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Multivariate toxicity screening of liposomal formulations on a human buccal cell line

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Abstract

The influence of various formulation factors on the *in vitro* cellular toxicity of liposomes on human buccal cells (TR146), were studied by using the concept of statistical experimental design and multivariate evaluation. The factors investigated were the type of main phospholipid (egg-PC, DMPC, DPPC), lipid concentration, the type of charge, liposome size, and amount and nature of the charged component (diacyl-PA, diacyl-PG, diacyl-PS, stearylamine (SA), diacyl-TAP) in the liposomes. Both full factorial design and D-optimal designs were created. Several significant main factors and interactions were revealed. Positively charged liposomes were shown to be toxic. The toxicity of negatively charged liposomes was relatively low. Diacyl-TAP was less toxic than SA, and DPPC was less toxic than DMPC. Low level of positively charged component was favourable and essential when using egg-PC as the main lipid. The amount of negatively charged component, the liposome size, and the total lipid concentration did not affect the toxicity within the experimental room. DPPC appeared to be a good candidate when formulating both positively and negatively charged liposomes with low cellular toxicity. The concept of statistical experimental design and multivariate evaluation was shown to be a useful approach in cell toxicity screening studies.

Keywords: Liposomes; Cellular toxicity; Buccal cell line; Multivariate data analysis

1. Introduction

The buccal region appears to be an attractive site for administration of drugs due to the good accessibility, the smooth and relatively immobile surface, the avoidance of possible degradation in the gastrointestinal tract, and avoidance of the first-pass metabolism in the liver. However, continuous saliva excretion and swallowing may lead to a very short residence time in the oral cavity (Rathbone et al., 1994). To overcome this problem novel bioadhesive dosage forms have been developed, such as bioadhesive tablets, bioadhesive patches, bioadhesive gels and ointments, and medicated chewing gums (Gandhi and Robinson, 1994; Hao and Heng, 2003; Birudaraj et al., 2005). Also, liposomes have been investigated as a delivery system in the oral cavity (Harsanyi et al., 1986; Sveinsson and Holbrook, 1993; Farshi et al., 1996; Petelin et al., 1998; Yang et al., 2002; Erjavec et al., 2006). Liposomes may be expected to protect the active ingredient from degradation in the oral cavity, they may act as a depot, and they may be designed to be bioadhesive. A bioadhesive formulation is intended to stay in the mouth for hours; hence, the toxicity of the formulation on the cells coming into close contact with the formulation is an important issue. Toxicity studies of liposomes on various cell lines have been reported in the literature (Layton et al., 1980; Mayhew et al., 1987; Filion and Phillips, 1997, 1998; Berrocal et al., 2000); the sensitivities of various cell lines to the same liposome formulation have been reported to vary. To our knowledge the toxicity to the cells in the oral cavity by liposomal formulations has not previously been addressed.

In this paper we study the toxicity of liposomal formulations using the human buccal cell line TR146. The TR146 cell line has been used as a model for the buccal epithelium in several studies on other substances in the literature (Burgalassi et al., 1996; Jacobsen et al., 1996, 1999; Pedersen et al., 1998; Eirheim et al.,

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2004; Boulmedarat et al., 2005a). All the liposomal formulation variables, e.g. type of the main lipid, the nature and amount of the charged component, and the liposome size, may be expected to influence the toxicity.

Toxicity studies on cell lines are usually carried out as univariate experiments, i.e. one factor is varied at a time. Evaluating the effect of a high number of variables and possible interactions will require a lot of experiments. In this paper, however, we have used the concept of statistical experimental design and multivariate evaluation. In such designs many factors are varied simultaneously in a systematic way, e.g. the influence of a high number of formulation variables can be studied at the same time and interactions between the variables can be detected. By this approach more information can be gained from a smaller number of experiments.

Thus, the objective of this paper was two-fold: (1) to identify important liposomal formulation factors influencing the toxicity on cells in the buccal region of the oral cavity using the TR146 buccal cell line a model, and identify significant interactions between the formulation variables, and (2) to investigate the potential of statistical experimental design and multivariate evaluation in cell toxicity studies. The factors investigated in this study were the type of the main phospholipid, the total lipid concentration, the type of the charge (positive, negative), the liposome size, the nature of the charged component, and the amount of the charged component in the liposomes. Both a full factorial screening design and D-optimal designs were created.

2. Materials and methods

2.1. Materials

Dimyristoylphosphatidylcholine (DMPC), dipalmitoylphosphatidylcholine (DPPC) and dimyristoylphosphatidylglycerol (DMPG) were kindly provided by Nattermann Phospholipids GmbH (Köln, Germany), egg-phosphatidylcholine (egg-PC) and egg-phosphatidylglycerol (egg-PG) were kindly provided by Lipoid GmbH (Ludwigshafen, Germany), dimyristoyl-trimethylammonium-propane (DMTAP), dipalmitoyl-trimethylammonium-propane (DPTAP), dioleyl-trimethylammonium-propane (DOTAP), dimyristoylphosphatidic acid (DMPA), egg-phosphatidic acid (egg-PA), dipalmitoylphosphatidylserine (DPPS) and dioleylphosphatidylserine (DOPS) were obtained from Avanti Polar Lipids, Inc. (Alabaster, AL, USA), dimyristoylphosphatidylserine (DMPS) was obtained from Genzyme Pharmaceuticals (Liestal, Switzerland), dipalmitoylphosphatidylglycerol (DPPG), dipalmitoylphosphatidic acid (DPPA), stearylamine (SA), and phenazine methosulfate (PMS) were purchased from Sigma Chemical Co. (St. Louis, MO, USA), MTS (3-(4,5-dimethylthiazol-2-yl)-5-(carboxymethoxyphenyl)-2-(4-sulphenyl)-2*H*-tetrazolium) was from Promega Corp. (Madison, WI, USA), Hank's balanced salt solution (HBSS) with and without calcium and magnesium were obtained from BioWhittaker Europe (Verviers, Belgium), and *ortho*-phosphoric acid and copper sulphate pentahydrate were from Merck (Darmstadt, Germany). Chloroform and methanol used for liposome preparation were of analytical grade.

The TR146 cell line is derived from a metastasis of a buccal carcinoma (Rupniak et al., 1985) and was provided by Cancer Research Technology Ltd. (London, UK).

2.2. Liposome preparation

Liposomes were prepared by the film-method as follows: the phospholipids were dissolved in chloroform:methanol (2:1), glass beads were added to the flask and the solution was evaporated to dryness in a rotary evaporator. The films were further dried in vacuum (<3 mbar) in a Christ Alpha 2–4 freeze drier (Christ, Osterode am Hatz, Germany) for 20 h. The thin film obtained was hydrated and gently shaken for 2 h at a temperature above the gel to liquid-crystalline phase transition temperature (T_c) with the aqueous solution (Hanks balanced salt solution (HBSS) with or without Ca and Mg) and kept in the refrigerator overnight. The samples were extruded at a temperature above T_c (Lipex extruder, Biomembranes Inc., Vancouver, Canada) using two stacked 800, 200, 100 or 50 nm polycarbonate membranes (Nucleopore®, Costar Corp., Cambridge, USA).

2.3. Liposome characterisation

The intensity mean diameter of the liposomes and the polydispersity index (PI) of the distribution were determined by photon correlation spectroscopy (PCS) at a 90° angle (25 °C) using Zetasizer 1000 (Malvern Instruments, Great Britain). The refractive index and viscosity of pure water were used as calculation parameters and each sample was measured five times using the unimodal model for size distribution. All samples were diluted with HBSS to an appropriate counting rate prior to analysis.

The zeta potential was measured by micro-electrophoresis at 25 °C (Zetasizer 2000 HS, Malvern Instruments, Great Britain). The viscosity and dielectric constant of pure water were used as calculation parameters. All samples were diluted with HBSS to an appropriate counting rate prior to analysis, and all samples were analysed in triplicate.

The lipid concentration in the samples was determined by high-performance thin-layer chromatography (HPTLC) as described elsewhere (Stensrud et al., 1996). In short, the samples were transferred to separate vials followed by freezing and lyophilisation in a Christ Alpha 2–4 freeze drier (Christ, Osterode am Hatz, Germany). The dry samples were dissolved in chloroform, applied to silica gel $60F_{254}$ HPTLC plates (E. Merck, Darmstadt, Germany) using Linomat IV sample applicator (CAMAG, Muttenz, Switzerland), developed in a horizontal developing chamber (CAMAG, Muttenz, Switzerland) using a mixture of chloroform:methanol:distilled water (32.5:12.5:2, v/v) as the mobile phase, and dried. The spots were visualised by immersing into a solution of 8% *ortho*-phosphoric acid and 10% copper sulphate pentahydrate. The plates were scanned at 510 nm using a dual-wavelength flying-spot scanner CS-9000 (Shimadzu, Kyoto, Japan). All samples were analysed in duplicate.

2.4. Cellular toxicity—MTS/PMS assay

TR146 cells of passage numbers 3 and 10 were cultured as described previously (Jacobsen et al., 1999).

Cellular toxicity of liposomal formulations was performed using a colorimetric method, a MTS/PMS assay, optimised for TR146 cells. Viable cells enzymaticly reduce the colourless tetrazolium salt MTS to intensively coloured MTS-formazan. The assay was carried out as described previously (Jacobsen et al., 1996) with the exception of a prolonged incubation time with formulations. Briefly, 2×10^4 TR146 cells/well were seeded in flat bottom 96-well plate (Corning Incorporated, Corning, NY, USA) and incubated for 24 h. After removing the culture medium, 100 μl of formulation/well or 100 μl HBSS/well (control, 100% viability) was applied and the plate was incubated at 37 °C for a period of 6 h. For each formulation we used wells with cells $n = 5$, and wells without cells $n = 3$ (background). For each plate we used HBSS as a control, wells with cells $n = 8$ (100% viability), and wells without cells $n = 8$ (background). Sodium dodecyl sulphate (SDS) (10 mM) was used as a positive control (0% survival), wells with cells $n = 5$, wells without cells $n = 3$. The formulations were sucked off, the wells were washed one time with HBSS in order to remove the formulations, 125 μl reagent (240 μg/ml MTS and 2.4 μg/ml PMS in PBS)/well were added and the plates were incubated for a period of 4 h. The absorbance was read at 492 nm on a micro-plate reader (Multiskan MS photometer type 352, Labsystems, Helsinki, Finland). The results are expressed as the mean cellular sensitivity (%, $n = 5$). Cellular sensitivity is the ratio between the optical density (OD) values for the cells treated with liposomes and cells treated with HBSS (control), both values corrected for background absorbance (Eq. (1) in Jacobsen et al., 1996).

2.5. Experimental design

The total experimental domain was studied through three separate designs.

In the first design the influence of four factors on the toxicity of the buccal cells were investigated in a full factorial screening design without centre points. The factors investigated were the type of main phospholipid, the type of charge (positive or negative), lipid concentration, and the liposome size ("small" extruded through 50 nm filter and "large" extruded through 200 nm filter). The factors and the levels are listed in Table 1.

Table 1
Experimental levels of the design variables in the full factorial screening design

Factor	Experimental levels	
	Low (−)	High (+)
Lipid (main lipid)	DMPC (saturated fatty acids)	Egg-PC (unsaturated)
Charge	Diacyl-PA (negative)	Diacyl-TAP (positive)
Lipid concentration (mM)	12	35
Filter pore size (nm)	50	200

Table 2
Experimental levels of the D-optimal design for the positively charged liposomes

Factor	Experimental levels
Lipid (main lipid)	DMPC, DPPC, egg-PC
Charged component (low, high)	SA, diacyl-TAP
Amount of charged component (mol%)	5, 20
Filter pore size (nm)	100, 800

As we chose to include only one type of charged lipid in each liposome batch, two separate designs were constructed in the next step; one for the negatively charged liposomes and one for the positively charged ones. In both designs the influence of the main phospholipid, the nature of the charged group, the amount of charged component in the liposomes, and the liposome size, were investigated in D-optimal designs comprising 12 samples for the positively charged liposomes and 18 samples for the negatively charged ones. The factors and the different levels are listed in Tables 2 and 3 for the positively and the negatively charged liposomes, respectively.

The lipids chosen are lipids commonly used for liposome preparation. It has been reported that gel phase and fluid phase liposomes show different ability to associate with cell membranes (Mayhew et al., 1980; Szoka et al., 1980). Therefore, both gel phase and fluid phase liposomes were included. DPPC was chosen as the main lipid to make gel phase liposomes, egg-PC to make fluid phase liposomes with unsaturated fatty acids and DMPC to make fluid phase (at 37 °C) liposomes with saturated fatty acids. To avoid influencing the T_c too much when including charged phospholipids in the liposomes, the same fatty acid part in the charged component as in the main lipid was used when possible. Egg-TAP and egg-PS were not available, and the synthetic unsaturated DOTAP and DOPS, respectively, were used in stead. The lowest lipid concentration (12 mM) was comparable to what has been used or recommended for mucosal delivery in the literature (Boulmedarat et al., 2005b; Erjavec et al., 2006). In the screening design we wanted to include a high concentration level as well, and we chose 35 mM.

The designs were created by the aid of a computer program (Modde 4.0, Umetri AB, Umeå, Sweden) and evaluated by partial least square regression (PLSR) (Unscrambler®, The Unscrambler 7.5, Camo ASA, Trondheim, Norway). All data were auto scaled and the models were evaluated by employing full cross-validation. The approximated uncertainty variance of the PLSR coefficients were estimated by the Jack-knife uncertainty test as described by Martens and Martens (2000). A description of the PLS methods as well as an introduction to interpreting typical graphs (e.g. plots of regression coefficients)

Table 3
Experimental levels of the D-optimal design for the negatively charged liposomes

Factor	Experimental levels
Lipid (main lipid)	DMPC, DPPC, egg-PC
Charged component	Diacyl-PG, diacyl-PS, diacyl-PA
Amount of charged component (mol%)	5, 20
Filter pore size (nm)	100, 800

can be found elsewhere (Martens and Næs, 1989; Esbensen et al., 2000).

3. Results

The OD values of the cells treated with HBSS (controls, 100% viability) were usually about 0.8. The OD values of the background (liposome formulation or HBSS without cells) were usually in the range 0.05–0.07. The cells treated with SDS (control, 0% survival) confirmed the method since the OD values were about equal to the blank. The OD values of cells exposed to liposomal formulations ranged from 0.09 to 0.93.

3.1. Full factorial screening design

In these experiments the amount of the charged lipid in the liposomes was chosen to be 20 mol%. The liposomes were prepared in HBSS containing calcium and magnesium, the same solution was used as blank in the toxicity experiments. The size of the liposomes was measured on the same day as the batches were extruded and again when the toxicity experiments were finished. The toxicity experiments were performed 2–4 days after the production of the batches. An overview of the samples and the results are given in Table 4. The sizes in the size groups "small" and "large" differed significantly ($p = 0.0008$) for the new batches. However, due to poor size stability of some of the batches the difference was no longer significant ($p = 0.68$) at the time of the experiment. In addition, a correlation between "size at experiment" and the type of main phospholipid (main lipid) was observed ($r = 0.816$). Size was therefore excluded as a factor in the modelling.

Due to lipid loss during the extrusion process, the real (measured) lipid concentration was employed in the modelling of the concentration effect.

In Fig. 1 a plot of the weighted regression coefficients from the PLSR analysis on the sensitivity of the buccal cells is shown. Uncertainty limits including zero render the regression coefficient not significant. The response variable "sensitivity" expresses cellular sensitivity relative to untreated cells. High sensitivity means low toxicity; consequently negative regression coefficients imply toxic effect.

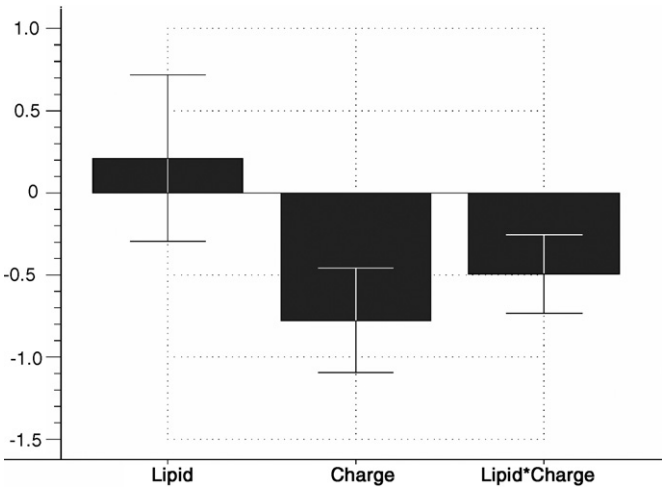

Fig. 1. Weighted regression coefficients (full factorial screening design). Bars represent the confidence intervals for the estimated regression coefficients. Response variable: sensitivity—high sensitivity means low toxicity. The levels of the design variables are listed in Table 1.

In the model 89% of the variation in the sensitivity is explained (1 PLSR component). One significant main factor was observed; that is the "charge" which is negatively correlated to the sensitivity. As positive charge is defined as high level in the model (Table 1), this means that positively charged liposomes reduces the sensitivity value and consequently are more toxic than negatively charged liposomes. The interaction between lipid and type of charge (lipid × charge) is significant indicating different effect of changes in charge among the two lipids. The egg-PC is much more sensitive to changes in the type of charge than DMPC (Fig. 2). The least toxic liposomes are the

Table 4
Full factorial screening design: characteristics of the samples

Sample	Main lipid (lipid)	Charged lipid (charge)	Size group	Size new batch		Size at experiment		Concentration level	Real concentration (mM)	Sensitivity (% of control)
				nm	PI	nm	PI			
N3	DMPC	DMPA (−)	Small	228	0.68	505	0.96	Low	6.9	56.8
N4	Egg-PC	Egg-PA (−)	Small	198	0.25	209	0.08	Low	5.1	89.6
N7	DMPC	DMPA (−)	Large	334	0.56	437	0.88	Low	6.8	82.0
N8	Egg-PC	Egg-PA (−)	Large	234	0.36	230	0.33	Low	8.5	94.2
N11	DMPC	DMPA (−)	Small	228	0.68	449	0.93	High	15.8	24.2
N12	Egg-PC	Egg-PA (−)	Small	198	0.25	195	0.22	High	21.1	114.0
N15	DMPC	DMPA (−)	Large	334	0.56	331	0.85	High	21.0	44.1
N16	Egg-PC	Egg-PA (−)	Large	234	0.36	214	0.34	High	26.5	115.7
P1	DMPC	DMTAP (+)	Small	190	0.36	255	0.67	Low	5.8	33.1
P2	Egg-PC	DOTAP (+)	Small	168	0.26	170	0.28	Low	8.4	13.5
P5	DMPC	DMTAP (+)	Large	248	0.22	m	m	Low	5.0	34.5
P6	Egg-PC	DOTAP (+)	Large	254	0.18	243	0.17	Low	9.6	8.7
P9	DMPC	DMTAP (+)	Small	190	0.36	291	0.72	High	12.2	32.9
P10	Egg-PC	DOTAP (+)	Small	168	0.26	168	0.27	High	21.7	13.0
P13	DMPC	DMTAP (+)	Large	248	0.22	440	0.69	High	16.5	23.5
P14	Egg-PC	DOTAP (+)	Large	254	0.18	245	0.17	High	26.1	4.9

The amount of charged lipid was 20 mol% in all the samples. Solvent was HBSS with Mg and Ca (PI: polydispersity index).

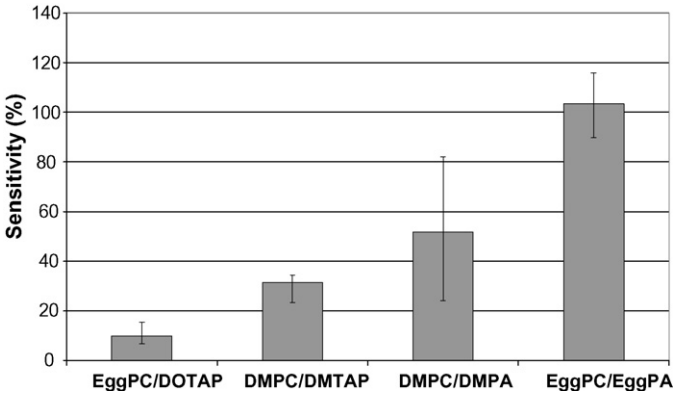

Fig. 2. Mean sensitivity values (full factorial screening design) of the different liposome formulations ($n = 4$). High sensitivity means low toxicity. Bars represent the highest and the lowest sensitivity value within the group.

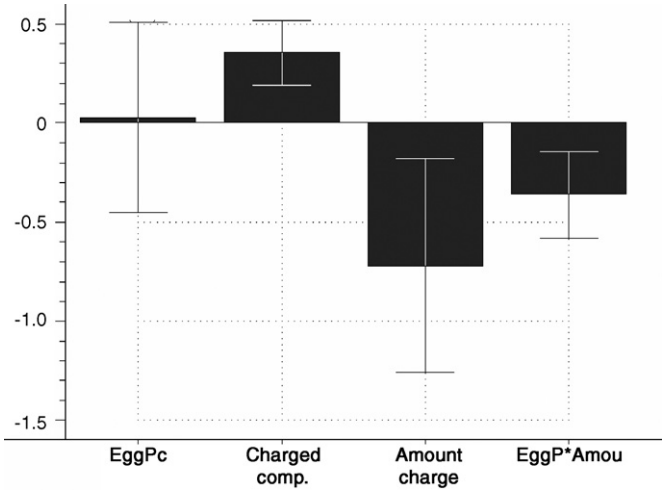

Fig. 3. Weighted regression coefficients (D-optimal design, positively charged liposomes). Bars represent the confidence intervals for the estimated regression coefficients. Response variable: sensitivity—high sensitivity means low toxicity. The levels of the design variables are listed in Table 2.

negatively charged egg-PC liposomes, while positively charged egg-PC liposomes are the most toxic ones, with DMPC liposomes showing intermediate toxicity. This can clearly be seen in Fig. 2. No effect of the lipid concentration was observed.

3.2. D-optimal design for positively charged liposomes

Since no effect of lipid concentration was observed in the screening experiment, the lipid concentration was kept at the low level (12 mM) in this second part.

In this part some of the liposome formulations had to be extruded at a relatively high temperature (70 °C) due to the high T_c of some of the components. However, at this temperature the calcium and/or magnesium in the HBSS sometimes precipitated. All the liposomes were therefore prepared in HBSS without magnesium and calcium. The same solvent was used as blank in the toxicity experiments. HBSS without calcium and magnesium was shown not to be toxic to the cell line before the liposome toxicity experiments were performed (data not shown).

Liposome size was included as a factor also in these experiments. Although the levels of liposome size were chosen further apart than in the screening design, poor size stability produced considerable overlap between the designed size groups. "Size at experiment" was, however, not correlated with any of the other factors, and could be included as a factor in the modelling allowing the possibility to reveal any hidden effects of the size.

Table 5 gives an overview of the samples and the results from the experiments. A plot of the weighted regression coefficients from the PLSR analysis with the sensitivity of the buccal cells as the response is shown in Fig. 3. The explained variance of the model was 78% (1 PLSR component). Two significant main effects appeared; the type and the amount of charged component. "Charged component" was positively correlated with the sensitivity and this shows that diacyl-TAP (defined as high level) was less toxic than SA (low level) under the experimental conditions. "Amount charge" was negatively correlated with the sensitivity. High mol% of charged lipid is therefore more toxic than low level (5 mol%).

The model revealed one significant interaction; a negative interaction between egg-PC and amount of charged component

Table 5
D-optimal design of positively charged liposomes: characteristics of the samples

Sample	Main lipid (lipid)	Charged component	Amount charged component (mol%)	Size group	Size new batch		Size at experiment		Sensitivity (% of control)	Zeta potential[a] (mV)
					nm	PI	nm	PI		
P1	DMPC	DMTAP	5	Small	104	0.1	108	0.15	93.2	13
P2	DMPC	DMTAP	20	Large	764	0.61	1700	1	43.8	34
P3	Egg-PC	DOTAP	5	Large	886	0.6	793	0.54	119.6	7
P4	Egg-PC	DOTAP	20	Small	105	0.09	108	0.06	5.3	25
P5	DPPC	DPTAP	5	Small	97	0.11	99	0.12	104.2	8
P6	DPPC	DPTAP	20	Large	816	0.88	4650	0.76	94.9	27
P7	DMPC	SA	5	Large	682	0.57	528	0.65	33.9	13
P8	DMPC	SA	20	Small	119	0.4	152	0.43	12.9	17
P9	Egg-PC	SA	5	Small	104	0.1	117	0.1	112.0	8
P10	Egg-PC	SA	20	Large	439	0.45	451	0.34	16.8	17
P11	DPPC	SA	5	Large	710	0.47	1590	0.18	91.4	9
P12	DPPC	SA	20	Small	139	0.6	2000	0.88	13.1	8

Lipid concentration 12 mM. Solvent HBSS without Mg and Ca (PI: polydispersity index).
[a] Corresponding formulation.

(egg-PC × amount charge). This shows that the toxicity of egg-PC liposomes is especially sensitive to the amount of the charged component; high levels of the positively charged component in egg-PC liposomes are especially toxic while low levels are favourable.

The liposome size did not appear to influence the sensitivity of the buccal cells.

3.3. D-optimal design for negatively charged liposomes

The liposomes were prepared in HBSS without calcium and magnesium for the same reason as described in Section 3.2 and the same medium was used as blank in the toxicity experiments. An overview of the samples and the results from the experiments is shown in Table 6. The correlation between "size new batch" and "size at experiment" was very good ($r = 0.933$), indicating good size stability of the batches. There was no correlation between "size at experiment" and any of the other factors and "size at experiment" could therefore be included in the modelling.

During the modelling sample N16 was excluded as an outlier due to its large deviation from corresponding samples (N15 and Table 4: N4, N8, N12, N16).

The weighted regression coefficients of the best model are shown in Fig. 4. All the samples showed high sensitivity, mostly within the range 90–115% (calculated relative to cells exposed to HBSS). The reduced range of sensitivity compared to the previous designs caused poorer models with an explained variance of only 48% (1 PLSR component) for the best model. However, some main factors and interactions appeared to be significant. DMPC and PA were negatively correlated to the sensitivity, i.e. they increase the toxicity. The regression coefficient of the interaction DPPC × PA points to the opposite direction compared to the regression coefficient of PA, and the DPPC × PG interaction coefficient points to the opposite direction of PG. This shows that the toxicity of DPPC is relatively insensitive to the type of charged group (PA or PG). This can easily be seen in Fig. 5; the slope of the trend line for the DPPC samples is low. The influence of the amount of charge and the liposome size did not appear to be significant factors in the experimental room.

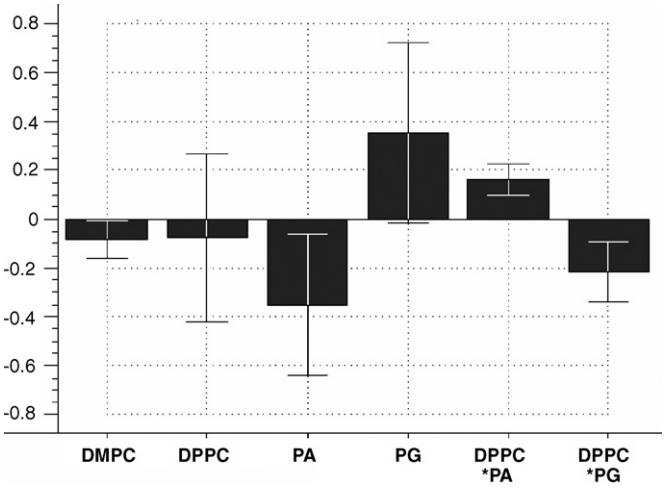

Fig. 4. Weighted regression coefficients (D-optimal design, negatively charged liposomes). Bars represent the confidence intervals for the estimated regression coefficients. Response variable: sensitivity—high sensitivity means low toxicity. The levels of the design variables are listed in Table 3.

4. Discussion

4.1. Influence on the toxicity of positively charged liposomes

The present study demonstrates that positively charged liposomes are toxic to the buccal cells. This is consistent with

Table 6
D-optimal design of negatively charged liposomes: characteristics of the samples

Sample	Main lipid (lipid)	Charged component	Amount charged component (mol%)	Size group	Size new batch		Size at experiment		Sensitivity (% of control)
					nm	PI	nm	PI	
N1	DMPC	DMPG	5	Small	106	0.05	110	0.06	111.8
N2	DMPC	DMPG	5	Large	515	0.46	488	0.37	119.4
N3	Egg-PC	egg-PG	5	Small	110	0.09	114	0.01	135.2
N4	Egg-PC	egg-PG	20	Large	512	0.43	474	0.47	115.9
N5	DPPC	DPPG	20	Small	102	0.09	110	0.18	101.4
N6	DPPC	DPPG	20	Large	385	0.43	450	0.53	113.3
N7	DMPC	DMPS	5	Large	535	0.53	521	0.52	123.4
N8	DMPC	DMPS	20	Small	88	0.05	96	0.13	95.4
N9	Egg-PC	DOPS	20	Small	97	0.08	94	0.08	116.4
N10	Egg-PC	DOPS	20	Large	499	0.51	454	0.31	93.7
N11	DPPC	DPPS	5	Small	99	0.05	105	0.10	115.7
N12	DPPC	DPPS	5	Large	513	0.49	692	0.38	98.8
N13	DMPC	DMPA	20	Small	87	0.09	205	0.72	99.3
N14	DMPC	DMPA	20	Large	353	0.56	1810	0.85	84.6
N15	Egg-PC	Egg-PA	5	Small	107	0.07	102	0.06	100.6
N16	Egg-PC	Egg-PA	5	Large	555	0.47	390	0.42	10.6
N17	DPPC	DPPA	5	Small	103	0.22	108	0.25	103.2
N18	DPPC	DPPA	20	Large	360	0.45	450	0.70	102.8

Lipid concentration 12 mM. Solvent HBSS without Mg and Ca (PI: polydispersity index).

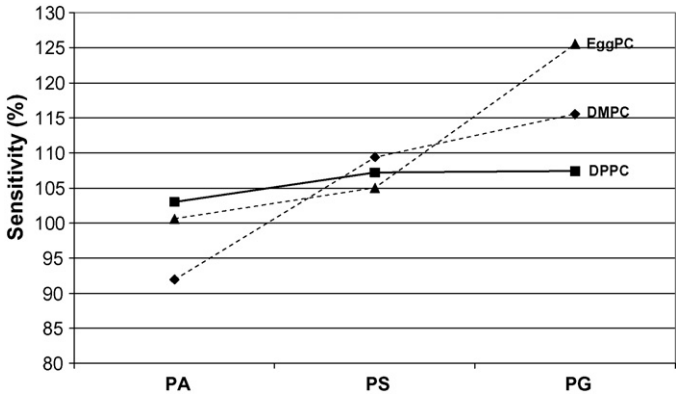

Fig. 5. Sensitivities of the negatively charged liposome formulations in the D-optimal design. Type of charged head group is indicated on the *X*-axis (PA: phosphatidic acid; PS: phosphatidylserine; PG: phosphatidylglycerol).

what has been reported in the literature to various other cell lines (Layton et al., 1980; Olson et al., 1982; Campbell, 1983; Mayhew et al., 1987; Filion and Phillips, 1997, 1998). SA was found to be more toxic than diacyl-TAP. The relatively high toxicity of SA has been suggested to be related to the fast desorption rate of the amine from the liposome (Parnham and Wetzig, 1993). Amines with two chains are expected to have slower desorption rate and lower toxicity; the lower toxicity was verified in the present study.

High level of the charged component was shown to increase the toxicity for positively charged liposomes. High amounts of charged component may be expected to increase the zeta potential of the liposomes. Due to instrumental problems we were not able to measure the zeta potential of the batches included in the toxicity experiments. However, new liposome batches with the same compositions were made later on and the zeta potential of these were determined (Table 5). The higher zeta potential of liposomes containing high level of charged component was verified. However, the results also showed that the zeta potentials of the diacyl-TAP batches with high amount of charged lipid were significantly higher than the zeta potentials of the corresponding SA batches. This suggests that the toxicity of the positively charged liposomes is not a result of the net liposomal charge alone but is also related to the properties of the charged lipid itself. This was also concluded by Filion and Philips who compared the toxicity of various positively charged liposomes on macrophages (Filion and Phillips, 1997).

The toxicity of egg-PC liposomes containing a low level (5 mol%) of positively charged component was found to be especially low. This may be explained by a possible lower degree of association of these liposomes with the cells. It has been shown that the fluidity of the lipid matrix may be more important in mediating the interaction with cells than the surface charge (liposomes containing 10 mol% of charged component were investigated) (Szoka et al., 1980). Also, it has been shown that gel phase liposomes associate with cells to a greater degree than fluid vesicles (Mayhew et al., 1980; Szoka et al., 1980). In our experiments all the egg-PC liposomes were in the fluid phase; T_c of egg-PC is in the range −5 to −15 °C (Cevc, 1993) and T_c of DOTAP has been reported to be −16.5 °C (Filion and Phillips, 1997). A low degree of association with the cells of the fluid egg-PC liposomes (5 mol% of charged component) may therefore be expected. At high content of charged component (20 mol%), however, it seems plausible that the electrostatic interaction with the negatively charged cell surface will mediate the association and improve the contact between the liposome and the cell. As unsaturated liposomes (soy- and egg-PC) have been shown to be more toxic to cells than saturated ones (DMPC, DPPC, distearoylphosphatidylcholine (DSPC)) (Juliano et al., 1987; Stensrud et al., 1999; Berrocal et al., 2000), the especially high toxic effect of egg-PC/DOTAP (20 mol%) observed in this study (Sections 3.1 and 3.2) may be a result of the combination of the effect of the positively charged lipid and the unsaturated egg-PC.

In the D-optimal design on the positively charged liposomes the type of main lipid was not a significant factor. The model included lipids with fatty acid chain length of C_{14} (DMPC), C_{16} (DPPC) and mixed chain length (egg-PC). During the modelling the factor DMPC all the time was close to be significant and all the time negatively correlated to the sensitivity. To investigate the influence of the chain length on the sensitivity a separate model was made on a subset of the data including only the saturated (DMPC, DPPC) positively charged (diacyl-TAP, SA) liposomes. The amount of charged lipid and liposome size (size at experiment) were included. The model showed three significant main factors; type of main lipid, the charged component and the amount of charged component (amount charge) (Fig. 6). Explained variance was 87% (2 PLSR components). The regression coefficient of the main lipid was positively correlated to the sensitivity and demonstrates that DMPC (defined as low level) was more toxic than the lipid containing the longer fatty acid chain length (DPPC) in our experiments. This is consistent with the literature showing that neutral DMPC liposomes are more toxic to macrophages than neutral DSPC liposomes (Stensrud et al., 1999). Also, DMTAP liposomes have been shown to be more toxic than DPTAP and distearoyl-trimethylammonium-propane

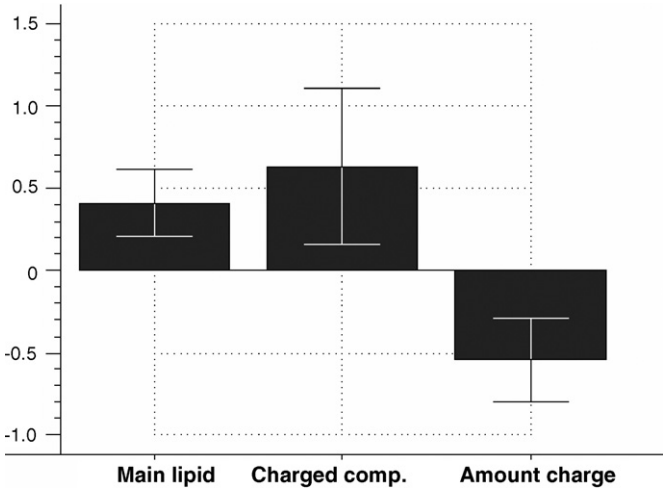

Fig. 6. Weighted regression coefficients (D-optimal design, positively charged liposomes). Subset model comprising only the liposomes containing DMPC or DPPC as the main phospholipid. Bars represent the confidence intervals for the estimated regression coefficients. Response variable: sensitivity—high sensitivity means low toxicity. The levels of the design variables are listed in Table 2.

(DSTAP) liposomes (other cell lines) (Filion and Phillips, 1997). Thus, the phospholipid fatty acid chain length seems to influence the cellular toxicity of liposomes based on saturated phospholipids.

The size of the liposomes was not found to be a significant factor for the positively charged liposomes (size range about 100–2000 nm) and this is consistent with findings on other cell lines (Filion and Phillips, 1997).

4.2. Influence on the toxicity of negatively charged liposomes

All in all the toxicity of the negatively charged liposomes on the buccal cells was low. This is consistent with previous reports on the toxicity of negative liposome formulations on other cell lines (Mönkkönen and Heath, 1993; Phillips et al., 1996; Stensrud et al., 1999). However, some significant factors and interactions were revealed; PA was shown to increase the toxicity but it was also shown that the toxicity of the DPPC liposomes was not affected by PA. This may be explained as follows: the negative DPPC formulations are in the gel phase for all the compositions investigated in this study, thus, the association ability for all these formulations may be expected to be about the same. If the toxicities of the charged head groups in fact are similar, then the toxicities of all the negative DPPC formulations should be similar. The significant interactions DPPC × PA and DPPC × PG suggest that this is true. When including DMPA in DMPC liposomes (T_c +50 and +23 °C, respectively, Cevc, 1993) the fluidity of the membrane will be affected and this may lead to improved ability to associate with the cells. DMPC was shown to exhibit some toxicity both in the D-optimal design for the negatively charged liposomes and in the sub-model with the positively charged ones. Higher toxicity of DMPC/DMPA liposomes compared to, e.g. DMPC/DMPG liposomes (T_c 23 °C, i.e. fluid phase) may therefore be expected even if the toxicities of the charged head groups are similar. Thus, the effect of PA observed in the D-optimal design may be a result of changed cell association ability and consequently an effect of the toxicity of DMPC rather than the toxicity of PA itself. The egg-PC/egg-PA liposomes are in the fluid phase (T_c of egg-PA is +18 °C, Cevc, 1993) and low association with the cells may be expected. The low toxicity of egg-PC/PA was verified both in the D-optimal design and in the screening design.

4.3. The multivariate approach

Usually cell toxicity studies are carried out by increasing the liposome concentration stepwise and determining the concentration or dose inhibiting 50% of the control (IC_{50} or ID_{50}) for every formulation. In a multivariate approach, however, specific factors are selected to determine the effect of these on the response. The IC_{50} for the different formulations cannot be compared. However, the specific factors influencing the response variable can be extracted and interactions between different factors can be revealed.

This work shows that positively charged liposomes are toxic to the buccal cells, that the toxicity of negatively charged liposomes is relatively low, that DMPC exhibit some toxicity, and also that the liposome size does not seem to influence the cell toxicity within the size range of about 100–1000 nm. Although different cell lines have been shown to vary significantly in their sensitivity to the same liposome formulation (Mayhew et al., 1987) our observations are consistent with what has been reported in the literature for other cell lines. This suggests that statistical experimental designs and multivariate evaluations are useful in cell toxicity screening studies and that the methods seem to be relatively robust.

An additional benefit of this approach is the possibility to reveal interactions. In our study several interactions were revealed: "lipid × charge" (Section 3.1) which shows that the type of charge (positive, negative) seems to be an extremely important factor for the toxicity of egg-PC liposomes, the interaction "egg-PC × amount charge" (Section 3.2) which shows that the toxicity of positively charged egg-PC liposomes was especially sensitive to the amount of charged component, and the interactions "DPPC × PA" and "DPPC × PG" (Section 3.3) which shows that the toxicity of negatively charged DPPC liposomes was relatively unaffected by the type of charged component. It has, however, to be mentioned that the interaction "lipid × charge" (Section 3.1) may include the liposome size instead of type of phospholipid because these factors were correlated. However, as size was not found to be a significant factor in the D-optimal designs, it seems most probable that the interaction is a result of lipid type and not the size.

In this study important factors influencing the toxicity on buccal cells have been extracted and should be taken into account when formulating liposomal delivery systems for use in the oral cavity. The conclusions are based on analysis of 46 liposomal formulations. Detecting these observations by univariate investigations would have required a lot of more experiments. The reduced number of samples necessary is an additional benefit of statistical experimental design and multivariate evaluation.

5. Conclusion

When formulating liposomes the choice of main lipid, the choice of charged component, and the amount of charged component in the liposomes are important issues and have to be considered to obtain liposomal formulations with minimum toxicity.

This work shows that positively charged liposomes are toxic to buccal cells in culture while the toxicity of negatively charged liposomes is relatively low.

When formulating positively charged liposomes a low amount of charged lipid is favourable, and diacyl-TAP is less toxic than SA. DPPC seems to be the best choice as the main lipid; DPPC is less toxic than DMPC. When using egg-PC as the main lipid in positively charged liposomes, a low level of charged lipid is essential. The most toxic combination in this study was egg-PC/positively charged lipid (20 mol%).

When formulating negatively charged liposomes, egg-PC may be used as the main lipid; the best formulation seems to be egg-PC/egg-PG. However, DPPC may also be a good choice as the toxicity of DPPC is relatively insensitive to the differ-

ent negatively charged head groups (DPPG, DPPS, DPPA). DMPC seems to exhibit some toxicity, and the combination DMPC/DMPA seems to be the least favourable. The amount of negatively charged component did not seem to affect the toxicity (experimental range 5–20 mol%).

The size of the liposomes and the total lipid concentration were not shown to be important within the experimental range (diameter within the size range of about 100–1000 nm, lipid concentration within 12–35 mM).

These conclusions have been drawn from toxicity screening experiments on a human buccal cell line by using a multivariate approach. In the literature different cell lines have been shown to vary significantly in their ID_{50} to the same liposome formulation, and such a screening may therefore be expected to be necessary for each cell line. However, our observations are consistent with what have been reported in the literature for other cell lines. This suggests that although the cell lines may have different ID_{50}, the trends may be comparable. This suggests that a multivariate approach is useful in cell toxicity screening studies. The benefits of this approach are the ability to reveal interactions between the design variables, and the reduced number of experiments necessary.

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