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Consumption kinetics of *N*-monosubstituted amino acid fullerene C_{60} derivatives in reactions with peroxy radicals in aqueous colloidal solutions

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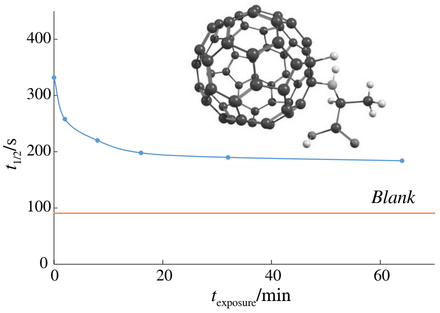
The kinetics of *N*-(monohydrofullerenyl)-L-alanine and *N*-(monohydrofullerenyl)-D-alanine consumption during interaction with alkylperoxy radicals in aqueous colloidal solutions were recorded. The covalent attachment of radicals to the fullerene 'core' was confirmed by IR spectroscopy. It was found that the antiradical activity of fullerene derivatives initially quickly declines but does not further tend to zero, remaining at a significant level for a long time, which is probably due to another inhibition mechanism realized through free radical adsorption on nanoparticles followed by recombination.



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It is known that fullerene C_{60} has a wide range of biological activities, including antioxidant properties.[1–3] However, the insolubility of the C_{60} fullerene molecule limits its use in biological systems. To impart water-soluble properties to this molecule, it can be modified, most often by functionalization through the addition of a water-soluble addend. It was found that various functionalized forms of fullerene C_{60} can inhibit superoxide anion radicals as well as hydroxyl and nitroxyl radicals.[4] However, the mechanism of antioxidant action of fullerene C_{60} and its various derivatives remains under discussion. Thus, some authors suggest that the antioxidant activity of the hydrated form of fullerene C_{60} is associated with an increase in the probability of recombination of free radicals in the surface layers of the hydration shell around the fullerene 'core'. According to the mechanism described for the hydrated form of fullerene C_{60} by Andrievsky *et al.*,[5] it is the ability to order water molecules near the surface that explains its unique antiradical activity. Identical types of free radicals, having specific structures of hydrated shells, are located, absorbed, and concentrated in those areas of the fullerene–water cluster where states of water resembling them in structure and order are present. This leads to an increase in free radical recombination probability with the formation of neutral molecules.[5–9]

Previously,[10] it was shown that the mechanism of antiradical activity of *N*-monosubstituted amino acid fullerene C_{60} derivatives (AAFDs, Figure 1) is not associated with electron donation or hydrogen atom transfer, and these compounds do not catalyze peroxide decomposition. In this work, we investigate whether AAFDs[†] can exhibit antiradical activity by the mechanism proposed by Andrievsky *et al*. When the compounds under consideration can exhibit activity according to the specified mechanism, AAFDs should not be consumed during the inhibition of radicals formed in the chemical model system. In order to find an answer to this question, we conducted a series of experiments to evaluate the consumption kinetics of AAFDs in the reaction with alkylperoxy radicals produced during the decomposition of 2,2′-azobis(2-amidinopropane) (AAPH). Fluorescein was used as a probe sensitive to free radical concentration in the model system. It has very intensive fluorescence even at nanomolar concentrations, but its reaction products with radicals lack fluorescent properties. Fullerene derivatives decrease the equilibrium radical concentration in the solution and, therefore, increase the half-life of fluorescein, which is a function of the fullerene derivative concentration and activity.

As a result of experiments using the colloidal solutions of H-C_{60}-L-Ala-OK and H-C_{60}-D-Ala-OK as inhibitors of free-radical reactions,[‡] it was found that preliminary exposure of

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[†] *N*-(monohydrofullerenyl)-D-alanine potassium salt (H-C_{60}-D-Ala-OK) and *N*-(monohydrofullerenyl)-L-alanine potassium salt (H-C_{60}-L-Ala-OK) were obtained by the one-stage synthesis with a direct attachment of the amino acid residue to the fullerene core according to the method described previously by Romanova *et al.*[11] The samples (water colloidal solutions) used here were the same as in our previous work[12] with the maxima of the nanoparticle size distribution with descending intensity at 2.2, 4.5, 8.5, and 17.5 nm in the case of H-C_{60}-L-Ala-OK and 3.5, 8.5, and 14 nm for H-C_{60}-D-Ala-OK.

[‡] Kinetic curves of the decrease in the fluorescence intensity of the fluorescein over time were measured on a Hitachi F-4010 spectrofluorimeter using a thermostated cuvette holder. The observed fluorescence was detected at a wavelength of 515 nm with an excitation wavelength of 491 nm. The sample temperature was maintained at 37 ± 0.5 °C using a liquid thermostat. Solutions of the fluorescein and 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH) (97% Sigma–Aldrich) were prepared in the

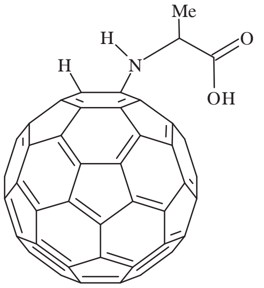

Figure 1 General formula of the studied AAFD.

AAFD to a medium with constant generation of free radicals for 16 min just before the fluorescein addition leads to a significant decrease in AAFD inhibitory activity determined by the fluorescein half-life measure (Figure 2). It is obvious that the consumption of AAFD in reactions with free radicals is due to the covalent addition of alkylperoxy radicals to fullerene and is not related to the mechanism of increasing the probability of radical recombination described by Andrievsky *et al.*[5] However, with a further increase in the AAFD exposure time before introducing the fluorescein into the reaction system, its half-conversion time does not decrease to the level of the control experiment performed without inhibitor addition into the reaction system. It is obvious that the antiradical activity mechanism, including the addition of free radicals to double bonds, is not the only one for these compounds. Fullerene-derived nanoparticles may probably act as nanowalls where absorption of free radicals takes place, accompanied by an increase in their recombination.

The initial AAFD concentrations in the reaction medium were chosen to reach the comparable fluorescein half-life values in both cases of fullerene derivatives. As stated earlier, fullerene C_{60} and its various derivatives, including AAFDs, are not true solutions in water, but colloidal systems, and their biological and chemical properties are strongly dependent on the size of nanoparticles. It was shown previously that a smaller average AAFD nanoparticle size correlates with a higher antiradical activity.[10] This explains the fact that the H-C_{60}-D-Ala-OK sample demonstrates activity comparable to that of H-C_{60}-L-Ala-OK only at a concentration significantly exceeding that of H-C_{60}-L-Ala-OK.

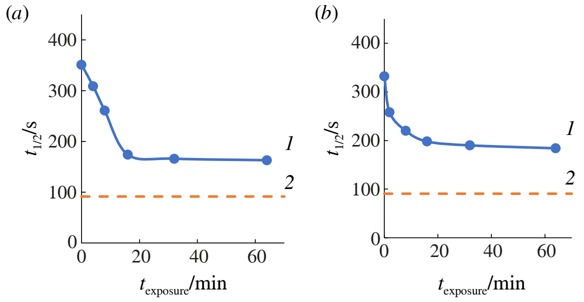

Figure 2 (*1*) Dependence of the fluorescein half-life on the incubation time of AAFDs with 9.6 mmol dm^{-3} AAPH at 37 ± 0.5 °C before fluorescein addition for (*a*) 3.1 μmol dm^{-3} H-C_{60}-L-Ala-OK and (*b*) 32.8 μmol dm^{-3} H-C_{60}-D-Ala-OK; (*2*) fluorescein half-life in the reaction with AAPH-produced peroxy radicals without inhibitor addition.

The conclusion that at least one of the AAFD antiradical activity mechanisms in aqueous colloidal solutions is related to direct radical addition to the double bonds in the fullerene 'core' is consistent with the data available in the literature on the interaction of fullerene C_{60} and its derivatives in the organic phase with various free radicals. For instance, the EPR method with a ^{13}C spin trap showed that benzyl radicals can be added to a C_{60} fullerene molecule in the toluene medium.[13] The existence of at least two intermediate radical adducts was found upon the addition of 3 benzyl radicals (R_3C_{60}•) and 5 benzyl radicals (R_5C_{60}•), which are stable at 50 °C. It was assumed that up to 15 benzyl radicals can be added to one fullerene molecule. It was also found by mass spectrometric methods that one fullerene molecule can add up to 34 methyl groups. In the work of Galimov *et al.*,[14] the product $C_{60}(OOC(CH_3)_2Ph)_2$ of the interaction of fullerene C_{60} with radicals formed during the liquid-phase oxidation of cumene was discovered and identified using the HPLC–ESI MS method.

Most experiments on radical reactions involving fullerene C_{60} and its various derivatives were conducted in organic solvents. However, there are only a few published data on water-soluble derivatives, which is probably due to the difficulties in detecting the products of radical interaction with water-soluble fullerene derivatives. For example, in the case of the derivatives used in this work, it was not possible to detect the reaction products of AAFDs with alkylperoxy radicals using MALDI-TOF MS and ESI MS, probably due to decomposition of the reaction products upon ionization and interaction of compounds with the substrate in MALDI-TOF, or lack of fullerene ionization in ESI MS.

However, it was possible to obtain confirmation of covalent radical attachment using IR spectroscopy.[§] Figure 3 shows the IR spectra of the azoinitiator AAPH before and after its partial thermal decomposition. No significant differences between these two spectra are observed, probably due to the low degree of AAPH decomposition under the selected conditions. However, it is possible to distinguish the appearance of several new absorption peaks (Figure 3, curve *2*): the peak at 3450 cm^{-1} may be related to the stretching vibrations of the N–H group of the pyrrole ring formed during the radical recombination reactions,[15] and the peak at 1590 cm^{-1} is probably referred to the vibrations of the pyrrole ring itself. The appearance of absorption peaks at 1166 and 1152 cm^{-1} can be attributed to the stretching vibrations of the C–O bond in either the C–O–O or C–O–H fragment, and the weak peak near 864 cm^{-1} can be attributed to the stretching vibrations of O–O in the peroxyl products of the oxygen pathway of radical recombination. Also, a decrease in the intensity of the peak

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phosphate buffer (75 mM, pH 7.4). To investigate the change in the antiradical activity of AAFDs, the AAPH solution was first placed in the cuvette and kept at 37 °C for 10 min until the equilibrium concentration of radicals in the system was established; then, the AAFD aqueous solution was added, and the fluorescein was introduced either immediately after AAFD, or after 2, 4, 8, 16, 32, and 64 min, keeping the sample with AAPH. The kinetic curve of the fluorescence intensity was recorded immediately after adding the fluorescein solution. The final concentrations of the mixture components in the cuvette were 9.6 mmol dm^{-3} (AAPH), 3.74 × 10^{-8} mol dm^{-3} (fluorescein), and 3.1 μmol dm^{-3} of H-C_{60}-L-Ala-OK and 32.8 μmol dm^{-3} of H-C_{60}-D-Ala-OK. At this initiator concentration and temperature, the rate of radical formation is 4.12 × 10^{-9} mol dm^{-3} s^{-1}.

[§] To prepare a sample of the products of AAFD interaction with alkylperoxy radicals, 1 ml of 2.73 mM solution of H-C_{60}-D-Ala-OK in distilled water was mixed with 0.2 ml of 0.45 mM solution of AAPH in distilled water; the mixture was stirred and placed into a thermostat at 65 °C for 3 h. Upon completion, a brown precipitate was formed. This precipitate was washed by centrifugation, decanting the supernatant, adding distilled water to the previous volume, and subsequent centrifugation. Washing was repeated 15 times. For water removal, finally acetone was added to the precipitate; after shaking, centrifugation, and decantation, the precipitate was dried at room temperature.

A solid AAFD sample for IR spectroscopic assay was obtained by evaporation of 3 ml of 2.73 mM H-C_{60}-D-Ala-OK solution in distilled water at room temperature.

To prepare the AAPH sample containing its thermal decomposition products, 1 ml of 0.45 mM AAPH solution in distilled water was placed in a thermostat for 3 h at 65 °C. Then it was dried at room temperature.

IR absorption spectra of samples pressed in NaCl tablets were recorded by a Perkin–Elmer 1760X spectrometer; the spectra were processed in the OPUS 7.2 program (Bruker).

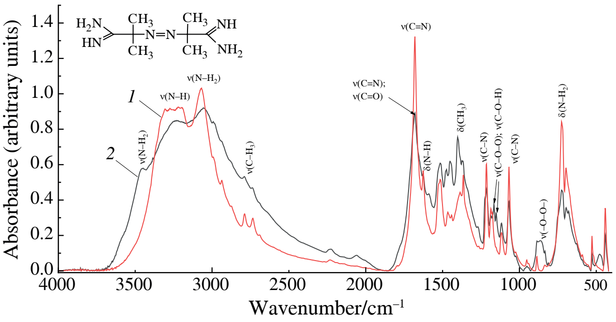

Figure 3 IR spectra of AAPH azoinitiator (*1*) before thermal decomposition and (*2*) after partial thermal decomposition at 65 °C for 3 h.

at 1680 cm^{-1}, corresponding to the stretching vibrations of the C=N bond, can be explained by partial hydrolytic deamination of AAPH and its products.[15] Additionally, the broadening of the 1680 cm^{-1} peak indicates the C=O bond formation, consistent with the thermal decomposition products reported by Werber *et al.*[15]

Figure 4 shows the IR spectra of H-C$_{60}$-D-Ala before and after interaction with alkylperoxy radicals formed during the thermal AAPH decomposition. In the IR spectrum of H-C$_{60}$-D-Ala (Figure 4, curve *1*), the absorption peaks at 1244, 1100, 894, 880, and 842 cm^{-1} are due to vibrations of the fullerene fragment; the peaks at 2920 and 2852 cm^{-1} are attributed to CH and CH$_3$ groups of the amino acid substituent; the peak at 1734 cm^{-1} is due to the carbonyl group of the amino acid fragment; and the peak at 1624 cm^{-1} is due to the carboxyl anion.[16] After repeated washing of the products of AAFD interaction with alkylperoxy radicals (15 times), the IR spectra (Figure 4, curve *3*) show the preservation of peaks attributed to the functional groups of azoinitiator AAPH, which indicates the covalent attachment of radicals to the fullerene core. At the same time, the preservation of the absorption peaks at 2920 and 2852 cm^{-1} indicates that the amino acid fragment remains linked to fullerene.

Thus, the investigated water-soluble *N*-monosubstituted amino acid derivatives of fullerene C$_{60}$ exhibit antiradical activity, the greatest contribution to which is made by the addition of radicals to numerous double bonds of the fullerene ‘core’. However, the complex nature of the dependence of antiradical activity of fullerene derivatives on the time of preliminary exposure in a medium with constant generation of alkylperoxy radicals indicates the existence of an alternative mechanism of inhibiting free radicals. We assume that fullerene derivatives in aqueous colloidal solutions may act as ‘nanowalls’ on which the death of radicals occurs as a result of their adsorption, leading to an increased probability of recombination.

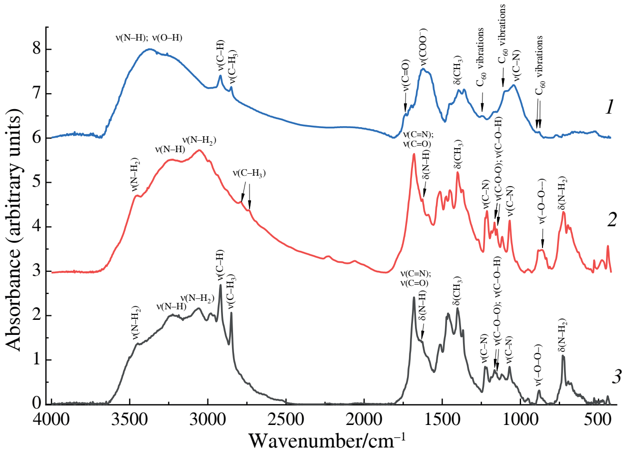

Figure 4 IR spectra of (*1*) H-C$_{60}$-D-Ala before reaction with alkylperoxy radicals; (*2*) AAPH after thermal decomposition at 65 °C for 3 h (absorbance is multiplied by 3 for ease of data presentation); (*3*) H-C$_{60}$-D-Ala after exposure to alkylperoxy radicals at 65 °C for 3 h (absorbance is multiplied by 30 for ease of data presentation).

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