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Formulation and evaluation of polysaccharide- and liposome-based nanosystems for improved targeting to the oral cavity

Sara Pistone

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CONTENTS

ABSTRACT

The burden of oral diseases has increased worldwide in the last decades, which indicates the need for implementation of treatments with increased efficacy. The development of bioadhesive formulations that provide sustained release might improve the efficacy of therapeutic agents with poor retention time in the oral cavity. Moreover, a formulation adhering onto the tooth surface could act as a physical barrier, thus protecting the teeth from environmental challenges. Based on these considerations, this project includes preliminary studies carried out for the development of novel nanoformulations for improving the prevention and the treatment of oral ailments, and, in specific, diseases of the tooth.

The nanoformulations investigated were charged polysaccharide-based nanoparticles prepared through ionic gelation, and charged liposomes both uncoated and coated with polysaccharides. The polysaccharides used for the preparation of the nanoparticles and for the coating of the liposomes were alginate, pectin, and chitosan.

Firstly, the formulations were optimized for obtaining colloidally stable polysaccharide nanoparticles and fully coated liposomes. The optimization was carried out by varying formulation factors known to be able to modify the physical characteristics of the nanosystem. The factors polysaccharide concentration, crosslinker concentration, and ionic strength of the solvent were investigated for the polysaccharide nanoparticles (paper I-III). The factor polysaccharide concentration was investigated for the polysaccharide coating of the liposomes (paper IV).

Secondly, selected nanoformulations, expected to be the most promising for oral targeting, were tested through *in vitro* experiments to determine possibilities and limitations of each type of investigated nanosystem concerning the intended application (paper III and IV). To this scope, the stability of the nanosystems was investigated in a simulated salivary fluid, the cytotoxicity was tested against the TR146 cell (model for the buccal epithelium), and the potential adhesion onto the tooth enamel was estimated using hydroxyapatite as enamel model. Most of the formulations were bioadhesive onto hydroxyapatite. However, the positively charged nanoformulations tended to be unstable in the simulated salivary fluid, and the polysaccharide-based nanoparticles presented some cytotoxicity due to the presence of positively charged components. The findings in this thesis provide the basis for further studies for development of improved nanosystems for oral cavity applications.

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Oslo, August 2016

Sara Pistone

LIST OF PAPERS

This thesis is based on the following papers, which in the text are referred to by their Roman numerals:

Paper I:

S. Pistone, D. Qoragllu, G. Smistad, M. Hiorth

Formulation and preparation of stable cross-linked alginate-zinc nanoparticles in the presence of a monovalent salt

Soft Matter 11, 5765-5774 (2015)

Paper II:

S. Pistone, D. Qoragllu, G. Smistad, M. Hiorth

Multivariate analysis for the optimization of polysaccharide-based nanoparticles prepared by self-assembly

Colloids and Surfaces B: Biointerfaces 146, 136-143 (2016)

Paper III:

S. Pistone, F. M. Goycoolea, A. Young, G. Smistad, M. Hiorth

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

European Journal of Pharmaceutical Sciences 96, 381-389 (2017)

Paper IV:

S. Pistone, M. Rykke, G. Smistad, M. Hiorth

Polysaccharide-coated liposomal formulations for dental targeting

International Journal of Pharmaceutics 516, 106-115 (2017)

LIST OF ABBREVIATIONS

AFM	Atomic force microscopy
AM pectin	Amidated, low methoxylated pectin
DA	Degree of amidation
DDA	Degree of deacetylation
DE	Degree of esterification
DLS	Dynamic light scattering
DOTAP	Dioleyl trimethylammoniumpropane
Egg-PC	Egg phosphatidylcholine
Egg-PG	Egg phosphatidylglycerol
G	Guluronic acid units in relation to the total number of monomers
HA	Hydroxyapatite
HM pectin	High methoxylated pectin
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
M_v	Viscosity average molecular weight
M_w	Weight average molecular weight
NBD-PC	Nitrobenzoxadiazol-4-yl-phosphocholine
PDI	Polydispersity index
PLS	Partial least squares regression
SEM	Scanning electron microscopy
TPP	Tripolyphosphate

1. INTRODUCTION

Oral diseases are major public health problems in all the regions of the world, collectively affecting nearly four billions people worldwide.[1, 2] The impact of oral conditions in terms of pain and suffering, impairment of function, and reduced quality of life is considerable. Dental caries and periodontal diseases are considered the most important global oral health burdens. In specific, dental caries affect 60-90% of school-aged children and the vast majority of adults, while severe periodontitis is found in 5-20% of most adult populations worldwide.[1]

Improved prevention measures are expected to play a primary role in the reduction of the incidence of oral conditions.[3] Therefore, the development of enhanced pharmaceutical systems for oral hygiene is of importance to increase the protection of the teeth and of the oral cavity from detrimental processes. The comprehension of the main oral problems and of the challenges related to the drug delivery in the oral environment constitutes the basis of the rational for developing pharmaceutical formulations that can provide an improved oral protection.

1.1. Background: the environment of the oral cavity

The oral cavity consists of various structures comprising both soft and hard tissues (Figure 1.1), continuously bathed in a complex fluid denominated whole saliva.

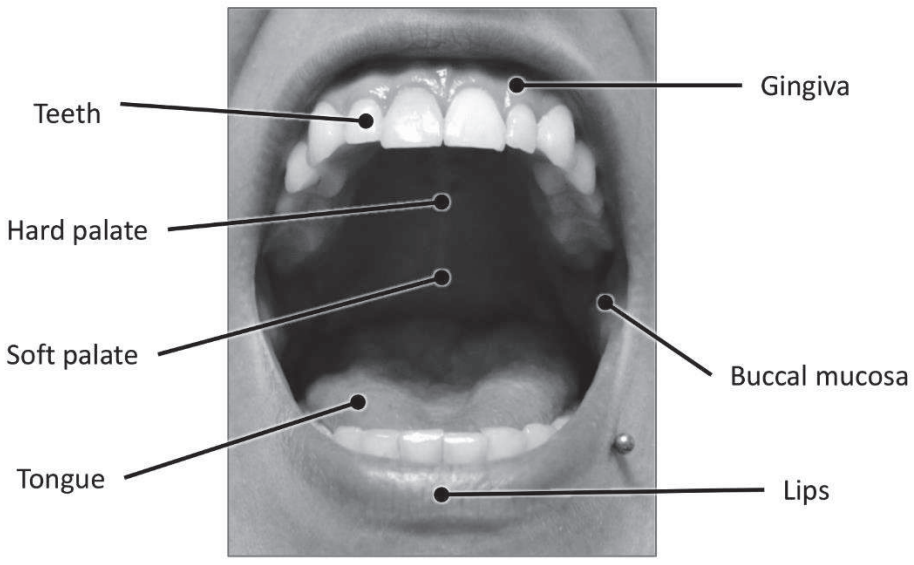


Figure 1.1. Structures included in the oral cavity.

1.1.1. The dental enamel

The dental enamel is the hard mineralized tissue that covers the external surface of the tooth crown. It is primarily constituted by inorganic substance (91 vol%), and contains also low amounts of water and organic material (proteins and lipids).[4, 5] The inorganic content includes mainly crystals of calcium phosphate $Ca_{10}(PO_4)_6(OH)_2$, referred to as calcium hydroxyapatite (HA).[5] The solubility of the enamel depends on the composition of the liquid to which the enamel is exposed (such as saliva).[4] In fact, the solution that submerges the enamel influences the equilibrium between the HA (the primary component of enamel) and its ions in solution:

$$Ca_{10}(PO_4)_6(OH)_2 \underset{\text{mineralization}}{\overset{\text{demineralization}}{\rightleftharpoons}} 10Ca^{2+} + 6PO_4^{3-} + 2OH^-$$

At the resting salivary pH (nearly neutral), tooth demineralization is discouraged, since saliva is supersaturated with respect to Ca^{2+} and PO_4^{3-}. The equilibrium concentrations vary markedly with pH. At acid pH, the concentrations of Ca^{2+} and PO_4^{3-} in saliva are too low to provide saturation, so HA dissolves to reach the equilibrium and the saturation condition. The consequence is enamel demineralization.[6] The supersaturation of Ca^{2+} and PO_4^{3-}, however, has been shown to lead to partial remineralization of HA and enamel surfaces after early damage induced by acid challenges.[7, 8]

Even though the inorganic substance of dental enamel includes mainly HA, "impurity" ions are also incorporated into the crystal during hard tissue formation and replace ions in the crystal structure of HA. Ca^{2+} can be replaced by Na^+ and Mg^{2+}, OH^- by F^- and CO_3^{2-}, and PO_4^{3-} by CO_3^{2-}. The presence of impurities generally increases the enamel solubility. However, fluoride is an exception; the inclusion of fluoride into the crystal forms fluoroapatite, which, being less soluble than HA, lowers the critical pH for unsaturation, thus strengthening the enamel against acid-induced dissolution.[4]

At the surface, the tooth enamel and HA expose both the negatively and the positively charged ions. In fact, HA is slightly soluble in aqueous environment, and the surface ions are in equilibrium with those in solution, which give rise to electrostatic potentials. The zeta potential of enamel and HA measured at neutral pH is negative, which indicates the prevalence of the negative charges of PO_4^{3-} at the surface.[9, 10] Counterions present in the fluid surrounding HA can adsorb onto the HA surface through ionic interactions, thus

creating an hydration layer of ions in dynamic equilibrium, which can modify the zeta potential at the solid surface. This zeta potential is, therefore, influenced by pH, ionic content and concentrations in the surrounding environment.[9, 11] In particular, Ca^{2+} and PO_4^{3-}, which are contained in saliva, are strong potential determining counterions.[9]

1.1.2. The saliva

The whole saliva constitutes the dominating oral environment in healthy individuals and comprises a mixture of fluids produced by the salivary glands together with many other components found in the oral cavity, such as bacteria, desquamated epithelial cells, blood cells, and food debris.[12] Saliva is produced by the salivary glands. It has a variable pH around 7 and contains 99% water and a variety of compounds, such as electrolytes, nitrogenous products, glucose, metabolites, hormones, vitamins and different kind of peptides and proteins (such as proline-rich proteins, mucins, amylase and other enzymes, and immunoglobulins).[13, 14] The flow of saliva is estimated to be 0.2-0.4 ml/min in basal conditions, while different stimuli can increase the rate up to 2.0-5.0 ml/min.[12] The composition and the flow of saliva are, however, highly variable among individuals and also varies in the same individual under different circumstances. The variation depends on several factors, such as the time of the day, gender, age, physiological conditions, stimulatory status of the glands, diseases, and medications.[13-15]

The presence of salivary fluid is critical for the protection and the maintenance of oral health.[16] The salivary flow (especially when high) and the swallowing enable to dilute and mechanically clear undesirable substances in the oral cavity, as non-adherent bacteria, cellular and food debris, and sugars that promote bacterial proliferation.[14, 16] The mucins in saliva are the main responsible for the formation of a seromucosal layer on the oral tissues (mucus).[17] The presence of mucus on the oral surfaces prevent dehydration, protects against irritating agents and modulate the adhesion of microorganisms, thus controlling bacterial and fungal colonization.[17]

Several components of saliva serve as buffering systems in order to retrieve a physiological pH value (6-8)[12, 13] when it drops following, for example, the consumption of acidic beverages or the acid production by the oral microbiota.[14, 16] The maintenance of a neutral pH prevents the creation of an optimal environment for bacterial colonization and discourages tooth demineralization. The carbonic acid-bicarbonate is the most important buffering system in saliva, while also phosphates, urea and sialin (a salivary peptide)

contribute to the buffering capacity of saliva. The tooth demineralization is also dampened by the presence of calcium and phosphate in saliva at supersaturated levels with respect to dental enamel, which help to maintain the integrity of the tooth enamel by facilitating remineralization after acid exposure.[8, 18]

The salivary proteins have several functions. For example, by binding Ca^{2+}, proline-rich proteins and statherins inhibit the spontaneous precipitation due to supersaturation of calcium phosphate both in the salivary glands and on the tooth surface.[19] Moreover, enzymes, such as amylase, lipase, and protease, exert digestion function; and immunoglobulins, lactoferrin, histatins and other proteins bear antimicrobial properties.[14, 16]

1.1.3. The acquired enamel pellicle

A thin organic layer, known as the acquired enamel pellicle, is observed on enamel surfaces exposed to saliva. The acquired enamel pellicle represents the interface between the dental enamel and the oral environment, and it is formed following a selective adsorption of specific salivary constituents onto the enamel.[20, 21] The pellicle offers protection against erosion to the enamel. In particular, its lubricating effect reduces the friction between teeth and the other oral surfaces.[22] Moreover, the pellicle layer retards enamel dissolution caused by acid attack by acting as a selective permeability barrier for ions.[23, 24] The transport of Ca^{2+} and PO_4^{3-} from the enamel to the surrounding environment is delayed, and the diffusion of acids toward the enamel, following acid challenges, is reduced.[25] The pellicle also facilitates a selective adsorption of harmless bacteria, thus reducing the damage induced by the presence of cariogenic microorganisms.[26]

The constituents of the pellicle are mainly proteins of salivary origin, such as mucins and proline-rich proteins, and also small quantities of carbohydrates and lipids can be present.[20, 21] A thin layer of pellicle is formed few minutes after exposure of the enamel to the salivary fluid, and its thickness has been reported to increase to a plateau after about 30 minutes.[27] The thickness of the pellicle has been shown to vary generally between 0.3 and 1 μm.[27, 28] The process of formation of the pellicle onto the enamel proceeds first with the adhesion of a layer of single proteins, then proteins in the form of globular micelle-like structures are incorporated.[27, 29] The main constituents of the globular micelle-like structures are the proteins lysozyme, lactoferrin, proline-rich proteins, secretory immunoglobulins A, mucin MG2, and amylase.[30, 31] Salivary calcium ions are necessary

for the integrity of the globular structures, which can also aggregate into multiglobular structures with a "raspberry-like" appearance.[31, 32] At physiological pH, the globular and multiglobular structures have a net negative zeta potential of about -9 mV[33] and their average size was reported to be around 50-500 nm.[31, 32]

The adsorption of the salivary constituents onto HA appears to be mainly based on ionic interactions between the ionic sites on the HA surface (Ca^{2+} and PO_4^{3-}) and the charged groups of the proteins adsorbed (*e.g.* phosphate, carboxyl, amino groups).[34] Due to the presence of salivary counterions adsorbed onto HA in the hydration layer (as described in Section 1.1.1), the adsorption of proteins and other macromolecules seems to involve ion exchange processes.[35] In other words, the cations dissolved in the saliva (*e.g.* Ca^{2+}) compete with the positively charged groups of the macromolecules for binding the negatively charged sites onto the HA surface (PO_4^{3-}); while the anions (*e.g.* PO_4^{3-}) compete with the negatively charged groups of the macromolecules for binding the Ca^{2+} sites onto the HA.[9] In theory, the net negative electrostatic potential of enamel should result in repulsion with negatively charged proteins, such as mucin, proline-rich proteins and salivary micelle-like globules. However, Ca^{2+} in the hydration layer can promote their adhesion by lowering the net negative surface potential of proteins and HA,[9] and acting as a ligand between their negatively charged groups.[29, 33, 34]

1.1.4. The oral mucosa

The oral mucosa identifies the soft tissues that line the oral cavity, and includes the buccal, sublingual, gingival, palatal, and labial mucosa.[36] The surface area of the oral mucosa is estimated to be about 170 cm^2, and includes ~80% of the oral surfaces.[37] The oral mucosa consists of different layers. The outermost layer, which represents the interface with the oral environment, is a stratified squamous epithelium.[36] The epithelium acts as a barrier to avoid penetration in the underlying tissue of potentially harmful agents. The turnover time for the buccal epithelium is 5-6 days, and the composition of the epithelium varies with location in the oral cavity. The epithelium of gingiva, hard palate and dorsum of the tongue, which tend to be subject to mechanical stress, is keratinized, while the other mucosal surfaces are non-keratinized.[36] Beside the epithelial cells, which constitute the majority of the cells in the epithelium, also melanocytes, Langerhans cells, Merkel cells, and lymphocytes can be present.[38]

A layer of highly viscoelastic and adhesive mucus covers the luminal face of the mucosal tissues. The mucus is mainly composed by entangled mucins (macromolecular glycoproteins),[17] which can be free or attached to certain regions on the cell surfaces. The components of mucus are secreted by salivary glands as part of the saliva. The mucus acts as lubricant to prevent abrasion and has a protective function.[17] Moreover, the mucus layer protects the epithelium since external agents can be physically entrapped or can adhere to the mucus layer, and are subsequently removed from the mucosa by the mucus turnover.[17]

1.2. Common oral diseases and local treatments

The Global Burden of Diseases Studies estimate levels and trends in disease and injury incidence, prevalence, and years lived with disability in the world population. In the study of 2010, untreated caries in permanent teeth ranked first in the list of the most prevalent conditions worldwide.[2] Dental caries are an infectious tooth disease, which is originated by oral bacteria adhered onto the acquired enamel pellicle or onto the bare tooth, that develop into a biofilm, known as the dental plaque.[5] The cariogenic bacteria included in the dental plaque, such as *Streptococcus mutans*,[39] lead to the production of acids as a result of the sugars' fermentation, thus causing a localized demineralization of dental enamel.[5] If the area is not remineralized, for example through the effect of the saliva, the demineralization can become permanent, and lead to dental caries.[5] Since the damage is irreversible, caries can be treated only through a professional restorative intervention, therefore the prevention of caries is essential.[40, 41]

Beside mechanical removal of the dental plaque (*e.g.* tooth brushing), preventive local treatments generally include the use of antimicrobials and enamel strengtheners.[40, 41] Commonly used antimicrobials are chlorhexidine, essential oils, triclosan, and zinc.[42-44] However, the administration of fluoride is considered to be the most effective preventive treatment.[4, 43, 45] For this reason, recently the World Health Organization included the effective use of fluoride among the priority action areas for the improvement of oral health worldwide.[3] Fluoride exerts an enamel strengthening action by promoting the formation of resistant fluorapatite on the teeth surface.[6, 43] Moreover, the presence of fluoride has also been shown to increase the remineralizing effect of saliva.[7, 8]

The enamel strengthening effect of fluoride is also exploited for the prevention of tooth wear.[6] Tooth wear is a dental disease whose prevalence has increased in the last decades, due mainly to changes of the life style, such as the increased consumption of acidic food

and beverages. Tooth wear includes tooth erosion induced by the decrease of the salivary pH level (following *e.g.* reflux or consumption of acidic beverages), and by mechanical abrasion or attrition of the hard tissue (*e.g.* due to use of abrasive toothpaste or bruxism).[5, 40]

Severe periodontitis is the second most common oral ailment and, in 2010, was estimated to be the sixth prevalent condition worldwide.[2] Periodontal diseases are oral conditions concerning gingival mucosa and the surrounding structures (periodontal ligament, cementum and alveolar bones). Gingivitis is the first step for the development of periodontitis, and represents the inflammation of the gingiva, induced by bacteria in the dental plaque. In the presence of a chronic inflammatory state of the gingiva, the disease can progress into periodontitis, which leads to alveolar bone deterioration and tooth loss.[40, 41] In addition to surgical and non-surgical plaque removal by dental professionals, the local periodontal treatments include the use of antimicrobials, such as chlorhexidine or other chemotherapeutic agents (*e.g.* metronidazole, minocycline, doxycycline), and sodium hypochlorite.[40, 41]

A well-functioning saliva secretion is of major importance for the maintenance of the oropharyngeal health, due to the several protecting functions of saliva.[16] For this reason, salivary dysfunctions (*e.g.* hyposalivation and xerostomia), occurring mainly as a consequence of medications, may lead to oral adverse complications, although not directly influencing the oral cavity function.[12, 46] In specific, hyposalivation causes the lack of saliva-induced remineralization and of protection exerted by the enamel pellicle, whose formation in this circumstance can be impaired. As a result, the susceptibility for caries and tooth erosion is increased.[12] Moreover, the loss of the antibacterial activity of saliva can increase the vulnerability to infections, while the loss of the protective mucus layer on the oral epithelium can induce inflammation of the oral mucosa.[46] The local treatments for the management of salivary dysfunctions generally involve the use of salivary substitutes that relieve the sensation of dry mouth by providing lubrication, and protect to a certain extent the teeth from demineralization.[12, 40, 47] Nevertheless, the efficacy of the common salivary substitutes is somewhat limited, since their formulation is aimed mainly to relieve the dry mouth symptoms,[47] but cannot fully replicate the essential protective functions of the natural saliva and of the enamel pellicle.

1.3. Challenges in local oral drug delivery and current approaches

The complex and dynamic environment in the oral cavity is challenging for local oral drug delivery. While the flushing action of the salivary flow, through the dilution of exogenous substances and swallowing, is of major importance for the maintenance of the oral health, it also leads to a rapid clearance of the therapeutic agents from the oral cavity.[44] In the same way, the consumption of food and beverages can induce the swallowing of the therapeutic agents. Moreover, both the whole saliva, and food and beverages may contain substances and microorganisms that, by interacting with the drug, could contribute to its inactivation. In addition, the abrasion due to the movements of the soft tissue, for example during the masticatory process or while speaking, may also remove the pharmaceutical formulation from its site of action. Therefore, the effects of the salivary flow, the consumption of food and beverage, and the abrasion by soft tissues render the oral cavity a harsh environment for the local application of therapeutic agents, which would decrease their retention time, and consequently their efficacy (Figure 1.2).

The most successful antimicrobials, such as chlorhexidine, are able to adhere onto the oral surfaces, thus creating a reservoir for a slow release of the agents.[44] This contributes to

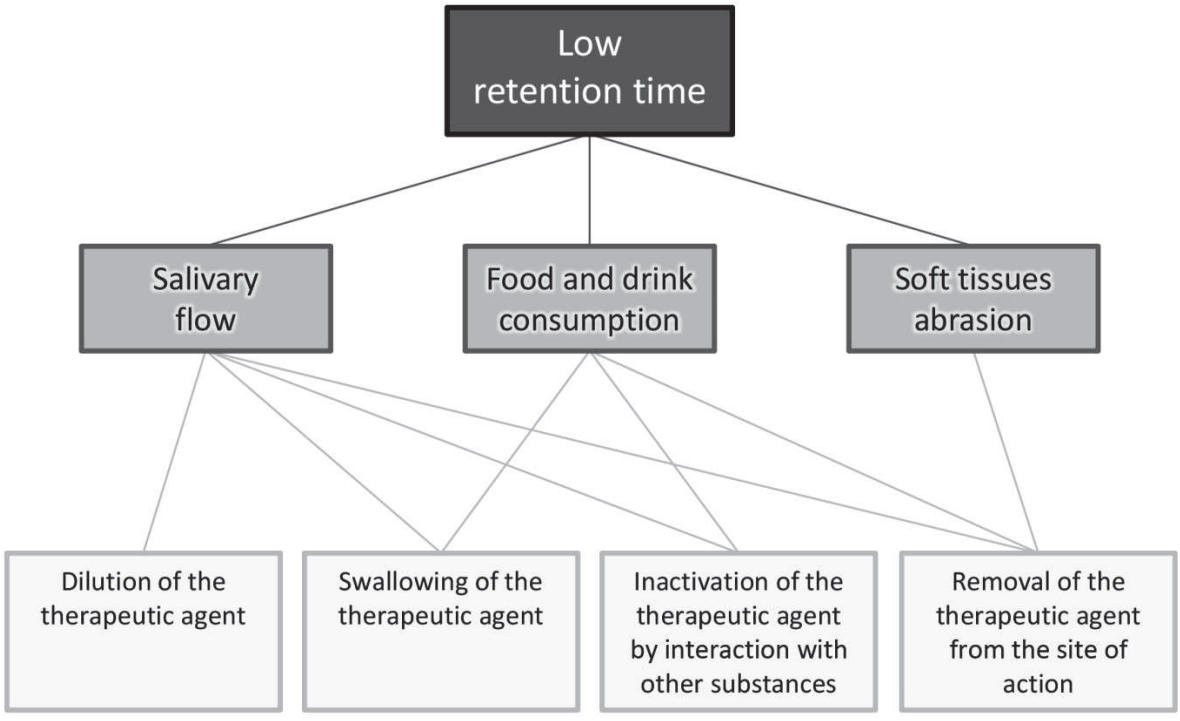


Figure 1.2. Main determinants of the low retention time of therapeutic agents in the oral cavity.

the increased retention time of the drug in the oral cavity and to keep concentration of the drug at the site of action at a therapeutic level for an extended period between two applications. Agents that cannot adhere onto oral surfaces act only for a relatively short period after application. Therefore, they must possess a sufficient biological activity to be effective in a short time, otherwise the clinical efficacy of the agent would be lost.[44] Frequent applications at high concentrations could therefore be required, which, on the other hand, might increment the side effects and would decrease the compliance of the patient. Consequently, a delayed clearance from the oral cavity is preferable in order to prolong the effectiveness of an active agent.

To resolve this limitation, research today focuses on the development of bioadhesive delivery systems, which would assure a prolonged retention time of the formulation. Unlike conventional formulations, bioadhesive drug carriers can improve the effectiveness of the therapy by adhering on oral surfaces and providing sustained release. Moreover, specifically designed drug delivery systems could target the desired site of action, which could result in a further increase of the effectiveness of the therapy.[48]

Bioadhesive delivery systems with sustained release can be beneficial not only to increase the potential of therapeutic compounds with poor efficacy, but could also be advantageous for effective therapeutic agents. For example, although fluoride is considered the most effective enamel strengthener, its long-term efficacy *in vivo* seems to be low,[49] therefore frequent applications are generally required to achieve satisfying results.[4] Therefore, a sustained release of fluoride could provide the significant advantage of reducing the application frequency, thus requiring a minimal patient compliance, which is important especially for high-risk groups (children, persons with handicap, elderly, etc).[50]

Beside the advantages offered by these advanced dosage forms, some aspects of importance to consider during the formulation of conventional drug delivery systems become even more relevant when developing bioadhesive formulations with sustained release. For example, due to their prolonged time of contact with oral tissues, it is important to thoroughly evaluate the potential toxicity of the formulation against oral tissues, especially when the protective mucus layer is reduced or absent. Moreover, since the active substance would be released in saliva, possible modifications on the release rate induced by the variable composition and pH of saliva need to be taken in consideration.

Bioadhesive dosage forms have mainly been studied for the specific targeting to the oral mucosa (mucoadhesive drug delivery systems). The mucoadhesive systems mostly studied for local oral delivery are adhesive tablets, patches and films for application onto the

buccal mucosa, adhesive semisolid systems (*e.g.* gels and ointments), and adhesive liquid systems.[48] The adhesive liquid systems are particularly of interest since their presence in the oral cavity do not tend to create discomfort to the patient. In addition, they can spread uniformly on the oral surfaces thus avoiding the presence of areas with too low or too high drug concentration.

Unlike mucoadhesion, the adhesion onto the teeth hard tissue has scarsely been investigated.[51-53] In *in vivo* conditions, tooth adhesive drug delivery systems are expected to adhere onto the acquired pellicle that covers the enamel. In the presence of salivary dysfunctions, the formulation could adhere onto a partially-formed pellicle or, in its absence, directly onto the enamel surface. Tooth adhesive drug delivery systems could be beneficial especially for improving the effectiveness of the treatments addressed to dental ailments (such as dental caries and erosion).

1.4. Nanosystems for local oral use

Nanomedicine has lately received considerable attention for the development of advanced systems addressed to the prevention and the treatment of oral conditions. Various applications of nanomedicine are investigated in dentistry from drug release systems, to nanoparticulate scaffolds for tissue formation, to nanorobots for diagnostics and therapeutics.[54] Nanosystems investigated for drug delivery purposes in the oral cavity include liposomes, polymeric nanoparticles and nanocapsules, solid nanoparticles and nanocrystals, dendrimers, and nanofibers.[55] They are usually administered in the form of aqueous dispersions with low viscosity, thus promoting a uniform distribution in the oral cavity, but they can also be incorporated in semisolid formulations.[55] Hereby the focus will be put only on polymeric nanoparticles and liposomes.

Polymeric nanoparticles are nanospheres consisting of curled up polymer chains bound to each other (Figure 1.3 A).[56] Therapeutic agents can be entrapped in the nanoparticles generally through interaction with the polymer chains.[57-61] The liposomes are the oldest and most studied nanosized drug delivery system. They are lipidic vesicles formed through spontaneous self-assembly of amphipathic phospholipids in the form of an external lipidic double layer with an inner aqueous core (Figure 1.3 B).[62] Liposomes may entrap molecules either in the hydrophilic core, or in the hydrophobic double layer, or in both, depending on the nature of the molecule.

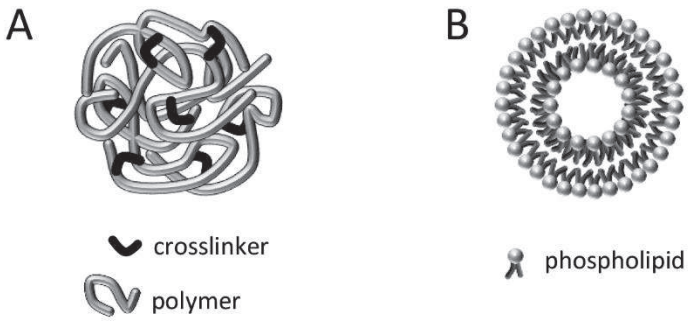


Figure 1.3. Schematic illustration of the basic structure of (A) polymeric nanoparticles and (B) liposomes.

Polymeric nanoparticles and liposomes are able to offer several advantages for oral local treatments and for drug delivery in general. They can, for instance, provide: sustained drug release,[61] or release induced by a specific environmental stimulus;[57, 60, 63, 64] selective action against a designated target;[60] bioadhesion with consequent improved drug bioavailability;[63, 64] protection of active substances from possible environmental inactivation;[57, 63, 64] reduction of the dose of a drug, while maintaining the same efficacy, which could possibly lead to a reduction in toxicity.[61]

The variation of the nanosystems' constituents, the variation of formulation factors, or the addition of other components to the basic structures of polymeric nanoparticles and liposomes can modulate their properties. This enables to tailor the characteristics of the nanosystems for a specific purpose. For example, the nanosystems can be decorated with different molecules in order to provide specific targeting,[60] enhancement of the nanosystem stability,[65-67] increase in bioadhesion and retention capacity,[64, 66] and modulation the drug entrapment efficiency and release.[66, 68]

The presence of bioadhesive molecules at the surface of the nanoformulations could be particularly useful for oral local applications, since they could provide bioadhesive capacity to the nanosystems, thus possibly improving the outcome of oral therapies. Beside the advantages of mucoadhesion, the retention of the nanosystems onto the teeth surface (tooth adhesion) could be especially beneficial for the treatment and prevention of dental pathologies. In fact, tooth adhesive nanosystems could not only lead to drug delivery at the site of action, but they could also mimic the micelle-like structures of the acquired enamel pellicle,[31, 32] thus possibly providing an analogous protecting effect. In this way, the nanoformulations, by adhering onto the enamel pellicle on the tooth surface, might strengthen the tooth protection exerted by the pellicle. For example, the aqueous content of

the nanosystems could provide further lubrication, or the particle layer could possibly discourage the bacterial adhesion. Moreover, if the enamel pellicle is absent or damaged, the nanosystems might replace its beneficial functions by creating an artificial pellicle on the tooth surface. This action could be of primary importance for the development of improved salivary substitutes for the management of hyposalivation.

1.4.1. Polysaccharides in nanosystems

Polysaccharides are often used in pharmaceutical formulations, since they are generally regarded as biodegradable, biocompatible and non-toxic.[69] Their presence at the surface of nanoparticulate formulations could increase their bioadhesion capacity.[63, 64] In addition, the polysaccharides chitosan, alginate, and pectin could be particularly of interest for oral cavity targeting, since they could provide further therapeutic effect that might work synergically with the substances entrapped in the nanosystems. In fact, chitosan, alginate, and pectin have been shown to be able to protect HA from tooth erosion caused by acid challenges,[70, 71] while chitosan has also antibacterial activity.[72]

Alginates are found in nature, and they are generally extracted from brown seaweed. They are linear block copolymers consisting of α-L-guluronates and β-D-mannuronates units linked through β(1→4)-glycosidic bonds. Mannuronates and guluronates are joined together in sections consisting of homopolymeric blocks of guluronate or mannuronate units, or heteropolymeric blocks of alterning mannuronates and guluronates. The percentage of guluronates (G) in relation to the total units characterizes the alginate.[73]

Pectins are also found in nature, and they are primarily extracted from citrus peel and apple pomaces. The main monomer included in the pectins is the D-galacturonate, which constitutes at least the 70% by weight of the pectin chains. The pectin chains comprise two types of regions. One is the linear region composed of a linear homopolymer of α(1→4)-linked D-galacturonate units. The other is a "hairy" region composed of ramified domains, consisting of a linear structure (only galacturonate units, or alternated galacturonate-rhamnose units) with side chains comprising units of different neutral sugars. The galacturonate monomers include a carboxylic group that can be methyl-esterified or amidated. The degree of methyl-esterification (DE) indicates the ratio between the methyl-esterified galacturonates to the total galacturonate units. Based on this, different forms of pectin are available; the high methyl-esterified pectin (HM pectin), with DE in the range 55-75%, and the low methyl-esterified pectin, with DE in the range 20-45%. Moreover, the

low methyl-esterified pectin can exist in an amidated form (AM pectin), and the degree of amidation (DA) indicates the ratio between methyl-esterified galacturonates to the total galacturonate units.[73] The carboxylic groups included in some monomer of pectin and in all the monomers of alginate confer, at neutral pH, a negative charge to the pectin and alginate backbone (pK_a 3-4).[73, 74]

Chitosans are obtained by deacetylation of the polysaccharide chitin, which is mainly found in nature in crab and shrimp shells. Chitosans consist of linear chains of β(1→4)-linked glucosamine and N-acetyl-D-glucosamine. The degree of deacetylation (DDA) characterizes the chitosan by representing the number of acetylated monomers in relation to the total units. The deacetylated glucosamine units include primary amine groups that, at acidic to neutral pH, confer positive charge to the chitosan chains (pK_a 6-7).[75]

In virtue of their charged nature, these three types of polysaccharides can modify the surface of oppositely charged liposomes by creating a layer through electrostatic interactions.[64, 65, 76, 77] Moreover, polymeric nanoparticles based on charged polysaccharides can be prepared through ionic crosslinking by using oppositely charged multivalent ions or molecules.[58, 59, 78, 79]

The negatively charged tripolyphosphate (TPP) is commonly used for crosslinking chitosan. Regarding alginate and pectin, calcium is most often used as the crosslinker; however, the divalent cation zinc could be an interesting alternative in virtue of its anti-halitosis, antibacterial, and enamel strengthening actions.[42, 80, 81] In fact, zinc is often used in local oral formulations to reduce the adherence of colonizing species, the bacterial growth and metabolism,[44] and it was reported to have synergistic effect with other therapeutic agents.[81]

1.4.2. Preparation of nanosystems

The physicochemical characteristics of the nanosystems are essential factors and will determine the nanoparticles' properties for pharmaceutical use. For example, the particle size can determine the ability of the nanosystem to penetrate through a tissue, the drug release rate, and the biodistribution;[68, 82-85] the type of charge at the surface of the nanosystem can influence its cytotoxicity[86] and bioadhesivity;[52, 64] and the charge density can determine the storage stability.[87] An important parameter to consider is also the polydispersity of the size of the nanosystem, which should be as narrow as possible in order to obtain nanoparticles with homogeneous characteristics and properties.

The physicochemical characteristics of the nanosystems can be varied by using different techniques of preparation, and by modifying formulation factors during their preparation.[57, 62, 88, 89] When developing a new nanoformulation, it is therefore important to study how the formulation factors influence the characteristics of the nanosystem, and how the characteristics of the nanosystem influence its properties for the specific application.

Methods for the preparation of polysaccharide-based nanoparticles

The two main methods available for the preparation of ionically crosslinked polysaccharide-based nanoparticles are the emulsification method, and the self-assembly method.[56, 90] In the emulsification method, two water-in-oil emulsions are first prepared, by using as the water phase an aqueous solution of the polymer or of the crosslinker. In this way, discrete homogeneously sized nano-droplets are formed. The formation of the nanoparticles is achieved by mixing energetically the two emulsions of the polymer and of the crosslinker to enhance collisions between the droplets, with consequent induction of the gelation and particle formation.

In the self-assembly method, the nanoparticles are prepared by adding, in controlled conditions, a solution of crosslinker into a dilute solution of polysaccharide (Figure 1.4). The nanoparticles are spontaneously formed after mixing due to the gelation induced by the crosslinker. The self-assembly method is fast and easy, and enables to prepare

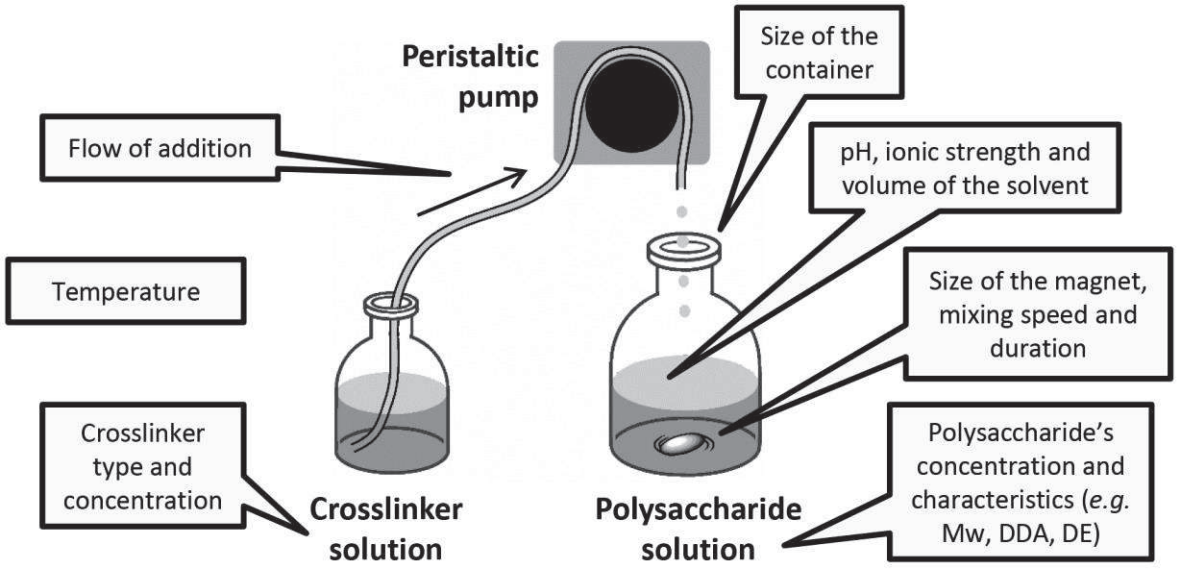


Figure 1.4. Schematic illustration of the preparation of polysaccharide based nanoparticles through self-assembly. The callouts indicate some of the formulation parameters that can influence the characteristics of the nanoparticles.

nanoparticles in mild conditions by using only aqueous media at room temperature, which would allow to entrap also sensitive material. The most studied polysaccharide-based nanoparticles prepared through ionic gelation by self-assembly are the chitosan-TPP nanoparticles.[58, 88, 91, 92]

The characteristics of nanoparticles prepared by the emulsification method and the self-assembly method can be modified by varying different formulation parameters. For example, in the emulsification method, the mechanical power input used to prepare the emulsion determines the particle size. Figure 1.4 lists some of the formulation parameters whose modification can induce variation in the characteristics of the particles, when using the self-assembly method.[57-59, 79, 82, 88, 91, 92]

Methods for the preparation of liposomes and for polysaccharide coating

Several methods are available for the preparation of liposomes.[62] One of the most commonly used is the thin film method. It consists of the formation of a phospholipid film through the evaporation of a lipidic solution prepared in an organic solvent, followed by hydration of the film with an aqueous medium. In this way, liposomes in the form of multilamellar vesicles are spontaneously assembled. In order to reduce the size of the liposomes and to increase their size homogeneity, the liposomes can be further processed, for example, by sonication or extrusion through polycarbonate filters, which enable the formation of small or large unilamellar vesicles. When performing extrusion, the size of the liposomes can be modulated by varying the size of the pores of the filters, and the size homogeneity can be increased by repeating the extrusion process several times.[62]

By using charged phospholipids, it is possible to obtain liposomes with a charged surface. Charged liposomes can be coated with oppositely charged polysaccharides by adding the liposomal suspension to a polysaccharide solution.[64, 65, 76, 77] The coating layer is spontaneously formed through electrostatic deposition.

Coating the entire liposomal surface is essential for maintaining the stability of the nanosystem.[65, 93] In fact, when the amount of polysaccharide is not sufficient to provide a complete coating, uncoated areas on the liposomal surface would interact electrostatically with the polysaccharide on the surface of other liposomes, with consequent aggregation. Aggregation could also occur when high amounts of polysaccharide are used, since the polysaccharide in excess would remain free in solution. High amounts of free polysaccharide would, in fact, create a gradient of osmotic pressure that moves the solvent

between the liposomes toward areas at higher polysaccharide concentrations, with consequent approaching and aggregation of the liposomes (depletion flocculation).[65, 93] In sum, stable coated liposomes can be prepared only by using polysaccharide concentrations in a specific range, which usually needs to be determined empirically.

2. AIM OF THE THESIS

The overall aim of this project was to develop and evaluate selected polysaccharide- and liposome-based nanosystems intended for improved local delivery to the oral cavity.

The objective was two-fold:

1. To develop nanosystems with good colloidal stability (papers I-IV). In specific, the goals were:
    - to determine how important formulation factors can influence the characteristics of the nanosystems and the colloidal stability;
    - to understand the processes underlying the formation of the nanosystems.
2. To evaluate the suitability of the different nanosystems as formulations addressed to the oral cavity by scrutinizing the following aspects (papers III-IV):
    - the stability of the nanosystems in the presence of artificial saliva;
    - the cytotoxicity of the nanosystems against cells of the buccal epithelium;
    - the potential adhesion of the nanosystems onto tooth enamel, by using hydroxyapatite as a model.

3. SUMMARY OF PAPERS

The papers included in this thesis reflect the steps taken towards the development of improved bioadhesive nanosystems designed for local treatment in the oral cavity, as summarized in the chart below (Figure 3.1).

Paper I:

The aim of this study was to investigate the possibility of preparing stable alginate nanoparticles through ionotropic gelation and self-assembly technique, by using only divalent cations (zinc) as the crosslinker. The samples were prepared by dripping a zinc solution into an alginate solution in controlled conditions. The influence of the variation of two preparation parameters (ionic strength of the solvent and zinc content) was investigated, and the obtained formulations were characterized by dynamic light scattering (DLS), zeta potential and pH measurements, and atomic force microscopy (AFM) imaging.

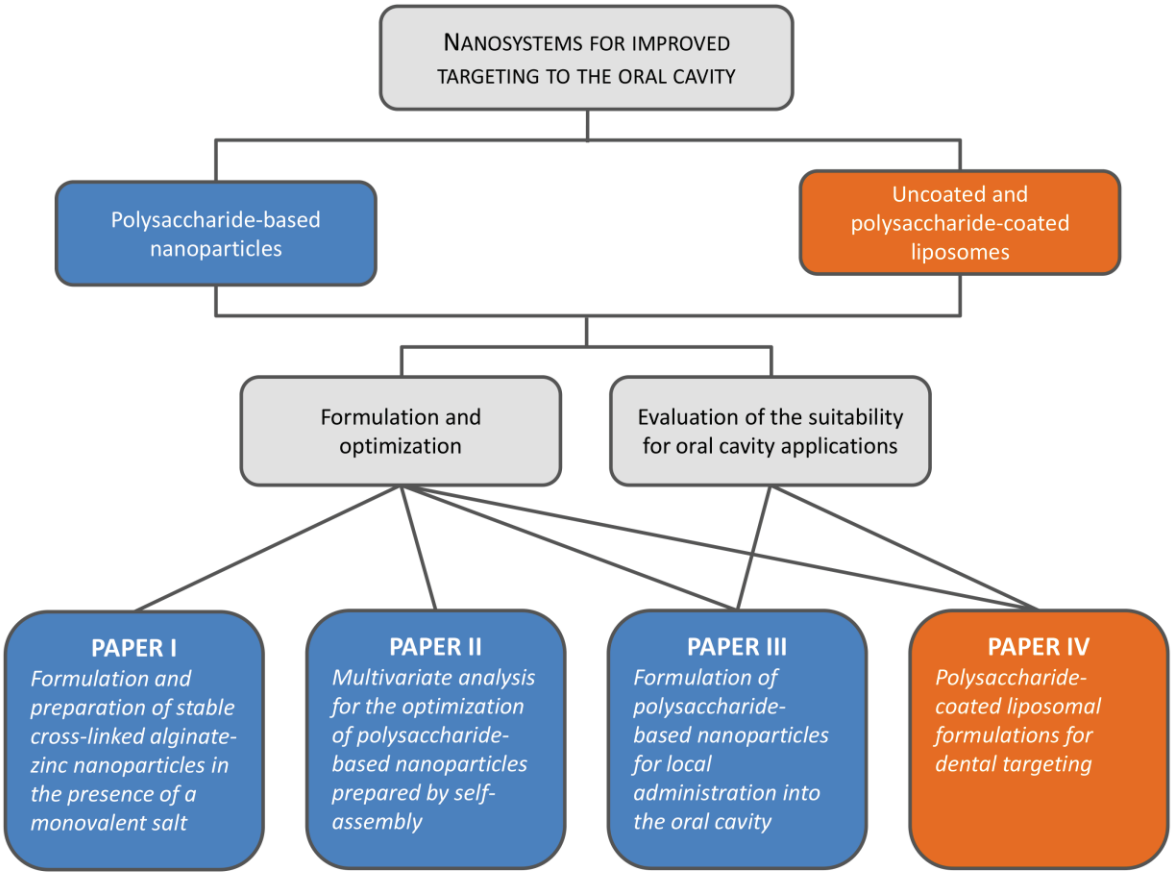


Figure 3.1. Outline of the thesis based on the investigated topics and the publications.

By increasing the zinc-to-alginate ratio, it was possible to disclose the process of formation of the alginate-zinc nanoparticles and to determine the critical zinc-to-alginate ratio required to obtain fully formed nanoparticles. Furthermore, an increase in the ionic strength of the solvent provided stable nanoparticles with considerably narrower size distributions compared to the samples prepared in water.

Paper II:

In this study, formulation factors of the alginate-zinc nanoparticles developed in paper I were optimized with respect to polydispersity index (PDI) by using multivariate evaluation, in specific partial least squares regression (PLS). Two full factorial (mixed level) designs were analyzed. The first design investigated the influence of the preparation factors alginate concentration and zinc concentration; nanoparticles with a low PDI were obtained by decreasing the alginate concentration when keeping constant the zinc concentration at a specific value. The second design investigated the possibility of obtaining low PDI values for formulations containing a high alginate concentration, by varying the preparation factors zinc concentration and ionic strength of the solvent (modified by changing the NaCl concentration in the solvent). At high alginate concentration, a reduction in PDI was obtained by increasing simultaneously the ionic strength of the solvent and the zinc concentration.

Paper III:

This study evaluated the suitability of three types of polysaccharide-based nanoparticles for local applications in the oral cavity. The nanoparticles were prepared by ionotropic gelation and self-assembly technique, by using the polysaccharides alginate, chitosan, or AM pectin. First, the process of formation at increasing crosslinker concentration was investigated for pectin-zinc and chitosan-TPP nanoparticles, and this allowed to select formulations with possibly desirable characteristics for local oral use. Further tests, carried out on all the three types of nanoparticles, indicated that the alginate nanoparticles were stable in a medium simulating saliva, while the chitosan nanoparticles formed aggregates, and the pectin nanoparticles were partially disintegrated. On the other hand, the chitosan nanoparticles were the most biocompatible with cells of the buccal epithelium, and alginate and pectin nanoparticles were revealed to be possibly toxic due to the presence of zinc in their formulations.

Paper IV:

In this study, the characteristics of liposomes coated with increasing concentrations of oppositely charged polysaccharide (chitosan, HM pectin, or alginate) provided the basis for selecting the optimal formulations (non-aggregated). Thereafter, the uncoated liposomes and the polysaccharide-coated liposomes were tested both for stability in a medium simulating saliva and for potential bioadhesion onto HA. The positive uncoated liposomes and especially the chitosan-coated liposomes formed aggregates in the artificial saliva. The coated negatively charged nanosystems (pectin-coated and alginate-coated liposomes) displayed some size variation in the presence of the artificial saliva, while no variations were observed for the negative uncoated liposomes. On the contrary, the positively charged systems displayed the highest adhesion capacity to HA. The adhesion capacity to HA of the negatively charged liposomes was moderate, and the presence of calcium, as in natural saliva, improved their adhesion.

4. EXPERIMENTAL SECTION

Materials and methods applied in this project were described in depth in the papers I-IV. The following is an overview of the procedures and experimental considerations as a background for the discussion in Chapter 5. A schematic presentation of the main materials, techniques of preparation, and analysis performed in this thesis is depicted in Figure 4.1.

All the polysaccharides used in the studies and their characteristics are listed in Table

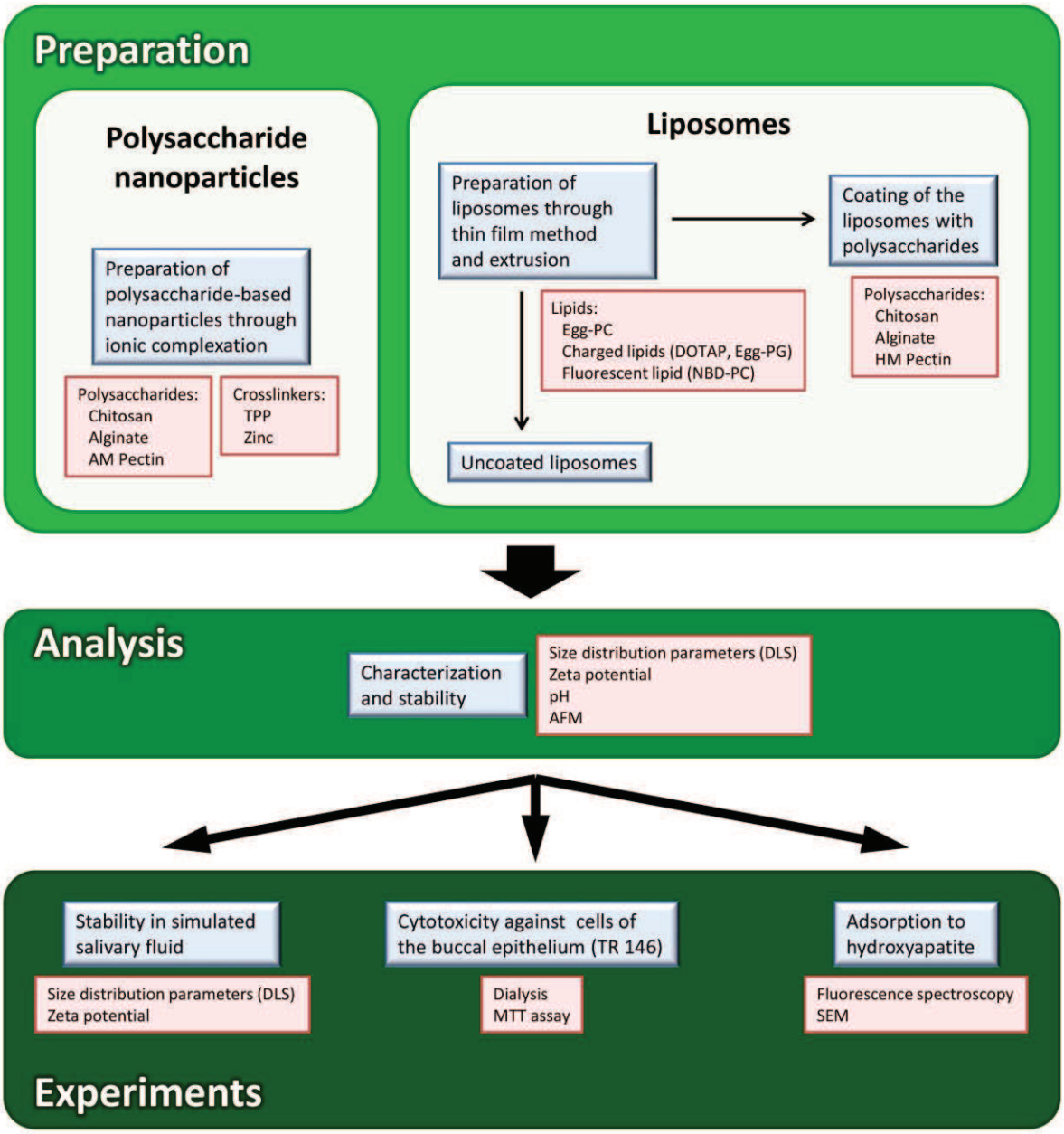
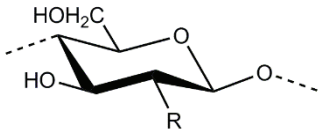
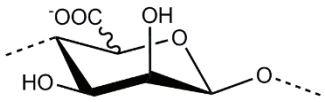
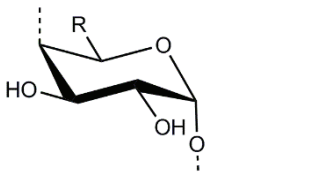
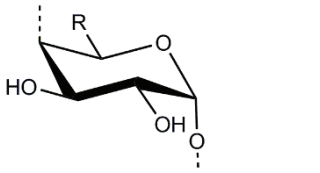


Figure 4.1. Overview of the main materials and methods used in the study.

Table 4.1. Schematic illustration of the chemical structure, type of charge at neutral pH, and main characteristics of all the polysaccharides used in this thesis.

Polysaccharide	Chemical structure	Type of charge	Characteristics
Chitosan (chloride)	 R = NH_3^+, or $NHCOCH_3$	Positive	M_w 307 kDa DDA 83%
Alginate (sodium salt)		Negative	M_v 147 kDa G 65–75%
AM pectin (sodium salt)	 R = COO^-, $COOCH_3$, or $COONH_2$ (simplified structure)	Negative	M_w 96 kDa DE 30% DA 19%
HM pectin (sodium salt)	 R = COO^-, or $COOCH_3$ (simplified structure)	Negative	M_w 110 kDa DE 70.2%

4.1. The polysaccharide-based nanoparticles were prepared through ionotropic gelation and self-assembly by mixing a diluted aqueous solution of polysaccharide with an aqueous solution of crosslinker (papers I-III). Three types of nanoparticles were prepared; alginate-zinc nanoparticles, AM pectin-zinc nanoparticles and chitosan-TPP nanoparticles.

The liposomes were prepared according to the thin film method followed by extrusion through 200 nm filters in order to obtain size reduction[62] (paper IV). The main lipid used for all the formulations was 89 mol% Egg-PC (egg phosphatidylcholine), and 1 mol% NBD-PC (nitrobenzoxadiazol-4-yl-phosphocholine) was added to provide fluorecence necessary for further analysis. The charged lipids DOTAP (dioleyl trimethylammoniumpropane) or Egg-PG (egg phosphatidylglycerol) were added at a concentration of 10 mol% in order to confer to the liposomes a positive or a negative

charge, respectively. The charged surface of the liposomes was modified through coating with oppositely charged polysaccharides, namely chitosan, alginate, or HM pectin.

The choice of the type of lipids and of polysaccharides used in the papers, and the choice of the formulation parameters were based on results of previous studies, in order to possibly obtain stable formulations with low toxicity,[52, 86] and high bioadhesion and tissue coverage.[76, 77, 79] The formulations were characterized through the determination of their size distribution and associated parameters (by DLS), and the measurement of the zeta potential, which are routinely used for the physical characterization of nanosystems.[62, 94] AFM images were also recorded for some formulations investigated for the preparation of alginate-zinc nanoparticles (paper I).

A selection of formulation variables (polysaccharide concentration for coated liposomes and polysaccharide nanoparticles; crosslinker concentration and ionic strength of the solvent for polysaccharide nanoparticles) was studied regarding the effect on the physicochemical properties of the nanosystems. In particular, for alginate-zinc nanoparticles, multivariate analysis was applied to reveal the effects of the formulation variables on the PDI, which represents a measure of the size polydispersity ($0 < \text{PDI} \leqslant 1$, where values close to 0 indicate homogeneously sized samples). The PDI was chosen as the response variable, since size homogeneity in a batch is important to obtain nanoparticles with consistent pharmaceutical properties. Multivariate analysis is advantageous compared to traditional univariate analysis, since it enables to gain more information.[95] For example, in the experimental region, PLS can estimate individual effects (both linear and curved) together with interactions between the factors; in addition, the developed model allows for the prediction of the response in the design space investigated.

Formulations with desirable characteristics for all the types of nanosystem scrutinized were selected and further tested *in vitro*. The stability in simulated salivary fluid (papers III and IV) was evaluated by monitoring over time the physical characteristics of the nanosystems at 37 °C in the presence of the artificial saliva formulated by Gal *et al*.[96] This artificial saliva was chosen in virtue of its high resemblance to the average values in natural saliva concerning ionic content, ionic concentrations, ionic strength, and pH.[13] For this experiment, the use of an artificial saliva was considered advantageous compared to natural saliva, because the composition variability typical of natural saliva could be avoided. Moreover, the use of an artificial saliva with a known composition can facilitate the understanding of the possible interactions between the salivary components and the nanosystems. Finally, the absence in the artificial saliva of nanosized components, such as

the micelle-like structures of natural saliva,[31, 32] could avoid interferences during the DLS characterization.

The cytotoxicity of the polysaccharide-based nanoparticles and their components was investigated against the human-derived cell line TR146. TR146 cells, obtained from a metastasis of a buccal carcinoma,[97] are commonly used as an *in vitro* model for determining the cytotoxicity against buccal cells, as a first approximation to the biocompatibility with the buccal epithelium. The cytotoxicity was assessed by MTT viability test,[98] which is a colorimetric assay that estimates the cytotoxicity by measuring the cellular metabolic activity after the treatment with potential agents. After a four-hour treatment of the cells with the samples, the particle cytotoxicity was evaluated by measuring the mean cell viability relative to the negative control. Prior to the test, the solvent of the nanosystems was replaced with media that the cells could tolerate, through an overnight dialysis. The cytotoxicity test for the polysaccharide-based nanoparticles is described in paper III.

The potential adhesion of the liposomal formulations onto the tooth enamel was assessed by using synthetic HA, which is commonly employed as a model for evaluating the adhesion of substances onto the dental enamel.[70, 99] The comparison between the adhesion capabilities of the different liposomal formulations is described in paper IV. The percentage of liposomes adhered onto different amounts of HA powder was measured by using an indirect method; the concentration of the non-adhered liposomes was measured through fluorescence spectroscopy after mixing the HA and the liposomal suspension. The media used for the experiments were phosphate buffer 5 mM, or, in order to simulate conditions closer to the oral cavity environment, artificial saliva (both pH 6.8).

Additional experiments

In order to get the whole picture, further experiments were performed in addition to the tests described in the papers:

- In paper I, the characteristics of alginate-zinc nanoparticles were evaluated at increasing zinc concentrations. When 0.05M NaCl was used as the solvent, the zinc (chloride) concentrations tested were in the range 0-0.033%, since such concentrations were sufficient to provide the full formation of the nanoparticles. In addition to the zinc concentrations investigated in the paper I, two further

concentrations were tested (0.041% and 0.050%). This allowed to evaluate the influence of the presence of an excess of crosslinker in the alginate-zinc formulations (in the same way as investigated in paper III for pectin-zinc and chitosan-TPP nanoparticles).

- All the liposomal formulations (both coated and uncoated) were investigated for cytotoxicity by using the same method as used for the evaluation of the cytotoxicity of the polysaccharide-based nanoparticles in paper III. In this experiment, the cells were treated with liposomal formulations at a concentration corresponding to 0.6 mM lipid.

- The potential adhesion of polysaccharide-based nanoparticles onto HA was assessed through a qualitative test, performed by using the method described in detail in the section below.

Adsorption of the polysaccharide-based nanoparticles onto HA discs

The adsorption of the polysaccharide-based nanoparticles onto HA was assessed qualitatively. HA discs (9.7 x 1.5 mm) were purchased from Clarkson Chromatography Products Inc. (USA). The HA discs were cleaned through rinsing with ethanol, followed by a 15 minutes sonication in MilliQ water and rinsing in MilliQ water. The cleaned HA disc was incubated for 5 minutes into 1 ml of nanoparticulate suspension (or MilliQ water for control) at room temperature. Thereafter, the HA disc was rinsed to eliminate the non-adhered particles by dipping in 1 ml of MilliQ water (1 second, twice). The HA disc was dried overnight in a desiccator at room temperature.

The adhesion of polysaccharide nanoparticles onto HA discs was assessed the following day through characterization of the morphology of the HA surface by scanning electron microscopy (SEM) imaging (Hitachi S-3000N SEM microscope, Japan) at an accelerating voltage of 20 kV. Prior to imaging, the HA disc surface was sputtered with a gold-layer (Emitech K550X Sputter Coating System, England).

5. DISCUSSION OF THE MAIN RESULTS

In the present thesis, eight types of nanosystems were investigated, which can be divided in two categories; polysaccharide-based nanoparticles, and liposomes. The three types of polysaccharide-based nanoparticles investigated were: alginate-zinc nanoparticles, chitosan-TPP nanoparticles, and AM pectin-zinc nanoparticles. The five types of liposomes investigated were: chitosan-coated liposomes, HM pectin-coated liposomes, alginate-coated liposomes, negative uncoated liposomes and positive uncoated liposomes.

The interest in using these types of nanosystems arises from their possibility of providing bioadhesion onto oral surfaces and also from their potential as drug carriers. In fact, the charge of the nanosystems is expected to improve the incorporation of oppositely charged active substances and modulate their release. Potential examples are the cationic bactericides chlorhexidine and cetylpyridinium, or fluoride anion, which have antierosion effect.[43, 44] Moreover, the lipidic bilayer of the liposomes could enable incorporation of non–ionic and hydrophobic substances, such as the antibacterial triclosan and antiseptic essential oils.[43, 44]

The surface charge of the nanosystems, and the strength of the ionic bonds that induce the formation of the coating around the liposomes and of the polysaccharide-based nanoparticles can vary depending on the pH and ionic strength of the medium. This could be advantageous since it could confer to the nanosystems the ability of responding to specific environmental stimuli. For example, following modifications of pH or ionic strength, a selective release of an entrapped substance could be achieved due to swelling or disintegration of polysaccharide nanoparticles.[57, 60, 63, 69, 82]

5.1. Formulation and optimization of the nanosystems

One part of this project was dedicated to the understanding of the interactions and the processes underlying the formation of the investigated nanosystems, which is an essential knowledge for the selection and the development of promising formulations. This was pursued by examining different preparation parameters able to affect the characteristics of the nanosystems. The information obtained allowed to select potentially optimal formulations for local oral usage for each type of nanosystem investigated.

5.1.1. Polysaccharide-based nanoparticles

Formulation and optimization of alginate-zinc nanoparticles

Alginate nanoparticles have been prepared in previous studies by ionic crosslinking through self-assembly.[57, 59, 63, 100] However, polycations have mostly been used as the crosslinkers, while divalent cations have been shown to form large entities that tended to aggregate.[57, 82] Nevertheless, the aim of this set of experiments was to test the possibility of preparing stable alginate-based nanoparticles crosslinked by using only zinc cations. Some disadvantage due to the use of polycations as the crosslinkers are, for example, the more complicated particle formulation,[57] and the higher cost compared to divalent cation salts. Moreover, avoiding the use of polycations would allow to include zinc in the formulation at the highest possible concentration, which could be advantageous for nanoparticles addressed to local oral use for the therapeutic effects of zinc. [42, 80, 81, 101]

The possibility of preparing stable nanoparticles by self-assembly and ionotropic gelation cannot be predicted, therefore it is generally determined empirically by varying formualtion factors that can influence the nanoparticle formation. For this reason, 42 different formulations combining different values for the alginate concentration, the zinc concentration and the ionic strength of the solvent (varied by using different concentrations of NaCl) were investigated in the papers I and II. These formulation factors were chosen since they have been shown to influence the characteristics of other charged polysaccharide-based nanoparticles.[78, 79, 88, 91, 92] This investigation enabled to study the possibility of preparing alginate-zinc nanoparticles and, furthermore, to optimize their formulation.

Initially, formulations containing an alginate concentration of 0.05% and zinc concentrations in the range 0-0.033% were prepared both in water and in 0.05M NaCl, and characterized (paper I). The results showed that the preparation of colloidally stable alginate-zinc nanoparticles was possible only in the solvent with the highest ionic strength (0.05M NaCl). In fact, all the samples prepared in water had broad size distributions and tended to form aggregates, as previously reported in the literature for alginate-calcium nanoformulations.[57, 82] However, when 0.05M NaCl was used as the solvent, stable alginate-zinc nanoparticles with narrower size distributions were formed. The presence of NaCl in the solvent has previously been shown to increase the colloidal stability and to provide narrower size distributions also for chitosan-TPP nanoparticles.[91, 92] Huang and

Lapitsky[91] suggested that the monovalent salt can compete with the crosslinker for the binding sites onto the polysaccharide, thus weakening the polysaccharide-crosslinker bond and slowing down its formation. The slow formation of the crosslinks leads to a more homogeneus dispersion of the crosslinker in solution, with the consequent formation of more homogeneousy sized particles. In addition, the particle aggregation induced by the crosslinker through inter-particle bridging could be discouraged by the weakening of the polysaccharide-crosslinker bonds (Figure 5.1).

The examination of different formulation factors allowed to determine the factors' levels that enabled the preparation of colloidally stable nanoparticles. Moreover, when the factors were fine tuned within the ranges where colloidally stable nanoparticles were formed, it was possible to obtain nanoparticles with specific characteristics.[88] Nanoparticles for drug delivery purposes should preferably have a low PDI in order to obtain particles with uniform properties, such as drug release rate, biodistribution and passive targeting.[68, 82-85] Moreover, a high zeta potential would discourage particle aggregation, thus promoting a high storage stability.[87] The optimal particle size depends on the target of the nanoparticles.[83] A size range of 50-500 nm could be advantageous for nanoparticles addressed to the oral cavity, since it might allow to mimic the salivary

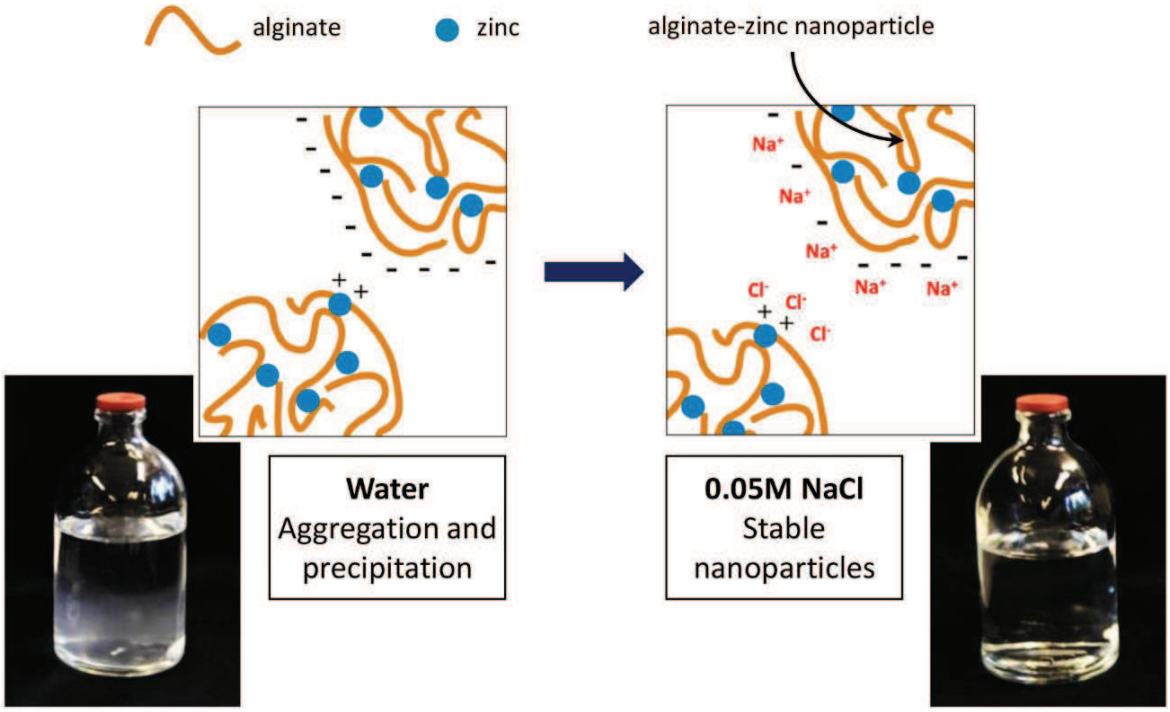


Figure 5.1. Schematic illustration of the particle aggregation induced by inter-particle crosslinking (in water), and of the stabilizing effect of the monovalent ions (in 0.05M NaCl).

micelle-like structures.[31, 32]

Several levels for each investigated factor (alginate concentration, zinc concentration, NaCl concentration in the solvent) were examined to disclose their possible effect on the nanoparticle characteristics (paper II). All three factors were revealed to be significant by PLS with respect to PDI modification; in addition, also size changes were recorded. The zeta potential, which is correlated to the charge of the colloidal entity, was about -30 mV for all the samples, which indicated a negative charge consistent with the type of charges on the alginate chains at the pH of the samples (4,5-5).

An increase in the alginate concentration in the formulation caused an increase in the PDI and in the particle size (Figure 5.2), which are both in accordance with previous studies regarding chitosan-TPP nanoparticles.[89, 92] The increase in PDI could be attributed to a faster process of formation of the nanoparticles, due to the increase of collisions between zinc and alginate at higher alginate concentrations. In fact, a faster nanoparticle formation has been correlated to the formation of particles less homogeneously sized.[91] The increase in the particle size at increasing alginate concentrations could be attibuted to

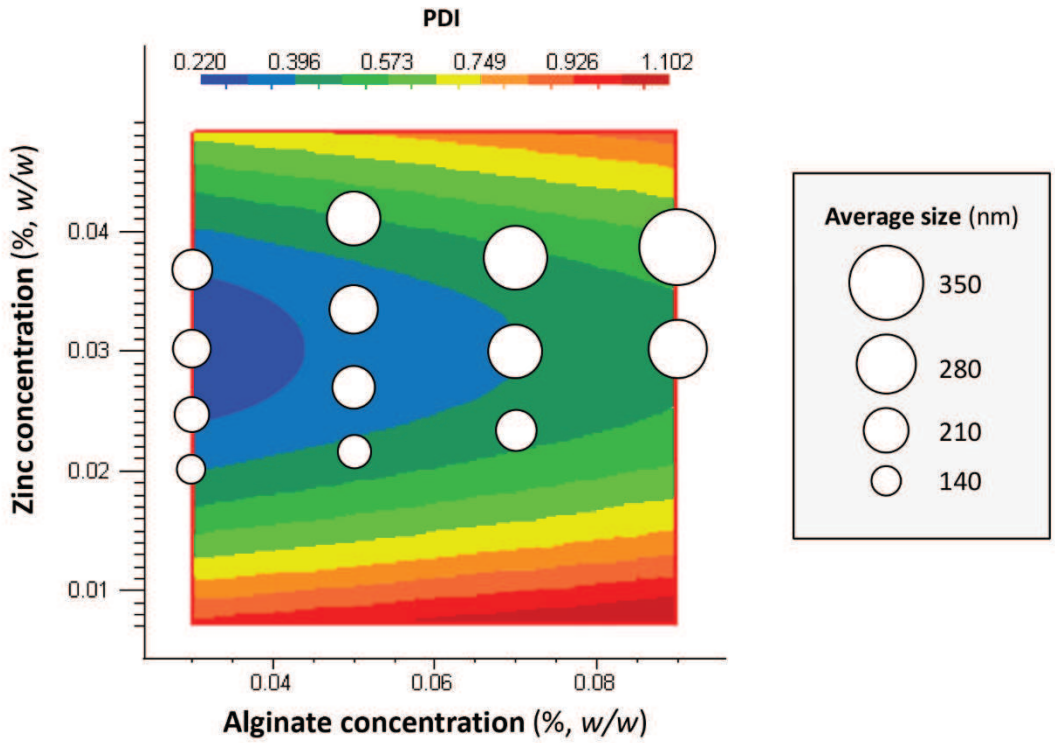


Figure 5.2. Plot illustrating the effect of the alginate concentration and zinc concentration on the PDI (response surface) and on the particle diameter (z-average) of the samples prepared in 0.05M NaCl. The particle diameter is indicated only for the samples with PDI < 0.5.

an increased overlap of the alginate chains in the initial polysaccharide solution. A high overlap of alginate chains could result in particles with a high aggregation number (number of chains in each particle),[92] which could contribute to the formation of large particles.

A decrease in the PDI and in the particle size was obtained when the NaCl concentration and the zinc concentration were increased simultaneously (Figure 5.3). The decrease in the particle size can possibly be attributed to shrinkage of the particles, since the screening of the charges on the alginate chains, induced by the presence of the sodium cations, might decrease the intra-particle repulsions.[92] Another possible explanation for the size reduction at high NaCl concentration could be the reduced formation of large aggegates, which occurs in the presence of solvents with low ionic strength (as explained above). The reduced formation of aggregates, which are heterogeneously sized, would in fact explain also the reduction in PDI at high concentrations of NaCl and zinc.[91, 92]

A previous study[91] suggested the existence of optimal NaCl concentrations for the preparation of nanoparticles ionically crosslinked; this was confirmed by the present findings. In fact, when considering a single zinc concentration, a low PDI was obtained

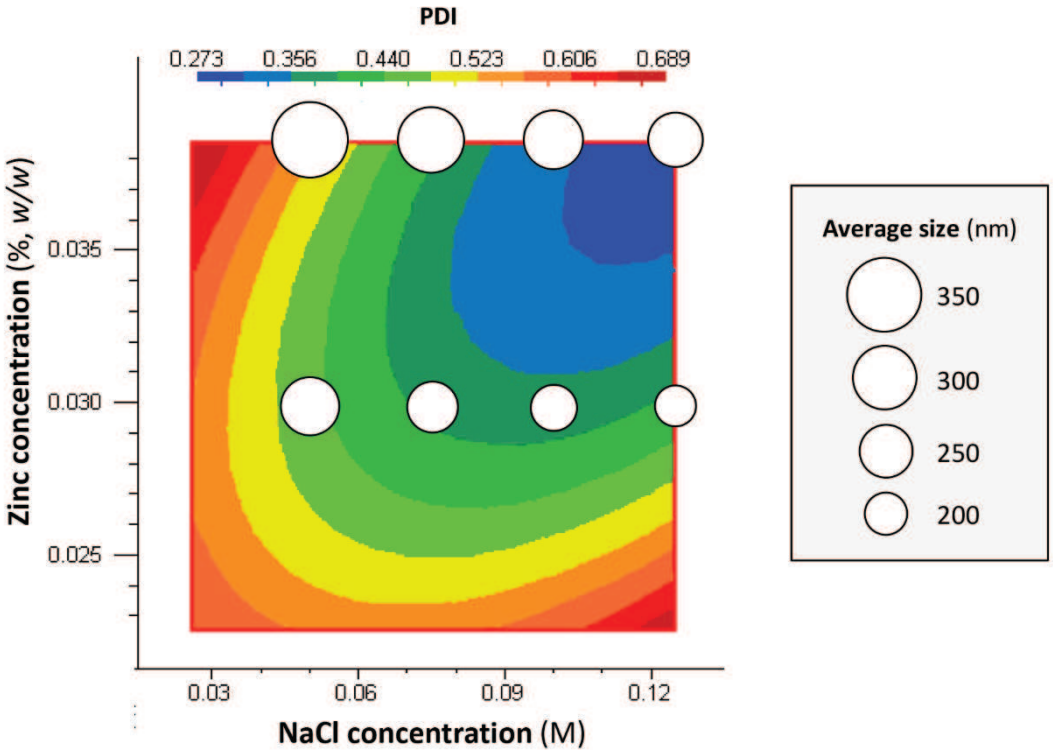


Figure 5.3. Plot illustrating the effect of the the concentration of NaCl in the solvent and the zinc concentration on the PDI (response surface) and on the particle diameter (z-average) of the samples prepared with 0.09% alginate. The particle diameter is indicated only for the samples with PDI < 0.5.

just in a specific range of NaCl concentrations,[91] which was different for each level of zinc concentration. A NaCl concentration below the optimal range could cause the formation of particle aggregates, and a NaCl concentration above the optimal range could inhibit the particle formation, due to competition of the sodium cation with zinc. Both cases would result in an increase in PDI.

The increase in zinc concentration led to an increase of the measured average size (Figure 5.2 and 5.3). This can be explained by a higher formation of crosslinks that could increase the number of alginate chains involved in the formation of the colloidal entities, or could cause bridging between the particles.[82, 92] Moreover, a decrease in the PDI value was recorded followed by its increase, when progressively increasing the zinc concentration (Figure 5.2 and 5.3). These variations in PDI are explained in specific in the following section.

Processes of formation of polysaccharide-based nanoparticles

The characteristics of the formulations based on alginate-zinc, chitosan-TPP, and AM pectin-zinc were determined at increasing crosslinker concentrations, while keeping constant the polysaccharide concentration (0.05%) and the ionic strength of the solvent (0.05M NaCl) (papers I-III). This allowed to identify the process of formation of the nanoparticles by combining the different parameters obtained by DLS measurements. In particular, previous studies suggested a relationship between the nanoparticle formation and the intensity of scattered light.[102, 103] The level of chain aggregation was therefore assessed, in the present investigation, by using the scattered intensity, in virtue of its correlation with the size and the compactness of the aggregates. The compactness is defined as the local polysaccharide concentration inside the aggregates.

First, the process of formation of alginate-zinc nanoparticles was investigated (paper I). The increase in crosslinker concentration induced only a slight neutralization of the negative zeta potential. In the absence of zinc and at low zinc concentration, the high polydispersity and the low scattered intensity denoted the absence of aggregates of alginate chains (Figure 5.4). When the zinc concentration was increased the polydispersity decreased, while the scattered intensity increased together with the apparent average size. This suggested a progressive alginate chain aggregation into constant sized colloidal entities. The lowest polydispersity was recorded at a zinc chloride concentration of 0.027%, together with the steepest increase in the scattered intensity, which indicated the highest

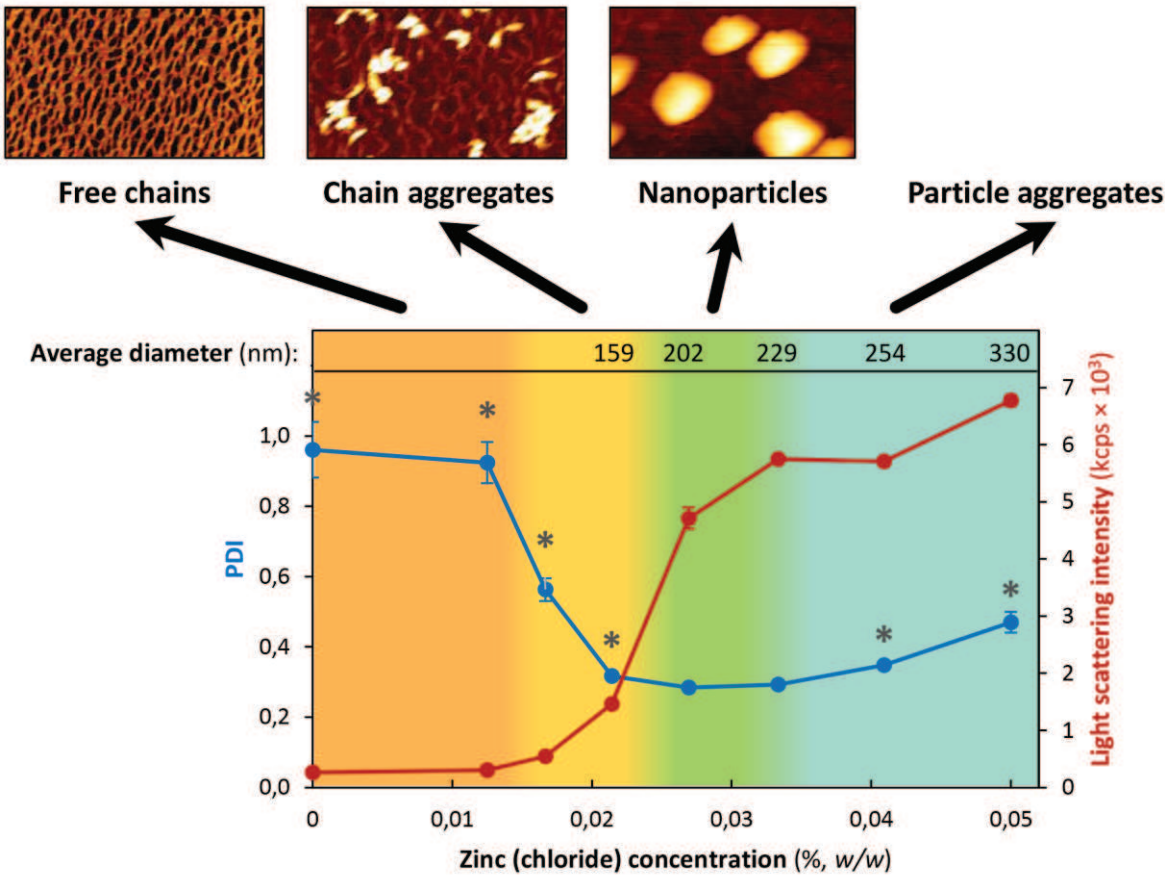


Figure 5.4. Stages of alginate-zinc nanoparticle formation at increasing zinc concentrations. AFM images (above) and plot indicating PDI, scattered intensity and z-average (below) at increasing zinc concentrations. * Multimodal size distributions.

formation of crosslinks. This zinc concentration can be considered as the concentration required to obtain the full formation of the nanoparticles, and was therefore used for the nanoparticles in further experiments. In fact, at higher zinc concentrations, a less steep increase in scattered intensity was recorded. A similar process of formation has previously been observed for chitosan-pyrophosphate nanoparticles[102], and the less steep increase in the scattered intensity, recorded after the highest increase, has been attributed to the saturation of the intra-particle binding sites of the polysaccharides' chains. This explanation could also be valid for the alginate-zinc formulations. By increasing the zinc concentration further beyond the supposed level of intra-particle saturation, inter-particle crosslinks and particle aggregates could be formed. This is indicated by the increase in scattered intensity combined with the increase in polydispersity and apparent average size (Figure 5.4 and 5.2, respectively).

The process of formation of chitosan-pyrophosphate nanoparticles has been reported to be independent of the chitosan concentrations used.[103] This was also observed for the alginate-zinc nanoparticles, since the same process of formation at increasing zinc concentrations was observed at different alginate concentrations (0.03-0.09%) using the data obtained from paper II (Figure 5.5). The zinc concentration that provided the full formation of the nanoparticles (optimum) was ~0.30% for all the different alginate concentrations tested. However, the crosslinker concentration required for the full formation of the chitosan-pyrophosphate nanoparticles have been shown to increase at increasing polysaccharide concentration.[103] The discrepancy observed between chitosan-pyrophosphate nanoparticles and alginate-zinc nanoparticles could origin from the alginate concentrations used in this study that were possibly too close to each other to be able to distinguish differences in the amount of crosslinker required for their formation.

The data obtained at increasing ionic strength (paper II) suggest that the same process of formation could also occur independently of the ionic strength of the solvent. However, the zinc concentration required for the complete nanoparticle formation increased when the ionic strength of the solvent was increased, which was probably due to the higher competition between the zinc and the sodium in the solvent.

When using different types of polymers or crosslinkers, the process of formation of the nanoparticles has been reported to vary.[102] This was confirmed by the results obtained for the AM pectin-zinc and the chitosan-TPP nanoparticles (paper III), which showed that the processes of formation at increasing crosslinker concentrations was different for each type of polysaccharide nanoparticle tested.

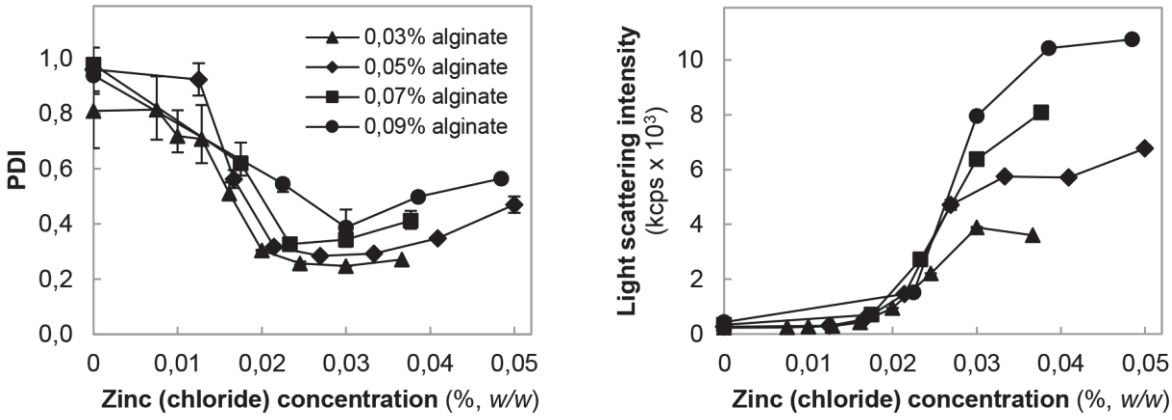


Figure 5.5. Plots showing similar trends for PDI (left) and scattered intensity (right) when the zinc concentration is increased at different alginate concentrations (0.03-0.09%).

The increase in the crosslinker concentration caused a progressive neutralization of the zeta potential for both chitosan-TPP and pectin-zinc formulations, whose zeta potential was positive and negative, respectively. Moreover, the relatively low polydispersity indicated that homogeneously sized entities were present in all the measured samples in the presence of the crosslinker. In the pectin-based formulations, homogeneously sized nanoparticles were also present in samples containing only AM pectin (in 0.05M NaCl), as indicated by the high scattered intensity (Figure 5.6) and the relatively low polydispersity (PDI ~0.3). The formation of nanoparticles in the presence of only pectin and monovalent ions has been previously observed, and has been attributed to inter- and intra-molecular interactions between the pectin chains, such as hydrogen bonding.[79] On the contrary, the chitosan alone had a low scattered intensity and a high polydispersity (PDI ~0.7), which indicated that the chitosan, in the same way as the alginate, required the presence of the crosslinker in order to be able to form nanoparticles.

By increasing the crosslinker concentration, the scattered intensity increased for both pectin- and chitosan-based formulations (Figure 5.6). In the pectin formulations, the simultaneous progressive decrease of the particle size suggested an increase in the particle compactness due to the crosslinks formation, which strengthened the intra-particle bonds. On the other hand, the size of the chitosan-TPP nanoparticles was nearly constant at low

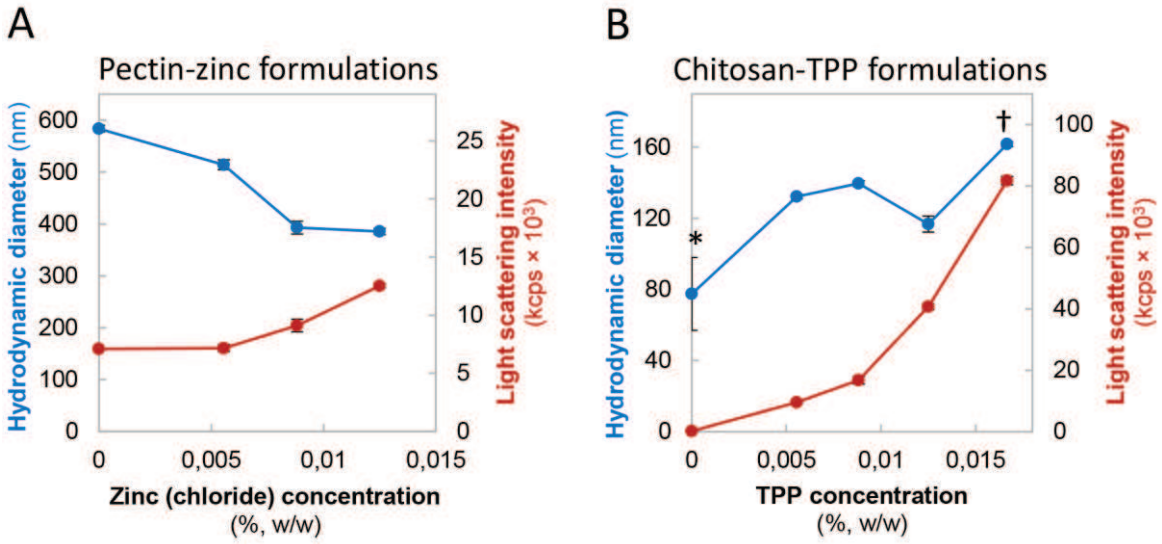


Figure 5.6. Variation of the size (z-average) and the intensity of the scattered light upon increase of the crosslinker concentration for pectin-based formulations (A) and for chitosan-based formulations (B). The results for the pectin-based formulation containing the highest zinc concentration (0.017%) are not reported, since it was excluded from measurement due to visible aggregation. * PDI > 0.5. † Macroscopic aggregation after one day.

TPP concentrations 0.006-0.009% (the size at 0% TPP cannot be considered reliable due to the high polydispersity). The increase in scattered intensity at nearly constant size indicated an increase in the compactness, probably induced by the incorporation into the nanoparticles of new chitosan chains (free at lower TPP concentration).[92] The particle size tended to decrease by further increasing the TPP concentration, as also observed in previous studies.[88, 92] The simultaneous increase in scattered intensity suggested an increase in compactness, in the same way as recorded for the pectin nanoparticles at increasing crosslinker concentrations. This could indicate that most of the chitosan chains were included into the nanoparticles, thus only shrinking due to the formation of intra-particle crosslinks could be detected.[92] High crosslinker concentrations have previously been reported to cause particle aggregation for chitosan nanoparticles and other types of nanoparticles formed by ionic crosslinking.[102] In fact, the highest crosslinker concentration tested for both pectin and chitosan formulations (0.017%) led to the formation of particle aggregates, as described above for the alginate nanoparticles. In fact, at high crosslinker concentration, the pectin formulation showed macroscopic aggregation; and the chitosan formulation showed an increase in size (Figure 5.6 B) and, after one day of storage, precipitation.

The process of formation of the three types of polysaccharide-based nanoparticles scrutinized is outlined in Figure 5.7. Optimal formulations for drug delivery purposes should provide fully formed and non-aggregated nanoparticles. The pectin-zinc

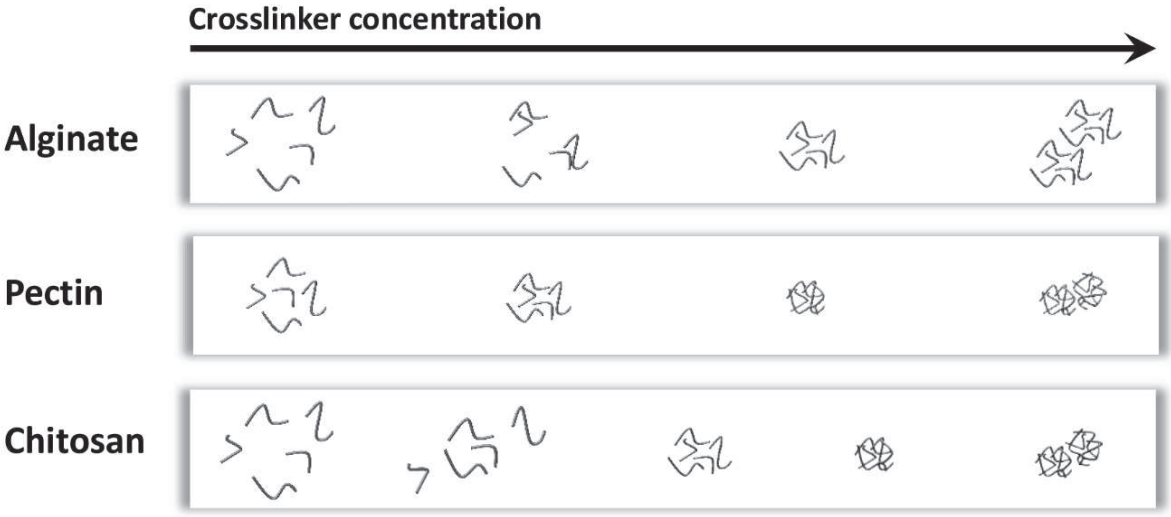


Figure 5.7. A schematic illustration of the stages identified in the process of formation of alginate, pectin, and chitosan nanoparticles at increasing crosslinker concentrations.

formulations corresponding to these requirements contained 0-0.13% zinc; the formulation containing zinc > 0.13% can be excluded due to aggregation (Figure 5.6 A). Regarding the chitosan-TPP formulations, the TPP concentrations that seemed to be the most suited for pharmaceutical use are 0.009-0.013%, since TPP > 0.013% caused aggregation and TPP < 0.009% seemed not sufficient for incorporating most of the chitosan chains in the nanoparticles (Figure 5.6 B).

Previous studies indicated that the bioadhesion of positively charged nanosystems on the negatively charged surface of teeth and mucosa is mainly driven by ionic interactions.[51, 52, 64] Therefore, a high positive charge of the nanoparticles could be advantageous for oral targeting, since it might promote stronger interactions with the oral surfaces. Consequently, a TPP concentration of 0.009% was chosen for further experiments for chitosan-TPP nanoparticles, because, in the range of the optimal TPP concentrations, it provided the particles with the highest positive zeta potential (paper III). Oppositely, for negatively charged nanoparticles a low charge could reduce the repulsion with the oral surfaces,[9, 64] and a high zinc content could be advantageous to exploit its therapeutic activity. Hence, the zinc concentration 0.013% was selected for the pectin-zinc formulations for further studies, due to the highest zinc content and the lowest negative charge density in the range of the optimal zinc concentrations.

5.1.2. Polysaccharide-coated liposomes

Colloidal entities with a charged surface can be coated with oppositely charged polymers by electrostatic deposition.[65, 76, 93, 104] A complete coverage of the liposomal surface is crucial for obtaining stable non-aggregating liposomes, and it can be achieved by using polymer concentrations only in a specific range.[65, 93] In paper IV, positively charged liposomes (Egg-PC + DOTAP) were coated at neutral pH by using the negatively charged polysaccharides HM pectin or alginate; while negatively charged liposomes (Egg-PC + Egg-PG) were coated with the positively charged chitosan. In order to determine the concentrations of the polysaccharides that could provide a complete coverage of the liposomes, increasing concentrations of the polysaccharide were mixed with liposomal suspensions at a constant concentration.

The presence of the coating layer can be verified by an increase in size and a reversal of the zeta potential.The average size presented an abrupt increase after adding the polysaccharides to the liposomes, and tended to decrease to a plateau while increasing the

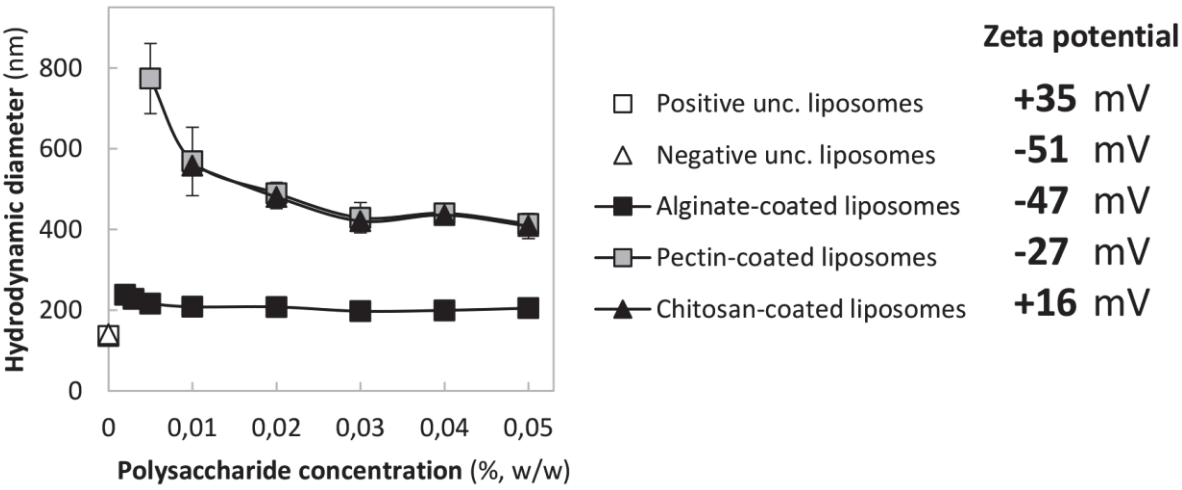


Figure 5.8. The mean particle diameter (z-average) of uncoated and polysaccharide-coated liposomes at increasing polysaccharide concentrations (%, in the initial polysaccharide solution). The zeta potential of the liposomes is indicated on the right (the zeta potential at the plateau is reported for the polysaccharide-coated liposomes).

polysaccharide concentration (Figure 5.8). At the plateau the size was constant when the polysaccharide concentration was increased, because the coating repelled further adsorption of polysaccharide with the same charge by creating an electrostatic barrier.[65] The size at the plateau represents the size of the liposomes completely coated with the polysaccharide. The large sizes recorded at the lowest polysaccharide concentrations were due to the presence of large liposomal aggregates. These aggregates can be attributed to a partial coverage of the liposomal surface with the polysaccharide; uncoated areas on a liposome bind electrostatically the coating on the surface of a different liposome, thus resulting in the so called "bridging flocculation" (Figure 5.9).[65, 93] At the plateau, the charge of the polysaccharide-coated liposomes was constant and reversed compared to the charge of the uncoated liposomes (Figure 5.8), thus confirming a successful coating process. Similar trends for zeta potential and average size have also been previously observed during the coating of negatively charged liposomes with chitosan.[65, 105, 106]

The thickness of the polysaccharide layer on the fully coated liposomal surface can be estimated by calculating the difference between the average radius of the coated liposomes and of the corresponding uncoated liposomes.[107] The alginate coating (~30 nm) was markedly thinner than the chitosan and the pectin coatings (~150 nm each). The thickness of the layer adsorbed onto the liposome is influenced by the amount, the conformation and the compactness of the polysaccharide adsorbed.[108] Polysaccharides with a high charge

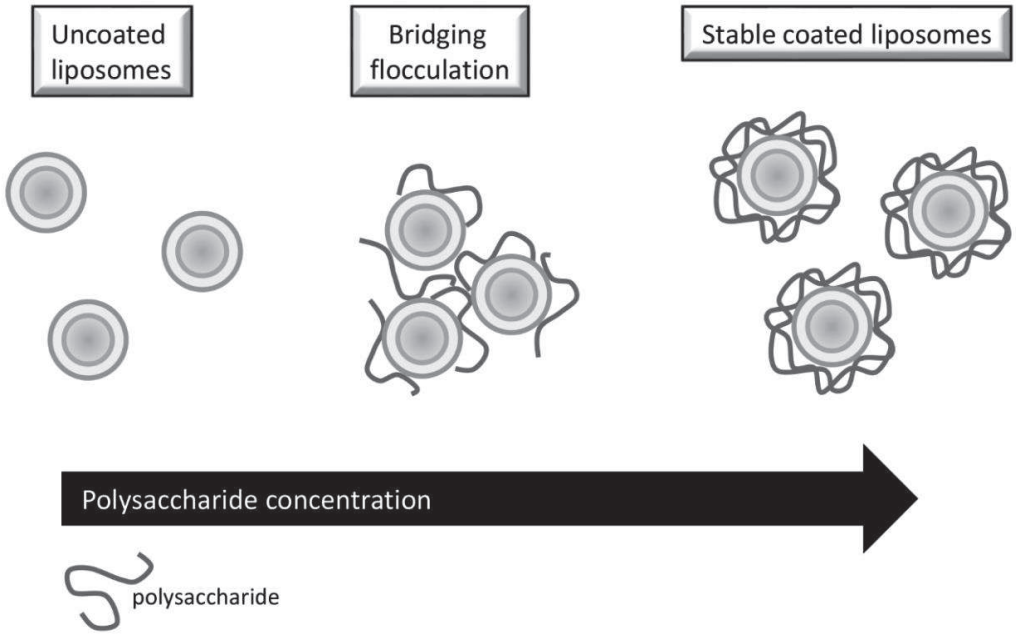


Figure 5.9. A schematic illustration of the coating of the liposomes at increasing polysaccharide concentrations.

density, such as algiante, tend to adhere in a flat conformation ("trains") to an oppositely charged surface.[108-110] Moreover, the high charge on the polysaccharide layer would promote a strong attraction with the liposomal surface and discourage the adhesion of further polysaccharide chains through electrostatic repulsion. The result is the formation of a thin and compact layer. On the other hand, polysaccharides with a lower charge density tend to form loops on the liposomal surface ("loops-and-tails" conformation) and loose thick layers.[108, 109] This could occur for chitosan and HM pectin coatings, as previously observed,[66, 77, 110] since, at neutral pH, such polysaccharides are more weakly charged than alginate. The low repulsion would also allow to adsorb higher amounts of polysaccharide that might also be present in overlapped layers.[107] In addition, the ramifications of the pectin could also have contributed to increase the thickness of the coatings.[77, 107]

A polysaccharide concentration (in the added solution) of 0.04% was chosen for the experiments carried out in the second part of the project (Section 5.2), since this concentration appeared to be in the range that provided a full coverage of the liposomes for all the polysaccharides tested.

5.2. Evaluation of the suitability of the nanosystems for oral cavity applications

The second part of the project examined possibilities and limitations of the eight potentially optimal formulations selected in the first part of the project, with regard to aspects relevant for their application as improved systems for local oral usage. In particular, stability in artificial saliva, cytotoxicity on a buccal cell line, and adsorption to HA were investigated. The average diameter of the chosen nanosystems ranged between about 140 and 440 nm, and was in the same size range as the salivary micelle-like structures.[31, 32]

5.2.1. Stability in simulated salivary fluid

An important aspect to evaluate when developing a new drug delivery system is the stability of the formulation in the physiological fluids present at the site of administration, such as saliva. Considering the charged nature of the investigated nanosystems and the electrostatic interactions involved in the formation of most of them, it is reasonable to presume that the electrolytic content, the pH, and the ionic strength of the saliva can influence the stability of the nanosystems.[60, 78, 82, 108, 111] For this reason, an artificial saliva mimicking these aspects of natural saliva was used for the stability study.[96] The physical stability in artificial saliva of polysaccharide-based nanoparticles was tested in paper III, and of liposomes in paper IV.

The most common causes of instability due to electrostatic intereference are depicted in Figure 5.10 for the three types of nanosystems investigated. Aggregation can occur as a result of reduced charge repulsion between the particles, or of ionic bridging due to the presence of multivalent oppositely charged ions.[78, 87, 111] The charged and hydrophilic nature of the polysaccharides renders the nanosystems susceptible also to other types of modification. Disintegration occurs when the electrostatic bonds between the polysaccharide and the crosslinker or the liposomal surface are broken.[78, 108] Regarding the polysaccharide-coated liposomes, the disintegration of the polysaccharide layer might result in bridging flocculation due to ionic bridging between the partially coated areas and the uncoated patches on different liposomes.[107] Shrinking and swelling are generally caused by variations in the charge density of the polysaccharide coating;[112] nevertheless, these are less dramatic modifications since single nanoparticles can still be discerned.

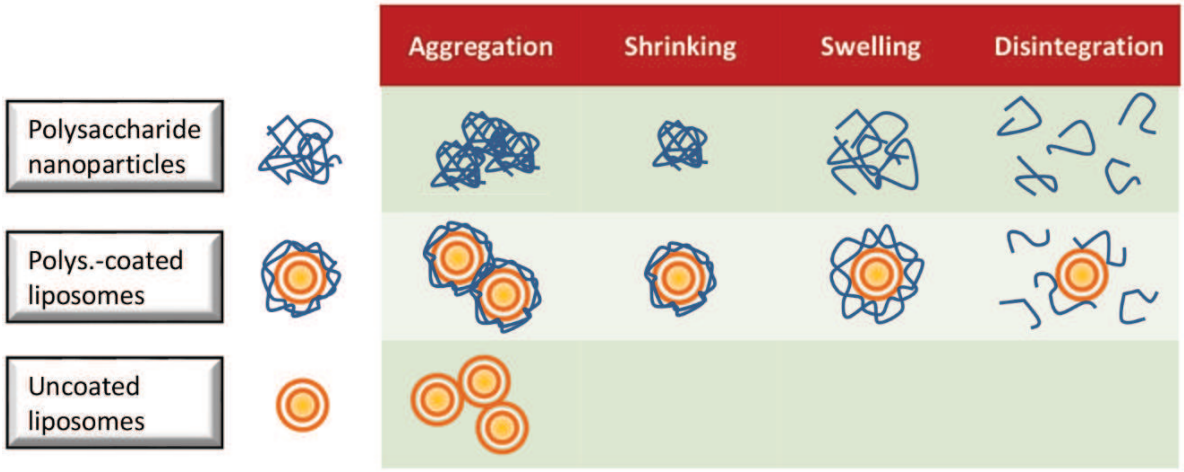


Figure 5.10. Potential causes of physical instability due to electrostatic interference for polysaccharide-based nanoparticles, polysaccharide-coated liposomes, and uncoated liposomes.

All the nanosystems with a positively charged surface (positive uncoated liposomes, chitosan-coated liposomes, and chitosan nanoparticles) formed aggregates in artificial saliva, as suggested by the increase in both PDI and average size. The same behaviour has also been recoded in a previous study for positively charged oil droplets in nanoemulsions.[111] The aggregation can be possibly attributed to the presence of mulitvalent anionic species in artificial saliva (sulfates, phosphates, and carbonates) that could lead to bridging between the particles and screen the particle charge with consequent reduction of their repulsion. Moreover, the reversal of the particle charge, recorded for the chitosan-coated liposomes, could originate from the partial desorption of the coating due to electrostatic competition with the dissolved electrolytes, thus leading to bridging flocculation. Previous studies documented the aggregation of positively charged nanoformulations also in the presence of natural saliva, which has been attributed to ionic interactions with negatively charged macromolecules present in the natural saliva.[113-115] However, the findings reported hereby indicate that also the electrolytic composition of saliva could have a role in their aggregation.

The nanosystems with a positively charged surface presented signs of instability immediately after contact with artificial saliva, except for the positive uncoated liposomes, which were stable for ~80 minutes before aggregation appeared. For this reason, the positive uncoated liposomes were the positively charged formulation that performed best in artificial saliva. Nevertheless, further studies are required to confirm a possible stability also in the presence of the negatively charged macromolecules found in the natural saliva.

The negatively charged nanosystems were generally more stable in artificial saliva compared to the positively charged ones. Negative uncoated liposomes and alginate nanoparticles were the most stable formulations, since they maintained the same size and PDI during the whole test. Pectin- and alginate-coated liposomes showed a constant PDI, and a reduction and an increase in size, respectively. Both of these variations could be attributed to variations of the coating layer induced by electrostatic screening.[108] The reduction in size was possibly caused by partial disintegration of the coating due to breakage of the ionic bonds between the pectin and the liposomal surface, as a result of the reduced charge of the pectin chains in artificial saliva.[107, 108] The increase in size of alginate-coated liposomes could be caused by the swelling of the alginate layer due to the breakage of some bonds between the alginate chains and the liposomal surface, thus increasing the porosity of the alginate layer. However, the breakage of the bonds between the alginate layer and the liposomal surface was not pronounced enough to cause the disintegration of the coating.[109, 110] The least stable negatively charged nanosystems in the artificial saliva were the pectin nanoparticles. Their reduction in size and increase in PDI suggested a partial disintegration, possibly caused by the chelation of the crosslinker zinc operated by the phosphate anions in the artificial saliva, as previously reported for alginate systems crosslinked with calcium.[116]

Previous experiments carried out in natural saliva have also showed that colloidal systems with a negatively charged surface tended to be more stable than positively charged liposomes.[113, 115] Nevertheless, the aggregation of some negatively charged formulation has been detected due to bridging induced by the presence in natural saliva of calcium cations.[113] In the present experiments, the presence of calcium did not seem to cause aggregation of the negatively charged formulations. This indicates that the stable negatively charged nanosystems (negative uncoated liposomes, and alginate nanoparticles) could be the most promising formulations for application in the oral environment in the presence of saliva. However, the other nanosystems might still find application for those patients suffering from a reduced production of saliva.

Due to the complexity and variability of natural saliva,[13, 15] it is necessary to keep in mind that the interactions occurring in natural saliva are more complicated than in an artificial saliva. Therefore, further investigations in the presence of other salivary components and in natural saliva would be appropriate in order to evaluate in more depth the stability of the nanosystems in an oral environment.

5.2.2. Cytotoxicity against cells of the buccal epithelium

The biocompatibility is an important aspect to consider, especially when designing bioadhesive formulations that are meant for long term administration and for prolonged retention in close contact with tissues. The cytotoxicity of the tested nanosystems and their components was, therefore, investigated *in vitro* against the cells of the human buccal epithelium (TR146)[97] as a first approximation to the biocompatibility with the buccal epithelium (paper III and additional experiments). Figure 5.11 shows the relative viability

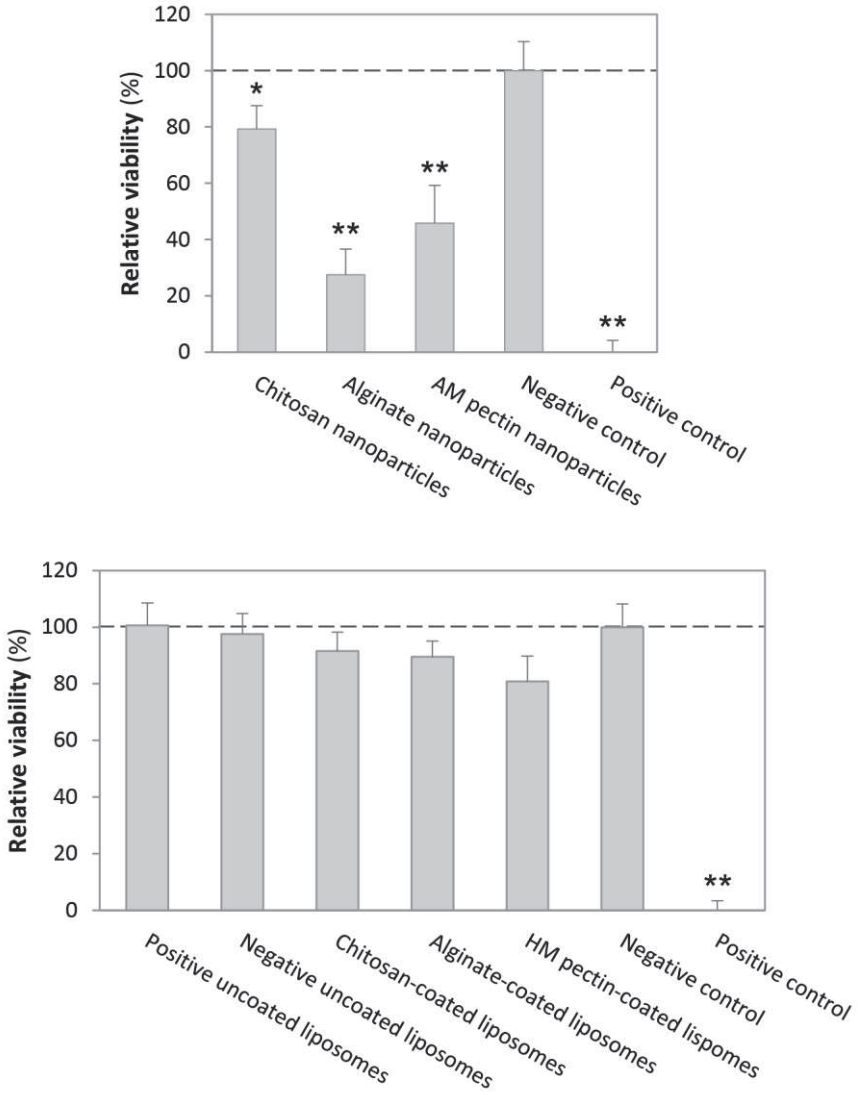


Figure 5.11. Relative viability of the TR146 cells after four-hour treatments with the polysaccharide-based nanoparticles (top) and with the liposomal nanosystems (bottom). The concentration of the polysaccharide-based nanoparticles corresponded to 0.05% polysaccharide, and the concentration of the liposomal formulations corresponded to 0.6 mM lipid (and 0.032% polysaccharide for the polysaccharide-coated liposomes). Viability significantly different from the negative control: * $p < 0.05$ and ** $p < 0.001$.

of the TR146 cells treated for four hours with each nanoformulation investigated. While interpreting the results, it is necessary to keep in mind that all the nanosystems with a positively charged surface tended to aggregate in the test medium, therefore their results must be considered with caution. All the polysaccharide-based nanoparticles displayed some cytotoxicity, especially the alginate-based and the pectin-based nanoparticles.

Cationic compounds and positively charged nanosystems have often been reported as more toxic than the negatively charged ones.[86, 117] For example, chitosan[118-120] and chitosan-based nanoparticles[119, 121, 122] caused toxicity and cell necrosis on various cell lines and *in vivo*. Unexpectedly, in the present results, the positively charged formulations (chitosan-based nanoparticles, positive uncoated liposomes, and chitosan-coated liposomes) displayed no or low cytotoxicity at the concentrations and time course investigated. However, an increase in cytotoxicity at higher concentrations (or prolonged time course) cannot be excluded, since in previous studies the cytotoxicity of chitosan and chitosan-based nanoparticles was reported to be dose-dependent.[118, 119, 121]

Interestingly, the free chitosan, at the same concentration as in the chitosan-based nanoparticles (0.05%), exhibited a considerably higher cytotoxicity (42% cell viability) than the chitosan-based nanoparticles. This is in accordance with a previous study that showed a higher cytotoxicity of the free chitosan compared to chitosan nanoparticles.[118] The cytotoxicity of chitosan has been attributed to the interaction of chitosan with components on the cellular membrane[122] and is correlated with the density of positive charge on the chitosan chains.[119, 122] This suggests that, when the chitosan is in a crosslinked form, the reduction in cytotoxicity could be attributed to the lower charge density on the chitosan chains of the particle (due to the interaction between the chitosan and ionic crosslinker). Moreover, the low cytotoxicity of the chitosan nanoparticles could also reflect a lower amount of chitosan interacting with the cell surface, since only the chitosan on the external surface of the particle could be available for the interaction.

Solutions of free alginate and free pectin at the same concentration as in the polysaccharide-based nanoparticles (0.05%) did not display any cytotoxicity. In the same way also the alginate-coated and the pectin-coated liposomes were not cytotoxic at the conditions of the test. Conversely, alginate-based and pectin-based nanoparticles were revealed cytotoxic, probably due to the presence of zinc in the formulations. In fact, the free zinc displayed a dose-dependent cytotoxicity in the range of the concentrations tested (0.006-0.027%).

The toxicity of the zinc and of the nanoparticles containing zinc could, however, be overestimated in the present *in vitro* test. In fact, compared to zinc concentrations used in oral hygiene products (9-17 mM),[44, 81, 123] the zinc concentration in the nanoparticle formulations was considerably lower (corresponding to about 1-2 mM). Therefore, the *in vitro* test might be oversensitive compared to *in vivo* situations,[124] where, for example, a layer of mucus protect the cells from external agents. However, even though oral hygiene products contain high zinc concentrations, the amount of zinc retained in the oral cavity or its retention time might be relatively low,[44] thus possibly reducing their toxicity.

All the liposomal formulations resulted less cytotoxic than the polysaccharide-based nanoparticles at the tested concentrations, (Figure 5.11). In fact, the relative viability calculated for each liposomal formulation did not differ significantly from the negative control ($p < 0.05$). This indicated that all the liposomal formulations were cytocompatible with TR146 cells at the concentrations and time course tested. Based on these results, all the liposomal formulations can be considered promising for local oral use with respect to potential cytotoxicity. However, further cytotoxicity tests that keep in consideration the amount of nanoformulation retained in the oral cavity and the retention time would be suggested for possibly obtaining results closer to *in vivo* conditions.

5.2.3. Adsorption to hydroxyapatite

The capacity of adhering onto oral tissues could be an advantage for the pharmaceutical formulations addressed to the oral cavity, since the increase of their retention time could improve their efficacy against oral ailments. The adsorption of colloidal particles onto the enamel surface could potentially provide not only a prolonged and targeted drug release, but the particles themselves could act as a physical protection for the enamel surface, similarly to the acquired enamel pellicle. For example, a previous study showed that specific milk proteins could be adsorbed onto teeth and inhibit the adhesion of cariogenic bacteria.[125] In the same way, colloidal particles adhered on the tooth enamel might inhibit the development of dental caries.

In paper IV and additional experiments, the adhesion of the nanosystems was evaluated using HA as a model for the surface of the dental enamel. The adhesion capacity of each liposomal formulation was evaluated by measuring the percentage of liposomes adhered onto determined amounts of HA powder. Positively and negatively charged nanosystems were investigated for HA adhesion, based on the known adsorption of both positively and

negatively charged proteins for the formation of the enamel pellicle[32, 34, 126] and of both positively and negatively charged polysaccharides onto HA and enamel.[70, 71] In fact, HA possesses amphoteric features since its surface is constituted of phosphate anions and calcium cations, which both allow for electrostatic interactions with oppositely charged entities. Nevertheless, the predominant charge of the HA surface has been reported to be negative at neutral pH, especially in the presence of phosphate ions in solution.[9] As a consequence, the positively charged liposomes presented a considerably higher adsorption capacity compared to the negatively charged ones due to electrostatic interactions (Figure 5.12), as also previously observed.[52]

The adsorption of the liposomes is driven by a subtle balance between attractive and repulsive forces that depend on the surface properties of HA. The surface properties of HA are highly dependent on the pH, ionic content and concentration of the surrounding environment, since ions can adsorb electrostatically onto the HA surface in a dynamic equilibrum.[9, 11] The phosphate ions are known to be able to adhere onto HA and enamel, thus lowering the zeta potential of their surface.[9, 10] For this reason, the use of phosphate buffer as the medium might have enhanced the adhesion of the positively charged liposomes, and diminished the adhesion of negatively charged liposomes due to electrostatic repulsion, as illustrated in Figure 5.12.

The presence of other ionic species and salivary components have previously been shown to influence the zeta potential of HA and enamel,[9, 10] which, in natural saliva, was less negative compared to the zeta potential measured in phosphate buffer.[10] In specific, the calcium cations seem to play a role in the adhesion to the enamel surface of the negatively charged proteins in natural saliva, by neutralizing the zeta potential of the enamel[9] and acting as a bridge between the negatively charged enamel and the proteins.[34] For this reason, the adhesion test was carried out also by using artificial saliva as the medium, since it could mimic the presence of the major ionic species detected in saliva in physiological concentrations. The adhesion capacity of the negatively charged liposomes was significantly increased in artificial saliva for all the formulations tested, due probably to the presence of the bridging calcium cations. The positively charged liposomes were not tested in the presence of artificial saliva due to their aggregation tendencies in its presence.

The considerably higher increase in adhesion observed for the alginate-coated liposomes compared to the negative uncoated liposomes and pectin-coated liposomes should probably be attributed also to other reasons. For example, the possibly higher porosity of the alginate coating, induced by the presence of artificial saliva, could promote

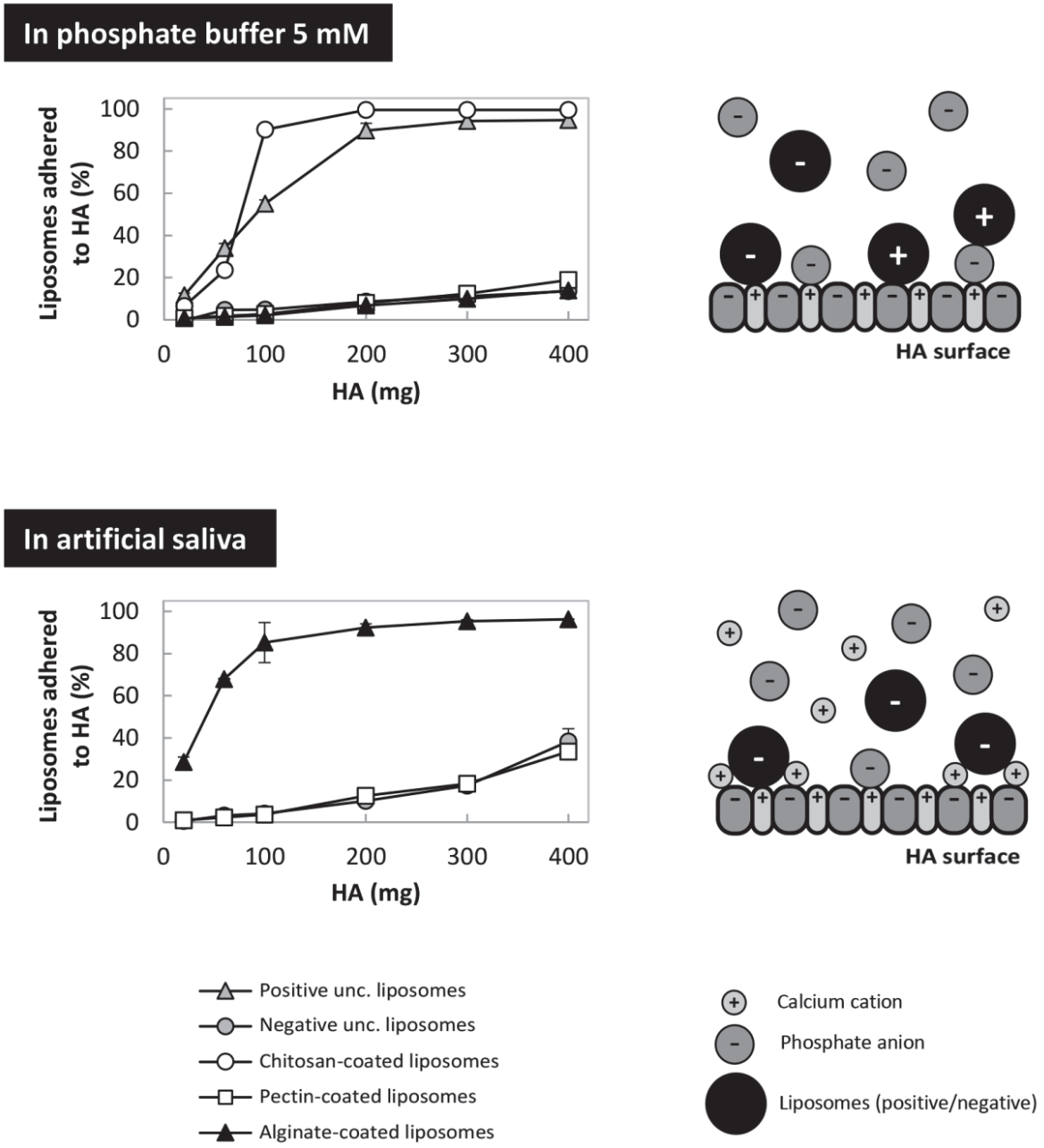


Figure 5.12. Adsorption of the liposomes on the HA surface both in phosphate buffer 5 mM (above) and in artificial saliva (below). The graphs indicate the percentage of liposomes adsorbed onto HA powder, while keeping constant the amount of liposomes and increasing the amounts of HA.

the interaction of the HA surface not only with the alginate layer, but also with some positive charges of the liposome underneath. This would therefore result in an adhesion capacity markedly higher than expected.

The presence of the polysaccharide coating improved, for some of the formulations, the adsorption onto the HA. In specific, an improved adhesion compared to the uncoated

liposomes (both positive and negative) was recorded for the alginate-coated liposomes (in artificial saliva only) and for the chitosan-coated liposomes.

In additional experiments (not included in the papers), the potential adsorption of the polysaccharide-based nanoparticles onto HA was evaluated through a qualitative test by visual determination of the presence of the nanoparticles on the surface of HA discs. The SEM images of the surface of HA discs treated with the polysaccharide-based nanoparticles are reported in Figure 5.13. The images indicate that the alginate-based and the pectin-based nanoparticles were capable of adhering onto the HA surface, since it was possible to visualize their presence at the HA surface. However, no particles were detected on the HA disc treated with chitosan-based nanoparticles, which therefore did not seem to adhere markedly onto HA. These results were unexpected, since, conversely, chitosan coated-liposomes had the highest adsorption capacity compared to alginate-coated and pectin-coated liposomes.

The adhesion of the alginate-based and pectin-based nanoparticles could have been promoted by the presence of zinc cations. In fact, the zinc cations could have acted as bridges between the HA surface and the negatively charged nanoparticles, in the same way described above for the calcium cations and the liposomes with negatively charged surface. Moreover, the absence of phosphate anions at the interface could have maintained a low electrostatic repulsion between the HA and the negatively charged nanoparticles.[9] In fact, the solvent in this set of experiments was constituted by 0.05M NaCl, whose presence have a lower influence on the zeta potential of the HA surface compared to phosphates.[9]

The most surprising result was obtained for the chitosan-based nanoparticles, which, despite the relatively high zeta potential, did not seem to adhere markedly onto the HA surface. This could probably be promoted by a low negative charge on the HA surface due to the absence of phosphates. Moreover, the presence of chloride anions in the solvent of the nanoparticles could have reduced the adhesion capacity of the chitosan-based nanoparticles. In fact, a previous study showed a marked reduction of the adhesion of chitosan microparticles onto mucosa (which presents a net negative charge at neutral pH, as HA) when monovalent anions (fluoride) were included in the formulation.[127]

Since this experiment represents only a qualitative test, further quantitative tests are required in order to confirm and complement the present observations for the adhesion of polysaccharide-based nanoparticles onto HA.

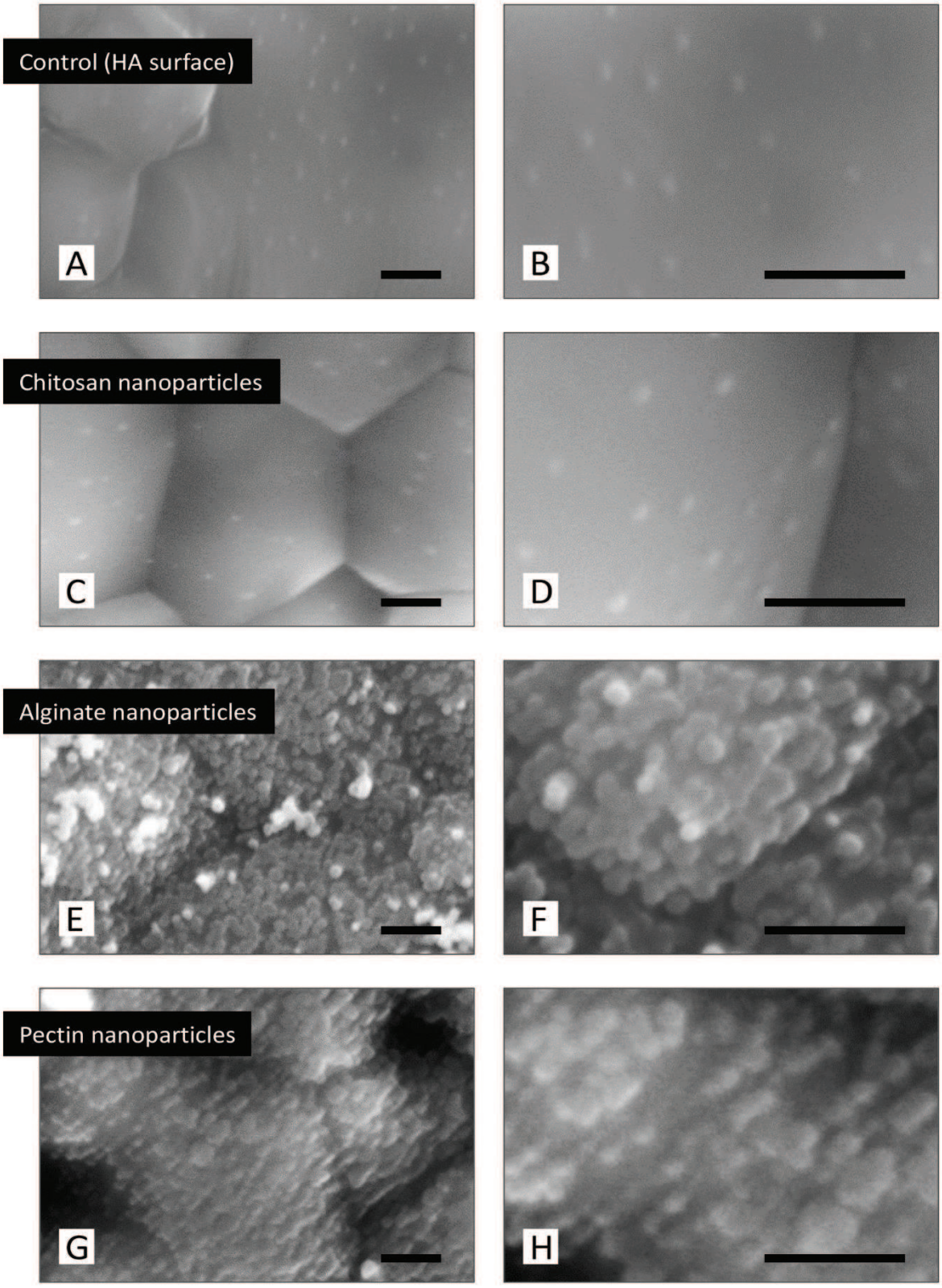


Figure 5.13. Qualitative evaluation of the adsorption to HA of the polysaccharide-based nanoparticles. SEM images of the HA surface after dipping in (A and B) water (control), (C and D) chitosan nanoparticles, (E and F) alginate nanoparticles, and (G and H) pectin nanoparticles. The black bar represents a length of 1 μm.

6. CONCLUSIONS

In this project, three types of charged polysaccharides were used for the preparation of nanoparticles and for coating liposomes. Specifically, 52 formulations of polysaccharide-based nanoparticles were investigated to determine how the concentration of the polysaccharide, the concentration of the crosslinker, and the ionic strength of the solvent influence the nanoparticle characteristics. Moreover, 22 formulations for polysaccharide-coated liposomes were prepared to determine the polysaccharide concentration that provide the full formation of the polysaccharide coating around the liposomes. This knowledge allowed to determine ranges of the values of the formulation factors that granted the formation of colloidally stable non-aggregated nanosystems. In addition, the process of formation of all the nanosystems was revealed, and enabled to establish six promising formulations for further studies expected to be the most suited for administration into the oral cavity. The six selected formulations, together with two types of uncoated liposomes (positively and negatively charged), were tested for preliminary *in vitro* studies in order to evidence possibilities and limitations for their potential application as local oral treatment (Table 6.1).

Table 6.1. Overview of the results, at the conditions of the tests, of the preliminary *in vitro* studies for the eight types of nanosystems scrutinized.

	Charge of the nanosystem	Stability in artificial saliva	Cytotoxicity	Adhesion to HA
Negative uncoated liposomes	-	*stable*	*safe*	*moderate adhesion*
Positive uncoated liposomes	+	*unstable*	*safe*‡	*high adhesion*
Alginate-coated liposomes	-	*stable**	*safe*	*high adhesion*
HM pectin-coated liposomes	-	*stable**	*safe*	*moderate adhesion*
Chitosan-coated liposomes	+	*unstable*	*safe*‡	*high adhesion*
Alginate-zinc nanoparticles	-	*stable*	*high toxicity*	*adhered* †
AM pectin-zinc nanoparticles	-	*unstable*	*high toxicity*	*adhered* †
Chitosan-TPP nanoparticles	+	*unstable*	*low toxicity*‡	*non-adhered* †

‡ Particle aggregation during the test; * Particle size variation, † Qualitative test.

The type of charge of the nanosystem was revealed to be of primary importance for the stability in artificial saliva and for the adhesion capacity onto HA. Generally, the negatively charged nanosystems were more stable in artificial saliva compared to the positively charged. The positively charged formulations displayed instead a higher adhesion capacity onto HA compared to the negatively charged formulations for the liposomal systems. However, the opposite trend seemed to be true for the HA adhesion of polysaccharide-based nanoparticulate formulations, even though further quantitative testing is required to possibly confirm this result. Regarding the cytotoxicity studies, the liposomal formulations were more cytocompatible compared to the polysaccharide-based nanoparticles at the tested concentrations. The cytotoxicity of the polysaccharide nanoparticles seemed to stem in the presence of their positively charged components chitosan or zinc.

Based on the results discussed in this thesis, the most suitable nanosystems for tooth protection are represented by the negative uncoated liposomes, pectin-coated and alginate-coated liposomes. This was in virtue of their stability in artificial saliva, moderate to high HA adhesion capacity, and non-cytotoxicity. However, the results of the studies enabled also to determine the possible causes of the poor performance of the other nanosystems. In this way, modifications of their formulations that might improve their outcome could be suggested. For example, the partial replacement of the crosslinker zinc in the alginate and pectin nanoparticles with another divalent cation, such as calcium, might reduce their cytotoxicity;[128] or the coating of the chitosan nanoparticles with a negatively charged polysaccharide might improve their stability in a salivary environment and their cytocompatibilty.

7. FUTURE PERSPECTIVES

The results obtained in this project constitute the basis for future investigations regarding the application of the tested nanosystems for local oral usage.

- Further studies could test and optimize the formulations of the investigated nanosystems for stability in the presence of other salivary components and in natural saliva.

- The amount of nanoformulation adsorbed onto human enamel and the strength of the adhesion in the presence of natural saliva could be determined quantitatively. The evaluation of the adhesion capacity at low pH values would also be important in order to simulate the environment created by cariogenic bacteria through the production of acids.

- The presence of the nanosystems at the tooth enamel surface is supposed to mimic and possibly strengthen the physical protective action of the acquired enamel pellicle. This effect could be verified, for example, by inducing acid challenges in order to test the capacity of preventing tooth erosion, or by determining if the presence of the nanoformulations could discourage the adhesion of cariogenic microorganisms to the enamel surface.

- In virtue of the antibacterial activity of chitosan and zinc,[42, 72] further investigations could also verify the potential antimicrobial action of the polysaccharide-based nanoparticles and the chitosan-coated liposomes against cariogenic bacteria.

- Liposomes and polysaccharide nanoparticles can entrap a wide variety of active substances and provide sustained release, which can improve the efficacy of specific treatments.[55] Studies on the encapsulation into the nanosystems of active substances relevant for the treatment of oral ailments could, therefore, be beneficial for complementing the potential physical tooth protection of the nanosystems with the chemical action of an active substance. In particular, the use of polysaccharide nanoparticles as drug carriers might be interesting, since the possible swelling or disintegration of the particles following variations of the oral pH might provide a stimuli selective drug release.[60, 63]

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