Electronic Supporting Information

Protein-Induced Low Molecular Weight Hydrogelator Self-Assembly Through a Self-Sustaining Process

Jennifer Rodon Fores, Miryam Criado-Gonzalez, Marc Schmutz, Christian Blanck, Pierre Schaaf,* Fouzia Boulmedais and Loïc Jierry*

MATERIALS AND METHODS

1. List of Chemicals and abbreviations

All reagents and solvents were purchased at the top commercial quality and used without further purification.

Name, acronym (abbreviation)	MW ($g.mol^{-1}$)	Supplier	CAS number
Bovine serum albumin (BSA)	66 000	Sigma-Aldrich	9048-46-8
Poly(ethylene imine) (PEI)	750 000	Alfa Aesar	9002-98-6
Poly(styrene sulfonate) (PSS)	70 000	Sigma Aldrich	25704-18-1
Deuterated Water (D_2O)	20.03	Sigma Aldrich	7789-20-0
Dimethylformamide (DMF)	73.09	Acros Organics	68-12-2
Dichloromethane (DCM)	84.93	Acros Organics	75-09-2
Trifluoroacetic acid (TFA)	114.02	Alfa Aesar	76-05-1
Deuterated DMSO (DMSO-d_6)	84.17	SDS	2206-27-1
N-Ethyldiisopropylamine (DIEA)	129.25	Alfa Aesar	7087-68-5
Alkaline Phosphatase from bovine intestinal mucosa (ALP)	170 000	Sigma Aldrich	9001-78-9
Fmoc-L-phenylalanine (Fmoc-F-OH)	387.43	Iris biotech	35661-40-6
Fmoc- L-Tyrosine 5-*tert*-butyl ester (Fmoc-Y(tBu)-OH)	483.41	Bachem	147762-53-6
Fmoc-L-Glycine (Fmoc-G-OH)	297.31	Iris biotech	29022-11-5
Fmoc-L-glutamic acid 5-*tert*-butyl ester (Fmoc-E(tBu)-OH	619.71	Iris biotech	109425-51-6
Glutathion reductase from baker's yeast (S. cerevisiae) (GR)	124 000	Sigma Aldrich	9001-48-3
Cystamine dihydrochloride	225.20	Sigma Aldrich	56-17-7
Triisopropylsilane (TIPS)	158.36	Sigma Aldrich	6485-79-6
Resin 2-chlorotrityl chloride (2-CTC)	-	Sigma Aldrich	42074-68-0
1-Hydroxybenzotriazole hydrate (HOBt)	135.12	Sigma Aldrich	123333-53-9

N,N,N',N'-Tetramethyl-o-(1H-benzotriazol-1-yl)uranium hexafluorophosphate (HBTU)	379.24	Alfa Aesar	94790-37-1
Diethyl ether	74.12	Acros Organics	60-29-7
Succinic acid	118.09	Sigma Aldrich	110-15-6
Poly-L-Lysine (PLL)	52 000	Sigma Aldrich	26124-78-7
Poly-L-Glutamic acid (PGA)	41 400	Sigma Aldrich	26247-79-0
Dithiothreitol (DTT)	154.25	Sigma Aldrich	3483-12-3
Glutathione	307.32	Sigma Aldrich	70-18-8

2. Hydrogel preparation in bulk and upside-down vial tests

All hydrogels were prepared in PBS buffer (pH 7.4). The PBS buffer is prepared the day of the hydrogel preparation.

General preparation of Fmoc-*A*-SS-*B* solution: the suitable amount of Fmoc-***A***-SS-***B*** was dissolved in PBS to get the suitable concentration (usually 1, 10 or 30 mg/mL). This solution is vortexed 2 – 5 minutes and sonicated in an ultrasound bath during 1 minute. The resulting Fmoc-***A***-SS-***B*** solution was thus used for all kind of analyses.

PBS Buffer (pH 7.4): one tablet of commercially available PBS (P4417 from Sigma Aldrich) was dissolved in 200 mL of ultrapure water (Milli-Q Plus system, Millipore, Billerica, MA) leading to 0.01 M phosphate buffer, 0.0027 M potassium chloride and 0.137 M sodium chloride. If necessary, the pH of this buffer is adjusted to 7.4 value by addition of few drops of HCl (0.01 M) or NaOH (0.01 M) solution. The pH value is monitored using a pH meter.

Hydrogel formation triggered with GR were obtained by dissolving Fmoc-***A***-SS-***B*** (10 mg/mL) in PBS buffer. This solution was vortexed during 2 min and then dipped into an ultrasound bath during 1 minute. 20 µL of commercial GR (G3664 from Sigma Aldrich) was added. The resulting hydrogel was obtained immediately.

Hydrogel formation triggered by BSA or AP: peptide solution was prepared by dissolving Fmoc-***A***-SS-***B*** in PBS buffer to get a concentration of 30 mg/mL. This solution was vortexed during 5 min and then dipped in an ultrasound bath during 5 min. BSA or AP was added to the Fmoc-***A***-SS-***B*** solution to get a concentration of 30 mg/mL and the resulting mixture was vortexed 10 seconds. The hydrogel is obtained in 20 min for both protein.

Upside-down vial test of TCEP and Fmoc-*A*-SS-*B* solution: Fmoc-***A***-SS-***B*** (30 mg/mL) was dissolved into a freshly prepared solution in PBS buffer. This mixture was vortexed (5 min) and then dipped in an ultrasound bath (5 min). Then 1 equivalent molar of TCEP was added as a single solid portion into the mixture. This solution was vortexed 10 seconds. The inverted vial test was done 5 and 20 minute later.

Upside-down vial test of Fmoc-*A*-SS-*B* and BSA mixture in presence of sodium citrate: the peptide solution was prepared by dissolving Fmoc-***A***-SS-***B*** (30 mg/mL) in PBS buffer. This solution was vortexed (5 min) and then dipped in an ultrasound bath (5 min). Then 1, 10

or 100 molar equivalents of sodium citrate were added and the mixture was vortexed during 30 seconds. Finally, BSA solution was added in PBS to get a concentration of 30 mg/mL) and the mixture was vortexed during 1 min. The inverted vial test was done 5 minute later.

Upside-down vial test of GR and *A*-SS-*B* mixture: the peptide solution was prepared by dissolving ***A***-SS-***B*** in PBS buffer. The solution was vortexed (2 min) and then dipped in an ultrasound bath (1 min). Then 20 µL of the commercial solution of GR was added to get a concentration of 10 mg/mL and the resulting mixture was vortexed during 10 seconds. The inverted vial test was done 5 minute later.

Upside-down vial test of BSA or AP and *A*-SS-*B* mixture: the peptide solution was prepared by dissolving ***A***-SS-***B*** (30 mg/mL) in PBS buffer. The solution was vortexed (2 min) and then dipped in an ultrasound bath (1 min). Then BSA or AP was added as a solid portion into to ***A***-SS-***B*** solution to get a concentration of 30 mg/mL. The resulting mixture was vortexed 10 seconds. The inverted vial test was done 20 minute later.

3. Multilayer film preparation and hydrogel self-assembly

All polyelectrolytes (1 mg/mL), enzymes (Gr 10 µl/mL; AP 1 mg/mL) and proteins (1 mg/mL) were prepared in PBS buffer freshly prepared as described in section 2. Different surfaces were used depending on the characterization techniques investigated: Gold coated quartz crystal for QCM-D, ZnSe crystal for ATR-FTIR experiments and glass slide for Cryo-SEM and Fluorescence emission assays (in SAFAS instrument) experiments.

The preparation of the hydrogel on surface was done as following: after the deposition of a PEI (1 mg/mL) precursor layer on the chosen surface by dipping, the multilayer film was built up by alternately exposing the surface to PSS (1 mg/mL in PBS buffer) and PEI (1 mg/mL in PBS buffer) solutions for 10 min with an intermediate rinsing step with PBS buffer during 5 min. AP (1 mg/mL), BSA (1 mg/mL) and GR (10 µL/mL), all prepared in PBS, were put in contact during 20 min followed by 5 min of rinsing step with PBS buffer. Finally, the peptide Fmoc-***A***-SS-***B*** solution (1 mg/mL in PBS buffer) was let in contact with the modified surface overnight. The volume of each solution used brought in contact with the substrate was 700 µL.

4. Quartz crystal microbalance with dissipation monitoring

QCM-D experiments were performed on a Q-Sense E1 apparatus (Q-Sense AB, Göteborg, Sweden) by monitoring the resonance frequencies of gold coated crystals, as well as the dissipation factors at four frequencies: the fundamental frequency at 5 MHz ($\upsilon = 1$) and the 3rd, 5th, and 7th harmonics ($\upsilon = 3$, 5 and 7 at 15, 25, and 35 MHz respectively). The QCM-D results give information on the adsorption process, as well as on viscoelastic properties of the adsorbed film[1]. Preparation of the multilayer film and formation of the hydrogel layer are described in point 3 just above.

5. Infrared spectroscopy

The Fourier Transform Infrared (FTIR) experiments were performed on a Vertex 70 spectrometer (Bruker, Germany) using a DTGS detector. Spectra, for the surface analysis, were recorded in the Attenuated Total Reflection (ATR) mode using a 45° trapezoidal ZnSe (internal reflection element) crystal (6 reflections, dimensions 72 × 10 × 6 mm^3) in ATR cell

(GRASEBY-SPECAC, England). Reference (bare ZnSe crystal in contact with ultrapure water) and sample spectra were taken 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). Multilayer films were assembled on ZnSe crystal by the dipping method as described above. PEI, PSS, AP, BSA, GR or Fmoc-***A***-SS-***B*** solutions were prepared as described previously (section 3, just above) but in deuterated PBS buffer to avoid the water signal in the amide I region.

Spectrums of the hydrogels made in solution were recorded in ATR mode using a diamond. All the hydrogels were formed in deuterated PBS buffer.

6. Scanning Electron Microscopy (SEM) and Cryo-SEM

To observe cross-sectioned gels, a specific cryo-holder was designed and manufactured by the mechanical facility of the Charles Sadron Institute (see Figure below). The glass slide, covered by enzymatic precursor film and the self-assembled gel, was inserted vertically in the jaws of the vise. The sample is plunged rapidly into liquid ethane and then fixed into the holder which was previously place inside nitrogen slush. Finally, the holder with the sample is placed inside the cryo preparation chamber of the Quorum PT 3010 machine. As the sample is free standing over the holder, during the plunging, the sample is rapidly frozen by direct contact with the liquid ethane. The sample is then transferred under vacuum into the chamber attached to the microscope and fractured with a razor blade. First, a sputtering step is realized (10 mA for 30s), then the surface is fracture. A slight etching at -90°C for 2 min is performed to render the fibers more visible followed by the deposition of a thin Pt layer (metallization step, 5 mA for 20s). The sample is then transferred in the FEG-cryoSEM (Hitachi SU8010) and observed at 1kV at -170°C.

Picture of the specific cryo-holder designed and built for the preparation of cross-sections. The “cross-section” holder is a vise like holder with two movable jaws. A screw compressing/uncompressing two springs moves the jaws forwards and backwards. By design the center of the holder is invariable as the two jaws can be displaced independently to adjust to the sample thickness by screws and springs. In that way, the sample will be in the optical axis of the SEM after introduction in the microscope and easily found under the beam.

7. Transmission Electronic Microscopy (TEM)

The TEM images were performed with sample prepared in liquid (diluted solutions (1 mg/mL of Fmoc-A-SS-B) or hydrogels (10 mg/mL of Fmoc-A-SS-B)). To make the observations 20 µL of the sample is dropped off on a shelf. Then, the sample is observed by a TEM Technai G2 machine in negative coloration. To make the observations, 5 microliters of the different

hydrogels are deposited onto a freshly glow discharged carbon-covered grid (400 mesh). The hydrogel is left for 2 minutes and the grid is negatively stained with 5 microliters uranyl acetate (2% in water) for another minute and finally dried using a filter paper. The grids were observed at 200kV with a Tecnai G2 (FEI) microscope. Images were acquired with a camera Eagle 2k (FEI) ssCD camera.

8. Analytic High-Performance Liquid Chromatography (HPLC)

Analytic High-Performance Liquid chromatography (HPLC) was carried out with a 1100 Series from Agilent technologies. The column is a Supelcosil ABZ + Plus with the following dimensions 15 cm × 4.6 mm, 3 µm. The eluent used for all analyses was acetonitrile/deionized water in ratio 80/10 in isocratic conditions, at 1 mL/min. Chromatograms were recorded by the software OpenLab Agilent 1100. All samples were observed in solution in diluted conditions (ten times under the gelation condition (1 mg/mL of Fmoc-***A***-SS-***B***).

Note: For the assays using 1 equivalent of TCEP in presence of Fmoc-***A***-SS-***B*** (1 mg/mL), the peptide is first solubilized then the TCEP is added. The 100% formation of Fmoc-A-SH peptide is checked by HPLC before the addition in this reaction mixture of the chosen protein (GR, AP or BSA). Solutions were prepared in the eluent ratio of ACN and water. And all solution are filtrated with a PTFE 0,2 µm filter before each injection.

9. Dynamic Light Scattering (DLS)

Dynamic Light Scattering (DLS) experiments were carried out with a Zetasizer from Malvern. The experiments were done in diluted system, which means the concentration of peptide is at 1 mg/mL with a concentration of enzyme at 1 mg/mL too and the concentration of TCEP is done for a 1/1 ratio between TCEP and Fmoc-***A***-SS-***B***. The kinetics is followed every 10 min. For the assays using a 1/1 ratio of TCEP with peptide, the peptide is solubilized first then the TCEP is added and after 1 min, the protein is added to be sure that the Fmoc-***A***-SS-***B*** is totally converted into Fmoc-***A***-SH before the addition of the protein. All solutions were freshly prepared in PBS buffer and filtrated with a PTFE 0.2 µm filter before each experiment.

10. Fluorescence emission spectroscopy

Microplate reader UV spectroscopy (FLX-Xenius®, SAFAS, Monaco) using $SP_{2000}V_7$ software was the main device entailed in the fluorescence measurement of the π-stacking. To follow the π-stacking of the Fmoc group, we excite our molecule at 280 nm and take our spectrum in the range 300-600 nm. The fluorescence assays were done for hydrogels in solution (Fmoc-***A***-SS-***B*** (10 mg/mL with GR and 30 mg/mL for AP and BSA), enzymes (20 µL/mL for GR and 30 mg/mL for BSA and AP) and TCEP in ratio 1/1 with the peptide), diluted solutions (peptide and enzymes at 1 mg/mL) and in surface by adding the glass slide at the bottom of the well and following the protocol describe in section 3. For the assays using a 1/1 ratio of TCEP with peptide, the peptide is solubilized first then the TCEP is added and after 1 min, the protein is added to be sure that the Fmoc-***A***-SS-***B*** is totally converted into Fmoc-**A**-SH before the addition of the protein.

11. Rheology measurements

Rheological properties were measured in a Kinexus Malvern rheometer using a plate geometry of 20 mm diameter and a gap of 0.5 mm. Firstly, 380 µL of Fmoc-A-SS-B (10 mg/mL) were mixed with 20 µL (20 µL/mL) of GR directly over the plate of the rheometer and the gelation process was followed at a fixed frequency of 0.3 Hz and 0.06 % strain for 30 min until a plateau was reached. Subsequently, strain measurements were carried out from 0.01% to 100% at 0.3Hz and frequency sweeps were performed from 0.01 Hz to 10 Hz at a fixed strain of 0.06%.

12. Nuclear Magnetic Resonance (NMR)

^{1}H-NMR spectra were recorded on a Bruker Avance 400 spectrometer at 400 MHz in the specified solvent at 25°C. The spectra were internally referenced to the residual solvent signal. The chemical shifts are given in ppm and coupling constants J are listed in Hz. The following notation is used for the ^{1}H-NMR spectral: singlet (*s*), doublet (*d*), triplet (*t*), multiplet (*m*).

Experimental set-up concerning the ^{1}H NMR measurement in D_2O:
Fmoc-***A***-SS-***B*** (30 mg) was dissolved in 1 mL of deuterated water, vortexed 5 min and treated in an ultrasound bath during 5 min. Then the solution was added to the NMR tube. BSA (30 mg/mL) or TCEP (1 molar equivalent) was added directly in the 30mg/mL of Fmoc-***A***-SS-***B*** solution. The mixture was vortexed before transferred inside an NMR tube for analysis. The ^{1}H NMR spectra of solely BSA was recorded as following: 30 mg of BSA was solubilized in 1 mL of deuterated water and vortexed. Then, the ^{1}H NMR spectra was recorded.
Addition of DMSO in D_2O and ^{1}H NMR analysis was done as following: 5 mg of Fmoc-***A***-SS-***B*** was solubilized in 1 mL D_2O in a vial, vortexed during 1 min and finally dipped in an ultrasound bath during 2 min. Then the solution is transferred into a NMR tube. The first spectra is taken. Then we added inside the NMR tube 10 µL per 10 µL of DMSO (in D_2O). The solution is homogenized by turning the NMR tube upside-down 3 times before ^{1}H NMR recording.

13. Circular Dichroism (CD)

Circular dichroism (CD) spectra were recorded using a Jasco J-1100 spectropolarimeter with a data pitch of 1 nm on the light wavelength. The CD spectra show the ellipticity expressed as an angle as a function of the wavelength. Liquid samples were inserted in a quartz cell of path length 1 mm. Solutions were maintained at 25°C using a Peltier apparatus with an accuracy of ±0.2 °C.

14. High Resolution Mass Spectrometry (HRMS)

All the spectrum were done by a platform from the Laboratoire de spectrométrie de masse Bio-organique (Département des sciences analytiques, Institut Pluridisciplinaire Hubert Curien, UMR 7178 (CNRS-UdS) ECPM, 25 rue Becquerel F67087-Strasbourg-Cedex 2).

For doing the mass analysis they performed them on a microTOF-Q mass spectrometer (Bruker Daltonics GmbH, Bremen, Germany). This instrument was used at a

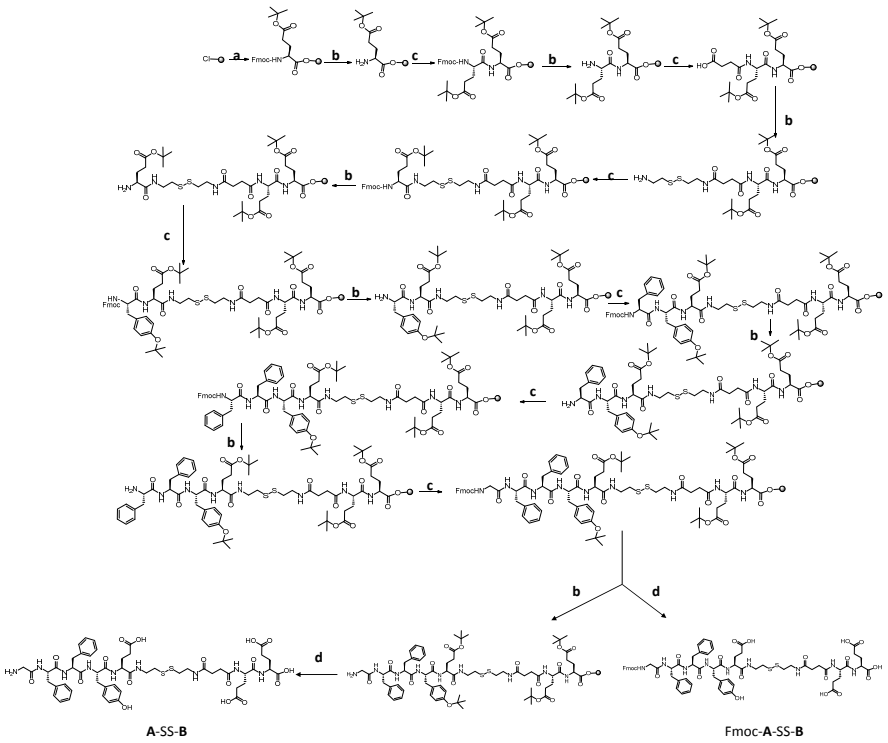
maximum accelerating potential of 20 kV in positive mode and was operated in linear mode at 19 kV. The delay extraction was fixed at 80 ns and the frequency of the laser (nitrogen 337 nm) was set at 5 Hz. Each raw spectrum was opened with flexAnalysis 2.4 build 11 (Bruker Daltonics GmbH, Bremen, Germany) software.

15. Syntheses of Fmoc-*A*-SS-*B* and *A*-SS-*B*

Both peptides Fmoc-***A***-SS-***B*** and ***A***-SS-***B*** were prepared using solid support chemistry. The "Fmoc strategy" was used based on 2-CTC resin.[1] The following synthetic pathway is given in the scheme below.

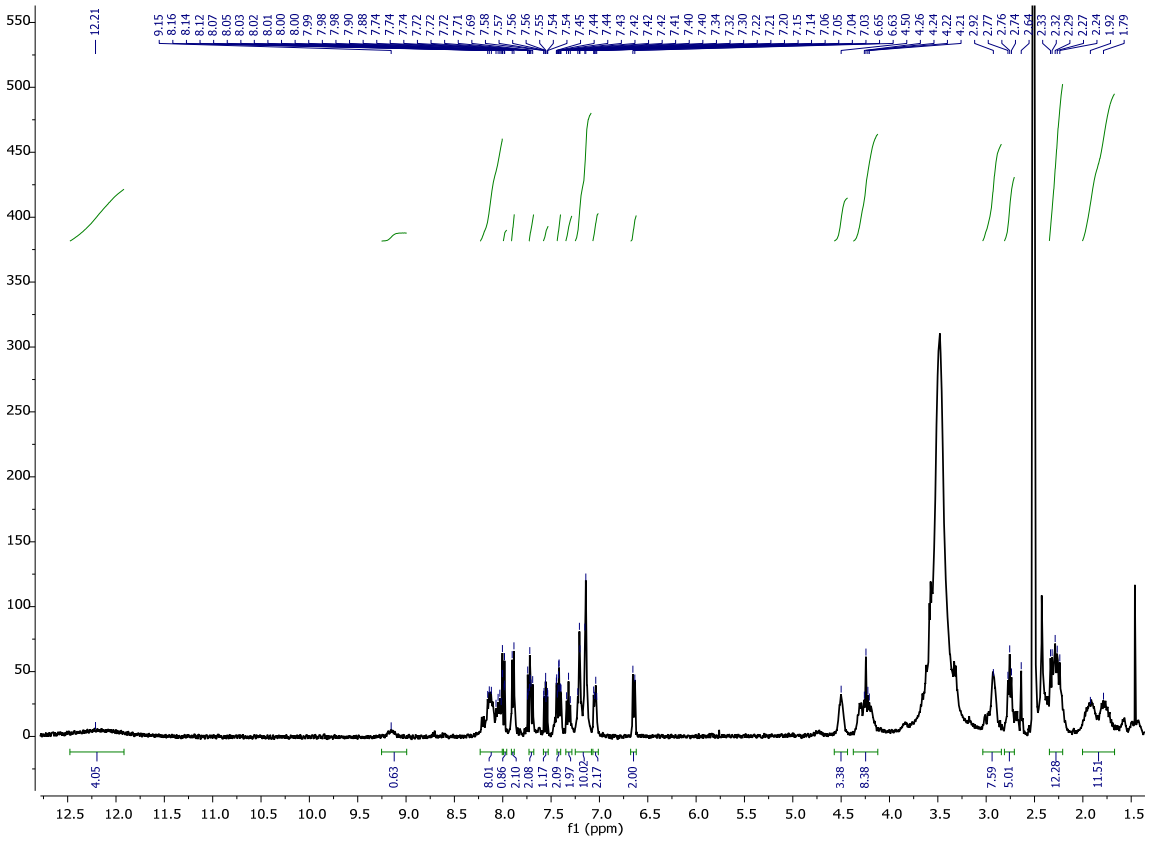
General procedure:

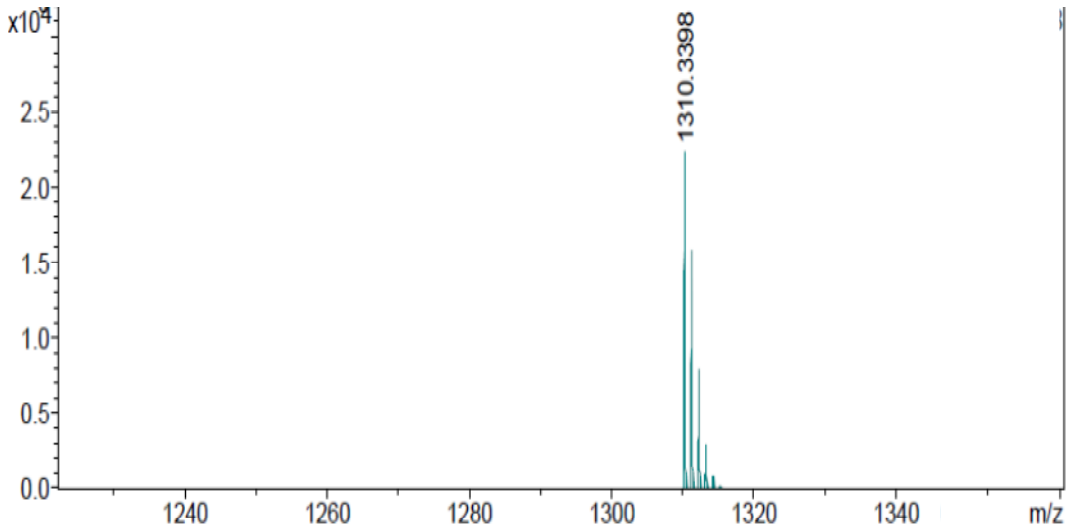
- **Step a**: loading of the resin. Addition of 3 eq/r of Fmoc-Glu(OtBu)-OH + 6 eq/r of DIEA in 3 mL of DMF for 300 mg of resin. The solution in contact with the resin is stirred at RT for 2 h. Then, the solution is removed and a solution of MeOH is added at RT for 1 h.
- **Step b**: Fmoc group deprotection: 3 mL of a 20% of piperidine in DMF solution is added and stirred at RT for 15 min.
- **Step c**: Coupling step: 3eq/r of Fmoc-amino acid + 3 eq/r of HOBt + 3 eq/r of HBTU + 6 eq/r of DIEA are added in 3 mL of DMF and let in contact with the resin at RT for 30 min.
- **Step d**: Cleavage of the resin and lateral chains deprotection: addition of 3 mL of a solution containing 95% TFA + 2.5 % H2O + 2.5 % triisopropylsilane it's stirred at RT for 2 h. Then the solution is filter. The solvent is then removed. Finally the product is precipitate by using a small amount of cold ether.
- Between each step **a**, **b**, **c** and **d**, a rinsing stage is executed by using 5 times 3 mL of DMF and then a Kaiser test is made to confirm the achievement of the coupling or deprotection steps.



Fmoc-***A***-SS-***B***:

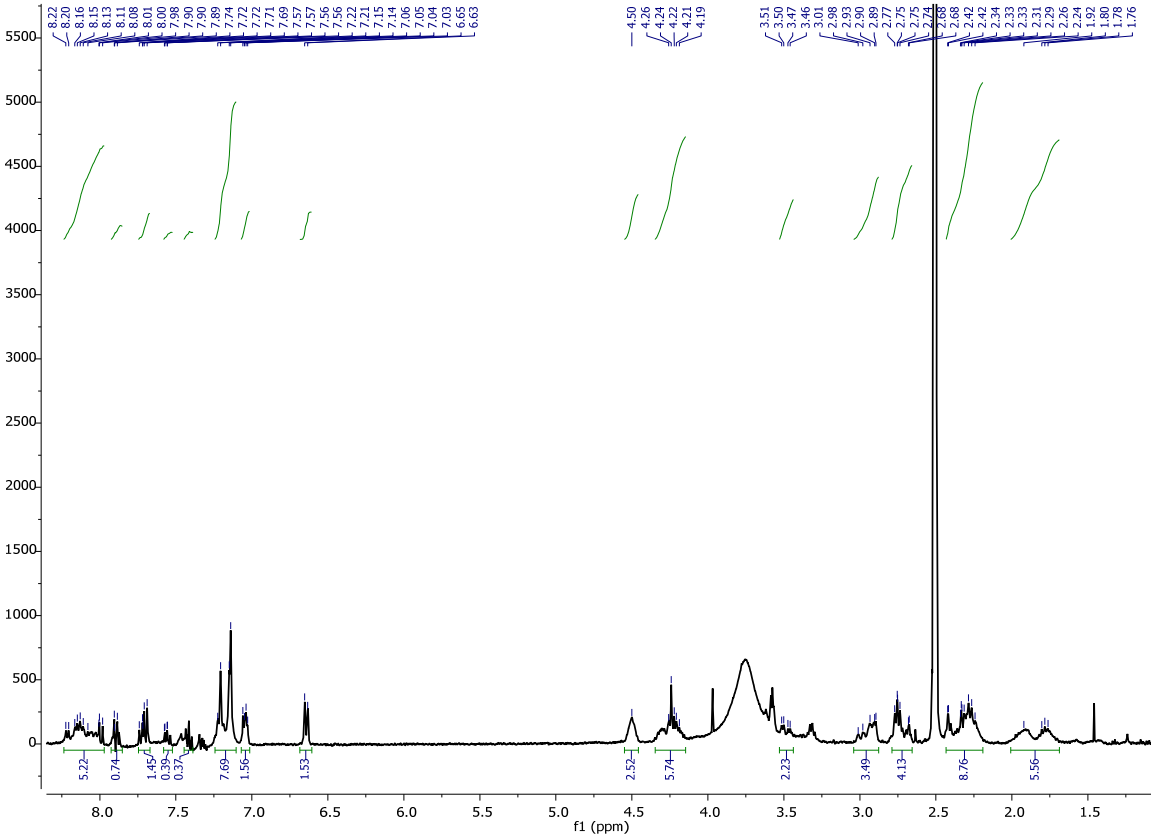
^{1}H NMR (400 MHz, DMSO-d_6): δ 1.92-2.02 (*m*, 12H), 2.15-2.64 (*m*, 12H), 2.67-2.81 (*m*, 5H), 2.90-3.04 (*m*, 8H), 4.06-4.12 (*m*, 8H), 4.18-4.28 (*m*, 3H), 6.63-6.65 (*d*, 2H, *J*=8.5 Hz), 7.02-7.04 (*d*, 2H, *J*=8.5 Hz), 7.09-7.25 (*m*, 10H), 7.29-7.33 (*t*, 2H, *J*=*J*=7.5 Hz), 7.39-7.44 (*t*, 2H, *J*= *J*=7.5 Hz), 7.46-7.48 (*t*, 1H, *J*=*J*=6.5 Hz), 7.68-7.70 (*d*, 2H, *J*= 7.5 Hz), 7.88-7.90 (*d*, 2H, *J*= 7.6 Hz), 7.97 (*s*, 1H), 8.06-8.20 (*m*, 8H); HRMS (ESI/micrOTOF-Q) m/z: $[M + H]^+$ Calcd for $C_{62}H_{67}N_9O_{19}S_2$: 1310.3317; Found 1310.3398; HPLC (ACN/Water=80/20): retention time (rt): Fmoc-***A***-SS-***B*** (15.43 min, 90% , 10% Fmoc-A-SH, rt: 15.63 min). UV-VIS spectra: max Abs: 280 nm. IR spectra: ν_{OH}= 3521 cm^{-1}; $\nu_{C=O\ acid}$= 1721 cm^{-1}; $\nu_{C=O\ amide}$= 1679 cm^{-1}; ν_{N-H}= 1648 cm^{-1}; ν_{C-O}= 1195 cm^{-1} and 1013 cm^{-1}.





A*-SS-*B:

^{1}H NMR (400 MHz, DMSO-d_6): δ 2.21-2.42 (*m*, 7H), 2.65-2.79 (*m*, 11H), 2.90-3.04 (*m*, 10H), 4.06-4.28 (*m*, 8H), 4.50 (*m*, 3H), 6.63-6.65 (*d*, 2H, *J*=8.5 Hz), 7.02-7.04 (*d*, 2H, *J*=8.5 Hz), 7.14-7.22 (*m*, 10H), 7.74 (m, 2H), 7.93 (m, 1H), 8.06-8.20 (*m*, 7H); HRMS (ESI/Q-TOF) m/z: $[M + H]^+$ Calcd for $C_{52}H_{67}N_9Na_1O_{17}S_2$: 1164.470; Found 1164.537; HPLC (80/20= ACN/Water): retention time (rt): ***A*-SS-*B*** (1.39 min, 98%, 5% A-SH, rt: 6.55 min).



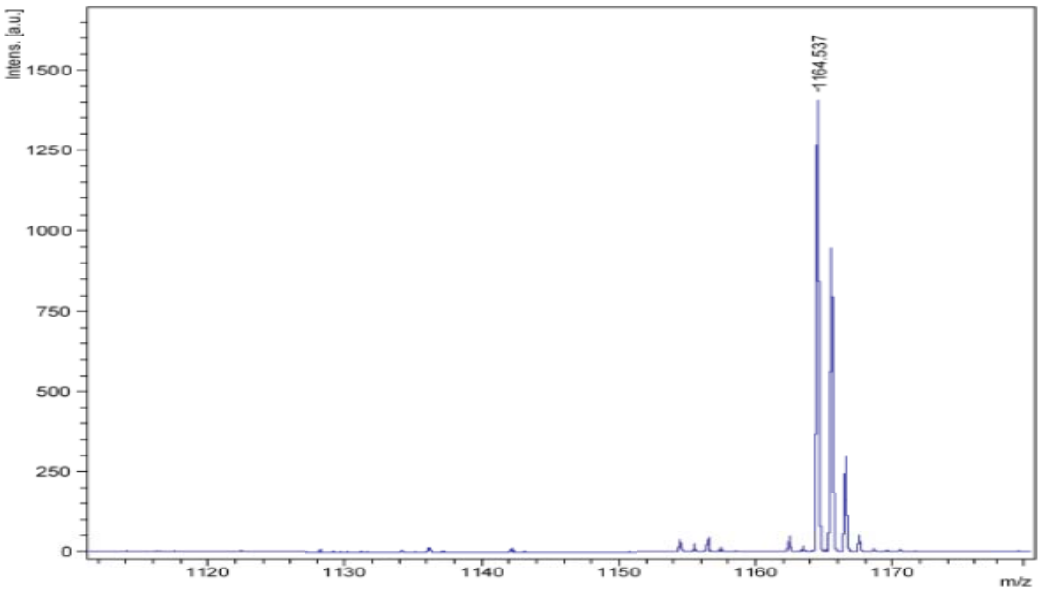


Figure S1: **Rheology measurement of the Fmoc-*A*-SS-*B* (10 mg/mL) hydrogel in presence of GR (20 µL/mL). The gelation process is monitored at a fixed strain (0.06%) and fixed frequency (0.3 Hz).**

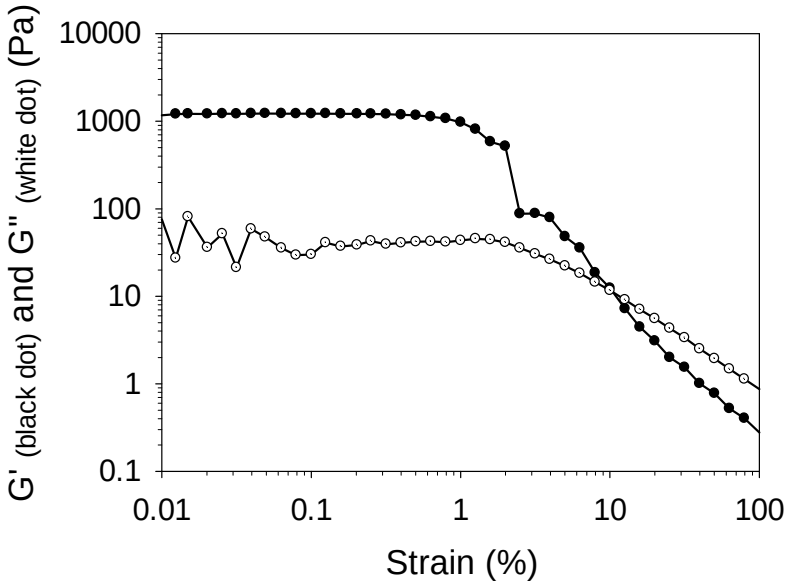


Figure S2: **Magnification of Figure 1e given in the manuscript. This image highlights nanofibers constituting the hydrogel formed from the addition of GR in Fmoc-*A*-SS-*B* solution (10 mg/mL).**

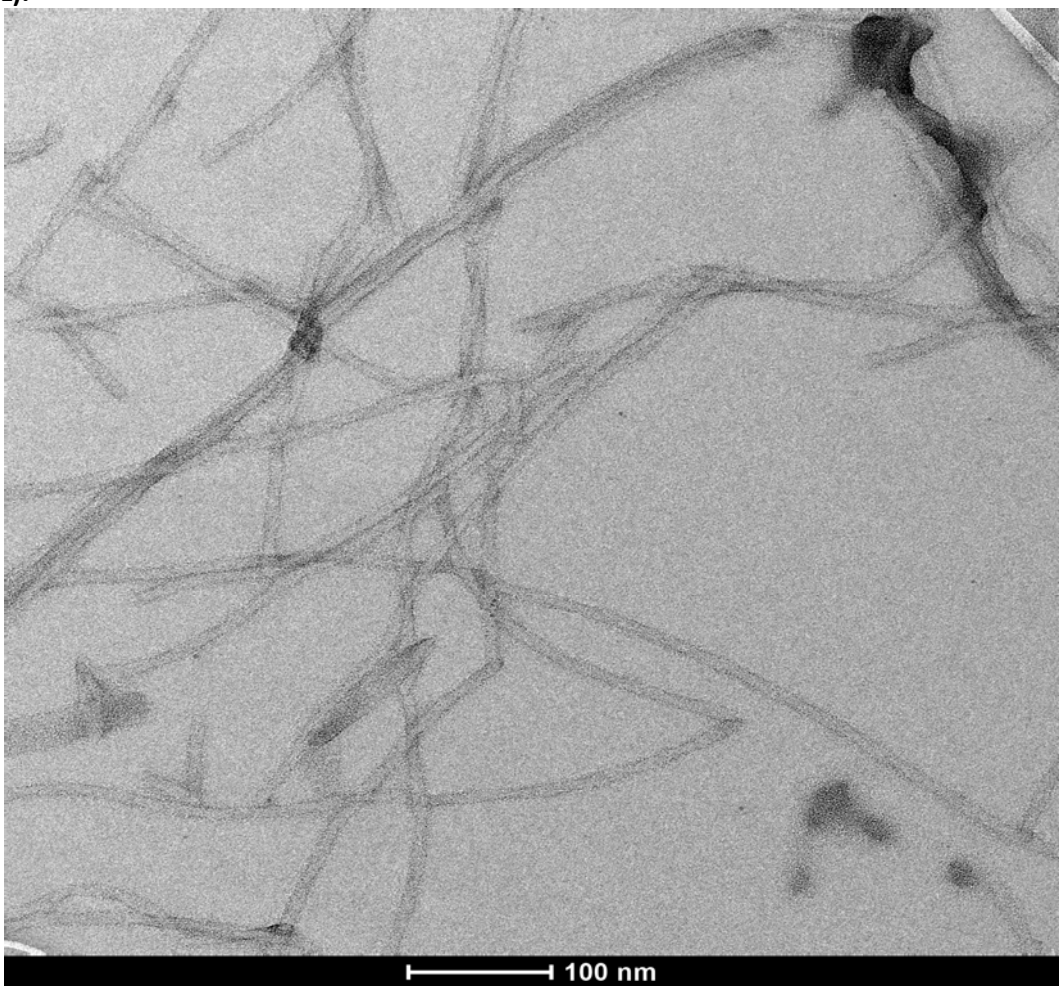


Figure S3: **Observations and HPLC monitoring of the evolution overtime of the Fmoc-*A*-SS-*B* solution (10 mg/mL) when a biomacromolecule or TCEP is added: GR (Sol.1), AP (Sol.2), GR (Sol.3) and TCEP (Sol.4).**

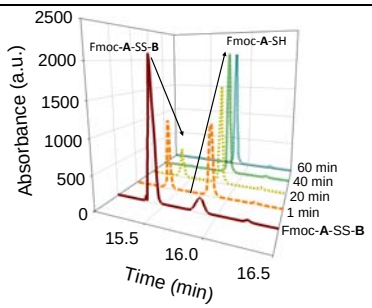
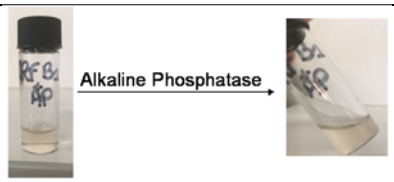
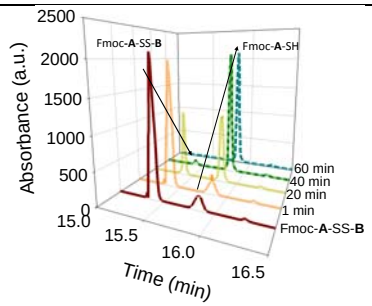
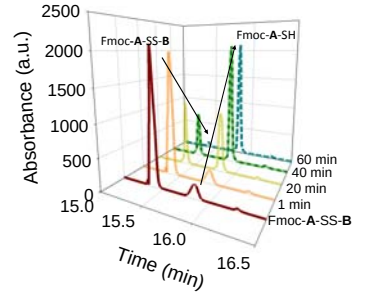
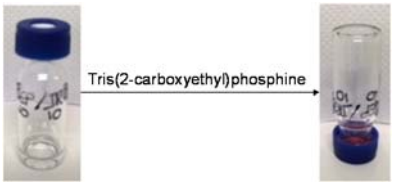
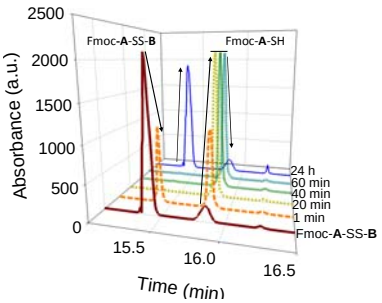
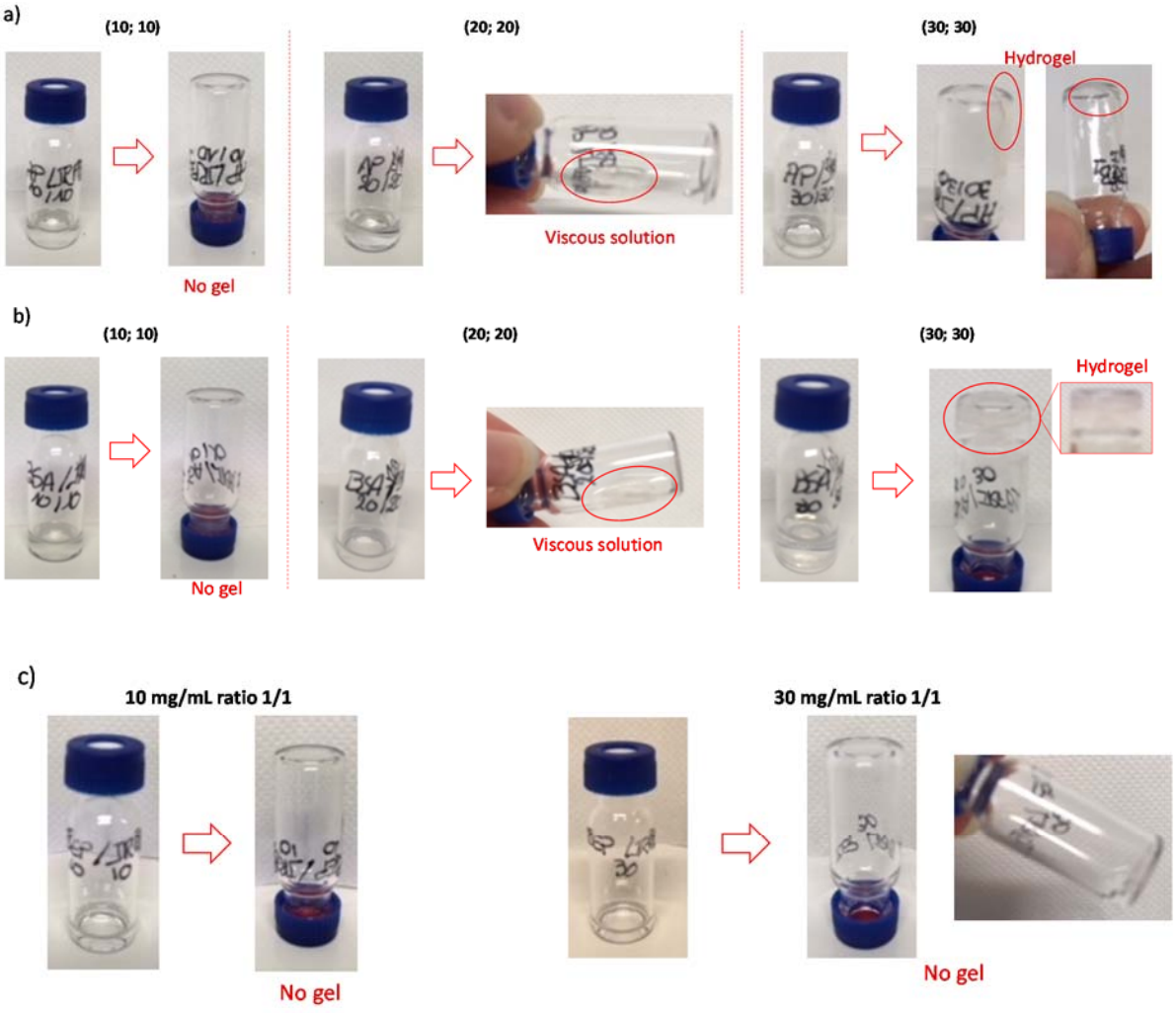
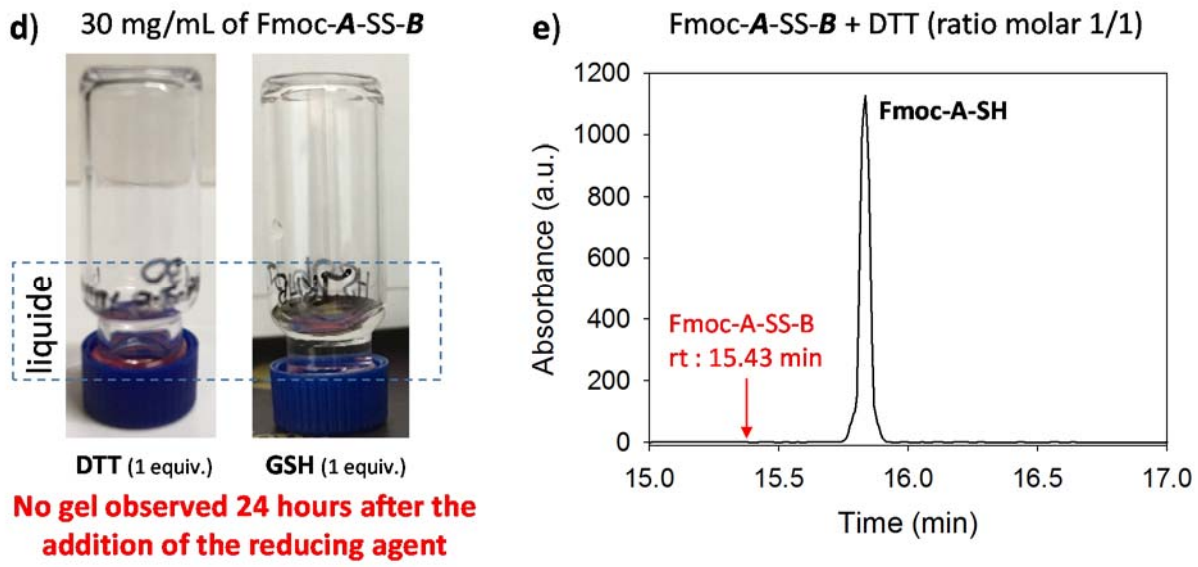
Addition at t = 0	Solution	Observations at t = 60 minutes	HPLC monitoring over time
GR (1 mg/mL)	Sol.1		
AP (1 mg/mL)	Sol.2		
BSA (1 mg/mL)	Sol.3		
TCEP (1 equiv.)	Sol.4		

Figure S4: **Observations of macroscopic hydrogel formation using the inversion tube test according to the concentration of Fmoc-*A*-SS-*B* and biomacromolecules used: 10, 20 and 30 mg/mL for both Fmoc-*A*-SS-*B* and the biomacromolecule. This ratio is mentioned (X; X) for X mg/mL of Fmoc-*A*-SS-*B* and biomacromolecule concentration. In case of solutions containing TCEP, 10 or 30 mg/mL of Fmoc-*A*-SS-*B* were used involving 1 equivalent of TCEP in both cases. The supramolecular hydrogel formation is potentially observed after the addition of the biomacromolecule or TCEP into the vial. In case of solution containing DTT or GSH, 30 mg/mL of Fmoc-*A*-SS-*B* were used involving 1 molar equivalent of the reducing agent in both cases (see next page).**





(**a**) Upside-down vial test in presence of (AP; Fmoc-***A***-SS-***B***) = (10;10), (20;20) or (30;30); (**b**) Upside-down vial test in presence of (BSA; Fmoc-***A***-SS-***B***) = (10;10), (20;20) or (30;30) and (c) upside-down vial test of Fmoc-***A***-SS-***B*** solution (10 and 30 mg/mL) in presence of 1 equivalent of TCEP; (**d**) Picture of the 30 $mg.mL^{-1}$ of Fmoc-***A***-SS-***B*** solution, 24 hours after the addition of 1 molar equivalent of DTT or GSH; (**e**) HPLC analysis of the mixture few minutes after the addition of 1 molar equivalent of DTT.

Figure S5: **CD and IR spectra of Fmoc-*A*-SS-*B* solution (1 mg/mL) in presence of 1 mg/mL of GR (Sol.1), AP (Sol.2), BSA (Sol.3) or 1 equivalent TCEP (Sol.4) after 60 min**

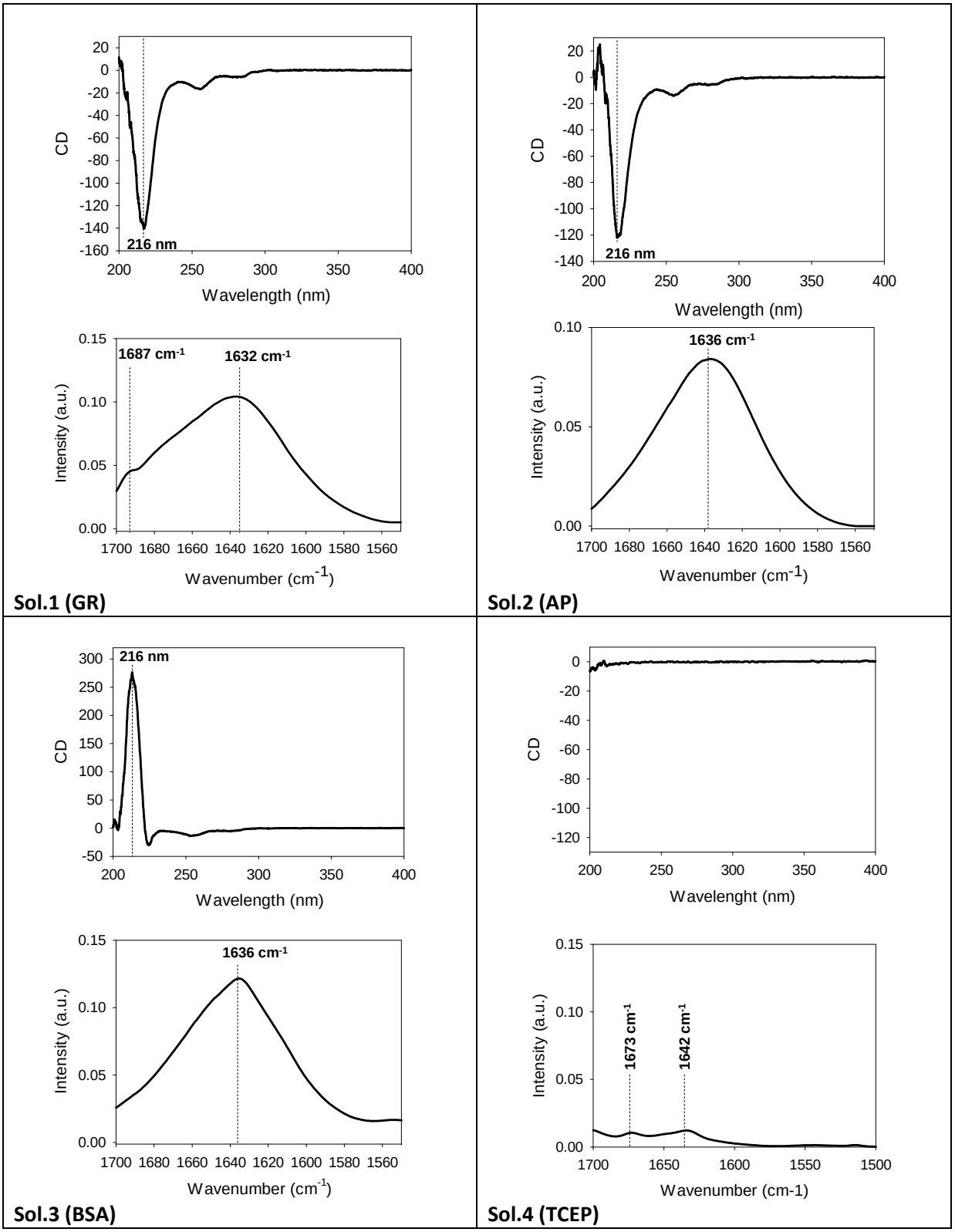


Figure S6: **DLS spectra evolution of the hydrodynamic diameter over time of GR (4 µL/mL), AP (0.4 mg/mL) and BSA (0.4 mg/mL) in presence of Fmoc-*A*-SS-*B* (1 mg/mL). Values between brackets up to each peak give the PDI. It must be take into account that the values herein reported are not accurate and only the trends in the evolution has to be considered.**

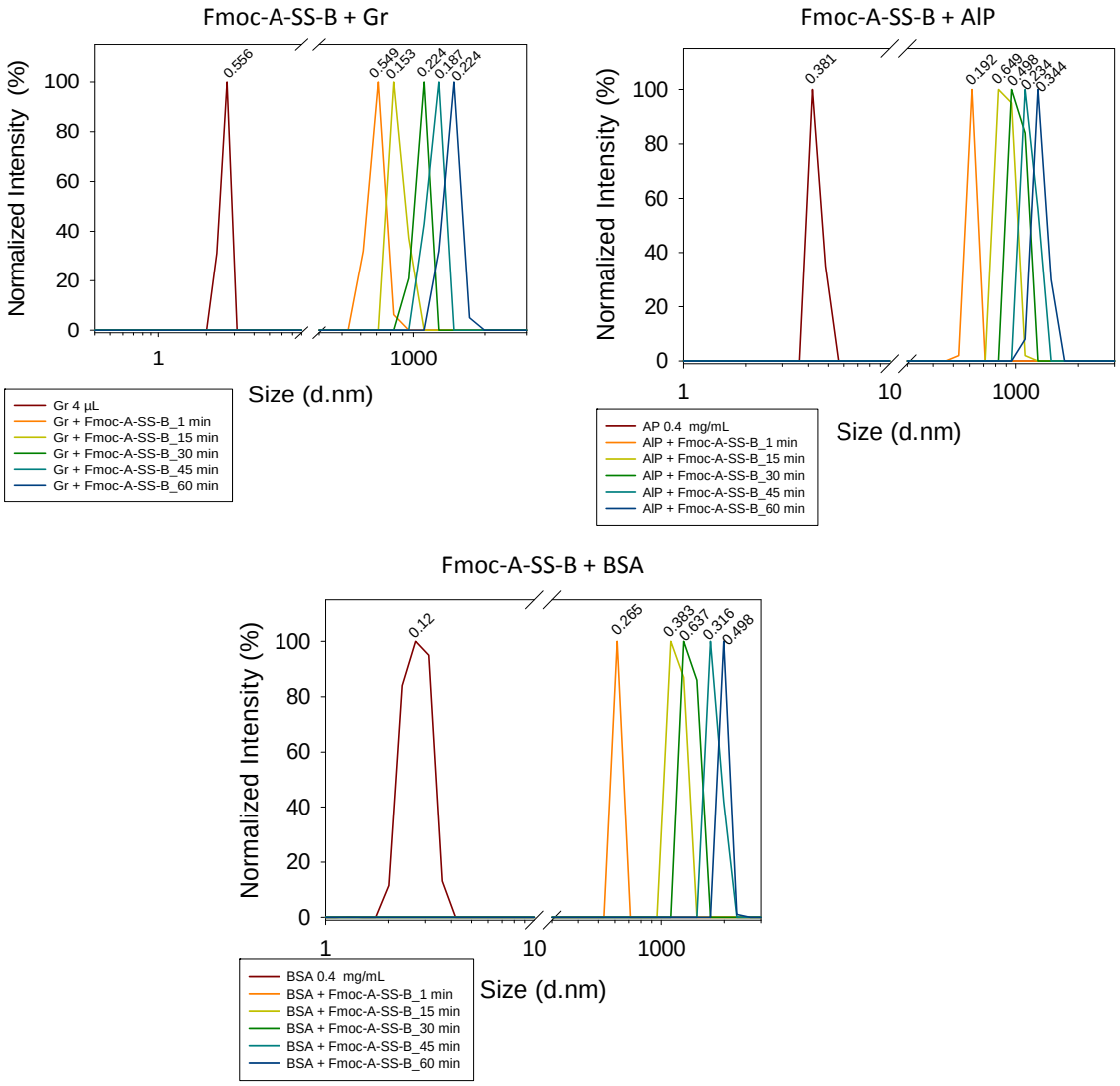
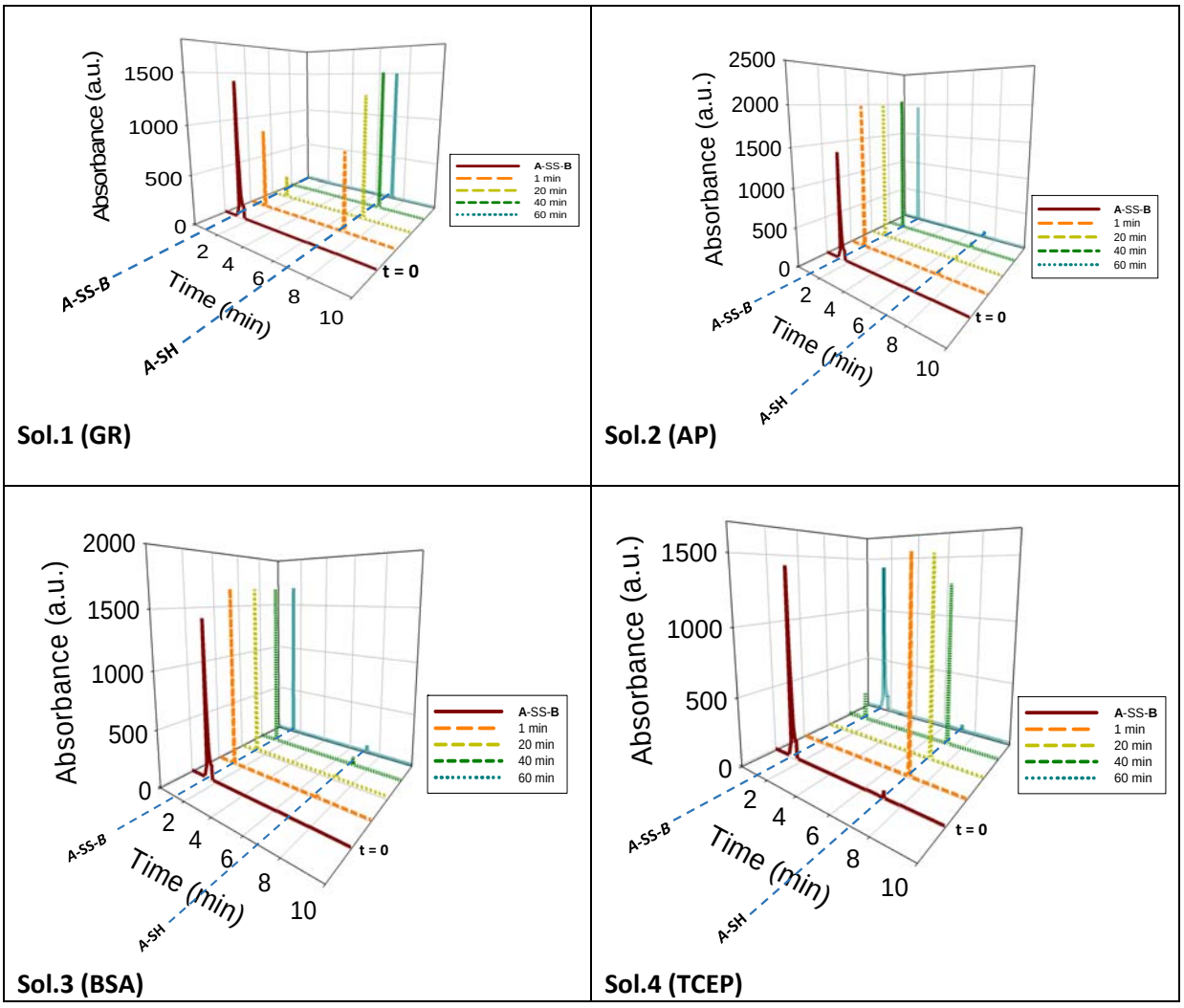


Figure S7: **HPLC monitoring of *A*-SS-*B* solution (1 mg/mL) in presence of 1mg/mL of GR (Sol.1), AP (Sol.2), BSA (Sol.3) or 1 equivalent of TCEP (Sol.4).**



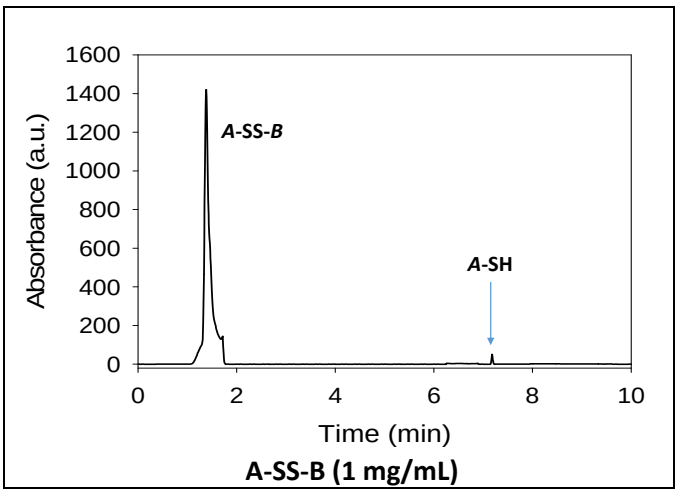


Figure S8: **HPLC monitoring and fluorescence emission intensity measured at 325 nm over time (λ_{ex} = 280nm) of a freshly prepared 100% Fmoc-*A*-SH solution (generated from the following mixture: Fmoc-*A*-SS-*B* + 1 equivalent of TCEP) after the addition of GR or AP.**

GR **AP**

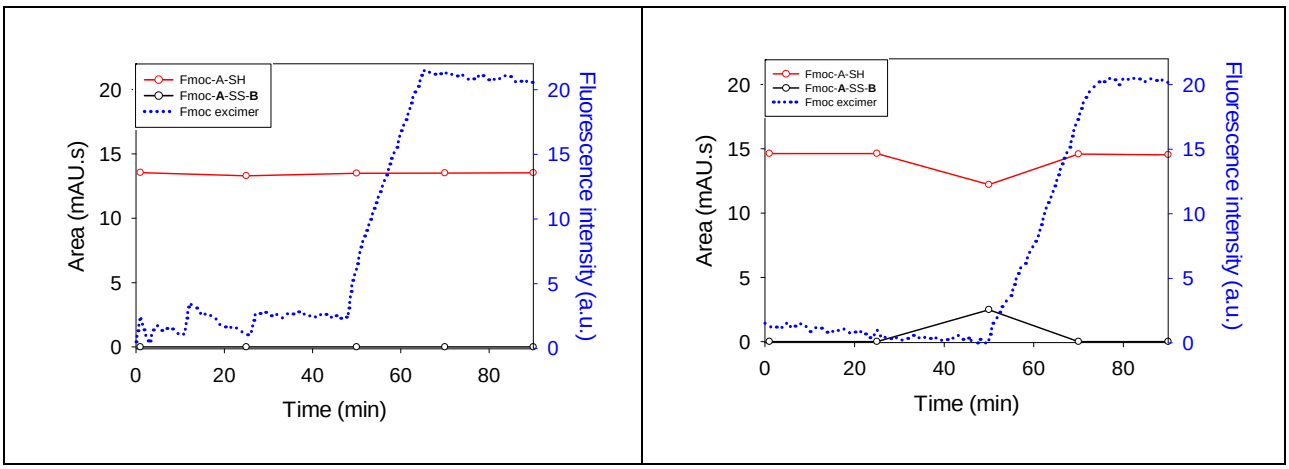


Figure S9: **Pictures of the Fmoc-*A*-SS-*B* (30 mg.ml^{-1}) in presence of 1, 10 or 100 equivalents of sodium citrate, 24 hours after the addition of BSA (30 mg.mL^{-1}).**

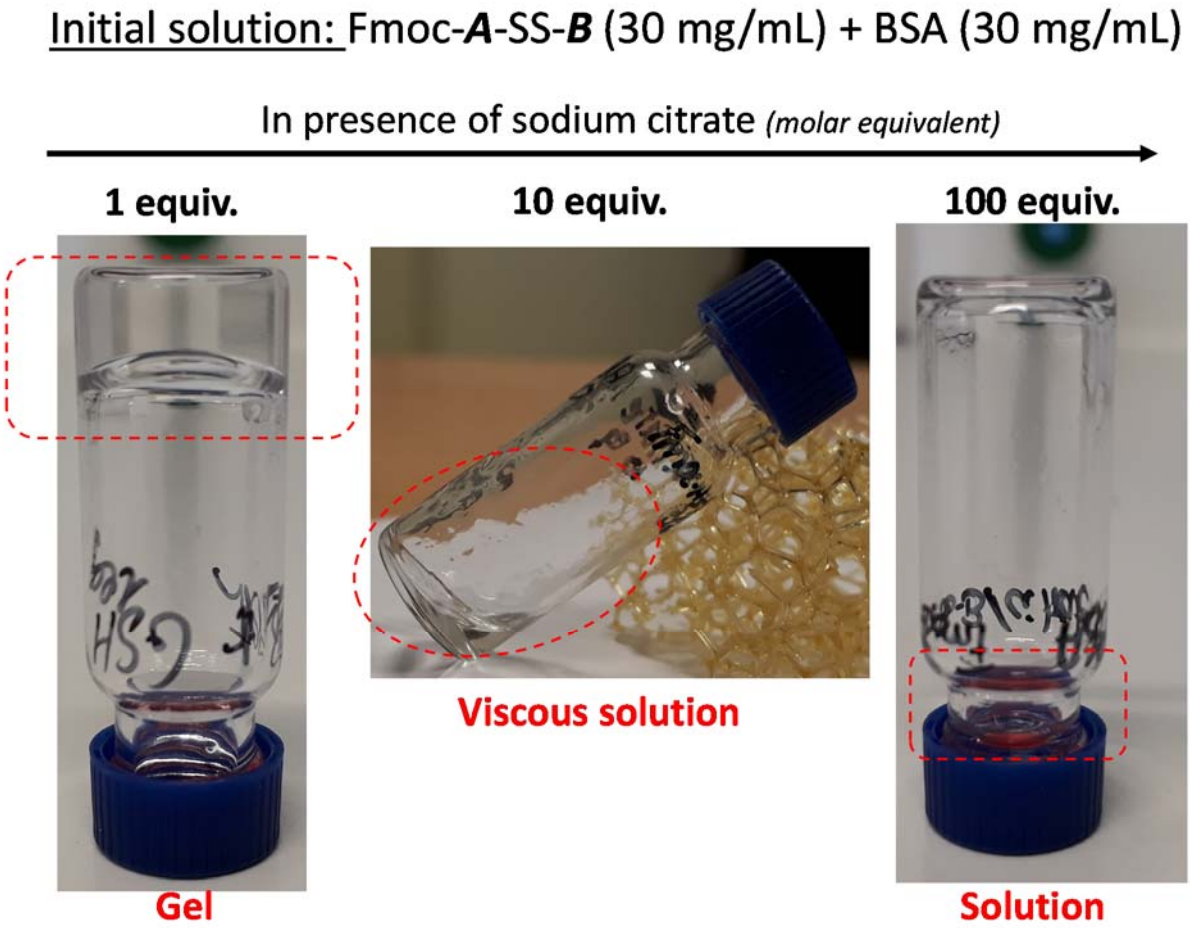


Figure S10: **^{1}H NMR spectra of the aromatic region corresponding to Fmoc-*A*-SS-*B* in 100% D_2O (black spectra), 90% D_2O/10% (blue spectra) DMSO-d_6 and 83% D_2O/17% DMSO-d_6 (green spectra).**

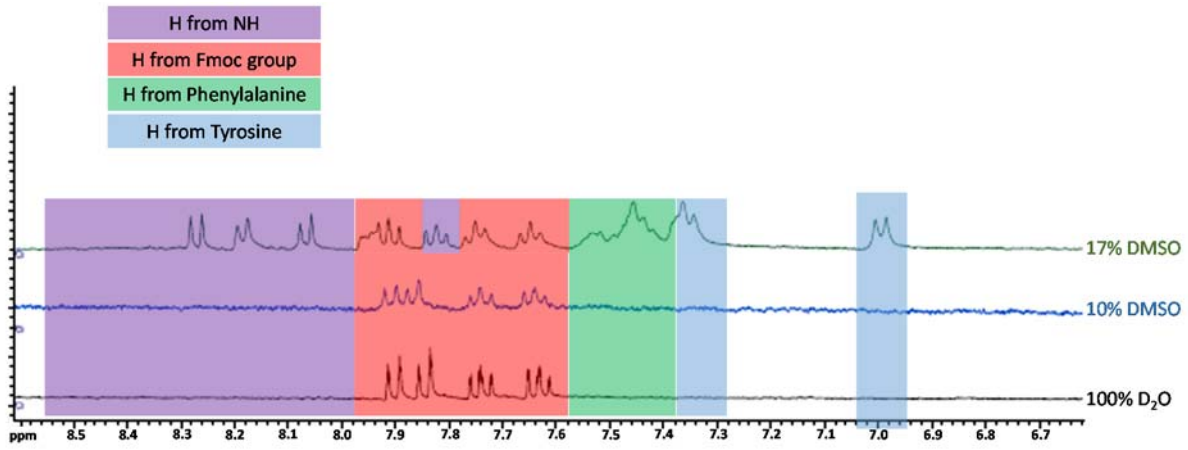


Figure S11: **^{1}H NMR spectra of Fmoc-*A*-SS-*B* in D_2O before (up) and after (down) the addition of 1 equivalent molar of TCEP.**

TCEP (1 equiv. molar) was added into a D_2O solution of Fmoc-***A***-SS-***B*** and ^{1}H NMR monitoring was carried out. The 2 minutes time to record the ^{1}H NMR spectra after the addition of TCEP is enough to cleave entirely the disulfide bridge of Fmoc-***A***-SS-***B*** as confirmed by HPLC. Particular attention was paid to the aromatic region of the ^{1}H NMR spectra. Before TCEP addition, Fmoc-***A***-SS-***B*** is pre-aggregated in D_2O and only the aromatic signals corresponding to the Fmoc groups can be observed. When 1 equivalent molar of TCEP is added into the D_2O solution only signals of protons in α and β positions of the sulfur atom are changing. The aromatic region does not change, and thus there is no appearance of the protons corresponding to Phe or Tyr residues. This means that despite the reduction of the disulfide bridge by TCEP, the resulting Fmoc-***A***-SH is maintained aggregated.

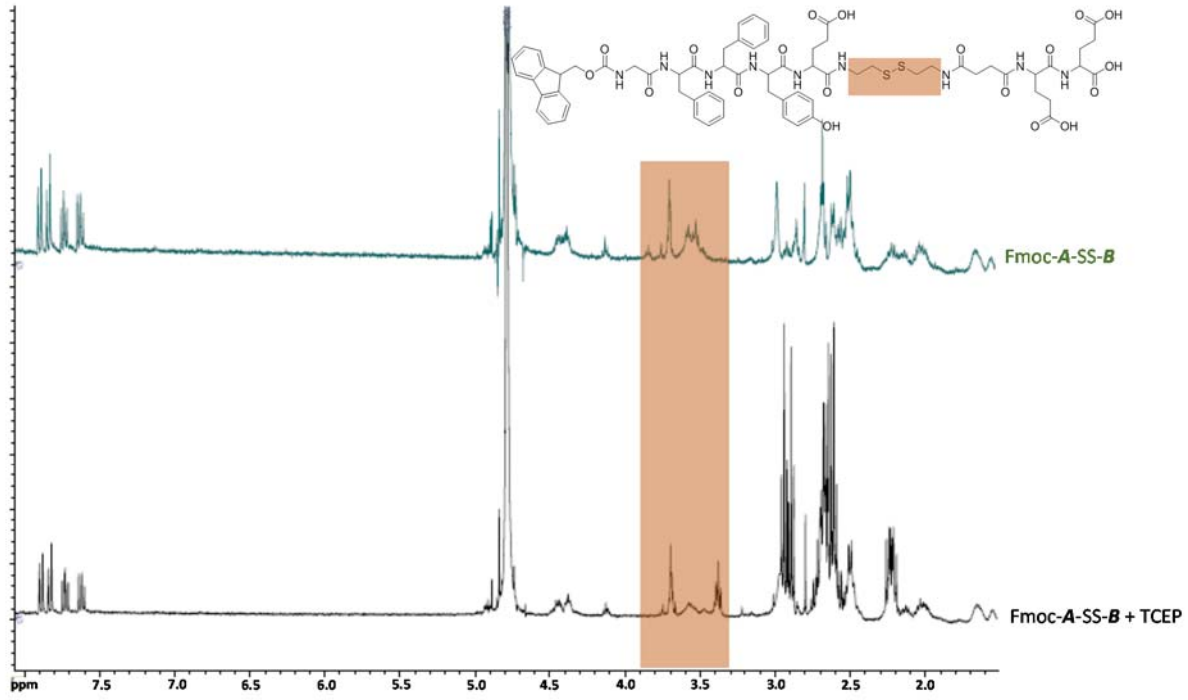


Figure S12: ^{1}H NMR (in D_2O) spectra of BSA (black curve), Fmoc-*A*-SS-*B* (green curve) and Fmoc-*A*-SS-*B* after the addition of BSA (red curve).

After the dissolution of Fmoc-***A***-SS-***B*** (10 mg.mL^{-1}) and the record of the NMR spectra, BSA (1 mg.mL^{-1}) was introduced into the NMR tube and spectra were immediately recorded.

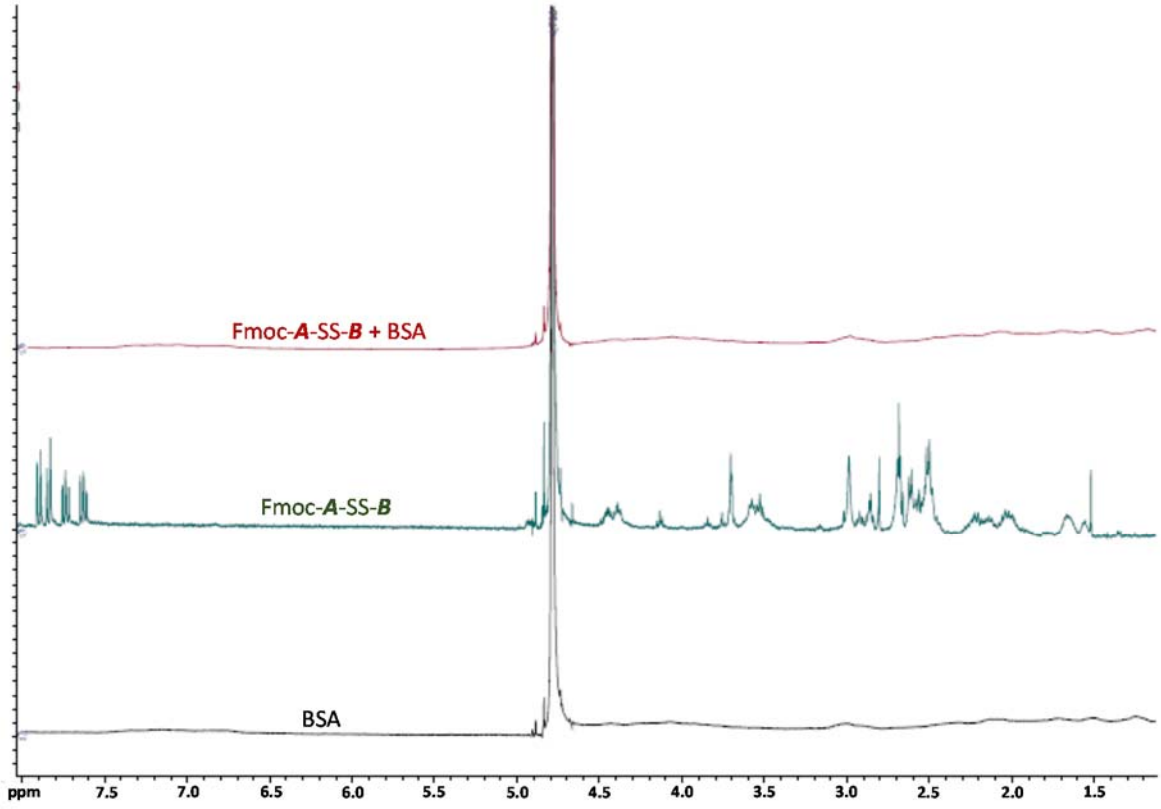
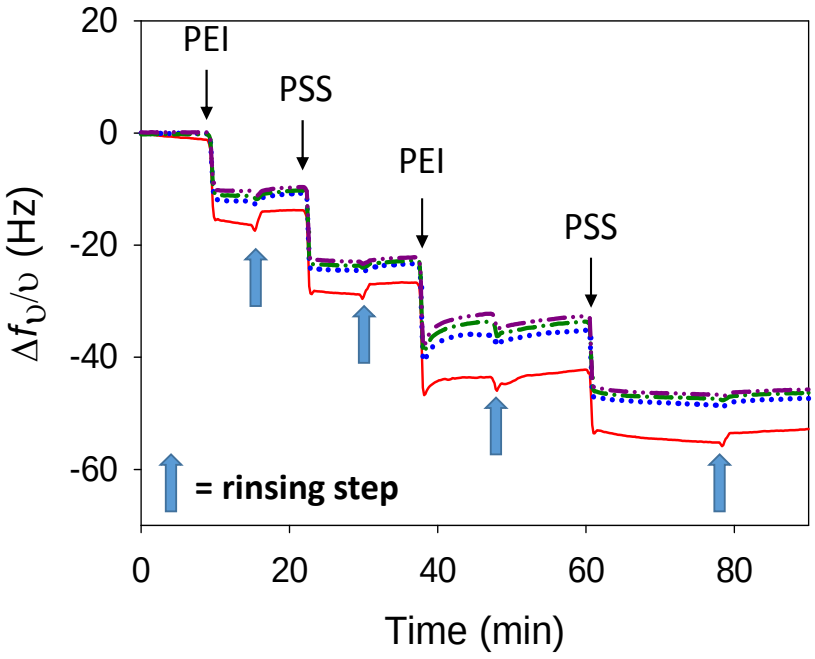
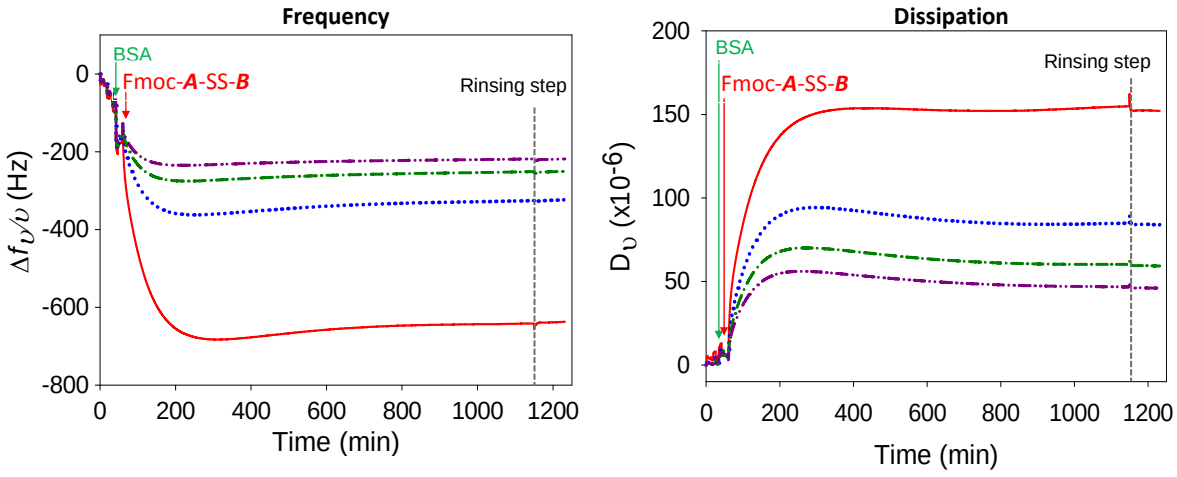


Figure S13: **QCM-D monitoring of the multilayer film buildup followed by the biomacromolecule adsorption, and the localized peptide self-assembly from this modified surface.**

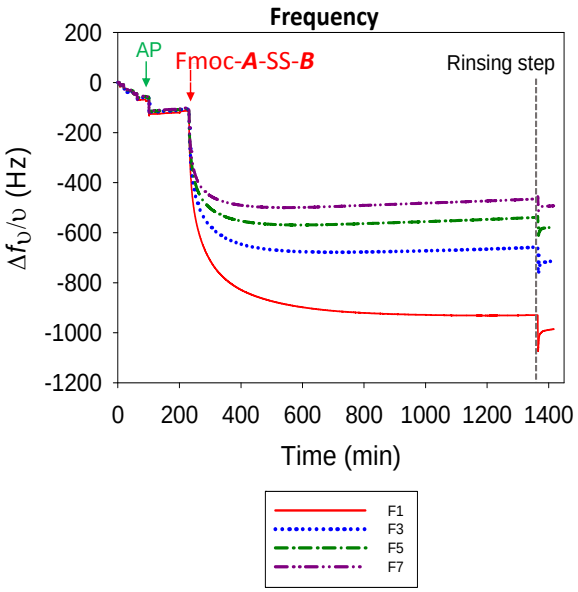
- **Multilayer film $(PEI/PSS)_2$ buildup monitored by QCM-D: Frequency shift (F1 – red curve, F3 – dotted blue, F5 – dotted green and F7 – dotted purple) measurements overtime:**



- **Adsorption of BSA on $(PEI/PSS)_2$ multilayer film followed by the contact with the Fmoc-*A*-SS-*B* solution (1 mg/mL): Frequency shifts (F1 – red curve, F3 – blue curve, F5 – green curve and F7 – purple curve) and corresponding dissipation factors measurements overtime:**



- **Adsorption of AP on $(PEI/PSS)_2$ multilayer film followed by the contact with the Fmoc-*A*-SS-*B* solution (1 mg/mL): Frequency shifts (F1 – red curve, F3 – blue curve, F5 – green curve and F7 – purple curve) and corresponding dissipation factors measurements overtime:**


Frequency
AP
Fmoc-A-SS-B
Rinsing step
Δfυ/υ (Hz)
Time (min)
F1
F3
F5
F7

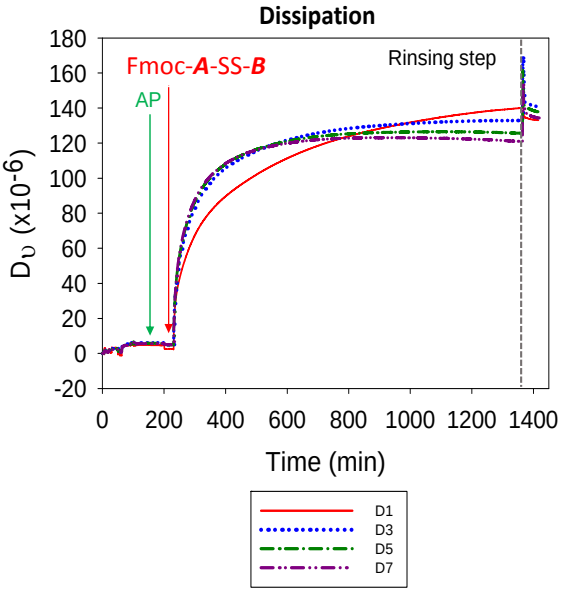

Dissipation
Fmoc-A-SS-B
AP
Rinsing step
Dυ (x10-6)
Time (min)
D1
D3
D5
D7

Figure S14: **Cryo-SEM image (z cross section) of the supramolecular hydrogel formed from a BSA layer adsorbed on a suitable multilayer supported on a glass substrate in contact with a Fmoc-*A*-SS-*B* solution (1 mg/mL).**

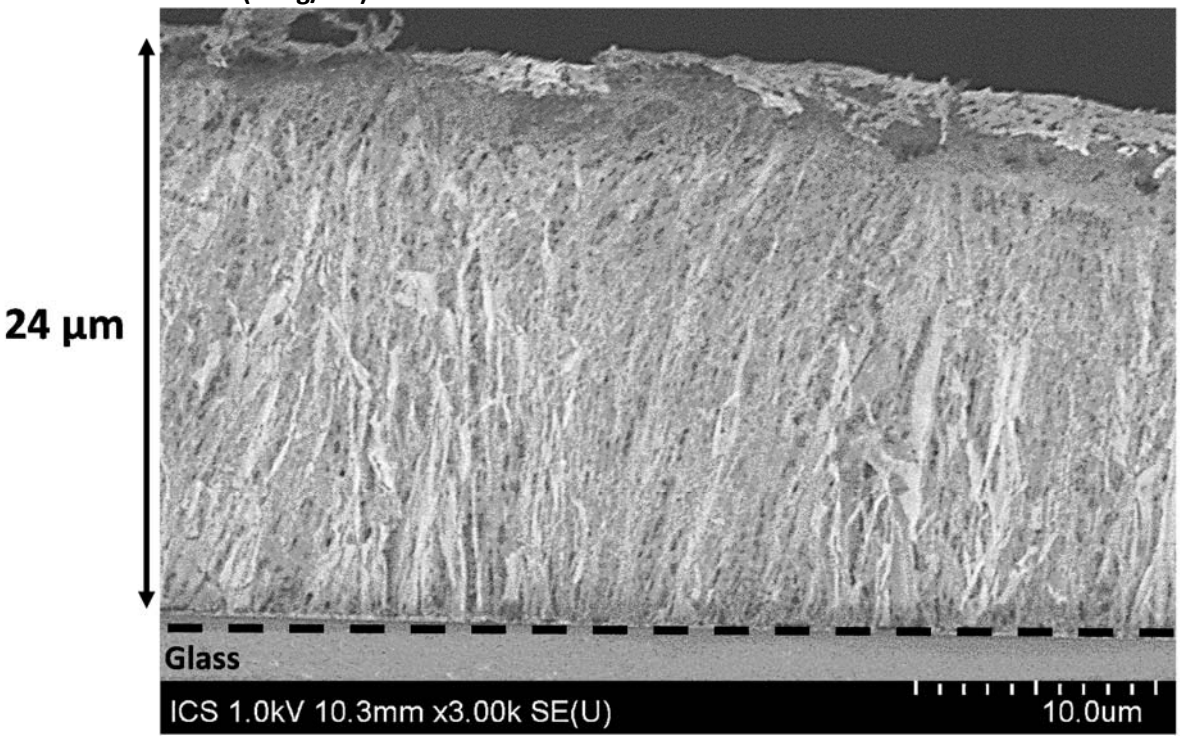


Figure S15: **IR and fluorescence emission intensity measurements monitored on the supramolecular hydrogels grew from BSA or AP modified substrate in presence of Fmoc-*A*-SS-*B* solution (1 mg/mL). The red arrow indicates the evolution of the IR spectra (ATR) recorded at different time from t=1 minute to t=12 hours.**

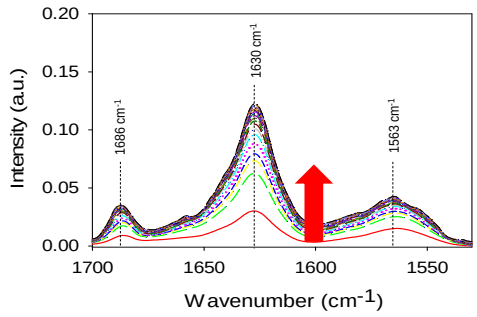
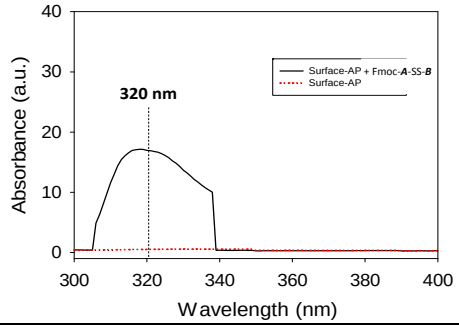
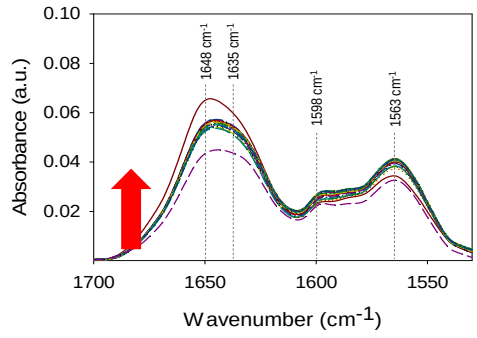
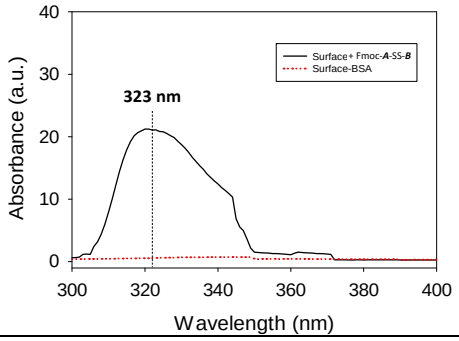
Multilayer film	IR (ATR)	Emission of fluorescence intensity when excited at 280 nm
$(PEI/PSS)_2/AP$		
$(PEI/PSS)_2/BSA$		

Figure S16: **QCM-D, IR, cryo-SEM and fluorescence emission intensity (λ_{ex}=280 nm) analyses of the supramolecular hydrogels grew from GR modified substrate in presence of Fmoc-*A*-SS-*B* solution (1 mg/mL). The red arrow indicates the evolution of the IR spectra recorded at different time from t=1 minute to t=12 hours.**

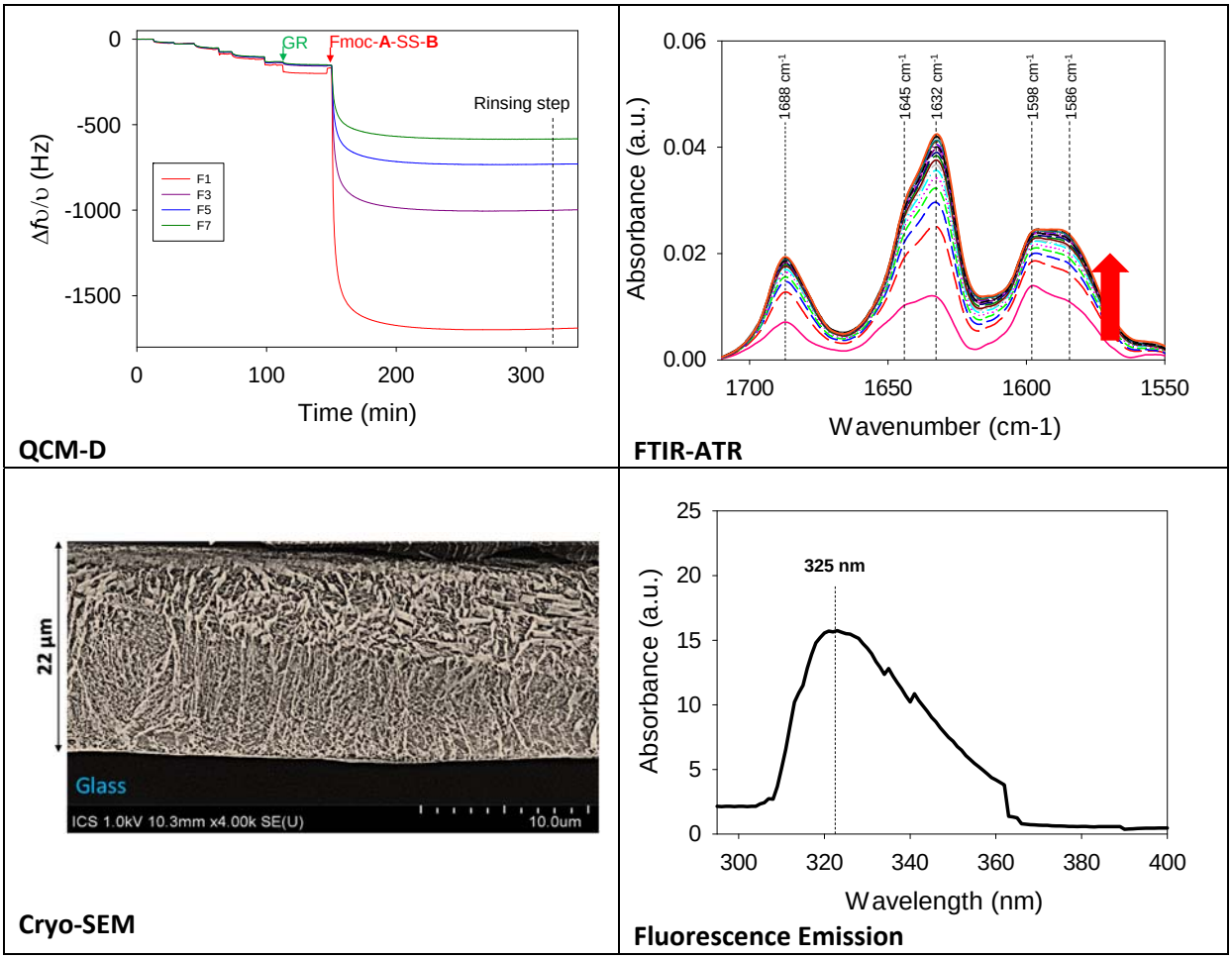


Figure S17: QCM monitoring (evolution of the 3rd harmonic v=3) when Fmoc-*A*-SS-*B* (1 mg.mL^{-1}) solution is brought in contact with P-film (green curve) and N-Film (red curve) at t=0.

Thus, to highlight the electrostatic interaction of the negatively charged peptide Fmoc-***A***-SS-***B*** (mainly due to the ***B*** sequence which contains two glutamic acid residues and the carboxylate group at the C-term position), we have modified the surface of a first substrate with poly-L-Lysine (PLL) and a second substrate with poly-L-glutamic acid (PGA). We brought each of them in contact with Fmoc-***A***-SS-***B*** solution freshly prepared. This has been monitored by quartz crystal microbalance with dissipation (QCM-D), which is an adequate tool to bring to light this kind of interaction. The surface modification of the QCM quartz substrate was done using the multilayer film strategy (as described in the submitted manuscript). Positively and negatively charged films were obtained through the buildup of the following multilayer architectures (and their effective structure monitored and confirmed by QCM-D):

- Positively charged film (**P**-Film): PEI/(PSS/**PLL**)$_2$
- Negatively charged film (**N**-Film): PEI/(PGA/PEI)/**PGA**

(Polycations: PEI=poly(ethylene imine), PLL=poly-L-lysine; Polyanions: PSS= poly(styrene sulfonate), PGA=poly-L-glutamic acid).

P-Film and N-Film are ended by poly-L-lysine (polycation) and poly-L-glutamic acid (polyanion) respectively, mimicking so protein charged patches of each electrostatic charge.

When N-Film is put in contact with a freshly prepared solution of Fmoc-***A***-SS-***B*** (1 mg.mL^{-1}), almost no decrease of the normalized frequency shift (v=3) from the quartz crystal is measured, meaning that no significant mass is adsorbed onto the N-Film, suggesting that no interaction occurs between Fmoc-***A***-SS-***B*** and the negatively charged polypeptide adsorbed onto the surface, even after several hours. When the P-film is put in contact with a freshly prepared solution of Fmoc-***A***-SS-***B*** (1 mg.mL^{-1}), a decrease of the normalized frequency shift (v=3) is measured. This decrease occurs immediately after contact between the Fmoc-***A***-SS-***B*** solution and the positively charged polypeptide layer. Despite a rinsing step, Fmoc-***A***-SS-***B*** stays onto the top of the P-film.

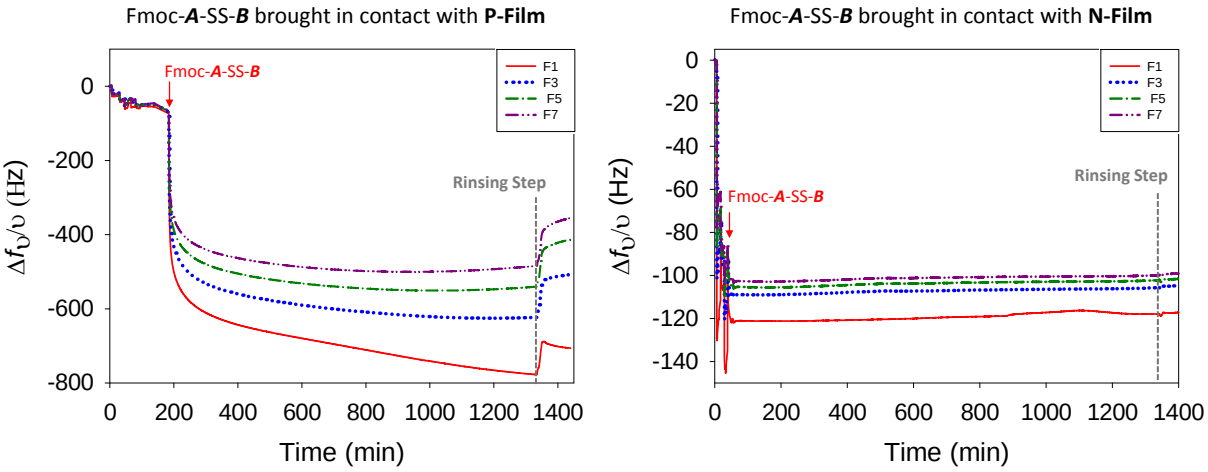


Table S1: **Evolution the hydrodynamic diameter over time measured by DLS of GR (4 µL/mL), AP (0.4 mg/mL) and BSA (0.4 mg/mL) in presence of Fmoc-*A*-SS-*B* (1 mg/mL). Values between brackets give the PDI. It must be take into account that the values herein reported are not accurate and only the trends in the evolution has to be considered.**

[Protein] / Time (min)	GR	AP	BSA
	Hydrodynamic diameter (nm) (*PDI*)		
1	734.9 (*0.549*)	616 (*0.192*)	615 (*0.265*)
15	873.6 (*0.153*)	894 (*0.649*)	1187 (*0.383*)
30	1065 (*0.224*)	1032 (*0.498*)	1314 (*0.637*)
45	1356 (*0.187*)	1154 (*0.234*)	1796 (*0.316*)
60	1405 (*0.224*)	1251 (*0.344*)	1924 (*0.498*)

References

[1] A. G. Kreutzer and P. J. Salveson, *Standard practices for Fmoc-based solid-phase peptide synthesis in the Nowick laboratory*, **2018**, *version 1.6.3*

[2] L. Lv, H. Liu, X. Chen and Z.Yang, *Colloids and Surfaces B: Biointerfaces*, 2013, **108**, 352.

[3] G. Sauerbrey, *Z. Phys*. 1959, **155**, 206.

[4] M. V. Voinova, M. Rodahl, M. Jonson, B. Kasemo, *Phys. Scr.* 1999, **59**, 391.; G. Sauerbrey, *Z. Phys.* 1959, *155*, 206-222; F. Höök et *al*., *Anal. Chem.* 2001, **73**, 5796.

[5] A. D. Martin, J. P. Wojciechowski, A. B. Robinson, C. Heu, C. J. Garvey, J. Ratcliffe, L. J. Waddington, J. Gardiner, P. Thordarson, *Sci. Rep.* 2017, 43947.