

EFFECT OF SURFACE CHARGE OF POLYMERIC MICELLES ON *IN VITRO* CELLULAR UPTAKE*

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Abstract This work focuses on the interaction between polymeric micelles with different charged surfaces and cancer cells in order to study the influence of surface charge on the *in vitro* cellular uptake efficiency. The amphiphilic diblock copolymers poly(ϵ -caprolactone)-*b*-poly(ethylene oxide) (PCL-*b*-PEO) with different functional groups at the end of hydrophilic block were synthesized. The functional groups endue the micelles with different charges on the surfaces. The cellular uptake of micelles to T-24 cells (human bladder tumor cells), HepG2 cells (human liver hepatocellular carcinoma cell line) and Hela cells (human epithelial cervical cancer cells) was studied by means of flow cytometer and confocal laser scanning microscopy. The results indicate that the surface charges showed great influence on zeta potential of micelles at different pH values. The *in vitro* cellular uptake efficiency of micelles with different charged surfaces demonstrated different cellular uptake patterns to three kinds of cancer cells.

Keywords: Polymeric micelles; Surface charge; pH value; Cellular uptake efficiency.

INTRODUCTION

Because of the particular physiological characteristics of tumor microenvironment and tumor angiogenesis, it is possible to design the target drug delivery systems that specifically transport the anti-cancer drugs to tumors^[1]. The interaction between cell membrane and drug delivery system plays significant role in the efficient intracellular uptake, drug delivery and therapeutic effect. The morphology^[2], composition^[3, 4], molecular weight, size^[5] and surface function^[6–8] of drug delivery system are the most notable elements which should be taken into account when designing an efficient drug delivery system. In addition to the most widely investigated route of conjugating targeting moieties to the surface of drug carrier for enhancing the intracellular uptake efficiency, the surface charge of drug carrier also plays an important role in regulating the cellular uptake efficiency and making further impact on the final therapeutic effect^[8–10]. It has been reported that the positive charged or negative charged surface possesses different interaction mechanisms with cell membrane, leading to the diverse cell adsorption modes and tissue retention of drug carrier^[11, 12]. Yong-Il Chung and his coworkers^[7] prepared the negatively and positively charged heparin or chitosan pluronic conjugated PLGA-based nanoparticles. Both

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functional nanoparticles were found to be stable even in the blood plasma condition. The chitosan and heparin-functionalized surfaces of PLGA nanoparticles provided effectively the opportunity for cell membrane adsorption and improved cellular uptake, which is the desirable form for the effective accumulation in tumor.

On the other hand, it was reported that the solid tumor and normal tissue possess different pH values which give us another inspiration for the design of drug delivery system^[13–15]. The typically extracellular pH value of normal tissue or blood is 7.4^[11], while the average pH value for readily accessible tumor areas like head, neck, chest and limbs for various solid tumors has been reported as 7.06^[16, 17]. In addition, 80% of all measured tumor extracellular pH values was reported in the range from 7.0 to 6.5^[18]. Moreover, the extracellular pH value of tumor decreases by 0.2–0.4 pH units when glucose is given orally for patients^[19].

However, little study was reported about how the pH value and surface charge affect the intracellular uptake and final accumulation of drug carriers in tumor site up to now. Herein the micelles with different surface charges based on the self-assembly of amphiphilic diblock copolymers poly(ϵ -caprolactone-*b*-ethylene oxide) (PCL-*b*-PEO) with different terminal groups at hydrophilic block were developed in this paper. The zeta potentials and size distribution at different pH environment were studied by dynamic light scattering. The interaction between micelles and cancerous cells was investigated by means of flow cytometer. Meanwhile, the internalization of micelles into those cells was observed by confocal laser scanning microscope. An efficient targeting drug delivery system was desired *via* the study on the influence of different surface charges of micelles on targeting efficiency.

EXPERIMENTAL

Materials

ϵ -Caprolactone (ϵ -CL, Acros Organics) was dried with calcium hydride (CaH_2) and then purified by vacuum distillation at 70 °C before use. Ethylene oxide (EO, BDH Limited Poole, England) was distilled in the presence of CaH_2 at 0 °C and then stored at –20 °C before use. Fluorescein isothiocyanate (FITC, Beijing Fanbo Science & Technology Co., Ltd), Dulbecco's Modification of Eagle's Medium (DMEM, high glucose), HAM'S/F12, penicillin, streptomycin and fetal bovine serum (FBS) (all from Gibco), human bladder tumor cells (T-24 cells), human liver hepatocellular carcinoma cell line (HepG2 cells) and human epithelial cervical cancer cells (Hela cells) (from Cell Resource Center, IBMS) were used as received without further treatment. Other reagents were commercially available chemicals and used as received.

Synthesis of Amphiphilic Diblock Copolymers PCL-*b*-PEO-NH₂ and Conjugation with FITC

The amphiphilic diblock copolymers with different terminal groups at the end of hydrophilic block PCL-*b*-PEO-FG in which FG is amino group, carboxyl group or methoxy group were synthesized as following: the PCL-*b*-PEO-NH₂ was synthesized first by ring-opening polymerization of CL and EO monomers with potassium bis(trimethylsilyl) amide ($[(\text{CH}_3)_3\text{Si}]_2\text{NK}$) as initiator. Then the terminal NH₂ group was transferred into carboxyl group *via* the reaction of succinic anhydride and amino group with the help of 4-dimethyl aminopyridine to get PCL-*b*-PEO-COOH^[20]. The methoxy-capped PCL-*b*-PEO-OCH₃ (number-average molecular weight $M_n = 9800$, molecular weight distribution $M_w/M_n = 1.10$) was synthesized according to literature^[21].

To conjugate FITC into block copolymers, 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 72 h. Then the mixed solution was dialyzed for another 72 h to get rid of the solvent and unreacted FITC. The final product was centrifuged, the supernatant fluid was discarded and the precipitate was freeze-dried to get the final product PCL-*b*-PEO-FITC.

Preparation and Characterization of FITC Labeled Micelles

The FITC labeled micelles were prepared *via* a dialysis method according to the previous reported^[22]. First, to prepare FITC labeled micelles, block copolymer (PCL-*b*-PEO-OCH₃, PCL-*b*-PEO-NH₂ or PCL-*b*-PEO-COOH) (0.005 g) and FITC labeled PCL-*b*-PEO-FITC (0.005 g) were dissolved in DMSO (1 mL), and then the mixed solution was slowly dispersed into aqueous phase with gentle stirring for 8 h to allow the block copolymers

self-assembled into micelles. The solution was then extensively dialyzed against distilled water for 3 d. Finally, the obtained micelles were centrifuged for 5 min at 5000 r/min, the precipitate was discarded to get the supernatant fluid and stored at 4 °C before use. The diameter and its distribution of micelles as well as zeta potential were measured by a dynamic light scattering measurer.

The Influence of pH and Surface Charge on Targeting Efficiency

T-24 cells, HepG2 cells and Hela cells were employed for *in vitro* experiment. T-24 cells were grown in F-12 medium supplemented with 10% FBS and 1% streptomycin. The DMEM (High glucose) supplemented with 10% FBS and 1% penicillin-streptomycin was utilized as the cell culture medium for HepG2 cells and Hela cells. All cells were maintained at 37 °C with 5% CO₂ in a tissue culture incubator.

For flow cytometer analysis, T-24 cells, HepG2 cells and Hela cells were seeded into 24-well plate at a density of 1×10^5 cells per well. After the cells reached 80% confluence, the medium was refreshed with new F-12 and DMEM (High glucose) culture medium without FBS. Then the FITC labeled micelles solution with different pH values and surface charges was added and continuing cultured for certain hours. After incubation, the micelles solution in the testing wells was removed and the wells were washed with 1 mL phosphate buffer saline (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 the cells were measured on a flow cytometer (Beckman) excited with an argon laser (488 nm) and the fluorescence was detected at 560 nm. About 20000 cells were counted for each sample.

For confocal microscope scanning, T-24 cells, HepG2 cells and Hela cells were cultured in a 35 mm cover-glass chamber (Costar) till 80% confluence. Then the cells were incubated with micelles solution with different pH values and surface charges for certain hours, respectively. Afterward the micelles solution was removed and the cells were washed with 1 mL PBS twice. Then the cells were fixed with 70% ethanol for 10 min and stained by Hoechst 33342 for 5 min, then sealed with 90% glycerin. The fixed cell monolayer was finally observed under a confocal laser scanning microscope (CLSM, Olympus Fluoview FV1000).

RESULTS AND DISCUSSION

Preparation and Characterization of Amphiphilic Diblock Copolymers and Micelles

Figure 1 shows the GPC traces of PCL-*b*-PEO-NH₂ before and after conjugation with FITC. It is found that the number-average molecular weight (M_n) of PCL-*b*-PEO-NH₂ is 10600 and the molecular weight distribution (M_w/M_n) is 1.13. After conjugation reaction, the molecular weight distributions of PCL-*b*-PEO-FITC became 1.17 which was still quite narrow, while the elution time was little ahead of PCL-*b*-PEO-NH₂, suggesting the slight increase of molecular weight of the copolymer after conjugation with FITC.

Figure 2 shows the ¹H-NMR spectra of PCL-*b*-PEO-FITC. The characteristic peaks of FITC were clearly observed. The appearance of the characteristic peaks at $\delta = 6.6$ and $\delta = 6.7$ assigned to FITC and the disappearance of the characteristic peak at $\delta = 2.78$ of CH₂NH₂ from PCL-*b*-PEO-NH₂ copolymers indicated that the FITC has been successfully conjugated into the copolymer by reaction with NH₂ group.

Figure 3 shows the size and morphology of micelles self-assembled from diblock copolymers with different functional groups. It was found that the micelles from the three different copolymers show spherical morphology with uniform size. The well dispersion in water with no aggregation was observed, indicating the stability of micelles with different functional groups on the surface.

It has been reported that the size of micelles plays an important role in the *in vivo* distribution, biological fate, toxicity and targeting ability because of the so called enhanced permeation and retention (EPR) effect^[23, 24] and ability of escaping the clearance by the reticular endothelial system (RES)^[25]. The size of micelles is affected greatly by the complicated physiological environments. Herein the size and its distribution of micelles with different surface properties were measured and studied.

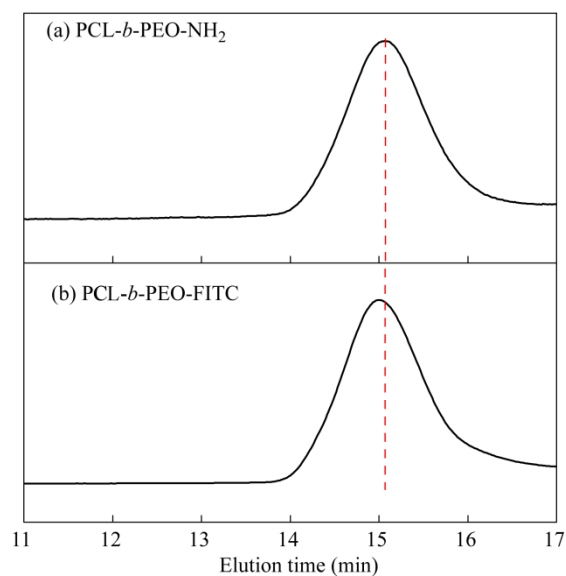


Fig. 1 GPC curves of (a) PCL-*b*-PEO-NH₂ and (b) PCL-*b*-PEO-FITC

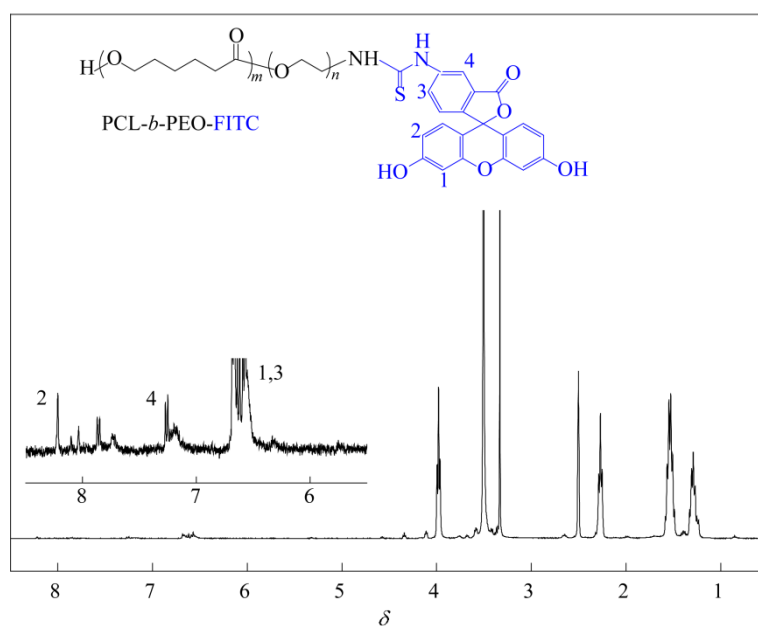


Fig. 2 ¹H-NMR spectra of PCL-*b*-PEO-NH₂ after conjugation with FITC

Figure 4 lists the sizes of micelles distribution and their changes with diverse pH values. As we can see, the size of the three different surface charged micelles does not change much and keeps a very narrow distribution between 80 nm and 120 nm at various pH values, indicating that micelles are stable at given conditions which are consistent with the results from Fig. 3.

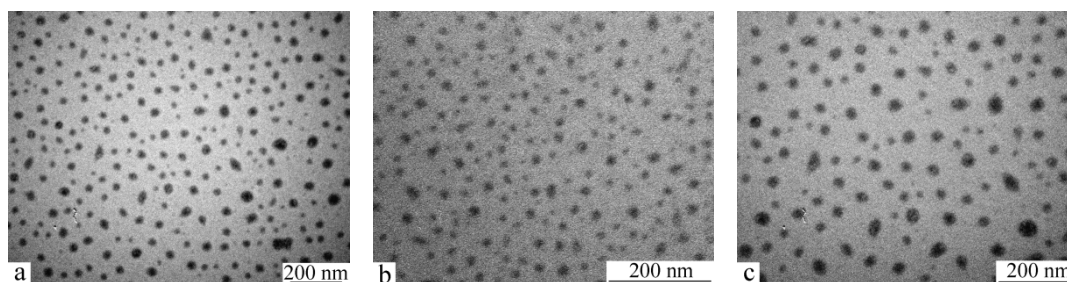


Fig. 3 TEM micrographs of micelles self-assembled from (a) PCL-*b*-PEO-OCH₃, (b) PCL-*b*-PEO-NH₂ and (c) PCL-*b*-PEO-COOH

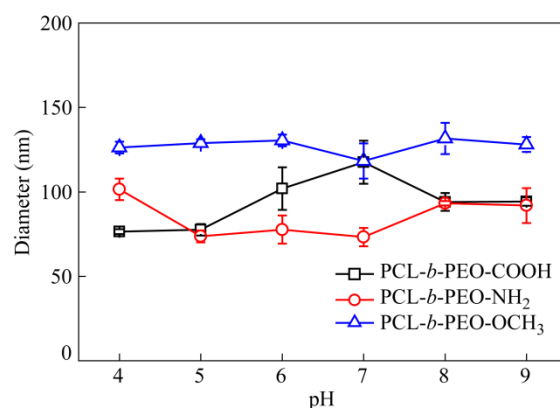


Fig. 4 Diameter of micelles with different charged surfaces at different pH values

Surface Charge of Micelles at Different pH Values

Figure 5 shows the zeta potential at different pH values for the micelles with different surfaces. It was found that the PCL-*b*-PEO micelles with different surface charges resulted in different zeta potential at various pH values. For the micelles self-assembled from methoxy group capped PCL-*b*-PEO-OCH₃, the zeta potential kept near zero in a wide range of pH values. However, when pH increased from 4 to 9, the zeta potential of the micelles with charged surfaces showed obvious decrease in zeta potentials. For the amino group capped PCL-*b*-PEO-NH₂ micelles with positive charges on the surface, the zeta potentials always kept above zero even in basic environment. However, the zeta potential also decreased with increasing pH value due to the loss of proton from micelles surface.

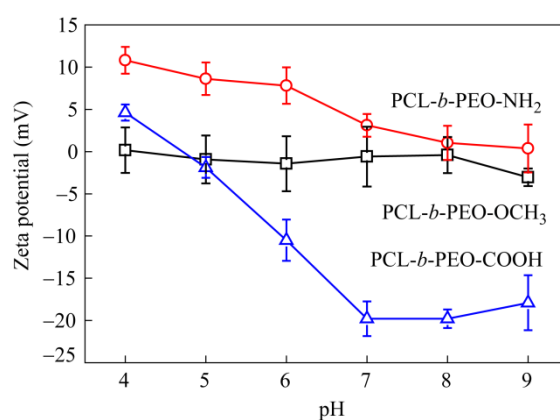


Fig. 5 Zeta potential of micelles with different charged surfaces at different pH values

For the carboxyl group capped PCL-*b*-PEO-COOH micelles with negative charges, they also showed zeta potentials above zero at strong acidic environment (*e.g.* pH = 4) due to the incorporation of micelle surface with free hydrogen ions. Compared to the PCL-*b*-PEO-NH₂ micelles, the PCL-*b*-PEO-COOH micelles showed rapid decrease in zeta potential from about +5 mV to about −20 mV when pH values increased from 4 to 7, and then kept almost unchanged when further increasing pH value from 7 to 9. Micelles were prone to be positive charged in acidic environment, whereas negative charged in basic environment. These results indicated that the micelles with negatively charged surface are much sensitive to the acidic environment than the micelles with positively charged surface.

Influence of PH and Surface Charge on in vitro Cellular Uptake without Serum

To better understand the influence of surface charge of micelles on the cellular uptake, *in vitro* cellular uptake efficiency of micelles with different surface charges at various pH values were studied by means of flow cytometer and confocal laser scanning microscopy.

Figure 6(a) shows the cellular uptake of bladder cancer cells (T-24 cells) when incubated with micelles with different surface charges for 2 h at various extracellular pH values. It was found that the PCL-*b*-PEO-COOH micelles with negatively charged surface exhibited the highest cellular uptake efficiency, while no clear difference was observed between PCL-*b*-PEO-NH₂ micelles and PCL-*b*-PEO-OCH₃ micelles. Furthermore, the cellular uptake efficiency of all micelles to T-24 cells decreased with increasing pH values.

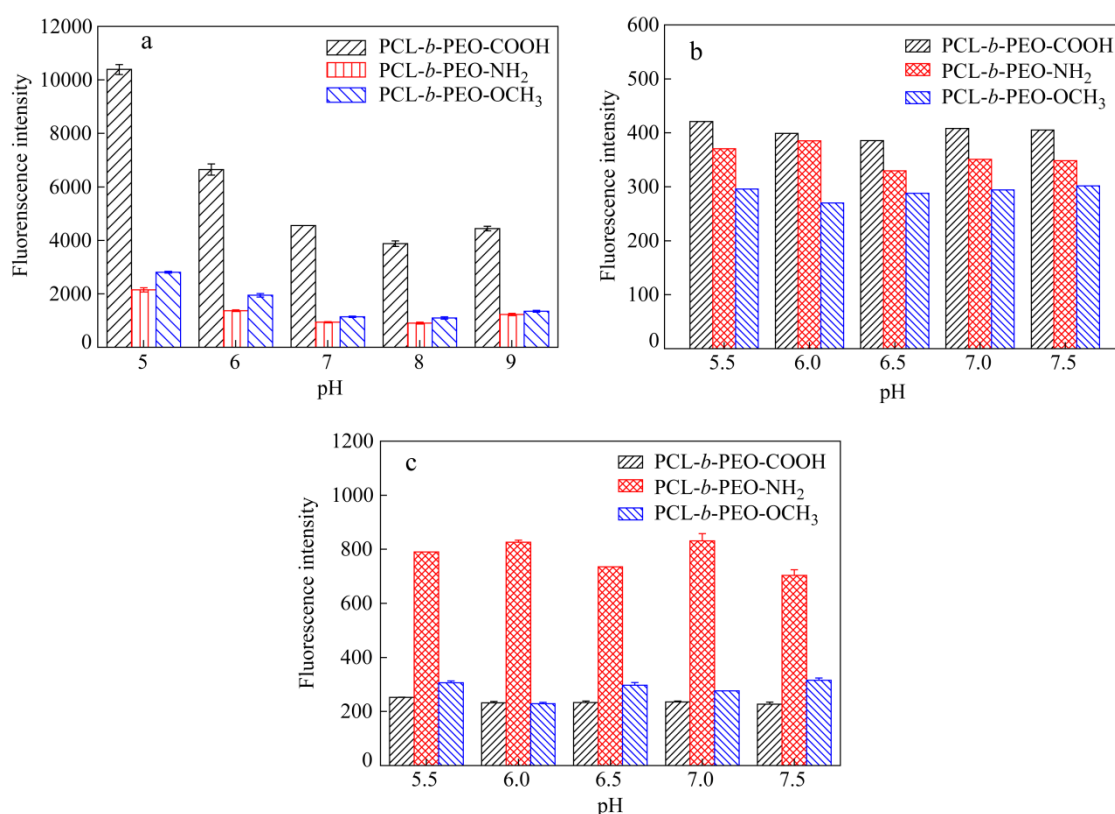


Fig. 6 Flow cytometer analysis of micelles with different charged surfaces after 2 h incubation with T-24 cells (a), HepG2 cells (b) and Hela cells (c)

Figure 7 shows the images of confocal laser scanning microscopy of T-24 cells after incubated with micelles for 2 h in weak acidic environment. The strongest green fluorescence was detected from T-24 cells after incubated with PCL-*b*-PEO-COOH micelles with negative charged surface, suggesting that the PCL-*b*-PEO-

COOH micelles are easier to enter T-24 cells at this condition. Meanwhile the relatively weak fluorescence intensity was observed from T-24 cells after incubated with either PCL-*b*-PEO-OCH₃ micelles or PCL-*b*-PEO-NH₂ micelles. This result was well consistent with flow cytometer result in Fig. 6(a).

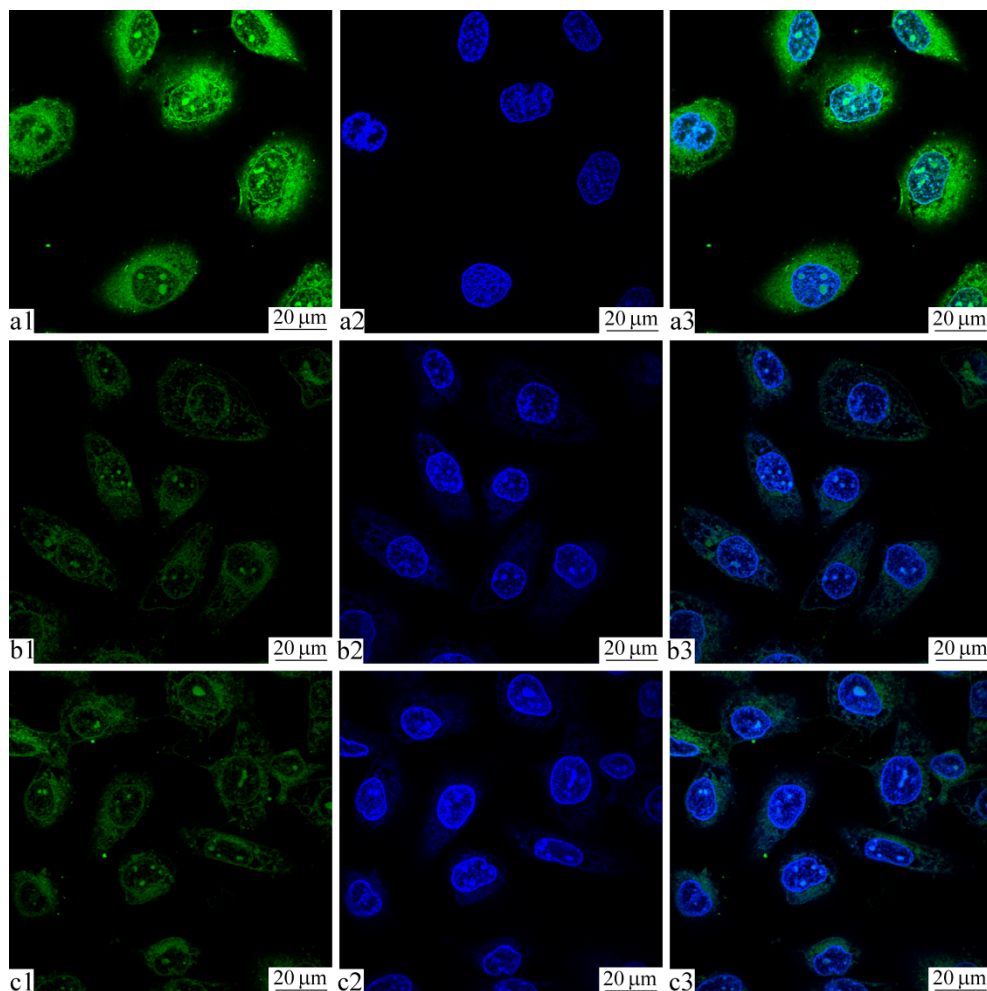


Fig. 7 Confocal laser scanning microscopy (CLSM) images of FITC-labeled micelles to T-24 cells after cultivation for 2 h at pH5.0

(a1), (a2) and (a3) are cells incubated with the FITC-labeled PCL-*b*-PEO-COOH micelles. (b1), (b2) and (b3) are cells incubated with the FITC-labeled PCL-*b*-PEO-OCH₃ micelles. (c1), (c2) and (c3) are cells incubated with the FITC-labeled PCL-*b*-PEO-NH₂ micelles. (a1), (b1) and (c1) are the FITC channels which showing the green fluorescence from FITC labeled micelles distributed in cytoplasm. (a2), (b2) and (c2) are the PI channels which showing blue fluorescence from PI stained nuclei. (a3), (b3) and (c3) are the merged channels of FITC and PI channels.

As we have already known that extracellular environment of tumor cells usually exhibited lower pH than normal tissue cells, often 6.5–7.0; the extracellular pH can even be lower to 6.0 at some special situation. So it could be concluded that negatively charged surfaces of PCL-*b*-PEO-COOH micelles greatly facilitated the uptake of micelles for T-24 cells. Therefore the changes of extracellular pH values gave us insight into the design and preparation of drug delivery system. However, specific cells may possess different cellular uptake properties. Here the HepG2 cells were utilized to study the *in vitro* cellular uptake behavior for the micelles with different surface properties.

Figure 6(b) shows the cellular uptake of HepG2 cells after incubation with micelles for 2 h. It was found that the PCL-*b*-PEO-COOH micelles showed the highest cellular uptake efficiency while the PCL-*b*-PEO-OCH₃ micelles showed the lowest efficiency among the three types of micelles. However, the cellular uptake efficiency for the three types of micelles did not show obvious difference and almost did not change with extracellular pH values. From the confocal images in Fig. 8, green fluorescence from the FITC-labeled PCL-*b*-PEO-COOH micelles in HepG2 cells after incubation for 2 h was little stronger than those from other two types of micelles.

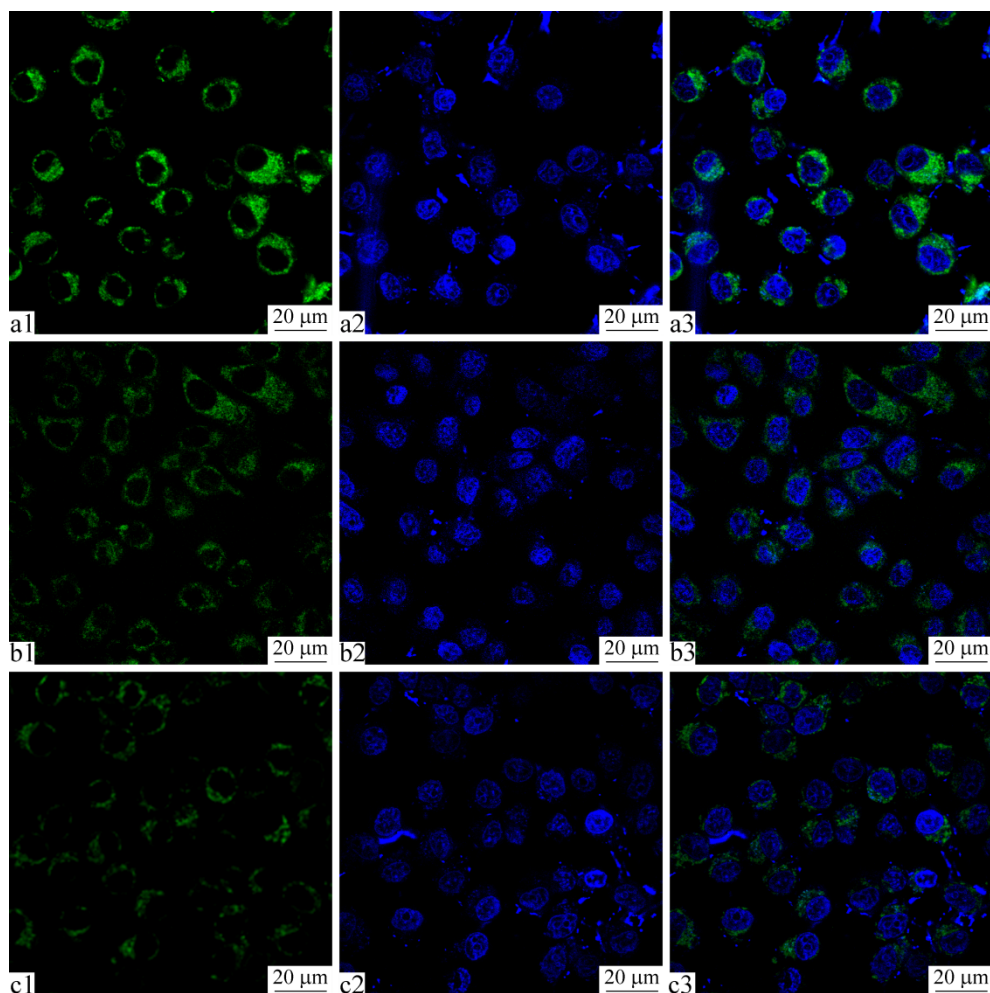


Fig. 8 Confocal laser scanning microscopy (CLSM) images of FITC-labeled micelles to HepG2 cells after cultivation for 2 h at pH7.5
(a1), (a2) and (a3) are cells incubated with the FITC-labeled PCL-*b*-PEO-COOH micelles. (b1), (b2) and (b3) are cells incubated with the FITC-labeled PCL-*b*-PEO-OCH₃ micelles. (c1), (c2) and (c3) are cells incubated with the FITC-labeled PCL-*b*-PEO-NH₂ micelles.

To further understand the effect of surface charges on *in vitro* cellular uptake efficiency, Hela cells was studied by means of flow cytometer and confocal microscopy. As shown in Fig. 6(c), after incubation with Hela cells for 2 h, the PCL-*b*-PEO-NH₂ micelles with positively charged surface showed the most notable cellular uptake among the three kinds of micelles, while the PCL-*b*-PEO-OCH₃ micelles and PCL-*b*-PEO-COOH micelles behaved the similar cellular uptake efficiency. Furthermore, the uptake efficiency of micelles with different charged surfaces to the Hela cells was much close in the range of pH values from 5.5 to 7.5.

Figure 9 shows the confocal laser scanning microscopy images of Hela cells after incubation with micelles for 2 h. The strongest green fluorescence can be observed in the cytoplasm of Hela cells after incubation with

FITC labeled PCL-*b*-PEO-NH₂ micelles, indicating that the micelles with positively charged surface facilitated *in vitro* cellular uptake to Hela cells. Furthermore, it can be clearly seen from Fig. 9 that the green fluorescence was mainly detected from cytoplasm but almost not detected in nucleus labeled by blue fluorescence, suggesting that the micelles were mainly accumulated in the cytoplasm of Hela cells.

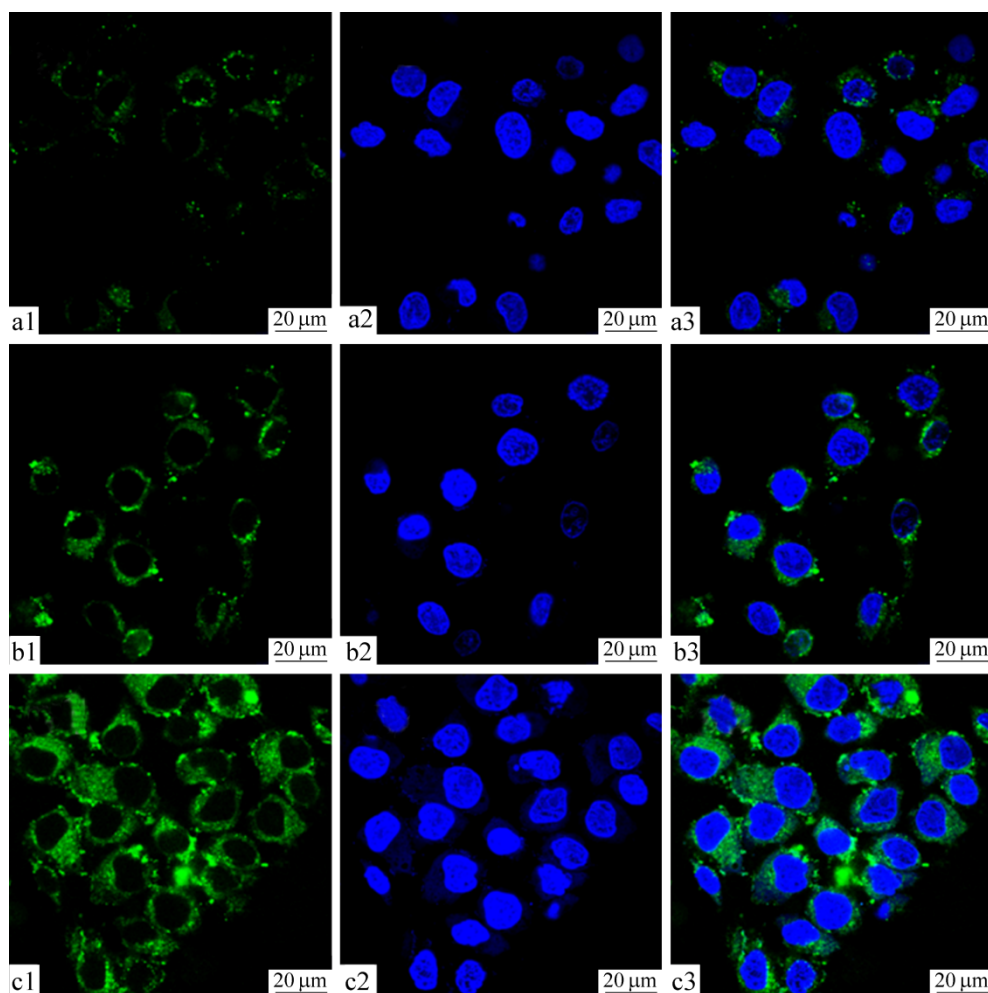


Fig. 9 Confocal laser scanning microscopy (CLSM) images of FITC-labeled micelles to Hela cells after cultivation for 2 h at pH7.5

(a1), (a2) and (a3) are cells incubated with the FITC-labeled PCL-*b*-PEO-COOH micelles. (b1), (b2) and (b3) are cells incubated with the FITC-labeled PCL-*b*-PEO-OCH₃ micelles. (c1), (c2) and (c3) are cells incubated with the FITC-labeled PCL-*b*-PEO-NH₂ micelles.

Considering the above results, it could be concluded that the PCL-*b*-PEO-NH₂ micelles with positively charged surface facilitate the *in vitro* cellular uptake into Hela cells. It has been reported that most tumor cells demonstrated negative charged^[1], so it is proposed that the favorable electrostatic interaction between the positively charged surface of PCL-*b*-PEO-NH₂ micelles and the negative cellular membrane of Hela cells could be the rationale behind such a high cellular uptake among three kinds of surface charged micelles. Of cause the penetration of cationic micelles through the cellular membrane by making transient holes without causing substantial membrane disruption may be the another reason^[26]. However, for the other two types of T-24 cells and HepG2 cells in this work, the PCL-*b*-PEO-COOH micelles show the highest *in vitro* cellular uptake efficiency in a wide range of pH values. The reason is still not clear, but the physiological properties of specific

cells in different environments may also play significant role in the interaction between cells and micelles in addition to the surface properties of micelles. Therefore, the further study is still needed for the complicated micelle system with charged surface. However, from the viewpoint of chemical modification, the control of surface charge of micelles can yet be regarded as effective method to study and promote the internalization and cellular uptake efficiency for the micelles with various tumor cells.

CONCLUSIONS

Three different surface charged micelles self-assembled from PCL-*b*-PEO-COOH, PCL-*b*-PEO-NH₂ and PCL-*b*-PEO-OCH₃ were characterized in terms of size and zeta potential. The *in vitro* cellular uptake efficiency of these micelles to different cells at different extracellular pH values was investigated. The pH values show great influence on the zeta potential of the micelles with different charged surfaces. The flow cytometer results and confocal laser scanning microscopy images indicated that the micelles with different charged surfaces facilitate different cellular internalization, depending on both the micelle surface and the specific tumor cells. Specifically, the PCL-*b*-PEO-NH₂ micelles with positively charged surface show the highest cellular uptake efficiency to Hela cells, while the PCL-*b*-PEO-COOH micelles with negatively charged surface show the highest cellular uptake efficiency to both T-24 cells and HepG2 cells.

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