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Experimental design for in vitro skin penetration study of liposomal superoxide dismutase

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Abstract

A computer-based technique using a $2^{(5-2)}$ fractional factorial design was applied for screening the factors affecting the penetration effectiveness of liposomal recombinant human-Cu/Zn-superoxide dismutase. Unilamellar liposomes, containing recombinant rh-Cu/Zn-SOD in the aqueous core, are aimed at enhancing the penetration of the drug applied topically. Factors that mainly influence the chemical and physical characteristics of liposomes such as charge, molar content of cholesterol, size, surfactant and lipid were evaluated at two levels. In vitro skin penetration studies with pigskin were carried out in Franz-type diffusion cells over a period of 4 and 8 h. The response variables, namely the amounts of rh-Cu/Zn-SOD penetrated into the different skin layers, were analyzed by ELISA (enzyme linked immunosorbent assay). Analysis of variance showed that the size and the cholesterol content of liposomes as well as the duration of the penetration studies have a statistically significant influence on the amount of protein found in deeper skin layers.

Keywords: Experimental design; In vitro skin penetration study; Liposome; Superoxide dismutase; Rh-Cu/Zn-SOD; Biotechnology; Drug delivery

0. Introduction

For medical purposes, biotechnology has produced a wide range of molecules which can be applied topically, i.e. delivered through the skin in a controlled manner.

At the Institute of Applied Microbiology, in cooperation with Polymun Scientific, great efforts were attempt to investigate a liposomal format of the recombinant human Cu/Zn superoxide dismutase (rh-Cu/Zn-SOD) for local treatment. One of our therapeutical approaches is the treatment of Peyronie's disease (IPP) characterized by a variety of symptoms that are consequent to structural alterations of the penile corpora carvernosa. In the past, IPP which is strongly related with an increased level of free oxygen radicals, was successfully treated with intralesional injection therapy of SOD. To afford a more convenient local therapy, we developed a liposomal rh-Cu/Zn-SOD format, which was successfully applied in a phase-2 pilot study followed by a randomized, placebo controlled multi centre study [1,2].

Topical application is an attractive alternative to pharmaceuticals, particularly for diseases located in the skin and related tissue. However, the skin prevents the uptake of hydrophilic proteins of high molecular weight, and therefore it is of great interest to develop new strategies to overcome the skin barrier.

One method of increasing the penetration rate of a drug across the skin is encapsulation of the drug in lipid vesicles. Methods of studying interaction between vesicles and skin include diffusion experiments, visualization by electron microscopy and fluoromicrography [3,4]. To date, the factors influencing the efficiency of drug transport when using liposomes have been investigated in various in vitro skin penetration studies [5–9].

Penetration of drugs across the skin is a very complex process and can vary widely unless all procedures and operating conditions are kept constant. To begin with, there are different models to carry out penetration studies, and skin characteristics play an important role in them. Other factors are methods of

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liposome preparation, size and lipophilicity of the component and type of formulation. The biochemical and biophysical characteristics of liposomes are determined by their composition and influence the efficiency of drug transport to a great extent. As a consequence of the numerous variables in previous studies, interpretation of their results is controversial.

As to liposomal composition, there would be numerous combinations to be studied when optimizing them for drug transport. Besides, preparing liposomes as well as performing penetration studies are time-consuming and require costly material. Therefore, it is useful to employ an experimental design for screening the factors affecting the efficiency of the carriers in terms of enhancing drug delivery. In a 2^k full factorial design, where all possible combinations are tested, all factors are examined at two levels. The use of a $2^{(k-p)}$ fractional factorial design can considerably reduce the number of preparations. As a consequence, information about two-way interactions partly gets lost but the most important factors can still be identified and isolated. By focusing further on these factors, employing techniques such as the fold over, the resolution of the design increases. So one should consider carefully which design is chosen for a certain problem to obtain the expected information.

Based on our clinical results with liposomes consisting of dipalmitoyl-phosphatidyl-choline (DPPC), cholesterol and stearylamine, with a molar ratio of 7/2/1, the aim of the present study is to investigate the influence of liposome composition on the transport of SOD into pigskin by using the Franz diffusion cell, cryo-homogenization and ELISA. The screening for the influencing factors was based on a $2^{(k-p)}$ fractional factorial design, namely the quarter fraction design. This quarter fraction design was chosen to evaluate the potency of our formulation in comparison to alternative formulations.

1. Materials and methods

1.1. Materials

Dipalmitoyl-phosphatidylcholine (DPPC) and egg-phosphatidylcholine (EPC) were obtained from Lipoid, cholesterol and phosphatidylglycerol (PG) were purchased from Solvay, stearylamine (SA) and sodium-cholate were obtained from Sigma, and Carbopol 981 NF was supplied by Noveon. Phosphate buffered saline (PBS, pH 7.2–7.4) was used as penetration buffer. The drug to be incorporated into the liposomes was recombinant human superoxide dismutase (rh-Cu/Zn-SOD), expressed in *Escherichia coli*. This protein is available from the Institute of Applied Microbiology (IAM), produced in clinical grade quality by the project sponsor Polymun Scientific GmbH.

1.2. $2^{(k-p)}$ fractional factorial design

In the present study, the design chosen was the quarter fraction design. If five factors are examined at two levels, as in our case, the number of runs is reduced from 32 to 8 ($2^{(5-2)}$). Five factors that mainly influence the biochemical and biophysical characteristics of liposomes were examined: charge, molar content of cholesterol, liposome size, surfactant and lipid. Table 1 shows the two levels for the chosen factors.

Table 1
High and low settings (levels) for the five examined factors

Factors	Factor setting	
	High (+)	Low (−)
Charge	10 mol% SA (pos.)	10 mol% PG (neg.)
Cholesterol content	40 mol%	10 mol%
Liposome size	500 nm	200 nm
Surfactant (sodium-cholate)	0.1% (w/v)	Non
Lipid	DPPC	EPC

Factors for the experimental design and the corresponding concentrations on the levels. Stearylamine (SA) and phosphatidylglycerol (PG) were chosen to charge the surface of the vesicles. Liposome diameters are given in nm with their polydispersity indices (PI).

The statistical software package Statgraphics Plus 5.0 with experimental design capabilities was used to create the design, which means that the composition of eight combinations was determined randomly and put in random order. Table 2 shows the product combinations according to which the liposome preparations were produced.

Statistical data analysis was carried out using the software package SPSS 8.0. Statistically significant differences were determined using ANOVA with *P*-values less than 0.05 as a minimal level of significance. For the analysis of variance, absolute as well as relative data were used. Absolute data refer to the weight of the corresponding biopsy and the buffer used for enzyme extraction. They elucidate the absolute protein amount that penetrated into a certain area of skin. Relative data illustrate the percentage penetrated to applied protein and thus the utilization of the applied drug.

1.3. Liposome preparation

Unilamellar liposomes were produced by the well-established crossflow triple injection technique [10,11]. Lipids were dissolved in 96% ethanol, the protein concentration in the buffer solution was 30.00 mg/ml. The liposomes were prepared according to the eight product combinations created by the experimental design (Table 2). The formation of liposomes, prepared by the crossflow injection technique, occurs directly when the ethanolic-lipid-phase impinges on the aqueous-phase. As an intermediate product, so called planar lipid fragments are formed, which associate to unilamellar vesicles enclosing the protein containing aqueous phase. By varying the lipid concentration in the ethanolic phase, the drill whole diameter of the injection unit and the injection pressure, liposomes with different diameters can be produced without further downsizing. In order to separate non-entrapped protein and the access of ethanol the liposome suspensions were ultra-/dia-filtrated using Amicon membranes YM 100 (cut off: 100 kDa, Ø 44.5 mm). By repeated buffer changes, protein and ethanol free suspensions were obtained. After characterization (size, cholesterol content, amount of encapsulated protein), 0.5% Carbopolgel 981 NF formulations in sterile water were produced. The gels were filled in sterile syringes and stored at $5 \pm 3\,^{\circ}C$.

Table 2
Liposomal formulations based on the ($2^{(5-2)}$) plan

Formulation	Charge	Cholesterol (mol%)	Size (nm)	Surfactant	Lipid
1	SA	40	220 ± 10 nm, PI 0.15	+	EPC
2	PG	10	220 ± 10 nm, PI 0.15	+	DPPC
3	PG	10	500 ± 20 nm, PI 0.20	+	EPC
4	PG	40	500 ± 20 nm, PI 0.20	−	EPC
5	SA	10	220 ± 10 nm, PI 0.15	−	EPC
6	SA	10	500 ± 20 nm, PI 0.20	−	DPPC
7	SA	40	500 ± 20 nm, PI 0.20	+	DPPC
8	PG	40	220 ± 10 nm, PI 0.15	−	DPPC

This table represents the experimental design ($2^{(5-2)}$ plan) generated by Statgraphics Plus 5.0. Eight samples, respectively formulations, according the chosen parameters were determined randomly.

1.4. Liposome characterization

According to the experimental design, the size and the cholesterol content were two of the factors being examined at two levels. The compliance with the predetermined levels was checked with qualified and validated analytical methods.

1.4.1. Liposomal size

Photon correlation spectroscopy (Precision Detectors) was used to measure the diameter of the unilamellar vesicles, and the polydispersity index (PI) was used to qualify unimodal size distribution. Liposomes with the setting high (+) were about 500 ± 20 nm in diameter with a polydispersity index of 0.20 and those with the setting low (−) were about 200 ± 10 nm with a PI of 0.15.

1.4.2. Cholesterol content

The cholesterol content of the liposomes in the suspensions as well as in the gels was analyzed by rp-HPLC [12]. Liposomes were dissolved in 2-propanol, analytical grade (1:1). Fifty microliters of the clear sample was injected. Analysis was performed with methanol, HPLC-grade, with a flow rate of 1ml/min on a reverse phase column (Spherisorb, C8, 5 μm ODS1, 80A, Waters) at 25 ± 1 °C. The HPLC apparatus was Agilent 1100 HPLC with a variable wavelength detector, detection wavelength 207 nm (Agilent). Data were processed with HP-Chemstation-software, version 07.01. Method validation, including the sample preparation was validated according the validation guidelines of the international conference on harmonization (ICH). Data demonstrated suitable accuracy, precision and linearity over the range of interest (10–300 μg/ml). Data demonstrated appropriate recovery from the sample matrix. Limit of detection and quantification were calculated. The recovery in the preparations was 95 ± 3% related to the weighed amount.

1.4.3. Rh-Cu/Zn-SOD content

To examine the encapsulation efficiency of rh-Cu/Zn-SOD, lipid bilayers of the liposomes were first disintegrated and solubilized by diluting the liposome suspension 1:2 with sodium cholate (240 mg/ml) and incubation for 2 h at 37 ± 3 °C. Then the protein content was analyzed by a sandwich ELISA, established at the IAM, based on two different monoclonal antibodies. The murine antibody IAM-SOD-M05 was selected as the capture antibody and the second murine antibody IAM-SOD-M08 was conjugated with alkaline phosphatase. *P*-nitrophenylphosphate-sodium salt (pNPP) was chosen as the substrate for detection. As standard rh-Cu/Zn-SOD quantified photometrically was used.

A total of 96 well, module plates (Maxisorp) were coated overnight at 5 ± 3 °C with capture antibody (100 μl/well, 2 μg/ml, in coating buffer: 8.4 g/l $NaHCO_3$, 4.2 g/l Na_2CO_3, pH 9.7). For each assay, we performed a duplicate standard curve by utilizing serial dilutions (1:2) of standard SOD (50–0.39 ng/ml) in sample dilution buffer [phosphate buffered saline plus 0.1% Tween 20 (PBS-T) and 1% bovine serum albumin (BSA)] to quantify sample concentrations. All samples were equally treated in 1:2 dilution steps. The test plates were washed four times with PBS-T. Then 50 μl/well of the sample and standard dilution series were applied and incubated for 1 h at 22 ± 3 °C. Plates were washed four times with PBS-T before applying the conjugated antibody, diluted to (1:800) in sample dilution buffer. After incubation for 1 h at 22 ± 3 °C, the plates were washed six times with PBS-T and 100 μl/well of p-NPP solution (1 mg/ml), diluted in 10 ml coating buffer, were applied to each well. The plates were incubated at 22 ± 3 °C. The optical density was read with an ELISA plate reader (Tecan Sunrise) at 405 nm with a reference wavelength of 692 nm. ELISA software Magellan was utilized for processing the standard curve and for calculation of the amount of rh-Cu/Zn-SOD in the samples. ELISA was validated for liposome suspensions, and liposome gel formulation. For all samples blank and spiked samples at different concentrations were analyzed. Parameters, as mentioned in Section 1.4.2, were evaluated. Data were suitable over the range of interest.

1.5. Skin

Full thickness skin of male and female pigs weighing 30 ± 2 kg was used. The skin sections were taken postmortem by means of a dermatome, wrapped in mull bandages that were soaked in Ringer solution and stored at ≤−20 °C. Before carrying out a penetration experiment the skin sections were thawed overnight at 5 ± 3 °C. Therefore, the wrapped skin sections were soaked in sterile RO–H_2O and placed on polystyrene. All this

was put in a plastic bag whose bottom was covered with sterile RO–H_2O. The thawed skin was unwrapped and disks of 25 mm in diameter were cut out. Skin thickness was measured with a sliding calliper.

It is critical to maintain the integrity of the stratum corneum during storage and preparation, because otherwise, permeability would be overestimated. Therefore, skin integrity was evaluated before starting the penetration experiment in order to eliminate damaged skin. Skin sections were checked visually for physical damage. After mounting the skin samples in the Franz diffusion cell and allowing the skin to equilibrate, a pre-study integrity evaluation was made by determining the specific conductivity as a measure for the electrical resistance.

$$\lambda = G\frac{L}{A} = \frac{1}{R}$$

λ is the specific conductivity, G the conductivity, L the length, A the area and R is the electrical resistance. As skin integrity may change because of physical wash-off procedures, hydration or effects of the test formulation, a post-study integrity evaluation was also carried out after rinsing off the excess of the test formulation. Specific conductivity of equilibrated, untreated skin was evaluated by repeated measurements, whereas, a relative average conductivity of 1000 $\mu S/cm^2$ was measured for intact skin. Therefore, penetration studies were only performed with skin samples of about this value. Post-study integrity shows neither increasing nor decreasing conductivity after penetration.

1.6. Penetration studies

1.6.1. Franz diffusion cell

The thawed skin sections were equilibrated in phosphate-buffered saline (PBS, pH 7.2–7.4) for 30 min at 22 ± 3 °C before mounting them on the Franz diffusion cell. The epidermal side of the skin was exposed to ambient conditions while the dermal side was bathed in PBS. The receptor fluid was mixed with a magnetic stirring bar. Buffer was kept at 32 ± 1 °C by a water jacket. After examining the integrity of the skin and drying the surface with a cotton bud, 170 mg of the liposomal gel formulations were applied non-occlusively onto the skin surface with a syringe. The amount was determined by weighing the syringe before and after applying the sample. All experiments were carried out in three parallel diffusion cells for a time period of 4 and 8 h, respectively, and were repeated with the skin of another pig. At the end of the penetration experiment, the surface of the skin was rinsed three times with PBS. Finally, the skin was once more examined for integrity.

1.7. Skin treatment and protein quantification

1.7.1. Biopsies and skin fractionation

After the post-study integrity examination, the skin was dried with cotton buds. Then an exact area of 0.95 cm^2 covered with the formulation during the penetration study was punched out of the section that had been placed in the diffusion cell. Next, the skin disks were placed on a heater at 60 ± 1 °C for 20 s and subsequently, using two forceps, the epidermis was separated from the dermis. The separated biopsies were weighed for further calculations and stored at ≤−20 °C.

1.7.2. Homogenization and protein extraction

The frozen biopsies were pulverized in liquid nitrogen in a Cryomill (microdismembrator, B. Braun Melsungen A.G). Before milling, 500 μl PBS were added so that the protein rh-Cu/Zn-SOD could be extracted in the same step. After centrifugation of the homogenized skin samples at 5 ± 3 °C, 10 min, 12,000 rpm, extracts containing rh-Cu/Zn-SOD were obtained and stored at ≤−20 °C. Untreated skin and skin spiked with liposomal and free rh-Cu/Zn-SOD were prepared with the same procedure for method validation.

1.7.3. Protein quantification

The rh-Cu/Zn-SOD content in the extracts of epidermis and dermis was quantified with a high sensitive sandwich ELISA established at the IAM, based on two different monoclonal antibodies. The murine antibody IAM-SOD-M05 was selected as the capture antibody. In contrast to the assay employed for quantification of the liposomal SOD the second antibody was biotinylated. Streptavidin-peroxidase-conjugate (500 U) with 1,2-*o*-phenylendiamine-dihydrochloride (OPD) as substrate was chosen for detection. Rh-Cu/Zn-SOD (2 μg/ml) was used as a standard.

A total of 96 well, module plates (Maxisorp) were coated overnight at 5 ± 3 °C with capture antibody (100 μl/well, 2 μg/ml, in coating buffer: 8.4 g/l $NaHCO_3$, 4.2 g/l Na_2CO_3, pH 9.7). For each assay, we performed a duplicate standard curve by utilizing serial dilutions (1:2) of standard SOD (4–0.03 ng/ml) in sample dilution buffer [phosphate buffered saline plus 0.1% Tween 20 (PBS-T) and 1% bovine serum albumin (BSA)] to quantify sample concentrations. All samples were equally treated in 1:2 dilution steps. Test plates were washed four times with PBS-T. Then 50 μl/well of the sample and standard dilution series were applied and incubated for 1 h at 22 ± 3 °C. Plates were washed four times with PBS-T before applying the biotinylated antibody, diluted 1:4000 in sample dilution buffer. After incubation for 1 h at 22 ± 3 °C, plates were washed four times with PBS-T. Fifty microliters of Streptavidin-peroxidase-conjugate, diluted 1:10,000 in sample dilution buffer, were applied to each well and incubated for 20 min at 22 ± 3 °C. After a final six-cycle washing procedure, 100 μl/well of staining solution were added (100 μl OPD, 10 ml staining buffer (7.3 g/l citric acid H_2O, 11.86 g/l $Na_2HPO_4{\cdot}2H_2O$, pH 5.0) and 10 μl H_2O_2, 35%) and incubated at 22 ± 3 °C. The enzymatic reaction was stopped by applying 100 μl of H_2SO_4 (2.5%) to each well. The optical density was read with an ELISA plate reader (Tecan Sunrise) at 492 nm with a reference wavelength of 620 nm. ELISA software Magellan was utilized for processing the standard curve and for calculation of the amount of rh-Cu/Zn-SOD in the samples. ELISA was validated for the skin samples and receptor fluids obtained in the skin penetration model. For all samples blank and spiked samples at different concentrations were analysed. Parameters, as mentioned in Section 1.4.2, were evaluated. Data were suitable over the range of interest.

2. Results

2.1. Content of rh-Cu/Zn-SOD in the skin layers

According to the experimental design (Table 2) eight liposomal formulations were produced and applied non-occlusively onto the surface of pig skin. In vitro skin penetration studies were carried out in a Franz diffusion cell with three parallel chambers. The experiments with each formulation were repeated with the skin of another pig. This is of statistical importance when certain experiments, in our case the experiments with the skin derived from one animal, are blocked in one group in order to see if there is a statistically significant difference between blocks. After skin fractionation, homogenization and extraction, the content of rh-Cu/Zn-SOD was analyzed in the skin extracts. Fig. 1 shows the mean absolute protein content in the skin for the epidermis and the dermis (the two response variables) after 4 and 8 h, penetration.

After 8 h, formulations No. 2 and No. 5 result in a high rh-Cu/Zn-SOD content, both in the epidermis and in the dermis. Fig. 2 represents the corresponding relative data, namely the percentage penetrated to applied protein amount.

2.2. Factor analysis

Five main factors that influence the biochemical and biophysical characteristics of liposomes were examined at two levels using a quarter fraction design. All experiments were carried out with the skin of two pigs (blocked into two sets) and over a time of 4 and 8 h. In order to yield a more restrictive design and higher statistical significance, data of all studies were evaluated together. Therefore, the statistical influence of the factors 'block' and 'duration' could also be calculated. The amounts of rh-Cu/Zn-SOD analyzed in epidermis and dermis represented the two response variables.

2.3. Evaluation of main effects

First of all, the main effects of the factors were calculated. Multivariate analysis of variance was carried out with absolute and relative amounts of rh-Cu/Zn-SOD. Absolute amounts refer to the weight of the corresponding biopsy. The percentage of penetrated vs. applied protein represents the relative data. Statistical significance of the factors was tested by *P*-values. *P*-values less than 0.05 denote that the corresponding factor has a statistically significant effect on the response variable at the 95.0% confidence level. The ANOVA results are shown in Tables 3 and 4.

In Table 3, all factors marked with a '+' have a statistical significance on the corresponding response variables.

The *P*-values of these factors are less than 0.05, as shown in Table 4. Factors marked with a '−' and with a *P*-value greater than 0.05 do not have a statistical significance on the corresponding response variables.

Whether or not a certain factor has a positive or negative effect on a response variable can be assessed by parameter estimate '*B*'. The parameter estimates are listed for the factors at the low level.

Table 3
Statistical significance of the main effects

	Absolute		Relative (%)		*B* for (−1)
	Epidermis	Dermis	Epidermis	Dermis	
Block	−	−	−	−	
Charge	−	+	−	−	(−)
Cholesterol	+	+	+	+	(+)
Size	+	+	+	+	(+)
Surfactant	−	+	−	+	(+)
Lipid	−	−	−	−	
Duration	+	+	+	+	(−)

Statistical significance of the main factors tested on account of the *P*-values. If there is a statistically significant difference, the signs of the parameter estimates (*B*) for the low level of the main factors are shown. '−' means no significance; '+' means significant; (−) means negative effect; (+) means positive effect.

A negative *B*-value denotes that the corresponding factor has a negative effect on the response variable. *B*-values for factors without a statistical significance are not listed because they are irrelevant. As the *B*-values were the same for the analysis of absolute and relative data, they are only shown in one column for both.

To examine if the individual skin has an influence on the penetration efficiency of rh-Cu/Zn-SOD, the experiments were repeated with the skin of another pig. It turned out that the block and thus the individual skin were statistically insignificant. On the contrary, cholesterol content, liposome size and the time of the penetration showed a statistically significant influence on the protein content in epidermis and dermis. Considering the parameter estimate, small liposomes, a molar cholesterol content of 10% and a penetration period of 8 h had a positive effect on the penetration of the drug. In addition, liposomes with negative charge as well as liposomes without surfactant showed a statistically significant positive effect on the protein content in the dermis.

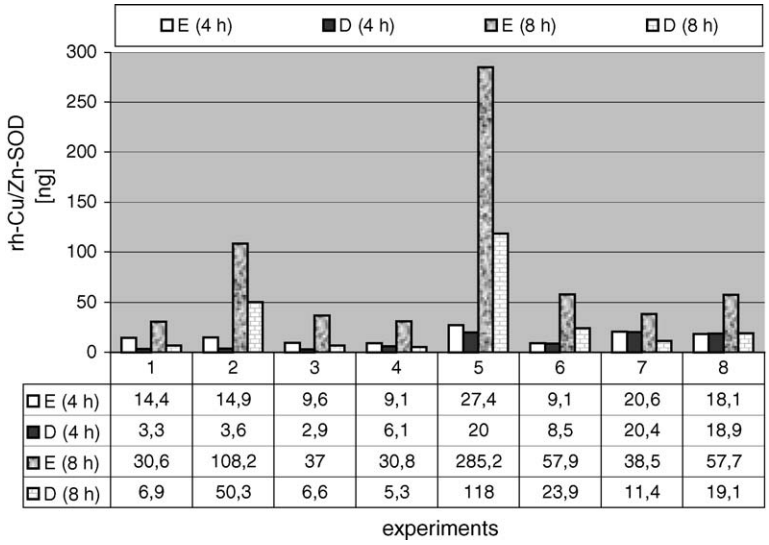
2.4. Two-way interactions

The present fractional factorial design is resolution III, so the main effects are confounded with two-factor interactions. As a consequence, only some two-way interactions can be calculated. Amongst these, statistical significance could be shown for interactions between the factors cholesterol × time, size × time and

Table 4
P-values and parameter

	Absolute		Relative (%)		*B* for (−1)
	Epidermis	Dermis	Epidermis	Dermis	
Block	0.661	0.830	0.850	0.176	
Charge	0.175	0.038	0.624	0.306	(−)
Cholesterol	0.020	0.003	0.005	0.002	(+)
Size	0.007	0.001	0.047	0.007	(+)
Surfactant	0.125	0.018	0.117	0.006	(+)
Lipid	0.304	0.725	0.985	0.082	
Duration	0.000	0.001	0.000	0.000	(−)

P-values and parameter estimates for the main effects. If there is a statistically significant difference, the signs of the parameter estimates (*B*) for the low level of the factors are shown. (−) means negative effect; (+) means positive effect.



	1	2	3	4	5	6	7	8
E (4 h)	14,4	14,9	9,6	9,1	27,4	9,1	20,6	18,1
D (4 h)	3,3	3,6	2,9	6,1	20	8,5	20,4	18,9
E (8 h)	30,6	108,2	37	30,8	285,2	57,9	38,5	57,7
D (8 h)	6,9	50,3	6,6	5,3	118	23,9	11,4	19,1

Fig. 1. Absolute SOD amount. Absolute SOD content analyzed in the epidermis (E) and dermis (D) after penetration over a time period of 4 and 8 h, respectively.

cholesterol × size. The *P*-values for the calculated interactions are shown in Table 5. If there is a statistically significant difference, the signs of the parameter estimates for the low level of both factors are shown.

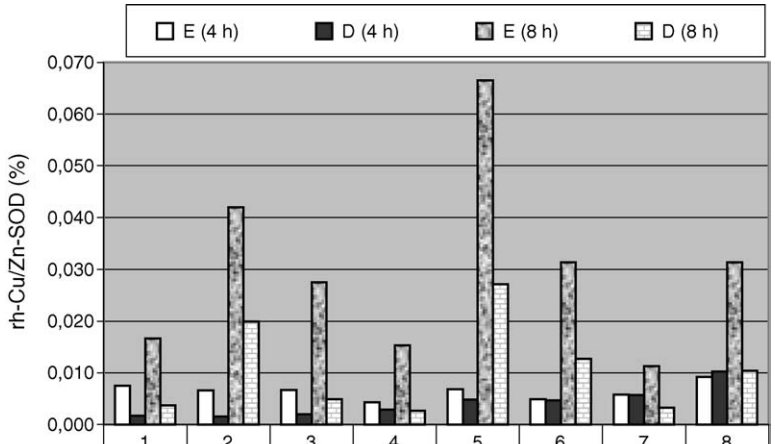
2.5. Box plot analysis

Box plots are graphical summaries of the presence of outlier in data for one or two variables. They are particularly useful for comparing parallel batches of data and divide the data into four equal areas of frequency. A box encloses the middle 50%, where the median is represented as a horizontal line inside the box. The vertical lines, called whiskers, extend from each end of the box to the highest and lowest values, excluding outlier. In

Table 5
P-values for certain two-way interactions

	Absolute		Relative (%)		*B* for (−1/−1)
	Epidermis	Dermis	Epidermis	Dermis	
Charge × duration	0.225	0.199	0.602	0.419	
Cholesterol × duration	0.010	0.000	0.003	0.000	(−)
Size × duration	0.024	0.002	0.107	0.014	(−)
Surfactant × duration	0.102	0.133	0.110	0.391	
Lipid × duration	0.346	0.256	0.901	0.953	
Cholesterol × size	0.020	0.001	0.687	0.331	(+)

P-values for certain two-way interactions. If there is a statistically significant difference, the signs of the parameter estimates for the low level of both factors are shown. (−) means negative effect; (+) means positive effect; × means two-way interaction.



	1	2	3	4	5	6	7	8
E (4 h)	0,008	0,007	0,007	0,004	0,007	0,005	0,006	0,009
D (4 h)	0,002	0,002	0,002	0,003	0,005	0,005	0,006	0,010
E (8 h)	0,017	0,042	0,028	0,015	0,067	0,031	0,011	0,031
D (8 h)	0,004	0,020	0,005	0,003	0,027	0,013	0,003	0,010

Fig. 2. Relative SOD amount. Relative SOD content analyzed in the epidermis (E) and dermis (D) after penetration over a time period of 4 and 8 h, respectively. Ratio between applied rh-Cu/Zn-SOD and concentration in the skin is given in percent.

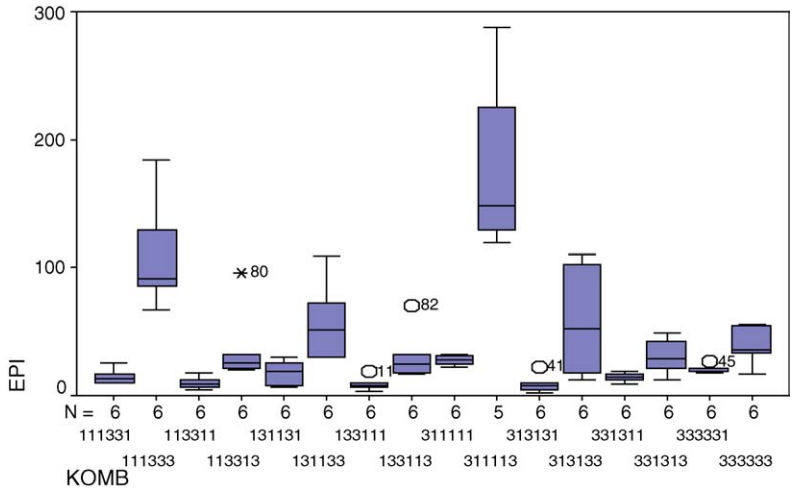


Fig. 3. Absolute rh-Cu/Zn-SOD content in the epidermis. Protein content in the epidermis, drawn in box plots for the eight formulations, applied over a period of 4 and 8 h each. EPI means epidermis; KOMB means combination of the factors charge, cholesterol, size, surfactant, lipid and duration; $N = 6$ means six single results of parallel experiments forming one box plot.

the following figures, box plots were drawn for the absolute and relative protein amount penetrated into epidermis and dermis. The plots allow to visually compare the efficiency of the different liposomal formulations. The code for the composition of the corresponding formulation and its incubation time is written below so one can assess immediately which combinations result in a high protein content in the different skin layers. The factors are put in the order charge, cholesterol, size, surfactant, lipid and time while '1' means low level and '3' means high level of the corresponding factor. Fig. 3 shows box plots for the absolute amounts of protein analyzed in the epidermis. It can be noticed that the two formulations containing small liposomes with a low content of cholesterol and being applied for 8 h (box plot 2 and 10) show considerably higher rh-Cu/Zn-SOD amounts in the epidermis than all other combinations.

The absolute amount of protein analyzed in the dermis is shown in Fig. 4. Again, the two formulations containing small liposomes with low cholesterol content applied over 8 h (box plot 2 and 10) show considerably higher protein content in the dermis. The box plots drawn by the relative protein content of the epidermis are shown in Fig. 5.

They illustrate that in addition to the two formulations described above (box plot 2 and 10), two further combinations (box plot 6 and 12) seem to result in a high amount of penetrated protein. Size, cholesterol content and time seem to be important, as in these box plots either size or cholesterol content and time are on the level that was shown to have a positive effect (Table 3).

Fig. 6 illustrates the relative protein amounts analyzed in the dermis, and a trend similar to the one for the relative protein content in the epidermis can be noticed.

3. Discussion

Topical application of drugs is very promising as it allows controlled and continuous delivery into the skin. Furthermore, it makes it possible for drugs to be applied locally, and thereby avoid systemic side effects. The most important limitation to the transdermal route is the skin barrier, mostly provided by the stratum corneum. Already the uptake of molecules with a molecular weight about 200–350 Da is very difficult and achieving the same task for molecules greater than 750 Da is practically impossible, even when these molecules have an ideal solubility in the skin [13,14]. Regarding the protein of interest, the rh-Cu/Zn-SOD with 33 kDa, penetration without any penetration enhancement must be excluded.

One strategy to overcome the skin barrier is the encapsulation of drugs into liposomes, vesicles consisting of a lipid bilayer that act as carriers. Interactions between liposomes and the stratum corneum have been studied thoroughly and have shown to enable penetration of drugs into the skin. To find out about the factors influencing the efficiency of drug transport, numerous penetration studies have been performed [5–9].

It turned out that parameters like the liposomal composition, the preparation method, the type and amount of applied formulation including additional penetration enhancers such as ethanol in the formulation, the duration of the penetration experiments and the origin of the skin play an important role and influence the penetration results to a great extent. Over the years, some encouraging data were reported on the dermal use of suspensions based on phospholipids. However, success is a relative matter, depending on the means by which lipid vesicles can improve the performance of the topical agent delivery. At least, depot mechanism, solubility increase, permeation enhancement and penetration-based delivery can be employed by these principles. As so many parameters were varied in the previous penetration studies, it is hard to draw general conclusions.

In the present study, a screening based on a $2^{(k-p)}$ fractional factorial design (Section 2.2) was carried out to investigate the influence of liposome composition on the transport of the hydrophilic protein rh-Cu/Zn-SOD into pig skin. The fractional factorial design was chosen to evaluate the liposomal penetration-based delivery exclusively for our target protein. Therefore, in the present penetration studies (Section 1.6),

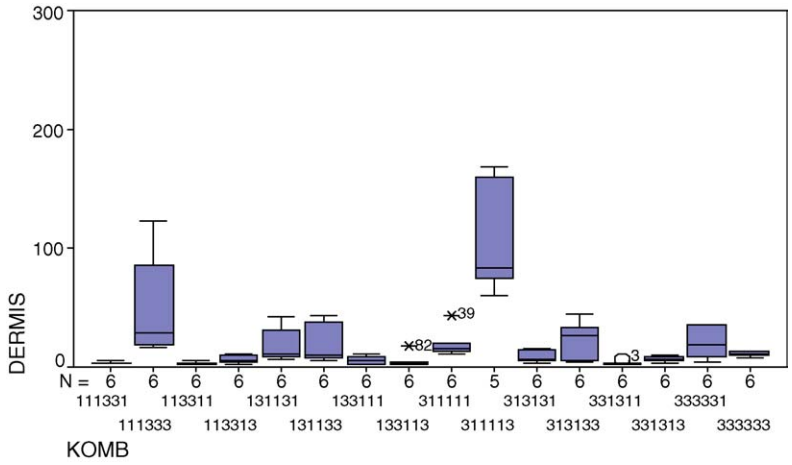


Fig. 4. Absolute rh-Cu/Zn-SOD content in the dermis. Protein content in the dermis, drawn in box plots for the eight formulations, applied over a period of 4 and 8 h each. KOMB means combination of the factors charge, cholesterol, size, surfactant, lipid and duration; $N = 6$ means six single results of parallel experiments forming one box plot.

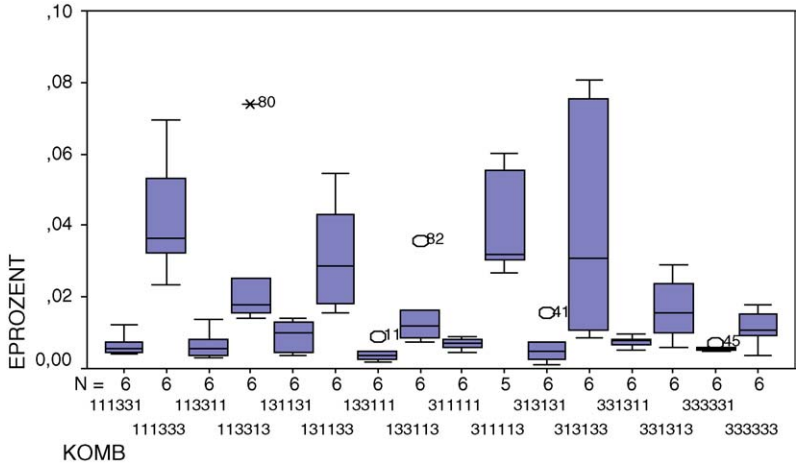


Fig. 5. Relative rh-Cu/Zn-SOD content in the epidermis. Relative protein content in the epidermis, drawn in box plots for the eight formulations, applied over a period of 4 and 8 h each. Eprozent means relative protein content in the epidermis; KOMB means combination of the factors charge, cholesterol, size, surfactant, lipid and duration; $N = 6$ means six single results of parallel experiments forming one box plot.

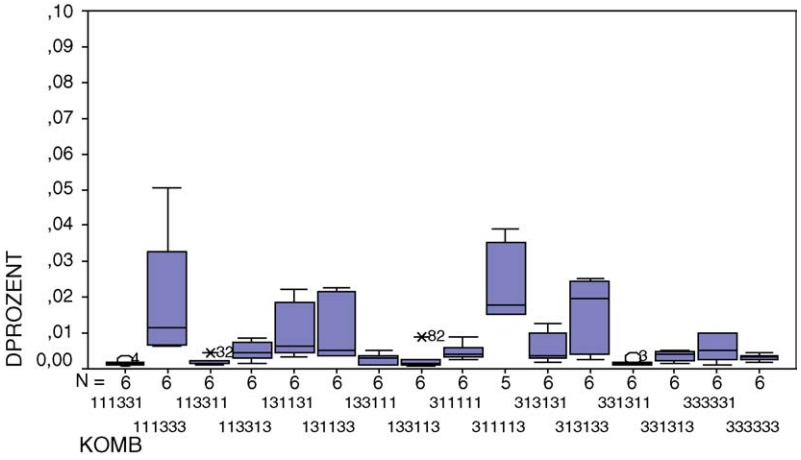


Fig. 6. Relative rh-Cu/Zn-SOD protein content in the dermis. Relative protein content in the dermis, drawn in box plots for the eight formulations, applied over a period of 4 and 8 h each. Dprozent means relative protein content in the dermis, KOMB means combination of the factors charge, cholesterol, size, surfactant, lipid and duration; $N = 6$ means six single results of parallel experiments forming one box plot.

all procedures and operating conditions were kept constant. In accordance with the experimental design (Table 2), eight liposome suspensions of varying composition were prepared by the crossflow triple injection technique [10,11]. This technique allows the continuous production of sterile liposomes, free of pyrogens without using organic solvents, all of these being prerequisites for medical use. By this method vesicles composed of various lipids and different diameters can be produced by varying the preparation conditions. Prior penetration, each liposome suspension was spread in a hydrophilic gel, Carbopol 981NF, which was prepared in the presence of sterile water.

For liposomes to effectively penetrate the skin and release the encapsulated drugs, they have to interact with the stratum corneum. One possible interaction can be described as follows: liposomes penetrate the stratum corneum and permeate into the lamellar lipid region that is located between the corneocytes. Along this tortuous pathway liposomes interact with neighbouring cells and release their content [15]. When penetrating intact skin, liposomes have to pass through a series of very fine pores. While vesicles with rigid membranes fail to do so, liposomes with flexible, high deformable membranes can squeeze themselves between the cells in the stratum corneum [16].

To develop a therapeutically efficient carrier system, the composition of liposomes has to be optimised because it is the crucial factor in delivering the incorporated drug. In our study, the factors charge, content of cholesterol, size, surfactant and lipid were examined at two levels. Each liposome suspension was produced with the cross flow injection technique, meaning that the lipids were dissolved in ethanol prior injection. The environmental ethanol, as well as the non-entrapped SOD, were removed quantitatively by repeated diafiltration. Nevertheless, the bilayer associated ethanol should be taken into account. Depending on the cholesterol concentration and temperature, more or less ethanol is partitioning into lipid bilayers, whereas the precise chemical structure is less important. Studies performed by Trandum et al. (2000), showed that the partitioning of ethanol into lipid bilayers reach a maximum in liposomes of low cholesterol concentration ($\leq$10%), whereas, cholesterol in large amount reduces the partitioning of ethanol significantly [17]. In addition, the interaction parameter is closely dependent on temperature. Interaction in low cholesterol systems decrease with temperature, while in high cholesterol systems interaction increase slightly. Liposomes produced by our crossflow injection technique were repeatedly analyzed with small angle X-ray technique to calculate the membrane thickness and distance between the phosphorous headgroups. In comparison to liposomes prepared without ethanol no difference is obvious, meaning that no interdigitation occurs. In addition, ethanol quantification of our liposome suspensions confirms that our present study is not influenced by penetration enhancement of ethanol.

A prerequisite for the medical use of liposomal formulations is a stable product. Depending on their composition and size, liposomes are more or less susceptible to chemical and physical degradation. Aggregation of neutral liposomes is brought about by Van der Waals interactions. The simplest way to overcome this is to include a small quantity of negative charge, e.g. 10% phosphatidylglycerol (PG) [18]. Compounds with positive charges such as stearylamine (SA) also decrease potential aggregation and eventual fusion of vesicles [19].

Concerning penetration efficiency, a positive surface potential might relevant as it enables interaction between liposomes and negatively charged skin and tissue cells. It is evident that charged compounds have a stabilizing effect on liposomes. Whether or not they play a role in penetration cannot be ascertained yet. According to Cevc et al. (1992), surface charge has been proved to influence drug penetration into deeper skin layers. However, our results did not indicate any statistically significant impact on the amount of rh-Cu/Zn-SOD penetrated into the skin.

Cholesterol is often used as a main component of the lipid membrane. It has been shown to modify the order and mobility of the phospholipids in the bilayer [20,21] and as a consequence, it alters the bilayer fluidity. Other studies indicate that cholesterol content might be of crucial importance for the effective delivery of liposome-entrapped substances into the skin [22].

Coderch et al. (2000) observed contrary effects of cholesterol depending on the degree of saturation of the employed lipid [23]. Fluidity of membranes containing unsaturated lipids gradually decreased as the amount of cholesterol increased. For membranes containing saturated lipids cholesterol had a fluidising effect at temperatures below the transition temperature T_m, while it had a condensing effect at temperatures above T_m. In the same study, a correlation between skin penetration results and membrane fluidity could be observed. In our study, cholesterol also had a statistically significant influence on the penetration efficiency of the drug, but in contrast to earlier findings, liposomes with a lower cholesterol content resulted in higher amounts of penetrated protein.

The influence of liposome size seems to be important, too, but all previous studies failed to clarify this subject. Du Plessis et al. (1994) showed that the intermediate particle size of 300 nm resulted in both the highest reservoir in the deeper skin layers, as well as the highest drug concentration in the reservoir, confirming that topical drug delivery is influenced by the size of liposomes [24]. Šentjurc et al. (1999) examined liposomes of different composition and observed that size was not of great significance for the transport of hydrophilic substances into the skin, as long as the diameter of the liposomes was larger than 200 nm [5]. For smaller liposomes transport was significantly decreased. However, Verma et al. (2003) showed that the penetration of a hydrophilic compound was inversely related to the size of the liposomes [25]. In the present study, liposomes including the hydrophilic drug rh-Cu/Zn-SOD with a size of 200 ± 10 and 500 ± 20 nm diameter were tested, and the results indicate that smaller liposomes has a positive effect on the penetration of rh-Cu/Zn-SOD across the skin. If much smaller liposomes would improve the penetration of rh-Cu/Zn-SOD was not studied. In fact, preparation of smaller liposomes with the cross flow injection technique is in principle possible, but the encapsulation efficiency of rh-Cu/Zn-SOD decreases beneath an economically justifiable value.

Addition of a surfactant during liposome preparation was another parameter being examined. Surfactants tend to fluidise membranes and make them very elastic, resulting in highly deformable vesicles. Cevc et al. (1996) showed that these flexible

vesicles can squeeze themselves between the cells in the stratum corneum and are thus able to deliver drugs into the skin with a very high efficiency [16]. El Maghraby et al. (2000) showed that incorporation of surfactants into vesicles reduced the main T_m, which indicates fluidization of the lipid bilayer [26]. The results suggested that there might be an optimum concentration for each surfactant to yield maximum drug delivery into the skin. For sodium cholate the refined concentration in vesicles containing phosphatidylcholine (PC) was 14.0% (w/w). We used sodium cholate concentrations of 0.1% (w/v), what is equivalent to 5.4% (w/w). When employing the crossflow triple injection technique, the vesicle forming process happens in one third of the total volume. In this case, the maximum surfactant concentration that allows stable vesicles to form is lower. In contrary to other studies [26,27] that showed enhanced drug penetration for surfactant containing, ultra-flexible liposomes, our results suggested more a negative than an effect on the protein content in the dermis when adding surfactants.

Lipids are the major structural components of liposomes and therefore also have a great influence on the fluidity characteristics of the liposomal membrane. Depending on the chain length and the degree of saturation, they show a different T_m. Usually phospholipids occurring in natural membranes are employed, which makes liposomes very safe vehicles for medical application.

For the purpose of this study, the lipids of choice were synthetic dipalmitoyl-phosphatidylcholine (DPPC), also used for our liposomes in the clinical trials, containing a saturated C16 fatty acid and forming rigid membranes, and naturally occurring egg-phosphatidylcholine (EPC), a mixture of fatty acids of different chain length and varying degree of saturation. Although the characteristics of these lipids differ widely, there was no statistically significant influence of the lipid on the amount of drug delivered. This result is in contrast to the works of Coderch et al. (2000), showing higher drug penetration into the stratum corneum for liposomes containing unsaturated PC [23].

4. Conclusion

In summary, evaluation of the main effects indicate that liposomes about 200 nm with a molar cholesterol content of 10% as well as a penetration time of 8 h had a statistically significant positive influence on the delivery of the model drug rh-Cu/Zn-SOD. The three parameters size, cholesterol and time also showed two-way interactions, which does not necessarily mean that positive effects can only be obtained when combining them on the level described. In general, all combinations resulted in a higher penetration rate after 8 h than after 4 h, which could be explained by the depot effect of liposomes [15].

Within the scope of our study, the use of surfactants, usually described as positive, yielded a negative effect on the content of protein in the dermis, although previous biochemical studies exclude any protein denaturing of the rh-Cu/Zn-SOD. This might be due to the choice of the surfactant concentration that had to be determined when setting up the experimental design. To clarify this subject, further studies based on the results obtained could be carried out using liposomes about 200 nm, a molar cholesterol content of 10% and varying amounts of surfactant.

It can be concluded that the experimental design, namely the quarter fraction design, was appropriate for screening. In our case, the data of the experimental design confirm the clinical observations. However, this technique is also suitable in basic research for screening factors influencing the efficiency of liposomes as drug carriers. Unfortunately, the use of such a design can hardly be found in literature, even though it is a very useful means of obtaining valid results while reducing the number of time-consuming and costly experiments.

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References

[1] C.R. Riedl, E. Plas, K. Vorauer, B. Vcelar, A. Wagner, H. Pflüger, Eur. Urol. 40 (2001) 343–349.
[2] C.R. Riedl, P. Sternig, G. Gallé, F. Langmann, B. Vcelar, K. Vorauer, A. Wagner, H. Katinger, H. Pflüger, Eur. Urol. 48 (2005) 656–661.
[3] M.E.M.J. Kuijk-Meuwissen van, H.E. Junginger, J.A. Bouwstra, Biochim. Biophys. Acta 1371 (1998) 31–39.
[4] R. Alvarez-Románn, G. Merino, Y.N. Naik, R.H. Guy, J. Pharm. Sci. 92 (2003) 1138–1146.
[5] M. Šentjurc, K. Vrhovnik, J. Kristl, J. Control. Release 59 (1999) 87–97.
[6] E. Touitou, N. Dayan, L. Bergelson, B. Godin, M. Eliaz, J. Control. Release 65 (1999) 403–418.
[7] G. Betz, R. Imboden, G. Imanidis, Int. J. Pharm. 229 (2001) 117–129.
[8] G. Betz, P. Nowbakht, R. Imboden, G. Imanidis, Int. J. Pharm. 228 (2001) 147–159.
[9] S.I. Simoes, T.C. Delgado, R.M. Lopes, S. Jesus, A.A. Ferreira, J.A. Morais, M.E. Cruz, M.L. Corvo, M.B. Martins, J. Control. Release 103 (2005) 419–434.
[10] A. Wagner, K. Vorauer-Uhl, G. Kreismayr, H. Katinger, J. Liposome Res. 12 (2002) 259–270.
[11] A. Wagner, K. Vorauer-Uhl, G. Kreismayr, H. Katinger, J. Liposome Res. 12 (2002) 271–283.
[12] J.K. Lang, J. Chromatogr. 507 (1990) 157–163.
[13] K.A. Walters, M.S. Roberts, in: K. Walters, J. Hadgraft (Eds.), Pharmaceutical Skin Penetration Enhancement, Marcel Dekker, New York, 1993, pp. 339–358.
[14] G. Cevc, Biochim. Biophys. Acta 1031 (1990) 311–382.
[15] J.A. Bouwstra, H.E.L. Hofland, F. Spies, G.S. Gooris, H.E. Junginger, in: O. Braun-Falco, H.C. Korting, H.I. Maibach (Eds.), Liposome Dermatics, Springer, Heidelberg, 1992, pp. 121–136.
[16] G. Cevc, G. Blume, A. Schätzlein, D. Gebauer, A. Paul, Adv. Drug Del. Rev. 18 (1996) 349–378.
[17] C. Trandum, P. Westh, K. Jorgensen, O.G. Mouritsen, Biophys. J. 78 (2000) 2486–2492.
[18] R.R.C. New, Liposomes, A Practical Approach, Oxford University Press, 1990, pp. 99–102.
[19] K. Vorauer-Uhl, A. Wagner, H. Katinger, Eur. J. Pharm. Biopharm. 54 (2002) 83–87.
[20] A. Nagumo, Y. Sato, Y. Suzuki, Chem. Pharm. Bull. 39 (1991) 3071–3074.
[21] R. Constanzo, T. De Paoli, J.E. Ihlo, A.A. Hager, H.A. Farach, C.P. Poole, J.M. Knight, Spectrochim. Acta 50A (1994) 203–208.

[22] K. Vrhovnik, J. Kristl, M. Sentjurc, J. Smid-Korbar, J. Pharm. Res. 15 (1998) 525–530.
[23] L. Coderch, J. Fonollosa, M. De Pera, J. Estelrich, A. De La Maza, J.L. Parra, J. Control. Release 68 (2000) 85–95.
[24] J. Du Plessis, C. Ramachandran, N. Weiner, D.G. Müller, Int. J. Pharm. 103 (1994) 277–282.
[25] D.D. Verma, S. Verma, G. Blume, A. Fahr, Int. J. Pharm. 258 (2003) 141–151.
[26] G.M.M. El Maghraby, A.C. Williams, B.W. Barry, Int. J. Pharm. 196 (2000) 63–74.
[27] G. Cevc, A. Schätzlein, G. Blume, J. Control. Release 36 (1995) 3–16.