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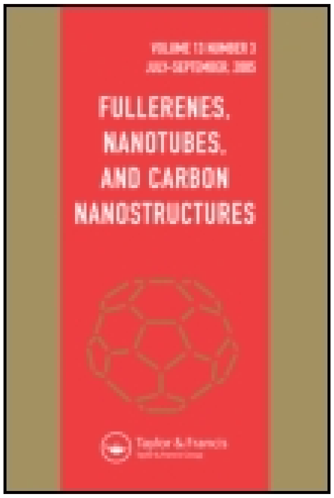

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Study of Fullerene Aqueous Dispersion Prepared by Novel Dialysis Method: Simple Way to Fullerene Aqueous Solution

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Study of Fullerene Aqueous Dispersion Prepared by Novel Dialysis Method: Simple Way to Fullerene Aqueous Solution

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A simple approach for large-scale production of aqueous dispersions of fullerene C60 (*n*C60) with good stability was developed by mixing a solution of crystalline fullerene in N-methylpyrrolidone (NMP) with water followed by exhaustive dialysis against water. Addition of amino acids or sugars at low concentration before dialysis increases the stability of the dispersion. Measurement of the size and ξ-potential of particles in *n*C60 showed that their average diameter is 100 nm and charge about –30 mV. IR spectra of the dried dispersions showed the presence of a broadband characteristic for C—O bond. Changing the solvent NMP to pyridine does not significantly affect the spectral characteristics of the resulting dispersion. Our data suggest that the fullerene molecule in *n*C60 is associated with MP and water molecules, and obviously is partially hydroxylated. The proposed method is promising for the preparation of aqueous solutions of endofullerenes.

Keywords: fullerene aqueous dispersion, N-methylpyrrolidone, FTIR, mass spectra, electronic microscopy

1. Introduction

Since the discovery of a preparative synthesis of the fullerene C_{60} in 1990 (1), numerous C_{60}-based compounds have been synthesized and some of them appeared to display a pronounced biological activity including antiviral, antibacterial, antioxidant, neuroprotective and radioprotective ones (2–6). This is attributable primarily to the unique structural and electronic properties of the C_{60} molecule that represents itself as a hydrophobic spherical cage with strong electron-acceptor ability and polarizability. The behavior and properties of aqueous fullerene dispersions are evidently of great interest from the viewpoint of their use in biological research and medical application. In particular, some studies have shown that aqueous dispersions of pristine fullerene C_{60} are capable of modulating a cytokine response and reduce an allergic inflammation (7–9). Many researches showed that C_{60} has no acute toxicity while the toxic effects sometimes observed could be attributed partly to chemicals used for preparation of C_{60} solutions (10). In particular, recent experiments carried out on rats indicated that a fullerene aqueous dispersion exhibited no chronic toxicity upon intragastrical routes of administration, and no statistically significant differences in hematological and biochemical parameters of control and treated rats were found after single and multiple administrations (11).

It is known that the crystalline fullerene (fullerite) is completely not soluble in aqueous medium (final concentration is less than 0.1 ng/ml) without a special treatment (12,13), and individual molecules of C_{60} are typically not present in water. In this connection, a simple and mild method for preparation of aqueous fullerene solutions has been a challenge for chemists for more than 15 years. Till date, well-known preparative procedures for production of water fullerene dispersions are based on the transfer of organic solution of C_{60} (in benzene, toluene or tetrahydrofuran) into the aqueous phase by using ultrasound treatment or long-term stirring or sonication in pure water and often combined with grinding in mortar ((14–16), see review in (17)). Other methods used include chemical attaching of hydrophilic groups to the fullerene cage, in particular, hydroxylation of the latter resulting in fullerenol, and charge-transfer complexation with hydrophilic polymers or surfactants (18). It was found that aerobic conditions and, especially in the presence of ozone, resulted in formation of epoxide on the surface of fullerene nanoparticles thereby increasing their hydrophylicity. However, the concentration of C_{60} solutions prepared by this method is rather low (19).

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As a rule, aqueous fullerene-containing solutions (dispersions) have typically a color that ranges from yellow to reddish-brown depending on concentration, and C_{60} molecules always assembled in hydrated negatively charged clusters whose dimensions may vary over a wide range, from few up to 1000 nm. The charge is known to play an important role in stabilization of C_{60} dispersions, and changes in pH and especially ionic strength can lead to the cluster aggregation and precipitation of the fullerene material. It should be noted that dried C_{60} dispersions irreversibly lose their solubility. Relatively low yields of fullerene conversion from the crystalline state to the solution, a necessity for prolonged stirring/sonication and heterogeneous process conditions, are the main causes of poor reproducibility and high cost of these methods. In addition, one of their drawbacks is that a sonication procedure used to accelerate C_{60} dissolution can sometimes lead to aggregate formation (20). On the other hand, commonly used aromatic solvents and tetrahydrofuran are toxic substances, and we do not know for certain whether they are completely removed from the dispersions due to their specific π-stacking and dispersion interaction with the C_{60} molecules (21,22).

In the present work, we describe a simple method for the preparation of stable C_{60} nanodispersions (nC_{60}) suitable for its application in biomedical purposes. The protocol designed includes a dissolution of crystalline fullerene in N-methylpyrrolidone (NMP), dilution of this solution with distilled water or aqueous solution of an amino acid (or other low molecular natural substance) used as a stabilizing agent (SA) with subsequent exhaustive dialysis against deionized water. Thus, the protocol excludes the use of toxic organic solvents as well as sonication, durable mixing, and heating, with all these operations being performed at room temperature.

2. Materials and Methods

2.1 Materials

Fullerene C_{60} (99.9%, SES Research, catalog 600–9969, USA) and NMP (99%, Panreac) were used without further purification; L-amino acids and other reagents were from Fluka and Sigma-Aldrich; dialysis tubes 6–8 kDa were from Spectra/Por (Spectrumlabs) and disposable sterile acetate 0.45 and 0.22 micron syringe filters (ArtChemicals.com) were used for filtration of fullerene dispersions.

2.2 Aqueous Fullerene C_{60} Dispersions (nC_{60})

Twenty milligrams of crystalline C_{60} were dissolved in 25 ml of NMP using a magnetic stirrer (up to 24 hr) and then the resulting dark brown-purple solution was mixed with distilled water (from 12.5 to 100 ml) or with a solution of 40 mg of SA (see Table 1) in 100 ml of deionized water. The dark-red transparent solution obtained was stirred for 1 hr and then subjected to exhaustive dialysis (cutoff 6–8 kDa) against deionized water (5 l × 4 changes). The dialysis solution was filtered through 0.45 μm filter resulting in a clear transparent solution with brownish-yellow color (Figure 1). The ratio NMP:H_2O or SA solution can be varied from 1:2 to 1:10 without fullerene precipitation. The solutions prepared were stored at 10°C protected from light.

2.3 Fullerene Extraction with Hexane from the nC_{60} ("Hexane Test")

An aliquot (10 ml) of nC_{60} was mixed with 5 ml of *n*-hexane by using vortex in a test tube for 1 hr. Then, the mixture was left to settle for 30 min to allow a phase separation and thereafter the hexane phase was transferred to a quartz cell. The C_{60} concentration in the extract was estimated on the basis of its UV-VIS absorbance at 328 nm ($\varepsilon = 52.5 \times 10^{-3}$ (23)).

2.4 Spectroscopy Measurements

UV-VIS absorption spectra were recorded on a double beam spectrophotometer Cary 100 (Agilent Technologies) in the range of 190–800 nm using a 1-cm quartz cell. Concentrations of C_{60} solutions were measured using the Beer's law and the experimental calibration. A direct correlation between the optical density and the concentration of C_{60} in the range of 1–50 mg/l at 340 nm was established giving $\varepsilon_{340} = 46452$. Fourier-transformed infrared spectroscopy (FTIR) spectra was recorded on a Bruker Alpha IR spectrometer (attenuated total reflectance (ATR) measurements) using freeze-dried or vacuum-dried samples of C_{60} dispersions or the samples prepared from the C_{60} dispersions deposited on an ultrafiltration membrane PLTK Dia NMWL 30 kDa (Millipore). In the latter case, the spectrum of the sample on a membrane is subtracted from one of the membranes. Mass spectra were recorded on Microflex^{TM} LT MALDI-TOF (Bruker Daltonics) using the samples taken as aqueous C_{60} solutions. Values of relative peak intensity were obtained by measuring of peak areas.

2.5 Size of Nanoparticles and ξ-Potential

Measurements of ξ-potential and nanoparticle sizes were performed using dynamic light scattering (DLS) on a Zeta Sizer Nano ZS instrument (Malvern Instruments, Worcestershire, UK).

2.6 Electron Microscopy

Measurements were taken on a Transmission Electron Microscope (TEM) equipped with a high-efficient autoemission cathode Libra 200 (Carl Zeiss) at an accelerating voltage of 200 kV. For the TEM analysis (images and electron diffraction pattern of selected areas) the nC_{60} dispersions (~100 μg/ml) were applied on a copper grid SPI ® with supporting formvar-carbon film and then dried under air at room temperature for 4 h. Prior to measurements, the sample in holder (Model 655, Gatan) was exposed to vacuum for 2–2.5 hr.

Table 1. Effect of stabilizing agent (SA) on the resulting C_{60} dispersion[a]

SA	Appearance[b]	SA	Appearance
Ala, Leu, Tyr, Trp, Phe, Gly, Pro, Met, Acp[e], His, Gln, Thr, Arg-Lys-Glu, L-Rhamnose, D-Glucose, Glycerol, Tris (base), PVP (10 kDa), PEG (1 kDa), without SA	Transparent solution	Asp, Glu, Arg, Lys/HCl, Lys, Cys, Lys-Trp-Lys (KWL), spermine, spermine/HCI, polyethyleneimine, acetic acid, benzoic acid, *p*-aminobenzoic acid, succinic acid, gelatin, BSA[d]	Settleable aggregates

[a] Solution of C_{60} (20 mg) in NMP (25 ml) were diluted with 100 ml of deionized water or SA (20 mg in 100 ml of water) followed by exhausted dialysis against deionized water.

[d] BSA—bovine serum albumin.

[e] Acp—ε-aminocaproic acid.

3. Results

3.1 Preparation of Aqueous Fullerene Dispersion

In this work, we have select NMP as a suitable organic solvent for initial dissolution of crystalline fullerene C_{60} (fullerite) due to its high hydrophility and very low toxicity taking into account the medical aspect (24). According to (25), a solubility of C_{60} in NMP is approximately 0.8 mg/ml, and in the very beginning of dissolution the solution has purple-cherry color but after storage it turns brown-cherry. Our first attempts to prepare an aqueous solution of C_{60} by using prolonged stirring and sonication of a mixture of NMP solution with water appeared to be practically unsuccessful because stability of the dispersion obtained significantly varied from bath to bath as judged by the formation of the C_{60} precipitate, and the resulting C_{60} content was very low. However, to our surprise, it was found that a saturated C_{60}/NMP solution can be diluted with water at almost any ratio without formation of any sediment and further it can be dialyzed against distilled water to form a clear stable solution. The time required for complete dissolution of NMP may be as much as 10–20 hr (upon stirring using magnetic plates) and the resulting solution upon dilution with water can be immediately dialyzed to remove NMP and any low molecular weight impurities (cutoff is 6–10 kDa). The volume of water used for mixing with solution of C_{60} in NMP prior to a dialysis significantly affects the final concentration of C_{60} and only to a slight extent the particle size in nC_{60}. The lesser the water, the higher the resulting concentration of C_{60} but here there is a certain limit due to the osmotic process. A maximal concentration of C_{60} in the solution prepared amounted up to 170 mg/l (calculated $\varepsilon_{340} = 46{,}450$) at a volume ratio of NMP solution to water equal to 2:1. At lower water content, the mixture becomes very muddy even after dialysis. However, we found that the dialysis against a concentrated solution of a high molecular polymer (e.g., dextran, polyvinylpyrrolidone) allowed a dramatic increase in the concentration of fullerene in dialysate. Anther way to increase the C_{60} concentration is vacuum evaporation; however, the maximum concentration that can be achieved without formation of flocks is 800–850 mg/l. Our efforts to concentrate the dispersion by ultrafiltration were unsuccessful due to adsorption of fullerene on the membrane.

During mixing the NMP/C_{60} solution with water, we observed a change in color of the resulting solution from dark to green-brown and then to brown that may serve as an indication of possible emergence of free radicals. The nC_{60} prepared after dialysis represents itself as a clear solution with pH 5–6 and color from yellow to brownish-orange with light opalescent indicating its colloid nature. This solution is rather stable because no precipitation was observed even after

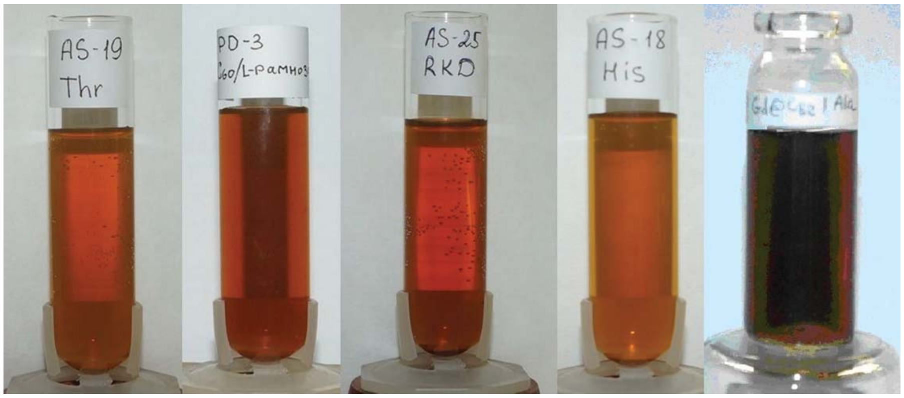

Fig. 1. Aqueous fullerene dispersions (from left to right): nC_{60}–Thr, nC_{60}–Rhamnose, nC_{60}–RKD (tripeptide), nC_{60}–His, and $nGd@C_{82}$.

at least 12 months storage at 10°C, and it easily passes through microfilters.

As noted, our first experiments with the use of the previous technique sometimes led to unstable solutions of C_{60} and emergence of loose precipitates after dialysis. In this connection, we put forward the hypothesis that increased stability of C_{60} in water may be reached by a weak noncovalent complexation of fullerene to amphiphilic molecule, but without a surfactant, and in this case a good candidate for such a role may be natural amino acids. It should be noted that recently published theoretical calculations performed by other researchers predict the possibility of such interaction (26). To select potential stabilizing agents (SA), we checked the efficiency of almost all natural amino acids (L-form) and a number of other agents, as peptides, proteins, saccharides, organic acids, and bases. Stock solution of C_{60} in NMP (0.8 mg/ml) was diluted with aqueous solution of SA (0.4 mg/ml) at a volume ratio of 1:4 and the resulting mixture exhaustively dialyzed against deionized water. A list of SAs and some characteristics of the dispersions obtained are given in Table 1 (designations: samples of fullerene dispersions prepared with SA are denoted as nC_{60}-SA, without SA as nC_{60}). Different SAs exhibited dramatically different effects: neutral and hydrophobic amino acids as well as saccharides all had a stabilizing effect leading to formation of clear stable solutions (stability was kept at least for 12 months), and their absorption spectra contained the peaks typical for aqueous C_{60} dispersions obtained by the solvent exchange procedure (Figure 1). At the same time, all the substances bearing positive or negative charges in aqueous media at neutral pH, such as basic and acidic amino acids, organic acids, including even more hydrophobic aromatic acids, resulted in emergence of loose sediments during dialysis. The incorporation of SA exerted some effect on particle size in nC_{60} evaluated as the degree of filterability through microfilter (0.45 μm) by measuring of the optical density (at 340 nm) before and after filtration. Use of amino acids, such as Tyr, His, Thr, and Met resulted in the dispersions with 100% passing.

3.2 *Effects of pH and Electrolytes*

The nC_{60} samples obtained after dialysis always had pH in the range of 5.8–6.8, whereas if the pH dropped up to 3—4, a marked formation of the precipitates began. This process, however, was reversible because the increase in pH led to dissolution of the precipitates. At the same, increasing the ionic strength with sodium chloride to concentrations of 10–40 mM at neutral pH also led to aggregation, and the salt concentration at which the precipitation started was dependent on the nature of the SA (data not shown). These findings allow us to conclude that the nC_{60} dispersions prepared exhibited the behavior well known and typical for colloid solutions of C_{60} (17,27,28).

3.3 *Particle Size and Charge*

A hydrodynamic particle size of six diluted C_{60} samples (10 μg/ml) measured by DLS appeared to average between 100 and 120 nm with polydispersity index (PI) of 0.17–0.37 indicating that the latter depends on the SA used. The ξ-potential measured for all nC_{60} samples was practically the same, –28 mV, while the nC_{60}–Ala particles exhibited slightly more negative value (Table 2). It should be emphasized that these parameters, namely size and ξ-potential, are very close to the ones for C_{60} dispersions obtained by other methods (13).

3.4 *Elemental Analysis*

Elemental analysis of some selected freeze-dried nC_{60}s (Table 3) showed the presence of nitrogen in all the samples analyzed (about 1–2%) that is obviously explained by an association of C_{60} and NMP molecules. This fact is also confirmed by the results of FTIR spectroscopy of dried nC_{60}, indicating that the band at 1650–1670 cm^{-1} is apparently related to the amide carbonyl group of NMP. Calculations on the molar ratio of NMP to fullerene displayed the value 0.6–1.3, while a calculation of the ratio H_2O to C_{60} based on the hydrogen content gave values from 5 to10. However, it must be noted that the presence of hydrogen in a sample may also be associated with the hydroxylation, the difference between the masses OH and H_2O is minimal, and **therefore** in Table 3 we present the ratio C_{60}:H_2O/OH. It also remains unclear whether NMP is associated with all the C_{60} molecules or only with these on the surface of C_{60} nanoparticles.

3.5 *UV-VIS Absorption Spectra*

Absorption spectra of all obtained nC_{60} samples are characterized by three intense maxima at 219, 265, and 344 nm and weak broadband between 400 and 500 nm, and they practically did not differ from those described by other authors (17,31,32). Unlike spectral characteristics of C_{60} in hexane (bands at 213, 257, and 328 nm and small sharp peak at

Table 2. The size and **ξ**-potential of selected nC_{60} measured by DLS method

nC_{60}-SA	Concentration mg/L	Average size nm	PDI	**ξ** mV
nC_{60}	10.8	103	0.177	−28.4
nC_{60}-Ala	10.9	110	0.176	−36.0
nC_{60}-rhamnose	10.7	124	0.172	−27.2
nC_{60}-RKD	10.1	100	0.210	−27.3
nC_{60}-His	10.0	112	0.377	−28.7

Table 3. Elemental analysis of freeze-dried nC_{60} and estimated content of NMP and H_2O/OH

Dispersion	N %	C %	H %	Calculated molar ratio	
				NMP:C_{60}	H_2O/OH:C_{60}
C_{60}-Ala	1.89	73.93	2.13	1.31	6.84
C_{60}-Thr	0.91	82.65	—	0.57	5.27
C_{60}-rhamnose	2.33	75.05	1.43	1.60	4.51
nC_{60}	1.75	77.27	1.50	1.29	10.22

405 nm (29)), the observed bands of nC_{60} are broader and less intense (Figure 2) and, in addition, a peak at 407 was absent and instead a broadband at 450 nm appears. According to the opinion of some authors, the last peak may be caused by the aggregation of C_{60} molecules (see review (17)). As noted earlier, a disappearance of free fullerene molecules in solution can be tested by the extraction of fullerene into the solvent not capable of mixing with NMP, for instance, such as hexane (20). In our case, the "hexane test" revealed the spectral changes inherent in nonaggregated molecules of C_{60}, namely a blue shift of peak from 343 nm to 328 nm as well as emergence of a weak band at 405 nm (Figure 2A) suggesting the presence of some number (approximately 8% of total amount) of single fullerene molecules in nC_{60}. However, this does not mean that these molecules are not associated with the NMP. Such a conclusion follows from the fact that the solution of C_{60} in NMP stored for 6 days and strongly diluted with hexane displayed the spectrum practically analogous to that of pristine C_{60} dissolved in hexane (data not shown). It is evident that the C_{60} must interact with NMP forming the charge-transfer complex (30) and the latter may most likely be transferred into hexane.

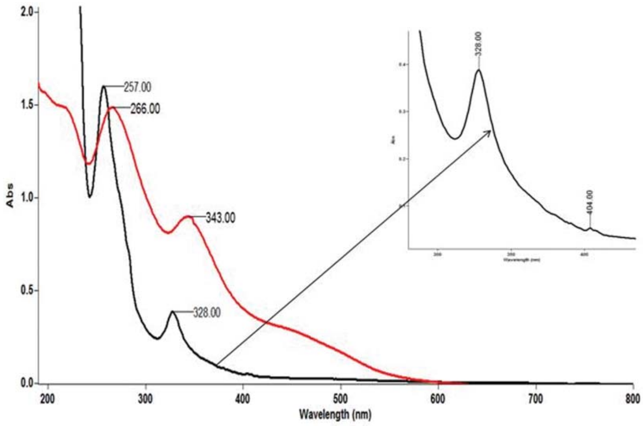

Fig. 2. (A) UV-VIS absorption spectra of nC_{60} (red) and hexane extract of nC_{60} (black); (B) absorption spectrum of nC_{60}–Gln.

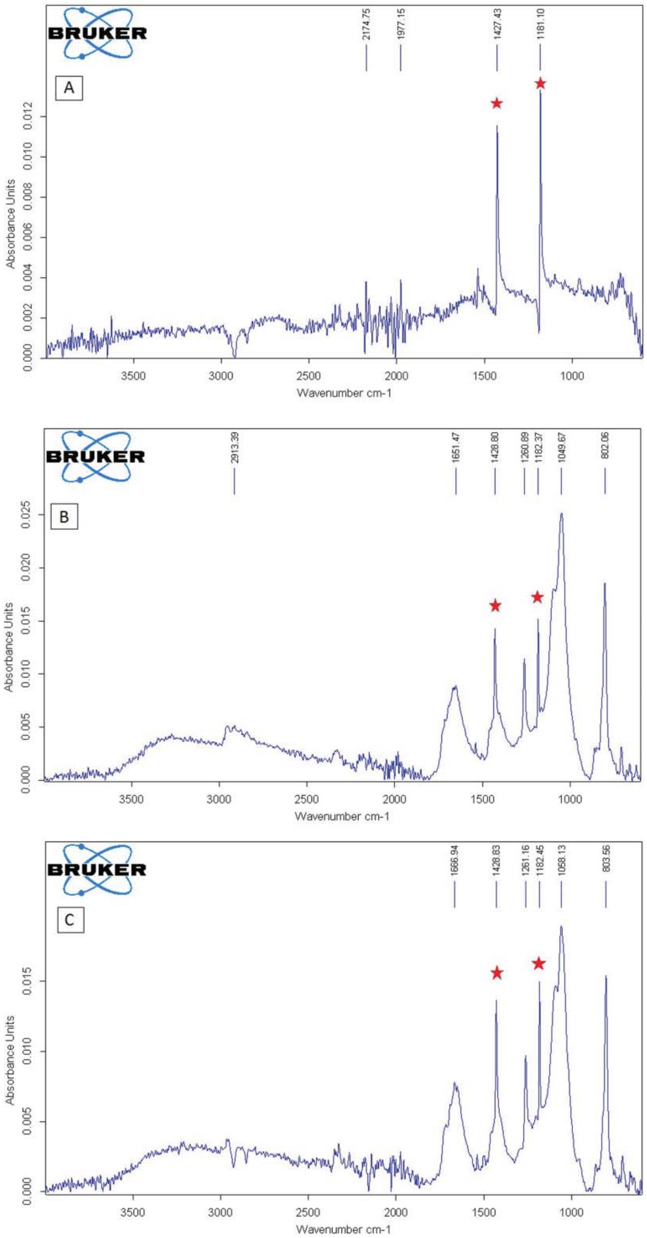

Fig. 3. FTIR spectra of crystalline C_{60} (A), freeze-dried nC_{60}-His (B) and d nC_{60}-Thr (C) depositing on an ultrafiltration membrane.

3.6 FTIR

Figure 3 shows ATR-FTIR spectra of dried nC_{60} samples prepared by freeze-drying or depositing on an ultrafiltration membrane. For comparison, the spectra of crystalline C_{60} (finely milled in a mortar) and NMP are also shown. Two IR vibration bands of free C_{60} molecules are clearly revealed in all spectra as narrow peaks at 1182 and 1428 cm^{-1} typical for C–C bonds although these are partly overlapped by other features. In addition, the spectra contain additional vibration bands in the ranges 3500–3200 and 1650–1660 cm^{-1} as well as a broadband at 1000–1100 cm^{-1} due to the obvious presence of O–H, C = O, and C–O groups, with the first two ones referring to bound and residual free water and NMP, respectively. At the same time, the presence in the spectra of the latter band is difficult to explain, for instance, they are unlikely due to the presence of amino acids (SA) since the ninhydrin test of nC_{60} samples were always negative. It should be noted,

Table 4. List of prominent peaks in mass spectra of nC_{60} dispersions

Sample	Supposed structure	Observed mass	Predicted mass	Relative peak intensity %
$n\mathrm{C}_{60}$	C_{60}	720.0	720	100
	$C_{60}(OH)_1$	736.9	737	14.6
	$C_{60}(OH)_5$	806.7	805	13.8
	$C_{60}(OH)_6$	823.5	822	13.8
	$C_{60}(OH)_7$	839.5	839	16.9
	C_{60}	720	720	100
	$C_{60}(OH)_1$	736.9	737	11.5
	$C_{60}(OH)_5$	806.7	805	11.9
	$C_{60}(OH)_{11}$	906.7	907	9.7
	$C_{60}(OH)_{12}$	923.4	924	32.5
$n\mathrm{C}_{60}$-Gln	C_{60}	720	720	100
$n\mathrm{C}_{60}$-Tyr	$C_{60}(OH)_5$	806.5	805	38.3
	$C_{60}(OH)_6$	822.4	822	22.3
	$C_{60}(OH)_{11}$	906.5	907	82.9
	$C_{60}(OH)_{12}$	923.3	924	147
	$C_{60}(OH)_{21}$	1076.0	1077	14.6

namely this fact, that the obvious presence of OH groups, had attracted special attention in an earlier work related to studies of aqueous C_{60} dispersions (33). It was speculated that the intense band at 1100 cm^{-1} is caused by the presence of alcohol groups at the surface of the C_{60} nanoparticles, but the mechanism responsible for their origin needs clarification. One of the possible proposed explanations is the oxidation occurring at prolonged sonication of C_{60} suspension (28,32,34). The acoustic cavitation phenomenon leads to collapse of bubbles in a liquid medium due to the extremely high temperatures (>5000 K) and pressures (>20 MPa), and sonolysis of the water molecules to H and OH radicals (35,36). However, in this work, though sonication or heating is not used, nonetheless all the samples are characterized by the presence of bands at 1000–1100 cm^{-1}. In addition, the use of anerobic conditions (argon atmosphere) did not change the spectral profile of the dried nC_{60} samples. Earlier experiments on hydroxylation of fullerene catalyzed by quaternary ammonium hydroxides showed that this reaction presents as a very slow reaction in the absence of oxygen, but even in this case (reaction under argon) it also produces fullerol with 10 hydroxyl groups per C_{60} molecule (37). We suggest that in our case, hydroxylation of fullerene also may occur since the mass spectra of nC_{60} samples provided some evidence for fullerene hydroxylation.

3.7 Mass Spectrometry

It is known that many fullerene-based adducts are thermally unstable and hence there is a need for a mild ionization method as a matrix-assisted laser desorption/ionization (MALDI) which allows to identify molecular ions with minimal fragmentation. Using this mode, we showed all the tested samples have common patterns showing the C_{60} molecular ion (m/z 720), dominant in all of the observed spectra. The spectra showed no attachment of amino acids, at the same time minor peaks were observed indicating the addition of oxygen atoms and/or hydroxyl groups. Note that these small peaks do not reflect impurities in starting substance since they are absent in the spectrum of crystalline C_{60}. Some of these peaks appeared to be closely matched with the mass of polyhydroxylated C_{60}, as m/z 806–808 ($+(OH)_5$), 907 ($+(OH)_{11}$), 924 ($+(OH)_{12}$), 1077 ($+(OH)_{21}$) (Table 4). It is noteworthy, there is certain regularity, and we see the peaks that correspond to a certain amount of OH groups attached to C_{60}: 1, 5, 6, 7, 11, 12, and 21. Thus, the data obtained by mass spectrometry and FTIR spectroscopy indicate, although indirectly, that fullerene hydroxylation occurs. It should be noted, that in the case of a high degree of hydroxylation, its UV spectrum would look like a smoothed curve and also four strong characteristic bands would be visible in the IR spectrum (at 3400, 1630, 1400, and 1080 cm^{-1}). Therefore, we assume that the data perhaps indicate the hydroxylation of C_{60} molecules lying only in the surface layer of nanoparticles.

3.8 Transmission Electron Microscopy (TEM)

TEM images of dried nC_{60}s are shown in Figure 4 (A, B, C, D). Large agglomerates of about 500 nm consisting of small particles with the size of about 20 nm were observed. Their microstructure appears to be similar to that of dried amorphous gels but there are the areas containing the orderly packed C_{60} structures as evidenced by the diffraction images, their size varies from 2–5 to 150–200 nm (data not shown). However, we do not know yet, whether fullerene crystals are present initially in nC_{60} dispersions or they are formed during sample preparation as a result of drying solutions on carbon grid. However, taking into account the information accumulated on aqueous C_{60} dispersions, we assume that the dispersions obtained in this work initially contain the particles with ordered arrangement of C_{60} molecules.

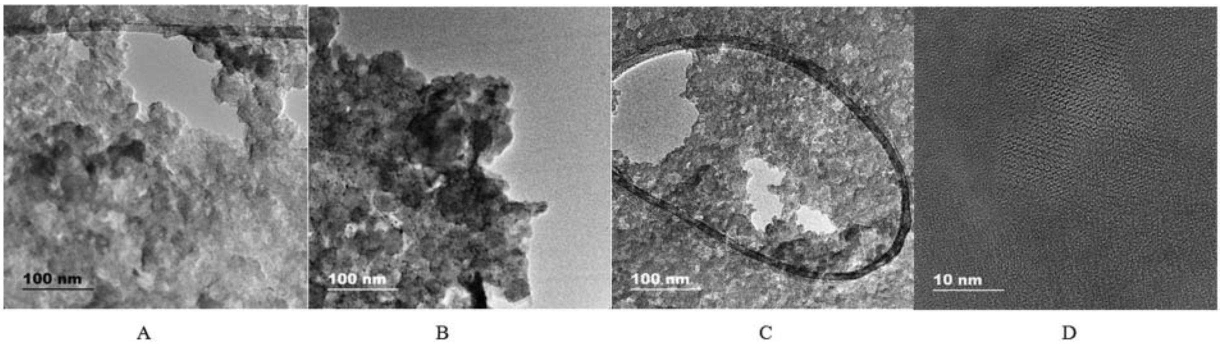

Fig. 4. TEM images of nC$_{60}$ dispersions dried on a copper grid: (A) nC$_{60}$, (B) nC$_{60}$–His , (C) nC$_{60}$–His, (D) nC$_{60}$–RKD.

3.9 *Effect of NMP Replacement*

It should be noted that the role of NMP in the nC$_{60}$ formation is not entirely clear, and we would like to explore whether the replacement of NMP to another solvent, but oxygen-free, can lead to C$_{60}$ dispersion with similar properties. With this in mind, we chose pyridine as the good solvent (solubility for C$_{60}$ is ~0.3 mg/ml) to prepare nC$_{60}$ using the same protocol as described earlier for NMP. Note, earlier it was shown that the fullerene dissolved in pyridine/water mixture forms monodispersive chemical inert 30 nm nanoparticles, and the absorption spectrum of the freshly prepared C$_{60}$/pyridine solution is almost identical to that of C$_{60}$ in n-hexane (38).

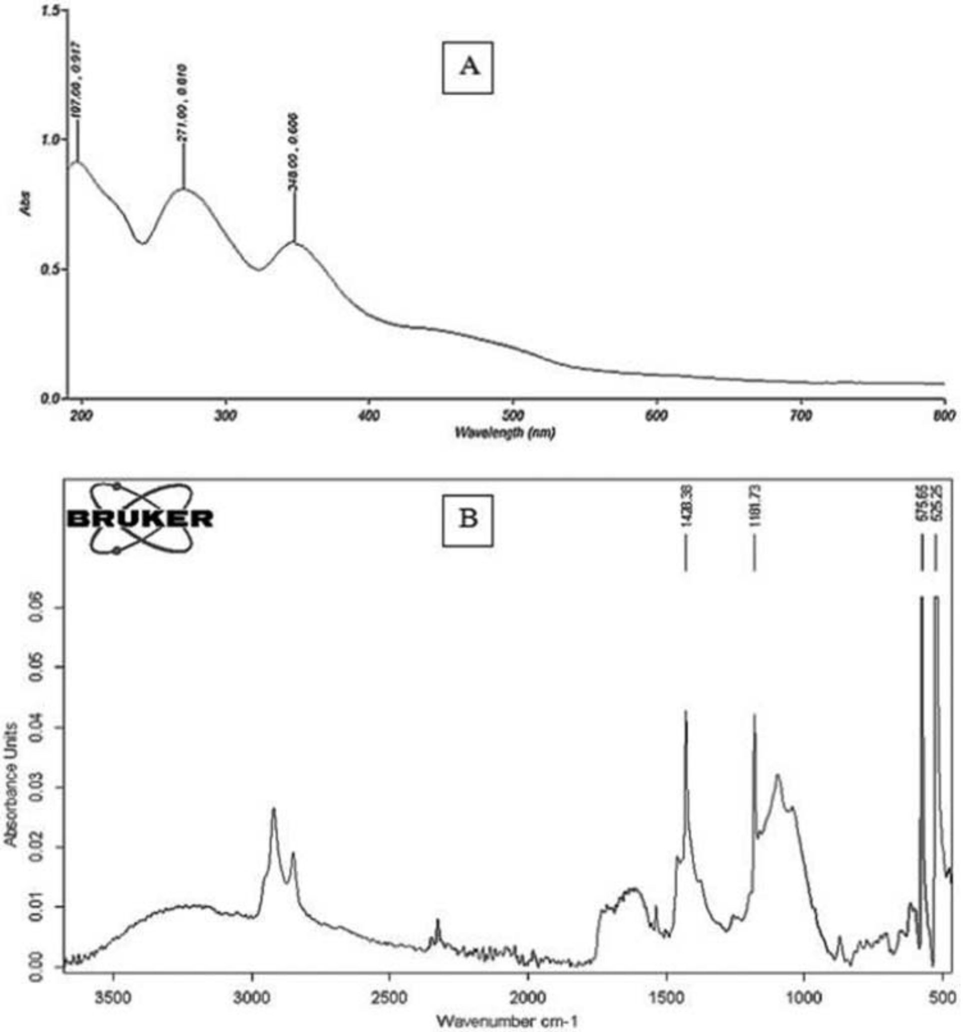

Fig. 5. UV-VIS (A) and FTIR (B, freeze-dried dispersion) spectra of nC$_{60}$ obtained after dialysis of C$_{60}$ solution in water/pyridine mixture.

The aqueous dispersion prepared by us by using pyridine represented a yellow strongly opalescent solution (nC$_{60}$/Pyr). Its absorption spectrum (Figure 5) was very similar to that of nC$_{60}$ described earlier but there was a slight red shift at about 5–6 nm. The FTIR spectrum showed sharp peaks characteristic for C_{60} (1428, 1182, 576, and 525 cm^{-1}) and additive peaks at 2921, 2849 (C-H modes), low intensive broadband at 1500–1700, and more intensive band at 1000–1150 cm^{-1} with distinguished peaks at 1097 and 1043 cm^{-1} (Figure 5B). Its mass spectrum, besides a main peak at 720, displayed minor peaks, which could be interpreted as products of C_{60} hydroxylation, $C_{60}(OH)_{11}$ and $C_{60}(OH)_{12}$. These observations suggest that C_{60} dispersions prepared for the dialysis method were similar and apparently did not depend on the structure of the organic solvent.

4. Discussion

This work suggests a new attainable and financially viable approach to produce stable aqueous fullerene solutions without the need for ultrasonic treatment and suitable for biomedical applications. The method is evidently also suitable for preparation of higher fullerene and endofullerene aqueous solutions.

The solvent used NMP which is known to have low toxicity by oral, dermal, and inhalation routes. In medicine, it has a long track record as a constituent in medical devices approved by the European Commission and FDA and thus can be considered as safe (39). Importantly, the exposure of nC$_{60}$ at 100°C for 1 hr did not lead to any noticeable visual changes except that of a slight reduction in the size of particles in nC$_{60}$ (data not shown). On the other hand, freezing and thawing of nC$_{60}$ samples leads to sedimentation.

According to our findings, natural amino acids and some other substances (SA) possess certain stabilizing effect on C_{60} dispersion, although the essence of stabilization mechanism is not known. It is possible that they in some way affect the C_{60} cluster reorganization in C_{60} dispersion. Based on the spectral and elemental analyses, we can speculate that the process underlying C_{60} solubilization can involve a formation of the complexes of C_{60} molecules or their clusters with NMP followed by partial hydroxylation of the nanoparticle surface that is capable of stabilizing the nC$_{60}$ aggregates. It is also obvious that these aggregates were surrounded by a firmly bound water envelope; the water is not removed on drying under high vacuum. The bond between C_{60} and NMP has a noncovalent character as evidenced by the results obtained by absorption, fluorescent, and electrooptical spectroscopy (30), while both NMR data and theoretical calculations provided evidence for the formation of the donor–acceptor bond between the NMP keto-group and the fragment of a C_{60} molecule (40). In addition, the C_{60}/NMP complex extracted from the solution and dissolved in methylene chloride was found to be characterized by all the features inherent in the C_{60} absorption spectrum which confirms a noncovalent linkage of fullerene in its new electronic state and also exhibits additional peaks at 435 and 460 nm typical for another donor–acceptor complex of C_{60} with the polymeric analog of NMP, PVP (30). Some theoretical calculations showed that fullerene C_{60} may attach covalently saturated molecules, including H_2O molecules, even in the absence of photoactivation, and this process can take place under mild conditions due to the lowering of the energy barrier at the six-membered transition state involving two molecules of water (41).

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