

## A MODEL BASED IN THE RADIUS OF VESICLES TO PREDICT THE NUMBER OF UNILAMELLAR LIPOSOMES

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### ABSTRACT

In particulate systems such as liposomes, concentration units are not enough to describe the drug distribution, as suspensions are not homogeneous. In certain *in vitro* assays, exposure to different number of particles introduces an extra variable regarding to contact phenomena. The aim is to achieve a rapid estimation of the number of unilamellar liposomes in a suspension. A simple mathematical method was developed; variables were the area and molecular weight of lipids, and the mean size of the liposomes. Unilamellar liposomes were prepared. Size was determined by dynamic light scattering, and then the number of particles were determined by tunable resistive pulse sensing. There was about a 90% coincidence between the theoretical results and the number of counted liposomes. This model could be useful for interpretation of *in vitro* experiments, when results could depend on the distribution of actives into different quantities of liposomes.

**Keywords:** liposomes, unilamellar, radius, equation, TRPS.

### INTRODUCTION

Liposomes can act as nano-drug delivery systems (nano-DDS) in which drugs are confined within each liposome, and therefore physically separated from the suspension media<sup>1, 2</sup>. Molarity, mass fraction or mass concentration are expressions not accurate enough to describe the drug distribution in systems where the drug is not homogeneously dissolved in the solvent but confined inside the inner aqueous phase of liposomes, and/or partitioned into their lipid bilayers. For the lipids themselves, as components of liposomal systems, the determination of their concentration does not provide a good description of the suspension without complementary data on size and lamellarity. The absence of a detailed knowledge about the composition of the system in those regards could lead to erroneous conclusions in comparative toxicity assays, cell response dynamics, and other *in vitro* assays<sup>3</sup>, since exposure of cells to different number of

particles could imply different uptake activities, endo/phagocytosis events, or other contact phenomena. Thus, similar to an *in vitro* infection test where the initial number of infecting parasites by cell is stated, the determination of *in vitro* activity of nano-DDS could include data on the number of liposomes that are available to a certain number of cells.

Upon extrusion -the passage of the suspension through a membrane with a narrow pore size-, the size and lamellarity of the liposomes are reduced<sup>4</sup>. Volume and area of a lipid bilayer (which respectively depends on the cube and the square of the radius) drastically decrease when the diameter is reduced by extrusion. This reduction in size and lamellarity implies an increment in the number of liposomes, due to the formation of new vesicles from the lipids that were excluded from the previously existent bilayers along the process<sup>5</sup>.

In this article, a theoretical model for the calculation of the number of unilamellar

liposomes based on the radius and the phospholipid mass has been developed. An empirical correlation was found by counting of liposomes by tunable resistive pulse sensing (TRPS). Its advantage compared to other calculation methods<sup>6, 7</sup> is the simplicity of the approximation and the obtained expressions. This could be used as a complement for interpretation of *in vitro* assays, in regard of the distribution of actives into drastically different quantities of liposomes.

## MATERIALS AND METHODS

### Materials

Soy phosphatidylcholine (SPC) (phospholipon 90 G, 92-98 % purity) was obtained as a gift from Phospholipid/Natterman (Germany). Other reagents were of analytic grade from Anedra (Argentina).

### Liposome preparation

Unilamellar liposomes (UL) were prepared as described in<sup>8</sup>. Briefly, liposomes were prepared by rehydration in Tris buffer (10 mM, 0.9 % w/v NaCl, pH 7.4) of a lipid film obtained by evaporation under rotary vacuum at 40°C in a round-bottom flask of a solution of SPC in chloroform. Liposomes were sonicated for 30 min and then extruded 25 times through 200 nm pore size membranes employing a Thermobarrel extruder (Northern Lipids, Burnaby, Canada) to render unilamellar 200 nm liposomes. The mean size of the liposomes was determined after 1:100 dilution by dynamic light scattering with a Zetasizer Nano ZS (Malvern Instruments Ltd., Malvern, Worcestershire, UK) and the phospholipids were quantified by a colorimetric method upon perchloric acid digestion<sup>9</sup>.

### Theoretical development of calculation

Assuming a monodisperse, unilamellar liposome population composed by spherical vesicles with a thin bilayer thickness -around 4 nm<sup>10</sup>

If  $r_L$  = liposome radius (*experimental data obtained by dynamic light scattering*), then the surface area of the liposome ( $S_L$ ) can be calculated according to

$$S_L = 4\pi r_L^2 \quad \text{Eq.1}$$

If, on the other hand,  $S_{PL}$  is the surface area of a phospholipid head, the contribution of this area to the total surface of the liposome can be calculated as follows; By assuming a spherical shape for the phospholipid head, its radius ( $r_{PL}$ ) can be calculated as:

$$r_{PL} = (S_{PL}/4\pi)^{1/2} \quad \text{Eq.2}$$

By assuming a compact lipid headgroup packing<sup>11</sup>, the surface area of a liposome should be the sum of the projections of the exposed area of each head. In addition, if the radius of the liposome is  $\gg$  than the radius of each headgroup, the area of each projected headgroup could be reduced to a square with length sides equal to the headgroup diameter, as it is shown in Figure 1, and then,

$$(2 \cdot r_{PL})^2 = S_{APL} \quad \text{Eq.3}$$

being  $S_{APL}$  the contribution of a single lipid to the liposomal surface area. Following, it can be stated that:

$$S_L/S_{APL} = N_{PL/C} \quad \text{Eq.4}$$

where  $N_{PL/C}$  is the number of lipids in the inner layer of one liposome (as the inner layer shows the most compact packing, with polar heads at a nearer distance from each other). The outer layer lipid content has been reported to be between the 50% of the total<sup>12</sup> and twice the phospholipids than the inner layer<sup>13</sup>. Although this ratio depends on the thickness of the bilayer and the liposome radius, an intermediate value of 1.5 was used for estimate the total number of lipid molecules in a liposome:

$$N_{PL/C} + 1.5 \cdot N_{PL/C} = 2.5 \cdot N_{PL/C} = N_{PL/L} \quad \text{Eq.5}$$

being  $N_{PL/L}$  the number of single lipids in one liposome (i.e., in the entire bilayer).

On the other hand, from the molecular weight of the phospholipid  $MW_{PL}$  and the mass of total phospholipids in liposomal suspension  $m_{PL}$  (obtained by weight measurement), can be calculated:

$$(m_{PL} \cdot N_{Avogadro})/MW_{PL} = N_{PL} \quad \text{Eq.6}$$

being  $N_{PL}$  the total number of phospholipid molecules ( $N_{PL}$  could be also obtained from a phosphate determination in the final suspension e.g. by a colorimetric measurement<sup>9</sup>).

Then, from the total number of phospholipids and the number of phospholipid in one liposome, can be determined:

$$N_{PL}/N_{PL/L} = N_L \quad \text{Eq.7}$$

where  $N_L$  is the number of liposomes in the suspension.

### Direct liposome counting by Tunable Resistive Pulse Sensing (TRPS)

A 1:200 dilution of the suspension was performed prior to analysis, and then an aliquot of 40  $\mu$ l of a 1:1,000 dilution of it (total:

1:200,000) was loaded into the NP200 nanopore membrane of the qViro (Izon Science Ltd, Christchurch, New Zealand) with a stretch of 46.99 mm. The voltage used was 0.22 V, and the pressure applied was of 14 cm of H<sub>2</sub>O. The sample was calibrated with a standard of 217 nm with a concentration of  $1.0 \times 10^{11}$  particles per ml. The assay was performed by triplicate, with a particle count of 500 particles per assay.

## RESULTS AND DISCUSSION

### Physicochemical characterization

The mean size for the liposomes of the suspension calculated in number was 208.0 nm  $\pm$  0.8, with polydispersion index of 0.04. Lipid concentration was determined as 40 mg/ml of SPC.

### Theoretical results

By the theoretical method, 1 ml of a suspension of liposomes of 208.0 nm diameter containing 40 mg of SPC (MW=775 g/mol,  $S_{PL} = 0,6 \text{ nm}^{214}$ ) would include  $3.99 \times 10^{13}$  unilamellar liposomes. It was calculated as follows:

$$r_L = 104. \text{ nm}$$

$$4\pi (104 \text{ nm})^2 = 1.36 \times 10^5 \text{ nm}^2 = S_L ,$$

being  $S_{PL} = 0,6 \text{ nm}^2$ :

$$(0,6 \text{ nm}^2 / 4. \pi)^{1/2} = 2.185 \times 10^{-1} \text{ nm} = r_{PL} ,$$

$$(2 \times 2.184 \times 10^{-1} \text{ nm})^2 = 4.37 \times 10^{-1} \text{ nm}^2 = S_{APL}$$

then,

$$1.36 \times 10^5 \text{ nm}^2 / 4.37 \times 10^{-1} \text{ nm}^2 = 3.11 \times 10^5 = N_{PL/C}$$

then,

$$2.5 \times 3.11 \times 10^5 = 7.78 \times 10^5 = N_{PL/L}$$

Now,

$$(0,04 \text{ g} \times 6.02 \times 10^{23}) / 775 \text{ g} = 3.107 \times 10^{19} = N_{PL}$$

$$3.107 \times 10^{19} / 7.78 \times 10^5 = 3.99 \times 10^{13} = N_L$$

### Liposome counting by TRPS

The measured mean concentration was of  $1.8 \times 10^8$  liposomes/ml, corresponding to an undiluted mean concentration of the aliquot of  $1.8 \times 10^{11}$  liposomes/ml. As there was a 1:200 dilution prior to TRPS, the particle concentration in the suspension was finally determined as  $3.6 \times 10^{13}$  liposomes/mL. The particle diameter had a mode of 207.9 nm, in concordance with DLS (Figure 2). The average current was 97.65 nA and the mean particle rate was of 31.4 particles per minute.

## DISCUSSION

In this work, the model prediction of the number of liposomes was contrasted against the number of liposomes determined by TRPS. The

predicted number had an accuracy of a 90 % in comparison to the estimation by direct counting. The aim was not to obtain exact quantitative predictions but a rapid way to know an approximation of the number of liposomes that will be present in a given suspension.

Any reduction in the mean size of the vesicles in a liposomal suspension will increase the number of liposomes obtained *de novo*. In fact, a moderate change in the mean size of the liposomal population -due to extrusion or sonication- will generate an increment in the quantity of liposomes in the suspension, by the formation of new and smaller structures from the larger liposomes under stress<sup>15</sup>. As the surface area of the unilamellar liposomes depends on the polar headgroups of the phospholipids, it can be stated that from a limited quantity of phospholipids there is a total surface area that they are able to cover. Being the surface of a sphere equal to  $4 \pi r^2$ , longer radii will generate much larger areas (as the area depends on the square of the increments in the radius). The smaller the area of a single sphere, the greater the total number of spheres that can be covered by the total area (i.e. the total amount of available phospholipids). As the number of vesicles depends inversely on the area, and the area depends directly on the square of the radius, the change in the number of vesicles depends inversely of the square of the change in the mean radius.

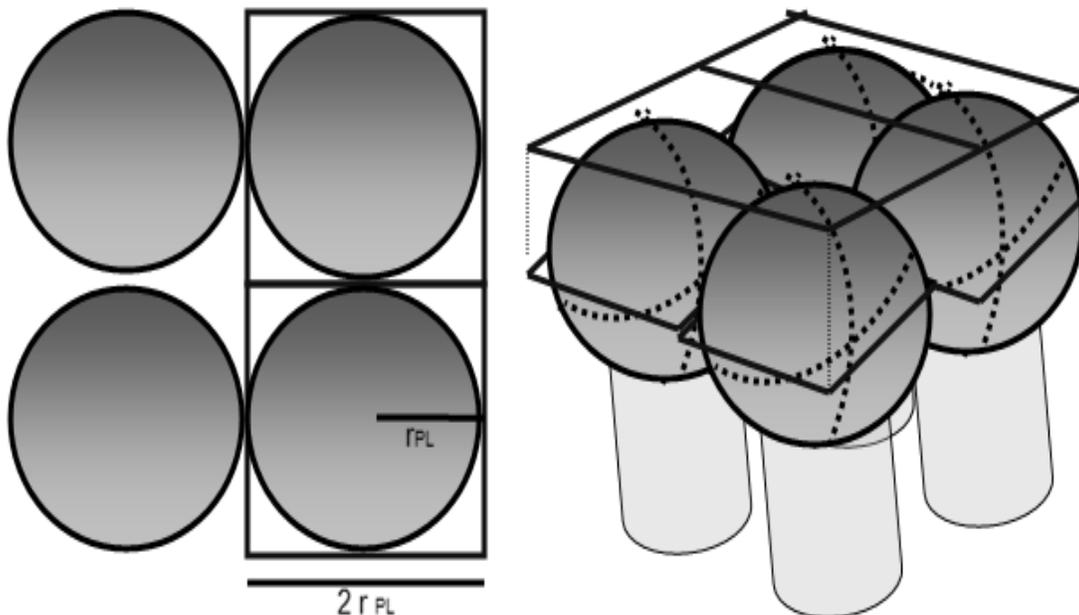
As the size of liposomes is reduced, their radii of curvature become smaller. Thus, lipid packing differences between inner and outer leaflets become increased<sup>16, 17</sup>, the outer possessing a higher number of lipids exhibiting a larger area per lipid headgroup than the inner leaflet<sup>13</sup>. The surface area covered by a single phospholipid calculated under several hydration and packing conditions was found to fluctuate within a small range<sup>18</sup>, being nearly  $0.60 \text{ nm}^2$  for phosphatidylcholine in liquid crystalline phase<sup>14</sup>. In addition, there is no significant contribution of the hydrophobic tails to the area of the horizontal section of the lipid; their vertical projections in fluid state do not extend beyond the projection of the polar head<sup>17</sup>. When considering systems with more than one type of amphiphilic lipid, if lipids are different in size, there will be more than one  $S_{APL}$  that would need to be calculated from its respective  $S_{PL}$  and  $r_{PL}$ , and a summation of terms consisting of the product of every  $S_{APL}$  by its molar fraction in respect to the total lipids. Thus, the  $N_{PL/C}$  shall

be the ratio between the  $S_L$  and the summation. Although there is asymmetry between the distribution of the mix compounds between the inner and the outer membrane, the equilibrium radius of the liposome is the same as if there was no asymmetry<sup>19</sup>, as also happens with its volume and the quantity of liposomes in a suspension.

In the work of Teeguarden<sup>3</sup> it is stated that, due to the low effect of the gravitational forces on the particles in suspensions, a monolayer of cells is not actually exposed to all the nano-objects present in an incubation medium (i.e. the nominal dose) but to a small fraction of them, with most of the particles remaining in the medium without contacting the cells at the bottom of the flask. Later, Lison<sup>20</sup> propose that, mainly due to the forces of convection existing in any liquid medium, virtually every particle in the suspension can establish a direct contact with the cells of the monolayer. With the aim of understanding better the influence of many

variables in the dose, this latter group performed some experiments, but when they tested the influence of increasing the number of particles in the dose, the mass and the surface area were also increased (because they used increasing volumes of a fixed concentration of particles). A complementary experiment using an increasing number of unilamellar nano-objects with a fixed total surface area and mass could be very useful to determine the influence of that variable in a new *ceteris paribus* condition.

It is well known that different internalization ways (phagocytosis, pinocytosis, endocytosis) depend on the size of the objects that are exposed to the cells (and of the ability of the cells to undergo each kind of uptake)<sup>21-23</sup>. The cell response also depends on the size and composition of the nanomaterial and on the type of cell<sup>24</sup>. In the work of Napierska<sup>25</sup> on the influence of the size of the nanoparticles in cytotoxicity, it is stated that the surface area is an important parameter regarding to the toxic effect.



**Fig. 1: Scheme of a small portion of a liposomal membrane. Spheres represent the phospholipid heads. Their vertical projection can be inscribed into a square whose sides are equal to the diameter of the head**

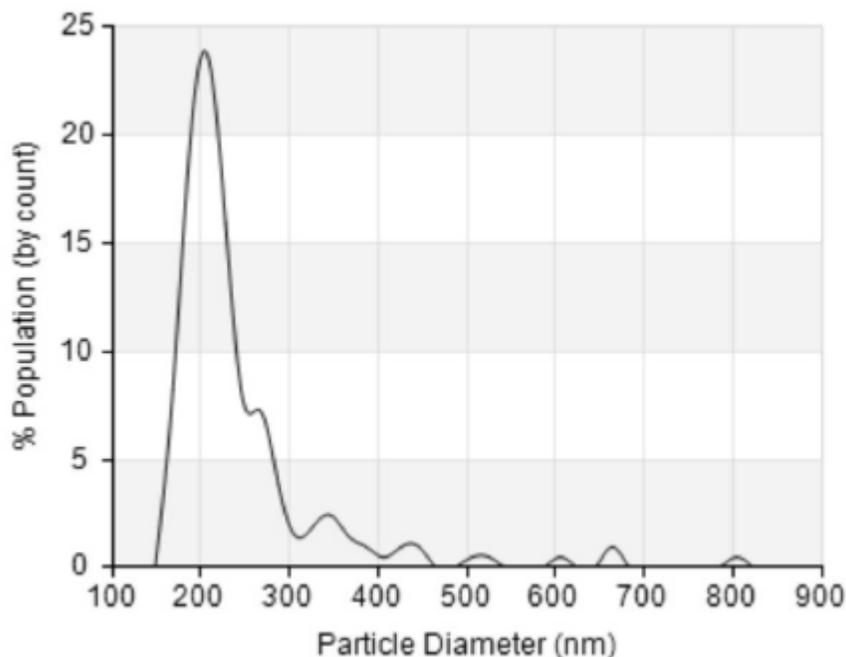


Fig. 2: Size histogram for the TRPS analysis of the liposome suspension

## CONCLUSION

This mathematical method could be useful to predict the change in the number of liposomes to which a cell culture is exposed when liposome suspensions are processed to render different mean sizes. Small changes in the mean size of the liposomes cause remarkable variations in the number of finally formed vesicles, which could lead to reinterpretate situations of apparently similar *in vitro* assays in which cell cultures would have been exposed to substantially different quantity of liposomes.

## ACKNOWLEDGMENTS

Jorge Montanari and Silvia Alonso are members of the Carrera del Investigador Científico of CONICET (National Council of Scientific and Technologic Research), Argentina. The authors thank biotechnologist Leandro J. Martinez for his help with TRPS.

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