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Complementary use of flow and sedimentation field-flow fractionation techniques for size characterizing biodegradable poly(lactic acid) nanospheres

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Abstract

Poly(lactic acid) nanoparticles were synthesized using a modified evaporation method, testing two different surfactants (sodium cholate and Pluronic F68) for the process. During their formulation the prodrug 5'-octanoyl-CPA (Oct-CPA) of the antiischemic N⁶-cyclopentyladenosine (CPA) was encapsulated. Three different purification methods were compared with respect to the influence of surfactant on the size characteristics of the final nanoparticle product.

Flow and sedimentation field-flow fractionation techniques (FIFFF and SdFFF, respectively) were used to size characterize the five poly(lactic acid) particle samples. Two different combinations of carrier solution (mobile phase) were employed in the FIFFF analyses, while a solution of poly(vinyl alcohol) was used as mobile phase for the SdFFF runs. The separation performances of the two techniques were compared and the particle size distributions, derived from the fractograms, were interpreted with the support of observations by scanning electron microscopy. Some critical aspects, such as the carrier choice and the channel thickness determination for the FIFFF, have been investigated. This is the first comprehensive comparison of the two FFF techniques for characterizing non standard particulate materials. The two FFF techniques proved to be complementary and gave good, congruent and very useful information on the size distributions of the five poly(lactic acid) particle samples.

Keywords

Sedimentation field-flow fractionation; Flow field-flow fractionation; Poly(lactic acid) nanospheres; Particle size distribution

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1. Introduction

Biodegradable polymer particles, both micro and nano sized, have become an important area of research in the field of drug delivery because of their ability of deliver a wide range of drugs to various areas of the body for sustained periods of time [1]. Quite recently, two new kinds of nanoparticles have attracted the attention of scientists as promising drug carriers, namely polymeric micelles and dendrimers [2]. However, poly(lactic acid) (PLA) remains one of the most commonly used polymers for drug delivery because its biodegradation leads to pharmacologically inactive substances, which are adsorbed by the body or removed by the metabolism [3].

The size determination of the particles is crucial because different organs are targeted by different sized particles [3]. Nanoparticles, for example, have several advantages over larger micro-particles since they have been proven to be better suited to intravenous delivery. There are also tremendously promising applications for nanoparticles in oral administration of vaccines and cancer therapies [4]. The drug efficacy and its release are strictly related to particle uptake, which is in turn greatly dependent on size, surface charge and other physicochemical properties of the nanosized colloidal drug carrier systems.

The particle dimensions can be controlled during their synthesis by adjusting several experimental parameters. For example, the stirring rate, the concentration of additives (like surface active agents), and the internal phase volume are important parameters that influence the diameter of the nanospheres in the emulsification – solvent evaporation synthesis technique [5].

The size characterization of pharmaceutical nano or micro spheres is commonly carried out by electron microscopy (both transmission and scanning, TEM and SEM, respectively). Light scattering techniques, such as photon correlation spectroscopy (PCS), also known as quasi-elastic light scattering (QELS), are also frequently used. These techniques are rapid but of low resolution since they are not fractionation methods [6] and measurements are made of the entire particle mixture. More useful are the fractionation methods, which include disc centrifugation, hydrodynamic chromatography, size exclusion chromatography [7] and more recently field-flow fractionation: higher resolution is achieved since different particle fractions are first separated and then sized.

The field-flow fractionation (FFF) techniques are members of an established family of elution techniques that differ in the types of fields used to induce separation [8,9]. FFF may be used for the measurement of numerous properties of macromolecules and colloidal particles, including particle mass, size, and density. The property characterized is that which interacts with the field applied. FFF techniques have been successfully applied to the size characterization of particles of pharmaceutical interest, such as liposomes [10–12], gelatin nanoparticles [13,14], nanospheres [15], and microspheres [16,17].

The aim of this study was to use both flow and sedimentation FFF techniques to size characterize different PLA nanosphere samples, encapsulating 5'-esters of N⁶-cyclopentyladenosine (CPA), a prodrug which has a neuroprotective role [18], and to critically examine the complementary nature of the data provided by the two techniques. For the first time, data obtained using flow FFF (FIFFF) are presented along with data obtained using sedimentation FFF (SdFFF) in order to check differences in the particle size distributions (PSDs) of nano-sized PLA samples. FIFFF is unique in its ability to separate components based solely on differences in their size, while SdFFF, which uses centrifugal acceleration, sorts components according to their buoyant mass. Both FIFFF and SdFFF elution profiles can be converted directly into PSDs, provided the experimental conditions are appropriate for the use of the FFF retention theory [9]. SdFFF has the highest size selectivity among the common FFF

techniques, but in order to determine the PSD, particle density must be acquired from other methods. The morphology of the nanoparticles was also examined using a scanning electron microscope (SEM).

The considered PLA samples differ by the surfactant used during their formulation and by the recovery-purification methods used. These parameters affect the PSD of the nanoparticles, the prodrug loading, its release modalities and the related stabilisation in whole blood. The prodrug 5'-esters of CPA have the advantage of being more stable in blood and are better able to diffuse through lipid barriers compared to adenosine and all its N⁶-substituted compounds. They are equally capable of activating the adenosine receptor subtype A₁. The A₁ agonist compounds have promising effects against SNC ischemic damage but their clinical use is limited since they are quickly degraded in the blood [18] and they are not able to reach the brain by a systemic pathway [19].

2. Theory

Field-flow fractionation is a family of chromatography-like elution techniques, whose theory is well documented in the literature [8,9]. Separation occurs inside a thin empty channel, usually of rectangular cross section of high aspect ratio, in which flows a liquid mobile phase. Under conditions of laminar flow, the liquid phase assumes a parabolic flow profile across the thin dimension with the highest velocity streamlines at the center, decreasing to zero velocity at the walls. The sample is injected as a small pulse into the inlet line using an injection loop and is transported into the channel by the flow of carrier liquid. The flow is temporarily interrupted and a field is applied across the thin dimension of the channel, perpendicular to the walls. The particles that interact strongly with the field, and/or possess small diffusion coefficients, form thin steady state layers that are located close to the so-called accumulation wall. Those that interact less strongly with the field, and/or have higher diffusion coefficients, form thicker, more diffuse steady state zones next to the wall. The concentration profiles of the analytes in the FFF channel are in dynamic steady state brought about by balance between the motion toward the wall induced by the field and the opposing motion of diffusion. The concentration profiles decay exponentially with distance from the accumulation wall, and the exponential decay constant is related to physicochemical characteristics that govern the two opposing motions. On resumption of the mobile phase flow, components forming thinner zones are confined to regions of slowly moving fluid and are transported more slowly than those forming thicker zones. It follows that the times of elution are related to the exponential decay constants of the concentration profiles and therefore to the physicochemical characteristics of the sample components.

The ratio of the distance ℓ between the accumulation wall and the center of gravity of an analyte zone and the channel thickness w is known as the FFF retention parameter λ , and is given by

$$\lambda = \frac{\ell}{w} = \frac{D}{|u|w} = \frac{kT}{|F|w} \quad (1)$$

where D is the particle diffusion coefficient, $|u|$ is the magnitude of the field-induced particle velocity, k is the Boltzmann constant, T is the absolute temperature, and $|F|$ is the magnitude of the force on a particle due to its interaction with the field. The concentration profile is then given by

$$c(x)=c_0\exp\left(-\frac{x}{\ell}\right)=c_0\exp\left(-\frac{x}{\lambda w}\right) \quad (2)$$

where x is the distance from the accumulation wall and c_0 is the concentration at the wall. The experimental factor that describes the retardation of an analytical zone caused by its compression by the field into the region of slower fluid velocities is the retention ratio R , defined by

$$R=\frac{v_{zone}}{\langle v \rangle} \quad (3)$$

where v_{zone} is the average velocity of the analyte, and $\langle v \rangle$ is the average velocity of the carrier solution. This is experimentally measured as

$$R=\frac{t^0}{t_r} \quad (4)$$

where t^0 is the elution time for a non retained material, or the void time, and t_r is the elution time for a retained analyte, or the retention time.

The fluid velocity profile in the thin channel is parabolic with maximum velocity at the center and zero velocity at the walls. Knowing the functional expressions for both the concentration and fluid velocity profiles, an analytical expression for R as defined by Eq. (3) may be derived. The final result, corrected to take into account the finite size of the sample particles, which are excluded from fluid streamlines adjacent to the wall due to their physical dimensions [20] is given by

$$R=6\left(\alpha-\alpha^2\right)+6\lambda[(1-2\alpha)\coth((1-2\alpha)/2\lambda)-2\lambda] \quad (5)$$

where α is equal to $d/2w$, with d the diameter of the particle. The steric exclusion factor α may be significant for particles larger than a few hundred nanometers in diameter. For well retained samples, there are approximate expressions of Eq. 5 of acceptable accuracy [21].

In FIFFF the “field” consists of a cross-flow of carrier liquid, and the transverse force on the particles is caused by the viscous drag of the fluid cross flow. To generate this kind of field, the channel walls must be semi-permeable, and a membrane acts as the accumulation wall, so that carrier liquid is allowed to pass but not the analytes. The explicit expression for the retention parameter λ (cf. Eq. 1) in FIFFF is given by

$$\lambda=\frac{D}{|u|w}=\frac{DV^0}{\dot{V}_c w^2}=\frac{kTV^0}{f\dot{V}_c w^2} \quad (6)$$

where V^0 is the geometric volume of the channel and $\dot{V}_c$ the volumetric cross-flow rate. The Nernst equation shows that the ratio of thermal energy kT to diffusion coefficient is equal to

the particle friction factor, i.e., $f = kT/D$. The final form on the right side of Eq. (6) follows by substitution for D . Note that in the case of FIFFF the field-induced particle velocity is simply equal to the cross flow velocity. It is evident from Eq. (6) that FIFFF separates colloids according to their diffusion coefficient, which can be related to their hydrodynamic diameter through Stokes' law $f = 3\pi\eta d_h$. The Stokes diameter, d_h , is approximately related to the observed retention time t_r by the simple expression valid for well-retained samples:

$$d_h = \frac{2kTV^0 t_r}{\pi\eta w^2 \dot{V}_c t^0} = \frac{2kT \dot{V} t_r}{\pi\eta w^2 \dot{V}_c} \quad (7)$$

in which $\dot{V} (= V^0 / t^0)$ is the longitudinal channel flow rate.

In SdFFF the channel is wrapped around the inside of a centrifuge basket with a radius of curvature r and is spun with an angular velocity ω . SdFFF separates colloids and particles on the basis of differences in their effective mass [22–27]. In the case of SdFFF, λ is given by the equation

$$\lambda = \frac{kT}{V_p \Delta\rho G w} = \frac{6kT}{\pi d^3 |\rho_p - \rho| \omega^2 r w} \quad (8)$$

in which V_p is the volume of a particle, $\Delta\rho = |\rho_p - \rho|$ is the density difference between the particle and suspending fluid, $G = \omega^2 r$ is the centrifugal acceleration, and d is the spherical particle diameter. In terms of particle diameter, SdFFF therefore exhibits a higher selectivity than FIFFF, and larger particles in a polydisperse sample may be very strongly retained compared to the smaller particles. It is therefore common practice to program a decay of field strength during an analysis so that the separation of a broad distribution of sample components can be accomplished in a reasonable time. The most common field programming for SdFFF is described by the power decay function [28]

$$S = S_0 \left(\frac{t_1 - t_a}{t - t_a} \right)^p \quad (9)$$

where the field strength S is held constant at an initial level S_0 for a pre-decay period t_1 and decreased according to a power function of elapsed time t . The second time constant t_a is generally set to $-p t_1$. The retention time for well retained analytes is then related to the field decay parameters as follows [28]

$$t_r = (p+1)t_1 \left\{ \frac{t^o}{6t_1\lambda_0} \right\}^{1/(p+1)} - p t_1 \quad (10)$$

where λ_0 is the value of λ at initial field strength S_0 . In the case of SdFFF, the power p is usually set to 8 in order to obtain constant fractionating power or relative resolving power [28].

In the case of SdFFF, the detector signal as a function of the retention time t_r , known as the fractogram, may be transformed into a size distribution plot using Eqs. 8 and 10 for elution

during field decay and the approximation $t_r \approx t^0 / 6\lambda_0$ for the pre-decay period. In practice, a more accurate approach is taken that involves numerical solution of the equation

$$t^0 = \int_0^{t_r} R dt \quad (11)$$

with R given by Eq. 5 and taking into account the actual field decay as monitored during the sample elution. The approach has been described in detail elsewhere [29], and is applicable to both SdFFF and FIFFF, as well as any other form of FFF for which an expression for R may be derived. The approach yields a more accurate result than would be obtained using the 6λ approximation for R , and also takes into account a first order correction for steric effects through the inclusion of terms in α . The calculations therefore yield an estimate for the position of steric inversion, discussed later, that equations such as Eqs. 7 and 10 cannot..

3. Experimental

3.1 Materials

Polystyrene – divinylbenzene microsphere standard, with nominal diameter of $7.0 \pm 0.2 \mu\text{m}$, and Nanosphere™ standard polystyrene samples with nominal diameters of $92 \pm 3.7 \text{ nm}$, $240 \pm 6 \text{ nm}$, and $350 \pm 7 \text{ nm}$, were obtained from Duke Scientific Co. (Palo Alto, CA, USA). The 1% w/v commercial suspensions were diluted in the respective carrier solutions prior their use in calibration of the FlowFFF system. A mixture of PS standard of 194, 258 and 360 nm was given by Postnova Analytics together with the SdFFF instrument to test it.

Polyvinyl alcohol 8–88 (PVA), degree of hydrolysis 86.7–88.7 mole %, having M_w of 67,000 g/mol (81383 Fluka Chemie GmbH, Buchs, Germany), FL-70 (# SF105-1, Fisher Scientific, Fair Lawn, NJ, USA), sodium dodecyl sulfate - SDS (# 02674-25, Fisher Scientific) and NaN_3 (EM Science, Gibbstown, NJ, USA) were used to prepare different mobile phase solutions for the FFF systems.

Deionized ($18 \text{ M}\Omega\text{cm}^2/\text{cm}$) water (Milli-Q system, Waters Corp., Milford, MA, USA) was used in the preparation of all the solutions and suspensions.

3.2 Nanoparticle preparation and purification

The poly(lactic acid) (PLA) nanoparticles were prepared according to the nanoprecipitation method [30], as detailed in a previous publication [31]. The particle synthesis was inspired by a method proposed by Fessi et al. [30] but was simplified in order to use just one emulsifier. Two different stabilizing agents were tested for the synthesis: the ionic surfactant sodium cholate (or SDS - Sigma, St. Louis, MO, USA), and the non-ionic Pluronic® F68 (BASF Corp., Mount Olive, NJ, USA). The density of PLA nanoparticles was assumed to be 1.25 g/ml , corresponding to that of high molecular weight PLA [32,33]. Three different methods were used to purify the nanospheres following synthesis: i) dialysis, ii) gel-filtration chromatography and iii) ultracentrifugation (see Table 1).

Dialysis was carried out by introducing 10 mL of nanoparticle suspension into a hermetically sealed dialysis tube (T3 Cellu-Sep membrane, Interchim, Montluçon, F: molecular weight cut-off 12,000) and dialyzing at 37°C against 500 ml of deionized water for 2 hours under magnetic stirring.

Gel-filtration chromatography was carried out using a column containing about 160 ml of Sepharose CL-4B gel (Sigma-Aldrich). The column had a length of 50 cm and an inner diameter

of 2 cm. The nanoparticles were eluted using deionised water at a flow rate of 1.5 ml/min. The appearance of the nanoparticles in the eluate was detected in about 20 minutes using a turbidimeter (model DRT 15-CE, HF Scientific Inc., Fort Meyers, FL, USA).

The nanosphere purification by ultracentrifugation was performed at 10,000 gravities for 10 minutes using a F38/36 rotor (DuPont). The nanospheres were centrifuged three times, resuspending them each time in 2 ml of deionized water.

In each case, the samples were prepared and purified in triplicate and subsequently freeze-dried for a period of 24 hours (Lyovac GT2; Leybold-Heraeus, Hanau, Germany) to obtain a fine powder.

3.3 FIFFF system

The FIFFF was a Model F-1000 symmetric FIFFF channel (Postnova Analytics, Salt Lake City, UT, USA). The channel was formed from a 0.0254 cm thick Mylar[®] spacer, and had a length of 29.5 cm (tip-to-tip) and breadth of 2 cm. Regenerated cellulose (RC) membranes, having a nominal cut-off of 10 kDa, were purchased from Postnova Analytics. The geometrical channel void volume was calculated to be $(1.41 \pm 0.03) \text{ cm}^3$. The extra column volume, due to the inlet and outlet connections to the injection valve and the detector, was estimated as 0.265 cm^3 .

Carrier liquid was delivered to the channel using an HPLC pump (model GP40 Gradient pump, Dionex Corp., Sunnyvale, CA, USA), while the cross flow was given by a pair of VersaPump6 pumps (Kloehn Ltd., Las Vegas, NV, USA).

An injection volume of 20 μl was used for the PS standard suspension and 50 μl for the PLA samples; different respective loop volumes were employed with a Rheodyne 7725i sample valve (Rheodyne LLC, Rohnert Park, CA, USA). The outlet tube from the channel was connected to a UV detector operating at a fixed wavelength of 254 nm (VUV-10, HyperQuan Inc., JMST Systems, Colorado Springs, CO, USA), whose cell had a volume of 32.4 μl and an optical path length of 10 mm. The UV signal was collected using a DI-154RS acquisition card (DATAQ Instruments, Inc., Akron, OH, USA), and stored using the included software on a PC-compatible computer. The fractograms were converted to particle size distributions (PSDs) using software developed in 1990 at the FFF Research Center (FFRC) (University of Utah, Salt Lake City, UT, USA).

3.4 SdFFF system

The SdFFF was a Model S101 (Postnova Analytics, Salt Lake City, UT, USA) with hastelloy C channel walls. The rotor radius r was 15.5 cm. Nominal channel dimensions, cut from a Mylar spacer, were 90 cm (tip-to-tip length), 2 cm (breadth) and 0.0254 cm (thickness). The void volume of 4.86 cm^3 was experimentally determined from injections of sodium benzoate.

An HPLC Pump model 422 Master (Kontron Instruments, Milan, Italy) was used to deliver the carrier solution. The outlet tube from the channel was connected to a UV detector operating at 240 nm (Uvidec 100, Jasco Ltd., Tokyo, Japan). The volume of the cell was 8 μl and the optical path 10 mm. Signal data were collected using an ACRO-900 12 bit I/O acquisition system (Acrosystems Co. Beverly, MS, USA) and stored on a PC-compatible computer. Data reduction of the fractograms was again carried out using appropriate software developed in 1990 at the FFFRC.

3.5 Other instruments

A Vortex-Genie Mixer, Model k-550-G (Scientific Industries, Inc., Bohemia, NY, USA) was used to distribute the particle suspensions before injection to the sample loop. A Branson B-300

Tabletop Ultrasonic bath, purchased from Fischer Scientific (Pittsburg, PA, USA) was employed to physically disperse the PLA suspensions. For the same purpose, a probe-type Sonicator cell disruptor Model W-220F (Heat Systems-Ultrasonics Inc., Farmingdale, NY, USA) was also used. This generated a power up to 200 watts at a frequency of 20 kHz.

A scanning electron microscope (SEM) (Philips XL40, FEI Company, Eindhoven, NL) was used to evaluate both size and morphology of the PLA nanospheres. The samples were mounted on aluminum stubs using double-sided sticky tape (TAAB Laboratories Equipment, Ltd. Aldermaston, Berkshire, UK). Before SEM analysis, samples were coated under argon atmosphere with gold palladium to a thickness of 10 nm (Emitech K550 Sputter Coater, Emitech, Ltd., Ashford, Kent, UK).

4. Results and Discussion

As mentioned previously, two different surfactants were used for particle synthesis. In each case the nanospheres produced were quite polydisperse in size, as shown by the diameter data determined by SEM and listed in Table 1. The nature of the surfactant was found to strongly influence the size of the particles. With use of SDS the nanospheres had a mean diameter of approximately 90 nm, while with Pluronic F68 the mean particle diameters were around 220–300 nm up to 390 nm, depending on the method used for purification (see Table 1). These results are in qualitative agreement with those reported previously in the literature [34], according to which the use of ionic surfactant produces considerably smaller nanoparticles (70 ± 2 nm) compared to a nonionic formulation (200 ± 5 nm).

As described earlier, the particles were purified using three different methods: dialysis, gel-filtration and ultracentrifugation. Dialysis allowed recovery of 100% of the mass (samples A and C), but this method has the drawback of also entrapping a fraction of the stabilizing agent. The residual amount of stabilizing agent was determined by mixing a known weight of dried particles with a given volume of dichloromethane and using a colorimetric method to determine the concentration of Pluronic F68, and an HPLC assay for the extracted SDS [31]. These data are also reported in Table 1. The gel-filtration and ultracentrifugation methods were more efficient at removing the stabilizing agent but they resulted in an average particle mass loss of 30% (gel-filtration gave the lowest recovery at 60–70%, samples B and D in Table 1).

Size characterization: FFF results and SEM

Accurate size characterization of the nanoparticles requires that they be analyzed immediately after their purification. This is to avoid changes in the size distribution due to degradation reactions (such as hydrolysis) or Ostwald ripening, for example. In fact, lipid nanoparticle dispersions are not particularly stable, and in most cases an increase in particle size is observed over a short period of time [34].

In this work, immediately following the particular purification method used, a part of each sample was subjected to SEM analysis and the remainder was freeze-dried (lyophilized) for better preservation over extended periods of time. Lyophilization guarantees better chemical and physical stability for the nanoparticles than would be obtained for their aqueous dispersions. Lyophilization is considered advantageous even though it is known that resuspension of the dried samples may be difficult. Resuspension may be difficult because it involves, at least in its initial stages, conditions which favor particle aggregation (high particle concentration and high osmotic pressure) [34].

Flow FFF

The flow FFF system was operated in the normal mode [35], for which the retention parameters are, in theory, quantitatively related to the range of diffusion coefficients exhibited by the sample, provided all the geometrical dimensions of the channel are accurately known. Under these ideal conditions, calibration is not required and the fractograms can be converted to PSDs without further experiments. The parameter that is susceptible to the highest uncertainty for this approach is the channel thickness w . This is because one of the channel walls is a membrane, and under different chemical conditions it may shrink or swell, resulting in change of the effective channel thickness. This phenomenon is particularly evident with the ultrafiltration membranes which are cast on compressible fabric backing material [36]. Also, when a membrane expands, w may not be constant over the wall surface, and if there is some variation in w across the channel breadth there would be non-uniform flows inside the channel. The membrane behaviour therefore has an influence on the choice of carrier solution composition. A suitable carrier composition should not produce an appreciable swelling of the membrane, it should not interfere with the detector response, it should be conducive to dispersion of the standards and of the samples, and it should not induce particle-wall interaction [37]. It has been observed experimentally that the greatest variations of w are caused by changes in pH and ionic strength of the solution. In order to get the most appropriate value of w , every time that the mobile phase is changed it is routine practice [38] to run a set of nanoparticle standards and back-calculate the value of w consistent with the observed retention.

The first set of experiments was produced under well proven experimental conditions for FIFFF [39]: a 10 kDa RC membrane and a carrier solution of 0.1% v/v FL-70 and 0.02% w/v NaN_3 . The measured pH of the carrier was 10.16 at 24.2 °C and, by taking into account only the contribution of NaN_3 , the ionic strength I was 3×10^{-3} M. An estimate of the effective channel void volume V^0 was obtained by injecting the supramicron PS beads ($d = 7 \mu\text{m}$), without applying any relaxation procedure or cross-flow. The longitudinal flow rate $\dot{V}$ was 1.05 ± 0.01 ml/min, and the channel void volume, computed by measuring the void time t^0 according to the breakthrough method [40], was found to be 0.763 ml. The back-calculated channel thickness was therefore equal to 0.0145 cm; 43% lower than the nominal value.

Figure 1a shows the fractogram obtained by injecting a mixture of three PS Nanosphere™ standards, setting the cross flow $\dot{V}_c$ at 0.76 ml/min and the longitudinal flow $\dot{V}$ at 1.72 ml/min. The selectivity plot (Figure 1c) yielded a best fit of $\log d = (-0.88 \pm 0.04) \log R + (0.64 \pm 0.10)$, for 95% confidence limits. The diameter selectivity S_d of 0.88 is close to the ideal, high retention limiting value of 1 (see Eq. 7). Assuming the nominal sizes of the PS beads are correct and taking retention times at the peak maxima, the back-calculated void volume V^0 was 1.10 mL and the correspondent channel thickness was found to be 0.0186 cm; 27% lower than the nominal value. The particle size distribution (PSD) shown in Figure 1b was calculated using this value for w . The agreements between the nominal values and the experimental data differed of -2.3% for the first standard, -6.5% for the second while an +7.3% difference was observed for the third. These results underline how the measurement of the channel thickness w , together with the void time t^0 , are of fundamental importance when the FFF techniques are used to obtain the PSD of a sample. A difference of the 27% in w corresponds to a difference of the 38% in d_h (see Eq. 7).

After the channel thickness calibration, the PLA samples were dispersed in carrier solution and, unless otherwise described, bath sonicated for 30 minutes prior to FFF analysis to break down any aggregates which could have formed during the purification and/or drying and resuspension processes.

Figure 2 shows examples of fractograms obtained for the samples A, C and D. The direct comparison of the fractograms in terms of retention time is not meaningful, since the

experimental set-up did not allow the use of identical flow rate conditions. Sample A, which was formulated using SDS, was separated by applying a cross flow $\dot{V}_c$ of 0.85 ml/min and a longitudinal flow $\dot{V}$ of 1.99 ml/min. A broad peak was obtained following the void time with all particles eluted within 30 minutes. The fractogram was converted into a PSD (also shown in Figure 2), assuming a channel thickness of 0.0186 cm. The majority of the fractionated particles were calculated to range from 50 nm up to approximately 400 nm. Two peak maxima at around 8 and 16 minutes correspond to particle diameters of 96 and 190 nm, respectively. Particles larger than about 220 nm were assumed to be aggregates since the SEM micrographs (Sample A, Figure 3) indicate that 80% of the particles should be smaller than 100 nm (see Table 1), with some particles of around 200 nm present.

The fractograms for samples C and D (Figure 2), which were both formulated using Pluronic F68 as stabilizing agent, show very broad peaks, ending after 50 minutes, well past the theoretical points of steric inversion [41] indicated on each plot, i.e., the points after which no particles should elute. This suggests that there must be significant interaction between the particles and the membrane surface. The conversion to PSD cannot take into account material that elutes after the steric inversion, and these fractions of the fractograms could not be incorporated into the calculations. Furthermore, the retention times for particles eluting before the theoretical inversion cannot be expected to correspond to ideal retention theory when there is significant membrane interaction. The PSDs, obtained by assuming $w = 0.0186$ cm and $\dot{V}^0 = 1.10$ ml, shown for samples C and D cannot therefore be considered to represent true distributions. Given the possible influence of particle-membrane interactions, there may also be some uncertainty associated with the PSD determined for sample A.

By inspecting the samples with SEM and by measuring an average of 500 particles for each, it was determined that sample C contains nanospheres of 220 ± 50 nm, with 60% smaller than 250 nm, and sample D spheres of 300 ± 40 nm with only 30% < 250 nm (Table 1). A small number of very large particles, up to 1 μ m in diameter, were seen to be present in both samples C and D, as may be seen in Figure 3.

To test the hypothesis that aggregation caused the broadening of the obtained PSDs, relative to the composition seen with the SEM, methods of improving the dispersion state of the PLA particles were explored. Specifically, application of mechanical force, such as a shear force or that provided by application of ultrasound, and variation of the physico-chemical environment by changing the pH and the ionic strength of the dispersion solution, were explored. The latter conditions play a significant role in controlling the attraction and repulsion that occurs between particles as well as between particles and the membrane.

The pH of the carrier was relatively high in these initial experiments. The pH of the solution has been reported to have an important role in the *in vitro* degradation of PLA, with degradation being accelerated in acidic or basic conditions [42]. Hydrolysis is favoured by high pH conditions, as in this case, and by increase of temperature, which occurs during sonication [43]. So as not to degrade or even dissolve the PLA nanoparticles, a bath sonicator was used to apply a relatively gentle sonication.

Figure 4 shows the effects of different mechanical actions used to disperse the PLA particles. Sample D was chosen for this study. The fractogram shown in Figure 4a was obtained after simply mixing the sample with a vortex for 5 minutes. The separation conditions were $\dot{V}_c = 0.70$ ml/min and $\dot{V} = 2.09$ ml/min. The UV signal was quite weak and even after an hour part of the sample continued to elute from the channel. The cross flow was removed after 65 minutes and a stable baseline was reached only after 100 minutes (not reported on the plot). The conclusions were that the vortex was too weak a tool for dispersing the sample. A stronger mechanical action was attempted by treating the suspension with a tip-type sonicator for 45–

50 sec, maintaining its output power at a nominal 120 W. The results are shown by the fractogram reported in Figure 4b; the flow conditions were $\dot{V}_c = 0.84$ ml/min and $\dot{V} = 2.00$ ml/min. The whole sample was eluted in 25 minutes without being resolved from the void peak. The signal was twice as intense compared to the vortexed sample (Figure 4a). Conversion to a PSD (Figure 4b) shows that almost all particles are calculated to have dimensions smaller than 300 nm. This is not in agreement with the SEM micrographs, one of which is shown in Figure 3, in which some particles are seen to be considerably larger. The conclusions were that the tip-probe sonicator was too powerful and it probably destroyed the fragile structure of the nanospheres.

In order to evaluate the influence of the carrier composition on the PLA particle dispersions, a carrier having a less basic pH was prepared: a solution of SDS (0.05% w/v) and NaN_3 (0.02% w/v). Carrier of this composition had previously been used successfully for analyzing supramicron PLA particles [17]. Also, SDS is a cationic surfactant in common with the SDS used in the formulation of samples A and B. The measured pH of the mobile phase was 6.89 at 24.4 °C, while the ionic strength I was $= 3 \times 10^{-3}$ M, neglecting the presence of SDS. The pH was therefore close to that of physiological fluids in which the PLA particles are used for drug delivery. A new 10 kDa RC membrane was placed in the channel on changing the carrier composition.

PS NanosphereTM standards were run in order to test the performance of channel under these new chemical conditions. The peaks were generally broader than those obtained with FL-70. This suggests that, in this medium, the physicochemical interactions between the negatively charged PS particles and the membrane are stronger than in the presence of FL-70. Figure 5a shows a fractogram obtained for the injection of PS 92 and 240 nm; a cross flow rate of 0.97 ml/min and channel flow rate of 1.83 ml/min were used. If this fractogram is compared with the fractogram shown in Figure 1a, it is evident that the UV signal is reduced which suggests some loss of sample due to adsorption in the channel. PS 350 nm was excluded from the calibration since the elution of this standard occurred always at unreasonably high retention times, even on changing the fractionation conditions. This experimental evidence suggested a strong interaction between the membrane and the PS particles, an interaction that increased with particles size. The PS fractogram was converted into a PSD distribution (see Figure 5b), and the good correspondence between the nominal size of the standards and those computed by FFF was achieved by assuming a channel thickness of 0.0228 cm (13% smaller than the nominal value). The apparent increase of the channel thickness compared to the combination of the FL-70 carrier and the same type of membrane could be due to either an effective reduction of membrane swelling or could be an artefact of stronger interactions of the PS particles with the membrane. The increase in the retention times affects the selectivity; it increased to 1.02 in the reported example (Figure 5c).

Variation of channel thickness with mobile phase composition is not a newly observed phenomenon. Literature reports examples in which membrane swelling decreased with increase of ionic strength [37]. In particular, for a spacer thickness of 102 μm , Du et al. [36] determined channel thicknesses ranging from 75 μm to 87 μm as ionic strength was increased from that of DI water to 1×10^{-4} M. In some cases, back-calculated channel thicknesses have been calculated that exceed the thickness of the Mylar spacer [12]. This may be due to the use of glue to prevent leaks put between the spacer and the plexiglass which contain the frit, i.e. the upper channel wall, or may be due to actual shrinkage of the membrane under particular experimental conditions. The consequence of assuming a higher value for the channel thickness than its true value is an underestimate of the particle sizes determined from the fractogram, as sometimes proved by other sizing techniques (e.g., PCS or SEM) [12].

The PLA suspensions appeared to be quite stable in the SDS carrier solution, especially samples A and B, and a mild mixing with the vortex was sufficient to keep the particles suspended for a longer period of time than in the previous eluent.

Figure 6(a) shows a fractogram obtained for sample A. The cross flow rate was lower than for the corresponding analysis in the presence of FL-70 ($\dot{V}_c = 0.78$ ml/min vs. 0.85 ml/min), but the longitudinal flow rate was the same ($\dot{V} = 1.99$ ml/min). The sample was vortexed and bath-sonicated for 15 minutes before the injection. The fractogram shows a very narrow peak after only 3 minutes and a broader peak ending after approximately 25 minutes. The fractionation of the whole sample was accomplished before the theoretical steric inversion point, so that the complete fractogram could be converted to a PSD. The PSD in Figure 6a was obtained on assuming $w = 0.0228$ cm, i.e., the channel thickness evaluated from the PS fractograms run in this carrier. The particle size range corresponds very well to that measured using SEM, with approximately 70% having diameters smaller than 100 nm (see Table 1). The excellent correspondence between the FIFFF results and the SEM observation was ascribed to the compatible chemical environment for the particles in a medium very similar to that in which they were synthesized.

Figure 6(b) shows the effect of a stronger ultrasound treatment of the sample suspension. Even though the pH was close to neutral and sonication was applied for half the time used in the case of the FL-70 carrier (20 sec vs. 35–40 sec.), it was suspected that particles may still be destroyed by this relatively intense sonication. A higher cross flow rate was therefore applied for this analysis ($\dot{V}_c = 0.95$ ml/min). The resultant peak was not resolved from the void peak and the PSD showed a greatly reduced particle size. Similar results were obtained for the other samples. Consequently, it was deduced that the tip sonicator was too powerful a tool for the mechanical dispersion of the PLA nanoparticles in the neutral pH solution as well as the basic solution.

Figure 7 shows the fractograms obtained for samples C and D, bath-sonicated for 15 minutes before analysis. The channel flow rate was the same in the two cases ($\dot{V} = 1.77$ ml/min) and the cross flow rate was almost identical (C: $\dot{V}_c = 1.00$ ml/min and D: $\dot{V}_c = 1.04$ ml/min). The direct comparison of the fractograms is therefore possible. In both cases the signal was quite weak. In both cases a very narrow, intense peak was evident at about 70–75 minutes (not included in the plots), corresponding to the elution of sample material when the cross flow was reduced to zero. This fraction of each sample must have been strongly retained in the channel under run conditions, and was released when the field was removed. They correspond, perhaps, to the larger particles in each sample.

Sample C produced a peak that was not completely resolved from the void time, had a maximum at about 18–20 minutes, and ended after about 50 minutes. The theoretical steric inversion point was determined to occur at about 48 minutes under these experimental conditions. The peak was eluted before this time, so the signal given by this part of sample could be converted to a PSD. We can assume that the particles represented in the PSD are relatively small, similar to those found in sample A, and were correctly fractionated. However, it has to be emphasized that probably half of the sample was eluted after interruption of the cross flow, and these larger particles were not included in the calculated PSDs.

Sample D generated a peak broader than sample C but better resolved from the void peak; the elution ended in approximately 60 minutes, which exceeds the theoretical steric inversion time by about 10 minutes. This small fraction of the peak was necessarily neglected by the software during the fractogram-PSD conversion. Sample D presented only a very small amount of particles smaller than 100 nm, if the first peak in the PSD is considered an artefact due to inclusion of part of the void peak. This is in agreement with the SEM observations and may

be explained by considering the two purification methods: sample C was recovered by dialysis, so that almost all the nanospheres generated during the synthesis were retained, while sample D was recovered by gel-filtration and the smallest nanoparticles may be lost due to their very slow elution. The elution of significant material after the removal of cross flow prevents a meaningful comparison of the upper region of the PSDs obtained for samples C and D.

On the basis of these experiments on samples C and D, sample E was not subjected to FIFFF, since its size composition, as observed by SEM, indicated the presence of very large particles. These would not be expected to elute in the normal mode. Even those not so large may elute at perturbed times, as did the 350 nm PS particles.

Sedimentation FFF

The SdFFF technique was used to complement the analyses obtained by FIFFF. PVA has been used as a supplementary surfactant in the preparation of PLA nanoparticles and is known to be compatible with the particles. This surfactant had been found to be incompatible with the RC membranes used in FIFFF resulting in a build-up of surfactant on the membrane, even when a 100 kDa cut-off membrane was used. Since the SdFFF instrument does not have a permeable membrane wall, a carrier solution of PVA 0.01 % w/v having a pH of 6.40 at 24.5 °C was tested. All samples were analysed using field programming, described by Eq. 9. The applied field and the decay parameters were: Initial field = 1000 RPM, $t_1 = 5$ minutes, $t_a = -40$ minutes, $p = 8$, final RPM = 20. All the samples were usually sonicated for 10 minutes in the ultrasonicator bath before being injected directly into the SdFFF channel using an HPLC syringe. The flow rate was 2 ml/min and a constant stop flow period of 10 minutes was applied for all samples in order to allow the particles to equilibrate with the applied field. The correct functioning of the SdFFF system was confirmed by running a PS standard mixture (194, 258 and 360 nm) and comparing the fractogram with that certified by the instrument supplier, Postnova Analytics. The separation of the PS standards was performed by using a solution of FI-70 (0.01% w/v) and NaN_3 (0.02% w/v) as carrier.

Figure 8(a–b) shows the fractograms obtained for samples A and B, together with the respective PSDs calculated for an assumed particle density of 1.25 g/ml [32–33, 42]. According to SEM, both samples have a mean particle size of 90 nm. Both fractograms exhibit a sharp void peak, followed by a small peak that elutes in the first 10 minutes, and then a broad peak that elutes between 10 and about 40 minutes.

If the fractograms are converted to PSDs, by removing first the void peak (in all cases, arbitrarily the end of the void peak was set at 5 minutes, i.e. for $t \cong 2t^0$), the signal profile generates a distribution showing a peak at around 90–100 nm and a second broad particle distribution ranging from 100 to 500 nm. This is not supported by the SEM observations. The nanoparticles were apparently not well dispersed. In order to test this hypothesis, sample A was sonicated for a longer period (30 minutes as opposed to 10 minutes) and the results of this experiment are shown in Figure 8(c). The appearance of two peaks at high retention time suggests a possible aggregation of the small particles, promoted by the increase of temperature that occurs during the sonication process. This quick check established that 10 minutes were a good compromise to partially break down the particle clusters created during the resuspension of the particles following freeze-drying without promoting a further agglomeration among the particles as experimentally observed in Figure 8(c).

The fractogram obtained by the injection of sample C shown in Figure 9 presents a very intense void peak and a broad peak ending at about 50 minutes. The corresponding PSD shows a very narrow peak at 93 nm, while the broad peak produces a PSD profile peaking at around 250 nm and extending up to 900 nm. This corresponds quite well with the SEM observations and with Figure 7a. The PSD plot includes a superimposed cumulative curve, that confirms that 64% of

the particles are calculated to be smaller than 250 nm, as observed by SEM, and confirms the presence of a few particles of up to about 700 nm in diameter.

A very different profile was found for sample D: the void volume was much smaller than in all previous cases and the peak due to the sample was much better defined than in the previous case. The corresponding PSD gave a distribution centered at around 260 nm and only 39% of the particles were calculated to be smaller than 250 nm. Also in this case, the experiments confirmed what had been deduced from the FIFFF analysis shown in Figure 7b and from the SEM micrographs, *i.e.*, there are few particles smaller than 100 nm in sample D that had been formulated using Pluronic F68 and purified by gel-filtration.

The last plots in Figure 9 were obtained for sample E. The fractogram had a narrow void peak and from the profile it is possible to identify the presence of two peaks at around 20 and 35 minutes, which correspond to the bi-modal particle size distribution reported in the PSD, peaking respectively at 238 nm and 461 nm. The fraction of particles smaller than 250 nm was calculated to be 39%. Again in this case, the SdFFF measurements confirmed the observations of SEM.

5. Conclusions

The flow and the sedimentation FFF techniques were used to determine size information of nanospheres loaded with the Oct-CPA prodrug. The value of this coupled approach is due to the complementary nature of the two separation techniques. The FIFFF has the advantage of being a very gentle separation technique, which is able to fractionate samples without altering their shape, also for very soft structures like liposomes. If the experimental separation conditions are chosen respecting the theory, the fractograms could be easily transformed to PSDs without knowing the density of the nanoparticles, provided the geometrical dimensions of the channel are carefully determined using standards. The presence of a membrane, and the well documented particle-wall interactions in this technique, can limit the choice of the mobile phase. However, by realizing optimum conditions, the derived information is very accurate, especially for nanoparticles smaller than 250–300 nm. In particular, in this work a perfect correspondence was found for sample A analyzed using a carrier solution containing SDS.

These limitations are compensated by the SdFFF technique, which has a theoretical resolving power, in terms of the size, three times higher than FIFFF and less restriction on the composition of the carrier solution. However, the fractogram conversion into a PSD can be achieved only with the knowledge of the density of the sample components.

In this work the synergetic use of these two techniques led to the establishment of the most suitable suspending media for the PLA particles for analysis in each case. Once these good experimental conditions were determined, the size information could be achieved in a more rapid and less tedious way than from SEM observations.

Having established optimum operating conditions, the two FFF techniques may be used routinely to check the chemical formulations of the samples immediately after their purification without lyophilizing and resuspension. They may be used as an alternative or to complement SEM or light scattering techniques, and have the advantage of low cost of analysis.

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Figure 1.

(a): FIFFF separation of a mixture of the 3 PS latex standards ($d = 92, 240, 350$ nm respectively) obtained at a channel flow rate $V = 1.72$ ml/min and with a cross flow rate $V_c = 0.76$ ml/min; UV range 0.005, injection volume 20 μ l, carrier FL-70 (0.1% v/v) and NaN_3 (0.02% w/v). Black line: original fractogram, gray line: smoothed fractogram. (b) Particle size distribution computed from the fractogram, assuming $w = 0.0186$ cm and $V^0 = 1.10$ mL. (c) Selectivity plot.

Figure 2.

Original FIFFF fractograms (black line) and smoothed fractograms (gray line) on the left; PSD obtained for PLA samples by assuming $w = 0.0186 \text{ cm}$ and $V^0 = 1.10 \text{ mL}$ elaborating the smoothed UV signals, on the right. Sample A: $V_c = 0.85 \text{ ml/min}$ and $V = 1.99 \text{ ml/min}$, sample C: $V_c = 0.98 \text{ ml/min}$ and $V = 1.85 \text{ ml/min}$, sample D: $V_c = 0.81 \text{ ml/min}$ and $V = 1.98 \text{ ml/min}$. For all the samples, injection volume was $50 \mu\text{L}$, UV range 0.005, stop-flow time 5 minutes, carrier FL-70 (0.1% v/v) and NaN_3 (0.02% w/v).

Figure 3. Scanning electron micrographs of the five samples of PLA nanoparticles in which the Oct-CPA was encapsulated.

Sample D

a) Vortex

b) tip-sonicator

Figure 4.

Mechanical mixing effect on sample D. **(a)** Original FIFFF fractogram obtained from the suspension vortex mixed. Conditions: $\dot{V}_c = 0.70$ ml/min and $\dot{V} = 2.09$ ml/min. **(b1)** Original FIFFF fractogram and smoothed signal due to the injection of a tip-probe sonicated suspension (45–50 seconds, power 120 W). Separation conditions: $\dot{V}_c = 0.84$ ml/min and $\dot{V} = 2.00$ ml/min. In both cases, injection volume was 50 μ l, UV range 0.005, stop-flow time 5 minutes; carrier FL-70 (0.1% v/v) and NaN_3 (0.02% w/v). **(b2)** PSD elaborated from the smoothed signal (gray line) by assuming $w = 0.0186$ cm and $V^0 = 1.10$ mL.

Figure 5.

(a) Original fractogram (Black line) obtained by FIFFF of a mixture of two PS latex standards (92 and 240 nm) with $\dot{V} = 1.83$ ml/min and $\dot{V}_c = 0.97$ ml/min, UV range 0.005, injection volume 50 μ l, stop-flow time 5 minutes; carrier SDS (0.05% w/v) and NaN_3 (0.02% w/v). The gray line is the smoothed fractogram (b) Particle size distribution computed from the fractogram, assuming $w = 0.0228$ cm and $V^0 = 1.345$ mL. (c) Selectivity plot.

Sample A

Figure 6.

(a) FIFFF fractograms for sample A fractionated in SDS (0.05% w/v) and NaN_3 (0.02% w/v), conditions $\dot{V}_c = 0.78$ ml/min and $\dot{V} = 1.99$ ml/min (original fractogram - black line, smoothed fractogram - gray line), and PDS obtained on assuming a channel thickness of 0.0228 cm and elaborating the smoothed signal. (b) Tip-probe sonication effect on sample A. Analysis conditions: $\dot{V}_c = 0.95$ ml/min, $\dot{V} = 1.88$ ml/min. In both cases: injection volume was 20 μl , UV range 0.005, stop-flow time 5 minutes.

a) Sample C

b) Sample D

Figure 7.

FIFFF fractograms (original fractogram - black line, smoothed fractogram – gray line) and PDSs. (a) Sample C ($V_c = 1.00$ ml/min and $V = 1.77$ ml/min) and (b) Sample D ($V_c = 1.05$ ml/min and $V = 1.77$ ml/min). For both: injection volume $20\ \mu\text{l}$, UV range 0.005, stop-flow 5 minutes, carrier SDS (0.05% w/v) and NaN_3 (0.02% w/v). PDSs obtained from the smoothed signal on assuming a channel thickness of 0.0228 cm.

Figure 8.

SdFFF fractograms (original fractogram - gray line, smoothed fractogram - black line) and PSDs for the samples A (a) and B (b) calculated from the smoothed signals and for an assumed density of 1.25 g/ml. Power programmed elution conditions: Initial field = 1000 RPM, relaxation time 10 minutes, $t_1 = 8$ minutes, $t_d = -64$ minutes, $p = 8$, final field = 20 RPM; carrier: PVA 0.01 % w/v, flow rate = 2 ml/min; Sonication time = 10 minutes. (c) SdFFF fractogram and PSD for Sample A; conditions the same as for (a), but sonication was applied for 30 minutes.

Figure 9.

SdFFF fractograms (original fractogram - gray line, smoothed fractogram – black line) and PSD for the samples C, D and E obtained by elaboration of the smoothed signals and assuming a density of 1.25 g/ml. Power programmed elution conditions: Initial field = 1000 RPM, relaxation time 10 minutes, $t_I = 8$ minutes, $t_a = -64$ minutes, $p = 8$, final field = 20 RPM; carrier: PVA 0.01 % w/v, flow rate = 2 ml/min. Sonication time = 10 minutes.

Table 1

Effects of the stabilizing agent and of the recovery/purification method on recovery percentage, stabilizing agent residuals and on the nanosphere sizes. SD= Standard deviation.²

Sample	Stabilizing agent	Recovery method	Recovery (%)	Residual of stabilizing agent $\pm$ SD (% w/w)	SEM Mean diameter $\pm$ SD (nm)	% of nanospheres
A	SDS*	Dialysis	100	20.0 $\pm$ 3.0	90 $\pm$ 25	80% < 100 nm
B	SDS*	Gel-Filtration	60	5.2 $\pm$ 1.8	90 $\pm$ 20	70% < 100 nm
C	Pluronic F68	Dialysis	100	21.0 $\pm$ 2.2	220 $\pm$ 50	60% < 250 nm
D	Pluronic F68	Gel-Filtration	70	0.5 $\pm$ 0.2	300 $\pm$ 40	30% < 250 nm
E	Pluronic F68	Ultra-Centrifugation	75	0.9 $\pm$ 0.3	390 $\pm$ 90	30% < 250 nm

* SDS (Sodium Dodecyl Sulfate) alias known as Sodium Cholate