



Peroxidative permeabilization of liposomes induced by cytochrome *c*/cardiolipin complex



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ABSTRACT

Interaction of cytochrome *c* with mitochondrial cardiolipin converting this electron transfer protein into peroxidase is accepted to play an essential role in apoptosis. Cytochrome *c*/cardiolipin peroxidase activity was found here to cause leakage of carboxyfluorescein, sulforhodamine B and 3-kDa (but not 10-kDa) fluorescent dextran from liposomes. A marked decrease in the amplitude of the autocorrelation function was detected with a fluorescence correlation spectroscopy setup upon incubation of dye-loaded cardiolipin-containing liposomes with cytochrome *c* and H₂O₂, thereby showing release of fluorescent markers from liposomes. The cytochrome *c*/H₂O₂-induced liposome leakage was suppressed upon increasing the ionic strength, in contrast to the leakage provoked by Fe/ascorbate, suggesting that the binding of cyt *c* to negatively-charged membranes was required for the permeabilization process. The cyt *c*/H₂O₂-induced liposome leakage was abolished by cyanide presumably competing with H₂O₂ for coordination with the central iron atom of the heme in cyt *c*. The cytochrome *c*/H₂O₂ permeabilization activity was substantially diminished by antioxidants (trolox, butylhydroxytoluene and quercetin) and was precluded if fully saturated tetramyristoyl-cardiolipin was substituted for bovine heart cardiolipin. These data favor the involvement of oxidized cardiolipin molecules in membrane permeabilization resulting from cytochrome *c*/cardiolipin peroxidase activity. In agreement with previous observations, high concentrations of cyt *c* induced liposome leakage in the absence of H₂O₂, however this process was not sensitive to antioxidants and cyanide suggesting direct membrane poration by the protein without the involvement of lipid peroxidation.

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1. Introduction

Peroxidation of membrane lipids [1–3] has long been considered as one of the harmful consequences of oxidative stress in living cells, manifesting itself, in particular, in perturbing the barrier function of cell membranes. In support of this common idea, experiments with model lipid membranes – planar bilayers and liposomes – have shown that lipid peroxidation results in augmented membrane permeability [4–17]. On the other hand, mechanistic studies of apoptosis have pointed to a key role of cardiolipin peroxidation in the process of cytochrome *c* (cyt *c*) release from mitochondria into cytosol [18–21]. Intriguingly, cardiolipin peroxidation has appeared to be catalyzed by cyt *c* [19, 22–24], the peroxidase activity of which [25–32] is dramatically enhanced upon binding to cardiolipin [19,33–35], partial proteolysis

[26,36], denaturation [31], dimerization [37], tyrosine nitration [38], methionine (met₈₀) oxidation [39,40] and residues 26 or 41 mutation [41–43]. Induction of lipid peroxidation by cyt *c* has also been reported for other unsaturated lipids [44–49].

In view of the above relationship between lipid peroxidation and membrane permeability, peroxidase activity of cyt *c*/cardiolipin complex may imply its propensity to permeabilize lipid membrane in the presence of hydrogen peroxide. Of note, cyt *c* by itself is also able to induce ion permeability of liposomal [50] and planar bilayer [51] membranes and even leakage of fluorescent dextran and carboxyfluorescein from liposomes, the latter being more pronounced with cardiolipin-containing vesicles [52]. Of relevance to the issue are the data on the cyt *c*-induced formation of non-bilayer structures [53] and morphological transitions [54] in cardiolipin-containing model membranes, as well as permeation of apocytochrome *c* across lipid bilayers [55] along with its ability to induce dye leakage from liposomes [56]. Previously, induction of electrical current across planar bilayer lipid membrane formed from the mixture of soybean phosphatidylcholine and tetraoleoyl-cardiolipin was reported upon the addition of hydrogen peroxide in the presence of cyt *c* [57,58]. To study in detail the membrane-permeabilizing activity of cyt *c* promoted by H₂O₂, we applied here a fluorescence dequenching assay and a fluorescence

Abbreviations: cyt *c*, cytochrome *c*; CF, 5(6)-carboxyfluorescein; SRB, sulforhodamine B; PC, soybean phosphatidylcholine; CL, bovine heart cardiolipin; TMCL, tetramyristoyl-cardiolipin; DPhPC, diphyanoylphosphatidylcholine; BHT, butylated hydroxytoluene; EDTA, ethylenediaminetetraacetic acid; FCS, fluorescence correlation spectroscopy; *G*(τ), autocorrelation function

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correlation spectroscopy (FCS) approach using dye-loaded large unilamellar vesicles of different lipid compositions. A combination of cyt *c* with hydrogen peroxide was found to induce dye release from liposomes, provided that both cardiolipin and bulk phosphatidylcholine components of liposomal membranes contained unsaturated acyl tails. Together with sensitivity to antioxidants, these results allowed to relate the cyt *c*/H₂O₂-induced dye leakage to formation of membrane structural defects involving oxidized cardiolipin molecules.

2. Materials and methods

2.1. Materials

Most chemicals including equine heart cytochrome *c*, bovine heart cardiolipin (CL), sulforhodamine B (SRB), 5(6)-carboxyfluorescein (CF), butylated hydroxytoluene (BHT), trolox and quercetin were from Sigma; 1,2-Diphytanoyl-sn-Glycero-3-Phosphocholine (DPhPC) and tetramyristoyl-cardiolipin (TMCL) were from Avanti Polar Lipids (Alabaster, AL).

2.2. Preparation of liposomes

Dye-loaded liposomes were prepared by evaporation under a stream of nitrogen of a 2% solution of a mixture of lipids in chloroform followed by hydration with a buffer solution containing appropriate fluorescent marker. Four different lipid mixtures were used: 1) PC, 5 mg soybean phosphatidylcholine (Sigma, Type II-S), 2) PC/CL, 4 mg soybean phosphatidylcholine and 1 mg bovine heart cardiolipin, 3) PC/TMCL, 4 mg soybean phosphatidylcholine and 1 mg tetramyristoyl-cardiolipin, 4) DPhPC/CL, 4 mg diphytanoylphosphatidylcholine and 1 mg bovine heart cardiolipin. Four different marker solutions (0.5 ml each) were used: 1) 100 mM CF adjusted to pH about 8 by Tris, 2) 1 mM SRB in 100 mM KCl, 10 mM Tris, 10 mM MES, pH 7.4, 3) 3 mg/ml rhodamine-labeled 3-kDa dextran in the same buffer, and 4) 10 mg/ml rhodamine-labeled 10-kDa dextran in the same buffer. The mixture was vortexed, passed through several cycles of freezing and thawing, and extruded through 0.1-μm pore size Nucleopore polycarbonate membranes using an Avanti Mini-Extruder. The unbound marker was then removed by passage through a Sephadex G-50 coarse column (for SRB) or through a Sephadex G-150 coarse column with a buffer solution containing 100 mM KCl, 10 mM Tris, 10 mM MES, and pH 7.4.

2.3. Fluorescence dequenching assay

Fluorescence of liposomes loaded with 100 mM CF was monitored at 520 nm (excitation at 490 nm) with a Panorama Fluorat 02 spectrofluorimeter (Lumex, Russia). The extent of CF efflux was calculated as $(F_t - F_0) / (F_{100} - F_0)$, where F_0 and F_t represent the initial fluorescence intensity and the fluorescence intensity at the time t , and F_{100} is the fluorescence intensity after complete disruption of liposomes by addition of the detergent Triton-X100 (final concentration, 0.1% w/w).

2.4. Fluorescence correlation spectroscopy

The home-made setup was described previously [59]. Briefly, fluorescence excitation and detection utilized a Nd:YAG solid state laser with a 532-nm beam attached to an Olympus IMT-2 epifluorescent inverted microscope equipped with a 40×, NA 1.2 water immersion objective (Carl Zeiss, Jena, Germany). The fluorescence light passed through an appropriate dichroic beam splitter and a long-pass filter and was imaged onto a 50-μm core fiber coupled to an avalanche photodiode (SPCM-AQR-13-FC, PerkinElmer Optoelectronics, Vaudreuil, Quebec, Canada). The signal from an output was sent to a PC using a fast interface card (Flex02-01D/C, Correlator.com, Bridgewater, NJ). The data acquisition time was 30 s. The fluorescence was recorded from the confocal volume located at about 50 μm above the coverslip

surface with 50 μl of the buffer solution added. Most of the data were collected under the conditions of stirring a suspension by a paddle-shaped 3-mm plastic bar rotated at 600 rpm. To calibrate the setup, we recorded the fluorescence autocorrelation function of Rhodamine 6G solution. Assuming the diffusion coefficient of the dye to be $2.5 \times 10^{-6} \text{ cm}^2/\text{s}$, the value of the confocal radius $\omega = 0.42 \mu\text{m}$ was obtained. The correlated fluorescence emission signals were fitted to the three-dimensional autocorrelation function [60,61]:

$$G(\tau) = \frac{1}{N} \left(\frac{1}{1 + \frac{\tau}{\tau_D}} \right) \left(\frac{1}{\sqrt{1 + \frac{\omega^2 \tau}{z_0^2 \tau_D}}} \right) \quad (1)$$

with τ_D being the characteristic correlation time during which a molecule resides in the observation volume of radius ω and length z_0 , given by $\tau_D = \omega^2/4D$, where D is the diffusion coefficient, N is the mean number of fluorescent particles in the confocal volume. The amplitude of the autocorrelation function is inversely proportional to the number of fluorescent particles ($N = 1/G(\tau \rightarrow 0)$), but is independent of the fluorescence intensity of a single particle (in a system of identical particles) and therefore does not depend on the number of fluorophores per vesicle. Particles can be any fluorescent “point objects” in comparison to the dimension of the observation volume (i.e. about 1 μm). Therefore, particles can be single molecules of dye (i.e. SRB), as well as liposomes carrying different numbers of dye molecules. Initially (before the leakage induction) the system has a limited number of particles per observation volume comprising predominantly several liposomes loaded with the dye. After the leakage, the number of particles increases tremendously, because every liposomal particle produces thousands of particles of free dye leading to a significant decrease in the parameter $G(\tau \rightarrow 0)$.

3. Results and discussion

3.1. Cyt *c*/H₂O₂-induced leakage of carboxyfluorescein from liposomes

Fig. 1A illustrates liposome leakage induced by cyt *c* and H₂O₂, as monitored by release of the fluorophore carboxyfluorescein encapsulated in liposomes at a self-quenching concentration [62], which manifested itself in an increase of CF fluorescence. It is seen that 100 nM cyt *c* in combination with 1.5 mM H₂O₂ caused leakage of CF entrapped in cardiolipin-containing liposomes at low (curve 2), but not at high (100 mM KCl) ionic strength (curve 6). Of note, the H₂O₂-induced change in CF fluorescence depended non-monotonically on cyt *c* concentration (Fig. 1B): the increase in fluorescence became smaller at 1 μM cyt *c* (curve 5), whereas at higher cyt *c* concentrations the addition of H₂O₂ elicited even a decrease in CF fluorescence (curve 6). Earlier the cyt *c*-dependent quenching of fluorescence and the corresponding absorbance loss associated with oxidative damage were observed with the cyanine dye diS-C₃-(5) [63]. Therefore, the cyt *c*/H₂O₂-induced reduction of CF fluorescence was most likely due to destruction of CF caused by cyt *c*-mediated lipid peroxidation. This assumption is supported by the fact that CF itself has been shown to serve as a substrate of a peroxidase [64]. Oxidative instability of CF was also observed previously upon photodynamically induced leakage from liposomes [16].

Thus, we found significant stimulation of cyt *c*-induced carboxyfluorescein leakage from liposomes by hydrogen peroxide. However, a quantitative study of cyt *c*/cardiolipin peroxidase effect on the fluorescence of liposomes loaded with CF is hampered by oxidative instability of this dye.

3.2. FCS study of cyt *c*/H₂O₂-induced liposome leakage

In recent publications [16,65–67], permeability of vesicle membranes to fluorescent dyes has been studied by FCS, the approach which does not require loading of liposomes with dyes at very high,

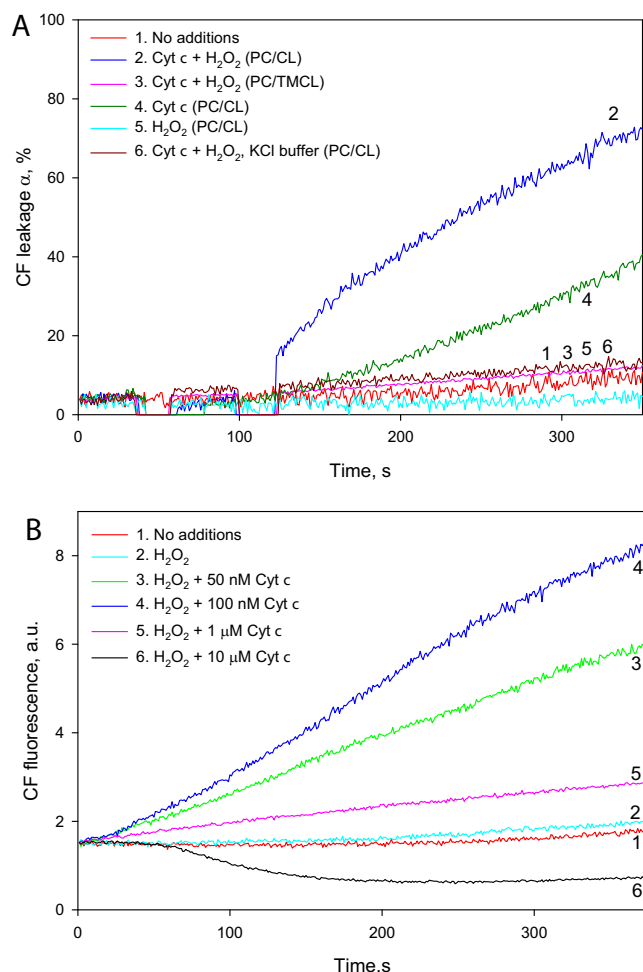


Fig. 1. A. Effect of cytochrome c and H₂O₂ on carboxyfluorescein leakage (α denotes the extent of the CF leakage) from liposomes composed of PC/CL (curves 2, 4, 5, 6) or PC/TMCL (curve 3). The concentration of cyt c was 100 nM, the concentration of H₂O₂ – 1.5 mM. B. Changes in fluorescence of CF-loaded PC/CL liposomes induced by the addition of H₂O₂ at different concentrations of cytochrome c. Curve 1, no additions; curve 2, 1.5 mM H₂O₂; curve 3, cyt c 50 nM and 1.5 mM H₂O₂; curve 4, cyt c 100 nM and 1.5 mM H₂O₂; curve 5, cyt c 1 μ M and 1.5 mM H₂O₂; curve 6, cyt c 10 μ M and 1.5 mM H₂O₂. The sucrose buffer contained 200 mM sucrose, 10 mM Tris, 10 mM MES, pH 7.4. The KCl buffer contained 100 mM KCl, 10 mM Tris, 10 mM MES, pH 7.4. Lipid concentration, 10 μ g/ml.

self-quenching concentrations. Here we studied the effect of a combination of H₂O₂ and cyt c on the fluorescence intensity autocorrelation function ($G(\tau)$) of the water-soluble dye sulforhodamine B encapsulated in cardiolipin-containing liposomes (Fig. 2A). Importantly, this fluorescent dye has been shown to be resistant to oxidative damage [16]. According to [60,61], the amplitude of $G(\tau)$ in the limit $\tau \rightarrow 0$ is determined by the reciprocal of a mean number (N) of fluorescent particles in the observation volume. In dye-leakage experiments, N comprises dye-loaded liposomes and free dye molecules released from liposomes. To measure $G(\tau \rightarrow 0)$ more precisely, we performed FCS experiments under stirring conditions [59]. As seen from Fig. 2A, the $G(\tau \rightarrow 0)$ amplitude of SRB-loaded cardiolipin-containing liposomes measured after incubation with H₂O₂ alone (curve 2) did not differ from the control level (curve 1). Incubation of SRB-loaded liposomes in the presence of cyt c led to a decrease in the $G(\tau \rightarrow 0)$ amplitude (curve 3), which was much more pronounced if cyt c was combined with H₂O₂ (curve 4). Disruption of liposomes by the addition of Triton X-100 resulted in a drop of $G(\tau \rightarrow 0)$ to nearly zero (curve 5). The reduction of the $G(\tau \rightarrow 0)$ amplitude apparently reflected an increase in the number of fluorescent particles due to SRB release from liposomes. Of note, the contribution of different fluorescent species (here dye-loaded liposomes and free dye molecules released

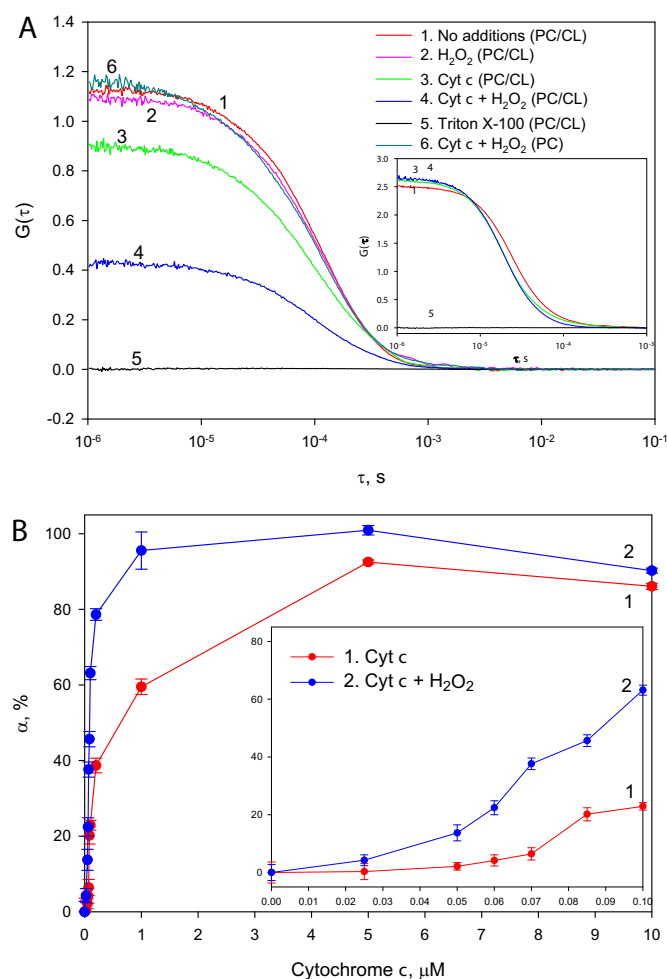


Fig. 2. Effect of cytochrome c and H₂O₂ on sulforhodamine B leakage from liposomes. A. Autocorrelation functions of SRB-loaded liposomes formed from PC/CL, 80/20% w/w (curves 1–5), or SRB-loaded liposomes formed from PC only (curve 6) measured after 30-min incubation with cyt c (100 nM) and/or H₂O₂ (1.5 mM) in sucrose buffer. Curve 1, no additions; curve 2, H₂O₂; curve 3, cyt c; curve 4, cyt c and H₂O₂; curve 5, 0.1% Triton X-100; curve 6, cyt c and H₂O₂. Inset: similar experiments performed in KCl buffer. B. The dependence of α (the extent of the SRB leakage) on the concentration of cytochrome c after 30-min incubation with (blue curve) and without (red curve) 1.5 mM H₂O₂. The sucrose buffer contained 200 mM sucrose, 10 mM Tris, 10 mM MES, pH 7.4. The KCl buffer contained 100 mM KCl, 10 mM Tris, 10 mM MES, pH 7.4. Lipid concentration, 2 μ g/ml.

from liposomes) to $G(\tau \rightarrow 0)$ is proportional to the square of their brightness [65,66]. With cardiolipin-lacking liposomes, no sizeable changes in the $G(\tau \rightarrow 0)$ amplitude was detected upon incubation with cyt c and H₂O₂ (Fig. 2A, curve 6).

Fig. 2B displays cyt c concentration dependences of the extent of liposome leakage α after 30-min incubation of liposomes with cyt c calculated from the Eq. (2) (see Appendix A):

$$\alpha(t) = 1 - \sqrt{\frac{G^t(\tau \rightarrow 0)}{G^0(\tau \rightarrow 0)}} \quad (2)$$

where $G^0(\tau \rightarrow 0)$ and $G^t(\tau \rightarrow 0)$ represent $G(\tau)$ in the limit $\tau \rightarrow 0$ at the moment of cyt c addition (zero time) and t min after the addition, respectively. It is seen that H₂O₂ markedly enhanced the dye-releasing effect of cyt c.

The non-linear profiles of the concentration dependence in Fig. 2B suggest the involvement of the interaction of cyt c molecules with each other in the permeabilization process. It is relevant that cyt c oligomerization has recently been shown to promote its binding to

negatively charged lipid membranes [68] and facilitate the induction of its peroxidase activity [37].

3.3. Comparison of cyt *c*/H₂O₂-induced and Fe/ascorbate-induced liposome leakage

To test specificity of the cyt *c*/H₂O₂ permeabilizing activity, we compared its sensitivity to cyanide (known to bind the cytochrome heme group), a ferrous chelator and ionic strength (Fig. 3A) with that of the Fe/ascorbate-induced membrane permeabilization (Fig. 3B). Similar to the effect of KCN on cyt *c*-dependent lipid peroxidation described earlier [28,46,47,69], the cyt *c*/H₂O₂-induced SRB release from liposomes was prevented by cyanide (Fig. 3A), whereas the Fe/ascorbate-provoked SRB release was completely insensitive to it (Fig. 3B). The free iron chelator EDTA had no effect on the cyt *c*/H₂O₂-induced liposome leakage (Fig. 3A), but inhibited the Fe/ascorbate-induced leakage (Fig. 3B). In line with the data on the ionic strength effect on cyt *c* binding to cardiolipin-containing lipid bilayers and monolayers [33,34,51,70–76], supplementing the medium with 100 mM KCl suppressed the effect of cyt *c* and H₂O₂ on $G(\tau \rightarrow 0)$ (Fig. 3A), which indicated that the membrane binding of cyt *c* is a key step in the cyt *c*/H₂O₂ permeabilizing activity. By contrast, a drop in the $G(\tau \rightarrow 0)$ amplitude caused by the Fe/ascorbate addition did not change upon increasing the ionic strength (Fig. 3B). These data excluded the possibility that the cyt *c*/H₂O₂-induced SRB release from liposomes was a result of heme decomposition by H₂O₂.

Fig. 4 shows the dependence of the cyt *c*/H₂O₂-induced SRB release from liposomes on the concentration of NaCN. The measurements were carried out after 30-min incubation in the presence of cyt *c*/H₂O₂. The inhibiting effect of cyanide could be related to CN binding to the heme group of cyt *c*. Following [69], we believe that cyt *c* with the CN coordinated to the central Fe atom of the heme is unable to perform peroxidative activity, presumably because of the involvement of the Fe atom of the heme in the binding of H₂O₂ to cyt *c*. The pattern of the dependence of the cyt *c*/H₂O₂-induced SRB release on the cyanide concentration could be associated with the protein heterogeneity regarding the depth of heme insertion into the lipid bilayer, which results in variation of heme accessibility to cyanide.

3.4. Dependence of cyt *c*/H₂O₂-induced liposome leakage on lipid composition

Time courses of SRB leakage induced by cyt *c* and H₂O₂ were measured for liposomes of different lipid compositions (Fig. 5). It appeared that either substitution of fully saturated tetramyristoyl cardiolipin

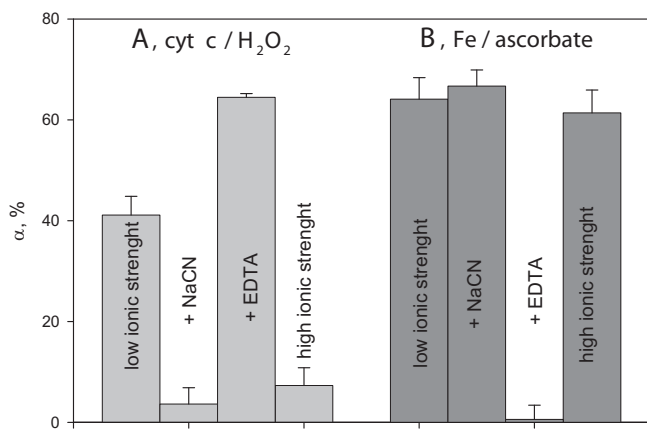


Fig. 3. Effect of sodium cyanide, EDTA and ionic strength on the SRB leakage from PC/CL liposomes induced by cytochrome *c*/H₂O₂ (A) or Fe/ascorbate (B). Lipid, 2 µg/ml; cyt *c*, 100 nM; H₂O₂, 1.5 mM; FeSO₄, 5 µM; ascorbate, 100 µM; NaCN, 1 mM; EDTA, 1 mM. The buffer solution of 10 mM Tris, 10 mM MES, pH 7.4 contained 200 mM sucrose for low ionic strength conditions or 100 mM KCl for high ionic strength conditions.

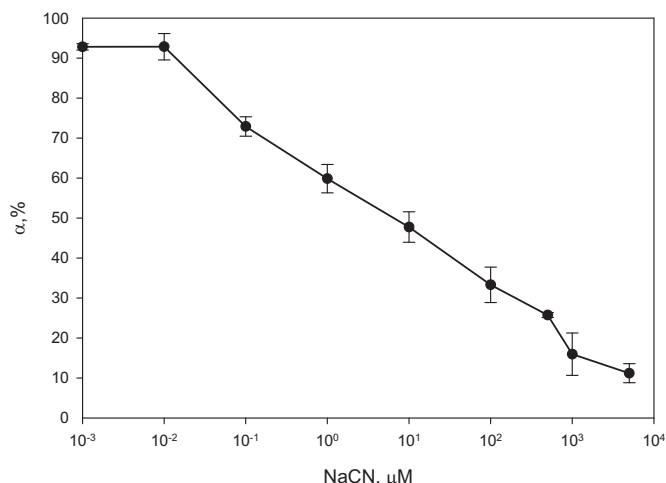


Fig. 4. Dependence of the cyt *c*/H₂O₂-induced SRB leakage on the concentration of sodium cyanide measured after 30-min incubation with cyt *c*/H₂O₂. Lipid, 2 µg/ml; cyt *c*, 100 nM; H₂O₂, 1.5 mM. The solution was 200 mM sucrose, 10 mM Tris, 10 mM MES, pH 7.4.

(curve 2) for bovine heart cardiolipin (predominantly containing tetralinoleyl-cardiolipin) (curve 1) or substitution of fully saturated diphytanoyl phosphatidylcholine (curve 3) for soybean phosphatidylcholine (curve 1) led to dramatic suppression of the cyt *c*-induced leakage, becoming close to that in the absence of cyt *c* and H₂O₂ (curve 4). Similar dependence on lipid composition was also found with CF-loaded liposomes (Fig. 1A). These effects of changing lipid composition on membrane permeabilization could not be associated with phase transitions, because diphytanoyl phosphatidylcholine, although fully saturated, is known to be in the fluid state at room temperature [77] and the addition of 20% of fully saturated TMCL to highly unsaturated egg PC could also hardly alter its fluid state. Thus, the requirement of the presence of unsaturated fatty-acid tails in both cardiolipin and diacyl phosphatidylcholine for cyt *c*/cardiolipin membrane-permeabilizing activity was most likely due to the involvement of lipid peroxidation in this activity.

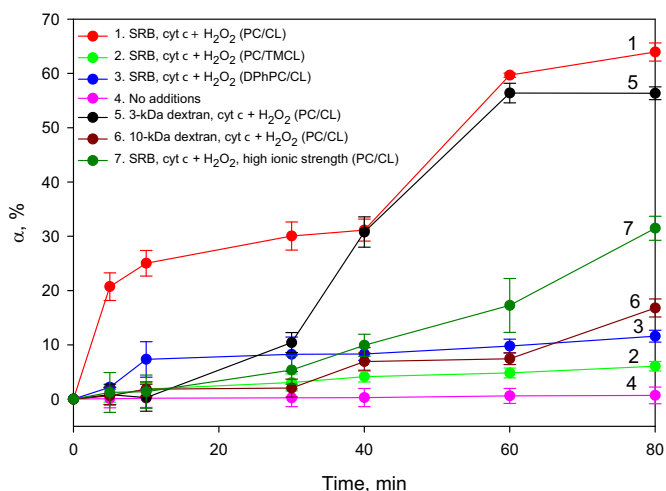


Fig. 5. Time courses of leakage of SRB (curves 1–4, 7), 3-kDa dextran (curve 5) and 10-kDa dextran (curve 6) from liposomes of different lipid compositions (PC/CL, DPhPC/CL, PC/TMCL) induced by cytochrome *c* and H₂O₂. Experiments were carried out in the low ionic strength buffer containing 200 mM sucrose, 10 mM Tris, 10 mM MES, pH 7.4, except for curve 2 where 100 mM KCl was used instead of 200 mM sucrose. Lipid, 2 µg/ml; cyt *c*, 100 nM; H₂O₂, 1.5 mM.

3.5. Cyt *c*/H₂O₂-induced leakage of fluorescent dextrans from liposomes

To estimate the size of conductive defects in membranes formed in the presence of cyt *c* and H₂O₂, we measured leakage of fluorescently labeled dextrans from cardiolipin-containing soybean phosphatidylcholine liposomes. Initially the leakage of 3-kDa dextran induced by the combination of cyt *c* and H₂O₂ was noticeably slower than that of SRB, although at longer time the extent of leakage for 3-kDa dextran became close to that for SRB (Fig. 5, curve 5). This observation could be related to lateral expansion of pores described for amphipathic antimicrobial peptides [78]. The leakage of 10-kDa dextran (2.4-nm Stokes radius [79]) was markedly suppressed (curve 6), suggesting the size of cyt *c*-induced membrane defects being approx. 5 nm.

3.6. Effect of antioxidants on cyt *c*/H₂O₂-induced liposome leakage

To test the relation of the cyt *c*/cardiolipin permeabilizing activity to lipid peroxidation, we studied the effect of various antioxidants on cyt *c*/H₂O₂-induced SRB leakage from liposomes. As follows from Fig. 6A, the addition of trolox, butylated hydroxytoluene (BHT) or quercetin significantly suppressed the SRB leakage. These free radical-scavengers were earlier found to inhibit cyt *c*/cardiolipin peroxidase

activity [35]. Panel B of Fig. 6 shows the effect of trolox and sodium cyanide on the SRB leakage induced by a high concentration of cyt *c* (1 μM) without H₂O₂. Both compounds did not affect the extent of the leakage under these conditions, thereby indicating that mechanisms of the leakage differed in the two cases.

3.7. Mechanism of cyt *c*/H₂O₂-induced liposome leakage: toroidal protein-lipid pore requiring oxidized cardiolipin versus pure lipidic pores

There has been an enormous body of evidence revealing changes in cyt *c* tertiary structure associated with extensive unfolding of the protein upon binding to lipid vesicles [80,81], especially those containing cardiolipin (see [82] and refs. therein). Furthermore, insertion or partial penetration of cyt *c* into membranes have been proposed [52,83–90]. It is the membrane-penetrating capacity of cyt *c* that presumably results in its ability to permeabilize lipid membranes in the absence of hydrogen peroxide [51,52]. Electrophysiological measurements revealed the cyt *c*-induced fast current flickering at low protein concentrations and stable giant pores at micromolar cyt *c* concentrations suggesting different mechanisms of the membrane permeabilization [51]. According to our data, this cyt *c*-induced leakage proved to be insensitive to antioxidants and cyanide (Fig. 6B). Therefore, it was not associated with peroxidase activity of cyt *c* and induction of lipid peroxidation.

Based on the obligatory presence of unsaturated alkyl chains in cardiolipin as a prerequisite to observe cyt *c*/H₂O₂-induced permeabilization (Fig. 5) and its sensitivity to antioxidants and cyanide (Figs. 3, 4, 6), it seems reasonable to suggest the involvement of oxidized cardiolipin molecules in the formation of membrane defects associated with cyt *c*/H₂O₂-induced perturbation of bilayer structure. According to electrophysiological data reported in [58], the combination of cyt *c* and H₂O₂ induced low-amplitude ion channels with a lifetime in a timescale of seconds, which differed substantially from those found with cyt *c* only [51]. Our experiments revealed the significant permeability of the liposomal membranes to 3-kDa dextran caused by cyt *c*/H₂O₂, which could be tentatively ascribed to evolution of structural defects into hydrophilic pores, presumably of the toroidal protein-lipid nature, with pore walls formed by protein helices intercalated by lipid head-groups [91–97,52] (see Fig. 7 depicting insertion of cyt *c* α-helices into a membrane driven by cyt *c*/cardiolipin peroxidase activity which results in toroidal pore formation). Favoring this model, lipid oxidation was shown to dramatically stimulate membrane pore formation induced by the water-soluble protein colicin E1 [98]. Alternatively, cyt *c*/H₂O₂-caused permeabilization may be ascribed to formation of pure lipidic pores involving oxidized lipids [16,99–101] or trivial membrane rupture resulting from lipid peroxidation. However, our FCS experiments with rhodamine-labeled liposomes showed that the cyt *c*-promoted dye leakage could proceed during a long time without membrane disruption (data not shown), and no stable pores were observed upon lipid peroxidation [102], which makes the pure oxidized lipid pore model of cyt *c*/H₂O₂-induced permeabilization less probable than the toroidal one involving protein helices. The striking differences in the sensitivity of cyt *c*/H₂O₂-induced and Fe/ascorbate-induced liposome leakage to cyanide (forming the cyanide-ferricytochrome *c* complex) and a ferrous chelator (Fig. 3) support direct participation of the protein in cyt *c*/H₂O₂-caused permeabilization. The preventing effect of cyanide on the cyt *c*/H₂O₂-induced permeabilization of liposomes could be attributed to the competitive binding of hydrogen peroxide and cyanide to the sixth ligand position of the heme iron in cyt *c*, as described in [69].

The importance of studying here the liposome leakage induced by Fe/ascorbate was associated with the possibility that H₂O₂ could provoke considerable damage to cyt *c* which might include iron release from the heme. Actually, this process was described in [103]. Of note, it was known from early studies that Fe/ascorbate could bring about an increase in membrane permeability resulting in dextran leakage from liposomes [7]. Our experiments with Fe/ascorbate and the applied

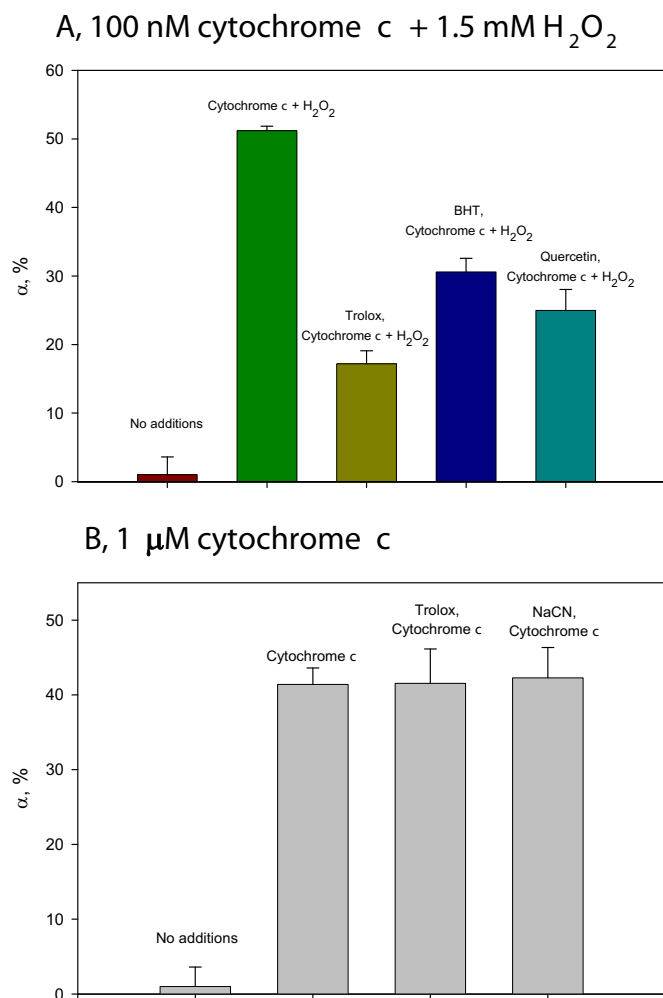


Fig. 6. A. Effect of antioxidants on the cytochrome *c*-induced SRB leakage from PC/CL liposomes in the presence of H₂O₂. Lipid, 2 μg/ml; cyt *c*, 100 nM; H₂O₂, 1.5 mM, trolox, 100 μM; BHT, 1 mM; quercetin, 20 μM. The solution was 200 mM sucrose, 10 mM Tris, 10 mM MES, pH 7.4. B. Effect of Trolox (100 μM) and KCN (1 mM) on the cytochrome *c*-induced SRB leakage from PC/CL liposomes without H₂O₂. Cyt *c* was 1 μM. Other conditions as in panel A.

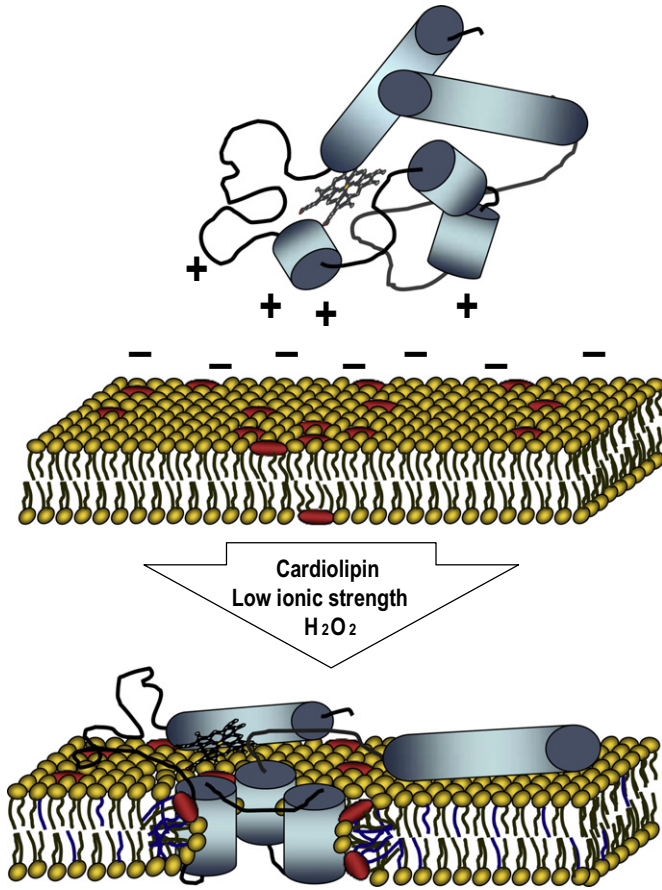


Fig. 7. Scheme of cardiolipin-dependent lipid membrane permeabilization by cytochrome *c* and H_2O_2 . Electrostatic binding of cyt *c* to cardiolipin-containing membranes followed by conformational changes of the protein leading to the induction of peroxidase activity, lipid oxidation and formation of a protein–lipid pore permeable for SRB.

inhibitory analysis proved that the possible release of iron was not involved in the liposome permeabilization process induced by cyt *c*/ H_2O_2 .

In summary, cyt *c*/cardiolipin peroxidase activity is shown to result in leakage of fluorescent markers from liposomes, inhibitable by cyanide and antioxidants. Requirement of unsaturated cardiolipin for the leakage points to the involvement of oxidized cardiolipin in the formation of cyt *c*/ H_2O_2 -induced membrane defects.

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Appendix A

According to [66], $G(\tau)$ of a system of a dye in and out of vesicles (V) can be described by the following equation (Eq. (4) of the reference):

$$G(\tau) = \frac{\langle N_{\text{dye}} \rangle}{(\langle N_{\text{dye}} \rangle + \tilde{B} \langle N_V \rangle)^2} \left[1 + \frac{\tau}{\tau_{\text{dye}}} \right]^{-1} \left[1 + \left(\frac{r_0}{z_0} \right)^2 \frac{\tau}{\tau_{\text{dye}}} \right]^{-\frac{1}{2}} + \frac{\tilde{B}^2 \langle N_V \rangle (1 + \tilde{B}^{-1})}{(\langle N_{\text{dye}} \rangle + \tilde{B} \langle N_V \rangle)^2} \left[1 + \frac{\tau}{\tau_V} \right]^{-1} \left[1 + \left(\frac{r_0}{z_0} \right)^2 \frac{\tau}{\tau_V} \right]^{-\frac{1}{2}}$$

Where N_{dye} and N_V are the number of dye and vesicle particles in the observation volume with dimensions r_0 and z_0 , τ_{dye} and τ_V are diffusion times of the dye and the vesicles, \tilde{B} is the mean number of dyes per vesicle at a given time. In the limit $\{\tau \rightarrow 0\}$ we have

$$G(\tau \rightarrow 0) = \frac{\langle N_{\text{dye}} \rangle}{(\langle N_{\text{dye}} \rangle + \tilde{B} \langle N_V \rangle)^2} + \frac{\tilde{B}^2 \langle N_V \rangle (1 + \tilde{B}^{-1})}{(\langle N_{\text{dye}} \rangle + \tilde{B} \langle N_V \rangle)^2} = \frac{\langle N_{\text{dye}} \rangle + \tilde{B}^2 \langle N_V \rangle (1 + \tilde{B}^{-1})}{(\langle N_{\text{dye}} \rangle + \tilde{B} \langle N_V \rangle)^2}.$$

Parameters N_{dye} and N_V are not independent. At the beginning of leakage experiments $N_{\text{dye}} = 0$ and $\tilde{B} = B_0$, afterwards

$$\langle N_{\text{dye}} \rangle = (B_0 - \tilde{B}) \langle N_V \rangle$$

because the appearance of the dye molecules in the solution is a consequence of their efflux from the vesicles. Therefore:

$$G(\tau \rightarrow 0) = \frac{(B_0 - \tilde{B}) \langle N_V \rangle + \tilde{B}^2 \langle N_V \rangle (1 + \tilde{B}^{-1})}{((B_0 - \tilde{B}) \langle N_V \rangle + \tilde{B} \langle N_V \rangle)^2} = \frac{1}{\langle N_V \rangle} \frac{B_0 + \tilde{B}^2}{\tilde{B}_0^2} = \frac{1}{\langle N_V \rangle} \left(\frac{1}{B_0} + \left(\frac{\tilde{B}}{B_0} \right)^2 \right).$$

$$\text{Since } G^{t=0}(\tau \rightarrow 0) = \frac{1}{\langle N_V \rangle},$$

$$G^t(\tau \rightarrow 0) = G^{t=0}(\tau \rightarrow 0) \left(\frac{1}{B_0} + \left(\frac{\tilde{B}}{B_0} \right)^2 \right) \quad \text{and} \quad \frac{\tilde{B}}{B_0} = \sqrt{\frac{G^t(\tau \rightarrow 0)}{G^{t=0}(\tau \rightarrow 0)} - \frac{1}{B_0}}.$$

The estimated value of B_0 (number of dye molecules per vesicle) in our experiments was about 10^3 . This estimation corresponds to the ratio of the initial value of $G(\tau \rightarrow 0)$ and the value after the addition of Triton X-100. This means that at low and intermediate leakage extents one can use the approximation

$$\frac{\tilde{B}}{B_0} \cong \sqrt{\frac{G^t(\tau \rightarrow 0)}{G^{t=0}(\tau \rightarrow 0)}}.$$

The conventional definition of the leakage extent α is a percentage of dye outside of vesicles, i.e.

$$\alpha = 1 - \frac{\tilde{B}}{B_0} = 1 - \sqrt{\frac{G^t(\tau \rightarrow 0)}{G^{t=0}(\tau \rightarrow 0)}}.$$

This equation gives a simple relationship between the decrease in $G(\tau \rightarrow 0)$ and the dye leakage. The assumption $1/B_0 \ll \tilde{B}/B_0$ makes the deviation from the equation less than 1% in the region of $\alpha < 50\%$ keeping in mind that $B_0 > 100$.

References

- [1] Y.A. Vladimirov, V.I. Olenev, T.B. Suslova, Z.P. Cheremisinina, Lipid peroxidation in mitochondrial membrane, *Adv. Lipid Res.* 17 (1980) 173–249.
- [2] A.W. Girotti, Photodynamic lipid peroxidation in biological systems, *Photochem. Photobiol.* 51 (1990) 497–509.
- [3] H. Yin, L. Xu, N.A. Porter, Free radical lipid peroxidation: mechanisms and analysis, *Chem. Rev.* 111 (2011) 5944–5972.
- [4] V.F. Antonov, Y.A. Vladimirov, A.N. Rossel's, L.G. Korkina, E.A. Korepanova, Effect of the products of peroxide oxidation of unsaturated fatty acids on ion transport through bimolecular phospholipid membranes, *Biofizika* 18 (1973) 668–673.

- [5] S.M. Anderson, N.I. Krinsky, M.J. Stone, D.C. Clagett, Effect of singlet oxygen quenchers on oxidative damage to liposomes initiated by photosensitization or by radiofrequency discharge, *Photochem. Photobiol.* 20 (1974) 65–69.
- [6] M.R. Deziel, A.W. Girotti, Photodynamic action of bilirubin on liposomes and erythrocyte membranes, *J. Biol. Chem.* 255 (1980) 8192–8198.
- [7] M. Kunimoto, K. Inoue, S. Nojima, Effect of ferrous ion and ascorbate-induced lipid peroxidation on liposomal membranes, *Biochim. Biophys. Acta* 646 (1981) 169–178.
- [8] R. Muller-Runkel, J. Blais, L.I. Grossweiner, Photodynamic damage to egg lecithin liposomes, *Photochem. Photobiol.* 33 (1981) 683–687.
- [9] A.V. Lebedev, D.O. Levitsky, V.A. Loginov, V.N. Smimov, The effect of primary products of lipid peroxidation on the transmembrane transport of calcium ions, *J. Mol. Cell. Cardiol.* 14 (Suppl. 3) (1982) 99–103.
- [10] S.N. Chatterjee, S. Agarwal, Liposomes as membrane model for study of lipid peroxidation, *Free Radic. Biol. Med.* 4 (1988) 51–72.
- [11] F. Tanfani, E. Bertoli, Permeability of oxidized phosphatidylcholine liposomes, *Biochem. Biophys. Res. Commun.* 163 (1989) 241–246.
- [12] V.M. Mirsky, I.N. Stozhkova, T.V. Szito, Photosensitized damage of bilayer lipid membrane in the presence of haematoporphyrin dimethylether, *J. Photochem. Photobiol. B* 8 (1991) 315–324.
- [13] T.I. Rokitskaya, Y.N. Antonenko, E.A. Kotova, The interaction of phthalocyanine with planar lipid bilayers — photodynamic inactivation of gramicidin channels, *FEBS Lett.* 329 (1993) 332–335.
- [14] P.I. Oteiza, E.J. Bechara, 5-aminolevulinic acid induces lipid peroxidation in cardiolipin-rich liposomes, *Arch. Biochem. Biophys.* 305 (1993) 282–287.
- [15] H. Mojzisova, S. Bonneau, P. Maillard, K. Berg, D. Brault, Photosensitizing properties of chlorins in solution and in membrane-mimicking systems, *Photochem. Photobiol. Sci.* 8 (2009) 778–787.
- [16] A. Pashkovskaya, E. Kotova, Y. Zorlu, F. Dumoulin, V. Ahsen, I. Agapov, Y. Antonenko, Light-triggered liposomal release: membrane permeabilization by photodynamic action, *Langmuir* 26 (2010) 5726–5733.
- [17] E.A. Kotova, A.V. Kuzevanov, A.A. Pashkovskaya, Y.N. Antonenko, Selective permeabilization of lipid membranes by photodynamic action via formation of hydrophobic defects or pre-pores, *Biochim. Biophys. Acta* 1808 (2011) 2252–2257.
- [18] Y. Shidoji, K. Hayashi, S. Komura, N. Ohishi, K. Yagi, Loss of molecular interaction between cytochrome c and cardiolipin due to lipid peroxidation, *Biochem. Biophys. Res. Commun.* 264 (1999) 343–347.
- [19] V.E. Kagan, V.A. Tyurin, J. Jiang, Y.Y. Tyurina, V.B. Ritov, A.A. Amoscato, A.N. Osipov, N.A. Belikova, A.A. Kapralov, V. Kini, I.I. Vlasova, Q. Zhao, M. Zou, P. Di, D.A. Svistunenko, I.V. Kurnikov, G.G. Borisenko, Cytochrome c acts as a cardiolipin oxygenase required for release of proapoptotic factors, *Nat. Chem. Biol.* 1 (2005) 223–232.
- [20] T. Kriska, W. Korytowski, A.W. Girotti, Role of mitochondrial cardiolipin peroxidation in apoptotic photokilling of 5-aminolevulinic-treated tumor cells, *Arch. Biochem. Biophys.* 433 (2005) 435–446.
- [21] V.E. Kagan, H.A. Bayir, N.A. Belikova, O. Kapralov, Y.Y. Tyurina, V.A. Tyurin, J. Jiang, D.A. Stoyanovsky, P. Wipf, P.M. Kochanek, J.S. Greenberger, B. Pitt, A.A. Shvedova, G. Borisenko, Cytochrome c/cardiolipin relations in mitochondria: a kiss of death, *Free Radic. Biol. Med.* 46 (2009) 1439–1453.
- [22] H. Iwase, T. Takatori, M. Nagao, K. Iwadate, M. Nakajima, Monoepoxide production from linoleic acid by cytochrome c in the presence of cardiolipin, *Biochem. Biophys. Res. Commun.* 222 (1996) 83–89.
- [23] Y.Y. Tyurina, V. Kini, V.A. Tyurin, I.I. Vlasova, J. Jiang, A.A. Kapralov, N.A. Belikova, J.C. Yalowich, I.V. Kurnikov, V.E. Kagan, Mechanisms of cardiolipin oxidation by cytochrome c: relevance to pro- and antiapoptotic functions of etoposide, *Mol. Pharmacol.* 70 (2006) 706–717.
- [24] I. Wiswedel, A. Gardemann, A. Storch, D. Peter, L. Schild, Degradation of phospholipids by oxidative stress—exceptional significance of cardiolipin, *Free Radic. Res.* 44 (2010) 135–145.
- [25] A.L. Tappel, The mechanism of the oxidation of unsaturated fatty acids catalyzed by hematin compounds, *Arch. Biochem. Biophys.* 44 (1953) 378–395.
- [26] S. Paleus, A. Ehrenberg, H. Tuppy, Study of a peptic degradation product of cytochrome c: investigation of the linkage between peptide moiety and prosthetic group, *Acta Chem. Scand.* 9 (1955) 365–374.
- [27] A.T. Tu, J.A. Reinos, Y.Y. Hsiao, Peroxidative activity of hemepeptides from horse heart cytochrome c, *Experientia* 24 (1968) 219–221.
- [28] E. Cadenas, A.I. Varsavsky, A. Boveris, B. Chance, Low level chemiluminescence of the cytochrome c-catalyzed decomposition of hydrogen peroxide, *FEBS Lett.* 113 (1980) 141–144.
- [29] R. Radi, L. Thomson, H. Rubbo, E. Prodanov, Cytochrome c-catalyzed oxidation of organic molecules by hydrogen peroxide, *Arch. Biochem. Biophys.* 288 (1991) 112–117.
- [30] I.L. Nantes, A. Faljoni-Alario, A.E. Vercesi, K.E. Santos, E.J. Bechara, Liposome effect on the cytochrome c-catalyzed peroxidation of carbonyl substrates to triplet species, *Free Radic. Biol. Med.* 25 (1998) 546–553.
- [31] R.E. Diederix, M. Ubbink, G.W. Canters, Peroxidase activity as a tool for studying the folding of c-type cytochromes, *Biochemistry* 41 (2002) 13067–13077.
- [32] A. Lawrence, C.M. Jones, P. Wardman, M.J. Burkitt, Evidence for the role of a peroxidase compound I-type intermediate in the oxidation of glutathione, NADH, ascorbate, and dichlorofluorescein by cytochrome c/H₂O₂. Implications for oxidative stress during apoptosis, *J. Biol. Chem.* 278 (2003) 29410–29419.
- [33] L.R. Brown, K. Wuthrich, A spin label study of lipid oxidation catalyzed by heme proteins, *Biochim. Biophys. Acta* 464 (1977) 356–369.
- [34] N.A. Belikova, Y.A. Vladimirov, A.N. Osipov, A.A. Kapralov, V.A. Tyurin, M.V. Potapovich, L.V. Basova, J. Peterson, I.V. Kurnikov, V.E. Kagan, Peroxidase activity and structural transitions of cytochrome c bound to cardiolipin-containing membranes, *Biochemistry* 45 (2006) 4998–5009.
- [35] Y.A. Vladimirov, E.V. Proskurnina, E.M. Demin, N.S. Matveeva, O.B. Lubitskiy, A.A. Novikov, D.Y. Izmailov, A.N. Osipov, V.P. Tikhonov, V.E. Kagan, Dihydroquercetin (taxifolin) and other flavonoids as inhibitors of free radical formation at key stages of apoptosis, *Biochemistry (Mosc)* 74 (2009) 301–307.
- [36] J. Everse, C.J. Liu, P.W. Coates, Physical and catalytic properties of a peroxidase derived from cytochrome c, *Biochim. Biophys. Acta* 1812 (2011) 1138–1145.
- [37] Z. Wang, T. Matsuo, S. Nagao, S. Hirota, Peroxidase activity enhancement of horse cytochrome c by dimerization, *Org. Biomol. Chem.* 9 (2011) 4766–4769.
- [38] L.A. Abriata, A. Cassina, V. Tortora, M. Marin, J.M. Souza, L. Castro, A.J. Vila, R. Radi, Nitration of solvent-exposed tyrosine 74 on cytochrome c triggers heme iron-methionine 80 bond disruption. Nuclear magnetic resonance and optical spectroscopy studies, *J. Biol. Chem.* 284 (2009) 17–26.
- [39] Y.R. Chen, L.J. Detering, B.E. Sturgeon, K.B. Tomer, R.P. Mason, Protein oxidation of cytochrome c by reactive halogen species enhances its peroxidase activity, *J. Biol. Chem.* 277 (2002) 29781–29791.
- [40] H.S. Aluri, D.C. Simpson, J.C. Allegood, Y. Hu, K. Szczepanek, S. Gronert, Q. Chen, E.J. Lesniewski, Electron flow into cytochrome c coupled with reactive oxygen species from the electron transport chain converts cytochrome c to a cardiolipin peroxidase: role during ischemia–reperfusion, *Biochim. Biophys. Acta* 1840 (2014) 3199–3207.
- [41] A. Patriarca, F. Polticelli, M.C. Piro, F. Sinibaldi, G. Mei, M. Bari, R. Santucci, L. Fiorucci, Conversion of cytochrome c into a peroxidase: inhibitory mechanisms and implication for neurodegenerative diseases, *Arch. Biochem. Biophys.* 522 (2012) 62–69.
- [42] B.S. Rajagopal, A.N. Edzuma, M.A. Hough, K.L. Blundell, V.E. Kagan, A.A. Kapralov, L.A. Fraser, J.N. Butt, G.G. Silkstone, M.T. Wilson, D.A. Svistunenko, J.A. Worrall, The hydrogen-peroxide-induced radical behaviour in human cytochrome c-phospholipid complexes: implications for the enhanced pro-apoptotic activity of the G41S mutant, *Biochem. J.* 456 (2013) 441–452.
- [43] T.M. Josephs, I.M. Morison, C.L. Day, S.M. Wilbanks, E.C. Ledgerwood, Enhancing the peroxidase activity of cytochrome c by mutation of residue 41: implications for the peroxidase mechanism and cytochrome c release, *Biochem. J.* 458 (2014) 259–265.
- [44] R.M. Kaschnitz, Y. Hatefi, Lipid oxidation in biological membranes. Electron transfer proteins as initiators of lipid autoxidation, *Arch. Biochem. Biophys.* 171 (1975) 292–304.
- [45] E. Cadenas, A. Boveris, B. Chance, Chemiluminescence of lipid vesicles supplemented with cytochrome c and hydroperoxide, *Biochem. J.* 188 (1980) 577–583.
- [46] F.M. Goni, M. Ondarroat, I. Azpiroz, J.M. Macarulla, Phospholipid oxidation catalyzed by cytochrome c in liposomes, *Biochim. Biophys. Acta* 835 (1985) 549–556.
- [47] R. Radi, J.F. Turrens, B.A. Freeman, Cytochrome c-catalyzed membrane lipid peroxidation by hydrogen peroxide, *Arch. Biochem. Biophys.* 288 (1991) 118–125.
- [48] R. Radi, K.M. Bush, B.A. Freeman, The role of cytochrome c and mitochondrial catalase in hydroperoxide-induced heart mitochondrial lipid peroxidation, *Arch. Biochem. Biophys.* 300 (1993) 409–415.
- [49] Y.Y. Tyurina, K. Kawai, V.A. Tyurin, S.X. Liu, V.E. Kagan, J.P. Fabisiak, The plasma membrane is the site of selective phosphatidylserine oxidation during apoptosis: role of cytochrome C, *Antioxid. Redox Signal.* 6 (2004) 209–225.
- [50] H.K. Kimelberg, D. Papahadjopoulos, Interactions of basic proteins with phospholipid membranes. Binding and changes in the sodium permeability of phosphatidylserine vesicles, *J. Biol. Chem.* 246 (1971) 1142–1148.
- [51] J. Xu, T.K. Vanderlick, P.A. Beales, Lytic and non-lytic permeabilization of cardiolipin-containing lipid bilayers induced by cytochrome c, *PLoS ONE* 8 (2013) e69492.
- [52] C.L. Bergstrom, P.A. Beales, Y. Lv, T.K. Vanderlick, J.T. Groves, Cytochrome c causes pore formation in cardiolipin-containing membranes, *Proc. Natl. Acad. Sci. U. S. A.* 110 (2013) 6269–6274.
- [53] B. de Kruijff, P.R. Cullis, Cytochrome c specifically induces non-bilayer structures in cardiolipin-containing model membranes, *Biochim. Biophys. Acta* 602 (1980) 477–490.
- [54] P.A. Beales, C.L. Bergstrom, N. Geerts, J.T. Groves, T.K. Vanderlick, Single vesicle observations of the cardiolipin–cytochrome c interaction: induction of membrane morphology changes, *Langmuir* 27 (2011) 6107–6115.
- [55] A. Rietveld, W. Jordi, B. de Kruijff, Studies on the lipid dependency and mechanism of the translocation of the mitochondrial precursor protein apocytochrome c across model membranes, *J. Biol. Chem.* 261 (1986) 3846–3856.
- [56] Q. Miao, X. Han, F. Yang, Phosphatidic acid-phosphatidylethanolamine interaction and apocytochrome c translocation across model membranes, *Biochem. J.* 354 (2001) 681–688.
- [57] M.N. Puchkov, R.A. Vassara, E.A. Korepanova, A.N. Osipov, Cytochrome c produces pores in cardiolipin-containing planar bilayer lipid membranes in the presence of hydrogen peroxide, *Biochim. Biophys. Acta* 1828 (2013) 208–212.
- [58] V.F. Antonov, M.N. Puchkov, E.A. Korepanova, O.Y. Nemchenko, V. Borodulin, Soft performation of cardiolipin-containing planar lipid bilayer membrane by cytochrome c and H₂O₂, *Eur. Biophys. J.* 43 (2014) 469–476.
- [59] I.V. Perevoshchikova, D.B. Zorov, Y.N. Antonenko, Peak intensity analysis as a method for estimation of fluorescent probe binding to artificial and natural nanoparticles: tetramethylrhodamine uptake by isolated mitochondria, *Biochim. Biophys. Acta* 1778 (2008) 2182–2190.
- [60] S.T. Hess, S. Huang, A.A. Heikal, W.W. Webb, Biological and chemical applications of fluorescence correlation spectroscopy: a review, *Biochemistry* 41 (2002) 697–705.
- [61] O. Krichinsky, G. Bonnet, Fluorescence correlation spectroscopy: the technique and its applications, *Rep. Prog. Phys.* 65 (2002) 251–297.

- [62] R.F. Chen, J.R. Knutson, Mechanism of fluorescence concentration quenching of carboxyfluorescein in liposomes: energy transfer to nonfluorescent dimers, *Anal. Biochem.* 172 (1988) 61–77.
- [63] A.P. Singh, G.A. Chanady, P. Nicholls, Interactions involving the cyanine dye, diS-C3-(5), cytochrome c and liposomes and their implications for estimations of delta psi in cytochrome c oxidase-reconstituted proteoliposomes, *J. Membr. Biol.* 84 (1985) 183–190.
- [64] Y. Tatsu, S. Yamamura, H. Yamamoto, S. Yoshikawa, Peroxidase-dependent fluorescence decrease of carboxyfluorescein and its enhancement by liposome encapsulation, *Anal. Bioanal. Chem.* 351 (1995) 782–785.
- [65] L. Yu, J.L. Ding, B. Ho, S.S. Feng, T. Wohland, Investigation of the mechanisms of antimicrobial peptides interacting with membranes by fluorescence correlation spectroscopy, *Open Chem. Phys.* 1 (2008) 62–80.
- [66] A. Blicher, K. Wodzinska, M. Fidorra, M. Winterhalter, T. Heimburg, The temperature dependence of lipid membrane permeability, its quantized nature, and the influence of anesthetics, *Biophys. J.* 96 (2009) 4581–4591.
- [67] A.I. Sorochkina, S.I. Kovalchuk, E.O. Omarova, A.A. Sobko, E.A. Kotova, Y.N. Antonenko, Peptide-induced membrane leakage by lysine derivatives of gramicidin A in liposomes, planar bilayers, and erythrocytes, *Biochim. Biophys. Acta* 1828 (2013) 2428–2435.
- [68] S. Junedi, K. Yasuhara, S. Nagao, J. Kikuchi, S. Hirota, Morphological change of cell membrane by interaction with domain-swapped cytochrome c oligomers, *Chembiochem* 15 (2014) 517–521.
- [69] E. Cadenas, A. Boveris, B. Chance, Low-level chemiluminescence of hydroperoxide-supplemented cytochrome c, *Biochem. J.* 187 (1980) 131–140.
- [70] P.J. Quinn, R.M. Dawson, Interaction of cytochrome c and [14C]-carboxymethylated cytochrome c with monolayers of phosphatidylcholine, phosphatidic acid and cardiolipin, *Biochem. J.* 115 (1969) 65–75.
- [71] P. Nicholls, Cytochrome c binding to enzymes and membranes, *Biochim. Biophys. Acta* 346 (1974) 261–310.
- [72] M. Rytomaa, P. Mustonen, P.K. Kinnunen, Reversible, nonionic, and pH-dependent association of cytochrome c with cardiolipin-phosphatidylcholine liposomes, *J. Biol. Chem.* 267 (1992) 22243–22248.
- [73] Z. Salamon, G. Tollin, Surface plasmon resonance studies of complex formation between cytochrome c and bovine cytochrome c oxidase incorporated into a supported planar lipid bilayer. I. Binding of cytochrome c to cardiolipin/phosphatidylcholine membranes in the absence of oxidase, *Biophys. J.* 71 (1996) 848–857.
- [74] I.L. Nantes, M.R. Zucchi, O.R. Nascimento, A. Faljoni-Alario, Effect of heme iron valence state on the conformation of cytochrome c and its association with membrane interfaces. A CD and EPR investigation, *J. Biol. Chem.* 276 (2001) 153–158.
- [75] F. Sinibaldi, L. Fiorucci, A. Patriarca, R. Lauceri, T. Ferri, M. Coletta, R. Santucci, Insights into cytochrome c–cardiolipin interaction. Role played by ionic strength, *Biochemistry* 47 (2008) 6928–6935.
- [76] J. Hanske, J.R. Toffey, A.M. Morenz, A.J. Bonilla, K.H. Schiavoni, E.V. Pletneva, Conformational properties of cardiolipin-bound cytochrome c, *Proc. Natl. Acad. Sci. U. S. A.* 109 (2012) 125–130.
- [77] N. Kucerka, M.P. Nieh, J. Katsaras, Fluid phase lipid areas and bilayer thicknesses of commonly used phosphatidylcholines as a function of temperature, *Biochim. Biophys. Acta* 1808 (2011) 2761–2771.
- [78] P.D. Rakowska, H. Jiang, S. Ray, A. Pyne, B. Lamarre, M. Carr, P.J. Judge, J. Ravi, U.I. Gerling, B. Kokschi, G.J. Martyna, B.W. Hoogenboom, A. Watts, J. Crain, C.R. Grovenor, M.G. Ryadnov, Nanoscale imaging reveals laterally expanding antimicrobial pores in lipid bilayers, *Proc. Natl. Acad. Sci. U. S. A.* 110 (2013) 8918–8923.
- [79] R.H. Pearce, B.J. Grimmer, Calibration of agarose columns for gel chromatography with commercially available dextran fractions. Application to the measurement of distributions of molecular radii of glycosaminoglycans, *J. Chromatogr.* 150 (1978) 548–553.
- [80] T. Heimburg, D. Marsh, Investigation of secondary and tertiary structural changes of cytochrome c in complexes with anionic lipids using amide hydrogen exchange measurements: an FTIR study, *Biophys. J.* 65 (1993) 2408–2417.
- [81] T.J. Pinheiro, G.A. Elove, A. Watts, H. Roder, Structural and kinetic description of cytochrome c unfolding induced by the interaction with lipid vesicles, *Biochemistry* 36 (1997) 13122–13132.
- [82] J. Muenzner, E.V. Pletneva, Structural transformations of cytochrome c upon interaction with cardiolipin, *Chem. Phys. Lipids* 179 (2014) 57–63.
- [83] J. Szebeni, G. Tollin, Interaction of cytochrome c with liposomes: covalent labeling of externally bound protein by the fluorescent probe, azidonaphthalenedisulfonic acid, enclosed in the inner aqueous compartment of unilamellar vesicles, *Biochim. Biophys. Acta* 932 (1988) 153–159.
- [84] F. Zhang, E.S. Rowe, Calorimetric studies of the interactions of cytochrome c with dioleoylphosphatidylglycerol extruded vesicles: ionic strength effects, *Biochim. Biophys. Acta* 1193 (1994) 219–225.
- [85] G.P. Gorbenko, Structure of cytochrome c complexes with phospholipids as revealed by resonance energy transfer, *Biochim. Biophys. Acta* 1420 (1999) 1–13.
- [86] T.J. Pinheiro, H. Cheng, S.H. Seeholzer, H. Roder, Direct evidence for the cooperative unfolding of cytochrome c in lipid membranes from H-(2)H exchange kinetics, *J. Mol. Biol.* 303 (2000) 617–626.
- [87] M.J. Zuckermann, T. Heimburg, Insertion and pore formation driven by adsorption of proteins onto lipid bilayer membrane-water interfaces, *Biophys. J.* 81 (2001) 2458–2472.
- [88] S. Oellerich, S. Lecomte, M. Paternostre, T. Heimburg, P. Hildebrandt, Peripheral and integral binding of cytochrome c to phospholipids vesicles, *J. Phys. Chem. B* 108 (2004) 3871–3878.
- [89] E.J. Choi, E.K. Dimitriadis, Cytochrome c adsorption to supported, anionic lipid bilayers studied via atomic force microscopy, *Biophys. J.* 87 (2004) 3234–3241.
- [90] S. Patariaia, Y. Liu, R. Lipowsky, R. Dimova, Effect of cytochrome c on the phase behavior of charged multicomponent lipid membranes, *Biochim. Biophys. Acta* 1838 (2014) 2036–2045.
- [91] S.J. Ludtke, K. He, W.T. Heller, T.A. Harroun, L. Yang, H.W. Huang, Membrane pores induced by magainin, *Biochemistry* 35 (1996) 13723–13728.
- [92] K. Matsuzaki, O. Murase, N. Fujii, K. Miyajima, An antimicrobial peptide, magainin 2, induced rapid flip-flop of phospholipids coupled with pore formation and peptide translocation, *Biochemistry* 35 (1996) 11361–11368.
- [93] V.V. Malev, L.V. Schagina, P.A. Gurnev, J.Y. Takemoto, E.M. Nestorovich, S.M. Bezrukov, Syringomycin E channel: a lipidic pore stabilized by lipopeptide? *Biophys. J.* 82 (2002) 1985–1994.
- [94] A.A. Sobko, E.A. Kotova, Y.N. Antonenko, S.D. Zakharov, W.A. Cramer, Effect of lipids with different spontaneous curvature on the channel activity of colicin E1: evidence in favor of a toroidal pore, *FEBS Lett.* 576 (2004) 205–210.
- [95] S.D. Zakharov, E.A. Kotova, Y.N. Antonenko, W.A. Cramer, On the role of lipid in colicin pore formation, *Biochim. Biophys. Acta* 1666 (2004) 239–249.
- [96] A.A. Sobko, E.A. Kotova, Y.N. Antonenko, S.D. Zakharov, W.A. Cramer, Lipid dependence of the channel properties of a colicin e1-lipid toroidal pore, *J. Biol. Chem.* 281 (2006) 14408–14416.
- [97] O. Terrones, B. Antonsson, H. Yamaguchi, H.G. Wang, J. Liu, R.M. Lee, A. Herrmann, G. Basanez, Lipidic pore formation by the concerted action of proapoptotic BAX and tBID, *J. Biol. Chem.* 279 (2004) 30081–30091.
- [98] A.A. Sobko, M.A. Vlasina, T.I. Rokitskaya, E.A. Kotova, S.D. Zakharov, W.A. Cramer, Y.N. Antonenko, Chemical and photochemical modification of colicin E1 and gramicidin A in bilayer lipid membranes, *J. Membr. Biol.* 199 (2004) 51–62.
- [99] A.V. Lebedev, Ion channels in bilayer lipid membranes dependent on oxygen, *Dokl. Akad. Nauk SSSR* 260 (1981) 757–761.
- [100] J. Wong-Ekkabut, Z. Xu, W. Triampo, I.M. Tang, D.P. Tieleman, L. Monticelli, Effect of lipid peroxidation on the properties of lipid bilayers: a molecular dynamics study, *Biophys. J.* 93 (2007) 4225–4236.
- [101] M. Lis, A. Wiert, M. Przybylo, M. Langner, J. Swiatek, P. Jungwirth, L. Cwiklik, The effect of lipid oxidation on the water permeability of phospholipids bilayers, *Phys. Chem. Chem. Phys.* 13 (2011) 17555–17563.
- [102] K. Anzai, K. Ogawa, Y. Goto, Y. Senzaki, T. Ozawa, H. Yamamoto, Oxidation-dependent changes in the stability and permeability of lipid bilayers, *Antioxid. Redox Signal.* 1 (1999) 339–347.
- [103] S. Harel, M.A. Salan, J. Kanner, Iron release from metmyoglobin, methaemoglobin and cytochrome c by a system generating hydrogen peroxide, *Free Radic. Res. Commun.* 5 (1988) 11–19.