

Thesis for the degree of *Philosophiae Doctor*  
Department of Pharmacy, School of Pharmacy  
University of Oslo - Norway

**FORMULATION OF LIPOSOMES FOR  
PROTECTIVE FUNCTIONS FOR THE HUMAN  
DENTAL ENAMEL**

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Oslo 2011

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## **To my parents**

"One should not pursue goals that are easily achieved. One must develop an instinct for what one can just barely achieve through one's greatest efforts."

Albert Einstein, 1915



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Sanko Nguyen

## ABSTRACT

The present work concerns the development of liposomal formulations that can adsorb to the human dental enamel. The overall aim of this pharmaceutical approach is to physically protect the teeth against detrimental processes, such as tooth wear, acidic challenges and dental caries. Adsorption experiments of different liposomal formulations to hydroxyapatite (HA), a model substance for the dental hard tissue, and the human dental enamel were performed.

To find which liposomal formulations are promising for the adsorption to teeth, formulation factors important for the interaction were initially mapped by the use of experimental design and multivariate analysis (Paper I). The type of surface charge became the most significant factor for the adsorption process. Positively charged liposomes adsorbed better than the negatively charged liposomes to HA in phosphate buffer, pH 6.8-7. However, the adsorption of positively charged liposomes to HA in a salivary environment was interfered as they were found to aggregate with components of saliva (Paper II).

To overcome problems related to the positively charged liposomes, the surface of the liposomes was modified with the polymer pectin. Three types of pectin were investigated for the surface coating of liposomes: LM-, HM- and amidated pectin (Paper III). Pectin coating of positively charged liposomes was successfully prepared, and a reproducible method was established. Pectin coated liposomes did not seem to interact with salivary components (Paper IV), and were therefore promising for use in the oral cavity.

Pectin coated liposomes adsorbed to HA in saliva, and liposomes coated with LM- and HM-pectin were selected for further investigation with the dental enamel (Paper IV). Both uncoated negatively charged liposomes and pectin coated liposomes adsorbed onto enamel specimens in a salivary environment (Paper IV), indicating their potential use in the protection of the teeth. The adsorption was examined by exposing a flow on the enamel surfaces for certain time intervals. Pectin coated liposomes seemed to retain better than uncoated negatively charged liposomes at longer time intervals. It was hypothesized that pectin may help to prolong the adhesion of liposomes on the tooth surfaces.



# LIST OF PAPERS

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

- I.** Nguyen S, Solheim L, Bye R, Rykke M, Hiorth M, Smistad G  
The influence of liposomal formulation factors on the interactions between liposomes and hydroxyapatite.  
*Colloids and Surfaces B: Biointerfaces* 76 (2010) 354-361
  
- II.** Nguyen S, Hiorth M, Rykke M, Smistad G  
The potential of liposomes as dental drug delivery systems.  
*European Journal of Pharmaceutics and Biopharmaceutics* 77 (2011) 75-83
  
- III.** Nguyen S, Alund SJ, Hiorth M, Kjøniksen AL, Smistad G  
Studies on pectin coating of liposomes for drug delivery  
*Colloids and Surfaces B: Biointerfaces* 88 (2011) 664-673
  
- IV.** Nguyen S, Hiorth M, Rykke M, Smistad G  
Adhesion of liposomes to the enamel surface for potential protective functions  
*Caries Research* (submitted)

## LIST OF ABBREVIATIONS

AFM	Atomic force microscopy
AM-pectin	Amidated pectin
DLS	Dynamic light scattering
DM-ethylPC	Dimyristoyl ethylphosphatidylcholine
DMPA	Dimyristoyl phosphatidic acid
DMPC	Dimyristoyl phosphatidylcholine
DMPG	Dimyristoyl phosphatidylglycerol
DMPS	Dimyristoyl phosphatidylserine
DMTAP	Dimyristoyl trimethylammoniumpropane
DOPS	Dioleoyl phosphatidylserine
DOTAP	Dioleoyl trimethylammoniumpropane
DP-ethylPC	Dipalmitoyl ethylphosphatidylcholine
DPPA	Dipalmitoyl phosphatidic acid
DPPC	Dipalmitoyl phosphatidylcholine
DPPG	Dipalmitoyl phosphatidylglycerol
DPPS	Dipalmitoyl phosphatidylserine
DPTAP	Dipalmitoyl trimethylammoniumpropane
Egg-PA	Egg phosphatidic acid
Egg-PC	Egg phosphatidylcholine
Egg-PG	Egg phosphatidylglycerol
HA	Hydroxyapatite
HM-pectin	High methoxylated pectin
LM-pectin	Low methoxylated pectin
NBD-PC	Nitrobenzoxadiazol-4-yl-phosphocholine
PI	Phosphatidyl inositol
PP	Pyrophosphate
PRP	Proline-rich protein

# 1. INTRODUCTION

The most common public health problems worldwide are dental ailments <sup>1</sup>. Dental problems can greatly impair an individual's well-being and thereby have a major impact on the individual's quality of life. Evidently, there is a need to implement preventive measures that can protect the teeth against detrimental processes such as tooth wear, acidic effects and dental caries. This thesis presents the development of pharmaceutical formulations, i.e. liposomes that can adsorb onto tooth surfaces, as a possible means for the physical protection of the dental enamel. The possibility of formulating liposomes of nanosize with the appropriate surface characteristics enables them to mimic the natural protective layer of the teeth; the acquired enamel pellicle. The concept of using liposomal formulations for their physical properties *per se* instead of their drug carrying capacity, as will be demonstrated here, has been scarcely investigated.

## 1.1. Dental problems – prevention and treatment options

Identification of the most common tooth related problems defines the background and the rationale for developing systems that can protect teeth and reduce the subsequent dental ailments. Knowledge of the available prevention and/or treatment procedures for these problems may also reveal the inadequacies related to the present methods. In this regard, present treatment options that require professional intervention will not be considered here.

Dental enamel is the outmost mineralized tissue that covers the crown of the tooth. It is this surface of the teeth that is in constant interactions with components of the oral cavity in normal *in vivo* conditions. In order to understand the behavior of the dental enamel in the oral environment, important surface characteristics must be known.

### 1.1.1. Human dental enamel – physicochemical parameters

Approximately 96% (by weight) of the human dental enamel is composed of inorganic substance, the remainder being organic material and water. Because the enamel is highly mineralized, it is the hardest substance in the human body and, consequently, has

high resistance to both shearing and impact forces. This enable the teeth to withstand the mechanical forces applied during normal tooth functioning. This hardness also makes the enamel brittle. However, the enamel can withstand fracture due to high modulus of elasticity in combination with the resilient support of the underlying tissue, i.e. the dentine <sup>2</sup>.

The inorganic content of enamel consists of crystalline calcium phosphate, also known as hydroxyapatite ( $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})^2$ ; HA). Ionic exchange can occur between the enamel and the environment of the oral cavity. Hydroxyl ions may be substituted by fluoride in the crystalline lattice, leading to a more stable and resistant structure against acidic dissolution. This ability is important considering the beneficial effects of fluoride in the remineralization of the dental enamel <sup>3</sup>.

The enamel surface is subjected to changes depending on the pH and ionic content of the surrounding medium. For instance, the pH affects the solubility of the enamel surface. At low pH (acidic conditions), the surrounding environment is unsaturated with respect to HA, and the mineral will tend to dissolve to reestablish the saturated condition <sup>4</sup>. This makes pH the driving force for the de- and remineralization processes at the enamel surface. However, the presence of fluorides in the surrounding medium strongly influences the pH at which the surrounding medium is unsaturated. The presence of fluorides lowers the critical pH for unsaturation and is, thus, probably one of the most successful factors in caries prevention.

At neutral pH, the enamel exhibits about 90% phosphate and about 10% calcium ions at the surface <sup>5,6</sup>. This means that the enamel expose both cations and anions at the solid surface. In the salivary environment of the oral cavity, a hydration layer is formed at the enamel surface. Since the enamel exhibits mainly negative phosphate groups at the surface, the hydration layer comprises mostly of attached calcium ions, acting as counterions to the phosphate ions of the enamel. Thus, possessing both phosphate and calcium sites at the enamel surface, the enamel has affinity to both acidic and basic substances. The zeta potential of human enamel has been estimated to be about -9 mV at neutral pH <sup>7</sup>. Apart from pH, the presence of calcium and phosphate in the surrounding environment also influences the zeta potential <sup>8</sup>.

## 1.1.2. Dental caries

The most prevalent and widespread dental problem today is caries. Although it has been observed a decline in dental caries in industrialized countries in the last two decades due to the effective use of fluoride and improved self-care practices, dental caries still remain a major oral health problem<sup>9-12</sup>. Additionally, there is an increased tendency of caries-related problems in developing countries. A growing consumption of sugars together with an inadequate exposure to fluorides are thought to be the main causes<sup>1</sup>.

Dental caries is characterized by the loss of enamel substance due to the demineralizing effects of organic acids (e.g. lactic acid) produced by bacteria in dental plaque. Acid production stem from the metabolism of simple dietary sugars, most notably glucose and sucrose, by cariogenic bacteria such as streptococci, lactobacilli and actinomycetes<sup>13</sup>. Since caries develops slowly in most cases, and restorative treatment has a tendency of short durability due to recurrent caries, more emphasis has been laid on the prevention of caries management<sup>14</sup>. For self-administered care, fluoride toothpaste has proven to be the most effective caries preventive approach. Fluoride can also be delivered in other forms such as gels, lozenges, varnishes or mouth rinses. To maintain a constant level of fluoride in the oral cavity, new formulations for sustained release of fluoride have been developed, e.g. new intra-oral devices such as bioadhesive tablets<sup>15, 16</sup>. Other common self-care preventive measures of dental caries are mechanical plaque removal (e.g. tooth brushing) and antimicrobial therapy (e.g. chlorhexidine mouth rinses).

It should be emphasized that although with improved oral hygiene, synchronously well-functioning saliva secretion is of major importance for an optimal prophylactic effect, especially in the early phase of caries lesion development. Saliva secretion offers continuous protection in its ability to clear cariogenic food substances, neutralize and dilute organic acids produced by plaque bacteria and detoxify or kill bacteria by its antibacterial components<sup>17, 18</sup>. Moreover, the ability to regulate demineralization and remineralization processes by virtue helps to prevent further hard tissue loss by means of caries processes and tooth wear. This should be borne in mind for patients with salivary hypofunction. Many patients suffer from xerostomia, also known as dry mouth, due to e.g. the side-effects of medical treatment, radiotherapy for cancer treatment in the head and neck region, and certain systemic diseases such as Sjögren's syndrome<sup>19-21</sup>. Although xerostomia does not directly influence the teeth, the lack of salivary protective functions may lead to adverse oral implications that affect the health of teeth<sup>22</sup>.

### 1.1.3. Tooth wear

Tooth wear is a general term used to describe the process of non-cariou enamel and dentin loss, i.e. not involving bacteria<sup>23</sup>. There are two distinct mechanisms of tooth wear; those of chemical origin (erosion) and those of physical origin (abrasion and attrition). The mechanical removal of dental hard tissue by abrasion is defined by a foreign material in repeated contact with the teeth, whereas attrition is caused by the direct contact between teeth without any foreign substance intervening<sup>24</sup>. Typical causes of abrasion are the ingestion of abrasive foods or excessive use of oral hygiene products (e.g. tooth brushing and dentifrices with abrasives), whereas the action of mastication or bruxism (pathologically intensified chewing) itself can be a cause of attrition. Tooth erosion is the loss of dental hard tissue following chemical dissolution by acid where the acid source is not derived from oral bacteria. Erosion may be caused by either intrinsic (e.g. gastric acid in medical conditions such as gastro esophageal reflux disease or eating disorders) or extrinsic (e.g. dietary such as acidic beverages) factors<sup>25</sup>. Erosive demineralization softens the enamel so that the tooth surface is more vulnerable to mechanical impacts, thereby able to enhance physical wear<sup>26</sup>.

The prevention and control of tooth erosion rely on the early recognition of enamel loss in combination with other signs associated with the aetiological factors<sup>25</sup>. Decreased salivary flow, excessive oral hygiene, behavioral factors such as eating and drinking habits, especially the frequent ingestion of acidic foods and beverages, are predisposing factors for erosive tooth wear<sup>27</sup>. Saliva hypofunction and/or the absence of dental pellicle can make individuals more susceptible to erosion or aggravate erosive lesions<sup>27</sup>. Methods to enhance salivary flow (e.g. chewing sugar-free gum, saliva substitutes) and strengthen the enamel by the delivery of fluoride have proven useful in the prevention and control of tooth erosion as well as in dental caries<sup>28</sup>.

In the clinics, tooth wear mostly is observed as combined results of erosive effects (acid softening the hard tissues) and mechanical forces (abrasion) easily removing the softened enamel. Tooth wear, therefore, presents a multifactorial process and often occurs as a result of the simultaneous and/or synergistic action of the wear mechanisms. Compared to dental caries, the concern of tooth wear is relatively recent. For the past twenty years, there has been an increased attention in tooth wear, in particular erosion, due to changes in life style, often associated with increased consumption of acidic foods and drinks<sup>29</sup>. Several studies on the prevalence of tooth wear show that there is a tendency to

develop more wear with age<sup>30-32</sup>. Along with the increased human longevity and that tooth wear is a cumulative life time process, there is clearly a need to protect the dentition against the progression of wear in the management of long-term oral health care.

## 1.2. The oral environment – from a pharmaceutical viewpoint

The local treatment of tissues in the oral cavity (Fig. 1), e.g. of teeth, is challenging due to the complex and dynamic intraoral environment.

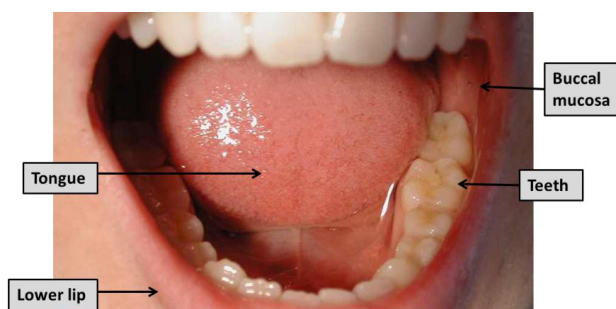


Fig. 1: The oral cavity consists of various structures; lips, buccal mucosa, tongue, teeth, gingiva and palate, representing both soft and hard tissues.

The oral surfaces are continuously bathed in a fluid, an intricate mixture of oral bacteria, leukocytes, desquamated epithelial cells, food debris, salivary secretions and crevicular fluid. This oral fluid is often termed whole saliva or mixed saliva. Because saliva is the main transporting vehicle within the oral environment, the salivary interaction of exogenous materials is unavoidable. This is an important aspect for the delivery of pharmaceuticals into the oral cavity as they will be influenced by saliva. Salivary clearance is beneficial for oral health since it can rapidly remove or reduce the concentration of both oral (e.g. desquamated cells) and exogenous, often harmful, substances (e.g. pathogenic bacteria/viruses, sugars and acids)<sup>33</sup>. Despite the advantages, the flushing action of saliva also clears away protective substances that are externally introduced, such as fluoride and other drugs. From a therapeutically point of view in the oral cavity, a slow or delayed clearance of the drug agents is highly preferable.

The healthy oral cavity is normally colonized by microorganisms like fungi, viruses and a tremendous high number of bacteria<sup>34</sup>. It has been estimated that over 700 bacterial

species reside in the oral cavity; some may be pathogenic, other may be beneficial for the host <sup>35</sup>. The oral bacteria can co-exist in complex populations in biofilms, rendering the microbial community increased protection and resistance. Formation of a biofilm on the tooth surface is known as dental plaque and is the primary cause of caries and other oral diseases <sup>36</sup>. Beside from adhering to the oral surfaces, oral bacteria may potentially attach to other substrates, e.g. therapeutic components, and interfere with their purpose in the oral cavity.

Anyway, foods and drinks, the salivary action and the diverse microbial flora all lead to a very harsh and hostile oral environment to foreign materials such as pharmaceutical formulations for dental applications. Furthermore, the environment in the oral cavity undergoes substantial changes due to fluctuations in the salivary secretions; the individual's eating and drinking behavior in combination with the overall state of health, time of day and physical activity. One way to endure and persist in the oral environment is to avoid the cleansing action of saliva by the development of bioadhesive formulations. Knowledge of saliva's role to maintain oral homeostasis can help to design an appropriate formulation that can overcome the obstacles present in the oral cavity.

### 1.2.1. Salivary variables

Saliva represents the immediate environment of the teeth, thus, influencing the properties of the solid surface of the teeth. Human saliva is mainly produced from three major paired glands; the parotid, the sublingual and the submandibular glands. Secretions from these glands differ in composition and their relative contributions in whole saliva may vary according to the degree or nature of stimulation. The secretion of saliva is exclusively under the control of the autonomic nervous system, in particular parasympathetic stimulation. The parotid and submandibular glands do not secrete saliva spontaneously, their secretion is entirely nerve-mediated and stimulation dependent <sup>37</sup>.

A precise account of the composition of saliva is difficult, because it is highly variable depending on a number of factors, including the type of gland, time of the day, the flow rate, the nature and duration of stimulation. Variation of the composition of saliva occurs also between different sites in the mouth as well as between individuals. Secretions from the parotid gland are more serous in nature, whereas those from submandibular and sublingual glands are viscous due to their glycoprotein content <sup>17</sup>. The continuous secretion of saliva exhibits a circadian rhythm with flow rate peaking in the afternoon and very low



levels during sleep<sup>38</sup>. The normal unstimulated flow rate of whole saliva is estimated to be 0.2-0.4 ml/min (resting saliva), whereas in a stimulated state the flow rate may increase to 2-5 ml/min<sup>39</sup>. It should be emphasized that these averaged values have a wide range within normality. Because of saliva's many variables, the total volume of saliva secreted per day has been much disputed; however, the range of 0.5-1.5 l/day covers the most values that have been reported in the literature.

Saliva contains over 99% water, the remaining small quantity is divided into organic (carbohydrates, lipids and proteins) and inorganic constituents (sodium, potassium, calcium, magnesium, hydrogen carbonate, phosphate, chloride and fluoride)<sup>37</sup>. Of the inorganic fraction in saliva, calcium and phosphate are of particular importance to teeth because they are supersaturated with respect to the dental enamel<sup>40</sup>. Although the range may vary widely, typical concentrations of calcium and phosphate in whole saliva are 1.4 mmol/l and 6 mmol/l, respectively, in unstimulated saliva, and 1.7 mmol/l and 4 mmol/l, respectively, in stimulated saliva<sup>17, 37</sup>. The supersaturation of these electrolytes helps to prevent the dissolution of the enamel surface and facilitates the remineralization of dental enamel after acidic challenges. By providing a reparative and stabilizing environment at the enamel surface, saliva is of major importance to maintain the integrity of the dental enamel.

Another important electrolyte involved in saliva's functions is hydrogen carbonate. The resting pH of whole saliva is in the range 6.7-7.4<sup>17</sup>. After consumption of foods or drinks containing fermentable carbohydrates, acid is produced by bacteria and the pH of the local environment within the oral cavity decrease. When the pH drops below the critical pH of the enamel (pH ~ 4.5-5.5), demineralization of the teeth may take place<sup>4</sup>. The rate of recovery to normal pH values is largely dependent on the increase of the hydrogen carbonate concentration, thus contributing to the buffering capacity of saliva, especially at high salivary flow rates<sup>41</sup>.

The large array of proteins constitutes the most important organic fraction of saliva as they are responsible for saliva's many physiological roles. The total protein content of stimulated whole saliva has been reported to be in the range 2.4-3 g/l<sup>17, 42</sup>. The functions of saliva can be regarded as three-sided; directed towards teeth (tissue coating and protection), food (alimentation) and microbes (regulation of the oral flora)<sup>43</sup>. Proline-rich proteins (PRPs) and statherin exert the protective role of saliva by binding to calcium and inhibiting spontaneous precipitation of calcium phosphate salts on teeth<sup>44</sup>. They can also selectively promote adhesion of some bacteria to tooth surfaces<sup>45</sup>. Lubrication of oral surfaces by

saliva is thought to be attributed to the viscoelastic nature of mucins. The digestive function is accomplished by the enzymes amylase, lipase and protease, while the antibacterial activity of saliva is ascribed to immunoglobulins, lactoferrin, and the antimicrobial enzymes lysozyme and lactoperoxidase<sup>43</sup>. Many of these proteins are multifunctional<sup>46</sup>. Mucins have antibacterial effects in concert with other factors in saliva. Together with PRPs and statherin, they have high affinity to enamel hydroxyapatite and can inhibit demineralization of the enamel by selective adsorption to tooth surfaces contributing to the formation of the acquired enamel pellicle<sup>47</sup>. Thus, the protection of the oral cavity by saliva is established in several ways (Fig. 2).

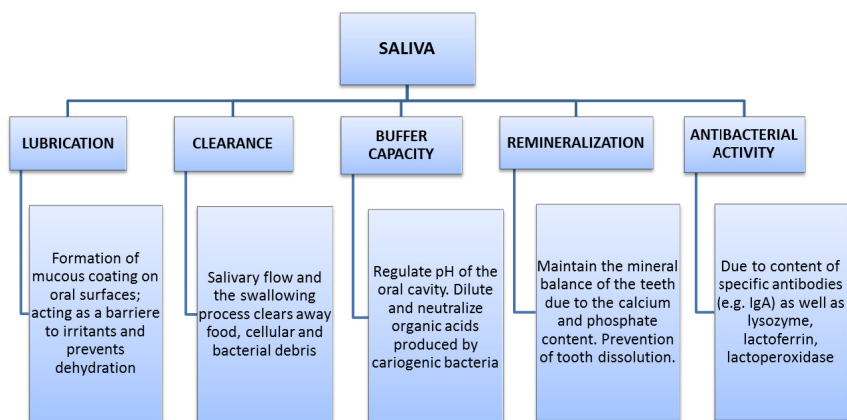


Fig. 2: Some of the protective mechanisms of saliva.

### 1.2.2. The acquired enamel pellicle

Saliva is rarely in direct contact with the teeth because of a thin layer of salivary origin, the acquired enamel pellicle, covering the tooth enamel surfaces. The term was first used by Dawes et al. in 1963<sup>48</sup>. Since then, numerous papers have given evidence for its formation by selective adsorption of salivary proteins on the enamel surface<sup>49-52</sup>. More recently, owing to advances in proteomics, the peptides and proteins components of the pellicle have been identified<sup>53-56</sup>. The acquired enamel pellicle is formed quickly after tooth eruption into the oral cavity or on tooth surfaces exposed to saliva after a thorough

cleansing procedure. The formation of the pellicle has been described to proceed in two stages<sup>57</sup>. The first stage constitutes the initial formation of an organic covering by the adsorption of discrete proteins. This occurs within minutes. The pellicle matures into the second stage when the proteins are assembled into globular micelle-like structures, and as such adsorbed to the enamel surfaces increasing the pellicle thickness. The growth of the pellicle reaches a maximum after 30 minutes and can be up to 1.0  $\mu\text{m}$  thick<sup>58</sup>. The previously mentioned selective adsorption of salivary proteins to enamel surfaces are now merely interpreted as adsorption of salivary structures selectively aggregated into micelle-like globules.

The proteins involved in the micelle-like globules are generally phosphoproteins with amphiphilic character, thus having the ability to associate into micellar structures with high affinity to the dental enamel<sup>59-61</sup>. Rykke et al.<sup>59,62</sup> determined the mean particle size of these protein globules and demonstrated an overall net negative charge at the surface of the globules. The particle size was in the size range 100-500 nm with zeta potential of about -9 mV at physiological pH (pH  $\sim$  7.8). The negative surface charge of these particles enabled electrostatic interactions with the enamel surface. It was also demonstrated that calcium was important to maintain the integrity of these structures.

The pellicle layer on the teeth has been thought to have protective functions by several mechanisms. The pellicle serves as a lubricant between teeth and other oral structures, thus preventing tooth wear<sup>63,64</sup>. The influence of the salivary pellicle on erosion, i.e. direct acid attack, has been demonstrated by Hannig and Balz<sup>65,66</sup> and more recent by Hara et al<sup>67</sup>. The pellicle was shown to be protective against mild erosive challenges, thus, limiting the damaging effects of erosion in the mouth. The pellicle also acts as a barrier, modifying acid diffusion and the exchange of calcium and phosphate at the enamel surface. This can prevent demineralization and facilitate remineralization<sup>68</sup>.

Pellicle components can mediate non-specific and selective bacterial adhesion and initial plaque formation, and also affect the attachment of cariogenic microorganisms to the enamel<sup>69-71</sup>. A recent study reported that adherent bacteria were present in the initial pellicle and suggested that the pellicle should be classified mainly as a proteinaceous layer with a considerable number of adherent bacteria, instead of a proteinaceous film free of bacteria as previously thought<sup>72</sup>. Thus, the pellicle layer may provide a base for the subsequent development of dental plaque (Fig. 3).

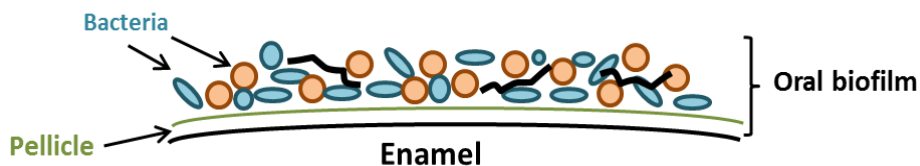


Fig. 3: A schematic illustration of the *in vivo* layers on the enamel surface.

As a second layer on the tooth surface, dental plaque can further separate the enamel surface from bulk saliva and may limit the ability of saliva to exert its protective effect. Thus, the fate of the enamel is driven by the interactions between saliva, salivary pellicle, dental plaque (oral biofilm) and the enamel surface<sup>73</sup>.

It appears that the salivary pellicle participates in all interfacial actions taking place in the oral cavity, including adsorption phenomena onto enamel surface. This means that adsorption by a pharmaceutical formulation for a protective function on the tooth surface is strictly not on the enamel, but on a pellicle-covered enamel in normal *in vivo* conditions. However, in situations where saliva secretion is greatly reduced, there may be insufficient or even incomplete or totally absent pellicle coating on the tooth surfaces. Following the tooth protection by the pellicle, developing formulations that can mimic and substitute, or be a part of the pellicle layer may offer advantages in cases where this protective coating is lost.

### 1.3. The potential of liposomes for protection of the dental enamel

Recently, nanotechnology and the use of biomimetic nanomaterials have been proposed as new strategies for the prevention and treatment in dentistry<sup>74, 75</sup>. Liposomes are biocompatible nanoparticles that offer innumerable possibilities in that they can be easily designed and tailored to suit a specific application. The use of liposomes for the delivery of drugs to the oral mucosa to treat oral ulcers, has been studied<sup>76-79</sup>. The adsorption of various liposomal formulations to oral bacteria and biofilms has been reported by Jones and coworkers<sup>80-82</sup>. They investigated the concept of using liposomes for the delivery of antimicrobial agents, such as Triclosan and chlorhexidine, and found that liposomes could be used to target oral bacteria. These studies demonstrate the potential use of liposomes in the intraoral environment.

Liposomes can be formulated in an attempt to overcome common problems associated with drug therapy in the oral cavity, such as salivary clearance and the non-uniform distribution within the oral cavity<sup>83-85</sup>. Liposomes can be formulated to have high affinity to the dental enamel to obtain direct targeting to teeth. To minimize salivary clearance, the liposomes should also be able to retain on the dental enamel (bioadhesive liposomes). This may reduce the frequency of administration. Liposomal preparations are easy to self-administer into the oral cavity and most likely impose little discomfort. All these factors may lead to increased patient compliance.

#### 1.3.1. Liposomes - formulation aspects

In the simplest form, liposomes are nanosize vesicles comprising of phospholipid bilayers of natural or synthetic origin. The lipid molecules, each of which typically consists of a hydrophilic headgroup and two hydrophobic hydrocarbon tails, spontaneously self-assemble in the presence of aqueous environment (Fig. 4). Hence, hydrophilic molecules can be entrapped in the aqueous core, while lipophilic molecules can be incorporated in the lipid bilayer. The amphipathic nature enables liposomes to carry drug molecules of different properties to the site of action as well as to protect them against degradation mechanisms, e.g. metabolism or inactivation, in the human body. The physicochemical properties of liposomes i.e. surface charge, hydrophobicity, particle size, bilayer rigidity and the packing of the lipid bilayers are important factors for their stability *in vivo* as well as *in vitro*. For example, the alkyl-chain length and degree of saturation play a major role in the rigidity and permeability of the bilayer as well as the chemical stability of the liposomes<sup>86</sup>. *In vivo* toxic effects of positively charged liposomes have been reported; the toxicity dependent on the concentration and charge density of the cationic lipid<sup>87</sup>. Therefore, to develop liposomes for a specific application, formulation factors should be studied initially, to find which factors are important and suitable for the intended purpose.

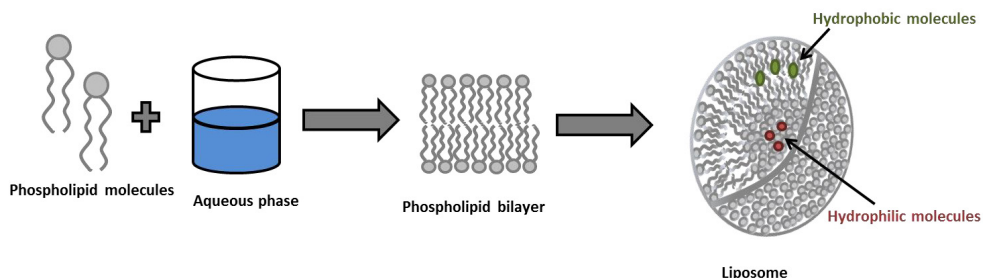


Fig. 4: The formation of liposomes.

Physical stability is one of the major hurdles encountered with liposomal preparations. Processes such as aggregation and precipitation, fusion and drug leakage may hamper the pharmaceutical development of liposomes<sup>86</sup>. Coating liposomes with natural polymers may improve the liposomal stability. Polysaccharides are attractive polymers for surface coating because of their availability and low cost, biodegradability, low toxicity, preventing binding of plasma proteins, and interaction in biological recognition processes through specific entities<sup>88</sup>. Many polysaccharides have been studied such as amylopectin, chitosan, dextran, mannan and pullulan<sup>89-91</sup>. These investigations show that the polymers are able to interact and strongly adhere to the liposomal membrane thereby execute their intended function. Furthermore, most natural polysaccharides have hydrophilic groups, such as hydroxyl and carboxyl groups, which can form non-covalent bonds with biological surfaces. The attachment of a macromolecule onto biological tissues is called bioadhesion. Bioadhesion onto mucosal membranes, i.e. mucoadhesion, has been extensively investigated to prolong the retention time of drug delivery systems and thereby improve drug bioavailability<sup>92</sup>. Liposomes coupled with bioadhesive polysaccharides can function as bioadhesive drug delivery systems. Pectin, which is a mucoadhesive polysaccharide<sup>93,94</sup>, has recently been investigated for coating onto liposomes to improve drug delivery through the gastrointestinal tract<sup>95</sup>. Bioadhesive liposomes may also be popular for local use in the oral cavity<sup>84</sup>. This is due to the rapid elimination of drugs owing to the flushing action of saliva. In this regard, liposomes that are bioadhesive against enamel surfaces of the teeth may offer potential in dental applications.

### 1.3.2. Physical protection of the dental enamel

The protection of the dental enamel against detrimental processes, such as dental caries and tooth wear, can occur by two principles: chemical and/or physical protection. The use of liposomes can potentially contribute to both types of protection. Liposomes may chemically protect the teeth by functioning as carriers for a variety of pharmacologically active substances (dental drug delivery systems). Depending on which type of drug is delivered, liposomal formulations may be used for prophylaxis (e.g. fluoride) or for therapeutic treatment (e.g. chlorhexidine). Targeting the delivery systems directly to the enamel for treatment of dental problems may increase the pharmacological effect of the encapsulated drug and reduce unfavorable side effects. However, in order to target and improve liposomal performance, the liposomes need to be physically adsorbed onto teeth prior to action. The adsorption of liposomes onto teeth *per se* may function as a protective layer for the enamel.

In normal *in vivo* conditions, salivary proteins, by forming the acquired enamel pellicle on the enamel surfaces, provide the natural protection from both chemical and mechanical challenges to the tooth surface. In conditions where there is a lack or reduced secretion of saliva, this physical layer on the teeth can be incomplete or totally lost or the protection is insufficient, increasing tooth wear and dental caries. To improve the oral health of affected individuals, liposomes can be formulated similar to the protein globules of the salivary pellicle, for adsorption to teeth where they can mimic, substitute or be a part of the natural pellicle layer. Thus, through the adsorption of liposomes, the mechanical protection of the teeth is exhibited. A liposomal covering on the dental enamel may reduce the frequency of contact with acids, increase the resistance of the dentition and thereby reduce tooth wear processes. Furthermore, the liposome layer may change the basis for bacterial accumulation of plaque bacteria on the tooth surface, thereby influencing the development of dental caries.

## 2. AIM OF THE THESIS

The overall aim of this thesis was to develop liposomal formulations that can adsorb to the human dental enamel, and thereby physically protect the teeth against tooth wear and dental caries.

The specific objectives in the investigations were:

- To prepare liposomes with different characteristics and to study the surface coating of liposomes by different types of the polymer pectin (Paper I and III).
- To find which liposomal formulation factors are important for the adsorption of liposomes onto the dental enamel by using the model substance hydroxyapatite (HA) and phosphate buffer, pH 6.8 - 7.0 (Paper I).
- To examine the interactions between liposomal formulations and components of the saliva (Paper II and IV).
- To evaluate the bioadhesion of selected liposomal formulations to the dental enamel in a salivary environment (Paper II and IV).



### 3. SUMMARY OF PAPERS (I-IV)

#### **PAPER I:**

The aim of this study was to find the most promising liposomal formulation for the *in vitro* adsorption to teeth. Formulation factors important for the interaction between liposomes and hydroxyapatite (HA), a model substance for the dental enamel, were mapped. Experimental design was employed for a systematic approach and multivariate analysis was used to evaluate the results. The type of charge on the liposomes (positive, negative), the type of main phospholipid (egg-PC, DMPC, DPPC), the type (diacyl-TAP, -ethylPC, -PA, -PG, -PS) and amount of charged lipid (2.5 and 10 mol%), and the inclusion of cholesterol were variables investigated. The type of charge became the most significant factor. Positively charged liposomes adsorbed better than negatively charged liposomes to HA in phosphate buffer, pH 6.8-7. Positively charged liposomes with DPPC as the main lipid were most stable during storage. Based on the results, formulations based on positively charged DPPC liposomes with 10 mol% charged lipid included seemed most promising for targeting to the teeth.

#### **PAPER II:**

To simulate oral-like conditions and examine the influence of saliva on the interaction between liposomes and HA, phosphate buffer was replaced by parotid saliva as adsorption medium in this study. Precipitation was observed in samples containing positively charged liposomes (DPPC/DPTAP) and parotid saliva as the only components. Turbidimetric measurements of mixtures liposomes-parotid saliva were employed to study this interaction. DPPC/DPTAP-parotid saliva resulted in very turbid sample, which precipitated and phase separated after about 30 min. In contrast, the turbidity of negatively charged liposomes was dependent on the nature of the charged lipid. DPPC/DPPG liposomes in parotid saliva were very turbid at a constant level, while DPPC/DPPA in parotid saliva exhibited low turbidity. The addition of pyrophosphate, a calcium sequestering agent, to the liposomes-parotid saliva mixtures, rendered a great fall in the turbidity of samples with DPPC/DPPG-liposomes, while only a small reduction was observed for samples with DPPC/DPTAP-liposomes. This indicated that calcium may play a role in the interaction between negatively charged DPPC/DPPG-liposomes and parotid saliva.

Negatively charged DPPC/DPPA liposomes were found most suitable for use in the oral cavity as they were not observed to react with components of parotid saliva.

### **PAPER III:**

This study investigated the surface coating of liposomes by three types of the polymer pectin; LM-, HM- and amidated pectin, each in two concentration levels (0.05 and 0.2 w/w %). The purpose of preparing pectin coated liposomes was to possibly improve the bioadhesion of liposomes to the dental enamel. Characterization of uncoated and pectin coated liposomes were based on particle size determinations and zeta potential measurements. The pectin coating on positively charged liposomes was verified by an increase in size and a shift in zeta potentials from positive to negative side of the pectin coated particles. A reproducible method for coating the liposomes was established. Pectin coating on the negatively charged liposomes could not be demonstrated as the results were inconclusive.

### **PAPER IV:**

This study examined the *in vitro* adsorption of uncoated and pectin coated liposomes onto human dental enamel in a salivary environment. Firstly, pectin coated liposomes were shown to adsorb to HA in phosphate buffer and parotid saliva. LM- and HM-pectin coated liposomes were selected for further investigations. As enamel specimens, the enamel crowns of extracted molars were used. A new, reproducible method for liposome adsorption was developed. In principle, the procedure makes use of a dipping device to immerse the enamel specimens in different liposomal suspensions. Uncoated positively charged liposomes exhibited the highest adsorption levels to the enamel specimens, while the adsorption of liposomes with a negative surface charge (uncoated and pectin coated) could not be discriminated. The adsorption was examined by exposing the enamel surface for a flow; simulating the flow rate of stimulated saliva secretion. The results indicated that pectin coated liposomes retained better than the uncoated liposomes on the dental enamel. This supports the hypothesis that pectin may help to prolong the adhesion of liposomes on the tooth surfaces.

## 4. GENERAL EXPERIMENTAL CONSIDERATIONS

### 4.1. Materials

#### 4.1.1. Lipids

A general structure of a phospholipid is illustrated in Fig. 5. All lipids used in the studies in this thesis are listed in Table 1 together with their molecular structure and some of the important properties. The lipids were used without further purification.

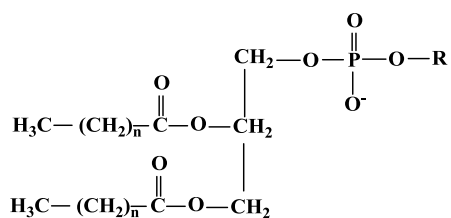


Fig. 5: General structure of a phospholipid.

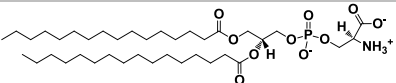
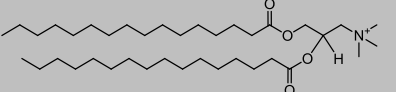
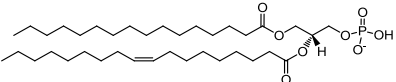
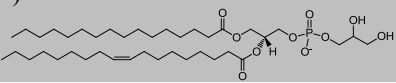
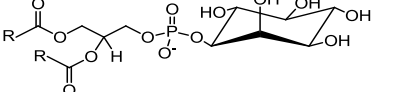
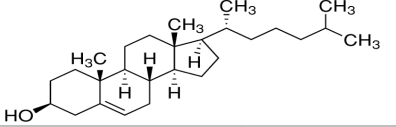
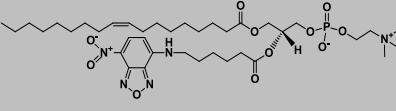
Table 1: The chain length, molecular weight ( $M_w$ ), main phase transition temperature ( $T_c$ ), type of charge together with the molecular structure of the lipids used in this thesis. The data are taken from Cevc<sup>96</sup> or from [www.avantilipids.com](http://www.avantilipids.com).<sup>a</sup> Adapted from [www.avantilipids.com](http://www.avantilipids.com).<sup>b</sup> Fluorescent lipid; fatty acid labeled.  
\*)Structure of predominant species. n.a.: Data not available

GENERAL EXPERIMENTAL CONSIDERATIONS

TYPE OF LIPID	CHAIN LENGTH	M <sub>w</sub> (Da)	T <sub>c</sub> (°C)	TYPE OF CHARGE	MOLECULAR STRUCTURE <sup>a</sup>	PAPER
<b>MAIN LIPIDS</b>						
DMPC	C <sub>14</sub>	678	23	Neutral		I
DPPC	C <sub>16</sub>	734	41.5	Neutral		I, II, III, IV
Egg-PC	C <sub>16</sub> -C <sub>22</sub>	~770	-10	Neutral		I
<b>CHARGED LIPIDS</b>						
DM-ethylPC	C <sub>14</sub>	742	23.6	Positive		I
DMPA	C <sub>14</sub>	615	50	Negative		I
DMPG	C <sub>14</sub>	689	23	Negative		I
DMPS	C <sub>14</sub>	702	35	Negative		I
DMTAP	C <sub>14</sub>	590	24.5	Positive		I
DOPS	C <sub>18</sub>	810	-11	Negative		I
DOTAP	C <sub>18</sub>	699	-12	Positive		I
DP-ethylPC	C <sub>16</sub>	799	42	Positive		I
DPPA	C <sub>16</sub>	671	67	Negative		I, II, IV
DPPG	C <sub>16</sub>	745	41	Negative		I, II, III

## GENERAL EXPERIMENTAL CONSIDERATIONS

(Continued)

TYPE OF LIPID	CHAIN LENGTH	M <sub>w</sub> (Da)	T <sub>c</sub> (°C)	TYPE OF CHARGE	MOLECULAR STRUCTURE <sup>a</sup>	PAPER
<b>CHARGED LIPIDS</b>						
DPPS	C <sub>16</sub>	758	54	Negative		I, II
DPTAP	C <sub>16</sub>	646	44.5	Positive		I, II, III, IV
Egg-PA	C <sub>16</sub> -C <sub>22</sub>	~706	18	Negative *)		I
Egg-PG	C <sub>16</sub> -C <sub>22</sub>	~782	< 0	Negative *)		I
PI; from wheat germ	C <sub>16</sub> -C <sub>18</sub>	~856	n.a.	Negative		II
<b>OTHER LIPIDS</b>						
Cholesterol; from porcine liver	Sterol	387	n.a.	Neutral		I
NBD-PC <sup>b</sup>	C <sub>18</sub> and C <sub>6</sub>	798	n.a.	Neutral		II, IV

## 4.1.2. Pectin

Pectin is a complex plant polysaccharide where the dominant feature is composed of galacturonic acid residues.  $pK_a$  of pectin is in the range 2.9-3.3<sup>97</sup>. The acid groups of the galacturonic units can be methoxylated or/and amidated in varying degree, giving rise to different types of pectin with different properties. For the surface modification of liposomes, three commercial types of pectin were employed (Paper III and IV): high-methoxylated (HM), low-methoxylated (LM) and amidated (AM) pectin, all of which were mainly derived from citrus peel (Fig. 6). The degree of esterification of LM-pectin is 34.8 % and of HM-pectin 70.2 %. Having the highest level of carboxylic acid in the structure, LM-pectin is the most acidic type of pectin among the three types of pectin examined. HM-pectin is highly substituted with methoxy groups (-OCH<sub>3</sub>) imparting a more hydrophobic structure than LM pectin. Due to the natural origin of pectin, batch-to-batch variation is high<sup>98</sup>. All the three types of pectin were purified by dialysis with molecular weight cut off 8000 Da, followed by characterization of the average molecular weight prior to use. Pectin solutions 0.05 and 0.2 % (w/w) in 5 mM phosphate buffer, pH  $7 \pm 0.1$ , were prepared for coating onto liposomes.

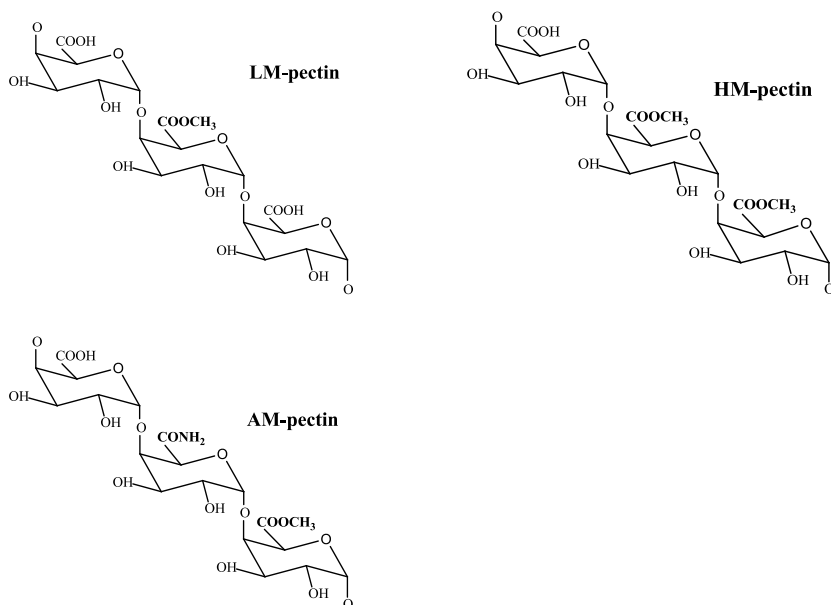


Fig. 6: A schematic illustration of the fundamental unit of the three types of pectin used (From Paper III).

## 4.2. Methods

### 4.2.1. Preparation and characterization of liposomes

Liposomes were prepared according to a standard thin film method<sup>99</sup>, followed by extrusion through double 200 nm membranes to get unilamellar vesicles with the appropriate particle size (Paper I-IV). All liposomes were prepared in phosphate buffer medium. To surface modify the liposomes by pectin, liposomes were added to purified pectin solutions in a controllable manner by means of a peristaltic pump to avoid aggregation and ensure reproducible results (Paper III).

Particle size determinations and zeta potential measurements are two techniques routinely used in the physical characterization of liposomes<sup>86</sup>. In the present work, these methods have been used:

- As a standard protocol to ensure that the preparation of liposomes has been successful; both in-process and final product control (Paper I-IV).
- To examine changes in the liposomal system (e.g. fusion or aggregation of the liposomes) during the stability study (Paper I).
- To verify changes when coating the liposomes with pectin (Paper III).

The mean hydrodynamic diameter of the nanoparticles was determined by means of dynamic light scattering (DLS) technique (a Zetasizer 1000 and a Coulter N4 Particle sizer), and the surface potential by microelectrophoresis. Calculation parameters for water were used, and the measurements were performed at 25°C. All samples were diluted with phosphate buffer to an appropriate counting rate prior to analysis. To avoid sample dilution before the measurements, the size determinations of pectin coated liposomes were performed by use of an additional instrument; an ALV-goniometer (Paper III). This instrument is also capable of yielding  $\beta$ -values which is a measure of the width of the distribution of the relaxation times in the correlation function.  $\beta$ -values close to 1 indicates relatively monodisperse samples (non-aggregated particles). This information was useful in the verification of pectin coating on the liposomes.

#### 4.2.2. Interactions between liposomes and saliva

Pure parotid saliva is the most readily obtained among the three main glandular secretions when considering sample collection techniques. The serous nature of parotid saliva makes it also easier to deal with in experiments. Parotid saliva is always supersaturated with respect to enamel hydroxyapatite; average calcium and phosphate concentration is 0.9 mmol/l and 3.5 mmol/l, respectively<sup>17, 100</sup>. Statherin and acidic PRPs constitute the major protein fractions of parotid saliva<sup>101, 102</sup>. Prior to use, parotid saliva was filtered 0.45µm with PVDF membranes (Millex-HV Durapore®) due to low protein binding capacity (Paper II and IV).

In order to minimize salivary variables, the saliva collection conditions were standardized. Acid is the most potent stimulus for salivary secretion, especially parotid saliva<sup>17</sup>. Sour candies, containing both citric and malic acid, were intensively sucked to stimulate secretion in the studies (Paper II and IV). The same type of sour candies was used in all studies in an attempt to standardize the flow rate and thus the salivary composition. However, the flow rate and the composition of saliva exhibit circadian rhythm. Protein concentrations peak in the late afternoon, while sodium and chloride levels peak in the morning<sup>38, 103</sup>. It was difficult to perform the collection of saliva at a certain time of the day due to many samples and parallels in the experiments. To overcome this potential variation in the results, the parallels of each sample were collected at different time of the day to achieve representative averages, and all samples were randomized to avoid biased results. One healthy female donor contributed to the collection of saliva. Rykke et al. have demonstrated that the amino acid composition of the acquired pellicle formed over 2h was very consistent both inter- and intraindividually<sup>52</sup>.

Saliva is sterile until it enters the oral cavity where it is continuously contaminated with oral microorganisms, desquamated epithelial cells, food remnants or other elements present in the oral cavity. In order to obtain pure parotid saliva, an individually fitted appliance was made to collect saliva directly from the parotid gland into test tubes (Fig. 7). To further avoid contamination, the first 1-2 ml of collected saliva was discarded. After filtration, parotid saliva was used immediately. The importance of using freshly collected saliva was two-fold: 1) To avoid exposure to the atmosphere as CO<sub>2</sub> will be released and saliva pH will artificially be elevated. 2) To reduce the continuous aggregation of the micelle-like globules in the samples prior to use<sup>59</sup>.



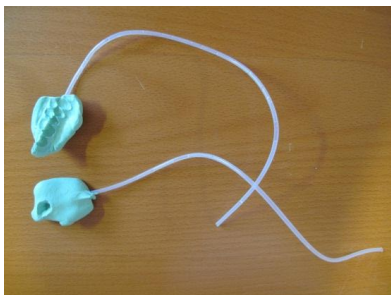


Fig. 7: The saliva collection device.

The effect of adding liposomal formulations or pectin solutions to parotid saliva was studied by turbidimetry with the aid of a spectrophotometer (Paper II and IV). These experiments were carried out to examine the aggregation behavior of both uncoated and pectin coated liposomes in parotid saliva. The turbidity was followed at 700 nm. Preliminary experiments showed low adsorption at this wavelength. Because the liposomes contained fluorescent lipids, a high wavelength was also found favorable to avoid any interference. The same wavelength has been employed by Young et al. in the spectrophotometric analyses of bacterial strains in saliva<sup>104</sup>.

The interactions between uncoated liposomes and salivary components (Paper II), and liposomes and the three types of pectin (Paper III) were visualized by the aid of atomic force microscopy (AFM). Some problems are related to the AFM technique. The problems are associated with the preparation of the specimens: the requirement of very low sample concentration, the removal of excess liquid by filter paper can give rise to different thickness of the sample layer, problems with liposomal stability because of air drying, and the random images produced are inherent in the method. For interpretations of the AFM images, it is necessary to have these potential problems in mind. Nevertheless, representative AFM images can give an impression of the situation in the samples and may provide supportive evidence to confirm a hypothesis.

#### 4.2.3. Bioadhesion of liposomes to hydroxyapatite and dental enamel

The surface properties of synthetic HA as well as the dental enamel are highly dependent on the surrounding environment<sup>8</sup>. Initially, phosphate buffer, pH 6.8-7, was used to have a controlled environment so that the mechanisms behind an interaction could

be better understood (Paper I). In the next step of the investigations, it was desirable to mimic the realistic conditions of the interaction. To simulate oral-like conditions, freshly collected parotid saliva was therefore employed (Paper II and IV).

HA powder is an easily supplied product and was used as a model substance for the human dental enamel in the initial experiments. In Paper I, HA powder was suspended in 5 mM phosphate buffer, pH  $6.8 \pm 0.1$ , stirred over night for hydration, before the adsorption experiments. In Paper II and IV, HA was first suspended in water for magnetic stirring overnight, evaporated to dryness and then used in the adsorption experiments. Since parotid saliva was used as adsorption medium and the volume of each sample was reduced in these studies, this pretreatment of HA was necessary in order to obtain about the same surface area of HA as in the previous study.

The adsorption of liposomes to HA was conducted either by adding HA suspended in phosphate buffer to liposomes (Paper I), or by adding liposomes to pretreated HA suspended in parotid saliva in test tubes (Paper II and IV). The procedure was changed in the latter experiments due to the reduced volume and instant use of parotid saliva. Corresponding references were prepared similarly without containing HA. Each tube was whirled shortly and placed on a rotator to ensure homogenous mixing (20 rpm, 35°C) for five minutes. To check if the time for liposome adsorption onto HA was appropriately chosen, varying time intervals for the adsorption were tested in a salivary environment at 35°C (Fig. 8).

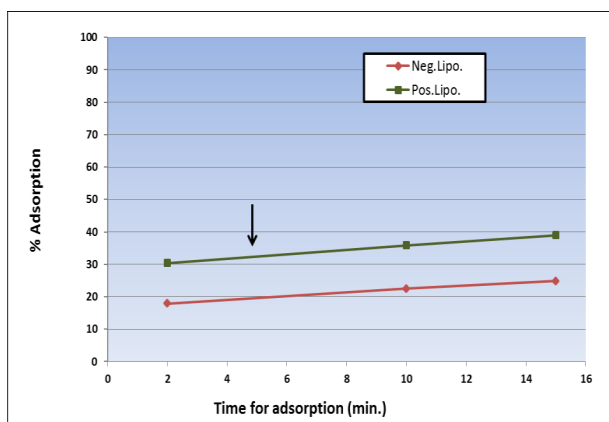


Fig. 8: Adsorption isotherm (20 rpm, 35°C) for charged liposomes to HA in a salivary environment. The black arrow denotes the five-minutes-point on the curves. (-■-) Positively charged liposomes: DPPC/10% DPTAP. (-◆-) Negatively charged liposomes: DPPC/2.5% DPPA.

The curve for both positively and negatively charged liposomes increased only slightly with increasing time. This shows that interactions between liposomes and HA occurs fast, and that five minutes is a sufficient time for liposome adsorption onto HA in a salivary environment. The continuous aggregation of micelle-like globules in collected saliva<sup>59</sup> may interfere with the *in vitro* adsorption process. To reduce these potential problems, a short adsorption time was therefore preferable.

After the adsorption to HA, the test tubes were centrifuged, the supernatants were transferred to glass vials and subjected to lipid quantification. Lipid quantification was performed by high-performance thin layer chromatography (HPTLC) analysis (Paper I) or by fluorescence spectroscopy (Paper II and IV). In the HPTLC-analysis, the supernatants were freeze dried and the residues dissolved in chloroform before they were applied on silica plates. Without elution, the silica plates were immersed in a detection reagent, cupric sulfate – phosphoric acid solution, dried and heated to develop the applied phospholipid bands. The mechanism behind this reaction is not clear, however, cupric sulfate tend to char the phospholipids, leaving stained bands on the silica plates for scanning by densitometry<sup>105</sup>. The amount of liposomes adsorbed to HA was calculated as the difference between the area under the curve of the sample and the corresponding reference in percent.

To be able to quantify liposomes by fluorescence spectroscopy, 1 mol% of the fatty acid labeled fluorescent lipid, NBD-PC, was incorporated in the liposomes investigated (Paper II and IV). The excitation wavelength of NBD-PC is 460 nm and the emission wavelength 534 nm. From each supernatant, samples were transferred to a microtiter plate and the fluorescence was measured in a plate reader. A non-ionic surfactant, Triton X-100, was used to induce the disintegration of the liposomes to improve the fluorescence detection. The amount of liposomes adsorbed to HA was calculated as the difference between the amount of fluorescence detected in the sample and the corresponding reference in percent.

Although synthetic HA has the same surface characteristics as the dental enamel, the powder form is not ideal to mimic the adsorption area of the enamel. The enamel crown of extracted human molars was therefore collected to obtain a more realistic surface for adsorption in Paper IV (Fig. 9).

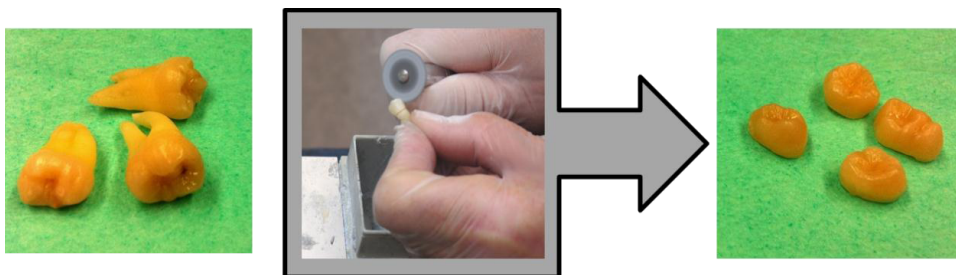


Fig. 9: The roots of molars were cut off at the cemento-enamel junction using a carborundum disc. These enamel crowns were used as enamel specimens in Paper IV.

With the use of enamel specimens, it was no longer possible to perform the adsorption in test tubes and it was necessary to change the experimental set-up (Fig. 10). A new adsorption method was developed (Paper IV). This method involved dipping the enamel specimens in liposomal solutions applied in a flat-bottomed cell culture plate. To obtain a reproducible dipping technique, an immersion device was utilized (Fig. 10). The dipping procedure was standardized by the following steps for each plate:

- 1) Equilibration of four enamel specimens in parotid saliva for five minutes.
- 2) Incubation of three enamel specimens in liposome sample (containing the fluorescent lipid NBD-PC) and one enamel specimen in phosphate buffer (control) for five minutes.
- 3) Washing all four enamel specimens by quick dipping in phosphate buffer.
- 4) Incubation in 2% (w/v) Triton X-100 of all four specimens for two minutes to solubilize the adsorbed liposomes for detection by fluorescence spectroscopy.

The amount of liposomes that have been adsorbed on the dental enamel is reflected by the fluorescence intensity detected in Triton X-100 solutions. The average fluorescence intensity for the three enamel specimens was calculated to obtain one representative value. Three plates were assayed for each type of liposomal formulation. A final average was calculated based on the three representative averages of each plate ( $n = 3$ ).

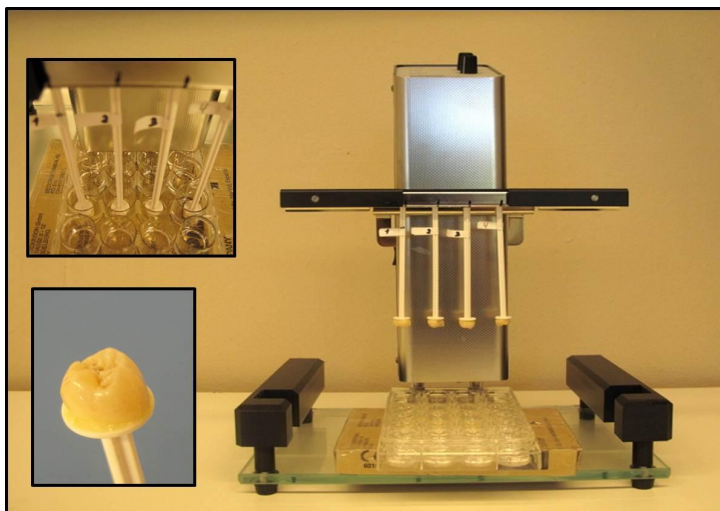


Fig. 10: The experimental set-up for the adsorption of liposomes to human dental enamel. The enamel specimens are attached to the clamping element of the immersion device. On the left: detail of the immersion of enamel specimens in sample solutions (top) and a representative enamel specimen (bottom).

To examine the duration of the liposome adhesion onto enamel surfaces, the enamel specimens were exposed to a flow after step 3 of the dipping procedure. A new experimental set-up was developed for this purpose (Paper IV). The enamel specimens were placed inside the syringes and a tubing pump was employed to generate flow of phosphate buffer (Fig. 11).

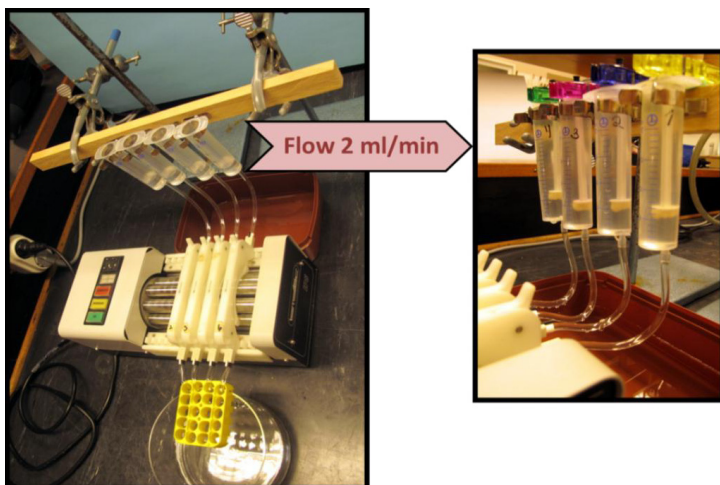


Fig. 11: The experimental set-up to investigate the retention of liposomes to human dental enamel. The flow rate used was 2 ml/min.

## GENERAL EXPERIMENTAL CONSIDERATIONS

After exposing to a flow rate of 2 ml/min for certain time intervals (5, 20 and 60 minutes), the enamel specimens were transferred back to the dipping system to execute step 4 of the dipping procedure. The test was not continuous and the whole procedure was repeated for each time point. The amount of liposomes that remained adsorbed on the dental enamel after a certain time is reflected by the fluorescence intensity detected in Triton X-100 solutions (step 4). The average fluorescence intensity for each plate at each time point was calculated in % relative to the intensity detected at time point 0, for that appropriate liposomal formulation. The time point 0 (= 100%) was where the dipping procedure was executed without any exposure to flow.

## 5. DISCUSSION OF RESULTS

### 5.1. Formulation of liposomes

To share the physicochemical properties of the enamel pellicle, the size of all liposomes have been tentatively prepared to be in the same size range as the salivary micelle-like globules (100-500 nm<sup>59</sup>). The mean particle size of uncoated liposomes have been measured to be in the range 90-180 nm (Paper I, II, IV), while that of pectin coated liposomes in the range 220-600 nm (Paper III and IV).

In the present thesis, 32 different liposomal formulations were investigated. The lipid composition was varied to yield different surface properties of the liposomes. Both positively and negatively charged liposomes were investigated for the potential adsorption onto dental enamel (Paper I, II and IV). This was based on the known adsorption of both type of charged proteins onto the enamel in the formation of the acquired enamel pellicle<sup>53, 106</sup>. The interest of using both types of charged liposomes for the adsorption to the dental enamel was also due to their potential as drug carriers. Several active substances for use in the oral cavity are charged. Potential examples are chlorhexidine which is a cationic bactericide<sup>107</sup> and fluoride which is an anionic anticaries agent<sup>16</sup>. Incorporation of cationic or anionic lipids in the liposome formulations are expected to improve entrapment efficiency of drugs and other substances which are of opposite charge. A wider range of active substances can then be entrapped in the liposomes; offering liposomes greater possibilities as drug delivery systems.

In addition, since the *in vitro* cellular toxicity of positively charged liposomes on human buccal cells are higher than the negatively charged liposomes<sup>108</sup>, a new formulation was added to the study namely pectin coated liposomes. A polymer coating around the liposomes would shield the positive charge at the surface and, thus, reduces the problem associated with the positively charged liposomes (Fig. 12). This would also still maintain the possibility of entrapping active substances of both type of charge. The chosen polymer in the present investigations was pectin (Paper III and IV).

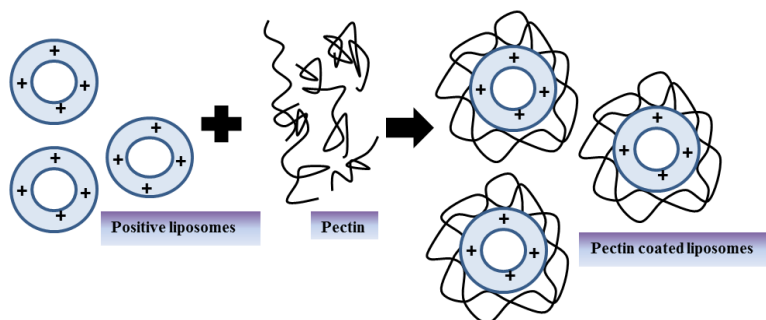


Fig. 12: A schematic illustration of pectin coating on positively charged liposomes.

Pectin is negatively charged at neutral pH due to the carboxylic acid groups in the galacturonic residues of the pectin chain. This feature of pectin enables the surface coating of positively charged particles, such as liposomes, by ionic interactions. Pectin is a substance generally recognized as safe (GRAS) by the American Food and Drug Administration (FDA), and was chosen based on its long and safe history in the food industry as a gelling agent or as a stabilizer<sup>97</sup>, and its mucoadhesive properties in drug delivery systems<sup>98</sup>. Although pectin has been mostly studied in systemic drug delivery systems, this polymer may as well be promising in the construct of drug carriers for the local use in the oral cavity<sup>109</sup>. Pectin coating on liposomes is advantageous as it may also improve the stability of liposomes *in vitro* as well as *in vivo*. Liposomes are physical unstable in dried conditions<sup>110, 111</sup>. Since the purpose of using liposomes may be to ameliorate xerostomic symptoms of the oral cavity, the liposomes may need to be protected against dehydration.

Three types of pectin, LM-, HM- and amidated pectin, were investigated for the surface coating of liposomes (Paper III). The pectin coating on positively charged liposomes was verified by an increase in size (from diameter about 200 nm to 220-550 nm) and a shift in the zeta potentials from positive to negative charge. From the DLS measurements, the  $\beta$ -values for the pectin coated liposomes (0.92-0.97) were very close to the  $\beta$ -values of the uncoated liposomes (0.98-1.00). The fact that the  $\beta$ -values for the pectin coated liposomes were so high, suggests that the increase in particle size after adding positively charged liposomes to pectin is not due to the clustering of particles, since large aggregates typically have a much broader distribution. Thus, it was believed that the



positively charged liposomes can individually adsorb polymer chains at their bilayer surface and that there is a low degree of particle aggregation. In contrast, it was difficult to verify complete coating of the negatively charged liposomes as the results were inconclusive (Paper III). Positively charged liposomes coated with HM-pectin (both concentrations) gave the largest pectin coated particles and the least negative zeta potential values (Fig. 13). HM-pectin coated liposomes may be preferred for a physical protection of teeth because of their large particle size. Less negative charge on the surface may help to minimize repulsive forces at the liposome-enamel interface.

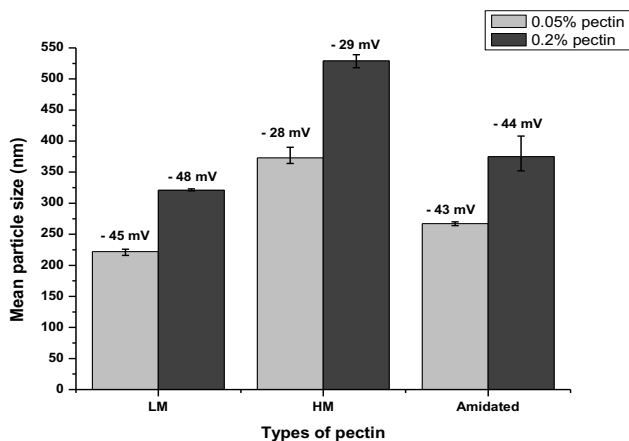


Fig. 13: The mean particle size (diameter) of the three types of pectin coated liposomes. Two concentrations of pectin (0.05 and 0.2 w/w %) were used to coat the positively charged liposomes (3mM DPPC/10 mol% DPTAP). The average zeta potential of each formulation is indicated above the bar.

High and low pectin concentration in the formulation did not reveal any differences in the zeta potentials for the respective pectin type (Fig. 13). This indicated that 0.05 % is a sufficient concentration to completely coat around the liposomes.

In order to manufacture a pharmaceutical product, the drug and the dosage form must express a sufficient shelf-life, thus, stability studies are required. Liposomal formulations are not an exception<sup>112</sup>. DPPC- and DMPC liposomes with 2.5 or 10 mol% charged lipid included were examined for their *in vitro* stability in phosphate buffer, pH 6.8-7 (Paper I). The particle size and zeta potentials of the liposomes were measured at specific time points during storage in refrigerator for 37 days. The results indicated that DPPC as the main lipid yielded the most physically stable formulations. In a screening study of liposomal formulations on buccal cell toxicity, the main lipid DPPC was found

less toxic than DMPC<sup>108</sup>. The stability data indicated that 10 mol% of positively charged liposomes were more stable than 2.5 mol% of the corresponding liposomes (Paper I). Thus, among the tested formulations, it appeared that 10 mol% of charged lipid with DPPC as the main lipid were the most promising liposomal formulations for further investigations. In order to use liposomes or pectin, or a combination of both, in the oral cavity, factors of the oral environment that may influence the formulation should be investigated. For example, the presence of pectinolytic enzymes in the oral cavity that can lead to unfavorable degradation of pectin<sup>97</sup>, or potential interactions with components of saliva.

## 5.2. Interaction studies of liposomes and saliva

The oral surfaces are constantly exposed to salivary secretions. Foreign agents that enter the oral cavity are also introduced to saliva for the subsequent distribution in the oral cavity. As saliva is the main oral transport medium, there is a possibility that liposomes introduced to the oral cavity will interact with saliva. To investigate how saliva will affect the *in vivo* performance of liposomes, turbidimetric measurements of the mixtures liposomes-parotid saliva over time were performed (Paper II and Paper IV). These investigations included both uncoated liposomes and pectin coated liposomes. The anionic lipids DPPA, DPPG, DPPS and PI and the cationic DPTAP (10 mol % of each charged lipid) were included in the uncoated liposomes (Paper II).

Saliva contains both charged organic components and electrolytes, enabling electrostatic interactions with oppositely charged liposomes. Salivary micelle-like globules, 100-500 nm in size, carry a net negative surface charge<sup>59, 62, 113</sup> and are capable to interact with the positively charged DPPC/DPTAP liposomes. Very turbid samples of the mixture DPPC/DPTAP liposomes-parotid saliva supported a strong interaction between the liposomes and components of saliva, and the formation of large aggregates (Paper II). Rykke et al. have reported a size increase of the globular structures with increasing time<sup>59</sup>. This supported the observation of an abrupt fall in the turbidity of this mixture after about 30 minutes (Fig. 14). The aggregates grew in size and became so large that they sedimented leading to phase separation of the mixture.

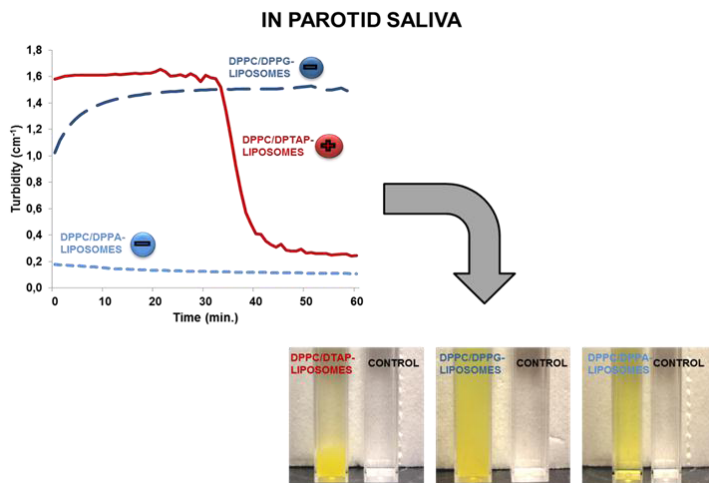


Fig. 14: A schematic illustration of the interactions between uncoated, charged liposomes and components of parotid saliva.

In the case of negatively charged liposomes, the turbidity of the mixtures DPPC/DPPA liposomes-parotid saliva remained low, whereas for the mixtures DPPC/DPPG liposomes-parotid saliva turbid samples were observed (Fig. 14). For the mixtures DPPC/PI liposomes-parotid saliva and DPPC/DPPS liposomes-parotid saliva the turbidities remained quite low with values of  $\tau \sim 0.3$  and  $0.5$ , respectively. Since the high level of turbidity of DPPC/DPPG liposomes was kept relatively constant during the measuring time, it was thought that calcium ions from saliva may be involved in this interaction. To confirm this, pyrophosphate (PP), which is a calcium sequestering agent, was added to the mixtures liposomes-parotid saliva. The addition of PP immediately cleared the mixture DPPC/DPPG liposomes-parotid saliva, and the turbidity of the sample fell almost to the control level (Fig. 15). In contrast, this phenomenon did not happen to the mixtures DPPC/DPPA liposomes-parotid saliva (Fig. 15). This mixture exhibited an initial very low turbidity, and the addition of PP did only render a minor effect. Thus, the most pronounced effect following the PP treatment among the negatively charged liposomes tested was in the order: DPPG > DPPS > PI/DPPA. It seemed therefore that the affinity to calcium ions is dependent on the nature of the negatively charged group. In the case of the mixtures DPPC/DPTAP liposomes-parotid saliva, the addition of PP did cause a small reduction in turbidity. This observation was thought to be due to the presence of calcium inherent in the salivary micelle-like structures. Rykke et al. has reported that calcium is

important for the maintenance of the micellar globules<sup>59</sup>, and the effect of PP was assumed to be caused by some degree of sequestration of the bound calcium of these structures.

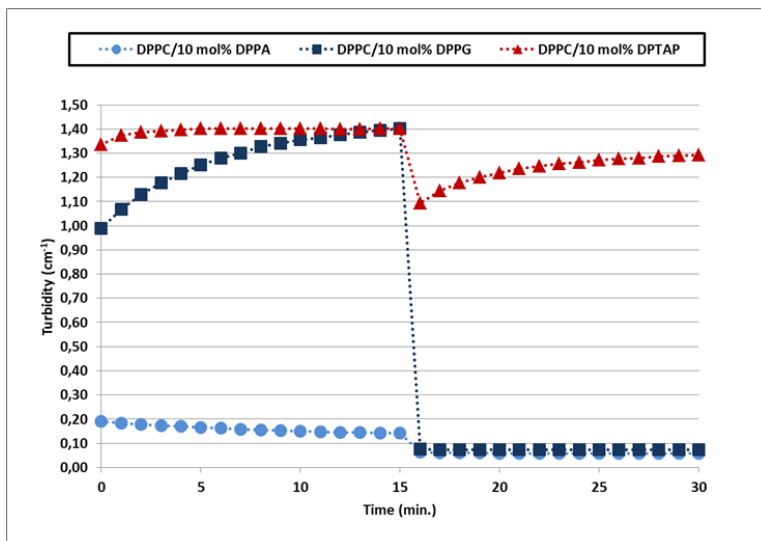


Fig. 15: The effect of PP addition (after time point 15 minutes) on the turbidity of the different mixtures of liposomes-parotid saliva.

The aggregates formed in the DPPC/DPTAP-parotid saliva mixtures are probably so large and dense that it was difficult for PP to penetrate and reach into the bound calcium of the micellar globules. Therefore, complete disintegration of the aggregates did not happen and only a minor drop in turbidity was observed after PP treatment.

The turbidity of the mixtures pectin coated liposomes-parotid saliva, and pure pectin solutions-parotid saliva was also examined in order to confirm the compatibility of pectin coated liposomes with saliva (Paper IV). No changes were observed for the mixtures, indicating no aggregation tendencies of pectin coated liposomes and parotid saliva.

It might seem like the positively charged liposomes (DPPC/DPTAP) are not favorable for use in the oral cavity due to aggregation reaction with salivary components. The formation of large aggregates may also cause too rapid clearance of the liposomes from the oral cavity. However, this disadvantage with positively charged liposomes is generalized, because only one type of cationic lipid, DPTAP, was induced in the liposomal formulations in these experiments. The nature of the charged lipid seemed to influence the surface properties of the negatively charged liposomes and, thus, the interaction

mechanisms. Hence, several types of cationic lipids should also be included in the liposomal formulations for interactions studies with saliva in order to strengthen this hypothesis. At this point, negatively charged DPPC/DPPA liposomes or positively charged liposomes coated with pectin seemed to be the most promising liposomal formulations as they are least likely to react with saliva.

### **5.3. Bioadhesion of liposomes to hydroxyapatite and dental enamel**

The use of synthetic hydroxyapatite (HA) as an analogue for the dental enamel in adsorption studies is commonly employed<sup>114,115</sup>. In the present papers (Paper I, II and IV), HA has been used in the initial experiments to screen potential liposomal formulations for the adsorption onto the dental enamel.

The surface charge, size, phase transition temperature and bilayer stability of liposomes are all dependent on the lipid composition<sup>116</sup>. In order to formulate liposomes that adsorb to HA, screening of various formulation factors for their influence on the interaction between liposomes and HA were therefore initially investigated. The type of surface charge, the type of main phospholipid, the type and amount of charged lipid, and the inclusion of cholesterol in the liposomal formulation were variables examined (Paper I). Two significant factors were revealed by multivariate analysis: the “type of charge” and the interaction “main lipid x type of charge”. It was not surprising that the “type of charge” of the liposomes was important for the adsorption onto HA. Synthetic HA as well as human dental enamel express negative surface charge at neutral pH due to phosphate groups<sup>7</sup>, allowing for electrostatic interactions. Thus, there is a strong attractive force towards positively charged species. This was confirmed in Paper I with a high level of adsorption onto HA for the positively charged liposomes (10 mol% -TAP as charged lipid). The type of main lipid played a role when positively charged liposomes adsorbed onto HA. Paper I showed that for positively charged liposomes, the main lipid DPPC adsorbed better to HA than DMPC, suggesting the use of positively charged DPPC-liposomes for further investigations. Moreover, DPPC is a gel state phospholipid (phase transition temperature ( $T_c$ ) 41.5°C<sup>96</sup>) and is rigid at body temperature, while in the same conditions DMPC is a fluid phase phospholipid ( $T_c$  23.5°C<sup>96</sup>). Szoka et al. have shown that the flexibility of the liposomes is important for the interaction with eukaryotic cells having a negative surface charge, and that solid vesicles adsorb to a greater extent than fluid vesicles. This study supports the choice on DPPC-liposomes.

The surface of HA possess amphoteric features in that calcium ions are also present at the surface (approximately 10%)<sup>6</sup>. This means that negatively charged species can also be attracted to HA. The adsorption of the negatively charged liposomes (2.5 and 10 mol% of -PG, -PA and -PS as charged lipids, respectively, Paper I) and pectin coated liposomes (Paper IV) onto HA was also demonstrated, however, these levels were quite low.

The surface properties of HA, and hence the interaction process, are highly dependent on the surrounding environment. The adsorption studies of charged liposomes in Paper I and in the initial experiments in Paper IV were performed in phosphate buffer, pH 6.8-7. It has been demonstrated in *in vitro* as well as *in vivo* studies that most pellicle precursor proteins carry a net negative charge, such as acidic PRPs and statherin<sup>55, 117, 118</sup>. Moreover, the early bacterial colonizers on the enamel surface possess a negative surface charge<sup>119</sup>. Thus, in the *in vivo* conditions of the oral cavity, negatively charged species have also high affinity to the dental enamel. Based on this theory, it was hypothesized that negatively charged liposomes adsorb better in the oral fluid of saliva than in phosphate buffer. This prompted to change the adsorption medium; phosphate buffer was replaced by freshly collected parotid saliva (Paper II and IV).

Young et al. have investigated the surface potential of human enamel and HA particles suspended in different buffers<sup>120</sup>. Human enamel and HA particles exhibited an overall net negative zeta potential, -17 to -30 mV. Among the tested media, phosphate buffer, pH 7.7-7.9, rendered the most negative surface potentials for both enamel and HA particles. The particles exhibited less negative zeta potential following incubation in saliva; -8 to -14 mV. Apart from pH, both calcium and phosphate ions have shown to be potential-determining factors for the enamel surface; presence of calcium ions makes the zeta potential more positive and phosphate ions makes it more negative<sup>7, 8</sup>. Young et al. proposed that the high content of calcium in saliva mediates to the adsorption of salivary proteins<sup>40</sup>, thus, leading to the less negative surface potential of the enamel and HA particles observed in the salivary environment. The calcium ions in saliva partly lowered the negative surface potentials of the enamel/HA, and partly acted as a bridge between negatively charged groups.

When it comes to the liposomes, the presence of calcium ions in saliva may promote the adsorption of negatively charged liposomes onto HA by a reduction in the electrostatic repulsion as proposed by Young et al. In phosphate buffer, the phosphate groups may contribute to a more negative enamel surface and, conversely, increase the repulsion towards the negatively charged liposomes. These mechanism are summarized in

Fig. 16. Paper II and IV demonstrated the adsorption of uncoated liposomes (DPPC/DPPA) and pectin coated liposomes (LM- and HM-pectin), all with negative surface charge, onto HA in parotid saliva, supporting the present hypothesis. Thus, it can be summarized that the adsorption of liposomes onto HA is driven by the subtle balance between attractive and repulsive forces, and is highly influenced by the surrounding environment (pH and the presence of electrolytes).

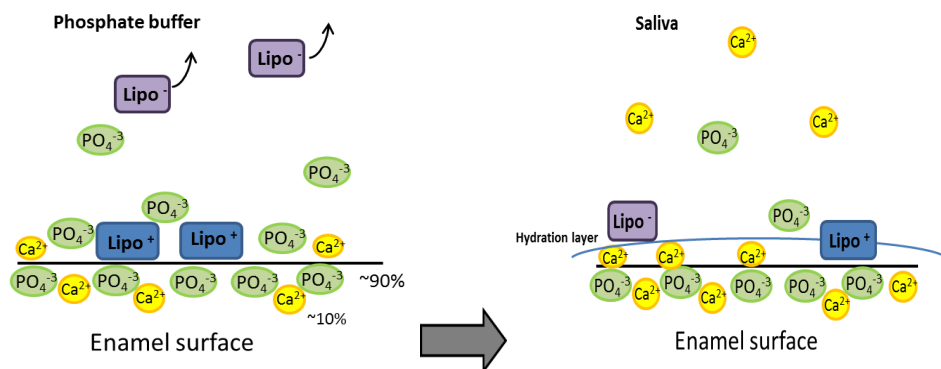


Fig. 16: The adsorption of charged liposomes on the enamel surfaces in phosphate buffer compared to that in saliva.

In Paper I, it was shown that positively charged liposomes adsorbed better than negatively charged liposomes to HA in phosphate buffer. The same result was obtained with the dental enamel in a salivary environment (Paper IV). This confirms that the type of surface charge on the liposomes play the primary role for the immediate attachment of liposomes onto enamel surfaces, independent of the surrounding environment. The liposomal formulations selected for the adsorption onto the dental enamel, both uncoated and pectin coated liposomes, have been shown to adsorb to HA in a salivary environment (Paper II and IV). Pectin has high affinity to calcium ions<sup>121</sup>. The presence of these cations in saliva was therefore thought to be involved in the immediate attachment of pectin coated liposomes to HA. The adsorption of pectin coated liposomes to HA in phosphate buffer, pH 6.8, showed that liposomes coated with LM- and HM-pectin exhibited slightly higher adsorption levels than liposomes coated with AM-pectin (Paper IV). This suggests that the affinity to HA may be affected by amide groups in the pectin structure. From the formulation work with pectin coated liposomes (Paper III), 0.05 % (w/w) pectin was found to be sufficient to completely coat around the liposomes. Based on these results, 0.05 %

LM- and 0.05 % HM-pectin coated liposomes were therefore selected for the adsorption to the dental enamel.

Before the adsorption experiments with the dental enamel, the enamel specimens were pre-incubated in parotid saliva (Paper IV). The results indicated that the adsorption of liposomes onto the dental enamel was unaffected by the presence of a pellicle layer on the enamel surface. This is important in the *in vivo* conditions, suggesting that liposomes are able to adsorb to tooth surfaces covered or partly covered with the acquired enamel pellicle. Milk contains casein molecules (phosphoproteins) that are able to form spherical complexes with calcium phosphate termed casein micelles<sup>122</sup>. These casein micelles structurally resemble the micelle-like globules in saliva. Devold et al. has studied the *in vitro* adsorption of milk proteins onto tooth enamel<sup>123</sup>. They found that enamel specimens pre-incubated in whole saliva did not inhibit or affect the formation of a “milk pellicle” on the enamel surface, and concluded that *in vivo* adsorption of milk proteins may occur on tooth surfaces in the oral cavity. This study supports the findings in Paper IV. Moreover, the adsorption of certain milk proteins have shown to inhibit the adhesion of cariogenic bacteria to HA<sup>124</sup>. Similarly, the idea of an adsorbed layer of liposomes on the tooth surface could potentially interfere with the initial bacterial adhesion of plaque formation, and thereby hamper the development of dental caries. The inhibitory effect of liposomal formulations on the adhesion of cariogenic bacteria on dental enamel remains to be further investigated.

Considerable fluctuations in the oral environment, mainly due to the secretion of saliva, may lead to rapid clearance of liposomes from the oral cavity. Moreover, movement of the oral soft tissues associated with speaking, eating and swallowing may be a problem for liposomes to remain in place. Formulation work today often concentrates on the design of formulations to increase their retention time at a certain location<sup>125</sup>. Delayed clearance from the oral cavity is preferable to prolong the effectiveness of the formulation. It was hypothesized that pectin coating on liposomes may improve the bioadhesion to teeth and, thus, retain liposomes on the teeth for a protective action. Furthermore, this could enhance the substantivity and efficacy of therapeutic agents if they were entrapped in the liposomes, by decreasing liposomal clearance from the oral cavity. The local concentrations of the active substance at site of action may also be increased.

In Paper IV, pectin coating on liposomes did not render enhanced level of adsorption onto the dental enamel. Positively (DPPC/DPTAP) and negatively charged (DPPC/DPPA) liposomes were compared against pectin coated liposomes (0.05 % LM-and



HM-pectin, respectively) in this experiment. However, when exposed to a dynamic flow (2 ml/min), pectin coated liposomes seemed to retain better on the enamel surfaces at longer time intervals than the uncoated negatively charged liposomes (Fig. 17). The level of retention seemed to be even better or comparable to the positively charged liposomes.

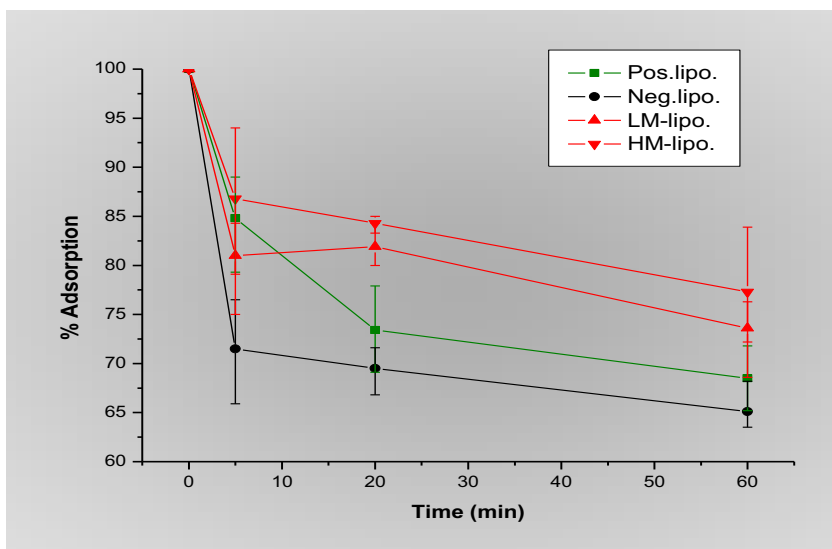


Fig. 17: Percent liposomes remaining adsorbed to the dental enamel after exposure to a flow rate of phosphate buffer 2 ml/min for different liposomal formulations. Positively charged liposomes: DPPC/DPTAP; negatively charged liposomes: DPPC/DPPA; LM-pectin coated liposomes: DPPC/DPTAP + 0.05 % (w/w) LM; HM-pectin coated liposomes: DPPC/DPTAP + 0.05 % (w/w) HM.

This supports the hypothesis that surface modification by pectin on liposomes helps to prolong the adsorption of liposomes on enamel surfaces. It was thought that a similar mechanism behind mucoadhesion is responsible for this bioadhesivity. The strengthened interaction between liposomes and the enamel was thought to be related to the secondary bindings of the pectin chains (van der Waals and hydrogen bindings). The adsorption and retention of pectin coated liposomes suggest that these formulations are promising for the protection of the enamel by a physical approach.

Recently, Wattanakorn et al. prepared pectin discs for buccal adhesion for the treatment of aphthous ulcers in the oral cavity. They concluded that pectin is a potential bioadhesive polymer for buccal drug delivery systems. The purpose of the pectin coated liposomes in the present thesis is the bioadhesion to teeth. Since pectin is also bioadhesive against oral mucosa, this may pose a problem for the specific targeting to teeth. One way to

## DISUCSSION OF RESULTS

overcome the problem is to develop a dosage form, e.g. varnish or “teeth spray”, that can deliver and retain the pectin coated liposomes directly on the enamel surfaces. However, the competitive binding between oral mucosa and the dental hard tissues needs to be examined to find the proper use of pectin coated liposomes in the oral cavity.

The strength and duration of the adsorption is an important perspective considering the dynamic environment of the oral cavity. As shown in Paper IV, the adsorption of liposomes was challenged by imposing a flow, similar to the flow rate of stimulated saliva secretion, at the enamel surface. There are other factors associated with the normal functions of the oral cavity that may also interfere with the adsorption and retention process. The production of acids by plaque bacteria when sugars are consumed may lead to local pH changes in the oral cavity. The influence of the surrounding environment on the liposomal adsorption has been a thread through this thesis (Paper I, II and IV). Therefore, it would be interesting to see the effects of low pH on the adsorption of liposomes to the dental enamel.

## 6. CONCLUSIONS

In the present thesis, 32 different liposomal formulations with both positive and negative surface charge were investigated. In addition, positively charged liposomes were surface modified with the polymer pectin. Three types of pectin were included in the study; low methoxylated, high methoxylated and amidated pectin. The  $\beta$ -values from the DLS analysis of the pectin coated liposomes revealed the formation of non-aggregated nanoparticles.

The type of surface charge on the liposomes was found to be the most important formulation factor for the adsorption onto the model substance hydroxyapatite in phosphate buffer, pH 6.8 - 7.0. Positively charged liposomes adsorbed better than liposomes with a negative surface charge. Among the pectin coated liposomal formulations tested, liposomes coated with 0.05 % (w/w) LM- and HM-pectin, respectively, adsorbed best onto hydroxyapatite.

Uncoated negatively charged liposomes together with pectin coated liposomes did not seem to interact with components of parotid saliva. In contrast, positively charged liposomes aggregated with salivary components and were therefore found incompatible with saliva.

Pectin coated liposomes and uncoated negatively charged liposomes adsorbed to the dental enamel in a salivary environment. Furthermore, pectin coated liposomes retained better on the dental enamel on exposure to flow using phosphate buffer. This indicates their possible use as bioadhesive liposomes in dental applications.

The results from the present thesis suggest that uncoated negatively charged liposomes (DPPC/10 mol% DPPA) together with positively charged liposomes (DPPC/10 mol% DPTAP) coated with 0.05 % (w/w) LM- and HM-pectin are the most promising liposomal formulations for use in the physical protection of teeth.

## 7. FUTURE PERSPECTIVES

The present thesis has suggested the use of liposomes in the physical protection of human teeth by the adsorption to dental hard tissues. To improve and explore their potential use in dental applications, future studies are proposed:

- Verify the protection of teeth by inducing challenges, such as acids, on a liposome covered enamel surface.
- Investigate the adsorption of liposomes to restorative materials.
- Improve the bioadhesion to teeth by investigating other biopolymers for coating on liposomes. For example, chitosan is a cationic polymer and may lead to strong electrostatic interactions with the enamel surface.
- Investigate the possibility of chemical protection by encapsulating drugs, such as chlorhexidine or fluoride, in the liposomes. Liposomes may act as reservoirs from which the active substance is gradually released.
- Investigate the potential of pH-responsive liposomes for use in caries prophylaxis. Liposomes may release entrapped fluoride in response to low pH due to the production of acids by cariogenic bacteria.

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