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RESEARCH PAPER

Targeted Polymeric Micelle System for Delivery of Combretastatin A4 to Tumor Vasculature *In Vitro*

Yiguang Wang • Tingyuan Yang • Xun Wang • Jiancheng Wang • Xuan Zhang • Qiang Zhang

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ABSTRACT

Purpose To develop an efficient tumor vasculature-targeted polymeric micelle delivery system for combretastatin A4 (CA4), a novel antivascular agent.

Methods CA4-loaded micelles were prepared from poly (ethylene glycol)-*b*-poly (d, l-lactide) copolymers. RGD peptides that target integrins αvβ3 and αvβ5, markers of angiogenic endothelial cells, were coupled to the surface of micelles. The micelles were characterized in terms of particle size, morphology, drug loading, and drug release. Cellular uptake of micelles was evaluated by fluorometric determination and confocal microscopy. Anti-proliferation of targeted micelles was also evaluated by SRB method.

Results The mean diameters of CA4-loaded targeted micelles were 25.9 ± 1.3 nm and spherical in shape. Approximately 4 mg/mL of micellar CA4 loading was obtained with an entrapment efficiency of 97.2 ± 1.4%. *In vitro* release studies revealed that targeted micelles release CA4 in a sustained-release manner within 48 h. *In vitro* cellular uptake studies demonstrated that targeted micelles significantly facilitated the intracellular delivery of the encapsulated agents via integrin-mediated endocytosis. Anti-proliferation studies showed that targeted micelles containing CA4 present superior efficacy over nontargeted micelles.

Conclusion These results suggested that RGD conjugated PEG-PLA micelles loading CA4 have potential as a new formulation for targeting angiogenic tumor vasculature.

Yiguang Wang and Tingyuan Yang contributed equally to this work.

Y. Wang • T. Yang • X. Wang • J. Wang • X. Zhang • Q. Zhang (✉)
State Key Laboratory of Natural and Biomimetic Drugs
School of Pharmaceutical Sciences, Peking University
38 Xueyuan Road
Beijing 100083, China
e-mail: zqdodo@bjmu.edu.cn

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INTRODUCTION

Antiangiogenic therapy has become an elegant modality for the suppression of tumor growth and metastasis. Destruction of functional tumor vasculature leads to tumor starvation and regression (1–3). Combretastatin A4 (CA4) is one of the most potent antiangiogenic drugs to undergo clinical trials. CA4 is a natural product isolated from the South African tree Combretum Caffrum that has been shown to prevent tubulin polymerization and induce irreversible tumor vascular shutdown (4). CA4 has been shown to induce apoptosis in human umbilical vein endothelial cells (HUVECs), impair HUVECs migration, and disrupt the endothelial cytoskeleton (5,6). CA4 has poor water solubility; thus, its hydrophilic prodrug, CA4 disodium phosphate, is used in clinical trials (7). However, this prodrug has several undesirable side effects on many normal tissues (8). These drawbacks can be decreased by targeting the drug specifically to the tumor vasculature.

Endothelial cells in the angiogenic vessels within solid tumor uniquely express several epitopes, which can function as targets for tumor-targeted therapy (9). Integrins αvβ3 and αvβ5 are over-expressed on actively proliferating tumor endothelial cells. Integrins αvβ3 and αvβ5 can specifically recognize the peptide motif RGD (Arg-Gly-Asp) (10). Recently, various integrin-mediated nanocarriers, such as RGD-modified liposomes, nanoparticles, conjugates and so on, have shown *in vitro* and *in vivo* efficacy (11–13). Therefore, it may be possible to develop a targeted nanoscale delivery system for combretastatin A to increase its antiangiogenic activity and alleviate its adverse effects.

Polymeric micelles are nanoscale shell-core structures constructed from amphiphilic block copolymer in aqueous solution. The hydrophobic core may serve as a nanoreservoir for hydrophobic drugs, while the hydrophilic shell allows the stabilization of micelles in aqueous media. Polymeric micelles have presented special interests because this carrier has some advantages, including easiness of preparation, small and uniform particle size (10–100 nm), high stability, high drug-loading capacity, biodegradability, controllable drug release profiles, long systemic circulation time, and enhanced accumulation in tumor via the enhanced permeability and retention (EPR) effect (14). Despite high accumulation of nanoscale micelles in solid tumor as a result of EPR effect, the level of the encapsulated drugs at the intracellular and subcellular molecular targets can not be guaranteed (15,16). To achieve more specific targeting, researchers have focused on the active targeting system of polymeric micelles. Several ligands, such as antibody, folate, and peptides, modified micellar anticancer drugs, have been shown to have increased cellular uptake, enhanced cytotoxicity, and improved anticancer activity as compared with nontargeted micelles (17–19).

Here, we developed a novel targeted polymeric micellar formulation of CA4 for enhanced intracellular delivery to tumor endothelial cells. The lipophilic antivascular drug, CA4, was entrapped into RGD-modified polymeric micelles using thin film hydration method. The targeted micelles were characterized in terms of particle size, morphology, drug loading, and release kinetics. The cellular uptake was evaluated quantitatively using fluorophotometer, and visualized using confocal microscopy in integrin-overexpressing human umbilical vein endothelial cells (HUVECs). Its effect on endothelial cell proliferation was evaluated *in vitro* using sulforhodamine B (SRB) assay.

MATERIALS AND METHODS

Materials

$MPEG_{2000}$-PLA_{2000} (Mw/Mn = 1.11) and α-carboxyl-ω-hydroxyl terminated PEG-PLA (HOOC-PEG_{3000}-PLA_{2000}, Mw/Mn = 1.19) were purchased from Advanced Polymer Materials Inc. (Montreal, QC, Canada). N-hydroxysuccinimide (NHS), dicyclohexylcarbodiimide (DCC), sulforhodamine B, heparin and endothelial cell growth supplement (ECGS) were from Sigma-Aldrich (St. Louis, MO, USA). Arginine-glycine-aspartic acid tripeptide (RGD) was obtained from Calbiochem Co. (Darmstadt, Germany). Cell culture media M199, penicillin-streptomycin, fetal bovine serum, L-glutamine, and HEPES buffer were from GIBCO (USA). Fluorescent probes, including DiI and Hoechst 33258, were purchased from Molecular Probes Inc. (USA). CA4 was kindly provided as a gift by FWD Chemicals Limited (Shanghai, China). All other solvents and reagents were of analytical grade and used as received.

Cell Culture

The target vascular endothelial cells, HUVECs were isolated from umbilical cords using collagenase digestion method described by Jaffe and coworkers (20). Briefly, freshly harvested cells were cultured in endothelial cell growth medium (ECGM) consisting of M199, 10 mM HEPES, 10% fetal bovine serum (FBS), 100 μg/mL of heparin, 50 μg/mL of endothelial cell growth supplements (ECGS), and penicillin/streptomycin. The culture flasks were coated with 0.5% gelatin (Sigma-Aldrich). After 24 h, nonadherent cells were removed by changing the culture media, and fresh medium was added to the adherent cells. HUVECs were cultured to confluence and were passaged twice. Endothelial cell was confirmed by immunofluorescence analysis of factor VIII-related antigen and CD31 (data not shown). Human epithelial cervical cancer cells (Hela) were cultured in Eagle's Minimum Essential Medium (EMEM) supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin in a humidified atmosphere containing 5% CO_2 at 37°C.

Synthesis of RGD-PEG-PLA Copolymer

The synthesis of RGD-PEG-PLA conjugate followed the method in reference (21). Briefly, 500 mg of NHS-PEG-PLA was reacted with 17.5 mg of RGD in 20 mL HEPES (0.1 M, pH = 7.5) for 4 h (molar ratio of NHS-PEG-PLA: RGD = 1:0.5). The reaction was traced by high performance liquid chromatography (HPLC). The resulting reaction mixture was dialyzed against deionized water for 24 h to remove unconjugated RGD. The final solution was lyophilized for further investigation.

Solubilization of CA4 by PEG-PLA Micelles

Water solubility of CA4 was determined by shaking an excess solute in phosphate-buffered saline (PBS, pH 7.4) at 25°C for 48 h. The suspension was then filtered through 0.45 μm cellulose membrane. CA4 amount in saturated solution was determined by HPLC using a reversed-phase C_{18} column (5 μm, 4.6 mm × 250 mm, Kromasil) with a mobile phase consisting of 68% methanol and 32% water at 295 nm at the flow rate of 1 mL/min.

CA4-loaded micelles were prepared using thin film hydration method (22). Briefly, PEG-PLA copolymers and CA4 were codissolved in 4 mL of acetonitrile over a period of 4 h. Drug/polymer feed ratios by weight were varied from 1:5 to 1:20. The solution was dried under reduced

pressure. The waxy film was warmed to 60°C, and 2 mL of PBS at 60°C was added and vortexed for 5 min. The resulting micellar solution was placed at room temperature for 6 h and centrifugated at 12,000 g for 5 min to remove precipitates. CA4 content in micelles was determined by HPLC.

Preparation of CA4-Loaded RGD-Modified Micelles

RGD-modified polymeric micelles encapsulating CA4 (targeted micelles) were prepared with a blend of RGD-PEG-PLA, MPEG-PLA and CA4 using thin film hydration method. The percentage of RGD-PEG-PLA in the total polymer content (RGD-PEG-PLA + PEG-PLA) is 20%. Briefly, MPEG-PLA (64 mg), RGD-PEG-PLA (16 mg), and CA4 (8 mg) were codissolved in 8 mL of acetonitrile over a period of 4 h. The solution was dried under reduced pressure. The waxy film was warmed to 60°C, and 2 mL of PBS at 60°C was added and vortexed for 5 min. The resulting micellar solution was placed at room temperature for 6 h and centrifugated at 12,000 g for 5 min to remove precipitates.

Plain PEG-PLA micelles containing CA4 (nontargeted micelles) were also prepared by the method described above, except that the RGD-PEG-PLA conjugate was substituted by the equal weight of MPEG-PLA. To investigate the cellular uptake of micelles by the target vascular endothelial cells, fluorescently labeled PEG-PLA micelles (~0.1% loading) were prepared (23).

Characterization of Micelles

The particle size distribution of targeted micelles containing CA4 was measured by dynamic light scattering (DLS) using Malvern Zetasizer Nano ZS with 633 nm laser at an angle of 90° and at a temperature of 25°C. The polymer concentration of the analyzed sample solution was diluted to 1 mg/mL using double-distilled water. The intensity-weighted mean value was obtained from triplicate samples. The morphology of targeted micelles was measured by a JEOL 100 CX electron microscope (JEOL USA, Inc. MA). Samples (1 mg/mL) were placed on a carbon-coated copper grid and negatively stained with 1% uranyl acetate solution, then dried at room temperature overnight. The observations were performed at an acceleration voltage of 120 kV.

Evaluation of Drug Entrapment in Micelles

CA4 loading content and entrapment efficiency in polymeric micelles were determined by disintegration of micelles in methanol. The CA4 concentrations in the micellar samples were quantified by HPLC. The CA4 loading content and entrapment efficiency were calculated using the following equations:

$$\text{CA4 content (w/w)} = \frac{\text{amount of loaded CA4 in mg}}{\text{amount of added polymer and CA4 in mg}} \times 100\%$$

$$\text{Entrapment Efficiency (\%)} = \frac{\text{amount of loaded CA4 in mg}}{\text{amount of added CA4 in mg}} \times 100\%$$

In Vitro Drug Release

The release of CA4 from targeted micelles was investigated using the dialysis method. Briefly, 1 mL of micelles solution was added to 1 mL of fetal bovine serum (FBS). The mixture was placed in a dialysis bag (MWCO 10 KD) and dialyzed against 100 mL of PBS containing 10% FBS at 37°C with gentle shaking. Aliquot of buffered solution was sampled from the incubation medium at designated time points and stored frozen for analysis. Released CA4 were quantified using HPLC method described above.

Cellular Uptake Studies

Aliquot of 1.0 mL of HUVECs (1×10^5cells/well) or Hela cells (5×10^4cells/well) suspension was plated in a 24-well tissue culture plate and cultured for 24 h at 37°C until cells were grown nearly confluent. Then, the medium was replaced with 1.0 mL DiI-labeled targeted or nontargeted micelles and diluted with complete medium; the plate was maintained at either 4°C or 37°C for 3 h. The final DiI and polymer concentration in each well was 0.5 μg/mL and 0.5 mg/mL, respectively. After incubation, the cell monolayer was washed with cold PBS three times and lysed by 1 mL of DMSO. The fluorescence intensity of the DiI in DMSO was determined using a fluorophotometer (Shimadzu, RF-5301, Japan) with excitation and emission wavelengths at 550 nm and 565 nm, respectively. The cell uptake was expressed as the fluorescence intensity normalized to the total protein concentration, which was determined by Bradford method.

In competition studies, HUVEC cells or Hela cells grown as monolayers in 12-well plates were pretreated with free RGD (10 mM) for 30 min and then incubated with DiI-loaded targeted and nontargeted micelles. The cellular uptake of nanocarriers was determined fluorometrically.

For confocal microscopy analysis, HUVECs were cultured into sterile 35 mm glass-base dishes at a cell density of 2.5×10^5 cells/well. The cells were incubated for 24 h to allow for adhesion. Then, the cells were incubated with DiI-loaded nontargeted or targeted micelles as described

above. After 3 h incubation, cells were washed with cold PBS three times. Then, the cells were fixed with 4% formaldehyde in PBS at room temperature for 10 min, followed by cell nuclei staining with Hoechst 33258 for 5 min. Cells were examined by a Leica SP2 confocal microscope (Heidelberg, Germany). DiI and Hoechst 33258 were excited at 543 nm and 352 nm, respectively. The emission wavelengths of DiI and Hoechst 33258 are 565 nm and 460 nm, respectively.

In Vitro Efficacy of CA4-Loaded Micelles

HUVECs were plated onto gelatin-coated 96-well plates at a density of 10,000 cells/well and were incubated in endothelial cell complete medium at 37°C, 5% CO_2/95% air to allow cell growth for 24 h. Then, the cells were exposed to increasing concentrations of CA4-loaded nontargeted micelles and targeted micelles for 2 h and washed three times with PBS, and the fresh medium was added into plates. The cells were incubated for 48 h before determination of cell viability. The viability of cells was measured using SRB method, as previously described (24). Briefly, after drug exposure, the cells were fixed with 10% trichloracetic acid and stained with 0.4% SRB solution for 30 min, after which the excess dye is removed by washing repeatedly with 1% acetic acid. The bound dye is redissolved in 10 mM Tris base solution for OD determination at 540 nm using a microplate reader. Dose-response curves were generated, and the concentrations of drug result in 50% cell-killing (IC_{50}) were calculated.

Statistical Analysis

All data were reported as means ± standard deviation (SD). Student's t-test was used for all statistical analysis between two groups. Multiple comparisons were performed using ANOVA. A p-value less than 0.05 was considered as statistically significant, a p-value less than 0.01 was considered as highly significant. All statistical tests were two-sided.

RESULTS AND DISCUSSION

Preparation and Characterization of Micelles

To develop CA4-loaded targeted micelles, we synthesized RGD-PEG-PLA conjugate. The conjugation efficiency of RGD to NHS-PEG-PLA determined by HPLC was 98.2% (based on RGD peptide). The characterization of RGD-PEG-PLA has been published by our group (21).

The antivascular drug CA4 is a lipophilic stilbene, and the measured water solubility of CA4 was 11.8±1.2 μg/mL. Therefore, we utilized the hydrophobicity to encapsulate the drug with high efficiency within the core of PEG-PLA micelles.

To obtain stable drug formulations, the loading and entrapment efficiency of CA4 in $MPEG_{2000}$-PLA_{2000}, $MPEG_{3000}$-PLA_{3000} and $MPEG_{5000}$-PLA_{5000} polymeric micelles was evaluated by varying the CA4/Polymer feed ratio by weight. The results are presented in Table I. As shown in Table I, the maximum CA4 loading efficiencies in three PEG-PLA micelles were obtained with CA4/Polymer ratios of 1:10. The maximal CA4 entrapment efficiency in $MPEG_{2000}$-PLA_{2000} micelles (98.14%) was much higher than in the other two PEG-PLA micelles. Moreover, CA4-loaded $MPEG_{2000}$-PLA_{2000} micelle at CA4/Polymer feed ratio of 1:10 was much more stable than other formulations. Thus, $MPEG_{2000}$-PLA_{2000} micelle was selected as carrier for further investigation.

After preparation of CA4-loaded targeted micelles, DLS analysis was performed to determine the particle size and distribution of targeted micelles and nontargeted micelles (Fig. 1A). The prepared targeted and nontargeted micelles were 25.9±1.3 nm and 27.0±1.2 nm with a unimodal distribution, respectively. These results indicated that the incorporation of RGD-PEG-PLA conjugates into MPEG-PLA micelles did not change the particle size of micelles. We also characterized the morphology of targeted micelles by transmission electron microscopy (Fig. 1B). The images showed that the micelles are a uniform spherical structure, and the diameter is about 20–30 nm.

Table I Characteristics of CA4-Loaded PEG-PLA Micelles ($n = 3$)

MPEG-PLA Copolymer	CA4/Polymer (w/w)	Entrapment efficiency (%)	CA4 loading (wt %)	Particle size (nm)	Polydispersity (PDI)
$MPEG_{2000}$-PLA_{2000}	1:5	77.44±9.50	13.41±1.65	29.34±0.55	0.175
	1:10	98.14±1.03	8.94±0.10	27.34±0.80	0.068
	1:20	94.88±1.60	4.53±0.08	27.09±1.01	0.054
$MPEG_{3000}$-PLA_{3000}	1:5	32.27±7.32	6.06±1.37	65.85±3.46	0.24
	1:10	95.85±3.72	8.75±0.34	64.05±5.26	0.162
	1:20	92.08±1.82	3.03±0.06	54.87±8.21	0.207
$MPEG_{5000}$-PLA_{5000}	1:5	15.26±2.44	2.96±0.47	64.16±4.97	0.195
	1:10	63.31±12.72	5.95±1.20	80.89±9.52	0.231
	1:20	54.17±11.56	2.64±0.56	70.14±8.68	0.202

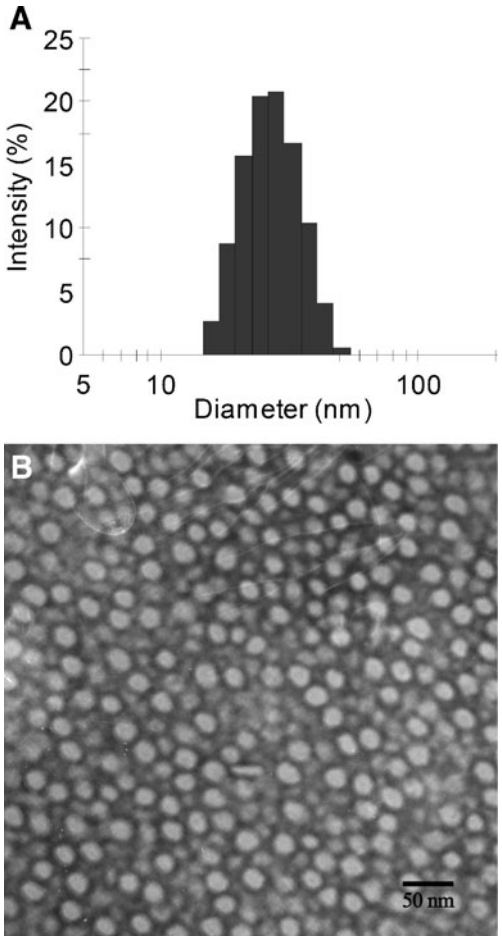

Fig. 1 Characterization of RGD conjugated PEG-PLA micellar combretastatin A4. **A** Representative particle size distribution analysis of RGD conjugated PEG-PLA micellar combretastatin A4 by dynamic light scattering. **B** A typical TEM image of RGD conjugated PEG-PLA micellar combretastatin A4 stained with 1% uranyl acetate. The concentration of targeted micelle solution is 1 mg/mL. Scale bar is 50 nm.

Using the same self-assembly method, CA4 was encapsulated into targeted micelles and nontargeted micelles. The level of encapsulated CA4 was measured by HPLC after destroying the micellar structure with methanol. Table II shows the results of CA4 loading using MPEG-PLA block copolymer or the mixture of MPEG-PLA and RGD-PEG-PLA at a weight ratio of 4:1. It was found that 40 mg/mL MPEG-PLA copolymer solubilized 3.92 mg/mL of CA4 when the initial weight ratio of drug to polymer was 1:10. The solubilization capacity of mixed micelles of MPEG-PLA and RGD-PEG-PLA at a weight ratio of 64 mg: 16 mg was further evaluated. Results indicated that 40 mg/mL mixed copolymer present a comparable solubilization capacity (3.89 mg/mL) as compared with MPEG-PLA copolymer. As shown in Table II, the CA4 loading content and entrapment efficiency of targeted micelles and nontargeted micelles was comparable (8.9% *vs* 8.8% for loading content; 97.9% *vs* 97.2% for entrapment efficiency). These results indicated that PEG-PLA polymeric micelles can solubilize the antivascular agent, CA4 to about 4 mg/mL in aqueous solution regardless of the addition of RGD-PEG-PLA conjugates.

Table II Characteristics of Prepared Polymeric Micelles ($n = 3$)

Preparations	Particle size (nm)	Polydispersity (PDI)	Loading content (w/w)	Entrapment efficiency (%)
Nontargeted micelles	27.0 ± 1.2	0.082	8.9 ± 0.1	97.9 ± 0.8
Targeted micelles	25.9 ± 1.3	0.099	8.8 ± 0.1	97.2 ± 1.4

In previous studies, surfactants or lipid vesicles have been used as the recipient phase in evaluating the *in-vitro* release kinetics of drugs from nanocarriers (25,26). However, these release media can not mimic the circulation systemic condition. Instead, fetal bovine serum (FBS) was used as a biomimetic recipient phase to maintain the sink condition for the release of CA4 from polymeric micelles. In addition, PBS (pH = 7.4) containing 10% FBS can also mimic the conditions employed for the cell culture. Fig. 2 includes the 48-h release profile for CA4 from targeted micelles and nontargeted micelles. The amount of cumulative drug release within 2 h was 13.6% and 12.0% for targeted micelles and nontargeted micelles, respectively. After 48 h, 92.7% and 91.2% of total drug were released from targeted micelles and nontargeted micelles, respectively. These results indicated that PEG-PLA micelles could effectively encapsulate CA4 into micelles and release drug with a sustained-release manner. We can predict that CA4 in micelles would be taken up into cells in the form of nanoparticles rather than as the free drug during the first 2 h incubation.

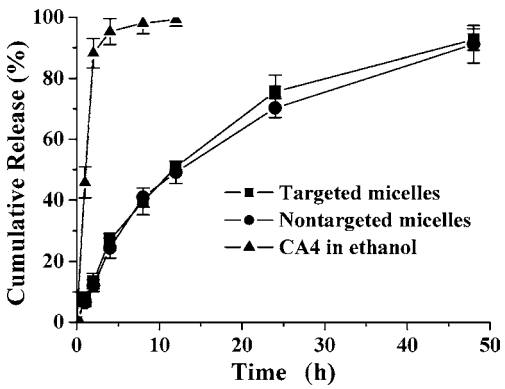

Fig. 2 *In vitro* release of combretastatin A4 from RGD conjugated PEG-PLA micelles (-■-, targeted micelles), MPEG-PLA micelles (-●-, nontargeted micelles) and CA4-ethanol solution (-▲-, CA4 in ethanol). Each point represents the mean ± SD of three samples.

Cellular Uptake in HUVECs

The cellular uptake of nontargeted micelles and targeted micelles was evaluated in human umbilical vein endothelial cells (HUVECs) and MCF-7 cells. HUVECs overexpress integrins αvβ3 and αvβ5 and have been previously used as an *in-vitro* tumor vascular endothelial cells model (11). Hela cells (integrin αvβ3 negative and integrin αvβ5 weakly positive) were used as a control cell line (27,28).

In order to investigate the micelle uptake by receptor-bearing cells, HUVECs and Hela cells were incubated with nontargeted and targeted micelles containing 0.5 mg/mL polymer and 0.5 μg/mL DiI at 4 and 37°C. Incubation at 4 and 37°C were used to distinguish between cell binding and internalization. Receptor-mediated internalization is expected to be inhibited at 4°C because of the suppression of energy-dependent uptake at this temperature, while both cell binding and internalization are expected to take place at 37°C (29). As shown in Fig. 3, cell uptake of targeted micelles by HUVECs at 37°C was significantly greater than that at 4°C (2,085±187 *vs* 1,199±174, $P<0.01$), and cell uptake of targeted micelles was significantly higher than that of nontargeted micelles at either 4 or 37°C (1,199±174 *vs* 468±169 at 4°C, $P<0.01$; 2,085±187 *vs* 747±11 at 37°C, $P<0.01$). However, there was no significant difference for the mean fluorescence intensities of Hela cells exposed to either targeted micelles or nontargeted micelles (398±38 *vs* 345±47 at 4°C, $P>0.05$; 765±108 *vs* 725±85 at 37°C, $P>0.05$).

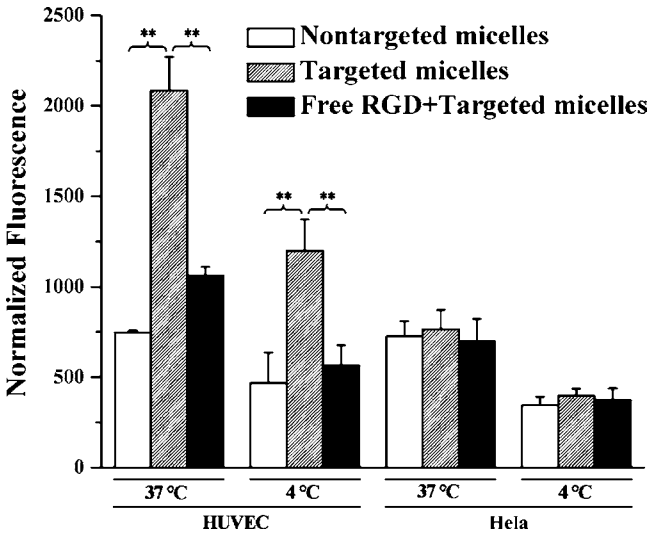

Fig. 3 Uptake of targeted micelles and nontargeted micelles by HUVECs and Hela cells. HUVECs or Hela cells were incubated with various micelle formulations containing 0.5 mg/mL polymer and 0.5 μg/mL fluorescent probes, DiI at 4°C and 37°C. In competition study, HUVECs or Hela cells were pretreated with free RGD (10 mM) for 30 min and the cellular uptake of micelles was evaluated. Error bars represent standard deviation ($n=3$). $^{**}P<0.01$.

To evaluate the role of RGD peptide in the cellular uptake of targeted micelles, a competitive binding assay was performed. For this experiment, HUVECs and Hela cells were pretreated with 10 mM free RGD in complete media to block the integrin. As shown in Fig. 3, 10 mM free RGD significantly reduced the micelles uptake by HUVECs incubated with targeted micelles at either 4 or 37°C (1,199±174 *vs* 565±111 at 4°C, $P<0.01$; 2,085±187 *vs* 1,063±47 at 37°C, $P<0.01$), whereas the uptake of targeted micelles by Hela cells was not inhibited by free RGD at either 4 or 37°C (398±38 *vs* 373±64 at 4°C, $P>0.05$; 765±108 *vs* 698±124 at 37°C, $P>0.05$). These results validated that free RGD peptide competitively inhibited the binding and uptake of targeted micelles to integrins-positive HUVECs, but not integrins-negative Hela cells.

The uptake of the targeted micelles by HUVECs was also observed using confocal microscopy. As shown in Fig. 4, after incubation at 37°C for 3 h, significantly increased intracellular DiI fluorescence intensity was observed with targeted micelles (Fig. 4B), whereas HUVEC cells incubated with nontargeted micelles had weak intracellular fluorescence (Fig. 4A). Moreover, the uptake of targeted micelles was significantly inhibited by free RGD peptide (Fig. 4D). This result was consistent with the quantitative result of cellular uptake by fluorometric method, indicating again that the targeted micelles enhanced the cellular uptake of encapsulated agent. Previous studies reported that encapsulated DiI in micelles were internalized into the cytoplasm as nanoparticles (16,23). Confocal images of nontargeted micelles and targeted micelles showed granular fluorescence, indicating the presence of internalized micelles in the endosomes. In addition, targeted micelles showed a significantly larger amount of speckled fluorescence in cytoplasm as compared with nontargeted micelles, suggesting that receptor-mediated endocytosis was involved in cell uptake of targeted micelles.

Overall, these results demonstrate that integrin is responsible for the binding and uptake of targeted micelles in angiogenesis endothelial cells, and the cellular uptake was governed by a receptor-mediated process.

Anti-proliferation Assay

To compare anti-proliferation activity of CA4-loaded nontargeted micelles and targeted micelles, HUVECs were exposed to a series of equivalent concentrations of CA4-loaded nontargeted micelles or targeted micelles for 48 h. The percentage of viable cells was determined using the SRB assay. The concentration of CA4 in targeted micelles that induced 50% killing was much lower than that of nontargeted micelles ($IC_{50}=0.52\pm0.11$ μM *vs* 1.13±0.18 μM, $P<0.05$) (Fig. 5 and Table III). The IC_{50} of free

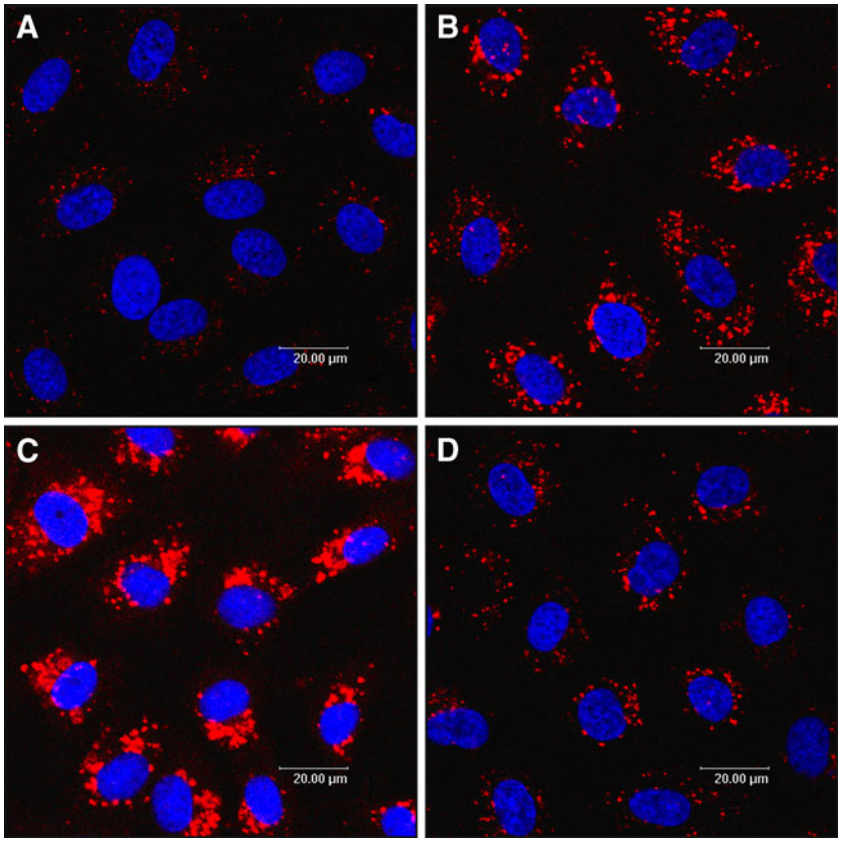

Fig. 4 Confocal microscopy images of HUVECs treated with (**A**) nontargeted micelles, (**B**) targeted micelles, (**C**) free DiI, and (**D**) targeted micelles (pretreated with 10 mM RGD for 30 min), for 3 h at 37°C. Each formulation has DiI concentration of 0.8 μM. Cells were fixed with 4% formaldehyde and incubated with Hoechst 33258 for nuclei staining. Red represents fluorescence of DiI. Blue represents fluorescence of Hoechst 33258.

CA4 on HUVECs is 0.25±0.07 μM. The difference in IC_{50} between free CA4 and micellar CA4 may be due to the hydrophilic surface of micellar CA4, which results in reduced cellular uptake of drug. But this advantage of free CA4 can not be achieved *in vivo* because of the broad distribution in normal tissue. Thus, these results indicate that conjugation of RGD on to the surface of micelles plays a pivotal role in the enhancement of anti-proliferation of CA4 on endothelial cells.

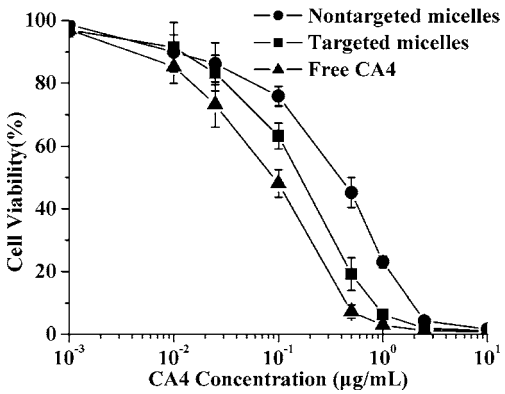

Fig. 5 The viability of HUVECs exposed to CA4-loaded targeted micelles (-■-), nontargeted micelles (-●-) and Free CA4 (-▲-). Error bars represent standard deviation ($n=3$).

CONCLUSIONS

RGD-conjugated PEG-PLA micelles encapsulating CA4 were successfully prepared and characterized. It was concluded that RGD-targeted micelles significantly enhanced the cell uptake of encapsulated drug in angiogenic tumor endothelial cells via integrin-mediated endocytosis process, which also resulted in increased anti-proliferation activity of antivascular agent. These results suggest that RGD-conjugated PEG-PLA micelles encapsulating CA4 have potential as a new formulation for targeting angiogenic tumor vasculature.

Table III Anti-proliferation Effect (IC_{50}) of CA4 Formulations on HUVECs After 48 h Treatment ($n=3$)

Formulation	IC_{50} (μM)
Free CA4	0.25±0.07
Nontargeted micelles	1.13±0.18
Targeted micelles	0.52±0.11* **

*$P<0.05$, *vs* Free CA4

**$P<0.05$, *vs* nontargeted micelles

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