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# Spacer driven morphological twist in Phe-Phe dipeptide conjugates

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ABSTRACT

Chemists and material scientists are interested in complex biological systems and morphologies for fabrication of new materials. Bioinspired design of short peptide conjugates produces various self-assembled structures, depending on their structural diversity. Phe-Phe aromatic dipeptide is an interesting candidate for designing variety of molecular scaffolds. This report describes the synthesis of two symmetrical peptide conjugates by a coupling with C2 and C3 symmetric functional linker's, which spontaneously generate different self-assembled ultra-structures. C2 Symmetric, ethylene diamine linker, conjugated two Phe-Phe dipeptides and produced Phe-Phe-*EDA*-Phe-Phe (**5**), which undergoes spontaneous self-assembly to form fibrillar morphology, while C3 symmetric tris(2-aminoethylamine) conjugated three Phe-Phe dipeptides, produced (Phe-Phe)$_3$TREN (**7**) resulted in spherical morphology structures interconnected by fibers. A rational design of two peptide conjugates, by introducing two different linker's, direct different self-assemble pathway, which result in distinct morphologies.



## 1. Introduction

The organization of molecules into highly ordered assemblies in chemistry and biology, is a phenomenon of great interest to researchers of both disciplines. The process 'autonomous organization' also known by the term 'molecular self-assembly'[1] is mediated through weak inter-molecular forces such as van der Waals, electrostatic interactions, hydrogen bonds and π–π stacking interactions. The combined effect of these weak non-covalent interactions plays an intriguing role in the fabrication of well-ordered supramolecular architectures. In recent years, extensive studies on the fabrication of new materials using natural building blocks, such as, nucleic acids, lipids, and amino acids have been performed.[2] Several studies have demonstrated that short peptides, which possess specific molecular recognition modules may form well organized assemblies such as tubes, tapes, and spheres.[3] These assemblies could be utilized for various applications, including molecular electronics, tissue engineering, and material science.[4] Recent reports showed that short aromatic dipeptides derived from natural fibril forming peptides form nanometric tubular structures, which have been used as a template for the fabrication of metallic nanowires and coaxial metal nano-cables.[5] The large surface area of these nanostructures was utilized for the formation of a sensitive sensor, which detects phenol,[6] as well as for superhydrophobic coatings for microfluidic devices and electrostatic ultracapacitors, which significantly extended the electrode capacitance.[7] In addition, various methodologies were used for the vertical and horizontal alignment of peptide nanotubes.[8] Moreover, extensive work was done to study the role of π-stacking interaction in the self-assembly process.[9] Another interesting aspect in designing the peptide based nanostructures is the fusion of multiple short peptides by an intelligent selection of spacers, which allows for high flexibility and conformational freedom in the construct. Interesting reports by our group and others showed that the use of symmetric and asymmetric bifunctional linkers as well as a trifunctional linkers between peptides affect the self-assembly process.[10] Here, we report the fascinating role of various spacers fused in between the short homo dipeptide, Phe-Phe, on the production of different self-assembled nanostructures.

## 2. Results and discussion

### 2.1. Synthesis of Phe-Phe peptide conjugates

Phe-Phe-*EDA*-Phe-Phe (Compound **5**) and (Phe-Phe)$_3$TREN conjugate (Compound **7**) were synthesized by the solution phase peptide synthesis method. Scheme 1 shows the stepwise synthesis of compounds **5** and **7**. Compound **1** was hydrolyzed with the 1 N NaOH in methanol to form compound **2**, which was converted to its active NHS ester resulting in compound **3**. Compound **3** was treated with the ethylene diamine to form compound **4**. Compound **4** was treated with the 1 N HCl in EtOAc resulting in compound **5**. For the

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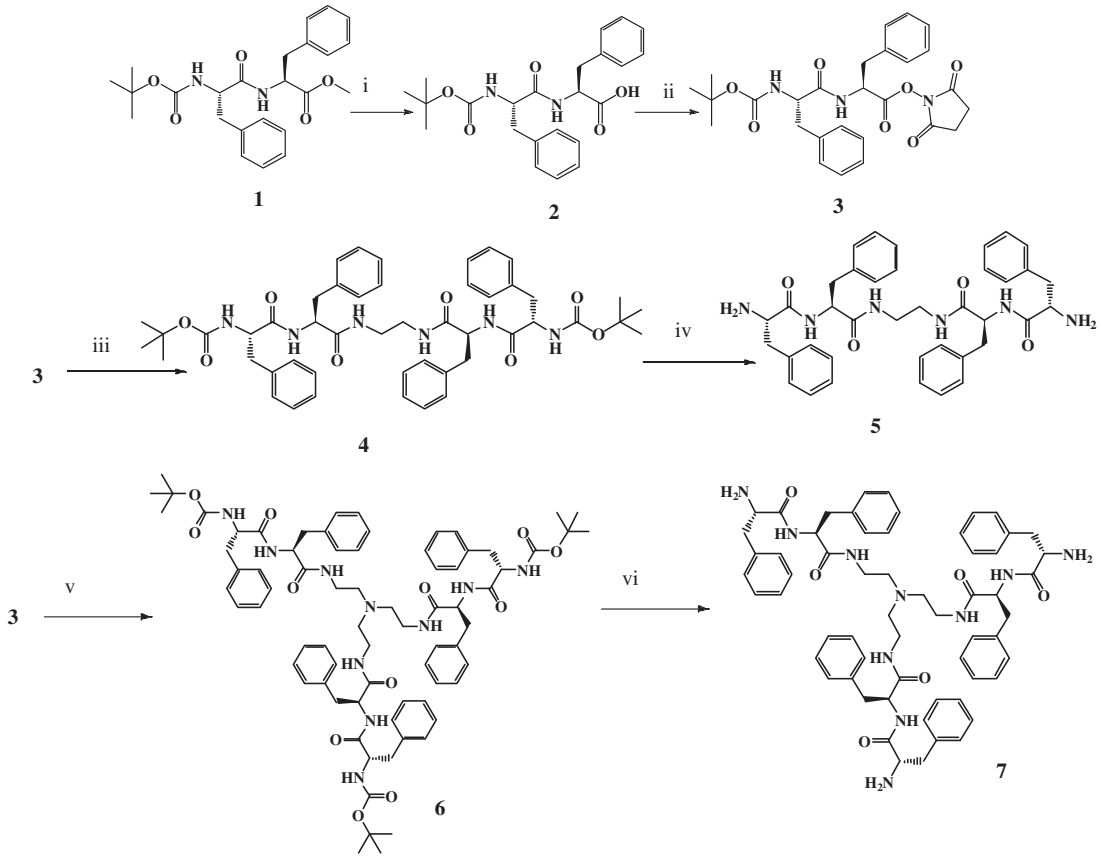


(i) 1 N NaOH, MeOH, RT, 24h; (ii) NHS, DCC, 1,2 Dimethoxyethane, 0 $^{0}$C, 3h; (iii) Ethylene diamine, DMF, RT, 24h; (iv) 1N HCl in EtOAc, 3h, ion exchange column; (v) TREN, DMF, RT, 24h; (vi) 1N HCl

**Scheme 1.** Synthesis of compound **5** and **7**, Phe-Phe-*EDA*-Phe-Phe and (Phe-Phe)$_3$TREN, respectively.

synthesizing of the triskelion derivative of Phe-Phe conjugate, compound **3** was again treated with tris(2-aminoethyl) amine to form compound **6**, which was further treated with 1 N HCl in EtOAc to form compound **7**. Detailed experimental procedures are given in the Experimental section.

### 2.2. Morphological structures characterization of Phe-Phe-*EDA*-Phe-Phe conjugate

As mentioned above, the Phe-Phe dipeptide forms discrete nanotube structures.[5] Here, we observe that two Phe-Phe conjugated with ethylene diamine, Phe-Phe-*EDA*-Phe-Phe (**5**) shows an extensive fiber formation in 60% methanol in water. Optical microscopy [OM] images show the extensive fiber formation in 60% methanol in water (Fig. 1a), and a magnified view of the OM image shows rod-shaped fibers (Fig. 1b). Atomic force microscopy images exhibit the fibrous bundles, and a higher magnification reveals the fibers approximated width of 60 nm (Fig. 1c, d). Transmission electron microscopy micrograph also shows the fibrous bundle with an averaged width of 10–20 nm (Fig. 1e). We have further investigated this fiber morphology in environmental scanning microscopy, which also confirmed the rod shaped fiber formation (Fig. 1f). Therefore, Phe-Phe-*EDA*-Phe-Phe in 60% methanol in water exhibited extensive nanofiber formation with a varied diameter of 10–500 nm.

### 2.3. Morphological structure characterization of the triskelion: (Phe-Phe)$_3$TREN conjugate

Interestingly, we have also investigated the morphology of the self-assembled triskelion structures, which composed of three diphenylalanine peptide modules. The triskelion dissolving in 60% methanol in water resulted in the formation of fibers emanating from the spherical structures. Scanning electron microscopy image reveals the fibers radiating from the spherical surface (Fig. 2a). Similar observation was obtained using atomic force microscopy, which exhibited spherical structures with a diameter ranging from 600 nm to 1 μm, and fibers radiating from the vesicular surface with an average diameter of 60–100 nm (Fig. 2b). In addition, it was also apparent that the fibers interconnect the spherical structures.

### 2.4. Secondary structures analysis of the self-assembled nanostructures

In order to study the secondary structures of the two compounds, we have performed Fourier-transform infrared (FTIR) spectroscopy of the conjugates in 60% methanol in water. The amide I spectral peak obtained for Phe-Phe-*EDA*-Phe-Phe conjugate at around 1652 cm$^{-1}$, indicates the typical α-helix structure, while (Phe-Phe)$_3$TREN conjugate peak appeared at 1643 cm$^{-1}$ indicates the typical $3_{10}$ helix structure (Fig. 3).[11]

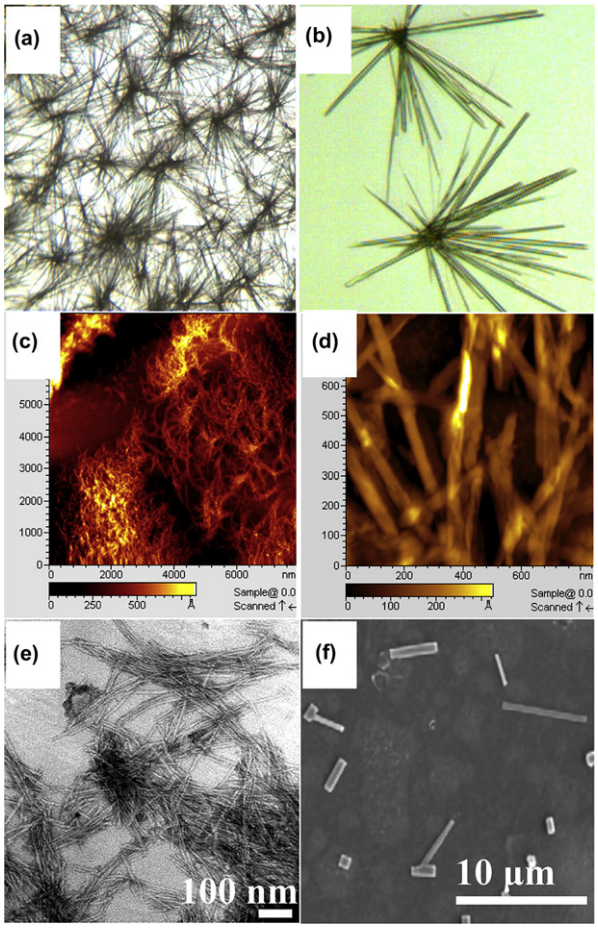


**Fig. 1.** Microscopy images of the Phe-Phe-*EDA*-Phe-Phe conjugates in 60% methanol in water: (a) optical microscopy image shows the extensive fiber formation; (b) high magnification image of the fibers (c) atomic force microscopy [AFM] image of fibrous aggregates; (d) high magnification AFM image of the fibers; (e) TEM image of the fibers aggregates; (f) ESEM image of the fibers.

## 2.5. Nanostructures stained with thioflavin T

Thioflavin T is a benzothiazole dye, which is well known for exhibiting enhanced fluorescence upon binding to amyloid fibrils and is commonly used to identify and quantify amyloid fibrils, both *ex vivo* and *in vitro*.[12] For investigating the ordered nature of the nanostructures, we have stained the fibers with thioflavin T. Phe-Phe-*EDA*-Phe-Phe stained with Thioflavin T shows green fluorescence fibers (Fig. 4a) while (Phe-Phe)$_3$TREN conjugate shows stained spherical structures (Fig. 4b).

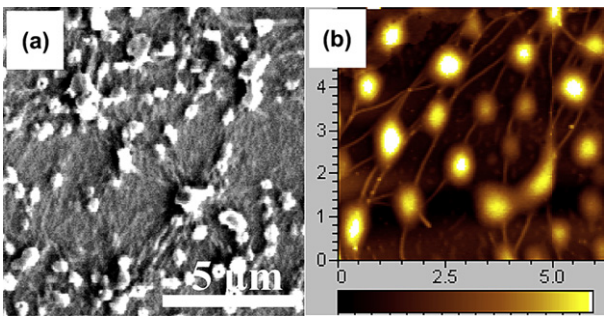


**Fig. 2.** Microscopy image of the (Phe-Phe)$_3$TREN conjugate in 60% methanol in water: (a) SEM image shows the fibers radiating from the vesicular surface; (b) atomic force microscopy image also shows the fibers radiating from the vesicular surface and interconnects to each other.

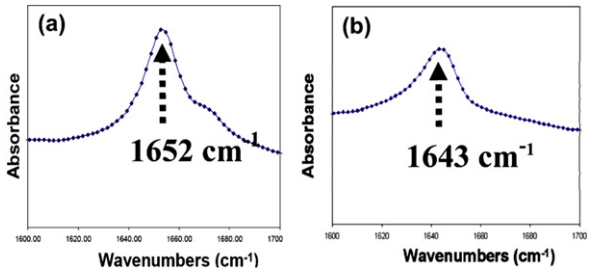


**Fig. 3.** FTIR spectroscopic studies of the Phe-Phe conjugate in 60% methanol in water: (a) absorption maxima of Phe-Phe-*EDA*-Phe-Phe at 1652 cm$^{-1}$ indicates the typical α-helix structures; (b) absorption maximum of (Phe-Phe)$_3$TREN at 1643 cm$^{-1}$ indicates the typical 3$_{10}$ helix structures.

The experimental results presented in the current article provide an interesting evidence for the morphological and structural features of the Phe-Phe conjugates. A key point in these simple systems is the ability to form well-ordered assemblies by the facilitation of the geometrically-restricted π–π stacking.[5,7,13] Following is a potential model for the mode of interaction of the peptide conjugate building-block (Fig. 5).

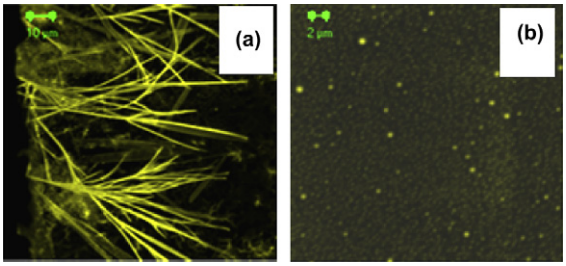


**Fig. 4.** Fluorescence microscopy image of Phe-Phe conjugates with Thioflavin T (ThT) in 60% methanol water: (a) Phe-Phe-*EDA*-Phe-Phe shows fiber morphology and (b) (Phe-Phe)$_3$TREN shows spherical structures.

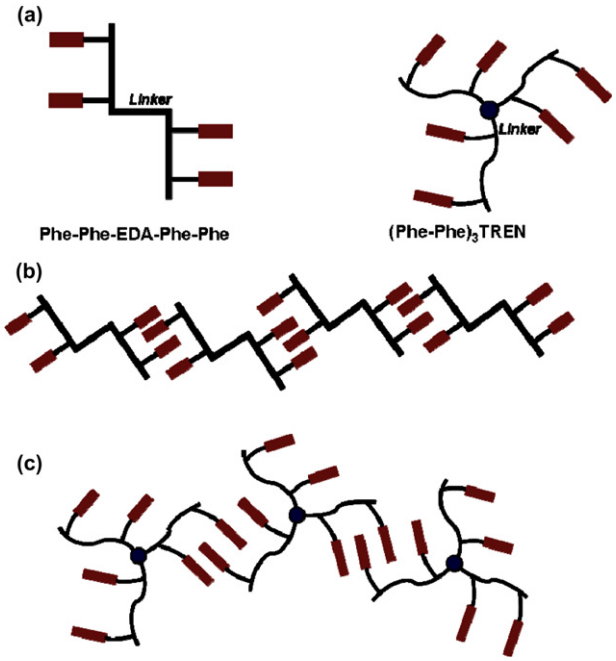


**Fig. 5.** Model for the molecular organization of the Phe-Phe conjugates. (a) Schematic illustration of Phe-Phe-*EDA*-Phe-Phe and (Phe-Phe)$_3$TREN. (b) Possible intermolecular interaction through π–π stacking in Phe-Phe-EDA-Phe-Phe. (c) Possible intermolecular interaction through π–π stacking in (Phe-Phe)$_3$TREN.

## 3. Conclusion

In conclusion we have reported and demonstrated novel design, synthesis and microscopic evaluation of self-assembled structures of two different peptide conjugates. Direction of self-assembly was shown to be solely dependent on the symmetry of linkers, and as a consequence, a morphological switch was observed. Bioinspired selection of linkers offers the design of novel peptide conjugates, which could open up new avenues of smart materials.

## 4. Experimental

### 4.1. General

Dichloromethane, *N*,*N*-dimethylformamide, methanol, triethylamine and 1,2-dimethoxy ethane were distilled following standard procedures prior to use. *N*, *N*'-dicyclohexylcarbodiimide (DCC), *N*-hydroxybenzotriazole (HOBT), *t*-butyloxycarbonyl carbonate, L-amino acids were purchased from Spectrochem, Mumbai, India, and used without further purification. Tris (2-aminoethyl) amine (TREN) was purchased from Sigma. $^{1}$H and $^{13}$C NMR spectra were recorded on JEOL-JNM LAMBDA 400 model operating at 400 and 100 MHz, respectively. HRMS spectra were recorded on Waters Q-TOF Premier Micromass MS Technology.

### 4.2. Synthesis of compound 1

(BOC)FF–OMe (**1**): *N*-(BOC)-L-Phenylalanine (5 g, 18.8 mmol), and *N*-Hydroxybenzotriazole (2.53 g, 18.8 mmol) were dissolved in dry DMF (25 ml) and reaction mixture was cooled to 0 °C under nitrogen atmosphere. Solution of DCC (4.26 g, 20.6 mmol) in dichloromethane was then added into the reaction mixture. The reaction mixture was stirred at 0 °C for 1 h. After which, L-Phenylalanine methyl ester hydrochloride (3.3 g, 18.8 mmol) was added into the reaction mixture followed by triethylamine (3.13 ml, 22.5 mmol) and the reaction mixture was stirred for 24 h at room temperature. Reaction mixture was filtered to remove DCU and filtrate was concentrated in reduced pressure. The residue was dissolved in dichloromethane and the organic layer was washed with 1 N HCl (3×30 ml), 10% $NaHCO_3$ (3×30 ml) and brine (30 ml). The organic layer was dried over anhydrous sodium sulfate and concentrated in reduced pressure. The crude compound was purified through silica gel column chromatography by using dichloromethane and methanol (94:6) solvent system to form the pure compound **1** (6.2 g, 14.5 mmol). mp=102–105 °C, $R_f$ [5% methanol in dichloromethane]=0.5, =−00.04 [*c* 0.46, methanol]. $^{1}$H NMR (400 MHz, $CDCl_3$, TMS, $\delta$ ppm): 1.32 (s, 9H); 2.93–2.99 (m, 4H); 3.6 (s, 3H); 4.2 (m, 1H); 4.70–4.71 (m, 1H); 6.89–6.91 (m, 2H); 7.08–7.23 (m, 10H, overlapped aromatic signal and $CDCl_3$ peak); $^{13}$C NMR (100 MHz; $CD_3OD$, $\delta$ ppm): 28.6, 38.4, 39.2, 52.6, 55.0, 57.1, 80.5, 127.6, 127.8, 129.3, 129.5, 130.3, 137.8, 138.5, 157.4, 173.0, 174.1; FTIR (KBr, $cm^{-1}$): 1523 (amide II); 1655 (amide I); 3329 (−NH str); FABMS (M+1): 427; Anal. Calcd for $C_{24}H_{30}N_2O_5$ C, 67.59; H, 7.09; N, 6.57; found, C, 67.19; H, 6.71; N, 6.23.

### 4.3. Synthesis of compound 2

(BOC)FF–OH (**2**): Compound **1**(3.0 g, 7.0 mmol) was dissolved in methanol (30 ml) and 1(N) NaOH (10 ml) was added into the solution. The reaction mixture was stirred for 3 h at room temperature. Reaction mixture was concentrated to completely remove the methanol under reduced pressure. The residue was acidified with 1 N HCl (15 ml) and extracted in dichloromethane (3×25 ml). The combined organic layer was washed with water followed by brine (20 ml), dried over anhydrous sodium sulfate and concentrated under reduced pressure to form compound **2** (2.3 g, 5.5 mmol). mp=82–84 °C, $R_f$ [5% methanol in dichloromethane]=0.3, =−00.02 [*c* 0.12, methanol]. $^{1}$H NMR (400 MHz, CD3OD, TMS, $\delta$ ppm): 1.23 (s, 9H); 2.5–2.6 (m, 1H); 2.70–2.95 (m, 2H); 3.0–3.2 (m, 1H); 4.15–4.18 (m, 1H); 4.53–4.57 (m, 1H); 7.09–7.15 (m, 10H); FTIR (KBr, $cm^{-1}$): 1520 (amide II); 1660 (amide I); FABMS (M+1): 413; Anal. Calcd for $C_{23}H_{28}N_2O_5$, C, 66.97; H, 6.84; N, 6.79; found, C, 67.11; H, 6.66; N, 6.12.

### 4.4. Synthesis of compound 3

(BOC)FF–NHS (**3**): Compound **2** (0.5 g, 1.2 mmol) and *N*-Hydroxy succinimide (0.153 g, 1.3 mmol) were dissolved in 1,2-dimethoxy ethane (10 ml) and reaction mixture was cooled to 0 °C under nitrogen atmosphere. A solution of DCC (0.274 g, 1.3 mmol) in 1,2-dimethoxy ethane (5 ml) was added into the reaction mixture dropwise and reaction mixture was stirred for 2 h at 0 °C. After which the reaction mixture was kept in a freezer overnight. The reaction mixture was filtered and filtrate was concentrated under reduced pressure. Solid material was washed with diethyl ether and dried under high vaccum. Crude compound **3** (0.6 g, 1.1 mmol) was directly used for synthesis of compounds **4** and **6**.

### 4.5. Synthesis of compound 4

[(BOC)FF]2EDA (**4**): Compound **3** (2.5 g, 4.9 mmol) was dissolved in dry DMF (30 ml) at room temperature under nitrogen atmosphere. Solution of ethylene diamine (0.144 g, 2.4 mmol) in dry DMF (1.0 ml) was added into the reaction mixture dropwise and stirred under nitrogen atmosphere at room temperature for 24 h. The reaction mixture was concentrated under reduced pressure and water was added. Solid material was washed with 1N HCl (3×15 ml), 10% $NaHCO_3$ (3×10 ml), water 20 ml and diethyl ether 20 ml and dried by vaccum to form pure compound **4** (2.0 g, 2.3 mmol). mp=202–204 °C, $R_f$ [5% methanol in dichloromethane]=0.5, =−00.02 [*c* 0.48, methanol]. $^{1}$H NMR (400 MHz, DMSO-$d_6$, TMS, $\delta$ ppm): 1.27 (s, 18H); 2.82–2.85 (m, 4H); 2.85–2.98 (m, 4H); 3.32 (4H, overlapped signal for linker's H and DMSO-$d_6$ peak); 4.03–4.10 (m, 2H); 4.44–4.49 (m, 2H); 6.88–6.90 (m, 2H); 7.10–7.24 (m, 20H); 7.95 (broad signal, 4H); $^{13}$C NMR (100 MHz; DMSO-$d_6$, $\delta$ ppm): 28.09, 33.35, 37.43, 38.01, 47.5, 53.8, 55.8, 78.1, 126.3, 127.9, 129.29, 137.4, 138.0, 155.12, 170.7, 171.2; FTIR (KBr, $cm^{-1}$): 1526 (amide II); 1648 (amide I); 3302 (−NH str); FABMS (M+1): 850; Anal. Calcd for $C_{48}H_{60}N_6O_8$, C, 67.90; H, 7.12; N, 9.90; found, C, 67.55; H, 6.89; N, 9.63.

### 4.6. Synthesis of compound 6

[(BOC)FF]$_3$TREN (**6**): Compound **3** (0.8 g, 1.5 mmol) was dissolved in dry DMF (4.5 ml) at room temperature under nitrogen atmosphere. Solution of Tris (2-aminoethyl) amine (0.0642 g, 0.43 mmol) in dry DMF (0.5 ml) was added into the reaction mixture dropwise and stirred under nitrogen atmosphere at room temperature for 24 h. The reaction mixture was concentrated under reduced pressure and water was added. Solid material was washed with 1 N HCl (3×15 ml), 10% $NaHCO_3$ (3×10 ml), water 20 ml and diethyl ether 20 ml and dried by vaccum to form pure compound **6** (0.55 g, 0.41 mmol). mp=178–180 °C, $R_f$ [10% methanol in dichloromethane]=0.6, =−00.02 [*c* 0.26, methanol]. $^{1}$H NMR (400 MHz, DMSO-$d_6$, TMS, $\delta$ ppm): 1.26 (s, 27H); 2.73–2.83 (m, 6H); 2.93–3.07 (m, 6H); 3.3 (s, overlapped signal for linker's 12H and DMSO-$d_6$ peak); 4.18–4.20 (m, 3H); 4.45–4.52 (m, 3H); 6.87–6.89 (d, 3H, $J$=8 Hz), 7.15–7.21 (m, 30H); 7.86 (broad s, 3H); 7.97–7.99 (m, 3H); $^{13}$C NMR (100 MHz; DMSO-$d_6$, $\delta$ ppm): 27.9, 33.6, 37.3, 38.2, 47.3, 53.8, 56.1, 78.1, 126.3, 127.8, 129.3, 137.4, 138.02, 155.12, 170.74, 171.29; FTIR (KBr, $cm^{-1}$): 1527 (amide II); 1648 (amide I);

3324 (−NH str); FABMS (M+1): 1331; Anal. Calcd for $C_{75}H_{96}N_{10}O_{12}$, C, 67.75; H, 7.28; N, 10.53; found, C, 67.55; H, 7.01; N, 9.99.

### 4.7. Synthesis of compound 5

$(FF)_2$EDA (**5**): Compound **4** (0.17 g, 0.20 mmol) and 1 N HCl in EtOAc (5.0 ml) were mixed and stirred for 3 h. After 3 h solid material was separated from the reaction mixture. Solvent was removed from the reaction mixture by decantation method and residue was washed with EtOAc twice, followed by two washes with diethyl ether. Solid material was dried in high vacuum pump and desolved in (90%) methanol in water passed through ion exchange column. Solvent was removed in reduced pressure and solid material was dried under high vacuum. Crude solid material was dissolved in methanol and precipitated out with diethyl ether. Solid material was dried under high vacuum to form pure compound **5** (0.05 g, 0.07 mmol). mp=above 210 °C, $R_f$ [10% methanol in dichloromethane]=0.5, =−00.02 [*c* 0.26, methanol]. $^1$H NMR (400 MHz, CD3OD, TMS, $\delta$ ppm): 2.86−3.13 (m, 8H); 3.20−3.22 (m, 4H, overlapped signal for $CD_3OD$ and linker's H); 4.06−4.09 (m, 2H); 4.48−4.52 (m, 2H); 7.18−7.32 (m, 20H); $^1$H NMR (400 MHz, DMSO-$d_6$, TMS, $\delta$ ppm): 2.84−3.13 (m, 8H); 3.15 (s, 4H, linker's H); 3.95−4.05 (broad signal, 2H); 4.42−4.48 (m, 2H); 7.18−7.29 (m, 20H); 8.15−8.18 (m, 6H, overlapped signal for amine's 4H and amide's 2H); 8.90−8.92 (m, 2H, amide); $^{13}$C NMR (100 MHz; $CD_3OD$, $\delta$ ppm): 38.54, 39.08, 39.79, 50.2, 55.49, 56.7, 127.9, 128.8, 129.6, 130.1, 130.3, 130.6, 135.49, 138.14, 169.4, 172.9; FABMS (M+1): 650; Anal. Calcd for $C_{38}H_{44}N_6O_4$, C, 70.35; H, 6.84; N, 12.95; found, C, 70.11; H, 6.95; N, 13.11.

### 4.8. Synthesis of compound 7

$(FF)_3$TREN (**7**) : Compound **6** (0.2 g, 0.15 mmol) and 1 N HCl in EtOAc (6.0 ml) were mixed and stirred for 3 h. After 3 h solid material was separated out from the reaction mixture. Solvent was removed from the reaction mixture by decantation method and residue was washed with the EtOAc twice, followed by the two washes with diethyl ether. Solid material was dried by high vacuum and dissolved in methanol water (90%) passed through ion exchange column. Solvent was removed in reduced pressure and solid material was dried under high vacuum. Crude solid material was dissolved in methanol and precipitated out with diethyl ether. Solid material was dried under high vacuum to form pure compound **7** (0.07 g, 0.068 mmol). mp=Hygroscopic Not Performed, $R_f$ [10% methanol in dichloromethane]=0.4, =−00.02 [*c* 0.23, methanol]. $^1$H NMR (400 MHz, $CD_3OD$, TMS, $\delta$ ppm): 2.83−2.92 (m, 6H); 2.98−3.03 (m, 6H); 3.19 (s, 12H, overlapped signal for linker's H and $CD_3OD$ peak); 3.97−4.00 (m, 3H); 4.44−4.48 (m, 3H); 7.10−7.23 (m, 30H); $^{13}$C NMR (100 MHz; $CD_3OD$, $\delta$ ppm): 36.5, 38.3, 39.5, 55.2, 56.4, 57.8, 129.05, 129.8, 130.6, 131.10, 131.12, 131.5, 136.4, 138.8, 170.84, 175.0; FABMS (M − 1): 1028; Anal. Calcd for $C_{60}H_{72}N_{10}O_6$, C, 70.01; H, 7.05; N, 13.61; found, C, 69.85; H, 6.95; N, 13.33.

## 5. Nanostructure sample preparation and imaging

### 5.1. Nanostructure preparation

The lyophilizes powder of Phe-Phe-*EDA*-Phe-Phe (Compound **5**) and (Phe-Phe)$_3$TREN conjugate (Compound **7**) were dissolved in 60% methanol in water at a concentration of 1 mM.

### 5.2. Optical microscopy

Solution of conjugate **5** in 60% methanol in water was loaded onto a glass slide and images were captured under cross polarized light by Labomed Digi 3 microscope.

### 5.3. Scanning electron microscopy

20 μL of the incubated solution of conjugates **5** and **7** in 60% methanol in water at a concentration of 1 mM were deposited on slides, and then coated with gold. SEM measurements were performed on either FEI QUANTA 200 Microscope equipped with a tungsten filament gun or a JSM JEOL 6300 SEM operating at 5 kV. Environmental scanning electron microscopy images were captured using Quanta 200 FEG Field Emission Gun ESEM operating at 10 kV.

### 5.4. Atomic force microscopy

Solutions of conjugates **5** and **7** were imaged with an atomic force microscope (Molecular Imaging, USA) operating under acoustic AC mode (AAC), with the aid of a cantilever (NSC 12(c) from MikroMasch). The force constant was 0.6 N/m, while the resonant frequency was 150 kHz. The images were taken in air at room temperature, with a scan speed of 1.5−2.2 lines/s. The data acquisition was done using PicoScan 5® software, while data was analyzed with the aid of visual SPM. The solutions of conjugates were incubated for 0−7 days in 60% methanol in water and micrographs were recorded for selected incubation periods. 10 μL of sample solution was transferred onto freshly cleaved mica surface and uniformly spread with the aid of a spin-coater operating at 200−500 rpm (PRS-4000). The sample-coated mica was dried for 30 min at room temperature, followed by AFM imaging.

### 5.5. Transmission electron microscopy

3 μL solutions of conjugates **5** and **7** in 60% methanol water were loaded on carbon coated copper grids. After 1 min, excess fluid was removed and the grids were stained with 2% uranyl acetate in water. Excess staining solution was removed from the grid after 2 min. Samples were viewed using a JEOL 1200EX electron microscope operating at 80 kV. High resolution samples were viewed and analyzed with a Philips Tecnai F20 Field Emission Gun electron microscope operating at 200 kV.

### 5.6. ThT staining and confocal laser microscopy imaging

10 μL ThT solution (2 mM, PBS buffer) was mixed with 10 μL of each of the two Phe-Phe conjugates solutions: Phe-Phe-*EDA*-Phe-Phe (Compound 5) and (Phe-Phe)$_3$TREN conjugate (Compound **7**). An LSM 510 confocal laser scanning microscope (Carl Zeiss Jena, Germany) was used at excitation and emission wavelengths of 440 and 485 nm, respectively.

### 5.7. Fourier-transform infrared spectroscopy

Infrared spectra were recorded using Nicolet Nexus 470 FTIR spectrometer with DTGS detector. Solution samples of Phe-Phe-*EDA*-Phe-Phe (Compound **5**) and (Phe-Phe)$_3$TREN conjugate (Compound **7**) were dried on $CaF_2$ plate to form thin film. The peptide deposits were resuspended with $D_2O$ and dried. The resuspension procedure was repeated twice to ensure maximal hydrogen to deuterium exchange. The measurements were taken using a 4 $cm^{-1}$ resolution and 2000 scans averaging. The transmittance minima values were determined by OMNIC analysis program (Nicolet).

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## References and notes


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