Published in: Journal of colloid and interface science (1995), vol. 173, iss. 1, pp. 55-65
Status: Postprint (Author's version)

Polylactide Microparticles Prepared by Double Emulsion-Evaporation

II. Effect of the Poly(Lactide-co-Glycolide) Composition on the Stability of the Primary and Secondary Emulsions

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Abstract

Polylactide and copolymers with glycolide have been used as coating polymers in the microencapsulation technique based on the $W_1/o/w_2$ double emulsion-evaporation method. Stability of the primary emulsion is of critical importance and clearly predetermines the internal morphology of the microspheres, in agreement with the very fast hardening of the oil phase (CH_2Cl_2) as soon as the primary emulsion is dispersed in water. When the peptide or' protein to be encapsulated is able to interact with the coating polymer and possibly with the surfactant, the stabilization mechanism of the primary emulsion is very complex. It has been shown that the interaction of BSA (bovine serum albumin) with the polyester is as strong as the glycolide content is high, which results in the formation of a solid film at the w_1/o interface and in a high emulsion stability. Addition of a surfactant, such as poloxamer 188, in the oil phase has a deleterious effect on both the emulsion stability and the internal structure of the microspheres. Use of a dye tracer in the internal aqueous phase was not useful in probing the double emulsion stability and the encapsulation efficiency, because of specific interactions between the dye (indigocarmine) and the coating polyester.

Key Words
microencapsulation; double emulsion-evaporation technique; controlled drug delivery; poly(lactide-co-glycolide) microsphere morphology; bovine serum albumin-polylactide interactions.

Introduction

Nowadays, a large range of industrial applications rely upon the microencapsulation of solids or liquids by polymer coating and entrapment into polymer matrices, This general technique is largely used in the pharmaceutical field, mainly in order to improve the stability, sustained release, and targeting of drugs. Various microencapsulation methods have been reported, which primarily depend on the drug solubility in the polymeric material. Immobilization of a lipophilic
drug within a hydrophobic polymer is easily carried out by the conventional oil-in-water emulsion-evaporation method (1,2).
For the last decade, increasing attention has been paid to the encapsulation of hydrophilic drugs by a hydrophobic polymer. At this time, improving the bioavailability of peptide derivatives by an efficient entrapment method is still quite a challenge,. A potential approach to this problem might be found in a nonaqueous phase separation technique (3-5), i.e., an oil-in-oil (o/o) emulsion followed by the selective solidification of the internal phase (6). More recently, a technique based on a double water-oil-water (w/o/w) emulsion has been proposed as an alternative technique. To describe it briefly, an aqueous solution of the hydrophilic compound is emulsified into an organic solution of the hydrophobic coating polymer. This primary water-in-oil emulsion (w/o) is then dispersed in water accompanied by the formation of a double water-oil-water emulsion. The final evaporation of the organic solvent leads to the microsphere formation, This microencapsulation technique was first patented by Vrancken and Clays (7) in 1970 and by de Jaeger and Tavemier in 1971 (8). Kitajima and Kondo have immobilized highly labile molecules, such as proteins, by using this technique (9). Later on, enzymes and corrosion inhibitors have been encapsulated by w/o/w emulsions within polystyrene microspheres (10, 11). In 1988, Ogawa *et al.* patented the double-emulsion technique in view of the production of injectable poly(lactide-co-glycolide) microcapsules for the sustained release of leuprolide acetate (12-14).

There are no reports in the literature on the effect of experimental variables of the w/o/w emulsion technique on the most representative characteristics of the final microspheres, such as drug content, particle size and morphology, and kinetics of drug release, To the best of our knowledge, only Alex and Bodmeier (15) and Bodmeier *et al.* (16, 17) have paid some attention to the mechanism of microsphere formation from a double emulsion, These authors have been interested in a possible relationship between some features of the microspheres, such as drug loading, porosity, and surface morphology, and the way the coating polymer is precipitated. Actually, porous microspheres result from a rapid polymer precipitation and, conversely, the porosity decreases as the solvent extraction is slowed.
A previous paper has highlighted the effect of the stability of the primary emulsion on the morphology of polylactide microspheres loaded with bovine serum albumin (BSA) (18). Although the conditions used for polymer precipitation are important (precipitation rate, coprecipitation with an in-terfacial compound, e.g., a protein or a surfactant), the stability of the primary emulsion has a decisive effect on the internal morphology of the microspheres. The surface and internal morphologies of the microspheres, as investigated by scanning electron microscopy (SEM) and mercury poros-imetry, depend on the demixing rate, the interfacial tension of the primary emulsion, and the size distribution of the internal aqueous phase.
This paper will focus on the effect of the lactide/glycolide composition of the coating copolyester on the stability of the primary and secondary emulsions and ultimately on the microparticle morphology.

Experimental part

Materials

(D,L) poly lactide [(D,L)PLA or Resomer^{R} (R206)] and poly(lactide-co-glycolide) containing 50 wt% lactide (Re-somer*: RG505, or PLGA 50/50) and 75 wt% lactide (Re-somer*. RG7.56, or PLGA 7.5/25), respectively, were supplied by Boehringer, Ingelheim (Germany). Inherent viscosities were provided by the supplier as follows: 0.7 dl/g for PLGA 50/50, 0.8 dl/g for PLGA 75/2.5, and 0.9 dl/g for PLA (solvent and temperature were not specified). Apparent number average molecular weight *(Mn)* was estimated by size exclusion chromatography (SEC) in THF at 30°C by using a Hewlett Packard chromatograph 1090 calibrated with polystyrene standards. *Mn* was found to be 20,000, 2.5,000, and 51,000 for PLGA .50/50, PLGA 75/2.5, and (D,L)PLA, respectively.
Methylene chloride (Merck, p.a.) was used as a polymer solvent. Indigocarmine (Merck, p.a.), selectively soluble in the dispersed aqueous phase, was used as a dye.
Pluronic F68, represented by the general formula H- $(O\text{-}CH_2\text{-}CH_2)_a$-$(O\text{-}CHCH_3\text{-}CH_2)_b$-$(O\text{-}CH_2\text{-}CH_2)_a$-OH (poloxamer 188) (MW = 8,300) (gift from ICI, Belgium) was used as a nonionic surfactant in the primary emulsion.
Bovine serum albumin (BSA, Sigma A-7906) was encapsulated in microspheres as a model drug.
Polyvinyl alcohol, or PVA, (Mowiol VP 3-83, gift from Hoechst, Belgium) contained 17 mol% vinyl acetate units (MW = 18,000). It was used as a stabilizer for the second emulsion.

Methods

Standard microencapsulation technique

One milliliter of an aqueous solution of BSA (concentration from 0.25 to 2.5 wt%) and indigocarmine (500 μg/ml) were emulsified in a polymer solution (9 wt%) in methylene chloride (5 g) by using an ultra-turrax (IKA, T25). Theoretical protein drug loading is thus in the range from 0.2 to 5.5%. This primary emulsion was stabilized by poloxamer 188 previously dissolved in the organic phase (concentration up to 10 wt% in the organic phase). For the sake of comparison, the emulsion was also prepared without any surfactant.
The primary emulsion was then dispersed in water (100 ml containing 2.5 wt% PVA) with stirring A four-pitched blade impeller (rod diameter: 6 mm, blade 8 X 20 mm pitched at 4.5°) was used at a rate of 800 rpm. The temperature was fixed at 0°C during the emulsification process Half a hour later, it was increased to 20°C for two h. Microspheres were finally collected by filtration, washed with water, and dried under reduced pressure. Microencapsulation was studied in relation to the aqueous concentration of BSA (from 0 to 2.5 wt%), and the concentration of poloxamer 188 in the organic

phase (0-10 wt%). This contribution was analyzed at two BSA concentrations in the aqueous phase, i.e., 0 and 0.5 wt%.

Stability of the primary emulsion: demixing rate

The demixing rate is representative of the emulsion stability in relation to coalescence, coagulation, creaming, and sedimentation phenomena (19). The primary emulsion prepared as reported in the previous section, was stored in 12 ml assay tubes fitted with a rubber septum. The time required for an initial macroscopic phase separation to occur was measured at room temperature.

FTIR analysis of a solid film formed at the primary emulsion interface

A solid film was systematically observed to form more or less rapidly at the interface of the water phase and the organic solution of the primary emulsion. In order to analyze the composition of this interfacial film and to clear up its origin, aqueous solutions of BSA (0 to 10 wt%) were spread out onto an organic solution of the polymer (9 wt%). When used, poloxamer 188 was dissolved in the oil phase. After 4 h of equilibrium, solid films were picked off with tweezers, carefully washed with water, and dried overnight under vacuum.
Film composition was analyzed by FTIR, since coating polyester, BSA, and poloxamer 188 display characteristic absorption bands when mixed together, i.e., 1756 cm^{-1} (ester of polymer), 16.56 cm^{-1} (amide of BSA), and 842 cm^{-1} (ether of poloxamer 188). Calibration curves were made available for each (co)polyester by grinding powders of polymer and protein, on one hand, and powders of polymer and poloxamer 188, on the other hand, with KBr. Various contents of BSA and poloxamer 188 (5, 10, 15, 20, 2.5, and 30 wt% relative to polymer) were considered, although the organic components over KBr weight ratio was kept constant (1/10). FTIR spectra of the films were recorded by immobilizing a piece of film between two KBr disks.

Stability of the secondary emulsion

Stability of the secondary emulsion was related to the efficiency of the dye encapsulation. Indigocarmine was dissolved in the internal aqueous phase of the double emulsion. Its release kinetics in the external aqueous solution were measured from the absorbance at 615 nm of an aliquot of the aqueous phase withdrawn 15 min after preparation of the double emulsion (Philips PU8780 series UV/visible spectrophotometer) The percentage of dye entrapped within microspheres was calculated from the initial amount of dye in the primary emulsion and the amount of dye released in the external aqueous phase of the double emulsion 1.5 min after formation (encapsulation effiency or EE).
Scanning electron microscopy, Microparticles and/or cross sections of them (performed with a razor blade under stereooptical microscopy) were metallized (Au-Pd Sputtering Balzer, SCP-20) and then examined by scanning electron microscopy with a 20 kV voltage tension (Jeol JSM-840, A Technics Co, Ltd, Tokyo).

Results and discussion

According to Bodmeier *et al,* the structure and porosity of microspheres prepared from double w/o/w emulsions strongly depend on the polymer precipitation rate (16, 17). Fast precipitation gives rise to porous structures as a result of the rapid hardening of the polymer surface which prevents the microdroplets from shrinking, In addition to this effect, the stability of the double emulsion is an obvious prerequisite for the successful encapsulation of hydrophilic drugs. In this regard, the main characteristic features of the emulsion, which are strongly related to the emulsion stability, foreshadows the size and porosity of the final microspheres (18). The purpose of this paper is to focus on the effect that the chemical composition of the coating polyester can have on the stability of the primary and secondary emulsions and ultimately on the morphology of the final microspheres. The system under investigation consists of a primary emulsion stabilized by either the model protein to be encapsulated (BSA), or poloxamer 188 dissolved in the organic phase. It is worth pointing out that BSA has proved to be a very efficient surfactant (18) which explains why emulsion stability has been studied in relation to BSA content. Poloxamer 188 was first selected as surfactant because of a known

biocompatibility (20), although a high HLB (HLB = 29) is not favorable to the formation of a primary water-in-oil emulsion. These two stabilizers have been combined to extend the range of the emulsion stability and to have a better insight on the effect of this characteristic feature on the final microspheres.

TABLE 1 Time Required for Phase Separation to Occur

A. Effect of BSA content in the inner aqueous phase

BSA (wt%/wt water)	PLGA 50/50	PLGA 75/25	PLA
0 .00	30 min	15 min	Immediate
0. 05	16 h	1 h 30 min	5 min
025	24 h	16 h	24 h
0.50	3 days	3 days	3 days
1.50	3 days	3 days	3 days

B Effect of polyoxamer 188 content in the organic phase

Poloxamer 188 (wt%/wt CH_2Cl_2)	PLGA 50/50	PLGA 75/25	PLA
0.0	30min	15 min	Immediate
0.5	30 min	2h	5 min
1.0	30 min	3h	10 min
3.0	2h	5h	lh
10.0	Immediate	1 h	1 h 30 min

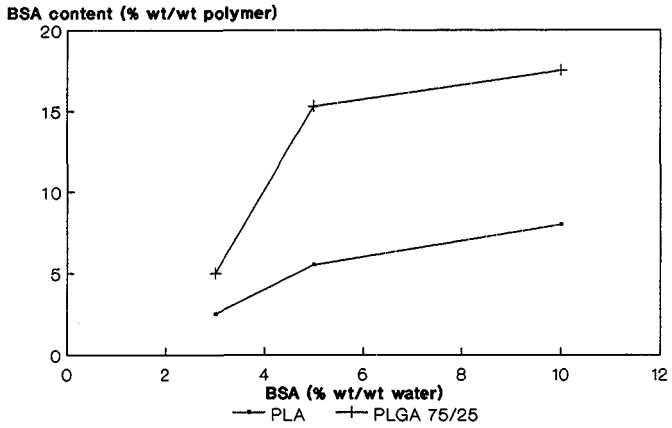
C. Effect of polyoxamer 188 content in the organic phase with 0.5% BSA in the inner aqueous phase

Poloxamer 188 (wt%/wt CH_2Cl_2) + 0.5% BSA	PLGA 50/50	PLGA 75/25	PLA
0.0	3 days	3 days	3 days
0.5	24 h	1 h	1 h
1.0	16 h	1 h 30 min	1 h
3.0	3h	1 h	30 min
10.0	Immediate	2h	Immediate

The secondary emulsion was stabilized by 2.5 wt% of PVA in the external aqueous phase.

Stability of the Primary Emulsion

Table 1 shows how the phase separation time depends on the surfactant, the surfactant concentration, and the coating polyester composition. In the absence of surfactant, primary emulsions prepared with the least hydrophobic polymer (PLGA 50/50) are the most stable, This result can be related to data reported elsewhere (4) on the CH_2Cl_2/water interfa-cial tension, that decreases as the hydrophobicity of the (co)-polyester decreases (thus from PLA to PLGA 50/50).
Emulsion stability is dramatically enhanced upon dissolution of small amounts of BSA in water. This effect is more pronounced as the coating polyester is more hydrophilic, in agreement with the aforementioned results. When the BSA concentration exceeds 0.25 wt%, no phase separation is observed until three days pass, whatever the coating polyester.
Poloxamer 188, a hydrophilic block copolymer surfactant (HLB = 29), is, as expected, much less efficient than BSA in stabilizing watei-in-oil emulsions.



FIG, 1. BaSA content of the interfacial film versus BSA concentration in the aqueous phase of a w/o emulsion A polyester was dissolved in the organic phase and no surfactant was used

The effect of poloxamer 188 concentration is complex, since stability goes through a maximum with the increase of the surfactant concentration in the organic phase, at least in the presence of PLGA 50/50 and 75/25. At a constant surfactant concentration of 3 wt% or less, the emulsion stability is the highest for the copolyester of an intermediate hydrophobicity (PLGA 75/2.5), In contrast, a more stable emulsion results from a less hydrophilic polyester (PLA) when a large amount of poloxamer 188 is used (10 wt%).

Very clearly, increasing the amount of poloxamer 188 in CH_2Cl_2 has a very detrimental effect on the stability promoted by 0,5 wt% of BSA in the aqueous phase. Table 1 shows that the dependence of the emulsion stability on the coating polyester is the same as for systems A and C.

As stated in a previous paper (18), the interfacial tension of the primary emulsion cannot be measured when the coating polymer is part of the emulsion, Indeed, a very thin film is systematically formed at the oil/water interface, which prevents any reliable measurement of the interfacial tension. The film formation is instantaneous when BSA and the (co)-polyester coexist in the emulsion. This process is however slowed (by ca, 2 h) by the addition of poloxamer 188 (0.5 to 3 wt%) to the organic phase. Even in the absence of BSA, the coating polyester is responsible for a film at the interface after ca. 3 h, Finally, when BSA and poloxamer 188 are associated in the emulsion, in the absence of any coating polymer, a precipitate is formed at the w/o interface after ca, 15 min. This precipitate results from BSA-poloxamer 188 interactions, as reported elsewhere (21). All these experimental observations indicate that a complex set of interactions occur among the main components of the primary emulsion and lead to the formation of insoluble associated species at the interface.

It is worth recalling that the interfacial tension between CH_2Cl_2 and an aqueous BSA solution (0,5 wt%) is slightly affected by the addition of poloxamer 188, This observation is in contradiction with results of Table 1 (systems C). This apparent discrepancy might, however, be reconciled if the emulsion stability is assumed to be promoted by an interfa-cial interaction between BSA and the polyester, as seems to occur (interfacial film) in systems A (Table 1). Then it is worth pointing out that, in agreement with our experimental observations, Law *et al.* (21) have reported the association of BSA with surfactants of the poloxamer type, including poloxamer 188. Moreover, according to these authors, BSA-poloxamer 188 complexes increase the stability of o/w emulsions in contrast to that of w/o emulsions. Thus the poor stability of systems C (Table 1) could be of threefold origin, Poloxamer 188 would successfully compete with the polyester for association with BSA. As a result, formation of the interfacial film (BSA-polyester complexes) might be inhibited. Furthermore, the BSA-poloxamer 188 interfacial association would favor a phase inversion of the w/o emulsion. Finally, an interaction between poloxamer 188 and polyester in methylene chloride solution has been emphasized by an associative thickening effect (18).

In order to attain deeper insight on the film formation, composition of the interfacial film has been analyzed by FTIR.

Chemical Composition of the Interfacial Film

The film formed at the interface of the primary emulsion when a polyester is dissolved in CH_2Cl_2 (together with poloxamer 188, or not) and BSA in water, has been collected and analyzed as described

in the Experimental Part, Composition data are reported in Fig. 1 and Table 2. The content of BSA and poloxamer 188 in the film cannot be measured accurately when the concentration of BSA in water and poloxamer 188 in CH_2Cl_2 are smaller than 3 wt%. Figure 1 shows that the dependence of BSA content in the film versus the BSA concentration in water strongly depends on the coating polyester.

TABLE 2 Chemical Composition of the Interfacial Film as Analyzedby FTIR

Polymer	F68in CH_2Cl_2 (wt%)	F68 in the film (wt%)	Mole of F68/gr of film	BSA in the film (wt%)	Mole of BSA/gr of film
PLA	3	2.38	2.9×10^{-6}	4.67	6.8×10^{-7}
	5	4.28	5.2×10^{-6}	5.16	7.5×10^{-7}
	10	10.15	$1\ 2 \times 10^{-5}$	8.06	1.2×10^{-6}
PLGA 75/25	3	1.10	$1,3 \times 10^{-6}$	21.57	3.2×10^{-6}
	5	3.24	3.9×10^{-6}	29.40	4.3×10^{-6}

Note. The film was formed at the interface of a BSA aqueous solution (0.5 wt%) and a polymer solution in CH_2Cl_2 (9 wt%).

More BSA is immobilized in the film when PLA is substituted by PLGA 75/25. Indeed, an increase in the BSA concentration in the water from 3 to 10 wt% results in a BSA content ranging from 2.5 to 8 wt% in a PLA film, compared to 5 to 17 wt% in a PLGA 7.5/25 film. The presence of BSA in the interfacial film might be attributed to an adsorption process at a liquid-liquid interface. Nevertheless, when expressed in μg BSA per film cm^2, the BSA content is exceedingly high (23 and 210 $\mu g/cm^2$ for 2,5 and 17 wt%, respectively) compared to values merely promoted by adsorption (1 or 2 $\mu g/cm^2$) (22). Therefore, the film formation should result from an interf acial coprecip-itation of BSA and the coating polymer. It is worth pointing out that the FTIR spectrum of the films clearly shows a broadening of the carbonyl absorption of the polyester at 1756 cm^{-1} when BSA is a film component. Undoubtedly, specific interactions occur between BSA and the polymer at the oil-water interface (23), which explains the insolubility of a film associating BSA and the polyester in each of the two liquid phases, The BSA/polyester interaction more likely originates from hydrophobic interactions and hydrogen bondings, and is possibly responsible for the protein denaturation.

As an additional piece of information, Table 2 shows that the BSA content in the film is substantially increased by poloxamer 188 in the oil phase, This effect is more pronounced when the polyester is more hydrophilic. Indeed, at a 5 wt% of poloxamer 188 in the oil phase, ca. 5 and 30 wt% of BSA are found in the PLA film and the PLGA 75/ 25 film, respectively. Increasing poloxamer 188 concentrations in CH_2Cl_2 results in higher contents of BSA and polox-amer 188 in the film, Nevertheless, there is as much polox-amer 188 as BSA (molar content) in the PLGA 7.5/2.5 films, although F68 dominates in PLA films. All in all, the experimental data in Fig. 1 and Table 2 confirm the complex interplay of interactions between the three components of the primary emulsion: BSA, poloxamer 188, and the coating polyester. This is tentatively schematized in Fig. 2.

It is obvious from Fig, 1 and Table 2 that BSA's interaction with the coating polyester are as favorable as the gly-colide content of the polyester is high, Poloxamei 188, which is a hydrophilic surfactant, also interacts with BSA as assessed by previously reported interfacial tensions (18) and the slow formation of a precipitate at the w/o interface,, It has a decisive effect on the BSA content in the film, since this content is higher, e.g., for emulsions containing only 0.5 wt% BSA in water plus poloxamer 188 in CH_2Cl_2 (Table 2), compared to emulsions consisting of 3 wt% BSA in water in the absence of poloxamer 188 (Fig. 1). This effect is in agreement with a decrease in the solubility of BSA when associated with the surfactant. The BSA/poloxamer 188 molar ratio in the film (Table 2) dramatically increases with the hydrophilicity of the polyester at a constant polox-amer 188 concentration in CH_2Cl_2.

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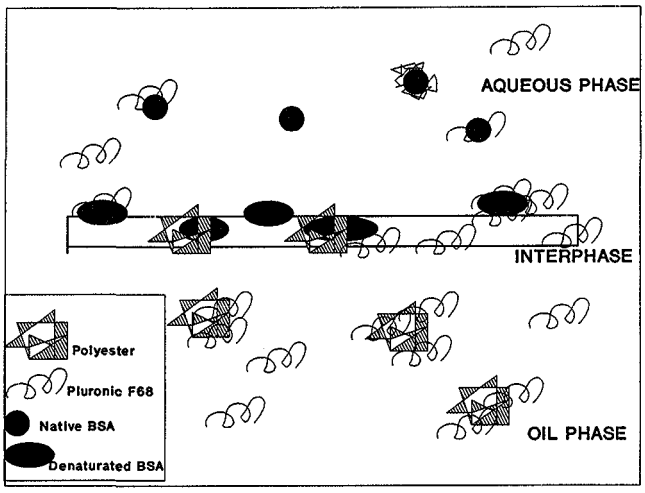

FIG, 2. Schematic picture of the interactions between BSA, poloxamer 188, and coating polyester and the corresponding repartition in the phases and at the interface of a watei/CH_2Cl_2 system.

Conversely, this ratio slightly decreases when more and more poloxamer 188 is dissolved in CH_2Cl_2, all other conditions remaining the same (coating polyester and BSA concentration in water), It must accordingly be concluded that the three major components of the primary emulsion (BSA, polyester, and surfactant) mutually interact in a complex manner, which clearly affects the remarkable ability of BSA to stabilize the emulsion either in an intrinsic manner and/or by changing deeply the formation kinetics and the chemical composition of the interf acial polymer film, As a rule, the instantaneous coprecipitation of BSA and the coating polyester has to do with the emulsion stability, Actually, more BSA coprecipitates with a more hydrophilic copolyester and this might explain the better emulsion stability observed for systems A and C (Table 1) when the glycolide content of the copolyester is increased, All the experimental data discussed until now show how complex the stabilization mechanism of an emulsion can be when a peptide or protein, i.e., an intrinsically surface-active compound, is dissolved in water and interacts with a coating polymer dissolved in the organic phase and possibly with a currently used surfactant. Clearly, any specific interactions between a drug to be encapsulated and the coating polymer have to be considered in the processing of a w/o/w double emulsion, particularly when the drug (peptide or protein) has intrinsic interfacial activity. Up to now, little data (24) has been published on the molecular interactions between a flexible-phase-forming polymer and a rigid globular protein. As suggested by Abbott *et al.* (24), an analogy might be found with the interactions between ionic micelles and polymers. Indeed, shape, size, and structure (a hydrophobic core surrounded by charges) are comparable for ionic micelles and proteins. This suggests possible interactions of surface charges and amino acid residues of the protein with the carbonyl dipoles of the coating polyester and the ether units of poloxamer 188, respectively. Moreover localization of the polymer-protein pair at the liquid interface can possibly perturb the protein conformation and favor interaction with poloxamer 188.

Stability of the Secondary Emulsion

Due to the complexity of a multiple emulsion, the study of the emulsion stability is quite a problem, Several techniques have been proposed in the scientific literature (25, 26) which rely upon the time dependence of the number and size of the emulsion droplets. Size has been measured by microphotography (27), the Coulter Counter method (28), and electron microscopy of freeze-etched samples. Matsu-moto *et al* (29) have also measured the change in viscosity of a w/o/w emulsion versus time, Unfortunately, all of these techniques cannot be applied in a reliable way to the system under consideration, Indeed, the lifetime of the secondary emulsion is very short (ca. a few seconds or tenths of second), due to the very fast hardening of the coating polymer at the H_2O/CH_2Cl_2 interface.

Although short in duration, the stability of the secondary emulsion is crucial, because it controls the efficacy of the internal aqueous phase entrapment within the oil droplets, Thus, exchange between internal and external aqueous phases should be restricted as much as possible during the second emulsification step. In this regard, analysis of the internal morphology of solid particles could provide indirect information on the emulsion stability, as reported elsewhere (18).

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An alternative way of studying the double emulsion stability has been proposed by Matsumoto *et al.* (30). This method consists of measuring the efflux rate of a tracer (glucose (25, 30), salt (25), marker (31)) initially dissolved in the internal aqueous phase. These authors have carried out the dialysis of a w/o/w emulsion against distilled water and they have measured the tracer concentration in the external aqueous phase by microanalysis. This method can only be used when the multiple emulsion does not change versus time (e.g., no hardening of the oil phase), It is however not very convenient because the emulsion has to be prepared independently of the dialysis equipment, and when the dialysis bag is filled with the emulsion, the system has to reach the diffusional regime before reliable data can be collected.

The hardening of the double emulsion investigated in this paper is so fast that there is no reason to use dialysis to measure the release of indigocarmine (i.e., a hydrophilic tracer dissolved in the internal aqueous phase at a 500 μg/ ml concentration) into the external aqueous solution. The encapsulation efficiency (EE) of indigocarmine has been calculated 15 min after the double emulsion preparation. Although the lifetime of the (liquid) double emulsion is extremely short, the amount of released indigocarmine cannot be measured until the solid particles have settled down, so that an aliquot of the external aqueous phase can be properly picked out and analyzed by colorimetry

The data collected in Fig. 3 are very surprising, since the most efficient encapsulation of indigocarmine is observed in a large range of experimental conditions, including surfactant (BSA and/or poloxamer 188)-free primary emulsions. This means that although the multiple emulsion is of very poor stability (Table 1 and Fig, 4), the indigocarmine EE is as high as 90-95%, whatever the coating polymer, The only explanation for this abnormal observation is to accept that the indigocarmine is trapped in the coating polyester by a completely different mechanism. To investigate this, a small quantity of polymer (100 mg) was suspended in an aqueous solution of indigocarmine (3 ml, 500 μg/ml) for 24 h. The concentration of indigocarmine was then measured by spectrophotometry. Although minimal under these heterogeneous conditions, the adsorption of indigocarmine onto the coating polymer increases from PLA (2% adsorption) to PLGA 50/50 (10% adsorption). After a few days, the indigocarmine solution becomes yellow in the presence of PLGA 50/50 and PLGA 7,5/25, in contrast to PLA. This observation confirms a stronger interaction between indigo-carmine and glycolide containing copolymers, most likely driven by H bonding between the NH groups of the dye and the stronger carbonyl dipoles of the glycolide subunits of the polymer.

Since the addition of 0.05 wt% BSA to the internal aqueous phase of the multiple emulsion significantly improves the primary emulsion stability (Table 1) although it decreases the dye EE, it is clear that BSA competes with indi-gocarmine for interactions with the coating polyester and accordingly favors the release of the tracer. Increasing further the amount of BSA remarkably stabilizes the emulsion, which can explain why a very high EE is rapidly restored (Fig. 3). It is also worth noting that the substitution of BSA by small amounts of poloxamer 188 as a surfactant does not perturb the EE of the dye, It is only when at least 1 wt% poloxamer 188 is dissolved in the oil phase that the EE drops, particularly in the case of PLA and in contrast to the emulsion stability. It can be speculated that either poloxamer 188 competes less efficiently with the dye than BSA does for interaction with the polyester, or when enough poloxamer 188 is dissolved in the oil phase, it favors the transfer of indigocarmine from the internal to the external aqueous phase of the multiple emulsion. Micelles of surfactant, particularly in the PLA-containing oil phase, could indeed solu-bilize and transport indigocarmine from the internal to the external aqueous phase. The effect of poloxamer 188 on the dye EE is qualitatively preserved when the primary emulsion is stabilized by 0.5 wt% BSA.

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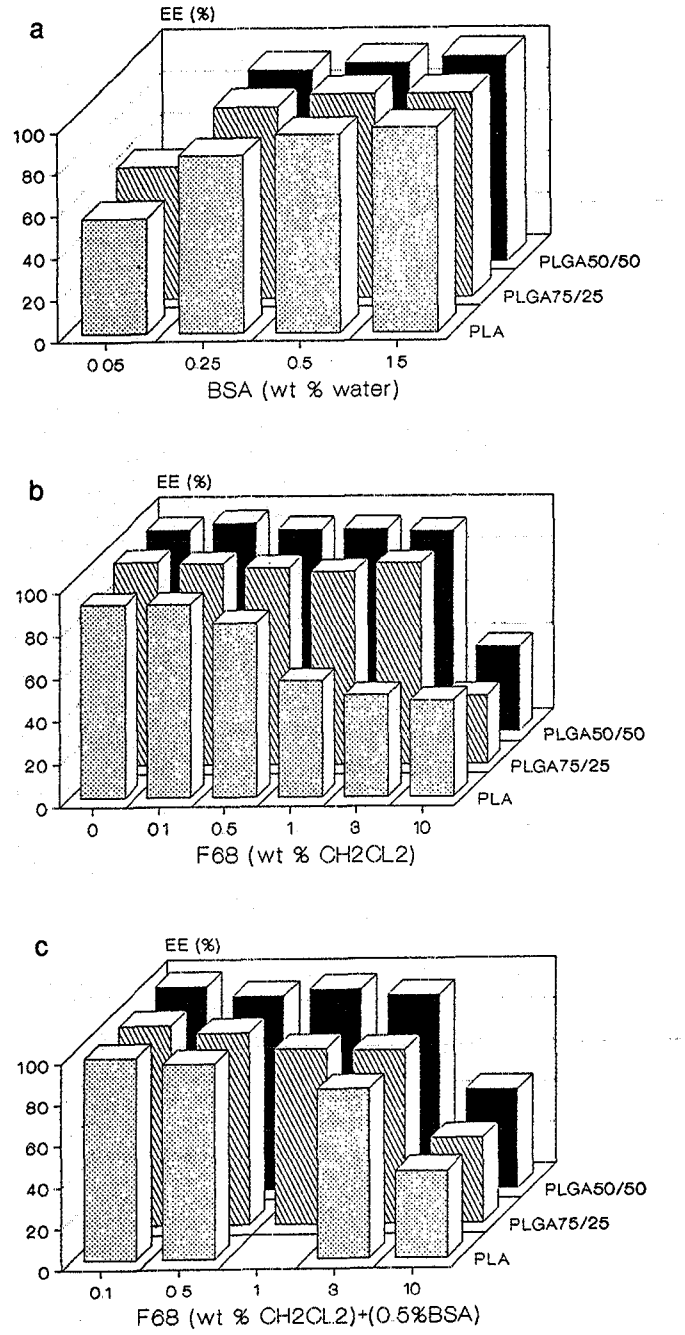

FIG. 3. Dependence of the encapsulation efficiency (EE, %) on: (a) BSA concentration in water (wt%); (b) poloxamer 188 concentration in CH_2Cl_2 (wt%); and (c) poloxamer 188 concentration in CH_2Cl_2 at a constant BSA concentration in water (0.5 wt%). EE was measured 15 min after the secondary emulsification step.

It must be concluded from Fig. 3 that encapsulation of a dye, such as indigocarmine, is a method which may not be recommended for probing the stability and internal structure of a w/o/w emulsion as the emulsion stability and the internal morphology of PLGA microparticles.

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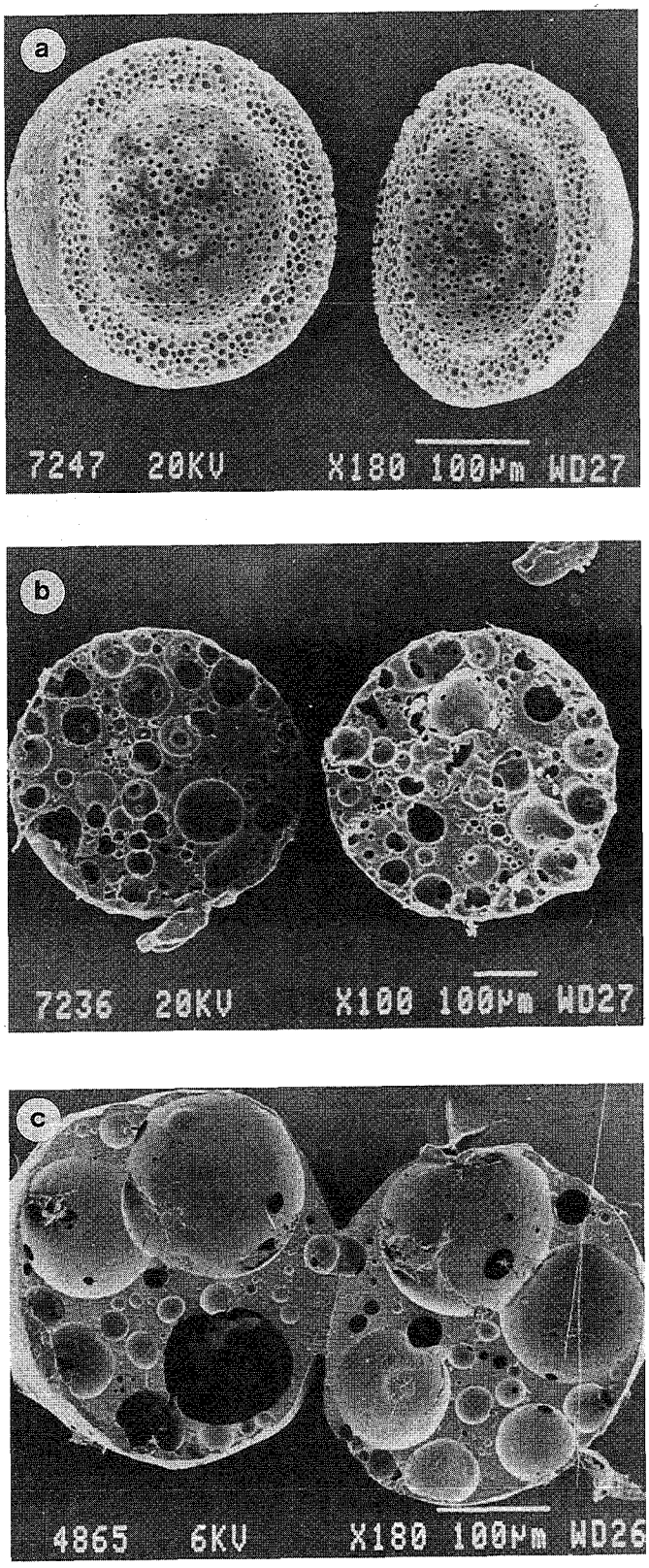

FIG. 4. SEM of microspheres of PLGA 50/50 (a), PLGA 75/25 (b), and PLA (c) stemmed from primary emulsions prepared without surfactant.

Published in: Journal of colloid and interface science (1995), vol. 173, iss. 1, pp. 55-65
Status: Postprint (Author's version)

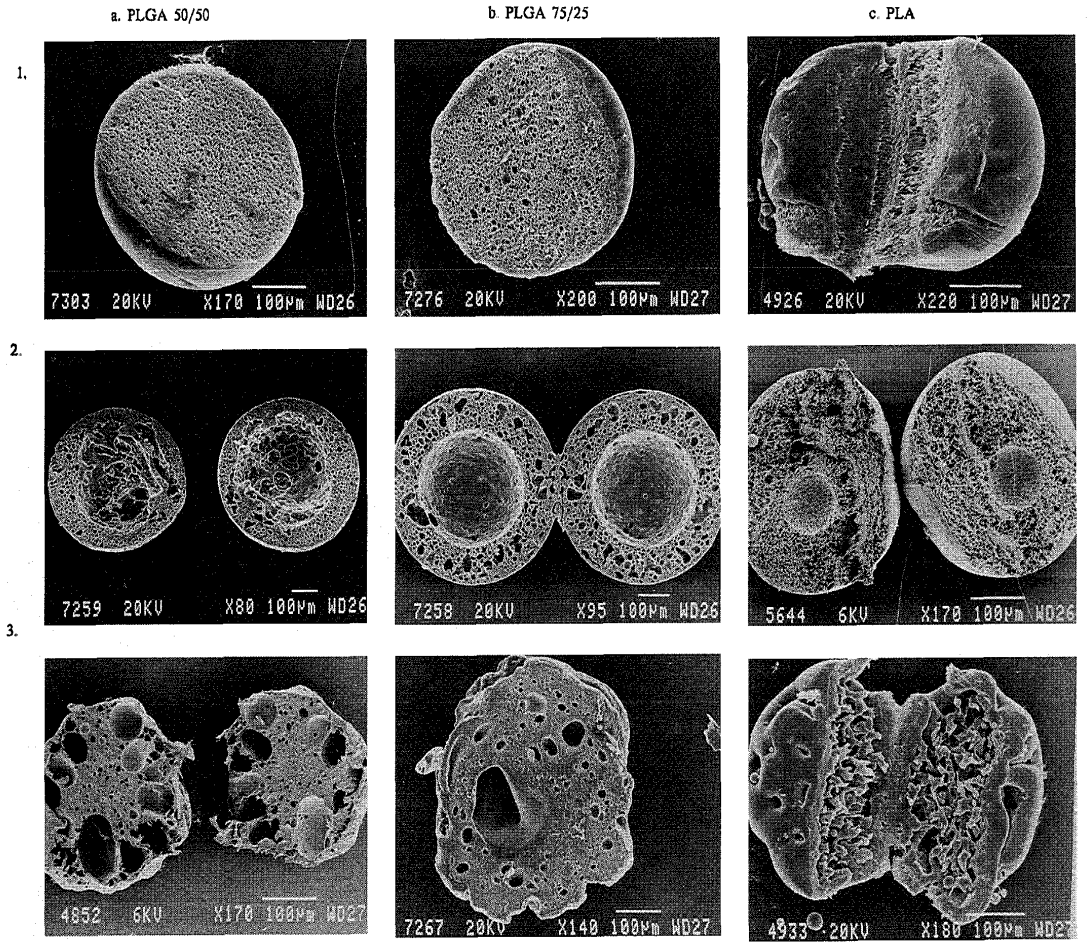

FIG. 7. SEM of microspheres of PLGA 50/50 (a), PLGA 75/25 (b), and PLA (c) stemmed from primary emulsions stabilized by (0.5 wt%) BSA in the aqueous phase and various amounts of F68 in the organic phase: 1, 0 wt%; 2, 3 wt%; 3, 10 wt%,

PLGA 50/50 particles dramatically shrink as a result of a very fast expulsion of the internal droplets. The morphology of PLGA 7.5/25 particles confirms an important coalescence of the internal aqueous phase to the point where microspheres are now macroporous. This general modification reported for PLGA 75/25 and 50/50 is in qualitative agreement with a phase inversion promoted by the well-known propensity of poloxamer 188 to stabilize o/w rather than w/o emulsions (32). The same tendency is not observed when PLGA copolymers are substituted by PLA in the oil phase as a partner of poloxamer 188. Then, things happen as though much less Pluronic is available at the w/o interface, which would restrict the ultimate destabiliza-tion of the o/w emulsion. The presence of surfactant micelles in the oil phase has been proposed under these experimental conditions in the previous section.
Figure 7 illustrates the deleterious effect of poloxamer 188 on the microporous internal morphology of microspheres containing 0.5 wt% BSA. These observations are quite consistent with data in Table 1.

Conclusions

This study has emphasized how complex a w/o/w double emulsion can be as the result of cross interactions between the main components, i.e., the coating polymer, the protein to be encapsulated, and the surfactant. These interactions can lead to the formation of insoluble complexes which can affect the stability of the emulsion. In the particular case investigated in this study, the protein (BSA) and the coating polyester (PLA or PLGA) interact instantaneously, forming an interfacial film which has a stabilizing effect on the primary emulsion. This film actually results from coprecipitation of BSA and the polyester and the amount of BSA incorporated is as high as the glycolide content of the polyester. Adding poloxamei 188 as a traditional surfactant would decrease the solubility of BSA, so accounting for a higher content of BSA in the interfacial film. Measurement of the encapsulation

efficiency of a dye has proved to be inappropriate to the analysis of the double emulsion stability, Indeed, specific interactions between the dye tracer (indigocarmine) and the coating polyester completely distort the results. In contrast, there is a good relationship between the stability of the primary emulsion and the internal structure of the final microspheres. Since the oil phase is hardened as soon as the primary emulsion is dispersed in water, the microsphere morphology foreshadows the main structural features of the parent primary emulsion, which cannot be otherwise made available.

Acknowledgments

The authors are vety much indebted to the "Services Federaux des Affaires Scientifiques, Techniques et Cultutelles" for financial support within the framework of the "Pôles d'Attraction Intemniversitaires: Polymeres" They thank ICI Belgium for the gift of a poloxamer 188 sample They are also grateful to Professor G Goffinet (ULg) for helpful assistance in the SEM One of us (S.C..) is grateful to IRSIA for a fellowship

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