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Low molecular weight protamine-functionalized nanoparticles for drug delivery to the brain after intranasal administration

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ABSTRACT

The development of new strategies for enhancing drug delivery to the brain is of great importance in diagnostics and therapeutics of central nervous diseases. Low-molecular-weight protamine (LMWP) as a cell-penetrating peptide possesses distinct advantages including high cell translocation potency, absence of toxicity of peptide itself, and the feasibility as an efficient carrier for delivering therapeutics. Therefore, it was hypothesized that brain delivery of nanoparticles conjugated with LMWP should be efficiently enhanced following intranasal administration. LMWP was functionalized to the surface of PEG-PLA nanoparticles (NP) via a maleimide-mediated covalent binding procedure. Important parameters such as particle size distribution, zeta potential and surface content were determined, which confirmed the conjugation of LMWP to the surface of nanoparticle. Using 16HBE14o- cells as the cell model, LMWP-NP was found to exhibit significantly enhanced cellular accumulation than that of unmodified NP via both lipid raft-mediated endocytosis and direct translocation processes without causing observable cytotoxic effects. Following intranasal administration of coumarin-6-loaded LMWP−NP, the $AUC_{0-8\ h}$ of the fluorescent probe detected in the rat cerebrum, cerebellum, olfactory tract and olfactory bulb was found to be 2.03, 2.55, 2.68 and 2.82 folds, respectively, compared to that of coumarin carried by NP. Brain distribution analysis suggested LMWP-NP after intranasal administration could be delivered to the central nervous system along both the olfactory and trigeminal nerves pathways. The findings clearly indicated that the brain delivery of nanoparticles could be greatly facilitated by LMWP and the LMWP-functionalized nanoparticles appears as a effective and safe carrier for nose-to-brain drug delivery in potential diagnostic and therapeutic applications.

1. Introduction

The presence of the blood–brain barrier (BBB), which limits the distribution of systemically administered therapeutics to the central nervous system (CNS), poses a major challenge to drug development efforts to combat the CNS disorders [1]. Therefore, the development of effective strategies to enhance drug delivery to the brain is of great interest in both clinical and pharmaceutical fields. Nowadays there are some potential approaches for brain drug delivery in either the invasive or non-invasive manners. The invasive approaches consist of a temporary disruption of BBB allowing the entry of a drug into the CNS, or of a direct drug delivery by means of intraventricular or intracerebral administration [2], while the non-invasive ones are made possible by the systemic application of colloidal drug carriers undergoing a receptor or adsorptive-mediated transcytosis mechanism [3], or by bypassing the BBB via intranasal delivery [4].

Intranasal delivery is considered as a promising alternative which could bypass the blood–brain barrier to rapidly deliver therapeutic agents to the brain for treating CNS disorders [5–12]. It provides the advantages including a large surface area for absorption, rapid achievement of target drug levels, avoidance of first pass metabolism and improvement of drug bioavailability; furthermore, this delivery route is needleless, maximizing patient comfort and compliance. It has been demonstrated that part of the therapeutics could be delivered directly to the CNS within minutes along both the olfactory and trigeminal nerves [13–15]. However, the total

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amount of drugs reported to access the brain was still low, especially for nasally applied biotech drugs such as peptides, proteins and DNA, which were poorly absorbed and highly susceptible to the harmful environment of the nasal cavity [16–18].

The application of nanoparticles offers an improvement to nose-to-brain drug delivery since they are able to protect the encapsulated drug from biological and/or chemical degradation, and extracellular transport by P-gp efflux proteins [19]. Despite these advances, the amount of nose-to-brain drug delivery mediated by nanoparticles is still not satisfied. A key mechanism to enhance nasal adsorption of nanoparticles is to improve their transmucosal transport, which may be facilitated by the surface modification with bioactive peptides such as cell-penetrating peptides (CPPs).

CPPs are relatively short peptides of 5–40 amino acids in length that derived from natural sources such as animal toxin [20] or from synthetically designed constructs. They hold remarkable capacity for membrane translocation and gaining access to the cell interior. CPPs such as TAT have been previously reported as efficient drug carriers to deliver many kinds of cargoes to the brain through the BBB [21–23]. Among the CPPs, low molecular weight protamine (LMWP) (CVSRRRRRRGGRRRR), which possess high arginine content and carry significant sequence similarity to that of the virus-derived TAT peptide, was found to be as potent as TAT in mediating cellular translocation of the attached cargos. In addition, unlike other cationic proteins/peptides, LMWPs were neither antigenic [24] nor mutagenic [25], and exhibited a much-reduced toxicity and thus an improved safety profile over protamine. Besides these advantages, while other CPPs must be chemically synthesized, LMWP can be produced in mass quantities direct from native protamine with limited processing time and cost [26]. Therefore, LMWP could be practically employed as an effective, safe and economical carrier for drug delivery. Indeed, it has been reported that LMWP has been utilized in facilitating anticancer [27,28] and percutaneous drug delivery [29]. As CPPs have been employed for the delivery of a wide variety of cargo including small molecules, nucleic acids, antibodies and nanoparticles, and the highly efficient translocation capacity of CPPs has been observed in a variety of cell lines [30–33]. In this study, we speculated that LWMP might serve as an effective and safe CPP for facilitating the nose-to-brain delivery of drug-loaded nanoparticles.

In order to justify this hypothesis, LWMP was functionalized to the surface of poly (ethyleneglycol)-poly (lactic acid) (PEG-PLA) nanoparticles, and brain delivery property of the developed nanoparticles was extensively studied following intranasal delivery. The nanoparticles (NP) were prepared with an emulsion/evaporation method, and functionalized with thiolated LMWP by taking advantage of the thiol group coupling activity of maleimide. The physicochemical characteristics of the nanoparticles were investigated by means of morphology, particle size, zeta potential and the surface elemental analysis. Coumarin-6 was used as a probe to study the brain-targeting efficiency of this system. Cellular association of LWMP-NP was evaluated on 16HBE14o- cells and compared with that of the unmodified ones. Endocytosis inhibition experiments were performed to clarify the mechanism of cellular association of LMWP-NP. *In vitro* cytotoxicity of LMWP-NP were analyzed by a cell counting kit-8 (CCK-8) assay and compared with that of NP to evaluate its safety as drug delivery carrier. Finally, brain biodistribution of the fluorescent marker associated to LMWP-NP following intranasal administration were qualitatively and quantitatively analyzed and compared with that of coumarin-6 carried by the unmodified NP. The possible pathway that LMWP-NP travels from the nasal cavity to the brain following intranasal administration was also discussed.

2. Experimental

2.1. Materials and animals

LMWP were synthesized by ChinaPeptides Co., Ltd (Shanghai, China). Methoxy poly(ethylene glycol) $_{3000}$-poly(lactic acid) $_{34000}$ (MePEG-PLA) and maleimide-poly (ethylene glycol) $_{3400}$-poly(lactic acid) $_{34000}$ (Male-PEG-PLA) were kindly provided by East China University of Science and Technology. Coumarin-6, coumarin-7 and DiR (1, 1′- dioctadecyl- 3,3,3′,3′- tetramethyl indotricarbocyanine Iodide) were purchased from Sigma–Aldrich (St. Louis, MO, USA). DAPI (4,6-diamidino-2-phenylindole) was obtained from Molecular Probes (Eugene, OR, USA), and cell counting kit-8 (CCK-8) from Dojindo Laboratories (Japan). Cell culture media, DMEM nutrient mix F12, certified fetal bovine serum (FBS), penicillin/streptomycin stock solutions and 0.25% Trypsin-EDTA were purchased from Invitrogen Co., USA. All the other chemicals were of analytical grades and used without further purification.

Male Sprague–Dawley rats weighing 200 ± 20 g were obtained from BK Lab Animal Ltd. (Shanghai, China) and maintained at 25 ± 1 °C with free access to food and water. The protocol of animal experiments was approved by the Animal Experimentation Ethics Committee of Fudan University.

2.2. Nanoparticles preparation and characterization

2.2.1. Preparation of NP and LMWP-NP

Unmodified nanoparticles (NP) loaded with coumarin-6 were prepared through an emulsion/solvent evaporation technique [34]. In brief, MePEG-PLA (22.5 mg), Male-PEG-PLA (2.5 mg) and 0.1% (w/w) of coumarin-6 were dissolved in 1 ml dichloromethane, and then added into a 2 ml of 1% sodium cholate aqueous solution with the mixture emulsified by sonication (280 w, 30 s) on ice using probe sonicator (Ningbo Scientz Biotechnology Co. Ltd., China). The O/W emulsion was diluted into an 8 ml of 0.5% sodium cholate aqueous solution under magnetic stirring for 5 min. After evaporating dichloromethane with a ZX-98 rotavapor (Shanghai Institute of Organic Chemistry, China) at 30 °C, the obtained nanoparticles were concentrated by centrifugation at 15000 rpm for 45 min using a TJ-25 centrifuge (Beckman Counter, USA). With the supernatant discarded, the nanoparticles were resuspended in double-distilled water, subjected to a 1.5 × 20 cm sepharose CL-4B column (Pharmacia Biotech, Inc., Sweden) and eluted with 0.01 M HEPES buffer (pH 7.0) to remove the unentrapped coumarin-6. Nanoparticles modified with LMWP (LMWP-NP) were prepared via a maleimide-thiol coupling reaction at room temperature for 8 h as described previously. The products were then eluted with 0.01 M HEPES buffer (pH 7.0) through the 1.5 × 20 cm sepharose CL-4B column to remove the unconjugated peptide.

2.2.2. Morphology, particle size, zeta potential and X-ray photo electron spectroscopy (XPS)

The morphological examination of nanoparticles was performed by transmission electron microscopy (TEM) (H-600, Hitachi, Japan) following negative stain with sodium phosphotungstate solution. Particle size and zeta potential were determined using Nicomp™ 380 XLS Zeta Potential/Particle Sizer (PSS·Nicomp, USA).

To determine the surface composition of NP and LMWP-NP, the samples were lyophilized using an ALPHA 2-4 Freeze Dryer (0.070 Mbar Vakuum, −80 °C, Martin Christ, Germany) and subjected to XPS analysis. The determination was performed on a RBD upgraded PHI-5000C ESCA system (Perkin Elmer) and the data analysis was carried out by using the RBD AugerScan 3.21 software provided by RBD Enterprises.

2.2.3. In vitro release of coumarin-6 from NP and LMWP-NP

To evaluate if the fluorescence probe remained associated with the particles during a 24 h incubation period, the *in vitro* release of coumarin-6 from the nanoparticles was investigated under sinking condition. Coumarin-6-loaded NP and LMWP-NP were incubated at 37 °C in pH 4 and pH 7.4 PBS, at a coumarin-6 concentration of 50 ng/ml with a shaking rate at 100 rpm [35]. One milliliter of nanoparticle samples was withdrawn at 0 min, 5 min, 15 min, 30 min, 1 h, 2 h, 4 h, 8 h, 12 h, 24 h ($n = 6$). Periodic samples were subject to centrifuged at 15000 rpm for 45 min and the supernatant was further diluted with methanol and analyzed for the released coumarin-6 by HPLC assay. The cumulative release percentage (CR %) of coumarin-6 from nanoparticles was calculated using the following equation [36,37]:

$$\mathrm{CR}(\%) = \frac{\text{amount of coumarin-6 in the supernatant}}{\text{total amount of coumarin-6}} \times 100\%$$

2.3. Cellular association of coumarin-6-labeled NP and LMWP-NP in 16HBE14o- cells

2.3.1. Cell culture

16HBE14o- cells, human bronchial epithelial cell line, were maintained in DMEM nutrient mix F12 containing L-glutamine, 15 mM HEPES, 10% fetal bovine