

# Evaluation of polyelectrolyte and emulsion covalent crosslink of chitosan for producing mesalazine loaded submicron particles

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This study evaluates various techniques for producing mesalamine (5ASA)-loaded particles employing chitosan as a biopolymer: (1) the polyelectrolyte complexation of chitosan with phthalate hypromelose (HP), (2) the chemical crosslinking of chitosan with genipin and (3) the water-in-oil emulsion method associated with chemical crosslinking with genipin. Systems were characterized by dynamic light scattering, zeta potential ( $\zeta$ ), powder X-ray diffraction (PXRD), Fourier transform infrared spectroscopy (FTIR) and a drug release profile. Method (1) was efficiently produced unloaded nanoparticles (491 nm, PDI=0.26 and  $\zeta = 23.2$ ), but the conditions for chitosan and HP cross-linking enhanced the precipitation of 5ASA. Method (2) caused the degradation of the drug. Method 3 produced sub-micron and microparticles, thereby varying the agitation method; 3 h magnetic agitation resulted in 2692 nm, PDI = 0.6 and  $\zeta = 46$ , while Ultra-Turrax, 5 min produced submicron particles (537 nm, PDI = 0.6). The percentage yield was approximately 50%, which is very satisfactory considering the impossibility of encapsulating 5ASA using other methods. FTIR showed the covalent interaction of chitosan and genipin. The drug release was rapid in acidic fluid, but in neutral pH a slower release was obtained in the initial stage, followed by rapid release, which may ensure the controlled release of 5ASA in the colon.

**Keywords:** Emulsion method. Polyelectrolyte crosslinking. Chemical cross-linking. Chitosan. Mesalazine.

## INTRODUCTION

Inflammatory bowel disease (IBD) is a generalized term for a group of chronic diseases affecting the gastrointestinal system, including ulcerative colitis (UC) and Crohn's disease (CD) (Hua *et al.*, 2015). Mesalamine, also called 5-aminosalicylic acid (5ASA), has been used to treat IBD for more than 70 years with proven efficacy. It is estimated that the total market value will reach \$ 6.2 billion in 2017, which emphasizes the importance of research in this sector (Talaie *et al.*, 2013). The general principle of pharmacotherapy in IBD is to induce remission events and prevent new episodes during remission (Pertuit *et al.*, 2007). The treatment of UC and CD depends on factors such as the severity and subtype of the disease, as

well as pre-existing diseases and patient tolerance to pharmacotherapy (Lautenschläger *et al.*, 2014). The most common classes of drugs are anti-inflammatory agents such as 5ASA, which are used mainly in mild to moderate conditions, and corticosteroid and immunosuppressive agents, which are more effective in the treatment of more severe forms of IBD (Talaie *et al.*, 2013).

The efficacy of many drugs is usually limited by low solubility, low stability, and a need for increasing potency, and in many cases, only a low fraction of the administered dose actually reaches the site of action; most of the drug diffuses through the body (Moura, Martins, Duarte, 2015a). The main obstacle faced by treatment with 5ASA in conventional dosage forms is to achieve local levels of the drug in the inflamed mucosa without the use of dosage forms that are less acceptable to patients, such as enemas. The low bioavailability of drugs and low patient compliance may result in hospitalizations and failure of the treatment (Viscido *et al.*, 2014).

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When administered orally, 5-ASA is rapidly and completely absorbed by the upper portion of the intestine and only reaches and remains in the colon at low levels. Therefore, it is of extreme importance to promote the local release of the drug for greater therapeutic success with fewer adverse effects and less drug loss (Pertuit *et al.*, 2007). Thus, gastroresistant drug delivery systems may delay the early release of the drug and allow enteric or colonic release. In addition to the early absorption of drugs, colonic drug delivery is affected by the mucous barrier: a thick and abundant layer of mucus is present in this pathway, which may prevent penetration of the drug delivery system or depuration by peristaltic movements and food bolus (Date, Hanes, Ensign, 2016).

Micro and nanoparticles can be designed to reach the colon using gastroresistant polymers. Due to the reduced size of the micro and nanoparticles, they can reach the firmly adhered mucus layer via passive diffusion without being eliminated with the peristaltic movements. In addition, submicronic particles (<300 nm) may favor capture by immune cells, which are highly proliferated in the inflamed colonic mucosa; this strategy targets the release of drug at the site of action (Lamprecht, Schäfer, Lehr, 2001; Moulari *et al.*, 2008).

The use of hydrophilic polymers, especially polymers with a positive charge such as chitosan, may favor the penetration of micro- and hydrophilic micro- and nanoparticles and their interaction with mucus glycoproteins, which are negatively charged, thereby increasing their retention in a phenomenon known as mucoadhesion (Carvalho *et al.*, 2012; Ensign, Cone, Hanes, 2012). In this manner, micro and nanoparticles can be promising colonic drug delivery systems due to the controlled release of drugs in the TGI, targeting capacity, mucoadhesion and consequent increase in bioavailability. All of these properties may allow the reduction of administered doses, as well as reduction of toxicity and adverse effects.

Another strategy to prolong release in TGI is the polysaccharide-based nanoparticles obtained by covalent cross-linking of chitosan with crosslinking agents such as glutaraldehyde or genipin, which can delay the premature disintegration in TGI (Kumar, Su, Velusamy, 2016; Muzzarelli *et al.*, 2016).

Based on the potential of chitosan in the development of mucoadhesive systems and the need of development of new drug delivery systems that target the colon, especially for IBD treatment, this work aims to develop chitosan micro and nanoparticles for 5-ASA incorporation. Some attempts to produce chitosan nanoparticles of 5ASA are found in the literature, such as the studies of Mura *et al.* (2012), Mladenovska *et al.* (2007) and Aguzzi *et al.*

(2011), which employed gastroresistant polymers in combination with spray dry techniques (Aguzzi *et al.*, 2011; Mladenovska *et al.*, 2007; Mura *et al.*, 2012).

In this study, our goal is to evaluate and compare different low cost techniques for producing 5ASA-loaded submicronic particles, taking into consideration the limited solubility of the drug in both organic and aqueous solvents, which limits many physical processes that requires solubilization of the drug prior to particle production. The novelty is the comparison of three approaches employing chitosan as a biopolymer to produce 5ASA particles that have not been tested before: polyelectrolyte complexation of chitosan with phthalate hypromellose (PH), chemical crosslinking of chitosan with genipin and water in oil emulsion (W/O) method associated with chemical crosslinking with genipin. Systems were physico-chemically characterized and the drug release profile varying the pH was performed as proof of concept. Since 5ASA is greatly limited pH-dependent solubility and oxidation, this study was very important to discuss better approaches to handling 5ASA during encapsulation.

## MATERIAL AND METHODS

### Material

Mesalamine (5ASA), low viscosity chitosan and HPLC-grade methanol were purchased from Sigma-Aldrich, St. Louis, USA. The phthalate hypromellose (PH) was from HP-55 Shin-Etsu Chemical, Tokyo, Japan. Polysorbate 80 (Tween 80) and sodium hydroxide were from Vetec, Duque de Caxias, Brazil. Choridric acid and glacial acetic acid were from Proquimios, Rio de Janeiro, Brazil.

### Experimental

#### *Method of polyelectrolyte complexation of chitosan*

Nanoparticles were produced by low-viscosity chitosan dispersion at 0.1% w/v in 1% acetic acid, pH=5.5 and hypromellose phthalate (HP) as ionic cross-linker at 0.5% w/v in NaOH 1 M (pH=5.5), and 5ASA at 5 mg/mL in HCl 1 M pH=5.5 was incorporated into the chitosan dispersion to have the drug at 5% w/w to the polymeric weigh. The HP dispersion (2.0 mL) was dropwise added to 1.2 mL of chitosan dispersion under magnetic stirring, resulting in 3:1 chitosan/HP opalescent suspension. Samples using this method were named Poly Electrolyte Complexes (PECs) (Barbi *et al.*, 2014). All procedures were performed in a dark room, and the samples were protected against light.

### Method of chemical crosslinking of chitosan with genipin

The method was adapted from Fonseca (2015). Chitosan was dispersed at 0.5% (w/v) in chloridric acid 1 M; then 0.1% w/v polysorbate 80 and 5 mg/ml of 5ASA were added. Separately, 1 mM of genipin was dissolved in 10 mL of ethanol, and this solution was dropwise added into 10 mL of chitosan dispersion using a syringe immersed into the dispersion, pressing the plunger at 1 mL/min (Fonsêca, 2015). Two types of stirring were tested for the crosslinking reaction: magnetic stirring at 800 rpm for 3 h and high-performance dispersing instrument (T 25 digital Ultra Turrax, Ika) at 3,000 rpm for 5 min. The resulting dispersions were centrifuged at 3500 rpm and suspended in water for characterization. Samples using this method were named as Magnetic Stirred Chemical Cross-Linked Particles (Chem\_Mag) and Ultra-Turrax Stirred Chemical Cross-Linked Particles (Chem\_UT). All procedures were performed in a dark room, and the samples were protected against light.

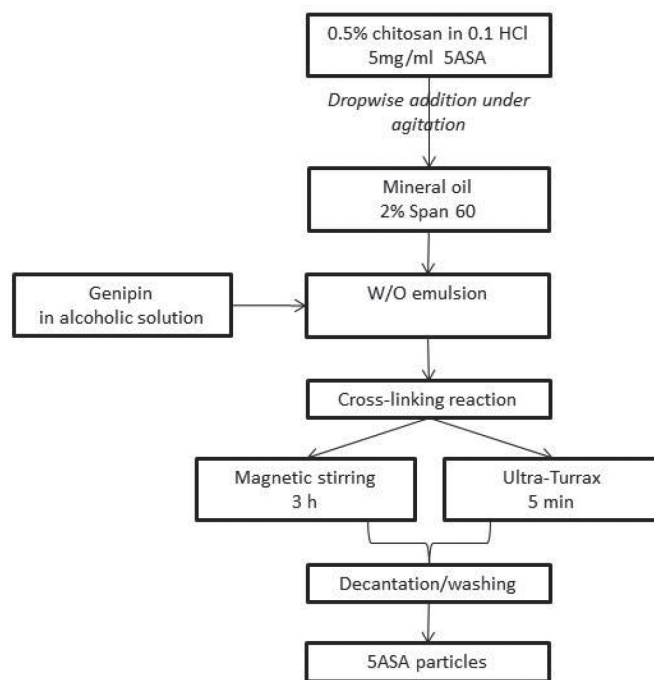
### Method of W/O emulsion followed by crosslinking of chitosan with genipin

The preparation procedure is similar to those reported by Moura *et al.* (2015) with minor modifications. Chitosan was dissolved at 0.5% p/v in aqueous HCl solution 0.1 M, and the resulting solution is the water-dispersed phase, in which 5ASA was dissolved at 5 mg/ml. A volume of 3 mL of this solution was then dispersed dropwise into a 40-mL mixture of mineral oil and Span 60 (2%, w/v), which was the organic phase, resulting in a water-to-oil phase ratio of 1/13.3 (v/v). The W/O emulsion formed, followed by homogenization with continuous stirring using a magnetic stirrer (Moura, Martins, Duarte, 2015).

Once the emulsion formed, a volume of 0.5 mL of genipin 70% (v/v) alcoholic solution was added dropwise to the W/O emulsion to obtain a solution with a ratio of genipin to chitosan of 1/6 (v/v). To cross-link particles, two stirring processes were tested: one using a magnetic stirrer at 800 rpm for 3 h, and a second using a high-performance dispersing instrument (T 25 digital Ultra Turrax, Ika) at 3,000 rpm for 5 min, both at room temperature (25 °C).

The resulting particles were separated from the continuous phase by decanting and submitted to washing with hexane and decanted three times. the particles were dried at room temperature. Samples using this method were named Magnetic Stirred Emulsified Cross-Linked Particles (Emul\_Mag) and Ultra-Turrax Stirred Chemical Cross-Linked Particles (Emul-UT). The complete procedure is schematically presented in Figure 1. All

procedures were performed in a dark room, and the samples were protected against light.



**FIGURE 1** - Organogram of method of production of submicron particles named as Magnetic Stirred Emulsified Cross-Linked Particles (Emul\_Mag) and Ultra-Turrax Stirred Chemical Cross-Linked Particles (Emul-UT).

### Physicochemical characterization

The samples were characterized by dynamic light scattering (DLS), polydispersity index (PdI) and electrophoretic mobility using a Zetasizer Nanoseries (Malvern Instruments, Ltd., Malvern, UK). The refraction indexes used were 1.33 and 1.55 for water and chitosan, respectively. All measurements were performed at 25 °C in triplicate. For each sample, the mean particle diameter, polydispersity and standard deviation of 10 determinations were calculated, applying multimodal analysis.

The shape of particles was examined via optical microscope coupled to a camera, and images were acquired using a scanning procedure. The microscope slide was divided into three sections in the longitudinal direction. An average of 50 images per slide were acquired following a sequence from left to right (Moura, Martins, Duarte, 2015).

### Fourier Transform Infrared Spectroscopy (FTIR)

Particles used in the analysis were previously lyophilized to produce fine powder. Then, the powder was mixed with KBr and pressed into a pellet. The FTIR spectra were recorded using a FTIR (Fourier transform

infrared spectrometer, Vertex 70, Bruker, Germany) operating at room temperature, in the frequency range 4000 e 400  $\text{cm}^{-1}$  and 4  $\text{cm}^{-1}$  resolution. FTIR spectra of pure polymers and drug were also recorded for differential comparison.

#### *Powder X-ray Diffraction analysis (PXRD)*

PXRD data were recorded at room temperature (293 K) using an Ultima IV diffractometer (Rigaku, Tokyo, Japan) with  $\theta$ - $2\theta$  geometry. Cu  $K\alpha$  radiation ( $\lambda = 1.5418 \text{ \AA}$ ) was generated using a sealed tube at 40 kV and 30 mA. The data were collected with a step size of  $0.02^\circ$  in  $2\theta$ . The speed of scanning was  $1^\circ 2\theta/\text{min}$ , from  $3^\circ$  to  $50^\circ$  in  $2\theta$ . The samples were finely ground and mounted onto a grooved glass slide employed as a sample holder. The experimental PXRD patterns were compared with those calculated by importing the crystal structures determined for the 5ASA free base, 5ASA hydrochloride and 5ASA hydrochloride monohydrate (CCDC code: SAQJAV02; SAQJEZ; PUVMAU) (Banić-Tomišić, Kojić-Prodić, Širola, 1997; Cherukuvada *et al.*, 2013) forms into Mercury (Macrae *et al.*, 2008).

#### *Analytical characterization*

A high-performance liquid chromatography (HPLC) analytical methodology, developed and validated, was used to measure drug concentration. Briefly, a Shimadzu HPLC system was used for the analyses, consisting of a CBM-20A Solvent Delivery Module, SPD-20A Spectrophotometric Detector (set at 254 nm) and a SIL-20AHT automatic injection module. A reverse-phase C18 column (Acqua, Phenomenex) of 150 mm, 4.6 mm I.D. and 5- $\mu\text{m}$  particle size was used. The isocratic mobile phase was a mixture of methanol/phosphate buffer 0.05 M, pH 6.6, at 9:1 (v/v), flowing at 0.6 mL/min. It was constructed calibration curve of standard solutions of 5ASA ranged from 1  $\mu\text{g/mL}$  to 50  $\mu\text{g/mL}$  dissolved in mobile phase, and the regression line obtained was  $y = 27324x - 1212.5$ , with  $r^2=0.999$ , precision of 2.5, 10 and 50  $\mu\text{g/mL}$  among 0.1-0.5% and accuracy in the range of 97.8-98.5%. LD and LQ of 0.0102  $\mu\text{g/mL}$  and 0.034  $\mu\text{g/mL}$ , respectively, and precision and accuracy in the acceptable range of ICH guidelines.

#### *Percentage yield*

An accurate weight of chitosan particles was dispersed in 15 mL of NaOH 1 M for chitosan precipitation and 5ASA solubilization. The system was centrifuged at 3600 rpm for 30 min, and the supernatant was diluted in mobile phase, filtered through 45  $\mu\text{m}$  membrane for HPLC injection.

Percentage yield was calculated from the weight of placebo, and 5ASA-encapsulated particles recovered from each batch in relation to the starting material were used in the preparation of chitosan particles. The percentage yield of prepared microspheres was determined using the formula as follows:

$$\text{Percentage yield} = \text{Practical yield/Theoretical yield} \times 100$$

#### *In vitro drug release*

The dissolution apparatus (299 Nova Ética, Brazil) was set up as follows: dissolution compartment volume 300 mL, stirred at 50 rpm. Synthetic cellulose acetate membrane with molar mass cut-off 12–14 kDa (Spectrum) previously treated with Milli-Q water at  $100^\circ\text{C}$  for 15 min. The membrane was cut into 6-cm cylinders filled with 2 mL of samples, and both sides of the bag were tied with a twine knot.

The experiment was performed for gastric dissolution of medium composed of chloridric acid buffer 0,1 M, pH=1.3 for 1 h, followed by enteric dissolution medium composed of monobasic phosphate buffer 0.05 M, pH=6.6, during 6 h. All experiments were performed at  $37^\circ\text{C}$ , and sink conditions were maintained. Samples of 1 mL were withdrawn from the receptor fluid and the volume withdrawn was replaced with fresh receptor fluid. Each formulation was analyzed in triplicate, with 5ASA at 4 mg/mL. 5ASA was quantitatively determined by HPLC with the UV-Vis detector at 254 nm.

The drug release kinetics were evaluated by different mathematical models, such as zero order, first order, Weibull, Korsmeyer-Peppas and Higuchi. To select the best model that fit the release profile obtained for the samples, the determination coefficient ( $R^2$ ) was used (Costa, Sousa Lobo, 2001; Fredenberg *et al.*, 2011).

## RESULTS AND DISCUSSION

### **Method of polyelectrolyte complexation of chitosan**

Unloaded nanoparticles were successfully produced using this method, resulting in a homogenous opalescent dispersion containing nanoparticles with a mean diameter of 491 nm, PDI 0.26 and zeta potential of + 23.2 mV (Table I). The incorporation of the drug into a chitosan solution during condensation was chosen due to the better stability in acidic than basic solution. Previous incorporation of 5ASA into HP solution resulted in a brown dispersion due to oxidation. However, even careful handling of 5ASA-loaded nanoparticles resulted

in a turbid dispersion with brown suspended particles, different from the unloaded nanoparticles, suggesting precipitation of the drug. Loaded samples were centrifuged for encapsulation efficiency determination and 5ASA was not detected in the supernatant. This could suggest total entrapment of the drug into nanoparticles, or the drug could not be detected because it had precipitated. Previous to polyelectrolyte complexation, the chitosan dispersion containing the drug was totally clear. The brown particles were observed after complexation. The 5ASA can exist in different ionic forms depending on the pH of the solution. Below  $pK_a=2.2$ , 5ASA is cationic; between  $pK_a$  2.02 and 5.8, it is zwitterionic; and above  $pK_a$  12, it is anionic (Zerrouk *et al.*, 1998). The pH of the nanoparticle suspensions is 5.5; the zwitterionic form predominates and may be less soluble than the ionic forms. These findings indicate that polyelectrolyte complexation is a method not recommended for 5ASA nano-encapsulation, since it depends on the drugs' solubility for complexation, and the conditions for chitosan and HP cross-linking enhance the precipitation of the 5ASA.

### Method of chemical crosslinking of chitosan with genipin

Chemical crosslinking of chitosan with genipin resulted in a dark opalescent dispersion of 5ASA-loaded samples. The resulted dispersions produced using both stirring methods, magnetic and Ultra-Turrax, presented the same aspect. The opalescence may indicate colloidal or nanoparticle formation (see size and zeta potential in Table I), but the color is problematic. One mechanism by which 5ASA could degrade appears to depend on the production of colored black imines (Zerrouk *et al.*, 1998). The oxidation of 5ASA is also light-sensitive, but all experiments were performed in a dark room or under UV light. Therefore, the conditions found for this crosslinking method enhanced degradation of the drug and are not recommended for 5ASA encapsulation.

### Method of W/O emulsion followed by crosslinking of chitosan with genipin

In this method, the variable parameter was the stirring process: either magnetic/long time and Ultra-Turrax/short time. The size distribution and zeta potential results are in Table I. The method resulted in sub-micron particles, which act as a mucous penetration system. The high zeta potential is desirable for colloidal stability.

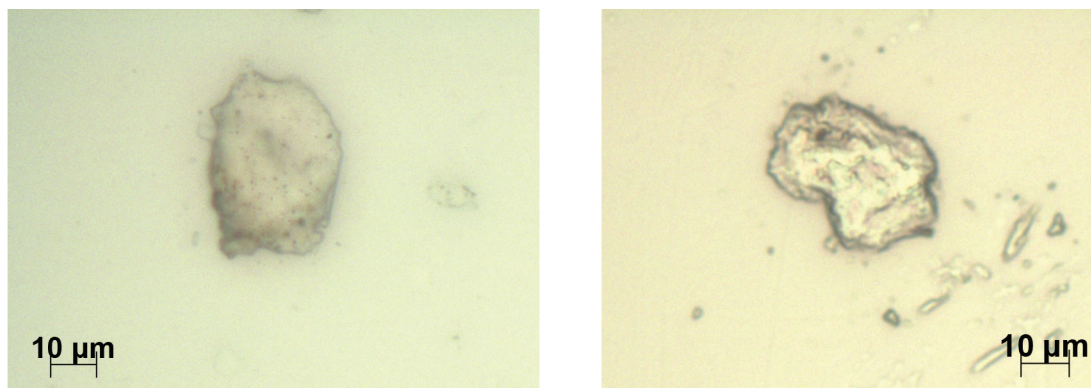
The incorporation of the drug was tested for Emul-samples, since they resulted in stable particles. The percentage yield was calculated based on the total particle weight, and the quantification of the drug into the particles followed their destruction. It was not possible to calculate the encapsulation efficiency, because it would be necessary to quantify the unloaded drug into the supernatant, and in case of emulsion method, the oily supernatant was washed, precluding the quantification of 5ASA. Percentage yield results are shown in Table I. It was found that ultra-turrax stirring was slightly more effective in loading 5ASA, suggesting that the intensity of agitation is more important than time of agitation, since ultra-turrax was more rapid (5 min) than magnetic stirring (3 h). The values found near 50% are very satisfactory, considering the impossibility of encapsulating 5ASA using polyelectrolyte, as well as chemical cross-linking. Notably, the incorporation of 5ASA is limited to its solubility in HCl, which was experimentally found as 5 mg/mL. Since we solubilized 5ASA at 5 mg/mL in the internal aqueous phase of emulsion, the maximum amount of 5ASA possible to incorporate in the system was used in this method.

The microscopic images of particles are shown in Figure 2. The figure shows that 5ASA-loaded and unloaded particles are nearly spherical and blue. Moura *et al.* (2015) attributed this color to the formation of cross-linked particles oxygen-radical-induced polymerization of genipin and its subsequent reaction with chitosan amino groups. DLS showed the high polydispersity of the systems, which correlates with the microscopy image.

**TABLE I** - Size, zeta potential and percentage yield of 5ASA-loaded particles produced by different methods of cross-link

	Size (nm)	PdI	Zeta potential (mV)	Percentage yield (% w/v)
PEC	532 nm ± 45	0.29 ± 0.2	25.7 ± 2.3	*
Unloaded PEC	491 nm ± 83	0.26 ± 0.1	23.2 ± 1.2	*
Chem-Mag	724± 209	0.36 ± 0.3	724± 209	*
Chem-UT	854 ± 121	0.63 ± 0.1	59.0 ± 2.5	*
Emul-Mag	2692 ± 717	0.58 ± 0.1	46.0 ± 5.4	47.21 ± 0.08
Emul-UT	537 ± 169	0.57 ± 0.1	43.3 ± 1.5	58.40 ± 4.62

\*not determined



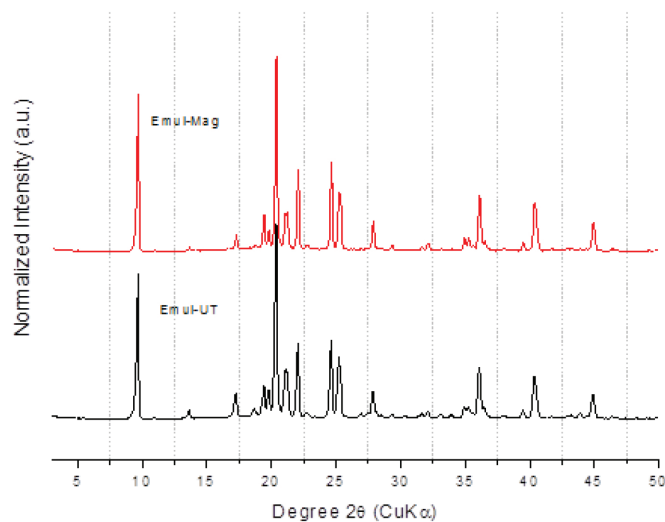
**FIGURE 2** - Optical microscope image of chitosan particles produced w/o emulsion followed by crosslinking of chitosan with genipin. Left: cross-linking under magnetic stirring for 3 h. Right: cross-linking under ultra turrax for 5 min. Images treated using AxioVision software.

Since a simple optical microscope was used, it was not possible to obtain images of smaller particles, which could be visualized by scanning electron microscopy (SEM). Although the image does not represent DLS behavior, it is presented to show the gel-like structure and the amorphous morphology of the particles.

### Powder X-ray Diffraction (PXRD)

Powder X-ray Diffraction (PXRD) analyzes were performed to characterize the initial polycrystalline material and submicron particles. Figure 3 shows the experimental diffractograms of the unloaded Emul-Mag and Emul-UT. The samples are observed to be crystalline materials, because they show diffraction peaks at different angles. Comparison of the two samples shows that the Bragg peaks appear at coincident angles, indicating that the stirring processes (magnetic and Ultra-Turrax) did not generate different crystalline materials.

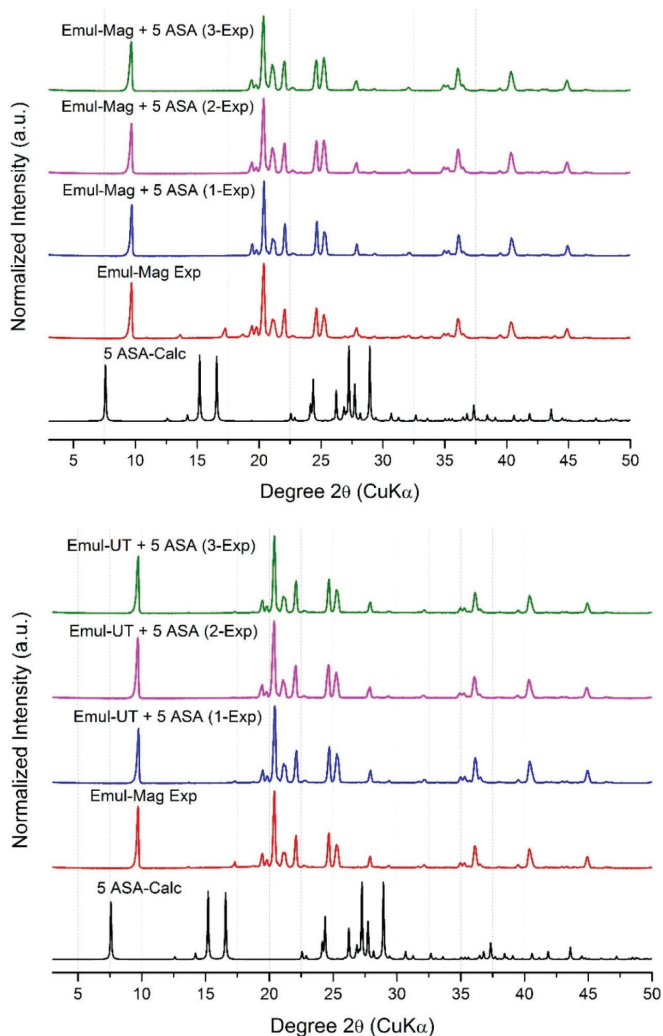
The experimental diffractograms of 5ASA loaded samples were compared with the experimental unloaded Emul-Mag and Emul-UT, and the calculated diffractogram from the CCDC CODE: SAQJAV02 was found in the database of crystalline organic structures (CCDC-CSD) for the free base of 5ASA (Cherukuvada *et al.*, 2013). These diffractograms are in Figure 4 and show that the Bragg peaks generated for the unloaded and loaded samples are coincident. In those samples, it was not possible to identify coincident angles of Bragg peaks related to the free base of 5ASA. These results indicate that 5ASA may have been fully incorporated into the system, or it was not possible to identify the drug phase in the sample, due to the low proportion in the systems (lower or equal to 5% w/w). A similar result was found in Nunes, Mahendrasingam and Suryanarayanan (2005) and in Varasteh *et al.* (2009). Another possibility is that



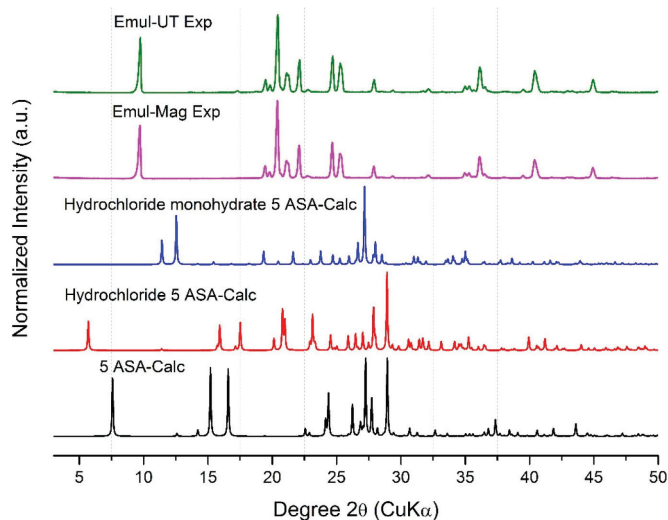
**FIGURE 3** - Experimental diffractograms generated for unloaded Emul-Mag and Emul-UT.

drug is dispersed in the amorphous state, and thus, there are no drug peaks in the particles. Moreover, there was no difference between the two types of agitation for the crystalline materials obtained.

The experimental diffractograms of the 5ASA-loaded Emul-Mag and Emul-UT were compared to the calculated diffractograms of 5ASA free base, 5ASA hydrochloride and 5ASA hydrochloride monohydrate (CCDC CODE: SAQJAV02; SAQJEZ; PUVMAU), respectively (Banić-Tomišić, Kojić-Prodić, Širola, 1997; Cherukuvada *et al.*, 2013; Dobson, Gerkin, 1998). This comparison was performed to evaluate whether there is a crystalline phase transition of the incorporated drug. As observed in Figure 5, there was no identification of Bragg peaks for submicron particles at the correspondent angles of crystalline forms of 5ASA, demonstrating no phase transition, and thus, the obtained material can be a new crystalline form or a phase mixture.



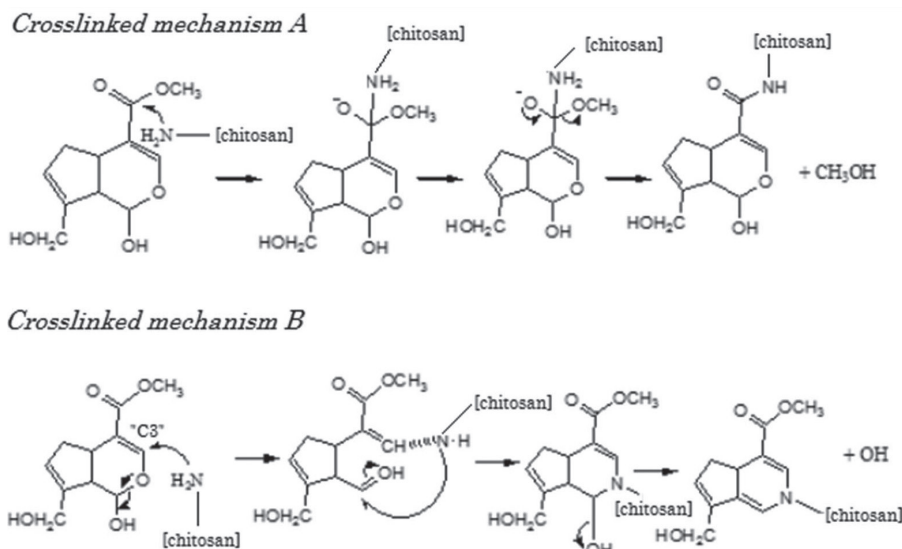
**FIGURE 4** - Experimental diffractograms of unloaded and 5ASA-loaded Emul-Mag (a) and Emul-UT (b) and 5ASA free base (CCDC CODE: SAQJAV02) (in a and b).



**FIGURE 5** - Experimental diffractograms of 5ASA-loaded Emul-Mag and Emul-UT compared to experimental and calculated diffractograms of 5ASA free base, 5ASA hydrochloride and 5ASA hydrochloride monohydrate (CCDC CODE: SAQJAV02; SAQJEZ; PUVMAU).

### FTIR

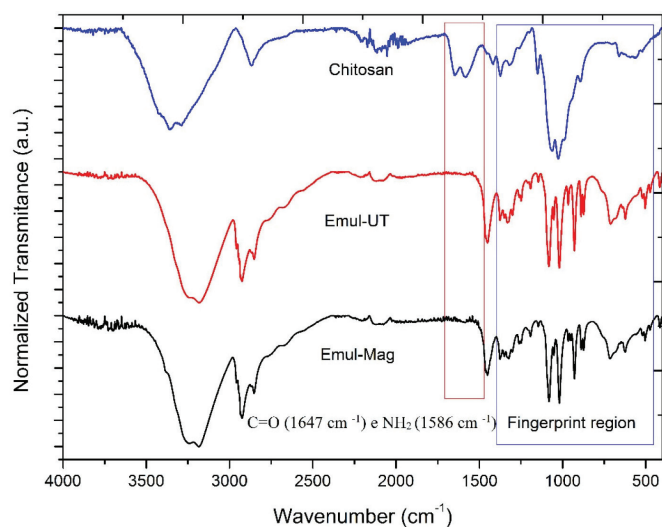
FTIR analyses show the bands and characteristic stretches of chitosan (Brugnerotto *et al.*, 2001). The literature proposes two distinct mechanisms for crosslinking between chitosan and genipin. One mechanism (mechanism A) is based on a slower reaction for nucleophilic substitution, which involves a nucleophilic attack on the carbon of the ester group of the genipin; substitution of this group is achieved with a secondary amide with the release of methanol (Figure 6). The second proposal (mechanism B) is related to the reaction in



**FIGURE 6** - Proposed mechanisms for chitosan and genipin cross-linking. Source: (Butler, Yiu-Fai, Pudney, 2003).

which a nucleophilic attack of the primary amine on the carbon "C3" of genipin forms an intermediate aldehyde. There is an opening of the heterocyclic ring of genipin, which is attacked by the secondary amine group to form a heterocyclic compound attached to the amine group, according to Figure 6 (Butler, Ng, Pudney, 2003).

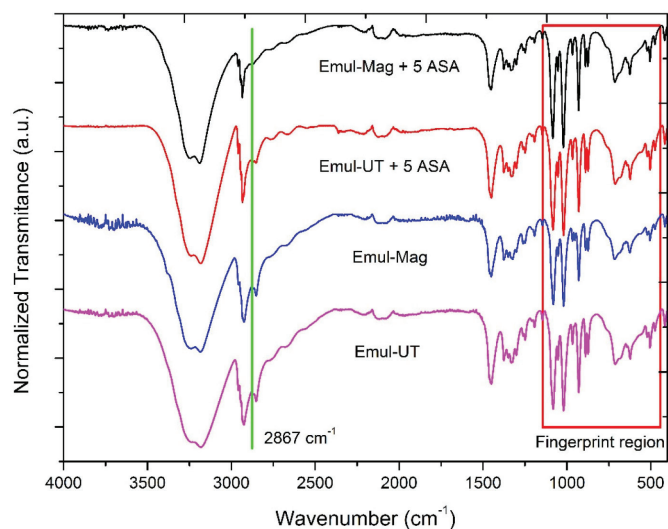
FTIR spectra were obtained for the pure chitosan and chitosan crosslinked with genipin are shown in Figure 7. The crosslinking probably occurred according to mechanism B, since there is no amide band ( $1650\text{ cm}^{-1}$ ) in the cross-linked material (Butler, Yiu-Fai, Pudney, 2003; Moura, Martins, Duarte, 2015). The  $\text{C}=\text{O}$  ( $1647\text{ cm}^{-1}$ ) and  $\text{NH}_2$  ( $1586\text{ cm}^{-1}$ ) bands presented in the spectrum of pure chitosan are no longer observed in the spectra of the crosslinked materials. The disappearance of these chitosan characteristic bands indicates that there was the attack of the amino group on the "C3" of genipin, generating the heterocyclic group with a tertiary amine. Moreover, there was a change in the chitosan fingerprint region for the particles, which corroborates the crosslinking process. Notably, the agitation procedures employed for particle production did not interfere in this characterization.



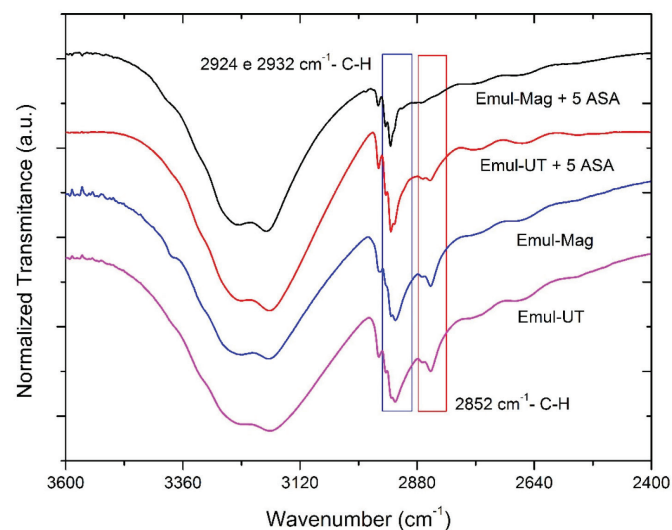
**FIGURE 7** - FTIR spectra of chitosan, Emul-Mag e Emul-UT.

In Figure 8, the fingerprint region of the molecules is highlighted in red. There were no changes between the loaded and unloaded particles. According to Brugnerotto *et al.* (2001), the band near  $2867\text{ cm}^{-1}$ , highlighted in green, as well as the absence of bands near  $1560\text{ cm}^{-1}$ , indicate the low acetylation of chitosan even in the presence of other materials (Brugnerotto *et al.*, 2001).

The FTIR spectra in Figure 9 show the likely point of molecular interaction between 5ASA and chitosan. The attenuation of the band near  $2852\text{ cm}^{-1}$  for 5ASA-loaded samples is highlighted in red, and the inversion



**FIGURE 8** - FTIR spectra of unloaded and 5ASA-loaded Emul-Mag and Emul-UT. Highlighted: fingerprint region (red) and band near  $2867\text{ cm}^{-1}$  (green).



**FIGURE 9** - FTIR Spectra of unloaded and 5ASA-loaded Emul-Mag and Emul-UT, highlighting C-H bands in  $2852\text{ cm}^{-1}$  (red) and band near  $2924$  and  $2932\text{ cm}^{-1}$  (blue).

of intensity of the bands present near  $2924$  and  $2932\text{ cm}^{-1}$  is in blue. There was no difference between the agitation processes. To demonstrate the interaction on the C sp of chitosan, the relative intensity for the C-H bands from the highest-intensity band (near  $3177\text{ cm}^{-1}$ ) was calculated and is shown in Table II.

The calculations demonstrated that the band near  $2852\text{ cm}^{-1}$  for the 5ASA-loaded Emul-UT sample was attenuated 39.59% relative to the unloaded sample. When comparing the relative intensities of the bands near  $2924$  and  $2932\text{ cm}^{-1}$  with those of unloaded particles, the attenuation was 31.78 and 26.10%, respectively. In addition to band attenuation, there was also an inversion of



**TABLE II** - Calculation of relative intensities of bands approximately 2852.3 and 2932  $\text{cm}^{-1}$  of unloaded and 5ASA-loaded Emul-Mag and Emul-UT

Emul-UT			5ASA-Emul-UT		
Intensity	$\text{cm}^{-1}$	Relative Intensity (%)	Intensity	$\text{cm}^{-1}$	Relative Intensity (%)
88.01	3177	100.00	74.95	3177	100.00
59.35	2852	67.44	20.87	2852	27.85
73.34	2924	83.33	38.64	2924	51.55
71.55	2932	81.30	41.37	2932	55.20
Emul-Mag			5ASA-Emul-Mag		
Intensity	$\text{cm}^{-1}$	Relative Intensity (%)	Intensity	$\text{cm}^{-1}$	Relative Intensity (%)
56.35	3177	100.00	49.55	3177	100.00
33.97	2852	60.28	14.65	2852	29.57
44.12	2924	78.30	22.00	2924	44.40
42.91	2932	76.15	25.87	2932	52.21

intensity. The relative intensities were 83.33 and 81.30% for the bands at 2924 and 2932  $\text{cm}^{-1}$ , respectively, while the values for 5ASA-loaded Emul-UT were inverted: at 2932  $\text{cm}^{-1}$  the intensities were greater (55.20%) than for the band at 2924  $\text{cm}^{-1}$  (51.55%).

The same was observed for 5ASA-loaded Emul-Mag. The attenuation of the 2852  $\text{cm}^{-1}$  band was 30.71% and the 2924 and 2932  $\text{cm}^{-1}$  bands were 33.90 and 23.94%, respectively. The inversion was also verified, since unloaded Emul-Mag showed relative intensities of 78.30 and 76.15%, respectively, while for 5ASA-loaded samples, the inversion of relative intensities is verified. The relative intensity calculated for the band near 2924  $\text{cm}^{-1}$  was 44.40%, which is lower than that found for the band near 2932  $\text{cm}^{-1}$  (52.21%). These results demonstrate that potential interactions between the drug and chitosan may occur at the C sp site of the chitosan molecule.

### ***In vitro* drug release**

In the drug release assay, it is important to ensure sink conditions, so that the concentration of the total components cannot achieve more than 10% in the release medium. This condition was assured consideration of the rate in the total weight of the components by the total volume of release medium, which in our case resulted in less than 1% w/v.

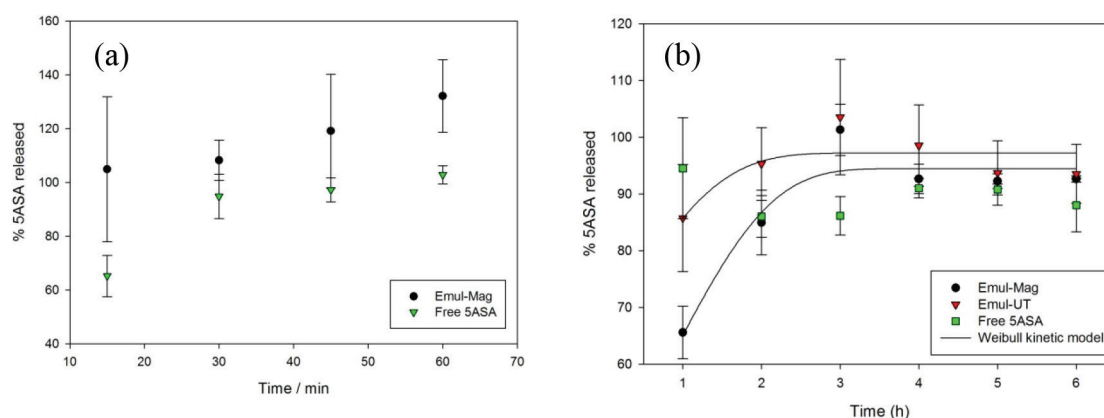
The amount of 5ASA released was expressed as a percentage, which is plotted against time in Figure 10. It can be seen in Figure 10a that 5ASA was totally released from submicron particles in acidic medium. This result

shows that covalent crosslinking cannot effectively promote gastro-resistance for the developed systems. For colonic delivery, the submicron particles produced here should be encapsulated into a gastro-resistance capsule, or a polymeric gastro-resistance coating may be tested.

The release profile obtained from colonic pH is shown in Figure 10b. There was fast release from free-5ASA and Emul-UT samples, and the release profiles are overlapped. Emul-Mag was more effective in controlling drug release, since in the first 1 h there was 60% drug release, while more than 80% had already been released from Emul-UT and free-5ASA.

Structurally, FTIR and PXRD show no difference between Emul-UT and Emul-Mag. The retardation in the initial release of Emul-Mag can be attributed to the type and time of stirring process during the production of submicron particles. Thus, magnetic stirring may result in more stable covalent bonds between genipin and chitosan, due to the longer time for reaction and milder agitation, which retards 5ASA release.

The profiles obtained in the drug delivery assay were analyzed by mathematical models to identify the mechanisms involved in 5ASA release, and the  $R^2$  values are shown in Table III. The Weibull model showed the highest  $R^2$  and was used thus to discuss the data. This model expresses the accumulated fraction of the drug in solution at a given time, from which the shape parameter,  $b$ , characterizes the curve as exponential ( $b=1$ ) (Case 1), sigmoid, S-shaped, with upward curvature followed by a turning point ( $b>1$ ) (Case 2), or parabolic, with a higher initial slope and afterwards, which is consistent with the



**FIGURE 10** - Drug release profile of submicron particles Emul-Mag, Emul-UT and free-5ASA: (a) simulated gastric fluid; (b) simulated colonic fluid.

**TABLE III** - Determination coefficients ( $R^2$ ) obtained from drug release mathematical models fitting, and shape parameters (b) calculated from Weibull model

Kinetic model	Determination coefficient ( $R^2$ )	
	Emul-Mag	Emul-UT
Zero order	0.2641	0.6487
First order	0.6330	0.8740
Higushi	0.0000	0.0000
Korsmeyer-Peppas	0.4703	0.7801
Weibull	0.7818	0.9536
b	18551.2	23828.4

exponential ( $b < 1$ ) (Case 3) (Costa, Sousa Lobo, 2001). Table III shows the b values derived from the fitting of Weibull model to the entire drug release curve data. Values of b were significantly greater than 1, indicating the sigmoid profile.

A sigmoidal release profile characterized by slower release in the initial stage (0–1 h) followed by rapid release during the later stage (3–6 h) may ensure targeted drug release to the colon (Ali Asghar *et al.*, 2009).

These results show controlled drug release in a neutral environment. Release in an acidic pH can be easily achieved by encapsulating the particles into gastro-resistant capsules.

The initial aim of our study was to obtain  $< 300$  nm, but the methods employed resulted in  $> 500$  nm particles. Therefore, immune system targeting based on particle size was not accomplished, but this study did show the difficulty in stabilizing 5ASA using different methods to produce sub-micron particles. The emulsion method showed more efficacy because it allows protection of the drug in an acidic environment surrounded by an oily phase that avoids 5ASA oxidation. In addition, the drug

release assay showed that Emul-samples have potential to control drug release in neutral pH. The polydispersity index was quite high, but the use of an appropriate scale-up equipment, such as high-pressure homogenizer, may result in monodispersed and smaller particles. Therefore, this study importantly outlines a strategy to produce sub-micron particles of 5ASA for oral administration and colonic-controlled delivery, resulting in sub-micron particles that have great potential to traverse the loosely adhered mucus layer combined with a mucoadhesive polymer that can promote intimate contact with the inflamed mucosa, releasing 5ASA in a controlled manner. These results can be a starting point for formulators to delineate future experiments to study the nano- and micro-encapsulation of 5ASA.

## CONCLUSION

This study is important because it shows the limitations of encapsulating 5ASA in nano- and micro-particles using conventional techniques such as polyelectrolyte complexation of chitosan via polyanion and chemical crosslinking of chitosan with genipin. The successful method was the method that protected the drug against the reactional medium, namely, the W/O emulsion followed by crosslinking of chitosan with genipin. It was possible to solubilize the drug at an appropriately stable pH (acidic) and to protect the drug in the internal phase of the emulsion, while the chitosan and genipin reaction may occur in the surface of the droplet. The agitation process in this method was varied, including conditions combining magnetic/long time and Ultra-Turrax/short time treatments. There was no significant difference in the percent yield, but magnetic/long time agitation more efficiently prolonged drug release in neutral pH. Release in acidic pH can be easily handled by encapsulating particles into gastro-resistant capsules. These finds are very

important for determining a suitable method to produce sub-micron particles for potential colonic release of 5ASA.

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## REFERENCES

- Aguzzi C, Ortega A, Bonferoni MC, Caramella C. Assessment of anti-inflammatory properties of microspheres prepared with chitosan and 5-Amino salicylic acid over inflamed caco-2 cells. *Carbohydr Polymers*. 2011;85(3):638-644.
- Ali Asghar LF, Azeemuddin M, Jain V, Chandran S. Design and in vitro evaluation of formulations with pH and transit time controlled sigmoidal release profile for colon-specific delivery. *Drug Deliv*. 2009;16(6):295-303.
- Banić-Tomišić Z, Kojić-Prodić B, Širola I. Hydrogen bonds in the crystal packings of mesalazine and mesalazine hydrochloride. *J Molecular Struct*. 1997;416(1-3):209-220.
- Barbi Mda S, Carvalho FC, Kiill CP, Barud Hda S, Santagneli SH, Ribeiro SJ, Gremião MP. Preparation and characterization of chitosan nanoparticles for azt nasal delivery. *J Nanosci Nanotechnol*. 2015;15(1):865-74.
- Brugnerotto J, Lizardi J, Goycoolea FM, Argülles-Monal W, Desbrières j, Rinaudo M. An infrared investigation in relation with chitin and chitosan characterization. *Polymer*. 2001;42(8):3569-80.
- BUTLER, M. F.; NG, Y.-F.; PUDNEY, P. D. A. Mechanism and kinetics of the crosslinking reaction between biopolymers containing primary amine groups and genipin. *J Pol Sci Part A: Pol Chem*. 2003;41(24):3941-53.
- Carvalho FC, Bruschi ML, Evangelista RC, Gremião MPD. Mucoadhesive drug delivery systems. *Braz J Pharm Sci*. 2012;46(1):89-100.
- Cherukuvada S, Bolla G, Sikligar K, Nangia A. 4-Aminosalicylic acid adducts. *Cryst Growth Des*. 2013;13(4):1551-57.
- Costa P, Sousa Lobo JM. Modeling and comparison of dissolution profiles. *Eur J Pharm Sci*. 2001;13(2):123-133.
- Date AA, Hanes J, Ensign LM. Nanoparticles for oral delivery: design, evaluation and state-of-the-art. *J Controll Release*. 2016;240:504-26.
- Dobson AJ, Gerkin RE. 5-Ammoniosalicylic acid chloride monohydrate. *Acta Crystallogr Sect C Cryst Struct Comm*. 1998;54(Pt 11):1632-4.
- Ensign LM, Cone R, Hanes J. Oral drug delivery with polymeric nanoparticles: the gastrointestinal mucus barriers. *Adv Drug Deliv Ver*. 2012;64(6):557-70.
- Fonsêca GD. Micropartículas contendo nanopartículas de quitosana usando sulfato e genipina para liberação modificada da triancinolona: obtenção, caracterização e estudo em células tumorais. [dissertação]. Natal: UFRN; 2015.
- Fredenberg S, Wahlgren M, Reslow M, Axelsson A. The mechanisms of drug release in Poly(Lactic-Co-Glycolic Acid)-based drug delivery systems - a review. *Int J Pharm*. 2011;415(1-2):34-52.
- Hua S, Marks E, Schneider JJ, Keely S. Advances in oral nano-delivery systems for colon targeted drug delivery in inflammatory bowel disease: selective targeting to diseased versus healthy tissue. *Nanomed Nanotechnol Biol Med*. 2015;11(5):1117-32.
- Kumar GV, Su CH, Velusamy P. Ciprofloxacin loaded genipin cross-linked chitosan/heparin nanoparticles for drug delivery application. *Mater Lett*. 2016;180:119-22.
- Lamprecht A, Schäfer U, Lehr CM. Size-dependent bioadhesion of micro- and nanoparticulate carriers to the inflamed colonic mucosa. *Pharm Res*. 2001;18(6):788-93.
- Lautenschläger C, Schmidt C, Fisher D, Stallmach A. Drug delivery strategies in the therapy of inflammatory bowel disease. *Adv Drug Deliv Rev*. 2014;71:58-76.
- Macrae CF, Bruno IJ, Chisholm JA, Edgington PR, McCabe P, Pidcock E et al. *Mercury Csd 2.0* – New features for the visualization and investigation of crystal structures. *J Appl Crystallogr*. 2008;41(2):466-70.

- Mladenovska K, Cruaud O, Richomme P, Belamie E, Raicki RS, Venier-Julienne MC, et al. 5-Asa loaded chitosan-calginate microparticles: preparation and physicochemical characterization. *Int J Pharm.* 2007;345(1-2):59-69.
- Moulari B, Pertuit D, Pellequer Y, Lamprecht A. The targeting of surface modified silica nanoparticles to inflamed tissue in experimental colitis. *Biomaterials.* 2008;29(34):4554-60.
- Moura MJ, Martins SP, Duarte BPM. Production of chitosan microparticles cross-linked with genipin - identification of factors influencing size and shape properties. *Biochem Eng J.* 2015a;104:82-90.
- Mura C, Nacher A, Merino V, Merino-Sanjuán M, Manconi M, Loy G, et al. Design, characterization and in vitro evaluation of 5-aminosalicylic acid loaded n-succinyl-chitosan microparticles for colon specific delivery. *Colloids Surf B Biointerfaces.* 2012;94:199-205.
- Muzzarelli RAA, El Mehtedi M, Bottegoni C, Gigante A. Physical properties imparted by genipin to chitosan for tissue regeneration with human stem cells: a review. *Int J Biol Macromolec.* 2016;93(Pt B):1366-81.
- Nunes C, Mahendrasingam A, Suryanarayanan R. Quantification of crystallinity in substantially amorphous materials by synchrotron x-ray powder diffractometry. *Pharm Res.* 2005;22(11):1942-53.
- Pertuit D, Moulari B, Betz T, Nadaradjane A, Neumann D, Ismaïli L, et al. 5-amino salicylic acid bound nanoparticles for the therapy of inflammatory bowel disease. *J Control Release.* 2007;123(3):211-8.
- Talaei F, Atyabi F, Azhdarzadeh M, Dinarvand R, Saadatzadeh A. Overcoming therapeutic obstacles in inflammatory bowel diseases: a comprehensive review on novel drug delivery strategies. *Eur J Pharm Sci.* 2013;49(4):712-22.
- Varasteh M, Deng Z, Hwang H, Kim YJ, Wong GB. Quantitative determination of polymorphic impurity by x-ray powder diffractometry in an OROS® formulation. *Int J Pharm.* 2009;366(1-2):74-81.
- Viscido A, Capannolo A, Latella G, Caprilli R, Frieri G. Nanotechnology in the treatment of inflammatory bowel diseases. *J Crohn's Colitis.* 2014;8(9):903-18.
- Zerrouk N, Ginès Dorado JM, Arnaud P, Chemtob C. Physical characteristics of inclusion compounds of 5-Asa in  $\alpha$  and  $\beta$  cyclodextrins. *Int J Pharm.* 1998;171(1):19-29.

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