Journal of Controlled Release 263 (2017) 4–17

Contents lists available at ScienceDirect

Journal of Controlled Release

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

Rational design of polyarginine nanocapsules intended to help peptides overcoming intestinal barriers

Zhigao Niu [a], Erik Tedesco [b], Federico Benetti [b], Aloïse Mabondzo [c], Isabella Monia Montagner [d], Ilaria Marigo [d], David Gonzalez-Touceda [e], Sulay Tovar [e], Carlos Diéguez [e], Manuel J. Santander-Ortega [a,f], María J. Alonso [a,*]

[a] Department of Pharmacy and Pharmaceutical Technology, CIMUS Research Institute, IDIS research Institute, University of Santiago de Compostela, 15782 Santiago de Compostela, Spain
[b] ECSIN-European Center for the Sustainable Impact of Nanotechnology, ECAMRICERT SRL, I-45100 Rovigo, Italy
[c] Service de Pharmacologie et d'Immunoanalyse, IBITECS, CEA, Université Paris-Saclay, F-91191 Gif-sur-Yvette, France
[d] Veneto Institute of Oncology, IOV-IRCCS, 35128 Padova, Italy
[e] Biomedical Research Group, Center for Research in Molecular Medicine and Chronic Diseases, University of Santiago de Compostela, 15782 Santiago de Compostela, Spain
[f] Cellular Neurobiology and Molecular Chemistry of the Central Nervous System Group, Universidad de Castilla-La Mancha, Spain

ARTICLE INFO

Article history:
Received 7 December 2016
Received in revised form 2 February 2017
Accepted 19 February 2017
Available online 21 February 2017

Keywords:
Polyarginine
Nanocapsule
Insulin
Permeation enhancer
Oral peptide delivery

ABSTRACT

The aim of this work was to rationally design and characterize nanocapsules (NCs) composed of an oily core and a polyarginine (PARG) shell, intended for oral peptide delivery. The cationic polyaminoacid, PARG, and the oily core components were selected based on their penetration enhancing properties. Insulin was adopted as a model peptide to assess the performance of the NCs. After screening numerous formulation variables, including different oils and surfactants, we defined a composition consisting of oleic acid, sodium deoxycholate (SDC) and Span 80. This selected NCs composition, produced by the solvent displacement technique, exhibited the following key features: (i) an average size of 180 nm and a low polydispersity (0.1), (ii) a high insulin association efficacy (80–90% AE), (iii) a good colloidal stability upon incubation in simulated intestinal fluids (SIF, FaSSIF-V2, FeSSIF-V2), and (iv) the capacity to control the release of the associated insulin for >4 h. Furthermore, using the Caco-2 model cell line, PARG nanocapsules were able to interact with the enterocytes, and reversibly modify the TEER of the monolayer. Both cell adhesion and membrane permeabilization could account for the pronounced transport of the NCs-associated insulin (3.54%). This improved interaction was also visualized by confocal fluorescent microscopy following oral administration of PARG nanocapsulesto mice. Finally, *in vivo* efficacy studies performed in normoglycemic rats showed a significant decrease in their plasma glucose levels after treatment. In conclusion, here we disclose key formulation elements for making possible the oral administration of peptides.

1. Introduction

Peptide therapeutics has drawn increasing interest in the pharmaceutical market since the 1970s. Over the last decades, the regulatory approval rate of these products has been shown to be superior to that of small molecules, and their global value has increased from 14.1 to 25.4 billion USD from 2011 to 2018 [1]. Currently, over 500 peptide drugs are under preclinical development, 140 are candidates in clinical trials and about 60 commercialized products have been approved by the FDA [2]. Due to their complex structure, these macromolecules offer high specificity, advanced drug potency, low toxicity and less interaction with other drugs when compared to small molecules. However, because of their physicochemical properties, their delivery to the subjects' body represents a critical challenge. In particular, these macromolecules are prone to lose their activity in a physiological environment and have great difficulties to cross biological barriers. Because of this, up to date, peptide therapeutics is principally administrated *via* injection, which is an easy but inconvenient way of medication.

Oral administration is the most attractive modality for the administration of peptide drugs. However, peptides administered by this route would have to overcome major barriers, including the harsh gastrointestinal environment, the mucus layer and the underlying epithelium barrier [3]. The approaches investigated so far to improve the intestinal absorption of peptides include the chemical modification of the peptide molecule, the co-administration of protease inhibitors and/or permeation enhancers, and the incorporation of the peptide formulation to delivery carriers [4,5]. A broad array of carriers including polymeric micro and nanoparticles, silica nanoparticles, liposomes, micelles, solid lipid nanoparticles, microemulsions, self-emulsifying drug delivery systems, nanoemulsions and NCs have been explored for the delivery of peptide drugs [6–8]. Among them, liver-targeted HDV-I liposomes of specific composition [9], bioadhesive calcium phosphate nanoparticles in an enteric capsule [10], and silica nanoparticles [11] have reached the clinical

* Corresponding author.
E-mail address: mariajalonso@usc.es (M.J. Alonso).

http://dx.doi.org/10.1016/j.jconrel.2017.02.024

development phase for the delivery of insulin. Although the basis for the success of these delivery carriers has not been described in detail, it is generally assumed that these carriers have the capacity to overcome the GI barriers.

Within this frame, our laboratory has contributed with the design of NCs consisting of an oily core surrounded by a shell made of chitosan [12–15]. These NCs allowed us to encapsulate the peptide salmon calcitonin (sCT), and the resulting nanocomposition was found to facilitate and prolong sCT absorption following oral administration. More recently, we have also explored the interaction of PARG nanocapsules based on Miglyol® core [16] with the Caco-2 monolayer [17] and concluded that PARG NC may have a potential for oral delivery. Taking into account this background information, the objective of this work was to engineer a new composition based on PARG NCs that would endow them with the capacity to load peptides, *i.e.* insulin, and to overcome the biological barriers associated with the oral modality of administration. Hence, the main criteria for this design were: i) efficient encapsulation and controlled release of the selected peptide, *i.e.* insulin; ii) stability in the intestinal fluids, in the presence of enzymes and bile salts; iii) capacity to diffuse across the mucus layer and, iv) capacity to interact with the intestinal epithelium and facilitate the transport of the associated peptide across it. With these criteria in mind, we performed a thoughtful analysis of the potential ingredients to form the lipid core, and selected oleic acid and SDC because of their permeation enhancing properties [18–22]. Besides, SDC is known to form complexes with insulin during the formulation process, thus improving the encapsulation of insulin [23,24]. On the other hand, we chose polyarginine (PARG) as the material to form the polymer shell. Finally, to facilitate the dispersion of the oily droplets and improve the stability of the NCs in the intestinal fluids, we selected surfactants with different HLB values, *i.e.* poloxamer 188 and Span®80 [25]. Once formulated, we determined the physicochemical properties of these NCs and their capacity to promote the absorption of insulin in different *in vitro* and *in vivo* intestinal models.

2. Materials and methodology

2.1. Materials

Recombinant human insulin hexamer Insuman® (Mw 5808 Da) was kindly provided by Sanofi (Paris, France). Poly-L-Arginine (Mw 26–37 kDa) was purchased from Polypeptide Therapeutic Solutions (PTS, Valencia, Spain). Analytical grade poloxamer 188, oleic acid, Span® 80, SDC and Triton™ X-100 were purchased from Sigma Aldrich (St. Louis, USA). Pharmaceutical grade poloxamer 188 was purchased from BASF (Ludwigshafen, Germany); pharmaceutical grade oleic acid and Span® 80 were purchased from Croda (Snaith, UK); pharmaceutical grade SDC was purchased from New Zealand Pharmaceuticals (Palmerston North, New Zealand). Pancreatin (8xUSP) was purchased from Biozym (Hamburg, Germany). Sephadex® G-50 was purchased from GE healthcare (Little Chalfont, UK). The 1,10-dioctadecyl-3,3,30,30-tetramethylindodicarbocyanine perchlorate fluorescent dye (DiD oil, Em 644 nm; Ex 663 nm) was obtained from Life Technologies (Eugene, USA). Human colorectal adenocarcinoma Caco-2 cells (ATCC® HTB37 ™) were purchased from American Type Culture Collection (Manassas, VA, USA). High glucose Dulbecco's modified eagle medium (DMEM) and non-essential amino acid (NEAA) solution were purchased from Sigma Aldrich (St. Louis, USA), while heat inactivated fetal bovine serum (FBS), penicillin-streptomycin solution, L-glutamine, phosphate-buffered saline (PBS), Dulbecco's phosphate-buffered saline with calcium and magnesium (DPBS) were purchased from Lonza (Basel, Switzerland). Reagents for cytotoxicity assays were MTS based CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay kit (Promega, Madison, USA), LDH cytotoxicity detection kit plus (Roche, Mannheim, Germany) and Neutral Red based *In Vitro* Toxicology Assay Kit, (Sigma Aldrich, St. Louis, USA). Ultrapurified water was obtained from Millipore Milli-Q Plus water purification system (Darmstadt, Germany). HCl and NaOH solutions are purchased from Scharlab (Barcelona, Spain). All the other chemicals were of analytical grade.

2.2. Preparation of the PARG NCs

Polyarginine based NCs were prepared by a modified solvent displacement technique previously developed by our laboratory [26,27]. Different oils (oleic acid or Miglyol® 812N) and surfactants (Span®80, Tween®80 or Labrasol®) at different concentrations were explored for the formation of the NC's core. Finally, at the optimum condition, insulin was dissolved in 0.01 N HCl (pH ~ 2.1) at a concentration of 15 mg/mL, and 0.1 mL of this solution was transferred to an organic phase composed by 62.5 μL oleic acid, 20 mg surfactant Span®80, 4.1 mL acetone and 0.8 mL ethanol with/without 2.5 mg SDC. This organic phase was mixed using a vortex agitator (VELP Scientifica, Usmate, Italy) and immediately poured onto 10 mL of ultrapure water or pH 5.5 acetate/citrate buffer. In the first case, the pH of the external aqueous phase was adjusted with 0.1 N NaOH. Alternatively, 10 mM, 20 mM, 30 mM and 50 mM pH 5.5 acetate or citrate buffer was used to prepare the aqueous phase to ensure the desired final formulation pH (pH 5.5). In all cases, the aqueous phase solution contained 0.05% (w/v) PARG and 0.25% (w/v) of poloxamer 188. After magnetic stirring for 10 min, the solvents were evaporated under vacuum, decreasing the volume of the final formulation from 15 mL to 5 mL in a rotavapor (Heidolph Hei-VAP Advantage, Schwabach, Germany). Oleic acid-based nanoemulsions, used as controls for some experiments, were prepared by the same method without incorporating PARG.

Fluorescent NCs to be used for *in vitro*/*in vivo* studies were also produced by adding 50 μg DiD to the organic phase. The absence of dye leakage was assessed upon incubation of the nanocapsules in PBS, at 37 °C for up to 4 h.

2.3. Physicochemical and morphological characterization of PARG NCs

Particle size distribution and PDI were determined by dynamic light scattering (DLS) and zeta potential was calculated from the electrophoretic mobility values determined by laser doppler anemometry (LDA). Both were obtained with a Malvern Zeta-sizer device (NanoZS, ZEN 3600, Malvern Instruments, Worcestershire, UK) equipped with a red laser light beam ($\lambda = 632.8$ nm). To measure the particle size and PDI, a volume of 50 μL of the formulations was diluted with 950 μL of ultrapure water. For the *Z*-potential measurements, the sample was diluted with 1 mM KCl solution in the same proportion. The analysis was performed at 25 °C with at least, three different batches and each batch was analyzed in triplicate. The morphological analysis of the NCs was carried out in a transmission electron microscope (TEM, CM12, Philips, Netherlands). The samples were stained with phosphotungstic acid (2%, w/v) solution and placed on cupper grids with Formvard® for TEM observation.

2.4. Association of insulin to PARG NCs

The AE of insulin to PARG NCs was determined upon separation of the NCs from the suspending aqueous medium. The analysis was done by both indirect and direct methods. Briefly, in the indirect method, 2 mL of NC formulation was ultracentrifuged (Beckman Coulter, Optima L-90K, Brea, USA) at 82,656 g for 1 h at 15 °C, and the amount of free insulin in the undernatant was determined using reverse phase isocratic HPLC (Agilent, 1100 Series, Santa Clara, USA) method. The phosphoric acid/sodium perchlorate buffer was mixed with acetonitrile at different volume phase ratios, in order to produce two different mobile phases (93:7 as phase A and 43:57 as phase B), and C18 column (Superspher® RP-18 endcapped) was used as stationary phase. The AE of insulin in