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Effect of aminophospholipid glycation on order parameter and hydration of phospholipid bilayer

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Abstract

The effect of aminophospholipid glycation on lipid order and lipid bilayer hydration was investigated using time-resolved fluorescence spectroscopy. The changes of lipid bilayer hydration were estimated both from its effect on the fluorescence lifetime of the 1-[4-(trimethylammonium)-phenyl]-6-phenylhexa-1,3,5-triene (TMA-DPH) and 1,6-diphenylhexa-1,3,5-triene (DPH) and using solvatochromic shift studies with 1-anilinonaphthalene-8-sulfonic acid. The head-group and acyl chain order were determined from time-resolved fluorescence anisotropy measurements of the TMA-DPH and DPH. The suspensions of small unilamellar vesicles (with phosphatidylethanolamine/phosphatidylcholine molar ratio 1:2.33) were incubated with glyceraldehyde and it was found that aminophospholipids react with glyceraldehyde to form products with the absorbance and the fluorescence properties typical for protein advanced glycation end products. The lipid glycation was accompanied by the progressive oxidative modification of unsaturated fatty acid residues. It was found that aminophospholipid glycation increased the head-group hydration and lipid order in both regions of the membrane. The lipid oxidation accompanying the lipid glycation affected mainly the lipid order, while the effect on the lipid hydration was small. The increase in the lipid order was presumably the result of two effects: (1) the modification of head-groups of phosphatidylethanolamine by glycation; and (2) the degradation of unsaturated fatty acid residues by oxidation.

Keywords: Aminophospholipid glycation; Lipid oxidation; Lipid order; Lipid hydration; Fluorescence spectroscopy; DPH

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1. Introduction

The reaction between reducing sugars and amino groups in amino acids, proteins, and other biomolecules (also called glycation) has been shown to proceed in living systems. This slow glycation is involved in the pathogenesis of aging [1,2], diabetes [3,4], cataract [5] and possibly in neurodegenerative amyloidal diseases such as Alzheimer's [6,7]. After the initial formation of a Schiff base adduct between the carbonyl and the amine moiety, the resulting aldimine rearranges to a more stable ketoamine or Amadori product. These 'early' glycation products then undergo a series of inter- and intramolecular rearrangements, dehydrations, cyclizations, fragmentations, and oxidation–reduction reactions to produce the 'late' glycation products termed advanced glycation end products (AGEs). Oxidation reactions, which are involved in Maillard reaction [8], can produce the reactive oxygen species ($O_2^{\cdot -}$, H_2O_2, and $^{\cdot}OH$). These reactive oxygen species are able to cause collateral damage to proteins, lipids and DNA.

Amine-containing phospholipids can react with glucose to initiate Maillard reaction, forming lipid-linked AGEs and promoting fatty acid oxidation [9,10]. It has been observed that both aminophospholipid glycation and lipid oxidation increase during the chronic hyperglycemia of diabetes. Ravandi et al. [11] have reported that 10–16% of ethanolamine phospholipids of the red cells and plasma of the diabetic subjects were found glycated. It has been also shown that glucosylated phosphatidylethanolamine (PE) is more susceptible to peroxidation than non-glucosylated PE [12]. This supports the concept that lipid glycation can play an important role in initiating lipid oxidation in vivo. Thus the glycation of a major proportion of the aminophospholipids would affect membrane functions and all the processes involving aminophospholipids (e.g. the activity of membrane-bound enzymes [13]). The effect of aminophospholipid glycation on the physical properties of the lipid bilayer, however, has never been studied.

The structural and dynamical properties of fluorescent lipid probes in membrane systems are considered to reflect the structural order and dynamics of the lipid molecules surrounding them. Time-dependent fluorescence decay and fluorescence anisotropy measurements are convenient methods of studying membrane lipid order dynamics, provided that the reorientational motions of the probe molecules take place on the timescale of the intrinsic fluorescence decay. 1,6-Diphenylhexa-1,3,5-triene (DPH) and its polar analogue, 1-[4-(trimethylammonium)phenyl]-6-phenylhexa-1,3,5-triene (TMA-DPH) have been used extensively in such fluorescence measurements [14,15]. Both molecules are insoluble in aqueous buffers. Hence they both associate with lipid bilayer, and it has been thought that DPH partitions into the acyl chain hydrophobic core of membrane. On the other hand TMA-DPH is thought to be anchored with the positively charged trimethylammonium group in the lipid water interface and the phenylhexatriene portion in the acyl chain interior.

The fluorescence lifetime of a fluorophore (such as TMA-DPH or DPH) in lipid bilayer depends on its surrounding dielectric environment provided that no excited state reactions such as excimer formation occur. Small alterations of the membrane hydration have been shown to affect significantly the fluorescence lifetime due to fluorophore excited state–water interactions [16–19]. Thus fluorescence lifetime measurements can be used to infer the change of lipid hydration in the immediate fluorophore vicinity.

The primary aim of this study was to investigate the effect of aminophospholipid glycation by glyceraldehyde on the lipid order and lipid hydration of the phospholipid bilayer. A biologically relevant and well-defined binary lipid mixture system, phosphatidylethanolamine/phosphatidylcholine (POPE/POPC), has been used. In order to determine the role of the lipid oxidation on the observed lipid bilayer changes we have used a hydrophobic antioxidant probucol to suppress the oxidative processes. The effects of aminophospholipid glycation have been compared with the effects of lipid oxidation induced by the system L-ascorbic acid/Fe^{2+}.

2. Materials and methods

2.1. Reagents

DL-Glyceraldehyde, 1-palmitoyl-2-oleoyl-3-*sn*-phosphatidylethanolamine (POPE), 1-palmitoyl-2-oleoyl-3-*sn*-phosphatidylcholine (POPC), malonaldehyde bis(diethyl acetal) (MDA), probucol®, ANS (1-anilinonaphthalene-8-sulfonic acid) and Tris {2-amino-2-(hydroxymethyl)-1,3-propanediol} were obtained from Sigma Chemical Co.

1,6-diphenylhexa-1,3,5-triene (DPH) and 1-[4-(trimethylammonium)phenyl]-6-phenylhexa-1,3,5-triene (TMA-DPH) were purchased from Molecular Probes (Eugene, OR, USA). The other chemicals were of the highest purity commercially available. All solvents used were of spectroscopic grade.

2.2. Small unilamellar vesicles (SUV) preparation

The suspensions of POPE/POPC SUVs without or with probucol were prepared. Molar ratio POPE/POPC was 1:2.33 and molar ratio phospholipids/probucol was 2×10^3:1. Small unilamellar vesicles (30–45 nm in diameter) were prepared by the method of Barenholz et al. [20,21], in which, phospholipids (and probucol) were suspended in buffer containing 20 mM Tris–HCl and 0.02% NaN_3, pH 7.4 and sonicated 30 min with 150-W sonicator (M.S.E., UK). After sonication, large multilamellar vesicles were removed by ultracentrifugation for 1 h at $100\,000 \times g$. Then suspensions of SUVs were diluted to 30 mM concentration.

2.3. Sugar–lipid incubations

Suspensions of SUVs were diluted into sterile scintillation vials (final lipid concentration was 15 mM) by buffer containing 20 mM Tris–HCl and 0.02% NaN_3, pH 7.4. Then the glyceraldehyde was added (final GCA concentration was 150 mM). The vials were incubated in the dark at 37°C.

2.4. Induction of lipid oxidation by the system L-ascorbic acid / Fe^{2+}

Suspension of SUVs (final lipid concentration was 15 mM) was incubated with 0.4 mM ascorbic acid and 0.1 mM $FeSO_4$ in 20 mM Tris–HCl, 0.02% NaN_3 buffer at pH 7.4 for 100 min at 37°C. The reaction was started by the addition of ascorbic acid and stopped by the addition of 5 mM EDTA.

2.5. Analysis of thiobarbituric acid (TBA)-reactive substances

Monounsaturated fatty acid residues are 10- to 30-fold less susceptible to oxidation than polyunsaturated fatty acids [22] but, once oxidized, can undergo β-scission to form reactive aldehydes that represent the initiation reactions. POPE and POPC used in our experiments contained esterified oleic acid (monounsaturated fatty acid) and palmitic acid (saturated fatty acid). Under defined conditions, TBA condenses with a variety of reactive aldehydes (e.g. lipid oxidation products) to form addition products that can be quantitated by reference to a standard solution of MDA. At different time intervals (0–35 days) the aliquots (1 ml) were analyzed for TBA-reactive substances content [23]. Lipids and lipid-soluble products were extracted into 500 μl of chloroform/methanol (2:1) to remove unreacted GCA or L-ascorbic acid and $FeSO_4$. The tubes were gently rocked for 15 min, the interface was cleared by centrifugation, and the extraction was then repeated two additional times. The solvent was evaporated under nitrogen, 1 ml of TBA solution was added, and tubes were incubated at 95°C for 45 min. After reaction with TBA, samples were extracted into 2 ml of 1-butanol prior to fluorescence measurement (emission 553 nm upon excitation at 515 nm). TBA-reactive substances were quantified by comparison of samples to a MDA standard curve (expressed as nmol of MDA equivalents per mg of lipids).

2.6. Thin layer chromatography

Lipids were extracted using chloroform/methanol (2/1, v/v). TLC was performed on a

Silica Gel 60 TLC plates (Merck, Darmstadt, Germany) in: chloroform/methanol/acetic acid/water (60:50:1:4, by vol.). The lipids were detected either with iodine vapor or under ultraviolet light illumination [24].

2.7. Absorption and steady-state fluorescence measurements

Absorption measurements were performed on a UVICON 810 spectrophotometer at 25°C. Steady-state fluorescence data were obtained on a Perkin-Elmer LS-5B fluorometer. All measurements were carried out at 25°C. TMA-DPH dissolved in methanol was added to vesicle suspension (roughly 1 μl of probe stock/ml of vesicle suspension), and these mixtures were incubated for 30 min in the dark under continuous stirring. The final probe to phospholipid ratio was 1:500. Excitation and emission wavelengths were 365 nm and 430 nm, respectively. Two Glan-Thompson polarizers were used for detection of steady-state anisotropy values.

2.8. Fluorescence quenching experiments

These experiments were carried out at 25°C with TMA-DPH and DPH molecules embedded in POPE/POPC vesicles. The final probe to phospholipid ratio was 1:500 and vesicles were dispersed in 20 mM Tris–HCl, 0.02% NaN_3 buffer at pH 7.4. Lipid concentration was 6×10^{-4} M. Aliquots of the quenching solution (40 μl of a 4-M KI solution) were added stepwise to the samples (2 ml of a lipid suspension). The final KI concentration was 0.7 M. The Stern–Volmer quenching constants were obtained using Stern–Volmer plot. The classical Stern–Volmer equation is expressed [25] by Eq. (1):

$$F_0/F = 1 + K_{SV}[Q], \quad (1)$$

where F_0 and F are fluorescence intensities without and with the quencher, respectively, [Q] is the concentration of the quencher. From Eq. (1), a plot of F_0/F vs. [Q] yields a straight line of slope K_{SV}.

2.9. Solvatochromic shift studies with 1-anilinonaphthalene-8-sulfonic acid (ANS)

All measurements were done at 25°C. ANS dissolved in water was added to vesicle suspension (roughly 1 μl of probe stock/ml of vesicle suspension), and these mixtures were incubated for 30 min in the dark under continuous stirring. The final probe to phospholipid ratio was 1:300. Excitation wavelength was 370 nm and emission spectra were recorded from 390 to 580 nm [26].

2.10. Dynamic fluorescence measurements and data analysis

The glycated or oxidized lipid suspensions were first of all dialyzed to remove unreacted aldehyde or L-ascorbic acid and $FeSO_4$. TMA-DPH and DPH dissolved in methanol and acetone, respectively, were added to vesicle suspension (roughly 1 μl of probe stock/ml of vesicle suspension), and these mixtures were incubated for 30 min in the dark under continuous stirring. The final probe to phospholipid ratio was 1:500.

Time-domain fluorescence decay measurements were carried out using the time-correlated single photon counting technique at the Institute of Physics, Charles University, Prague. The excitation source consisted of a cavity dumped dye laser (model 375, Spectra Physics, USA) synchronously pumped by the argon ion laser (model 171, Spectra Physics, USA) and a frequency doubler. The excitation pulses (FWHM approx. 10 ps) were generated at 356 nm with Pyridine 1 as a laser dye. The required emission wavelength (430 nm) was selected by a monochromator with a proper cut-off filter in front of the input slit. The two components I_{vv} and I_{vh} were recorded, and the total fluorescence decay corresponds to the weighted sum of the two components:

$$I(t) = I_{vv}(t) + [2\beta I_{vh}(t)] \quad (2)$$

where β is a correction factor for the transmission of polarized light by the monochromator at a given wavelength. The apparatus response function was determined by a REF procedure de-

scribed by Vecer et al. [27] with fluorescein and KI quenched fluorescein as a pair of monoexponential reference compounds. All experiments were repeated with an unlabelled sample in order to check for background fluorescence and light scattering. The decay data were analyzed by a nonlinear least-squares deconvolution procedure. All measurements were performed at 25°C and each measurement was repeated three times on separate preparations.

We used the 'wobble in a cone' model to describe the decay of fluorescence anisotropy [28]. The parameters that describe the probe motion are as follows: ϕ, the rotational correlation time, which is the exponential decay constant describing the decay of fluorescence anisotropy to its asymptotic value, r_∞, after a pulsed excitation. The value of r_∞ approaches 0.4 for very small apical cone angles and approaches zero for un-inhibited rotation. Basically, it provides an order parameter for the orientation of TMA-DPH (or DPH) relative to the normal to the bilayer [29]. A useful approximation of this decay behavior is

$$r(t) = r_\infty + (r_0 - r_\infty)\exp(-t/\phi) \quad (3)$$

where $r(t)$ is the fluorescence anisotropy at time t after the flash and ϕ is the rotational correlation time. The values of r_∞ and ϕ were obtained by non-linear, least-squares fitting.

An order parameter S was used to characterize the lipid molecules structural order from anisotropy measurements on TMA-DPH and DPH and was calculated according to Eq. (4):

$$S = (r_\infty/r_0)^{1/2} \quad (4)$$

3. Results

3.1. Lipid-derived glycofluorophores formation

The suspensions of POPE/POPC SUVs (15 mM) with or without probucol were incubated with glyceraldehyde (150 mM) at physiological pH and temperature for increasing time intervals. All samples were observed to react with GCA to form products with the absorbance and the fluorescence properties which are typical for protein AGEs (Fig. 1). It was seen that the absorption and fluorescence intensity of glycofluorophores in POPE/POPC vesicles was enhanced as the time of incubation increased. The GCA-lipid glycation products showed an excitation maximum at 335 nm and an emission maximum at 439 nm.

Progress curves for the development of lipid-derived glycofluorophores are shown in Fig. 2. Glycation of probucol (hydrophobic antioxidant)

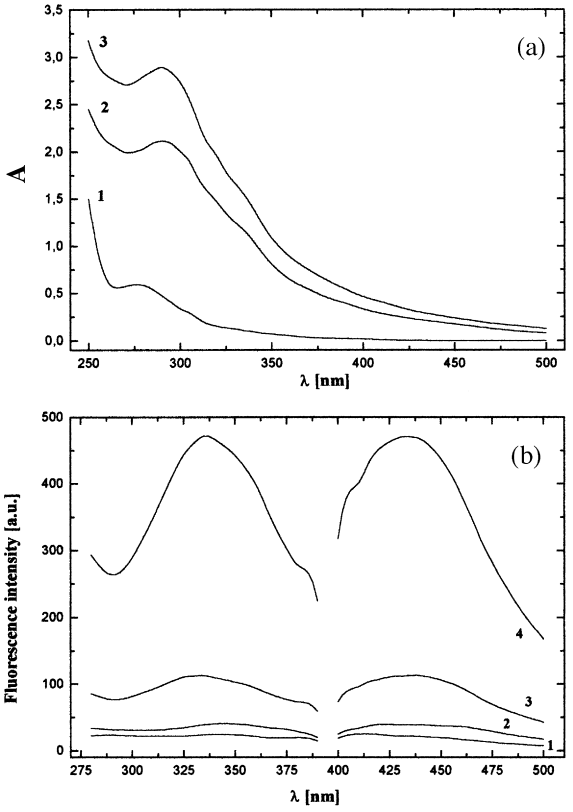

Fig. 1. (a) Time progress of the absorption spectrum of aminophospholipid AGEs. Spectrum (1) the beginning of the treatment, spectrum (2) after 21 days of the treatment, spectrum (3) after 35 days of the treatment. (b) Time progress of uncorrected excitation and emission fluorescence spectra of aminophospholipid-derived AGEs. The excitation and emission wavelengths were 335 nm and 425 nm, respectively. Spectrum (1) corresponds with the beginning of the treatment, spectrum (2) after 7 days of the treatment, spectrum (3) after 21 days of the treatment and spectrum (4) after 21 days of the treatment.

containing vesicles gave a reduced glycofluorophores formation, as expected for the system in which the glycooxidation reactions are suppressed. Oxidation of vesicles with or without probucol by the system L-ascorbic acid/Fe^{2+} produced only negligible amount of fluorophores (data not shown). Degree of aminophospholipid (POPE) glycation was simultaneously monitored using thin layer chromatography (not shown). We have observed that the glycated POPE showed higher chromatographic mobility in comparison with control POPE and could be detected under ultraviolet light illumination. We have observed that after 28 days of incubation 53% and after 42 days 82% of POPE molecules was glycated. The presence of probucol reduced the level of modified POPE molecules after 28 days of treatment on 43% and after 45 days on 70%. The retention factor of POPC extracted from the mixture of glycated lipids was unchanged and no fluorescence signal under ultraviolet light illumination was observed.

3.2. Lipid oxidation

Fig. 3A,B show the time dependence of MDA generation for vesicles treated by glyceraldehyde or by the system L-ascorbic acid/Fe^{2+}. It was seen that in the case of POPE glycation the lipid oxidation products were formed at a rate higher but parallel to the rate of glycofluorophores formation. Both glycation and oxidation of vesicles containing probucol produced smaller amount of MDA in comparison with SUVs without probucol.

3.3. Changes of the steady-state fluorescence anisotropy of TMA-DPH

In Fig. 3C,D we present the effect of the glycation or oxidation on the steady-state fluorescence anisotropy of TMA-DPH. The steady-state fluorescence anisotropy of TMA-DPH increased with the time of incubation for both glycated and oxidized vesicles. Observed values for the steady-state fluorescence anisotropy of TMA-DPH for SUVs containing probucol were slightly lower than for vesicles without probucol.

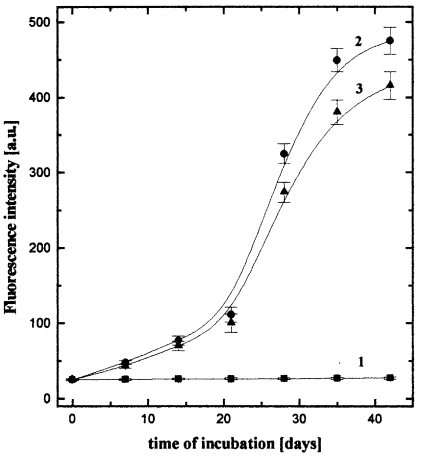

Fig. 2. Progress curves for the development of aminophospholipid-derived AGEs. Fluorescence emission at 425 nm (335 nm excitation) was used as a reflection of glycofluorophores formation. Curve (1) shows control (non-glycated) POPE/POPC SUVs, curve (2) glycated POPE/POPC SUVs and curve (3) glycated POPE/POPC/probucol SUVs. Means ± S.D. were calculated from the three independent measurements.

3.4. Quenching of TMA-DPH and DPH emission

To obtain more information about the effects of SUVs glycation or oxidation on lipid–lipid interactions, we followed the collisional quenching of the TMA-DPH and DPH emissions. The Stern–Volmer quenching constants (K_{SV}) for fluorescence quenching of the TMA-DPH and DPH emissions with KI are presented in Table 1. As it can be seen from these data the TMA-DPH probe proved to be more efficiently quenched then DPH probe. The aminophospholipid glycation influenced the Stern–Volmer quenching constants of both probes. The quenching constant of the TMA-DPH decreased but the quenching constant of DPH increased with the time of incubation (Table 1). On the other hand, lipid oxidation triggered by the system L-ascorbic acid/Fe^{2+} had only the small effect on the Stern–Volmer quenching constants of TMA-DPH and DPH.

3.5. Solvatochromic shift studies with ANS

Solvatochromic shift studies with ANS (1-anilinonaphthalene-8-sulfonic acid) incorporated

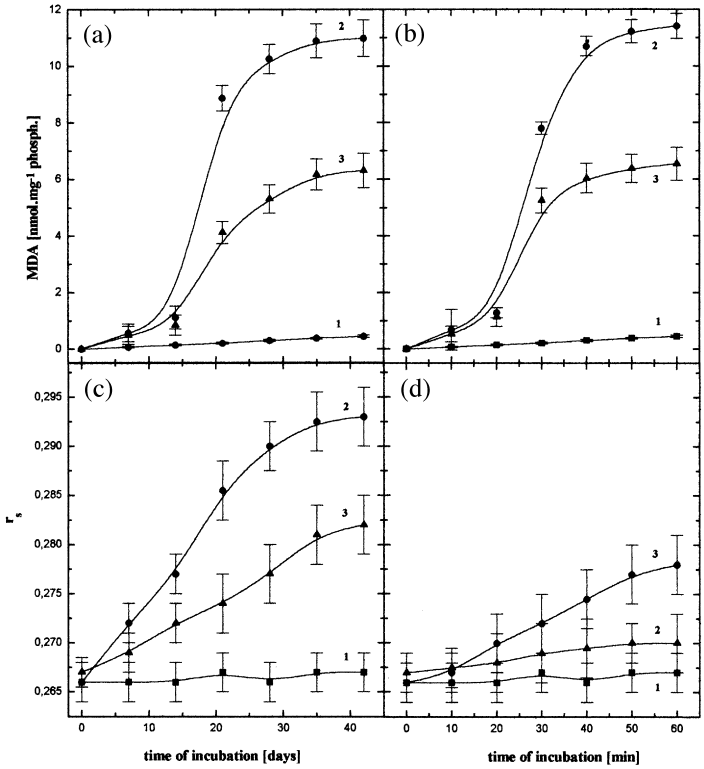

Fig. 3. Progress curves for the generation of malondialdehyde (MDA) and steady-state fluorescence anisotropy of TMA-DPH as the function of the incubation time of vesicles with glyceraldehyde (a,c) or oxidation by the system L-ascorbic acid/Fe^{2+} (b,d). Probe to phospholipid ratio was 1:500. Excitation and emission wavelengths were 365 nm and 430 nm, respectively. Curves (1) show control (non-treated) POPE/POPC SUVs, curves (2) POPE/POPC SUVs, and curves (3) POPE/POPC/probucol SUVs. Means ± S.D. were calculated from the three independent measurements.

in both glycated and oxidized vesicles were performed to study the changes of polarity in the head-group region (binding site of ANS) of the lipid bilayer. We have observed that glycation by GCA (but not oxidation by the system L-ascorbic acid/Fe^{2+}) decreased the quantum yield of ANS fluorescence together with a red shift of fluorescence maximum (Fig. 4). ANS incorporated in non-treated control vesicles revealed a fluorescence emission maximum at 474 nm. After 28 and 42 days of glycation the emission maximum of ANS fluorescence was 485 nm and 491 nm, respectively. The presence of probucol in the glycated or oxidized vesicles did not significantly influence the emission maximum of ANS fluorescence.

3.6. Dynamic fluorescence measurements of TMA-DPH

In order to improve the definition of the lipid bilayer structural alterations induced by glycation or oxidation, we attempted to measure the dynamics of TMA-DPH and DPH fluorescence. For elucidation of the effects of glycation on the lipid bilayer hydration, the principle parameter in question was the excited state lifetime. It was found that fluorescence decay of TMA-DPH and DPH could be described adequately by assuming two lifetime components. These two-lifetime descriptions for TMA-DPH are summarized in Table 2. The time of SUVs oxidation by the system

Table 1
Stern–Volmer quenching constants for TMA-DPH and DPH fluorescence quenching by KI in glycated (by glyceraldehyde) and oxidized (by the system L-ascorbic acid/Fe^{2+}) vesicles[a]

	Probe	Time of treatment	K_{SV} (mol^{-1}) Control	K_{SV} (mol^{-1}) Treated
Glycation	TMA-DPH	0 days	0.829 ± 0.007	0.829 ± 0.007
		7 days	0.824 ± 0.009	0.741 ± 0.016
		21 days	0.825 ± 0.007	0.670 ± 0.021
		35 days	0.821 ± 0.008	0.551 ± 0.025
	DPH	0 days	0.349 ± 0.019	0.349 ± 0.019
		7 days	0.350 ± 0.020	0.433 ± 0.023
		21 days	0.355 ± 0.018	0.442 ± 0.017
		35 days	0.356 ± 0.022	0.480 ± 0.026
Oxidation	TMA-DPH	0 min	0.829 ± 0.007	0.829 ± 0.007
		40 min	0.829 ± 0.007	0.792 ± 0.013
		80 min	0.829 ± 0.008	0.735 ± 0.015
		120 min	0.830 ± 0.007	0.701 ± 0.011
	DPH	0 min	0.349 ± 0.019	0.349 ± 0.019
		40 min	0.349 ± 0.020	0.378 ± 0.016
		80 min	0.349 ± 0.020	0.396 ± 0.016
		120 min	0.348 ± 0.020	0.409 ± 0.018

[a] Means ± S.D. were calculated from the three independent experiments.

L-ascorbic acid/Fe^{2+} was set to obtain roughly the same lipid oxidation as for glycated SUVs but this did not affect significantly the average fluorescence lifetime of TMA-DPH.

In comparison with vesicles without probucol the presence of this hydrophobic antioxidant in the lipid bilayer increased the contribution of the long lifetime component (f_2) for both the glycated and oxidized SUVs. The lifetime associated with this component decreased, however, only for glycated vesicles.

Lipid order in head-group region was determined from the time-resolved fluorescence anisotropy measurements of TMA-DPH. The effects of aminophospholipid glycation on the head-group order parameter are shown in Table 2 as well. The increase in the order parameter S was observed only for glycated vesicles. The presence of probucol did not significantly reduce the order parameter. The rotational correlation time ϕ for all systems remained practically unchanged.

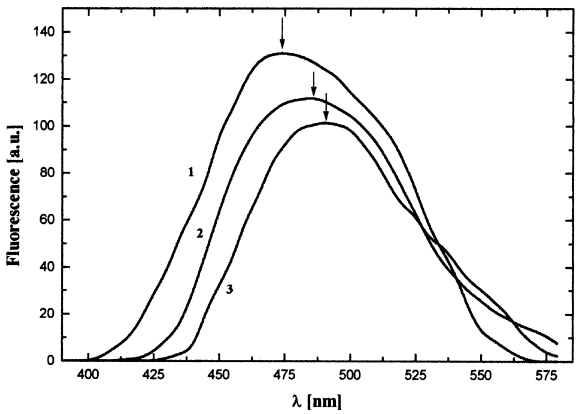

Fig. 4. Uncorrected fluorescence spectra of ANS in glycated vesicles. The excitation wavelength was 370 nm. The decrease in the quantum yield is accompanied by a red shift of fluorescence maximum (shown by arrows) as the time of glycation increases. Spectrum (1) corresponds with the beginning of the treatment, spectrum (2) after 28 days of the treatment and spectrum (3) after 42 days of the treatment.

3.7. Dynamic fluorescence measurements of DPH

Aminophospholipid glycation but not the lipid oxidation (triggered by the system L-ascorbic acid/Fe^{2+}) decreased significantly the average fluorescence lifetime of DPH as shown in Table

Table 2
Summary of TMA-DPH lifetimes and dynamics in control, glycated and oxidized vesicles with and without probucol[a]

	Control	Control + probucol	GCA	GCA + probucol	AA/Fe^{2+}	AA/Fe^{2+} + probucol
f_1	0.385	0.383	0.458	0.37	0.499	0.422
τ_1/ns	1.61	1.61	1.22	1.06	1.59	1.62
f_2	0.615	0.617	0.542	0.63	0.501	0.578
τ_2/ns	5.08	5.1	4.17	3.57	5.05	5.06
$\langle\tau\rangle$/ns	3.74 ± 0.23	3.76 ± 0.25	2.81 ± 0.28	2.64 ± 0.31	3.32 ± 0.26	3.61 ± 0.29
χ^2	1.09	1.11	1.18	1.17	1.12	1.17
r_∞	0.124 ± 0.003	0.126 ± 0.004	0.143 ± 0.004	0.136 ± 0.004	0.129 ± 0.003	0.124 ± 0.004
S	0.564 ± 0.007	0.568 ± 0.009	0.606 ± 0.008	0.591 ± 0.009	0.575 ± 0.007	0.564 ± 0.009
ϕ/ns	2.72 ± 0.35	2.70 ± 0.38	2.98 ± 0.43	2.85 ± 0.41	2.79 ± 0.35	2.74 ± 0.40

[a] The SUVs were glycated by incubation with glyceraldehyde for 35 days in the dark at 37°C. To oxidize the vesicles on the same level as glycation by GCA, the time of incubation of SUVs with oxidative system L-ascorbic acid/Fe^{2+} was 100 min. Component lifetimes (τ_i) were obtained by non-linear, least-squares analysis (f_i is the fractional intensity of ith component $\langle\tau\rangle = \Sigma f_i\tau_i$ is the average excited-state lifetime, r_∞ is the limiting fluorescence anisotropy and ϕ is rotational correlation time). Quality of the fit is indicated by the reduced mean squared deviations calculated from experimental values (χ^2); values close to 1 indicate an adequate description of the data. Parameter uncertainties are standard deviations based on the three independent measurements.

3. Both the glycation and lipid oxidation were found to increase the order parameter of DPH, while the rotational correlation time ϕ stayed practically unchanged. The presence of probucol did not influence the average lifetime of DPH but significantly decreased the order parameter for only oxidized vesicles.

4. Discussion

In the present work, we have investigated the effect of aminophospholipid glycation both on order and hydration of the lipid bilayer of small unilamellar vesicles by fluorescence approach. The POPE/POPC and POPE/POPC/probucol vesicles were either glycated by glyceraldehyde (GCA) or oxidized by the system L-ascorbic acid/Fe^{2+}. It was found that GCA reacted with POPE, forming lipid-linked glycofluorophores with the absorbance and fluorescence properties of protein-linked AGEs (Fig. 1). The modification of POPE molecules was proved by TLC chromatography (not shown). TLC analyses also showed that after 42 days of glycation approximately 82% of POPE molecules were modified. The lipid glycation was accompanied by the progressive oxidative modification of unsaturated fatty acid residues. MDA, main product of lipid oxidation was formed at a rate that was parallel but higher to the rate of glycofluorophores formation (Figs. 2 and 3A). It means that the lipid oxidation was initiated mainly during the 'early' phase of the glycation reaction, when Amadori products were predominant products of aminophospholipid glycation. These results are in accordance with an earlier observations reported by Bucala et al. [9].

Probucol is a hydrophobic antioxidant that blocks chain-propagating radical reactions in the lipid phase [30,31]. We have prepared SUVs with probucol to elucidate the role of oxidative processes on the observed phenomena. The presence of probucol in vesicles reduced both the glycofluorophores formation and lipid oxidation (Fig. 3), as expected for a system in which the oxidative processes were suppressed. The inhibition of glycofluorophores formation by probucol indicates that glycoxidative reactions participate in the lipid derived AGEs production [8,9].

Steady-state fluorescence measurements of TMA-DPH represented the first attempt at detecting distinctive structural perturbations induced by lipid glycation or oxidation. The steady-state fluorescence anisotropy of TMA-DPH increased with the time of incubation, presumably

Table 3
Summary of DPH lifetimes and dynamics in control, glycated and oxidized vesicles with and without probucol[a]

	Control	Control + probucol	GCA	GCA + probucol	AA/Fe^{2+}	AA/Fe^{2+} + probucol
f_1	0.071	0.067	0.206	0.215	0.088	0.093
τ_1/ns	2.95	2.93	2.35	2.26	2.73	2.78
f_2	0.929	0.923	0.794	0.785	0.912	0.907
τ_2/ns	9.19	9.17	7.29	7.08	8.99	9.10
$\langle\tau\rangle$/ns	8.75 ± 0.25	8.66 ± 0.27	6.27 ± 0.32	6.04 ± 0.30	8.44 ± 0.27	8.51 ± 0.30
χ^2	1.07	1.11	1.19	1.16	1.10	1.15
r_∞	0.073 ± 0.003	0.074 ± 0.004	0.088 ± 0.005	0.080 ± 0.005	0.089 ± 0.004	0.079 ± 0.004
S	0.433 ± 0.009	0.436 ± 0.012	0.475 ± 0.014	0.453 ± 0.014	0.478 ± 0.011	0.450 ± 0.011
ϕ/ns	2.37 ± 0.36	2.33 ± 0.41	2.58 ± 0.45	2.50 ± 0.44	2.52 ± 0.38	2.48 ± 0.41

[a] The SUVs were glycated by incubation with glyceraldehyde for 35 days in the dark at 37°C. To oxidize the vesicles on the same level as glycation by GCA, the time of incubation of SUVs with oxidative system L-ascorbic acid/Fe^{2+} was 100 min. Component lifetimes (τ_i) were obtained by nonlinear, least-squares analysis (f_i is the fractional intensity of ith component, $\langle\tau\rangle = \Sigma f_i\tau_i$ is the average excited-state lifetime, r_∞ is the limiting fluorescence anisotropy and ϕ is rotational correlation time). Quality of the fit is indicated by the reduced mean squared deviations calculated from experimental values (χ^2); values close to 1 indicate an adequate description of the data. Parameter uncertainties are standard deviations based on the three independent measurements.

because of the increased lipid order of the bilayer. The glycation affects especially the aminophospholipid head groups [9,10], while the lipid oxidation perturbs the membrane core [32]. Both these processes can change the volume occupied per phospholipid molecule in bilayer and thus the lipid order [32,33]. Probucol in the lipid bilayer significantly decreased the steady-state fluorescence anisotropy of TMA-DPH for both glycation and oxidation. This effect can be explained by the reduced lipid oxidation, which is known as an effective factor influencing the lipid order [32] and thus the steady-state fluorescence anisotropy of TMA-DPH.

Modification of polar head-groups of POPE together with fatty acid residues degradation induced by glycation probably affect lipid–lipid interactions. In order to obtain more information about these effects, we measured the collisional quenching of the TMA-DPH and DPH emissions. Iodide ion is a very efficient quencher of excited state species (by a spin-orbit coupling mechanism), highly soluble in aqueous environments, and much less soluble in the apolar environment of the bilayer [25]. In addition, iodide has a low effectiveness in causing flocculation of phospholipid vesicles, because of its small hydrated radius. The efficiency of quenching was used here to assess the change of accessibility of TMA-DPH and DPH in the bilayer. In both cases quenching was inefficient requiring molar concentration of iodide ion to significantly decrease the fluorescence yield. The TMA-DPH probe proved to be more efficiently quenched then DPH probe. This is due to the positive charge of TMA-DPH probe and its position which is closer to the surface, as expected because of its polar nature. Inspection of the Stern–Volmer constants (Table 1) enables clear conclusion that aminophospholipid glycation decreased the accessibility of TMA-DPH probe and increased accessibility of DPH probe. On the other hand, the effect of lipid oxidation initiated by the system L-ascorbic acid/Fe^{2+} was much less pronounced (Table 1). These results can be interpreted on the basis of increasing polarity of the lipid bilayer which probably affected the depth of DPH probe and thus increased the DPH accessibility. However, the decrease in quenching constant of TMA-DPH could be result either of redistribution of the TMA-DPH molecules in lipid bilayer (because of its positive charge) and/or increasing order in head-group region of the bilayer which can protect molecules of TMA-DPH against collisions with iodide ions.

To study the changes of the lipid bilayer polarity in the head-group region we have performed solvatochromic shift studies with ANS (Fig. 4) [26]. These measurements clearly revealed in-

creased polarity in the head-group region (where ANS is bound) of the glycated vesicles. Lipid oxidation by the system L-ascorbic acid/Fe^{2+} did not alter the polarity in this region. From these results it appears that only glycation of POPE molecules was able to change the polarity in head-group region of the bilayer.

In order to improve the definition of these effects, we attempted to measure the dynamics of TMA-DPH and DPH fluorescence. It was found that lipid glycation decreased the fluorescence lifetime of both probes (Tables 2 and 3). The fluorescence lifetime of the fluorophore in lipid bilayer depends on its surrounding dielectric environment provided that no excited state reactions such as excimer formation occur. Small alterations in the degree of hydration have been shown to significantly affect the fluorescence lifetime due to the fluorophore excited state–water interactions [16–19]. We assume that the effect of glycation was to increase the hydration in the head-group and probably also in the interchain region of the lipid bilayer as probed by the decrease in average TMA-DPH and DPH fluorescence lifetimes. This is in the contrast with the effect of the oxidation by the system L-ascorbic acid/Fe^{2+}, which had a small effect on the membrane hydration.

Aminophospholipid glycation, but not oxidation by the system L-ascorbic acid/Fe^{2+}, was found to increase the order of the head-group region of the membrane. This difference could be explained by the glycation-mediated modification of head-groups of POPE molecules which is accompanied by the increase in the steric lipid head-group interactions [33]. The similar effect of glycation and oxidation on the acyl chain order is not surprising because the level of unsaturated fatty acid residues degradation (which affects mainly the acyl chain region of the bilayer [32]) was roughly the same for both systems.

The lipid order and lipid hydration are not simply related quantities [16]. In this study, it was found that aminophospholipid glycation increased both the polarity and the lipid order in the head-group part of the membrane (Fig. 4 and Tables 2 and 3). The polar groups of lipid glycation products can efficiently interact with the excited state of TMA-DPH and thus reduce the fluorescence lifetime, while the head-groups of glycated POPE molecules (which are more bulky in comparison with non-modified POPE molecules) order this membrane region. The head-group glycation can also introduce packing defects into the lipid bilayer, which can accommodate more water. The higher acyl chain order should have the opposite effect on the interchain hydration [16,34,35]. It seems, that in the case of POPE glycation by GCA, this effect cannot prevent the penetration of water into the bilayer. Other factor reducing the DPH lifetime in glycated vesicles could be the interactions between lipid oxidation products and excited state of DPH as well.

The probucol mediated inhibition of the lipid oxidation of glycated vesicles had only a weak effect on both the average fluorescence lifetime (Tables 2 and 3) and the lipid order parameter of DPH. In the case of lipid oxidation triggered by the system L-ascorbic acid/Fe^{2+} probucol reduced the concentration of MDA by 43%. Nevertheless this fact had no effect on the average lifetime, although the lipid order of DPH was decreased. It means that the lipid oxidation, which accompanies the lipid glycation can affect mainly lipid order, but not lipid hydration. This supports the recent hypothesis that the lipid order cannot exactly predict the functional effects on membrane proteins, which may also be affected by altered polarity of the bilayer [16].

5. Conclusions

1. POPE/POPC vesicles can react with glyceraldehyde to form products with the absorbance and the fluorescence properties of protein AGEs.
2. The POPE glycation increased the lipid order in both regions of bilayer and head-group hydration.
3. The lipid oxidation which accompanies the lipid glycation affected mainly the acyl chains order, while the effect on lipid hydration was negligible.
4. The increase of the lipid order at both re-

gions of the membrane was presumably the superposition of two effects: (1) the modification of head-groups of POPE by glycation; and (2) the degradation of unsaturated fatty acid residues by oxidation, which accompanies the lipid glycation.

5. The present results show that aminophospholipid glycation can affect the physical properties of the lipid bilayer and thus could influence the conformation of integral membrane proteins, which may be influenced by altered dielectric constant and lipid order at both regions of the bilayer.

6. Nomenclature

POPC:	1-Palmitoyl-2-oleoyl-3-*sn*-phosphatidylcholine
POPE:	1-Palmitoyl-2-oleoyl-3-*sn*-phosphatidylethanolamine
PE:	Phosphatidylethanolamine
TMA-DPH:	1-[4-(trimethylammonium)phenyl]-6-phenylhexa-1,3,5-triene
DPH:	1,6-Diphenylhexa-1,3,5-triene
ANS:	1-Anilinonaphthalene-8-sulfonic acid
MDA:	Malonaldehyde bis(diethyl acetal)
SUVs:	Small unilamellar vesicles
GCA:	D,L-glyceraldehyde
AGE:	Advanced glycation end product
TBA:	Thiobarbituric acid
f_I:	Fractional intensity of the ith component
τ_I:	Lifetime of ith component
$\langle\tau\rangle$:	Average lifetime
χ_R^2:	Goodness of fit
S:	Order parameter
r_∞:	Limiting fluorescence anisotropy
ϕ:	Rotational correlation time
K_{SV}:	Stern–Volmer quenching constant

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