

**SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO
DIRECCIÓN DE NUEVAS CREACIONES**

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Abogado
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SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO



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	Trámite	002
	Evento	001
	Actuación	430
	Oficio	12
	Folios	12

18302

Patente de Invención



Patente de Modelo de Utilidad

Vía Nacional

Vía PCT (Fase Nacional)



Capítulo I



Capítulo II

No. Solicitud Internacional

PCT/JP2008/055730

Fecha de Presentación Internacional

26/Marzo/2008

Como resultado del Examen de Fondo realizado, con fundamento en el Artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se comunica:

1. Documentos estudiados

Descripción:	Folios 53 a 83 y 87	26/Octubre/2009
Reivindicaciones:	Folios 84 a 86	26/Octubre/2009
Dibujos:	Folios 88 a 89	26/Octubre/2009
Prioridad:	JP 2007-079944	30/Mayo/2007

2. Análisis de la Prioridad

Se acepta la reivindicación de Prioridad porque esta solicitud fue presentada dentro del plazo establecido (Art. 9 D 486) y su contenido técnico corresponde con el de la prioridad reivindicada.

3. Objeto de la invención

Título de la solicitud: "Composición portador de suministro de ácido nucleico" (folio 01).

El problema planteado en esta solicitud consiste en la necesidad de suministrar eficientemente un ácido nucleico en las células, tal como se administra siARN a células derivadas de animal(es), con baja toxicidad y alta seguridad.

Para resolver este problema técnico la solicitud en estudio proporciona una composición portadora para suministro de ácido nucleico.

Expediente: 09-120191 1er Art 45

El objeto de la presente Solicitud se relaciona con la Clasificación Internacional de Patentes (CIP): A61K 47/24; A61K 31/7088; A61K 48/0

4. Excepción a la Patentabilidad (Art 20 D 486 literales (a) – (d))

El objeto de las reivindicaciones 12-13 no es patentable de acuerdo con el Art citado.

5. Reivindicaciones relativas a un uso o a un segundo uso (Art 14 y 21 D 486)

El uso de las reivindicaciones 14-15 no es patentable, de acuerdo con el Art 14.

6. De la Solicitud de Patente

Reivindicaciones (Art 30 D 486, Art 22 PCT)

Las Reivindicaciones 1-24, 30-35 no son claras porque:

- 1- El término “por lo menos” es relativo por lo cual no permite distinguir la invención del estado de la técnica. Se sugiere sustituirlo por un término o rango concreto.
- 2- Se recomienda especificar en la reivindicación 1, que la composición de liberación, es para RNA interferente (siRNA).

Se requiere al Solicitante hacer las aclaraciones necesarias.

7. Determinación del Estado de la Técnica

El Estado de la Técnica se determinó con base en la Fecha de Presentación de la Solicitud de Prioridad: Marzo 26 de 2007 y se encontraron los siguientes documentos que anticipan el objeto de esta solicitud:

Ítem	Documento	Título	Fecha de Publicación*
D1	European Journal of Pharmaceutical Sciences 9, (2000), 245-252	Stability of SUV liposomes in the present of cholate salts and pancreatic lipases: effect of lipid composition	Febrero/ 2000
D2	WO 2005/007196	Lipid encapsulated interfering RNA	27/Enero/2005

D1 <http://www.sciencedirect.com/science/journal/09280987/9> divulga (resumen, introducción, Pág. 246, materiales y métodos) composiciones liposomales de liberación a base de sales de colato y lipasas pancreáticas.

D2 [http://patentscope.wipo.int/search/en/detail.jsf?docId=WO2005007196&recNum=1&docAn=CA2004001051&queryString=FP:\(wo2005007196\)&maxRec=1](http://patentscope.wipo.int/search/en/detail.jsf?docId=WO2005007196&recNum=1&docAn=CA2004001051&queryString=FP:(wo2005007196)&maxRec=1) divulga (resumen, reivindicación 1 y 7) composiciones liposomales para RNA interferente.

8. Examen de Patentabilidad (Art 14 D486)

Nivel Inventivo (Art18 D486)

La reivindicación 1 consiste en:

Una composición de portador para suministro de ácido nucleico, que comprende (A) una diacilfosfatidilcolina, (B) por lo menos un miembro seleccionado del grupo que

consiste del grupo que consiste de colesterol y derivados del mismo y (C) una amina primaria alifática.

Comparación de las características técnicas **esenciales**, con las del Estado de la Técnica:

Características esenciales	D1	D2
Una composición de portador para suministro de ácido nucleico	Una composición en portadora de material genético (En liposoma)	Una composición de portador para suministro de ácido nucleico siRNA (En liposoma)
Una diacilfosfatidilcolina	Una diacilfosfatidilcolina	Una diacilfosfatidilcolina
Un miembro seleccionado del grupo que consiste del grupo que consiste de colesterol y derivados del mismo	Colesterol	Colesterol
Una amina primaria alifática.	Una amina primaria alifática.	No se menciona

Los documentos D1 y D2 se consideran el estado de la técnica cercano a la invención definida en la reivindicación 1, porque divulgan composiciones portadoras para la liberación de moléculas tales como agentes activos tal como siRNA, y que comprenden una diacilfosfatidilcolina, colesterol y una amina primaria alifática. (Ver resumen, Pág. 246, materiales y métodos-D1) (Ver resumen, reivindicación 1 y 7-D2)

La diferencia entre la invención, definida en la reivindicación 1 y la composición portadora del documento D1, es que se emplea para la liberación de siRNA. En D1, se emplea material genético.

La diferencia entre la invención, definida en la reivindicación 1 y la composición portadora del documento D2, es que se emplea además una amina primaria. En D2, se emplean lípidos catiónicos y lípidos conjugados que inhiben la agregación de partículas.

El efecto que logra el incluir ácidos nucleicos como siRNA, es que se obtienen composiciones para liberación de ácidos nucleicos en células; de baja toxicidad y altamente seguras.

Por lo tanto, el problema técnico objetivo que pretende resolver esta invención se puede formular así: “cómo modificar la composición portadora previamente conocida en el documento de D1 para que “pueda liberarse el ácido nucleico en células, y las composiciones sean de baja toxicidad y altamente seguras”.

Sin embargo, la utilización de siRNA en composiciones portadoras con diacilfosfatidilcolina y colesterol, ya está divulgada en el documento D2, que se refiere a una composición portadora de liberación de siRNA en células. (Ver resumen, reivindicación 1 y 7)

En consecuencia, la persona del oficio normalmente versada en la materia, incluiría como agente activo un siRNA con diacilfosfatidilcolina y colesterol del documento de D2 en las composiciones portadoras del documento D1 para llegar así al objeto de la reivindicación 1 de la solicitud. Por lo cual se considera obvia.

Conclusión:
La reivindicación 1 cumple el requisito de novedad (Art. 16), pero no cumple con el requisito de nivel inventivo (Art. 18).

9. Conclusión

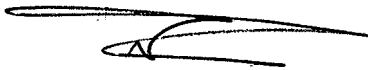
Los requisitos de Patentabilidad de la presente Solicitud están afectados así:

Ítem	Documento	Reivindicaciones afectadas	Requisito que afecta
D1	European Journal of Pharmaceutical Sciences 9, (2000), 245-252	1-11	Nivel Inventivo
D2	WO 2005/007196	1-11	Nivel Inventivo

En cumplimiento del artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se le indica que debe dar respuesta dentro del término de sesenta (60) días contados a partir de la fecha de notificación de la presente comunicación. En caso de no dar respuesta al presente requerimiento, o si a pesar de la respuesta subsistieran los impedimentos para la concesión, se denegará la patente.

Se invita al solicitante se sirva indicar, si así lo considera, su renuncia al término faltante para los 60 días y presentarla por escrito.

Notifíquese el presente oficio conforme a lo dispuesto en el numeral 5.2, literal e), del capítulo quinto del título primero de la Circular Única No. 10 de 2001.



JOSÉ LUIS SALAZAR LÓPEZ
Director de Nuevas Creaciones (C)

El anterior requerimiento fue notificado por fijación en lista, de fecha 16 OCT. 2012

Elaborado por: Adriana P Camelo
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Stability of SUV liposomes in the presence of cholate salts and pancreatic lipases: effect of lipid composition

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Abstract

The effect of bile salts (sodium cholate and sodium taurocholate), and pancreatic lipases on the structural integrity of SUV liposomes of different lipid compositions was studied. Liposomal membrane integrity was judged by bile salt or pancreatin-induced release of vesicle encapsulated 5,6-carboxyfluorescein, and vesicle size distribution before and after incubations. Bile salt concentration was 10 mM, while a saturated solution of pancreatin (mixed with equal volume of liposomes) was utilized. Results agree with earlier studies, demonstrating the instability of liposomes composed of lipids with low transition temperatures (PC and DMPC) in presence of cholates. Addition of cholesterol (1:1 lipid:chol molar ratio) does not substantially increase the encapsulated molecule retention. Nevertheless, liposomes composed of lipids with high transition temperatures (DPPC, DSPC and SM), retain significantly higher amounts of encapsulated material, under all conditions studied. Furthermore, the vesicles formed by mixing cholesterol with these lipids will possibly be sufficiently stable in the gastrointestinal tract for long periods of time. Sizing results reveal that in most cases release of encapsulated molecules is mainly caused by their leakage through holes formed on the lipid bilayer. However, in stearylamine containing DPPC and DSPC vesicles, the cholate-induced drastic decrease in vesicle size suggests total liposome disruption as the possible mechanism of encapsulated material immediate release. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Liposome; Stability; Bile salt; Pancreatic lipases; Oral administration

1. Introduction

In the last three decades liposomes have been extensively studied as possible delivery vehicles for drugs, enzymes, antibodies and genetic material (see Gregoriadis, 1995; Storm and Crommelin, 1998, for reviews). The majority of these studies involve the administration of liposomes intravenously or intraperitoneally. The potential for delivery of liposomal drugs by the oral route has not been well elucidated despite the fact that in the past there has been an increased interest in the use of liposomes as a highly convenient orally administered delivery system. Many agents such as insulin (Dapergolas and Gregoriadis, 1976; Patel and Ryman, 1981; Woodley, 1985; Choudhari et al., 1994; Takeuchi et al., 1996), and other proteins or vitamins (Fukunaga et al., 1991; Arien et al., 1993; Kirilenko and Gregoriadis, 1993; Maitani et al., 1996; Tsume et al., 1996) have been tested and though they were found to reach the blood circulation after oral administra-

tion of their liposomal preparations, their absorption results were mixed and unpredictable in most cases (especially in the earlier studies for insulin). However, the results of several studies involving oral administration of liposomal insulin indicate the occurrence of a marked hypoglycemic response. This has been linked, by some researchers, to the uptake of liposomes by enterocytes, which has been demonstrated *in vitro* by Rowland and Woodley (1981), while others claim that liposomes act as a barrier, protecting encapsulated molecules from degrading agents present in the gastrointestinal tract. Regardless of the controversy, a major prerequisite for effective oral administration of liposomal formulations, is their survival in the hostile environment of the gastrointestinal (GI) tract. In previous studies (Arien et al., 1993; Sehgal and Rogers, 1995; Ramaldes et al., 1996a; Ouadahi et al., 1998), the stability of liposomes in media mimicking the conditions of the GI tract, has been seen to increase when saturated phospholipids or high amounts of cholesterol were used for liposome preparation. Although most liposomal formulations previously studied have been found stable in media with different pH values and in the presence of pancreatic

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lipases, they were rapidly losing their entrapped material in the presence of cholate salts. In most of these studies the stability of liposomes was tested only for a short period of time (0.5 or 1 h), though it is well known that the mean residence time of a formulation administered orally is well over 2 h. In addition, most of the previous studies involved multilamellar vesicles which are expected to be more stable (when stability is judged by retention of entrapped molecules) due to the many bilayers surrounding the encapsulated material. Also, these liposomes are larger in size; a factor that has been linked with particle uptake by enterocytes (Eldridge et al., 1990; Jani et al., 1990; Florence, 1997) as well as absorption of recombinant human erythropoietin after oral administration of liposomal formulations (Maitani et al., 1996). In the present study the membrane integrity of small unilamellar liposomes with different lipid compositions (measured as their ability to retain a small hydrophilic molecule, 5,6-carboxyfluorescein) was evaluated in the presence of cholic salts (sodium cholate and sodium taurocholate) and a mixture of pancreatic lipases, for a period of at least 5 h. The size distribution of several of the liposomes studied was measured before and after sodium cholate addition in the samples and also after a 24-h incubation period at 37°C, in order to further elucidate the mechanism of CF release from liposomes.

2. Materials and methods

2.1. Materials

Egg L- α -phosphatidylcholine (PC) (grade 1), dipalmitoyl-phosphatidylcholine (DPPC) (synthetic, grade 1), distearoyl-phosphatidylcholine (DSPC) (synthetic, grade 1), dimyristoyl-phosphatidylcholine (DMPC) (synthetic, grade 1), and sphingomyelin (SM) (from bovine spinal cord), were obtained from Lipid Products, Nutfield, UK. The 99% purity of the lipids was verified by thin layer chromatography as described by New (1990). All lipids used gave single spots on TLC plates. Cholesterol (Chol) (pure), stearylamine (SA), pancreatin (from porcine pancreas), and the bile salts, cholic acid and taurocholic acid as sodium salts, were obtained from Sigma. 5,6-Carboxyfluorescein (CF) was purchased from Eastman Kodak, and was used after purification, as previously described (New, 1990). All the gels used for gel chromatography were obtained by Pharmacia. All other reagents used in the study were of analytical grade and were purchased from Sigma (Poole, UK).

All the measurements of sample fluorescence intensity were performed with a Shimadzu RF-1501 Spectrophotometer thermostated at $37 \pm 0.1^\circ\text{C}$ by a Julabo FS18 refrigerating and heating circulator. Samples were incubated at $37 \pm 0.5^\circ\text{C}$ in a Julabo SW-20C shaking incubator.

2.2. Liposome composition

PC, DMPC, DPPC, DSPC and SM were used alone or mixed with cholesterol in a 1:1 molar ratio, with SA in a 9:1 (lipid:SA) molar ratio, or with cholesterol and SA in a 7:2:1 (lipid:Chol:SA) molar ratio.

2.3. Preparation of liposomes

Small unilamellar liposomes were prepared by dissolving lipids alone, or with SA (9:1 molar ratio) or cholesterol (1:1 molar ratio) or cholesterol and SA (7:2:1 molar ratio), where appropriate, in a chloroform:methanol (2:1) solution. The solution was dried by rotary evaporation of the organic solvent to a thin film on the walls of a 50-ml round-bottomed flask, and any traces of solvent were removed under a stream of nitrogen and by overnight connection to a vacuum pump. The lipid thin film was hydrated by vortex agitation, with 1 ml of Tris buffer, pH 7.4 (5 mM Tris), containing 20 mM NaCl and 100 mM CF. The large liposome suspension initially produced was sonicated using a vibra cell sonicator (Sonics and Materials, UK), equipped with a tapered micro tip, for at least two 10-min cycles. In all cases, the initially turbid liposomal suspension was well clarified after sonication. Following sonication, the liposome suspensions were left to stand for 2 h at a temperature higher than the transition temperature of the lipid used in each case, in order to anneal any structural defects. The Ti-fragments and any multilamellar vesicles or liposomal aggregates were removed by centrifugation at $10\,000 \times g$ for 15 min.

Non-encapsulated CF was separated from liposomes on a Sephadex G-50 column (1×35 cm) eluted with Tris-buffered saline, pH 7.4 (5 mM Tris, 150 mM NaCl), which rendered the liposomes osmotically stable (Cleland and Allen, 1981). The column was presaturated with the lipid used in each case and therefore, the lipid recovery in all cases was well over 95%, as calculated by a colorimetric assay for phospholipids (Stewart, 1980). The final lipid concentration in the incubations was adjusted to 2.5 mM.

2.4. Preparation of bile salt and pancreatin solutions and liposome incubation conditions

The bile salt solutions (20 mM) were prepared by dissolving sodium salts of cholic or taurocholic acid in a Tris buffer, pH 7.4, containing NaCl in the amount needed for osmolarity adjustment in each case. The pancreatin solution was prepared by dissolving 10 mg of pancreatin per 100 ml of a Tris buffer, pH 7.4, containing 5 mM CaCl_2 and NaCl for osmolarity adjustment.

The effect of bile salts or pancreatin on each liposome type was determined by incubating equal volumes of liposomes and the appropriate solution at 37°C under mild agitation (40 rpm) in a thermostated incubator. At appropriate time points, samples were drawn from each vial for

the determination of the percentage of CF release. Each experiment was repeated at least four times.

For each liposome type a control experiment in which liposomes were incubated with plain buffer was performed in triplicate and the results from the control experiments were used for the estimation of CF release in the samples.

2.5. Determination of CF release by fluorescence

The release of CF was taken as a measure of the liposome structural integrity after incubating the liposomes in the media described above, and was calculated as reported elsewhere (Senior and Gregoriadis, 1982). In brief, 10 μ l were drawn out from each incubation tube and diluted with 4 ml of Tris-buffered saline, pH 7.4. The fluorescence intensity of the samples was then measured on a Shimadzu fluorescence spectrophotometer (emission 470 nm, excitation 520 nm) before and after the addition of Triton X-100 at a final concentration of 1% (v/v).

The percent of CF release (% release) was determined at the experimental conditions, from the equation:

$$\% \text{ Release} = \frac{F - F_i}{F_T - F_i} \times 100$$

where F and F_T are the CF fluorescence of the sample in the absence and presence of 1% Triton X-100 (final concentration), respectively, and F_i is the initial quenched fluorescence of the same liposome sample before addition of the incubation media in TBS buffer, pH 7.4 (values measured after mixing the samples with the incubation media and after Triton addition were corrected accordingly for dilution).

2.6. Liposome sizing

A 20- μ l quantity of the liposome dispersions was diluted with filtered buffer (0.22 μ m pore size, polycarbonate filters, Millipore, UK) and sized immediately and also right after mixing and 24 h after incubating in sodium cholate (as described above). The samples were sized by photon correlation spectroscopy (Zetasizer 5000, Malvern Instruments, Malvern, UK). Measurements were performed at 25°C with a fixed angle of 90° and sizes quoted are the z-average mean (dz) for the liposomal hydrodynamic diameter.

3. Results

3.1. Liposome stability measured by CF release

In general the stability of liposomes in cholate and taurocholate salts is low, while most of the lipid compositions studied are stable in the presence of pancreatin. High amounts of the encapsulated CF (approaching in some cases 100%) were released from some of the

liposomes studied, even after 1 h of incubation in the presence of cholates (both cholic and taurocholic salts).

More specifically, all liposomes containing DMPC are very unstable (data not shown) in the presence of cholates and pancreatin. The addition of stearylamine and/or cholesterol did not seem to significantly enhance the retention of CF in DMPC liposomes. Liposomes composed of plain PC (Fig. 1(I–III)) were also found very unstable in the presence of cholates (both cholic and taurocholic salts), releasing about 80–90% of the encapsulated CF in 1 h, but not so leaky in the presence of pancreatin (~25% CF released after 1 h of incubation at 37°C). This was expected since liposomes composed of plain lecithin are unstable and leaky even when incubated in plain buffer. The inclusion of stearylamine alone or with a small amount of cholesterol (7:2 (lipid:chol) molar ratio), did not seem to significantly enhance CF retention in the media studied (Figs. 1(I–III)), while mixing with an equimolar amount of cholesterol (1:1 molar ratio), significantly decreased the amount of CF released immediately after incubation of liposomes with cholate solutions. But even so, after 1 h of incubation most of the entrapped CF had leaked out.

In agreement with previous studies, liposomes composed of lipids with higher transition temperatures, as DPPC, DSPC and SM, were found to exhibit significantly higher stabilities in the conditions studied. Although, in all

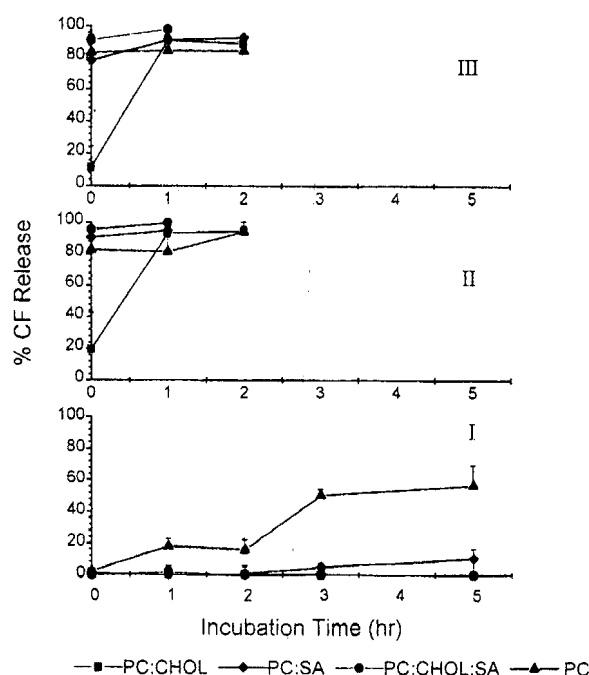


Fig. 1. Release of encapsulated carboxyfluorescein from PC-based liposomes after incubation at 37°C in the presence of: (I) pancreatin, (II) 10 mM sodium cholate and (III) 10 mM sodium taurocholate. Each point is the mean of at least four experiments (bars represent SD values). Key: triangles, plain lipid; squares, lipid:chol; circles, lipid:chol:SA; diamonds, lipid:SA.

cases, plain lipid liposomes (SM, DPPC and DSPC) released most of the entrapped CF (70–90%) after 1 h of incubation in cholic or taurocholic salts (Figs. 2–4(II and III)), these values were significantly lower from the respective values of plain PC and DMPC liposomes. In any event, the CF retention values of plain SM, DPPC and DSPC liposomes are not high enough for efficient oral delivery of low-molecular weight hydrophilic drugs. However, although the addition of a positive charge on these liposomes (SA) does not seem to improve their stability even in the presence of a small amount of cholesterol (with the exception of DPPC:SA:chol liposomes), when an equimolar amount of cholesterol is used for liposome preparation, all three lipids produce liposomes which are comparably stable in the presence of both cholic salts studied. Indeed, the values of CF release in presence of cholates are comparatively low, ranging from 20 to 40% for taurocholic salts, and 20–55% for cholate salts after 1 h of incubation. These values remain more or less stable up to 5 h, and even 24 h (not shown) of incubation, indicating that CF release is initially a very fast process which after a burst effect levels off.

In the presence of pancreatin, CF retention in liposomes composed of lipids with high transition temperatures (SM, DSPC) is very high even after 5 h and also after 24 h of incubation (not shown) (Figs. 2(I) and 4(I)), with the exception of plain DPPC liposomes which are not stable in

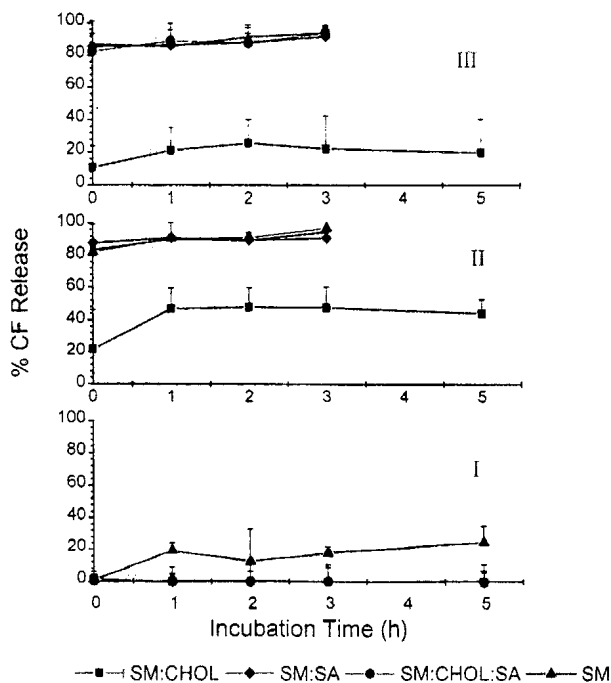


Fig. 2. Release of encapsulated carboxyfluorescein from SM-based liposomes after incubation at 37°C in the presence of: (I) pancreatin, (II) 10 mM sodium cholate and (III) 10 mM sodium taurocholate. Each point is the mean of at least four experiments (bars represent SD values). Key: triangles, plain lipid; squares, lipid:chol; circles, lipid:chol:SA; diamonds.

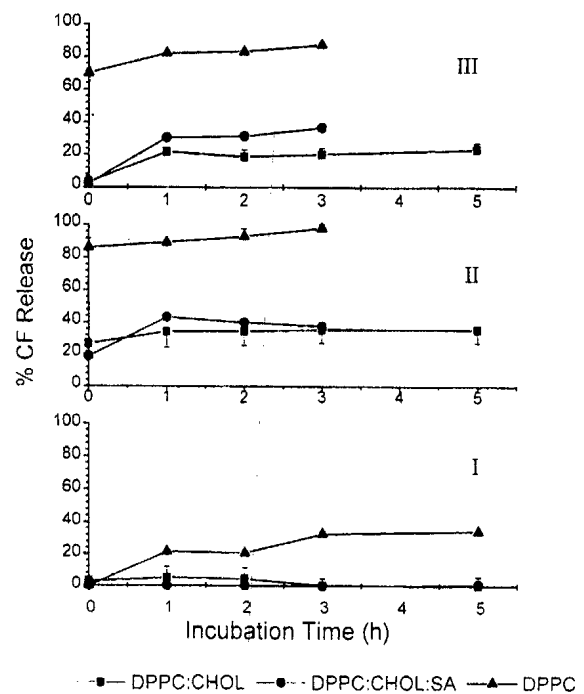


Fig. 3. Release of encapsulated carboxyfluorescein from DPPC-based liposomes after incubation at 37°C in the presence of: (I) pancreatin, (II) 10 mM sodium cholate and (III) 10 mM sodium taurocholate. Each point is the mean of at least four experiments (bars represent SD values). Key: triangles, plain lipid; squares, lipid:chol; circles, lipid:chol:SA.

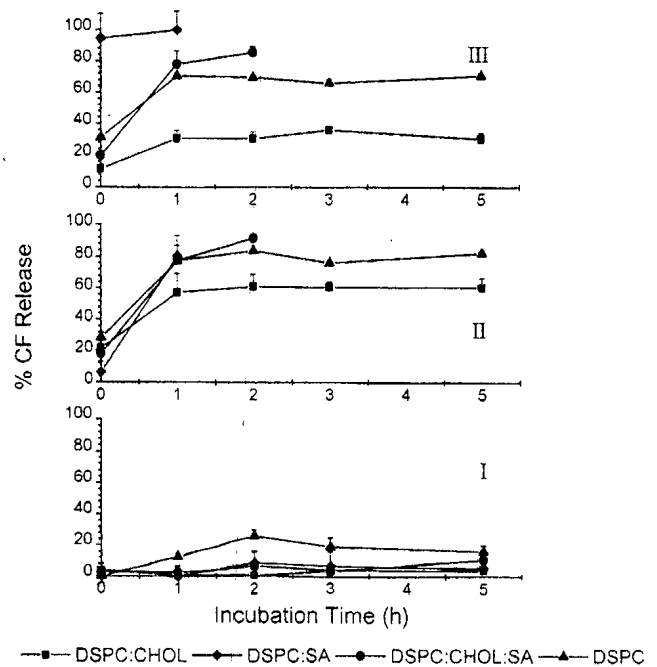


Fig. 4. Release of encapsulated carboxyfluorescein from DSPC based liposomes after incubation at 37°C in the presence of: (I) pancreatin, (II) 10 mM sodium cholate and (III) 10 mM sodium taurocholate. Each point is the mean of at least four experiments (bars represent SD values). Key: triangles, plain lipid; squares, lipid:chol; circles, lipid:chol:SA; diamonds.

pancreatin if cholesterol is not present in their membrane (Fig. 3(I)).

3.2. Liposome sizing before and after incubation in cholate salts

The size distribution of PC, DPPC and DSPC liposomes containing or not cholesterol (1:1 lipid/chol molar ratio) or stearylamine (9:1 lipid/SA molar ratio) in TBS buffer and also in the presence of 10 mM sodium cholate was estimated before mixing, at time 0 (immediately after mixing) and after 24 h of incubation in sodium cholate at 37°C. The results of the sizing experiments performed by photon correlation spectroscopy, are presented in Table 1. For plain PC liposomes, although there is no significant change in mean size when cholate is added or after 24 h of incubation, immediately after the addition of cholate the mean diameter and standard deviation, as well as the mean polydispersity index of the samples, are significantly higher. The equivalent values after the 24 h incubation are in the same range as those of the initial measurements (before cholate addition). The mean diameter of the cholesterol containing PC liposomes is decreased by approximately 20% when cholate is added and does not change any further, while SA containing PC liposomes do not show any change in size or size homogeneity even after the 24 h incubation in sodium cholate.

For DPPC-based liposomes, plain lipid vesicles seem to be disrupted, their mean diameter being reduced by 50% after the 24 h incubation in cholate. This effect is not immediate, since the liposomal size is not significantly decreased immediately after addition of the cholate salt. With the addition of cholesterol however, the size of vesicles formed (DPPC:chol) is practically unaffected by incubation in cholate. This is also true for DSPC:chol liposomes. In fact, even plain DSPC liposomes do not change in size by cholate incubation.

On the other hand, addition of positive charge to DPPC and DSPC liposomes, by adding stearylamine, has a dramatic effect on vesicle size. The mean diameter of liposomes in both cases (for DPPC and DSPC), is decreased to less than half of the initial, and this disruption is immediate. In fact, for DPPC the vesicle diameter is not further decreased whilst for DSPC an additional large decrease in vesicle mean diameter is observed after 24 h of incubation. These particular suspensions were optically clear after the 24 h incubation in cholate and a loose pellet was precipitated, suggesting that large scale disruption of these liposomes had occurred, while other liposomal samples (especially those containing high amounts of cholesterol) remained slightly turbid due to light scattering by vesicles.

4. Discussion

The main objective of this study was to determine the possibility of formulating liposomes, which will (fully or partly) retain their integrity after oral administration. Therefore the membrane integrity of liposomes composed of several different lipids (with transition temperatures below and above 37°C) was followed after incubation in cholate or taurocholate salts, and also in a mixture of pancreatic lipases. Liposomes composed of plain lipids or mixtures of lipids with cholesterol, which is known to enhance membrane integrity (Papahadjopoulos et al., 1972, 1973; Jonas and Maine, 1979), were prepared and tested. The effect of positive charge on vesicle stability, was studied by incorporating SA in liposomal preparations. A final concentration of 10 mM bile salts, which may be encountered in the GI tract after oral administration of liposomal formulations, was used.

Experimental results reveal that almost all of the liposomal compositions studied are very stable in presence

Table 1
Mean diameter of liposomes and mean polydispersity index values of liposomal dispersions before and after incubation in the presence of 10 mM sodium cholate

Lipid composition	Initial size (nm)	Sodium cholate ^a (0 h) ^b (nm)	Sodium cholate ^a (24 h) ^c (nm)
PC	80.2 (±13.2) (<i>P</i> 0.59) ^d	125.3 (±34.0) (<i>P</i> 0.78)	85.3 (±6.22) (<i>P</i> 0.58)
PC:chol	147.8 (±13.4) (<i>P</i> 0.56)	128.7 (±9.33) (<i>P</i> 0.33)	127.1 (±11.4) (<i>P</i> 0.31)
PC:SA	85.6 (±21.8) (<i>P</i> 0.55)	87.9 (±29.3) (<i>P</i> 0.46)	81.2 (±11.2) (<i>P</i> 0.43)
DPPC	107.1 (±20.3) (<i>P</i> 0.51)	99.0 (±28.7) (<i>P</i> 0.51)	54.6 (±9.80) (<i>P</i> 0.43)
DPPC:chol	101.2 (±6.11) (<i>P</i> 0.36)	99.3 (±3.92) (<i>P</i> 0.36)	119.8 (±9.33) (<i>P</i> 0.41)
DPPC:SA	117.3 (±13.7) (<i>P</i> 0.75)	60.1 (±36.4) (<i>P</i> 0.64)	58.2 (±52.4) (<i>P</i> 0.59)
DSPC	107.6 (±27.2) (<i>P</i> 0.87)	121.6 (±32.0) (<i>P</i> 0.79)	112.8 (±25.2) (<i>P</i> 0.83)
DSPC:chol	111.9 (±7.48) (<i>P</i> 0.41)	107.3 (±5.22) (<i>P</i> 0.40)	123.1 (±10.9) (<i>P</i> 0.48)
DSPC:SA	120.7 (±29.2) (<i>P</i> 0.70)	65.4 (±26.2) (<i>P</i> 0.55)	20.8 (±14.3) (<i>P</i> 0.85)

^a All values are means of at least three different samples (±SD).
^b Measured immediately after mixing the liposome samples with sodium cholate (10 mM final cholate concentration).
^c Measured after 24 h of incubation of the liposome samples with sodium cholate at 37°C (10 mM final cholate concentration).
^d *P*. polydispersity index means from at least three different samples. Polydispersity Index is a measure of the homogeneity of the vesicle dispersions. This index ranges from 0.00 (for an entire homogeneous dispersion) to 1.00 (for a completely heterogeneous dispersion).

of pancreatin. The demonstrated enzyme-induced instability of DPPC liposomes is in good agreement with earlier studies in which pancreatin was found to hydrolyze lipids only at temperatures approaching their transition temperature (Op Dem Kamp et al., 1974; Wilschut et al., 1979). Indeed, this is very important for DPPC liposomes ($T_c = 41^\circ\text{C}$) and especially sonicated ones, as used in our studies, for which broadening of lipid transition temperatures has been demonstrated (Lentz et al., 1976; Van Dijck et al., 1978). Cholesterol, even at a low molar concentration (lipid/chol ratio of 7:2) prevents enzyme attack (Fig. 3(III)), correlating well with the claimed by many researchers abolishment of T_c when cholesterol is added in the liposomal membrane.

Liposomes composed of lipids with transition temperatures below 37°C (PC and DMPC), release most if not all of their encapsulated CF immediately after mixing with cholate salts (Fig. 1(II and III, time 0)). In PC:chol liposomes initial CF retention is higher, but overall stability is not significantly enhanced. Considering the fact that in our studies the lipid to detergent molar ratio was equal to 0.25, the observed CF release values correlate well with previous results for PC and PC:chol liposomes (Ruiz et al., 1988; De la Maza and Parra, 1997). Despite the devastating stability results for PC and DMPC liposomes, in some cases, DPPC, DSPC and SM (with transition temperatures above 37°C) form vesicles which retain significant amounts of encapsulated CF in presence of cholate salts, especially when cholesterol is included in their bilayers (Figs. 2–4). The higher rigidity and order in packing of these lipids, in comparison with PC, is possibly the cause of their enhanced resistance against cholate salts. Our results for DSPC:chol liposomes agree with those obtained by other groups (Rowland and Woodley, 1980; Ouadahi et al., 1998). However, the case of DPPC:chol liposomes has been contradictory (Dapergolas and Gregoriadis, 1976; Rowland and Woodley, 1980) and, to our knowledge, liposomes composed solely of SM have not been tested by others.

As for the mechanism of encapsulated material release, in most cases our CF release and sizing results are consistent with the theory that transient and rapid-resealing membrane holes form after bile salt addition (Schubert and Schmidt, 1988). This is probably the case for most of the liposome types studied, in which vesicle size is practically unaffected by cholates. The fact that in all these cases cholate-induced CF release was initially fast, followed by a significantly slower process, further substantiates the former hypothesis suggesting that incubation conditions do not cause massive disruption of liposomes. However, in the cases of stearylamine-containing cholesterol-free DPPC and DSPC liposomes and plain DPPC liposomes, for which cholates induced rapid release of encapsulated CF as well as significant decrease in vesicle diameter (Figs. 3(II,III) and 4(II,III) and Table 1), continuous permeation through a permanently disrupted membrane is the most

likely mechanism of CF release. The observation that these samples are optically clear after incubation, suggests that liposomes are breaking down and forming micelles (possibly mixed micelles with cholates). Especially in the case of SA containing liposomes ionic interactions between negatively charged cholates and positively charged SA, may be contributing to this phenomenon, since in our case SA is not interacting with any negatively charged encapsulated molecule.

According to previous reports (Schurtenberger et al., 1985; Schubert and Schmidt, 1988; Ramaltes et al., 1996b; De la Maza and Parra, 1997), mixed micelles may form between PC and cholates when cholate concentration is above the CMC of the surfactant in the specific media. The onset and rate of the micellization process may be influenced by several parameters, as the specific media and conditions applied (salt concentration, temperature, etc.), as well as the mode of cholate addition. In our study, the 10 mM concentration of sodium cholate was not sufficient for solubilization of 2.5 mM PC liposomes, as judged by the sizing experiment results (Table 1). In order to clarify if the demonstrated cholate-induced initial slight increase in plain PC vesicle mean diameter is due to partitioning of surfactant molecules into the lipid bilayer (indicative of initialization of micelle formation (Ramaltes et al., 1996b; De la Maza and Parra, 1997)), the light scattering of plain PC liposomes in increasing sodium cholate concentrations was measured. Indeed the results demonstrate that in the conditions applied, 7 mM sodium cholate dissolve 1 mM PC vesicles, while for complete breakdown of a 2.5 mM dispersion of PC vesicles, 12 mM sodium cholate are needed (Fig. 5). The estimated cholate to lipid ratio required for 50% vesicle solubilization is approximately 0.17, close to the value (0.14) estimated by Ruiz et al., 1988.

When cholesterol was included in the membranes of DPPC, DSPC and SM liposomes a protective effect against cholate salt attack was observed (Figs. 2–4(II,III), filled squares in the figures). This protection was greater for sodium taurocholate as reported also by others (Schubert and Schmidt, 1988).

Summarizing, we conclude that liposomes composed of DPPC, DSPC or SM mixed with high amounts of cholesterol are good choices for orally administered liposomal formulations of drugs.

Of course, since the release of a very small and highly hydrophilic molecule was used as a measure of liposome stability in our studies, we cannot reject the hypothesis that larger molecules (like proteins) will be significantly retained by rich in cholesterol PC liposomes even in the presence of cholates, for an application relevant time period, or that these liposomes can substantially protect encapsulated protein or peptide molecules from digestive enzyme attack. Also, we cannot reject the theory that in presence of bile salts, molecules encapsulated in liposomes may form mixed micelles or different complexes with

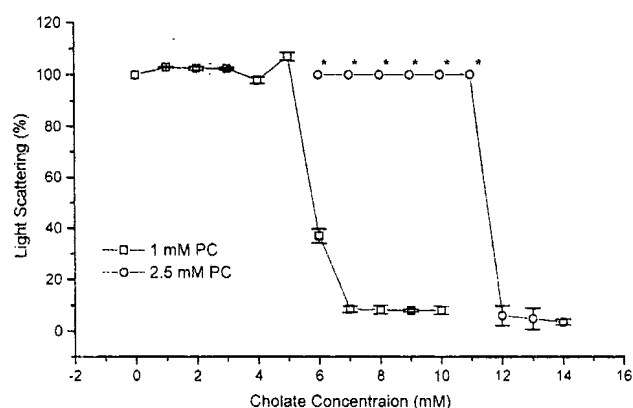


Fig. 5. Percentage of light scattering of PC small unilamellar liposomes (lipid concentration, 1 and 2.5 mM) in the presence of increasing concentrations of sodium cholate. Liposomes (2 ml) in TBS, pH 7.4, were placed in a magnetically stirred thermostated cuvette ($37 \pm 0.1^\circ\text{C}$) and light scattering measurements were performed with a Shimadzu RF-1501 Spectrophotometer (both emission and excitation set at 500 nm), immediately after each addition of cholate (20 μl of a 100 mM sodium cholate solution in TBS, pH 7.4). Each experiment was duplicated (bars represent SD values). *In the case of the 2.5 mM liposome dispersion, fluorescence intensity values were above the saturation value of the instrument for all points measured prior to complete vesicle disruption. Key: squares, 1 mM PC; circles, 2.5 mM PC.

lipids and cholates, with favoring results for the bioavailability of the specific compound after oral administration of its liposomal formulation, as postulated in previous studies (Arien et al., 1995; Ramaldes et al., 1996a). Additionally, in some cases, the presence of a specific lipophilic drug molecule in the lipid bilayer may modify membrane characteristics. This has been observed by Kirilenko and Gregoriadis (1993) for liposomes incorporating lipophilic vitamins in presence of cholate salts, and also for liposomes incorporating drug molecules in their bilayer in presence of plasma (Fatouros and Antimisariis, unpublished results). Nevertheless, it should be kept in mind that specific interactions of each drug molecule with liposomal components, as well as the size and lipophilicity of the drug, may influence the stability of the specific formulation (or else, retention of drug in vesicles) in the environment of the gastrointestinal tract.

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