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Phosphatidylcholine with conjugated linoleic acid in fabrication of novel lipid nanocarriers

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ABSTRACT

Exploration of novel lipid-based formulations has been an aim for researchers over the last decades. Lipid nanoparticles have been extensively studied as bioactive agent carriers in the pharmaceutical and biomedical fields. In our studies, we have provided properties of two different types of lipid nanocarriers, i.e. solid lipid nanoparticles (SLN) and nanostructured lipid carriers (NLC) prepared via high pressure homogenization. The choice of surfactants in the structural design is one of the most significant approaches to achieve nanocarriers with desirable parameters that acquire both good stability and requested physical state.

The present work has been carried out to explore the potential for fabrication of stable and nearly monodisperse lipid nanoparticles stabilized by functionalized phosphatidylcholine, i.e., 1,2-di(conjugated) linoleoyl-*sn*-glycero-3-phosphocholine ((CLA)PC) which may comprise a potential lipid prodrug. Additionally, 1,2-distearoyl-*sn*-glycero-3-phosphocholine ((SA)PC) was taken into account as the surfactant to be compared. The sizes of the studied nanosystems (about 130–230 nm in diameter D_H) along with the size distribution (polydiyspersity index PdI below 0.3) were determined by dynamic light scattering (DLS), while the morphology was imaged by atomic force microscopy (AFM). The studied nanoparticles physical state was characterized by differential scanning calorimetry (DSC) and X-ray diffraction (XRD). The NLCs have shown less ordered crystalline structure than the SLNs nanoproducts, this observation being conferred by the inclusion of a liquid lipid. The *in vitro* cytotoxicity studies were performed on human cancer epidermoid carcinoma (A431) and MeWo cells using trypan blue staining and MTT assays. Nanocarriers labeled by cyanine IR-780 were efficient in bioimaging (MeWo). Our results give new insights into the lipid nanoparticles containing the pharmaceutically promising (CLA)PC and may serve as guidelines for design and preparation of new effective delivery nanosystems, including anti-cancer applications.

1. Introduction

Phospholipids – composed of a glycerol backbone with phosphate group attached at *sn*-3 position and two fatty acid moieties at *sn*-1 and *sn*-2 positions – are substances considered as 'generally recognized as safe' (GRAS) for drug delivery by the Food and Drug Administration (FDA) and European Medicines Agency (EMA) [1,2]. Due to their amphipatic structure, they have wide application as emulsifiers in the food formulations, pharmaceuticals as well as in the cosmetic industry. These prominent amphiphilic agents have gained a special attention because of some essential health benefits such as improvement of memory, liver protection, cholesterol reduction and treating neurological disorders [3,4]. In nature, phospholipids are the main components of cell membranes in animals, plants and microorganisms. Moreover, they form semipermeable membranes playing important roles in separation, protection, and transportation of cellular constituents, as well as in cellular integrity and signaling [5].

Synthesis of new functionalized phospholipids by enzymatic or chemical routes, through varying fatty acid residues, might be an interesting approach to study these amphiphilic compounds with new, desired properties. The synthesis of lysophosphatidylcholine – docosahexaenoic acid conjugate (1-lyso-2-DHA-PC) and evaluation of their ability to protect ARPE-19 cell lines from carbonyl stress were described [6]. In a separate study, phosphatidylcholine was enzymatically modified with ethyl ferulate yielding feruloylated lysophospholipid [7]. Another report described chemo-enzymatic synthesis of novel

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structured phenolic phospholipids (with ferulic, sinapic, vanillic, syringic acids) having potential applications as novel antioxidants [8]. Recently, we presented a work on the synthesis of phosphatidylcholine with a mixture of predominantly *cis*-9,*trans*-11 and *trans*-10,*cis*-12 conjugated linoleic acid isomers, which had improved favourable bioavailability in comparison with CLA itself and exhibited cytotoxicity against three human cancer cell lines (HL-60, MCF-7, and HT-29) [9,10].

Lipid-origin formulations extent a wide range of potential compositions and include varied delivery systems ranging from lipid subcutaneous depots, intravenous emulsions or liposomal formulations to topical cosmetics. The majority of the mentioned formulations are ultimately filled into soft or sealed hard polysaccharide capsules for medical and commercial application, but may also be used as the liquid fill containers during preclinical development [11]. Several strategies using a phospholipid have been described, including liposomes, lipospheres, pharmacosomes, mixed micelles, micro- and nanoemulsions, self-emulsifying drug delivery systems, phytosomes, suspensions, solid lipid nanoparticles, nanostructured lipid carriers, vesicular phospholipid gels and phospholipid-drug complexes [12,13]. Phospholipids are frequently used in the design delivery carriers because of their active surface and penetration enhancing properties, as well as their capacity to self-assemble [14].

Colloidal delivery systems like solid lipid nanoparticles (SLN) and nanostructured lipid carriers (NLC) have been receiving considerable attention for several decades. SLNs are one of the most interesting colloidal systems, produced using biodegradable and biocompatible lipids which are solid at room and body temperature. Furthermore, if these lipid nanoparticles are produced by partially interchanging solid lipid with liquid lipid they form nanocarriers with more internal defects such as NLC. Among the methods for the preparation of lipid nanoparticles, the most widely described in the literature are the high pressure homogenization (HPH), high shear homogenization and ultrasound, solvent emulsification/evaporation, and the microemulsion-based approaches [15,16]. The achievement of physical and chemical stability of lipid nanoparticles – a major point of interest in the formulation of thermodynamically durable systems – requires careful selection of the surfactant and its concentration. The surfactant composition in colloidal systems has a major influence on the physical stability of the nanoparticles as well as their permeability into the cell. Furthermore, it can contribute to the safety of nanocarriers during administration in the body [17]. Despite biocompatibility and amphiphilic properties of phospholipids, the stabilization of a nanoparticulate formulation usually requires adding another surfactant beside the phosphatidylcholine, i.e.: Tweens, Poloxamers or bile salts [18].

As a continuation of our search for new efficacious drug delivery nanocarriers, their fabrication, desired physicochemical behavior and amenable biological evaluation [19–23], we focused our present studies on biodegradable lipid nanoparticles fabricated by HPH. The main aim of the present contribution is to describe potential of biologically active 1,2-di(conjugated)linoleoyl-*sn*-glycero-3-phosphocholine ((CLA)PC) in its efficiency to stabilize two different types of carriers, i.e. SLNs and NLCs with the most unimodal distribution in size, exhibiting sufficient chemical and colloidal stability and preservation of their structural state in the lipid matrix. A saturated phospholipid – 1,2-distearoyl-*sn*-glycero-3-phosphocholine ((SA)PC) – was taken for comparisons with ((CLA)PC) as nanoparticle stabilizing agent. For the structures and abbreviations of both phospholipids see Fig. 1. Biological impact of the best systems was tested on two human cell lines: epidermoid carcinoma (A431) and skin melanoma (MeWo) in order to evaluate the cytotoxicity of the tested compounds and the obtained nanosystems. The selected nanocarriers were labeled with hydrophobic dye, IR-780 to study intracellular localization in melanoma cells (MeWo), which revealed the best response for applied nanosystems.

2. Materials and methods

2.1. Materials

Solid lipid, cetyl palmitate (CRODAMOL; CP) with melting point 52–56 °C was provided by CRODA Inc. (England). The medium chain triglyceride oil (Miglyol 812 N; M812) was purchased from Cremer, Oleo Division (Germany). Stearic acid (> 95%), linoleic acid (> 99%), Tween 80 (polyoxyethylene sorbitan monooleate; T80), used as the nonionic surfactant and hydrophobic indocyanine diagnostic agent IR-780 (2-[2-[2-Chloro-3-[(1,3-dihydro-3,3-dimethyl-1-propyl-2H-indol-2-ylidene)ethylidene]-1-cyclohexen-1-yl]ethenyl]-3,3-dimethyl-1-propylindolium iodide; IR-780) were supplied by Sigma-Aldrich (USA). Water used for all experiments was doubly distilled and purified by means of a Millipore (Bedford, MA) Milli-Q purification system.

2.2. Synthesis of phosphatidylcholine-type surfactants

The studied phospholipids, 1,2-di(conjugated)linoleoyl-*sn*-glycero-3-phosphocholine ((CLA)PC), Fig. 1a) and1,2-distearoyl-*sn*-glycero-3-phosphocholine ((SA)PC), Fig. 1b), were prepared from cadmium chloride complex of *sn*-glicero-3-phosphocholine (GPC x $CdCl_2$) and stearic acid or conjugated linoleic acid (CLA) according to the method published previously [9]. CLA contained a mixture of isomers (percentage according to GC): 46% *cis*-9,*trans*-11, 45% *trans*-10,*cis*-12, 9% other CLA isomers and was prepared from linoleic acid according to the earlier described procedure [24].

In brief, the GPC x $CdCl_2$ (1.53 g, 2.7 mmol), 4-dimethylaminopyridine (DMAP, 662 mg, 5.4 mmol), and *N,N*'-dicyclohexylcarbodiimide (DCC, 2.35 g, 11.4 mmol) were dissolved in 65 mL of CH_2Cl_2 and then a solution of stearic acid or conjugated linoleic acid (10.3 mmol, 35 mL CH_2Cl_2) were added to start the reaction. The reaction mixture was protected from light and stirred magnetically at room temperature under nitrogen atmosphere. After 40 h, the reaction mixture was filtered and ion-exchange resin (DOWEX 50W X8, H^+ form) was used to dislodge the cadmium chloride and DMAP. The crude phosphatidylcholines were purified on a silica-gel column (Kieselgel 60, 230–400 mesh, Merck) using the following eluent: 65:25:4 $CHCl_3$/CH_3OH/H_2O (v/v/v). Phosphatidylcholines were obtained in very good yield (> 96%).

The chemical structure and purity of the synthesized compounds ((SA)PC, (CLA)PC) were confirmed by NMR spectroscopy (Bruker Avance DRX 300 spectrometer) and HPLC analysis according to the previously reported methods [10]. The spectral data of (SA)PC: δ_H (300 MHz, $CDCl_3$) 5.19 (m; 1H, H-2), 4.38 (m; 1H; one of H-1), 4.35–4.25 (m, 2H, H-α), 4.12 (dd, J 11.9 and 7.4, 1H, one of H-1), 3.98–3.86 (m, 2H, H-3), 3.85–3.77 (m, 2H, H-β), 3.36 (s, 9H, $N(CH_3)_3$), 2.35–2.21 (m, 4H, H-2a), 1.64–1.50 (m, 4H, H-3a), 1.36–1.15 (m, 56H, H-4a–16a); 0,88 (t, J 6.7, 6H, H-18a). δ_C (75 MHz, $CDCl_3$) 173.72 (C-1a in *sn*-2), 173.37 (C-1a in *sn*-1), 70.63 (d, $J_{C\text{-}P}$ 7.3, C-2), 66.50 (m, C-β), 63.58 (d, $J_{C\text{-}P}$ 3.9, C-3), 63.14 (C-1), 59.49 (d, $J_{C\text{-}P}$ 3.9, C-α), 54.55 (-N$(CH_3)_3$), 34.48 (C-2a in *sn*-2), 34.29 (C-2a in *sn*-1), 32.08 (C-16a), 30.13–29.16 (C-4a–15a), 25.14 (C-3a in *sn*-2), 25.05 (C-3a in *sn*-1), 22.83 (C-17a), 14.25 (C-18a). ('a' and 'α,β' denote signals from stearoyl moiety and the choline moiety, respectively). δ_P (121 MHz, $CDCl_3$): −0.13. High resolution electrospray ionization (HR-ESI) mass spectra were obtained on a Bruker micrOTOF-Q. HR-ESI calculated for (SA)PC was 790.6320 $[M+H]^+$ and the result was found at 790.6332.

The spectral data of (CLA)PC: δ_H (300 MHz, 2:1 $CDCl_3$/CD_3OD (v/v)) 6.06 (m, 2H, H-11a, and H-11b), 5.71 (m, 2H, H-10a, and H-12b), 5.41 (dtd, J 9.7, 6.9, 2.7, 2H, H-12a, and H-10b), 5.11–4.95 (m, 3H, H-9a, H-13b, and H-2), 4.19 (m, 1H, one of H-1), 4.07–3.97 (m, 2H, H-α), 3.93 (dd, J 12.0, 7.0, 1H, one of H-1), 3.83–3.72 (m, 2H, H-3), 3.41–3.33 (m, 2H, H-β), 2.98 (s, 9H, $N(CH_3)_3$), 2.15–2.04 (m, 4H, H-2a, and H-2b), 1.97–1.77 (m, 8H, H-8a, H-13a, H-9b, and H-14b), 1.45–1.29 (m, 4H, H-3a, and H-3b), 1.21–1.00 (m, 32H, H-4a–7a, H-