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COMMUNICATION

Bulky Toroidal and Vesicular Self-Assembled Nanostructures from Fullerene End-Capped Rod-Like Polymers

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In this work, we present novel fullerene (C₆₀) end-capped rod-like polypeptide-polymers, obtained by one-pot thiol-ene chemistry. These systems are able to self-assemble in water creating precise bulky microstructures of toroidal or vesicular shapes. Independent molecular dynamics simulations supported the observed experimental results.

One of the novel themes in areas such as materials science, nanochemistry and biomimetic chemistry take as target the construction of supramolecular architectures characterized by well-distinct shapes and functions.^{1,2} Undoubtedly, the self-assembly process of polymers through non-covalent interactions including hydrophilic and hydrophobic effects, electrostatic contacts, hydrogen bonding, microphase segregation and shape effects, has a great potential for creating such supramolecular architectures.³⁻⁶ Recently, the improvement in synthetic methodologies associated with the use of designed conformational constraints, led to the ability to perform nanometric control, on both size and shape, even at the single polymer molecule level.⁷ In the literature many examples of supramolecular vesicles and micelles formed by block copolymers containing at least one high-molecular weight polymeric peptide domain,⁸⁻¹⁰ as well as by small- or medium-size peptides conjugated with molecules of nonpeptidic nature,^{9,11} can be found. Rarely, homo-polypeptides alone were reported as precursor of self-assembled microstructures of spherical shape. Polypeptides prepared by the ring-opening polymerization (ROP) of α -amino acid N-carboxyanhydrides (NCAs), which are known since many decades,¹² are nowadays emerging biomaterials¹³ that are gaining an increasing attention for various applications including drug delivery, gene therapy, antibiotics and tissue engineering.¹⁴ Polypeptides can offer considerable chemical diversity: beyond the twenty canonical amino

acids alone, non-canonical amino acids are readily accessible from commercial sources or could be designed and synthesized depending on the finalities. Moreover, polypeptides are fascinating and unique for their ability to self-assemble into ordered structures,¹⁵ making them attractive in those many applications where well-organized architectures are essential to accomplish a function. Substantial progresses have been made in the NCA/polypeptides field in the past decade¹⁴ and today several innovative polypeptide-based materials have been produced and studied.¹⁶ Apart from the ability to assemble in a wide range of different supramolecular structures of nanoscale dimensions,¹⁷ another unique characteristic of rod-polypeptidic segments is the possibility to endow various functionalities such as photophysical and electrochemical properties to the supramolecular materials. An example of well-known rod-like polypeptide is poly(γ -benzyl-L-glutamate) (PBLG). PBLG has intramolecular hydrogen bonds that stabilize the secondary α -helical structure and shows mesoscopic liquid crystal order because of its good solubility and rod-like structure.¹⁸ Inspired by the recent work of Yagci and co-workers,¹⁹ we decided to functionalize both N- and C-termini of PBLG using thiol-ene chemistry²⁰ to prepare C₆₀-PBLG (C₆₀, fullerene) conjugates in a way that only C or C/N-terminus mono-addition products are formed without any contamination of ill-defined derivatives. In particular, herein we report the one-pot synthesis of two C₆₀-PBLG derivatives, namely NH₂-PBLG-C₆₀ (**3**) and C₆₀-PBLG-C₆₀ (**6**) (Figure 1) and their controlled self-assembly in water.²¹

BLG-NCA (**4** mmol) was polymerized in dioxane for 24 hrs at room temperature, using Trt-cysteamine (Trt, triphenylmethyl) as initiator (0.04 mmol) (Scheme 1). Polymer **1** was obtained after precipitation with methanol in an 81% yield ($M_w = 19100$, PDI 1.11 by SEC). Subsequently, the Trt-protecting group was removed yielding polymer **2** (not shown in the Scheme 1). Next, **2** (0.01 mmol) was dissolved in DMF and added to toluene. Then, fullerene (0.05 mmol) and AIBN (0.15 mmol) were added (in this order) to the previous solution and the subsequent homogeneous mixture was heated at

90 °C under stirring for 5 hrs. Subsequently, the solvent was removed and the resulting solid was suspended in DMF, passed through a 20 μm filter, and then precipitated by addition of methanol, giving polymer **3** as a brown solid in 85 % yield ($M_w = 19550$, PDI 1.09 by SEC).

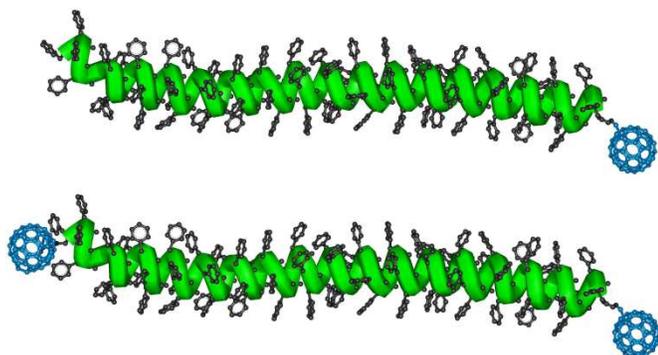
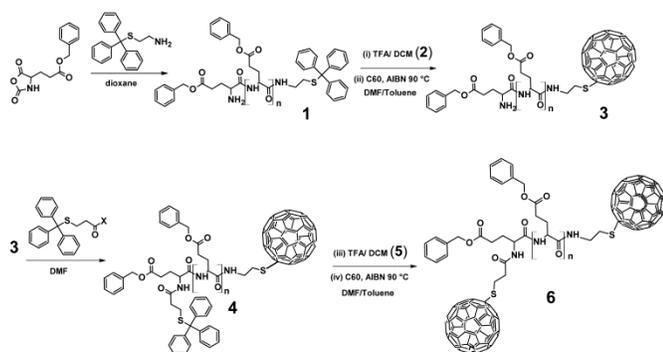


Fig. 1 Molecular structures of $\text{NH}_2\text{-PBLG-C}_{60}$ (**3**) and $\text{C}_{60}\text{-PBLG-C}_{60}$ (**6**).

Moreover, polymer **3** was successively N-end capped with a large excess of pre-activated Trt-mercaptopropionic acid, yielding polymer **4** (not shown in the Scheme 1). Polymer **4** was C-terminus Trt-deprotected (as described before, yielding polymer **5**, not shown in Scheme 1) and placed to react in the same conditions described above for the synthesis of polymer **3**, thus to generate the bisconjugate $\text{C}_{60}\text{-PBLG-C}_{60}$, polymer **6** in 78 % yield ($M_w = 20300$, PDI 1.11 by SEC). Since all the synthesized polymers possess the same PBLG part (as it comes from polymer **1**), the differences in M_w s determined by SEC constitute a first evidence for the addition of one or two fullerenes.



Scheme 1. Synthesis of polymers $\text{NH}_2\text{-PBLG-C}_{60}$ (**3**) and $\text{C}_{60}\text{-PBLG-C}_{60}$ (**6**).

A second evidence for the covalently attachment of fullerenes arises from the absorption spectra of **1**, **3** and **6**, which were recorded in THF solvent and are reported in Figure 2A. The observed patterns are close to that of fullerene, which shows high UV absorptions around $\lambda = 287, 330$ nm and tails spanning over the 600 nm region. We assign the observed UV absorptions in **1** and **2** *mono* and *bis* fullerene-conjugates to the fullerene moiety¹⁹ considering the small differences in the resonance frequencies with respect to the isolated fullerene due to perturbations of the electronic structure caused by the bonding with the PBLG polymer. The third indication of the formation of PBLG-conjugated fullerenes come from the thermal stability of **1**, **2** and **3** that was investigated *via* thermogravimetric analysis (TGA; 100-700 °C) under nitrogen flow with a heating rate

of 10 °C min^{-1} (Figure 2B). Accordingly, PBLG polymers undergo a thermal decomposition between 250-350 °C, whereas the thermally stable fullerene component keeps its stability up to almost 600 °C.¹⁹ The weight percentages of the components (polymer **3**, $\text{C}_{60}/\text{PBLG}$ 4:96 %; polymers **6**, $\text{C}_{60}/\text{PBLG}$ 7:93 %) determined by TGA are in agreement to those of SEC analysis. All of the experimental results suggested an average number of BLG amino acids, for polymer unit, close to 85. The secondary structure of polymers **1**, **3** and **6** was analyzed by ECD (electronic circular dichroism), which confirmed the preservation of the α -helical conformation for all the synthesized compounds (Fig. S1, Electronic Supporting Information, ESI), that in turn implies the rod-like shape of the polymers. Synthetic details, SEC traces, ¹³C-NMR, ¹H-NMR (these last two characterizations do not allow the detection of C_{60} due to the large amount of PBLG respect to C_{60}) and solid state IR characterizations of the polymers (**1**, **3** and **6**) are reported in ESI, Figures S2-5 respectively.

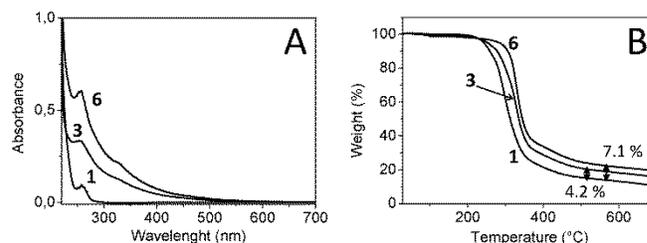


Fig. 2 A: UV/Vis spectra of polymers **1**, **3** and **6** in THF solution (concentration: 0.01 mg/mL). B: Thermogravimetric analysis comparison of polymers **1**, **3** and **6**.

From polymer **3**, donut-like morphologies were generated, in form of milk-like suspension, starting from a 20 mg (3:7, v/v) DMF/THF solution (10 mL) dialyzed (membrane cutoff, 12 KD) against ultrapure water (48 hours) (Figure 3).

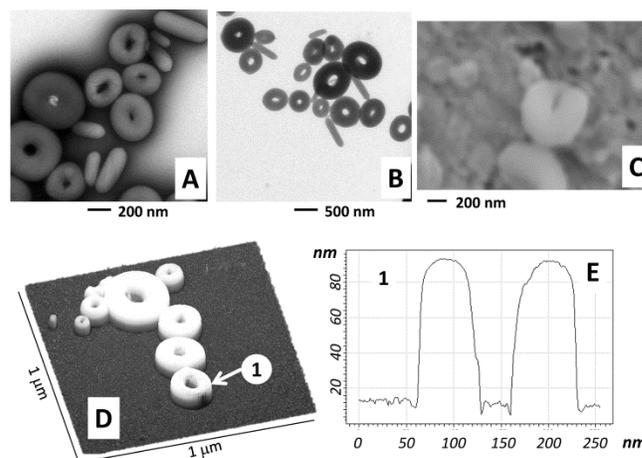


Fig. 3 A and B: TEM (A, stained and B, unstained) images of the resulting self-assembled nanostructures obtained from polymer **3**. C: SEM image from the very same prepared sample used in TEM analysis. D and E: AFM micrograph and high-profile of the selected (**1**) toroidal nanostructure.

TEM (transmission electron microscopy) images showed toroidal shapes with an external diameter ranging between 200 nm and 650 nm (Figures 3, A and B, stained and unstained samples,

respectively), additionally confirmed by SEM (scanning electron microscopy) analysis (Figure 3C). Moreover, AFM (atomic force microscopy) details of the selected nanodonor reported in Figure 3C, revealed a diameter of 200 nm, a thickness of 70 nm and a hole of 30 nm (Figure 3D). Additionally, after running the same self-assembly process but starting from a more dilute solution of **3** (2-5 mg in 10 ml), smaller but uniform in size (60 nm diameter) toroidal nanostructures were obtained as detected by AFM (Fig. S6, ESI). Sphere-shaped aggregates were generated from polymer **6** under the same condition described above for polymer **3** (Figure 4). TEM, unstained images, revealed the formation of deep-dark round morphologies (Figure 4A) with diameters ranging between 250-700 nm. Moreover, these aggregates displayed an internal cavity as shown from the TEM image reported in Figure 4B; thus, the observed spherical morphologies can be attributed to hollow vesicle-like microstructures. These results were additionally confirmed by AFM (Figure 4C) and SEM analyses (Figure 4D). While polymers **3** and **6** were able to self-assemble into ordered structures, polymer **1**, bearing a Trt group instead of fullerene, did not give any specific nano-structuration under similar conditions. To get polymer **3** from **1**, the Trt protecting group was removed in acidic conditions, thus in polymer **3** the N-terminus amine is protonated (NH_3^+). Importantly, when NaOH 0.2 M was added to a solution containing the toroidal objects obtained by self-assembly of **3**, an immediate precipitation of the polymer was achieved. On the other hand, the addition of HCl 0.2 M did not affect the self-assembled structures, since donut-like structures could still be detected by TEM. This information suggested that: (i) fullerene is necessary for the formation of ordered nanostructures and (ii) the NH_3^+ group is mandatory for the formation of the toroid-shaped structures.

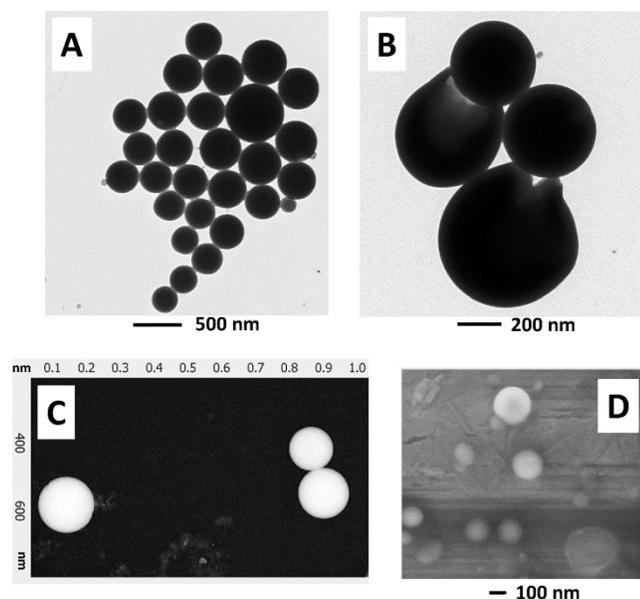


Fig. 4 **A** and **B**: TEM (unstained) images of the resulting self-assembled nanostructures obtained from polymer **6**. **C** and **D**: AFM and SEM images, respectively, from the very same sample used for TEM analysis.

Since, at fixed volume, a toroidal shape exposes a larger surface with respect to a spherical shape, our hypothesis is that to generate self-assembled toroid forms in water (starting from the hydrophobic polymer **1**) the single polymer chain may firstly undergo a tail-to-tail

dimerization and successively a large scale, coil-coil spiral-like, self-assembly process. According to this hypothesis, all of the fullerenes could be aligned along an internal axle, thus to result buried by the PBLG hydrophobic helices, while the charged NH_3^+ groups may be exposed to water solution (a schematic representation is reported in Figure S7, ESI) in according with previously reported similar, but much smaller, structures.²² This hypothesis is in agreement with the pH-depending experiments described above. Moreover, the same but shorter functionalized PBLG-polymer series was also synthesized using a BLG-NCA/initiator ratio of 25:1. After the self-assembly assay, the shorter **1** and **3** analogues, did not provide any specific microstructure propensities while the shorter **6** analogue afforded smaller spherical structures (10-35 nm diameter) compared to the previously described longer one (Fig. S8, ESI).

Calculus was used to support experimental observations and in particular to investigate the atomistic structure of the vesicles formed by polymer **6**. To this purpose we employed a bottom-up coarse graining protocol with the intent to set up a simulation able to independently recover the experimental observations, i.e. we did not use the experimental results to bias the parameterization of the model. To limit the computational weight of the whole procedure we used as model molecule polymer **6** constituted by 25 BLG units. The coarse-graining representation sees each of the BLG units and the two terminal moieties as single beads. Differently from systems recently reported in the literature,²³ in which a balance between attractive and repulsive forces of the building blocks drives the assembly, here the driving force of the self-assembly is the low affinity of the hydrophobic PBLG molecules with water. Thus, in our simulations we needed to include explicitly the medium to observe aggregation of polymer **6** molecules. We employed a coarse-grained 4-water model for the solvent, in which one bead is used to represent a cluster of 4 water molecules.^{24,25} Details on the coarse graining procedure and the parameterization of the bead-bead energy function are provided in the ESI. We simulated a system containing 1600 polymer molecules and 1600608 water beads (see the ESI for details). The starting configuration, in which the polymer molecules are randomly placed and oriented in space, is shown in Figure 5A (water was omitted for clarity).

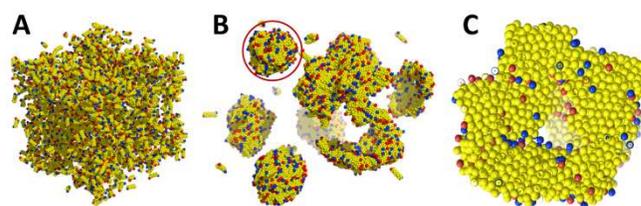


Fig. 5 The color scheme is: yellow to represent BLG beads, red for the N-terminal group, and blue for C-terminal group. Beads radii are set to 0.43 nm for the BLG units and 0.53 nm for the terminal moieties. These values have been determined from the moieties average translational diffusion tensor calculated using a hydrodynamic approach.²⁶ Water molecules have been omitted for clarity. **A**: Initial random configuration of the 1600 PBLG molecules constituted by 25 BLG amino acids. **B**: Final configuration of the system after 5 ns of simulation time. **C**: Details of a selected spherical aggregate with front and rear clipping planes applied to highlight the hollow part of the spherical aggregate.

The situation just after 5 ns of simulation time is shown in Figure 5B. The simulation box results populated by a small number of

aggregates with different shapes. Most of them are spherical aggregates of dimensions ranging from 15 to 20 nm of diameter. However, a couple of assemblies are present showing a bent layer of about 4 nm of thickness (very close to the length of one polymer molecule) that can be thought to be a piece of a larger vesicle that could not be formed in this simulation due to an insufficient number of polymer molecules. A number of important observations can be drawn from the simulations. The first and most important observation reflecting the experiments, is that in water the polymer molecules aggregate into ordered structures instead of creating an amorphous precipitate. Second, all the aggregates are formed by layers of limited size ranging from 4 to 6 nm, which is in the order of the length of the polymers. Third, the layer is not structured in a simple way as it happens in phospholipids: couples of polymers form coiled-coil super structures, which in turn create a network giving origin to the layer, with the C₆₀ termini preferentially exposed to the solvent. We shall also notice that, while being quite compact, the spherical structures obtained in the simulation are hollow (Figure 5C). The obtained aggregates are in quite good accord with those observed experimentally for the shorter 6 analogue.

Conclusions

In this work, we reported the one-pot synthesis of two novel PBLG-polymers C- and C/N-terminus capped with fullerenes, by means of thiol-ene chemistry. The self-assembly in water of such rod-like systems led to the formation of bulky, toroid-like or vesicle-like, nanostructures depending on the fullerene position over the main chain of the polymer. Based on experimental results, we suggested a mechanism for the formation of the toroidal like-nanostructures and we explored vesicle-like systems by means of computational modelling. In conclusion, such polymer-fullerene conjugates maybe interesting systems to prepare organized, at nanoscale level, fullerene-based devices.

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Notes and references

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† Electronic Supplementary Information (ESI) available: Synthetic details, SEC traces, ¹³C-NMR, ¹H-NMR and solid state IR characterizations of the polymers (1, 3 and 6) are reported in ESI. See DOI: 10.1039/c000000x/

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