European Journal of Pharmaceutical Sciences 85 (2016) 39–46

Contents lists available at ScienceDirect

European Journal of Pharmaceutical Sciences

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

The cellular internalization of liposome encapsulated protoporphyrin IX by HeLa cells

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ARTICLE INFO

Article history:
Received 1 October 2015
Received in revised form 19 January 2016
Accepted 27 January 2016
Available online 28 January 2016

Keywords:
Liposomes
Cellular internalization
Colocalization
Protoporphyrin IX
Confocal microscopy

ABSTRACT

The proper lipid composition of liposomes designed to carry drugs determines their surface properties ensuring their accumulation within selected tissue. The electrostatic potential and surface topology of liposomes affect the internalization by single cells. The high-resolution imaging of cancer cells and the distribution of protoporphyrin-loaded liposomes within the cytoplasm and its dependence on the liposome surface properties are presented. In the paper, HeLa cells were used to investigate the uptake of porphyrin-loaded liposomes and liposomes alone by means of confocal and differential interference contrast microscopies. The effect of liposomes surface electrostatic potential and surface topology on their intracellular distribution was evaluated. The time evolution of the intracellular distribution of liposomes labelled with Rhodamine-PE was examined on HeLa cells. These studies allow for the identification of the liposome lipid composition so the efficient delivery of the active substance to cancer cells will be achieved. The obtained results showed that neutral PC-liposomes are the most efficiently internalized by HeLa cells. Moreover, results showed that properties of liposomes affect not only the internalization efficiency of the photosensitizer but also its distribution within the cells, as revealed by colocalization measurements.

1. Introduction

Liposome formulations of drugs used in anti-cancer therapies allow for the extensive modification of their pharmacokinetics. This makes possible improvement of the active ingredient efficacy and/or simultaneous reduction of its toxicity (Van Leengoed et al., 1994). Liposomes can be used as autonomous delivery systems of active ingredients or as externally triggered active substance depot as in the case of the photodynamic therapy (PDT) (Chatterjee and Yong, 2008; Chatterjee et al., 2008; Fleige et al., 2012; Thakor and Gambhir, 2013). Photosensitizers as a source of toxicity to tumour tissues cause harmful effects on healthy cells as well. Therefore the application of liposomes as carriers of those compounds combined with the illumination-triggered release is a promising pharmacological strategy. In addition, lipid phase of liposomes may serve as a safe solvent for hydrophobic photosensitizers (Drummond et al., 1999). In literature, many studies on bio-distribution of substances used in PDT and their liposomal formulations can be found (Damoiseau et al., 2001; Richter et al., 1993; Wang et al., 1999). There are a large number of compounds intended for photodynamic therapy on various level of development from proof-of-principle level to clinical studies (Boumédine and Roy, 2005). The latest, third generation photosensitizer (5,10,15,20-tetrakis(m-hydroxyphenyl)chlorine) uses DPPC/DPPG liposomes for directed targeting and controlled release (Bonnett et al., 2001). Series of study on different photosensitizers confirmed that liposomal formulations are characterized by improved overall performance, including reduced toxicity, when compared to the photosensitizer in proper solutions (Buchholz et al., 2005; Kiesslich et al., 2007; Kosobe et al., 2005; Lassalle et al., 2009). Studies presented in the paper are aiming at identification of liposomes having physicochemical properties, which ensure their effective internalization by HeLa cells. Selected liposomes were subsequently tested as a new delivery vehicle ensuring high intracellular concentration of the photosensitizer.

2. Materials and methods

2.1. Liposome preparation

Four populations of empty liposomes with different physical properties were prepared by the extrusion method as described elsewhere (Senior, 1987). Vesicles were composed of egg phosphatidylcholine (PC) alone or with addition of DOTAP (N-[1-(2,3-Dioleoyloxy)propyl]-N,N,N-trimethylammonium methyl-sulphate) or PS (1-stearoyl-2-oleoyl-sn-glycero −3-[phospho-L-serine]) to appropriate molar ratios. Liposomes were labelled with 1 mol% of Rhodamine-PE. For the colocalization studies liposomes composed of egg phosphatidylcholine were labelled with 1 mol% of NBD-C6. All lipids for liposome preparation and fluorescent probes were purchased from Avanti Polar Lipids.

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http://dx.doi.org/10.1016/j.ejps.2016.01.028
0928-0987/

Protoporphyrin IX (PpIX) was purchased from Sigma. PpIX stock solution was obtained by addition of PpIX dissolved in DMSO to the aqueous buffer, which was later used to treat cells. Liposomes were produced by the hydration of a dry film method. Specifically, lipids, PpIX and fluorescent probes were mixed in chloroform. The organic solvent was removed with the stream of argon and the residues of chloroform were eliminated under the overnight, low-pressure storage. The resulting lipid film was hydrated with DMEM-F12 buffer containing 1% serum to provide a final lipid concentration 3 mg/ml, corresponding to 3.9 mM (770 average MW – Avanti Lipids) with 2 mol% of the fluorescent dye and 100 μg/ml PpIX (0178 mM). In liposomes, PpIX is a component of the lipid bilayer and its location in the membrane was measured by EPR spectroscopy. The hydrated lipid film was afterwards extruded 20 times through a 0.1 μm polycarbonate membrane (Nuclepore Corp., Pleasanton, CA) in order to obtain large unilamellar vesicles (LUV's). Final photosensitizer to lipid ratio was 1:21 (approximately 4.5 mol% of PpIX in liposomes). For cell culture studies, liposomal – PpIX formulations were diluted ten times in order to obtain 10 μg/ml PpIX concentration. Size distribution of liposomes alone and that containing photosensitizers was determined using dynamic light scattering (DLS) with individually determined values of viscosity for each aqueous solution. The concentration of lipid in the measured samples was 0.2 mg/ml. For the viscosity evaluation a suspension of beads with well-defined size (66 nm in diameter) was used. In accordance with current standards, measurements using dynamic light scattering were conducted in water. For this purpose 10 μl of studied formulation was added to 990 μl of MiliQ water and gently stirred to obtain visually homogeneous suspension, free of air bubbles and droplets on the walls of cuvette. The size distributions of liposomes alone and liposomes containing PpIX with corresponding correlation functions are presented in Table 1 and in Fig. 1S (Supplementary Materials).

2.2. Cell cultures

HeLa cells were cultured on 20 mm diameter, 0.17 mm thick coverslips (Menzel Gläser, Braunschweig, Germany) placed in tissue culture Petri dishes (TPP, Switzerland). DMEM (Sigma, Poznan, Poland) supplemented with 1% fatal bovine serum (Gibson, U.K.) and antibiotics were used. HeLa cells (Scherer et al., 1953) were cultured in DMEM medium at 37 °C and 5% CO_2 to the level of confluence >85%. Confluent monolayers (two days after seeding) were used in all experiments. The cell density was typically of the order of 1100 cells/mm^2. For confocal microscopy observations cells were transferred into the measurement chambers following by 12 h exposure to the suspension of liposomes at 37 °C. Cells were incubated with photosensitizer for 1, 2, 4, 12 or 24 h for microscopic imaging. For the purposes of imaging cells were transferred to microscope slides by trypsinization and incubated until measurement at 37 °C (5% CO_2). Cells incubated for 1 up to 4 h were embedded with 3% paraformaldehyde solution before imaging. Emission spectra obtained from spectrofluorometric measurements showed no significant differences between examined chromophores without and in the presence of paraformaldehyde. Therefore cells preservation does not interfere with the fluorescence of photosensitizers.

Table 1
Z-ave [nm], PdI, Zeta Potential [mV] are demonstrated.

Sample ID	Z-ave [nm]	SD* [nm]	PdI	SD PdI	Zeta potential [mV]	SD [mV]
	[nm]	[nm]		[−]	[mV]	[mV]
eggPC/NBD-C6	128	0.81	0.045	0.019	−3.6	2.32
eggPC/NBD-C6/PpIX	123	0.21	0.044	0.021	−2.4	3.18
eggPC/PpIX	124	0.85	0.06	0.021	−4.2	3.03
eggPC/PS (4:1)/Rhodamine PE	117	1.93	0.083	0.021	−54.4	3.06
eggPC/DOTAP (4:1)/Rhodamine PE	148	0.17	0.19	0.01	63.2	1.24
eggPC/Rhodamine PE	126	1.26	0.088	0.01	−7.73	0.457

* SD – standard deviation was calculated from the average of three measurements of the same sample (reflects sample stability).

2.3. Image acquisition

Empty liposomes internalization was monitored with Bio-Rad MRC1024 (Bio-Rad Microscience, Hemel Hempstead, U.K.) confocal system equipped with a Nikon Diaphot 300 microscope, 60 PlanApo oil immersion objective lens (NA 1.4), a 15 mW krypton–argon laser (ALC, Salt Lake City, UT, U.S.A.) and a 100 mW argon ion laser (ILT, Salt Lake City, UT, U.S.A.). For comparison and determination of cell organelles locations images in the transmission mode were also collected and later overlapped with the fluorescence scans. Liposomes containing PpIX was imaged with Carl Zeiss ConfoCor 3 confocal microscope equipped with argon and HeNe laser, halogen lamp to conduct observations in DIC mode, thermo-regulated ring on the surface of glass slide and chamber with CO_2 control gas flow were used. C-Apochromat water immersion objective with numerical aperture NA = 1.2 and magnification 40× was used for cells imaging. Microscope was operated by ZEN programme (Carl Zeiss). The level of autofluorescence was less than 1% of the fluorescence signal. PpIX and fluorescent labels were characterized using Evolution 100 Hellma spectrophotometer and Carry Eclipse (Varian) spectrofluorometer.

3. Results

3.1. The effect of liposome surface properties on their internalization by cells

The fate of a particulate formulation of an active ingredient is determined by the surface properties of the formulation. Two surface modifications, electrostatic charge and grafted polymers, are used for the alteration of the particulate bio-distribution and pharmacokinetics (Gabizon et al., 1990). The surface electrostatic charge is a potent biological signal leading to the rapid particle identification followed by efficient elimination (Gabizon and Papahadjopoulos, 1992). The surface electrostatic charge also affects the particulate internalization by targeted cell population (Raz et al., 1981). To test the effect of surface electrostatic charge on the internalization of vesicles by HeLa cells the liposomes were formed from mixtures of neutral (PC) and charged cationic (DOTAP) or anionic (PS) lipids. The head group of phosphatidylcholine (PC) contains negatively charged phosphate group and a positively charged choline group, therefore the head group has no net charge in physiological pH. 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) is a cationic synthetic lipid, frequently used as an element of non-viral vectors in gene therapies, and phosphatidylserine (PS) is an anionic phospholipid naturally occurring in biological membranes of eukaryotic cells. Phosphatidylserine has three ionisable groups (phosphate carboxyl and the amino group).

3.2. Internalization of phosphatidylcholine liposomes

HeLa cells incubated with phosphatidylcholine liposomes produced images, which indicate that aggregates of liposomes are localized within the cytoplasm (Fig. 1C and D) as compared with transmission images (Fig. 1A and B). The fluorescence is unevenly distributed indicating that the fluorescence probe (Rhodamine-PE) does not partition into the cell membrane structure. The pattern of fluorescence object distribution within the cell volume suggests that liposomes are internalized by the endomembrane system, which is located in the whole cytoplasm except nucleus. Presented images show therefore those liposomes and/or their aggregates are localized in endosomal vesicles and that the internalization of liposomal lipids as limited since fluorescence probe remains in liposomes. There are limited diffused fluorescence from intracellular and plasma membranes indicating limited fusion or intermembrane exchange.