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Proteins as Supramolecular Hosts for C₆₀: A True Solution of C₆₀ in Water

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Proteins as Supramolecular Hosts for C₆₀:

A True Solution of C₆₀ in Water.

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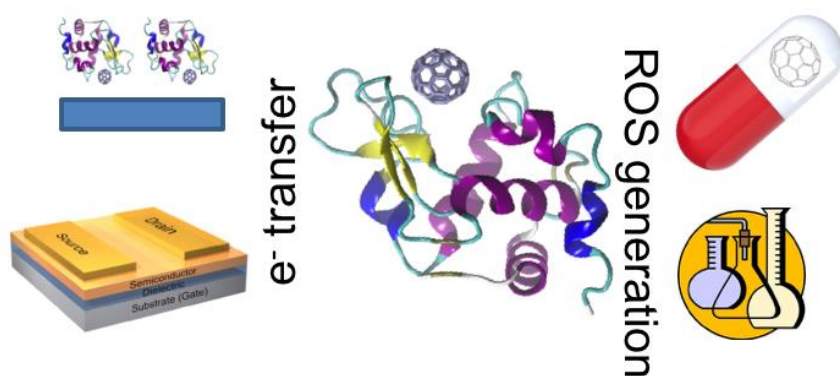
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ABSTRACT

Hybrid systems have great potential for a wide range of applications in chemistry, physics and materials science. Conjugation of a biosystem to a molecular material can tune the properties of the components or make new ones arise. As a workhorse, here we take C₆₀@lysozyme hybrid. We show that lysozyme recognizes and disperses fullerene in water. AFM, Cryo-TEM and high resolution X-ray powder diffraction show that the C₆₀ dispersion is monomolecular. The adduct is biocompatible, stable in physiological and technologically-relevant environments, and easily storable.

Hybridization with lysozyme preserves the electrochemical properties of C_{60} . EPR spin-trapping experiments show that the C_{60} @lysozyme hybrid produces ROS following both the type I and type II mechanisms. Due to the shielding effect of the protein, the adduct generates significant amounts of 1O_2 also in aqueous solution. In the case of the type I mechanism, the protein residues provide the electron and the hybrid does not require addition of external electron donors. The preparation and the properties of C_{60} @lysozyme are general and can be expected to be similar in other C_{60} @protein systems. It is envisaged that the properties of the C_{60} @protein hybrids will pave the way for a host of applications in nanomedicine, nanotechnology, and photocatalysis.

TOC



INTRODUCTION

C₆₀ retains a privileged status in the carbon family and continues to stimulate the development of new platforms for producing advanced materials. Versatile technological applications of fullerenes have been proposed and are in continuous development in different fields that cover lubricants, superconductors, sensors, solar cells, pharmaceutical scaffold, contrast agents, therapeutic agents in cancer therapy, and, in general, new applications for nanotechnology¹⁻¹¹ and nanomedicine.^{4,12-17}

The insolubility of fullerene in a physiological environment and the formation of fullerene aggregates, also in organic solvents, hampers its exploitation.¹⁸ The photophysical and photochemical properties of C₆₀ depend strictly on the nature of the fullerene dispersion and a strict control of the dispersion is truly necessary for technological applications.^{19,20}

Different approaches have been used to increase the dispersion of fullerenes, each with its own drawbacks:

- i) Mechanical dispersion-stabilization of C₆₀,²¹ either through ultrasonication²² or by solvent-exchange methods.²³ These mechano-physical approaches generate only metastable dispersions of fullerenes, that eventually re-aggregate. These harsh treatments may determine uncontrolled modifications of the chemical surface of C₆₀.²⁴
- ii) Synthesis of water-soluble fullerene derivatives by chemical functionalization with hydrophilic groups of pristine fullerene.^{4,25,26} The chemical functionalization of the fullerene shows limitations due to fact that soft derivatization processes maintain the tendency of these amphiphilic C₆₀ derivatives to aggregate,²⁷ while multiple functionalization leads to the alterations of the unique structure of fullerene and has a

negative influence on its peculiar properties; thus, restricting the potential for applications.

iii) Use of dispersants such as surfactants, block copolymers, amphiphilic polymers, micelles and liposomes.^{28,29} The use of dispersants is effective and a large quantity of C₆₀ can be dispersed also in water. The resulting solutions are characterized by polydispersion of fullerene aggregates of different sizes, because the fullerenes may exist in the form of both small aggregates solubilized within the hydrophobic core of the nanostructures formed by the dispersants and large aggregates stabilized by surface adsorption of the dispersants.^{28,29}

iv) Dispersion of C₆₀ by suitable carriers endowed with hydrophobic cores, such as γ -cyclodextrins, calixarenes, and other macrocyclic receptors or molecular tweezers.³⁰⁻³² The supramolecular approach is the most effective way to obtain monodispersed pristine fullerenes. Subsequent aggregation of the inclusion complexes³³ is common and for some biological application the fullerene host may be toxic.

Many proteins are known to interact with fullerenes.³⁴⁻⁶⁶ Few are the studies that aim to exploit the protein-C₆₀ recognition process for technological application. When proteins were used to disperse fullerene in water, they were simply used in lieu of surfactant molecules,^{67,68} leading to fullerene aggregates of various sizes.^{67,68} We recently proposed an innovative approach, using a protein, namely lysozyme, to disperse with a 1:1 stoichiometry C₆₀.^{36,44} The recognition is well-defined and the fullerene binds selectively in the protein substrate binding pocket.^{36,44} Lysozyme, as many proteins, follows, as a supramolecular host, the set of rules defined by Martin and Perez^{69,70} to recognize efficiently carbon nanomaterials^{36,71} which entail i) the presence of a nonpolar cavity, featuring aromatic recognizing units (Trp residues), of

the appropriate size to fit the fullerene cage; ii) a recognition process based on concave-convex complementarity; iii) a high degree of pre-organization of the host (due to the 3D structure of the protein) that lowers the entropic cost for guest binding.

RESULTS AND DISCUSSION

C₆₀@lysozyme hybrids were prepared in an Eppendorf by adding C₆₀ powder in a solution of lysozyme. The Eppendorf was ultrasonicated and after centrifugation and collection of the supernatant, a brown solution was obtained. UV-vis spectrum of the solution (Figure 1) clearly demonstrates the presence of fullerene in water, in particular notice the diagnostic band at 341 nm. This dispersion system is more efficient than the commonly used γ -cyclodextrin, that represents the most popular way to disperse C₆₀ in water (Figure 1). The absorbance at 341 nm for C₆₀@lysozyme (0.515) is 1.76 higher than C₆₀ dispersed with γ -cyclodextrin (0.293).

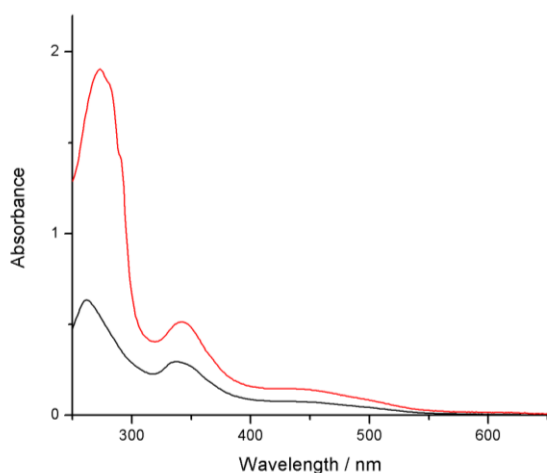


Figure 1. UV-visible spectra of C₆₀@lysozyme hybrids (red line) and C₆₀@2 γ -cyclodextrin (black line).

AFM Imaging of C₆₀@Lysozyme hybrids. NMR experiments have already shown the formation of a 1:1 stoichiometric adduct between lysozyme and the fullerene.³⁶ A

direct measure of the dispersion of the C₆₀@lysozyme hybrid is provided by atomic force microscopy (AFM). In Figure 2a the C₆₀@lysozyme hybrids appear monomolecularly dispersed over a mica surface. Height distribution analysis (Figure 2b) of the AFM images is consistent with that of a singly adsorbed C₆₀@lysozyme monodispersed and confirms the lack of presence of C₆₀@lysozyme aggregates, or nC₆₀ aggregates dispersed by the protein.

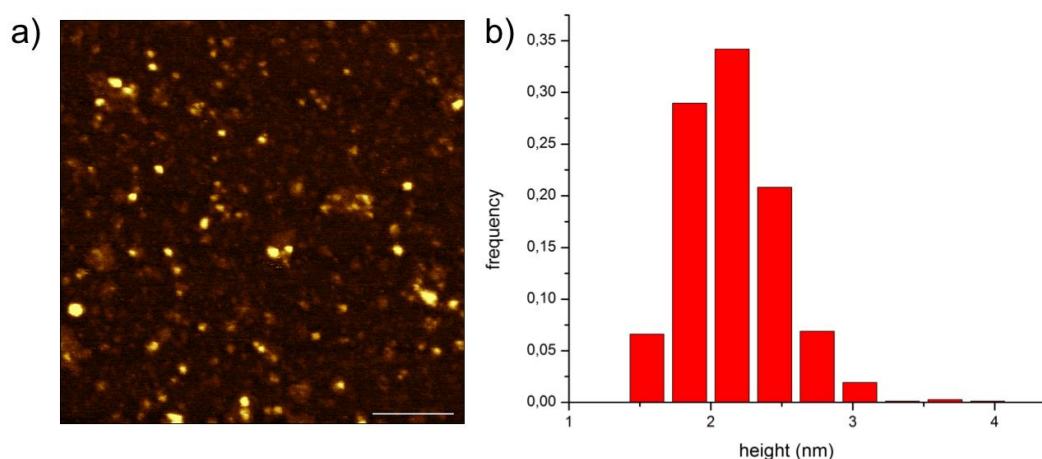


Figure 2. a) AFM image of C₆₀@lysozyme, top view (500 nm x 500 nm), scale bar 100 nm; b) Height distribution analysis of the AFM images.

To eliminate possible surface-effects due to adsorption of proteins on a surface, cryo-TEM experiments were carried out on the C₆₀@lysozyme solution. Cryo-TEM images allowed direct visualization of the dispersion state in solution of the C₆₀@lysozyme hybrids (Figure 3a).⁷² The particle size distribution (Figure 3b) shows that the C₆₀@lysozyme are monodispersed and there is no presence of nC₆₀ aggregates. These aggregates were previously observed when proteins or other surfactants were used as dispersants. They showed faceted, high-contrast particles, characteristic of crystalline aggregates.^{68,69,73}

Furthermore, when fullerene aggregates (nC₆₀) are present in solution, X-ray

diffraction patterns show peaks that evidence the presence of crystalline aggregates of fullerene.^{74,75} The high resolution X-ray powder diffraction pattern of a freeze-dried C₆₀@lysozyme solution does not show any diffraction peak (Figure 3c).

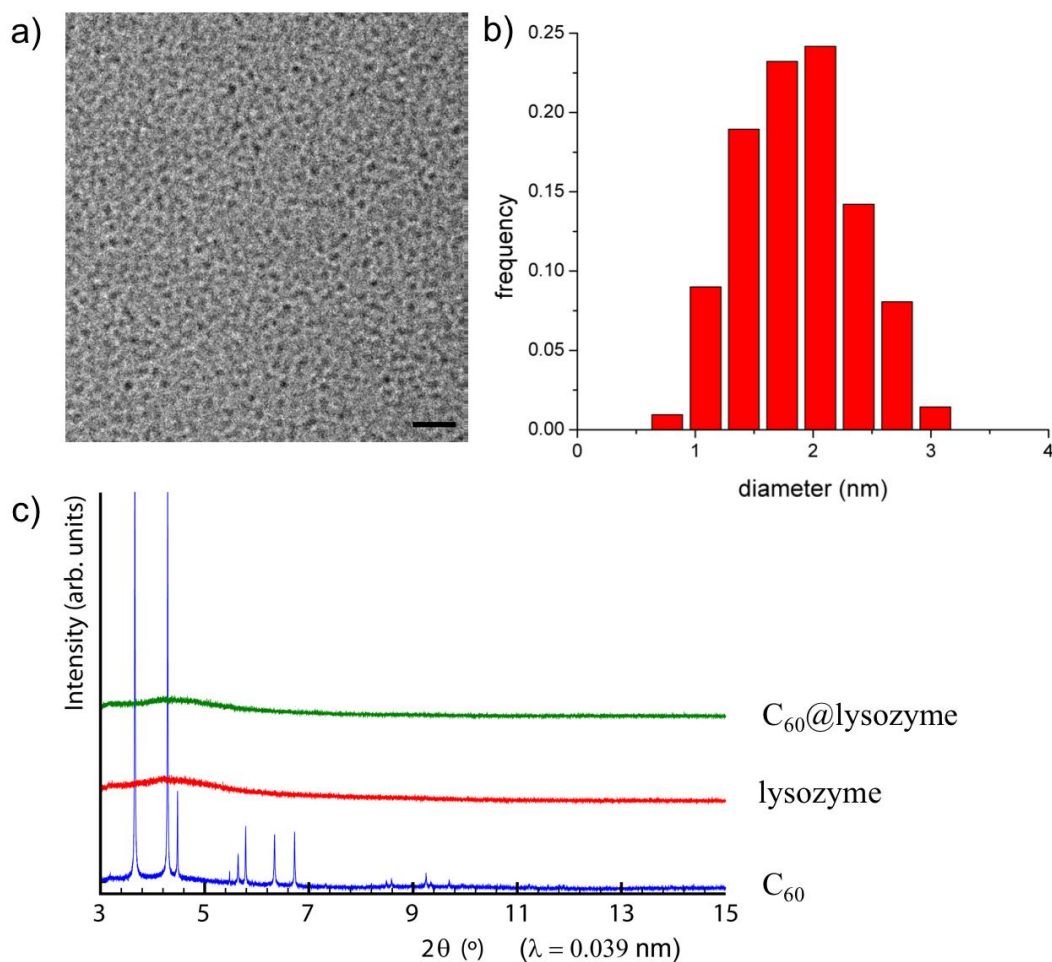


Figure 3. a) Cryo-TEM image of C₆₀@lysozyme solution, scale bar 20 nm; b) Particle size distribution of the C₆₀@lysozyme; c) High resolution X-ray powder diffraction of C₆₀ (blue line), lysozyme (red line) and C₆₀@lysozyme (green line).

These data confirm the absence of C₆₀ crystalline aggregates in solution, the ability of lysozyme to disperse monomolecularly C₆₀ and more importantly provide a route for the storage of monodispersed fullerene by lyophilization. In fact, re-dissolution of the lyophilized powder gives back a solution with the same characteristic of the starting

C₆₀@lysozyme solution.

Stability of C₆₀@lysozyme hybrids. The analysis of the stability of C₆₀@lysozyme at different pH's shows that the solution is stable up to the isoelectric point of lysozyme (pH 10.7), where aggregation occurs (Figure 4a).

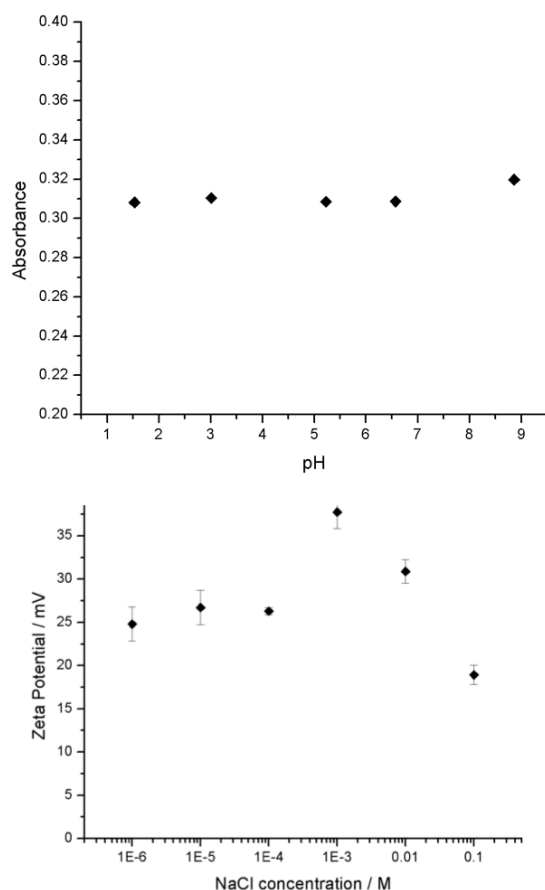


Figure 4. C₆₀@lysozyme: a) Absorbance vs pH; b) zeta potential at different NaCl concentrations.

The C₆₀@lysozyme adduct is stable also at different saline concentrations, up to physiological concentration (Figure 4b), while usually C₆₀ dispersed by γ -cyclodextrins or nC₆₀ rapidly precipitates when NaCl is added.²³ This means that the C₆₀ solubility is governed entirely by the protein. The solutions of C₆₀@lysozyme remain stable for months in water. The formation of monodispersions and stability of

C₆₀@lysozyme, in water and in saline solutions, open the way for technological applications able to exploit the chemical-physics properties of C₆₀ in physiological and technological relevant environments.

Electrochemical characterization of C₆₀@lysozyme hybrids

In Figure 5a the cyclic voltammetry recorded for the C₆₀@lysozyme hybrids and for lysozyme are shown.

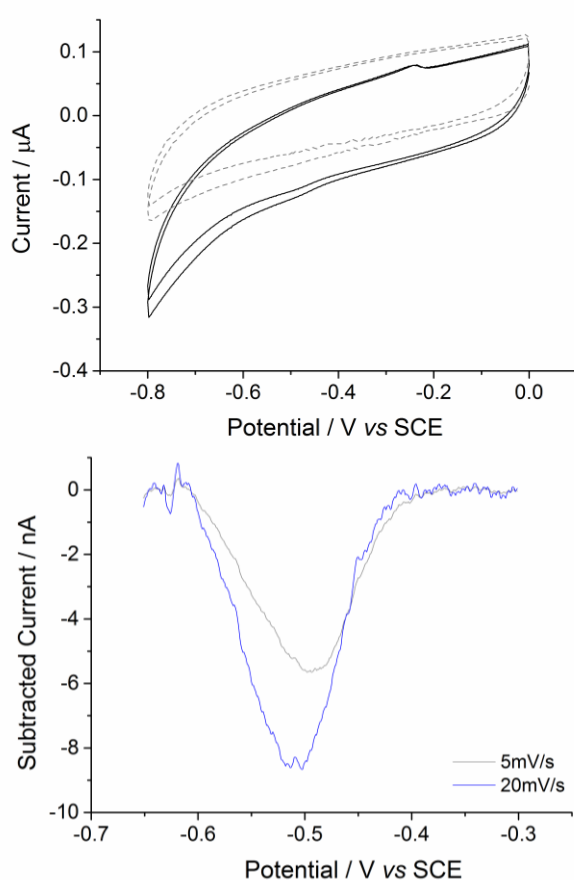


Figure 5. a) Cyclic voltammograms of C₆₀@lysozyme hybrids (bold line) and pristine lysozyme (dashed line), scan rate 5 mV/s. b) C₆₀@lysozyme reduction peak baselines subtracted scan rate 5 mV/s (gray line), 20mV/s (blue line). A glassy carbon was used as working electrode in 10 mM NaCl aqueous solution, the potentials are reported towards SCE reference electrode.

C₆₀@lysozyme shows a reduction peak at -0.49 V vs SCE and an oxidation peak at -0.24 V vs SCE. No peaks were observed in the same potential window for the pristine lysozyme. The peak-peak separation is 250 mV in accordance with a slow charge transfer probably due to the hindrance of the C₆₀ charge transfer to the electrode caused by the protein. In Figure 5b, the reduction peaks of C₆₀@lysozyme, subtracted for the baseline recorded at 5 and 20 mV/s, are reported. The potential of the reduction peak is around -0.5 V vs SCE. This potential is more negative than the one recorded for C₆₀ in organic solvent, a behavior that is due to the partial charge transfer of the amino acids constituting the binding site to the C₆₀ moiety. We already showed that two tryptophan residues tightly interact with the C₆₀ cage in the binding site³⁶ and that they establish a charge transfer process toward C₆₀.³⁶

C₆₀ presents six possible reduction waves, anyway they are only rarely observed in ultra-dry solvents, typically 2-3 quasi-reversible reduction waves are observed in organic solvents. Here, we report for the first time the electrochemical behaviour of a C₆₀@protein complex monodispersed in water, which retain the first reduction wave of C₆₀. This process appears to be irreversible in the investigated conditions. The voltammetric study confirms that the first reduction of C₆₀ is observable even when the carbon cage is confined in the protein binding pocket. In turn, modification of the pocket may tune C₆₀ redox potential. Since fullerenes are ideal electron mediators, C₆₀@protein hybrids can lead to new applications of C₆₀ in electrochemical catalytic systems or in biosensing.¹⁰⁻¹²

Photoinduced reactive oxygen species (ROS) generation by C₆₀@lysozyme hybrids

C₆₀ shows a significant visible light-induced generation of ROS, that can be exploited in photodynamic therapy⁷⁶⁻⁸⁷ or photocatalysis.⁸⁸⁻⁹¹ However, the use of C₆₀ molecules in water still presents important restrictions in its application due to both the dependency of C₆₀ properties on the physiological environment and the related aggregation phenomena⁹² and C₆₀ poor solubility.¹⁸ Aggregation is a well-known factor that deactivates the electronically excited states of photosensitizers, drastically decreasing the long-lived triplet excited state lifetime, and consequently reducing the ROS production efficiency.^{19,20,92,93} When fullerene aggregates are present, ³C₆₀^{*} is rapidly quenched by the surrounding C₆₀ or by other ³C₆₀^{*}.²⁰ In addition, aggregation reduces the active surface area of C₆₀ in contact with oxygen molecules for ROS production. The possibility to use C₆₀@lysozyme may overcome the current limitations.

Two different pathways can be identified for the production of ROS upon light absorption by C₆₀. In the simpler pathway (also known as type II energy transfer), the singlet excited state of C₆₀, initially formed, is converted to the long lived triplet state through intersystem crossing with a quantum yield close to unity. ³C₆₀ can be efficiently quenched by molecular oxygen (³O₂) to generate large amounts of singlet oxygen (¹O₂). In the more complex pathway (also known as type I electron transfer), ³C₆₀ in the presence of electron donors gives the C₆₀ radical anion that can readily transfer the electron to molecular oxygen forming the superoxide anion radical (O₂^{•-}) or hydroxyl radical (•OH). EPR measurements with the use of a spin trap can be used to verify the presence of the two pathways.

¹O₂ production was detected by EPR using 2,2,6,6-tetramethyl-4-piperidone (4-oxo-TEMP) as a probe. Actually, 4-oxo-TEMP reacts with ¹O₂ to afford 4-oxo-TEMPO nitroxide radical (see Figure 6a) that can be detected by EPR. The measurements were

carried out by irradiating with visible light the aqueous solution containing lysozyme, C₆₀@lysozyme, and C₆₀@2 γ -cyclodextrin. All solutions were prepared at the same fullerene concentration (identified by same intensity of absorption at 341 nm). When lysozyme and C₆₀@2 γ -cyclodextrin were irradiated up to 15 minutes in the presence of 4-oxo-TEMP no significant increase of the signal due to 4-oxo-TEMPO was detected by EPR. On the contrary, the specific signals of 4-oxo-TEMPO significantly increases when C₆₀@lysozyme is photoirradiated for 15 min (see Figure 6b). This result suggests that a significant amount of ¹O₂ is generated in the aqueous solution during irradiation of C₆₀@lysozyme and that the protein environment strongly reduces the quenching of the singlet oxygen by water molecules.

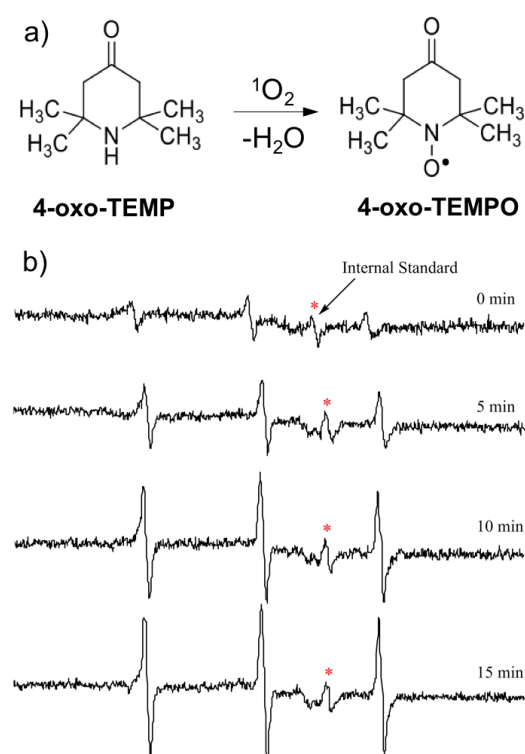


Figure 6. a) Reaction scheme for the generation of 4-oxo-TEMPO by the reaction of ¹O₂ with 4-oxo-TEMP; b) X-band EPR spectra of 4-oxo-TEMPO formed in aqueous solution of C₆₀@lysozyme complex at different irradiation times.

The pathway for ROS generation was also investigated by using EPR with 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) as a spin-trapping reagent. The generation of $\cdot\text{OH}$ was detected through an EPR signal corresponding to DMPO-OH, formed by the reaction of $\cdot\text{OH}$ with DMPO (figure 7a).

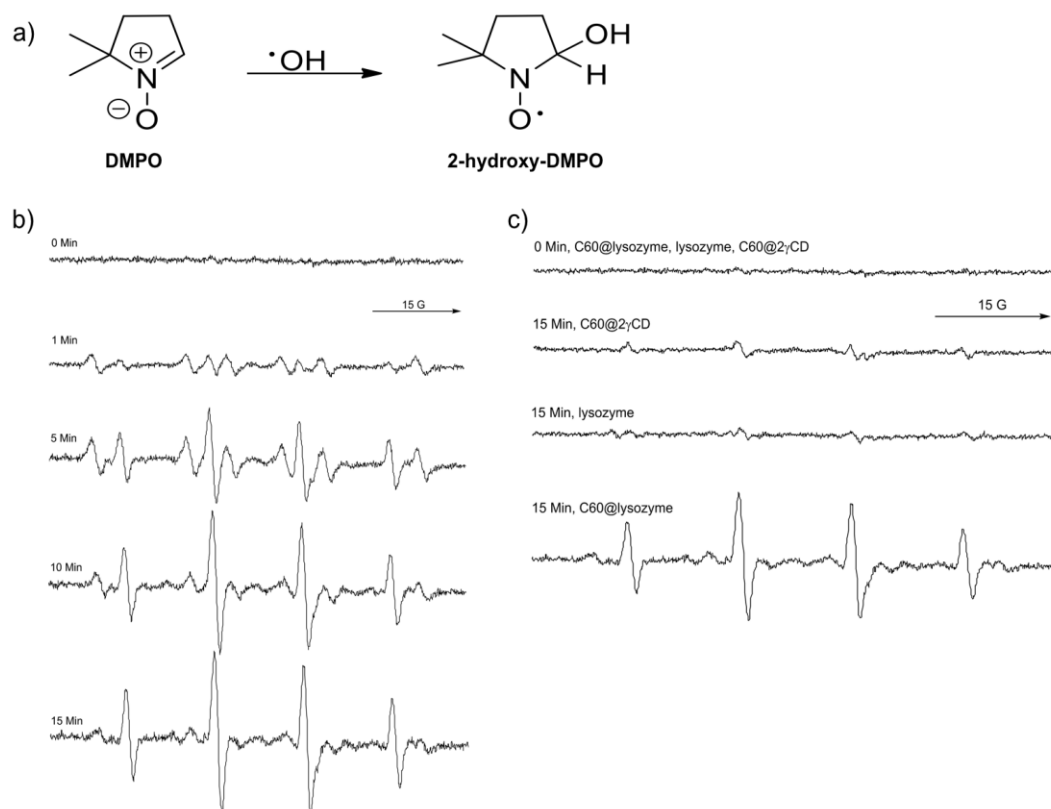


Figure 7. a) Reaction scheme for the generation of DMPO-OH by the reaction of $\cdot\text{OH}$ with DEMPO; b) X-band EPR spectra of DMPO-OH formed in aqueous solution of C_{60} @lysozyme complex at different irradiation times; c) X-band EPR spectra of DMPO-OH at time 0 and after 15 minutes of photoirradiation for C_{60} @2 γ -cyclodextrin, lysozyme and C_{60} @lysozyme.

Figure 7b shows clearly the specific signals of DMPO-OH ($a_{\text{N}}=a_{\text{H}}=14.75$ G), which was produced upon irradiation by the reaction of DMPO with the radical oxygen species generated by C_{60} @lysozyme. During the initial minutes of irradiation, the spectrum shows also the presence of an alkyl adduct to DMPO ($a_{\text{N}} = 15.80$ G, $a_{\text{H}} =$

22.52 G). Extension of irradiation, however, leads to a significant decrease in the intensity of this signal.

With $C_{60}@2\gamma$ -cyclodextrin or lysozyme the amount of radical oxygen species generated by irradiation of the aqueous solution was significantly smaller, as indicated by the very weak EPR signal recorded in these conditions (figure 7c).

Importantly, in the case of the type I mechanism, measurements involving C_{60} need the addition of a sacrificial electron donor, as for example NADH or triethylamine. C_{60} @lysozyme hybrid, however, does not need any external electron donors because the protein residues do this work. This means that the type I mechanism is self-activated in the C_{60} @lysozyme hybrid, due to the presence of the protein itself.

The high performances of C_{60} @lysozyme in the visible light-induced generation of ROS in water suggest the potential use of this hybrid as an agent for photodynamic therapy.⁷⁶⁻⁸⁷ Photogenerated singlet oxygen can be used also in synthetic organic chemistry. Even though singlet oxygen is a short-lived metastable excited state of molecular oxygen, it is a practical reagent for compound oxidation and can form carbon-oxygen and heteroatom-oxygen bonds.⁸⁸⁻⁹¹

CONCLUSION

In this paper we demonstrated that lysozyme can be used as a host molecule to recognize and disperse fullerene in water. AFM, Cryo-TEM and high resolution X-ray powder diffraction showed that the C_{60} dispersion is monomolecular. The adduct is biocompatible, stable in physiological and technologically-relevant environments, and easily storable. The electrochemical properties of C_{60} are preserved in the C_{60} @proteins hybrid suggesting new applications in electrochemical catalytic systems, biosensing or bioelectronics. EPR spin-trapping experiments showed that the

C₆₀@lysozyme hybrid is able to produce a considerable amount of ROS following both the type I and type II mechanism. This behavior suggests potential application of C₆₀@proteins in photodynamic therapy or in organic synthesis as singlet oxygen generator. Considering the use of C₆₀ in nanomedicine, C₆₀@proteins hybrids may be also potentially used for sonodynamic therapy, enhanced microwave and radiowave induced hyperthermia, photothermal treatment and optoacoustic imaging.

In addition, the different chemical groups offered by the protein platform allow an easy route for the functionalization of the hybrid, without altering the structure and properties of fullerene. C₆₀@protein hybrids could be augmented by using 1) targeting tags able to improve the cell/bacterial selectivity and promote the uptake of the C₆₀@proteins hybrid in cancer or antimicrobial therapy; 2) imaging tags to create an innovative, protein based theranostic platform; 3) light-harvesting antennae to extend the absorption spectrum of the C₆₀@proteins hybrid further into the red. Furthermore, these hybrids could be used to create stable and ordered C₆₀ suprastructures on surfaces with potential applications in nanotechnology, ranging from sensors and photovoltaic cells to nanostructured devices for advanced electronic applications.

Proteins other than lysozyme can be exploited in the future to tune the C₆₀ properties⁹⁴ and to shape a variety of C₆₀ spatial arrangements on surfaces, controlling the C₆₀ molecular assembly. C₆₀ has not yet exhausted its role as a leading material, because its integration in biomolecules will pave the way to new innovative applications.

MATERIALS AND METHODS

Synthesis of C₆₀@lysozyme and C₆₀@2γ-cyclodextrin hybrids.

The adduct of C₆₀@2γ-cyclodextrin and C₆₀@lysozyme was synthesized as described previously.³⁶ C₆₀ powder (Sigma-Aldrich, Cat. no. 483036) was used in 2:1 excess

with respect to the stoichiometric relationship to 1 mL of a 1 mM solution of lysozyme (lysozyme from chicken egg white lyophilized powder, Cat. no. L6876) or γ -cyclodextrin (Sigma-Aldrich, Cat. no. C4892) in Milli-Q water. After sonication for 60 min using a probe tip sonicator (Hielscher Ultrasonic Processor UP200St, equipped with a sonotrode S26d7, used at 45% of the maximum amplitude) in an ice bath, C₆₀ was dispersed in the solution forming a dark brown mixture. A dark-brown solution was obtained after centrifugation at 10 600g for 10 min and the supernatant was collected. In order to eliminate possible ROS generated during the sonication process, the solution has been washed four times with the 3K Amicon Ultra-0.5 Centrifugal Filter Units, using a volume of 500 mL and spinning at 14 000 g for 20 minutes, obtaining a concentration factor of 8. After each spin the volume of the concentrated solution has been brought back to its original value using Milli-Q water. UV-vis absorption spectra were recorded at 25 C° by means of Agilent Cary 60 UV-Vis Spectrophotometer. Zeta potential measurements were carried out using a Malvern Zetasizer Nano ZS.

AFM measurements. AFM experiments were performed at the SPM@ISMN microscopy facility in Bologna. AFM (Digital Instruments, Multimode VIII equipped with a Nanoscope V) operated in ScanAsyst mode was used to analyze the dispersion state of the proteins. The samples for AFM measurements were prepared by drop casting 5 μ l of C₆₀@lysozyme hybrids solution onto a freshly cleaved mica substrate for 10 min then rinsed with milliQ water and dried with a stream of N₂. Height distribution from AFM images was calculated using Gwyddion.

Cryo-TEM microscopy. Sample vitrification was performed using an automated vitrification robot (FEI Vitrobot™ Mark III) for plunging in liquid ethane. TEM Cu Quantifoil grids, R2/2 (Quantifoil Micro Tools GmbH) were surface plasma cleaned for

40 s using a Cressington 208 carbon coater prior to use. The samples were studied on the TU/e Cryo Titan (FEI, <http://www.cryotem.nl>), equipped with a field emission gun (FEG) operating at 300 kV and a post column Gatan energy filter (GIF). Images were recorded using a post-GIF 2k x 2k Gatan CCD camera in low dose conditions. Particle diameters were measured with the Measure_lengths script from the CMEM toolbox. As spherical symmetry was assumed, the diameter was averaged over the 'length' and the 'width'. For each image, 6 regions were selected, in which approx. 33 particles were measured. Data was analyzed with Origin. The data was binned in bins with sizes set equal to the pixel size (0,2814 nm).

X-ray powder diffraction measurements. High resolution X-ray powder diffraction (HRPXRD) measurements were collected with a dedicated high-resolution powder diffraction synchrotron beamline (ID22 at the European Synchrotron Radiation Facility (ESRF), Grenoble, France) using as wavelength 0.039 nm. The C₆₀@protein water dispersions were lyophilized before the HRPXRD measurements.

Electrochemical characterization of C₆₀@lysozyme hybrids.

Instrumentation. The electrochemical measurements were performed using a CHI900B bipotentiostat (from CH Instruments Inc. Austin, TX) in a typical three-electrode configuration cell. A glassy carbon ($\varnothing = 1$ mm) was used as working electrode (WE), a platinum wire as counter electrode (CE) and a saturated calomel electrode (SCE) as reference electrode (RE). The working electrode was carefully polished before its use by mechanical polishing employing 0.05 mm alumina paste and successively cleaned ultrasonically in distilled water for 5 min.

Measurement. The solution (PBS 1x) was degassed by bubbling Ar for at least 30 min before measuring and the cyclic voltammetry was carried out by cycling the

potential range from 0.0 to -0.8 V at different scan rate: 5 mV/s– 10mV/s – 20mV/s – 50mV/s – 100mV/s.

EPR spectroscopy. EPR spectra were recorded at 298 K using an ELEXYS E500 spectrometer equipped with an NMR gaussmeter for the calibration of the magnetic field and a frequency counter for the determination of g-factors that were corrected against that of the perylene radical cation in concentrated sulfuric acid ($g = 2.002583$). Light irradiation and EPR measurements were carried out on the sample in a capillary tubes (1 mm i.d.). Measurement conditions: modulation amplitude = 1.0 G; conversion time = 163.84 ms; time constant = 163.84 ms; modulation frequency 100 kHz; microwave power = 6.4 mW; microwave frequency 9.375 GHz.

¹O₂ generation by the EPR spin-trapping method. ¹O₂ was detected by EPR using 2,2,6,6-tetramethyl-4-piperidone (4-oxo-TEMP, Sigma-Aldrich, Cat. no. 459119) as probe. To 0.1 mM C₆₀@lysozyme (40 μL) in milliQ water, 0.5 M 4-oxo-TEMP (20 μL), and milliQ water (140 μL) were added and mixed well. The mixed solution was introduced into a capillary tube, deaerated under nitrogen flux, sealed and placed inside an EPR tube. The sample was irradiated by a UV-filtered 500 W high pressure mercury lamp and subjected to EPR measurements. The generation of singlet oxygen was detected by the three line EPR signal ($a_N = 16.13$ G) corresponding to 4-oxo-TEMPO, formed by the reaction of ¹O₂ with 4-oxo-TEMP.

·OH generation by the EPR spin-trapping method. ·OH was detected by EPR using 5,5-dimethyl-1-pyrroline-N-oxide (DMPO, Sigma-Aldrich, Cat. no. 92688) as a spin-trapping reagent. To a 0.1 mM C₆₀@lysozyme (40 μL) in milliQ water (20 μL), 0.1 mM DMPO (2 μL), and milli Q water (180 μL) were added and mixed well. The mixed solution was introduced into a capillary tube, deaerated under nitrogen flux,

sealed and placed inside an EPR tube. The sample was irradiated by a UV-filtered 500 W high pressure mercury lamp and subjected to EPR measurements. The generation of $\cdot\text{OH}$ was detected as five line signal corresponding to DMPO-OH, formed by the reaction of $\cdot\text{OH}$ with DMPO ($a_{\text{N}} = a_{\text{H}} = 14.75$ G).

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