European Journal of Pharmaceutics and Biopharmaceutics 107 (2016) 120–129

Contents lists available at ScienceDirect

European Journal of Pharmaceutics and Biopharmaceutics

journal homepage: www.elsevier.com/locate/ejpb

Research paper

A nanoformulation containing a scFv reactive to electronegative LDL inhibits atherosclerosis in LDL receptor knockout mice

Marcela Frota Cavalcante [a,1], Soraya Megumi Kazuma [a,1], Eduardo André Bender [b], Márcia Duarte Adorne [b], Mayara Ullian [a], Mariana Matera Veras [c], Paulo Hilário Nascimento Saldiva [c], Andrea Queiroz Maranhão [d], Silvia Stanisçuaski Guterres [e], Adriana Raffin Pohlmann [b], Dulcineia Saes Parra Abdalla [a,*]

[a] Department of Clinical and Toxicological Analyses, Faculty of Pharmaceutical Sciences, University of Sao Paulo, Sao Paulo, SP, Brazil
[b] Department of Organic Chemistry, Chemistry Institute, Federal University of Rio Grande do Sul, Porto Alegre, RS, Brazil
[c] LIM5, Department of Pathology, Medicine School, University of Sao Paulo, Sao Paulo, SP, Brazil
[d] Molecular Immunology Laboratory, Department of Cell Biology, University of Brasilia, Brasilia, Distrito Federal, Brazil
[e] Department of Production and Control of Medicines, Faculty of Pharmacy, Federal University of Rio Grande do Sul, Porto Alegre, RS, Brazil

ARTICLE INFO

Article history:
Received 31 January 2016
Revised 24 June 2016
Accepted in revised form 1 July 2016
Available online 1 July 2016

Keywords:
Atherosclerosis
Nanocapsules
Single chain fragment variable
Electronegative LDL
Macrophage
Foam cell

ABSTRACT

Atherosclerosis is a chronic inflammatory disease responsible for the majority of cases of myocardial infarction and ischemic stroke. The electronegative low-density lipoprotein, a modified subfraction of native LDL, is pro-inflammatory and plays an important role in atherogenesis. To investigate the effects of a nanoformulation (scFv anti-LDL(−)-MCMN-Zn) containing a scFv reactive to LDL(−) on the inhibition of atherosclerosis, its toxicity was evaluated *in vitro* and *in vivo* and further it was also administered weekly to LDL receptor knockout mice. The scFv anti-LDL(−)-MCMN-Zn nanoformulation did not induce cell death in RAW 264.7 macrophages and HUVECs. The 5 mg/kg dose of scFv anti-LDL(−)-MCMN-Zn did not cause any typical signs of toxicity and it was chosen for the evaluation of its atheroprotective effect in $Ldlr^{-/-}$ mice. This nanoformulation significantly decreased the atherosclerotic lesion area at the aortic sinus, compared with that in untreated mice. In addition, the *Il1b* mRNA expression and CD14 protein expression were downregulated in the atherosclerotic lesions at the aortic arch of $Ldlr^{-/-}$ mice treated with scFv anti-LDL(−)-MCMN-Zn. Thus, the scFv anti-LDL(−)-MCMN-Zn nanoformulation inhibited the progression of atherosclerotic lesions, indicating its potential use in a future therapeutic strategy for atherosclerosis.

1. Introduction

Cardiovascular disease is the most frequent cause of death worldwide [1], accounting for almost 30% of all deaths [2]. Although several diseases can affect the cardiovascular system, atherosclerosis predominates as the major cause of myocardial infarction and ischemic stroke [3]. Atherosclerosis originates from a chronic inflammation into large- and medium-sized arteries, as a result of the accumulation of modified cholesterol-rich lipids in the arterial wall that mediate an immune-inflammatory response [4]. Upon accumulation in the arterial intima, modified low-density lipoprotein (LDL) particles can activate the endothelium to express leukocyte adhesion molecules and chemokines, leading to the recruitment of immune cells, such as monocytes and T cells. In the presence of macrophage-colony stimulating factor (M-CSF),

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; ATCC, American Type Culture Collection; CD14, cluster of differentiation 14; CD36, cluster of differentiation 36; DLS, dynamic light scattering; DMSO, dimethylsulfoxide; DTT, dithiothreitol; Fab, monovalent antigen-binding; Fab'_2, divalent antigen-binding; HC, hypercholesterolemic; HDL-C, high-density lipoprotein cholesterol; HUVEC, human umbilical vein endothelial cells; IL-1β, interleukin 1 beta; LDE, laser Doppler electrophoresis; LDL, low-density lipoprotein; LDL(−), electronegative low-density lipoprotein; LDL-C, low-density lipoprotein cholesterol; MCMN, metal-complex multi-wall nanocapsules; M-CSF, macrophage-colony stimulating factor; MDA, malondialdehyde; nLDL, native low-density lipoprotein; PBS, phosphate buffered saline; PDI, polydispersity index; PGs, proteoglycans; scFv, single chain fragment variable; SDS, sodium dodecyl sulfate; TC, total cholesterol; TG, triglyceride; TLR-4, toll-like receptor 4; VLDL-C, very low-density lipoprotein cholesterol.

* Corresponding author.
E-mail address: dspabdalla@gmail.com (D.S.P. Abdalla).
[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.ejpb.2016.07.002
0939-6411/

monocytes differentiate into macrophages and, in association with the up-regulation of scavenger and Toll-like receptors, mediate the uptake of modified LDL particles [5]. This process leads to one of the key events on atherosclerosis, the formation of foam cells. Further, the death of these cholesterol-overloaded macrophages promotes the formation of a necrotic and lipid core, and the proliferation and migration of vascular smooth muscle cells to the intima layer promotes the growth of atherosclerotic lesions [6].

The electronegative LDL [LDL(−)], a subfraction of the native LDL that can be found in both blood plasma [7] and the subendothelial space [8] is strongly associated with the development of atherosclerosis [9]. LDL(−) has demonstrated pro-inflammatory and pro-angiogenic properties leading to the production of pro-inflammatory cytokines and adhesion molecules by endothelial cells [10] that mediate leukocytes recruitment into the intima [11–13], and to increased differentiation of monocytes to macrophages expressing scavenger receptors, such as CD36, the main responsible for the recognition and internalization of oxidized LDL (oxLDL) by these cells [14].

The use of monoclonal antibodies and their fragments (Fab, Fab_2 and scFv) as therapeutic agents for atherosclerosis has been reported [15]. Treatment of male $apoE^{-/-}$ mice with a scFv with specificity for malondialdehyde (MDA)-modified apoB-100-derived peptides significantly reduced the extent of atherosclerosis and the plaque content of oxidized LDL epitopes and macrophages [16]. Moreover, a Fab and an anti-oxLDL scFv (single chain fragment variable) inhibited the *in vitro* formation of macrophage-derived foam cells and decreased the progression of atherosclerotic lesions in $Ldlr^{-/-}$ mice by blocking the binding and subsequent uptake of oxLDL particles by macrophages [17]. The scFv described here has high specificity and affinity for LDL(−) [18]. It has been demonstrated that treatment with the 2C7 scFv anti-electronegative LDL inhibited the LDL(−) uptake by murine macrophages and decreased the expression of *Cd36*, *Tlr4* and *Cox2* mRNA. In addition, the *in vivo* treatment of $Ldlr^{-/-}$ mice with this scFv decreased the atherosclerotic lesion area at the aortic sinus [18], indicating its potential as a therapeutic tool in atherosclerosis.

Although the use of peptides, antibodies and proteins is a promising approach for the prevention and treatment of diseases, such as atherosclerosis and cancer [19], this therapy still has some disadvantages, such as their short half-life, lack of stability and, in some cases, potential immunogenicity [20]. To improve the effectiveness of these potential biodrugs, some strategies have emerged in the nanotechnology field such as the use of synthetic polymer nanoparticles and nanoparticle-protein conjugates as nanocarriers [21]. These strategies have provided extremely promising characteristics, such as precise control of the pharmacokinetics and bioavailability and improved stability of drugs and biodrugs [22], greater cell interaction, due to both their larger contact surface [23], and the better targeting for drug delivery [24], and the ability to use nanodevices, a combination of nanomaterials and bioactive molecules.

In an effort to contribute to this therapeutic arsenal, a nanoformulation containing the 2C7 scFv anti-electronegative LDL has been developed [25]. This nanoformulation is composed of biodegradable multiwall polymeric nanocapsules with a functionalized surface completely covered with 2C7 scFv anti-LDL(−) molecules and is named scFv anti-LDL(−)-MCMN-Zn [25]. The innovative approach to surface functionalization is based on an organometallic complex chitosan-zinc II-scFv anti-LDL(−) formed *in situ* at the nanocapsule-water interface. The synthesis is an easy and fast process with no need of any further purification step. Here, we demonstrate the atheroprotective effect of scFv anti-LDL(−)-MCMN-Zn in LDL receptor knockout mice, indicating its potential promising use as a therapeutic nanodevice.

2. Materials and methods

2.1. Materials

Poly(ε-caprolactone) (PCL) (density 1.146 g mL^{-1}, α,ω-dihydroxy functional polymer; Mn 10,000 g mol^{-1}, Mw 14,000 g mol^{-1}), sorbitan monostearate (Span 60®, density 1.00 g mL^{-1}), low molar weight chitosan (Mw 50,000–190,000 g mol^{-1}, 75–85% deacetylated polymer) and zinc acetate were supplied by Sigma-Aldrich (Sao Paulo, Brazil). Caprylic/capric triglyceride (density 0.98 g mL^{-1}) and polysorbate 80 were delivered by Delaware (Porto Alegre, Brazil). Lipoid S75 (soybean lecithin) was obtained from Lipoid (Ludwigshafen, Germany). All aqueous solutions were prepared using deionized water (resistivity of 18.2 MΩ) obtained from a Millipore Direct-Q® system (Merck Millipore, Darmstadt, Germany). The solvents, acetone (analytical grade) and ethanol (analytical grade), were obtained from Nuclear (Porto Alegre, Brazil). All reagents and solvents were used as received.

2.2. Nanoparticle design

2.2.1. Preparation of scFv anti-LDL(−)-surface functionalized nanocapsules

The nanocapsules were prepared by the interfacial deposition of the preformed polymer method, as previously described [25]. In one flask, Lipoid S75 (0.075 g) was dissolved in ethanol (10 mL) (Solution A). In another flask, PCL (0.250 g), sorbitan monostearate (0.100 g) and caprylic/capric triglyceride (0.300 mL) were dissolved in acetone (62.5 mL) under magnetic stirring at 40 °C (Solution B). Solution A was added to Solution B under magnetic stirring at 40 °C. Then, this organic phase was injected into Solution C which contained polysorbate 80 (0.200 g) dispersed in ultrapure water (125 mL) under magnetic stirring at 40 °C. After 10 min, the turbid solution was evaporated under reduced pressure at 40 °C to approximately 23 mL, eliminating the organic solvents (acetone and ethanol) and concentrating the formulation by partial removal of water. The final volume of the formulation was adjusted to 25.0 mL in a volumetric flask (lipid-core nanocapsules in aqueous dispersion, LNC).

A 3 mg mL^{-1} chitosan solution in 1% acetic acid (10 mL) was prepared and filtered (filter unit, 0.45 μm, Millipore®, Merck Millipore, Darmstadt, Germany) (Solution D). Separately, 9 mL of LNC was added dropwise of 1 mL of Solution D under high magnetic stirring (900 rpm) at room temperature. The resulting mixture remained under magnetic stirring for 2 h at room temperature (20 °C). The theoretical concentration of chitosan in the multiwall nanocapsules [chitosan-coated lipid-core nanocapsules (MN)] formulation is 0.3 mg mL^{-1}.

A 1 mg mL^{-1} Zn^{2+} solution (Solution E) was prepared by dissolving 28 mg of zinc acetate in ultrapure water (10 mL). Solution E (50 μL) was added into 1.950 mL of MN aqueous dispersion, under magnetic stirring (900 rpm) at room temperature (MCMN-Zn). After 1 min, 567 μL of MCMN-ZN aqueous dispersion was transferred to an amber flask for immediate addition to 433 μL of 462.39 μg mL^{-1} 2C7 scFv anti-LDL(−) aqueous solution under magnetic stirring. The aqueous dispersion of scFv anti-LDL(−)-MCMN-Zn remained under moderate magnetic stirring (400 rpm) for 5 min at room temperature (20 °C). The theoretical concentrations of Zn^{2+} and 2C7 scFv anti-LDL(−) in the formulation are 25 μg mL^{-1} and 200 μg mL^{-1}, respectively. A control formulation was prepared as described, but phenylalanine was reacted with Zn^{2+} instead of 2C7 scFv anti-LDL(−). This formulation was termed Phe-MCMN-Zn.