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Lipid nanoparticles assessment by flow cytometry

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ABSTRACT

Background: Liposomes are promising carriers for drugs and bioactive compounds. Size and structure are their crucial parameters. Thus, it is essential to assess individual vesicles as prepared. Currently available techniques fail to measure liposome's size and structure simultaneously, with a high throughput. To solve this problem, we have developed a novel, flow cytometric method quantifying liposomes.
Methods: Firstly, the following fluorescent staining combinations were tested: DiD/TO, Rh123/DiD, Syto9/DiD. Further, chosen fluorochromes were used to compare three populations of vesicles: raw (R), obtained by thin film hydration and extruded ones (populations E10 and E21). Dynamic light scattering (DLS) was used for determination of average diameter and size distribution of nanocarriers. Structural differences between the raw and the extruded liposomes, as well as additional information concerning vesicles size were acquired employing atomic force microscopy (AFM).
Results: DLS analysis indicated that, three distinct populations of vesicles were obtained. Liposomes were characterized by mean diameter of 323 nm, 220 nm and 170 nm for population R, E10 and E21 respectively. All the populations were stable and revealed zeta potential of -29 mV. AFM confirmed that raw and extruded liposomes were differed in structure.
Conclusions and general significance: DiD/TO was the optimal fluorochrome combination that enabled to resolve distinctly the sub-populations of liposomes. Results obtained by flow cytometry were in a good agreement with those from DLS and AFM. It was proved that, flow cytometry, when proper fluorescent dyes are used, is an adequate method for liposomes assessment. The proposed method enables fast and reliable analysis of liposomes in their native environment.

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1. Introduction

Liposomes are defined as spherical vesicles ranging in diameter from 30 nm to several micrometres. They consist of one or more lipid bilayers enclosing aqueous compartments. Liposomes vary mostly in terms of size and lamellarity (Wagner and Vorauer-Uhl, 2011). They are generally recognised as very promising carriers for bioactive compounds (Akbarzadeh et al., 2013). A desired liposomal carrier for hydrophilic drug should consist of a single lipid bilayer enclosing relatively sizable aqueous compartment. Contrarily, hydrophobic drugs require multilamellar structures to maximise incorporating area. At the same time, liposomal formulation, like all of the colloidal dispersions, should be as homogenous as possible towards an adequate stability. Thus, both lamellarity and size are crucial as far as application is concerned (Johnston et al., 2007; Hofheinz et al., 2005).

Numbers of methods for liposomes characterization are developed. Fundamental properties to be assessed are: membrane fluidity, particles charge, encapsulation efficiency, structure, particles diameter and size distribution (Wagner and Vorauer-

Abbreviations: ANOVA, one-way analysis of variance; DLS, dynamic lights scattering; AFM, atomic force microscopy; ICM, intermittent contact mode; SEC, size exclusion chromatography; FFF, field-flow fractionation; ISO, International Organization of Standardization; PDI, polydispersity index; NMR, nuclear magnetic resonance; DMSO, dimethyl sulfoxide; DiD, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindodicarbocyanine perchlorate; TO, Thiazole Orange (1-Methyl-4-[(3-methyl-2(3H)-benzothiazolylidene)methyl]quinolinium p-tosylate); Rh123, Rhodamine 123 (2-(6-Amino-3-imino-3H-xanthen-9-yl)benzoic acid methyl ester); Syto9, Syto®9; BODIPY, 4,4-difluoro-4-bora-3a,4a-diaza-s-indacene; EGFP, enhanced green fluorescence protein; EV, extracellular vesicles; CV, coefficient of variation; PBS, phosphate buffered saline; MLV, multilamellar vesicles; SUV, small unilamellar vesicles; FSC, forward scatter channel; SSC, side scatter channel; FITC, fluorescein 5(6)-isothiocyanate green fluorescence signal; APC, red fluorescence signalallophycocyanine; RFU, relative fluorescence unit.

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Uhl, 2011). Among them, calorimetry, EPR spectroscopy or micro turbidometry (Sułkowski et al., 2006; Neunert et al., 2015), microscopy techniques (Bibi et al., 2011), size exclusion chromatography (SEC) (Gabrielle-Madelmont et al., 2003), field-flow fractionation (FFF) (Arifin and Palmer, 2003), dynamic light scattering (DLS) (Palmer et al., 2003), 31P NMR, and small angle X-ray scattering (Ulrich, 2002) can be named.

Despite numerous techniques are available, they fail as far as fast and reliable multiparametric liposomes analysis is concerned. Flow cytometry could meet those requirements. It is an analytical method that enables cell counting, characterization and sorting. The technique was designed for cell biology, and as such is not optimal for liposomes, which are submicron vesicles and reveal similar to water refractive index (Childers et al., 1989). Nevertheless, the detection problem could be overcome by fluorescent labelling. Flow cytometry is employed in liposome technology for size distribution and average internal and membrane volume assessment (Oku et al., 1982; Vorauer-Uhl. et al., 2000). Vorauer-Uhl proved that, using fluorescence-labelled latex beads of a known diameter as a reference, liposomes size and size distribution can be determined. The technique is proved to be reliable, of high throughput and reproducibility. However, it is adequate only for particles in the range of 100 nm-1000 nm and positively charged (Anabousi et al., 2005a). Lipoplex particles (a complex of plasmids and lipids) are also studied by flow cytometry, when both lipoid and DNA content were quantified in individual liposomes as well as membrane integrity (Pozharski and MacDonald, 2005). Moreover, structural quantification of liposomes by flow cytometry was presented by Sato. The method involves the use of BODIPY-RED-UA combined with EGFP. Double-labelled liposomes were assessed in terms of internal and membrane volume, the intrinsic properties for drug encapsulation. Afterwards, vesicles were sorted according to a desired property, what may open the way for development of minimal cells. Nevertheless, BODIPY is defined as a fluorochrome, which binds to neutral lipids (Cirulis et al., 2012; Kacmar et al., 2006). At the same time, liposomes are often prepared also from positively and negatively charged phospholipids. Additionally, method proposed by Sato assumes staining during liposomes preparation, so this procedure cannot be applied in a post-formation way (Sato et al., 2006).

A concept of post-formation liposomes' assessment, adequate for vesicles prepared from different lipids is presented in this work. The idea was to label liposomes both with lipophilic dye which can bind to the lipid bilayer, and with hydrophilic dye which can enter aqueous compartments. Accordingly, a feedback on crucial properties of liposomes would be obtained in a fast and reliable manner. Furthermore, neither severe manipulation of sample's environment nor complex preparation step would be required. The purpose of the presented work was to select a unique fluorochromes combination and adapt flow cytometry to assess structure and size distribution within liposomal population and to compare them.

2. Materials

Soybean lecithin – PC, PE, PI molar ratio 2:1.5:2 respectively (Brenntag, Poland), chloroform (POCH SA, Poland), 1% phosphate buffered saline (PBS) of pH = 7.4 (Sigma Aldrich, Poland) were used for liposomes preparation. DiD Lipophilic Carbocyanine $DiIC_{18}$, Vybrant® Multicolor Cell-Labelling Kit, 1 mM solution in ethanol from Thermo Fischer Scientific, Poland; Thiazole Orange (TO), 1 μM solution in dimethylsulfoxide (DMSO) from Sigma Aldrich, Poland; Rhodamine 123 (Rh123), 1 μM solution in dimethylsulfoxide (DMSO) from Sigma Aldrich, Poland; Syto®9 (Syto9), 5 mM solution in dimethylsulfoxide (DMSO) from Thermo Fischer Scientific, Poland were used for staining of vesicles towards flow cytometry analysis.

3. Methods

3.1. Liposomes preparation

In order to obtain liposomes lecithin was dissolved in chloroform (20 mg/1 ml), in a round bottom flask. Solvent was further removed at room temperature to complete dryness using a rotary evaporator. Thus, a thin lipid film was formed. The lipid film was hydrated for 2 h at 42 °C, by adding 4 ml of an aqueous solution (1% PBS buffer). Hydration step was performed in a rotary evaporator, without vacuum, in a warm water bath. Obtained sample was divided into three parts: first one was collected as raw (R) vesicles, the second one was filtered 10 times through a 100 nm ISOPORE® membrane filter (LiposoFast by Avestin, Germany) giving a population E10, and the third one was similarly extruded, but for 21 times (population E21).

3.2. Zeta potential and size distribution

Liposome populations were assessed in terms of size distribution and zeta potential using Zetasizer apparatus equipped with a 5 mW helium/neon laser (Zetasizer Nano ZS090, Malvern Instrument, England). All of the presented measurements were done at 21 °C for 200 s, applying the detection angle equal to 90°. Cumulant technique with normal Gaussian distribution was applied for calculations. Results were given as the following parameters: Z-average size – a mean value of the thermodynamic diameter of analysed particles, PDI – a measure of the width of particles size distribution, Zeta potential – electrokinetic potential characterizing colloidal dispersions. Liposomes were assessed directly after preparation. The measurement was performed thrice. One-way analysis of variance (ANOVA) was realized independently for each value. Post-hoc Tukey HSD multiple comparison test was used to identify statistically homogeneous subsets a = 0.05. Statistical analysis with Statistica 10 (StatSoft, Inc., 2011) software was used.

3.3. Atomic force microscopy

Atomic force microscopy studies were conducted with a VeecoInnova AFM apparatus (Veeco Instruments Inc., New York, US). To minimize destructive lateral forces, that occur while scanning delicate vesicles in full atomic contact, a soft Intermittent Contact Mode (ICM) in air atmosphere, at the ambient pressure (about 760 mmHg) and temperature (20 °C) conditions, was used. A droplet of 15 μl of PBS buffer that contained only one type of the vesicles (population R, E10 or E21), with concentration of 10 mg/ml, was deposited on freshly cleaved muscovite mica surface (1.5 cm^2). All the measurements were started immediately after the buffer evaporation. A rectangular PPP-NCLR (Nanosensors – NanoWorld AG, Neuchatel, Switzerland) silicon μ-cantilevers, ~160 kHz and ~48 N/m, with a nominal tip radius less than 10 nm, were used. AFM was utilized to collect information about the specimen's surface morphology by means of the topography and ICM phase data. The latter bring an information which enables a distinction in various parts of a sample with respect to their different mechanical properties. All AFM output files were analysed in Gwyddion 2.43 – a free data processing software (Nečas and Klapetek, 2012).

3.4. Flow cytometric analysis

The diversity of liposome's populations was studied using a flow cytometer BD FACS Aria™III (Becton Dickinson), equipped

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