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Reverse micelle-loaded lipid nano-emulsions: New technology for nano-encapsulation of hydrophilic materials

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ABSTRACT

This study presents novel, recently patented technology for encapsulating hydrophilic species in lipid nano-emulsions. The method is based on the phase-inversion temperature method (the so-called PIT method), which follows a low-energy and solvent-free process. The nano-emulsions formed are stable for months, and exhibit droplet sizes ranging from 10 to 200 nm. Hydrophilic model molecules of fluorescein sodium salt are encapsulated in the oily core of these nano-emulsion droplets through their solubilisation in the reverse micellar system. As a result, original, multi-scaled nano-objects are generated with a 'hydrophilic molecule in a reverse-micelles-in-oil-in-water' structure. Once fluorescein has been encapsulated it remains stable, for thermodynamic reasons, and the encapsulation yields can reach 90%. The reason why such complex objects can be formed is due to the *soft method* used (PIT method) which allows the conservation of the structure of the reverse micelles throughout the formulation process, up to their entrapment in the nano-emulsion droplets. In this study, we focus the investigation on the process itself, revealing its potential and limits. Since the formulation of nanocarriers for the encapsulation of hydrophilic substances still remains a challenge, this study may constitute a significant advance in this field.

1. Introduction

Over the last few decades, research focused on the 'low-energy' processes generating nano-emulsions has seen a real explosion. This is due (Salager et al., 2004; Tadros et al., 2004; Solans et al., 2005) to both the great potential of such technology regarding industrial transposition and their large number of applications in fields such as nanomedicine, drug delivery, cosmetics, etc. (Anton et al., 2008). Nano-emulsions are defined as nanometric-scaled emulsions (up to 300 nm) and constitute an extremely stable dispersion since their submicron droplet sizes prevent droplet flocculation (Tadros et al., 2004). Moreover, the destabilisation process of nano-emulsions is only attributed to Ostwald ripening (Taylor, 1998; Tadros et al., 2004), which explains their stability for months in solution, and their stability against dilution and temperature changes. It is important to note here that these systems are fundamentally different from microemulsions, since they are kinetically metastable.

Nano-emulsion generating processes are divided into two groups (Solans et al., 2005; Anton et al., 2008). The first gathers together the 'high-energy' processes which use high mechanical shear to reach the very small droplet sizes, whereas the second 'low-energy' process benefits from the intrinsic physico-chemical properties of surfactants for generating nano-emulsions. These low-energy methods have aroused considerable interest, insofar as they involve a very low amount of energy for the same results. Furthermore, these soft methods help prevent the potential degradation of encapsulated drugs during processing. Droplet sizes are simply controlled by the system composition (Anton et al., 2007a). Among these low-energy methods we can distinguish (i) the spontaneous nano-emulsification method (Bouchemal et al., 2004; Anton et al., 2008), and (ii) the phase-inversion temperature method (PIT method) (Forster et al., 1995; Morales et al., 2003; Anton et al., 2008). We have recently shown (Anton and Vandamme, 2009) that these two methods are very closed systems and that they obey a universal formulation process. However, in this article we focus on the PIT

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method, which uses polyethoxylated non-ionic surfactants for their particular properties which allow their solubility in water and oil, this being controlled by temperature. The resulting nano-emulsions are stable, solvent-free, polymer-free, and compatible with parenteral administration when formulated with suitable pharmaceutical excipients (Lamprecht et al., 2002; Heurtault et al., 2002; Lamprecht and Benoit, 2006). Furthermore, these colloidal nano-systems formulated with pegylated surfactants generally include stealth properties as far as the immune system is concerned (Béduneau et al., 2006).

However, the PIT method has only been shown to produce *direct* (e.g. oil-in-water) nano-emulsions, and thus are only able to encapsulate hydrophobic molecules. Besides the numerous advantages presented above, this can constitute one important drawback, since numerous therapeutic principles are hydrophilic, e.g. nucleic acids, proteins and some anti-cancer molecules (doxorubicine, gemcitabine). The purpose of this work is to adapt this low-energy, phase-inversion process in order to allow the encapsulation of hydrophilic species into these lipid nano-emulsions. This strategy (patent pending (Saulnier et al., 2008)) involves the solubilisation of hydrophilic molecules (in this case, a model, fluorescent dye) in oil, through a stable, reverse-micellar system. Given that to date only a few examples have been reported in this field, and that these have involved the extensive use of polymers, organic solvents or high-energy methods (Lambert et al., 2000a,b; Hillaireau et al., 2006; Perez et al., 2001; Ruysschaert et al., 2006; Gomes et al., 2006; Almeida and Souto, 2007), the nano-encapsulation of hydrophilic materials is actually still a real challenge, and this unprecedented study may propose a simple and efficient solution.

2. Materials and methods

2.1. Materials

Non-ionic, polyethoxylated surfactant, Solutol HS 15®, is polyoxyethylene-660-12-hydroxy stearate, kindly supplied by BASF (Ludwigshafen, Germany). This surfactant is of technical grade and is a mixture of different oligomers of molecular weights based on the those announced by the manufacturer (in most cases (Salager et al., 2004)), say around 960 g mol^{-1}. Oil phase, Labrafac CC®, is composed of medium-chain triglycerides (caprilic triglycerides), and was provided by Gattefoss (Saint-Priest, France). Ultrapure water was obtained by the MilliQ® filtration system (Millipore, Saint-Quentin-en-Yvelines, France). Fluorescein sodium salt, sodium chloride and Span 80®, were purchased from Sigma (Saint-Louis, USA). Bio-Gel P6®, used for size exclusion chromatography, was obtained from Bio-Rad (Marnes-la-Coquette, France).

2.2. Methods

2.2.1. Reverse micelles-loaded lipid nano-emulsions by the PIT method

Two representative formulations have been selected which typically exhibit different ranges of droplet sizes. In the first case (designated formulation A), the surfactant amount within the nano-emulsion is fixed at 5.6 wt.%, whereas in the second case (designated formulation B), it is increased to 13.0 wt.%. The water/oil weight ratio, defined as $\mathrm{WOR} = 100 \times w_{\mathrm{water}}/(w_{\mathrm{water}} + w_{\mathrm{oil}})$ in both cases is fixed at 70.0%, and the NaCl at 1.8 wt.%. The respective role of the different components on the nano-emulsion formulation is discussed in the literature (Béduneau et al., 2006; Anton et al., 2007a,b, 2008). The dilution is performed with a water volume of three-times the volume of the ternary system.

On the other hand, above the critical micellisation concentration in oil (cmc), thermodynamically stable, reverse micelles were formed with lipophilic surfactants (such as the Span 80 that we used). Such micelles are able to solubilise a given amount of hydrophilic drug. In this study, fluorescein salt was chosen as a model and labile fluorescent dye. The fluorescein-loaded micellar suspension is previously prepared in oil simply by incubating an empty micellar suspension with excess crystalline fluorescein sodium salt until micelle saturation (2 h). Next, a quick centrifugation of the sample is performed in order to eliminate the excess solid fluorescein salt remaining in suspension in the oil. Afterwards, a selected volume of this fluorescent oil (the values are given in Section 3) is added under weak, magnetic stirring to the formulation at ~5 °C above the PIT.

To summarise, surfactant, water, NaCl, and oil are mixed at the proportions noted above, which are: formulation A: $w_{\mathrm{surfactant}} = 1.12$ g, $w^{\mathrm{A}}_{\mathrm{NaCl}} = 0.36$ g, $w^{\mathrm{A}}_{\mathrm{water}} = 12.96$ g, $w^{\mathrm{A}}_{\mathrm{oil}} = 5.56$ g; formulation B: $w_{\mathrm{surfactant}} = 2.60$ g, $w^{\mathrm{A}}_{\mathrm{NaCl}} = 0.36$ g, $w^{\mathrm{A}}_{\mathrm{water}} = 11.93$ g, $w^{\mathrm{A}}_{\mathrm{oil}} = 5.11$ g. Next, the temperature is gradually increased to ~85 °C (the PIT was determined by conductimetry at around ~8 °C, by following the electrical conductivity of the emulsion as a function of the temperature; a method we have described in detail elsewhere (Anton et al., 2007a,b)). The reverse micelle-loaded oil is injected into the warm system (for which the volume depends upon the experiment chosen), and the micelle-loaded oil is homogeneously incorporated in the bulk oil phase (since $T >$ PIT) in a few seconds. Finally, a sudden dilution with 40 g of water at room temperature is performed, resulting in the generation of highly, monodispersed, fluorescein-loaded nano-emulsions smaller than 100 nm.

2.2.2. Fluorescence characterisation

Once the loaded nano-emulsion has been generated, the free and encapsulated fluorescein molecules are separated by size exclusion chromatography (Bio-Gel P6, with hydrated beads from 90 to 180 μm); the deposited volume is 50 μL. Elution fractions are then set up in the 96-well plate to be analysed by spectrofluorimetry (Fluoroskan Ascent, Saint-Herblain, France). The excitation and emission light passes through band-pass filters at 485 ± 14 and 542 ± 13 nm, respectively. Moreover, fluorescent excitation and emission maxima in water and in micellar suspension in oil, are separately and precisely determined by spectrofluorimetry (Aminco Bowman Series 2 spectrofluorimeter). They are: $\lambda^{\mathrm{water}}_{\mathrm{exc.}} = 488$ nm, $\lambda^{\mathrm{water}}_{\mathrm{em.}} = 514$ nm, $\lambda^{\mathrm{oil}}_{\mathrm{exc.}} = 493$ nm and $\lambda^{\mathrm{oil}}_{\mathrm{em.}} = 520$ nm.

In this way, encapsulated and free fluorescein are physically and suitably separated, their respective quantification being possible by analysing each peak separately (with its respective quantification curve). The fluorescence behaviour of fluorescein depends on the solubilising medium, and appears rather different between water and oil. Thus, the two calibration curves for these two solubilising media are established and reported in Fig. 1.

Fluorescence appears weakly inhibited in oil; the comparison between the two signals can thus be made by using the calibration curves slope ratio, given by Fig. 1 at 7.09.

The stability of the fluorescein encapsulation, i.e. the follow-up of a potential fluorescein leakage with time was assessed as follows: the representative formulation A was chosen and set up in a thermostatted vessel at 37 °C under weak magnetic stirring (500 rpm). At regular time intervals, encapsulation yields of aliquots were evaluated, separating free and encapsulated fluorescein as carried out above.

2.2.3. Dynamic light scattering

The hydrodynamic diameter and polydispersity index (PDI) of the nano-emulsions were measured by photon-correlation spectroscopy using a Nano ZS apparatus, Malvern Instrument (Orsay, France). The Helium–Neon laser was operated at 4 mW, 633 nm, at

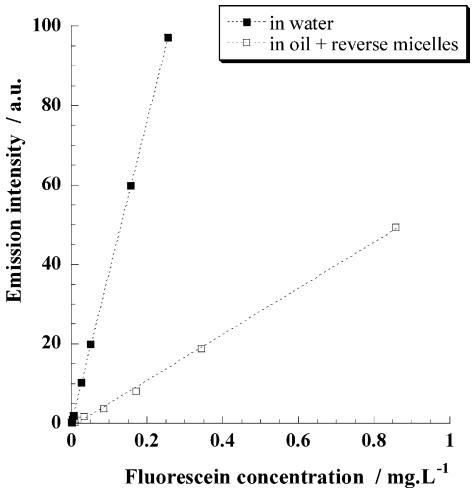

Fig. 1. Calibration curves of fluorescein solubilised in water and in the reverse micellar suspension in oil.

a scatter angle fixed at 173°. The temperature was maintained at 25 °C. A polydispersity index is a mathematical definition accounting for the relative error between curve fit and experimental values. It discloses the quality of the dispersion: overall, values of ≤0.1 reflect suitable measurements and good monodispersity of the nano-emulsions. All measurements were performed in the standard suitable conditions to validate the results, in terms of sample dilution and at constant temperature. Likewise, the apparatus systematically and automatically adapts to the sample, both the intensity of the laser and the attenuator of the photomultiplier (diaphragm). This ensures the reproducibility of the experimental measurement conditions.

3. Results and discussion

3.1. Nano-emulsion formulation

The phase-inversion, temperature method (PIT method) is recognised as being a very efficient and easy way for generating monodispersed nano-emulsions (Salager et al., 2004; Tadros et al., 2004; Solans et al., 2005; Anton et al., 2008). The process is based on the fact that non-ionic surfactant affinities for the two non-miscible phases (partitioning coefficient), are simply controlled by the temperature. As a result, for the lower temperatures, amphiphiles are water-soluble and stabilise oil-in-water (o/w) macro-emulsions, and on the other hand, for higher temperatures, the surfactants become oil-soluble and stabilise water-in-oil (w/o) macro-emulsions. We recently showed (Anton and Vandamme, 2009) that the key point of the process lies in the fast migration of the amphiphiles, from the oily to the aqueous phase, following the sudden temperature change. In other words, when the system is initially at a temperature higher than the PIT (T > PIT), and when it is suddenly diluted with water at room temperature (i.e. at a temperature significantly lower than the PIT), this dilution will induce a very fast change of surfactant solubility (being lipophilic at high temperatures and becoming hydrophilic), which finally results in their sudden displacement towards the aqueous phase. A highly monodispersed o/w nano-emulsion is immediately generated.

Nano-emulsions are kinetically stable, oil droplets dispersed in water, and reverse micelles are thermodynamically stable systems. Thus, by combining both forms, a kinetically stable 'reverse-micelles-in-oil-droplets-in-water' nano-emulsion is obtained. The whole formulation process is summarised in Fig. 2, and finally shows that the fluorescein molecules should be spread in the lipid droplets in a very homogeneous way.

In this way, the amount of reverse micelle-loaded added oil has two direct impacts: (i) The first one is on the formulation mechanism itself, and (ii) the second one is on the fluorescein quantity and encapsulation. These two points will be looked at in detail below by studying the influence of this formulation parameter defined as 'relative oil volume' (which refers to the relative proportions between the initial and reverse micelle-loaded oil volumes).

The first result we present regards the impact of the added oil volume on droplet formulation, in particular their size and polydispersity. These results are summarised in Fig. 3 for the formulations A and B, and clearly show an increase of the sizes along with the increase of oil volume.

Each point is labelled by the average PDI value, and thus indicates that whatever the volume of added florescein-loaded oil, the resulting nano-emulsions keep a very narrow monodispersity value. This latter point shows that this oil injection step does not affect the nano-droplet formation mechanism. The important fact highlighted in Fig. 3 is that the PDI values remain very low even if the injected oil amount is increased: this means that all the added oil participates in the formation of the nano-droplets, and thus is homogeneously spread into these nano-emulsion droplets (in the opposite case, the PDI should dramatically be increased). The gradual growth of the nano-droplets formed actually fits a behaviour we had previously observed with low-energy, nano-emulsification (Anton and Vandamme, 2009), for which, as is the case here, the nano-emulsion droplet diameters increase as the sur-

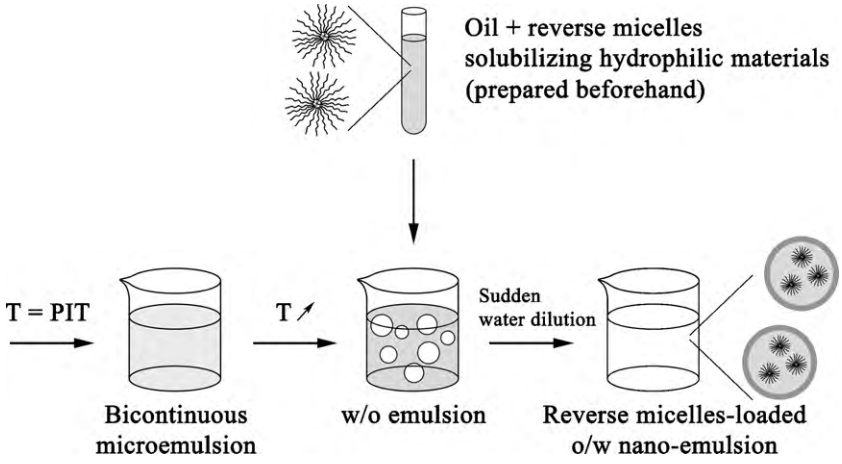

Fig. 2. Diagram of the formulation of hydrophilic materials, reverse micelle-loaded w/o nano-emulsions by the PIT method.

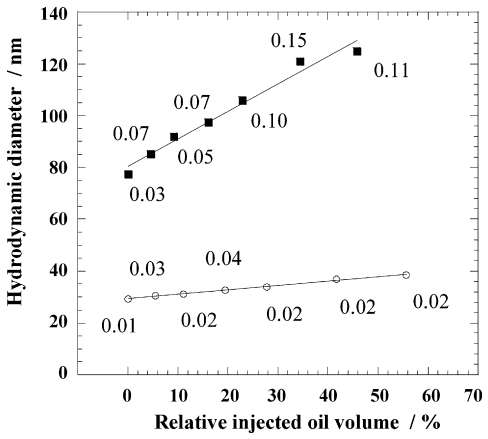

Fig. 3. Nano-emulsion droplet diameters in function of the injected oil volume, expressed as a volume relative to the initial volume. The points are labeled by the average polydispersity indices for each measurement. The filled squares show data for formulation A and the open circles the data for formulation B.

factant/oil weight ratio decreases. All these results on both sizes and PDI values indicate that, even if the loaded oil is added to the ternary mixture before dilution, the droplet formation mechanism is not affected, and follows the known mechanisms of low-energy nano-emulsification. It therefore follows that the reverse micelles should be homogeneously spread into the droplet population. As a final remark, we also observed a difference in the straight line slopes between the two systems A and B. It still remains coherent compared to the literature (Anton and Vandamme, 2009) owing to their differences in surfactant quantities, respectively 5.6 and 13.0%.

3.2. Fluorescein sodium salt encapsulation

Fluorescein sodium salt was encapsulated in these multi-scaled, nano-objects, within the two formulations A and B. Their compositions were carefully chosen to strictly contain the same fluorescein concentration, i.e. the relative oil volume was fixed at 23%. The encapsulation yields were obtained by size-exclusion chromatography, after which the fluorescence-emission intensity was plotted against the elution volume, as reported in Fig. 4.

The first peak shows the passage of the nano-emulsion droplets, and the narrower, second one is the free fluorescein in water. In order to compare the amount of encapsulated fluorescein with the free one, the fluorescence emission was corrected according to the ratio between the slopes of the calibration curves (see Fig. 1, and noted as *comparable data* in Fig. 4). The encapsulation yields are found as 81.1 and 90.0% for the formulations A and B, respectively.

These results show without doubt that the fluorescein, being a low, molecular-weight hydrophilic substance, is encapsulated into the oily medium of the nano-emulsion droplets. This result occurs due to the low-energy emulsification process which actually preserves the multi-scale structure, i.e. encapsulating the thermodynamically stable micelles into a kinetically stable emulsion. On the other hand, the non-aggressive formulation method not only prevents the potential degradation of fragile molecules during processing (Anton et al., 2007a,b, 2008; Anton and Vandamme, 2009), but also prevents destruction of the internal micellar assemblies. A fluorescein leakage towards the aqueous phase still exists however, and gives rise to encapsulation yields lower than 100%, but, since these initial encapsulation yields remain stable with time (which will be presented below), this fluorescein loss in the aqueous phase was only attributed to its leakage during the formulation process.

Besides this first important result of the capability of encapsulating hydrophilic substances into lipid nano-droplets, the study

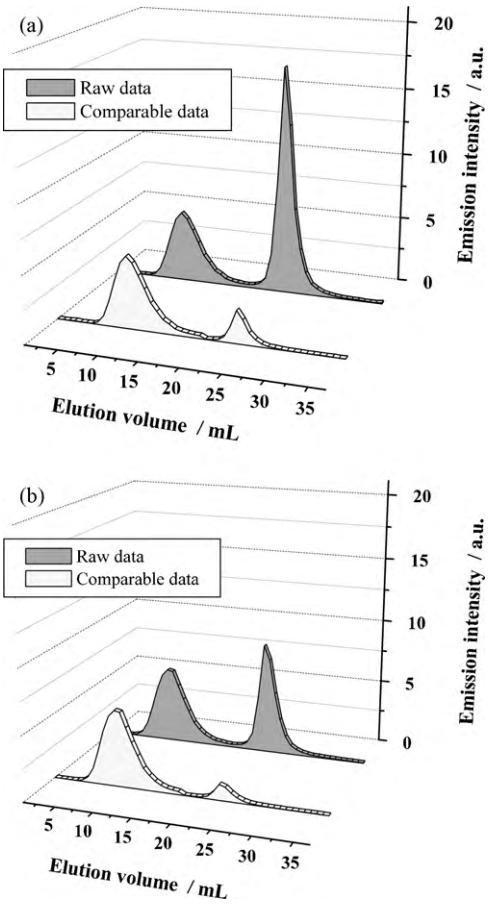

Fig. 4. Experimental values of the emission fluorescence intensity as a function of the size exclusion chromatography elution volume. The deposited volume of nano-emulsion is 50 μL. (a) Formulation A, WOR = 70, surfactant amount = 5.6 wt.%, relative oil volume = 23%. (b) Formulation B, WOR = 70, surfactant amount = 13.0 wt%, relative oil volume = 23%. 'Raw data' refer to the raw fluorescence emission, and 'comparable data' take into account the fluorescence inhibition in oil (see Section 2.2.2 for details).

also investigates the influence of the formulation parameters on the nano-emulsion properties and encapsulation yields. A first series of experiments reported in Fig. 5 regarded the impact of the fluorescein concentration dispersed in oil (micelles plus oil)

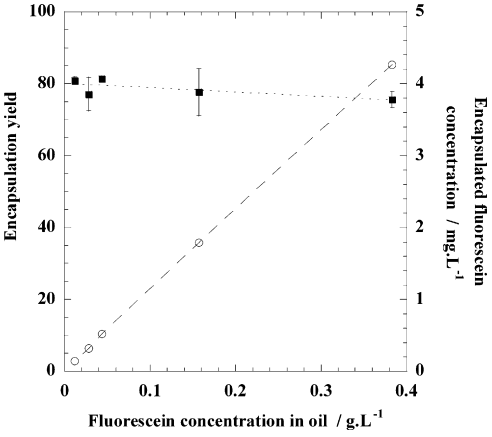

Fig. 5. Encapsulation yields (filled squares) and encapsulated fluorescein concentration (open circles) as a function of the concentration of fluorescein solubilised in oil (injected in the formulation). Experiments done for the formulation A, WOR = 70, surfactant amount = 5.6 wt.%, for a relative injected oil volume of 23% of the initial oil volume.

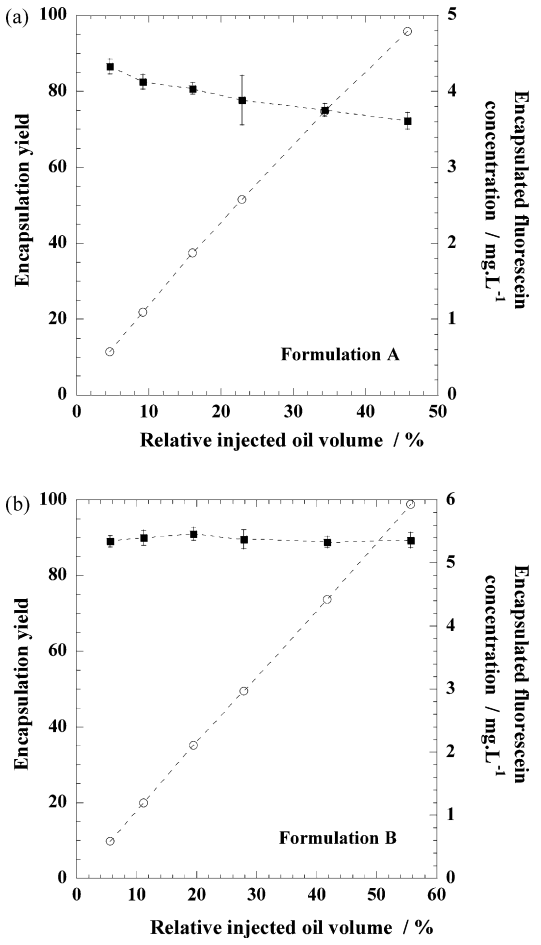

Fig. 6. Encapsulation yields (filled squares) and encapsulated fluorescein concentration (open circles) in function of the injected oil volume, expressed as relative volume respective to the initial volume. (a) Formulation A and (b) formulation B, and in both cases fluorescein concentration in oil was fixed at 0.16 g L^{-1}.

on both the encapsulation yield and the fluorescein concentration encapsulated into the resulting nano-emulsion droplets. Different representative fluorescein concentrations in oil were obtained by playing on the Span 80 flexibility, with the upper limit attained at 0.38 g L^{-1}.

It appears that the encapsulation yields remain unchanged, whatever the quantity of fluorescein. Accordingly, the encapsulation concentration (defined by {total fluorescein concentration} × {encapsulation yield}) increases in a linear fashion, up to the limit of fluorescein saturation in reverse micelles. In addition, the sizes of the generated droplets remain very similar (data not shown), being equal to those presented in the same case in Fig. 3.

It therefore follows that increasing the fluorescein concentration does not impact (i) on the formulation process, i.e. on the nano-emulsion properties (droplet size, morphology), as well as (ii) on their ability to maintain the hydrophilic molecules in oil, keeping constant the encapsulation yields along an increasing concentration of encapsulated florescein.

The following results, presented in Fig. 6, regard the influence of the reverse micelle-loaded oil amount introduced in the formulation on both the encapsulation yield and the concentration of encapsulated fluorescein. The fluorescein quantities in the formulations, as well as the droplet sizes, increase along with the volume of oil added (see Fig. 3). This time, the dye concentration in reverse micelle remains constant and the volume of oil added in the formulation is gradually increased between the different experiments. Fig. 6(a) and (b) respectively refer to the formulations A and B.

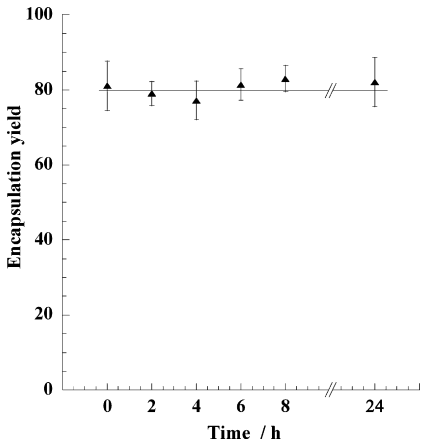

Fig. 7. Stability study performed on the sample of Fig. 4(a), formulation A, WOR = 70 and surfactant amount = 5.6 wt.%. The temperature is maintained constant in a vessel at 37 °C, under constant magnetic stirring.

In both cases, the encapsulated fluorescein concentration increases with the loaded-oil quantity, each following a very similar, linear trend. In addition, the encapsulation yield for the two formulations still exhibits significantly high values, showing that their properties are conserved with the gradual modification of the process. However, whereas the smaller, nano-emulsion particles of formulation B keep an encapsulation yield strictly constant at around 90% with the injected oil, the larger droplets of formulation A show a gradual yield decrease from 86% to values as low as 73%. This result, along with the fact that between the two formulations the surfactant amount also differs (since they induce the differences in size), shows the complexity of the phenomena involved in this nano-emulsion-generating process. Overall, the trends highlighted in Figs. 5 and 6 reveal the extent of the formulation process developed by studying the possible range values and limits of the formulation variables.

A final important point regards the stability of the nanocarriers; besides the stability of the oil nano-droplets, which are by definition stable for months, we have also followed the potential release of the encapsulated fluorescent dye. The results within 24 h are reported in Fig. 7, showing a finite stability without any fluorescein leakage, which was confirmed with additional measurements at one, two and three months (kept in a thermostatted vessel) with no further change.

This result is quite surprising, and could be related to the affinity of the fluorescein with its micellar, oily, solubilising medium. Actually, its solubilisation corresponds to a thermodynamic equilibrium state, which can be maintained once formulated in nano-emulsion, if the micellar structure stays stable; this could constitute the more plausible explanation. Finally, the potential of this new technology is even more interesting in that, thanks to this relevant stability, the high encapsulation efficiencies of hydrophilic species will be conserved, bringing an optimised molecule amount to the therapeutic target.

4. Conclusion

This study proposes an original process for encapsulating hydrophilic materials (fluorescein sodium salt) into lipid nano-droplets. The incorporation of water-soluble compounds in oil is possible thanks to a reverse-micellar system. The lipid, nano-emulsion formulation containing this loaded oil is achieved following the low-energy, phase-inversion temperature method. This soft method actually plays a fundamental role in preventing the destruction of micellar assemblies during processing, and

thus ensures encapsulation of the dye. Throughout this study, the impact of formulation parameters on the resulting nanocarrier size distributions, polydispersity, encapsulation yield and stability were investigated, and allowed us to understand the potential and limits of such new technology. These particles appear to be very promising systems, able to fulfill numerous specifications in fields including drug delivery and controlled release. The low-energy processes involved make this technology compatible with an easy industrial scaling-up process. The nanoparticle structure proposed also enables the simultaneous encapsulation of hydrophilic and lipophilic compounds in the same nanocarrier. Even if this study presents the encapsulation of a model, hydrophilic dye, this technology has also been shown to be efficient for the encapsulation of various, low molecular-weight molecules such as other dyes (e.g. methylene blue), bioactive molecules such as anticancer drugs (e.g. doxorubicin chlorhydrate), or larger molecules such as oligonucleotides. These alternatives will be presented in further studies using this technology. To conclude, this study should open the door to the advantages of the phase-inversion processes to generate nano-emulsions for the nano-encapsulation of hydrophilic molecules, involving numerous potential applications.

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