International Journal of Pharmaceutics 645 (2023) 123407

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Stability and cytotoxicity of biopolymer-coated liposomes for use in the oral cavity

Julie Frigaard [a,*], Janicke Liaaen Jensen [a], Hilde Kanli Galtung [b], Marianne Hiorth [c]

[a] Department of Oral Surgery and Oral Medicine, University of Oslo, Norway, Institute of Clinical Dentistry, Faculty of Dentistry, Box 1109 Blindern, 0317 Oslo, Norway
[b] Institute of Oral Biology, Faculty of Dentistry, University of Oslo, Norway, Box 1052 Blindern, 0316 Oslo, Norway
[c] Section for Pharmaceutics and Social Pharmacy, Department of Pharmacy, The Faculty of Mathematics and Natural Sciences, University of Oslo, Norway, Box 1068 Blindern, 0316 Oslo, Norway

ARTICLE INFO

Keywords:
Xerostomia
Dry mouth
Liposome
Biopolymers
Cytotoxicity
Cell viability

ABSTRACT

This study investigates the stability and cytotoxicity of biopolymer-coated liposomes for use in the oral cavity. Liposomes (3 mM and 6 mM) were prepared by the thin film method and hydrated with phosphate buffer (PB) or glycerol phosphate buffer (G-PB). For coating, liposomes were added to a biopolymer solution of opposite charge. Particle stability was evaluated by measuring the size, polydispersity index, and zeta potential for up to 60 weeks. *In vitro* interaction of fluorescent-labelled biopolymer-coated liposomes and dysplastic oral keratinocytes was analyzed by confocal microscopy. Potential cytotoxicity was assessed in dysplastic oral keratinocytes by cell proliferation and cell viability. All three biopolymers showed good coating abilities for both concentrations and hydration media. The alginate coated liposomes in PB, 3 mM chitosan-coated liposomes in PB, and chitosan-coated liposomes in G-PB were stable for up to 60 weeks. *In vitro* studies demonstrated low cytotoxicity for all coated liposomes and non-specific cellular uptake of biopolymer-coated liposomes, independent of biopolymer, surface charge, lipid concentration and hydration media. All three formulations demonstrated low cytotoxicity and were considered safe. Alginate- and chitosan-coated liposomes demonstrated good stability over time and may be promising agents for use in the oral cavity and should be investigated further.

1. Introduction

Saliva plays an essential role in maintaining good oral health through lubrication of the oral mucosa thereby facilitating oral functions such as speech and swallowing. The flow of saliva contributes to clearance of food particles and bacteria from the oral mucosa and the teeth. Saliva also plays a role in the digestion of food, by dissolving taste substances, moisturizing and adding digestive enzymes to the bolus, and regulating the pH of the oral cavity. In contrast, reduced salivary flow may lead to dry mouth and oral diseases like candidiasis and dental caries, and may negatively impact on functions like talking, eating, and swallowing. In turn, nutrition may be compromised leading to reduced general health and reduced quality of life (Dynesen, 2015; Hahnel et al., 2014).

Side-effects of prescribed drugs (e.g., antihypertensives, antihistamines, antidepressants) (Tan et al., 2018; Tiisanoja et al., 2018), autoimmune disorders (Sjögren's syndrome, diabetes mellitus) (Borgnakke, 2019; Ngo et al., 2016) and damage to salivary glands after head- and neck irradiation (Connor et al., 2006; Westgaard et al., 2021) are well-known causes of dry mouth (Carpenter, 2015). As the prevalence of dry mouth is highly dependent on the patient group explored, numbers in the literature vary from 22% to 39% (Agostini et al., 2018; Carpenter, 2015; Johansson et al., 2020; Liu et al., 2012; Nederfors et al., 1997). The incidence seems to be higher in women and increases with age and with the use of medications, and the highest incidence is seen in patients who have undergone radiation to salivary glands due to cancer in the head and neck region (Agostini et al., 2018; Dynesen, 2015).

Non-pharmaceutical treatment of dry mouth includes products like chewing gums and lozenges to stimulate salivary secretion in the presence of functioning salivary gland tissue. Saliva substitutes as sprays or gels are topically applied to lubricate the oral mucosa in the absence of functioning salivary gland tissue (Gil-Montoya et al., 2016). Due to practical considerations, saliva substitutes may be more convenient during nighttime for all dry mouth patients. Besides lacking several natural salivary components such as mucins and electrolytes, the main drawback of salivary substitutes is the short residence time in the oral cavity, hence the need for repeated application (Vinke et al., 2020).

* Corresponding author.
E-mail address: j.m.frigaard@odont.uio.no (J. Frigaard).

https://doi.org/10.1016/j.ijpharm.2023.123407
Received 6 June 2023; Received in revised form 7 September 2023; Accepted 10 September 2023
Available online 12 September 2023

Consequently, there has been an increasing interest in mucoadhesive agents for prolonged effect of the local administration of hydration in the oral cavity (Kockisch et al., 2005; Tsibouklis et al., 2013).

From the first discovery of liposomes by Bangham in 1965, the idea of encapsulating drugs into these small spherically shaped vesicles has been introduced (Bangham, 1963). Since then, several products with liposomes as drug carrying vehicles have been approved by the FDA (Bulbake et al., 2017). Liposomes have the ability to encapsulate and release for example hydrating substances in a controlled manner over time, making liposomes highly relevant in the treatment of dry mouth (Bozzuto & Molinari, 2015). Improvement of the mucoadhesive properties of liposomes may enhance the delivery time of a hydrating substance and provide an effective oral hydration. Furthermore, previous studies have shown enhanced mucoadhesive properties and higher water absorption capacity when liposomes are coated with biopolymers such as chitosan and alginate (Adamczak et al., 2016; Adamczak et al., 2017; Karn et al., 2011).

Alginate is a natural anionic biopolymer that originates from marine brown algae. It is a linear copolymer composed of blocks of D-mannuronic acid and L-guluronic acid residuals (pKa 3.38 and 3.65, respectively) (Draget et al., 1994; Lee & Mooney, 2012). Alginate is commonly used as a texture thickener and stabilizer in food and medicines. This substance is a well-known biocompatible biopolymer with mucoadhesive properties that can absorb large quantities of water and may therefore be a promising coating material for liposomes used in the treatment of dry mouth (Adamczak et al., 2016; Liu & Krishnan, 1999; Tentor et al., 2020).

Chitosan is a cationic biopolymer derived from the natural polysaccharide chitin found in crustaceans. It is a heteropolysaccharide with randomly distributed D-glucosamine and N-acetyl glucosamine units (pKa 6.5) (Mohammed et al., 2017). Chitosan is widely used in the food industry, biomedical applications, and tissue engineering (Mohammed et al., 2017). Chitosan is one of the few cationic polymers, and as such can interact electrostatically with negatively charged molecules including those in the cell membranes. As the retention time at site will be enhanced, chitosan is of particular interest in drug delivery systems (Frigaard et al., 2022; Karn et al., 2011; Tentor et al., 2020). Also, a lubricating property of chitosan-coated liposomes has been demonstrated (Hiorth et al., 2023). In that study, it was shown that the enhanced lubricating properties of polymer-coated liposomes were dependent on charge while size was of minor importance.

Finally, gellan gum is a natural anionic biopolymer produced by the bacteria *Sphingomonas elodea*, and consists of repeating units of D-glucose, L-rhamnose and D-glucuronate units (pKa 3.5) (Cassanelli et al., 2018). Gellan gum can incorporate high volumes of hydrating substance due to excellent swelling properties. It is approved for use in food, cosmetics and pharmaceuticals as thickener, stabilizer and emulsifier as well as applied in pharmaceutical technology to achieve mucoadhesive properties in ocular drug delivery systems (Blocadren®) (Dhanka et al., 2018; Osmałek et al., 2014).

The aim of this study was to assess the potential of biopolymer-coated liposomes for use in the oral cavity by scrutinizing the physicochemical properties and stability of three types of biopolymer-coated liposomes in two different hydration media (PB and G-PB) and lipid concentrations (3 mM and 6 mM) and evaluating *in vitro* cell interaction and cytotoxicity in a relevant cell model.

2. Materials and method

2.1. Materials

Phosphatidylcholine (SoyPC) was a kind gift from Lipoid GmbH (Ludwigshafen, Germany). Dioleoyl trimethylammoniumpropane (DOTAP), phosphatidylglycerol (EggPG) and fluorescent lipid 1-oleoyl-2-{6-[(7-nitro-2–1.3-benzoxadiazol-4-yl)amino]hexanoyl}h-*sn*-glycero-3-phospcholine (NBDPC), were purchased from Avanti Polar Lipids, Inc. (Alabaster, USA). Chitosan of ultrapure grade (Protasan UP CL 213, DDA 85%, MW 3.1×10^5 Da, Novamatrix) and sodium alginate (Protanal LF 10/60, MW 1.47×10^5 Da (Pistone et al., 2015), FMC Biopolymer) was obtained from FMC Biopolymer AS (Sandvika, Norway). Gellan Gum (Gelzan 1.0×10^3 kDa, Sigma Aldrich), Dulbecco's modified eagle medium/nutrient mixture F-12 (DMEM/F12) (Gibco), fetal bovine serum (FBS) (Gibco), phosphate-buffered saline (PBS) (Gibco), Insulin-Transferrin-Selenium (Gibco) and tryphan blue 0.4% (Invitrogen) were purchased from Thermo Fisher Scientific, USA. Epidermal growth factor (Sigma), hydrocortisone (Sigma), ascorbic acid (Sigma), L-glutamin (Sigma), antibiotic–antimycotic solution (Sigma), WST-1 reagent (4-[3-(4-Iodophenyl)-2-(4-nitro-phenyl)–2H-5-tetrazolio]-1,3-benzene sulfonate) (Cellpro-ro, Roche), 4% paraformaldehyde (PFA) (Sigma) and trypsin (Sigma) were purchased from Merck KGa, Germany. DAPI Fluoromount-G (SouthernBiotech) was purchased from AH Diagnostics AS, Oslo, Norway. Milli-Q water was purified by a Milli-Q system with 0.22 μm Millipak® 40 filter (Millipore®, Ireland).

2.2. Cell culture

The dysplastic oral keratinocytes (DOK) (ECACC 94122104) were purchased from the European Collection of Authenticated Cell Cultures (ECACC, Salisbury, UK) and used between passages 3 to 20. Cell cultures were grown using 25 or 75 cm^2 cell culture flask (Sarstedt, Sarstedt AG & Co. KG, Germany) and incubated at 37 °C in a 5% CO_2 atmosphere. Cell culture medium was 450 ml Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (1:1), with 50 ml foetal bovine serum, 5 μl epidermal growth factor (10 ng/mL), 400 μl hydrocortisone (0.4 μg/mL), 500 μl ascorbic acid (50 μg/mL), 10 ml L-glutamine (200 mM), 250 μl Insulin-Transferrin-Selenium 100x and 5 ml antibiotic–antimycotic solution. Medium was exchanged every third day and cells were passaged using trypsin when confluent.

2.3. Preparation of liposomes

The liposomes were prepared using the thin film method previously described (Nguyen et al., 2011). In short, 90 mol% of the neutral lipid SoyPC and 10 mol% of the charged lipid (DOTAP or EggPG) were dissolved in chloroform. The lipid film was formed through rotary evaporation (Heidolph W 2001 rotavapor, Heidolph Instruments GmbH Co. KG, Kelheim, Germany) at 90 rpm and 40 °C at 20 kPa until the solvent was visually evaporated, then held at 6 – 6.5 kPa for 20 min, before the films were dried under vacuum (25 °C, < 100 kPa) (Christ Alpha 2–4 freeze drier, Christ, Osterode am Harz, Germany) overnight to remove the last residues of chloroform. The lipid film was hydrated by careful addition of PB or G-PB to the dry lipid film, followed by 10 min of rotation at 90 rpm, and 2 h of soft swirling every 30 min before overnight storage at 4 °C. To obtain monolayered liposomes of homogenous size, the lipids were extruded (Lipex extruder, Lipex Biomembranes Inc. Canada) ten times through two polycarbonate filters of 200 nm (Nucleopore®, Costar Corp., USA) before coating. Liposomes were prepared in 3 mM and 6 mM lipid concentrations, in both PB and G-PB. NBDPC-labelled liposomes were prepared in the same manner, except that 1 mol% of SoyPC was replaced with NBDPC.

2.4. Preparation of biopolymer solutions

Commercially available alginate and gellan gum had previously been purified to remove soluble residuals with low MW (such as sodium and chloride ions) with dialysis prior to its use as previously described (Pistone et al., 2015). In short, the polymers were dissolved in water (concentration 1.5 w/w%) and stirred overnight in room temperature. The solutions were then dialyzed against distilled water for eight days using a Spectra/Por® dialysis membrane (Spectrum Laboratories Inc., CA, USA, 8000 Dalton molecular weight cutoff). The dialyzed solution was freeze-dried (Christ Alpha 2–4 freeze drier, Christ, Germany) and

stored in the refrigerator.

The biopolymer solutions were prepared by dissolving the polymers (purified alginate and gellan gum, chitosan as from supplier) in PB and G-PB (5 mM, pH 6.8), at 0.125 w/w% and 0.250 w/w% concentrations, by overnight stirring at room temperature. The biopolymer solution was filtrated through a 5 μm syringe filter (Nucleopore®, Costar Corp., Cambridge, USA) prior to use.

2.5. Preparation of biopolymer-coated liposomes

The liposomes were added dropwise to the biopolymer solution in a 1:4 ratio under magnetic stirring using a peristaltic pump, followed by 5 min of stirring. The liposomes of 3 mM were coated with 0.125 w/w% biopolymer and the liposomes of 6 mM were coated with 0.250 w/w% biopolymer, in both PB and G-PB. The biopolymer-coated liposomes were flushed with N_2, sealed, and stored at 4 °C in between measurements. For the biopolymer-coated liposomes the final concentrations of lipids were 0.6 mM and 1.2 mM, and the final concentration of biopolymers were 0.1 w/w% and 0.2 w/w%. For reasons of simplicity the original concentration of lipids and biopolymers before coating is used to identify the different formulations in this paper. A schematic illustration of the twelve produced formulations is presented in Fig. 1.

2.6. Size and zeta potential of the formulations

The hydrodynamic diameter (size) and size distribution (polydispersity index, PDI) of the particles in the formulations were determined by dynamic light scattering, while zeta potential (z-potential) was calculated using phase analysis light scattering (PALS) and multi-frequency measurement. Both measurements were carried out using a Malvern Zetasizer Nano ZS (Malvern instruments Ltd. Worcestershire, UK) at 25 °C, with laser beam ($\lambda = 632.8$ nm) at angle 173°. The refractive index and viscosity of pure water and glycerol at 25 °C were used as constant parameters.

Each sample was diluted with the corresponding buffer 1:9 and size and z-potential measurements were repeated three and five times within the same sample, respectively. The biopolymer-coated liposomes were measured in triplicates. The formulations were visually inspected and the size, PDI, and z-potential were determined regularly for a period up to 60 weeks to evaluate the physical stability of the formulations. Samples with visible precipitation were excluded for further measurements, as well as samples with PDI > 0.3 as these samples were regarded to have insufficient homogenous particle size distribution (Danaei et al., 2018). Between measurements, the samples were flushed with N_2, sealed, and stored in the refrigerator.

2.7. Cell studies

Based on preliminary tests on chitosan-coated liposomes, 6.25% was determined as the highest possible test concentration in the dye exclusion experiment, and 12.5% as the highest concentration in the formazan reduction experiment (see below). For alginate and gellan gum-coated liposomes, the test concentration was 50% for all experiments. To assure sufficient nutrition for the cells, additional FBS was added to maintain 10% FBS in the total volume whenever the cell medium was diluted with the formulations. Only formulations hydrated in PB were investigated in the cell studies because the high osmolality of G-PB interfered with the osmotic requirements of the cells.

2.7.1. Cell viability test

Cell viability was evaluated by the tryphan blue dye exclusion test after 4 and 24 h of exposure to biopolymer-coated liposomes. The DOK cells were preincubated in 12-well plates at a density of 2×10^5 cells/well for 24 h before cell growth and morphology were visually inspected in the light microscope (Axiovert 25, Zeiss). The cell culture medium was discharged and replaced by 0.5 ml of fresh medium containing biopolymer-coated nanoparticles of the predetermined concentrations. Cell culture medium alone was added as a control. After 4 and 24 h incubation, the cells were inspected in the microscope before they were washed with PBS and treated with trypsin to detach them from the wells. The cell-containing medium was stained with tryphan blue and immediately added to the cell counting chamber slide (Invitrogen™ Countess™ Cell Counting Chamber Slides; Thermo Fisher Scientific, USA) and inserted into the automated cell counter (Invitrogen™ Countess™ II; Thermo Fisher Scientific, USA). The formulations were tested in duplicates and in three parallels; each test was repeated twice. The results are expressed as percentage relative to the control with cell culture medium alone. A clinical relevant concentration was chosen for the *in vitro* studies based on recommendations from the Biological Evaluation of Medical Devices (ISO 10993–5) and $< 30\%$ cell viability reduction was assessed as non-cytotoxic effect (International Organization for Standardization (ISO) 2009).

2.7.2. Cell proliferation assay

Cell proliferation was evaluated by formazan reduction using the WST-1 reagent. The DOK cells were preincubated in 96-well plates at a

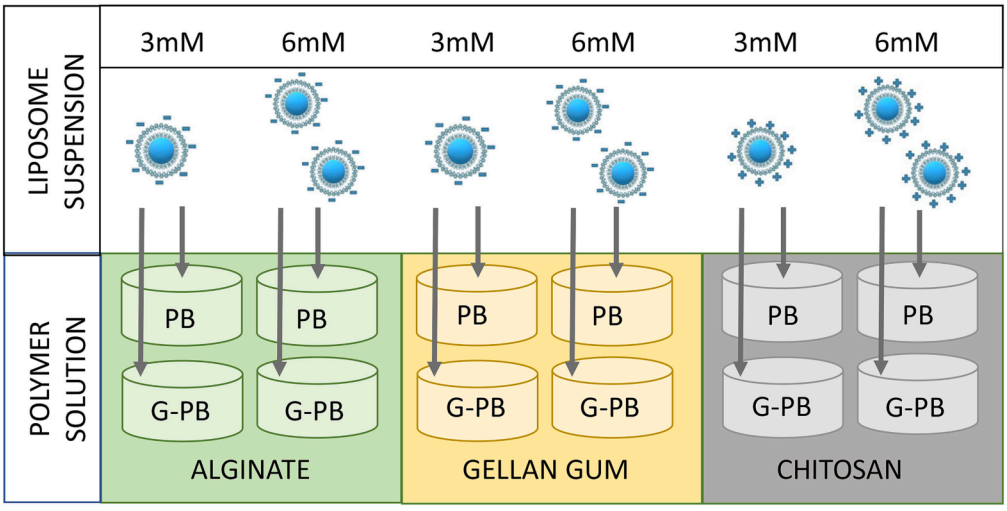


Fig. 1. Schematic representation of the experimental setup. The twelve different polymer-coated liposome formulations produced in this experiment. PB: phosphate buffer. G-PB: glycerol phosphate buffer.

density of 5 × 10^3 cells/well for 24 h, the cells were inspected in the microscope and the culture medium was discharged and replaced by 100 µl fresh cell culture medium containing biopolymer-coated liposomes. Predetermined concentrations of the most stable biopolymer-coated liposome formulations (1.56 – 50% for alginate-coated liposomes, and 1.56 – 25% for chitosan-coated liposomes) were evaluated. Cell culture medium and diluted cell culture medium (medium/PB 1:1 for alginate-coated liposomes, and medium/PB 2:1 for chitosan-coated liposomes) were included as negative controls, and cell-free wells (blanks) where included for calculation of baseline absorbance. After 4 and 24 h of exposure, 10 µl of WST-1 reagent was added to each well, and the cells were incubated for another 2 h. The plates were shaken thoroughly for 1 min using a shaker (VIBRAX VXR basic, IKA®-Werke GmbH & Co. KG, Staufen, Germany) before absorbance was measured at 450 nm by a plate reader (Microplate Spectrophotometer, Epoch Agilent, California, USA). The assay was performed three times for each formulation, and each experiment contained five replicates per concentration. The results are expressed as percentages relative to the control containing only cell culture medium.

2.7.3. Confocal microscopy

Cellular uptake of biopolymer-coated fluorescence-labeled liposomes was investigated by confocal microscopy (gated stimulated emission depletion microscopy, gSTED, LEICA) at the Core Facility for Advanced Light Microscopy, University of Oslo, Norway. Using this method, three-dimensional images of the samples are created and can assist in determining if substances have entered the cell. Cells were seeded on a coverslip (VWR, VWR International), and incubated at 37 °C in a 5 % CO_2 atmosphere for 24 h. The cells were visually inspected in the light microscope and each coverslip was washed three times with PBS before 100 µl fresh medium containing NBDPC-labelled biopolymer-coated liposomes (50% for alginate- and gellan gum-coated liposomes, 6.25% for chitosan-coated liposomes) were added to the cells and incubated for 4 h. Furthermore, the cells were washed three times with PBS and fixed with PFA in a biological safety cabinet. PFA was removed after 15 min and the coverslip was washed three times with PBS for 5 min. A drop of mounting medium with blue fluorescent DNA-stain (DAPI Fluoromount-G®) was placed on the coverslip and overturned to mount to the glass slide. The mounted slides were placed in a biological safety cabinet for 48 h before they were investigated further with confocal microscopy (HC PL APO CS2 40x/1.3 oil objective, 405 nm, and 470 nm) according to the manufacturer's protocol. All samples were produced in duplicates.

3. Results

3.1. Particle size and zeta potential

3.1.1. Uncoated liposomes

The freshly prepared uncoated liposome formulations showed similar particle size independent of lipid concentration and hydration medium. SoyPC/DOTAP liposomes ranged from 120 to 131 nm, the z-potential from 31.9 to 37.7 mV, and the PDI was 0.1. The size of SoyPC/EggPG liposomes ranged from 127 to 135 nm, the z-potential from −38.8 to −46.7 mV, and the PDI was 0.1.

3.1.2. Biopolymer-coated liposomes

The particle size for the biopolymer-coated liposomes ranged from 200 to 591 nm; the alginate-coated liposomes were the smallest, and the chitosan-coated liposomes the biggest (Table 1). The PDI ranged from 0.2 to 0.4 and the z-potential was reversed compared to the original charge of the uncoated liposomes in all formulations. Gellan gum-coated liposomes (6 mM) precipitated as visually observed immediately after coating in both hydration media, in contrast to the lowest concentration (3 mM), where no precipitation was seen.

The alginate-coated liposomes were the smallest in both hydration media and concentrations, with a two-fold increase compared to the uncoated liposomes. The chitosan- and gellan gum-coated liposomes demonstrated a three- to four- fold increase in size compared to the uncoated liposomes.

3.2. Formulation stability

3.2.1. Uncoated liposomes

All the negatively charged uncoated liposomes (SoyPC/DOTAP) remained stable in size at week 24. After 24 weeks the size decreased considerably for both formulations in G-PB (119 to 60 nm for 3 mM, and 126 to 13 nm for 6 mM). The formulations in PB remained steady for up to 44 weeks, but at week 60 the size was drastically increased for 3 mM (130 to 5988 nm) and decreased for 6 mM (133 to 14 nm).

All the positively charged uncoated liposomes (SoyPC/EggPG) remained stable in size at week 8, they showed a small decrease in size at week 25 – 26 (week 16 for 3 mM in G-PB), followed by a drastic increase. PDI and z-potential remained steady for all uncoated formulations for as long as the size was stable.

3.2.2. Coated liposomes

All three parameters (size, PDI and z-potential) of the two alginate-coated liposome formulations (3 mM and 6 mM) hydrated in PB, remained steady for up to 60 weeks (Fig. 2). This contrasts with the alginate-coated liposome formulations hydrated in G-PB. The 6 mM formulation had an increasing size trend from week one, and although

Table 1
Characterization of the biopolymer-coated liposomes. Size, polydispersity index (PDI) and z-potential of freshly prepared biopolymer-coated liposomes using two different lipid concentrations (3 mM and 6 mM) and hydration media (PB and G-PB). Results are presented as mean values ± standard deviation. Visually precipitated samples (PR) were not characterized. PB: Phosphate buffer. G-PB: Glycerol-phosphate buffer.

Lipids	Lipid concentration before coating	Polymer	Hydration medium	Size (nm)	PDI	Z-potential (mV)
SoyPC/DOTAP	3 mM	Alginate	PB	206 ± 7	0.20 ± 0.01	−49.5 ± 4.7
	6 mM			236 ± 3	0.24 ± 0.01	−51.3 ± 4.5
	3 mM		G-PB	200 ± 4	0.20 ± 0.01	−43.0 ± 0.1
	6 mM			253 ± 6	0.25 ± 0.00	−45.3 ± 0.1
SoyPC/DOTAP	3 mM	Gellan Gum	PB	423 ± 13	0.29 ± 0.01	−28.8 ± 0.1
	6 mM			PR	PR	PR
	3 mM		G-PB	415 ± 8	0.37 ± 0.06	−29.3 ± 0.2
	6 mM			PR	PR	PR
SoyPC/EggPG	3 mM	Chitosan	PB	454 ± 11	0.22 ± 0.00	17.3 ± 0.4
	6 mM			591 ± 23	0.25 ± 0.01	18.9 ± 0.1
	3 mM		G-PB	428 ± 16	0.29 ± 0.00	17.1 ± 0.3
	6 mM			448 ± 73	0.30 ± 0.04	19.2 ± 0.5

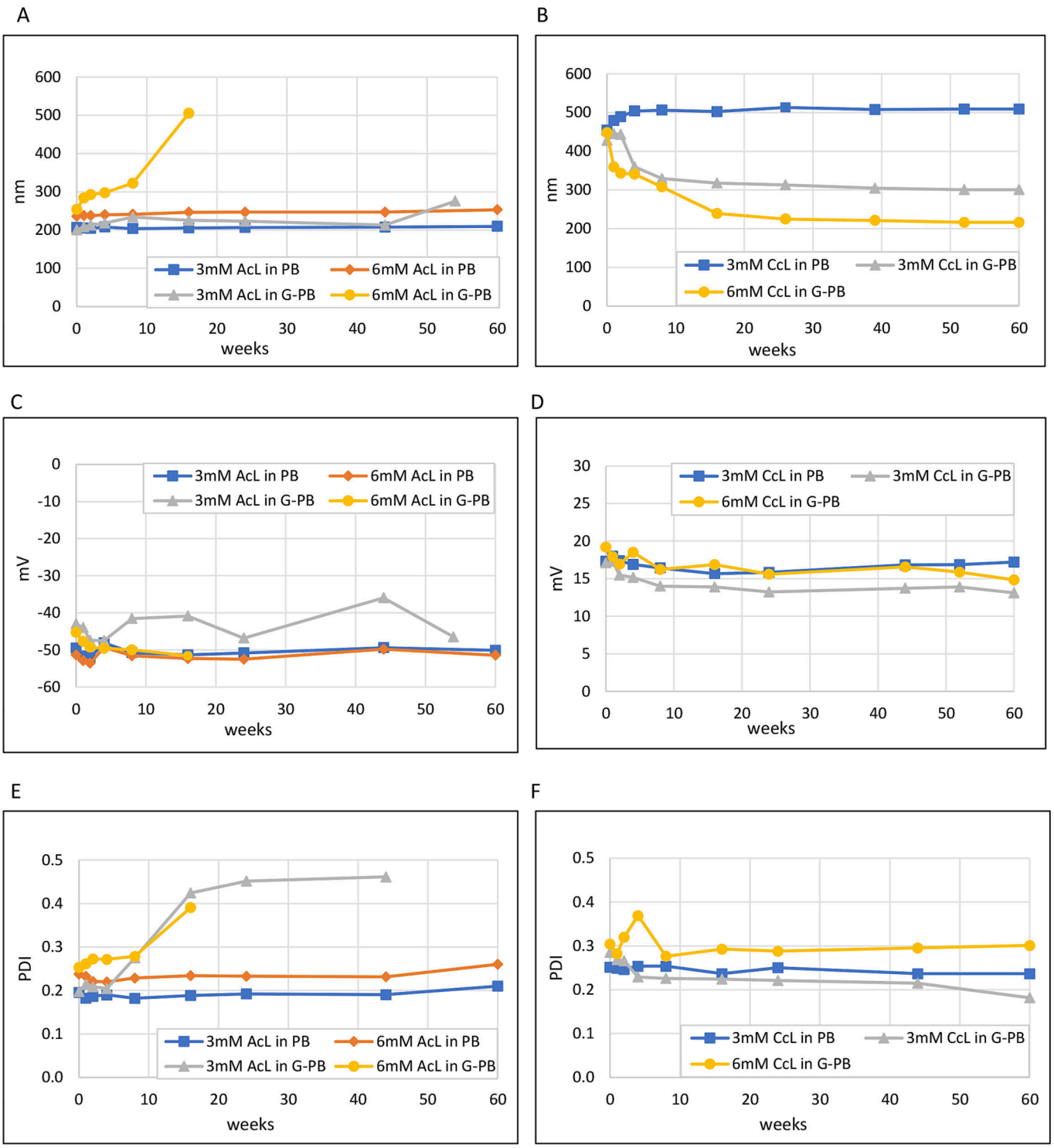


Fig. 2. Characterization of alginate- and chitosan-coated liposomes over time. Size (nm), z-potential (mV) and polydispersity index (PDI) of alginate-coated liposomes (AcL) (A, C, and E, respectively) and chitosan-coated liposomes (CcL) (B, D, and F, respectively). Measurements were terminated when the particle size and PDI were above the chosen threshold value. 3 mM and 6 mM: Lipid concentration before coating. PB: Phosphate buffer. G-PB: Glycerol phosphate buffer.

the 3 mM formulation showed a stable size up to week 44, both concentrations had an increase in PDI from 0.3 to 0.4 between week eight and week 16; consequently, these formulations were considered unstable and further measurements were not carried out.

The 3 mM gellan gum-coated liposomes in PB had a size of 445 nm and a PDI of 0.3 at week 16. The PDI increased to 0.4 in week 24, thus, further measurements were not performed. The 3 mM gellan gum-coated liposome formulation in G-PB had high PDI (0.4) at day of production, and since the PDI did not decrease during the two following two weeks, measurements were not continued (results not shown). The 6 mM gellan gum-coated liposome formulations (PB and G-PB) precipitated at day of production (observed visually); thus, no measurements were carried out.

The chitosan-coated liposomes, both 3 mM and 6 mM, in G-PB had a size reduction of 100 – 200 nm the first 8 to 16 weeks, followed by a period where the size remained stable for up to 60 weeks. The PDI

remained < 0.3 for 60 weeks, except from one measurement of 6 mM in G-PB in week four with PDI 0.4 (Fig. 2). The PDI remained < 0.3 for the lowest concentration of chitosan-coated liposomes in PB, although a size increase of approximately 50 nm was seen during the first four weeks, following a size stable period up to 60 weeks. Due to visual precipitation of the 6 mM samples in PB, measurements were discontinued after week one.

3.3. Cell viability and proliferation

Cell viability was ≥ 70% (relative to the control) after exposure to each of the biopolymer-coated liposome formulations (alginate, gellan gum and chitosan) at both 4 and 24 h (Fig. 3). Cells exposed to alginate-coated liposomes expressed the highest cell viability, closely followed by cells exposed to gellan gum-coated liposomes and chitosan-coated liposomes. Cell viability decreased for all formulations after 24 h compared to after 4 h.

DOK cells exposed to alginate-coated liposomes showed > 70% cell proliferation at 24 h compared to the control (except for 50% alginate-coated liposomes) (Fig. 4). The highest cell proliferation was seen for 12.5% liposome formulation. A small concentration-dependent increase in cell proliferation was detected at 24 h for concentrations from 1.56 to 25%, as opposed to 4 h where the cell proliferation was more alike. The highest concentration (50%) showed a reduced proliferation, corresponding to the control (without alginate-coated liposomes).

Cell proliferation after exposure to chitosan-coated liposomes was above 100% (compared to the control) for all concentrations (1.56 to 25%) except for cells exposed to 1.56% chitosan-coated liposomes at 24 h (Fig. 4). The highest cell proliferation was seen for 12.5% chitosan-coated liposomes. A small concentration dependent increase in cell proliferation was detected at 24 h for concentrations 1.56 to 12.5%. A dissimilarity in cell proliferation was seen between cells exposed to 25% chitosan-coated liposomes and cells exposed to the control (without chitosan-coated liposomes) in that a decline in cell proliferation was seen for the chitosan-coated liposomes at 4 h while an increase was seen at 24 h.

3.4. Cellular liposome uptake

The uptake of fluorescently labelled liposomes by DOK cells was studied by confocal microscopy. All formulations seemed to be incorporated into the cell cytoplasm, independent of biopolymer coating, surface charge, lipid concentration, and hydration media (Fig. 5). The images of fluorescence-labelled biopolymer-coated liposomes (green color) indicated that the liposomes were assembled in the cytoplasm and not in the nucleus (blue color). The controls showed the absence of autofluorescence in cells, cell media and hydration media.

4. Discussion

In this study we have explored the coating ability of alginate, gellan gum and chitosan to liposomes of two different lipid concentrations and hydration media and scrutinized their stability, cytotoxicity, and cell uptake. We have demonstrated that biopolymer-coated liposomes were successfully produced, that they had low cytotoxicity and were incorporated into DOK cells, and that five of the twelve formulations remained size stable for up to 60 weeks. These are important steps towards a more effective product to treat dry mouth, as the liposomal formulations may be presented in a range of topical applications such as a mouth spray, mouthwash or a gel.

4.1. Coating of the liposomes

According to the coating theory (McClements, 2005), there is an optimal concentration of polymer for optimal coating and by such increased stability. Previous studies have shown that for chitosan and alginate this concentration after coating lays between 0.05 w/w% and 0.1 w/w% when the lipid concentration is 3 mM (Pistone et al., 2017). Therefore 0.1 w/w% of coating concentration was chosen in the present study. The increase in size and size variation after coating liposomes with biopolymers were in accordance with these previous studies (Adamczak et al., 2016; Diebold et al., 2007; Klemetsrud et al., 2018), and is due to an augmented hydrodynamic volume when a biopolymer layer is covering the liposome surface. All formulations coated with the highest polymer concentration (0.250 w/w%) showed an increase in size compared to the lowest concentration (0.125 w/w%), suggesting a thicker coating layer of biopolymer on the surface of the liposomes. This is supported by the results of increased z-potential for the positively charged biopolymer-coated liposomes and decreased z-potential for the negatively charged biopolymer-coated liposomes, probably due to increased density of the specific biopolymer when increasing the concentration (Guo et al., 2003).

4.2. Stability

Stability in the system is essential for the quality and shelf life when developing a new product and is a well-known challenge in liposome production because the particles are susceptible to oxidation during storage, since they have a high tendency to aggregate and fuse due to natural random Brownian motions causing constant particle collisions (Maherani & Wattraint, 2017; Shah et al., 2015; U.S. Department of Health and Human Services 2018). We found that five of the 12 biopolymer-coated liposome formulations (3 mM and 6 mM chitosan-coated liposomes in G-PB, 3 mM chitosan-coated liposomes in PB, 3 and 6 mM alginate-coated liposomes in PB) demonstrated prolonged

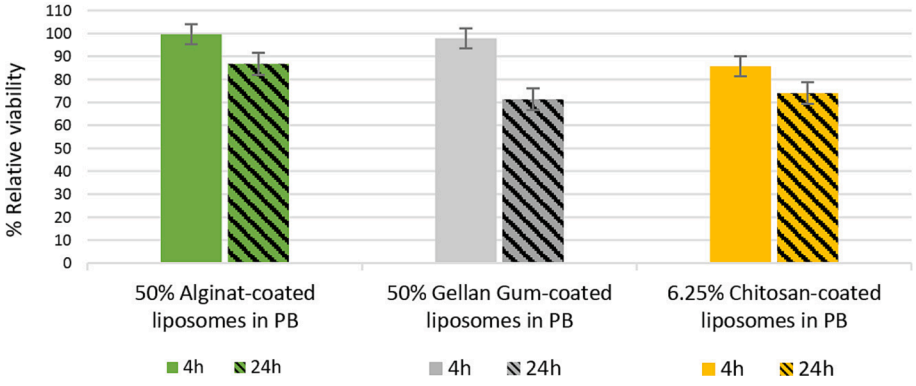


Fig. 3. Relative cell viability after exposure to biopolymer-coated liposomes. Relative viability of dysplastic oral keratinocytes (DOK cells) after 4 h and 24 h of exposure to biopolymer-coated liposomes diluted in cell media: 50% of 3 mM alginate-coated liposomes, 50% of 3 mM gellan gum-coated liposomes and 6.25% of 3 mM chitosan-coated liposomes, all hydrated in PB. Results presented as mean ± standard error of the mean (SEM) relative to control set to 100%. N = 3.

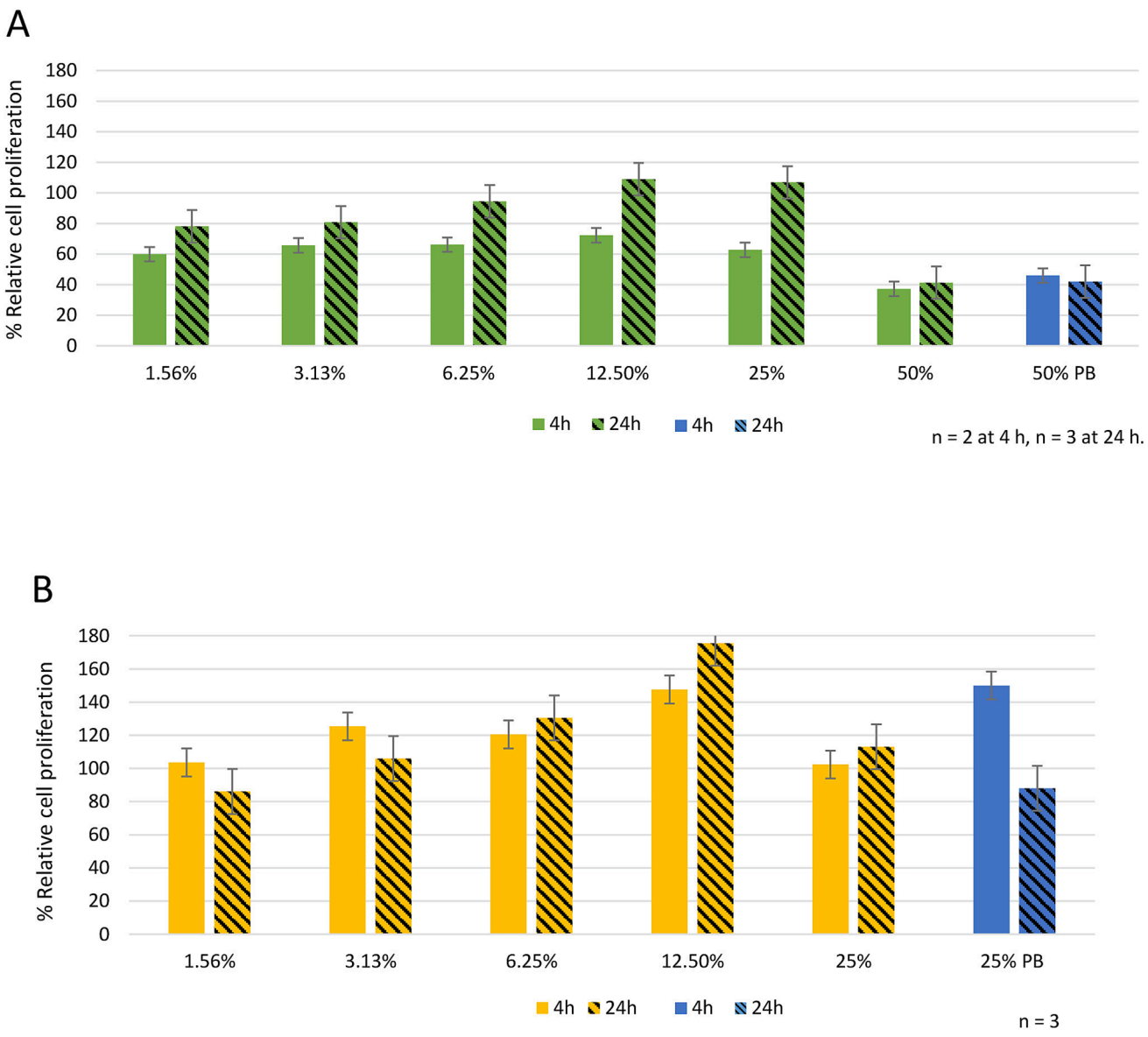


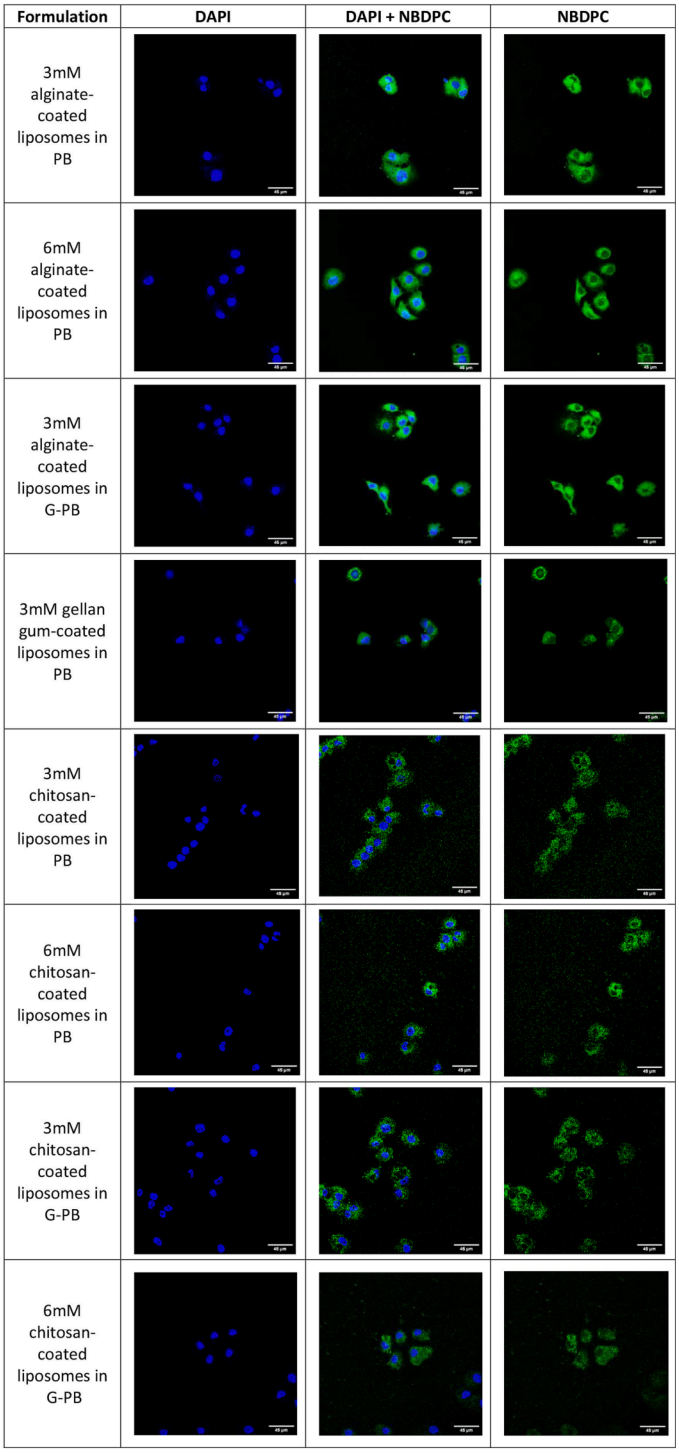
Fig. 4. Relative cell proliferation after exposure to biopolymer-coated liposomes. Relative cell proliferation of dysplastic oral keratinocytes (DOK cells) at 4 h and 24 h of exposure to alginate-coated liposomes of concentrations from 1.56% to 50% (A) and chitosan-coated liposomes of concentrations from 1.56% to 25% (B). Sample PB (phosphate buffer) is without liposomes. Results presented as mean ± standard error of the mean (SEM) relative to control set to 100%.

stability compared to the uncoated liposome formulations. This is in accordance with previous studies where alginate- and chitosan-coated liposomes demonstrated stable size and PDI for a longer period than the uncoated liposomes (Laye et al., 2008; Liu et al., 2016; Yang et al., 2013; Zamani Ghaleshahi & Rajabzadeh, 2020). In our study, the biopolymer-coated liposomes seemed to be protected from hydrolysis but were more susceptible to aggregation, as a size increase was seen rather than a decrease as the system turned unstable. A suggestion may be that the natural collisions caused the particles to stick to each other, thus turning the system thermodynamically unstable and more susceptible to aggregation. For the uncoated liposomes, however, a size reduction, followed by an increase for SoyPC/DOTAP was seen, suggesting that the uncoated liposomes underwent a structural rearrangement from lamellar to a micellar system due to hydrolysis (Zuidam et al., 1995). The stabilizing properties of alginate- and chitosan-coating seen in our study make these formulations highly promising in further development of a more effective product to treat dry mouth.

Alginate-coated liposomes hydrated in PB were stable in size for a longer period compared to when hydrated in G-PB. This may be due to the difference in z-potential, as a lower z-potential was detected for the alginate-coated liposomes in PB than in G-PB. A lower (or higher) electrical charge may prevent aggregation because of the repellent forces among the particles (Honary & Zahir, 2013; Nemeth et al., 2022), but other factors may also have affected the system such as the addition of glycerol, since glycerol is affecting the ability to form hydrogen bonds (Nie et al., 2008). Thus, this should be explored in further studies. To our knowledge, no other studies have scrutinized the characteristics of biopolymer-coated liposomes in G-PB over time, but Manca et al. found that diclofenac-loaded liposomes were stable longer than the unloaded liposomes when hydrated in increasing glycerol concentrations (Manca et al., 2013). The results were explained by the difference in z-potential, as the unloaded liposomes were close to zero, and the loaded liposomes were more negative (Manca et al., 2013).

Gellan gum-coated liposomes seemed to aggregate very fast as both (PB and G-PB) 6 mM formulations precipitated at day of production, and the PDI and size increased the first weeks for both 3 mM formulations. This may be due to instability in the system from early on as the particle size after coating was twice the size compared to the alginate-coated

A



B

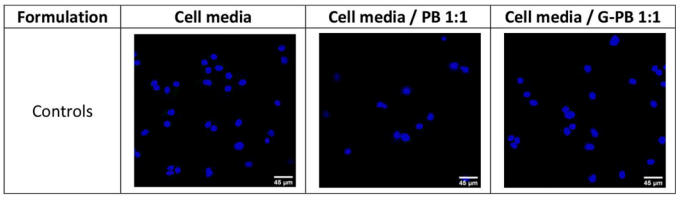


Fig. 5. Confocal microscopic imaging demonstrating cellular uptake of fluorescent-labelled biopolymer-coated liposomes. Fluorescence confocal microscopic images demonstrating uptake of fluorescent-labelled liposomes coated with biopolymers alginate, gellan gum or chitosan, with variable lipid concentrations (3 mM and 6 mM) and hydration media (PB and G-PB) (A). Fluorescence confocal microscopic images of negative controls demonstrating the absence of autofluorescence (B). DAPI (blue): fluorescent DNA-stain indicating nucleus. NBDPC (green): fluorescent lipid indicating liposomes. PB: phosphate buffer. G-PB: glycerol phosphate buffer. Scale bar = 45 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

liposomes and may suggest that the biopolymer coating was less firmly attached, thus triggering aggregation. The molecular weight of gellan gum is higher than both alginate and chitosan and due to the natural structure of gellan gum with long polymer chains, it can be assumed that only parts of the chains bind to the liposome surface, resulting in loose ends and loops reaching out of the surface area and encouraging bridging and aggregation.

Despite relatively low surface charge, the chitosan-coated liposomes seemed to avoid aggregation in both hydration media, except for 6 mM in G-PB where visual precipitation was seen at day of production. Although the size of the chitosan-coated liposomes was comparable to the gellan gum-coated liposomes, the chain length of the chitosan molecule is much shorter, hence the coating of the liposome may be more compact, with fewer polymer chains protruding from the liposome surface. Chitosan-coated liposomes demonstrated a size reduction of 100–200 nm in G-PB the first weeks before it stabilized, possibly due to rearrangement of the three-dimensional network to a more compact structure surrounding the liposome, or chitosan chains with poor binding falling off during the first weeks due to an overload of chitosan chains in multiple layers. Nonetheless, a thick adsorptive layer has been shown to keep the particles apart and lead to stabilization (Guo et al., 2013).

4.3. Cell studies

During the preliminary cell studies, two limitations were observed: Firstly, aggregation in the cell medium and adherence to plastic and glass surfaces were seen when chitosan-coated liposome formulations were tested in 50% concentration with cell culture medium. The aggregation was probably due the neutral pH of the cell medium since chitosan precipitates at pH $>$ 6. The adherence of chitosan-coated liposomes to plastic and glass surfaces may be due to the natural positive charge of chitosan, which attracts to negatively charged surfaces such as glass and plastic. These problems were solved by lowering the concentration of chitosan-coated liposomes in cell medium to 6.25% for all the cell work in our study.

Secondly, the formulations hydrated in G-PB had a very high osmolality compared to the cell medium, 1310 mOsm vs. 291 mOsm (50% formulation/cell medium vs. 100% cell medium, respectively). The G-PB exposed cells appeared normal at visual inspection after the incubation period, but after de-attaching the cells from the culture wells/flasks by trypsinization, the cells visually vanished and the cell counter results indicated complete cell death. Further investigation was not performed in the present work, but a previous study found that the ability of cells to withstand hyperosmotic environment was anchorage-dependent, and that cells in suspension were much more sensitive to osmotic conditions compared to cells in monolayers (Mironescu, 1977). It can be hypothesized that when the DOK cells in monolayers were trypsinated into suspension, the hyperosmotic environment became too harsh and led to apoptosis of the cells (Clouzeau et al., 2012; Terada et al., 2001). To overcome this problem, only the formulations hydrated in PB were evaluated for cell viability and cell proliferation, as glycerol is already well-known as a non-toxic substance widely used as food additive as well as in cosmetics and oral products (Archives and Code, 2023; National Center for Biotechnology Information 2023).

A clinical relevant concentration of the formulation was chosen for the *in vitro* studies based on recommendations from the Biological Evaluation of Medical Devices (ISO 10993–5) (ISO, 2009). Although the relatively high concentration applied caused some technical difficulties for the chitosan-coated liposomes, the concentration was maintained for alginate- and gellan gum-coated liposomes to meet the clinically relevant concentration in patients with extreme hyposalivation. Regardless of the challenges mentioned above, all three formulations showed $<$ 30% reduction in cell viability after 24 h, which is considered a non-cytotoxic effect by the ISO 10993–5 (ISO, 2009). An over-proliferation was seen for the chitosan-coated liposomes and for the PB sample and may be due to that more cells were seeded in the specific wells compared to the control wells but may also indicate that the formulation contained substances that led to an increase in metabolic activity. At any rate, no cytotoxic effect was detected for neither alginate-coated liposomes nor chitosan-coated liposomes as both formulations showed comparable cell proliferation results as the PB control (with no biopolymer-coated liposomes).

The cell uptake studies showed no obvious difference in cell interaction between the biopolymer-coated liposomes, despite their different size and surface charge. This is in accordance with previous studies where liposomes of positively, negatively, and neutral charge showed cellular uptake within minutes, in both cancer cells and normal cells (Kang et al., 2017). The mechanism of cellular uptake has previously been identified as via endocytosis, while the specific endocytic pathway seems to differentiate between cell type, particle size and surface charge (Düzgüneş & Nir, 1999; Fröhlich, 2012; Kang et al., 2017).

5. Conclusion

In summary, we successfully coated liposomes with alginate, gellan gum and chitosan using two different lipid concentrations and hydration media. Alginate- and chitosan-coated liposomes demonstrated good stability over time. Alginate was most stable in phosphate buffer, while chitosan was most stable in glycerol phosphate buffer. All three formulations demonstrated uniform cell uptake and low cytotoxicity and were considered safe. Alginate- and chitosan-coated liposomes may be promising agents for use in the oral cavity and should be investigated further.

Formatting of founding sources

This work was supported by the University of Oslo, Norway.

CRediT authorship contribution statement

Julie Frigaard: Investigation, Visualization, Writing – original draft. **Janicke Liaaen Jensen:** Conceptualization, Writing – review & editing, Supervision, Project administration. **Hilde Kanli Galtung:** Conceptualization, Methodology, Validation, Resources, Writing – review & editing, Supervision, Project administration. **Marianne Hiorth:** Conceptualization, Methodology, Validation, Resources, Writing – review & editing, Supervision, Project administration.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgement

We thank Head Engineer Tove Larsen at the Section for Pharmaceutics and Social Pharmacy, Department of Pharmacy, Faculty of Mathematics and Natural Sciences, and the Head Engineer Ann-Kristin Ruus at the Institute of Oral Biology, Faculty of Dentistry, both at the University of Oslo, Norway, for professional support and training. We also thank professor Dipak Sapkota at the Institute of Oral Biology, Faculty of Dentistry, University of Oslo, Norway, for the generous donation of DOK cells and the essential instructions that enabled the *in vitro* experiments.

We thank the Core Facility for Advanced Light Microscopy, Faculty of Medicine, University of Oslo, Norway, for technical support.

References

Adamczak, M.I., Hagesaether, E., Smistad, G., Hiorth, M., 2016. An in vitro study of mucoadhesion and biocompatibility of polymer coated liposomes on HT29-MTX mucus-producing cells. Int. J. Pharm. 498 (1), 225–233. https://doi.org/10.1016/j.ijpharm.2015.12.030.

Adamczak, M.I., Martinsen, Ø.G., Smistad, G., Hiorth, M., 2017. Polymer coated mucoadhesive liposomes intended for the management of xerostomia. Int. J. Pharm. 527 (1), 72–78. https://doi.org/10.1016/j.ijpharm.2017.05.032.

Agostini, B.A., Cericato, G.O., Silveira, E.R.d., Nascimento, G.G., Costa, F.D.S., Thomson, W.M., Demarco, F.F., 2018. How Common is Dry Mouth? Systematic Review and Meta-Regression Analysis of Prevalence Estimates. Braz. Dent. J. 29 (6), 606–618.

National Archives - Code of Federal Regulations *eCFR - 21 CFR 182.1320 - Substances Generally Recognized as Safe*. Retrieved 04 June 2023 from https://www.ecfr.gov/current/title-21/chapter-I/subchapter-B/part-182/subpart-B/section-182.1320.

Bangham, A.D., 1963. Physical structure and behavior of lipids and lipid enzymes. Adv. Lipid Res. 1, 65–104.

Borgnakke, W.S., 2019. IDF Diabetes Atlas: Diabetes and oral health – A two-way relationship of clinical importance. Diabetes Res. Clin. Pract. 157, 107839. https://doi.org/10.1016/j.diabres.2019.107839.

Bozzuto, G., Molinari, A., 2015. Liposomes as nanomedical devices. Int. J. Nanomed. 10, 975–999. https://doi.org/10.2147/IJN.S68861.

Bulbake, U., Doppalapudi, S., Kommineni, N., Khan, W., 2017. Liposomal Formulations in Clinical Use: An Updated Review. Pharmaceutics 9 (2), 12. https://doi.org/10.3390/pharmaceutics9020012.

Carpenter, G., 2015. Introduction. In: Carpenter, G. (Ed.), Dry Mouth: A ClinicAl Guide on CAuses, Effects and TreAtments. Springer, Berlin Heidelberg, pp. 3–4. https://doi.org/10.1007/978-3-642-55154-3_1.

Cassanelli, M., Prosapio, V., Norton, I., Mills, T., 2018. Acidified/basified gellan gum gels: The role of the structure in drying/rehydration mechanisms. Food Hydrocoll. 82, 346–354. https://doi.org/10.1016/j.foodhyd.2018.04.024.

Clouzeau, C., Godefroy, D., Riancho, L., Rostène, W., Baudouin, C., Brignole-Baudouin, F., 2012. Hyperosmolarity potentiates toxic effects of benzalkonium chloride on conjunctival epithelial cells in vitro. Mol. Vis. 18 (90–91), 851–863.

Connor, N.P., Cohen, S.B., Kammer, R.E., Sullivan, P.A., Brewer, K.A., Hong, T.S., Chappell, R.J., Harari, P.M., 2006. Impact of conventional radiotherapy on health-related quality of life and critical functions of the head and neck. Int. J. Radiat. Oncol. Biol. Phys. 65 (4), 1051–1062. https://doi.org/10.1016/j.ijrobp.2006.01.054.

Danaei, M., Dehghankhold, M., Ataei, S., Hasanzadeh Davarani, F., Javanmard, R., Dokhani, A., Khorasani, S., Mozafari, M.R., 2018. Impact of particle size and polydispersity index on the clinical applications of lipidic nanocarrier systems. Pharmaceutics 10 (2), 57. https://doi.org/10.3390/pharmaceutics10020057.

Dhanka, M., Shetty, C., Srivastava, R., 2018. Methotrexate loaded gellan gum microparticles for drug delivery. Int. J. Biol. Macromol. 110, 346–356. https://doi.org/10.1016/j.ijbiomac.2017.12.026.

Diebold, Y., Jarrín, M., Sáez, V., Carvalho, E.L.S., Orea, M., Calonge, M., Seijo, B., Alonso, M.J., 2007. Ocular drug delivery by liposome–chitosan nanoparticle complexes (LCS-NP). Biomaterials 28 (8), 1553–1564. https://doi.org/10.1016/j.biomaterials.2006.11.028.

Draget, K.I., Skjåk Bræk, G., Smidsrød, O., 1994. Alginic acid gels: the effect of alginate chemical composition and molecular weight. Carbohydr. Polym. 25 (1), 31–38. https://doi.org/10.1016/0144-8617(94)90159-7.

Düzgüneş, N., Nir, S., 1999. Mechanisms and kinetics of liposome–cell interactions. Adv. Drug Deliv. Rev. 40 (1), 3–18. https://doi.org/10.1016/S0169-409X(99)00037-X.

Dynesen, A.W., 2015. Oral Dryness, Dietary Intake, and Alterations in Taste. In: Carpenter, G. (Ed.), Dry Mouth: A ClinicAl Guide on CAuses, Effects and TreAtments. Springer, Berlin Heidelberg, pp. 69–80. https://doi.org/10.1007/978-3-642-55154-3_5.

Frigaard, J., Jensen, J.L., Galtung, H.K., Hiort, M., 2022. The Potential of Chitosan in Nanomedicine: An Overview of the Cytotoxicity of Chitosan Based Nanoparticles. Front. Pharmacol. 13, 880377. https://doi.org/10.3389/fphar.2022.880377.

Fröhlich, E., 2012. The role of surface charge in cellular uptake and cytotoxicity of medical nanoparticles. Int. J. Nanomed. 7, 5577–5591. https://doi.org/10.2147/IJN.S36111.

Gil-Montoya, J.A., Silvestre, F.J., Barrios, R., Silvestre-Rangil, J., 2016. Treatment of xerostomia and hyposalivation in the elderly: A systematic review. Medicina oral, patologia oral y cirugia bucal 21 (3), e355–e366. https://doi.org/10.4317/medoral.20969.

Guo, J., Ping, Q., Jiang, G., Huang, L., Tong, Y., 2003. Chitosan-coated liposomes: characterization and interaction with leuprolide. Int. J. Pharm. 260 (2), 167–173. https://doi.org/10.1016/S0378-5173(03)00254-0.

Guo, M., Rong, W.T., Hou, J., Wang, D.F., Lu, Y., Wang, Y., Yu, S.Q., Xu, Q., 2013. Mechanisms of chitosan-coated poly(lactic-co-glycolic acid) nanoparticles for improving oral absorption of 7-ethyl-10-hydroxycamptothecin. Nanotechnology 24 (24), 245101. https://doi.org/10.1088/0957-4484/24/24/245101.

Hahnel, S., Schwarz, S., Zeman, F., Schäfer, L., Behr, M., 2014. Prevalence of xerostomia and hyposalivation and their association with quality of life in elderly patients in dependence on dental status and prosthetic rehabilitation: A pilot study. J. Dent. 42 (6), 664–670. https://doi.org/10.1016/j.jdent.2014.03.003.

Hiorth, M., Mihailovic, L., Adamczak, M., Goycoolea, F.M., Sarkar, A., 2023. Lubricating Performance of Polymer-Coated Liposomes. Lubricating Performance of Polymer-Coated Liposomes. Biotribology (Oxford) 35-36, 100239.

Honary, S., Zahir, F., 2013. Effect of zeta potential on the properties of nano-drug delivery systems - A review (Part 2). Trop. J. Pharm. Res. 12 (2), 265–273. https://doi.org/10.4314/tjpr.v12i2.20.

International Organization for Standardization (ISO) (2009). ISO 10993-5:2009 Biological evaluation of medical devices. In *Part 5 Tests for in vitro cytotoxicity*.

Johansson, A.-K., Johansson, A., Unell, L., Ekbäck, G., Ordell, S., Carlsson, G.E., 2020. Self-reported dry mouth in 50- to 80-year-old Swedes: Longitudinal and cross-sectional population studies. J. Oral Rehabil. 47 (2), 246–254.

Kang, J.H., Jang, W.Y., Ko, Y.T., 2017. The Effect of Surface Charges on the Cellular Uptake of Liposomes Investigated by Live Cell Imaging. Pharm. Res. 34 (4), 704–717. https://doi.org/10.1007/s11095-017-2097-3.

Karn, P.R., Vani, Z., Pepi, I., Škalko-Basnet, N., 2011. Mucoadhesive liposomal delivery systems: the choice of coating material. Drug Dev. Ind. Pharm. 37 (4), 482–488. https://doi.org/10.3109/03639045.2010.523425.

Klemetsrud, T., Kjøniksen, A.-L., Hiorth, M., Jacobsen, J., Smistad, G., 2018. Polymer coated liposomes for use in the oral cavity – a study of the in vitro toxicity, effect on cell permeability and interaction with mucin. J. Liposome Res. 28 (1), 62–73. https://doi.org/10.1080/08982104.2016.1255640.

Kockisch, S., Rees, G.D., Tsibouklis, J., Smart, J.D., 2005. Mucoadhesive, triclosan-loaded polymer microspheres for application to the oral cavity: preparation and controlled release characteristics. Eur. J. Pharm. Biopharm. 59 (1), 207–216. https://doi.org/10.1016/j.ejpb.2004.07.007.

Laye, C., McClements, D.J., Weiss, J., 2008. Formation of Biopolymer-Coated Liposomes by Electrostatic Deposition of Chitosan. J. Food Sci. 73 (5), N7–N15. https://doi.org/10.1111/j.1750-3841.2008.00747.x.

Lee, K.Y., Mooney, D.J., 2012. Alginate: Properties and biomedical applications. Prog. Polym. Sci. 37 (1), 106–126. https://doi.org/10.1016/j.progpolymsci.2011.06.003.

Liu, B., Dion, M.R., Jurasic, M.M., Gibson, G., Jones, J.A., 2012. Xerostomia and salivary hypofunction in vulnerable elders: prevalence and etiology. Oral Surg Oral Med Oral Pathol Oral Radiol 114 (1), 52–60. https://doi.org/10.1016/j.oooo.2011.11.014.

Liu, P., Krishnan, T.R., 1999. Alginate-pectin-poly-L-lysine particulate as a potential controlled release formulation. J. Pharm. Pharmacol. 51 (2), 141–149. https://doi.org/10.1211/0022357991772259.

Liu, W., Liu, W., Ye, A., Peng, S., Wei, F., Liu, C., Han, J., 2016. Environmental stress stability of microencapsules based on liposomes decorated with chitosan and sodium alginate. Food Chem. 196, 396–404. https://doi.org/10.1016/j.foodchem.2015.09.050.

Maherani, B., Wattraint, O., 2017. Liposomal structure: A comparative study on light scattering and chromatography techniques. J. Dispers. Sci. Technol. 38 (11), 1633–1639. https://doi.org/10.1080/01932691.2016.1269651.

Manca, M.L., Zaru, M., Manconi, M., Lai, F., Valenti, D., Sinico, C., Fadda, A.M., 2013. Glycerosomes: A new tool for effective dermal and transdermal drug delivery. Int. J. Pharm. 455 (1), 66–74. https://doi.org/10.1016/j.ijpharm.2013.07.060.

McClements, D.J., 2005. Theoretical Analysis of Factors Affecting the Formation and Stability of Multilayered Colloidal Dispersions. Langmuir 21 (21), 9777–9785. https://doi.org/10.1021/la0512603.

Mironescu, S., 1977. Hyperosmotic injury in mammalian cells 1. Survival of CHO cells in unprotected and DMSO-treated cultures. Cryobiology 14 (4), 451–465. https://doi.org/10.1016/0011-2240(77)90007-4.

Mohammed, M.A., Syeda, J.T.M., Wasan, K.M., Wasan, E.K., 2017. An Overview of Chitosan Nanoparticles and Its Application in Non-Parenteral Drug Delivery [Review]. Pharmaceutics 9 (4), 20. https://doi.org/10.3390/pharmaceutics9040053.

National Center for Biotechnology Information, 2023. PubChem Compound Summary for CID 753, Glycerin. Retrieved 4 June 2023 from. https://pubchem.ncbi.nlm.nih.gov/compound/Glycerol.

Nederfors, T., Isaksson, R., Mörnstad, H., Dahlöf, C., 1997. Prevalence of perceived symptoms of dry mouth in an adult Swedish population - relation to age, sex and pharmacotherapy. Commun. Dent. Oral Epidemiol. 25 (3), 211–216. https://doi.org/10.1111/j.1600-0528.1997.tb00928.x.

Nemeth, Z., Csoka, I., Jazani, R.S., Sipos, B., Haspel, H., Kozma, G., Konya, Z., Dobo, D. G., 2022. Quality by Design-Driven Zeta Potential Optimisation Study of Liposomes with Charge Imparting Membrane Additives. Pharmaceutics 14 (9), 1798. https://doi.org/10.3390/pharmaceutics14091798.

Ngo, D.Y.J., Thomson, W.M., Nolan, A., Ferguson, S., 2016. The lived experience of Sjögren's Syndrome. BMC Oral Health 16 (1), 7. https://doi.org/10.1186/s12903-016-0165-4.

Nguyen, S., Alund, S.J., Hiorth, M., Kjøniksen, A.-L., Smistad, G., 2011. Studies on pectin coating of liposomes for drug delivery. Colloids Surf. B Biointerfaces 88 (2), 664–673. https://doi.org/10.1016/j.colsurfb.2011.07.058.

Nie, H., He, A., Zheng, J., Xu, S., Li, J., Han, C.C., 2008. Effects of Chain Conformation and Entanglement on the Electrospinning of Pure Alginate. Biomacromolecules 9 (5), 1362–1365. https://doi.org/10.1021/bm701349j.

Osmałek, T., Froelich, A., Tasarek, S., 2014. Application of gellan gum in pharmacy and medicine. Int. J. Pharm. 466 (1–2), 328–340. https://doi.org/10.1016/j.ijpharm.2014.03.038.

Pistone, S., Qoragllu, D., Smistad, G., Hiorth, M., 2015. Formulation and preparation of stable cross-linked alginate–zinc nanoparticles in the presence of a monovalent salt [10.1039/C5SM00700C]. Soft Matter 11 (28), 5765–5774. https://doi.org/10.1039/C5SM00700C.

Pistone, S., Rykke, M., Smistad, G., Hiorth, M., 2017. Polysaccharide-coated liposomal formulations for dental targeting. Int. J. Pharm. 516 (1), 106–115. https://doi.org/10.1016/j.ijpharm.2016.11.028.

Shah, R., Eldridge, D., Palombo, E., Harding, I., 2015. Lipid Nanoparticles: Production, Characterization and Stability, (1st ed.). Springer International Publishing : Imprint: Springer.

Tan, E.C.K., Lexomboon, D., Sandborgh-Englund, G., Haasum, Y., Johnell, K., 2018. Medications That Cause Dry Mouth As an Adverse Effect in Older People: A Systematic Review and Metaanalysis. J. Am. Geriatr. Soc. 66 (1), 76–84. https://doi.org/10.1111/jgs.15151.

Tentor, F., Siccardi, G., Sacco, P., Demarchi, D., Marsich, E., Almdal, K., Bose Goswami, S., Boisen, A., 2020. Long lasting mucoadhesive membrane based on alginate and chitosan for intravaginal drug delivery. J. Mater. Sci. - Mater. Med. 31 (3), 25. https://doi.org/10.1007/s10856-020-6359-y.

Terada, Y., Inoshita, S., Hanada, S., Shimamura, H., Kuwahara, M., Ogawa, W., Kasuga, M., Sasaki, S., Marumo, F., 2001. Hyperosmolality activates Akt and regulates apoptosis in renal tubular cells. Kidney Int. 60 (2), 553–567. https://doi.org/10.1046/j.1523-1755.2001.060002553.x.

Tiisanoja, A., Syrjälä, A.M., Komulainen, K., Lampela, P., Hartikainen, S., Taipale, H., Knuuttila, M., Ylöstalo, P., 2018. Anticholinergic burden and dry mouth among Finnish, community-dwelling older adults. Gerodontology 35 (1), 3–10. https://doi.org/10.1111/ger.12304.

Tsibouklis, J., Middleton, A.M., Patel, N., Pratten, J., 2013. Toward mucoadhesive hydrogel formulations for the management of xerostomia: The physicochemical, Biological, and Pharmacological Considerations. J. Biomed. Mater. Res. A 101 (11), 3327–3338. https://doi.org/10.1002/jbm.a.34626.

U.S. Department of Health and Human Services (2018). *Liposome Drug Products; chemistry, manufacturing and controls; human pharmacokinetics and bioavailability; labeling documentation - Guidance for industry* (FDA-2016-D-2817). https://www.fda.gov/media/70837/download.

Vinke, J., Kaper, H.J., Vissink, A., Sharma, P.K., 2020. Dry mouth: saliva substitutes which adsorb and modify existing salivary condition films improve oral lubrication (vol 15, pg 318, 2020). Clin. Oral Invest. 24 (11), 4019–4030. https://doi.org/10.1007/s00784-020-03272-x.

Westgaard, K.L., Hynne, H., Amdal, C.D., Young, A., Singh, P.B., Chen, X., Rykke, M., Hove, L.H., Aqrawi, L.A., Utheim, T.P., Herlofson, B.B., Jensen, J.L., 2021. Oral and ocular late effects in head and neck cancer patients treated with radiotherapy. Sci. Rep. 11 (1), 4026. https://doi.org/10.1038/s41598-021-83635-w.

Yang, K.K., Kong, M., Wei, Y.N., Liu, Y., Cheng, X.J., Li, J., Park, H.J., Chen, X.G., 2013. Folate-modified–chitosan-coated liposomes for tumor-targeted drug delivery. J. Mater. Sci. 48 (4), 1717–1728. https://doi.org/10.1007/s10853-012-6930-0.

Zamani Ghaleshahi, A., Rajabzadeh, G., 2020. The influence of sodium alginate and genipin on physico-chemical properties and stability of WPI coated liposomes. Food Res. Int. 130, 108966. https://doi.org/10.1016/j.foodres.2019.108966.

Zuidam, N.J., Gouw, H.K.M.E., Barenholz, Y., Crommelin, D.J.A., 1995. Physical (in) stability of liposomes upon chemical hydrolysis: the role of lysophospholipids and fatty acids. BBA-Biomembranes 1240 (1), 101–110. https://doi.org/10.1016/0005-2736(95)00180-5.