Available online at www.sciencedirect.com
SCIENCE DIRECT®
International Journal of Pharmaceutics 312 (2006) 144–150
INTERNATIONAL JOURNAL OF PHARMACEUTICS
www.elsevier.com/locate/ijpharm

Effects of sterilizing-grade filters on the physico-chemical properties of onion-like vesicles

Antoine Richard [a,b], Julie Delvaux [a], Line Bourel-Bonnet [a,*]

[a] *UMR 8525 CNRS/Université de Lille 2, Faculté de Pharmacie, 3, rue du Pr Laguesse, BP 83, 59006 Lille Cedex, France*
[b] *ETHYPHARM S.A., 194, Bureaux de la Colline, 92213 Saint-Cloud, France*

Received 10 October 2005; received in revised form 3 January 2006; accepted 9 January 2006
Available online 15 February 2006

Abstract

Spherulites™ are new promising multilamellar vesicles that we study in a drug delivery context. The sterilization of spherulites™ suspensions is a necessary step before biological tests and later, before pharmaceutical applications (for example, parenteral or local injections). Among all sterilizing operations, the filtration through 0.22 μm sterilizing-grade filters (of the type Millex® (Ø 4 mm) by Millipore) is easy and rapid, and we decided to study it as a mean to obtain sterile suspensions. The spherulites™ diameter is usually comprised between 0.2 and 0.5 μm but bigger vesicles occur and reach Ø 1 μm. The effects of such filters on vesicles' size and lipids' concentration were then compromised. After examination of this challenging operation, results proved that the sterilizing filtration had no effect on these two parameters whatever the formulation chosen. Then, the possible release of amaranth, an encapsulated hydrophilic dye was followed. With the formulations and in spite of a filter diameter inferior to that of the vesicles, the encapsulation yields were not significantly different before and after the filtration and no leakage could be detected. Finally, the spherulites™, functionality after sterilizing filtration was studied under the chemical angle: vesicles containing an amphiphilic reactive anchor ($CholE_3ONH_2$) were still able to bind covalently a peptidic molecular recognition pattern. The ligation was quantified by fluorimetry as high as for non-filtrated suspensions. Thus, though spherulites™ can present a diameter superior to that of the sterilizing filters, their passage through them do not alter the physico-chemical properties of these vesicles.

Keywords: Vesicles; Encapsulation; Sterilizing filtration; Cell targeting; Drug delivery

1. Introduction

Spherulites™ are multilamellar vesicles with no aqueous core obtained by shearing a lipidic lamellar phase (Diat et al., 1993; Gulik-Krzywicki et al., 1996) and whose diameter is comprised between 200 nm and 1 μm. Easy to produce both at the laboratory and the industrial scale, these vesicles were first designed and studied (Diat and Roux, 1993) and further developed industrially.[1] Spherulites™ are constituted by an alternance of lipidic bilayers and aqueous layers and therefore display an onion-like structure (Fig. 1).

Phosphatidylcholine (PC), cholesterol and various co-surfactants (see Table 1) constitute their lipidic bilayers. A water layer is comprised between two bilayers to form a lamellar phase. The shearing of the latter followed by the dispersion in aqueous solutions gives the onion-like vesicles (Scheme 1). Their size is related to their formulation and the shearing's strength and duration.

Theoretically, spherulites™ can encapsulate either hydrophilic (Mignet et al., 2000; Pott and Roux, 2002; Simard et al., 2005) or hydrophobic active compounds (Freund, 1998;

Abbreviations: Boc, tertiobutyloxycarbonyl; Chol, cholesterol; CryoTEM, transmission electronic microscopy; DIEA, diisopropylethylamine; DLS, dynamic light scattering; F, filtration; Fmoc, 9-fluorenylmethyloxycarbonyle; HOBt, *N*-hydroxybenzotriazole; MALDI-TOF, matrix assisted laser desorption ionisation-time of flight; Mo, Monooleyl glycerol; MRP, molecular recognition pattern; Pbf, 2,2,4,6,7-pentamethyl-dihydrobenzofurane-5-sulfonyle; PC, phosphatidylcholine; PTFE, polytetrafluorethylene; PVDF, polyvinylidene fluoride; SF, sterilizing filtration; Sim, Simulsol 2599; Sol, Solutol HS15; T80, Tween® 80; TBTU, 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate; *t*Bu, tertiobutyl; TFA, trifluoroacetic acid; TS, titring solution

* Corresponding author. Tel.: +33 3 20 96 49 49; fax: +33 3 20 96 47 09.
E-mail address: lbourel@pharma.univ-lille2.fr (L. Bourel-Bonnet).

[1] Industrially developed by Capsulis for non-pharmaceutical applications (cosmetology, detergents, . . .) and Ethypharm for pharmaceutical applications.

0378-5173/$ – see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijpharm.2006.01.007

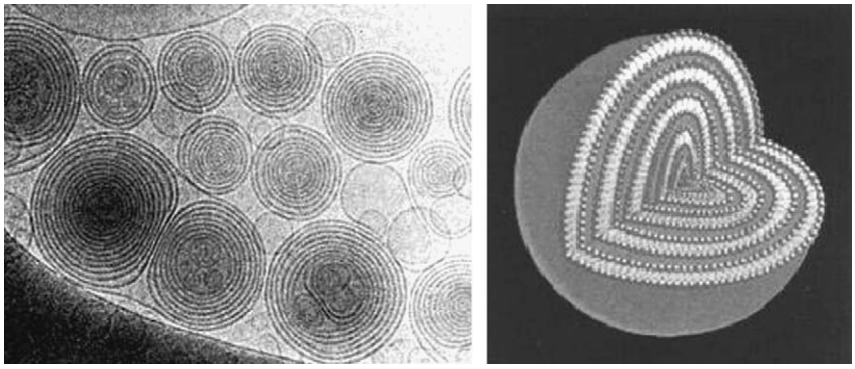


Fig. 1. Spherulites™. Left, by CryoTEM and right, 3D-scheme (courteously given by Chenevier, 2001).

Table 1
Massic composition (%, w/w) of spherulites™

	S1	S2	S3	S4	S5
PC	23.2	22.5	49.4	44.9	42.2
Co-surfactant	T80: 13.6	T80: 13.2	Mo: 2.6	Sim: 20.1	Sol: 10
Chol	3.2	0	0	0	12.8
$CholE_3ONH_2$	0	4.3	0	0	0
Water	60	60	48	35	35

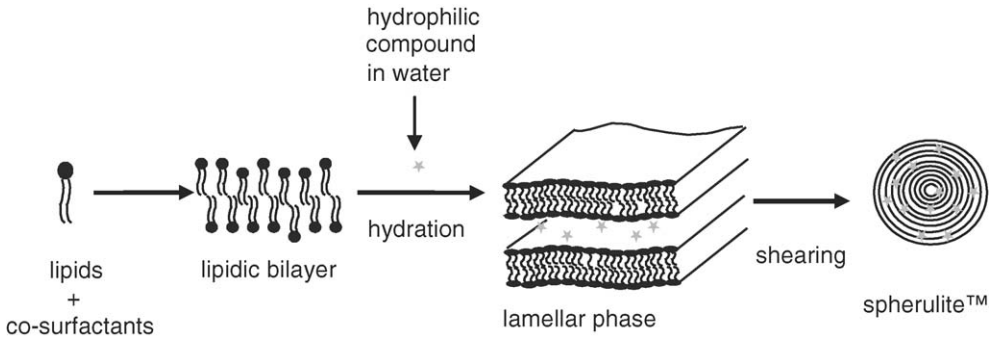
Richard, 2005), in the aqueous phase or in the lipidic bilayers, respectively. This indicates their potential interest in drug encapsulation and further, in drug targeting (Freund et al., 2000; Chenevier et al., 2002, 2003). We thus envisaged the use of spherulites™ in that field and especially chose the cancer cells targeting as a way to validate these vesicles (Richard, 2005). In Scheme 1, the introduction of an hydrophilic compound simultaneous to the hydratation step is illustrated, and as an example, we chose a non-toxic dye, amaranth, to replace hydrophilic anticancer drugs for the following of the present study.

To obtain a specific targeting, we previously developed a ligation strategy to immobilize molecular recognition patterns (MRPs) on the spherulites™, outer membrane. A cholesterol-like anchor of the type $CholE_3ONH_2$ was designed and synthesized to achieve this goal, and some complementary functionalized targeting glyoxylyl peptides were prepared. Synthetic data, thermodynamic and kinetical aspects of the ligation were reported (Richard and Bourel-Bonnet, 2005).

As spherulites™ are designed to contain and deliver drugs by local or parenteral injection, they are supposed to be sterile. Among all sterilization methods described in the literature, terminal heat sterilization, gamma-irradiation, UV-irradiation, ethylene oxide treatment, high pressure sterilization and sterilizing filtration have been reported, sometimes in liposome suspensions (like gamma-irradiation, Zuidam et al., 1996). The latter, sterilizing filtration (Goldbach et al., 1995; Endruschat and Henschke, 2000; Walsh, 1998), is a pharmaceutical operation aimed at eliminating particles and microorganisms within a liquid by a porous filter to obtain a sterilized solution or suspension. It has been acknowledged as one of the most suitable method for liposome formulations consisting of small unilamellar vesicles (SUVs), typically Ø 100 nm or smaller, so we decided to consider it for the sterilization of spherulites™.

Sterilizing-grade filters are usually thin highly porous membranes designed to minimize non-specific adsorption and acting as screens (Le Hir, 2001). Several types of filters are commercially available: hydrophobic PTFE membranes to filter organic solutions, hydrophilic PTFE membranes to filter aqueous or aqueous–organic solutions, hydrophilic PVDF membranes for the filtration of aqueous solutions where the proteic adsorption is minimized. We chose the latter for the filtration of spherulites™.

We especially investigated the physico-chemical aspects of sterilizing filtration onto our multilamellar onion-like vesicles at the laboratory scale. This operation could have an impact on the integrity, the leakage and the chemical functionality of our vesi-



Scheme 1. Spherulites™, preparation.

cles, especially those whose diameter was superior to that of the filters' pores. We studied all these parameters in the following. Such work, although already studied in the case of multilamellar liposomes (Endruschat and Henschke, 2000), is unprecedented in the spherulites™' field and needed to be tackled for the following of our researches.

2. Material and methods

2.1. Chemicals and peptide syntheses

All Fmoc protected amino acids (see "Abbreviations") (L-Arg(Pbf), L-Asp(O*t*Bu), L-Glu(O*t*Bu), L-Ser(*t*Bu), L-Thr(*t*Bu), L-Trp(*t*Boc), L-Tyr(*t*Bu) coupling agents and the resin (PEGA®) were purchased by Novabiochem. Chemicals were from Sigma–Aldrich. Solvents were from Acros. P1 (H-PLRTAWPG-$NH(CH_2)_3NH$-CO-CHO) was synthesized according to a Fmoc/*t*Bu solid phase peptide strategy on a Pioneer synthesizer (Applied Biosystems, UK). The solid phase synthesis, whose protocol was already reported (Fields and Noble, 1990; Mikolajczyk et al., 1994; Melnyk et al., 2001) was performed with a 9 eq in situ activation of Fmoc amino acid in the presence of TBTU, HOBt and DIEA. RP-HPLC analyses were performed on a Shimadzu SCL-6A (3 mL min^{-1}) and a WATERS 2695 (1 mL min^{-1}) for preparative and analytical separations, respectively (RP-HPLC eluents: A: TFA 0.05% in H_2O; B: TFA 0.05% in CH_3CN/H_2O: 80/20, v/v; Column C18 Nucleosil® at 50 °C). Detection was performed both at 215 and 254 nm. The mass spectra were acquired on a Perspective Biosystems Voyager-DE™ STR, Biospectrometry™ Workstation MALDI-TOF spectrometer and measures were acquired after deposition on a dihydroxybenzoic acid (DHB) matrix. P1: yield 20%, purity 84% (HPLC 215 nm), $m/z = 1009.4$ ($[M+H]^+$ calcd: 1009.2).

2.2. Vesicles' formulation and preparation

Membranes of spherulites™ were made of soy lecithin (Phospholipon 90, Natterman, GmbH, also called PC in the text), and either Tween® 80 (Sigma), or Monooleyl glycerol, or Simulsol 2599 (Seppic), or Solutol HS 15 (a gift from BASF) and cholesterol (Sigma) and/or $CholE_3ONH_2$ (Richard and Bourel-Bonnet, 2005). Five basal formulations were studied (see Table 1 for massic proportions). In each case, lipids were solubilized and mixed into dichloromethane in a glass flask. After evaporation under reduced pressure, water (or amaranth 10 mg mL^{-1} in water) was dropped onto the lipidic film before a strong homogenization. Glass flasks were centrifuged (stopper up) at 3500 rpm for 5 min at 20 °C. Suspensions were rehomogenized by vigorous shaking. Glass flasks were then centrifuged (stopper down) at 1500 rpm for 5 min at 20 °C and again centrifuged (stopper up) at 3500 rpm for 15 min at 20 °C and finally rehomogenized. The cycle was repeated three times and the last cycle comprised a final centrifugation (stopper up) at 3500 rpm for 30 min at 20 °C. The lamellar phase was left over for 12 h at 4 °C. Then it was sequentially sheared and dispersed within distilled water drop by drop to obtain a final 8% or 4% (w/w) suspension.

2.3. Ligation of a peptide onto functionalized spherulites™

A solution of P1 at 6.4 mM was prepared in distilled water. 300 μL of P1 solution was added to 300 μL of spherulites™ suspension (8%, w/w) under stirring during 48 h to get a final 4% (w/w) concentration.

2.4. Gel filtration

A NAP-5™ column (Sephadex™ G-25 as stationary phase, Amersham Biosciences) was emptied and stabilized with 3 × 3 mL of distilled water. Then 300 μL of a suspension of spherulites™ in water 4% (w/w) were dropped on the column. Three hundred microlitre of water were added to complete the dead volume of the column. Then 900 μL of distilled water eluted the vesicles that were collected at the end of the column.

2.5. Filtration

A 1 mL syringe was equipped with a Millex® filter (Ø 4 mm) (Millipore) whose pores' size was either 0.45 or 0.22 μm. Five hundred microlitre of spherulites™ suspension were put in the syringe and the filtration occurred slowly with a light manual pressure on the piston. The syringe and the filter were purged three times with air.

2.6. Phospholipids' dosage

2.7 g of $FeCl_3$ hexahydrate and 3.0 g of ammonium thiocyanate were dissolved in 100 mL of distilled water to obtain the titring solution (TS). 3, 5 and 7 μL of spherulites™ suspension were successively solubilized in 1.1 mL of a methanol/chloroform (0.1/1.0, v/v) mixture. 0.8 mL of TS was added and the mixture was stirred for 1 min and centrifuged at 20 °C for 5 min at 2000 rpm. The organic phase of each tube was withdrawn and its UV absorbance at 480 nm was measured on a UV–visible Uvikon 933 spectrometer.

2.7. Fluorimetry

A Quantamaster C60 fluorimeter was used. The samples were placed in a quartz vessel (2 mm × 10 mm). Emission spectra were recorded between 310 and 500 nm, with excitation wavelength at 295 nm.

2.8. Vesicles' size

This parameter was measured by dynamic light scattering on a DynaProMS800. Spherulites™ suspensions were diluted at 1/1000 (w/w) or 1/2000 (w/w) in distilled water filtrated over 0.22 μm filters to remove dust particles and microorganisms. Mean results are given for 100 measurements.

2.9. Bacteriology

One hundred microlitre of suspension S5 (4%, w/w, in water) was seeded before or after filtration (respectively, through Ø

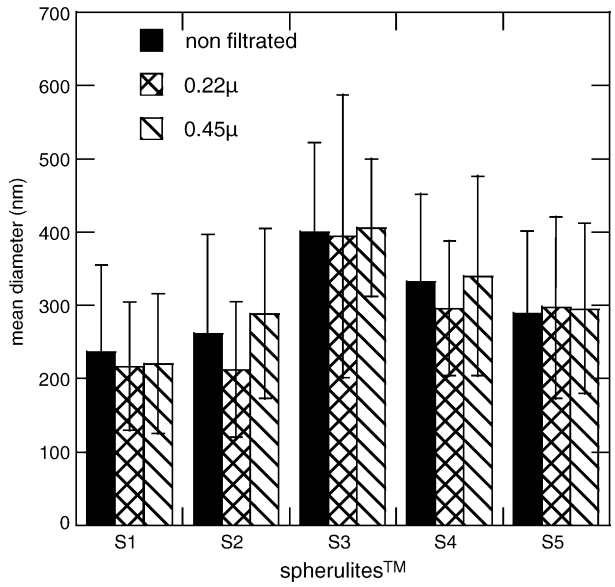


Fig. 2. Diameters of spherulites™ after sterilizing filtration. Error bars represent the polydispersity.

0.45 and Ø 0.22 μm filters) on blood agar. The seeding was then incubated 48 h either at 25 °C or at 37 °C. The number of colonies was evaluated visually.

3. Results and discussion

3.1. Sterilizing filtration of spherulites™

With the formulations, the spherulites™ diameter is generally comprised between 0.2 and 1 μm, and precisely in our formulations (Table 1), mostly between 200 and 500 nm (Fig. 2). On the other hand, sterilizing filtration (SF) is permitted by exerting a pressure on the suspension. We wondered if spherulites™ were supple enough to get out of shape under mechanical constraints and return to their initial form and properties. Whatever the filter diameter (and especially when it is inferior to the vesicles' one), several questions are raised. (a) Does SF eliminate the big spherulites™ (Ø > 500 nm) or could a 'cake' layer be formed on the membrane surface? (b) Does SF alter or destroy a part of our vesicles and is there a leakage of previously encapsulated compounds? (c) Is there a loss of chemical functionality? (Scheme 2). To answer these questions, we studied the influence of filtration through sterilizing-grade filters on spherulites™ in terms of size, phosphatidyl choline quantification, encapsulation and leakage, and ligation capabilities.

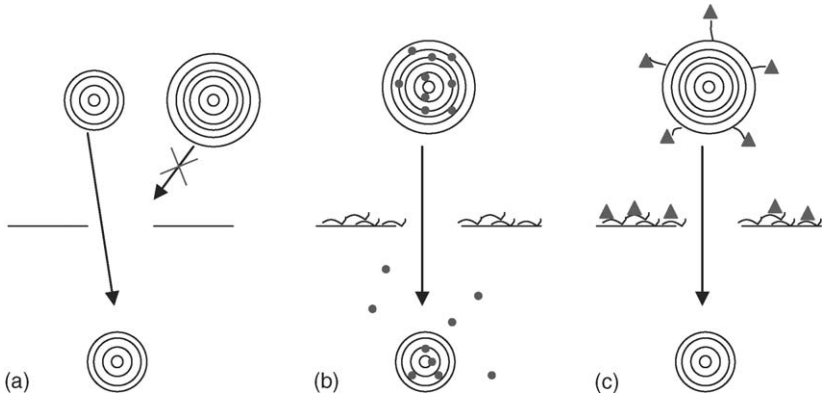
The experiments were performed on several formulations (Table 1). For suspension S1, phosphatidyl choline (PC), the major component, Tween® 80 (T80) and cholesterol (Chol) constituted the lipidic bilayers of the spherulites™. In S2 formulations, cholesterol was totally replaced by an anchor of the type $CholE_3ONH_2$. This amphiphilic reactive compound inserted in the bilayers was aimed at immobilizing recognition patterns onto the vesicles via an α-oxo oxime bond (Richard and Bourel-Bonnet, 2005). For S1 and S2 the mean diameter was between 200 and 300 nm. Furthermore, some other formulations were studied where Monooleyl glycerol (Mo), Simulsol 2599 (Sim) or Solutol HS 15 (Sol) were the co-surfactants (respectively, S3, S4 and S5). S3 generally make bigger vesicles whose diameter is around 400 nm. S4 and S5's diameter is usually around 300 nm.

3.2. Filtration and vesicles' size

We measured the mean diameter of spherulites™ by dynamic light scattering (DLS) before and after filtration to determine the impact of this operation on their size.

As we can see in Fig. 2, the filtration operation did not diminish significantly the vesicles' mean diameter, as well as the polydispersity of size distribution, whatever the filter used (0.22 or 0.45 μm). Mean diameters remained centered on a value comprised between 200 and 300 nm for S1 and S2 and between 300 and 400 nm for S3, S4 and S5.

Viewing these results, we can suppose that the filtration do not stop the spherulites™ that are bigger than the pore size. Indeed, if it was the case, the mean diameter would diminish and the polydispersity become narrow. This, and the absence of 'cake' layer clogging the filter, will be confirmed by the phosphatidyl choline quantification presented in the following.



Scheme 2. Hypothetical phenomenoms that can occur along sterilizing filtration of spherulites™. (a) Selection of small-sized spherulites™. (b) Disruption of external bilayers and leakage of a previously encapsulated compound. (c) Disruption of external bilayers and loss of the MRP and/or chemical functionality.

Table 2
Phosphatidyl choline concentration (±0.2 mg mL^{-1}) in various spherulites^{TM} formulations after filtration experiments through Ø 0.22 and Ø 0.45 μm filters

	S1	S2	S3	S4	S5
Non-filtrated	3.1	3.0	4.5	5.0	4.7
0.22 μm	3.0	3.0	4.0	5.1	4.8
0.45 μm	2.9	2.8	4.0	5.0	4.9

Spherulites^{TM} were gel filtrated and dispersed at 1.33% (w/w) in water.

3.3. Dosage of phosphatidyl choline in vesicles and extrapolation to lipidic bilayers' integrity

A colorimetric method (Stewart, 1980), first reported to quantify soy phosphatidyl choline, was developed to quantify the amount of lipids present in a suspension of spherulites. This was tuned and further applied to study the influence of diverse operations (gel filtration, ligation, ... (Richard, 2005) and here sterilizing filtration) onto vesicles. The method consists in the quantification of the phosphatidyl choline in a precise volume of vesicles' suspension by complexation of $Fe(SCN)_3$ to the phosphate head in organic phase.

Iron thiocyanate in aqueous solution is intensively red, thus its absorbance can be measured in the visible range at 480 nm in weakly concentrated solutions. By a phase transfer mechanism, a complexation occurs between an aqueous titring solution of $Fe(SCN)_3$ in large excess and a chloroform organic phase where the lipids are soluble whereas the free $Fe(SCN)_3$ is not. Thus, in the presence of phosphatidyl choline, $Fe(SCN)_3$ is transferred from the aqueous to the organic compartment and the absorbance of the complex in organic phase is measured by visible spectroscopy (Table 2).

The results presented in Table 2 show that no significant loss of matter could be detected except for the spherulites^{TM} S3 that lost 10% of their initial amount of PC. Filtration is thus a non-harmful method for our vesicles. Knowing the initial percentage of phosphatidyl choline in the formulation and assuming that the relative percentage of all the lipids is constant during the operations, a calcul of the total quantity of lipids in the suspension, its loss or its constancy, is permitted and an extrapolation to the integrity of the lipid bilayers is possible. Here, it can be extrapolated, for every formulations except S3, that PC's constancy is an evidence of: (1) the lipidic bilayers' and the vesicles' integrity during the filtration operation and (2) the absence of clogging by the biggest vesicles.

3.4. Filtration, encapsulation and release

Whereas no significant alteration in terms of size and PC concentration was detected when performing a filtration, notably through sterilizing-grade filters, there was a need to study the influence of filtration on the encapsulation and the leakage of interesting compounds. Thus, amaranth, a non-toxic hydrophilic azoic dye whose formula is displayed Fig. 3, was chosen as a 'witness' of events occurring in the aqueous compartment when applying the above-mentioned operation. Amaranth absorbs strongly at 521 nm so that its quantification is permitted by visible spectroscopy.

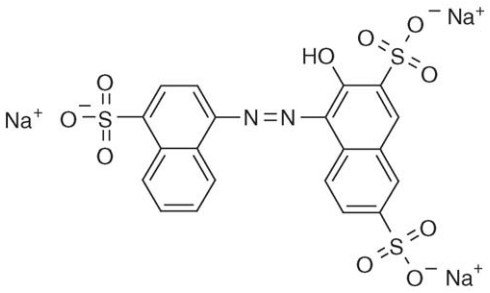


Fig. 3. Amaranth is a monoazoic dye (E123, 2-hydroxy-(4-sulfo-1-naphtylazo)-1-naphtalene-3,6-disulfonic acid trisodium salt, MW 604.48 g mol^{-1}, $C_{20}H_{11}N_2Na_3O_{10}S_3$).

After amaranth encapsulation, a gel filtration removed the non-encapsulated dye. By measuring the absorbance at 521 nm before and after gel filtration, the encapsulation yield was calculated. Then, every suspensions were submitted to a filtration step, respectively, at 0.22 μm (sterilizing-grade) and 0.45 μm. The loss of amaranth was quantified identically, by measuring the absorbance of the suspensions at 521 nm after gel filtration (Table 3).

Table 3 shows that the loss of amaranth is negligible in formulations S1, S2 and S5 and very weak in formulation S3 and S4 that are the biggest spherulites^{TM} in the study. Sterilizing filtration over 0.22 μm filters is thus a good mean to filter suspensions of spherulites^{TM} containing a compound of interest encapsulated in the aqueous compartment. To summarize, in spherulites^{TM}, not only the lipidic bilayers but also the hydrophilic compartment are left intact by the filtration, especially that over sterilizing-grade filters.

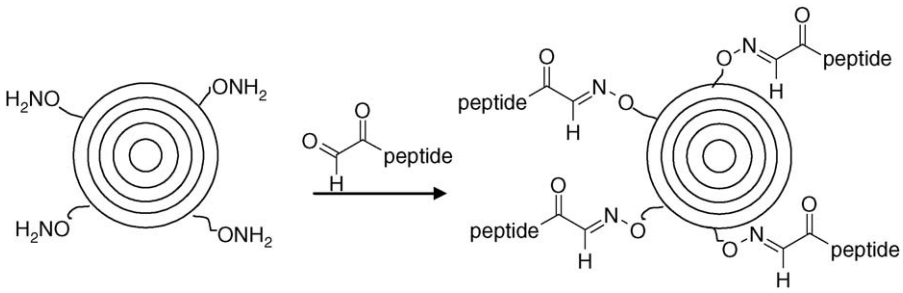
3.5. Sterilizing filtration and spherulites^{TM}' chemical functionality

With a targeting aim in mind, the spherulites^{TM} S2 (containing the anchor $CholE_3ONH_2$) reacted with a model glyoxylyl peptide to study the formation of an α-oxo oxime bond at their surface. Excesses of peptide were removed by gel filtration (Scheme 3).

Before and after the oxime ligation, the vesicles' suspensions were filtrated through a sterilizing-grade filter (0.22 μm). In every cases, the oxime formation was followed by fluorimetry, a selective and precise analytical mean. The studied peptide, P1, of the type H-PLRTAWPG-NH$(CH_2)_3$NH-CO-CHO, con-

Table 3
Encapsulation yields and loss of amaranth in percentage, respectively, at 0.22 and 0.45 μm

Susp.	S1	S2	S3	S4	S5
Encapsulation yield (%)	34	44	81	20	85
Loss SF 0.22 μm (%)	<5	<5	9	12	<5
Loss F 0.45 μm (%)	<5	<5	5	14	<5



Scheme 3. Functionalization of spherulites^{TM} S2, that contain the anchor $CholE_3ONH_2$, by a glyoxylyl peptide.

tained a tryptophan residue to quantify the ligation capability of the suspension.

As we can see Fig. 4, no significant change was measured, the quantity of peptide fixed did not change whether the filtration was carried out before or after the chemical ligation. Thus, fluorimetry measurements demonstrate that SF does not alter the amount of peptide anchored on the vesicles S2. These other results demonstrate that SF does not disturb the chemical functionality of spherulites^{TM}. It is a supplementary proof of the integrity of the lipidic bilayers and further, of the vesicles.

3.6. Bacteriological impact of sterilization of spherulites^{TM}' suspension

We rapidly evaluated the bacteriological impact of this operation by the seeding of the filtrated and non-filtrated suspensions of spherulites^{TM} S5 and found that, logically, the most efficient way to decontaminate the suspension was the filtration through a 0.22 μm filter that removed all the germs initially present before filtration (Table 4). The filtration through 0.45 μm removed only half the germs.

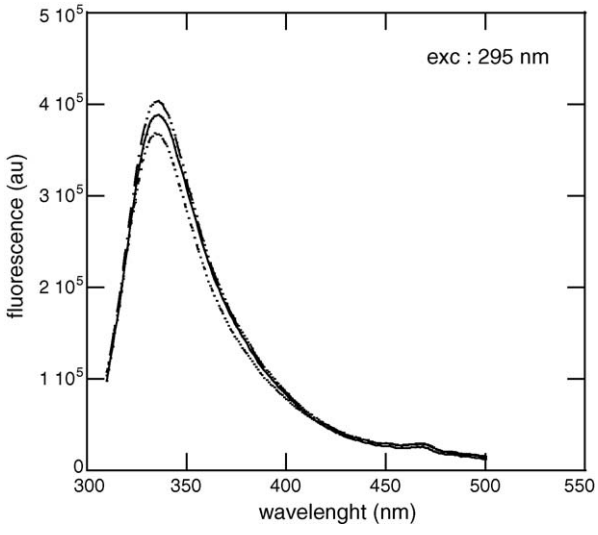


Fig. 4. Fluorescence emission spectra of spherulites^{TM} S2 functionalized by peptide P1 before or after SF on 0.22 μm filters. Excitation at 295 nm. Measurements were performed after gel filtration to remove the peptide's surplus. (– · · –) Spherulites^{TM} filtrated before the peptide ligation, (—) spherulites^{TM} functionalized with the peptide and not filtrated and (···) spherulites^{TM} firstly functionalized and then filtrated through a 0.22 μm filter.

Table 4
Number of germs in 100 μL of a suspension of spherulites^{TM} S5 (4%, w/w) filtrated or not

	Non-filtrated S5	Filtrated S5 on 0.45 μm	Filtrated S5 on 0.22 μm
Incubation at 25 °C	1000 germs	500 germs	0 germ
Incubation at 37 °C	1000 germs	500 germs	0 germ

Spherulites^{TM} were seed on blood agar either at 25 °C or at 37 °C during 48 h.

The fact that the number of germs was not dependent on the temperature indicated that the contamination came from materials or environment (the spherulites^{TM} were not prepared in sterile conditions for this rapid evaluation) and not from the experimentator.

4. Conclusion

To summarize, for every formulations excepting those containing Monooleyl glycerol or Simulsol and whose diameter is superior to 300 nm, the filtration of spherulites^{TM} through sterilizing-grade filters does not alter their size, their lipid bilayers nor their aqueous compartment. Their chemical functionality is also kept after this operation. For the above-mentioned S3 and S4, both size and weak elasticity can be charged for a slight loss of PC and amaranth. Thus, we chose to work on S1, S2 and S5 for further experiments to be reported in due course. In these three cases and from the physico-chemical point of view, sterilizing filtration is a non-harmful and easy pharmaceutical operation that can be applied to spherulites^{TM}.

Acknowledgements

This work was supported by a CIFRE fellowship of ETHYPHARM S.A. We thank Drs. Didier Roux (C.R.P.P., CNRS, Pessac, France), René Laversanne, Corinne Degert (ETHYPHARM S.A., Pessac, France), Denis Requier (ETHYPHARM S.A., St-Cloud, France) and Didier Hoarau (ETHYPHARM S.A., Laval, Canada) for fruitful scientific discussions, Annick Blanpain and Albane Decarsin (UMR 8525) for technical assistance, Hervé Drobecq (UMR 8525) for MALDI-TOF analyses and Dr. Christel Neut (Faculté de Pharmacie de Lille) for bacteriological experiments. We gratefully acknowledge financial support from Ministère de la Recherche (LBB received a young scientist 'ACI jeune chercheur' financial support in 2001), Université de Lille 2, Centre National de la Recherche Scientifique (C.N.R.S.).

References

Chenevier, P., 2001. Ph.D. Thesis. University of Bordeaux 1.

Chenevier, P., Grandjean, C., Loing, E., Malingue, F., Angyalosi, G., Gras-Masse, H., Roux, D., Melnyk, O., Bourel-Bonnet, L., 2002. Grafting of synthetic mannose receptor-ligands onto onion vectors for the human dendritic cells' targeting. Chem. Commun., 2446–2447.

Chenevier, P., Bourel-Bonnet, L., Roux, D., 2003. Chemical characterization of alpha-oxo hydrazone ligation on colloids: toward grafting molecular addresses onto biological vectors. J. Am. Chem. Soc. 125, 16261–16270.

Diat, O., Roux, D., 1993. Preparation of monodisperse multilayer vesicles of controlled size and high encapsulation ratio. J. Phys. II France 3, 9–14.

Diat, O., Roux, D., Nallet, F., 1993. Effect of shear on a lyotropic lamellar phase. J. Phys. II France 3, 1427–1452.

Endruschat, J., Henschke, K., 2000. Bench-scale manufacture of multilamellar liposomes using a newly developed multistage pressure filtration device. Int. J. Pharm. 196, 151–153.

Fields, G.B., Noble, R.L., 1990. Solid phase peptide synthesis utilizing 9-fluorenylmethoxycarbonyl amino acids. Int. J. Pept. Protein Res. 35, 161–214.

Freund, O., 1998. Ph.D. Thesis. University of Bordeaux 2.

Freund, O., Amédee, J., Roux, D., Laversanne, R., 2000. In vitro and in vivo stability of new multilamellar vesicles. Life Sci. 67, 411–419.

Goldbach, P., Brochart, H., Wehrlé, P., Stamm, A., 1995. Sterile filtration of liposomes: retention of encapsulated carboxyfluorescein. Int. J. Pharm. 117, 225–230.

Gulik-Krzywicki, T., Dedieu, J.C., Roux, D., Degert, C., Laversanne, R., 1996. Freeze-fracture electron microscopy of sheared lamellar phase. Langmuir 12, 4668–4671.

Simard, P., Hoarau, D., Khalid, M.N., Roux, E., Leroux, J.C., 2005. Preparation and in vivo evaluation of PEGylated spherulite formulations. Biochim. Biophys. Acta 1715, 37–48.

Le Hir, A., 2001. Abrégé de Pharmacie Galénique, Bonnes Pratiques de Fabrication des Médicaments, 8ème éd. Masson, Paris, p. 408.

Melnyk, O., Fruchart, J.S., Grandjean, C., Gras-Masse, H., 2001. Tartric acid-based linker for the solid-phase synthesis of C-terminal peptide alpha-oxo aldehydes. J. Org. Chem. 66, 4153–4160.

Mignet, N., Brun, A., Degert, C., Delord, B., Roux, D., Hélène, C., Laversanne, R., François, J.C., 2000. The Spherulites^{TM}: a promising carrier for oligonucleotide delivery. Nucl. Acids Res. 28, 3134–3142.

Mikolajczyk, S.D., Meyer, D.L., Starling, J.J., Law, K.L., Rose, K., Dufour, B., Offord, R.E., 1994. High yield, site-specific coupling of N-terminally modified beta-lactamase to a proteolytically derived single-sulfhydryl murine fab'. Bioconjugate Chem. 5, 636–646.

Pott, T., Roux, D., 2002. DNA intercalation in neutral multilamellar membranes. FEBS Lett. 511, 150–154.

Richard, A., Bourel-Bonnet, L., 2005. Internalisation of a peptide into multilamellar vesicles assisted by the formation of a alpha-oxo oxime bond. Chem. Eur. J. 11, 7315–7321.

Richard, A., 2005. Ph.D. Thesis. University of Lille 2.

Stewart, J.C.M., 1980. Colorimetric determination of phospholipids with ammonium ferrothiocyanate. Anal. Biochem. 104, 10–14.

Walsh, G., 1998. Biopharmaceuticals: Biochemistry and Biotechnology. Wiley, Chichester, pp. 123–124.

Zuidam, N.J., Versluis, C., Vernooy, E.A.A.M., Crommelin, D.J.A., 1996. Gamma-irradiation of liposomes composed of saturated phospholipids. Effect of bilayer composition, size, concentration and absorbed dose on chemical degradation and physical destabilization of liposomes. Biochim. Biophys. Acta—Biomembr. 1280, 135–148.