Electronic Supplementary Information (ESI)

Fluorescent homopolypeptide toroids

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1. Materials

Triphosgene (99%), γ-benzyl-L-glutamate (BLG, 97%), α-pinene (98%), diphenylmethanone (99%), 4-aminobenzophenone (98%), titanium tetrachloride ($TiCl_4$, 99%), zinc powder (99%), sodium bicarbonate ($NaHCO_3$, 99%), and anhydrous $MgSO_4$, *n*-hexylamine (99%) were purchased from Aladdin Bio-Chem Technology Co., Ltd. (Shanghai, China) and used directly without further purification. Phosphate-buffered saline (PBS), glutathione (GSH), Roswell Park Memorial Institute (RPMI), and fetal bovine serum (FBS) were purchased from Thermo Fisher Co., Ltd. (Shanghai, China). L02 cells (a normal liver cell line) were obtained from Gaining Biological Technology Co., Ltd. (Shanghai, China). Tetrahydrofuran (THF), *n*-hexane, dimethylformamide (DMF), ethyl acetate (EtOAc), and diethyl ether were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). THF and DMF were dried with sodium to remove traces of water prior to use. $CDCl_3$ and DMSO-d_6 were purchased from J&K Scientific Ltd. (Shanghai, China).

2. Characterization

2.1 Proton nuclear magnetic resonance (^{1}H NMR)

Spectra were recorded using a Bruker AV 400 MHz spectrometer at room temperature (25 °C) using $CDCl_3$ or DMSO-d_6 as the solvent.

2.2 Dynamic light scattering (DLS) and zeta potential (ζ)

Measurements were performed on a ZETASIZER Nano series instrument (Malvern S7 Instruments ZS 90) at a fixed scattering angle of 90°. The hydrodynamic diameters (D_h) and polydispersity (PD) of nanoparticles were determined using cumulant analysis of the experimental correlation function and calculated from the computed diffusion coefficients using the Stokes-Einstein equation. Zeta potential (ζ) studies were conducted at 25 °C. Each reported measurement is an average from three times that consist of 10 runs (10 seconds for each run).

2.3 Size exclusion chromatography (SEC)

The molecular weights (M_n) and polydispersity ($Đ$) of homopolypeptides were evaluated using a DMF SEC conducted by an Agilent 1260 Infinity SEC analysis

system with two Shedex SEC KD series columns with HPLC grade DMF (0.05 M LiBr) as the eluent at a flow rate of 1.0 mL min^{-1} at 50 °C.

2.4 Transmission electron microscopy (TEM)

The nanoparticle dispersions (8.0 μL) were added dropwise onto a carbon-coated copper grid and dried at ambient temperature (25 °C) and analyzed without staining. The intermediate morphologies during the self-assembly process were quenched in an excess amount of deionized water prior to TEM sample preparation. All TEM images were recorded on a JEOL JEM-2100F instrument at 200 kV equipped with a Gatan 894 Ultrascan 1k CCD camera.

2.5 Scanning electron microscopy (SEM)

A fresh silicon wafer was washed in acetone under ultrasound for 10 min. 10.0 μL of sample solution was dropped onto the silicon wafer and dried at 25 °C. Samples were coated with platinum and viewed by an FEI Quanta 250 FEG electron microscope (USA) or Nova Nano-SEM 450 electron microscope (USA). All images were recorded using a digital camera.

2.6 Atomic force microscopy (AFM)

A fresh silicon wafer was washed in acetone under ultrasound for 10 min. 10.0 μL of diluted sample solution was dropped onto the silicon wafer and dried at 25 °C. The AFM analysis was conducted on a Dimension Icon instrument operating in tapping mode at 200-400 kHz drive frequency.

2.7 Ultraviolet-visible spectroscopy (UV-vis)

UV-vis spectra of the homopolypeptides and the corresponding assemblies were acquired using a UV759S UV-vis spectrophotometer (Shanghai Precision & Scientific Instrument Co., Ltd.). All samples were analyzed using quartz cuvettes. THF and water were used as measurement background.

2.8 Fluorescence spectroscopy

Fluorescence spectra of samples were obtained using a Lumina fluorescence spectrometer (Thermo Fisher Scientific Co., Ltd.). All samples were analyzed using quartz cuvettes at an excitation wavelength of 330 nm.

2.9 Fourier-transform infrared spectroscopy (FTIR)

The FTIR spectra of the corresponding freeze-dried homopolypeptides powders were obtained using a thermo Bruker Equinoxss/Hyperion2000 FTIR spectrometer.

2.10 Differential scanning calorimetry (DSC)

The DSC traces of freeze-dried homopolypeptides powders were obtained using a Q100 DSC (TA Instruments, USA). The samples were kept for 5 min at –40 °C and heated afterwards at a rate of 10 °C min^{-1} to 120 °C. The process was performed for three cycles for the sample. The data was obtained from the last run.

2.11 Confocal laser scanning microscopy (CLSM)

The fluorescent images of cells were obtained using confocal laser scanning microscope (Leica TCS SP8).

3. Experimental and discussion

3.1 Synthesis of initiator 4-(1,2,2-triphenylvinyl)aniline (TPE-NH_2)

The initiator TPE-NH_2 was prepared by the following procedure: diphenylmethanone (1.84 g, 10.0 mmol), 4-aminobenzophenone (2.00 g, 10.0 mmol) and zinc powder (5.80 g, 88.0 mmol) were dispersed in 100 mL of anhydrous THF in a three-necked round-bottomed flask (250 mL). Oxygen in the flask was removed by purging with argon for 60 min. Then $TiCl_4$ (2.50 mL, 22.0 mmol) was slowly added to the mixture at ice bath condition using a microliter syringe. The reaction solution was stirred at room temperature for 30 min and then refluxed at 80 °C for 48 h. The crude mixture was subsequently poured into a beaker and quenched with an aqueous 1.0 M HCl solution (100 mL). The crude product was extracted with ethyl acetate (50 mL) and the organic layer was washed with a saturated $NaHCO_3$ (3 × 50 mL) and a saturated aqueous NaCl solution (3 × 50 mL). After dried over anhydrous $MgSO_4$, the organic solvent was removed using a rotary evaporator at 25 °C. The product was purified by column chromatography ($V_{n\text{-hexane}}$: V_{EtOAc} = 4 : 1) to yield 2.51 g of the final product, an orange/yellow solid. Yield: ~50%.

3.2 Synthesis of *γ*-benzyl-L-glutamate *N*-carboxyanhydride (BLG-NCA)

BLG-NCA monomer was synthesized as follows: *γ*-benzyl-L-glutamate (5.00 g,

21.1 mmol) and *α*-pinene (5.81 g, 42.6 mmol) were dissolved in 120 mL of anhydrous THF in a two-necked round-bottomed flask. The triphosgene (6.50 g, 21.9 mmol) in anhydrous THF was added into the solution dropwise under the protection of nitrogen. The mixture was heated to 50 °C and the clear solution formed within two hours. After stirring for another hour, the concentrated solution was poured into 500 mL of petroleum ether, leading to white flocculent precipitation. The resulting suspension was then stored at –20 °C overnight and filtered. The above process was repeated three times to remove unnecessary impurities. The product was dried under high vacuum by a rotary evaporator to yield white powder. Yield: ~85%.

3.3 Synthesis of homopolypeptides

The general synthesis of homopolypeptides was prepared in an anhydrous DMF solution using ring-opening polymerization of BLG-NCA initiated by initiator TPE-NH_2. The target degree of polymerization (DP) of the homopolypeptides was controlled by adjusting the molar ratio of the BLG-NCA monomer to the initiator. Taking one homopolypeptide as example, the TPE-NH_2 (10.0 mg, 30.0 μmol) and the BLG-NCA monomer (250 mg, 0.930 mmol) were dissolved in 5.0 mL anhydrous DMF in a round-bottomed flask equipped with a drying tube and a magnetic stir bar. The reaction was performed at 30 °C for 48 h using pump for removing CO_2, to ensure the formation of polymers with molecular weights in the range of theoretical degree of polymerization. Subsequently, the solvent was removed under reduced pressure and the concentrated reaction solution was precipitated into anhydrous diethyl ether. The product was dried under vacuum and then purified at least twice by repeating the precipitation procedure from DMF.

HEX-PBLG homopolypeptide was synthesized *via* ring-opening polymerization of BLG-NCA using the *n*-hexylamine initiator following a similar procedure.

3.4 Self-assembly of TPE-PBLG into toroids

TPE-$PBLG_{28}$ was dissolved in THF with a final concentration of 0.2 mg mL^{-1}. Then deionized water was added into the solution dropwise under stirring at 25 °C (V_{water} : V_{THF} = 3 : 1). Upon the addition of water, the colorless solution gradually

became slightly blue, which indicates the formation of assemblies. THF was removed by dialyzing against deionized water for three days. The self-assembly procedure of other homopolypeptides (TPE-PBLG$_{17}$ and TPE-PBLG$_{40}$) was under the same condition.

3.5 Self-assembly of TPE-PBLG$_{28}$ with different water contents

TPE-PBLG$_{28}$ was dissolved in THF and self-assembled with the same solvent-switching method described above. The solutions (100 μL) were taken out at different water contents and analyzed by Fluorescence Spectroscopy, UV-vis Spectroscopy, TEM, and SEM.

3.6 Self-assembly of TPE-PBLG$_{28}$ at different self-assembly temperatures (T_s)

TPE-PBLG$_{28}$ was dissolved in THF with a final concentration of 0.2 mg mL^{-1} and balanced at 5, 10, 15, 20, 25, 30 and 40 °C for 1 h before self-assembly, that is, after dissolving the polymer, polymer solution was stand still for 1 h under corresponding temperature. Then deionized water (V_{water} : V_{THF} = 3 : 1) was added into the solution dropwise under stirring at different T_s. All experiments were conducted at corresponding T_s. Upon the addition of water, the colorless solution gradually became slightly blue, which indicates the formation of assemblies. THF was removed by dialysis against deionized water for three days. The processes of adding water and dialysis were performed at corresponding T_s.[1]

3.7 Cell viability evaluated *in vitro*

The cytotoxicity of AIE nanoparticles should be critical consideration for biological applications. We used the CCK-8 assay to evaluate the cell viability. L02 cells were cultured in 96-well plates at a density of 10000 cells per well. After overnight incubation in a 5% CO_2 and humidity incubator at 37 °C, medium in each well were replaced by fresh medium containing 100 μL different concentrations dispersions (The control experiment used equal volume phosphate buffer solution). After 24 hours of culturing, 10.0 μL CCK-8 solution was added into each well, then the absorbance of each well at 450 nm was recorded. Each of the experiments was conducted at least 3 times as a parallel test.

3.8 Cell culturing and imaging

L02 cells were cultured in 40 mL RPMI (containing 10 mL FBS, 500 μL GSH and 500 μL AA) in a 5% CO_2 and humidity incubator at 37 °C. The liver L02 cells were stained with 125 μg mL^{-1} toroids dispersion (0.1 mL) for 15, 30 min and 1 h in culture medium. The cells were imaged under a CLSM using 405 nm excitation wavelength.

4. Figures, tables and schemes

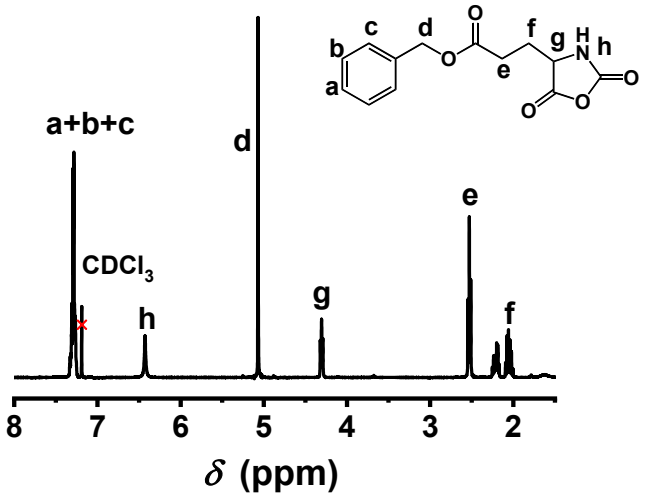


Fig. S1. ^{1}H NMR spectrum of BLG-NCA monomer in $CDCl_3$.

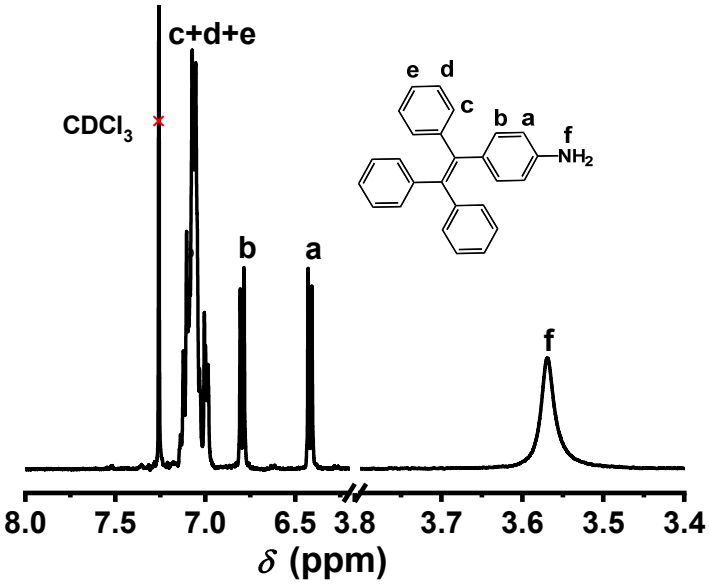


Fig. S2. ^{1}H NMR spectrum of TPE-NH_2 initiator in $CDCl_3$.

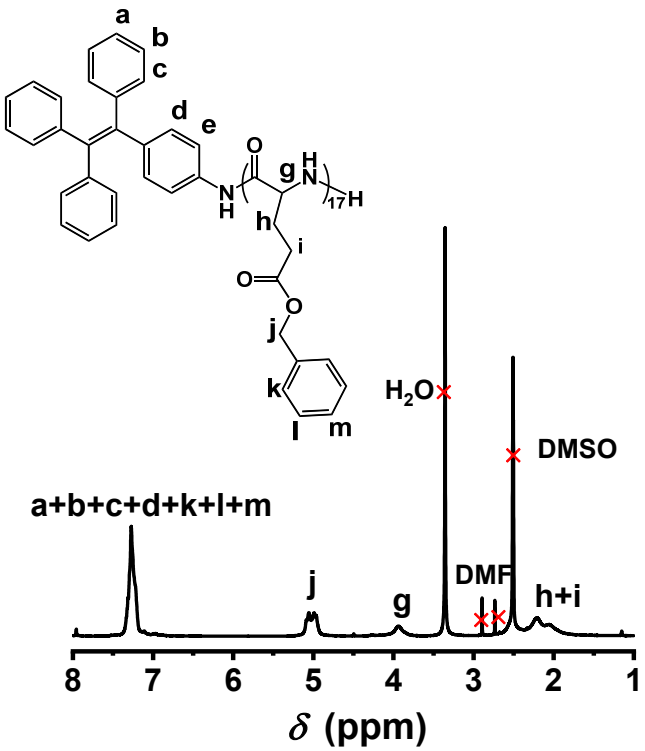


Fig. S3. ^{1}H NMR spectrum of TPE-PBLG$_{17}$ in DMSO-d_6.

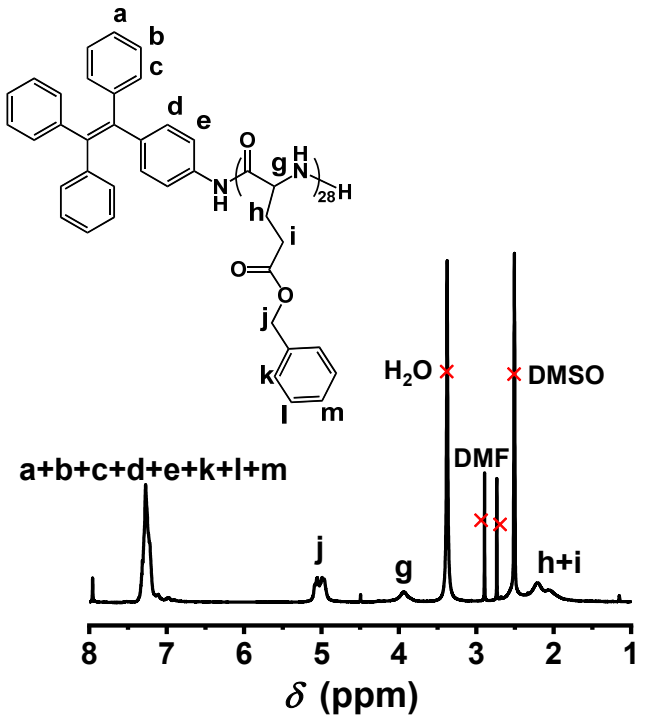


Fig. S4. ^{1}H NMR spectrum of TPE-PBLG$_{28}$ in DMSO-d_6.

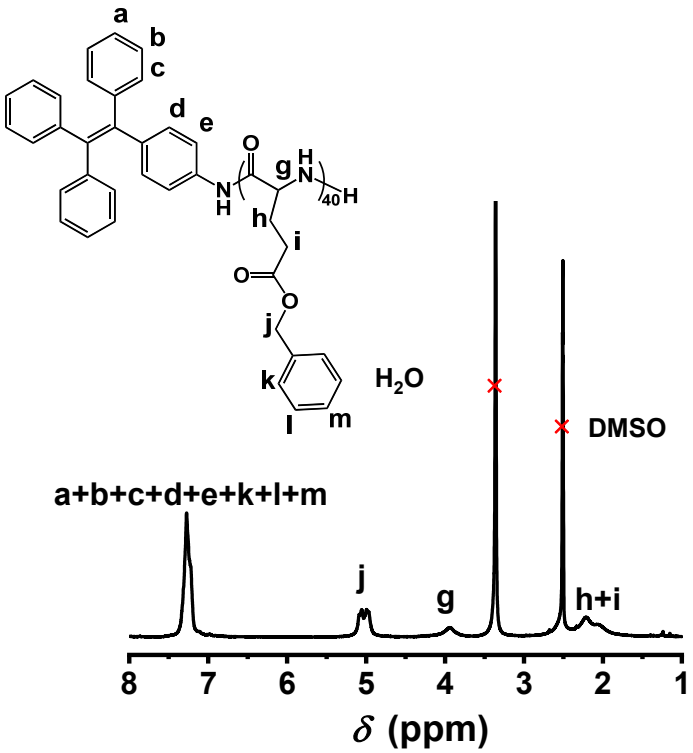


Fig. S5. ^{1}H NMR spectrum of TPE-PBLG$_{40}$ in DMSO-d_6.

Calculation of the degree of polymerization from ^{1}H NMR spectrum:

The degree of polymerization was obtained by integrating the aromatic signal of the TPE end-group and the aromatic group from the repeat units (*a, b, c, d, e, k, l, m*), these signals were compared to the integra of group *j*. To calculate the DP of TPE-PBLG, the total integral area of peaks is defined as A_{abcdeklm} and set as 100, with which the integral area of peak *j*, defined as A_{j}, can be therefore obtained. Then we set the DP of TPE-PBLG as *n*, which should satisfy the following equation:

$$\frac{A_{\mathrm{abcdeklm}}}{A_{\mathrm{j}}} = \frac{19 + 5n}{2n} \quad (\boldsymbol{Equation\ 1})$$

For TPE-PBLG$_{17}$, $A_{\mathrm{j}} = 32.51$, $n = 17$

For TPE-PBLG$_{28}$, $A_{\mathrm{j}} = 35.19$, $n = 28$

For TPE-PBLG$_{40}$, $A_{\mathrm{j}} = 36.58$, $n = 40$

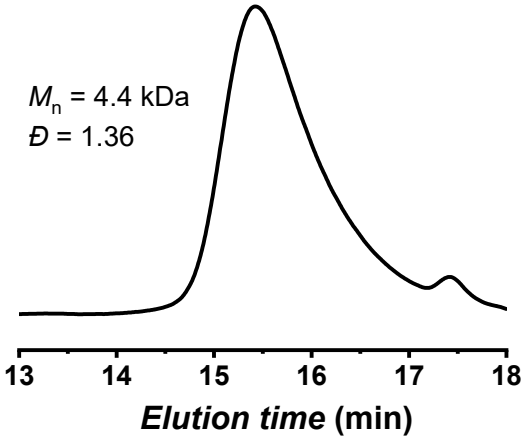


Fig. S6. DMF SEC trace of TPE-PBLG$_{17}$.

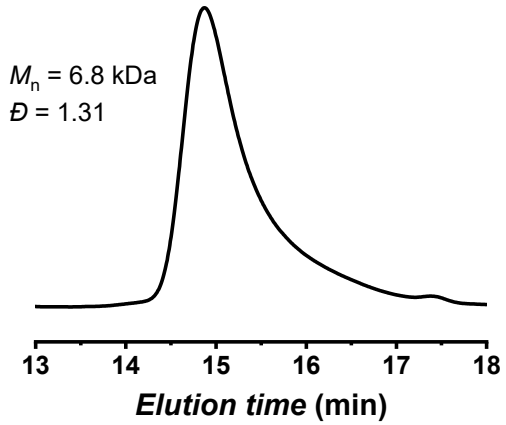


Fig. S7. DMF SEC trace of TPE-PBLG$_{28}$.

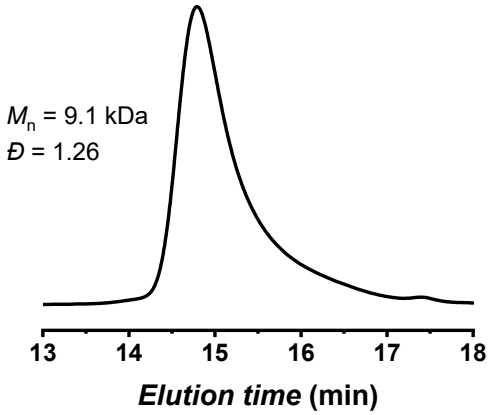


Fig. S8. DMF SEC trace of TPE-PBLG$_{40}$.

In ring-opening polymerizations of NCA using conventional amine initiators with low nucleophilicity (R–NH_2), it is difficult to achieve a living polymerization once an R–NH_2 initiator reacts with an NCA monomer, leading to relatively broad molecular weight distribution.[2]

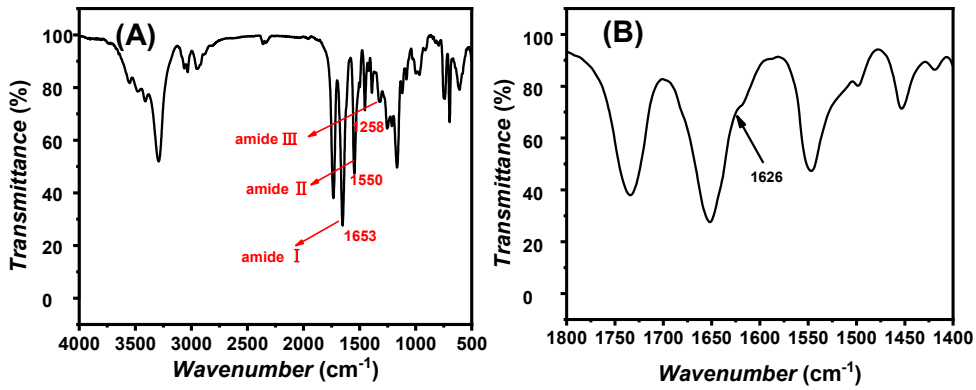


Fig. S9. FTIR spectra of freeze-dried powder of TPE-PBLG$_{28}$.

The absorbance bands around 1650, 1550, and 1250 cm^{-1} can be ascribed to the amide I, II, and III bands of polypeptide. The small peak at 1626 cm^{-1} was assigned to β-sheets, which indicated TPE-PBLG$_{28}$ adopts imperfect α-helix structure.

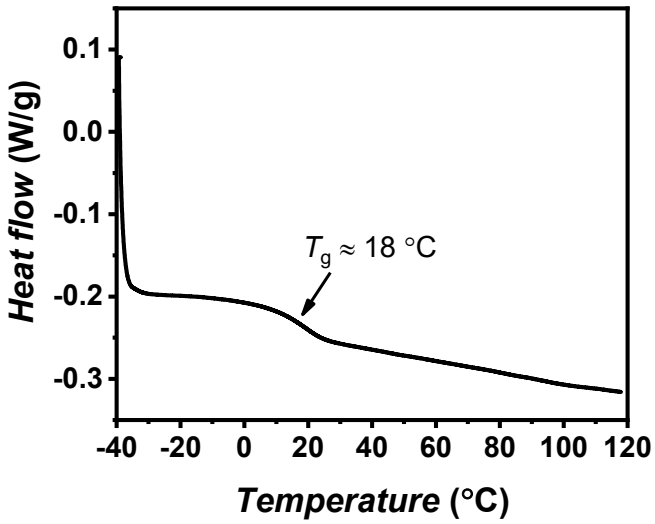


Fig. S10. DSC trace for freeze-dried TPE-PBLG$_{28}$ powder. The T_g was obtained from the last run of DSC traces.

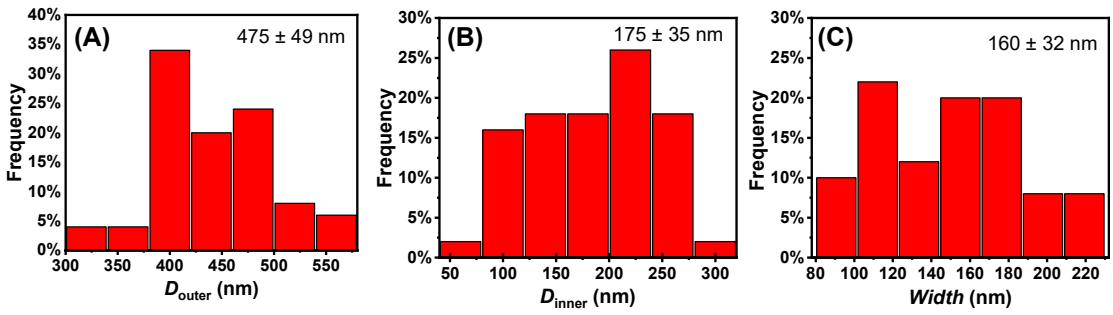


Fig. S11. Statistical average size distribution of (A) outer diameter (D_{outer}), (B) inner diameter (D_{inner}) and (C) width (W) of the toroids self-assembled from TPE-PBLG$_{28}$, determined by SEM analysis.

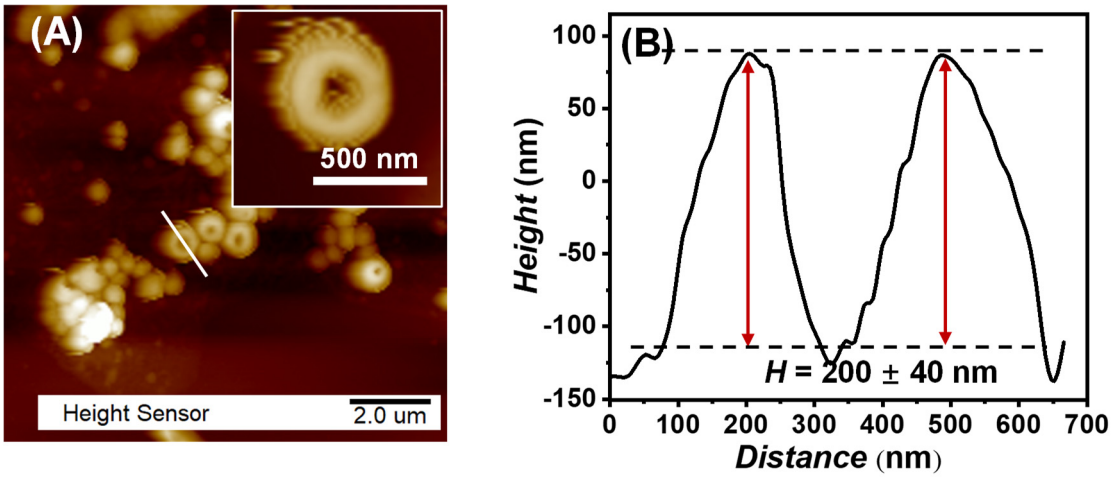


Fig. S12. (A) AFM image of toroids. (B) AFM height profile along the white line in (A). T_s = 25 °C and V_{water} : V_{THF} = 3 : 1, the assemblies were dialyzed against deionized water for removing THF.

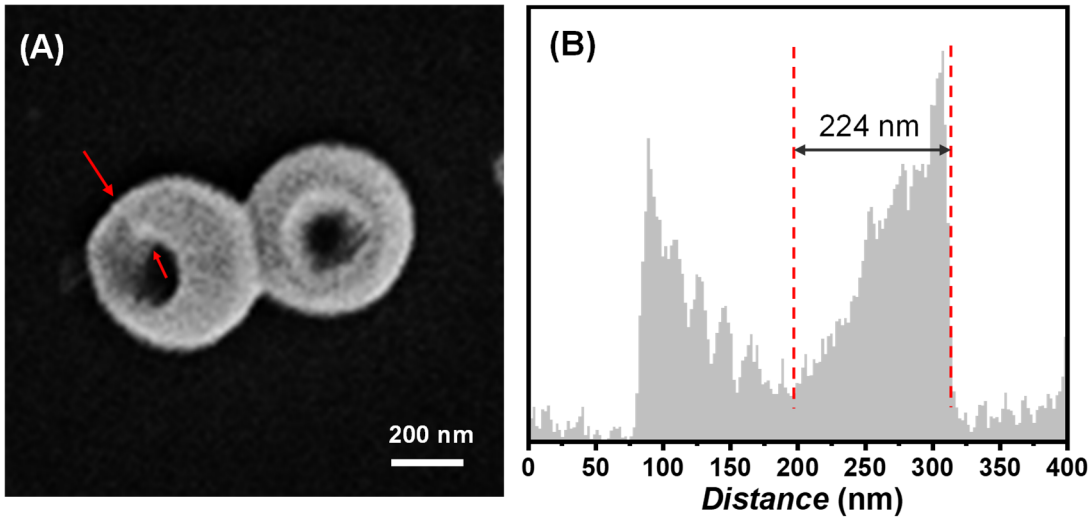


Fig. S13. (A, B) Analysis of the height of toroids self-assembled from TPE-PBLG$_{28}$, as measured by SEM across the toroids. The red arrow displays the height of toroids. T_s = 25 °C and V_{water} : V_{THF} = 3 : 1, the assemblies were dialyzed against deionized water for removing THF.

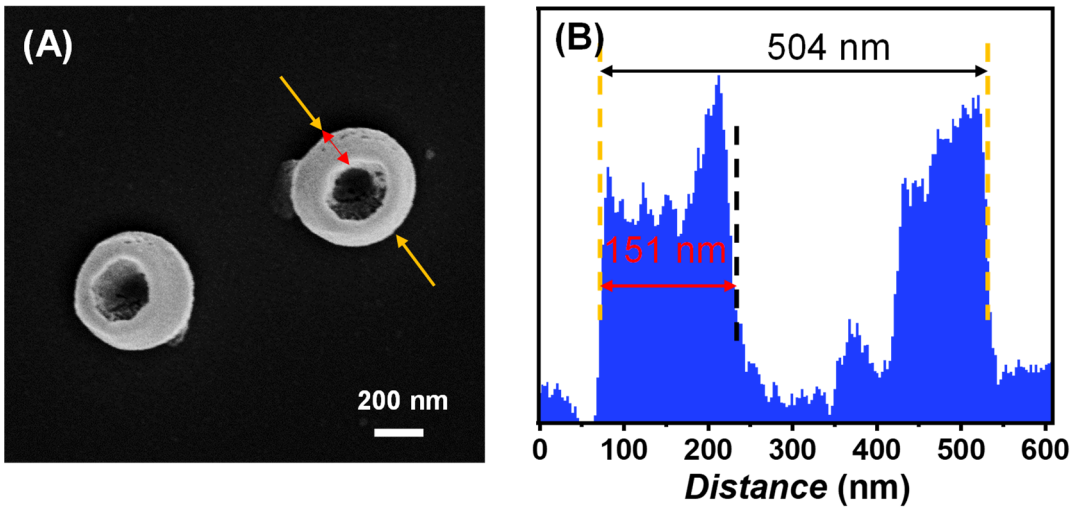


Fig. S14. (A) SEM image of toroids. (B) Intensity profiles of D_{outer} and width of the toroid. The yellow arrows represent the D_{outer} and the red arrow displays the width of the toroid. T_s = 25 °C and V_{water} : V_{THF} = 3 : 1. The assemblies were dialyzed against deionized water for removing THF.

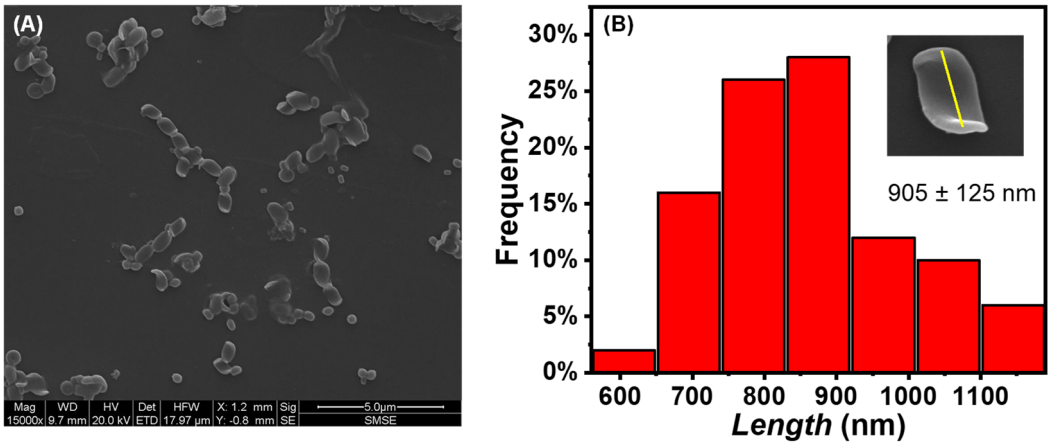


Fig. S15. (A) SEM image of twisted nanoparticles. (B) Statistical average length of twisted nanoparticles from (A); the yellow line in the insert represents the length from the edge of two end-heads in twisted nanoparticles.

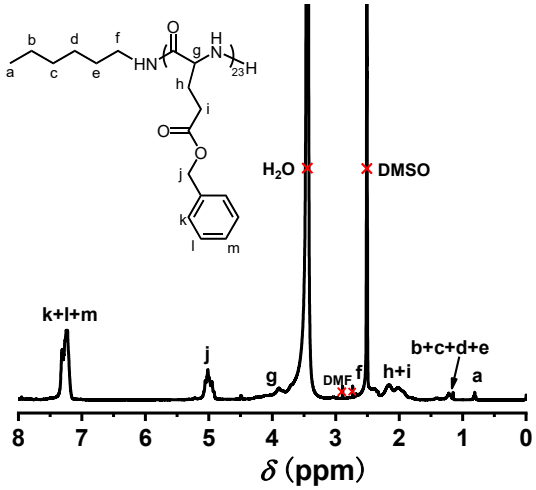


Fig. S16. ^{1}H NMR spectrum of HEX-PBLG$_{23}$ in DMSO-d_6.

The calculation of the DP of HEX-PBLG: the integral area of peak *a* (defined as A_a) is set as 3.00, with which the integral area of peaks *k*, *l*, *m* (defined as A_{klm}) is obtained as 113.25. As a consequence, the DP of HEX-PBLG$_n$ is calculated as:

$$n = \frac{A_{klm}}{5} = \frac{113.25}{5} \approx 23 (\boldsymbol{Equation\ 2})$$

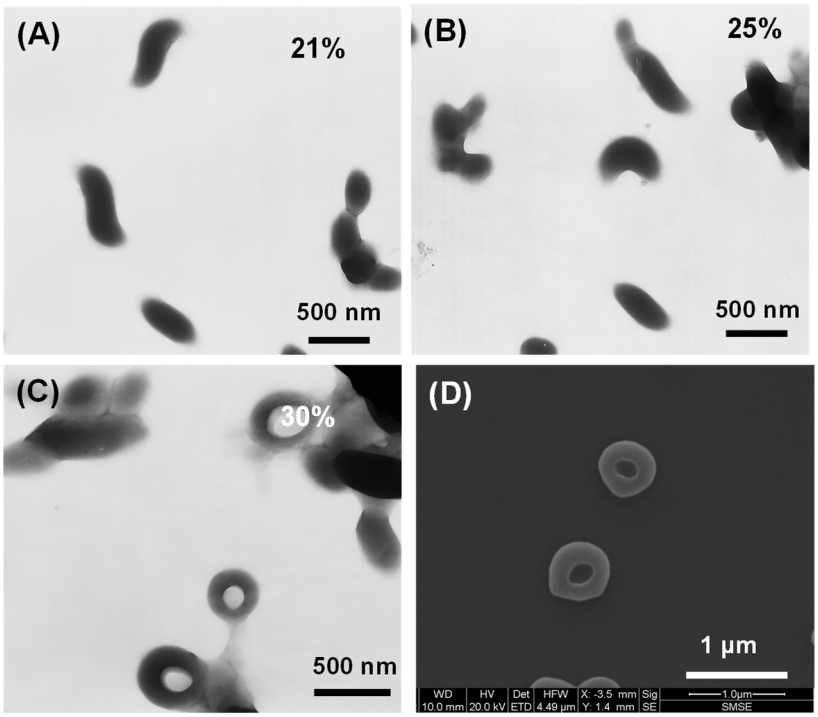


Fig. S17. (A-C) TEM images of nanoparticles self-assembled from HEX-PBLG$_{23}$ at different water contents. (D) SEM image of toroids.

TEM images of different nanoparticles confirmed that the toroids are also formed by end-to-end closure of twisted nanoparticles, suggesting that the end group TPE is not essential for the formation of toroids.

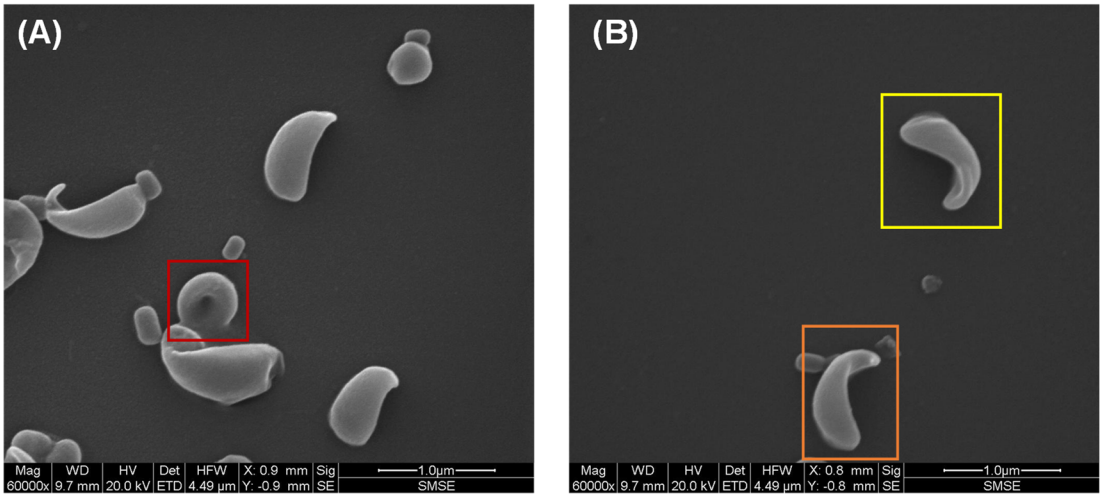


Fig. S18. (A) SEM image of twisted nanoparticles self-assembled from TPE-PBLG$_{28}$ at 25% water content; the red frame highlights a coexisting toroid. (B) Side view of the twisted micelle in the orange frame and the curved micelle in the yellow frame. The end-heads of twisted nanoparticle shows twisted feature but gradually close in curved nanoparticle.

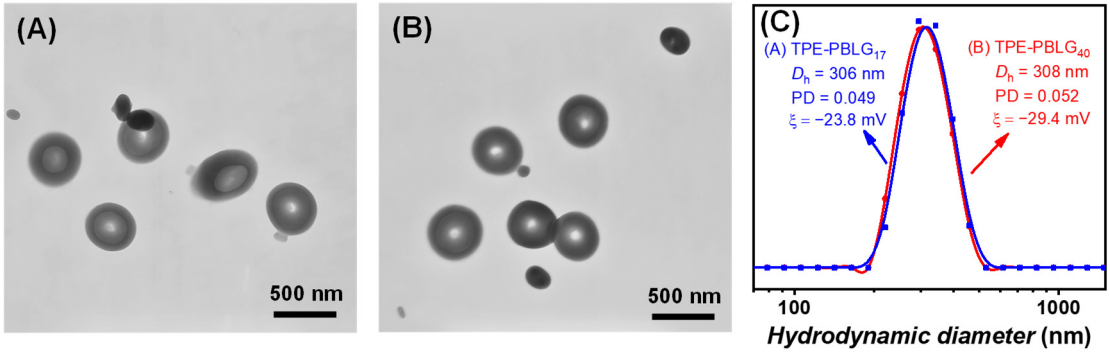


Fig. S19. (A, B) TEM images and (C) DLS analysis of the toroids self-assembled from TPE-PBLG$_{17}$ and TPE-PBLG$_{40}$, respectively. T_s = 25 °C and V_{water} : V_{THF} = 3 : 1. The assemblies were dialyzed against deionized water for removing THF.

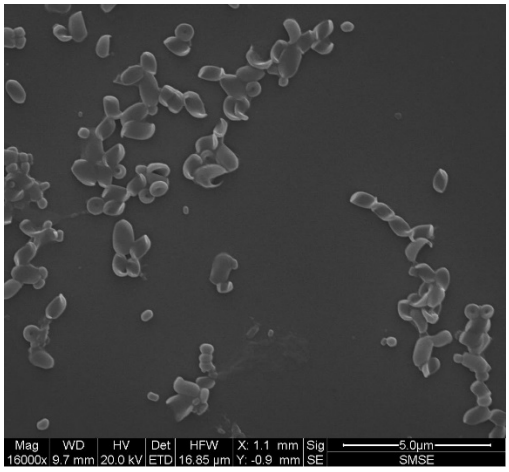


Fig. S20. The twisted nanoparticles were self-assembled from TPE-PBLG$_{28}$. T_s = 15 °C, V_{water} : V_{THF} = 3 : 1. Fig. 4C in the main text is a part of this figure.

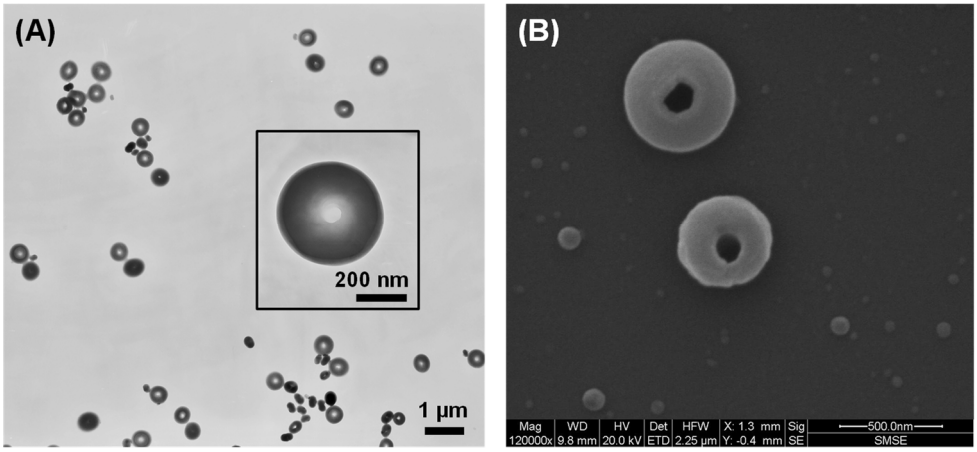
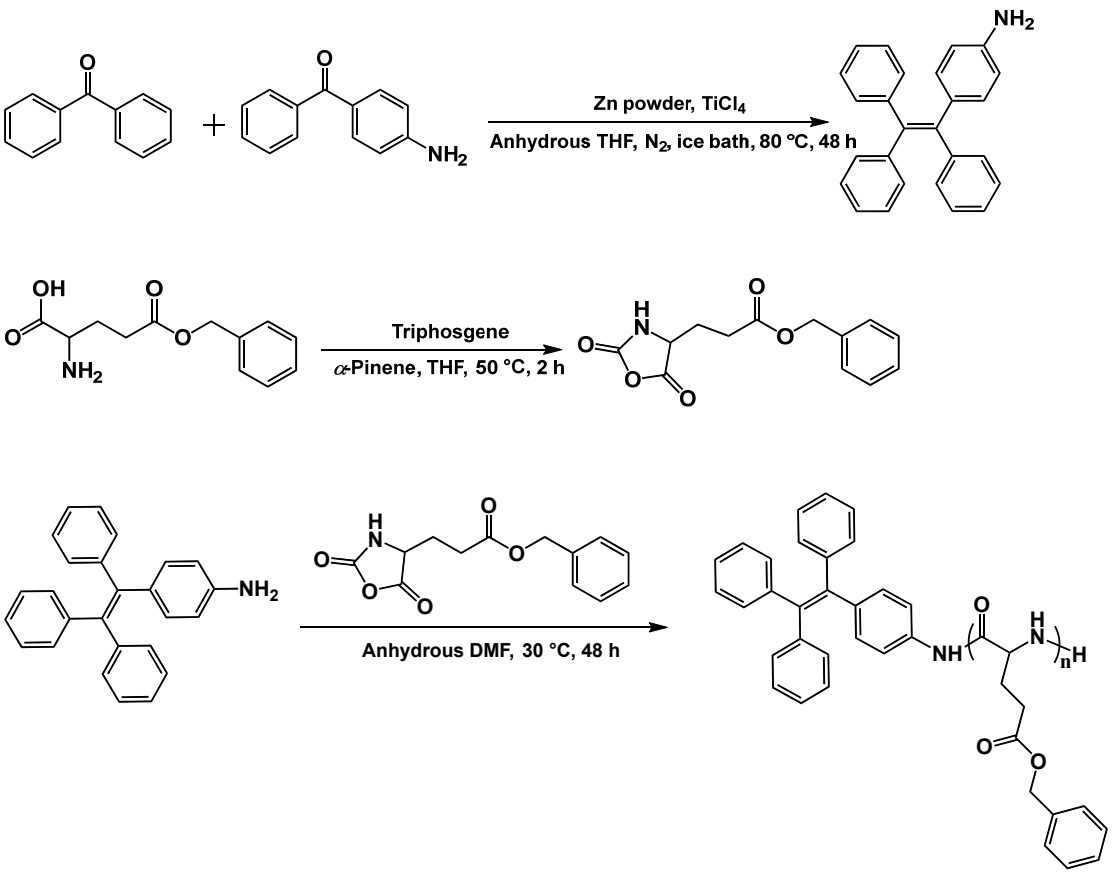


Fig. S21. (A) TEM and (B) SEM images of toroids self-assembled from TPE-PBLG$_{28}$ at T_s of 40 °C (above 25 °C). The assemblies were dialyzed against deionized water for removing THF.

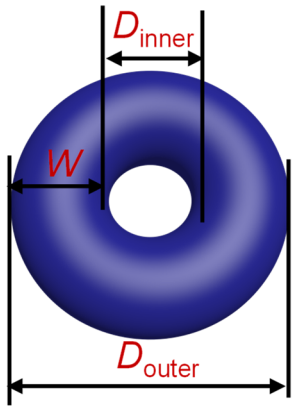
Table S1. Morphologies of nanoparticles self-assembled from TPE-PBLG$_{28}$ at different self-assembly temperatures (T_s) at a fixed water content, V_{water} : V_{THF} = 3 : 1.

Self-assembly temperature (T_s, °C)	Main/coexisting morphologies	Fraction (%)[a]
5	Ellipsoids	85.4
10	Ellipsoids / Oblate-ellipsoids	22.5 / 56.4
15	Ellipsoids / Twisted nanoparticles	23.2 / 62.1
20	Twisted nanoparticles / Curved nanoparticles	20.4 / 64.8
25	Curved nanoparticles / Toroids	11.4 / 66.3
30	Toroids / Nanospheres	64.2 / 35.6

[a] Measured from TEM and SEM images and the proportion is the number fraction.



Scheme S1. Synthesis of initiator TPE-NH_2, BLG-NCA monomer and homopolypeptides TPE-$PBLG_n$ (n =17, 28, 40).

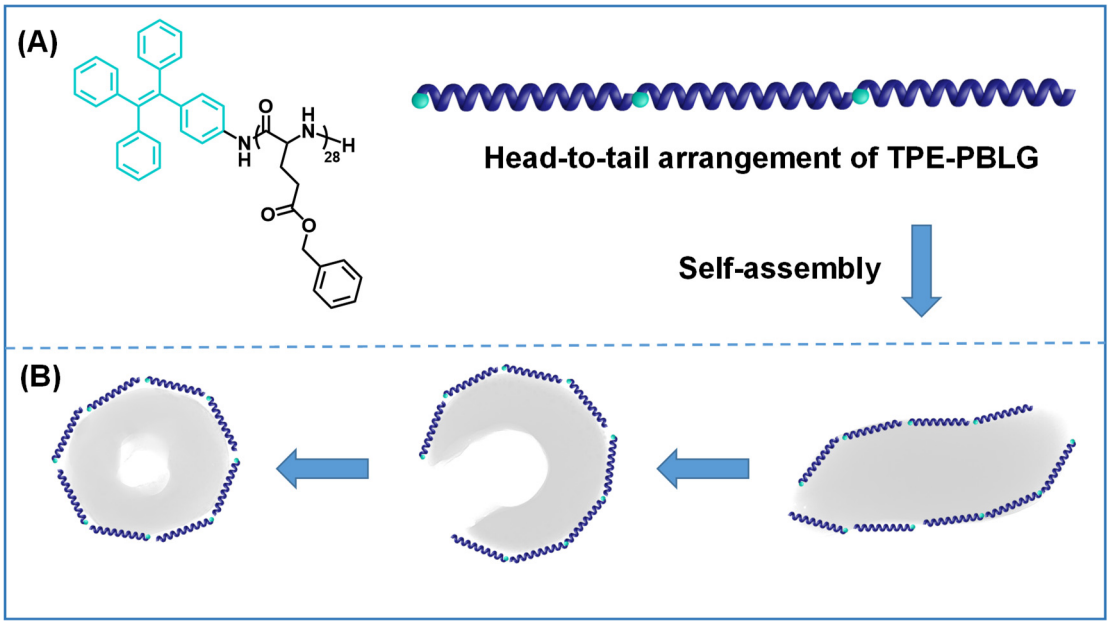


Scheme S2. Graphic illustration about parameters of outer diameter (D_{outer}), inner diameter (D_{inner}) and width (W) for a toroid.

The circumference of per toroid is roughly calculated by the following equation:

$$C = \pi(D_{outer} - W) \qquad (\boldsymbol{Equation\ 3})$$

For SEM analysis: D_{outer} = 475 ± 49 nm, W = 160 ± 32 nm, C = 989 ± 51 nm.



Scheme S3. (A) Illustration of head-to-tail arrangement of TPE-PBLG along the direction of its rod axis in helicogenic solvent THF. (B) Illustration of arrangement of TPE-PBLG for different nanoparticles such as twisted, curved, and toroidal nanoparticles determined by water content and T_s.

5. References

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