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International Journal of Pharmaceutics 279 (2004) 67–79
international journal of pharmaceutics
www.elsevier.com/locate/ijpharm

Studies on molecular interactions between nalidixic acid and liposomes

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Received 16 December 2003; received in revised form 24 March 2004; accepted 4 April 2004

Available online 2 June 2004

Abstract

The interaction between nalidixic acid sodium salt (NANa) and liposomes prepared from α-L-dipalmitoyl-phosphatidylcholine (DPPC) or from its binary mixture with dioleoyl-phosphatidylcholine (DOPC) was studied with differential scanning calorimetry (DSC) and electron paramagnetic resonance (EPR) spectroscopy. We evaluated the role of broadband ultraviolet-B (UV-B) irradiation on the molecular interactions between the lipids and the NANa, and determined the decay-kinetics of the incorporated spin labeled fatty-acid free radicals. Multilamellar and unilamellar vesicles were prepared by sonication and extrusion. The entrapment efficiencies were determined spectrophotometrically. The size-distribution of the liposomes and its change in time was checked by dynamic light scattering (DLS). Our results indicate that NANa mainly interacts with lipid head groups. However, its effect and presumably the formation of the free radicals, induced by broadband ultraviolet-B, is not localized only to the head group region of the lipid molecules. Depending on DOPC content, interaction between the NANa and the lipids modifies the phase-transition parameters of the liposome dispersions.

Keywords: Liposomes; Nalidixic acid; DSC; DLS; Zeta-potential; EPR

1. Introduction

The nalidixic acid was introduced into the clinical practice in the early 1960s. The pharmacological agent has only modest Gram-negative activity and low oral absorption with a peak serum level of less than 0.5 mg/l and was not, therefore, a suitable candidate for the treatment of systemic infections. Urinary concentrations, however, are high, and the compound is still used for urinary tract infections.

In the early 1970s, many cases of phototoxicity, limiting the use of the nalidixic acid, were reported in the clinical practice (Bases, 1968; Louis et al., 1973; Brauner, 1975; Ljunggren and Bjellerup, 1986). To minimize the side effects, a number of structurally related, highly potent, broad-spectrum antibacterial agents have been synthesized over the last decades (Chu and Fernandes, 1991; Reynolds, 1993; Gibbs, 2001; Turel, 2002).

Nalidixic acid has several structural features retained by many of the newer compounds, and is based

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doi:10.1016/j.ijpharm.2004.04.008

on a 4-oxo-1,8-naphthyridin-3-carboxylic acid nucleus (Domagala, 1994; Ball et al., 1999; Appelbaum and Hunter, 2000). All first-generation quinolones (cinoxacin, nalidixic acid, oxolinic acid, pipemidic acid, rosoxacin) and enoxacin, fleroxacin, ofloxacin provoke photohaemolysis (Przybilla et al., 1990) that seems to be oxygen dependent (Fernández et al., 1987; Fernández and Cárdenas, 1990).

Number of studies indicated the photosensitizing properties of the nalidixic acid and its derivatives, which lead to phototoxic responses both in human and in animal subjects (Boisvert and Barbeau, 1981; Halkin, 1988; Shelley and Shelley, 1988; Vermeersch et al., 1989; Patterson, 1991; Robertson et al., 1991; Wagai and Tawara, 1991; Allen, 1993; Ferguson and Johnson, 1993; Goldstein, 1996; Reano et al., 1997; Martinez et al., 1998). Comparisons of the in vitro results show that the new quinolones (norfloxacin, ciprofloxacin) are considerably less phototoxic than nalidixic acid (Johnson et al., 1986; Ferguson and Johnson, 1990). Alterations of the cationic permeability of red blood cell membranes induced by nalidixic acid were demonstrated. It is postulated that the increased cation permeability induced by the NANa cannot be attributed to cholesterol oxidation or to lipid peroxidation; a more probable mechanism is photooxidation of amino acid residues of the membrane proteins (Cardenas et al., 1992).

Using lipid peroxidation as an index of phototoxicity, the effects of some quinolones were studied. It is likely that reactive oxygen species (ROS), generated by the interaction between UV-sensitized drug molecules and oxygen molecules mediate lipid peroxidation in erythrocyte membrane. The lipid peroxidation was entirely dependent on both the ultraviolet light (UV) and the drug; moreover, it could completely be inhibited by sodium azide or by phenyl-*N*-*tert*-butylnitrone (PBN) (Wada et al., 1994).

In vitro and in vivo studies related the phototoxicity to the generation of reactive oxygen species, including hydrogen peroxide, superoxide, singlet oxygen and hydroxyl radical (Wagai and Tawara, 1992; Chetelat et al., 1996; Verna et al., 1998). Singlet oxygen production by irradiated quinolones can be responsible for secondary changes, including an increase in thiobarbituric acid products, lipid peroxidation and ultimately results in cellular lysis. Also, according to Fujita and Matsuo (1994), the non-singlet-oxygen mechanism is operative, in addition to the singlet oxygen mechanism.

Some suggest that cyclooxygenase products may dominate the development of quinolone phototoxicity rather than ROS in vivo (Shimoda, 1998). Membrane-dependent reactions may play a major role in nalidixic acid phototoxicity and the phototoxicity of newer quinolones may be mediated more through an intracellular target (Ferguson and Johnson, 1990).

Many drugs can adsorb/bind to the (liposomal) membrane because of energetic, covalent binding, as well as entropic, hydrophobic effects. The interactions appear to be “specific”, i.e. sensitive to the choice of the host membrane phospholipid (Cevc, 1990). A variety of chemotherapeutic agents strongly interacts with the phospholipid components of cellular bilayer membranes. More detailed information about the type of various interactions between (phototoxic) drugs and lipid molecules may direct attention to the rational design of new liposomal drug-delivery systems (Sharma and Sharma, 1997; Fresta et al., 2002).

To obtain further pieces of knowledge of the photo-processes responsible for the phototoxicity of NANa, we have investigated the possible interactions between the nalidixic acid and the lipid molecules of the liposomal membrane in the presence and absence of ultraviolet light. In our present study, liposomes were prepared from the most generally used lipid molecules—α-L-dipalmitoyl-phosphatidylcholine (DPPC) and dioleoyl-phosphatidylcholine (DOPC). We addressed the question on how the amount of DOPC with its unsaturated bonds influences the nature of molecular interactions. The amount of entrapped nalidixic acid was determined in case of small unilamellar (SUV) and multilamellar vesicles (MLV). The size-distribution and the stability of liposomal preparations were checked by dynamic light scattering. In addition, the zeta-potential was examined. We also studied, how the physicochemical properties of liposomal membrane alter, if the nalidixic acid treatment is combined with different doses of ultraviolet-B (UV-B) irradiation. Using differential scanning calorimetry (DSC), we observed the changes of the enthalpy and phase-transition temperatures for the pre- and main-transitions. With electron paramagnetic spectroscopy (EPR), we tried to localize the NANa in the liposomal membrane. Decay-kinetics of the spin labeled stearic acid free radicals, incorpo-

rated into different depths of the membrane, were also studied in the presence of NANa and different doses of the UV-B irradiation. We addressed the question, if the position of the photosensitizing molecule in the artificial membrane gives an explanation to the latter process.

2. Materials and methods

2.1. Preparation of liposomal nalidixic acid

2.1.1. Preparation of multilamellar vesicles

MLVs were prepared using the thin-film hydration method. Ten milligrams lipids, α-L-DPPC (Sigma) in different mixtures with DOPC (Sigma) (0, 5, 10, 20, 30 % (w/w) DOPC) were dissolved in absolute ethanol (Merck). The mixture was dried to thin-film under nitrogen stream. The hydrating solution contained nalidixic acid sodium salt (NANa) (Sigma) in a concentration of 1 mM dissolved in PBS solution (2.86 g $Na_2HPO_4 \times 2\ H_2O$, 0.27 g KH_2PO_4, 8.0 g NaCl per 1000 ml distilled water; pH 7.3). Thin films of lipid were hydrated at ∼50 °C, above the main-transition temperature of the DPPC and the DOPC, with 1 ml of the hydrating solution resulting in a lipid/NANa molar ratio of ∼13:1. The final lipid concentration was 10 mg/ml (∼13.6 mmol/l).

2.1.2. Preparation of small unilamellar vesicles with sonication

To get a small and uniformly sized population of SUVs, the MLV suspension was sonicated. For sonication, a Soniprep 150 MSE device (frequency of 20 kHz and wave amplitude of 8 μm) was used with two times 10 min sonication, and 10 min pause between the sonications.

2.1.3. Preparation of small unilamellar vesicles with extrusion

MLV samples were prepared similarly as above, but the lipid concentration was 1 mg/ml. Using an Avestin type extruder (Avestin Inc., Canada), the heterogeneous population of liposomes was passed through Avestin polycarbonate membrane filters, with a diameter of 19 mm. The samples were extruded through filters with pore sizes of 400, 100, and 50 nm, repeating the extrusion 50, 100, and 50 or 150 times, respectively.

We measured the amount of encapsulated nalidixic acid in case of pure DPPC–SUVs, pure DPPC–MLVs, and for SUVs prepared from a mixture of DPPC and DOPC in the ratio of 70:30 % (w/w). SUVs were prepared with the sonication method. In each case, 400 μl of freshly prepared samples was centrifuged with an Eppendorf centrifuge (type 5804R, 11,000 × *g*, 20 min) through Microcon YM-10 Centrifugal Filter Devices (Millipore) with a cut-off value of 10 kD. The amount of NANa in the hydrating and in the centrifuged solutions—the latter representing the amount of NANa out of the liposomes—was determined spectrophotometrically. The absorbances were directly measured by a Unicam 3.40 UV–Vis spectrophotometer at the wavelengths of 258 and 334 nm after the European Pharmacopoeia Ed. 4. (Ph. Eur. Ed. 4.).

2.2. Sample preparation and DSC measurements

For DSC measurements, dispersions prepared from 10 mg of lipids/40 μl PBS were used. The lipid mixture contained different amounts of DOPC (0, 5, 10, 20, 30 % (w/w)) and DPPC. Ten milligrams of lipids were dissolved in 150 μl of pure alcohol. The organic solvent was removed by nitrogen stream forming thin layer on the vessel wall. Multilamellar vesicles were prepared by hydrating the lipid-film with 40 μl of PBS at 50 °C and further dispersed by 20 min shaking in water bath at 50 °C. In case of the samples with nalidixic acid, the hydrating solution contained NANa (FW: 254.2) in a 10:1 final molar concentration of lipid to NANa. Thus, in case of DSC measurements, the lipid concentration was 250 mg/ml, with a NANa concentration of about 7 mg/ml. About 5.0–8.0 mg of samples was encapsulated in hermetically sealed standard aluminum DSC pans of 40 μl, without removing the free NANa. In irradiation experiments, samples were irradiated in opened DSC pans, under quartz glass to inhibit the evaporation. For irradiation, broadband UV-B lamp, equipped with a 40 W FS-20 tube was used. The intensity and dose of irradiation were measured with a VLX-3W dosimeter equipped with a CX-312 sensor measuring at the wavelength 312 nm. Intensity was kept at 1.4 mW/cm^2, and the dose was measured continuously during the irradiation. The maximal dose used in this work was 10 kJ/m^2. For

DSC measurements a Du-Pont 990 differential scanning calorimeter, in a temperature range of 20–50 °C was used, with a scan rate and sensitivity of 5 °C/min and 5 mV/cm, respectively. In each case, samples were measured in six separate experiments.

2.3. Sample preparation and EPR measurements

SUVs were prepared as above, adding to the ethanol solution either 5-doxyl-stearic acid (Sigma), or 12-doxyl-stearic acid (Sigma) or 16-doxyl-stearic acid (Sigma) spin probes. The molar ratio of the spin probe to lipid molecules was 1:100. Spectra were registered with an EMX6 Bruker X-band on-line spectrometer. Temperature dependence of the spectra was measured by controlling the temperature within the sample with a precision of ±0.1 °C. To characterize the fluidity of the lipid membrane the outer-peak separation ($2A_{max}$) was determined in the temperature range of 2–50 °C. 100 kHz modulation frequency and maximally 2 gauss modulation amplitude were used throughout the measurements. The applied microwave power was 15 mW; scan speeds of 167.77 or 335.5 s with 2048 points on 100 gauss field interval were used. We determined the outer-peak separation ($2A_{max}$) for control and for NANa-treated DPPC SUVs. In case of NANa treatment, the hydrating solution contained NANa in a concentration of 1 mM, corresponding to a lipid/NANa molar ratio of 13. To study the effect of UV-B irradiation on the kinetics of the free radical reaction, the decrease of the signal amplitude of the spin labeled stearic acid was determined. In irradiation experiments, liposome dispersions were irradiated in quartz cuvettes using the broadband UV-B source given above. The applied doses were between 0 and 15 kJ/m^2; maintaining the intensity at 1.4 mW/cm^2. Both the intensity and the accumulated dose were measured continuously during the irradiation.

2.4. Measurement of size-distribution and zeta-potential

Mean diameter, size-distribution, and polydispersity index of the liposomal preparations were monitored by dynamic light scattering (DLS) (Zetasizer 3000 HS_A Malvern Instruments). The instrument was equipped with a He–Ne laser operating at a wavelength of 633 nm. The size analysis was made at a lipid concentration of 0.05 mg/ml, at 25 °C. To test the stability of liposomal preparations the analysis was repeated at different storage times (up to 3–4 weeks). Samples were stored at 4 °C between the measurements. In each case the hydrating PBS solution was sterile filtered through Osmonics filter (Herba Chemosan) with a pore size of 220 nm. The zeta-potential for pure DPPC-, and for 30/70 % (w/w) DOPC/DPPC–MLVs were determined in the absence and presence of NANa in distilled water and in PBS (both were filtered and autoclaved). The lipid concentration of the examined diluted samples was 0.1 mg/ml. The measurements were done with a Zetasizer 3000 HS_A (Malvern Instruments).

3. Results and discussion

3.1. Liposomal nalidixic acid

The amount of entrapped NANa was determined from the absorbance of the NANa in the hydrating solution and in the solution not entrapped into liposomes. In the preliminary experiments, we determined that the concentration of the hydrating solution does not change during centrifugation; NANa does not adsorb to filter. The loading capacity of liposomal formulations containing NANa was expressed as μmol of NANa per μmol of phospholipid (Table 1). Our results indicate that the encapsulation efficiency was 15.6 ± 1.0% (average ± S.E.M.) and 10.9 ± 4.9% for DPPC and DPPC–DOPC SUVs, respectively. Statistical analysis of the entrapment efficiencies has shown no significant difference between the different types

Table 1
Encapsulation of nalidixic acid sodium salt into liposomes

Liposome formulation	Loading capacity (×100)	Encapsulation (%)
DPPC (SUV)	1.15 ± 0.076[a]	15.6 ± 1.0[a]
DPPC (MLV)	2.00 ± 0.69	27.2 ± 9.4
DPPC:DOPC/70:30 (SUV)	0.801 ± 0.3655	10.9 ± 4.9

Loading capacity is given in μmol of NANa per μmol of phospholipid. Encapsulation efficiency is determined as the percentage difference in the absorbance of the hydrating solution and the supernatant after having centrifuged the liposomal suspension.

[a] Values are given with SEM of at least four parallels.

of liposomes. Thus, 30% presence of the DOPC in binary mixture of DPPC–DOPC SUVs has a negligible influence on the encapsulation efficiency. Supposing homogeneous size-distribution of the liposomes—our size-distribution measurements revealed an average diameter of about 80 nm—we calculated that the volume of liposomes corresponds only to ∼3% of the total volume of the dispersion. Taking into account the encapsulation efficiency and the liposomes' volume, the liposomal—entrapped solution, and the bilayer together—concentration of the NANa inside the liposomes is about seven times higher than outside of the liposomes. The apolar character of the quinolone-ring of the molecules would suggest a higher degree of encapsulation. However, the sodium salt makes nalidixic acid more water soluble than the basic compound, thus leading to a smaller, apparent encapsulation into the liposomes. Earlier observations on encapsulation of charged molecules into lipid membranes have shown that the charged form of such molecules will bind to the polar headgroup region of the lipids (Boulanger et al., 1981; Lee and Schreier, 2000). On the contrary, the uncharged drugs favor to penetrate deeper into the membrane relative to the aqueous phase of liposomes (Kelusky and Smith, 1984; Betageri and Parsons, 1992).

As given in Table 1, MLVs prepared from DPPC, show higher entrapment efficiency, 27.2 ± 9.4%, than SUV samples. This observation may be consequence of the multilamellar structure. In earlier measurements (Marsh et al., 1976), it has been found that ascorbate treatment can be used to determine the internal volume of the liposomes. Reducing the EPR signal out of the liposomes by ascorbate, Marsh et al. (1976) found that the internal volume of the liposomes in MLVs is about 2%. In our preliminary experiments, the EPR signals out of the liposomes were quenched by paramagnetic relaxation, and we observed for the incorporated volumes about 7 and 2 percentages in case of MLVs and SUVs, respectively. Since, the entrapped total volume within the multilamellar liposomes is greater than in case of SUVs: the NANa molecules, which situate within the inter-bilayer space, are also encapsulated in the MLV.

3.2. DSC measurements

In our experiments, phase-transition parameters of the DPPC–DOPC dispersions, with and without NANa, in the presence and the absence of UV-B irradiation, were determined and are collected in Tables 2, 3 and 4. Concentrations of the DOPC were 0, 5, 10, 20, 30 % (w/w).

As characteristics, we used the main-transition temperature (T_m), the half-width ($\Delta T_{1/2}$) and the enthalpy (ΔH_m) of the main-transition. The amount of DOPC contained in the liposomes substantially influenced the main-transition temperature (Table 2). The larger was the amount of DOPC the lower was the main-transition temperature. In case of pure DPPC, T_m was 42.56 ± 0.25 °C. As the consequence of the unsaturated bonds, this value decreased to 36.08 ± 0.14 °C, when the liposomes contained 30 % (w/w) of DOPC. Evaluation of the changes in T_m, in the presence of NANa and/or irradiation with UV-B, did not show significant changes compared to the controls (*P*

Table 2
Main-transition temperatures of the liposomes with different DOPC content

Percentage (% (w/w)) DOPC content	0	5	10	20	30
Control liposomes	42.56 ± 0.25	41.69 ± 0.24	40.75 ± 0.16	38.42 ± 0.24	36.08 ± 0.14
NANa-liposomes[a]	43.01 ± 0.21	41.25 ± 0.22	40.64 ± 0.24	38.19 ± 0.43	35.74 ± 0.49
Irradiated liposomes[b]	42.56 ± 0.3	41.63 ± 0.04	41.11 ± 0.29	37.95 ± 0.51	35.61 ± 0.09
Irradiated NANa-liposomes[c]	43.08 ± 0.2	41.13 ± 0.24	40.54 ± 0.08	38.30 ± 0.25	35.87 ± 0.26

Effect of NANa-treatment and/or irradiation. Values given in the table are the main-transition temperatures ± standard error of mean (°C). In each case, six separate samples were measured.

[a] Liposomes were prepared by hydrating the lipid-film, formed on the vessel wall after evaporating the ethanol, with 7 mg/ml of NANa as given in the text.

[b] Liposomes were irradiated with a broadband UV-B lamp with a dose of 10 kJ/m^2. Intensity was kept at 1.4 mW/cm^2 during the irradiation.

[c] Liposomes were supplied with NANa as above, and irradiated with a maximal UV-B dose of 10 kJ/m^2.

Table 3
Effect of NANa-treatment and/or irradiation on the half-width of the main-transition

Percentage (% (w/w)) DOPC content	$\Delta T_{1/2}$ (°C)				
	0	5	10	20	30
Control liposome	1.32 ± 0.09	2.37 ± 0.12	3.37 ± 0.30	4.05 ± 0.31	4.41 ± 0.34
NANa-liposomes[a]	1.30 ± 0.10	2.37 ± 0.19	3.36 ± 0.47*	4.04 ± 0.36*	4.08 ± 0.41
Irradiated liposomes[b]	1.35 ± 0.15	2.43 ± 0.06	4.16 ± 0.23*	5.15 ± 0.33*	4.75 ± 0.45
Irradiated NANa-liposomes[c]	1.40 ± 0.20	2.58 ± 0.19	4.18 ± 0.35	5.15 ± 0.28	4.29 ± 0.19

Fields with * show significant ($P < 0.05$) changes in the values, evaluated by two samples *t*-test with unequal standard deviation.
Values in the table are given as average ± standard error of mean (°C). In each case, six separate samples were measured.
[a] Liposomes were prepared by hydrating the lipid-film, formed on the vessel wall after evaporating the ethanol, with 7 mg/ml of NANa as given in the text.
[b] Liposomes were irradiated with a broadband UV-B lamp with a dose of 10 kJ/m^2. Intensity was kept at 1.4 mW/cm^2 during the irradiation.
[c] Liposomes were supplied with NANa as above, and irradiated with a maximal UV-B dose of 10 kJ/m^2.

Table 4
Effect of NANa-treatment and/or irradiation on the enthalpy of the main-transition

Percentage (% (w/w)) DOPC content	ΔH_m (mJ/mg)				
	0	5	10	20	30
Control liposomes	43.43 ± 3.65	47.74 ± 5.57	45.15 ± 1.28	33.32 ± 5.41	32.72 ± 4.14*
NANa-liposomes[a]	44.80 ± 2.60	47.31 ± 3.29	42.80 ± 3.65*	32.99 ± 7.08*	25.74 ± 1.07*
Irradiated liposomes[a]	42.20 ± 2.10	46.96 ± 3.92	38.77 ± 2.65	29.14 ± 4.10	20.20 ± 1.97
Irradiated NANa-liposomes[a]	42.20 ± 1.10	42.24 ± 1.30	35.70 ± 3.84	23.39 ± 2.76	8.51 ± 0.26

Fields with * show significant ($P < 0.05$) changes in the values, evaluated by two samples *t*-test with unequal standard deviation.
[a] For NANa-treatment and UV-B irradiation see the legend to Table 3.

> 0.05). Studying the half-width of the main-transition, $\Delta T_{1/2}$, which is related to the cooperativity of the hydrocarbon chains in the phase-transition, we observed a detectable reduction in the cooperativity between the fatty-acid chains ($P < 0.05$; Table 3) at all DOPC concentrations compared to the DPPC control. According to Table 3. (rows one and two), $\Delta T_{1/2}$ values of the NANa-treated DOPC–DPPC binary liposomes do not differ significantly from the corresponding case control. Similarly, neither irradiation alone, nor combination of irradiation and NANa-treatment cause significant change in the system cooperativity, if the concentration of the DOPC is below 10 or greater than/equal to 30 % (w/w). Irradiation alone or combined with NANa-treatment changes significantly the lipid chain cooperativity in the concentration-range of the DOPC between 10 and 20 % (w/w). Comparing values in a given row of Table 3, it can be seen that increasing the concentration of the DOPC decreases the cooperativity (increasing half-width). Hence, the homogeneity in the interactions between the different lipids, palmitoyl and oleoyl, should be smaller than in the case of a homogeneous system. It is, however, not the NANa treatment alone, which provokes a further change in the cooperativity, as can be judged by comparing the values with/without NANa treatment. We suppose that in the narrow range, where the DOPC concentration is already high enough to get significant change upon UV-B irradiation, but not too high to shade this effect, one can observe a further decrease in the cooperativity between the different lipids in the mixed liposomes.

From the fact that NANa and/or UV-B irradiation do not have significant influence on the T_m, we can conclude that free radical formation does not substantially modify the overall interaction of the lipid molecules in the liposomes. Thus, the suggested lipid-peroxidation (Wagai and Tawara, 1992; Wada et al., 1994) that occurs in presence of NANa and UV-B radiation does not affect the main transition temper-

ature of lipids. In contrast to the above findings with the non-ionizing UV-B radiation, gamma-irradiation changed significantly the phase-transition parameters of liposomes prepared from DPPC, and the shape of the calorimetric peaks due to oxidative damage (Albertini et al., 1985). Using the same saturated lipid, DPPC, it was found, that after gamma-irradiation the fluidity and permeability of the DPPC liposomes altered—gamma-irradiation induced lipid bilayer fluidization (Marathe and Mishra, 2002).

According to our experiments, at higher DOPC content (20 and 30%), decrease of the phase-transition enthalpy ($P < 0.05$; Table 4) can be observed, compared to the pure DPPC samples. In case of binary liposomes containing 30 % (w/w) DOPC, the NANa or UV-B alone decreased significantly the ΔH_m, but the enthalpies measured were not significantly different from each other ($P > 0.05$). Simultaneous NANa and UV-B treatment, however, resulted in further decrease of the ΔH_m, and the effect of the combined treatment was observable beginning from 10 % (w/w) DOPC content. The effect of NANa and UV-B treatment on the decrease of ΔH_m is more substantial with increasing DOPC concentration. After combined NANa and UV-B treatment, the ΔH_m values of the liposomes with 10, 20 and 30 % (w/w) DOPC content decrease by about 21, 30 and 74%, respectively, relative to the enthalpies determined for the corresponding controls.

We concluded that the free radicals produced by UV-B irradiation up to 10 kJ/m^2 in the presence/absence of the NANa do not modify the thermodynamic properties of the pure DPPC MLVs. On the contrary, UV-B irradiation leads to decrease in cooperativity of the lipid phase-transition in both presence and absence of NANa, compared to unirradiated samples, if the DOPC concentration is in the range between ∼10 and <30 % (w/w).

3.3. EPR spectroscopy

Samples of DPPC SUVs were prepared as given above. All the samples were freshly prepared and measured on the subsequent 1–2 days. To check the possible sensitive range along the lipid molecule, we used spin labeled stearic acids, 5-doxyl-stearic acid (SL-5), and 16-doxyl-stearic acid (SL-16) spin probes. In EPR measurements, we determined the outer-peak separation of the spectra, $2A_{max}$ (Fig. 1)—an increase

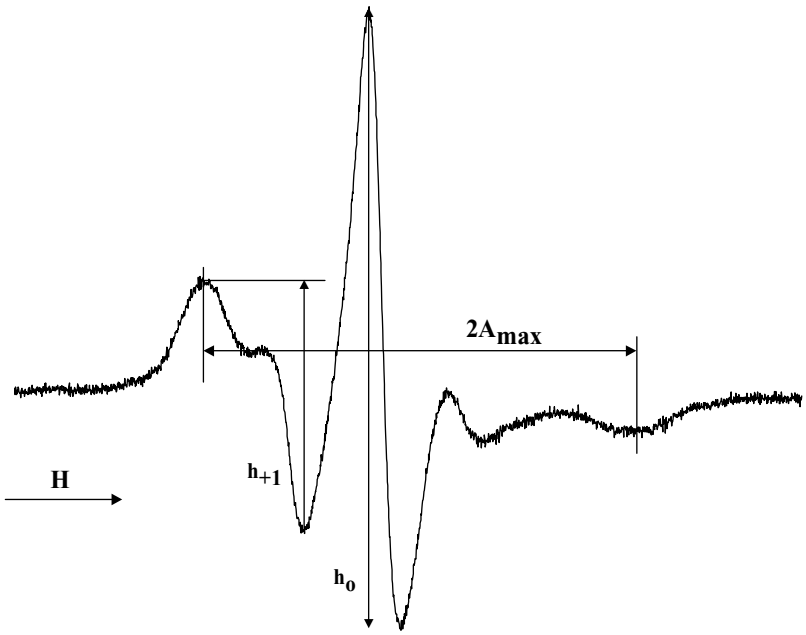

Fig. 1. EPR spectrum of SL-5 stearic acid derivative in DPPC SUVs labeled at 100:1 lipid to SL-5 molar ratio (13.6 mM DPPC/0.13 mM SL-5). Measurement temperature 23 °C. $2A_{max}$ denotes the outer-peak separation, and h_0 shows the amplitude of the EPR signal used to observe the decay-kinetics.

in the $2A_{max}$ value indicates a decrease of membrane fluidity (Marsh, 1981). To characterize the fluidity of the samples, labeled with SL-16, the ratio of the heights h_{+1}/h_0 (Fig. 1) can be used. In this case, an increase of the ratio indicates increased membrane fluidity. Doxyl group of the 5-doxyl-stearic acid is situated closest to the polar head of the lipids, while it localizes close to the apolar end of fatty-acid chains in the 16-doxyl-stearic acid spin label. Thus, these spin labeled compounds can be used to detect molecular motions, and changes in the attractive/repulsive forces between the polar head groups of the DPPC molecules and between the lipid chains, respectively. We determined the $2A_{max}$ values for pure DPPC and for treated DPPC liposomes. Fig. 2 demonstrates the temperature dependence of the outer-peak separation measured with SL-5 spin label. Regarding the level of experimental uncertainty (±0.3 gauss), we concluded that close to the head group region there is a significant difference in the temperature dependence of the pure and the NANa-treated samples. The difference can be observed in the temperature range between 2 and 35 °C, while above 35 °C, the curves coincide. In agreement with this result, our DSC measurements showed no difference either in T_m, $\Delta T_{1/2}$ or in ΔH_m between NANa-treated and pure DPPC liposomes. Using SL-16, in agreement with earlier observations (Marsh, 1981), a higher fluidity was observed for the control liposomes than with SL-5. This finding coincides with the recent results on the fluidity map along the lipids' fatty-acid chains (Hoffmann et al., 2000). However, according to our EPR measurements, the presence of NANa does not cause further significant change in the temperature dependence of h_{+1}/h_0 (figure not shown) compared to the control sample. Thus, we concluded that NANa can situate close to the polar phospho-diester groups, close to the outer surface of the liposomal membrane, resulting in pronounced decrease of the membrane fluidity in that membrane region. On the basis of the EPR measurements with SL-16, we can conclude that NANa either does not enter the hydrocarbon chain region or its perturbation is not strong enough to be detectable in the membrane fluidity at the end of the hydrocarbon chains.

Our observation that the fluidity changes could only be detected close to the lipid head groups involved the question—whether the free radical formation, and its effect, due to UV radiation in the presence of NANa would also be limited close to the upper region of the bilayer, or can it be detected further along the hydrocarbon chain too? Free radicals formed due to combined effect of the UV-B radiation and the NANa can

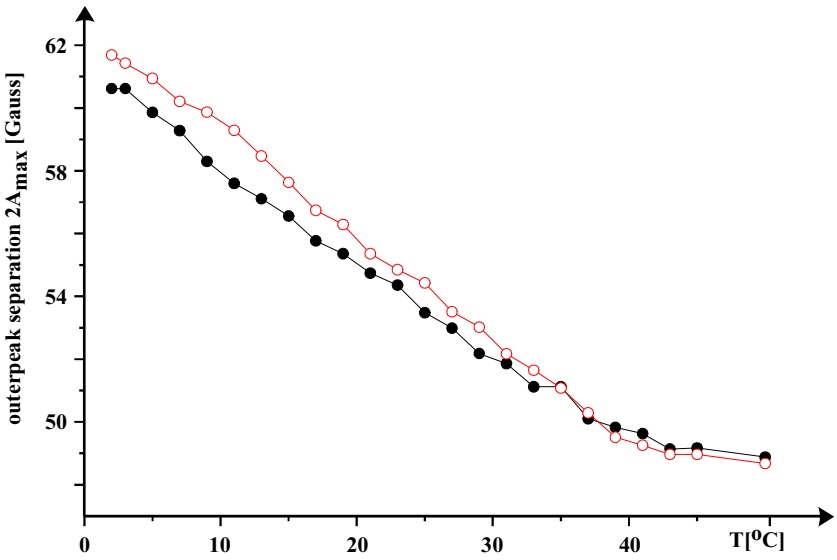

Fig. 2. Effect of NANa-treatment on the fluidity of DPPC SUVs observed by the EPR spectra of SL-5 incorporated into the vesicles. Concentration of the NANa was 1 mM, which was added during the liposome preparation. Closed and opened symbols denote values determined for the control and the NANa-treated samples, respectively.

react with the nitroxide-stearic acid derivatives incorporated into the membrane. This reaction leads to consumption of the incorporated nitroxide radicals converting them to an EPR-silent oxym. Thus, in this case the decay of the EPR signal amplitude can be used as a measure of the free radical formation. To determine the kinetics of free radical production we measured the decrease of the EPR signal amplitude (h_0). To exclude the possibility that in aqueous milieu the broadband UV-B radiation would cause a significant decrease of the nitroxide radicals, we measured the decay of the SL-5 amplitude. Fig. 3 shows that the relative change of the EPR signal remained within the level of experimental uncertainty. Similarly, we checked that the UV-B radiation did not lead to a significant decrease of the EPR signal amplitude in case of irradiated DPPC SUVs (data not shown). In agreement to expectation, the UV-B irradiation of doxyl-stearic acid spin probes in PBS solution containing NANa in a concentration of 1 mM affected the signal amplitudes of adequate spin probes (only the SL-5 case is shown). In case of 5-doxyl-stearic acid, an irradiation dose of 10 kJ/m^2 caused about 20% decrease in the signal amplitude. Irradiation of spin labeled DPPC SUV samples containing NANa of 1 mM produced a more intensive free radical formation, thereby a more pronounced decrease of the spin labels' EPR signal amplitude than without SUVs. The presence of DPPC liposomes enhanced the reduction rate compared to cases without liposomes. Enhancement of the reduction rate was observed at all three levels of the incorporated spin labels—close to the head groups (SL-5), at the middle, and at the end of the hydrocarbon chain (SL-12, and SL-16). According to our measurements, reduction rates almost coincide in different depths of liposomal membrane (5, 12, 16th carbon atoms). At 10 kJ/m^2 the reduction is almost complete for SL-5 and SL-16, and more than 80% of the incorporated SL-12 labels was reduced. In conjunction with the active role of the NANa, in producing free radicals due to UV-B irradiation, two possible pathways can lead to the above observations. One of them is the NANa mediated lipidperoxidation, which can occur also in case of saturated fatty-acid chains of DPPC. The other one is the known, increased lifetime of the generated free radicals in the

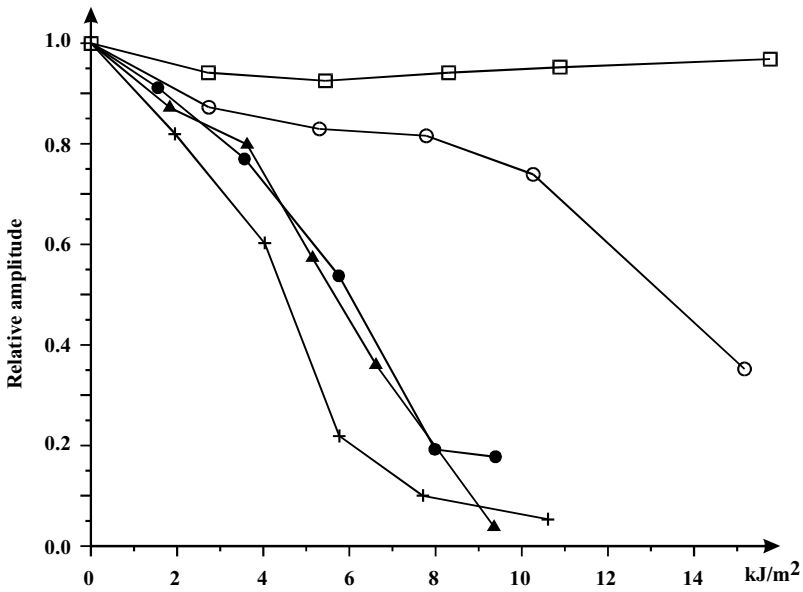

Fig. 3. Kinetics of the nitroxide consumption as the function of broadband UV-B radiation. Relative intensities are determined from the amplitudes (h_0) of the middle-peak at the given UV-B dose relative to the unirradiated samples. The spin label concentration in each of the liposome samples was 1.3×10^{-4} M, with 1:100 spin label to lipid molar ratio. Open square: SL-5 (2.6×10^{-4} M) in PBS. Open circle: SL-5 (2.6×10^{-4} M) in PBS in the presence of 1 mM NANa. Solid triangle: SL-5 labeled DPPC SUV + 1 mM NANa. Solid circle: SL-12 labeled DPPC SUV + 1 mM NANa. Cross: SL-16 labeled DPPC SUV + 1 mM NANa.

apolar inter-space of the hydrocarbon chains. The free radicals, produced in the upper regions of the membrane close to the NANa, can move down to the apolar region leading to the reduction of spin probes (SL-12, and SL-16) in this depth.

Our observation may direct attention to investigate how different derivatives of drug molecules (mainly drugs with photosensibilizing property) can influence the stability of liposomal preparations. In case of NANa, and other fluoroquinolone drug molecules can adversely influence the stability of the liposomal formulation causing more intensive free radical production during preparation and storage (if the effect of UV irradiation cannot be eliminated, e.g. sterilization with germicidal lamps).

3.4. Size-distribution and surface potential

The stability of preparation, the ability of liposomes to fusion or aggregation depends on the surface potential. The pH of the hydrating phase, the acidic or basic properties of the drug molecules, interacting with the bilayer, can influence the integrity, and size-distribution of liposomes.

We checked the cumulant mean (or hydrodynamic diameter) as the best single figure to describe the sample size and the polydispersity index of the liposomes as well. These two parameters were used to follow up possible changes of the liposomal preparation. We addressed the question, if the method of preparation (extrusion or sonication), the composition of liposomes (pure DPPC or 30/70 % (w/w) DOPC/DPPC), the incubation with NANa and/or UV-B light have an influence on the size-distribution, and the stability of the liposome dispersion (Table 5). We observed that the extrusion method enables to get more homogeneous distribution of particles, than sonication. Extrusion method provided liposome samples with polydispersity index (PDI) of about 0.09–0.22, while ultrasound resulted in samples of PDI values between 0.28 and 0.43. On the contrary, sonication gives SUVs with slightly smaller mean diameter than extrusion (Table 5). In case of extrusion, we observed that there exists a lowest limit of the hydrodynamic diameter for both types of lipids used for preparing liposomes. Increasing number of extrusions through filters (with a pore size of 50 nm) did not cause significant decrease in the size of prepared liposomes. The smallest diameter we obtained, for both DPPC and 30/70 % (w/w) DOPC/DPPC liposomes, was about 93–96 nm. Vesicle size of extruded DPPC–liposomes, containing NANa was significantly higher, about 160–170 nm. However, similar increase of the hydrodynamic diameter was not found for the liposomes prepared from 30/70 % (w/w) DOPC/DPPC. UV-B irradiation (applied dose 5 kJ/m^2) of liposomes prepared in the presence of NANa resulted in an increase of the average size of vesicles compared to control liposomes. The increase was found to be more pronounced for DPPC liposomes (from 99.9 to 157.2 nm) than for DOPC/DPPC liposomes (from 99.9 to 104.6 nm). Comparing the vesicle sizes of NANa-treated to UV-B + NANa-treated samples, reveals that mostly the NANa-treatment determines the size increase.

Checking the PDIs obtained for extruded liposomes, we observed relative high values only for NANa containing DPPC SUVs. Both pure DPPC and its DOPC mixture liposomes show an increase in the PDI value,

Table 5

	Day of measurement	
	1st	21st
	Mean diameter ± S.D. (nm) and (PDI)	
DPPC-control		
Extrusion	99.9 ± 6.3 (0.111)	119.5 ± 7.4 (0.207)
Sonicated	85.2 ± 6.9 (0.339)	125.5 ± 7.4 (0.568)
30% DOPC-control[a]		
Extrusion	93.3 ± 1.3 (0.094)	109.8 ± 11.4 (0.255)
Sonicated	79.9 ± 7.7 (0.431)	92.6 ± 16.6 (0.668)
NANa-DPPC		
Extrusion	170.5 ± 18.8 (0.227)	nd (1)
Sonicated	91.9 ± 6.7 (0.288)	127.8 ± 10.2 (0.495)
NANa-30% DOPC[a]		
Extrusion[b]	97.1 ± 5.0 (0.1)	113.3 ± 3.9 (0.109)
Irradiated DPPC		
Extrusion	96.7 ± 4.9 (0.113)	140.2 ± 11.5 (0.287)
Sonicated	87.8 ± 8.8 (0.354)	150.3 ± 17.1 (0.654)
Irradiated 30% DOPC[a]		
Extrusion	99.4 ± 1.4 (0.102)	130.3 ± 1.6 (0.318)
Sonicated	71.4 ± 1.2 (0.358)	81.7 ± 12.4 (0.607)
Irradiated NANa-DPPC		
Extrusion[b]	157.2 ± 6.6 (0.222)	nd (1)
Irradiated NANa-30% DOPC[a]		
Extrusion[b]	104.6 ± 1.0 (0.122)	114.2 ± 8.3 (0.206)

nd: value not measured.

[a] Liposomes of 30/70 % (w/w) DOPC/DPPC ratio.

[b] Sonicated samples were not measured.

after 21 days storage, with no characteristic difference in the relative changes. Alike to the observation on hydrodynamic diameter, the PDI values show significant change only in case of extruded DPPC SUVs treated with NANa. In these samples, intensive growth was observed in the particle size, and after 6–7 days the liposome dispersion became polydisperse (PDI = 1). Thus, the presence of NANa has pronounced impact on the size-distribution, and on the polydispersity index of the SUVs prepared from pure DPPC, and this effect is attenuated by the presence of 30 % (w/w) DOPC. Earlier DSC results and theoretical considerations (Heimburg, 1998, 2000) have shown that there are differences in molecular properties of the liposomes prepared by sonication or extrusion. It has also been shown, that structural transitions involve changes in mean curvature, and they are linked to lipid chain melting in lipid membranes. According to our observations, NANa interacts with the lipid molecules close to the head group region, but it can, to a lesser extent, affect also the interaction between the lipid chains. On the other side, DOPC differs from the DPPC in the lipid chain composition, which can then influence the mechanical aspects of the lipid chains, i.e. it can have impact on the mean diameter when preparing liposomes under identical condition either from DPPC alone or from a mixture of DPPC/DOPC.

We aimed to evaluate the possible effect of NANa on the surface potential, which can lead to an increase in the fusion behavior of membranes composed of pure DPPC or 30/70 % (w/w) DOPC/DPPC (Sentjurc et al., 1999). According to our experiments, NANa does not possess such a property. The control and treated samples did not exhibit significantly different zeta potentials; the presence of NANa does not cause a significant change in the surface charge in any cases studied ($P > 0.05$). In distilled water (pH 6.9) the surface potentials were found to be slightly negative between −13 and −19 mV. In PBS solution the zeta-potential of the liposomes was already neutralized due to the influence of the buffer salts.

4. Conclusions

Results of our DSC and EPR measurements show evidences that NANa mainly interacts with lipid head groups of the DPPC molecules. This impact increases the rigidity of the lipid bilayer at this level of the membrane. NANa and UV-B induced free radical production does not influence the thermodynamic parameters of the lipid vesicles, in case of lower than 30% DOPC content. Increased nitroxide reduction due to UV-B irradiation of the NANa-treated samples revealed the effect of the free radical production along the whole lipid chain. It can suggest lipid peroxidation and/or increased lifetime of the generated free radicals in the apolar hydrocarbon inter-space. Incorporation of NANa in the liposomes provokes long-term destabilization of pure DPPC SUVs, which can be attenuated by 30 % (w/w) DOPC.

Acknowledgements

The authors are grateful to B. Deutschmann, E. Jauk, and Gy. Bányay for their valuable work. The work was partially supported by the grant no.: ETT 229/2000 from the Hungarian Ministry of Welfare and Health and from the National Research Foundation OTKA M-041729, OTKA M-045181.

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