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Designing chitosan-tripolyphosphate microparticles with desired size  
for specific pharmaceutical or forensic applications

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30 **Highlights**

- 31       ▪ CS: TPP microparticles were prepared using an experimental design
- 32       ▪ Variable factors were pH, ionic strength and CS: TPP ratio
- 33       ▪ Physical properties ( $[\eta]$ ,  $\zeta$ -potential and  $D_{[4,3]}$ ) were measured
- 34       ▪ Equations were generated to predict physical properties of the microparticles
- 35       ▪ Potential to design tuneable CS-TPP microparticles for specific applications

36

37 **Abstract**

38 Chitosan (CS) is a natural cationic polymer obtained by the partial *N*-deacetylation of chitin.  
39 Chitosan microparticles can be prepared by cross-linking with tripolyphosphate (TPP) via the  
40 ionic interaction between positively charged amino groups (CS) and negatively charged  
41 counter ions (TPP). This can be controlled by the charge density of CS and TPP, which depend  
42 on the pH and ionic strength of the solution. The purpose of this study is to investigate the  
43 combined effects of three independent variables (pH, ionic strength and CS: TPP ratio) on three  
44 important physico-chemical properties (viscosity, zeta potential and particle size) during the  
45 preparation of microparticles. CS: TPP microparticles were prepared using experimental  
46 design and equations were generated and used to predict relative viscosity, zeta potential and  
47 particle size under different conditions. This gives us the ability to design tuneable CS-TPP  
48 microparticles with desired size for specific pharmaceutical or forensic applications *e.g.* latent  
49 fingerprint visualisation.

50

51 *Keywords:* Chitosan-Tripolyphosphate Microparticles; Ionic gelation; Experimental design

52

## 53 1. Introduction

54 Chitosan refers to a family of linear copolymer polysaccharides consisting of  $\beta$  (1-4)-linked 2-  
55 amino-2-deoxy-D-glucopyranose (D-glucosamine) and 2-acetamido-2-deoxy-D-glucose (*N*-  
56 acetyl-D-glucosamine) units with different fractions of acetylated units [1]. An acetyl group  
57 may be present on some units (*N*-acetyl- D-glucosamine), which determines the degree of  
58 deacetylation (DD). Moreover, the DD of commercial chitosan is approximately 66 - 95 %,  
59 and the molecular weight ( $M_w$ ) approximately 10000 – 1000000 g/mol [2]. The structural units  
60 of chitosan have one reactive primary amino group (-NH<sub>2</sub>) on the C-2 position of each D-  
61 glucosamine unit, and two reactive free hydroxyl groups (-OH) for each C-6 and C-3 position  
62 building unit (glucosamine and *N*-acetyl-D-glucosamine). These groups (both amino and  
63 hydroxyl) can be modified to obtain different chitosan derivatives, and provide opportunities  
64 for chemical modification to impart useful physicochemical properties and distinctive  
65 biological functions [3]. In addition, the advantage of chitosan over other polysaccharides is  
66 that its chemical structure allows specific modifications at the C-2 position without too many  
67 difficulties [4]. Chitosan has been investigated widely for its potential in the development of  
68 drug delivery systems and pharmaceutical applications [5] and more recently for its forensic  
69 applications [6].

70

71 In latent fingerprint visualisation it is now accepted that particles adhere to fingermarks due to  
72 the mechanical attraction with the oily subcutaneous residues [7]. The factors with influence  
73 this interaction are particle size, particle charge, particle shape and relative surface area [7, 8]  
74 all of which are controlled by processing parameters such as chitosan concentration, pH and  
75 ionic strength of the dissolution media, temperature of cross-linking, stirring rate, *etc* [9].

76

77 Various techniques have been developed to prepare chitosan micro/nanoparticles, such as ionic  
78 gelation, emulsion droplet, spray drying, coacervation and self-assembly chemical  
79 modification [10]. Among those methods, the ionic gelation method (also known as ionotropic  
80 gelation) with the non-toxic multivalent polyanion tripolyphosphate (TPP) is the most widely  
81 used approach to physical cross-linking. Ionic cross-linking can occur inside the network via  
82 interactions between the negative charges of the cross-linker such as TPP and the positively  
83 charged amino groups of chitosan molecules [11-14]. This method is advantageous as the  
84 reaction is simple and the conditions are relatively mild and do not require the use of organic  
85 solvents or high temperatures [1, 15]. Other advantages from the point of view of drug delivery  
86 and latent fingerprint enhancement are that particle size and (positive) charge can be easily

87 controlled and microparticle formulations have previously demonstrated the ability to associate  
88 with peptides, proteins [16] and with subcutaneous secretions in fingerprints [6].

89  
90 Knowledge of viscosity, zeta potential, particle size and shape has an influence on potential  
91 applications of chitosan-TPP microparticles in drug delivery [9] or in forensic applications [6].  
92 It is therefore the purpose of the present study is to investigate the systematic manipulation of  
93 three independent processing parameters (pH, ionic strength and CS: TPP ratio) on three  
94 important physico-chemical properties (relative viscosity, zeta potential and particle size)  
95 during the preparation of chitosan-TPP (CS-TPP) microparticles. This will then enable the use  
96 of mathematical models obtained to predict the relative viscosity, zeta potential (net surface  
97 charge) and particle size under different conditions to obtain predictable and programmable  
98 microparticle properties in relation to, for example, latent fingerprint enhancement, drug  
99 release kinetics or mucoadhesion.

100

## 101 **2. Materials and Methods**

### 102 **2.1. Materials**

103 Chitosan of medium molecular weight ( $M_n \sim 295\ 000\ \text{g/mol}$ ) was obtained from Sigma-  
104 Aldrich (Gillingham, UK) and reported to have an average degree of deacetylation (DD) of  
105  $\sim 75\text{--}85\%$ . Glacial acetic acid, sodium acetate trihydrate and tripolyphosphate (TPP) sodium  
106 salt were obtained from Sigma-Aldrich (Gillingham, UK) and red food colouring was from  
107 Silver Spoon (Peterborough, UK). All materials were used without any further purification.

108

### 109 **2.2. Sample preparation**

110 Nine different acetate buffers (AB) including AB-1, AB-2, AB-3, AB-4, AB-5, AB-6, AB-7,  
111 AB-8, and AB-9 were prepared (**Table 1**) in order to investigate the effect of three independent  
112 variables: pH value, ionic strength and volumetric ratio of chitosan to TPP on the physico-  
113 chemical properties of CS-TPP microparticles.

114

115

116 **Table 1.** Acetate buffers of varying ionic strength and pH. Buffers AB-1 to AB-9 were used to  
117 create generate model equations and buffer AB-10 to AB-13 were used in model validation.

Acetate buffer (AB)	pH	Ionic strength (IS)
AB-1	3.3	0.1 M
AB-2	3.3	0.3 M
AB-3	3.3	0.5 M
AB-4	4.3	0.1 M
AB-5	4.3	0.3 M
AB-6	4.3	0.5 M
AB-7	5.3	0.1 M
AB-8	5.3	0.3 M
AB-9	5.3	0.5 M
AB-10	3.8	0.2 M
AB-11	3.8	0.4 M
AB-12	4.8	0.2 M
AB-13	4.8	0.4 M

118

119 **2.2.1. Preparation of chitosan and TPP samples with different ionic strengths and pH**  
120 **value (Acetate buffers AB-1 to AB-9)**

121 Nine different chitosan solutions were prepared by dissolving 2 g of chitosan powder in 1 L of  
122 acetate buffers (AB-1 to AB-9) to prepare chitosan solutions (2.0 mg/mL). The chitosan  
123 solutions were stirred overnight at room temperature using a magnetic stirrer. The TPP powder  
124 (1.680 g) was dissolved in 2 L of the acetate buffers (AB) to prepare nine samples of TPP  
125 solution (0.84 mg/mL) [17, 18].

126

127 **2.2.2. Preparation of CS: TPP microparticles**

128 To prepare the CS:TPP microparticles, an appropriate volume of the TPP solution was added  
129 drop wise to the appropriate volume of the chitosan solution make seven ratios of CS: TPP  
130 microparticles (6:1, 4:1, 2:1, 1:1, 1:2, 1:2, 1:4 and 1:6), and the samples were then stirred at  
131 600 rpm for 60 minutes at room temperature. The resultant microparticles spontaneously  
132 formed due to the ionic crosslinking of chitosan by sodium tripolyphosphate. Then 30 drops (~  
133 2 mL) of red dye added to all ratios to make the particles clearly visible and more amenable in  
134 latent fingerprint visualisation. The resultant microparticles were left standing overnight at  
135 room temperature before being subjected to further analysis.

136

137

138 **2.2.3. Model validation (prediction method)**

139 Chitosan solutions were prepared by dissolving 2 mg/mL of polymer in a further four different  
140 acetate buffers (AB-10, AB-11, AB-12 and AB-13) (**Table 1**) and TPP solutions were prepared  
141 by dissolving TPP at a concentration of 0.84 mg/mL in the same acetate buffers (AB-10, AB-  
142 11, AB-12 and AB-13). The resultant solutions were as in section 2.2.2 to give CS: TPP volume  
143 ratios (v/v) of 6: 1, 4: 1, 2: 1, 1: 1, 1: 2, 1: 4 and 1: 6 respectively.

144

145 **2.3. Characterisation of chitosan microparticles**

146 **2.3.1. Fourier transform infrared (FTIR) spectroscopy**

147 FTIR spectra of chitosan, TPP and chitosan microparticles were recorded using a Fourier  
148 transform infrared spectrophotometer (Thermo Nicolet 380 FT-IR spectrometer, Thermo  
149 Electron Corporation), operating from 4000 to 500 cm<sup>-1</sup>.

150

151 **2.3.2. Powder X-Ray diffraction (XRD) study**

152 A crystallinity study was carried out by comparing XRD spectrum of microparticles using  
153 Bruker AXS diffractometer (D2 PHASER) with Cu K $\alpha$  radiation to characterise chitosan, TPP  
154 and CS/TPP microparticles. The data was recorded at 2 $\theta$  range of 5° to 100° at a scanning rate  
155 of 4°/min.

156

157 **2.3.3. Determination of relative viscosities**

158 All rheological measurements (solutions and reference solvents) were performed using a  
159 Bohlin Gemini HR Nano Rheometer (Malvern Instruments, Worcester-shire, UK) using 1 mm  
160 gap and 55 mm parallel plate geometry at a constant shear rate of 500 s<sup>-1</sup> under precise  
161 temperature control (25.0  $\pm$  0.1°C). All measurements were performed in triplicate.

162

163 
$$\eta_{rel} = \left( \frac{\eta}{\eta_0} \right) \tag{1}$$

164

165 where  $\eta$  is the average ( $n = 3$ ) viscosity of the CS: TPP microparticles and,  $\eta_0$  is the viscosity  
166 for the appropriate acetate buffer [19].

167

168



169 **2.3.4. Determination of zeta potential**

170 Zeta potential was measured for each volume ratio using a Malvern Zetasizer NANO-Z  
171 (Malvern Instruments Limited, Malvern, UK). All Measurements were performed in the  
172 appropriate buffers in triplicate by using a folded capillary cell at  $25.0 \pm 0.1$  °C and refractive  
173 index of the CS: TPP microparticles was set at 1.6 - 1.8 [20] and no significant effect of  
174 refractive index was identified. Each data value is an average of three measurements with a  
175 refractive index of 1.8.

176

177 **2.3.5 Determination of particle size**

178 The particle size distributions of the resultant chitosan particles were measured directly by a  
179 dynamic light scattering using a Malvern Mastersizer 2000 (Malvern Instruments Ltd.,  
180 Malvern, UK).The microparticles were dispersed in deionized water. Refractive index of  
181 particles and dispersion medium (water) was set to 1.8 (see section 2.3.4) and 1.330,  
182 respectively. The size was described using the volume-weighted mean diameter  $D_{[4,3]}$ . The  
183 intensity of scattered light was transformed into the diffusion factor, the mean value of 10  
184 measurements was obtained and each formulation and was repeated three times.

185

186 **2.3.6. Scanning electron microscopy (SEM)**

187 The surface microparticle morphology was characterised using scanning electron microscopy  
188 (SEM). The microparticles were vacuum dried, coated with gold palladium and observed  
189 microscopically (JEOL JSM 6060 LV - Oxford instruments, Abingdon, UK). Images were  
190 taken by applying an electron beam accelerating voltage of 20 kV. Images were then analysed  
191 using Image J software (version 1.42q, National Institute of Health, Bethesda, USA) to estimate  
192 particle surface areas.

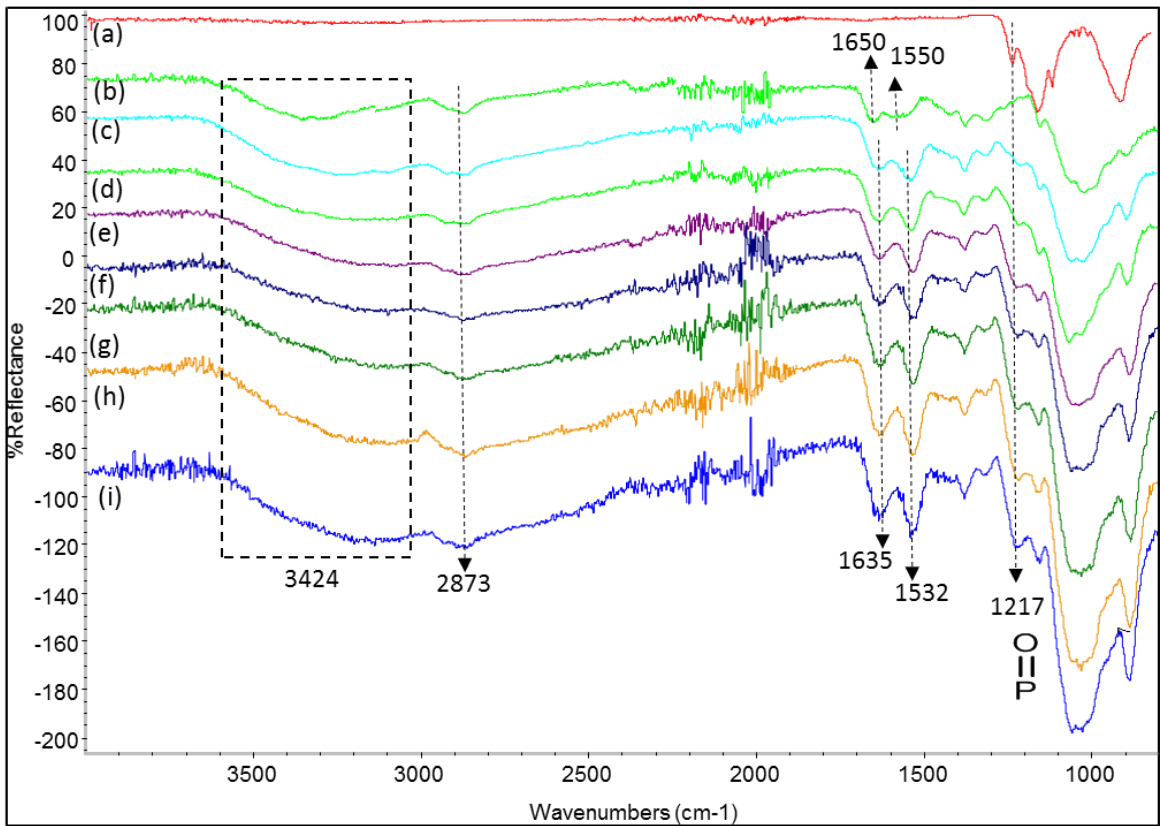
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194 **3. Results and Discussion**

195 **3.1. FTIR analysis**

196 The FTIR spectrum of pure TPP (**Figure 1a**) showed characteristic bands at  $1217\text{ cm}^{-1}$  which  
197 indicates P= O stretching [21],  $1138\text{ cm}^{-1}$  which indicates symmetrical and asymmetric  
198 stretching vibration of the  $\text{PO}_2$  groups ,  $1094\text{ cm}^{-1}$  which indicates symmetric and asymmetric  
199 stretching vibration of the  $\text{PO}_3$  groups and  $892\text{ cm}^{-1}$  (P- O- P) asymmetric stretching [22]. As  
200 ca be seen in **Figure 1b** the spectrum of CS exhibits characteristic absorption bands at  $3424$   
201  $\text{cm}^{-1}$  indicates the combined broad non-symmetric band of the -NH and -OH group stretching  
202 vibration of functional groups involved in hydrogen bonds, and the peak at  $2873\text{ cm}^{-1}$  indicates

203 the  $-CH$  stretching vibration [21, 23]. The peak at  $1650\text{ cm}^{-1}$  indicates  $C=O$  stretching in  
 204 amide I vibration group ( $CONH_2$ ), and  $1560\text{ cm}^{-1}$  which indicates N-H deformation in amide  
 205 II group vibration ( $NH_2$ ) [24]. Peaks at  $1377\text{ cm}^{-1}$  and  $1322\text{ cm}^{-1}$  might be attributed to O-H  
 206 deformation of  $-CH_2-OH$  and  $-CH-OH$ , and absorption bands at  $1151\text{ cm}^{-1}$  indicates  
 207 asymmetric bridge oxygen ( $C-O-C$ ) stretching [24].



208  
 209 **Figure 1.** FTIR spectrum of (a) TPP, (b) CS, (c) CS: TPP (6:1), (d) CS: TPP (4:1), (e) CS: TPP  
 210 (2:1), (f) CS: TPP (1:1), (g) CS: TPP (1:2), (h) CS: TPP (1:4), (i) CS: TPP (1:6) in buffer AB-  
 211 1.

212  
 213 The CS: TPP particles were characterized through FTIR spectroscopy, and the spectra are  
 214 presented in **Figures 1c – 1i**. Crosslinking process in the spectra of all CS:TPP ratios samples  
 215 the band of  $3424\text{ cm}^{-1}$  becomes wider, this indicates that hydrogen bonding is enhanced [25].  
 216 In addition in microparticles the band of  $1650\text{ cm}^{-1}$  disappears and there appears a new band  
 217 at  $1635\text{ cm}^{-1}$ . This band can be assigned to anti-symmetric deformation N-H vibrations in  
 218  $NH_3^+$  ion. The  $1560\text{ cm}^{-1}$  peak in pure chitosan shifts to a new sharp peak at  $1532\text{ cm}^{-1}$  [25].  
 219 These two new peaks as mentioned above ( $1635\text{ cm}^{-1}$  and  $1535\text{ cm}^{-1}$ ) show that a the linkage  
 220 between the ammonium ions and phosphate ions [26]. In other words, the new  $NH_3^+-PO^-$  bond  
 221 is formed due to one hydrogen atom of the amino group in chitosan is substituted by the

222 phosphate group. It further provides that the amino group is the only reactive functional group  
223 chitosan. Moreover, the characteristic peaks of the hydroxyl groups at  $1377\text{ cm}^{-1}$  and  $1322\text{ cm}^{-1}$   
224 <sup>1</sup> mentioned above do not change [24]. The cross-linked microparticles also show a new peak  
225 at  $1217\text{ cm}^{-1}$  which may be attributed to the P=O stretching from TPP [27]. Therefore clearly  
226 indicating that the protonated amino groups of chitosan are linked with negatively charged  
227 triphosphate groups of TPP, clearly demonstrating the formation of CS: TPP particles.

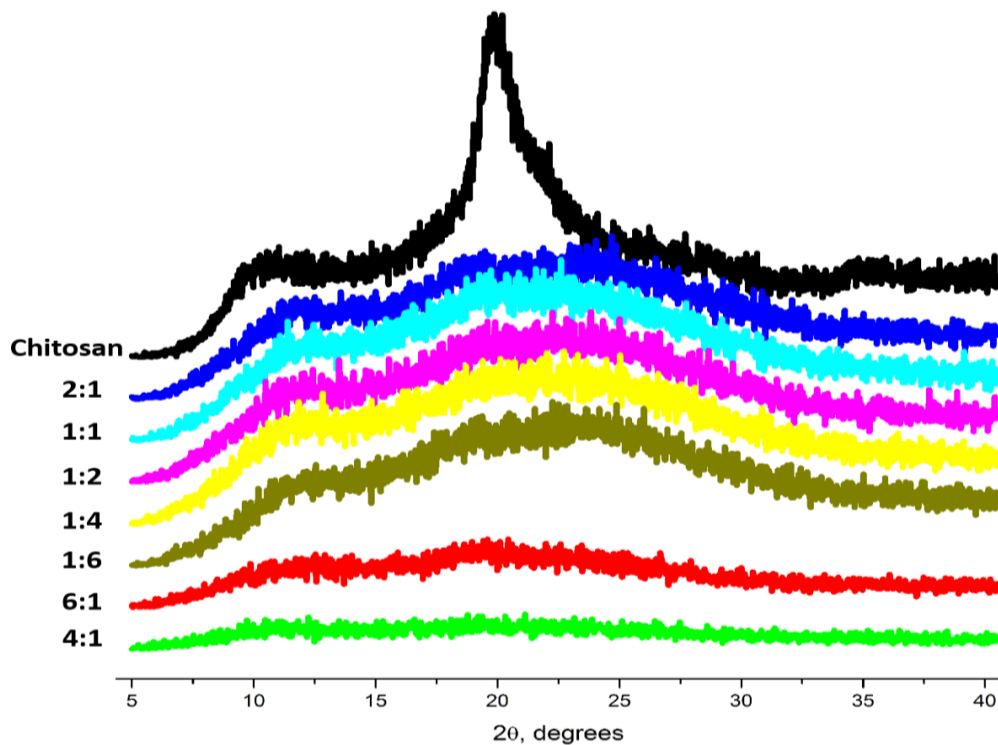
228

### 229 3.2. Crystallographic characterisation

230 Crystallographic structure of chitosan powder and chitosan microparticles were determined by  
231 X-Ray Diffraction (XRD). The XRD spectra of the chitosan microparticles were characteristic  
232 of amorphous structures. As can be seen in **Figure 2** there are two strong characteristic peaks  
233 in the diffractogram of chitosan powder at  $2\theta = 10^\circ$  (amine I “-N-CO-CH<sub>3</sub>” of chitosan) and  
234  $2\theta = 20^\circ$  (amine II “-NH<sub>2</sub>” of chitosan), indicating the some degree of crystallinity of chitosan  
235 chains [28]. The peak at  $10^\circ$  is due to the integration of water molecules into the hydrated  
236 chitosan crystal structure and the latter peak at  $20^\circ$  is assigned to the crystal lattice of the  
237 chitosan orthorhombic unit cell (110) [29], furthermore there is no indication of impurities in  
238 the chitosan formulation [30]. It is known that the width of X-ray diffraction peak is related to  
239 the size of crystallite and an increase in the amorphous nature of the material [31]. Imperfect  
240 crystals usually lead to a broadened peak [32]. After ionic cross-linking with TPP, a shift of  
241 peak positions, significant reduction in the intensity of characteristic peaks of chitosan (at  $2\theta$   
242  $= 20^\circ$ ), and broadening of peaks were observed, reflecting the destruction of the native chitosan  
243 packing structure [33]. **Figure 2** also highlights similarity between the CS: TPP ratios 2:1, 1:1,  
244 1:2, 1:4 and 1:6. Therefore the broad peak of the chitosan microparticles is due to ionic cross-  
245 linking interaction between amino groups on chitosan and the TPP, which is known to destroy  
246 the crystalline structure of chitosan [33]. Integration of the two crystalline peaks ( $2\theta = 10$  and  
247  $20^\circ$ ) as a proportion of the total integrated area gives an approximate estimate of the degree of  
248 crystallinity in each of the samples. Based on this calculation the degree of crystallinity of the  
249 native chitosan is  $\sim 30\%$  and the degrees of crystallinity of the TPP-chitosan microparticles  
250 are all  $\sim 10\%$ , this is almost entirely due to the decrease in the chitosan orthorhombic unit cell  
251 reflection (110) at  $\sim 20^\circ$ . Other than for 6:1 and 4:1 the reflection (020) at  $\sim 10^\circ$  remains  
252 unchanged during ionotropic gelation with TPP. Changes in chitosan crystallinity is important  
253 in terms of polymer degradation, tensile strength, moisture content, cell responses in *in vivo*  
254 applications and contact angles which are important during hydration. All of these are factors

255 are important to consider when developing novel chitosan-based formulations for forensic or  
256 pharmaceutical applications.

257



258

259 **Figure 2.** X-ray diffraction pattern of chitosan and of CS: TPP microparticles of different ratios  
260 in buffer AB-1. Only the diffraction pattern from  $2\theta = 5 - 40^\circ$  is shown for clarity.

261

262 The cross linking of chitosan with a higher concentrations of TPP shows less intense and  
263 broader crystalline peaks (6:1 and 4:1) which may be due to a greater amorphisation as  
264 compared with those of less TPP 2:1, 1:1, 1:2, 1:4 and 1:6 [28]. The distinct differences in the  
265 diffractogram of chitosan and cross-linked chitosan might be attributed to chemical  
266 modification in the arrangement of molecules in the crystal lattice [26] and this is also in  
267 agreement with FT-IR as to the absence of “native” chitosan.

268

### 269 3.3. Relative viscosity and zeta potential for varying chitosan solutions

270 Chitosan when in solution is a polycation which is influenced by the presence of electrolytes  
271 [34]. Thus, the effect of ionic strength and pH value on nine different solutions of chitosan was  
272 studied. It can be seen from **Figure 3A** that the relative viscosity of nine chitosan solutions,  
273 with fixed pH including AB-1, AB-2 and AB-3; AB-4, AB-5 and AB-6; AB-7, AB-8 and AB-  
274 9 decreased with increasing ionic strength solution.

275 Chain flexibility of chitosan molecules in solution can be manipulated by using chitosan with  
276 differing solution pH and/or ionic strength. Furthermore, it is known that in acidic media the  
277 amino groups of chitosan,  $-\text{NH}_2$ , are protonated to  $-\text{NH}_3^+$  groups. This causes electrostatic  
278 repulsion between chitosan molecules; meanwhile, there also exists inter-chain hydrogen  
279 bonding interactions between chitosan molecules. The hydrogen bonding occurs between the  
280 amino and hydroxyl groups [35].

281

282 In low ionic strength solutions (0.1 M), the intramolecular electrostatic repulsion effect, also  
283 called the third electroviscous effect, dominates in which the chitosan molecule exists in an  
284 extended conformation [35, 36]. Therefore, more inter-molecular hydrogen bonding occurs in  
285 low ionic strength solution [37]. This causes a high resistance to the flow or mobility of the  
286 polymer molecules and consequently a high relative viscosity is observed. However, in high  
287 ionic strength solutions (0.5 M), the concentration of acetate ions ( $\text{CH}_3\text{COO}^-$ ) is raised which  
288 neutralises more  $-\text{NH}_3^+$  groups. This leads to less dissolution of chitosan and weaker  
289 intermolecular electrostatic repulsion, causing the chitosan polymer chains to become more  
290 contracted and lowers the resistance to the flow or mobility of the polymers, resulting in a  
291 lower relative viscosity [38]. In addition, the relative viscosity of chitosan also decreased with  
292 increasing pH in solutions with fixed ionic strength. The number of positive charges on CS at  
293 I.S 0.1 M will be greater at pH 3.3 of the solvent, leading to a higher degree of expansion of  
294 chitosan and a rigid conformation due to electrostatic repulsions [39]. Information on chain  
295 expansion of chitosan used in the formulation of microparticles enables the possibility to better  
296 control microparticle properties by selecting suitable preparation conditions or starting polymer  
297 [40]. Because of this, the chitosan molecules disrupt the streamlining of the flow and increases  
298 viscosity, which will have an influence on the size (and shape) of any chitosan microparticles  
299 formed under these conditions [41].

300

301 Zeta potential measurement is important to gain knowledge on the surface charge. This charge  
302 can affect the interaction between chitosan polymer chains in phenomena such as swelling, in  
303 the interaction with TPP during gelation [42] or during the interaction with oily subcutaneous  
304 residues [7]. The ionic strength and pH value of the chitosan solution affect this interaction.

305

306 The effect of pH value and ionic strength of the chitosan solution on zeta potential may be seen:

307

308 (i) At variable pH value and fixed ionic strength

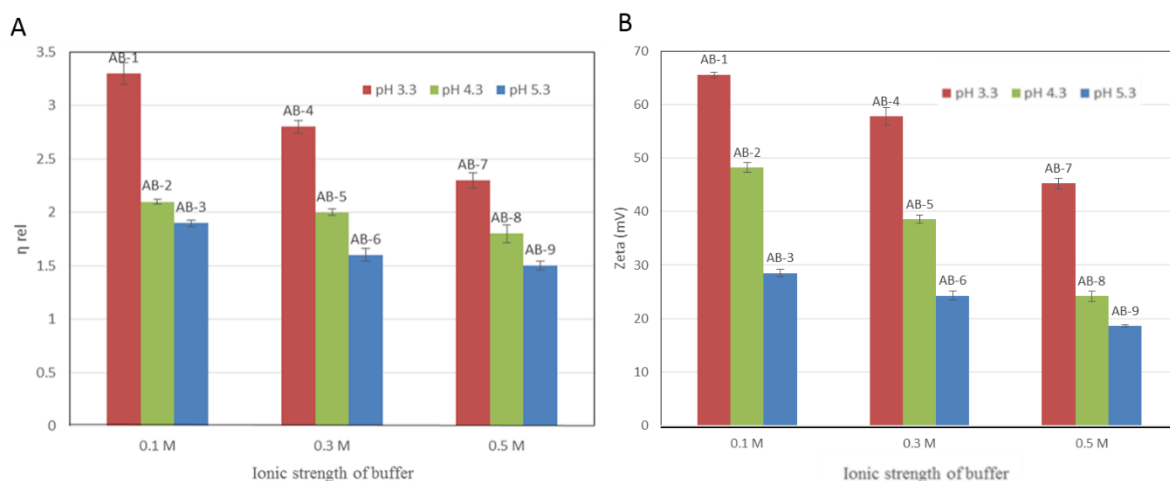
309 It can be seen from **Figure 3B** that the zeta potential decreases as the pH value increases from  
310 3.3 to 5.3. At pH 3.3, the primary amine groups  $-NH_2$  of chitosan are more strongly protonated  
311 as  $-NH_3^+$  in acetate buffer solution and therefore increased zeta potential. On the other hand, at  
312 an increased pH value of 5.3 the  $-NH_3^+$  on the chitosan molecules were more neutralised  
313 resulting in a decreased zeta potential.

314

315 (ii) At the fixed pH value and variable ionic strength (0.1, 0.3 and 0.5 M)

316 It can be seen from **Figure 3B** that the zeta potential decreased with an increase in the ionic  
317 strength from 0.1 M to 0.5 M. At ionic strength 0.1 M, the primary amine groups  $-NH_2$  of  
318 chitosan are protonated as  $-NH_3^+$  in acetate buffer solution and therefore an increased zeta  
319 potential is seen. Conversely, with an increased ionic strength at 0.5 M, the  $-NH_3^+$  on the  
320 chitosan molecules were charge screened by acetate ions ( $CH_3COO^-$ ) leading to a decreased  
321 zeta potential. This is important in terms of the conformation of chitosan chains and how that  
322 might influence their interactions with TPP polyanions during ionotropic gelation where the  
323 change in zeta potential of chitosan (and indeed all polyelectrolyte biopolymers) can be used  
324 to estimate chain stiffness [36].

325



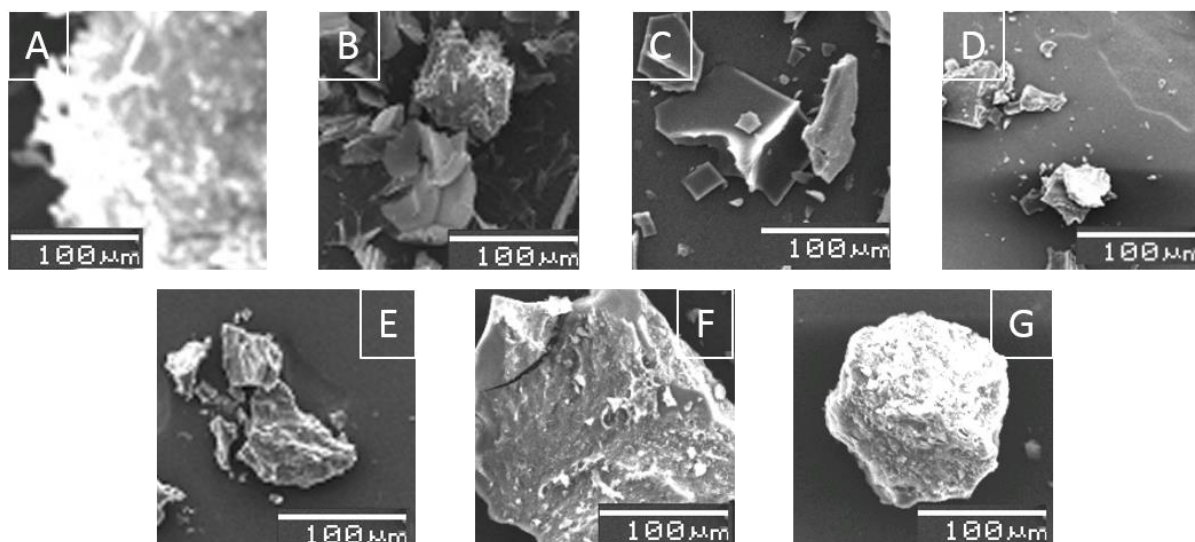
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327 **Figure 3.** Relative viscosities (**A**) and zeta potentials (**B**) of nine different chitosan solutions  
328 (using AB-1 to AB-9) at varying ionic strength and pH values at  $25.0 \pm 0.1$  °C (mean  $\pm$  STDEV,  
329  $n = 3$ ).

330

### 331 **3.4. Analysis of different ratios of CS: TPP microparticles with different acetate** 332 **buffers**

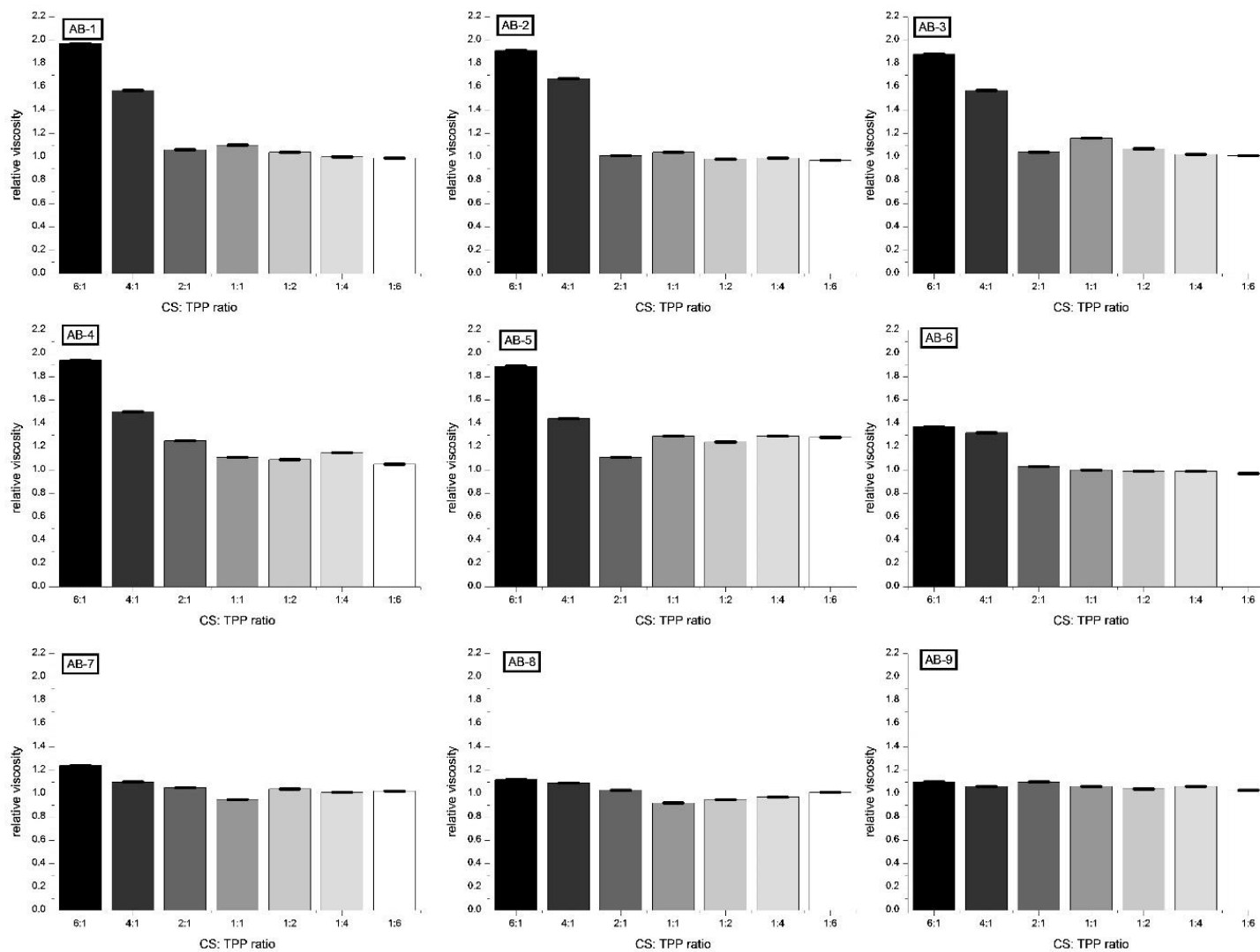
333 In this study CS: TPP microparticles formed by ionotropic gelation, were prepared at various  
334 ratios, (loaded with red dye for visualisation purposes), by the mixing CS solution with TPP  
335 solution under stirring. The particles formed at each ratio were shown to have different  
336 chemical and physical properties (**Figure 4 A-G**). As can be seen in **Figure 4**, microparticles  
337 prepared with AB-12 at the higher CS: TPP ratios and therefore at higher viscosity and surface  
338 charge had more porous surfaces than those of microparticles prepared with the lower CS: TPP  
339 ratios which had irregular angular surfaces, this is expected to have an influence strength of  
340 interaction and therefore integrity of the particle “walls” and therefore their size and shape [43].  
341 The availability of TPP is of course limited at high chitosan ratios and in excess in those with  
342 lower chitosan ratios and this influences the cross-linking density which again has an effect on  
343 size, shape and morphology of the particles [43]. Furthermore, although it may appear as  
344 though some of the particles are fragments of precipitated chitosan this is not the case as this  
345 inconsistent with both the FT-IR and XRD data above. In terms of potential applications of  
346 non-spherical particles it has been previously reported that flake-like metal particles  
347 (aluminium, copper, *etc.*) are more effective than spherical particles in latent fingerprint  
348 development [8] due to increased surface: volume ratios [7], therefore samples with a 2:1 CS:  
349 TPP ratio were used for further forensic studies in latent fingerprint visualisation with  
350 encouraging results (Hejjaji, Smith and Morris, submitted), this will depend on total particle  
351 surface area, which ranges from  $\sim 7000 \mu\text{m}^2$  (**Figure 4D**) to  $>30000 \mu\text{m}^2$  (**Figure 4A**) and on  
352 the number of particles per unit area. In the case of the irregular particles previous research has  
353 shown that the shape of CS: TPP microparticles depends on the pH at which chitosan and TPP  
354 are mixed and the molecular weight (viscosity) of the chitosan [44], furthermore in terms of  
355 pharmaceutical applications irregular particles with angular features have been shown to  
356 decrease drug dissolution [45], have a higher drug loading efficiency [46], influence  
357 phagocytosis [47] and to have a greater probability of adhering to cancer cell surfaces [48]  
358 which suggests that CS: TPP microparticles formed in this way may be have potential in drug  
359 delivery formulations. Although due to the inherent difficulties in measuring particles with  
360 different morphologies the impact of shape has not been studied to a great extent.



361  
 362 **Figure 4.** Example SEM images at 20 kV of chitosan microparticles CS: TPP using AB-12 (A)  
 363 6:1, (B) 4:1, (C) 2:1, (D) 1:1, (E) 1:2, (F) 1:4 and (G) 1:6. Where the scale bar is 100  $\mu\text{m}$  and  
 364 the estimated total surface areas of the particles are approximately  $\sim 30000$ , 14000, 8000, 7000,  
 365 8000, 32000 and 15000  $\mu\text{m}^2$ , respectively.

366  
 367 The relative viscosity of the CS: TPP microparticle suspension is shown in **Figure 5A**, which  
 368 indicates that neither pH nor ionic strength have a large influence the relative viscosity at ratios  
 369 CS: TPP 1:6, 1:4, 1:2, 1:1 and 2:1. It can be attributed to its lesser resistance towards flow due  
 370 the relatively low charge on chitosan microparticles. At higher ratios (4:1; 6:1), the relative  
 371 viscosity is higher with an increase in the CS: TPP ratio in the mixture. Moreover, at the fixed  
 372 pH value and different ionic strength (0.1, 0.3 and 0.5 M), the relative viscosity increased with  
 373 a decrease in ionic strength. This behaviour may arise because of the decrease in the repulsion  
 374 force between charges for the solvent and polymers and not unsurprisingly is dominated by the  
 375 amount of chitosan in the microparticles.

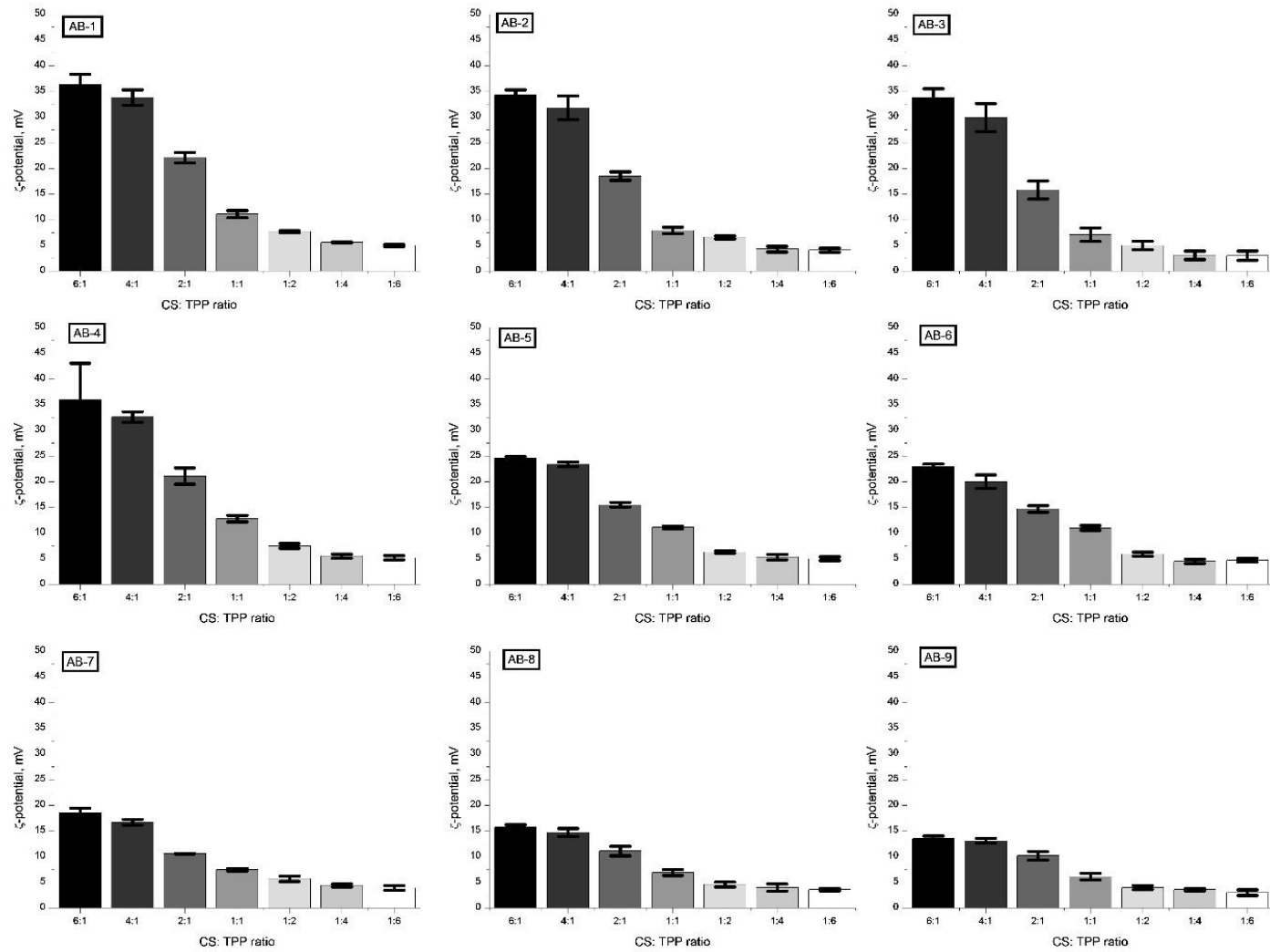




376

377 **Figure 5A.** Relative viscosities of CS: TPP microparticles solutions (using AB-1 to AB-9) at varying ionic strength and pH values at  $25.0 \pm 0.1$

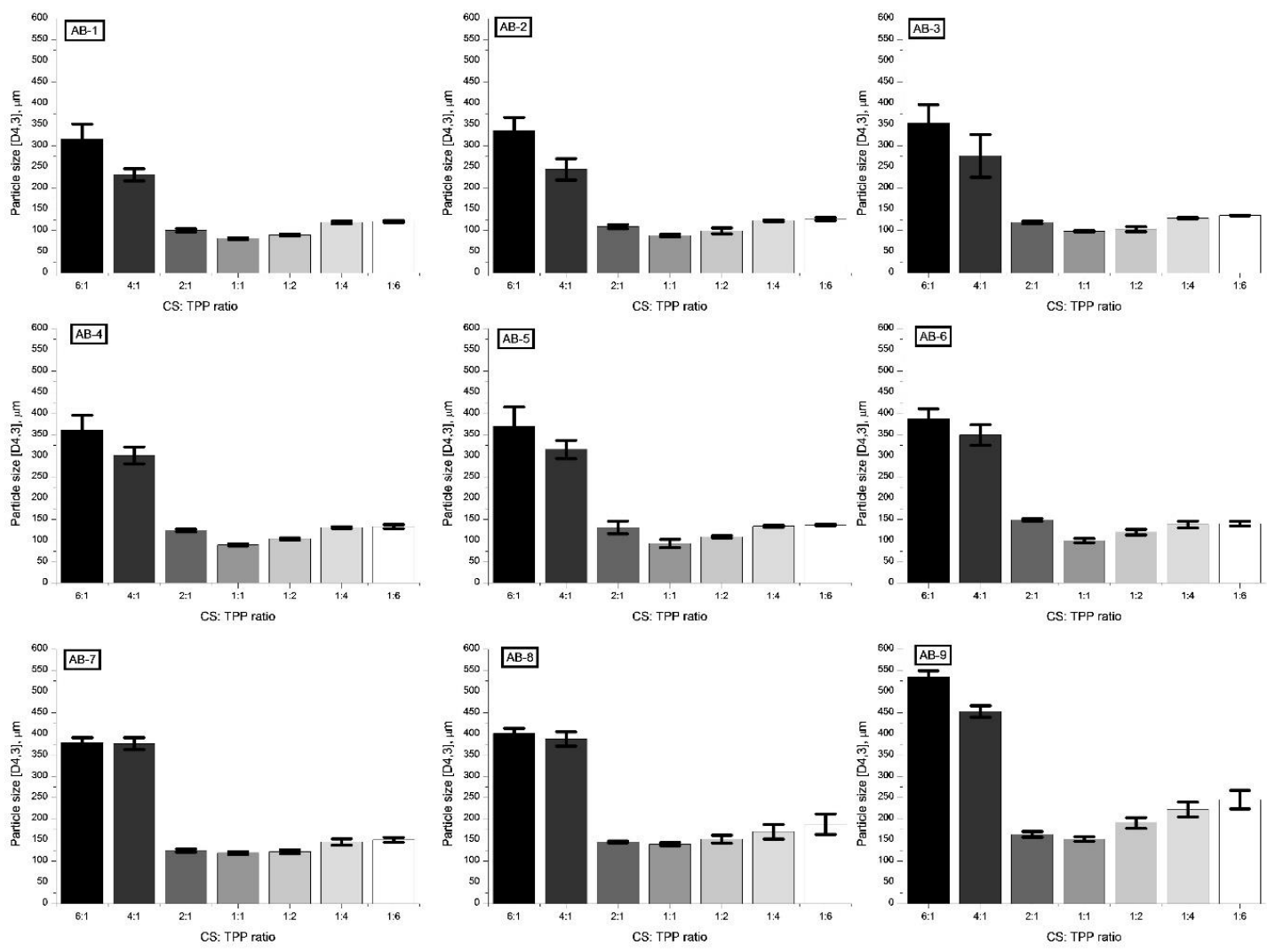
378  $^{\circ}\text{C}$  (mean  $\pm$  STDEV,  $n = 3$ ).



379

380 **Figure 5B.** Zeta potentials of CS: TPP microparticles solutions (using AB-1 to AB-9) at varying ionic strength and pH values at  $25.0 \pm 0.1$  °C

381 (mean  $\pm$  STDEV, n = 3).



382

383 **Figure 5C.** Particle size ( $D_{[4,3]}$ ) of CS: TPP microparticles solutions (using AB-1 to AB-9) at varying ionic strength and pH values at  $25.0 \pm 0.1$

384  $^{\circ}\text{C}$  (mean  $\pm$  STDEV,  $n = 3$ ).

385 The effect of ionic strength and pH value on the zeta potential of nine chitosan microparticle  
386 formulations was investigated as shown in **Figure 5B**. When chitosan and TPP were mixed  
387 with each other in an acetate buffer, they spontaneously formed microparticles (diameters were  
388 in the range 28-445  $\mu\text{m}$ ) with an overall positive surface charge which are at least partially  
389 within the size range of particles which have been demonstrated to be effective in latent  
390 fingerprint visualisation  $\sim 1\text{-}50\ \mu\text{m}$  [8] and may have potential in pulmonary [40] or colonic  
391 drug delivery systems[49] . The more positively or negatively charged the particles, the more  
392 they repel each other and therefore at values of  $\pm 30\ \text{mV}$  are required for optimal stability [50].  
393 As the CS: TPP ratio decreased from 6:1 to 1:6 the zeta potential values decreased from for  
394 example 36.4 mV to 5 mV in buffer AB-1 or from 13.5 mV to 3 mV in buffer AB-9 (**Figure**  
395 **5B**). It was also observed that with a decrease in the concentration of chitosan the appearance  
396 of the system changed from clear viscous liquid to milky dispersion prior to precipitation.

397

398 It was demonstrated that, there was no significant difference in the zeta potential values of CS:  
399 TPP from 1:2 to 1:6, indicating neutralization of the protonated amino groups on the surface  
400 of chitosan microparticles and subsequent loss of repulsive force which led to precipitation of  
401 the particles. On the other hand, as the CS: TPP ratio increased from 1:2 to 6:1 the zeta potential  
402 increased almost linearly. The large positive surface charge due to the high degree of  
403 deacetylation and protonation causes the chitosan molecules to have a large number of potential  
404 cross-linking sites. The presence of higher positive charge on the particles indicated that free  
405 (non-cross-linked) amino groups remained on the particle surface [35, 51] which is consistent  
406 with an increased viscosity in solution.

407

408 When the CS: TPP ratio was high at 6:1 and 4:1 (the available quantity of TPP was small) the  
409 reaction solution was clear, indicating that the amount of phosphate groups was inadequate to  
410 lead to the full cross-linking with the chitosan amino groups [52]. As the CS: TPP ratio  
411 decreased from 6:1 to 1:1, the particle size decreased due to increased intramolecular and  
412 intermolecular cross-linking density between chitosan amino groups and the TPP groups  
413 (**Figure 5C**), this is also due to the decrease in viscosity (**Figure 5A**) which leads weaker  
414 networks and therefore assuming there is no change in shear forces (stirring rate was constant  
415 at 600 rpm in all cases) smaller particles [41]. It can be inferred that chitosan molecules were  
416 almost fully cross-linked at CS: TPP (1:1), which coincided with the smallest particle size range  
417 measured. As the CS: TPP ratio decreases further from 1:1 to 1:6 the particle size increased, as

418 more TPP molecules are involved in the formation of the microparticles. This increased  
 419 concentration of TPP promotes aggregation due to inter-particle cross-linking (bridging  
 420 effects) which leads to a lower surface charge density of the particles resulting in precipitation  
 421 [52]. As we can see in **Figure 4** the CS: TPP microparticles are in some cases non-spherical,  
 422 with aspect ratios ranging from 1:1 to 13:1 and as particle size analysis treats particles as  
 423 equivalent spheres there is potential for minor discrepancies in the absolute particle sizes, these  
 424 are expected to be minimal although this will depend on the type of material being measured  
 425 [53].

426

427 Using multiple regression analysis, the responses (relative viscosity, zeta potential and particle  
 428 size) were correlated with the three variables studied using second-order polynomials. The  
 429 coefficients of the model equation and their statistical significance were evaluated using  
 430 Minitab® 17.1.0 software (Minitab Inc., Philadelphia, U.S.A.). The regression model for the  
 431 responses to relative viscosity ( $Y_1$ ), zeta potential ( $Y_2$ ) and particle size ( $Y_3$ ) in terms of coded  
 432 factors is given by Equations 2 - 4 respectively.

433

$$434 \quad Y_1 = -0.251 + 0.575 X_1 - 0.136 X_2 + 0.3315 X_3 - 0.0631 X_1^2 + 0.008 X_1X_2 - 0.0676 X_1X_3 \\ 435 \quad + 0.232 X_2^2 - 0.178 X_2 X_3 + 0.01397 X_3^2 + 0.0213 X_1X_2X_3 \quad (2)$$

436

$$437 \quad Y_2 = -25.54 + 14.89 X_1 - 35.8 X_2 + 15.00 X_3 - 1.812 X_1^2 + 6.88 X_1X_2 - 1.606 X_1X_3 \\ 438 \quad + 16.5 X_2^2 + 2.32 X_2 X_3 - 0.5282 X_3^2 - 1.446 X_1X_2X_3 \quad (3)$$

439

$$440 \quad Y_3 = 299 - 98.3 X_1 - 271 X_2 - 9.9 X_3 + 12.4 X_1^2 + 54.5 X_1X_2 + 4.23 X_1X_3 + 167 X_2^2 - 12.2 X_2 X_3 \\ 441 \quad + 5.50 X_3^2 + 7.1 X_1X_2X_3 \quad (4)$$

442

443 The equations were applied the response, to describe the principal effects and interactions  
 444 amongst the identified variables pH ( $X_1$ ), ionic strength ( $X_2$ ) and ratio ( $X_3$ ).

445

446 The coefficients with one factor represent the effect of the particular factor, while the  
 447 coefficients with two factors, three factors and those with second order terms represent the  
 448 interaction between the two factors, three factors and quadratic effect, respectively. The  
 449 positive sign in front of the terms indicates synergistic effect, while negative sign indicates  
 450 antagonistic effect on the responses.

451

452 **3.5. Model validation of relative viscosity, zeta potential and particle size**

453 Four different chitosan microparticles formulations were prepared in different acetate buffers  
454 including AB-10, AB-11, AB-12 and AB-13. The relative viscosities and zeta potential of the  
455 four chitosan microparticles were measured (**Table 2**). The regression equations were obtained  
456 for equations 2 - 4 which suggests the empirical relationship between the value of response and  
457 the independent variable. Therefore, the predicted values were calculated using mathematical  
458 model from equations 2 - 4.

459

460 For validation of relative viscosity, zeta potential and particle size results, the experimental  
461 values of the responses were compared with that of the predicted values **Table 2**. Moreover,  
462 **Table 2** indicates that ionic strength, pH and Chitosan: TPP ratio are suitable in predicting  
463 viscosity, zeta potential and particle size due to a high values of  $r^2$ , ( $r^2 = 0.91$ ,  $r^2 = 0.96$  and  
464  $0.86$  respectively) and can therefore be used in future studies to design tuneable microparticles  
465 for specific applications.

466

467 **Table 2.** Observed (Exp.) responses and predicted (Pred.) values for relative viscosity ( $Y_1$ ),  
 468 zeta potential ( $Y_2$ ) and particle size ( $Y_3$ )

Dependant Variables			$Y_1$ Relative viscosity		$Y_2$ Zeta potential (mV)		$Y_3$ : Particle size [D <sub>4,3</sub> ] ( $\mu\text{m}$ )	
$X_1$ pH	$X_2$ I.S.	$X_3$ (CS:TPP) Ratio	Exp.	Pred.	Exp.	Pred.	Exp.	Pred.
3.8	0.2	6:1	1.66 ± 0.01	1.85	35.2 ± 1.3	34.2	354 ± 40	351
3.8	0.2	4:1	1.34 ± 0.01	1.46	29.7 ± 1.1	28.2	226 ± 21	223
3.8	0.2	2:1	1.07 ± 0.01	1.18	19.0 ± 1.6	18.0	135 ± 6	139
3.8	0.2	1:1	1.11 ± 0.01	1.08	11.8 ± 0.9	11.3	111 ± 3	113
3.8	0.2	1:2	1.05 ± 0.01	1.04	8.5 ± 1.3	7.6	119 ± 2	104
3.8	0.2	1:4	1.04 ± 0.01	1.03	6.1 ± 0.9	5.6	124 ± 3	101
3.8	0.2	1:6	1.04 ± 0.01	1.02	5.8 ± 0.4	5.0	128 ± 6	100
3.8	0.4	6:1	1.66 ± 0.01	1.74	32.6 ± 2.9	30.4	379 ± 49	376
3.8	0.4	4:1	1.45 ± 0.01	1.38	27.0 ± 2.7	25.7	248 ± 41	242
3.8	0.4	2:1	1.04 ± 0.01	1.15	17.0 ± 0.6	16.8	146 ± 5	152
3.8	0.4	1:1	1.00 ± 0.01	1.07	10.0 ± 0.7	10.8	121 ± 2	123
3.8	0.4	1:2	1.02 ± 0.01	1.04	8.0 ± 1.2	7.3	129 ± 5	113
3.8	0.4	1:4	1.02 ± 0.01	1.03	6.0 ± 1.4	5.5	132 ± 4	109
3.8	0.4	1:6	1.02 ± 0.01	1.02	4.3 ± 0.7	4.9	135 ± 5	108
4.8	0.2	6:1	1.45 ± 0.01	1.50	21.3 ± 0.4	23.5	407 ± 50	404
4.8	0.2	4:1	1.24 ± 0.01	1.24	19.9 ± 0.8	21.3	267 ± 31	265
4.8	0.2	2:1	1.09 ± 0.01	1.08	14.3 ± 0.6	14.9	171 ± 4	169
4.8	0.2	1:1	1.03 ± 0.01	1.05	9.7 ± 0.5	10.1	135 ± 2	138
4.8	0.2	1:2	0.98 ± 0.01	1.04	8.0 ± 0.1	7.3	138 ± 2	126
4.8	0.2	1:4	1.07 ± 0.01	1.04	6.9 ± 0.4	5.8	139 ± 2	122
4.8	0.2	1:6	0.97 ± 0.01	1.04	4.8 ± 0.1	5.4	142 ± 6	120
4.8	0.4	6:1	1.38 ± 0.01	1.42	18.6 ± 0.6	19.4	451 ± 31	445
4.8	0.4	4:1	1.21 ± 0.01	1.18	17.3 ± 0.1	19.0	306 ± 29	300
4.8	0.4	2:1	1.06 ± 0.01	1.06	10.3 ± 0.3	14.5	194 ± 11	196
4.8	0.4	1:1	1.02 ± 0.01	1.04	9.0 ± 0.5	10.6	158 ± 8	161
4.8	0.4	1:2	0.98 ± 0.01	1.05	6.8 ± 0.3	8.3	164 ± 10	147
4.8	0.4	1:4	1.02 ± 0.01	1.05	5.7 ± 0.2	7.0	167 ± 23	141
4.8	0.4	1:6	0.98 ± 0.01	1.05	4.2 ± 0.5	6.6	171 ± 15	139

469

#### 470 **4. Conclusions**

471 In this study, chitosan microparticles of different morphologies were successfully formed by  
 472 the ionotropic gelation method at different CS: TPP ratios and pH/Ionic strength conditions.  
 473 The particles were characterized by relative viscosity, zeta potential, particle size, FTIR  
 474 spectroscopy and XRD. Using experimental design, the relative viscosity, particle size and zeta  
 475 potential of CS: TPP microparticles under different conditions could be predicted using the

476 mathematical models. The mathematical models obtained showed good relationships between  
477 independent variables (pH, ionic strength and CS: TPP ratio) and dependent variables (relative  
478 viscosity, zeta potential and particle size) for prediction. This gives us the ability to design  
479 tuneable CS-TPP microparticles for specific pharmaceutical or forensic applications more  
480 specifically latent fingerprint visualisation.

481

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485 **6. References**

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