Evaluation of the mucoadhesive properties of chitosan nanoparticles prepared using different chitosan to tripolyphosphate (CS:TPP) ratios

Ezzeddin M. A. Hejjaji[a,b], Alan M. Smith[b] and Gordon A. Morris[a, ✉]

[a]Department of Chemical Sciences, School of Applied Sciences, University of Huddersfield, Huddersfield HD1 3DH, UK

[b]Department of Pharmacy, School of Applied Sciences, University of Huddersfield, Huddersfield HD1 3DH, UK

✉Corresponding author

Tel: +44 (0) 1484 473871

Fax: +44 (0) 1484 472182

Email: g.morris@hud.ac.uk

Highlights

- the interactions between CS:TPP nanoparticles and mucin were evaluated
- the degree of interaction depends on the CS:TPP ratio, ζ-potential and viscosity
- a CS:TPP ratio of 4:1 displayed the strongest interaction with mucin
- greater mucin binding efficiencies achieved at CS:TPP ratios of 4:1 and higher
- a minimum CS:TPP ratio of 4:1 is required for stable interactions with mucin

Abstract

Mucoadhesive molecules such as chitosan, can allow targeting of a particular tissue to prolong residence time and subsequently improve bioavailability. The purpose of this study was to investigate chitosan-tripolyphosphate (CS:TPP) nanoparticles and to evaluate the interaction between nanoparticles of different CS:TPP ratios with mucin using viscosity, particle size analysis and ζ-potential. For all CS:TPP ratios examined, a minimum value of viscosity was reached for a 3:1 CS:TPP ratio, however chitosan nanoparticles at this ratio were not stable (< +30 mV), whereas a CS:TPP ratio of 4:1 displayed the strongest interaction. This suggests a minimum CS:TPP ratio of 4:1 is required to produce stable nanoparticles able to form strong interactions, which is consistent with a greater mucin binding efficiencies at CS:TPP ratios of 4:1 and higher, which were quantified using a colorimetric assay**.** Further analysis of similar systems could lead potentially to tuneable chitosan nanoparticles for specific applications.

Keywords: chitosan-tripolyphosphate (CS:TPP) nanoparticles; tuneable ionotropic gelation; mucoadhesiveness

1. Introduction

A large number of polymers both natural (*e.g*. chitosan, alginate, guar gum, xanthan gum, pectin, *etc*.) and synthetic (*e.g.* Poly(ethylene oxide), Poly(acrylic acid), Poly(vinyl amine), *etc*.) have been studied for their mucoadhesive properties. Some of the reported advantages of these systems include targeting and localisation of the drug dosage form to a specific site, providing and area of intimate contact between the dosage form and the mucosa [1] and disadvantages include cytotoxicity, natural variations in both composition and molecular weight [2]. However, these disadvantages could be mitigated by the preparation of nanoparticles of controlled size and composition from an appropriate natural source for example chitosan.

Chitosan (CS), is the second most abundant naturally occurring polymer on the planet, it is a nontoxic, biocompatible, biodegradable, cationic polysaccharide, which can be easily cross-linked with tripolyphosphate (TPP polyanions) under mild conditions to form nanoparticles. Chitosan and chitosan nanoparticles (CSNPs) have been of particular interest in the pharmaceutical industry, for use in drug delivery systems especially in those, which target specific delivery sites as they demonstrate mucoadhesive characteristics. Amongst the different methods developed for the preparation of nanoparticles, ionotropic gelation with TPP is perhaps the simplest process and it can be easily optimised [3-7] to produce nanoparticles of the required particle size and charge such that the nanoparticles and any encapsulated drugs could penetrate the epithelial membrane [8] or interact with mucin in a controlled manner. Furthermore, the pH dependent solubility of chitosan enables tuneable pH-dependent release [9], which can be further tailored by derivatisation [10] or cross-linking (with TPP for example) as this would change the electrophoretic properties (net charge) [11] and nanoparticle size. Tuning the physical properties of nanoparticles can therefore, influence the pharmacokinetic profile of a loaded drug [12] and have an influence on the degree of mucin binding.

Chitosan has great potential as a drug delivery vehicle due to its mucoadhesive behaviour, however the interactions of chitosan with mucus are complicated. In general, mucoadhesive drug delivery systems are designed with the aim of increasing the residence time of drugs at a specific site of absorption/action, to sustain drug release over a prolonged period and to minimize the degradation of drugs [13]. However, for specific applications it may be desirable to minimise nanoparticle-mucus interactions where the aim is for example, to rapidly penetrate mucosal barriers [14]. Therefore, controlling the physico-chemical properties of chitosan

nanoparticles (viscosity, charge and particle size, for example) via ionotropic cross-linking can be used as a method of controlling the strength of chitosan-mucus interactions [14].

The components of mucus which are responsible for the viscous and elastic gel-like properties are mucins [15]. Mucins are a family of complex high molecular weight (5 - 20 x 10^5 g/mol) glycoproteins secreted by the epithelia of the intestinal, respiratory and urogenital tracts, consisting of linear or branched oligosaccharides attached to the protein core. These glycoproteins are mostly carbohydrate, which can account for 60 - 80 % of its weight. The carbohydrate fraction consists of five sugars N-acetyl-glucosamine, N-acetyl-galactosamine, galactose, and fucose and sialic acid (N-acetyl-neuraminic), as well as traces of mannose and sulfate esters [16, 17].

The rigidity of the structure of mucin is mostly due to the high sialic acid and sulfate ester contents which leads to a negative charge on mucin which is the main reason for its gelling and mucoadhesive properties [18], although interactions with chitosan can vary depending on biological target tissues [19, 20]. Sialic acid, which is distributed throughout human tissues, is present in several fluids, including, cerebrospinal fluid, serum, urine, amniotic fluid saliva, and breast milk. Depending on the physiological conditions and physiochemical properties such as pH, the carboxylate group of sialic acid residues on mucin can interact with the positive charge on the chitosan particles, due to the protonated amino group (NH_3^+) to form electrostatic and hydrogen bonds [21]. Hydrophobic and hydrophilic interactions are also very important [22]. It is therefore clear that the surface charge (ζ-potential), the size and occupied space (viscosity) of the nanoparticles will have an influence on their interactions with mucin. Previous studies which have attempted to alter the charge on chitosan nanoparticles have involved modification with poly-ethylene glycol for example [23] and have clearly demonstrated that mucin binding and drug release are influenced by nanoparticle charge [14, 23].

In this study, once the different ratios (CS:TPP) of chitosan nanoparticles (CSNPs) were formulated their physico-chemical properties (viscosity, ζ-potential, particle size and particle size distribution) were measured prior to being mixed with mucin. The physico-chemical properties were then determined after mixing, in order to examine the interaction between the chitosan nanoparticles and mucin. This provided an indication into how CS:TPP nanoparticles may act *in vivo* and which ratios of CS:TPP show potential as drug delivery vehicles. Moreover,

the mucoadhesiveness was then evaluated by measuring the mucin binding efficiency. It is therefore our hypothesis that by preparing CS:TPP nanoparticles of controlled viscosity, size and charge [3-7] it will be possible to determine whether a minimum CS:TPP ratio or net charge is required for mucoadhesion and from this gain a greater understanding of the mucoadhesive process. This will provide important information which would be in essential in designing tuneable mucoadhesive systems for specific applications, where for example, the degree of mucin binding should be controlled.

2 Materials and Methods

2.1 Materials

Chitosan of low molar mass (LMW) of ~ 50 000 g/mol as determined by capillary viscosity using equations 2-5 (see section **2.2.4.2**), was purchased from Sigma–Aldrich (Gillingham, UK) and has an average degree of deacetylation (DD) of ~90 % as determined by FT-IR using the equation 7 (see section **2.2.6**). Glacial acetic acid and tripolyphosphate in the sodium salt form were also obtained from Sigma–Aldrich (Gillingham, UK). Extensively degraded pig gastric mucin was a kind gift from Biofac A/S (Kastrup, Denmark) and has been fully characterized previously in our group [17]. This product may differ slightly from the native porcine mucus gel due to the manufacturing process. However, off-the-shelf mucin formulations are often used research as they have similar functionality [24, 25] and it is expected that batch-to-batch variability would be less of an issue compared to freshly prepared material [24]. All materials were used without any further purification.

2.2 Experimental

2.2.1 Preparation of chitosan and TPP solutions

A 1.0 mg/mL solution of TPP was prepared in deionized water and the pH of this solution was adjusted by adding 0.1 N hydrochloric acid (HCl) until a final pH of 5.0 was obtained. A 3.0 mg/mL solution of chitosan was dissolved in dilute acetic acid (0.5 %) and this was left stirring overnight at room temperature. The solution was then filtered under Gooch crucible (AG 1 X 3) vacuum filtration to discard any trace insoluble material then pH was adjusted to 5.0 using 0.1 N sodium hydroxide (NaOH).

2.2.2 Preparation of chitosan: TPP nanoparticles

CS:TPP nanoparticles were produced by the drop wise addition of appropriate volumes of chitosan solutions to appropriate volumes of TPP solutions under magnetic stirring at 600 rpm

for 60 min, resulting in CS:TPP ratios of 3:1, 4:1, 5:1, 6:1 and 7:1 respectively. All operations above were conducted at room temperature and mixtures were sonicated for 5 min (the cycle and amplitude was adjusted to 0.5 and 80 % respectively; Hielscher Ultrasonics GmbH, Teltow, Germany) before being subjected to further analysis.

2.2.3 Mucin sample preparation

A 3.5 % (w/v) mucin stock solution was formulated using 3.5 g of mucin to 100 mL of deionised water (pH 4.2). This was magnetically stirred overnight at room temperature (~20 °C). The solution was then filtered through filter paper (Whatman No.1, Sigma–Aldrich Gillingham, UK).

2.2.4 Evaluation of the mucoadhesive properties of chitosan-TPP nanoparticles

2.2.4.1 Adsorption of mucin on to chitosan-TPP nanoparticles

Mucin solution (1 mL) was added to each CS:TPP nanoparticle preparation (19 mL), with magnetic stirring at 600 rpm and mixtures were incubated at 37 °C for 1 h prior to analysis. The mucin-nanoparticle mixtures were then centrifuged at 40 000 x g for 60 min and the supernatant was used to measure the free mucin concentration using the standard calibration curve (see section 2.2.5). In addition, the mucoadhesiveness was expressed as the mucin binding efficiency of the nanoparticles and was calculated from the following equation:

$$Mucin\ binding\ efficiency\ (\%) = \frac{(Co - Cs)}{Co} \times 100 \qquad \text{Eq. (1)}$$

where C_o is the initial concentration of mucin used for incubation, and C_S is the concentration of free mucin in the supernatant [26, 27].

2.2.4.2 Viscosity analysis of chitosan nanoparticles-mucin mixtures

The relative viscosity (η_{rel}) of all samples (chitosan solution, mucin solution and chitosan nanoparticle-mucin mixtures) were tested at 37.0 ± 0.1 °C by a Bohlin Gemini HR Nano Rheometer (Malvern Instruments, Worcestershire, UK) using 1 mm gap and 55 mm parallel plate geometry at a constant shear rate of 500 s^{-1} under precise temperature control, according to the following equation:

$$\eta_{rel} = \left(\frac{\eta}{\eta_0} \right) \quad \text{Eq. (2)}$$

where η is the average viscosity of the samples and, η_o is the average viscosity for the reference solvent *i.e.* dilute acetic acid (pH 5). All measurements were performed in triplicate.

The specific (η_{sp}), viscosity is defined as follows:

$$\eta_{sp} = \eta_{rel} - 1 \quad \text{Eq. (3)}$$

A useful method for measuring the intrinsic viscosity is to calculate the relative and specific viscosities at one concentration and utilise the Solomon-Ciutâ approximation [28, 29]. The intrinsic viscosity can then be accurately estimated (error generally ~1 %) by a single measurement at low concentration approximately ≤ 0.5 %.

$$[\eta] \approx \frac{\left(2\eta_{sp} - 2\ln(\eta_{rel})\right)^{1/2}}{c} \quad \text{Eq. (4)}$$

We can then convert intrinsic viscosities to molar mass using the following Mark–Houwink–Kuhn–Sakurada (MHKS) power law relationship [30]:

$$Mw = \left(\frac{[\eta]}{0.0074}\right)^{1/0.95} \quad \text{Eq. (5)}$$

A change in relative viscosity of nanoparticles indicates the interaction with mucin [31]. This information can then be used to give a quantitative estimation of the degree of interaction, by comparing the value for the relative viscosity with a theoretical value based on there being no interaction between the nanoparticles and mucin calculated using equation 6. In order to gain a fuller understanding of this interaction samples were not filtered prior to measurement.

$\eta_{rel\ (theoretical)} = (\eta_{rel\ (mucin)}\ x$ fraction of mucin) + ($\eta_{rel\ (nanoparticles)}\ x$ fraction of nanoparticles)

Eq. (6)

where the fraction of mucin ranged from 0.49 - 0.57 and the fraction of nanoparticles was 0.43 - 0.51 based on the amount of chitosan in the formulation.

In the case of no interaction the difference between the theoretical and calculated relative viscosity is zero [31]. The relative deviation from the theoretical additive line (or line of "no interaction") gives a quantitative estimate of mucoadhesion [32], although the formation of insoluble complexes/ coacervates can lead to difficulty in interpretation [33].

2.2.4.3 Zeta potential of chitosan nanoparticles-mucin mixtures

The ζ-potential of the chitosan solution (0.3 %), mucin solution (3.5 %) and chitosan nanoparticle-mucin mixtures were measured with a Malvern Zetasizer Nano-Z (Malvern Instruments Limited, Malvern, UK) using the capillary cell. All measurements were taken at 37.0 ± 0.1 °C and the mean values and standard deviations of triplicate measurements were calculated. Mucin solutions were measured at pH 4.2 and CS:TPP nanoparticle-mucin mixtures were at pH 5.

2.2.4.4 Particle Size analysis of chitosan nanoparticles-mucin mixtures

The particle diameter of mucin solution (3.5 %) and chitosan nanoparticle-mucin mixtures were measured by dynamic light scattering (DLS) using a Malvern Zetasizer Nano-Z (Malvern Instruments Limited, Malvern, UK). The dispersion medium (water) and refractive index of particles was set at 1.330 and 1.6 respectively. A glass cuvette was used and the angle of scattering was 173°. The samples were measured in triplicate and the results represent the mean particle diameter at 37.0 ± 0.1 °C. Mucin solutions were measured at pH 4.2 and CS:TPP nanoparticle-mucin mixtures were at pH 5.

2.2.5 Mucin adsorption assay

Mucin adsorption was studied using a periodic acid /Schiff colorimetric method described by Mantle and Allen [34] to determine the free mucin concentration following incubation with chitosan nanoparticles. This method was divided into two steps: firstly, periodic acid reagent was prepared by adding 10 µL of 50 % of periodic acid solution to 7 mL of 7 % acetic acid

solution. Secondly, the preparation of the Schiff reagent was prepared by adding 1 % basic Fuschin aqueous solution to 20 mL of 1 M HCl, and twice mixing the resulting solution with 300 mg of activated charcoal. Sodium metabisulfite (0.1 g per 6 mL of Schiff reagent) was added directly before use and the resultant solution was incubated at 37 °C until it became colourless or pale yellow (about 90 min). A standard calibration curve was constructed by adding 200 µL of freshly prepared periodic acid reagent to 2 mL of mucin standard solutions (0.01 % – 0.08 %), solutions then were incubated at 37 °C for 2 h in a water bath to complete periodate oxidation. Then 200 µL of the colourless Schiff reagent was added at room temperature in order to react with the aldehyde (from first step) to form a pink coloured solution. Colour development was complete after 30 min and the absorbance of the standard solutions was measured at 555 nm using UV-Vis spectrophotometer (Shimadzu UV-160A, Wolverton, UK). Samples were prepared for analysis as per section **2.2.4.1**.

2.2.6 Fourier transform infrared (FTIR)

Spectroscopy FTIR spectra of chitosan, were recorded using a Fourier transform infrared spectrophotometer (Thermo Nicolet 380 FT-IR spectrometer, Thermo Fisher Scientific, Loughborough, UK), operating from 4000 to 400 cm^{-1}. The degree of deacetylation (DD) was calculated from equation 7 [35].

$$DD\ (\%) = 100 - \left(\frac{\left(\frac{A1655cm^{-1}}{A3450cm^{-1}}\right)x\ 100}{1.33}\right) \qquad \text{(Eq. 7)}$$

where $A_{1655\ cm^{-1}}$ and $A_{3450\ cm^{-1}}$ are the areas under the absorbance peaks for the C=O and OH stretches, respectively.

2.3 Statistical analysis

All experiments were expressed as the mean value ± standard deviations (SD) of at least three readings. Statistical significance ($p < 0.05$) between test groups was performed by one-way analysis of variance (ANOVA) and a Tukey post-hoc test.

3 Results and discussion

Chitosan was dissolved in glacial acetic acid at pH 5, below its pKa 6.2–6.5, to produce a reactive positively charged ammonium group (NH_3^+) (protonated amine D-glucosamine

monomeric unit). Due the different pKa values 0.9, 1.9, 5.3, 7.7 and 9.5 ([36, 37], multiple anions ($P_3O_{10}^{5-}$, $HP_3O_{10}^{4-}$, $H_2P_3O_{10}^{3-}$, $H_3P_3O_{10}^{2-}$ and $H_4P_3O_{10}^{-}$) may be present in solution depending on the pH when TPP is dissolved in water, which is undesirable as they can competitively react with the protonated ammonium groups (NH_3^+) of chitosan solution (pH 5.0). Therefore, the pH of TPP was adjusted to 5.0 to make sure that predominantly $H_2P_3O_{10}^{3-}$ (~67%) ions exist in solution; this is also beneficial in producing less polydisperse nanoparticles [38]. The formation of chitosan nanoparticles (CSNPs) by ionic gelation occurs spontaneously upon the interaction with the TPP anion solution with the cationic chitosan solution (ammonium groups, NH_3^+). Five different ratios of (CS:TPP) nanoparticles (3:1, 4:1, 5:1, 6:1 and 7:1) were spontaneously obtained upon addition of a TPP (polyanion) solution to the chitosan solution (polycation). These formulations resulted in positively charged chitosan nanoparticles, which should facilitate electrostatic interactions with the negatively charged carboxylic acid groups of the mucin. Studies on native pig gastric mucin have previously shown an isoelectric point at ~pH 2–2.5 [39] and sialic acid has a pKa of 2.6 [40]. Therefore, there is potential to interact with the positive charged amino groups of chitosan nanoparticles [41] when both sialic acid and chitosan are oppositely charged at therefore ~ pH 4.5 - 5. However, other contributions from, for example, hydrogen bonding and hydrophobic interactions may also have an effect.

3.1 Mucoadhesion studies

The mucoadhesive properties and the influences of different CS:TPP ratio at 3:1, 4:1, 5:1, 6:1 and 7:1 nanoparticles were evaluated by measuring the relative viscosity, particle size and change of ζ-potential on interaction with negatively charge mucin. Chitosan nanoparticle mixing with mucin was used to assess the stability and interaction of nanoparticles prepared using various CS:TPP ratios and mucin which could lay the foundations for potential future use as a drug carrier and/ or other pharmaceutical applications. All formulations were based on the measurements of the viscosity, ζ-potential (surface charge) and particle size of chitosan nanoparticles before and after incubation with mucin at 37 °C under moderate stirring.

3.1.1 Assessment of chitosan nanoparticle-mucin interactions by relative viscosity

The interactions between chitosan nanoparticles (CS:TPP) and mucin were initially studied by relative viscosity (η_{rel}). Chitosan solution (0.3 %) and mucin solution (3.5 %) were prepared in order to produce a relative viscosity (η_{rel}) close to 1.8. Relative viscosities of this order of

magnitude are required as at higher relative viscosities for example >2 the onset in polymer entanglement is observed. Therefore, the relative viscosities were kept below two with the aim of minimising these polymer entanglement effects, which would obscure changes in viscosity due to interactions with mucin [42].

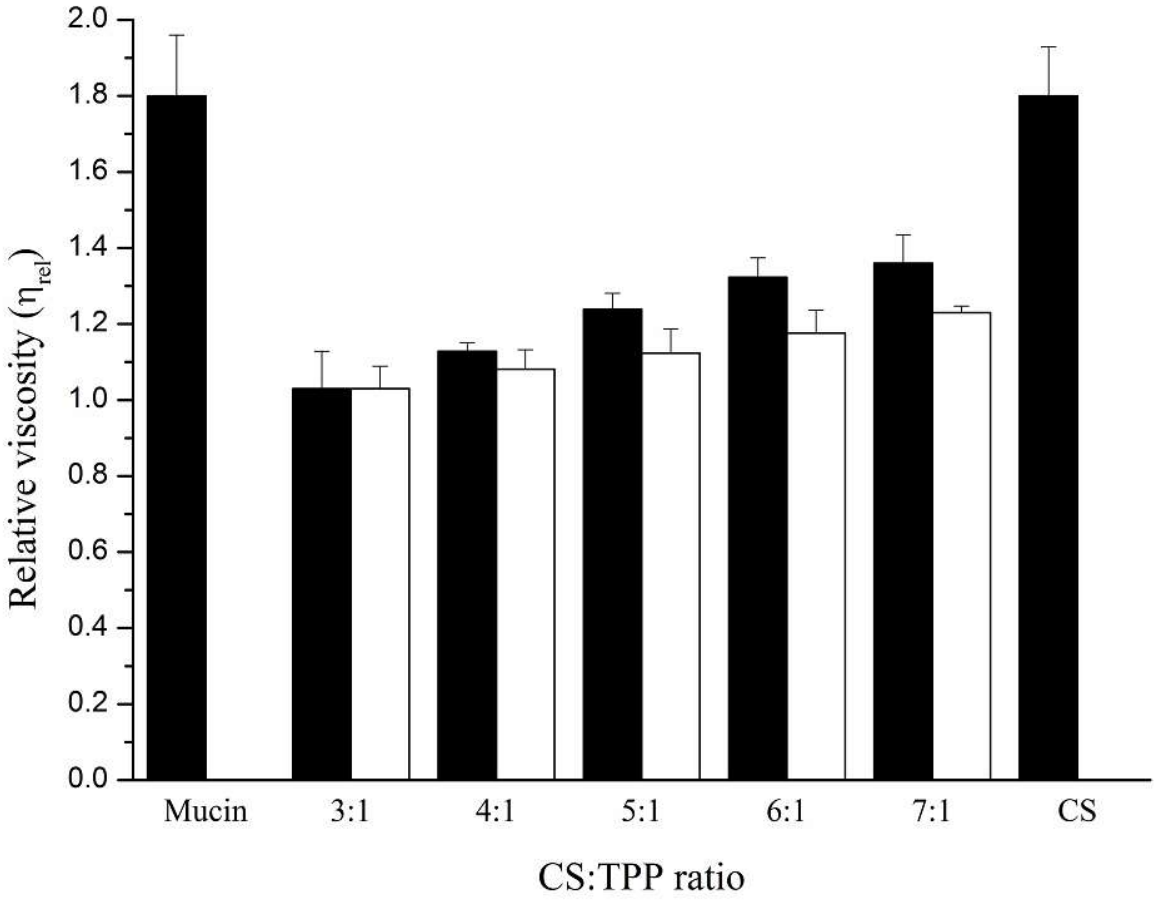

Figure 1: Relative viscosity of mucin, chitosan, native chitosan nanoparticles (black columns) and chitosan nanoparticle-mucin mixtures (white columns) at 37 °C. All values represent the mean ± 2SD (n = 3). The deviations from the theoretical viscosity of no interaction were -27.7, -25.5, -24.9, -23.3 and -20.6 % for CS: TPP ratios of 3:1, 4:1, 5:1, 6:1 and 7:1, respectively.

When chitosan nanoparticles were mixed with mucin at different CS:TPP ratios (3:1, 4:1, 5:1, 6:1 and 7:1), the formation of chitosan nanoparticles-mucin interaction products were determined on the basis of the changes in relative viscosities of the nanoparticle-mucin mixtures [43]. The relative viscosity of chitosan nanoparticle (CS:TPP)-mucin mixtures increased with increasing CS:TPP ratios (**Figure 1**).

Increasing the CS:TPP ratio (no mucin), caused an increase in relative viscosity which was expected due to the increased concentration of chitosan used (higher charge on chitosan

nanoparticles) [44, 45]. It was observed however, that chitosan nanoparticle-mucin mixtures decreased the relative viscosities compared with CS:TPP nanoparticles (blank). This could be due to the electrostatic interactions between positively charged ammonium group on the chitosan nanoparticles and the negatively charged sialic acid residue on mucin, perhaps with a contribution from hydrogen bonds. The difference between the native chitosan nanoparticle and chitosan nanoparticle-mucin mixture was not statistically significant ($p > 0.05$) on relative viscosity at (CS:TPP) 3:1. This could be attributed to a small amount of chitosan nanoparticles interacting with the mucin causing a limited viscosity change. However, other CS:TPP ratios (4:1, 5:1, 6:1 and 7:1) did significantly affect relative viscosity ($p < 0.05$), where the largest absolute decrease in relative viscosity is at CS:TPP ratio of 6:1. The percentage deviation from theoretical line of no interaction is similar for all ratios (~ -25 %), albeit the deviation is less in nanoparticles with higher CS:TPP ratios. A percentage deviation of ~ -25 % is consistent with native chitosan-mucin interactions in the presence of salt [31]. This suggests an important interaction occurring between chitosan nanoparticles and mucin. This observed reduction in relative viscosity and the increased deviation from the "no interaction" line is consistent with a reduction in the concentration of macromolecules in solution (*e.g.* the formation of a precipitate [46], which was observed when mucin was mixed with CS:TPP nanoparticles in larger amounts (data not shown)). However, in this case it is most likely due to a conformational change to a more compact structure (*i.e.* a reduction in the hydrodynamic volume [32]) for one or more of the macromolecules due to an interaction [6, 31, 32]. The latter would be consistent with a decrease in net charge/ ζ-potential [47]. A decrease in viscosity may also be advantageous from a formulation point of view, for example in ocular delivery systems where an increase in viscosity would be unacceptable due the blink process requiring low shear viscosities in order to avoid unnecessary damage to the corneal epithelium [41], low viscosity aids the sprayability of liquid nasal formulations [21, 32]. Although not explicitly evaluated, changes in viscosity are related to the swelling and stiffness of polymeric systems and could therefore be probed further, this would be influenced greatly by the CS:TPP ratio and the pH at which nanoparticles were formed [37] where chain stiffness has been shown to influence mucin interactions [31, 32].

3.1.2 Zeta potential of chitosan nanoparticles-mucin mixtures

In order to further support the interactions between chitosan nanoparticles and mucin, ζ-potential were investigated. Determination of the ζ-potential of chitosan nanoparticles in the

presence of mucin has been demonstrated to be a good means of studying the mucoadhesive interactions of the chitosan nanoparticle–mucin mixtures [24, 25, 31]. Furthermore, ζ-potentials of less than +30 mV indicate lower chitosan nanoparticle stability due to lower electrostatic repulsion, however, ζ-potential of native chitosan nanoparticles increased linearly ($r^2 > 0.99$) as the CS:TPP ratio is increased (**Figure 2**) which is a highly attractive property amongst nanoparticles. Furthermore, this would theoretically allow the preparation of nanoparticles of controlled ζ-potential in the range +34 - +42 mV by varying the CS:TPP ratio. Nanoparticles outside of this range could be prepared under different pH conditions or by using different CS:TPP ratios, for example.

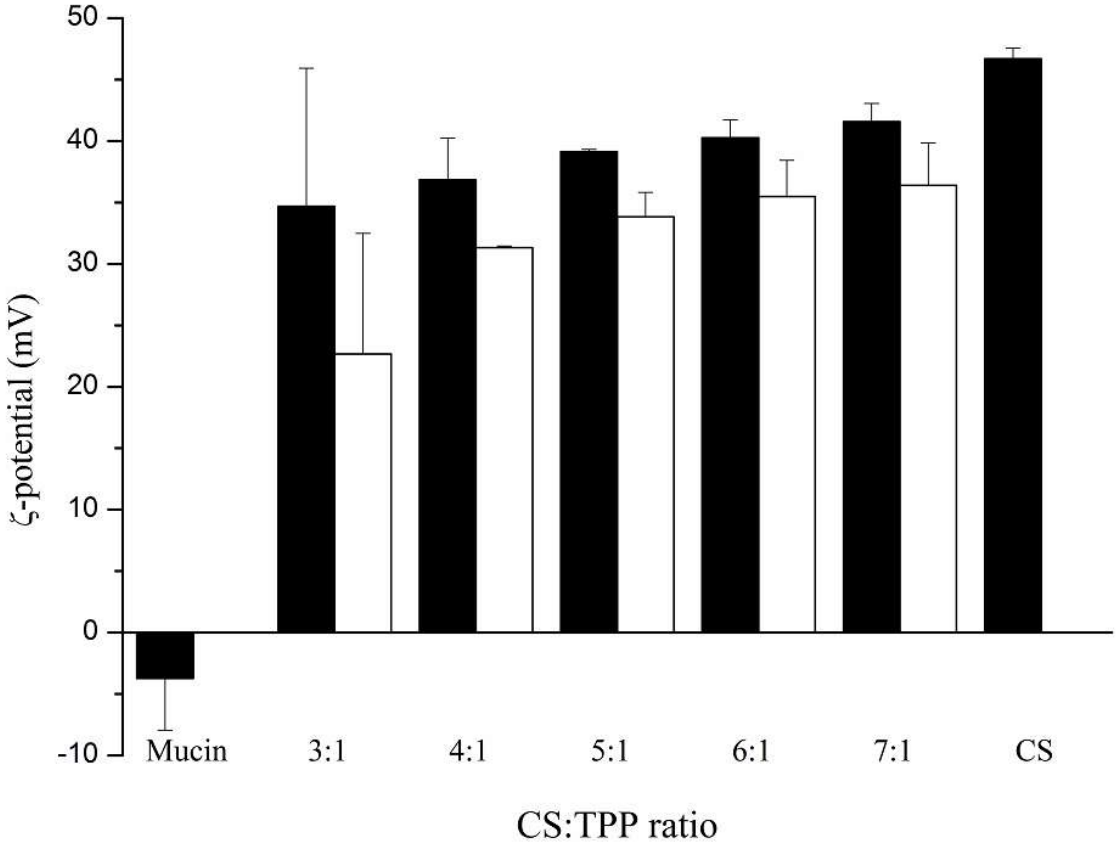

Figure 2: Zeta potential values obtained for mucin, chitosan and native chitosan nanoparticles (black columns) and chitosan nanoparticle-mucin mixtures (white columns) at 37 °C. All values represent the mean ± 2SD (n = 3).

Chitosan has a mucoadhesive properties, therefore it would be expected that the surface charge of chitosan nanoparticles might be changed by the adhesion of mucin and in this case a decrease in ζ-potential was observed upon mixing with mucin at all CS:TPP ratios (**Figure 2**). The occurrence of such change was detected by measuring the changes in ζ-potential of chitosan

nanoparticle-mucin mixtures with different of CS:TPP ratios. The ζ-potential of mucin and chitosan were -4 ± 3 and +46.7 ± 0.4 mV, respectively. It is known that chitosan has positive charge at this pH due to presence of ammonium ions (NH_3^+) [48]. The negative charge, however, of mucin is as a result of the ionization of sialic acid (COO^-). Therefore, chitosan nanoparticles could lead to a strong electrostatic interaction with the mucin. An addition of mucin to the different ratios of CS:TPP nanoparticles results in a significant ($p < 0.05$) decrease in ζ-potential for all CS:TPP ratios, except 3:1 **(Figure 2)**. The reduction of ζ-potential could be due to the ionic interaction between negatively charged sialic acid in mucin and positively charged amino groups in chitosan nanoparticles [49]. Moreover, as shown in **Figure 2**, the ζ-potential decreased sharply as the CS:TPP ratio decreased from mixtures 4:1 to 3:1, which might be caused by the decreased amount of chitosan in these nanoparticles which leads to most of the NH_3^+ groups interacting with COO^- groups on the sialic acid. These results were in agreement with lowest relative viscosity of 3:1 mixture (**Figure 1**). Furthermore, this may be attributed to the fact that native (3:1) ratio nanoparticles had lower ζ-potential values (+34.7 mV) than other ratios including 4:1, 5:1, 6:1 and 7:1. On the other hand, a small increase in ζ-potential of chitosan nanoparticle-mucin mixtures was observed as the CS:TPP ratio increased from 5:1 to 7:1, which might be due to increase positive charge surfaces on the particles. This may also be confirmed by the changes in particle size and polydispersity index (PDI) of native CS:TPP nanoparticles. At all CS:TPP ratios greater than 3:1 the ζ-potential decreased by ~ 5 mV which is in the range of the overall charge on mucin and may be indicative of the majority of the mucin being bound to the nanoparticles at these ratios.

3.1.3 Particle size of chitosan nanoparticle-mucin mixtures

In all cases there is an increase in particle size upon addition of CS:TPP nanoparticles to mucin, clearly indicating an interaction (**Table 1**), although this increase in particle size is generally not as pronounced when the CS:TPP nanoparticles are larger *i.e.* those containing greater amounts of chitosan [2]. This is consistent with the smaller deviation from the no interaction line for CS:TPP nanoparticles containing larger amounts of chitosan. The change in ζ-potential is related to a change particle size [21] and it is clear that as the CS:TPP ratio increases both the ζ-potential and particle size also increase. This is true both in the presence and absence of mucin which is related to the reduction in TPP available to interact with chitosan and therefore a decrease in the density internal cross-linking and hence larger particles [50] (**Table 1**). Nanoparticles at all ratios, other than 3:1, were in the optimal range (200 – 500 nm) for mucosal

interaction [51]. When the mucin solutions were mixed with different CS:TPP ratios from 3:1 to 7:1, the particle size increased significantly **(Table 1)**, This is probably due to adsorption (binding) of mucin on the chitosan nanoparticle surfaces [24]. In addition, the increase in particle size, together with a decrease in ζ-potential demonstrates that the mucin is binding to the surface of the chitosan nanoparticles, when the CS:TPP ratios varied from 3:1 to 7:1. This result may be attributed to the overall negative charge of mucin, due to the presence of sialic acid, and the overall positive charge of the chitosan nanoparticles. The interaction at the CS:TPP ratio (4:1) between CSNPs and mucin was the strongest as the nanoparticles binding the mucin more strongly and hence reducing the particle size.

Table 1: Particle size and polydispersity index of chitosan TPP nanoparticles (CSNPs) in the presence and absence of mucin.

Sample	Mean particle size (nm) ± 2 standard deviations	Polydispersity index (PDI) ± 2 standard deviations
Mucin	41 ± 5	0.48 ± 0.02
CSNPs		
3:1	195 ± 16^{g}	$0.30 \pm 0.02^{b,c}$
4:1	216 ± 8^{g}	0.22 ± 0.03^{c}
5:1	247 ± 4^{f}	$0.24 \pm 0.00^{b,c}$
6:1	283 ± 8^{e}	$0.25 \pm 0.02^{b,c}$
7:1	$293 \pm 22^{d,e}$	$0.27 \pm 0.03^{b,c}$
CSNPs with mucin		
3:1	$336 \pm 14^{b,c}$	0.44 ± 0.05^{a}
4:1	305 ± 19^{d}	$0.29 \pm 0.08^{b,c}$
5:1	332 ± 12^{c}	$0.31 \pm 0.07^{b,c}$
6:1	356 ± 8^{b}	$0.30 \pm 0.02^{b,c}$
7:1	387 ± 30^{a}	$0.35 \pm 0.05^{a,b}$

Means sharing the same letters in a column are not significantly ($p > 0.05$).

Furthermore, this increase in interaction is important in respect to potential applications as increased interaction with mucin is indicative of increased mucoadhesion, which depending on

the specific application may not always be optimum. At the lowest CS:TPP ratio (3:1) mucin in mixture is potentially available in greater amounts. Therefore, we would expect more mucin to be adsorbed on to the chitosan nanoparticle surface, which would increase aggregation due to, for example, the formation of mucin bridges between chitosan nanoparticles [24], or the aggregation of nanoparticles due to their instability the latter is consistent with the low ζ-potential value of the 3:1 mixture (+22.6 mV). The PDI value was used as a reflection of uniformity and stability of particles [50]. Moreover, the highest value (0.44) of PDI at CS:TPP ratio 3:1 mixture indicated a wider range of particle size distribution in chitosan nanoparticle-mucin mixtures relative to other CS:TPP ratios. At a CS:TPP ratio of 4:1 mucin mixture, chitosan nanoparticles with the smallest size and lower PDI were formed. Above the CS:TPP ratio 4:1 mixture, a clear increase in chitosan nanoparticle-mucin mixture size is obtained, thereby confirming the adsorption of negative mucin onto the surface of the chitosan nanoparticles. In addition, as is shown in **Table 1**, the CS:TPP ratios from 5:1 to 7:1 mixtures have PDI values of 0.30 - 0.35, indicating a narrow size range and a homogenous dispersion of chitosan nanoparticle-mucin mixtures were obtained [52]. This is important in terms of the movement/ diffusion of nanoparticles through a mucosal layer *in vivo* [53]. On the basis of these observations, the strongest interaction, which can be related to the smallest particle size was a CS:TPP ratio of 4:1.

3.2 Mucin binding test (adsorption) as indicator of mucoadhesiveness

3.2.1 Mucin colorimetric assay and calibration curve

The colorimetric assay described in section 2.2.5 was used to provide an effective method of detection and analysis of mucin glycoproteins. This assay showed a high sensitivity with LOD and LOQ being <0.01 and <0.02 %, respectively with a high linearity (R^2 > 0.99) and reproducibility: RSD < 3.7 % (data not shown).

Mucin is predicted to spontaneously adsorb onto the surface of the chitosan nanoparticles [52]. The mucoadhesive behaviour of chitosan nanoparticles was assessed by the suspension of different CS:TPP ratios in a fixed amount of mucin in aqueous solution at 37 °C. Furthermore, the amount of mucin adsorbed was measured from the change in free concentration of mucin in the reaction mixtures according to Eq. 1.

After confirming the high surface charge (ζ-potential > +30 mV) for all native CS:TPP ratios (**Figure 2**), a mucin binding efficiency assay was applied to confirm the system's adhesiveness (**Figure 3**).

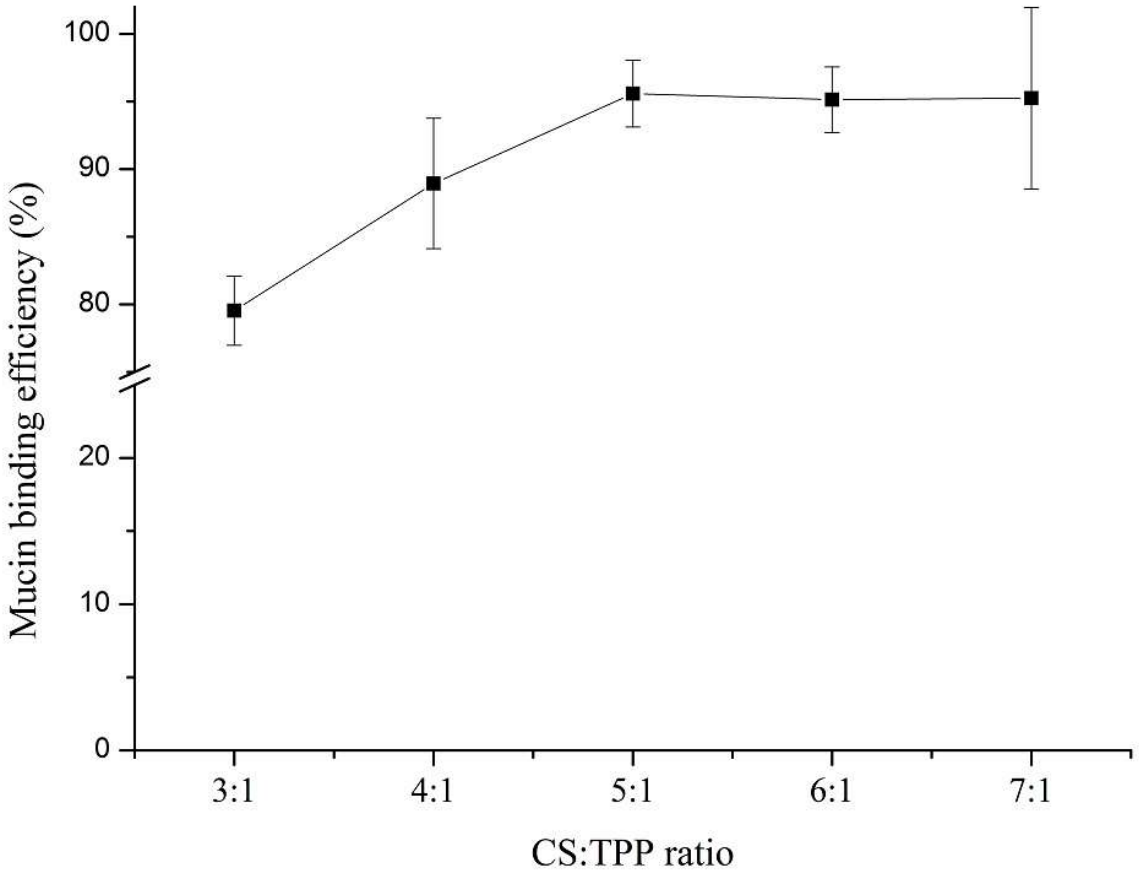

Figure 3: Mucin binding efficiency (adsorption) of chitosan nanoparticles of different CS:TPP ratios. All values represent the mean ± 2SD (n = 3).

The mucin binding efficiency (mucin adsorbed onto the chitosan nanoparticle surface) increased from 80 ± 3 % to 89 ± 5 % ($p < 0.05$) as CS:TPP ratio increased from 3:1 to 4:1 **(Figure 3)**. However, it was demonstrated that, there were no significant differences ($p > 0.05$) in the mucin binding efficiency values (~95 %) when increase in CS:TPP ratios from 5:1 to 7:1. This result may be attributed to more NH_3^+ functional groups being present to interact with the sialic acid residues on mucin. This also agrees with the findings from ζ-potential which suggests a large amount of mucin has been bound to the nanoparticles at CS:TPP ratios greater than 3:1 and that native CS:TPP nanoparticles of ratios from 5:1 to 7:1 were shown to have more available surface charges (> +39 mV). Based on these observations the chemical interaction between the CS: TPP nanoparticles is shown diagrammatically in **Figure 4**. In this

very simplistic model the CS:TPP is shown as a spherical particle, although this is generally not the case [54].

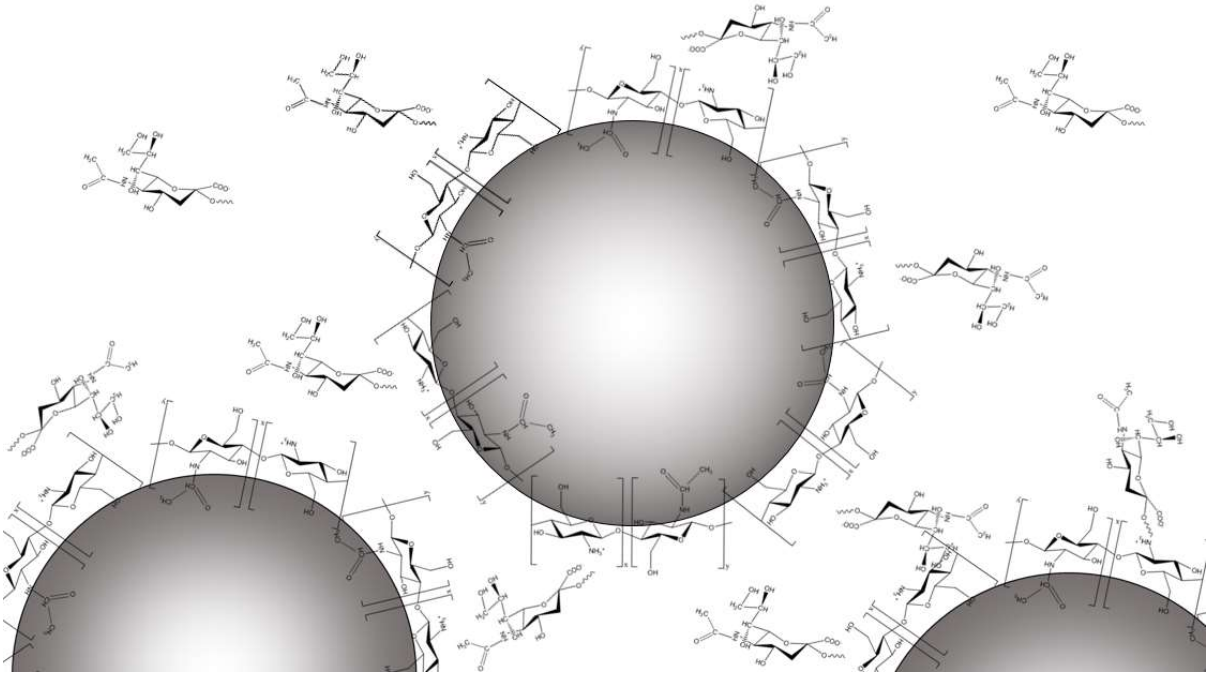

Figure 4: Diagrammatic representation of the interaction between CS:TPP nanoparticles and mucin. For simplicity and clarity, the CS:TPP nanoparticles are represented as spheres with chitosan residues on the exterior and only the negatively charged sialic acid residues are shown in the mucin (*N.B.* sizes of residues and nanoparticles are not to scale). Adapted from [55]. At lower CS:TPP ratios, for example 3:1 there will be less chitosan (positive charge) on the nanoparticle surface which may lead to an excess of mucin and potentially aggregation due to mucin bridges between nanoparticles [56] or due to nanoparticle instability if the zeta potential is in the range -30 mV – +30 mV.

It is known that the smaller particles are able to penetrate to the sub-mucosal layers whereas the larger particles are localised in the epithelial lining [57]. Based on our results an optimal minimum chitosan nanoparticle CS:TPP ratio of 4:1 is required to interact with mucin, nanoparticles with lower amounts of chitosan are unstable and prone to aggregation. The ζ-potential of 4:1 mixture is +31.3 mV (**Figure 2**), its particle size is the smallest and has lowest PDI value (**Table 1**). At 4:1 there are sufficient levels of CS:TPP particles for the mucin to interact with. This result may be attributed to a critical point of binding to sialic acid being saturated at the CS:TPP ratio of 4:1 and all of the mucin being adsorbed on to the particles. Although not significant statistically the CS:TPP ratio of 6:1 appears to be particularly interesting as at this ratio there is the greatest difference in relative viscosity upon the

interaction with mucin (although not the greatest deviation from the additive line), and there is also the smallest change in zeta potential, particle size and polydispersity index and may be related to decreased mucoadhesion at larger particle sizes [2].

4 Conclusions

In this study, CS:TPP nanoparticles of different ratios, prepared by the ionotropic gelation method, were evaluated for their mucoadhesive properties for potential use as in pharmaceutical applications. The incubation (at 37 °C) of CS:TPP nanoparticles solutions of different CS:TPP ratios from 3:1 - 7:1 with mucin led to a modification in their physiochemical properties such as decreased ζ-potential and increased particle size.

For all CS:TPP ratios examined, a minimum value of viscosity was reached for a 3:1 CS:TPP ratio, however chitosan nanoparticles at this ratio (3:1) were not stable (ζ-potential < +30 mV), whereas a CS:TPP ratio of 4:1 displayed the strongest interaction with mucin (greatest deviation from the interaction additive line). Taken all together we can conclude that a minimum CS:TPP ratio of 4:1 is required to produce stable nanoparticles able to form strong interactions with mucin, which is consistent with a greater mucin binding efficiency at CS:TPP ratios of 4:1 and higher. Clearly, the potential ability to fine tune the physico-chemical properties (viscosity, charge and size) of chitosan nanoparticles demonstrates that cross-linking with tripolyphosphate could lead to advances in transmucosal drug delivery. Further analysis, including for example drug release kinetics and swelling properties of CS:TPP nanoparticles should be the next step in this process and would ideally lead to tuneable chitosan nanoparticles for specific drug delivery applications.

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