Self-assembled 2D Free-Standing Janus Nanosheets with Single-Layer Thickness

Yiyang Lin, Michael R. Thomas, Amy Gelmi, Vincent Leonardo, E. Thomas Pashuck, Stephanie A. Maynard, Ye Wang, Molly M. Stevens*

Department of Materials, Department of Bioengineering and Institute of Biomedical Engineering, Imperial College London, Exhibition Road, London SW7 2AZ, UK.
E-mail: m.stevens@imperial.ac.uk

Experimental Section

Reagents and materials. All Fmoc-protected amino acids, Rink Amide resin, and 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) were purchased from Anaspec, Inc. Qdot 605 ITK Streptavidin Conjugate Kit was obtained from Life Technologies (U.K.). Avidin D, horseradish peroxidase (Av-HRP) was obtained from Vector Laboratories Ltd. Fmoc-11-aminoundecanoic acid was synthesized according to the method reported previously.[1] Fmoc-6-Ahx-OH was obtained from Iris Biotech GmbH and used without further purification. Thiol terminated polyethylene glycol (HS-PEG, HS-PEG-COOH, and HS-PEG-NH_2) of molecular weight 5000 were purchased from Nanocs Inc. Citrate-coated AuNPs were purchased from BBI Solutions (U.K.). All the other reagents were used as received.

Solid phase peptide synthesis (SPPS). Peptide amphiphiles were synthesized using standard 9-fluorenylmethoxycarbonyl (Fmoc) solid state peptide synthesis using Rink amide resin. Protected amino acids were added to the growing peptide chain with the activating reagent HBTU. Amino acid coupling was carried out using a coupling mixture of amino acid/HBTU/DIEA (4:3.95:6 relative to the resin) in DMF. Fmoc-11-aminoundecanoic acid and Fmoc-6-Ahx-OH were used in peptide synthesis to insert a hydrophobic tail. Upon the addition of the N-terminal amino acid, the FMOC group was removed using a standard 20 v/v% piperidine in DMF deprotection solution for 10

minutes, repeated twice. The peptides were cleaved using a trifluoroacetic acid/triisopropylsilane/water (95:2.5:2.5, v/v/v) cleavage solution for 3 hours. Excess TFA was removed by rotary evaporation. The crude peptide was precipitated by cold diethyl ether several times and purified using preparative reverse phase high performance liquid chromatography (HPLC, Shimadzu). A water-acetonitrile gradient was used (95% water to 5%) with 0.1% NH_4OH added to the mobile phase. The Phenomenex C18 Gemini NX column was 150 x 21.2 mm and had a 5 μm particle size and 110 Å pore size. Matrix-assisted laser desorption spectroscopy (MALDI; Waters) was used to confirm the expected *m/z* ratio and *α*-Cyano-4-hydroxycinnamic acid was used as the MALDI matrix substance.

Preparation of 2D peptide nanosheets. Stock solution of 10 mM **F6C11** was prepared in hexafluoro-2-propanol (HFIP) to completely break the hydrogen-bonding between peptide molecules. Typically, 10 μL of peptide stock solution was injected quickly into 390 μL of phosphate buffer (10 mM, pH 7). The mixture was vortexed for 10 seconds and stored at room temperature overnight. The solution will become cloudy once the nanosheets are formed.

Preparation of AuNR on nanosheet via copper-free click chemistry. (1) AuNRs were prepared using the aromatic additive modified synthesis of Ye *et al*.[2] with adjustments as described by Thomas *et al*.[3] As-prepared AuNRs were centrifuged twice at 10,000 rpm to remove excess surfactants. The GNRs were surface modified using two different thiol-terminated polyethylene glycols (1:1 molar ratio), HS-PEG (Mw 5000) and HS-PEG-COOH (Mw 5000). The mixture was further centrifuged at 10,000 rpm to remove free surfactant and unbound thiol ligands. The AuNRs were re-dispersed in 0.5 mL DMSO containing 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride/N-hydroxysuccinimide (EDC/NHS, 1 mg/1 mg) and dibenzocyclooctyne-amine (DBCO-NH_2, 1 mg). The mixture was incubated at room temperature overnight. The sample was purified by washing with DMSO and phosphate buffer. (2) The azide-displaying nanosheets were prepared by a co-assembly method. Typically, 5 μL of 10 mM **F6C11** in HFIP were mixed with 5 μL of 2 mM **Azide-F6C11** in HFIP, followed by the addition of

390 μL of phosphate buffer (10 mM, pH 7). (3) To prepare AuNR@nanosheet hybrid materials, 20 μL AuNRs were added into nanosheet solution containing 0.125 mM **F6C11** and 0.025 mM **Azide-F6C11** (Figure S1). The mixture was incubated overnight before TEM measurement.

Preparation of AuNP on nanosheet via thiol-Au bonding. (1) The thiol-displaying nanosheets were prepared by a co-assembly method as described above. (2) To prepare AuNP@nanosheets, 100 μL of citrate-coated AuNPs (OD = 1.0) were added to 50 μL of nanosheet solution containing 0.125 mM of **F6C11** and 0.25 mM **F6C11-Cys** (Figure S1). The mixture was vortexed for 10 seconds and incubated at r.t. overnight before TEM measurement.

Preparation of AuNP on nanosheet via electrostatic attraction. (1) To prepare positively charged AuNPs, 40 μL of HS-PEG (1 mg/mL) and 40 μL of HS-PEG-NH_2 (1 mg/mL) were added to 1 mL of citrate-coated AuNPs (15 nm, OD 1.0). After vortexing for 10 seconds, the solution was stored at 4 ^{o}C overnight. The mixture was centrifuged at 13,000 rpm for 20 minutes and washed twice with water. The final pellet was re-dispersed in 1 mL of phosphate buffer (10 mM, pH 7) and stored at 4 ^{o}C before use. (2) To prepare AuNP on nanosheet hybrid materials, 20 μL positive AuNPs were added into 10 μL **F6C11** nanosheet solution (0.25 mM). The mixture was incubated overnight before TEM measurement.

Preparation of QD on nanosheet via biotin-streptavidin interaction. To prepare QD@nanosheet, 10 μL of streptavidin coated QDs (0.2 μM) was incubated with 10 μL of peptide solution containing 0.125 mM of **F6C11** and 0.025 mM **Biotin-F6C11**. The mixture was incubated overnight before TEM measurement.

Transmission electron microscopy (TEM). TEM measurements were performed on an JEM 2100F with an acceleration voltage of 200 kV, and the images were recorded by Orius camera. TEM samples were prepared using the negative-staining method. Briefly, a drop was deposited onto the carbon-coated 200 mesh copper grid for 5 min. The excess

of solution was wiped away using a filter paper. Subsequently, the grid was stained with a drop of uranyl acetate (1.0 wt%) solution for 3 min. Excess staining agent was removed using a filter paper and the sample dried in air.

Small angle X-ray scattering (SAXS). Small angle X-ray scattering measurements were performed at the Diamond Light Source on beamline I22 with an X-ray energy of 18 keV. The geometry was calibrated using powdered silver behenate. A 0.5 mM suspension of peptide nanosheets in 10 mM phosphate buffer (pH 7.0) was measured in a 1.7 mm quartz glass capillary and the scattered X-ray intensity collected.

The 2-D scattered intensity was masked, calibrated and azimuthally integrated automatically on I22. The resulting plots of scattered X-ray intensity with Q were then generated by subtracting a solvent filled capillary measurement from the sample measurement prior to analysis. The scattering is immediately reminiscent of plate-like structures with an approximately Q^{-2} decay in the Guinier region which agreed with the structures observed by TEM and AFM. A quantitative assessment of the thickness of the structures was achieved by fitting the scattering data using the SasView fitting software with the LamellarModel which is appropriate for plate-like structures with dimensions much greater than the thickness. The background, lamellar thickness and a scaling factor were allowed to vary during fitting of the scattering data in the region between $Q = 0.022$ and 0.3 $Å^{-1}$. The parameters for the fit can be seen in Table S1.

Table S1. Fitting parameters for the Lamellar Model. The background, thickness and scale factors were allowed to vary during the fitting. The SLDs were fixed at the default values and the PD[ratio] of thickness was fixed at 0.1. The Chi^2/Npts for the fit was 0.0022.

Background	Thickness / nm	Scale	SLD_lamellar	SLD_solvent	PD[ratio] of thickness
0.10 ± 0.05	4.8 ± 0.3	0.0117 ± 0.0005	$1x10^6$	$6.3x10^6$	0.1

Atomic force microscopy (AFM). AFM was performed on an AFM 5500 microscope (Keysight technologies, previously Agilent). The measurements were performed in ambient atmosphere. An HQ:NSC15/Al BS tip (μmasch) was used (tip radius < 8 nm, force constant of 40 N/m, resonance frequency of 325 kHz) in tapping mode.

Fluorescence measurements. (1) ThT assay was performed in a 384-well plate with a total volume of 80 μL. The peptide solutions were incubated with 10 μM ThT overnight before measurement. Fluorescence measurements were performed on a SpectraMax M5 plate reader with the excitation of 440 nm. (2) Nile Red[4] is an environment-sensitive hydrophobic probe, exhibiting an enhancement of fluorescence and the blue-shift of its emission peak when it is solubilized in hydrophobic domains (with higher microviscosity and lower micropolarity). Nile Red assay was performed in a 384-well plate with a total volume of 80 μL. The peptide solutions were incubated with 2.5 μM Nile Red overnight before measurement. Fluorescence spectra were recorded on a SpectraMax M5 plate reader with the excitation of 575 nm. (3) Fluorescence polarization (FP) was measured on SpectraMax M5 plate reader using 1,6-diphenyl-hexa-1,3,5-triene (DPH) as the fluorescence probe.[5] The value of FP was determined from the measurement of fluorescence intensity parallel to the plane of linearly polarized excitation light ($I_{\parallel}$), and that perpendicular to the excitation plane ($I_{\perp}$), which was expressed as:

$$FP = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + I_{\perp}}$$

To measure the fluorescence polarization, 5 μM of DPH was added to peptide solution and the mixture was incubated overnight before FP measurement (Ex = 350 nm, Em = 430 nm).

Circular dichroism (CD). CD spectra were recorded using a Jasco-715 circular dichroism spectrophotometer at 20 ^{o}C. The samples were loaded into a rectangular quartz cell with the light path length of 1 mm.

Structured illumination microscopy (SIM) of nanosheets. Nanosheets were diluted in phosphate buffered saline and absorbed on a positive charge microscope slide

(SUPERFROST PLUS, Thermo Scientific) for 5 minutes. The samples were mounted with 22 mm high precision coverslips (Thermo Scientific) and sealed with clear nail varnish and were analysed on a Zeiss Elyra PS1 inverted microscope (Zeiss, Germany).

HRP catalytic activity test. (1) To prepare biotin-displaying nanosheets, 5 μL of **F6C11** solution in HFIP (10 mM) was mixed with 5 μL of **Biotin-F6C11** in HFIP (2 mM), followed by the addition of 390 μL phosphate buffer (10 mM, pH 7). The mixture was incubated at r.t. overnight to reach equilibrium. The free peptide amphiphiles were removed by centrifugation and the nanosheets were re-dispersed in phosphate buffer. (2) 200 μL of the above nanosheet solution was incubated with 200 μL of HRP-Avidin D solution (0.2 nM) for 3 hours to ensure the complete binding of HRP-Avidin D to biotin-displaying nanosheets. Since the biotin-avidin affinity is very strong (dissociation constant of avidin is measured to be $K_D \approx 10^{-15}$ M), we expect that most of the HRP-Avidin D was absorbed onto peptide nanosheets. Indeed, the unbound HRP-Avidin D was determined to be 3% by measuring the enzyme activity in the supernatant. (3) To test the HRP activity, 1-Step™ Ultra TMB-ELISA Substrate Solution (Thermo Fisher Scientific) was used as the enzyme substrate. Typically, 40 μL of substrate solution was mixed with 40 μL of HRP-AvD bound nanosheets containing 0.125 mM **F6C11** and 0.025 mM **Biotin-F6C11** in a 384-well plate. The absorbance at 650 nm was recorded on a SpectraMax M5 plate reader. (4) In a control experiment, 40 μL of substrate solution were mixed with 40 μL of 0.2 nM HRP-AvD and the absorbance at 650 nm was monitored. (5) To test the intrinsic catalytic activity of peptide nanosheets, 40 μL of substrate solution was mixed with 40 μL of peptide solution containing 0.125 mM **F6C11** and 0.025 mM **Biotin-F6C11** in a 384-well plate. The absorbance at 650 nm was recorded on SpectraMax M5 plate reader. (6) To test the enzyme activity in ABTS ((2,2'-Azinobis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt)), 40 μL of 1-Step™ ABTS Substrate Solution (Thermo Fisher Scientific) was mixed with 40 μL of HRP-AvD bound nanosheets (0.125 mM **F6C11** and 0.025 mM **Biotin-F6C11**) in a 384-well plate. The absorbance at 650 nm was recorded on a SpectraMax M5 plate reader.

Surface functionalization of silicon wafer. (1) Silicon wafers were treated for 30 min in hot piranha solution (3:1, H_2SO_4/H_2O_2) and washed with water. After drying in N_2 stream, the substrate was treated with air plasma for 5 min. (2) To functionalize the surface with amine groups, the fresh substrate was immersed in 2 w/v% (3-aminopropyl)triethoxysilane solution in toluene at 60 ^oC for 30 min. After that, the substrate was washed with toluene and ethanol. (3) To display maleimide groups on the surface, the substrate was immersed into a solution containing succinimidyl-4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (1 mg/mL in DMF) for 60 min. The surface was washed with DMF and ethanol.

Surface hetero-functionalization of peptide nanosheets. The nanosheets with cysteine and biotin on the same face were prepared via a co-assembly method. Typically, 5 μL of 10 mM **F6C11**, 2.5 μL of 2 mM **Biotin-F6C11** and 2.5 μL of 2 mM **Cys-F6C11** were mixed in HFIP. To this mixture, 390 μL of phosphate buffer (10 mM, pH 7.0) was added and the solution was incubated overnight. Similarly, the nanosheets with cysteine and biotin on opposing face were prepared in a similar way except using **F6C11-Cys** instead of **Cys-F6C11**. To attach nanosheets on silicon wafer, 50 μL of nanosheets solution were dropped onto maleimide-displaying silicon wafer. After incubating for 5 hours, the surface was blocked with 1 mM HS-PEG6 in phosphate buffer for 1 hour. After that, 50 μL of streptavidin-coated AuNPs (OD= 1) was added to the silicon surface. The unbound AuNPs were removed by gentle washing with phosphate buffer (10 mM, pH 7.0).

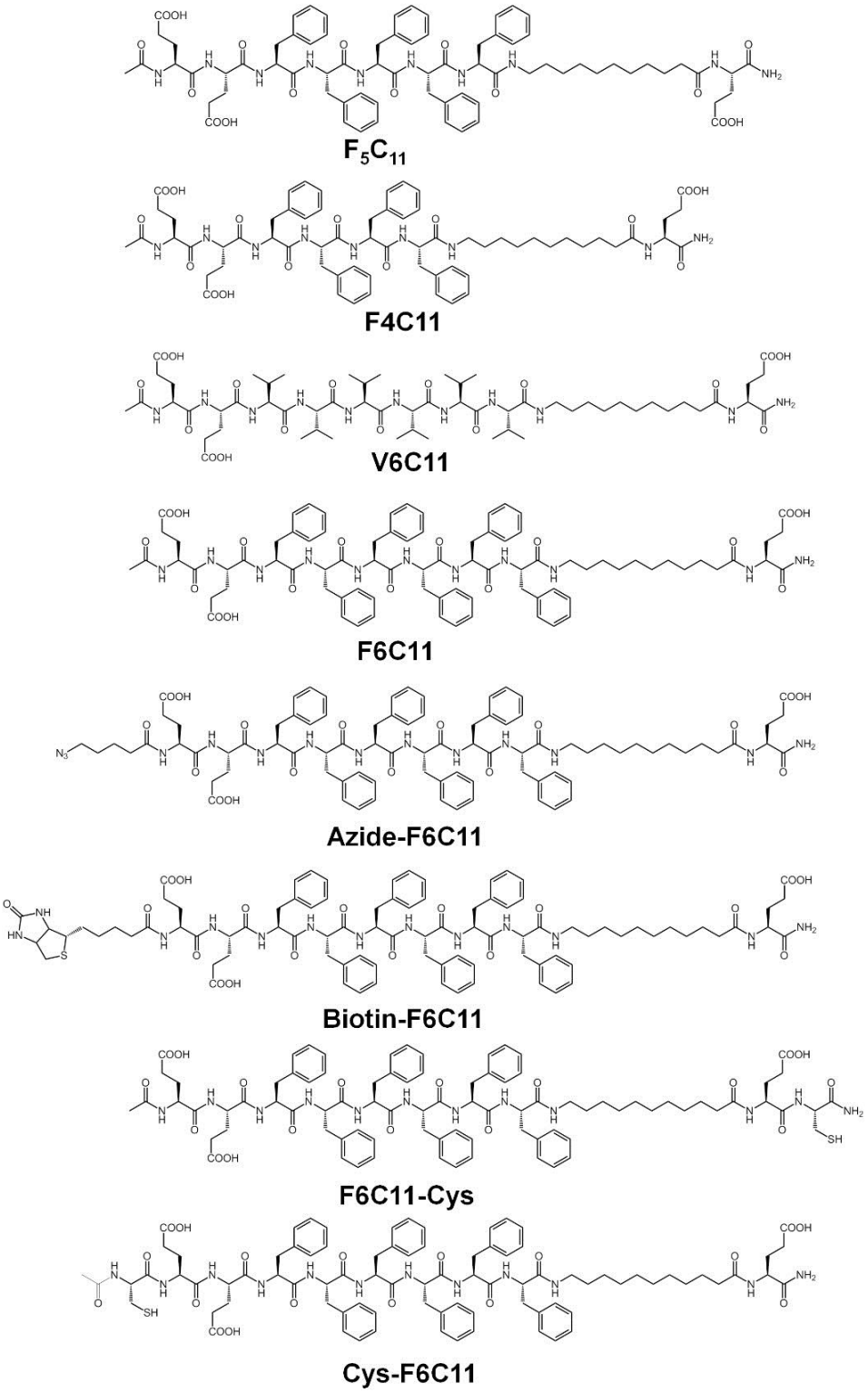


Figure S1. Molecular structures of peptide amphiphiles studied in this work.

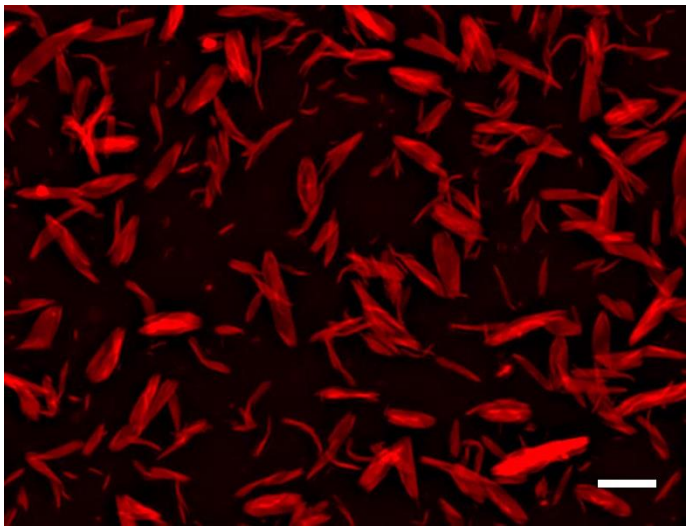


Figure S2. SIM image of **F6C11** supramolecular nanosheets, which were stained with Nile Red (2.5 μM). Scale bar: 2 μm.

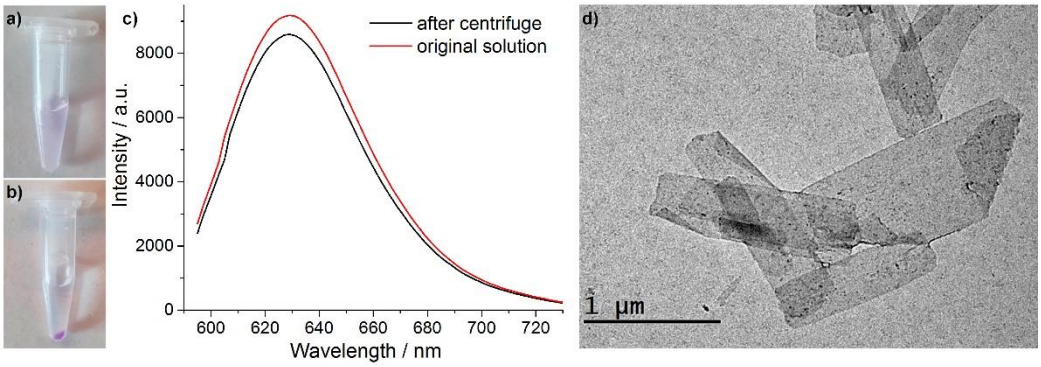


Figure S3. Macroscopic appearance of Nile Red (2.5 μM) encapsulated **F6C11** peptide nanosheet solution (a) before and (b) after centrifugation. The enrichment of peptide nanosheets was noted from the pink pellet at the bottom. (c) Fluorescence emission spectra of Nile Red loaded peptide nanosheets before and after centrifugation treatments (Ex=570 nm). The recovery rate was determined to be ~92 % by calculating the fluorescence of Nile Red before and after centrifugation. (d) TEM image of **F6C11** nanosheets (0.25 mM) recovered from centrifugation. The sample was stained with 1.0 % uranyl acetate before TEM measurement.

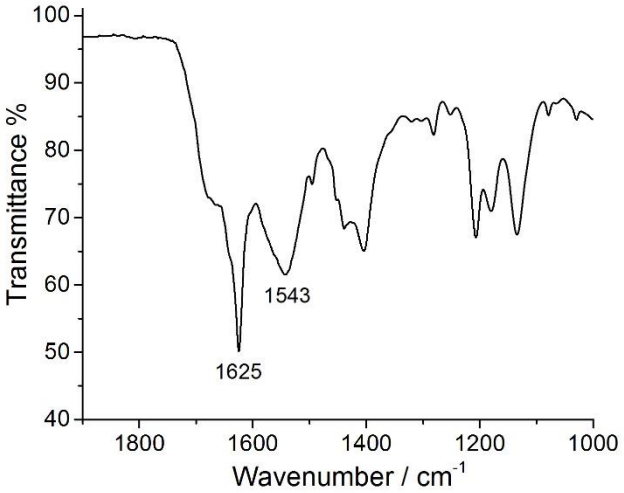


Figure S4. FTIR spectrum of **F6C11** nanosheets. The peaks at 1622 cm^{-1} (amide I) and 1543 cm^{-1} (amide II) indicate the existence of parallel β-sheet. The strong absorbance at 3274 cm^{-1}, 2931 cm^{-1} and 2855 cm^{-1} correspond to the N-H stretching, $-CH_3/-CH_2$ antisymmetric and symmetric stretching, respectively.

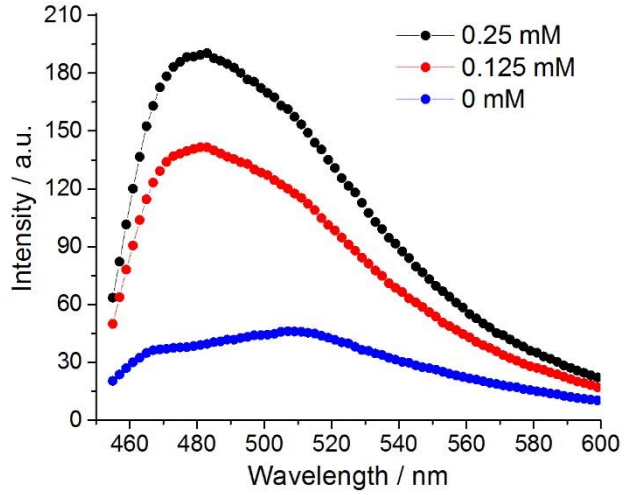


Figure S5. Fluorescence emission spectra of ThT in the absence or presence of 0.125 mM and 0.25 mM of **F6C11** (Ex = 440 nm). The enhancement of fluorescence intensity confirms the location of ThT inside rigid β-sheets.

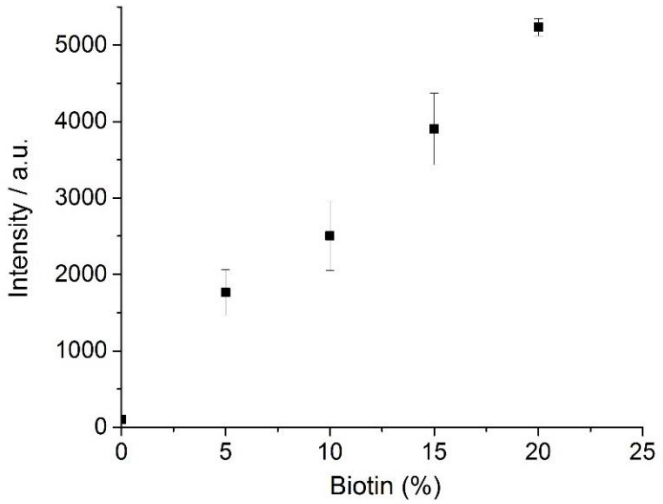


Figure S6. Fluorescence of streptavidin-Cy3 binding to **F6C11/biotin-F6C11** nanosheets. The molar ratio of **biotin-F6C11**/**F6C11** was 0, 5%, 10%, 15% and 20%. An increase of Cy3 fluorescence was noted, indicating the almost quantitative adsorption of streptavidin to nanosheets. The error bar represents the standard deviation, N = 2.

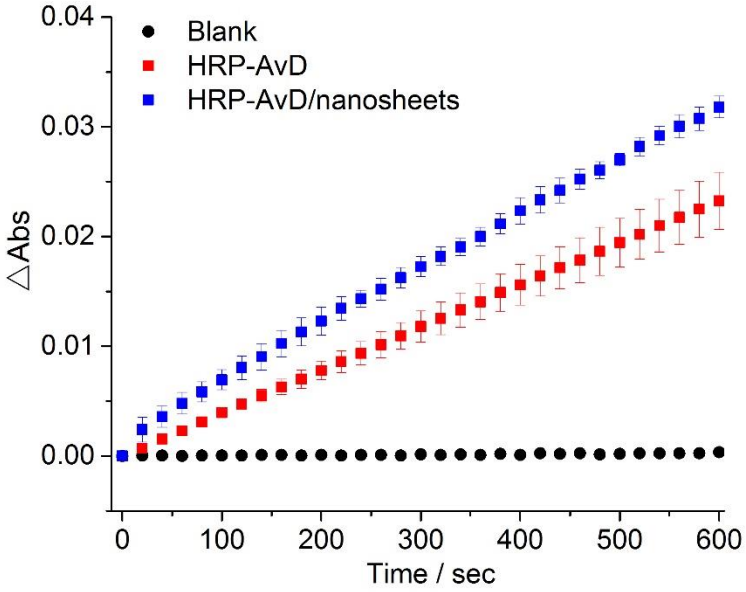


Figure S7. Kinetics of HRP-catalyzed ABTS oxidation by H_2O_2 in the presence and absence of biotinylated peptide nanosheets. HRP concentration was 0.1 nM. The absorbance of oxidized ABTS was monitored at the wavelength of 650 nm. The error bar represents the standard deviation, N = 2.

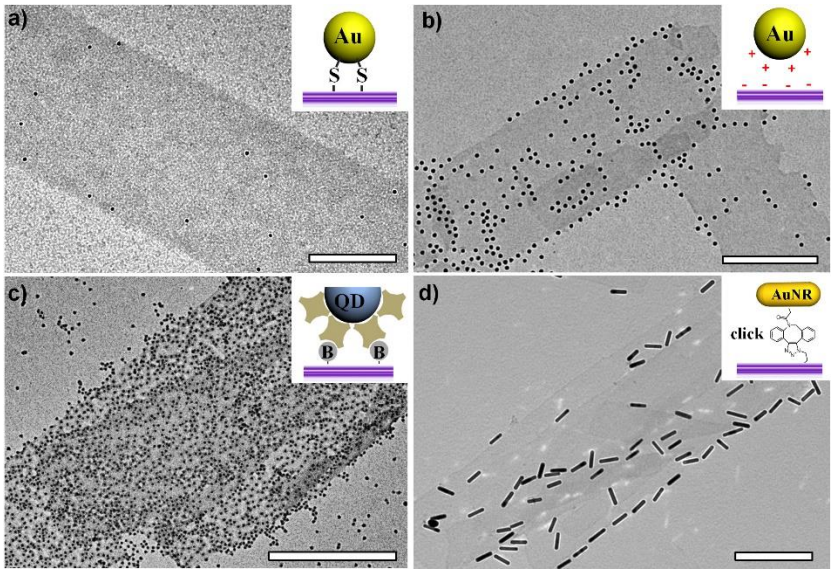


Figure S8. Surface functionalization of nanosheets: (a) binding of citrate-coated AuNPs on cysteine-displaying nanosheets (**F6C11**/**F6C11-Cys**) *via* thiol-Au bonding; (b) binding of positive AuNPs *via* electrostatic interaction; (c) QD adsorption *via* biotin-streptavidin recognition; (d) AuNR assembly *via* copper-free click chemistry. Scale bar: (a) 200 nm; (b-d) 500 nm.

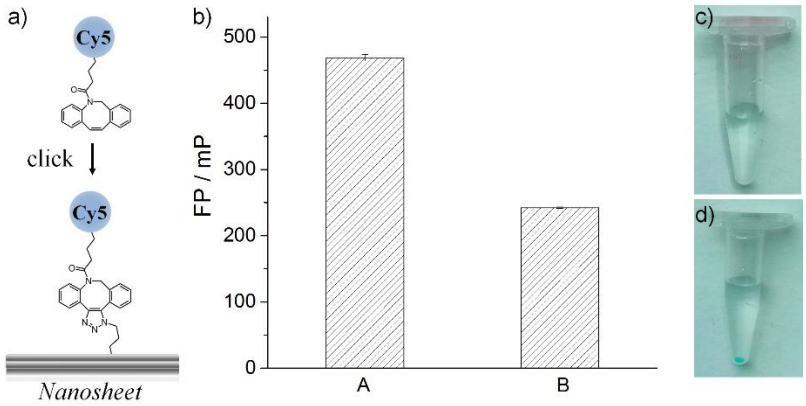


Figure S9. (a) Schematic of surface conjugation of Cy5 on a peptide nanosheet using copper-free click chemistry between DBCO and azide. (b) Fluorescence polarization (FP) of Cy5: (A) DBCO-Cy5 anchored on nanosheet surface and (B) DBCO-Cy5 in solution. The higher FP of sample A confirmed the conjugation of DBCO-Cy5 on nanosheets, which restricts the rotation and diffusion movement (Ex=600 nm, Em=685 nm). (c, d) **F6C11/Azide-F6C11** nanosheets incubating with DBCO-Cy5: (c) before and (d) after

centrifugation. A blue pellet can be found in (d), indicating the attachment Cy5 on peptide nanosheet.

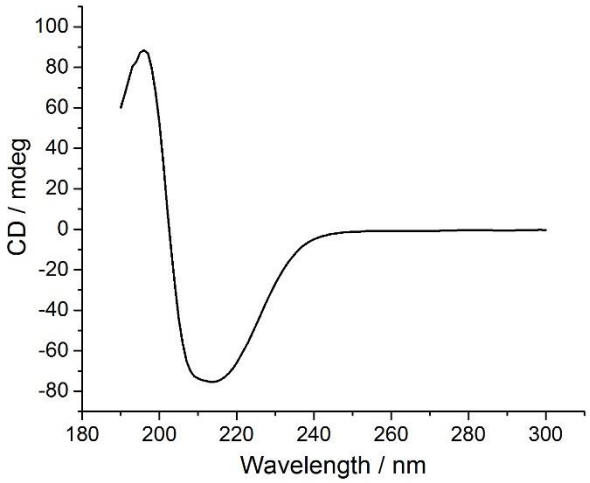


Figure S10. CD spectrum of **V6C11** solution in phosphate buffer (10 mM, pH 7.0). The minimum at 214 nm and the maximum at 196 nm are representative of typical β-sheets.

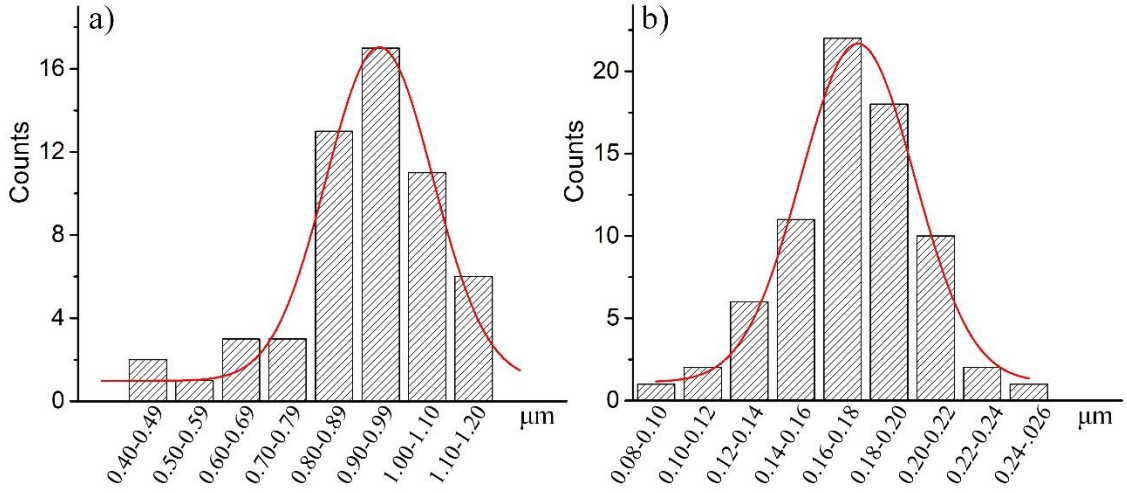


Figure S11. (a) Width and (b) length distribution of 2D structures assembled from **F6C6**. The Gaussian fitting showing the average width and length was 178 nm and 1.05 μm, respectively.

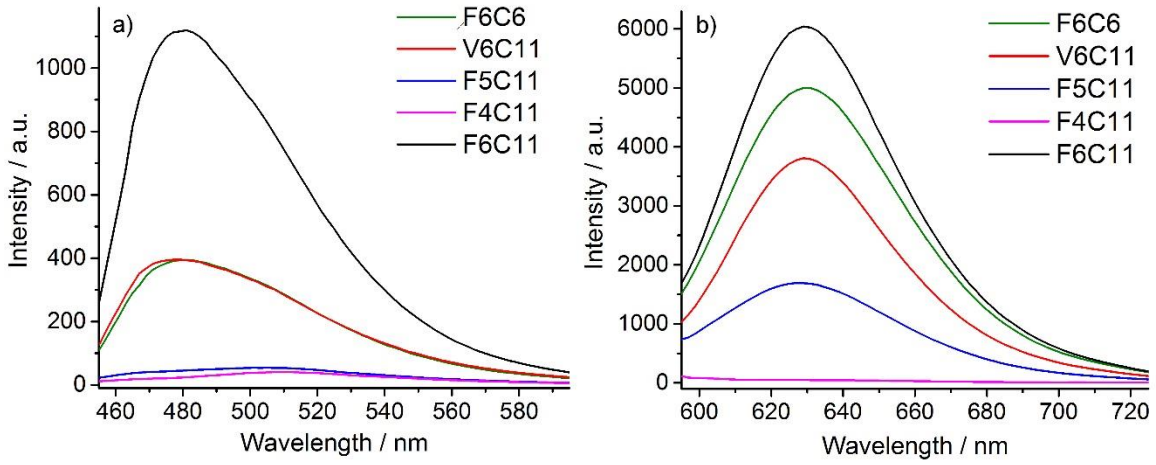
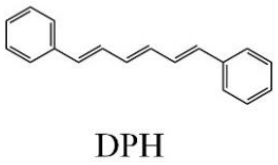


Figure S12. (a) Fluorescence spectra of ThT showing the *β*-sheet strength[6] of different peptide sequences: **F6C11** > **V6C11** ≈ **F6C6** > **F5C11** > **F4C11.** (b) Fluorescence spectra of Nile Red showing the micropolarity[4] of peptide nanostructures: **F6C11** > **F6C6** > **V6C11** > **F5C11** > **F4C11.** The concentration of peptide solutions was 0.25 mM in phosphate buffer (10 mM, pH 7). The concentration of ThT and Nile Red was 10 μM and 2.5 μM, respectively.



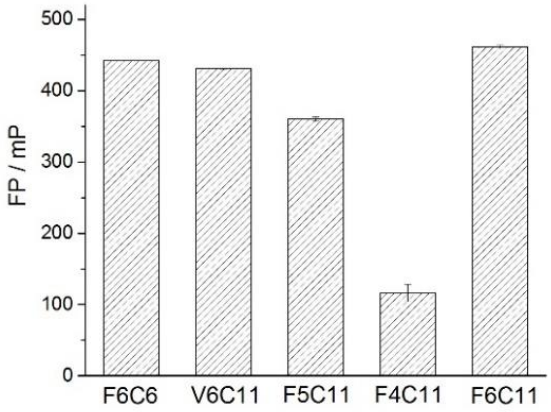


Figure S13. Fluorescence polarization (FP) of 1,6-diphenyl-l,3,5-hexatriene (DPH) in the presence of different peptide solutions (0.25 mM). The DPH concentration is 5 μM (Ex = 350 nm, Em = 430 nm).

[1] S. A. Low, J. Yang, J. Kopeček, *Bioconjug. Chem.* **2014**, *25*, 2012.
[2] X. Ye, L. Jin, H. Caglayan, J. Chen, G. Xing, C. Zheng, V. Doan-Nguyen, Y. Kang, N. Engheta, C. R. Kagan, C. B. Murray, *ACS Nano* **2012**, *6*, 2804.

[3] M. R. Thomas, J. E. Hallett, S. Klein, S. Mann, A. W. Perriman, R. M. Richardson, *Mol. Cryst. Liq. Cryst.* **2015**, *610*, 44.
[4] M. C. A. Stuart, J. C. van de Pas, J. B. F. N. Engberts, *J. Phys. Org. Chem.* **2005**, *18*, 929.
[5] D. M. Jameson, J. A. Ross, *Chem. Rev.* **2010**, *110*, 2685.
[6] H. Levine, *Protein Sci.* **1993**, *2*, 404.