ºC respectively, below the normal body temperature. With the increase of pH of the external environment, the carboxylic acid groups in acrylic acid segments (pKa of acrylic acid = 4.25) are more easily de-protonated, increasing the hydrophilicity of acrylic acid segments. This leads to an increase in the LCST of the nanoparticles. Since the LCST of the nanoparticles is higher than the normal body temperature in the physiological environment (pH 7.4) but lower than the normal body temperature in acidic environments (pH 4 and 5), these nanoparticles can be used for intracellular drug delivery. In the endosomes or lysosomes, the nanoparticles can adsorb protons and the shell of the nanoparticles becomes hydrophobic due to the decrease in the LCST. These may help to break down the endosome or lysosome membrane, releasing the enclosed drug molecules into cytoplasm.

4.4. Size, morphology and stability of the nanoparticles

The size and morphology of the nanoparticles play an important role in evaluating the possibility of their use as a drug carrier. Table 1 lists the size and size distribution of the nanoparticles fabricated in DI water. All the nanoparticles have a relatively narrow size distribution and the average effective diameter of the nanoaprticles ranges from 147 to 253 nm. A typical TEM picture indicates that the paclitaxel-loaded nanoparticles are spherical in nature (Figure 5). The particle size observed from the TEM picture is in good agreement with that measured by dynamic light scattering. From Table 1, polymer concentration has a significant effect on particle size. An increased polymer concentration yields a more viscous solution, decreasing the solvent exchange rate and thus resulting in larger nanoparticles (16, 17). Physical stability of the nanoparticles is another important aspect to be considered for clinical applications because the aggregation of nanoparticles may cause blood vessel occlusion and make them more susceptible to clearance by the RES. The shell of the nanoparticles consists of PNIPAAm and PAA segments. The hydrophilic nature of the shell prevents the nanoparticles from aggregation. As shown in Figure 6, there is no much variation in the size of nanoparticles in PBS (pH 7.4) after 14 days. Moreover, the carboxylic acid groups are de-protonated in the physiological environment (pH 7.4), rendering the negatively charged surface of the nanoparticles. Therefore, the size of the nanoparticles does not change significantly in PBS buffer containing 5% and 10% bovine Serum

Table 1 Encapsulation efficiency, size and size distribution of paclitaxel-loaded nanoparticles

Sample No.	Mass of drug (mg)	Mass of polymer (mg)	Encapsulation efficiency (%)	Effective diameter ±S.E. (nm)	Polydispersity±S.E. (nm)
N1	1.0	3.0	36	147 ± 2	0.28 ± 0.01
N2	1.0	5.0	38	208 ± 1	0.16 ± 0.02
N3	1.0	10.0	47	253 ± 3	0.20 ± 0.01
N4	2.0	6.0	23	170 ± 2	0.18 ± 0.01
N5	2.0	8.0	24	181 ± 3	0.25 ± 0.01
N6	2.0	10.0	37	194 ± 2	0.23 ± 0.01
N7	2.0	12.0	38	244 ± 1	0.20 ± 0.01
N8	3.3	10.0	17	225 ± 1	0.22 ± 0.01

albumin. From this, it is anticipated that the nanoparticles would have good physical stability *in vivo*.

4.5. Encapsulation efficiency of paclitaxel

Drug encapsulation efficiency is affected by many fabrication variables, including fabrication temperature, polymer concentration and initial drug loading. At room temperature (20ºC), the nanoparticles have low encapsulation efficiency of paclitaxel. However, at 4ºC, much greater encapsulation efficiency of paclitaxel is achieved (36% *versus* 15%). The paclitaxel-loaded nanoparticles are fabricated by a dialysis process, which involves a solvent exchange mechanism. The polymer and paclitaxel are dissolved in DMF. Upon exposure to water, solvent exchange occurs and the amphiphilic copolymer self-assembles into a core-shell structure. At the lower temperature, the solvent exchange rate is lower so that paclitaxel molecules could have enough time to assemble into the core of the nanoparticles (6). Furthermore, the dialysis membrane has a molecular weight cut-off of 2,000, larger than the molecular weight of paclitaxel (Mw 853). Therefore, it is expected that paclitaxel molecules diffuse into the external aqueous phase during the solvent removal process. A reduced fabrication temperature results in slower diffusion loss of paclitaxel so that more paclitaxel molecules could be encapsulated into the core of the nanoparticles, yielding greater encapsulation efficiency. In the following studies, all the paclitaxel-loaded nanoparticles are fabricated at 4ºC.

The effects of polymer concentration and initial drug loading level are also investigated. From Table 1, an increased initial drug loading yields lower encapsulation efficiency (Samples N3, N6 and N8). An increase in the initial drug loading yields greater drug concentration gradient between the drug solution inside the dialysis membrane and the external aqueous phase, leading to more diffusion loss of drug. Another possible reason is that the drug has precipitated at higher concentrations. With the initial drug mass of 3.3 mg (Sample N8), the solution inside the dialysis membrane appears to be turbid after 4 hours dialysis. However, with the initial drug loading of 2 mg, turbidity of the solution occurs after 20 hours with lower turbidity extent. Furthermore, at the initial drug loading of 1 mg, the colloidal solution remains clear through the whole fabrication process. These phenomena indicate that the initial drug loadings of 2 mg and 3 mg have exceeded the maximum capacity of the nanoparticle core, resulting in lower encapsulation efficiency. From Table 1, it can also be seen that an increased polymer concentration yields higher encapsulation efficiency. This is because greater polymer concentrations lead to bigger nanoparticles, providing a larger hydrophobic microdomain for the incorporation of drug molecules.

4.6. *In vitro* drug release

In vitro paclitaxel release from the nanoparticles is studied under a physiological condition (PBS, pH 7.4) and in an acidic environment (pH 4.0 and 5.0) to simulate the pH of the lysosome and the endosome respectively. As shown in Figure 7, all the release profiles are characterized with an initial burst followed by a non-release phase. The drug release from the nanoparticles in pH 7.4 at 37ºC is considerably slow, with an initial burst of about 35%. The initial burst release of paclitaxel is attributed to paclitaxel molecules located within the corona or at the interface between the micelle core and corona. On the contrary, the drug release is much faster at pH 4.0 and 5.0 at 37ºC, with approximately 92% and 72% of the drug released within 48 hours. In addition, it is observed that the paclitaxel-loaded nanoparticles are well dispersed in the buffer at pH 7.4 but aggregate and settle at the bottom of the dialysis bag at pH 4.0 and 5.0. As discussed in Section 3.3, at pH 5.0 and 4.0, the LCST of the nanoparticles decreases to a value below the normal body temperature, leading to hydrophobic shells of the nanoparticles. The loss of hydrophilicity/hydrophobicity balance of the core-shell nanoparticles leads to the deformation of the core-shell structure, releasing the enclosed drug molecules.

4.7. Cellular uptake and *in vitro* cytotoxicity

Doxorubicin is used as a model compound to study cellular uptake and intracellular drug release. MDA-MB-435S cells are incubated with free doxorubicin or doxorubicin-loaded nanoparticles for one hour. For free doxorubicin, most doxorubicin molecules are found in the nucleus but the signal is weak (Figure 8a). Free doxorubicin molecules are transported into the cell *via* a passive diffusion pathway, and some of the molecules may be pumped out of the cells because of a multi-drug resistance effect (18). On the contrary, strong fluorescence is observed in the cytoplasm of the cells incubated with paclitaxel-loaded nanoparticles (Figure 8b). The doxorubicin-loaded nanoparticles are internalized by the cells through non-specific endocytosis (19), and then escape from the endosome and/or lysosome to enter the cytoplasm. In addition, fluorescence also appears in the nucleus of the cells, which is attributed to the doxorubicin molecules released from the nanoparticles.

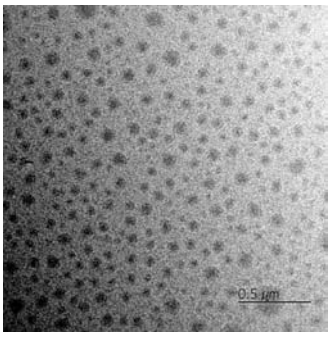

Figure 6. A typical TEM picture of the paclitaxel-loaded nanoparticles (Sample N1).

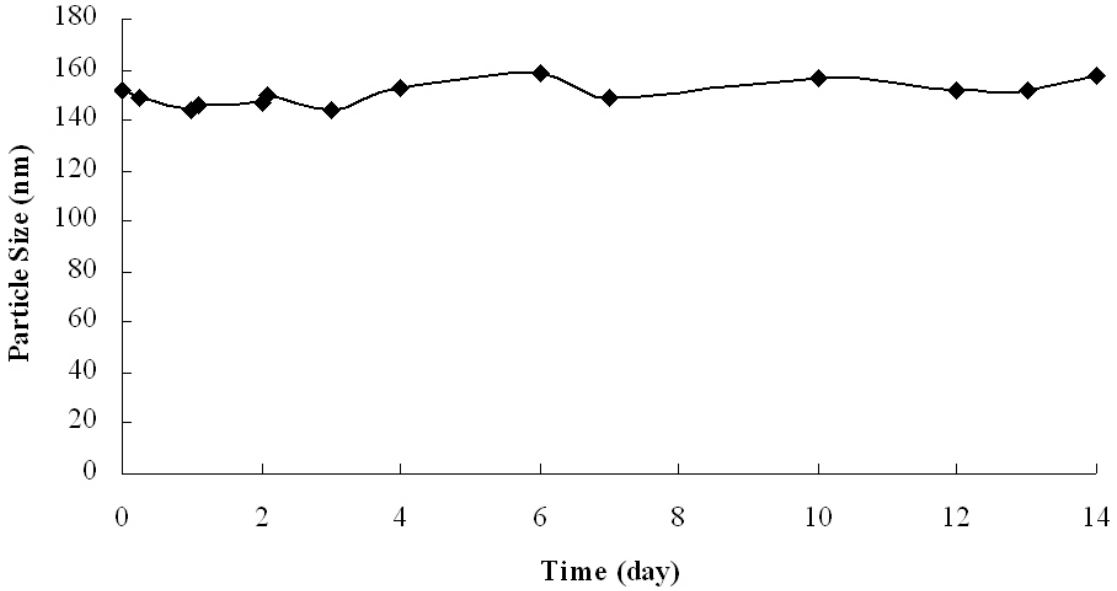

Figure 7. Size of the blank nanoparticles changes as a function of time (PBS, pH 7.4).

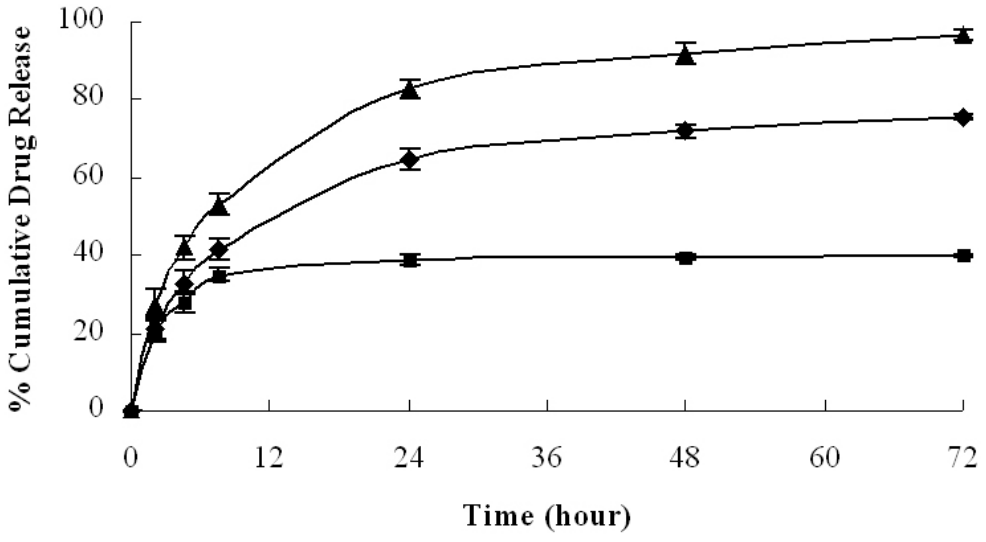

Figure 8. *In vitro* release profiles of the paclitaxel-loaded nanoparticles (Sample N6) at 37ºC, but at varying pH (▲ pH 4.0 ● pH 5.0 ■ pH 7.4).

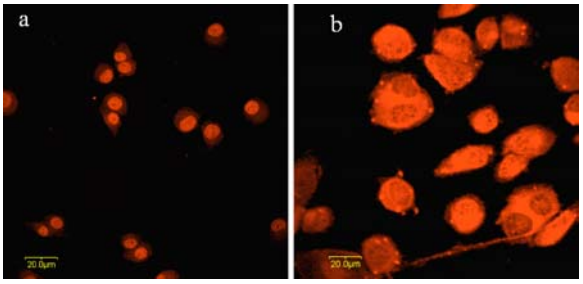

Figure 9. Confocal images of MDA-MB-435S cells incubated with (a) free doxorubicin and (b) doxorubicin-loaded nanoparticles at 37°C. (doxorubicin concentration = 5 mg/L; incubation time: one hour)

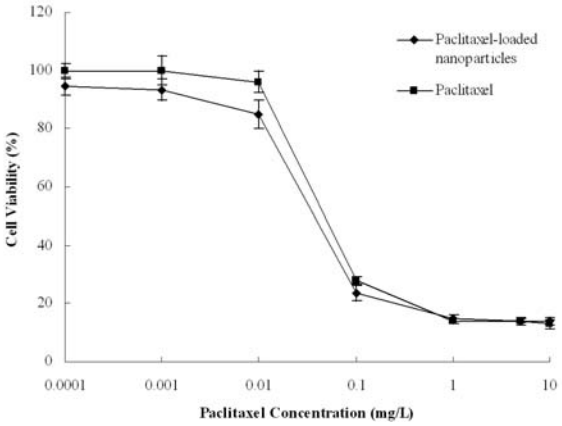

Figure 10. Viability of MDA-MB-435S cells incubated with free paclitaxel and paclitaxel-loaded nanoparticles at 37ºC for 48 hours.

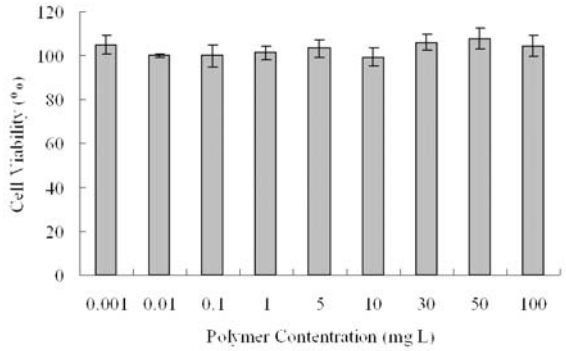

Figure 11.. Cytotoxicity of P(NIPAAM-*co*-AA-*co*-CHA) against MDA-MB-435S cells

The cytotoxicity of free paclitaxel and paclitaxel-loaded nanoparticles against MDA-MB-435S cells is examined. Paclitaxel-loaded nanoparticles show a slightly higher cytotoxicity when compared to free paclitaxel because of the increased cellular uptake of paclitaxel-loaded nanoparticles (IC_{50}: 61 μg/L *versus* 70 μg/L) (Figure 9). This finding also suggests that paclitaxel released from the nanoparticles remains bioactive. It should be noted that the blank nanoparticles do not show cytotoxcicity at a concentration of up to 100 mg/L (Figure 10).

5. CONCLUSION

Temperature- and pH-sensitive amphiphilic copolymer poly(*N*-isopropylacrylamide-*co*-acrylic acid-*co*-cholesteryl acrylate) has been synthesized and used to make core-shell nanoparticles. A highly hydrophobic anticancer drug, paclitaxel, can be easily encapsulated into the nanoparticles under the optimized fabrication conditions. The size of paclitaxel-loaded nanoparticles can be controlled below 200 nm. The nanoparticles have a pH-dependent LCST. In the physiological environment (pH 7.4), the core-shell structure of the nanoparticles is stable but it deforms, releasing the enclosed drug molecules in an acidic environment (e.g. endosomes and lysosomes). This unique property can be used for intracellular delivery of anticancer drugs. The paclitaxel-loaded nanoparticles exhibit greater cellular uptake and slightly higher cytotoxicity against MDA-MB-435S cells than free paclitaxel but the blank nanoparticles are not cytotoxic at a concentration of up to 100 mg/L. These nanoparticles may make an efficient carrier for anticancer drug delivery.

6. REFERENCES

1. K. Kataoka, A. Harada, Y. Nagasaki. Block copolymer micelles for drug delivery: design, characterization and biological significance. *Advanced Drug Delivery Rev* 47113-131 (2001)
2. C. Allen, D. Maysinger, A. Eisenberg. Nano-engineering block copolymer aggregates for drug delivery. *Coll. Surf. B: Biointerfaces* 16 3-27 (1999)
3. Y. Matsumura, H. Maeda. A new concept for macromolecular therapeutics in cancer chemotherapy: mechanism of tumoritropic accumulation of proteins and the antitumor agent smancs. *Cancer Res* 46 6387-6392(1986)
4. V. P. Torchilin. Protein- and peptide-mediated transduction: mechanisms and implications for drug delivery. *Adv. Drug Delivery Rev.* 57 489-490. (2005)
5. E. S. Lee, H. J. Shin, K. Na, Y. H. Bae. Poly(L-histidine)-PEG block copolymer micelles and pH-induced destabilization. *J. Control. Rel*. 90 363-374. (2003)
6. C. S. Chaw, K. W. Chooi, X. M. Liu, C. W. Tan, L. Wang, Y. Y. Yang. Thermally responsive core-shell nanoparticles self-assembled from cholesteryl end-capped and grafted polyacrylamides: drug incorporation and *in vitro* release. *Biomaterials* 25 4297-4308 (2004)
7. K. S. Soppimath, C. W. Tan, Y. Y. Yang. pH-Triggered thermally responsive polymer core-shell nanoparticles for targeted drug delivery. *Advanced Materials* 17 318-323(2005)
8. C. M. Spencer, D. Faulds. Paclitaxel: a review of its pharmacodynamic and pharmacokinetic properties and therapeutic potential in the treatment of cancer. *Drugs* 48 794-847(1994)
9. S. C. Kim, D. W. Kim, Y. H. Shim, J. S. Bang, H. S. Oh, S. W. Kim, M. H. Seo. *In vivo* evaluation of polymeric micellar paclitaxel formulation: toxicity and efficiency. *J. Control. Rel.* 72 191-202(2001)
10. R. B. Weiss, R. C. Donehower, P. H. Wiernik, T. Ohnuma, R. J. Gralla, D. L. Trump, J. R. Baker, D. A.

Vanecho, D. D. Vonhoff, B. Leyland-Jones. Hypersentivity reactions from taxol. *J Clin Oncol* 8 1263-1268(1990)
11. M. Kongshaug, L. S. Cheng, J. Moan, C. Rimington. Interaction of Cremophor-EL with human plasma. *Intl J Biochem* 23473-478 (1991)
12. D. C. Dong, M. A. Winnik. The Py scale of solvent polarities. *Can. J. Chem.* 62 2560-2565(1984)
13. C. Kim, S. C. Lee, S. W. Kang. Synthesis and the micellar characteristics of poly(ethylene oxide)-deoxycholic acid conjugates. *Langmuir* 16 4792-4797. (2000)
14. G. Chen, A. S. Hoffman. Graft-copolymers that exhibit temperature-induced phase transitions over a wide range of pH. *Nature* 373 49-52. (1995)
15. S. Q. Liu, Y. W. Tong, Y. Y. Yang. Incorporation and *in vitro* release of doxorubicin in thermally sensitive micelles made from poly(*N*-isopropylacrylamide-*co*-*N,N*-dimethylacrylamide)-*b*-poly(D,L-lactide-co-glycolide. with varying compositions. *Biomaterials* 26 5064-5074. (2005)
16. A. L. Le Roy Boehm, R. Zerrouk, H. Fessi. Poly epsilon-caprolactone nanoparticles containing a poorly soluble pesticides: formulation and stability study. *J. Microencapsulation* 17195-205. (2000)
17. J. Molpeceres, M. Guzman, M. R. Aberturas, M. Chacon, L. Berges. Application of central composite design to the preparation of polycaprolactone nanoparticles by solvent displacement. *J. Pharm. Sci.* 85206-213. (1996)
18. H. S. Yoo, T. G. Park. Folate-receptor-targeted delivery of doxorubicin nano-aggregates stabilized by doxorubicin-PEG-folate conjugate. *J. Control. Rel.* 100 247-256. (2004)
19. R. Savic, L. Luo, A. Eisenberg, D. Maysinger. Micellar nanocontainers distribute to defined cytoplasmic organelles. *Science* 300 615-618. (2003)

Key Words: Temperature, pH, Sensitive; Core-Shell, Nanoparticles, Paclitaxel, *In vitro*, Release

Send correspondence to: Dr Yi-Yan Yang, Institute of Bioengineering and Nanotechnology, 31 Biopolis Way, The Nanos, Singapore 138669, Tel: 65-68247106, Fax: 65-64789084, E-mail: yyyang@ibn.a-star.edu.sg

http://www.bioscience.org/current/vol10.htm