# Didodecyldimethylammonium Bromide Role in Anchoring Gold Nanoparticles onto Liposome Surface for Triggering the Drug Release

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## ABSTRACT

**Introduction.** Nowadays, liposomes with their possibility of loading gold nanoparticles (AuNPs) onto their surface are used in terms of detection and imaging in several pathologies such as cancer disease. The objective of this work was first to optimize vesicle composition to reinforce the anchoring process of AuNPs onto liposomes by using cationic agents, and then, be study how the local temperature and the vesicle size affect to drug release.

**Materials and methods.** A Plackett–Burman design was applied to find out the optimal composition to anchor AuNPs. A comprehensive study about the influence of lipid bilayer composition on the surface charge, size and PDI of liposomes was carried out. Afterwards, in vitro release studies were developed by dialysis and several release parameters were calculated

**Results and discussion.** Cholesterol was fixed as rigid-agent and Didodecyldimethylammonium bromide (DDAB) was selected as cationic lipid into

liposome bilayer. Images from Transmission Electron Microscopy (TEM) revealed that AuNPs were anchored onto the liposomal surface, mostly in the presence of DDAB. The modification of incubating temperature revealed that the anchoring of AuNPs to the liposome surface provided an enhancement of calcein release, overall in extruded samples, in magnitude and in rate. The effect of surface available of vesicles on drug release was also studied in extruded samples (0.8 and 0.2 μm), demonstrating that calcein release increased as vesicle surface was higher (the anchoring process was also improved).

**Conclusion.** This interesting contribution may be taken into account with regard to design this lipid nanostructured system with controlled release properties for anticancer drugs.

## 1. INTRODUCTION

Targeted drug delivery constitutes an interesting alternative in order to overcome the current limitations of drugs in many different therapies. Nowadays, a continuous interest exists in developing highly localized and specific drug delivery systems. Among them, nanocarriers with enhanced functionality and smart responsiveness are being promoted [1].

**In cancer research, the physiological changes suffered in surrounding tumor area have been exploited to promote the accumulation of the drug carrier in this zone in order to reduce the unfavorable side effects in normal tissue** [2]. The well-known Enhanced Permeability and Retention (EPR) effect promotes this effect by diffusion and convection processes [3]. However, it is widely accepted that this effect is not enough to control the side effects of cytotoxic drugs [4]. Therefore, the development of composite nanosystems which are triggered by stimuli (i.e., pH, enzyme, temperature and light) is being exploited [5].

Among these systems, thermal sensitive liposomes constitute a potential method to produce triggered systems for controlled drug delivery [6]. Besides considering liposomes as attractive nanocarriers by virtue of their high biocompatibility and non-toxicity, some strategies for a triggering purpose include: to adjust the lipid composition of liposomes, in order to modify the temperature required for gel-to-liquid crystalline phase transition, or to use thermosensitive polymers, acoustically active liposomes and laser light sensitive liposomes [7].

In recent years, noble metal nanoparticles are being exploited in virtue of their physical properties. Among them, gold nanoparticles (AuNPs) are well known for their capacity to exhibit surface plasmon resonance when irradiated with infrared electromagnetic radiation, converting most of the absorbed energy to heat [8]. These properties make AuNPs well

suitable for widespread biomedical applications [9, 10]. AuNPs can be engineered as diagnosis agents and/or as targeting systems to specific tissues [11, 12]. They possess widespread desirable properties in terms of long-term stability, high surface area-to-volume ratio, providing size-dependent optical, electric and magnetic properties [13].

However, in vivo evaluation of AuNPs has revealed short biological half-life and cellular toxicity. In addition, the accumulation of these nanoparticles at the tumor site in cancer is limited [14] and they tend to aggregate and lose their unique photo-properties. These drawbacks can be overcome with the anchoring of AuNPs onto liposomes [15, 16]. Regarding the numerous applications as biophysical and biochemical tools of liposomes, the possibility of loading AuNPs onto their surface provides several advantages in terms of detection and imaging [17, 18]. They are used to activate the drug release in virtue of local temperature changes next to the nanoparticle. This property of producing light-induced heating of these nanoparticles has been widely used as generating cell damage, for example, in cancer disease [19-21].

Gold nanoparticles can be combined with liposomes by following different strategies, as postulated by Paasonen et al. [22]. Hydrophobic nanoparticles can be embedded into the lipid bilayer, whereas charged hydrophilic nanoparticles can be entrapped into the liposomal core. Finally, lipid functionalized gold nanoparticles can be localized onto liposome surface.

To reinforce the anchoring process of these nanoparticles onto liposomes, it is desirable to develop an adequate modification of bilayer composition in order to provide suitable surface charge characteristics of the vesicle; incorporation of cholesterol, which contributes to bilayer rearrangement and its dynamic [23]. Besides to take into account the lipid concentration, molecular interaction between the drug and the lipid bilayer membrane plays

an important role in liposome formation and drug encapsulation, as was reported by Villasmil-Sánchez et al. [24].

The homolog double-chain liposome-forming cationic lipid didodecyldimethylammonium bromide (DDAB), a synthetic cationic lipid, has been widely used in the study of vesicles and other biomembrane models [25, 26] and liposome formation in aqueous solutions with this lipid is extensively reported [27, 28].

In this study, we prepared the complex of AuNPs with liposomes by physical adsorption. We have used negatively charged nanoparticles, stabilized by citrate buffer. Anionic nanoparticles have been reported to be less cytotoxic than cationic nanoparticles, [29]. So, we have developed a cationic liposome in order to improve the anchoring junction with the anionic AuNPs, as previously proposed Balazs and Godbey [30]. We hypothesized that the key role of the cationic lipid will be to provide an electrostatic attraction between the positively charged liposome and the negatively charged AuNPs. For this, a first study will be focused on obtaining a vesicle lipid composition suitable for further anchor AuNPs onto the liposome surface.

Calcein was used as a model drug to monitor the effect of anchoring AuNPs onto liposomes. This model substance has the property of increasing the fluorescence intensity when it is released from the vesicles to the dissolution medium [31], which will be used for tracking the drug release. Afterward, we will also study the response of AuNPs liposomes and liposomes to the temperature changes concerning the calcein release. Finally, the influence of vesicle size on calcein release will be evaluated.

## 2. MATERIALS AND METHODS

### 2.1. Materials

L-α-phosphatidylcholine from egg yolk (EPC) and stearylamine (SA) were purchased from Fluka (Switzerland). Cholesterol (Ch), Cholesteryl hemisuccinate (Chems) and gold nanoparticle solution were obtained from Nanovex Biotecnología (Asturias, Spain). Chloroform was provided by Panreac Chemistry (Barcelona, Spain). **Calcein (Lot N. 127K1057)** and didodecyldimethylammonium bromide (DDAB) were provided **by Sigma–Aldrich (Italy).** The buffer solution used for the preparation of liposomes was PBS adjusted to pH 7.4.

**2.2. Liposome preparation**

*2.2.1. Thin-Layer Evaporation (TLE).* Multilamellar vesicles were prepared according to the method previously described [32, 33]. Briefly, different ratios of EPC, Ch and the inducer-charge substance (SA or DDAB) containing 14.1 – 14.6 mmol total lipids were dissolved in chloroform. The sample was rotaevaporated (Büchi, R-200) at 58°C to remove the organic solvent until obtaining a thin lipid film, which was then, hydrated by adding 3 mL of buffer PBS pH 7.4. Multilamellar liposomes (MLV) were formed after five vortexing cycles consisting in stirring for 2 minutes and heating at 58 ºC for 5 minutes until vesicles were formed.

For studying drug release, a solution of calcein in PBS (1 mg/mL) was added to the hydrating solution.

*2.2.2. Freezing and Thawing (FAT).* Frozen and thawed MLV (FATMLV) were obtained placing MLV colloidal dispersion in a pyrex tube, being accomplished subsequent freezing and thawing cycles by freezing each preparation of liposomes at −196 °C. After that, samples were immersed in liquid nitrogen for 30 s and thawed for 30 seconds at 58 °C in a water bath. The sequence was repeated nine times [34].

*2.2.3. Large (LUV) or small (SUV) unilamellar vesicles* were obtained by the extrusion technique [35]. According to this method, 2 mL of FATMLV dispersion were placed in a Lipex Thermobarrel extruder (Northern Lipids Inc., Burnaby, Canada) under air flow, thermostated at 58 ºC and extruded-through a 0.8 and 0.2 µm polycarbonate membrane for 6 times each, as previously reported [36].

### 2.3. Screening the significant parameters by a Plackett–Burman design

A Plackett-Burman design (PBD) was used to identify the significant variables of the formulation on size, polydispersity index (PdI) and zeta potential. This study was focused on the selection of the most appropriate liposomal formulation for the future anchoring process of AuNPs. PBD assumes the independence of each factor and a first-order model can describe it:

$$Y = \beta_0 + \sum \beta_i \cdot X_i$$

where $Y$ is the predicted target response (vesicle size, PdI and zeta potential), $\beta_0$ is the intercept, $\beta_i$ is the regression coefficient and $X_i$ is the independent variable.

Sixteen experiments including five independent variables related to the structure of liposome bilayer, were planned. Each factor was studied at two levels: high (+1) and low (−1). The five independent variables were rigidity-inducer lipid ($X1$), cationic lipid ($X2$), method of production ($X3$), the concentration of cationic lipid ($X4$) and extrusion ($X5$). The input variables with their levels are described in Table 1A. Based on preliminary studies, mean vesicle size (Y1), PdI (Y2) and zeta potential (Y3) were selected as the response variables [36].

The effect of each variable on the different responses was calculated by using the following equation:

$$E_{(X_i)} = \frac{\sum Y_{(+)_i} - \sum Y_{(-)_i}}{L/2}$$

where E(Xi) was the effect of the tested variable, Y(+)i and Y(-)i were the response values for higher and lower levels of the variables tested, and L was the number of experiments realized. When the effect value (E(Xi)) is positive (>0), the influence of the variable is greater at the higher level, while when it is negative (<0), the influence of the variable is greater at the lower level [37].

The experimental data were analyzed by ANOVA and F-test. The determination coefficient ($R^2$) and the F-value were applied for statistical evaluation.

Based on the Pareto chart and analysis of variance (ANOVA) results, we established the variables that exhibited significant main effects on the selected responses.

**2.4. AuNPs anchoring**

AuNPs-anchored liposomes (AuNPs-liposomes) were obtained by adding a volume of nanoparticles (size: 10 nm, zeta potential: -30 mV) to the liposome dispersion, which was previously optimized in composition. AuNPs solution was added in different ratios maintaining the stirring for 1 min.

**2.5. Characterization studies**

*2.5.1. Size and surface charge*

Particle size and PdI values were obtained by dynamic light scattering (DLS) by using the Zetasizer Nano-S equipment (Malvern Instruments, UK) at room temperature as previously described [36].

Zeta potential was determined from electrophoretic mobility (μ), which was converted to Z by the Smoluchowski equation, as was previously reported [36].

Diluted formulations (1/20 v/v) were used for both measurements.

*2.5.2. Morphological analysis*

Samples were analyzed by transmission electron microscopy (TEM, Philips CM 10, USA). The methodology employed for sample preparation has been previously described [33]. In this study, an aqueous solution of uranyl acetate (2% w/v) was used as a negative staining agent.

**2.6. In vitro calcein release**

Calcein release from AuNPs-liposomes was carried out by a dialysis method. Previously, non-entrapped calcein was removed from samples by centrifugation (Eppendorf Centrifuge 5804R) where samples were subjected to 10000 rpm, 4 ºC during 45 min. The resulting residue was hydrated with PBS solution pH 7.4. Before the experiments, the dispersion of calcein-entrapped liposomes was stocked under 4 ºC and in dark conditions. Afterward, the release of calcein from liposome samples was carried out placing 0.5 mL of each sample in a dialysis bag (molecular cut-off of 10 kD) which was sealed in both ends with a dialysis clip. After a conditioning stage (artificial membrane in 30 mL of PBS pH 7.4), the whole system was placed in an automated shaker (IKA Magnetic Stirrer RT 10) maintained at 100 rpm and temperature 37 ºC or 42 ºC depending of the experiment. At scheduled times, a fixed volume of dissolution medium was collected and replaced with an equal volume of fresh medium. Fluorescence emission of calcein was quantified using Synergy 2 (BioTek) at 485 nm and 520 nm for excitation and emission, respectively. Then, the aliquots were transferred to a black plate (Fluotrac 200, Greiner Bio-One, Monroe, NC 28110) for

measuring the fluorescence. Standard curves were generated to express relative fluorescence units into the amount of released calcein.

The amount of calcein released after time t was calculated according to the equation [38]:

$$RF(\%) = 100 \cdot \frac{I_t - I_o}{I_{max} - I_o}$$

where RF is the fraction of calcein released, $I_0$, $I_t$ and $I_{max}$ are the fluorescence intensities measured at the beginning of the experiment, at time t and the fluorescence intensity of the total amount of calcein added to the sample, respectively.

Release profiles were obtained by plotting the cumulative amount of calcein released (normalized values in percent per unit). Release parameters such as the cumulative drug released at 60 ($Q_{60}$), 120 ($Q_{120}$), 240 ($Q_{240}$) and 480 ($Q_{480}$) min, were calculated. In addition, the area under the curve of dissolution profiles (AUCDP) was calculated by the trapezoidal method for all samples. Finally, the time required for dissolution of 50% of the dose was selected as rate-indicating parameter ($t_{50\%}$).

The influence of the temperature of the dissolution medium (37 ºC or 42 ºC) and vesicle size (non-extruded samples, 800 nm or 200 nm-**e**xtruded samples) on calcein release parameters, were analyzed.

### 2.7. Statistical analysis

Student's t-test was used to evaluate the statistical significance. The differences were considered significant when the p-values were less than 0.05.

## 3. RESULTS AND DISCUSSION

### 3.1. Screening step

In this study, a two-level PBD comprising 16 experiments was introduced with the aim to screen those variables that significantly affect the surface characteristics of liposomes [39].

Variables selected for this study (Table 1A) were considered to potentially affect the available area and surface properties of vesicles for further proceed to anchor the AuNPs. This selection was carried out based on previous works in which liposomes with stearylamine prepared by different manufacturing techniques gave rise to cationic liposomes with high drug entrapment efficiency [34, 40]. Also, it is widely demonstrated the capacity of Ch to provide rigidity to liposome bilayer [33, 41].

As shown in Table 1B, the selected variables exhibited a wide range of values, so suggesting that the independent factors had a significant effect on the response chosen.

The statistical test F was used to validate the good fit of the model (Figure 1A). When F values were compared with the theoretical values (($F\alpha\ (p-1, N-p)$, being $\alpha$ the chosen risk, p the number of terms of the model and N the number of the experiments), we can conclude that there are statistically significant differences among the factors. The experimental test statistic was much higher (68.72; 65.41 and 849.9 for Y1, Y2 and Y3 respectively) than the critical value (F0.05 (4, 15) is 3.056); therefore, good linearity between the predicted and the observed values was suggested.

In addition, the standardized Pareto charts ($P < 0.05$) of main effects were illustrated in Figure 1B. The decision limits representing the statistically significant effect of factors at a 95% confidence level were 11.1, 0.0107 and 0.149 for vesicle size, PdI and zeta potential, respectively. Effects above this critical limit are significant and effects below this value are not likely to be significant.

Once obtained these diagrams, it is very easy to predict the suitable conditions and formulation composition in order to minimize the vesicle size and PdI, and to maximize the zeta potential. Results are shown in Figure 1C.

A clear explanation and discussion about the influence of the most significant factors (Pareto charts) on the responses evaluated will be carried out in the next sections.

*3.1.1. Influence of preparation method (X3 and X5)*

Reduction in the vesicle size is considered an important strategy to enhance the anchoring of AuNPs onto the liposomal surface since the available surface area increases [**42**]. In this regard, many methods are widely used to reduce the vesicle size including sonication, freezing and thawing and extrusion [43].

In this study, the mean vesicle size of liposomes ranged from 235.6 to 1511.33 nm and PdI from 0.1 to 0.6 (Table 1B). In general, the multilamellar structure of vesicles prepared by TLE method gives rise to liposomes largely heterogeneous in size and with a relatively small volume of hydrophilic phase. When these vesicles are submitted to sonication, single-layer liposomes are obtained. Also, this mechanical dispersion method results in low internal volume/encapsulation efficacy vesicles, due to the reduced size (44). In addition, this commonly used procedure in all formulations resulted in higher sizes in several batches, probably because aggregation phenomena occur, as was reported by Riaz [45]. Probably, a tendency to undergo concentration-dependent aggregation in this relatively low polarity environment has contributed to the generation of hydrogen bonds between the drug and lipid bilayer, giving rise to self-aggregation, limited drug loading and poor shelf stability [46].

In order to analyze the influence of freezing and thawing procedure on vesicle properties, certain sonicated samples were subjected to cycles of freeze-thawing to make unilamellar vesicles. Generally, dehydration of water molecules that bind to the hydrophilic head of liposomal lipids occurs during the freezing stage. This causes the breakdown of the lipid bilayer, which is reconstructed by fusion in the next step of thawing. Vesicle

fragmentations and deformations produce that larger vesicles are divided into smaller **regular** and irregular vesicles, exhibiting a size reduction with a broad PdI, as was shown in Figure 1B1 and 1B2, respectively [47]. In this figure, the application of freezing and thawing cycles to the sonicated samples gave rise to vesicles with lower sizes and higher values of PdI. As the freeze-thawing process was repeated, the number of lipid layers' also decreases, despite the fact that some studies have demonstrated that multilayered vesicles are formed after freeze-thawing was applied, due to the interaction forces between the drug and the bilayer components [48].

On the other hand, Pareto charts (Figures 1B1) and 1B2)) indicated that both the vesicle size and PdI were reduced when the extrusion process was applied to the samples (X5). It is well known that this mechanical methodology is widely used to produce homogeneous liposomes with a controlled average size [49]. So, unilamellar liposomes can be obtained after extruding the frozen and thawed MLVs, resulting in monodispersed samples with an inner volume higher than LUVs prepared by extrusion of MLVs. In this binary process, the maintenance of temperature (during thawing and extrusion) above the temperature of transition of the main phospholipid was critical to obtain an improvement of aqueous entrapment volume.

As expected, Pareto chart revealed that extrusion had a maximum standardized effect at a 95% confidence interval on the vesicle size and PdI, while this factor did not have any significant effect on surface charge. PdI is a homogeneity-indicating parameter and Figure 1B showed that the samples were more homogeneous in size (Figure 1B2) when they were extruded (X5) without submitting to the freezing-and-thawed processes (X3), as expected.

*3.1.2. Influence of bilayer composition (X1, X2 and X4)*

In this study, we have selected the rigidity-inducer lipid (X1), the cationic lipid (X2) and its concentration (X4) as variable factors in the experimental design, in order to select the most adequate combined formulation in terms of reduced size and PdI, and higher zeta potential values.

Pareto charts indicated that a reduction in liposome size was achieved when Chems and DDAB (15 mol%) were added into the lipid bilayer, probably as a consequence of the rearrangement of these molecules with the phospholipid within the bilayer. However, this lack of rigidity in the structure becomes to increase the heterogeneity in terms of size, making the PdI more favourable when Chems and stearylamine (15 mol%) were added (Figure 1B2). The combined mixture of Chems and SA favoured the curvature angle to produce samples that are more homogeneous: Chems is less rigid than Ch and acts a membrane stabilizer in the preparation of liposomes [50], whereas SA is a rigid lipid with a small and linear structure that acts stabilizing the liposome [51].

Zeta potential is a surface charge-indicating parameter that is related to vesicle stability. According to the obtained results, the zeta potential was maximized (more positive values) by adding Ch and DDAB at a concentration of 15 mol%. Vesicle stabilizing effect of DDAB and Ch has been previously demonstrated by the authors in liposomes formed with the 3:1 DMPC:DDAB ratio and fixing the ratio of Ch after registering the Langmuir isotherms of lipid monolayers containing different Ch molar fractions [**52**]. Therefore, DDAB has demonstrated to have an important role in stabilizing the vesicles.

As a conclusion of this section and regarding the obtained results (Figure 1C), the starting composition to be used in next studies was: bilayer composed by EPC:Ch:DDAB in a molar ratio of 16:1:3 (80:5:15 mol%). Liposomes will be synthesized from TLE methodology following sonication and extrusion.

315 **3.2. Anchoring AuNPs onto DDAB-charged liposomes**

316 Among the different types of AuNPs, in this study, we have used synthetic anionic
317 nanoparticles because of their physical stability. AuNPs have – 30 mV of zeta potential. So,
318 the synthesis of cationic liposomes was firstly planned by adding positively-charged lipid
319 into the bilayer. As concluded in the previous section, we have selected DDAB as cationic
320 lipid forming the lipid bilayer with EPC and Ch. The cationic net charge of liposome
321 surface at physiological pH 7.4 should make it as a suitable formulation to anchor AuNPs
322 by electrostatic interaction.

323 In addition, samples were extruded through a 0.4 μm membrane filter in order to provide a
324 more uniform liposome size without affecting surface charge as we have previously
325 obtained in Figure 1B, factor X5.

326 3.2.1. Effect of DDAB on vesicle stability

327 It is known that zeta potential parameter predicts the potential stability of a colloidal
328 system: as zeta potential increases (absolute values), the repulsion between vesicles will be
329 greater, leading to a more stable colloidal dispersion [53]. It is well recognized that the
330 introduction of ionic surfactants into liposome bilayer changes liposome properties. In this
331 study, DDAB was selected as a cationic surfactant, in different concentrations, able to
332 modify the surface charge of vesicles and so, the zeta potential.

333 Liposomes without DDAB showed a slight negative zeta potential, in agreement with the
334 observations of previous studies. In order to clarify the influence of DDAB concentration
335 on the surface charge and stability of lipid vesicles, formulations with increased DDAB
336 mol% were made. From Table 2, we can see that the increase of its concentration gives rise
337 to liposomes with a higher cationic surface charge, in accordance with reported by other
338 authors [54], showing the disposable lipid with the polar head onto the surface structure, as

339 vesicles or as micelles. However, only between 14A and 14B and between 14C and 14D,
340 were significant the differences (p:0.0023 and p:0.0048, respectively).
341 Regarding the size of these samples, the significant differences were non-existent in the
342 concentration range (22.02 to 31.44 mol%) of the DDAB. In addition, an important
343 decrease of this parameter was obtained from the EPC:Ch:DDAB ratio (59.91:3.01:37.09),
344 showing significant differences with the previous ($p<0.0001$). This result may be related to
345 the arrangement of this lipid forming micelles above its critical concentration, creating
346 structures with lower dimensions [55]. In this study, the same tendency was observed for
347 PdI decrease.
348 Based on results obtained after increasing DDAB percentage into the bilayer, we can
349 conclude that this surfactant should be used for further studies in 22.02 mol% since this
350 concentration provides a significant increase of surface charge with lower sizes. Therefore,
351 the following ratio of lipid components will be selected for the next step: EPC:Ch:DDAB
352 (74.20:3.78:22.02 mol%).
353 Afterwards, AuNPs anchoring onto the cationic liposome surface was performed by using
354 three different ratios AuNPs:liposomes v/v of the working dispersions.
355 Results showed in Table 3 evidenced an increase in size in vesicles containing DDAB
356 (compared with control without AuNPs) when the ratio of AuNPs was also increased,
357 intuiting that AuNPs have been located onto liposome surface after the anchoring process.
358 Moreover, the anchoring process was homogeneously realized since PdI values were not
359 significantly affected.
360 Regarding the surface charge of DDAB samples, it is clear that all liposome batches had
361 cationic zeta potential values, ranging from 26.2 in control batch to 24.73 mV after adding
362 AuNPs onto the liposomal surface at the higher ratio (3:08 v:v AuNP:liposomes). This

significant decrease in zeta potential values ($p=0.0244$) was indicative of the neutralizing effect of AuNPs after the anchoring process has been performed. Therefore, electrostatic interaction between opposite charges has been proposed as the main surface adsorption mechanism AuNP-ammonium polar head of DDAB.

As revealed TEM images of AuNPs/EPC liposomes (Figure 2), unilamellar liposomes containing DDAB may be physically associated with the AuNPs at the surface without disturbing the membrane packing (Figure 2A). Results suggest that AuNPs could be entrapped in the hydrophilic surface region of the bilayer because the darker colour of liposomes is due to the presence of AuNPs on the surface. Therefore, AuNPs were observed at the boundary surface on the liposomal assembly. Kojima et al. [42] reported that the head group of phosphatidylcholine was associated with the AuNPs via physical adsorption. Taken together, we suggest that liposomes-loading DDAB were physically associated with the AuNPs at the surface without disturbing the membrane.

This behavior was compared with vesicles containing SA (Figure 2B), where TEM images showed much-undefined vesicle structures, probably due to a disturbing effect of AuNPs on the membrane. This last result can also be confirmed from the characterization properties of SA liposomes in terms of size and zeta potential after AuNPs have been surface-anchored (Table 3).

3.2.2. Effect of anchoring AuNPs, liposome size and temperature on calcein release from liposomes

Calcein release studies from these nanocarriers were performed based on three main parameters that have a relevant effect on calcein release: temperature of release medium, liposome size and the presence of anchored AuNPs. Release tests were carried out at 37 ºC and 42 ºC, with the aim to mimic the physiological and tumor local area, respectively. In

addition, formulations corresponding to non-extruded (NE) and extruded by 0.8 or 0.2 μm were also analyzed in order to evaluate the effect of size and type of vesicles on calcein release.

Release profiles were compared using several dissolution parameters: the area under the curve of dissolution profile (AUCDP), cumulative amount (percent per unit) of calcein released at 60 ($Q_{60}$), 120 ($Q_{120}$), 240 ($Q_{240}$) and 480 ($Q_{480}$) minutes, which values are collected in Table 4. From them, we can obtain a clear idea about calcein release from the different formulations.

In agreement with the obtained results, the effect of temperature on calcein release is crucial on the dissolution of samples containing AuNPs anchored. The presence of AuNPs onto the liposome surface increased the amount of calcein release at 60, 120 and 240 minutes when the assay was realized at 42 ºC. This fact contributed to obtaining higher AUCDP values in these formulations. On the other hand, Table 4 reveals an interesting contribution to the release rate of calcein from these nanocarriers. $t_{50\%}$ results at 42ºC showed lower values than at 37ºC in those samples with AuNPs (Figure 3), supporting again the relevance of the effect of temperature on these metal nanoparticles, which accelerate the drug release at the temperature characteristic of cancer cells.

The relative enhancement of the release rate of calcein from liposomes with AuNPs only appears with extruded samples and is slightly higher for the samples AuE0.2 than for the samples AuE0.8. On the contrary, calcein release from samples without AuNPs was faster at 37ºC than at 42ºC for the samples NE and E0.8, just the opposite of was expected, while no temperature influence in the release rate is observed for the samples E0.2. According to other authors [56, 18], this could be justified by a homogeneous distribution of the temperature into the lipid bilayers of liposomes; however, in AuNPs-anchored liposomes,

411 specific zones are created at different temperatures due to the thermal conductivity and the
412 heat capacity of the Au, and this leads to defects in the bilayer.
413 This result can be checked in Figure 4. Certainly, AuNPs anchoring process onto the
414 liposome surface provides significant differences in the calcein release when release assay
415 was carried out at 37 ºC or 42 ºC in the case of liposomes extruded at 0.2 μm (Figure 4A).
416 However, the difference in calcein release from liposomes of the same size in the absence
417 of AuNPs was inappreciable (Figure 4B). These results are explained because the
418 anchoring of AuNPs onto the bilayer may cause local changes in lipid packing, causing an
419 increase in fluidity [55]. AuNPs work as localized heat sources transferring it to the
420 surrounding microenvironment. Since the bilayers associated AuNPs are in direct contact
421 with the lipids, heat is conducted more efficiently to the lipid molecules with higher
422 available specific area, undergoing the lamellar lipid lattice a structural change to the
423 crystalline liquid state, thereby inducing the phase transition and calcein release, as
424 demonstrated Paasonen et al. [22].
425 At this point, vesicle size constitutes an important parameter to be studied in order to
426 establish a relationship among size, anchoring capacity and calcein release with
427 temperature. This hypothesis about the influence of vesicle size on the anchoring process
428 and so, on the calcein release was corroborated after realizing release test to samples
429 containing non-extruded vesicles and extruded samples (0.2 μm and 0.8 μm). The
430 comparative study (Figure 5) provides interesting information about the influence of the
431 surface area of vesicles on the calcein release behavior. As it is generally accepted, the
432 reduction of vesicle size increases the dissolution rate due to the subsequent increase in
433 their specific surface area, though with some exceptions [57].

As concluding remarks, we can emphasize that the anchoring of AuNPs onto vesicle surface-sized 200 nm will improve the calcein release from these nanocarriers in the tumoral area.

## 4. CONCLUSIONS

In this study, we have obtained an optimal liposome formulation to anchor AuNPs, emphasizing the role of DDAB in obtaining stabilized cationic liposomes. TEM images and DLS study demonstrated that the anchoring of AuNPs to liposomes occurred successfully.

Release studies revealed an improvement of calcein release in AuNPs liposomes, overall at 42 ºC and smallest sizes. This fact becomes interesting for using these nanostructured carriers in future studies with anticancer drugs.

Therefore, we can conclude that AuNPs provides an interesting approach to design thermally sensitive liposomes. Their combination with lipid nanostructured systems such as liposomes might be a useful tool in drug delivery system.

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