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FULLERENES, NANOTUBES AND CARBON NANOSTRUCTURES
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Synthesis of hybrid nanostructures based on *e*-carboxy-dihydroxycobinamide and *N*-(monohydrofullerenyl)-L-fluorophenylalanines

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ABSTRACT

Mono-derivatives of fullerene C_{60} with L-phenylalanine, *o*-fluoro-L-phenylalanine and *p*-fluoro-L-phenylalanine, as well as their hybrid nanostructures with derivatives of vitamin B_{12}, have been synthesised. The structure of the compounds was determined by IR, UV and CD-spectroscopy. The IR spectra of the obtained compounds contain all bands characteristic of the original compounds, with the exception of the band at 1227 cm^{-1}, which is related to the free carboxyl group in the fullerene fragment. In the UV spectra of hybrid nanostructures contain smoothed peaks in the region of 360 nm, confirming the presence of corrin ligand in the original and obtained compounds. Using the CD method, it was found that all spectra have a characteristic maximum in the region of 430 nm, confirming the presence in all hybrids nanostructures of a fragment of vitamin B_{12}: *e*-COOH-Cbi$(OH)_2$. The radiuses of nanoparticles of hybrid nanostructures, calculated by the dynamic light scattering method, range from 90 to 120 nm. The degree of association in the complexes ranges from 400 to 600 thousand molecules. It was shown that the resulting hybrid nanostructure retains catalytic activity in the autoxidation reaction of ascorbic acid, inherent in derivatives of vitamin B_{12}.

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1. Introduction

Recently, the attention of researchers has been attracted by the use of nanostructured agents in biology, medicine and biotechnological processes, which can exhibit unexpected effects on biological objects[1].

It is known that fullerene derivatives have a wide range of biological activities[2,3]. It is due to the unique structure of the carbon frame and its physicochemical properties[4,5]. Water-soluble C_{60} derivatives exhibit antioxidant, membranotropic, neuroprotective, antiviral, antibacterial and antitumour properties, and also act as effective low-toxic means of drug delivery to targets for various diseases[1,6].

The creation of biological products based on fullerenes is difficult due to the extremely low solubility of fullerene-containing compounds in water. The solution to this problem is associated with the production of water-soluble forms, in particular, functionalised water-soluble fullerenes. However, as follows from[6], when obtaining fullerenols by various methods, complex mixtures of products with a poorly defined structure are formed, differing in solubility and biological effects, which is the reason for poor data reproducibility.

We were the first to develop a method for the synthesis of monoamino acid and peptide derivatives of fullerene Previously, we have proposed a method for the synthesis of monoamino acid and peptide derivatives of fullerene (ADF)[7]. According to this technique, fullerene was modified by attaching an amino acid/peptide to the fullerene core by breaking the double bond of the fullerene and forming a bond with the amino group[8–10]. It has been established that these compounds do not exhibit toxicity, are easily excreted from the body and have various types of biological activity.

Our group and colleagues have been conducting research on the biological activity of natural vitamin B_{12} derivatives for a number of years (Figure 1). As a result, the effectiveness of the approach to method of catalytic therapy of oncological diseases proposed by Academician Volpin using the "Askor" combination, consisting of catalytically active hydroxocobalamin and ascorbic acid as a natural reducing agent, was confirmed. Vitamin B_{12} derivatives, according to the approach are capable of accumulating in tumour tissue and catalysing the formation of reactive oxygen species (ROS) there, which causes the death of cancer cells[11].

It is known that cobalamins can be used as a means of delivering drugs to a tumor[12]. However, the vitamin B_{12} molecules do not pass through cell membranes well. This can be compensated by attaching it to a hydrophobic compound, for example, a C_{60} fullerene derivative. Previously, we showed the possibility of obtaining new compounds with potential biological activity based on the conjugate of ADF and vitamin B_{12} derivatives[13] which have catalytic activity and are capable of forming reactive oxygen species (ROS)[14]. Indeed, fullerene derivatives not only have a

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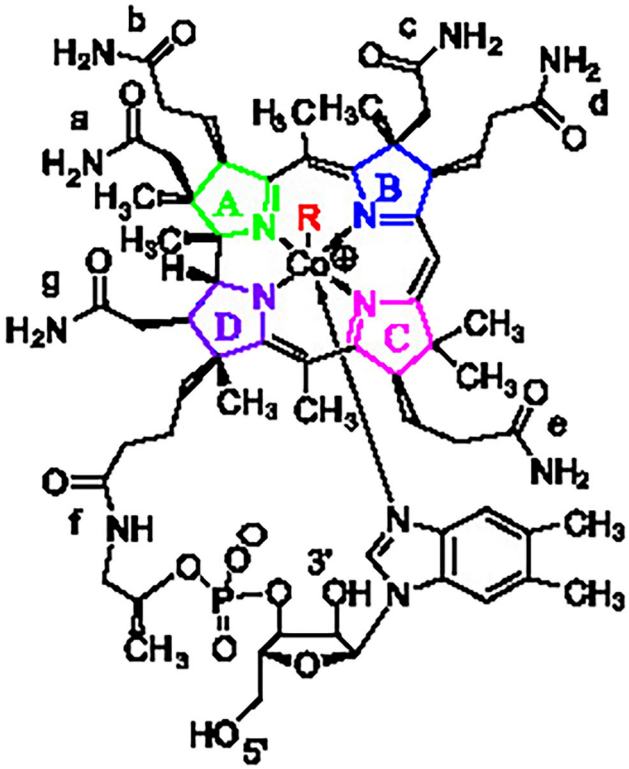


Figure 1. General chemical structure of natural vitamin B12 derivatives.

variety of biological activities, but also serve as a means of delivering drugs to targets. Thus, a fullerene conjugate with ε-aminocaproic acid was used for selective delivery of hexamethonium, since the C_{60} derivative formed a complex with it based on ion-pair interaction[15].

It is known that the manifestation of the biological activity of the resulting conjugate depends on the type of the amino acid included in the composition[9].

The introduction of a fluorine atom into an amino acid molecule usually imparts new biological properties to the final product[16–19]. Therefore, to expand the range of these properties of ACE, close analogues of L-phenylalanine, namely *o*-fluoro-L-phenylalanine and *p*-fluoro-L-phenylalanine were used as starting compounds for the synthesis of water-soluble derivatives of C_{60} fullerene.

Thus, the purpose of this work is to develop a synthesis method, obtain and study the properties of new hybrid nanostructures based on L-phenylalanine derivatives of fullerene C_{60} and a derivative of vitamin B_{12} *e*-carboxydihydroxycobinamide.

2. Materials and methods

IR spectra were measured on a Bruker Tensor 37 Fourier spectrometer in the range 4000–400 cm^{-1} for samples in the form of tablets with KBr. CD spectra were obtained on a SKD-2 dichrograph (joint development of the IMB and IS RAS) in the wavelength range 200–600 nm at 23 °C. All measurements were carried out in a 1 cm thick quartz cuvette with a spectral resolution of 3 nm, acquisition time of 2.4 s and scanning speed of 35 nm/min. The intensity of the extremes of the Cotton effect (EC) in the CD spectra was calibrated using an aqueous solution of D-10-camphorsulfonic acid. In all figures, dichroism values are given in ΔA (a value expressing the difference in light absorption corresponding to right and left circular polarization). UV–Vis spectra were recorded on a V-780 spectrophotometer (Japan) in 1 cm quartz cuvettes in aqueous solutions containing $5{\cdot}10^{-5}$ mol of the test substance at 23 °C. Wavelength range 220–700 nm. Scanning speed 400 nm/min, scanning interval 0.5 nm.

Catalytic activity in the oxidation reaction of ascorbic acid was studied on a V-780 spectrophotometer (Japan) in 1 cm quartz cuvettes in 0.05 M phosphate buffer at pH 6.0 at room temperature. The working wavelength was 262 nm, the number of cycles was 5. The substrate: complex ratio was 10:1, and the working concentrations of substrate and complex were $5{\cdot}10^{-5}$ M and $5{\cdot}10^{-6}$ M, respectively.

2.1. Laser dynamic light scattering method

The method of laser dynamic light scattering (photon correlation spectroscopy) was used to determine the hydrodynamic radius of ASK-53 particles dispersed in an aqueous solution at a concentration of 100 μg/ml. The measurements were carried out on a "Photocor Compact-Z" analyzer (Russia), equipped with a thermally stabilised AlGaInP diode laser with a wavelength $\lambda = 637.4$ nm and a power of 30 mW, with a built-in multichannel correlator "Photocor-FC", obtaining a correlation function of fluctuations in the intensity of scattered light and the integral scattering intensity. The correlation function was processed using DynaLS software from Alango Ltd (Israel)[20,21]. The range of permissible measurable sizes of nanoparticles is from fractions of a nm to 5-10 microns.

An aqueous solution of 0.420 g (2.07 mmol) of the potassium salt of L-phenylalanine or 0.449 g (2.07 mmol) of its fluorine derivatives was added to a solution of 0.03 g (0.0414 mmol) of fullerene in 5 ml of ***o***-dichlorobenzene and 0.540 g (2.07 mmol) 18-crown-6. The reaction mass was stirred for 8 hrs at 60 °C. Then the solvents were distilled off, the residue was treated with a saturated solution of KCl, and the residue of the fullerene derivative was washed with water. The yields of compounds **1, 2, 3** were 0.0281 g (93.7%), 0.0278 g (92.7%), 0.0284 g (94.6%), respectively. The resulting *N*-(monohydro)fullerene-L-phenylalanine acids are soluble in dimethyl sulfoxide, dimethylformamide, and pyridine.

The synthesis of *e*-COOH-Cbi$(OH)_2$ (4) was carried out according to the procedure described in[14].

To a solution of 0.0378 g ($3.64{\cdot}10^{-5}$ mol) of compound **4** in 5 ml of DMF was added 0.0290 g ($3.64{\cdot}10^{-4}$ mol) of ethylene chlorohydrin and 0.0091 g ($4.37{\cdot}10^{-5}$ mol) (1.2-fold excess) dicyclohexylcarbodiimide (DCC). The mixture was stirred for 2 days at room temperature. Dialysis was performed against chloroform for two days. Then 0.0491 g ($5.46{\cdot}10^{-5}$ mol) (1.5-fold excess) of *N*-(monohydro-fullerenyl)-L-phenylalanine methyl ester or 0.050 g ($5.46{\cdot}10^{-5}$ mol) (1.5-fold excess) of its fluorine derivatives in 5 ml of pyridine and the mixture was stirred at room temperature for

8 hrs, then left overnight. Next, the solution was dialysed against water. Excess *N*-(monohydrofullerenyl)-L-phenylalanine methyl ester and the resulting dicyclohexylurea precipitated, and unreacted *e*-carboxy-dihydroxycobinamide penetrated the dialysis bag, turning the water pink. Dialysis was carried out for two days until the dialysis water was completely discoloured. The solution was then removed from the dialysis bag and the precipitate was filtered off. An aqueous solution of compounds **5, 6, 7** was obtained with yields of 0.0354 g (93.7%), 0.0348 g (92.1%), 0.0350 g (92.6%), respectively.

3. Results and discussion

3.1. Preparation of N-amino acid derivatives of fullerene

The first stage of the work was the synthesis of hybrid nanostructures of fullerene with L-phenylalanine, *o*-fluoro-L-phenylalanine and *p*-fluoro-L-phenylalanine (**1–3**). L-Phenylalanine derivatives of fullerene C_{60} were synthesised (Scheme 1) according to the method we previously proposed[7].

The structure of the obtained derivatives was studied using IR spectroscopy methods. A characteristic feature for amino acid derivatives of fullerene is the appearance in the spectra of a group of third bands, which are attributed to vibrations in the C_{60} molecule, where substitution occurs[22]. The wavelengths of this triad (about 1108 cm^{-1}, about 960 cm^{-1}, about 840 cm^{-1}) are the same in all amino acids derivatives of fullerene. Their position and intensity depend little on the structure of a particular amino acid. All spectra (Figures 2–4) contain strong absorption bands in the region of 1590–1620 cm^{-1} and 1350–1420 cm^{-1}. Such absorption bands are characteristic of asymmetric and symmetric stretching vibrations of the carboxyl anion, which confirms the presence of an amino acid fragment in the molecule, where the vibrations of the C_{60} molecule as an integral part of the amino acid derivative of fullerene are of particular interest.

3.2. Preparation of hybrid nanostructures

The next step was to combine the previously obtained nanostructures of *N*-(monohydrofullerenyl)-L-phenylalanines and a vitamin B_{12} derivative, *e*-carboxy-dihydroxycobinamide **4**. Since complex **4** contains several free hydroxyl groups in its structure, the addition cannot be carried out using thionyl chloride. Therefore, a scheme using an ethylene spacer and DCC as a condensing agent was chosen (Scheme 2).

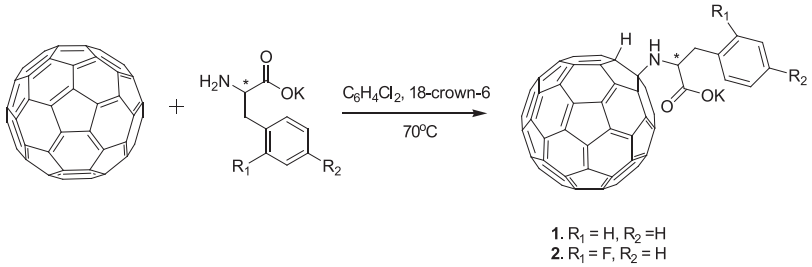
Isolation of the resulting products was carried out by dialysis against chloroform. Dialysis of the reaction mixture against water turned out to be impossible, since along with the excess ethylene chlorohydrin, the resulting product also penetrated through the dialysis bag. In this case, $Cbi(OH)_2$-*e*-$C(O)OCH_2CH_2Cl$ remained in the dialysis bag, and the excess ethylene chlorohydrin left along with the solvent. An excess of the corresponding compound **1–3** in pyridine was then added to the solution and stirred at room temperature for 8 hrs. Conjugates **5–7** were dialysed against water. Excess ACE methyl ester and dicyclohexylurea precipitated. The final hybrid nanostructure remained in water. Dialysis was carried out until complete discolouration. The solution was then removed from the dialysis bag and the precipitate was filtered off. The substances were obtained in solution with a yield of more than 90%.

3.3. Study of the structure of hybrid compounds

The structures of the obtained derivatives were studied using IR, UV spectroscopy, circular dichroism (CD), and the sizes of nanoparticles were determined by dynamic light scattering. The IR spectra of compounds **5–7** (Figures 5–7) contain almost all the bands characteristic of compound **2**, with the exception of the band at 1227 cm^{-1}, which belongs to the free carboxyl group. Also in the spectrum of *e*-COOH-$Cbi(OH)_2$ the following characteristic features are observed: intense broad bands at 3314 cm^{-1} with a shoulder at 3187 cm^{-1}, which confirms the assignment of other bands to the stretching vibrations of the OH group of compound **2**. In addition, in its spectrum an intense broad band is observed at 1661 cm^{-1}. Almost the same band is present in the spectra of hybrid nanostructures: **5**—1627 cm^{-1}, **6**—1663 cm^{-1}, **7**—1663 cm^{-1}, it can be attributed to the stretching vibrations of the CO group of the *e*-COOH-$Cbi(OH)_2$ molecule. In the spectra of compounds **6** and **7** in the same region of carbonyl vibrations, in addition to the band at 1663 cm^{-1}, there is also a weak shoulder at 1702–1709 cm^{-1}, which can be attributed to the ester group of the fullerene fragment.

Using the circular dichroism method, it was found that all spectra have a characteristic maximum in the region of 430 nm, confirming the presence of a fragment of vitamin B_{12}—*e*-COOH-$Cbi(OH)_2$ in all hybrid nanostructures (Figures 8–11).

The dynamic light scattering method makes it possible to determine the size of particles in a solution[20,21]. As can be



1. R_1 = H, R_2 = H
2. R_1 = F, R_2 = H
3. R_1 = H, R_2 = F

Q3 **Scheme 1.** Synthesis of fullerene-L-phenylalanine acids (1–3).

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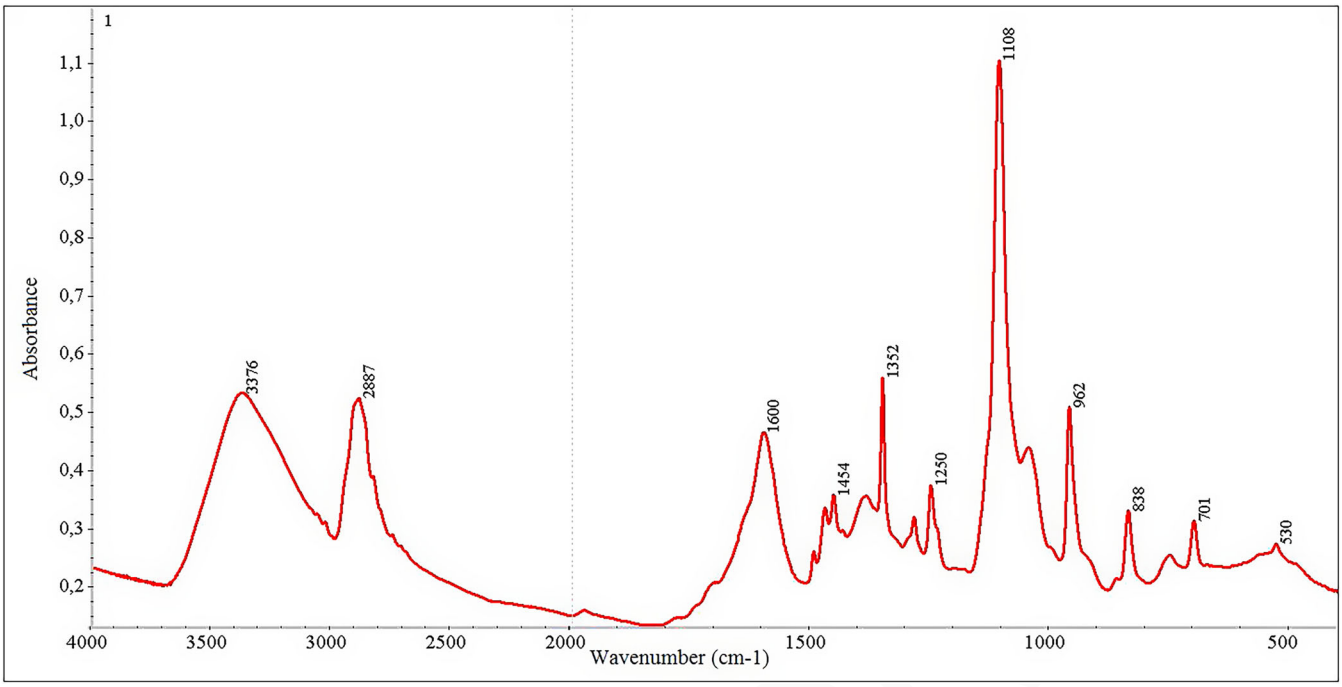

Figure 2. IR-spectra of *N*-(monohydrofullerenyl)-L-phenylalanine (**1**).

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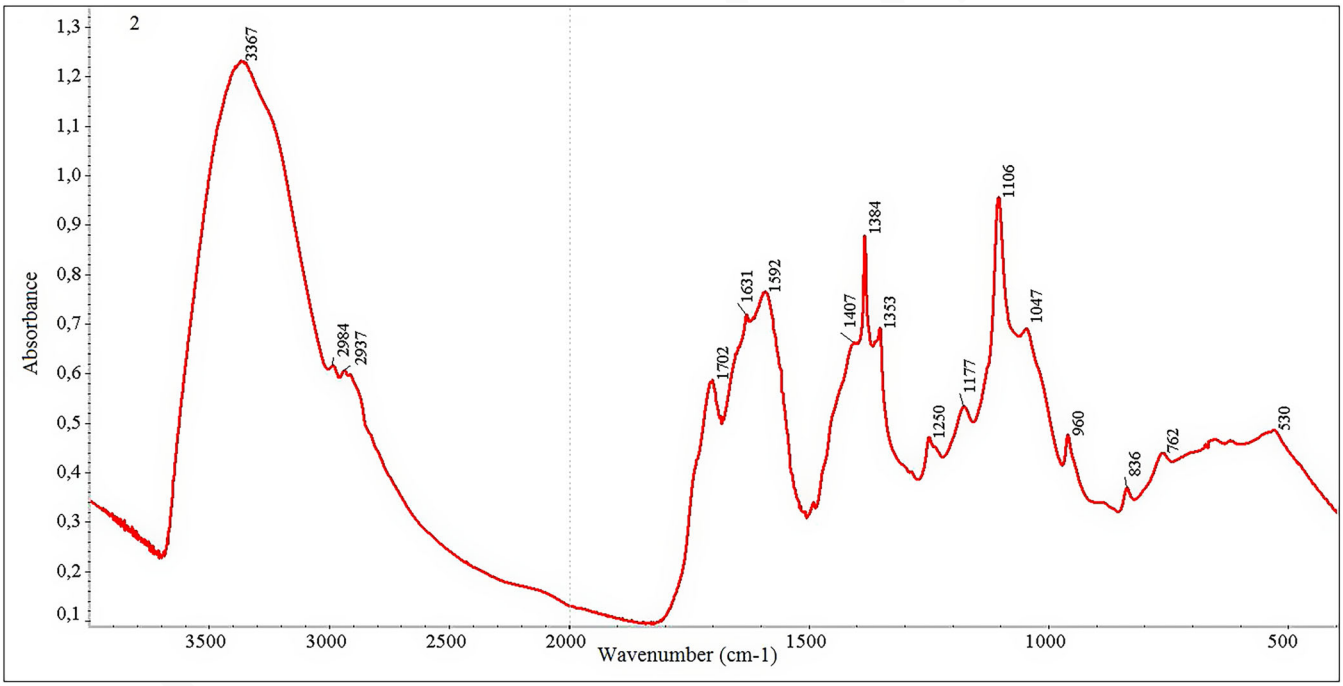

Figure 3. IR-spectra of *N*-(monohydrofullerenyl)-*o*-F-L-phenylalanine (**2**).

seen from the data obtained (Figures 12–14), the most associated complex was with the *o*-fluoro derivative of L-phenylalanine **6** (Figure 13), while the conjugate with L-phenylalanine **5** was the least associated (Figure 12). Based on previously obtained data using diffusion, electron and tunnel microscopy methods[23–25], which showed that associates of fullerene derivatives in solutions have a shape close to a sphere, it is possible to calculate the number of molecules in the resulting associates. The particle radii of the resulting compounds range from 90 to 120 nm. The degree of association in the least associated complex is about 400 thousand molecules in the per associate (Figure 12), and in the most associated (Figure 13) it is about 600 thousand. Thus, the presence of fluorine in the amino acid fragment enhances the association of the corresponding conjugates.

3.4. Study of the catalytic properties of hybrid compounds in autoxidation of ascorbic acid

The results of studying the oxidative transformation of biological substrates under the influence of cobalt corrin complexes, in particular derivatives of natural vitamin B_{12}, indicate

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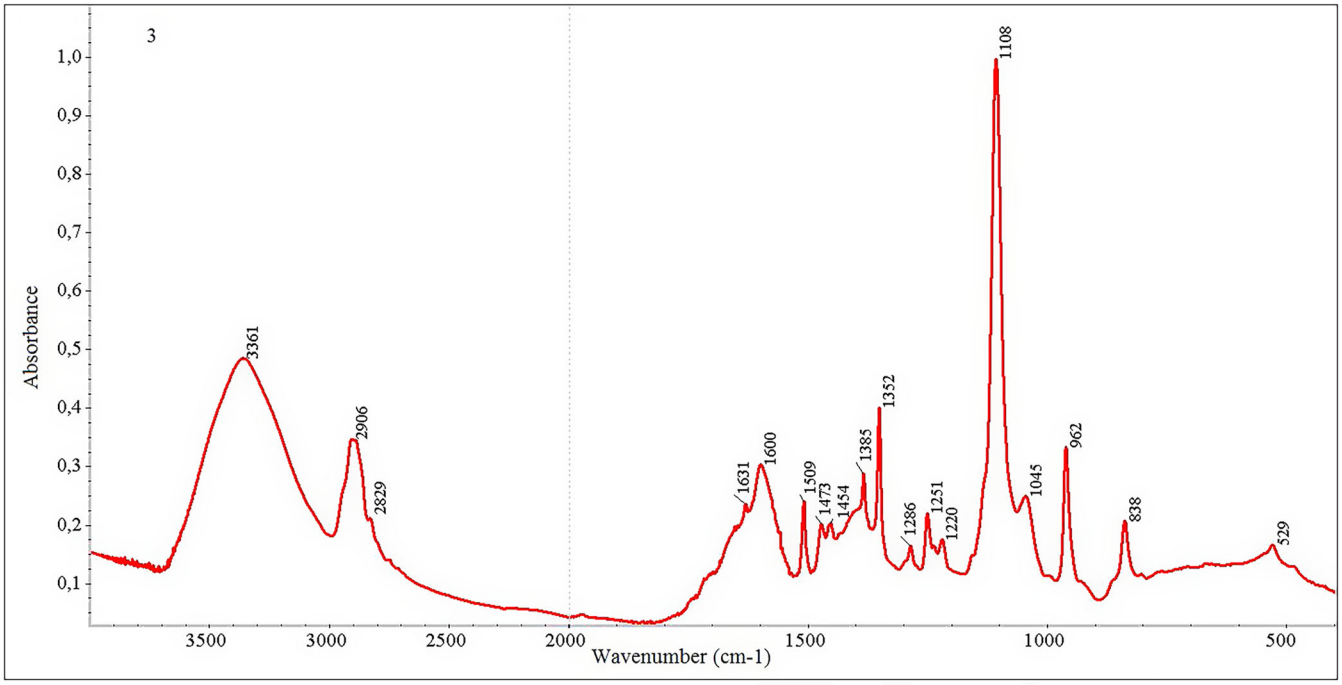

Figure 4. IR-spectra of *N*-(monohydrofullerenyl)-*p*-F-L-phenylalanine (**3**).

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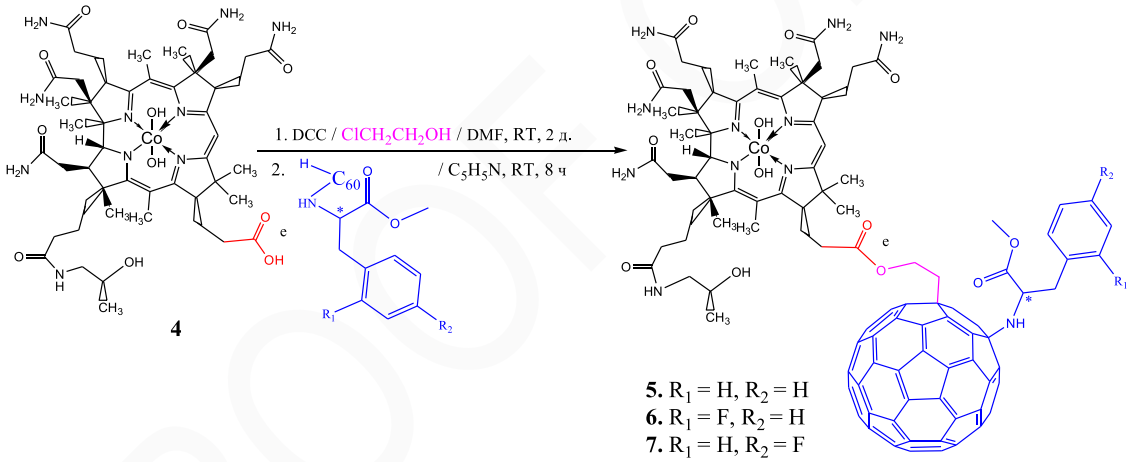

Scheme 2. Synthesis of *N*-(monohydrofullerenyl)-L-phenylalanine and *e*-carboxy-dihydroxycobinamide derivatives (**5–7**).

the possibility of using the latter as catalytic generators of reactive oxygen radicals (ROS), capable of damaging cellular targets[26]. The consideration of the catalytic activity of cobalt corrin complexes in the processes of oxidative cleavage of nucleic acids deserves special attention. Similar processes underlie mutagenesis caused by DNA destruction. To elucidate the mechanism of oxidative DNA destruction in living organisms, a model approach using chemical systems and, in particular, transition metal complexes as chemical sources of ROS is useful. It was shown[27] that a number of coordination compounds are capable of inducing oxidative cleavage of the phosphodiester bond of DNA in the presence of molecular oxygen and a reducing agent; photolysis of DNA in the presence of porphyrins and their metal complexes in this case is due to the photogeneration of active oxygen.

Early in work[11] were proposed "Askor" system (hydroxycobalamin + ascorbic acid) as a potential antitumour drug (Scheme 3), based on the catalytic activity of hydroxocobalamin and its ability to form reactive oxygen species in the presence of a substrate[11].

It has been shown that catalytically active corrin metal complexes capable of forming ROS may be promising antitumour reagents. In mice, high efficiency was demonstrated in suppressing the growth of epidermoid carcinoma of the Lewis lung by 60%, lymphocytic leukaemia P-388 by 57%, and in the life expectancy of mice with ascitic Ehrlich tumour (AET)—an increase of 100%, and in the case of lymphocytic leukaemia P-388—by 25%[11].

We hypothesised that the addition of ACE to the catalytically active derivative of vitamin B_{12}, *e*-(COOH)-dioxycobinamide, may improve catalytic activity of the original complex into the cell. For this purpose, the autoxidation of ascorbic acid (AH_2) was studied, as well as changes in this process in the presence of *e*-COOH-Cbi-$(OH)_2$ **4** and

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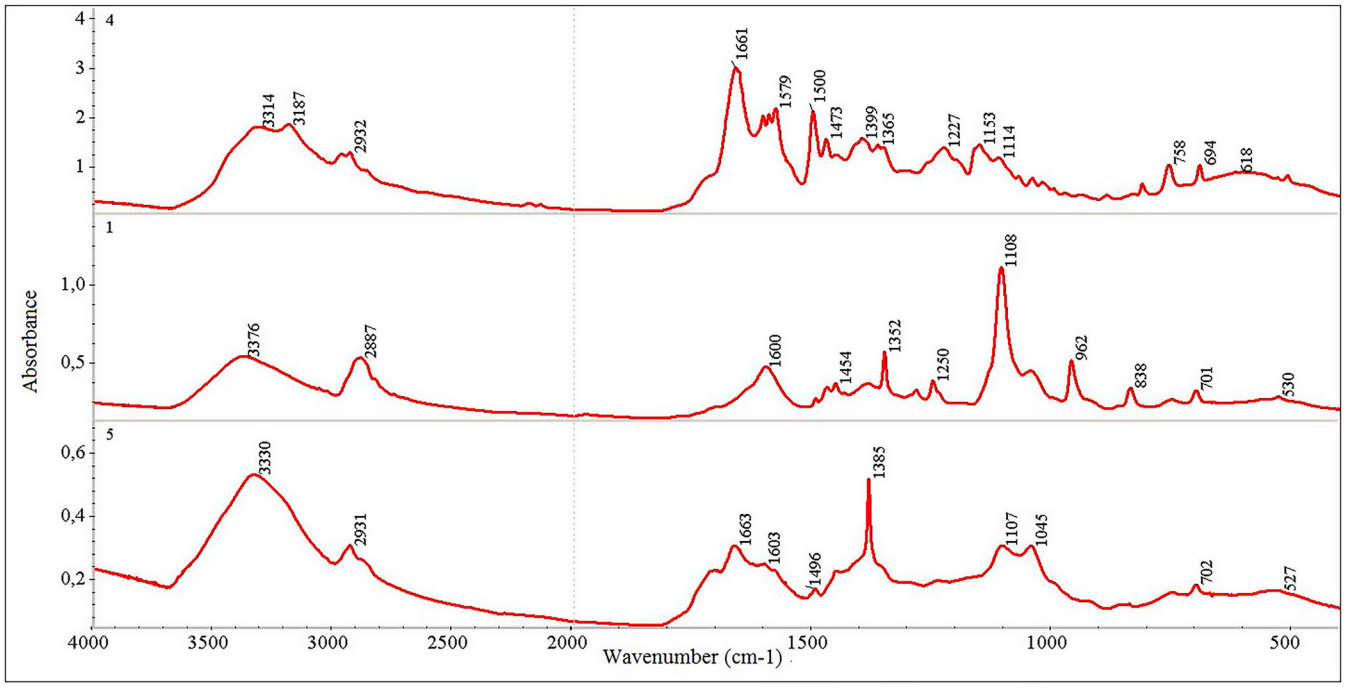

Figure 5. IR-spectra of: (**4**) e-COOH-Cbi(OH)$_2$; (**1**) *N*-(monohydrofullerenyl)-L-phenylalanine; (**5**) hybride nanostructure based on (**4**) and (**1**).

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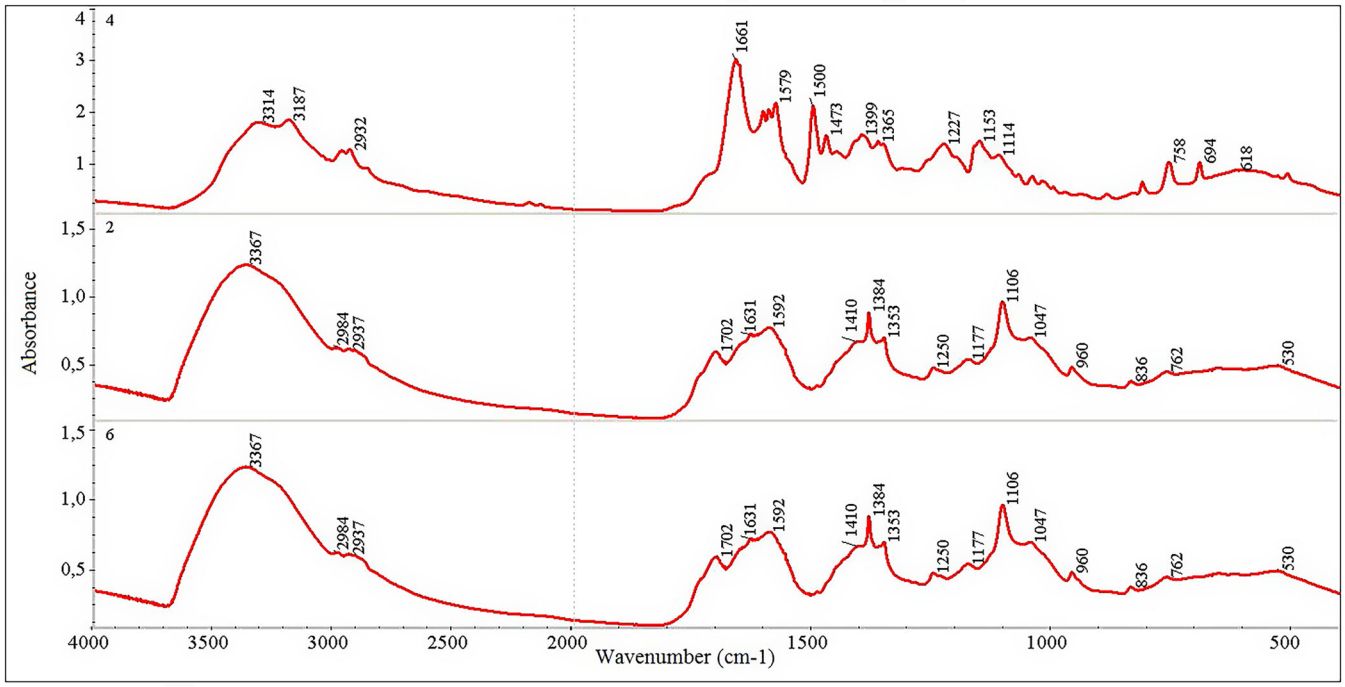

Figure 6. IR-spectra of: (**4**) e-COOH-Cbi(OH)$_2$; (**2**) *N*-(monohydrofullerenyl)-o-F-L-phenylalanine; (**6**) hybride nanostructure based on (**4**) and (**2**).

N-(monohydrofullerenyl)-L-phenylalanine derivatives **5–7**. Autoxidation of AH_2 in the presence of cobalamins can be schematically represented by reactions (1) and (1′):

Previously obtained results[11] show that under these conditions one of the most effective catalysts was the synthetic complex *e*-(COOH)-dioxycobinamide e-COOH-Cbi-(OH)$_2$.

From Table 1 it can be seen that the catalytic system generating reactive oxygen species, including, in addition to AH_2, the resulting hybrid nanostructure: **5–7** has approximately 2 times less activity than that based on the Cbi(OH)$_2$ complex. This is probably characterised by quenching of the excited singlet state upon electron transfer to the fullerene nucleus. The data obtained are consistent with the results obtained for similar structures based on pure chlorine and its fullerene derivative[10]. In the resulting hybrid nanostructure, there is a slight decrease in the catalytic activity of the vitamin B_{12} fragment in the autoxidation reaction of ascorbic acid. This can be explained by a decrease in the relative concentration of the vitamin B_{12} fragment in the molecule of the resulting hybrid nanostructure and the partial deactivation of the resulting ROS by the fullerene framework. The presence of a fullerene fragment in the resulting conjugate

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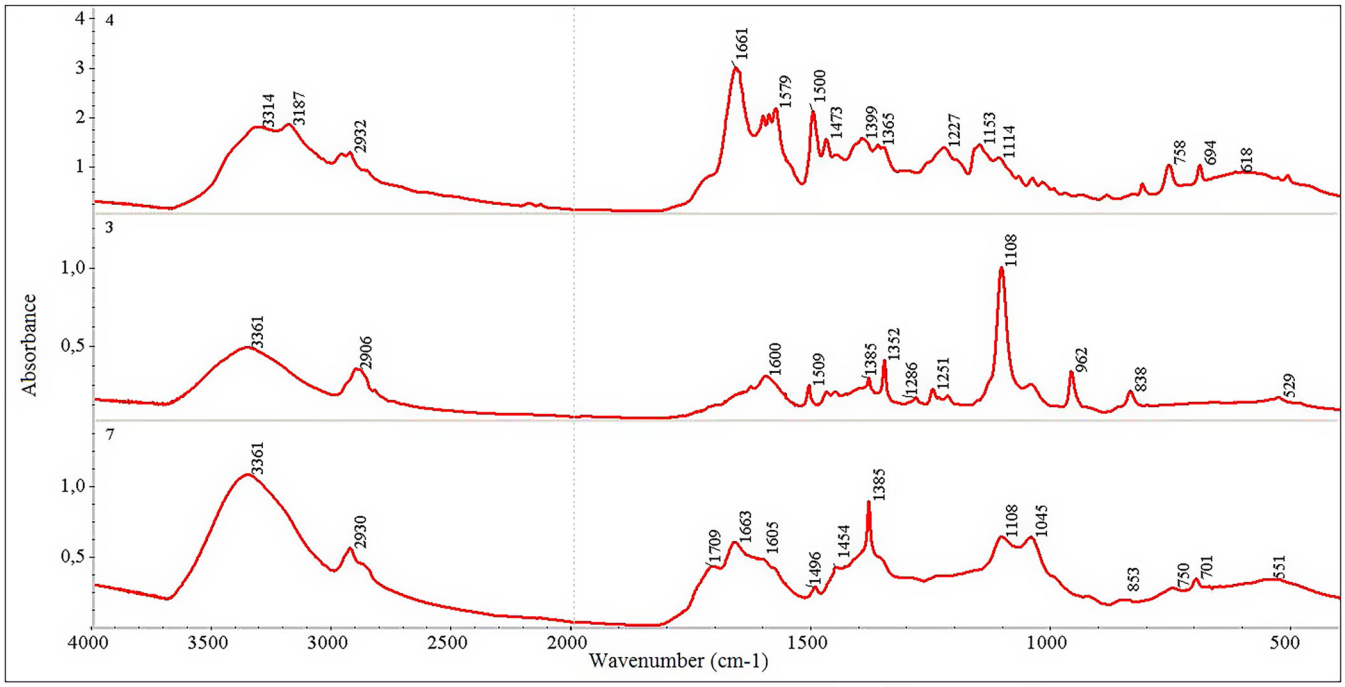

Figure 7. IR-spectra of: (**4**) e-COOH-Cbi(OH)$_2$; (**3**) *N*-(monohydrofullerenyl)-*p*-F-L-phenylalanine; (**7**) hybride nanostructure based on (**4**) and (**3**).

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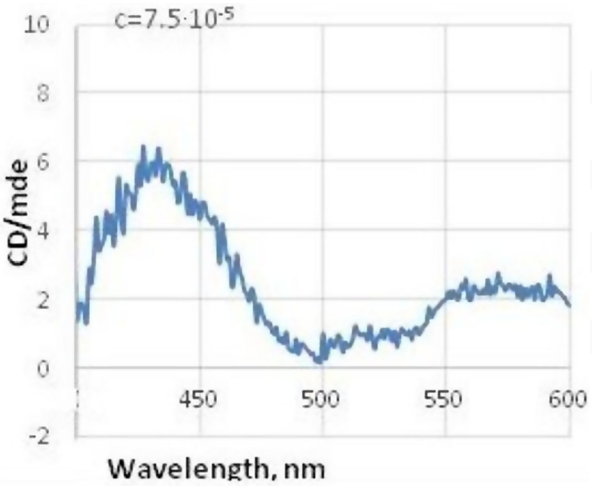

Figure 8. CD-spectra of hybride nanostructure (**5**) based on (**4**) and (**1**).

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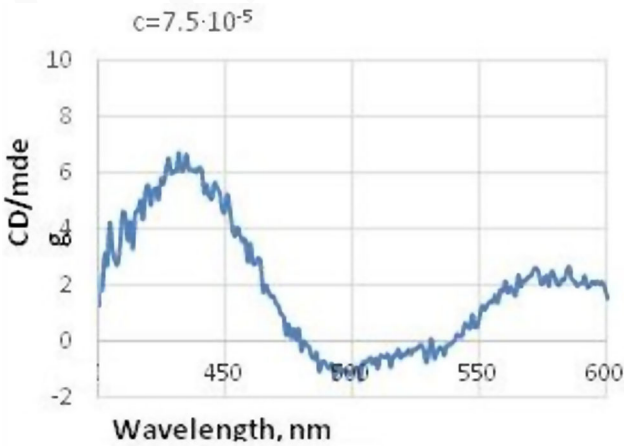

Figure 10. CD-spectra of hybride nanostructure (**7**) based on (**4**) and (**3**).

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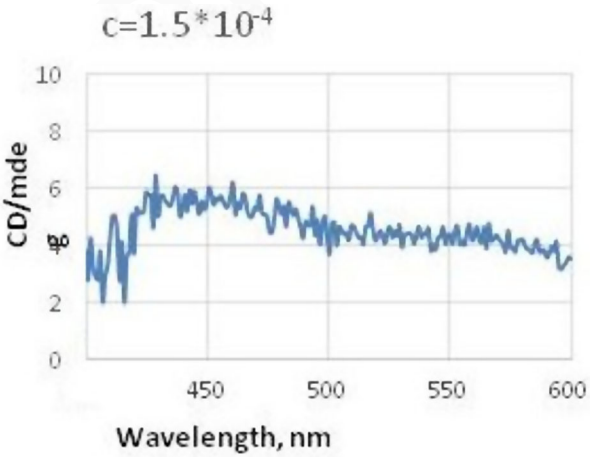

Figure 9. CD-spectra of hybride nanostructure (**6**) based on (**4**) and (**2**).

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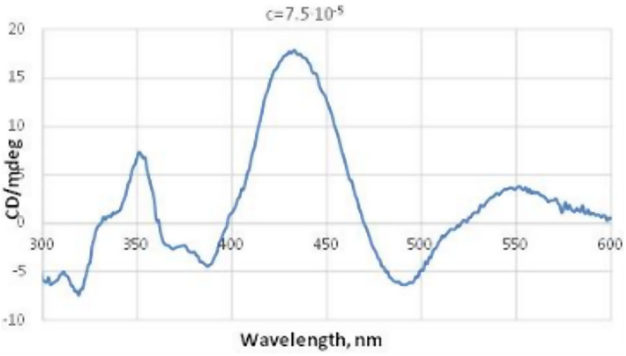

Figure 11. CD-spectra of (**4**) *e*-COOH-Cbi(OH)$_2$.

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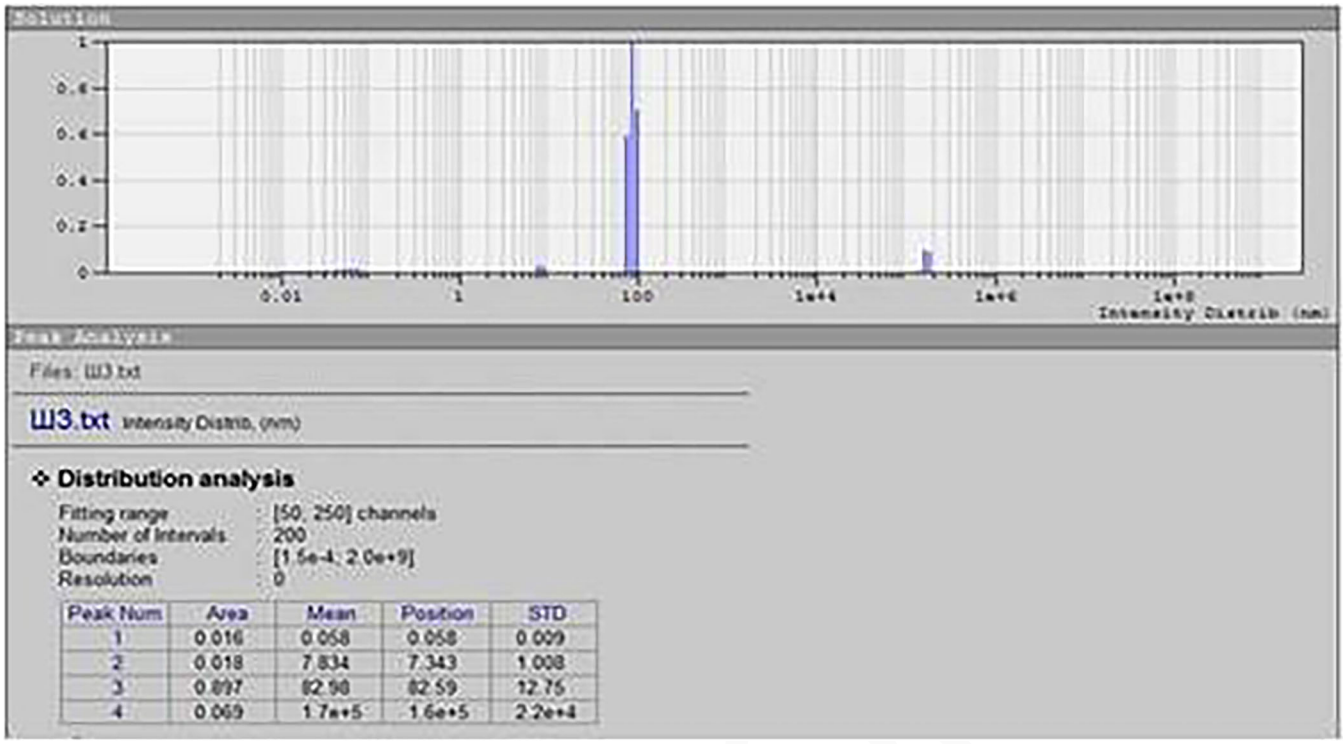

Figure 12. The results of laser dynamic light scattering of hybride nanostructure (**5**) based on (**4**) and (**1**).

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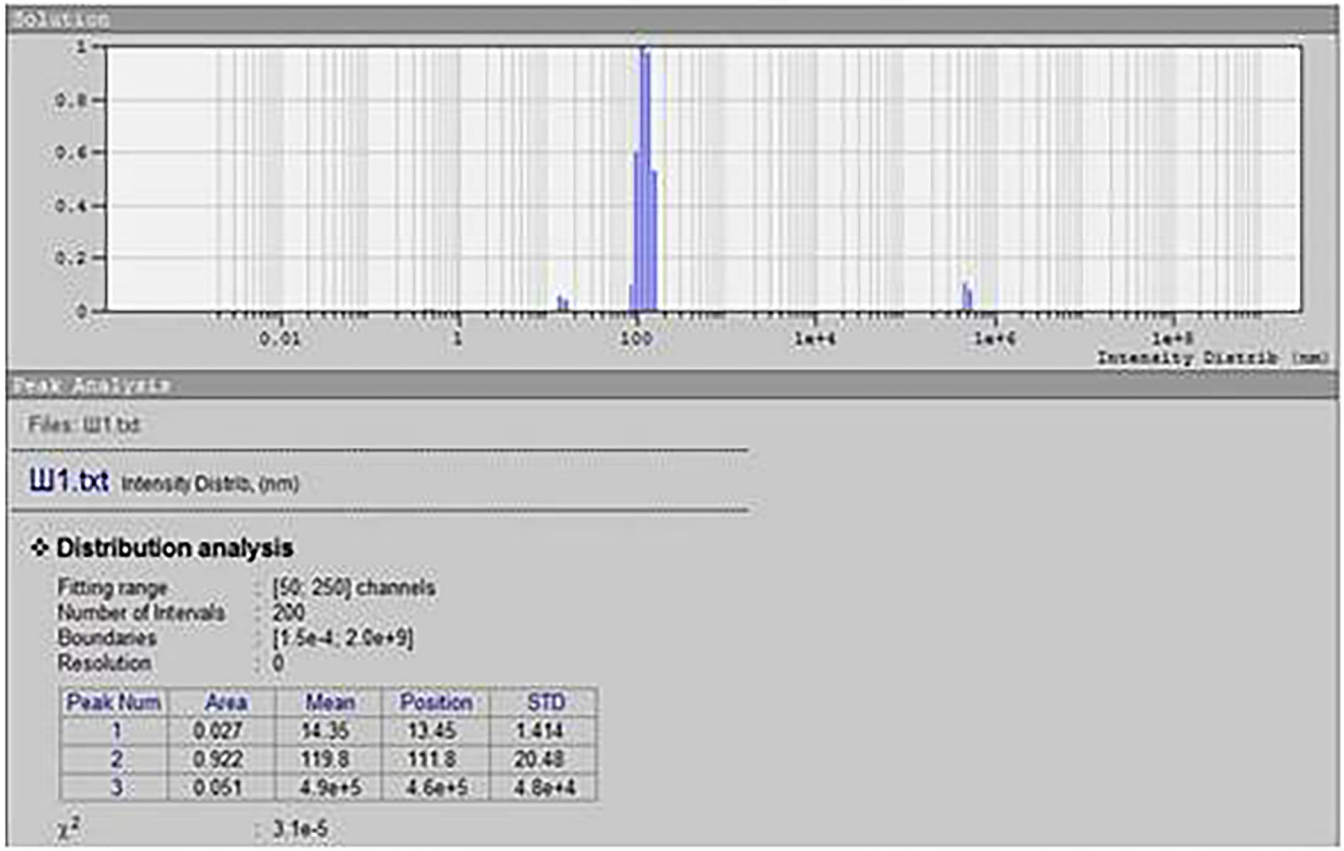

Figure 13. The results of laser dynamic light scattering of hybride nanostructure (**6**) based on (**4**) and (**2**).

can probably compensate for this by imparting lipophilic properties to the complex, which is facilitated by amino acid derivatives of fullerene.

4. Conclusion

In summary, we have developed an efficient chemical strategy for the synthesis of hybrid nanostructures based on

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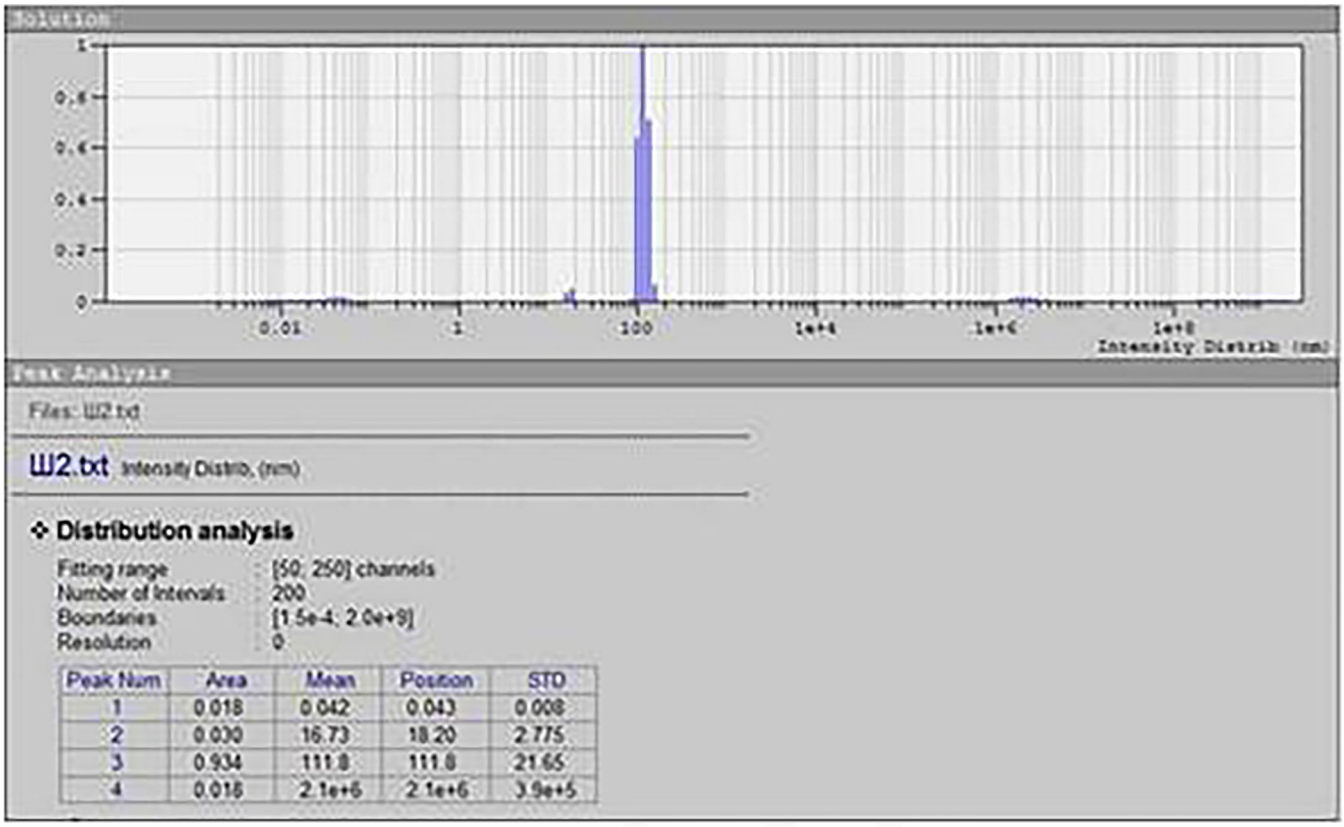

Figure 14. The results of laser dynamic light scattering of hybride nanostructure (**7**) based on (**4**) and (**3**).

$$[Co^{2+}] + O_2 \longrightarrow [Co^{2+}\cdot O_2] \longrightarrow [Co^{3+}] + O_2$$

$$2[Co^{2+}] + O_2 \xrightarrow{2H^+} 2[Co^{3+}] + H_2O_2$$

$$[Co^{2+}] + H_2O_2 \longrightarrow [Co^{3+}] + OH^- + OH^\cdot$$

$$2[Co^{3+}] + H_2A \longrightarrow 2[Co^{2+}] + 2A^- + 4H^+$$

Scheme 3. •••

$$AH_2 + O_2 \xrightarrow{[M]} A + H_2O_2 \quad (1)$$

$$2AH_2 + O_2 \xrightarrow{[M]} 2A + 2H_2O \quad (1')$$

Q4 **Scheme 4.** •••

Table 1. Autoxidation of ascorbic acid in the presence of compounds **4–7**.

System	α (α = ΔD/D·100%) %
AH_2	14.87
AH_2 + **4**	45.59
AH_2+ **5**	22.04
AH_2 + **6**	19.24
AH_2+ **7**	17.13

e-carboxy-dihydroxycobinamide and *N*-(monohydrofullerenyl)-L-phenylalanines and their fluorine derivatives. We were the first to obtain and characterise mono-derivatives of fullerene C_{60} with L-phenylalanine, o-fluoro-L-phenylalanine and *p*-fluoro-L-phenylalanine, as well as their hybrid nanostructures with vitamin B_{12} derivatives. The presence of catalytic activity of the obtained nanostructures in the autoxidation reaction of ascorbic acid has been established. In the future, it is planned to test the obtained compounds as neuroprotective drugs.

Authors contribution

Conceptualization; V.S.R.; methodology, V.S.R.; supervision, K.A.K.; project administration, N.Y.S.; investigation (chemistry), V.S.R., N.Y.S. All authors have read and agreed to the published version of the manuscript.

Q2

Disclosure statement

The authors declare that this research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest. Informed consent was obtained from all subjects involved in the study.

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