**Electronic supplementary materials** *Mendeleev Commun.*, 2025, **35**, 589–591

# Fullerene $C_{60}$ conjugated with L- and D-alanine: integration into lipid bilayer and protection of cell membrane against oxidative stress

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## Synthesis of AAFDs

*N*-monosubstituted amino acid fullerene $C_{60}$ derivatives (AAFDs) with α-amino acids L- and D-alanine (Acros Organics 99+%) were prepared by one-step synthesis by direct addition of the amino acid residue to the fullerene $C_{60}$ molecule by the method described previously by Romanova *et al.*[S1]

## Materials and methods

All lipids for experiments were obtained from Avanti Polar Lipids (Alabaster, AL, USA): 1,2-dioleoyl-*sn-glycero*-3-phosphocholine (DOPC), 1,2-dioleoyl-*sn-glycero*-3-phospho-L-serine (sodium salt) (DOPS), 1,2-dioleoyl-*sn-glycero*-3-phosphoethanolamine (DOPE), and cholesterol (Chol). Chloroform (>99.0%) from Sigma-Aldrich (St. Louis, MO, USA) was used as a solvent for the lipids. KCl (Sigma-Aldrich, Saint-Louis, MO, USA), MES (Sigma-Aldrich, Saint-Louis, MO, USA) were dissolved in deionized water with a resistivity of 18.2 MΩ cm to use as a working buffer (WB, pH 7.0).

To obtain a lipid stock solution, lipids DOPC, DOPS, DOPE, cholesterol were dissolved in chloroform to a final concentration of 10 g/L. To prepare lipid films all components were combined in a molar ratio 40:30:10:20 in glass round bottom flask. Then this mixture was evaporated under vacuum at 37 °C for 40 min. The resulted lipid film was then dissolved in a WB to a final concentration of 1 g/L. For small vesicle formation the lipid suspension in the vial was sonicated for 20 min while maintaining a temperature of 55 °C. To obtain liposomes with AAFDs the prepared lipid films was dissolved in mixture of water solution of AAFDs and WB in a ratio of 1:1 (v/v). The vesicle suspension was used immediately after preparation.

## Atomic force microscopy

AFM experiments were performed with a Multimode Nanoscope V (Bruker, Billerica, MA, USA) setup using an electrochemical fluid cell and ultra-sharp SiN3 cantilevers SNL-10 (Bruker, Billerica, MA, USA) with a tip radius of about 2 nm and nominal spring constant of 0.06 N/m. A 100 μL aliquot of the liposome or liposome/AAFDs suspension was applied to freshly cleaved mica and incubated for 15 min to allow the formation of a lipid film on the mica surface. The resulted supported lipid bilayer was then washed seven times with WB. Subsequently, 111 μL of the aqueous solutions of fullerene adducts with L-alanine ($C = 7.67 \times 10^{-6}$ M) and D-alanine ($C = 2.73 \times 10^{-6}$ M) were applied to the substrates containing the lipid bilayer (adsorption was carried out only for a pure lipid mixture). These substrates were then examined by atomic force microscopy to observe the adsorption of nanoparticles onto the lipid bilayer.

Similarly, aqueous solutions of AAFD were applied to the mica substrate without a deposited lipid bilayer, and the adsorption of AAFD nanoparticles on the substrate was observed using atomic force microscopy.

All images were scanned at dimensions of 3×3 μm$^2$ and subsequently processed using WSxM software.[S2]

## Fluorescent probe method

A 0.8 mL sample of erythrocytes was collected from blood obtained from a healthy donor. The erythrocytes were separated into components by centrifugation and washed with isotonic PBS (pH 7.40) using the following procedure: the erythrocytes were resuspended in PBS to a total volume of 1.5 mL, centrifuged for four minutes at 8000 rpm, and the supernatant was decanted. The erythrocyte pellet was then resuspended in PBS. This process was repeated until the supernatant became transparent after centrifugation, ensuring the erythrocyte mass was free of hemolyzed cells. A suspension of erythrocytes in PBS was prepared with a hematocrit of 0.02%.

A pyrene solution in ethanol was prepared at a concentration of 35 mM. Subsequently, 0.4 mL of this pyrene solution was diluted in 41.1 mL of PBS to obtain a working solution of pyrene in PBS.

For the experiment, 2.7 mL of the erythrocyte suspension was transferred to a cuvette. The studied AAFDs were added to the suspension and incubated for 15 min at 25 °C. Then, 43 μL of the pyrene working solution in PBS was added to the cuvette to achieve a final pyrene concentration of 5 μM. The total volume was adjusted to 2.9 mL with isotonic PBS. Before recording the fluorescence spectrum, pyrene was incubated with the erythrocytes and the fullerene derivatives for 10 min.

Fluorescence spectra were recorded using a Perkin Elmer LS-50 spectrofluorimeter at 25 °C. The fluorescence intensity of pyrene was measured at excitation wavelengths of 280 nm and 337 nm. As a control, the fluorescence of pyrene in erythrocytes was recorded in the absence of the studied fullerene derivatives.

**AAPH-induced hemolysis of human blood erythrocytes**

A suspension of erythrocytes was prepared in isotonic PBS with a hematocrit of 0.2%. To induce hemolysis, the water-soluble azo initiator 2,2'-azobis(2-methylpropionamidine) dihydrochloride (AAPH) was used as an oxidative stress inducer. A solution of AAPH in PBS was prepared at a concentration of 350 mM, resulting in a final concentration of 50 mM in the cuvette.

For the experiment, 2.9 mL of the erythrocyte suspension was transferred to a cuvette. The studied AAFDs were added to the suspension to achieve a final concentration of 10 μM. The total volume in the cuvette was adjusted to 3.0 mL, and the mixture was thermostatted for 15 min at 37 °C. Simultaneously, the AAPH solution was thermostatted for 15 min at 37 °C. After incubation, 500 μL of the AAPH solution was added to the cuvette containing the erythrocyte suspension and the fullerene derivatives. The kinetic curves of the change in optical density were immediately recorded with periodic stirring, and measurements were taken every 10 min. The temperature was maintained at 37 °C throughout the experiment using a cuvette compartment thermostatted at 37 ± 0.2 °C.

Hemolysis was monitored by measuring the decrease in optical density of the erythrocyte suspension at a wavelength of $\lambda$ = 650 nm using a Specord M40 spectrophotometer. A control experiment was performed in the absence of the fullerene derivatives.

**References**

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