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Development of electrosprayed mucoadhesive chitosan microparticles

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Abstract

The efficacy of chitosan (CS) to be used as drug delivery carrier has previously been reported. However, limited work has been pursued to produce stable and mucoadhesive CS electrosprayed particles for oral drug delivery, which is the aim of this study. Various CS types with different molecular weight (MW), degree of deacetylation (DD), and degree of polymerization (DP) were assessed. In addition, the effect of the solvent composition was also investigated. Results showed that stable CS electrosprayed particles can be produced by dissolving 3% w/v of low MW CS in mixtures of aqueous acetic acid and ethanol (50/50% v/v). The stable CS particles displayed diameters of approximately 1 μm as determined by dynamic light scattering. The zeta potential of these particles was found to be approximately 40 mV confirming the mucoadhesion properties of these CS electrosprayed particles and its potential to be used as drug delivery carrier.

Keywords

Electrospray; Microparticles; Chitosan; Mucoadhesion; Electrohydrodynamics; Polysaccharide

1. Introduction

Oral administration of drugs is the preferred route due to increased patient compliance compared to invasive routes. The challenges related to oral drug delivery can, among others, be the low pH and enzymes of the stomach, that can degrade the released drugs before reaching the desired place of absorption in the intestine (Perioli, D'Alba, & Pagano, 2012). Therefore, it can often be necessary to encapsulate the drug using a particulate drug delivery system (McClements, 2015). A particulate carrier can enable the entry of a drug into the body with the advantages of enhancing the efficacy and safety of the drug by controlling the rate, time and site of release (Zamani, Prabhakaran, & Ramakrishna, 2013). During the past few decades, biopolymeric nano- and micro-particles have gained interest as particulates as they can increase the dissolution rate of a drug and thereby, possibly also the bioavailability (Zamani et al., 2013), but also function as a protective shell for the drug for sustained periods of time (Hans & Lowman, 2002; Lee, Mei, Bai, Zhao, & Chen, 2010).

Chitosan (CS) is a polysaccharide made of glucosamine and N-acetyl glucosamine, typically derived by partial or full deacetylation of chitin, a naturally occurring polysaccharide mostly extracted from marine crustaceans, shrimps or crabs (Rinaudo, 2006). CS is a hydrophilic, pH-sensitive and cationic polymer (Rinaudo, 2006), and it gained much attention in the pharmaceutical area due to its non-toxic mucoadhesive properties (Van Der Lubben, Verhoef, Van Aelst, Borchard, & Junginger, 2001), as well as its bioactive properties including antibacterial and anti-mycotic (Carmona-Ribeiro, 2014; Songsurang, Praphairaksit, Siraleartmukul, & Muangsin, 2011). The properties of CS are known to be dependent on the degree of deacetylation (DD), the distribution of the acetyl groups along the chain and also the molecular weight (MW) of the polysaccharide (Rinaudo, 2006). Therefore, due to the versatile chemistry of chitosan and bioactive properties, it has been processed into several nano-micro structures for drug delivery including fibers (Mendes, Gorzelanny, Halter, Schneider, & Chronakis, 2016; Mendes, Strohmenger, Goycoolea, & Chronakis, 2017), hydrogels (Bhattarai, Gunn, & Zhang, 2010; Mendes, Shekarforoush et al., 2016) and particles (Chen et al., 2013).

Nano- and micro-particles of CS have shown to be able to reduce side effects and to extend the release time of drugs mainly due to its mucoadhesive properties (Van Der Lubben et al., 2001). Mucoadhesive CS particles can extend their residence time in the mucosal areas, where the absorption takes place (e.g. small intestine) and thereby, prolong the drug absorption and result in an enhanced bioavailability (Ensign, Cone, & Hanes, 2013). CS nano- and micro- particles for oral drug delivery purposes can for example be prepared by ionotropic gelation (Carmona-Ribeiro, 2014), emulsion (Carmona-Ribeiro, 2014), spray drying (Harris, Lecumberri, Mengíbar, & Heras, 2011) or reverse micellar method (Mitra & Dey, 2011). However, these methods generally need chemical cross-linkers and/or long durations of time, and often result in low drug-loading efficiencies and limitations to scale up (Songsurang et al., 2011; Zamani et al., 2013).

Electrospray is a electrohydrodynamic technique referring to a one-step process using electrostatic forces as the driving force through electrically charged fluid jet to produce particles from the nano- to micro size-range, suitable for oral drug delivery (Bugarski, Li, Goosen, Poncelet, & Neufeld, 1994; Zamani et al., 2013). Electrospraying has shown advantages in particle preparation, where controlled and narrow size distribution of the particles is often observed together with a high drug-loading efficiency (Jaworek, 2007; Zamani et al., 2013). One of the other advantages of electrospray is that it is a gentle method, and therefore, biopharmaceuticals have been reported not to be degraded during the encapsulation (Xie & Wang, 2007).

The morphology, size and composition of the particles can be adjusted through process parameters and choice of material, making electrospray a versatile method, also being easily scaled up for mass production of the particles (Lee et al., 2010; Zamani et al., 2013). Previously, CS particles have been produced using electrospraying where doxorubicin was encapsulated resulting in spherical particles with a narrow size distribution, and with an encapsulation efficiency of the drug of more than 60% (Songsurang et al., 2011). Furthermore, electrosprayed CS microparticles have also led to an improvement in bioavailability of the small drug compound, verapamil by prolonging the drug residence time in the gastrointestinal tract (Netsomboon & Bernkop-Schnürch, 2016). Various publications have covered the effect of types of CS, electrospraying and formulation parameters for the development of CS particles (Arya, Chakraborty, Dube, & Katti, 2009; Geng, Kwon, & Jang, 2005; Gómez-Mascaraque, Sanchez, & López-Rubio, 2016; Jaworek, 2007; Sreekumar, Lemke, Moerschbacher, Torres-

Giner, & Lagaron, 2017; Zhang & Kawakami, 2010). There is an agreement on that the optimal operational conditions and concentration of components for the particle formation will depend on the type of CS used for the particles (Arya et al., 2009; Geng et al., 2005; Gómez-Mascaraque et al., 2016; Jaworek, 2007; Sreekumar et al., 2017; Zhang & Kawakami, 2010). In addition, it has previously been found that the MW of CS has a great impact on its sprayability (Gómez-Mascaraque et al., 2016) but also many other properties will influence the CS particle formation. However, these nanoparticles are often dissolving in aqueous medium in a few seconds, which limits its applications such as oral drug delivery. Moreover, there has been a lack of focus on the effect of the physical and chemical properties of CS on the mucoadhesive properties being very important for developing oral drug delivery systems and for achieving a good oral bioavailability (Gebril, Alsaadi, Acevedo, Mullen, & Ferro, 2012). CS has been studied as a coating (Llabot et al., 2011; Lupo et al., 2017) for nanoparticles or has been cross-linked with other materials (Marin Briceño, & Caballero-George, 2013; Oliveira et al., 2017; Rodrigues, Dionísio, López, & Grenha, 2012; Schattling, Taipaleenmäki, Zhang, & Städler, 2017) to increase the mucoadhesive properties of drug carriers to improve bioavailability. Pawar et al. have reported on a good mucoadhesion of CS coated particles in the rat gut, which supplements other studies assuring the mucoadhesive properties of CS (Pawar, Asthana, Mishra, Chaurasia, & Chourasia, 2013).

Therefore, the aim of this study was to investigate various types of CS with different MW and DD percentage together with the effect of solvent for the ability to create stable microparticles using the method of electrospray. Furthermore, the mucoadhesive properties of the stable particles were further examined for the evaluation of the CS particles to be utilized in the future as an oral drug delivery system.

2. Materials and methods

2.1. Materials

CS with different DD and MW were supplied by Heppe Medical Chitosan GmbH (HMC+, Halle (Saale), Germany) or purchased from Sigma-Aldrich (Steinheim, Germany) (Table 1). Ethanol 96% (EtOH) was obtained from VWR International (Darmstadt, Germany). Phosphate buffer solution (PBS) tablets were acquired from Sigma-Aldrich (Saint Louis, MO, USA), while fasted state simulated intestinal fluid (FaSSIF) was obtained from Biorelevant.com (London, UK). Fresh pig intestine was obtained from the Meat Trade College in Roskilde, Denmark, whereas acetic acid (AA), >99%, stomach porcine mucin and periodic acid and Schiff's reagent for aldehydes were obtained from Sigma-Aldrich (Steinheim, Germany).

Table 1. Properties of the various CS used in this study.

Company	Name of the chitosan[a]	DD (%)	MW (kDa[a])	DP[b]	IP[b]	Sample/formulation designation
Sigma	Low Molecular (LMW)	89	28	175	1.7	5–18
HMC	80/3000	76	386	2262	1.9	1–2
HMC	90/2500	91	213	1292	1.3	3
HMC	85/2500	89	187	1129	1.2	4
HMC	70/5	88	81	491	1.4	19–20
HMC	85/5	94	18	108	1.4	21–22
HMC	70/10	82	49	293	1.8	23
HMC	90/10	97	28	177	1.5	24

[a] The name refers to what is provided by the company. HMC+ uses as product nomenclature "degree of deacetylation percentage/Viscosity", i.e. HMC+ 80/3000, 80% average DD and 3000 is the viscosity in cP at 1% w/v.
[b] Abbreviations: DD = degree of deacetylation (actual DD), MW = Molecular weight, DP = degree of polymerization and IP = Index of polymerization.

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2.2. Preparation of CS solutions

CS solutions were prepared at concentration of 0.5, 1 or 3% w/v in aqueous AA (either of 10, 40 or 70% v/v, AA:H_2O) and EtOH in the ratio of either 0, 10, 30 and 50% v/v, EtOH:AA, and was evaluated for their ability to be electrosprayed and create CS particles in the micrometer size. For the preparation of the solutions, CS was dispersed in water, followed by the addition of the AA. Subsequently, the solution was vortexed for approximately 3 min until being homogeneous, followed by the addition of EtOH (when required). This final solution was mixed until obtaining a homogeneous solution.

2.3. Characterization of CS solutions

2.3.1. Conductivity

The conductivity was calculated based on the electrical resistance of the prepared CS solutions measured by electrochemical impedance spectroscopy. 300 µL of each solution was poured into a 6 × 10 × 5 mm polymethylmethacrylate chamber with two copper electrodes, and the modular electrochemical workstation IM6 (Zahner Elektronic, Kronach, Germany) measured the resistivity in a frequency of 5.447 kHz. The measurements were performed in triplicates. By the use of Pouillet's law and the definition of conductivity, the equation used to calculate conductivity of the solutions was:

(1)$\sigma = l/(R*A)$

where σ stands for conductivity, l for length, R for resistivity and A for cross-sectional area.

2.3.2. Viscosity of solutions

The dynamic viscosities of the CS solutions were measured with an AMVn automated rolling ball viscometer from Anton Paar (Graz, Austria). Each sample was measured three times, each as the average of four technical replicates with an internal standard deviation (SD) of less than 0.5% using a calibrated glass capillary (d = 1.6 mm) and a steel ball (d = 1.5 mm, ρ = 7.67 g/cm^3). All measurements were performed at 25 °C and an angle of inclination of 80°. The data was recorded with the Visiolab software from Anton Paar version 1.70 (Graz, Austria).

2.4. Electrospray of CS solution

Each of the prepared CS solutions were electrosprayed using a flow rate of 16.4 or 20 µL/min, and the distance from tip of needle to the collector was either 11 or 15 cm. Furthermore, the voltage was set in the range of 19–24 kV, according to the stability of the electrospray process, and a needle-size of 18 G or 19 G was utilized. The relative humidity was kept at 20% during the electrospray process using a polycarbonate chamber, and the temperature was 18–20 °C. The needle was connected to a high-voltage direct-current power supply and the processed particles were collected horizontally on aluminum foil or a glass surface.

2.5. Morphology of electrosprayed particles

The CS particles were transferred and mounted on aluminum stubs and sputter-coated with gold, generating a thin film of a thickness of 9 nm using a Q150R Rotary-Pumped Sputter

Coater from Fedelco S.L (East Sussex, United Kingdom) prior visualization. The particles were imaged on a Scanning Electron Microscope (SEM) Quanta FEG 200 ESEM with an Everhart-Thornley Detector (Hillsboro, OR US), using a magnification of 30,000× and a voltage of 10 kV.

2.6. Size, zeta potential and stability of the CS particles

For the first stability tests and the size distribution analysis, PBS solution was prepared according to the protocol from the manufacturer using purified water followed by adjusting pH to 6.8. Dry CS particles were dispersed in PBS at a concentration of 1 mg/mL for the dynamic light scattering (DLS) analysis, using a Zetasizer Nano ZS from Malvern Instruments (Malvern, UK). The size of the particles was measured at time 0 and after 1 h and 24 h of dispersion in PBS. Stable CS particles after 24 h in PBS (size did not decrease) were further tested in FaSSIF at pH 6.5 in order to investigate their stability in an in vivorelevant medium. These samples were dispersed at a concentration of 1 mg/mL in the medium, and the size was measured using DLS at time 0, 3 h after dispersion and after 24 h.

The zeta potential of the particles was measured in purified water in a concentration of 1 mg/mL using a Zetasizer Nano ZS. For these measurements, the particles were dispersed inside a capillary cell. All the measurements were performed in triplicates.

2.7. Mucoadhesive properties of CS particles

2.7.1. Ex vivo assay using texture analyzer

The mucoadhesive properties of the stable electrosprayed particles was carried out using a texture analyzer (Stable Micro Systems, UK) with a 50 N load-cell equipped with a mucoadhesive test ring. Electrosprayed CS particles collected on aluminum foil were pasted on a probe (1 cm diameter) using carbon pads. Fresh pig mucosal tissue was rinsed with PBS at pH 6.8 and cut into pieces of 2.5 × 2.5 cm. The tissues were placed in the mucoadhesion test ring and equilibrated at 37 °C in PBS, pH 6.8 to keep the tissue moist throughout the measurements, and further the medium was pumped in and out using a peristaltic pump to keep the temperature constant.

The probe with the CS particles was put in contact with the mucosa tissue with 20g of force for 1 min, and then withdrawn. The work of adhesion necessary to separate CS particles from the mucosal tissue was calculated using the area under the curve of force versus distance, obtained from the software (Exponent, Stable Micro Systems, UK) of the texture analyzer.

Teflon films of 1 cm in diameter were used as control samples. All the samples were tested five times in three different fresh sections of the pig tissue.

2.7.2. Periodic acid schiff (PAS) assay

PAS assay was developed by Mantle and Allen (1978) and adapted by Kilcoyne, Gerlach, Farrell, Bhavanandan, and Joshi (2011), and used with slight modifications in this study. In triplicates, 5 mg/mL particles were dispersed in a solution of 0.025% w/v mucin in 0.1 M PBS at pH 6.5 and vortexed for 2 h at 37 °C. The samples were centrifuged at 12,000 rpm for 2 min and 25 μL of the supernatant (unbounded mucin) was mixed in a 96-well microplate with 120 μL of 0.06% w/v periodic acid in 7% v/v AA and incubated for 1.5 h at 37 °C. Subsequently, at room temperature, 100 μL of Schiff's reagent was added to the 96-well plate and incubated for 40 min. The absorbance was read at 550 nm and correlated with a standard curve with mucin solutions.

2.8. Statistical analysis

The data are expressed as mean ± SD. Where appropriate, statistical analysis were carried out using Student t-tests using Microsoft Excel 2017 version 15.36. P-values below 5% ($p < 0.05$) were considered statistically significant.

3. Results and discussion

3.1. Usage of high MW CS in the electrospraying process

High viscous solutions were obtained mainly with high MW CS (MW > 100 kDa) (formulations 1, 2, 3 and 4). When high MW solutions were electrosprayed using a flow rate of 16.4 μL/min, a distance from tip to collector of 15 cm and 24 kV the formation of a Taylor cone, which is essential for the production of droplets to be electrosprayed (Fukui et al., 2010) was not observed. Gómez-Mascaraque et al. suggested that when using higher MW CS, the CS concentration needs to be decreased in order to make the solution electrosprayable (Gómez-Mascaraque et al., 2016). CS concentration was decreased from 3 to 0.5% w/v of the HMC^{+} 80/3000 (formulations 1 and 2) reducing both conductivity and viscosity from 0.34 to 0.16 mS/cm and from 622.38 to 51.83 mPa s, respectively (Fig. 1) with no particle formation.

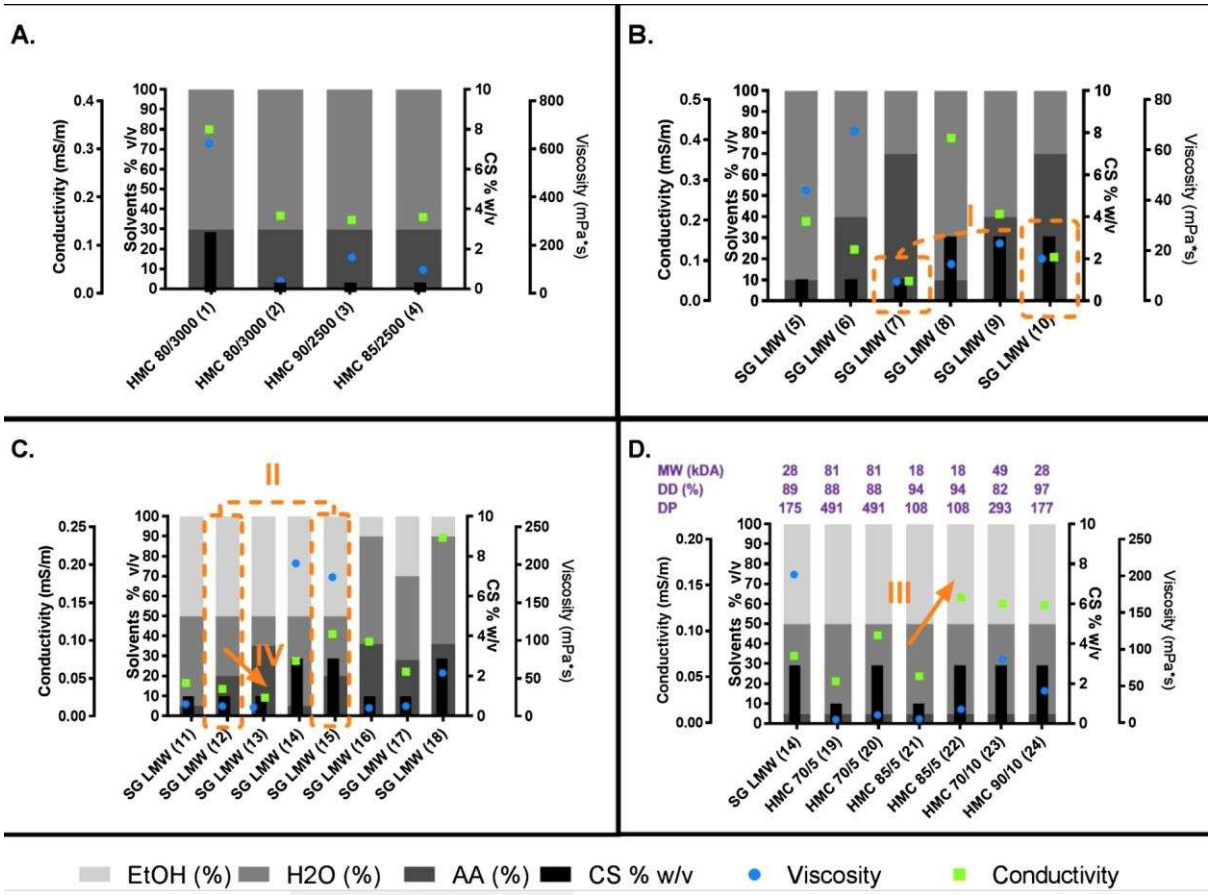

Fig. 1. The effect of the solvent (AA/EtOH) content, CS type and concentration on viscosity and conductivity of the different CS solutions using: A) high MW CS and different CS concentration B) low MW CS concentration and different AA concentration C) low MW CS concentration and different AA/EtOH concentrations D) different CS concentrations, DD and MW. The conductivity and viscosity are indicated in green and blue dots, respectively. The data represents means ± SD, n = 3. Abbreviations: EtOH = Ethanol, AA = Acetic Acid, CS = chitosan, DD = Degree of Deacetylation (%), DP = degree of polymerization and SG LMW = Sigma Low Molecular weight chitosan.

When having a concentration of 0.5% w/v CS of HMC⁺ 90/2500 and 85/2500, there was no particle formation even though, the electrospray process seemed stable. The conductivity remained similar in value (0.15 mS/cm), but the viscosities varied from 149.61 to 98.65 mPa s, respectively. This variability could be explained by the higher MW and DP of CS HMC⁺ 90/2500 (MW 213 KDa, DP 1292) comparatively to HMC 85/2500 (MW 187 kDa, DP 1129). In literature, it has been reported that the viscosity of CS in aqueous AA-solutions is more affected by the decrease of DD, rather than the concentration of CS. This is because of the increase in the molecular interactions due to the charge density along the chain backbone leading to entanglements (Mucha, 1997; Wang & Xu, 1994).

3.2. Effect of AA, EtOH and CS concentration on the size of electrosprayed particles

To investigate the effect of AA and EtOH content on the conductivity and viscosity of CS solutions, low MW CS (MW < 100 kDa) were dissolved in mixtures of AA/EtOH using CS with different DA, DP (Fig. 1B and C). Overall, the results suggest that an increase in concentration of AA to the CS solution decreased the conductivity leading to the formation of larger particles, while the values of their viscosity did not change significantly. Increasing AA concentration do not affect significantly the viscosity and this is due to the low degree of dissociation of the AA (Zhang & Kawakami, 2010). Previously, Zhang et al. reported that there is a minimum concentration of AA required to keep the viscosity and conductivity in a electrosprayable range (Zhang & Kawakami, 2010), which is dependent on the type and concentration of CS. The increment of CS concentration in the AA solution increased the conductivity and viscosity, leading to the increase of the particles size (Fig. 1, Fig. 2). For example (Fig. 1B, trend I), formulation 7 (1% w/v CS in 70% AA) had a viscosity of 7.61 ± 0.10 mPa s and a conductivity of 0.049 mS/cm producing particles of 773 ± 79 nm. When the concentration was increased to 3% w/v of CS keeping the AA in 70% (formulation 10), the viscosity raised to 16.73 ± 0.09 mPa s, a conductivity of 0.108 mS/cm and the particle size to 855 ± 67 nm. Incrementing CS concentration, as reported in literature, has an effect in particle size because based on mass conservation, is roughly proportional to the power of 1/3 of concentration of CS. Although, there are also other factors influencing the size of the particles and make the slope of this proportion steeper (Gómez-Mascaraque et al., 2016; Sreekumar et al., 2017; Zhang & Kawakami, 2010).

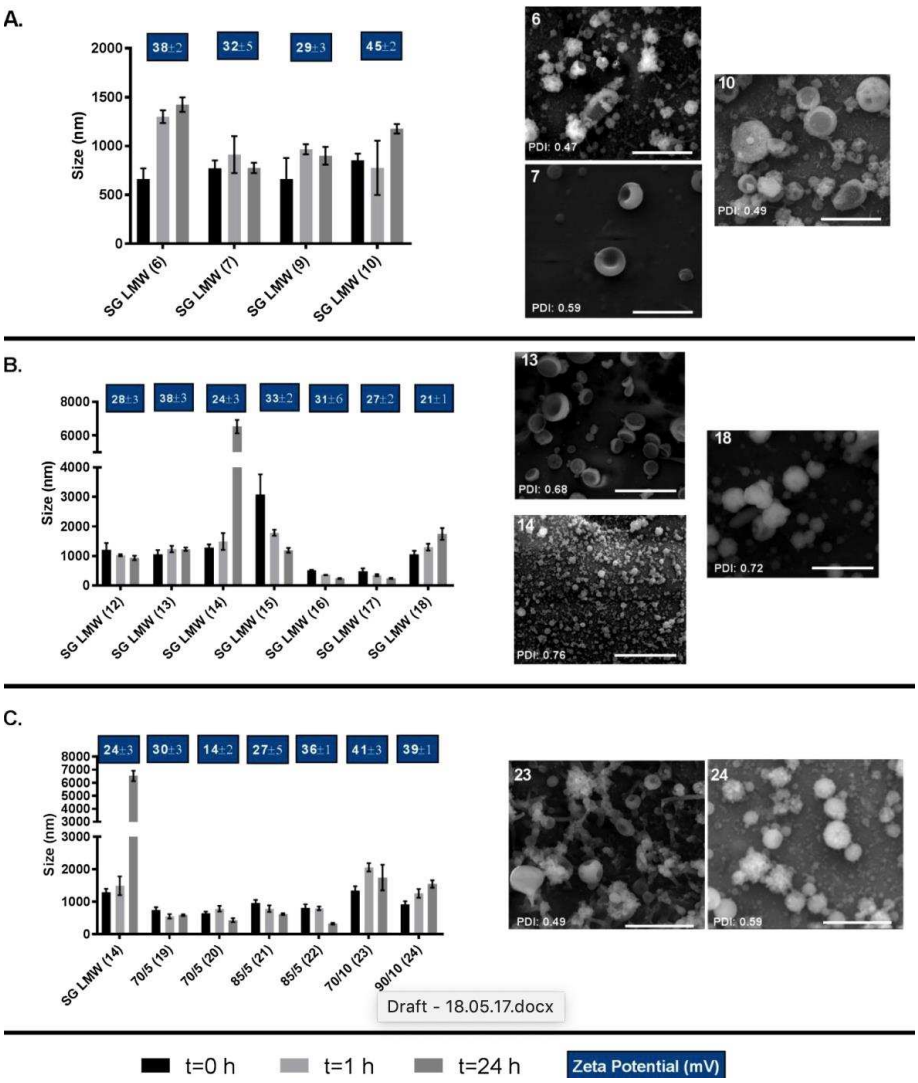

Fig. 2. Size, zeta potential of the electrosprayed particles and SEM images of the particles which remained stable after 24 h: A) The effect of AA content using low MW CS, B) The effect of AA and EtOH content using low MW CS; C) The effect of CS concentration, DD and MW. The scale bar represents 3 μm. The data represents mean ± SD, n = 3. All shown zeta potential values had a positive charge.

However, when increasing AA concentration in presence of EtOH, as seen for formulation 12 and 13, (both 1% w/v CS in 50% EtOH) (Fig. 1C, trend IV), a decrease in viscosity from 13.21± to 11.56± mPa s, conductivity from 0.03 to 0.02 mS/m and the particle size from 1.20 ± 0.22 μm to 1.06 ± 0.135 μm was observed. It was confirmed, as reported in literature, that the presence of EtOH helps to stabilize the electrospraying process as the conductivity and viscosity of the solutions are decreased (Sun et al., 2010). The addition of 50% v/v EtOH, resulted in the increase of the particles size from 1.21 ± 0.2 μm (formulation 12 without EtOH) to 3.08 ± 0.7 μm (formulation 15) (Fig. 1C, trend II and Fig. 2). The effect of using EtOH on the particle size was clearly seen when increasing the ratio from 0 to 50% in 1% w/v CS (Fig. 1,

formulation 6, 16, 17 and 12). EtOH decreased the conductivity of the solutions from 0.127 to 0.036 mS/m. For formulation 6, 1% w/v (no EtOH present) had a viscosity of 67.46 ± 0.02 mPa s, and when adding 10% v/v EtOH, the conductivity decreased to 10.66 ± 0.1 mPa s (formulation 16). Furthermore, it slightly changed to 13.21 ± 0.1 mPa s when increasing EtOH up to 50% v/v (formulations 12). The size had smooth variation from 0% to 30% v/v EtOH (formulation 6, 16 and 17) and increased abruptly at 50% v/v EtOH being 661 ± 108 nm, 498 ± 89 nm and 1217 ± 222 nm, respectively. In contrast, 3% w/v SG LMW CS showed a growth in viscosity, when increasing EtOH from 0 to 50% v/v (formulation 9, 18 and 15, respectively), and the size of the particles increased as well, but the conductivity remained in the range of 0.1–0.2 mS/m. Formulations 5 (1% w/v CS), 8 (3% w/v CS) and 11 (1% w/v CS) using SG LMW CS were unable to produce CS particles due to low content of AA (10% v/v or 5% v/v) in the solvent.

3.3. Effect of DD, MW and DP in the particle size of electrosprayed particles

Formulations with the same concentration of solvents and same electrospraying parameters (16.4 μL/min, distance of 15 cm and voltage of 24 kV), but different MW, DD and DP were compared (Fig. 1D). It is known that DD, MW and DP affect the particle formation, as it has been previously reported in literature (Gómez-Mascaraque et al., 2016; Sreekumar et al., 2017). Therefore, it is important to take all three factors into account when predicting how the electrospraying of the CS particles will proceed. The results showed that when comparing formulation 14 (MW = 28 kDa, DP = 175 and DD = 89%) with formulation 24 (MW = 28 kDa, DP = 177 and DD = 97%), the conductivity of formulation 24 was higher (0.07 and 0.12 ± 0.01 mS/cm) and the viscosity of 24 was lower (201.83 ± 9.2 and 43.36 ± 0.2 mPa S), as well as the particle size, due to the higher DD of the formulation 24. However, when comparing formulation 14 (MW = 28 kDa, DP = 175 and DD = 89%) with formulation 20 (MW: 81 KDa, DP of 491 and DD of 88%) or formulation 23 (MW = 49 kDa, DP = 293 and DD of 82%), which have a similar DD in magnitude, no clear trend was observed to suggest which of the three factors have a higher impact on conductivity and viscosity and therefore, on size and zeta potential.

3.4. Morphology of electrosprayed particles

The electrosprayed particles showed mainly three types of morphology (Fig. 2); either rounded spheres with porous surface (Fig. 2A, sample 10), bowl-like particles (Fig. 2B, sample 13) or elongated spheres (Fig. 2C, sample 23). The morphology of electrosprayed particles is related

with the droplet drying rate and the shrinkage caused by evaporation of the solvents (Sreekumar et al., 2017; Zhang & Kawakami, 2010). Zhang et al. reported that an increase of EtOH concentration in the CS solution resulted in more homogeneous particles due to a stabilizing effect of EtOH on the electrospraying process (Zhang & Kawakami, 2010). For formulation 6, 7 and 10, there was no EtOH and hence, a higher variability of shapes can be observed (Fig. 2A) compared to the other SEM samples (Fig. 2B and C), where EtOH was used.

3.5. Size, zeta potential and stability of the CS particles in PBS pH 6.8

CS is a cationic polymer (Pancholi, Ahras, Stride, & Edirisinghe, 2009) and it was therefore, expected that all particles showed a positive charge. Particles with a positively charged surface, are good candidates for oral drug delivery applications, since they can promote adhesion towards mucosal cells, i.e. mucins, which are normally negatively charged (Ensign et al., 2013; Honary & Zahir, 2013). It has been reported that particles with a zeta potential of approximately +30 mV are stable, whereas in the range of +20 mV, the particles show short-term stability. Below +20 mV, it is reported that the particles might aggregate rapidly (Honary & Zahir, 2013). It was found that the zeta potential of the particles increased from $+28 \pm 3$ to $+38 \pm 3$ mV at concentrations of AA ranging from 20 to 35% v/v, as it can be seen for formulation 12 and 13 (both 1% w/v CS in 50% v/v EtOH) (Figs. Figure 1C and Figure 2B). Moreover, the increase of EtOH from 0 to 10, 30 to 50% v/v (formulation 6, 16, 17 and 12, respectively) led to a decrease in the zeta potential from 38.2 ± 2 to 31.77 ± 5, 27.38 ± 2 to 28.06 ± 3 mV, respectively. From our study, where several parameters were tested simultaneously, there is no clear evidence of a correlation between the particle size and the type of CS in relation to the zeta potential.

For testing the physical stability of the particle, the CS particles were dispersed in PBS at pH of 6.8 and the size was measured at different time points (0, 3 and 24 h) by DLS (Fig. 2). The initial size of the particles ($t = 0$) measured by DLS was in agreement with the size of the CS particles measured in the SEM images (Fig. 2). After 3 h, it was observed that some particles increased their size, but at 24 h decreased in size (i.e. formulation 7 or 9, Fig. 2A). This could suggest agglomeration among particles or swelling after 3 h with further disintegration after 24 h. Moreover, some particles decreased in size after 3 h and 24 h (i.e. formulation 15 or 16, Fig. 2B) showing that these particles are not able to sustain their size. In other formulations, the particles did not vary significantly over time (i.e. formulation 13, Fig. 2A) or the size over time was not observed to decrease (i.e. formulation 24, Fig. 2C) suggesting that those particle formulations are stable.

Accordingly, the formulations 6, 10, 13, 14, 18, 23 and 24 were considered stable. In case of formulation 14, there was a pronounced peak after 24 h suggesting that the sample aggregated and the size changes over time are not due to swelling. As the zeta potential was found to be +24 ± 3 mV, below the required zeta potential to maintain capsules stability (+30 mV), it suggested that there was no sufficient number of charges to stabilize the particles (Honary & Zahir, 2013).

3.6. Stability of particles in simulated intestinal medium

Performing gastrointestinal simulated tests are essential to predict in vivo behavior and to decrease the size of human studies (Karuppiah, Kannappan, & Manavalan, 2012). Therefore, the stability of the particles, whose size was not observed to decrease after 24 h in PBS, was further investigated in FaSSIF at pH 6.5 over time. Overall, Fig. 3 shows an increase in CS particles size in FaSSIF after 3 h compared to their initial size. This was expected due to the composition of the medium, where the bile salts and surfactants of FaSSIF would favor either swelling or agglomeration of the particles. However, for some of the particles, the increase of the size was observed to be abrupt (formulations 13, 14, 18) followed by a drastic decrease in size measured after 24 h as result of dissolution of these particles. Particles made from formulations 6 and 7 did not change significantly their size after 3 h in FaSSIF, but they were observed to disintegrate after 24 h.

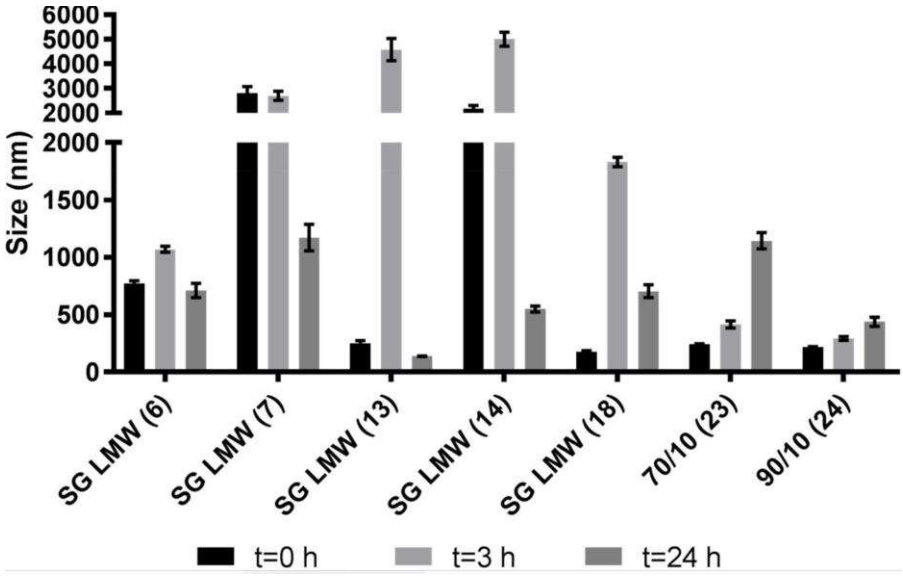

Fig. 3. Size of the electrosprayed particles after 0, 3 and 24 h in FaSSIF at pH 6.5 measured by DLS. Data represents mean ± SD in triplicates.

For formulations 23 and 24, HMC$^+$ 70/10 and 90/10 with 3% w/v CS in 50% EtOH and 5% AA, it was not observed a decrease in size from 3 to 24 h, suggesting that they are stable in FaSSIF. Both samples had a zeta potential >+ 38 mV, which according to literature might promote physical stability of the electrosprayed CS particles (Honary & Zahir, 2013). Therefore, these formulations were further tested for their mucoadhesive properties.

3.7. Mucoadhesive properties of CS particles

The mucoadhesive properties of the CS particles made of CS HMC$^+$ 70/10, DD = 82% (formulation 23) and CS HMC$^+$ 90/10, DD = 97% (formulation 24) were initially tested using the well stablish Periodic acid–Schiff (PAS) assay (Kilcoyne et al., 2011). This is a staining method measuring the interaction between mucins and the particles by calculating the bonding degree (Kongsong, Songsurang, Sangvanich, Siralertmukul, & Muangsin, 2014). The results from the PAS assay (Fig. 4A) showed that the unbounded mucin of formulation 23 and 24, were 49 ± 1.41% and 42.3 ± 1.88%, respectively, giving a significant difference between the two formulations ($p < 0.001$). The higher affinity of formulation 24 to bind to mucin comparatively to formulation 23 is related with its higher DD = 97%. It is reported that the mucoadhesive capacity is proportional to the DD due to the increased number of charged amino groups available to interact with the negative charges of the mucus (Rodrigues et al., 2012). Although, formulation 24 had a larger interaction to mucin than formulation 23, both formulations showed strong mucoadhesive properties. It has previously been reported that CS microparticles (DD = 85% and 500 kDa) had large mucin interaction using the same PAS assay (Kongsong et al., 2014).

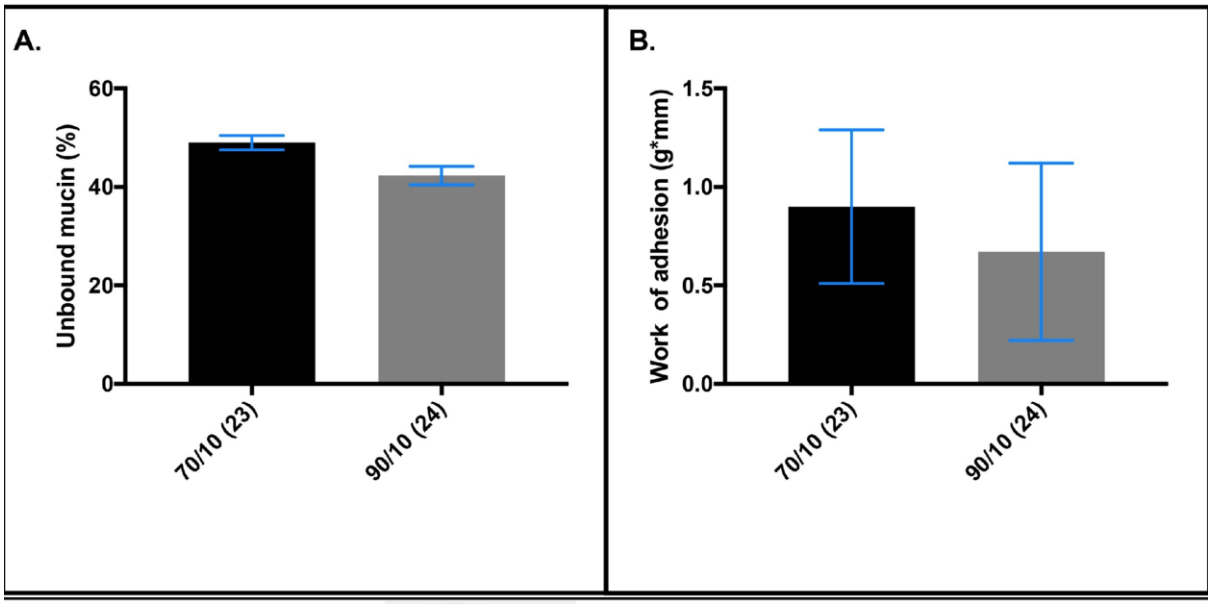

Fig. 4. A) Unbounded particles percentage of mucin from interaction with CS particles. B) Work of adhesion of CS particles with pig small intestine, normalized with Teflon pads as a control. Results are expressed as means ± standard deviation, and are performed in triplicates.

Mucoadhesive properties of CS particles were also investigated by determining the work of adhesion resulted by the compression of the particles against a section of pig small intestine in physiological conditions. Fig. 4B shows that formulation 23 display higher work of adhesion (0.90 ± 0.39 g mm), compared to formulation 24 (0.67 ± 0.45 g mm), however, this difference is not statistically different ($p = 0.22$). Such result can be related with similarity of the diameter of the particles, as observed on Fig. 2, but also with the variability and complexity of pig tissues samples of the small intestine. The result indicates that HMC^{+}90/10 (formulation 24) has a higher affinity to mucin, although, when tested in intestinal tissue, no significant difference was observed between the two types of CS. This suggests that beside the mucin interaction, the rest of the compounds contained in the mucus layer of the intestine interact electrostatically with the particles and modify the mucoadhesive properties, including hydrogen bonding, electrostatic and hydrophobic interactions (Crater & Carrier, 2010).

4. Conclusion

Water stable and mucoadhesive CS particles were produced by electrospray, using mixtures of EtOH and aqueous AA as solvents. The electrospray of CS was observed to be dependent on the viscosity and conductivity of the CS solutions, which varied according to the ratio

EtOH/AA and the type of CS used. Increasing the content of AA resulted in a decrease of conductivity of the solution necessary to produce the CS particles. The addition of EtOH was observed to decrease the conductivity and viscosity of the CS solutions facilitating the electrospraying process and the production of more homogeneous CS particles. The simultaneous study of DA, MW and DP did not show a clear evidence of a correlation of such properties to the particle size or zeta potential of the electrosprayed particles.

Stable CS electrosprayed particles were produced by dissolving 3% w/v of low MW CS (28–49 KDa) with DD of 82–97% and DP of 177–292, in mixtures of aqueous acetic acid and ethanol (50/50% v/v).

Electrosprayed particles produced with Mw 49, DD 82, DP 292 exhibited size and zeta potential of 1.34 ± 0.12 μm and $+41.15 \pm 2.69$ mV, while CS Mw28, DD 97 and DP 177 produced particles with diameters of 920 ± 96 nm and zeta potential of $+39.82 \pm 1.31$ mV. Furthermore, it was found that these two formulations displayed mucoadhesive properties, confirming the potential of these electrosprayed CS particles to be used in drug delivery applications.

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