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RESEARCH PAPER

Extraction of PLGA-Microencapsulated Proteins Using a Two-Immiscible Liquid Phases System Containing Surfactants

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ABSTRACT

Purpose The extraction of proteins from PLGA/PLA microspheres by a two-immiscible liquid phases system with the addition of surfactants was investigated.

Methods First, the extraction without surfactants and the interaction between proteins (IFN-α2b and EGF) and empty microspheres (PLGA or PLA) was studied. Next, proteins stability in presence of different surfactants was evaluated by: (1) bicinchoninic acid protein assay, (2) reversed phase-high performance liquid chromatography, and (3) enzyme-linked immunosorbent assay. Then, proteins were extracted with PBS/dichloromethane including selected surfactants and characterized by the above mentioned techniques, biological activity tests, sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electrospray ionization mass spectrometry.

Results Without surfactants, protein recovery was only 27–43% for IFN-α2b and 58–73% for EGF. Protein content in solutions incubated with blank microspheres decreased to 66% for IFN-α2b and 86% for EGF. It was only possible to quantify the EGF and IFN-α2b in the same manner as in PBS alone when the surfactant added was Pluronic F-68 and SDS, respectively. Addition of these surfactants allowed the complete isolation of both biomolecules from the microspheres. The extraction procedure did not affect the encapsulated proteins.

Conclusion Proteins can be quantitatively extracted, without changes, from PLGA/PLA microspheres using PBS/dichloromethane system that include an appropriate surfactant.

KEY WORDS epidermal growth factor · interferon · protein extraction · microspheres · poly (lactide-co-glycolide)

ABBREVIATIONS

BCA	bicinchoninic acid
EGF	epidermal growth factor
ELISA	enzyme-linked immunosorbent assay
ESI-MS	electrospray ionization mass spectrometry
IFN	interferon
mAb	monoclonal antibody
PLA	poly (L-lactide)
PLGA	poly (DL-lactide-co-glycolide)
PVA	polyvinyl alcohol
RP-HPLC	reversed-phase high performance liquid chromatography
SDS-PAGE	sodium dodecylsulfate-polyacrylamide gel electrophoresis

INTRODUCTION

Several peptides and proteins have been encapsulated into polymeric matrixes with different purposes (1,2). Numerous studies have been addressed to obtain an acceptable *in vitro* release of the encapsulated substance but one of the challenges when these biomolecules are encapsulated is to

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conserve its physicochemical and/or biological characteristics. This is because proteins could become modified during the microencapsulation processes due to the presence of organic solvents and large interfaces, vigorous stirring and/or interactions with polymeric matrixes. The most frequent modifications comprise denaturing, covalent and non-covalent aggregation, deamidation, oxidation and the incorrect assembly of disulphide bonds (3–5). The presence of any of these modified products should be avoided because: (i) their properties, like biological activity, are often affected, and (ii) they are unacceptable since the regulatory point of view. Consequently, to develop a successful delivery system based on the microencapsulation of a protein, it is necessary to guarantee both: an adequate release of the encapsulated molecule and the conservation of its physicochemical and biological properties.

To characterize the proteins encapsulated into PLGA microspheres, they should be previously extracted from the particles because these matrixes are non-water soluble. Such procedures ought to be quantitative and should not change the characteristics of the encapsulated protein. Several methods have been evaluated to extract encapsulated proteins from the PLGA microspheres. The extraction by precipitation of the protein in a solvent in which the polymer is soluble has been used by Johnson *et al.* for characterizing human growth hormone encapsulated into PLGA microspheres (6). Our group used this method as well to extract interferon alpha 2b from PLGA microparticles but the protein recovery was only about 50% (7). Other researchers have used electrophoresis to extract the protein from the microspheres to the gel (8,9), but the use of this technique has been limited to determine the presence of degradation products or aggregates of the protein and moreover the complete recovery of the protein could be unachievable if the encapsulated protein molecules are surrounded by a dense polymer structure (10). A two-immiscible liquid phases system has also been used. This method involves the dissolution of the polymeric matrix in an organic solvent, followed by the extraction of the encapsulated protein into an aqueous phase. Several encapsulated peptides have been successfully characterized using the last extraction procedure (11,12). In contrast, some researchers have found that protein recovery can be negatively affected because the protein tends to be distributed between the interface and the aqueous phase (13–15). On the other hand, some protein-polymer interactions formed throughout the encapsulation process could persist during the extraction. This could be another factor that difficult the complete recovery of the protein. The addition of some substances, such as surfactants, could enhance the extraction because they reduce the adsorption of proteins at the water/methylene chloride interface (16) and also could eliminate or attenuate protein-polymer interactions (17,18).

This work was aimed to evaluate the utility of a two-immiscible liquid phases system, that includes a surfactant in the aqueous phase, to extract interferon alpha 2b and epidermal growth factor from PLGA microspheres for their physicochemical and biological characterization.

MATERIALS AND METHODS

The therapeutic proteins (interferon alpha 2b (IFN-α2b) and epidermal growth factor (EGF)) and monoclonal antibodies for ELISA assays (anti-recombinant IFN-a2b CBIFNA2.3 mAb, anti-recombinant IFN-a2b CBIFNA2.4-HRP conjugate, anti-recombinant EGF CB EGF1 mAb, and anti-EGF CB EGF2-HRP conjugate) were commercial products supplied by CIGB (Center for Genetic Engineering and Biotechnology, Havana, Cuba). This EGF is a mixture of molecules having 51 and 52 amino acids produced as a clinical grade preparation with high purity and biological activity (19,20). Poly (L-lactide) (PLA), poly (DL-lactide-co-glycolide) (PLGA), polyvinyl alcohol 30,000-70,000 (PVA), sodium dodecylsulphate (SDS), Nonidet® P 40 (NP-40), Triton® X-100, Pluronic F-68 and Pluronic F-127 were acquired from Sigma (St. Louis, Missouri, USA). Tween 20, Tween 80 and all other reagents were obtained from Merck (Darmstadt, Germany).

Preparation and Characterization of Microspheres Containing IFN-α2b or EGF

Microspheres were prepared using the double emulsion-solvent evaporation method. Specifically microparticles containing IFN-α2b (MS-IFN-α2b) were obtained as Saez *et al.* described in 2008 (7) while EGF-loaded microspheres (MS-EGF) were fabricated following the procedure detailed by Han *et al.* (17) with some modifications. Briefly, 100 μl of 10 mM saline sodium phosphate, pH 7.2 (PBS) containing 2 mg of IFN-α2b were sonicated for 30 s into 1 ml of 10% (*w*/*v*) PLGA in dichloromethane (DCM) using an IKA-SONIC U 200 S ultrasonic homogenizer (IKA Labortechnik, Germany). While 100 μL of 10 mM sodium phosphate, pH 7.2 (PB) containing 2.7 mg of EGF was dispersed in the polymeric solution (3 ml of 5% (*w*/*v*) PLA in DCM) with a high-speed homogenizer Ultraturrax T8 (IKA Labortechnik, Germany) at 14,000 rpm for 2 min. The resulting primary (water-in-oil (w/o)) emulsion was dispersed in the external aqueous solution (1% (*w*/*v*) PVA) with an Ultraturrax T8 at 14,000 rpm for 3 min. The double emulsion was poured into another volume of the PVA solution and stirred to extract and evaporate the dichloromethane. Finally, the formed microspheres were collected by filtration through a 0.45 μm cellulose nitrate membrane (Sartorius GmbH, Germany), washed 5 times with distilled water and dried in a freeze-dryer (Edwards, UK) during 16 h (T=−40° C, P=10^{-3}mbar). Microspheres were stored at 4°C. Blank microspheres were prepared with PBS or PB instead of the interferon or EGF solution, respectively.

The particle size distribution of the microspheres containing IFN-α2b or EGF was determined with a laser diffraction particle size analyzer Coulter LS 230 (Coulter, USA). Encapsulation efficiency and protein loading were measured by: 1) digesting the particles by partial hydrolysis with 1 N NaOH, 2) neutralizing the resulting solution with 1 N HCl, and 3) quantifying protein by the bicinchoninic acid method using the microBCA protein assay reagent kit (Pierce, Rockford, IL, USA).

Extraction and Characterization of the Encapsulated Protein

Extraction of the Encapsulated Protein from the PLGA Microspheres

Encapsulated protein was extracted from the microspheres using a system of two immiscible liquid phases. Dichloromethane was used as the organic phase while PBS solution was used as the aqueous phase. Briefly, 10 mg of microspheres were mixed with 0.5 ml of dichloromethane, vortexed for 1 min, and then gently agitated for another 20 min in a Roto-Shake Genie shaker (Scientific Industries Inc., Bohemia, NY, USA). A volume of the aqueous phase (250 μl) was then added to the resulting sample and the mixture was vigorously vortexed for 30 s. The emulsion was centrifuged at 5,000 rpm in a Hettich centrifuge (Tuttlingen, Germany) for 15 min. The aqueous phase (upper) was then recovered, transferred to a vial and stored at 4°C. The rest of the sample was extracted again with 250 μl of the aqueous phase. The solutions obtained after the extractions were then mixed and analyzed. Protein recovery was calculated as the percentage of the extracted protein in relation to the expected value. The expected protein concentration was determined considering the protein loaded onto the extracted microspheres and the volume of the aqueous phase recuperated from the extraction procedure.

In the indicated cases, surfactants were added to PBS in order to increase the recovery of the protein in the aqueous phase of the extraction system.

Effect of the Extraction Phase Composition on the Protein Quantification and Integrity

EGF or IFN-α2b was diluted in the different aqueous phases to be examined as candidates to extract the encapsulated molecule from the microspheres. Such aqueous phases contain PBS, pH 7.2 and a surfactant. PBS alone was used as a reference. Seven surfactants were evaluated: SDS, Triton X-100, NP-40, Tween 20, Tween 80, Pluronic F-68 and Pluronic F-127. All of them were added at 0.1% (*w/v*).

The protein concentration in the solutions prepared was assessed by the microBCA assay and the characteristics of EGF or IFN-α2b were evaluated by enzyme-linked immunosorbent assay (ELISA), reverse phase high performance liquid chromatography (RP-HPLC) and biological activity test. Four replicates of all aqueous phases were prepared and each one was analyzed twice.

Utility of Two-Immiscible Liquid System Including Surfactant in Aqueous Phase for Extraction of Encapsulated Protein

Encapsulated protein was extracted by the two-immiscible liquid phases system with or without the surfactant in the aqueous phase. The protein recovery was calculated as the percentage of the protein in the aqueous solution (quantified by microBCA and ELISA) with respect to the theoretical value.

The procedure was performed in samples constructed by mixing empty microspheres with an exact quantity of EGF or IFN-α2b to confirm that the surfactant does not modify EGF or IFN-α2b during extraction. The protein recovery was determined as mentioned above. The characteristics of the extracted protein were evaluated by ELISA, RP-HPLC and biological activity test.

Characterization of Encapsulated Proteins in Experimental Batches of Microspheres

Two experimental batches of microspheres containing IFN-α2b were treated by the extraction system having PBS/SDS while two experimental batches of EGF-loaded microspheres were extracted by PBS/Pluronic F-68 system. Both surfactants were used at 0.1% (*w/v*). The proteins recovered in the aqueous phase was quantified by microBCA and evaluated by ELISA, RP-HPLC and biological activity test. Additionally, samples of IFN-α2b extracted from the microspheres were evaluated by SDS-PAGE.

Statistical Analysis

Statistical analysis of data was carried out with application of StatGraphics Plus 5.1 Software (Statistical Graphics Corp., EEUU). The results are expressed as the mean ± standard deviation. Analysis of variance (ANOVA) and the Duncan test were used when appropriate for comparison of the variables between the different groups studied. The results were considered significant if $p<0.05$.

Characterization of IFN-α2b

RP-HPLC

RP-HPLC analysis was performed on a Vydac (Hesperia, CA, USA) wide pore octyl C8 column (5 μm, 250 mm x 4.6 mm). The elution was made, in 47 min, using a gradient from 15 to 80% of A in B (A: 0.05% trifluoroacetic acid in

acetonitrile; B: 0.1% trifluoroacetic acid in water). The flow rate was 0.8 ml/min. Detection of IFN-α2b was performed at 226 nm with automatic data processing, by means of D-7000 Multi HSM software (Merck, Darmstadt, Germany).

ELISA

This analysis was made as described by Santana *et al.* (21). Each well of the microtiter plate was coated with 1.5 μg of anti-recombinant IFN-α2b CBIFNA 2.3 mAb in 0.1 ml coating buffer (0.05 M Na_2CO_3, 0.05 M $NaHCO_3$, pH 9.6) and incubated for 3 h at 37°C. The coated plate was washed twice with washing solution (PBS, pH 7.2, containing 0.05% (*v*/*v*) Tween 20). Then, 100 μl-diluted samples were added per well in the assay buffer (PBS containing 0.5% (*w*/*v*) skim milk). After incubation for 30 min at 37°C, the plate was washed five times with PBS/Tween 20. Then, 70 μl of the conjugated second antibody (anti-IFN-α2b CBIFNA 2.4 mAb) labeled with horseradish peroxidase, diluted 1:10 000 (*v*/*v*) in the assay buffer, were added and incubated at 23°C for 1 h. The plate was washed eight times with PBS/Tween 20, and then incubated for 15 min at 23°C with 100 μl of the substrate solution. The substrate solution contained 0.1 M Na_2HPO_4, 0.048 M citric acid, pH 5.5, 0.45 g o-phenylenediamine/l plus 0.3 g H_2O_2/l. The reaction was stopped by adding 50 μl of 2.5 M H_2SO_4. Absorbance was measured at 492 nm by using an ELISA-plate reader (SensIdent Scan; Merck, Darmstadt, Germany).

Antiviral Activity Assay

Antiviral activity of IFN-α2b was assayed by inhibition of the cytopathic effect produced by the Mengo virus with Hep-2 cells (ATCC No. CCL23) (22). Interferon samples were serially diluted 1:2 (*v*/*v*) in the Minimum Essential Medium containing 2% (*v*/*v*) fetal calf serum and 40 μg/ml gentamicin and were mixed with cell monolayers in 96-well microtiter plates. The plates were then incubated at 37°C for 24 h, under 5% CO_2 and 95% humidity. After adding the virus (10^7 TCID), the plates were incubated until the cytopathic effect (90% cell lysis) was evident (approximately 18–20 h) in the virus control wells (without IFN-α2b). Staining the remaining cells with crystal violet measured the degree of cell destruction. A microplate reader (Tecnosuma, Havana, Cuba) was used to determine the cytopathic effect. The resulting data were transformed into a linear regression through a probit transformation with the ParLin 50 software (Center for Genetic and Biotechnology, Havana, Cuba), which is validated and registered. The unit of antiviral activity was defined as the reciprocal of the sample dilution that yields 50% protection of cells against the Mengo-induced cytopathic effect. The potency of each sample was expressed in IU compared to a secondary reference calibrated against the 69/19 International World Health Organization IFN-α2b standard.

SDS-PAGE

The IFN-α2b samples (recovered by passive diffusion-controlled release or solvent-assisted extraction) were diluted 1:5 (*v*/*v*) in 625 mM Tris–HCl buffer (pH 6.8) containing 5% (*w*/*v*) sodium dodecyl sulphate, 50% (*v*/*v*) glycerol and 0.025% (*w*/*v*) bromophenol blue. Then, they were run in a 12.5% (*w*/*v*) acrylamide gel, as described by (23). The electrophoresed gels were stained for protein with Coomassie brilliant blue, and scanned with a ScanJet 4c/T scanner (Hewlett Packard, Palo Alto, CA, USA).

Characterization of EGF

RP-HPLC

RP-HPLC analysis was performed on a Vydac (Hesperia, CA, USA) wide pore octyl C8 column (5 μm, 250 mm x 4.6 mm). EGF was eluted using a gradient (in 32 min) from 20 to 80% of A in B (A: 0.05% trifluoroacetic acid in acetonitrile, B: 0.1% trifluoroacetic acid in water), with a flow rate of 0.8 ml/min. Detection was performed at 226 nm with automatic data processing, by means of D-7000 Multi HSM software (Merck, Darmstadt, Germany).

ELISA

This analysis was made as described by Martínez *et al.* (24). Each well of the microtiter plate was coated with 0.2 μg of anti-recombinant EGF CB EGF1 monoclonal antibody in 0.1 ml coating buffer (0.05 M Na_2CO_3, 0.05 M $NaHCO_3$, pH 9.6) and incubated for 3 h at 37°C. The coated plate was washed once with washing solution (0.05% (*v*/*v*) Tween 20 in distilled water). Then, 100 μl-diluted samples were added per well in the assay buffer (PBS containing 0.5% (*w*/*v*) skim milk). After incubation for 1 h at 37°C, the plate was washed five times with Tween 20. Then, 80 μl of the conjugated second antibody (anti-EGF CB EGF2 monoclonal antibody) labeled with horseradish peroxidase, diluted 1:8000 (*v*/*v*) in the assay buffer, were added and incubated at 23°C for 1 h. The plate was washed eight times with Tween 20, and then incubated for 10 min at 23°C with 100 μl of the substrate solution. The substrate solution contained 0.1 M Na_2HPO_4, 0.048 M citric acid, pH 5.5, 0.45 g *o*-phenylenediamine/l plus 0.3 g H_2O_2/l. The reaction was stopped by adding 50 μl of 2.5 M H_2SO_4. Absorbance was measured at 492 nm by using an ELISA-plate reader (SensIdent Scan; Merck, Darmstadt, Germany).

Biological Activity Test

Biological activity of EGF was assayed by the evaluation of the inducing activity for the proliferation of 3 T3 A31 cells. The degree of cell proliferation was measured by staining the cells with crystal violet. The result was expressed in international units (IU), compared to a secondary reference which was calibrated against the 91/530 NIBSC EGF standard.

Mass Spectrometry

Buffer exchange of protein samples to MilliQ water, was performed by using ultra filtration units with a cut-off of 1000 Da (Millipore, Bedford, UK), followed by 20-fold dilution in 60/40(*v*/*v*) acetonitrile/MilliQ water + 1% formic acid.

The electrospray ionization mass spectrometry (ESI-MS) analysis were made in a hybrid quadrupole-time-of-flight instrument (QTOF1, Waters, USA) fitted with a Z-spray nanoflow electrospray ion source. The applied voltage on the needle was 900 V and the sample cone voltage 35 V. All spectra were mass calibrated using an aqueous solution of cesium and sodium iodide.

RESULTS AND DISCUSSION

The microparticles using in this investigation were spherical with smooth surfaces. Particle sizes and encapsulation efficiencies of microspheres were 28.1 ± 0.4 μm and 65 ± 3% for MS-IFN-α2b and 18.0 ± 0.1 μm and 36 ± 2% for MS-EGF, respectively. In preliminary studies, we found that the recovery of EGF and IFN-α2b from microspheres after the extraction using two immiscible phases (DCM as organic phase and PBS as aqueous phase) was not quantitative. Only about 58–73% of the encapsulated EGF could be extracted to the aqueous phase and approximately 27–43% of the IFN-α2b was recovered from the microspheres.

This incomplete extraction could be due to the adhesion of the protein to the interface or to the interaction between protein and polymer. Other authors have shown that this interaction might cause incomplete release profiles of proteins encapsulated into PLGA microspheres (25,26). In order to investigate the presence of these interactions in our systems, each protein was incubated in PBS alone or together with blank microspheres. After 14 days, only 66 ± 6% of IFN-α2b was detected in the solution incubated with blank microspheres. Conversely, no difference was detected between IFN-α2b content in the same solution incubated without particles. The decrement in concentration was less remarkable for EGF whose content dropped to 86 ± 3% when the solution was incubated with microspheres. As happened for IFN-α2b, the EGF content in the solution used as reference (incubated without particles) did not change during the incubation period. Consequently, we hypothesized that the inclusion of a surfactant in the aqueous phase of the extraction system could help to achieve complete extraction because surfactants can eliminate or attenuate such interactions. Surfactants could also favour the distribution of the protein to the aqueous phase. Several surfactants were tested; however it was necessary to perform a previous study for the evaluation of the influence of these substances on protein quantification and integrity.

Effect of Different Surfactants on Protein Quantification and Integrity

Samples of each protein diluted in PBS alone or with different surfactants were prepared for evaluating the effect of the surfactants on protein quantification and integrity.

An analysis of variance (ANOVA) illustrated significant differences between the IFN-α2b concentration in the mixtures assessed by microBCA (p=0.0000) and ELISA (p= 0.0000). A multiple range Duncan test showed that it is only possible to quantify the IFN-α2b by microBCA or ELISA in the same manner as in PBS alone when the surfactant added was SDS (Table I).

The differences between EGF concentration in the solutions evaluated by microBCA (p=0.0001) and ELISA (p= 0.0001) were detected by ANOVA as well. A Duncan test confirmed that it is only possible to quantify the EGF by microBCA in the same manner as in PBS alone when the surfactant added was SDS or Pluronic F-68. On the other hand, the concentration of EGF assessed by ELISA only was different from the reference when Pluronic F-127 was used (Table II).

These ELISA assays detect conformational changes. IFN-α2b is recognized by the mAbs IFN-α2bCBIFNA2.3 and IFN-α2bCBIFNA2.4 (21). The CBIFNA2.3 mAb tightly binds, and, *in vitro*, neutralizes the Mengo virus biological activity of IFN-α2b, while the CBIFNA2.4 mAb has a high affinity for solid phase-adsorbed IFN-α2b. The CBIFNA2.4 mAb does not compete with the CBIFNA2.3 mAb. These IgG1-subclass mAbs recognize conformational epitopes of IFN-α2b, which are exposed when the cytokine is correctly folded. A previously developed ELISA assay based on these mAbs accurately quantifies biologically active IFN-α2b even in the presence of Escherichia coli-derived protein mixtures, while at the same time discriminating from incorrectly folded IFN-α2b molecular species from disulfide-bonded species (21). Consequently, this ELISA not only measures low concentrations (down to 0.2 ng/ml) of IFN-α2b, but may also show IFN integrity and has been used in the process and quality control of large-scale production of IFN-α2b (21). While, EGF is recognized by the mAbs CB-

Table I Duncan Homogeneous Groups for the IFN-α2b Concentration Assessed by microBCA and ELISA in the 8 Aqueous Phases under Evaluation

Experimental Group	microBCA					ELISA				
	IFN-α2b (μg/ml)	Homogeneous groups				IFN-α2b (μg/ml)	Homogeneous groups			
PBS	1.1 ± 0.1	×				1.2 ± 0.1	×			
PBS + SDS	1.1 ± 0.1	×				1.3 ± 0.1	×			
PBS + Pluronic F-68	1.2 ± 0.2		×			1.5 ± 0.1		×		
PBS + Pluronic F-127	1.3 ± 0.1		×	×		1.6 ± 0.2		×		
PBS + Tween 20	1.4 ± 0.1			×	×	1.7 ± 0.1				×
PBS + NP-40	1.4 ± 0.1			×	×	1.8 ± 0.2				×
PBS+ Triton X-100	1.4 ± 0.1			×	×	1.8 ± 0.2				×
BS + Tween 80	1.4 ± 0.2				×	1.8 ± 0.2				×

EGF1 and CB-EGF2. Both mAbs recognize conformational epitopes and, consequently, they only detect well-folded EGF (27). Specifically, the epitope recognized by the CB-EGF1 MAb comprises aminoacids S9, H10, Y13, K28, E40 and R41 (28). This conformational epitope includes the Y13 and R41 residues, essential for the EGF-EGF_receptor interaction and defining the neutralizing activity of the MAb.

Furthermore, the addition of the evaluated surfactants in the aqueous phase did not affected the RP-HPLC profile of the proteins with the exception of SDS that caused a great distortion in the signals of EGF (Fig. 1).

This results point to SDS and Pluronic F-68 as good candidates for evaluating their effect in increasing the recovery of IFN-α2b and EGF respectively, during the extraction using the two-immiscible liquid phases system.

Utility of Two-immiscible Liquids System Including Surfactant in Aqueous Phase for Extraction of Encapsulated Proteins

The addition of 0.1% SDS to the aqueous phase of the extraction system increased the extraction of encapsulated IFN-α2b from 35 ± 8% to 100 ± 6% while the inclusion of 0.1% Pluronic F-68 enhanced the EGF extracted from 66 ± 9% to 100 ± 8%. Both surfactants, like others, may possibly disrupt the interactions between the proteins and the polymeric matrix (17). Consequently, they could enhance the extraction of IFN-α2b and EGF into the aqueous phase. These substances can also help to increase the efficiency of extraction because they compete with the protein for the water/chloroform interface, preventing their adsorption, and besides, surfactants may enlarge the interfacial area during the extraction process (18).

The main disadvantage of the procedure for extraction of encapsulated proteins into PLGA microspheres based on two immiscible liquid phases is the incomplete recovery of the protein (29). In our case, this limitation was solved by including a surfactant in the aqueous phase. However some authors did not exploit this method because it can cause significant protein adsorption at the interface followed by unfolding and aggregation (10,29). Some of these changes could alter the biological activity of the protein. For example, Woo *et al.* found different enzymatic activity between horseradish peroxidase released and extracted from PLGA microspheres with a DCM/PBS system. They attributed this discrepancy to the negative effect that could be exerted on the protein by the extraction procedure (30). However it is really difficult to discriminate the contribution of the extraction procedure or the encapsulation process to the changes that suffer the recovered protein. This fact could

Table II Duncan Homogeneous Groups for the EGF Concentration assessed by microBCA and ELISA in the 8 Aqueous Phases Under Evaluation

Experimental Group	microBCA				ELISA			
	EGF (μg/ml)	Homogeneous groups			EGF (μg/ml)	Homogeneous groups		
PBS	0.8 ± 0.1	×			1.03 ± 0.04			×
PBS + SDS	0.9 ± 0.1	×	×		1.05 ± 0.04			×
PBS+ Pluronic F-68	0.9 ± 0.3	×	×		1.03 ± 0.04			×
PBS + Triton X-100	1.0 ± 0.1		×	×	1.05 ± 0.07			×
PBS+ Pluronic F-127	1.0 ± 0.1		×	×	0.91 ± 0.07	×		
PBS + Tween 20	1.0 ± 0.1		×	×	1.08 ± 0.08			×
PBS+ NP-40	1.1 ± 0.1			×	1.07 ± 0.08			×
PBS + Tween 80	1.1 ± 0.2			×	1.03 ± 0.04			×

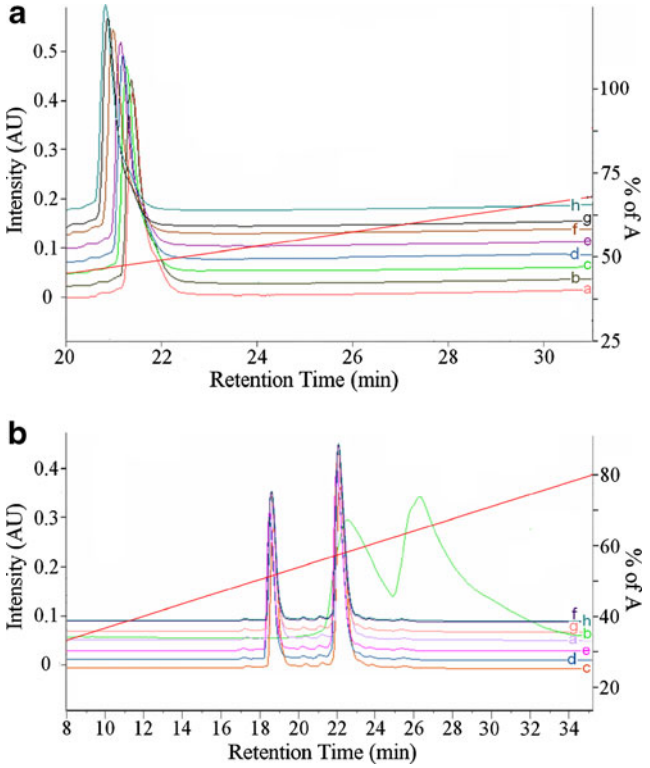

Fig. 1 RP-HPLC profile of IFN-α2b (**a**) and EGF (**b**) in PBS alone (*a*) or with different surfactants: SDS (*b*), Triton X-100 (*c*), NP-40 (*d*), Tween 20 (*e*), Tween 80 (*f*), Pluronic F-68 (*g*), Pluronic F-127 (*h*).

lead to erroneous interpretations attributing the changes in the protein to the encapsulation process instead of the extraction procedure.

To confirm that the surfactants selected as candidates for increasing the IFN-α2b or EGF recovery do not modify the proteins during the extraction, the procedure was performed in samples constructed by mixing empty microspheres with an exact mass of free IFN-α2b or EGF (MS + IFN-α2b and MS + EGF). The protein recovered in the aqueous phase of the extraction system was then quantified by microBCA and examined by ELISA, RP-HPLC and biological activity test. Table III shows that the proteins could be recovered completely in the aqueous phase, they were entirely immunorecognized by the two monoclonal antibodies of the corresponding ELISA system and their biological activity was preserved. As it was explained above, these monoclonal antibodies only recognize well-folded IFN-α2b and EGF, respectively.

In addition, the RP-HPLC and ESI-MS profiles of the samples extracted remained unchanged with respect to free IFN-α2b or EGF used as a reference (Figs. 2, 3 and 4).

These results illustrate that the properties of IFN-α2b and EGF do not change as a consequence of the extraction method, thus the procedures designed here are adequate for the quantitative extraction and characterization of the encapsulated IFN-α2b and EGF.

Table III Protein Recovery and Properties of the Extracted Protein from Samples Comprising Empty Microspheres with an Exact Quantity of IFN-α2b or EGF (MS + IFN-α2b or MS + EGF) and Microspheres Containing IFN-α2b or EGF (MS-IFN-α2b and MS-EGF)

Sample	R[1] (%)	IR[2] (%)	Specific activity (IU/mg)	
			Limit[3]	Real
MS + IFN-α2b	100 ± 10	100 ± 10		2.1×10^8
MS-IFN-α2b (S1)	100 ± 10	60 ± 20	2×10^8	1.3×10^8
MS-IFN-α2b (S2)	95 ± 5	50 ± 20		1.4×10^8
MS + EGF	110 ± 10	110 ± 10		5.4×10^5
MS-EGF (S1)	100 ± 10	110 ± 10	5×10^5	6.8×10^5
MS-EGF (S2)	110 ± 20	90 ± 10		6.5×10^5

[1] R: Recovery calculated as the percentage of the extracted protein in relation to the expected value. The expected protein concentration was calculated considering the quantity of the biomolecule added to the empty microspheres or the protein content of the loaded microspheres and the volume of the aqueous phase of the extraction procedure

[2] IR: Immunorecognition, defined as the percentage of the protein quantified by ELISA in relation with the protein assessed by the microBCA assay

[3] This value is the limit for considering that the protein has an adequate biological activity

S1 and S2 refer to different experimental batches of microspheres containing IFN-α2b or EGF

Characterization of Extracted Proteins from Experimental Batches of Microspheres

Samples of IFN-α2b and EGF-loaded microspheres (MS-IFN-α2b and MS-EGF) were extracted with the corresponding procedures. No changes were detected in the RP-HPLC profiles of encapsulated IFN-α2b (Fig. 2) or EGF (Fig. 3) and in the ESI-MS analysis (Fig. 4). On the other hand, Table III shows that the EGF extracted from

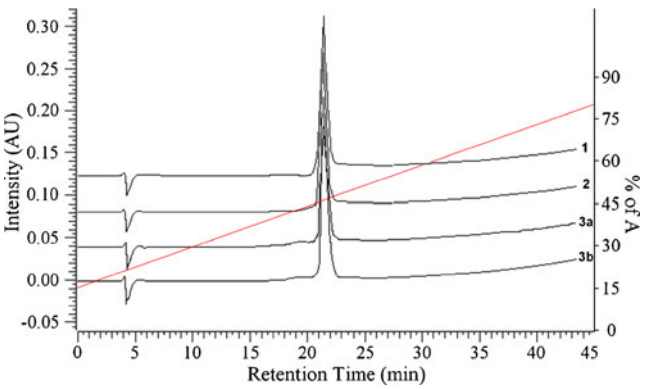

Fig. 2 RP-HPLC profile of IFN-α2b in aqueous solution (*1*) and extracted from: *a*) a mixture of empty microspheres with an exact quantity of IFN-α2b (*2*) and *b*) microspheres containing IFN-α2b (*3a* and *3b*). The extraction procedure was performed using DCM as organic phase and PBS/0.1% SDS as aqueous phase.

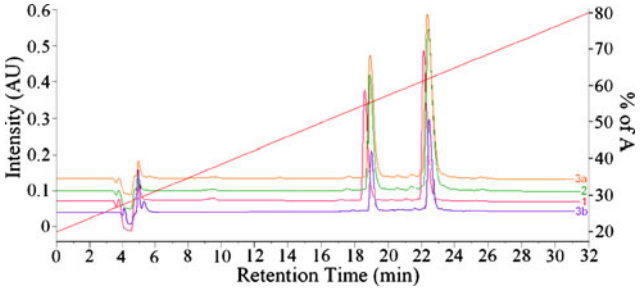

Fig. 3 RP-HPLC profile of EGF in aqueous solution (*1*) and extracted from: a) a mixture of empty microspheres with an exact quantity of EGF (*2*) and b) microspheres containing EGF (*3a* and *3b*). The extraction procedure was performed using DCM as organic phase and PBS/0.1% Pluronic F-68 as aqueous phase.

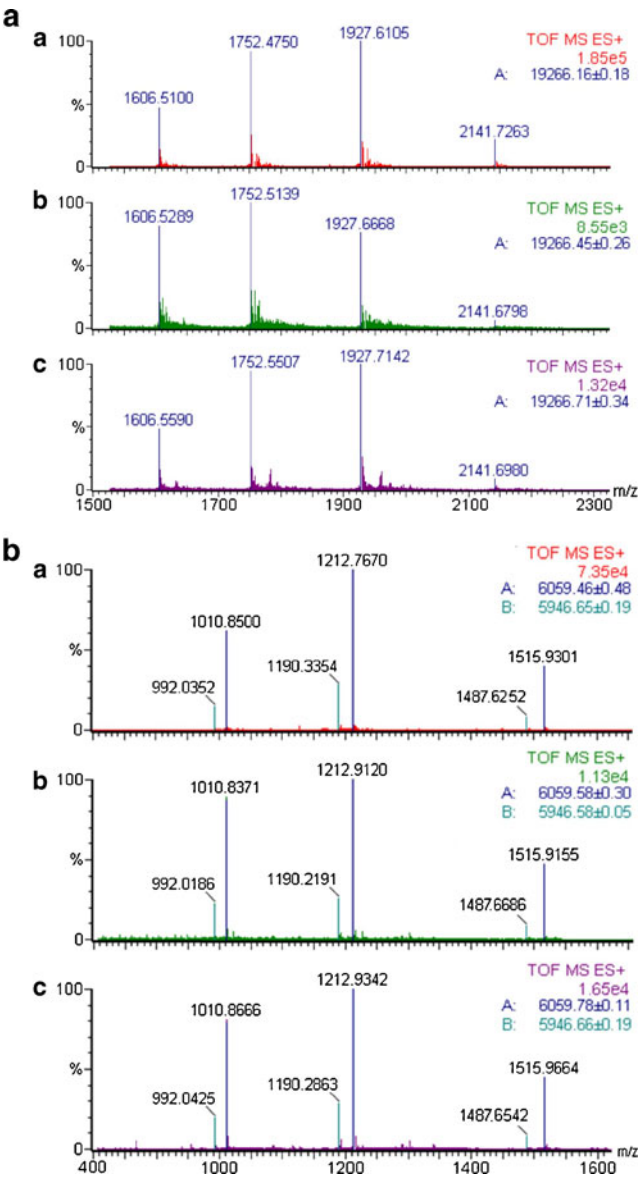

Fig. 4 Analysis by ESI-MS of IFN-α2b (**a**) and EGF (**b**). Samples were native protein in aqueous solution (*a*) and extracted from a mixture of empty microspheres with an exact quantity of protein (*b*) and microspheres containing protein (*c*).

the microspheres could be recognized by the monoclonal antibodies of the ELISA system and conserved its biological activity, while the IFN-α2b extracted from microparticles showed a decrease in both properties: immunorecognition and antiviral activity. In a previous work, our group found similar results, after the extraction of encapsulated IFN-α2b by dissolving the particles with a mixture of dichloromethane and acetone, followed by suspension of precipitated IFN with phosphate-buffered saline (7). A negative effect of the microencapsulation process over IFN-α2b was also described by Zhou *et al.* when the protein was encapsulated into magnetic PLA or PLGA microspheres by the double emulsion - solvent evaporation technique (31). They found that the minimum effective concentration of antiviral activity of IFN-α2b extracted, by DCM/double distilled water,

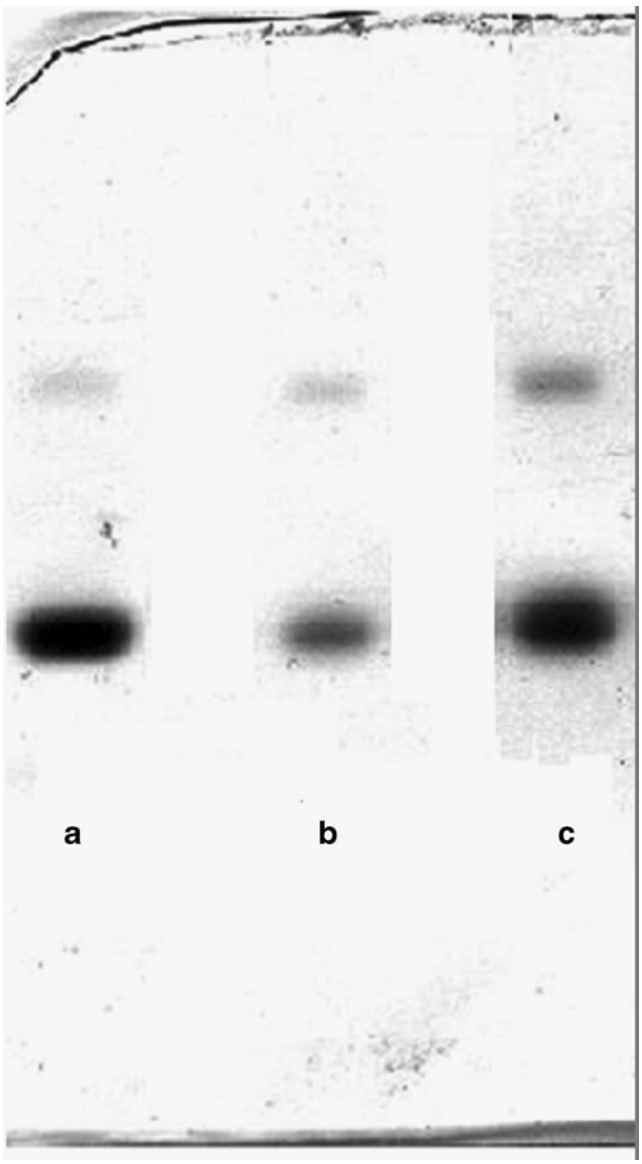

Fig. 5 Analysis of aggregation by slab-SDS-PAGE (15% gel). (b and c) samples isolated from microspheres, (a) non encapsulated IFN-α2b.

from magnetic PLA and PLGA microspheres (2.269 μg/ml and 0.724 μg/ml, respectively) was higher than that of pure protein (0.488 μg/ml).

Additionally, the samples of IFN-α2b extracted from the microspheres were evaluated by SDS-PAGE. Figure 5 shows that the protein extracted from microspheres have more aggregates than the non encapsulated cytokine. Conversely, EGF isolated from microspheres did not exhibit aggregation (data not shown).

It was demonstrated that EGF preserves its physicochemical and biological properties after the encapsulation process used here to obtain the microparticles. Al Haushey *et al.* reported that EGF encapsulated into poly-ε-caprolactone microspheres was inmmunorecognized once extracted by acetone/McIlvaine buffer but the authors also suspected that EGF quantity entrapped in microparticles may have been under-estimated by the solvent extraction method (32). The present investigation provides preliminary evidences indicating that IFN-α2b is modified after the encapsulation process while the microencapsulated EGF remains intact. According to our analyses (ELISA with MAbs that recognize conformational epitopes, SDS-PAGE, ESI-MS, and RP-HPLC), IFN modifications were probably on the secondary structure of the protein and they could be the cause of aggregation of the protein. Perhaps this discrepancy is related to the structural differences between these biomolecules. Interferon α2b is constituted by 165 amino acids with a 3D structure characterized by two intramolecular disulfide bonds (Cys1-Cys98 and Cys29-Cys138) (33) while the EGF used in this investigation is a shorter molecule that have 51–52 amino acids with three disulfide bonds (Cys6-Cys20, Cys14-Cys31 and Cys33-Cys42) (34). Consequently, it is possible that the growth factor could have a more compact structure than IFN-α2b making EGF more resistant to the harsh conditions of the encapsulation process.

CONCLUSIONS

This paper demonstrated that a two-phase liquid system comprising DCM, PBS pH 7.2 and a proper surfactant, being 0.1% of SDS for interferon alpha 2b and 0.1% of Pluronic F-68 for epidermal growth factor, allows extracting quantitatively both proteins from PLGA microspheres. Furthermore the extraction methodology is adequate to know the physicochemical and biological characteristics of the encapsulated proteins because additional changes to the molecule properties during the extraction do not occur in the studied cases. Finally it was demonstrated that the biological properties of IFN-α2b are affected by the encapsulation procedure while EGF conserved its physicochemical and biological attributes making easier the route for develop a microsphere-based delivery system with this growth factor. For extending this procedure to the extraction and characterization of other encapsulated proteins three main aspects have to be verified: 1) the stability of the protein when they are mixed with the surfactant, 2) the correct response of the analytical techniques used to quantify or characterize the protein when the surfactant is present and 3) the absence of undesired changes in the protein characteristics introduced by the extraction procedure.

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