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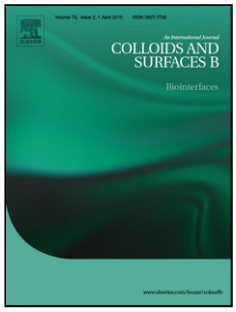

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Author: Amirhossein Sahebkar Ali Badiee Mahdi Hatamipour Majid Ghayour-Mobarhan Mahmoud Reza Jaafari

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Apolipoprotein B-100-Targeted Negatively Charged Nanoliposomes for the Treatment of Dyslipidemia

Amirhossein Sahebkar, PhD[a,b], Ali Badiee, PhD[c], Mahdi Hatamipour, MSc[c], Majid Ghayour-Mobarhan MD,[d] Mahmoud Reza Jaafari, PhD[a,c*]

[a]*Biotechnology Research Center, Mashhad University of Medical Sciences, Mashhad, Iran*

[b]*Metabolic Research Centre, Royal Perth Hospital, School of Medicine and Pharmacology, University of Western Australia, Perth, Australia*

[c]*Nanotechnology Research Center, School of Pharmacy, Mashhad University of Medical Sciences, Mashhad, Iran*

[d]*Cardiovascular Research Center, Mashhad University of Medical Sciences, Mashhad, Iran*

**** Correspondence:*** Professor Mahmoud Reza Jaafari, Biotechnology Research Center, School of Pharmacy, Mashhad University of Medical Sciences, Mashhad 91775-1365, jafarimr@mums.ac.irIran. Tel: +985118823255; Fax: +985118823251; E-mail:

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Abstract

Background: Anionic nanoliposomes can interact with serum lipoproteins and regulate lipid metabolism through several mechanisms. This study aimed to evaluate the lipid-modifying effects of anionic immunoliposomes targeted against apoB, an important component of atherogenic lipoproteins.

Methods: Two sets of nanoliposomes (20 mM) were prepared with low (including soy phosphatidylcholine [SPC] and egg phosphatidylglycerol [EPG]) and high (including hydrogenated soy phosphatidylcholine [HSPC] and distearoyl phosphatidylglycerol [DSPG]) phase transition temperature values without cholesterol. In each set, the anionic phospholipid (EPG or DSPG) constituted 75% of total phospholipid content. Immunoliposomes were prepared by conjugating a monoclonal antibody against apoB-100 to the liposomal surface using a post-insertion technique. Fluorescently-labeled immunoliposomes were assessed for their uptake by *J774.A1* macrophages. Lipid-modifying effects of immunoliposomes were tested at different doses (50, 100 or 200 μmole/g weight) using a tyloxapol-induced hyperlipidemic mouse model. Blood sampling was performed 1 h after the injection of each immunoliposomal formulation.

Results: ApoB-targeted HSPC/DSPG and SPC/EPG nanoliposomes were both taken up by cultured macrophages but the uptake rate was higher with the former formulation. Both immunoliposomal formulations significantly reduced serum LDL-cholesterol concentrations of hyperlipidemic animals at all tested doses ($p < 0.001$) and this effect lasted for at least 48 h. Significant reductions of serum levels of apoB, non-HDL-C, total cholesterol and triglycerides, and elevations of HDL-C levels were also observed.

Conclusion: Intravenous injection of a single dose of apoB-targeted anionic nanoliposomes improves serum lipid profile parameters. These findings might have implications for the treatment of patients with severe dyslipidemias or statin intolerance.

Keywords: Dyslipidemia; Liposome; Phospholipid; Monoclonal antibody; Cholesterol

Introduction

Coronary artery disease (CAD) is a major cause of mortality and morbidity worldwide (1). Dyslipidemia is an established risk factor for atherosclerosis and CAD. Inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA), known as statins, are the most widely prescribed and the most potent drugs for lowering low-density lipoprotein cholesterol (LDL-C) concentrations. In spite of proven lipid-lowering efficacy and a number of pleiotropic benefits, statins have moderate effects on plasma triglycerides and high-density lipoprotein (HDL-C) concentrations. Moreover, findings from the landmark trials have indicated that monotherapy with statins leads to ~27% reduction in the overall cardiovascular (CV) risk whilst combination of statins with other classes of anti-dyslipidemic agents leads to a further risk reduction to 71% (2). Therefore, there is a significant residual CV risk even among patients who are receiving statin therapy (3), and this risk necessitates further research to find novel lipid-lowering therapies with potent effects on plasma triglycerides and HDL-C levels.

Recent guidelines from the National Cholesterol Education Program-Adult Treatment Panel III (NCEP-ATP III) have specified LDL-C targets at < 100 mg/dL and < 70 mg/dL for patients at high and very high CV risk, respectively (4). Achieving these targets requires new and potent agents as currently available anti-dyslipidemic agents can lower serum LDL-C by up to 30-50% (5). Moreover, aggressive reduction of serum LDL-C by high-intensity statin therapy increases the risk of developing adverse events such as myopathies, hepatotoxicity and life-threatening rhabdomyolysis, which is another limitation for the use of statins in patients with severe dyslipidemias.

Phospholipids are an emerging class of anti-dyslipidemic agents (6-8). These bioactive lipids constitute around 1-10% of total daily fat intake which is equivalent to 2-8 g/day (7). Hitherto,

several types of phospholipid preparations such as soy lecithin (mixture of phosphatidylcholine [PC], phosphatidylethanolamine [PE], phosphatidylinositol [PI] and phosphatidic acid), and isolated PC, PE and PI have been investigated for the treatment of dyslipidemia, atherosclerosis and non-alcoholic fatty liver disease. Both animal as well as clinical trials have confirmed that oral administration of these products significantly reduces serum concentrations of LDL-cholesterol and triglycerides, and increases those of HDL-C (9-15). Moreover, anionic phospholipids such as PI and PE have been the subject of particular interest for their hypolipidemic activity owing to their important roles in molecular signaling and interaction with receptors involved in the metabolism, secretion and clearance of lipoproteins (16). However, it remains elusive whether intravenous administration of phospholipids in the form of targeted nanoliposomes may cause an efficient and rapid reduction of serum LDL-C concentrations.

There is evidence indicating that liposomes containing at least 75% anionic phospholipids in their composition can coalesce with LDL particles, resulting in the formation of particles that are cleared through either hepatocyte LDL receptors or macrophages (17-19). In addition, cholesterol-poor liposomes are known to interact with plasma lipoproteins and serve as a sink for cholesterol in particular from LDL as the main carrier of plasma cholesterol (20). Recently, we have shown that intravenous administration of anionic nanoliposomes ameliorates serum lipid levels in an acute hyperlipidemic mouse model (21). In this study, we hypothesized that active targeting of nanoliposomes to LDL particles would enhance such liposome-lipoprotein interactions and provide a useful tool for the clearance of plasma cholesterol owing to the efficient and rapid uptake of nanoliposomes by liver (20). This hypothesis was verified using apoB-targeted anionic nanoliposomes.

Materials and Methods

Chemicals

Hydrogenated soy phosphatidylcholine (HSPC), soy phosphatidylcholine (SPC), Egg phosphatidylglycerol (EPG) and 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 (Grand Island, NY, USA). Tyloxapol and simvastatin were from Sigma (Germany) and Sami Saz Pharmaceutical Company (Iran), respectively. Alamar Blue was purchased from Biosource International Inc. (Camarillo, CA, USA). Monoclonal apolipoprotein B-100 antibody (apoB-100) was purchased from Meridian Life science, Inc. (Memphis, TN, USA).

Preparation of nanoliposomes

Conventional liposomes were prepared from different phospholipids (HSPC, SPC, EPG and DSPG) by the method of dried lipid-film hydration plus extrusion. Phospholipids were different in their liquid crystalline transition temperature (*Tm*) and charge (see **Figure S1** in supplement). Briefly, two sets of liposomal formulations (**Table 1**) were prepared with low (SPC and EPG), and high (HSPC and DSPG) T_m values at a final lipid concentration of 20 mM. Both SPC/EPG and HSPC/DSPG preparations 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 (10mM)/sucrose (10%) buffer (pH 6.5). The size and lamellarity of resultant multilamellar dispersions were reduced by vortexing, sonication

and passing through a high-pressure homogenizer (EmulsiFlex-C3, Avestin, Canada) for three consecutive cycles at 20,000 psi.

Preparation of immunoliposomes

In order to conjugate the antibodies to the liposomes, apoB-100 monoclonal antibody (4.5 mg/mL) was mixed with a heterobifunctional reagent *N*-Succinimidyl-3-(2-pyridyldithio)-propionate (SPDP; 6.25 mg/ml; SPDP/Mab molar ratio = 10:1) and incubated for 30 min at room temperature to prepare pyridyldithiopropionated IgG (PDP-IgG). The mixture was passed immediately through a PD-10 column (GE Healthcare Biosciences) equilibrated with acetate buffer (0.1M NaCl, 100mM sodium acetate, pH 4.5) to separate PDP-IgG from excess SPDP. Fractions containing PDP-IgG conjugates (assessed by absorbance in 280 nm) were pooled and reduced with 50mM dithiothreitol (DTT) to produce thiolated-IgG (IgG-SH). The IgG-SH was separated from excess DTT by a PD-10 column equilibrated with HEPES buffer. The IgG-SH was mixed immediately with MPEG2000-DSPE-maleimide micelles (1 mM; micelle/Mab molar ratio = 10:1) and stirred in the dark at room temperature for an overnight. Resulting micelle-IgG complexes were then mixed with liposomes at 1:1000 molar ratio, and incubated for an hour at 60°C (22) (see **Figure S2** in supplement).

Characterization of immunoliposomes

The particle diameter of each sample together with its polydispersity index was measured in triplicate using Dynamic Light Scattering method (Nano-ZS; Malvern, UK). The zeta potential of nanoliposomes was determined on the same machine using the zeta potential mode as the average of 20 measurements (23).

Phospholipid assay

The phospholipid concentration of formulations was determined according to Bartlette test (24). Briefly, portions of each formulation, equivalent to 80 ± 50 nmoles 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. The sample was heated at 195-210˚C for 60 minutes using a hot-plate apparatus under a fume hood. After allowing to cool down for about 10 minutes at room temperature, 0.1 mL of 10% H_2O_2 was added to each sample tube followed by heating for 10 minutes at 190-210˚C. After cooling the tubes for about 10 minutes, 4.7 ml of molybdate reagent and 0.5 ml of 10% ascorbic acid were added to each tube and vortexed immediately for 10 seconds. Samples were then heated at 100˚C for 10 to 20 minutes, quickly cooled and finally were measured for optical density at 800 nm. Phospholipid concentration of samples was calculated according to the phosphate standard curve.

Immunoliposome uptake by macrophages

J774.A1 macrophages were seeded in 12-well tissue culture plates at 3.0×10^5 cells/well in RPMI 1640 supplemented with 10% fetal calf serum and antibiotics (100 U/mL penicillin and 100 µg/mL streptomycin). Treatment of *J774.A1*cells was performed with two different doses of each immunoliposome i.e. final phospholipid concentrations were set in each well at 0.1 and 0.2 mM. After 3, 10 and 24 h of incubation, cells were washed with cold phosphate buffered saline (PBS) to remove unbound immunoliposomes and then lysed using 1% Triton X-100. Flow cytometric analyses were performed on the obtained lysate by means of a Partec® instrument (Münster, Germany) at FL2 mode. Data were analyzed using WinMDI version 2.8 software. For

each sample, the percentage of stained events together with mean fluorescence intensity was determined.

In-vivo hypolipidemic assay

Male albino mice (20-30 g) were obtained from the animal house of the School of Pharmacy, Mashhad University of Medical Sciences (Mashhad, Iran), and used for *in-vivo* experiments. The animals were housed in constant room temperature (23-25°C), kept in plastic cages with sawdust and had free access to food and water. Mice were starved for ~ 6 h prior to experiment. For the screening tests, animals were randomly divided into 8 groups of four mice each. Hyperlipidemia was induced in all groups via injection of a single dose of tyloxapol (Triton WR-1339) at a dose of 300 mg/kg mouse weight. Simvastatin (80 mg/kg) and saline were used as positive and negative control, respectively. Immunoliposomal formulations were intravenously injected through tail vein at three doses of 50, 100 and 200 µmole phospholipid/kg. Prior to injection, all solutions were sterilized by passing through a 0.22 µ filter. Blood collection was performed 1 h post-injection via cardiac puncture, following anesthesia with ketamine/xylazine combination. To determine the pattern of changes over time, serum LDL-C levels were additionally determined at 12-h and 48-h time points.

Lipoprotein measurements

After being allowed to clot at room temperature, collected blood was centrifuged at 14000 rpm for 10 min to obtain serum. A complete lipid profile comprising LDL-C, HDL-C, total cholesterol, triglycerides, non-HDL-C, apoA and apoB was determined in serum samples. Lipoprotein measurements were performed based on the routine enzymatic methods using

commercial kits (Biosystems, Spain). Atherogenic index was calculated using two routinely used equations i.e. LDL/HDL (AI_1) and log (triglycerides/HDL-C) (AI_2).

Cytotoxicity assessment

Cytotoxicity of immunoliposomes was assessed using Alamar Blue®. *J774.A1* cells were seeded in 96-well plates, allowed to adhere and grow for an overnight at 37°C and 5% CO_2, and then used in the cytotoxicity experiments. A total of three doses (5, 10 and 15 µL of the 20 mM immunoliposomal preparation); and three different time points (3, 12 and 24 hrs) were used to determine cytotoxicity of nanoliposomes. Alamar Blue® solution was directly added to the medium resulting in a final concentration of 10% in each well. After 4 hrs of incubation with Alamar Blue®, the absorbance was read at 545 nm using a microplate reader according to the manufacturer leaflet.

Statistical analysis

Statistical analyses were performed using SPSS software (version 13.0). Values were expressed as mean ± SEM. Between-group comparisons of serum LDL-C and macrophage uptake measures were performed using one-way analysis of variance (ANOVA) with Dunnett's test for *post-hoc* multiple comparisons. A two-sided *p*-value of < 0.05 was considered as statistically significant.

Results

Characterization of prepared formulations

Prepared liposomal formulations had a diameter around 70 nm. Based on DLS findings, conjugation of liposomes with apo B-100 antibodies increases the size of HSPC/DSPG and SPC/EPG liposomes to around 100 and 150 nm, respectively. All liposomal and immunoliposomal formulations had high negative charge and PDI values of ≤ 0.5. Characterization parameters of formulations are summarized in **Table 1**.

Macrophage uptake assay

HSPC/DSPG and SPC/EPG immunoliposomes were subjected to flow cytometry for comparison of the uptake rate by *J774.A1* macrophages. Cellular uptake rate was assessed in terms of percentage of positively stained events and mean fluorescence intensity. The uptake of both HSPC/DSPG immunoliposomes was higher than those of SPC/EPG immunoliposomes at all assessed time points i.e. 3, 10 and 24 h of incubation ($p < 0.001$). The uptake of all formulations was enhanced with increasing concentration and incubation time (**Figures 1**).

In-vivo lipid-lowering activity

LDL-C

Intravenous administration of both SPC/EPG and HSPC/DSPG immunoliposmes significantly reduced serum LDL-C concentrations at all tested doses ($p < 0.001$ versus the saline group). The LDL-lowering efficacy of HSPC/DSPG immunoliposomes was higher than that of SPC/EPG-based formulations (**Figure 2**).

As low-doses of both immunoliposomal preparations showed higher efficacy, a separate experiment was performed at this dose with serum LDL-C determination at additional time points i.e. 12 h and 48 h post-injection. The results indicated that reductions (versus the saline group) observed after 1 h of injection of both immunoliposomal formulations are sustained for at least 48 h (**Figure 3**).

Total cholesterol

Tested immunoliposomes reduced serum total cholesterol levels – compared with the saline group – to varying degrees. The reduction in serum total cholesterol reached statistical significance only for HSPC/DSPG ($p < 0.01$ for the low and high doses and $p < 0.001$ for the medium dose) but not SPC/EPG ($p > 0.05$) immunoliposomes (**Figure 2**).

HDL-C

The lowest dose of both HSPC/DSPG ($p < 0.05$) and SPC/EPG ($p < 0.01$) immunoliposomes significantly elevated serum HDL-C concentrations compared with the saline group (**Figure 2**).

Non-HDL-C

Serum non-HDL-C concentrations were significantly reduced by both immunoliposomal preparations at all tested doses ($p < 0.001$ versus the saline group) (**Figure 2**).

Triglycerides

Efficient reductions in serum triglycerides concentrations were observed with both HSPC/DSPG ($p < 0.01$) and SPC/EPG ($p < 0.01$ for the low and high dose and $p < 0.05$ for the medium dose) immunoliposomes compared with the saline group (**Figure 2**).

Atherogenic indices

Both evaluated immunoliposomal preparations significantly reduced AI_1 at all tested doses ($p < 0.001$ versus the saline group). In the same manner, AI_2 was reduced (compared with the saline group) following administration of both HSPC/DSPG and SPC/EPG formulations at low ($p < 0.01$), medium ($p < 0.05$) and high ($p < 0.001$) dose (**Figure 4**).

Apolipoproteins

Serum apoB concentrations were significantly reduced following injection of HSPC/DSPG ($p < 0.05$) and SPC/EPG ($p < 0.01$) immunoliposomes compared with the saline-injected group, whilst serum apoA levels remained statistically unaltered ($p > 0.05$). There was also a significant elevation in serum apoA/apoB following treatment with SPC/EPG immunoliposomes ($p < 0.05$ versus the saline grpup) (**Figure 5**). However, elevations in serum apoA/apoB observed in the simvastatin and HSPC/DSPG groups did not reach statistical significance when compared with the saline group (**Figure 5**).

Cytotoxicity assays

Cytotoxicity of the HSPC/DSPG and SPC/EPG formulations was dose- and time-dependently evaluated using Alamar Blue® assay. Several doses (5, 10 and 15 µL) were used, and cell viability was determined after 3, 12 and 24 h of incubation. Overall, the viability of cultured

macrophages was almost completely maintained in the presence of both tested immunoliposomal preparations regardless of dose and duration of incubation. The results of cytotoxicity assays are summarized in the supplement (**Figure S3)**.

Discussion

This study produced results which corroborate our hypothesis on the lipid-modifying effects of apoB-targeted nanoliposomes. In the current research, both tested immunoliposomal formulations improved lipid profile by reducing serum concentrations of LDL-C, apoB, total cholesterol, non-HDL-C and triglycerides, and elevating HDL-C levels. HSPC/DSPG-based formulation had a higher efficacy in reducing LDL-C, which can be attributed to the higher *Tm* value of this formulation that makes the resulting cholesterol rich particles more stable in delivering their cholesterol cargo to the liver.

Efficient reduction of serum LDL-C concentrations by immunoliposomes represents a potentially relevant approach for the treatment of severe dyslipidemias. Most of the currently available anti-hyperlipidemic medications cannot reduce plasma LDL-C levels by > 50%, unless high doses of potent statins are administered for several weeks. As referred earlier, administration of high doses of statins increases the risk of muscular adverse events. In contrast, intravenous administration of the anionic immunoliposomes could effectively and rapidly lower serum LDL-Cconcentrations. Another important feature of the apoB-targeted nanoliposomes, particularly at their lowest tested dose, is their promising impact on triglycerides and HDL-C concentrations. Many patients with mixed types of hyperlipidemia are treated with statins in combination with niacin (to raise HDL-C levels) and/or fibrates (to lower triglycerides). Such a combination therapy is usually practiced for patients with diabetes and metabolic syndrome in whom

atherogenic dyslipidemia (characterized by elevated triglyceride and low HDL-C concentrations) is common. Therefore, introduction of newer agents that can ameliorate different lipid indices in an effective and rapid manner is highly demanded and would reduce the need for multiple drug therapy (25-30).

The efficacy of dietary phospholipids to regulate hepatic lipid metabolism and ameliorate serum lipid profile has been confirmed in several previous studies (7, 31). However, previous studies have mainly tested the effects of complex phospholipid preparations such as soy lecithin, egg lecithin or PC-based preparations (13). However, more recent *in-vitro* and *in-vivo* studies have shown that negatively-charged phospholipids, in particular PI, may play the major role in the anti-dyslipidemic activity of soy lecithin or other mixed phospholipid preparations (32, 33). Previous studies have shown that anionic liposomes (containing at least 75% PG) can interact with LDL particles, either through direct coalescence (17, 18) or an electrostatic interaction between negatively-charged phospholipids and apoB-100 as a highly cationic protein (34, 35). Removal of resulting liposome-LDL complexes would be mediated by LDL receptor or macrophages (17-19). This simple mechanism enhances the delivery of serum cholesterol to the hepatic tissue where cholesterol can be excreted ion. We hypothesize that the immunoliposomes tested in the present study can reduce serum LDL-C by at least two additional mechanisms: First, incorporation of apoB-100 monoclonal antibody to the surface of nanoliposomes would target them to LDL as the main cholesterol containing lipoprotein and thus enhance the liposome-LDL interactions. Second, cholesterol-free nanoliposomes can serve as cholesterol acceptors from cholesterol-rich LDL particles and deliver the absorbed cholesterol to the liver (20).

Anionic phospholipids can exert their lipid-modifying effects through several mechanisms. Anionic phospholipids enhance reverse cholesterol transport via induction of both ATP binding

cassette transporter A1 (ABCA1) and apo A-I expression and secretion (33). These effects increase the net flux of cholesterol from peripheral tissues to HDL and subsequent clearance of cholesterol by liver (32, 33). Intravenous administration of PI has also been shown to inhibit lecithin-cholesterol acyltransferase (LCAT) and cholesterylester transfer protein (CETP), two important enzymes involved in cholesterol metabolism (36, 37). Inhibition of these enzymes by anionic phospholipids results in the inhibition of production, transfer and storage of cholesterol in the apoB-containing lipoprotein pool (32, 38). Anionic phospholipids such as PI and PG may also regulate triglyceride metabolism by stimulation of triglyceride lipases including hepatic-, lipoprotein- and pancreatic lipase. It has been suggested that anionic phospholipids increase the clearance of triglycerides by reducing hepatic lipase interaction with HDL, while increasing the enzyme affinity for triglyceride-rich lipoproteins (16, 39, 40).

A number of limitations need to be considered when interpreting the results of this study: First, this study only addressed changes in lipid concentration following administration of immunoliposomes. It remains unclear if the observed effects on lipid indices would lead to a relevant change in the size and composition of atherosclerotic plaques as well as the survival of hypercholesterolemic animals. Second, the animal model that was used in the present study represented an acute hyperlipidemic state. This model was used to replicate elevated levels of serum lipids as seen in patients with severe dyslipidemias. Nevertheless, it is required to evaluate the lipid-modifying effects of immunoliposomes in standard models of chronic dyslipidemia i.e. high fat-fed $LDLr^{-/-}$ or $apoE^{-/-}$ mice. Finally, PG was the only anionic phospholipid that was tested in the present study. Hence, it remains elusive if formulation of nanoliposomes with other anionic phospholipids can alter serum lipid concentrations.

Conclusions

The results of this investigation indicated that intravenous injection of a single dose of apoB-targeted anionic nanoliposomes reduces serum LDL-C, apoB, non-HDL-C, total cholesterol and triglycerides, and elevates HDL-C concentrations. Since immunoliposomes are biocompatible and biodegradable, these results might have implications for the treatment of patients with severe dyslipidemias (e.g. familial hypercholesterolemia) or statin-intolerance. Future studies are warranted to confirm the present findings in other models of dyslipidemia and to evaluate the impact of anionic nanoliposomes on clinical endpoints of atherosclerosis.

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Graphical abstract

Injection of cholesterol-free immunoliposomes targeted against apolipoprotein B-100 improves lipid profile in tyloxapol-induced hyperlipidemic mice. Immunoliposomes can interact with serum lipoproteins via lipid exchange between the vesicles, and direct capture of atherogenic lipoproteins via apoB-100 monoclonal antibodies on the liposome surface. Resulting liposome-lipoprotein complexes will be efficiently cleared from serum by liver.

Figure legends

Figure 1. Comparison of immunoliposomal formulations in terms of uptake rate by *J774.A1* macrophages following 3 (upper row), 10 (middle row) and 24 h (lower row) of incubation. The results are shown as % positively stained events (left) and mean fluorescence intensity (right) according to flow cytometric data. Treatment of *J774.A1*cells were performed with two different concentrations of each immunoliposome i.e. final phospholipid concentrations were set in each well at 0.1 (L) and 0.2 mM (H). Values are expressed as mean ± SEM, n = 3.

Figure 2. *In-vivo* effects of different doses of anionic immunoliposomes on serum low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), non-HDL-C, total cholesterol, and triglyceride concentrations. H: high concentration (200 μmole/kg); M: medium concentration (100 μmole/kg); L: low concentration (50 μmole/kg). Values are expressed as mean ± SEM. *p*-values refer to the statistical comparison of immunoliposomal preparations and simvastatin compared with saline. No significant difference was found between liposomal preparations and simvastatin.

Figure 3. Changes in serum LDL-C concentrations at different time points following injection of anionic immunoliposomes at a dose of 50 μmole/kg. LDL-C concentrations were significantly lower at 12-h and 48-h time points in the HSPC/DSPG and SPC/EPG groups compared with the saline group ($p < 0.05$).

Figure 4. *In-vivo* effects of different doses of anionic immunoliposomes on AI_1 (calculated as LDL-C/HDL-C) and AI_2 [calculated as log (triglycerides/HDL-C)]. H: high concentration (200 μmole/kg); M: medium concentration (100 μmole/kg); L: low concentration (50 μmole/kg). Values are expressed as mean ± SEM. *p*-values refer to the statistical comparison of immunoliposomal preparations and simvastatin compared with saline.

Figure 5. *In-vivo* effects of different doses of anionic immunoliposomes on serum apoA apoB, and apoA/apoB. Evaluations were performed using the immunoliposomal dose of 50 μmole/kg. Values are expressed as mean ± SEM. *p*-values refer to the statistical comparison of immunoliposomal preparations and simvastatin compared with saline.

Supplementary material

Figure S1. Chemical structures of phospholipids used for the preparation of immunoliposomes.

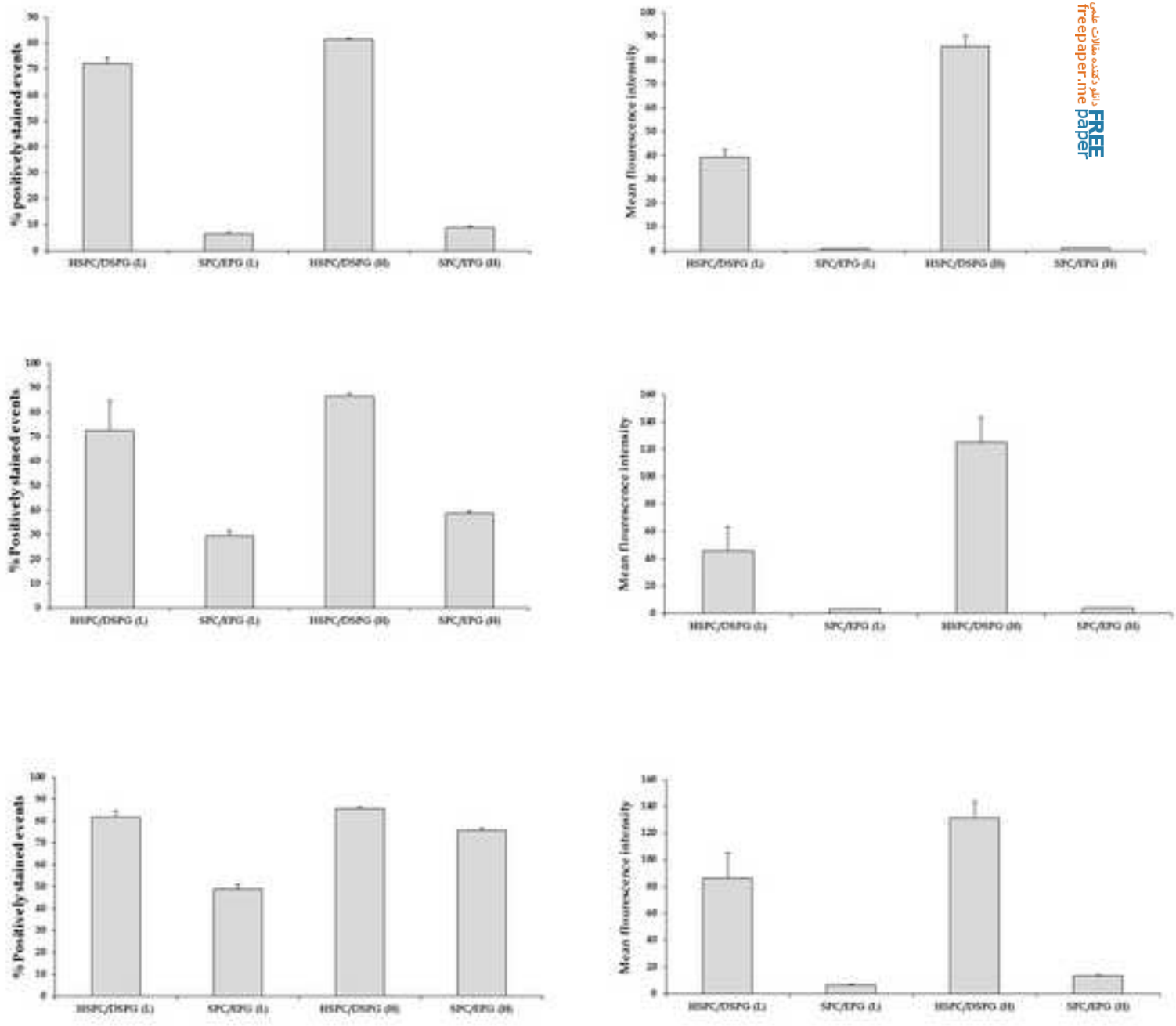
Figure S2. A summary of reactions in the conjugation of apoB-100 monoclonal antibody to the liposomes using post-insertion technique. SPDP: *N*-succinimidyl 3-(2-pyridyldithio) propionate; DTT: dithiotheritl; RT: room temperature.

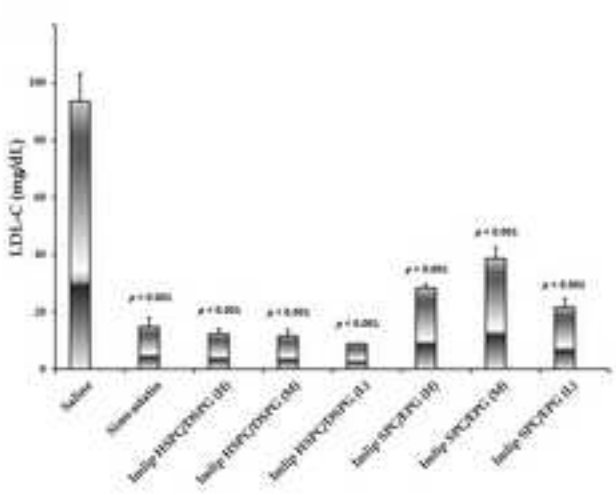
Figure S3. Dose- and time-dependent evaluation of the viability of *J774*.A1 macrophages in the presence of HSPC/DSPG (left) and SPC/EPG (right) immunoliposomes. Values are expressed as mean ± SEM.

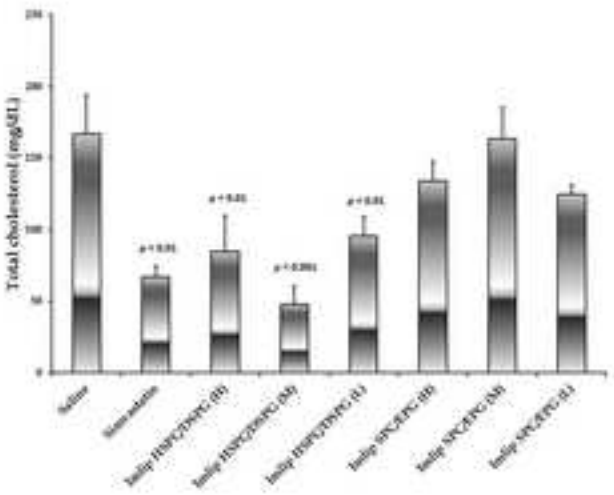
Highlights

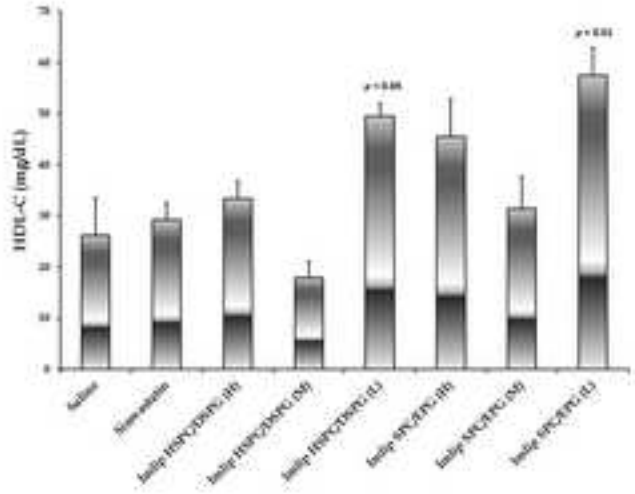
- Apolipoprotein B-100 (apoB) is the major protein in the structure of atherogenic lipoproteins.
- There is evidence on the strong interaction between cholesterol-free nanoliposomes and lipoproteins.
- Anionic nanoliposomes are known to be efficiently cleared from plasma by liver.
- ApoB-targeted cholesterol-free anionic nanoliposomes were prepared and administered to hyperlipidemic mice.
- Anionic nanoliposomes lowered serum LDL-C, triglycerides, apoB and non-HDL-C, and elevated HDL-C concentrations.
- Anionic nanoliposomes might serve as an effective, safe and rapid-acting treatment for dyslipidemia.

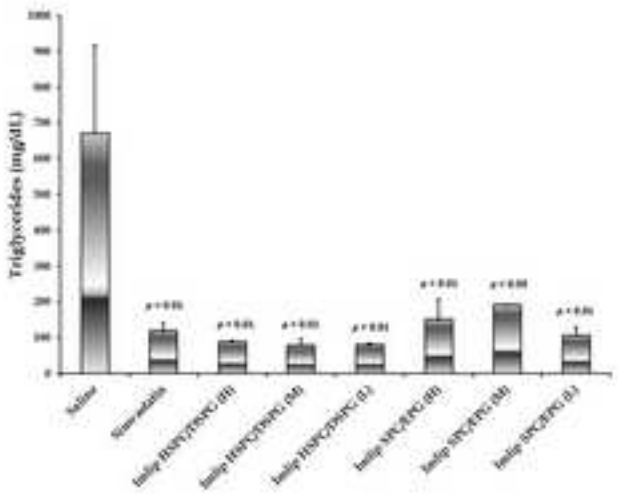
Figure 1











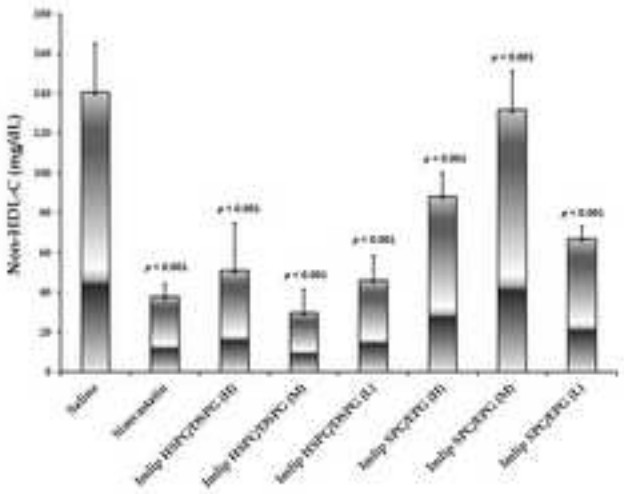


Figure 3

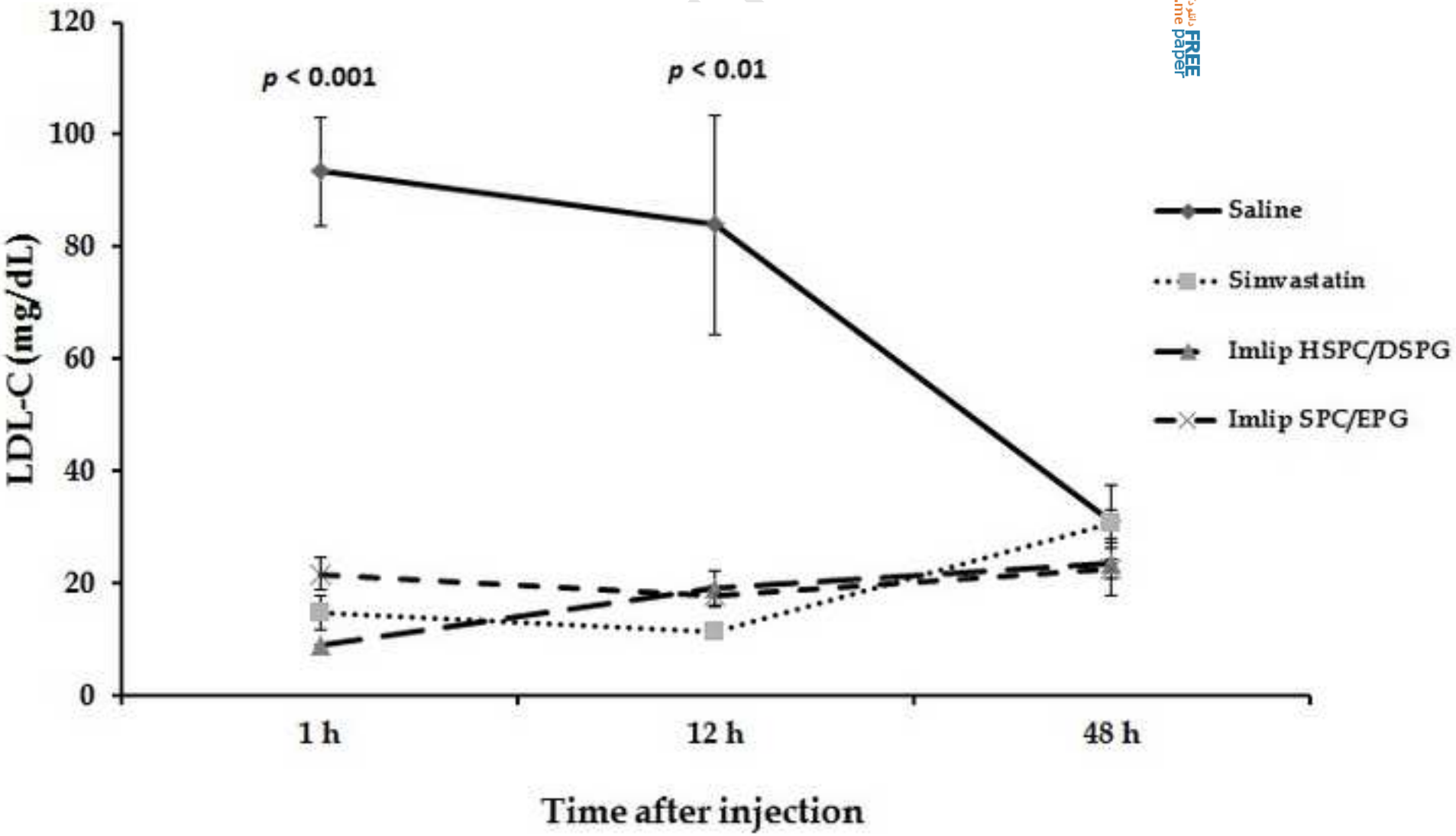
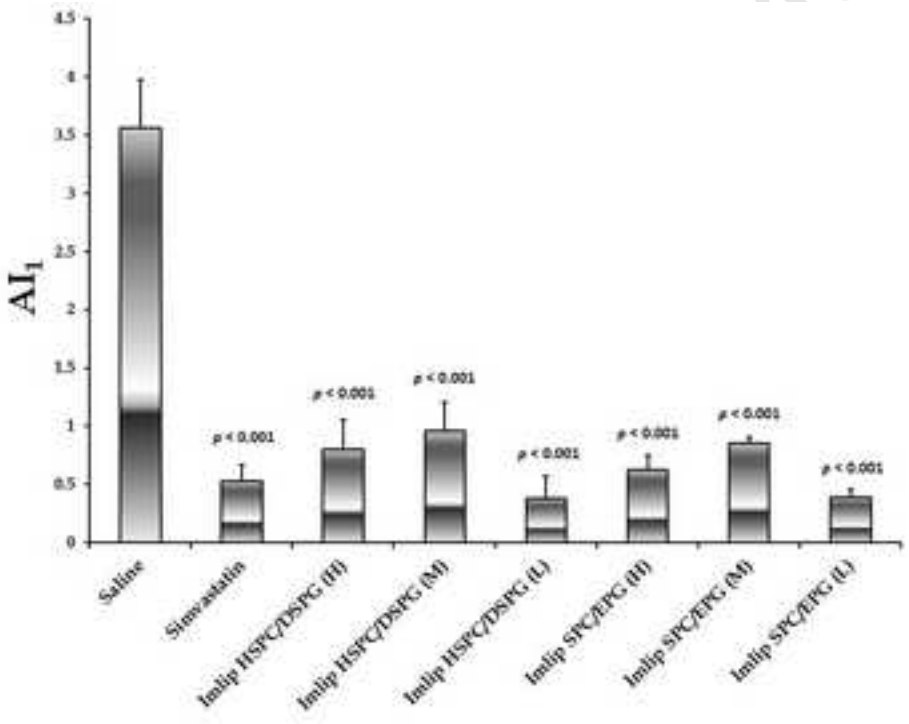


Figure 4



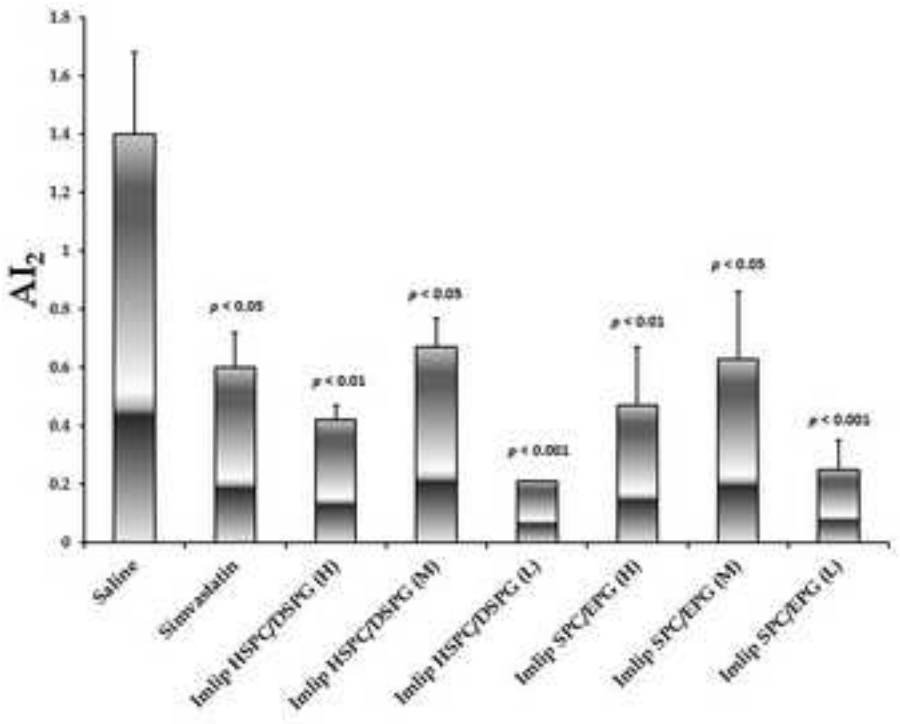
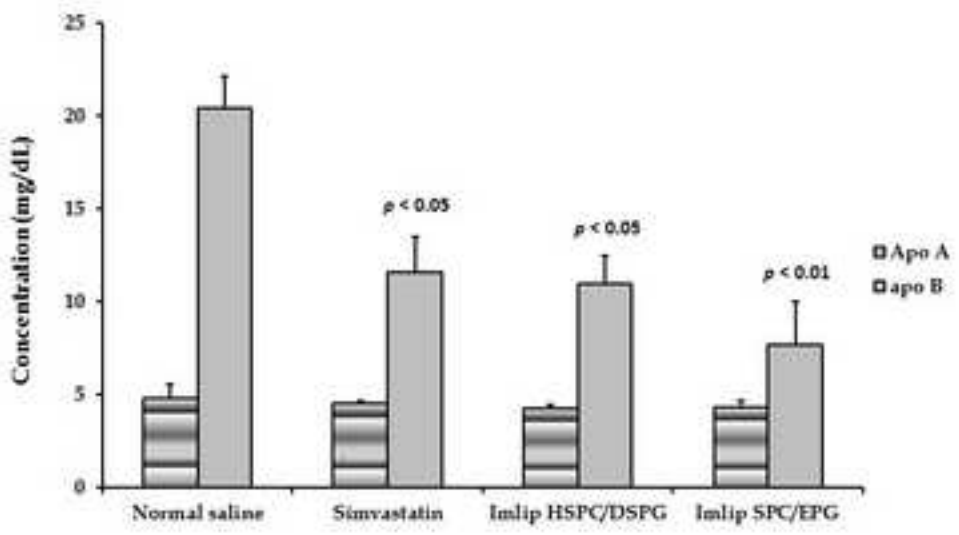


Figure 5



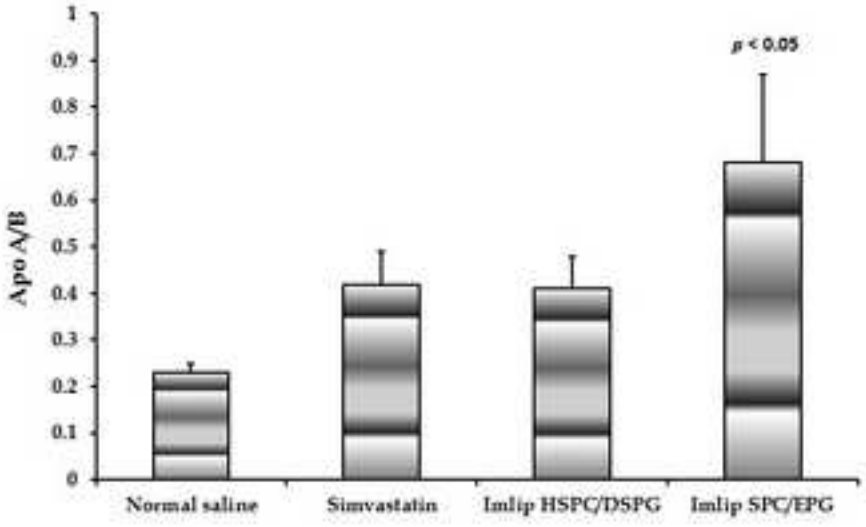


Table 1. Particle size distribution, zeta potential and polydispersity of liposomal formulations.

Formulation	Molar ratio	Z-Average (d.nm)	PDI	Zeta potential (mV)
HSPC/DSPG	**1/3**	72.33±0.70	0.34±0.003	-60.6±8.99
SPC/EPG	**1/3**	71.53±0.63	0.24±0.01	-63.5±11.7
Apo B-100 targeted immunoliposomes				
HSPC/DSPG	**1/3**	99.03±0.82	0.13±0.007	-23.70±0.71
SPC/EPG	**1/3**	148.13±4.68	0.52±0.01	-39.4±0.77

PDI: poly dispersity index. Values are expressed as mean ± SD, n = 3.

Graphical Abstract (for review)