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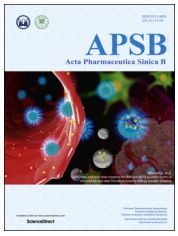

ORIGINAL ARTICLE

Liposomes and lipid disks traverse the BBB and BBTB as intact forms as revealed by two-step Förster resonance energy transfer imaging

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Abstract The blood–brain barrier (BBB) and the blood–brain tumor barrier (BBTB) prevent drug and nano-drug delivery systems from entering the brain. However, ligand-mediated nano-drug delivery systems have significantly enhanced the therapeutic treatment of glioma. In this study we investigated the mechanism especially the integrity of liposomes and lipid disks while traversing the BBB and BBTB both *in vitro* and *in vivo*. Fluorophores (DiO, DiI and DiD) were loaded into liposomes and lipid disks to form Förster resonance energy transfer (FRET) nano-drug delivery systems. Using brain capillary endothelial cells as a BBB model, we show that liposomes and disks are present in the cytoplasm as their intact forms and traverse the BBB with a ratio of 0.68‰ and 1.67‰, respectively. Using human umbilical vein endothelial cells as BBTB model, liposomes and disks remained intact and traversed the BBTB with a ratio of 2.31‰ and 8.32‰ at 3 h. *Ex vivo* imaging and immunohistochemical results revealed that liposomes and disks could traverse the BBB and BBTB *in vivo* as intact forms. In conclusion, these observations explain in part the mechanism by which nano-drug delivery systems increase the therapeutic treatment of glioma.

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1. Introduction

The blood–brain barrier (BBB), which mainly consists of capillary endothelial cells, prevents approximately 98% of small molecule drugs and nearly 100% of large molecule drugs from reaching the central nervous system[1–3]. Moreover, the blood–brain tumor barrier (BBTB), formed by specialized endothelial cells in tumors such as gliomas, prevents the transport of drugs or drug delivery systems into the cancerous tissue[4–6].

Transcytosis mediated by receptors has been utilized as an effective pathway to circumvent the BBB[7–12] and BBTB[13,14]. Nicotinic acetylcholine receptors (nAChRs) are extensively expressed on brain capillary endothelial cells and could mediate delivery of various drugs to the brain[15,16]. The peptide ^{D}CDX ($G^{D}R^{D}E^{D}I^{D}R^{D}TG^{D}R^{D}A^{D}E^{D}R^{D}W^{D}S^{D}E^{D}K^{D}F$) demonstrates excellent binding affinity to nAChRs and enables nano-drug delivery systems to target the brain[17,18]. This peptide was reported to be resistant to proteolytic degradation as compared to the ^{L}CDX peptide. Hence, ^{D}CDX was adopted as the brain-targeting ligand in this study.

As previously reported, the adhesion receptor integrin $\alpha_v\beta_3$ is overexpressed on the BBTB and on glioma cells. It plays a vital role in neovasculature formation[19,20]. The cyclic RGD peptide (cRGDyK) selectively targets integrin $\alpha_v\beta_3$ and enhances BBTB transport and tumor cell uptake[21].

Active-target ligands can significantly enhance the therapeutic efficacy of drug-loaded nano-drug delivery systems in central nervous system diseases[13,17,22,23]. It has not been established that nano-drug delivery systems can traverse BBB and BBTB as their intact forms, which could profoundly impact their ability to target diseases such as glioma.

With regard to *in vivo* distribution, inorganic nanoparticles such as iron oxide nanoparticles and gold nanoparticles can be easily measured due to their imaging properties[24]. However, tracking organic nano-drug delivery systems such as liposomes is more difficult.

Förster resonance energy transfer (FRET) is a type of fluorescence imaging which involves energy transfer from excited donors to acceptor molecules. It is widely applied in biological investigations on protein interactions, protein conformational change and enzyme activity[25]. The distance-dependent FRET signal endows it with the ability to monitor nanoparticle integrity by the loading of FRET pairs. For instance, this technique has been widely adopted to monitor the interaction of nanoparticles with the cell membrane[26] as well as polymeric nanoparticle stability in serum[27].

In this study, we designed a method of detecting the integrity of nano-drug delivery systems using DiO, DiI and DiD loaded into nano-drug delivery systems. These three fluorophores are in close proximity in nano-drug delivery systems. When excited at the DiO absorption band (488 nm), The presence or absence of a FRET signal (DiD) would indicate the integrity or dissociation of the nano-drug delivery systems. We also investigated the possibility of nano-drug delivery systems traversing BBB and BBTB as their intact forms both *in vitro* and *in vivo*.

2. Materials and methods

2.1. Materials

The fluorophores DiO, DiI and DiD were purchased from Invitrogen (Grand Island, NY, USA). 4′,6-Diamidino-2-phenylindole (DAPI) was from Roche (Basel, Switzerland). $mPEG_{2000}$–DSPE, HSPC (hydrogenated soy phosphatidylcholine) and POPC (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine) were supplied by Lipoid GmbH (Ludwigshafen, Germany). Cholesterol was purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Mal–PEG_{3400}–DSPE was provided by Laysan Bio Co. (Arab, AL, USA). EBM-2 was from Lonza (Visp, Switzerland).

U87 (human glioblastoma cells) and HUVECs (human umbilical vein endothelial cells) were provided by Shanghai Institute of Cell Biology. Both cell lines were cultured in Dulbecco's modified Eagle medium (DMEM, Gibco) containing 10% fetal bovine serum (FBS, Gibco) at 37 °C in a humidified atmosphere containing 5% CO_2. Male ICR mice and BALB/c nude mice of 6–8 week age were supplied by Shanghai SLAC laboratory animal Co., Ltd. (Shanghai, China). All animal experiments were performed in accordance with guidelines approved by the ethics committee of Fudan University, Shanghai, China.

2.2. Synthesis of peptide and functional materials

The peptides $G^{D}R^{D}E^{D}I^{D}R^{D}TG^{D}R^{D}A^{D}E^{D}R^{D}W^{D}S^{D}E^{D}K^{D}F$ (CDX) and c(RGDyK) (RGD) were synthesized by GL Biochem (Shanghai) Ltd. CDX–PEG_{3400}–DSPE was synthesized *via* the sulfhydryl-maleimide coupling method. In short, 20 mg of maleimide–PEG_{3400}–DSPE (mal–PEG_{3400}–DSPE) was dissolved in *N*,*N*-dimethylformamide (DMF) and 10 mg of CDX–Cys was dissolved in phosphate buffer (0.1 mol/L, pH = 7.4). The solutions were mixed and stirred at room temperature for 2 h. Excessive CDX–Cys was removed by dialysis against distilled water. The solution was lyophilized to obtain pure CDX–PEG_{3400}–DSPE. RGD–PEG_{3400}–DSPE was also prepared according to the above method. Both were characterized by ^{1}H NMR.

2.3. Preparation and characterization of nano-drug delivery systems

2.3.1. Preparation of liposomes

Liposomes loaded with 3D (DiO, DiI and DiD), including liposomes without any targeting moiety (LS/3D), liposomes decorated with CDX (CDX–LS/3D), liposomes modified with RGD (RGD–LS/3D) and those modified with both CDX and RGD (CDX+RGD–LS/3D), were prepared by the thin-film hydration and extrusion method[28]. For blank liposomes, the ratio of components is shown in Table 1, and were dissolved in $CHCl_3$ solution and then rotary evaporated to form a thin film. The dried lipid film was subsequently hydrated in saline at 65 °C for 2 h. The lipid dispersion then was extruded through a series of polycarbonate membranes with pore size ranging from 200 to 50 nm using an Avanti Mini Extruder (Avanti Polar Lipids).

2.3.2. Preparation of disks

Lipid disks loaded with DiO, DiI and DiD, including disks without any targeting moiety (Disks/3D), disks decorated with CDX (CDX–Disks/3D), disks modified with RGD (RGD–Disks/3D) and those modified with both CDX and RGD (CDX+RGD–Disks/3D), were prepared by the thin-film hydration and ultrasound method[29]. The ratio of components in the different forms of blank disks are presented in Table 2. A mixture of the indicated materials in chloroform was rotary-evaporated to form a thin film. The lipid film was dried under vacuum overnight and hydrated in phosphate-buffered saline (PBS) for 1 h at 37 °C. Disks were subsequently