pH-Sensitive Polymer Nanospheres for Use as a Potential Drug Delivery Vehicle

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We report the development and characterization of pH-sensitive poly(2-tetrahydropyranyl methacrylate) [poly-(THPMA)] nanospheres and demonstrate their feasibility as an effective drug delivery vehicle. Poly(THPMA) nanospheres were prepared using either the double emulsion or single emulsion method for the encapsulation of, respectively, water soluble (rhodamine B) or organic soluble (paclitaxel) payloads. The resulting nanospheres showed pH-dependent dissolution behavior, resulting in significant morphologic changes and loss of nanoparticle mass under mild acidic conditions (pH 5.1) with a half-life of 3.3 days, as compared to physiologic condition (pH 7.4) with a half-life of 6.2 days. The in vitro drug release profile of the paclitaxel-loaded poly(THPMA) nanospheres revealed that the rate of drug release in pH 5.1 acetate buffer was relatively faster than that in pH 7.4 HEPES buffer. Furthermore, poly(THPMA) nanospheres showed lower cytotoxicity and higher cellular uptake as compared to the FDA-approved PLGA-based nanospheres currently in clinical practice.

Introduction

The ability to encapsulate and release drugs in response to an acidic environment is an exciting area of investigation, allowing for regulated release of drug in a cell- or tissue-specific manner. Several examples of pH-sensitive polymer-based drug encapsulated nano- or microspheres have been described for effective cancer- or antigen-presenting cells (APCs) targeting.[1−4] Additionally pH-responsive diblock copolymer micelles have shown promise as drug delivery vehicles.[5,6] Similar drug delivery systems have also been constructed for a myriad of oncology indications by linking drugs through a pH-cleavable bond such as hydrazone or acetal to amphiphilic polymers to develop pH-responsive micelles or dendrimers.[7−9] Since pH in areas of tumor interstitium is known to be relatively acidic (pH 5.5−6.5) as compared to normal tissues, it is possible to use a pH-responsive drug-encapsulated nanospheres to enhance the efficacy of current cancer therapeutics through their delivery and release directly near or inside of the cancer cells.[10,11] Furthermore, since the drug is expected to be released much faster at the tumor site as compared to the surrounding normal tissues which maintains a physiological pH (∼7.4), it is expected that the delivery of chemotherapeutic drugs via these systems may also reduce their adverse effects which in some cases can be severely debilitating.[12] For polymeric nano- or microspheres to possess controlled release properties in response to acidic pH, it is necessary for the polymer to contain acid-labile or degradable functional group in its backbone or side chains which can facilitate the surface or bulk erosion of nanospheres and result in pH-responsive drug release.[13] Nanospheres made of poly(ortho esters) or its derivatives are typical examples showing pH-mediated drug release at acidic environment, in which the ortho ester part of the polymer backbone acts as an acid-labile functional group, leading to degradation.[3,14] Several favorable attributes of poly(ortho esters)-derived nanospheres have made them suitable as a drug delivery vehicles. These include (1) pH-responsive drug release, (2) biocompatibility, and (3) low cytotoxicity.[15] To date, however, few examples of pH-sensitive polymer nano- or microspheres have been developed which meet these three requirements. Here we report a new pH-sensitive polymer system and demonstrate its potential use as a nanosphere drug delivery vehicle. We used the poly(2-tetrahydropyranyl methacrylate) [referred to as poly(THPMA)] polymer system to develop our drug delivery system.[16,17] This polymer has been studied as a photoresist material for photolithography in the semiconductor industry.[18] Briefly, when the poly-(THPMA) film containing a catalytic amount of photoacid precursor is exposed to UV through a photomask having a pattern, the generated photoacid undergoes acid-mediated degradation of THP side chain to leave a water-soluble poly-(methacrylic acid) at UV-exposed areas and finally generates a replica of initial pattern after a washing step with basic aqueous solution. The fact that water-insoluble poly(THPMA) is converted to water-soluble polymer at acidic pH made us reinvestigate this polymer system for use as a potential drug delivery vehicle possessing pH-sensitivity. Despite these characteristics that make the poly(THPMA) polymer system highly suitable for pH-sensitive drug delivery, we are not aware of a prior report demonstrating this concept. The structure of this polymer, the resulting drug encapsulated nanospheres that can be developed from it, and the proposed mechanism for controlled release of drug are depicted in Figure 1. The poly(THPMA) contains an acid-labile acetal bond as a side chain, and after exposure to mild acidic aqueous solution, the THP side chain of the polymer is cleaved to generate polymethacrylic acid (PMAA) and tetrahydropyran-2-ol (Figure 1a) which are known to be nontoxic.[19] As depicted in Figure 1b, this pH-mediated dissolution characteristic is particularly useful for drug delivery applications since the release of encapsulated drug from the polymer matrix takes place upon dissolution (not degradation) of the polymer, and its rate can be controlled depending on the pH of its surrounding environment. In this report, we further discuss the synthesis, characterization, pH-sensitivity, cytotoxicity, and cellular uptake of these nanospheres.

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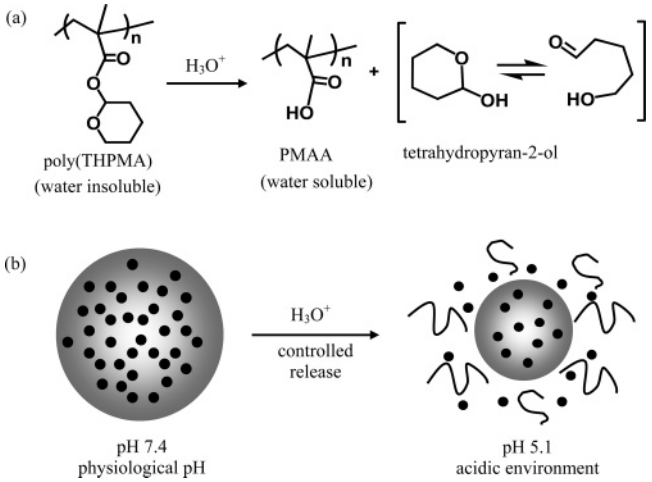

Figure 1. Schematic illustration for (a) hydrolysis mechanism of poly(THPMA) and (b) pH-mediated controlled release of its payload from poly(THPMA) nanospheres.

Experimental Section

Materials. Methacrylic acid, 3,4-dihydro-2*H*-pyran (DHP), polyvinyl alcohol (PVA; Mw 13 000−23 000), azobisisobutyronitrile (AIBN), 3-[4,5-dimethylthiaol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), and rhodamine B were purchased from Sigma (Sigma Chemical Co., St. Louis, MO). Poly (D,L-lactide-co-glycolide) 50/50 (PLGA, inherent viscosity range of 0.55−0.75 dL/g in hexafluoro-2-propanol solution) was purchased from Birmingham Polymers (Birmingham, AL). Paclitaxel (PTX) was obtained from Samyang Genex Co. (Seoul, Korea). The B16F10 melanoma cancer cell lines were purchased from the American Type Culture Collection (ATCC, Manassas, VA). The NCI-H358 cancer cell was purchased from the Korean Cell Line Bank (Seoul, Korea). Cells were cultured in DMEM medium, each supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco, Gramd Island, NY) and 1% penicillin and streptomycin at 37 °C in a humidified atmosphere of 5% CO_2−95% air. All other reagents and organic solvents were used as chemical grade.

Polymer Synthesis and Characterization. Poly(2-tetrahydropyranyl methacrylate) (poly(THPMA)) was synthesized by free radical reaction of 2-tetrahydropyranyl methacrylate (THPMA) using AIBN as a free radical initiator.[20] Briefly, THPMA (1.7 g, 10 mmol) and AIBN (8.2 mg, 0.05 mmol) were placed in a Teflon-capped vial (20 mL) and dissolved in anhydrous THF (5 mL), after which the resulting solution was degassed using N_2 gas flow for 15 min. After the vial was sealed with a Teflon-lined screw-cap, the vial was heated to 70 °C to initiate radical reaction and stirred for 12 h. After the reaction mixture was cooled to room temperature, diethyl ether (15 mL) was poured into the mixture to precipitate polymer while vigorously stirring. The polymer product was collected in white solid and dried under vacuum. ^{1}H NMR ($CDCl_3$, 300 MHz): δ 5.80−6.07 (br, 1H), 3.59−4.08 (br, 2H), 0.77−2.27 (br, 11H) ppm; FT-IR: 2942 (ν(C−H)), 1735 (ν(C═O)) cm^{-1}. Molecular weight of poly(THPMA) was measured by gel permeation chromatography (GPC Breeze system, Waters Corp., Milford, MA) using THF as an eluent: M_n = 33 828 with polydispersity index (PDI = 2.02) relative to polystyrene standards. Thermogravimetric analysis (TGA 2050, TA Instruments, New Castle, DE) showed that poly(THPMA) started to decompose at 128 °C. No glass transition temperature (T_g) was observed over temperature range studied (10 to 120 °C) by differential scanning calorimetry (DSC 2010, TA Instruments).

Preparation of Rhodamine@poly(THPMA) Nanospheres. Poly(THPMA) nanospheres encapsulating rhodamine B were prepared using double emulsion method (w/o/w). Briefly, an aqueous solution of rhodamine B (50 μL, 10 mg/mL in distilled water) was suspended in a solution of poly(THPMA) (50 mg) in CH_2Cl_2 (1 mL), and then the mixture was emulsified at 80 W for 1 min using a probe sonicator (VCX-500, Sonic & Materials Inc., Newtown, CT) to form a primary emulsion. As soon as the cloudy pink emulsion was added to aqueous solution of polyvinyl alcohol (3 mL, 1% w/v in water), the mixture was emulsified again at 80 W for 1 min using the same probe sonicator above to form the secondary emulsion that was soon added to second aqueous PVA solution (50 mL, 0.3% w/v) and stirred at room temperature until evaporation of the organic solvent was complete. The resulting nanospheres were collected by centrifugation at 13 000 rpm for 20 min (Combi-514R, Hanil, Korea) at 4 °C, resuspended in cold water, and centrifuged again to remove excess PVA. Finally, the nanospheres were lyophilized to yield a pink, fluffy powder. Blank@poly(THPMA) (without rhodamine B), blank@PLGA, and rhodamine@PLGA nanospheres were also prepared by the same method.

Preparation of PTX@poly(THPMA) and PTX@PLGA Nanospheres. Poly(THPMA) nanospheres encapsulating PTX were prepared using single emulsion method (o/w). Briefly, PTX (5 mg) and poly(THPMA) (50 mg) were dissolved in CH_2Cl_2 (1 mL). The mixture was added to aqueous solution of polyvinyl alcohol (3 mL, 1% w/v in distilled water) and subsequently emulsified using a probe sonicator at 80 W for 1 min to form primary emulsion that was soon added to second aqueous PVA solution (50 mL, 0.3% w/w), followed by stirring at room temperature for 3 h until evaporation of the organic solvent was complete. The remaining procedures were as same as those for the preparation of rhodamine@poly(THPMA) nanospheres. PTX@PLGA nanospheres were prepared by the same procedure as described above using PLGA instead of poly(THPMA).

Determination of the Loading Efficiency. Drug encapsulation efficiency was determined by measuring the amount of free drug remained in the supernatant solution, not in the nanospheres after collection of nanospheres by centrifugation. The amounts of free rhodamine B and PTX in the supernatant were measured using a fluorescence spectrometer (RF-5301PC, Shimadzu, Japan) and HPLC (Shimazu, Kyoto, Japan), respectively. For detection of rhodamine B, excitation wavelength of 535 nm was used and emission spectra were recorded from 540 to 680 nm. The detection of PTX using HPLC was described in the in vitro drug release experiment mentioned below.

Encapsulation efficiency (EE, %) = (the amount of drug entrapped in the nanospheres)/(the actual amount of drug used) × 100; where the actual amount of drug used = the initial amount of drug used − the loss of drug during overall double emulsion procedures (in the absence of polymer).

Characterization of Poly(THPMA) Nanospheres: Size, Zeta Potential, and Morphology. The size and zeta potential of the nanospheres were determined using Electrophoretic Light Scattering Spectrophotometer (ELS 8000, Otsuka Electronics Co., Ltd, Japan). Once the lyophilized nanospheres were dispersed in distilled water, the mean particle size was measured. The morphology change of nanospheres was monitored using scanning electron microscopy (SEM, S4700, Hitachi Ltd, Japan). For sample preparation for SEM measurement, a drop of the nanospheres suspension was transferred onto a small silicon wafer and dried in air. The resulting sample was coated with Pt for 30 s using a sputter to obtain approximately 10 nm thick of coating layer.

Monitoring of Weight Loss of Poly(THPMA) and PTX@poly(THPMA) Nanospheres at Different pHs. Weight loss of the blank@poly(THPMA) and PTX@poly(THPMA) nanospheres with times was monitored upon incubation in pH 5.1 acetate buffer (100 mM) and pH 7.4 HEPES buffer (100 mM), respectively. Briefly, the nanospheres (60 mg) were dispersed in each buffer at a concentration of 2 mg/mL. The suspensions were incubated at 37 °C with gentle mixing using Intelli-Mixer SLRM-2M (Seoulin Co., Korea) for 1 week. At predetermined time intervals (1, 24, 48, 96, and 168 h), 5 mL of the suspension was withdrawn and centrifuged for 20 min at 13 000 rpm. The pellets collected in bottom were washed with cold water and centrifuged again to collect. After lyophilization of the collected pellets, their weights were measured to calculate the percent weight loss relative to the initial weight at time 0.

Table 1. Mean Particle Size, Zeta-Potential, and Encapsulation Efficiency of Nanospheres Prepared by Either the Double Emulsion or the Single Emulsion Methods

nanospheres	preparation method	particle size (nm)[a]	zeta-potential (mV)[a]	encapsulation efficiency (%)[a]
blank@poly(THPMA)	double emulsion	199 ± 52	−34 ± 3.0	-
blank@PLGA	double emulsion	220 ± 47	−26 ± 0.3	-
rhodamine@poly(THPMA)	double emulsion	228 ± 63	−33 ± 5.0	58 ± 25
rhodamine@PLGA	double emulsion	214 ± 64	−34 ± 0.1	67 ± 16
PTX@poly(THPMA)	single emulsion	314 ± 77	−21 ± 0.4	96 ± 2
PTX@PLGA	single emulsion	337 ± 68	−25 ± 2.0	92 ± 3

[a] Mean ± SD ($N = 3$).

In Vitro Drug Release from PTX@poly(THPMA) Nanospheres. For the in vitro release experiment, the PTX@poly(THPMA) nanospheres (20 mg) were put into a dialysis bag (Spectra/Por membranes, MWCO 50 000, Spectrum Medical Industries, Los Angeles, CA) and the bag was sealed and immersed in 30 mL of HEPES (pH 7.4) and acetate (pH 5.1) buffers (30 mL) with 0.1% (w/v) Tween 20 at 37 °C. The tubes were incubated in a shaking water bath (Hanbaek, Korea) at 37 °C at a shaking frequency of 70 rpm. The release medium was exchanged totally with fresh HEPES or acetate buffer solution of an equal volume when the concentration of the released drugs was determined. The release solution (200 μL) was extracted with methylene chloride (800 μL) to collect free PTX released. The organic layer was evaporated and then reconstituted in mobile phase (1 mL, CH_3CN:H_2O = 50/50 (v/v)). The amounts of PTX in the samples were measured using HPLC (Shimazu, Kyoto, Japan) under the following analysis conditions: Nova-Pak C_{18} Column (4 μm, 60 Å, 3.9 × 150 mm, Waters Corp.) and the mobile phase (CH_3CN:H_2O = 50/50 (v/v)) was used. The column effluent was monitored at 227 nm by using an UV/vis detector. Quantitative value for PTX amounts was determined from a standard curve of PTX. Each batch of samples was measured in triploid.

Cytotoxicity Study of Nanospheres by MTT Assay. Cytotoxicity of poly(THPMA) and PLGA nanospheres was determined using MTT assay. NCI-H358 (human non-small cell lung cancer) cells were seeded onto a 96-well plate at density of 2×10^4 cells/well in 100 μL of cell culture medium and incubated for 1 day (37 °C, 5% CO_2). The culture medium of each well was then replaced by 100 μL of fresh medium containing various concentrations of the nanospheres. The tests were conducted in replicates of four at each concentration. After the cells were incubated for 12 h, the medium was aspirated and 100 μL of fresh medium was added, followed by addition of 10 μL of MTT solution (5 mg/mL). After the cells were incubated for additional 4 h at 37 °C, the medium was carefully aspirated. DMSO (100 μL) was added and the absorbance of each well was measured at 570 nm using a microplate reader (FL600, Bio-Tek Inc., Winooski, VT).

Cellular Uptake of Nanospheres. B16F10 cells were seeded at a density of 1×10^4 cells/well onto a 24-well plate containing a cover slip (round shape, 12 mm in diameter). After incubation overnight (37 °C, 5% CO_2), the medium was carefully aspirated and replaced with 1 mL of fresh medium containing rhodamine@poly(THPMA) and rhodamine@PLGA nanospheres (50 μg/mL). After incubation for 4 h, the medium was carefully removed and cells were washed twice with PBS buffer (pH 7.4) to remove unbound nanospheres. The remaining cells were fixed for 10 min using 4% paraformaldehyde (200 μL). After each well was rinsed three times with PBS buffer, the cover slip in each well was transferred to glass slides which were covered with a mounting medium (Permafluor, Thermo Electron Co., Waltham, MA). Cells were visualized and analyzed by light transmission and fluorescent microscopy using a confocal laser scanning microscope (FV 1000, Olympus Optical Co. Ltd., Tokyo, Japan). Individual cell image along *z*-axis was taken at 100× magnification with 0.45-μm intervals.

Cytotoxicity Study of PTX@poly(THPMA) and PTX@PLGA Nanospheres. B16F10 cells were seeded onto 96-well plates at a density of 5×10^3 cells/well in 100 μL of cell culture medium and incubated 1 day (37 °C, 5% CO_2). The culture medium of each well was then replaced by 100 μL of fresh medium containing various concentrations (μg/mL) of PTX@PLGA and PTX@poly(THPMA) nanospheres, respectively. The tests were conducted in replicates of four at each concentration. After the cells were incubated for 12 h, the medium was aspirated to remove free, unbound nanospheres and 100 μL of fresh medium was added. After the cells were incubated for additional 18 h, the cell viability was measured using MTT assay as described above.

Results and Discussion

Preparation and Characterization of Poly(THPMA) Nanospheres. To examine the feasibility of poly(THPMA) as a potential drug delivery vehicle, we prepared several nanospheres encapsulating model drugs. Rhodamine B and PTX were selected as a model water soluble and organic soluble drug, respectively. Because double and single emulsion are a typical method for the preparation of nanospheres encapsulating water soluble and organic soluble drugs, respectively, we prepared nanospheres encapsulating rhodamine B (referred to as rhodamine@poly(THPMA)) by double emulsion and paclitaxel (PTX@poly(THPMA)) by single emulsion.[21,22] Control nanospheres without drug encapsulation (referred to as blank@poly(THPMA)) were also prepared by double emulsion method to study the pH-mediated dissolution behavior by SEM. For cytotoxicity and cell-based uptake studies we used the widely described PLGA polymer system to develop similar nanospheres for comparison with the poly(THPMA) nanospheres. Specifically, we prepared PLGA nanospheres without a drug (blank@PLGA) and encapsulating rhodamine B and PTX (rhodamine@PLGA and PTX@PLGA, respectively) for comparative studies. The mean particle size, zeta potential, and drug encapsulation efficiency were summarized in Table 1. The nanospheres prepared from poly(THPMA) by double emulsion had a ca. 200−220 nm in size, similar to those of control PLGA nanospheres. The size of blank@poly(THPMA) nanospheres measured by elastic light scattering (ELS8000, Ozuka Electronics Inc.) was consistent with that obtained from scanning electron microscope (SEM) (see Figure 2). The use of single emulsion to develop PTX@poly(THPMA) nanospheres resulted in a larger particles (314 ± 77 nm; mean ± SD) as compared to rhodamine- and blank@poly(THPMA) that were developed by the double emulsion. The drug encapsulation efficiency, however, was significantly higher for the single emulsion (96 ± 2% versus 58 ± 25%; mean ± SD), which is presumably due to the good solubility of PTX in the polymer matrix and the absence of second emulsion step. The similar results were observed for the PLGA-based nanospheres. Both polymer-based nanospheres had a negative zeta potential ranging from −25 to −33 mV, indicative of the presence of carboxylic acid on their surfaces from the expected trace hydrolysis of these polymers during the nanosphere preparation step. The negative surface charge of the poly(THPMA) nanospheres may also contribute

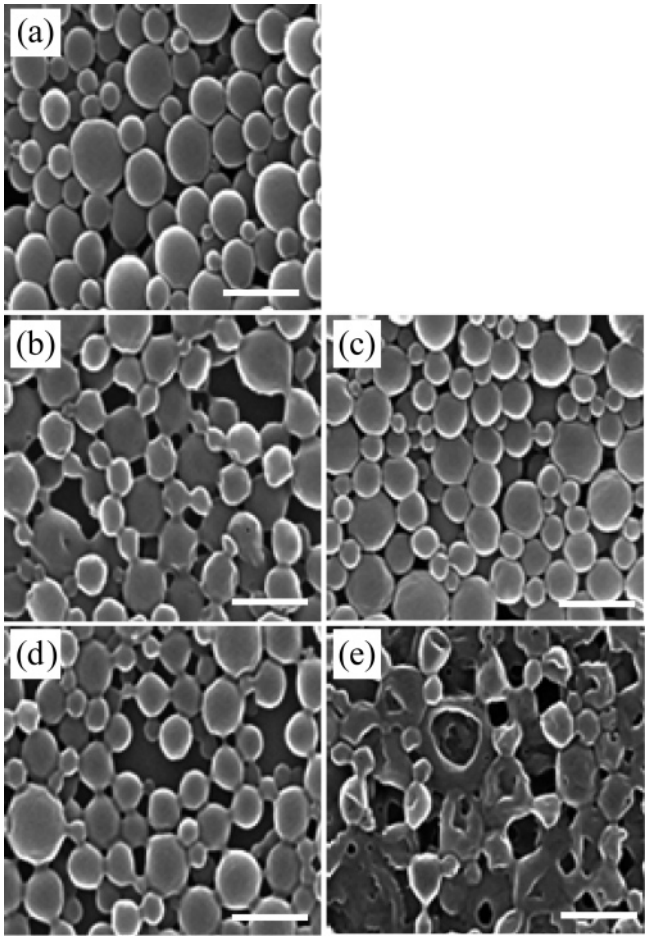

Figure 2. Scanning electron micrographic (SEM) images of blank@poly(THPMA) nanospheres upon incubation in different pH buffers (pH 7.4 HEPES and pH 5.1 acetate, respectively) at 37 °C for 0 day (as-prepared) (a); 2 days in pH 7.4 buffer (b) and in pH 5.1 buffer (c); 4 days in pH 7.4 buffer (d) and in pH 5.1 buffer (e). Scale bars denote 500 nm.

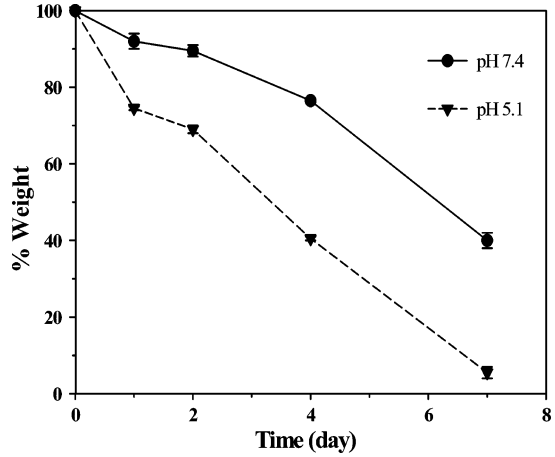

Figure 3. Profiles of weight loss of blank@poly(THPMA) nanospheres with times upon incubation in acetate (pH 5.1) and HEPES (pH 7.4) buffers at 37 °C. A percent weight loss was calculated using the following equation: weight loss = $(W_{time=0} - W_{time=T})/W_{time=0} \times 100\%$.

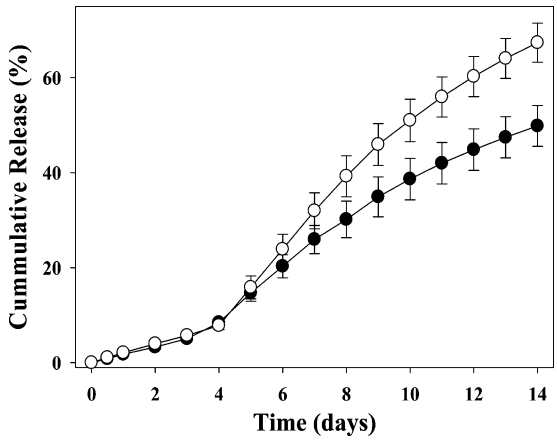

Figure 4. In vitro release of paclitaxel from PTX@poly(THPMA) nanospheres upon incubation in acetate (pH 5.1, open circle) and HEPES (pH 7.4, closed circle) buffers at 37 °C.

to the low levels of aggregation that was observed since particles are expected to be well dispersed in water presumably due to repulsion between nanospheres. Such good dispersibility in water is desirable for nanosphere delivery by parenteral injection routes.

pH-Sensitivity of Poly(THPMA) Nanospheres: Morphology Change and Weight Loss. To verify whether or not the poly(THPMA) nanospheres possess the pH-mediated dissolution property, we incubated the blank nanospheres in different pHs of buffer solutions such as HEPES buffer (pH 7.4) and acetate buffer (pH 5.1) and monitored the morphologic changes of the nanospheres over time by SEM. As shown in Figure 2, there were slight differences in terms of shape or morphology of the nanospheres during the first 2 days of incubation in both pH 7.4 and 5.1 as compared to day 0. The nanospheres appeared to have a smooth surface as visualized by the resolution limits of SEM. However, by day 4 the nanospheres that were incubated in pH 5.1 acetate buffer had significant morphologic changes presumably due to dissolution of the polymer matrix, whereas those incubated in pH 7.4 HEPES buffer had maintained their original spherical shape. These results suggests that the nanospheres made of poly(THPMA) possess a pH-sensitive dissolution property which may be exploited to engineer drug delivery vehicles that can release their payloads under mild acidic condition in a controlled manner. To further confirm the acid-mediated dissolution of the polymer particles, the extent of weight loss was measured upon incubation of the nanospheres in both HEPES (pH 7.4) and acetate (pH 5.1) buffer solutions. Figure 3 showed the % weight loss of the blank nanospheres as a function of time. As expected, the nanospheres in acetate buffer were dissolved much faster than those in HEPES buffer with a half-life of 3.3 and 6.2 days, respectively. Complete dissolution was observed after 7 days in the case of pH 5.1 buffer, whereas ~40% of nanoparticles in weight still remained in pH 7.4 buffer at the same time point of measurement. These results clearly indicate that the water-insoluble poly(THPMA) is converted to water-soluble PMAA, which occurs more efficiently in acidic pH than neutral pH and lead to dissolution of the nanospheres.

In Vitro Release of Drug from PTX@poly(THPMA) Nanospheres. Since we confirmed pH-mediated dissolution property of the poly(THPMA) nanospheres, we next examined the drug release characteristic using PTX@poly(THPMA) nanospheres in vitro. Figure 4 shows the release profile of PTX from the nanospheres incubated in acetate buffer (pH 5.1) and in HEPES buffer (pH 7.4). Unlike our expectation, the similar release rate of PTX was observed for both pHs up to day 4. Beyond that time point, however, faster release of PTX was seen in pH 5.1 buffer compared to pH 7.4, showing ~67.3 ± 4.1% and ~49.8 ± 4.2% of PTX release in day 14, respectively. In addition, no burst release was observed and a pseudo first-

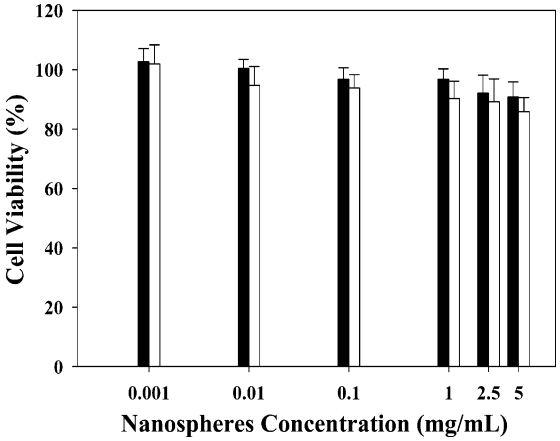

Figure 5. Cytotoxicity of blank@poly(THPMA) (filled bar) and blank@PLGA (unfilled bar) nanospheres against NCI-H358 cells after 12 h incubation.

order release profile was attained, which are two favorable characteristics for controlled release drug delivery. According to both SEM and % weight loss data, relatively faster drug release is expected in pH 5.1 even at day 2, but the actual profile showed a lagged release of 2−3 days. This phenomenon may be attributed to some fractions of partially degraded nanoparticles that may exist in the supernatant due to the decreased size, not in the pellet, even after centrifugation. Thus, such nanoparticles in the supernatant are excluded in the calculation for the weight of the pellet in the % weight loss experiment. They still remained, however, in a dialysis bag during the drug release experiment. In other words, the data of % weight loss would not be exactly matched to those of the drug release. On the other hand, ~70% of PTX release was observed in pH 5.1 even at day 14, although almost PTX@poly(THPMA) nanospheres are assumed to be dissolved within that time (see Figure S1 in Supporting Information). We attributed this imperfect PTX release to the extreme hydrophobicity of PTX with water solubility of ~0.3 μg/mL. In general, highly hydrophobic drugs such as PTX do not show complete release from particles.[23] According to a previous report, a similar release profile of PTX was also observed from PTX@PLGA nanospheres.[24] Despite the fact that drug release characteristics may be varied from drug to drug, this result indicates that poly(THPMA) nanospheres possess several favorable characteristics: pH-sensitive dissolution properties, no burst release, and a first-order release profile; thus, this nanosphere system hold a promise as a controlled release delivery vehicle for potential cancer therapy.

Cytotoxicity of Poly(THPMA) and PLGA Nanospheres. Since polymer and its degradation products should be nontoxic for use as drug delivery carriers, in vitro cytotoxicity of the blank@poly(THPMA) nanospheres was measured on NCI-H358 (human non-small cell lung cancer) cell line using the MTT assay[25] (Figure 5). PLGA nanospheres were used as positive control to compare the relative cytotoxicity of the poly(THPMA) polymer with that of the FDA-approved PLGA polymer.[26] Both polymeric nanospheres showed high cell viability even at relatively high polymer concentration: for example, over 80% of viability was observed at concentration of 5 mg/mL. It appears that the poly(THPMA) is slightly less toxic than the clinically used PLGA and thus may hold a promise for use as biomaterials for clinical applications.

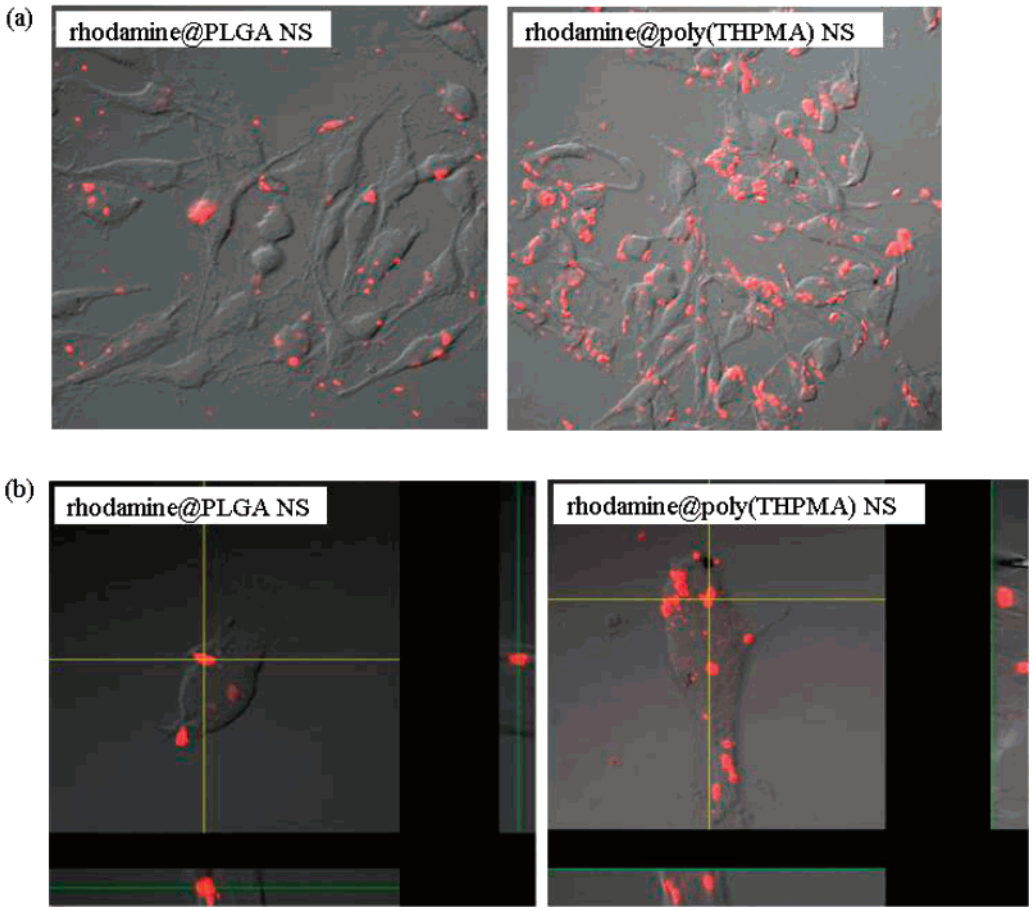

Figure 6. Overlay of light transmission and fluorescence images of B16F10 cells (a) and individual cell images along the *z*-axis at a 0.45-μm position from the bottom (b) taken after 4 h incubation with rhodamine@poly(THPMA) and rhodamine@PLGA nanospheres (50 μg/mL).

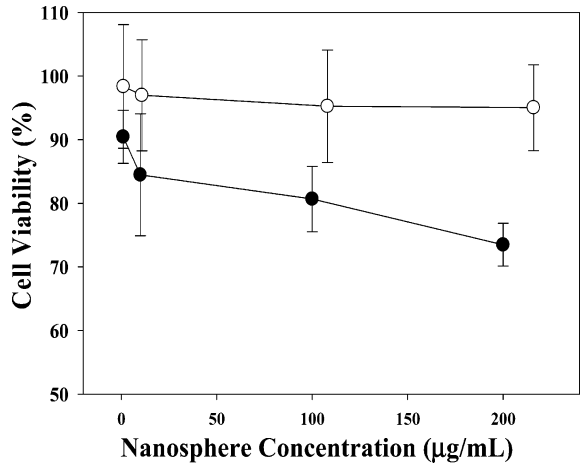

Figure 7. Cytotoxicity of PTX@poly(THPMA) (closed circle) and PTX@PLGA nanospheres (open circle) against B16F10 cells, which was measured at 18 h postincubation after removal of unbound, free nanospheres from the cells that were preincubated with various concentrations of each nanosphere for 12 h.

Comparison on Cellular Uptake of Poly(THPMA) and PLGA Nanospheres. Whether drug-loaded nanospheres can be taken up by cells is an important consideration in treatment of diseases, especially for cancer therapy by intratumoral injection because higher uptake of nanospheres by cancer cells may result in better therapeutic outcomes.[27] We examined the cellular uptake of poly(THPMA) nanospheres by cancer cells and compared the efficiency with conventional PLGA nanospheres that are known to show low uptake unless their surface is properly engineered by targeting ligands.[27] Overlay of phase contrast and fluorescence images of B16F10 melanoma cells (Figure 6a) showed that a much higher extent of binding with cells was achieved with rhodamine@poly(THPMA) than rhodamine@PLGA nanospheres (~80% and ~20%, respectively). More importantly, individual cell images taken along *z*-axis (Figure 6b) clearly reveals that rhodamine@poly(THPMA) nanospheres seen in Figure 6a were largely internalized into cells compared to rhodamine@PLGA nanospheres. The snap shot images of the individual cells along the *z*-axis were provided in Figure S2 and S3, which also indicate the position or localization of nanospheres inside cell. This attribute may ensure better therapeutic outcome of poly(THPMA) nanospheres compared to the PLGA nanospheres. Once taken up by endocytosis, the pH-sensitive poly(THPMA) nanospheres may start to be dissolved in endosomes or lysosomes releasing their payloads directly inside the cells. In this respect, we are currently studying the antitumor effect of the present system in vivo using animal models. Although the size and zeta potential of both nanospheres differ slightly, the extent of cellular uptake was significantly different. One of plausible explanation would be that the hydrophobicity or morphology difference of the particles' surface at the nanolevel may affect the interaction between particles and cell membrane, but the mechanism is still unclear.

Because the poly(THPMA) nanospheres showed higher cellular uptake than that of PLGA nanospheres, we expected that the former encapsulating anticancer drugs might have better efficacy in killing cancer cells than the latter. For this experiment, both PTX@poly(THPMA) and PTX@PLGA nanospheres containing a similar amount of PTX were used. After B16F10 melanoma cells that were used for the cellular uptake experiment above were incubated for 12 h with various concentrations of each PTX-loaded nanosphere, the free, unbound nanospheres were removed from the cell surface, followed by MTT assay after 18 h additional incubation to measure the cytotoxicity of PTX released from each nanosphere remained in the cells (Figure 7). A similar trend of result was observed with the case of 12 h additional incubation (data not shown). As seen in Figure 7, the PTX@poly(THPMA) showed significantly higher cytotoxicity than the PLGA counterpart throughout the nanosphere concentrations, which may be attributed to the higher cellular uptake of the former ones. Taken together, it is anticipated that the present poly(THPMA) nanospheres may be able to show better therapeutic efficacy as a drug delivery vehicle for treatment of cancers in vivo compared to the popular PLGA nanospheres.

Conclusion

We have demonstrated that poly(THPMA) nanospheres possess many desirable attributes of a potential drug delivery vehicle, including pH-sensitive dissolution, pH-sensitive drug release, low cytotoxicity, and high cellular uptake. The polymer nanospheres were dissolved faster in mild acidic buffer solution (pH 5.1) than at a neutral pH of 7.4, suggesting that this pH-sensitivity may be a useful property for drug delivery to tumors whose environment is more acidic than normal tissue, and for DNA or antigen delivery to antigen-presenting cells in which fast release of such payloads from pH-sensitive nanospheres is expected because of the mild acidic intracellular environment of endosomes and phagosomes. The fact that poly(THPMA) nanospheres showed lower toxicity as well as higher cellular uptake compared to conventional FDA-approved PLGA nanospheres suggests that the present polymer system may be a useful biomaterial for many biological or clinical applications. We are currently investigating the in vivo efficacy of the PTX@poly(THPMA) nanospheres using animal models of melanoma and other cancers.

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Supporting Information Available. ^{1}H NMR and FT-IR spectra, DSC and TGA profiles of poly(THPMA), % weight loss of PTX@poly(THPMA) at pH 5.1 and pH 7.4 buffers with times, and the snap shots of *z*-axis CLSM images of rhodamine@PLGA and rhodamine@poly(THPMA) taken up by B16F10 melanoma cell. This material is available free of charge via the Internet at http://pubs.acs.org.

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