Journal of Peptide Science
*J. Pept. Sci.* 2008; **14**: 1003–1009
Published online 21 April 2008 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/psc.1036

JOURNAL OF PEPTIDE SCIENCE

# A fluorescence approach of the gamma radiation effects on gramicidin A inserted in liposomes‡

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*Received 30 June 2007; Revised 7 January 2008; Accepted 14 February 2008*

**Abstract:** The fluorescence of tryptophan residues of gramicidin A (gA), bound to phosphatidylcholine liposomes contains valuable information about local changes in the environment of the molecule induced by gamma radiation. With this work, we aim to demonstrate that the gamma radiation effect on the peptide involves the action of free radicals, derived from water radiolysis and the process of lipid peroxidation. Basically, the methodology consists of the analysis of UV and fluorescence emission spectra of the peptide liposome complexes under control conditions and upon gamma irradiation. Free radical production was impaired by the removal of molecular oxygen or the presence of ethanol in the liposome suspension. The intensity of the tryptophan fluorescence was recorded as a function of the gamma radiation dose in the range of 0–250 Gy and the data were fitted with a single decay exponential function containing an additional constant term (named residual fluorescence). The correlation between the decrease in tryptophan fluorescence emission ($D_c = 80 \pm 10$ Gy) and increase in gamma radiation dose indicates the partial damage of the tryptophan side chains of gA. $O_2$ removal or ethanol addition partially reduced the decay of the tryptophan fluorescence emission involving an indirect action of gamma radiation via a water radiolysis mechanism. The residual fluorescence emission ($A_0$) increases in $O_2$-free buffer ($98 \pm 13$) and in 10% ethanol-containing buffer ($74 \pm 34$) compared to control conditions ($23 \pm 5$). Varying the dose rate between 1–10 Gy/min at a constant dose of 50 Gy, an inverse dose-rate effect was observed. Thus, our study provides evidence for the lipid peroxidation effect on the tryptophan fluorescence. In conclusion, this article sustains the hypothesis of the connection between the lipid peroxidation and structural changes of membrane proteins induced by gamma radiation. Copyright © 2008 European Peptide Society and John Wiley & Sons, Ltd.

**Keywords:** ionizing radiation; gramicidin A; lipid peroxides; free radicals; liposomes

## INTRODUCTION

The investigation of the cellular effects of the ionizing radiation (IR) is an important part of biomedical research as well as of basic biophysical research. Our experiments deal with the gamma radiation effects on a model system: gramicidin A (gA) doped lipid vesicles. Despite intense efforts devoted to investigate the genetic effects of IRs, much fewer studies were targeted to elucidate the mechanisms involved in the radiation interaction with the membrane components and processes.

Ion channels are transmembrane proteins that regulate ionic permeability in cell membranes and connect the inside of the cell to its outside in a selective fashion. The linear peptide gA forms ion channels specific for monovalent cations and has been extensively used to study the organization, dynamics, and function of pore-forming ion channels [1–3]. An important aspect of its conformation is the membrane interfacial location of the tryptophan residues, a common feature of many transmembrane helices [4–6]. The gA has four tryptophan residues in positions 9, 11, 13, and 15. Several spectroscopic techniques, such as red-edge excitation shift (REES), the parallax method, or CD spectroscopy, have been used to investigate the organization and the dynamics of the functionally important tryptophan residues of gramicidin inserted in membranes [7,8].

The ionic transport through membranes is perturbed by the IR effect. IR disturbs the activity of Na, K–ATP-ase both in neuron and in glial cells [9]. $K^+$ and pH homeostasis in the developing rat spinal cord is impaired by early postnatal X-irradiation [10]. A transient increase in the cytosolic free calcium influx is detected in human epithelial cells [11]. Generation of the reactive species gives rise to a decay of the membrane potential, to an inactivation of $K^+$-channels, and to an increase of the leak conductance of the

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‡ This article is part of the Special Issue of the Journal of Peptide Science entitled "2nd workshop on biophysics of membrane-active peptides".

membrane [12]. Pore-forming proteins, such as gA, amphotericin B etc., inserted in artificial lipid bilayers show a decrease of their conductance upon X-ray irradiation in the 0–500 Gy dose range [12,13]. Lipids from model and natural membrane are drastically affected by radiation (e.g. lipid peroxidation processes), a phenomenon which is prevented by radical scavengers [14–16].

The most important pathway of the radiation effects on biological systems is initiated by water radiolysis which produces a series of reactive oxygen species (ROS) with high chemical reactivity. ROS effects on membrane functionality are extensively studied in photodynamic therapy. The sensitivity of gramicidin channels to ROS can be used for evaluation of photodynamic efficacy of different photosensitizers [17]. The photo-suppression of the gramicidin-mediated current across a lipid bilayer has proven to be highly specific, as it is caused by selective damage to tryptophan residues located near the channel gate [18].

Lipid peroxidation can be defined as the reaction of lipids with molecular oxygen leading to the formation of lipid hydroperoxides. Uncatalyzed lipid peroxidation is spin-forbidden, but this rule can be overcome in the presence of free radicals. Primary radical OH· resulting from water radiolysis, some organic radicals, and metal ions (i.e. $Fe^{3+}$) act as promoters of the lipid peroxidation chain reaction. This process is most efficient for polyunsaturated fatty acid residues. Considering the cyclic character of the described process, it can be concluded that once started the lipid peroxidation will develop until all the lipid molecules are oxidized. Additionally, there is a limiting factor of the process consisting in the reaction between two peroxyl radicals. The concentration of the peroxyl radical is increased by the dose rate (at a constant dose value) of the IR. As a consequence, lipid peroxidation chain reaction is more efficient at low dose rate, because the peroxyl radical concentration is lower [19]. This is the so-called *inverse dose-rate effect* which was described for the first time by Mead in 1952 [20].

Upon irradiation the conductance of ion channels (i.e. gA pore) inserted in lipid membranes is strongly reduced in the presence of protector molecules, such as $\alpha$-tocopherol [21], ethanol [22], calcium antagonists, and the antagonists of receptors H2 [23].

The aim of our study was to characterize the effects of gamma radiation on the structure of ion channels using a model system: gA doped lipid vesicles. In order to quantify the effects of irradiation, the fluorescence of tryptophan (Trp) residues of gA was investigated.

## MATERIALS AND METHODS

### Materials

The compounds 3-*sn*-phosphatidylcholine (from fresh egg; 99% TLC), NaCl, $Na_2HPO_4{\cdot}2H_2O$, $KH_2PO_4$ anhydrous were purchased from Sigma-Aldrich, USA, and gA from *Bacillus brevis* (90% HPLC) was purchased from Fluka Chemie GmbH. Typical lots of egg yolk phosphatidylcholine have fatty acid contents of approximately 33% C 16:0 (palmitic), 13% C 18:0 (stearic), 31% C 18:1 (oleic), and 15% C 18:2 (linoleic) (other fatty acids being minor contributors), which would give an average molecular weight of approximately 768 (P 2772; http://www.sigmaaldrich.com). Thus, it can be emphasized that the total amount of saturated fatty acids is about 46%, and that unsaturated fatty acids represent 46%, without any precise indication of the saturation degree of the remaining 8% lipids. Methanol and chloroform were purchased from Merck, Darmstadt, Germany. Lipids are used without further purification. Phosphate buffer saline (PBS) was used at a concentration of 10 mM, pH = 7.2, and contains 123 mM NaCl, 10 mM $Na_2HPO_4 \cdot 2H_2O$, and 2.4 mM $KH_2PO_4$.

The gamma irradiation was performed using a $Co^{60}$ source, for a dose range of 0–250 Gy and for a dose-rate range of 1–10 Gy/min.

### Liposome Procedure

Small unilamellar vesicles (SUVs) of L-$\alpha$-Phosphatidylcholine (egg yolk) were prepared by sonication method as previously described [24]. Final phospholipid concentration was 0.2 mM. Stock solutions were prepared from lipids in chloroform (10 mg/ml) and gA in methanol (2 mg/ml) and preserved at −20 °C. For liposomes preparation lipid stock solution was diluted in 1:1 (v/v) methanol/chloroform solution at a concentration of 0.4 mg/ml. Multilamellar lipid vesicles (MLVs) were obtained using the following steps: (i) drying of the lipid film under nitrogen flow, (ii) hydration of the lipids in a phosphate saline buffer 10 mM, pH = 7.2, and (iii) vigorous shacking. SUV suspension was obtained after freezing–thawing of the MLV suspension three times followed by sonication (30 min) at room temperature in a common bath sonicator (80 W). The gA was incorporated into the phosphatidylcholine vesicles at a molar ratio of 50:1 lipid/gA.

### Spectroscopic Measurements

The presence of vesicles and of vesicle-bound gramicidin molecules was proved by recording the UV spectra of the suspensions against PBS ($\lambda = 200$–$400$ nm) using a Varian Cary 100 UV-VIS spectrophotometer.

The state of the gA was checked by recording the tryptophan fluorescence emission spectrum ($\lambda_{ex} = 270$ nm, $\lambda_{em} = 320$–$400$ nm) using a steady-state spectrofluorimeter (FluoroMax 3, Horiba Jobin Yvon).

### Data Analysis

The data are presented as the percentage relative change of the maximum intensity of emission: $I_{max}/I_{max\,0}$, where $I_{max\,0}$ is the control emission intensity of the nonirradiated gA doped suspension. Our fluorescence data were fitted with a first order exponential decay function:

$$I_{max}/I_{max\,0} = A_0 + A_1 \times \exp(D/D_c) \quad (1)$$

where $A_0$ is the residual fluorescence emission, $A_1 = 100 - A_0$, $D$ is the dose and $D_c$ is the decay constant. Means ± SEM are plotted for $n = 3$.



DOI: 10.1002/psc

## RESULTS

The typical UV absorption spectra of SUVs and SUVs doped with gA are presented in Figure 1. One can observe both the light scattering on the vesicles (solid curves) and the gA specific absorption (dotted curves). The specific absorption of the tryptophan ring ($\lambda = 230$ and 290 nm) can be observed in the spectrum of SUV doped with gA (see the arrows). These spectra (representative of all the SUV suspensions doped with gA) prove both the presence of the vesicles (the specific signal produced by the light scattering [25]) and the presence of the gA molecules within the suspension (see arrows in Figure 1).

The fluorescence emission spectra of the liposomes doped with gA present a maximal intensity at 340–342 nm (data not shown), in good agreement with other reported values for the peak value from the tryptophan emission spectra of gA inserted into phosphatidylcholine bilayers [8,26]. A blue shift of the peak position occurs comparing with the tryptophan emission in hydrophilic media (347 nm) due to the lipidic environment [27], which proves the insertion of gA molecules into the bilayer.

In Figure 2 the results concerning the radiosensitivity of this experimental model to gamma irradiation are presented. The maximum intensity of the fluorescence emission decreased with an increase of the irradiation dose for the range used in this experiment.

The changes in the fluorescence emission spectra of gA can be considered as an evidence of modifications in the properties of trytophan residues of gA. The maximal fluorescence intensity data are well fitted by an exponential decay curve with a residual term (Figure 2). The decay constant $D_c$ was found to be $75 \pm 10$ Gy, much smaller than $D_{37-direct} = 6 \cdot 10^7 - 6 \cdot 10^5$ Gy, the dose necessary to reach the same effect by direct action of radiation on the peptide molecule [19]. It should

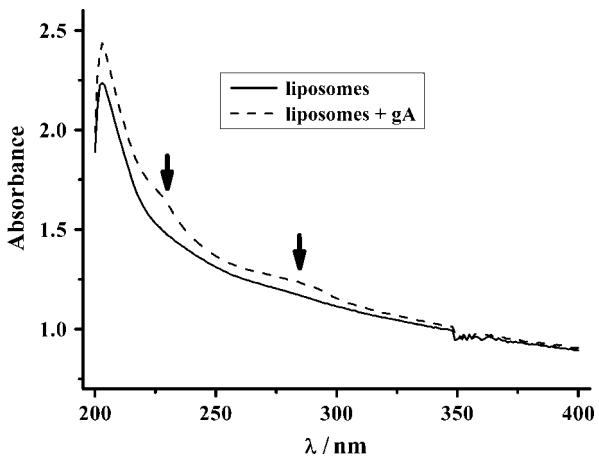


**Figure 1** UV spectra for two samples (A and B) from the same preparation: the solid curves plot the vesicles suspension turbidity and the dotted curves plot the absorption of the vesicles doped with gA.

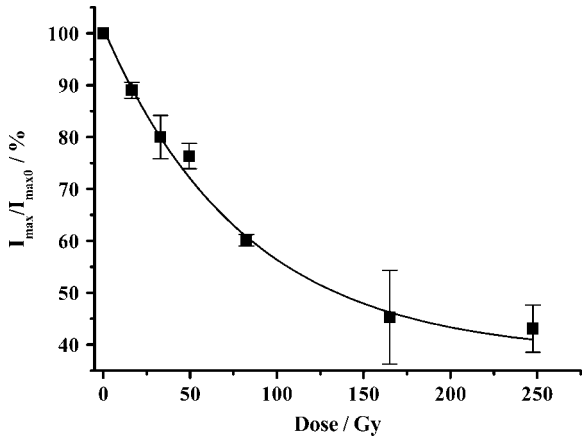


**Figure 2** The dose - effect response for gamma irradiated gA doped vesicles (dose-rate 9 Gy/min); the solid curve represents the fit with a first order exponential decay: $I_{max}/I_{max0} = A_0 + A_1{}^*\exp(D/D_c)$, where $A_0$ is the background relative emission, $A_1 = 100\% - A_0$, $D$ is the dose and $D_c$ is the decay constant ($A_0 = 38 \pm 6$, $A_1 = 63 \pm 5$, $D_c = 80 \pm 16$Gy).

be pointed out that there is a net difference between $D_c$ and $D_{37}$. $D_c$ is obtained by fitting the experimental data with an exponential decay function which contains a residual term ($A_0$), while $D_{37}$ is a theoretical value evaluated under the prerequisite that gamma radiation interacts only directly with the peptides.

The indirect action of gamma radiation (by means of the free radicals generated in the water radiolysis processes) has been confirmed in experiments with oxygen-free buffers. In this case, the buffer used for the liposome suspension was initially bubbled with a nitrogen flow in order to remove molecular oxygen. During all the subsequent steps of the experiment, the vesicle suspensions were maintained under nitrogen. The results of this experiment are presented in Figure 3

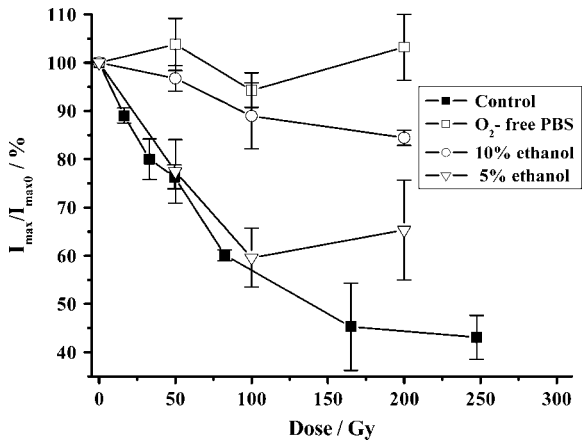


**Figure 3** The dose - effect response of gA doped vesicles when the effects of the free radicals were prevented (dose-rate 9 Gy/min): vesicles prepared with free oxygen buffer (open squares), vesicles prepared with buffer containing ethanol as scavenger (down triangle - 5% of ethanol, up-triangle - 10% of ethanol). The filled symbols plot is the same as in Figure 2.




DOI: 10.1002/psc

(open squares). In comparison, the decay curve in the presence of oxygen (filled squares) is presented. Apparently, the radiation effect upon the tryptophan fluorescence is completely abolished in oxygen-free suspensions.

Another way to partially prevent the effects of free radicals produced by radiolysis of water molecules is to add scavenger molecules into the vesicle suspension. In our experiments, ethanol was used as a scavenger [22]. The results obtained with two different concentrations (5 and 10% of ethanol) are presented in Figure 3 (open circle and triangle symbols). The effect of radiation is dependent on the ethanol concentration. The residual fluorescence emission ($A_0$) increases for $O_2$-free buffer ($98 \pm 13$) and for 5% ($58 \pm 10$) and 10% ethanol-containing buffer ($74 \pm 34$) compared to control conditions ($23 \pm 5$). The results suggest that 10% ethanol produces almost the same effect as $O_2$ removal from the buffer.

In the last set of experiments, the same procedure was used to evidence the inverse dose-rate effect. This phenomenon consists of an increase in radiation effect with a decrease in the dose rate of radiation at constant total dose [19]. The effect is attributed to the cyclic production of lipid hydroperoxides upon irradiation of the lipid membrane [19].

The direct interaction between free radicals (derived from water radiolysis) and Trp residues do not imply an inverse dose-rate effect. Our experiments are an explicit argument for the involvement of lipid oxidation products in the changes of the fluorescence properties of gA, in particular, of Trp residues. In Figure 4 the results of two different experiments are shown. The relative decrease of the maximum emission intensity is linearly related to the dose rate in the investigated range of 1–10 Gy/min at the same dose (50 Gy).

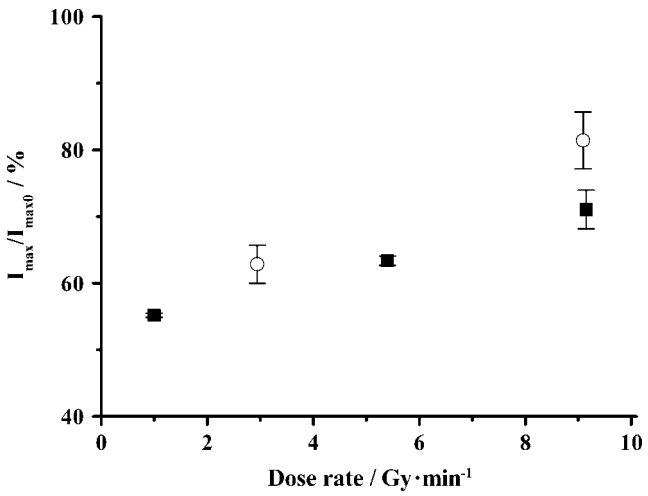


**Figure 4** The inverse dose-rate effects (for the total dose of 50 Gy) in two different experiments (the empty and filed symbols).

## DISCUSSIONS

Primarily, the radiation produces local changes (e.g. excitations, breakages) of macromolecules and also water molecule radiolysis. Among the products of water radiolysis are many radicals, such as hydrated electrons, hydroxyl and hydrogen radicals. These highly reactive species trigger processes of chemical reactions that lead to final products that differ from the normal natural molecules and in this way perturb the cellular homeostasis. They have influence on the functional capacity of the macromolecules, such as DNA being the most sensitive cellular component [14].

A secondary cellular response to radiation is triggered by transduction signals at the level of the plasma membrane. An external stress factor (usually a chemical one) induces a change at the level of membrane proteins, modifying their recognition or transport activities and thus producing a secondary response on the internal face of the cellular membrane. As we already stated, radiation can act directly or indirectly by water-free radicals on the membrane components (proteins and lipids) [19]. The resulting functional changes can be further translated in a secondary response of the cell. For this reason, our experimental approach of the radiation effects at the membrane level is of high scientific interest.

In our experiments, the irradiated samples (excepting the experiments done on the suspension prepared with the oxygen-free buffer) have a maximum emission smaller than the control. This is a direct proof of the irradiation-induced peptide modification at the level of the tryptophan residues. The shape of the curve fitting the relationship between the relative decrease of the maximum emission intensity and the radiation dose (Figure 2) suggests that not all the four Trp residues of the gA are accessible to the radiation-produced free radicals. Consequently, at the highest dose used in our experiments there is still a residual Trp emission. This is in agreement with the decrease of the fluorescence of BSA irradiated in the presence of lipid vesicles [28] or of irradiated creatine kinase [29].

Tryptophan residue substitution on gA by phenylalanine (gM), tyrosine (gT), or naphthylalanine (gN) results in a decrease in channel conductivity [30–32]. In addition, UV irradiation or chemical modification of the Trp side chains have been shown to induce comparable changes in the gA channel conductivity [21,33]. Both radiolysis and photolysis (light absorption triggers a direct effect on the Trp residues) effects are reduced by several orders of magnitude by diminishing the number of Trp residues per gramicidin monomer [34], and as a consequence the channels are completely inactivated in the case of only one Trp per monomer. This could explain the fact that by measuring the damage of gA by Trp fluorescence, a residual signal term ($A_0$) occurs.



DOI: 10.1002/psc

By contrast, no residual term is necessary for the analysis of the membrane conductance decrease with the radiation dose.

The decay constant value ($D_c$) in the normal buffer is higher compared to the measured $D_{37}$ values published in the literature (a few Grays only; [19]). Differences in the pH provide the explanation. We worked at physiological pH values (pH = 7.2) in contrast to the very low pH values used in the cited reference (pH = 3). On the other hand, the same authors observed an increase of approximately two orders of magnitude for the $D_{37}$ values in the case of gA inactivation by the X-rays if the pH switches from low values (pH = 3) to high values (pH = 9) [19]. Interpolating our values, the results are in agreement with this observation.

Experiments using different radical scavengers [35] have indicated that channel inactivation by radiolysis is due to a subsequent reaction of OH and $HO_2$ radicals with Trp residues of gA. Ethanol is frequently used to chemically block some species of free radicals produced by irradiation [22]. In our experiments a concentration of 10% of ethanol in the buffer used to prepare the liposomes almost completely prevented the effect of irradiation, in good agreement with the results of Bonnefont-Rousselot *et al.* [22]. This observation suggests the important role of the OH radical in gA inactivation by gamma irradiation.

Natural membranes are composed of phospholipids consisting in both saturated and polyunsaturated fatty-acyl chains. Phosphatidylcholines are one of the major components of eukaryotic biological membranes. They are a heterogeneous group of components whose fatty acid constituents vary in chain length and degree of saturation. Most naturally occurring phosphatidylcholines (such as in egg yolk) contain several amounts of polyunsaturated fatty acids, which are particularly susceptible to oxidation mediated by free radicals [19]. In our study, 3-*sn*-phosphatidylcholine (from fresh egg; 99% TLC, Sigma-Aldrich), that contains approximately the same amount of saturated and unsaturated fatty acids was used. It is now well admitted that lipids are nonenzymatically peroxidized through two types of reaction: (i) autoxidation or photooxygenation and (ii) enzymatic peroxidation. In our experiments, lipid peroxidation (produced by autoxidation) was probed by the increase of the local microviscosity of the bilayer, using the diphenylhexatriene (DPH) fluorescence depolarization method (unpublished results). Similar reports from the literature indicate the increase of DPH and *N*, *N*, *N*-Trimethyl-4-(6-phenyl-1,3,5-hexatrien-1-yl)phenylammonium *p*-toluenesulfonate (TMA-DPH) fluorescence anisotropy following the lipid peroxidation in SUV. Another phenomenon associated with radiation-induced lipid peroxidation was the increase of membrane capacitance [18] explained in terms of increase of the membrane dielectrical constant. This small change of the dielectrical constant can be the cause of the large increase in membrane conductance in the presence of macrocyclic ion carriers of the valinomycin type [35].

The yield of the cyclic chemical reactions involved in the lipid peroxidation triggered by irradiation is dependent of the dose rate. At low dose rate the yield of lipid peroxides production is higher and results in the inverse effect of the dose rate [19].

In our study, the inverse dose-rate effect was confirmed by measuring the Trp residues fluorescence in the gA structure. In this way, we proved the fact that lipidic environment is one of the main causes of the gA inactivation. In the literature, a similar inverse dose-rate effect has been demonstrated by conductance modification in other ion channels, such as amphotericin B [13]. In addition, the Trp fluorescence in hydrophilic proteins (BSA) in the presence of lipid vesicles also showed an inverse dose-rate effect [28,29]. We consider these results as strong evidence sustaining the hypothesis that lipid peroxidation is one of the main pathways to produce protein damage by irradiation.

## CONCLUSIONS

In our experiments, the change of molecular structure of gA was produced preferentially by an indirect radiation effect. The free radicals (produced by the lysis of water molecules) and lipid peroxidation products induced by gamma radiation at the membrane level interact with the gA molecules and can induce changes in tryptophan emission either through direct action on the indole ring [36] or by changing the local environment of the tryptophan residue. In reports [12,19] regarding gA channel conductance, a rapid inactivation of the channel activity was found. On the other hand, using MS, fragmentation of gramicidin molecules was observed in photolysis conditions [34]. Such modifications of the gA structure could be causing the changes in the tryptophan emission spectrum observed in our experimental approach. Apparently, not all tryptophan residues are affected by the irradiation. Besides the direct action of free radicals on the Trp residues, another important pathway of Trp damage involves the lipid peroxidation chain reaction. This fact has been proved in our study by the inverse dose-rate effect. To characterize in detail the radiation-induced modification in the gA structure, further experiments with gA analogs, formed by varying the number of Trp residues [34,36] are necessary. Another interesting approach would be to modify the degree of saturation for the fatty acid chains or to vary the position of the double bonds in the chain, and to extensively analyze the effect of lipid



DOI: 10.1002/psc

peroxidation on gA structure and membrane fluidity. Intercalation of cholesterol between different types of phosphatidylcholine chains is also a very important issue to investigate.

Related to this last aspect, it is well recognized that lipid rafts as membrane structural and functional platforms, play a crucial role in the initiation and organization of signaling cascades, since they spatially concentrate or exclude components of the signaling machinery. Cholesterol and sphingomyelin are important constituents of lipid rafts. Lipid peroxidation process could be a perturbing factor modulating the dynamics of lipid rafts [37]. Some recent reports suggest the importance of lipid rafts in DNA repair after radiation of living cells [38]. Considering these reported results, our future studies will focus on the influence of IR on microdomains in model membranes and the effects on the protein-embedded functionality.

## Acknowledgements

This study was supported by the Romanian Ministry of Research grants CERES 4-220 and CEEX 6/2005 AGRAL.

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DOI: 10.1002/psc