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Title: Ultradeformable liposome loaded with zinc phthalocyanine and $[\text{Ru}(\text{NH.NHq})(\text{tpy})\text{NO}]^{3+}$ for photodynamic therapy by topical application

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**Ultradeformable liposome loaded with zinc phthalocyanine and
[Ru(NH.NHq)(tpy)NO]³⁺ for photodynamic therapy by
topical application**

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Graphical Abstract

Research highlights

- √ Tween 20 as edge activator in DOPC:DMPC liposome is six times more deformable than conventional liposome;
- √ Substantial skin permeation of the photosensitizer, ZnPc encapsulated in UDLs;
- √ UDL ZnPc/RuNO is toxic for melanoma cells when irradiated at 675 nm but not in the absence of light.

Abstract

Ultradeformable liposomes (UDLs) as a drug delivery system (DDS), prepared from the unsaturated phospholipid, dioleoylphosphocholine (DOPC), and containing the non-ionic surfactant Tween 20 as edge activator, have been explored as topical vehicles for zinc phthalocyanine (ZnPc) and the nitrosyl ruthenium complex $[\text{Ru}(\text{NH.NHq})(\text{tpy})\text{NO}]^{3+}$ (RuNO) as a photosensitizers for co-generation of $^1\text{O}_2$ and NO as reactive species, respectively. However, in order to ensure that ZnPc was present in the UDLs in its monomeric form - essential for maximal ZnPc photophysical properties - it was necessary to replace 40 wt% of the DOPC with the saturated phospholipid, dimyristoylphosphocholine (DMPC). The resultant ZnPc and complex $[\text{Ru}(\text{NH.NHq})(\text{tpy})\text{NO}]^{3+}$ containing UDLs were stable for at least a month when stored at 4 °C, six times more elastic/deformable than conventional liposome (c-Ls), i.e. liposome prepared using the same weight ratio of lipids but in the absence of Tween 20, and to significantly enhance the *in vitro* permeation of ZnPc across fresh pig ear skin. The UDLs DDS incorporating ZnPc and $[\text{Ru}(\text{NH.NHq})(\text{tpy})\text{NO}]^{3+}$ were toxic (by the MTT assay) towards B16-F10 melanoma cells when irradiated with visible light at 670 nm, the maximum absorption of ZnPc, and at a dose of 3.18 J/cm², but not when applied in the absence of light

as expected. Based on these results it is proposed that the novel topical UDLs formulation developed is a suitable delivery vehicle for photodynamic therapy.

Keywords: ultradeformable liposome; nitrosyl ruthenium complex; zinc phthalocyanine; photodynamic therapy

1.Introduction

In a solid tumor, the high rate of cell proliferation and abnormal vasculature network result in a highly heterogeneous microenvironment which means that some regions of the tumor are well perfused while others are starved of oxygen - a phenomenon called as hypoxia [1-2]. Hypoxia is known to be the most important cause of clinical radioresistance. The low levels of oxygenation in a tumor is responsible for many side effect problems compromising the success of photodynamic therapy (PDT), which requires the presence of oxygen for the effective generation of reactive oxygen species (ROS) during the process, that will induce a cascade of events (apoptosis and necrosis), leading to the cell death.

In order to overcome the poor oxygenation levels in tumors, it has been proposed that a combination between the zinc phthalocyanine (ZnPc), known to produce ROS upon the application of visible light, and a nitric oxide (NO) donating agent, such as a nitrosyl ruthenium complex [3-7], should be used to significantly improve the likelihood of PDT being successful [8-9]. The advantage of including an NO donating agent is because it (NO) is an efficient radiosensitizer under hypoxic conditions, mimicking the effects of oxygen radiation-induced DNA damage [2, 10-11]. Motivated for the use nitrosyl ruthenium complexes as NO agent donor

by light irradiation on the therapeutic window (600-800 nm), proposed a combination of both, an ROS producing agent like zinc phthalocyanine compound, and a NO-donating compound as ruthenium nitrosyl complex, acting synergistically in death of the neoplastic cells [8,12-13].

PDT is a potentially exciting, non-invasive treatment for dermatological conditions such as melanoma, if the main barrier namely the *stratum corneum* (SC), which limits the permeability of many therapeutically active compounds across the skin, can be overcome to the successful delivery of compounds into the skin [14-15]. A potential means to overcome the *stratum corneum* (SC) is to deliver the PDT agents in ultradeformable liposomes (UDLs) [16-17]. UDLs are generally prepared from a mixture of conventional (vesicle-forming) phospholipids (generally in their fluid phase) combined with a molecule known as an edge activator, typically a surfactant (e.g. a bile salt or a Span) or a co-solvent (e.g., ethanol). The presence of an edge activator allows the UDLs to undergo strong, spontaneous fluctuations in structure [16,18-22], so that UDLs can deform sufficiently to penetrate pores 1/5th of their own size, and as a result, traverse the water-filled gaps between the lipid layers separating the skin cells. The high flexibility is due to the fact that elastic energy of the UDLs is of the order of the ambient thermal energy (kT), and is approximately 20 times lower than that of conventional liposomes (c-Ls) i.e. liposomes with one or more bilayers of phospholipids prepared in the absence of an edge activator [16, 23-24]. The transport of UDLs into the skin is greatly enhanced if the UDLs are not occluded upon application to the surface of the skin, so that the transepidermal hydration gradient which results as a consequence can drive the passage of the UDLs into the skin. UDLs are currently being investigated as carriers for the transdermal delivery of a range of drug molecules including non-steroidal anti-inflammatory drugs [25] and steroids [26].

The hypothesis in the present study, is the transdermal application of two compounds formulated in the same DDS, the zinc phthalocyanine, which produces reactive oxygen

species (ROS) by Type I and Type II mechanism [27], and a nitrosyl ruthenium complex which generates reactive nitric oxide species (RNOs). Both compounds packaged in a UDLs formulation should provide a very efficient means of destroying skin melanomas after irradiation of the skin with visible light of an appropriate wavelength.

The main objective of the present research was, therefore, to develop and characterize, from a physico-chemical and cellular viewpoint, the potential of a UDLs system loaded with zinc phthalocyanine, ZnPc and the nitrosyl ruthenium complex, $[\text{Ru}(\text{NH.NHq})(\text{tpy})\text{NO}]^{3+}$, (Figure 1) for use in PDT from treatment of skin melanomas.

INSERT FIGURE 1A

INSERT FIGURE 1B

As previous studies [28] in our group, have shown that UDLs can be successfully prepared from fluid-phase phospholipids such as DOPC in combination with a non-ionic surfactant such as the pharmaceutically acceptable Tween 20 (Figure 2), it was decided in the present study to prepare UDLs from DOPC in combination with Tween 20 as vehicles for ZnPc and the nitrosyl ruthenium complex, $[\text{Ru}(\text{NH.NHq})(\text{tpy})\text{NO}]^{3+}$ intended for use in PDT.

INSERT FIGURE 2

2. Experimental

2.1. Chemical and Reagents

Zinc phthalocyanine (ZnPc) was obtained from Aldrich (St. Louis, USA). The complex $[\text{Ru}(\text{NH.NHq})(\text{tpy})\text{NO}](\text{PF}_6)_3$ (RuNO) was synthesized by a procedure similar to that described by de Lima and co-workers [7]. In brief, the precursor complex $[\text{RuCl}(\text{NH.NHq})(\text{tpy})]\text{Cl}$ was

dissolved in water with sodium nitrite (NaNO_2) and refluxed under an atmosphere of argon for 1 h. After this period, hexafluorophosphate acid (HPF_6) was added to the solution with stirring and the resulting brown precipitate, namely the complex $[\text{Ru}(\text{NH.NHq})(\text{tpy})\text{NO}](\text{PF}_6)_3$, was isolated by filtration, washed with diethyl ether, and stored in the dark under vacuum until use. The complex $[\text{Ru}(\text{NH.NHq})(\text{tpy})\text{NO}](\text{PF}_6)_3$ (RuNO) was crystallized immediately prior to use. Dimyristoyl- and dioleoyl-phosphatidylcholine (DMPC and DOPC, respectively), purity >99%, were purchased from LIPOID (Germany) and Avanti (USA), respectively. Tween 20 (polyoxyethylene (20) sorbitan monolaurate) was acquired from Fluka (UK). Chloroform and ethanol (analytical grade) were supplied by Merck (UK). All chemicals were the highest grade available and used without further purification. Ultrapure water (resistivity equal $18.0 \text{ M}\Omega\cdot\text{cm}$) (Option 3-Maxima HPLC Ultra Pure Water, ELGA, UK) was used throughout the study and was filtered through a 200 nm polycarbonate filter (Millipore, UK) prior to use, in order to minimize interference from any extraneous particulate matter.

2.2. Spectroscopic measurements

Absorption measurements were performed over a wavelength range of 300 – 800 nm using a double beam Perkin-Elmer Lambda 20 spectrophotometer (Perkin-Elmer, US). The concentration of the ZnPc and RuNO were determined spectrophotometrically at the λ_{max} using $\epsilon_{670} = 2.04 \times 10^5 \text{ L}^{-1} \text{ mol cm}^{-1}$ for ZnPc in an organic solvent, namely DMF/DMSO (ZnPc is a hydrophobic compound) [8], and $\epsilon_{355} = 9550 \text{ L}^{-1} \text{ mol cm}^{-1}$ for RuNO in aqueous solution [7].

2.3. Preparation of the ultradeformable liposomes

Ultradeformable liposomes (UDLs) composed of a 60:40 wt% ratio of dioleoylphosphatidylcholine (DOPC) and dimyristoylphosphatidylcholine (DMPC), with and without Tween 20, as well as liposomes composed solely of DOPC, were prepared according

to the injection method [29]. Regardless of type, all liposomes were prepared by the injection of 360 μL of an ethanolic solution, containing sufficient lipid/lipid mixture for a final concentration of 2 mg/mL, at a flow rate of 1 $\mu\text{L/s}$, into 5 mL of a stirred solution of phosphate buffer (pH 7.4) at 40 $^{\circ}\text{C}$. Where appropriate, the ethanolic solution also contained enough Tween 20 for a final concentration of 5 mmol L^{-1} , equivalent to a concentration of 6 mg/mL. When preparing the ZnPc-containing liposomes, 50 μL of ZnPc in a 1:1 (vol%) DMF/DMSO mixture was typically added to ethanol (360 μL), so that a concentration of 5 $\mu\text{mol L}^{-1}$ ZnPc would be achieved in the final liposome suspension, although other concentrations of ZnPc were also examined in the study. For the RuNO-containing liposomes, RuNO complex was incorporated by dissolving the required amount in aqueous phase to result in a concentration of 50 $\mu\text{mol L}^{-1}$ in the liposomal suspension [8]. For associated ZnPc/RuNO UDLs formulation, firstly, the ZnPC-containing liposome were prepared like above, and after the RuNO complex was incorporated by dissolving the required amount in liposome suspension.

2.4. Determination Entrapping Efficiency (%EE)

The percentage of RuNO complex encapsulated in the liposomes was determined by measuring the amount of free/entrapped complex in the supernatant after ultracentrifugation of the suspension [30]. In brief, immediately after preparation the liposomal suspensions were stored at 4 $^{\circ}\text{C}$ overnight before ultracentrifugation at 40,000 rpm for 40 min using a L8-70M Ultracentrifuge (Beckman, UK). Free RuNO was then assayed by determining the UV spectrum of the supernatant using wavelength at 355 nm ($\epsilon_{355\text{ nm}} = 9550\text{ L}^{-1}\text{ mol cm}^{-1}$), when measured over the wavelength range of 300 – 800 nm using a Perkin-Elmer Lambda 20 spectrophotometer (Perkin-Elmer, US). The percentage RuNO entrapment was calculated from the equation 1:

$$\%EE = \frac{Q_t - Q_s}{Q_t} \times 100 \quad (1)$$

where %EE is the entrapment efficiency, Q_t is the amount of RuNO that was added during UDLs preparation, and Q_s is the amount of RuNO detected only in the supernatant after ultracentrifugation. The %EE data were obtained from three different liposomal preparations.

2.5. Determination of the solubility of the nitrosyl ruthenium complex

The aqueous solubility of RuNO was determined using a slight modification of the procedure of Al-Maaieh and Flanagan [31]. In brief, excess RuNO was added to 1 mL of water contained in a 1.5 mL centrifuge tube, which was sonicated at ambient temperature (22 °C) for 1 minute and centrifuged for 1 h at 10,000 rpm to sediment excess RuNO (Microfuge, UK). The RuNO-saturated supernatant was then removed, filtered through a 50 nm cellulose acetate membrane (Sartorius, Germany) under an external pressure of 14.5 psi (or 1 bar), diluted in water and at the $\lambda_{\max}$ of the absorbance of the resulting solution measured over the wavelength range of 300-800 nm using a double beam Perkin-Elmer Lambda 20 spectrophotometer (Perkin-Elmer, US).

2.6. Dynamic light scattering

The apparent hydrodynamic size of the various species present in the mixed lipid:Tween 20 systems were measured using dynamic light scattering (DLS). The DLS measurements were performed using an ALV-5000 system comprising a compact goniometer system and a multi- τ real-time digital correlator (AL-Laser Vertriebsgesellschaft m.b.H, Langen, Germany). The instrumentation was equipped with a 22 mW He-Ne laser source, operating at a wavelength of 632.8 nm. The duration of a single-size measurement was typically 30 s, averaged over a minimum number of three runs, until a statistically significant result was obtained. The apparent hydrodynamic size of the particulate species in the sample, and the total intensity of the scattered light were measured at a temperature of 25 °C, and at

angles ranging from 30 to 150°. However, unless otherwise stated, the apparent hydrodynamic size obtained at 90° is quoted. Samples for examination by DLS were placed in cylindrical light scattering cuvette (Pyrex disposable culture tubes, Corning Inc., Corning, NY, USA). In order to minimize dust contamination, all operations involved in the preparation of the samples for light scattering were performed in a laminar airflow cabinet. In the current study, the intensity-weight distribution of the apparent hydrodynamic radius was determined from the autocorrelation function using a Laplace inversion routine, which employs the constrained regularization method (CONTIN program).

2.7. Apparent hydrodynamic size and zeta potential

The apparent hydrodynamic size, polydispersity index (PDI), and zeta potential of c-LS and UDLs were determined by dynamic light scattering with Zetasizer Nano-ZS equipment (Malvern Instruments, Malvern, UK). All measurement were performed at room temperature (25 °C), at least two independent fresh samples were taken.

2.8. Stability of liposome formulations

The apparent hydrodynamic size-stability of the liposome formulations during storage was determined after 1 month storage at 4 °C in a darkened room and undiluted.

2.9. Quantitative determination of phospholipids

The concentration of phospholipid in a liposomal suspension before, and after its extrusion through a polycarbonate membrane (pore size either 50 or 100 nm, Millipore, UK) in a cycle of 3 times at ambient temperature (22 °C) was determined using an inorganic phosphate assay [32]. In brief, 0.4 mL of the liposome suspension, before and after its passage through the polycarbonate membrane was freeze-dried for 24 h and reconstituted in 1 mL of chloroform. To this lipidic chloroform solution, 1 mL of a 0.1 mol L⁻¹ ammonium ferrothiocyanate solution (prepared using 270.3 g/L ferric chloride hexahydrate and 30.4 g/L

ammonium thiocyanate in double-distilled water) was added, and the resulting mixture vigorously vortexed for 15 s followed by 10 min centrifugation at 2,000 rpm, in order to separate the aqueous and organic (chloroform) layers.

0.3 mL of the organic layer was diluted with 3 mL of chloroform, and the absorbance of the resulting chloroform solution measured over the wavelength range 300-800 nm, $\lambda_{\max}$ at 485 nm, after correction for the chloroform solvent, using a double beam Perkin-Elmer Lambda 20 spectrophotometer (Perkin-Elmer, US). The concentration of lipid in the chloroform solution was determined by comparison with a standard curve constructed from samples, prepared in the same way, but containing known amounts of phospholipid.

2.10. Deformability tests

In order to evaluate the deformability of the UDLs and conventional liposomes (c-Ls), their permeation at ambient temperature (22 °C), under pressure, across an artificial membrane of known pore size was determined. 1 mL of liposome suspension was passed, under external pressure of 14.5 psi (or 1 bar), through either a 100 or 50 nm polycarbonate membrane filter (Millipore, UK) held in a stainless steel pressure filter holder (Satorius, Germany). The time taken for 1 mL of an UDLs suspension to pass through 100 and 50 nm pore size filters was 20 and 30 s, respectively. The apparent hydrodynamic size of liposomes before and after passage across the filters was measured as described below.

2.11. Calculation of the deformability index

The deformability index [14] was defined from the equation 2:

$$DI = V * m * \left(\frac{r_v}{r_p} \right)^2 \quad (2)$$

where V is the ratio of the volume of liposomal suspension passed, m is the ratio of the phospholipid crossing the membrane (calculated by dividing the concentration of phospholipid in the filtrate by that in the initial liposome suspension), and $(r_v/r_p)^2$ is a factor related to the liposome size where r_v is the liposome size after filtration and r_p is the radius of the membrane pores.

2.12. Degree of retention of entrapped solute after extrusion

The degree of retention (DR) of ZnPc and RuNO after passage of UDLs containing these solutes through polycarbonate filters of 100 and 50 nm pore size, at ambient temperature (22 °C), was determined using UV/vis. analysis of the filtrate using a Perkin-Elmer Lambda 20 spectrophotometer (Perkin-Elmer, US). The absorbance was corrected for background using matched quartz cuvette and for light scattering due to the presence of liposomes using the method of Arvinte and co-workers [33]. The DR was calculated from the equation 3:

$$DR = \frac{Abs_{100nm}}{Abs_0} \times 100 \quad (3)$$

where Abs_{100nm} is the absorbance of the liposome suspension after passage through filters of 100 nm pore size, and Abs_0 is the absorbance of the liposome suspension before passage through the nanoporous polycarbonate filters. The DR were obtained from the measurement of two different liposome preparations ($n = 2$).

2.13. Permeation across fresh pig ear skin

The fresh pig ear skin was used as a model of human skin in the present study. To this end, the fresh pig ear skin was isolated and treated as previously described [34-35]. The

thickness of the pig ear skin was normalized at 75 mm using a dermatome (Robins Instruments, USA). After isolation of the skin, it was either used immediately or frozen under storing at $-20\text{ }^{\circ}\text{C}$ for a maximum of 30 days prior to use.

The *in vitro* skin profile release and retention studies were performed using the fresh pig ear skin fixed in Franz diffusion cells of surface area of 1.77 cm^2 . The receptor compartment was 7 mL of phosphate buffered saline, pH 7.4, containing 20% (v/v) ethanol to aid solubility of the hydrophobic ZnPc [36], and was maintained at $32 - 37.0\text{ }^{\circ}\text{C}$ [23] by means of an external circulating water bath. 400 μL of a UDLs formulation containing ZnPc (without RuNO) was applied (under non-occlusive conditions) to the exterior surface of the skin and the permeation of ZnPc across the skin measured over 7 h by assaying the receptor fluid its presence of ZnPc.

The Franz cells were dismantled 12 h after the application of the liposomal formulations and the fresh pig ear skin removed for determination of the amount of ZnPc present. Firstly, the skin was carefully rinsed with distilled water to remove residual formulation on the skin and dried with filter paper. After the skin samples had been attached and fixed on a smooth surface, the *stratum corneum* (SC) was removed using adhesive tape, and stripping 15 times (Scotch 3 M, Brazil). The 15 tape-strips were placed in 5 mL of DMSO in a glass tube, stored overnight, and then stirred for 1 min before the DMSO was filtered using filter paper. The remaining skin (viable *epidermis* and *dermis*) were cut into small pieces used for the extraction process. In the extraction process, 5 mL of DMSO were added to *epidermis/dermis* (ED) pieces. These samples were stirred for 20 min in ultrasonic sonicated bath (Brasonic 1800 model, 40 Hz). After filtration through a $0.45\text{ }\mu\text{m}$ polycarbonate membrane, the supernatant containing amount of ZnPc in the *epidermis* and *dermis* were determined using fluorescence spectroscopy. Fluorescence measurements were performed using a Fluorolog–Spex spectrofluorometer (Ivon–Jobin SPEX, USA), using an excitation

wavelength (λ_{ex}) of 610 nm and emission wavelength (λ_{em}) of 680 nm; slits were adjusted to 5 and 5, respectively. A calibration curve for ZnPc in 1:1 (vol%) phosphate buffered saline pH 7.4 containing 20% (v/v) ethanol/acetonitrile was prepared in the concentration range of 0.035-0.22 $\mu\text{mol L}^{-1}$.

2.14. Cell viability

The methodology employed to evaluate the toxicity of the UDLs formulations was based on the classical MTT assay [38], in which the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide after mitochondrial reduction produce the formazan colorimetric compounds used as an indicator of cell viability. B16-F10, murine melanoma cells, maintained in RPMI-1640 medium (Gibco, Brazil) complemented with 10% fetal bovine serum (Gibco, Brazil) and 1% penicillin streptomycin (Gibco, Brazil), were cultured in 60 cm^2 flasks at 37 °C in humidified air containing 5% CO_2 . The melanoma cells were used when in the logarithmic phase of their growth.

B16-F10 cells (plated at 5×10^4 cells/well in 96-well plates) were incubated with 100 μL either empty UDLs or UDLs formulations containing either RuNO (5 $\mu\text{mol L}^{-1}$), ZnPc (0.5 $\mu\text{mol L}^{-1}$) or ZnPc/RuNO (0.5 $\mu\text{mol L}^{-1}$ and 5 $\mu\text{mol L}^{-1}$) at 37.0 °C. The aqueous micellar solutions of Tween 20 in different concentration were available. After 3 h incubation, the cells were washed with 200 μL Hank's buffer (Gibco, Brazil) to remove the UDLs formulations and 200 μL of RPMI-1640 medium added to each well and the cells incubated at 37°C for a further 24 h after which time the cells were washed again with a further 200 μL of Hank's buffer and 100 μL of a 0.5 mg/mL MTT solution added to each well. After 4 h incubation at 37°C, the MTT solution was removed and any insoluble formazan crystals dissolved in isopropanol. The absorbance of the isopropanol solution was measured at 570 nm and 690 nm in a microplate

reader (ELISA, Corning, USA). The percentage cell viability was calculated with respect to control cells treated in the same way but in the absence of a UDLs formulation. All experiments were performed in triplicate with an average of 12 wells being used for each test condition. The statistical significance (P-value) of the results was determined by Student's t-test, using Igor-Pro for statistical analysis.

2.15. Phototoxicity assay

B16-F10 cells were incubated for 3 h under identical conditions to those used described above in the cell viability assay. The B16-F10 cells were incubated with 100 μL of a UDLs formulation containing ZnPC ($0.5 \mu\text{mol L}^{-1}$), and other ZnPc/RuNO ($0.5 \mu\text{mol L}^{-1}$ and $5 \mu\text{mol L}^{-1}$). After the 3 h incubation the cells were irradiated for 5 s with visible laser. The PDT protocol used: the cells were irradiated for 5 s with light of a wavelength of 675 nm (Eagle Laser, Quantum Tech, São Carlos, Brazil), potency of 0.4 W, energy of 10 J, and dose of 3.18 J/cm^2 . Immediately after exposure to the light, the cells were washed Hank's buffer and incubated with 200 μL RPMI 1640 for 24 h at 37°C and cell viability determined after this period using the MTT assay as described above. Control cells were incubated with RPMI 1640 medium for 3h and irradiated using the same conditions. All experiments were performed in triplicate with an average of 12 wells use for each test condition. The statistical significance (P-value) of the results was determined by Student's t- test and Prism Biostatistical software.

3. Results and Discussion

3.1. Selection of the composition of the ultradeformable liposome

Using the extrusion method, our group has previously demonstrated that liposomes prepared from DOPC in combination with Tween 20 exhibited a high degree of deformability

at room temperature (22 °C) [28]. In contrast, DOPC vesicles prepared in the absence of Tween 20 were far less flexible despite the fact that DOPC exhibits a phase transition of – 15.5 °C [39], illustrating the need for the presence of an edge activator when preparing UDLs. As a consequence of these results our initial formulation studies expose the potential of Tween 20-containing DOPC liposomes as a feasible DDS for the topical delivery to the combination of photoactive compounds, ZnPc and RuNO.

Based on the physico-chemical properties of each of the drugs, it was expected that ZnPc would be one of the best candidate to be incorporated into the lipid bilayer of the liposome [41]. ZnPc exhibit hydrophobic nature confirmed by *n*-octanol/water partition coefficient (Log P= 2), which ZnPc has a *n*-octanol/water ratio equal 100 [40], and complete absence of water solubility. While the RuNO compound, show high aqueous solubility (determined in the present study as 1.73 g/L) and lower trapping efficiency (%EE = 19.0 ± 3.2 (n=3)). The RuNO complex can be dissolved in the aqueous space present in the liposome, i.e. in the core or the aqueous layers between bilayers in the case of any multilamellar vesicles formed [8].

DOPC liposome was prepared by the ethanolic injection method, where were analyzed the ability of stabilize ZnPc and RuNO in monomeric form of these molecules in liposomal vesicle using UV-visible spectroscopy. The spectroscopy studies were demonstrated that DOPC liposomes were unsuitable for entrapping ZnPc due indications of aggregations.

Figure 3 shows the absorption spectrum (solid black line) of an organic solution of DMSO/DMF containing $5 \mu\text{mol L}^{-1}$ of the photosensitizer, ZnPc. The ZnPc spectrum displays an absorption maximum at 670 nm, i.e. a Q-band arising from a $\pi\text{-}\pi^*$ transition, indicating that at this concentration, ZnPc was present in the DMSO/DMF solution in its monomeric form [42-44].

The spectral profile of a freshly DOPC liposomal preparation containing ZnPc ($5 \mu\text{mol L}^{-1}$) and RuNO ($50 \mu\text{mol L}^{-1}$) were evaluated for fresh preparation and after 48 hours (Figure 3). Significantly for this part of the study, the UV-vis. spectrum of freshly prepared liposome in the presence of ZnPc and RuNO (Figure 3, dashed line) exhibits the Q-band at 670 nm, suggesting that, at least initially, the ZnPc is present in the DOPC liposomes in its monomeric form, c.f. the UV-vis. spectrum obtained for ZnPc in DMSO:DMF (Figure 3, solid black line). In addition, the UV-vis. spectrum of the ZnPc and RuNO containing-liposomes displays a shoulder at 355 nm due to the presence of RuNO and is assignable to a metal-ligand charge transfer arising from the $d_{\pi}(\text{Ru}^{\text{II}}) \rightarrow \pi^*(\text{NO})$ transition [7-8].

INSERT FIGURE 3

As can be seen from Figure 3 the UV-vis. spectra of the DOPC liposomes containing both ZnPc and RuNO changes over 48 h when stored at $25.0 \pm 2.0 \text{ }^{\circ}\text{C}$, suggesting that there is a change occurring within the formulation. In particular, there was a decrease in the intensity of the Q-bands at 606, 641, and 672 nm, an effect which is characteristic of the aggregation of ZnPc in the system under study [44]. Interestingly a similar aggregation over time has also been observed for other photosensitizer molecules encapsulated into liposomes containing DOPC [44-45]. These results suggest that DOPC liposomes are unsuitable vehicles for the delivery of ZnPc, at least at the desired ZnPc concentration of $5 \mu\text{mol L}^{-1}$. Aggregated form of photosensitizers are generally much less effective in PDT compared with their monomeric counterparts due to poor photosensitizing properties [44-45]. For example, Zimcik and co-workers [44] found that PC-dimers (phthalocyanines in dimeric form) exhibit a reduced cellular uptake, increased release from plasma, and a high level of accumulation of the photosensitizer in the liver, due to its fast release. Although it was not explored in the

present study, it may be possible to prepare DOPC liposomes containing ZnPc in the monomeric form by adding a lower amount of ZnPc.

Phthalocyanines as photosensitizer have been reported to be more soluble in their monomeric form in liposomes prepared from chain-saturated phospholipids, such as DMPC and DPPC, where solubilities of 7mg ~ ZnPc/g lipid have been reported [24,45]. Liposome prepared from chain-unsaturated phospholipids, such as soybean and DOPC, present phthalocyanines solubility of 1.7 mg/g lipid and 1.8 mg/g lipid, respectively [16,44]. On the basis of these results, it was decided to explore the possibility of encapsulating 5 $\mu\text{mol L}^{-1}$ of ZnPc in the monomeric form in liposomes prepared from a mixture of unsaturated and saturated lipids. DMPC was selected as the saturated phospholipid for study because it readily allows the formation of vesicles, and possesses a relatively low phase-transition of $\sim 24.0\text{ }^{\circ}\text{C}$ [46] (c.f. to $-15.5\text{ }^{\circ}\text{C}$ for DOPC [39]). As a consequence, it should be possible to retain the deformability of the liposomal formulation, while, at the same time, increasing the amount of monomeric ZnPc incorporated. A mixture of 60:40 wt% DOPC:DMPC was selected for study, and while it may be possible that a lower concentration of the DMPC could successfully be used, this was not examined in the present study.

The aggregation state of 5 $\mu\text{mol L}^{-1}$ ZnPc encapsulated in mixed unsaturated/saturated DOPC:DMPC liposomes was determined by examining the UV-vis. absorbance of the liposomal suspension over a period of 24 h (Figure 4) for any changes indicative of the aggregation of ZnPc – however, none were seen. By measuring the UV-vis. absorption spectra of DOPC:DMPC (60:40 wt%) liposomes containing a range of ZnPc concentrations (i.e. 1-25 $\mu\text{mol L}^{-1}$), it was possible to establish the maximum concentration of ZnPc that could be incorporated into the liposomal membrane in the monomeric state, using the Beer Lambert's law [42]. These measurements were performed on freshly prepared liposomal preparations. The UV-vis. spectra obtained for DOPC:DMPC liposomes containing different

amounts of ZnPc are shown in Figure S1 (Supplementary Material), with the insert showing the variation in peak intensity at 672 nm as a function of the concentration of entrapped ZnPc. These results indicated that as much as 5.00 ± 0.05 mg ZnPc/g lipid could be incorporated before aggregation was detected. Figure 4 confirms this result and shows that liposomes prepared from a 60:40 wt% DOPC:DMPC could incorporate 5.00 mg ZnPc/g without any aggregation being detected. Furthermore, no aggregation of ZnPc was observed in these liposomes at 48 h after preparation.

INSERT FIGURE 4

When DOPC liposomes were prepared, the solubility of the monomeric form of ZnPc was established to be only 2.00 ± 0.09 mg ZnPc/g DOPC - a result that agrees with data previously published by Zimcik and co-workers [44]. The higher solubility of monomeric ZnPc in the DOPC:DMPC liposomes c.f. to its solubility in the DOPC liposomes is a consequence of the higher solubility of monomeric ZnPc in saturated phospholipids. It is worth noting that the UV-vis. spectra revealed no change in the peaks attributable to RuNO over time in either the DOPC or the DOPC:DMPC liposomes, suggesting the attainment of an equilibrium solubility of ZnPc in the liposomes.

A light scattering study was performed to investigate how much Tween 20 could be incorporated into the liposomes prepared from a 60:40 wt% ratio of DOPC:DMPC at a total lipid concentration of 2 mg/mL. These studies are based in the addition of different amounts of Tween 20 (range from 0.9 to 25 mmol L⁻¹) to the ethanolic lipid solution used to prepare the liposomes and, using light scattering to establish the Tween 20 concentration at which the formation of aggregates other than liposomes was observed in the liposome suspension. Figure 5(A) shows the variation in the % of each species of aggregate present in a Tween

20/lipid mixture containing 2 mg/mL of a 60:40 wt% DOPC:DPMC as a function of Tween 20 concentration as determined by CONTIN analysis of the light scattering data. Figure 5(B) gives the apparent mean hydrodynamic radius of the aggregates after filtration through a 200 nm pore size filter as a function of Tween 20 concentration.

INSERT FIGURE 5A

INSERT FIGURE 5B

Significantly, as is clear from Figure 5(A), it was possible to add Tween 20 to the DOPC:DMPD at concentrations of up to 5 mmol L⁻¹, and still observe only one species of aggregate using dynamic light scattering. As can be seen from Figure 5(B), the addition of 15 mmol L⁻¹ Tween 20 to the DOPC/DMPD liposomes resulted in an increase in apparent hydrodynamic radius from 26.7 ± 1.5 to 52.7 ± 2.5 nm. The increase in apparent liposome size upon the addition of Tween 20 is in line with the observations of Morandat and Kirat [47] who have suggested that the increase in apparent size is due to the partitioning of the surfactant into the liposomal bilayer [47]. At Tween 20 concentrations of 15 mmol L⁻¹ or less there was no angular dependence observed in the apparent hydrodynamic size or total scattering of the liposomes suggesting that there was no large change in the shape of the liposomes under these conditions, supporting the assumption that the liposomes were likely to be spherical in shape (Figure S2 Supplementary Material). This result demonstrates that below to 15 mmol L⁻¹ of Tween 20, i.e. 250 times the critical micelle concentration (CMC) of Tween 20 in water at 0.059 mmol L⁻¹ [48], can be accommodated within the bilayer of the DOPC:DMPD liposomes before the need for the formation of other aggregates in solution.

At Tween 20 concentrations of 25 mmol L⁻¹, two species of aggregates were observed using light scattering – one species of an approximate apparent hydrodynamic radius of 6.7

nm (Figure 5A), assumed to be Tween 20 micelles, possibly containing a small amount of phospholipid, and a second very large, polydisperse species of a mean apparent hydrodynamic radius of 161 nm (Figure 5A). Very large, polydisperse aggregates were also observed by Lim and Lawrence when determining the effect of the addition of various Tweens (i.e. Tween 20, 40, 60, and 80) to phospholipid vesicles [28]. These large aggregates seen at added Tween 20 concentrations of 25 mmol L⁻¹ were found to exhibit an angular dependence in both their apparent hydrodynamic radius and their total scattering, with the largest apparent hydrodynamic radius and highest total scattering being observed at the lowest scattering angles (data not shown).

A corresponding study was performed on liposomes prepared using DOPC to evaluate the influence of Tween 20 on the DOPC solubilization. Interestingly, as can be seen in Figure 6(A), the DOPC liposomes were found to be slightly less resistant to solubilization by Tween 20, since two species of aggregates could be observed at added Tween 20 concentrations of 15 mmol L⁻¹ as opposed to 25 mmol L⁻¹ when the liposomes were prepared from a 60:40 wt% DOPC:DMPC. Furthermore, the mean apparent hydrodynamic radius of the DOPC liposomes in the presence of an added Tween 20 concentration of 15 mmol L⁻¹ was ~ 127 nm as opposed to 52.7 nm when prepared using a 60:40 wt% DOPC:DMPC.

These results suggest that a 9:1 wt% ratio of Tween 20:DOPC (final phospholipid concentration of 2 mg/mL) is required to, at least partially, destroy the liposomes. While in the 60:40 wt% DOPC:DMPC formulation this relation is bigger (15:1 wt% ratio of Tween 20:DOPC). Morondat and Kirat [47], made a similar observation that liposomes prepared from phospholipid mixtures were less resistant to solubilisation by surfactant than those made using a single lipid species.

INSERT FIGURE 6

As a result of establishing how much Tween 20 can be incorporated into the liposomes prepared using 2 mg/mL of DOPC:DMPC liposomes at a weight ratio of 60:40 wt%, it was decided to prepare DOPC:DMPC UDLs containing 5 mmol L⁻¹ Tween 20 for the delivery of ZnPc and RuNO. The corresponding DOPC/DMPC liposomes prepared in the absence of Tween 20 (denoted conventional liposomes, c-Ls) were used as controls. All following studies were performed using liposomes of these two compositions.

3.2. Physico-chemical characterization of liposomal preparations

The apparent hydrodynamic radius of empty and metal-loaded c-Ls and UDLs, determined using dynamic light scattering, is shown in Table 1. In agreement with our earlier study, the mean apparent hydrodynamic size of the UDLs is approximately twice that of the c-L, undoubtedly a consequence of the presence of the surfactant, Tween 20, swelling the liposomal bilayer. It can be seen that the apparent hydrodynamic size of neither of the c-Ls and UDLs changed in the presence of ZnPc and/or RuNO.

INSERT TABLE 1

Regardless of their composition, and the presence or otherwise of one or more of the metal compounds, all UDL formulations were very stable with their apparent hydrodynamic size when stored at 4 °C for a month in the dark (Table S1 in Supplementary Material). This result is in contrast to the apparent hydrodynamic size instability of DMPC liposomes, which have been reported to double in apparent hydrodynamic size within 24 h of their preparation [28]. The high stability of the liposomes in the present study is undoubtedly a consequence of

the presence of DOPC which is known to form stable liposomal apparent hydrodynamic preparations.

The zeta potential of all UDLs formulations in Table 1 were in the range 1.4 (for empty UDLs) to -5.5 mV for UDLs containing RuNO. These negatively charged liposomal formulations indicating electrostatic stabilization between the liposomes under the present experimental conditions, in which showed physical stability during 1 month. Polydispersity index (PDI) of all UDLs formulations was less than 0.4 indicating a narrow size distribution of these liposomes [49]. DOPC and DMPC are zwitterionic phospholipids, where in the study conditions (pH 7.4) the vesicles had an overall negative charge [50].

3.3. Liposome deformability

Deformability index (DI) results of the UDLs formulations were compared with the deformability of the c-Ls preparations, and the results are shown in Table 1. Regardless of the presence or absence of a metal compound within the liposomal preparation, all the UDLs formulations more rapidly crossed a 100 nm pore filter than any of the c-Ls formulations, for example 0.97 mL of a 1 mL empty UDLs suspension crossed the filter in 20 sec as opposed 0.3 mL of a c-Ls suspension which crossed in 5 min. In addition, the UDLs preparations crossing the 50 nm pore size membrane filter very readily, differently to c-Ls formulations. Multi-angle dynamic light scattering showed that UDLs preparation there was no change in either the apparent hydrodynamic radius and/or shape of the liposomes subsequent to their crossing of either the 100 or 50 nm pore size polycarbonate filters. Differently the c-Ls passed the 100 nm filter, albeit very slowly, they did not cross the 50 nm pore size filter - most likely because the pressure applied to the liposome suspension was insufficient to deform the liposomes to force their passage through the filters.

Interestingly, neither the presence of one or both of the metal compounds nor the pore size of the filter employed altered the DI determined for the UDLs suspensions. Table 1 shows that the DI value obtained for c-Ls upon passing through a 100 nm pore size filter was approximately 6 times lower than the DI measured for the UDLs. Oh and co-workers [21], reported that UDLs prepared from a mixture of egg yolk lecithin and cholesterol in the presence of Tween 20 possessed a DI 8.5 times that of the c-Ls (i.e. the corresponding c-Ls in the absence of Tween 20). The same group of co-workers reported that the nature of the edge activator significantly affected the DI value, with UDLs containing Tween 20 exhibiting the highest DI. The DI values obtained for the UDLs in the present study are higher than that reported by Silva and co-workers [24] for UDLs intended for chloroaluminium phthalocyanine delivery and prepared using soybean phosphatidylcholine and containing Tween 80, Span 80 or sodium deoxycholate. The reason for these differences results is dependent of the method of UDLs preparation. The UDLs produced in the present paper were made using the ethanolic injection method whereas Silva and co-workers [24] used to thin film hydration method. Ascenco and co-workers [23] determined that the presence of ethanol and surfactant acts synergically on the flexibility of the lipid bilayer membrane and thus on the liposome's deformability.

The high value obtained for the UDLs in the present study indicate that they should be rapidly able to penetrate the skin [23,51], carrying their payload into the skin. In contrast the low DI values achieved for the c-Ls suggest that these preparations are unlikely to pass into the skin to any significant extent.

3.4. Degree of retention of entrapped material after liposome extrusion

The degree of retention (RD), expressed as percentage, of the metal compounds after the liposomes passage under pressure through the nanoporous polycarbonate filters at

ambient temperature (22 °C) was determined. When UDLs were passed through a 100 nm pore size filter, the filtrate was found to contain 97.3 ± 1.3 % RuNO and 98.5 ± 2.3 % ZnPc, while after passage through a 50 nm pore size filter, slightly smaller amounts of the metal compounds were detected in the filtrate, namely 93.6 ± 1.5 % RuNO and 90.5 ± 2.8 % ZnPc.

3.5. Skin permeation studies

In order to determine whether UDLs containing ZnPc could enhance the transdermal delivery of encapsulated ZnPc, permeation studies were performed using full thickness fresh pig ears skin. Note that it has been previously reported that when ZnPc is trapped into c-Ls it does not cross the skin in detectable amounts after 24 h [22]. Indeed it is well established that c-Ls are of little value as carriers for transdermal delivery of therapeutic molecules. Figure 7(A) shows the release profile of ZnPc permeated across the fresh pig ear skin over 7 h in the present study while Figure 7(B) shows the amount of ZnPc retained in the *stratum corneum* (SC) and the epidermis and dermis at 12 h, i.e. the end of the skin permeation experiments.

INSERT FIGURE 7A

INSERT FIGURE 7B

About 400 μL of a 5 $\mu\text{mol L}^{-1}$ ZnPc UDLs formulation was applied in the donor compartment (1.77 cm^2) in the Franz cell under non-occlusive conditions. After 7 h, a total of 540 ng/cm^2 of the applied dose of ZnPc had crossed the pig's ear skin and was detected in the receptor phase of the Franz cells. While after 12 h of application, only 60 ng/cm^2 of the applied ZnPc dose was retained within the pig skin. The high level of ZnPc determined in the receptor phase of the Franz cells suggests that ZnPc is being very efficiently transported into the skin by the UDLs, thus indicating that the UDLs were sufficiently deformable to cross the

skin and should therefore effectively deliver any entrapped payload into the skin, particularly when that payload is a highly hydrophobic drug molecule.

3.6. Cell viability

The effect of UDLs containing ZnPc and/or RuNO, when stored either in the dark or under visible irradiation, on the viability of B16-F10 cells was analyzed by means of the MTT assay. In the absence of irradiation, the viability of the B16-F10 cell line was unaffected by the presence of empty UDLs (Figure 9), showing that the relatively high concentration of Tween 20 used in the preparation of the UDLs does not affect the viability of the B16-F10 cells. In contrast the maximum concentration of free Tween 20 that can be added to the B16-F10 cells without toxicity being observed is 0.3 mmol L⁻¹ (Figure S3 in Supplementary Material). Significantly, the viability of the B16-F10 cells were not affected by UDLs containing 5 μmol L⁻¹ RuNO, however, a reduced cell viabilities were observed when UDLs containing ZnPc (0.5 μmol L⁻¹) (28%) and ZnPc/RuNO (0.5 μmol L⁻¹ + 5 μmol L⁻¹) (36.5%) were evaluated (Figure 8). This observation suggests that ZnPc exhibits some toxicity towards the B16-F10 cells even in the absence of light activation.

INSERT FIGURE 8

When the cell viability experiments were repeated under irradiation at 675 nm, it can be seen (Figure 9) that while the empty UDLs had no effect on cell viability, cell viability decreased to approximately 10% that of the control in the presence of UDLs containing ZnPc, both in the presence and absence of RuNO. Interestingly the results obtained in this study show that the UDLs containing ZnPc and UDLs containing both ZnPc and RuNO decrease cell viability to 10 times a lower concentration than when the same compounds are

incorporated in stealth liposomes [8]. UDLs system revealed more behaved that conventional liposome, with accentuated skin permeation profile and decreasing of 90 % in the cellular viability under light irradiation. These results point as excellent candidate in PDT.

INSERT FIGURE 9

3.7. Conclusion

Dynamic light scattering results indicated that the surfactant Tween 20 might be a highly effective edge activator for the preparation of UDLs. Moreover, the substantial skin permeation of the photosensitizer, ZnPc when encapsulated in UDLs, observed after topical application suggested that UDLs prepared using Tween 20 could be of use in the transdermal delivery of either hydrophobic or hydrophilic photosensitizers. Additionally, the reduction in the viability of neoplastic cells upon treatment using UDLs containing ZnPc and RuNO occurred at a lower concentration than when encapsulated in stealth liposomes.

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Figure 1: Structure of the photosensitizer ZnPc (ciane: carbon, blue: nitrogen, white: hydrogen and grey: zinc) (A) and the nitrosyl ruthenium complex $[\text{Ru}(\text{NH.NHq})(\text{tpy})\text{NO}]^{3+}$ (ciane: carbon, blue:nitrogen, red: oxygen, white: hydrogen and purple: ruthenium) (B).

Figure 2: Structure of the polyoxyethylene sorbitan monoester (Tween 20).

Figure 3: UV-visible absorption spectra of $5 \mu\text{mol L}^{-1}$ ZnPc in a solution of DMSO:DMF (solid line); ZnPc ($5 \mu\text{mol L}^{-1}$) + RuNO ($50 \mu\text{mol L}^{-1}$) loaded in DOPC liposomes (2 mg/mL) over a wavelength range $270 - 800 \text{ nm}$ at $25.0 \pm 2.0 \text{ }^\circ\text{C}$. The dashed line represents the data obtained from a freshly prepared liposome sample, while the dotted line is the data of the same 48 h after preparation. Inset: Magnification over a wavelength range $500 - 800 \text{ nm}$ at $25.0 \pm 2.0 \text{ }^\circ\text{C}$.

Figure 4: UV-visible absorption spectra of $5\ \mu\text{mol L}^{-1}$ ZnPc in a 1:1 vol% DMSO:DMF solution (solid line); $5\ \mu\text{mol L}^{-1}$ ZnPc + $50\ \mu\text{mol L}^{-1}$ RuNO loaded in 60:40 wt% DOPC:DMPC vesicles over the wavelength range 270 – 800 nm at $25.0 \pm 2.0\ ^\circ\text{C}$, immediately after preparation (dashed line) and 48 h after preparation (dotted line). Inset: magnification of the data over the wavelength range 500 – 800 nm.

Figure 5: (A) The percent population of aggregate species present in a lipid mixture containing 2 mg/mL of a 60:40 wt% DOPC:DPMC as a function of added Tween 20 concentration (expressed as mmol L⁻¹) as determined by CONTIN analysis of the light scattering data) and (B) the apparent mean hydrodynamic radius of the aggregates after filtration through a 200 nm pore size filter as a function of Tween 20 concentration (expressed as mmol L⁻¹).

Figure 6: The percent population of aggregate species present in a lipid solution containing 2 mg/mL of DOPC as a function of added Tween 20 concentration (expressed as mmol L^{-1}) as determined by CONTIN analysis of the light scattering data).

Figure 7: (A) Permeation of ZnPc over time across dermatomed ear pig skin mounted in Franz diffusion cells and (B) in vitro profile of ZnPc accumulation in skin layers 12 h after topical application. Statistical parameters: $n = 6$, $p < 0.05$.

Figure 8: Cellular viability of B16-F10 cells when stored in the dark. 1 = control; 2 = empty UDLs; 3 = UDLs-ZnPc ($0.5 \mu\text{mol L}^{-1}$); 4 = UDLs-[Ru(NH.NHq)(tpy)NO] $^{3+}$ ($5 \mu\text{mol L}^{-1}$); 5 =

UDLs-ZnPc/ [Ru(NH.NHq)(tpy)NO]³⁺ (0.5 $\mu\text{mol L}^{-1}$ /5 $\mu\text{mol L}^{-1}$). Statistical parameters: n = 3, * p > 0.05 and ** p < 0.05.

Figure 9: In vitro phototoxicity against B16-F10 cells. 1 = control; 2 = UDL-ZnPc (0.5 $\mu\text{mol L}^{-1}$); 3 = UDL-ZnPc/[Ru(NH.NHq)(tpy)NO]³⁺ (0.5 $\mu\text{mol L}^{-1}$ /5 $\mu\text{mol L}^{-1}$). Statistical parameters: n = 3, *** p > 0.001.

List of Table

Table 1: Hydrodynamic radius (nm), deformability index (DI) and zeta potential (mV) for empty and metal-loaded c-Ls and UDLs after passage through polycarbonate membrane filter of either 100 or 50 nm.

UDLs	–	Apparent hydrodynamic radius (nm)/zeta potential (mV)	Deformability index (100 nm filter)	Deformability index (50 nm filter)
DOPC:DMPC				
empty		51.2 ± 4.9 (n = 2)/ 1.44 ± 0.30 (n=2)	3.2 ± 0.2 (n = 2)	3.1 ± 0.2 (n = 2)
RuNO		48.3 ± 5.6 (n = 2)/ -5.50 ± 0.50 (n=2)	2.9 ± 0.4 (n = 2)	2.3 ± 0.2 (n = 2)
ZnPc		56.0 ± 7.6 (n = 2)/ -2.37 ± 0.52 (n=2)	3.6 ± 0.5 (n = 2)	3.5 ± 0.7 (n = 2)
ZnPc/RuNO		49.3 ± 8.2 (n = 2)/ -4.74 ± 0.35 (n=2)	3.2 ± 0.1 (n = 2)	3.6 ± 0.2 (n = 2)
c-L	-	Apparent hydrodynamic radius (nm)	Deformability index (100 nm filter)	Deformability index (50 nm filter)
DOPC:DMPC				
empty		22.7 ± 1.4 (n = 3)	0.55 ± 0.11 (n = 2)	-
RuNO		23.0 ± 1.4 (n = 3)	0.54 ± 0.07 (n = 2)	-

ZnPc	21.7 ± 2.1 (n = 3)	0.47 ± 0.03 (n = 2)	-
ZnPc/RuNO	21.9 ± 1.9 (n = 3)	0.42 ± 0.08 (n = 2)	-