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Hydrolysable core crosslinked particle for receptor-mediated pH-sensitive anticancer drug delivery

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Biodegradable micelle systems with both extracellular stabilities and specific targeting properties are highly desirable for anti-cancer drug delivery. Here, we report a biodegradable and crosslinkable poly(propylene fumarate)-*co*-poly(lactide-*co*-glycolide)-*co*-poly(ethylene glycol) (PPF-PLGA-PEG) copolymer conjugated with folate (FA) molecules for receptor-mediated delivery of doxorubicin. Micelles with folate ligands on surface and fumarate bonds within the core were self-assembled and crosslinked, which exhibited better stability against potential physiological conditions during and after drug administration. A pH sensitive drug release profile was observed showing robust release at acidic environment due to the ester hydrolysis of PLGA (50:50). Further, micelles with folate ligands on surface showed strong targeting ability and therapeutic efficacy through receptor-mediated endocytosis, as evidenced by efficacious cancer killing and fatal DNA damage. These results imply promising potential for ligand-conjugated core crosslinked PPF-PLGA-PEG-FA micelles as carrier system for targeted anti-cancer drug delivery.

Introduction

Delivery of drugs in carrier systems offers multiple advantages such as minimizing damages to normal tissues, prolonging drug circulation time, improving hydrophobic drug water solubility, reinforcing drug bioavailability, and enhancing target-ability using cancer specific-ligands.^{1,2} A variety of delivery systems have been reported in recent years, e.g., liposomes,³ dendrimers⁴ quantum dots⁵ and self-assembled nanoparticles.⁶⁻⁹ Self-assembly of amphiphilic copolymers containing hydrophobic and hydrophilic segments is a facile and well established method in forming micelles at the micron and nano level.¹⁰ For example, Qi *et al* successfully self-assembled cationic copolymers and showed excellent cellular uptake and therapeutic effects to human liver cancer cells.^{11,12} Multiple hydrophobic polymers, including poly(ϵ -caprolactone) (PCL),¹³⁻¹⁶ poly(propylene fumarate) (PPF)¹⁷⁻¹⁹, poly(lactic-*co*-glycolic acid) (PLGA)^{20,21} and their copolymers,^{22,23} are accepted to be biocompatible and widely used in biomedical fields. PPF is a unique polymer with double bonds and tunable mechanical properties, and is applied extensively for medical purposes, e.g., tissue regeneration and cancer targeted imaging.^{23,24} PLGA is being widely used in drug delivery benefiting from its fast degradation property.²⁵ Under an acidic environment, PLGA chain hydrolysis accelerates to an even

faster speed,²⁶ which makes it a favorable candidate material for controlled drug release in endosomes/lysosomes (pH 4.5-6.5) after endocytosis. Poly(ethylene glycol) (PEG) is a hydrophilic polymer that can be applied to the outer layer of micelles because of its excellent biocompatibility and prevention of nanoparticle aggregation.^{27,28}

However, micellar drug delivery always associate one practical challenge of inferior *in vivo* stability.²⁹ Self assembled polymeric micelles were reported to be eliminated from circulation and achieve weak tumor accumulation due to disintegration *in vivo*.³⁰ After intravenous administration, a severe condition happens to drug-loaded micelles, e.g., co-existence with human blood components (serum, NaCl) and magnificent dilution, which will substantially cause micelle disassociation and instant release of encapsulated drug, with concomitant undesired *in vivo* distribution profile.^{30,31} Therefore, a strong interaction that is able to resist the physiological destabilisation forces *in vivo* conditions, is highly desired for polymer micelles that aim at sustained and controllable *in vivo* drug delivery. To address this issue, micelles with a stabilization function, e.g., forming crosslinked bonds after capsuling drugs, thus attracted intensified interests. In recent years, several micelle systems were reported incorporating cross-linkable properties to enhance micelle stabilities.³² The crosslinking of bonds could occur inside the hydrophobic core, the hydrophilic shell, or the core shell interface.³³⁻³⁵ In order to gain cancer specific delivery of chemotherapeutics, micelles were designed with targeting ligands including antibodies, peptides, aptamers, or folate.³⁶⁻³⁹ Folate receptors are a type of membrane molecule that are overexpressed in a majority of human cancer cells.⁴⁰⁻⁴³ Therefore, the design of folate into the micelle surface is expected to enhance the binding and transportation of micelles into cancer cells through receptor-mediated endocytosis.

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In this study, we report novel folate-decorated core crosslinkable biodegradable PPF-PLGA-PEG-FA block copolymer micelles for enhanced cancer-targeting delivery of doxorubicin (DOX), as demonstrated in Fig. 1. PPF segments was designed to offer crosslinkability for the micelles, and thus enhance the particle stability. FA ligand was introduced to provide cancer targetability for these crosslinked particles. Uncrosslinked and crosslinked micelles, micelles with or without incorporated folate ligands, were fully evaluated against possible physiological variables, including 1000 dilution, 0.9% NaCl, 10% FBS, and size changes for 5 hours in a strong acid environment. The release profile of micelles in neutral (pH 7.4) and acidic (pH 5.0) were evaluated. Further therapeutic efficiency to cancer cells is determined by cell viability, cellular and nuclei morphology.

Experimental

Materials

Fumaryl chloride, D,L-lactide (3,6-dimethyl-1,4-dioxane-2,5-dione), propylene glycol, glycolide (1,4-dioxane-2,5-dione), dimethyl sulfoxide (DMSO), 4-(dimethylamino) pyridine (DMAP, $\geq 99\%$), *N,N'*-dicyclohexylcarbodiimide (DCC, $\geq 99\%$), folate and Tween 80 were purchased from Sigma Aldrich Co. (Milwaukee, WI) and used as received. Doxorubicin hydrochloride was purchased from LC Laboratories (Woburn, MA). Organic solvents at reagent grade were purchased from Fisher (Pittsburgh, PA). UV initiator 1-[4-(2-hydroxyethoxy)-phenyl]-2-hydroxy-2-methyl-1-propanone (Irgacure 2959) was purchased from Ciba Specialty Chemicals (Tarrytown, NY). Anhydrous dichloromethane (CH_2Cl_2) was distilled over calcium hydride (CaH_2) prior to use. All other chemicals or reagents used in this study were purchased from Sigma or Fisher unless noted otherwise.

Characterizations

The synthesized polymers were confirmed by ^1H NMR spectroscopy (300 MHz Varian NMR) using $\text{DMSO-}d_6$ or CDCl_3 as solvent. Molecular weights were monitored by gel permeation chromatography (GPC) on a Viscotek GPCMax/VE 2001 GPC machine (Malvern Instruments, Inc.) using tetrahydrofuran (THF) as

eluent. Thermal properties of copolymer chains analysed using Differential Scanning Calorimetry (DSC, TA Instruments) measurements with temperature increased from -50 to 100 $^\circ\text{C}$ at a rate of 5 $^\circ\text{C}/\text{min}$. Thermal stabilities of polymers were analyzed using Thermogravimetric Analysis (TGA, TA Instruments) with samples heated up from room temperature to 700 $^\circ\text{C}$ at a rate of 20 $^\circ\text{C}/\text{min}$.

Polymer synthesis

Crosslinkable PPF-PLGA. PPF was synthesized from diethyl fumarate and 1,2-propylene glycol monomers as described in our previous report.⁴⁴ PPF-PLGA copolymer was further obtained by ring opening of D,L-lactide and glycolide monomer at 140 $^\circ\text{C}$ for 24 h using stannous octoate ($\text{Sn}(\text{Oct})_2$) as catalyst and PPF as initiator.

Amphiphilic PPF-PLGA-PEG. PPF-PLGA was reacted with an excess amount of oxalyl chloride to obtain PPF-PLGA-COCl. Unreacted oxalyl chloride was removed by rotary evaporation under reduced pressure, PPF-PLGA-COCl was then reacted with an excess amount of HO-PEG-OH to obtain PPF-PLGA-PEG copolymer. After reaction, the polymer mixture was repeatedly dissolved in methylene chloride and precipitated in methanol to remove unreacted PEG chains for at least 5 times.

Folate functionalized PPF-PLGA-PEG-FA. Folate was coupled to the hydroxyl-terminated PPF-PLGA-PEG copolymer chains in the presence of dehydrant DCC and DMAP catalyst using DMSO as solvent. After reaction, the mixture were then filtrated to remove *N,N'*-dicyclohexylurea (DCU) and washed five times in methanol/diethyl ether (1/15, v/v) to remove unreacted FA molecules. For ease of reference in the article, PPF-PLGA-PEG copolymers are abbreviated as C, and functionalized PPF-PLGA-PEG-FA copolymers are abbreviated as C-FA.

Polymer micelles preparation

Two types of micelles were prepared as C and C/C-FA (containing 20 wt% of C-FA) by self-assembly. Briefly, 50 mg polymer (C, or C/C-FA) together with 2.5 mg photo-initiator Irgacure 2959 (2.5 wt% to polymer) dissolved in 5 mL of acetone under stirring at ambient temperature. Then 25 mL of 0.3% poly(vinyl alcohol)

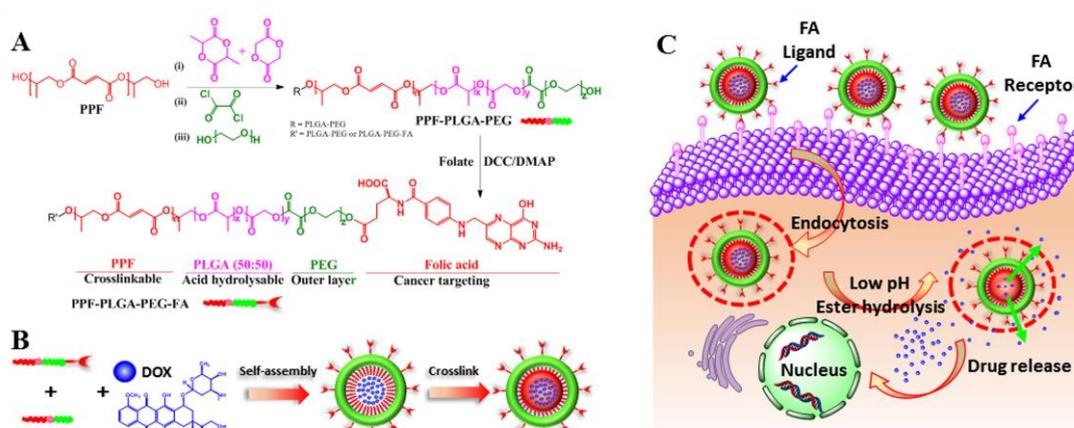


Fig. 1 Schematic illustration of the (A) synthesis, (B) self-assembly, and (C) cancer killing mechanism of PPF-PLGA-PEG-FA micelle system.

(PVA) solution was added slowly. Residual acetone was removed under reduced pressure. Final micelle concentration was 2.0 mg mL⁻¹. Crosslinked micelles were prepared by irradiating under 365 nm UV light (Black-Ray Model 100AP, Upland, CA) for 100 seconds. Hydrodynamic sizes of formed micelles were determined by dynamic light scattering (DLS) performed on Zetasizer Nano ZS (Malvern Instruments). The morphology of micelles was observed by JEOL 1400 Transmission Electronic Microscopy (TEM) at a voltage of 80 kV.

Loading of DOX into micelles

Doxorubicin was extracted from doxorubicin hydrochloride with triethylamine and methylene chloride according to a previous study.⁴⁵ Doxorubicin (1 mg) and polymer (20 mg) were dissolved in 2 mL of acetone, and 10 mL of 0.3% PVA/water solution was slowly added following stirring at room temperature. Crosslinked micelles were prepared by UV irradiation as described above. Exposure time is essential thus need to be precisely controlled in this step because DOX is photosensitive and too long exposure may weaken its inherent fluorescence intensity. Chemical crosslinking using ammonium persulfate and tetramethylethylenediamine solution can be an alternative in this step. After crosslinking, the obtained micelles were collected by centrifuging at 15,000 rpm for 10 min. The concentration of DOX left in the supernatants was collected after centrifugation and washed 3 times then quantified using a UV-vis absorbance microplate reader (SpectraMax Plus 384, Molecular Devices, Sunnyvale, CA) with a detection wavelength set at 490 nm. The drug loading content (DLC) and drug loading efficiency (DLE) of DOX in micelles was calculated according to the following equation, as previously described.⁴⁶

$$\text{DLC (\%)} = [\text{weight of drug capsuled} / (\text{weight of drug loaded polymers})] \times 100\%$$

$$\text{DLE (\%)} = [\text{weight of drug capsuled} / \text{weight of drug in feed}] \times 100\%$$

In vitro release profile of DOX from the micelles

Doxorubicin-loaded uncrosslinked and crosslinked C and C/C-FA micelles were studied at pH 7.4 and pH 5.0. Briefly, DOX loaded micelles obtained by self-assembly of 20 mg polymer and 1 mg DOX were collected and resuspended in 5 mL phosphate-buffered saline (pH 7.4, 0.5% Tween 80) or acid-buffered solution (pH 5.0, 0.5% Tween 80) and stirred at 100 rpm under 37 °C. At a certain time point, 300 µL medium was withdrawn and centrifuged at 15,000 rpm for collecting micelles. The concentrations of released DOX in the supernatant were quantified using UV-vis absorbance microplate reader with a wavelength of 490 nm.

Cell viability

HeLa cells were suspended in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 100 U mL⁻¹ penicillin, and 0.1 mg mL⁻¹ streptomycin. Cells were then seeded to 48-well tissue culture polystyrene (TCPS) plates at a density of 10 000 cells cm⁻² and then cultured for 24 h to ensure cell adhesion. For cytotoxicity studies, empty micelles without loaded drugs were collected, washed three times with PBS, sterilized with 70% ethanol and dried under vacuum. Dried particles were then incubated with cells at varied concentrations of 0.1, 0.5, 1.0 and 2.0 mg mL⁻¹. Wells seeded with the same density of cells while no nanoparticles added were used as positive controls. At 3-day point, cell numbers in each group were determined by MTS assay (CellTiter 96 Aqueous One Solution, Promega, Madison, WI). For micelles loaded with drugs, the same procedures were applied by using drug-loaded uncrosslinked and crosslinked C or C/C-FA micelles, or free drugs at concentrations of 0.01, 0.1, 0.5, 1, 5, 10 and 50 µg mL⁻¹. Cell viability (%) after co-culture for 70 hours was calculated by comparing the OD value in each group to that of positive controls (set as 100%).

Fluorescent imaging of cells and nuclei

HeLa cells treated with DOX-loaded micelles at a drug concentration of 5 µg mL⁻¹ for 3 days were washed three times with DPBS and fixed by 4% paraformaldehyde for 10 min. Then paraformaldehyde was removed by washing three times with DPBS and cells were stained with Rhodamine-Phalloidin (RP, Life Technologies) for 1 hour at 37 °C. Cell nuclei were stained with DAPI (4',6-diamidino-2-phenylindole) for 2 min at room temperature. Cells and cellular nuclei were visualized and photographed with Axiovert 25 Zeiss light microscope (Carl Zeiss, Germany).

Results and discussion

Polymer synthesis and characterization

PPF-co-PLGA copolymer was synthesized by ring-opening reaction of D,L-lactide and glycolide monomer using PPF as initiator. The number average molecular weights (M_n), weight average molecular weights (M_w) and polydispersity index (PDI) were determined to be 4400 g mol⁻¹, 10900 g mol⁻¹, 2.4 and 13200 g mol⁻¹, 37400 g mol⁻¹, 2.8 for PPF and PPF-PLGA copolymers, respectively. After linking with PEG chains and further conjugating with folate ligand, copolymers were determined to have M_n , M_w and PDI of 14400 g mol⁻¹, 32700 g mol⁻¹, 2.3 and 14900 g mol⁻¹, 33000 g mol⁻¹, 2.2 for PPF-PLGA-PEG and PPF-PLGA-PEG-FA (Table S1), respectively. Chemical structure of synthesized polymer was confirmed by ¹H

Table 1 Characterizations of micelles composed of varied chain contents before and after drug loading.

	Empty micelle			DOX-loaded			DLC (%)	DLE (%)
	Size (nm)	PDI	Zeta (mV)	Size (nm)	PDI	Zeta (mV)		
Uncross-C	136.1 ± 1.7	0.07	-41.4 ± 0.6	207.6 ± 3.4	0.17	-39.5 ± 1.9	2.3	48.2
Cross-C	135.6 ± 2.7	0.10	-36.4 ± 0.3	193.6 ± 0.6	0.08	-24.6 ± 0.6	2.4	50.8
Uncross-C/C-FA	141.1 ± 1.8	0.09	-34.8 ± 0.6	219.2 ± 3.1	0.18	-40.5 ± 1.3	2.1	43.5
Cross-C/C-FA	141.0 ± 1.6	0.10	-32.5 ± 1.4	206.4 ± 1.9	0.16	-26.5 ± 1.6	2.2	45.9

NMR using DMSO- d_6 as solvents, as demonstrated in Fig. S1. Thermal properties determined by DSC measurements (Fig. S2) showed glass transition temperatures (T_g) of 9.7, 38.8, 27.5 and 33.8 °C for PPF, PPF-PLGA, PPF-PLGA-PEG and PPF-PLGA-PEG-FA polymers (Table S1), respectively. Heat stabilities of these polymers were analyzed by TGA, and degradation temperatures (T_d) of 390.2, 275.8, 358.5 and 359.0 °C were determined for PPF, PPF-PLGA, PPF-PLGA-PEG and PPF-PLGA-PEG-FA polymers, respectively (Fig. S3).

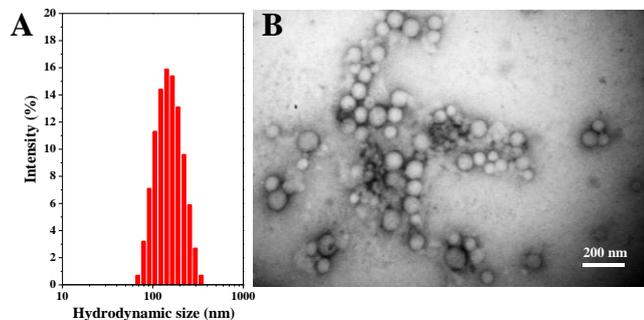


Fig. 2 (A) size distribution and (B) TEM images of crosslinked C/C-FA micelles.

Core crosslinked micelle preparation and properties

Micelles with varied chain components of C and C/C-FA were readily prepared through self-assembly and crosslinked by UV irradiation using biocompatible photo initiator Irgacure 2959. DLS measurements showed average diameters of 136.1 ± 1.7 nm, 135.6 ± 2.7 nm and PDI of 0.07 and 0.10 for uncrosslinked and crosslinked C micelles (Table 1), respectively. Size distribution of all these micelles were also characterized and summarized (Fig. 2 and Fig. S4). After the incorporation of 20 wt% of chains that conjugated with folate ligand, a slight increase in micelle sizes were detected with hydrodynamic size values of 141.1 ± 1.8 nm and 141.0 ± 1.6 nm for uncrosslinked and crosslinked C/C-FA, respectively. The size distribution of crosslinked C/C-FA was presented in Fig. 2A. The morphological images observed by TEM demonstrated consistent values in sizes from DLS, as demonstrated in Fig. 2B. Slightly negative surface charges from -41.4 ± 0.6 to -32.5 ± 1.4 was detected by zeta potential measurements for all these crosslinked or uncrosslinked C and C/C-FA micelles (Table 1).

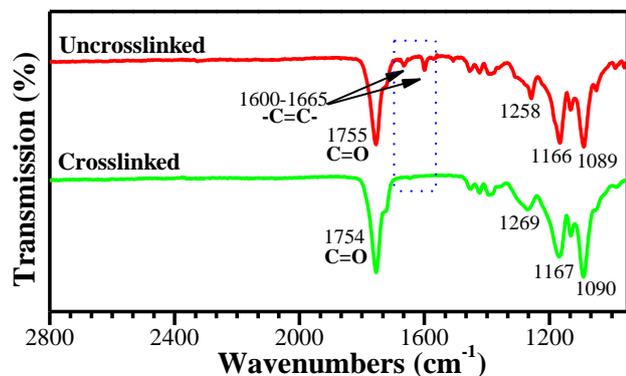


Fig. 3 The FTIR spectra of uncrosslinked and crosslinked self-assembled C/C-FA micelles.

Fourier transform infrared spectroscopy (FTIR) spectra were determined to confirm the opening of double bonds and the crosslinking among polymer chains. As demonstrated in Fig. 3, peaks representing $-C=C-$ double bonds in the synthesized crosslinkable chains exhibited at wavenumbers of $1500 \sim 1700$ cm^{-1} . However, after a photo-crosslink process under UV light, no further peaks for double bonds could be detected, confirming a success in the bonds opening and crosslinking.

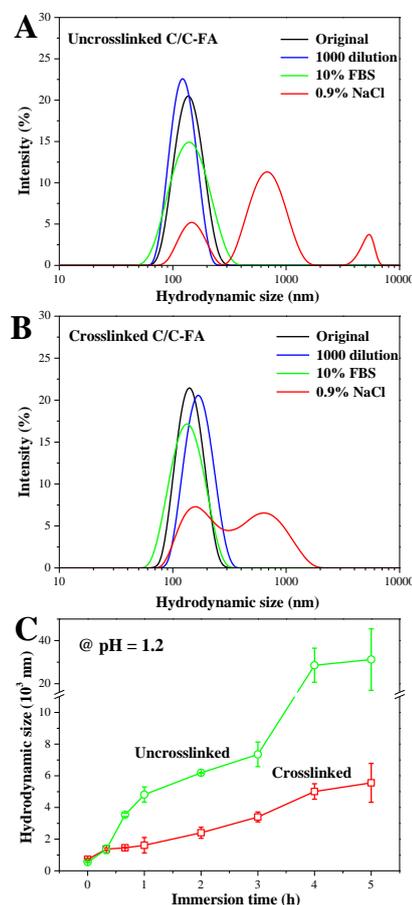


Fig. 4 Stabilities of (A) uncrosslinked and (B) crosslinked micelles under 1000 times dilution, 10% FBS and 0.9% NaCl conditions. (C) Size changes of uncrosslinked and crosslinked micelles under strong acidic (pH 1.2) environment.

The stability studies of micelles with or without crosslinked cores were evaluated using C/C-FA micelles under 1000 times dilution (mimicking injection to blood), 10% FBS (mimicking blood serum condition), and 0.9% NaCl (mimicking physiological saline condition). As demonstrated in Fig. 4A and B, both crosslinked and uncrosslinked micelles maintained similar size distribution upon 1000 times dilution or 10% FBS exposure. However, under the condition of 0.9% NaCl, a majority of the uncrosslinked micelles altered sizes and three populations of particles were detected in DLS with average size distribution of 480.7 ± 37.3 nm. In contrast, for the crosslinked micelles, two populations were observed while large percent of micelles maintained their size. The average hydrodynamic size for crosslinked micelles under 0.9% NaCl was 291.2 ± 8.9 nm, much smaller than the uncrosslinked particles. In addition, the crosslinked micelles were shown to be more stable under a strong

acid environment with aggregates in micron size were determined for uncrosslinked micelles in a given 5 h time period, as demonstrated in Fig. 4C. After 4 hours, there were large sized aggregates can be observed by naked eyes for uncrosslinked particles and precipitates can be seen at 5 hours incubation in pH 1.2 acid solution. For crosslinked micelle, no obvious precipitates were observed at the same time point. Taken together, the photo-crosslinking of hydrophobic cores are believed to minimize the particle disassembly during the blood injection process and prevent the elimination of particles from body circulation process. This extracellular stable property is highly favored in drug delivery process and thus enhanced the potential of crosslinked micelles as promising drug carriers.

DOX loading

DOX is a potent anticancer drug widely used for the treatment of various malignant tumors through inhibition of nucleic acid synthesis. In this study, the DOX-loaded micelles were readily prepared and characterized by DLS, as listed in Table 1. After the loading of drugs, the uncrosslinked and crosslinked C or C/C-FA micelles were determined to have hydrodynamic sizes of 207.6 ± 3.4 , 193.6 ± 0.6 , 219.2 ± 3.1 and 206.4 ± 1.9 nm, respectively. Compared with their empty counterparts, there is an increase in the micelle size due to the drug encapsulation. Zeta potential measurements showed values between -24.6 and -40.5 mV, implying negative surface charges for the four types of DOX-loaded micelles (Table 1). DLC of DOX in the micelles were determined to be 2.3, 2.4, 2.1 and 2.2% and DLE were calculated to be 48.2, 50.8, 43.5, and 45.9% (Table 1) for uncrosslinked and crosslinked C or C/C-FA micelles, respectively. These results indicate that the loading of DOX into micelles slightly enlarged the micelle size while no noticeable alternation in surface charges.

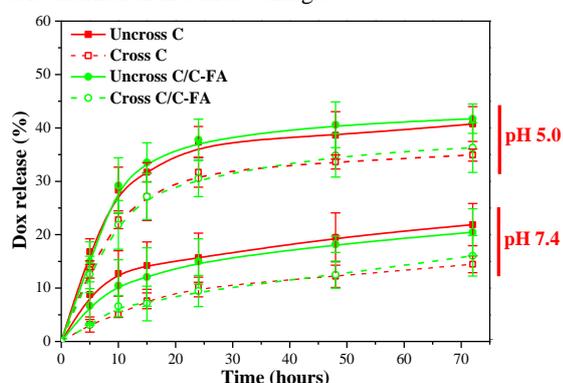


Fig. 5 Release profile of DOX from uncrosslinked and crosslinked C-DOX and C/C-FA-DOX micelles with varied chain components at different pH solutions.

In vitro release

Release of DOX from C or C/C-FA micelles was investigated under both physiological (pH 7.4) and acidic conditions (pH 5.0, mimicking the acidic environments in lysosomes), respectively (Fig. 5). At a releasing time scale of 72 h, all the micelles displayed a sustained release profile. However, a substantial higher amount of DOX was released under acidic environment (pH 5.0) than that of physiological condition (pH 7.4). At physiological conditions, $22 \pm$

4% of DOX loaded in the uncrosslinked C micelles were determined to release to the medium at 37 °C in the presence of 0.5% Tween-80 surfactant. After UV irradiation, crosslinked C micelles showed lower drug release of $14 \pm 2\%$ largely due to the constraint from crosslinks. C/C-FA micelles showed similar release profile of $20 \pm 4\%$ and $16 \pm 4\%$ for uncrosslinked and crosslinked particles. Nevertheless, under an acidic environment, the DOX release almost doubled for all the micelles. The DOX determined in medium increased to $41 \pm 3\%$ and $35 \pm 1\%$ for uncrosslinked and crosslinked C micelles at pH 5.0 condition, as shown in Fig. 5. Similar trends with values of $42 \pm 3\%$ and $36 \pm 5\%$ were determined for uncrosslinked and crosslinked C/C-FA particles, respectively.

The higher DOX release phenomenon may largely due to the polymer hydrolysis, especially for PLGA chains. PLGA (50:50) is widely acknowledged to have a fast degradation rate, e.g., around 1-2 months at neutral conditions.⁴⁷ The acidic conditions further catalyzed the hydrolysis process and accelerated chain degradation.^{48,49} After endocytosis, the pH values in endosomes/lysosomes decrease to the 4.5-6.5 range,⁵⁰ which will cause a fast hydrolysis of PLGA chains and lead to a robust release of anti-cancer drugs. Under real physiological conditions, the situation is much more complicated. There are multiple categories of enzymes could help to digest and disassemble the micelles besides pure pH change. In addition, the total time for micelles to trap in tumors through enhanced permeability and retention (EPR) effect and digest inside cancer cells, may be longer than estimated in the *in vitro* study here, which will also result enhanced release of drugs. Therefore, this pH sensitive degradation property and usage of EPR effect could be beneficial for our micelle system in potential tumor therapy applications.

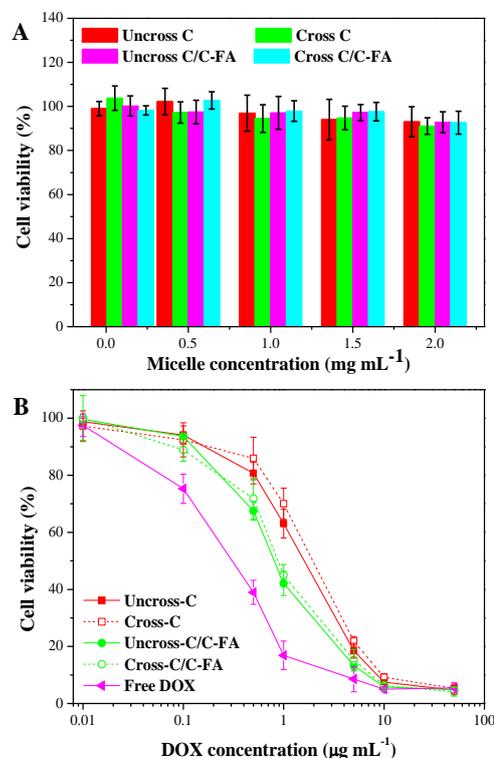


Fig. 6 Cell viability under co-culture with (A) empty micelles and (B) varied concentrations of drug-loaded C-DOX or C/C-FA-DOX micelles, and free drugs.

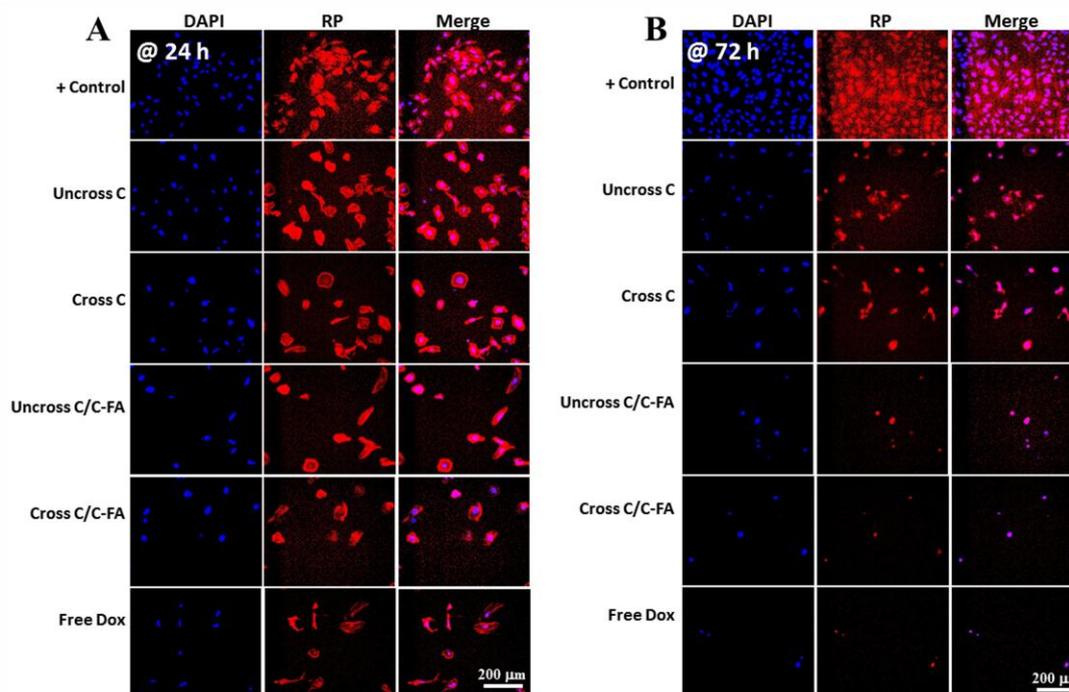


Fig. 7 Fluorescent images of HeLa cancer cells incubated with uncrosslinked and crosslinked C-DOX and C/C-FA-DOX micelles at a DOX concentration of $5 \mu\text{g mL}^{-1}$ for 24 and 72 h.

In vitro cytotoxicity

The cytotoxicity of empty micelles and DOX-loaded micelles were evaluated using HeLa cancer cells (Fig. 6). No apparent cytotoxicity for empty micelles was observed, as shown in Fig. 6A. High viability of cells was determined by MTS after 3 days culturing with micelles at different concentrations of 0.1, 0.5, 1.0, 1.5 and 2.0 mg mL^{-1} . As a biodegradable polymer, PPF-PLGA-PEG-FA chains mainly degrade through hydrolysis of ester bonds. Since the PPF, PLGA and PEG segments in the PPF-PLGA-PEG backbone chain are all biocompatible and nontoxic, it is reasonable for the copolymers to have a low cytotoxicity. However, after loading of DOX into the micelles, they showed inhibition effect to cells even at low concentrations. As demonstrated in Fig. 6B, under the same concentration of DOX administration, C/C-FA with cancer targeting folate ligands on surface showed remarkable better cancer killing effects than that of C particles without targeting ligands. It is acknowledged that the cellular uptake of large molecules or particles generally takes place by endocytosis through endosomes and further lysosomes.^{51,52} The incorporation of folate ligands on particle surfaces is widely accepted to enhance binding of nanoparticles to the cells via ligand-receptor interaction. Compared with passive taken or non-receptor taken, this receptor-mediated endocytosis is more effective and result in enhanced DOX delivery, as confirmed by our results. As a positive control, free DOX showed the lowest cell viability *in vitro* experiments. It is understandable because the DOX encapsulated in micelles need to go through a releasing process, which resulted a delay in cancer cell killing effects, while free DOX does not require the release process and could kill cancer cells immediately after the administration.

Together with the discussion in the release section, our micelle system has acidic sensitive drug releasing properties due to the fast hydrolysis of PLGA segments in PPF-PLGA-PEG-FA polymer chain. After endocytosis, for micelles swallowed in the intracellular organelles especially late lysosomes, their surrounding environment becomes acidic. Therefore, it is expected that our DOX-loaded micelles will release DOX in a time-effective manner, which will result in robust cancer-killing effects as evidenced by low cancer cell viability in Fig. 6B.

Cell and nuclei morphology

To further substantiate the cancer killing effects of our micelle system, HeLa cellular morphology and nuclei morphology were visualized and photographed after culturing for 24 h and 72 h in the presence of multiple micelles with DOX concentration of $5 \mu\text{g mL}^{-1}$. As shown in Fig. 7A, at 24 h, the positive group without DOX administration showed the highest cell numbers and free DOX showed the lowest cell numbers, as consistent with the above cell viability tests. For the groups cultured with DOX-loaded micelles, a noteworthy trend that lower cell numbers, i.e., better cancer inhibition effects, was observed on the C/C-FA micelles with folate ligands. When the culture time was prolonged to 72 h, cells in the positive group almost grow confluent, whereas the cells treated with free DOX almost fully inhibited to death (Fig. 7B). The groups cultured with DOX-loaded micelles, however, showed apparent differences in cell killing effects. There are substantial cells in the DOX-C micelle treated group, whereas few fluorescence dots could be observed in groups treated with DOX-C/C-FA micelles. To ascertain the results observed in fluorescence microscopy, further MTS studies were conducted at DOX concentration of $5 \mu\text{g mL}^{-1}$. Significant differences were established by One-way ANOVA

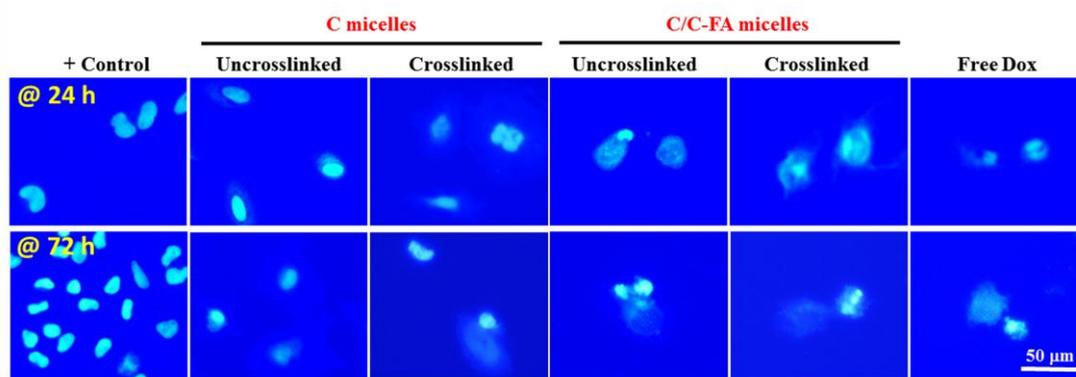


Fig. 8 The DNA profile in HeLa cancer cells after incubation with uncrosslinked and crosslinked C-DOX and C/C-FA-DOX micelles at a DOX concentration of $5 \mu\text{g mL}^{-1}$ for 24 and 72 h.

analysis between groups treated with C and C/C-FA micelles (Fig. S5 and Fig. S6).

Doxorubicin released from micelles will finally permeate nuclear membranes and accumulates in the nucleus, where it interferes with DNA replication and causes cancer cell apoptosis.⁵³ A majority of free DOX in tumors was reported to quickly bind to nuclear DNA within 1 day after injection, whereas DOX releasing from carriers entered nuclei slowly but accumulated at a higher concentration in long term.⁵⁴ Here, the changes in cell nuclei were monitored using DAPI, a dye that can effectively bind to A-T rich regions in DNA. As can be noted from Fig. 8, DOX was delivered and released into the nuclei of HeLa cells following incubation time of 24 h and 72 h. At 24 h, positive cells showed round and regular shaped nuclei. In contrast, cells incubated with free DOX or DOX-loaded micelles, showed abnormal nuclei with irregular shapes. The same trend was observed at a prolonged incubation time of 72 h. In particular, cells incubated with C/C-FA micelles demonstrated more severe damage in the nuclei, as evidenced by the severely deformed shapes of nuclei. Further, the fluorescence intensity in the nuclei revealed that there is an existence of DNA pieces all surrounding the nuclei region, which imply fatal damage made by delivered DOX to cancer nuclei. These findings, taken as a whole, put the basis for a better receptor-mediated delivery of anti-cancer doxorubicin to cancer cells based on the introduction of crosslinkable polymeric core and incorporation of cancer targeting ligands.

Conclusions

Biodegradable crosslinkable copolymer micelle systems were developed and crosslinked. Favorable extracellular stabilities were achieved for micelles against potential severe conditions, including large volume dilution, salt, serum, and low pH condition. Desired targetability was obtained by incorporating cancer targeting folate ligands. After drug loading, the DOX release showed a pH sensitive profile through acidic degradation of polymer chains, in particular, PLGA (50:50) chains. Cell studies showed lower cancer cell number in groups treated with FA decorated micelles, indicating a higher targeting ability through receptor-mediated endocytosis of micelles. Further cellular morphology and nuclei staining showed consistent results as cell viability studies, demonstrating an enhanced killing of cancer cells through inhibiting nucleic acids synthesis. Our results

provide essential implication for cancer targeted drug delivery using this stabilized core crosslinked micelle system.

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Extracellular stable micelle with folate conjugated surface, crosslinked core and pH-sensitive hydrolysable bonds was developed for cancer targeted drug delivery.