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# Incorporation of a model protein into chitosan–bile salt microparticles

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**Abstract**

In order to develop a mucosal delivery system based on biocompatible polymers, a new methodology for production of protein-loaded microparticles is developed. Chitosan anionic precipitation/coacervation is accomplished by the addition of sodium deoxycholate (DCA). These microparticles were prepared under mild conditions, where bovine serum albumin (BSA) and DCA were simply dipped into a chitosan solution under stirring. Platelet-like and/or spherical microparticles, having high protein loading efficiency and relatively low protein external exposure, are obtained. To achieve a better compaction of the microparticle matrix, block copolymers and other non-ionic surfactants are added to the formulation. BCA analysis and fluorescence quenching were used to assess the degree of protein exposure. BSA release profiles for chitosan–DCA formulations in PBS pH 7.4 and HCl 0.1N revealed, in most cases, an initial burst release, but more than 55% of the BSA remains protected inside the microparticles. It is also observed that in acidic environment (HCl 0.1N) the protein is better shielded from the environment. Some of the formulations show good properties for mucosal protein delivery, and one of those here developed is now being tested in vivo, for mucosal administration of an adenovirus vaccine.


*Keywords:* Microparticles; Chitosan; Protein delivery; Mucosal drug delivery; Bile salts

## 1. Introduction

Recombinant oligopeptides and proteins have been increasingly used in applications in the pharmaceutical field along the last decade. This development constitutes one of the critical trends in present-day pharmaceutical technology with particular impact in the field of vaccine formulation. However, protein formulations present serious stability problems and are classically administrated by parenteral route. The alternative mucosal administration, although convenient, is prone to bioactive degradation, and its efficiency may be compromised due to deficient transport across the epithelial barrier (Chen, 2000). To overcome these problems, some authors have developed methods of encapsulation based on colloidal polymeric matrices, which are adequate for different routes of mucosal administration, protect the protein and enhance its absorption (Merkus et al., 1993; Chen, 2000).

In the case of vaccines, the oral and nasal routes are very attractive, leading to both, mucosal and systemic immune response stimulation (Merkus et al., 1993; Janes et al., 2001). This response can be modulated by the size of the particle loaded with the immunologic agent. For instance, particles smaller than 200 nm are said to be taken up by the M cells, while larger ones remain at the surface of the epithelium, releasing the macromolecule by enzymatic digestion (Thanou et al., 2001; van der Lubben et al., 2001). However, when particles are larger than 20 μm, they are prone to be washed out, being inefficient for mucosal delivery.

We are interested in developing a method suitable for the microencapsulation of an adenovirus for cattle vaccination by either nasal or oral delivery. In the present work we develop a methodology for encapsulation of a model protein, bovine serum albumin (BSA, 69 kDa), under mild conditions, as a preliminary step for future application to adenoviral delivery. Attending to the objectives, microencapsulation should involve cheap materials and methodology, and result in an effective protection against adverse temperature and humidity conditions.

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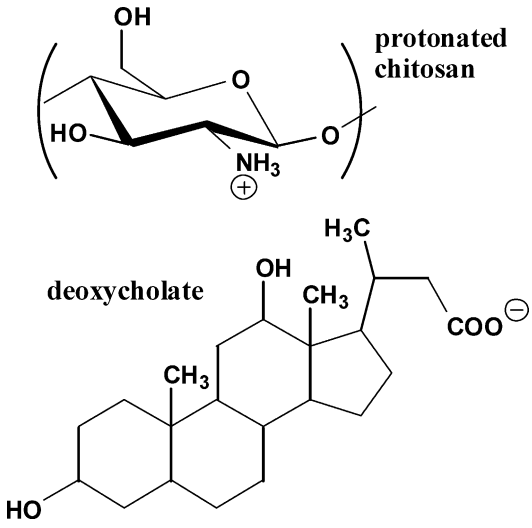
Several polymers were until now used to encapsulate proteins. In this work we will focus on chitosan matrixes whose advantages are already well described in the literature (Illum, 1998). Chitosan is a natural hydrophilic polymer obtained by partial deacetylation of chitin, an abundant polysaccharide present in crustacean shells (Illum, 1998) and is one of the polymers that were proposed for preparing protein-stabilizing matrixes (Carrara and Rubiolo, 1994). Chitosan promotes tight junction permeability (Thanou et al., 2001; Ranaldi et al., 2002), has strong mucoadhesive properties (Lehr et al., 1992; Bernkop-Schurch et al., 1998) and is used as mucosal immunoadjuvant and immunopromoter (van der Lubben et al., 2001). The positively charged amino groups on chitosan position C2 interact with the negatively charged cell surface, facilitating paracellular penetration of hydrophilic macromolecules due to tight junction opening (van der Lubben et al., 2001). Also, mucoadhesion of chitosan microparticles to Peyer's patches has been related to the enhancement of bioactive uptake (van der Lubben et al., 2002). Being biocompatible and biodegradable (its biodegradation is catalyzed by some human enzymes as lysozymes and chitinases produced by macrophages; Muzzarelli, 1997), it is an ideal carrier for bioactive molecules. Additionally, chitosan as a polycationic polyelectrolyte, has already been applied for DNA (polyanionic) complexation, and proved efficiency as a non-viral transfection agent (Mao et al., 2001; Liu and Yao, 2002).

A great advantage of chitosan relative to other encapsulation polymers is its high hydrosolubility at pH lower than 6.4 due to the protonation of the glucosamine groups. These properties enable interaction with negatively charged polymers, macromolecules and polyanions in aqueous environment (Janes et al., 2001). This is particularly interesting, since organic solvents and phase separation processes are not needed in chitosan formulations for encapsulation of macromolecules, avoiding problems related with protein denaturation during processing and regulatory specifications approvals (Chen, 2000).

Different colloidal devices for protein delivery using chitosan as a polymer have been described, as for example, spray-dried microparticles (Illum, 1998; Xu and Du, 2003), covalently cross-linked particles (Janes et al., 2001; Sinha et al., 2004), ionically coacervated particles (Aydin and Akbuga, 1996; Calvo et al., 1997; Dumitriu and Chornet, 1998; de Kruif and Tuinier, 2001; Janes et al., 2001; Ozbas-Turan et al., 2002; Shu and Zhu, 2002), chitosan-coated anionic core systems (Janes et al., 2001), thermal cross-linking, emulsification methodologies, among others (Agnihotri et al., 2004; Sinha et al., 2004).

Coacervation of chitosan can be done with several anions, namely sulfate, citrate, alginate and tri-polyphosphate as described in the literature (Calvo et al., 1997; Shu and Zhu, 2002). In this work we study the ability of bile salts to coacervate chitosan. Although not directly comparable with our study, it has been observed that chitosan derivatized with bile salts, forms hydrophobic (micelle-like) microdomains in aqueous solution (Lee et al., 1998a,b), and some authors have proposed the use of these hydrophobically modified polymers for DNA delivery (Lee et al., 1998b). Based on these data, it is predictable that bile salts may induce the aggregation of chitosan chains. Furthermore, bile salts are known to interact with lipid membranes by intercalation, increasing the permeability (Merkus et al., 1993). Therefore, it may be interesting for mucosal administration of proteins to associate bile salt to chitosan, since both are known to be good absorption enhancers.

In this study the model protein, BSA, is encapsulated into chitosan microparticles coacervated with bile salt, sulfate and citrate (Shu and Zhu, 2002; van der Lubben et al., 2002). Characterization and comparison of those microparticles, in which concerns the efficiency of coating and the release of the protein from the matrix are presented. Criteria followed for the comparative evaluation will focus on the following points: microparticle morphology, protein protection, incorporation yield, protein integrity during the process and inside the matrix and suitable drug release profile for mucosal administration (van der Lubben et al., 2001).




## 2. Materials and methods

### 2.1. Materials

Chitosan medium molecular weight (86.2% deacetylation degree, 400,000 Da average molecular weight) and the fluorescence-labeling reagent, fluorescein isothiocyanate isomer I (FITC), were purchased from Aldrich (Steinheim, Germany). Deoxycholic acid sodium salt (for microbiology), trehalose (for biochemistry), BSA (Albumin Fraction V from bovine serum, 69,000 Da), polysorbate 80 (Tween® 80), tri-sodium citrate dihydrate (GR), sodium sulfate dihydrate (GR), potassium iodide and all PBS buffer salts (GR) were purchased from Merck (Darmstadt, Germany). Pluronic® F68 and Pluronic® F127 (PEO–PPO–PEO triblock copolymers) were from BASF (Mount Olive, NJ, USA). All cellular media and supplements were purchased to GIBCO, Invitrogen (Scotland, UK). All reagents were used as acquired.

### 2.2. Protein-fluorescein conjugation

For detection and quantification purposes, BSA was labeled with FITC. Fluorescence probe conjugation of BSA was achieved according to a previously described methodology

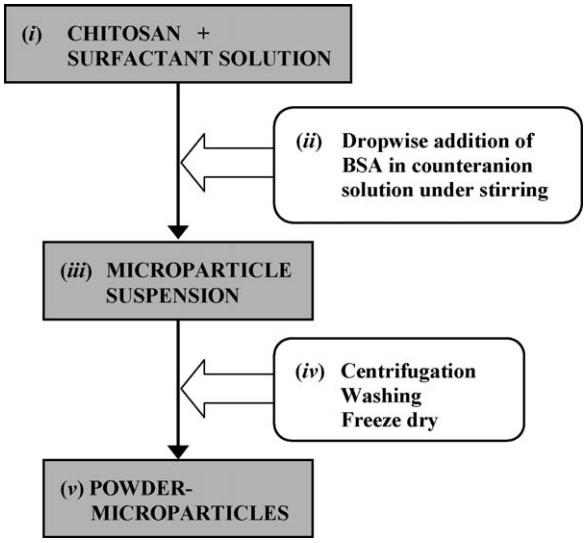



Fig. 1. Schematic representation of the microencapsulation process.

(Schauenstein et al., 1978) for amino reactive probes. Analytical quantification of BSA-FITC was done in a Spex Fluorolog 212 spectrofluorimeter at 90° geometry and the steady-state fluorescence measured at the maximum of the emission band (excitation at 500 nm).

### 2.3. Preparation of microparticles

The preparation of microparticles was carried out according to the scheme presented in Fig. 1. Two different solutions were prepared. One is a solution of 5 mg of BSA-FITC in 800 μl of an aqueous solution of counterion. Depending on which counterion is desired for chitosan coacervation, we will use DCA (0.7 and 0.18%, w/v), sodium sulfate 10% (w/v) or sodium citrate 10% (w/v). The other solution is prepared in the following way: first, 0.1 g of chitosan was dissolved in 10 ml of acetic acid 1% (v/v) containing surfactant 4% (w/v); then, this solution is diluted with PBS until 40 ml and the pH adjusted to 6.4 with NaOH. Surfactants used for the preparation of this solution were Pluronic® F68, Pluronic® F127 or Tween® 80. The first solution is added to the last one dropwise under stirring, resulting in the formation of microparticles. The suspension of microparticles so obtained was magnetically stirred for 1 h at room temperature to allow stabilization. Microparticles were collected by centrifugation (30 min at 5000 × *g*), resuspended twice in PBS and finally in 5 ml of 4% (w/v) trehalose solution. This suspension was frozen in liquid nitrogen and lyophilized overnight in a Savant Modulyo freeze-dryer. The microparticles were then kept in glass vials at 4 °C.

### 2.4. Polyacrylamide gel electrophoresis in SDS (SDS-PAGE)

In order to evaluate the harmfulness of the encapsulation process for the protein stability, protein samples from each step of the protein production scheme (Fig. 1) were analyzed by SDS-PAGE electrophoresis, with controls. Protein integrity was analyzed in samples collected during the microparticle production (steps (ii), (iii) and (iv)). Positive control, as a model of protein aggregation/degradation was obtained by heating a 10 mg/ml BSA solution in PBS at 80 °C during 15 min (Arakawa and Kita, 2000). Analysis was performed on both, the samples of the production procedure, and protein resulting from the destruction of microparticles (step (v)) with NAOH 1N. As control, we have used a solution of BSA in PBS submitted to the same procedure. The negative control used 1 mg/ml of a fresh BSA solution in PBS. All samples were either concentrated (using Centricon® tubes) or diluted in order to obtain 5 μg of protein per electrophoresis well. Protein concentration was determined by BCA analysis. SDS-PAGE electrophoresis was performed using homogeneous (12.5%) polyacrylamide slab gels on a Mini-Protean 3 Electrophoresis cell (Bio-Rad, CA, USA) with settings according to the manufacturer's instructions for our experimental conditions. Proteins were stained with Coomassie Brilliant blue R-250, and we use the Precision Plus calibration mixture (Bio-Rad) with molecular masses ranging from 10 to 250 kDa as standards.

### 2.5. Protein loading efficiency

Protein load of microparticles was calculated according to Eq. (1), where $w_t$ is the total amount of BSA-FITC used for microparticles preparation and $w_w$ is the total amount of BSA-FITC not incorporated (sum of what is left in the waste and washing solutions). Loss of protein by adsorption to glassware was found to be negligible. Calculations were done upon three replicates.

$$\text{Loading}\,(\%) = \frac{w_t - w_w}{w_t} \times 100 \quad (1)$$

### 2.6. Protein fraction exposed to the external environment

The fraction of BSA-FITC exposed to the external media in chitosan–DCA microparticles (exp (%)) was calculated according to Eq. (2), in which $w_b$ and $w_a$ correspond to the amount of BSA-FITC quantified, respectively, before and after microparticles destruction with NaOH 1 M. Protein quantification was made by the colorimetric bicinchoninic acid (BCA) method (MicroBCA, PIERCE® protein analysis kit) following the absorbance at 562 nm in a Varian Cary 3E spectrophotometer. Due to the strong interference caused by Tween 80, this method could not be applied to formulations with this surfactant. For each microparticles formulation three samples of ca. 10 mg each were analyzed.

$$\text{Exp}\,(\%) = \frac{w_b}{w_a} \times 100 \quad (2)$$

### 2.7. Fluorescence quenching assays

Another evaluation of the degree of protein exposure to the external media was carried out by the analysis of the quenching of BSA-FITC fluorescence by iodide added to the aqueous suspension. Fluorescence emission intensity of BSA-FITC

from microparticles (7 mg/ml) dispersed in a mixture of 50:50 PBS/bicarbonate pH 9.3 buffer and excited at 500 nm, $F_0$, is compared with the emission intensity after addition of iodide, $F$. In the presence of a single population of fluorophores and quencher, the conventional Stern-Volmer kinetics, Eq. (3), is observed.

$$\frac{F_0}{F} = 1 + K_{SV}[I^-] \tag{3}$$

In this equation, $K_{SV}$ is the Stern-Volmer constant that will depend on the location of the protein, which will determine the local concentration of quencher and its diffusion coefficient in the vicinity of the probe. In principle, this will allow us to distinguish between proteins at the surface or buried into the matrix. In comparison with the BCA method, that requires alkaline conditions with the risk of microparticle damage, the Stern-Volmer method is safer and, in principle, more accurate.

When the BSA-FITC is retained inside the polymer aggregates, the quenching efficiency depends of the partition of the quencher into the matrix. The Stern-Volmer equation should accordingly, be modified by substituting the quencher concentration in Eq. (3) by the local quencher concentration, $[I_I^-]_I$, related to the total iodide concentration, $[I^-]_T$, by the partition coefficient between matrix and aqueous external media, $K_P = [I_I^-]_I/[I_E^-]_E$ and fraction of the total volume occupied by the particles, $X_I$. When $X_I$ is not negligible in comparison to the total volume, as it happens in this work, Eq. (3) may be rewritten as

$$\frac{F_0}{F} = 1 + \frac{K_{SV}K_P}{1 - X_I + X_I K_P}[I^-]_T \tag{4}$$

By changing $X_I$ and $[I^-]_T$ it is possible to obtain both $K_{SV}$ and $K_P$. In practice, Stern-Volmer type plots are performed for several microparticle dilutions (several $X_I$) and the data globally fitted to Eq. (4), following the same principle currently used for partition constant determination developed by Encinas and Lissi (1982).

When a deviation from Eq. (3) or (4) is observed, namely when the Stern-Volmer plots are non-linear, it indicates the existence of two populations with different quencher accessibilities. Other reasons for non-linearity can be discarded given the low concentrations of quencher used, dimension of the particles and short lifetime of the probe (Almgren, 1991). A modified Stern-Volmer equation may be easily derived if we take into account that the fluorescence intensity of the internal and external fractions is additive. The fraction of BSA-FITC accessible to the iodide in the external medium, $x_E$, is quenched with a Stern-Volmer constant, $K_{SV\,E}$, that is, in principle, different from the one for the internally located BSA-FITC, $K_{SV\,I}$. Supposing the molar absorption coefficient the same in both media, Eq. (5) is obtained.

$$\frac{F_0}{F} = \frac{1 - x_E + (\Phi_E/\Phi_I)x_E}{\frac{(\Phi_E/\Phi_I)x_E}{1+K_{SV\,E}[I_E^-]_E} + \frac{1-x_E}{1+K_{SV\,I}[I_I^-]_I}} \tag{5}$$

Eq. (5) can be further simplified in the case of the fluorescein dianion (the species present at pH 9.3) because its fluorescence quantum yield, $\Phi$, is quite insensitive to solvent properties and it can be predicted that $\Phi_E \simeq \Phi_I$ (Magde et al., 2002). Although we do not known the local internal and external concentrations of iodide, $[I_I^-]_I$ and $[I_E^-]_E$, respectively, they are, for the same volume fraction occupied by the particles core, $X_I$, proportional to the total concentration of iodide referred to the total volume, $[I^-]_T$. This way, Eq. (5) becomes

$$\frac{F_0}{F} = \frac{1}{x_E/(1 + K'_{SV\,E}[I^-]_T) + (1 - x_E)/(1 + K'_{SV\,I}[I^-]_T)} \tag{6}$$

where $K'_{SV}$ are constants that include the Stern-Volmer constant, $K_{SV\,E}$ or $K_{SV\,I}$ and the correction for local concentration given by $X_I$ and $K_P$.

### 2.8. Contrast and fluorescence microscopy analysis

Morphological characterization and size analysis of the microparticles dispersed were performed by microscopy. A small amount of microparticles (1 mg) was suspended in 1 ml of Tween® 80 1% solution, and this suspension observed with a Leica DM RB fluorescence and contrast microscope coupled to a Leica® DC 350F CCD camera. Fluorescence was observed using a Leica L5 filter.

### 2.9. Evaluation of BSA-FITC release from microparticles

In vitro release studies were performed by suspending 10 mg of microparticles in glass vials filled with 2 ml of PBS pH 7.4 or in HCl 0.1 M. All vials were then incubated at 37 °C under planetary continuous stirring (150 rpm) maintaining the particles in suspension (sink conditions). To determine the amount of BSA-FITC released after a given time, the sample was centrifuged for 15 min at 3000 rpm in a Beckman GS-6R bench centrifuge, and the fluorescence of the supernatant measured. The pellet was resuspended, and the procedure repeated at several time intervals to obtain the release profile as a function of time. The assay was done for, at least, three replicates.

For some of the formulations, microparticles were collected after 6, 24 and 240 h of release, washed with PBS and fluorescence quenching assays carried out.

### 2.10. Accelerated stability assay

Samples of each microparticles formulation were exposed in closed vials to 50 °C in an oven (without humidity control) and their release properties compared with those of the related sample kept at 4 °C.

### 2.11. Microparticles degradation

The cellular degradation of the microparticles was studied by inoculation of Caco-2 and 293 cells with BSA-FITC loaded particles. Extracellular samples were collected, centrifuged (10 min at 2000 × $g$) and diluted 1:1 in carbonate buffer pH 9.0. Quantification of BSA-FITC was done by monitoring the fluorescence emission at 520 nm upon excitation at 500 nm in a

SLM-AMINCO 8100 spectrofluorimeter. Caco-2 and 293 cell lines were purchased from the American Type Culture Collection and were maintained in minimum essential medium (MEM) supplemented with 2 mM of glutamine, 1 mM of sodium pyruvate and heat-inactivated fetal bovine serum (10% for Caco-2 and 5% for the 293 cells) at 37 °C in an atmosphere with 5% of $CO_2$. For the fluorescence quantification assay, Dulbecco's modified eagle medium (DMEM) without phenol red was used to avoid interference, and additional supplementation was performed with 4.5 g/l of glucose.

## 3. Results and discussion

### 3.1. Microparticles formation

In Table 1 we present the different formulations studied and the abbreviations used to hereafter nominate them. All formulations departed from the same amount of chitosan (100 mg) and BSA-FITC (5 mg), prepared as described in Section 2. In the absence of counterion there is not precipitation of chitosan. When polyvalent anions, sulfate and citrate or DCA are used, precipitation occurs. In the absence of a surfactant only DCA induces formation of microsized particles. Sulfate and citrate, alone, lead to larger aggregates that are not suitable to our purposes, formulations SULF-0 and CIT-0. Precipitation due to coulombic cross-linking of chitosan cationic chains by polyvalent anions is a well-known process (van der Lubben et al., 2002; Shu and Zhu, 2002). Since monovalent hydrophilic anions (e.g. acetate) do not lead to chitosan precipitation, the mechanism of DCA-induced precipitation should involve the interaction, for one side of the hydrophobic moieties of DCA (cmc = 3 mM) (Coello et al., 1996), and for the other a coulombic interaction with chitosan, in a similar way to that already described by other authors for chitosan–SDS interaction (Dédinaité and Ernstsson, 2003). According to the content of protonated glucosamine even the smaller amount of DCA used in this study is larger than the number of non-neutralized positive charges in the polyelectrolyte chains. This number was determined based on the amount of NaOH added to maintain pH 6.4 and did not exceed 16% of the glucosamine units (total concentration 6.3 mM).

It is known that addition of a non-ionic surfactant to the chitosan solution induces microparticle formation with sulfate and citrate. We have studied the effect of the addition of non-ionic amphiphiles for all the three counteranions and compared the results (Table 1) with and without the addition of surfactant. As amphiphiles we have used 1% weight of Tween® 80, Pluronic® F68 and Pluronic® F127. Concerning the counteranion concentrations we used for sulfate and citrate those referred as optimal by other authors (van der Lubben et al., 2002). For DCA, we tried two concentrations, 0.18 and 0.7%. This last concentration was chosen such that the number of charges equals those used in the formulation with sulfate as counterion.

Depending on the formulation, different amounts of microparticles are obtained, as presented in Table 1. Although a reasonable yield of microparticles formation is desirable, the degree of protection against degradation due to external agents conferred by the matrix is the most relevant property to take into account. This property may be evaluated, in a first approach, by the loading efficiency and the microparticle/protein weight ratio, also presented in the same table.

The quite different values obtained for the microparticle recovery are hard to interpret because they result from very different masses of starting material. Anyhow, they were important to decide if the composition leads to the formation of particles. In the course of our experiments many formulations were abandoned because of their excessive low yield.

Table 1
Weight of the particles recovered (weight), protein loading efficiency (loading (%)) and microparticle to protein weight ratio (weight ratio), as a function of the nature and amount of chitosan counter anion and additional surfactant for each formulation

| Formulation | Counterion | Surfactant (1%, w/v) | Weight (mg)[b] | Loading (%)[a] | Weight ratio[b] |
|---|---|---|---|---|---|
| DCA-0-1 | DCA 0.7% | – | 107.1 | 94.0 ± 4.2 | 23.7 |
| DCA-0-2 | DCA 0.18% | – | 25.0 | 99.0 ± 1.1 | 5.3 |
| DCA-PF127-1 | DCA 0.7% | Pluronic F127 | 112.7 | 87.7 ± 4.3 | 26.8 |
| DCA-PF127-2 | DCA 0.18% | Pluronic F127 | 42.0 | 64.6 ± 5.2 | 13.5 |
| DCA-PF68-1 | DCA 0.7% | Pluronic F68 | 187.2 | 98.2 ± 1.5 | 39.7 |
| DCA-PF68-2 | DCA 0.18% | Pluronic F68 | 61.5 | 94.5 ± 2.7 | 13.6 |
| DCA-TW-1 | DCA 0.7% | Tween 80 | 168.2 | 88.3 ± 4.4 | 39.7 |
| DCA-TW-2 | DCA 0.18% | Tween 80 | 42.3 | 65.2 ± 5.8 | 13.5 |
| SULF-0 | $Na_2SO_4$ 0.2% | – | 19.7 | 24.9 ± 2.2 | 16.5 |
| SULF-PF127 | $Na_2SO_4$ 0.2% | Pluronic F127 | 35.0 | 34.0 ± 2.1 | 21.4 |
| SULF-PF68 | $Na_2SO_4$ 0.2% | Pluronic F68 | 56.9 | 46.8 ± 4.2 | 25.3 |
| SULF-TW | $Na_2SO_4$ 0.2% | Tween 80 | 45.3 | 43.6 ± 3.9 | 21.6 |
| CIT-0 | Na citrate 0.2% | – | 20.8 | 30.9 ± 2.6 | 14.0 |
| CIT-PF127 | Na citrate 0.2% | Pluronic F127 | 64.3 | 36.5 ± 2.5 | 36.7 |
| CIT-PF68 | Na citrate 0.2% | Pluronic F68 | 79.0 | 39.9 ± 2.4 | 41.2 |
| CIT-TW | Na citrate 0.2% | Tween 80 | 97.7 | 35.7 ± 2.3 | 57.0 |

The abbreviated formulation name used in the text is given in the first column.
[a] Errors presented are calculated based in the values obtained with three replicates and are not weighted by the standard deviation of the method.
[b] Due to insufficient number of replicates for most formulations, errors are not presented.

### 3.2. Protein loading efficiency

The values obtained for the BSA-FITC loading efficiency for each formulation are presented in Table 1 and were calculated according to Eq. (1). It is noticeable a clear superiority of the DCA formulations in what concerns BSA-FITC encapsulation. This larger efficiency has to be the result of the nature of the coacervation mechanism. While polyanions are directly connecting two or more protonated glucosamine groups, in this way promoting chitosan coacervation, DCA is electrically neutralized by a single glucosamine group, and coacervation results from the hydrophobic interaction of cholesteric rings.

The particle/protein weight ratio, as shown in Table 1, may be a first approach for the evaluation of the amount of the polymeric matrix that protects BSA-FITC molecules from the environment. Only considering the formulations with better loading efficiency, higher particle/protein ratio were obtained in the cases of DCA 0.7% microparticles with Pluronic® F68 and Tween® 80 as surfactant. This preliminary evidence of protein protection will be tested by direct determination of the protein exposure ratio by BCA analysis.

### 3.3. Fraction of protein exposed to the external media

The protein loading efficiency accounts for all the protein associated with a particle that may be encapsulated in the matrix or just adsorbed at the particle surface. In an attempt to distinguish these two possible protein locations, we quantified the number of peptide links available for reduction by BCA reagent. This quantification was made for dispersions of microparticles in PBS, and the result compared with the amount determined in the same dispersion after disruption of the chitosan matrix with NaOH 1N (Eq. (2)). With this method only a rough estimation of the fraction of exposed protein is obtained, because: (i) partially exposed protein may exist, (ii) some protein may be released from the matrix during the determination and (iii) the BCA reagent may infiltrate through the matrix and react with incorporated protein. In the experimental conditions used, some microparticle degradation is noticeable, so we expect that the values obtained are a majorant of the real exposed fraction of the protein, case (iii). If this test does not present other virtue, at least it measures the fraction of the peptidic links that are unprotected against an external chemical aggression in aqueous (or high humidity) conditions. This quantification could not be carried out in the case of particles containing Tween® 80, due to interference of this surfactant with the BCA method.

In Table 2 the values of the fraction of protein exposed obtained by this method are shown. All the formulations analyzed, present approximately the same fraction exposed (around 45%). An exception is the formulation DCA-PF68-1 with 30% exposure. We conclude that in all cases, at least 55% of the

Table 2
BSA-FITC exposure fraction determined by the BCA method, and linear and non-linear Stern-Volmer, SV, parameters for each DCA-microparticle formulation and time of release

| Formulation | Time of release (h) | BCA method | Linear SV | | Non-linear SV | |
|---|---|---|---|---|---|---|
| | | Exp (%) | $K'_{SV}$ ($M^{-1}$) | $X_E$ | $K'_{SV\,E}$ ($M^{-1}$) | $K'_{SV\,I}$ ($M^{-1}$) |
| BSA in solution | – | – | 98 | – | – | – |
| DCA-PF127-1 | 6 | 0.44 | – | 0.36 | 1100 | 4.4 |
| | 24 | | 15 | – | – | – |
| | 240 | | 41 | – | – | – |
| DCA-PF127-2 | 6 | 0.46 | – | 0.092 | 1500 | 18 |
| | 24 | | – | 0.11 | 2150 | 36 |
| | 240 | | 19 | – | – | – |
| DCA-PF68-1 | 6 | 0.29 | – | 0.21 | 1190 | 18 |
| | 24 | | 21 | – | – | – |
| | 240 | | 17 | – | – | – |
| DCA-PF68-2 | 6 | 0.43 | – | 0.085 | 575 | 15 |
| | 24 | | – | 0.14 | 450 | 5.5 |
| | 240 | | 25 | – | – | – |
| DCA-TW-1 | 6 | – | 20 | – | – | – |
| | 24 | | – | 0.36 | 665 | 19 |
| | 240 | | 19 | – | – | – |
| DCA-TW-2 | 6 | – | – | 0.076 | 500 | 8 |
| | 24 | | 88 | – | – | – |
| | 240 | | 26 | – | – | – |
| DCA-0-1 | 6 | 0.40 | 45 | – | – | – |
| | 24 | | 69 | – | – | – |
| | 240 | | 34 | – | – | – |
| DCA-0-2 | 6 | 0.45 | 44 | – | – | – |
| | 24 | | – | 0.58 | 525 | 7.1 |
| | 240 | | 31 | – | – | – |

loaded protein is actually inside the microparticles and is not accessible to the BCA reagent.

Based on the evaluation of encapsulation efficiency and protein protection the formulation containing 0.7% DCA and 1% Pluronic® F68, formulation DCA-PF68-1, seems to be the one with less protein exposed to the external media.

### 3.4. Contrast and fluorescence microscopy analysis

While particles of most formulations give the visual impression of being micron-sized, the morphological characterization and direct proof that BSA-FITC is associated to the particles could only be obtained by microscopy. Microphotographs of the microparticles obtained with formulations CIT-TW, SULF-TW and DCA-PF68-1 are shown in Fig. 2. Contrast images of all formulations reveal the irregular shape of the particles typical from random aggregation of polymer. Those of DCA-PF68-1 (Fig. 2e) are more spherical (less platelet-like) than CIT-TW (Fig. 2c) and SULF-TW (Fig. 2a) particles. In average, particles of CIT-TW are larger, about 30 μm, and those of SULF-TW are around 20 μm but the size distribution is much larger. The smaller particles were obtained for DCA-PF68-1 with a mean diameter 8–10 μm.

Fluorescence microscopy allows evaluating the distribution of BSA-FITC in the particles. The overlap of the images from contrast and fluorescence microscopies confirms that BSA is associated with the chitosan particles and not forming self-aggregates independent of the particles. Better uniformity is apparent for CIT-TW (Fig. 2d) and DCA-PF68-1 (Fig. 2f) than for SULF-TW (Fig. 2b). In all cases, the size and morphologic

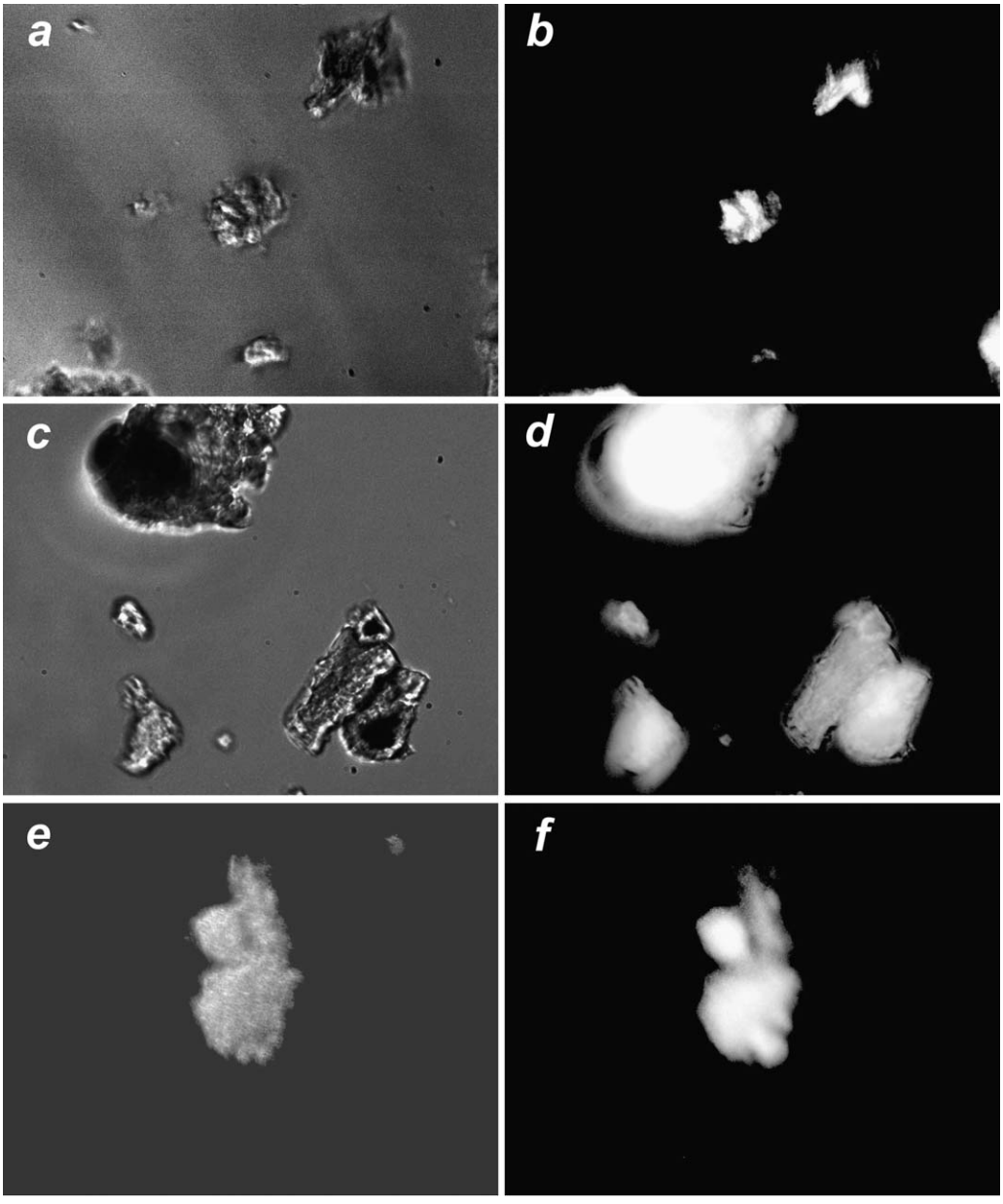



Fig. 2. Contrast (a, c and e) and fluorescence (b, d and f) micrographs of 1 mg/ml chitosan microparticles dispersions. Chitosan microparticles pertain to formulations SULF-TW (panels a and b), CIT-TW (panels c and d) and DCA-PF68-1 formulations (panels e and f). Micrographs (a–d) are 4000× amplified and (e and f) 10,000×.

characteristics of the particles obtained with and without protein are similar (not shown).

### 3.5. Analysis of the protein stability during the process of microparticle formation and in the microparticles, by SDS-PAGE electrophoresis

Integrity of BSA-FITC along the encapsulation process was screened by electrophoresis from steps (i) to (v) as described in Fig. 1. Formulation DC-PF68-1, taken as a model formulation, was analyzed, and the resulting electrophoresis is presented in Fig. 3. In steps (ii)–(iv) (lanes B, D and E), no addition of bile salt was done in order to better evaluate the effect of each physical/chemical agent in the protein stability. The evaluation of step (v) (lane I, Fig. 3) was performed by disrupting the microparticles with NaOH, and compared to a control of protein submitted to the same conditions used for disruption of microparticles (lane F, Fig. 3).

We may observe in all lanes that the strongest band is located at 60–70 kDa, which is in agreement with the molecular weight of BSA (69 kDa). That means that in all microencapsulation steps the protein is in its majority integer.

In lane H, the positive control for aggregation and hydrolysis, we may observe two other weak bands above and under the strongest common band. The bands under the main one indicate the existence of fractions of the protein resulting from hydrolysis and the two above result from the aggregation of the protein. None of the hydrolysis bands are present on the other lanes and, only lane F presents a dim band at 100–150 kDa that corresponds to aggregation of BSA in dimers. However, this band is not so pronounced as the one in lane H. Lane F corresponds to the control under the condition for the disruption of the microparticles, and even if a light sign of aggregation is present in this control, no similar bands are observed in lane I, that corresponds to the sample of BSA-FITC after disruption of the DC-PF68-1 microparticles. So, we may conclude that there is no evidence of aggregation of the protein inside the microparticle, and that the process of microencapsulation by itself (lanes B–E) is mild to the protein and do not promote its degradation.

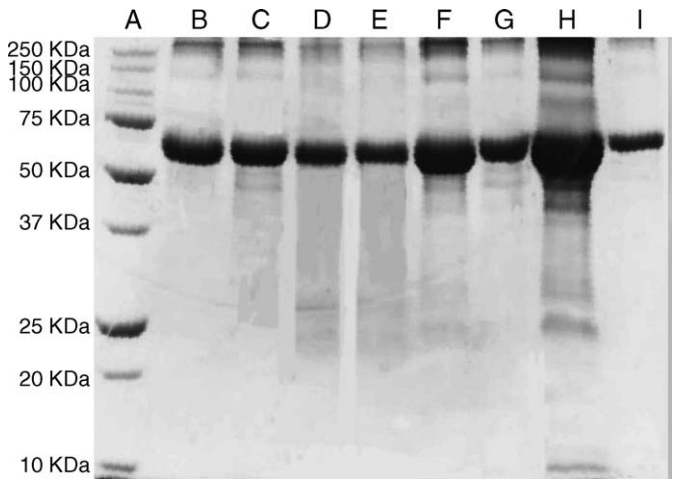



Fig. 3. SDS-PAGE electrophoresis of BSA-FITC samples collected during several steps of the encapsulation process and after microparticle disruption. Microparticles were prepared according to the formulation DCA-PF68-1. Lane A—molecular standards, lane B—BSA-FITC in DCA solution (step (ii), Fig. 1), lane C—BSA-FITC in the chitosan solution (step (i), Fig. 1), lane D—BSA-FITC in the chitosan solution under stirring (step (iii)), lane E—BSA-FITC in the chitosan solution after centrifugation, washing and freeze-drying (step (iv)), lane F—BSA-FITC standard exposed to NaOH under the microparticle disruption conditions (control 1), lane G—BSA-FITC standard in PBS (negative control), lane H—BSA-FITC in PBS exposed at 80 °C during 15 min (positive control of aggregation), lane I—BSA-FITC obtained after DCA-PF68-1 microparticle disruption with NaOH (step (v)).

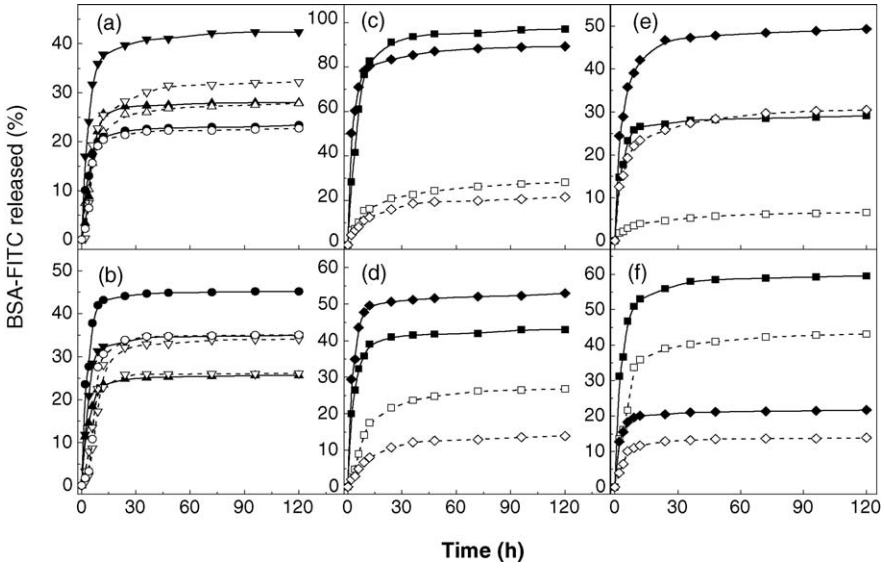



Fig. 4. Release profiles of BSA-FITC from chitosan microparticles in PBS pH 7.4 (solid symbols) and in HCl 0.1 M (open symbols), with stirring rate 150 rpm, under sink conditions. Release profiles were studied with the following formulations: (a) all sulfate-chitosan formulations, (b) all citrate-chitosan formulations, (c) DCA-without surfactant, (d) DCA-Pluronic F127, (e) DCA-Pluronic F68 and (f) DCA-Tween 80. For sulfate and citrate formulations (panels a and b), up triangles correspond to formulations with Pluronic F127, down triangles to Pluronic F68 and circles to Tween 80. For DCA formulations, squares correspond to formulations with 0.7% of DCA and diamonds to formulations with 0.18% DCA. Lines are only presented for eye guidance and do not derive from a model.

There is strong indication that BSA-FITC maintains its native form in the presence of DCA, in the concentrations used by us, and after encapsulation. When BSA-FITC is denatured in the presence of SDS we observe a hypsocromic shift of the fluorescence emission. However, no shift is observed either when DCA is added to the protein, or when the protein is encapsulated. Additionally, denaturation usually leads to aggregation of BSA, what was not evidenced in our SDS-PAGE results. In any case it is well known that non-ionic amphiphiles, such as the ones used, usually do not induce protein denaturation presumably because their bulky apolar head groups and rigid structure cannot readily penetrate into the protein molecule (Volkin and Klibanov, 1989).

### 3.6. Evaluation of BSA-FITC release from the microparticles

In Fig. 4 we show the release profiles of BSA-FITC from the microparticles for several of the formulations in physiologic and gastric release media, PBS 7.4 and HCl 0.1 M, respectively. All plots exhibit an initial burst release, whose extent is strongly dependent of the formulation and dispersing media, followed by a stagnation of the exit of BSA-FITC.

Irrespective from the surfactant or pH of the release media used, we do not observe major differences between the release profiles obtained for the formulations with sulfate as counterion, panel a of Fig. 4. All formulations have reasonable release properties, and are insensitive to pH except for the one containing Pluronic F68. Other authors described a similar behavior, but in their experiments, BSA was only adsorbed to preformed chitosan microparticles (van der Lubben et al., 2002).

With formulations coacervated by citrate, Fig. 4b, the pattern is similar to that of sulfate formulations yet the amount of BSA-FITC released is marginally larger.

The poor loading efficiency of formulations containing sulfate and citrate led us to give no further attention to them. Conversely, the formulations with DCA without surfactant, Fig. 4c, release the incorporated BSA-FITC in PBS almost completely in the first few hours. These particles tend to gellify after about 260 h (time after which we stopped the assay). This gellification is not observed in the case of HCl 0.1 M, as well as when a non-ionic surfactant is added, panels d–f. In general, formulations with DCA and surfactant show a burst release followed by a plateau and good response to acidic media. The protein remaining in the particles seems to be well protected, and is not released to the external medium even after 1000 h (data not shown). From all the formulations the one with DCA 0.7% and Pluronic® F68, confers a better general performance considering both pHs.

The kinetics of initial release in HCl 0.1 M is, in general, slower than in PBS 7.4 and for most of the tested formulations the release is less extensive in HCl than in PBS. These differences in the release profiles are more pronounced for DCA formulations. To analyze this pH sensitivity we must consider the isoelectric point of the protein, 4.7, the protonation state of chitosan at pH 7.4 that makes it water-insoluble, the p$K_a$ = 6.2 of the amphiphilic counterion DCA and the p$K_a$s of citrate making it a trianion in PBS and neutral in HCl 0.1 M. The difference

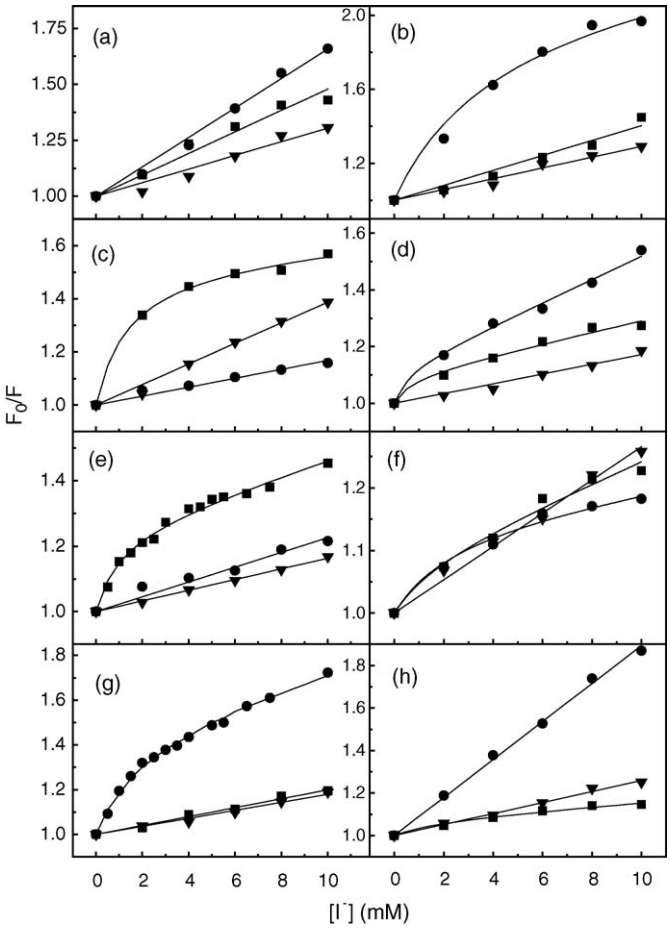



Fig. 5. Representations of the Stern-Volmer plots for the formulations: (a) DCA-0-1, (b) DCA-0-2, (c) DCA-PF127-1, (d) DCA-PF127-2, (e) DCA-PF68-1, (f) DCA-PF68-2, (g) DCA-TW-1 and (h) DCA-TW-2. Squares, circles and triangles correspond to microparticles after 6, 24 and 240 h of release, respectively. Lines presented are obtained from fitting to the linear or non-linear Stern-Volmer equations, Eqs. (3) and (6), respectively. The fitting parameters recovered are presented in Table 2.

between citrate/sulfate and DCA behavior seems to stem on the water solubility of these components in the presence of HCl 0.1 M; a lower water solubility of the counterion conducing to a better stability of the aggregate structure. Conversely, the high solubility of chitosan and the neutrality of citrate or DCA in HCl 0.1 M do not seem to be of significance for aggregate dispersion. We do not understand this last behavior, neither the apparently irrelevant effect of the change in the charge of the protein as a result of pH change. However, what is presented in Fig. 5 is the release of protein that may, or may not, be accompanied by a solubilization of chitosan. To check if chitosan is released from the matrix we quantified, with all DCA formulations, the weight loss and the amount of chitosan in the acidic release media after 24 h. The quantification of free chitosan in the release media was done forcing its precipitation from the supernatant by increasing the pH, after having removed the particles by centrifugation. The amount obtained never exceeds 7% of the weight of the particles. So, chitosan is not significantly released from the matrix.

In the case of Pluronic® formulations, panels d–e, the initial release of BSA-FITC agrees with the previously determined

externally exposed protein (Table 2). This led us to believe that the protein released is the one that was exposed in the initial matrix. The same behavior might occur for formulations with Tween (panel f), although for these systems the protein exposure ratio could not be measured.

Test of the release of the microparticles after exposure for 15 days to 50 °C do not lead to significant differences in behavior, data not shown. The particles containing Pluronic® do evidence unprotection of part of the protein and, consequently, a larger initial release (45–60%). The opposite was observed with Tween®. Since the particles remain solid at least until 140 °C the differences in behavior can only be ascribed to the characteristics of the surfactant, eventually leading to protein/surfactant separation.

### 3.7. Protein exposure by fluorescence quenching assays

The quenching of BSA-FITC fluorescence by iodide is an alternative to the BCA assay giving more detailed information concerning protein exposure. The analysis made at three different times of release may give more information on protein protection and mechanism of release from the polymer–DCA matrix.

The Stern-Volmer plot of the quenching of fluorescence of free BSA-FITC by iodide in aqueous homogeneous solution is perfectly linear, showing no evidence of differences in accessibility of the chromophores or other peculiarities (data not shown).

After 240 h of release all the Stern-Volmer plots are linear, Eq. (3), indicating that all the BSA-FITC is equally accessible to iodide as a result of the exposed protein being already removed. In all cases the slopes are lower than in water, $K_{SV}$ (water) = 98 $M^{-1}$ (Table 2). Notice that the plots (Fig. 5) are made with $F_0/F$ as a function of the global iodide concentration, $[I^-]_T$, that may differ from the concentration at the vicinity of the internally located protein, $[I_I^-]_I$. BSA-FITC buried in the matrix feels a different quencher concentration, $[I_I^-]_I$, and a presumably higher viscosity. Taking advantage of the linearity of the Stern-Volmer for the formulation DCA-PF68-1 after 24 h of release indicating that all BSA-FITC is equally accessible, we evaluated the partition constant of the iodide to the matrix using Eq. (4) as described in Section 2. Iodide prefers the polymeric matrix, with $K_P = 109$ (and $K_{SV} = 0.47\ M^{-1}$), and the rigidity of the matrix around the protein accounts for a Stern-Volmer constant about 200 times smaller than in water. This preference of iodide by the chitosan matrix should be general, so, the small values of $K'_{SV}$ (or $K'_{SVI}$) generally obtained for the internal quenching indicates a very rigid/viscous protein environment. This is particularly true for formulations DCA-PF127-2, DCA-PF68-1 and DCA-TW-1, that have the lowest Stern-Volmer slopes for the quenching of the residual BSA-FITC, possibly those that better protect the protein from water (Table 2 and Fig. 5, panels d, e and g).

Several plots of Fig. 5 have a downward curvature that is probably the consequence of fluorophores not being equally accessible to the quencher due to both, local concentration and viscosity. Taking for example formulation DCA-TW-1, panel g, 6 h after release beginning, a low slope linear plot indicates a rather compact and uniform matrix. After 240 h an identical slope is obtained indicating that 40% of the original BSA-FITC remaining is retained in a matrix with a density similar to the original. For the same formulation at 24 h the curvature indicates the coexistence of BSA-FITC in different environments. A fit of this plot to Eq. (6) gives a fraction of 0.36 exposed protein and $K'_{SVE}$ (24 h) = 665 $M^{-1}$ and $K'_{SVI}$ (24 h) = 19 $M^{-1}$ identical, within the experimental error, to the value obtained for the linear plots at 6 and 240 h $K'_{SV}$ (240 h) = 19 $M^{-1}$ $K'_{SV}$ (6 h) = 20 $M^{-1}$ (Table 2). The matrix that protects BSA-FITC seems to be uniform and very similar in which concerns density, at both, 6 and 240 h after beginning of release; at 24 h two regions are distinguishable, one of them with the same characteristic quenching efficiency. The 36% exposed protein should be located at the interface due to removal of an external protecting layer and disappears during the release process. For this and all other formulations quenching of the most exposed fluorophores, $K'_{SVE}$, is more efficient than in water. This can only be explained by a higher local concentration of iodide due to the interfacial position of the BSA-FITC.

A not too different pattern comes out from the analysis of the other formulations as presented in Table 2 and Fig. 5. The formulation already mentioned as having better properties, formulation DCA-PF68-1, presents an amount of exposed protein after 6 h of release (21%) that seems to progressively disappear at longer times.

### 3.8. Microparticles degradation by animal cells

In order to evaluate the degradation of the microparticles in vivo, digestion tests were performed with direct inoculation of BSA-FITC loaded microparticles from the formulation DC-PF68-1 (1.5 mg/ml) into cultures of animal cells seeded in six well plates and compared with the release profile of identical amounts of these microparticles in PBS. Two cell lines were used in this study, 293 and Caco-2. Fluorescence of BSA-FITC released was recorded at given times after inoculation, and measured by fluorescence emission in cellular supernatant. The release profiles are depicted in Fig. 6. As previously seen, the

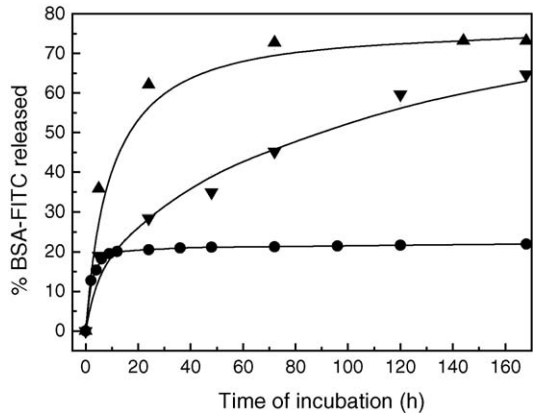



Fig. 6. BSA-FITC release profiles of microparticles prepared according to DCA-PF68-1 formulation in PBS 7.4 (circles), after inoculation in 293 cell culture (down triangles) and Caco-2 cells (up triangles). Lines are presented only for eye-guidance purpose and do not derive from any model.

release of BSA-FITC in PBS attains its maximum of about 20% after 24 h, and it was previously demonstrated that this released quantity corresponds to the most exposed protein fraction. With 293 cells, the release increases, without sign of a plateau, attaining 60% after 168 h (Fig. 6). In the experiments with Caco-2 cells the release process was faster than with 293 cells, reaching a plateau for 70% after 68 h. This evidences that these two cell lines have chitosanase activity, promoting the release of the bioactive encapsulated. We may foresee a similar in vivo behavior, since mucosal cells are known to have chitosanase activity (Muzzarelli, 1997), making this formulation a possible proposal for mucosal administration of bioactive molecules.

## 4. Conclusions

Simple addition of DCA to a chitosan solution in mild conditions showed to be highly effective in the formation of microparticles with good protein encapsulation efficiency and appropriate morphological characteristics, namely size and acceptable shape for mucosal delivery purposes. Yet, by itself, this system does not allow a satisfactory protein release pattern; this difficulty can be overcome by the addition of a biocompatible non-ionic surfactant to the formulation, e.g. Pluronics® or Tween®. The encapsulation process does not seem to be harmful for the protein, and at least in the case of BSA its integrity is maintained, as shown by SDS-PAGE electrophoresis.

The microparticles obtained, especially in the case of DCA-TW-1 and DCA-PF68-1 formulations, have a particle to protein weight ratio approaching 40 and the matrix protects 55–80% of the protein. We can consider this fraction of protein protected as very acceptable. Furthermore, the matrix seems very robust, maintaining the protein encapsulated for several weeks when microparticles are suspended in aqueous media (PBS or HCl 0.1 M). These microparticles release most of the encapsulated BSA-FITC after digestion by animal cells. So, protein delivery depends of the chitosanase activity on the delivery site.

In view of the results obtained, some of these formulations seem to have good properties for mucosal protein delivery and are adequate for the preparation of microparticulate systems for drug administration. A variation of the method, suitable for the encapsulation of an adenoviral vector for mucosal administration, is presently under development, and its efficiency is being studied in vivo (Ferreira et al., 2005).

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