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In vivo study of liposomes as drug carriers to oral mucosa using EPR oximetry

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Abstract

The purpose of this study was to select the best types of liposomes for use as drug carriers for topical treatment of oral mucosal lesions. Electron paramagnetic resonance (EPR) oximetry, using the paramagnetic probe lithium phthalocyanine, was used in vivo to measure the effects of a hyperemic drug, benzyl nicotinate (BN) which was incorporated into liposomes of varying size and composition. The liposomes were made from either hydrogenated or non-hydrogenated soy lecithin and mixed with polymethyl methacrylate ointment for application. EPR oximetry was used to measure the partial pressure of oxygen (pO_2) in the oral mucosa before and after application of liposomes. It was found that the most pronounced changes of pO_2 in oral mucosa and also the longest action of the drug occurred after the topical application of BN in multi-lamellar liposomes made from hydrogenated soy lecithin ($p < 0.0001$). When these liposomes were applied to oral mucosa over 3 successive days it was found that pO_2 increased the most on the first day, the effect gradually decreased following application on the second and third days. The duration of the resulting hyperemia was the longest on the second day ($p < 0.01$). Among the examined carriers, multi-lamellar liposomes made from hydrogenated soy lecithin appear to be the most appropriate for local drug delivery to oral mucosa.
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Keywords: Drug delivery; Liposomes; In vivo EPR oximetry; Oral mucosa; Rat

1. Introduction

One of the characteristics of the oral mucosa is its selective permeability, which permits its use for local or systemic delivery of some drugs (Hicks, 1973; Squier and Johnson, 1975; Galey et al., 1976; Pimlott and Addy, 1985; Barsuhn et al., 1988; Harris and Robinson, 1992; Sveinsson and Holbrook, 1993). In man, there are numerous applications for local delivery of drugs to oral lesions, such as in the treatment of aphthous stomatitis, lichen planus, bacterial and fungal infections and periodontal disease (Harris and Robinson, 1992; Petelin et al., 1998; Shojaei, 1998). Many drugs are absorbed through damaged mucosa, but the success of topical treatment of lesions with intact oral mucosa depends on the selection of: (i) a suitable active ingredient in an appropriate carrier, (ii) the rate of penetration through the mucosa and (iii) the residence time of the active ingredient in oral mucosa. An appropriate vehicle is essential for successful application of local drug delivery systems in the oral cavity as the vehicle influences availability of the active ingredients for absorption, their rate of penetration and their residence time in the tissues. For example, ointments used for local drug delivery to the oral mucosa need to have excellent muco-adhesive properties (Bremecker et al., 1984).

Topical treatment of ulcerative inflammatory diseases is associated with several general disadvantages, like the high permeability of damaged oral mucosa which may permit uncontrolled absorption of drugs into the circulation with unwanted local and distant side effects (Squier and Johnson, 1975; Bremecker

Abbreviations: ASC, sodium ascorbate; ASL, *N*-1-oxyl-2,2,6,6-tetramethylammonium iodide; AUC, area under the curve; BN, benzyl nicotinate; EPR, electron paramagnetic resonance; EXT, extruded liposomes; HSL, hydrogenated soy lecithin; LiPc, lithium phthalocyanine; MLV, non-extruded liposomes; mT, milliTesla; mW, milliWatt; NSL, non-hydrogenated soy lecithin; PI, polydispersity index; PMM, polimethyl methacrylate; pO_2, partial pressure of oxygen; PSC, photon correlation spectroscopy

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et al., 1984). Liposomal formulations have been used to both enhance absorption and to regulate release of incorporated drugs, thus localizing the effect of the drugs (Harsayi et al., 1986), both enhancing local and decreasing systemic drug concentrations (Mazei and Gulasekharam, 1982; Harsayi et al., 1986).

The aim of this study was to investigate liposome formulations of varying composition and size to identify a suitable carrier for drug delivery to oral mucosal lesions by assessing the effects of a hyperaemic drug on the oral mucosa using in vivo EPR oximetry.

2. Materials and methods

The study protocol was submitted to and approved by the Veterinary administration of the Republic of Slovenia (No. 323-02-76/01).

2.1. Animals and implantation of the paramagnetic probe

The Faculty of Medicine Laboratory of Pathology, Ljubljana, Slovenia supplied adult female Wistar rats between 7 and 9 weeks old, weighing 200–250 g. Anaesthesia was induced using intraperitoneal injection of a mixture containing xylazine hydrochloride, 10 mg/kg (Rompun, Bayer, Leverkusen, Germany) and ketamine hydrochloride, 75 mg/kg (Ketanest 50, Parke-Davis, Berlin, Germany). Anaesthesia was maintained for the necessary 2 h period by administration of a further increment of the drug mixture, at half the initial dosage, after 1 h. Twenty-four hours prior to starting electron paramagnetic resonance (EPR) measurements the rats were anaesthetized and the paramagnetic probes, lithium phthalocyanine (LiPc) crystals (a generous gift from the EPR centre for viable tissues, Dartmouth College of Medicine, Hanover, New Hampshire, USA), were implanted beneath the buccal mucosal epithelium through a 23 gauge injection needle (Microlance, Becton Dickinson, Fraga, Spain). The needle tip was filled with approximately 0.1 mm^3 of LiPc microcrystals (the approximate volume of individual crystals was 0.5 μm^3). The needle was inserted 2 mm into the buccal submucosa laterally from the injection site and the microcrystals deposited about 1 mm below the mucosa surface using a stylette. Measurements were started 24 h after the implantation of the paramagnetic probe, permitting time for the oxygen concentration to equilibrate between the probe and the surrounding tissue and to minimize the risk of the initial stress and tissue injury from the implantation procedure affecting the results. The delay also permitted the injection site to seal, reducing the likelihood that either the drug or carrier would permeate directly through the wound. Each rat was anaesthetised and the EPR spectral line-width measured for 15 min to obtain the baseline partial pressure of oxygen (pO_2) in the tissue before application of drug. As the body temperature tends to decrease during anaesthesia (Petelin et al., 2004), temperature homeostasis was maintained at 36.5 ± 0.5 °C throughout anaesthesia by use of a thermostatically controlled warm air 'blanket' linked to a rectally placed glass encased thermocouple.

2.2. Liposomes with encapsulated hyperaemic drug

Liposomes were prepared by the thin film method (Lasic, 1993) from cholesterol and either hydrogenated or non-hydrogenated soy lecithin (HSL or NSL) in a weight ratio of three parts cholesterol to seven parts lecithin, with or without the addition of the active drug. The lipophilic phase of the liposomes, containing phospholipid, cholesterol and when appropriate the hyperaemic drug—benzyl nicotinate (BN) (Lek; Ljubljana, Slovenia), was dissolved in dichloromethane for NSL or in a chloroform/methanol mixture (1:1, v/v) for HSL due to their differing properties. The solvent was removed in a rotary evaporator to give a thin film on the wall of the flask. Remaining solvent was removed completely under vacuum (10–15 min at 40 °C and pressure 100 Pa). The dry film was hydrated with distilled water at approximately 80 °C for HSL (i.e. above its phase transition temperature) and at room temperature (22 °C) for NSL. The flask was shaken until the film was completely removed from the walls. The liposome dispersion was stabilized by stirring for 2 h on a magnetic stirrer (300 rpm) at room temperature. A 1 ml sample of the final active liposome dispersion contained 25 mg of lipids and 12.5 mg of BN. Higher BN concentrations could not be used as they inhibited formation of liposomes.

A Liposofast extruder (Avestin, Ottawa, Canada) was used to grade multi-lamellar liposomes (MLV) at slightly above the phase transition temperature. The samples were extruded through a series of polycarbonate membranes with defined pore diameter (Nucleopore Corporation, Pleasation, CA), starting with the 800 nm and subsequently going to 100 nm pore diameter.

2.3. Characterization of liposomes

Liposome size and polydispersity index (PI) were determined by both photon correlation spectroscopy (PCS; Zetasizer 3000, Malvern, UK) at a fixed angle of 90° and by the laser diffraction method (Malvern analyzer; Mastersizer X, UK) in order to detect the larger particles in the liposome dispersion. For PCS, the samples were diluted with dust-free water to give the recommended scattering intensity of approximately 100,000.

For stability measurements liposomes were prepared as described above except that instead of water the liposomes were hydrated with a 10 mM aqueous solution of the spin probe *N*-1-oxyl-2,2,6,6-tetramethyl-ammonium iodide (ASL) so that this became incorporated into the liposomes. The charged ASL molecules do not easily penetrate intact liposome membranes. Uncaptured spin probe was removed by overnight dialysis at 4 °C (Petelin et al., 1998). Liposome stability was measured by the EPR response of the ASL incorporated into the liposomes. The liposome dispersion was mixed with a 0.1 M aqueous solution of sodium ascorbate (ASC), another charged molecule that does not easily penetrate intact liposomes. ASC is a reducing agent, so on exposure to ASC any free ASL paramagnetic spin probe is converted into the corresponding inactive hydroxylamine. EPR spectral intensity was measured over time following preparation of liposomes or after mixing the liposomes with

the carrier (detailed below). As long as the liposome membrane is stable EPR spectra intensity remains unchanged, however, after disintegration ASL is released from the liposomes and is reduced by ASC. Decay in EPR spectral intensity with time is therefore a measure of liposome instability (Petelin et al., 1998).

2.4. Formulation for application

A muco-adhesive polymethyl methacrylate (neutralized co-polymer of methacrylic acid and methyl methacrylate, Sigma–Aldrich, Steinheim, Germany) based ointment (PMM) was used for the application of the liposomal formulations to the buccal mucosa. PMM was prepared as described elsewhere (Sveinsson and Holbrook, 1993; Petelin et al., 1998). Briefly, 9.99 g of methacrylic acid and 4.28 g of methyl methacrylate were mixed and sufficient of an 11.5 g/50 ml solution of NaOH added to achieve a pH of 6.5. Gelatin, 0.69 g, was incorporated to ensure proper PMM formulation and water was added to bring the total mass up to 129.8 g. The mixture was then warmed to and maintained at 50 °C for 30 min. Liposome suspension with encapsulated BN was mixed with PMM in a weight ratio of 2:3, resulting in a final concentration of 0.5% BN (w/w) in the final PMM formulation. The prepared liposome containing PMM was used within 24 h of mixing to minimize any effects of loss of activity on storage.

The effect of PMM alone and PMM mixed with BN having already been tested (Petelin et al., 2004), PMM containing empty liposomes (i.e. prepared without BN) was used as a negative control.

2.5. EPR measurements

In vivo EPR oximetry measurements were obtained from the oral cavity as has been described in detail elsewhere (Petelin et al., 2004). Briefly, 0.05 ml of the prepared liposomal formulation was applied to the surface of the buccal mucosa over the LiPc implantation site using a dispensing pipette.

Measurements were made by placing the surface coil of an extended loop resonator (11 mm diameter) over the implanted area and EPR spectra recorded on a Varian E-9 EPR spectrometer with a custom-made low frequency microwave bridge (designed by Dr. T. Walczak, Dartmouth college of Medicine, Hanover, N.H., USA), operating at 1.1 GHz. Recordings were made under the following conditions: magnetic field 44–45 milliTesla (mT), modulation amplitude 2.5×10^{-3} mT and microwave power 20 milliWatt (mW). The EPR spectral line-width (ΔB), which is proportional to local oxygen partial pressure (pO_2), was measured and converted to mucosal pO_2 according to the calibration curve for LiPc as published elsewhere (Sentjurc et al., 2001, 2004). Since the calibration curve is linear the pO_2 could be calculated from the equation:

$$\Delta B\,(\text{mT}) = 0.0059 + 3.9 \times 10^{-4} \times pO_2\,(\text{mmHg})$$

Each experimental formulation, including the negative control was tested in eight or nine rats. In order to obtain basal pO_2 in the oral mucosa, five EPR spectra were recorded before the application of the test formulation and the mean value was taken as a basal pO_2. The local pO_2 changes were then measured over 90 min (at 2–5 min intervals) following application of the test material. As the basal pO_2 value varied from animal to animal in the range of 18–25 mmHg, the difference in pO_2 with respect to the basal value was used rather than absolute values. Efficiency of both drug absorption and action were evaluated for each type of liposomes by recording the following time points: onset of increasing tissue oxygenation (lag time, t_{lag}), the maximal pO_2 (ΔpO_{2max}), the time when pO_{2max} was reached (t_{max}), the time of return to the basal pO_2 levels (t_{end}) and area under the curve (AUC). The measurements were repeated on 3 successive days.

2.6. Statistical analysis

The hypothesis of average equality in different groups was tested with one-way single factor ANOVA. GLM procedure for non-balanced data was used for the analysis of data. When the analysis of variance test was significant, post-test analysis was performed using the Duncan test in order to find the specific difference. p-Values of less than 0.05 were accepted as statistically significant.

3. Results

3.1. Liposome size

From laser diffraction experiments the portion of particles larger than 0.8 μm is estimated to be less than 0.1%, therefore their contribution can be neglected.

Plots of PCS measurements of liposome size distribution (intensity of light scattering against the size of liposomes) typically shows two populations of vesicles with different mean diameter, which are presented in Table 1. In non-extruded liposomes, the proportion of both populations is similar, while in the extruded liposomes more than 90% of the population has diameters in the region of 260 and 175 nm for HSL and NSL liposomes, respectively.

3.2. Stability of liposome dispersion

Liposomes in water solution were stable for more than 1 week. In 1 week, the EPR spectral intensity decrease was less than 10% for all types of liposomes. However, after mixing with PMM the EPR spectral intensity decreased by 60% for non-extruded HSL and by 40% for extruded HSL. After the initial decrease the EPR spectra remains stable indicating that after initial destruction of a proportion of the liposomes those that remain are stable for more than 100 h. On the other hand, for NSL, immediately after mixing with PMM the intensity decays only by about 20%, and in extruded liposomes remains unchanged after that, but gradually decreases in non-extruded liposomes. Our results show that NSL are more stable with respect to the mixing with PMM than HSL. This is not surprising taking into account that at both room and physiological temperatures HSL are in the more rigid gel phase, while NSL are in the liquid crystal phase, being much more elastic, at same temperatures.

Table 1
Mean diameter of hydrogenated soy lecithin (HSL) and non-hydrogenated soy lecithin (NSL) liposomes before (MLV) and after extrusion (EXT)

Type of liposomes	Mean diameter (nm)		Proportion of each population		Width of the distribution (nm)	
	Peak 1	Peak 2	Peak 1	Peak 2	Peak 1	Peak 2
HSL						
MLV	415	1820	43	57	83	324
EXT	260	1100	97	3	107	460
NSL						
MLV	245	890	56	44	104	155
EXT	51	175	7	93	25	104

Typically two populations of liposomes are visible, with different mean diameters. The estimated portion of liposomes for each population and the width of the size distribution within the two populations are also presented.

3.3. Effect of anaesthesia on pO_2 in oral mucosa

In preliminary experiments where only basal pO_2 was measured, it was found that tissue oxygenation is influenced by anaesthesia. After the administration of the anaesthetic combination the pO_2 in oral mucosa decreased and only stabilised after about 30 min. After 1 h, as anaesthesia lightened, the pO_2 started to rise again (Fig. 1). As measurements of local pO_2 changes after the application of liposomal formulation were performed over a 90 min period. As anaesthesia had to be maintained for approximately 2 h, an additional incremental dose of the anaesthetic combination was given 1 h after the initial dose (without the animal being moved). This was found satisfactory to maintain the anaesthesia to the completion of measurements and also to stabilize the pO_2 throughout much of this time. With respect to the initial influence of anaesthesia the formulations were not applied to the mucosa until the basal pO_2 had stabilized following induction of anaesthesia.

3.4. Effect of different types of liposomes on pO_2 in oral mucosa

It was previously found that PMM alone has no influence on oral mucosa oxygenation (Petelin et al., 2004). Control studies show that inclusion of empty liposomes also has no effect on pO_2 (Figs. 2 and 3). For the test substances the lag time (t_{lag}), the maximal relative increase of pO_2 after the application of the liposomes and the time when this was reached (ΔpO_{2max}, t_{max}), the area under the curve (AUC), and the time when BN stops acting (t_{end}) were determined from the individual pO_2 curves. The influence on the

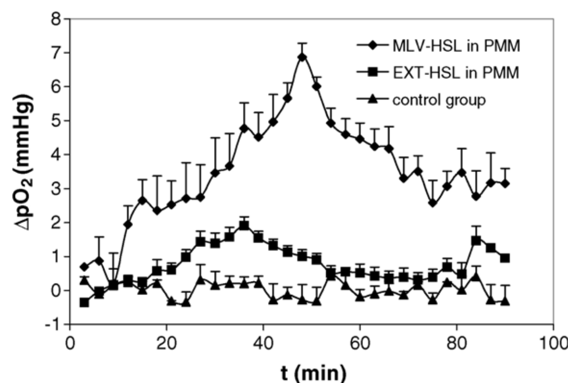

Fig. 2. The time-course of oxygen level variation (ΔpO_2) in rat oral mucosa on the first day of measurements after the first application of benzyl nicotinate in HSL liposomes of different sizes: (♦) non-extruded HSL liposomes (MLV-HSL), (■) extruded HSL liposomes (EXT–HSL) and (▲) control group (PMM with empty liposomes). Each point represents mean value ± S.D. of eight to nine measurements.

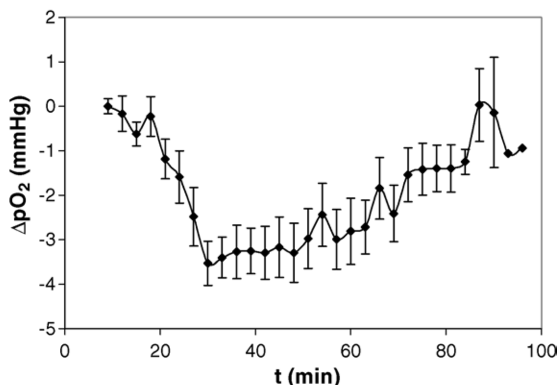

Fig. 1. Effect of a single anaesthetic dose on partial pressure of oxygen (pO_2) in rat oral mucosa (mean value ± S.D. of four measurements). There was a steady decrease in the pO_2 in oral mucosa during the first 30 min following administration of the anaesthetic mixture. The level then remained approximately constant for next 30 min before starting to increase as the anaesthetic effects wore off.

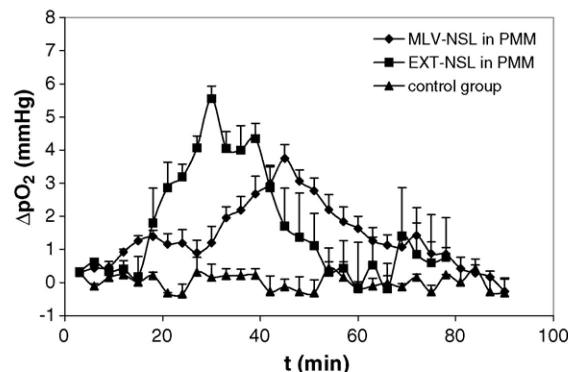

Fig. 3. The time-course of oxygen level variation (ΔpO_2) in rat oral mucosa on the first day of measurements after the first application of benzyl nicotinate in NSL liposomes of different sizes: (♦) non-extruded (MLV–NSL) liposomes, (■) extruded (EXT–NSL) liposomes and (▲) control group (PMM with empty liposomes). Each point represents mean value ± S.D. of eight to nine measurements.

Table 2
The effect of topical application of benzyl nicotinate (BN) incorporated in liposomes of various size and composition on oxygenation of the rat oral mucosa

Liposomes	N	ΔpO_{2max} (mmHg)	t_{max} (min)	t_{lag} (min)	AUC (mmHg min)	t_{end} (min)
MLV–HSL	8	6.87 ± 0.78a	48 ± 11e	13.63 ± 7.59	267.72 ± 103.87*	[a]
EXT–HSL	9	1.91 ± 0.56b	36 ± 7f	12.00 ± 4.50	53.59 ± 37.37	50.33 ± 22.59
MLV–NSL	9	3.75 ± 1.01c	45 ± 10e	16.22 ± 8.35	107.87 ± 48.99	57.22 ± 14.84
EXT–NSL	8	5.56 ± 1.02d	30 ± 6f	12.00 ± 7.35	86.87 ± 8.00	40.25 ± 10.40

Number of measurements (N), the maximal relative increase of pO_2 after application of the liposomes (ΔpO_{2max}), the time when pO_{2max} was reached (t_{max}), the time when the BN starts to act (lag time, t_{lag}), area under the curve (AUC), the time when BN stops to act (t_{end}) after the application of BN incorporated in different carriers.
Different letters (a–d) denote statistically significant difference between different carriers (analysis of variance, Duncan's test; $p < 0.0001$).
Different letters (e and f) denote statistically significant difference (analysis of variance, Duncan's test; $p < 0.01$). Each value represents the mean ± S.D. of measurements from the first day of measurements.
* Statistically significant difference from the other carriers (analysis of variance, Duncan's test; $p < 0.0001$).
[a] The effect lasted more than the time of measurements.

oxygenation of the oral mucosa of BN incorporated in HSL or NSL liposomes is shown in Figs. 2 and 3. The influence of the different carriers investigated was significant. The major changes of pO_2 in oral mucosa occurred after the application of non-extruded liposomes made from hydrogenated soy lecithin (MLV–HSL) ($p < 0.0001$), which is expressed in maximal changes of pO_2 as well as in the AUC. Also the effect of the drug lasted the longest, for this type of liposomes ($p < 0.0001$). The average pO_2 remained above the basal value more than 90 min, i.e. beyond the measurements period. The least effect on rat oral mucosal oxygenation was observed for extruded liposomes from hydrogenated soy lecithin (EXT–HSL), this being lower than for liposomes from non-hydrogenated soy lecithin. When BN was incorporated in liposomes made from non-hydrogenated soy lecithin, the effectiveness was more pronounced when incorporated in multi-lamellar (MLV–NSL) compared to the extruded (EXT–NSL) liposomes, although the differences are much less noticeable than with HSL liposomes. Maximal pO_2 is greater for extruded liposomes, while the time required for maximal increase in pO_2 to be achieved was longer, as was the duration of effects, for non-extruded liposomes.

The analysis of variance test for non-balanced data showed significant differences ($F = 54.37, p < 0.0001$) in the average values of the maximal increase of pO_2 (ΔpO_{2max}) for all four carriers (Table 2). Statistically significant differences ($F = 4.47$, $p = 0.01$) in the values of t_{max} were observed between extruded and non-extruded liposomes of both types (NSL and HSL). The maximal change in pO_2 in oral mucosa was achieved sooner for extruded than for the non-extruded NSL and HSL liposomes, while the differences in t_{lag} between different carriers were not significant. However, the most pronounced effect was observed for MLV–HSL liposomes, where the pO_2 remained above the baseline in the time exceeding the time of measurement. The effectiveness of the BN expressed as AUC, was the most pronounced when applied in MLV–HSL (Table 2) ($F = 17.65$, $p < 0.0001$) while for other formulations the differences in AUC were not significant.

When the liposome preparations were applied to oral mucosa over 3 successive days, it was found that the recorded pO_2 increased most on the first day. The duration of the effect of BN (AUC) was the longest for MLV–HSL ($p < 0.01$), after the second application of liposomes (Table 3). For NSL liposomes, the effectiveness decreases with successive treatments. At the

Table 3
The effect of daily repeated topical application of benzyl nicotinate (BN) incorporated in different types of liposomes on oxygenation of the rat oral mucosa over a period of 3 days

Liposomes	N	Time (days)	ΔpO_{2max} (mmHg)	t_{max} (min)	t_{lag} (min)	AUC (mmHg min)	t_{end} (min)
MLV–HSL	3	1	7.55 ± 1.8	30 ± 7	18 ± 3	182 ± 42	[a]
MLV–HSL	4	2	6.47 ± 1.2	33 ± 6	18 ± 5.2	278.1 ± 74	[a]
MLV–HSL	3	3	5.80 ± 0.9	24 ± 8	6 ± 2	155.2 ± 39	[a]
EXT–HSL	4	1	1.58 ± 0.3	7 ± 6.9	7 ± 1.8	24.7 ± 8	42 ± 8.2
EXT–HSL	4	2	2.37 ± 0.4	9 ± 8	10 ± 2.1	52.2 ± 12	42 ± 6
EXT–HSL	3	3	1.10 ± 0.1	19 ± 9	12 ± 3.1	21.8 ± 7	39 ± 7
MLV–NSL	4	1	5.30 ± 1.6	21 ± 7	15 ± 3	138.3 ± 69	57 ± 12.4
MLV–NSL	4	2	4.60 ± 1.4	27 ± 8	9 ± 2	117.1 ± 44	42 ± 6.9
MLV–NSL	3	3	3.20 ± 0.8	21 ± 6.8	12 ± 3	58.9 ± 8	27 ± 5
EXT–NSL	3	1	6.90 ± 2.1	18 ± 9.2	6 ± 2	114 ± 54	30 ± 4.9
EXT–NSL	4	2	5.70 ± 1.8	15 ± 6	6 ± 2.1	90 ± 33	27 ± 3.6
EXT–NSL	4	3	4.45 ± 1.5	30 ± 7.5	21 ± 5.3	75 ± 37	42 ± 12

Number of measurements (N), the maximal relative increase of pO_2 after the application of the liposomes (ΔpO_{2max}), the time when pO_{2max} was reached (t_{max}), the time when the BN starts to act (lag time, t_{lag}), area under the curve (AUC), the time when BN stops to act (t_{end}) after the application of BN incorporated in different carriers.
[a] The effect lasted more than 90 min.

third application of the extruded liposomes (of both types) the lag-time and the time when the maximal increase in pO_2 was achieved were significantly longer than for the first two applications.

4. Discussion

Oral mucosa is composed of a stratified squamous epithelium, on the surface of which the intercellular spaces are filled with lipids extruded from intracellular granules during maturation. The lipids may be organised into lamellae and they constitute the principal barrier against molecular diffusion through the mucosa. Keratinized areas in the oral cavity are generally more permeable than the skin because the intercellular lipids are less well structured. They exist mainly in discrete lamellar domains and there are fewer structural contributions from lipids (ceramides) covalently bound to the corneocyte surface (Wertz and Squier, 1991; Wertz et al., 1993; Squier and Wertz, 1996). In man there are considerable differences in permeability of the different oral mucosae (Shojaei, 1998). In non-keratinized regions (e.g. cheek, floor of the mouth and lips), the chemical nature of the intercellular material is less well defined and the barrier is less efficient than that in the keratinized epithelia (Wertz et al., 1993), such as the gingiva and hard palate. In general, the permeability of the oral mucosa decreases in the order of sublingual greater than buccal and buccal greater than palatal (Harris and Robinson, 1992).

The permeability of the oral mucosa is estimated to be 4–4000 times greater than that of the skin (Galey et al., 1976; Harris and Robinson, 1992; Shojaei, 1998). The oral epithelia of a number of experimental animals are entirely keratinised (Harris and Robinson, 1992), and the rat has a buccal mucosa with a very thick, keratinised surface layer (Shojaei, 1998). Human oral lining mucosa is thin and non-keratinised. From the point of view of human mucosal drug delivery, carriers tested in rats are expected to be even more effective in the thin non-keratinized human buccal mucosa. Inflammatory infiltrate in submucosal connective tissue increases epithelial permeability (Squier and Johnson, 1975), and an ulcerated surface, i.e. without the epithelial barrier provides an easier entry, but also an easier exit, for a drug (Harsayi et al., 1986).

Mechanical movements of the tongue and continuous salivary flow may prevent long-term adhesion of carriers to the oral mucosa and lead to dilution of the active drug content. It has been found that among the different hydrophilic polymers which have been investigated, PMM is the most appropriate muco-adhesive ointment for local liposome application in the oral cavity as they are most stable in this polymer, and enhanced penetration of the incorporated substance into the oral mucosa or gingiva was found when PMM was used (Petelin et al., 1998). Therefore, PMM was chosen as the vehicle for application of liposomes, with and without the entrapped hyperaemic drug (BN), for this study. BN increases local blood flow indirectly through release of nicotinic acid, stimulating prostaglandin D2 formation (Wilkin et al., 1985; Morrow et al., 1992). The increase of blood flow leads to an elevation of tissue oxygenation, pO_2, which can be measured by EPR oximetry. Increase in pO_2 with time after the application of formulations containing BN was measured and the overall effectiveness of the incorporated drug in different types of liposomes was determined.

It was found that multi-lamellar liposomes made from hydrogenated soy lecithin were the most effective carriers among the liposomes investigated in this study. Several studies show that liposome composition and, to a lesser extent, liposome size influence the rate of transport and effectiveness of drug action in skin (Kirjavainen et al., 1999; Sentjurc et al., 1999; Coderch et al., 2000; Honzak et al., 2000). The effect of free BN in PMM has been evaluated previously, the drug effect increasing linearly with BN concentration up to 3%, higher concentration having no greater effect, indicating that the saturation had been achieved (Petelin et al., 2004). If the concentration of the hyperaemic drug was smaller than 1% no local changes of pO_2 were observed (Petelin et al., 2004). As in our study the concentration of BN in the formulation was 0.5% and we got the significant effect with all four liposomal formulations we can conclude that the encapsulation of a drug into liposomes enhances its delivery into oral mucosa.

Non-extruded liposomes made from HSL were the most effective carriers for BN in this study, also producing the longest period of action (Table 2). That HSL liposomes cause greater effect than NSL liposomes is in agreement with previously obtained in vitro and in vivo results which showed that a hydrophilic probe, when applied entrapped in NSL liposomes did not penetrate deeper than 100 μm, while the HSL liposomes enabled penetration into the deeper layers of the skin (Sentjurc et al., 1999; Honzak et al., 2000). We studied the influence of a lipophilic substance which can penetrate into the skin even if it is not entrapped in liposomes. Therefore, the enhanced effect of all four formulations is not surprising. However, in accordance with previous findings which show that liposomes allow controlled and continuous release of drug over a longer period of time (Sentjurc and Gabrijelcic, 1996; Krzic et al., 2001), non-extruded BN containing HSL liposomes caused the most prolonged and pronounced increase in pO_2 during our study. The pO_2 remained above the base line even after 90 min when measurements were terminated. Further study using a prolonged recording time is necessary to determine the actual duration of effect of this formulation. The population of non-extruded liposomes is very heterogeneous in size and lamellar structure; therefore, they release the entrapped substance more evenly over a prolonged period. On the contrary, after initial increase in pO_2 a decrease was observed after 35 min with the extruded liposomes. We suppose that the extruded liposomes, which are smaller, more homogeneous in size and with fewer layers, or presumably with only one layer in their structure are not only absorbed quickly, but release drug more uniformly and rapidly, therefore having a shorter effect. We assume, from published data, that in any population of extruded liposomes a significant proportion are uni-lamellar and so likely to break down on the surface of the oral mucosa in a similar manner to what is found in skin (Sentjurc et al., 1999). In this situation, the released free BN then penetrates the mucosa as if it had been applied directly.

The effect of repeated application of the liposomal formulations once a day over 3 successive days showed that BN action

was the most pronounced on the first or the second day of application for HSL, while for NSL it decreases with repeated applications. It should be stressed that these repeat studies were difficult to perform since in some animals the LiPc became insensitive by the fourth day after its implantation into the oral mucosa so the lesser effects seen on the third day might partially represent loss of LiPc activity rather than reduced effectiveness of the applied liposome formulation. Although some studies (Swartz et al., 1991; Norby et al., 1998) report that LiPc appears to elicit only a minor inflammatory response, a prolonged or more major inflammatory response could shorten the period during which the marker is sensitive to pO_2. The reduction in sensitivity to oxygen has been observed previously in some other studies (Liu et al., 1993; Smirnov et al., 1993; Smirnov et al., 1994a,b; Norby et al., 1998) but the reason has not been determined.

Anaesthetic agents typically affect tissue pO_2 either through effect on the respiratory centre or on the peripheral circulation, for example by causing vasoconstriction (Liu et al., 1995). Xylazine hydrochloride is an agonist for α2-adrenoceptors with profound sedative and muscle relaxant activity and is commonly used in veterinary medicine in combination with other agents to provide anaesthesia in small animals (Greene and Thurmon, 1988). Xylazine decreases the heart rate, causes a biphasic change in mean blood pressure (transient hypertension followed by hypotension), decreases cerebral venous blood volume thus reducing intracranial pressure, and it depresses the central nervous system (Hsu, 1981; McCormick et al., 1993; Liu et al., 1995) so the drop seen in pO_2 after administration of the anaesthetic mixture and its later return towards normal as drug effects wear off is not unexpected. During the initial stage of hypertension there is peripheral vasoconstriction reducing blood flow through capillaries. This varies with the type of α2-adrenoceptors present in the tissue, the dose and the route of administration. The later stage of vasodilation results in lowering of the blood pressure. Reduced peripheral blood flow, together with the decreased arterial oxygen content, account for the remarkably reduced oral mucosa oxygenation in ketamine–xylazine hydrochloride anaesthetised rats. Using this anaesthetic regime it is impossible to avoid the influence of anaesthesia during measurements in vivo. However, a reasonably stable baseline was achieved by: (i) waiting for the side effects to become apparent, (ii) allowing them to stabilise before undertaking experimental measurements and (iii) administration of a 'top-up' dose of the anaesthetic mixture before anaesthesia lightened. There was some fluctuation of pO_2 measurements so the baseline value was obtained by averaging five EPR spectra line-width measurements taken after the anaesthetic effects stabilised but before application of PMM.

In this study we have used EPR oximetry to show that, in the rat, topical application of liposome preparations with entrapped BN, facilitates penetration of BN through the oral mucosa in vivo; the absorbed drug inducing significantly increased oxygenation of the oral mucosa in the area where it is applied at dosages below those required to obtain effects using the free drug. Liposome composition and size played an important role in drug penetration. The study indicates that non-extruded hydrogenated soy lecithin liposomes (which are heterogeneous in size and lamellar in structure) are much more effective at carrying BN into oral mucosa than are non-hydrogenated liposomes or extruded liposomes of the same type. The application of liposome preparations over 3 successive days yielded the greatest recorded effects on the first day with successive decrease after that. In vivo use of EPR oximetry is a safe non-invasive (after the initial implantation of the pO_2 sensitive marker) method for precise monitoring of the effects of BN absorbed into the oral mucosa by following the physiological response to the drug. The technique permits repeated application of the active substance at the same location over a period of several days. The method can be widely applied to test the influence of different vehicles on the physiological action of BN and possibly other dissolved lipophilic active ingredients. In addition, oral mucosal pO_2 is altered by body temperature and anaesthetic agents, but compensation for these effects is both possible and practical.

Among the examined carriers, multi-lamellar liposomes made from hydrogenated soy lecithin appear to be the most appropriate for local drug delivery to oral mucosa.

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References

Barsuhn, C.L., Olanoff, L.S., Gleason, D.D., Adkins, E.L., Ho, N.F., 1988. Human buccal absorption of flurbiprofen. Clin. Pharmacol. Ther. 44, 225–231.

Bremecker, K.D., Strempel, H., Klein, G., 1984. Novel concept for a mucosal adhesive ointment. J. Pharm. Sci. 73, 548–552.

Coderch, L., Fonollosa, J., De Pera, M., Estelrich, J., De La Maza, A., Parra, J.L., 2000. Influence of cholesterol on liposome fluidity by EPR. Relationship with percutaneous absorption. J. Controlled Release 68, 85–95.

Galey, W.R., Lonsdale, H.K., Nacht, S., 1976. The in vitro permeability of skin and buccal mucosa to selected drugs and tritiated water. J. Invest. Dermatol. 67, 713–717.

Greene, S.A., Thurmon, J.C., 1988. Xylazine-a review of its pharmacology and use in veterinary medicine. J. Vet. Pharmacol. Ther. 11, 295–313.

Harris, D., Robinson, J.R., 1992. Drug delivery via the mucous membranes of the oral cavity. J. Pharm. Sci. 81, 1–10.

Harsayi, B.B., Hilchie, J.C., Mazei, M., 1986. Liposomes as drug carriers for oral ulcers. J. Dent. Res. 65, 1133–1141.

Hicks, D.C., 1973. The buccal absorption of some adrenoceptor blocking drugs. Br. J. Pharmacol. 47, 680–681.

Honzak, L., Sentjurc, M., Swartz, H.M., 2000. In vivo EPR of topical delivery of a hydrophilic substance encapsulated in multilamellar liposomes applied to the skin of hairless and normal mice. J. Controlled Release 66, 221–228.

Hsu, W.H., 1981. Xylazine-induced depression and its antagonism by alpha adrenergic blocking agents. J. Pharmacol. Exp. Ther. 218, 188–192.

Kirjavainen, M., Monkkonen, J., Saukkosaari, M., Valjakka-Koskela, R., Kiesvaara, J., Urtti, A., 1999. Phospholipids affect stratum corneum lipid bilayer fluidity and drug partitioning into the bilayers. J. Controlled Release 58, 207–214.

Krzic, M., Sentjurc, M., Kristl, J., 2001. Improved skin oxygenation after benzyl nicotinate application in different carriers as measured by EPR oximetry in vivo. J. Controlled Release 70, 203–211.

Lasic, D.D., 1993. Liposomes: From Physics to Application. Elsevier, Amsterdam, London, p. 67.

Liu, K.J., Gast, P., Moussavi, M., Norby, S.W., Vahidi, N., Walczak, T., Wu, M., Swartz, H.M., 1993. Lithium phthalocyanine: a probe for electron paramagnetic resonance oximetry in viable biological systems. Proc. Natl. Acad. Sci. U.S.A. 90, 5438–5442.

Liu, K.J., Bacic, G., Hoopes, P.J., Jiang, J., Du, H., Ou, L.C., Dunn, J.F., Swartz, H.M., 1995. Assessment of cerebral pO_2 by EPR oximetry in rodents: effects of anesthesia, ischemia, and breathing gas. Brain Res. 685, 91–98.

Mazei, M., Gulasekharam, V., 1982. Liposomes: a selective drug delivery system for the topical route of administration. Gel dosage form. J. Pharm. Pharmacol. 34, 473–474.

McCormick, J.M., McCormick, P.W., Zabramski, J.M., Spetzler, R.F., 1993. Intracranial pressure reduction by a central alpha-2 adrenoreceptor agonist after subarachnoid hemorrhage. Neurosurgery 32, 974–979.

Morrow, J.D., Adwad, J.A., Oates, J.A., Roberts, L.J., 1992. Identification of skin as a major site of prostaglandin D2 release following oral administration of niacin in humans. J. Invest. Dermatol. 98, 812–815.

Norby, S.W., Swartz, H.M., Clarkson, R.B., 1998. Electron and light microscopy studies on particulate EPR spin probes lithium phthalocyanine, fusinite and synthetic chars. J. Microsc. 192, 172–185.

Petelin, M., Sentjurc, M., Stolic, Z., Skaleric, U., 1998. EPR study of mucoadhesive ointments for delivery of liposomes into the oral mucosa. Int. J. Pharm. 173, 193–202.

Petelin, M., Pavlica, Z., Bizimoska, S., Sentjurc, M., 2004. In vivo study of different ointments for drug delivery into oral mucosa by EPR oximetry. Int. J. Pharm. 270, 83–91.

Pimlott, S.J., Addy, M., 1985. A study into the mucosal absorption of isosorbide dinitrate at different intraoral sites. Oral Surg. Oral. Med. Oral Pathol. 59, 145–148.

Sentjurc, M., Gabrijelcic, V., 1996. Transport of liposome—entrapped molecules into the skin as studied by electron paramagnetic resonance imaging methods. In: Lasic, D.D. (Ed.), Handbook of Nonmedical Applications of Liposomes, vol. 4. CRC Press, Boca Raton, pp. 91–114.

Sentjurc, M., Vrhovnik, K., Kristl, J., 1999. Liposomes as a topical delivery system: the role of size on transport studied by the EPR imaging method. J. Controlled Release 59, 87–97.

Sentjurc, M., Krzic, M., Kristl, J., Grinberg, O., 2001. Swartz H.M, EPR Oximetry in vivo in mouse skin. Pol. J. Med. Phys. Eng. 7, 165–174.

Sentjurc, M., Kristl, J., Abramovic, Z., 2004. Transport of liposome entrapped substances into skin as measured by electronic paramagnetic resonance oxymetry in vivo. Methods Enzymol. 387, 267–287.

Shojaei, A.H., 1998. Buccal mucosa as a route for systemic drug delivery: a review. J. Pharm. Pharm. Sci. 1, 15–30.

Smirnov, A.I., Norby, S.W., Clarkson, R.B., Walczak, T., Swartz, H.M., 1993. Simultaneous multi-site EPR spectroscopy in vivo. Magn. Reson. Med. 30, 213–220.

Smirnov, A.I., Norby, S.W., Weyhenmeyer, J.A., Clarkson, R.B., 1994a. The effect of temperature on the respiration of cultured neural cells as studied by a novel electron paramagnetic resonance technique. Biochem. Biophys. Acta 1200, 205–214.

Smirnov, A.I., Norby, S.W., Walczak, T., Liu, K.J., Swartz, H.M., 1994b. Physical and instrumental considerations in the use of lithium phthalocyanine for measurements of the concentration of the oxygen. Magn. Reson. B103, 95–102.

Squier, C.A., Johnson, N.W., 1975. Permeability of oral mucosa. Br. Med. Bull. 31, 169–175.

Squier, C.A., Wertz, P.W., 1996. Structure and function of the oral mucosa and implications for drug delivery. In: Rathbone, M.J. (Ed.), Oral Mucosa Drug Delivery. Marcel Dekker, New York, pp. 1–25.

Sveinsson, S.J., Holbrook, W.P., 1993. Oral mucosal adhesive ointment containing liposomal corticosteroid. Int. J. Pharm. 95, 105–109.

Swartz, H.M., Boyer, S., Gast, P., et al., 1991. Measurements of pertinent concentrations of oxygen in vivo. Magn. Reson. Med. 20, 333–339.

Wertz, P.W., Squier, C.A., 1991. Cellular and molecular basis of barrier function in oral epithelium. Crit. Rev. Ther. Drug Carrier Syst. 8, 237–269.

Wertz, P.W., Swartzendruber, D.C., Squier, C.A., 1993. Regional variation in the structure and permeability of oral mucosa and skin. Adv. Drug Deliv. Rev. 12, 1–12.

Wilkin, J.K., Fortner, G., Reinhardt, L.A., Flowers, O.V., Klipatrick, S.J., Steeter, W.C., 1985. Prostaglandins and nicotinate-provoked increase in cutaneous blood flow. Clin. Pharmacol. Ther. 38, 273–277.