Electronic Supplementary Information

Enzyme-Assisted Self-Assembly Within a Hydrogel Induced by Peptide Diffusion

Miryam Criado-Gonzalez[a,b,c], Jennifer Rodon Fores[a], Déborah Wagner[a], André Pierre Schroder[a], Alain Carvalho[a], Marc Schmutz[a], Eva Harth[d], Pierre Schaaf*[a,b,c], Loïc Jierry*[a], Fouzia Boulmedais*[a]

a. Université de Strasbourg, CNRS, Institut Charles Sadron UPR 22, 67034 Strasbourg, France

b. Institut National de la Santé et de la Recherche Médicale, UMR-S 1121, "Biomatériaux et Bioingénierie", 67087 Strasbourg, France

c. Université de Strasbourg, Faculté de Chirurgie Dentaire, Fédération de Médecine Translationnelle de Strasbourg (FMTS), and Fédération des Matériaux et Nanoscience d'Alsace (FMNA), 67000 Strasbourg, France.

d. Department of Chemistry, Center of Excellence in Polymer Chemistry (CEPC), University of Houston, Houston, Texas 77030, United States

Summary

1. Materials and methods

List of chemicals and abbreviations

All chemicals used in this work are gathered in the following table:

Name, acronym (abbreviation)	MW (g/mol)	Supplier	CAS number
4-arm PEG maleimide (4-arm PEG Mal)	10000	Seebio Biotech, Inc	
HS-PEG-SH (bis-SH PEG)	2000	Interchim	
Fmoc-FF*p*Y	777.54	PepMic	
Phosphatase alkaline (AP)	160000	Sigma Aldrich	9001-78-9
Sodium tetraborate anhydrous (borax)	201.22	Acros Organics	1330-43-4
p-Nitrophenyl Phosphate Liquid Substrate System (p-NPP)	263.05	Sigma Aldrich	4264-83-9
Fmoc-G	297.30	Iris Biotech	29022-11-5
Thioflavin T	318.86	Sigma Aldrich	2390-54-7

Gelation's conditions

Preparation of Fmoc-FFY self-assembled hydrogel

All solutions were prepared in borax buffer (25 mM, pH = 9.5). Fmoc-FFY self-assembled hydrogel was obtained by mixing in a vial 0.04 U/mL of AP solution and 2.5 mg/mL of Fmoc-FF*p*Y solution. After 2 h, the self-assembled gel is formed.

Preparation of AP-PEG gel and diffusion of Fmoc-FFpY within this host gel

AP-PEG gels, *i.e.* PEG gels with physically entrapped AP, were prepared by mixing at 4°C in a first vial 37 µL of 4-arm PEG maleimide (8.1 mM) and 13 µL of AP (0.04 U/mL) and in a second vial 38 µL of Bis-SH PEG (13.2 mM) and 12 µL of AP (0.04 U/mL). The two prepared vials were mixed keeping constant the temperature at 4°C and shaking manually. After 2 h, the gel was formed. Subsequently, 25 µL of Fmoc-FF*p*Y (1 or 2.5 mg/mL) in borax buffer [*or 25 µL of Fmoc-G (2.5 mg/mL) in case of negative control experiments*] was deposit on the surface of the AP-PEG gels. These 25 µL diffuse into the host hydrogel in few minutes and then it is let 24 h at room temperature before further investigations.

UV spectroscopy

The enzymatic activity was measured in a microplate reader UV spectroscopy (FLX-Xenius®, SAFAS, Monaco) using a 96 well plate. The AP activity from AP-PEG gel was measured by incubation of the substrate, *para*-nitrophenyl-phosphate (PNP) (200 µL at 1 mM in Borax buffer). Concentration and volume ensure a large excess of substrate for the enzymatic reaction. PNP is a colourless AP substrate that by sequential enzymatic hydrolysis of the phosphate substituent of PNP in presence of AP yields a yellow absorbance at λ = 405 nm.

Infrared spectroscopy

All solutions were prepared in borax buffer using deuterated water (25 mM, pH = 9.5). AP-PEG gels were prepared as follows by mixing at 4°C in a first vial 37 µL of 4-arm maleimide (8.1 mM) with 13 µL of AP (0.04 U/mL) and in a second one 38 µL of 2-arm thiol (13.2 mM) and 12 µL of AP (0.04 U/mL). The two prepared vials were mixed keeping constant the temperature at 4°C and shaking manually to get homogeneous gels. After 2 h the gel was completely formed. Subsequently, 25 µL of Fmoc-FF*p*Y (2.5 mg/mL) was put in contact with AP-PEG gels for 24 h at room temperature. The Fourier Transform Infrared (FTIR) experiments were performed on a Vertex 70 spectrometer (Bruker, Germany) using DTGS detector. FTIR Spectra were recorded in the Attenuated Total Reflection (ATR) mode by collecting 128 interferograms between 800 and 4000 cm^{-1} at 2 cm^{-1} resolution, using Blackman-Harris three-term apodization and the standard Bruker OPUS/IR software (version 7.5).

Fluorescence spectroscopy

All fluorescence intensities were measured by using the microreader fluorescence spectroscopy (FLX-Xenius®, SAFAS, Monaco) at an excitation wavelength of 290 nm by recording the spectra between 300-440 nm. A special 96 well plate was used to prepare the samples and measure the fluorescence during the self-assembly of Fmoc-FFY peptide gel when AP-PEG gels were put in contact with Fmoc-FF*p*Y solutions at 1.0 and 2.5 mg/mL and Fmoc-G (2.5 mg/mL) solution.

Confocal laser scanning microscopy

All solutions were prepared in borax buffer (25 mM, pH = 9.5). Thioflavin T-AP-PEG gels, named ThT-AP–PEG gels, were prepared as follows by mixing at 4°C in a first vial 25 µL of 4-arm maleimide (12 mM) with 13 µL of AP (0.04 U/mL) and 12 µL of Thioflavin T (0.5 mM) and in a second one 25 µL of 2-arm thiol (20 mM) with 12 µL of AP (0.04 U/mL) and 13 µL of Thioflavin T (0.5 mM). The two prepared vials were mixed keeping constant the temperature at 4°C and shaking manually to get homogeneous gels. After 2 h the gel was completely formed. Subsequently, 25 µL of different solutions, *i.e.* borax buffer, Fmoc-FF*p*Y (2.5 mg/mL) and Fmoc-G (2.5 mg/mL), were put in contact with Thioflavin T-AP-PEG gels for 24 h at room temperature. Confocal laser scanning microscopy (CLSM) images were acquired using an inverted TE2000 microscope (Nikon, Japan) equipped with a C1-Si scan-head. EZ-C1 software (Nikon, version 3.50) was used for image capture. Samples were excited using an argon-ion laser (Melles-Griot) at 488 nm. Thanks to its flatness, gel sample extracted from its containing vial was observed immediately after it was gently placed on an adapted coverslip. 100x oil immersion/1.3NA Plan Apo DIC objective was used for imaging. Objective working distance enabled to observe samples up to circa 100 µm above the coverslip.

Scanning Electron Microscopy (SEM) and Cryo-SEM

To observe the morphology of the gels, a specific cryo-holder and a cryo preparation chamber were designed and manufactured by the mechanical facility of the Charles Sadron Institute (Vigier-Carriere et *al*. Langmuir **2017**, *33*, 8267). A piece of the gel was placed on the cryo-holder to be quickly plunged into an ethane slush. As the sample is free standing over the holder, the sample is rapidly frozen during the plunging by direct contact with the liquid ethane. Subsequently, the sample is transferred into the Quorum PT 3010 chamber attached to the microscope. There, the frozen sample is coated with a thin Pt layer (by sputter deposition) and fractured with a razor blade. A slight etching at -90°C is performed to render the fibres more visible. The sample is eventually transferred in the FEG-cryoSEM (Hitachi SU8010) and observed at 1kV at -150°C.

Rheology measurements

All solutions were prepared in Borax buffer (25 mM, pH = 9.5). AP-PEG gels were prepared as follows: by mixing in a first vial 225 µL of 4-arm maleimide (8.1 mM) with 75 µL of AP (0.04 U/mL) at 4°C and in a second vial 225 µL of 2-arm thiol (13.2 mM) and 75 µL of AP (0.04 U/mL) at 4°C. The two prepared vials were mixed keeping constant the temperature at 4°C and shaking manually to get homogeneous gels. After 2 h the gel was completely formed. Subsequently, 150 µL of different solutions, *i.e.* borax buffer, Fmoc-FF*p*Y (1.0 and 2.5 mg/mL) and Fmoc-G (2.5 mg/mL), were put in

contact with AP-PEG gels for 24 h at room temperature. Rheological properties were measured in a Kinexus Malvern rheometer using a plate geometry of 20 mm diameter and a gap of 0.5 mm. Strain measurements were carried out from 0.01% to 100% at 0.3Hz. Frequency sweeps were performed from 0.01 Hz to 10 Hz at a fixed strain of 0.06%.

2. Supplementary Figures

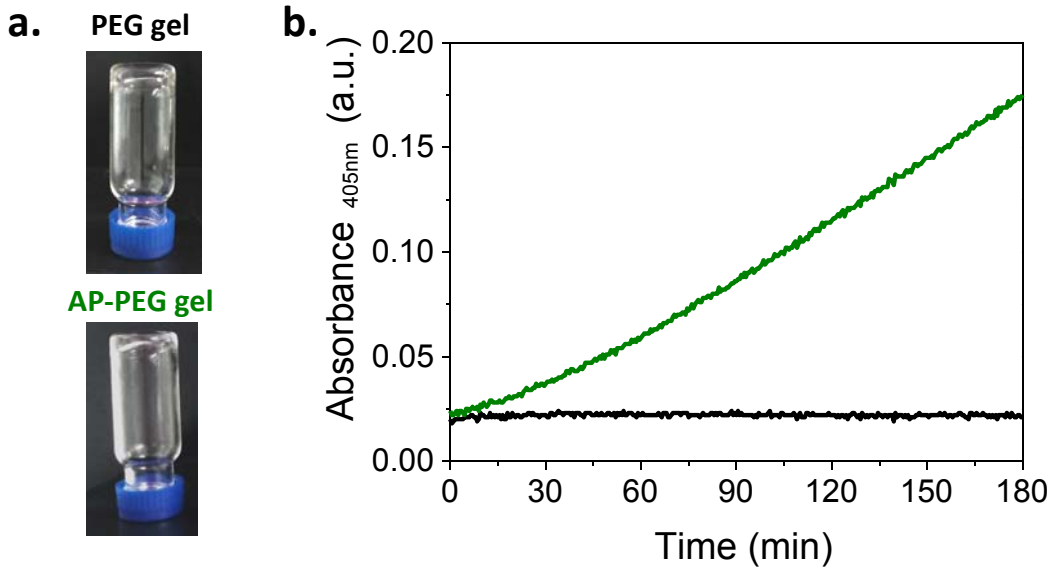

Figure S1: (**a**) Inverted tube test of PEG and AP-PEG gels after 24 h; (**b**) Evolution of the optical density (OD) as a function of time of a *para*-nitrophenyl phosphate (PNP) solution brought in contact with PEG gel (black curve) and AP-PEG gel (green curve). The absorbance was measured at 405 nm. PNP is transformed into *para*-nitrophenol (λ_{max} = 405 nm) and phosphate ions by AP embedded into the gel. The measurement was carried out in a microplate reader (see Materials and Methods section, described above). The value of the slopes are 0 and 8.6×10^{-4} min^{-1} for PEG and AP-PEG gels, respectively.

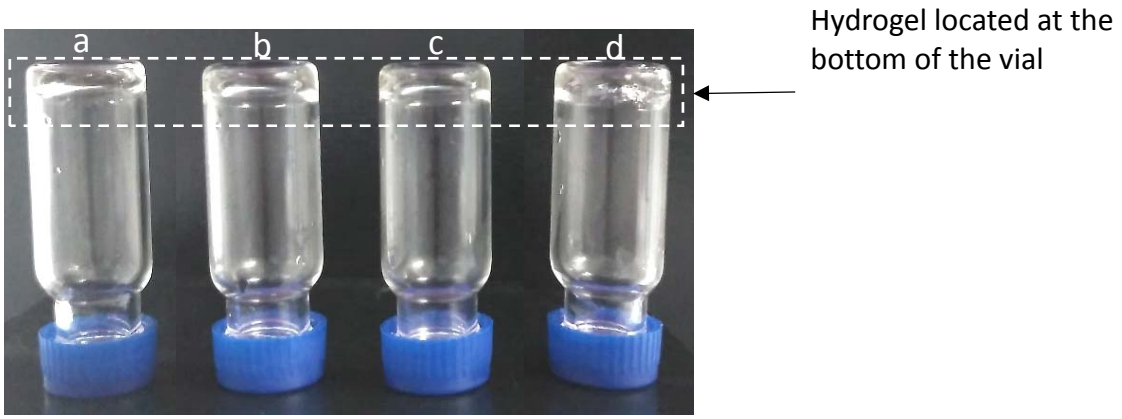

Figure S2: Inverted tube tests of AP-PEG gels after 24 h in contact with (**a**) borax solution (buffer), (**b**) 1 and (**c**) 2.5 mg/mL of Fmoc-FF*p*Y and (**d**) 2.5 mg/mL of Fmoc-G.

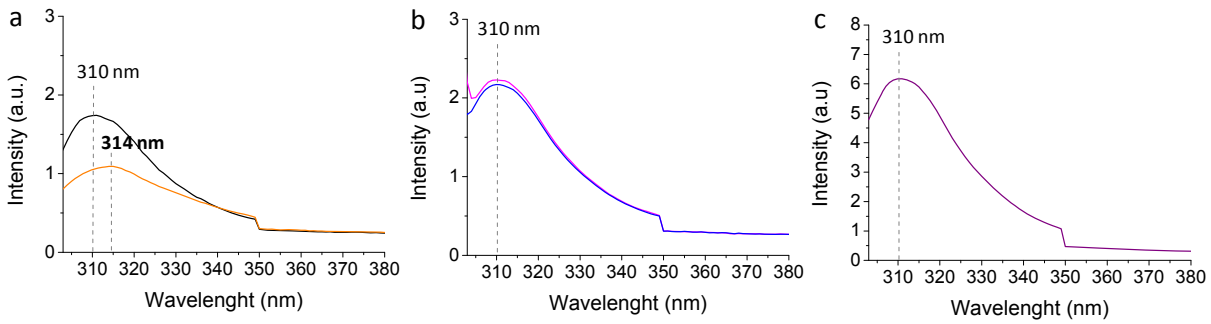

Figure S3: Fluorescence spectra obtained at an excitation wavelength of 290 nm of: (**a**) Fmoc-FF*p*Y 2.5 mg/mL (black line) and AP/Fmoc-FF*p*Y mixture with 2.5 mg/mL of Fmoc-FF*p*Y in borax buffer (orange line), (**b**) Fmoc-G 2.5 mg/mL (pink line) and AP/Fmoc-G mixture with 2.5 mg/mL Fmoc-G in borax buffer (blue line) and (**c**) AP-PEG gel after 24 h contact with Fmoc-G (2.5mg/mL) solution.

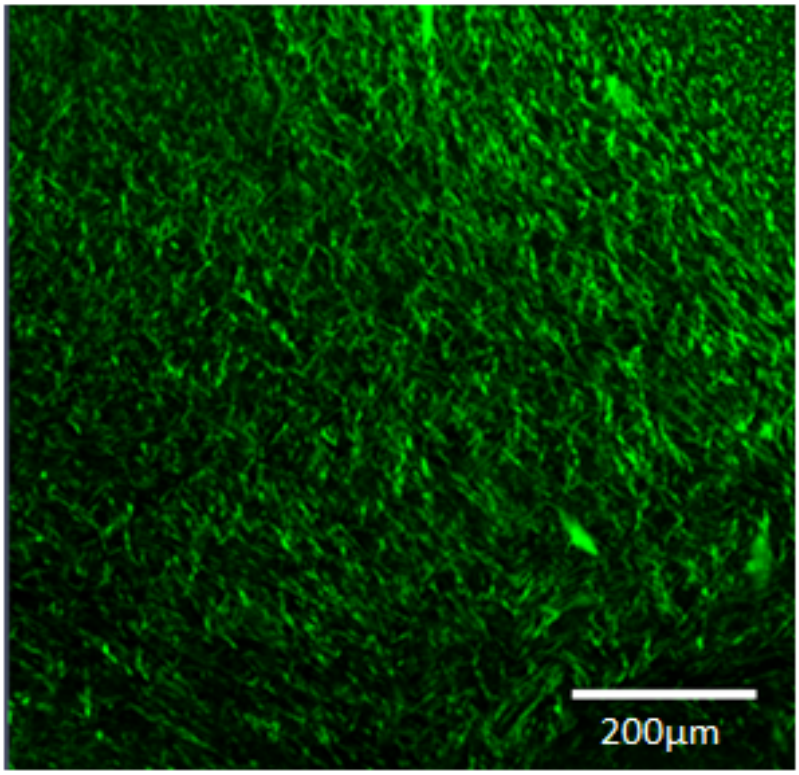

Figure S4: Confocal image of AP-PEG hydrogel containing ThT, 24h after the diffusion of Fmoc-FF*p*Y (25 µL, 2.5 mg/mL).

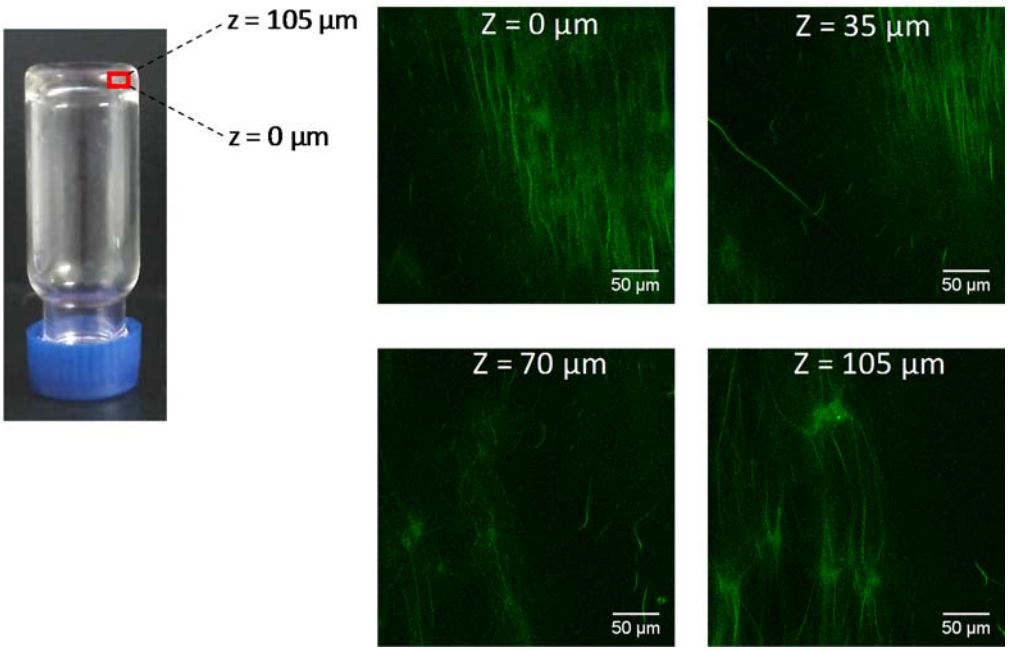

Figure S5: Confocal images taken along the z-axis penetrating inside the AP-PEG hydrogel, 24 hours after the diffusion of Fmoc-FF*p*Y solution (25 µL, 2.5 mg/mL): z = 0 µm (interface), z = 50 µm, z = 70 µm and z = 105 µm.

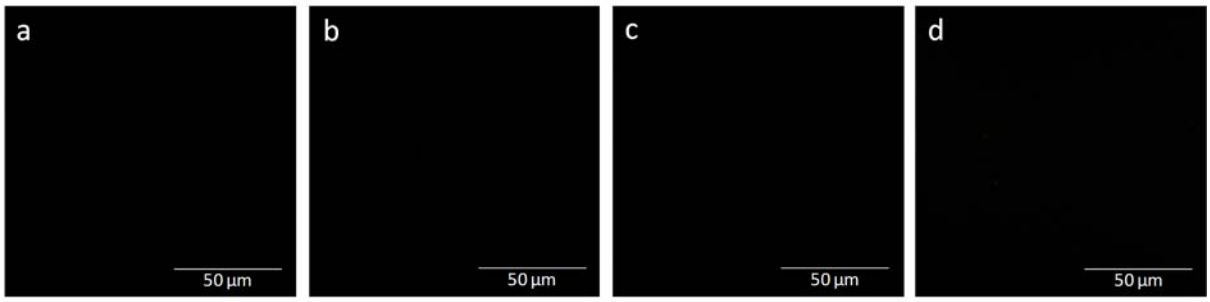

Figure S6: Confocal images of PEG host hydrogel containing ThT (without AP), 24 h after the diffusion of the following solution (25 µL): (**a**) borax buffer and (**b**) Fmoc-FF*p*Y solution (2.5 mg/mL). Confocal images of AP-PEG host hydrogel containing ThT, 24 h after the diffusion of the following solution (25 µL): (**c**) borax buffer and (**d**) Fmoc-G solution (2.5 mg/mL).

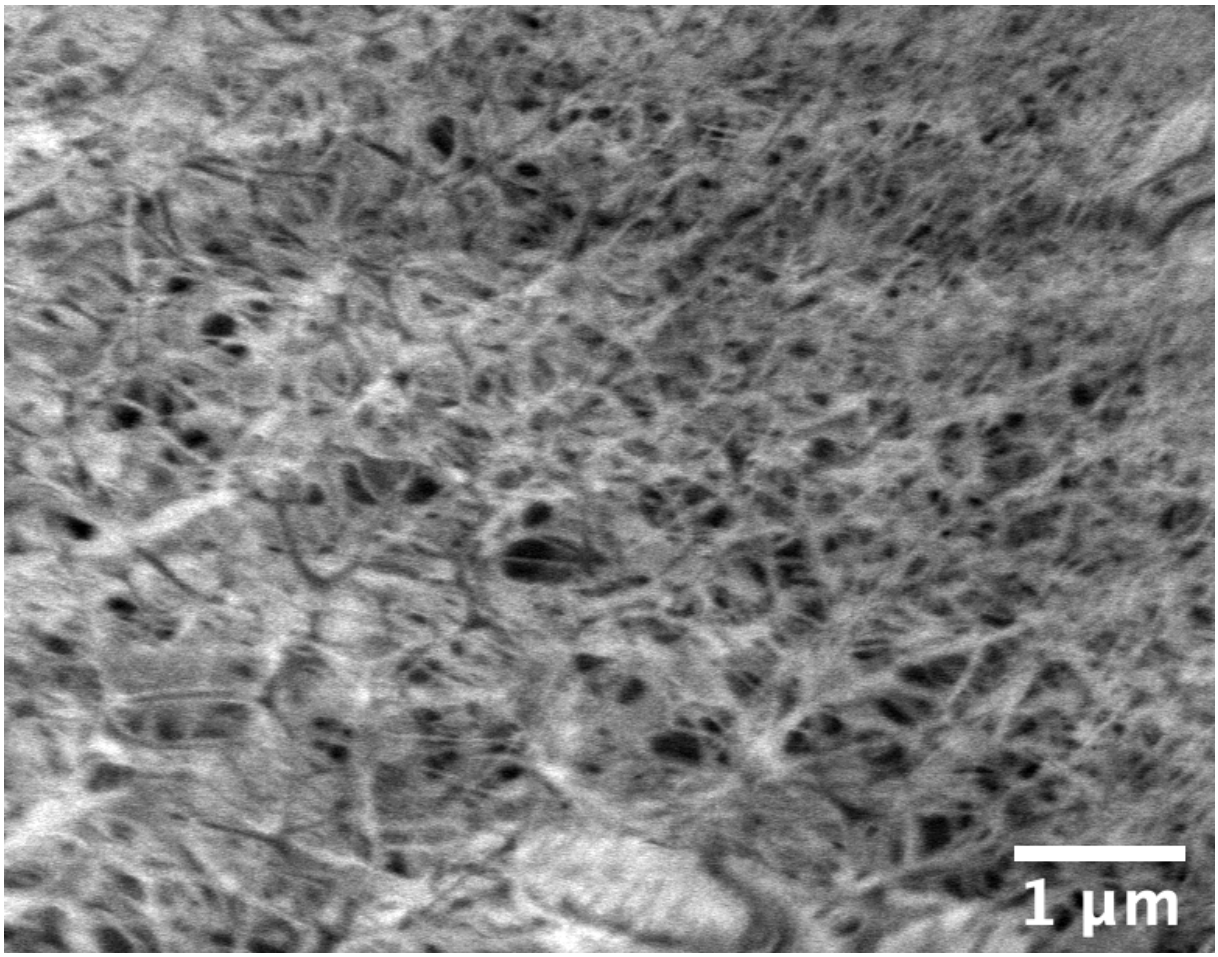

Figure S7: Cryo-SEM image of Fmoc-FFY self-assembly obtained by mixing 0.1 U/mL AP and 2.5 mg/mL Fmoc-FF*p*Y solution.

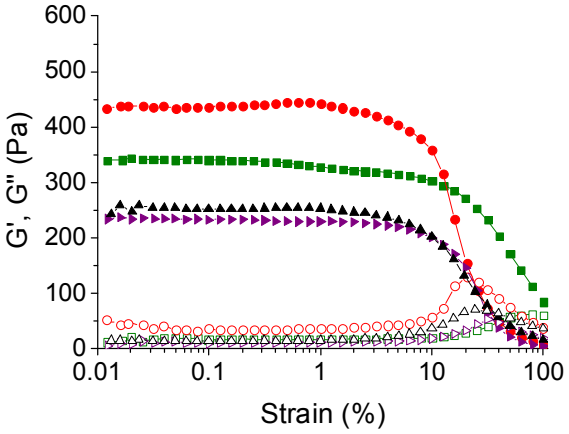

Figure S8: Storage modulus (G' –solid symbols) and loss modulus (G'' – hollow symbols) as a function of strain for AP-PEG gel (■) before and after 24 h contact with (▲) 1 mg/mL Fmoc-FF*p*Y, (●) 2.5 mg/mL Fmoc-FF*p*Y and (►) 2.5 mg/mL of Fmoc-G solutions.

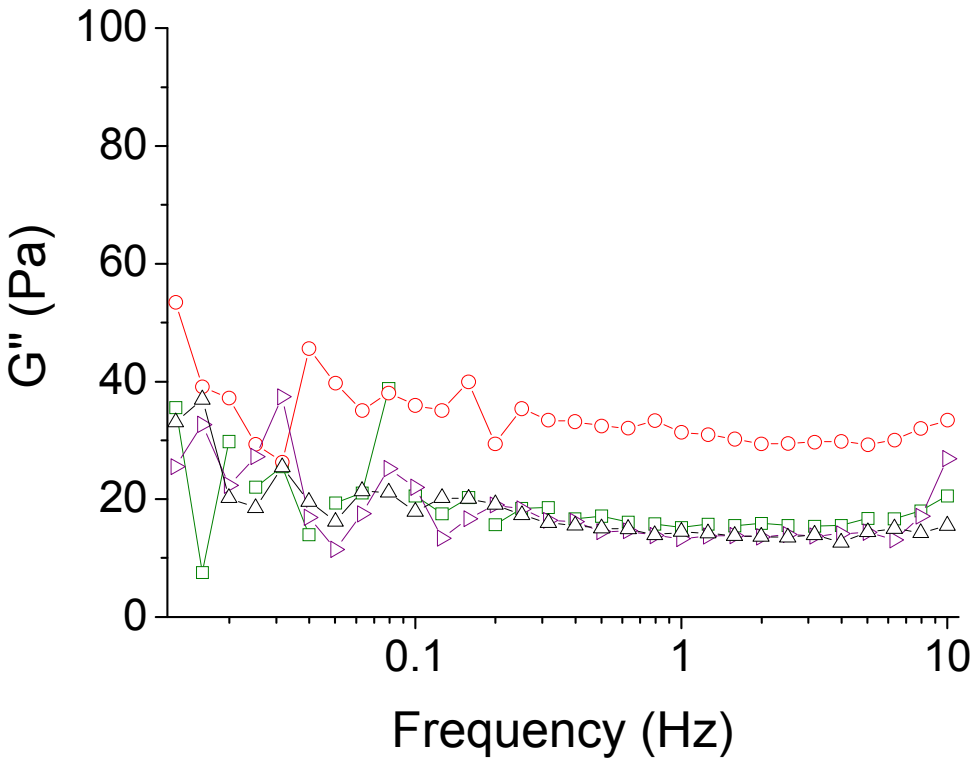

Figure S9: Magnification of Figure 4a (in the manuscript) highlighting the evolution of loss modulus G'' as a function of strain for AP-PEG gel (■) before and after 24 h contact with (▲) 1 mg/mL Fmoc-FF*p*Y, (●) 2.5 mg/mL Fmoc-FF*p*Y and (►) 2.5 mg/mL of Fmoc-G solutions.

Table S1: Storage modulus (G') and loss modulus (G'') values at 1 Hz for AP-PEG gels, 24 hours after the diffusion of peptide solutions mentioned below.

Sample	**G' (Pa) at 1Hz**	**G'' (Pa) at 1Hz**
AP-PEG gel	317.6 ± 15.1	15.1 ± 0.1
AP-PEG gels - Fmoc-G 2.5 mg/mL	281.9 ± 55.9	12.0 ± 1.8
AP-PEG gels - Fmoc-FF*p*Y 1 mg/mL	284.4 ± 15.1	14.8 ± 0.5
AP-PEG gels - Fmoc-FF*p*Y 2.5 mg/mL	422.9 ± 38.2	29.3 ± 3.0