Water-soluble modifications of fullerene C_{60} easy penetrate through cellular membranes

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Fullerene C_{60} and its derivatives (FDs) exert substantial biological activity in a variety of cells and tissues However, mechanism of interaction of FDs with the cellular membranes are poorly understood, and the reports available reflect largely visual observations or their effects on artificial membranes.

In this work, a penetration dynamics of fullerene C_{60} in hydrated molecular colloidal form (FMC) and various C_{60} water-soluble derivatives (FDs) through membranes of human erythrocytes and platelets and plant symbiosomes were assayed. FDs bearing amino acid were capable of resulting in pronounced depolarization of symbiosomal membrane energized with the Mg-ATP. In human erythrocytes and platelets incubated in K^+-free medium in the presence of FCCP, a known protonophore, FDs with malonic acid pendants promoted acidification of intracellular medium thereby simulating an effect of the K^+-ionophore valinomycin. Dissipation of ΔpH artificially induced on the plasma membrane of these cells was observed in the presence of C_{60}-?-amino-butiric acid, with the latter compound strongly stimulated Mg-ATP-dependent generation of membrane potential on the symbiosomal membrane. C_{60}-Arg was shown to dissipate K^+-diffusion potential on the erythrocyte membrane induced by valinomycin. Fullerene used as FMC also entered symbiosomes and platelets as evidenced by quenching the fluorescence of Ca^{2+} indicator chlorotetracycline which is exclusively localized within Ca-stores of these cells. These findings provide evidence for easy permeation of fullerene-based compounds through biological membranes of different type cells that may be important for our understanding the molecular mechanisms responsible for delivery of such compounds to their cellular targets.

The myth about toxicity of pure fullerenes is irreversibly destroyed

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In our report we present the facts which were the reason for erroneous conclusions about high toxicity of water nanodispersions of pristine fullerenes and which in 2004-2005 have intimidated the world with some toxic properties of fullerenes [1-7] that continues to mislead still both the scientific world and the public.

Scrupulous analysis of such facts testifies that authors who have discovered 'special' toxic properties of C_{60} fullerenes, actually investigated the biological activity of both crystal-solvated forms of pristine C_{60} which contained toxic molecules of organic solvent (THF) and/or products catalytic degradations of the last as well as chemically modified (oxidized) C_{60} nanoparicles, but not pure fullerenes [7-16].

The criticism of hasty conclusions about fullerenes toxicity [8,11] stimulated new investigations, which have in essence denied previous statements about danger of fullerenes for living organisms and an environment [12-16].

Moreover, investigating pure fullerenes and their aqueous nanodispersions in the course of more than 12 years, we are constantly observing the unique and only positive effects of their biological activity [8 and http://fullwater.com.ua] that finds the strict confirmations in works made previously and now by other independent experts in fullerene sciences [11, 16-19].

As a whole, pristine fullerenes do not constitute a few danger for living organisms, including the hazardous influence on environmental, and it is recommended to handle pure fullerenes in the same way as with usual carbon black.

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Concerning the generation of antibodies against fullerene C_{60} core

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The purpose of this study is to investigate the immunogenic and allergenic activities of water-soluble modifications of fullerene C_{60} including its hydrated molecular-colloidal form (FMC). Various C_{60} compounds containing amino acid pendants (Ala, Ser, ?-Acp, ?-Aba, and Arg), proteins (BSA, KLH, ovalbumin, aldolase) as well as palmitoylated proteins, conjugated via ?-Acp-spacer were synthesized to use their as intraperitoneal immunogens in mice. Assay of anti-mouse sera using an immunoenzyme technique (ELISA) demonstrated no specific anti-C_{60} antibody production for both IgG and IgE classes. In contrast, marked antibody production to pendant compounds and their structural analogues was noted. For instance, the antibody response observed for the C_{60}-protein conjugates was related to the immunogenic activity of spacer molecule ?-aminocaproic acid (?-Acp). There was no IgE-inducing or histamine-releasing activity of fullerene amino acid derivatives when assessed by specific IgE and the fiberglass-based histamine release assay used as an indicator of allergenic activity. Thus, the C_{60} core evidently does not form a specific epitope. Our many attempts to reveal immunostimulating (adjuvant) effect as result of the fullerene covalent conjugation or non-covalent complexation to proteins were unsuccessful. Thus, fullerene core does not appear to be immunogenic site. Taking in consideration of good membrane penetration activity and the absence of immune toxicity, the water-soluble fullerene compounds are potentially ideal measure for drug and vaccine delivery.

Nitroxyde malonate methanofullerenes: interaction of Langmiur monolayers and thin films with radical antioxidants

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Mono- and dinitroxyde malonate methanofullerenes (referred as I and II, respectively) are fullerene C_{60} derivatives which are applicable in the successful production of new materials and synthesis of biologically active compounds [1], for example, they are perspective compounds used as a photodynamic sensors in oncology therapy.

In the present work stable radical mono- and dinitroxyde methanofullerenes I and II were used to study monolayers and thin films properties in presence of polyphenolic flavonoid antioxidants such as dihydroquercetin (DHQ) and quercetin (Q) in subphase. Mixed Langmiur monolayers of nitroxyde methanofullerene and radical sponge such as three-2,4,6-tret-butylphenol (TTBP), 2-methyl-2-nitrosopropane (MNP), and 2,2,6,6-tetramethylpiperidine oxyl(TEMPO) were investigated too by measuring surface pressure-area isotherms. Thin films of mixed system with nitroxyde metrhanofullerenes after water treatment were examined through AFM images. The feature of isotherms depends on a composition of water subphase and on nature of radical sponge (acceptor or donor) in mixed monolayers. The limiting area per molecule A0 extrapolated to ?=0 in monolayers of methanofullerenes I or II is increased twice from 0.55 nm^2 (subphase – water) to 1.00 nm^2 (subphase – polyphenolic antioxidants Q and DHQ). The same increasing A_0 was found when studying mixed monolayers of methanofullerenes and radical sponge (TTBP, MNP, and TEMPO). In both cases monolayers of I and II exhibit as condensed phases like liquid-solid films which are characterized by gently sloping rise of the isotherms upon compression.

AFM images show a looser and more regular structure of the films obtained in the presence of antioxidants and radical sponge.

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Generation of nitrogen monoxide by amino acid fullerene derivatives in mitochondrion

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It is known that biotransformation of exogenous donors of nitrogen monooxide (NO) is realized in mitochondrion. Efficiency of NO generation by amino acid fullerene $\tilde{N}_{60}$ derivatives in mitochondria of rat cerebrum was studied by monitoring the changes in catalytic activity of cytochrome c oxidase. It is known that two copper atoms and two hemes are present in the active center of cytochrome c oxidase. It is also known that NO reacts with copper ions and hemes to inhibit catalytic activity of cytochrome c oxidase. This fact allowed us to use this enzyme to study the formation of NO in mitochondrion. It has been shown that nitroxy derivatives of $\tilde{N}_{60}$ as well as arginine derivative perform as competitive inhibitors of catalytic activity of the enzyme that is evidence of that they generate NO. The constants of cytochrome c oxidase inhibition by amino acid fullerene $\tilde{N}_{60}$ derivatives in mitochondrion have been determined. The analysis of the experimental data allowed us to find the most efficient donors of NO in the family of amino acid fullerene $\tilde{N}_{60}$ derivatives.

The influence of fullerene on adsorption properties of silica gel with respect to peptides of middle molecular mass

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Our recent studies of low-density lipoprotein (LDL) adsorption revealed the significant change in adsorption properties of silica gel and adsorption mechanism in the presence of C_{60} fullerene molecules. These results provide the possibility to use silica gel modified with fullerene for treatment and prophylaxis of atherosclerosis [1-3].

In this work we continue the investigation of selective adsorption of blood plasma components; it is aimed at obtaining adsorbent for efferent therapy at acute ischemic states of myocardium. The objects of adsorption were peptides with middle molecular mass, which appear in blood plasma in abundance at many pathological states. They are the products of protein degradation and act as secondary endotoxins, causing depressions and disorder of different physiological processes.

For the preparation of adsorbent we used amorphous silica gel MCA with pore diameter of 100 nm, specific surface equal to 150 m^2/g, and total pore volume equal to 0.89 cm^3/g ("Fullerene Technologies Ltd.", 99.5wt.% grade). Two methods of introduction of fullerene into ailica gel were compared: in solid and liquid phase. We studied solutions of peptides isolated from blood plasma after adsorption and desorption from adsorbent.

Optical absorption spectra were taken using spectrophotometer SF 2000, molecular masses of peptides were determined using mass-spectrometer MX 5303 equipped with electrospray ionization device and time-of-flight mass analyzer, connected with microcolumn liquid chromatograph.

Analysis of optical absorption spectra of peptides revealed the influence of fullerene and method of its introduction onto adsorption propeties of silica gel. It was shown that silica gels after solid-phase introduction of fullerene possess maximum adsorption capacity and specificity. The change in absorption binding of peptides with silica gel was observed. The role of chemoadsorption arises after solid-phase introduction of fullerene.

From the comparison of mass-spectra it was shown that during adsorption the formation of new peptides with low mass takes place, presumably as a result of sequencing of initial peptides.

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Modification of polymeric materials with nanostructured surface by fulleropyrrolidines for medical applications

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Biomaterials with nanostructured surface (NSS) represent a novel interesting class of materials with a rich spectrum of possible applications in biology and medicine (for example, surface coatings of implants, cell cultivation media, and materials for the industry of drugs and medical systems for external administration). Here we report first successful modification of NSS polymers with bioactive organic derivatives of fullerenes.

The original polymeric material, polyethylene terephthalate, was treated with ion beams of chemically active gas mixture (O_2+N_2) in order to create NSS. Further modification of NSS comprised two stages. On the first stage, NSS polymers were modified by means of vapor deposition of carbon films (10, 20, 50 and 100 nm thick). The second stage involved fulleropyrrolidines deposition by means of *spin coating* technique. Fulleropyrrolidines with indole, quinoline and 2H-tetraphenylporphyrin moieties were obtained via the Prato reaction [1].

Investigation of antimicrobial activity of polymeric materials with NSS modified by fulleropyrrolidines with indole and quinoline moieties was carried out with the use of various cultures, namely *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa* and *Candida albicans*. In most cases we observed either appearance or improvement of antimicrobial properties of obtained materials, as compared to the original polymers without fullerene modifiers.

Preliminary experiments have showed that polymeric materials with NSS modified by fulleropyrrolidine with 2H-tetraphenylporphyrin moiety possess cytotoxicity against breast cancer cells. And, moreover, the immunological investigation of fulleropyrrolidine with 2H-tetraphenylporphyrin moiety showed that this fullerene derivative possesses immunopotentiating activity. Combination of such material properties gives wide perspectives for therapy of a cancer.

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Pro- and antioxidant properties of C_{60}/PVP complex and fullerene coated surface.

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Various fullerene preparations show the wide spectrum of biological action, from which it is possible to extract pro- and antioxidant activities [1]. We have investigated the ability to reveal these properties by two such preparations, namely, water soluble C_{60}/PVP complex and solid fullerene at the surface.

Antioxidant action of C_{60}/PVP complex was described earlier [2]. But in non-biological systems C_{60}/PVP complex also distinctly shows prooxidant activity. At the same time this complex does not reveal the prooxidant activity at its addition to the medium during the cell cultivation, and C_{60}/PVP complex itself does not have any apparent influence at the growing cells (cell cultures MA-104 and HepG2).

Fullerene associates at the surface in darkness reveal evident antioxidant activity, while at the illumination of the visible light they become powerful prooxidants. Death of cell cultures in this case is caused by the presence of reactive oxygen species (ROS), formed in the medium due to photoexcited C_{60} molecules. The analysis of the nature of ROS showed that the most important role in this case belongs to the singlet oxygen. Different cell cultures show various sensibility to the ROS, which are formed in the medium at the illumination at the presence of fullerene C_{60}. In general, malignant cells are more sensitive to the affective activity of ROS than the normal ones (types of cells).

The obtained data allow us to state that the varying of the ways of fullerene C_{60} introduction into the biological medium gives us the possibility to use different mechanisms of its activity on the biological objects – both pro- and antioxidant activities, as well as the influence on the biological membranes.

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Effects of hydrated forms of C_{60} fullerene on amyloids of X-protein and A?-peptides

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Amyloidoses are characterized by deposits of insoluble protein fibrils in different organs and tissues which arise as a result of inherited or acquired abnormality of protein folding. The search for drugs for therapy of amyloidoses is actual task. Senile plaques composed mainly by beta-amyloid (Abeta) protein are one of the pathological hallmarks of Alzheimer's disease (AD). Trying to decrease the production of Abeta is envisaged as a promising approach to prevent neurodegeneration in AD. We discovered new amyloid muscle proteins of titin family (X-, C-, H-proteins) [1] and showed their capacity to form amyloid fibrils similar to those found in the AD brain. We demonstrated also that tetracycline disrupted amyloids of the proteins and those of Abeta peptides. This confirms our idea of the possibility of similar approaches to the disruption of different amyloids due to the similarity of their physicochemical properties [1]. Here, using EM we demonstrate that under the same conditions, $\tilde{N}_{60}$ HyFn disrupts amyloid fibrils in short protofibrils and small aggregates. We noted that $\tilde{N}_{60}$ HyFn had the strongest anti-amyloidogenic effect on amyloids of X-protein and Abeta(25-35) peptide. The effect of $\tilde{N}_{60}$ HyFn on Abeta(1-42) peptide was a little weaker. Probably, higher doses and/or more prolonged treatment by $\tilde{N}_{60}$ HyFn may be required for its more effective action. Further testing of anti-amyloidogenic properties of $\tilde{N}_{60}$ HyFn in different amyloid systems will favor the creation of new nanotechnology in therapy of amyloidoses.

The work was supported by grants RFBR ¹ 06-04-48896; RF Education 1.1.06; RF President «Outstanding scientific schools» ¹ 4981.2006.4 and by RAS Presidium Program «Basic sciences for medicine» 2006.

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Effects of hydrated C_{60} fullerene on animal models of alzheimer's disease

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The effect of A? peptides (A?$_{1-40}$ and A?$_{1-42}$) is one the most important factors of pathogenesis of Alzheimer's disease (AD), a neurodegenerative disorder associated with the progressive memory impairment and dementia. Neurons and glial cells of the hippocampus and the cortex are targets for A? peptides.

The risk of AD dramatically increases in individuals beyond the age of 70. There is no effective therapy for AD. The prevention of the formation and/or the disruption of A? aggregates are the most promising approaches to the therapy of the disease.

Here we first demonstrated using transmission electron microscopy that hydrated fullerene (C_{60}HyFn) and its small clusters strongly inhibit the formation of A? peptide fibrils *in vitro*. C_{60}HyFn (1 μM, 1 h) did not possess any toxic effect in hippocampal slices *in vitro*.

Our *in vivo* investigations in rats showed that a single intracerebroventricular injection of C_{60}HyFn significantly improved the performance of the complicated cognitive task in normalcy and prevented the impairment of performance of the cognitive task induced by amyloid-?$_{25-35}$.

Our data allow one to suppose that unmodified fullerenes can be useful in the therapy of cognitive impairments and early stages of AD.

This study was supported by grants of the Russian Foundation for Basic Research (project no. 05-04-49331), the Program "Basic Research for Medicine" of the Presidium of the Russian Academy of Sciences, and the grant "Leading scientific schools" (no. 4981.2006.4).

Influence of Ñ$_{60}$/PVP complex on the healing of wounds and the toxisity in the experiments *in vivo*

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Main part of the published data concerning the biological activity of fullerence deal with the results of investigations in vitro, and only several works describe the experiments in vivo, for the whole organism.

Taking into account for the wound process the increase of the content of the products of peroxide oxidation of lipids and free radicals, which possess the injuring effect, it seemed reasonable to study the influence of fullerene C_{60} on the healing of wounds. The obtained data concerning the proportionality of antioxidant activity of Ñ$_{60}$/PVP complex to its concentration allowed us to chose precisely this water-soluble form of fullerene Ñ$_{60}$ for the further investigations.

White rats of the Wistar line (the weight 160–180 g were used in the experiments. Two methods of application were used: either the powdering of the dry Ñ$_{60}$/PVP complex on the wound, or its injection into the spongy wound covering. The influence of Ñ$_{60}$/PVP complex on the wound healing has been studied at aseptic tangential, full-thickness skin, purulent wounds, as well as for granulation wounds after deep thermal burns. It was found that Ñ$_{60}$/PVP complex stimulates actively rehabilitation processes in the wound (the formation of granulations, marginatum epithalamica, cicatrisation) and decreases the wound healing for 30%. These data allows us to make the conclusion about the perspectives of using Ñ$_{60}$/PVP complex as a wound healing preparation.

That is why the activity of this complex was also estimated for intra-abdominal injection of its 10% and 25% aqueous solution to rats. Structure-morphological changes and the activity of enzymes were investigated in dynamics in viscera of abdominal and thoracic cavities, kidneys and brain. It was found that Ñ$_{60}$/PVP complex does not show general toxicity - does not change the activity, the weight of the animals and their general state.

The obtained data testify the validity and perspectives of the use of Ñ$_{60}$/PVP complex, immobilized in wound coating, in purulent surgery and the surgery of deep burns.

Antioxidant properties of nanocarbon materials *in vitro*

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Until today significant scientific interest cause the representatives of the new allotropic forms of carbon - fullerenes and nanotubes, which are characterized by large reduction potential and ability to recover free radicals. Because of these properties carbon nanostructures have significant antioxidant potential and are capable of preventing the oxidizing stress, by which the development of many pathologic states is accompanied.

Influence of C_{60} fullerenes (10^{-5} and $2 \cdot 10^{-5}$ Ì) on the viability of thymocytes, subjected to the action of 100 μM Í $_2$Î $_2$ as the inducter of oxidizing stress and apoptosis, is studied. The viability of thymocytes, isolated from the thymus of the Wistar rats, was evaluated by MTT-test, that reflects the activity of the electron- transport chain of mitochondria (ETC). The rate of MTT reduction was determined after addition of Í $_2$Î $_2$ to the incubation medium in the different periods of time. It is established that the rate of MTT reduction in thymocytes treated Í $_2$Î $_2$ decreases and composes 74, 62 and 53% in 1, 3, and 24 h of incubation respectively in comparison with the control.

After simultaneous introduction of Í $_2$Î $_2$ and C_{60} fullerenes into the incubation medium MTT reduction remained at decreased level in 1 and 3 h, but increased in 24 h, reaching 75% from control.

The more expressed protecting effect of C_{60} fullerenes ($2 \cdot 10^{-5}$ Ì) was observed in condition of preliminary 1 h incubation of thymocytes in the presence of C_{60} fullerenes before introduction of Í $_2$Î $_2$. In this case the rate of MTT reduction in thymocytes in 1 h after Í $_2$Î $_2$ treatment was not changed, and in 24 h it is equaled to 80% as compared with control.

The influence of carbon nanotubes on the viability of thymocytes and the hemolysis of erythrocytes is also studied. The possible mechanisms of the biological effect of C_{60} fullerenes and carbon nanotubes are discussed.

S.V.P. is grateful to the INTAS (N 05-109-4328) for the support.

C_{60} hydrated fullerenes stimulate the plant growth at early stage

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Till now the biological effects of aqueous molecular-colloidal solutions of C_{60} hydrated fullerene (C_{60}HyFn) known as "FWS" [1, 2] were generally investigated using *in vitro* and *in vivo* animal models [3-5]. There is lack of information concerning its affection of living plants. The aim of present study was to investigate growth parameters and peroxidase activity of Zea mays *L.* (Dneprovsky-310 breed) seeds treated with C_{60}HyFn. Seeds were replaced on medium contained C_{60} in concentration of 1.4×10^{-8} M/l. Seeds couched on distilled water were taken as control. The length of both primary roots and germs (in mm), protein content and peroxidase activity were measured at 5-, 7-, 9- and 11-th days after emergence of seedlings. C_{60}HyFn-treated germs have displayed more rapid growth of roots compared with control at 5-th and 7-th days (100.5±1.4 vs. 77.1±1.7 and 133.4±1.5 vs. 125.2±3.0 correspondingly). The increases of germ length of C_{60}HyFn-treated seeds were statistically non-significant compared to control. Enhancement of root growth induced by C_{60}HyFn was correlated with evaluation of peroxidase activity at the same period in 2.6 and 2.37 times correspondingly as well as protein content at the 5-th day (in 2.33 times). These data demonstrate that $C_{60}H_yF_n$ treatment promotes metabolic activity of growing seeds at the early stage of germination. It is most probably that $C_{60}H_yF_n$ does not operate as phytohormone itself. It is observed that C_{60}FWS was consummated by growing seeds more readily than distilled water and thus is supposed to activate the enzyme mechanisms responsible for auxin synthesis and cell growth, affecting both cell division and cellular expansion. Enhancement of electron transport chain activity may result in increase of both H_2O_2 and reactive oxygen specie levels and triggering enzyme antioxidant system. Taken together these results indicate beneficial effects of FWS with smallest C_{60}HyFn concentration at early ontogenesis of Zea mays and may be useful for further application of Nano-Structured Water [see e.g. http://www.docoop.com] in agricultural cultivation, plant technology, nanobiotethnology etc.

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