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# Formulation of polysaccharide-based nanoparticles for administration in the oral cavity

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## ABSTRACT

This study describes the evaluation of polysaccharide-based nanosystems addressed to oral cavity, as possible formulations for improving the treatment of oral ailments. Nanoparticles based on chitosan (Chit-NP), alginate (Alg-NP) or pectin (Pec-NP) were prepared through self-assembly by ionotropic gelation using tripolyphosphate and zinc as cross-linkers. Characteristics of nanoparticles at increasing cross-linker concentration provided the basis for selecting the most suitable formulations. The nanoparticles were tested for cytotoxicity against buccal cells (TR146) and for stability in a medium simulating pH, ionic strength, electrolyte composition and concentration of saliva. Alg-NP were the most stable in the salivary environment, while Chit-NP were the most cytocompatible. Alg-NP and Pec-NP revealed possible cytotoxicity due to the presence of zinc. This knowledge is important in the earlydesign of biopolymer-based nanoparticles for oral usage and for potential improving of the biocompatibility of the investigated nanoparticles with the oral environment.

## BACKGROUND

A common problem of conventional pharmaceutical formulations for the treatment of oral diseases is the short residence time in the oral cavity due to the saliva clearance and to the oral muscular function. Formulations based on natural polysaccharides, which are generally regarded as biodegradable and biocompatible, may assure a prolonged effect through their bio- and mucoadhesive properties and the possibility of providing controlled release.[1] Furthermore, polysaccharides in the form of nanoparticulate carriers could be beneficial due to their small size allowing them to reach areas that are inaccessible to other types of delivery systems.[2] Despite the possible advantages of this type of formulation, only few studies have so far been focused on the possible application of polysaccharide-based nanoparticles as local drug delivery systems addressed to the oral cavity.[3, 4]

The families of polysaccharides chitosan, pectin and alginate are commonly used in pharmaceutical formulations and medical devices. Chitosan consists of linear chains of β(1→4)-linked glucosamine and N-acetyl-D-glucosamine, and is characterized by the amount of deacetylated monomers in relation to the total, regarded as the degree of deacetylation (DDA%) or as the degree of acetylation (DA%=100 – DDA%). The amine groups on the glucosamine can be protonated at acidic pH (pKa ≈ 6.0-7.0),[5] thus rendering the polymer positively charged. Alginate and pectin are polyuronates, hence their monomers contain carboxylic groups that can confer a negative charge to the polymer (pKa 3-4).[6, 7] Alginate is a linear block copolymer constituted by D-guluronates (G) and L-mannuronates (M), whose amounts characterize the polymer. Pectin is a ramified polymer constituted mostly by D-galacturonate units. The carboxylic group of the galacturonate monomers can be methylesterified or amidated, and the degree of esterification (DE) and of amidation (DA) characterizes the pectin.

Nanoparticles can be prepared from polysaccharides in dilute or semi-dilute solution through ionotropic gelation with oppositely charged cross-linkers, such as tripolyphosphate for chitosan,[8-10] and zinc (Zn) for alginate and pectin.[11-13] Zn is commonly used in formulations for oral hygiene due to its antibacterial and anti-halitosis actions;[14-16] therefore, the inclusion of Zn could possibly confer a further beneficial effect to particle formulations intended for buccal administration. The polysaccharide nanoparticles that have been mostly investigated are chitosan-based, while fewer studies have examined particles prepared with negatively charged polysaccharides. A convenient technique for the preparation of "soft" polysaccharide nanoparticles is by non-covalent self-assembly method. The characteristics of the nanoparticles thus obtained can vary depending on preparation factors, such as the cross-linker concentration, the coil overlap concentration, among other.[8, 12, 13] Therefore, by fine tuning the preparation factors, it is possible to design nanoparticles on a rational basis suited for the intended application.

Electrolytes dissolved in the medium where the nanoparticles are dispersed may interact with the charged components of the particles' surface and interfere with their electrostatic bonding and hydration forces,[13, 17] refthus causing instability. It was therefore considered important to perform a study looking at the influence of the biological fluid present at the site of administration on the particles colloidal stability, namely in the simulated conditions of the saliva of the oral cavity. Natural saliva has a complex and variable composition,[18, 19] therefore, the use of an artificial saliva with known composition should facilitate the understanding of the influence of the salivary constituents on the colloidal systems. In this work, the artificial saliva formula proposed by Gal and coworkers[20] was chosen due to the great resemblance to natural saliva with regard to the type and concentrations of ionic species, the ionic strength, the pH, and the buffering capacity.[18] Moreover, the presence of only electrolytes allows characterization of the nanoparticulate systems through dynamic light

scattering. This would be impossible with natural saliva due to the presence of components that can interfere with the measurement, such as nanosized micelle-like globules formed by salivary proteins.[21]

Another important aspect regarding possible clinical application of the nanoparticles is the evaluation of their toxicity at the site of administration. This becomes especially important when delivery systems are designed to obtaining a long retention time.

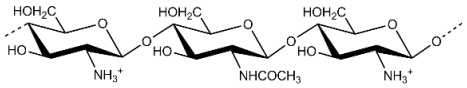
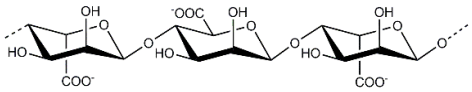
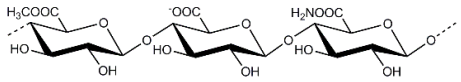
This study aimed to formulate nanoparticles based on chitosan, alginate and pectin as possible drug delivery systems for the oral cavity. Their potential oral biocompatibility was investigated from the stability of the particles in the salivary environment and their cytotoxicity towards a buccal cell line.

## METHODS

### Preparation of nanoparticles

The nanoparticles tested were prepared through self-assembly by ionotropic gelation. The characteristics of the polysaccharides used in the study are provided in Table 1; pectin and alginate were purified prior to utilization as previously described.[12, 22] Alginate nanoparticles (Alg-NP) and pectin nanoparticles (Pec-NP) were prepared through ionic cross-linking of the negatively charged polysaccharides with the cation $Zn^{2+}$ (zinc chloride, Merck, Germany), and chitosan nanoparticles (Chit-NP) were prepared with the positively charged chitosan cross-linked with the anion TPP (sodium tripolyphosphate pentabasic, Sigma-Aldrich, Germany). The detailed description of the protocol of preparation for Chit-NP, Alg-NP, and Pec-NP can systems is available in Supplementary Material.

**Table 1.** Schematic representation of the chemical structure, major characteristics and source of the polysaccharides used in the study.

| Polysaccharide | Chemical structure | Characteristics | Supplier |
|---|---|---|---|
| Chitosan chloride (Protasan™ UP CL 213) |  | Mw 307 kDa* DDA 83%§ | Novamatrix - FMC Biopolymer (Norway) |
| Sodium alginate (Protanal® LF 10/60) |  | Mv 147 kDa† G 65–75%§ | FMC BioPolymer (Norway) |
| Pectin (Genu® pectin LM-102 AS) |  | Mw 96 kDa‡ DE 30%§ DA 19%§ | CPKelco (Denmark) |

* Jonassen et al. (2012)[10]
† Pistone et al. (2015)[12]
‡ Nguyen et al. (2011)[22]
§ information given by the supplier

For comparative purposes, the final concentration of the polysaccharide in the test samples (0.05%, *w/w*) and the solvent (0.05M NaCl) were kept constant throughout the formulations; the cross-linker amount was varied as indicated in Table 2. All the test samples were prepared at least in duplicate. Solutions of chitosan and pectin in the absence of the cross-linker were also prepared (0.05% polysaccharide in 0.05M NaCl).

**Table 2.** Test formulations.

| Nanosystem | Cross-linker : polysaccharide ratio (*w:w*) |
|---|---|
| Chit-NP | 10:90, 15:85*, 20:80, 25:75 |
| Pec-NP | 10:90, 15:85, 20:80*, 25:75 |

| | |
|---|---|
| Alg-NP | 65:35* |

* Formulations used for further testing.

## Physical characterization

The particles were characterized by dynamic light scattering with non-invasive back scattering (DLS-NIBS). From the cumulants fit of the autocorrelation data, the size distribution and associated parameters were derived, namely the intensity-based size distribution plots, mean Z-average diameter, polydispersity index (PDI), and the intensity of the scattered light (measured as the derived count rate). The refractive index, viscosity, and the dielectric constant of pure water at the relevant temperature (either 25 or 37 °C), were given and used by the equipment's software in the calculations. The surface zeta potential ($\zeta$) was determined by mixed laser Doppler electrophoresis and phase analysis light scattering (M3-PALS). Both type of measurements were recorded using a Malvern Zetasizer NanoZS (ZEN 3600, Malvern Instruments, Worcestershire, U.K.) fitted with a red laser light beam ($\lambda$ = 632.8 nm). All the characterization determinations were conducted at least in triplicate and at 25 °C. The pH was measured at room temperature. More detailed information can be found in the Supplementary material.

## Stability in simulated salivary fluid

Artificial saliva was prepared, as described by Gal et al.,[20] . Briefly, the following salts (mg) were dissolved in 1 L of MilliQ water: 125.6 NaCl, 963.9 KCl, 189.2 KSCN, 654.5 $KH_2PO_4$, 200.0 urea, 336.5 $Na_2SO_4$, 178.0 $NH_4Cl$, 227.8 $CaCl_2 \cdot 2H_2O$, and 630.8 $NaHCO_3$. The pH was adjusted to 6.8 by bubbling $CO_2$ gas through the solution before each experiment. An aliquot

of 750 μl of artificial saliva (or pure water for control) was mixed with 250 μl of each nanoparticulate suspension and kept at 37 °C for 2 h. The z-average, PDI and size distributions were recorded every ~2 min while the zeta potential every 30 min by DLS-NIBS and M3-PALS, respectively, as described above. The first measurement of each parameter was recorded five min after mixing, so as to allow for temperature equilibration. All measurements were collected in triplicate.

**Cytotoxicity studies**

**Changing the medium of the nanoparticulate systems.** Prior to cell studies, the original solvent of the nanoformulations was replaced with the media that the cells were found to tolerate, namely HBSS (Hank's balanced salt solution modified with calcium and magnesium, Sigma SAFC Biosciences Ltd., UK) or a mixture of HBSS and 0.05M NaCl (1:1, *v:v*).. To this end, the particles suspension was dialyzed overnight as described in the Supplementary Materials.. Solutions of polysaccharides and cross-linkers were prepared directly in HBSS.

**MTT assay.** The cell line TR146 (Sigma-Aldrich GmbH, Steinheim, Germany) was used for the cytotoxicity studies as a model cell line for the buccal epithelium.[23] The cells were cultured as described previously,[24] and cells of passage number 29-33 were used. In each well of a 96-well culture plate, 100 μl of suspension of TR146 cells (~$10^4$ cells) was seeded. The plate was incubated 24 h at 37 °C with 5% $CO_2$ (Sanyo MCO-19AIC, Panasonic Biomedical Sales Europe BV, AZ Etten Leur, Netherlands) to allow for the attachment of the cells. After the removal of the culture medium, the cells were rinsed twice with 100 μl/well of HBSS. Test samples or control solutions (100 μl/well, $n$ = 8 wells) were incubated for 4 h. The media of the test samples were used as negative controls (100% viability), and a solution of 4% Triton X-100 (t-Octylphenoxypolyethoxyethanol, Sigma-Aldrich, Germany) in phosphate buffer saline was used as positive control (0% survival). Then, the samples and control

solutions were removed and replaced with 100 µl of supplement-free medium. Twentyfive µl MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) solution, containing a concentration of 5 mg/ml of thiazolyl blue tetrazolium bromide, was added to each well, and incubated for 4 h at 37 °C. Thereafter, the medium was removed and the resulting dye crystals were dissolved in 100 µl DMSO. The absorbance (A) was quantified at λ = 570 nm in a microplate reader (Safire, Tecan AG, Salzburg, Austria) after orbital shaking at 300 rpm for 10 min. The result for each sample was calculated as the mean relative cell viability ($n$ = 8) relative to the negative control, as follows:

$$Relative\ cell\ viability\ (\%) = \frac{A_{sample} - A_{positive\ control}}{A_{negative\ control} - A_{positive\ control}} \cdot 100$$

The data are presented as the mean of three independent measurements carried out on different days and the pooled standard deviation (SDp obtained by pooling the standard deviation from each experimental day). Student's $t$-test ($p < 0.05$) was used to compare the relative viability of the tested formulations with the negative control.

Both nanoparticles and their single components were investigated for cytotoxicity. The samples tested are listed in Table 3. The ratios of cross-linker to polysaccharide in the nanoparticulate formulations tested corresponded to the following cross-linker concentrations (*w/w*): 0.009% TPP in Chit-NP (15:85), 0.013% Zn in Pec-NP (20:80), and 0.027% Zn in Alg-NP (65:35).

**Table 3.** Formulations tested in cytotoxicity studies.

| **Samples** | **Concentrations** (*w/w*) |
|---|---|
| Chit-NP, Alg-NP, Pec-NP | Undiluted, diluted 3:5 (*v:v*) in the media |
| Chitosan, alginate, pectin | 0.05% |
| TPP | 0.009% |
| Zn | 0.006%, 0.013%, 0.020%, 0.027% |

## RESULTS

### Characterization of the formulations

**Alginate-Zn formulations.** Systems at increasing Zn to alginate ratios (w/w) have already been developed and characterized, and a suitable formulation for buccal administration have already been established in a previous work by our group.[12] These formulations were used in the stability and biological studies of the present study for comparison with the chitosan- and pectin-based formulations.

**Chitosan-TPP formulations.** The samples comprising only chitosan in solution displayed a low scattered intensity (~200 kcps), high PDI (0.70) and a multimodal size distribution (results not shown). The physical characteristics of the chitosan-TPP formulations developed in the present study at increasing TPP to chitosan ratio are illustrated in Figure 1. The PDI was low (~0.2) and the size distributions were monomodal for all the tested formulations. The

zeta potential of the particles decreased with increasing TPP to chitosan ratio. The average size of the particles was similar at low TPP to chitosan ratios (10:90 and 15:85), and went through a minimum at a ratio of 20:80. In turn, at the highest TPP to chitosan ratio 25:75 the particles attained their largest size and after one day of storage they aggregated. In turn, the scattered intensity increased exponentially with increasing TPP to chitosan ratio.

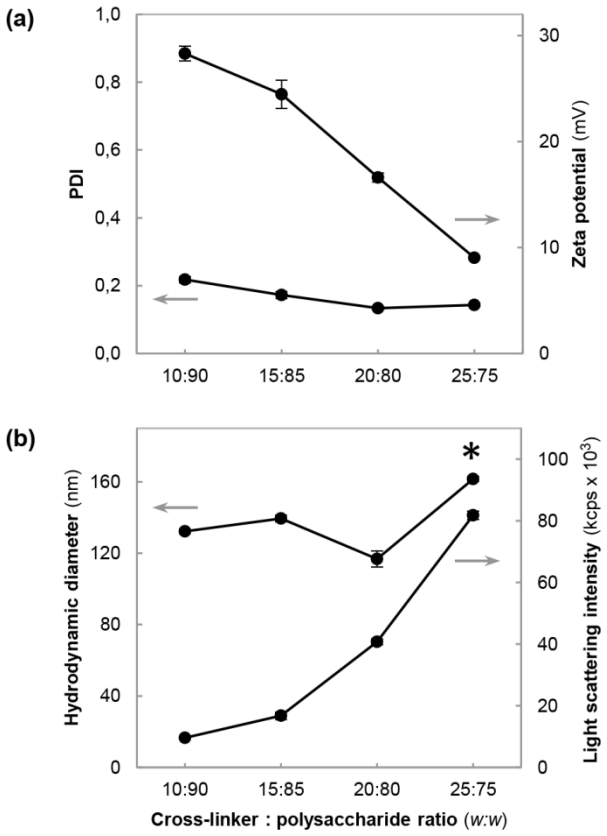



**Figure 1.** Physical characteristics of chitosan-TPP formulations as a function of the crosslinking ratio: (a) Polydispersity index (PDI) and zeta potential, and (b) Z-average hydrodynamic diameter and scattered intensity of chitosan-TPP formulations at increasing TPP to chitosan ratios (w/w). *Macroscopic aggregation after one day. The error bars denote standard deviations, and the points without error bars have standard deviations equal to or smaller than the size of the markers.

**Pectin-Zn formulations.** The samples containing only pectin in solution presented a low PDI (0.275), a monomodal size distribution (average size 584 ± 6 nm) and a relatively high scattered intensity (~7100 kcps). The characteristics of the pectin-Zn nanoparticle formulations at increasing Zn to chitosan ratios are illustrated in Figure 2. The results for the formulation at Zn to pectin ratio 25:75 Zn were not recorded due to immediate aggregation and precipitation. At increasing crosslinking ratios, the zeta potential became less negative, the average size decreased and the scattered intensity increased. The size distributions were monomodal and the PDI was relatively constant for all the test formulations.

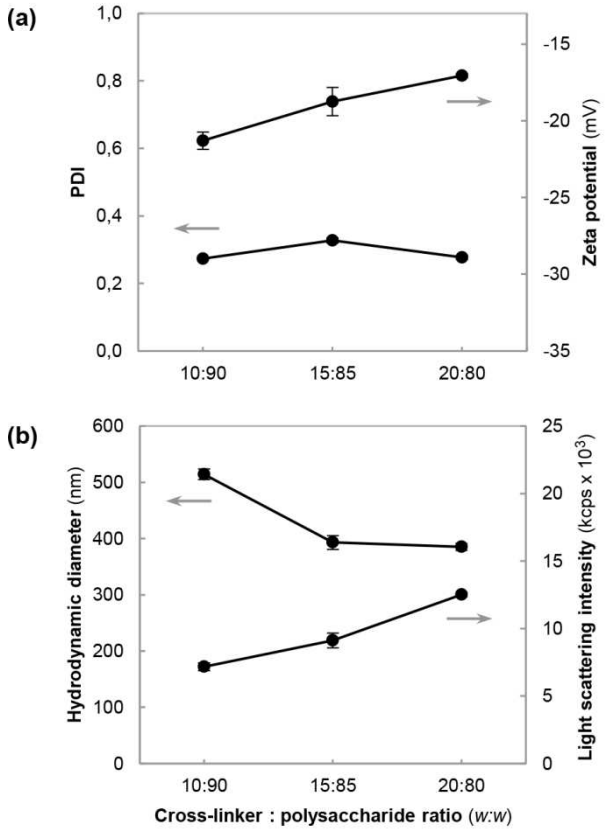


**Figure 2.** Physical characteristics of pectin-Zn formulations as a function of crosslinking ratio: (a) PDI and zeta potential, and (b) average diameter (z-average) and scattered intensity of pectin-Zn formulations at increasing Zn to pectin ratios. The error bars are standard deviations, and the points without error bars have standard deviations equal to or smaller than the size of the markers.

**Stability of the particles in a salivary environment**

The stability of the nanoparticles in the salivary environment was monitored through DLS measurements. The pH of the three formulations selected for stability studies in artificial saliva was adjusted to pH 6.0, and their characteristics, measured before mixing with artificial saliva, is depicted in Figure 3 (black markers). A control test was performed by mixing the nanoparticulate systems with pure water instead of artificial saliva in order to check the influence of the increase in temperature to 37 °C or the dilution on the characteristics of the formulations. Negligible variations occurred during the control test confirming that the variations observed were exclusively due to the composition of the artificial saliva.

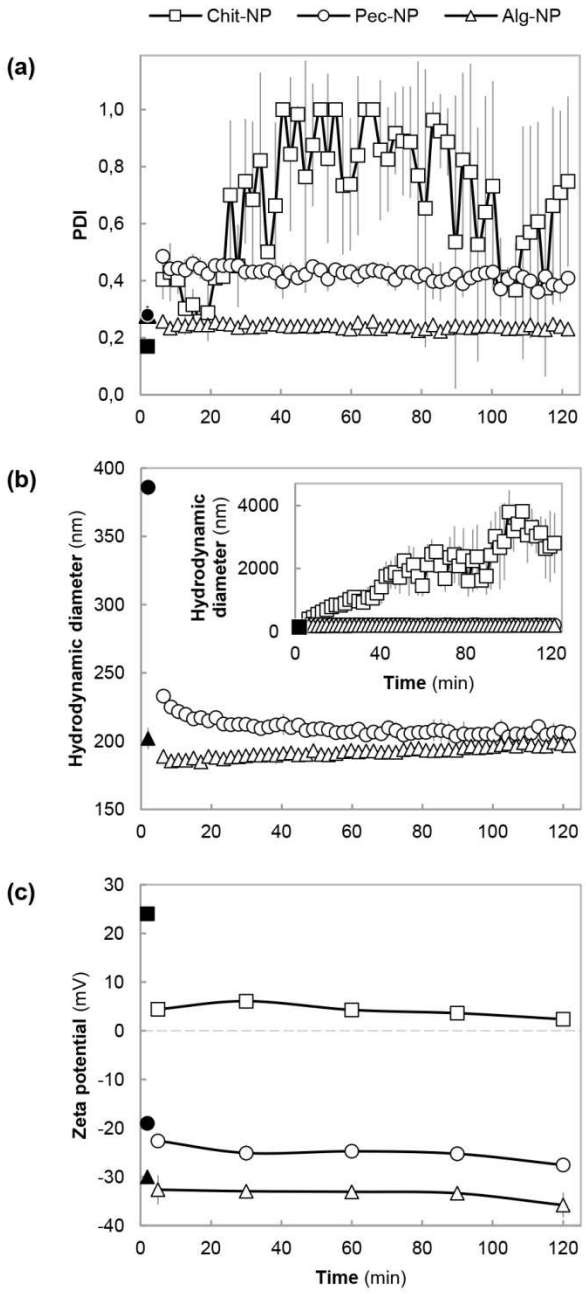



**Figure 3.** Stability of chitosan-TPP (□, ■), pectin-Zn (□, ●), and alginate-Zn (Δ,♦) formulations during incubation in artificial saliva (37°C). (a) PDI, (b) average diameter, and (c) zeta potential of the nanoparticles during the stability test in artificial saliva (white markers). The filled markers represent the original values measured before the mixing of the particles with artificial saliva. The error bars represent the standard deviation and the points without error bars have standard deviations equal to or smaller than the size of the markers.

Figure 3 displays the PDI, the average size and the zeta potential of the nanoparticles when mixed with artificial saliva (empty markers). For Alg-NP, the measurements of PDI, average size and zeta potential measured during the two-hour test were constant and similar to the values measured before mixing with artificial saliva; the size distributions remained monomodal at all the time points.

The PDI of the Pec-NP remained constant during the two-hour test, however, its average value (0.42) was significantly higher than the average value recorded for the particles before the mixing with artificial saliva, and the size distributions were multimodal at all time points. Moreover, the particle size underwent a pronounced decrease in the order of ~150 nm upon addition of Pec-NP to artificial saliva and the size was further reduced by ~30 nm during the test, until a plateau was reached (Figure 3b). A slight increase in zeta potential (absolute value) was recorded.

The PDI of the Chit-NP increased markedly immediately after mixing and, even though the measured values were too high to suggest reliable measurements, there was a trend showing a large progressive increase in the z-average size during the test. At the end of the test it was possible to observe macroscopic aggregates and precipitates. Even though the zeta potential was constant for the whole test, its value decreased from +25 mV to nearly neutral upon mixing the Chit-NP with artificial saliva.

**Cytotoxicity study**

The three selected formulations were also tested for cytotoxicity against TR146 cells, a buccal epithelial cell line. Figure 4a shows the cell viability after the treatment with Chit-NP or the separate components. The solution of free chitosan (0.05%) significantly reduced the

viability, while the TPP alone did not affect the viability compared to the negative control. The viability of the cells treated with Chit-NP was doubled compared to the viability of the cells treated with chitosan alone and, when the Chit-NP were diluted on a 3:5 ratio (v/v), the viability further increased and was not significantly different from the negative control.

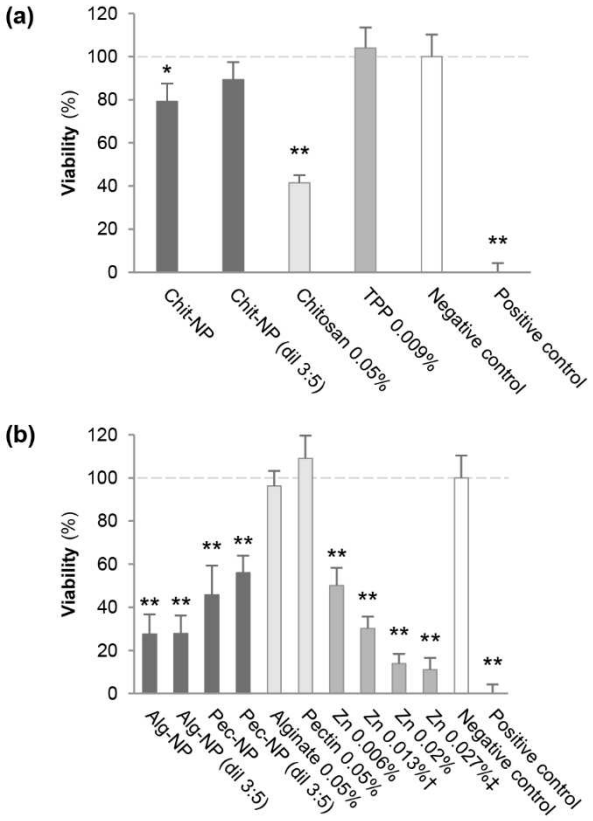



**Figure 4.** Relative viability assessed by MTT assay of TR146 cells treated with the samples for 4 h: (a) Chit-NP and their components; (b) Alg-NP, Pec-NP, and their components. The error bars indicate the pooled standard deviations. † and ‡ Zn concentration as in the undiluted Pec-NP and Alg-NP, respectively. Viability significantly different from the negative control: * $p < 0.05$ and ** $p < 0.001$.

Figure 4b shows the cell viability after treatment with Alg-NP, Pec-NP or the separate components during 4 h. Neither of the solutions of free alginate and pectin (0.05%) changed the viability compared to the negative control. By contrast, in the range of concentrations and time course tested, all the solutions containing free Zn reduced the cell viability in a dose-dependent manner. Alg-NP and Pec-NP also significantly reduced the cell viability both when applied at the full concentration and when diluted 3 to 5. The dilution of the particles did not significantly increase the viability for both Alg-NP and Pec-NP. Moreover, the cells treated with diluted samples of Pec-NP displayed a significantly higher viability ($p < 0.05$) compared to the diluted Alg-NP.

## DISCUSSION

### Formulation of the nanoparticles

The cross-linker amount has previously been shown to be of importance when preparing polysaccharide-based nanoparticles.[8, 12, 13]ref In this study, the cross-linker to polysaccharide ratio of the formulations was investigated with the aim to reveal the process of formation of the nanoparticles, and consequently enable to establish formulations most suited as drug delivery carriers addressed to the oral cavity. Both positively and negatively charged particles were studied due to their different ability to promote bio- and mucoadhesion to the oral cavity. Positively charged particles have been reported to adhere strongly to the negatively charged surfaces of the oral tissues due to the formations of electrostatic bonds.[25-27] While, negatively charged particles with a low charge are less repelled by the mucosa compared to particles highly charged.[26] At the same time, it is known that both chitosans of varying DDA and Mw (ref), and also negatively charged polysaccharides, including alginate and pectin (ref), are able to interact with pig's gastric mucin and reduce its viscosity in solution.

The results for the characterization of the sample containing only chitosan in solution suggested that the chitosan chains in the absence of the cross-linker (TPP) adopted… free or in form of randomly sized loosely packed aggregates, as previously observed for alginate.[12] As soon as the TPP was added the nanoparticles formed spontaneously, the low polydispersity indicated the presence of homogenously sized particles. An increase in scattered intensity can be caused by an increase in the size or in the compactness of the particles. Therefore, the increase in scattered intensity and the fact that the size remained nearly constant when the TPP to chitosan ratio was increased form 10:90 to 15:85, could also be diagnostic of the increase in the compactness. This could be due to the addition of an excess of chitosan disordered chains being free in solution or in form of loose aggregates at 10:90 TPP to chitosan ratio.[9] A similar result was observed in a previous study,[10] where, by increasing the TPP to chitosan ratio, an increase in the aggregation number (the number of polysaccharide chains that associated together form a particle) was recorded. Consequently, at 10:90 TPP to chitosan ratio, surplus free chitosan chains that were not involved in the formation of the nanoparticles, were probably present in solution.

The decrease in size when the TPP to chitosan ratio increased to 20:80 was probably due to the formation of intra-particle cross-links, thus reducing the electrostatic repulsion between the chitosan chains and increasing the ionic cross-link density within the particles.[28] As a consequence, the particles would shrink and become more compact, as evidenced by the increase in scattered intensity. The formation of mainly intra-particles bonds upon increase of TPP to chitosan ratio to 20:80 could indicate that at a 15:85 ratio most of the chitosan chains in solution was comprised within the nanoparticles. The size increase and the following macroscopic aggregation at a ratio of 25:75 was probably due to the excessive inter-particle cross-linking[8, 28] and to reduced electrostatic repulsion between the Chit-NP since the pH of the solvent also increased as more basic TPP was added.[8]

The formulations expected to be most suited for drug delivery applications, were those that contained non-aggregated particles and the majority of chitosan chains cross-linked in form of nanoparticles. Hence, such formulations contained a TPP ratio of 15:85 or 20:80. Since a high positive charge on the particle surface would be advantageous for nanoformulations addressed to the oral cavity, the formulation with the highest zeta potential (15:85 ratio) was chosen for further studies.

Unlike the chitosan solutions, the low polydispersity of the pectin solution in the absence of the cross-linker revealed the presence of homogeneously sized polymer chains, expected to occur as disordered random coils. The aggregation of pectin chains into nanoparticles in the absence of Zn was previously explained by inter and intra-molecular interactions, such as hydrogen bonding.[11] Moreover, the size reduction and the increase in scattered intensity at increasing Zn to pectin ratios could indicate that the cross-links formed by Zn caused a simultaneous shrink and increase in compactness of the particles previously formed in its absence. This could indicate a different mechanism of formation of the Pec-NP compared to the Chit-NP. The precipitation observed at the highest Zn to pectin ratio (25:75) was probably due to the formation of inter-particle cross-links and to the particle neutralization caused by the addition of acidic Zn solution.

All the pectin-based formulations tested, except the formulations containing 25:75 Zn to pectin ratio, could be suitable for drug delivery purposes. The formulation containing a Zn to pectin ratio of 20:80 was chosen for further investigation due both to the lowest zeta potential and to the highest content of Zn that could be advantageous for a drug delivery system addressed to the oral cavity.

The characterization of Alg-NP at increasing Zn to alginate ratios have been thoroughly described in a previous study by our group,[12] where at Zn to alginate ratio below 35:65 the

particles were not fully formed, while higher ratios caused particle aggregation.[12, 13] Differently from Chit-NP and Pec-NP, no shrinking was detected by increasing the cross-linker to polysaccharide ratio. This might be attributed to a possible high compactness of the particles, or to the stiffness of the alginate chains that would discourage changes in the conformation. Since at 35:65 Zn to alginate ratio resulted in the formation of stable nanoparticles, this ratio was considered as the most suitable for drug-delivery purposes.

In sum, the formulations obtained with increasing amounts of cross-linker in the three addressed systems (alginate, chitosan and pectin) revealed that the process of formation of the nanoparticles might be different depending on the type of polysaccharide employed. Moreover, the process of formation of the particles yielded important information in order to select the most suitable formulations for drug delivery purposes.

**Stability of the particles in a salivary environment**

Important causes of the instability of ionically cross-linked nanoparticles are the aggregation of the nanoparticles, and the dissolution of the particles caused by the disaggregation of the particle-forming polysaccharide chains. Even though less dramatic, the potential role of shrinking and swelling of the particles, resulting in changes in particle size, cannot be ruled out.

Alg-NP were the most stable in artificial saliva. This was unexpected because the cross-links between alginate and divalent cations, such as Zn, can be broken due to the presence of phosphates that can chelate the divalent cations.[29] However, the presence of calcium in the artificial saliva could have prevented the degradation of the alginate-Zn bonds,[29] since calcium can be chelated by phosphates and could also form stabilizing cross-links on the Alg-NP.[30]

An abrupt reduction in average size upon mixing with artificial saliva, as observed for Pec-NP, can be the consequence of shrinking of the particles or to their partial dissociation. Shrinking can occur if further cross-links are formed (Figure 2b) or when screening the charges on the polysaccharide chains. In both of these scenarios, a decrease of the zeta potential (absolute value) would have been expected, but this was not observed (Figure 3c). In addition, the increase in polydispersity could confirm the partial dissociation, as would be expected from the emergence of new populations of particles of varying sizes. A cause for the dissolution of Pec-NP might stem in the interaction of the cationic cross-linker with the anionic species in solution (such as phosphates) which could have caused its partial displacement until an equilibrium was reached.[29]

Even though both Pec-NP and Alg-NP were constituted by polyuronates cross-linked with Zn, the stability of the Alg-NP was superior to the Pec-NP. This might be attributed to a higher alginate-Zn affinity as compared to pectin-Zn, that could prevent the displacement of Zn (*e.g.*, by phosphate) in the Alg-NP. Moreover, due to its structure alginate possesses a higher charge density than pectin and thus a higher amount of Zn was used for the preparation of Alg-NP compared to Pec-NP. This probably caused the formation of a higher number of cross-links in Alg-NP which might have contributed to their higher stability. A possible strategy to avoid the destabilization of Pec-NP in the saliva environment could be the addition of a polycation, such as chitosan, as a second cross-linker.[30]

The Chit-NP were the least stable formulation in artificial saliva. This could be due to the low zeta potential attained in artificial saliva after mixing (Figure 3c). In fact, a low particle charge could reduce the repulsion between the particles thus promoting aggregation. The reduction in the zeta potential could be a consequence of the increase in the pH from 6.0 to 6.8, and of the presence of anions (*e.g.*, sulfates, phosphates and carbonates) that could bind to the charged groups of chitosan, leading to bridging between the particles.[31]

**Cytotoxicity study**

Biocompatibility should always be investigated when designing new drug delivery biomaterials. Particularly, if they are intended for long-term administration. In this study, the human buccal cell line TR146, derived from a metastasis of a buccal carcinoma,[23] was used as a model for studying the cytocompatibility of the samples, as a first approximation to the biocompatibility with the buccal epithelium.

It needs to be noted that trial tests prior the experiment showed that Chit-NP tended to form aggregates in the cell medium (HBSS). This was probably due to the high pH of HBSS (~7.3), since the particles were stable in HBSS when the pH was reduced to 6.0 - 6.5. Due to this experimental constraint, the MTT test could not be carried out under pH conditions that would be suitable for both the stability of the particles and the cells viability and hence, it was conducted at the normal pH of HBSS (~7.3). For this reason, it was not possible to draw conclusions with certainty about the cytotoxicity of the particles with respect to their original particle size. However, a previous study has shown that the cytotoxicity of Chit-NP against other cell types was independent of their size;[32] in addition, the size of other types of positively charged nanoparticles was reported to be a negligible factor with respect to the cytotoxicity against TR146 cell line.[33] Therefore, the results of the cytotoxicity studies for Chit-NP must be considered with caution.

Chitosan is widely regarded as a biocompatible polysaccharide; however, the present study pinpointed out the possibility for the free chitosan to be cytotoxic against the cells of the buccal epithelium. Previous studies were in accordance with these results, since dose-dependent toxicity of chitosan has been reported against other types of cells.[34-37] The cytotoxicity of chitosan have been attributed to its positively charged groups that would

interact with the negatively charged components on the cellular membrane,[35, 36] causing neutralization and damage of the cell surface with consequent cell death.[38]

Chitosan in free soluble form was markedly more cytotoxic than the chitosan incorporated into the particles (Figure 4a), as also observed previously for chitosan nanocomplexes against a different cell line.[37] This difference of cytotoxicity may be ascribed to a different interaction of the two systems with the cell membranes. For example, by using Chit-NP, a lower amount of chitosan's charged groups could interact with the cell surface, because only the chitosan on the external surface of the particle would be available for the interaction. Moreover, since the cross-linker binds chitosan's positive charges, it is expected the addition of TPP to reduce the charge density on the chitosan chains, which would result in a lower interaction with the cells compared to free chitosan. The low cytotoxicity of Chit-NP indicated that Chit-NP could be relatively safe, and the decreased cytotoxicity when the concentration of the Chit-NP was reduced suggested a dose-dependent response.

Since the cytotoxicity of chitosan has been reported to be proportional to its density of positive charge,[39, 40] a strategy to further decrease the toxicity of the Chit-NP could be the reduction of the charge density on the particle surface. Nevertheless, this would represent a compromise with particle stability and bioadhesion that are promoted by a high charge density. A reduction of the charge density on the Chit-NP can be achieved, for example, by using higher cross-linker concentrations during the preparation of Chit-NP (as shown by the trend of the zeta potential in Figure 1) or by using chitosan with a lower DDA.

The biocompatibility of the formulations based on the negatively charged polysaccharides has been far less investigated than that of chitosan-based systems. In this study, Alg-NP and Pec-NP were shown to be cytotoxic (Figure 4b). However, alginate coated liposomes and pectin coated liposomes were previously reported to be non-cytotoxic against another cell line,[25] and

alginate nanoparticles cross-linked with calcium have been shown to have a good cyto- and biocompatibility.[41] Considering also the good cytocompatibility of the free polysaccharides detected in this study, the observed cytotoxicity of the particles was probably caused by the presence of Zn crosslinker. In fact, all the solutions tested containing free Zn presented a dose-dependent cytotoxicity. The higher cytotoxicity of the Alg-NP as compared to the Pec-NP could be ascribed to the higher concentration of Zn present in the Alg-NP. Zn toxicity has previously been documented in various kinds of cells,[42-44] and the expression of Zn cytotoxicity has been shown to occur following the uptake of the free Zn ions into the cells.[42, 44] In the case of the nanoparticles, the uptake of Zn into the cells could occur in its free form, if present partially free in the nanoparticulate formulations[13] or if released during the test. Alternatively, the nanoparticles could be uptaken into the cells and the Zn might be released intracellularly. We do not have at present experimental evidence to support either of these mechanisms, but future studies can address this aspect.

In the light of these considerations, strategies to reduce the toxicity of Alg-NP and Pec-NP could be developed. For instance, removing the solvent, possibly containing unbound Zn ions, could be replaced with Zn-free solvent; the toxicity of Pec-NP could be reduced also by preparing the nanoparticles with a lower Zn to polysaccharide ratio (Figure 2); calcium, which has been demonstrated to be safer than Zn,[45] could replace Zn as the cross-linker, or the replacement could also be on a partial basis in order to possibly retain some of the Zn antibacterial action. For example, in a previous study,[45] the toxicity of alginate-Zn microparticles was reduced by mixing batches of particles cross-linked with Zn and batches cross-linked with calcium.

Even though *in vitro* proof of principle plays a significant role in risk assessment, these tests cannot fully replicate clinical conditions, and Alg-NP and Pec-NP could be less toxic than they appear in the *in vitro* assay. In fact, the mucus layer present *in vivo* on the surface of the

cells was not present on the TR146 cells during the test. The mucus layer has been reported to delay the internalization of substances into the cells,[46] hence it could protect the cells from the toxicity of Zn. Moreover, the cells in the oral cavity present a rapid turnover, therefore by using the nanoparticle formulation for short periods of times, the damage could be not permanent in healthy individuals. In addition, the concentrations of Zn used in oral care products and in clinical trials are normally considerably higher [14, 47, 48] compared to the Zn concentrations tested in this experiment.

In conclusion, this study has shown that the cytotoxicity of the particles could be influenced not only by their concentration, but also by the amount retained and by the retention time in the oral cavity. Therefore, further investigations aimed at addressing such variables could be recommended to obtain more accurate indications about the possible toxicity of the Alg-NP and the Pec-NP in the oral cavity and also to confirm the possible safety of the Chit-NP.

In the present work, preliminary *in vitro* proof-of-concept studies were carried out to assess the possible application of polysaccharide-based nanoparticles as drug delivery systems addressed to the oral cavity, and solutions for the improvement of the particles investigated were suggested. This knowledge could possibly be extended to nanoparticles prepared with other ionically cross-linked polymers, thus facilitating the formulation of stable and safe nanoparticles for the oral environment.

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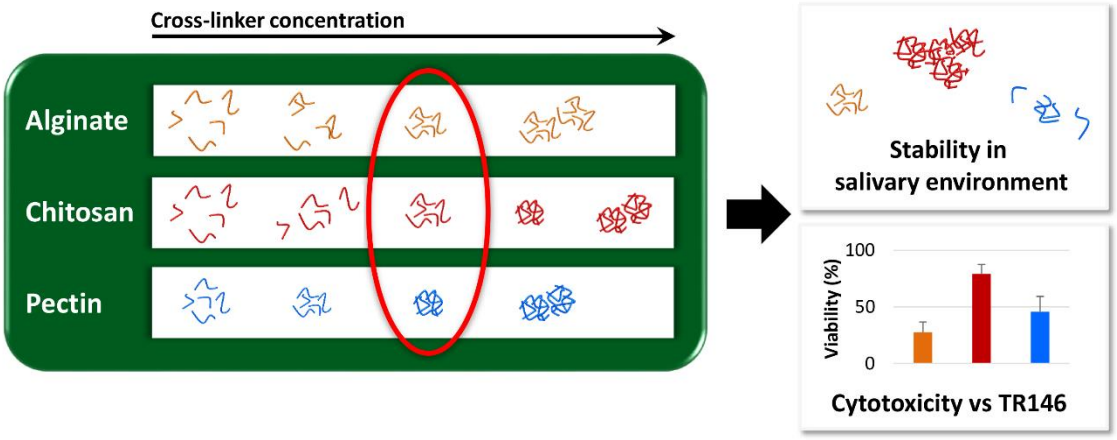
## GRAPHICAL ABSTRACT




For the first time, polysaccharide-based nanoparticles were studied as carriers addressed to the oral cavity. The particles were prepared through self-assembly. The mechanism of formation of the nanoparticles at increasing cross-linker concentration was disclosed in order to determine the formulations most suitable for the desired application. Moreover, the stability in a salivary environment and the cytotoxicity against the cells of the buccal epithelium were investigated and discussed. The alginate nanoparticles were the most stable in the salivary environment and the chitosan nanoparticles were the least toxic.