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A novel lactoferrin-modified β-cyclodextrin nanocarrier for brain-targeting drug delivery

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ABSTRACT

The blood–brain barrier (BBB) restricts the transfer and delivery of most drug substances to brain. In this study, a novel nano-drug delivery system for brain-targeting was developed and investigated *in vitro* and *in vivo*. Lactoferrin (Lf) was selected as a brain-targeting ligand and conjugated to β-cyclodextrin (β-CD) *via* the heterobifunctional polyethyleneglycol (PEG) linker NHS-PEG-MAL, yielding Lf conjugated β-cyclodextrin (Lf-CD). UV–vis, FTIR, NMR and transmission electron microscopy (TEM) techniques clearly demonstrated the successful synthesis of Lf-CD nanoparticles with the average diameter of 92.9 ± 16.5 nm. Using near-infrared fluorescent dye IR-775 chloride (IR) as a model compound of poorly water-soluble drugs, IR-loaded Lf-CD nanoparticles (Lf-CD/IR) were successfully prepared with a high entrapment efficiency of 98.1 ± 4.8%. Biodistribution and pharmacokinetics of Lf-CD/IR were evaluated in KM mice after intravenous administration. The results of tissue distribution studies revealed that Lf-CD/IR treatment showed greatly improved BBB transport efficiency. In addition, $AUC_{0-2\ h}$ of IR in brain after Lf-CD/IR treatment was seven fold higher compared with that of IR treatment without Lf-CD nanocarriers, demonstrating that the introduction of Lf-CD drug-delivery system positively resulted in a higher AUC located in brain tissue. These results provide evidence that Lf-CD nanoparticles could be exploited as a potential brain-targeting drug delivery system for hydrophobic drugs and diagnostic reagents which normally fail to pass through the BBB.

1. Introduction

The blood–brain barrier (BBB), formed by brain vessel endothelial cells linked together by tight junctions, represents a major obstacle to the delivery of drugs, diagnostic reagents, and biomacromolecules. It restricts exchange of charged molecules and hydrophilic compounds between blood plasma and the central nervous system (Chen and Liu, 2012). Tremendous efforts and attention have been focused on constructing drug transport systems for brain targeting *via* different approaches including tight junction opening (Monnaert et al., 2004; Vykhodtseva et al., 2008), adsorptive-mediated transcytosis (Adenot et al., 2007; Deshayes et al., 2005), inhibition of efflux pumps (Fromm, 2000; Kabanov et al., 2003), construction of nanocarriers (Bhaskar et al., 2010; Garcia-Garcia et al., 2005), and endogenous receptor-mediated transcytosis (Boado et al., 2007; Ulbrich et al., 2009). The cell-mediated transcytosis is also a relatively new approach for transporting therapeutical materials that emerged in last decade (Qin et al., 2007).

Endogenous receptor-mediated transcytosis is an efficient and site-specific method of drug transport to brain, since brain endothelial cell contain receptors for recognizing special ligands including insulin (Zhang et al., 2003a), low-density lipoprotein (LDL) related proteins 1 and 2 (Blasi et al., 2007), diphtheria toxin (Gaillard et al., 2005), and transferrin (van Rooy et al., 2011). Drug delivery systems containing those ligands are recognized by corresponding receptors on the membranes of brain endothelial cell, and trigger receptor-mediated endocytosis, resulting in drug transport across the BBB.

For endogenous receptor-mediated transcytosis, lactoferrin receptor (LfR)-mediated endocytosis is among the most efficient cellular uptake pathways (Hu et al., 2009). Lactoferrin (Lf), a single-chain mammalian cationic iron-binding glycoprotein belonging to the transferrin (Tf) family, consists of a polypeptide chain of

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about 690 amino acids folded into two globular lobes, each of which contains one iron-binding site. It is a multifunctional protein which plays important roles in anti-inflammatory, immunomodulatory, antimicrobial, and anticarcinogenic activities (Vogel, 2012). Evidences from immunohistochemistry and reverse transcriptase-polymerase chain reaction have indicated the presence of specific LfR in the brain endothelial capillary cells, cerebral microvasculature, and glial cells. The LfR-mediated transcytosis through the BBB has also been demonstrated *in vitro* and *in vivo* (Hu et al., 2011). These results proved that Lf is a promising targeting molecule for improving brain delivery which inspired us to construct a brain delivery system employing the brain targeting ability of Lf.

Other than brain targeting ability, the solubility and loading efficiency of a drug are also problems to be considered in a drug delivery system. β-cyclodextrins (β-CD) and its derivatives are used widely as distinct solubilizers in pharmaceutical applications, giving prominence to their low bio-toxicity and high biocompatibility. Many studies have also confirmed that adding β-CD or its derivatives can improve the loading efficiency of drugs and reduce the drug release rates (Liu and Zhu, 2007; Marques et al., 1990).

The objective of this paper is to develop a novel nano-drug delivery system for brain-targeting. The thiolated Lf reacted with the maleimide (MAL) group of the difunctional polyethyleneglycol (PEG) linker, NHS-PEG-MAL, to construct the resulting Lf conjugated β-CD derivative (Lf-CD). We explored the potential of this new carrier for drug delivery to brain with IR-775 chloride (Fig. 1A) as a model compound of poorly water-soluble drugs. The IR loaded Lf-CD nanoparticles (Lf-CD/IR) were investigated *in vitro* for physicochemical properties and cytotoxicity. The tissue distribution and pharmacokinetic behaviors were studied *in vivo*.

2. Materials and methods

2.1. Chemicals and reagents

β-cyclodextrin was purchased from Sinopharm Chemical Reagent Co., Ltd. *p*-toluenesulfonyl chloride was obtained from Shanghai Haiqu Chemical Co., Ltd. Ethylenediamine was purchased from Shanghai Lingfeng Chemical Reagent Co., Ltd. Transferrin human was obtained from Shanghai Richu Bioscience Co., Ltd. 2-Iminothiolane hydrochloride (Traut's reagent), IR-775 chloride (IR), and lactoferrin from bovine milk were purchased from Sigma–Aldrich Chemicals (St Louis, MO, USA). 5, 5-Dithiobis(2-nitrobenzoic acid) (Ellmann's reagent) was obtained from Shanghai Suolaibao Bio-technology Co., Ltd. α-Malemidyl-ω-N-hydroxysuccinimidyl polyethyleneglycol (NHS-PEG-MAL, Mw = 5000 Da) was purchased from Beijing JenKem Technology Co., Ltd. Dulbecco's modified eagle's medium (DMEM), penicillin, streptomycin, horse serum and fetal bovine serum (FBS) were purchased from Invitrogen (Carlsbad, CA, USA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetra-zolium bromide (MTT) was obtained from Sigma–Aldrich Chemicals (St Louis, MO, USA). The SiO_2 particles (80 nm) used as a positive control in this study were supplied by the Laboratory for Ultrafine Materials (East China University of Science and Technology, Shanghai, China). All other chemicals and reagents were of analytical grade.

2.2. Synthesis of mono-6-deoxy-6-(*p*-tolylsulfonyl)-β-cyclodextrin (6-OTs-β-CD)

β-cyclodextrin (30.0 g, 26.5 mmol) was dissolved at room temperature in 250 ml of water, and 10 ml NaOH (8.25 M) was added dropwise over 6 min. The suspension became homogeneous and slightly yellow after the addition was complete. *p*-toluenesulfonyl chloride (TsCl, 7.5 g, 39.4 mmol) dissolved in 15 ml of acetonitrile was then added dropwise over 8 min, causing the immediate formation of a white precipitate. After stirring for 2 h at 25 °C, the precipitate was removed by filtration. The filtrate was acidified to about pH 8–9 with 1 M HCl and the product was refrigerated at 4 °C overnight. The resulting white precipitate was recovered by filtration. The combined filter cake was recrystallized with water as the solvent for 3 times and dried for 6 h at 50 °C to afford the final product 3.1 g (9% yield). ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.75 (d, J = 8.1 Hz, 2H), 7.43 (d, J = 8.1 Hz, 2H), 5.72 (bs, 14H), 4.84 (s, 6H), 4.77 (s, 1H), 4.51 (bs, 6H), 4.32 (d, J = 10.3 Hz, 1H), 4.18 (dd, J = 10.3, 6.4 Hz, 1H), 3.15–3.80 (m, 40H), 2.42 (s, 3H); ESI-MS (m/z): calculated for $C_{49}H_{76}O_{37}S$, 1288.4; found, 1311.4 for $[M+Na]^+$.

2.3. Synthesis of mono-6-deoxy-(6-aminoethylamino)-β-cyclodextrin (EDA-β-CD)

The dried 6-OTs-β-CD (3.0 g, 2.4 mmol) was dissolved in 20 ml of anhydrous ethylenediamine (EDA) and then stirred at 80 °C for 4 h under N_2. After the reaction was complete, the mixture was allowed to cool to room temperature. Subsequently, a large amount of cold acetone was added and the precipitate was collected by filtration. To further remove unreacted EDA in the precipitate, it was first redissolved in water, reprecipitated by addition of cold acetone, and then recovered by centrifuging at 12,000 rpm, 4 °C for 15 min. The same purification procedure was repeated for three times. The final precipitate was dried under vacuum to afford 2.3 g (80% yield) white solid. ^{1}H NMR (400 MHz, D_2O) δ 5.05 (s, 7H), 3.94 (t, J = 9.4 Hz, 7H), 3.85 (bs, 14H), 3.62 (dd, J = 7.2, 2.9 Hz, 6H), 3.56 (t, J = 8.8 Hz, 6H), 3.42 (t, J = 9.3 Hz, 1H), 3.03 (d, J = 11.9 Hz, 1H), 2.55–2.85 (m, 4H); ESI-MS (m/z): calculated for $C_{44}H_{76}O_{34}N_2$, 1176.4; found, 1177.2 for $[M+H]^+$.

2.4. Synthesis of Lf/Tf-modified EDA-β-CD

Lactoferrin-modified β-cyclodextrin derivatives (Lf-CD) or transferrin-modified β-cyclodextrin derivatives (Tf-CD) were synthesized by the following method: First, a conjugate of EDA-β-CD (3 mg) and NHS-PEG-MAL (6 mg) was prepared *via* a reaction between the primary amino group of EDA-β-CD and the N-hydroxy succinimide (NHS) group of the bifunctional PEG derivative. The reaction was performed in phosphate-buffered solution (PBS, pH 8.0) for 30 min at 4 °C. The resulting conjugate CD-PEG-MAL was purified by ultrafiltration (cutoff molecular weight 5 kDa, Sartorius AG, Germany) to remove unreacted EDA-β-CD. At the same time, Lf or Tf was dissolved in borate-EDTA buffer (0.15 M sodium borate, 0.1 mM EDTA, pH 8.0) containing Traut's reagent for 1.5 h at 4 °C under constant shaking (550 rpm) in the dark. Thereafter, the buffer was exchanged with PBS (pH 7.0) by centrifugation. Ellmann's reagent was used to determine the extent of thiolation (Ellmann, 1959). The mole ratio of thiol group to protein maintained around 1/1.22. Then, the thiolated proteins were mixed with CD-PEG-MAL in PBS (pH 7.0) for 24 h in the dark at 4 °C. The mole ratio of thiolated proteins to CD-PEG-MAL was kept at 1:10. The MAL group of CD-PEG-MAL was specifically reacted with the thiol group of thiolated proteins. Finally, the product was purified and freeze-dried to obtain the conjugate Lf-CD or Tf-CD in powder form.

2.5. Characterization of Lf-CD

Characterization of Lf-CD was analyzed by UV–vis, Fourier transform infrared (FTIR) spectra and nuclear magnetic resonance (NMR) spectroscopy. For the UV–vis study, three samples (Lf-CD, CD-PEG-MAL and CD-PEG-MAL plus free Lf) were scanned in the range of 200–600 nm using a UV 2401PC spectrophotometer (Shimadzu, Japan). FTIR spectra of EDA-CD and CD-PEG-MAL were recorded