Biomaterials 34 (2013) 3870–3881

Contents lists available at SciVerse ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

Lactoferrin-modified PEG-co-PCL nanoparticles for enhanced brain delivery of NAP peptide following intranasal administration

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ARTICLE INFO

Article history:
Received 5 December 2012
Accepted 1 February 2013
Available online 27 February 2013

Keywords:
Brain targeting
Lactoferrin
Nanoparticle
NAP
Alzheimer's disease
Intranasal administration

ABSTRACT

Development of effective non-invasive drug delivery systems is of great importance to the treatment of Alzheimer's diseases and has made great progress in recent years. In this work, lactoferrin (Lf), a natural iron binding protein, whose receptor is highly expressed in both respiratory epithelial cells and neurons is here utilized to facilitate the nose-to-brain drug delivery of neuroprotection peptides. The Lf-conjugated PEG-PCL nanoparticle (Lf-NP) was constructed via a maleimide-thiol reaction with the Lf conjugation confirmed by CBQCA Protein Quantitation and XPS analysis. Other important parameters such as particle size distribution, zeta potential and *in vitro* release of fluorescent probes were also characterized. Compared with unmodified nanoparticles (NP), Lf-NP exhibited a significantly enhanced cellular accumulation in 16HBE14o-cells through both caveolae-/clathrin-mediated endocytosis and direct translocation. Following intranasal administration, Lf-NP facilitated the brain distribution of the coumarin-6 incorporated with the AUC_{0-8h} in rat cerebrum (with hippocampus removed), cerebellum, olfactory tract, olfactory bulb and hippocampus 1.36, 1.53, 1.70, 1.57 and 1.23 times higher than that of coumarin-6 carried by NP, respectively. Using a neuroprotective peptide – NAPVSIPQ (NAP) as the model drug, the neuroprotective and memory improvement effect of Lf-NP was observed even at lower dose than that of NP in a Morris water maze experiment, which was also confirmed by the evaluation of acetylcholinesterase, choline acetyltransferase activity and neuronal degeneration in the mice hippocampus. In conclusion, Lf-NP may serve as a promising nose-to-brain drug delivery carrier especially for peptides and proteins.

1. Introduction

Alzheimer's disease (AD), a devastating neurodegenerative disorder characterized by cortical amyloidogenesis, loss of neurons particularly in those regions associated with cognitive functions [1], are now representing one of the largest and fastest growing area of unmet medical need [2,3]. Today, 36 million people worldwide are living with dementia, with numbers doubling every 20 years to 66 million by 2030, and 115 million by 2050 [4]. Neurotrophic biomacromolecules such as nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) and insulin have showed neuroprotective effects on neurodegenerative diseases, and represent promising therapeutics to the treatment of AD [5]. However, the challenge to their clinical application is that most of them are not orally bioavailable and the blood–brain barrier (BBB) greatly limits their penetration for action in the brain following parenteral administration [5].

Intranasal administration provides a non-invasive alternative to the brain delivery of bioactive agents which could bypass the BBB and allow direct access of the therapeutic substances to the brain. The advantages include its rich vasculature, large surface area and highly permeable membrane for rapid absorption and avoidance of first pass metabolism; in addition, this delivery route is needleless, maximizing patient comfort and compliance [6–8]; more importantly, part of the therapeutics even stem cells [9] absorbed nasally could be delivered directly to the central nervous system (CNS) within minutes along both the olfactory and trigeminal nerves [10]. Actually, non-invasive intranasal delivery of peptide therapeutics to treat AD has already been done successfully in humans with

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http://dx.doi.org/10.1016/j.biomaterials.2013.02.003

demonstrated therapeutic benefits [11,12]. Despite these advantages, the nose-to-brain absorption of most biomacromolecules (peptides, proteins and DNA) was still quite low due to their limit permeability and high susceptibility to the nasal cavity environment [10]. One promising strategy to improve the nose-to-brain delivery of these agents is to encapsulate them in poly (ethylene glycol) (PEG)-coated nanoparticles.

Over the last decade, PEG-coated polyester nanoparticles have attracted increasing attention as a drug delivery system (DDS) due to their favorable biological properties. Besides their biocompatibility, biodegradability and long-circulating behavior, PEG-coated polyester nanoparticles are able to enhance drug interaction with the mucus barriers and protect it from biological and/or chemical degradation [13]. However, the system still has its drawbacks; the surface PEG chains could probably inhibit its interaction with cell surfaces [14]. A key mechanism to obtain higher nasal adsorption of nanoparticles is modifying the nanocarrier with biological ligands that with receptors highly expression in the nasal cavity.

Lactoferrin (Lf), a natural iron binding cationic glycoprotein of the transferrin family, weighs 80 kDa, consists of a single-chain glycoprotein folded into two globular lobes, is expressed in various tissues and involved in various physiological processes [15–18]. Extensive histological studies showed that Lf receptor (LfR) was highly expressed on the apical surface of respiratory epithelial cells [19], and also in the brain cells such as brain endothelial cells and neurons [20,21]. Besides, LfR has also been demonstrated to be overexpressed in the CNS associate with age-related neurodegenerative diseases including AD, Parkinson's disease, Huntington's disease and amyotrophic lateral sclerosis [22]. Furthermore, Lf has shown higher brain uptake than transferrin (Tf) and OX-26, an anti-Tf receptor antibody [23]. Based on this information, we speculated that Lf might serve as a suitable ligand for mediating enhanced nose-to-brain delivery of nanoparticles following intranasal administration.

NAP (NAPVSIPQ), an 8-amino acid neuropeptide fragment derived from the activity-dependent neuroprotective protein (ADNP) family, is currently in Phase II clinical trials, which showed neuroprotection effects at low concentration (ranging from 10^{-17} to 10^{-10} M), and considered as a promising candidate for the treatment of AD [24–26]. It exhibited neurotrophic/neuroprotective activity in various *in vitro* neuronal cell cultures, protecting cells against the neurotoxicity induced by β-amyloid, electrical blockade by tetrodotoxin and oxidative stress by hydrogen peroxide [27–29]. *In vivo* NAP protected animals against traumatic brain injury, oxidative stress and apolipoprotein E-deficiency-associated cholinergic dysfunction and learning/memory impairments [29–31]. However, the nasal absorption of NAP is still limited by its rapid enzymatic degradation by nasal cytochrome P450/peptidases/proteases, low permeability via the nasal mucosa and rapid mucociliary clearance [32].

Therefore, the aim of this study was to determine the potential of Lf-conjugated poly(ethyleneglycol)-poly (ε-caprolactone) nanoparticle (Lf-NP) for delivering neuroprotective agents to the treatment of AD. Fluorescently labeled nanoparticles were used to study the *in vitro* cellular interaction of Lf-NP and its *in vivo* biodistribution and brain targeting efficiency following intranasal administration. Using NAP as the model drug, neuroprotective effects of the Lf-NP formulation was evaluated in AD mice model.

2. Experimental

2.1. Materials

Methoxyl poly(ethylene glycol)-co-poly(ε-caprolactone) copolymer (Me-PEG-PCL, 15 kDa) and Maleimidyl-poly(ethylene glycol)-co-poly(ε-caprolactone) copolymer (Maleimide-PEG-PCL, 18 kDa) were kindly gifted by East China University of Science and Technology. Coumarin-6, Ibotenic acid (IBO), and β-amyloid$_{1-40}$ (Aβ$_{1-40}$) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Mono-reactive hydroxysuccinimide ester of Cy5.5 (NHS-Cy5.5) was purchased from Amersham Bioscience (Piscataway, NJ, USA). DAPI (4, 6-diamidino-2-phenylindole) was obtained from Molecular Probes (Eugene, OR, USA). Penicillin-streptomycin, Dulbecco's modified Eagle's medium (high glucose) (DMEM), fetal bovine serum (FBS) and 0.25% (w/v) trypsin solution were purchased from Gibco BRL (Gaithersberg, MD, USA). NAP (NAPVSIPQ) was synthesized by the ChinaPeptides Co., Ltd (Shanghai, China). Quantity Protein assay kits, acetylcholinesterase (AChE) and choline acetyltransferase (ChAT) activity assay kits were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). All the other chemicals were of analytical grades and used without further purification.

2.2. Cell line

16HBE14o-cells, human bronchial epithelial cell line, were cultured in DMEM medium, supplemented with 10% FBS, 100 IU/ml penicillin and 100 μg/ml streptomycin sulfate. All the cells were cultured in incubators at 37 °C, 5% CO_2.

2.3. Animals

Male ICR mice (4–5 weeks, 20 ± 2 g) and male SD rats (8–10 weeks, 220 ± 20 g) were supplied by Department of Experimental Animals, Fudan University (Shanghai, China), and acclimated at 25 ± 1 °C, 55% of humidity under natural light/dark conditions for 1 week before experiment. All the animal experiments were carried out in accordance with guidelines evaluated and approved by the ethics committee of Fudan University (Shanghai, China).

2.4. Preparation and characterization of nanoparticles

2.4.1. Preparation of nanoparticles

Unmodified nanoparticles (NP) loaded with coumarin-6 or NAP were prepared respectively for different purposes using the emulsion/solvent evaporation technique [33]. For coumarin-6-loaded NP, MePEG-PCL (22.5 mg), Male-PEG-PCL (2.5 mg) and 0.1% (w/w) of coumarin-6 were dissolved in 1 ml dichloromethane, and then added with 50 μl of deionized water as inner phase of the w/o primary emulsion, which was produced by sonication (160 W, 30 s) on ice using a probe sonicator (Ningbo Scientz Biotechnology Co. Ltd., China). The primary emulsion was then emulsified by sonication (220 W, 30 s) on ice in a 2 ml of 1% aqueous sodium cholate solution. The resulted w/o/w emulsion was further diluted into 8 ml of a 0.5% aqueous sodium cholate solution and then stirred for 5 min at room temperature. After that, the organic solvent was evaporated by a ZX-98 rotavapor (Shanghai Institute of Organic Chemistry, China). The formed nanoparticles were concentrated by centrifugation at 15,000 rpm for 45 min using a TJ-25 centrifuge (Beckman Counter, USA) at 4 °C. After the supernatant discarded, the pellets were resuspended in 2 ml HEPES buffer (pH 7.0) and purified with a 1.5 × 20 cm Sepharose CL-4B column (Pharmacia Biotech, Inc., Sweden). The nanoparticles loaded with NAP were prepared with the same way using 50 μl of NAP solution (25 mg/ml) as the inner phase of the primary emulsion. All the procedures were conducted in darkness.

2.4.2. Preparation of lactoferrin-conjugated nanoparticles

Lf was thiolated by reaction for 60 min with a 40:1 M excess of 2-iminothiolane (Traut's reagent) according to Huwyler's method [34]. The product was desalted with a Hitrap™ Desalting column (Amersham Pharmacia Biotech AB, Sweden). The amount and stability of the introduced thiol groups were determined spectrophotometrically (λ = 412 nm) with Ellmann's reagent [35]. The maleimide-functionalized nanoparticles were reacted with the purified thiolated Lf via a maleimide-thiol coupling reaction in HEPES buffer (pH 7.0) at room temperature for 9 h. The product was then subjected to a 1.5 × 20 cm sepharose CL-4B column and eluted with 0.01 M HEPES buffer (pH 7.0) to remove the unconjugated protein.

2.4.3. Morphology, particle size and zeta potential

The morphological examinations of nanoparticles were studied by transmission electron microscopy (TEM) (H-600, Hitachi, Japan) after negative staining with sodium phosphotungstate solution (2%, w/v). The average size and zeta potential of the nanoparticles were determined with a Zetasizer Nano ZS (ZEN3600, Malvern Instruments).

2.4.4. Lf conjugation efficiency, Lf density on nanoparticle surface and X-ray photo electron spectroscopy

The Lf conjugation efficiency (CE) was determined via a CBQCA Protein Quantitation Kit (Molecular Probes), a rapid and highly sensitive method for the quantitation of peptides and proteins. The calculation formula is as follow: CE (%) = (amount of Lf conjugated to the nanoparticle surface/total amount of Lf added) × 100%.

The average Lf number on each nanoparticle was calculated by dividing the number of Lf molecules by the calculated average number (n) of nanoparticles using the methods described previously [36]: $n = 6m/(\pi \times D^3 \times \rho)$ (m, the nanoparticle