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Microencapsulation of peptide: a study of the phase separation of poly(D,L-lactic acid–co-glycolic acid) copolymers 50/50 by silicone oil

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Summary

In this work, the phase separation of different poly(D,L-lactic acid–co-glycolic acid) batches induced by the addition of silicone oil was studied, for peptide microencapsulation purposes. The phase separation phenomena can be divided in 4 steps according to the amount of incompatible polymer added. But the stabilization of the coacervate droplets and, consequently, the formation of the microspheres, can only be obtained in the third step defined as the stability window. Two experimental parameters influencing the presence, the width and the displacement of the stability window inside ternary diagrams, have been studied: the physicochemical nature of the copolymers and the viscosity of the silicone oil. The results are discussed with respect to the presence of low molecular weight compounds in the studied polymer batches. It is concluded that this characteristic dramatically affects the phase separation of the copolymers by modifying their overall hydrophobicity.

Introduction

In the last few years, major advances have been performed in therapeutics with the development of biotechnologies, allowing the synthesis of peptides, on the industrial scale (Nestor et al., 1982; Siegel and Langer, 1984). The high pharmacological potency of these molecules is counterbalanced by their poor penetration through the physiological barriers, their fragility together with their very short biological half-life which, in turn, limit their therapeutical use (Szekerke and Driscoll, 1977; Lee, 1986). Different strategies have been proposed to overcome these problems. Thus, various routes of administration such as the nasal or the transdermal approach have been investigated to facilitate the resorption of some proteins and peptides (Illum, 1987; Siddiqui et al., 1987).

From the formulation point of view, when considering the bioresorbable characteristics of polyesters such as those of homo- and copolymers of lactic and glycolic acids (Vert and Chabot, 1981; Fong et al., 1986), some implantable controlled-release pharmaceutical systems based on the use of

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these materials as surgical sutures could be prepared, including single- (Sanders et al., 1986; Hutchinson and Furr, 1987) and multiple-unit dosage forms (Beck et al., 1983; Sanders et al., 1984). Besides the sustained release, such a dosage form design also provided increased drug stability.

Unfortunately, little information is available about the preparation of these rather sophisticated pharmaceutical forms. With regard to the microencapsulation of a luteinizing hormone releasing hormone (LHRH) analogue, i.e. triptoreline (Tice et al., 1984), this study was carried out to determine the experimental parameters leading to well-defined microspheres and to show, to some extent, their influence on the in vitro drug release.

Materials and Methods

Materials

The 4 batches of poly(D,L-lactic acid–co-glycolic acid) copolymers (PLGA) which were used for this work, were obtained at 180 °C by the ring-opening polymerization of dry, freshly prepared acid dimers, D,L-lactide and glycolide, by using $SnCl_2 \cdot 2H_2O$ as catalyst. The gross composition of the various poly(α-hydroxy acid) chains was 50% D,L-lactic units and 50% glycolic units. The polymer average molecular weights (Mgpc) ranged from 35,500 to 55,000 (G_3 column). All the molecular weight distributions varied from 1.8 to 2.2.

However, the solutions from Polymers 3 and 4 were filtered before GPC assay, because of their poor solubility in THF. In these cases, the given Mgpc and polydispersity values were underestimated. Relative amounts of glycolic (GA) and lactic acid (LA) units were determined by ^{1}H-NMR. Polymers 1, 2, 3 and 4 had respectively the following compositions: LA 56.4% GA 43.6%; LA 57.0% GA 43%; LA 55.6% GA 44.4%; and LA 50% GA 50%. Silicone oils with different viscosity grades (20; 350; 1000; 10,000; 12,500 cs) were obtained from Dow Corning (Valbonne, France).

D-Trp^6-LHRH (triptoreline) was supplied by Bachem CH (Budendorf, Switzerland) as a lyophilisate powder.

The dye was purchased from Prolabo (Bleu Patente Violet, Prolabo, Paris, France).

Methylene chloride (Rectapur, Prolabo, Paris, France or SDS, Vitry-sur-Seine, France) was used as received.

Methods

Preparation of phase diagram

Polymer solutions in methylene chloride, with different concentrations (2.5, 5 and 10% w/v) were poured in centrifuge tubes equipped with screw stoppers. The dye was then dispersed in the organic solution.

Aliquots of 1 ml silicone oil were progressively added. After each addition, the tubes were vigorously shaken with a vortex mixer (Genie K550 GE model, Scientific Industries, Bohemia, NY, U.S.A.). Then, a sample was observed under an optical microscope equipped with a coaterless instant pack film (Olympus BH_2, Tokyo, Japan). The phase diagram was established using a 10 ml PLGA solution at 20 °C.

Preparation of microsphere batches

Preparation of microspheres was made by polymer phase separation. Triptoreline (2.5% w/w) was suspended in PLGA methylene chloride solution under stirring (1500 rpm). Silicone oil was added to precipitate out the polymer around the peptide particles. The suspension of semi-formed microspheres was transferred to a non-solvent solution to cause them to harden. The microspheres were then filtered, washed and dried. The core loading as determined by HPLC assay ranged from 1.58% to 1.79% (w/w).

Triptoreline content

40 mg of triptoreline-loaded microspheres were dissolved in 5 ml methylene chloride. The peptide was extracted by 5 ml acetic acid and subjected to HPLC analysis. The HPLC operating conditions have been described in the publication by Nestor et al. (1982).

Size distribution analysis

This was determined using a Coulter Counter TAII model (Coultronics, Margency, France).

In vitro release studies

They were performed by incubating 50 mg microspheres in 20 ml phosphate buffer (pH 7.4) at 37 °C. After 1, 2, 4 and 6 h, an aliquot of the supernatant was taken and assayed by HPLC according to the experimental conditions previously described.

Determinations of acid, saponification, ester and hydroxyl values

The determinations were made according to the protocols of the European Pharmacopoeia (1980).

Polymer molecular weight determinations

Two experiments by gel permeation chromatography were carried out.

First, polymer molecular weights were determined using a G_3 type column (P.L mixed gel 10 μm, 60 × 0.7 cm, Polymer Laboratories, Church Stretton, Shropshire, U.K.). 150 μl of a 0.5% polymer solution were injected in a GPC apparatus equipped with a refractometric detector (401 model Millipore division Waters, St Quentin en Yvelines, France). The flow rate of THF mobile phase was 1 $ml \cdot min^{-1}$.

Calibration was made with alcane and polystyrene standards.

Polymer molecular weights were also determined by using a C_3 type column set (5 columns micro-styragel 10 nm, 3 × 50 nm, 100 nm, 30 × 0.7 cm, Millipore Division Waters, St Quentin en Yvelines, France) in order to gain more insight into the presence of monomers and oligomers in the polymer samples. The operating conditions were identical to these previously described.

All the molecular weights given in this paper correspond to maxima of GPC peaks.

Results

On the ternary diagram, each point corresponds to a well-defined weight percentage of methylene chloride, PLGA and silicone oil. The specific gravity of methylene chloride is 1.325 (Merck Index, 1983).

TABLE 1

Specific gravities of silicone oil versus its viscosity at 25 °C according to Dow Corning data

Viscosity (cs)	Specific gravities (d^{25C})
20	0.949
350	0.970
1000	0.971
10000	0.975
12500	0.975

As far as silicone oil is concerned, Table 1 gives its specific gravity versus its viscosity. Fig. 1 illustrates the sequence of events occurring with a 5% Polymer 2 solution following the progressive addition of 350 cs grade silicone oil. The process can be divided in 4 steps.

- In step 1 (Fig. 1A), the amount of phase inducer added to the solution is low (1–5% v/v). Silicone oil seems to form a pseudo-emulsion in the organic phase.
- In step 2 (Fig. 1B), for a higher amount of silicone oil introduced in the medium, a beginning of phase separation appears. The droplets of coacervate are unstable, merge together to give bigger structures which burst.
- In step 3 (Fig. 1C), the added quantity of polymer is sufficient to allow a stable dispersion of PLGA coacervate droplets.
- Finally, step 4 (Fig. 1D) is characterized by an extensive aggregation of coacervate droplets which precipitate. To make well-individualized microspheres, step 3 must be attained by carefully controlling the addition of phase inducer to avoid a possible evolution towards a massive aggregation. This step will be designated "stability window" in the following.

Influence of the physico-chemical nature of PLGA on the stability window

Fig. 2 shows the width of the stability window in ternary diagrams expressing the respective weight proportions of PLGA, silicone oil and methylene chloride.

A 350 cs viscosity grade silicone oil was chosen in this study. It clearly shows significant differences in the areas of the stability window, PLGA

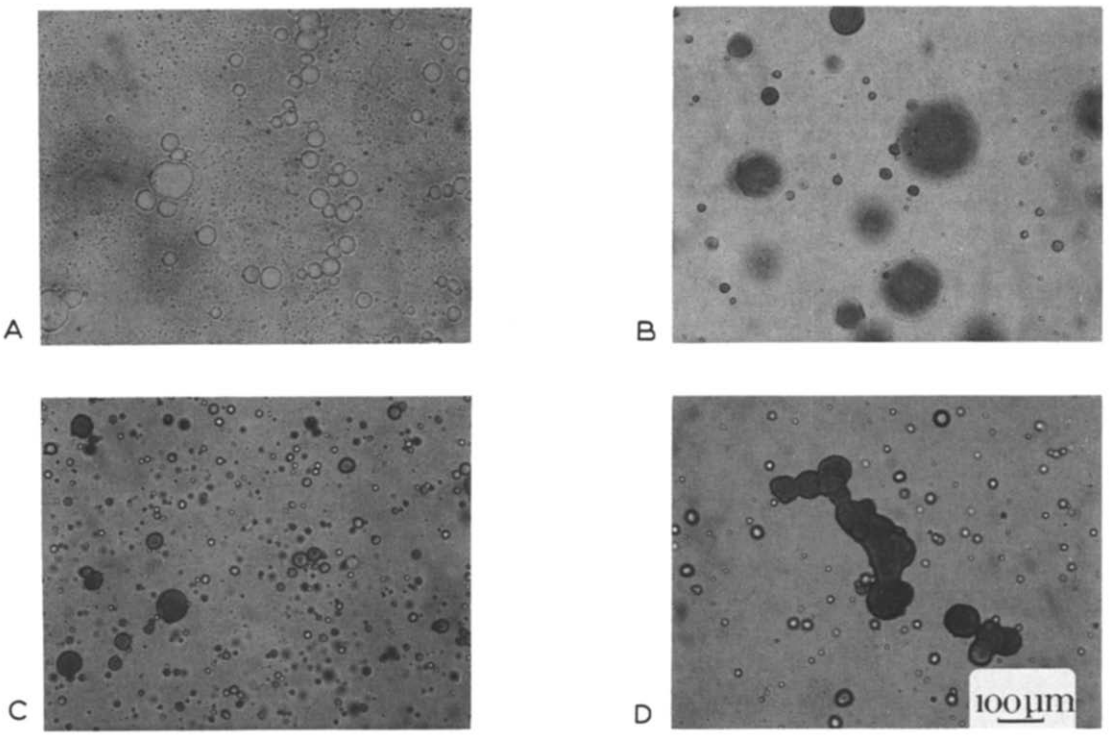

Fig. 1. Sequence of events occurring with a 5% Polymer 2 solution following the addition of 350 cs grade silicone oil.

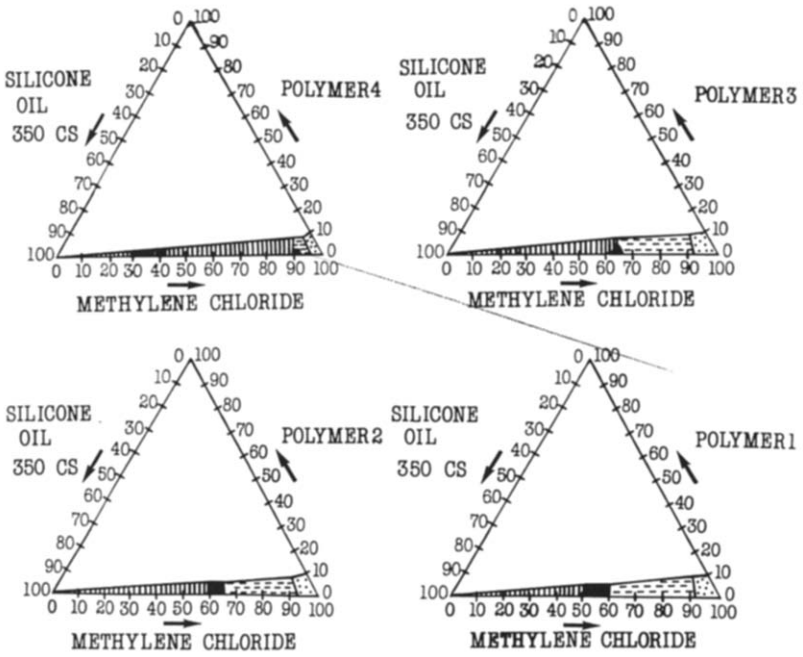

Fig. 2. Phase diagrams for the coacervation of different PLGA batches. ▨, step 1; ▤, step 2; ■, step 3 (stability window); ▥, step 4.

TABLE 2

Phase diagram for the coacervation of different batches of PLGA: respective percentages of the 3 compounds corresponding to the middle of the stability window

Polymer type	Percentage (w/w) of Polymer: A	Percentage (w/w) of methylene chloride: B	Percentage (w/w) of silicone oil: C
1	1.0	53.0	46.0
2	1.1	56.9	42.0
3	1.3	66.7	32.0
4	1.5	91.5	7.0

1 leading to the largest surface corresponding to step 3, PLGA 3 and 4 exhibiting the narrowest window.

In Table 2, the respective percentages of the 3 compounds of the medium corresponding to the middle of the stability window are reported. Table 2 and Fig. 2 indicate that increasing amounts of silicone oil must be added to the PLGA solution to induce the formation of stable coacervate droplets according to the following sequence: Polymer 4, Polymer 3, Polymer 2, Polymer 1.

The preparation of a phase diagram for the microencapsulation allows one to determine the volume of silicone oil, z (ml), leading to the formation of microspheres, according to the following expressions:

$$C = \frac{0.97z}{x + 1.325y + 0.97z}$$

therefore

$$z = \frac{C(1.325y + x)}{0.97(1 - C)}$$

where C is the percentage (w/w) of 350 cs silicone oil corresponding to the middle of the stability window; x is the weight of PLGA (g); and y is the volume of methylene chloride (ml).

Influence of the added volume of silicone oil on the characteristics of the microspheres

To define the influence of the added volume of silicone oil, z (ml), in the suspension on the characteristics of the peptide-loaded microspheres, the ratio z/y was calculated. As far as Polymer 2

TABLE 3

Influence of the added volume of 350 cs silicone oil (expressed as C and z / y) on the size, specific surface, the core loading and the initial in vitro release profile of triptoreline-loaded microspheres

Polymer type	C value % (w/w)	z/y	Average diameter (μm)	Standard deviation (μm)	Calculated specific surface ($cm^2 \cdot g^{-1}$)	Core loading % (w/w)	Amount released over 1 h % (w/w)	Amount released over 2 h % (w/w)	Amount released over 4 h % (w/w)	Amount released over 6 h % (w/w)
2	28.1	55	34.7	15.3	1520	1.58	26.41	26.70	28.07	31.44
			39.8	20.7	1326	1.53				
	32.0	65	41.6	18.0	1226	1.70	9.03	9.74	10.34	13.60
			49.0	24.2	1067	1.65				
	36.6	80	47.4	19.8	1087	1.69	5.44	5.48	5.78	7.48
			57.0	28.6	897	1.71				
	42.1	100	58.9	23.2	960	1.78	2.67	2.72	3.38	5.59
			58.8	26.4	952	1.78				
	46.3	120	Aggregation	–	–	–	–	–	–	–
3	22.1	40	35.1	14.6	1573	1.59	43.83	44.60	47.35	56.77
			43.2	16.4	1116	1.49				
	26.2	50	39.4	21.5	1337	1.74	7.90	9.00	12.25	20.49
			46.1	19.3	1067	1.64				
	30.7	61	42.0	18.3	1276	1.79	5.00	5.10	8.42	15.27
			52.9	17.9	903	1.70				
	36.6	80	Aggregation	–	–	–	–	–	–	–

TABLE 4

Mean values of the different PLGA batches (± S.D.) used to establish the ternary diagrams

Polymer type	Acid value [a]	Saponification value [a]	Ester value [a]	Hydroxyl value [a]	Polydispersity value [b]
1	4.2 ± 1.37	940.2 ± 18.02	936	7.1 ± 0.82	1.51
2	6.8 ± 0.38	892.2 ± 16.29	885.4	12.2 ± 0.59	1.58
3	8.0 ± 0.43	883.2 ± 23.36	875.2	12.5 ± 0.53	1.62
4	21.0 ± 2.61	889.7 ± 6.29	868.7	28.0 ± 0.69	2.10

[a] Average of 3 measurements.
[b] Determined on C_3 column.

and Polymer 3 are concerned, Table 3 shows the relationship existing between z/y, the microsphere average diameter, the specific surface, the core loading and the amount of triptoreline released in vitro over 1, 2, 4 and 6 h.

As the added volume of phase inducer increases, the microsphere average diameter increases in parallel and the calculated specific surface ($cm^2 \cdot g^{-1}$) decreases; consequently, the core loading augments and the initial burst effect lowers.

Table 3 illustrates also how the width of stability window varies with the type of polymer. With Polymer 2, microspheres are obtained for a broad range of C values, in contrast to Polymer 3. To gain more insight into the physicochemical nature of the PLGA used to establish the ternary diagrams, determinations of the acid, saponification, ester and hydroxyl values were performed (Table 4). Certain values allow one to classify, to some extent, the polymers according to their respective hydrophobicity.

The differences between the saponification and the ester values are barely significant. But, large variations are recorded for the acid and hydroxyl values. They show that Polymer 4 is much less hydrophobic than Polymers 2 and 3. On the other hand, Polymer 1 appears to be the most hydrophobic compound.

The polydispersities recorded on Table 4 were calculated using experimental conditions appropriate to study low molecular weight compounds (C_3 column).

It is clear that, among the 4 polymers, Polymer 4 exhibits the highest polydispersity value. This is evidence that this batch contains a fairly high amount of oligomers. Indeed, based on the GPC charts (Fig. 3), it was possible to calculate the percentage of low molecular weight compounds existing in each batch of polymers.

Polymers 1, 2, 3 and 4 contained respectively 2%, 3.1%, 4.5%, and 40% of oligomers expressed as area ratio. Although the determined percentages are overestimated for Polymers 3 and 4, due to solubility problems in THF, this point is consistent with high acid and hydroxyl values found for Sample 4.

Influence of the phase inducer viscosity on the stability window

Fig. 4 magnifies the role of the silicone oil viscosity on the formation of stable coacervate droplets of Polymer 1. For a low viscosity grade

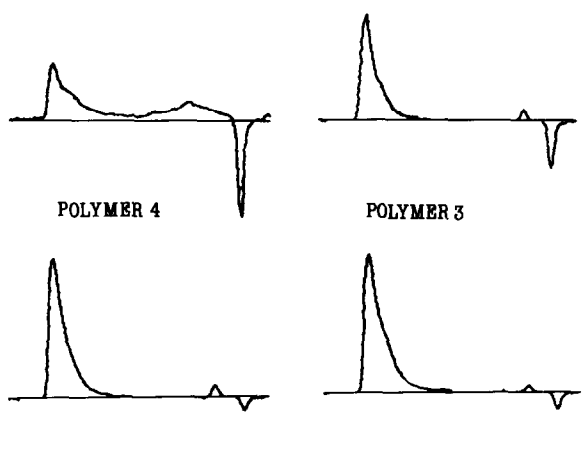

Fig. 3. Gel permeation chromatography charts (column C_3) of different copolymers studied.

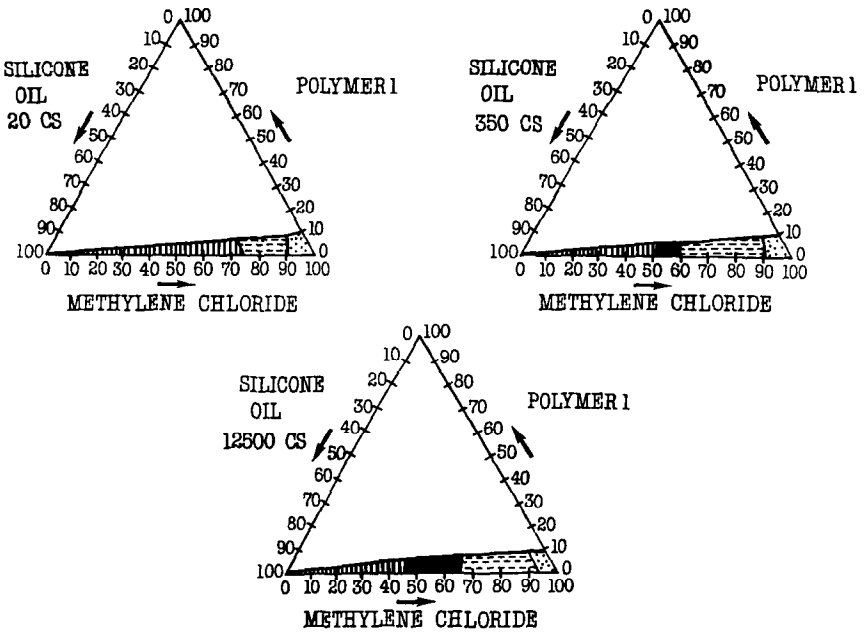

Fig. 4. Phase diagrams for the coacervation of Polymer 1. Influence of the phase inducer viscosity on the stability window. ▨, step 1; ▤, step 2; ■, step 3 (stability window); ▥, step 4.

silicone oil (i.e. 20 cs), no stability window could be detected. This was true for all the polymers tested. By increasing the viscosity of the phase inducer, it was possible to enlarge the stability window until a value of 12,500 cs. Above this limit, the silicone oil could not be handled for microencapsulation purposes. The evolution of the stability window versus the phase inducer viscosity is common for all the polymers studied, although less marked for Polymer 2.

Discussion

The results concerning the influence of the physicochemical nature of PLGA on the stability window show the relationship existing between

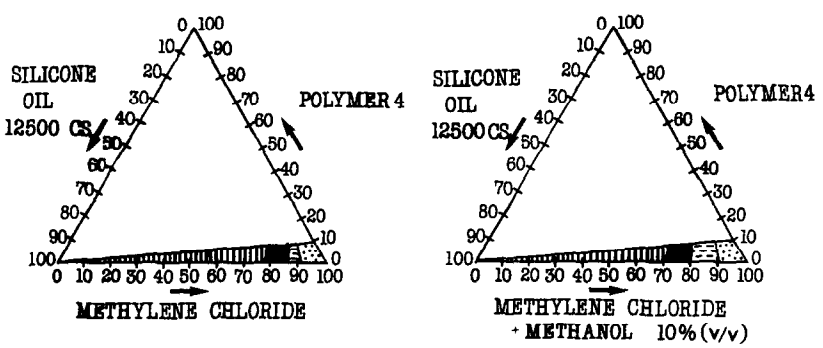

Fig. 5. Phase diagrams for the coacervation of Polymer 4. Displacement of the stability window versus the chemical nature of the solvent. ▨, step 1; ▤, step 2; ■, step 3 (stability window); ▥, step 4.

the amounts of silicone oil added to induce the formation of stable coacervate droplets and the hydrophobicity of polymers involved in the phase separation. The more the copolymer is hydrophobic, the more methylene chloride is a good solvent for the coating material and the more silicone oil is needed to desolvate the copolymer. Thus, Polymer 4, which is the least hydrophobic among the copolymers studied, does not easily dissolve in methylene chloride. In other words, it requires low amounts of silicone oil to precipitate. But, if the solubility of the copolymer is enhanced in the organic phase by adding 10% (v/v) methanol, more silicone oil is needed to reach the stability window and, in parallel to induce the appearance of stable PLGA droplets (Fig. 5). Based on the chemical and polydispersity values of the copolymers, it was possible to classify them in three categories.

- Copolymers with a high hydrophobic affinity due to a small ratio of low molecular weight compounds having free carboxyl and hydroxyl groups at their ends. Polymer 1 can be sorted out in this category.
- Copolymers with an intermediate hydrophobic affinity exhibiting a higher ratio of oligomers: Polymers 2 and 3.
- Finally, copolymers with a relatively low hydrophobic affinity owing to the presence of a high percentage of oligomers, such as Polymer 4.

As mentioned earlier, the amounts of silicone oil added to precipitate the coating material in a controlled manner vary with the degree of hydrophobicity of the copolymers.

From the formulation point of view, the width of the stability window can be modulated by changing the viscosity of the silicone oil used.

A 20 cs viscosity of the phase inducer leads either to a low apparent viscosity of the medium, unable to stabilize the formed coacervate droplets or to a very rapid solvation of the silicone oil involving, in turn, an uncontrolled precipitation of the wall material. These two assumed phenomena might simultaneously occur, although it becomes very difficult to demonstrate it. Higher viscosity grades of silicone oil produce reverse effects and allow one to extend the area of the stability window.

Conclusion

The presence of low molecular weight compounds in the different polymer batches was shown to affect the overall hydrophobicity of the matrix-forming copolymers, and consequently, the experimental conditions of the phase separation. However, since the presence of free carboxyl groups in the polymer bulk was known to catalyze the degradation rate of such systems (Makino et al., 1985), it is likely that the microspheres prepared from the copolymers studied in this work will have different in vivo behaviors and will exhibit different long-term release profiles. This remains to be defined.

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