Polysaccharide-coated liposomal formulations for dental targeting

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ABSTRACT

The efficacy of treatments of oral ailments is often challenged by a low residence time of the conventional pharmaceutical formulations in the oral cavity, which could be improved by using bioadhesive formulations. This *in vitro* study investigated charged liposomes, both uncoated and coated through electrostatic deposition with polysaccharides (chitosan, alginate and pectin), as bioadhesive systems for the oral cavity. First, formulations that provided liposomes fully coated with polysaccharide were selected. Thereafter, the stability of both the uncoated and the polysaccharide-coated liposomes was investigated in artificial saliva simulating pH, ionic strength, and ionic content of natural saliva. Additionally, adsorption to hydroxyapatite (model for tooth enamel) was tested. The surface charge was of high importance for both the stability in salivary environment and bioadhesion. In artificial saliva, the negatively charged liposomes were the most stable, and the stability of the positively charged liposomes was improved through coating with a negatively charged polysaccharide. On the contrary, the positively charged liposomes were the most bioadhesive, although a moderate adsorption was recorded for the negatively charged liposomes. Based on the present results, the negatively charged liposomes seem to be the most promising formulations used as a tooth adhesive nanosystem and could as such provide improved treatment of tooth ailments.

KEYWORDS: dental drug delivery; polymer-coated liposome; oral cavity; hydroxyapatite; adsorption; artificial saliva.

ABBREVIATIONS: Alg-Lip, alginate-coated liposomes; Chit-Lip, chitosan-coated liposomes; HA, hydroxyapatite; Neg-Lip, negatively charged uncoated liposomes; PDI, polydispersity index; Pec-Lip, pectin-coated liposomes; Pos-Lip, positively charged uncoated liposomes.

1. INTRODUCTION

The burden of oral conditions seems to have increased worldwide in the last decades (Marcenes et al., 2013). Oral diseases are usually treated or prevented through local administration of therapeutic agents (Silk, 2014). However, conventional local treatments are often challenged by a low residence time in the oral cavity due to salivary secretion, swallowing, food intake and abrasive actions of the soft tissue. For this reason, substances that cannot adhere to oral surfaces are effective only for a limited time after application (Cummins and Creeth, 1992). However, bioadhesive formulations might improve the retention time of active substances in the oral cavity, and consequently, as such improve the efficacy of the treatments (Paderni et al., 2012).

Several types of bioadhesive delivery systems for the treatment of oral ailments have been extensively investigated (Paderni et al., 2012). In particular, bioadhesive nanosystems, such as liposomes, have been shown to be advantageous since their small size could allow to reach sites inaccessible to other types of formulations, and furthermore, can be formulated for site specific targeting (Ozak and Ozkan, 2013; Zupancic et al., 2015). The oral mucosa is the most studied adhesion site for bioadhesive formulations in the oral cavity (Paderni et al., 2012). Even though less investigated, the tooth surfaces could represent another important targeting site especially for the treatment of tooth ailments, such as tooth erosion and dental caries (Beyer et al., 2010; Lee et al., 2012; Ozak and Ozkan, 2013). Liposomal formulations could be particularly of interest for tooth targeting, since, in addition to site specific drug delivery, they could also provide physical protection through a biomimetic effect. In fact, in physiological conditions, components of saliva are selectively adsorbed onto tooth enamel surfaces, thus forming the acquired enamel pellicle, which provides protection *e.g.* against acid attacks, mineral loss and abrasive factors (Siqueira et al., 2012). Similarly, the liposomes adsorbed onto the tooth surface could provide physical tooth protection.

Little information is available in the literature regarding the adsorption of liposomes onto the tooth surface. Previous studies indicated that the adsorption of liposomes onto hydroxyapatite (HA), the main component of the tooth enamel, is mainly based on electrostatic interactions, and it can be influenced by the presence of saliva (Nguyen et al., 2013; Nguyen et al., 2010). Moreover, saliva has previously been shown to influence also the stability of the liposomal suspensions (Nguyen et al., 2011b, 2013). This knowledge can be complemented by investigations regarding how the salivary components influence the adsorption of liposomes to HA and the liposomal stability in saliva, and this information could provide the rational for the improvement of tooth adhesive formulations. Nevertheless, natural saliva is an extremely complex and variable fluid (Lentner, 1981; Nunes et al., 2015). Therefore, the understanding of the specific influence of the salivary constituents can be facilitated by using artificial saliva with a relatively simple composition and salivary components at known concentrations, as in previous studies regarding other nanosystems (Pistone et al., 2017; Sarkar et al., 2009).

The application of liposomes as drug carriers is well known and their coating with specific molecules has been exploited for improving their properties. For example, coating of liposomes with polymers have been reported to increase the bioadhesive capacity (Filipović-Grčić et al., 2001; Takeuchi et al., 2003), modulate the release and the entrapment efficiency of drugs (Filipović-Grčić et al., 2001), as well as improve the stability of the system by preventing liposomal oxidation or aggregation (Mady et al., 2009; Meland et al., 2014). In addition, the influence of pectin coatings on tooth adhesion has previously been studied (Nguyen et al., 2013), and has been suggested to improve the strength of adhesion between the liposomes and the tooth enamel. Charged polysaccharides, such as chitosan, pectin and alginate, could be a rational choice for the coating of liposomes for oral applications in virtue of their biocompatibility, bioadhesive and HA adhesive properties (Beyer et al., 2010; Lee et al., 2012).

On this basis, in the present study liposomal coatings consisting of chitosan, pectin and alginate were investigated. Liposomes coated with charged polysaccharides can be prepared through ionic complexation between charged liposomes and an oppositely charged polysaccharide. In order to obtain stable coated liposomes, only polysaccharide concentrations in a specific range can be used (McClements, 2005), which are generally determined through empirical studies. While chitosan-coated liposomes have been widely studied (Chun et al., 2013; Filipović-Grčić et al., 2001; Guo et al., 2003; Laye et al., 2008; Mady et al., 2009; Quemeneur et al., 2010; Takeuchi et al., 2003), pectin- and especially alginate-coated liposomes have received limited attention (Alund et al., 2013; Klemetsrud et al., 2013; Nguyen et al., 2011a; Nguyen et al., 2013; Sriamornsak et al., 2008).

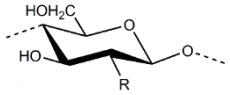
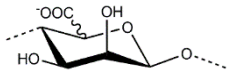
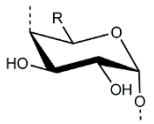
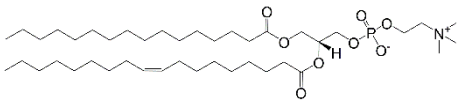
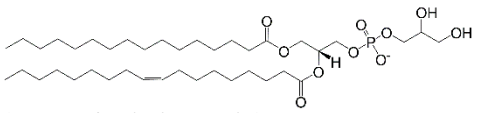
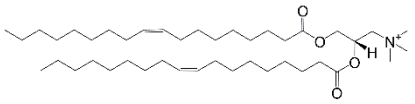
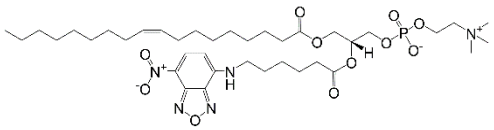
The aim of the present study was to investigate the preparation and the characteristics of liposomes coated with chitosan, pectin, or alginate. Thereafter, the stability of the liposomal formulations was tested in an artificial saliva containing only salivary electrolytes in physiological concentrations (Gal et al., 2001). Furthermore, the formulations were tested for *in vitro* adsorption onto HA in the presence of either phosphate buffer or artificial saliva.

2. MATERIALS AND METHODS

2.1 Materials

The polysaccharides and lipids used in the study are listed in Table 1. The pectin and the alginate were purified prior to utilization as previously described (Nguyen et al., 2011a; Pistone et al., 2015). The chloroform used for the liposome preparation was of analytical grade. A Millipore Milli-Q system with 0.22µm Millipak® 40 filter (Millipore™, Ireland) was used to purify the water. Triton X-100 (t-Octylphenoxypolyethoxyethanol) was obtained from Sigma-Aldrich GmbH (Germany). Spray-dried HA powder with homogeneous particle diameter of 10.0 ± 2.0 µm was purchased from Fluidinova (Portugal).

Table 1. Polysaccharides and lipids used in the study.

	Name	Chemical structure and characteristics		Supplier
Polysaccharides	Chitosan chloride (Protasan™ UP CL 213)	 R = NH_3^+, or $NHCOCH_3$	Mw 307 kDa[a] DDA 83%[d]	Novamatrix (FMC Biopolymer, Norway)
	Sodium alginate (Protanal® LF 10/60)		Mv 147 kDa[b] G 65–75%[d]	FMC BioPolymer (Norway)
	Pectin sodium salt (Genu® pectin 150 USA-SAG)	 R = COO^-, or $COOCH_3$ (Simplified structure)	Mw 110 kDa[c] DE 70.2%[d]	CPKelco (Germany)
Lipids	Egg-PC (Egg-phosphatidylcholine)	 (Structure of predominant species)		Lipoid GmbH (Germany)
	Egg-PG (egg L-α-phosphatidylglycerol)	 (Structure of predominant species)		Lipoid GmbH (Germany)
	DOTAP (Dioleyl trimethylammoniumpropane)			Avanti Polar Lipids, Inc. (USA)
	NBD-PC (Nitrobenzoxadiazol-4-yl-phosphocholine)			Avanti Polar Lipids, Inc. (USA)

[a] Jonassen et al. (2012)
[b] Pistone et al. (2015)
[c] Nguyen et al. (2011a)
[d] information given by the supplier

2.2 Preparation of the liposomes

The liposomes were prepared according to the thin-film method as previously described (Nguyen et al., 2011a). In short, the lipid film was formed through rotary evaporation of a lipid solution in chloroform at 40 °C (Heidolph W 2001 rotavapor, Heidolph Instruments GmbH & Co. KG, Germany). The lipid solution contained 89 mol% of egg-PC; 1 mol% of NBD-PC, a fatty acid labeled fluorescent phospholipid; and 10 mol% of the charged lipids, egg-PG or DOTAP, for the preparation of negatively and positively charged liposomes, respectively. In order to remove thoroughly the chloroform, the film was freeze dried overnight (Christ Alpha 2–4 freeze drier, Christ, Germany). The film was then hydrated with a 5 mM phosphate buffer (pH 6.8) for two hours at room temperature, and stored overnight in the refrigerator. The size reduction was obtained by extrusion (Lipex extruder, Lipex Biomembranes Inc., Canada) repeated ten times through two-stacked 200 nm polycarbonate membranes (Nucleopore®, Costar Corp., USA) at room temperature. The samples were stored overnight in the refrigerator under nitrogen atmosphere, to avoid oxidation of the lipids, before characterization, coating, and further experiments. The lipid concentration in the final preparations was 3 mM.

2.3 Coating of the liposomes with polysaccharides

The coating of the charged liposomes was achieved through electrostatic deposition by mixing the liposomal dispersion with a solution of oppositely charged polysaccharide. The liposomes were coated by dripping 2 ml of liposomal dispersion into 8 ml of polysaccharide solution (prepared in phosphate buffer 5 mM, pH 6.8) in a 10 ml vial (2.2 cm diameter), under constant magnetic stirring (cylindrical stirring bar 12 x 2.5 mm) at about 480 rpm. The addition of the liposomal dispersion was performed using a peristaltic pump at a constant flow of 1.7 ml min^{-1}. The samples were stirred for ten minutes and characterized after storage overnight in the refrigerator under nitrogen

atmosphere. The lipid concentration in the final preparations was 0.6 mM. Both uncoated and coated liposomes were prepared at least in triplicate. All the liposomal dispersions were diluted to a concentration of 0.3 mM with a 5 mM phosphate buffer immediately prior to characterization (the uncoated liposomes were diluted 1:10 and the polysaccharide coated liposomes 1:2).

2.4 Characterization of the liposomes

Size determination. A Zetasizer Nano ZS (ZEN3600, Malvern Instruments Ltd., UK) with a red light laser (λ = 633nm) was used for determination of the size through dynamic light scattering (DLS) at 25 °C. A backscattered detection was used at a scattering angle of 173°. The refractive index and the viscosity of pure water at 25 °C were employed as constant parameters in the calculations. The autocorrelation function, obtained through the fluctuations of the intensity of the scattered light, was fitted by the Zetasizer software (version 7.11) with the general purpose fitting method. The Stockes-Einstein equation was used to calculate the hydrodynamic diameters of the liposomes. The software provided the mean size (z-average) and the polydispersity index (PDI). The measurement was carried out after temperature equilibration. The size and PDI obtained for each batch were the average of three measurements on the same sample aliquot.

Zeta potential. The Zetasizer Nano ZS also determined the zeta potentials of the liposomes at 25 °C through the laser Doppler electrophoresis technique. The zeta potential (ζ) was calculated using the Smoluchowski approximation for Henry equation: $U = \varepsilon\zeta/\eta$, where U is the electrophoretic mobility of the particles, measured by the instrument after the application of an electric field. The dielectric constant (ε) and the viscosity (η) of pure water at 25 °C were used for the calculations. The measurement was carried out after temperature equilibration. The result obtained for each batch was the average of five measurements on the same sample aliquot.

pH. The pH of the samples was measured at room temperature using a 744 Metrohm pH meter (Metrohm, Switzerland), calibrated between pH 4 and 7.

2.5 Determination of the optimal polysaccharide concentrations for the liposome coating

The negatively charged liposomes (Neg-Lip) were coated with chitosan, and the positively charged liposomes (Pos-Lip) were coated with alginate or pectin. The concentrations of the different polysaccharides used for coating are listed in Table 2 and represent the concentration of the solutions before the coating process.

Table 2. Polysaccharide concentrations investigated for the coating of the liposomes.

Concentration (%, *w/w*)	**Alginate**	**Pectin**	**Chitosan**
0.001	•		
0.002	•		
0.003	•	•	
0.005	•	•	•
0.01	•	•	•
0.02	•	•	•
0.03	•	•	•
0.04	•	•	•
0.05	•	•	•

2.6 Stability of the liposomes in artificial saliva

Artificial saliva was prepared according to Gal et al. (2001) by dissolving the following salts in 1 L of MilliQ water: 125.6 mg NaCl, 963.9 mg KCl, 189.2 mg KSCN, 654.5 mg KH_2PO_4, 200.0 mg

urea, 336.5 mg Na_2SO_4, 178.0 mg NH_4Cl, 227.8 mg $CaCl_2 \cdot 2H_2O$, and 630.8 mg $NaHCO_3$. The pH was adjusted to 6.8 by bubbling CO_2 gas before each experiment.

Cuvettes were prepared containing 750 µl artificial saliva or pure water (as a control) mixed with 250 µl of liposomal dispersion (lipid concentration of 0.6 mM, polysaccharide concentration 0 or 0.04%). The samples were characterized during storage at 37 °C for two hours. No further dilution was performed prior to characterization. The first measurement was performed five minutes after mixing for temperature equilibration. The data were collected in triplicate for both samples and controls. The size was determined every second minute on the same cuvette (for each repetition). The zeta potential was measured every 30 minutes using a different cuvette for each time point. The refractive index, viscosity, and dielectric constant of pure water at 37 °C were used for calculations.

2.7 Adsorption of the liposomes to HA powder

Spray-dried HA powder was weighed in glass tubes (0 for blank, 20, 60, 100, 200, 300, and 400 mg) and then 2 ml of either a 5 mM phosphate buffer or artificial saliva were added as medium. The suspensions were mixed using a rotator for about 30 minutes at room temperature (5 rpm, rotator model LD-79, Labinco BV, The Netherlands) and then stored overnight for equilibration. The following day the tubes were mixed on the rotator (5 rpm) in a heating cabinet at 35°C to re-suspend the powder and equilibrate the temperature. Further 2 ml of the medium and 2 ml of the liposomal dispersion (lipid concentration of 0.6 mM, polysaccharide concentration 0 or 0.04%), both at 35 °C, were added to each tube of suspended HA, followed by mixing on the rotator at 35 °C for 5 minutes (20 rpm). The tubes were then centrifuged for 10 minutes at 1100 rpm (centrifuge 5430 R and rotor F-35-6-30, Eppendorf AG, Germany) and the supernatants, containing the non-adsorbed liposomes, were collected.

The supernatant of the blank, representing 100% non-adsorbed liposomes, was diluted with the medium in order to prepare the references for a six-point calibration curve. Aliquots of 100 µl from each supernatant and each reference for the calibration curve were transferred to a 96-well plate (Nunc™, Denmark) and were then mixed with 100 µl of 2% (w/w) Triton in water. After 30 minutes, the fluorescence was measured at 535 nm (λ_{ex} 485 nm) using a plate reader (Victor^3 Multilabel, PerkinElmer, Finland). The percentage of liposomes adsorbed onto HA was calculated as follows:

Adhered liposomes (%) = 100 – *non-adhered liposomes* (%)

The tests were carried out in triplicate for each liposomal preparation.

3. RESULTS AND DISCUSSION

3.1 Determination of the optimal polysaccharide concentrations for coating of the liposomes

The surface of liposomes can be coated with oppositely charged polymers through ionic complexation (Chun et al., 2013; Laye et al., 2008; McClements, 2005; Nguyen et al., 2011a; Sriamornsak et al., 2008). A complete coating of the liposome is crucial for the stability of the colloidal system. In fact, a complete saturation of the particle surface, achieved by using a polymer concentration above a specific value leads to reversal of the particle charge and, thus, to stabilization through electrostatic repulsions. When the concentration of the polymer is too low, the particles tend to aggregate through bridging flocculation (McClements, 2005). A high excess of polymer could also lead to instability of the system when attractive depletion forces are strong enough to overcome the repulsive forces (depletion flocculation). For this reasons, the purpose of this experiment was to determine the range of polysaccharide concentrations that provided stable coated liposomes.

The adsorption of the polysaccharides onto the liposomal surface can be monitored through the measurement of variations in the zeta potential and the particle size before and after the coating process (Guzey and McClements, 2006). Figure 1a and 1b show the zeta potentials and the mean size of the particles, respectively, before and after mixing of the uncoated liposomes with increasing concentrations of the polysaccharides. The uncoated liposomes (Neg-Lip and Pos-Lip) presented the same size of ~140 nm and an opposite zeta potential (-51 mV and +35 mV, respectively). The samples prepared with the lowest polysaccharide concentrations for each type of polysaccharide (0.005% chitosan, 0.001% alginate, 0.003% pectin) were excluded from characterization since macroscopic aggregation and precipitation occurred immediately after preparation. These polysaccharide concentrations could correspond to the concentrations needed for neutralization of the charge on the liposomal surface resulting in aggregation and flocculation, as previously observed during the coating of liposomes with chitosan (Chun et al., 2013; Laye et al., 2008).

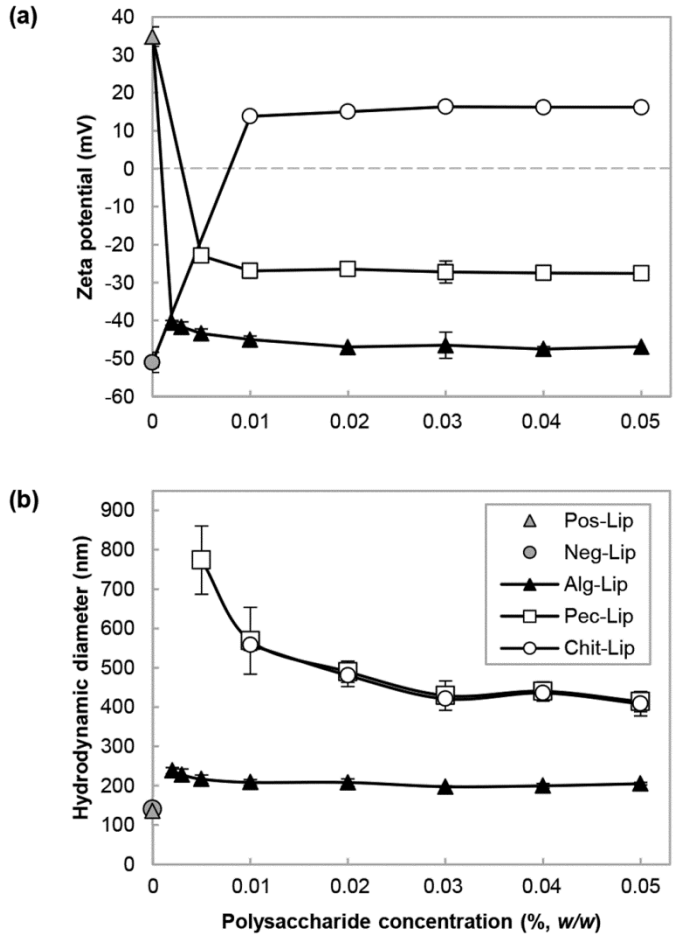


Figure 1. Coating of the liposomes with polysaccharides. Influence of the polysaccharide concentration (a) on the zeta potential and (b) on the mean diameter (z-average) of the polysaccharide-coated liposomes (final lipid concentration of 0.6 mM). The PDI was below 0.45 for all the formulations characterized. 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.

The success of the coating process was proven by the reversal of the zeta potentials of the uncoated liposomes resulting from the deposition of a layer of oppositely charged polysaccharide on the surfaces. When the polysaccharide concentration was increased, the zeta potential reached a plateau for all the types of coated liposomes (Figure 1a). The zeta potential is correlated to the charge density on the surfaces of the particles (Guzey and McClements, 2007). The highest charge density

at the plateau was observed for the alginate-coated liposomes (Alg-Lip) whereas a lower charge density was observed for the chitosan-coated liposomes (Chit-Lip) and the pectin-coated liposomes (Pec-Lip). This may be correlated to the charge density of the free polysaccharides, since at pH 6.8 the chitosan chains were weakly charged (pKa 6.5-7) (Rinaudo et al., 1999) and the alginate chains strongly charged (pKa 3-4) (Imeson, 2011). This was confirmed by the zeta potentials of the polysaccharide solutions (0.04%) in 5 mM phosphate buffer, which were -53 mV for alginate and +19 mV for chitosan. Pectin has a pKa similar to alginate (Imeson, 2011). However the different monomer compositions of pectin and alginate, with more charged groups on alginate, might have induced a lower charge density on pectin, as indicated by the less negative zeta potential (-28 mV).

The largest mean size of the coated liposomes was recorded in the samples with the lowest concentrations of polysaccharide. When the polysaccharide concentration was further increased, the apparent size tended to decrease until a plateau was reached (Figure 1b). This was probably due to the adsorption of polysaccharide chains increasing the coverage of the liposomal surface, thus causing a decrease of the apparent size by diminished bridging flocculation (McClements, 2005). Full surface saturation probably prevented further polysaccharide adsorption by creating an electrostatic barrier, therefore a relatively constant size value, a plateau, was observed. The size of the liposomes in the presence of polysaccharide at the plateau was larger than the size measured for the corresponding uncoated liposomes. This supported the hypothesis of the presence of an adsorbed layer of polysaccharide onto the liposomal surface. At the highest polysaccharide concentrations aggregation did not occur, therefore, depletion flocculation was not observed.

The size and hence the thickness of the polysaccharide layer was markedly smaller for the Alg-Lip compared to the Chit-Lip and the Pec-Lip (Figure 1b). The thickness of the layer may be influenced by various characteristics of the polysaccharide, such as chain length, charge density, charge distribution, rigidity, and degree of branching (Guzey and McClements, 2006). These parameters

are expected to influence the amount and the conformation of the adsorbed polysaccharide, and also the compactness of the layer.

In solution, a highly charged polysaccharide has an extended conformation and tends to be adsorbed onto an oppositely charged surface in this form, thus lying totally flat ("trains") (Bajpai, 1997). The strong electrostatic attraction between the polysaccharide and the surface, and the repulsion between different polysaccharide chains would promote the formation of a thin and compact layer. Since alginate has a strong negative charge, it implies the formation of a flat coating, confirmed by the low size increase. A polysaccharide with a low charge density tends to take the conformation of a random coil in solution and will be adsorbed to the surface in such a state, thus the polysaccharide chain forms loops anchored to the surface by only fractions of its length ("loops-and-tails") (Bajpai, 1997) and higher amounts of polysaccharide can be adsorbed. As a consequence, a thick and loose polysaccharide layer is formed (Guzey and McClements, 2006). In accordance with previous studies (Claesson and Ninham, 1992; Filipović-Grčić et al., 2001; Guzey and McClements, 2007; Klemetsrud et al., 2013), chitosan and pectin could coat the liposomes by this mechanism since they have a lower net charge than alginate. Moreover the branched structure of the pectin (Klemetsrud et al., 2013) could also have contributed to the formation of a thick polysaccharide layer on the liposomal surfaces.

The PDI of both types of uncoated liposomes was 0.11-0.12, while the PDI at the plateau was 0.15 for the Alg-Lip and 0.24-0.25 for the Pec-Lip and the Chit-Lip. This indicates that all types of liposomes exhibited a relatively narrow size distribution, even though the coated liposomes had a size distribution somewhat broader compared to the uncoated. A reason for this behavior could be the irregular shape of the coated liposomes caused by the presence of polysaccharide. In addition, the low increase of PDI for the Alg-Lip may be explained by a flatter conformation of alginate on the liposomal surface, thus preserving the original spherical shape of the liposomes.

The reproducibility of the data for both the particle size and the zeta potential showed that the coating procedure was suitable for the preparation of polysaccharide-coated liposomes. To be inside the polysaccharide concentration range that provides fully coated liposomes, a polysaccharide concentration of 0.04% (in the added solution) was chosen for all formulations used for further experiments.

3.2 Stability of the liposomes in artificial saliva

Human saliva has a complex and variable composition, containing 98-99% water and a variety of components, such as electrolytes, nitrogenous products, glucose, metabolites, hormones, vitamins and different peptides and proteins (Lentner, 1981; Nunes et al., 2015), and might as such be regarded as an ultrafiltrate of serum. The ions present in the physiological environment could influence the stability of the liposomal formulations, due to the charged nature of the liposomes and to the major role that the ionic interactions play in the deposition of the polysaccharide coating onto the liposomes (Bajpai, 1997; Guzey and McClements, 2006; Guzey and McClements, 2007). For this reason, the stability of the liposomal systems in an artificial saliva containing the major salivary electrolytes, with a concentration, ionic strength, and pH that mimicked natural saliva, was studied (Gal et al., 2001; Lentner, 1981). None of the components of artificial saliva interfered with the DLS measurements, in contrary to components of natural saliva (Rykke et al., 1995). Therefore, the stability of the liposomes could be monitored by measuring the size, polydispersity and zeta potentials. Negligible variations of the characteristic of the liposomes were observed in the control tests performed by mixing the liposomes with water instead of artificial saliva. This indicated that the increase in temperature to 37 °C and the dilution of the samples during the tests did not affect the characteristics of the formulations, which confirmed that the liposome modifications observed in artificial saliva were induced only by the composition of the artificial saliva itself.

One of the most common causes for instability of colloidal particles is aggregation. Aggregation can be initiated, for example, by the reduction of repulsion forces between the liposomes, by bridging due to cross-linking of the charged surfaces, or to partial desorption of the polysaccharide coating thus leading to bridging flocculation. Another modification of the characteristics of the liposomes, even though less dramatic compared to aggregation, is represented by changes in the size of the single liposomes. For example, the size of the polysaccharide-coated liposomes can vary due to modifications of the thickness of the coating layer.

The characteristics of the liposomes measured during the test are illustrated in Figure 2. In general, the largest change of the liposomal characteristics was recorded immediately after mixing with artificial saliva, suggesting that the interactions between the electrolytes and the liposomes occurred rapidly. The zeta potentials measured in artificial saliva remained relatively constant for at least two hours for all formulations (except Chit-Lip) (Figure 2a and b). However, the zeta potential (absolute value) measured in artificial saliva for all the formulations was lower compared to the zeta potential measured before mixing the liposomes with artificial saliva. This can be attributed to the electrostatic screening of the liposomal surface caused by the electrolytes present in artificial saliva (Guzey and McClements, 2007; Sarkar et al., 2009).

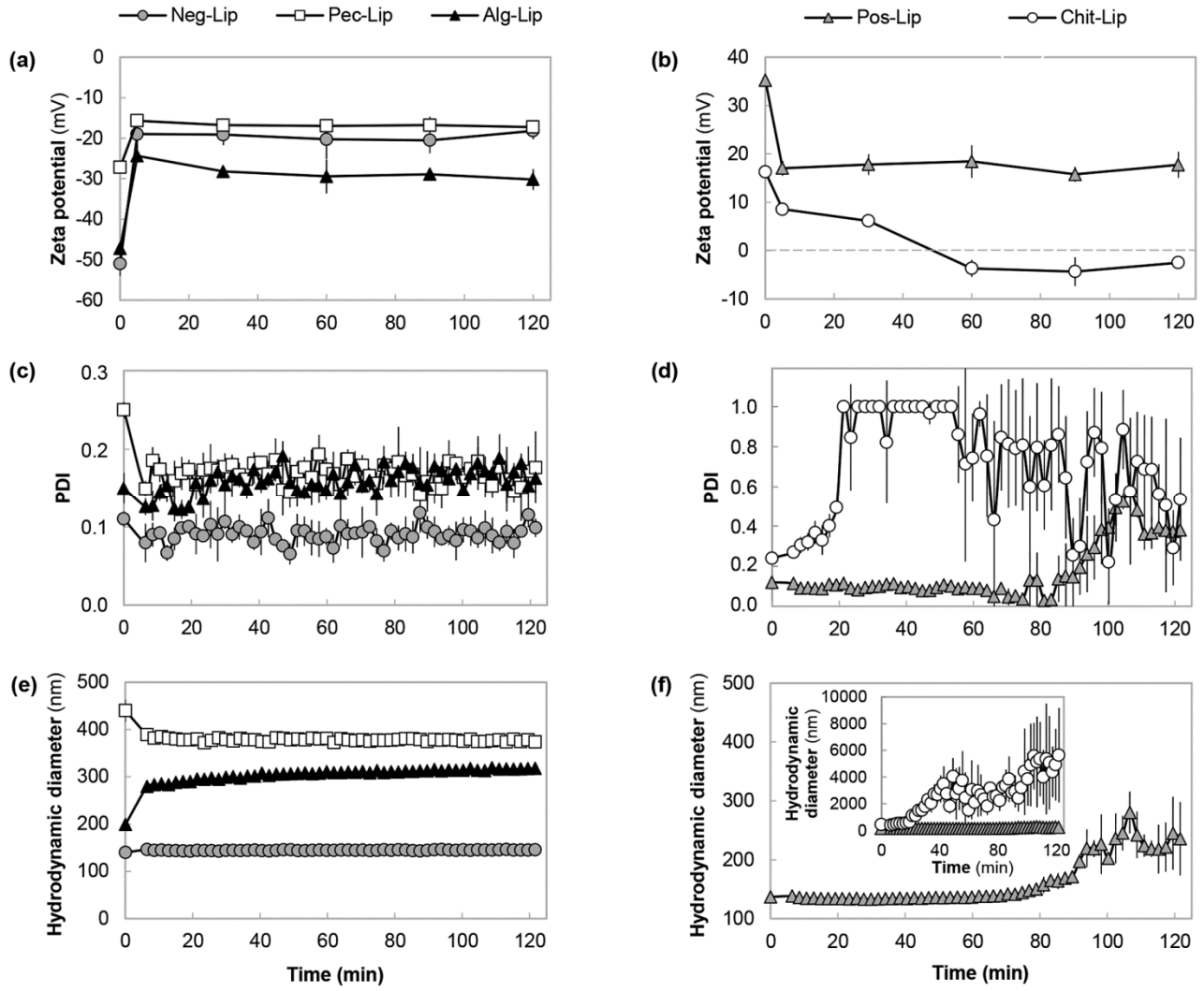


Figure 2. Stability in artificial saliva. Zeta potential (a and b), PDI (c and d), and average diameter (e and f) in artificial saliva over time for the liposomes with a negative surface (left) and a positive surface (right), respectively. At 0 min the characteristics measured before mixing with artificial saliva are indicated. The average of three separate experiments for each sample is shown and the error bars represent standard deviation. The points without error bars have standard deviations equal to or smaller than the size of the markers.

The Neg-Lip were the most stable formulations, since they maintained the same PDI and size as recorded before the test for at least two hours after mixing with artificial saliva (Figure 2c and 2e). This also indicated that the reduction in the surface charge was not sufficient to reduce the repulsion forces between the liposomes and cause aggregation.

During the tests, the Alg-Lip maintained for at least two hours the same PDI value as recorded before mixing with artificial saliva. Nonetheless, the average size increased ~80 nm immediately after mixing with artificial saliva, and further increased ~40 nm to a plateau during the following two hours. The constant low PDI suggests that this increase in size was not caused by the formation of randomly sized aggregates of liposomes; rather, a change in the conformation of the alginate adsorbed onto the liposomal surface could have explained the size increase (Guzey and McClements, 2006). In fact, the electrostatic screening, occurring after mixing with artificial saliva, could reduce the charge both on the alginate backbone and on the surface of the liposomes. As a consequence, the formation of a less compact coating with increased loop-and-tail fractions could explain the increased thickness of the coating layer (Bajpai, 1997; Claesson and Ninham, 1992; Guzey and McClements, 2006).

During the two hours of incubation in artificial saliva, the Pec-Lip maintained the same size and PDI; however, the size after mixing with artificial saliva was ~60 nm smaller and the PDI was ~0.1 lower (Figure 2e and 2c). Similarly, a decrease in size has previously been observed when mixing pectin-coated nanoemulsion droplets with saline (Guzey and McClements, 2007). This size reduction has been explained by the weakening of the electrostatic repulsion between the pectin molecules in the adsorbed layer through electrostatic screening, which allowed the pectin chains to pack more closely. This explanation could also be relevant for the size reduction of the Pec-Lip in the present study. Alternatively, the electronic screening in artificial saliva could have reduced the charges on the pectin and caused its partial desorption from the liposomal surface (Guzey and McClements, 2006; Guzey and McClements, 2007). Bridging flocculation could have been avoided

due to the possible presence of several layers of pectin on the surfaces of the liposomes (Guzey and McClements, 2007) or for stabilization induced by the reduction of the charges on the pectin and on the liposomal surface due to electrostatic screening (Pistone et al., 2015). The swell of the alginate layer on Alg-Lip in artificial saliva, explained by the loss of bonds between the liposome and the alginate, could support the hypothesis of the partial desorption of pectin from Pec-Lip. However, partial desorption for the Alg-Lip was possibly limited by the higher charge of the alginate chains compared to pectin, which would have maintained the alginate chains bound to the liposomal surface.

The type of charge (positive or negative) on the liposomal surface was shown to be important to avoid aggregation in artificial saliva. In fact, unlike the negatively charged liposomes, the positively charged liposomes (Pos-Lip and Chit-Lip) aggregated in artificial saliva. This aggregation was indicated by the simultaneous increase in the average PDI and size caused by the formation of randomly sized liposome clusters (Figure 2d and f), and by the presence of macroscopic precipitates. Coating with alginate or pectin could, therefore, represent a strategy to avoid the aggregation of the Pos-Lip, since Alg-Lip and Pec-Lip did not present signs of aggregation during the tests. A previous study described the possibility for negatively charged liposomes to aggregate in natural saliva depending on the type of negative group on the liposomes (Nguyen et al., 2011b). This aggregation was ascribed to the interaction with divalent cationic calcium in solution. In the present study, the calcium concentration available for binding was possibly higher than in natural saliva, due to the absence of calcium binding proteins in artificial saliva (Bennick et al., 1981; Gal et al., 2001). Nonetheless, the liposomes with the negative surfaces did not present signs of aggregation, hence they could be promising for local use in the oral cavity.

Previous studies have observed that negatively charged colloidal particles were superior compared to positively charged with respect to stability in human saliva. In fact, the presence of negatively charged molecules in human saliva, such as mucins and proline-rich proteins, has been suggested to

initiate aggregation of positively charged colloidal particles (Nguyen et al., 2011b; Silletti et al., 2007a, b). However, the results in the present study indicate that the electrolytes in saliva also could play a role in the stability of nanoparticulate systems, since the positively charged liposomes were shown to aggregate also in the presence of the salivary electrolytes only.

The Pos-Lip were more stable in artificial saliva than the Chit-Lip, as the size and PDI in artificial saliva remained the same as measured before mixing until after ~80 minutes, when aggregation occurred. The zeta potential of the Pos-Lip was relatively high in artificial saliva, comparable to the zeta potential (absolute value) of the stable Neg-Lip. For this reason, the aggregation cannot be attributed to a reduction of the surface charge. Instead, the multivalent anionic species present in artificial saliva (such as sulfates, carbonates and phosphates) could have led to electrostatic bridging between the liposomes.

Regarding the Chit-Lip, the aggregation could also derive from a weakened electrostatic repulsion between the liposomes, as indicated by the low zeta potential measured after mixing with artificial saliva (Guzey and McClements, 2007). Moreover, a charge reversal occurred on the Chit-Lip after ~60 minutes, which probably originated from a progressive desorption of chitosan from the liposome surfaces due to electrostatic competition (Guzey and McClements, 2006). This could have led to aggregation through bridging flocculation between the uncoated and the coated areas of different liposomes (Guzey and McClements, 2007). Consequently, the stability of the Chit-Lip in artificial saliva might be improved by increasing the charge density of the coating, thus maintaining the repulsion forces between the liposomes and preventing desorption of the coating. This can be achieved, for example, by using a chitosan with a higher degree of deacetylation, or by increasing the concentration of both the negative lipid and the chitosan in the formulations (Alund et al., 2013). Nevertheless, possible interactions with other negatively charged components of human saliva, such as mucins, might be promoted.

3.3 Adsorption of the liposomes to HA powder

The coating of liposomes is usually exploited in drug delivery to improve their bioadhesive properties (Takeuchi et al., 2003). In the present study, the adsorption of both uncoated and polysaccharide-coated liposomes was tested against HA, a widely used model substance for the dental enamel (Beyer et al., 2010; Faraj et al., 2007; Lee et al., 2012; Liu et al., 2007; Nguyen et al., 2013; Nguyen et al., 2010). The HA powder used for the adsorption test was in the form of homogeneously sized microspheres to have a constant HA surface per weighed amount and to possibly avoid variations of surface roughness that could influence the adsorption of liposomes.

The amount and concentration of liposomes in the test samples was kept constant and the amount of HA powder was increased. The percentages of liposomes adsorbed to HA in phosphate buffer are illustrated in Figure 3. All the formulations showed a linear increase in the adsorption of liposomes to HA when the HA amount was increased, and the Pos-Lip and the Chit-Lip presented also a plateau corresponding to the complete adsorption of the liposomes present in the sample. This indicated that all types of liposomes were able to adsorb to some extent to the HA surfaces. A small excess of free polysaccharide might be present in the preparations of polysaccharide-coated liposomes (see section 3.1). The free polysaccharide could compete with the liposomes for the sites of adsorption onto the HA, therefore the adsorption capacity of the polysaccharide-coated liposomes might be slightly underestimated.

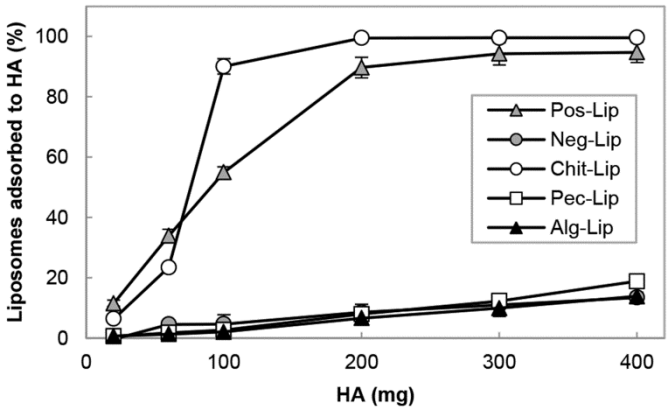


Figure 3. Adsorption to HA in phosphate buffer. Percentage of liposomes adsorbed to increasing amounts of hydroxyapatite powder (HA) in a 5 mM phosphate buffer. The average of three separate experiments for each sample is shown and the error bars represent the standard deviations. The points without error bars have standard deviations equal to or smaller than the size of the markers.

The slope indicated by the linear increase in the adsorption of liposomes at increasing HA amounts (Figure 3), determines the adsorption capacity for each type of liposome. The highest slope was observed for the Chit-Lip, indicating the highest adsorption to HA. All the Chit-Lip in the samples were adsorbed when at least 200 mg HA were used in the samples. The slope for the Pos-Lip was less steep compared to the Chit-Lip, indicating a lower adsorption, and all the Pos-Lip in the samples were adsorbed when at least 300 mg of HA were used. The liposomes with a net negative surface charge (Neg-Lip, Alg-Lip, Pec-Lip) presented lower slopes, thus indicating that the negatively charged liposomes had a lower adsorption capacity compared to the positively charged liposomes. The different types of negatively charged liposomes had similar slopes, hence similar adsorption capacities. For all of them, an adsorption of 14-19% was observed when using the highest amount of HA (400 mg).

The binding sites on the HA surface involved in the adsorption processes are positively charged calcium ions and negatively charged phosphate groups that can interact electrostatically with

oppositely charged groups or electrolytes. However, at neutral pH the HA surface is primarily dominated by the negatively charged phosphate groups (Yin et al., 2002; Young et al., 1997). Therefore, the higher adsorption of the positively charged liposomes (Chit-Lip, Pos-Lip) compared to the negatively charged liposomes indicated that the adsorption onto HA was primarily based on ionic interactions, which is consistent with findings in previous works (Nguyen et al., 2010; Yin et al., 2002).

The charge density on the surfaces of the liposomes, however, did not seem to influence the adsorption, since the highly charged Pos-Lip were less adsorbed than the more weakly charged Chit-Lip. Moreover, since small liposomes take up less space than larger liposomes, a higher amount of the small liposomes was expected to be adsorbed (Nguyen et al., 2010). Nevertheless, the small size of the Pos-Lip did not seem to promote their adsorption compared to the larger sized Chit-Lip. The difference in adsorption capacity between Chit-Lip and Pos-Lip could rather be attributed to other reasons. It has previously been reported a higher adsorption of chitosan-coated liposomes to mucosa compared to positive uncoated liposomes (Takeuchi et al., 2003), indicating that physical entanglement between the mucosa and the chitosan on the liposomal surface is of importance to facilitate mucoadhesion. Similarly, the flexibility and the spreading of chitosan chains of Chit-Lip on the HA surface could facilitate the adsorption. In addition, the capacity of chitosan to bind calcium ions (Bravo-Osuna et al., 2007) could have enhanced the adsorption of Chit-Lip through binding on further calcium sites of HA, probably increasing their adsorption capacity as compared to the Pos-Lip. Moreover, the low charge density on the Chit-Lip could have improved the adsorption since the Chit-Lip would have been less repelled by the presence of others Chit-Lip already adhered onto the HA surface; while the high positive charge of the Pos-Lip adsorbed onto the surface of HA might have hampered further Pos-Lip adsorption.

Some affinity to HA was also observed for the negatively charged liposomes (Neg-Lip, Alg-Lip, Pec-Lip), as previously reported for negatively charged liposomes prepared with other lipids and

also coated with pectin (Nguyen et al., 2013; Nguyen et al., 2010). Free alginate and pectin have previously been reported to adsorb onto tooth enamel surfaces through binding onto the calcium sites (Beyer et al., 2010). Similarly, negatively charged groups on the surface of the liposomes could have formed ionic bonds with the calcium sites, as well as hydrogen bonds with the hydroxyl groups at the HA surface, respectively. However, repulsion forces due to the presence of negative phosphate ions on the HA might have prevented an extensive adsorption of the liposomes. Also for the negatively charged liposomes, the adsorption was independent of the amount of charge and the liposomal size, as the adsorption levels were the same for the three different formulations. This could be explained with a comparable affinity of alginate, pectin, and Egg-PG (in the uncoated liposomes) to the calcium sites of the HA surface. A previous study (Nguyen et al., 2013), however, showed that uncoated liposomes prepared with other lipids might have a higher adsorption capacity compared to pectin-coated liposomes in phosphate buffer.

The environment in which HA is investigated can affect the surface properties and consequently the interaction processes. In fact, since the HA surface is highly polar, the presence of electrolytes in an aqueous medium will generate a hydration layer covering the solid surface and consisting of counter ions in dynamic equilibrium with the HA surface (Yin et al., 2002). In these experiments, phosphate buffer was used as the medium, hence the phosphate anions could have rendered the HA surface more negative by adsorbing onto the cationic calcium at the surfaces (Nguyen et al., 2010; Yin et al., 2002). Consequently, the more negative zeta potential of the HA could further have reduced the adsorption of the negatively charged liposomes in these experiments.

In the oral environment, however, the negative zeta potential of the HA surface would rather become more neutral, due to the predominant adsorption of calcium cations from saliva (Young et al., 1997). This would most likely reduce the repulsion forces between the negatively charged liposomes and the HA surfaces. Moreover, calcium cations in the hydration layer are known to contribute through ionic bridging to the adsorption onto tooth surfaces of negatively charged

components of saliva, as salivary proteins and mucins (Tanizawa et al., 2004). Similarly, it may be assumed that liposomes with a net negative surface could have a higher adsorption profile in physiological conditions than in the present experiments in phosphate buffer. For this reason, the adsorption tests were performed also with artificial saliva as the medium, which contained calcium cations in physiological concentrations (Gal et al., 2001). Due to the instability of the Pos-Lip and the Chit-Lip in artificial saliva, these formulations were excluded from the test. The results of the adsorption experiment in artificial saliva are depicted in Figure 4.

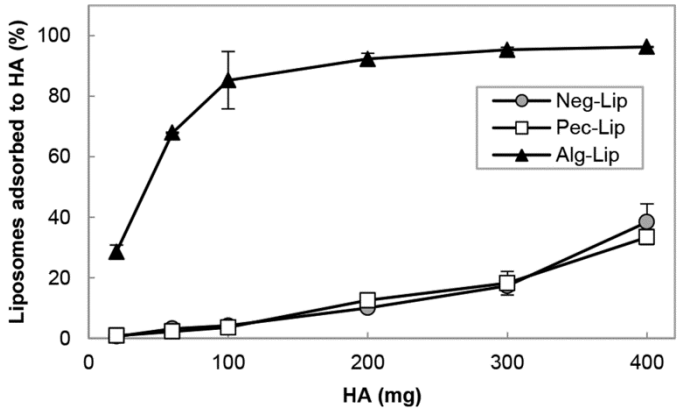


Figure 4. Adsorption to HA in artificial saliva. Percentage of liposomes adsorbed to increasing amounts of hydroxyapatite powder (HA) in artificial saliva. The average of three separate experiments for each sample is shown and the error bars represent the standard deviations. The points without error bars have standard deviations equal to or smaller than the size of the markers.

An increase in the adsorption capacity was recorded for all the negatively charged liposomes in the presence of artificial saliva, thus possibly confirming the hypothesis that in physiological conditions the presence of calcium could increase the adsorption. The Neg-Lip and the Pec-Lip presented again similar adsorption capacities, which were doubled compared to the previous experiment in

phosphate buffer. For example, the highest adsorption of Neg-Lip and Pec-Lip shifted from 14-19% in phosphate buffer to 33-38% in artificial saliva, when using the highest amount of HA (400 mg).

Alg-Lip, however, presented an interestingly high increase in adsorption to HA in the presence of artificial saliva, resulting in an adsorption capacity comparable to the one of Chit-Lip in phosphate buffer. In fact, 200 mg of HA were sufficient to provide the total adsorption of the Alg-Lip. The zeta potential of the HA might become slightly positive following adsorption of calcium cations (Yin et al., 2002). Thus, the higher negative charge of the Alg-Lip compared to the Neg-Lip and the Pec-Lip in artificial saliva, could have favored the higher adsorption of the Alg-Lip. Nevertheless, this explanation may not be sufficient to justify such a high adsorption increase. Therefore, it is reasonable to assume that the positive liposomal surface underneath the alginate layer might also interact in some way with the phosphate anions at the HA surface, thus strengthening the liposome-HA bond. This might occur following an increased porosity of the alginate coating in artificial saliva due to an increase in electrostatic screening, as described in section 3.2 (Guzey and McClements, 2006). This double interaction of HA with both the alginate layer and the liposomal surface underneath could explain the higher adsorption capacity of Alg-Lip in artificial saliva, compared to the adsorption capacity of Alg-Lip or Pos-Lip in phosphate buffer. The marked difference of the results for Alg-Lip in artificial saliva and in phosphate buffer underlines that the adsorption to HA can be greatly influenced by modifications of the surface properties of both liposomes and HA induced by the salivary components.

4. CONCLUSIONS

Liposomes, both uncoated and coated with polysaccharides, were tested in pre formulation studies for future application as delivery systems addressed to teeth. The negatively charged liposomes (both uncoated, and coated with alginate or pectin) did not aggregate in artificial saliva. Their

adsorption to HA was moderate and could be increased by the presence of calcium cations, as in the physiological environment. This indicates that the composition of the medium is of importance since it can influence the adsorption process.

The positively charged liposomes (both uncoated and chitosan-coated) presented a higher adhesion capacity to HA compared to the negatively charged liposomes. However, the positively charged liposomes aggregated in artificial saliva, thus suggesting that the application of an outer layer of negatively charged polysaccharide could improve the stability.

These results indicate that the negatively charged liposomes could be the most promising formulations for dental applications. Nevertheless, the positively charged liposomes might find use for patients with reduced salivary secretion. The results obtained in the present study can be considered as the basis for further studies developing liposomal formulations for dental targeting.

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The authors declare no conflict of interest.

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