

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

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## **ABSTRACT**

The efficacy of treatments for oral ailments is often challenged by a low residence time of the conventional pharmaceutical formulations in the oral cavity. The residence time in the oral cavity could be improved by using bioadhesive formulations, such as preparations based on polysaccharides. This study describes the formulation and the evaluation of polysaccharide-based nanosystems as drug delivery systems addressed to the oral cavity. Nanoparticles based on chitosan, alginate or pectin were prepared through self-assembly by ionotropic gelation using oppositely charged crosslinkers (tripolyphosphate or zinc). Characteristics of nanoparticles at increasing crosslinker 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. Alginate nanoparticles were the most stable in the salivary environment, while chitosan nanoparticles were the most cytocompatible. Alginate nanoparticles and pectin nanoparticles revealed possible cytotoxicity due to the presence of zinc. This knowledge is important in the early design of polymer-based nanoparticles for oral usage and for potential improving of the biocompatibility of the investigated nanoparticles with the oral environment.

**Keywords:** nanoparticle; ionic gelation; oral cavity; drug delivery; artificial saliva; cytotoxicity.

**Abbreviations:** Alg-NP, alginate-based nanoparticles; Chit-NP, chitosan-based nanoparticles; HBSS, Hank's balanced salt solution; PDI, polydispersity index; Pec-NP, pectin-based nanoparticles; TPP, tripolyphosphate;  $Zn^{2+}$ , zinc cation.

## 1. INTRODUCTION

A common problem of conventional pharmaceutical formulations for the treatment of oral diseases is the short residence time in the oral cavity due to saliva clearance and oral muscular function. Formulations based on natural polysaccharides, which are generally regarded as biodegradable and biocompatible, may assure a prolonged effect of the agent due to their bio- and mucoadhesive properties and the possibility of providing controlled release (Pedro et al., 2009). 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 (Jayakaran and Arjunker, 2013). Despite the possible advantages of this type of formulation, only few studies have so far focused on the possible application of polysaccharide-based nanoparticles as local drug delivery systems designed for the oral cavity (Dung et al., 2007; Liu et al., 2007). Polysaccharide nanoparticles could be incorporated in conventional formulations (*e.g.* mouthwashes, dental varnishes, oral sprays) and for instance if covering the oral surfaces, they are expected to provide physical protection from environmental challenges.

The families of polysaccharides chitosan, pectin and alginate are commonly used in pharmaceutical formulations and medical devices. Chitosan consists of linear chains of glucosamine and N-acetyl-D-glucosamine, and is characterized by the number of acetylated monomers in relation to the total units, regarded as the degree of deacetylation (DDA%). The amine groups on the glucosamine can be protonated at acidic pH ( $pK_a \approx 6-7$ ) (Rinaudo et al., 1999), 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 ( $pK_a \approx 3-4$ ) (Imeson, 2011). Alginate is a linear block copolymer consisting of L-guluronates (G) and D-mannuronates (M), whose percentage in relation to the total characterize the polymer. Pectin is a ramified polymer consisting mostly of 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 crosslinkers, such as tripolyphosphate (TPP) for chitosan (Fan et al., 2012; Jonassen et al., 2012; Kleine-Brueggeney et al., 2015), and zinc cation ( $Zn^{2+}$ ) for alginate and pectin (Jonassen et al., 2013; Pistone et al., 2015, 2016).  $Zn^{2+}$  is commonly used in formulations for oral hygiene due to its antibacterial and anti-halitosis actions (Gjeramo and Saxton, 1991; Schmidt and Tarbet, 1978). Therefore, the inclusion of  $Zn^{2+}$  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 formulation factors, such as the crosslinker concentration (Fan et al., 2012; Kleine-Brueggeney et al., 2015; Pistone et al., 2015, 2016). Therefore, by fine tuning the formulation 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 can interact with the charged components of the particles and interfere with their electrostatic bonding and hydration forces (De and Robinson, 2003; Pistone et al., 2016; Santander-Ortega et al., 2011), thus causing instability. Since body fluids have different ionic strengths and compositions (Lentner, 1981), it was considered important to perform a study looking at the influence on the particles colloidal stability of the biological fluid present at the site of administration, namely in the simulated conditions of the saliva of the oral cavity. Natural saliva has a

complex and variable composition (Lentner, 1981; Nunes et al., 2015), and the use of an artificial saliva with known composition should therefore facilitate the understanding of the influence of the salivary constituents on the colloidal systems. In this work, the artificial saliva formula proposed by Gal et al. (2001) 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 (Lentner, 1981). 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 (Rykke et al., 1995).

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 a salivary environment and their cytotoxicity towards a buccal cell line. In fact, the evaluation of the toxicity at the site of administration is an important aspect regarding possible clinical application, especially when a delivery systems is designed to assure a long retention time.

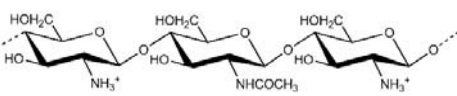
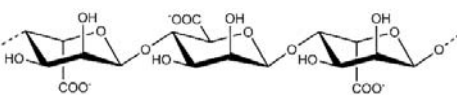
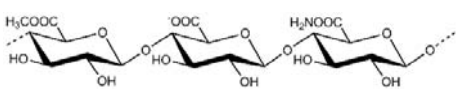
## **2. MATERIALS AND METHODS**

### **2.1 Materials**

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 (Nguyen et al., 2011; Pistone et al., 2015). Sodium tripolyphosphate pentabasic (purity  $\geq 98.0\%$ ) and Triton X-100 (t-Octylphenoxyethoxyethanol) were supplied by Sigma-Aldrich (Germany), and zinc

chloride (purity  $\geq 98.0\%$ ) by Merck (Germany). Hank's balanced salt solution modified with calcium and magnesium (HBSS) was purchased from Sigma SAFC Biosciences Ltd. (UK). The water was purified through a Millipore Milli-Q system with 0.22 $\mu\text{m}$  Millipak® 40 filter (Millipore™, Ireland).

**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% <sup>§</sup>	Novamatrix - FMC Biopolymer (Norway)
Sodium alginate (Protanal® LF 10/60)		Mv 147 kDa <sup>†</sup> G 65–75% <sup>§</sup>	FMC BioPolymer (Norway)
Pectin sodium salt (Genu® pectin LM-102 AS)		Mw 96 kDa <sup>‡</sup> DE 30% <sup>§</sup> DA 19% <sup>§</sup>	CPKelco (Denmark)

\* Jonassen et al. (2012)

<sup>†</sup> Pistone et al. (2015)

<sup>‡</sup> Nguyen et al. (2011)

<sup>§</sup> Information given by the supplier

## 2.2 Preparation of nanoparticles

The nanoparticles tested were prepared using self-assembly by ionotropic gelation. Alginate nanoparticles (Alg-NP) and pectin nanoparticles (Pec-NP) were prepared through ionic crosslinking of the negatively charged polysaccharides with the  $\text{Zn}^{2+}$ , and chitosan nanoparticles (Chit-NP) were prepared with the positively charged chitosan crosslinked with the anion tripolyphosphate (TPP). A solution of the polysaccharide (0.063%, w/w) and a solution of the crosslinker (0.03 - 0.13 %, w/w) were prepared in 0.05 M NaCl and filtered with 0.80  $\mu\text{m}$  Millex® AA and 0.22  $\mu\text{m}$  Millex® GV syringe filters (Millipore™, Ireland), respectively. Fifteen ml of crosslinker solution was dripped into 60 ml of polysaccharide

solution in a 100 ml vial (5 cm diameter) under magnetic stirring at 600 rpm (cylindrical stirring bar 6 mm × 20 mm) at room temperature. The mixed amounts of polysaccharide and crosslinker solution were the same for all the formulations tested. The flow of the crosslinker solution, controlled using a peristaltic pump, was 9.3 and 3.5 ml min<sup>-1</sup> for Zn<sup>2+</sup> and TPP solutions, respectively. The samples were stirred for ten minutes and stored overnight at room temperature for equilibration. They were characterized and used for further experiments without dilution. The pH of the formulations tested for stability in artificial saliva was adjusted to 6.0 ± 0.1 by adding small amounts of 0.02 M NaOH.

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 crosslinker amount was varied (Table 2) by changing its concentration in the added crosslinker solution. All the test samples were prepared at least in duplicate. Solutions of chitosan and pectin in the absence of the crosslinker were also prepared (0.05% polysaccharide in 0.05M NaCl).

**Table 2.** Test formulations.

Nanosystem	Crosslinker : 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*

\* Used for stability studies in artificial saliva and toxicity studies.

### 2.3 Physical characterization

The formulations were characterized according to size and zeta potential at 25 °C (unless differently indicated) using a Malvern Zetasizer Nano ZS (ZEN3600, Malvern Instruments,

UK) fitted with a red laser light beam ( $\lambda = 632.8$  nm). The size was determined by dynamic light scattering with non-invasive back scattering (DLS-NIBS) at a scattering angle of  $173^\circ$ . From the cumulants fit of the autocorrelation data, the size distributions 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 autocorrelation function was fitted with the general purpose fitting method by the Zetasizer software (version 7.11), and the hydrodynamic diameters of the particles were calculated using the Stokes-Einstein equation.

The surface zeta potential ( $\zeta$ ) of the nanoparticles was determined by mixed laser Doppler electrophoresis and phase analysis light scattering (M3-PALS). The Smoluchowski approximation for the Henry equation was used to calculate the zeta potential:  $U = \epsilon\zeta/\eta$ , with U as the electrophoretic mobility of the particles after the application of an electric field.

The refractive index, the viscosity ( $\eta$ ), and the dielectric constant ( $\epsilon$ ) of pure water at the relevant temperature (either 25 or 37 °C), were given and used as constant parameters by the equipment's software in the calculations. The data obtained were the average of three and five measurements on the same sample aliquot for each batch, for the determination of the size and the zeta potential, respectively. No dilution of the sample was performed prior to the measurement.

A 744 Metrohm pH meter (Metrohm, Switzerland) and a MP 220 Mettler Toledo pH meter (Mettler Toledo, Switzerland) were used for measuring the pH. The pH measurements were carried out at room temperature after calibration of the pH meter between pH 4 and 7 or between pH 2 and 4, depending on the pH of the sample.



## 2.4 Stability in simulated salivary fluid

Artificial saliva was prepared, as described by Gal et al. (2001). Briefly, the following salts (mg) were dissolved in 1 L of MilliQ water: 125.6 NaCl, 963.9 KCl, 189.2 KSCN, 654.5  $\text{KH}_2\text{PO}_4$ , 200.0 urea, 336.5  $\text{Na}_2\text{SO}_4$ , 178.0  $\text{NH}_4\text{Cl}$ , 227.8  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , and 630.8  $\text{NaHCO}_3$ . The pH was adjusted to 6.8 by bubbling  $\text{CO}_2$  gas through the solution before each experiment.

An aliquot of 750  $\mu\text{l}$  of artificial saliva (or pure water for control) was mixed with 250  $\mu\text{l}$  of each nanoparticulate suspension and kept at 37 °C for 2 h. During this time, z-average, PDI and size distributions were recorded every  $\sim 2$  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 5 min after mixing in order to achieve temperature equilibration. The refractive index, the viscosity, and the dielectric constant of pure water at 37 °C were used for calculations. All data were collected in triplicate.

## 2.5 Cytotoxicity studies

**Changing the medium of the nanoparticulate systems.** Prior to the 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 solvent of the nanoparticles was substituted with cell medium through dialyzation overnight of 1 ml of nanoparticulate formulation against 6 ml of medium at room temperature under constant magnetic stirring. The dialysis membrane (Spectra/Por<sup>®</sup> Float-A-Lyzer<sup>®</sup> G2, Spectrum Laboratories Inc., USA) had a molecular weight cut-off of 20 kDa. Sample dilution due to osmotic stress could be avoided by dissolution of 300 mg dextran (Sigma-Aldrich

GmbH, Germany) into the medium prior to dialysis (Mw declared by the producer 512 kDa). The presence of dextran caused a slight up-concentration of the nanoparticulate suspension (Vauthier et al., 2008), and the original concentration of the sample was restored through dilution with the medium. Solutions of polysaccharides and crosslinkers were prepared directly in HBSS.

**MTT assay.** The cell line TR146 (Sigma-Aldrich GmbH, Germany) was used for the cytotoxicity studies as a model cell line for the buccal epithelium (Rupniak et al., 1985). The cells were cultured as described previously (Kaiser et al., 2015), and cells of passage number 29-33 were used. One hundred  $\mu\text{l}$  of suspension of TR146 ( $\sim 10^4$  cells) was seeded in each well of a 96-well culture plate. The plate was incubated 24 h at 37 °C with 5% CO<sub>2</sub> (Sanyo MCO-19AIC, Panasonic Biomedical Sales Europe BV, Netherlands) to allow for the attachment of the cells. After the removal of the culture medium, the cells were rinsed twice with 100  $\mu\text{l}$ /well HBSS. Test samples or control solutions (100  $\mu\text{l}$ /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-Octylphenoxyethoxyethanol, Sigma-Aldrich, Germany) in phosphate buffer saline was used as positive control (0% survival). The samples and control solutions were then removed and replaced with 100  $\mu\text{l}$  of supplement-free medium. Twenty-five  $\mu\text{l}$  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 the plate was incubated for 4 h at 37 °C. Thereafter, the medium was removed and the resulting dye crystals were dissolved in 100  $\mu\text{l}$  DMSO. The absorbance (A) was quantified at  $\lambda = 570$  nm in a microplate reader (Safire, Tecan AG, 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:

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

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 nanoparticle formulations and their single components were investigated for cytotoxicity. The samples tested are listed in Table 3. The ratios of crosslinker to polysaccharide in the nanoparticulate formulations tested corresponded to the following crosslinker concentrations (*w/w*): 0.009% TPP in Chit-NP (15:85), 0.013% Zn<sup>2+</sup> in Pec-NP (20:80), and 0.027% Zn<sup>2+</sup> in Alg-NP (65:35).

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

Samples	Concentrations (% , <i>w/w</i> )
Chit-NP, Alg-NP, Pec-NP	Undiluted, diluted 3 : 5 ( <i>v:v</i> ) in the media
Chitosan, alginate, pectin	0.05%
TPP	0.009%
Zn <sup>2+</sup>	0.006%, 0.013%, 0.020%, 0.027%

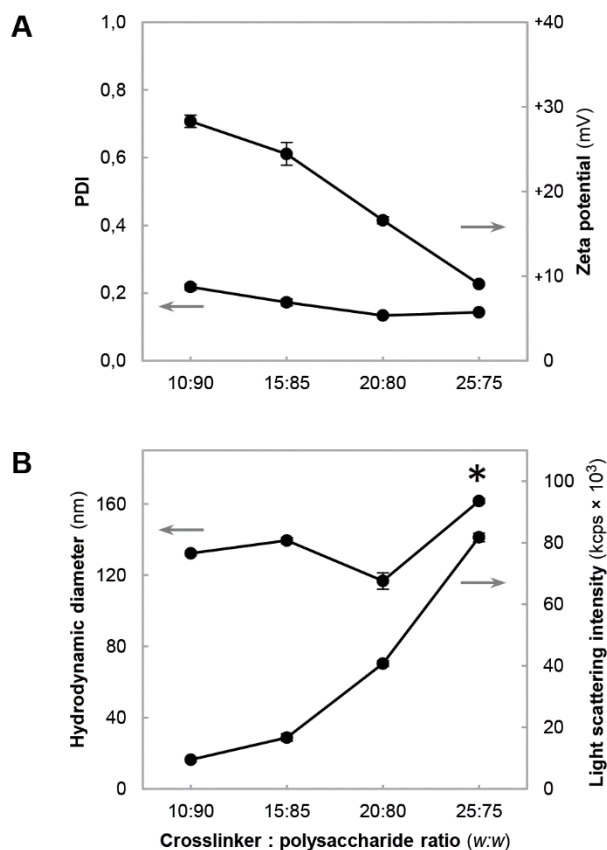
### 3. RESULTS AND DISCUSSION

#### 3.1 Formulation of the nanoparticles

The crosslinker amount has previously been shown to be of importance when preparing polysaccharide-based nanoparticles (Fan et al., 2012; Kleine-Brueggeney et al., 2015; Pistone

et al., 2015, 2016). In this study, the crosslinker-to-polysaccharide ratio of the formulations was investigated with the aim to examine the process of formation of the nanoparticles, and consequently enable to establish the formulations that are most suited as drug delivery carriers for the oral cavity. Both positively and negatively charged nanoparticles were studied due to their differing abilities to promote bio- and mucoadhesion in the oral cavity. Positively charged nanoparticles and chitosan have been reported to adhere strongly to the negatively charged surfaces of teeth, mucosa, and mucin due to the formations of electrostatic bonds (Adamczak et al., 2016; Menchicchi et al., 2014; Nguyen et al., 2010; Takeuchi et al., 2003). On the other hand, negatively charged particles with a low charge are less repelled by the mucosa than highly negatively charged particles (Takeuchi et al., 2003).

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). This suggested that, in the absence of the crosslinker (TPP), the chitosan chains were free, or in the form of randomly sized, loosely packed aggregates, as previously observed for alginate (Pistone et al., 2015). The physical characteristics of the chitosan-TPP formulations at increasing ratio of TPP to chitosan are illustrated in Figure 1. As soon as the TPP was added, the nanoparticles formed spontaneously, as indicated by the low polydispersity (PDI 0.22 and monomodal size distribution) which confirmed the presence of homogenously sized particles.



**Figure 1.** Physical characteristics of chitosan-TPP formulations as a function of the crosslinker-to-polysaccharide ratio of the undiluted samples measured at 25°C: (A) Polydispersity index (PDI) and zeta potential, and (B) z-average hydrodynamic diameter and scattered intensity of chitosan-TPP formulations at increasing ratios of TPP to chitosan (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.

The scattered intensity increased exponentially with increasing ratio of TPP to chitosan. An increase in scattered intensity can be due to an increase in either the size or the compactness of the particles. Therefore, the increase in scattered intensity combined with a nearly constant particle size when the TPP-to-chitosan ratio was increased from 10:90 to 15:85 (Figure 1B)

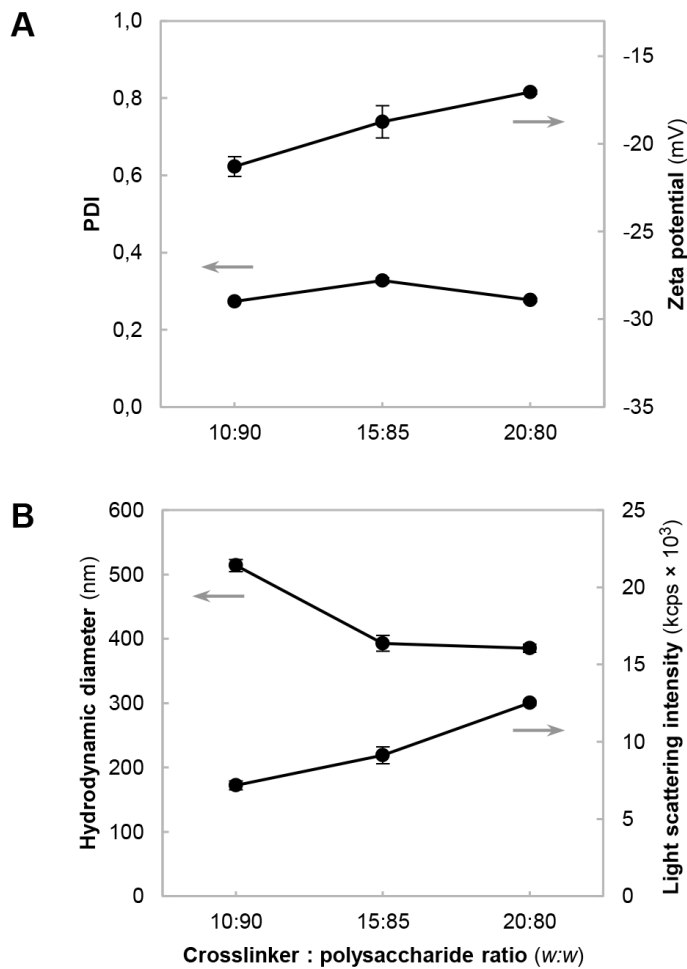
could also be diagnostic of the increase in the compactness of the particles. This could be due to the addition into the nanoparticles of chitosan chains that, at 10:90 TPP-to-chitosan ratio, were in excess and free in solution. A similar result was observed in a previous study (Jonassen et al., 2012), where, by increasing the TPP-to-chitosan ratio, the aggregation number (number of polysaccharide chains associated together to form a particle) increased. 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 particle size when the TPP-to-chitosan ratio increased to 20:80 was probably due to the formation of intra-particle crosslinks, thus reducing the electrostatic repulsion between the chitosan chains and increasing the ionic crosslink density within the particles (Huang and Lapitsky, 2011). Consequently, the particles shrank and became more compact, as evidenced by the increase in scattered intensity. The formation of mainly intra-particle 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. At a ratio of 25:75, the size increase and the macroscopic aggregation after one day of storage were probably due to the excessive inter-particle crosslinking (Fan et al., 2012; Huang and Lapitsky, 2011) and to reduced electrostatic repulsion between the Chit-NP, since the pH of the solvent also increased as more basic TPP was added (Fan et al., 2012).

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

The samples containing only pectin in solution presented a low PDI (0.28), a monomodal size distribution (average size  $584 \pm 6$  nm) and a relatively high scattered intensity ( $\sim 7100$  kcps). Unlike the chitosan solutions, these results revealed the presence of homogeneously sized particles. The aggregation of pectin chains into nanoparticles in the absence of  $Zn^{2+}$  (when using 0.05M NaCl as the solvent) has previously been discussed (Jonassen et al., 2013) and was explained by inter and intra-molecular interactions, such as hydrogen bonding.

The characteristics of the pectin- $Zn^{2+}$  nanoparticle formulations at increasing ratios of  $Zn^{2+}$  to pectin are illustrated in Figure 2. The size distributions were monomodal and the PDI was relatively constant for all the pectin- $Zn^{2+}$  formulations. The particle size reduction and the increase in scattered intensity at increasing ratios of  $Zn^{2+}$  to pectin (Figure 2B) could indicate that the crosslinks formed by  $Zn^{2+}$  caused a simultaneous shrink in size and an increase in compactness of the particles previously formed in its absence. This could indicate a different mechanism of formation for Pec-NP compared to the Chit-NP. The results for the formulation containing at  $Zn^{2+}$ -to-pectin ratio 25:75 were not recorded due to immediate aggregation and precipitation, which was probably due to the formation of inter-particle crosslinks and to particle neutralization caused by the addition of acidic  $Zn^{2+}$  solution.



**Figure 2.** Physical characteristics of pectin-Zn<sup>2+</sup> formulations as a function of the crosslinker-to-polysaccharide ratio of the undiluted samples measured at 25°C: (A) PDI and zeta potential, and (B) average diameter (z-average) and scattered intensity of pectin-Zn<sup>2+</sup> formulations at increasing Zn<sup>2+</sup>-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.

All the pectin-based formulations tested, except the formulations containing 25:75 Zn<sup>2+</sup>-to-pectin ratio, could be suitable for drug delivery purposes. At increasing Zn<sup>2+</sup>-to-pectin ratios, the zeta potential became less negative. The formulation containing a Zn<sup>2+</sup>-to-pectin ratio of 20:80 was chosen for further investigation due both to the least negative zeta potential and to



the highest content of  $Zn^{2+}$  that could be advantageous for a drug delivery system targeted at the oral cavity.

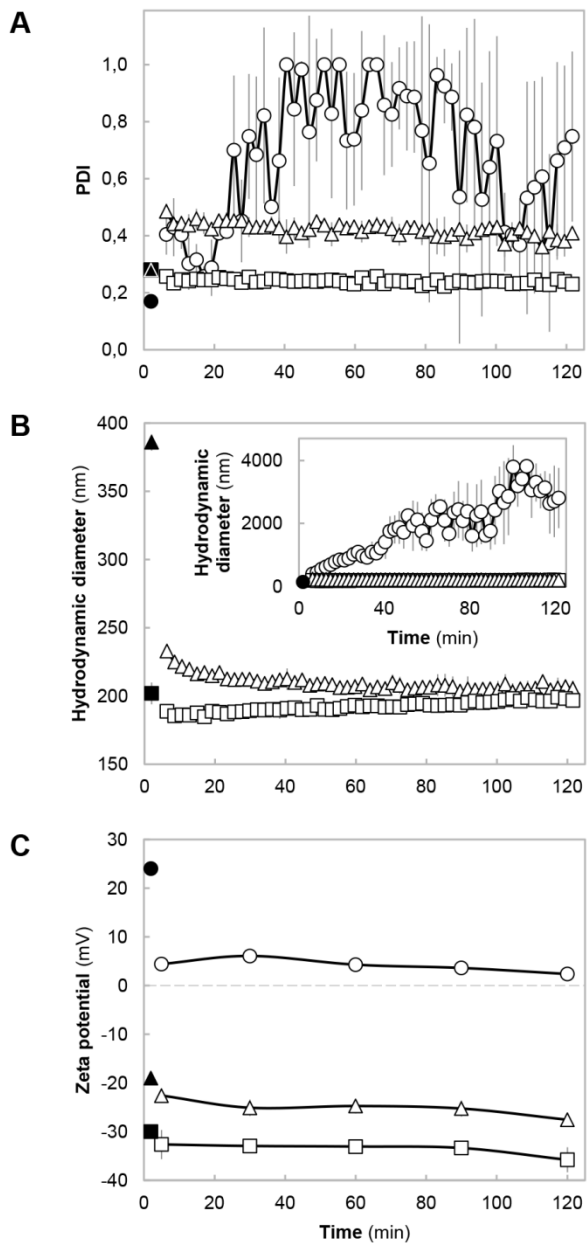
The characterization of Alg-NP at increasing  $Zn^{2+}$ -to-alginate ratios have been thoroughly described in a previous study by our group (Pistone et al., 2015), where at a  $Zn^{2+}$ -to-alginate ratio below 35:65 the particles were not fully formed, while higher ratios caused particle aggregation (Pistone et al., 2015, 2016). In contrast to the situation for Chit-NP and Pec-NP, no shrinking was detected when increasing the crosslinker-to-polysaccharide ratio. This might be attributed to a possible high compactness of the particles, or to the stiffness of the alginate chains that could discourage changes in the conformation. Since fully formed and non-aggregated particles were present at 35:65  $Zn^{2+}$ -to-alginate ratio, this ratio was considered as the most suitable for drug-delivery purposes.

In summary, the formulations obtained with increasing amounts of crosslinker in the three addressed systems (alginate, chitosan, and pectin) suggested 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. The selected formulations also showed a good reproducibility, as indicated by the low standard deviations obtained for all the characteristics of the nanoparticles (Figures 1 and 2).

### **3.2 Stability of the particles in simulated salivary fluid**

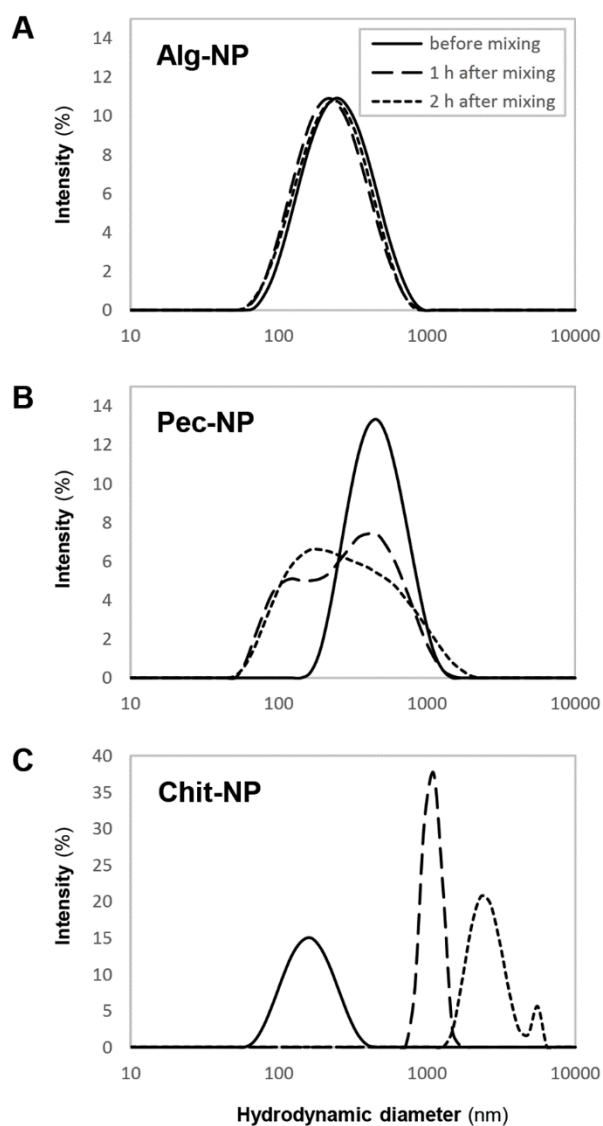
The most important causes of the instability of ionically crosslinked nanoparticles are the aggregation of the nanoparticles, and disintegration due to breakage of the crosslinks. Even though less dramatic, the potential role of shrinking and swelling of the particles, resulting in

changes in particle size, cannot be ruled out. 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, are depicted in Figure 3 (black markers). A control test was performed by mixing the nanoparticulate systems with MilliQ water instead of artificial saliva in order to check the influence of 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 observed variations were exclusively due to the content of the artificial saliva.



**Figure 3.** Stability of alginate-Zn<sup>2+</sup> (□), pectin-Zn<sup>2+</sup> (Δ), and chitosan-TPP (○) 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). Alg-NP were the most stable in artificial saliva. In fact, their 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 and relatively narrow at all the time points (Figure 4A). This was unexpected because the crosslinks between alginate and divalent cations, such as  $Zn^{2+}$ , can be broken in the presence of phosphates that can chelate the divalent cations (Gombotz and Wee, 2012). However, the presence of calcium in the artificial saliva could have prevented the removal of  $Zn^{2+}$  (Gombotz and Wee, 2012), since calcium itself can be chelated by phosphates and could also form stabilizing crosslinks on the Alg-NP (Sarmiento et al., 2007).



**Figure 4.** Exemplary plots of the intensity-based size distributions of (A) Alg-NP, (B) Pec-NP, and (C) Chit-NP measured prior to the mixing with artificial saliva, and 1 and 2 hours after mixing.

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 (Figure 3A), and the size distributions were broad at all time points (Figure 4B). 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). 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 of their partial disintegration. Shrinking can occur if further crosslinks are formed (Figure 2B) or when the charges are screened 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 suggest a partial disintegration, as would be expected from the emergence of new populations of particles of varying sizes. The disintegration of the Pec-NP may stem in the interaction of the cationic crosslinker with the anionic species in the solution (such as phosphates) which may have led to removal of the  $Zn^{2+}$  until an equilibrium was reached.

Even though both Pec-NP and Alg-NP consisted of polyuronates crosslinked with  $Zn^{2+}$ , the stability of the Alg-NP was superior to the Pec-NP. This result might be attributed to a higher alginate- $Zn^{2+}$  affinity, as compared to pectin- $Zn^{2+}$ , that could prevent the displacement of  $Zn^{2+}$  (*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  $Zn^{2+}$  was used for the preparation of Alg-NP compared to Pec-NP. This probably caused the formation of a larger number of crosslinks in Alg-NP, which might have contributed to their higher stability. A possible strategy to avoid the disintegration of Pec-NP in the saliva environment could be the use of a polycation (*e.g.* chitosan) as a second crosslinker or as the only crosslinker, as commonly done for alginate-based formulations (Gombotz and Wee, 2012; Sarmiento et al., 2007).

The Chit-NP were the least stable formulation in artificial saliva. The PDI of the Chit-NP increased markedly immediately after mixing (Figure 3A) 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 (Figure 3B and 4C). 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 (Figure 3C); the low zeta potential could be the cause of the low stability. In fact, a low particle charge could reduce 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, (Fernandes et al., 2005) leading to screening of charges and bridging between the particles.

Beside ions, other components of natural saliva could possibly interact with nanoparticles, such as charged molecules. In particular, mucins, which are glycoproteins negatively charged at neutral pH, could interact electrostatically with the positively charged Chit-NP, thus promoting further aggregation in natural saliva (Menchicchi et al., 2014). Pectin and alginate are expected to present weaker interactions with mucin compared to chitosan, nevertheless mucin might still influence the stability of Alg-NP and Pec-NP in natural saliva. For example, the presence of mucin could lead to depletion flocculation (Sarkar et al., 2009) or the chains of mucin could bind the negatively charged nanoparticles through electrostatic bridging mediated by *e.g.* calcium cations. For this reason, further investigations in the presence of other salivary components and in natural saliva are recommended.

### **3.3 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 (Rupniak et

al., 1985), was used as an *in vitro* model for studying the cytocompatibility of the samples, as a first approximation to the biocompatibility with the buccal epithelium.

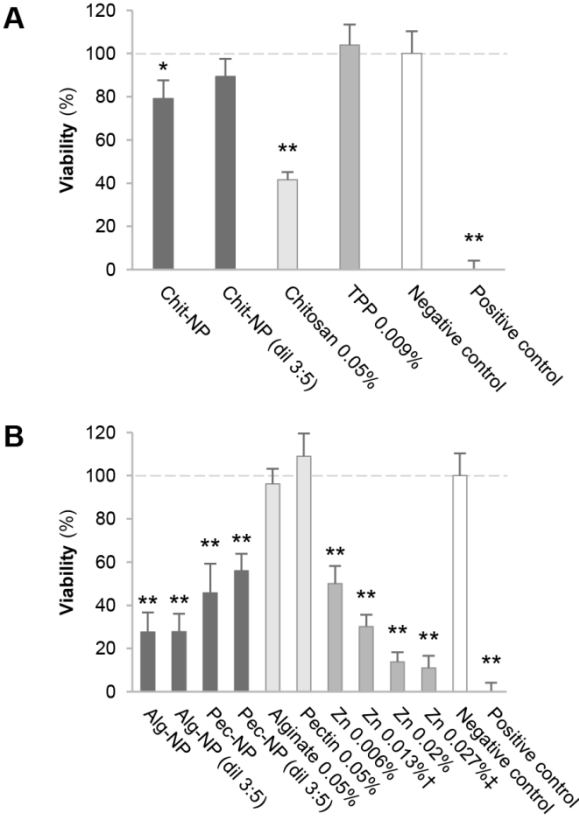
It should be noted that the trial tests prior to the experiment showed that, while Alg-NP and Pec-NP were stable in the cell medium (HBSS or HBSS dilution), Chit-NP tended to form aggregates. This was probably due to the high pH of HBSS (~7.3), since the Chit-NP were stable in HBSS when the pH was reduced 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 nanoparticles and cells viability, and hence, it was conducted at the normal pH of HBSS. For this reason, it was not possible to draw any certain conclusions 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 (Nasti et al., 2009). In addition, the size of other types of positively charged nanosystems was reported to be a negligible factor with respect to the cytotoxicity against TR146 cell line (Smistad et al., 2007). Therefore, despite the formation of aggregates during the test, the results of the cytotoxicity studies for Chit-NP could still possibly be considered somewhat meaningful, but must be considered with caution.

Figure 5A 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. 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.

These results are in accordance with results of previous studies where dose-dependent toxicity of chitosan has been reported against other types of cells (Carreño-Gómez and Duncan, 1997; Hafner et al., 2015; Hasegawa et al., 2001; Huang et al., 2004). The cytotoxicity of chitosan, proportional to its density of positive charge (Kean and Thanou, 2010; Wei et al, 2015), has



been attributed to the interaction of the positively charged groups with the negatively charged components on the cellular membrane (Carreño-Gómez and Duncan, 1997; Huang et al., 2004).



**Figure 5.** Relative viability assessed by MTT assay of TR146 cells treated with the samples for 4 h: (A) Chit-NP and their components, and (B) Alg-NP, Pec-NP and their components. The error bars indicate the pooled standard deviations. † and ‡ Zn<sup>2+</sup> 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.

The viability of the cells treated with Chit-NP was doubled compared to the viability of the cells treated with chitosan alone. Therefore, chitosan in free soluble form was markedly more cytotoxic than the chitosan incorporated into the particles (Figure 5A), as has also been observed previously for chitosan nanocomplexes against a different cell line (Hafner et al., 2015). In fact, the presence of the cross-linker in the Chit-NP is expected to reduce the charge density on the chitosan chains of the particles, thus resulting in a lower cytotoxicity.

Moreover, the difference in cytotoxicity between Chit-NP and free chitosan could also be ascribed to a different interaction of the two systems with the cell membranes. For example, by using Chit-NP, a smaller 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. The low cytotoxicity of Chit-NP indicated that these particles could be relatively safe, and the viability not significantly different from the negative control recorded when the Chit-NP were diluted on a 3:5 ratio (v/v) suggested a dose-dependent response.

A strategy to further decrease the toxicity of the Chit-NP could to reduce the charge density on the particle surface, *e.g.* by using higher crosslinker concentrations (Figure 1) or chitosan with a lower degree of deacetylation. Nevertheless, this would represent a compromise since particle stability and bioadhesion are usually promoted by a high charge density.

The biocompatibility of the formulations based on the negatively charged polysaccharides has been far less investigated than that of chitosan-based systems. Figure 5B shows the cell viability after treatment with Alg-NP, Pec-NP or the separate components during 4 h. Alg-NP and Pec-NP were cytotoxic, since they significantly reduced the cell viability both when applied at the full concentration and when diluted 3 to 5, and the dilution of the particles did not significantly increase the viability for both Alg-NP and Pec-NP. However, neither of the solutions of free alginate or pectin (0.05%) changed the viability compared to the negative control. Moreover, alginate coated liposomes and pectin coated liposomes were previously

reported to be non-cytotoxic against another cell line (Adamczak et al., 2016), and alginate nanoparticles crosslinked with calcium have been shown to have a good cytocompatibility (Zhang et al., 2009). Consequently, the observed cytotoxicity of the particles was probably caused by the presence of  $Zn^{2+}$  crosslinker. In fact, in the range of concentrations and time course tested, all the solutions containing free  $Zn^{2+}$  reduced the cell viability in a dose-dependent manner. The cells treated with diluted samples of Pec-NP displayed a significantly higher viability ( $p < 0.05$ ) compared to the diluted Alg-NP. This could be ascribed to the higher concentration of  $Zn^{2+}$  present in the Alg-NP.

$Zn^{2+}$  toxicity has previously been documented in various kinds of cells (Borovanský and Riley, 1989; Kappus and Reinhold, 1994; Shen et al., 2013), and the expression of  $Zn^{2+}$  cytotoxicity has been shown to occur following the uptake of the free  $Zn^{2+}$  into the cells (Borovanský and Riley, 1989; Shen et al., 2013). In the case of the nanoparticles, the uptake of  $Zn^{2+}$  into the cells could occur in its free form, if the  $Zn^{2+}$  was present as partially free in the nanoparticulate formulations (Pistone et al., 2016) or if it was released during the test. Alternatively, the nanoparticles could be taken up into the cells and the  $Zn^{2+}$  might be released intracellularly. We do not have at present experimental evidence to support either of these mechanisms, and future studies can address this aspect. Nevertheless, the decrease of the  $Zn^{2+}$  concentration in the formulations, *e.g.* through replacement with the less cytotoxic calcium (Jay and Saltzman, 2009), is expected to restrain the cytotoxicity of Alg-NP and Pec-NP.

Even though *in vitro* proof of principle plays a significant role in risk assessment, these tests cannot fully replicate clinical conditions and, in some case, has been found to be overly sensitive compared to *in vivo* situations (Czajkowska-Kośnik et al., 2015). Consequently, 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 oral mucosal cells was not present on the

TR146 cells during the test. The mucus layer has been reported to delay the internalization of substances into mucosal cells (Diebold et al., 2007), hence it could protect the cells from the toxicity of  $Zn^{2+}$ . Moreover, the cells in the oral cavity are subject to rapid turnover, therefore by using the nanoparticle formulation for short periods of times, the damage would be not permanent in healthy individuals. In addition, the concentrations of  $Zn^{2+}$  used in oral care products and in clinical trials are normally considerably higher (Gombotz and Wee, 2012; Mehdipour et al., 2011; Saxton et al., 1986) compared to the  $Zn^{2+}$  concentrations tested in this experiment.

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 to confirm the possible safety of the Chit-NP.

#### **4. CONCLUSIONS**

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 targeted to the oral cavity. The preparations obtained with increasing amounts of cross-linker disclosed that the process of formation of the nanoparticles might be different depending on the type of polysaccharide employed. The process of formation of the particles was revealed to be important information in order to select the formulations expected to be the most suitable for drug delivery purposes.

In addition, the stability in a salivary environment and the toxicity against the cells of the buccal epithelium were also assessed. The Alg-NP were the most stable in artificial saliva (at

least two hours), while Pec-NP and especially Chit-NP were unstable. On the other hand, the most cytocompatible preparations were the Chit-NP, while both Alg-NP and Pec-NP displayed cytotoxicity at the concentrations and time course investigated. Their cytotoxicity seemed to be caused by the presence of  $Zn^{2+}$ .

All the tested types of particles presented advantages and weaknesses for use in the oral cavity, therefore further optimization and refinement is required for all the formulations. The results of the study enabled to determine possible causes in case of poor performance of the nanosystem, therefore ideas for the improvement of the particles investigated could be suggested. This knowledge could possibly be extended to nanoparticles prepared with other ionically crosslinked polymers, thus facilitating the formulation of stable and safe nanoparticles for the oral environment.

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The authors declare no conflict of interest.

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