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ApoE-modified solid lipid nanoparticles: A feasible strategy to cross the blood-brain barrier

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ABSTRACT

Solid lipid nanoparticles (SLN) are colloidal drug delivery systems characterized by higher entrapment efficiency, good scalability of the preparation process and increased sustained prolonged release of the payload compared to other nanocarriers. The possibility to functionalize the surface of SLN with ligands to achieve a site specific targeting makes them attractive to overcome the limited blood-brain barrier (BBB) penetration of therapeutic compounds. SLN are prepared for brain targeting by exploiting the adaptability of warm microemulsion process for the covalent surface modification with an Apolipoprotein E-derived peptide (SLN-mApoE). Furthermore, the influence of the administration route on SLN-mApoE brain bioavailability is here evaluated. SLN-mApoE are able to cross intact a BBB in vitro model. The pulmonary administration of SLN-mApoE is related to a higher confinement in the brain of Balb/c mice compared to the intravenous and intraperitoneal administration routes, without inducing any acute inflammatory reaction in the lungs. These results promote the pulmonary administration of brain-targeted SLN as a feasible strategy for improving brain delivery of therapeutics.

1. Introduction

In the last decades, nanotechnologies have continuously evolved, raising great interest in pharmacological and biomedical field [1]. Recent applications in nanomedicine focus on nanoparticles (NPs) as they are promising tools for site-specific delivery of drugs and diagnostic agents, through the possibility to functionalize their surface with target-specific ligands (active targeting). Nowadays, brain drug development remains a challenging task due to the presence of the blood-brain barrier (BBB), a very restrictive barrier mainly composed of tightly sealed endothelial cells [2,3]. Indeed, due to its anatomy and physiology, the BBB strictly regulates the brain access and clearance of endogenous and exogenous molecules from the systemic circulation [4,5]. During the last three decades, different approaches have been developed to circumvent the BBB. Among others, embedding drugs within nanoparticulate matter able to cross the BBB is considered one of the most promising [6–8]. Solid lipid nanoparticles (SLN) are very interesting colloidal systems that have been studied for almost twenty years [9]. Historically, the origin of SLN can be traced back to the introduction in clinics of lipid emulsions for parenteral nutrition in the 1970s. Comparing to precursor oil based emulsions, SLN are aqueous nanoscale suspensions prepared mainly with phospholipids and solid lipids, such as fatty acids or triglycerides, phospholipids or synthetic surfactants with good physiological tolerability [2,10]. These ingredients are generally considered safe, biocompatible and biodegradable [10,11]. Moreover, they provide better colloidal stability thus creating the opportunity for sustained drug release and reducing the dosing and the frequency of the treatment [12,13]. Compared with liposomes, SLN have a higher stability, may incorporate both lipophilic and hydrophilic drugs and scalable production methods are already available [14]. Moreover, for the preparation of SLN neither organic solvents nor expensive excipients are needed as in the case of polymeric NPs [13].

Among the possible preparation methods available nowadays for SLN production, warm microemulsion technique focuses on specific set up of composition of the interface between lipids and water: this process allows great flexibility in design of surface characteristics of the final product and lets the introduction of different functional groups to be used for further bio-conjugation of SLN with targeting ligands. Stealth agents such as PEG-derivatized lipids are commonly used to obtain sterically stabilized SLN to avoid recognition by macrophages and

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to prolong the circulation time of SLN [15,16]. Several colloidal systems have been designed for CNS targeting by exploiting specific surface modifications with targeting ligands capable of taking advantage of specific receptors or nonspecific pathways [17–19]. Nevertheless, it has been widely emphasized the efficacy of the surface modification with less attention given to the comparison between the results obtained by administering the colloidal systems by different routes [20]. Although delivery to the blood circulation has the potential benefit of providing access to any vascularized tissue in the body, this pervasive approach also increases the likelihood that unintended tissues will be targeted. It is typically observed that systemic delivery of nanoparticles results in substantial accumulation in both the liver and the spleen as a result of immune clearance by the mononuclear phagocyte system. This off-target accumulation is a major impediment to achieving specificity with systemic delivery [21]. SLN have been virtually proposed for all administration routes [22] e.g., parenteral [23], oral [24], dermal [25] and ocular [26]. Among the different administration routes, pulmonary delivery is a field of increasing interest not only for the local treatment of airway diseases but also for the systemic administration of drugs [27]. Indeed lungs are an attractive target for the pulmonary administration of active pharmaceutical ingredients in the form of various drug delivery systems [12,28–30]. Additionally, this route offers many advantages over conventional per oral administration, such as the circumvention of the first pass effect and a high surface area with rapid absorption due to an extensive vascularization [31,32]. Since the capability of a synthetic decapeptide derived from the receptor binding region of human apolipoprotein E (mApoE) to enhance liposomes (LIP) penetration in the central nervous system (CNS) has already been demonstrated, [33–35] as well as the feasibility of the pulmonary administration of mApoE-functionalized LIP as an efficient strategy to reach the brain [36], this work aims at optimizing the preparation of SLN for brain drug targeting by exploiting the pulmonary route. We evaluated the adaptability of warm microemulsion process for ligand surface modification of SLN with mApoE to target the BBB. Moreover, we investigated how the different administration routes affected the SLN-mApoE brain bioavailability.

2. Materials and methods

2.1. Materials

Dynasan 116 was a gift from Cremer Oleo Division (Hamburg, Germany), epikuron 200 (soybean lecithin with a phosphatidylcholine content of at least 92% - Cargill) was purchased from AVG (Italy), sodium taurocholate (bile salt) was purchased from PCA (Italy), 1-butanol was purchased from Carlo Erba (Milan, Italy). 1,2-Distearoyl-sn-*glycero*-3-phosphoethanolamine-*N*-[maleimide(polyethylene glycol)-2000] (DPM) and 1,2-dipalmitoyl-sn-*glycero*-3-phosphatidic acid sodium salt (DPPA) were purchased from NOF Corporation (Tokyo, Japan). [^{14}C]-Sucrose, [^{3}H]-cholesteryl hexadecyl ether (CE), [^{14}C]-DPPA and Ultima Gold scintillation cocktail were from PerkinElmer (Italy). L-Cysteine HCl was purchased from Thermo Fisher Scientific (Waltham, MA, USA). 3,3′-Dioctadecyloxacarbocyanine perchlorate (DiO) and 1,1′-Dioctadecyl-3,3,3′,3′-Tetramethylindotricarbocyanine Iodide (DiR) dyes were from VWR (Italy). Transwell permeable supports 0.4 μm polyester membrane 12 mm insert, 12 well plates, were from Corning (NY, USA). All the media and supplements for cell culture were purchased from Life Technologies, Thermo Fisher Scientific. Phalloidin was from Molecular Probes, Thermo Fisher Scientific. Rabbit anti-LAMP1, rabbit anti-Niemann Pick C1 and rabbit anti-Rab11 antibodies were from Abcam (Science Park, Cambridge, UK). Mouse anti-EEA1 was from BD Transduction laboratories (BD Biosciences, San Jose, CA, USA). IT instillation was performed using a MicroSprayer Aerosolizer system (Penn Century, USA). All other chemicals were purchased from Sigma-Aldrich.

2.2. Preparation of SLN

SLN were prepared by Nanovector srl (Turin, Italy) by oil/water warm microemulsion technique. The microemulsion was prepared at 60–64 °C by heating dynasan 116, epikuron 200, short chain alcohols, and then adding an aqueous solution of bile salts heated at the same temperature. The warm microemulsion was dispersed in cold water to obtain an aqueous dispersion of SLN. The surface of SLN was functionalized with DPM to perform conjugation with the peptide CWG-LRKLRKRLLR (mApoE; residues 141–150 of human apolipoprotein E) exploiting thiol-maleimide reaction, by substituting 0.4% molar percentage of phosphatidylcholine (PC) with the compound. The peptide CWG was added at the C-term of mApoE to assess the yield of coupling reaction. Fluorescent SLN were prepared by adding the fluorescent lipophilic probes DiO (λex\λem = 484\501 nm) or DiR (λex\λem = 750\780 nm) to the oil phase of the microemulsion. Dually radiolabelled SLN were prepared using two radioactive compounds: [^{3}H]-CE and [^{14}C]-DPPA (. SLN incorporating fluorescent probes or radioactive markers were purified by tangential ultrafiltration (repeated four times) and using Vivaflow 50 system (Sartorius, Germany) equipped with regenerated cellulose membrane 100000 MWCO.

2.3. Preparation and characterization of SLN functionalized with ApoE peptide

Fluorescent and radiolabelled SLN were incubated with 50 μM mApoE overnight at 4 °C, protected from light. The final mApoE:DPM molar ratio was 1:1. After the incubation, the reaction mixture was purified by tangential ultrafiltration to remove unbound peptide by using a 300 kDa membrane. The yield of coupling reaction between SLN and mApoE was assessed by fluorescence spectroscopy of tryptophan residue at the C-terminal of mApoE, as previously described [29]. As a control, SLN-cys were prepared by incubating the SLN dispersion with cysteine in order to block maleimide reactive site. SLN were characterized in terms of dimension, ζ-potential and polydispersity index by means of Dynamic Light Scattering (DLS) analysis.

2.4. Culture of hCMEC/D3 cells

Human cerebral microvascular endothelial cells (hCMEC/D3) were obtained from Institut Cochin (INSERM, Paris, France). Cells at passage between 27 and 37 were grown on tissue culture flasks, covered with 0.1 mg/mL rat tail collagen type 1, in EBM-2 medium supplemented with 5% fetal bovine serum (FBS), 1% Penicillin-Streptomycin, 1.4 μM hydrocortisone, 5 μg/mL ascorbic acid, 1/100 chemically defined lipid concentrate, 10 mM HEPES and 1 ng/mL basic fibroblast growth factor (FGF). Cells were seeded at a density of 24,000–33,000 cells/cm^2 and cultured at 37 °C, 5% CO_2. For permeability assays, cells were seeded on 12-well transwell inserts coated with rat tail collagen type 1; cell culture medium was changed every 2 days. For uptake studies by confocal microscopy, hCMEC/D3 were grown on 25 mm glass coverslips precoated with collagen; confluent hCMEC/D3 monolayers were obtained typically by day 3. For flow cytometry analysis, cells were cultured on type 1 collagen-coated 12-wells plates; confluent hCMEC/D3 monolayers were obtained typically by day 3.

2.5. Cytotoxicity assay

Cell viability was assessed by means of PrestoBlue assay (Invitrogen). hCMEC/D3 were incubated with SLN-cys or SLN-mApoE at the final lipid concentrations of 0.05 mg/mL, 0.1 mg/mL, 0.5 mg/mL or 1 mg/mL at 37 °C, 5% CO_2. After 24 h, culture medium was removed, PrestoBlue solution was added to each well and incubated at 37 °C. Absorbance was measured at different time points until a plateau was reached. Values were measured as OD readings at 570/600 nm using FLUOStar Omega Multidetection Microplate reader (BMG LABTECH).