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Cell drug delivery of fluorescein loaded ApoB100 functionalized liposomes

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ABSTRACT

Liposomes are often modified and functionalized with different ligands to control their biological properties, such as targeting ability and intracellular penetration, in a desired fashion. Thus functionalized liposomes are taken into account for selective targeting of cancer cells. The aim of this study is to check whether drug loaded ApoB100 functionalized liposomes have ability to be loaded with hidrophilic molecules, and to test their carrier properties for drugs by incubation with cultured cells. The model has been tested and validated *in vitro*, where functionalized liposomes are internalized by clatrine coated endocytosis into malignant A375 cells. This drug delivery model involves the interaction of liposomes with cells via selective anchoring of ApoB100 ligand to the LDL receptor exposed on cell membrane. The process is then followed by endocytosis of the liposome into the cell. This study was focused on preparation of fluorescein loaded liposomes, incubation with cultured cell and performing spectroscopic examination of cell lysates. Results clearly showed that fluorescein loaded ApoB100-functionalized liposomes are recognized by LDL-R on cell surface and uptaken into the malignant cell *via* endocytosis at high rate. This justifies that ApoB100 functionalized liposomes are suitable for loading, carrying and delivering drugs to the target site, and further used as drug delivery systems for cancer therapy.

Keywords: *Liposomes, drug delivery, fluorescein, cell surface.*

1. INTRODUCTION

Nowadays cancer disease is one of the most severe health problems and is currently the third most common cause of death in the world after heart and infectious diseases [1]. Novel therapies must be developed and trialed because current anticancer therapies exhibit non-ideal pharmacological properties and are distributed non-specifically throughout the body. Therefore, cancer therapy do not targeted malignant cells alone, but induce a side effects harming healthy organs and tissues [2]. Furthermore, several problems such as low bioavailability of the drugs, low drug concentrations at the site of action, lack of drug specificity and drug-resistance also cause many restrictions on clinical applications of these drugs in the tumor therapy [3].

Different types of the liposomal formulations have been used in medicine due to their distinctive advantages associated with their structural flexibility in the encapsulation of various agents with different physicochemical properties [4, 5]. They can deliver drug to the appropriate cell type, targeting subcellular compartment [6, 7].

Selective targeting of cancer cells is a critical step for cancer drug delivery therapy [8]. To address this need, ligand functionalized liposomes have attracted significant attention as possible targeting ligands [9, 10]. As drug delivery systems liposomes are biocompatible, biodegradable, have a longer half-life, and can be rerouted.

LDL is a lipoprotein particle that present ApoB100 surface protein. LDL specifically transports cholesterol to cells expressing LDL-Rs. Because the LDL-R exhibits binding specificity, mimicking naturally occurring LDL particles with liposomes, these can be used to target drugs to site-specific cells.

The goal of this study is to check whether drug loaded ApoB100 functionalized liposomes are suitable for carrying hydrophilic substances and used further as drug delivery systems. Thus were focused on preparation of functionalized liposomes and examined their ability to be loaded with hydrophilic drugs as well to act as a carrier for drugs into the cell.

Liposomes were prepared containing fluorescein hidrophylic dye as a payload and functionalized with ApoB100 protein, a surface component of native LDL with strong binding affinity for cellular LDL-Receptors that are overexpressed in cancer cells. The overexpression of the LDLRs in various tumor cells has been attributed to the large quantities of cholesterol and fatty acids required for supporting rapid proliferation [11]. The entry of liposomes into cells is mediated by several mechanisms, including clathrin-mediated endocytosis, caveolae-dependent transport and macropinocytotic uptake [12].

The properties of ApoB100 functionalized liposomes, such as small size, amphiphilic surface chemistry, and receptor-mediated uptake make them ideal candidates for therapeutics and drug delivery vehicles.

2. MATERIALS AND METHODS

Phospholipids 1,2-dioleoyl-sn-glycero-3-phosphocholine (18:1 DOPC), dimethyl-ethanolamine (DMEA) and cholesterol (CHO) were purchased from Avanti Polar Lipids and used as received. ApoB100 protein from human plasma was purchased

from Sigma. PBS buffer (137 mM NaCl, 2.7 mM KCl, 8 mM Na2HPO4, 1.46 mM KH2PO4, pH 7.4) was prepared with Milli-Q Plus (Millipore) purified water.

A lipid stock solution containing DOPC/CHO/DMEA (68:30:10, molar ratio) was prepared in chloroform at an overall concentration of 1 mM [13]. For control samples – *unfunctionalized liposomes*, the stock solution was diluted with methanol at a chloroform:methanol ratio 6:4 (v:v). The sample containing *ApoB100 functionalized liposomed*, ApoB100 protein was dissolved in methanol solution (10^{-3} mM) and then mixed with the lipid solution at the same choloroform:methanol ratio[14]. The stock solution of fluorescein in a concentration of 50 μM was prepared in 0.1 M TRIS buffer and kept in the dark at room temperature. Samples were dried in a glass round-bottomed flask of a rotary evaporator (Heidolph Instruments) under nitrogen flush for 30 min, at 100 rot/min and 37 °C. The dry lipid film was hydrated using 5ml of fluorescein solution 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).

These two samples (*Fl-L* fluorescein loaded liposome and *ApoB-Fl-L* ApoB100 functionalized fluorescein loaded liposomed) were carefully transferred into dialysis membrane, and further placed in 1L PBS solution (pH 7.4) and left 24 hours in the dark at room temperature, with continuous stirring. Fresh liposome solutions were prepared prior to the beginning of each experiment.

A375 cultured cells were used to performe the study. Cells were grown for 48 h in DMEM medium (4.5 g/LGlucose, 1% L-Glutamine, Phenol Red, Sigma), supplemented with antibiotics (Penicillin/Neomycin, Sigma) and 10% Fetal Bovine Serum (Sigma). Cells were detached using Trypsin (0.25 g/L Porcine Trypsin – 0.2 g/L EDTA-4Na in 0.09%NaCl, Sigma).

Prior to each experiment, the cells were transferred to 6 well plates and preincubated for 1 hour with DMEM medium supplemented with 10% bovine serum albumin, 1% antibiotics, 1% L-glutamate. After 60 minutes when cells adhere to the surface, the medium was removed, cells were washed 2 times with sterile PBS and added to starvation medium with low glucose concentration (DMEM with 1.5 g/L Glucose) [15].

After 12 hours of incubation, starvation medium was removed and proceeded to incubation with samples. 1 ml of Fl-L and 1 ml of starvation medium were placed in the first two wells; 1 ml of ApoB100-Fl-L and 1 ml of starvation medium were distributed in the next two wells, another two wells were incubated serving as a control for cell viability (Table 1).

Incubation periods were 2 and 4 hours after which the medium was removed from each well and washed 5 times with PBS solution, then 0.25% trypsin solution was added in each well and left for 1 min in the incubator at 37 ºC.

Table 1. Experimental set-up for incubation of A375 cultured cells in the 6 well plates. We used two sapmples: fluorescein loaded unfunctionalized liposomes Fl-L; fluorescein loaded ApoB100 functionalized liposomes ApoB-Fl-l, two wells was used as control for cell viability; incubation periods were set for 2 and 4 hours.

	Incubation periods	
Sample 1 Fl–L	2 h	4 h
Sample 2 ApoB-Fl-L	2 h	4 h
Control	2 h	4 h

Cells were further gently detached, placed in test tube and centrifuged for 3 minutes at 200 RPM. The supernatant was removed, 3 ml of PBS solution was placed instead and agitated for 10 times to homogenize the cell suspension. Over each sample a solution of 100 mL with 5% Triton100 was placed, with gentle shaking for 30 seconds until the sample became clear. The samples were further centrifuged for 3 minutes at 200 RPM. The superntant was transferred into test tubes and subjected for spectrometric determination. Spectrometric recording was performed using HORIBA Jobin-Yvon spectrofluorimeter (with a mercury lamp).

3. RESULTS SECTION

At first it was performed a spectroscopic examination of fluorescein loaded liposome: firstly, the analysis was for buffer sample, the second was the suspension of liposomes and the third was for the suspension of liposomes treated with Triton100 (detergent that disolve lipid membranes). A spectroscopic determination of fluorescein loaded ApoB100 functionalized liposome was performed in the same manner. It was observed a growth of fluorescein absorption spectra for Triton100 treated liposome sample due to lipid membranes rupture and difusion of fluorescein into the buffer as is shown in table 3. Same results were obtained for ApoB100 functionalized liposomes as is shown in table 3.

The obtained result provide us a clear evidence that liposomes are able to act as a drug loaded carriers and have the capacity to store concentrated hidrophilic subtances inside.

To test the capacity and efficiency of fluorescein loaded liposomes to be uptaken by A375 cells in culture via receptor mediated endocytosis, a spectroscopic determination of the liposome incubated cell lysates treated with detergent was performed. For this set-up, 2 and 4 hour incubation periods set.

Spectra of the cell lysates treated with Triton100 showed that fluorescein loaded ApoB100 functionalized liposomes were transported into the cell in higher rate versus unfunctionalized liposomes. Fluorescein emission spectra present a clear peak at the

515nm for 2 and 4 hour incubation period as presented in table 4 and 5.

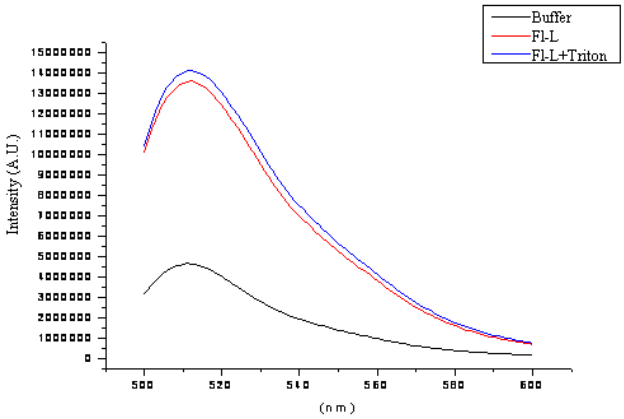

Figure 2. Fluorescein loaded unfunctionalized liposomes spectra analysis. Growth of fluorescein absorption spectra for Triton100 treated liposomes (blue line) is observed and shows that this liposomes are able to be loaded and suitable for carrying hidrophilic substances.

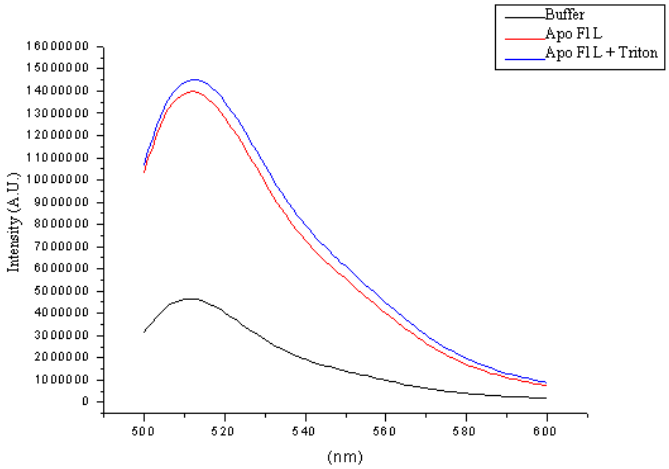

Figure 3. Fluorescein loaded ApoB100 functionalized liposomes spectra analysis. Growth of fluorescein absorption spectra for Triton100 treated functionalized liposomes demonstrate that this liposomes are able to be loaded and suitable for carrying hidrophilic substances.

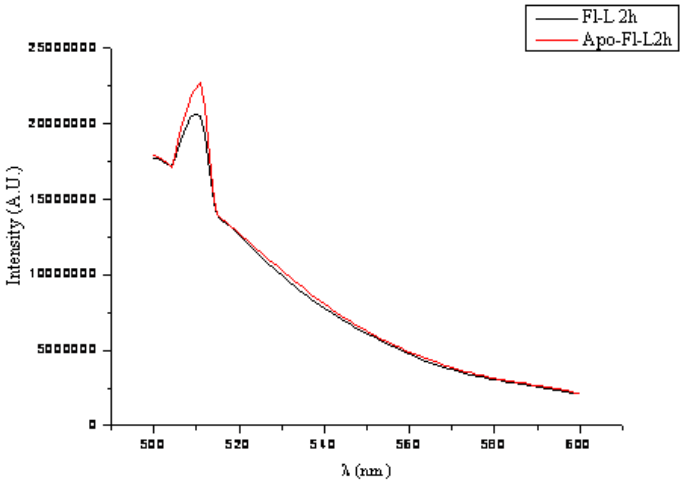

Figure 4. Cell lysates treated with Triton100 spectra analysis, 2 hour incubation period with fluorescein loaded ApoB100 functionalized liposomes and fluorescein loaded unfunctionalized liposomes. Fluorescein absorption spectra for cell lysates incubated with Apo-Fl-L liposomes is much greater than incubation with Fl-l liposomes.

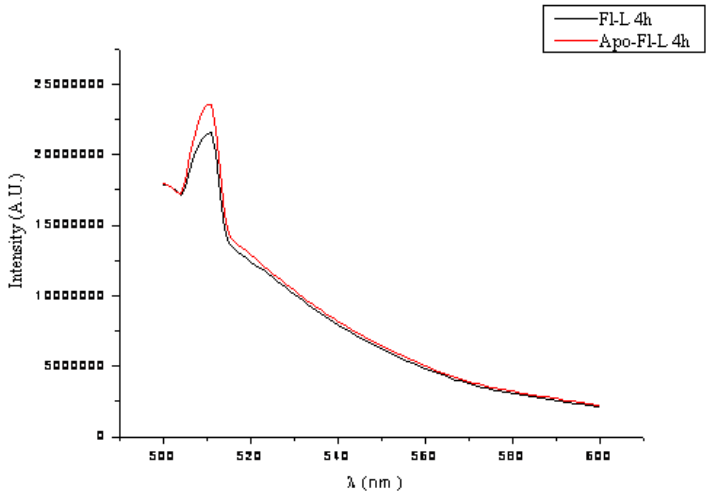

Figure 5. Cell lysates treated with Triton100 spectra analysis, 4 hour incubation period with fluorescein loaded ApoB100 functionalized liposomes and fluorescein loaded unfunctionalized liposomes. Fluorescein absorption spectra for cell lysates incubated with Apo-Fl-L liposomes is much greater than incubation with Fl-l liposomes.

4. CONCLUSIONS

The *in vitro* study performed on A375 cell line, suggests that fluorescein loaded ApoB100-functionalized liposomes can recognize LDL-R on cell surface, and therefore enable drug delivery with high specificity into the cell.

Loading the liposomes with the fluorescein dye has been shown to be effective and was confirmed by measurements of the absorption spectra of the liposome suspension. We found the maximum point value of the absorption spectrum of the after treatment the liposome detergent solution Triton100 was much higher than the maximum absorption spectrum for the untreated liposomes. This concludes that functionalized liposomes have the ability to be loaded and act as transporter for hydrophilic molecules.

After 2 and 4 hour incubation period, cell lysates treated with Triton100 spectra analysis demonstrates that ApoB100-functionalized liposomes are transported into the cell at higher rate versus nonfunctionalized liposomes. This indicates that receptor mediated endocytosis of functional liposomes is taking place.

For this experimental set-up, we should not overlook cellular pinocytosis phenomena [16]. This is a non-specific process when cells introduce substances from extracelular space and may be overrated for our A375 starved cells [17](due to incubation in starvation medium with low glucose concentration). This applies to fluorescein loaded unfunctionalized liposomes (Fl-L) to be introduced into the cell via nonspecific pathways (table 4).

Nevertheless LDL receptor mediated endocytosis efface pinocytosis, because cancer cells overexpress surface LDL receptor [18]. Binding of ApoB100 to LDL-R trigger activation of receptor mediated endocytosis and introduction of fluorescein loaded ApoB100 functionalized liposomes into the cell.

This results are very promising and encouraging for further experiments. Thus active molecules with antitimoral - proapoptotic action, will be used and loaded in the liposomes. Performing this will further demonstrate that ApoB100 functionalized liposomes are able to carry and transport

antitumoral drugs is safe way; to be endocytosed by the cancer cells, as well as to deliver active molecules to the target site and destroy malignant cells.

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