A Novel Phase Inversion-Based Process for the Preparation of Lipid Nanocarriers

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Purpose. To develop and subsequently evaluate a novel phase inversion-based method used to formulate lipidic nanocapsules.

Methods. Mechanical properties of emulsions prepared by multi-inversion phase processes were investigated using a drop tensiometer. Based on the results obtained, a formulation process was developed and a new type of nanocarrier was prepared. These particulates were sized by photon correlation spectroscopy and were visualized by atomic force microscopy and transmission electronic microscopy. Differential scanning calorimetry was also performed.

Results. The marginally cohesive but stable interfacial properties of the initial system led to the formulation of lipidic nanocapsules that were composed of a liquid core surrounded by a cohesive interface and were dispersed in an aqueous medium. These related suspensions were stable upon dilution for several months. The control of the formulation parameters allowed an adjustment of the particle mean diameter in the range of 25–100 nm with a monodisperse size distribution.

Conclusions. A novel and convenient process for the preparation of lipidic nanocapsules is described. The structure of these particulates resembles a hybrid between polymeric nanocapsules and liposomes. Such nanocapsules display a strong potential for drug delivery.

KEY WORDS: phase inversion process; elasticity; lipidic nanocapsules.

INTRODUCTION

The development of micrometric or nanometric drug carriers is frequently based on the use of dispersed systems in which solid or liquid phases are dispersed in fluid medium to constitute embryos of the final particles. Single emulsion systems (i.e., water-in-oil (w/o) or oil-in-water (o/w)) (1) as well as multiple emulsion systems (i.e., water-in-oil-in-water) (2) (typically prepared via homogenization (3)) have been widely used to encapsulate drugs into polymeric particles. This is, in part, due to the ease with which their droplet size can be controlled and stabilized.

Unfortunately, the solubilization of the coating material (i.e., polymers and lipids) in an organic solvent is frequently required in the emulsion step. Furthermore, to decrease the size characteristics and to avoid the droplet coalescence, high quantities of surfactants and cosurfactants, like butanol, are often used (4). These residues represent a potential toxicity for human use (5).

The aim of this study was to avoid the use of solvents, using pharmaceutically acceptable excipients in the formulation process. The approach developed in this article is to prepare nanocarriers based on the breaking of a bicontinuous sponge-like organization in which volumetric repetitive units could be precursor systems of the final objects. Moreover, this initial organization should display interesting mechanical properties, which should allow a fragmentation under various physicochemical changes. Such fragmentation should generate very small objects.

The potential of this solvent-free original approach to producing nanoparticulate objects has been evaluated subsequently by rheologic studies and quasi-elastic diffusion of light. The nanoparticulate structure then was explored by scanning probe atomic force microscopy (AFM), transmission electronic microscopy (TEM) and differential scanning calorimetry (DSC).

MATERIALS AND METHODS

The lipophilic Labrafac® WL 1349 (caprylic-capric acid triglycerides; European Pharmacopeia, IVth, 2002) was kindly provided by Gattefossé S.A. (Saint-Priest, France). Lipoïd S75-3 (soybean lecithin at 69% of phosphatidylcholine,) and Solutol HS 15 (mixture of free polyethylene glycol 660 and polyethylene glycol 660 hydroxystearate, European Pharmacopeia, IVth, 2002) were gifts from Lipoïd Gmbh (Ludwigshafen, Germany) and BASF (Ludwigshafen, Germany), respectively. Due to the complex composition of each product, the brand names will be used in the following text. NaCl, D (+)-glucose, mannitol, and D (+)-trehalose were obtained from Prolabo (Fontenay-sous-Bois, France). Water was obtained from a Milli RO System (Millipore, Paris, France).

Conductivity Measurements

The conductivity was determined using a conductimeter LF 325B (WTW, Weilheim, Germany) with two platinum plate attachments. Conductivity was measured at 2°C intervals, between 60°C and 85°C, under magnetic stirring.

Rheologic Study

The drop tensiometer (Tracker, IT Concept, Longessaigne, France) allowed the determination of the interfacial tension by analyzing the axisymmetric shape of the rising drop of the oil (Labrafac®; d = 0.945) in an aqueous Solutol® solution. The determination of surface elasticities was performed by assessing the response of the interfacial tension to several sinusoidal area variations of the oily drop (characterized by the pulsation ω). These experiments were performed when the surface pressure π had reached its equilibrium value. Subsequently, a complex transfer function, G(ω), was calculated (6). This transfer function can be detailed by the sum of a real part (G'(ω)) and an imaginary part (G"(ω)). According to (6), E_e, the equilibrium elasticity, described all lateral interactions between all the molecules in the interfacial zone. E_{ne}, the nonequilibrium elasticity, characterized the dissipation of the rheologic perturbation energy, especially

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ABBREVIATIONS: AFM, atomic force microscopy; DSC, differential scanning calorimetry; o/w, oil-in-water; PCS, photon correlation spectroscopy; PIZ, phase inversion zone; Tcd, temperature of cooling-dilution; TEM, transmission electronic microscopy; w/o, water-in-oil.

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the part related to the departure of tensioactive molecules to the bulk phase. These elastic constants were not dependent on the experimental conditions. E_e was obtained by extrapolating the curve $G' = f(\omega)$ for $\omega = 0$, and E_{ne} was calculated from the maximal value of $G'(\omega) = f(\omega)$ corresponding to $E_{ne} + E_e$ at $\omega = \infty$.

A rising drop of Labrafac® (9 μL) was formed using a G18 needle inside a trough of hydrophilic surfactant at 0.1 mol/l, corresponding to the concentration used in step I of the particle formulation process. At this concentration, 2 h of stabilization were necessary. ω was in the range of 0.039–3.140 rad/s, corresponding to a period *T* of 160–2 s.

Ternary Diagram

The concentration of Lipoïd® S75-3 and NaCl in water before the dilution step were fixed at 1.50% (w/w) and 1.75% (w/w), respectively. A ternary diagram was used to plot all the possible ratios of saline, hydrophilic surfactant, and oil. Forty-one formulations over the whole diagram were consequently tested in triplicate to define the zone where the mixture led to nanoparticulate systems. This related domain was named the ′feasibility domain.′ When particles were formed, their size range was characterized by photon correlation spectroscopy (PCS).

Characterization of the Nanocarriers

Size Measurements

The average volume diameters and the polydispersity index of nanocarriers were determined by PCS using a Malvern Autosizer 4700 (Malvern Instruments S.A., Worcestershire, United Kingdom) fitted with a 488-nm laser beam at a fixed angle (90°) at 25°C. A polydispersity index above 0.3 indicated a broad distribution. A 1:400 dilution of the nanoparticle suspension in distilled water was achieved to enable measurements (performed in triplicate).

AFM

AFM was carried out with an Autoprobe CP fitted with a 2-μm cantilever and a monocrystalline silicium tip Ultralever UL020 (Park Scientific Instrument, Geneva, Switzerland). The linear scanning rate was 1 Hz. Droplets of steady volume (20 μl of the final suspension) were deposited onto freshly cleaved mica. After the drop was dried, the contact or the noncontact mode was used at room temperature. The force was adjusted to 10 nN. The measurements were performed in triplicate in different sample locations. Furthermore, similarity between the contact and noncontact modes confirmed that the method did not induce any modification of the nanoparticle shape.

TEM

TEM analysis was performed using a Jeol 100CX instrument (JEOL-France, Paris, France). Before analysis, the nanoparticle suspensions were stained by a 2% phosphotungstic acid aqueous solution and were sprayed onto copper grids overlayed with 1% formwar in chloroform (Mikross, Paris, France).

DSC

DSC was obtained using a Mettler Toledo Star System (Mettler-Toledo, Viroflay, France). Each sample was studied at 5°C/min in the range of –50 to 150°C. The same study was performed on the particles after lyophilization in presence of a cryoprotectant (trehalose).

Stability

The produced particle dispersion was divided into different batches that were stored at different temperature conditions (6°C and 37°C) and were protected from light. Particle size measurements were performed by PCS every day for each sample.

Freeze-Drying

Nanocarrier aqueous suspensions were freeze-dried (RP2V, SGD, Le Coudray Saint Germer, France) for 24 h. The influence of various cryoprotectants (e.g., glucose, mannitol, and trehalose) added to the suspensions was studied at three different concentrations (5%, 10%, and 15% w/w).

RESULTS AND DISCUSSION

Conductivity measurements were performed after mixing all the components, corresponding to an oily phase (Labrafac) and a water phase in the presence of tensioactive molecules (Lipoïd® and Solutol®). Three temperature cycles ranging from 60°C to 85°C were necessary to obtain stable and reproducible curves. Figure 1A describes the conductivity

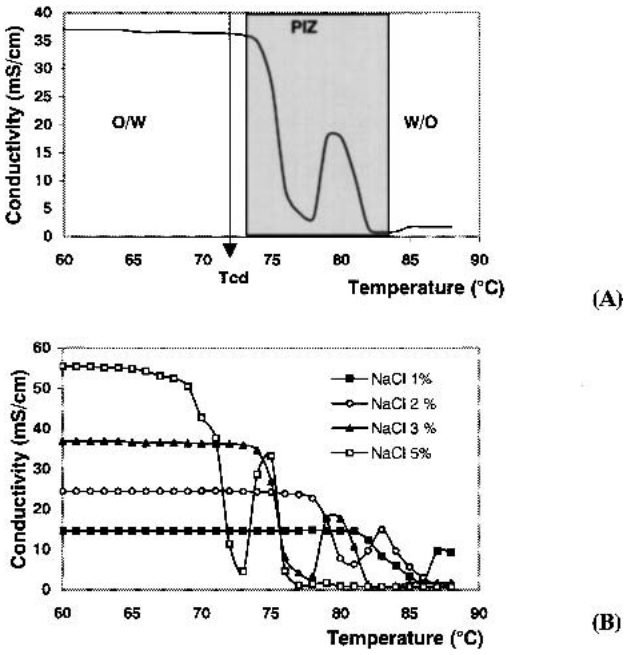

Fig. 1. Evolution of the conductivity as a function of the temperature before cooling-dilution. The amounts of Labrafac and Solutol were 20.6% and 16.9%, respectively. (A) For a sample containing 3% of NaCl, the PIZ (in gray on the graph) is characterized by the evolution of the conductivity from important values to 0 mS/cm via a narrow peak of conductivity. (B) For NaCl proportions between 1 and 5% (w/w), a shift of the PIZs from high to lower values of temperature is observed.

evolution as a function of the temperature. At low temperature, the emulsion led to a high conductivity value (35 mS/cm), which was characteristic of the aqueous continuous phase of an o/w emulsion. While increasing the temperature, a rapid conductivity decrease was observed, and it approached 0 mS/cm at 84°C. This is suggestive of a phase inversion from an o/w to a w/o emulsion. The hydrophilic/lipophilic balance of Solutol was probably changed with temperature, as was the case for the ethoxylated surfactant (7), becoming less hydrophilic on heating. This change took place in the phase inversion zone (PIZ) (gray area in Fig. 1A). In this region, the system appeared translucent with blue glints, which is representative of microemulsions. A conductivity peak was reported in the PIZ, corresponding to a narrow domain where a liquid crystalline lamellar phase should be present (8–10). This increase in conductivity was explained by the presence of a lamellar phase allowing some degree of surface conductivity (11). When the temperature increases (in this particular zone), the oily droplet size decreases from about 50 to 5 μm (data not shown) until we reach the beginning of the PIZ where no dispersed and continuous phases can be observed (microemulsion state). Figure 1B shows clearly that the salinity of the medium can change the thermal range of the PIZ. The PIZ began at 65°C for 5% (w/w) of NaCl compared to 81°C for 1% (w/w). The more that salinity increased, the more conductivity increased (7). Subsequently, when enhancing the NaCl concentration before dilution (from 1 to 5% w/w), the PIZ could be shifted from high to lower temperatures.

When considering the tensiometer results, the rheologic interfacial properties of a Labrafac® drop in a tensioactive solution (Solutol®) showed particular properties at the Labrafac®-water interface. The drop surface was considered here as a model of the bicontinuous organization interfaces. It was characterized, after a sinusoidal volume constraint was applied, by low and closed equilibrium and nonequilibrium elasticities at the same time (Ee = 1.4 ± mN/m; and Ene = 1.1 ± mN/m). The interactions between the surfactant molecules are minimized (low E_e), corresponding to a low interface rigidity, whereas surfactant molecules stayed at the interface after a constraint (low E_{ne}). Usually, a high value of E_{ne} can be hypothesized when E_e is low because of the lowered lateral interactions facilitating the departure of molecules to the surrounding bulk phase. In our case, this mechanical ability to be slightly cohesive, while keeping the initial structure, is interesting because such a system could support its own break (low E_e) without generating a coalescence phenomenon due to the surfactant departure (low E_{ne}). The hydrophilic interactions between the ethylene oxide units of Solutol® at the interface should explain this result. Consequently, such elasticity properties, which are characteristic of this structure, should favor the regular breaking of droplets into nanostructures using a physical shock. Nanoparticles thus can be expected to form.

A formulation process (Fig. 2) based on these results was subsequently developed (12). It involved two steps. Step I resulted in the formulation of a suitable system (i.e., a system that exhibited the previously described elasticity properties). This procedure consisted of the magnetic stirring of all the components, the proportions of which would be defined for each study (i.e., Lipoïd® S75-3, Solutol® HS 15, Labrafac® WL 1349, NaCl, and water), with a rise from room temperature to 85°C at a rate of 4°C/min (Fig. 1). A progressive cooling from 85°C to 60°C at a rate of 4°C/min then was performed. Three temperature cycles (85–60–85–60–85°C) were applied to reach the inversion process. The temperature before dilution then could be determined at the beginning of the inversion process. This temperature was named Tcd and was set at 1–3°C from the beginning of the PIZ in the o/w emulsion (72°C on Fig. 1).

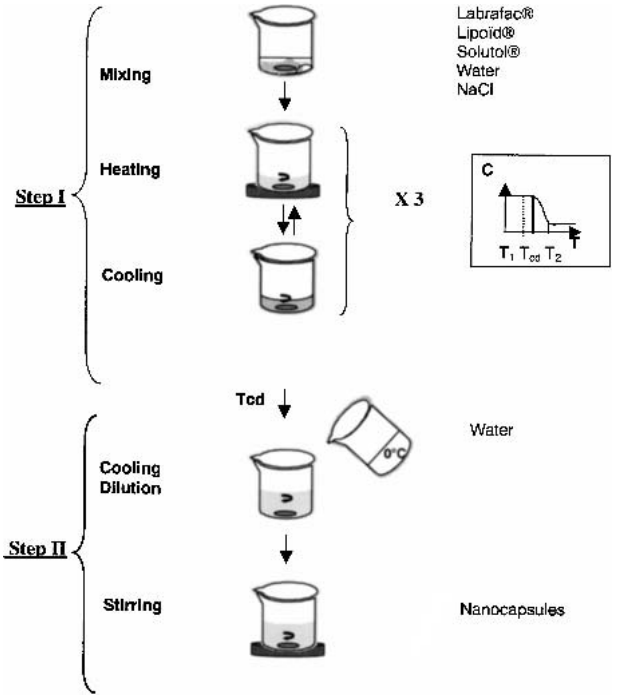

Fig. 2. Formulation process of lipidic nanocapsules. Step I corresponds to the determination of the cooling-dilution temperature (T_{cd}) after applying three temperature cycles to the system. Step II leads to the formation of nanocapsules by the fast addition of cold water.

Step II was an irreversible shock induced by dilution with cold water to the mixture maintained at the previously defined temperature (Tcd). This was done to break the system. This fast cooling-dilution process with cold distilled water (approaching 0°C) led to nanoobjects. Afterward, a slow magnetic stirring was applied to the suspension for 5 min (Fig. 2). The ability to modify the temperature range with salinity (Fig. 1B), and consequently the Tcd, are of prime importance for the encapsulation of thermolabile drugs. Drug degradation was expected to be limited because of the short heating period.

A ternary diagram was established to optimize the constituent proportions (w/w) before the cooling dilution, which could favor the formation of nanoparticular objects. The region was termed the ′Ob′ domain (Fig. 3). The experimental protocol, previously described, was applied for each point of the diagram. We observed that the preparation of optimal nano-objects (i.e., those belonging to the nanometer range, with monodisperse size characteristics, that were stable upon dilution) was strongly dependent on the proportions of compounds. When particles were obtained, the average volume sizes measured by PCS ranged between 20 and 100 nm, and were a function of the formulation composition. Nanoparticles also exhibited good monodispersity ($P < 0.3$). It was not possible to obtain a suitable formulation outside the "Ob"

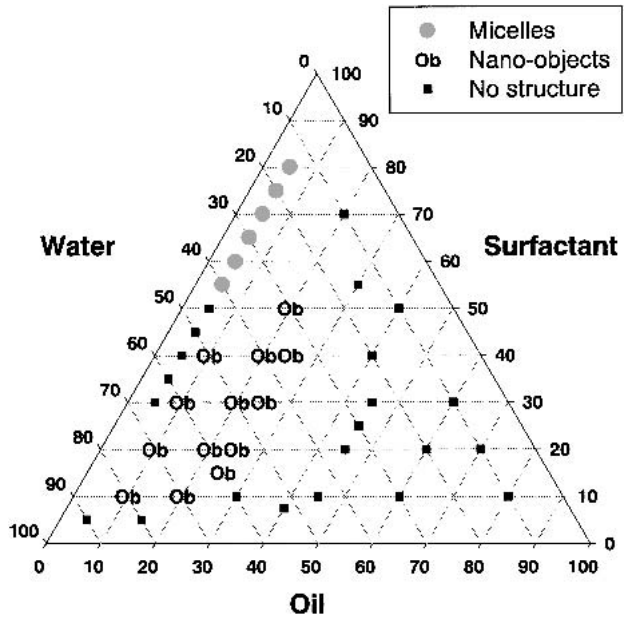

Fig. 3. Ternary diagram allowing the determination of the feasibility zone (Ob domain) of nanocapsules. Amounts between 10% and 40% (w/w) of hydrophilic surfactant, 50% and 80% (w/w) of water, and 10% and 25% (w/w) of oil led to particle formation.

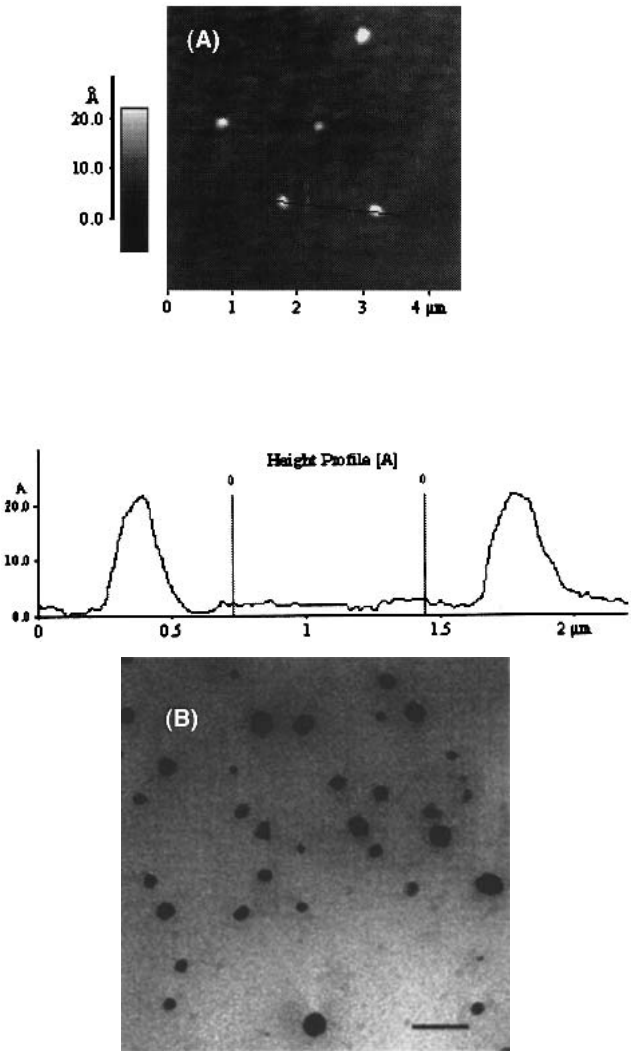

Fig. 4. AFM (A) of nanocapsules deposited and dried on mica plate and observed by the noncontact mode. The particle profile following the black line is represented below. TEM (B) of nanocapsules after they were stained by phosphotungstic acid. The black scale bar represents 200 nm.

domain, because when using a high quantity of hydrophilic surfactant in water, micellar systems were formed (Fig. 3). These micelles were not stable by dilution and were characterized by a particle size of <10 nm. On the other hand, the formed emulsion was not stable (phase separation) for a low hydrophilic surfactant concentration, again preventing the formation of nano-objects. Furthermore, inside the feasibility domain, each formulation was characterized by a wide range of diameters that were reproducible with the same composition. A parallelogram could plot the "Ob" domain (Fig. 3) using the limited data between 10% and 40% (w/w) hydrophilic surfactant, 35% and 80% (w/w) water, and 10% and 25% (w/w) oil.

For example, PCS analysis was performed on a suspension composed of Labrafac, Solutol, and salt water (3% w/w) in relative amounts of 21, 17, and 62% (w/w), respectively (before cooling-dilution). It led to an average PCS size of 51 ± 12 nm. This sample was studied by both TEM and AFM (Fig. 4). Particles with sizes from 20 to 70 nm, as determined by TEM, were observed (Fig. 4B). For AFM, after the spreading of the suspension on a mica plate, the particle shape looked like a cylinder, characterized by a 2-nm height and a 275-nm diameter (equivalent to a volume of about 0.1 μm^3). The lateral diameter differed from those given by PCS (50 ± 10 nm) or TEM (20–70 nm). This particle shape is attributed to the evaporation process (Fig. 4A). However, this volume was analogous to that of a sphere with a diameter of 61 nm and, thus, was similar to the results obtained by PCS and TEM. A nonoptimized contact force for F > 10 nN (data not shown) generated some sample deformations by the tip that were similar to those obtained by Soletti *et al.* (13) on liposomes. But, they did not fuse together, compared to what was observed on AFM images of liposomes that were spread on aminopropylsilane mica. These findings clearly demonstrated the fact that particles possessed a stability that was sufficient to avoid any fusion. Furthermore, even after a long time of contact with mica (i.e., >48 h), nanoparticles did not form a layer on the surface like that of liposomes (14). This result can be correlated to a sufficient rigidity, limiting their destructuration and coalescence on mica. As a consequence, the rigid property of the nano-object surface seemed directly inherent to the surfactant shell structure.

The DSC study on freeze-dried particles provided some answers about how compounds interacted in nanoparticles (Table 1). When Labrafac® was individually studied, its phase transition appeared at –7.5°C with an enthalpy of –64.6 J/g. Solutol® presented two peaks at 16.6°C and 27.9°C with enthalpy values of –46.4 and –11.3 J/g, respectively. Upon simple mixing of Solutol® and Labrafac®, the disappearance of the Solutol® peak at 16.6°C was observed because of the likely association of the main lipophilic components of Solutol® with Labrafac®. In the particle sample, the two peaks of Solutol® were still present, as if the compound was considered to be alone. Solutol® behavior was probably not affected by the

Table I. Enthalpies and Phase Transition Temperatures of Labrafac®, Lipoïd® S75-3, and Solutol®[a]

Samples	Labrafac®		Solutol®		Lipoïd®	
	Temperature (°C)	Enthalpy (J/g)	Temperature (°C)	Enthalpy (J/g)	Temperature (°C)	Enthalpy (J/g)
Labrafac®	–7.5	–64.6				
Lipoïd®					75.1	47.3
Solutol®			16.6	–46.4		
			27.9	–11.3		
Labrafac® + Solutol®	–7.3	–29.5	20.3	–21.9		
Lipoïd® + Labrafac®	–7.2	77.9			74.3	45.3
Nanocapsules	–7.8	–49.9	17.2	–42.5	69.0	45.0
			29.3	–10.7		

[a] Nanocapsules were composed by Labrafac®, Solutol®, and salt water in relative amounts, before the dilution step, of 25%, 15%, and 60% (w/w), respectively.

presence of Labrafac®. Consequently, the Solutol® molecules likely were oriented to the external aqueous phase. On the other hand, the correlation between the average diameter of the particle and the oil (Labrafac®) proportion in the formulation has been clearly established (data not shown). Furthermore, the fusion enthalpy of Labrafac® (proportional to its free amount in nanoparticles) increased with its proportion in particles to become close to its normal value without a modification of the fusion temperature. Thus, the liquid state of Labrafac® was supposedly maintained after encapsulation. All these results concurred to define a nanocapsule structure as described in Fig. 5. The decrease of Lipoid® enthalpy in the nano-objects and in the presence of pure Labrafac® indicated an interaction between these two components. Inside the objects, Lipoïd® was obviously in contact with Labrafac®. Therefore, it could be concluded that the system was constituted by an oily liquid core corresponding to free Labrafac®, that was surrounded by a tensioactive rigid shell that was made from a mixture of Lipoïd® and Solutol®. The first component (Lipoid®) was anchored in the oily phase, whereas the second component (Solutol®) was oriented toward the water phase. This is clearly illustrated in Fig. 5.

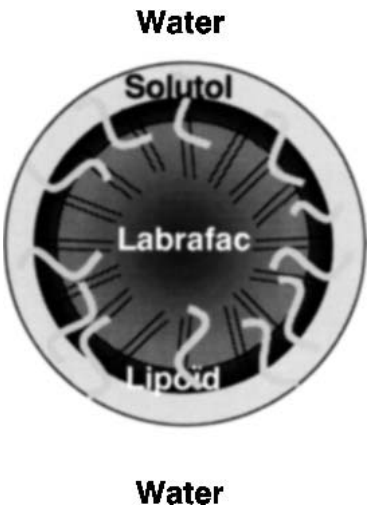

Fig. 5. Schematic representation of capsules formed by a tensioactive shell composed by the association of hydroxystearate of poly(ethylene glycol) and phosphatidylcholine protecting an oily core (medium chain triglycerides).

These new carriers showed an improved stability with time, in the same range as that of lipidic nanoparticles described in the literature (15). The sample stored at 4°C was perfectly stable for at least 18 months. At 37°C, the same sample was stable for 1.5 months. Freeze-drying assays then were performed. Nevertheless, in the absence of cryoprotectants and after rehydration, the sample became polydispersed ($P = 0.600$) as a result of the aggregation of particles.

More suitable conditions for sample characteristic preservation were observed when cryoprotectants were used. In the presence of mannitol, the polydispersity was still higher than 0.3 ($P = 0.350$). With glucose, an important increase of size occurred (30–60 nm). Trehalose seemed to be the best additive because of a limited increase in size (30–42 nm), with the preservation of the monodisperse characteristics. Furthermore, concentrations of trehalose higher than 5% (w/w) did not improve the stability of the nanocapsules (results not shown). These results were in complete agreement with other studies on liposomes (16) and solid lipid nanoparticles (17,18) lyophilization using trehalose successfully.

CONCLUSION

In this study, we have shown that the transformation of an o/w to a w/o emulsion, via a PIZ, was brought about by mechanical properties that could be described by an interfacial rheologic technique (i.e., drop tensiometry). Such properties led to the development of a new, solvent-free, lipidic nanoparticulate formulation. A fast cooling and dilution of the initial mixture in a state close to the beginning of the PIZ resulted in the formulation of novel lipidic systems in a nanometer-size range. Their structure was found to consist of an oily liquid triglyceride core surrounded by a tensioactive cohesive interface (i.e., a nanocapsule system). With this new preparation method, no heavy material was necessary, and small particles were formed whatever the process parameters. These particles represent an alternative system to polymeric nanoparticles, emulsions, liposomes, and current lipid nanoparticles.

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REFERENCES

1. J. P. Benoit, H. Marchais, H. Rolland, and V. Vande Velde. Biodegradable microspheres: Advances in production technology. In S. Benita (ed.), *Microencapsulation: Methods And Industrial Applications*, Marcel Dekker Inc., New-York, 1996 pp. 35–72.
2. M. Boisdron-Celle, P. Menei, and J. P. Benoit. Preparation and characterization of 5-fluorouracil-loaded microparticles as biodegradable anticancer drug carriers. *J. Pharm. Pharmacol.* **47**:108–114 (1995).
3. W. Mehnert and K. Mader. Solid lipid nanoparticles: production, characterization and applications. *Adv. Drug Deliv. Rev.* **47**:165–196 (2001).
4. M. R. Gasco. Solid lipid nanospheres from warm microemulsions. *Pharm. Technol. Eur.* **9**:52–59 (1997).
5. C. Witschi and E. Doelker. Residual solvents in pharmaceutical products: Acceptable limits, influences on physicochemical properties, analytical methods and documented values. *Eur. J. Pharm. Biopharm.* **43**:215–242 (1994).
6. P. Saulnier, F. Boury, A. Malzert, B. Heurtault, T. Ivanova, A. Cagna, I. Panaïotov, and J. E. Proust. Rheological model for the study of dilational properties of monolayers: Comportment of dipalmitoylphosphatidylcholine (DPPC) at the dichloromethane/water interface under ramp type or sinusoidal perturbations. *Langmuir* **17**:8104–8111 (2001).
7. D. J. Miller, T. Henning, and W. Grünbein. Phase inversion of w/o emulsions by adding hydrophilic surfactant: A technique for making cosmetics products. *Colloids Surf.* **183–185**:681–688 (2001).
8. A. J. Sing. Thèse pour le grade de docteur en physique, University of Pau, France, 1997.
9. T. Förster, F. Schambil, and H. Tesmann. Emulsification by the phase inversion temperature method: The role of self-bodying agents and the influence of oil polarity. *Int. J. Cosmet. Sci.* **12**: 217–227 (1990).
10. T. Förster, F. Schambil, and W. von Rybinski. Production of fine disperse and long-term stable oil-in-water emulsions by the phase inversion temperature method. *J. Dispers. Sci. Technol.* **13**:183–193 (1992).
11. K. Shinoda and H. Takeda. Effect of added salts in water on the hydrophile-lipophile balance of non-ionic surfactants: The effect of added salts on the phase inversion temperature of emulsions. *J. Colloid Interface Sci.* **32**:647–651 (1970).
12. B. Heurtault, P. Saulnier, B. Pech, J. E. Proust, J. Richard, and J. P. Benoit. Nanocapsules lipidiques, procédé de préparation et utilisation comme médicament, Patent No. 0002688000, 2000.
13. J. M. Solletti, M. Botreau, F. Sommer, W. L. Brunat, S. Kasas, T. M. Duc, and M. R. Celio. Elaboration and characterization of phospholipid Langmuir-Blodgett films. *Langmuir* **12**:5379–5386 (1996).
14. N. H. Thomson, I. Collin, M. C. Davies, K. Palin, D. Parkins, C. J. Roberts, S. J. B. Tendler, and P. M. Williams. Atomic force microscopy of cationic liposomes. *Langmuir* **16**:4813–4818 (2000).
15. C. Freitas, W. Mehnert, and R. H. Müller. Effect of storage conditions on long-term stability of solid lipid nanoparticles (SLN) in aqueous dispersion. *Eur. J. Pharm. Sci.* **2**:177 (1994).
16. L. M. Crowe, J. H. Crowe, R. Rudolph, C. Womersley, and L. Appel. Preservation of freeze-dried liposomes by trehalose. *Arch. Biochem. Biophys.* **242**:240–247 (1985).
17. R. Cavalli, O. Caputo, M. E. Carlotti, M. Trotta, C. Scarnecchia, and M. R. Gasco. Sterilization and freeze-drying of drug-free and drug-loaded solid lipid nanoparticles. *Int. J. Pharm.* **148**:47–54 (1997).
18. C. Schwarz and W. Mehnert. Freeze-drying of drug-free and drug-loaded solid lipid nanoparticles (SLN). *Int. J. Pharm.* **157**: 171–179 (1997).