Membranotropic properties of the water soluble amino acid and peptide derivatives of fullerene C_{60}

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Abstract The modifying effects of the products of the equimolar addition of DL-alanine and DL-alanyl-DL-alanine to fullerene C_{60} on the structure and permeability of the lipid bilayer of phosphatidylcholine liposomes has been studied using the luminescence probe technique. It is shown that these water soluble amino acid and dipeptide derivatives of fullerene (C_{60}-AD) are quenchers of pyrene fluorescence and erythrosine phosphorescence of in both a water solution and liposomes. To study the permeability of the lipid bilayer a procedure based on the triplet probe technique has been developed. It has been found that the C_{60}-AD derivatives under study are able to localize inside the artificial membrane, to penetrate into the liposomes through the lipid bilayer and to perform activated transmembrane transport of bivalent metal ions.

Key words: Liposome; Fullerene; Fluorescence; Triplet probe

1. Introduction

The unique chemical nature of fullerenes accounts for the considerable interest in the study of their effects on biological substrates In this connection, the development of procedures for the solubilization of fullerenes and the preparation of their water soluble derivatives are of special significance.

There is some evidence that fullerenes can be transferred to the water phase by incorporating them into micelles and liposomes [1] or by preparing the water soluble complexes with polyvinylpyrrolidone [2]. This has made it possible to study the action of fullerenes on the respiratory activity of liver cells and on the hydrophobicity of the microsomal membranes of hepatocytes [2], as well as to demonstrate neurobiological activity of C_{60}/(C_{70}-fullerenes [3].

However, studies on the synthesis of water soluble derivatives of fullerenes and their properties are more promising for the preparation of new biologically active compounds. It has been shown that these derivatives of fullerene C_{60} can affect the activity of some enzymes [4] and even inhibit AIDS virus [5

Recently, a method has been devised for the direct equimolar addition of various amino acids and dipeptides to fullerene C_{60} leading to the formation of the corresponding C_{60}-AD derivatives [6]. These derivatives are found to dissolve in water to give colloidal or true water solutions as dictated by their concentration [7]. It has also been demonstrated that C_{60}-AD substantially activates enzymes involved in the oxidative deamination of biogenic amines [8].

The membranotropic properties of C_{60}-AD, their effect on the structure and permeability of the lipid bilayer of phosphatidylcholine liposomes as well as the transmembrane transport of bivalent metal ions have been studied in the present paper for the first time. The data obtained allow one to put forth some proposals on the mechanism of action of C_{60}-AD toward membrane-bound enzymes.

2. Materials and methods

Water soluble products of the equimolar addition of alanine and alanylalanine to fullerene C_{60} (C_{60}-A and C_{60}-AA) have been studied in this work. Phosphatidylcholine liposomes were obtained by ultrasonic treatment of a mixture containing 0.5 ml of a 10% ethanol solution of phosphatidylcholine (Serva) and 9.5 ml Tris-HCl buffer (0.1 M, pH 7.2) under a flow of nitrogen at 4°C. The fluorescence probe employed was pyrene (Aldrich), the ethanol solution of which (10^{-5} M) was added to the resulting liposomes; the pyrene fluorescence spectra were then recorded on an Aminco-Bowman spectrofluorimeter. The C_{60}-AD solution (1.5×10^{-5} M) was sequentially added, 0.02 ml at a time; the sample was excited at λ = 340 nm.

Erythrosine (Sigma) excited by a pulsed nitrogen laser (λ = 337 nm) was applied as a triplet probe. Kinetic measurements of the phosphorescence quenching were performed using a pulsed laser equipment based on a DL-912 transient recorder. The time it takes for the phosphorescence intensity to decrease by a factor of e was taken to be the phosphorescence quenching time τ_{phosph}. Phosphorescence was registered after prior oxygen removal by an enzymatic method. For this purpose, 1 mg of glucose (Sigma), 100 μg of glucose oxidase, and 100 μg of catalase (Sigma) were added to the samples.

3. Results and discussion

The effects of C_{60}-AD on the structure and permeability of phosphatidylcholine liposomes were studied both from the changes in the fluorescence spectra of pyrene and using the kinetic measurements of phosphorescence quenching of erythrosine triplet probes. It is known that pyrene introduced into liposomes localizes inside the lipid bilayer. In this case the fluorescence spectrum of pyrene contains two typical bands at 390 and 460 nm, due to the monomer and excimer states of pyrene molecules, respectively. The spectral maximal intensities allow one to characterize the probe microenvironment.

The addition of 10^{-5} M C_{60}-AD water solution to a suspension of liposomes leads to the quenching of pyrene fluorescence. In this case the same decrease in intensity of the monomer and excimer bands is observed in the spectrum,

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Abbreviations: C_{60}-AD, water soluble amino acid derivatives of fullerene C_{60}; C_{60}-A, DL-alanine derivatives of C_{60}; C_{60}-AA, DL-alanyl-DL-alanine derivatives of C_{60}

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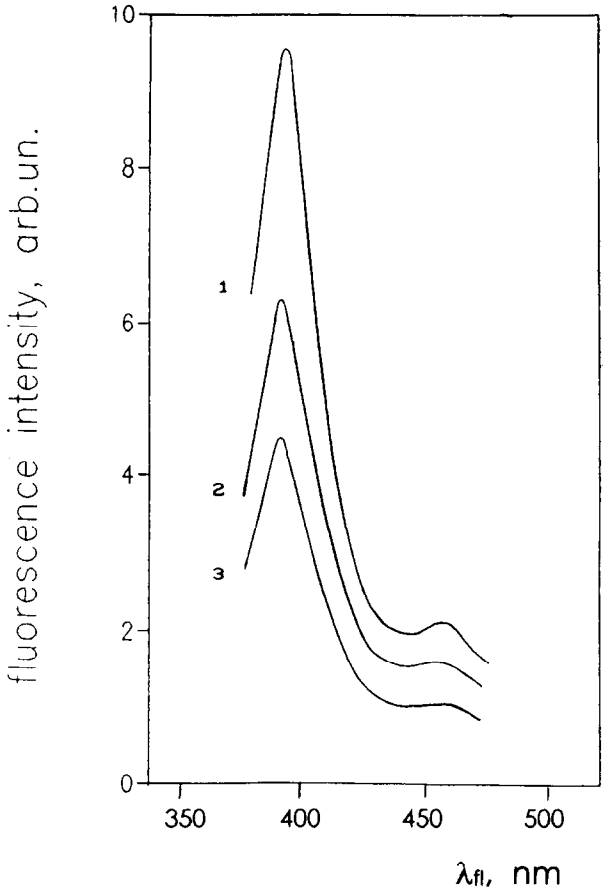

Fig. 1. Effect of C_{60}-AA on the fluorescence spectra of pyrene, introduced into the lipid bilayer of liposomes: 1, without C_{60}-AA; 2, 3.6×10^{-5} M C_{60}-AA; 3, 1.4×10^{-4} M C_{60}-AA.

i.e. their intensity ratio does not change (Fig. 1). This demonstrates the ability of C_{60}-AD to penetrate into the pyrene localization region without essentially changing the structure of the lipid bilayer.

A method based on the triplet probe technique has been developed to study the permeability of the lipid bilayer of phosphatidylcholine liposomes to chemical compounds and the effect of these compounds on liposome integrity.

The greater lifetime of the excited triplet states of the probes in the solution ($\sim10^{-4}$ s) as compared with the fluorescence quenching times ($\sim10^{-8}$ s) provides a much higher sensitivity of measurement of the diffusive collision between triplet probe molecules and those of the quenchers [9–11]. A number of organic and inorganic molecules provide quenching of the triplet states via energy-transfer or charge-transfer mechanisms [11,12]. Organic molecules possessing a lower triplet level, weak oxidants and reductants, paramagnetic ions and their complexes, and nitroxyl radicals are capable of quenching the triplet states. The quenching rate constants are close to the diffusion constants in such cases. This fact allows one to detect the quenchers under study at about 10^{-6} M in water solutions while in a membrane at a probe/lipid ratio of 10^{-4} [9–11].

It is known that the xanthene dye erythrosine introduced into a solution containing liposomes localizes in the region of the polar phospholipid groups, i.e. in the liposomal membrane surface layer [9]. Once the suspension, containing phosphatidylcholine and erythrosine (10^{-5} M), has been subjected to ultrasonic treatment, the probe molecules locate on both the external and internal surface of vesicles. Addition of the water soluble quenchers to these liposomes leads to the complete quenching of excited erythrosine both in the water solution (if erythrosine is present there) and on the external membrane surface. In this case, only the phosphorescence of erythrosine localized on the internal membrane surface is observed.

Since the quenching of erytrosine phosphorescence at the inner membrane side can be achieved only on probe-quencher diffusional contact, this clearly demonstrates that quencher molecules penetrate through the lipid bilayer. In the case of the amino acid derivatives of fullerene C_{60}, this approach provides opportunities for an investigation of the permeability of the lipid bilayer for the compounds under consideration, if they are quenchers of the triplet probes.

When the compound itself is not a phosphorescence quencher, one can observe its effect on the membrane by preintroducing into the external liposome volume an efficient phosphorescence quencher (for example, Co^{2+} ions) which is incapable of penetrating through the membrane on its own. The introduction of Co^{2+} ions into the external volume leads to the complete quenching of erythrosine phosphorescence both in the water solution and on the external surface of the membranes, while that at the inner membrane surface is retained. In this case, even insignifcant changes in membrane integrity due to the effect of the studied chemical compounds will lead to a drastic decrease in the phosphorescence intensity of the internal probes.

The ability of water soluble C_{60}-A and C_{60}-AA to quench erythrosine phosphorescence has been studied in the present work. They are shown to bring about efficient quenching of the excited probe in water solutions (Fig. 2). In the case of erythrosine phosphorescence the quenching rate constants K_q are comparable for the given compounds, amounting to $\sim0.4\times10^9$ M^{-1} s^{-1}.

The quenching of the triplet states of erythrosine can be due both to charge transfer and energy transfer. Since the tripet level of excited C_{60} is lower (12 600 cm^{-1}) [13] than that of the

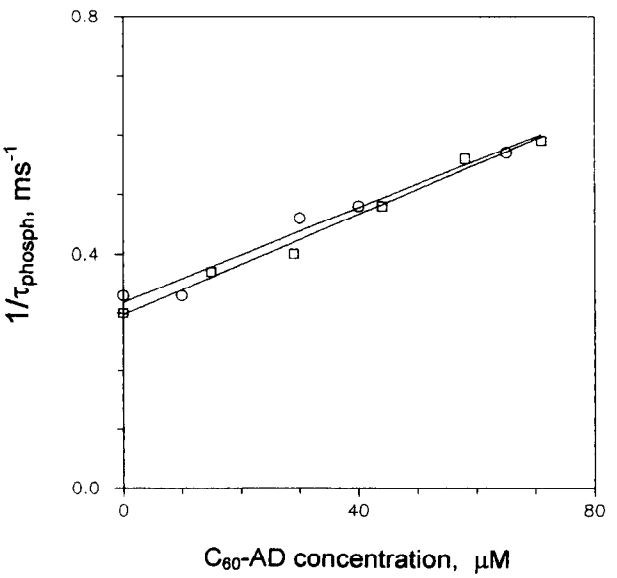

Fig. 2. Effect of C_{60}-A (○) and C_{60}-AA (□) on the guenching rate $1/\tau_{phosph}$ of erythrosine phosphorescence in Tris-HCl buffer solution (0.1 M, pH 7.2).

triplet excited erythrosine (14 300 cm^{-1}) [12], the energy-transfer mechanism is quite possible. It is also known that excited fullerene C_{60} interacts with amines, hydrocarbons, nitroxyl radicals and inorganic ions through a charge-transfer mechanism [14].

Thus, C_{60}-A and C_{60}-AA are efficient quenchers of erythrosine phosphorescence in water solutions. In our study of the effect of C_{60}-AD on liposomes, it has been demonstrated that C_{60}-AD are efficient quenchers of the excited triplet state of erythrosine in liposomes (K_q = 1.4×10^9 M^{-1} s^{-1} for C_{60}-A and 0.9×10^9 M^{-1} s^{-1} for C_{60}-AA). The increase in the values of the quenching rate constants of the phosphorescence probe in the membrane compared to water solution is probably due to the increase in the local concentration of the fullerene derivatives in the lipid bilayer.

It has been found that the phosphorescence intensity of the probe decreases by a factor of 20 with increasing concentration of C_{60}-A and C_{60}-AA up to 10^{-4} M in the liposome suspension. This suggests that C_{60}-A and C_{60}-AA quench the phosphorescence of erythrosine on both the external and internal surfaces of vesicles, which may be the result of liposomal damage or penetration of the given compounds through the lipid bilayer without membrane destruction.

The effect of C_{60}-A and C_{60}-AA on the lipid bilayer in the presence of $CoCl_2$ was studied in order to understand the mechanism of this phenomenon.

As has been mentioned above, Co^{2+} ions are efficient quenchers of excited triplet probes. At the same time Co^{2+} ions do not travel on their own through the lipid matrix of the membrane. $CoCl_2$ was added to 10^{-3} M to the suspension of liposomes, containing the erythrosine molecules embedded in liposomes by the procedure mentioned above (Fig. 3). In this case the phosphorescence of the erythrosine probe molecules absorbed on the external surface of liposomes is completely quenched. Starting with 4×10^{-4} M concentration of $CoCl_2$ the subsequent addition of Co^{2+} does not change the intensity of the erythrosine phosphorescence. The observed phosphorescence accounting for 50% of the starting intensity is determined only by erythrosine molecules, located inside liposomes.

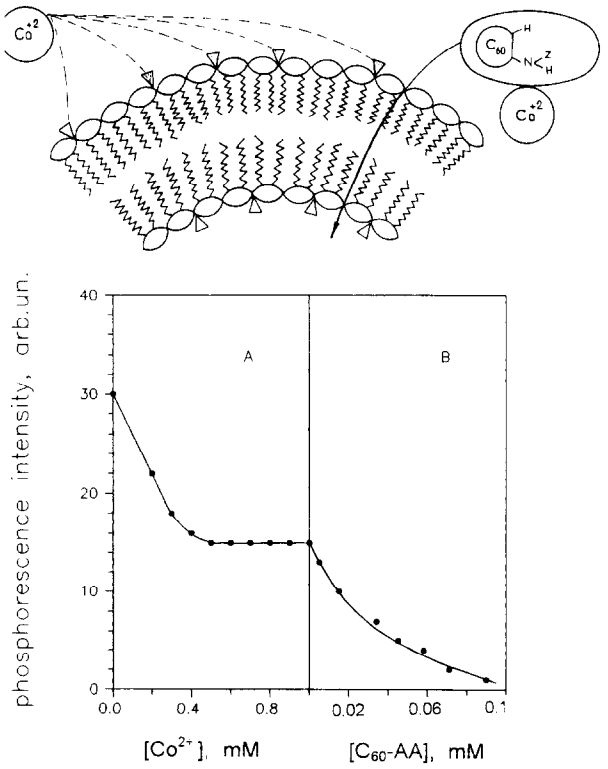

Fig. 3. Phosphorescence intensity dependence of erythrosine in the liposome structure on sequential treatment with Co^{2+} ions (A) and C_{60}-A (B). The phosphorescence intensity was measured at 20 ms after the laser impulse. In the upper scheme the erythrosine molecules are represented as triangles.

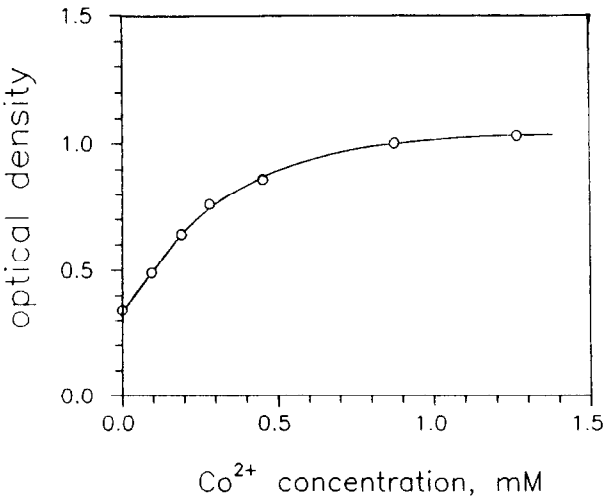

Fig. 4. Effect of Co^{2+} ions on the optical density of C_{60}-A at λ = 270 nm (concentration 1.5×10^{-5}M) in Tris-HCl buffer solution (0.1 M, pH 7).

On subsequent titration of the liposome suspension by C_{60}-A or C_{60}-AA solutions the intensity of erythrosine phosphorescence decreases (Fig. 3). This fact attests to substantial permeability of the lipid bilayer to C_{60}-A and C_{60}-AA without violating liposomal integrity. In the case of the liposome disruption the complete quenching of erythrosine phosphorescence due to Co^{2+} ions transferred inside the liposomes would be observed.

As can be seen from Fig. 4, the absorption spectral intensity of C_{60}-A and C_{60}-AA at λ = 270 nm grows with increasing concentration of Co^{2+} ions. This attests to the formation of complexes of C_{60}-AD with Co^{2+} ions in the solution. It should be noted that in the presence of Co^{2+} the values of K_q for C_{60}-A and C_{60}-AA equal 1.6×10^9 and 1.8×10^9 M^{-1} s^{-1}, respectively. These values are greater than those cited above for the same compounds in the absence of Co^{2+}. This also demonstrates that Co^{2+} ions form complexes with C_{60}-AD, resulting in an increase in the quenching efficiency of the phosphorescence of erythrosine embedded in liposomes as compared with the initial compounds. Seemingly, Co^{2+} ions are transferred through the liposome membrane by the amino acid derivatives of fullerene due to complex formation. In this connection such complexes are efficient quenchers of excited triplet states of erythrosine.

Thus, the studies of the effect of C_{60}-A and C_{60}-AA on the structure and permeability of the lipid bilayer of phosphatidylcholine liposomes allowed us to demonstrate the membranotropic properties of the C_{60}-AD derivatives, their ability to localize in the membrane, to penetrate inside liposomes

through the lipid bilayer and to bring about the activated transport of bivalent transition metal ions.

The observed properties of C_{60}-A and C_{60}-AA suggest that they are capable of affecting cyto- or endoplasmic membranes causing a change in the activity of the membrane-bound enzymes.

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References

[1] Besanson, R.V., Bienvenue, E., Dellinger, M., Leach, S. and Seta, P. (1994) J. Phys. Chem. 98, 3492–3500.
[2] Andrievsky, G.V., Samokhina, L.M., Zhmuro, A.V., Raslyahov, A.D. and Suhach, A.N. (1995) Abstracts of International Workshop on Fullerenes and Atomic Clusters (Grigor'yants, V.G. and Solov'eva, E.A., eds.) p. 172, St. Petersburg.
[3] Savateeva, T.N., Shevtchuk, M.K. and Jakautseni, P.P. (1995) Abstracts of International Workshop on Fullerenes and Atomic Clusters (Grigor'yants, V.G. and Solov'eva, E.A., eds.) p. 173. St. Petersburg.
[4] Tokuyama, H., Yamago, S., Nakamura, E., Shiraki, T. and Sugiura, Y. (1993) J. Am. Chem. Soc. 115, 7918–7919.
[5] Schinazi, R.F., Sybesma, R., Srdanov, G., Hill, C.L. and Wudl, F. (1993) Antimicrob. Agents Chemother. 37, 1707.
[6] Romanova, V.S., Tsyryaphin, V.A., Lyakhovetsky, Yu.I., Parnes, Z.N. and Vol'pin, M.E. (1994) Izv. Akad. Nauk Ser. Khim. 6, 1154–1155.
[7] Vol'pin, M.E., Belavtseva, E.M., Romanova, V.S., Lapshin, A.I., Aref'eva, L.I. and Parnes, Z.N. (1995) Mendeleev Communication 4, 129–131.
[8] Kotelnikova, R.A., Hazeeva, E.V. and Bogdanov, G.N. (1995) Abstracts of International Workshop on Fullerenes and Atomic Clusters (Grigor'yants, V.G. and Solov'eva, E.A., eds.) p. 157, St. Petersburg.
[9] Mekler, V.M., Kotelnikov, A.I., Likhtenshtein, G.I. and Berkovitch, M.A. (1982) Biofizika 27, 641–645.
[10] Kotelnikov, A.I., Fogel, V.R., Mekler, V.M., Kochetkov, V.V. and Likhtenshtein, G.I. (1982) Stud. Biophys. 91, 53–54.
[11] Likhtenshtein, G.I., Kulikov, A.V., Kotelnikov, A.I. and Levchenko, L.A. (1986) J. Biochem. Biophys. Methods 12, 1–28.
[12] Korobov, V.E. and Chibisov, A.K. (1983) Uspekhi Khimii 52, 43–71.
[13] Zeng, Y., Biczok, L. and Linschitz, H. (1992) J. Phys. Chem. 96, 5237–5239.
[14] Dimitrijevic', N.M. and Kamat, P.V. (1993) J. Phys. Chem. 97, 7623–7626.