ApoB100 FUNCTIONALIZED LIPOSOMES FOR TARGETED DELIVERY TO MALIGNANT CELLS

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Abstract. Liposomes can be modified and functionalized with different ligands to control their biological properties, such as longevity, targeting ability, and intracellular penetration, in a desired fashion (for example in photodynamic therapy). The aim of this study was to obtain functionalized liposomes with apolipoprotein ApoB100 ligand to achieve active intracellular targeted delivery *via* LDL-receptor, by naturally occurring endocytotic pathway. Human malignant melanoma A375 cells were used as model cells for *in vitro* evaluation of cellular endocytosis efficiency. The functionalized ApoB100-liposomes were incubated with cultured cells. Incubation periods of 2 and 4 hours were used to obtain ligand-receptor cellular endocytosis and fluorescence expression was evaluated. The functionalized liposomes displayed remarkably higher intracellular transfection efficiency comparatively to simple liposomal suspension. Liposomes functionalized with ApoB100 protein could function as excellent active targeting ligands and could be promising vectors for active targeted photosensitizer delivery in photodynamic therapy.

Key words: liposome delivery, active targeting, functionalized liposomes, ApoB100, endocytosis.

INTRODUCTION

Currently, the nanoscale triggered intracellular drug release requires carrier transfer through several biological barriers, which remains a formidable challenge to overcome. Liposomes are phospholipid vesicles with a bilayered membrane structure, widely used as pharmaceutical carriers for drugs and for targeting malignant cells, in particular in photodynamic therapy [6]. To enhance the intracellular penetration efficacy of the liposomal drugs, the drug-loaded liposomes may enter the cell by two ways: *passive targeting*, based on enhanced permeability, longevity and accumulation of liposomes in malignant cells [1], and *active targeting*, based on the attachment of specific ligands to the liposomal surface in order to bind certain antigens on the target cells. Ligand-targeted liposomes loaded with specific drugs demonstrate high potential for medical applications [17].

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Indeed, receptor-targeted drug delivery has been shown to improve cellular responses both *in vitro* and *in vivo* [12, 20].

A variety of ligands have been investigated including folate, transferrin, antibodies, peptides and aptamers. Multiple functionalities can be incorporated into the design of lipid vesicles, e.g., to enable imaging and triggered intracellular drug release [2].

In our work we are studying the *in vitro* mechanisms involved in the photosensitizer drug delivery, making use of a naturally occurring endocytosis process, which transports lipids through the blood *via* lipoproteins into the cells [18]. Lipoprotein is an endogenous particle which contains apolipoprotein B-100 (ApoB100) ligand which is recognized and taken up *via* a specific receptor, the low density lipoprotein receptor (LDLR) [19].

We propose a protocol for the preparation of ApoB100 functionalized liposomes. ApoB100-liposome (ApoB100-NBD-L) carrier is a biocompatible protein-lipid based complex which can be an excellent candidate for the targeted delivery of drugs to various malignant tissues, due to the up-regulated status of the LDLR to fulfill the elevated needs of the cell activity [9, 13].

The physiological role of the LDLR is to transport cholesterol-carrying lipoprotein particles into cells. The primary ligand for the receptor is the low-density lipoprotein (LDL) which contains a single copy of ApoB100 [3].

Receptor-ligand complexes enter the cell by endocytosis at clathrin-coated pits, where receptor molecules are clustering on the cell surface. Bound lipoprotein particles are subsequently released in the low-pH milieu of the endosome; the receptors then return to the cell surface in a process called receptor recycling [5].

The goals of our study were: 1) to set up a protocol for preparing functionalized unilamellar liposomes with ApoB100 protein; 2) to show that protein secondary structure is unchanged and the ligand maintains lipid-associating properties and 3) to demonstrate, using fluorescent microscopy, that functionalized liposomes enhance receptor mediated endocytosis.

MATERIALS AND METHODS

MATERIALS

Phospholipids 1,2-dioleoyl-sn-glycero-3-phosphocholine (18:1 DOPC), 1-oleoyl-2-[12-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]dodecanoyl]-sn-glycero-3-phosphocholine (18:1-12:0 NBD-PC) (λ_{ex} = 466 nm, λ_{em} = 539 nm) and cholesterol were purchased from Avanti Polar Lipids and used as received. LDL from human plasma, Dil complex (Dil-LDL) (λ_{ex} = 554 nm, λ_{em} = 571 nm) was purchased from Molecular Probes. ApoB100 from human plasma was purchased from Sigma. PBS buffer (137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 1.46 mM KH_2PO_4, pH 7.4) was prepared using Milli-Q Plus (Millipore) purified water.

LIPOSOME PREPARATION

A lipid stock solution containing DOPC/cholesterol/NBD-PC (68:30:2, molar ratio) was prepared in chloroform at an overall concentration of 1 mM [10]. For control samples, the stock solution was diluted with methanol at a chloroform:methanol ratio 6:4 (v:v). For ApoB100 containing sample, the protein was dissolved in methanol (10^{-3} mM) and then mixed with the lipid solution at the same choloroform:methanol ratio.

Samples were dried in a glass round-bottomed flask of a rotary evaporator (Heidolph Instruments) under nitrogen flush for 30 min, 100 rot/min, at 37 °C. The dry lipid film was hydrated using PBS buffer and further rotated, for 30 min, to ensure complete mixing of the sample constituents. The mixture was then sonicated for 5 min in an ultrasonic bath (Branson 1510), transferred in a plastic test tube and sonicated for 20 min, with continuous cycle, 100% power mode, at 4 ºC (Bandelin Sonoplus HD-2070) until a clear suspension of lipids was obtained (final lipid concentration: 1 mg/mL). Fresh liposome solutions were prepared prior to the beginning of each experiment.

CELL CULTURES

A375 human malignant amelanotic melanoma cell line was used. A375 cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented with 10% bovine serum albumin (BSA), 1% antibiotic, 1% L-Glutamate. All cells were grown at 37 °C in a humidified atmosphere containing 5% CO_2. Cultures were then washed with PBS, detached from the plate using 0.25% trypsin/EDTA (Invitrogen) incubation at 37 °C for 1 min and further cultured into 25 mm Petri dishes. Cells were allowed to attach for 30 min at 37 °C in DMEM medium 10% BSA and washed with sterile PBS. Due to differences in the growth rates, some cells did not attach and were washed away. In order to enhance the cellular over expression of LDLR, a starvation medium (Dulbecco's modified Eagle's medium with low glucose level of 1g/L and lack of Phenol Red and L-Glutamate) was used for 12 hours of incubation. The medium was then removed, fresh medium containing either simple liposomes or ApoB100-NBD-L was added and cells were allowed to incubate for 2 or 4 hours. After this period, the medium was removed and cells were washed five times with sterile PBS.

CIRCULAR DICHROISM ANALYSIS

Circular dichroism (CD) is a method for estimating secondary structure of proteins. In our experiments, CD data were collected using a Jasco810 instrument. 10^{-3} mM ApoB100 in methanol solution was used [14]. Each spectrum was an

average of four scans taken in a 1 cm optical path cuvette with a 5 s averaging time at each wavelength. CD scans of buffer solution were used for background correction [7].

FLUORESCENCE MICROSCOPY

Transmission and fluorescence microscopy (Axiovert-100, Zeiss, Germany) were used to examine liposomes distribution and endocytosis (with appropriate dichroic mirrors for Dil and NBD respectively).

RESULTS

DOPC was chosen as the primary lipid in our studies because of its low phase transition temperature (−20 °C) [15], ensuring that the resulting lipid bilayers are fluid at room temperature. The fluorescently labelled lipid used (NBD-PC, 18:1−12:0) was chosen, to ensure a structural similarity with the phospholipid to which it is tethered (DOPC, 18:1). In addition, there is a significant body of knowledge on the properties of this chromophore [4, 11], making it an attractive choice for probing the used structures.

In this work we were primarily concerned with three issues: the physical properties of liposomes such as unilamellarity of the lipid bilayer, the state of ApoB100 protein during the preparation protocol, and the cellular distribution of liposomes after incubation with cells. We are considering each of these issues individually.

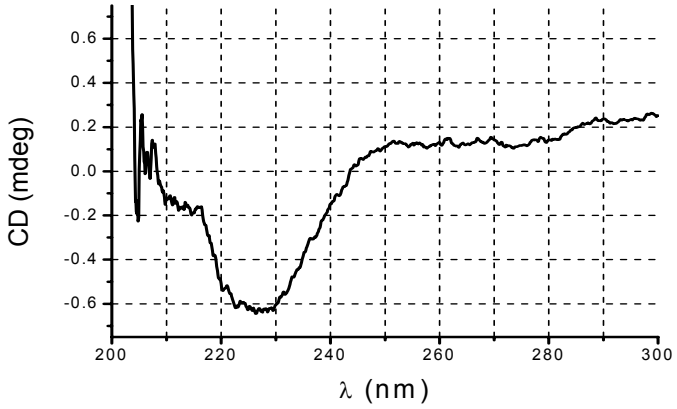


Fig. 1. CD spectrum of ApoB100 protein in methanol buffer.

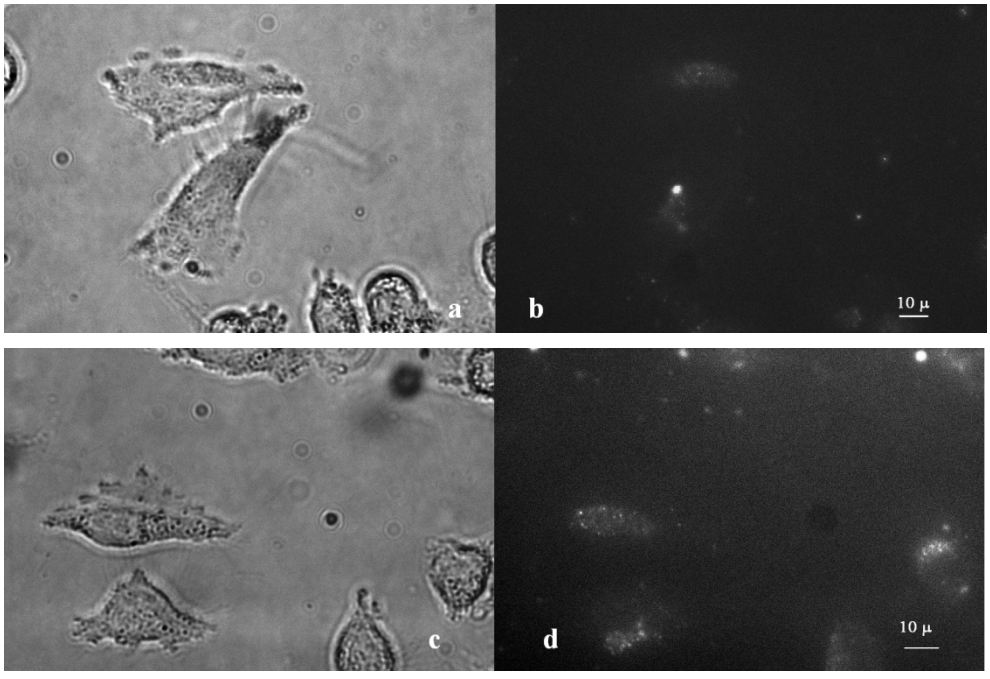
The physical attributes of liposomes obtained during the preparation are the key elements to obtain liposomes suitable for this experiment. During sonication,

the cavitation (caused by oscillating micro bubbles) produces shear fields. Large liposomes which enter these fields form long tube-like appendages that can pinch-off into smaller unilamellar liposomes. Due to the cavitation process we expect that a reorganization of the lipid bilayer occurs, allowing ApoB100 to insert into the membrane. It is well documented that the B apolipoproteins are highly insoluble in aqueous solutions [8]. They display five distinct domains composed of structural motifs responsible for the lipid-associating properties: three amphipathic α-helical domains alternating with two amphipathic β-strand domains [16]. These amphipathic sequences are the basic building blocks to achieve hydrophobic interactions with the unilamellar particles.

Using CD analysis, the molecular scale environment of ApoB100 protein during and after the preparation was studied. In Fig. 1, a typical CD spectrum of ApoB100 in methanol can be seen.

The presence of a minimum at 220–230 nm is characteristic for the existence of a predominantly α-helical secondary protein structure. This confirms that the secondary structure of ApoB100 is conserved during the liposomes preparation.

The endocytotic process was monitored by fluorescence microscopy. In Figs. 2 a–h, fluorescence images of cells incubated for 2 and 4 hours with NBD-L and ApoB100-NBD-L are shown. LDL-Dil was used as positive control for the endocytotic process of the A375 cellular line, presented in Fig. 3.



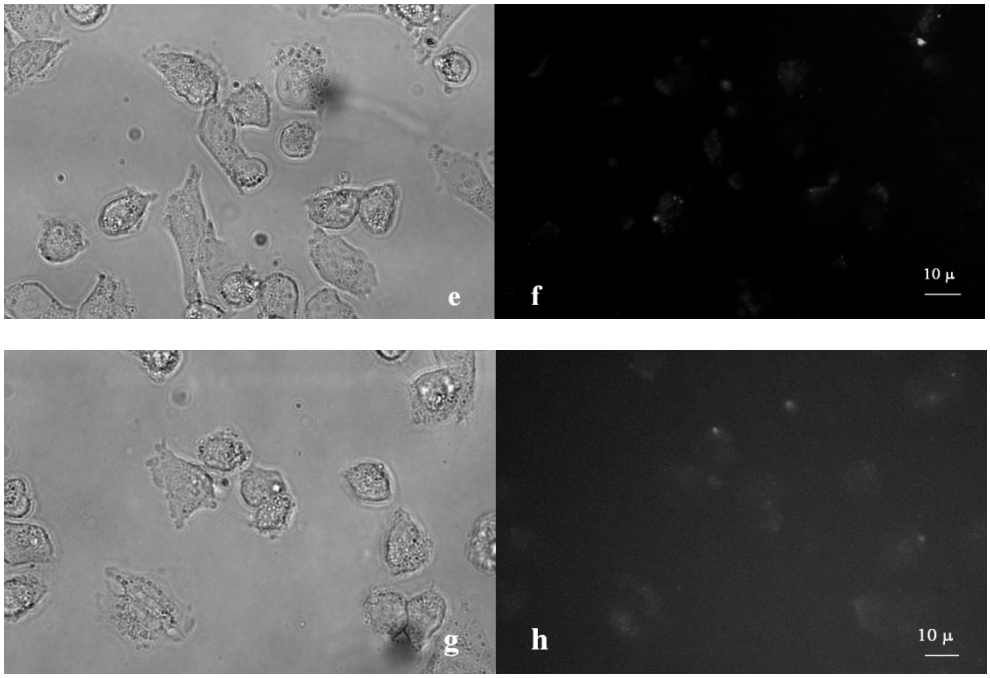


Fig. 2. Transmission (left column) and fluorescence (right column) microscopy images of A375 cultured cells (40 × magnification). a, b) 2 hour incubation period for ApoB100-NBD; c, d) 4 hour incubation period for ApoB100-NBD-L; e, f) 2 hour incubation period for NBD-L; g, h) 4 hour incubation period for NBD-L.

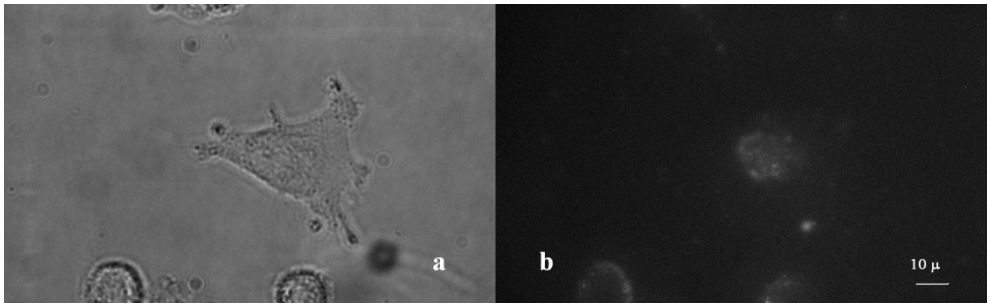


Fig. 3. Transmission (left column) and fluorescence (right column) microscopy images of A375 cultured cells (40 × magnification), positive control for endocytosis using LDL-Dil.

It can be seen that, in the case of the cells incubated with ApoB100-NBD-L (Fig. 2 b and d), the loading efficiency was higher compared to the incubation of cells with NBD-L (Fig. 2 f and h). As a positive control (Fig. 3) the aspect of the image has the same pattern as in the case of the use of ApoB100-NBD-L.

CONCLUSIONS

The goal of this study was to check whether the presence of ApoB100 within the liposomal membranes enhances the capability of the cells to endocytose the liposomes. Our findings demonstrate that, while the simple liposomes were found only on the cellular surface, the functionalized liposomes were located inside the cell as small aggregates.

Our data clearly show that the preparation protocol of the liposome functionalization with ApoB100 leaves unperturbed the protein secondary structure conformation and the active site, promoting ApoB100-NBD-L docking and binding to the LDLR, regulating direct receptor activation.

These findings indicate that the proposed protocol for preparation of functionalized liposomes which enhance receptor mediated endocytosis is a suitable procedure to prepare drug delivery systems.

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