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Article

C_{60} Bioconjugation with Proteins: Towards a Palette of Carriers for All pH Ranges

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Abstract: The high hydrophobicity of fullerenes and the resulting formation of aggregates in aqueous solutions hamper the possibility of their exploitation in many technological applications. Noncovalent bioconjugation of fullerenes with proteins is an emerging approach for their dispersion in aqueous media. Contrary to covalent functionalization, bioconjugation preserves the physicochemical properties of the carbon nanostructure. The unique photophysical and photochemical properties of fullerenes are then fully accessible for applications in nanomedicine, sensoristic, biocatalysis and materials science fields. And yet, proteins are not universal carriers. Their stability depends on the biological conditions for which they have evolved.

Here we present two model systems based on pepsin and trypsin. These proteins have opposite net charge at physiological pH. They recognize and disperse C_{60} in water. UV-Vis spectroscopy, zeta-potential and atomic force microscopy analysis demonstrates that the hybrids are well dispersed and stable in a wide range of pH's and ionic strengths. A previously validated modelling approach identifies the protein binding pocket involved in the interaction with C_{60}. Computational predictions, combined with experimental investigations, provide powerful tools to design tailor-made C_{60}@proteins bioconjugates for specific applications.

Keywords: fullerenes; nanohybrids; nanobiotechnology; bioconjugation; chemical stability

1. Introduction

C_{60}, the most representative member of the fullerenes family, has steadily attracted interest for its possible use in various fields, including nanomedicine [1–7]. A plethora of fullerene-based compounds have been synthesized with different targets. They display a range of biological activities that are potentially useful in anticancer therapy, antimicrobial therapy, enzyme inhibition, controlled drug delivery, and contrast or radioactivity-based diagnostic imaging [8–13,7]. Noteworthy is the possibility of their use in photodynamic and photothermal therapies [8,14,15]. The photophysical and electrochemical properties of C_{60} depend on their dispersion and a strict control of their disaggregation is truly necessary for nanotechnological applications [16,17]. To date two main approaches have been followed to tackle fullerene insolubility in water:

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i) the covalent approach is the more used method to prevent fullerene aggregation. The benefits obtained by functionalization are often offset by reduced photophysical performances [18];
ii) the noncovalent approach requires the use of supramolecular hosts that are amphipathic molecules able to interact with a single fullerene and to screen it from the aqueous environment. A variety of hosts is capable of interacting with fullerenes. They include surfactants, synthetic polymers, biopolymers, cyclodextrin [19], to name a few. In all cases, they stabilize small clusters of fullerenes [20]. In recent years, also proteins have become used as dispersing agents of fullerenes[21–24], CNTs [25–29] and graphene [30]. Proteins are naturally amphiphilic. This feature may avoid complicated synthetic procedures or the use of organic solvents. Most proteins are also pH responsive, which is an advantage for some manipulations [26]. Steric hindrance and electrostatic repulsion are the key factors determining the stability of the dispersion of carbon nanomaterials-protein complexes in aqueous solutions [31].
From the biological point of view, encapsulation of fullerenes by proteins may control and possibly decrease the cytotoxicity. Well-dispersed CNTs are less toxic than their agglomerates [32]. Protein binding can also alter the cellular pathways of interaction with carbon nanomaterials. Ultimately, coating of carbon nanomaterials with proteins can confer them a new biological identity [33].
We recently proposed the use of lysozyme to disperse with a 1:1 stoichiometry C_{60} in water [22]. The hybrid is well-defined and the fullerene binds selectively in the protein-substrate binding pocket. The protein-based supramolecular adduct preserves the photophysical properties of C_{60} and allows the exploitation of C_{60} as a photosensitizer for photodynamic treatments [34].

In this work, we evaluate the stability of C_{60}@protein complexes in biologically relevant conditions. Two proteins characterized by opposite net charges in physiological conditions were used as model systems and the role of the electrostatic contribution to the stability of their adducts with C_{60} is identified. Applications of docking protocols and MMPBSA calculations [35,36] further provide accurate description of the C_{60} binding pocket involved in the interaction between protein and C_{60}.

2. Materials and Methods

Trypsin from porcine pancreas (Cat. no. T0303), pepsin from porcine gastric mucosa (Cat. no. P7012), fullerene C_{60} (Cat. no. 483036) were purchased from Sigma Aldrich. They were used without further purifications. Phosphate buffered saline solutions were prepared dissolving the tablets purchased from Sigma Aldrich (Cat. no. P4417) in milliQ water.

2.1 C_{60}@Protein Synthesis

The C_{60}@protein hybrids were prepared mixing an excess of fullerene powder with a 0.3 mM solution of each protein (5 mL), with a 2:1 stoichiometry. NaOH and HCl 1M were used to adjust pH of the protein solutions. The heterogeneous mixtures were then sonicated in a vial for 120 minutes using a probe tip ultrasonicator (Hielscher Ultrasonic Processor UP200St, equipped with a sonotrode S26d7, used at 40% of the maximum amplitude). During the process, the sample was refrigerated with an ice bath. The dark brown turbid mixture obtained after the sonication was centrifuged at 10 kRCF. The resulting supernatant was then collected and characterized.

2.2 C_{60}@Protein Characterization

UV-Vis absorption spectra were recorded at 25 °C by means of Agilent Cary 60 UV-Vis Spectrophotometer. Surface charge analysis of the hybrids were estimated measuring the zeta-potential at 25 °C by means of Malvern Nano ZS.
AFM experiments were performed at the SPM@ISMN microscopy facility in Bologna. AFM analysis (Digital Instruments, Multimode VIII equipped with a Nanoscope V) operated in ScanAsyst mode were performed to evaluate the quality of the monodispersion of the bioconjugates. The

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samples were prepared by drop casting 10 µL of C_{60}@protein solution onto a freshly cleaved mica substrate for 10 minutes then rinsed with milliQ water and dried under nitrogen flux/stream.

2.3 Computational protocol

Generation of the poses. Docking models were obtained using the PatchDock algorithm [37]. PatchDock takes as input two molecules and computes three-dimensional transformations of one of the molecules with respect to the other with the aim of maximizing surface shape complementarity, while minimizing the number of steric clashes.

Scoring of the poses. Accurate rescoring of the complexes is then carried out using FireDock program [38]. This method simultaneously targets the problem of flexibility and scoring of solutions produced by fast rigid-body docking algorithms. Sidechain flexibility is modeled by rotamers and Monte Carlo minimization [39]. Following the rearrangement of the side-chains, the relative position of the docking partners is refined by Monte Carlo minimization of the binding score function. Free energy of solvation/desolvation in the binding process is taken into account by a solvation model that uses estimated effective atomic contact energies (ACE) [40]. All the candidates are ranked by a binding score [40]. This score includes, in addition to atomic contact energy used to estimate the desolvation energies [40], van der Waals interactions, partial electrostatics, explicit hydrogen and disulfide bonds contribution. In addition, three components to the total binding score are added: $E_{\pi-\pi}$ for the calculation of the π–π interactions, $E_{cation-\pi}$ for the calculation of the cation–π interactions and E_{aliph} for the calculation of hydrophobic interactions.

Minimizing the pose. The best poses for every selected protein were full minimized by AMBER 12 [41]. The ff12SB force field [41] was used to model the proteins, while the fullerene atoms were modeled as uncharged Lennard–Jones particles by using the CA atom type (sp2 aromatic carbon parameter), also from the AMBER force field. The minimization was carried out with sander, using the GB (Generalized Born) model [42] for the solvation and no cut-off for van der Waals and electrostatic was used.

MM-GBSA analysis. In order to identify the residues responsible for the binding of the proteins to C_{60}, we carried out a decomposition analysis of the optimized structure according to the MM-GBSA scheme [35,36]. The per-residue decomposition analysis provides the contribution of the individual amino acids to the binding.

3. Results and discussions

The ability of C_{60} to interact with proteins is a recent subject of investigation. Collectively, van der Waals, hydrophobic and electrostatic interactions must cooperate to establish energetically favorable interactions between a protein and a fullerene in order to allow the formation of a stable complex [43]. Geometrical complementarity also plays a primary role to maximize the effect of the stabilizing contributes [44]. Crucial for the understanding of protein-fullerene interactions is the identification of the fullerene-binding site together with the possible subsequent proteins structural modification [45]. It should also be further assessed if the interaction occurs between a single fullerene with a single protein or if fullerenes clusters are surrounded by a number of proteins.

Pepsin (pI = 2.2 - 3) [46] and trypsin (pI = 10.2 - 10.8) [47] are proteins characterized by very different values of isoelectric point, which makes one negatively and the other positively charged in physiological conditions. Sonication of C_{60} with each protein was performed in acidic (pH 2), neutral, and basic pH (pH 12) of unbuffered aqueous solutions. Pepsin was able to disperse fullerene in water only at basic pH, where the protein is negatively charged, while trypsin showed the best performances at acidic pH.

The two batches of hybrids were synthetized under optimized conditions. After sonication and centrifugation, the supernatants were collected and characterized. UV-Vis spectra of the solutions (Figure 1) show the diagnostic absorption bands of C_{60} at 341 nm and the overlap of C_{60} and protein absorption bands between 260-290 nm. Based on the extinction coefficients of both components of the

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adducts [48], the absorption spectra suggest a 1:1 stoichiometry between C_{60} and trypsin, while 1:2 stoichiometry can be estimated for the C_{60} and pepsin complex. UV-Vis spectra also suggest that the presence of particle aggregates, observed prior to centrifugation, was completely removed since scattering is not exhibited.

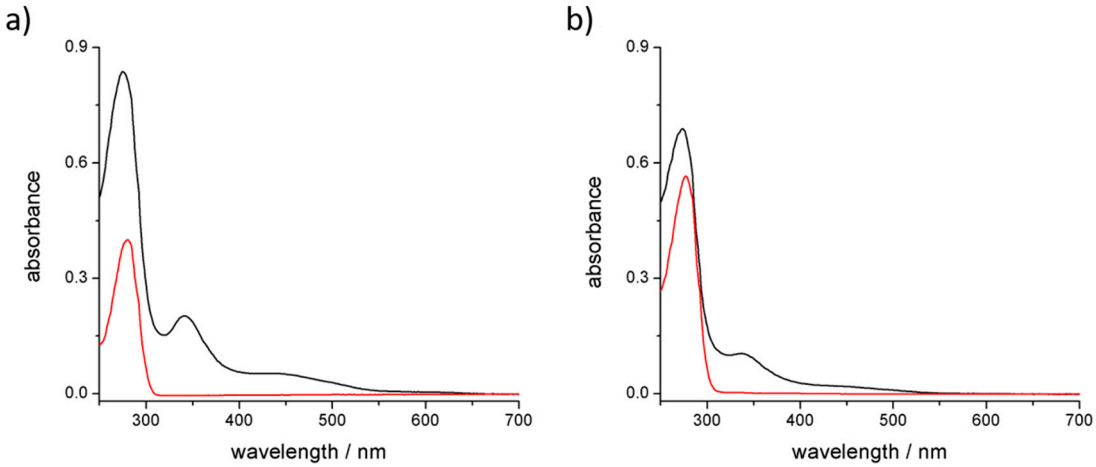

Figure 1. UV-visible spectra of (**a**) C_{60}@trypsin (black line) and trypsin (red line); (**b**) C_{60}@pepsin (black line) and pepsin (red line).

3.1. C_{60}@pepsin – C_{60}@trypsin, an atomistic view

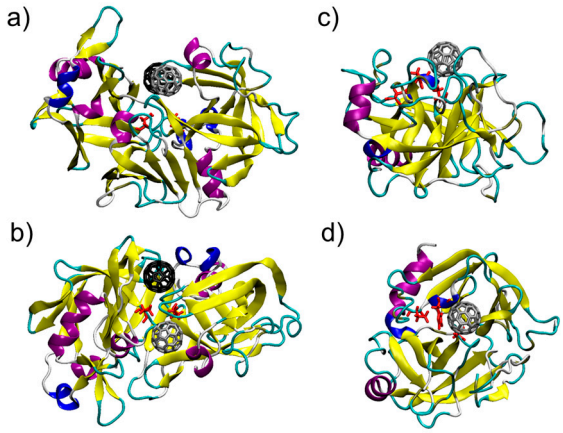

Figure 2. Two perspectives of C_{60}@pepsin (**a, b**) and C_{60}@trypsin (**c, d**). In red, the catalytic residues of the two proteins.

Surface complementarity between the proteins and the C_{60} surface appears. The results of the docking protocol explain the stoichometry observed by the UV-visible spectra. Pepsin is characterized by a dimeric interface region. In this region, two fullerene binding pockets are identifiable and are able to bind two C_{60} cages (figure 2a and 2b). The binding between C_{60} and pepsin is not surprising, since pepsin is an aspartic protease and structurally strongly correlates to HIV protease: fullerenes are well known inhibitors of HIV-1 protease [49–52]. In pepsin, as in the HIV protease, fullerenes block the large active site groove [49–52]. C_{60} is also a known serine protease inhibitor [53], and in fact C_{60} binds in the trypsin active site: a single, well defined binding pocket is identified by the docking protocol in this region (figure 2c and 2d). For the two C_{60}@protein hybrids tested here, MM-GBSA analysis of the structures in their optimized geometries provides a quantitative description of the C_{60} binding pocket and identifies the more effectively interacting residues. Table 1 shows the 10 largest

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interactions between the residues of the proteins and C_{60}. The three most interacting residue for binding pocket are represented in Figure 3a-c.

Table 1. Interaction energies (kcal mol^{-1}) of the top 10 residues interacting with C_{60}.

C_{60}@Pepsin-Binding pocket 1	Phe 111 = -5.7	Leu 112 = -3.1	Thr 218 = -3.0	Ser 219 = -2.9	Thr 12 = -2.8
	Glu 13 = -2.8	Phe 117 = -2.6	Ile 30 = -2.5	Tyr 75 = -2.5	Thr 77 = -2.2
C_{60}@Pepsin-Binding pocket 2	Val 291 = -4.9	Thr 74 = -4.3	Pro 292 = -3.7	Tyr 75 = -3.4	Gly 76 = -2.7
	Met 289 = -1.4	Thr 293 = -1.3	Tyr 189 = -1.2	Asp 290 = -1.0	Leu 298 = -0.6
C_{60}@Trypsin	His 57 = -4.9	Phe 41 = -4.2	Gln 192 = -3.5	Cys 58 = -3.4	Cys 42 = -2.7
	Gly 193 = -1.8	Ser 195 = -1.7	Asp 194 = -0.8	Tyr 151 = -0.6	Leu 99 = -0.4

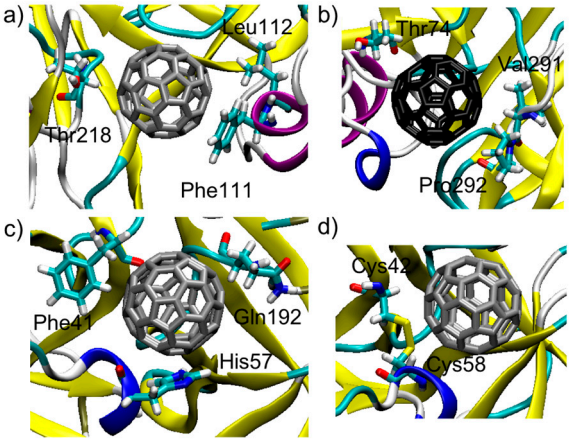

Figure 3. Top 3 residues interacting with C_{60} in the (**a**) pepsin binding pocket 1, (**b**) pepsin binding pocket 2; (**c**) Top 3 residues interacting with the C_{60} in the trypsin binding pocket; (**d**) Interaction in the trypsin binding pocket between C_{60} and a disulfide bridge (Cys42-Cys58).

From Table 1 and Figure 3 it appears that proteins are able to interact with C_{60} via:

i) π–π stacking interactions that are established between aromatic residues (phenylalanine, tyrosine, histidine) and C_{60} surface [25,54];

ii) Hydrophobic interactions (leucine, isoleucine, methionine, proline, glycine) that are established in water between aliphatic residues and C_{60} surface [25];

iii) Surfactant-like interactions where amphiphilic residues (threonine, serine, aspartate) behave similarly to surfactants and solvate C_{60}. The hydrophobic aliphatic chains of these residues interact with C_{60} surface, whereas the hydrophilic groups point out toward water [25,55,56].

In the case of trypsin, of interest is the interaction between a disulfide bridge (Cys42-Cys58) and C_{60} (Figure 3d). This kind of interaction was recently highlight by Hirano and coworkers for carbon nanotubes [57,58].

3.2. AFM analysis of C_{60}@protein hybrids

UV-Vis spectra and molecular modelling exhibit the expected stoichiometry between C_{60} and proteins. They do not give information about the possible aggregation of the adducts. Atomic force microscopy is a direct technique to evaluate the size distribution of particles.

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In Figure 4a, the C_{60}@trypsin hybrids are monomoleculary dispersed when deposited on a negatively charged mica surface. C_{60}@trypsin is positively charged, hence an electrostatic interaction takes place with the surface. The profile analysis (Figure 4b) of both C_{60}@trypsin and the trypsin reference (obtained in the same conditions) shows an average height of ~1.5 nm, which is slightly lower than the expected value. This behavior is a consequence of the strong electrostatic interaction, which squashes the proteins over the surface in order to maximize the attractive electrostatic contacts. Conversely, negatively charged pepsin hybrid (Figure 4c) shows an average height, which is slightly higher than the average size of the protein.

These results mainly originate from the combination of different forces: i) the pepsin tendency to self-associate; ii) the electrostatic repulsion between the pepsin and the surface, which reduces the number of interactions, as confirmed also by the small number of the particles deposited on the mica which repels the adduct. The AFM analysis demonstrates the absence of C_{60}@proteins aggregates, or nC_{60} clusters dispersed by the proteins.

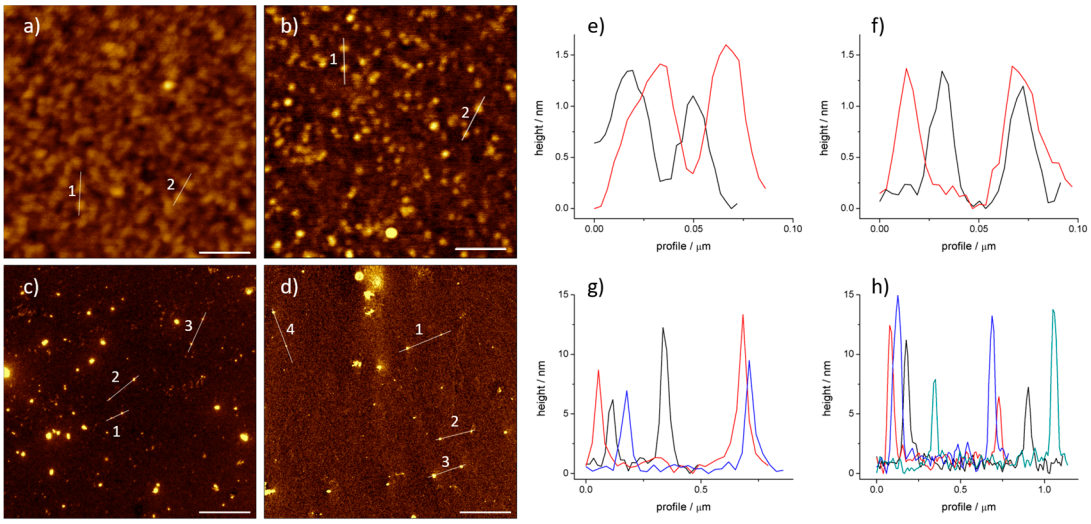

Figure 4. AFM images of (**a**) C_{60}@trypsin; (**b**) trypsin; (**c**) C_{60}@pepsin; (**d**) pepsin. Profile analysis of the height of (**e**) C_{60}@trypsin; (**f**) trypsin; (**g**) C_{60}@pepsin; (**h**) pepsin. Scale bar (**a,b**) 100 nm; (**c,d**) 1 µm. The lines in the AFM provide the numbering of the AFM profiles: profile 1 in black, profile 2 in red, profile 3 in blue and profile 4 in green.

3.3. Stability of the complex in aqueous media

Compared to the chemical functionalization of the fullerenes, one of the advantages from the use of host-guest system is the possibility to tune the stability of the complex in aqueous media. The tuning can be achieved by acting only on the host system, that is the protein. Evaluating the behavior of C_{60}@proteins at different pH's and physiological conditions, it was found that the stability of the hybrid in aqueous media was completely governed by the protein. To understand if proteins pH sensitivity was retained, acid-basic titration was performed. Zeta potential and UV-Vis spectra were obtained. The correlation between zeta potential and pH gives information about the behavior of the complex for possible future *in vivo* experiments, since pH varies in different compartments of the organisms. Moreover, the greater the range of pH stability the wider the conditions for subsequent manipulation of the adduct. pH dependent zeta potential trends of C_{60}@trypsin and C_{60}@pepsin are shown in Figure 5.

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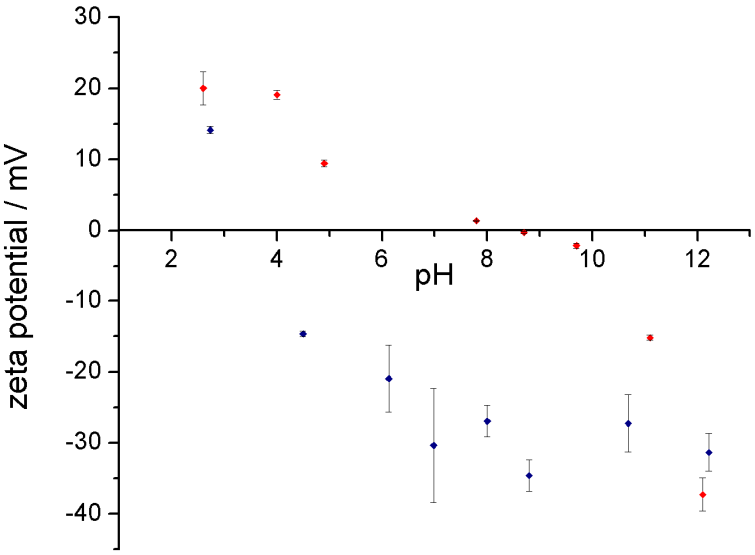

Figure 5. Zeta potential of C_{60}@trypsin (in red) and C_{60}@pepsin (in blue) hybrids as a function of the pH in aqueous solution. Standard deviations are shown in the error bars.

The isoelectric points (IEP) of both adducts resulted slightly shifted to values of pH's closer to neutrality with respect to IEP of the pristine proteins. This phenomenon can be attributed to a reduced accessibility to pH sensitive groups upon fullerene complexation. A further effect is related to the local change of the environment polarity, which could slightly perturb the pKa of few charged residues. For pH values closer to the IEP, the electrostatic repulsion between the proteins/adducts becomes minimal. The stability of possible aggregates is governed only by steric hindrance.

For C_{60}@pepsin complexes at pH values close to IEP (2.7 and 4.5), aggregation phenomena indeed occurred after few minutes. C_{60}@trypsin complexes did not aggregate also for pH values close to the IEP. The maximum stability for individual C_{60}@pepsin complexes was obtained in neutral and basic conditions. Absorption spectra performed on the same samples did not show changes of shape and intensity (Figure 6) between the different samples. In both the cases, the complex resulted stable for (at least) one week.

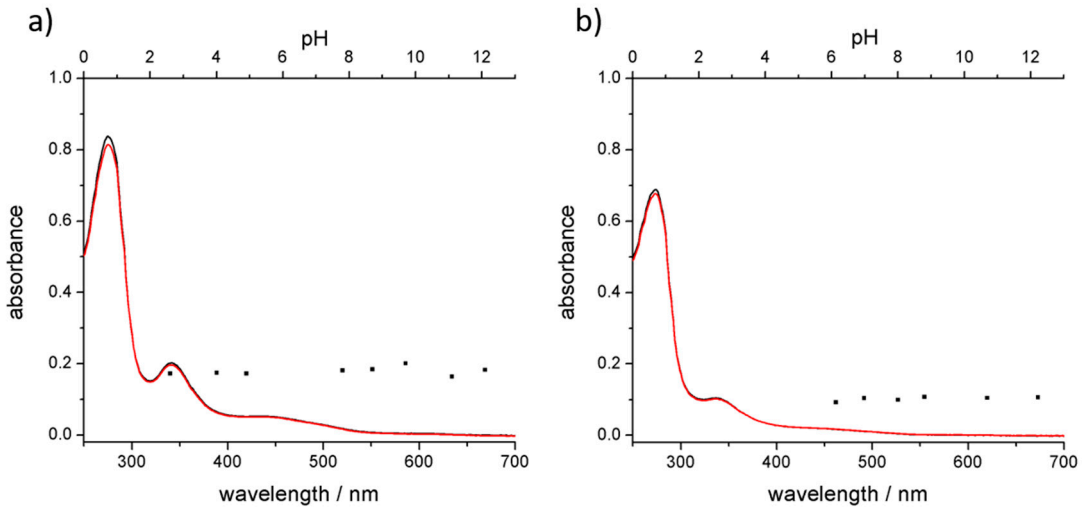

Figure 6. UV-Vis absorption spectra of (**a**) C_{60}@trypsin and (**b**) C_{60}@pepsin in water (black lines) and PBS (red lines). Black dots represent the absorbance of fullerene diagnostic band (341 nm) of the hybrids at different pH values (top axis).

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Comparison of the absorption spectra of C_{60}@trypsin (Figure 6a) and C_{60}@pepsin (Figure 6b) in water and PBS shows that the hybrids are stable also in physiologically relevant conditions (represented by PBS). This is an important difference with other C_{60} adducts, for instance fullerenes dispersed by cyclodextrins rapidly precipitates when NaCl is added [59].

These results suggest that fine-tuning of the net charge of the complex is possible and therefore it should also be possible to take advantage of the nature of each protein to create optimal C_{60}-protein systems as a function of the pH. Tuning the net charge of the protein used to host the C_{60} molecule it is possible to governs its interactions with cellular and bacterial surface, controlling C_{60} toxicity [60–63].

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