Physicochemical Parameters Associated with Nanoparticle Formation in the Salting-out, Emulsification-Diffusion, and Nanoprecipitation Methods

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Purpose. The aim of this work was to relate the physicochemical properties of the aqueous and organic phases used for nanoparticle (NP) preparation to the formation of NP produced by salting-out, emulsification-diffusion, and nanoprecipitation.

Methods. Methacrylic acid copolymer and poly(vinyl alcohol) (PVAL) were selected as NP polymer and emulsifying agent, respectively. Salting-out and emulsification-diffusion NP batches were prepared modifying the PVAL content in the aqueous phase. For nanoprecipitation, NP were produced with variation of the polymer content and type of solvent in the organic phase.

Results. For salting-out and emulsification-diffusion, NP formation was discussed in terms of the emulsification theory. The nanoemulsion obtained during NP preparation was visualized by scanning electron microscopy. Aqueous and organic phases used for NP preparation were characterized by their viscosity and surface tension. NP characteristics such as particle mean size, residual surfactant, suspendability in water after freeze-drying, and morphology were explained in terms of these properties. For nanoprecipitation, NP formation was analyzed considering the diffusion-stranding phenomenon.

Conclusions. NP formation by salting-out and emulsification-diffusion was related to PVAL chain interactions at the droplet interface (e.g., reduction in the interfacial tension, mechanical stabilization, and steric stabilization) and in the bulk solution (hydrodynamic stabilization). For nanoprecipitation, $\chi_{\text{solvent-water}}$ and $\Delta\delta_{\text{solvent-water}}$ of the organic phase solvents were well related to the NP characteristics.

KEY WORDS: emulsification-diffusion; interaction parameter; nanoemulsion; nanoparticles; nanoprecipitation; poly(vinyl alcohol); salting-out; solubility parameter.

INTRODUCTION

During the past few decades, there has been considerable interest in the use of nanoparticles (NP) as potential drug delivery systems. These colloidal carriers may offer some advantages such as protection of drugs against degradation, targeting of drugs to specific sites of action (organ or tissue), and delivery of biological molecules such as proteins, peptides and oligonucleotides. Depending on their composition and intended use, they can be administered orally, parenterally, or locally (1–4). A number of different strategies have been proposed in order to modify the physicochemical characteristics of the NP and, thus, their interactions within a biological environment. For example, it is possible to change the chemical nature of the polymeric matrix of the NP and thereby alter certain biological phenomena such as biorecognition, biodistribution, bioadhesion, biocompatibility, and biodegradation. Some polymeric materials used for this purpose are gelatin, chitosan, sodium alginate, poly(alkyl cyanoacrylates), poly(lactic acid), poly(lactic-*co*-glycolic acid), poly[ethylene glycol-*co*-(lactic-glycolic acid)], poly(ε-caprolactone), and poly(methyl methacrylate) (4–6). A second approach to modify the biological response is based on incorporation of adjuvants in the NP. In this case, proteins (e.g., albumin, invasins, and lectins) and polymers (e.g., polyethylene glycol, poloxamines, and poloxamers) are some of the most commonly tested molecules (1,3,7). Finally, different NP manufacturing methods can be developed that enable modification of the physicochemical characteristics such as size, structure, morphology, surface texture, and composition.

Conventionally, two groups of manufacturing techniques have been reported for producing NP. The first involves polymerization of monomers, whereas the second is based on the dispersion of preformed polymers. The salting-out (8), emulsification-diffusion (9), and nanoprecipitation (10) methods can be cited as typical examples for the second group. Not only do these techniques allow a modulation of the morphology, internal structure, and physicochemical characteristics of the NP, but the drug loading, drug encapsulation efficiency, and release kinetics can also be modulated. Certainly, all of these changes depend on a range of parameters involved directly in the manufacturing processes. It is important to note that a deeper understanding of the physicochemical phenomena involved during NP formation is also necessary. Specifically, the relationship between physicochemical parameters and their quantitative effects on NP features could be an invaluable tool in the controlled engineering of particles. Knowledge of these fundamental relationships would allow NP to be designed with defined size and surface characteristics for delivery to specific cells or organs without requiring exhaustive experimental procedures. Therefore, the objective of the current work was to study the influence of certain physicochemical properties of the aqueous and organic phases used during NP preparation, and their effect on the characteristics of NP produced by the salting-out, emulsification-diffusion and nanoprecipitation methods.

MATERIALS AND METHODS

Materials

Poly(vinyl alcohol), with a molecular weight of 26,000 and a hydrolysis degree of 88% (Mowiol 4-88), was a gift from Omya AG (Oftringen, Switzerland). Methacrylic acid copolymer Type C NF/USP (Eudragit L 100-55), was a gift from Röhm GmbH & Co. KG (Darmstadt, Germany). All other chemicals used were of reagent grade.

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Methods

Preparation of Nanoparticles

Nanoparticles were prepared according to procedures of the salting-out (8), emulsification-diffusion (9), and nanoprecipitation (10) methods. In some cases, these general procedures were modified in order to study different formulation factors. Eudragit L 100-55 (E L100-55) and poly(vinyl alcohol) (PVAL) were selected as the NP polymer and emulsifying agent, respectively.

Salting-out. Typically, 50 g of aqueous solution of magnesium chloride hexahydrate (30.4%, w/w) and PVAL (5-21%, w/w) were added under mechanical stirring to 30 g of an organic phase containing 9.0% (w/w) of E L100-55 in acetone. Stirring was maintained at 2000 rpm for 15 min. After emulsification, 50 g of pure water were added to induce the diffusion of the organic solvent in water and the formation of NP.

Emulsification-Diffusion. For this method, 30 g of an aqueous solution of PVAL (7–21%, w/w) was added under stirring to 21 g of an organic solution of polymer in benzyl alcohol (14.3%, w/w). The resulting o/w emulsion was stirred continuously at 2000 rpm for 15 min, and then, 660 g of water were introduced in order to allow the diffusion of the organic solvent into the water, leading to the formation of the NP.

Nanoprecipitation. The organic phase was prepared by dissolving the E L100-55 (360–810 mg) in 25 ml of organic solvent (acetone, dimethyl sulfoxide, isopropyl alcohol, ethanol, or ethyl lactate). The organic solution was added into the aqueous phase (50 ml) containing PVAL (0.4%, w/w) and stirred magnetically.

Nanoparticle Purification

Following the preparation stage, for the salting-out and emulsification-diffusion methods, the NP in the raw suspension were separated from free surfactant by centrifugation or cross-flow filtration. In the centrifugation technique, the sedimentation conditions were adapted depending on the viscosity of the NP raw dispersion. They ranged from 19,000 × *g* to 270,000 × *g* and the spinning times varied from 15 to 120 min (Centrifuge mod. Avanti 30 and Ultracentrifuge mod. LF, both of Beckman Instruments, Palo Alto, CA, USA). The NP were washed three times using water adjusted to pH = 3.0 (with concentrated HCl) and finally with deionized water. Using the cross-flow filtration, NP cleaning was carried out using a Sartocon Mini device (Sartorius, Göttingen, Germany) mounted with a polyolefin membrane with a pore size of 0.1 μm. Water adjusted to pH = 3.0 (with concentrated HCl) was used to clean the NP. After recovery, the purified NP were freeze-dried at 0.05 hPa and –60°C during 24 h (LSL Secfroid, model Lyolab BII, Switzerland).

Determination of Particle Size Distribution

Nanoparticle mean size and polydispersity index (P.I.) of the raw and freeze-dried NP were measured using a Zetasizer 3000HS (Malvern Instruments Ltd., Worcestershire, UK) and dispersing the NP in deionized water. The P.I. values are given in brackets and range from 0 to 1; a higher value indicates a less homogeneous NP size distribution. Three measurements on three different batches of each NP formulation were made to determine the mean particle diameter and standard deviation.

Residual PVAL Determination

Residual PVAL refers to the amount of PVAL that remains associated with the NP even after the purification process. Nanoparticles were assayed for residual PVAL using a previously reported method (11), which is based on the formation of a stable complex of PVAL-iodine in the presence of boric acid. First, 20 mg of lyophilized NP were dissolved in 0.1 N NaOH. Subsequently, E L100-55 was precipitated by the addition of 0.1 N HCl and the suspension centrifuged at 5,500 × *g* for 10 min at 10°C. For the PVAL determination, an aliquot of supernatant was treated with 7.5 ml of boric acid solution (4.0%, w/v) and 1.5 ml of iodine solution (1.27% iodine and 2.50% potassium iodide in distilled water, w/v), adjusting the volume to 25.0 ml with water. Finally, the absorbance was measured at 644 nm (Hewlett Packard 8453 Spectrophotometer, Waldbrom, Germany), and the percentage of residual PVAL was calculated.

Viscosity Determination

Viscosities of aqueous and organic phases, used for NP preparation by the salting-out and emulsification-diffusion methods, were determined at 25°C using a cone-plate system (Viscometer Contraves, Rheomat 15 T, Zurich, Switzerland). Three measurement cylinders were used A ($Ø_{int}$ = 48.1 mm), B ($Ø_{int}$ = 37.8 mm), and C ($Ø_{int}$ = 19.8 mm), for which the sample volumes were 143, 91, and 20 ml, respectively. Each determination was made in triplicate.

Measurements of Surface Tension

The ring method was used for surface tension determination (Digital Tensiometer K10, Krüss, Hamburg, Germany). The samples analyzed corresponded to aqueous and organic phases mentioned under the section "Preparation of Nanoparticles," above. Each measurement was made in triplicate at 25°C.

Scanning Electron Microscopy

Nanoparticle Characterization. The morphology and surface of the NP were observed using a scanning electron microscope (JEOL JSM-6400, Jeol Ltd., Tokyo, Japan). Samples of freeze-dried NP were dispersed in water, air-dried over metallic studs, and coated with gold.

Nanoemulsion Visualization. Scanning electron microscopy (SEM) was used to assess the droplet size of the emulsions formed during NP preparation by the salting-out method. Briefly, an emulsion was prepared using the typical organic and aqueous phases, the latter containing 12% PVAL. After stirring, the emulsion was divided into two parts. The first was immediately diluted with distilled water to obtain a dispersion of NP, which was purified by centrifugation and freeze-dried before being analyzed by SEM. The second emulsion sample was diluted with a saturated solution of $MgCl_2$ and then frozen in liquid nitrogen. Congealed emulsion was then freeze-dried at 0.05 hPa and –60°C during 96 h. After recovery, samples of the NP and the "dry emulsion" were investigated by SEM with regard to size and morphology.

RESULTS AND DISCUSSION

Considering that an important aspect in the preparation of NP, from both fundamental and technological viewpoints, is the production of a desired droplet size with a narrow size distribution, all methods were compared by analyzing the physicochemical variables that could affect these NP characteristics. Owing to the similarities in the preparation processes, the salting-out and emulsification-diffusion methods were first compared. The second part of the discussion was focused on data analysis from the nanoprecipitation technique.

Physicochemical Parameters Associated with NP Formation in the Emulsion-Based Methods

Nanoemulsion Imaging During NP Preparation by Salting-out

Although it is well-known that an emulsion is formed during NP preparation by the salting-out and emulsification-diffusion methods, the size range of the droplets is not clearly defined. In this study, an original method was developed to enable analysis by SEM of the emulsion formed during NP preparation by salting-out. The emulsion was prepared using an aqueous phase containing 12% of PVAL. In a first step, dilution of the emulsion was achieved using a saturated solution of $MgCl_2$. Here, the solvent diffusion was inhibited by the presence of $MgCl_2$, which induced a salting-out effect. Then, freezing of the diluted emulsion with liquid nitrogen led to the droplets passing from a liquid state to a solid state. Finally, freeze-drying allowed solvent removal from the droplets and water removal from the external phase. The result was a system containing "dried droplets" embedded in a solid continuous phase of PVAL and $MgCl_2$. With this technique, liquid-liquid diffusion of solvent into the external phase did not occur, thus avoiding a size alteration when the droplets were transformed to "dried droplets." Strictly, these "dried droplets" are formed of polymer so they should be considered as solid particles. However, to distinguish them from the NP obtained by the classical diffusion process of salting-out, they will be called "dried droplets."

Images of SEM show that emulsion droplets formed during emulsification (Figs. 1A and 1B) have similar characteristics to the NP obtained after the solvent diffusion with water (Figs. 1C and 1D). In both cases, spherical entities with a size ranging between 180 and 250 nm were observed. This remarkable similarity in size and morphology between the "dried droplets" and NP prepared after solvent diffusion strongly suggests that (i) one NP is formed from one emulsion droplet, and thereby (ii) the emulsion droplet size directly determined the NP size. In general, it means that the NP produced by the

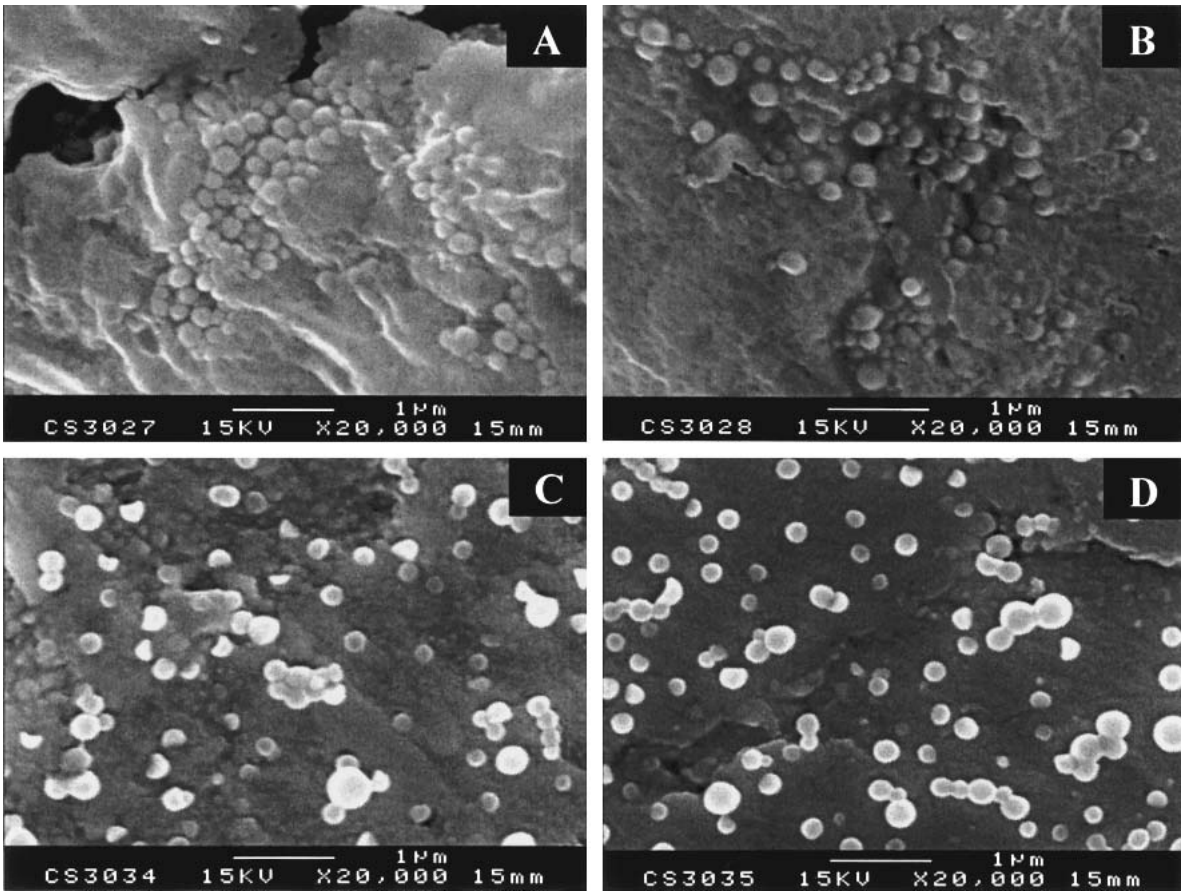

Fig. 1. Scanning electron micrographs (two different batches) of the nanoemulsion droplets and nanoparticles obtained by the salting-out method. "Dried droplets" of nanoemulsion diluted with a saturated solution of $MgCl_2$ and freeze dried (A and B) and nanoparticles purified by centrifugation and freeze-dried (C and D).

salting-out method are intimately influenced by all parameters associated with the preparation of emulsions. Therefore, considering that a nanoemulsion is formed during NP preparation by both salting-out and emulsification-diffusion, it is appropriate to analyze the results using the theory of emulsification. Basically, it is assumed that the quality of the droplets contained in the nanoemulsion is directly related to features of the final product, the NP.

Influence of PVAL Content in the Aqueous Phase on the NP Formation

The effect of the emulsifier concentration on the mean size of NP, for the salting-out and emulsification-diffusion methods, was studied at stirring rate of 2000 rpm using aqueous dispersion phases containing different concentrations of PVAL. In both cases, a broad size distribution of NP was obtained (Table I). For salting-out, mean diameter varied from 123 nm [0.071] to 710 nm [0.853], while for emulsification-diffusion it ranged from 108 nm [0.268] to 715 nm [0.615]. In general, the higher PVAL concentration in the external aqueous phase, the smaller the mean NP size. Emulsion droplet formation using a macromolecule as emulsifier is a complex process because of the numerous potential interactions of the polymer chains and their ability to adopt different conformations. For PVAL, a hydrophilic copolymer, various stabilization mechanisms may be involved in its action as an emulsifier (Fig. 2). On the one hand, when the polymer chains interact at the droplet interface, they can induce reduction in the interfacial tension, mechanical stabilization and steric stabilization. On the other hand, the nonadsorbed chains contained in the bulk solution can have an influence on the continuous phase viscosity to control the disruption kinetics and promote hydrodynamic stabilization. All of these mechanisms will be considered to explain variations in the droplet size during emulsification, and hence, in the corresponding NP mean size.

Table I. Influence of PVAL Concentration in the Aqueous Phase on the Size of Nanoparticles Prepared by the Salting-Out and Emulsification-Diffusion Methods

	Mean diameter* (nm) and polydispersity index†			
	Salting-out		Emulsification-diffusion	
PVAL (% w/w)	Raw NP	Freeze dried NP	Raw NP	Freeze dried NP
5	710 [0.853]‡	713 [0.523]‡	—	—
7	441 [0.519]§	483 [0.411]§	>2000	>2000
9	285 [0.173]	294 [0.109]	715 [0.615]¶	773 [0.363]¶
10	245 [0.198]	270 [0.098]	527 [0.430]	571 [0.384]
12	237 [0.045]	237 [0.089]	294 [0.153]	328 [0.136]
15	158 [0.056]	166 [0.077]	184 [0.113]	211 [0.092]
17	138 [0.053]	141 [0.062]	143 [0.048]	171 [0.127]
19	125 [0.089]	129 [0.076]	116 [0.090]	129 [0.101]
21	123 [0.071]	125 [0.074]	108 [0.268]‖	121 [0.193]‖

NP, nanoparticle; PVAL, poly(vinyl alcohol).

* Mean particle size results of three determinations from three different batches (Zetasizer 3000HS, Malvern Instruments).

† Polydispersity index values (P.I.), given in brackets, range from 0 to 1; a higher value indicates a less homogeneous NP size distribution.

‡,§,¶ Particles larger than 1 μm correspond to 34,† 17,§ and 19%¶ of all particles, respectively.

‖ Two populations of nanoparticles with mean sizes ~90 and 220 nm corresponding to 80% and 20% of all particles, respectively.

PVAL Interaction at the Droplet Interface. One of the most useful properties of a tensioactive agent is its capacity to lower the interfacial tension, which results in a significant reduction in the energy required during emulsification. The amphiphilic character, due to the presence of acetate groups (hydrophobic part) and hydroxyl groups (hydrophilic part) in the chains, means that PVAL can be adsorbed and oriented at liquid-liquid interfaces to reduce efficiently the interfacial tension and to promote the formation of a disperse system. In order to investigate the surface activity of PVAL, the surface tension of the aqueous phases used in the salting-out and emulsification-diffusion methods was measured. Taking the surface tension of pure water as a reference ($\gamma_{water}^{25°C}$ = 72.8 mN/m), it is clear from Fig. 3 that introducing even small amounts of PVAL, as low as 5%, causes a significant reduction in surface tension at the air/water interface. Gradually increasing PVAL concentration from 5 to 21% reduced the surface tension for the salting-out aqueous phases from 46.8 to 26.7 mN/m. For emulsification-diffusion, aqueous phases containing of 7 to 21% of PVAL displayed surface tension values in the range of 43.2 to 35.8 mN/m. The differences between the two profiles are attributed to changes in the configuration of polymer chains (a structural transition) when the polymer concentration increases in the bulk solution (12–14); however, this will not be discussed further in this paper. These results, in full agreement with those of other studies (15–17), show that PVAL chains adsorb at the air–water interface and present surface-active properties at this interface. Consequently, considering that PVAL polymer chains can interact at an organic solvent/water interface in the same way, a reduction in the interfacial tension of acetone/aqueous phase and benzyl alcohol/aqueous phase interfaces is also expected.

For a non-ionic copolymer like PVAL located at the droplet interface, besides reduction of interfacial tension, other stabilization criteria include (i) complete coverage of the droplets, (ii) firm anchoring at the interface, (iii) formation of a thick film, and (iv) optimal conformation of polymer chains into the external phase. These phenomena have been investigated for PVAL at organic solvent/water interfaces. In fact, two important conclusions were drawn from these studies: (i) interpenetration is the mechanism proposed to explain PVAL binding at the emulsion droplet interface, and (ii) the strong interaction of PVAL at the interface leads to an irreversible adsorption of PVAL at the NP surface (18,19). Data of residual PVAL in the NP, which are presented and extensively discussed in the following paragraphs, demonstrate the presence of nonremovable PVAL in the NP. This suggests that PVAL–E L100-55 interpenetration might occur at droplet interface during emulsification (Fig. 2). So, it is assumed that the compact packing of chains resulting from interpenetration leads to formation of a rigid film at the droplet interface which resists shear and imparts mechanical stability to the emulsion droplets during emulsification (15,20).

In addition, the steric effect of the PVAL chains adsorbed at the emulsion droplets surface, which induce short-range entropic repulsion between droplets, also contributes to droplet stabilization during emulsification. It is recognized that the efficiency of the steric stabilization depends on the

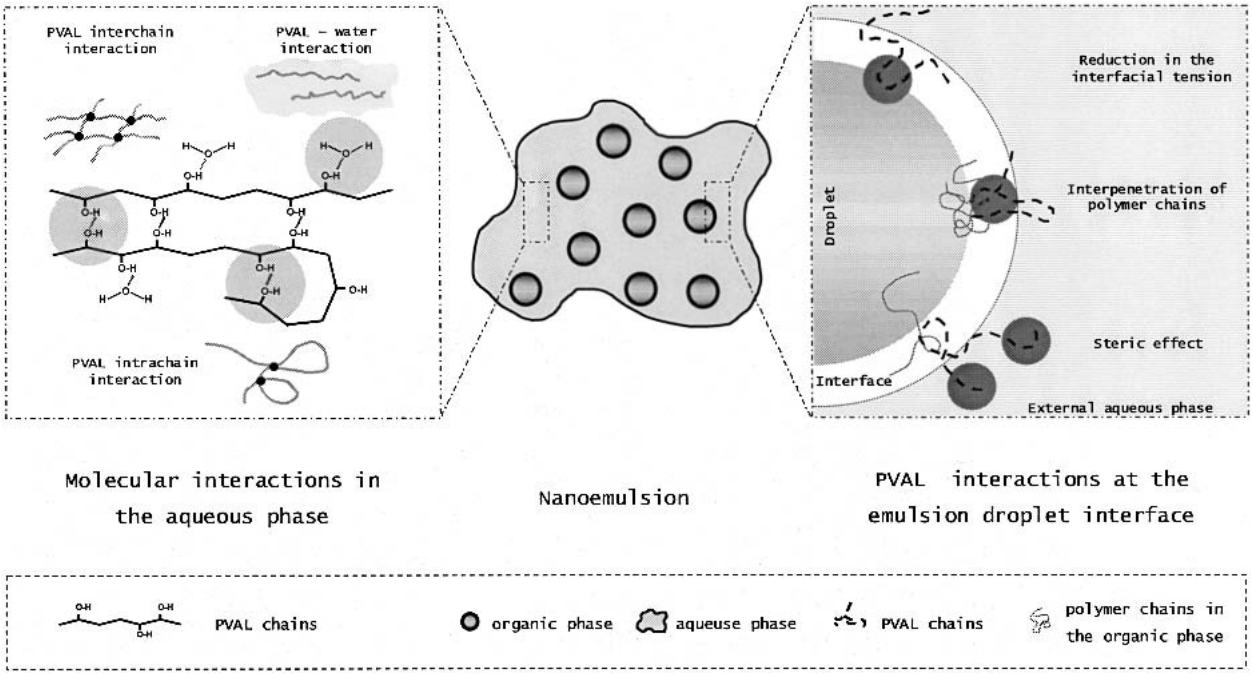

Fig. 2. PVAL interaction modes during the emulsification process.

conformation adopted by the polymer chains at the interface which is governed by the number and the type of functional groups that constitute the polymer. For partially hydrolyzed PVAL, acetate groups are also responsible for the conformation of the polymer at the organic solvent/water interface. These groups are anchored to the droplet surface and thus they determine the number and the length of loops and tails that will form during adsorption of the PVAL chains (21,22). Consequently, the hydrated chain segments that extend into the continuous phase adopt a definite conformation to induce steric stabilization during emulsification (18,23,24). From our study, increasing PVAL concentration in the aqueous phase results in more PVAL molecules being available for sterically stabilizing the more extended surfaces generated from the smaller emulsion droplets.

In particular, the importance of an optimal PVAL concentration in the aqueous phase during emulsification is illustrated at low PVAL concentrations. A minimal concentration

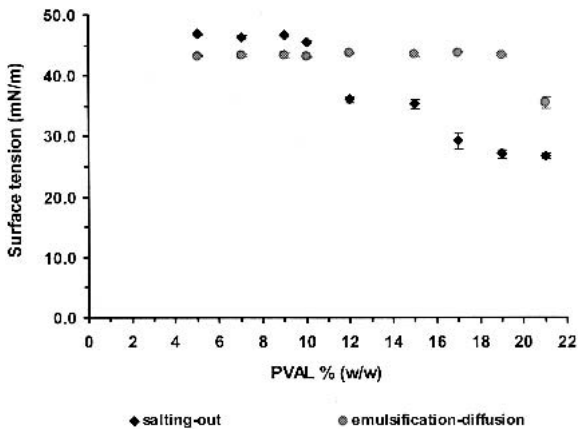

Fig. 3. Surface tensions of PVAL aqueous solutions used for the salting-out and emulsification-diffusion methods (mean ± SD, n = 3).

of PVAL is necessary to shift the droplet size from the micron range to the nanometer range. At low PVAL content, 5% and 7% for salting-out and 7% and 9% for emulsification-diffusion, the number of polymer chains in solution was insufficient to produce uniform emulsion droplets with sizes in the nanometer range (Table I). It was probably due to droplet coalescence, more than droplet rupture, that predominated during emulsification process. At these concentrations, the NP mean size distributions show evidence of coexistence of particles sized in the nanometer and micrometer ranges. For example, batches at 7% of PVAL for salting-out show a NP mean size of 441 and a P.I. of 0.519. In addition, using the CONTIN mode of Zetasizer 3000HS, the high P.I. value revealed not only a broad particle size distribution but also particles larger than 1 μm (17% of all particles). In the emulsification-diffusion case, at 9% PVAL content, particles with size larger than 1 μm were also present and they corresponded to 19% of all particles. Gradual increase of the PVAL concentration in the external aqueous phase resulted in both a size reduction and a lower P.I. (Table I). This suggests that the presence of sufficient polymer chains of PVAL leads to an excellent coverage and adequate stabilization of emulsion droplets which allows homogeneous NP distributions to be obtained.

Therefore, the important stabilization role of PVAL adsorbed at the droplet interface can be summarized as follows: (i) At low PVAL content in the aqueous phase, when there are insufficient polymer chains to cover completely the droplets, coalescence occurs and very heterogeneous emulsions and NP result; and, (ii) Above a critical concentration (C_{crit}), at which there is sufficient PVAL present to give excellent surface coverage, the coalescence efficiency decreases and the droplet size remains steady leading to uniform size distributions of NP. Here, it is assumed that all stabilization mechanisms of adsorbed PVAL, such as reduction in the surface tension and both mechanical and steric stabilizations, are in

force acting. The PVAL C_{crit} corresponded to 9% and 12% for the salting-out and emulsification-diffusion methods, respectively.

Interaction of Nonadsorbed PVAL in the Bulk Solution. The behavior of PVAL as emulsifier is not simply related to its interaction at the droplet interface. Nonadsorbed PVAL, defined as the polymer that is present in the bulk aqueous solution or not adhering to the droplet surface, also plays an important role during the emulsification process. For PVAL, the hydroxyl groups present in its structure are relevant because, through the formation of hydrogen bonds, they are directly associated to polymer properties such as solubility, viscosity, and crystallinity. The hydrogen bonds can be formed between the hydroxyl groups of the PVAL chains (intra- and interchain bonds) or between hydroxyl groups of polymer chains and water molecules. In particular, the balance between the polymer auto-association and polymer-water interaction plays an important role in determining the rheological behavior of PVAL solutions (Fig. 2). So, at low PVAL concentration in the aqueous phase (i.e., PVAL concentrations ≤10%), the intramolecular segments exclude one another to extend the PVAL chains and reduce the frequency of collision of these segments. In contrast, at high PVAL concentration (i.e., PVAL concentrations ≥15%), contracted coils are formed in the solution because of stronger intramolecular segment–segment interaction which results in an increase of the aqueous phase viscosity (25–28).

The viscosity of the PVAL solution is a key parameter because it is associated with hydrodynamic stabilization, which is considered as another mechanism of droplet stabilization during the emulsification process. Figures 4A and 4B illustrate the relationship among the PVAL concentration in the aqueous phase, aqueous phase viscosity, and NP mean size. For the salting-out method, there is an evident reduction

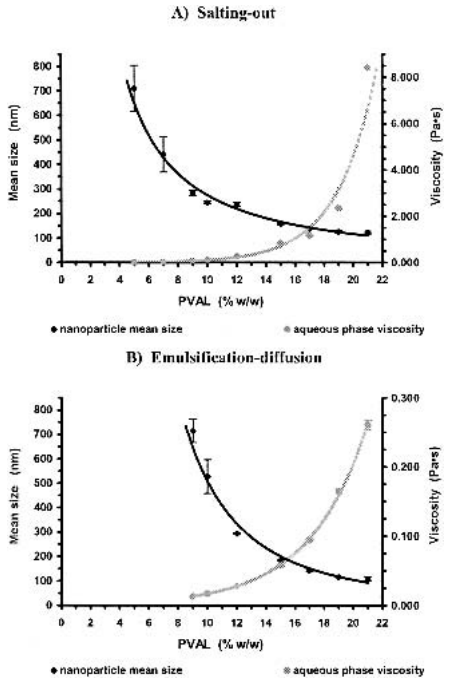

Fig. 4. Influence of PVAL concentration on the aqueous phase viscosity and mean size of nanoparticles prepared by (A) salting-out and (B) emulsification-diffusion (mean ± SD, n = 3).

of the NP mean size, from 710 to 123 nm, when the viscosity in the aqueous phase increased from 0.015 to 8.412 Pa•s, corresponding to PVAL concentrations from 5% to 21%. The same behavior was observed for emulsification-diffusion; NP mean size decreasing from 715 to 108 nm when the aqueous phase viscosity varied from 0.013 to 0.261 Pa•s, for PVAL concentrations between 9% and 21%. The concept of energy density is used to explain these results (29,30). At a constant energy density (e.g., energy input per unit volume), the mean diameter of the emulsion decreases with increasing viscosity. The reason is the lower probability of coalescence, because at higher viscosity of the continuous phase the drainage time is extended, whereas the collision time remains the same. Therefore, as a higher PVAL concentration corresponded to a higher viscosity of the external solution, it is conceivable that the viscosity of the external aqueous phase might prevent the coalescence of the internal phase droplets during the emulsification process.

However, it was observed that viscosity can also negatively affect NP formation. In the salting-out method, the mean particle size decreased by increasing the PVAL concentration in the external phase with the smallest size being obtained at 19% of PVAL. Higher PVAL concentrations did not, in real terms, reduce the NP mean size (Table I). Therefore, this concentration may be critical in determining when the viscosity of the aqueous phase (2.346 Pa•s) starts to have a negative effect on the mechanism of NP formation. It might be attributed to a reduction of the shear stress during the homogenization process resulting from a higher viscosity of the aqueous phase, which might lead to a less favorable mixing efficiency. Similar results were found for the emulsification-diffusion method. Nineteen percent of PVAL in the aqueous phase, with a viscosity of 0.165 Pa•s, was the concentration that allowed small NP (116 nm) with a relatively low P.I. [0.090] to be obtained. Above this concentration, aqueous phase viscosity was high enough to hinder the breakdown of the dispersed phase into small homogeneous droplets. In fact, the aqueous phase containing 21% of PVAL, and having a viscosity of 0.261 Pa•s, produced small NP (108 nm) but with a higher P.I. [0.268]. In this way, a more detailed analysis using the CONTIN mode of Zetasizer 3000HS showed that the higher P.I. was a result of the heterogeneous distribution of NP mean size, which was composed of two populations of particles with mean sizes of 90 and 220 nm, corresponding to 80% and 20% of all particles, respectively. In order to test the hypothesis that bringing more energy during emulsification leads to higher mixing efficiency, some batches were prepared with stirring at 2500 rpm. Aqueous phases containing 19% and 21% of PVAL were used for salting-out and emulsification-diffusion batches, respectively. For salting-out, NP mean size decreased from 125 nm [0.089] to 109 nm [0.077] when the stirring rate of emulsification increased from 2000 to 2500 rpm. In the emulsification-diffusion case, the mean size decreased from 108 nm [0.268] to 85 nm [0.090]. Clearly, the lower P.I. corresponds to a population of NP with a more homogeneous mean size distribution. In both cases, not only the NP mean size, but also the P.I. decreased. It suggests that a higher agitation intensity counteracts the negative effect of viscosity at high PVAL concentration in the aqueous phases.

Summarizing, an increase of PVAL content increases the aqueous phase viscosity and improves hydrodynamic stabili-

zation during the emulsification process. This mechanism is more pronounced at medium and high PVAL concentrations: from 10% to 19% PVAL for salting-out and from 12% to 19% for emulsification-diffusion. In these intervals, viscosity diminishes the interdrop contact time and reduces the coalescence. However, in both methods, viscosity has a negative effect on NP formation at PVAL concentrations higher than the optimum value of 19%. Beyond that value, the hydrodynamic stabilization neither gives finer droplets nor improves the P.I.

Nonremovable PVAL on the NP. As mentioned above, it has been also established that PVAL adsorption at the emulsion droplet surface leads to the formation of a permanent binding on the NP surface. Basically, this binding to the particle surface is likely to happen when the organic solvent diffuses into the aqueous phase and, at the droplet/aqueous medium interface, a hardening of the interpenetrated polymer chains of PVAL and E L100-55 occurs. In our study, the presence of PVAL on NP was confirmed from determinations of residual PVAL carried out with sub-300 nm NP. In a first study, raw NP were separated from free surfactant by centrifugation. Then, they were freeze-dried, and their PVAL content was determined. Using centrifugation as a cleaning process, residual PVAL values were 4.5 and 5.3% (w/w) for salting-out-NP and emulsification-diffusion-NP, respectively. Although this approach suggests that PVAL was strongly associated to the NP surface, a further experiment using cross flow filtration was undertaken in order to possibly eliminate more residual PVAL from NP. Compared to centrifugation, 5-fold greater volume of water was used. After washing, PVAL content in NP was not reduced significantly, corresponding to 3.5% and 4.8% (w/w) for salting-out-NP and emulsification-diffusion-NP, respectively. The resistance of PVAL to complete removal suggests that it was physically incorporated onto the NP surface. This has also been proposed by other authors (18). This PVAL layer on the NP is advantageous because, as shown in Table I, it promoted an almost instantaneous redispersion of the freeze-dried particles in water. In general, although all batches of freeze dried NP practically recovered their original sizes, a slight increase in the size of NP was observed after freeze-drying. It is due to solvation of PVAL chains incorporated at the NP surface. After cleaning, PVAL chains can interact with the surrounding water molecules to form hydrogen bonds and adopt an extended conformation. This unfolding of the chains leads to an effective increase in the volume of the particles, and hence in their hydrodynamic radius; thus, photon correlation spectroscopy measurements showed the apparent increase in the mean size of NP.

Comparison of NP Prepared by Salting-out and Emulsification-Diffusion at the Same PVAL Content in the Aqueous Phases

In general, although most clearly at low and medium PVAL concentrations, salting-out yielded significantly smaller NP than emulsification-diffusion. Differences between NP prepared by salting-out and emulsification-diffusion, at the same PVAL concentration in the aqueous phase, were related to physicochemical properties of external and internal phases.

Influence of the Internal Phase on the NP Mean Size. This first approach is based on the emulsification theory involving mechanical energy, which considers that the interface between the two immiscible phases is deformed to break up the internal phase and form the droplets. This deformation is opposed by the Laplace pressure to the viscosity and surface tension of the internal phase. In this study, NP prepared at 12% of PVAL can illustrate this influence (Table I). The mean size of NP obtained by salting-out and emulsification-diffusion were 237 nm [0.045] and 294 nm [0.153], respectively. On the other hand, the organic phase for the salting-out method showed a viscosity of 0.012 Pa•s and a surface tension of 24.8 mN/m, whereas for emulsification-diffusion, the values were 2.177 Pa•s and 39.8 mN/m, respectively. As can be noted, the highest values of both properties corresponded to the organic phase of the emulsification-diffusion method, which could explain the larger sizes of these NP as compared to those obtained by salting-out. An internal phase with higher values of viscosity and superficial tension tends to resist the turbulent flow and the pressure forces, and this results in a reduction in droplet breakup.

Influence of the Aqueous Phase on the NP Mean Size. Using aqueous phases with the same PVAL content, mean sizes of NP prepared by the salting-out method were smaller than those prepared by the emulsification-diffusion method. As comparison was done at the same concentration of PVAL in both aqueous phases, the number of PVAL molecules was not a determinant factor. In contrast, these differences could be related to the aqueous phase viscosities because, as can be observed in Figs. 4A and 4B, at the same PVAL concentration, the salting-out aqueous phases were always more viscous than those of emulsification-diffusion. Basically, the higher viscosities in salting-out aqueous phases are attributed to the effect of $MgCl_2$ on the PVAL polymers chains. The salt effect can be analyzed as the resultant of the following interactions: polymer-polymer, polymer-water and water-ion. Here, although PVAL and ions vie with one another for water molecules, the most effective are essentially the ion-water interactions which, by means of the salting-out effect, reduce the PVAL-water interactions. This relatively poor affinity of water to PVAL increases the intra- and interchain interactions in PVAL which augments hydrodynamic friction, and then, the viscosity of polymer solution (31–34). As $MgCl_2$ is absent in emulsification-diffusion aqueous phases, their viscosities are lower than those for the salting-out aqueous phases at the same PVAL content. So, as mentioned earlier, the higher viscosities of the aqueous phases used in salting-out promoted higher hydrodynamic stabilization and subsequently smaller emulsion droplets.

Besides its influence on the PVAL solution viscosity, other contributions of $MgCl_2$ to the emulsion formation should be considered. Fist, its influence on surface activity of a polymer solution should be taken into account. Some studies have shown that surface tension of a PVAL aqueous solution decreases drastically with increasing salt concentration (17). The behavior can be attributed to the increased adsorption of polymer molecules at the interfaces. In essence, salts make the aqueous phase less favorable for the polymer molecules, causing more molecules to go to the interface and consequently reducing the surface and interfacial tensions. Second, since $MgCl_2$ dissociates in solution, it could contribute to the stabilization of emulsions by means of an effect of

electrostatic origin known as short-range hydration repulsion. This phenomenon is most pronounced when strongly hydrated counterions, such as Mg^{2+} and Li^{+}, are present in solution (35,36). The resulting short-range repulsion can be attributed to the volume excluded by the hydrated counterions in the vicinity of the interface. In other words, the hydrated counterions form a protective shell around the emulsion droplets, which impedes their flocculation and coalescence on collision. Resuming, it can be concluded that the presence of $MgCl_2$ in the aqueous phase used in salting-out might improve the emulsification process by means of all these contributions.

Nanoprecipitation Method

Nanoprecipitation differs from the emulsification-diffusion and salting-out methods in that formally no precursor emulsion is formed during NP preparation. In fact, this process can be achieved with or without mechanical work. Basically, NP formation is explained in terms of the interfacial turbulence and the "diffusion-stranding" processes between two unequilibrated liquid phases (37,38). In the standard procedure, the organic phase containing the polymer that will form the NP is poured into the aqueous phase under slight magnetic stirring. When both phases are in contact, it is assumed that solvent diffuses from the organic phase into the water and carries with it some polymer chains which are still in solution. Then, as the solvent diffuses further into the water, the associated polymer chains aggregate forming NP. As can be noted, the mechanism of NP formation can be described based on the water-solvent, water-polymer and solvent-polymer interactions. Experimentally, the study of this method was carried out by varying the polymer content and the type of solvent in the organic phase.

Influence of Polymer Content in the Organic Phase

This effect was investigated using polymer concentrations in the organic phase ranging from 14.4 to 32.4 mg/ml. In all cases, increasing the polymer concentration in the organic phase resulted in an increase of the particle mean size (Fig. 5). However, for each solvent, above a critical concentration of polymer (batches marked with [*] in Fig. 5), large amorphous polymer aggregates were formed in addition to the NP. A further analysis concerning the critical concentration of E L100-55 in the different solvents will be given later. Then, the general trend for all solvents can be clearly illustrated considering the NP prepared with dimethyl sulfoxide as reference solvent (Fig. 5). Polymer concentrations from 14.4 to 32.4 mg/ml led to NP in a range of 98 nm [0.111] to 232 nm [0.224]. The critical concentration at which polymer forms amorphous aggregates was found at 32.4 mg/ml. This behavior might be explained by considering two facts: (i) the number of polymer chains per unit volume of solvent and (ii) the influence of polymer concentration on the viscosity. First, as a consequence of the greater number of polymer chains per unit volume of solvent, the solvent diffusing into the aqueous phase carries out more polymer chains which aggregate and thus form larger NP. This phenomenon is also favored by the fact that increasing polymer concentration increases polymer-polymer interactions which means that more polymer chains remain associated during the diffusion process. On the other hand, the influence of polymer concentration on the viscosity of the organic phase is also taken into account. On increasing the polymer concentration, a more viscous organic phase is obtained, which provides a higher mass transfer resistance; thus the diffusion of polymer-solvent phase into the external aqueous phase is reduced and larger NP are formed. In contrast, a diminution in the polymer concentration decreases the viscosity of the organic phase, which increases the distribution efficiency of the polymer-solvent phase into the external phase leading to formation of smaller NP. Here, an increase of the organic phase viscosity is related to an increase in the polymer-polymer and polymer-solvent interactions.

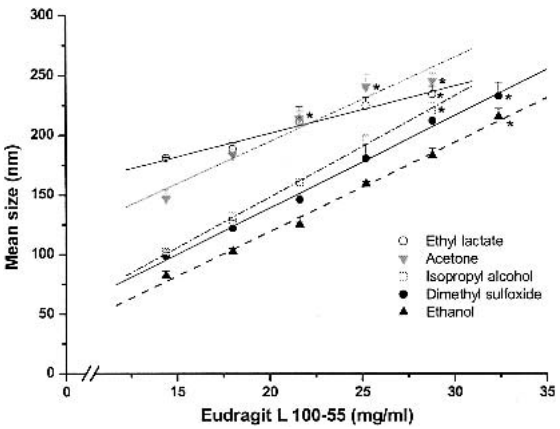

Fig. 5. Effect of type of organic solvent and polymer concentration in the organic phase on the mean size of nanoparticles prepared by nanoprecipitation. In batches marked with (*), the polymer forms nanoparticles and amorphous aggregates (mean ± SD, n = 3).

Influence of the Solvent in the Organic Phase on NP Formation

The nanoprecipitation method was also studied by modifying the nature of the solvent that constitutes the organic phase. Dimethyl sulfoxide, acetone, isopropyl alcohol, ethanol, and ethyl lactate were used for this purpose. Due to differences in the polymer-solvent and water-solvent interactions, it was supposed that the diffusion-stranding process might be altered, thus inducing changes in the mean size. Results are first analyzed regarding the influence of the organic solvent on the mean size of NP. Second, discussion is focused on the relationship between the type of solvent and the corresponding range of mean sizes of NP obtained at different polymer concentrations.

The profiles of the NP mean sizes, obtained using different solvents, are shown in Fig. 5. At polymer concentrations where aggregation was not apparent, the mean size was clearly dependent on the nature of the solvent. The mean size was shown to increase in the following order: ethanol < dimethyl sulfoxide < isopropyl alcohol < acetone < ethyl lactate. Considering that solvent-water interactions play an important role during the diffusion-stranding process, the solubility parameters of the solvents were initially used to explain this tendency. It is well-known that the most widely used application of solubility parameters is for the prediction of the solubility of a substance in a solvent. In effect, they express the affinity between like molecules. A first requirement of mutual solubility is that the solubility parameter of the solute (δ_{solute}) and that of the solvent ($\delta_{solvent}$) do not differ too much. The smaller the difference, the higher the affinity.

Table II. Physicochemical Characteristics of the Solvents Used in the Nanoprecipitation Method

Solvent	$\Delta\delta_{solvent\text{-}water}$* ($MPa^{1/2}$)	χ solvent-water†
Water	0.0	0.0
Ethanol	27.0	11.0
Dimethyl sulfoxide	28.7	13.3
Isopropyl alcohol	31.0	18.7
Acetone	34.4	23.7
Ethyl lactate	32.2	32.5

*,† Calculated from Eqs. 1 and 2, respectively.

For this study, the $\Delta\delta$ of organic solvent-water systems ($\Delta\delta_{solvent\text{-}water}$) were calculated from (39):

$$\Delta\delta_{solvent-water} = [(\delta_{d,S} - \delta_{d,W})^2 + (\delta_{p,S} - \delta_{p,W})^2 + (\delta_{h,S} - \delta_{h,W})^2]^{1/2} \quad (1)$$

where δ_d is the dispersion force component, δ_p is the polar component, and δ_h is the hydrogen bonding component of the total solubility parameter. Subscripts S and W are assigned to these partial solubility parameters of the organic solvent and water, respectively. Table II shows the $\Delta\delta_{solvent\text{-}water}$ values, which increase in the following order: ethanol < dimethyl sulfoxide < isopropyl alcohol < ethyl lactate < acetone. Comparing at the same polymer concentration (e.g., 14.4 mg/ml), the mean size of NP prepared using these solvents increases almost in the same order: ethanol < dimethyl sulfoxide < isopropyl alcohol < acetone < ethyl lactate. Ethanol, showing the smallest $\Delta\delta$ value (27.0 $MPa^{1/2}$), produced the smallest NP. Conversely, acetone, with the highest $\Delta\delta$ value (34.4 $MPa^{1/2}$), gave NP with a much larger mean size. In general, this first approach suggests that a high solvent-water affinity, corresponding to a smaller $\Delta\delta_{solvent\text{-}water}$ value, might improve the diffusion of solvent into the aqueous external phase, and hence lead to smaller NP being obtained.

Because $\Delta\delta_{solvent\text{-}polymer}$ does not completely explain the relationship between the mean sizes of NP produced with different solvents and the solvent-water affinity, a further analysis was made determining the solvent-water interaction parameter ($\chi_{solvent\text{-}water}$) calculated from (40):

$$\chi_{solvent-water} = \frac{V_{solvent}}{RT}(\delta_{solvent} - \delta_{water})^2 \quad (2)$$

where V is the molar volume of the organic solvent, R is the gas constant, T is the temperature, and $\delta_{solvent}$ and δ_{water} are the total solubility parameters of solvent and water, respectively. In comparison with $\Delta\delta_{solvent\text{-}water}$, the $\chi_{solvent\text{-}water}$ (Table II) exhibits a better correlation with the mean sizes of NP obtained by nanoprecipitation using different solvents. The lower the $\chi_{solvent\text{-}water}$ of the solvent, the smaller the NP mean size. Only considering polymer concentration free of aggregates (e.g., 18.0 mg/ml), mean size of NP increases as follows: ethanol < dimethyl sulfoxide < isopropyl alcohol < acetone < ethyl lactate (Fig. 5). The $\chi_{solvent\text{-}water}$ increases exactly in the same order (Table II). This correlation confirms that the solvent-water interaction plays an important role in the diffusion-stranding phenomenon and thus in the formation of NP by nanoprecipitation. Solvents having high affinity for water, which is evidenced by low $\chi_{solvent\text{-}water}$ values, tend to promote solvent diffusion and polymer chain partition into the aqueous phase. This leads to formation of smaller NP.

In the nanoprecipitation method, organic solvent performance can also be evaluated regarding the critical concentration of E L100-55 in each solvent ($C_{E\ L100\text{-}55\text{-}solvent}$). It is identified as the polymer concentration at which polymer starts to form amorphous aggregates during the nanoprecipitation process. Because the presence of aggregates affects greatly the yield of NP, experiments for each solvent were limited at the critical concentration. Only for acetone were batches prepared above the critical concentration. In Fig. 5, $C_{E\ L100\text{-}55\text{-}solvent}$ correspond to batches marked with [*] and appeared at 32.4 mg/ml for ethanol and dimethyl sulfoxide, 28.8 mg/ml for isopropyl alcohol and ethyl lactate, and finally 18.0 mg/ml for acetone. Taking into account the NP size ranges free of amorphous aggregates, the broadest ranges corresponded to ethanol and dimethyl sulfoxide, whereas the narrowest was with acetone. Obviously, because the amorphous aggregates have an adverse effect on NP recovery, the solvents with the broadest range of NP mean size were considered as the most advantageous for NP preparation. Searching to relate this trend to the characteristics of organic solvents, it is noted that the $\chi_{solvent\text{-}water}$ is quite correlated to the interval of NP mean sizes obtained for each solvent. In general, the smaller the $\chi_{solvent\text{-}water}$, the broader the range of NP mean sizes. The $\chi_{solvent\text{-}water}$ increases in the following order: ethanol < dimethyl sulfoxide < isopropyl alcohol < acetone < ethyl lactate (Table II). With the exception of acetone, the

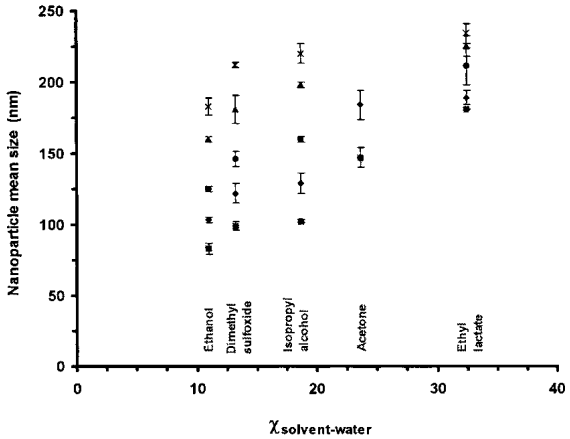

Fig. 6. Relationship between the $\chi_{solvent\text{-}water}$ and the mean size of nanoparticles prepared by nanoprecipitation using different organic solvents. Polymer concentration (mg/ml) in the organic phase 14.4 (■), 18.0 (♦), 21.6 (●), 25.2 (▲), and 28.8 (*) (mean ± SD, n = 3).

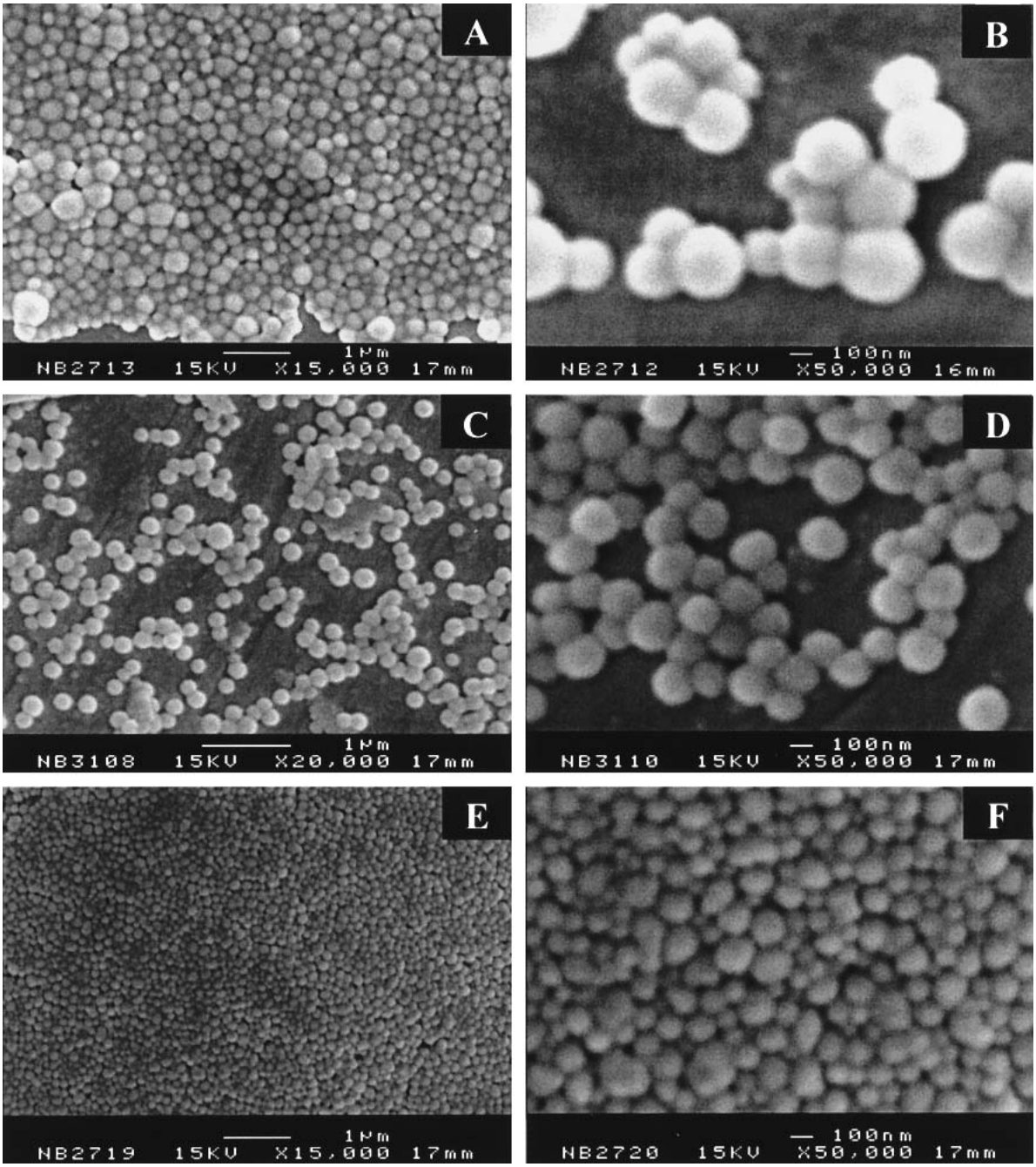

Fig. 7. Micrographs (at two different magnifications) of nanoparticles prepared by the salting-out (A and B), nanoprecipitation (C and D), and emulsification-diffusion (E and F) methods.

range of NP mean sizes increased in the same order (Figs. 5 and 6). Solvents with a low $\chi_{solvent\text{-}water}$, such as ethanol and dimethyl sulfoxide, give broad ranges of NP mean sizes. In contrast, when solvents exhibited high values of the $\chi_{solvent\text{-}water,}$ such as acetone, narrow ranges of NP mean sizes were obtained. This trend also supports our view that solvent-water interaction is an important factor related to NP formation using the nanoprecipitation method.

Finally, regarding the diffusion-stranding phenomenon, when the organic solvent is poured into the aqueous phase during NP preparation, a higher solvent-water affinity might facilitate the solvent distribution into the aqueous phase. Moreover, because polymer is transported by the solvent, it is assumed that polymer chains will be partitioned more efficiently in the external phase where they aggregate forming NP. Therefore, considering this analysis, it can be postulated that other organic solvents with similar properties would also be good candidates to prepare NP by the nanoprecipitation method. In addition to miscibility in water, the $\chi_{solvent\text{-}water}$ (or the $\Delta\delta_{solvent\text{-}water}$) might also be considered. Thus for E L100-55, the solvent of the organic phase must have optimally a $\chi_{solvent\text{-}water} \approx 11$ (or a $\Delta\delta_{solvent\text{-}water} \approx 27$ $MPa^{1/2}$). This enables NP with a wide range of mean sizes to be obtained by the nanoprecipitation method. From the point of view of for-

mulation, this is particularly interesting because it allows us to have a wide selection of solvents with which to formulate a greater number of polymers and drugs. Moreover, using different solvents to prepare NP, it is possible to modify not only the particle mean size, but also the internal and external structure of the NP (e.g. porosity and roughness). All of these NP properties lead to improve drug loading, control the release kinetics and eventually change the biological behavior of NP.

Acetone often showed a nonconsistent correlation with the physicochemical parameters studied. This may be due to the fact that acetone interacts with E L100-55 as a "poor solvent" in contrast to the other solvents that interact as "good solvents." This fact was evidenced by the turbid aspect of the organic phase constituted by the acetone and polymer, in contrast to the other E L100-55-organic solvent systems, which always showed transparent solutions. In a "good solvent," polymer chains are more disentangled from one another, and hence are extensively solvated. Conversely, in a "poor solvent," chains of polymer are more shrunken and their solvation is limited. Logically, because solvent diffusion and E L100-55 aggregation occur differently for a "good solvent" and a "poor solvent," it is highly probable that acetone will not behave like the other solvents. However, acetone was included in the analysis because it is one of the most widely used solvents in the nanoprecipitation method.

Finally, it should be pointed out that other physicochemical parameters were also considered. For example, the diffusion coefficients of solvent in water and water in solvent, as well as their ratio, were calculated from the Tyn and Calus model (41). However, no satisfactory correlations were found, neither with the NP mean size nor with the range of mean sizes of NP obtained by nanoprecipitation using different organic solvents. Polymer-solvent interaction was not considered in this study because of the impossibility to calculate theoretically the total solubility parameter of polymer ($\delta_{polymer}$) and thus the $\Delta\delta_{polymer\text{-}solvent}$ and $\chi_{polymer\text{-}solvent}$ values. The E L100-55 characteristics, basically its non-random distribution of the repeating units, did not allow accurate determination of the $\delta_{polymer}$ through the group contribution method.

Scanning Electron Microscopy

Representative scanning electron micrographs of NP obtained from the three methods are presented in Fig. 7. The NP produced by salting-out (275 nm [0.189]), using an aqueous phase with a 9.0% of PVAL content, show a spherical shape as well as a homogeneous particle size distribution (Figs. 7A and 7B). Then, micrographs of nanoprecipitation NP (Figs. 7C and 7D) also show relatively monodispersed smooth NP. These NP (211 nm [0.072]) were prepared using ethyl lactate in the internal phase and a polymer concentration of 14.4 mg/ml. Finally, although the NP prepared by emulsification-diffusion are not totally spherical (Figs. 7E and 7F), they appear to be homogeneous in size under the SEM. The asymmetry is probably due to the effect of the vacuum during freeze drying stage on the formed NP. In this case, NP were prepared with an aqueous phase containing 15% of PVAL and the mean particle size was of 173 nm [0.139].

CONCLUSIONS

For the three methods, NP mean size could be systematically varied and under most conditions narrow size distributions were observed. The size ranges of NP were broader for salting-out (123–710 nm) and emulsification-diffusion (108–715 nm) methods than for nanoprecipitation (147–245 nm). For each method, the variation in the NP mean size was associated with the physicochemical properties of the organic and aqueous phases used for NP preparation. For the salting-out and emulsification-diffusion methods, NP formation was analyzed using the theory of emulsification. First of all, the nanoemulsions formed during NP preparation methods were visualized by SEM. Secondly, the role of PVAL as emulsifier during the emulsification process was widely described. Various stabilization mechanisms were involved: (i) at the droplet interface polymer chains can not only reduce the interfacial tension, but also induce both mechanical and steric stabilization, and (ii) in the bulk solution PVAL chains can increase the external phase viscosity inducing a hydrodynamic stabilization.

Nanoparticle formation by the nanoprecipitation method was studied considering the diffusion-stranding phenomenon. Physicochemical characteristics of solvents such as the solubility parameter and the interaction parameter were used to relate the solvent properties to NP formation. In general, $\chi_{solvent\text{-}water}$ as well as $\Delta\delta_{solvent\text{-}water}$ were found to be proportional to the size of NP. Likewise, using E L100-55 as polymer, solvents with $\chi_{solvent\text{-}water} \approx 11$ (or $\Delta\delta_{solvent\text{-}water} \approx 27$ $MPa^{1/2}$) allowed NP with a wide range of particle mean sizes to be obtained. This demonstrates that water-solvent interaction, and thus the diffusion motion of the solvent, play an important role in explaining the variation of the NP size during NP preparation by the nanoprecipitation method.

Finally, these experimental results showed not only the role of each basic component (solvents, polymers, and salts) on the physicochemical properties of aqueous and organic phases (viscosity, surface tension and solubility), but also the effect of such properties on NP formation. It is important to point out that such knowledge is crucial because a rational control of the parameters or variables that influence NP formation leads to the design of NP with specific characteristics (size, surface properties, drug loading) for the targeting of cells and organs.

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