Kinetics of radiation- and cytochrome *c*-induced modifications in liposomes analysed by FT–Raman spectroscopy

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Abstract

Fourier transform Raman spectroscopy on artificial lipid membranes was used to study radiation-induced peroxidation processes as a function of time after radiation exposure. The time dependent intensity changes of the Raman lines of various C=C bondings were compared to results obtained by measuring conjugated dienes and by the thiobarbituric acid test for malondialdehydes. The results show that mainly the *cis* C=C bonds of the lipid chains are involved and, therefore, indicate that γ-radiation induces conformational changes in the lipid chain while the mobility of the lipid chains is reduced. New Raman bands can be assigned to aldehyde products induced at the end of the peroxidation process. The immediate decrease of the =CH vibration lines was directly correlated with the formation of conjugated C=C double bonds suggesting that these vibration lines are in contrast to the C=C lines solely Raman active, when isolated C=C bonds are present. Cytochrome *c* (ox.) incorporated into the bilayer of the artificial membranes induced autooxidation processes not influenced by γ-radiation. It was observed that cytochrome *c* (ox.)-induced changes of the relative intensity of the C=C bonds differ from those induced by γ-radiation. These results of cytochrome *c* together with the inhibitory effects of the antioxidant α-tocopherol suggest that the radical species involved in the cytochrome *c* induced process might be different from the free radicals involved in the γ-radiation-induced process. © 1997 Elsevier Science B.V.

1. Introduction

Lipid peroxidation processes play an important role in the 'oxidative stress' response and, in general, are responsible for various kind of damages in the cell membrane. These processes can cause impairment of enzyme activities [1], chemical induced toxicity [2] as well as damage in the genetic material because of the close adherence of DNA to the nuclear membrane [3].

Peroxidation processes cause chain reactions which lead to the formation of intermediate lipid hydroperoxides. Studies on model membranes have shown that polyunsaturated hydrocarbon moieties are the main target in this reaction [4]. In the further propagation process, a breakdown of lipid hydroperoxides occurs and reactive products are generated [5]. Even when it is likely that free radicals like O_2^-, OH^- or HO_2^- are involved in the peroxidation process induced by radiation [6,7], the specific mechanisms of the oxidation in lipids are not yet clearly understood. Additionally, also hemeproteins are able to induce lipid autooxidation, a process which is also not well

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understood. Several authors have studied the influence of cytochrome *c* on model membranes, because this protein is of special interest in view of its close association to the membrane. A catalysed uptake of O_2 was found in the presence of oxidised cytochrome *c* [8] and a catalysed process of the homolytic scission of preformed fatty acid hydroperoxides was suggested [8,9].

Verma and co-workers [4,10] have for the first time studied the effect of peroxidation processes to the status of the C=C double bonds in liposomes by Raman spectroscopy. Additionally, these authors have measured the influence of various proteins and have found that some of them inhibit the formation of radiation-induced lipid peroxides while other proteins have been found to catalyse this effect. Recently, variations of characteristic vibration lines of the C=C double bonds of lipids induced by γ-radiation were also studied by IR spectroscopy [11].

In the present paper, the kinetics of modifications in artificial membranes caused either by radiation or by cytochrome *c* were measured by FT–Raman spectroscopy. Since alterations in molecular structure lead to changes in the Raman spectrum, it is possible to characterise chemical changes as well as configurational modifications. Changes in intensity of the lines of the C=C and CH_2-lines are discussed in order to describe the state of these bonds by correlating the Raman data with the diene test and with the production of thiobarbituric acid-reactive material. Variations in the radiation- and cytochrome *c*-induced Raman spectra of liposomes and the role of the anti-oxidant α-tocopherol are taken into consideration to discuss the possible mechanism of cytochrome *c* in relation to lipid peroxidation.

2. Materials and methods

2.1. Preparation of liposomes for Raman spectroscopy

The phospholipids dilinoleoyl phosphatidyl choline (DLPC) and dilinoleoyl phosphatidyl serine (DLPS) were obtained from Avanti Polar Lipids (Alabaster, AL). The protein cytochrome *c* (oxidised) as well as the antioxidant α-tocopherol were obtained from Sigma (Deisenhofen, Germany) at the highest purity grade. All samples were used without further purification.

Negatively-charged small unilaminar vesicles (SUV) composed of DLPC and DLPS at a molar ratio of 5:1 were prepared according to Huang [12] with minor modifications. The lipid solution was evaporated to obtain a dry lipid film. 1 ml of a 5 mM phosphate buffer (pH 7.3) was added to the dry film that the final lipid concentration amounted to 18 mg ml^{-1}. In some experiments, 2 μM of α-tocopherol was added additionally. The suspension was sonicated for 10 min with a Sonifier 250 (Branson Sonic Power, Carouge-Geneve, Switzerland) at 20% of maximum power under argon.

Negatively charged DLPS lipids were used in addition, because cytochrome *c* can only be incorporated into the bilayer of negatively charged liposomes [13,14]. 20% of DLPS was sufficient to obtain a protein concentration of 100 μM. Therefore, a 5 mM solution of cytochrome *c* was added to the liposomes. To remove free cytochrome *c*, liposomes were centrifuged through Sephadex G-50 columns (Sigma, Deisenhofen, Germany) [15]. The final concentration of cytochrome *c*, determined by the method of Lowry [16], was 70 μM. For measuring the yield of oxidised and degenerated cytochrome *c*, the absorption spectrum of the liposomes with incorporated cytochrome *c* was measured on a diode-array spectrometer (Hewlett Packard, Type 8452A, USA) at 532 nm. Oxidised cytochrome *c* shows an absorption line at 532 nm, while degenerated cytochrome *c* has no absorption line in this region. The peak value at time 0 was taken as 100% oxidised cytochrome *c* assuming that only oxidised cytochrome *c* was present.

The samples were irradiated with 100 Gy using a ^{137}Cs γ-source (0.9 Gy min^{-1}) at ca. 25°C under atmospheric pressure of oxygen.

2.2. Measurement of peroxidation products

The various peroxidation products were measured using the absorption spectra for the yield of dieneconjugates and the thiobarbituric acid test for malondialdehydes. For measuring the absorption spectra of the lipids, 10 μl of the samples were dissolved in 1 ml ethanol [17] and the absorption spectra were registered on the diode-array spectrometer from 190 to

400 nm. The maximum value of the ratio at 234/195 nm was normalized to 1. For the thiobarbituric acid test, 50 μl of the liposome solution and 150 μl of the phosphate buffer (5 mM) were mixed with 0.1 ml of 0.025% butylated hydroxytoluene solution (BHT solution) to stop the reaction and to achieve a constant reaction time. The samples were mixed with 1.5 ml of 20% trichloroacetic acid and 1.5 ml of 0.8% thiobarbituric acid. The pH was adjusted to 3.8 with 1 mM NaOH. The reaction mixtures were incubated at 95°C for 40 min. The samples were then cooled to room temperature and 1 ml of distilled water and 5 ml of n-butanol was added. The well mixed samples were centrifuged for 10 min and the optical density of the upper layer was measured at 532 nm. The maximum value of the optical density was normalized to 1.

2.3. Fourier-transform (FT)–Raman spectroscopy and data analysis

FT–Raman spectra were obtained using an IFS-55 FT–IR spectrometer coupled to a FRA-106 Raman accessory (Bruker Instruments, Karlsruhe, Germany). Raman spectra were excited with the 1064 nm line of a continuous-wave diode-pumped Nd:YAG laser in backscattering geometry with around 600 mW at the sample. For signal detecting, a liquid nitrogen-cooled germanium detector was used. The resolution of the instrument was 4 cm^{-1}. The spectra were recorded at room temperature. Heating of the sample possibly produced by the laser illumination was carried off by a running water system. For each spectrum, an average of 2 spectra with 500 scans were taken. The signal-to-noise ratio for prominent Raman bands was found to be better than 40. The signals of the solvent were subtracted from the Raman spectrum of the liposome solution. The Raman frequencies of all described lines were accurate within ± 2 cm^{-1}.

As an internal intensity standard, the Raman line of the C–C vibration at 1444 cm^{-1} was used, because this line was found not to be sensitive to the peroxidation processes [4]. To describe the changes of the =CH wagging vibration and the –CH$_2$ twisting vibration, the intensity ratio at 1266 and 1303 cm^{-1} was used.

The uncertainties of the computed Raman intensity changes were evaluated by using the total standard deviation σ_{tot}, resulting from the different standard deviations of the reference normalisation, the RMS noise of the specific Raman band frequency ν and the baseline. The uncertainties of Raman band intensities were found to be less then $\pm 7\%$ for prominent bands and less then $\pm 13\%$ for weak bands.

The Raman band between 1575 and 1775 cm^{-1} was fitted with a simulated curve by minimising the weighted sum square by the Levenberg–Marquard method. The line shape was assumed to be Gaussian, the background was eliminated by fitting a separated base line. The Raman lines of the *cis* and *trans* C=C vibration were fixed at 1658 and 1673 cm^{-1}, respectively.

3. Results

3.1. Fourier-transform–Raman spectrum of artificial membranes

In Fig. 1, the FT–Raman spectrum of liposomes containing DLPC (80%) and DLPS (20%) is shown in the region from 600 to 1800 cm^{-1} and from 2800 to 3100 cm^{-1}. Raman bands can be assigned to three different regions of the lipids: The polar headgroup, the hydrophobic chains and the area between these two regions. The various Raman lines are shown in Table 1 and will be described in the following.

In the region between 2800 and 3100 cm^{-1}, the Raman bands of the aliphatic CH$_2$ stretching vibrations are located. The symmetric and antisymmetric CH$_2$ stretching modes of double unsaturated lipids are located at 2932 and 2963 cm^{-1}. The CH$_3$ stretching modes are found at 2932 and 2963 cm^{-1}. Additionally, the symmetric stretching mode of the =CH bond is located at 3012 cm^{-1} [4,22].

The C–C region between 1050 and 1150 cm^{-1} shows three prominent bands at around 1130, 1100 and 1062 cm^{-1}, assigned to *trans*, randomly oriented *gauche*, and *trans* C–C bonds, respectively. At room temperature, these lines cannot be resolved anymore and a broad band of the *gauche* conformation of the C–C stretching vibration between 1085 and 1090 cm^{-1} overlaps [18].

The stretching vibrations of the C=C double bonds are located at 1620 and 1680 cm^{-1}. The band at 1658 cm^{-1} is assigned to the *cis* C=C bond, the *trans*
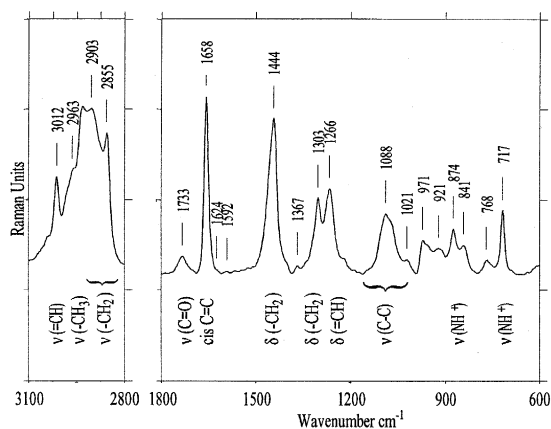
Fig. 1. FT–Raman spectra of artificial membranes containing DLPC (80%) and DLPS (20%) in the region between 600 and 1800 cm^{-1} and between 2800 and 3100 cm^{-1}. Assignments to various molecule vibrations in the liposomes are given.

C=C bond lies at around 1673 cm^{-1} [23]. The CH$_2$ scissoring mode of the methyl group shows a line at 1444 cm^{-1} and the Raman band at 1303 cm^{-1} can be assigned to the CH$_2$ twisting vibration [19]. Liposomes from double unsaturated lipids show additionally a =CH wagging mode at 1266 cm^{-1}. Therefore, the ratio of the lines at 1303 and 1266 cm^{-1} was used by some authors as a measure of the saturation of the lipid chains [19,24].

The lines at 717 and 874 cm^{-1} can be assigned to the C–N stretching vibrations of the headgroup. The energy position in the presented spectrum indicates a *gauche* conformation of the O–C–C–N$^+$ segment [18]. The line at 1733 cm^{-1} results from the C=O stretching mode [18].

3.2. Radiation-induced changes in the FT–Raman spectrum of liposomes

Irradiation of lipids induces a long process that can continue for many hours. Therefore, no changes could be detected during the first few hours immediately after irradiation.

Table 1
Assignments of Raman lines to vibrations of artificial lipid membranes

Raman line (cm^{-1})		Assignments	Ratiation-induced changes
Measurements	Ref.		
717	717 [a]	ν_{sym}(C–N)	
862			Int. ↑ − ↓
874	~ 870 [a]	ν_{sym}(C–N)$_{gauche}$ and δ(CH$_2$)	
971	970 [b]	δ(=CH)$_{wag}$	Int. ↓
	~ 1050 [a]	ν(P–O–C)	
	1062 [a]	ν(C–C)$_{trans}$	
1088	~ 1087 [a]	ν(C–C)$_{gauche}$	
	1100 [a]	ν(C–C)$_{trans}$	
	1130 [a]	ν(C–C)$_{trans}$	
1266	1266 [c]	δ(=CH)	Int. ↓
1303	1298 [d]	δ(CH$_2$)$_{twist}$	Int. ↑ →
1444	1445 [d]	δ(–CH$_2$)	Marker
1620			Int. ↑
1658	1658 [d]	ν(C=C)$_{cis}$	Int. ↑ − ↓
1673	1672 [b]	ν(C=C)$_{trans}$	Int. ↑
1733	1731 [d]	ν(C=O)	
2855	2850 [d]	ν_{sym}(–CH$_2$)	Int. ↓ and WZ →
2903	2890 [d]	ν_{asym}(–CH$_2$)	
2932	2930 [d]	ν_{sym}(–CH$_3$)	
2963	2967 [c]	ν_{asym}(–CH$_3$)	
3012	3012 [c]	ν_{sym}(=C–H)	Int. ↓

[a] See Ref. [18]. [b] See Ref. [20]. [c] See Ref. [19]. [d] See Ref. [21].
ν: stretching vibration; δ: deformation vibration; sym: symmetric; asym: asymmetric; wag: wagging; twist: twisting; Int. ↑: intensity increase; Int. ↓: intensity decrease; WZ → or WZ ← : frequency shift to higher and lower wavenumbers, respectively.
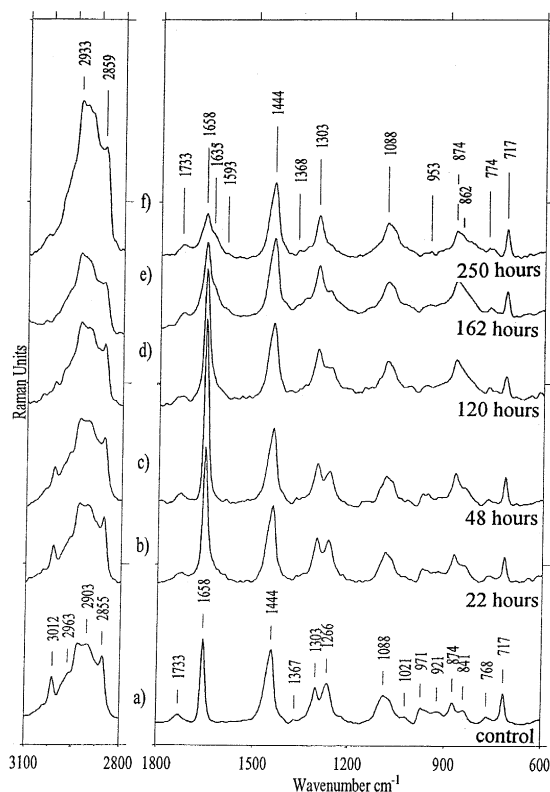
Fig. 2. FT–Raman spectra of artificial membranes containing DLPC (80%) and DLPS (20%) in the region between 600 and 1800 cm^{-1} and between 2800 and 3100 cm^{-1}. (a) Control (without irradiation), (b)–(f) Irradiated with 100 Gy. The spectra were recorded at various time intervals after irradiation.

The time interval for the appearance of the first radiation-induced modifications in the Raman spectra was found to depend on the concentration of the liposomes and on the dose rate. A comparison of the velocity of the peroxidation process in liposomes irradiated with a ^{137}Cs source (dose rate 0.9 Gy min^{-1}) and a ^{60}Co source (dose rate 120 Gy min^{-1}) demonstrated the well known dose rate effect (not shown, see, i.e., Ref. [24]). The results presented here were, therefore, obtained after irradiating liposomes with the ^{137}Cs source. At a lipid concentration of 18 mg ml^{-1} the radiation-induced peroxidation process continued for some days and it was possible to easily follow its time dependence with FT–Raman spectroscopy.

Fig. 2a–f show FT–Raman spectra of liposomes measured at various time intervals after irradiation as well as the control spectrum (Fig. 1). For unirradiated

samples, no changes could be detected during the investigated time interval. The various radiation-induced changes in the Raman spectra will be described separately in the following paragraphs.

3.2.1. C=C stretching region

Fig. 2 shows that in the region between 1620 and 1680 cm^{-1} distinct perturbations were induced by radiation. The line of the *cis* C=C stretching vibration at 1658 cm^{-1} increased in intensity during the first 50 h. After a short period at maximal value, the intensity decreased again continuously. The relative intensity change of the Raman line at 1658 cm^{-1} normalized to the C–C Raman band at 1444 cm^{-1} is shown in Fig. 4a as a function of time after irradiation. This normalization can be done, because no changes at 1444 cm^{-1} could be observed during the whole peroxidation process [4]. The half width of the whole band around 1658 cm^{-1} decreased during the peroxidation process. For a better understanding of this region, a simulation of the Raman bands observed as a function of time between 1575 and 1775 cm^{-1} was performed (Fig. 3). The measured data were fitted with a simulated curve by minimising the sum of weighted squared residuals by the Levenberg–Marquard method. Beside the *cis* C=C vibration at 1658 cm^{-1}, new peaks of the *trans* C=C-mode at 1673 cm^{-1} as well as at around 1640 (irradiated liposomes) and 1649 cm^{-1} (unirradiated liposomes),
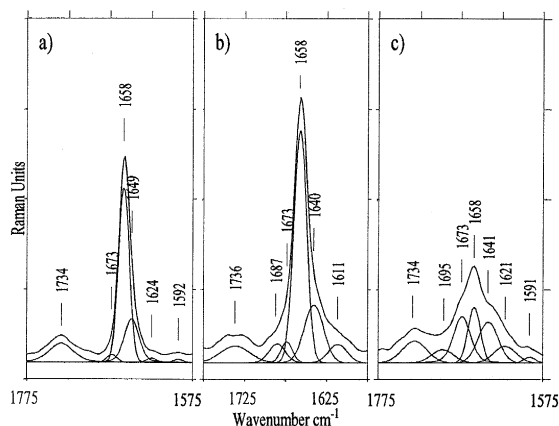

Fig. 3. FT–Raman spectra of liposomes in the region between 1575 cm^{-1} and 1775 cm^{-1}. (a) Control (without irradiation); (b) 162 h after irradiation with 100 Gy; (c) 250 h after irradiation with 100 Gy. A simulated function is fitted to all spectra as described in the text.

respectively, appeared as a function of time. In addition to the time dependent changes of the *cis* C=C stretching mode and the frequency shift of the not yet assigned peak at 1649 cm^{-1}, the Raman band of the *trans* C=C mode at 1673 cm^{-1} appeared and increased in intensity in the second step of the peroxidation process. Additionally, other new lines at 1611 (Fig. 3b) or 1620 cm^{-1} (Fig. 3c) and at 1687 (Fig. 3b) or 1695 cm^{-1} (Fig. 3c) show strong perturbations in the C=C region. These changes could be explained by decomposition of the peroxides and the development of stable endproducts like malondialdehydes (MDA) and the various hydroxyalkenals [5]. The C=C bonds of these products can be assigned to various functional molecule groups which differ from those of the lipid chains.

3.2.2. = CH vibrations

The line at 1303 cm^{-1}, assigned to the CH$_2$ twisting mode, increased in intensity only marginally during the first 20 h. On the other hand, the Raman band at 1266 cm^{-1}, resulting from the =CH wagging vibration, continuously decreased in intensity. Fig. 4b shows the time dependence of the intensity ratio $I(1266 \text{ cm}^{-1})/I(1303 \text{ cm}^{-1})$ after irradiation.

The =CH stretching vibration at 3012 cm^{-1} showed the most obvious variation in the region

between 2800 and 3100 cm^{-1}. The intensity of this line decreased continuously as a function of time after irradiation until its complete disappearance at around 170 h (Fig. 4c). The immediate decrease in intensity, similar to the =CH wagging vibration at 1266 cm^{-1}, indicates that the intensity changes of these two lines result from the same physical effect.

A new peak at around 862 cm^{-1} overlaps the C–N stretching vibrations at 874 and 841 cm^{-1}. This new line reached its maximum in intensity after around 150 h and decreased afterwards only marginally (Fig. 4d). The Raman line of the O–O stretching vibration of the aliphatic peroxides is located at 865 cm^{-1}. This indicates that the new line could be assigned to the –OOH group which is known to occur during a peroxidation process. The intensity decrease after around 170 h can be explained by decomposition of the lipids during the last steps of the peroxidation process.

3.3. Dienconjugations and malondialdehydes

The kinetics of radiation-induced dienes was determined by measuring the relative intensity of the absorption lines at 234 and 195 nm ($I(234/195)$) of the lipids. The yield of conjugated C=C bonds was found to increase during the first 70 h after irradia-
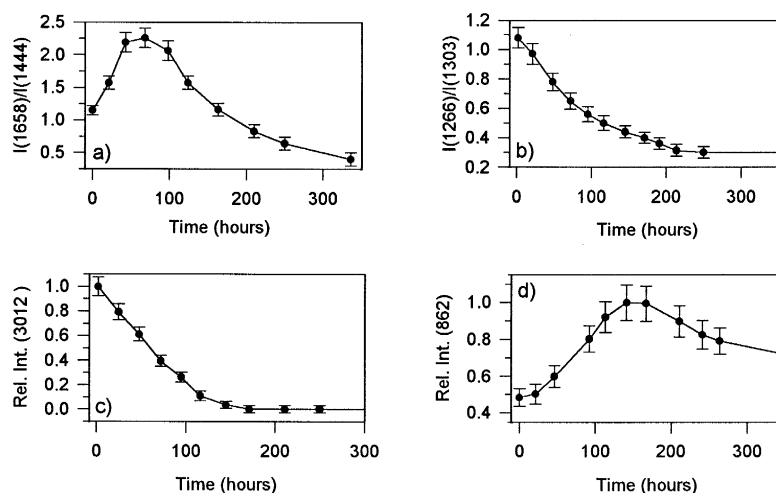

Fig. 4. Various intensity ratios of Raman lines as a function of time after irradiation with 100 Gy. (a) Intensity ratio of the *cis* C=C vibration line at 1658 cm^{-1} and the –CH$_2$ deformation vibration line. (b) Intensity ratio of the =CH wagging vibration line at 1266 cm^{-1} and of the –CH$_2$ deformation vibration line at 1303 cm^{-1}. (c) Relative intensity of the =CH stretching vibration line at 3012 cm^{-1} (maximum value normalized to 1). (d) Relative intensity of the Raman line at 862 cm^{-1} (maximum value normalized to 1).
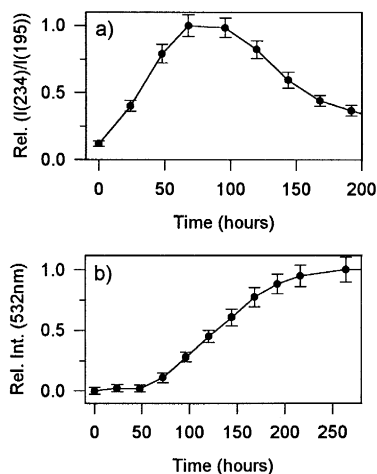
Fig. 5. (a) Yield of conjugated double bonds: Relative intensity of the UV absorption peaks at 195 and 234 nm as a function of time after irradiation (maximum value normalized to 1). (b) Yield of malonaldehydes: Relative intensity of the absorption line at 532 nm as a function of the time after irradiation (maximum value normalized to 1).

tion. After 100 h, the yield of diene conjugation decreased again (Fig. 5a). At the same time, other products like malondialdehydes (MDA) and 4-hydroxyalkenale are produced [5]. Fig. 5b shows the time dependence of the yield of MDA induced by the peroxidation process. It can be seen that 60 h after irradiation the first MDAs were produced. The yield of MDAs increased continuously and reached a saturation value after about 300 h. This shows that reactive molecules appear while the lipid hydroperoxides are decomposed again.

3.4. Autooxidation by cytochrome c (ox.)

Freshly prepared liposomes composed of DLPC (80%) and DLPS (20%) with and without incorporated cytochrome c show similar Raman spectra, because in the recorded frequency area Raman lines of cytochrome c can only be detected with resonance or preresonance Raman spectroscopy. Without irradiation cytochrome c alone was found to cause time dependent changes similar to those induced by γ-radiation in liposomes without cytochrome c. This indicates that cytochrome c either induced lipid peroxidation processes or catalysed an extremely slow process of self-oxidation [25].

The cytochrome c-induced intensity increase of the Raman line of the C=C stretching mode at 1658 cm^{-1}, however, differed from the radiation-induced changes of this line. Fig. 6a shows the intensity ratio $I(1658$ cm$^{-1})/I(1444$ cm$^{-1})$ as a function of time. The maximum value of this ratio is about 1.7 compared to a ratio of 2.1 observed in irradiated liposomes (Fig. 4a). The speed of the peroxidation process did not depend on the concentration of the liposomes and could not be influenced by an additional radiation exposure (not shown). Also, the maximal intensity ratio $I(1658$ cm$^{-1})/I(1444$ cm$^{-1})$ could not be increased by additional irradiation. The results of the TBA test and the diene test were found to be similar to the results obtained with irradiated
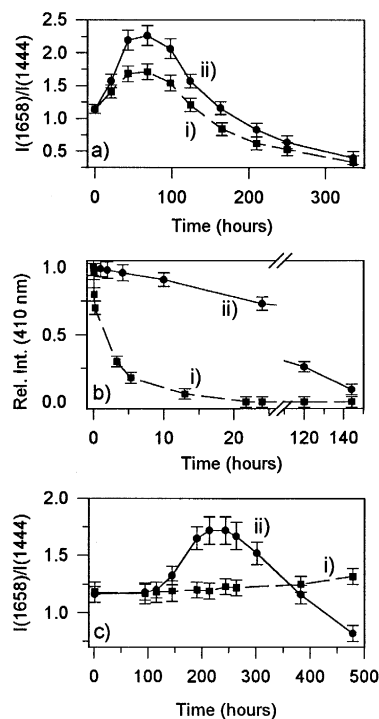

Fig. 6. (a) Intensity ratio of the *cis* C=C vibration line at 1658 cm^{-1} and the –CH$_2$ deformation vibration line as a function of time (i) after adding cytochrome c (ox.) to the liposomes; (ii) after irradiation with 100 Gy; (b) relative intensity change of the absorption band of cytochrome c (ox.) after incorporation in: (i) Irradiated liposomes; (ii) unirradiated liposomes; (c) intensity ratio of the *cis* C=C vibration line at 1658 cm^{-1} and the –CH$_2$ deformation vibration line in the presence of α-tocopherol as a function of time: (i) After irradiation of pure liposomes with 100 Gy; (ii) after adding cytochrome c (ox.) to the liposomes.

liposomes (pure lipids) and confirm the results of the Raman measurements that cytochrome c initiates the process of peroxidation.

During the cytochrome c-induced peroxidation process, the Soret absorption band of cytochrome c (ox.) at 410 nm decreased continuously until its complete disappearance after about 140 h (Fig. 6b). This means that cytochrome c degenerated during the peroxidation process [26]. When cytochrome c was added to liposomes irradiated 75 h earlier, the intensity decrease of the Soret band was much faster and the band disappeared already after about 20 h (Fig. 6b). This demonstrates the interaction of peroxidated liposomes with cytochrome c (ox.).

3.5. Protection effect of the antioxidant α-tocopherol

The effect of α-tocopherol on lipid peroxidation was studied by both using liposomes of pure lipids and liposomes with incorporated cytochrome c (ox.). 2 μM of α-tocopherol was incorporated into the liposomes. In both systems, the scavenging effect of α-tocopherol was obvious. In pure liposomes no changes in the Raman spectrum were detected even after 400 h (Fig. 6c shows the intensity ratio $I(1658/1444 \text{ cm}^{-1})$) and no dienes or malondialdehydes could be detected (not shown). Also the cytochrome c (ox.)-induced peroxidation process was inhibited by α-tocopherol for around 120 h. Later, however, the autooxidation process started in the same way as without α-tocopherol (Fig. 6a).

4. Discussion

4.1. Radiation-induced peroxidation of pure lipids

The results presented here show that FT–Raman spectroscopy can be used to study in detail the kinetics of peroxidation processes induced in artificial membranes by ionizing radiation and cytochrome c, respectively. Beside chemical modifications, structural changes of the lipid chains were observed. The Raman lines of the acyl chain vibrations show time dependent modifications which demonstrate the main

role of the cis double bonds in the peroxidation process.

The kinetics of the intensities of the cis C=C vibration (Fig. 4a, Fig. 6a) and the =CH wagging vibration (Fig. 4b–c) indicate that in addition to the chemical changes in the C–C-bonds also modifications in the structure of the lipids were induced. Previous experiments [27] showed different intensities of the C=C stretching mode arising from different crystalline structures of linoleic acid. The different conformations were achieved by rapidly cooling to −45°C (intensity ratio $I(1661 \text{ cm}^{-1}/I(1445 \text{ cm}^{-1}) = 0.95$) and by annealing (intensity ratio $I(1661 \text{ cm}^{-1})/I(1445 \text{ cm}^{-1}) = 1.29$). The authors attributed the intensity changes to alterations in the conformation around the sp^2, C–C axis. Furthermore, Faiman and Long [28] showed that the Raman intensities of the C=C stretching bonds in egg lecithin–water systems depend on the water content and the temperature. They explained these changes in intensity by modifications of the conformation of the egg lecithin–water system. At a $skew, skew$ conformation (found at a lower water content (1–2.5%, 3°C)) the intensity was found to be lower in comparison to a $skew, skew'$ conformation (found at a higher water content (5–8%, 3°C)). The same phenomena could be the base for the increase of the intensity ratio $I(1658 \text{ cm}^{-1})/I(1444 \text{ cm}^{-1})$ during the first period of the peroxidation process (Fig. 4a). This demonstrates that the cis C=C double bonds were not destroyed immediately during the first step of peroxidation but indicates structural changes in the lipid chains. The intensity increase may be explained by changes in the internal rotation around the sp^2, C–C axes at each end of the cis double bond.

The continuous decrease of the intensity ratio $I(1661 \text{ cm}^{-1})/I(1440 \text{ cm}^{-1})$ after around 70 h (Fig. 4a, Fig. 6a) demonstrates further changes in the cis C=C bonds which could be explained by the formation of final products like malonaldehydes or 4-hydroxyalkenales which are formed when lipid hydroperoxides break down. The functional groups around the C=C bonds of these highly reactive products differ from those of the lipid chains and are produced only in the last step of the peroxidation process. Additionally, the appearance of the $trans$ C=C Raman line at 1673 cm^{-1} as well as the lines at 1640, 1620 and 1695 cm^{-1} (Fig. 3) which could not yet be

assigned to a precise conformation of the C=C band, indicate perturbations of the structure of the lipid chains.

Radiation-induced intensity changes of the C=C stretching band in DPPC/DLPC liposomes were also found by Verma and Rastogi [4]. However, they only measured a decrease of the intensity ratio $I(1661$ $cm^{-1})/I(1440 \text{ cm}^{-1})$ after a defined time of 24 h postirradiation. We measured a decrease of this intensity ratio only after a time interval of 70–80 h (see above). Before this time interval, an increase of this intensity ratio was found. This could indicate that these authors measured the irradiated samples at a time interval that allowed them to detect only the final modifications of the peroxidation process.

In our experiments, the intensity ratio $I(1266$ $cm^{-1})/I(1303 \text{ cm}^{-1})$ as well as the intensity of the stretching vibration line at 3012 cm^{-1} decreased continuously even when the measurements of the C=C double bond at 1658 cm^{-1} show that these bonds were not destroyed immediately (see discussion above). In contrast to these observations, several authors described the intensity ratio of the =CH deformation vibration at 1266 cm^{-1} and of the $-CH_2$ vibration at 1303 cm^{-1} as an indicator for the saturation of the lipid chains [19,29]: they found a linear relation between the ratio $I(1266 \text{ cm}^{-1})/I(1303$ $cm^{-1})$ and the ratio of =CH to $-CH_2$ bonds. However, the investigations of these authors were performed using lipids with isolated C=C double bonds only. On the other hand, the diene test ([17], Fig. 4a) shows that in the first step of the peroxidation process, conjugated double bonds are formed. This suggests that the Raman vibration lines of the =CH deformation vibration at 1266 cm^{-1} as well as of the =CH stretching vibration at 3012 cm^{-1} are only Raman-active for isolated C=C bonds, what was also suggested for the corresponding IR-lines [11]. Therefore, the intensity change of these lines could be considered as an indicator for the yield of dienes, formed during the first step of the peroxidation process. Additionally, a change in the physical state of the lipid chains may contribute to the decrease of the intensity ratio $I(1266 \text{ cm}^{-1})/I(1303 \text{ cm}^{-1})$ as suggested by Verma et al. [10]. An increase of the chain length is followed by a decrease of the intensity ratio $I(1266 \text{ cm}^{-1})/I(1303 \text{ cm}^{-1})$. Liposomes with longer lipid chains have a higher phase transition tempera-

ture and, therefore, a different physical state. Verma et al. [10] explained this modification in irradiated liposomes with a decrease of the mobility of the C=C group.

4.2. Influence of cytochrome c (ox.) and protection effects of α-tocopherol

Modifications in the Raman spectrum of liposomes induced by added cytochrome c were similar to radiation-induced changes. This shows that cytochrome c induces lipid autooxidation as already suggested by other authors [8,30]. The lower increase in intensity of the cis C=C double bond at 1658 cm^{-1} in comparison to the –CH deformation vibration at 1444 cm^{-1} shows that the modifications of lipid conformation differ during the first peroxidation step (Fig. 6a). A possible influence of polymeric impurities of cytochrome c also does not enhance the maximum intensity ratio. Additional irradiation did not induce any catalysing effect to the cytochrome c-induced peroxidation. These observations might suggest that in cytochrome c- and radiation-induced peroxidation processes different radical species are involved. Furthermore, the measurements also could indicate a kind of protection effect of cytochrome c against radiation-induced free radicals, produced in water solution, which neither could increase the intensity ratio $I(1658 \text{ cm}^{-1})/I(1444 \text{ cm}^{-1})$ nor increase the speed of the process.

The double bonds of unsaturated acyl chains have π-electrons and, therefore, a lower hydrophobicity than saturated acyl bonds [30]. Thus C=C bonds provide an environment with slightly polar pockets. This could explain the strong interaction of the protein with unsaturated C=C bonds [31].

More details of the effect of cytochrome c (ox.) during peroxidation processes could be obtained by studying the results of the radical scavenger α-tocopherol. Radiation-induced peroxidation could be inhibited completely in liposomes containing only DLPC and DLPS lipids, when the concentration of α-tocopherol was sufficient. The peroxidation process did not start even after a time interval of at least 400 h after irradiation (Fig. 6c). This means that the radiation-induced radicals could be scavenged effectively by the radical scavenger α-tocopherol. α-Tocopherol was also sufficient in quenching radical

species produced in the presence of cytochrome *c* (ox.), while α-tocopherol molecules themselves were oxidised with a rate that depended on the protein concentration. During this time, no cytochrome *c*-induced autooxidation could be observed. But after a lag time, dependent on the radical density, the autooxidation process of lipids started in the presence of cytochrome *c* (ox.) (Fig. 6c). This supports the assumption, made above, that the lipid oxidation may occur by radical species induced by cytochrome *c* (ox.), which are sensitive to the quencher α-tocopherol.

Acknowledgements

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