

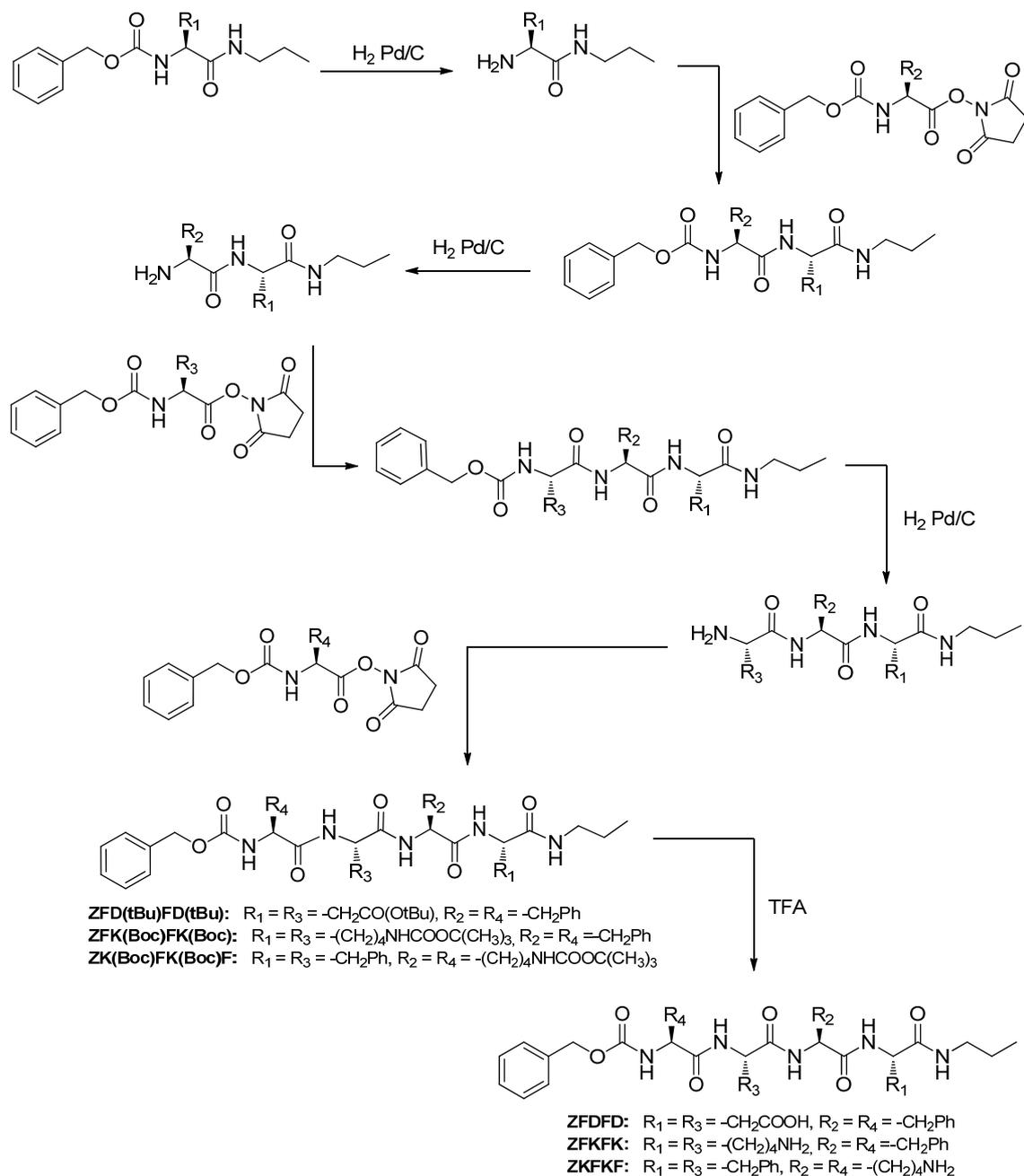
**Co-assembly of Tetrapeptides into Complex pH-Responsive Molecular Hydrogel Networks\*\***

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## Synthesis and characterization of compounds 1, 2 and 3

Compounds, **ZFDFD (1)** and **ZFKFK (2)** and **ZKFKF (3)** were prepared as follows using a step by step procedure. The general conditions for the different reactions are shown below:



### General procedure for amide coupling.

The N-hydroxysuccinimide ester (8.7 mmol) was dissolved in DME (50 mL). The deprotected amine (8.7 mmol) dissolved in DME (15 mL) was added drop wise and the resulting solution was stirred at room temperature for 18 hours and then warmed for 2 hours at 40-50°C. The solvent was evaporated under vacuum. The resulting solid was

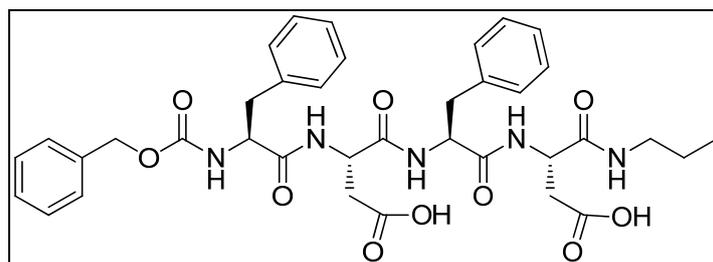
washed with saturated sodium carbonate, HCl 0.1 M and water. The final product was dried at 40°C under vacuum.

### ***General procedure for N-benzyloxycarbonyl deprotection***

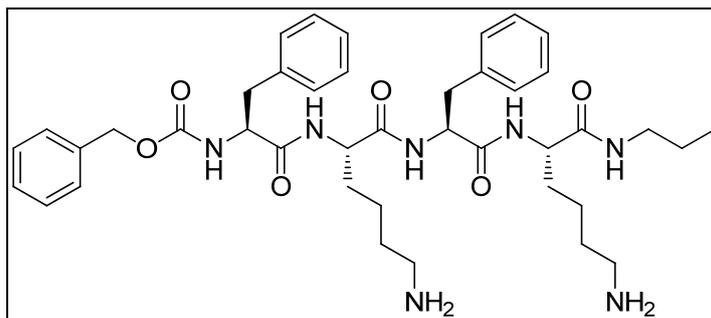
The corresponding N-benzyloxycarbonyl protected peptide derivative (7.8 mmol) and a catalytic amount of Pd over activated carbon (5-10% w/w) were placed in a two necked round bottom flask and suspended in MeOH (50 mL). The system was purged to remove the air with N<sub>2</sub> and connected to H<sub>2</sub> atmosphere. The pasty grey suspension was stirred for several hours until it turned completely black (also checked with TLC and revealed with ninhydrin). The black suspension was filtered over celite and the solvent was evaporated under reduced pressure. The resulting oil was dried further in vacuum pump for 24 hours.

### ***General procedure t-butyl ester or t-butyl carbamate group deprotection***

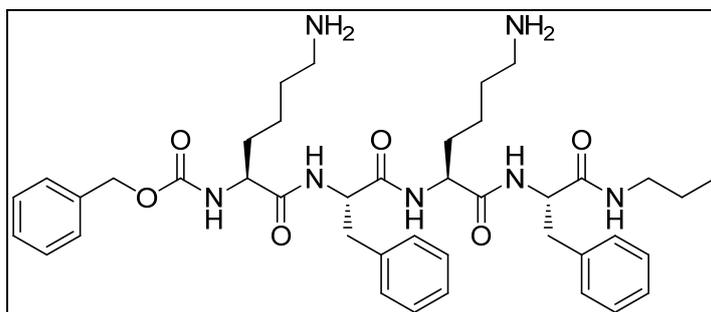
A solution of t-butyl ester or t-butyl carbamate (3.0 mmol) in a mixture of trifluoroacetic acid (TFA) (12 mL) and dichloromethane (12 mL) was stirred for 40 min at room temperature. Solvents were evaporated under reduced pressure and the residue was co-distilled three times with diethyl ether. A white solid was formed that was filtered and washed with diethyl ether.



**Synthesis of compound 1 (ZFDFD):** A white solid was obtained (Overall yield 51 %). <sup>1</sup>H NMR (500 MHz, 30 °C, d<sub>6</sub>-DMSO) δ 8.36 (d, *J* = 7.5 Hz, 1H), 8.11 (d, *J* = 8.0 Hz, 1H), 7.96 (d, *J* = 12.0 Hz, 1H), 7.40 – 7.50 (m, 2H), 7.37 – 7.03 (m, 15H), 4.98 – 4.83 (m, 2H), 4.61 – 4.53 (m, 1H), 4.52 – 4.45 (m, 1H), 4.44 – 4.37 (m, 1H), 4.30 – 4.19 (m, 1H), 3.05 – 2.80 (m, 5H), 2.77 – 2.57 (m, 3H), 2.55 – 2.41 (m, 2H), 1.44 – 1.33 (m, 2H), 0.80 (t, *J* = 7.4 Hz, 3H). <sup>13</sup>C NMR (126 MHz, 30 °C, d<sub>6</sub>-DMSO) δ 172.3, 172.2, 172.1, 171.20, 170.9, 170.3, 156.3, 138.5, 137.8, 137.4, 129.6, 129.6, 128.7, 128.5, 128.4, 128.1, 127.8, 126.7, 126.6, 65.6, 56.3, 54.8, 50.2, 50.0, 40.9, 37.9, 37.5, 36.6, 36.4, 22.6, 11.7. (ESI-TOF, positive mode) *m/z* exp [M + H]<sup>+</sup> calcd for C<sub>37</sub>H<sub>43</sub>N<sub>5</sub>O<sub>10</sub><sup>+</sup> 718.3088 ; found, 718.3093 [M + H]<sup>+</sup>, (Δ = 0.7 ppm).



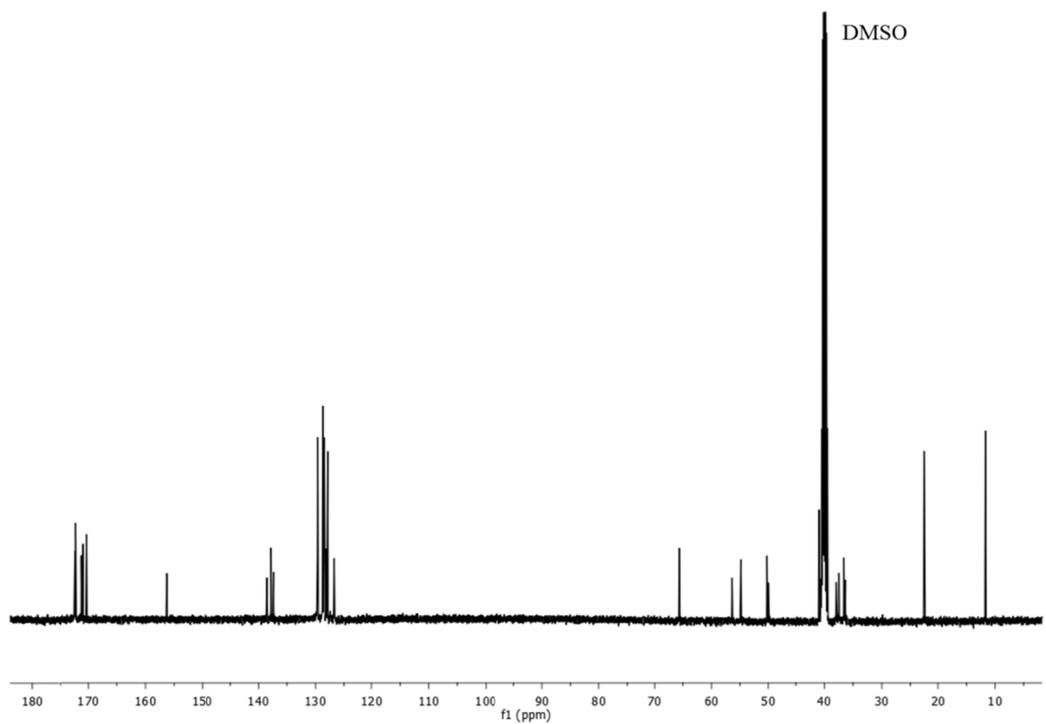
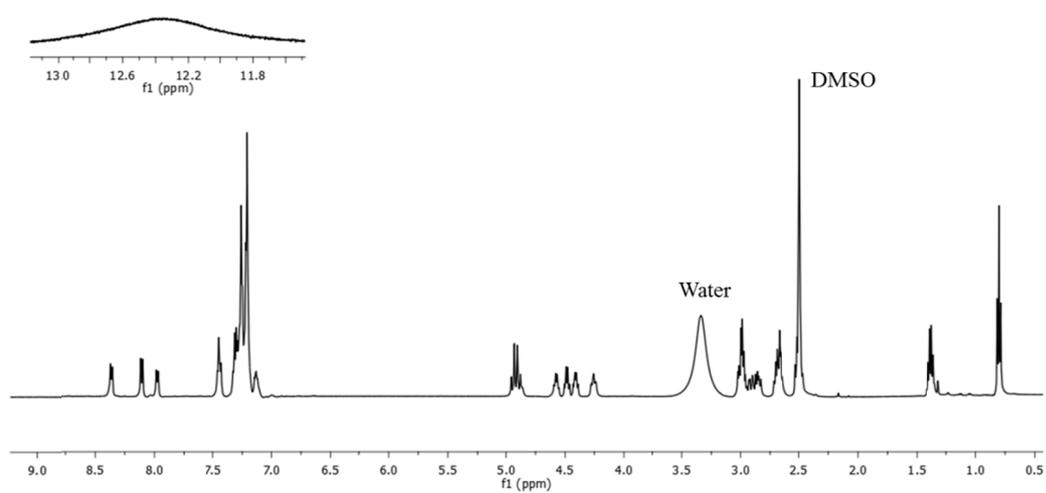
**Synthesis of compound 2 (ZFKFK):** A white solid was obtained (TFA salt, overall yield 35%).  $^1\text{H}$  NMR (300 MHz, 30 °C,  $\text{d}_6$ -DMSO)  $\delta$  8.14 (d,  $J = 8.0$  Hz, 1H), 8.03 (d,  $J = 8.0$  Hz, 1H), 7.95 (d,  $J = 7.9$  Hz, 1H), 7.82 (s, 6H), 7.76 (t,  $J = 5.8$  Hz, 1H), 7.45 (d,  $J = 8.5$  Hz, 1H), 7.41 – 7.10 (m, 15H), 5.00 – 4.83 (s 2H), 4.54 (m, 1H), 4.32 – 4.09 (m, 3H), 3.13 – 2.62 (m, 10H), 1.72 – 1.09 (m, 14H), 0.81 (t,  $J = 7.4$  Hz, 3H).  $^{13}\text{C}$  NMR (75 MHz, 30 °C  $\text{d}_6$ -DMSO)  $\delta$  172.0, 171.7, 171.4, 171.0, 158.2, 156.3, 138.5, 137.9, 137.4, 129.6, 128.7, 128.4, 128.1, 127.9, 126.7, 65.7, 56.5, 54.1, 53.0, 52.9, 40.7, 37.8, 37.8, 32.1, 31.8, 27.1, 22.7, 22.6, 11.7. (ESI-TOF, positive mode)  $m/z$  exp  $[\text{M} + \text{H}]^+$  calcd for  $\text{C}_{41}\text{H}_{58}\text{N}_7\text{O}_6^+$  744.4449 ; found, 744.4452  $[\text{M} + \text{H}]^+$ , ( $\Delta = 0.4$  ppm).



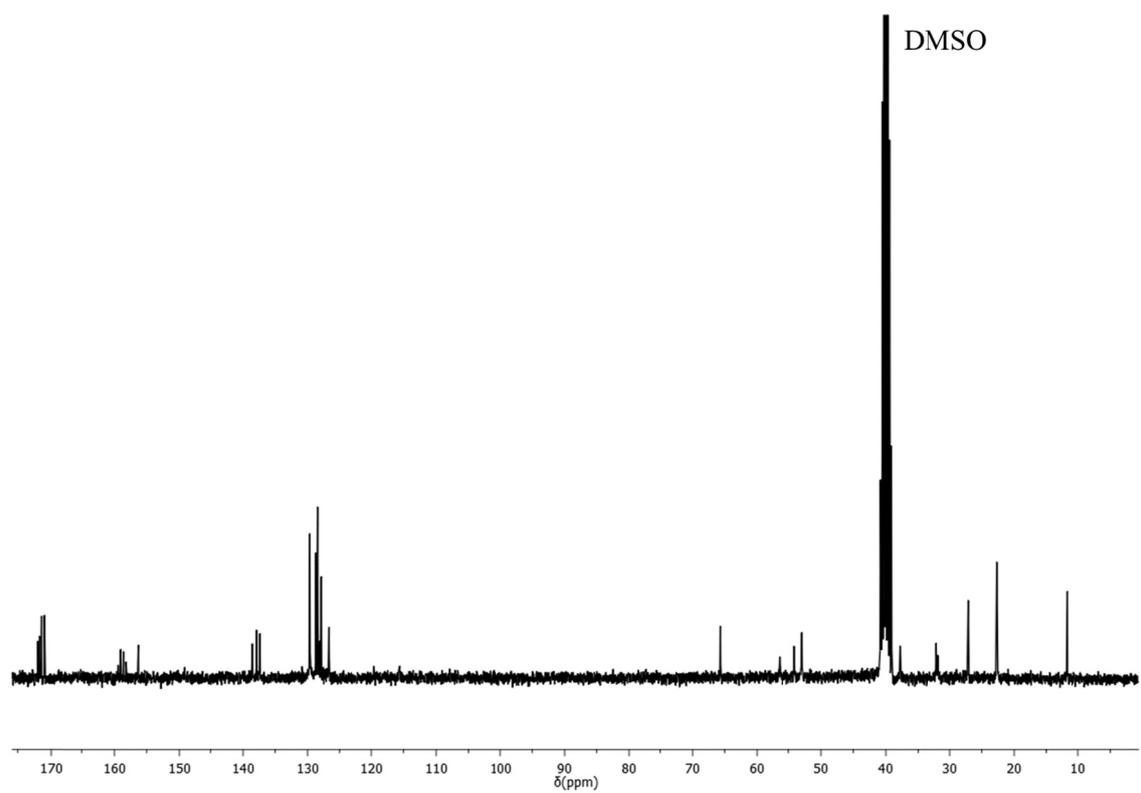
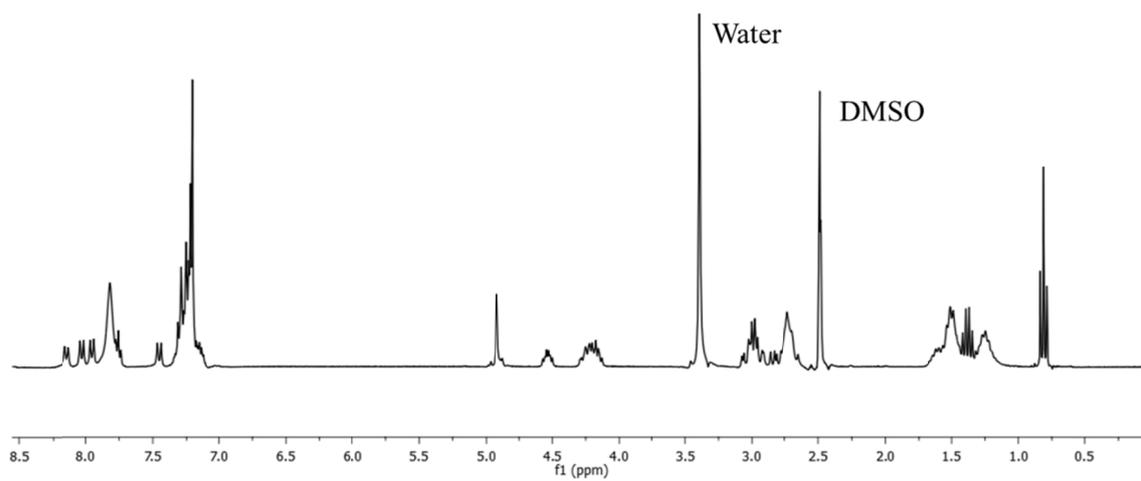
**Synthesis of compound 3 (ZKFKF):** A white solid was obtained (TFA salt, overall yield 35%).  $^1\text{H}$  NMR (500 MHz, 30 °C,  $\text{d}_6$ -DMSO)  $\delta$  8.14 (d,  $J = 7.6$  Hz, 1H), 7.92 (m, 2H), 7.84 (d,  $J = 7.5$  Hz, 1H), 7.68 (s, 6H), 7.45 – 7.09 (m, 16H), 5.00 (s, 2H), 4.57 (m, 1H), 4.49 (dd,  $J = 14.3, 8.0$  Hz, 1H), 4.22 (dd,  $J = 13.7, 7.9$  Hz, 1H), 3.90 (d,  $J = 5.3$  Hz, 1H), 3.09 – 2.68 (m, 10H), 1.67 – 1.15 (m, 14H), 0.78 (t,  $J = 7.4$  Hz, 3H).  $^{13}\text{C}$  NMR (126 MHz, 30 °C,  $\text{d}_6$ -DMSO)  $\delta$  172.2, 171.5, 171.4, 170.9, 158.2, 156.4, 138.1, 138.0, 137.4, 129.7, 129.6, 128.8, 128.5, 128.4, 128.3, 128.2, 126.7, 126.7, 66.0, 55.3, 54.4, 53.9, 53.1, 40.8, 39.2, 39.1, 38.3, 37.8, 31.8, 27.1, 27.1, 22.8, 22.6, 22.6, 11.8. ESI-TOF, positive mode)  $m/z$  exp  $[\text{M} + \text{H}]^+$  calcd for  $\text{C}_{41}\text{H}_{58}\text{N}_7\text{O}_6^+$  744.4449 ; found, 744.4453  $[\text{M} + \text{H}]^+$ , ( $\Delta = 0.5$  ppm).

# $^1\text{H}$ and $^{13}\text{C}$ NMR spectra

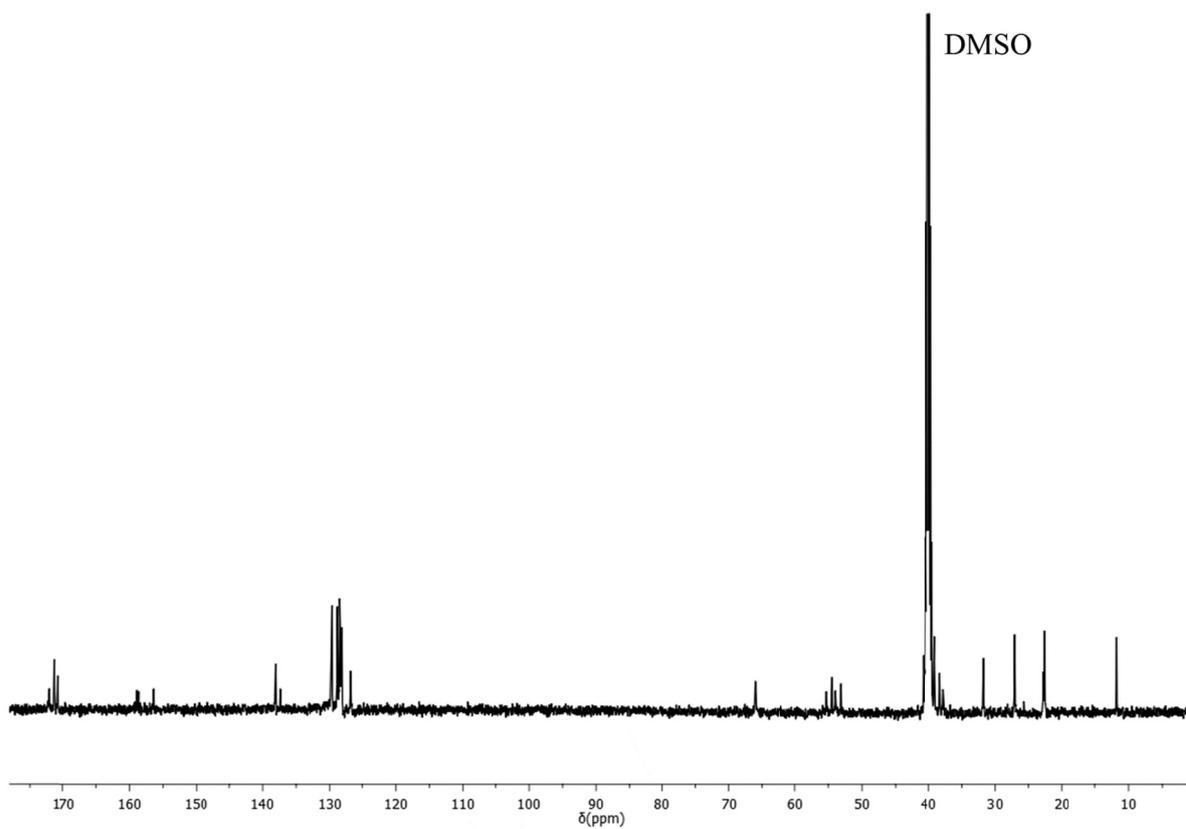
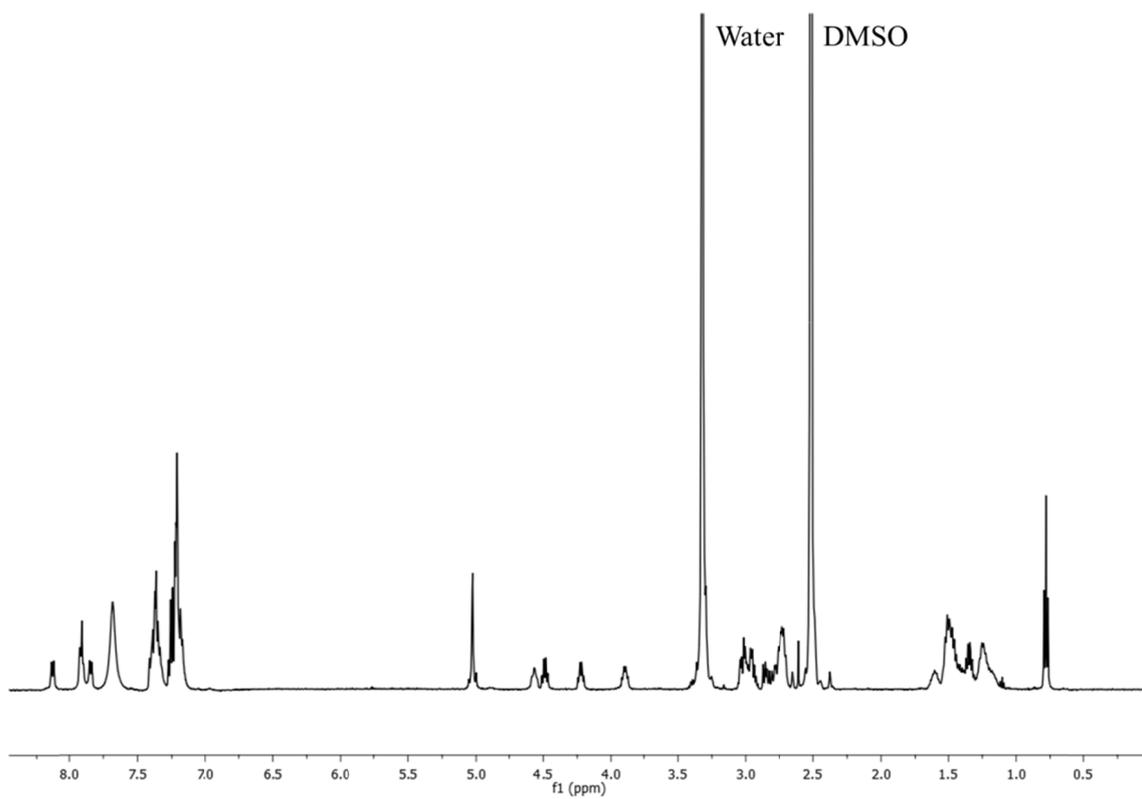
## Compound 1



## Compound 2



### Compound 3



## Hydrogel formation

Typically 0.5 mL of compound **1** at 5 mM in Na<sub>2</sub>CO<sub>3</sub> (10 mM) was added over 0.5 mL of compound **2** or compound **3** at 5 mM in HCl (10 mM). The gel was formed immediately with a final pH value between 6.5 and 7. The addition of aqueous solution of HCl and Na<sub>2</sub>CO<sub>3</sub> led to new hydrogels with pH values of 1.5 and 10.5 respectively.

## pH titration experiments

For the titration experiments the hydrogelators were dissolved in water as their dianionic form (P<sup>2-</sup>) by addition of an excess of triethylamine and then titrated with aqueous HCl. Thermodynamic constants reflected for the species in solution could be calculated for all the compounds using the titration data previous to experimental aggregation onset. On the other hand, apparent acidity constants and the solubility product, were estimated stopping the titration in the pH plateau zone corresponding to the fibrillization process and measuring pH values after 24 h, when the thermodynamic equilibrium was reached. For these calculations it was assumed that the solid network is formed exclusively by the neutral species PH<sub>2</sub>. The software Hyperquad2013 and Hyss2009 were used to determine these values.

## Thioflavine T (ThT) fluorescence assays.

Typically 10 μL of ThT 0.5 mM in water were added over 1 mL of **1** (ZFDFD) 5 mM in Na<sub>2</sub>CO<sub>3</sub> 10 mM (*Sample 1*). After 15 min the fluorescence intensity of the sample was measured. In the same way 10 μL of ThT 0.5 mM in water were added over 1 mL of **2** (ZFKFK) 5 mM and **3** (ZKFKF) 5 mM in HCl 10 mM (*Sample 2* and *Sample 3* respectively). The fluorescence intensity was measured 15 min after the addition (see Figure S1).

Then 0.5 mL of *Sample 1* were added over 0.5 mL of *Sample 2* and a gel was formed immediately. The fluorescence of this gel was measured after 20 min of its formation ( $\lambda_{\text{ex}}=450$  nm). The same procedure was used for the gel formed by *sample 1* and *sample 3*.

## Circular Dichroism.

Co-aggregates at 1 mM total concentration were prepared directly into the 1 mm quartz cell using the methodology mentioned above. Measurements were performed in a JASCO J-810 instrument, at room temperature and the spectra were recorded from 190 to 450 nm with 1.0 nm step, 1 nm bandwidth and 3 accumulations. Final spectra were the average of 3 measurements.

## **Transmission Electron Microscopy (TEM)**

Samples were applied directly onto a 200 mesh carbon coated copper grids. Excess solvent was carefully removed by capillary action using filter paper. After that one drop of distilled water was added in order to remove some salts and excess solvent was removed again in like manner. The grids were immediately stained with one drop of phosphotungstic acid 1 % for 1 min. Excess stain was removed by capillarity. TEM images were recorded in a Transmission Electron Microscopy JEOL 2100.

## **Rheology**

Rheological measurements were carried out on a TA AR-1000-N rheometer using an aluminium parallel plate-to-plate geometry (25 mm diameter). The gap distance was fixed at 1000  $\mu\text{m}$ . The gels were prepared under the desired conditions and aged for 24h. A homogeneous layer of gel was placed between the two plates. Frequency and stress sweep steps were performed at 23°C. Viscoelastic properties were studied under oscillatory experiments. All the measurements were carried out within the linear viscoelastic regime. For this purpose the experimental conditions to achieve a linear viscoelastic regime (LVR) were determined by running a stress sweep step (oscillatory Stress 0.1-500 Pa at 1Hz) and a frequency sweep step (0.1-100 Hz at 1 Pa). The storage and loss modulus independence with frequency and oscillatory stress applied defined the LVR. For the stress sweep step the  $G'$  and  $G''$  values were constant up to the yield stress point (gel break).

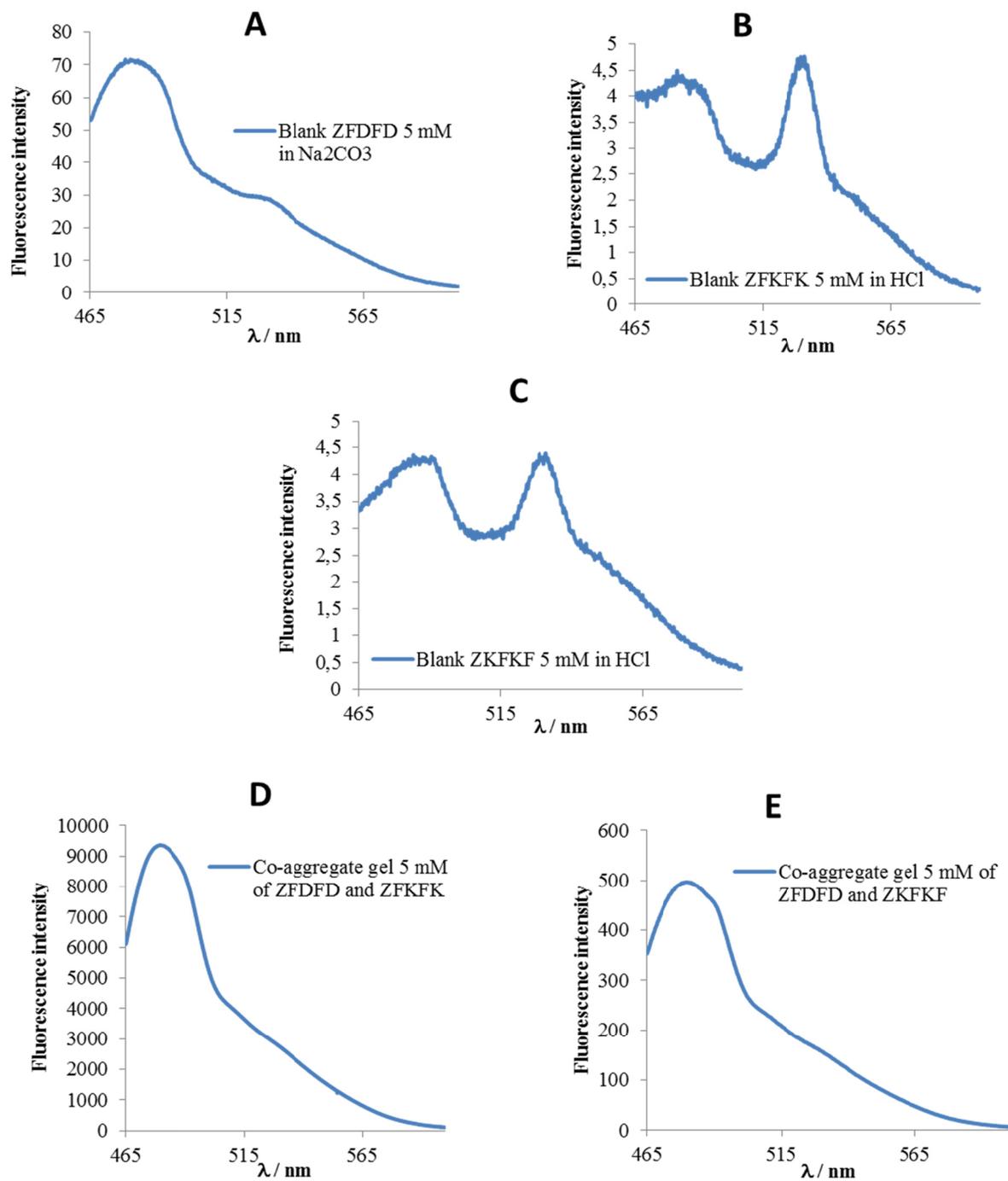


Figure S1. ThT binding assays of blank solutions of compounds **1** (A), **2** (B) and **3** (C) and co-aggregates **1:2**(D) and **1:3** (E).

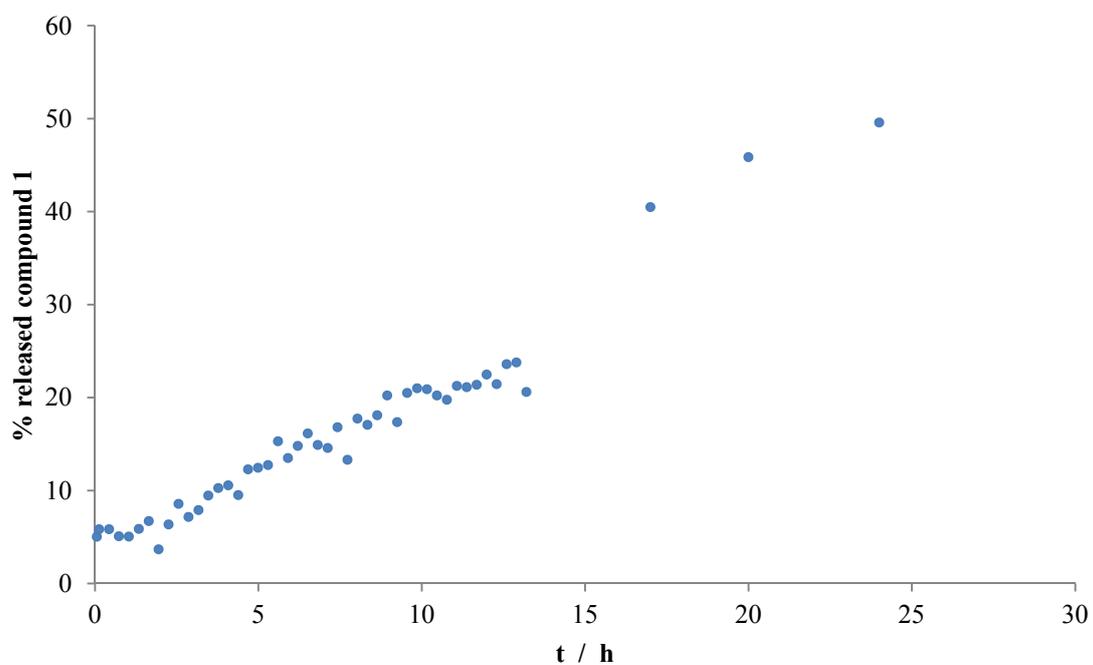


Figure S2. Kinetics of release of deprotonated compound **1** after the addition of  $\text{Na}_2\text{CO}_3$  to a two-component hydrogel **1:2** (5 mM) monitored by  $^1\text{H}$  NMR. The gel was formed inside the NMR tube at a total concentration of 5 mM. After 24 h of stabilization, 50  $\mu\text{L}$  of  $\text{Na}_2\text{CO}_3$  were added.

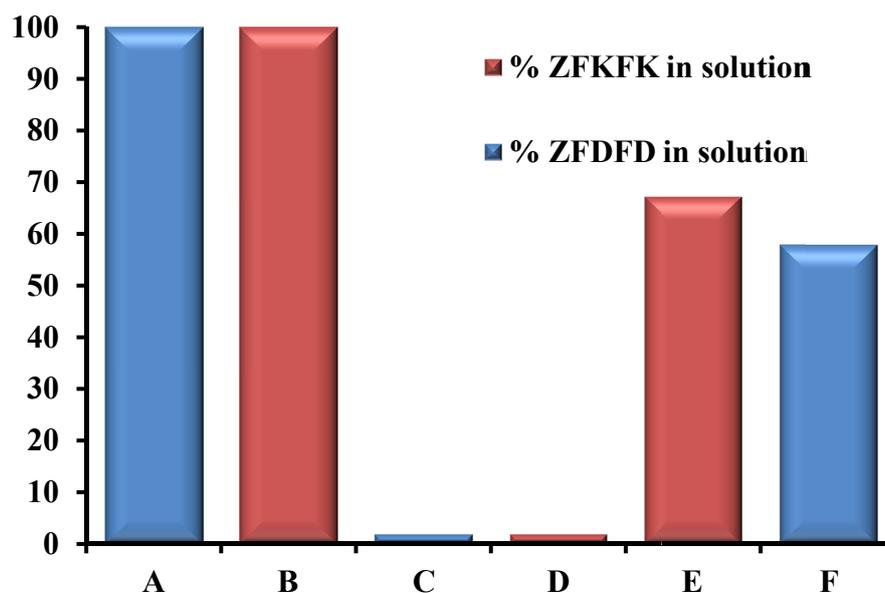


Figure S3. Amount of compounds **1 (ZFDFD)** and **2 (ZFKFK)** in solution under different experimental conditions (after 24 h, total concentration 1 mM). A) **1** in  $\text{Na}_2\text{CO}_3$ , B) **2** in HCl, C) **1** in HCl, D) **2** in  $\text{Na}_2\text{CO}_3$ , E) acidification of **1:2** co-aggregate and F) basification of **1:2** co-aggregate.

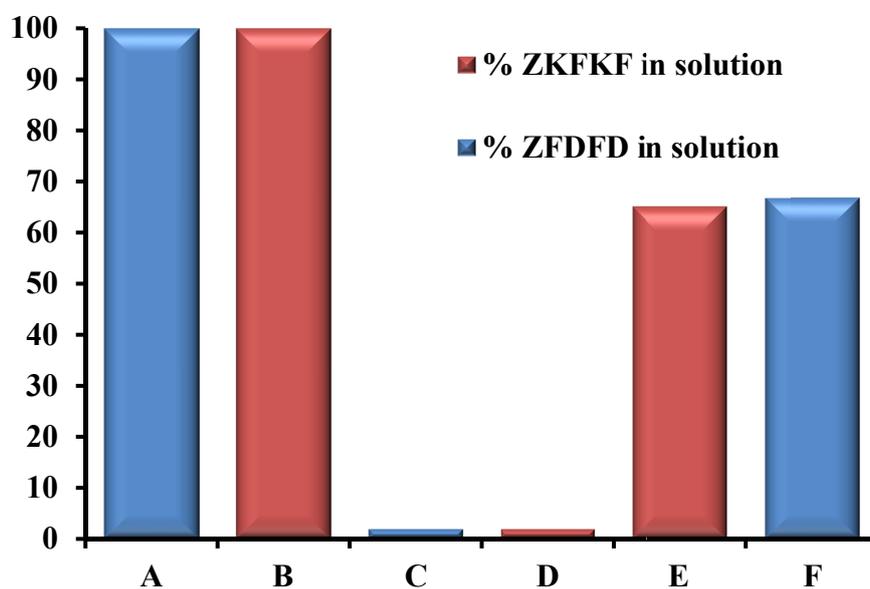


Figure S4. Amount of compounds **1 (ZFDFD)** and **3 (ZKFKF)** in solution under different experimental conditions (after 24 h, total concentration 1 mM). A) **1** in  $\text{Na}_2\text{CO}_3$ , B) **3** in HCl, C) **1** in HCl, D) **3** in  $\text{Na}_2\text{CO}_3$ , E) acidification of **1:3** co-aggregate and F) basification of **1:3** co-aggregate.

A)

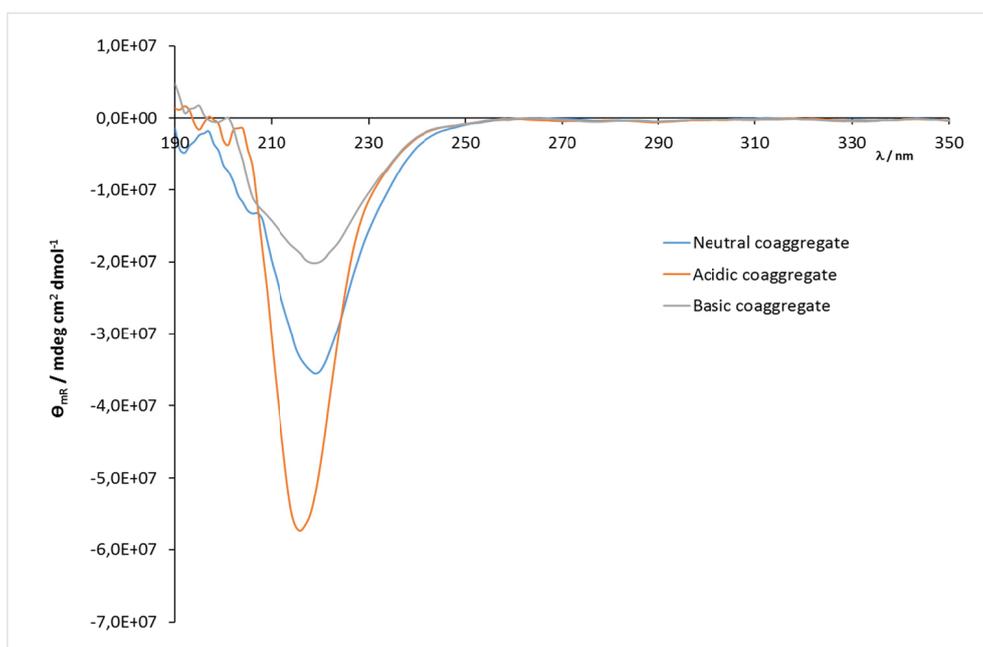


Figure S5. CD spectra of co-assembly 1:2 at different pH.

B)

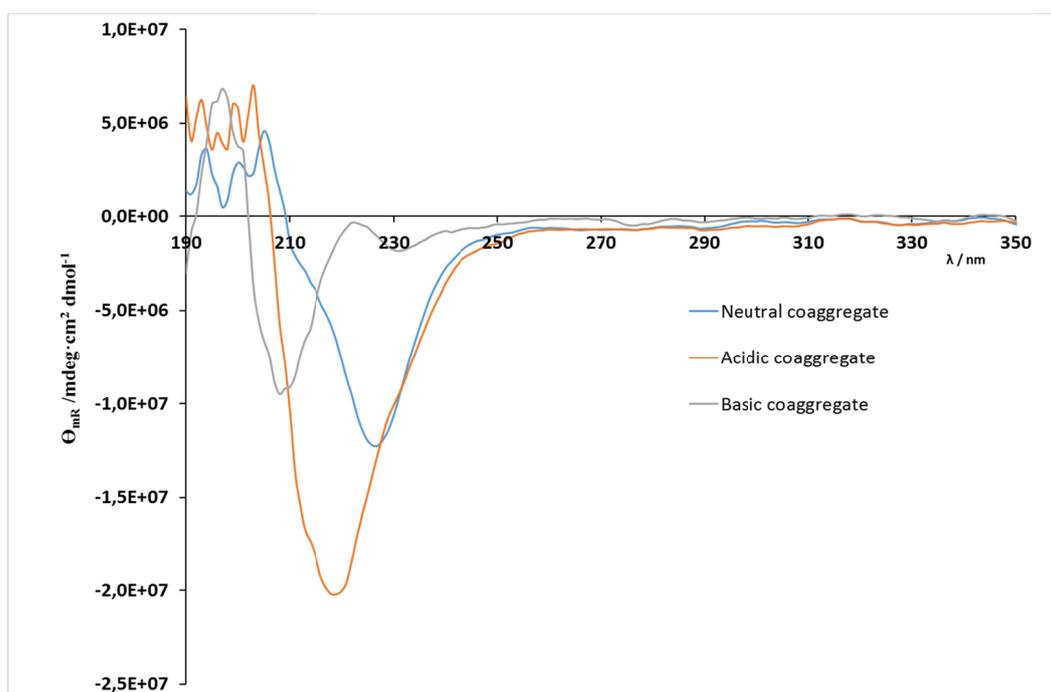
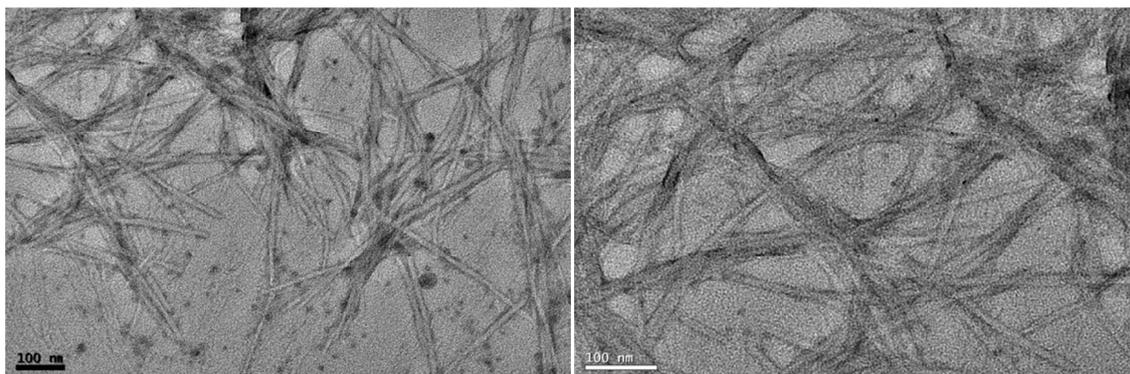
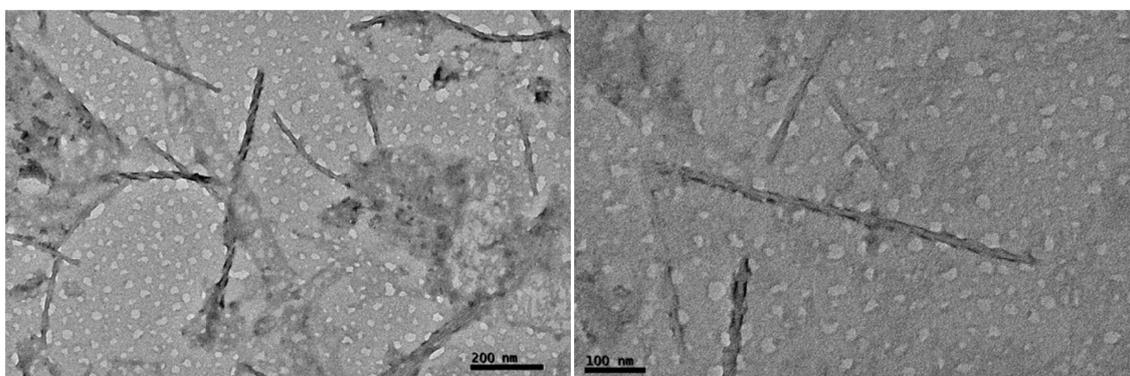


Figure S6. CD spectra of co-assembly 1:3 at different pH.

**A**



**B**



**C**

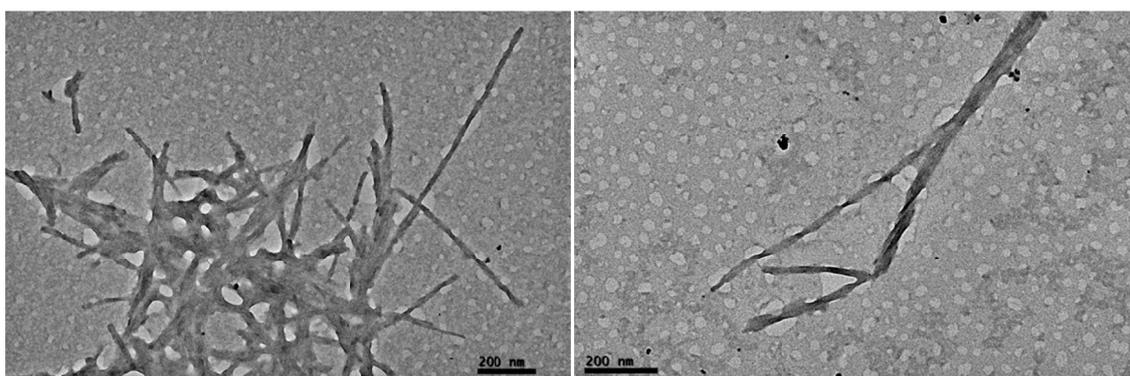


Figure S7. TEM images of A) acidic aggregates of **1** in HCl 10 mM, B) basic aggregates of **2** in Na<sub>2</sub>CO<sub>3</sub> 10 mM and C) basic aggregates of **3** in Na<sub>2</sub>CO<sub>3</sub> 10 mM.

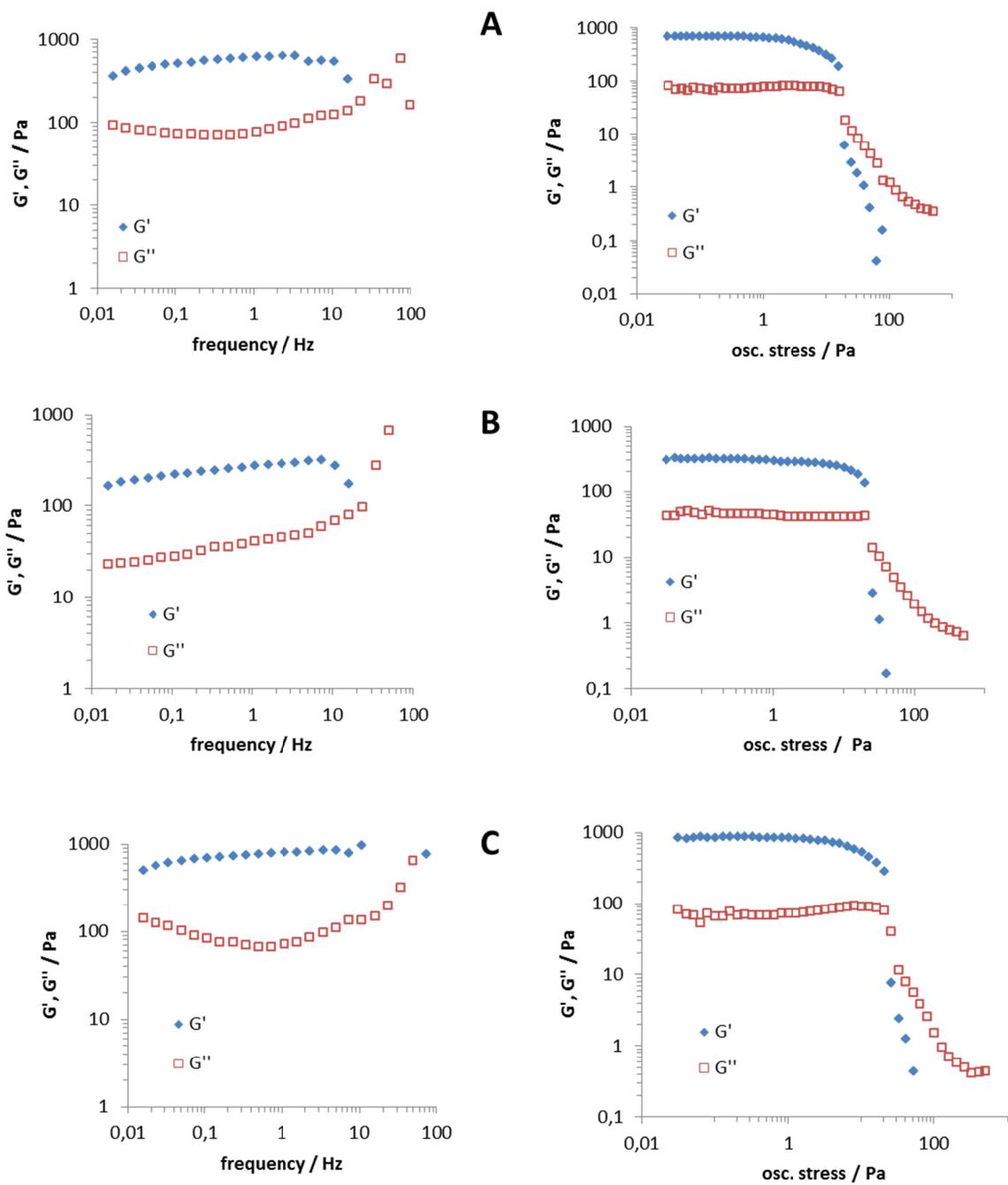


Figure S8. Frequency sweep at 1 Pa (left) and stress sweep at 1 Hz (right) for samples of co-aggregate 1:2 at A) neutral, B) acidic and C) basic pH.

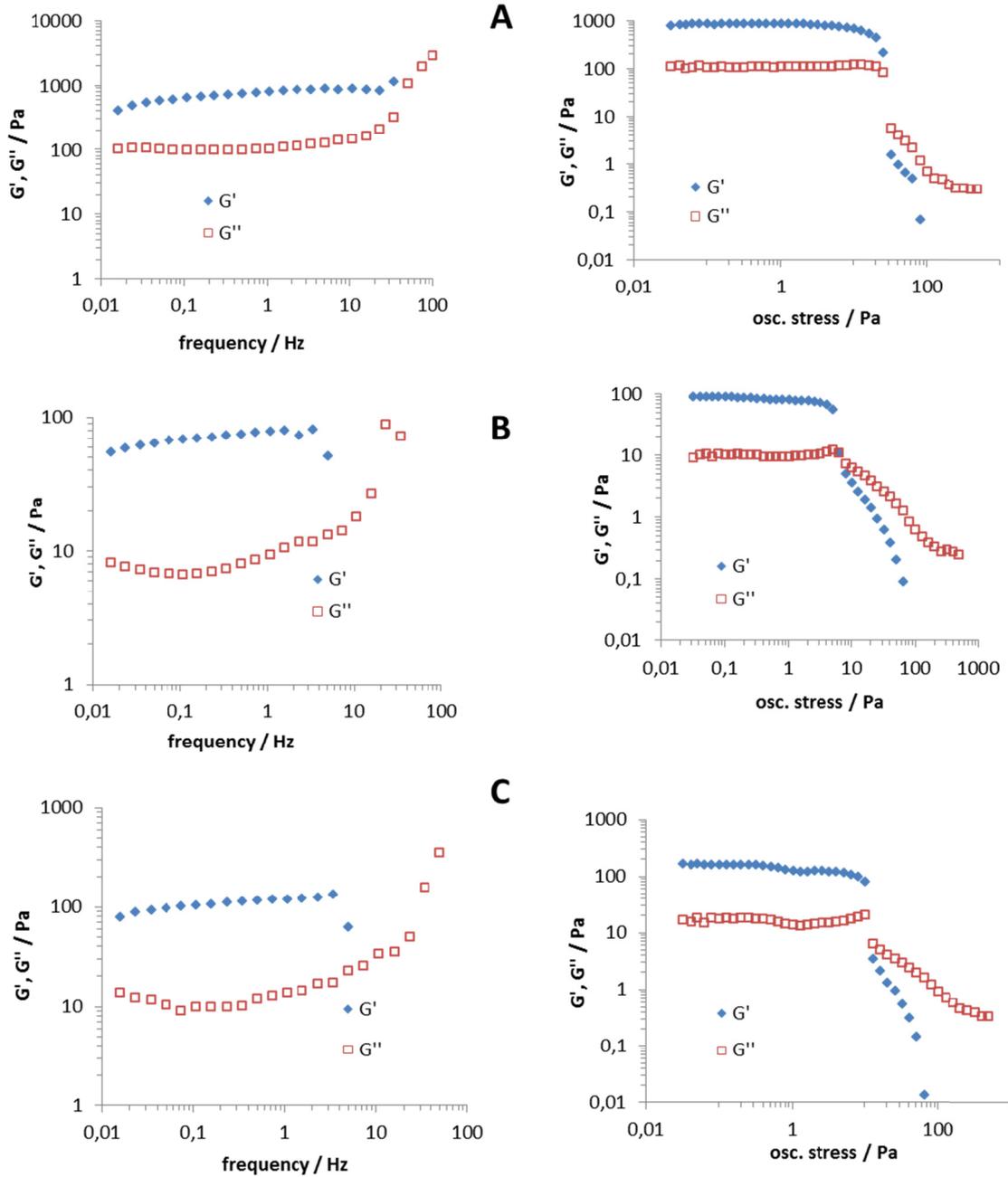


Figure S9. Frequency sweep at 1 Pa (left) and stress sweep at 1 Hz (right) for samples of co-aggregate **1:3** at A) neutral, B) acidic and C) basic pH.

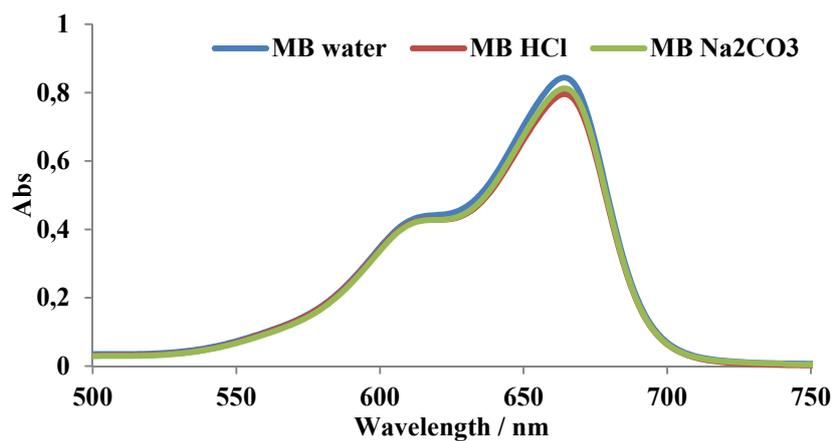


Figure S10. Absorbance spectra of blank solutions of methylene blue (25  $\mu\text{m}$ ) at different pH.

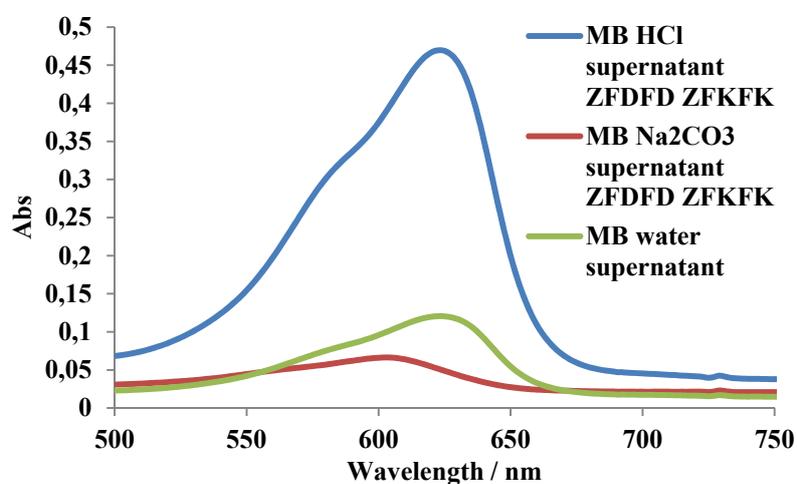


Figure S11. Absorbance spectra of supernatants of hydrogels 1:2 at different pH.

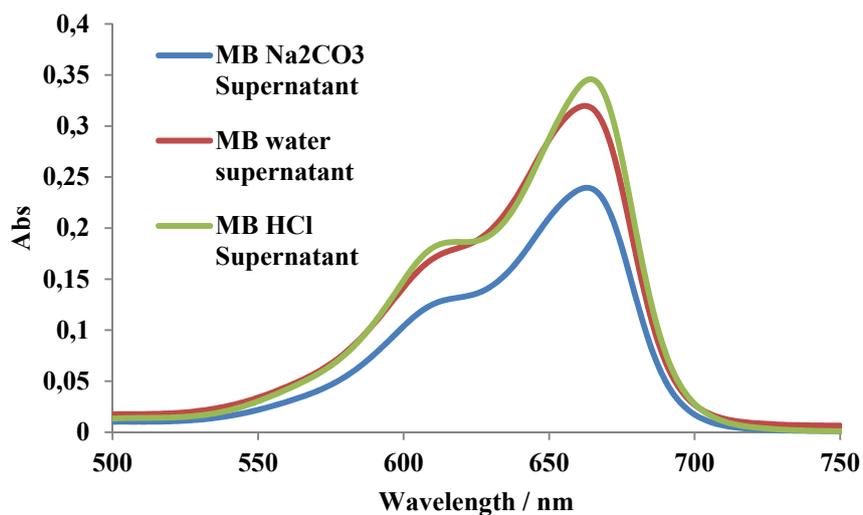


Figure S12. Absorbance spectra of supernatants of hydrogels 1:3 at different pH.

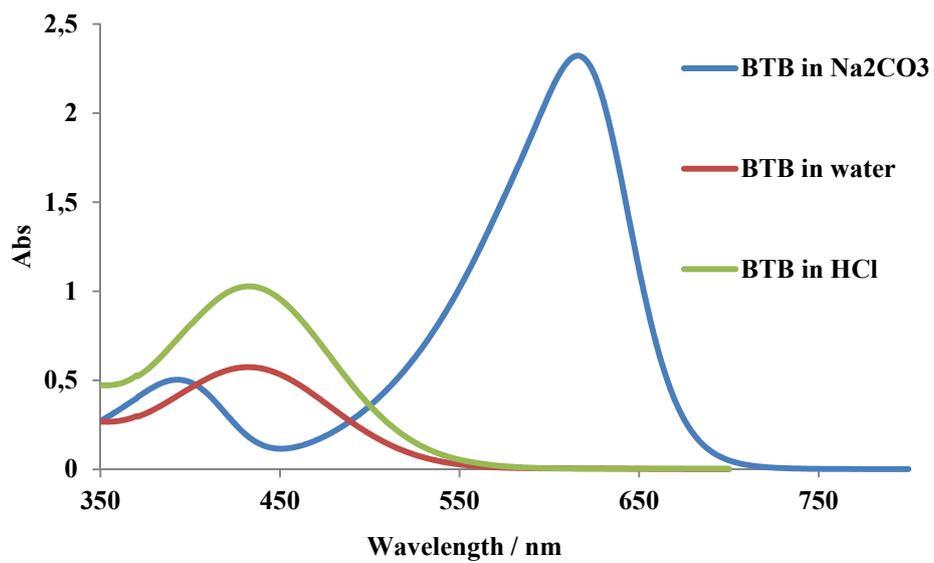


Figure S13. Absorbance spectra of blank solutions of bromothymol blue (64  $\mu\text{m}$ ) at different pH.