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# Colloids and Surfaces B: Biointerfaces

# Lipid-coated ruthenium dendrimer conjugated with doxorubicin in anti-cancer drug delivery: Introducing protocols

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## ARTICLE INFO

## ABSTRACT

One of the major limitations for the treatment of many diseases is an inability of drugs to cross the cell membrane barrier. Different kinds of carriers are being investigated to improve drug bioavailability. Among them, lipid or polymer-based systems are of special interest due to their biocompatibility. In our study, we combined dendritic and liposomal carriers and analysed the biochemical and biophysical properties of these formulations. Two preparation methods of Liposomal Locked-in Dendrimers (LLDs) systems have been established and compared. Carbosilane ruthenium metallodendrimer was complexed with an anti-cancer drug (doxorubicin) and locked in a liposomal structure, using both techniques. The LLDs systems formed by hydrophilic locking had more efficient transfection profiles and interacted with the erythrocyte membrane better than systems using the hydrophobic method. The results indicate these systems have improved transfection properties when compared to non-complexed components. The coating of dendrimers with lipids significantly reduced their hemotoxicity and cytotoxicity. The nanometric size, low polydispersity index and reduced positive zeta potential of such complexes made them attractive for future application in drug delivery. The formulations prepared by the hydrophobic locking protocol were not effective and will not be considered furthermore as prospective drug delivery systems. In contrast, the formulations formed by the hydrophilic loading method have shown promising results where the cytotoxicity of LLD systems with doxorubicin was more effective against cancer than normal cells.

## 1. Introduction

For many years pharmaceutical scientists have been looking for an efficient non-viral drug or gene delivery system. Presently, despite designing numerous new nanoparticles, efficiency is still low. Good vehicles for the delivery of medical biomolecules should be both non-

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toxic and of low immunogenicity. Drug delivery systems should cope with the transporting and loading of molecules into pathologically changed tissues [1,2]. Important properties of such vehicles are the regulation of solubility, bioavailability and biodistribution of the transported biomolecules [3–5]. It is especially important because most drugs have a low molecular weight [6]. Controlled drug delivery can avoid serious drug-related side-effects and improve the efficacy of therapy [4].

For efficient delivery of nucleic acids, effective carriers should protect gene material, especially as siRNA can be exposed to degradation by nucleases [2,7,8]. Moreover, the delivery of siRNA to the cytosol encounters a range of physical and immunologic barriers [4]. Nucleic acids are usually negatively charged [2,9], therefore, transport of naked genes across the negatively charged membrane into the cell is impeded [2,7,8, 10].

Different liposomes, dendrimers, polymers and polymeric micelles were tested as non-viral drug and gene delivery systems [5,8,9,11–17]. Among them, the dendrimers have attracted attention for many years. High flexibility in the design, well-defined architecture, low polydispersity, and the presence of numerous terminal groups make dendrimers promising drugs for gene delivery [5,18–21]. Different kinds of biomolecules can be attached to the surface of dendrimers [17]. Special attention should be paid to cationic dendrimers, due to their ability to create stable complexes with genetic material [2,9,22,23]. Additionally, in such complexes, the genes are protected against degradation by nucleases [2,7,8,10,23]. These systems are positively charged, therefore, they can cross the membrane barrier and be internalized into the cell [2, 8,10,24]. On the other hand, positively charged dendrimers interact with biological membranes and form micro-holes, inducing cell death that increases their cytotoxicity [2,17,25,26]. Therefore, the better solution seems to be a surface modification of dendrimers by covering them with, e.g., polyethylene glycol (PEG) or another surfactant copolymer. However, such modifications can result in a loss of the desired properties of dendrimers [27,28].

Liposomes are the second group of nanoparticles suggested as an alternative non-viral delivery system [16,29,30]. Liposomes are spherical vesicles, with an aqueous core and one or more concentric phospholipid bilayers, which have many applications in biomedical fields [31]. As nanocarriers, they offer several advantages, including biocompatibility regarding phospholipid composition and self-assembly [30]. Liposomes can carry large drug payloads. Physicochemical and biophysical properties (lipid composition, size, volume and surface charge) can be modified to control their biological characteristics [29, 30]. The use of liposomes significantly improves the pharmacokinetics and pharmacological profile of many pharmaceutical biomolecules [16, 29,30,32], however their application is limited mainly by problems with their thermodynamic instability [29].

The nano-systems that combine the advantages of dendrimers and liposomes, along with the LLDs technology (Liposomal locked in dendrimers), are relatively new in the field of drug delivery [32,33]. This idea was previously applied for poly(ethylene glycol) (PEG) hydroxyl terminated and poly(amidoamine) (PAMAM) dendrimers. The combining of dendrimers with liposomes increased the absorption, bioavailability and pharmacokinetic effects of loaded drugs [32,34].

As an additional benefit that can improve, e.g., the anti-cancer properties of a delivery system, seems to be the use of dendrimers containing ruthenium atoms. Ruthenium is the metal that has great potential in tumour treatment [4,20,35–38]. It binds strongly to DNA and thus destroys cancer cells, with reduced toxicity to normal cells and decreased cross-resistance compared to platinum-based anti-cancer drugs [9]. Therefore, in our study we combined 2 delivery agents - the ruthenium dendrimers and liposomes - to create a new LLDs drug delivery system with improved anti-cancer properties. Carbosilane ruthenium dendrimers labelled with fluorescent G1-{[[NCPh(o-N)Ru ($\eta^6$-*p*-cymene)Cl]Cl]₃[FITC]} (namely CRD13-FITC) were chosen to visually detect the dendrimer molecules in the cells. Metallodendrimer

CRD13-FITC had been considered earlier due to its high potential as an anti-cancer agent [36,39], previous results indicate that this dendrimer had higher cytotoxic effects on cancer cells compared to normal cells [4, 36–38]. Existing research and data regarding the CRD13 dendrimer indicate its ability to form conjugates with anti-cancer drugs, including doxorubicin [40]. Therefore, it appears that metallodendrimers have the potential to be effective vehicles for drug delivery in clinical anti-cancer treatments. Additionally, the presence of the fluorescence dye FITC in the dendrimer scaffold makes it possible to track its internalization into cells. The tracking is important to monitor liposomal system stability and risk of premature drug release including drug leakage [36]. Taking these findings into consideration, we decided to combine the individual benefits of dendrimers and liposomes, and find the appropriate method to produce a dendrimer/liposomal system for possible application in anti-cancer drug delivery. Optimization of the proper preparation technique of the LLDs system is important for increasing its delivery efficacy and minimizing post-synthesis processing, preventing potential damage or release of loaded material. Two methods of the LLDs system preparation were: 1) hydrophilic loading, and 2) hydrophobic loading of CRD13-FITC and CRD13-FITC complexed with doxorubicin into the liposomes. The effect of the LLD systems contained anti-cancer drug (doxorubicin) has also been evaluated regarding normal HEK 293 and cancer MCF-7 cells lines.

## 2. Materials and methods

### 2.1. Lipids, dendrimers and drugs

The first generation of fluorescently labelled cationic ruthenium-terminated carbosilane dendrimer, named CRD13-FITC has been applied an this work. CRD13-FITC contains 3 cationic surface imino-pyridine terminal groups. Metallodendrimer G1-{[[NCPh(o-N)Ru($\eta$6-*p*-cymene)Cl]Cl]₃[FITC]} namely CRD13-FITC, was prepared as previously described [34]. The structure of CRD13-FITC (Chemical formula, $C_{101}H_{142}Cl_6N_8O_5Ru_3SSi_5$, Mw = 2234.66 g/mol) is shown in Fig. 1.

Commercially available phospholipid, 1,2-dimyristoyl-sn-glycero-3-phosphocholine (**DMPC**) and the drug doxorubicin (Dox) used in this study were purchased from Sigma Aldrich, St. Louis, MO, USA.

### 2.2. Liposome preparation

Liposomes were prepared by a hydration method using **DMPC** phospholipid (1,2-dimyristoyl-sn-glycero-3-phosphocholine). 500 μl 30 mmol/L **DMPC** dissolved in chloroform was dried on a vacuum evaporator. To hydrate the dried lipid film 1.5 mL of 10 mmol/L Na-
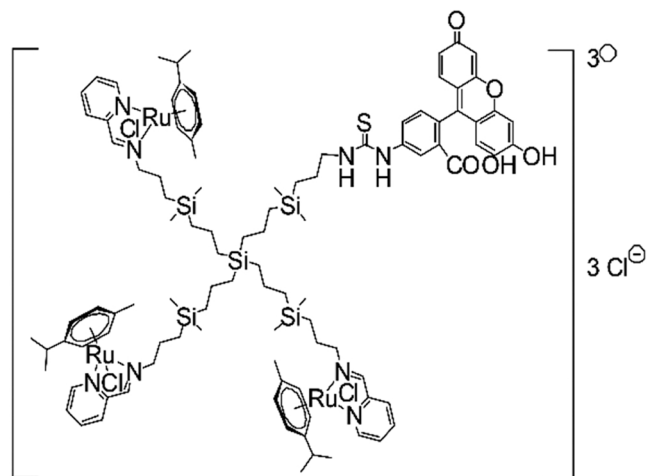


**Fig. 1.** Molecular structure of 1st generation of ruthenium terminated carbosilane Ru(II) metallodendrimer fluorescently labelled with FITC (**CRD13-FITC**).

phosphate buffer, pH 7.4, was added. After 30 min incubation at 37 °C, the hydrated lipid film was vortexed at 1000 rpm to form a multi-lamellar hydrated lipid suspension. Subsequently, lipid suspension was sonicated for 10 min and extruded at least 21 times using Avanti extruder (Avanti Polar Lipids, Alabaster, USA) through a polycarbonate membrane of a pore size 400 nm.

### 2.3. Preparation of lipid coated dendrimers

LLDs - liposomal locked in dendrimers systems were prepared in 2 ways: (A) hydrophilic loading and (B) hydrophobic loading. The main difference between these 2 techniques is the way of introducing the dendrimer or dendrimer complexed with doxorubicin into the lipid suspension. In the first method, the components were added in the buffer solution and applied for the lipid film hydration. In the second method, an appropriate volume of dendrimer or dendrimer/doxorubicin complexes was introduced into the chloroform solution of lipids. The schemas of both methods is shown in Fig. 2. In both methods, the chloroform was completely evaporated before hydration. Prepared lipid-dendrimer suspension was vortexed, sonicated in a bath sonicator and extruded to obtain the LLDs.

Prepared liposomal suspension was washed with Na-phosphate buffer solution and centrifuged 5 times for 30 min at, 2000 rpm to remove residual dendrimer molecules. The pellet was resuspended in 1 mL Na-phosphate buffer. The fluorescence intensity of FITC labelled dendrimers encapsulated in the liposomes was analysed to monitor the purification process of formed LLDs systems. A washed LLDs suspension was centrifugated, the pellet was resuspended, then added into the black-well plate and the fluorescence intensity of each sample measured using a BioTek plate-reader. Excitation and emission wavelength were 490 nm and 550 nm, respectively.

### 2.4. Zeta size measurements

A dynamic light-scattering technique was used to estimate the size of LLDs formulations. Malvern Zetasizer Nano-S90 (Malvern Instruments Limited, Worcestershire, UK) was used for the analyses. DLS measures Brownian motion and relates this to the size of the particles. Brownian motion of particles or molecules in suspension causes laser light to be scattered at different intensities. The time-dependent fluctuations in intensity of scattered light can then be analysed, and yielding the velocity of the Brownian motion then called as translational diffusion co-efficient D. This coefficient can be converted into the hydrodynamic diameter, $D_H$, using the Stoked-Einstein equation [41],

$$D_H = \frac{k_B T}{3\pi\eta D_{trans}}$$

Samples were prepared in 10 mmol/L Na-phosphate buffer, pH 7.4. Wavelength was set at 633 nm, a detection angle of 90°, and the refraction factor was 1.33. The 7 measurements in 7 cycles were collected and averaged. The experiments were performed at 25 °C. The data of average size and polydispersity index (PDI) were analysed using Malvern software.

### 2.5. Zeta potential measurements

The zeta potential of prepared LLDs formulations was measured using a Photon Correlation spectrometer, Zetasizer Nano S90 (Malvern Instruments Limited, Worcestershire, UK). Samples were suspended in 10 mmol/L Na-phosphate buffer, pH 7.4. Seven measurements in 7 cycles were collected and averaged, the experiments being done at 25 °C. The zeta potential values were calculated using the Henry equation [42].

$$U_E = \frac{2\varepsilon\zeta}{3\eta}f(Ka)$$

### 2.6. Transmission electron microscopy

The visualization of LLDs formulations involved transmission electron microscopy (JEOL-1010, JEOL, Tokyo, Japan). Sample solutions (10 µl) were put on 200-mesh copper grids with a carbon surface and stained with uranyl acetate solution for 20 min. The grids were washed with deionized water several times and finally dried at room



**co-dispersion in hydration buffer (hydrophilic loading/method A)**

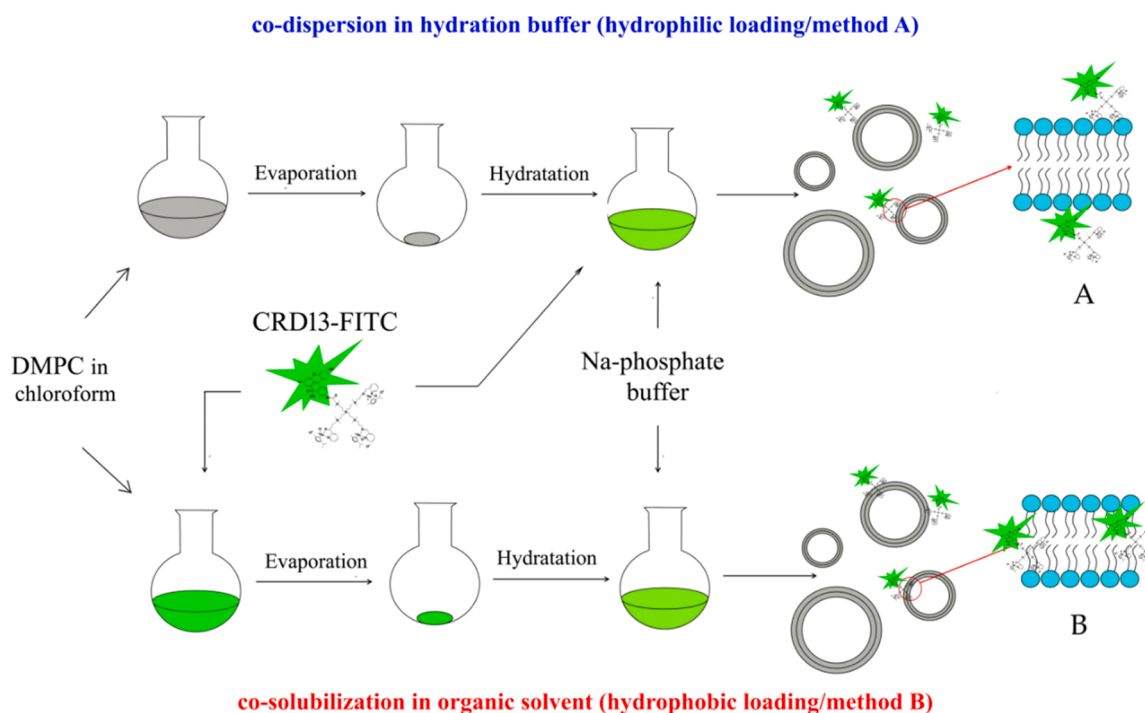**co-solubilization in organic solvent (hydrophobic loading/method B)**

**Fig. 2.** Schematic representation of preparation LLDs - liposomal locked in dendrimers systems by co-dispersion in hydration buffer (hydrophilic loading/method, **A**) and co-solubilization in organic solvent (hydrophobic loading/method, **B**). Possible localisation of the dendrimers in a liposomal membrane.

temperature.

## 2.7. Hemotoxicity analysis

To analyse the LLDs hemotoxicity profiles, the haemolysis of red blood cells technique was used. The blood of healthy adult donors was obtained from Central Blood Bank in Lodz (Poland) and used in the experiments. Erythrocytes were isolated by blood washing in phosphate-buffered saline (PBS), 10 mmol/L at pH 7.4 followed by centrifugation at 4 °C. LLDs at different concentrations were added in isolated erythrocyte suspension (haematocrit 14%). These samples were incubated at 37 °C for 24 h, before their absorbance being measured at 540 nm, using a Jasco V-650 spectrophotometer. The following formula was used to calculate the percentage of haemolysis:

$$\text{Haemolysis (\%)} = (A_{sample}\ 540\ nm\ /\ A_{water}\ 540\ nm) \times 100\%$$

## 2.8. Fluorescence anisotropy of DPH and TMA-DPH

To evaluate the interaction between LLDs and erythrocyte membranes, DPH and TMA-DPH fluorescent probes were applied. The erythrocyte membranes were isolated by washing of isolated erythrocytes with hypotonic 10 mmol/L Na-phosphate buffer, pH 7.4 at 4 °C diluted with deionised water (1:1). The membrane protein concentration was determined by the Lowry method. Interaction of LLDs with erythrocyte membranes was assessed by the analysis of changes in the fluorescence anisotropy of DPH and TMA-DPH probes incorporated in the lipid bilayer. Fluorescence anisotropy (r) can be then evaluated according to the equation:

$$r = \frac{I_{VV} - I_{VH}G}{I_{VV}G},$$

where $I_{VV}$ and $I_{VH}$ are the intensities of the fluorescence emitted parallel and perpendicular to the direction of the vertically polarized excitation light, and $G$ is the correction factor for the polarization bias of the detection system. A high degree of fluorescence anisotropy values pointed to a high structural order or low cell membrane fluidity.

The fluorescent probes were used at 1 μmol/L. The excitation wavelengths were 348 nm and 358 nm and emission wavelength were 426 nm and 428 nm for DPH and TMA-DPH, respectively. An LS-50B (Perkin-Elmer, UK) spectrofluorometer was used to detect anisotropy changes.

## 2.9. Cell cultures

To analyse the cytotoxicity, profiles and cellular uptake of LLDs systems, MCF-7 (human breast cancer cell line, ATCC, Manassas, Virginia, USA) cells and normal cells HEK 293, (human embryonic kidney cells, ATCC, Manassas, Virginia, USA) were used. Cell culture was carried out in plastic tissue culture flasks (Falcon, GE Healthcare Life Sciences, Chicago, Illinois, USA) at 37 °C in a humidified atmosphere containing 5%, $CO_2$ and 95% air. DMEM (Gibco, Thermo-Fisher Scientific, Waltham, MA, USA) with 10% heat-inactivated fetal bovine serum (FBS, HyClone, GE Healthcare Life Sciences, Chicago, Illinois, USA) containing 1% antibiotic were used for cell cultures.

## 2.10. Viability assay

HEK 293 and MCF-7 cells were seeded in 96-well plates at $1 \times 10^4$ per well and incubated in 37 °C at 5% $CO_2$ for 24 h and 72 h. The LLDs systems were added to the cells at ricing concentrations. Both LLDs system contained ruthenium dendrimer and DMPC lipids were prepared with a molar ratio 1:100 (1 molecule of dendrimer/100 molecules of lipid). The dendrimer/lipid molar ratio in both considered LLDs systems

was kept during the experiments, but the concentration of LLDs system was variable. If the dendrimer concentration in LLDs system was increased, the concentration of lipid was increased as well to keep the dendrimer/lipid molar ratio 1:100.

The systems containing doxorubicin were prepared at a molar ratio of 1:1:100 (1 molecule of dendrimer /1 molecule of drug/100 molecules of lipid). The following lipid concentrations were used: 50, 200, 500, 1000 μmol/L. The concentration of dendrimers or drugs in these systems consisted accordingly: 0.5, 2, an, 10 μmol/L, providing the constantly same dendrimer/drug/lipid molar ratio at 1:1:100.

The LLDs systems were incubated with cells for 24 and 72 h. The cells were then washed with phosphate-buffered saline (PBS). The MTT test used (3-(4,5-Dimethylthiazol-2-yl)− 2,5-Diphenyltetrazolium Bromide; Thermo Fisher Scientific (UK), which was dissolved in phosphate-buffered saline (PBS) and added to each sample. After 4 h incubation, MTT was removed. To dissolve any formed formazan crystals, DMSO solution was added. A BioTek plat- reader was used for the absorbance measurements. The wavelength was 580 nm, and the reference wavelength was 720 nm. Cell viability was calculated from the equation:

$$\text{\% viability} = (A_s/A_c) \times 100\%$$

where $A_c$ - absorbance of control, $A_s$ - absorbance of sample. The results are average SD of from least of the 3 independent experiments.

## 2.11. Cellular uptake study

To determine the cellular uptake profiles of applied LLDs systems, we used a confocal microscopy technique. Cells were seeded on 24-well glass bottom plates in DMEM containing 10% of fetal bovine serum and 1% of antibiotic (penicillin/streptomycin) at $1 \times 10^5$ cells per well. After 3, 24 and 72 h incubation with LLDs samples, cells were washed 2 times with phosphate-buffered saline (PBS) and fixed with 4% formalin for 20 min. After washing with phosphate-buffered saline (PBS,) the nuclei were stained with DAPI at μg/mL for 5 min

The confocal laser scanning microscopy platform TCS SP8 (Leica Microsystems, Wetzlar, Germany) with an objective 63 × /1.40 (HC PL APO CS2, Leica Microsystems, Germany) was used for this analysis. Samples were imaged with the following excitation and emission wavelength values: 489 nm and 490–590 nm for CRD13-FITC, and 405 nm and 430–480 nm for DAPI. For fluorescence intensity analysis, Leica Application Suite X software (LAS X, Leica Microsystems, Wetzlar, Germany) was used.

## 2.12. Statistical analysis

The data were summarized and evaluated using descriptive statistics. Continuous variables were first checked for normality using graphical methods and the Shapiro-Wilk test. In the case of responses expressed as fractions (the relative change from baseline), differences in the mean percent change were analysed. Ordinary or repeated measurement analysis of variance (ANOVA) were used, depending on the type of experimental design. Statistics and graphical representation used Microsoft Office Excel 2016 (Microsoft Corporation) and StatsDirect® 3.3.5 (StatsDirect Ltd., Cheshire, UK). Nonlinear regression analysis used the GraphPad Prism version 7.0 (GraphPad Software, Inc., US). All tests were 2-tailed, and analysed using a set level of significance of α = 0.05. P-values were rounded to 3 decimal places (Note, P-values that rounded to 0.000 have been presented as "< 0.001".).

## 3. Results

### 3.1. Biophysical characterisation of LLDs systems

To characterize the biophysical properties of formed LLDs formulations, their zeta average size and polydispersity indexes (PDI) were

firstly analysed using dynamic light-scattering technique. The zeta average sizes of both LLDs nanosystems prepared by hydrophilic or hydrophobic loading methods were larger than the size of non-complexed liposomes (Fig. 3A, Table S1). The average size of the liposomes was $421.2 \pm 45.8$ nm. The hydrodynamic diameter of LLDs prepared by the hydrophilic loading method was $475.4 \pm 2.3$ nm, whereas formulations prepared by the hydrophobic loading technique were larger, with an average size of $596.7 \pm 32.0$ nm. Polydispersity index (PDI) that showed sample homogeneity was different for the considered LLDs systems (omnibus ANOVA test $P < 0.001$). While the PDI of liposomes was $0.74 \pm 0.04$, LLDs were $0.17 \pm 0.08$ and $0.40 \pm 0.08$ for LLDs systems prepared by hydrophilic and by hydrophobic methods, respectively (Fig. 3B, Table S1).

The zeta potential technique was applied to analyse the surface charge values of the nanosystems under consideration. While the zeta potential of uncomplexed liposomes was around "1" mV, the liposome-dendrimer complexes were positively charged with zeta potential of $7.5 \pm 1.2$ mV (hydrophilic technique) and $7.35 \pm 1.2$ mV (hydrophobic method). The zeta potential for both systems did not differ significantly (Fig. 3C, Table S1).

To analyse morphological structure of complexes, transmission electron microscopy was used. Fig. 3D shows naked liposomes visible as spherical vesicles with size of ~100 nm. Similarly, the systems with dendrimers formed by hydrophilic or hydrophobic loading protocols were seen as spherical vesicles, with larger sizes of ~150 and 200 nm, respectively. In the case of LLDs systems formed by the hydrophilic loading method, small electron dense formulations with a size of 7–10 nm were also detected.

The confocal microscopy images of both studied LLDs formulations are demonstrated in Fig. 4. The microphotographs indicate localization of fluorescently labelled dendrimers inside multilamellar vesicles before the extrusion and other post-processing steps. This confirms our assumption that dendrimers are incorporated into the liposomal structure consisting of several lamellae forming vesicles with the size in the micrometres range, regardless of the methods used in the LLDs system preparation. These measurements were made for multilamellar dispersions before liposome extrusion and purification cycles. The dendrimers labelled with FITC were visible not only in the vesicles, but in the outer solution (Fig. 4).

### 3.2. LLDs interaction with erythrocyte membrane

The interaction of prepared LLDs nanosystems with the erythrocyte membrane was analysed using the hemotoxicity test. The effects of noncomplexed dendrimers and free liposomes were also considered. In general, the damage to erythrocyte membranes was influenced by the ligand releases proteins, including hemoglobin. Fig. 5A gives the percentage of hemolysis caused by free dendrimers, liposomes and LLDs systems. Noncomplexed liposomes did not show any significant hemolytic activity within the concentration range ($P > 0.05$ for all comparisons). On the other hand, free dendrimers had the strongest effect, increasing hemolysis up to 72% at 5 $\mu$mol/L was observed ($P = 0.003$). However, dendrimers "covered" by lipids were less hemotoxic in comparison with naked dendrimers. This effect occurred in both systems either prepared by hydrophilic or hydrophobic methods, but it was less pronounced for the former method ($P = 0.091$ vs. 0.013, respectively). Hemotoxic effect of dendrimers and LLDs systems was concentration dependent. The percentage of hemolysis monotonously increased with elevated sample concentration for all groups apart from liposomes ($P < 0.001$). The LLDs nanosystems were less hemotoxic than naked dendrimers overall with increasing concentration. Membrane fluidity was tested to get a better understanding of the peculiarities of interaction between biological membranes and LLDs systems. Two fluorescent probes, DPH and TMA-DPH, were separately incorporated into erythrocyte membranes. Interaction/incorporation of ligands into the hydrophobic or hydrophilic region of the lipid bilayer changes DPH or TMA-DPH anisotropy. Addition of LLDs nanosystems into the erythrocyte suspension containing DPH or TMA-DPH probes influenced the
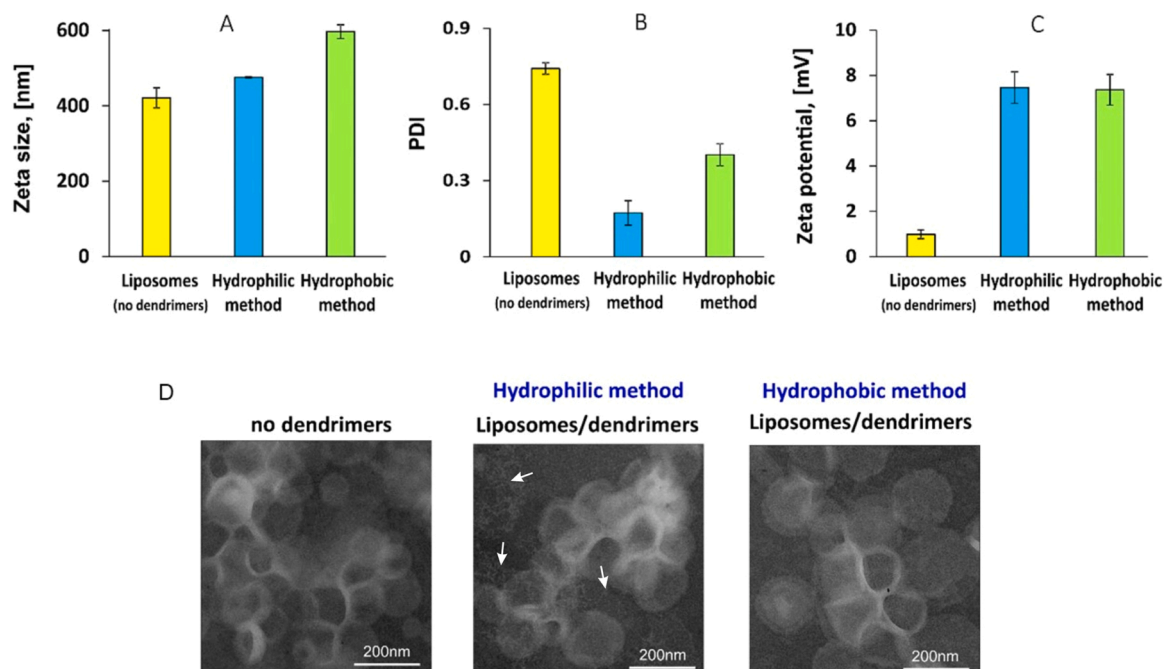


**Fig. 3.** Biophysical characterisation of LLDs systems prepared by hydrophilic or hydrophobic loading methods of LLDs preparation. (A) - zeta average size, DLS assay, (B) – polydispersity index, (C) – zeta potential. The results are presented as averages together with their standard deviations obtained from 7 measurements performed in 7 scans for each one. (D) - Transmission electron microscopy images of naked liposomes (left), LLD systems prepared by hydrophilic method (middle), and LLD systems prepared by hydrophobic method (right). Na-phosphate buffer 10 mmol/L, pH 7.4, lipid/CRR13-FITC molar ratio 1:100. Magnification of 100,000 × , Bar = 200 nm. To obtain the greater contrast the colors of images have been inverted.
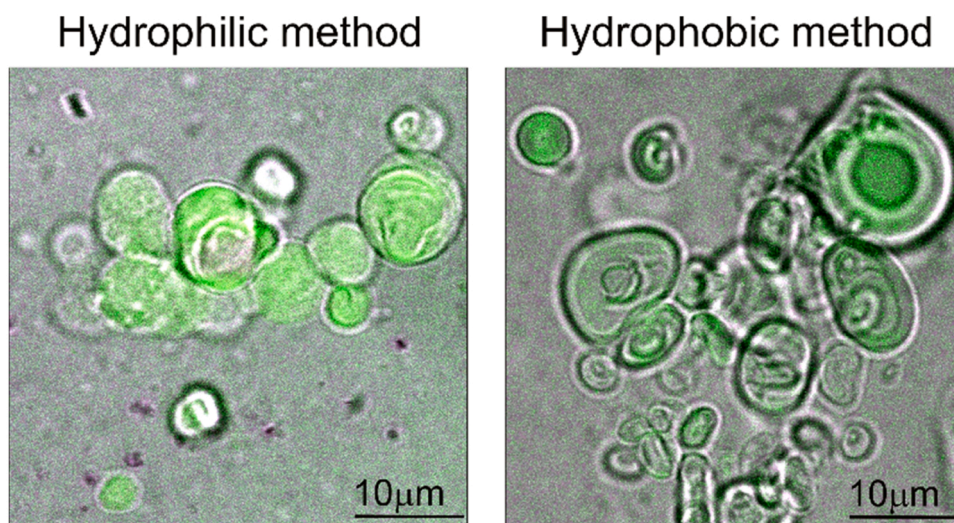
**Fig. 4.** Confocal microscopic images of the fluorescence of FITC-labelled dendrimers encapsulated into multilamellar vesicles. The samples were taken for microscopy analysis before the extrusion process. Hydrophilic method (left), hydrophobic method (right). Lipid/CRD13-FITC molar ratio 1:100. Bar = 10 μm.
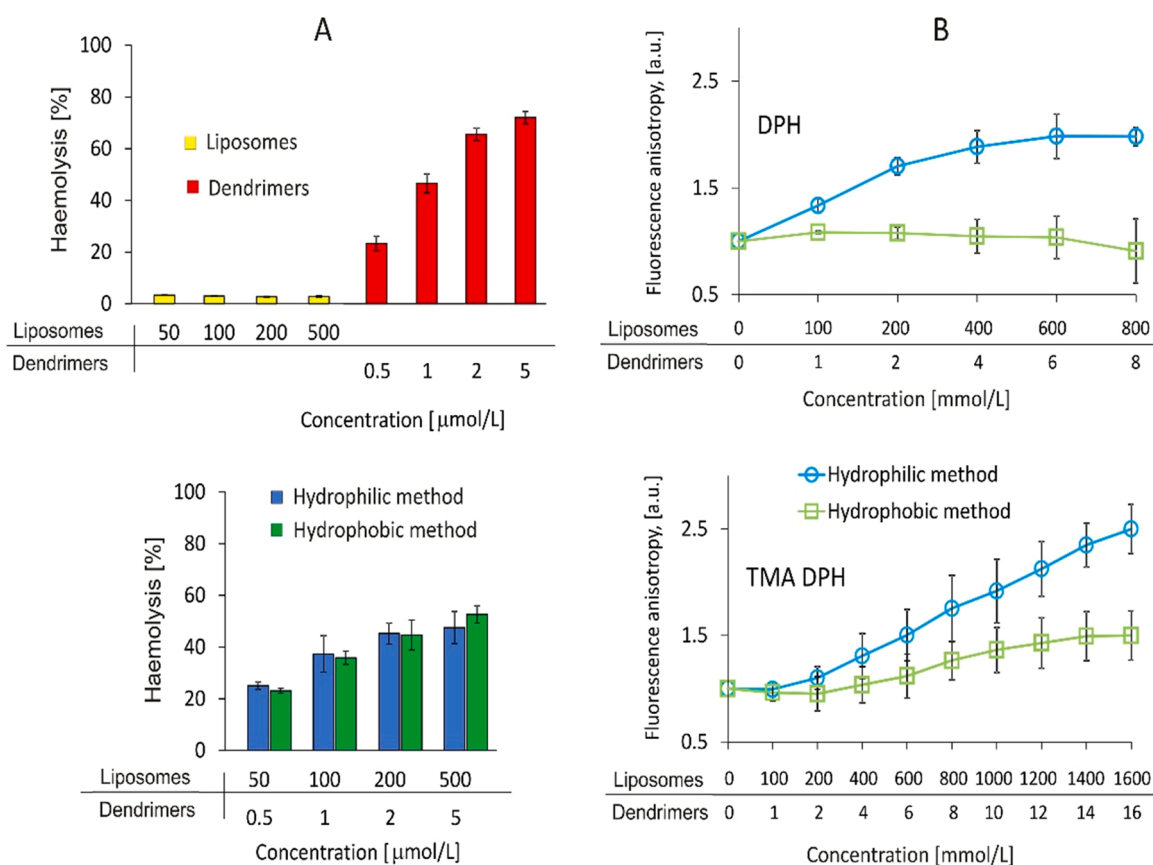


**Fig. 5.** Influence of LLD systems on human erythrocyte membranes. (A) - erythrocyte hemolysis induced by dendrimers or naked liposomes (top) and LLDs systems (bottom), hematocrit 7%, 10 mmol/L phosphate saline buffer (PBS), pH 7.4. (B) - fluorescence anisotropy of DPH or TMA-DPH fluorescent probes in membranes in the presence of LLDs systems prepared by hydrophilic or hydrophobic dendrimer loading protocols. The results are averages and their standard deviation obtained from 2 to 3 independent measurements.

fluorescence anisotropy values depending on the method of loading (Fig. 5B). LLDs nanosystems prepared by the hydrophilic loading technique interacted more strongly with both parts of the membranes compared with LLDs prepared by the hydrophobic loading method (P < 0.001). Interestingly, "hydrophobic locking" of the LLDs systems made the membrane more rigid near the phospholipid head groups.

TMA-DPH was also sensitive to increases in the area of the plasma membrane surface area, but did not significantly influence the hydrophobic part of the lipid bilayer.

### 3.3. Cytotoxicity of LLDs systems containing dendrimers

Since the previously described method showed the hemotoxic effects of studied LLDs nanosystems, their cytotoxic profiles have also been analysed. MTT assay was used to determine cell viability in the presence of LLDs systems.

Two cell lines, HEK 293 (human embryonic kidney cell line) and MCF-7 (human breast cancer cell line) were chosen for the tests. There was a non-selective pattern against the non-cancer cell line due to the systems affecting the non-tumour cells equally after 24 h (P = 0.648) and 72 h of incubation (P = 0.905) irrespective of the method of loading (Fig. 6, Table S2). The cytotoxic effect vs control cells was found for complexes created by both systems, even after 72 h incubation at the highest concentrations of the system's components: lipids 1000 μmol/L and dendrimers 10 μmol/L, with a molar ratio of 1:100. Concentration per se was the only significant factor (P < 0.003).

### 3.4. Cellular uptake

The internalisation capacity of the liposomes generated by the cell could be influenced by the method of preparation of the liposomes (hydrophilic or hydrophobic locking). The investigation was to determine whether the LLD systems due to the 2 different locking protocols might be useful in anti-cancer drug delivery.

Internalization experiments used confocal microscopy for MCF-7 cells. The FITC green signal was found in all cells treated with LLDs. The results suggest that the internalisation profiles of the LLDs system formed by hydrophilic loading were more efficient than those of the hydrophobic system (P < 0.001; Fig. 7A). This effect was time-dependent (P < 0.001). The fluorescence intensity of FITC labelled dendrimers decreased during the time, being maximal after 3 h and minimal after 72 h (Fig. 7B).

### 3.5. Cytotoxicity of LLD systems containing dendrimer-complexed doxorubicin

The results on cellular uptake showed that the hydrophilic LLD systems efficiently loaded dendrimers into MCF-7 cells. Previous data had indicated that carbosilane dendrimers could form stable complexes with anti-cancer drugs [4]. Thus, it was determined whether complexing dendrimers with doxorubicin were loaded into liposomes of cancer and normal cells, and their effect on cell viability. Fig. 8 indicates that LLD
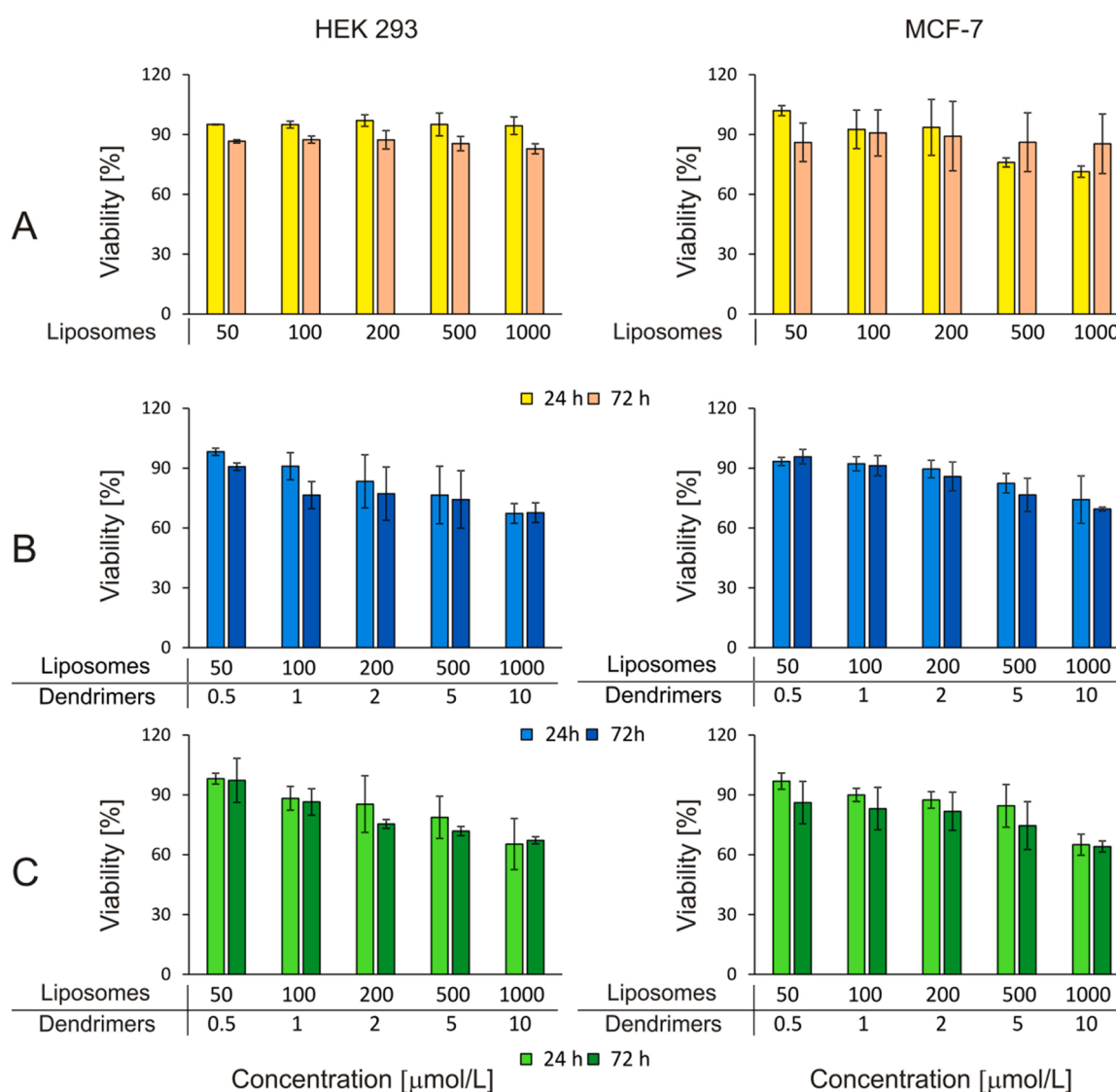


**Fig. 6.** Effect of LLDs systems on the viability of HEK 293 and MCF-7 cells after 24 and 72 h. (A) – liposomes, (B) – LLDs systems prepared by the hydrophilic locking method, (C) - LLDs systems prepared by the hydrophobic loading method. The results are averages with one standard deviation from 2 to 3 independent measurements.
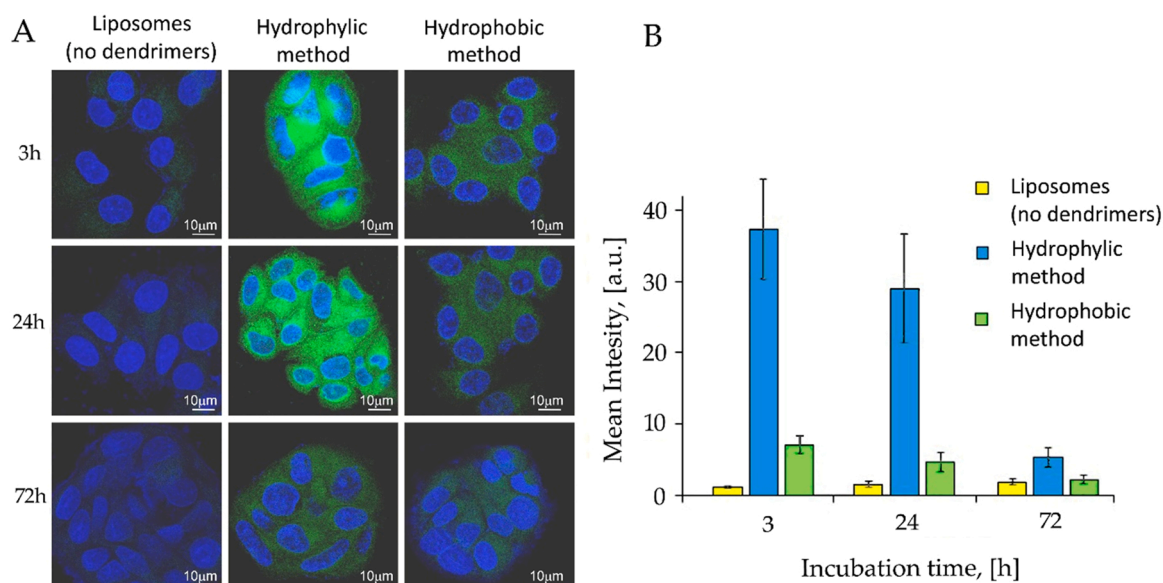
**Fig. 7.** (A) – Confocal microscopy images of MCF-7 cells incubated with liposomes or LLDs systems formed by hydrophilic or hydrophobic loading methods. (B) - fluorescence intensity of CRD13-FITC as a part of LLDs system internalized into MCF-7 cells after 3, 24 and 72 h incubation.

systems with dendrimers and doxorubicin were more cytotoxic against cancer than normal cells in a concentration-dependent manner (P < 0.001) at both incubation times (P < 0.003). Unexpectedly, the main effect of the treatment was highly significant for both of the protocols; however, the hydrophilic method proved better than the hydrophobic method – a significance level of P = 0.117 for the doxorubicin and P = 0.008 for the dendrimer-doxorubicin complex.

The viability of MCF-7 cells after 72 h incubation with LLDs contained doxorubicin (no dendrimers) decreased by up to $67.4 \pm 7.9\%$ vs the control for the hydrophobic protocol and up to $48.05 \pm 1.2\%$ for the hydrophilic protocol. When MCF-7 were treated with LLDs containing Dendrimer/Dox complexes, cell viability dropped by up to $46.6 \pm 1.2\%$ (hydrophobic method) and up to $44.1 \pm 0.8\%$ (hydrophilic method) vs the controls (Fig. 8, Table S3). The IC50 values of tested nanosystems are given in Table 1.

## 4. Discussion

One of the most important issues in the treatment of numerous diseases, especially cancer, is finding a good delivery system of therapeutic biomolecules [2,7,8,17,24,29,32,33,43,44]. To date, there are still numerous drawbacks for known drug carriers, including high toxicity and low effectiveness [1,26,45]. Cationic dendrimers have a great potential in forming complexes with drugs and genes, and thus can assist their transport across the biological barriers (membranes) [2,8,17,26]. On the other hand and due to their positive charge, they can form micropores in membranes that aids their cytotoxicity [17,20,26,27]. Nevertheless, the enormous flexibility in dendrimer design makes them as one of the more promising drug and gene delivery agents. Currently, metallodendrimers - including ruthenium dendrimers - are seen as potential agents in cancer therapy [2,4,10,20,35–39]. Therefore, combining their properties with the advantages of lipid systems by encapsulating them in micelles or liposomes can improve their effectiveness as therapeutic biomolecules [32–34]. For this purpose, it is desirable to develop an optimal experimental protocol to form an effective lipid/dendrimer drug delivery system. In this context, we investigated dendrimer/liposomal LLD systems prepared by 2 different methods of dendrimers encapsulation: co-dispersion in hydration buffer (hydrophilic loading) and co-solubilization in organic solvent (hydrophobic loading). Both systems were first analysed regarding their biophysical properties, e.g., surface potential or size and the polydispersity

index (PDI). The size of the nanosystem recommended for drug delivery depends on the method of application and destination. For example, the most approximate particle size for drug deposition in intravenous and intramuscular administration is 100–3000 nm, and 10–600 nm for transdermal application [45]. The size of nanocarriers in cancer therapies should be < 200 nm [46].

The sizes of the nanosystems considered here were measured by dynamic light scattering and transmission electron microscopy techniques. While the hydrodynamic diameter of LLDs systems formed by the hydrophilic and hydrophobic locking methods were $475.4 \pm 2.3$ nm and $596.7 \pm 32.0$ nm, respectively, their sizes measured by TEM were 150 nm and 200 nm, respectively. These differences could be explained by the different methods of sample preparation. For DLS measurements, the samples were formed in aqueous medium, whereas for transmission electron microscopy they were dried. The size and morphology of CRD13-FITC had been characterized earlier. From the electron micrographs, dendrimers were visible as single structures of 2–10 nm and as 50–500 nm in the aggregated form [36], which agrees with data in the literature for similar dendritic systems [47]. However, some reports indicate that such nanocomplexes consist of the carbosilane, PAMAM or phosphorus dendrimers can be efficiently internalized by cells [24,48].

Another parameter characterizing the quality of a drug carrier is its polydispersity index (PDI). The safety and stability of therapeutic compounds usually depend on their correct distribution [45]. At this point, the monodispersity of these systems becomes crucial. The optimal PDI for lipid-based carriers is < 0.3 [45]. Our results show that PDI for LLDs was 0.4 and 0.2 for the hydrophobic loading and hydrophilic loading methods, respectively.

Another important parameter which characterizes the ability of nanoparticle application in drug delivery is surface charge. DMPC lipid formulations usually have a neutral charge at physiological pH. However, adding dendrimers to the system can increase or decrease this parameter depending on the nature of groups situated in the structure or surface the dendrimer. Our results indicate that the presence of CRD13-FITC dendrimers in DMPC liposomes increased their initial zeta potential from 1 mV to $7.5 \pm 1.2$ and $7.35 \pm 1.2$ mV for LLDs prepared by hydrophilic locking and hydrophobic loading techniques, respectively, due to the surface positive charge. Previous studies [36] indicated that naked CRD13-FITC dendrimers have a higher zeta potential of $25.8 \pm 3.2$ mV. The reduced zeta potential of CRD13-FITC dendrimers upon their incorporation into the DMPC lipid vesicles imply less exposure of
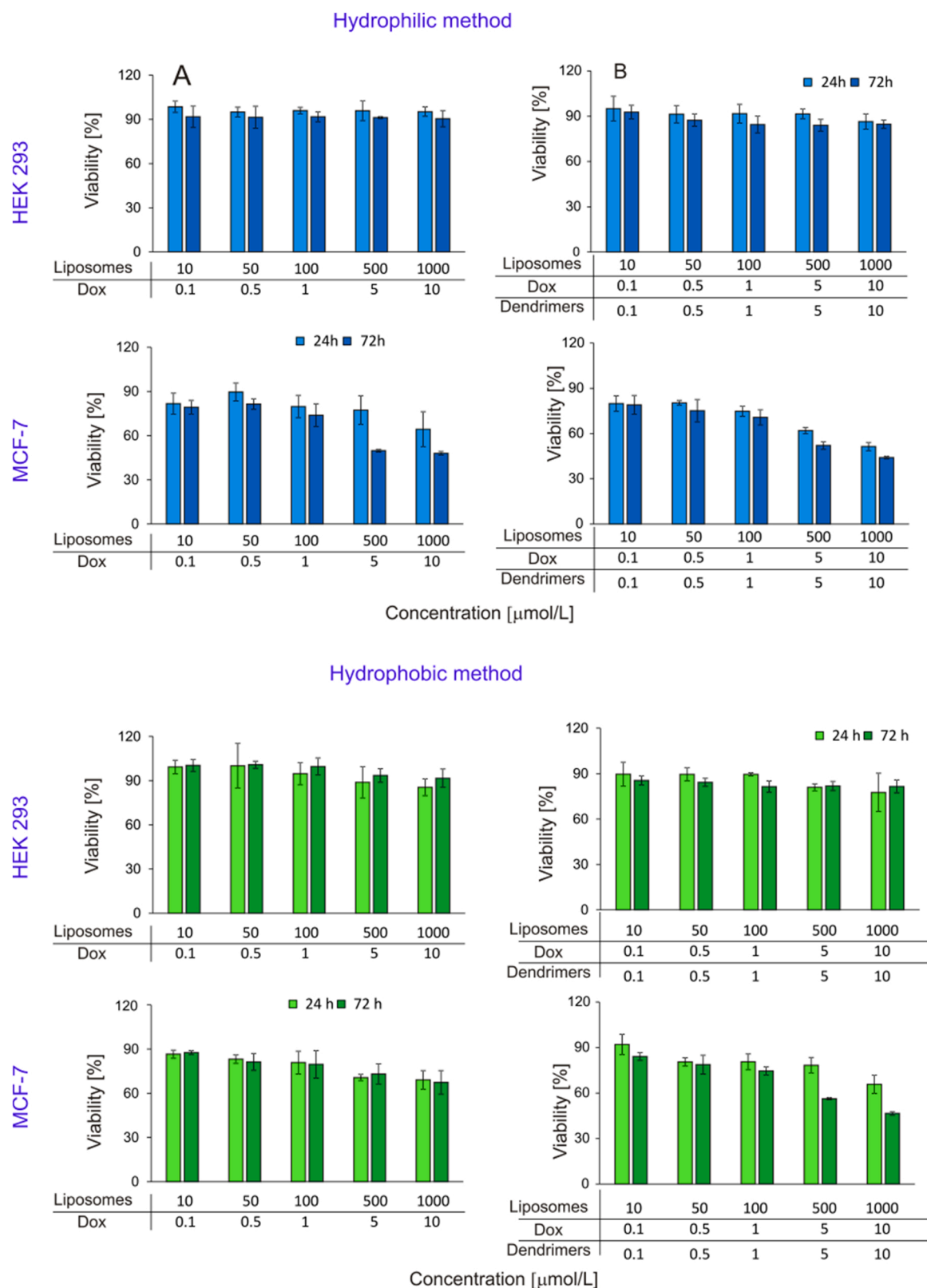
**Fig. 8.** Viability of HEK 293 and MCF-7 cells after 24 and 72 h incubation with LLDs systems containing doxorubicin (A) or dendrimer/doxorubicin complexes. (B) upper panels hydrophilic locking, lower panels hydrophobic locking. The results are averages and one standard deviation from 3 independent experiments.

the positive charges, which are partly responsible for cytotoxicity, and can explain their lower cytotoxicity seen in different experiments that were carried out.

To analyse the toxicity profiles of LLDs systems, we first evaluated

their hemolytic effects, which demonstrate how ligands interact with membranes. In this sense, while naked liposomes were essentially non-hemotoxic, the presence of dendrimers or LLDs systems significantly increased hemolysis in a concentration-dependent manner. However,

**Table 1**

IC50 values of MCF-7 cells treated with LLD systems contained Doxorubicin or Doxorubicin/dendrimer complexes showed in MTT assay, after 24 h and 72 h incubation. The results are shown as mean and standard deviation.

|  | MCF-7 | | | |
|---|---|---|---|---|
|  | hydrophilic method | | hydrophobic method | |
|  | 24 h | 72 h | 24 h | 72 h |
| LLDs containing DOX | $10.37 \pm 4.46$ | $4.79 \pm 0.13$ | $8.70 \pm 1.20$ | $7.70 \pm 0.35$ |
| LLDs containing Dendrimer/DOX complexes | $5.69 \pm 0.55$ | $4.54 \pm 0.05$ | $8.48 \pm 1.15$ | $4.85 \pm 0.11$ |

other studies have shown the dose-dependent hemolytic effect of different dendrimers containing metal atoms [1,20,36]. However, as suggested previously, the encapsulation of dendrimers in the liposomal matrix could reduce their hemotoxicity. Additionally, in the present study the influence of LLDs systems on membrane fluidity was investigated. Changes in the fluorescence anisotropy of TMA-DPH and DPH probes by being incorporated in the lipid bilayer suggest alterations in a hydrophilic and hydrophobic parts of the membrane, respectively. The presence of hydrophilic locked LLDs nanosystems in an erythrocyte membrane suspension increased the DPH and TMA-DPH fluorescence anisotropy, indicating decreased membrane fluidity in both regions of the membranes which made them more rigid. A similar effect was reported previously for CRD13, CRD13-FITC, copper and viologen-phosphorus dendrimers [35,36,41,46,47].

To evaluate their potential as carriers in cancer treatments, we have established cytotoxic profiles against normal and cancer cells. Again, the results indicate that the localisation of metallodendrimers within the liposome, decreases the cytotoxicity of the metallodendrimer for both normal (HEK293) and cancer (MCF-7) cells. This is relative to literature data in treatment with naked CRD13, CRD13-FITC, and other ruthenium dendrimers against different cell lines as previously reported [35–38].

In studying possible applications of dendrimer-liposome conjugates in anti-cancer drug delivery, analyses of their internalization capacity were based on confocal microscopy; the findings show that most effective cellular uptake profiles were with the LLDs system prepared by the hydrophilic locking protocol after 3 h treatment. Internalization efficacy of naked CRD13-FITC decreased with increasing incubation time [36]. Prolongation of the incubation time in the presence of free liposomes decreased the efficiency. Similar to the present study, previous results indicate that dendrimers were evenly distributed throughout the cells [36]. Copper [8] or ruthenium dendrimer nanocomplexes [2,10] were seen as separate dots. In contrast, the PAMAM dendriplexes were visible as numerous fine grains [24].

To improve the anti-cancer activity of new liposomal systems, we took into account that carbosilane ruthenium dendrimer can form complexes with anti-cancer drugs [4]. We had previously explored the efficiency of ruthenium dendrimers complexed with doxorubicin against MCF-7 (breast cancer) and HEK-293 (normal) cells. These complexes were weakly toxic to HEK-293, whereas the viability of MCF-7 cells was significantly decreased. Interestingly, naked doxorubicin at the same concentrations was reported as very toxic [49,50], with harmful side effects [51–53]. However, in our case, the concentrations of dendrimer and Dox were defined initially, whereas during all post-processing steps including purification of the final amount of components in the formed systems, probably were lower. Therefore, the results seem to be promising, and will need further investigations of LLDs systems.

## 5. Conclusions

This work describes 2 production protocols of LLDs systems for their possible application as smart drug delivery systems that combine the advantages of lipid and polymer formulations. Both methods were

carefully analysed and compared. The biophysical studies of the properties of these systems, as well as their interaction with erythrocytes and cells, indicate that nanosystems formed by the hydrophilic loading protocol are more suitable as drug carriers in comparison to systems formed by the hydrophobic loading method. The hydrophilic loaded formulations had an appropriate PDI index desired for drug carriers and were adequately efficient as transfectants. Therefore, we consider the LLD systems prepared by hydrophilic locking protocol should be further examined regarding their possible application.

The drug administration with the use of appropriate drug carriers can be very important in cancer treatment as it possesses some advantages over traditional approaches by combining the benefits of drugs and their carriers and allowing direct delivery into target cells and tissues. While such a system avoids possible side effects by reducing drug toxicity to normal cells, the smart delivery system should overcome natural barriers, e.g., cell membranes. This requires the finding of appropriate carriers that differ from others used as delivery vehicles for non-toxic drugs. A lot of different vectors have been considered for their potential application in anti-cancer drug delivery. However, each nanosystem has positive and negative properties. A closer examination of the current findings suggests that evaluated LLDs systems may serve as suitable agents for delivery of drugs in clinical applications aimed to combat cancer. Our findings suggest that the LLD systems obtained with hydrophilic protocol might be an optimal choice for future research, as this approach can cover a wide range of various applications in respect of their effective internalization, drug protection, release properties and reduced toxicity.

## CRediT authorship contribution statement

**Sylwia Michlewska**: Conceptualization, Data curation, Formal analysis, Methodology, Investigation, Visualization, Writing − original draft, Writing − review & editing. **Zuzana Garaiova**: Methodology, Investigation, Writing − review & editing. **Veronika Šubjakova**: Methodology, Investigation, Writing − review & editing. **Marcin Hołota**: Investigation. **Małgorzata Kubczak**: Investigation. **Marika Grodzicka**: Investigation. **Elżbieta Okła**: Investigation. **Nikolaos Naziris**: Investigation. **L.Łucja Balcerzak**: Investigation. **Paula Ortega**: Writing − review & editing, Sources. **Francisco Javier de la Mata**: Writing − review & editing, Sources. **Tibor Hianik**: Writing − review & editing, Sources. **Iveta Waczulikova**: Writing − review & editing, Methodology, Software, Data curation, Formal analysis. **Maria Bryszewska**: Funding acquisition, Data curation, Writing − review & editing. **Maksim Ionov**: Conceptualization, Writing − review & editing, Project administration, Data curation, Formal analysis, Supervision, Funding acquisition.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Data availability

Data will be made available on request.

## Acknowledgements

## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.colsurfb.2023.113371.

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