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Research paper

The potential of liposomes as dental drug delivery systems

Sanko Nguyen [a,*], Marianne Hiorth [a], Morten Rykke [b], Gro Smistad [a]

[a] Department of Pharmacy, School of Pharmacy, University of Oslo, Oslo, Norway
[b] Department of Cariology, Faculty of Dentistry, University of Oslo, Oslo, Norway

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ABSTRACT

The potential of liposomes as a drug delivery system for use in the oral cavity has been investigated. Specifically targeting for the teeth, the *in vitro* adsorption of charged liposomal formulations to hydroxyapatite (HA), a common model substance for the dental enamel, has been conducted. The experiments were performed in human parotid saliva to simulate oral-like conditions. It was observed, however, that precipitation occurred in tubes containing DPPC/DPTAP or DPPC/DPPG-liposomes in parotid saliva with no HA present, indicating that constituents of parotid saliva reacted with the liposomes.

The aggregation reactions of liposome–parotid saliva mixtures were examined by turbidimetry and by atomic force microscopy. Negatively charged DPPC/DPPS and DPPC/PI-liposomes were additionally included in these experiments. The initial turbidity of positive DPPC/DPTAP-liposomes in parotid saliva was very high, but decreased markedly after 30 min. AFM images showed large aggregates of micelle-like globules known to be present in saliva. The turbidity of the various negatively charged liposome and parotid saliva mixtures stayed relatively constant throughout the measuring time; however, their initial turbidities were different; mixtures with DPPC/DPPG-liposomes were the most turbid and DPPC/DPPA-liposomes the least. Pyrophosphate (PP) was added to the various liposome–parotid saliva mixtures to examine the effect of Ca^{2+} on the interactions. The effect of PP treatment of the negatively charged liposome–parotid saliva mixtures was most pronounced with DPPC/DPPG-liposome mixtures where it caused a sudden drop in turbidity. For positive DPPC/DPTAP liposome and parotid saliva mixtures, the effect of PP was minimal.

These experiments showed that saliva constituents may interact with liposomes. An appropriate liposomal drug delivery system intended for use in the oral cavity seems to be dependent on the liposomal formulation. Based on the present results, negatively charged DPPC/DPPA-liposomes seem to be most suitable for use in the oral cavity as they were found to be the least reactive with the components of parotid saliva.

1. Introduction

Caries and periodontal diseases, such as gingivitis and periodontitis, are the most prevalent dental ailments in humans [1]. Many pharmaceutical dosage forms have been developed for the local therapy of dental problems and diseases affecting the oral cavity; dentifrices and mouthrinses as the most common [2,3]. The disadvantage of these conventional systems is the short retention time in the oral cavity because of salivation, the intermittent swallowing, food and beverage intake as well as abrasion by soft tissue movements. Using liposomes as a dental drug delivery system is a new approach that might overcome this problem. Various liposomal formulations have been used as carriers to deliver bactericides to inhibit the growth of biofilms [4,5], and *in vitro* experiments have proven that liposomes adsorb to hydroxyapatite (HA) [6], a commonly accepted model substance for tooth enamel. Liposomes can thus be designed to be bioadhesive, e.g. being retained on enamel surfaces to increase the contact time, thereby prolonging the residence time in the oral cavity. In addition to its encapsulating ability of active pharmaceutical ingredients, e.g. antibacterial or anti-plaque agents affecting the attachment of cariogenic microorganisms onto the enamel, liposomes may protect the enamel against deterioration by physically covering the enamel surfaces. The initial adsorption of bacteria to dental enamel is the basis for dental plaque formation which in later and mature stages can give rise to plaque-related diseases such as caries and periodontal diseases, as previously mentioned.

To develop a pharmaceutical dosage form for delivery in the oral cavity, it is necessary to address the influence of the oral environment on the dosage form. The oral surfaces are constantly exposed to the dynamic and complex fluid saliva. The total volume of salivary secretion in humans has been much debated. It has been

* Corresponding author. Department of Pharmacy, School of Pharmacy, University of Oslo, Oslo, Norway. Tel.: +47 22856589; fax: +47 22854402.
E-mail address: s.h.nguyen@farmasi.uio.no (S. Nguyen).

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estimated to be from 620 ml/day, although much higher amounts (1–1.5 l/day) have been stated [7]. Saliva has many important functions to maintain oral health; it rinses the oral cavity, it protects the oral mucosa and teeth against mechanical abrasion and chemical damage, it acts as a lubricant to facilitate swallowing and clear speech, it contains digestive enzymes and antibacterial substances, it can act as a buffering agent, and it mediates taste sensation. Although saliva contains carbohydrate and lipidic components, as well as inorganic constituents such as phosphate and calcium ions, many of the important properties are due to the proteinaceous fraction of the salivary secretions [8,9].

The presence of amphiphilic proteins in human parotid saliva and their inherent surface activity leads to the formation of a proteinaceous covering on the dental enamel, called the acquired enamel pellicle [10]. It has been demonstrated that this organic layer consists of phosphoproteins associated into micelle-like globules in the size range 100–500 nm and with a net negative surface potential [11,12]. Calcium ions are also thought to play an important role in the structure of these salivary particles [11]. Some of the main roles of the acquired pellicle are to protect the enamel from acid attack, wear and demineralization, as well as crystallization of calcium phosphate salts onto the enamel surface [10,13]. It is widely known that human saliva is supersaturated with respect to calcium phosphate salts to provide a protective and reparative environment for the teeth [14–16]. Thus, saliva is an important contributor for dental as well as oral health.

Because of the complex composition of saliva, this oral fluid will have an impact on any foreign agent introduced to the oral cavity, e.g. liposomes, causing specific or non-specific interactions. Hence, the influence of salivary constituents on a drug delivery system like liposomes is important to investigate. The aim of this study was to examine the *in vitro* adsorption of charged liposomes onto HA in human parotid saliva and to investigate the influence of parotid saliva on the liposomal formulations. *In vitro* experiments of liposomes performed in phosphate buffer have shown to adhere to HA [6]. It was found that the positively charged liposomes adsorbed better than the negatively charged ones in the buffered milieu. In the present study, parotid saliva was used to simulate oral-like conditions. The interaction between liposomes and constituents of parotid saliva was demonstrated at the macroscopic level by turbidimetric measurements and at the microscopic level by imaging with atomic force microscopy.

2. Material and methods

2.1. Materials

The main lipid dipalmitoyl phosphatidylcholine (DPPC) was purchased from Lipoid GmbH (Ludwigshafen, Germany). The anionic lipids dipalmitoyl phosphatidylglycerol (DPPG) and dipalmitoyl phosphatidic acid (DPPA) were kindly provided from Lipoid GmbH (Ludwigshafen, Germany), and dipalmitoyl phosphatidylserine (DPPS) was obtained from Genzyme Pharmaceuticals (Liestal, Switzerland), and phosphatidyl inositol (PI) from wheat germ was obtained from Lipid Products (Surrey, England). The cationic lipid dipalmitoyl trimethylammoniumpropane (DPTAP) and the fatty acid labeled fluorescent phospholipid, 1-oleoyl-2-{6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]hexanoyl}-*sn*-glycero-3-phosphocholine (NBD-PC), were both purchased from Avanti Polar Lipids Inc. (Alabaster, AL, USA). Chloroform and methanol used for liposome preparation were of analytical grade from Merck (Darmstadt, Germany). Hydroxyapatite powder (Bio-Gel® HTP gels) was purchased from Bio-Rad Laboratories (Hercules, CA, USA) and tetrasodium pyrophosphate ($Na_4P_2O_7$), purum, p.a., from Fluka Chemie AG (Buchs, Switzerland).

2.2. Preparation and characterization of liposomes

Liposomes were made according to the film method as follows: the phospholipids were dissolved in a chloroform/methanol (2:1 v/v) mixture, and the solution was evaporated to dryness in a rotary evaporator. The films were further dried in vacuum in a Christ Alpha 2-4 freeze drier (Christ, Osterode am Harz, Germany) for 20 h to remove organic residues. The thin films obtained were hydrated and swelled for 2 h, gently shaken intermittently, at a temperature above the gel to liquid–crystalline phase transition temperature (T_c) with Milli-Q water and kept in the refrigerator overnight. Size reduction was performed at a temperature above T_c by extrusion with a Lipex extruder (Lipex Biomembranes Inc., Vancouver, Canada) using two-stacked 200 nm polycarbonate membranes (Nuclepore®, Costar Corp., Cambridge, USA). Final liposome concentrations for use in further experiments were 6 mM. The concentration of the fluorescent phospholipid used in all liposomal formulations was 1.0 mol%. The liposome dispersions were stored in the refrigerator (4 °C).

The liposomes were characterized by size determinations and zeta potential measurements the same day they were extruded. The zeta potential was measured by laser doppler micro-electrophoresis at 25 °C (Zetasizer 2000, Malvern Instruments Ltd., Worcestershire, United Kingdom) after dilution in the hydration medium to an appropriate counting rate. Three measurements were performed for each sample. The intensity mean diameter of liposomes was determined by photon correlation spectroscopy using Zetasizer 1000HSA (Malvern Instruments Ltd., Worcestershire, United Kingdom) at 25 °C with 90° scattering angle. The refractive index and viscosity of pure water were used as calculation parameters, and each sample was measured in triplicate using the Contin analysis model for size distribution. All samples were diluted with filtered hydration medium to an appropriate counting rate prior to analysis.

2.3. Collection of human saliva

Human parotid saliva was collected from a healthy female by an individually fitted bilateral appliance made of Provil®-P impression material (Bayer Dental, Germany). The impression material was fitted to the entire buccal vestibulum, and drainage was obtained through plastic tubes from excavations at the parotid papillae. The parotid saliva was collected from both sides simultaneously following stimulation with sour candy (Dragster 2000, Jämtgott AB, Sweden), and the first 1–2 ml was discarded. The saliva samples were filtered 0.45 μm with Millex-HV Durapore® membranes (Millipore Corp., MA, USA) and used immediately.

2.4. Adsorption to hydroxyapatite

Hydroxyapatite powder was pretreated prior to the adsorption experiment as follows: HA was suspended in Milli-Q water, placed on a magnetic stirrer at roomtemperature for 20 h and then evaporated to dryness in a drying oven (Termaks AS, Bergen, Norway). Parotid saliva was added to pretreated HA powder in centrifugal tubes to make up 40 mg/ml suspensions and equilibrated 2 min on a rotator (Model LD-79, Labinco BV, The Netherlands) at room temperature (20 rpm). The adsorption of liposomes to hydroxyapatite was conducted by adding liposomal solutions to the HA suspension samples. The liposomal concentration during the adsorption experiment for each sample was 1 mM. Corresponding references were prepared in a similar manner, but adding the liposomes to pure parotid saliva instead of HA suspension. Two parallel samples were prepared from each liposome dispersion. Each tube was then whirlmixed shortly, placed on a rotator for 5 min (20 rpm, 35 °C) and centrifuged (Centra MP4, International Equipment Co., MA,

USA) for 10 min at roomtemperature (1100 rcf). After centrifugation, the supernatants were transferred to glass vials and subjected to lipid quantification by fluorescence spectroscopy. 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.

2.5. Lipid quantification by fluorescence spectroscopy

The lipid concentration in the supernatant was determined as follows: From each supernatant, three samples, 100 μl each, were transferred to a white 96-well flat-bottomed microtiter plate (Nunc™, Denmark). Three samples of HA in parotid saliva without any liposomes, 100 μl each, were applied on the same plate to measure the background. The average fluorescence of the background was eliminated before any calculations. All samples were measured at an emission wavelength of 535 nm and at an excitation wavelength of 485 nm using Victor^3 Multilabel plate reader (PerkinElmer Life Sciences, Turku, Finland).

2.6. Turbidimetric measurements

To make up samples, 2.5 ml of fresh, filtered parotid saliva together with 0.5 ml liposomes were mixed on a rotator for 5 min (20 rpm, 35 °C) in centrifugal tubes. This step was carried out as such to get the same conditions in the mixtures as those applied in the adsorption experiment. The spectrophotometer was autozeroed with references containing parotid saliva and pure water instead of liposomes. The parotid saliva used in the samples and the references were of the same batch collection for each measurement. As controls for the liposome–parotid saliva combinations, pure solutions of parotid saliva and each type of the liposomal formulation were measured separately. Pure water was used to dilute each control solution to the same concentration as its respective sample solution. For the control measurements, the spectrophotometer was auto-zeroed with pure water.

The effect of pyrophosphate (PP) on the liposome–parotid saliva mixture was examined as follows: New sets of samples and references were prepared as described above. For each liposomal formulation, two cuvettes were prepared simultaneously; one functioning as sample for PP addition and the other functioning as the control. First, the sample was measured for 15 min. Filtered (0.2 μm) 0.5 ml PP-solution (50 mM in water) was then added to the sample. At the same time, 0.5 ml of water was added to the control. The PP-treated sample was measured for 15 min after which it was replaced with the control which was measured for another 15 min. The measurements were performed in this succession for each liposomal formulation. Additionally, three parallels of the combination 2.5 ml parotid saliva and 0.5 ml of 50 mM PP-solution were measured referenced against parotid saliva plus water in the same concentration.

Per cent transmittance of all the samples, controls and references were measured as a function of time in disposable cuvettes at ambient temperature using a Shimadzu UV 2550 spectrophotometer at 700 nm [17]. The measurements were repeated three times for each liposomal batch, and each time freshly collected parotid saliva was used. To calculate the turbidity (τ) from the measured transmittance, the following standard equation was used: $\tau = (-1/L)\ln(I_t/I_0)$, where L is the light path length in the cell (1 cm), I_t is the transmitted light intensity and I_0 is the incident light intensity (I_0 = 100%).

2.7. Imaging by atomic force microscopy (AFM)

Ten microliters of the sample was pipetted onto freshly cleaved mica. After 20 s adsorption time, the excess liquid was removed with a filter paper, and the sample was allowed to air-dry at room temperature to the next day (in a petri dish). AFM imaging was performed using The NanoWizard® AFM (JPK Instruments AG, Berlin, Germany). The stage was mounted onto a Nikon Eclipse TE2000-S inverted optical microscope placed on a Halcyonics antivibration table MOD-1M plus (Accurion GmbH, Germany). Intermittent contact mode in air was applied for imaging using NSC35/AlBS Ultrasharp Silicon Cantilevers (MicroMasch, Spain). Both undiluted samples and samples diluted 10 μl sample plus 290 μl of Milli-Q water were studied.

Table 1
Characteristics of the liposomal formulations. The main phospholipid used in all the formulations was DPPC.

Charged lipid	Mol% of charged lipid	Size (Zave, nm)	Zeta potential (mV)
DPPG	2.5	142	−24 ± 1
DPPG	10	149	−56 ± 2
DPPA	2.5	150	−21 ± 1
DPPA	10	144	−50 ± 7
DPTAP	10	176	+41 ± 1

3. Results

3.1. Adsorption to HA

Table 1 shows the liposomal formulations tested and their characteristics. The results from the adsorption experiments showed that both the positive liposomes charged with DPTAP and the negative liposomes charged with DPPA expressed an adsorption level of 16–25% to HA in parotid saliva. The adsorption of negative liposomes charged with DPPG onto HA in parotid saliva surprisingly expressed negative adsorption values. In an attempt to explain the negative results with DPPC/DPPG-liposomes but not with DPPC/DPPA-liposomes, a closer look on the raw data from the fluorescence spectroscopy was performed. As can be seen from Fig. 1, the fluorescence detected in the reference tubes (containing no HA) of both concentrations of DPPC/DPPG-liposomes was lower than in their respective sample tubes. This phenomenon did not occur with DPPC/DPPA-liposomes. Moreover, it was observed visually that a yellow sediment was formed after centrifugation in the reference tubes with the negative DPPC/DPPG-liposomes (both charge concentrations) as with the positive DPPC/DPTAP-liposomes. In contrast, in the reference tubes containing negative DPPC/DPPA-liposomes (both charge concentrations), no such precipitation was observed (Fig. 2).

3.2. Interactions with salivary components

The formation and deposition of aggregates were studied more closely by turbidimetric measurements of the references in the adsorption experiment, i.e. liposome–parotid saliva mixture without HA. Only the 10 mol% concentration of the charged group of all the liposomal batches was investigated. Additionally, two different types of negatively charged liposomal formulations were included in these measurements; DPPC with 10 mol% DPPS and10 mol% PI as the charged groups, respectively.[1] The turbidimetric results are presented in Fig. 3. For the positive DPTAP-liposomes, the liposome–parotid saliva mixtures was initially very turbid ($\tau \sim 1.6$), but the turbidity decreased drastically after about 30 min and leveled out after 50 min ($\tau \sim 0.2$). At 60 min, the mixture had completely phase separated with a clear supernatant and a yellow

[1] Only the type of charged group of the liposomes are indicated in the further text since the main lipid (DPPC) is the same in all formulations.

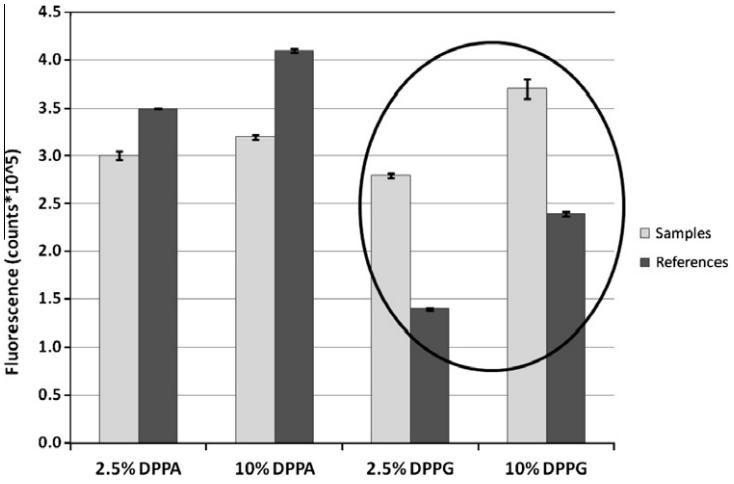

Fig. 1. Amount of fluorescence detected in the supernatant of samples and references of the negatively charged liposomal formulations after the adsorption experiment. DPPC is the main lipid in all formulations; the different type and amount of charged group (mol%) included in the formulations are indicated. The error bars indicate maximum and minimum fluorescence values.

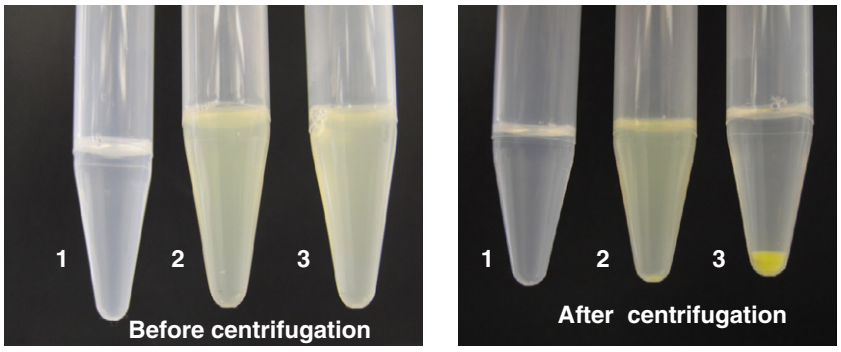

Fig. 2. Reference tubes after the adsorption experiments: (1) parotid saliva + water (control), (2) parotid saliva + negative liposomes (DPPC/10 mol% DPPA), (3) parotid saliva + positive liposomes (DPPC/10 mol% DPTAP). Total volume in each tube is 1.50 ml. Only in the mixture parotid saliva with DPTAP-liposomes (3) total phase separation was observed after centrifugation. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

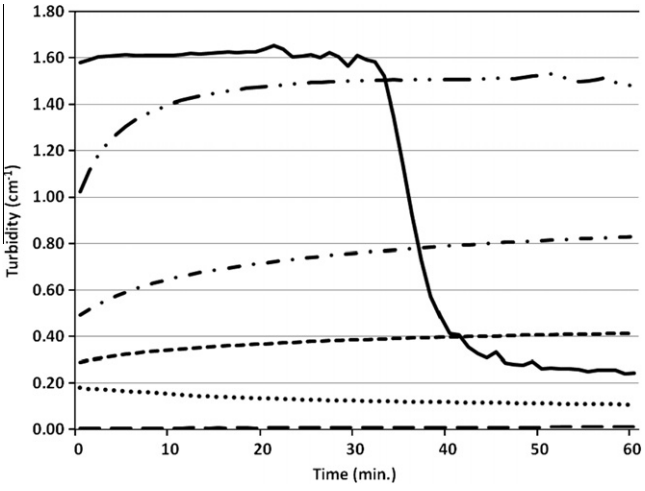

Fig. 3. Changes in turbidity of the mixtures liposome–parotid saliva and parotid saliva alone with time; (—) DPPC/10 mol% DPTAP, (—··) DPPC/10 mol% DPPG, (–·–) DPPC/10 mol% DPPS, (- - -) DPPC/10 mol% PI, (···) DPPC/10 mol% DPPA, (– – –) pure parotid saliva. The average of three separate experiments of each sample is shown.

precipitate. Among the negatively charged liposomes, the biggest difference was observed between the DPPG-liposomes and DPPA-liposomes as depicted in Fig. 3. Although the turbidity of both the DPPG and the DPPA-liposome–parotid saliva mixtures was relatively constant, the initial turbidity values were very different; $\tau \sim 0.2$ for DPPA-liposome compared to $\tau \sim 1.0$ for DPPG-liposome mixtures. Additionally for DPPG-liposome–parotid saliva mixtures, the turbidity increased somewhat the first 10 min to a level of 1.5 after which it remained constant for the rest of the measuring period. At the end of the measuring time, the DPPA-liposome–parotid saliva mixtures were still clear and transparent in contrast to the mixtures with DPPG-liposomes which appeared opaque. No phase separation was observed in either case. For the mixtures with the negatively charged PI-liposomes and DPPS-liposomes, the initial turbidities were quite low with values of $\tau \sim 0.3$ and 0.5, respectively. For both cases, the turbidities increased slowly and after 30 min the values were maintained at $\tau \sim 0.4$ and 0.8, respectively (Fig. 3). The control measurements of pure solutions of parotid saliva showed turbidity values $\tau \sim 0$ throughout the measuring time. Similarly, pure dispersions of each liposomal formulation were also measured yielding turbidity values <0.1 (data not shown).

To examine whether calcium ions were involved in the interactions, a potent calcium-complexing agent, tetrasodium pyrophosphate (PP), was used. The most extreme effects of PP addition on the liposome–parotid saliva mixtures are shown in Fig. 4. A sudden drop in turbidity after PP treatment was observed for both negatively and positively charged liposome–parotid saliva mixtures,

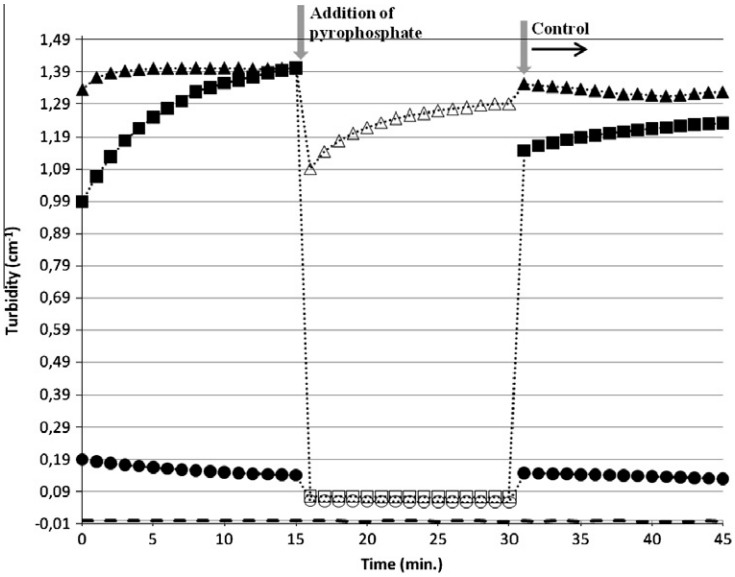

Fig. 4. The effect of pyrophosphate (PP) addition on the liposome–parotid saliva mixtures and parotid saliva alone as a function of time. Filled symbols indicate no added PP, and open symbols indicate addition of PP; (●) DPPC/10 mol% DPPA-liposomes, (▲) DPPC/10 mol% DPTAP-liposomes and (■) DPPC/10 mol% DPPG-liposomes, (- - -) pure parotid saliva + PP. The average of three separate experiments of each sample is shown.

but the phenomenon occurred to a different extent. The most drastic drop in turbidity was observed for the negative DPPG-liposome–parotid saliva mixture; from $\tau \sim 1.4$ to about 0.1. The mixture became immediately clear and transparent after the addition of PP. The same trend was observed in mixtures with the other types of negatively charged liposomes, although the fall in turbidity was less. The effect of PP decreased with DPPS and PI/DPPA, respectively (data for DPPS-liposomes and PI-liposomes not shown). For both PI and DPPA-liposomal mixtures, the effect of PP on the mixtures was minimal; a decrease of less than 0.1 cm^{-1}. Addition of PP to positive DPTAP liposome–parotid saliva mixtures did also result in a reduction in turbidity; however, the effect was not as extensive as for negative DPPG-liposome mixtures as shown in Fig. 4. τ was zero for three control solutions of the combination of parotid saliva and PP.

The main findings from the AFM examinations are presented in Figs. 5–8. Because of the deposition of large aggregates, some samples were diluted before being applied onto the mica. As shown in Fig. 5a, undiluted samples of pure, freshly collected parotid saliva have a "raspberry-like" appearance consistent with earlier findings of micelle-like structures in human parotid saliva as demonstrated by Rykke et al. [11,18]. There was a distinct difference between diluted samples of the mixtures positive DPTAP-liposomes + parotid saliva and negative DPPA-liposomes + parotid saliva as shown on the three-dimensional surface profile in Fig. 6. An even layer of small particles, with a maximum height of less than 8 nm, could be seen in the DPPA-liposome + parotid saliva mixture (Fig. 6b). On the contrary, particles seemed to be clustered together into large aggregates with the maximum height of 250 nm in the mixture with DPTAP-liposomes + parotid saliva (Fig. 6a). Images of these large aggregates (Fig. 7) showed strong resemblance to the "raspberry-like" structures found in pure parotid saliva. The visualization by AFM of the negatively charged liposomes was conducted on the mixtures of parotid saliva with DPPA and DPPG-liposomes. Undiluted samples of the mixtures were compared with undiluted, pure parotid saliva samples (Fig. 8). For the mixtures with DPPG-liposomes, it appeared that these particles are more closely associated compared to the mixture with DPPA-liposomes; however, the differences were not clearly discernible. The image of the mixture of DPPA-liposomes and parotid saliva seemed to be quite similar to the image of the pure parotid saliva.

4. Discussion

4.1. Adsorption to HA

Based on the nature of electrostatic forces, positive liposomes are expected to adsorb better to HA than the negatively charged liposomes. However, as a potential drug delivery system, the extent of adsorption will be dependent on the physiological *in vivo* environment as well, e.g. proteins in parotid saliva may compete with the liposomes for the same adsorption sites on HA. As the results showed, components of parotid saliva itself interacted with the liposomes and caused aggregation and sedimentation due to centrifugation, providing erroneous adsorption values of the charged liposomes onto HA. The results from the adsorption experiments were therefore not reliable as the samples were compared against references which contained false low levels of free liposomes. This may explain the negative adsorption values exhibited by the negatively charged liposomes with DPPG. Furthermore, sedimentation occurred for the DPPG-liposomes but not for the DPPA- liposomes in the salivary environment. This was unexpected as both liposomal formulations were negatively charged. In the present experiments, it was not possible to differentiate the liposomes adsorbed to HA from the liposomes aggregated with substances in saliva in the precipitates of the sample tubes. Nevertheless, the adsorption results suggest that different charged groups on the liposomes may cause different type and degree of interaction with the components of parotid saliva. Thus, the prevalence and stability of liposomes in this oral fluid will be influenced by their lipid composition. The interaction between liposomes and components of parotid saliva was therefore further investigated.

4.2. Interactions with salivary components

The application of spectrophotometric analysis to measure a solution's or a mixture's turbidity can give quantitative

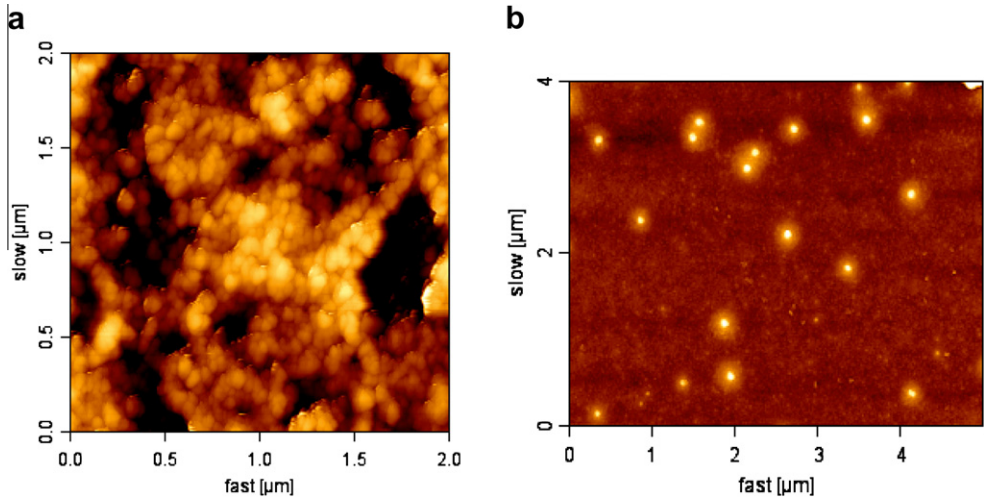

Fig. 5. (a) Undiluted sample of pure parotid saliva showing aggregates of salivary micelle-like structures. (b). Diluted sample (1:30) of pure parotid saliva showing scattered micelle-like structures. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

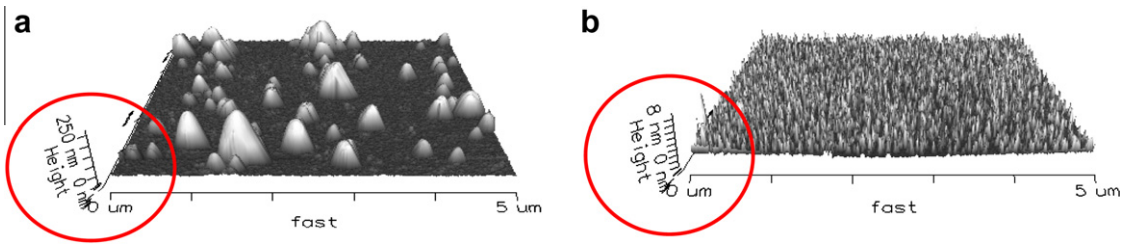

Fig. 6. 3D surface profile of diluted samples of liposome–parotid saliva mixtures showing large aggregates in the image with positively charged liposomes (a; parotid saliva + DPPC/10 mol% DPTAP) but not in the image with negatively charged liposomes (b; parotid saliva + DPPC/10 mol% DPPA). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

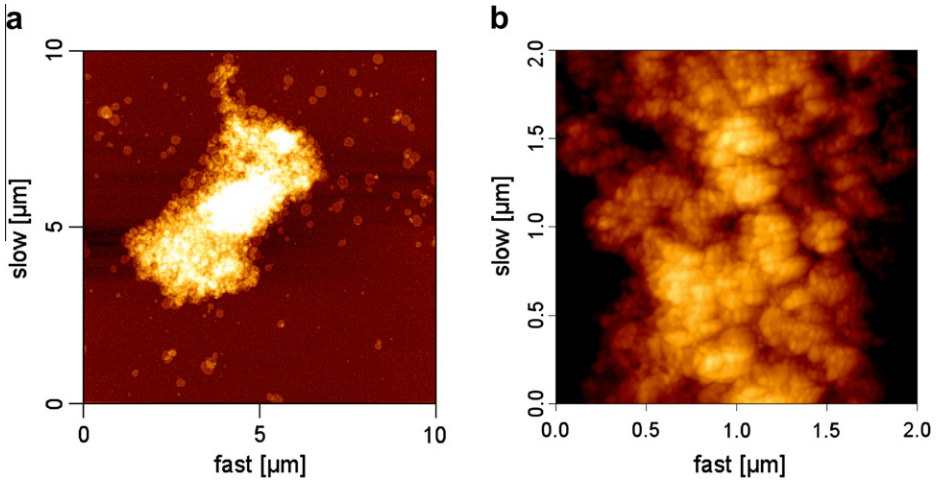

Fig. 7. Aggregates in diluted samples of the mixture parotid saliva + DPPC/10 mol% DPTAP-liposomes. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

information about aggregation reactions based on the intensity of transmitted light. Not surprisingly, the most obvious difference in the initial turbidity of the mixtures was between the positively and the negatively charged liposomes where the mixture of positive DPTAP-liposomes and parotid saliva was the most turbid. The major human salivary glands (parotid, submandibular and sublingual) produce different profiles with respect to the composition of saliva, and the protein content of any type of saliva is a highly important aspect as the proteins are related to the main protective functions of the saliva [19,20]. Pure parotid saliva is readily obtained, as described. The secretion is serous in nature with a high content of acidic phosphoproteins, e.g. proline-rich proteins (PRPs), which are likely to associate and form negatively charged micelle-like globules [21,22]. It has been demonstrated that the salivary micelle-like globules promote the agglutination of oral bacteria and that calcium-dependent, electrostatic and

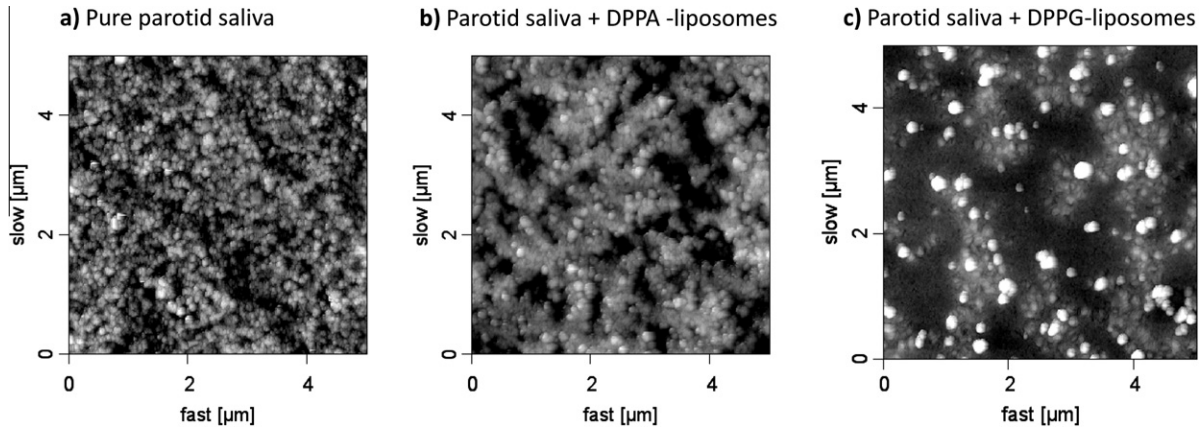

Fig. 8. Representative images of parotid saliva alone and the mixtures of parotid saliva with different negatively charged liposomes. Undiluted samples of pure parotid saliva (a), the mixture parotid saliva and DPPC/10 mol% DPPA-liposomes (b) and the mixture parotid saliva and DPPC/10 mol% DPPG-liposomes (c).

hydrophobic interactions are thought to be involved [17]. This phenomenon is thought to partly be responsible for the protection of the oral cavity by modifying microbial adsorption and subsequent colonization, i.e. plaque formation. It is therefore highly likely that these amphiphilic structures in parotid saliva can electrostatically complex with positive DPTAP-liposomes causing aggregation reaction and high turbidity of the solution. This reaction seemed to be instant since the solution became turbid immediately after mixing. The reason for the sudden drop in turbidity may be explained by the growing of the aggregates followed by sedimentation with time. Rykke et al. reported that salivary micelle-like globules increased in size with time due to continuous aggregation of the micellar units subsequently implicated in the formation of the protective acquired enamel pellicle [11]. The aggregates in the present experiments, probably consisting of micelle-like globules and positively charged DPTAP-liposomes, became eventually so large that they consequently sedimented out of the mixture. This may be due to the growing of the micelle-like structures itself, as reported by Rykke et al. [11] in combination with the attractive forces exhibited by the DPTAP-liposomes. The fact that the mixture phase separated without any additional centrifugal force (as used in the adsorption experiments) indicates how large and heavy these structures are. Proline-rich proteins have been identified in the micellar components of whole saliva in a recent study [23]. Moreover, Young et al. found that the globular micelle-like structures are primarily present in human whole saliva and only in small amounts in parotid saliva [24]. Whole saliva is a product of bacteria, leukocytes, desquamated epithelial cells and crevicular fluid in a mixture of secretions mainly from the three major salivary glands and, as such, provides the dominating oral environment under physiological circumstances. It is therefore likely to assume that the observed interactions between DPTAP-liposomes and the salivary micelle-like globules will be more pronounced in an *in vivo* environment and thus that these type of liposomes probably not are suitable for use in the oral cavity.

In contrast, for the negatively charged liposomes, no such drop in turbidity or phase separation was observed in the mixtures for the same time course. Although the initial turbidities were different for the various types of negatively charged groups of the liposomes, the turbidity levels were relatively constant with time for all the mixtures. These results suggest that the negative liposomes also bind to the components of saliva, like the positive liposomes, however, involved with a different type of interaction. As saliva is supersaturated with calcium phosphates, it was hypothesized that calcium is the substance responsible for the aggregation observed when adding the negative liposomes. This reaction can be attributed to the electrostatic binding of calcium ions to negatively charged liposomes forming small aggregates as a ligand between two negatively charged groups. These do not grow and reach the critical size of sedimentation in the same manner as the aggregates comprising the micelle-like structures. Several studies have shown that divalent cations (Ca^{2+}, Mg^{2+}, Sr^{2+}) interact with negatively charged liposomes [25,26]. The exact mechanism of the cation-induced aggregation is still not clear, but it has been assumed to be dependent on the fatty acid composition of the phospholipid in addition to the polar headgroup structure [27]. Negative DPPS-liposomes and PI-liposomes were included in this experiment to demonstrate that various types of negatively charged groups can have very different affinity to calcium as expressed in the different initial turbidity levels. Among the negative liposomes, the most turbid mixture in parotid saliva was observed for DPPG-liposomes probably having the strongest affinity to calcium. The affinity of calcium ions to the negative liposomes tested in parotid saliva may be arranged in the order: DPPG > DPPS > PI > DPPA. The proposed order is, however, not in agreement with Rosenberg et al. [28]. According to these investigators, the first step in the fusion reaction is aggregation and they showed that the calcium-induced fusion of phosphatidylglycerol (PG) liposomes required a higher calcium concentration than that for phosphatidylserine (PS) liposomes. This indicated a higher affinity to calcium for PS liposomes than for PG liposomes. The difference in the order of calcium affinity to negative liposomes may be explained by the fact that the calcium-induced aggregation of the negative liposomes in the present experiment was performed in the salivary environment, which is more complex than a buffered milieu. Furthermore, the mean total calcium concentration in stimulated parotid saliva is 1.6 ± 0.8 mM where only about 50% of the calcium is present in an ionic form [29]. This means that the calcium concentration responsible for the aggregation reactions of negative liposomes in parotid saliva is much lower than those examined by Rosenberg et al. [28] who used calcium concentrations in the range 2–30 mM.

To confirm that calcium ions are involved in the aggregates consisting of negative liposomes, pyrophosphate was added in the mixtures in a similar turbidity experiment. Pyrophosphate strongly sequesters calcium ions and will compete with the negative liposomes for the same binding sites. PP will assumedly pull out any calcium involved in the interaction with negative liposomes and induce deaggregation accompanied by a reduction in turbidity. For the mixtures with negative DPPG-liposome and parotid saliva, a deaggregation occurred by the addition of PP, as demonstrated by a marked decrease in the turbidity (Fig. 4). This strongly indicates a Ca^{2+}-dependent aggregation of these liposomes in the salivary

environment. In contrast, a similar change in turbidity was not observed with the mixtures of negative PI-liposomes or DPPA-liposomes. The most pronounced effect following the treatment with PP among the negative liposomes tested was in the order: DPPG > DPPS > PI/DPPA. These results support the sequence of calcium affinity among the negative liposomes previously mentioned. It has been reported that phosphate enhanced the calcium-induced fusion of PS vesicles [30]. The concentrations of calcium and phosphate examined in this study are analogous to those current in stimulated parotid saliva. Fusion reactions were thought to not occur in the present experiment, because the addition of pyrophosphate to negative liposomes in parotid saliva reduced the turbidity significantly. As fusion of the phospholipid membranes is an irreversible process, an effect of pyrophosphate addition on the turbidity of negative liposomes in parotid saliva would not be expected. Free calcium was assumed not to be the main factor in the aggregation of positive DPTAP-liposomes with salivary micelle-like globules. Nevertheless, in the mixtures with positive DPTAP liposome–parotid saliva, a small reduction in turbidity was observed after the PP treatment of these mixtures. This observation may be explained by the presence of calcium inherent in the salivary micelle-like structures. Rykke et al. reported that calcium is important in the maintenance of the micellar globules [11], and the effect of PP may be due to some degree of sequestration of the bound calcium of these structures. The effect of PP on the positive DPTAP-liposomes and parotid saliva mixtures was only minor (Fig. 4). The aggregates formed in these mixtures are probably so large and dense that it is difficult for PP to penetrate to the calcium ions involved in the micelle-like structures. Consequently, the aggregates consisting of positive DPTAP-liposomes and micelle-like globules did not completely disintegrate after PP treatment. Overall, these results are in accordance with the assumption of calcium involvement in the interactions between substances of parotid saliva and negative liposomes, but not with positive liposomes.

Representative AFM images can give a visualization of the situation and possible interactions in the salivary samples. Together with the turbidimetric measurements, this may strengthen the hypothesis of different interactions of positively charged liposomes versus negatively charged liposomes with salivary constituents. The AFM images substantiated the results as obtained by the turbidimetric data. Large aggregates were observed in images from diluted saliva mixtures containing positively charged DPTAP-liposomes (Fig. 7), whereas no such aggregates appeared in the images of diluted saliva alone (Fig. 5b) or in saliva mixtures containing the negatively charged liposomes (Fig. 8b and c). This finding was confirmed by 3D surface profiles of parotid saliva mixtures and differently charged liposomes (Fig. 6), indicating that the interactions of negatively charged liposomes and salivary constituents may be minimal. The globular aggregates, observed in the images from the mixtures of positive DPTAP-liposomes in parotid saliva (Fig. 7), are similar to the "raspberry-like" appearance of micelle-like globules observed in pure parotid saliva (Fig. 5a). This indicates that the aggregates formed in the mixtures parotid saliva and DPTAP-liposomes, causing the solutions to be initial turbid, mostly are comprised of large aggregates of the proteinaceous micelle-like salivary globules not disintegrating due to the dilution. As such, aggregates were not observed in the images of similarly diluted pure parotid saliva, and this indicates that the positively charged liposomes are involved in the aggregation reactions. In earlier reports, morphologically similar globular structures have been visualized by transmission electron microscopy of human parotid saliva and have been demonstrated to be the negatively charged protein micelles comprising parts of the acquired enamel pellicle [11,18]. Furthermore, images of diluted samples of pure parotid saliva showed only scattered particles (Fig. 5b), compared to the densely packed "raspberry-like" structures appearing in clusters in the undiluted samples (Fig. 5a). The calcium concentrations in diluted samples were reduced, suggesting that calcium ions are important mediators in the formation of these salivary micelle-like structures and the subsequent clustering. This is in agreement with the previous report by Rykke et al. [11].

5. Conclusions

These present experiments showed that salivary substances can interact with liposomal formulations. The type and the degree of interaction are dependent on the various compositional factors of the liposomes, such as the type of charge (positive/negative) and type of charged group. Positively charged liposomes with DPTAP as the charged group are not suitable for use as a drug delivery system to the oral cavity due to aggregation reactions with salivary constituents. The reactivity of negatively charged liposomes is dependent on the type of charged group bound to them. It seemed that calcium in parotid saliva is a prerequisite for the interplay with negatively charged liposomes, and their affinity to the cations determines the degree of interaction. Among the negatively charged liposomes, the liposomes charged with DPPA were the least interfered by the salivary environment. Therefore, it seems likely that this type of liposomal formulation has a high potential for use in the oral cavity. Thus, by tailoring the structural characteristics of liposomes, minimum interference with physiological substances in the oral cavity can be obtained and the behavior of liposomal formulations in the oral environment can be predicted.

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