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A simple and effective approach for the treatment of dyslipidemia using anionic nanoliposomes

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ABSTRACT

The present study was undertaken to evaluate the anti-dyslipidemic effects of nanoliposomes with different phospholipid compositions. Three sets of liposomal formulations (20 Mm; 100 nM in size) were prepared with low (SPC), medium (POPC) and high (HSPC) phase transition temperature values with and without cholesterol and anionic phosphatidyl glycerol (HSPC/DSPG; POPC/DMPG; SPC/EPG). The liposomal preparations were characterized for their size and zeta potential (dynamic light scattering), *J774A.1* macrophages uptake (flow cytometry) and lipid-modifying effects (tyloxapol-induced hyperlipidemic mouse model). Anionic formulations displayed the highest rate of uptake by macrophages. Among them, HSPC/DSPG and SPC/EPG liposomes had the best lipid-modifying activity. These two formulations exerted favorable impact on all lipid profile parameters by reducing LDL-C (by up to 76% [HSPC/DSPG] and 86% [SPC/EPG]), total cholesterol (by up to 52% [HSPC/DSPG] and 68% [SPC/EPG]), triglycerides (by up to 88% [HSPC/DSPG] and 73% [SPC/EPG]), apoB (by up to 44% [HSPC/DSPG] and 35% [SPC/EPG]) and elevating HDL-C (by up to 85% [HSPC/DSPG] and 75% [SPC/EPG]) concentrations. Atherogenic indices were also effectively reduced following HSPC/DSPG (by up to 69%) and SPC/EPG (by up to 79%) injections. Empty, cholesterol-free nanoliposomal formulations containing 75% anionic phospholipid (PG) might serve as effective and rapid acting anti-dyslipidemic agents. Further research is warranted to confirm the observed anti-dyslipidemic effects of anionic nanoliposomes in diet-induced hyperlipidemic models, and also to evaluate the potential protective effects in regressing atheromatous lesions.

1. Introduction

Coronary artery disease (CAD) is the leading cause of mortality worldwide. Annually, CAD imposes significant financial burden and casualties on healthcare systems. Therefore, the current focus of many health policy-making authorities is to find effective ways for the optimal prevention and management of CAD. Among the most important modifiable risk factors for CAD is dyslipidemia, which is characterized by elevated levels of low-density lipoprotein cholesterol (LDL-C) and decreased concentrations of high-density lipoprotein cholesterol (HDL-C). Findings of several landmark trials have confirmed the beneficial impact of lowering LDL-C levels on both primary and secondary prevention of cardiovascular disease (CVD) [1–4]. Most of the international guidelines recommend reduction of LDL-C to <70 mg/dL as an optimal target for primary prevention of patients at very high risk of CAD [5–9]. However, statins – as the most potent LDL-lowering class of drugs – are able to reduce serum LDL-C by up to 30–50% in most cases [10]. Besides, such a reduction would be achieved with aggressive therapy which may itself predispose the patient to adverse events such as myopathies and hepatotoxicity. Combination of low HDL-C and elevated triglycerides, known as atherogenic dyslipidemia, is another dyslipidemic phenotype that is frequently seen in patients with diabetes mellitus and metabolic syndrome, and is also a definite contributor to atherosclerotic CVD [11,12]. In addition to their inadequate potency for decreasing serum LDL-C to the optimal level, statins have also limited effect on serum HDL-C and triglycerides concentrations [13]. Owing to these limitations, there

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has been an increasing attempt to find novel lipid-modifying agents with improved efficacy and safety [14–22].

Liposomes are artificial phospholipid bilayers that have been used as carriers to enhance potency and reduce toxicity of drugs. These constructs have generated great interest for biomedical purposes owing to their biocompatibility, biodegradability, safety and lack of immunogenicity [23]. Nanoliposomes are known to have a short half-life in the circulation. Biodistribution studies have shown that nanoliposomes are efficiently taken up by the hepatic tissue within a few hours of intravenous injection [24]. This uptake has been shown to be performed through receptor mediated endocytosis, a process which normally occurs for the clearance of LDL from the circulation (Fig. S1) [25]. On the other hand, there has been evidence indicating the coalescence of liposomes containing 75–100% anionic phospholipids with LDL and the uptake of resulting complexes via either LDL receptors or macrophages [26–31]. In addition, liposomes have been shown to undergo vast lipid exchange with plasma lipoproteins, an interaction that promotes reverse cholesterol transport from peripheral tissues to liver [32–34]. In spite of these promising mechanisms, the potential of liposomes as anti-dyslipidemic agents has not been well clarified. To this end, the present study aimed to evaluate the impact of intravenous administration of different phospholipid liposomes on serum lipid profile.

Supplementary Fig. S1 related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.colsurfb.2014.07.045.

2. Materials and methods

2.1. Chemicals

Hydrogenated soy phosphatidylcholine (HSPC), soy phosphatidylcholine (SPC), Egg phosphatidylglycerol (EPG), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), 1,2-dimiristoyl-*sn*-glycero-3-phosphoglycerol (DMPG), 1,2-distearoyl-*sn*-glycero-3-phosphoglycerol (DSPG) were purchased from Lipoid GmbH (Germany). The fluorescent dye 1,1-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI) was from Invitrogen (USA). Tyloxapol and simvastatin were from Sigma (Germany) and Sami Saz Pharmaceutical Company (Iran), respectively. Alamar Blue was purchased from Biosource (International Inc., USA).

2.2. Preparation of liposomes

Conventional liposomes were prepared from different phospholipids (HSPC, SPC, POPC, DMPG, EPG or DMPG) by the method of dried lipid-film hydration plus extrusion. Phospholipids were different in their liquid crystalline transition temperature (T_m) and charge. Briefly, three sets of liposomal formulations (Table 1) were prepared with low (soy phosphatidylcholine, SPC; $T_m < 0\,^{\circ}C$), medium (palmitoyl-oleoyl-phosphatidylcholine, POPC; $T_m = 0\,^{\circ}C$) and high (hydrogenated soy phosphatidylcholine, HSPC; $T_m = 55\,^{\circ}C$) T_m values with (HSPC/chol; POPC/chol; SPC/chol) and without cholesterol (HSPC; POPC; SPC) and anionic phosphatidyl glycerol (HSPC/DSPG; POPC/DMPG; SPC/EPG) at final lipid concentration of 20 mM. The anionic formulations were cholesterol-free and contained an anionic phospholipid amounting to 75% of total phospholipid content. Fluorescence labeling of liposomes was performed using DiI (~0.2 mol% of phospholipid). After vacuum and freeze drying, the obtained thin lipid film was hydrated with histidine (10 mM)/sucrose (10%) buffer (pH 6.5). The resultant multilamellar dispersions were reduced in size lamellarity by vortexing, sonication and extrusion through 200 nm (5 cycles) and 100 nm (11 cycles) polycarbonate membranes using a thermobarrel extruder (Lipex Biomembranes Inc., Canada) [35].

2.3. Characterization of the liposomes

The particle diameter of each sample together with its polydispersity index was measured in triplicate using Dynamic Light Scattering Instrument (Nano-ZS; Malvern, UK). The zeta potential of liposomes was determined on the same machine using the zeta potential mode as the average of 20 measurements [36].

2.4. Phospholipid assay

The phospholipid concentration of formulations was determined according to Bartlette test [37]. Briefly, liposomal formulations (80 ± 50 nmol of phosphate containing lipid) were added into disposable borosilicate glass tubes. Then, 0.4 mL of 10 N H_2SO_4 was added to each tube. In the fume hood, the sample was digested at 195–210 °C for 60 min using a hot-plate apparatus. The tubes were then cooled for about 10 min at room temperature. Afterwards, 0.1 mL of 10% H_2O_2 was added to each sample and tubes were again heated for 10 min at 190–210 °C. After cooling the tubes for about 10 min, 4.7 mL of molybdate reagent and 0.5 mL of 10% ascorbic acid were added to each tube and vortexed immediately for 10 s. Samples were then heated at 100 °C for 10–20 min, quickly cooled and finally were measured for optical density at 800 nm. Phospholipid concentration of samples was calculated according to the phosphate standard curve.

2.5. Liposome uptake by macrophages

J774.A1 macrophages were seeded in 12-well tissue culture plates at 3.0 × 105 cells/well in RPMI 1640 supplemented with 10% fetal calf serum and antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin). Cells were incubated with different liposomal preparations (final phospholipid concentration in each well was set at 0.1 mM). After 3, 10 and 24 h of incubation, cells were washed with cold phosphate buffered saline (PBS) and then lysed using 1% Triton X-100. Flow cytometric analyses were performed on the obtained lysate by means of a Partec® instrument (Münster,

Table 1
Particle size distribution, zeta potential and polydispersity of liposomal formulations.

Formulation	Molar ratio	Z-average (d nm)	PDI	Zeta potential (mV)
HSPC	–	129.6 ± 2.76	0.175 ± 0.11	−10.2 ± 4.92
HSPC/Chol	5/1	121.7 ± 26.52	0.172 ± 0.12	−9.76 ± 8.19
HSPC/DSPG	1/3	108 ± 1.01	0.34 ± 0.01	−50.9 ± 14.50
SPC	–	81.64 ± 3.57	0.295 ± 0.04	−10.1 ± 0.57
SPC/Chol	5/1	82.40 ± 1.75	0.26 ± 0.03	−11.30 ± 1.70
SPC/EPG	1/3	95.31 ± 35.77	0.20 ± 0.02	−49.7 ± 15.8
POPC	–	119.30 ± 15.56	0.18 ± 0.02	−10.02 ± 2.65
POPC/Chol	5/1	121.98 ± 31.28	0.17 ± 0.10	−9.56 ± 0.11
POPC/DMPG	1/3	98.97 ± 7.96	0.25 ± 0.17	−49.55 ± 3.23

PDI, polydispersity index. Values are expressed as mean ± SD, $n = 3$.

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