molecular pharmaceutics

Article

pubs.acs.org/molecularpharmaceutics

Retro-Inverso Isomer of Angiopep-2: A Stable D-Peptide Ligand Inspires Brain-Targeted Drug Delivery

Xiaoli Wei,[†,‡] Changyou Zhan,*[,†,‡,§] Xishan Chen,[†,‡] Jiapeng Hou,[†,‡] Cao Xie,[†,‡] and Weiyue Lu*[,†,‡,∥,⊥]

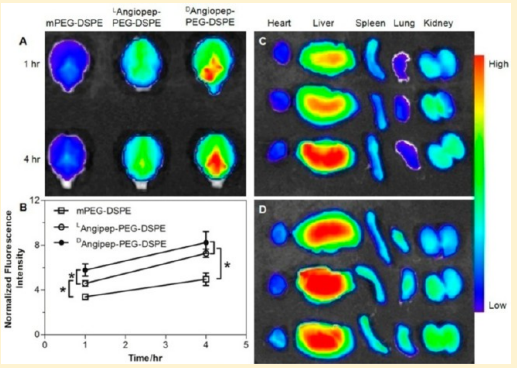
[†]Department of Pharmaceutics, School of Pharmacy, Fudan University, Shanghai, 201203, P. R. China

[‡]Key Laboratory of Smart Drug Delivery (Fudan University), Ministry of Education, Shanghai, 201203, P. R. China

[∥]State Key Laboratory of Molecular Engineering of Polymers, Fudan University, Shanghai, 200433, P. R. China

[⊥]State Key Laboratory of Medical Neurology, Fudan University, Shanghai, 200032, P. R. China

ABSTRACT: The blood–brain barrier (BBB) prevents most drugs from reaching the site of central nervous system (CNS) diseases, intensively confining the therapeutic efficiency. Angiopep-2 (here termed LAngiopep), which is a 19-mer peptide derived from human Kunitz domain, can trigger transcytosis and traverse the BBB by recognizing low density lipoprotein-related protein 1 (LRP-1) expressed on the brain capillary endothelial cells. Various enzymes in the blood and the BBB, however, present multiple metabolic barriers to peptide-inspired brain-targeted drug delivery. Here we designed a retro-inverso isomer of LAngiopep, termed DAngiopep, to inspire brain-targeted drug delivery. Both DAngiopep and LAngiopep displayed high uptake capacity in LRP-1 overexpressed cells, including bEnd.3 and U87 cells. DAngiopep demonstrated lower uptake efficiency in both cell lines than did LAngiopep, suggestive of lower binding affinity to LRP-1 of the D-peptide. DAngiopep was resistant to proteolysis in fresh rat blood serum, while more than 85% of LAngiopep disappeared within 2 h. Endocytosed DAngiopep and LAngiopep were found to be colocalized with lysosomal compartments of bEnd.3 cells, indicating that susceptibility to proteolysis of LAngiopep in the BBB may further attenuate its transcytosis efficiency. *In vivo*, DAngiopep modified PEG-DSPE micelles displayed high distribution in normal brain and intracranial glioblastoma. Due to the expression of LRP-1 on the BBB and glioblastoma cells, proteolytically stable DAngiopep holds much potential for designing two-order brain tumor targeted delivery systems.



KEYWORDS: *retro-inverso isomer, Angiopep-2, brain-targeted drug delivery, PEG-DSPE micelle, glioblastoma*

1. INTRODUCTION

The blood–brain barrier (BBB) is a key challenge in the development of drugs for diseases of the central nervous system (CNS). It prevents drugs and drug delivery systems from reaching the site of disease due to tight junction and lack of fenestration.[1,2] The design of brain-targeted drug delivery system has attracted extensive attention. Receptor-mediated transcytosis has been emerging as a successful method to traverse the BBB and increase brain transport.[3] A variety of receptors expressed on the BBB, including transferrin receptors,[4] low density lipoprotein-related protein 1 (LRP-1),[5,6] and nicotine acetylcholine receptors,[7−9] have been utilized to facilitate receptor-mediated transcytosis. Blood-borne ligands target luminal receptors and trigger receptor-mediated transcytosis. When modified on the surface of nanocarriers, those ligands inspire brain-targeted drug delivery and possess high potential for the diagnosis and therapy of CNS diseases. Peptide-based ligands identified by using structure-based design[10] and phage display[11,12] are capable of targeting the corresponding receptors with high affinity and specificity. In addition, peptide-based ligands have been widely used to facilitate brain-targeted drug delivery for the ease of functionalization.

Angiopep-2, which is a 19-mer peptide derived from human Kunitz domain, possesses high potential for inspiring brain-targeted drug delivery.[13] When conjugated with paclitaxel[14,15] or modified on the surface of nanocarriers,[16−19] Angiopep-2 can significantly increase the brain distribution of conjugates. Angiopep-2 transport and accumulation in brain endothelial cells can be blocked by α_2-macroglobulin, a specific ligand for LRP-1, indicating that Angiopep-2 facilitates the brain-targeted drug delivery through LRP-1-mediated transcytosis.[10] In addition, Angiopep-2 is promising for targeting glioblastoma due to overexpression of LRP-1 in glioblastoma cells. Angiopep-2 modified nanoparticles have been proposed to realize dual targeting for the chemotherapy of glioblastoma,

Special Issue: Recent Molecular Pharmaceutical Development in China

Received: January 28, 2014
Revised: March 5, 2014
Accepted: March 18, 2014

dx.doi.org/10.1021/mp500086e | *Mol. Pharmaceutics* XXXX, XXX, XXX−XXX

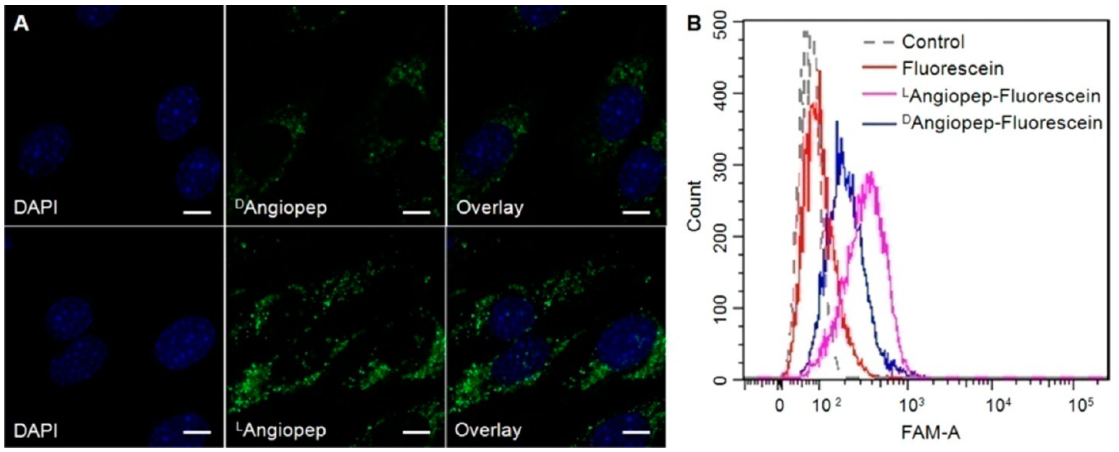


Figure 1. Cellular uptake of ^{D}Angiopep and ^{L}Angiopep by bEnd.3 cells. Cells were incubated with 5 μM fluorescein labeled peptide at 37 °C for 4 h, followed by DAPI staining and rinse with phosphate buffered saline. Intracellular fluorescence was detected by confocal laser scanning microscope (A) and flow cytometer (B). (Scale bar = 10 μm.)

wherein Angiopep-2 traversed the BBB and further targeted tumor cells.[20,21] However, the presence of multiple metabolic barriers may restrict the application of such peptide-based ligand for targeted drug delivery *in vivo*. Peptide ligand in conjugates or on the surface of nanocarriers is subject to proteolysis in the blood after systemic administration.[22] In addition, the BBB has been reported as a metabolic barrier due to the presence of various enzymes in brain capillary endothelial cells.[23] If the transcytosis of Angiopep-2 involves enzymatic organelles, degradation in the BBB may further deactivate the ligand.

In this study, we designed a stable D-peptide isomer of Angiopep-2 by using retro-inverso isomerization. We investigated brain targeting efficiency of the D-peptide *in vitro* and *in vivo*. We also constructed peptide modified micelles to evaluate the potential of D-peptide for inspiring brain targeted drug delivery and to discuss potential applications for the therapy of CNS diseases.

2. EXPERIMENTAL SECTION

2.1. Materials. mPEG$_{2000}$-DSPE was purchased from Lipoid GmbH (Germany). Mal-PEG$_{3400}$-DSPE was from Laysan Bio Co. (Arab, AL). Coumarin-6 and Sephadex G50 were purchased from Sigma (St. Louis, MO). Fluorescein-5-maleimide was acquired from Fanbo Biochemicals (Beijing, China). Near infrared dye DiR (1,1′-dioctadecyl-3,3,3′,3′-tetramethyl indotricarbocyanine iodide) and LysoTracker Red DND-99 were from Invitrogen (Grand Island, NY). 4′,6-Diamidino-2-phenylindole (DAPI) was purchased from Roche (Switzerland). Anti-EEA1 antibody, Anti-Mannose 6 Phosphate Receptor (Anti-M6PR) antibody, and Goat Anti-Rabbit IgG H&L (Alexa Fluor 594) were from Abcam (Cambridge, MA).

U87 cells were obtained from Shanghai Institute of Cell Biology, and brain capillary endothelial cells (bEnd.3) were acquired from ATCC (Manassas, VA). Both cell lines were maintained in Dulbecco's modified Eagle medium (Gibco) supplemented with 10% FBS (Gibco), 100 U/mL penicillin, and 100 μg/mL streptomycin at 37 °C under a humidified atmosphere containing 5% CO_2. ICR mice and BALB/c nude mice of 4−6 weeks age were purchased from Shanghai SLAC Laboratory Animal Co. LTD (Shanghai, China) and kept under SPF conditions. All animal experiments were carried out in accordance with guidelines evaluated and approved by the ethics committee of Fudan University.

2.2. Peptide Stability in Blood and Liver Lysosome Homogenate. 100 μL of ^{D}Angiopep or ^{L}Angiopep (1 mg/mL) was incubated with 0.9 mL of 25% fresh rat serum. After 0.25, 0.5, 1, 2, 4, and 8 h incubation at 37 °C, 20 μL of 15% trichloroacetic acid (TCA) was added into 100 μL of reaction mixture, followed by storage at 4 °C for 20 min and centrifugation at 12000 rpm for 10 min. 20 μL of supernatant was analyzed by RP-HPLC to monitor and quantify peptide hydrolysis. The degradation of peptides in rat liver lysosomal homogenate was determined as follows: 100 μL of 1 mg/mL lysosomal homogenate and 100 μL of 1 mg/mL angiopep were added into 800 μL of 0.2 M sodium acetate buffer (pH 5.0). After 2 and 15 min incubation at 37 °C, an aliquot of 100 μL was withdrawn and quenched with 20 μL of 15% TCA. The mixture was centrifuged at 12000 rpm for 10 min, and 20 μL of supernatant was subject to HPLC analysis.

2.3. Cell Uptake. bEnd.3 cells and U87 cells were seeded into confocal dishes or 12-well plates at a density of 10^5 cells per well. After 24 h incubation, the culture medium was changed with 5 μM ^{D}Angiopep-fluorescein or ^{L}Angiopep-fluorescein in DMEM supplemented with 10% FBS and the cells were incubated at 37 °C for 4 h. The fluorescent intensity was captured by laser scanning confocal microscope or flow cytometry after staining of nuclei by DAPI and three rinses with phosphate buffered saline (PBS). For the study of intracellular distribution, cells were fixed by formaldehyde and the immunofluorescence staining was carried out before imaging capture.

2.4. Preparation and Characterization of Peptide Modified PEG-DSPE. ^{D}Angiopep-PEG-DSPE and ^{L}Angiopep-PEG-DSPE were synthesized through covalent conjugation between thiolated peptide and mal-PEG-DSPE.[24] In brief, mal-PEG-DSPE dissolved in DMF was added into ^{D}Angiopep or ^{L}Angiopep dissolved in PBS (pH = 7.2) and the reaction was monitored by HPLC. The excessive peptide was removed by dialysis (MWCO 3.5 kDa) against distilled water and confirmed by disappearance of the peak in the HPLC chromatogram. For the preparation of micelles, mPEG$_{2000}$-DSPE or a mixture of mPEG$_{2000}$-DSPE/^{D}Angiopep-PEG-DSPE (95/5, by mol) or mPEG$_{2000}$-DSPE/^{L}Angiopep-PEG-DSPE (95/5, by mol) in chloroform was rotary evaporated to form a thin film, which

dx.doi.org/10.1021/mp500086e | *Mol. Pharmaceutics* XXXX, XXX, XXX−XXX

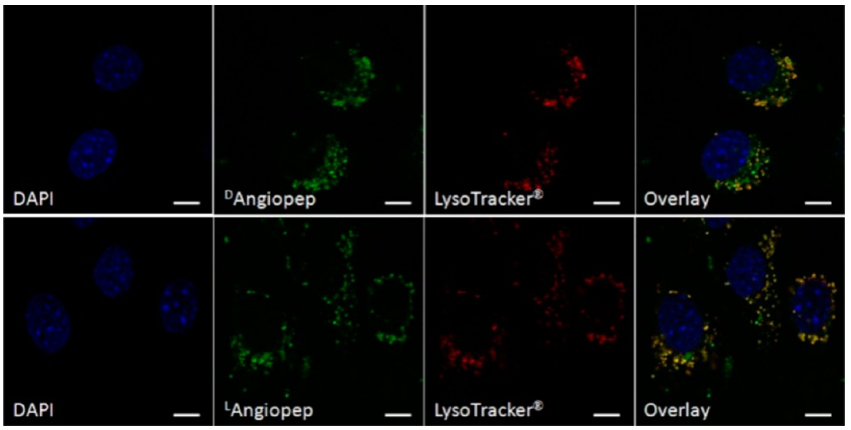


Figure 2. Intracellular distribution of endocytosed DAngiopep and LAngiopep in bEnd.3 cells. 5 μM fluorescein labeled DAngiopep or LAngiopep was incubated with cells at 37 °C for 4 h, followed by lysosome staining and confocal laser microscopy imaging. (Scale bar = 10 μm.)

was subsequently hydrated with HEPES buffer (pH 7.4) after the removal of residual chloroform under vacuum. For the loading of coumarin-6 or DiR, fluorophores were dissolved in methanol or acetonitrile before the formation of thin film and the free dyes were removed by gel filtration over a Sephadex G-50 column eluting with HEPES buffer.[25]

2.5. Cell Uptake of Micelles Loading Coumarin-6. bEnd.3 cells and U87 cells were seeded into 12-well plates at a density of 10^5 cells per well. After 24 h incubation, cells were incubated with micelles loading coumarin-6 (60 ng/mL) in DMEM supplemented with 10% FBS for one hour. Fluorescence intensity was captured by microscope after nuclei staining with DAPI and three rinses with PBS.

2.6. Biodistribution of Micelles *in Vivo*. DiR loaded micelles were injected into ICR mice through the tail vein (five rats for each group). At 1 and 4 h postinjection, the mice were sacrificed and organs were harvested for imaging by using an animal imaging system (IVIS Spectrum, PerkinElmer, Waltham, MA).

2.7. Intracranial Glioblastoma Model. The orthotropic glioblastoma model was established as described previously with slight modification.[26] In brief, BALB/c nude mice were anesthetized with chloral hydrate and U87 cells (8×10^5 cells suspended in 5 μL PBS) were implanted into the right brain (1.8 mm lateral, 0.6 mm anterior to the bregma with 3 mm depth) with the help of a stereotactic apparatus.

3. RESULTS

3.1. Retro-Inverso Isomerization of Angiopep. Angiopep-2 (here termed LAngiopep), which is a 19-residue peptide derived from human Kunitz domain, has been used as a ligand of LRP-1 to inspire receptor-mediated transcytosis. When modified on the surface of micelle[27] or nanoparticle,[28] LAngiopep efficiently facilitates brain-targeted drug delivery. The retro-inverso isomer of LAngiopep, termed DAngiopep (cyeetkfnnrkGrsGGyfft), is made up of D-amino acids in a reversed sequence of LAngiopep and assumes a side chain topology similar to that of the parent molecule.[29−31] A cysteine was added into N-terminal of the sequence for further conjugation with maleimide group. DAngiopep and LAngiopep were synthesized by solid phase peptide synthesis with Fmoc-protected amino acids and purified by prep reverse-phase HPLC to homogeneity. Peptide purity and molecular weight have been ascertained by analytic HPLC and ESI-MS spectrometry, respectively.

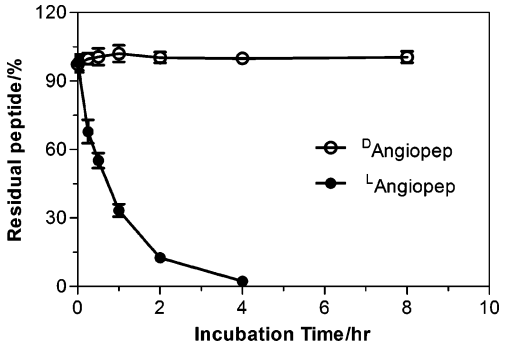


Figure 3. Stability of DAngiopep and LAngiopep in rat serum. Fresh rat serum was diluted with phosphate buffered saline and incubated with DAngiopep and LAngiopep at a concentration of 0.1 mg/mL. After 0.25, 0.5, 1, 2, 4, and 8 h incubation at 37 °C, 20 μL of 15% TCA was added into 100 μL of reaction mixture. The mixture was stored at 4 °C for 20 min and then centrifuged at 12000 rpm for 10 min. 20 μL of supernatant was analyzed by RP-HPLC to monitor and quantify peptide hydrolysis.

3.2. Cellular Uptake. We hypothesize in this study that DAngiopep possesses comparable bioactivity as the parent L-peptide along with exceptional stability against proteolysis. To investigate whether or not DAngiopep is capable of interacting with brain capillary endothelial cells, uptake of DAngiopep by bEnd.3 cells has been conducted. DAngiopep was labeled with fluorescein (DAngiopep-fluorescein) by Michael addition between the thiol group in peptide and maleimide group in fluoresein (fluorescein-5-maleimide). bEnd.3 cells were incubated with 5 μM of DAngiopep-fluorescein for 4 h at 37 °C, followed by confocal laser microscopy imaging and flow cytometry. As shown in Figure 1A, significant DAngiopep and LAngiopep were taken up by bEnd.3 cells and distributed in cytoplasm. The result of flow cytometry (Figure 1B) indicated that 46% bEnd.3 cells were fluorescence-positive with the treatment of DAngiopep-fluorescein, while the treatment of LAngiopep-fluorescein resulted in 81% fluorescence-positive cells. LRP-1 is overexpressed in brain capillary endothelial cells.[10] The uptake of DAngiopep by bEnd.3 may be explained by the specific interaction between D-peptide and LRP-1 on cell membrane. When compared with LAngiopep, less uptake of DAngiopep may have been related to its lower binding affinity with LRP-1.

dx.doi.org/10.1021/mp500086e | *Mol. Pharmaceutics* XXXX, XXX, XXX−XXX

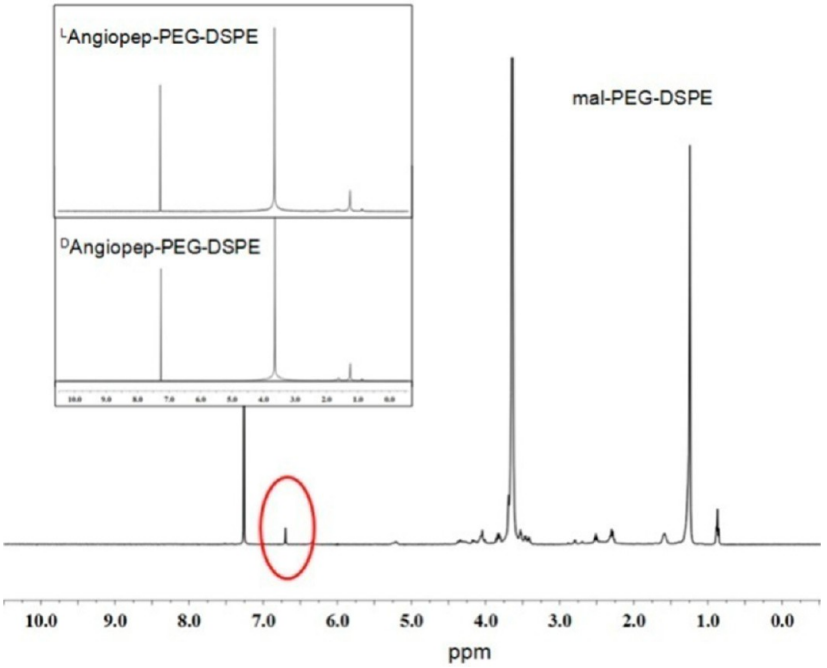


Figure 4. NMR spectra of mal-PEG-DSPE, ^{D}Angiopep-PEG-DSPE, and ^{L}Angiopep-PEG-DSPE in $CDCl_3$. The red circle highlights the characteristic peak of maleimide at 6.7 ppm in mal-PEG-DSPE, which disappeared in the spectra of ^{D}Angiopep-PEG-DSPE and ^{L}Angiopep-PEG-DSPE.

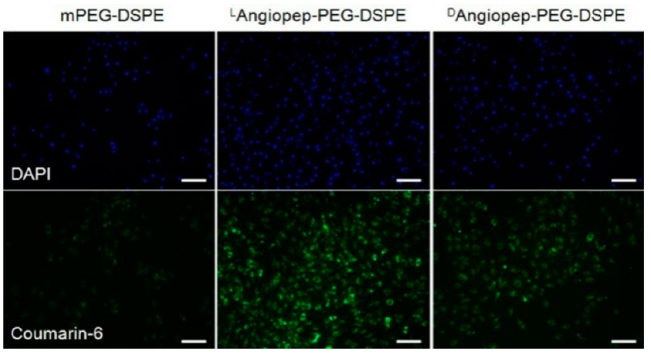


Figure 5. Cellular uptake of micelles loading coumarin-6 by bEnd.3 cells. bEnd.3 cells were incubated with mPEG-DSPE, ^{D}Angiopep-PEG-DSPE, or ^{L}Angiopep-PEG-DSPE micelles containing 60 ng/mL coumarin-6 at 37 °C for 1 h, followed by DAPI staining and rinses with phosphate buffered saline. Fluorescence microscopy imaging was conducted to detect cellular uptake of micelles. (Scale bar = 100 μm.)

3.3. Intracellular Pathway. Mixed pathways have been proposed to illustrate the process of receptor-mediated transcytosis.[32] It was postulated that transferrin[33] and lactoferrin[5] bypassed lysosomal compartment via receptor mediation and were exocytosed without degradation; however, anti-TfR antibody (OX-26) modified immunoliposome was reported to traverse *in vitro* BBB monolayer through lysosomal pathway.[34] Albeit ^{L}Angiopep has been applied to facilitating brain-targeted transport of prodrug conjugates or nanocarriers, intracellular pathway of the endocytosed peptide has been unknown. If the enzymatic intracellular organelles (such as lysosomal compartment) are involved in this process, susceptibility to proteolytic degradation of ^{L}Angiopep may attenuate transcytosis efficiency. To assess the trafficking route of ^{D}Angiopep and ^{L}Angiopep upon internalization, we studied the intracellular distribution of endocytosed peptides. bEnd.3 cells were incubated with fluorescein labeled peptides and were chased after 4 h uptake. A significant number of endocytosed ^{D}Angiopep and ^{L}Angiopep colocalized with lysosome (Figure 2), suggestive of the involvement of lysosomal compartment during endocytosis. We have prepared rat liver lysosomal homogenate to verify the stability of both peptides in lysosome. 100% of ^{L}Angiopep disappeared within 15 min while ^{D}Angiopep displayed no perceptible degradation. Lysosome is an intracellular reservoir of enzymes and capable of digesting L-peptide based ligand; intercellular susceptibility to lysosomal degradation may confine the efficiency of ^{L}Angiopep for brain-targeted transport.

3.4. Stability of D-Peptide in the Blood. Peptide-based ligands have attracted extensive attention for designing targeted drug delivery due to their high binding affinity and specificity to targets. The presence of fancy techniques for peptide identification, including structure-guided peptide design and phage display, further accelerates the extensive application of peptide ligand in the research field of molecular pharmaceutics. The main obstacle to such applications, however, is the instability of ligands due to their general susceptibility to proteolysis in the blood. Herein, we investigated the stability of ^{D}Angiopep and ^{L}Angiopep during blood circulation. Both ^{D}Angiopep and ^{L}Angiopep were incubated at body temperature with fresh rat serum. Based on reverse-phase HPLC analyses (Figure 3), it was evident that 50% of ^{L}Angiopep was degraded after 30 min incubation and more than 85% intact peptide disappeared after 2 h incubation. In conspicuous contrast, ^{D}Angiopep displayed nearly no degradation after 8 h incubation under the same conditions. When conjugated with drug or decorated on the surface of drug delivery system, ^{L}Angiopep, at least in part, will be degraded and deactivated by plasma proteases, thus attenuating brain targeting efficiency to some extent. ^{D}Angiopep holds the potential for extending brain targeting efficiency due to its resistance to proteolysis.

3.5. Preparation and Characterization of Micelles. ^{D}Angiopep-PEG-DSPE and ^{L}Angiopep-PEG-DSPE were syn-

dx.doi.org/10.1021/mp500086e | *Mol. Pharmaceutics* XXXX, XXX, XXX−XXX

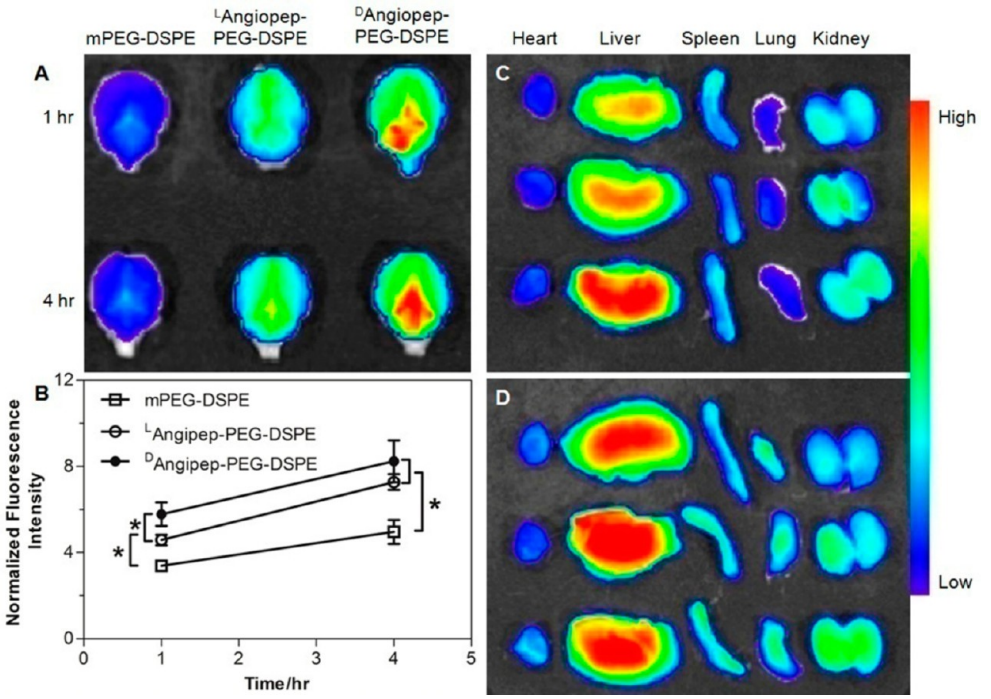


Figure 6. Biodistribution of micelles *in vivo*. mPEG-DSPE, ^{D}Angiopep-PEG-DSPE, or ^{L}Angiopep-PEG-DSPE micelles labeled with DiR were injected into the tail vein of ICR mice. At 1 and 4 h postinjection, all mice were sacrificed (n = 5) and the brain was dissected for *ex vivo* fluorescence imaging (A). Fluorescence intensity of five brains in each group was normalized (B). Other main organs, including heart, liver, spleen, lung, and kidney, were also dissected at 1 (C) and 4 (D) hours postinjection. In panels C and D, organs from top to bottom belong to mice treated with mPEG-DSPE, ^{L}Angiopep-PEG-DSPE, and ^{D}Angiopep-PEG-DSPE micelles, respectively. * $p < 0.05$. (The difference of brain distribution was not statistically significant between ^{L}Angiopep-PEG-DSPE and ^{D}Angiopep-PEG-DSPE groups at 4 h postinjection.)

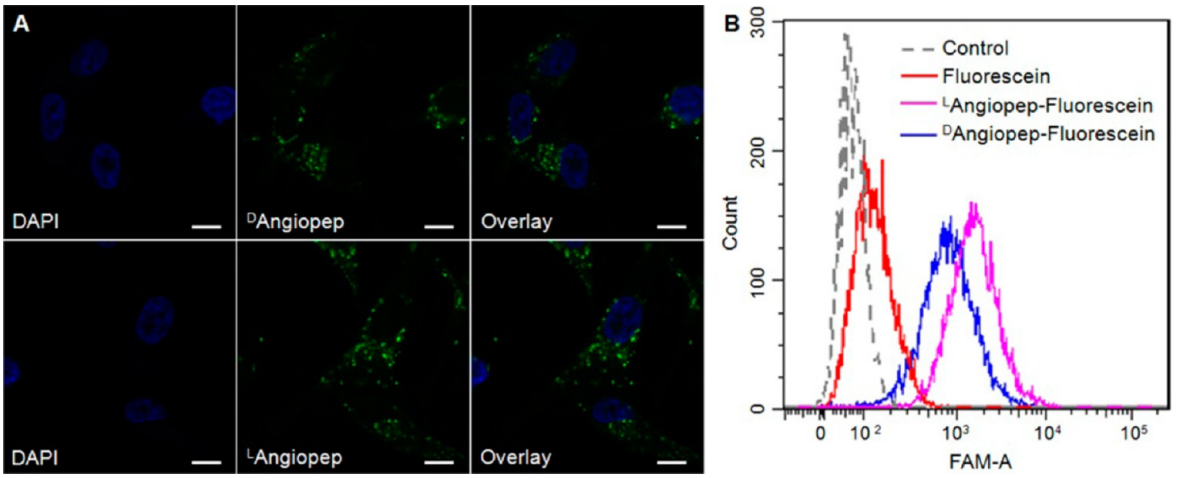


Figure 7. Cellular uptake of ^{D}Angiopep and ^{L}Angiopep by glioblastoma cells. U87 cells were incubated with 5 μM fluorescein labeled peptide at 37 °C for 4 h, followed by DAPI staining and rinses with phosphate buffered saline. Intracellular fluorescence was detected by confocal laser scanning microscope (A) and flow cytometer (B). (Scale bar = 10 μm.)

thesized through covalent conjugation between thiol group of peptide and maleimide group of mal-PEG$_{3400}$-DSPE. After removal of excessive peptide by dialysis, ^{D}Angiopep-PEG-DSPE and ^{L}Angiopep-PEG-DSPE were lyophilized and subjected to NMR spectrometry. As shown in Figure 4, the characteristic peak of maleimide group at 6.7 ppm disappeared in NMR spectra of ^{D}Angiopep-PEG-DSPE and ^{L}Angiopep-PEG-DSPE, suggestive of the occurrence of Michael addition between thiol group and maleimide group. Plain micelle and peptide modified micelles were prepared by using thin-film method.[35,36] mPEG$_{2000}$-DSPE (or mixed with 5% of peptide modified PEG$_{3400}$-DSPE) were dissolved in chloroform and rotary evaporated under vacuum at 40 °C. After removal of residue organic solvent, thin films were hydrated with HEPES buffer at 37 °C. All micelles were with mean diameters of 12−16 nm and narrow size distributions as analyzed by dynamic light scattering (DLS).

3.6. Cellular Uptake of Micelles. To investigate whether or not ^{D}Angiopep can enhance cellular uptake of PEG-DSPE micelles, we labeled mPEG-DSPE, ^{D}Angiopep-PEG-DSPE, and ^{L}Angiopep-PEG-DSPE micelles with coumarin-6 and studied cellular uptake by incubating micelles with bEnd.3 cells at 37

dx.doi.org/10.1021/mp500086e | *Mol. Pharmaceutics* XXXX, XXX, XXX−XXX

°C for one hour. As shown in Figure 5, both ^{D}Angiopep and ^{L}Angiopep modification improved uptake efficiency of micelles. The cellular uptake of ^{D}Angiopep-PEG-DSPE micelles displayed less efficiency than that of ^{L}Angiopep-PEG-DSPE micelles, consistent with the result of peptide uptake in bEnd.3 cells.

3.7. Brain-Targeted Delivery of Micelles *in Vivo*. To study *in vivo* brain targeting efficiency of ^{D}Angiopep-PEG-DSPE micelle, we compared brain distributions by using *ex vivo* fluorescence imaging technique. ^{D}Angiopep-PEG-DSPE and ^{L}Angiopep-PEG-DSPE micelles, as well as mPEG-DSPE micelles, were fluorescently labeled with a near-infrared dye (DiR). 100 μL of each labeled micelle was injected via mouse tail vein; and all organs were harvested and subjected to *ex vivo* fluorescent imaging at 1 and 4 h after injection (Figure 6). Both ^{D}Angiopep and ^{L}Angiopep modification significantly increased the distribution of dye in the brain. No perceptive difference of dye distribution has been recognized in other organs (Figures 6C and 6D). It was evident that ^{D}Angiopep-PEG-DSPE micelles generated the highest brain distribution at 1 and 4 h postinjection. In this study, ^{D}Angiopep displayed less cellular uptake efficiency than ^{L}Angiopep but was fully resistant to proteolysis in the blood and lysosomal homogenate. High brain distribution of ^{D}Angiopep-PEG-DSPE micelles may have been related to the unique stability of D-peptide *in vivo*.

4. DISCUSSION

^{L}Angiopep is a peptide ligand of LRP-1 and capable of initiating receptor-mediated transcytosis. ^{L}Angiopep modified paclitaxel prodrug has demonstrated a significant antiglioblastoma effect *in vivo*. When modified on the surface of nanocarriers, ^{L}Angiopep has been proposed to facilitate dual targeting of gliomas, wherein ^{L}Angiopep targets LRP-1 expressed on brain capillary endothelial cells and triggers receptor-mediated transcytosis, and the exocytosed ^{L}Angiopep can further target brain tumor due to the overexpression of LRP-1 in gliomas. Susceptibility to proteolysis of ^{L}Angiopep, however, has given birth to the present study. The presence of multiple metabolic barriers *in vivo*, including various enzymes in the blood, confines the targeting efficiency of peptide ligands. As to brain-targeted delivery, intracellular metabolic environment of the brain capillary endothelial cells may further challenge the transcytosis efficiency. We hypothesize that a stable D-peptide harboring high binding affinity to LRP-1 may generate enhanced brain targeting efficiency.

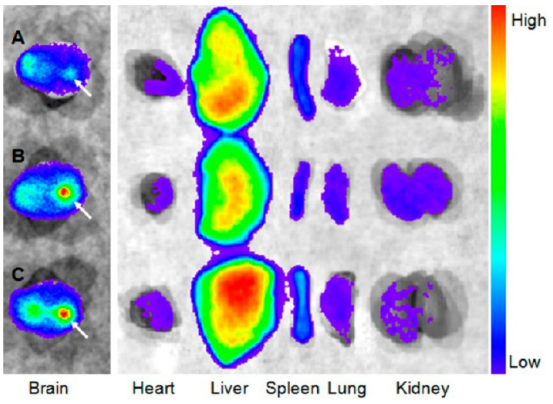


Figure 8. Glioblastoma targeting of micelles *in vivo*. mPEG-DSPE (A), ^{D}Angiopep-PEG-DSPE (B), and ^{L}Angiopep-PEG-DSPE (C) micelles labeled with DiR were injected into the tail vein of nude mice with intracranial glioblastoma (indicated by arrow) at 16 days post tumor implantation. After 12 h, mice were sacrificed and the brain, heart, liver, spleen, lung, and kidney were dissected (from left to right) for *ex vivo* imaging.

Herein, ^{D}Angiopep has been developed by using a retro-inverso isomerization technique. ^{L}Angiopep has demonstrated high binding affinity to LRP-1 and targets brain capillary endothelial cells via LRP-1-mediated endocytosis. D-Peptide displayed high cellular uptake efficiency, suggesting that the retro-inverso isomer may bind LRP-1 with high affinity. ^{L}Angiopep not only displayed high instability in fresh rat serum but also was colocalized after endocytosis with lysosomal compartment of bEnd.3 cells. We speculated that the proteolysis of ^{L}Angiopep in lysosome may further attenuate the transcytosis efficiency. When modified on the surface of micelles, both ^{D}Angiopep and ^{L}Angiopep could efficiently inspire the brain-targeted delivery *in vivo*. ^{D}Angiopep-PEG-DSPE micelles displayed relatively higher brain targeting efficiency in comparison to ^{L}Angiopep-PEG-DSPE micelles. Since ^{D}Angiopep demonstrated lower cellular uptake efficiency than did ^{L}Angiopep, we deduce that lower brain targeting efficiency of ^{L}Angiopep *in vivo* may have been related to its instability in the blood and lysosomal compartment of brain capillary endothelial cells.

U87 cells also displayed high uptake capacity toward ^{D}Angiopep and ^{L}Angiopep owing to the overexpression of LRP-1 on cell membrane (Figure 7A). As shown in Figure 7B, ^{D}Angiopep demonstrated lower efficiency than did ^{L}Angiopep, consistent with the results of bEnd.3 cellular uptake. Due to the stability and targeting efficacy to LRP-1, we envision that ^{D}Angiopep may be promising for designing a glioblastoma-targeted delivery system. In the early stage of glioblastoma, ^{D}Angiopep can facilitate brain-targeted drug delivery with higher efficiency than ^{L}Angiopep due to resistance to proteolysis in the blood and the BBB. With the tumor progression, the blood−brain barrier may be compromised in the tumor area with angiogenesis.[37,38] Both ^{D}Angiopep-PEG-DSPE and ^{L}Angiopep-PEG-DSPE micelles targeted glioblastoma, resulting in much higher distribution in tumor than plain micelles (Figure 8). Since both peptides could directly target glioblastoma without the presence of metabolic barriers in the BBB, ^{D}Angiopep and ^{L}Angiopep modification led to similar targeting capacity of micelles in intracranial glioblastoma.

5. CONCLUSION

In the present study, we designed a D-peptide ligand of LRP-1 by using retro-inverso isomerization. The resultant peptide, ^{D}Angiopep, displayed high uptake efficiency within LRP-1 expressed cells, including bEnd.3 cells and U87 cells. ^{D}Angiopep demonstrated high stability in the blood and lysosomal homogenate. When modified on the surface of PEG-DSPE micelles, ^{D}Angiopep efficiently inspired brain- and tumor-targeted drug delivery, suggestive of its promising in the treatment of brain cancers and other central nervous system diseases.

AUTHOR INFORMATION

Corresponding Authors

*Tel/fax: (86)-21-51980090. E-mail: zcy439@hotmail.com (C.Z.).

*E-mail: wylu@shmu.edu.cn (W.L).

Present Address

[§]Laboratory for Biomaterials and Drug Delivery, Department of Anesthesiology, Children's Hospital Boston, Harvard Medical School, Boston, MA 02115.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This work was supported by National Basic Research Program of China (973 Program) 2013CB932500, National Natural Science Foundation of China 81273458, and National Science & Technology Major Project 2012ZX09304004.

■ REFERENCES

(1) Hawkins, B. T.; Davis, T. P. The blood-brain barrier/neurovascular unit in health and disease. *Pharmacol. Rev.* **2005**, *57* (2), 173−85.

(2) Hutchinson, E. Blood-brain barrier: Plugging the leak. *Nat. Rev. Neurosci.* **2010**, *11* (12), 789.

(3) Fishman, J. B.; Rubin, J. B.; Handrahan, J. V.; Connor, J. R.; Fine, R. E. Receptor-mediated transcytosis of transferrin across the blood-brain barrier. *J. Neurosci. Res.* **1987**, *18* (2), 299−304.

(4) Pardridge, W. M.; Eisenberg, J.; Yang, J. Human blood-brain barrier transferrin receptor. *Metabolism* **1987**, *36* (9), 892−5.

(5) Fillebeen, C.; Descamps, L.; Dehouck, M. P.; Fenart, L.; Benaissa, M.; Spik, G.; Cecchelli, R.; Pierce, A. Receptor-mediated transcytosis of lactoferrin through the blood-brain barrier. *J. Biol. Chem.* **1999**, *274* (11), 7011−7.

(6) Dehouck, B.; Fenart, L.; Dehouck, M. P.; Pierce, A.; Torpier, G.; Cecchelli, R. A new function for the LDL receptor: transcytosis of LDL across the blood-brain barrier. *J. Cell Biol.* **1997**, *138* (4), 877−89.

(7) Kumar, P.; Wu, H.; McBride, J. L.; Jung, K. E.; Kim, M. H.; Davidson, B. L.; Lee, S. K.; Shankar, P.; Manjunath, N. Transvascular delivery of small interfering RNA to the central nervous system. *Nature* **2007**, *448* (7149), 39−43.

(8) Son, S.; Hwang do, W.; Singha, K.; Jeong, J. H.; Park, T. G.; Lee, D. S.; Kim, W. J. RVG peptide tethered bioreducible polyethylenimine for gene delivery to brain. *J. Controlled Release* **2011**, *155* (1), 18−25.

(9) Zhan, C.; Li, B.; Hu, L.; Wei, X.; Feng, L.; Fu, W.; Lu, W. Micelle-based brain-targeted drug delivery enabled by a nicotine acetylcholine receptor ligand. *Angew. Chem., Int. Ed.* **2011**, *50* (24), 5482−5.

(10) Demeule, M.; Currie, J. C.; Bertrand, Y.; Che, C.; Nguyen, T.; Regina, A.; Gabathuler, R.; Castaigne, J. P.; Beliveau, R. Involvement of the low-density lipoprotein receptor-related protein in the transcytosis of the brain delivery vector angiopep-2. *J. Neurochem.* **2008**, *106* (4), 1534−44.

(11) Li, J.; Zhang, C.; Fan, L.; Jiang, X.; Chen, J.; Pang, Z.; Zhang, Q. Brain delivery of NAP with PEG-PLGA nanoparticles modified with phage display peptides. *Pharm. Res.* **2013**, *30* (7), 1813−23.

(12) Li, J.; Feng, L.; Fan, L.; Zha, Y.; Guo, L.; Zhang, Q.; Chen, J.; Pang, Z.; Wang, Y.; Jiang, X.; Yang, V. C.; Wen, L. Targeting the brain with PEG-PLGA nanoparticles modified with phage-displayed peptides. *Biomaterials* **2011**, *32* (21), 4943−50.

(13) Demeule, M.; Regina, A.; Che, C.; Poirier, J.; Nguyen, T.; Gabathuler, R.; Castaigne, J. P.; Beliveau, R. Identification and design of peptides as a new drug delivery system for the brain. *J. Pharmacol. Exp. Ther.* **2008**, *324* (3), 1064−72.

(14) Regina, A.; Demeule, M.; Che, C.; Lavallee, I.; Poirier, J.; Gabathuler, R.; Beliveau, R.; Castaigne, J. P. Antitumour activity of ANG1005, a conjugate between paclitaxel and the new brain delivery vector Angiopep-2. *Br. J. Pharmacol.* **2008**, *155* (2), 185−97.

(15) Drappatz, J.; Brenner, A.; Wong, E. T.; Eichler, A.; Schiff, D.; Groves, M. D.; Mikkelsen, T.; Rosenfeld, S.; Sarantopoulos, J.; Meyers, C. A.; Fielding, R. M.; Elian, K.; Wang, X.; Lawrence, B.; Shing, M.; Kelsey, S.; Castaigne, J. P.; Wen, P. Y. Phase I study of GRN1005 in recurrent malignant glioma. *Clin. Cancer Res.* **2013**, *19* (6), 1567−76.

(16) Shao, K.; Huang, R.; Li, J.; Han, L.; Ye, L.; Lou, J.; Jiang, C. Angiopep-2 modified PE-PEG based polymeric micelles for amphotericin B delivery targeted to the brain. *J. Controlled Release* **2010**, *147* (1), 118−26.

(17) Xin, H.; Jiang, X.; Gu, J.; Sha, X.; Chen, L.; Law, K.; Chen, Y.; Wang, X.; Jiang, Y.; Fang, X. Angiopep-conjugated poly(ethylene glycol)-co-poly(epsilon-caprolactone) nanoparticles as dual-targeting drug delivery system for brain glioma. *Biomaterials* **2011**, *32* (18), 4293−305.

(18) Xin, H.; Sha, X.; Jiang, X.; Zhang, W.; Chen, L.; Fang, X. Anti-glioblastoma efficacy and safety of paclitaxel-loading Angiopep-conjugated dual targeting PEG-PCL nanoparticles. *Biomaterials* **2012**, *33* (32), 8167−76.

(19) Huang, R.; Ma, H.; Guo, Y.; Liu, S.; Kuang, Y.; Shao, K.; Li, J.; Liu, Y.; Han, L.; Huang, S.; An, S.; Ye, L.; Lou, J.; Jiang, C. Angiopep-conjugated nanoparticles for targeted long-term gene therapy of Parkinson's disease. *Pharm. Res.* **2013**, *30* (10), 2549−59.

(20) Huang, S.; Li, J.; Han, L.; Liu, S.; Ma, H.; Huang, R.; Jiang, C. Dual targeting effect of Angiopep-2-modified, DNA-loaded nanoparticles for glioma. *Biomaterials* **2011**, *32* (28), 6832−8.

(21) Yan, H.; Wang, L.; Wang, J.; Weng, X.; Lei, H.; Wang, X.; Jiang, L.; Zhu, J.; Lu, W.; Wei, X.; Li, C. Two-order targeted brain tumor imaging by using an optical/paramagnetic nanoprobe across the blood brain barrier. *ACS Nano* **2012**, *6* (1), 410−20.

(22) Li, Y.; Lei, Y.; Wagner, E.; Xie, C.; Lu, W.; Zhu, J.; Shen, J.; Wang, J.; Liu, M. Potent retro-inverso D-peptide for simultaneous targeting of angiogenic blood vasculature and tumor cells. *Bioconjugate Chem.* **2013**, *24* (1), 133−43.

(23) el-Bacha, R. S.; Minn, A. Drug metabolizing enzymes in cerebrovascular endothelial cells afford a metabolic protection to the brain. *Cell. Mol. Biol. (Noisy-le-grand)* **1999**, *45* (1), 15−23.

(24) Yan, Z.; Wang, F.; Wen, Z.; Zhan, C.; Feng, L.; Liu, Y.; Wei, X.; Xie, C.; Lu, W. LyP-1-conjugated PEGylated liposomes: a carrier system for targeted therapy of lymphatic metastatic tumor. *J. Controlled Release* **2012**, *157* (1), 118−25.

(25) Zhan, C.; Yan, Z.; Xie, C.; Lu, W. Loop 2 of Ophiophagus hannah toxin b binds with neuronal nicotinic acetylcholine receptors and enhances intracranial drug delivery. *Mol. Pharmaceutics* **2010**, *7* (6), 1940−7.

(26) Zhan, C.; Meng, Q.; Li, Q.; Feng, L.; Zhu, J.; Lu, W. Cyclic RGD-polyethylene glycol-polyethylenimine for intracranial glioblastoma-targeted gene delivery. *Chem. Asian J.* **2012**, *7* (1), 91−6.

(27) Shen, J.; Zhan, C.; Xie, C.; Meng, Q.; Gu, B.; Li, C.; Zhang, Y.; Lu, W. Poly(ethylene glycol)-block-poly(D,L-lactide acid) micelles anchored with angiopep-2 for brain-targeting delivery. *J. Drug Targeting* **2011**, *19* (3), 197−203.

(28) Gao, X.; Qian, J.; Zheng, S.; Xiong, Y.; Man, J.; Cao, B.; Wang, L.; Ju, S.; Li, C. Up-regulating blood brain barrier permeability of nanoparticles via multivalent effect. *Pharm. Res.* **2013**, *30* (10), 2538−48.

(29) Li, C.; Pazgier, M.; Li, J.; Liu, M.; Zou, G.; Li, Z.; Chen, J.; Tarasov, S. G.; Lu, W. Y.; Lu, W. Limitations of peptide retro-inverso isomerization in molecular mimicry. *J. Biol. Chem.* **2010**, *285* (25), 19572−81.

(30) Li, C.; Zhan, C.; Zhao, L.; Chen, X.; Lu, W. Y.; Lu, W. Functional consequences of retro-inverso isomerization of a miniature protein inhibitor of the p53-MDM2 interaction. *Bioorg. Med. Chem.* **2013**, *21* (14), 4045−50.

(31) Chaturvedi, N.; Goodman, M.; Bowers, C. Topochemically related hormone structures. Synthesis of partial retro-inverso analogs of LH-RH. *Int. J. Pept. Protein Res.* **1981**, *17* (1), 72−88.

(32) Bickel, U.; Yoshikawa, T.; Pardridge, W. M. Delivery of peptides and proteins through the blood-brain barrier. *Adv Drug Deliv Rev* **2001**, *46* (1−3), 247−79.

(33) Broadwell, R. D.; Baker-Cairns, B. J.; Friden, P. M.; Oliver, C.; Villegas, J. C. Transcytosis of protein through the mammalian cerebral epithelium and endothelium. III. Receptor-mediated transcytosis through the blood-brain barrier of blood-borne transferrin and

antibody against the transferrin receptor. *Exp. Neurol.* **1996**, *142* (1), 47−65.
(34) Markoutsa, E.; Pampalakis, G.; Niarakis, A.; Romero, I. A.; Weksler, B.; Couraud, P. O.; Antimisiaris, S. G. Uptake and permeability studies of BBB-targeting immunoliposomes using the hCMEC/D3 cell line. *Eur. J. Pharm. Biopharm.* **2011**, *77* (2), 265−74.
(35) Zhan, C.; Wei, X.; Qian, J.; Feng, L.; Zhu, J.; Lu, W. Co-delivery of TRAIL gene enhances the anti-glioblastoma effect of paclitaxel in vitro and in vivo. *J. Controlled Release* **2012**, *160* (3), 630−6.
(36) Zhan, C.; Gu, B.; Xie, C.; Li, J.; Liu, Y.; Lu, W. Cyclic RGD conjugated poly(ethylene glycol)-co-poly(lactic acid) micelle enhances paclitaxel anti-glioblastoma effect. *J. Controlled Release* **2010**, *143* (1), 136−42.
(37) Cheng, Y.; Morshed, R. A.; Auffinger, B.; Tobias, A. L.; Lesniak, M. S. Multifunctional nanoparticles for brain tumor imaging and therapy. *Adv. Drug Delivery Rev.* **2014**, *66*, 42−57.
(38) Zhan, C.; Lu, W. The blood-brain/tumor barriers: challenges and chances for malignant gliomas targeted drug delivery. *Curr. Pharm. Biotechnol.* **2012**, *13* (12), 2380−7.