

Folic Acid Modified Polymeric Micelles for Intravesical Instilled Chemotherapy

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Abstract In this study, a targeting micellar drug delivery system was developed for intravesical instilled chemotherapy of bladder cancer. The amphiphilic diblock copolymer poly(ϵ -caprolactone)-block-poly(ethylene glycol) (PCL-*b*-PEO) with functional amino group (NH₂) at the end of PEO block was synthesized. Then the copolymer was conjugated with folic acid (FA) and fluorescein isothiocyanate (FITC) via the PEO-NH₂ terminus, and then assembled into micelles with the target moiety and fluorescence labeling. In addition, drug loaded micelles were also fabricated with anticancer drug doxorubicin (DOX) encapsulated in the hydrophobic core. The micelles were characterized in terms of size, drug loaded efficiency and critical micellization concentration (CMC) by means of DLS, UV and fluorescence spectra. *In vitro* cellular uptake and cytotoxicity studies showed that FA modified PCL-*b*-PEO-FA micelles have a greater targeting efficiency to human bladder cancer cell (T-24 cell) compared to PCL-*b*-PEO-NH₂ micelles due to the conjugation of FA on the surface, while no targeting effect to normal tissue originated human embryonic kidney 293 (HEK-293) cells was observed, enabling the micelles a promising drug carrier for intravesical instilled chemotherapy of bladder cancer.

Keywords Targeted drug delivery; Superficial bladder cancer; Intravesical instilled chemotherapy; Folic acid; Micelles

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INTRODUCTION

Cancer is always a leading cause of death around the world. The World Health Organization reports that over 2.8 million people died of cancer in China in 2015. Among the great efforts in recent years, the nanotechnology has become a great attractive technology for cancer diagnosis and therapy^[1, 2], of which the nanoparticles and nanovehicles are playing a vital role nowadays. These nanoparticles include self-assembled polymeric nanoconstructs (e.g., micelle, nanosphere, liposome, etc.), functionalized carbon nanotubes, nanomachines (e.g., constructed from interchangeable DNA parts and DNA scaffolds), nanofibers, nanomembranes, and nano-sized silicon chips. The traditional administration of these vehicles are usually through vein injection or oral administration in forms of liquids, tablets, pills and capsules, etc., which have to be involved with the complicated blood circulation and immunologic system, resulting in the disassembly of vehicles, the burst release of the encapsulated drugs, and the rapid clearance by the immunologic system and metabolized before these vehicles reach the tumor site

and perform their clinical effect. These problems lead to the short retention time, decreased efficiency and severe side effects.

As a specific case to avoid the above shortages of nanovehicles for the administration of anticancer drug via systemic circulation, the intravesical instillation for superficial bladder cancer possesses great advantages, such as the direct exposure of drug to tumor site, high drug concentrations at tumor site, the controllable exposure time, no influences by the complicated blood circulation and lymphatic system, and the minimum systemic toxicity^[3]. Immediate intravesical instillation of chemotherapy agents is widely recommended as a complementary treatment for bladder cancer patients after traditional transurethral resection (TUR) for superficial bladder cancer.

As we know, the bladder cancer has become the 9th most common malignancy and the 13th most common cause of cancer death worldwide^[4]. Approximately 3.56×10^5 new bladder cancer cases (2.74×10^5 males and 8.3×10^4 females) occurred worldwide in 2002^[5]. Among them 60%–80% bladder cancers are superficial cancer^[6, 7]. According to the clinical effect, the chemotherapeutic agents show some efficacy. However they are eliminated with urine easily and approximately 60%–70% patients experience a recurrence after TUR, resulting in the burden either economically or

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physiologically for the patients^[8, 9]. As a result, frequent instillation is required to keep high drug concentration in tumor site, causing a series of problems such as increased cost, side effects and tumor drug-resistance *etc.* Hence the critical issue in the chemotherapy of superficial bladder cancer *via* intravesical instillation is to design an effective drug delivery system that can carry anti-cancer drugs to tumor site effectively and have none or low side effect for normal tissues.

In recent decades, targeting delivery of anticancer drugs including active targeting and passive targeting has been widely investigated^[10] due to its ability to deliver the drugs specifically to tumor sites^[11]. Active targeting can be realized by conjugating a cancerous tissue or cell-specific ligand into the delivery system, while passive targeting is mainly realized by the enhanced permeation and retention (EPR) effect of nanocarriers with large size^[11]. Nanoparticles have been studied extensively as a targeting drug delivery system over the past decades due to the excellent characteristics such as the nanoscaled size that well meets the demands of EPR, easily functionalized surface, solubilization for drugs, and the controlled and sustained drug release behavior^[12, 13]. Nanoparticles have also been studied in intravesically instilled chemotherapy of superficial bladder cancer^[14–16]. However, the results were not so satisfactory due to lack of selectivity between tumor and normal tissues. Therefore, we can expect a wonderful progress if the targeting ability is introduced to the nanoparticles for the treatment of bladder cancer.

Folic acid (FA) has been recognized as a prominent targeting moiety capable of specific interaction with the folate receptor (FR) which is vastly overexpressed in several human tumors and has little expression in normal tissues^[17–19]. In consideration of the specific interaction, FA has often been covalently attached to drug delivery carriers for constructing the targeting delivery system. Among them, polymeric micelles with FA functionalization have been extensively studied for actively targeting cancer cells^[20, 21].

Therefore, our present work focused on the development of micellar drug delivery system with tumor targeting ability for the chemotherapy of superficial bladder cancer by means of intravesical instillation. The micelles were constructed by amphiphilic diblock copolymer poly(ϵ -caprolactone)-block-poly(ethylene oxide) (PCL-*b*-PEO) for its biocompatibility, biodegradability, and FDA approval. The surface functionalization was realized by conjugating FA and the fluorescence label molecule fluorescein-5-isothiocyanate (FITC) onto the end of hydrophilic PEO block of copolymer *via* the amino group. After encapsulating the anticancer drug doxorubicin (DOX), the surface functionalized micelles were well studied on the intracellular uptake. *In vitro* cytotoxicity and targeting against the human bladder cancer (T-24 cell) were also evaluated.

EXPERIMENTAL

Materials

Ethylene oxide (EO, BDH Limited Poole, England) was distilled in the presence of calcium hydride (CaH₂) at 0 °C.

ϵ -Caprolactone (ϵ -CL, Acros Organics) was dried with CaH₂ and purified by vacuum distillation at 70 °C before use. Tetrahydrofuran (THF) was refluxed in the presence of metal sodium under the protection of argon atmosphere and distilled before use. Potassium bis(trimethylsilyl) amide ([$(\text{CH}_3)_3\text{Si}$]₂NK, Alfa Aesar), *N*-ethyl-*N'*-(3-dimethylaminopropyl) carbodiimide (EDC, Alfa Aesar), *N*-hydroxy-succinimide (NHS, Alfa Aesar), fluorescein-5-isothiocyanate (FITC, Beijing Fanbo Science & Technology Co., Ltd), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, from Alfa Aesar), Dulbecco's modified eagle medium (DMEM, high glucose), HAM'S/F12, penicillin, streptomycin and fetal bovine serum (FBS) (all from Gibco) were used as received without further treatment. Other reagents were commercially available chemicals and used as received. T-24 (human bladder cancer cells), HEK-293 (human embryonic kidney 293 cells) were obtained from Cell Resource Center, IBMS.

Synthesis of PCL-*b*-PEO-NH₂ Block Copolymer

PCL-*b*-PEO-NH₂ was synthesized by anionic ring-opening polymerization of CL and EO monomers in the presence of [$(\text{CH}_3)_3\text{Si}$]₂NK as an initiator^[22]. Briefly, PEO was firstly synthesized in THF solution under stirring in a previously flamed and nitrogen-purged glass reactor at 40 °C for 28 h. After that, quantitatively CL in THF was slowly added and further reacted for 0.5 h. Finally, formic acid was added to terminate the reaction and the solution was poured to ten-fold excess diethyl ether (pre-cold) to precipitate the product.

Conjugation of Folic Acid and FITC to PCL-*b*-PEO-NH₂

Folic acid (FA, 0.0609 g), NHS (0.016 g) and EDC (0.039 g) were dissolved in dimethyl sulfoxide (DMSO) (5 mL) and stirred at room temperature for 0.5 h, then PCL-*b*-PEO-NH₂ (0.500 g) was added to the solution and reacted for another 24 h to allow the conjugation of FA to the copolymer *via* NH₂ group. Afterwards, the mixture was dialyzed against deionized water and centrifuged to separate EDC and NHS. Then the precipitate was re-dissolved in acetone and centrifuged thrice to remove the unreacted FA. Finally, the supernatant fluid was rotarily evaporated to get the PCL-*b*-PEO-FA conjugate.

To conjugate FITC to the block copolymer, PCL-*b*-PEO-NH₂ (0.220 g) and FITC (0.012 g) were dissolved in anhydrous DMSO (5 mL) at room temperature and stirred for 24 h. Then the mixed solution was dialyzed for another 72 h. The final production was centrifuged, the supernatant was discarded and the precipitate was freeze-dried to get the final product.

The structural confirmation of PCL-*b*-PEO-FA and PCL-*b*-PEO-FITC was carried out by proton NMR in DMSO-*d*₆ at 400 MHz. The molecular weight and polydispersity were determined by gel permeation chromatography (GPC, Waters).

Preparation and Characterization of Micelles

The DOX loaded micelles were prepared *via* a dialysis method as previously reported^[23, 24]. Briefly, 5 mg of diblock copolymer (PCL-*b*-PEO-NH₂ or PCL-*b*-PEO-FA) and 5 mg of free DOX were dissolved in 1 mL of DMSO in the presence

of 10 μL triethylamine (TEA), then the organic phase was slowly dispersed into aqueous phase with gentle stirring for 8 h. Un-trapped DOX and TEA were removed by extensive dialysis against distilled water. The blank micelles were also prepared as above without loading DOX. For the preparation of FITC labeled micelles, similar procedure was adopted except that the diblock copolymers (PCL-*b*-PEO-NH₂ or PCL-*b*-PEO-FA) were mixed with PCL-*b*-PEO-FITC at weight ratio of 1:1 and no DOX was added.

The micelle size and size distribution were measured by a dynamic light scattering (DLS). Encapsulation efficiency (EE) was determined as the ratio of amount of DOX encapsulated in micelles and total amount of drug originally used in the encapsulation process by measuring the UV absorbance at 480 nm (UV spectrophotometry). The critical micelle concentration (CMC) was measured through fluorescence spectroscopy as described previously^[23].

In Vitro Cellular Uptake

Human bladder cancer cells (T-24 cell) and human embryonic kidney cells (HEK-293) were employed for *in vitro* experiment. T-24 cells were grown in F-12 medium supplemented with 10% FBS and 1% streptomycin while the DMEM (high glucose) supplemented with 10% FBS and 1% penicillin-streptomycin were utilized as the cell culture medium for HEK-293 cells. All cells were maintained at 37 °C with 5% CO₂ in a tissue culture incubator.

For quantitative analysis of cellular uptake, T-24 cells were seeded into a 24-well plate at a density of 1×10^5 cells per well. After the cells reached 80% confluence, the medium was changed to FITC labeled FA modified PCL-*b*-PEO-FA micelles or non-modified PCL-*b*-PEO-NH₂ micelles solution at a concentration of 0.15, 0.3 and 0.45 mg/mL, respectively. After incubation for a certain time, the micelle solution in the testing wells was removed and the wells were washed with 1 mL of PBS thrice to remove the micelles outside the cells. After that, 1 mL of NaOH solution (0.05 mol/L) was added to lyse the cells and then cell solution was transferred to the 96-well black plate. A multifunctional microplate reader (Genios, Tecan, Switzerland) was used to measure the fluorescence intensity from cells incubated with FITC labeled micelles in the desired wells with excitation wavelength of 488 nm and emission wavelength of 535 nm. The cellular uptake efficiency was expressed as the percentage of the fluorescence of the testing wells over that of the control wells. To eliminate the influence of cell numbers, final cellular uptake efficiency was normalized by protein amounts. Protein concentration of the cell lysates was determined by the micro BCA Protein Assay Reagent Kit (PIERCE, Rockford).

To further confirm the targeting selectivity of micelles between cancer cells and normal cells, HEK-293 cells were also seeded into 6-well plate at a density of 1×10^5 cells per well. After the cells reached 80% confluence, the medium was changed to FITC labeled FA modified PCL-*b*-PEO-FA micelles or non-modified PCL-*b*-PEO-NH₂ micelles solution at the same concentration and continuingly cultured for another 0.5, 1 and 2 h, respectively. After incubation, the micelle solution in the testing wells was removed and the wells were washed with 1 mL of PBS thrice to remove the

micelles outside the cells. After that, cells were harvested by trypsin with 0.02% EDTA and re-suspended in PBS. Then they were measured on a flow cytometer (Beckman) excited with an argon laser (488 nm) and fluorescence was detected at 535 nm. About 20000 cells were counted for each sample.

For confocal microscopic study, T-24 cells and HEK-293 cells were cultured in the 35 mm cover glass chamber (Costar) till 70% confluence. The micelle solution was removed and the wells were washed with 1 mL of PBS twice and the cells were stained by Hoechst 33342. Then the cells were fixed with 70% ethanol and sealed with 90% glycerin. The fixed cell monolayer was finally observed by confocal laser scanning microscope (CLSM, Olympus Fluoview FV1000).

In Vitro Cell Cytotoxicity by MTT Assay

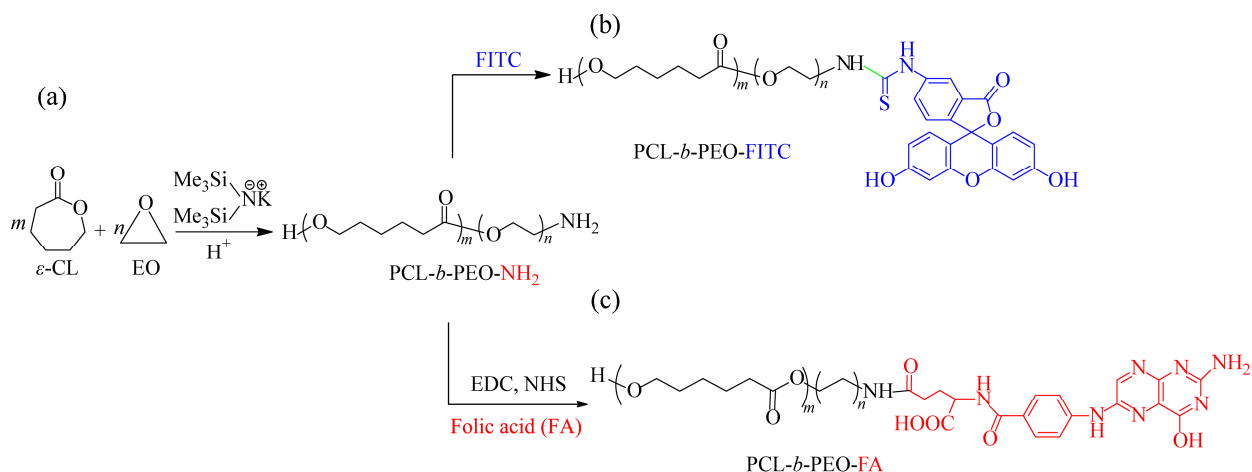
In vitro cytotoxicity of different DOX formulations was determined by MTT assay. T-24 cells were seeded in the 96-well plate at a density of 5×10^3 cells per well and incubated for 24 h to allow the cells to grow to 80% confluence. DOX loaded FA modified PCL-*b*-PEO-FA or non-modified PCL-*b*-PEO-NH₂ micelles with various concentrations from 0.11 mg/mL to 0.22 mg/mL were added and incubated with cells for 72 h. Cells incubated without micelles were set as a control while the wells without cells were set as zero wells. Then the wells were refreshed with 200 μL fresh F-12 without FBS but containing 20 μL of MTT solution (the concentration of MTT solution was 5 mg/mL). After 4 h incubation, the medium of each well was replaced with 200 μL of DMSO to dissolve the formazan crystals. Finally, the cell viability was analyzed by the multifunctional micro-plate reader at 490 nm. The results were expressed as the percentage of the absorbance in DOX treated cells comparing to that in the control cells.

RESULTS AND DISCUSSION

PCL-*b*-PEO-NH₂ Diblock Copolymer and Conjugation with FA and FITC

Scheme 1 shows the synthesis route of PCL-*b*-PEO-NH₂ diblock copolymer and its conjugation with FA and FITC. The PCL-*b*-PEO-NH₂ amphiphilic block copolymer was synthesized by sequence anionic ring-opening polymerization of EO and CL, as reported in our previous work^[22, 25]. Through the terminal amino group NH₂ of the copolymer, the target moiety FA and fluorescence label molecule FITC were conjugated to the end of hydrophilic block of the copolymer, respectively.

Figure 1 shows the GPC traces of PCL-*b*-PEO-NH₂ and its conjugates with FA and FITC. The number-average molecular weight (M_n) and its distribution (PDI) of PCL-*b*-PEO-NH₂ were 1.06×10^4 and 1.17, respectively, which were much close to the predesigned value of 1.0×10^4 . The M_n value of the copolymer was also consistent with the result measured by ¹H-NMR. After conjugation, the PDI of PCL-*b*-PEO-FITC and PCL-*b*-PEO-FA became 1.18 and 1.21, respectively, both a little wider than that of the original PCL-*b*-PEO-NH₂. The chemical structures of PCL-*b*-PEO-FA and PCL-*b*-PEO-FITC were analyzed by ¹H-NMR and the results are shown in Fig. 2. The peaks at 6.6 and 7.6 ppm



Scheme 1 Synthesis of (a) PCL-*b*-PEO-NH₂, (b) PCL-*b*-PEO-FITC, and (c) PCL-*b*-PEO-FA

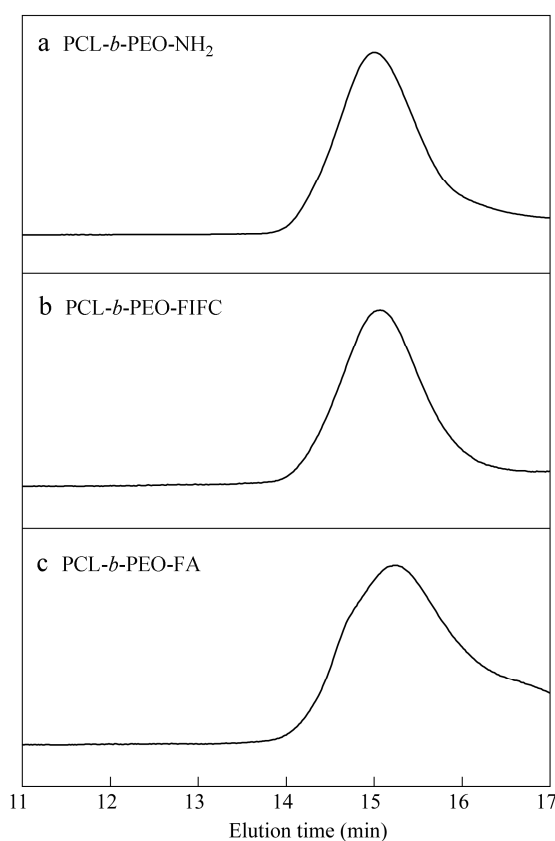


Fig. 1 GPC curves of (a) PCL-*b*-PEO-NH₂, (b) PCL-*b*-PEO-FITC and (c) PCL-*b*-PEO-FA

from folic acid^[26] and the peak at 6.6 and 6.7 ppm from FITC^[27] were clearly observed, while the peak at 2.78 ppm from CH₂NH₂ of the original PCL-*b*-PEO-NH₂ disappeared in the spectra of PCL-*b*-PEO-FA and PCL-*b*-PEO-FITC copolymers. The results from both GPC and ¹H-NMR analyses indicated the successful conjugation of FA and FITC to the PCL-*b*-PEO-NH₂ copolymer, leading to the formation of PCL-*b*-PEO-FA and PCL-*b*-PEO-FITC, respectively.

Preparation and Characterization of Micelles

Micelles with different surface functions were prepared by dispersing the mixture of PEO-*b*-PCL-FA or PCL-*b*-PEO-

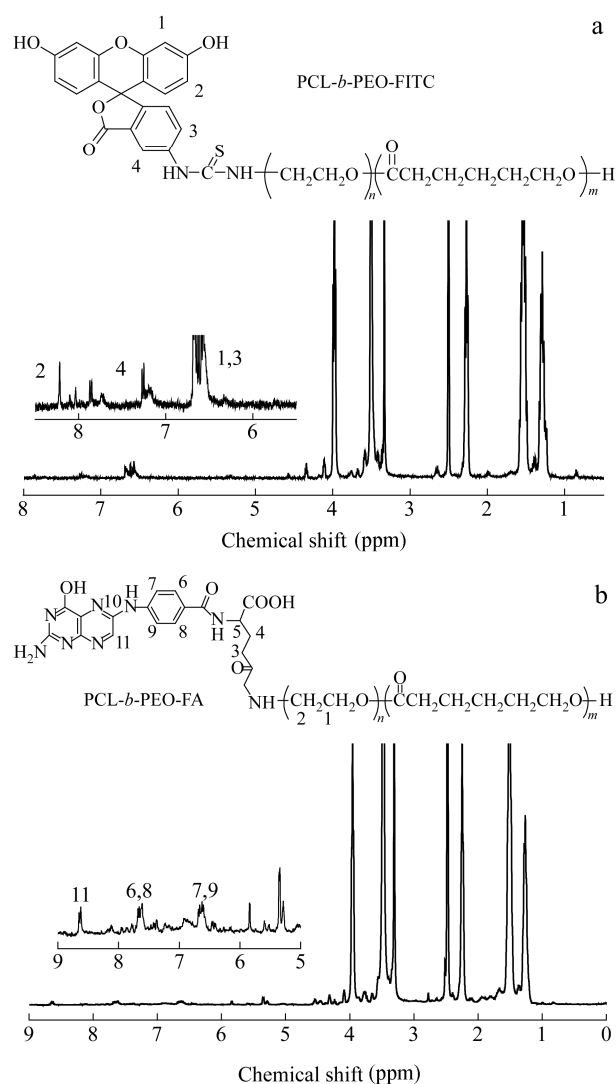
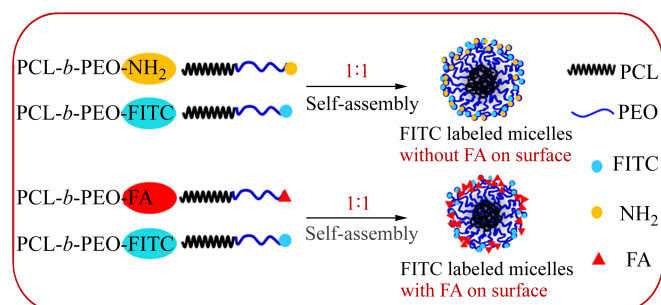


Fig. 2 ¹H-NMR spectra of (a) PCL-*b*-PEO-FITC and (b) PCL-*b*-PEO-FA

NH₂ with PCL-*b*-PEO-FITC at weight ratio of 1:1 in deionized water as shown in Scheme 2. The target moiety FA and fluorescence molecule FITC which conjugated to the

hydrophilic PEO block were on the surface of micelles. The drug loaded micelles were fabricated by adding DOX in the polymer solution so that the DOX was trapped in the hydrophobic cores of micelles.



Scheme 2 FITC labeled non-modified PCL-*b*-PEO-NH₂ and FA modified PCL-*b*-PEO-FA micelles prepared by dispersing both block copolymers in deionized water

Figure 3 shows the DLS results of the micelles with and without drug loading. It was found that the sizes of micelles from PCL-*b*-PEO-NH₂ and PCL-*b*-PEO-FA without DOX loading were 89 and 62 nm, respectively. After DOX was loaded into the above micelles, the size of micelles increased to 204 and 190 nm. The size increase of drug-loaded micelles is the result of the swelling of PCL core due to the entrapped DOX. As shown in Table 1, the critical micellization concentration (CMC) of PCL-*b*-PEO-NH₂ and PCL-*b*-PEO-FA micelles was 0.006 and 0.028 mg/mL, respectively. The lower CMC value of PCL-*b*-PEO-NH₂ polymer implied the easier formation of micelles compared to PCL-*b*-PEO-FA polymer. Both the micelles with and without FA targeting moiety showed relatively high encapsulation efficiency (EE) of above 60% and drug loading efficiency of above 13%.

In Vitro Cellular Uptake

In order to check the function of the targeting and selectivity of folic acid to bladder cancer cells and normal tissue cells, the qualitative and quantitative analyses of cellular uptake for targeting moiety FA modified PCL-*b*-PEO-FA micelles and non-modified PCL-*b*-PEO-NH₂ micelles were performed *via* the incubation with T-24 cells and HEK-293 cells.

Figure 4 shows the CLSM images of FA modified and non-modified micelles after incubated with T-24 cells for 2 h. The green fluorescence from two types of micelles was observed around the cells, indicating that the micelles have been accumulated nearby the cells. More importantly, stronger fluorescent intensity was detected from T-24 cells after incubation with FA modified micelles, while the fluorescent intensity for the cells after incubation with non-modified micelles was very weak. These results

suggested that more FA modified PCL-*b*-PEO-FA micelles were endocytosized into bladder cancer cells, indicating the strong targeting efficiency of FA moiety on the micelles surface from PCL-*b*-PEO-FA copolymers.

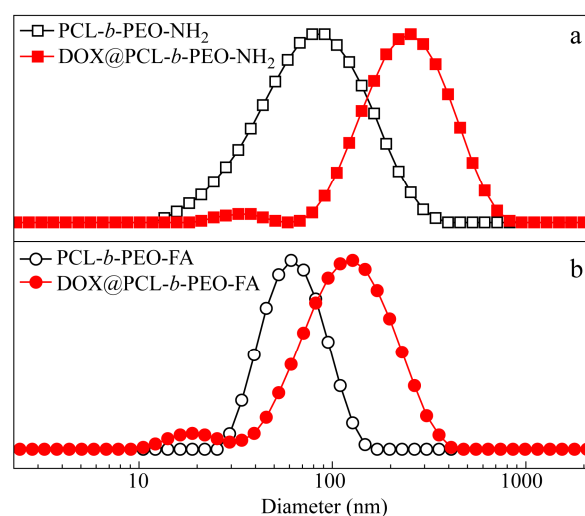


Fig. 3 DLS results of micelles with or without doxorubicin (DOX) loading: (a) DOX loading micelles DOX@PCL-*b*-PEO-NH₂ self-assembly from PCL-*b*-PEO-NH₂ and blank micelles from PCL-*b*-PEO-NH₂; (b) DOX-loading micelles DOX@PCL-*b*-PEO-FA self-assembly from PCL-*b*-PEO-FA and blank micelles from PCL-*b*-PEO-FA

To further investigate the targeting efficiency of FA modified micelles to T-24 cells, cellular uptake efficiencies of FA modified PCL-*b*-PEO-FA micelles and non-modified PCL-*b*-PEO-NH₂ micelles were evaluated by measuring percentage of fluorescence intensity from endocytosed micelles to the fluorescence intensity of original micelles used in incubation on a multifunctional micro-plate reader.

Figure 5 shows the quantitative analysis results of cellular uptake efficiency of micelles after incubation with T-24 cells for 0.5 and 2 h. It was clearly found that much more FA modified micelles can be accumulated into T-24 tumor cells than the non-modified micelles at the same conditions. For example, after 2 h incubation, the cellular uptake efficiency of non-modified PCL-*b*-PEO-NH₂ micelles was only 3.93%, 10.48% and 12.68% when the micelle concentration was 0.15, 0.3 and 0.45 mg/mL respectively, while the cellular uptake efficiency reached 18.29%, 28.60% and 49.52% for the FA modified micelles at the same micelle concentration, about 4 times higher than those of the non-modified micelles. These results provided us strong evidence that FA modified micelles showed stronger targeting ability to the bladder cancer cells and higher accumulation in the tumor cells. For

Table 1 Characterization of micelles^a

	Particle size ^b (nm)	Encapsulation efficiency ^c (%)	Drug loading efficiency ^c (%)	CMC ^d (mg/mL)
PCL- <i>b</i> -PEO-NH ₂ micelles	89	—	—	0.006
PCL- <i>b</i> -PEO-FA micelles	62	—	—	0.028
DOX@PCL- <i>b</i> -PEO-NH ₂ micelles	204	60.14	13.54	—
DOX@PCL- <i>b</i> -PEO-FA micelles	190	61.18	13.93	—

^a Date represent mean $\pm$ SD, $n = 3$; ^b Determined by DLS; ^c Determined by UV; ^d CMC is determined by fluorescence spectra

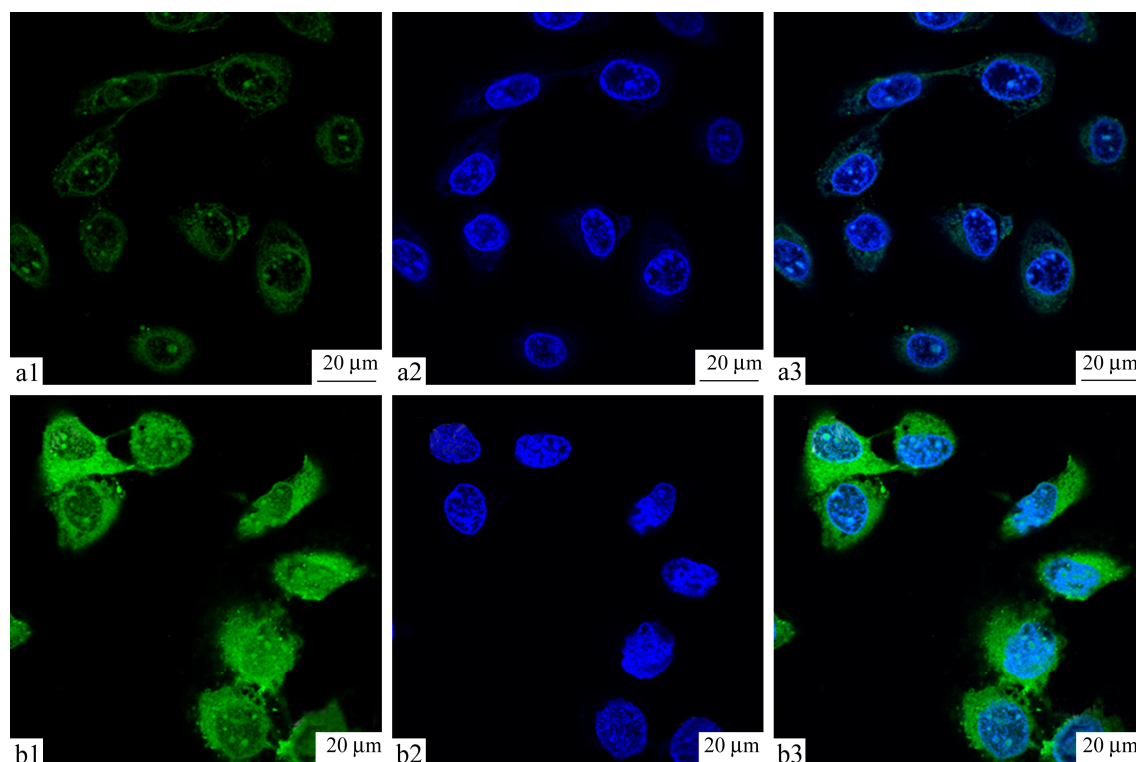


Fig. 4 Confocal laser scanning microscopy (CLSM) images showing the internalization of fluorescent micelles in T-24 cells (2 h incubation): (a1, a2, a3) images of cells incubated with micelles assembled from non-modified PCL-*b*-PEO-NH₂ block copolymers, and (b1, b2, b3) images of cells incubated with micelles from FA modified PCL-*b*-PEO-FA, with (a1, b1) the Hoechst33342 channels showing blue fluorescence from Hoechst33342 stained nuclei, (a2, b2) the FITC channels showing the green fluorescence from FITC labeled micelles, and (a3, b3) the merged channels of FITC and Hoechst33342 channels

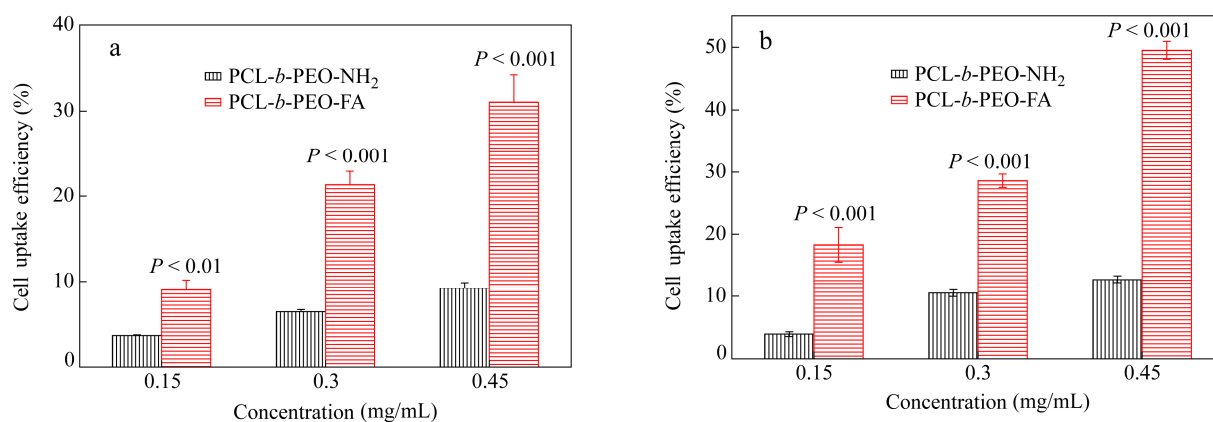


Fig. 5 Cellular uptake efficiency of FITC labeled FA modified PCL-*b*-PEO-FA and non-modified PCL-*b*-PEO-NH₂ micelles in T-24 cells (Cells were incubated with different treatments for (a) 0.5 h and (b) 2 h, respectively. The cells were rinsed, lysed and FITC accumulation was measured with a microplate fluorometer and normalized by content of protein. ($n = 8$))

the specific bladder cancer cells, our results revealed great targeting efficiency of FA to the T-24 tumor cells. The high affinity between FA and T-24 cells is due to the folate receptor-mediated mechanism, as reported for other tumor cells^[28].

The above results have shown the high target efficiency of FA modified micelles to the bladder cancer cells. Yet further investigation is necessary to reveal the interaction between micelles and normal tissue cells. Here the HEK-293 cells were used as the normal cell model for this work.

Figure 6 presents the CLSM images of micelles after incubation with HEK-293 cells for 2 h. It was found that there was no obvious difference in fluorescence intensity between FA modified micelles and non-modified micelles. The quantitative analysis results of cellular uptake after incubation for 0.5, 1 and 2 h are shown in Fig. 7. It could be found that the FA modified micelles showed slightly lower fluorescence intensity in HEK-293 cells than the non-modified micelles. In other words, FA modification of micelles did not increase the endocytosis of micelles by normal cells compared to the

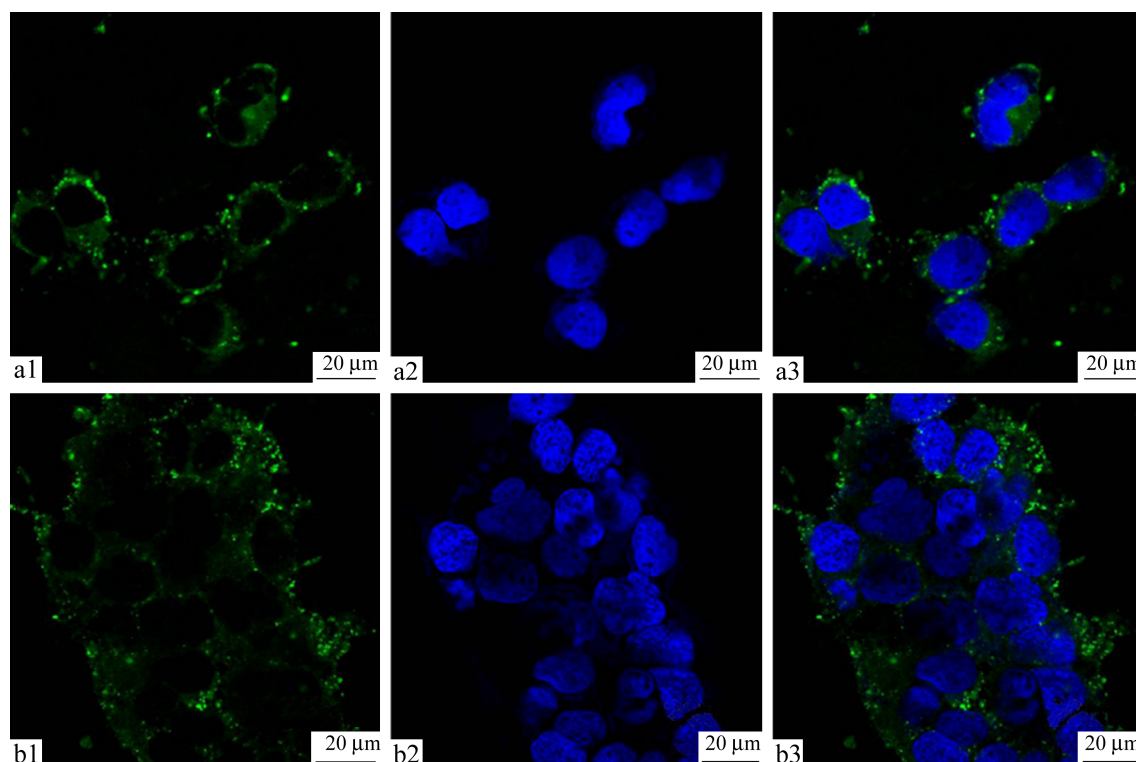


Fig. 6 Confocal laser scanning microscopy (CLSM) images showing the internalization of fluorescent micelles in HEK-293 cells (2 h incubation): (a1, a2, a3) cells incubated with micelles assembled from FA modified PCL-*b*-PEO-FA block copolymers and (b1, b2, b3) cells incubated with micelles from non-modified PCL-*b*-PEO-NH₂, with (a1, b1) the FITC channels showing the green fluorescence from FITC labeled micelles, (a2, b2) the Hoechst33342 channels showing blue fluorescence from Hoechst33342 stained nuclei, (a3, b3) the merged channels of FITC and Hoechst33342 channels

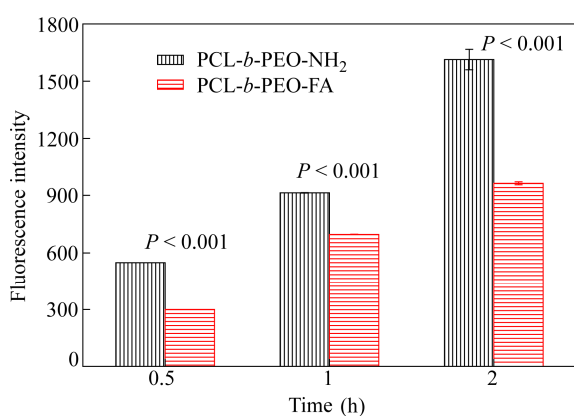


Fig. 7 The diagram of *in vitro* cellular uptake efficiency from flow cytometer at 0.5, 1 and 2 h incubation for HEK-293 cells

non-modified micelles.

Based on the above results from Figs. 4–7, it has been proven that more FA modified micelles can accumulate into T-24 tumor cells than normal micelles without FA modification under the same conditions. Meanwhile, the cellular uptake efficiency of FA modified micelles was a little lower for normal tissue original HEK-293 cells than that of non-modified micelles. Therefore, it could be concluded that FA modified micelles showed efficient targeting capacity to bladder tumor cells.

It has been reported that folic acid is able to be efficiently internalized into the tumor cells through the folate

receptor-mediated endocytosis mechanism^[29, 30]. Our work revealed that the receptor-mediated endocytosis mechanism of folic acid was very effective for the bladder cancer cells. In consideration of the specific administration method, the FA modified micelles promised good candidates for targeted drug delivery system for intravesical instillation of superficial bladder cancer.

***In Vitro* Cell Cytotoxicity by MTT Assay**

Figure 8 shows the *in vitro* cytotoxicity of polymer micelles with and without doxorubicin (DOX) loading against T-24 cells determined by MTT assay. It was found that the blank micelles without drug loading showed no significant cytotoxic effect against T-24 cells after incubation for 72 h at various concentrations, which indicated that the diblock copolymers PCL-*b*-PEO-NH₂ and PCL-*b*-PEO-FA were biocompatible and safe for clinical application.

However, the micelles with different functional surface showed different cytotoxicity after DOX were loaded under the two different concentrations. Without targeting moiety FA, the cell viability decreased to 61.72% and 31.35% for DOX loading micelles from PCL-*b*-PEO-NH₂ when the concentration was 0.1 and 0.2 mg/mL. However, when the DOX was loaded into FA modified micelles, the cell viability decreased to 43.47% and 16.46% for the same concentration change. These two values were almost equivalent to that of the free DOX drug. The much lower *in vitro* cell viability of FA modified micelles with DOX loading against the T-24 cells than the DOX-loaded micelles without FA modification

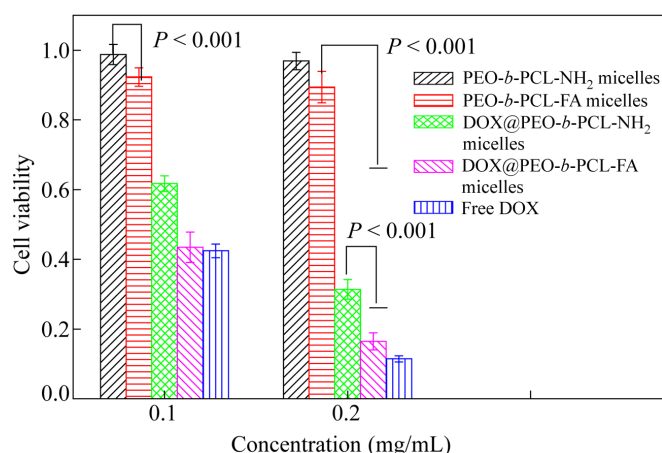


Fig. 8 *In vitro* cytotoxicity effect of various DOX formulations against T-24 cells after 72 h treatment

indicated the higher tumor killing efficacy, which arose from the FA targeting and its receptor-mediated endocytosis mechanism. The lower drug concentration and the similar cytotoxicity to the free DOX ensured the FA modified micelles great potential in raising therapeutic effect and reducing side effects in clinical application.

CONCLUSIONS

The targeted drug delivery system for intravesical instilled chemotherapy of cancer, *i.e.* polymeric micelles with modified surface self-assembled from FA and FITC conjugated PCL-*b*-PEO-NH₂ copolymers, was successfully developed in this work. The micelles have a size ranging from 60 nm to 200 nm, a rather low CMC and high drug loading efficiency. *In vitro* cellular uptake and cytotoxicity studies show that FA modified PCL-*b*-PEO-FA micelles have higher cellular uptake for T-24 cells compared with PCL-*b*-PEO-NH₂ micelles without FA modification due to the folate-receptor-mediated endocytosis mechanism, resulting in enhanced *in vitro* cytotoxicity. Meanwhile, FA modified PCL-*b*-PEO-FA micelles demonstrated rather lower cellular uptake efficiency in normal HEK-293 cells. Hence, it is concluded that the polymeric micellar system designed in this study is effective on targeting to the bladder cancer cells and possesses great capability in killing T-24 cells while low side effect on normal tissues are also expected. All these results ensured an effective targeting drug delivery system for raising therapeutic effect and reducing side effects in future clinical application. Further studies are planned to elucidate the *in vivo* behavior of FA modified PCL-*b*-PEO-FA micelles in animal model for a better optimization of the intravesical instillation chemotherapy of various cancers.

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