Spray Freeze-Drying as an Alternative to the Ionic Gelation Method to Produce Chitosan and Alginate Nano-Particles Targeted to the Colon

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ABSTRACT: Chitosan and alginate nano-composite (NP) carriers intended for colonic delivery containing prednisolone and inulin were obtained by two processes. Spray freeze-drying using chitosan (SFDC) or alginate (SFDA) was proposed as an alternative to the traditional chitosan–tripolyphosphate platform (CTPP). NPs were fully characterised and assessed for their yield of particles; level of prednisolone and inulin release in phosphate and Krebs buffers; and sensitivity to degradation by lysozyme, bacteria and faecal slurry. NPs based on chitosan showed similar properties (size, structure, viscoelastic behaviour), but those based on SFDC showed a higher mean release of both active ingredients, with similar efficiency of encapsulation and loading capacity for prednisolone but lower for inulin. SFDC was less degraded in the presence of lysozyme and *E. coli* and was degraded by *B. thetaiotaomicron* but not by faecal slurry. The results obtained with SFDA were promising because this NP showed good encapsulation parameters for both active ingredients and biological degradability by *E. coli* and faecal slurry. However, it will be necessary to use alginate derivatives to reduce its solubility and improve its mechanical behaviour.
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INTRODUCTION

Inflammatory bowel disease (IBD) is a chronic inflammatory disease that occurs in the middle or lower part of the gastrointestinal tract. IBD includes ulcerative colitis and Crohn's disease. The drugs commonly used are anti-inflammatory agents, 5-aminosalicylates and corticosteroids for the treatment of moderate and severe IBD and immunosuppressive agents for the treatment of the disease in severe stages. A major challenge in the therapy of IBD is the prevention and reduction of drug-related side effects.[1] Chitosan and alginate have been widely studied as drug delivery platforms targeted to the colon because the drug release from these systems is triggered by the enzymatic activity of the colonic resident micro-biota. Additionally, chitosan shows high mucoadhesive properties that have been used to prolong chitosan residence in the gastrointestinal tract.[2–6]

Patients with ulcerative colitis have exhibited both diminished mucus-layer barrier properties because of overall depletion of goblet cells and higher permeability of the intestinal epithelium, allowing nano-composites (NPs) to be more easily transported into the mucosa. Therefore, mucoadhesive NPs would be particularly appropriate for site-specific drug delivery in IBD patients. In addition, the efficient diffusion of NPs through the mucus is critical for drug effectiveness. The advantages of NPs include a tendency to be phagocytosed by macrophages and neutrophils at sites of inflammation and during interactions with the mucosal layer, which is thinner in inflamed tissues.[7–9] Size and charge are major determinants of the particles ability to passively target the inflamed intestinal mucosa and reach maximum retention times in the tissue. A size-dependent accumulation pattern of micro- and nanoparticles has been reported in rats with induced ulcerative colitis, specifically in inflamed intestinal regions. Within the small intestine and the colon, 15% of the 0.1-μm particles were found to adhere to inflamed areas, whereas approximately 5% of particles were found in non-inflamed areas. The same distribution was present but less pronounced for 1-μm beads, and 10-μm particle distribution showed only a minor resemblance to this pattern.[10] NPs based on polivinyl alcohol-polylactide encapsulated in a mixture of chitosan alginate loaded with Lys-Pro-Val (KPV), an anti-inflammatory tripeptide, were produced and evaluated in a mouse model colitis. NPs of 400 nm can overcome physiologic barriers and target KPV to inflamed areas.[11] However, recent studies in human IBD patients who received a rectal enema consisting of either 10^{10} micro-particles or 10^{13} nano-particles demonstrated practically no accumulation of NPs (250 nm) in the inflamed mucosa, though deposition of micro-particles (3.0 μm) was noted.[12] Similar findings were obtained with PLGA-based nano- (300 nm) and microparticles (3.0 μm) where the targeting efficiencies in terms of particle translocation and deposition were investigated in Ussing chamber experiments using intestinal tissue from human IBD patients.[13] The reason for the discrepancy between animal and human studies is unclear.[12] Another relevant aspect to be considered is the surface charges of the NPs, which affect their mobility through the mucus. The diffusion rate of anionic NPs is shown to be 20–30 times faster than that of cationic particles. In contrast, positive chitosan charges support adhesion to the mucins, as described above. Thus, a balance between

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mucoadhesive and mucus-penetrating properties of the particles is important for efficient delivery of the drug to the inflamed mucosa.[7,8]

The most common procedure for the preparation of chitosan NPs is ionic gelation with tripolyphosphate (TPP) because only mild temperature and pH conditions are used and because the NP size can be controlled by varying the ratio of chitosan to TPP, the pH or the molar mass of chitosan. NPs prepared by dropping a TPP solution in CS showed a *Z*-average of 350–400 nm with a mass ratio of 3:1 to 4:1 (CS:TPP), a CS MW of 350 kDa and DD > 75%.[14] Using CS of MW 150 kDa and a mass ratio of 5:1–6:1 (CS:TPP) yielded NPs with a *Z*-average of 200 nm and a zeta potential of 25 mV.[15] In another procedure, the CS was dropped into the TPP solution. NPs of 120–150 nm measured by transmission electron microscopy (TEM) were obtained with LMW CS and a mass ratio of 5:1 (CS:TPP).[16] The effect of different initial sizes of CS–TPP nano-particles and storage conditions using a phosphate buffer at different pH values (5.5, 7.5 and 9.9) and on storage stability in a phosphate buffer of pH 7.5 at 25°C was evaluated. The size of the nano-particles decreased with the increase of the pH in the solution because CS–TPP NPs have a metastable structure that changes easily with the pH and ionic strength of the solutions.[17]

Spray freeze-drying (SFD) is a method developed to enhance the wetting and dissolution properties of water-insoluble active pharmaceutical ingredients (APIs). SFD is a simple process based on the atomisation of a feed solution containing APIs and/or excipients above the cryogenic liquid surface to produce frozen NPs, which are subsequently lyophilised. The ultra-rapid freezing rate prevents the phase separation of solutes within the feed solution and induces the formation of amorphous structures with high surface areas.[18] This method has not been used in the manufacture of chitosan and alginate NPs and in the present study we propose SFD as an alternative to ionic gelation.

The aim of this work was to compare NPs obtained by the SFD procedure using chitosan (SFDC) and alginate (SFDA) as encapsulating agents with chitosan–TPP (CTPP) NPs obtained by ionic gelation by examining: (1) the encapsulation parameters of prednisolone and inulin; (2) the physicochemical properties of NPs; (3) the release of the active ingredients in compendial and bio-relevant buffers; and (4) the degradation of NPs by lysozyme, bacteria (*E. coli* and Bacteroides) and faecal slurry.

MATERIALS AND METHODS

Materials

Low-molecular-weight chitosan [reduced viscosity ($\eta_{sp/c}$) = 203 (mL/g), viscosity average molar mass (Mv) = 269 kDa and degree of acetylation (DA) = 21.7% determined using conditions described elsewhere[19]], alginic acid sodium salt of low viscosity from *Macrocystis pyrifera* (viscosity of the 2% solution at 25°C 100–300 mPa) and inulin–fluorescein isothiocyanate (FITC) were purchased from Sigma–Aldrich Inc. (St. Louis, Missouri). Prednisolone was obtained from Indukern (Mainland, China). Lysozyme (81,989 units/mg) from chicken egg white was purchased from Fluka (Bornem, Belgium). *E. coli* ATCC25022 (Biomedical Laboratory Reference National Public Health Institute, Santiago, Chile) and *Bacteroides thetaiotaomicron* ATCC 29741 (Medica Tec, S.A., Santiago, Chile) were used in bacterial experiments. All other chemicals used were analytical grade.

NP Preparation

Preparation of CTPP NPs by Ionic Gelation

Twenty-five milligrams of the non-ionic, surfactant polyol Pluronic F-127, 25 mg of FITC-labelled inulin, and 2.5 mL of prednisolone, previously dissolved in ethanol (10 mg/mL), were added to 10 mL of a 1% (w/v) chitosan–acetic acid solution and diluted with distilled water to reach 100 mL. The mixture was left stirring overnight and then filtered through a 0.45-μm membrane. The solution was loaded into two 50-mL syringes mounted on an infusion pump (Model KDS200; KD Scientific©, Holliston, Massachusetts). The solution was pumped at a rate of 1.8 mL/min over 50 mL of an aqueous solution of sodium TPP at 0.3% (w/v). The resulting suspension was centrifuged at 24,000*xg* for 30 min. The pellet was separated from the supernatant and both fractions were separately frozen (−30°C for 1 day) and subsequently freeze dried (−55°C and 6.7 Pa) for 2 days before further characterisation.

Preparation of Chitosan NPs by SFD (SFDC)

The same chitosan solution used in preparation of CTPP was applied to an HPLC pump (Model 510; Waters, Milford, Massachusetts) using the tip of a flattened PEEK tube (polyether ether ketone, length 7 cm, internal diameter 50 μm) to produce the spray solution. One hundred millilitres of the chitosan solution was sprayed onto 200 mL of liquid nitrogen contained in a 400-mL beaker immersed in bucket containing 300 mL of liquid nitrogen. Chitosan solution was sprayed on the surface of liquid nitrogen at a distance of approximately 4 cm at a flow rate of 6.0 mL/min and a fixed pressure of 2.07×10^7 Pa. Liquid N_2 agitations was adjusted to produce a vortex of approximately 4 cm in height. Once all the solution was sprayed, the vessel containing the product was removed and left to evaporate excess N_2. The solid product obtained was extracted from the vessel using a spatula, placed in a plastic Petri dish of 13.5-cm diameter, covered with a layer of aluminium foil with holes to facilitate lyophilisation (−55°C and 6.7 Pa) for a period of 2 days. Subsequently, the sample was ground in a porcelain mortar and then stored at 4°C.

Preparation of Alginate NPs by SFD (SFDA)

Twenty-five milligrams of Pluronic F-127, 25 mg of inulin–FITC, and 2.5 mL of prednisolone, previously dissolved in ethanol (10 mg/mL), were added to 10 mL of an alginate solution in water 1% (w/v), and diluted with water to reach 100 mL. The mixture was left overnight with stirring and then filtered through a 0.45-μm membrane. One hundred millilitres of the alginate solution was applied to an HPLC pump using the same procedure described for SFDC preparation.

Characterisation of NPs

Zeta Potential, Polydispersity Index and Particle Hydrodynamic Diameter

Nano-composites were measured at 25°C using a Zetasizer Nano ZS-20 (Malvern Instruments, UK) operating at 4.0 mW and 633 nm with a fixed scattering angle of 173°. Thirty milligrams of NPs were dispersed in 50 mL of distilled water, left in an orbital shaker at 25 ± 0.1°C and 50 ± 5 rpm for 1–3–6

and 24 h and then sonicated for 1 h. The suspension of NPs was filtered under pressure through a 0.45-μm membrane and analysed directly on the zeta sizer. The lyophilised supernatant was characterised only for CTPP.

Morphological Characterisation by TEM

Nano-composites were observed by TEM (Philips Tecnai 12 Bio Twin microscope, Eindhoven, The Netherlands). One drop of NP dispersion was spread onto a coated copper grid, which was then dried at room temperature prior to TEM analysis.

Fourier Transform Mid-Infrared Region Spectroscopy

Fourier transform infrared (FT-IR) spectroscopy measurements were made using a Spectrum 400 (PerkinElmer, Beaconsfield, United Kingdom) in the mid-infrared region. Approximately 10 mg of the sample were ground thoroughly with 500 mg KBr, and a pellet was formed using a manual press (Pike Technologies, Madison, Wisconsin). The characteristic absorption bands for prednisolone, inulin–FITC and the physical mixtures (PMs) and formulations were measured. The spectra were obtained by averaging 10 scans in the spectral range of 450–4000 cm^{-1}.

Powder X-ray Diffraction

Nano-composite crystallinity was evaluated with a Bruker D8 Advance instrument (Bruker, Germany) using CuKα radiation (λ = 1.5406 Å, 40 kV, 40 mA). Diffractions were measured in the angular range of 5°–50° (2θ).

FT-Raman Spectroscopy

Raman spectra were acquired with a LabRAM-010 instrument (HORIBA Jobin Yvon, Longjumeau, France) using a 633-nm He–Ne laser at 5.5 mW.

Dynamic Oscillatory Test

Nano-composites were suspended at a concentration of 0.1% (w/w) in phosphate buffer, pH 7.4 or Krebs buffer, pH 7.4 for 10 min at room temperature with gentle hand-shaking. The swollen NPs were then placed in a rheometer (Carri Med CSL2 100; TA Instruments, Crawley, England) using a 40-mm steel parallel plate. The lower plate was equipped with a Peltier temperature controller. The measuring gap between plates was in range of 1500–2000 μm. The sample was allowed to relax and equilibrate at 37°C for 5 min prior to assessing the rheological properties. The oscillation test was performed in the linear viscoelastic response zone. The frequency was varied from 0.1 to 40 Hz and with a deformation of 0.7%, and the storage (G') and loss (G'') moduli were measured in duplicate at 37°C.

Solubility and Drug Release Studies

Solubility was evaluated for prednisolone and inulin–FITC in water and phosphate buffer pH 7.4. A saturated solution was placed in orbital shaker at 37 ± 0.1°C and 50 ± 5 rpm for 24 h. Aliquots were filtered and analysed by HPLC. For prednisolone, the following conditions were used: a Symmetry C18 column (150 × 3.9 mm^2, 100 Å pore size, 5 μm); mobile phase = acetonitrile–0.1% acetic acid (v/v, in water) (40:60); flow rate = 1.0 mL/min; UV Photodiode Array Detector model 996 set to 245 nm (Waters); injection volume = 20 μL at room temperature. For inulin–FITC, the following conditions were used: a Symmetry300 C18 column (250 × 4.6 mm^2, 300 Å pore size, 5 μm); mobile phase = acetonitrile–0.1% acetic acid (v/v, in water) (50:50); flow rate = 1.0 mL/min; fluorescence detector model 2475 set to 485 nm excitation and 530 nm emission (Waters); injection volume = 10 μL at room temperature. A calibration curve was prepared from 10 to 200 ppm for prednisolone and inulin–FITC, and a correlation coefficient (R^2) of greater than 0.999 was obtained for both cases.

In the drug release studies, 10 mg of NPs were placed on a VMWP-type membrane filter (0.05 μm) in a stainless steel basket normally used in the USP Dissolution Apparatus 1. The basket was properly fitted to a 100-mL beaker and immersed in 100 mL of phosphate buffer (0.02 M KH_2PO_4, adjusted with 3 M NaOH solution to pH 7.4) or Krebs buffer (1.18 mM KH_2PO_4, 24 mM $NaHCO_3$, 118.07 mM NaCl, 4.69 mM KCl, 2.52 mM $CaCl_2$, 1.18 mM $MgSO_4 \cdot 7H_2O$, pH 7.4). The assay was performed in triplicate in an orbital shaker at 37 ± 0.1°C and 50 ± 5 rpm. Aliquots of 1.5 mL were obtained at multiple time points ranging between 5 min and 7 h and were replaced with an equal volume of the medium. A 5-mL layer of liquid Vaseline was added to the Krebs buffer solution to avoid pH fluctuations during the experiment. At each time point, the release of prednisolone and inulin–FITC was measured by HPLC as described above.

Loading Capacity, Yield of Encapsulation and Yield of NPs

For the determination of loading capacity (LC) of prednisolone and inulin–FITC, samples were taken at 24 h in both dissolution media. Preliminary studies performed at 24, 48 and 72 h showed that prednisolone and inulin–FTIC were completely released at 24 h. LC was calculated using equation:

$$\mathrm{LC}\,(\%) = \frac{\mathrm{MAI}_{24\,\mathrm{h}}}{\mathrm{MP}} \times 100 \tag{1}$$

where $MAI_{24\,h}$ is the amount of active ingredient released at 24 h and MP is the total amount of particles (mg).

The yield of encapsulation (YE) was calculated using equation:

$$\mathrm{YE}\,(\%) = \frac{\mathrm{LC} \times \mathrm{MP}}{\mathrm{MAI}} \tag{2}$$

where LC is the loading capacity of active ingredient, MP is the total amount of particles and MAI is the total amount of active ingredient (mg).

The yield of particles (YPs) was calculated using the equation:

$$\mathrm{YP}\,(\%) = \frac{\mathrm{MP}}{\mathrm{MP}_0} \times 100 \tag{3}$$

where MP is the total amount of particles and MP_0 is the sum of the initial dry weight of the ingredients.

Dissolution Studies with Lysozyme

Fifteen milligrams of lysozyme from chicken egg white with an activity of 81,989 units/mg was weighed and dissolved in 300 mL of 0.05 M KH_2PO_4 buffer, pH 7.4. The assay was applied to the CTPP, SFDC, and SFDA NPs in triplicate along with respective controls without enzymes under the same experimental conditions and equipment as described for the drug release studies.

Microbiological Assay and Growth Conditions

B. thetaiotaomicron ATCC 29741 was cultured on brain heart infusion (BHI)–agar plates for 7 days under anaerobic conditions (Gaspak Genebox System®). For the assay, a sample of colonies was re-suspended and cultured in BHI broth for 4 days under anaerobic conditions until the OD_{600} reached 0.8 (corresponding to a concentration of 6.0×10^8 CFU/mL).

E. coli ATCC 25922 was grown at 37°C in Luria Bertani (LB) broth with shaking until the OD_{600} reached 0.7 (corresponding to a concentration of 5.5×10^8 CFU/mL). Ten milligrams of NPs was added to 40 mL of each strain culture (at an initial pH of 6.5), and the suspensions were incubated at 37°C under constant stirring (50 rpm). One millilitre samples were harvested at 30, 60, 120 and 180 min (with a final pH of 5.5). To determine the release of prednisolone by HPLC, the samples were centrifuged at 3000 rpm for 15 min. Sterile BHI and LB culture media were used as controls. Each experiment was performed in triplicate.

Faecal Slurry Assay

A batch culture system carried out in Biostat bioreactor (B.Braun) was used to simulate the conditions of the colon according to the procedure described by Basit and Lacey.[20] Ten milligrams of NPs was introduced into 100-mL freshly voided human faeces (10%, w/v). The faeces were obtained and pooled from three healthy female volunteers and homogenised with a stomacher (Seward 400; Lab Blender, Worthing, West Sussex, UK) in phosphate buffer 0.02 M pH 7.4, which was previously boiled for at least 15 min for salt dissolution and oxygen removal. The faecal slurry was passed through a 500-μm sieve to remove any unhomogenised fibrous material. The fermenters were sealed under positive nitrogen pressure to establish an anaerobic environment and then incubated at 37°C. Each formulation was analysed in triplicate. A control was performed in parallel with the NPs in the presence of buffer. Aliquots of 300 μL were removed at 30, 60, 120 and 180 min and were precipitated with acetonitrile (900 μL). The samples were centrifuged at 6,000*xg* for 10 min, filtered and injected onto the HPLC. The analytical conditions were obtained from literature[21] with minor modifications. The system consisted of an HPLC (Alliance 2695e Model; Waters) equipped with a PDA detector (996 Model; Waters) and a Symmetry C18 (250×4.6 mm^2, 300 Å pore size, 5 μm) column from the same manufacturer. The temperature was maintained at 40°C and the mobile phase was water–tetrahydrofuran–methanol (68.8:25:6.2) pumped at a flow rate of 1 mL/min. The volume of injection was 20 μL and the detector was configured to 254 nm. A calibration curve was prepared from 2 to 20 ppm for quantitation of the samples, and a correlation coefficient (R^2) of greater than 0.999 was obtained.

Statistical Analysis

The experimental data were analysed by analysis of variance (ANOVA) and significance of differences between means by Tukey's multiple range tests (STATGRAPHICS Centurion XV.II; Statpoint Technologies, Inc. Warrenton, Virginia). A p value under 0.05 was considered significant.

RESULTS AND DISCUSSION

Characterisation of NPs

The effect of the time dispersion in water at 1, 3, 6 and 24 h on NPs' hydrodynamic size, PDI and ξ potential was evaluated (see Table 1). The NPs showed no significant time effect over *Z*-average except for the SFDC between 1 h (643 nm) and 24 h (484 nm). However, it was observed for all NPs that the *Z*-average at 24 h was lower compared with the value at shorter times of dispersion. Additionally, the *Z*-average of NPs based on alginate (SFDA) was significantly smaller (138 nm) compared with NPs based on chitosan (481–484 nm). A significant effect on PDI values was observed for SFDA at 1 h, where the PDI value was 0.71, compared with approximately 0.46 at 3, 6 and 24 h. In the case of NPs based on chitosan, no significant time effect was observed, but the PDI values were higher compared with SFDA. Regarding the surface charge, chitosan NPs were positive (around +21–24 mV), and alginate NPs were negative (−41 mV) as expected. In chitosan NPs, there is a tendency to increase the ξ potential when the time of dispersion is increased. SFDC showed a significantly higher ξ potential at 6 and 24 h (24–28 mV) compared with 1 and 3 h (16–20 mV). Moreover, ξ potential values were higher for SFDC (24–28 mV) compared with CTPP (15–21 mV), and significant differences were obtained at 3 and 6 h. In the case of SFDA, ξ potential value was not affected by the time of dispersion in water.

It has been described that the concentrations of chitosan and TPP and the CS–TPP mass ratio are critical variables on the size of the NPs obtained. The mean hydrodynamic size for CTPP varied from 200 to 745 nm.[22] In this work, CTPP was prepared from 1.0 mg/mL of LMW chitosan in an acetic acid solution (Mv = 269 kDa and DD = 78.3%) dropped over a 0.3-mg/mL TPP solution with a CS–TPP mass ratio of 6:1. The NPs showed a *Z*-average value between 481 and 549 and broad unimodal dispersion that was similar to the results described by Koukaras et al.[14] *Z*-average = 498 ± 7 nm at the same CS–TPP mass ratio using CS of MW 350 kDa and DD > 75%. However,

Table 1. Characterisation of NPs of CTPP, SFDC, SFD, *Z*-Average, PDI and ξ

NPs/Time	CTPP			SFDC			SFDA		
h	*Z*-Average	PDI	ξ	*Z*-Average	PDI	ξ	*Z*-Average	PDI	ξ
	nm ± SD		mV ± SD	nm ± SD		mV ± SD	nm ± SD		mV ± SD
1	549 ± 32^{aA}	0.57 ± 0.04^{aC}	15.8 ± 1.4^{aE}	643 ± 32^{aA}	0.57 ± 0.04^{aC}	16.0 ± 1.4^{aE}	262 ± 32^{aB}	0.71 ± 0.04^{aC}	-40.0 ± 1.4^{aG}
3	639 ± 32^{aA}	0.70 ± 0.04^{aC}	10.9 ± 1.4^{bE}	611 ± 32^{aA}	0.60 ± 0.04^{aC}	19.7 ± 1.4^{aF}	232 ± 32^{aB}	0.46 ± 0.04^{bD}	-41.5 ± 1.4^{aG}
6	608 ± 46^{aA}	0.62 ± 0.06^{aC}	15.2 ± 1.9^{aE}	539 ± 32^{aA}	0.71 ± 0.04^{aC}	27.9 ± 1.4^{bF}	175 ± 32^{aB}	0.47 ± 0.04^{bD}	-41.1 ± 1.4^{aG}
24	481 ± 32^{aA}	0.48 ± 0.04^{aC}	20.7 ± 1.4^{aE}	484 ± 32^{bA}	0.63 ± 0.04^{aC}	24.3 ± 1.4^{bE}	138 ± 32^{aB}	0.46 ± 0.04^{bC}	-41.4 ± 1.4^{aG}

Different superscript lowercase letters mean significant differences between rows $p < 0.05$; different superscript capital letters mean significant differences between columns $p < 0.05$.

Rampino et al.[15] described a *Z*-average of 193 ± 28 nm at the same CS–TPP ratio using CS of MW 150 kDa; the DD value was not provided. This lower *Z*-average could be attributed to the low molecular weight of CS. It has been suggested that low molecular weight CS would form smaller particles because of shorter polymer chains that are easily penetrated in the partially formed CS–TPP associates to produce smaller and denser beads.[22] The ξ potential of CTPP obtained in this work was 20.7 ± 1.4 mV and was in the range of 25 ± 3 mV described in the literature under similar conditions.[15]

In the case of chitosan NPs, the effect of time of dispersion in water over the *Z*-average and ξ potential was based on the metastable nature of this nano-gel. It has been described that the structure of NPs easily changes with different environmental conditions, such as solution pH and ionic strength. Because of lower protonation, CS molecules in water shrink and form more inter- or intra-molecular hydrogen bonds, thus leading to a smaller hydrodynamic size of the particles.[22,23]

The nano-structure of NPs was observed by TEM because of its high resolution and easy preparation of the sample. Gokce et al.[24] used TEM to characterise freeze-dried chitosan NPs that were ultra-sonicated in water for 20 min. TEM images of reconstituted formulations were obtained. Figures 1a–1c show spherical NPs of approximately 200 nm for CTPP and SFDC. These values were lower than those determined by DLS because TEM directly measures dried samples, whereas the Zetasizer system determines the size by first measuring by DLS the random movement of particles in a liquid because of bombardment by the surrounding particles (Brownian motion) in a sample and then extrapolating a size from this movement using the Stokes–Einstein equation.[25] Instead, SFDA showed many large amorphous areas and very small particles, indicating that the NPs were completely disaggregated. After freeze-drying, particles were agglomerated, as observed by SEM (results not shown), probably because of hydrogen bonding, as noted by FT-IR.

The interactions between the different components present in the NPs were evaluated by infrared spectroscopy in six different regions (R1–R6) of the spectrum (see Figs. 2a–2c). In R1, a broad and intense band indicated that the OH groups were involved in hydrogen bonding. The increasing intensity of this band in the SFDC formulation (see Fig. 2a) compared with that of chitosan could be explained by the possible association through hydrogen bonds between the inulin–FITC and chitosan.[26–29] In R2, the change in the appearance of the band at approximately 3000 cm^{-1} (CH stretching) indicated the presence of hydrophobic interactions between chitosan and inulin–FITC; this was confirmed by displacement of the absorptions in the R4 region. These changes have been described as results of hydrophobic interactions in biomolecules, mainly van der Waals forces,[30] and suggest miscibility between chitosan and inulin–FITC[30] to stabilise the NP. The interactions described above are weak in contrast to those observed in R3 (solid blue arrow), in which NH bending in chitosan at 1596 cm^{-1} is shifted to 1560 cm^{-1} in SFDC, implying the presence of an -NH_3^+ group that can interact electrostatically with the acetate anions of the formulation. The integrity of the inulin–FITC can be verified in R5 by the presence of the CH_2 vibration out of the plane at 948 cm^{-1} in SFDC (dashed black arrow), which is characteristic of inulin–FITC.[31] Prednisolone (Fig. 2b) showed characteristic signals in R3 at 1711, 1655 and 1613 cm^{-1}, associated with the C=O stretching unconjugated to C-20 and the C=O stretching

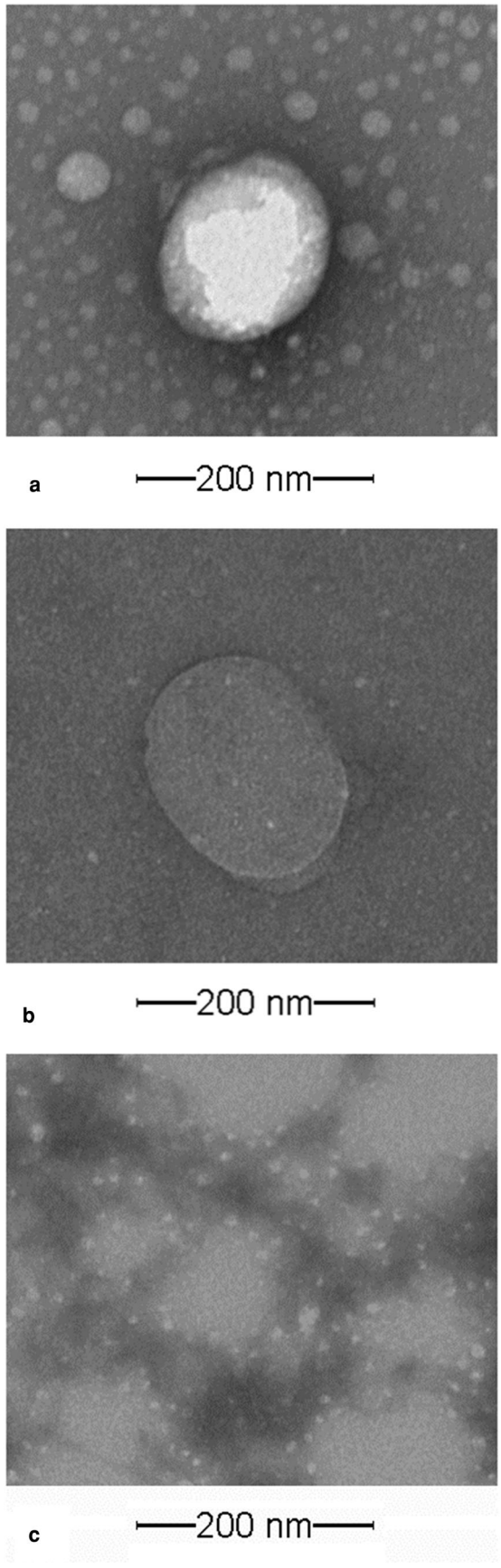

Figure 1. Transmission electron microscopy images of NPs suspended in buffer phosphate pH 7.4: (a) CTPP, (b) SFDC and (c) SFDA.

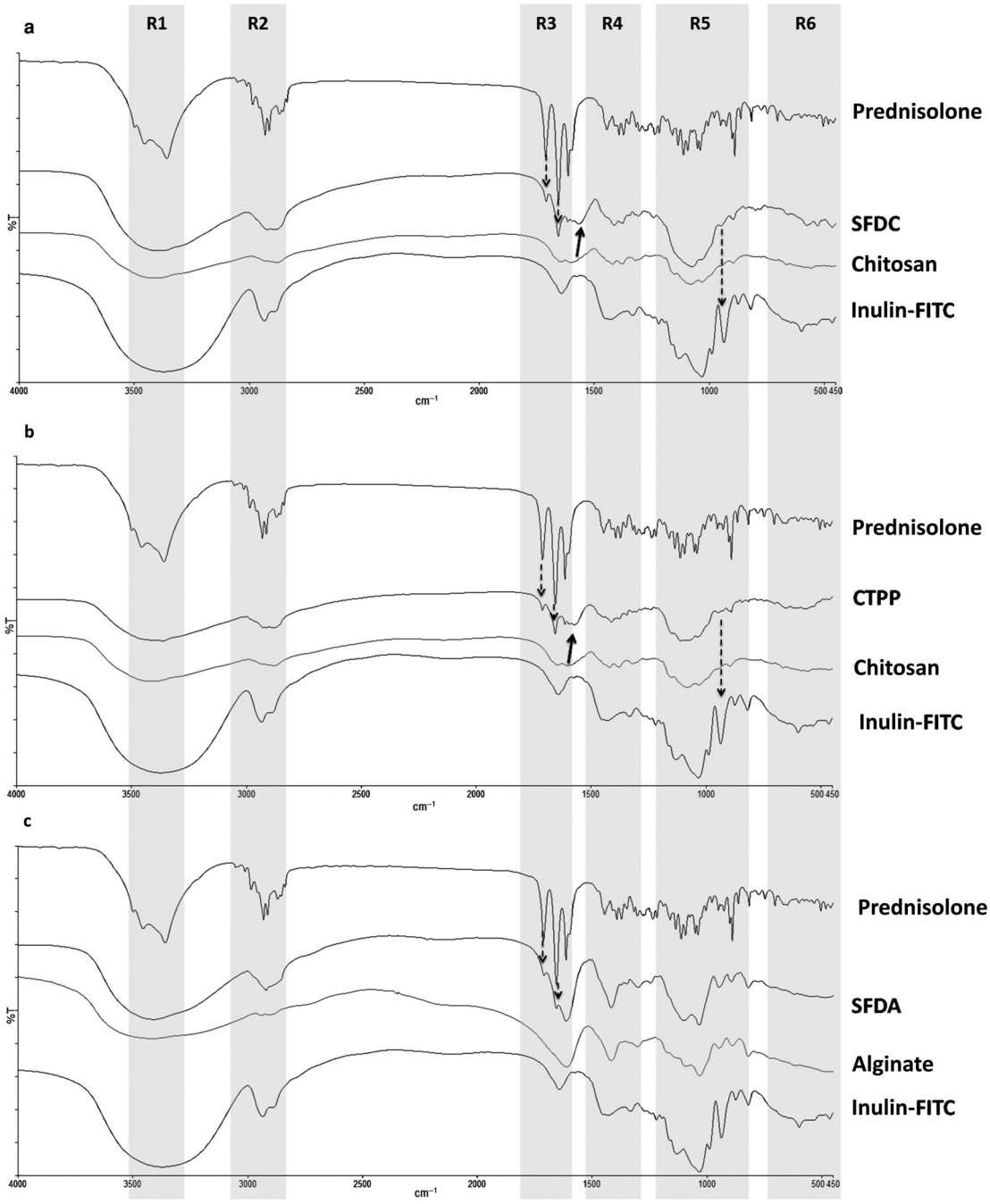

Figure 2. Fourier transform infrared spectra: (a) prednisolone, SFDC, chitosan and inulin–FITC; (b) prednisolone, CTPP, chitosan and inulin–FITC; (c) prednisolone, SFDA, alginate and inulin–FITC.

conjugate of the C-3 and C=C alkene groups, respectively.[32] These signals (dashed arrow) appear at the same wave number in SFDC, which indicates that there is no chemical modification or covalent bond formation when prednisolone is included in the matrix formulation. Figure 2b shows the spectrum for the CTPP. The interaction between the NH_3^+ group of chitosan with TPP can be corroborated by the shift of this signal from 1596 to 1548 cm^{-1} in CTPP (solid arrow), indicating a strong association between chitosan and its counter ion in the formation of the matrix.[16] As observed in the spectra of prednisolone and inulin–FITC, the incorporation of this active ingredient occurs without chemical modifications. In Figure 2c, the spectrum of alginate has two characteristic signals in R3 and R4 at 1614 and 1416 cm^{-1}, associated with the asymmetric and symmetric stretching of the carboxylate groups, respectively.[29] These signals are also present in SFDA at the same wave number, indicating no chemical changes of the product after processing. As for the other NPs, the addition of prednisolone or inulin–FITC did not produce covalent modifications.

Figure 3a shows the Raman spectra for the NPs. The spectrum of prednisolone shows a distinctive signal at 1653 cm^{-1} associated with the carbonyl group, in accordance with the

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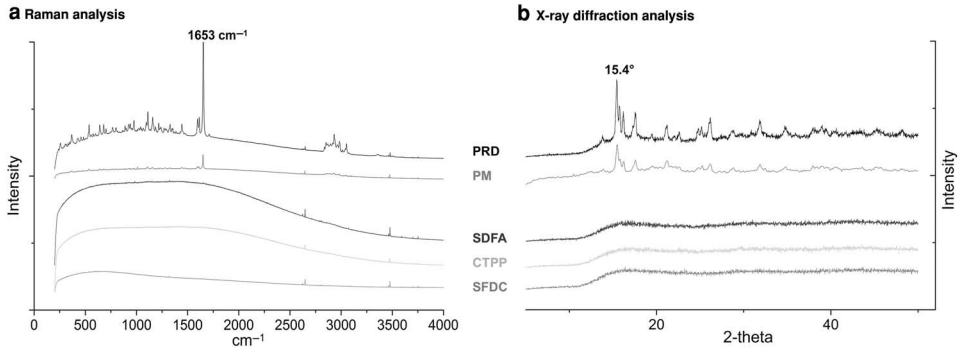

Figure 3. Raman and X-ray diffraction analysis of prednisolone (PRD), PM and formulations (SFDA, CTPP and SFDC).

literature.[33] This absorption is present in the PM without shift, similar to what was found with infrared analysis. However, in the Raman spectra of SFDA, CTPP and SFDC formulations, the intensity of this signal is low, appearing from 1649 to 1650 cm^{-1}, and no other characteristic bands of prednisolone are present. This type of phenomenon is associated with an amorphous drug dispersion.[34,35]

Figure 3b shows the diffractograms of NPs. The diffractogram of prednisolone shows distinctive signals at 2θ angles 15.4°, 15.7°, 16.2°, 17.6°, 21.2°, 26.1°, 31.6° and 34.8°, associated with its crystalline nature.[32,36–38] These peaks are also present in the PM; however, they are absent in SFDA, CTPP and SFDC. This type of phenomenon is associated with drug dispersion in an amorphous form.[36–38]

Small-amplitude oscillatory shear tests were performed to investigate the effect of the buffer composition on the dynamic rheological properties of the NPs. Figure 4 shows the effect of the buffer composition (phosphate and Krebs at pH 7.4) on G' and G'' moduli for each formulation. All the formulations showed that $G' > G''$ and both moduli diminished in the following order: CTPP > SFDC > SFDA. The mechanical spectrum observed for chitosan-based NPs in phosphate and Krebs buffers showed values of $G' > G''$, typical of a true gel, and both moduli showed flat mechanical spectra with slight frequency dependence under low-strain conditions.[39] This result indicates that CTPP and SFDC were in a "true gel" state under the tested conditions. CTPP showed higher G' and G'' values compared with SFDC and no dependence of the buffer composition. SFDC showed higher G' and G'' values in the Krebs buffer compared with the phosphate buffer. In the case of SFDA, the gel structure was very weak; thus, in the phosphate buffer the mechanical spectra cannot be measured despite the low-strain conditions used. In the Krebs buffer, very low G' and G'' values were observed, and in the high-frequency region, G' decreased, whereas G'' increased. It is not surprising that SFDA showed more viscous liquid behaviour because alginate is fully ionised at pH 7.4. In contrast, chitosan-based NPs showed a more solid elastic behaviour at this pH. SFDC was a weaker gel than CTPP because it had lower G' and G'' values and its mechanical behaviour was dependent on the buffer composition.

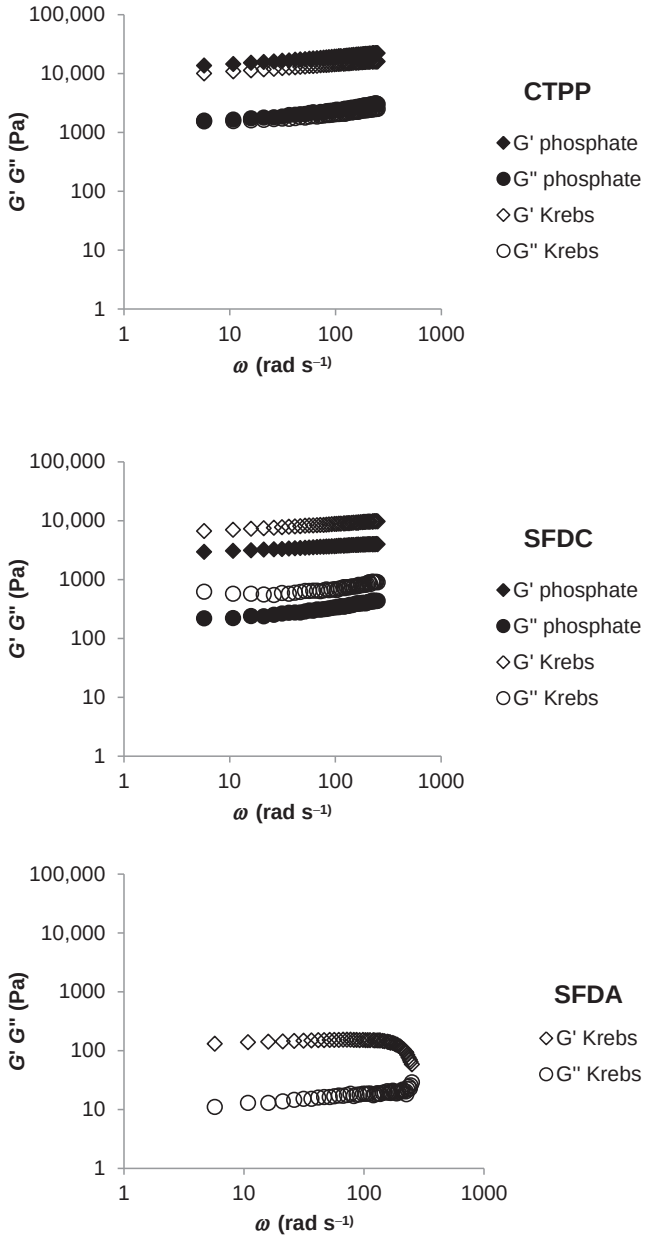

Figure 4. Mechanical spectra as a function of oscillatory frequency of CTPP, SFDC and SFDA. All samples were tested under 0.7% of strain at 37°C.

Solubility and Drug Release Studies

The solubility of prednisolone measured in water (0.295 mg/mL) and in phosphate buffer at pH 7.4 (0.2974 mg/mL) was very similar and also close to previously reported values, where the

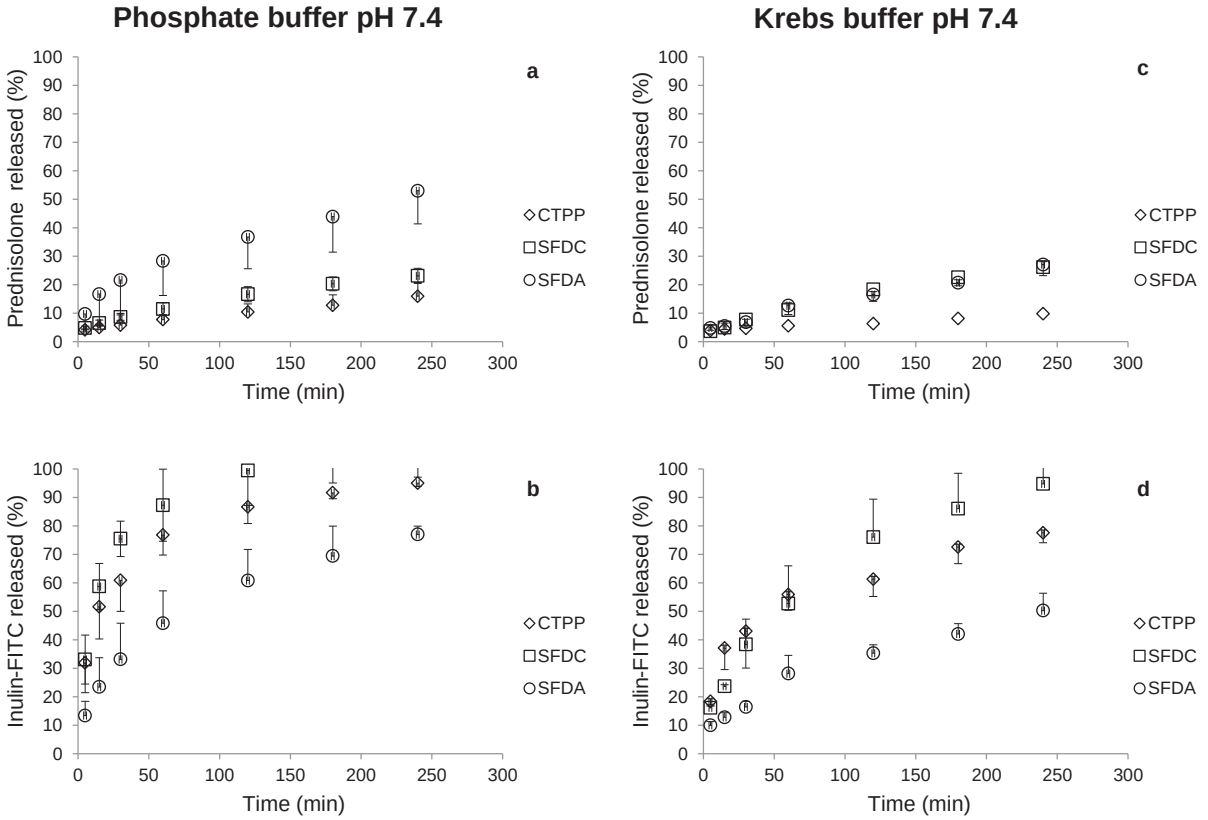

Figure 5. Prednisolone and inulin–FITC release from nano-particles formulations in phosphate buffer pH 7.4 (a and b) and Krebs buffer pH 7.4 (c and d).

solubility of prednisolone in water, phosphate buffer at pH 7.4, and Hanks buffer at pH 7.4 (which has a similar ionic composition to Krebs) was 0.223 mg/mL.[40] For inulin–FITC, the measured values were 4.2 mg/mL in water and 3.1 mg/mL in the phosphate buffer at pH 7.4. Figures 5a and 5b show the dissolution profiles for prednisolone, and Figures 5c and 5d) show the profiles for inulin in phosphate and Krebs buffers at pH 7.4 for each formulation. Clearly, the amount of inulin released in both dissolution media is significantly higher than prednisolone. Additionally, the apparent kinetics of the prednisolone (linear) and inulin (exponential) releases was different. The solubility of inulin–FITC is 10 times higher, and its molecular weight is 11 times higher than that of prednisolone.[41] Thus, the higher mass transfer rate of inulin in relation to prednisolone can be explained by the higher inulin solubility compared with prednisolone if the process is controlled mainly by diffusion.

A multifactorial ANOVA was performed for each active ingredient using the percentage change of the active ingredient released in response to the formulation, dissolution media and time. In the case of prednisolone, the mean release levels were: SFDA ($21.8 \pm 0.8\%$) > SFDC ($13.3 \pm 0.8\%$) > CTPP ($7.4 \pm 0.8\%$). The dissolution media had no effect on the mean prednisolone released for the chitosan-based NPs, CTPP and SFDC. However, for the alginate-based NP formulation, SFDA, the mean drug level released was higher in the phosphate buffer (30.0 ± 1.1%) compared with the Krebs buffer (13.5 ± 1.1%) ($p < 0.05$). For inulin, the mean release levels were: SFDC (70.9 ± 1.6%) > CTPP (61.4 ± 1.3%) > SFDA (37.0 ± 1.3%). In all the formulations, the percentage of inulin released was higher in the phosphate buffer than in the Krebs buffer ($p < 0.0031$). In the case of inulin, the highest mean release was obtained with chitosan-based NPs.

Alginate and chitosan show different solubilities at pH 7.4. Alginate is a block copolymer comprising homopolymeric regions of α-D-mannuronate (M) and R-L-guluronate (G), termed M- and G-blocks, respectively, and interspersed with regions of alternating structure (MG-blocks). The dissociation constants (p*K*a) for mannuronic and guluronic acid monomers are 3.38 and 3.65, respectively. Thus, sodium alginate-based SFDA is fully ionised and soluble at pH 7.4. However, chitosan, a copolymer of N-acetyl-D-glucosamine and D-glucosamine, is a weak base with a p*K*a value of the D-glucosamine residue of approximately 6.2–7.0.[42] At a pH above its p*K*a, chitosan molecules shrink because of lower protonation and/or form more inter- or intra-molecular hydrogen bonds, thus leading to smaller hydrodynamic volume NPs.[17] It has been reported that the release of bovine serum albumin (BSA) from CTPP at pH 7.5 was slower compared with that at pH 1.2 because the NPs are partially collapsed at pH 7.5, with BSA trapped inside and interacting with chitosan via hydrogen bonding and ionic interactions.[22] Another important factor to consider is the effect of ionic strength in the swelling-dissolution behaviour of the polyelectrolyte gels. Fadda et al.[43] have reported that the Krebs buffer (pH 7.4) has a higher ionic strength (0.161) than the phosphate buffer (pH 7.4) (0.129). Thus, different ionic environments can influence the tortuosity and porosity of the matrix and, therefore, the drug diffusion. In fact, the effects of buffer composition were

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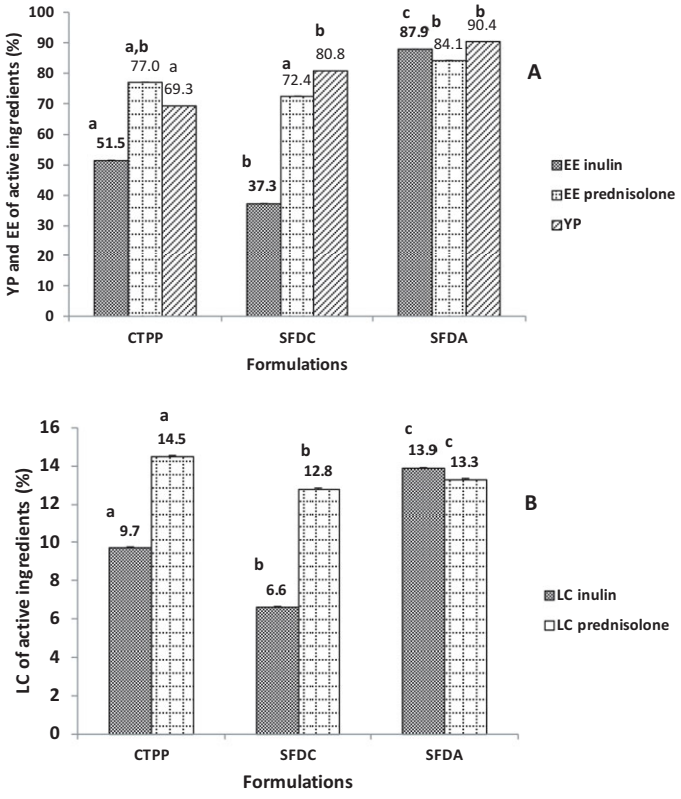

Figure 6. Yield of particles and EE of prednisolone and inulin (A), different letters indicate statistical significance relative to EE inulin, EE prednisolone and YP ($p < 0.05$); and LC of prednisolone and inulin of nano-particle formulations (B), different letters indicate statistical significance relative to LC inulin and LC prednisolone ($p < 0.05$).

observed on inulin release for all the formulations. The mean of inulin released was higher in the phosphate buffer compared with the Krebs buffer. For prednisolone, this effect was only observed in SFDA, which could be explained by the very weak structure of the gel. Thus, it seems that a lower ionic strength increases drug dissolution from NPs. It has been noted in alginate gels that higher ionic strength can affect the solute mobility gels by decreasing the gel Donnan potential, leading to decreased partitioning of solutes within the gel.[44] In the case of chitosan-based NPs, CTPP has an isoelectric point of approximately 7.5, which could explain the low mean release of prednisolone, in addition to the effect of the ionic strength causing a decrease of the gel partitioning.[23] This ionic effect results in the gel Donnan potential being higher in the phosphate buffer compared with the Krebs buffer, thereby explaining the higher mean release of inulin (CTPP, SFDC, SFDA) in the phosphate buffer compared with the Krebs buffer.

LC, Yield of Encapsulation and YPs

Figure 6a compares the YPs and efficiency of encapsulation (EEs) for inulin and prednisolone obtained by three methods of NP preparation. The highest YP was obtained by the SFD method, with significant differences ($p < 0.0060$) between alginate (SFDA, 90.4 ± 3.3%, $n = 3$) and chitosan (SFDC, 80.8 ± 3.3%, $n = 3$). The lowest YP corresponded to the ionic gelation method (CTPP, 69.3 ± 2.9%, $n = 4$). It has been reported that the YPs and their distribution in sediment and supernatant after separation of the CTPP by centrifugation depended on the CS–TPP ratio. At a CS–TPP ratio of 6:1, the YP in the supernatant was in in the range of 40%–50%, and the distribution of the particles was similar in the sediment. Both fractions had the same particle size for CS–TPP (6:1 and 9:1). For mass ratios higher than 9:1, bigger particles were obtained in the sediment.[45] In our experiments, the YP was 69.3% in the supernatant and 22.1% in the sediment. For the SFD procedure, there is no information in the literature about chitosan and alginate NPs obtained by this method. However, it is expected that the YP will be higher because there is no additional phase separation step by centrifugation as there is in the ionic gelation method.

The highest EE for both prednisolone and inulin was observed for SFDA (84.1 ± 3.3% and 87.9 ± 3.0%, respectively). In contrast, the EE of prednisolone in both CTPP (76.9 ± 2.9%) and SFDC (72.4 ± 3.3%) was much higher than that of inulin (51.5 ± 2.6% and 37.3 ± 3.0%, respectively). Figure 6b compares the LC of both active ingredients in the NPs. The highest LC for both prednisolone (13.3 ± 0.0%) and inulin (13.9 ± 0.0%) was detected in SFDA and the highest differences in LC between prednisolone and inulin were in SFDC (12.8 ± 0.0% and 6.6 ± 0.0%, respectively) and CTPP (14.5 ± 0.0% and 9.7 ± 0.0%, respectively). The lowest EE and LC were for inulin in SFDC (37.3 ± 3.0% and 6.6 ± 0.0%, respectively). Our previous work in micro-particles based on an alginate core coated with chitosan containing prednisolone and inulin as active ingredients showed the same results of higher EE and LC for prednisolone compared with inulin. Although inulin has higher solubility than prednisolone, it was necessary to include Pluronic F-127 to improve its encapsulation.[41]

Dissolution Studies with Lysozyme Enzyme

The effect of lysozyme (0.5 mg/mL) on prednisolone release from NPs was evaluated. In fact, several reports mention that the concentration of lysozyme is higher in IBD patients (Crohn's disease and ulcerative colitis) than in healthy subjects, and this condition has been reported for lysozyme in serum[46], faeces[47] and colonic epithelial cells.[48] Figure 7 shows the profile of prednisolone release from NPs in the presence or absence of lysozyme. It is observed that chitosan-based NPs were degraded by lysozyme, whereas SFDA did not show any differences in prednisolone release compared with the control. To evaluate the sensitivity of the chitosan-based NPs (CTPP, SFDC) to lysozyme degradation, the difference of mean prednisolone released in the presence of the enzyme and the control was estimated. The results show that CTPP (19.7 ± 5.8%) was significantly more degraded than SFDC (10.1 ± 2.0%) in the presence of lysozyme ($p < 0.05$). The degradation of chitosan-based NPs was expected because lysozyme has higher affinity for substrates with alternated units of N-acetylglucosamine and N-acetylmuramic acid and for glycan backbones such as chitin and partially deacetylated chitosan, which are polymers of glucosamine and N-acetylglucosamine.[49] The degradation of chitosan and glycol chitosan NPs in the presence of lysozyme has also been reported previously.[50]

Microbiological Assays

The colon contains a complex and dynamic microbial ecosystem with a high bacterial concentration of up to 10^{11} or 10^{12} microorganisms/g of luminal content, which influences the luminal environment and therefore drug and dosage form behaviour.

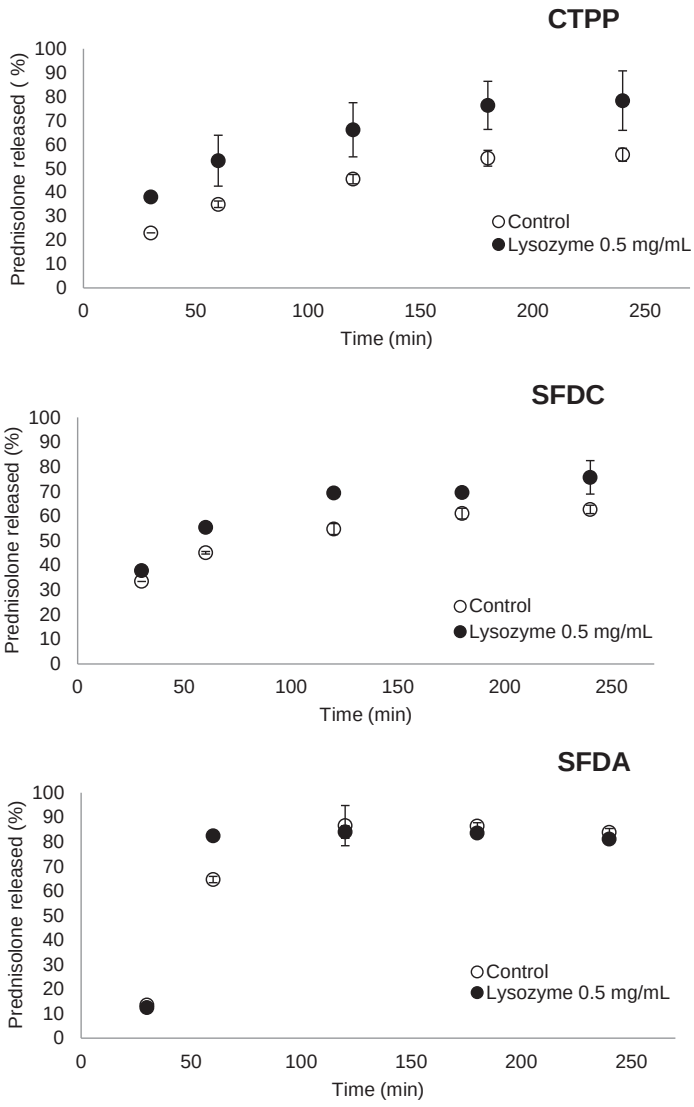

Figure 7. Prednisolone released from NPs formulations in the presence of lysozyme 0.5 mg/mL and control.

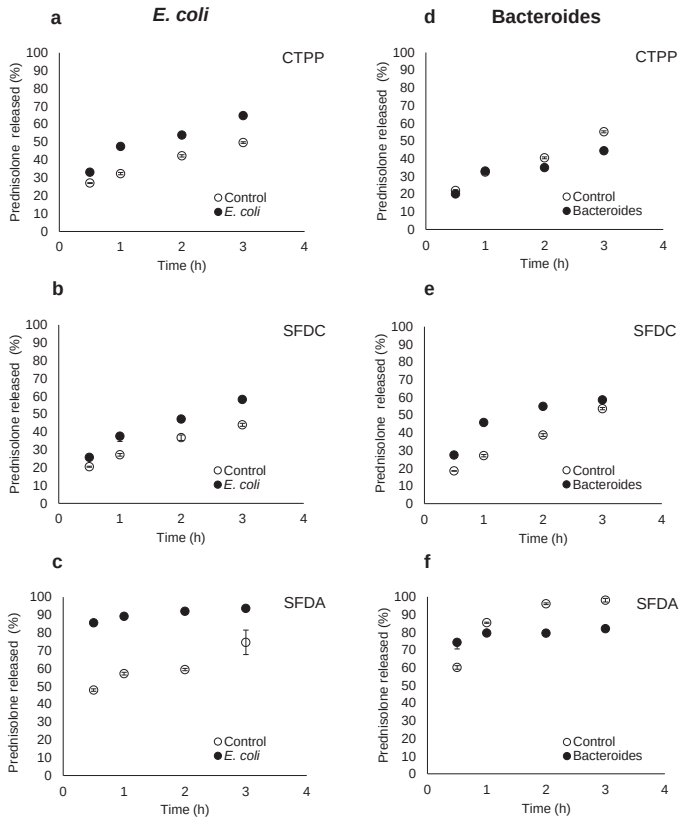

Figure 8. Prednisolone released from NPs formulations in the presence of *E. coli* (a–c) and *Bacteroides* (d–f).

Bacteroides, *Bifidobacterium*, *Eubacterium*, *Clostridium*, *Peptococcus*, *Peptostreptococcus* and *Ruminococcus* are considered dominant bacterial genera in the human micro-biota, whereas aerobic bacteria (facultative anaerobes) such as *Escherichia*, *Enterobacter*, *Enterococcus*, *Klebsiella*, *Lactobacillus* and *Proteus* are generally subdominant. *Bacteroides* spp. are among the most abundant bacteria in the distal human intestine. This group of anaerobes can degrade and ferment a wide variety of polysaccharides. It has been reported that patients with IBD have higher amounts of bacteria attached to these epithelial surfaces than healthy people do.[51] In Crohn's disease, concentrations of *Bacteroides*, *Eubacteria* and *Peptostreptococcus* are increased, whereas *Bifidobacterium* numbers are reduced, and in ulcerative colitis, the number of facultative anaerobes is increased.[52]

The effects of two relevant micro-organisms of the colonic micro-biota, *E. coli* (a facultative anaerobe) and *B. thetaiotaomicron* (Bacteroides), on the release of prednisolone from the NPs were evaluated. Figures 8a–8c show that all NPs were degraded when cultured in the presence *E. coli*, allowing the release of prednisolone. SFDA was the system most degraded by *E. coli* ($30.4 \pm 2.4\%$), whereas in the case of chitosan-based NPs, CTPP ($4.3 \pm 0.2\%$) was more degraded than SFDC ($2.5 \pm 0.3\%$). We have previously reported on the degradation of calcium alginate chitosan-coated particles of different sizes (pellets and microparticles) by *E. coli*.[41] Thus, it seems that alginate-based NPs are more easily degraded than chitosan-based NPs.

When the NPs were incubated in the presence of *B. thetaiotaomicron*, only SFDC showed a higher mean prednisolone release than the control ($12.2 \pm 0.5\%$, $p < 0.05$; see Figs. 8d–8f). *B. thetaiotaomicron*, a prominent member of human distal intestinal micro-biota, has been reported to degrade hyaluronic acid to disaccharides via the action of two chondroitin lyases, both of which are located in the periplasm. Hyaluronic acid (a polysaccharide comprising D-glucuronic acid and D-N-acetylglucosamine linked via alternating β-1,4 and β-1,3 glycosidic bonds) has a backbone similar to that of chitosan (a copolymer of glucosamine and N-acetylglucosamine linked through β-1,4 glycosidic linkages). The β-glucuronidase that cleaves the resulting disaccharides into mono-saccharides is located in the cytoplasm.[53] The degradation of alginate by *B. ovatus* isolated from human stools has also been described,[54] but the enzymes involved in the degradation were not described.

Human Faecal Slurry Assay

In vitro cultures of faecal samples in bioreactors are widely used as a model of the colonic ecosystem and to investigate the fermentation of non-starch polysaccharides.[2,55] Figure 9 shows the pattern of prednisolone release from NPs when incubated

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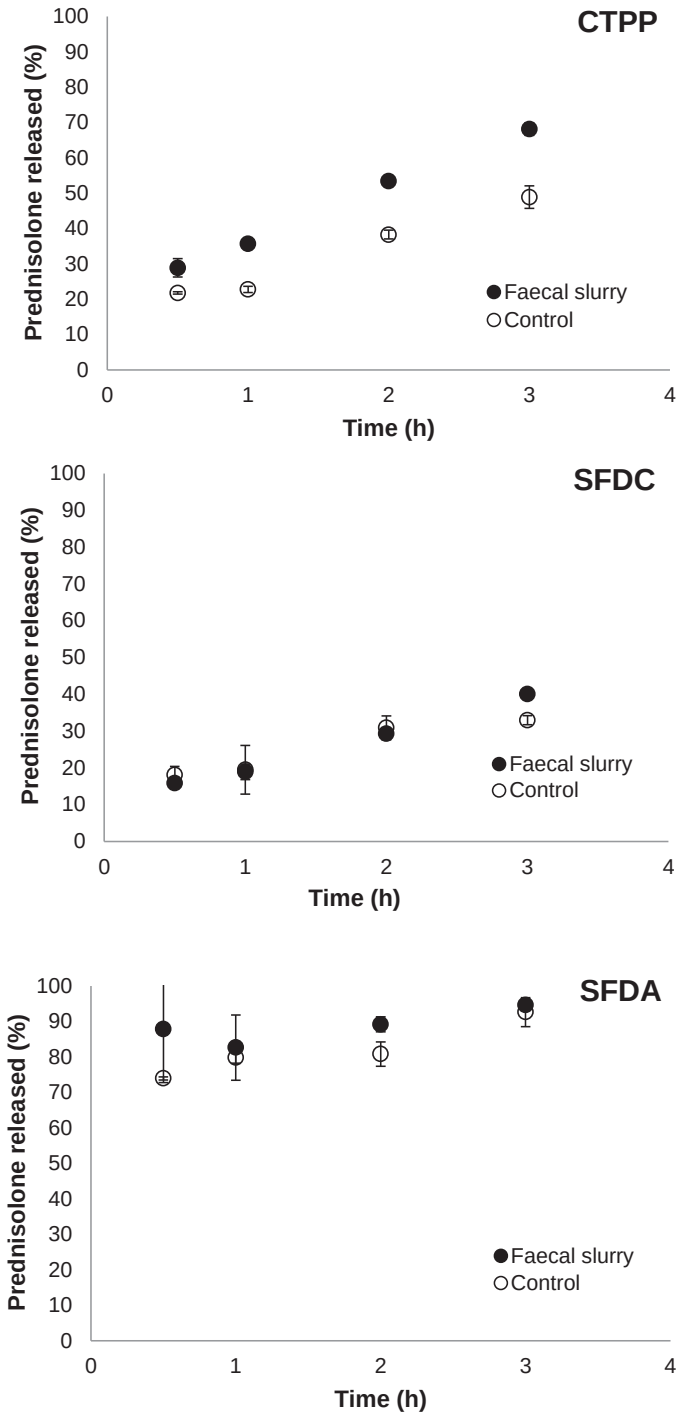

Figure 9. Prednisolone released from NPs formulations in the presence of faecal slurry and control.

with faecal slurry. An effect of the faecal slurry on prednisolone release was observed for CTPP (13.6 ± 1.5%), estimated as the difference between mean prednisolone released in the presence of faecal slurry and in the control ($p < 0.05$), but not for SFDC. In the case of SFDA, even though the prednisolone was released very quickly because of the dissolution of the NP, the faecal slurry had a significant effect (6.8 ± 5.7%). It is known that the glycosidase activity of the colon is mainly because of bacterial glycosidases, with the highest activities being α-L-arabinofuranosidase, β-D-xylosidase, β-D-galactosidase and β-D-glucosidases.[53] Thus, it was expected that chitosan would be more sensitive to the faecal slurry than alginate because its structure is degraded by β-D-glucosidases. Because of their complex structure (copolymers of a 1,4-linked α-L-galacturonic and β-D-mannuronic acids), alginates are resistant to degradation by human endogenous enzymes, but *in vitro* fermentation by human faecal bacteria showed 57% degradability.[56] It has been reported that films of chitosan cross-linked with TPP are easily digested by faecal enzymes but are more resistant to pancreatic enzymes. Instead, non-cross-linked chitosan polymers are digested by both faecal and pancreatic enzymes.[2]

CONCLUSIONS

In the case of NPs based on CS, SFDC showed a higher positive ξ potential than CTPP but had a similar *Z*-average and nanostructure. In contrast, SFDA showed a significantly smaller *Z*-average (138 nm) and negative ξ potential (−41 mV) compared with NPs based on chitosan (481–484 nm), and its nanostructure showed large amorphous areas and very small particles, indicating that the NPs are disaggregated in water. The interactions of the active ingredients, prednisolone and inulin, with chitosan or alginate were not affected by the manufacturing procedure. Chitosan-based NPs in phosphate and Krebs buffers showed values of $G' > G''$, typical of a true gel. However, SFDC was a weaker gel than CTPP because it had lower G' and G'' values and because its mechanical behaviour was dependent on the buffer composition. Thus, SFDA showed a very weak gel structure. The mean release of both active ingredients from SFDC was significantly higher compared with CTPP. SFDA showed a higher mean release of prednisolone compared with NPs based on CS but a lower mean release of inulin. The SFD procedure showed a higher YP compared with ionic gelation. SFDA showed the highest EE and LC for both active ingredients. SFDC showed similar EE and LC of prednisolone but lower for inulin compared with CTPP. CTPP was significantly more degraded than SFDC in the presence of lysozyme. SFDA was the system most degraded by *E. coli* (30.4 ± 2.4%), whereas in the case of chitosan-based NPs, CTPP (4.3 ± 0.2%) was more degraded than SFDC (2.5 ± 0.3%). Only SFDC in the presence of *B. thetaiotaomicron* showed a higher mean prednisolone release than the control (12.2 ± 0.5%, $p < 0.05$). An effect of the faecal slurry on prednisolone release was observed for CTPP (13.6 ± 1.5%) but not for SFDC. In the case of SFDA, even though the prednisolone was released very quickly because of the dissolution of the NP, the faecal slurry still had a significant effect (6.8 ± 5.7%).

The SFD procedure was studied as an alternative to the ionic gelation procedure. NPs based on CS showed similar NPs properties (size, structure, viscoelastic behaviour), but SFDC showed a higher mean release of both active ingredients with similar EE and LC for prednisolone but lower for inulin. SFDC was less degraded in the presence of lysozyme and *E. coli* and was degraded by *B. thetaiotaomicron*, but not by faecal slurry. The results obtained with SFDA were promising because this NP shows good encapsulation parameters for both active ingredients and biological degradability by *E. coli* and faecal slurry, but it will be necessary to use alginate derivatives to reduce its solubility and improve its mechanical behaviour.

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