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Development and characterization of a cationic lipid nanocarrier as non-viral vector for gene therapy

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ABSTRACT

The aim of the present work was to produce a cationic solid lipid nanoparticle (SLN) as non-viral vector for protein delivery. Cationic SLN were produced by double emulsion method, composed of softisan® 100, cetyltrimethylammonium bromide (CTAB), Tween® 80, Span® 80, glycerol and lipoid® S75 loading insulin as model protein. The formulation was characterized in terms of mean hydrodynamic diameter (z-ave), polydispersity index (PI), zeta potential (ZP), stability during storage time, stability after lyophilization, effect of toxicity and transfection ability in HeLa cells, *in vitro* release profile and morphology. SLN were stable for 30 days and showed minimal changes in their physicochemical properties after lyophilization. The particles exhibited a relatively slow release, spherical morphology and were able to transfect HeLa cells, but toxicity remained an obstacle. Results suggest that SLN are nevertheless promising for delivery of proteins or nucleic acids for gene therapy.

1. Introduction

New perspectives for diagnostic, treatment and prevention of untreatable diseases are emerging with gene therapy (Jiang et al., 2012). Non-viral transfection agents have recently been investigated for *in vitro* and *in vivo* applications in gene delivery. These systems should be stable, non-toxic, biocompatible, have low cost, and able to be delivered (targeted) to a specific site, transfer high amounts of material, and have high therapeutic efficacy (Tabatt et al., 2004a). Nano-vectors have received some attention due to their biocompatibility, safety (Liu et al., 2009), less immunogenic, easy methods of production on a large scale, and ability to be surface-modified (Boulaiz et al., 2005; Jiang et al., 2012; Torchilin, 2006). However, non-viral nano-vectors usually show low transfection efficiencies (Liu et al., 2009). Innovative cationic lipid nanocarriers, such as liposomes (Brgles et al., 2012), emulsions (Bozelli et al., 2012), and solid lipid nanoparticles (Choi et al., 2008; Tabatt et al., 2004a; Vighi et al., 2012), have been therefore tested in the transfection of proteins and nucleic acids.

Cationic SLN are promising systems to produced non-viral vectors because the former are easy to produce in large scale, are cost-effective and have reduced production time (Severino et al., 2012b). In the past decade, various groups have been working on the topic, and many publications have showed that cationic SLN are promising systems to use in transfection, indeed they are the most studied non-viral vectors (e.g. Vighi et al. (2012), Vighi et al. (2010), Olbrich et al. (2001), Rudolph and Rosenecker (in press)). But, it is known that the transfection efficiency with non-viral vectors is limited by several factors, e.g., internalization, disruption of the membrane of endosomes, and material delivery towards the nucleus (Vighi et al., 2012).

To transfect cells, SLN need cationic surface properties, which can be provided by adding benzalkonium chloride (Severino et al., 2013), cetylpyridinium chloride (Taveira et al., 2012), cetyltrimethylammonium bromide (CTAB) (Fangueiro et al., 2014), dimethyldioctadecylammonium (DDAB) and N-[1-(2,3-dioleoyloxy)-propyl]-N,N,N-trimethylammonium methyl-sulfate (DOTAP)

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(Carbone et al., 2012), hexadecyl-PTA iodide and octadecyl-PTA iodide (Cortesi et al., 2012; Tabatt et al., 2004b). Cationic surface properties were shown to determine the physical and chemical characteristics of SLN, increase the bioavailability of the loaded drug, increase intracellular penetration, influencing the targeting properties in blood circulation, and the rate of clearance (Doktorovova et al., 2012, 2011; Juillerat-Jeanneret and Schmitt, 2007).

Severino et al. has development a SLN formulation by double emulsion method, and the results of its physicochemical analysis has shown that this SLN could be a promising non-viral vector. The purpose of the present study was to use CTAB as cationic lipid in the development of cationic SLN and their characterization for transfection activity. The cationic SLN were loaded with insulin as model protein and were characterized in terms of mean hydrodynamic diameter (z-ave), polydispersity index (PI), zeta potential (ZP), morphology, encapsulation efficiency (EE), storage stability, and stability after lyophilization, toxicity effect and ability to transfect HeLa cells.

2. Materials and methods

2.1. Materials

Hydrogenated coconut-glycerides (Softisan® 100) were a gift from Sasol GmbH (Germany). Polysorbate 80 (Tween® 80) and Coomassie blue were obtained from Sigma (Portugal). Mono/oleate sorbitan oleate (Span® 80) was donated by Croda (Brazil), hydrogenated soya lecitin (Lipoid® S75) was a gift from Lipoid (Germany); Cetyltrimethylammonium bromide (CTAB) was obtained from Vetec Química Fina Ltda. (Brazil). DMEM/F-12 culture medium and fetal bovine serum (FBS) were obtained from Gibco. Insulin (Humulin®R Regular) was obtained from Pfizer Ltda (Sao Paulo, Brazil). Double distilled water was used after filtration in a Millipore system (home supplied).

2.2. Methods

2.2.1. SLN preparation

The method of double emulsion (w/o/w) was chosen for the SLN production to avoid high temperatures that contribute to protein denaturation. For the production of internal aqueous phase (IP) (insulin and water) and the lipid phase (LP) (490 mg Softisan® 100, 10 mg CTAB, 5 mL glycerol, 220 mg Span® 80, 100 mg Lipoid® S75) were, separately, heated to a temperature 10 °C above the lipid phase transition. The IP was added into the LP and homogenized with high shear homogenization (Ultra-Turrax®, IKA, T25, German) for 10 min, with intensity of 10,000 rpm and maintained at constant temperature. A part of external aqueous phase (EP) cooled (0.25 g Tween® 80 and 40 mL water) was added to the emulsion (w/o) previously formed. It was maintained by high shear homogenization for 2 min at 10,000 rpm. Then, it was transferred to magnetic stirring (Tecnal, TE-0851, Brazil), and added to the other part of the EP and kept under stirring for additional 20 min.

2.2.2. Particle size, polydispersity, and zeta potential analysis

The SLN was evaluated with respect to the hydrodynamic mean size (z-ave), polydispersity index (PI), and zeta potential (ZP). The mean size was determined by Dynamic Light Scattering (DLS; Zetasizer Nano NS, Malvern, UK). The samples were diluted with ultrapurified water to weaken the opalescence before particle size measurements. ZP was analyzed in 0.9% (w/v) NaCl solution, adjusting conductivity to 50 μS/cm. Radomska-Soukharev suggests the use of distilled water or water with very low conductivity to check ZP (Radomska-Soukharev, 2007). The ZP was calculated from the electrophoretic mobility using the Helmholtz–Smoluchowski equation. The analysis was performed using the software included in the system.

2.2.3. Encapsulation efficiency (EE)

The protein encapsulated into SLN was quantified by the Bradford method (Bradford, 1976), (using bovine serum albumin (BSA; Sigma, Portugal) as a standard. The absorbance reading was held in spectrophotometer at a wavelength of 595 nm, using a quartz cuvette. The calibration curve was performed with concentrations of 0, 10, 15, 20, 25, 30, 40, 50, 70, 90, 110, 140, 170, 200, 230, 270, 310, 350, 400, 450, 500, 600 μg/mL. To determine the EE of SLN samples were subjected to ultra-filtration (Amicon, Beverly, USA) using ultra-filtration membranes (Millipore, NMWL 30,000, Billerica, USA), after centrifugation (12,500 rpm during 30 min (ScanSpeed mini centrifuge from Scanlaf, Denmark)) and 500 μL of the supernatant was added to a solution of Coomassie Brilliant blue (Bradford reagent), followed by agitation. Then EE was calculated using Eq. (1).

$$\text{EE}\ (\%) = \frac{(\text{total of protein IP}) - (\text{total of protein supernatant})}{(\text{total of protein IP})} \times 100 \quad (1)$$

2.2.4. Stability testing

SLN was stored in amber flask at 4 °C. Particle parameters were monitored z-ave, PI and ZP. The parameters were analyzed at time zero, 24 h, 15 and 30 days.

2.2.5. Stability after lyophilization

Approximately 5.0 g of formulation were placed into a flask and frozen at −80 °C, after freezing the samples were lyophilized for 48 h using a vacuum pump followed by a steam condenser. After lyophilization, the formulation was reconstituted in Milli'Q water and sonicated for 60 min (Cole-Parmer, 8890, Illinois, USA). The average z-ave, PI, ZP and EE were determined.

2.2.6. In vitro release experiments

The protein release from SLN was evaluated using the dialysis bag diffusion method (Yang et al., 2013). The dialysis bag retains SLN and allows the released free protein to diffuse into dissolution media. The bags were soaked in Milli-Q water for 12 h before use. Two milliliters of SLN were poured into the dialysis bag and the two ends of the bag were sealed by clamps. The bags were then placed in a vessel containing 200 mL of phosphate buffer (pH 7.4), the receiving phase. The vessel was placed in a thermostatic shaker (New Brunswick, USA), at 37 °C, with agitation at a rate of 140 rpm. At predetermined time intervals (0–24 h), 1 mL samples were withdrawn and centrifuged 20,000 rpm for 20 min (Eppendorf, 5417R, USA). The supernatant was assayed for protein release, by using the Bradford method. All samples were analyzed in triplicate.

2.2.7. WST-1 cytotoxicity test

The biocompatibility of cationic SLN in HeLa cells was assessed by using the Cell Proliferation Reagent WST-1 (Roche Applied Science, USA) following the manufacturer's instructions. WST-1 is a colorimetric assay for quantification of cell proliferation and cytotoxicity based on the cleavage of the tetrazolium salt WST-1 by mitochondrial dehydrogenases in viable cells. Firstly, HeLa cells were cultivated in flasks with complete medium (Ham's/F-12 nutrient mixture containing 10% (v/v) fetal bovine serum, growth medium) and incubated at 37 °C, in a 5% CO_2 atmosphere. For sub-culturing, the supernatant was removed, cells were washed with PBS, and trypsin was added to cells which were incubated