Colloid Polym Sci (2003) 281: 1184–1190
DOI 10.1007/s00396-003-0894-1

ORIGINAL CONTRIBUTION

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Poly(D,L-lactic acid) nanoparticle preparation and colloidal characterization

Received: 1 June 2002
Accepted: 17 January 2003
Published online: 16 April 2003

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Abstract Nano size-ranged poly (D,L-lactic acid) (PLA) particles were obtained from 2 and 10 w% PLA solution in water-saturated ethyl acetate by the emulsification-diffusion method. An increase in the PLA solution concentration resulted in an increase of the particle mean size from ca 260 nm to 530 nm with a broadening of the distribution. After fractionation by centrifugation, the particle mean size was 980 nm. The Pluronic F68 used in the formulations at concentration ranging from 0.5 to 5% w/v, was shown to adsorb at the interface at 0.5 mg/m^2 for both particle sizes. The molecular occupied area of 24 nm^2/molecule suggested an expanded conformation of the surfactant at the interface, though an incomplete coverage could not be excluded. Coagulation kinetics measurements revealed a critical coagulation concentration (CCC) of 1.5 M sodium chloride, indicating a steric stabilization of the colloids by the adsorbed triblock copolymer. Nonetheless, variations in zeta potential with increasing salt concentration were observed, suggesting that the surface carboxylate groups were still accessible for further modifications.

Keywords Nanoparticles · Emulsification-diffusion · Surface charge · Colloidal stability

Introduction

Over these past years, biodegradable polymer nanoparticles have appeared to be promising forms for delivery of many drugs, either hydrophobic [1, 2] or more hydrophilic ones such as proteins [3] or DNA [4, 5]. Among them, poly(D,L-lactide) or poly(D,L-lactide-co-glycolide)-based particles have received particular attention due to the biocompatibility and complete degradability of the polymers.

Most of the techniques applied for the production of particles from those preformed polymers involve the use of solvents as acetone for the nanoprecipitation [6] or salting-out [7] method, or chlorinated solvents for classical emulsification-evaporation. Recently, a new technique called emulsification-diffusion [8] has been described as presenting some advantages such a high reproducibility and a relative particle size control [9]. This process involves the formation of an O/W emulsion from a partially water-soluble solvent, which contains the biodegradable polymer, and an aqueous phase containing either a stabilizer (polyvinyl alcohol) or a surfactant (Pluronic F68). The subsequent addition of water to the system causes the diffusion of the solvent into the external aqueous phase. On diffusing, ethyl acetate carries some polymer molecules, thus forming local regions of polymer supersaturation (Fig. 1). From these regions, new globules are created (b) which further desolvate to hard particles (c), as described in [10] and interpreted in terms of thermodynamics in [11]. The role of the surfactant will be to prevent coalescence of the droplets among themselves to allow the formation of nanoparticles.

The aim of this work is to investigate the experimental conditions allowing the production, by emulsification-

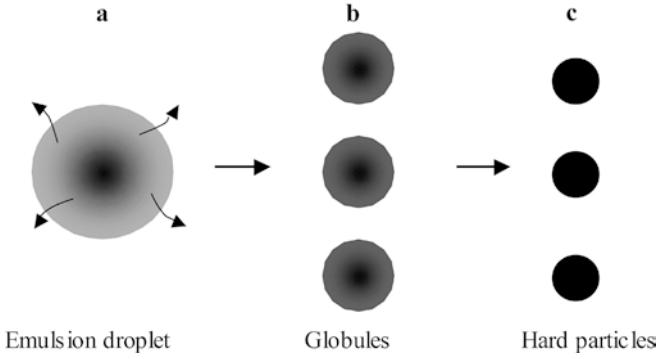

Fig. 1 Schematic representation of the emulsification-diffusion mechanism

diffusion, of poly(D,L-lactic acid) particles of two different mean sizes (300 and 1000 nm) and to characterize their interfacial and colloidal properties. Such a characterization, not described in details until now, to our knowledge, for particles produced by emulsification-diffusion, is essential for further investigations on the delivery of bioactive molecules.

Materials and methods

Materials

Poly(D,L-lactic acid) PLA50 ($M_n = 30\,000$ g/mole with $Mw/Mn = 1.7$ as the molecular weight distribution) was purchased from Phusis (Grenoble, France) and made according to [12]. Triblock-copolymer poly(oxyethylene)-poly(propylene)-poly(oxyethylene) (PEO_{76}-PPO_{29}-PEO_{76}) named Pluronic-F68 (PF68) from Sigma (USA) was used as such. The polymers were controlled by ^{1}H NMR (200 MHz, Bruker). Ethyl acetate was from SDS (France) and used as received. Milli-Q water was purified by a Millipore system Q-TM (USA).

PLA particle preparation

Ethyl acetate and water were mutually saturated before use. Two PLA solutions were prepared by dissolving 0.4 or 2 g of polymer in 20 mL of water-saturated ethyl acetate (corresponding respectively to 2% or 10%w/v). One or other of these organic solutions (internal phase) was emulsified with 40 mL of an ethyl acetate-saturated aqueous solution of PF68 at various w/v ratios (external phase), using a high-speed homogenizer (Ultra-turrax, IKA) at 8000 rpm for 5 min. The O/W emulsion obtained was then added to 215 mL of pure water under moderate stirring in order to induce diffusion of ethyl acetate into the continuous water phase (1 mL of ethyl acetate is completely miscible in 9 mL of water). This diffusion step led to the precipitation of PLA under the form of nanospheres. Ethyl acetate and a part of water were removed under reduced pressure at 40 °C. The colloidal dispersion was thus concentrated to reach a typical final PLA content of 1% w/v.

The PF68 remaining free in solution was removed by centrifugation/redispersion cycles (speed rate of 5000 or 500 g, for 20 or 5 min, respectively, depending on the mean diameter of the nanoparticles). After the centrifugation step, particles were resuspended by a short ultrasonic treatment of the dispersion. The obtained colloids were stored at 4 °C.

Hydrodynamic particle size

The hydrodynamic particle size was determined at 20 °C by quasi-elastic light scattering (QELS) (Zetasizer 3000 HS from Malvern instruments, UK) using highly diluted colloidal dispersion in 10^{-3} M NaCl solution. Each value is the average of at least three measurements.

Scanning electron microscopy (SEM)

The colloidal dispersions were first diluted (typically 0.1% solid content), then one droplet was deposited onto an aluminum grid and dried under vacuum. After sputtering with gold, the samples were observed by SEM using a S800 Hitachi microscope (at Centre de Microscopie Electronique Appliquée à la Biologie et à la Géologie, CMEABG-Claude Bernard University, Lyon 1, France).

Determination of residual Pluronic-F68 adsorbed onto particles

The cleaned particles were collected by centrifugation and dried under vacuum. The pellet obtained was dissolved in acetone-D6 for ^{1}H-NMR analysis. The amount of residual PF68 adsorbed onto particles was experimentally estimated according to the following equation:

$$N_{mg/g} = \frac{M_{PF68}}{M_{PLA}} \frac{I_{PF68}/695}{I_{PLA}} \times 1000 \quad (1)$$

in which, I_{PLA} is the peak integral of the –CH groups of the lactide unit ($\delta = 5.2$ ppm) corresponding to 1 proton, I_{PF68} is the peak integral of the $-CH_2$ and –CH groups of the PF68 ($\delta = 3.4$–3.7 ppm) corresponding to 695 protons, M_{PLA} and M_{PF68} are the molecular weights of the lactide unit (72 g/mol) and PF68 (8350 g/mol), respectively.

Surface characterization of the PLA particles

The electrophoretic mobilities of PLA particles were measured with the Zetasizer 3000 HS (from Malvern Instrument, UK). The measurements were performed at 20 °C using highly diluted dispersions as a function of salt concentration at a constant pH of 6, and also as a function of pH at a constant ionic strength. In the case of the effect of pH, an automated titration system was used. The dispersion was first diluted in 10^{-3} M HCl solution and an alkaline solution (10^{-2} M NaOH) was added in order to adjust the pH before each electrophoretic mobility measurement. All the reported values in this work are the average of triplicate measurements. The measured electrophoretic mobilities (μ) were converted to zeta-potentials (ζ) using the Smoluchowski equation [13]:

$$\mu = \frac{\varepsilon_0 \varepsilon_r}{\eta} \zeta \quad (2)$$

where ϵ_o and ϵ_r are the permittivity of vacuum and the relative permittivity of the medium, respectively, η is the viscosity of the medium and ζ is the zeta potential.

Colloidal stability

The colloidal stability of the particles was investigated by measuring the hydrodynamic particle size by QELS (as described above) instantaneously after dilution in NaCl solutions of variable concentrations, at constant pH 6.

For the smallest particles, colloidal stability was further investigated as a function of salt concentration (at pH 6) by measuring the aggregation kinetics via turbidity measurements at 500 nm wavelength using a UV spectrophotometer, (UV-mc2 from SAFAS, Monte Carlo, Monaco). Experimentally, 100 μL of dispersion (0.01%) were added to 100 μL of NaCl solution at a given concentration in a small measurement tank. After rapid homogenization, the turbidity (τ) was measured as a function of time (t) and the aggregation kinetic constant was calculated from the initial slope of τ vs. t. The critical coagulation concentration of salt was deduced from the stability factor (W) variation as function of ionic strength plotted in log-log scale, with W defined by the following ratio of fast $(\delta\tau/\delta t)_f$ and slow $(\delta\tau/\delta t)_s$ aggregation constants:

$$W = \frac{(\partial\tau/\partial t)_f}{(\partial\tau/\partial t)_s} \quad (3)$$

Results and discussion

The preparation of the particles is first described upon varying the concentration of the PLA and the polymeric surfactant (PF68). Then, the particle characterization is reported with particular emphasis on colloidal stability and interfacial properties.

PLA particle elaboration

PLA particles were prepared by the emulsification-diffusion method [8] as described in Materials and methods. After the particle formation, the organic solvent and a part of the water were removed under reduced pressure in order to concentrate the colloidal dispersion.

The standard formulation, consisting in a concentration of PF68 in the external phase of 5% w/v and that of PLA in the internal phase of 2% w/v, resulted in the formation of particles exhibiting a mean size around 250 nm, with good experimental reproducibility (Table 1). The slightly different size observed for run NP3 (compared to run NP1 and NP2) could be attributed to the fact that NP3 preparation was performed with doubled volumes with respect to that described in Materials and methods.

In order to produce particles of a larger mean size, two parameters were modulated: the PF68 concentration in the external phase and that of PLA in the internal phase, which have been previously described as affecting the colloidal dimension [9].

The PF68 concentration in the external phase was reduced down to 1% and 0.5% for NP4 and NP5, respectively, but the average particle size remained relatively constant. This is not in accordance with a previous investigation using propylene carbonate as a solvent: at 0.5% w/v, a marked increase in particle size was observed by the authors [9]. However, total suppression of the surfactant in run NP0 (Table 1) resulted in the formation of large particles (average value of 1400 nm) suggesting that the role of the PF68 is to stabilize the forming particles during the diffusion process. Moreover, a partial aggregation of NP0 was observed with time, suggesting that PF68 was also involved in the long-term stability. Because of this lack of stability, NP0 particles were not further investigated.

The effect on the final particle size distribution of the PLA concentration in the internal phase was investigated. As shown in Table 1, a significant increase of the particle mean size was observed with a 5-fold increase of the PLA concentration (about 550 nm, NP6/NP7), together with a broader size distribution, as attested by the QELS polydispersity index (> 0.3) and scanning electron microscopy (data not shown). Interestingly, this observed mean size was significantly higher than that of NP4 despite a same PF68/PLA formulation weight ratio (equal to 1). Thus, it can be assumed that only the increase of solid content (i.e., PLA concentration) allows an increase in the particle diameter.

The dispersions were washed by two cycles of centrifugation-redispersion (Table 2). For NP6/NP7 preparations, centrifugation at 500×*g* for 5 min and redispersion (twice) yielded a dispersion with an increased particle

Table 1 Preparation recipies and properties of crude nanoparticles produced by the emulsification-diffusion method (measurements were done at least in triplicate, SD ± 3%)

Crude dispersion code	PF68 in external phase (%)	PLA in internal phase (%)	Diameter (nm)[a]	PI[b]
NP1	5	2	229	0.07
NP2	5	2	230	0.07
NP3	5	2	260	0.09
NP4	1	2	262	0.09
NP5	0.5	2	265	0.13
NP0	0	2	1400	0.10
NP6	5	10	530	0.35
NP7	5	10	560	0.36

[a]Average hydrodynamic particle size
[b]Polydispersity index given by QELS: when PI is lower than 0.05, the dispersion can be considered as narrowly size distributed. Whereas, for PI > 0.05, the system is polydisperse in nature

Table 2 Effect of cleaning on the particle mean diameter

Dispersion code[a]	Mean diameter before cleaning (nm)	Mean diameter after cleaning (nm)
NP3	260 (0.09)[b]	280 (0.08)
NP5	265 (0.1)	265 (0.1)
NP6	530 (0.35)	975 (0.08)
NP7	560 (0.36)	985 (0.1)

[a]Particles were submitted to two centrifugation (5000×*g* or 500×*g* according to the initial size)-redispersion cycles. NP3 and NP5 were obtained from a 2% PLA solution with respectively 5 or 0.5% of PF68 in the external phase. NP6 and NP7 were obtained from a 10% PLA solution with 5% of PF68 in the external phase
[b]Figures in brackets, PI given by QELS

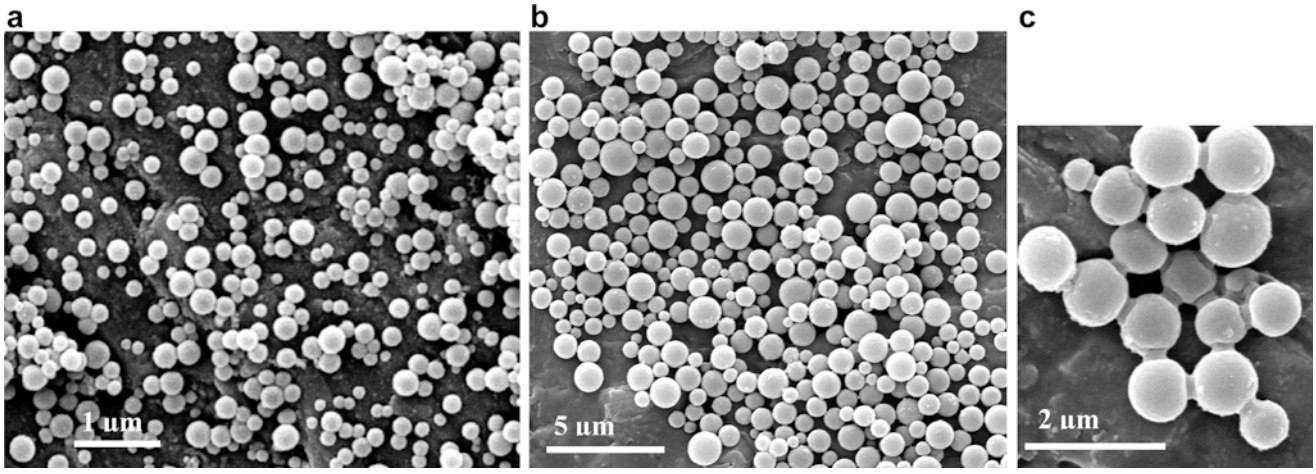

Fig. 2a–c Scanning electron micrographs of the PLA nanoparticles: **a** NP3, **b** and **c** NP6

mean diameter of 980 nm, and a polydispersity index reduced from 0.3 down to 0.1, as measured by QELS. This result suggests that particles of smaller diameter have been removed (as indeed the supernatant was turbid, with particles of 250 nm in diameter) and that only larger particles were recovered after redispersion. NP3 and NP5 particle dispersions were centrifuged at 5000×*g* for 20 min and the cleaning steps did not significantly affect the colloid mean diameter (Table 2).

Particle examination by scanning electron microscopy

The cleaned NP3 and NP6 dispersions as observed by SEM (Fig. 2) exhibited a regular spherical form and a smooth morphology. In addition, the measured particle size corroborated the hydrodynamic size given by QELS as reported in Table 2. The micrograph (Fig. 2) also revealed a relatively homogeneous distribution which confirms the QELS polydispersity index values (Table 2).

Adsorbed amount of PF68 onto PLA particles

The particles were separated by centrifugation, the supernatant was removed, and an identical amount of water was added for redispersion. This process was repeated twice. After dissolution of the cleaned particles in acetone-D_6, ^{1}H-NMR analysis was performed, leading to a spectrum on which PLA and PF68 peaks appeared, as shown in Fig. 3 for NP3. The amount of PF68 was then calculated according to Eq. 1, and the results are reported in Table 3. The residual amount of PF68 for dispersions NP3 and NP5 was close to 9 mg/g,

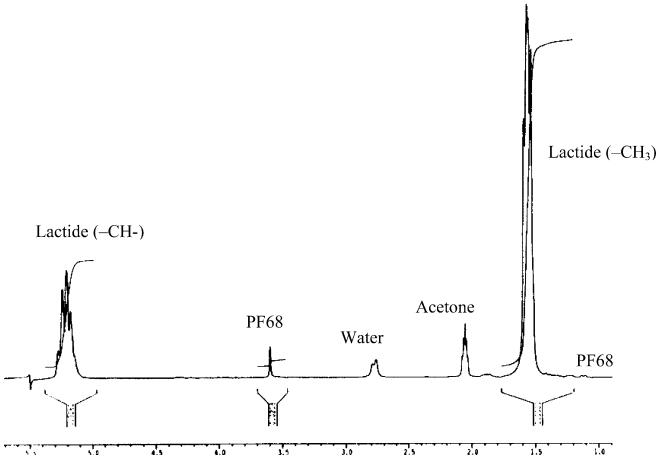

Fig. 3 ^{1}H-NMR spectrum of the cleaned NP3 particule pellet solubilized in acetone-D_6

Table 3 Residual amount of adsorbed PF68 after cleaning deduced from^{1}H-NMR analysis (Fig. 3)

Dispersion code[a]	Average hydrodynamic diameter (nm)	Adsorbed PF68 (N) (mg/g)	Specific area[b] (m^2/g)	Adsorbed PF68 (mg/m^2)
NP3	280	9.9	17.1	0.57
NP5	265	8.5	18.1	0.48
NP7	985	2.8	4.9	0.57

[a]NP3 and NP5 were obtained from a 2% PLA solution with respectively 5 or 0.5% of PF68 in the external phase. NP7 were obtained from a 10% PLA solution with 5% of PF68 in the external phase.
[b]Calculated by using hydrodynamic particle size and by assuming a monosized colloidal dispersion

hence, the weight ratio of residual PF68 to PLA is close to 0.01. In the formulations of NP3 and NP5, the initial PF68/PLA weight ratios were respectively of 5 and 0.5, i.e. 500 and 50 times higher than recovered adsorbed at the interfaces. This result suggests that the PF68 amount involved in the stabilization of the droplets during the emulsification-diffusion process was rather low. To confirm this result, the same ^{1}H NMR analyses were performed on the supernatants resulting from the centrifugations. In the first supernatant, 96% of the initial PF 68 used for NP3 elaboration was recovered. In the second supernatant, 96% of the remaining PF68 was again found in the continuous phase and, when a third cycle was performed, no PF68 could be detected (up to ^{1}H NMR sensitivity). Considering that the initial PF68/PLA weight ratio for NP3 particles is 5, we can calculate the residual PF68/PLA weight ratio using the results obtained on the supernatants. The value obtained is $5\times(0.04)^2 = 0.008$, which is quite in accordance with 0.01, the value directly determined by NMR on the dissolved particles. Hence, using two independent methods the same result was obtained.

Assuming the dispersions are narrowly size distributed, as a first approximation, the residual amount of adsorbed PF68, expressed in surface units, was similar for all particles NP3, NP5, NP7 ($\sim$0.50 mg/m^2, Table 3), thus being independent of the initial PF68 concentrations and of particle diameters. From this result, the area occupied by one copolymer molecule onto the particles was found to be 24 nm^2/molecule. For comparison, Kayes et al. reported values of 15 and 24 nm^2/molecule for PF68 and PF108 respectively, at saturation of polystyrene latexes [14]. Considering a hydrodynamic radius of 2.3 nm for the PF68 molecule [15], the theoretical area occupied by one molecule should be 16.5 nm^2/molecule, which is much smaller than we measured, suggesting quite a low packing density. This low packing density can be explained by the triblock structure of PF68, whose hydrophobic central PPO block is only 29 units, and the hydrophilic segment rather long, 2×76 EO units. Furthermore, Coombes et al. showed that PEO segments could interact with PLA [16], which would be in favor of an expanded conformation onto the particles.

Electrokinetic study of PLA particles

The effect of pH on the surface charge density of the prepared PLA particles was investigated by measuring the zeta-potential versus pH, as shown in Fig. 4. For NP3, the zeta-potential is negative in the investigated pH range (at 10^{-3} M NaCl solution), revealing the negative character of the colloidal particles. The zeta-potential reached a plateau value ($\sim$-45 mV) above pH 4.5, whereas it decreased at acidic pH. This may be attributed to the protonation of carboxylate groups of the PLA chain end.

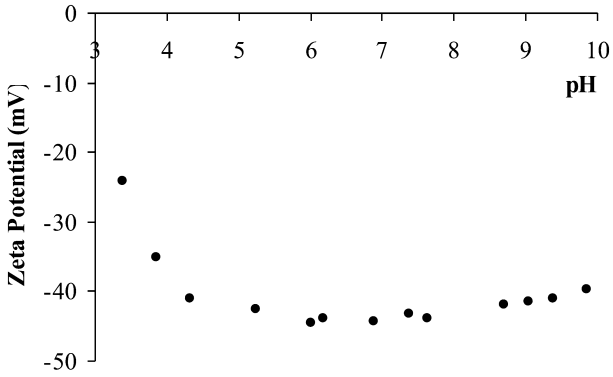

Fig. 4 Zeta potential variations with pH of NP3 dispersion

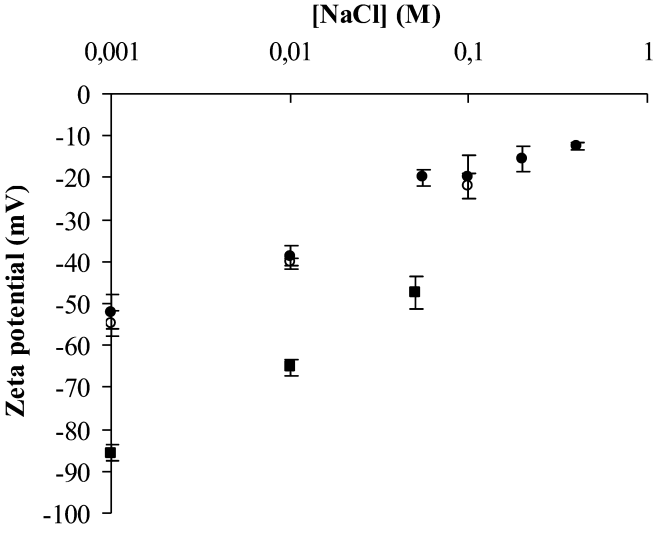

Fig. 5 Zeta potential as a function of ionic strength for NP3 (●), NP7 (○), and NP0 (■) particles. NP0: no PF68, 2% PLA organic solution. NP3 and NP7 were obtained respectively from a 2% or 10% PLA organic solution with 5% of PF68 in the external aqueous phase

The influence of ionic strength on the particle surface charge was also examined at pH 6, for NP3, NP7, and NP0 particles. As seen in Fig. 5, the zeta-potentials (in absolute value) were found to decrease with increasing ionic strength. Moreover, for NP0, the zeta-potential was higher in absolute value than for NP3 and NP7 samples, because of a partial screening of surface charges of the latter induced by the presence of PF68 at their interfaces. The similar behaviors for NP3 and NP7 observed in Fig. 5 (as some points overlap) probably arose from the presence of the same amount of adsorbed Pluronic as previously shown in Table 3.

In order to estimate the surface potential (Ψ_0), the Eversole and Boardman equation [17] was used by

Table 4 Surface potential ψ_0 and surface charge density σ_o of NP3, NP7 and NP0 particles

Dispersion code[a]	Surface potential ψ_0 (mV)	σ_o[b] ($\mu C\ cm^{-2}$)
NP0	−92	1.10
NP3	−52	0.45
NP7	−60	0.54

[a]NP0 : no PF 68, 2% PLA. NP3 and NP7 were obtained respectively from a 2% or 10% PLA solution with 5% of PF68 in the external phase
[b]Surface charge density ($\mu C\ cm^{-2}$) estimated from the surface potential using Gouy-Chapman's equation for 1 mM NaCl

plotting -Ln[tanh(eζ/4kT)] versus κ ($\kappa = 3.3C_s^{\frac{1}{2}}$ the reciprocal of Debye length in nm^{-1} where C_s is the salinity concentration). The complete equation is:

$$\tanh\left(\frac{e\varsigma}{4kT}\right) = \tanh\left(\frac{e\Psi_0}{4kT}\right)\exp(-\kappa\Delta) \quad (4)$$

where ζ is the zeta potential in V, Δ is the distance from the particle surface to the shear plan (in nm), e is the electron charge, κ is the Boltzman constant and T is the absolute temperature in K. The surface potential Ψ_0 could be estimated at the zero value of κ. The values obtained are reported in Table 4, together with the surface charge density σ_0, deduced from surface potential Ψ_0 using a plane surface model equation (Gouy-Chapman), adapted for colloids by Carrion et al. [18].

The relationship between surface charge density and surface potential (Ψ_0) is given by the following equation:

$$\sigma_0 = \left[(8kTn\varepsilon_0\varepsilon_r)^{\frac{1}{2}}\right] sh\left(\frac{ze\Psi_0}{2kT}\right) \quad (C\ m^{-2}) \quad (5)$$

where n is the ion number per volume unity (ion $.m^{-3}$), ϵ_r is the dielectric constant of water and ϵ_0 is the dielectric permittivity (8.85×10^{-12} C V^{-1} m^{-1}).

The surface potential and charge density values (in absolute value), for NP0 appeared to be much higher than those obtained for NP3 and NP7, as deduced from the zeta-potential variation versus NaCl concentration. The deduced surface potentials were found to be close to the zeta-potential as generally observed for moderately charged colloid systems. The dependence of the surface potentials on the interfacial Pluronic concentration clearly appears on comparing NP0 to NP3 and NP7.

Colloidal stability

The particle mean diameter was determinated by QELS as a function of ionic strength in order to appreciate the particle colloidal stability. As shown in Fig. 6, the aggregation occured at a higher salinity for NP3 and NP7 particles (more than 1 M NaCl) than for NP0 (about 0.2 M), confirming our previous naked eye observations and suggesting the contribution of PF68 to steric stabilization of the particles.

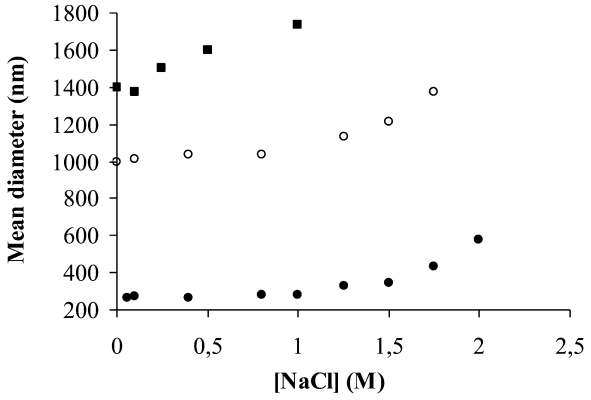

Fig. 6 Particle mean diameter (QELS) as a function of ionic strength for NP3 (●), NP7 (○), and NP0 (■) particles (at pH 6). NP0: no PF68, 2% PLA. NP3 and NP7 were obtained respectively from a 2% or 10% PLA solution with 5% of PF68 in the external phase

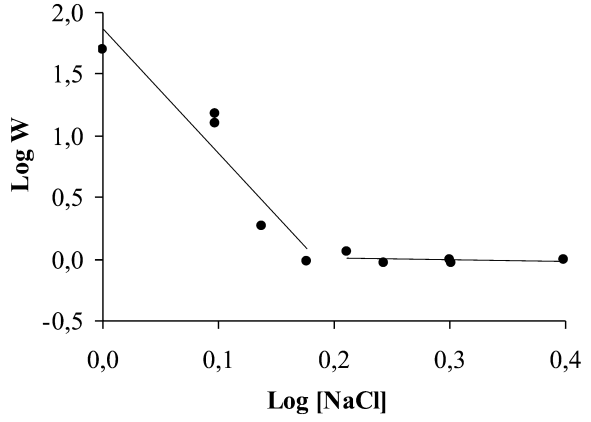

Fig. 7 Stability factor (W) as a function of electrolyte concentration for NP3 particles (at pH 6)

The colloidal stability of NP3 particles was then further investigated by studying the aggregation kinetics by turbidimetry as a function of ionic strength (at constant pH = 6) and by plotting the stability ratio (W) versus salt concentration in log-log scale as given in Fig. 7. As shown, two straight lines, as theoretically predicted, were observed: the first line exhibits a negative slope and the second line is horizontal ($W = 1$) above a given salinity concentration. The critical coagulation concentration (CCC) was determined from the junction point between both lines. The CCC value deduced from Fig. 7 was found to be around 1.5 M, confirming the results obtained by size measurements as a function of ionic strength (Fig. 6).

This relatively high CCC value strongly suggested that the colloidal dispersions prepared in the presence

of PF68 are indeed principally sterically stabilized. Whereas, the colloidal stability of the NP0 sample (prepared in the absence of Pluronic) is ensured by repulsive electrostatic interactions, as evidenced from the high zeta-potential values and the low salinity required for particle flocculation. Such a colloidal stability enhancement due to the interfacial Pluronics has already been observed for various similar systems, such as PLGA particles bearing Poloxamer-407 exhibiting a flocculation point around 4 M NaCl compared to 0.1 M for bare particles [19].

Conclusion

This work focused on the synthesis and characterization of biodegradable PLA nanospheres. The emulsification technique allowed the synthesis of different mean sizes of particles by changing the PLA concentration. Surface characterization showed that about 0.5 mg/m^2 of PF68 remained efficiently anchored at the surface of the particles, which conferred a steric stabilization. This corresponds to a surface area occupied by one molecule of 24 nm^2, suggesting either a relatively poor coverage or an expanded conformation of the surfactant at the interface, due to its chemical composition. Nevertheless, the surface charges remained available as shown by the decrease in zeta potential with increasing salinity. This is an important result for further use of these particles. Thus, the adsorption of proteins or nucleic acids can be envisioned, with a prior surface particle modification with a cationic agent. Moreover, the stealth aspect and reinforced stability of the particles conferred by the residual adsorbed PF68 could be of a beneficial interest in the case of in vivo applications.

Acknowledgements. The authors are grateful to C. Novat and CMEABG (University Claude Bernard, Lyon) for scanning electron microscopy and to Fondation Mérieux for funding T.T.

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