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Dual ligand immunoliposomes for drug delivery to the brain

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ABSTRACT

Drug delivery systems that can reach brain areas affected by amyloid deposits are still underdeveloped. We propose pegylated liposomes functionalized with two antibodies, the anti-transferrin receptor monoclonal antibody (OX26MAb) and the anti-amyloid beta peptide antibody (19B8MAb), as nanocarriers of drugs for Alzheimer's disease therapy. Two distinct conjugation methods are investigated. In one formulation, the OX26MAb is conjugated to the tip of polyethylene glycol molecules through the maleimide group and the 19B8MAb is bound through the streptavidin–biotin complex. In the second system the conjugation reagents are swapped between the antibodies. Fluorescence spectroscopy experiments on porcine brain capillary endothelial cells show that the cellular uptake of the immunoliposomes is substantially more efficient if OX26MAb antibody is conjugated through the streptavidin–biotin complex instead of the maleimide group. The ability of the immunoliposomes to cross the blood brain barrier was established by in vivo studies in wild type rats. Our results demonstrate the importance of the conjugation method used to bind the antibody that targets the blood brain barrier to immunoliposomes for efficient drug delivery to the brain.

1. Introduction

Targeting drugs across the blood brain barrier (BBB) is dependent on efficient delivery systems [1–4]. Certain drugs such as short peptide sequences show promising, medically relevant, biological responses for the treatment of Alzheimer's disease (AD) but they have limited ability of reaching the brain [5–7]. AD is a neurodegenerative disorder of the human brain that causes a decline in cognitive abilities [8,9]. It is the most common cause of dementia. The current therapies only alleviate the symptoms, while doing very little to slow down the progression of the disease [10]. Neuropathologically, the disease is characterized by the presence of neuritic plaques and neurofibrillary tangles in regions of the brain particularly related to memory and cognition. The neuritic plaques contain amyloid fibrils, whose main component is the amyloid β-peptide. The neurofibrillary tangles are helical paired filaments composed largely of abnormal microtubular tau protein. The amyloid β-peptide (Aβ) has 40 or 42 amino acids and is a normal metabolic product of the enzymatic processing of a transmembranar protein, the amyloid precursor protein [11–14]. According to the amyloid cascade hypothesis, one plausible therapeutic strategy for the treatment of AD is the inhibition of Aβ aggregation [14–16]. However, the delivery of drugs to the brain is particularly difficult because of the presence of the BBB. To circumvent this barrier, we propose the use of pegylated immunoliposomes targeted with anti-transferrin receptor and anti-Aβ monoclonal antibodies. Liposomes have several advantages as drug delivery systems. They are biodegradable, over the course of few days, biocompatible and are easy to functionalize [17–19]. We prepared liposomes coated with polyethylene glycol (PEG) and conjugated the anti-transferrin receptor OX26MAb and the anti-Aβ 19B8MAb to the tips of some of the PEG molecules. PEG is known to reduce the immunogenicity and to prolong the half-life time of nanoparticles and liposomes [20]. Transferrin receptor has been reported to be

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selectively enriched at the brain capillary endothelium [21]. We used two common bioconjugate reagents to couple the MAbs to the liposome surface, the maleimide and the streptavidin–biotin complex, and determined their influence on the cellular uptake of the nanocarriers. Our study shows that the cellular uptake efficiency of immunoliposomes depends on the method of conjugation.

2. Experimental

2.1. Preparation of liposomes

The lipids DSPC (1,2-distearoyl-sn-glycero-3-phosphocholine), Chol (cholesterol ovine wool), DSPE-PEG2000 (1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)-2000]), DSPE-PEG2000-maleimide (1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[maleimide(polyethylene glycol)-2000]), DSPE-PEG2000-biotin (1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[biotin(poly ethylene glycol)-2000]) and LissRhod-PE (1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl)) were purchased from Avanti Polar Lipids. Pegylated liposomes were prepared by the lipid film hydration method. DSPC, Chol, DSPE-PEG2000, DSPE-PEG2000-maleimide, DSPE-PEG2000 -biotin and LissRhod-PE were dissolved in chloroform (Sigma–Aldrich) at a molar ratio of 52:45:3:0.015:0.015:0.01. The solvent was evaporated using a nitrogen stream in a rotary evaporator and the resultant dried lipid film was dispersed in PBS buffer, pH 7.4 (phosphate buffered saline, Sigma–Aldrich) with a final lipid concentration of 0.8 mM. The mixture was vortexed, frozen-thawed (10 cycles), and then extruded through successively smaller sized pore polycarbonate membranes (200 nm, 100 nm and 50 nm) at least 10 times each using a Lipex™ extruder (Northern Lipids Inc.). The fluorescence intensity of each system was determined using a Fluoroskan Ascent (Labsystems) and was similar for all stock solutions (the excitation wavelength was 540 nm and the emission wavelength was 625 nm).

2.2. Conjugation of antibodies

The OX26MAb and 19B8MAb were obtained from, respectively, AbD Serotec and Abcam. The conjugation of the antibodies to the PEG terminus end was based on functionalized PEG with a chemically reactive end-group, the PEG-maleimide (thiol reactive) or PEG-biotin (streptavidin–biotin method). For the maleimide-MAb conjugation, the MAb was first activated by a twenty times molar excess of Traut's reagent (2-iminothiolane hydrochloride, Sigma–Aldrich). A drop of EDTA 0.28 M (ethylenediaminetetraacetic acid, Sigma-Aldrich) was added to prevent metal catalyzed oxidation of sulfhydryl groups [22]. The unreacted complex EDTA/2-iminothiolane was removed by size exclusion chromatography using a Sephadex column PD-Mini Trap G25 (GE Healthcare). The second MAb was coupled to a biotin molecule using the EZ-Link micro sulfo NHS-LC-biotin (Thermo Scientific) kit. The MAb-biotin was then linked to the functionalized PEG-biotin through streptavidin (from Streptomyces avidinii, Sigma–Aldrich) at a molar ratio of 1:1. Both antibodies were added to the liposomes at a molar ratio of 1:1 (MAb: functionalized PEG). Each antibody was incubated with the liposomes at RT for 1 h and then at 5 °C for 8 h. Unreacted material was removed by gel chromatography. The unbound antibody was analysed by SDS polyacrylamide gel electrophoresis (relative quantitation) to certify that all formulations contained the same amount of each type of antibody. The stability of the immunoliposomes was assessed by size (dynamic light scattering) and zeta potential (laser Doppler velocimetry method) measurements over time using a Zetasizer Nano ZS instrument (Malvern Instruments Ltd.).

2.3. Enzyme-linked immunosorbent assay (ELISA)

The functional activity of the antibodies conjugated to the liposomes was analyzed by the enzyme-linked immunosorbent assay (ELISA). The surface of 96 well plates (flat-bottom Nunc MaxiSorp®) was coated with either transferrin receptor (Abcam) or amyloid beta peptide (β-Amyloid (1–42) Human, purity >95%, GenScript) during 1 h at 37 °C. After blocking with bovine serum albumin (BSA), the immunoliposomes were added to each well. After a washing step, the secondary antibody conjugated with peroxidase (Goat anti-Mouse IgG (H + L) Cross Adsorbed Secondary Antibody, HRP conjugate, Thermo Scientific-Pierce Antibodies) was allowed to react for 45 min at RT. To reveal the presence of the antibodies, a solution with citric acid (Sigma–Aldrich), ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt, Sigma–Aldrich) and H_2O_2 (hydrogen peroxide solution, Sigma–Aldrich) was used. The intensity of the color was measured by spectrometry using a Biotek Synergy 2 spectrometer. Liposomes without MAb were used as control.

2.4. Immunoblot assay

Amyloid beta peptide was first dissolved in 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) (Sigma–Aldrich) and kept at room temperature (RT) overnight. HFIP was then removed under a stream of nitrogen until a clear film adsorbed on the Eppendorf tube walls was formed. The peptide film was then dissolved in dimethyl sulfoxide (DMSO, Sigma–Aldrich) at 2 mM concentration. Amyloid beta (50 μM) was incubated in the absence or presence of immunoliposomes for different periods of time. The final concentration of the liposomes was 0.78 mM. An aliquot of 2.5 μl of each sample was hand-spotted onto nitrocellulose membranes (GE Healthcare) and blocked with 10% non-fat milk in phosphate – buffered saline (PBS) for 1 h at RT. The membranes were then washed and incubated overnight with antibody mouse monoclonal anti-amyloid beta (clone BAM-10, 1:1000, sigma) in 3% milk/PBS at 4 °C. The membranes were washed again and incubated with secondary antibody horseradish peroxidase-conjugated anti-mouse Ig diluted 1:5000 in milk/PBS for 1 h at RT and the detection was performed with enhanced chemiluminescence (BIO-RAD).

2.5. Cellular uptake assays

Porcine brain capillary endothelial cells were incubated at 37 °C in Plating-Medium–Earls Medium 199 supplemented with L-glutamine (0.70 mM), penicillin/streptomycin (1%), gentamicin (1%) and newborn calf serum – in a 96 well plate (Corning) previously coated with Collagen G (all components were purchased from Biochrom). The culture medium was replaced every 48 h. Normally, apparent confluence of the monolayers was reached after 4 days (approximately $6–8 \times 10^4$ cells per well). At day 4 the medium was replaced by a solution of Krebs Ringer buffer (KRB) containing the immunoliposomes. KRB is composed by NaCl (sodium chloride p. A., AppliChem), KCl (potassium chloride, AppliChem), H_2KO_4P (potassium dihydrogen phosphate p.A., AppliChem), HEPES (ROTH), D-Glucose (D(+)-glucose anhydrous for biochemistry, MERCK), $MgCl_2.6H_2O$ (magnesium chloride hexahydrate p.A., AppliChem) and $CaCl_2.2H_2O$ (calcium chloride dehydrate p.A., AppliChem). The cells were incubated with the immunoliposomes at 37 °C for 2 h. After removal of the suspension by aspiration, the cells were rinsed three times with medium at 4 °C, then washed with an acid buffer pH 3 (26 mM of sodium citrate, 9.2 mM citric acid monohydrated, 90.1 mM NaCl and 30 mM KCl) for 5 min