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Protein-loaded PLGA microparticles engineered by flow focusing: Physicochemical characterization and protein detection by reversed-phase HPLC

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ABSTRACT

In the present study, a novel synthesis technique based on the flow focusing (FF) technology is investigated for the preparation of green fluorescent protein (GFP)-loaded poly(D,L-lactide-*co*-glycolide) (PLGA) microparticles. To our knowledge, this novel technique has never been applied to the formulation of proteins in polymeric systems. A simple, specific and rapid reversed-phase HPLC (RP-HPLC) method was validated for the determination of GFP in PLGA microparticles with the best chromatographic peak resolution, reduced run time and low cost of analysis. In order to achieve the finest GFP-loaded polymeric particles, experimental parameters mainly associated to the FF device were studied (liquid flow rate and pressure of the focusing air). Very high GFP encapsulation values (>90%) were obtained by this technique, and the electrokinetic characterization of these systems suggested that this protein was incorporated into the polymeric matrix. This study is intended to offer information on which to base the development of high molecular weight protein-loaded polymeric delivery systems prepared by FF.

1. Introduction

Protein and peptide delivery methods have evolved significantly over the past years, focusing the major research efforts on the delivery of high molecular weight proteins. These biomolecules are characterized by a poor absorption into the blood stream when administered orally and by a short half-life, which determines the need of a frequent administration in high doses to achieve a therapeutic effect. Even more, these macromolecules are very sensitive to environmental conditions (e.g., acidic pH of the stomach) and usually lead to toxic side effects after systemic administration in high doses. In view of the fact, drug delivery systems involving the use of polymers have become the major focus of this research in order to enhance the therapeutic index of this biomolecules (effectiveness and safety) by means of a localized and sustained release at the target site, without resulting in undesirable side effects. However, a great deal of research is still required to make most of these delivery methods feasible for commercialization (Arias, 2008; Dai et al., 2005).

Recently, we have developed a flow focusing (FF) method for the microencapsulation of drugs. This technique allows the easy formulation of well-formed polymeric systems with very suitable drug carrying properties: great entrapment efficiencies and drug loading values, and very slow (biphasic) drug release profiles (Holgado et al., 2008). FF has been suggested to enable the control of the size, the surface characteristics and the internal structure of the synthesized systems. Additionally, FF has many general advantages including: (i) it is a simple and scalable one-step approach, making additional purification and separation procedures unnecessary; (ii) it allows for the encapsulation of labile compounds, unlike many of the high-energy physical approaches of generating monodisperse droplets through the breaking up of larger droplets; (iii) it is compatible with different fluid mixtures (liquid–liquid, liquid–gas) and does not require surfactants, although their use might support droplet formation; (iv) particle size can be adjusted by changing the fluid flow velocity of the two phases; (v) droplet size is not limited by the injector and orifice size, i.e., droplets can be much smaller than the orifice size; and (vi) offers the generation of droplets and microspheres at low costs. Compared to other preparation procedures, such as spray drying or emulsion/solvent evaporation, FF lacks an overall high throughput. However, this inconvenience can be overcome by scaling up of the FF process into arrays of hundreds or even thousands of fluid streams (He, 2008; Holgado et al., 2008; Martín-Banderas et al., 2005, 2006; Schneider et al., 2008; Xu et al., 2009).

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In this work, we study the utility and versatility of FF in the preparation of polymer-based delivery systems loaded with either peptides or proteins, and intended for non-parenteral routes of administration, mainly the oral route, to obtain a local and/or systemic effect. With that aim, we engineered PLGA particles loaded with green fluorescent protein (GFP) by FF. To our knowledge, this novel technique has never been applied to the formulation of proteins in polymeric systems. The vehiculization of this fluorescent protein will facilitate this purpose, because its incorporation and distribution inside the polymer particles will be easily checked by confocal laser scanning microscopy (CLSM). GFP is a 26.9 kDa protein characterized by a tightly packed "β can" tertiary structure made up of 238 amino acids (Niwa et al., 1996; Yang et al., 1996; Bilati et al., 2005; McRae et al., 2005). Poly(D,L-lactide-*co*-glycolide) (PLGA) is a biocompatible and biodegradable polyester that was approved by the FDA for drug delivery and that is commonly used for the microencapsulation of therapeutics and antigens (Anderson and Shive, 1997; Jain, 2000; Kumar et al., 2001; Fu et al., 2005; Arias, 2008).

In order to obtain GFP-loaded PLGA particles with a narrow size distribution and the best loading properties, we investigated two important parameters related to the FF device: the liquid flow rate and the pressure of the focusing air. Furthermore, a specific and rapid reversed-phase HPLC (RP-HPLC) method coupled with fluorescence detection was validated for the quantification of GFP loading into PLGA microparticles. RP-HPLC is a powerful and sensitive technique for the separation and determination of peptides and proteins in both artificial and biological environments (Sarmento et al., 2006), that to our knowledge has never been applied to GFP determination in polymeric systems.

2. Materials and methods

2.1. Materials

Green fluorescent protein was purchased from Biomedal S.L. (Spain). Poly(D,L-lactide-*co*-glycolide) (PLGA 50:50) Resomer® RG 502 (Mw: 12,000; inherent viscosity: 0.24 dL/g) was obtained from Boehringer Ingelheim (Germany). All other chemicals used were of analytical quality from Panreac (Spain), except for trifluoroacetic acid (TFA, obtained from Sigma–Aldrich, Germany) and Span® 60 (Acofarma®, Spain). Water used in the experiments was deionized and filtered (Milli-Q Academic, Millipore, France).

The RP-HPLC analysis was carried out on a Hitachi LaChrom® (D-7000) Series HPLC system equipped with a L-7200 automatic injector, an interphase D-7000 and a quaternary pump (model L-7100), and coupled with a L-2485 fluorescence detector (Merck-Hitachi, Germany). A Waters Symmetry Shield® RP18 column (USA, 3.5 μm, 4.6 mm × 150 mm) kept at 15.0 ± 0.1 °C (L-2350 column oven, Elite LaChrom®) was used in this analysis. Data collection and calculation were done by using HSM software (Merck-Hitachi, Germany).

2.2. Preparation of the GFP-loaded PLGA microparticles

A water-in-oil emulsion was prepared by mixing for 6 min 357.7 μL of a GFP aqueous solution (0.27%, w/v) and 9.66 μL of a 4% (w/v) PLGA solution in ethyl acetate containing Span® 60 (0.5%, w/v) (Branson 5200E4 ultrasonic 188 bath, USA, operating at 40 kHz with a sonic power of 100 W). This emulsion was sprayed inside a chamber with an inlet temperature of 50 ± 5 °C by using a standard FF nozzle [model Advant 2 (D = 100 μm), Ingeniatrics Tecnologías S.L., Spain] fixed at different flow rates and pressures (Table 1). The polymeric particles were collected at the bottom of the chamber as a dry powder on a plate, and freeze-dried [frozen in liquid nitrogen and lyophilized (−80.0 ± 0.5 °C, and 0.057 mbar; Telstar Cryodos, Spain)] and stored at 4.0 ± 0.5 °C, in order to increase their physicochemical stability but without significantly modifying the characteristics of the PLGA-based delivery system. All the formulations were prepared in nonaplicate (n = 9).

Table 1
Formulations of GFP-loaded PLGA microparticles obtained by flow focusing (FF) under different preparation conditions.

Formulation	Flow rate (mL/h)	Pressure (mbar)
1	4	50
2	6	50
3	8	50
4	4	100
5	6	100
6	8	100
7	4	200
8	6	200
9	8	200

2.3. Characterization methods

The mean particle size and particle size distributions of GFP-loaded PLGA microparticles were measured at 25.0 ± 0.5 °C by a laser scattering technique (Malvern Mastersizer, 2000 laser light scattering instrument, Malvern Instruments Ltd., UK), after 60 min of mechanical stirring (50 rpm). The selected angle was 90° and the measurement was made after dilution of the protein-loaded polymer aqueous suspensions. In order to confirm these results, the mean particle diameters were also measured from scanning electron microscopy (SEM) pictures of 100 particles by using a Zeiss DSM 950 (Germany) scanning electron microscope set at 80 kV accelerating voltage. Prior to observation, a dilute microparticle suspension (≈0.1%, w/v) was sonicated for 5 min, and drops of the suspension were placed on copper grids with formvar film. The grids were then dried at 35.0 ± 0.5 °C in a convection oven.

The analysis of the presence and distribution of GFP in PLGA microparticles was carried out by confocal laser scanning microscopy (CLSM) (DM-IRE2 microscope, Leica Microsystems, Germany). In order to extensively characterize the type of GFP loading (surface adsorption or absorption inside the polymeric matrix) to PLGA particles, the zeta potential (ζ)-pH trend and ζ-ionic strength dependence were investigated in all the GFP-loaded PLGA formulations and in non-loaded PLGA particles (Holgado et al., 2008). Briefly, the surface electrical properties of these microparticle suspensions (≈0.1%, w/v) were analyzed by electrophoresis as a function of both pH (adjusted with either HNO_3 or KOH) and KNO_3 concentration using a Malvern Zetasizer, 2000 electrophoresis device (Malvern Instruments Ltd., UK). Measurements were performed at 25.0 ± 0.5 °C, after 24 h of contact at this temperature under mechanical stirring (50 rpm). The experimental uncertainty of the measurements was <5%. The theory of O'Brien and White (1978) was used to convert the electrophoretic mobility (u_e) into ζ values. Finally, this data was also used in the investigation of the influence of changes in the parameters of PLGA production by FF (flow rate and pressure) on the surface electrical properties (electrokinetics) of the PLGA microparticles.

2.4. Chromatographic conditions

The introduction of new HPLC methods for routine quality control of pharmaceutical preparations starts with the establishment of the optimal measurement conditions, and provides the maximum relevant information by analyzing the experimental data (García et al., 2003; Pérez-Lozano et al., 2004; Zaxariou and Panderi, 2004; Salas et al., 2008).

The mobile phase consisted of ACN 0.04% TFA/H_2O 0.04% TFA. The mobile phase was filtered through a 0.22 μm nitrocellulose-membrane filter (Millipore, Barcelona) and degassed under vacuum prior to use. The flow rate was 0.5 mL/min.

A linear gradient of:

- 5–24% of ACN in 6 min;
- 24–50% of ACN in 4 min;
- 50–5% of ACN in 17 min.

Eluent was pumped at a flow rate of 0.5 mL/min and the injection volume was 50 μL. GFP was detected at an excitation wavelength of 503 nm and at an emission wavelength of 573 nm. All the measurements were carried out at room temperature (25.0 ± 0.5 °C) and the total peak area was used to quantify the protein.

2.5. Preparation of the GFP solutions

A primary stock solution of GFP (2.7 mg/mL) was accurately prepared and filtered through a 0.45 μm nylon membrane filter (Millipore, Spain), following by rigorous dilution to obtain secondary standard solutions ranging from 0.5 to 1.5 mg/mL. These solutions were protected against ambient light, wrapping all glassware with aluminium foil, and stored at 4.0 ± 0.5 °C. No GFP precipitation or aggregation was observed after eight months, as was confirmed by RP-HPLC. Freshly prepared solutions were used in all the determinations.

The preparation of GFP-loaded PLGA microparticle samples for RP-HPLC involved the addition of an adequate amount of GFP-loaded polymer (equivalent to 5 mg of GFP) to 2 mL of an ACN solution in a volumetric flask of 5 mL. This solution was sonicated for 15 min, diluted with 2 mL of methanol, and filtered through a 0.45 μm nylon membrane filter (Millipore, Spain).

2.6. Validation of the RP-HPLC method

The method was validated in agreement with the International Conference on Harmonization guidelines (ICH, 1996) by using linearity, precision, accuracy, specificity, and detection and quantification limits as analytical parameters. System and method linearity were evaluated by assessing a regression line using the least squares method. Calibration curves were obtained by preparing three separate batches of five GFP concentrations (0.5, 0.75, 1, 1.25 and 1.5 mg/mL). The samples used in the determination of method linearity were ACN solutions of GFP-loaded PLGA microparticles with the same content in GFP as the GFP solutions used in the analysis of the system linearity (0.5, 0.75, 1, 1.25 and 1.5 mg/mL).

Instrumental precision was determined by testing 6 consecutive times in the same day the repeatability of a standard GFP solution (1.6 mg/mL). Acceptance criterion was set at a relative standard deviation (RSD) ≤ 1.5%. Method repeatability was determined by using the results obtained in the accuracy test (acceptance criterion: RSD ≤ 2.0%). Intermediate precision was evaluated by analyzing the same sample by different analysts in two different days (Peroza Meza et al., 2006).

The accuracy characterizes the proximity between the obtained experimental results and the real results and can be assessed by the determination of the percentage recovery of a known amount of GFP. The accuracy of the method was first tested by calculating the percentage recoveries of the mean of three determinations of GFP in samples where a known amount of protein was added to a blank PLGA solution (GFP concentrations: 0.5, 0.75, 1, 1.25 and 1.5 mg/mL, corresponding respectively to the concentration levels: 50, 75, 100, 125 and 150%). The accuracy was also assessed by determining the RSD.

In order to determine the capacity of this analytical technique to identify interferences coming from impurities, degradation products or any formulation component, the specificity of the method was investigated. Specificity was checked by running GFP standard solutions (2.7 mg/mL), ACN solutions of GFP-loaded PLGA microparticles (2 mg/mL) and ACN solutions of blank PLGA microparticles (2 mg/mL). All the preparations contained the same GFP concentration or the same PLGA content, and the determinations were performed under the same conditions. The specificity of the RP-HPLC method was also checked under different protein degradation conditions (pH, light and temperature) in triplicate. The effect of pH was studied after contact during 24 h of GFP standard solutions with appropriate [H^+] (0.5N HNO_3) or [OH^-] (0.5N KOH). The effect of exposure to light was studied by placing one set of glassware under ambient light during 24 h. To check the effect of temperature, samples were kept at 60.0 ± 0.5 °C during 24 h.

The detection limit (DL) can be defined as the lowest concentration of analyzed substance in a certain sample that can be detected under certain conditions by a given method. The quantitation limit (QL) is the lowest concentration that can be determined at an acceptable precision and accuracy. These parameters were determined by using the following expressions:

$$\mathrm{DL} = \frac{3.3\sigma}{S} \quad (1)$$

$$\mathrm{QL} = \frac{10\sigma}{S} \quad (2)$$

where σ is the standard deviation of the response and S is the slope of the calibration curve (GFP concentrations ranged from 0.01 to 0.4 mg/mL).

2.7. GFP loading of PLGA microparticles

Samples from the protein-loaded PLGA microparticles obtained under different synthesis conditions (Table 1) were added to 2 mL of ACN. This solution was sonicated for 15 min, diluted with 2 mL of methanol, and filtered through a 0.45 μm nylon membrane filter (Millipore, Spain) before RP-HPLC analysis. GFP incorporation to PLGA microparticles was expressed in terms of GFP entrapment efficiency (EE, %) [encapsulated GFP (mg)/total GFP in the colloidal suspension (mg) × 100] (Brigger et al., 2004). In order to determine the variations in particle size when PLGA was loaded with GFP, statistical analysis was performed by the use of the Student's t-test. Values with $p < 0.05$ and $p < 0.01$ were considered as significantly different.

3. Results and discussion

3.1. Chromatographic study

We have developed a simple RP-HPLC method for the determination of GFP in PLGA microparticles with the best chromatographic peak resolution, reduced run time and low cost of analysis. This method permits the analysis of a large series of samples, avoiding possible degradation associated to a long analysis time. Fig. 1 shows representative chromatograms of a GFP standard solution and of ACN solutions of GFP-loaded PLGA microparticles. As can be observed in this figure, the relatively symmetrical GFP peak has a retention time of ≈12.2 min. No peaks from possible degradation products were observed in the chromatograms, probing the purity of the GFP used in the study and its stability in stock solutions.

3.2. Method validation

Good system linearity was obtained in the range of the study (Fig. 2a). The calibration curve was $y = 892{,}754 \cdot x + 4251$, where y

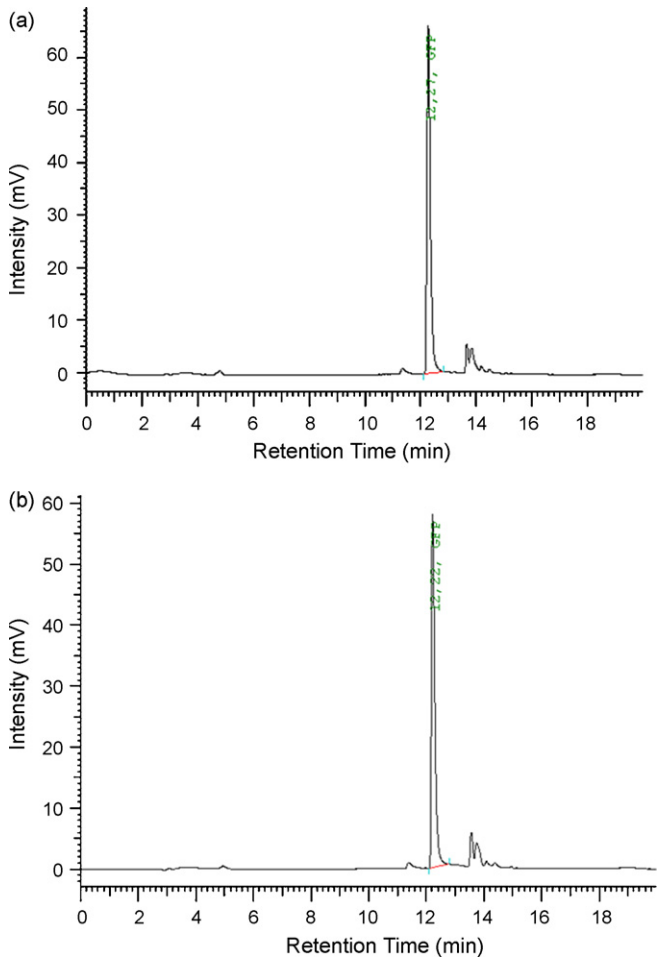

Fig. 1. Representative chromatograms of GFP detection in (a) standard solutions, and (b) ACN solutions of GFP-loaded PLGA microparticles.

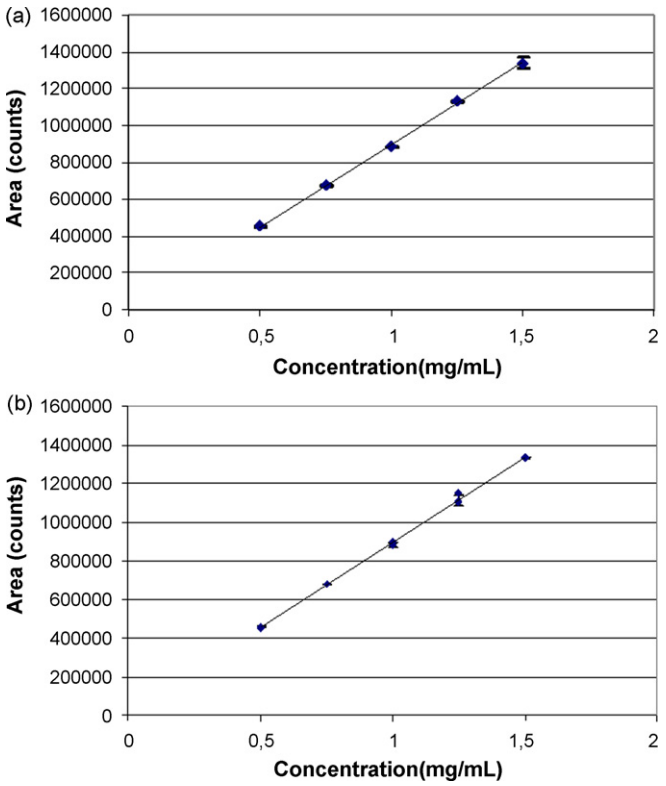

Fig. 2. Calibration curves of GFP solutions (a), and ACN solutions of GFP-loaded PLGA microparticles (b). GFP concentration range: 0.5–1.5 mg/mL.

represents the peak area and x represents the GFP concentration (mg/mL). The standard error was 13,654 and the correlation coefficient (R^2) was 0.9992 ($n = 15$). With respect to the linearity of the method (Fig. 2b), the calibration curve was $y = 881{,}712 \cdot x + 12{,}686$ and the R^2 was 0.9993 ($n = 15$). In both cases, the R^2 was $\geq$0.999, indicating a good linearity in the GFP concentration range (0.5–1.5 mg/mL) (Table 2).

Instrumental precision was determined after testing 6 consecutive times a standard GFP solution (1.6 mg/mL). The mean peak area was 868,318 ± 1336 (RSD = 0.154%) and the mean retention time was 12.8 min (RSD = 0.24%). According to these results, the instrumental precision is satisfactory (RSD < 1.5%). Method repeatability was considered valid as the RSD values were ≤2.0% (Table 3). Intermediate precision was evaluated by analyzing the same sample by different analysts in two different days. The results obtained in this analysis are collected in Table 4 and, as can be observed, the RSD values were under the limit of acceptance (RSD < 2.0%). This

Table 2
Statistic report for the system and method linearities.

ANOVA	d.f.	SS	MS	F	Significance F	
Statistic report for the system linearity						
Regression	1	1.49439×10^{12}	1.49439×10^{12}	8015.637427	1.5754×10^{-19}	
Residual	13	2,423,650,760	186,434,673.9			
Total	14	1.49682×10^{12}				
Intercept	4251.4[a]	10,576.42682[b]	0.401969405[c]	0.694237146[d]	−18,597.58096[e]	27,100.381[f]
Area	892,753.8667[a]	9971.550836[b]	89.5300923[c]	1.5754×10^{-19}[d]	871,211.6408[e]	914,296.093[f]
Statistic report for the method linearity						
Regression	1	1.49439×10^{12}	1.49439×10^{12}	8015.637427	1.5754×10^{-19}	
Residual	13	2,423,650,760	186,434,673.9			
Total	14	1.49682×10^{12}				
Intercept	12,685.86667[a]	9623.553732[b]	1.318210198[c]	0.210188338[d]	−8104.55714[e]	33,476.2905[f]
Area	881,712.6667[a]	9073.173471[b]	97.17797962[c]	5.43563×10^{-20}[d]	862,111.2671[e]	901,314.066[f]

[a] Coefficient.
[b] Standard error.
[c] t-Stat.
[d] p-Value.
[e] Lower 95%.
[f] Upper 95%.

Table 3
Recovery values of ACN solutions of GFP-loaded PLGA microparticles. Concentration range: 0.5–1.5 mg/mL (level I: 0.5 mg/mL; level II: 0.75 mg/mL; level III: 1 mg/mL; level IV: 1.25 mg/mL; level V: 1.5 mg/mL).

Level	Theoretical GFP content (mg)	Peak area	Experimental GFP content (mg)	Recovery (%)
I	0.5	454,529 ± 3675	0.5044 ± 0.0041	100.87 ± 0.82
	RSD	0.8086	0.8162	0.8162
II	0.75	675,213 ± 368	0.7516 ± 0.0004	100.21 ± 0.06
	RSD	0.0545	0.0548	0.0548
III	1	886,934 ± 11,601	0.9887 ± 0.0129	98.87 ± 1.29
	RSD	1.3079	1.3143	1.3143
IV	1.25	1,122,083 ± 26,014	1.2521 ± 0.0291	100.17
	RSD	2.3184	2.3272	2.3272
V	1.5	1,333,235 ± 2423	1.4886 ± 0.0027	99.24 ± 0.18
	RSD	0.1818	0.1824	0.1824

Table 4
Method repeatability evaluated by analyzing the same sample by different analysts in two different days.

Analyst/day (sample)	Theoretical GFP content (mg)	Peak area	Experimental GFP content (mg)	Mean value	RSD	Recovery (%)
1/1 (1)	0.47	413,751	0.46			97.59
1/1 (2)	0.46	415,506	0.46	0.46 ± 0.01	0.30	100.14
2/1 (1)	0.48	424,962	0.47			98.18
2/1 (2)	0.47	423,318	0.47	0.47 ± 0.01	0.28	99.87
1/2 (1)	0.48	421,112	0.47			97.28
1/2 (2)	0.48	422,444	0.47	0.47 ± 0.01	0.23	97.59
2/2 (1)	0.48	419,864	0.47			96.99
2/2 (2)	0.49	423,841	0.47	0.47 ± 0.01	0.67	95.92
Mean value	0.48 ± 0.01	420,599 ± 4035	0.47 ± 0.01	0.47 ± 0.01	0.37	97.95 ± 1.43
RSD	1.92	0.96	0.97			1.46

proved that the variations settled in the test did not influence the experimental method and showed the good precision of the analytical method. Moreover, the MANOVA statistical test confirmed that no statistical differences occurred between days and analysts ($p = 0.778$).

The investigation of the accuracy of the method revealed that recovery values (%) were between 98.12% and 101.84% (Table 3) and that the mean RSD was 0.934%. It was not needed to carry out any other statistical test since the ICH limit value for pharmaceutical formulations establishes a recovery between 98% and 102% (equivalent to ±2.0% of the relative error). These results demonstrated the coincidence between experimental and theoretical values and, therefore, it can be concluded that the RP-HPLC method is accurate.

The specificity of the method was verified by analyzing potential interfering peaks of the formulation components at GFP retention time. The RP-HPLC method was found to be specific as no interfering peaks were observed in the chromatogram with a similar retention time to that of the protein. The three chromatograms measured at different times were within the established threshold for the GFP peak. Furthermore, the specificity of the RP-HPLC method was also confirmed by studying the possible interference of the protein degradation products (coming from the exposure of GFP to different pH, light and temperature conditions) with the GFP peak. Under these degradation conditions, it can be said that the protein is almost completely degraded, giving rise to high amounts of degradation products that did not interfere with the GFP peak (Table 5).

Table 5
Peak areas and degradation degree (%) corresponding to GFP standard solutions (1.6 mg/mL) under different degradation conditions.

Sample	Peak area	Degradation (%)
Fresh GFP standard solution	621,353 ± 14,013	–
After light exposure	8696 ± 88	98.6
After heating (60.0 ± 0.5 °C)	40,834 ± 724	93.4
After contact with 0.5N HNO_3	91,836 ± 1622	85.2
After contact with 0.5N KOH	19,860 ± 21	96.8

The detection limit (DL) and the quantitation limit (QL) were found to be 0.1 and 0.2 mg/mL, respectively. These values are slightly lower in comparison to the ones obtained for other important biomedical proteins such as insulin by using other RP-HPLC methods (Sarmento et al., 2006), which could be pointed as an advantage compared with them.

3.3. Characterization of the GFP-loaded PLGA microparticles

3.3.1. Particle geometry

GFP-loaded PLGA microparticles were of spherical shape and with a smooth surface. This particle morphology was not significantly influenced by the FF conditions. All the formulations were in the colloidal size range and moderately monodisperse. Fig. 3a shows, as an example, a scanning electron microscopy (SEM) picture of microparticles from formulation 9. As it was observed by CLSM (Fig. 3b), GFP was uniformly distributed inside the microparticles in all the formulations.

The size (mean diameter ± SD) of the GFP-loaded PLGA formulations can be compared in Table 6 with the corresponding theoretical values that were calculated by using a mathematical model that considers the main geometrical parameters and the flow param-

Table 6
Theoretical and experimental sizes, and protein entrapment efficiency of the formulations of GFP-loaded PLGA microparticles obtained by flow focusing.

Formulation	Theoretical size (μm)	Experimental size (μm)	Entrapment efficiency (%)
1	10.9	11.5 ± 0.7	91.9 ± 1.2
2	12.7	12.3 ± 1.2	92.1 ± 1.8
3	14.1	13.9 ± 0.8	91.8 ± 2.0
4	8.1	7.8 ± 0.9	90.5 ± 1.6
5	9.2	8.4 ± 0.5	92.7 ± 2.2
6	10.2	10.5 ± 0.8	90.8 ± 1.9
7	5.8	4.2 ± 0.3	90.2 ± 1.9
8	6.6	4.9 ± 0.2	92.5 ± 1.2
9	7.3	7.1 ± 0.5	92.1 ± 2.1

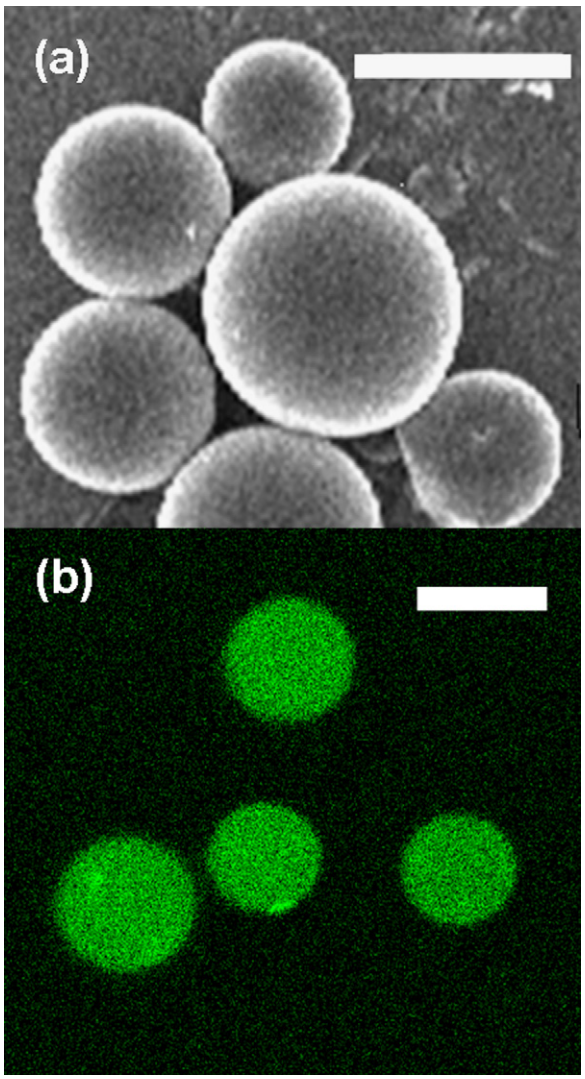

Fig. 3. Scanning electron microscopy (SEM) (a) and confocal laser scanning microscopy (CLSM) (b) pictures of GFP-loaded PLGA microparticles (formulation 9). Bar lengths: 7 μm.

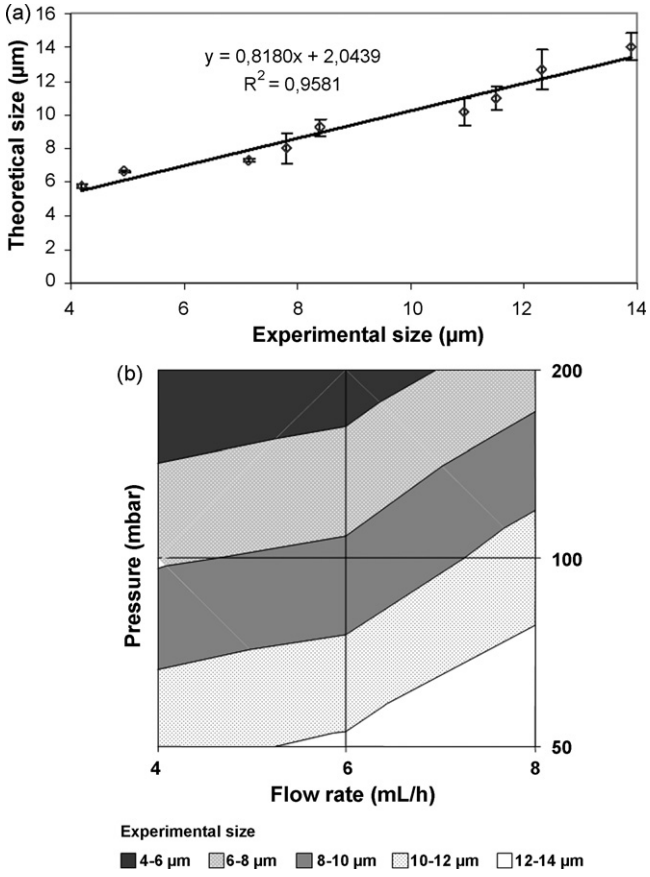

Fig. 4. Relation between the theoretical size and the experimental size of the GFP-loaded PLGA microparticles (a), and (b) between the flow parameters (liquid flow rate and pressure of the focusing air) and the experimental size of the GFP-loaded PLGA microparticles.

eters (liquid flow rate and pressure of the focusing air) of the FF device, and the properties of the fluid (density, viscosity and surface tension) (Gañán-Calvo, 1998; Martín-Banderas et al., 2006). Briefly, this mathematical model defined a simple and universal expression for the jet diameter at the hole exit of the flow focusing device:

$$d_j \cong \left(\frac{8\rho_l}{\pi^2 \Delta P_g}\right)^{1/4} Q^{1/2} \quad (3)$$

where d_j is the mean particle diameter, ρ_l is the liquid density, ΔP_g is the pressure gradient and Q is the flow rate.

Taking into account the statistical analysis that was done, a good linear relationship between theoretical and experimental values was found ($n = 9$; $F = 160.01$; $p < 0.0001$) (Fig. 4a) and, thus, it could be theoretically calculated the experimental conditions (flow rate and pressure) needed to obtain a given size (Fig. 4b). This is particularly important since these experimental conditions are determined by the properties of the fluid (Gañán-Calvo et al., 2006; Sakai et al., 2006; Ong et al., 2007; He, 2008). Therefore, FF technique allows to control and, even more, to predict the size of the GFP-loaded PLGA microparticles. As it can be checked in Table 6, the liquid flow rate and the pressure of the focusing air seems to determine the size of the GFP-loaded PLGA microparticles. An increase in the flow rate (at a constant pressure) induced the formation of microparticles with a bigger size. On the opposite, an increase in the pressure (keeping the flow rate constant) determined a significant reduction in the particle size and a narrow size distribution.

3.3.2. Electrokinetic properties

We first focussed our study on the effect of pH on the zeta potential, ζ, of the non-loaded PLGA and GFP-loaded PLGA microparticles. Thus, Fig. 5a shows this quantity as a function of pH in the presence of 10^{-3} M KNO_3. Note that ζ is negative for almost the whole pH interval investigated, and that it rises in absolute value as the pH increased. As it is observed, both non-loaded PLGA and GFP-loaded PLGA microparticles show a well defined isoelectric point (pH_{iep} or pH of zero potential) close to pH ≈ 2.5–2.8. This behaviour can be explained on the basis of the charge generation mechanism at the polymer/solution interface: we can expect that the chemical species responsible for the generation of the negative surface charge on PLGA are ionized weak acid groups, presumably carboxylic-end groups (Mu and Feng, 2001; Teixeira et al., 2005; Musumeci et al., 2006; Okassa et al., 2007). In addition, this negative charge could also be due to strong groups corresponding to dissociated end molecules of the polysorbate surfactant Span® 60 used in the synthesis, that remain adsorbed on the particle surface even after the cleaning procedure. The increasingly negative values and charge density may be explainable by the effect of increasing OH^- ion concentration in the solution, which tends to favour a gain in protons. In contrast, a decrease in absolute ζ as pH becomes more acidic may be explainable by neutralization of the negative regions as a result

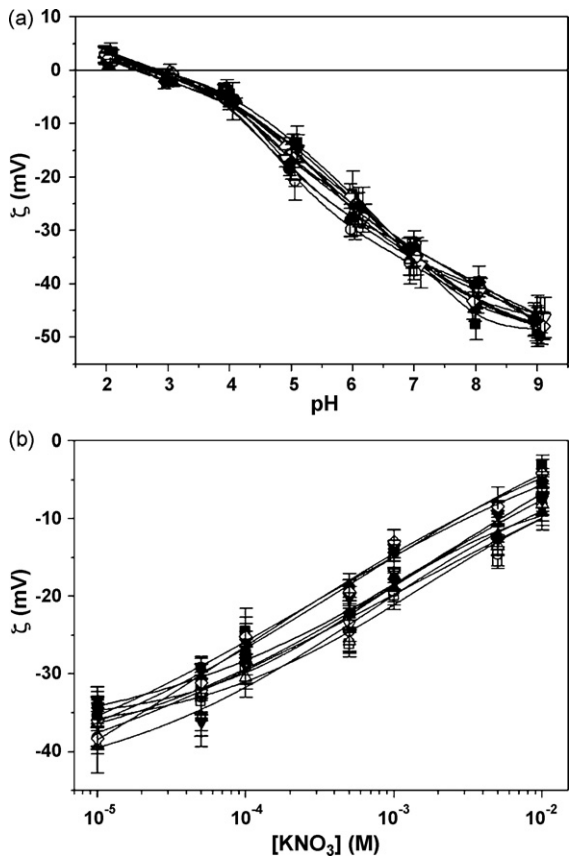

Fig. 5. Zeta potential of blank PLGA (■), formulation 1 (□), formulation 2 (●), formulation 3 (○), formulation 4 (▲), formulation 5 (△), formulation 6 (▼), formulation 7 (▽), formulation 8 (♦) and formulation 9 (◊), as a function of (a) pH in the presence of 10^{-3} M KNO_3, and (b) KNO_3 concentration at pH 5. The lines are guides to the eye.

of chemical adsorption of increasing numbers of H^+ ions (Ruiz et al., 2004; Arias et al., 2007).

In order to confirm these results, we also measured ζ as a function of KNO_3 concentration at a constant pH 5. ζ is negative for the whole ionic strength interval investigated, and falls in absolute value as the KNO_3 concentration increased, due to the double-layer compression (Fig. 5b). The counterions accumulate closer to the particle surface, such that the double layer shrinks as concentration increases. This leads to a lower electrical potential in the shear plane (or slip surface) that limits the value of the zeta potential (Dillen et al., 2004).

Finally, the results of this analysis, plotted in Fig. 5, clearly showed how similar are the electrokinetics of non-loaded PLGA particles and the electrokinetics of all the formulations of GFP-loaded PLGA microparticles. This points out that the protein was not adsorbed onto the polymeric surface and, consequently, a very efficient GFP entrapment have led to GFP-loaded PLGA particles which, from an electrokinetic point of view, are indistinguishable from non-loaded PLGA (Nicoli et al., 2001; Dillen et al., 2004; Teixeira et al., 2005; Díez and Tros de Ilarduya, 2006; Gómez-Gaete et al., 2007). Furthermore, this data clearly demonstrated that a modification in the parameters of particle production by FF (flow rate and pressure) did not influence the surface electrical properties of the particles.

3.3.3. GFP loading to PLGA microparticles

The proposed RP-HPLC method was successfully applied to the determination of the amount of GFP incorporated to the PLGA microparticles. It was demonstrated that none of other microparticle components interfered with the GFP analysis, but a filtration step (through a 0.45 μm nylon membrane filter) is needed to protect the column from contamination and delay pre-column obstruction.

FF allows obtaining high GFP entrapment efficiencies in PLGA microparticles (>90%), whatever the synthesis conditions (liquid flow rate and pressure of the focusing air) (Table 6). Furthermore, in comparison to non-loaded PLGA (data not shown for brevity), particle geometry (morphology, surface and size) did not vary significantly when GFP was loaded to this polymer, as was previously observed with other biomolecules (Teixeira et al., 2005; Holgado et al., 2008).

4. Conclusions

A novel synthesis procedure (flow focusing) has been developed for the preparation of protein-loaded PLGA microparticles by using GFP as a model protein. This technique allowed obtaining polymeric microparticles with a controllable size and an optimum GFP loading. A mathematical model was satisfactorily applied for the determination of the formulation conditions to achieve the desired particle size. GFP determination was carried out by a fast RP-HPLC method that was validated according to ICH guidelines. These very interesting results encourage further investigations to formulate peptide- and protein-loaded PLGA microparticles by FF, and to study the possibilities of controlling particle size without reducing the protein loading. Work is undergoing for the preparation of insulin-loaded PLGA microparticles by this novel technique.

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