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Cationic lipid nanosystems as carriers for nucleic acids

Q1 Rita Cortesi[1], Matteo Campioni[1], Laura Ravani[1], Markus Drechsler[2], Mirko Pinotti[1] and Elisabetta Esposito[1]

[1] Department of Life Sciences and Biotechnology, University of Ferrara, Ferrara, Italy
[2] Macromolecular Chemistry II, University of Bayreuth, Germany

Solid lipid nanoparticles (SLNs) consisting of tristearin or tribehenin, and monoolein aqueous dispersions (MADs) consisting of glyceryl-monoolein have been studied as potential nanocarriers for nucleic acids. The cationic character of nanocarriers was obtained by adding cationic surfactants, such as diisobutylphenoxyethyl-dimethylbenzyl ammonium chloride (DEBDA) or PEG-15 Cocopolyamine (PCPA), to the lipid composition. The products were characterised in terms of size and morphology by Cryo-TEM and PCS. The charge properties were determined by measuring the zeta potential. Our experimental protocol enabled us to obtain homogeneous and stable cationic nanosystems within 3–6 months of production. Assessment of cytotoxicity on HepG2 cells by MTT assays indicated that MAD preparations were less toxic than SLN, and in general PCPA-containing formulations are less cytotoxic than DEBDA-containing ones. The formation of electrostatic complexes with salmon sperm or plasmid DNA, used as model nucleic acids, was evaluated by electrophoresis on agarose gel. The results confirmed that all the formulations studied are able to form the complex. Finally, we investigated the ability of SLN and MAD to deliver DNA into HepG2 cells, and to this purpose we exploited expression plasmids for green fluorescent protein or firefly luciferase. Although with reduced efficiency, the results showed that the produced nanocarriers are able to convey plasmids into cells. The data obtained encourage further study aimed at improving these new formulations and proposing them as novel *in vitro* transfection reagents with potential application to *in vivo* delivery of nucleic acids.

Introduction

Over the last decade, gene transfer has received enormous attention as a therapeutic strategy for a large number of pathologies including genetic, neoplastic and infectious diseases [1–5]. In the substitutive gene therapy, a normal copy of the affected gene, or better its coding sequence, is delivered into target cells to restore normal gene expression and thus physiological function. Other therapeutic strategies are based on delivery of oligonucleotides [6] or vectors driving the expression of regulatory/antisense RNAs [7,8]. In all cases, a major issue is the design of an efficient system for their delivery into target cells and assuring their stability for a sufficient period of time to exert a pharmacological effect [9–11]. Various approaches, including the use of viral vectors, have been proposed [9,12,13], each possessing advantages and drawbacks. However, the use of lipid dispersions as gene delivery systems has attracted wide attention of worldwide formulators due to their potential applications [14,15].

Among lipid dispersions, solid lipid nanoparticles (SLN) represent a new generation of delivery systems. These sub-micron colloidal carriers are composed of physiological lipids, dispersed in water or in aqueous surfactant solution. Nanodisperse phase has a solid matrix of crystalline solid lipids, able to protect

Corresponding author: Cortesi, R. (crt@unife.it)

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encapsulated molecules from degradation and to modulate their release [14,16,17]. It is well known that mixtures of surfactants and lipids are able to form a variety of assemblies determined by packing parameter or spontaneous curvature (i.e. micelles, liquid crystalline phase) [18]. Moreover, the emulsification in water of surfactant-like lipid leads to the formation of aqueous nanostructured dispersions of a complex lyotropic liquid crystalline phase that can be characterised by lamellar, hexagonal or cubic structure [18]. The predominance of one species over the other depends on temperature and water content of the system [19].

Monoolein aqueous dispersions (MAD) typify a newer, attractive delivery system. In particular MAD stabilised by the addition of a block copolymer like poloxamer 407, are mainly constituted of dispersed nanoparticles, such as cubosomes and hexosomes, often in coexistence with vesicles [19,20]. Cubosomes are nanostructured particles of cubic liquid crystalline phases dispersed in water and characterised by an inner structure with a cubic crystallographic symmetry [21]. On the other hand, hexasomes are particles of hexagonal shape with an inner structure with hexagonal symmetry and/or curved concentric striations [22]. The methods of MAD production [23–25] and the inner structure of dispersed nanoparticles [26–29] have been widely investigated [23–29].

It should be pointed out that cationic nanosystems are able to bind DNA molecules on their surface by ionic interactions. The surface of these preformed cationic nanoparticles is indeed positively charged due to the presence of cationic molecules within the nanosystem composition. In this way, negatively charged nucleic acid is complexed to the surface of preformed cationic nanocarriers, namely MAD and SLN. In the present paper, as cationic molecules we explored two different cationic surfactants, namely diisobutylphenoxyethyl-dimethylbenzyl ammonium chloride (DEBDA) and PEG-15 Cocopolyamine (PCPA). The objective was to investigate the potential of new positively charged lipid nanocarriers (MAD and SLN) to convey nucleic acids. In particular, the specific aims were (a) the preparation and characterisation of positively charged nanocarriers, namely MAD and SLN by using DEBDA or PCPA; (b) to test their ability to complex DNA; (c) to assess their effects on cell proliferation of *in vitro* cultured human hepatocellular carcinoma HepG2 cells; and finally (d) to evaluate the ability of these cationic nanosystems to transfect DNA into HepG2 cells.

Materials and methods

Materials

The glyceryl monooleate RYLO MG 19 (MO) was a gift from Danisco Cultor (Grindsted, Denmark). Poloxamer 407 (Pluronic F127) (PEO98-POP67-PEO98) and poloxamer 188 (Lutrol F 68), oxirane, methyl-, polymer with oxirane (75:30) were obtained from BASF (Ludwigshafen, Germany). Compritol 888 ATO, a mixture of approximately 15% mono-, 50% di- and 35% triglycerides of behenic acid (tribehenin), was provided by Gattefossé (Saint Priest-France). Salmon sperm DNA (SS-DNA) was obtained from Sigma–Aldrich (Milano, Italy). The pGL3-control vector, having the firefly luciferase coding region under the control of the SV40 promoter, was obtained by PROMEGA (Madison, WI, USA). The pCMV5-FIX and pPKCβ-GFP are eukaryotic expression vectors in which the coding sequence for coagulation factor IX (FIX) or for the chimaeric protein kinase PKCß – green fluorescent protein (GFP), respectively, are cloned under the control of the CMV promoter [30,31]. Hyamine 1622 (diisobutylphenoxyethyl-dimethylbenzyl ammonium chloride, DEDBA), Polyquart H81 (PEG-15 Cocopolyamine, PCPA) and Tristearin (stearic triglyceride) were purchased from Fluka (Buchs, Switzerland). All chemicals were used as received.

MAD preparation

Production of dispersions was based on the emulsification of MO (4.5%, w/w) and Poloxamer 407 (0.5%, w/w) in water (90%, w/w), as described by Esposito et al. [32]. In the present study, after emulsification, the dispersion was subjected to homogenisation (15,000 rev min^{-1}, Ultra Turrax, Janke & Kunkel, Ika-Werke, Sardo, Italy) at 60 °C for 1 min, then cooled and maintained at room temperature in glass vials.

The dispersion was then filtered through Whatman™ mixed esters cellulose membrane (0.6 μm pore size) (Sigma–Aldrich, Milano, Italy) in order to separate big MO/poloxamer aggregates. Dispersion characterisation of the MO dispersions as well as *in vitro* experiments were performed after filtration, without taking into account the fraction of larger particles whose dimensions have been measured by laser diffraction (Horiba, LA-920, Horiba Ltd., Tokyo, Japan).

SLN preparation

SLN were prepared by stirring, followed by ultrasonication [33]. Briefly, 1 g of lipid consisting in tristearin or tribehenin in mixture with the cationic surfactant DEBDA or PCPA at the indicated concentrations, was melted at 75 °C. The fused lipid phase was dispersed in 19 ml of an aqueous poloxamer 188 solution (2.5% w/w). The obtained emulsion was subjected to ultrasonication (Microson TM, Ultrasonic cell Disruptor) at 6.75 kHz for 15 min and then cooled down to room temperature by placing it in a water bath at 22 °C. SLN dispersions were stored at room temperature.

Characterisation of lipid dispersions

Submicron particle size analysis was performed using a Zetasizer 3000 PCS (Malvern Instr., Malvern, England) equipped with a 5 mW helium neon laser with a wavelength output of 633 nm. Measurements were made at 25 °C at an angle of 90°. Data were interpreted using the 'method of cumulants' [32,33]. Samples were diluted with MilliQ water to an adequate scattering intensity prior the measurement. The results are presented as intensity weighted average (*Z*-ave) value obtained from three measurements (10 runs each) with corresponding standard deviation. Each experimental value results from three independent experiments performed in triplicate.

The electrophoretic mobility of cSLN/DNA complexes was measured at room temperature by mean of a Zetasizer 3000 PCS (Malvern Instr., Malvern, England) in 1 mM NaCl solutionto avoid the fluctuation in the ζ potential due to variations in the conductivity of purified water. Samples were injected in a glass capillary cell and analysed under a constant voltage after focusing with a 5 mW helium neon laser. The ζ potential, in mV, was automatically calculated from the electrophoretic mobility based on the Helmholtz–Smolukowski equation. Each sample was measured three times then mean value and standard deviation (SD) are presented.

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