Highly Stable Core-Surface-Crosslinked Micelles as Drug Carriers for Cancer Chemotherapy

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ABSTRACT:

Stealth nanoparticles made of polymer micelles are ideal drug carriers for targeted drug delivery, but their stability is critical for intravenous applications. We found that core-surface-crosslinked nanoparticles (SCNp) made from amphiphilic brush copolymers had greatly enhanced stability. The surface of the SCNps can be easily tailored by using different chains. The amphiphilic brush copolymers consisted of hydrophobic poly(caprolactone) (PCL) and hydrophilic poly(ethylene glycol) (PEG) or poly(2-(dimethylaminoethyl methacrylate) (PDMA) chains were used to fabricate SCNps. Positively charged PCL/PDMA SCNps could be internalized more efficiently by ovarian cancer cells, leading to an enhanced anticancer drug efficacy.

Introduction

Over the past decades, targeted drug delivery has been widely explored to deliver drugs specifically to tissues, especially cancerous tissues, for enhanced therapeutic efficacy and reduced systemic toxicity. For example, polymer–drug conjugates,[1-3] dendrimers,[4-6] liposomes,[7-9] polymer micelles,[10-15] and polymer nanoparticles[16-19] have been demonstrated to preferentially carry drugs to cancerous tissues, resulting in much improved therapeutic efficacies. Cancer targeting was achieved by the enhanced permeability and retention (EPR) effect of tumors: tumor's permeable capillaries and poor lymphatic drainage cause trapping of macromolecules and colloidal particles in cancerous tissues.[20-22] Hence, the resulting drug concentrations in tumors could be as much as an order of magnitude higher than that in healthy tissues.[20-22]

One special type of nanoparticles is polymer micelle formed from amphiphilic copolymers. Amphiphilic copolymers composed of hydrophilic and hydrophobic segments form a micellar structure in water with a hydrophobic compact inner core and a hydrophilic outer

corona. The size depends on the lengths of the hydrophilic and hydrophobic chains, but generally are in the submicron range.[10,17] The hydrophilic outer layer acts as a shelter to protect the inner hydrophobic core from being recognized and cleaned by RES and other clearing organs such as the kidneys, liver, spleen, and lung.[17] The hydrophilic corona is stable on the core surface due to the covalent linking. Thus, these type nanoparticles, also named "stealth nanoparticles", have a long circulation time necessary for passive targeting to cancerous tissues via the EPR effect.[17]

For intravenous application, it is critical that the micelles are stable, i.e. have low critical micelle concentration (CMC); Otherwise, the micelles will disassociate into unimers upon dilution in the bloodstream, causing non-targeted drug release and toxicity. Chemically crosslinking the cores after the core formation was used to increase the stability of micelles (Figure 1 a).[23] Such micelles do not disassociate, but chemical reactions in the cores are undesirable because they may alter the structures and properties of the encapsulated drugs.

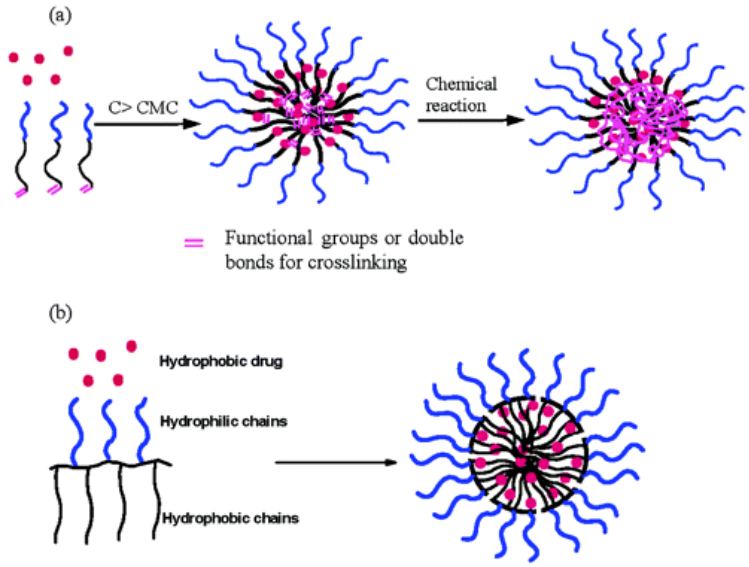


Figure 1. Formation of core-cross-linked micelles by chemical reactions (a) and core-surface cross-linked micelles (SCNs) from amphiphilic brush polymers(b)

We found that amphiphilic polymer brushes could form highly stable core-surface-crosslinked micelles (nanoparticles) (SCNs) in which the backbones of the polymer brushes

acted as crosslinks on the hydrophobic core surface to greatly enhance the stability of the micelles (Figure 1 b) without using chemical reaction in the core.[24] These highly stable SCNs can be used as drug carriers for cancer chemotherapy.

Experimental

Materials

ε-Caprolactone (ε-CL) (Aldrich) was dried over calcium hydride with stirring for 24 h at room temperature. PCL macromonomer (m-PCL: Mn =3.8 × 10^3; Mw/Mn = 1.07) was synthesized according to litersture.[25] Poly(ethylene glycol) methyl ether methacrylate 50% solution in water (Aldrich) (mPEG, Mn = 2080) was dried under high vacuum with rotary evaporator. Tetrahydrofuran (THF) was dried by refluxing over calcium hydride. CuBr was stirred in glacial acetic acid, filtered, and washed with absolute ethanol as well as diethyl ether. 1,1,4,7,10,10-Hexamethyltriethylenetetramine (HMTETA, 97%), methyl α-bromophenylacetate (MBP, 97%), acryloyl chloride (96%), triethylamine (Et_3N, 99.5%), N,N-dimethylformamide (DMF, 99.8%), N-phenyl-1-naphthylamine (PNA), 2-(N,N-dimethylamino)ethyl methacrylate (DMA), and cis-diammineplatinum (II) dichloride (cisplatin, 99.99%), all from Aldrich, were used as received without further purification.

Instrumentation

Gel permeation chromatography (GPC) was used to determine polymer molecular weights and molecular weight distribution (PDI) using polystyrene standards (Polysciences Corporation). The measurements were operated on a Waters SEC equipped with a Waters 2414 refractive index detector and two 300 mm Solvent-Saving GPC Columns (molecular weight ranges: 5×10^2-3×10^4, 5×10^3-6×10^5) using THF as solvent at a flow rate of 0.30 mL/min at 30˚C. Data were recorded and processed using Waters software package. ^{1}H NMR spectra were recorded on a Bruker Avance DRX-400 spectrometer using $CDCl_3$ as solvent. Chemical shifts were reported downfield from 0.00 ppm using TMS as internal reference. Micelle sizes were determined by laser dynamic light scattering (DLS, Nicomp 370 particle sizer). The measurement of the PNA excitation was carried out with the fluorescence spectroscopy (SPECTRAmax GEMINI XS spectrofluorometer, Molecular Devices). Excitation wavelength was 340 nm for the measurement of emission spectra. Sensitivity was set as 10. The emission intensity at 418 nm was recorded with the cutoff wavelength at 420 nm to estimate CMC.

Synthesis of ω-methacryloyl poly(2-(*N,N*-dimethylamino)ethyl methacrylate) macromonomer (mPDMA): mPDMA was prepared by two step reactions: the synthesis of ω-hydroxy-PDMA by atom transfer radical polymerization (ATRP)[26] of DMA and subsequent reaction with methacrylic acid.[24] The brush copolymers were synthesized by free radical polymerization or atom transfer radical polymerization.[24]

Micelle preparation of and characterization:

The micelles were prepared using solvent displacement method with an acetone-water system. In a typical procedure, the copolymer (10 mg) was dissolved in 2.5 mL of acetone with stirring about 2 h. The polymer acetone solution was dropwise added into 20 mL of deionized water with stirring, and the mixture was continually stirred overnight to form micelles. The suspension was stirred under reduced pressure for 8 h to remove acetone. The solution was filtrated through 0.2 μm syringe filters before any measurement.

Micelle size measurement:

The particle size was measured by dynamic light scattering (DLS), which was performed on a Nicomp 370 particle sizer with wavelength of 514.5 nm at a fixed scattering angle of 90 º. Data were analyzed by Nicomp software (version 12.3) with volume-weighted Gaussian/Nicomp analysis mode, which gave histograms of relative volume vs. mean diameter.

Critical micelle concentrations (CMC) measurement:

CMCs of the copolymers were estimated by fluorescence spectroscopic method using N-phenyl-1-naphthylamine (PNA) as the fluorescence probe. Typically, PNA solutions in acetone were added to each brown bottle. The acetone was evaporated, leaving 1.0×10^{-8} mole of PNA in each bottle. PCL/PEG or PCL/PDMA brush copolymer in acetone (10 mL) at concentrations ranging from 0.25 g/L to 0.05 mg/L were added. The mixtures were stirred at 60 ºC for 3 h. They were sonicated for 1 min before measurement. The spectroscopy measurement was carried out at the emission wavelength of 418 nm and excitation wavelength of 340 nm.[28]

Preparation of cisplatin-loaded SCNp for in vitro cytotoxicity assessment: SCNs loaded with cisplatin were prepared using solvent displacement method with an acetone-water system. In a typical procedure, the copolymer (2.5 mg) was dissolved in 2.5 mL of acetone with stirring about 2 h, then 166 μL of cisplatin acetone solution at the concentration of 1×10^{-3} M was added with stirring. The polymer-drug acetone solution was dropwise added into the 5 mL of RPMI

1640 media under stirring, and the mixture was continually stirred overnight to form micelles. The suspension was stirred under reduced pressure for 8 h to remove acetone.

Cell culture: SKOV-3 adenocarcinoma cells were obtained from American Type Culture Collection (Rockville, MD). Cells were propagated to confluence in T-75 flasks (Corning Costar, Cambridge, MA) at 37 °C under an atmosphere of 5% CO_2 in 15 mL RPMI-1640 medium supplemented with 10% fetal calf serum (FCS), 10 μg/mL insulin, and 1% antibiotic/antimycotic solution (Sigma A9909). Cells were harvested from flasks with 0.25% trypsin/0.03% EDTA.

Seeding of cancer cells and nanoparticle uptake: Cells were transferred (1mL/well at 2×10^5mL) into 12-well flat-bottom plates (Corning Costar) and incubated 24 h. A polylysine coated coverslip (12mm, Becton Dickinson Biocoat 354086) was placed in each well, and then incubated for another 24h. Coverslips were then inverted and transferred to another 12 well plate containing 0.9 mL of fresh media. Nanoparticles (encapsulated with 1-pyrenecarboxaldehyde) were added at 0.1 mL per well in RPMI media. After incubations for 1 h, the coverslips were rinsed with PBS, fixed with ice cold acetone and rinsed with distilled water. Coverslips were mounted on slides with Crystal/Mount (Biomedia M02) and viewed by confocal fluorescent microscopy.

The samples were examined under an inverted Confocal Laser Scanning Microscope (Bio-Rad Radiance 2100) Upright Nikon E800 microscope. The samples were excited using blue diode laser (405 nm).

Cell in vitro morphology: Cells were transferred (1mL/well at 2×10^5mL) into 12-well flat-bottom plates (Corning Costar) and incubated 24 h. The original media was removed and 975 μL fresh media was added. Nanoparticles were added at 25 μL per well in RPMI media. After 2h incubations, the plate was viewed with phase contrast microscopy.

Cytotoxicity test: MTT Cell Proliferation Assay was employed to estimate the cytotoxicity of SCNp encapsulated with cisplatin. Cells were first transferred (200μL/well at 7.5×10^4mL) into 96 well plates (Evergreen) and incubated for 24 h. The original media was removed and 100 μL fresh media was added. SCNps were added in RPMI media to achieve dosages from 0.005 μg/mL to 0.5 μg/mL and incubate for 2 hr. MTT reagent 10 μL was added to each well and

incubated for 2 hr at 37 °C under an atmosphere of 5% CO_2 until a purple precipitate was visible. Detergent reagent (100 μL) was added to each well then the plate placed in the 37 °C incubator for 2 h until all the crystals were dissolved. The absorbance intensity at 570 nm was recorded.

Results and Discussion

Micelle formation

The formation of micelles of the brush copolymers was detected using N-phenyl-1-naphthylamine (PNA) as fluorescent probe. PNA has high fluorescence activity in nonpolar environments but polar solvents such as water can quench its fluorescence.[30] We found that PNA was a much better fluorescent probe than pyrene in terms of reproducibility (Figure 2). The measured CMCs of the micelles of the brush copolymers are shown in Table 1. The CMCs of the block copolymers with the same chain lengths of PCL and PEG or PDMA as those in the brush copolymers were also measured for comparison. The PCL/PEG ratio only slightly affected the CMCs. With more PEG chains, the CMC of the brush copolymer only increased slightly (Table 1). The CMC of the copolymer with PCL/PEG ratio of 1/3 was 3.8 mg/L, compared with 1.0 mg/L of the copolymer with the ratio of 1/1. This makes it possible to tailor the hydrophilic chain density on the nanoparticle surface, which strongly affects its pharmacokinetics and biodistribution,[17] without changing its stability.

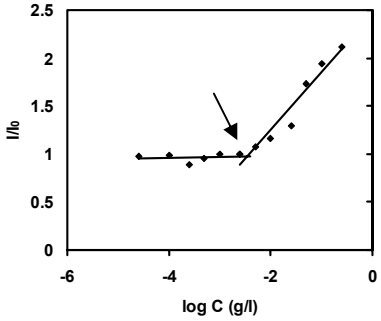


Figure 2. The intensity ratio I/I_0 in PNA fluorescence emission spectra as a function of logc of the PCL/PEG (1/3) brush copolymer in distilled water. [PNA] = 1.0×10^{-6} M; (I/I_0 is the relative fluorescence intensity in the presence of PNA (I) and the absence (I_0) of PNA).

Table 1. Critical micelles concentrations (CMCs) of copolymers of PCL (Mn: 7.0×10^3) with PEG (Mn: 2.08×10^3) or PDMA (Mn: 8.0×10^3) with different compositions.

Copolymers	PCL/PEG or PCL/PDMA chain ratio	Mn ($\times 10^3$)	CMC (g/L) ($\times 10^{-3}$)	CMC (mole/L) ($\times 10^{-8}$)
PCL-PEG block copolymer	1:1.0	8.2	40	480
PCL/PEG brush copolymer	1:0.93	21	1.0	5.0
PCL/PEG brush copolymer	1:3.0	48	3.8	7.9
PCL/PDMA brush copolymer	1:0.42	18	6.3	35

Most significantly, the brush copolymers have much lower CMCs than corresponding block copolymers. For example, the CMC of the brush copolymer with PCL/PEG chain ratio of 1/0.93 was almost 40 times (100 times if using molar concentration) lower than that of the block copolymer (chain ratio of 1/1). Even the brush copolymer with more PEG (e.g. PCL/PEG = 1/3) had much lower CMC than that of the block copolymer, indicative of much improved stability of the micelles formed from the brush copolymers. We believe that the improved stability of the micelles from the brush copolymer is derived from the partially crosslinking on the core surface, as shown in Figure 1 b. Table 1 shows that the Mn of the brush copolymers was about 3 to 6 times of that of the block copolymer. This was equivalent to 3 to 6 block copolymer chains linked together by the copolymer backbone on the micelle surface, entailing much improved stability of the micelles. Thus, the micelles less likely disassociate in the bloodstream. Meanwhile, there is no chemical reaction involved in the core and thus the properties of drugs encapsulated in the cores will not be altered.

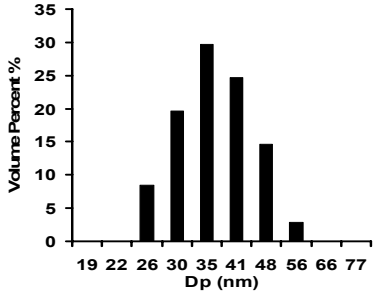


Figure 3. Micelles size distribution of brush copolymer (PCL/PEG = 1/1.09 (chain ratio); Mn: 9.2 × 10^3; mPCL (Mn: 3.8 × 10^3), mPEG (Mn: 2.08×10^3)).

Micelle Size

Solvent-displacement method was used to fabricate the micelles from the synthesized polymer brushes. Figure 3 shows that the micelles had very narrow size distribution. The diameter of micelles depends on the hydrophobic/hydrophilic chain ratio: it increased with increasing PCL/PEG chain ratio. For example, the micelles formed from the copolymer with PCL/PEG ratio of 2.81 had a diameter of 198 nm, while the diameter of the micelles was 27.4 nm at the PCL/PEG ratio of 0.73. Similarly, the micelle had 54.7 nm diameter at PCL/PDMA ratio of 2.38. Thus, the size of micelles of the brush copolymers can be easily tailored by changing the hydrophobic/ hydrophilic chain ratio without sacrificing micelle stability because it only slightly alters the CMCs.

In addition, PDMA is partially positively-charged in water. The micelles formed by PCL/PDMA brush copolymer thereby had a positively charged hydrophilic corona, which may be used to facilitate the cellular uptake of the nanoparticles by electrostatic interaction with the negatively-charged cell membrane. The surface property of the nanoparticles thus can be changed simply by using different hydrophilic chains.

Cellular uptake of the SCNs

Figure 4 shows the DIC and fluorescence images of the cells cultured with fluorescence-dye loaded PCL/PDMA SCNps. The bright fluorescence emitted from the inner of the cell indicates that the SCNps formed from the brush copolymers can be taken up efficiently by cancer cells.

DIC **Fluorescence**

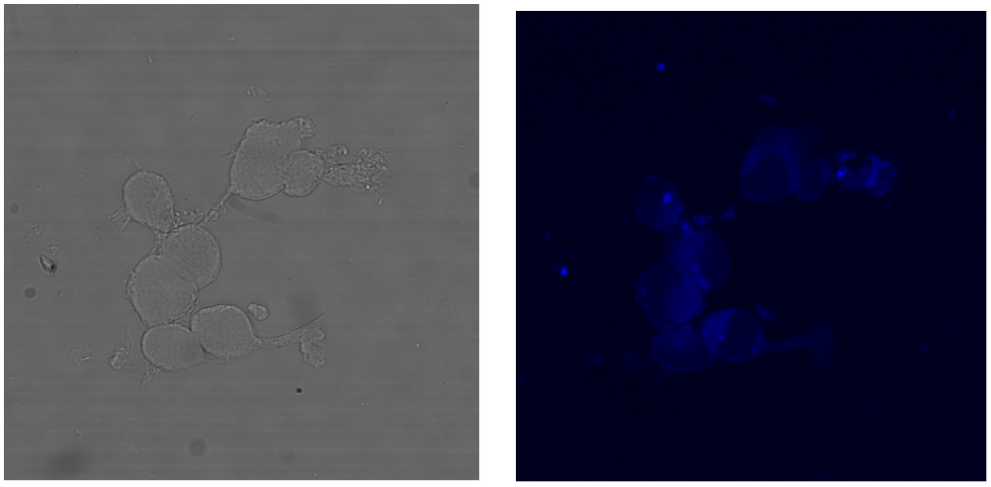
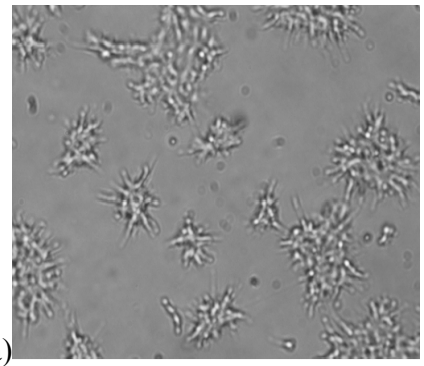
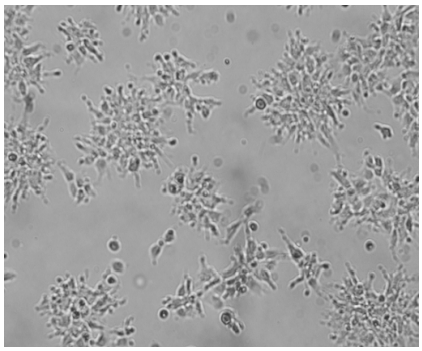


Figure 4. Differential interference contrast (DIC) and fluorescence confocal images of 1-pyrenecarboxaldehyde. Cell uptake and intracellular distribution in SKOV-3 adenocarcinoma cells cultured with PCL/PDMA SCNps containing 1-pyrenecarboxaldehyde. Original magnification 200 ×.

Cytotoxicity of SCNs loaded with cisplatin

Figure 5 shows that the morphology of cells treated with PCL/PEG SCNps and free cisplatin is not much different from that of the control, while a significant morphological change was observed for the cells treated with PCL/PDMA nanoparticles. In Figure 5 (e), cancer cells shrinked in the presence of PCL/PDMA nanoparticles.

At a cisplatin dosage of 0.25 μg/mL, free cisplatin drug killed about 60% cancer cells, but when encapsulated in the nanoparticle, its efficacy could be greatly enhanced, especially when encapsulated in PCL/PDMA SCNps, which killed more than 90% cancer cells (Figure 6). The IC_{50} of the cisplatin encapsulated in PCL/PDMA nanoparticles was about 0.01 μg/mL, which was much lower than that of cisplatin encapsulated in PCL/PEG SCNps and that of cisplatin free drug. (Figure 7). This proves that positively charged PCL/PDMA facilitated the adsorption of the SCNps onto the negatively-charged tumor cell surface and subsequently cellular uptake.

(a)  (b) 

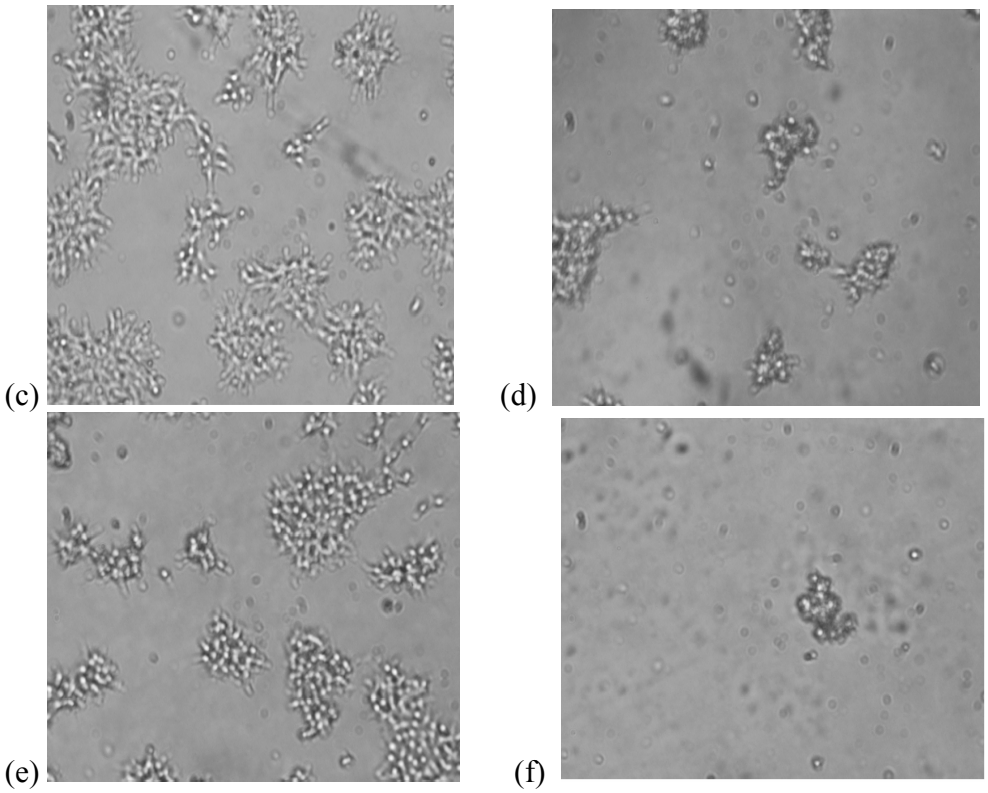


Figure 5. SKOV-3 adenocarcinoma cell morphology with or without SCNps after 2 h treatment. (a) Control : cultured with pure RPMI media; (b) Cisplatin control: cultured with cisplatin in RPMI media; (c) Cultured with PCL/PEG SCNp in RPMI media; (d) Cultured with PCL/PEG SCNp encapsulated with cisplatin in RPMI media; (e) Cultured with PCL/PDMA SCNp in RPMI media; (f) Cultured with PCL/PDMA nanoparticles encapsulated cisplatin in RPMI media.

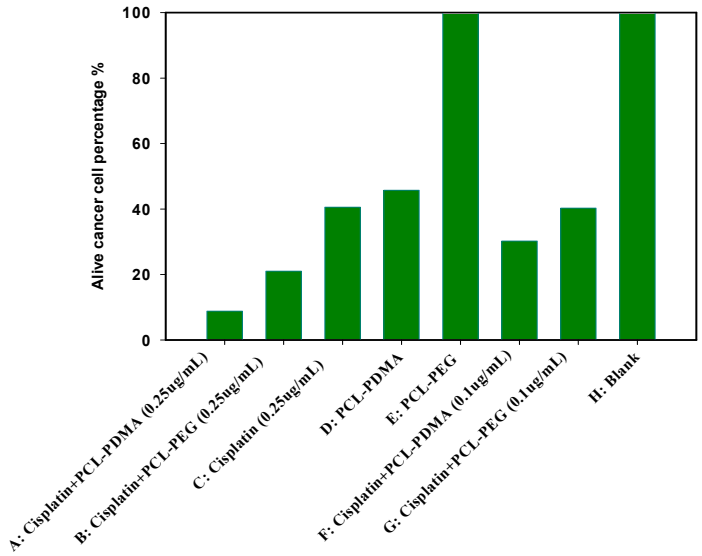


Figure 6. SKOV-3 adenocarcinoma cancer cells with 2 hr treatment. The concentrations of nanoparticle were 12.5 μg/mL in A, B, D, and E, and 5 μg/mL in F, and G were (cisplatin dose shown in the figure).

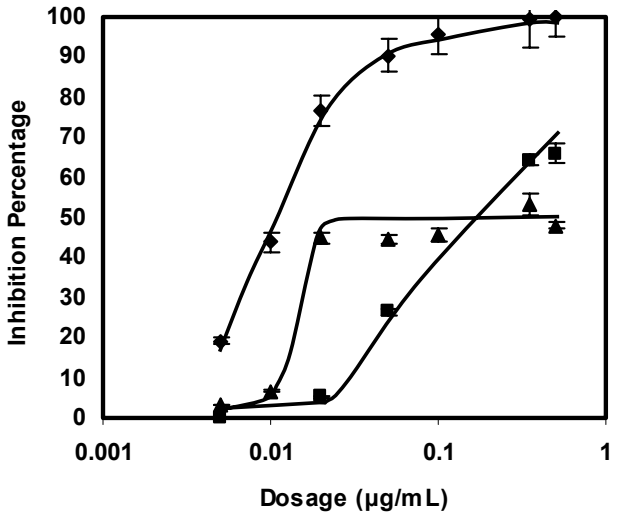


Figure 7. IC_{50} determination: SKOV-3 adenocarcinoma cancer cells with 2 h treatment, (Cisplatin in PCL/PDMA SCNps (♦), Cisplatin in PCL/PEG SCNps (■), free Cisplatin(▲)).

Conclusions

We demonstrate that core-surface crosslinked micelles fabricated from amphiphilic brush copolymers had much improved stability with easily adjustable sizes and surface properties by the hydrophobic/hydrophilic chain ratio and the type of the hydrophilic chains. The in vitro cytotoxicity test proved that this method provided a new approach to design "stealth" stable nanoparticles without chemical reaction in the cores for targeted drug delivery.

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