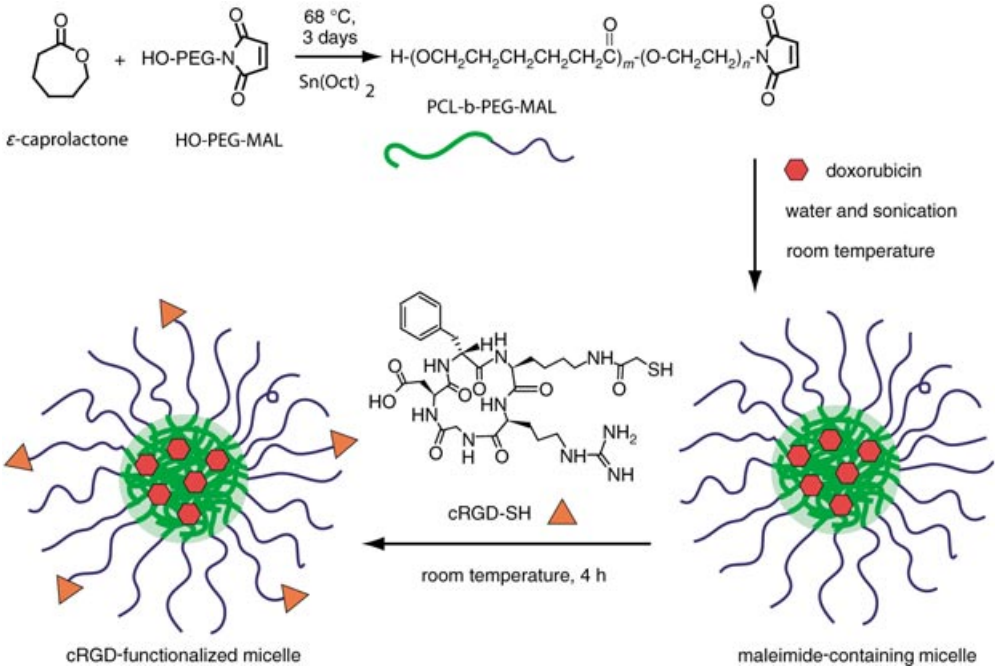
Bioorganic Chemistry

cRGD-Functionalized Polymer Micelles for Targeted Doxorubicin Delivery**

*Norased Nasongkla, Xintao Shuai, Hua Ai, Brent D. Weinberg, John Pink, David A. Boothman, and Jinming Gao**

Low water solubility, rapid phagocytic and renal clearance, and systemic toxicity represent three major barriers that limit the therapeutic use of many hydrophobic antitumor agents such as doxorubicin (DOXO) and paclitaxel.[1] Various drug-delivery systems, among which polymeric micelles have emerged as a very important system, have been developed to overcome these limitations and deliver various drugs with remarkable in vitro and in vivo success.[2,3] Polymeric micelles are nanoscopic (10 to 100 nm) colloidal particles self-assembled from amphiphilic block or graft copolymers in aqueous media. The hydrophobic core of the micelles is a carrier compartment that accommodates antitumor drugs, and the shell consists of a brushlike protective corona that stabilizes the nanoparticles in aqueous solution.[4] The basic requirements for polymeric micelles in drug-delivery applications include high drug-loading capacity, biodegradability, long blood circulation times, and controllable drug-release profiles. Research on micelles has been greatly advanced in the past decade.[5–8] However, the ability to achieve high targeting efficiency at the tumor site and associated cells remains a significant challenge for the development of micelle-mediated drug-delivery systems. Although nanosized micelles are known to spontaneously accumulate in tumors with leaky vasculature by an enhanced permeability and retention (EPR) effect,[9] micelles are also observed to accumulate quite significantly in reticuloendothelial sites such as the liver, spleen, and kidney.[10] Consequently, insufficient uptake in tumor sites will decrease the therapeutic effect of the administered drug dose, and nonspecific spreading to healthy tissues will lead to serious side effects and limit the dosage that can be applied. These limitations prevent these drugs from achieving the potential cures that they might otherwise attain.[11]

One strategy to achieve cancer-targeted drug delivery is the utilization of unique molecular markers that are specifically overexpressed in the cancerous tissues. It is well known that tumor endothelial cells show increased expression of



Scheme 1. Synthesis of MAL-PEG-PCL copolymer and preparation of cRGD-functionalized, DOXO-loaded micelles.

[*] N. Nasongkla,[+] Dr. X. Shuai,[+] Dr. H. Ai, B. D. Weinberg, Prof. Dr. J. Gao
Department of Biomedical Engineering
Case Western Reserve University, 10900 Euclid Avenue
Cleveland, Ohio 44106 (USA)
Tel: (+1) 216-368-1083
Fax: (+1) 216-368-4969
E-mail: jinming.gao@case.edu
Dr. J. Pink, Prof. D. A. Boothman
Department of Radiation Oncology
Case Western Reserve University, 10900 Euclid Avenue
Cleveland, Ohio 44106 (USA)
[+] These authors contributed equally to this work

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several cell-surface molecules that potentiate cell invasion and proliferation during tumor vascular remodeling and angiogenesis.[12,13] One such molecule is the $\alpha_v\beta_3$ integrin, which plays a key role in endothelial cell survival during angiogenesis.[14] Enlightened by the early discovery that viruses, such as rotavirus and adenovirus, can utilize this receptor to facilitate gene transfer by selective recognition between $\alpha_v\beta_3$ on the targeted cell membrane and the viral surface, researchers have exploited ligands such as the Arg-Gly-Asp (RGD) peptide. In fact, $\alpha_v\beta_3$ was recently used as an endothelial cell target in several therapeutic approaches such as nonviral gene delivery.[15,16]

Unfortunately, ligand-directed delivery of hydrophobic drugs with polymeric micelles has been reported only in a few cases.[17,18] Herein, we develop polymeric micelles that can selectively deliver hydrophobic drugs, such as doxorubicin, to angiogenic tumor endothelial cells with over-expressed $\alpha_v\beta_3$ integrins. To this end, we attached a cyclic pentapeptide c(Arg-Gly-Asp-D-Phe-Lys) (cRGDfK, also referred to as cRGD herein) as an $\alpha_v\beta_3$ ligand to the surface of doxorubicin-loaded poly(ε-caprolactone)-poly(ethylene glycol) (PCL-PEG) micelles. cRGDfK was selected as the targeting ligand since it can selectively bind to the $\alpha_v\beta_3$ integrin with high affinity.[19,20] Although the coupling of doxorubicin to RGD peptides (for example, RGD-4C) was found to be able to target tumor blood vessels,[21] RGD-directed doxorubicin delivery using polymeric micelles has not been reported to date.

The synthesis of the maleimide-terminated block copolymer (MAL-PEG-PCL, $M_n = 5.5$ kD) and preparation of the cRGDfK-functionalized micelles with DOXO loading are outlined in Scheme 1. In contrast to the reported procedure for the polymerization of ε-caprolactone with stannous(II) octoate as a catalyst,[22] the synthesis of MAL-PEG-PCL must be conducted at a lower temperature because of the thermal susceptibility of the maleimide end groups. We found that reaction at 68 °C led to PCL segments of the desired molecular weights (for example, 2.4 kD), while reducing the thermal decomposition of maleimide to a negligible level (see the Supporting Information). DOXO-loaded MAL-PEG-PCL micelles were prepared by a solvent-evaporation method.[23] Different amounts of methoxy-terminated MPEG-PCL copolymer were also introduced to control the density of maleimide at the micelle surface, which subsequently controls the cRGD density (5, 16, and 76% of all PEG chains). The typical DOXO loading content (DLC) in the micelle preparations was 3.10 wt%.

Figure 1a shows the ^{1}H NMR spectrum of MAL-PEG-PCL copolymer in $CDCl_3$. Resonances of the PEG methylene protons (mainly at $\delta = 3.64$ ppm) and PCL protons ($\delta = 1.38$, 1.65, 2.31, and 4.06 ppm) were observed. The small triplet at $\delta = 4.2$ ppm was attributed to the proton resonance of the methyleneoxyl group linking the PCL and PEG blocks. The intensity of the integrals for the maleimide vinylic protons at $\delta = 6.74$ ppm confirms that the maleimide group in MAL-PEG-PCL copolymers remained intact, as in the MAL-PEG-OH. These data strongly demonstrated that the desired block copolymers were successfully synthesized. The number-averaged molecular weight of PCL blocks was calculated to be

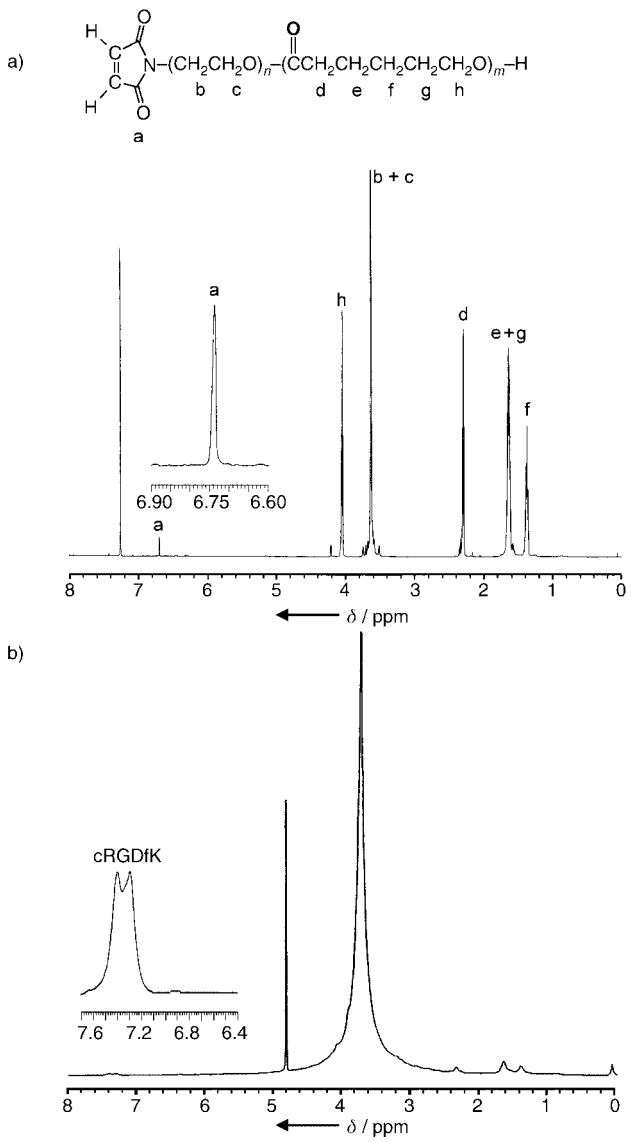

Figure 1. ^{1}H NMR spectra (600 MHz, 25 °C) of a) MAL-PEG-PCL copolymer in $CDCl_3$ and b) cRGD-functionalized, DOXO-loaded micelles in D_2O. The inset in (a) shows the proton signal from maleimide groups ($\delta = 6.74$ ppm), and the inset in (b) shows the absence of the maleimide signal and the presence of aromatic protons from the Phe residue in cRGDfK, thus indicating a complete conversion of the maleimide group after cRGD conjugation.

2.4 kD from the integral of the PCL protons at $\delta = 2.31$ ppm versus that of the PEG proton at $\delta = 3.64$ ppm. A postmicellar modification strategy was used to prepare cRGD-functionalized micelles (Scheme 1). The NMR spectrum of the freeze-dried micelles in D_2O strongly suggests the formation of the core-shell structure of DOXO-loaded micelles (Figure 1b). The micelle corona (shells) consisting of PEG blocks were solvated to a high degree in D_2O and showed clear ^{1}H NMR signals. In contrast, DOXO was loaded inside the solid PCL cores of micelles, and thus the resonances of both the PCL blocks and DOXO molecules were significantly reduced because of their insufficient mobility in D_2O. Moreover, successful conjugation of cRGDfK onto the solvated PEG

shells was verified by the resonances of the phenyl protons of cRGDfK at $\delta = 7.4$ ppm and complete disappearance of the maleimide signal at $\delta = 6.74$ ppm (Figure 1b).

It is well known that the size of nanocarriers plays an important role in cellular internalization process. Before evaluating the cellular uptake of non- and cRGD-functionalized micelles, we studied the size and morphology of the micelles by atomic force microscopy (AFM) and dynamic light scattering (DLS). AFM studies showed that micelles with 76% cRGD attachment had a mean size (43.2 ± 3.9 nm) similar to that of cRGD-free micelles (37.5 ± 2.6 nm). Both unfunctionalized and 76% cRGD-containing micelles appeared to be discrete and round-shaped nanoparticles. Aggregation of micelle particles was not observed. The DLS measurements showed that the size of the cRGD-functionalized micelles was also close to that of the unfunctionalized micelles (20.9 ± 1.7 and 24.4 ± 2.7 nm for non- and cRGD-functionalized micelles, respectively; Figure 2). Two major factors may have contributed to the larger diameter of the micelle determined by AFM measurement. First, the height of the micelle particles was approximately 5 nm by AFM, which is significantly smaller than the diameter of the micelle determined by DLS (20–25 nm). The decreased height in the solid state indicates that particle-flattening events occur during the sample dewetting process on the mica surface, which can increase the apparent particle diameter in the x,y plane for AFM measurement. Second, it is well known that AFM can give an overestimation of particle size as a result of the AFM tip-broadening effect.[24] The magnitude of the tip dilation effect depends on the height of the object, the ambient humidity, and the size and shape of the AFM tip.[25,26] The morphology reconstruction method can potentially be applied to correct the dilation effect and provide a more accurate determination of the particle sizes.[26]

Finally, we used flow cytometry and confocal laser scanning microscopy to study the uptake of micelles into SLK tumor endothelial cells (derived from human Kaposi's sarcoma) that over-express the $\alpha_v\beta_3$ integrin.[27] We quantified the cellular internalization efficiency of the cRGD-micelles by measuring the increase in fluorescence intensity after the DOXO-loaded micelles had been transported into the cells. Figure 3a shows the percentage of SLK cells with micelle uptake as a function of cRGD density on the polymer micelles after 2 h incubation. We observed a remarkable increase in the uptake of micelles in the cells upon attachment of cRGD molecules to the micelle surface. A higher density of cRGD molecules led to a higher level of cellular internalization of these micelles over the entire cRGD density range (0–76%) examined. A maximum 30-fold enhancement was achieved with 76% cRGD-functionalized DOXO-loaded micelles relative to unfunctionalized DOXO-loaded micelles. It is noteworthy that Kissel and co-workers recently reported that linear RGD molecules attached to DNA/PEI-PEG (PEI = polyethyleneimine) nanocomplexes through PEG spacers did

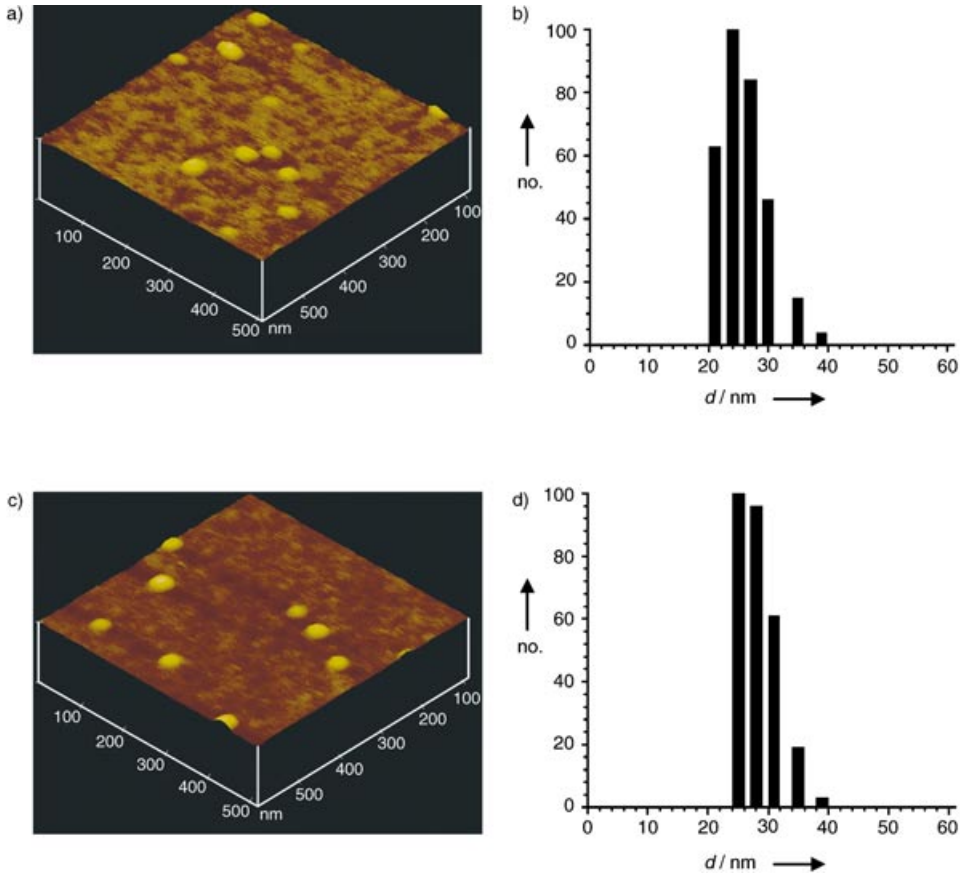

Figure 2. Size characterization of 0% (a, b) and 76% (c, d) cRGD-functionalized DOXO-loaded micelles by atomic force microscopy (a, c) and dynamic light scattering (b, d).

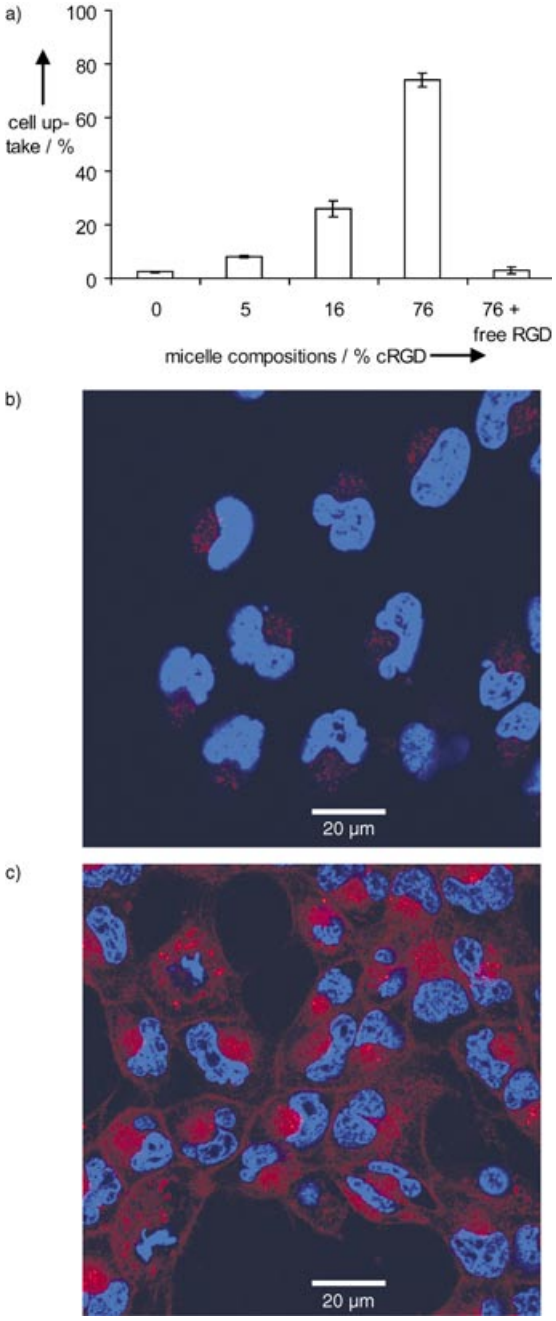

Figure 3. a) Percentage of micelle uptake in SLK tumor endothelial cells measured by flow cytometry as a function of cRGD density (0–76%) on the micelle surface. The last bar shows that the cell uptake of 76% cRGD-functionalized micelles is inhibited by the presence of free RGD ligands (9 mM) in solution. b, c) Confocal laser scanning microscopy images of SLK cells treated with 0% (b) and 16% (c) cRGD-functionalized micelles after incubation for 2 h. Cell nuclei were stained blue by Hoechst 33342 ($\lambda_{ex}=352$ nm, $\lambda_{em}=455$ nm) and overlaid with DOXO fluorescent images ($\lambda_{ex}=485$ nm, $\lambda_{em}=595$ nm).

not lead to effective targeting to $\alpha_v\beta_3$-expressing cells.[28] We believe the higher affinity of cyclic RGDfK to $\alpha_v\beta_3$ over the linear RGD peptides (>200 times)[19] in our current system has primarily contributed to the higher targeting efficiency by our cRGD micelles.

This cRGD-directed micelle targeting to $\alpha_v\beta_3$-overexpressed tumor endothelial cells was further demonstrated in a control experiment, in which SLK cells were first incubated with a free AARGDY blocking ligand (9 mM), and then co-incubated with 76% cRGD-functionalized micelles (see the Supporting Information). The cellular uptake level of cRGD-functionalized micelles in the presence of blocking ligand was dramatically reduced and became essentially equivalent to that of unfunctionalized micelles (Figure 3a). These data demonstrate that the $\alpha_v\beta_3$ receptor is essential for the uptake of cRGD-functionalized micelles in SLK tumor endothelial cells.

Confocal laser scanning microscopy was then used to characterize and compare the cell uptake and intracellular distribution of cRGD-free and 16% cRGD-functionalized DOXO micelles after incubation for two hours (Figure 3b,c). Significantly increased intracellular DOXO fluorescence intensity was observed with cRGD-functionalized micelles, thus demonstrating again the enhanced cellular uptake by the cRGD-$\alpha_v\beta_3$-mediated endocytosis as shown by flow cytometry. Similar to cRGD-free micelles, cRGD-functionalized micelles were localized in the cytoplasm rather than the nuclei where free DOXO accumulated quickly after membrane diffusion.[8,23] Recently, Maysinger and co-workers detected intracellular localization of modified PCL-PEG micelles (20 to 45 nm) in several intracellular organelles including mitochondria, Golgi apparatus, and acidic organelles such as lysosomes in PC12 and NIH 3T3 cells. They suggested the multiple cytoplasmic-targeting of modified PCL-PEG micelles.[4] On the basis of the $\alpha_v\beta_3$ receptor mediated endocytosis pathway of the cRGD-functionalized micelles, we suggest that in our system the cRGD-functionalized micelles were more likely entrapped in the endosomal compartments. Indeed, as shown in Figure 3b, a large amount of dot-shaped DOXO fluorescence was observed in the cytoplasm of treated cells, which suggests the presence of internalized micelles in the endosomes.[8]

In summary, a very effective $\alpha_v\beta_3$ ligand (cRGDfK) was successfully conjugated to DOXO-loaded PEG-PCL micelles by using a postmicelle modification method. Attachment of the cyclic RGD ligand greatly enhanced internalization of the micelles in tumor endothelial cells that overexpress $\alpha_v\beta_3$ integrins, apparently through receptor-mediated endocytosis. Although preliminary, our results illustrate the tremendous potential of cRGD-functionalized micelles for targeting the tumor neovasculature. To verify this hypothesis, we are conducting in vitro cytotoxicity tests and animal studies with cRGD-functionalized, DOXO-loaded micelles.

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