Journal of Materials Chemistry B

Accepted Manuscript

ROYAL SOCIETY OF CHEMISTRY

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Journal Name

RSCPublishing

ARTICLE

Combinatorial Targeting Polymeric Micelles for Anti-Tumor Drug Delivery

Cite this: DOI: 10.1039/x0xx00000x

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Received 00th January 2012,
Accepted 00th January 2012

DOI: 10.1039/x0xx00000x

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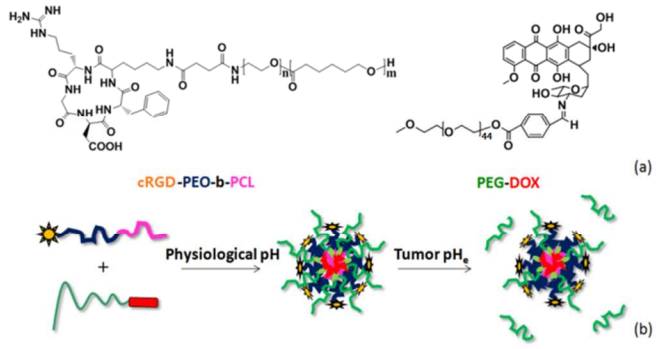
Polymeric micelles of amphiphilic block copolymers have been studied for decades for the application as targeting delivery agent of anti-tumor drugs. However, the targeting micelles may cause immunological response because of the surface distributed ligands. In this work, mixed micelles were developed to improve the specificity of cancer cell uptake under tumor-acidic condition. This was achieved by the co-assembly of an active targeting amphiphilic polymer, i.e. cyclic (Arg-Gly-Asp-D-Phe-Lys) c(RGDfK) functionalized poly(ethylene oxide)-b-poly(ε-caprolactone) (cRGD-PEO-b-PCL), and a pH sensitive drug conjugate, i.e. benzoic-mine linked PEGylated doxorubicin (PEG-DOX). Because the PEG-DOX is cleavable at the extracellular pH of solid tumor, the characteristics of the mixed micelles turns from "stealthy" to cancer cell-affinitive due to the detachment of PEG from the micelle surface and hence allows the action of the c(RGDfK) in the cRGD-PEO-b-PCL with cell membrane. Besides, the mixed micelles exhibited the capacity to encapsulate hydrophobic drugs such as paclitaxel to form a combination formulation. Our results indicate that co-assembly is a facile but efficient strategy to coordinate the characteristics of each individual component and thus provide combinatorial functions to the delivery system.

Introduction

Polymeric micelles have attracted great interest in pharmaceutical field as carrier for the delivery of therapeutic agents for the treatment of serious diseases such as cancer.[1-6] For instance, various kinds of anti-tumor drugs having different physical properties such as cisplatin, doxorubicin and paclitaxel were incorporated by polymeric micelles, and the formulations have shown characteristics to overcome the biological barriers including the reticuloendothelial system and drug resistance response, and thus gain reduced toxicity and enhanced bioavailability.[7-10] To improve the specificity of the delivery, targeting ligands as well as stimuli moieties that can response the biological differences and physiochemical signals between normal and illness tissues or extracellular and intercellular environments of cells have been utilized to functionalize the polymeric systems.[11-15] While folic acid and RGD peptide are commonly used for targeting tumor cells,[16-18] pH sensitive segments and reduction responsive disulfide bond were introduced to polymer systems to accelerate the intracellular delivery.[19,20] Furthermore, the combination of the biological and chemical stimuli was also applied to endow mutilfunctionality. As an example, folate functionalized poly(ethylene glycol)-poly(ε-caprolactone) with a hydrazone end was synthesized to conjugate doxorubicin.[21] The polymeric micelles performed a pH-triggered intracellular release after a ligand-receptor mediated endocytosis.

However, with targeting ligands distributed on the surface, typical polymeric micelles may cause immunological response resulting in fast clearance.[22] Smart carriers are thus proposed to have stealthy performance in blood but enable to recognize the extracellular environment of illness tissue, e.g. solid tumor. As a model suggested by Tochilin, it manages to temporally shield the surface targeting moieties by a PEG layer. Whenever the carrier reached the tumor site, the PEG chains could cleave to expose the ligands for a targeting effect to the cancer cells.[22] To achieve this, it requires the carriers to be very sensitive that can recognize the microenviroment of inside a tumor tissue, such as the subtle pH gradient between physiological pH (7.4) in blood and the extracellular pH of solid tumor (pH_e, 6.5-6.8).[23] So far some tumor pH_e responsive polymeric micelles were synthesized by using pH responsive monomers, e.g. histidine, β-amino ester, or the inclusion of low pH cleavable bonds.[24-26] However in case to endow multiple functionalities for the regime, the synthesis will be challengeable along sequential conjugation of additional functional moieties onto an individual polymer chain.

Co-assembly of amphiphilic polymers provides a facile way to achieve multifunctional polymeric micelles by combining the functionalities of the micelle-forming polymers.[27-29] We previously designed an amphiphilic polymer-drug conjugate, i.e. benzoic-imine linked PEGylated doxorubicin (PEG-DOX). As a dynamic covalent bond, benzoic-imine is capable to hydrolyze at tumor pH_e but more stable at physiological pH.[30-33] The co-assembly behavior of the PEG-DOX was also tested with a comb-structured amphiphilic polymer, i.e. cholate grafted poly-L-lysine. It was found that the microstructure of the co-assemblies was tuneable from micelle to vesicle by alternating the solution pH. And the cell uptake of the co-assemblies enhanced at tumor pH_e even though there was no biological ligands being conjugated.[34] Nevertheless, for engineering multifunctional co-assemblies, in addition to overlap the properties of every individuals, problem remains on regulating the performance of each components to achieve a synergistic effect. In this paper, we demonstrate a strategy for the generation of combinatorial targeting ability from co-assembly of the PEG-DOX with an active targeting block copolymer, i.e. cyclic (Arg-Gly-Asp-D-Phe-Lys) (c(RGDfK)) modified poly(ethylene oxide)-b-poly(ε-caprolactone) (cRGD-PEO-b-PCL). c(RGDfK) has been introduced into many delivery agents for targeting cancer cells.[35] And PEO-b-PCL is considered as a biocompatible amphiphilic copolymer for the encapsulation of hydrophobic drugs.[36,37] Our recent work used the cRGD-PEO-b-PCL micelle as a topical delivery agent for intravesical instilled chemotherapy of superficial bladder cancer.[38] However the c(RGDfK) related immunogenicity and the protein adsorption capacity is considered hampering the application of the cRGD-PEO-b-PCL for systematic administration.[35] In this work, by co-assembling the block copolymer with a tumor pH_e sensitive molecules, i.e. the PEG-DOX, we intend to harvest mixed micelles for triggered targeting activity of the c(RGDfK) only under the tumor-acidic condition through an ordered action of the two micelle-forming polymers (Scheme 1).



Scheme 1. (a) Chemical structure of cRGD-PEO-b-PCL and PEG-DOX. (b) Illustration of mixed micelle formation by cRGD-PEO-b-PCL and PEG-DOX, and the pH induced structural change. At physiological pH, the mixed micelle is expected to exhibit "stealth" property, in case the PEG chains of the PEG-DOX enable to shield the c(RGDfK) peptide on the cRGD-PEO-b-PCL. At the extracellular pH of solid tumor (tumor pH_e), the mild acidic condition can cause the hydrolysis of the benzoic-imine linker and hence the cleavage of PEG-DOX, which exposes the c(RGDfK) to guide the micelle to target cancer cells.

Herein the co-assembly behavior of cRGD-PEO-b-PCL and PEG-DOX is investigated to clarify the parameters that influence the property of the mixed micelles. It is worthy noticing that polymer-drug conjugates have been extensively reported for the improvement of therapeutic efficiency of the drug by changing its pharmacokinetics.[39,40] However in this regime, the use of cleavable PEG-DOX renders additional benefits to the mixed micelle on the construction of combination formulation. Since the co-dose of DOX and paclitaxel (PTX) has become a valid option in the chemotherapy of various kinds of cancers,[41-44] the activity of PTX loaded mixed micelles were formulated and evaluated in vitro.

Experimental section

Materials

Ethylene oxide (EO, BDH Limited Poole, England) and ε-caprolactone (ε-CL, Acros Organics) were dried with calcium hydride (CaH_2) and distilled before use. Potassium bis(trimethylsilyl) amide ($(CH_3)_3Si_2NK$) was purchased from Alfa Aesar. Doxorubicin hydrochloride (DOX) and paclitaxel (PTX) were obtained from Beijing Fanbo S&T. Cyclic (Arg-Gly-Asp-D-Phe-Lys) was purchased from China Peptides Co. Cell Counting Kit-8 (CCK-8) was purchased from Dojindo Molecular Technologies. PEG-DOX was synthesized according to previous report (Scheme S1, Figure S1).[34] Other compounds were obtained from J&K Chemical and used without further purification.

Synthesis of c(RGDfK) functionalized PEO-b-PCL (cRGD-PEO-b-PCL)

c(RGDfK) functionalized PEO-b-PCL was synthesized by a sequential ring-opening polymerization procedure.[38] Silicane protected amine-terminated poly(ethylene oxide) ($(CH_3)_3Si_2N$-PEO) was synthesized by initiating EO with $(CH_3)_3Si_2NK$ in anhydrous THF. ε-CL was added and polymerized as the second segment to get $(CH_3)_3Si_2N$-PEO-b-PCL. The polymer was isolated by precipitation in cold diethyl ether for three times and drying in vacuum. Three PEO-b-PCL samples with varied molecular weight were synthesized (yield 75-90%). The protection group of $(CH_3)_3Si_2N$-PEO-b-PCL was removed by adding moderate acetic acid, achieving H_2N-PEO-b-PCL. The amine- group was further terminated with carboxylic group after reaction with excess amount of succinic anhydride (4 equal) and DMAP (4 equal) for 48 h in THF. HOOC-PEO-b-PCL was purified by precipitation with cold diethyl ether for three times, and then re-dissolved in DMSO for the further conjugation of c(RGDfK). The reaction was carried out with EDC (4 equal) and NHS (4 equal) for 48 h. cRGD-PEO-b-PCL was purified by dialysis and freeze-drying (yield 89%). The synthesis route and the chemical characterization are detailed in ESI (Scheme S2 and Figure S1).

Synthesis of FITC labelled PEO-b-PCL (FITC-PEO-b-PCL)

H_2N-PEO-b-PCL (0.1 mmol) and FITC (0.2 mmol) were dissolved in DMSO (15 mL) under stirring at room temperature. The reaction was carried out in dark for 48 h. The product was obtained after dialysis against deionized water for 72 h and freeze dried (yield 87%). The chemical structure was determined by ^{1}H NMR (Figure S1).

Preparation of cRGD-PEO-b-PCL/PEG-DOX mixed micelles

Targeting mixed micelles of cRGD-PEO-b-PCL/PEG-DOX (named as TM micelles) were prepared by solvent exchange method. Desire amount of cRGD-PEO-b-PCL and 2 mg of PEG-DOX were dissolved in 0.5 mL of DMSO and then added to 2.0 mL of phosphate buffer (PBS, 10 mM, pH=7.4). The dispersion was stirred and dialyzed against 2 L of PBS with 6 changes in 24 h. The dialysate was diluted to 4 mL with PBS buffer. For fluorescence measurements, FITC-PEO-b-PCL was also mixed with the cRGD-PEO-b-PCL at 1 mol% to fluorescently label the micelles.

Characterizations

Bruker Advance 400 MHz NMR spectrometer (Bruker Instrument, Switzerland) was used to characterize chemical structure of the polymers. Molecular weight and polydispersity index (PDI) of the polymers were determined by a Waters 515 gel permeation chromatography (GPC) instrument equipped with three linear Styragel columns and a 2411 refractive index detector at 40 °C with THF at a flow rate of 1.0 mL/min. Critical micellization concentration (CMC) of the polymers was determined by using pyrene as a probe. Excitation spectra of pyrene were recorded using a fluorescence spectrophotometer (Cary Eclipse, Varian) at emission wavelength of 390 nm. The first inflection point on the plot of the intensity ratio of I_{335}/I_{338} against the polymer concentration was estimated as a CMC value. Size distribution of the polymer dispersions was measured using a Zetasizer (Nano Series, Malvern Instruments, UK) at 25 °C. The morphology of the micelles was observed on a JEM-1011 microscope at an operating voltage of 100 kV. The samples were stained by 5% phosphotungstic acid before the observation.

Drug loading

PTX was loaded to the cRGD-PEO-b-PCL and TM micelles by adding desired amount of the drug to the polymer DMSO solution during preparing the micelles as described above. To determine the drug content, the formulation was filtered through a 450 nm membrane to remove free drugs.[45] An aliquot of the filtrate was mixed with acetonitrile (1:4 v/v) to break micelles. PTX amount was determined by HPLC (Agilent Technologies 1200 Series) equipped with a UV detector set at 227 nm. A mobile phase of acetonitrile and water (1:1 v/v) was used at 1 mL/min at 30 °C. The content of DOX was measured using Shimadzu UV-2450 UV-vis spectrophotometer at 488 nm.

In vitro drug release

Drug release was carried out at 37 °C at three pHs, i.e. 7.4, 6.5 and 5.5. 1 mL of formulation was pipetted into dialysis tube with molecular weight cut-off of 2 kDa and put into 20 mL of release media and shaked at 37 °C and 140 rpm. The release media were 10 mM PBS buffer at pH 7.4 and 6.5, and 10 mM acetate buffer at pH 5.5 for DOX release. 0.1 % of Tween-80 was added to these buffers for testing the release of PTX.[46] At given times, 5 mL of release media was taken out and replenished with the same volume of fresh media. DOX and PTX concentration was determined by UV-vis spectrophotometer and HPLC. The experiments were conducted in triplicates.

Confocal laser scanning microscopy (CLSM)

U87MG and HEK293 cells (Cell Resource Center, IBMS) were cultured using Dulbecco's Modified Eagle's medium (DMEM, Gibco Invitrogen Corp.) supplemented with 10 % fetal bovine serum (FBS), 1 % L-glutamine, antibiotics, penicillin and streptomycin. The cells were plated on microscope slides in a culture dish at 2×10^4 cells/well. After 24 h, the culture medium was replaced with FITC labelled micelle dispersions in medium (containing 100 μg/mL of PEG-DOX) at a given pH (7.4 or 6.5). After incubation at 37 °C and 5 % CO_2 for 5 h, the cells were washed three times with PBS, fixed with 4 % formaldehyde for 20 min, and washed with cold PBS for three times. Then the cell nucleus was counterstained with DAPI for 15 min and washed with PBS again. CLSM microimages of cells were observed using a FV 1000-IX81 confocal laser scanning microscope (Olympus, Japan), with an excitation wavelength of 488 nm and emission wavelength of 500-520 nm for FITC and 560-620 nm for DOX.

Cytotoxicity assay

The cytotoxicity of the TM micelles and the PTX-loaded TM micelles were evaluated by CCK assay with free DOX, PEG-DOX as well as c(RGDfK) free mixed micelles which were composed of H_2N-PEO-b-PCL and PEG-DOX (named as NM micelles) as control samples. The cells were seeded in 96-well plates at a density of 2×10^4 cells/well and incubated for 24 h. Then the culture medium were replaced by the sample solutions containing desired amount of DOX and PTX with solution pH being adjusted to pH 7.4 or 6.5 by adding small volume of 1 M HCl or NaOH. After an incubation of 24 h, the sample dispersions were replaced by 100 μL of 10 % CCK-8 reagent solution. The cells were further incubated for 2 h before the absorbance at 450 nm was measured using a microplate reader (Thermo MULTISKAN MK3).

Statistics analysis. Statistical significance was assessed using Student's t-test. The difference was considered to be statistically significant if the probability value was less than 0.05 ($p < 0.05$).

Results and discussion

Co-assembly behavior of cRGD-PEO-b-PCL and PEG-DOX

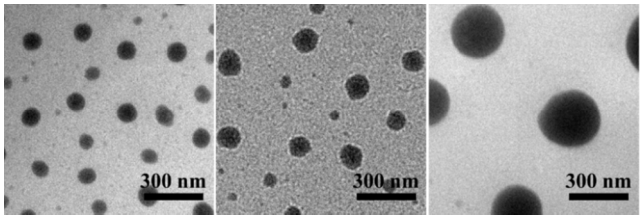
Three cRGD-PEO-b-PCLs with different molecular weights were synthesized and characterized using ^{1}H NMR (Figure S1)

and GPC (Figure S2), which are named as cRGD-PEO_{1K}-b-PCL_{1K}, cRGD-PEO_{2K}-b-PCL_{3K} and cRGD-PEO_{5K}-b-PCL_{5K} (Table 1). The cRGD-PEO-b-PCLs as well as the PEG-DOX self-assemble into micelles in PBS buffer due to their amphiphilic nature. The thermostability of the cRGD-PEO-b-PCL micelles is higher than that of the PEG-DOX assemblies by displaying low CMC values by two orders of magnitude (Table 1).[47] Nevertheless, the cRGD-PEO-b-PCLs can co-assemble with PEG-DOX at physiological pH (7.4) at given mixing molar ratios of 1.2:1.0, 1.0:2.5 and 1.0:5.0. The mixed micelles are named as TM1k, TM2k and TM5k corresponding to the component of cRGD-PEO_{1K}-b-PCL_{1K}, cRGD-PEO_{2K}-b-PCL_{3K} and cRGD-PEO_{5K}-b-PCL_{5K} respectively. Spheral shape morphology was observed from all of the samples by TEM (Figure 1). DLS shows that the co-assembly of TM1k, TM2k gains narrower dispersed aggregates at low PEG-DOX content, e.g. with a cRGD-PEO-b-PCL/PEG-DOX of 1.2:1.0 (mol/mol), while the size distribution increases with the PEG-DOX proportion (Figure 2a, b). Especially at a cRGD-PEO-b-PCL/PEG-DOX ratio of 1.0:5.0 mol/mol, the two co-assemblies give a multimodal distribution diagram (Figure 2a, b), which implies that portion of PEG-DOX was not involved in the co-assemblies but forms mono-component aggregates.[34] Differently, the DLS diagrams of TM5k show single peak at all tested mixing ratios (Figure 2c). And the particle size and distribution decreases with the increase of PEG-DOX. The results infer that in this particular system, since the free energy ($\Delta G_{micellization}$) for PEO-b-PCL micelle formation is much lower than for the PEG-DOX, the co-assembly of the two polymers could be regarded as a solubilization process of the DOX moiety by the PCL phase in cRGD-PEO-b-PCL aggregates. This can be evidenced by that the apparent CMC of the mixed micelles is more close to the values of the cRGD-PEO-b-PCL (Table 2). Thus, at fixed molar ratio, the cRGD-PEO-b-PCL with shorter PCL chain will have poorer capability for loading PEG-DOX, which could be the reason for part of the PEG-DOX molecules being not incorporated by the cRGD-PEO_{1K}-b-PCL_{1K} and cRGD-PEO_{2K}-b-PCL_{3K} at high PEG-DOX content. On the other hand, the TM1k, TM2k micelles with low PEG-DOX contents show lower CMC value when compared to the neat copolymers, indicating an increase of hydrophobic interaction and hence more negative $\Delta G_{micellization}$ for the mixed system, which means that the thermostability of colloidal drug carriers could be adjusted via the co-assembly.

Table 1. Molecular weight, CMC and particle size of cRGD-PEO-b-PCL and PEG-DOX

	Molecular weight [a]		Molecular weight [b]			CMC [c] ($\times10^{-3}$ mg/mL)	Size [d] (nm)
	$M_{n\ PEO}$ (kDa)	$M_{n\ PCL}$ (kDa)	M_n (kDa)	M_w (kDa)	M_w/M_n		
cRGD-PEO_{1K}-b-PCL_{1K}	0.9	1.0	3.2	4.1	1.3	5.1	126.6±0.8
cRGD-PEO_{2K}-b-PCL_{3K}	2.5	2.9	5.4	7.2	1.3	4.4	96.6±0.2
cRGD-PEO_{5K}-b-PCL_{5K}	4.2	5.3	9.3	11.2	1.2	2.7	131.6±0.1
PEG-DOX	2.0	-	3.2	3.4	1.1	130.0	70.4±0.4

[a] MW of blocks determined by ^{1}H NMR. [b] MW of polymer determined by GPC. [c] Determined by using fluorescence of pyrene. [d] Measured using DLS.



(a) (b) (c)

Figure 1. TEM image of TM1k (a), TM2k (b) and TM5k (c) micelle dispersions at a cRGD-PEO-b-PCL/PEG-DOX molar ratio of 1.2: 1.0.

Table 2. CMC of cRGD-PEO-b-PCL and PEG-DOX mixed system ($\times10^{-3}$ mg/mL)

Polymer ratio (mol/mol)	TM1k	TM2k	TM5k
1.2:1.0	2.0	1.1	1.7
1.0:2.5	3.0	2.2	3.0
1.0:5.0	30.3	4.1	3.4

Due to the acid-labile characteristic of the PEG-DOX, the microstructure of the mixed micelles changes with drop of solution pH. As listed in Table 3, the particle size of TM1k, TM2k micelles decreases slightly at pH 6.5 but significantly at pH 5.5, attributed to the extent of hydrolysis of the PEG-DOX.[34] At pH 6.5, partial PEG-DOX cleaves and therefore the co-assembly structure could be reserved by the cRGD-PEO-b-PCL and the residue PEG-DOX molecules. However, with a further decrease of pH to 5.5, the complete hydrolysis of PEG-DOX left only cRGD-PEO-b-PCL aggregates in solution (Table 3). Differently, the size of the TM5k micelles has only minor change from pH 7.4 to 5.0, probably due to the long PCL chain led to the formation of solid cores in the mixed micelle [48] and thus fixed the aggregates with no disassociation occurred even though the PEG-DOX component hydrolyzed at acidic pHs.

Table 3. Particle size of TM micelles at different pH and polymer molar ratio

pH	TM1k		TM2k		TM5k	
	1.2:1.0	1.0:5.0	1.2:1.0	1.0:5.0	1.2:1.0	1.0:5.0
7.4	127.5±1.5	50.8±1.6 (36.7%)[a] 295.3±6.1 (63.3%)[a]	164.2±2.8	39.4±4.5 (41.9%)[a] 198.1±1.7 (58.1%)[a]	246.3±3.3	148.3±0.5
6.5	115.2±1.3	190.2±4.2	123.3±1.9	125.5±1.1	249.5±0.3	158.6±0.2
5.5	71.6±3.0	33.9±0.3	70.4±0.1	54.3±0.3	249.0±0.5	156.6±0.5

[a] Percentage of intensity calculated from DLS diagrams showing multiple peaks (Figure 1).

Cell uptake

The TM1k and TM2k micelles were selected to test the cell uptake on U87MG, a human glioblastoma multiforme cell line. FITC-PEO-b-PCL was synthesized to label the micelles, by mixing with the cRGD-PEO-b-PCL in 1 mol%, in order to

monitor the internalization of the two micelle-forming components using LCSM. With a cRGD-PEO-b-PCL/PEG-DOX molar ratio of 1.2:1.0, the LCSM images show that the TM1k micelles were taken up by the U87MG cells at much higher level at pH 6.5 than at 7.4 after 5 hour incubation (Figure 3a). In contrast, strong fluorescence was viewed in cytoplasm by treating with the TM2k micelle at both pHs. A integration of fluorescence in the cells confirms that at pH 6.5 the TM1k micelle gains two times higher DOX fluorescence and six times higher FITC fluorescence than that at pH 7.4, whereas the fluorescence in the TM2k treated cells shows no pH dependence and keeps at a high level (Figure 3b). The data suggest that only in the TM1k micelle the c(RGDfK) can be blinded by the PEG-DOX at physiological pH, attributed to the PEO block of PEO_{1K}-b-PCL_{1K} is relatively shorter. For the TM2k micelle, the hydrophilic chains in the cRGD-PEO-b-PCL and PEG-DOX have close molecular weight and thus extend to similar distance to form the outlayer. Therefore the hydrophilic c(RGDfK) will still locate on the micelle surface and interacts with the receptors on the cancer cell membrane at pH 7.4.

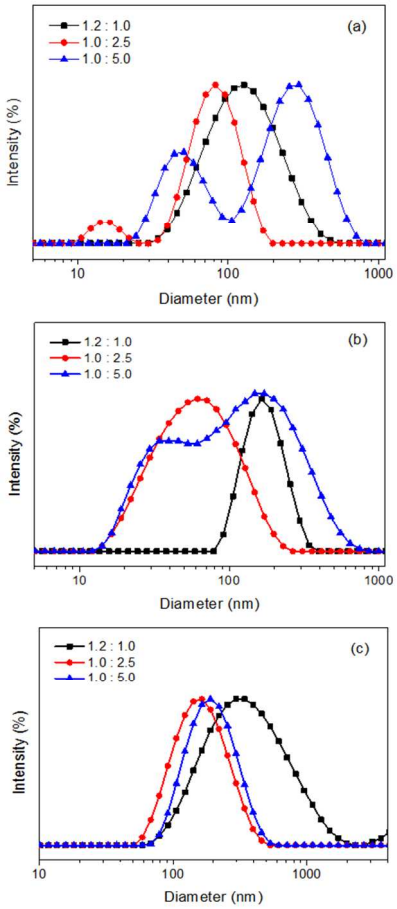


Figure 2. DLS diagrams of TM1k (a), TM2k (b) and TM5k (c) micelle dispersions in PBS at different cRGD-PEO-b-PCL/PEG-DOX molar ratios.

At tumor pH_e, the targeting activity of TM1k was recovered because the hydrolysis of the PEG-DOX has deshielded the c(RGDfK). To prove the function of c(RGDfK), a control experiment was done by using c(RGDfK) free mixed micelle, i.e. H_2N-PEO_{2k}-b-PCL_{3k}/PEG-DOX (NM2k micelle), to treat the U87MG cells. As expected, weak fluorescence was recorded at both incubation pHs (Figure 3a). The results confirm that the internalization of the TM1k micelle at the tumor-acidic pH is driven by a synergetic action of the two micelle-forming polymers, i.e. the cleavage of PEG-DOX and the receptor-mediated endocytosis proceeded by the cRGD-PEO_{1K}-b-PCL_{1K} (Scheme 1). The endocytosis pathway can be indicated by that both the FITC and DOX fluorescence mainly locates in the cytoplasm as shown in the LCSM images in Figure 3a, which were taken at 5-hour of incubation. And with longer incubation time e.g. 12h, the endocytosed DOX molecules would diffuse into the nuclei region (Figure S3).

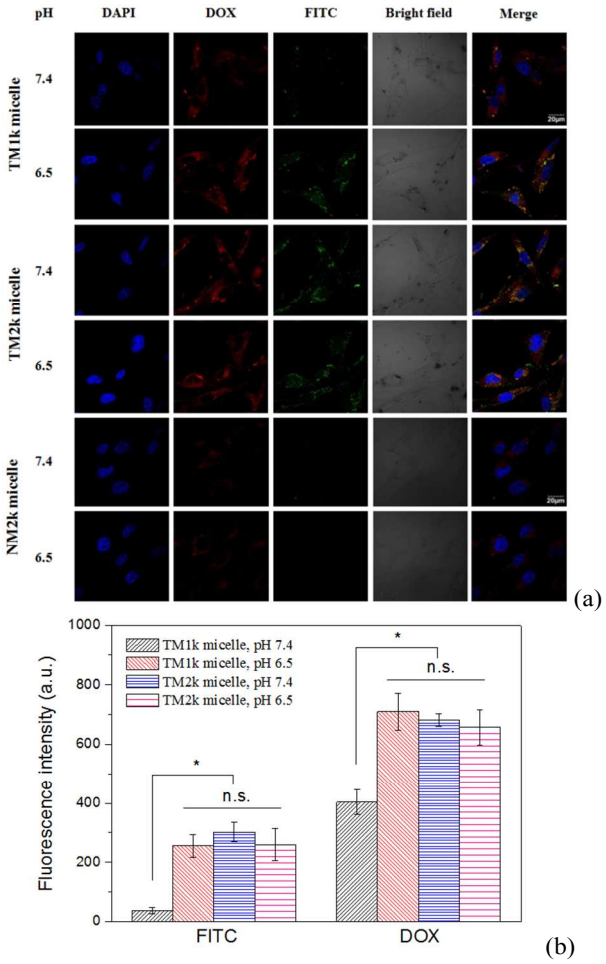


Figure 3. (a) LCSM image of U87MG cells after treated by the TM micelles and the c(RGDfK) free NM2k micelle for 5 h at pH 7.4 or 6.5. (b) Average fluorescence intensity in the cell region. Statistic analysis: * $p < 0.05$, n.s. not significant.

Carrier property of TM micelles and in vitro drug release

PEO-b-PCL has been used as a solubilizer of various kinds of lipophilic drugs.[49] In this work we checked the carrier property of the TM micelles for solubilizing hydrophobic drug paclitaxel. The loading capacity (LC) and encapsulation efficiency (EE) are listed in Table 4. It shows that the LC of the TM_{1k} and TM_{2k} micelles (polymer ratio 1.2:1.0 mol/mol) have

no significant difference in comparison with that of the parent cRGD-PEO-b-PCL micelles at a loading ratio of 5 wt% (Table 4). Nevertheless, it is noticed that the LC of PTX in TM2k is slightly poorer than that in the TM1k micelle, although the hydrophobicity of the former is considered stronger as its CMC value is lower (Table 2). The phenomenon is mainly attributed to the formation procedure, despite the efficiency of drug encapsulation in polymeric micelles can be influenced by various factors.[50] During the solvent evaporation process, since polymer molar ratio of the mixed micelles was constant, the core forming of TM2k is considered faster, as in this dispersion both the polymer concentration and the MW of the PCL moiety is higher. The rapid separation would leave more PTX molecules in the solvent phase. On the other hand, DOX is a component of the TM micelles. Its content can be adjusted by changing the composition of the mixed micelle. For example, at a cRGD-PEO-b-PCL/PEG-DOX molar ratio of 1.2:1.0, the DOX content was measured to be 8.1 % and 4.5 % (w/w) for TM1k and TM2k respectively. It is known that the physical properties of drugs such as solubility can influence their pharmacokinetics, and hence the efficiency of a combination therapy.[44] Therefore a matrix that can simultaneously carry both DOX and PTX will favor the delivery of the two therapeutics to same place and at same time.[51]

Table 4. Carrier property of cRGD-PEO-b-PCL and the TM micelles [a]

	LC (%)	EE (%)	Size (nm)
cRGD-PEO_{1K}-b-PCL_{1K}	4.4±0.4	91.2±4.1	137.5±0.4
cRGD-PEO_{2K}-b-PCL_{3K}	4.0±0.2	83.2±2.4	106.1±0.1
TM1k[b]	4.5±0.1	94.5±2.7	82.1±2.0
TM2k[b]	3.8±0.2	74.6±0.6	61.2±1.8

[a] PTX loading was 5 % to the total amount of polymer mass. [b] Polymer molar ratio was 1.2:1.0 (mol/mol).

The in vitro release of DOX and PTX was monitored at three pHs, i.e. 7.4, 6.5 and 5.5, corresponding to the physiological, tumoral and endosomal environments. Figure 4 shows pH dependent profiles of DOX release in which approximately 40, 60 and 80% of DOX were released after 24 h from the TM1k micelles at pH 7.4, 6.5 and 5.5, whereas 10, 20 and 50% were released for the TM2k micelles. While the pH response of the release can be attributed to the dynamics of the benzoic-imine hydrolysis in the PEG-DOX, the difference on the release rate between the two formulations is due to the molecular weight, i.e. the chain length, and the concentration of PCL in the TM micelles. Because the TM2k micelle contains longer and more portion of PCL blocks, it would obtain more hydrophobic inner cores, which could prohibit the water molecules from bonding the benzoic-imine bonds at the core-shell interface of the micelles, thus slow down the hydrolysis of the PEG-DOX and decreased the diffusion rate of the DOX molecules to the aqueous phase after the drug conjugate was eventually hydrolyzed.

For the PTX release, the TM micelles also shows a trend of release rate with the media pH, i.e. pH 7.4 < pH 6.5 < pH 5.5 (Figure 5), whereas no difference was observed from the cRGD-PEO-b-PCL formulations (Figure S4). This is understandable because the PTX was physically entrapped. And therefore its release could be accelerated following the structural change of the TM micelles with the drop of solution pH.

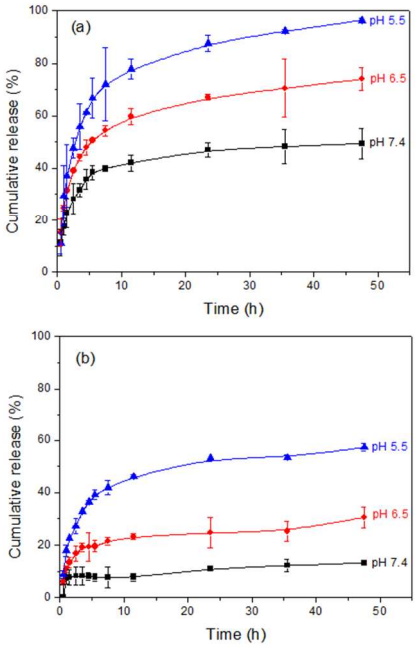


Figure 4. Cumulative release of DOX from the PTX loaded TM1k (a) and TM2k (b) micelles (polymer molar ratio 1.2:1.0 mol/mol) at different solution pHs.

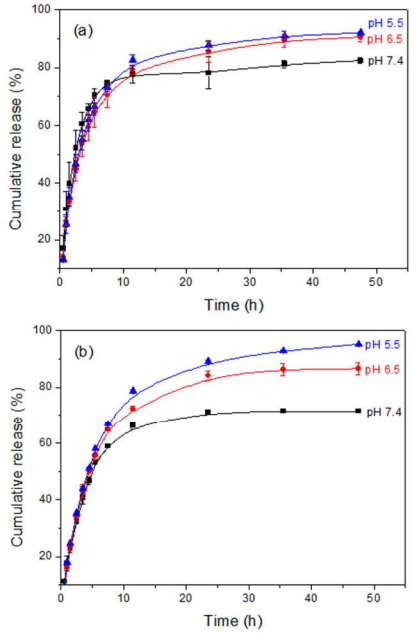


Figure 5. Cumulative release of PTX from the TM1k (a) and TM2k (b) micelles (polymer molar ratio 1.2:1.0 mol/mol) at different solution pH. The media contains 0.1 % v/v of Tween 80 to facilitate the solution of PTX.

Cytotoxicity

The cytotoxicity of the TM micelles was evaluated by CCK-8 assay. cRGD-PEO-b-PCL was first proven to be nontoxic to the U87MG cells with a polymer concentration up to 0.36 mg/mL and 0.9 mg/mL for cRGD-PEO_{1k}-b-PCL_{1k} and cRGD-PEO_{2k}-b-PCL_{3k} (0.18 mmol) respectively (Figure 6a). The formulations of the TM1k and the TM2k micelles (polymer molar ratio 1.2:1.0) were then tested on the U87MG and HEK293 cells. HEK293 is a normal human embryonic kidney cell line without RGD receptor. As shown in figure 6b, the mixed micelles caused cell death of HEK293 in a DOX concentration range of 20-60 μg/mL and the cell viability decreases with the amount of the drug. However the micelle formulations still show much less cytotoxicity when compared to that caused by free DOX, and the cell viability is not pH dependent. Furthermore, by comparing the cytotoxicity of the TM micelles with the c(RGDfK) free mixed micelle, i.e. H_2N-PEO_{1k}-b-PCL_{1k}/PEG-DOX (NM1k) micelle, it suggests that the inclusion of c(RGDfK) in the TM micelles did not improve their toxicity on the normal cell line. Without the mediation of ligand-receptor interaction, the cell uptake of micellar systems follows the fluid-phase endocytosis pathway, which is less effective and slower than the diffusion of free DOX.[52]

However to the U87MG cell line, it is seen that a treatment with the TM2k micelle resulted in much higher toxicity than that of the c(RGDfK) free formulations especially at higher drug concentrations, i.e. 40 and 60 μg/mL, showing cell viability values close to that of free DOX and having no pH dependency (Figure 6c). Compared to these, the cytotoxicity of the TM1k micelle is pH dependent. The treatment by TM1k micelle at pH 7.4 gave significant higher viability than that at pH 6.5. In contrast, the c(RGDfK) free NM1k micelle has low toxicity at both pH 7.4 and 6.5. The results are well-consisted with the LCSM observation related to the cell uptake performance of the TM micelles, and again indicate that both the cleavage of PEG-DOX and the existence of c(RGDfK) are the necessary conditions for the TM1k micelle to cause the apoptosis of cancer cells.

The cytotoxicity of the PTX loaded TM1k micelle was then evaluated. Figure 6d shows that free PTX shows dose-dependent cell viability, and the combination treatment of free DOX and PTX results in increase of cytotoxicity to the U87MG cells compared to that caused by either free DOX or free PTX, although the extent is very different. Similar phenomenon was reported in literature on other cell lines,[44] which is attributed to the different therapeutic mechanism of the two drugs.[42] Meanwhile, the PTX loaded mixed micelle formulations also demonstrate improved inhibition of cancer cell proliferation (Figure 6d). And among them, pH dependent cytotoxicity is again observed on the cells treated by the TM1k micelles. At an apparent DOX concentration of 20 μg/mL, the cell viability decreases to 60% and 45% for the dual drug formulation containing 11 μg/mL of PTX at pH 7.4 and 6.5 respectively (Figure 6d) , whereas it was ca. 70% and 60% for the neat TM1k micelle (Figure 6c). These confirm that the mixed micelles can be used for constructing combined drug formulation and transporting the carried drugs into cancer cells favourably under the tumor-acidic condition.

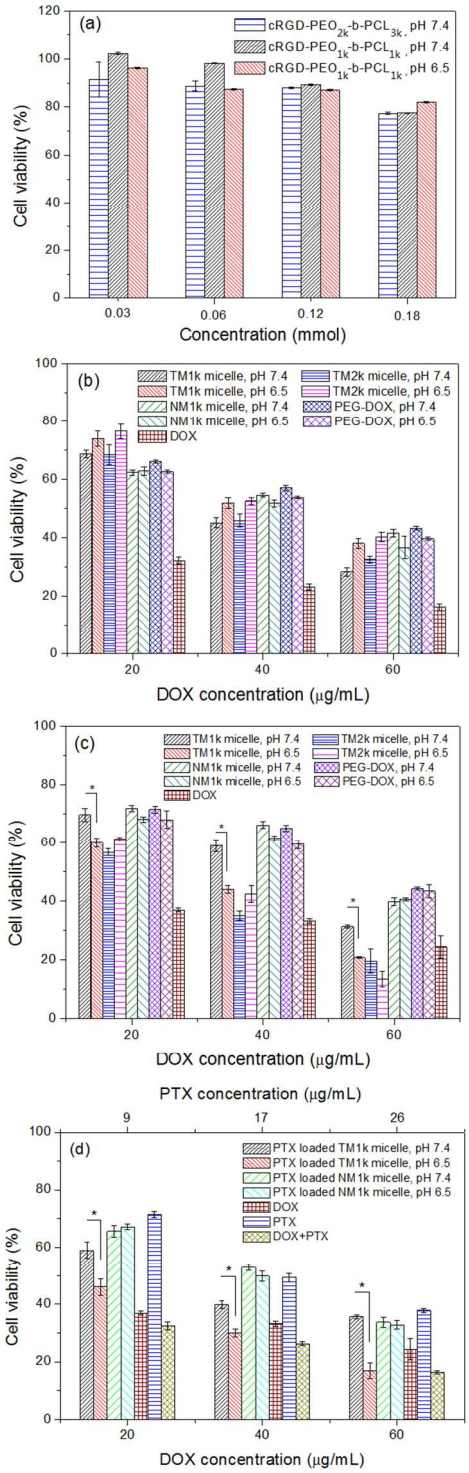


Figure 6. Cytotoxicity measurements of the cRGD-PEO-b-PCL copolymers on U87MG cells (a), TM and NM micelles on HEK 293 (b) and U87MG cells (c), and PTX loaded TM and NM micelles on U87MG cells (d). Statistic analysis: * $p < 0.05$, pH 7.4 vs. pH 6.5.

Conclusions

In summary, we demonstrate that the co-assembly of cRGD-PEO-b-PCL and benzoic-imine linked PEGylated doxorubicin endows combinatorial targeting activity of the mixed micelles to cancer cells. The TM1k micelle exhibits stealthy property at physiological pH and becomes cancer cell-affinitive at tumor pH_e, attributed to the sequential actions of the two components, i.e. the cleavage of PEG-DOX followed by the active targeting of the cRGD-PEO-b-PCL. These performances accelerated the internalization of the micelle under the tumor-acidic condition. Besides, the use of PEG-DOX favours the construction of combination therapeutic systems. PTX was loaded as the second drug in the TM micelles and gained improved cytotoxicity on U87MG cell line. The results suggest that the TM1k formulations are promising for cancer treatment and we also hope this work would benefit the future design of multifunctional delivery systems by a co-assembly method.

Acknowledgements

This work is financially supported by the National Natural Science Foundation of China (21274155, 51233007, 51473169), and MOST (2012CB933200).

Notes and references

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† Electronic Supplementary Information (ESI) available: Supplementary schemes, figures of ^{1}H NMR, GPC and PTX release profiles. See DOI: 10.1039/b000000x/

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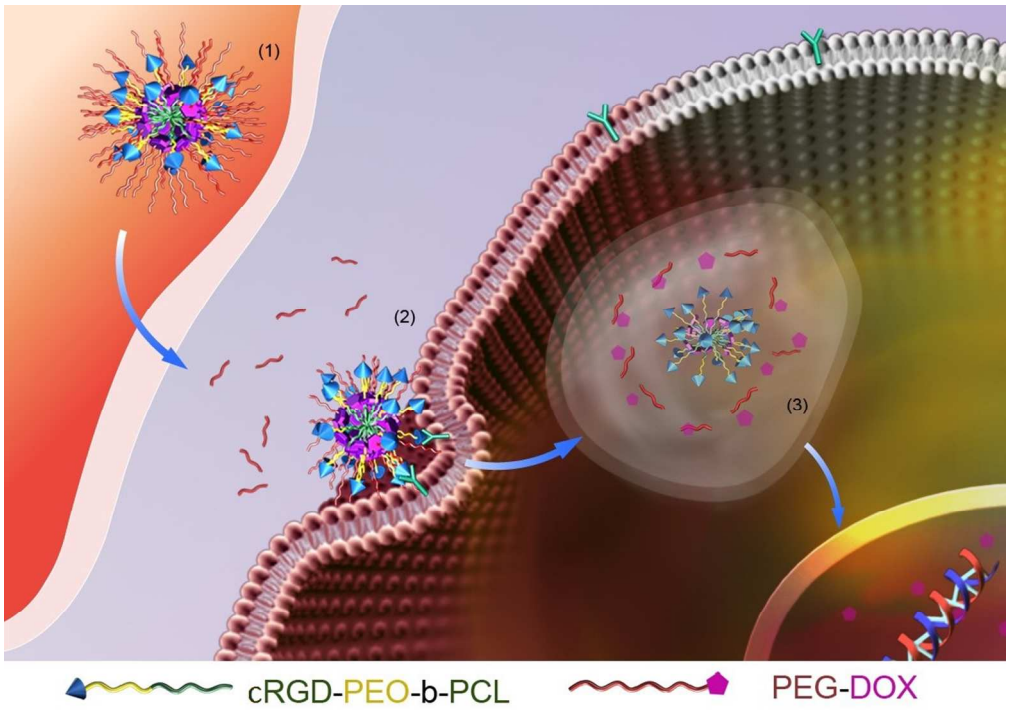

Polymeric micelles with combinatorial targeting ability to cancer cells was achieved by co-assembly of cRGD-PEO-b-PCL and tumor pH sensitive PEG-DOX.
100x71mm (299 x 299 DPI)