Protein-Directed Crystalline 2D Fullerene Assemblies

Repeat proteins with engineered tyrosine clamps provide a platform for fullerene assembly into 2D crystalline materials with long range molecular order and photogenerated charge carrier capacity. Thus, the self-assembling hybrid material allows to utilise the innate properties of fullerenes, demonstrating the potential of engineered protein-based functional materials.
Water soluble 2D crystalline monolayers of fullerenes grow on planar assemblies of engineered consensus tetratricopeptide repeat proteins. Designed fullerene-coordinating tyrosine clamps on the protein introduce specific fullerene binding sites, which facilitate fullerene nucleation. Through reciprocal interactions between the components, the hybrid material assemblies into two-dimensional 2 nm thick structures with crystalline order, that conduct photo-generated charges. Thus, the protein-fullerene hybrid material is a demonstration of the developments toward functional materials with protein-based precision control of functional elements.

Introduction

In nature, proteins are the main components of cell structure, catalysts and cell regulators, due to the chemical stability and the structural diversity. These properties also make protein-based materials very appealing for different biotechnological and nanotechnological applications, such as optoelectronics, cell signalling, plasmonics, and catalysis, among others. As the performance of hybrid materials relies heavily on precise organization of the functional elements within the materials and the emergent properties, many research efforts have been focussed on the design of encoded building blocks, which would allow reliable control over the arrangement of the active components as well as maintain long-range order. However, control of the coupling specificity between the active elements and engineered protein-based biomaterials remains a great challenge.

Despite the advances in understanding of protein manipulation and creation of artificial constructions with enhanced properties, rational protein design is still far from straightforward due to the limited understanding of the sequence-structure-function relationship. The problem is somewhat mitigated when working with small repeat proteins, that are amenable to modular design and thus allow local modifications without affecting global structure. Consensus tetratricopeptide repeat (CTPR) protein has been particularly useful in the synthesis of hybrid materials. CTPR proteins are made of modules of 34 amino acids, folded into a helix-turn-helix motif; however, the fold is defined by only 8 conserved residues, thus allowing a lot of freedom for protein engineering. The modules generally appear repeated in tandem, from 2 to 20 repeats, generating a rigid right-handed superhelical structure that is able to function as stable scaffold in both biological and laboratory settings. Thus, CTPR proteins have been successfully used to build photoconductive and electroactive systems, combining covalent modification and non-covalent interactions, as well as stabilise fluorescent and electroactive clusters.

In the current work, the CTPR proteins were engineered into scaffolds for C60 fullerene assembly. Carbon nanoforms combined with biomimetic nanomaterials have driven the advancement in nanoscience, biochemistry and materials chemistry in the recent years. Carbon fullerene, since its discovery, has been of particular interest due to its unique chemical and physical properties. The hydrophobic nature of the carbon species allows the fullerene to form complexes with proteins, leading to biological activity. A systematic computational analysis suggested the fullerene cage is able to scan the protein surface and identify a suitable hydrophobic pocket to accommodate. Recently, fullerenes were co-crystallised with short helical peptides coordinating the C60 spheres through a single strategically placed aromatic residue, showing that the fullerene binding sites can be precisely defined. In the current work, tyrosine-based fullerene binding sites were engineered onto CTPR protein for fullerene coordination along the protein surface. The resulting protein-fullerene hybrid material assemblies into two-dimension structures with long-range crystalline organisation in aqueous medium and improved photoconductivity through stabilisation and transmission of photo-generated charge carriers.
Results and Discussion

CTPR proteins were identified as good candidates for hosting fullerenes due to high abundance of tyrosine (6 residues per repeat), that could form π-π stacking interactions with the fullerenes (Fig. 1a). Protein composed of eight repeats comprising a full superhelical turn, CTPR8, was chosen as the scaffold, given the stability and the well characterised properties of the eight-repeat protein. In addition to the shape compatibility to accommodate carbon nanoforms in the hydrophobic inner cavity,13 the CTPR protein also contains inward-pointing lysine residues that can further stabilise the protein-C60 conjugates.27-29

Fullerene C60 was suspended in a solution of CTPR8 and the suspension was sonicated using a tip sonicator, after which the reaction was centrifuged to remove excess fullerene particles. Initial observations were promising, as the supernatant retained some of the dark colour, signifying that some of the otherwise completely water-insoluble fullerene was solubilised through interactions with the protein. The CTPR8-C60 hybrid material was purified from the supernatant by fast protein liquid chromatography (FPLC) (Fig. 1b). The absorption features of this new assembly confirmed the presence of the fullerene, with the appearance of the characteristic absorption bands at 266 and 343 nm and a shoulder around 450 nm (Fig. 1c). However, a substantial amount of free CTPR8 protein also eluted. The low extent of complex formation was attributed to poor dispersion of the fullerene in aqueous solutions even under sonication, thus coupling was attempted with a more water-soluble fullerene derivative C60 pyrrolidine tris-acid (C60A) (Fig. 1b). In this case, a higher degree of functionalisation was achieved, clearly observed with just the naked eye from the intense brown-solution. UV/vis spectra of the CTPR8-C60A co-assembly clearly showed a depletion of the characteristic C60 fullerene band at 330 nm and the loss of the feature band at 430 nm associated with the saturation of a C=C double bond in the C60 fullerene monoadduct, as well as considerable broadening of absorbance bands along the spectra (Fig. 1c). These absorption features of fullerene-based materials are typically attributed to interactions between fullerenes, e.g. in fullerene based colloids,10, 31 C60 films and aggregates.12-34 In both CTPR8-C60 and CTPR8-C60A assemblies, the α-helical structure of the protein was unaffected by fullerene binding.

Fig. 1. Schematic for CTPR8 and fullerene derivatives and characterisation of co-assemblies by FPLC chromatograms and UV-vis spectra. (a) The structures of CTPR8 (PDB ID: 2HY2) and the fullerene derivatives; tyrosines (red) and lysines (blue) are highlighted. (b) FPLC chromatograms of CTPR8-C60 (red) and CTPR8-C60A (blue) hybrid materials and free CTPR8 (gray). (c) UV-visual spectra of CTPR8-C60 (red) and CTPR8-C60A (blue) hybrid materials and free CTPR8 (gray).
The CTPR proteins were then reengineered to exert more specific interactions with the fullerene surface. Two additional tyrosine residues were introduced in positions 2 and 9 of the A helix of every repeat, thus positioning two aromatic rings ca. 1 nm apart, optimal to clamp a fullerene molecule (Fig. 2a). The modified protein, CTPR8Y16, expressed well and showed the same helical structure as the original protein, based on CD spectroscopy (Fig. 2b). The mutant protein also showed improved fullerene binding, resulting in significantly greater amounts of hybrid material, with no free protein recovered in the case of the more water-soluble C60 during purification, as determined by gel filtration chromatography (Fig. 2c).

The CTPR8Y16-C60 and CTPR8Y16-C60A hybrids also retained the α-helical structure of the protein (Fig. 2b). More significantly, fullerene-bound protein became more resistant to thermal denaturation. With incrementally increased temperature, the free protein CTPR8Y16 reached midpoint denaturation at Tm = 70.5 °C. The pristine C60-based conjugate CTPR8Y16-C60 was marginally more stable, with Tm = 73.0 °C, while the midpoint denaturation temperature of CTPR8Y16-C60A could not be directly determined, as the protein was not completely unfolded even at 95 °C (Fig. S1). Thermal denaturation of the complex was then monitored in the presence of increasing concentrations of urea, and Tm for CTPR8Y16-C60A was extrapolated to 90.0 °C (Fig. S2). The observed enhancement of protein thermal stability can likely be explained by protein core stabilisation through C60 binding.

The CTPR8Y16-C60A assembly was then investigated by Raman scattering spectroscopy. For pristine C60 and C60A the Raman spectra showed the characteristic A1g(2) pentagonal pinch mode located at 1466 and 1462 cm⁻¹, respectively. In the CTPR8Y16-C60A co-assembly, however, the A1g(2) mode was shifted towards lower frequencies at 1457 cm⁻¹ (Fig. S3). This significant downshift of the Raman A1g(2) peak for the CTPR8Y16-C60A hybrid material suggests possible electronic interactions between the protein and the electron-acceptor C60, which is in agreement with previous findings for the non-covalent modification of fullerene with electron donor units. In the current case the presence of electron donor aromatic residues in the protein favour the interaction with the fullerene surface through π-π and OH···π interactions.

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Fig. 2. Schematic of modified protein and characterisation of co-assemblies by different techniques. (a) The model of CTPR8Y16 showing the newly introduced tyrosine clamps. (b) CD spectra of CTPR8Y16-C60 (purple) and CTPR8Y16-C60A (green) co-assemblies compared with CTPR8Y16 (gray). (c) FPLC gel filtration chromatograms of CTPR8Y16-C60 (purple) and CTPR8Y16-C60A (green) hybrid materials and free CTPR8Y16 (gray). (d) UV-visual spectra of CTPR8Y16-C60 (purple) and CTPR8Y16-C60A (green) hybrid materials and free CTPR8Y16 (gray).
Fig. 3. Cryo-electron microscopy analysis of the hybrid materials. Cryo-electron micrographs of (a) CTPR8Y_{16}-C_{60}A, (b) CTPR8-C_{60}A, (c) CTPR8Y_{16}-C_{60} and (d) CTPR8-C_{60} hybrid materials. (e) Close-up images of the areas marked with black squares in A-D. (f) A tilt series of a selected region of CTPR8Y_{16}-C_{60}A hybrid material at different degrees of sample tilt, showing the selected assembly from different perspectives. (g) Representative crystallites of CTPR8Y_{16}-C_{60}A hybrid material and the corresponding FFT images, showing the diffraction patterns of crystalline assemblies. The scale bar is 20 nm in all cases.
Cryo-electron microscopy was employed to gain structural insights into the protein-fullerene assemblies. Well-defined structures with highly distinct features were visible in the micrographs of the CTPR8Y$_{16}$-C$_{60}$A assembly (Fig. 3a). 3D analysis of the structures revealed that the protein-fullerene hybrids assemble into thin 2D sheets of irregular shape, that are mostly flat, but may have a small twist (Fig. 3f, S4). The sheets are ca. 2 nm thick, with dimensions of 20 nm × 20 nm on average, although reaching up to 50 nm (more precise measurements are not possible, as it is impossible to determine the precise orientation of the objects in vitrified drops). From a side view, the sheets appear made up of two parallel high contrast bands of ca. 0.7 nm, with a low contrast band of similar thickness between. The thickness of the film is consistent with a monolayer of CTPR protein, and the high contrast bands suggest the fullerene are (predominantly) assembling on the surfaces of the protein monolayers. From face view, the plaques have very low contrast due to their thickness, but they are identifiable due to a very strong diffraction pattern, which indicates that the assemblies are ordered on the molecular level. As the particles are dispersed in the vitrified fluid, diffraction patterns from individual aggregates can be studied (Fig. 3g). The observed interplanar spacing can be divided into two groups, localized in the 0.85-0.79 nm region and the region between 0.50 nm and 0.40 nm. The observed interplanar distances are consistent with the diffraction signal from ordered fullerenes, as the distances are far too big for salt crystals, but far too small to originate from crystallised protein. The observed variability of diffraction distances could be a consequence of differing orientations of the ordered assemblies within the vitrified fluid, exposing diffraction patterns from different planes, or could signify that slightly different arrangements are possible for the fullerenes.

The CTPR8-C$_{60}$A assembly also formed similar 2D objects, but more sparsely and of smaller size, and without the two distinct bands in the side view, but rather of uniform contrast along the 2 nm thickness (Fig. 3b). The sample also produced similar diffraction patterns, although generally of lower intensity. This result strongly suggests that although the CTPR fold alone is able to facilitate fullerene nucleation, the “tyrosine clamps” direct fullerene assembly with much higher specificity, resulting in more extensive arrangements.

In the cases of CTPR8Y$_{16}$-C$_{60}$ and CTPR8-C$_{60}$ where pristine fullerene was used, the thin sheets were not free-floating, but instead clumped into large clusters, with various independent and very strong diffraction patterns in different parts of the plaques (Fig. 3c,d, S5). This behaviour is also explained by the proposed fullerene binding mode: when bound on the surface of the protein sheets, C$_{60}$A orient themselves to expose the hydrophilic tris-acid appendages to the solvent, thus contributing to the solubility of the superstructures, whereas pristine C$_{60}$ fullerenes introduce hydrophobic patches of carbon, causing the sheets to coalesce. The diffraction patterns of the C$_{60}$ assemblies showed on average smaller spacing (0.82-0.77 nm) than those of C$_{60}$A (vide supra), which could be an indication that greater spacing is required to accommodate the pyrrolidine-tris acid groups (Fig. S5).

To elucidate the molecular basis of the conjugation process, binding events were reconstructed with Molecular Dynamics (MD) simulations. Five replicas of 800 ns of MD were performed in the presence of eight [60]fullerene (either C$_{60}$ or C$_{60}$A) molecules and one CTPR8 unit (either CTPR8 or CTPR8Y$_{16}$), simulating the CTPR8-C$_{60}$, CTPR8-C$_{60}$A, CTPR8Y$_{16}$-C$_{60}$ and CTPR8Y$_{16}$-C$_{60}$A co-assemblies. To characterise the spontaneous binding process, all [60]fullerene species were initially placed in the solvent at least 10 Å away from the protein surface and were allowed to freely diffuse from the solvent to the protein surface along the MD trajectory. From these spontaneous binding MD simulations, the interactions established between the fullerene species and CTPR8 units were monitored along time to identify the most visited binding sites (hot spots) on the protein surface. In all cases, [60]fullerene species rapidly collapsed over the protein surface, establishing transient interactions with the side chains of superficial protein residues. The initial recognition with CTPR8 took place in few nanoseconds of simulation time, and the fullerene species were then displaced over the protein surface until they identified a binding site (Fig. 4), where commonly they remained for the rest of the simulation. Both C$_{60}$ and C$_{60}$A prefer to interact with the CTPR modules located at both extremes of the CTPR8 unit, while permanent interactions with the inner hydrophobic cavity were observed less frequently. To identify the most visited binding sites, representative snapshots from the last 100 ns of each replica of the MD simulations were extracted and the position of each fullerene with respect to the protein surface was plotted (colour spheres in Fig. 4). CTPR8-C$_{60}$, CTPR8-C$_{60}$A and CTPR8Y$_{16}$-C$_{60}$ assemblies showed a wide dispersion of fullerene molecules all over the protein surface. These results indicate that although the fullerene species tend to be in contact with protein residues, these interactions are not stable along the MD simulation and among replicas. On the contrary, for the CTPR8Y$_{16}$-C$_{60}$A co-assembly, fullerene molecules accumulate in selected hot spots independently of which MD simulation is analysed. These results indicate that the new tyrosine residues favour the association and stabilisation of C$_{60}$A at specific binding sites on the protein surface. These observations are in line with the highest fullerene/protein ratio experimentally observed for the CTPR8Y$_{16}$-C$_{60}$A co-assembly.
To explore the binding mechanism of CTPR8Y16-C60A in more detail, the distance between the centre of mass of CTPR8Y16 and the centre of mass of each of the eight C60A molecules along the MD trajectory was monitored (Fig. 5a). As shown in Fig. 5a and 5b, all fullerenes are recognised by CTPR8Y16 in less than 100 ns of MD simulation time, reaching the final binding site after 300 ns, where C60A remain for the rest of the simulation. Six out of the eight fullerene molecules bind hot spots located more than 25 Å far away from the CTPR8Y16 centre of mass, indicating that C60A prefer to interact with the CTPR modules located at the N- and C-terminal domains of CTPR8Y16. Close examination of molecular interactions showed predominantly π-π interactions between C60A and some of the introduced tyrosine residues (Fig. 5c). Thus, the introduced tyrosine residues enhance supramolecular interactions with the aromatic surface of the fullerene, fixing the fullerene molecule in a particular binding site. In some hot spots, the fullerene is stabilised by π-stacking interactions with a total of six tyrosine residues, i.e. Tyr209, Tyr213, Tyr216, Tyr240, Tyr243, and Tyr247 (A in Fig. 5c), located at the extreme of the CTPR8 unit. These interactions are able to retain the C60A molecule in the hot spot for the rest of the simulation time. Additional binding sites are located at the inner hydrophobic cavity and display a broader range of interactions (B in Fig. 5c). The simulations reveal that Arg203 establishes cation···π interactions with C60A, the introduced Tyr138 on helix A displays OH···π interactions with the fullerene, while Lys81 and Lys115 stabilise the carboxylate groups of C60A by salt bridge interactions. Such strong interactions are not observed in the simulations where pristine C60 is used, with the fullerene more delocalised all over the protein surface. To gain a deeper insight into the recognition and binding processes, five replicas of 500 ns of MD simulations with 24 C60A molecules were performed for CTPR8Y16. Increasing the number of fullerenes led to their aggregation in groups of 6-8 molecules mediated by sodium cations. Interestingly, fullerene aggregates accumulate at the same binding sites observed previously with a smaller number of C60A (Fig. 5d).

Finally, the potential role of C60A molecules on CTPR8Y16 aggregation was explored. To this end, five replicas of 200 ns of MD simulations were carried out with 24 C60A molecules...
and three CTPR8Y₁₆ protein units. The simulations showed that fullerene molecules first aggregate and then contribute to the formation of CTPR8Y₁₆ aggregates, acting as bridge molecules between CTPR8Y₁₆ units (Fig. 5e). Despite the short simulation time of these simulations and the model system used, MD simulations revealed a direct contribution of C₆₀A to the formation of CTPR8Y₁₆ assemblies.

These filaments, however, have no uniform structure and produce no electron diffraction; the assembly is thus likely governed by non-specific interactions observed in the simulations. The contacts with the ordered surface of CTPR protein, however, seem to serve as nucleation points for crystalline-ordered fullerene assemblies. Reciprocally, the fullerene appears to mediate and strengthen the interactions between the proteins that normally manifest in protein assemblies in solid state.4, 39 While during the simulations with 8 or 24 fullerene molecules preferential binding was observed, the more exposed binding sites at the termini of the protein, the large excess of fullerene present during the conjugation process would result in the occupation of all or nearly all possible binding sites, effectively coating the protein surface. The sonication, that could not be easily simulated, would further increase the mobility of the fullerene along the surface of the protein, facilitating the migration of the fullerene into the more hindered binding sites.

Fullerene C₆₀ crystallises in fcc lattice (a = 1.42 Å),40 transitioning into sc below 249 K (-24 °C),41, 42 however, depending on the additives used during crystallisation, a range of different lattices and spatial arrangements have been achieved.43-47 As the protein and the fullerene both actively contribute to the overall order of the hybrid assemblies, it is difficult to suggest the exact arrangement, and further research is needed to fully characterise the crystalline order. The analysis is greatly complicated by the low electron doses needed for cryo-electron microscopy, potentially resulting in loss of some of the finer diffraction peaks. Nevertheless, the thickness of the assemblies suggests the fullerene assembly exclusively in a monolayer, with the arrangement at least partially driven by fullerene interactions, rather than being entirely imposed by the protein. Furthermore, the crystalline arrangement of the hybrid material emerges and is stable in aqueous solution, with the fullerene present on the surface of the assemblies in contact with the aqueous phase. Under the right conditions, it is conceivable that the 2D lattice could be grown beyond the nanoscale, allowing to apply the potential of the carbonaceous assembly on a larger setting. Indeed, as the innate fullerene-fullerene contacts are maintained alongside with protein interactions, this new composite material is far more likely to retain some of the intrinsic electronic and photophysical properties of the constituent fullerene.48, 49

Hence, transient photoconductivity of CTPR8Y₁₆-C₆₀A was assessed using the flash-photolysis time-resolved microwave conductivity (FP-TRMC) system.50-53 The electrode-less method, based on microwave dielectric spectroscopy, allows to measure conductivity due to local-scale motions of charge carriers, and is not limited by deep traps of charges at grain boundaries. A drop-cast film of CTPR8Y₁₆-C₆₀A was photo-excited by 355 nm pulses, allowing to record time-dependent conductivity (Fig. 6a) from transiently photo-generated charge carriers (electrons and holes); the decay of the curve indicates the extinction of charges due to charge trapping and/or recombination processes. As expected, the maximum values of the transients are linearly dependent on the absorbed photon density, with slight deviation from a perfectly linear relationship caused by more frequent charge recombination as a result of high concentration of generated charges in the conductive material.
Conclusions

The described system provides a rare example of protein-directed ordered assembly of fullerenes. The new-engineered CTPR proteins with tyrosine clamps allows to steer the assembly of fullerenes into crystalline arrangements, while promoting innate fullerene interactions. As a result, the hybrid material supports strong photocurrents, allowing photo-generated charge carriers to move through fullerene contacts. Thus, the presented system brings protein research one step closer to supramolecular assemblies governed by rationally designed proteins. In addition, to the best of our knowledge the CTPR-fullerene hybrid assemblies present a first instance of crystalline assemblies observed in liquid phase. Despite the advances in successful protein design, it is noteworthy that correct choice of a starting structure plays a crucial role. Based on the current and multiple other supramolecular assemblies, using repeat proteins as a scaffold for supramolecular ordering seems to be the winning strategy.

Conflicts of interest

There are no conflicts to declare.

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SUPPORTING INFORMATION

Protein-directed crystalline 2D fullerene assemblies

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Experimental

Materials
Reagents, including [60] fullerene derivatives, were purchased from Sigma-Aldrich and were used without further purification.

Protein design and production
Molecular model of tyrosine mutations was build using Pymol. E2Y, N6A and N9Y point mutations constituting the mutated residues were introduced into C1 consensus repeat through QuikChange site-directed mutagenesis. CTPR8 proteins were constructed by sequential ligation of consensus and mutated C1 sequences into pPro-EX-HTa vector. Proteins were expressed in E.coli C41 (DE3) cells with IPTG induction at 30 °C or 37 °C overnight. Histag-IMAC purification was carried out using HisTrap™ HP columns from GE Healthcare. The hexahistidine tags were then cleaved using TEV purification was carried out using HisTrap™ H® columns from GE Healthcare. The hexahistidine tags were then cleaved using TEV

Circular dichroism (CD) measurements
CD spectra were recorded using a Jasco J-815 CD Spectrometer in a 0.1 cm path length quartz cuvette, acquired with a bandwidth of 1 nm at 1 nm increments and 10 s average time. Thermal denaturation was performed in a 0.1 cm path length quartz cuvette in MilliQ water or, in the case of CTP8Y e-Co6A, in 3, 4, 5 and 6 M urea solutions. The denaturation curves were monitored by following the CD signal at 222 nm wavelength as a function of temperature from 10 °C to 95 °C.

Transmission electron microscopy (TEM)
The samples for cryo-electron microscopy (cryo-TEM) experiments were vitrified on freshly glow-discharged (high vacuum coating system MED 020 BALTEC) holey carbon grids (Holey-carbon film on Copper 200 mesh; QUANTIFOIL R 2/2). The grids were held with ultra-thin tweezers inside the chamber of a Vitrobot Mark II (FEI Company, USA), maintained at 8 °C temperature and with relative humidity close to saturation (90% rh) to prevent drying artefacts in the blotting process. Four microliters of the sample solution were adsorbed onto the grid for 30 seconds and most of the liquid in the grid was removed by automatically blotting the grid (blot time = 3 seconds, number of blots = 1, drain time = zero and blot offset = -3 mm) with absorbent standard Vitrobot filter paper (Ø55/20mm, Grade 595, Thermo Fisher Scientific FEI) to create an “ultra-thin liquid film” (i.e., typically bellow ~100 nm film thicknesses). After the blotting, the grid was abruptly plunged into a liquid ethane bath, previously cooled with liquid nitrogen to approximately -180 °C. Vitrified grids were then removed from the plunger and stored under liquid nitrogen.

High resolution zero tilt two-dimensional (2D) images were collected on a JEM-2200FS/CF (JEOL, Ltd.) field emission gun (FEG) transmission electron microscope, operated at 200 kV at liquid nitrogen temperature, using a 626 DH cryo transfer holder (Gatan Inc.). An in-column Q energy filter was used to record images with improved signal to noise ratio by zero-loss filtering, with the energy slit width set at 15 eV. Digital images were recorded under low-dose conditions (on the order of 20-30 electrons/Å2 per exposure) with an under-focus range from 2.0 to 5.0 μm using DigitalMicrograph™ (Gatan) software with a 4K × 4K Ultracam4000™ CCD camera (Gatan Inc.). For electron tomography, a single-axis tilt series of ± 64° with 2° increments was recorded with under-focus values ranging from 5 to 8 μm, using a 914 high tilt liquid nitrogen cryo transfer tomography holder (Gatan Inc.). Semi-automatic data acquisition software SerialEM2 was used for data collection. Vitrified grids contained 10 nm gold nanoparticles for image alignment. Other conditions as described above.

Computational Methods. Molecular Dynamics Simulations
The parameters for the [60] fullerene species, Co6 or Co6A, for the MD simulations were generated within the ANTECHAMBER module of AMBER 16, using the general AMBER force field (GAFF)3, with partial charges set to fit the electrostatic potential generated at the HF/6-31G(d) level by the RESP model. The charges were calculated according to the Merz-Singh-Kollman scheme6,7 using Gaussian 09.8 MD simulations of CTPR8 were carried out using PDB 2HYZ as a reference, removing the crystallised samarium ion. The starting structure for the MD simulations of CTPR8 was prepared by manually introducing the abovementioned tyrosine mutations with the mutagenesis tool from PyMOL software using the CTPR8 crystal structure (PDB 2HYZ) as starting point. In all cases, i.e. CTPR8–Co6,
CTPR8-C60A, CTPR8Y16-C60, and CTPR8Y24-C60A simulations, [60]fullerenes were placed in arbitrary positions in the solvent region (more than 10 Å away from the protein surface and 20 Å far from other [60]fullerenes). In cases where more than one CTPR8Y16 unit was included in the simulation, the proteins were separated by at least 40 Å. From these coordinates, conventional MD simulations were used to allow the fullerene molecules to diffuse freely until spontaneous association with the surface of the protein, and, finally, interaction with CTPR8 binding sites.9 Each system was immersed in a pre-equilibrated truncated octahedral box of water molecules with an initial offset distance of 10 Å, using the LEAP module.10 All systems were neutralised with explicit counterions (Na+ or Cl−). A two-stage geometry optimisation approach was performed. First, a short minimisation of the positions of water molecules with positional restraints on solute by a harmonic potential with a force constant of 500 kcal mol−1 Å−2 was done. The second stage was an unrestrained minimisation of all the atoms in the simulation cell. Then, the systems were gently heated in six 50 ps steps, increasing the temperature by 50 K each step (0-300 K) under constant-volume, periodic-boundary conditions and the particle-mesh Ewald approach11 to introduce long-range electrostatic effects. For these steps, a 10 Å cut-off was applied to Lennard-Jones and electrostatic interactions. Bonds involving hydrogen were constrained with the SHAKE algorithm.12 Harmonic restraints of 10 kcal mol−1 were applied to the solute, and the Langevin equilibration scheme was used to control and equalise the temperature.13 The time step was kept at 2 fs during the heating stages, allowing potential inhomogeneities to self-adjust. Each system was then equilibrated for 2 ns with a 2 fs timestep at a constant pressure of 1 atm. Finally, conventional MD trajectories at constant volume and temperature (300 K) were collected. In total, five replicas of 800 ns MD simulations for CTPR8 in the presence of eight C60 were carried out, five replicas of 800 ns MD simulations for CTPR8 in the presence of eight C80A, five replicas of 800 ns MD simulations for CTPR8Y16 in the presence of eight C60, five replicas of 800 ns MD simulations for CTPR8Y16 in the presence of twenty-four C60A, and five replicas of 200 ns MD simulations for three CTPR8Y16 units in the presence of eight C60A; gathering a total of 4 μs for each CTPR8-C60, CTPR8-C80A, CTPR8Y16-C60 and CTPR8Y16-C80A co-assemblies, 2.5 μs for CTPR8Y16-24C60A co-assemble, and 1 μs for 3CTPR8Y16-24C60A co-assemble.

Flash-Photolysis Time-Resolved Microwave Conductivity (FP-TRMC)

Nanosecond laser pulses from a Nd:YAG laser of Spectra-Physics INDI-HG (full width at half maximum (fwhm) of 5-8 ns) was used as an excitation light source, and the third harmonic generation (THG) (355 nm) were exposed to the cast films. The laser power density was set at 1, 2, 4, 7, and 10 mW (0.91, 1.8, 3.7, 6.4, and 9.1 × 10¹⁵ photons cm⁻² s⁻¹, respectively). The microwave frequency and power were set at approximately 9.1 GHz and 3 mW, respectively, and the TRMC signal was evolved in a diode (rise time < 1 ns), and output signal was led into a digital oscilloscope of Tektronix TDS 3032B. All experiments were conducted at room temperature. Reflectance power change ratio (ΔP/P) of microwave from the cavity in FP-TRMC apparatus is in relation with the total loss (Δ(1/Q)) of microwave by the photo-induced transient species in the cavity as follows:

$$\Delta P = A\frac{Q}{\omega_0} = \left(\frac{1}{Q}\right) \left(\frac{1}{2Q}\right)^{-\Delta \left(\frac{1}{Q}\right)}$$

where \(\omega_0\) and \(\Delta \omega\) are the resonant frequency of 9.1 GHz and its shift by the photo-induced transient species. The loss and the frequency shift of the microwave are expressed as a function of complex conductivity \((\Delta \sigma + i \Delta \sigma')\) of the transient species by:

$$\Delta \left(\frac{1}{Q}\right) = i \frac{2\Delta \omega}{\omega_0} = F \left(\Delta \sigma + i \Delta \sigma'\right)$$

where \(F\) is a calibration factor derived from the measurements of total loss of microwave in the cavity loaded with materials with well-known conductivity values. The value of \(\Delta P/P\) is proportional to the sum of the mobilities \(1/\mu\) of charged species in case of negligibly small \(\Delta \alpha\)

$$\Delta \sigma = \sum N \phi \mu = A \frac{\Delta P}{P}$$

Notes and references

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Supporting images

**Fig. S1.** CD characterisation of CTPR8Y$_{16}$ and fullerene assemblies. Thermal denaturation profiles of CTPR8Y$_{16}$ (gray), CTPR8Y$_{16}$-C$_{60}$ (green) and CTPR8Y$_{16}$-C$_{60}$A (purple) co-assemblies, monitored by CD at 222 nm.

**Fig. S2.** Thermal stability analysis of CTPR8Y$_{16}$-C$_{60}$A. (a) Thermal denaturation curves in the presence of varying concentrations of urea, monitored by CD at 222 nm. (b) Relationship of T$_m$ with the concentration of urea from part (a), allowing to extrapolate the T$_m$ without urea.
**Fig. S3.** Raman spectra of $C_{60}$ (grey), $C_{60}A$ (green) and CTPR8Y$_{16}$-C$_{60}$A co-assembly (purple), excited at 532 nm.

**Fig. S4.** Two additional series of cryo-electron micrographs of CTPR8Y$_{16}$-C$_{60}$A hybrid material at different degrees of sample tilt, showing the assemblies from different perspectives. The scale bar is 20 nm in all cases.
Fig. S5. Cryo-electron micrographs of (a) CTPR8Y,16-C$_{60}$ and (b) CTPR8-C$_{60}$ hybrid materials (left) and the corresponding FFT images (right), showing the diffraction patterns of the hybrid materials.

Fig. S6. Cryo-electron micrograph of fullerene-pyrrolidine tris-acid C$_{60}$A.
