An *in vitro* study of mucoadhesion and biocompatibility of polymer coated liposomes on HT29-MTX mucus-producing cells

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Abstract

Drug delivery to the oral cavity poses a significant challenge due to the short residence time of the formulations at the site of action. From this point of view, nanoparticulate drug delivery systems with ability to adhere to the oral mucosa are advantageous as they could increase the effectiveness of the therapy. Positively, negatively and neutrally charged liposomes were coated with four different types of polymers: alginate, low-ester pectin, chitosan and hydrophobically modified ethyl hydroxyethyl cellulose. The mucoadhesion was studied using a novel *in vitro* method allowing the liposomes to interact with a mucus-producing confluent HT29-MTX cell-line without applying any external force. MTT viability and paracellular permeability tests were conducted on the same cell-line. The alginate-coated liposomes achieved a high specific (genuine) mucin interaction, with a low potential of cell-irritation. The positively charged uncoated liposomes achieved the highest initial mucoadhesion, but also displayed a higher probability of cell-irritation. The chitosan-coated liposomes displayed the

highest potential for long lasting mucoadhesion, but with the drawback of a higher general adhesion (tack) and a higher potential for irritating the cells.

Keywords: liposomes, polymers, mucoadhesion, MTT viability test, paracellular permeation, HT29-MTX cells

1. Introduction

Liposomes are sphere-shaped, nanosized vesicles consisting of an aqueous core surrounded by one or several phospholipid bilayers. Possibility of encapsulation of active ingredient within liposomes offers many advantages for pharmaceutical formulations and in drug delivery sciences. Liposomes can protect the active component from *in vivo* degradation, regulate release rate and reduce the toxicity of the encapsulated drug (Han et al., 2012). Liposomes are composed of various lipids and can additionally be coated with different types of polymers, which introduces a great degree of flexibility to the properties of the obtained nanocarriers (Alund et al., 2013; Smistad et al., 2012). A commercially successful example of a coated liposome formulation is Caelyx®; an intravenous injection containing doxorubicin for cancer treatment.

In the case of mucosal drug delivery, liposomes can also prove to be particularly useful. There are several formulations intended for nasal, ocular, pulmonary or vaginal delivery (Berginc et al., 2014; Heurtault et al., 2010; Huang and Wang, 2006; Jain and Shastri, 2011). The buccal mucosa has emerged as a promising administration site due to its accessibility and low enzymatic degradation avoiding first pass metabolism. As most mucosal membranes, the buccosa is constantly rinsed for protection purposes. Therefore, increasing the contact time by introducing mucoadhesive properties to the liposomes can be crucial for achieving an acceptable bioavailability. Also in the case of local treatment of diseases such as inflammation, infections or dryness, achieving a long lasting effect by combining sustained drug delivery with mucoadhesion should be advantageous.

Xerostomia, also referred to as dry mouth syndrome, is a condition caused by for example age-related salivary gland dysfunction, radiotherapy treatment or side effects of medication (Alimi, 2015;

Liu et al., 2012). Xerostomia is a bothersome condition which increases the susceptibility to candida infections, dental caries and tooth wear. In addition, it contributes to the difficulties in chewing, swallowing and speaking. Liposomes could have a potential in relieving the symptoms of dry mouth due to the presence of an aqueous compartment, which is expected to slowly release the hydration medium and thus provide prolonged moisture protection. The liposomes should then adhere to the mucosa, and in addition the toxicity must be low.

Monolayers of HT29-MTX cells have previously been used as a permeability model for studying the effect of mucus on the permeation of drugs (Hagesaether et al., 2013; Pontier et al., 2001). A co-culture of Caco-2 and HT29-MTX cells has been suggested as more realistic in-vitro models of the human intestine, due to mucus secretion and adjustable paracellular and P-gp mediated transport (Antoine et al., 2015; Woitski et al., 2011).

In this work of Antoine et al., the apical mucus barrier was also cell protective. This is relevant since the *in vitro* tests for studying toxicity have been found to be overly sensitive, and often poorly correlate with the *in vivo* situation (Czajkowska-Kosnik et al., 2014). The toxicity of poly(isobutyl cyanoacrylate) nanoparticles has recently been found to be highly cell line-dependent: a lower toxicity was reported using human fully-differentiated enterocyte-like Caco-2/TC7, and fully-differentiated mucus-secreting HT-29/MTX cells forming monolayer in culture, compared to undifferentiated human cervix epithelial HeLa cells. This was attributed to a resistance against internalization by the robust monolayers by tight assembly of polarized cells, mimicking the intestinal epithelial barrier (Pradines et al., 2015).

The aim of this study was to measure the mucoadhesive properties and toxicity of seven different types of uncoated and polymer-coated liposomes, possessing positive, negative or neutral charge. The following polymers were used for coating: alginate and low-ester pectin (both hydrophilic and negatively charged), chitosan (hydrophilic and positively charged) and hydrophobically modified ethyl hydroxyethyl cellulose (amphiphilic and neutrally charged).

A novel *in vitro* method for measuring the mucoadhesion was used. Liposomes diffusing freely in the samples were allowed to interact spontaneously with a confluent mucin-producing HT29-MTX cell monolayer. The amount of liposomes adhering was subsequently detected both directly on the cell monolayer and indirectly by measuring the difference in liposome concentration before and after incubation and rinsing. No external force was applied for attachment purposes, and no detachment force was applied, only gentle rinsing. We believe that this mimics the *in vivo* situation better than for example texture analyzers.

The same cell-line was used to assess the potential toxicity of the liposomes. The MTT viability test was conducted on a confluent mucus-secreting monolayer simulating the *in vivo* situation and on a diluted cell sample in exponential growth phase without mucus. In addition, the paracellular permeation of the hydrophilic marker carboxyfluorescein was measured on the confluent cell-layer.

2. Materials and methods

2.1. Materials

The main lipid, soybean phosphatidylcholine (SoyaPC), was a kind gift from Lipoid GmbH (Ludwigshafen, Germany). The anionic lipid phosphatidylglycerol (Egg-PG), cationic lipid dioleoyl trimethylammoniumpropane (DOTAP) and fluorescent lipid 1-oleoyl-2-{6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]hexanoyl-sn-glycero-3-phosphatidylcholine(NBD-PC) were obtained from Avanti Polar Lipids, Inc. (Alabaster, USA). Low-methoxylated pectin (LM-pectin, Genu® pectin LM12 CG-Z, degree of methoxylation 34.8 %, $M_W = 7.6 \times 10^4$) was obtained from CPKelco (Großenbrode, Germany) (Nguyen et al., 2011). Chitosan (Protasan UPCL 213, Novamatrix, degree of deacetylation 83 %, $M_W = 3.1 \times 10^5$) was obtained from FMC Biopolymer AS (Sandvika, Norway) (Jonassen et al., 2012). Sodium alginate (Protanal LF 10/60) was provided by FMC BioPolymer, Norway, while hydrophobically modified ethyl hydroxyethyl cellulose (HM-EHEC) was a kind gift from AkzoNobel Chemicals AS (Stenungsund, Sweden).

Chloroform used for liposome preparation, as well as sodium dihydrogen phosphate monohydrate and disodium hydrogen phosphate dihydrate used for preparation of phosphate buffer was of analytical grade from Merck (Darmstadt, Germany).

The *In Vitro* Toxicology Assay Kit, MTT based, and the paracellular marker 5(6)-carboxyfluorescein were purchased from Sigma-Aldrich.

The HT29-MTX cell-line was kindly provided by Dr. Thécla Lesuffleur (INSERM UMR S 938, Paris, France). These mucus-secreting cells have been adapted to and cultured for several passages in medium containing 10^{-6} M MTX and reversed for several passages in drug-free medium (Lesuffleur et al., 1993). They do not need to be maintained in media containing MTX in order to differentiate after confluency.

Medium for cell growth: Dulbecco`s Modified Eagle`s Medium with high glucose (DMEM), GlutaMAX™, sodium pyruvate and phenol red, pH 6.8-7.2 (sodium bicarbonate buffer) (Invitrogen), further supplemented with 10 % inactivated Fetal Bovine Serum (Sigma-Aldrich) and Penicillin (100 units/ml) + Streptomycin (100 µg/ml) (Sigma-Aldrich).

Medium for experiments: Hank`s Balanced Salt Solution (HBSS), modified, with 1.26 mM $CaCl_2$ x 2 H_2O, 0.81 mM $MgSO_4$ and 4.17 mM $NaHCO_3$, pH=7.2-7.6 (Sigma-Aldrich).

2.2. Methods

2.2.1. Preparation of liposomes

Neutral, positively and negatively charged liposomes were prepared by the thin film method (Nguyen et al., 2011). The phospholipids were dissolved in chloroform in order to obtain a homogeneous mixture. Later on chloroform was removed by rotary evaporation (Heidolph W 2001 rotavapor,

Heidolph Instruments GmbH & Co. KG, Kelheim, Germany) and the lipid films were thoroughly dried under vacuum overnight (Christ Alpha 2-4 freeze drier, Christ, Osterode am Harz, Germany). The resulting films were hydrated with phosphate buffer solution (5 mM, pH 6.8) and gently agitated for 2 hours at room temperature and overnight at 4°C. The hydrated films were extruded (Lipex extruder, Lipex Biomembranes Inc., Vancouver, Canada) through two polycarbonate membranes with pore size of 200 nm (Nucleopore®, Costar Corp., Cambridge, USA). The final concentration of the lipid in the samples was 3 mM. The liposome dispersions were stored in the refrigerator before further use.

2.2.2. Purification of polymers and preparation of polymer solutions

Commercially available LM-pectin, HM-EHEC and alginate were additionally purified in a three-step process involving centrifugation, dialysis and freeze-drying of the solutions as described elsewhere (Klemetsrud et al., 2013). Chitosan, alginate, HM-EHEC and LM-pectin solutions were prepared by dissolving polymers in phosphate buffer at a concentration of 0.125 % (w/v). Solutions were allowed to stir overnight at a room temperature and subsequently filtered through a 2 µm polycarbonate membrane (Nucleopore®, Costar Corp., Cambridge, USA).

2.2.3. Coating of liposomes

Positively charged liposomes were coated with LM pectin or alginate, while negatively charged liposomes were coated with chitosan and neutral liposomes were coated with HM-EHEC. The liposomes were added to the polymer solutions in a dropwise manner (Watson-Marlow peristaltic pump, 20 rpm) and at a ratio of 1:4, under magnetic stirring. The samples were additionally stirred for 5 minutes. The final concentrations of the components in the samples were 0.6 mM lipid and 0.1 % polymer. Later on, samples were concentrated by centrifugation in Spin-X® UF concentrator tubes with MWCO = 30,000 (Corning, USA). Two different types of samples were obtained: solutions with a lipid concentration of 1.2 mM and a polymer concentration of 0.2 %, as well as a lipid concentration of 6 mM and a polymer concentration of 1 %.

2.2.4. Characterization of liposomes

The size (hydrodynamic diameter) and the zeta potential (electrophoretic mobility) of the liposomes were determined using the dynamic light scattering (DLS) and the microelectrophoretic method, respectively (Zetasizer Nano Series, Malvern Instruments Ltd., Worcestershire, United Kingdom). The measurements were conducted at 25 °C with 173° backscatter angle. The obtained values of the size were counted as an average of the three subsequent runs with 10 measurements, while the zeta potential was an average value from the three runs with 20 measurements.

2.2.5. Cell culturing

The cells were incubated at 37°C under 5 % CO_2 atmosphere. For the maintenance, the cells were passaged before reaching 80 % confluency with Trypsin-EDTA solution (Sigma-Aldrich), and the medium was changed every second day. Before the experiments, the cells were counted using a hemocytometer and seeded at an initial density of 60 000 cells/cm^2. The medium was changed every second day during the first week and then every day.

For the mucoadhesion experiments, cells from the passage number 27 were seeded on white polystyrene Nunclon™ 96 well plates with a culturing area of 0.36 cm^2 and on transparent polystyrene Nunclon™ cell culture dishes with a culturing area of 1.8 cm^2 (Nunc A/S, Denmark). For the MTT viability test, cells were seeded on the polystyrene Nunclon™ cell culture dishes with a culturing area of 1.8 cm^2. For the paracellular permeation experiments, cells were seeded on uncoated polycarbonate Transwell filter inserts (Costar, Corning) with a 0.4 µm pore size and a cell growth area of 1.12 cm^2. The volume of the donor chamber and the acceptor chamber were 0.5 and 1.5 ml, respectively.

Experiments were carried out after 2 weeks of cell culturing. The MTT viability test was additionally conducted on a diluted cell sample grown for only 24 hours.

2.2.6. Preparation of liposome samples for cell tests

In order to prepare samples for the mucoadhesion tests, liposome dispersions with lipid concentration 1.2 mM were mixed with HBSS to a final concentration of 0.6 mM. HBSS ensured acceptable conditions for the cells during the 2 hours experimental time.

The fluorescence of the final liposome samples was measured using a plate reader (1420 Multilabel counter Victor^{3} ™ from Perkin Elmer, Waltham, Massachusetts, USA) at λ_{ex} 485 and λ_{em} 535, 0.1 s, on black optical bottom polystyrene Nunclon™ cell culture dishes (Nunc A/S, Denmark) with a well diameter of 6.5 mm. The blank consisting of HBSS was deducted. Each 200 µl sample was measured in a triplicate. The relative standard deviations of the triplicates were less than 2 %. The averages were therefore used, and the variances disregarded, in further calculations.

Samples for measuring the general adhesion were prepared in the same way. The fluorescence was measured in duplicate, and the relative standard deviations were always lower than 3.8 % (in most cases lower than 1 %).

Liposome samples for the MTT viability and paracellular permeation tests were mixed in ratio 1:1 with medium for cell growth (DMEM) in order to ensure acceptable conditions for the cells during the 24 hours incubation time. The final concentration of lipid in the MTT viability and paracellular permeation tests were 0.6 mM and 3.0 mM, respectively.

2.2.7. Mucoadhesion tests

The cell monolayers were washed and then incubated with the liposome test samples and a HBSS control sample in a shaking incubator, 37°C and 60 rpm. Each sample was incubated in triplicate. The sample volumes were 500 µl and 200 µl for transparent and white culture dishes, respectively.

After 2 hours the samples were removed by gently tilting the plates to approx. 45° and collecting the solutions gathering in the lower part of the well using a pipette. The fluorescence of the supernatants (F_S) was measured in the same way as for the starting liposome samples (F_0) and the results (F) were expressed as a percentage of the same starting liposome sample:

$$F = F_s/F_0 \times 100\% \qquad \text{(Eq. 1)}$$

The supernatants of the transparent wells were measured in duplicate and the averages were used.

In addition, the fluorescence was measured directly on the surface of the cells grown on the white culture dishes, before and after washing the cell surface with 200 µl of HBSS. These values were expressed as a percentage of the fluorescence of the same starting liposome sample, for standardization purposes. In all cases, the values of the HBSS control samples were deducted as blank.

The general adhesion was measured in the same way, but without cell layers in the wells. The only difference was that the 500 µl samples were incubated in duplicate, instead of triplicate.

2.2.8. MTT viability tests

The cell monolayers were incubated for 24 hours with 1 ml liposome test samples and three different control samples consisting of (a) 1 ml medium for cell growth, (b) 0.5 ml medium, or (c) 0.5 ml medium + 0.5 ml HBSS. Liposome samples and control samples were incubated in duplicate on 2 weeks old, confluent mucus-secreting monolayers. Additionally, MTT test was performed on the cells in the exponential growth phase (1 day after culturing); liposomes and control samples were incubated in triplicate or duplicate, respectively. The cell monolayers were subsequently washed and incubated with 100 µl reconstituted MTT (15 mg MTT in 3 ml HBSS) in a shaking incubator, 37°C and 60 rpm. After 2 hours, the resulting formazan crystals were dissolved by adding 900 µl/1.0 ml of MTT solubilizing solution (acidic isopropanol). The amount of formazan was quantified spectrophotometrically by measuring 200 µl samples transferred to optical bottom polystyrene Nunclon™ cell culture dishes at 590 nm using the plate reader Victor^{3} ™. The UV absorbance was usually measured only once. When measured twice, as done for two of the control samples, the relative standard deviations between the measurements were only 1-2 %. The different control samples yielded the same results and were therefore pooled.

2.2.9. Paracellular permeation experiments

The cell monolayers were incubated with 0.5 ml of liposome test samples or control samples consisting of 0.5 ml medium for cell growth and 0.25 ml medium + 0.25 ml HBSS in the donor chamber. Each sample was incubated in triplicate. The 1.5 ml acceptor chamber consisted of medium for cell growth in all cases. After 24 hours both the filters containing the cell monolayers, as well as the acceptor and donor chambers, were washed. 1.5 ml of HBSS and 0.5 ml of a 15 µM solution of carboxyfluorescein in HBSS were subsequently introduced to the acceptor and donor chambers, respectively. The plates were placed in a shaking incubator, 37°C and 60 rpm. After 2 hours the amount of permeated carboxyfluorescein appearing in each acceptor chamber was measured in triplicate using the fluorescence plate reader Victor^{3} ™ at λ_{ex} 485 and λ_{em} 535, 0.1 s, on the black optical bottom polystyrene Nunclon™ cell culture dishes, holding 200 µl of solution. The averages of these three measurements were used. The relative standard deviations were less than 5 %. The different control samples yielded the same results and were therefore pooled.

2.2.10. Statistics

The results are presented as the mean and standard deviation (S.D.) of the parallels. Statistically significant differences are discussed at $p < 0.05$.

3. Results and discussion

3.1. The model membrane

A relevant *in vivo* model membrane is advantageous when performing *in vitro* mucoadhesion and toxicity studies. The model membrane chosen in this work was a cell monolayer consisting of methotrexate (MTX) treated HT29 (HT29-MTX) cells. The HT29 cell-line is, like Caco-2 cells, a human colon adenocarcinoma cell-line that spontaneously differentiates into monolayers of polarized enterocytes connected by tight-junctions. MTX treated clones have been shown to postconfluently

differentiate into a mixed population of mucus-secreting goblet cells and enterocytes (Lesuffleur et al., 1990).

The cells were grown for 2 weeks on cell culture dishes and Transwell filters, which is long enough to form confluent monolayer secreting/expressing mucus (Hagesaether, 2011; Hagesaether et al., 2013). This is in line with previously reported observations, visualizing gradual development of mucus on HT29-MTX cells and reaching a maximum thickness and coverage (22 µm, 75 – 80 % coverage) after approximately 22 – 23 days (Pontier et al., 2001; Wikman et al., 1993). Figure 1 shows the HT29-MTX cells 2 and 14 days after passaging, respectively.

The membrane consisting of the mucus producing HT29-MTX cells can also be valuable for *in vitro* mucoadhesion experiments, since both commercially available mucin and animal mucosa have limitations when it comes to *in vivo* relevance. Animal mucosa is thicker than human mucosa, and can therefore potentially overestimate the mucoadhesion. Moreover, measurements on animal mucosal tissue show high standard deviations because of varying surface properties, which make it difficult to receive reproducible and reliable results. An appropriate mucosal tissue or a replacement of the tissue has therefore been requested as an alternative for animal mucosa, which is also of interest regarding ethical aspects (Woertz et al., 2013). In line with us, Pepić et al. suggested employing available mucus-producing cell lines for *in vivo* relevant *in vitro* investigations of nanoparticle interactions with the epithelial surface (Pepić et al., 2013).

3.2. Mucoadhesion

Table 1 presents the liposomal formulations tested for mucoadhesion and their characteristics, such as lipid composition, type of coating, size and zeta potential.

The mucoadhesion of the liposomes was detected by both direct measurement of fluorescence on the monolayers, and indirectly by measuring the difference in fluorescent liposome concentration of the supernatant before and after incubation.

Table 1: Characteristics of the different types of liposomes

Liposomal formulations	Lipid composition [mol %]	Coating	Size [nm]	PDI	Zeta potential [mV]
Neu LP (0)	SoyaPC: 99 NBD-PC: 1	No	174	0.12	-1.6
Neg LP (-)	SoyaPC: 89 Egg-PG: 10 NBD-PC: 1	No	132	0.12	-42.9
Pos LP (+)	SoyaPC: 89 DOTAP: 10 NBD-PC: 1	No	132	0.12	32.7
Alg cLP (-)	= Pos LP (+)	Alginate	258	0.24	-50.6
LMpect cLP (-)	= Pos LP (+)	LM pectin	300	0.15	-53.4
Chit cLP (+)	= Neg LP (-)	Chitosan	280	0.42	18.3
HM-EHEC cLP (0)	= Neu LP (0)	HM-EHEC	357	0.24	-0.2

The results from the direct detection method, both the initial mucoadhesion and the remaining mucoadhesion after the monolayers were washed once, are compiled in Figure 2. The results from

the general adhesion experiment, presenting the ability of the liposomes to adhere to a surface without any cells, are included for comparison purposes.

The initial fluorescence of the different liposome samples varied slightly. Therefore, the results from the direct measurement methods are expressed as a percentage of this initial value, which is the most accurate for comparison purposes. Since the fluorescence signal coming from the samples in the 'dry' state (after removing the solution) was generally stronger than the fluorescence of liposome test samples, in some cases the obtained fluorescence values exceeded 100 %.

Before washing, Neg LP (-) and Pos LP (+), as well as Alg cLP (-) and HM-EHEC cLP (0) showed a higher mucoadhesion than the general adhesion, implying a specific (genuine) mucin interaction. However, this difference was statistically significant only for Pos LP (+) and Alg cLP (-). The initial mucoadhesion was also highest for Pos LP (+), followed by Alg cLP (-) and then Chit cLP (+). The measured mucoadhesion for the other liposomes was low in comparison. Although the measured mucoadhesion of Chit cLP (+) was high, the general adhesion was even higher, implying a high tack, and possibly less specific mucin interaction.

The amount of liposomes adhering to the monolayer typically decreased 50 % after washing. The exception was Chit cLP (+), which percent wise decreased much less, implying that the chitosan coated liposomes have the ability to resist washing and stay on the mucosa for prolonged periods of time. A prolonged retention time of calcitonin-loaded chitosan coated liposomes compared to uncoated liposomes has previously been reported in rats intestine (Takeuchi et al., 2005).

The significance of a positive charge in binding negatively charged groups on the cell surface (e.g. sialic acid) is well known (Verma and Stellaci, 2010). These negatively charged groups are also present in mucin, explaining the promising mucoadhesion results found for Pos LP (+) and Chit cLP (+), both positively charged. Particularly the mucoadhesive properties of chitosan have previously been described, using different *in vitro* methods (Takeuchi et al., 2005). The same group also reported the highest mucoadhesion for chitosan coated liposomes, followed by positively charged

uncoated liposomes and negatively charged uncoated liposomes, in rats. There was some correlation with the pharmacological effect. These results imply that a physical entanglement between the polymer and mucosal layer is an important factor facilitating mucoadhesion (Takeuchi et al., 2003). The superior mucoadhesion of chitosan coating was not reflected in our results, except for the ability to resist washing.

The positive effect of coating the liposomes with chitosan on mucoadhesion has also been confirmed by other groups, this time compared to neutrally charged uncoated liposomes on an isolated bovine mucosa (Berginc et al., 2014) or compared to negatively charged uncoated liposomes using commercial mucin from Sigma-Aldrich (Han et al., 2012). This is in line with our results. In the last example, the improved mucoadhesion was reflected in an increased oral bioavailability of alendronate in rats. Also negatively charged uncoated liposomes increased the oral bioavailability compared to alendronate solutions.

Alg cLP (-) showed a high mucoadhesion and specific mucin interaction, neither of which was the case with LMpect cLP (-). These results were slightly surprising since we have previously characterized the mucoadhesive properties of different types of pectin using various *in vitro* methods. Although alginate is generally recognized as a substance possessing excellent mucoadhesive properties (Duchêne et al., 1988), we have found LM pectin to perform better as a solution (Hagesaether and Sande, 2007) and as plasticized and unplasticized cast films (Hagesaether and Sande, 2008). The high specific mucin interaction of LM pectin has also been demonstrated directly on a molecular scale using atomic force spectroscopy and surface Plasmon resonance (Joergensen et al., 2011).

However, when LM pectin was formulated as cross-linked beads, the mucoadhesive properties diminished, and LM pectin was no longer superior to alginate (Hagesæther et al., 2008). This effect might be even further accentuated when LM pectin is attached to the liposomes, explaining our findings. This is however not in line with Thirawong et al., who demonstrated strong *in-vivo*

mucoadhesive properties of self-assembling LM pectin–liposome nanocomplexes in rats (Thirawong et al., 2008). The discrepancy between results most probably reflects the differences between rat and human mucus, as well as differences between the formulations tested. The exact comparison of native mucus and mucus secreted from HT29-MTX cells at different time intervals has not, to our knowledge, been published. It is therefore also possible that the development of mature , fully lipophilic mucus takes longer than 14 days for HT29-MTX cells.

The results from the direct measurements can give a reliable rank order of mucoadhesion, but cannot quantify the amount of mucoadhesion. Therefore, the fluorescence signal of liposomes recovered from the supernatant after 2 hours incubation was measured and compared to the initial liposome sample. The difference between these two values defines percentage mucoadhesion. The results are compiled in Table 2.

Table 2: Percentage mucoadhesion of liposomes, expressed as the difference between fluorescence of initial liposome sample and signal recovered from supernatant after 2 hours incubation time.

	Neu LP (0)	Neg LP (-)	Pos LP (+)	Alg cLP (-)	LMpect cLP (-)	Chit cLP (+)	HM-EHEC cLP (0)
	General adhesion, incubated without cells.						
Average (n=3)	12.1 %	17.0 %	14.9 %	3.1 %	5.7 %	23.7 %	2.9 %
STD (n=3)	0.5 %	1.4 %	0.9 %	0.8 %	0.9 %	0.3 %	5.6 %
	Mucoadhesion, incubated with HT29-MTX cell monolayer.						
Average (n=3)	16.7 %	21.9 %	23.9%	16.8 %	9.3 %	14 %	5.7 %

STD (n=3)	3.9 %	1.8 %	1.9 %	1.5 %	0.8 %	1.3 %	8.4 %

The correlation between the results from the direct (Figure 2) detection method and the indirect (Table 2) detection method was high, but with a few exceptions. The high mucoadhesion of Pos LP (+) was reflected in both methods. Only about 76 % of the liposomes could be recovered from the supernatant after incubation on the HT29-MTX cell monolayer. Additionally, the value of mucoadhesion was statistically higher than the general adhesion, indicating specific (genuine) mucin interaction. This was also the situation for Alg cLP (-) where the general adhesion was very low and a statistically significant higher mucoadhesion of about 17 % (about 83 % recovered) was detected, indicating specific mucin interaction. For Chit cLP (+) the high general adhesion and statistically significantly lower mucoadhesion, indicating lack of specific mucin interaction, was also confirmed.

The most striking deviation between these two methods can be found for Neg LP (-), which displayed a high general adhesion in addition to a significantly higher mucoadhesion, implying a small, but genuine, mucin interaction. The high values of adhesion were not reflected in the direct method, suggesting the liposomes adhering to for example the plate wall or plastic pipettes. This finding also illustrated the advantage of combining the two methods of detection, both direct and indirect, to avoid potentially misleading results.

Smaller deviations are the high mucoadhesion of Neu LP (0) and, to a lesser degree, LMpect cLP (-), although this can probably be explained by methodically uncertainty, as neither were significantly different from the detected general adhesion.

The same experiment was repeated with bigger cell culture dishes, displaying more bottom surface compared to wall surface. This change in dimensions should also increase the effect of stirring. This experiment confirmed the high general adhesion of Neg LP (-) (9.8 % ± 0.8 %) and Chit cLP (+) (31.4 % ± 10.8 %), the genuine mucin interaction of Pos LP (+) (a mucoadhesion of 37.8 % ± 6.3 % being statistically significantly higher than the general adhesion) and Alg cLP (-) (a mucoadhesion of 12.8 %

± 0.7 % being statistically significantly higher than the general adhesion). The higher general adhesion compared to mucoadhesion (14.0 % ± 4.9 %) of Chit cLP (+) was confirmed as well. In this case, the general adhesion and mucoadhesion of Neg LP (-) were similar (10 % ± 1 %).

3.3. Biocompatibility

The biocompatibility of a formulation can be assessed on a cellular scale using at least three fundamentally different methods: cell viability, monolayer integrity and plasma membrane integrity (by measuring the release of cytosolic proteins or ATP) (Pepić et al., 2013). In our study the biocompatibility of the different types of liposomes was assessed by the MTT viability test. It was performed on 2 weeks old, confluent mucus-covered cell monolayer, as well as on diluted HT29-MTX cells in exponential growth phase. The biocompatibility was also assessed by the monolayer integrity test, measuring the permeability of the paracellular marker carboxyfluorescein through a confluent mucus-covered cell monolayer.

The MTT viability test is based on the ability of cellular oxidoreductase enzymes to reduce the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to formazan, which can be detected spectrophotometrically due to its purple color. The activity of the enzymes may reflect the number of viable cells.

The results from the confluent mucus-covered monolayers are compiled in Figure 3.

The conversion of MTT to formazan reflects the metabolic activity of the cells, which can be elevated upon certain types of stress. Therefore, absorbance values higher than for the control are not unusual (Kozlovskaya et al., 2015). We also observed another interesting effect when the monolayers were inspected under a microscope. First of all, the cells were not very metabolically active, and the values for control were therefore low. Secondly, the conversion was not uniformly distributed, but rather localized in particular areas (Figure 4). The majority of formazan crystals were present along the linings of monolayer damage/holes, indicating that cells in these areas were more metabolically

active. We therefore chose to consider any deviation from the control, whether positive or negative, as an irritation of the cell-layer.

According to the guidelines from the regulatory authorities (USP/ISO) , toxicity is considered as significant if more than 30 % reduction in the cell metabolic activity has occurred (Kozlovskaya et al., 2015). None of the liposome samples deviated as much as 30 %, but HM-EHEC cLP (0) showed a significant reduction of more than 20 %, while Pos LP (+) and Chit cLP (+) displayed a significant increase of more than 20 %. Neg LP (-) showed a smaller, but statistically significant reduction. Hence, the lowest biocompatibility was found for the liposomes coated with an amphiphilic polymer and the positively charged liposomes.

Sigma-Aldrich states that the MTT viability assay should be employed using cells in exponential growth phase with a density not exceeding 10^6 cells/cm^2. Compared to the robust confluent monolayer, this situation changed our results dramatically. The absorbance measured for the control cells decreased (0.16 ± 0.02), and the absorbances measured for Neg LP (-), Pos LP (+), Alg cLP (-), Chit cLP (+) and HM-EHEC cLP (0) were close and not significantly different from this value. This indicates that neither the amphiphilic coated (HM-EHEC cLP (0)) nor the positively charged liposomes (Pos LP (+) and Chit cLP (+)) affected cell growth. On the other hand, the neutral uncoated (Neu LP (0)) and LM pectin coated (LMpect cLP (-)) liposomes displayed values significantly lower than the control (0.12 ± 0.01 and 0.10 ± 0.002, respectively). To our knowledge, neutral liposomes are not known to be toxic, and we therefore speculate that something else was detected. Growing HT29-MTX cells are mobile, so one hypothesis might be some attachment of cells to the liposomes with subsequent discharge during washing, or hypothetically a different orientation of the cells when the liposomes were present.

Anyhow, the MTT viability test has some pitfalls, and the results can sometimes be difficult to interpret. Therefore, we used a second method for assessing biocompatibility: the permeation of a

paracellular marker after the confluent monolayers had been incubated with the liposome test samples for 24 hours. The results are compiled in Figure 5.

After incubation with Chit cLP (+) and HM-EHEC cLP (0) , the permeation of the marker statistically increased. However, only for HM-EHEC cLP (0) was this increase higher than 20 %. The increase inflicted by Chit cLP (+) was comparable to the influence of Pos LP (+) and Alg cLP (-).

In general, the different types of liposomes showed a high degree of biocompatibility, indicating low toxicity. Although permeability enhancement is not the same as toxicity *per se*, a correlation between the two properties is not uncommon (Aungst, 2000). Combining the results from the MTT viability and permeability test on confluent cell-layers, the following rank order of cell irritating potential can be suggested: HM-EHEC cLP (0) >> Pos LP (+), Chit cLP (+) > Alg cLP (-) > Neg LP (-), followed by no indication of toxicity for Neu LP (0) and LMpect cLP (-). The high biocompatibility was also confirmed on rapidly growing HT29-MTX cells, which surprisingly was only affected by Neu LP (0) and LMpect cLP (-).

EHEC is an amphiphilic polymer, which is generally known to possess permeation enhancing properties, often reflected in increased toxicity (Aungst, 2000). Positive charge might also irritate cells, and particularly chitosan has been extensively studied in this respect. In earlier work we have reported on the pronounced permeability enhancing effect of chitosan solutions (Hagesaether, 2011), but this effect seems to diminish when liposomes are coated with chitosan, in line with earlier studies on chitosan nanoparticles (Hafner et al., 2015). This effect might also have been reflected in the biocompatibility, as chitosan in a free soluble form is reported to be much more cytotoxic than when it is incorporated in a nanosystem, arguing for an acceptable cytotoxicity profile of chitosan nanoparticles (Hafner et al., 2015; Pradines et al., 2015).

Less is known about the biocompatibility of negative charge. Negatively charged polymers like alginate and LM pectin can affect tight-junctions by chelating calcium, and thereby increase the paracellular permeability (Aungst, 2000). Increased paracellular permeability was not found for LM

pectin coated liposomes, in line with results earlier reported by us on pectin solutions (Hagesaether, 2011). Alginate displayed some permeation enhancing properties, but the permeability was not significantly different from the control.

4. Conclusions:

This work has presented new detailed knowledge about the mucoadhesion and biocompatibility of differently charged uncoated and polymer coated liposomes, using an *in vivo* relevant model membrane and experimental set-up. Positive charge resulted in higher mucoadhesion, displayed both by uncoated and chitosan coated liposomes. The uncoated positively charged liposomes displayed the highest initial mucoadhesion, as well as significant specific mucin interaction. The chitosan coated liposomes displayed a very high general adhesion (tack) camouflaging any genuine mucin interaction. Still, chitosan prolonged the mucoadhesion of the liposomes. Unfortunately this high mucoadhesion was accompanied by lower biocompatibility, although no dramatic effect was seen. Nevertheless, alginate coated liposomes can be considered as an interesting option for use in chronic diseases, taking into account their high mucosal biocompatibility, specific mucin interactions, and moderate overall mucoadhesion.

This knowledge allows choosing between a high initial mucoadhesion, a long-lasting mucoadhesion or a high specific mucin interaction, with variable degrees of biocompatibility, depending on the desired clinical outcome. Our project is focused on relieving the symptoms of xerostomia, but our findings are also relevant in relation to both systemic and local drug delivery to the buccal mucosa.

Acknowledgements

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Figures

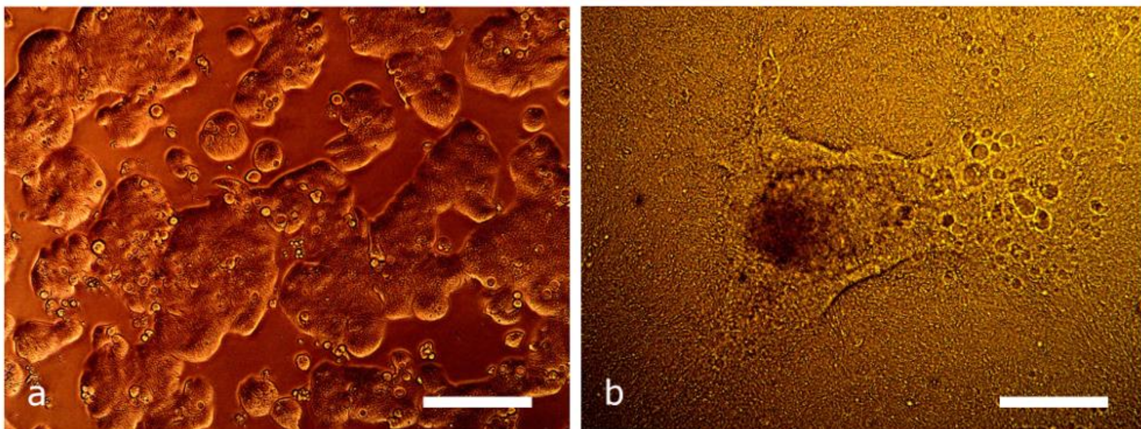

Figure 1. Morphology of the HT29-MTX cells (a) 2 days and (b) 14 days after passaging. The images were taken under the optical microscope with obj. 20x. The scale bar equals 100 µm.

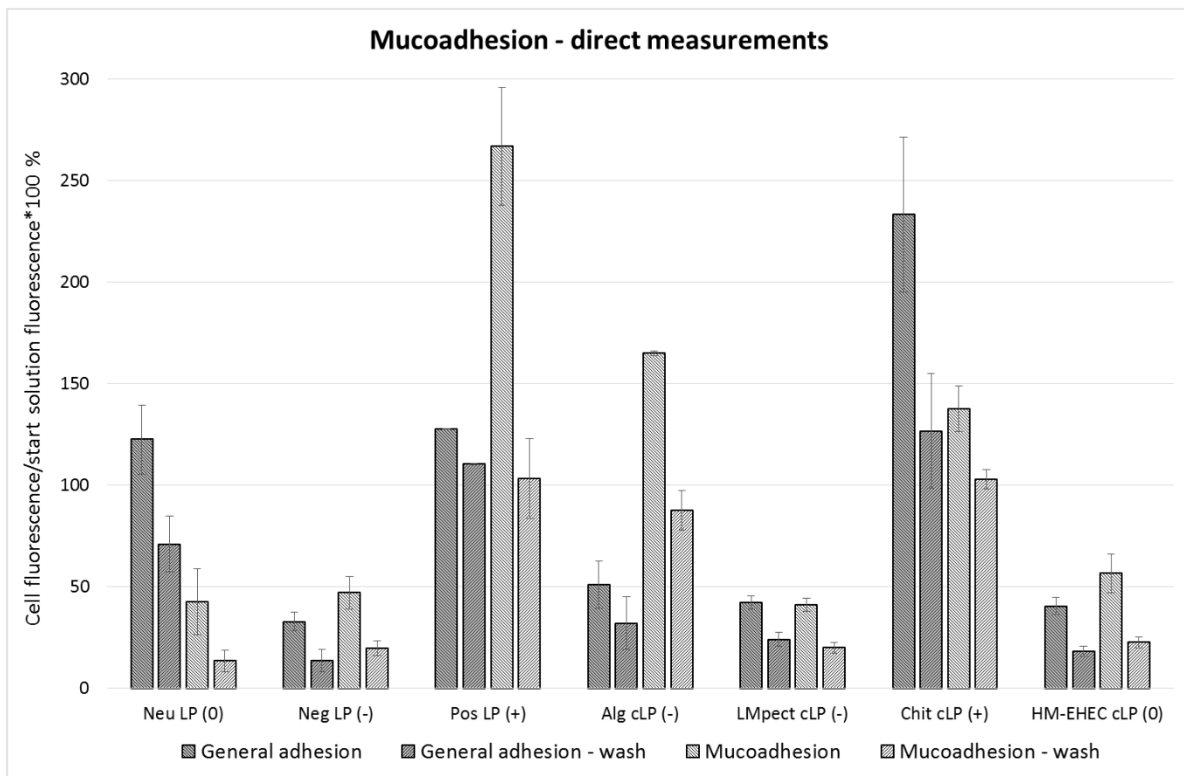

Figure 2. The fluorescence measured directly on the cell culture dishes incubated with the liposome test samples. The results are normalized with respect to the fluorescence of the same starting liposome sample. Results are expressed as the mean with the bar showing S.D. (n=3).

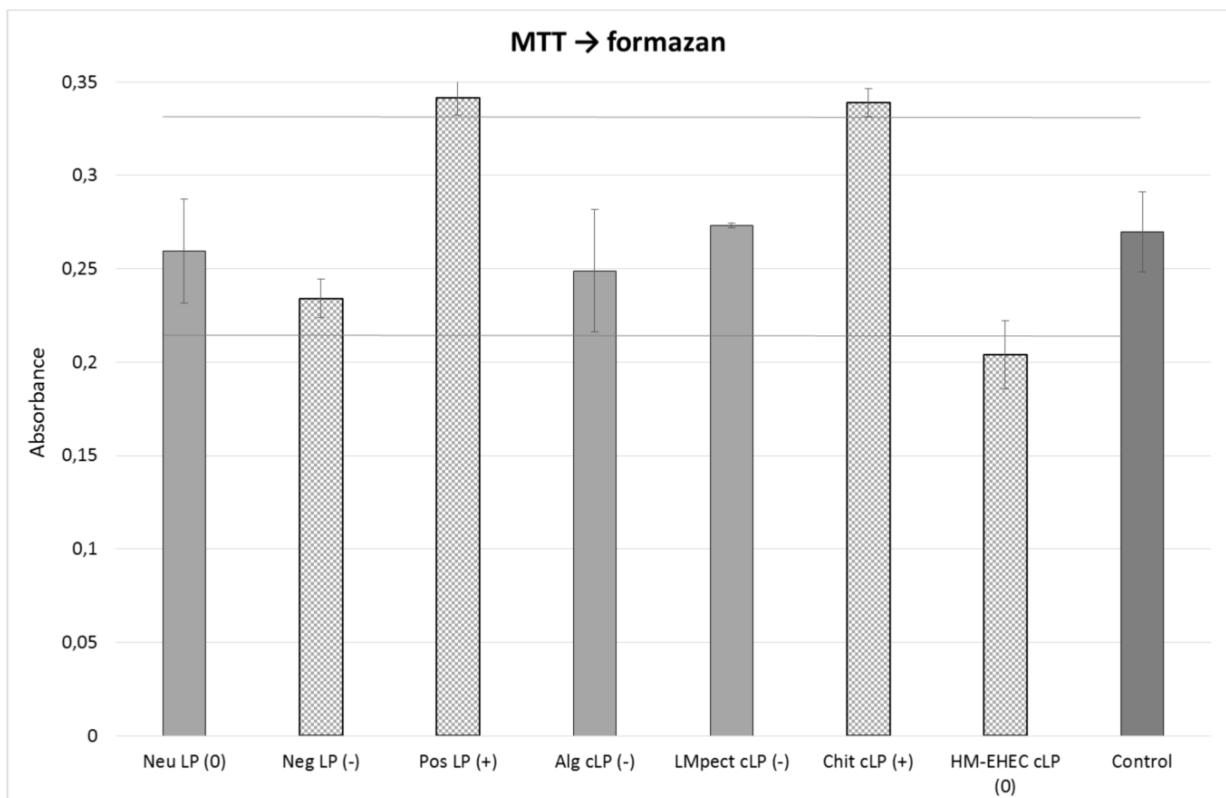

Figure 3. The measured absorbance representing formazan produced by the confluent mucus-covered monolayers. The checkered bars represent samples that are significantly different from the

control. The lines represent the value of the control ± 20 %. Results are expressed as the mean with the bar showing S.D. (n=2 for liposome test sample, n=6 for control).

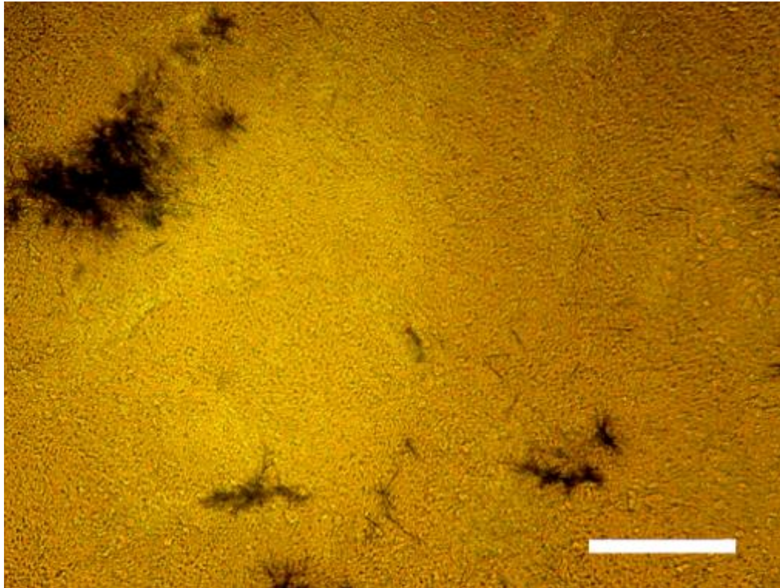

Figure 4. HT29-MTX cells after incubation with Chit cLP (+), washing and incubation with MTT, displaying nonuniform distribution of formazan crystals. The images were taken under the optical microscope with obj. 10x. The scale bar equals 200 µm.

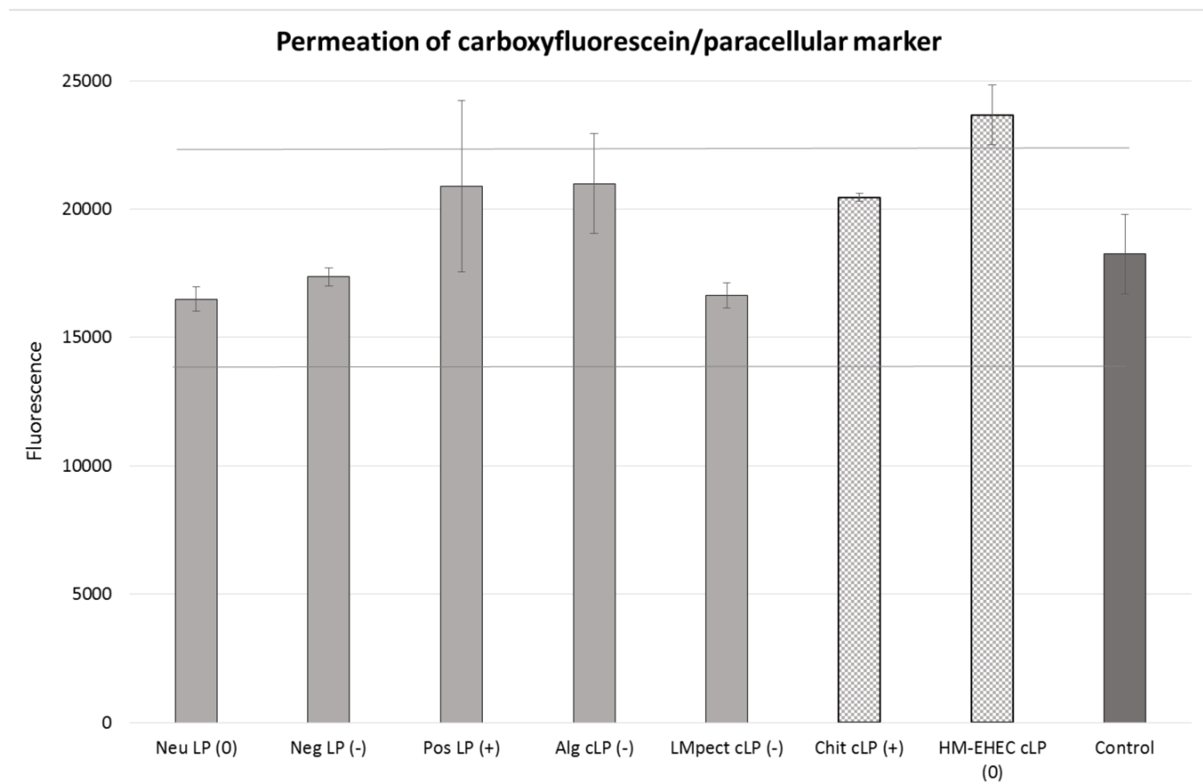

Figure 5. The measured fluorescence representing permeated paracellular marker carboxyfluorescein through the confluent mucus-covered monolayers. The checkered bars represent sample that are significantly different from the control. The lines represent the value of the control ± 20 %. Results are expressed as the mean with the bar showing S.D. (n=3 for liposome test sample, n=5 for control).