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Chitosan-pectin hybrid nanoparticles prepared by coating and blending techniques

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34 ABSTRACT

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The preparation of chitosan nanoparticles in combination with pectins, as additional mucoadhesive 36 biopolymers, was investigated. Pectin from apple and from citrus fruit were considered; 37 polygalacturonic acid was taken as a reference. Tripolyphosphate was used as an anionic cross-38 linker. Two different techniques were compared, namely the coating and the blending. Coated 39 nanoparticles (NPs) in the ratio pectin:NPs from 2:1 to 5:1 evidenced that the size of NPs increased 40 as the amount of pectin (both from apple and citrus fruit) was increased. In particular, for NPs 41 coated with pectin from citrus fruit the size ranges from 200 to 260 nm; while for NPs coated with 42 pectin from apple the size ranges from 330 to 450 nm. A minimum value of Z-potential around -35 43 mV was obtained for the ratio pectin:NPs 4:1, while further addition of pectin did not decrease the 44 Z-potential. Also blended NPs showed a dependence of the size on the ratio of the components: for 45 46 a given ratio pectin:tripolyphosphate the size increases as the fraction of chitosan increases; for a low ratio chitosan:pectin a high amount of tripolyphosphate was needed to obtain a compact 47 48 structure. The effect of the additional presence of loaded proteins in chitosan-pectin nanoparticles was also investigated, since proteins contribute to alter the electrostatic interactions among charged 49 species. FT-IR and DSC characterization are presented to confirm the interactions between 50 biopolymers. Finally, the biocompatibility of the used materials was assessed by the chorioallantoic 51 membrane assay, confirming the safety of the materials. 52

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54

55 **Keywords:** Chitosan; pectin; hybrid nanoparticles; ionotropic gelation; coating and blending 56 technique.

57

59 1. INTRODUCTION

Microspheres and microcapsules based on chitosan were developed for pharmaceutical applications 60 and the importance of mucoadhesive properties for site specific drug delivery were described (He et 61 al., 1998). The great potentiality of the use of chitosan as drug carrier is amply recognized. Various 62 techniques have been described in literature for the preparation of chitosan microcarriers, as well as 63 the parameters affecting drug release (Sinha et al., 2004). Different forms of chitosan based 64 nanomaterials have been also reported in recent reviews (Borgogna et al., 2011; Shukla et al., 65 2012). The nanotechnological approach for the development of nanoparticle-based drug delivery 66 systems has gained increase attention in the recent years (Desai, 2012). Several examples are 67 reported in literature on the use of chitosan nanoparticles. More recently, rivastigmine loaded 68 69 chitosan nanoparticles were investigated for intranasal delivery in case of Alzheimer's disease (Fazil et al., 2012); verapamil HCl loaded chitosan microspheres were studied for intranasal 70 71 administration (Abdel Mouez et al., 2014); gemcitabine loaded in chitosan NPs was studied for oral delivery by Derakhshandeh and Fathi (2012). 72

73 One of the widely used techniques for the preparation of chitosan nanoparticles (NPs) is the ionic gelation, which is relatively simple and mild; it allows the successful encapsulation of labile 74 molecules, such as proteins, since it avoids the use of organic solvents and high temperatures (Al-75 Qadi et al., 2012; Berger et al., 2004; Nasti et al., 2009; Xu and Du, 2003). The preparation of 76 chitosan NPs following the method described by Calvo et al. (1997), was recently investigated 77 focusing the attention on the effect of the ratio between chitosan and TPP (tripolyphosphate) on the 78 loading of protein, being all charged species (Rampino et al., 2013). This study and other literature 79 results (Papadimitiou et al., 2008; Bagre et al., 2013) show that small nanoparticles are obtained 80 with a ratio of chitosan to TPP of 5:1. Moreover, chitosan is widely used for the preparation of 81 several carriers due to its bioadhesive properties, its film forming abilities and low toxicity as well 82 as its abundance in nature. Chitosan can interact with the constituents of the mucosal glycoprotein 83 layer thus prolonging the residence time at the absorption site, increasing drug bioavailability 84 (Sogias et al., 2012). The basic mechanisms of mucoadhesion have been reported and extensively 85 86 commented (Andrews et al., 2009; Serra et al., 2009).

Pectin is another natural polysaccharide of pharmaceutical interest, whose properties mainly depend on the esterification degree. Its increasing use in the pharmaceutical field is due to its high availability in nature, its low or non toxicity nature and above all its mucoadhesive properties together with resistance to degradation by proteases and amylases. Such features make pectin attractive for the formulation of drug delivery carriers for many administration routes (Marras-

Marquez et al., 2015). In particular, the correlation between the mucoadhesive properties and the 92 degree of methoxylation of different types of pectin has been investigated by Hagesaether et al. 93 (2008). The same authors paid attention also to investigate the effect of formulation, i.e., when 94 specific ions are added to the pectin solution. Indeed, on one side cross-linking can reduce the 95 polymer mobility, therefore hampering its diffusion and interpenetration within mucin molecules; 96 on the other side, particles of very small sizes have better potential to penetrate the mucus layer. In 97 general, optimal size characteristics are required in order to achieve mucoadhesion by mean of the 98 increased residence time and closer contact with mucosa. The possibility of obtaining hybrid 99 100 nanoparticles, containing not only chitosan and TPP, but also other poly- or oligo-saccharides has been described in literature. Such hybrid systems are characterized by improved physical properties 101 and better performances when used as drug delivery carriers (Goycoolea et al., 2009 and references 102 therein). 103

The aim of this work was the investigation of two techniques, named coating and blending, for thepreparation of hybrid pectin-chitosan nanoparticles.

106 Characterization of PEC properties in terms of size, charge, and surface morphology, shows the strict dependence on the macromolecular parameters of the polyions used (in addition to 107 108 concentration, ionic strength, pH) and on the operative mixing conditions (interaction under resting or streaming mixing). For linear polymers the charge density (number of charges per unit length) 109 defines not only the value for counterion condensation, but also the conditions for chain pairing. 110 These comments may help the understanding of pectin-chitosan interaction with the co-presence of 111 TPP, under the hypothesis of thermodynamic equilibrium. The practical effects of addition of pectin 112 are investigated following the coating technique (post-synthesis of NPs) or the blending technique 113 114 (during synthesis of NPs).

On one side the coating technique allows to create a core consisting of a polymer and a protein, thus ideally the protein is more protected from the outside environment due to the presence of an outer shell obtained by subsequent coating with a polymer. This is not presumably obtained by a blending technique where the protein is dispersed throughout the polymeric matrix. On the other side one of the main advantages of the blending technique is the possibility of the one-step formulation, that is not possible with the coating technique.

121 Thus, it has been possible to exploit the well known capability of chitosan to form nanoparticles, to 122 obtain a drug delivery system based on the combination of two classes of mucoadhesive 123 biopolymers.

124 2. MATERIALS AND METHODS

125

126 **2.1 MATERIALS**

Low molecular weight (LMW) chitosan (MW 150 kDa; $[\eta] = 2.37$ dL/g; degree of acetylation DA 127 = 13%), polygalacturonic acid from orange, MW 18 kDa, degree of esterification (DE) 10,6% 128 (Cesàro et al., 1982), pectin from citrus fruit (MW 17 kDa, DE 22%), pectin from apple (MW 30-129 100 kDa, degree of esterification 71%), bovine serum albumin (BSA), albumin from chicken egg 130 albumen (OVA), technical grade pentasodium tripolyphosphate (TPP), sodium acetate, sodium 131 132 hydroxide, and sodium chloride were all purchased from Sigma-Aldrich Co. (St. Louis, Missouri, USA). Acetic acid and hydrochloric acid were obtained from Carlo Erba Reagents (Carlo Erba, 133 134 Milan, Italy). All other chemicals were of the highest purity grade commercially available and used without further purification. 135

The commercial chitosan sample was purified and characterized as reported elsewhere (Donati et al., 2005). The intrinsic viscosity of chitosan was measured by employing a Schott–Geräte AVS/G automatic apparatus and an Ubbelohde type viscometer (in acetate buffer 0.25 M, pH 4.7), as reported in the previous paper (Rampino et al., 2013).

140

141 **2.2 METHODS**

142 2.2.1 Nanoparticles preparation

143 Chitosan nanoparticles (NPs) were prepared using the ionotropic gelation method (Calvo et al., 144 1997; Rampino et al., 2013). A 0.25% w/v chitosan solution was prepared by dissolving LMW 145 chitosan in 0.05% v/v acetic acid solution for 24 hours under stirring. The pH of the solution was 146 adjusted to 5.5 with a sodium hydroxide solution while deionized water was added to obtain the 147 desired final concentration. TPP was dissolved in deionized water at a concentration of 0.25% (w/v) 148 and subsequently diluted to obtain solutions at different concentrations.

The TPP and chitosan solutions were filtered through a 0.45 μm mixed cellulose esters membrane
(Millipore, Massachusetts, USA) to remove any insoluble matter. TPP solution was added drop
wise to the chitosan solution under magnetic stirring at room temperature (Rampino et al., 2013).

152 Chitosan NPs have been loaded with two different model proteins: BSA and OVA, whose 153 isoelectric points are 4.8 and 4.7, respectively. The protein were dissolved in deionized water 154 (concentration 4 mg/mL) and added directly to the chitosan solution under magnetic stirring. 155 Batches with different theoretical loading were prepared by adding different volumes of the protein 156 stock solution, to obtain final protein concentrations of 200, 400, and 600 µg/mL. The solution, 157 containing chitosan and protein, was then diluted to a final volume of 5 mL using deionized water. 158 After dropping the TPP solution, the dispersion was left under constant stirring for 30 min at room 159 temperature. The suspension was centrifuged for 2 hours at 3270 RCF to remove the excess of 160 chitosan and protein. The supernatant was collected separately while the sedimented particles were 161 re-dispersed in deionized water, analyzed for their size and surface charge and then lyophilized.

162

163 **2.2.2 Pectin-chitosan nanoparticles**

Each pectin sample was dissolved in deionized water adjusting the pH between 6 and 7, thus forming the sodium salt. Hybrid pectin-chitosan NPs were prepared following two different procedures named coating (Borges et al., 2005) and blending (Alonso et al., 2006).

167 *Coating*

LMW Chitosan NPs suspension was added to a pectin solution drop wise at different ratios under magnetic stirring at room temperature. The suspension of coated particles was centrifuged for 2 h at 3270 RCF and the supernatant was discarded. Particles were re-dispersed in deionized water, characterized and freeze dried. Protein loaded NPs were prepared according to the procedure reported for sole chitosan NPs (Rampino et al., 2013). Pectin-coated NPs were prepared by using sodium polygalacturonate, pectin from citrus fruit and pectin from apple.

174 Blending

175 Chitosan and TPP solutions were prepared as previously described. TPP solution was mixed under 176 magnetic stirring to pectin solution at different volume ratios. NPs formed spontaneously upon drop 177 wise addition of the cationic solution to the anionic one under stirring. NPs were isolated by 178 centrifuging at 3270 RCF for 2 h, the supernatant was discarded and the pellet re-dispersed in 179 deionized water, characterized and freeze dried. Loaded NPs were prepared dissolving the model 180 protein directly in the anionic solution containing TPP and pectin, and then the particles were 181 prepared as previously described. Blended NPs were prepared by using pectin from apple.

182

183 2.2.3 Nanoparticle characterization: size and zeta potential

The size of the NPs and the surface zeta potential were determined using a Malvern Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK). Measurements were carried out at 25 °C in replicate of three.

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- 189

190 **2.2.4 Determination of protein loading efficiency**

- Six BSA and OVA standard solutions at different concentrations were prepared using a known 191 amount of protein and analyzed with a bicinchoninic acid protein assay kit (BCATM) Sigma-Aldrich 192 Co. (St. Louis, Missouri, USA) to build a calibration curve. 25 µL of each standard and sample 193 were placed into a 96-well microplate and 200 µL of reagent was added. Each analysis was 194 performed in a replicate of three. The microplate was incubated at 37 °C for 30 minutes after being 195 thoroughly mixed for 30 seconds by a microplate shaker. The microplate was then cooled to room 196 temperature, and the absorbance measured on a plate reader (Pierce, Illinois, USA). 197 198 Spectrophotometric measurements were carried out at 562 nm, which does not overlap with polymer spectra. Unloaded NPs were used as control. 199
- The amount of protein loaded in NPs was calculated as the difference between the protein total amount and the protein recovered in the supernatant after centrifugation, assuming that the amount not found in the supernatant was encapsulated in the NPs.
- The loading efficiency experiments were carried out on native NPs containing chitosan:TPP 5:1 w/w, NPs coated with pectin from apple in the ratio from 2:1 to 5:1, loaded with BSA. Pectin from apple was used in blended NPs with a pectin:TPP ratio 4:1 w/w and a ratio between chitosan and the anionic components (pectin, TPP and protein) of 1.2:1 and 2:1, loaded with OVA.
- 207

208 2.2.5 Fourier transform-infrared (FT-IR) spectroscopy

- IR spectra were recorded on lyophilized samples using a Vertex 70 (Bruker Optics GmbH) spectrophotometer (spectral resolution of 4 cm⁻¹) equipped with a MIRacleTM ATR devices (Pike Optics) with a single reflection diamond crystal (1.8 mm spot size) and using a MCT detector (HgCdTe, mercury-cadmium-tellurium) cooled with liquid nitrogen.
- Samples of the raw materials and freeze dried NPs were placed on the top of the diamond crystal and stopped with a high-pressure clamp. Spectra were recorded in the range 5000-600 cm⁻¹.
- FT-IR investigations were carried out on starting materials, chitosan:TPP NPs, coated NPs with pectin from apple and citrus fruit, blended NPs with pectin from apple.
- 217

218 **2.2.6 Differential Scanning Calorimetry (DSC)**

DSC analysis of raw materials, freeze dried uncoated, coated and blended pectin-chitosan NPs was
carried out by using a PerkinElmer[®] DSC 6 calorimeter (PerkinElmer, Massachusetts, USA).
Samples (about 2 mg) were accurately weighed into open aluminum pan and heated from 20 °C to
350 °C at a scanning rate of 10 °C/min under a nitrogen flow of 20 mL/min.

DSC investigations were carried out on on starting materials, chitosan:TPP NPs, coated NPs with pectin from apple and citrus fruit, blended NPs with pectin from apple.

225 2.2.7 Chorioallantoic membrane assay

The safety of raw materials (chitosan and pectin from apple), chitosan NPs and chitosan NPs coated 226 with pectin from apple in the ratio pectin:NPs 2:1 w/w was evaluated in vivo by using the chick 227 embryo chorioallantoic membrane (CAM) assay (Saw et al., 2008; Schoubben et al., 2013). 228 Fertilized eggs were disinfected with alcohol 70° and placed in an incubator at 38 °C with 60% 229 relative humidity. At incubation day 3, a window opening was punctured at the blunt end of the egg 230 and living embryos were selected for the experiment. The opening was then covered with a 231 polyethylene film glued with albumen to avoid water loss and microbial contamination. At 232 incubation day 6, solid samples (6 mm tablet) were applied directly on the CAM. A Leica WILD 233 M32 stereomicroscope (equipped with a WILD PLAN 1X lens), connected to a Leica DFC 320 234 camera system, was used to follow the evolution of any effect on the materials on the CAM. After 235 24 hours, all the eggs were examined again and all the acquired images were compared with those 236 at time 0 and with the controls for qualitative acute toxicity evolution (Vargas et al., 2007). 237

239 **3. RESULTS AND DISCUSSION**

240

3.1. Chitosan and pectin samples used in nanoparticle preparation.

LMW chitosan NPs, produced following a previous optimized methodology, had a mean size and 242 surface charge of 200 ± 24 nm and 25 ± 3 mV, respectively (Rampino et al., 2013). The production 243 of hybrid pectin-chitosan NPs as drug delivery systems was investigated in view of the well-known 244 mucoadhesive properties of pectins. The pectin samples were characterized by different values of 245 molecular weight and degree of esterification (MW and DE, respectively): polygalacturonic acid 246 (18 kDa and 10.6%), pectin from citrus fruit (17 kDa and 22%), pectin from apple (30-100 kDa and 247 71%). These macromolecular properties of the pectins and of chitosan sample used (MW 140 kDa 248 and DE 13%) in addition to the coating or blending procedure used, govern and control the final 249 characteristics of the hybrid NPs that can be effectively defined as poly-electrolyte complexes 250 251 (PEC).

252

253 **3.2.** Pectin-chitosan nanoparticle preparation and characterization

254 **3.2.1. Coating technique**

255 Chitosan NPs, obtained using a chitosan:TPP ratio of 5:1 w/w, were centrifuged, re-dispersed and 256 added to the pectin solution. Since chitosan NPs are characterized by a net positive surface charge 257 and pectin is negatively charged at the pH (6-7) employed for the coating, the electrostatic 258 interaction between opposite charges favors the complexation of pectin on the NPs outer surface. 259 This is seen by the dependence of NPs size and Z-potential on the degree of esterification, 260 molecular weight and concentration of the pectin used, as reported in **Fig. 1**.



Fig. 1: mean size (a) and Z-potential (b) of chitosan NPs (chitosan:TPP ratio 5:1 w/w) coated with sodium polygalacturonate (\blacksquare), pectin from citrus fruit (\bullet) and pectin from apple (\blacktriangle).

265

In all cases, an increase of the mean size is observed and, supposing that pectin is confined in the outer layer (coating), the thickness of the coating depended on the type and amount of pectin used. A remarkable size increase was recorded when apple pectin was used to coat chitosan NPs (**Fig. 1a**), while a smaller increase in NPs size is produced with sodium polygalacturonate or citrus pectin. The behavior of apple pectin coating is clearly due to its higher MW and higher DE as compared with the other two pectin samples that show similar behavior due to the quite similar values of molecular weight and charge density of the two macromolecules.

As a further general comment, the larger the amount of pectin, the higher was the increase in particle size. However, this effect is particularly evident for the apple pectin, since the presence of a higher amount of low-charged polymer increases the coating thickness. As a speculative observation (to be confirmed with the other results here reported), a more swollen coating wouldoccur with apple pectin that has the highest DE.

As the pectin concentration is concerned, at pectin:NPs ratio less than 2:1, both apple and citrus pectins led to the formation of aggregates (data not shown), probably because of the low surface charge. Indeed, upon increasing pectin concentration the surface charge of NPs changes from positive to slightly negative producing particles with a Z-potential of -6 mV (data not shown), while for NPs stability a high charged surface is required to avoid their aggregation. It has been shown that such a low absolute value of Z-potential does not produce an efficient electrostatic repulsion between NPs leading to aggregation (Gonzalez-Mira et al., 2010).

Fig. 1b reports the NPs surface charge as a function of the pectin:NP ratio. At low pectin 285 concentration (2:1) the value of Z-potential is very negative for sodium polygalacturonate (ca -45 286 mV), while for citrus pectin and for apple pectin is about -25 and -15 mV, respectively. Upon 287 288 increasing pectin concentration, no changes in Z-potential are observed with sodium polygalacturonate, while both apple and citrus pectins reached a minimum value of about -35 mV at 289 290 pectin:NPs ratio of 4:1 (Fig. 1b). These findings suggest that an optimal level of coating (as derived from the value of Z-potential due to polymers complexation) can be identified and that a further 291 292 addition of pectin to NPs only produces an increase in size.

293

3.2.2. Blending technique

The preparation of NPs by blending technique implies that chitosan is added to the anionic solution of pectin containing TPP, with the important advantage of a one-step procedure. The effect of the amount of pectin on NPs size has been investigated both as a function of the mass ratio between the cationic (chitosan) and anionic (pectin+TPP) components, and as a function of the ratio between pectin and TPP (**Fig. 2**).

For a given ratio between pectin and TPP, it was generally observed a size increase as the mass 300 fraction of chitosan increased. A remarkable size increase was observed when a high amount of 301 pectin was used and the ratio between pectin and TPP was high. A concomitant reduction of 302 chitosan and pectin and an increase of TPP amount provoked a reduction of particle size. This 303 might be due to the strong effect of TPP that produced a compact structure with chitosan that 304 interacts with pectin. Thus, the dependence of the complexation stoichiometry on the reaction of the 305 $[-COO^{-}]$ and $[-NH_3^{+}]$ functional groups of both polyelectrolytes was established, expressed as the 306 ratio of the chitosan concentration in the NPs relative to the total pectin concentration. 307



Fig. 2: mean size of chitosan NPs blended with apple pectin in different ratios with TPP. Ratio pectin:TPP 1:1 (\blacksquare), 2:1 (\bullet), 4:1 (\blacktriangle), 6:1 (\blacktriangledown) and 8:1 (\bullet). The mean size of NPs in the absence of pectin is in the range of 200 nm.

309

For the same chitosan mass fraction, NPs with different size can be obtained depending on the ratio 314 between pectin and TPP. A low mean size of NPs was obtained for formulations with a low 315 pectin: TPP ratio. This is not surprising since a high concentration of TPP, available for ionic 316 complexation, produces a highly compact polyelectrolytes network. When the concentration of 317 pectin increased, the electrostatic interactions with less accessible -NH3⁺ groups resulted in the 318 formation of more swollen structures; this is due to the increase in the promotion of un-complexed 319 stretches of the pectin chains. A trend for NPs size can be recognized suggesting that the lowest size 320 321 of NPs (less than 300 nm) is obtained for chitosan mass fractions in the range between 0.3 and 0.5, and with pectin:TPP ratio 1:1 to 4:1. 322

A general decrease of the Z-potential was found as the amount of pectin was increased reaching values between -20 and -29 mV. For the same fraction of chitosan, NPs with different surface charge can be obtained depending on the ratio between pectin and TPP. For a given ratio, the lowest surface charge is obtained with the highest ratio pectin to TPP. This aspect is particularly importantin view of modulating NP surface properties.

As mentioned, the blending technique is preferred from the operational point of view since tedious steps, such as centrifugation and re-dispersion, are not necessary. In addition, the blending technique resulted suitable to prepare NPs even at chitosan:TPP ratio lower than 5:1 w/w, while this was not possible using the coating technique because of aggregation.

332

333 **3.3 Effect of protein loading**

The effect of two model proteins, BSA and OVA, was investigated since the addition of proteins to the formulation, either blending or coating, can further modify the equilibria established among chitosan, pectin and TPP, in relation to the charged groups on the protein surface (Yu et al., 2009). OVA and BSA are medium size globular protein with a molar mass of 45 and 66.5 kDa, respectively, a similar isoelectric point (4.9 and 4.7) and equal absolute negative charge of about -11*e* at pH 7. Therefore, the only difference would reside in the dimensions, being OVA 2/3 smaller that BSA (Stokes radius 3.1 and 3.5, respectively).

The loading efficiency of experiments were carried out on native NPs containing chitosan: TPP 5:1 341 w/w and NPs coated with pectin from apple in the ratio from 2:1 to 5:1. With the coating technique, 342 the loading efficiency for BSA was similar to that found for uncoated NPs (chitosan:TPP 5:1 w/w), 343 and ranging between 40% and 60% (Rampino et al. 2013), suggesting that the final step of coating 344 345 did not induce protein loss. Furthermore, while the presence of protein did not significantly increase the size of the NPs, however, a relevant increase of the dimensions was detected after the coating 346 347 with pectin. A direct correlation was found with the amount of pectin added, loaded NP mean diameter ranging from 700 nm to 1250 nm for ratio of pectin to NPs from 2:1 to 5:1 w/w. Given the 348 349 pI of OVA and BSA (both slightly negative charged), an electrostatic interaction occurs between the positively charged chitosan and the negatively charged proteins, therefore screening chitosan for 350 351 further interaction with pectin and producing much less compact polymer particles. Loaded NPs were characterized by Z-potential values slightly lower than unloaded NPs. In addition, the decrease 352 of the Z-potential was as pronounced as the loading increased. The subsequent addition of pectin 353 completely changes the surface charge from positive to negative. The Z-potential was around -38 354 mV, sufficient to maintain a permanent electrostatic repulsion and avoid the aggregation. 355

Pectin from apple was used in blended NPs with a pectin:TPP ratio 4:1 w/w and a ratio between chitosan and the anionic components (pectin, TPP and protein) of 1.2:1 and 2:1. Regarding the blending technique, experiments with OVA suggested a possible competition between pectin and ovalbumin. Indeed, the loading efficiency, ranging between 16% and 27%, was lower than the corresponding unblended; pure chitosan NPs had a loading efficiency between 48% and 76% (Rampino et al. 2013), depending on the initial amount. A decrease of the loading efficiency was found as the amount of pectin increased and minor effects were observed on NPs size (an increase of about 50 nm was observed), while the value of Z-potential was similar to that of unblended NPs.

364

365 **3.4 FTIR-ATR spectroscopic evidence for polymer interaction**

FT-IR investigations were carried out coated NPs with pectin from apple and citrus fruit and 366 blended NPs with pectin from apple; for comparison starting materials and native chitosan: TPP NPs 367 are also reported. The solid state studies (FT-IR and DSC) have been conducted on empty NPs. The 368 presence of a protein would have added another variable for the experiments. Chitosan spectrum 369 shows a broad absorption between 3350 and 3270 cm⁻¹ (Fig. 3a) previously attributed to a 370 combination of stretching modes of O-H and N-H bonds in chitosan and to hydrogen bonds among 371 polysaccharide chains. Fig. 3a shows that the same band becomes broader and shifted to lower 372 wavenumbers in the sample of chitosan NPs, thus indicating an enhancement of the hydrogen bonds 373 system (Mishra et al., 2008). The main peaks recognized for the chitosan sample were related to 374 C=O stretching amide I at 1635 cm⁻¹ and to amide II at 1539 cm⁻¹ (Woranuch and Yoksan, 2013). 375 These peaks slightly shifted to 1633 cm⁻¹ and to 1543 cm⁻¹ in the chitosan NPs sample, with an 376 increase of intensity for the latter. The presence of this intense band at 1543 cm⁻¹ is attributed to the 377 bond formation between the amino groups of chitosan and TPP (Azevedo et al., 2011). Similar 378 considerations were done by Xu and Du (2003) studying chitosan film treated with NaH₂PO₄ 379 (Knaul et al., 1999). Other characteristic bands of chitosan NPs are the peak at 1213 cm⁻¹ attributed 380 to the P=O of the TPP, the intense band around 1070 cm⁻¹ corresponding to C-O stretching and the 381 pyranose ring at 890 cm⁻¹ (Woranuch and Yoksan, 2013). 382

Fig. 3b-d reports the FTIR spectra of chitosan NPs coated with the three different pectin considered (sodium polygalacturonate, apple pectin and citrus fruit pectin) or blended with apple pectin. The spectra are reported in the range between 600 cm⁻¹ and 1800 cm⁻¹, since major changes occur within this range. The region 1000 - 1200 cm⁻¹ contains skeletal C-O and C-C vibration bands of glycosidic bonds and pyranose ring (Synytsya et al., 2003). The main changes were in the region 1500-1700 cm⁻¹ corresponding to the stretching vibrations of amide bond and indeed the interaction of chitosan with pectin involves amine groups on chitosan (not yet involved in bonds with TPP) and carboxylic groups of pectin (**Fig. 3b**).

Polygalacturonate shows only one peak around 1608 cm^{-1} ; the two characteristic peaks of chitosan 391 NPs disappeared giving only one peak at 1593 cm⁻¹ with a small shoulder after the coating with 392 polygalacturonate. A peak at 1410 cm⁻¹ was identified and attributed to C-OH stretching of the 393 carboxylic group (Synytsya et al., 2003), as similarly observed for alginate (Sarmento et al., 2006a). 394 Apple pectin and citrus fruit pectin (Fig. 3c-3d) are characterized by the C=O stretching at 1608 395 and 1600 cm⁻¹ respectively and by an additional peak, respectively at 1741 and 1743 cm⁻¹, 396 corresponding to the C=O stretching in the ester form (Synytsya et al., 2003). In the apple pectin 397 sample the two peaks of C=O stretching have comparable intensity, due to the high degree of 398 esterification. As similarly observed for the polygalacturonate, the coating with apple pectin and 399 citrus fruit pectin gives rise to a single peak, respectively at 1597 and 1591 cm⁻¹, due to the 400 presence of the pectin on the surface on the NPs, and a small shoulder, in both cases at 1535 cm⁻¹, 401 as already commented for the chitosan NPs formation. The peak around 1400 cm⁻¹, that was a 402 double peak in the chitosan NPs, becomes a single peak and it is shifted to 1410 cm⁻¹ after coating. 403 404 The peaks attributed to the stretching of C=O in the ester form are still visible, but less intense. The shifts observed in the two pectins vary only slightly from one to another, probably suggesting that 405 the different degree of esterification does not affect the number of interactions that are formed with 406 amino groups of chitosan. 407





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Fig. 3. FTIR spectra of a) chitosan (dash line) and chitosan NPs (full line); b) chitosan NPs (black full line), polygalacturonate (red dash line), polygalacturonate coated NPs (blue full line); c) chitosan NPs (black full line), apple pectin (red dash line), apple pectin coated NPs (blue full line) and apple pectin blended NPs (blue dot line); d) chitosan NPs (black full line), citrus fruit pectin (red dash line), citrus fruit pectin coated NPs (blue full line).

The effect of the shift is more pronounced for the sample coated with polygalacturonate. The important outcome is that the shift of these bands indicates a change in the environment of amino and carboxyl groups through the mutual interaction (Bigucci et al., 2008). From the presence of peaks different from those of the uncoated NPs it was possible to confirm the existence of a coating layer of pectin around the NPs, as also commented for alginate (Borges et al., 2005).

Fig. 3c reports the spectra of pectin-chitosan NPs obtained by blending technique. It has to be noticed that the bands are broader than that found for the corresponding coated NPs. A weak peak at 1741 cm^{-1} corresponding to the C=O stretching of the ester group of the pectin is still observed.

The peak at 1608 cm⁻¹ corresponding to the asymmetric stretching of carboxylate group shifts to 1597 cm⁻¹ in case of the coated NPs; in case of the blended NPs it is still possible to identify the two peaks characterizing the chitosan NPs, at 1635 cm⁻¹ and 1542 cm⁻¹, but these peaks are shifted of a few cm⁻¹ (to 1616 cm⁻¹ and to 1564 cm⁻¹) and are of comparable intensity. The shift of the band at 1564 cm⁻¹ (attributed to N-H bending) is greater than what has been observed in the coated samples. This could be due to the fact that the bending modes are sensitive to the changes in the environment of the group and so are more affected by a perturbative surrounding than the stretching modes. Therefore this shift of the N-H bending band could be indicative of a greater number of interaction between the amino groups of chitosan and the carboxyl groups of pectin, compared to the coated samples (Bigucci et al., 2008).

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435 **3.5 Differential scanning calorimetry**

DSC investigations were carried out on coated NPs with pectin from apple and citrus fruit and on 436 blended NPs with pectin from apple; for comparison starting materials and native chitosan: TPP NPs 437 are also reported. DSC data of pure chitosan are characterised by an endothermal event (peak temp 438 at 57.2 °C) corresponding to the dehydration and by an exothermal event that begins at 252 °C with 439 a peak around 320 °C corresponding to the thermal degradation (Sarmento et al., 2006a; Sarmento 440 et al., 2006b). In addition to the dehydration endothermic event (peak at 54.3 °C), chitosan NP DSC 441 data revealed another endothermic event at 225 °C that has been ascribed to the breakdown of 442 unspecific electrostatic interactions by Borges et al. (2005). The authors reported also a second 443 endothermic peak of minor intensity, related to the cleavage of electrostatic interactions between 444 chitosan and the counterion (sulphate ions in that case). In our case (TPP as counterion) a similar 445 second peak was observed, and it is similarly attributed to the cleavage of such interactions. Indeed, 446 447 the thermogram of TPP alone evidenced an endothermic peak at 192 °C; therefore, it could also derive from the TPP, although shifted to higher temperatures in NPs. The exothermic event 448 beginning at 243.8 °C with a peak around 310 °C, and corresponding to thermal degradation, is 449 slightly shifted to lower temperatures (Bagre et al., 2013) as a result of the interaction of chitosan 450 451 with TPP (Azevedo et al., 2011). The intensity of the exothermal event for chitosan NPs is lower 452 than that of the native chitosan (similar weight are compared); this is reasonably due to the fact that 453 a fraction of the chitosan chain is cross-linked with TPP and therefore differently susceptible to 454 degradation.

Pectin from apple is characterized by the endothermal event corresponding to dehydration and by two sharp exothermal peaks at 255 °C and 328 °C, corresponding to degradation (**Fig. 4b**). Apart from the endothermal event of dehydration, chitosan NPs coated with apple pectin are characterized by an exothermal event starting at 273 °C with a peak at 324 °C, suggesting a thermal stabilization due to the interaction of chitosan with pectin and not only to TPP. The interaction is also confirmed by the absence of any characteristic peaks of the pectin. The thermogram of pectin chitosan blended NPs did not reveal any characteristic peak of the apple pectin; the only difference observed was that the height of the exothermal event is far higher than that of the chitosan NPs and it is similar to chitosan alone, so not cross-linked with TPP. A lower interaction between chitosan and TPP, reasonably due to a competition between TPP and pectin, both negatively charged, would explain the recorded data.

Pectin from citrus fruit is characterized by an exothermal event between 225 and 250 °C with two distinct peaks at 232 and 248 °C, and another exothermal sharp peak at 331 °C (**Fig. 4c**). The thermogram of chitosan NPs coated with citrus fruit pectin is characterized by an exothermic event with a peak at 318 °C, similar to that of NPs coated with apple pectin. As previously commented for apple pectin samples, characteristic peaks of the citrus fruit pectin were not found, confirming the interaction of pectin with chitosan as already evidenced by surface charge measurements.





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Fig. 4: DSC curves of a) chitosan (dash line) and chitosan NPs (straight line); b) apple pectin (dash
line), coated NPs (straight line) with pectin and blended NPs (dot line) with pectin; c) citrus fruit
pectin (dash line) and coated NPs (straight line) with pectin.

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479 **3.6** Chorioallontoic membrane assay (CAM)

CAM of 6 days old chicken embryos are shown in **Fig. 5a-d**. Because of eggs incubation in vertical position (convex pole in upper position), CAM floats over the yolk sack and, while growing, will cover all the air-exposed surface adhering to most of the testaceous membrane internal surface. Intrinsic CAM characteristics (e.g., high transparency, high vascularization and capillarity, sensitivity to physical and chemical insults) give this structure the right features for the direct and continuous evaluation of acute inflammatory response (Vargas et al., 2007; Saw et al., 2008).

The tested materials showed different behaviors in terms of dissolution/dispersion in the albumen 486 wetting CAM surface. After deposition on CAM surface, pectin from apple swelled and 487 polysaccharide chains rapidly disperse in the albumen film covering the membrane (Fig. 5a' and 488 5a"). The material was not visible 24 hours after deposition. On the contrary, 24 hours after 489 deposition, chitosan, chitosan NPs, and chitosan-pectin NPs swelled generating transparent gels that 490 did not disperse in the albumen film (Fig. 5). Transparency allowed to visualize easily the portion 491 of CAM under the material and not just the space around the implant. A careful observation of 492 treated embryos (1 day after treatment) evidences the complete absence of the signs ascribable to 493 494 acute toxicity, inflammation or pro-angiogenic effect, and no substantial differences were evidenced between the raw materials and NPs (Fig. 5). The same results are obtained with saline, the negative 495 control routinely employed in our laboratories in this experimental setup (Blasi et al., 2013) and 496 confirmed here (Fig. 5). TPP, being ionically bound to chitosan amino groups, does not impair the 497 biocompatibility of the NPs evaluated. TPP has shown to be extremely toxic with the same 498 experimental setup (Rampino et al., 2013). Membrane opacity, bleeding, vessel rupture, CAM 499 500 corrosion, capillary and vessel overgrowth, are the classical reactions to substances or materials provoking a toxic insult or an inflammation on this extraembryonic membrane. Additionally, a 501 502 substance having pro- or anti-angiogenic effect is responsible of the increase or decrease of capillaries/vessels density under and/or around the treated zone. These effects are experienced by 503 deposing aggressive surfactants, such as sodium dodecilsulphate (Blasi et al., 2013), or 504 polysaccarides, such as λ carrageenan (Fig. 5), that are used as positive controls. 505

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Fig. 5 Chicken embryos at different stages of development observed during acute toxicity experiments. a, embryo before treatment; a', after material (apple pectin) deposition; a" after 24 hours - b, embryo before treatment; b', after material (chitosan) deposition; b" after 24 hours - c, embryo before treatment; c', after material (chit/pect NPs) deposition; c" after 24 hours - d, embryo before treatment; d', after material (chit NPs) deposition; d" after 24 hours - e, embryo before treatment; e', after saline deposition (negative control); e" after 24 hours - f, embryo before treatment; f', after material (λ carrageenan) deposition (positive control); f" after 24 hours.

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520 **4. CONCLUSIONS**

521 Chitosan in combination with pectins confirmed to be a valuable biopolymer for the preparation of 522 carriers interesting for drug delivery applications, due to the possibility to tailor the physico-523 chemical characteristics of the final NPs.

The most relevant outcome is the large number of potentially useful nanoparticles prepared with the 524 two procedures, which, however, has been a challenge for selecting a few batches for 525 characterization and testing studies. A rationalization would imply that the actual composition of 526 NPs and not simply the starting composition is taken as a characterizing parameter of the products. 527 528 Unfortunately, to the best of our knowledge, this approach is not yet reported in literature. The results here presented evidenced that it was possible to prepare pectin:chitosan NPs with both 529 techniques, as already reported for other polymers. Two main advantages resulted from the 530 blending technique: the first is the one-step preparation, that is highly desirable especially in view 531 532 of a scale-up process, the second one is the possibility to tune the size and Z-potential by properly selecting the ratio of chitosan, pectin and TPP. The coating technique did not allow such a 533 534 modulation of size, since the addition of pectin as external NPs coating always increases the size. It is worth noticing that the addition of a protein requires to take into account the competition among 535 charged species. Indeed, a decrease of the loading of BSA and OVA was found in case of the 536 blending technique due to the electrostatic interactions of chitosan with protein and pectin, both 537 negatively charged. This brings to the conclusion that the most suitable technique depends on the 538 physico-chemical characteristics of the species involved, i.e. polymer and protein. 539

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656 **CAPTION TO FIGURES**:

Fig. 1: mean size (a) and Z-potential (b) of chitosan NPs (chitosan:TPP ratio 5:1 w/w) coated with sodium polygalacturonate (\blacksquare), pectin from citrus fruit (\bullet) and pectin from apple (\blacktriangle).

Fig. 2: mean size of chitosan NPs blended with apple pectin in different ratios with TPP. Ratio pectin:TPP 1:1 (\blacksquare), 2:1 (\bullet), 4:1 (\blacktriangle), 6:1 (\triangledown) and 8:1 (\bullet). The mean size of NPs in the absence of pectin is in the range of 200 nm.

Fig. 3: FTIR spectra of a) chitosan (dash line) and chitosan NPs (full line); b) chitosan NPs (black full line), polygalacturonate (red dash line), polygalacturonate coated NPs (blue full line); c) chitosan NPs (black full line), apple pectin (red dash line), apple pectin coated NPs (blue full line) and apple pectin blended NPs (blue dot line); d) chitosan NPs (black full line), citrus fruit pectin (red dash line), citrus fruit pectin coated NPs (blue full line).

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Fig. 4: DSC curves of a) chitosan (dash line) and chitosan NPs (straight line); b) apple pectin (dash
line), coated NPs (straight line) with pectin and blended NPs (dot line) with pectin; c) citrus fruit
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Fig. 5: Chicken embryos at different stages of development observed during acute toxicity experiments. a, embryo before treatment; a', after material (apple pectin) deposition; a'' after 24 hours - b, embryo before treatment; b', after material (chitosan) deposition; b'' after 24 hours - c, embryo before treatment; c', after material (chit/pect NPs) deposition; c'' after 24 hours - d, embryo before treatment; d', after material (chit NPs) deposition; d'' after 24 hours - e, embryo before treatment; e', after saline deposition (negative control); e'' after 24 hours - f, embryo before treatment; f', after material (λ carrageenan) deposition (positive control); f'' after 24 hours.

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