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# Thermo and pH Dual-Responsive Nanoparticles for Anti-Cancer Drug Delivery**

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The emergence of stimuli-responsive drug carriers has opened the door to a new generation of anti-cancer drug delivery systems that are more intelligent and more effective than those conventional ones.[1–6] Certain malignancies can cause two stimuli around tumor site at the same time. One is a slight local temperature increase[7] and the other is a minor decrease in extracellular pH (6.8–7.2).[8] Thus, properly designed nanocarriers that are dual-responsive to both temperature and pH may be able to intelligently distinguish between normal and pathological tissues, achieving better targeting efficiency and treatment efficacy. Indeed, efforts for the preparation of dual-responsive drug carriers have been made for some time. Kim's group did some pioneering work on the temperature and pH dual-responsive polymeric delivery system for targeted oral delivery of insulin.[9] Recently, dual-responsive systems are gradually being extended to anti-cancer drug delivery. For example, Soppimath et al. prepared pH-triggered thermo responsive nanoparticles made of terpolymer P(*N*-isopropylacrylamide-*co*-*N*,*N*-dimethylarylamide-*co*-10-undecenoic acid) (P(NIPAAm-*co*-DMAAm-*co*-UA)).[2] They found that weak acidic environment (pH 6.6) could cause nanoparticle aggregation at body temperature and trigger the release of the loaded anti-cancer drug doxorubicin (DOX). Unfortunately, the drug loading capacity was quite low (2.7 %), which limits their practical usefulness. Kang et al. fabricated DOX-loaded thermo and pH responsive nanoparticles based on copolymers of NIPAAm, DMAAm and a pH sensitive telomer sulfamethoxypyridazine.[3] Similarly, this system also suffered from poor drug loading capacity and encapsulation efficiency, less than 3 % and 10 %, respectively. Lo et al. synthesized thermo and pH sensitive poly(D,L-lactide)-*g*-poly(NIPAAm-*co*-methacrylic acid) nanoparticles entrapping 5-fluorouracil of anti-cancer drug.[4] However, at body temperature, their system could only work in a pH window from 4.5 to 5.5, whereas the extracellular pH around tumor site is only slightly lower than 7.4.[8] Besides, the preparation process was complicated. Therefore, although some light has been shed on the development of thermo and pH-responsive nanocarriers for anti-cancer drug delivery, an easy-to-fabricate nanoparticulate system that combines appropriate responsiveness to pathological microenvironment, satisfactory drug loading, good controllability and biocompatibility is still lacking and remains highly desirable.

Herein, we report a kind of thermo and pH dual-responsive nanoparticles with appropriate response to pathological environment and with high payload of anti-cancer drug. The nanoparticles were assembled from a diblock copolymer comprised of a thermo-responsive hydrophilic P(*N*-isopropylacrylamide-*co*-acrylic acid) block and a biocompatible hydrophobic polycaprolactone (PCL) block, which is denoted as P(NIPAAm-*co*-AA)-*b*-PCL. Paclitaxel (PTX), a very potent yet highly water-insoluble anti-cancer drug,[10] was loaded into P(NIPAAm-*co*-AA)-*b*-PCL nanoparticles by modified nano-precipitation method. The obtained nanoparticles had a core–shell structure and tunable size ranging from 100 to 230 nm. The payload of PTX in nanoparticles was high up to 30 % with high drug encapsulation efficiency. More encouragingly, the nanoparticles aggregated at body temperature under a slightly acidic pH (pH 6.9), and a faster drug release was found to be associated with higher temperature and lower pH. This property may lead to selective accumulation of these nanoparticles and subsequent drug release in tumor tissues, which is advantageous in targeted anti-caner drug delivery.

P(*N*-isopropylacrylamide) (PNIPAAm) has been commonly used as the building block for the construction of thermo-responsive materials owing to its well-known water-soluble to water-insoluble transition at the lower critical solution temperature (LCST) of about 32 °C.[11] Incorporation of pH sensitive moiety such as acrylic acid (AA) would impart pH-response to the PNIPAAm copolymer.[12] PCL was chosen as the hydrophobic block because of its good drug encapsulation ability, favorable biocompatibility and biodegradability.[13,14] In order to synthesize P(NIPAAm-*co*-AA)-*b*-PCL copolymer, hydroxyl-terminated copolymer of NIPAAm and AA, P(NIPAAm-*co*-AA)-OH, was firstly prepared by free radical copolymerization using 2-hydroxylethanethiol (HESH) as the chain transfer agent to introduce the terminal hydroxyl. The

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molar ratio of the AA in the obtained copolymer was 10.8 % as calculated from $^1$H-NMR spectrum, which was similar to the result obtained by titration method (data not shown). A weight average molecular weight ($M_w$) of 18 000 and a number average one ($M_n$) of 7 900 were determined by gel permeation chromatography (GPC). P(NIPAAm-*co*-AA)-OH copolymer was then employed as a macromolecular initiator to prepare P(NIPAAm-*co*-AA)-*b*-PCL diblock copolymer by ring opening polymerization of caprolactone (CL). The obtained diblock copolymer had an $M_w$ of 27 000 and an $M_n$ of 18 000, and the hydrophobic/hydrophilic block ratio was estimated to be 1.1 (number-averaged) (details in Supporting Information).

PTX loaded nanoparticles were prepared by a modified nano-precipitation method. A predetermined amount of PTX and P(NIPAAm-*co*-AA)-*b*-PCL were thoroughly dissolved in hot ethanol (60 °C), a relatively environment-friendly and physiologically-acceptable solvent, to yield a clear solution. Quick injection of hot water (pH 8.5, 50 °C) into the ethanol solution precipitated the water-insoluble PTX immediately and, in the mean while, induced a rapid precipitation of the hydrophobic PCL block of the amphiphilic block copolymer, resulting in spontaneous formation of drug-entrapped polymeric nanoparticles. The pH value of the injected water was adjusted to 8.5 beforehand to ensure that the P(NIPAAm-*co*-AA) block remained water-soluble during the preparation process at 50 °C, as will be discussed in the following text. Therefore, P(NIPAAm-*co*-AA) block could serve as the hydrophilic stabilization shell, whereas the hydrophobic PCL block constructed the core entrapping precipitated PTX. The core–shell structure was later evidenced by $^1$H-NMR spectroscopy method as proposed by Hrkach et al.[15]

Table 1 summarized the major properties of several batches of PTX loaded nanoparticles prepared with varied feeding ratio of drug to copolymer. By varying drug feeding, the drug loading content of the nanoparticles can be conveniently adjusted from 3 % to 30 %. The prepared nanoparticles had relatively narrow size distribution (polydispersity < 0.15) and negative zeta potential ranging from –25 mV to –36 mV due to the ionization of the AA residues on the particle surface.

**Table 1.** PTX loaded P(NIPAAm-co-AA)-b-PCL nanoparticles with varying drug feeding [a]

| PTX feeding (copolymer based w/w) | Diameter (nm) | PDI [b] | D.L. [c] (%) | E.E. [d] (%) | Zeta Potential (mV) |
|---|---|---|---|---|---|
| 0 | 138.3 | 0.064 | 0.0 | N.A. | –30.6 |
| 0.05 | 136.2 | 0.125 | 3.3 | 69.1 | –27.9 |
| 0.125 | 135.8 | 0.133 | 8.9 | 77.7 | –25.2 |
| 0.25 | 134.3 | 0.089 | 19.7 | 98.2 | –34.2 |
| 0.50 | 138.7 | 0.145 | 29.5 | 83.8 | –36.0 |

[a] The copolymer concentration in the initial ethanol solution was kept at 20 mg/mL. [b] PDI = Polydispersity index, which stands for the width of the particle size distribution. PDI = $\mu_2/G^2$, where $\mu_2 = (D^2\text{-}D^{*2})q^4$, $G = Dq^2$. D is the translational diffusion coefficient, D* is the average diffusion coefficient and q is the scattering vector. [c] D.L. = Drug loading content. [d] E.E. = Encapsulation efficiency.

Transmission electron microscopic (TEM) image shows that the prepared nanoparticles had a near-spherical shape with minor deformation (Fig. 1a). Atomic force microscopic (AFM) result shown in Figure 1b indicates that the nanoparticle surface was very smooth with no visible drug crystals. A subsequent X-ray diffraction (XRD) analysis of the freeze-dried nanoparticles confirmed that no drug crystals existed, suggesting that PTX was amorphously dispersed inside the nanoparticles, which is preferable for a controlled release system.[16]

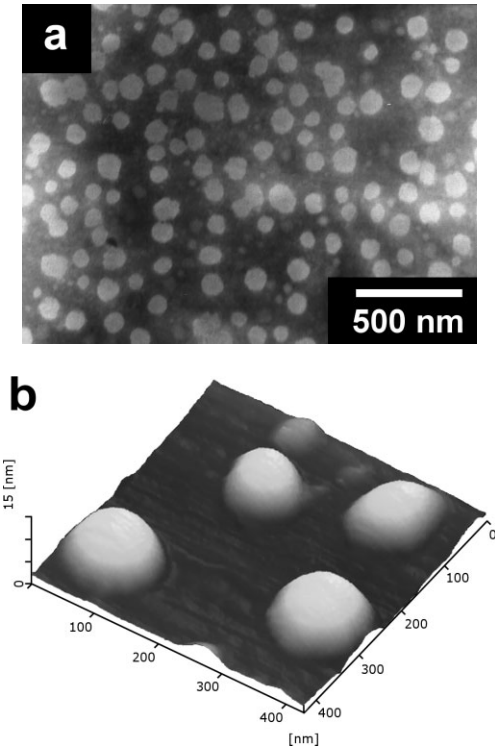


**Figure 1.** TEM image a) and AFM micrograph b) of PTX loaded nanoparticles with 20 % drug loading.

More importantly, satisfactory drug loading content (up to 30 %) as well as surprisingly high entrapment efficiency (up to 98 %) can be achieved (Table 1), implying that the encapsulation method we developed is highly effective for PTX loading. We speculate that after the injection of hot water, the speed of PTX precipitation from ethanol solution matched quite well with that of copolymer precipitation. In other words, the freshly precipitated PTX could act as the nucleation site for subsequent block copolymer precipitation and be entrapped into the hydrophobic core of the newly formed nanoparticles just in time, so that most of the drug was immobilized within the particles, leading to very high entrapment efficiency as well as drug loading content.

Particle size has a crucial impact on the in vivo fate of a colloidal drug delivery system;[17] therefore easy and effective control over the carrier size is of great importance. In our case, the copolymer concentration in the ethanol phase was

  

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found to be a key factor that determined the particle size. As shown in Figure 2, particle size increased monotonously, from about 100 nm to 230 nm, with the increase of copolymer concentration in the ethanol solution. A pseudo-linear relationship between the concentration and particle size could be established, which is similar to the result observed in our previous study.[18] Higher copolymer concentration led to higher viscosity and corresponsively slower diffusion of the organic phase into aqueous phase, hence bigger globules of organic phase containing polymer got solidified before they were disrupted into smaller ones. This result indicates that particle size adjustment could be easily achieved by simple varying the copolymer concentration in ethanol, which endows these nanocarriers with high controllability in size.

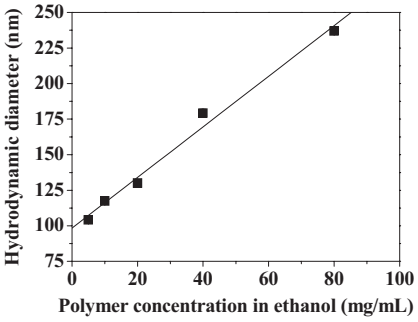


**Figure 2.** Dependence of particle size on the initial copolymer concentration in ethanol solution.

The LCST of PNIPAAm based copolymers can be tuned by introducing other monomers. Copolymerization with hydrophilic monomers increases the LCST, while hydrophobic ones lower the transition temperature.[19] Deprotonated AA is much more hydrophilic compared with its protonated counterpart and the deprotonation degree of AA is affected by pH. As a result, P(NIPAAm-*co*-AA)-OH displayed a pH-dependent thermo sensitivity as shown in Figure 3a. At pH lower than 3.5, the LCST of P(NIPAAm-*co*-AA)-OH was near to that of the PNIPAAm homopolymer (32 °C)[11] since AA moiety was in its relatively hydrophobic protonated form ($pK_a$ of AA monomer is 4.26[20]). When the pH entered the range of 4–6, where the deprotonation of AA occurs, LCST began to rise sharply. No LCST was observable when pH got higher than 6.5. For PTX loaded P(NIPAAm-*co*-AA)-*b*-PCL nanoparticles, which had a shell comprised of P(NIPAAm-*co*-AA) block, it is rational that these nanoparticles should also be dual-responsive to temperature and pH. This is confirmed by the changes of size and relative scattering intensity of nanoparticles as functions of temperature with different medium pH value, as shown in Figure 3b and c. It is apparent that these nanoparticles have a pH dependent transition temperature above which inter-particle aggregation would take place due to the dehydration of the P(NIPAAm-*co*-AA) block, as indicated by the steep increase in both size and scattering intensity. It is noteworthy that such inter-particle aggregation observed would turn into macroscopic aggregates finally. Figure 3 also shows that at the same pH, PTX loaded P(NIPAAm-*co*-AA)-*b*-PCL nanoparticles exhibited a transition temperature much lower than P(NIPAAm-*co*-AA)-OH. However, the transition temperature of the PTX loaded nanoparticles increased with the increase of medium pH, from 16 °C (pH 4) to 25 °C (pH 5.9) and more importantly to about 37 °C at a slightly acidic pH of 6.9. In contrast, at a neutral pH of 7.4, no severe aggregation of the particles could be observed and the particle size was still less than 200 nm even when the temperature was 40 °C. We attribute the difference between the transition temperature of P(NIPAAm-*co*-AA)-OH and P(NIPAAm-*co*-AA)-*b*-PCL nanoparticles to the following factors. First, introducing a hydrophobic PCL block may considerably decrease the transition temperature of P(NIPAAm-*co*-AA)-*b*-PCL nanoparticles. Though Okano et

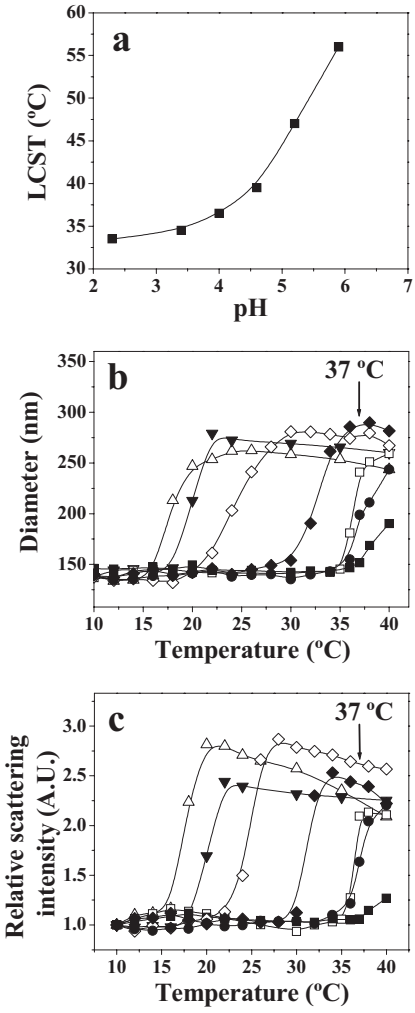


**Figure 3.** a) The effect of medium pH on the LCST of P(NIPAAm-*co*-AA)-OH copolymer. b) Diameter and c) relative scattering intensity of P(NIPAAm-*co*-AA)-*b*-PCL nanoparticles (20 % drug loading) as functions of temperature with different medium pH values. The relative scattering intensity is defined as the ratio of detected scattering intensity to the initial one at 10 °C. Legend in (b) and (c): △ pH 4.0. ▼ pH 4.9, ◇ pH 5.9, ◆ pH 6.7, □ pH 6.9, ● pH 7.2, ■ pH 7.4.

al. observed that poly(*N*-isopropylacrylamide-*b*-DL-lactide) micelles exhibited the same LCST as free linear PNIPAAm chains,[6] our case is different in that the length ratio of hydrophobic block to hydrophilic one is much larger than that in their case. Thus, larger size of the hydrophobic core could then exert a more noticeable influence on the thermo-sensitive behavior of P(NIPAAm-*co*-AA) shell. Second, the conformational freedom of the solubilized P(NIPAAm-*co*-AA) chains was greatly restricted due to covalent linking with the insoluble PCL core. Therefore, for P(NIPAAm-*co*-AA) blocks, the intrachain and interchain interactions, such as hydrogen-bonding,[21] would become more prominent whereas the solvent-polymer (i.e., water and P(NIPAAm-*co*-AA)) interaction might be weakened. Accordingly, it would be more difficult for the AA moieties in the P(NIPAAm-*co*-AA) block to dissociate, resulting in a higher and broader distribution of the apparent p$K_a$ of AA moieties in our system, which is confirmed by titration experiment (details in Supporting Information). As a consequence, these nanoparticles were able to maintain their pH-dependent thermo-sensitivity even when pH got higher than 6.9. Thus, as long as these dual-responsive drug carriers are distributed around normal tissues with normal pH and temperature, the small size combined with their hydrophilic shell may confer long-circulating property on the nanoparticles so that they have enough circulation time to reach pathological site in a passive targeting manner through the so-called enhance permeability and retention (EPR) effect.[17] Once these nanocarriers reach a tumor site with slightly higher temperature and lower extracellular pH (usually at a level of 6.8–7.2),[8] they could aggregate and accumulate there, resulting in a semi-active tumor targeting effect.

Figure 4a shows the cumulative in vitro release profiles of PTX loaded P(NIPAAm-*co*-AA)-*b*-PCL nanoparticles under different release conditions. It is apparent that the in vitro release showed certain thermo and pH sensitivity too. In order to amplify the influences of pH and temperature, we also included release tests at pH 4 and at 20 °C. When at pH 4, the nanoparticles aggregated at both investigated temperatures as mentioned earlier due to the collapse of the P(NIPAAm-*co*-AA) block. This conformational transition would produce a shrinkage of the particle shell and would deform or squeeze the hydrophobic core,[5] facilitating the drug release from the polymer matrix. Therefore at either temperature, release speed at pH 4 was higher than at pH 7.4. Similarly, releases at pH 6.8 and 7.2 were also slightly faster than at pH 7.4 at 37 °C, signifying that the collapse of the P(NIPAAm-*co*-AA) block may be favorable for drug release. Moreover, a higher temperature was also found to be helpful for drug release because the hydrophobic core becomes more mobile at higher temperature, which makes drug movement within the polymer matrix easier. Therefore, if these nanocarriers accumulate at a site with lower pH, such as tumor and cell interior,[22] along with an elevated temperature, a faster local drug release may be expected. Taking into consideration that PNIPAAm at a temperature above its LCST exhibited better cell adherence,[23]

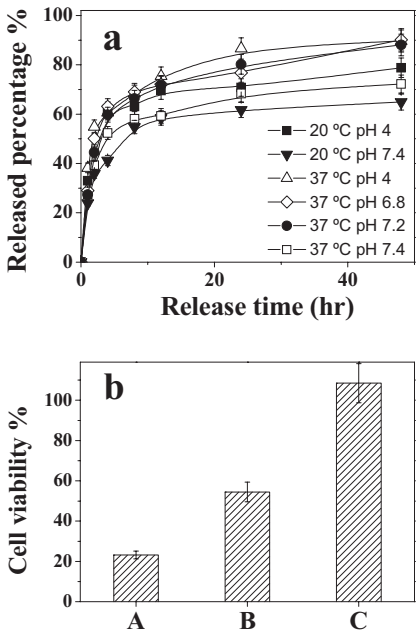


**Figure 4.** a) In vitro release profiles of PTX loaded nanoparticles (20 % drug loading) at 20 °C and 37 °C in media with different pH. b) In vitro cytotoxicity of PTX loaded nanoparticles (B) in comparison with free PTX (A) and empty nanocarriers (C).

the thermo and pH dual-responsive aggregation and drug release behaviors of the PTX loaded nanoparticles would be of great value for anti-cancer drug delivery.

To verify whether the released PTX was still pharmacologically active, and to evaluate the cytotoxicity of the nanoparticulate carriers, in vitro cytotoxicity tests against HepG2 cells were conducted. The result in Figure 4b revealed that PTX loaded P(NIPAAm-*co*-AA)-*b*-PCL nanoparticles exhibited a cytotoxic activity half of the free PTX after a 24-hour exposure time, whereas the empty nanocarriers were found nontoxic at the same polymer concentration as that of the drug-loaded ones. Considering that about only 60 % of the loaded PTX was released in 24 h, the difference in cytotoxicity between free PTX and drug-loaded nanoparticles could be easily understood.

In summary, the obtained P(NIPAAm-*co*-AA)-*b*-PCL core–shell nanoparticles have been demonstrated to be dual-responsive to both thermo and pH in a suitable window for targeted anti-cancer drug delivery. Furthermore, the system holds advantages in terms of simple preparation process, high drug loading capacity and convenient size control. Thus, these nanoparticles may serve as a promising prototype intelligent anti-cancer drug delivery system which could preferably accumulate at tumor site to release the loaded drug in response to the weak acidic extracellular pH and the slight temperature increase around malignancies. In addition, the carboxyl groups on the particle shell may be exploited in subsequent chemical modification to further widen the application of this dual-responsive nanoparticulate system.

  

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## Experimental

P(NIPAAm-*co*-AA)-OH was prepared by radical polymerization of NIPAAm and AA at a ratio of (10:1) using AIBN as initiator and HESH as the chain transfer agent in ethanol at 70 °C for 8 h. P(NIPAAm-*co*-AA)-*b*-PCL diblock copolymer was synthesized by ring-opening polymerization of CL using P(NIPAAm-*co*-AA)-OH as the initiator in the presence of stannous octoate (0.1 % w/w monomer based) as the catalyst. The polymerization was carried out at 80 °C for 120 h.

To prepare drug-loaded nanoparticles, 20 mg of P(NIPAAm-*co*-AA)-*b*-PCL and a predetermined amount of PTX were dissolved in an aliquot of ethanol by heating to 60 °C. Then 10 mL of water (pH 8.5, 50 °C) were quickly injected into the hot ethanol solution through a syringe. The solution turned bluish immediately due to the formation of nanoparticles, and the ethanol in the solution was removed by evaporation under gentle stirring.

The concentration of PTX was assayed on a Shimadzu LC-10AD (Shimadzu, Japan) HPLC system using Lichrospher C-18, 5 μ, 200 mm × 4.6 mm RP-HPLC analytical column. The mobile phase was consisting of 43/57 acetonitrile (spectral grade, Fisher, Germany)/water, and the wavelength of the ultraviolet detector was set at 227 nm.

Mean diameter and size distribution of the nanoparticles were determined by dynamic light scattering (DLS) method using a Brookhaven BI-9000 AT instrument (Brookhaven Instruments Corporation, USA). Zeta potential of the nanoparticles was obtained using Zeta-plus (Brookhaven Instruments Corporation, USA). Each sample of the nanoparticle suspensions was adjusted to a concentration of 0.05 % (w/v) in filtered water or in 0.01 M NaCl solution in the case of zeta potential examination. The thermo and pH responsive behaviors of P(NIPAAm-*co*-AA)-OH copolymer as well as P(NIPAAm-*co*-AA)-*b*-PCL nanoparticles were assessed using DLS method too by monitoring the change of particle size and scattering intensity at different temperatures with varied medium pH. The samples were allowed to equilibrate for 10 mins at each tested temperature before measuring. Morphological examination of the nanoparticles was carried out using a JEM-100S (Japan) transmission electron microscope after negative staining with phosphotungstic sodium solution (1 % w/v). AFM (SPI3800, Seiko Instruments Inc, Japan) was used to study the surface morphology of nanoparticles in a greater detail.

In vitro release of PTX from nanoparticles was studied using a dialysis bag diffusion technique. Periodically, 1 mL of the release medium (20 mL total) was withdrawn and then 1 mL of fresh release buffer was added to the system. Then PTX in the sampled release medium was extracted with 0.3 mL of $CH_2Cl_2$ and PTX concentration was subsequently determined by HPLC.

In vitro cytotoxicity of PTX loaded nanoparticles against human HepG2 cells was evaluated by MTT assay at pH 7.4, 37 °C. HepG2 cells were incubated in the presence of free PTX and PTX loaded nanoparticles at the same concentration of 80 ng mL$^{-1}$ (PTX eq.) for 24 h before the addition of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT). The cytotoxicity of empty nanoparticles was also assessed in the same way and the concentration was the same as that of the PTX loaded nanoparticles.

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