Supporting Information

Tripeptide self-assembled hydrogels: unexpected twists of chirality.

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Materials

Phe-Wang resin, Val-Wang resin, *O*-Benzotriazole-*N*,*N*,*N*,*N*'-tetramethyl-uronium-hexafluoro-phosphate (HBTU), and Fmoc protected L-valine and L-phenylalanine were purchased from GL Biochem (Shanghai) Ltd. Fmoc-protected D-valine and D-phenylalanine were purchased form Mimotopes (Australia). All solvents were purchased of analytical grade from Merck (Australia). Piperidine, trifluoroacetic acid (TFA), diisopropyl ethyl amine (DIPEA), triisopropyl silane (TIPS) were from Acros (Australia). Sodium dihydrogen phosphate and disodium hydrogen phosphate were from BDH AnalaR (Australia). High purity Milli-Q-water (MQ water) with a resistivity greater than 18 M Ω cm was obtained from an in-line

Millipore RiOs/Origin system. Silicon wafers (M.M.R.C Pty Ltd., Australia) were cleaned by ultrasonication (1 h) in a surfactant solution of 2% ethanol with 2% RBS 35 (Pierce, USA) followed by rinsing with copious amounts of MQ water and dried with nitrogen, then cleaned for 1h in a UV/ozone Procleaner™ (Bioforce Nanosciences). ^{1}H-NMR spectra were recorded at 400 MHz and ^{13}C-NMR spectra were recorded at 100 MHz on a Bruker Instrument Biospin Av400H. Chemical shifts are reported in ppm relative to TMS. Low resolution ESI-MS spectra were acquired with a Shimadzu LCMS-2010EV mass spectrometer using a cone voltage of 50V and the source was maintained at 80°C. The solvent used was methanol containing 0.1% formic acid with a flow rate of 0.1ml/min.

Peptide synthesis and purification

All the peptides were synthesized using standard Fmoc solid phase peptide synthesis with HBTU activation. Briefly, Fmoc-amino acid deprotection was performed in a sintered funnel, with occasional stirring, in 25% piperidine in *N*,*N*-dimethyl formamide (DMF) for 15-20 minutes until both ninhydrin and 2,4,6-trinitrobenzene sulfonic acid (TNBS) tests were positive. HBTU activation was performed with 3 eq. of Fmoc-amino acid and 2.5 eq. of HBTU in DMF (4 ml for every gram of resin), with DIPEA (1 ml of a 1M solution in DMF for every gram of resin). Coupling was performed at RT for 1 h in a sintered funnel with occasional stirring, and completeness was monitored by both ninhydrin and TNBS tests after thorough washes with DMF and DCM (dichloromethane). Final cleavage was obtained using a mixture of TFA/TIPS/water (95:2.5:2.5). Crude peptides were too hydrophobic to be precipitated in cold ether, thus the majority of TFA was evaporated under nitrogen flow, and the remaining oil was redissolved in a mixture of acetonitrile/water, and then freeze-dried overnight. The freeze-dried product was then purified by reverse-phase HPLC (Agilent Technologies). The HPLC was equipped with a preparative gradient pump (1100/1200), preparative C-18 column (Luna, 10 microns, 100 Å, 150 x 21.20 mm, Phenomenex), autosampler (G2260), Diode Array detector (G1365D). The gradient used consisted of acetonitrile (AcN) / water with 0.1% TFA with the following program: t = 0-3 min. 25% AcN; t = 18 min. 95% AcN; t = 18-21 min. 95% AcN (t_R= 10-11 min). Compounds were then freeze-dried and their purity verified by HPLC with the same equipment as described above, and the following gradient: t = 0-3 min. 25% AcN; t = 18 min. 50% AcN; t = 18-20 min 55% AcN, t = 22 min. 25% AcN (t_R shown in the chromatogram in the results section for each peptide). Peptide identity was verified by ESI-MS, ^{1}H-NMR and ^{13}C-NMR.

Sample preparation

Samples prepared with sonication: 4 mg of peptide were dissolved in 300 µL of 0.1M sodium phosphate, pH 12, sonicated for 5 min., then diluted 1:1 with another 300 µL of 0.1M sodium phosphate buffer, pH 5.7-6 to yield a final pH of 7.4 and sonicated for another 5 min. Samples prepared without sonication: the procedure is identical to the one described above, but without the use of sonication.

Circular Dichroism Spectroscopy

The secondary structure of the peptides was analysed using a 0.1 cm quartz cell on a Jasco J815 Spectropolarimeter, with 1s integrations, 1 accumulation and a step size of 1 nm with a band width of 1 nm over a range of wavelengths from 200 to 280 nm. Freshly prepared and sonicated peptide samples were immediately transferred into the quartz cell, and spectra were recorded after 15 min. Measurements were repeated at least 5 times, and to reduce the noise near 200 nm, their average is plotted.

Infrared Spectroscopy

IR spectra were collected on a Nicolet 6700 Fourier Transform Infrared Spectrometer in iTR mode. Freshly prepared sonicated peptide samples were left to settle and gel for 3 days in a glass vial. A small portion of the gel was then transferred on a clean piece of silicon wafer (1cm x 1cm), then gently spread over the surface by pressing a coverslip on top, and dried under vacuum for 24h. Dried samples on the silicon wafers were placed directly to the iTR crystal, facing down. Scans were between 1800 and 1500 cm^{-1} with 80 accumulations at a resolution of 4 cm^{-1}.

Rheometry

Dynamic time sweep rheological analysis was conducted on an Ares rheometer (TA Instruments, USA) with a 25 mm aluminium parallel plate geometry. A Peltier temperature controller was also connected to the rheometer to maintain a temperature of 25°C. Freshly prepared peptide sample solutions were immediately dispensed on the rheometer without prior sonication. Sonicated samples were dispensed with the aid of a spatula after the initial 5 minutes in the sonicator where gelation may already start to occur. A gap of 300 μm was set, a constant strain of 2% applied, and a frequency of 10 rad/s was used. Strain sweeps were recorded using a frequency of 10 rad/s. Likewise frequency sweeps were recorded using a controlled strain of 2%.

Atomic force Microscopy

An Asylum Research MFP-3D atomic force microscope (Santa Barbara, CA, USA) was used to measure surface topography in tapping mode with ultrasharp silicon nitride tips (NSC15 noncontact silicon cantilevers, MikroMasch, Spain). The tips used in this study had a typical force constant of 40 N/m and a resonant frequency of 320 kHz. Typical scan settings involved the use of an applied piezo deflection voltage of 0.6 – 0.7 V at a scan rate of 0.7 Hz. All images were processed (1st order flattening algorithm) and linescans generated using Igor Pro software. Freshly prepared peptide solutions without prior sonication ere spread onto the silicon wafer by gently pressing a glass coverslip on top. Samples were then dried in a vacuum oven at room temperature for 24 h. Alternatively, freshly prepared peptide samples were left to settle and gel for 3 days, after which the gel structure was disrupted by tapping against the glass vial, and ca. 30 μL were transferred onto a square piece of silicon wafer (1 cm x 1 cm) which was previously cleaned (as described above). The peptide mixture was spread onto the silicon wafer by gently pressing a glass coverslip on top. Samples were then dried in a vacuum oven at RT for 24 h.

Cryo-TEM

A laboratory-built humidity-controlled vitrification system was used to prepare the hydrogels for imaging in a thin layer of vitrified ice using cryo-TEM. Humidity was kept close to 80% for all experiments, and ambient temperature was 22°C. 200-mesh copper grids coated with perforated carbon film (Lacey carbon film: ProSciTech, Qld, Australia) were used for all experiments. Grids were glow discharged in nitrogen for 5 seconds immediately before use. Hydrogels were prepared as described above with sonication. After 3 days, the hydrogels were disrupted by tapping against the glass vial, and samples were agitated with the pipette tip in order to liquefy them if possible. Approximately 4 μl aliquots of sample were pipetted onto each grid prior to plunging. In the case of samples which could not be liquefied adequately, the gel was smeared gently onto the grid. After 30 seconds adsorption time the grid was blotted manually using Whatman 541 filter paper, for approximately 2 seconds. Blotting time was optimised for each sample. The grid was then plunged into liquid ethane cooled by liquid nitrogen. Frozen grids were stored in liquid nitrogen until required. The samples were examined

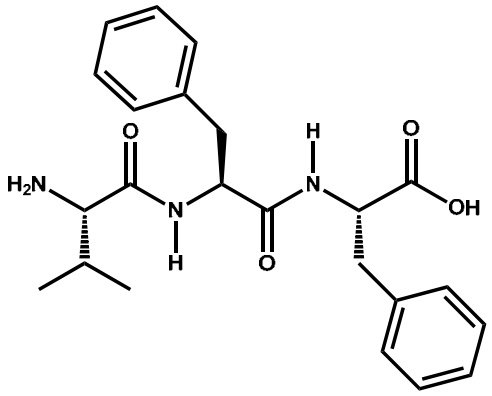
using a Gatan 626 cryoholder (Gatan, Pleasanton, CA, USA) and Tecnai 12 Transmission Electron Microscope (FEI, Eindhoven, The Netherlands) at an operating voltage of 120KV. At all times low dose procedures were followed, using an electron dose of 8-10 electrons/Å2 for all imaging. Images were recorded using a Megaview III CCD camera and AnalySIS camera control software (Olympus.) using magnifications in the range 40 000x to 110 000x.

Thioflavin T fluorescence

Gel precursor solutions were prepared as indicated above and 25 µl were immediately placed on wells of a "µ-Slide Angiogenesis" uncoated (Ibidi, Germany, through DKSH Australia). The solutions were left to gelate for 1 h. 25 µl of a solution of Thioflavin T (200 µM in 50 mM Glycine-NaOH pH 7.5, filtered) were placed on top. After 15 minutes the slides were imaged on a Leica SP5 microscope (63x water immersion objective, NA 1.2, 6x zoom, excitation 458 nm, emission 468-600 nm). One section of 0.5 microns thickness is reproduced for each sample. Autoquant X2 was used for single plane noise reduction.

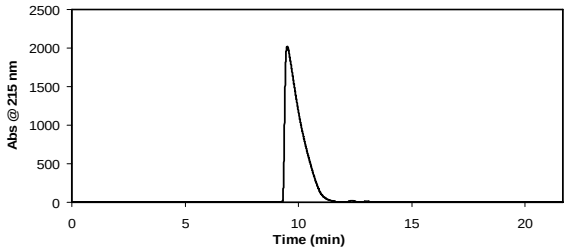
II – Results - Analytical Characterization of Peptides

a. L-Val-L-Phe-L-Phe

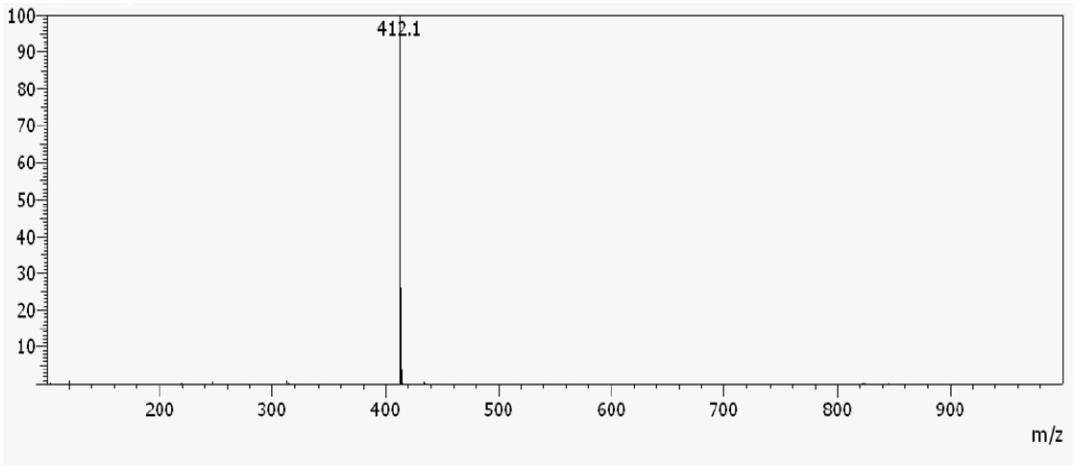


^{1}H-NMR (400 MHz, DMSO, TMS): δ 12.79 (1H, s (br), COOH), 8.50 (d, *J* = 8 Hz, 1H, NH), 8.48 (d, *J* = 8 Hz, 1H, NH) 7.96 (s (br), 3H, NH_3^+), 7.24-7.13 (m, 10H, Ar), 4.61 (ddd, *J* = 4 Hz, 8 Hz, 8 Hz, 1H, αCH), 4.44 (ddd, *J* = 4 Hz, 8 Hz, 8 Hz, 1H, αCH), 3.54 (m, 1H, αCH), 3.04 (dd, *J* = 6 Hz, *Jgem* = - 14 Hz, 1H, βCH_2), 2.96 (dd, *J* = 6 Hz, *Jgem* = - 14 Hz, 1H, βCH_2), 2.87 (dd, *J* = 8 Hz, *Jgem* = - 12 Hz, 1H, βCH_2), 2.75 (dd, *J* = 8 Hz, *Jgem* = - 12 Hz, 1H, βCH_2), 2.01 (m, 1H, βCH), 0.83 (d, J = 4 Hz, 3H, γCH_3), 0.78 (d, J = 4 Hz, 3H, γCH_3). ^{13}C-NMR (100 MHz, DMSO, TMS): δ (ppm) 173.5, 171.6, 168.7 (3 x CO); 138.3, 130.1, 129.9, 129.1, 129.3 (Ar); 58.0, 54.8, 54.2 (3 x αC); 38.3, 37.6 (2 x βCH_2); 30.8 (βCH); 19.3, 18.0 (2 x γCH_3). ESI-MS: m/z 412.1 $(M+H)^+$ $C_{23}H_{29}N_3O_4$ requires 412.2.

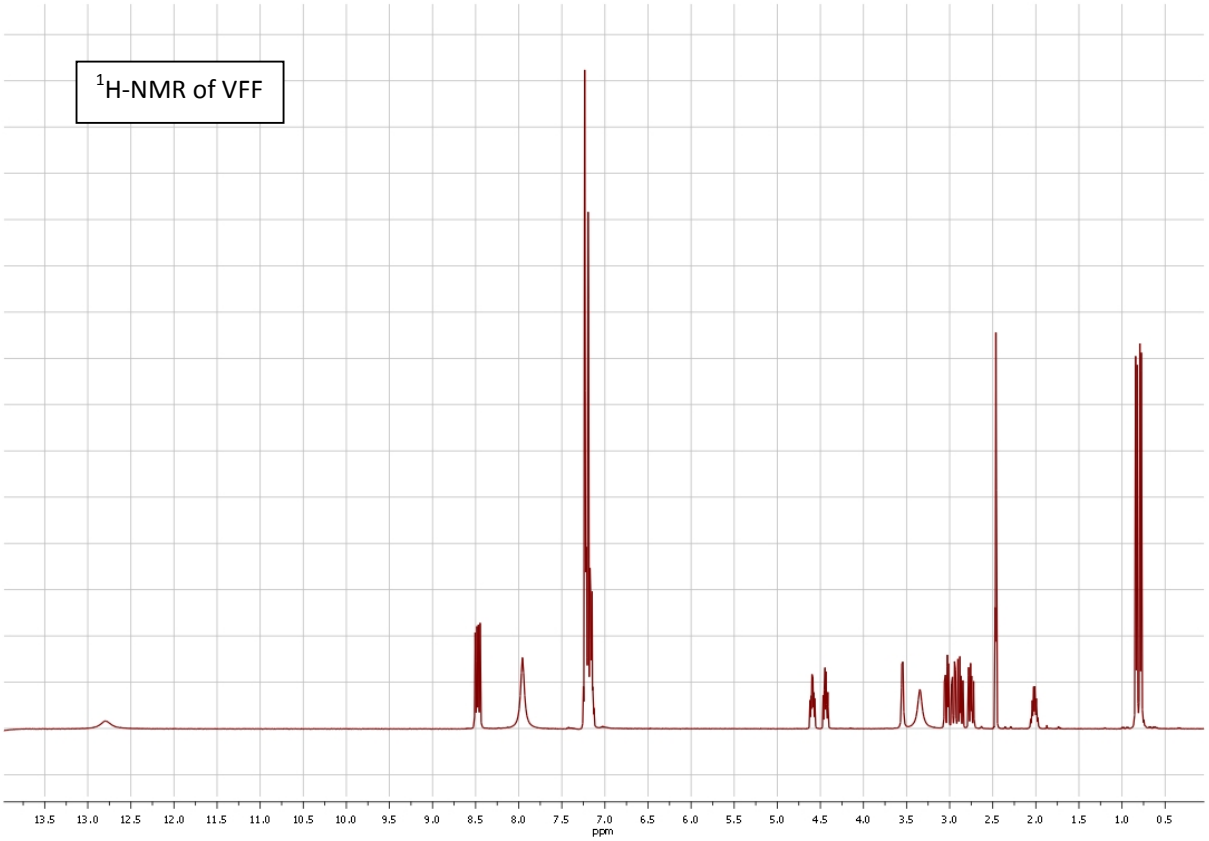
HPLC



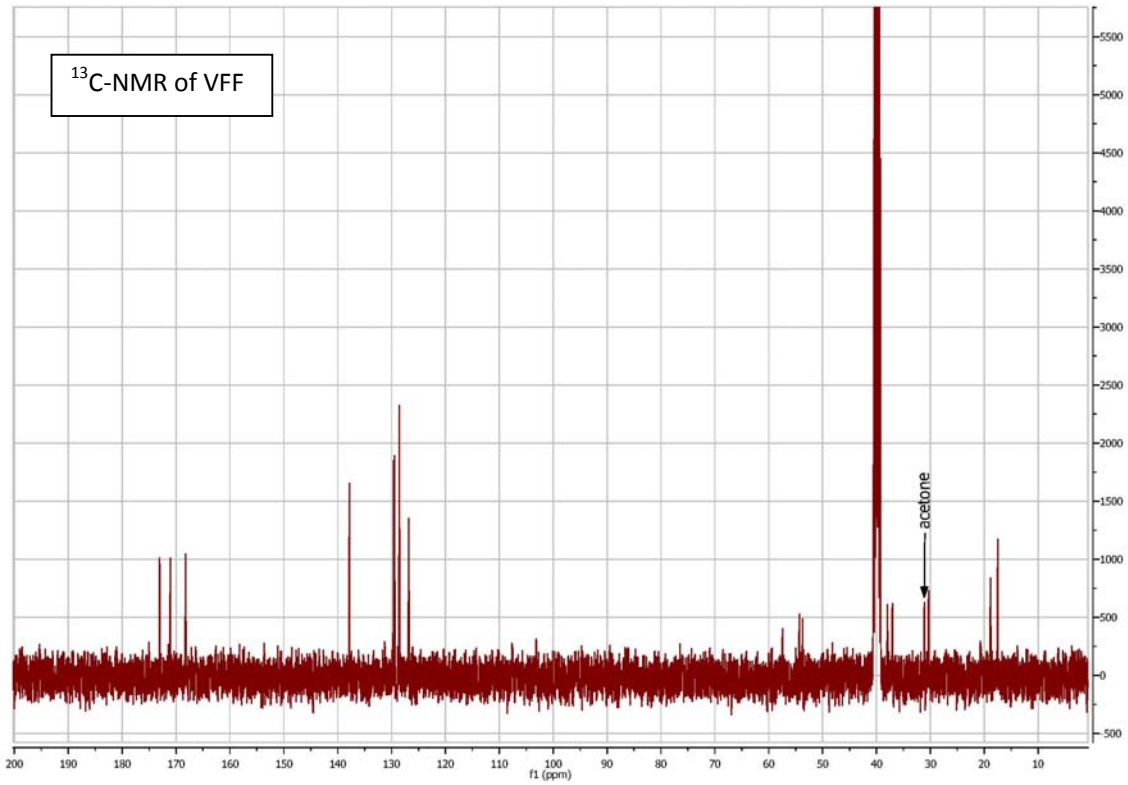
ESI-MS



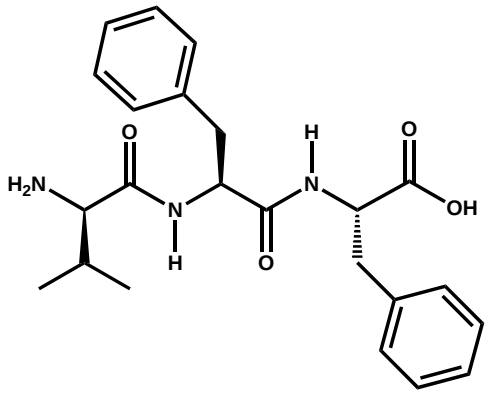
^{1}H-NMR of VFF



^{13}C-NMR of VFF

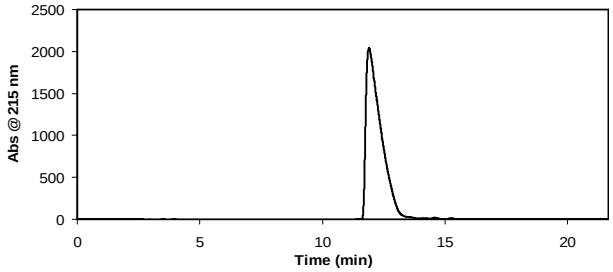


b. D-Val-L-Phe-L-Phe

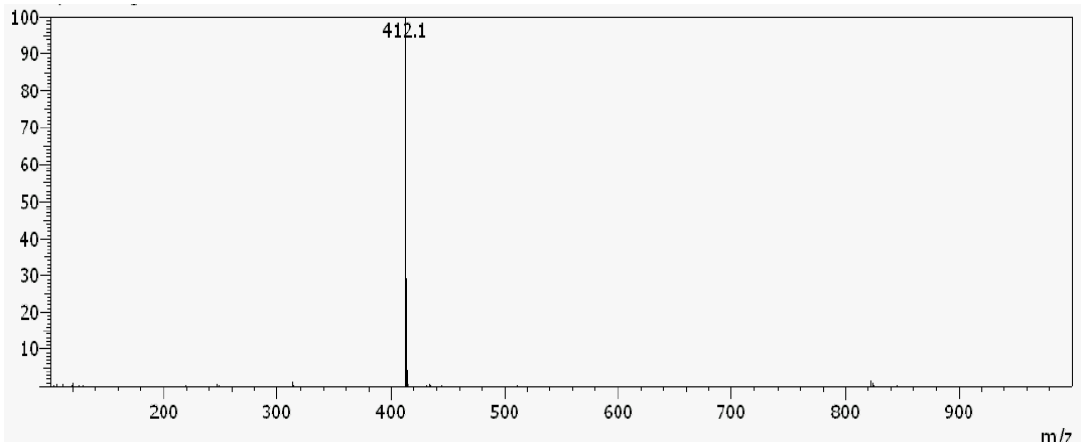


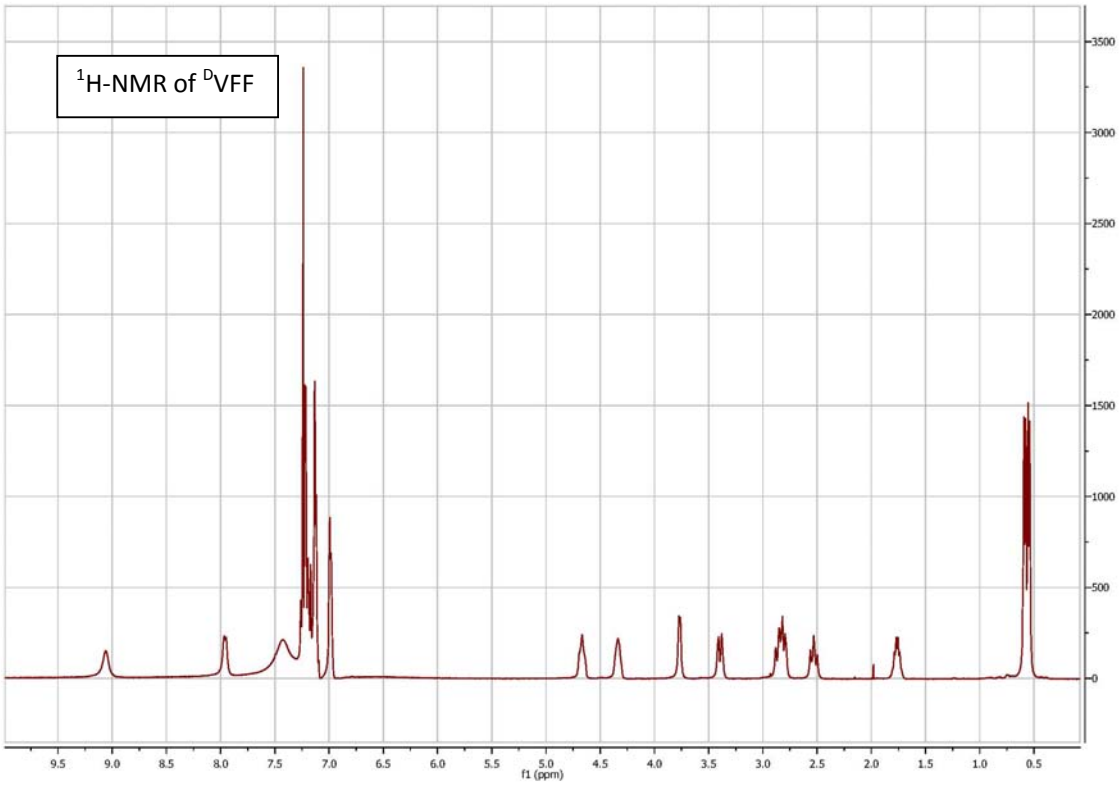
^{1}H-NMR (400 MHz, $CDCl_3$, TMS): δ (ppm) 9.08 (s (br), 1H, NH), 7.99 (d, 1H, NH) 7.4 (s (br), 3H, NH3+), 7.28-7.01(m, 10H, Ar), 4.69 (m, 1H, αCH), 4.36 (m, 1H, αCH), 3.79 (m, 1H, αCH), 3.42 (dd, *Jgem* = -12 Hz, 1H, βCH_2), 2.87 (dd, *Jgem* = -12 Hz, 1H, βCH_2), 2.83 (dd, *J* = 8 Hz,, *Jgem* = -8 Hz, 2H, βCH_2), 2.55 (dd, *J* = 8 Hz, *Jgem* = - 8 Hz, 1H, βCH_2), 1.77 (m, 1H, βCH), 0.60 (d, *J* = 6 Hz, 3H, γCH_3), 0.58 (d, *J* = 6 Hz, 3H, γCH_3) cxc). ^{13}C-NMR (100MHz, DMSO 40.45, TMS): δ (ppm) 173.7, 172.2, 168.5 (3 x CO); 138.5, 138.3, 130.1, 129.2, 129.0, 127.4, 127.3, (Ar); 58.2, 54.8, 54.6, (3 x αC); 39.1, 37.5 (2 x βCH_2); 30.5 (βCH); 19.1, 17.3 (2 x γCH_3). ESI-MS: m/z 412.1 $(M+H)^+$ $C_{23}H_{29}N_3O_4$ requires 412.2.

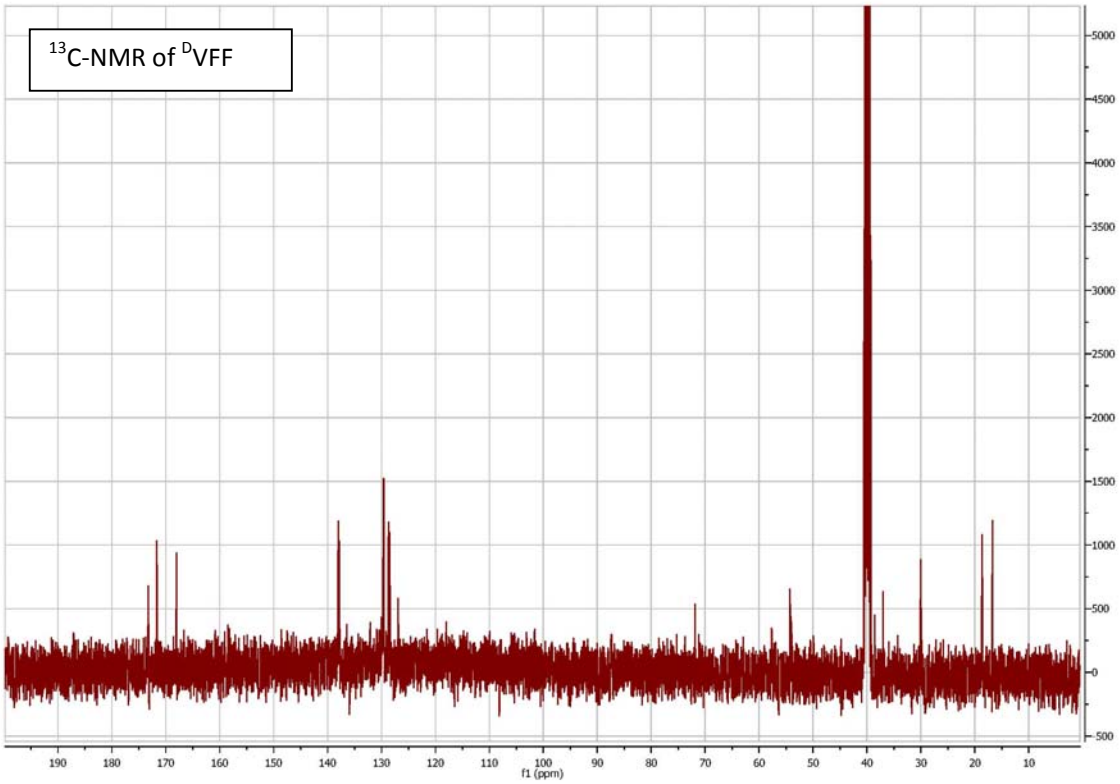
HPLC



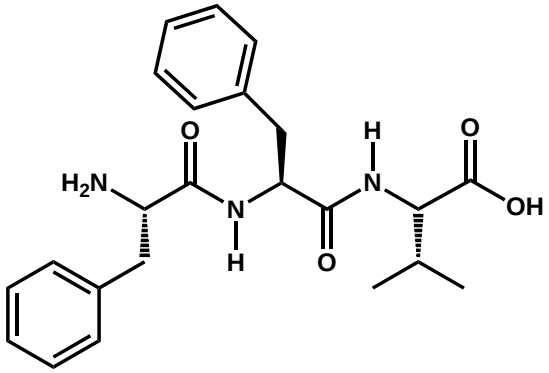
ESI-MS





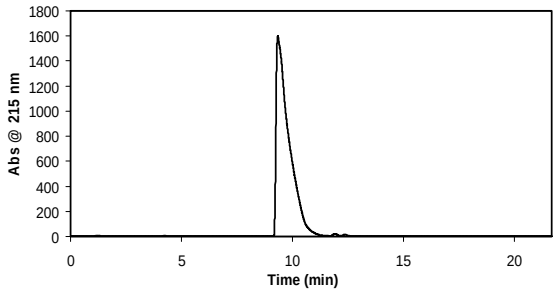


c. L-Phe-L-Phe-L-Val

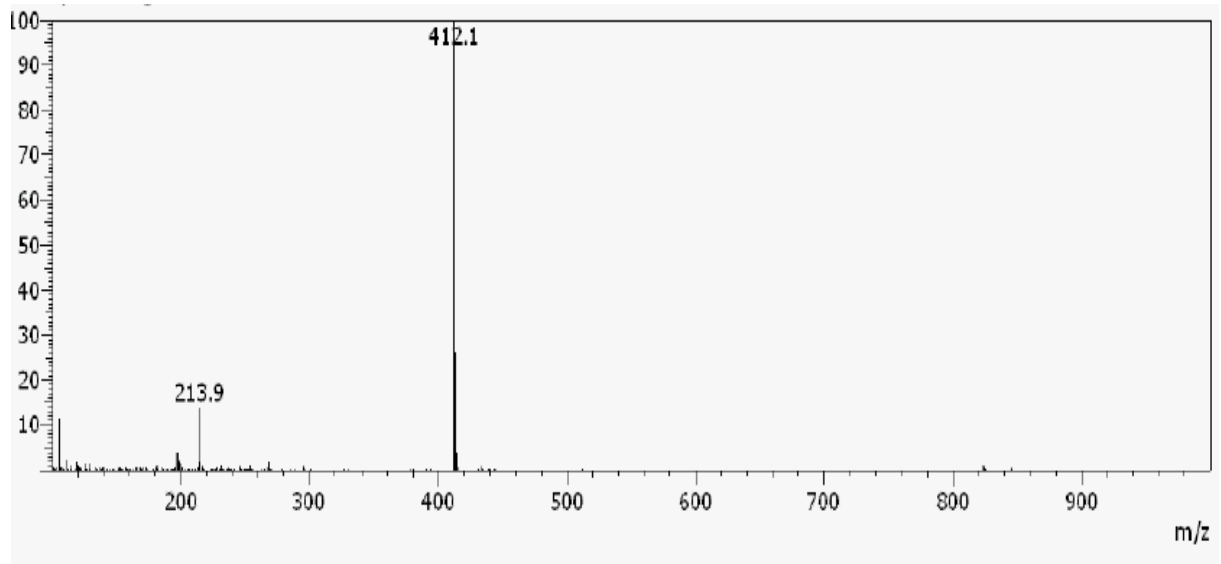


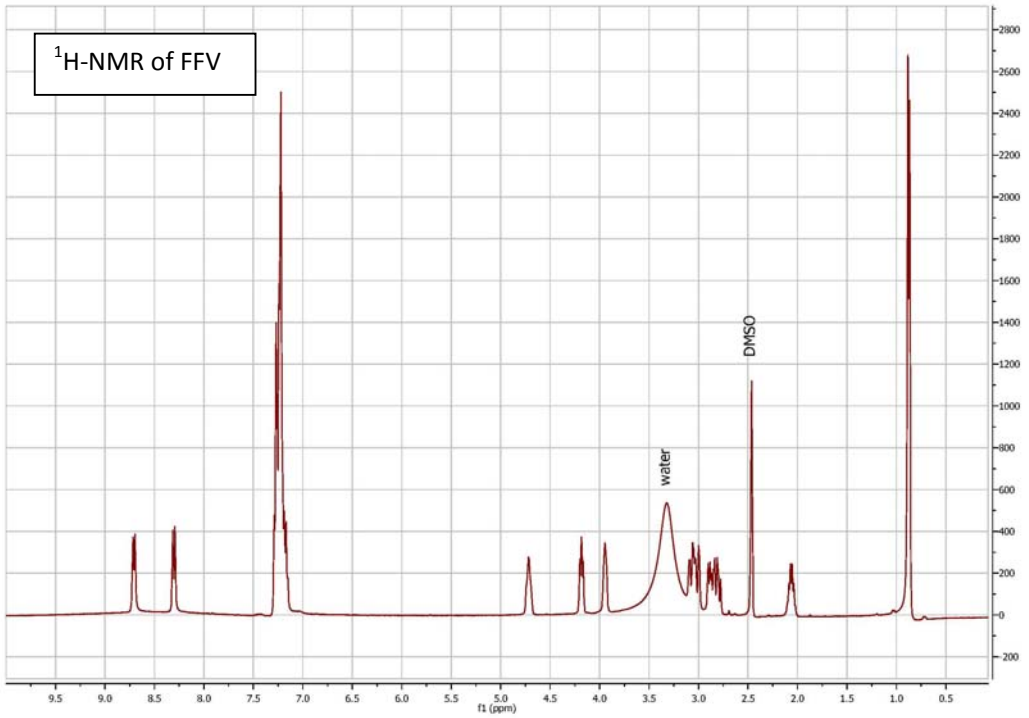
^{1}H-NMR (400 MHz, DMSO, TMS): δ 8.79 (d, *J* = 8 Hz, 1H, NH), 8.38 (d, *J* = 8 Hz, 1H, NH), 7.35-7.25 (m, 10H, Ar), 4.80 (m, 1H, αCH), 4.26 (m, 1H, αCH), 4.03 (m, 1H, αCH), 3.18-2.88 (m, 4H, 2 x βCH_2), 2.13 (m, 1H, βCH), 0.95 (d, *J* = 4 Hz, 3H, γCH_3), 0.95 (d, *J* = 4 Hz, 3H, γCH_3). ^{13}C-NMR (100MHz, DMSO): δ (ppm) 173.7, 171.9, 169.1 (3 x CO); 138.4, 135.7, 130.6, 135.7, 130.6, 130.2, 129.4, 129.0, 128.0, 127.4, 127.3, (Ar); 58.1, 54.7, 54.0, (3 x αC); 38.5, 37.9 (2 x βCH_2); 30.9 (βCH); 20.0, 18.9 (2 x γCH_3). ESI-MS: m/z 412.1 $(M+H)^+$ $C_{23}H_{29}N_3O_4$ requires 412.2.

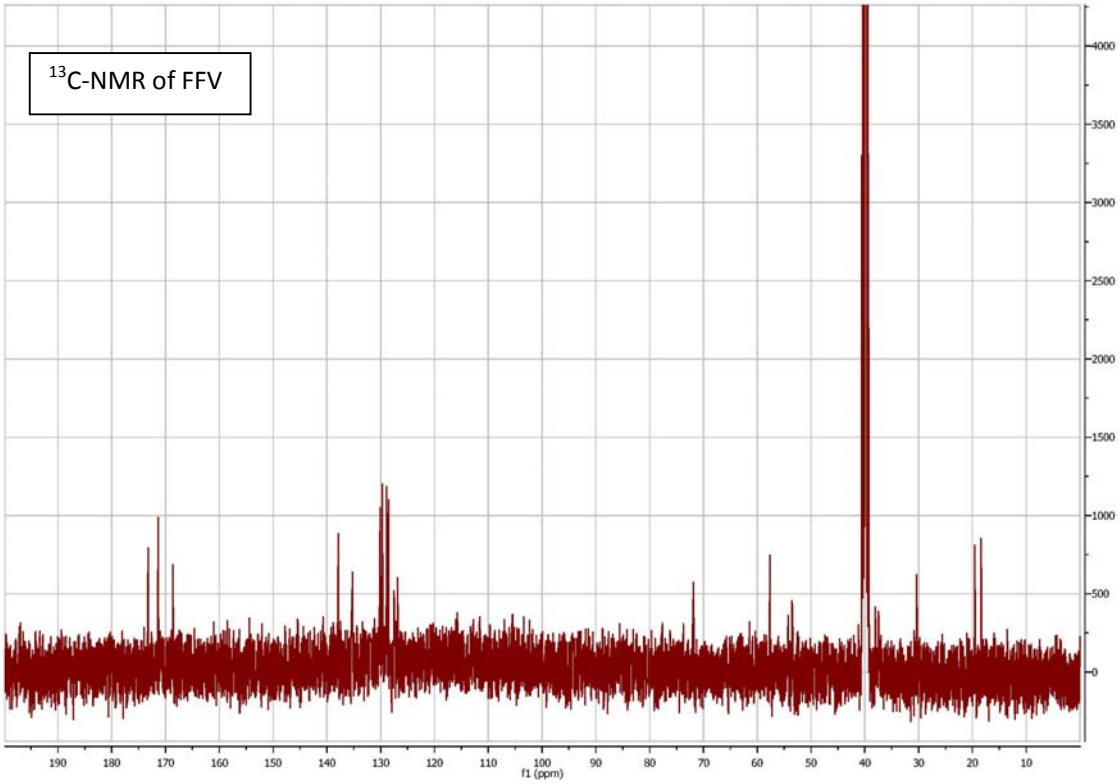
HPLC



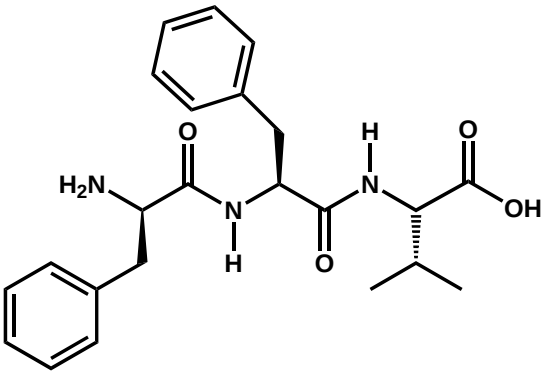
ESI-MS




^{1}H-NMR of FFV
water
DMSO
f1 (ppm)

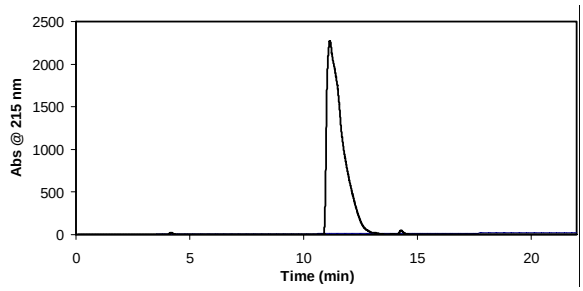

^{13}C-NMR of FFV
f1 (ppm)

d. D-Phe-L-Phe-L-Val

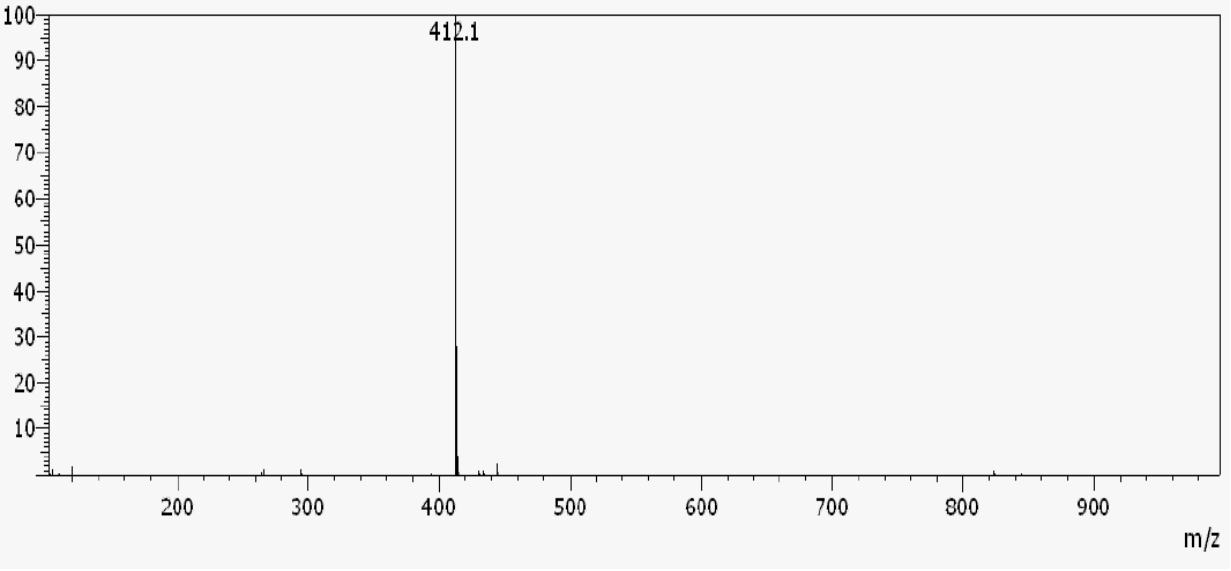


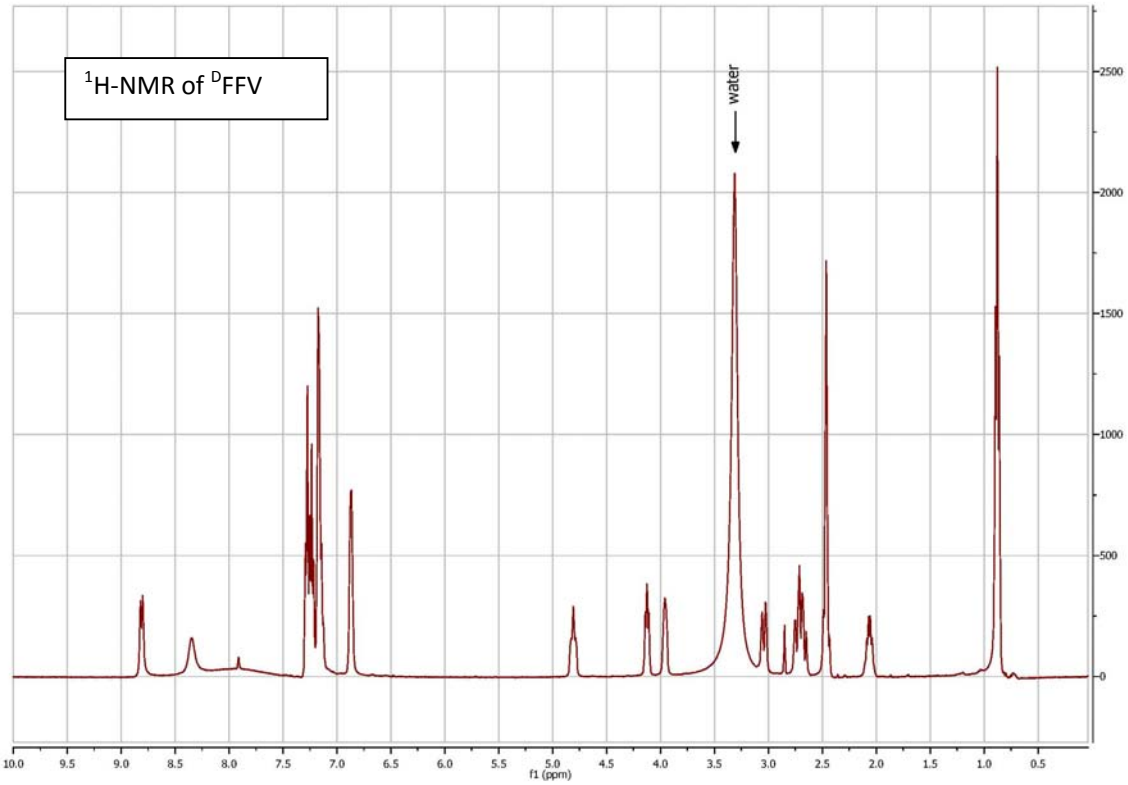
^{1}H-NMR (400 MHz, DMSO, TMS): δ 8.88 (d, J = 8 Hz, 1H, NH), 8.43 (s (br), 1H, NH), 7.35-6.95(m, 10H, Ar), 4.89 (m, 1H, αCH), 4.21 (m, 1H, αCH), 4.04 (m, 1H, αCH), 3.14-2.73 (m, 4H, 2 x βCH$_2$), 2.14 (m, 1H, βCH), 0.95 (d, *J* = 8 Hz, 3H, γCH$_3$), 0.95 (d, *J* = 8 Hz, 3H, γCH$_3$). ^{13}C-NMR (100MHz, DMSO, TMS): δ (ppm) 173.2, 172.4, 168.1 (3 x CO); 136.7, 133.8, 128.9, 128.6, 128.2, 127.4, 126.6 (Ar); 57.9, 54.6, 54.0 (3 x αC); 37.7, 37.0 (2 x βCH$_2$); 30.3 (βCH); 18.2, 17.0 (2 x γCH$_3$). ESI-MS: m/z 412.1 $(M+H)^+$ $C_{23}H_{29}N_3O_4$ requires 412.2.

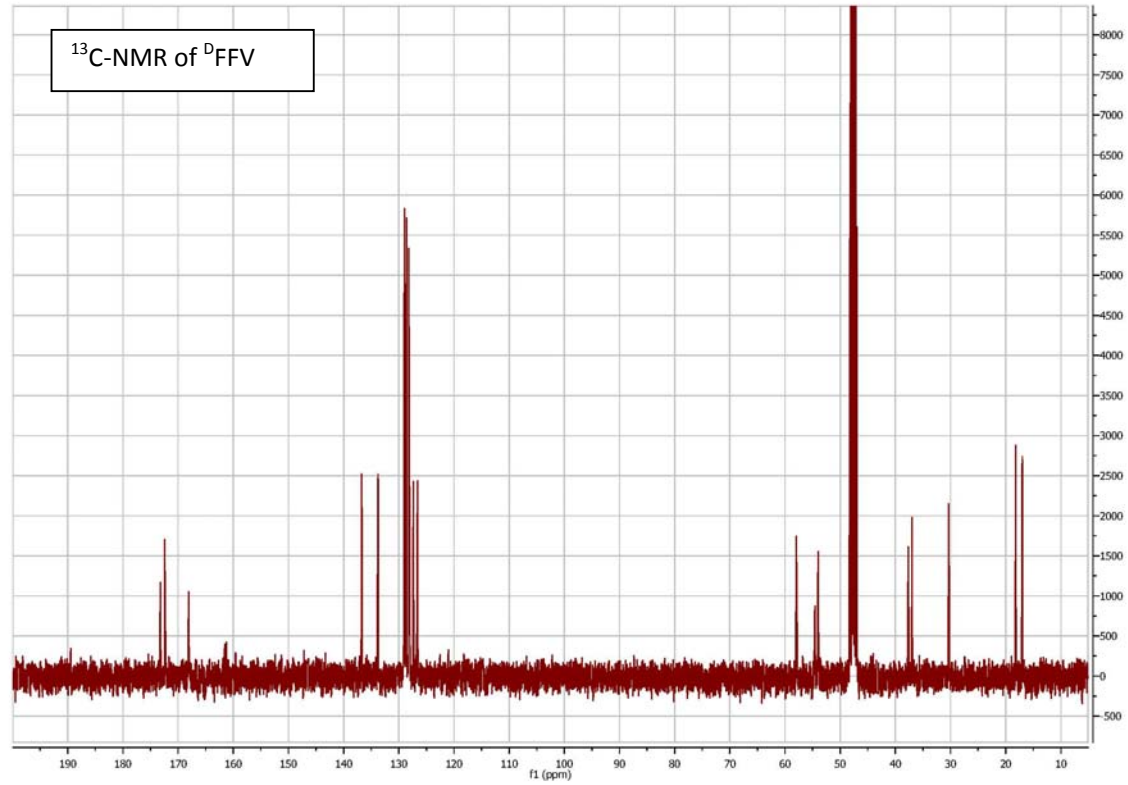
HPLC



ESI-MS



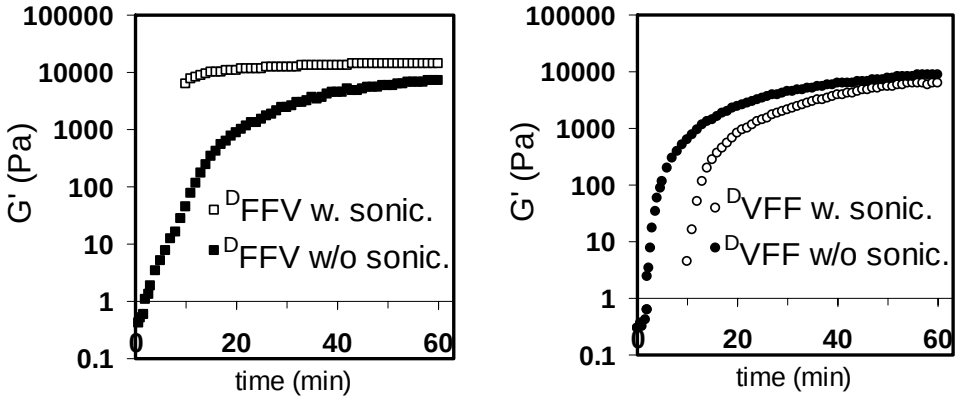

^{1}H-NMR of ^{D}FFV
water
f1 (ppm)


^{13}C-NMR of ^{D}FFV
f1 (ppm)

III- Results- Rheometry

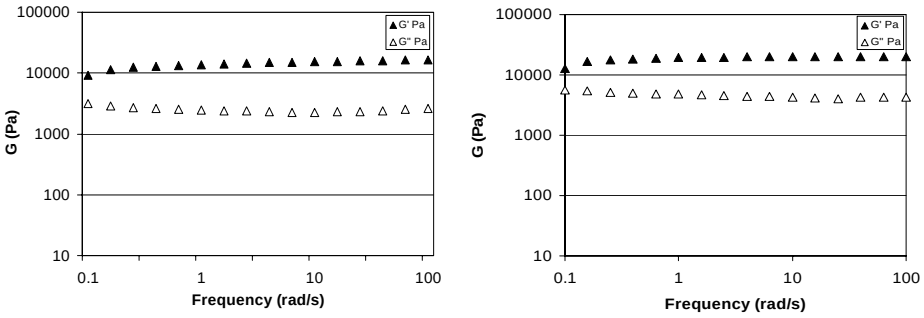
Rheometrical measurements for the samples containing the L-analogues VFF and FFV recorded only noise for both G' and G" and therefore the corresponding graphs are omitted.

Dynamic time sweep rheology data (2% strain, 10 rad/s frequency) for ^{D}FFV with (□) and without (■) sonication (left) and ^{D}VFF with (○) and without (●) sonication (right) during the first hour after preparing the sample are shown below. The initial time data points for the sonicated samples are missing as the samples were in the sonicator for the first 5 min., and then were transferred to the rheometer. It is interesting to note that for sample ^{D}FFV sonication visibly accelerates gelation. In contrast, gelation of ^{D}VFF is delayed by sonication, but only of a few minutes.

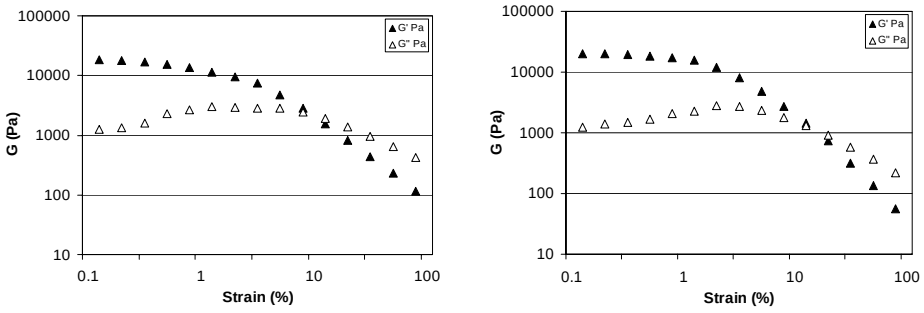


Dynamic frequency and strain sweeps after 24 h show that sonication determines a slight increase in G' for both samples (see below). This increase in G' becomes more evident after 3 days only for ^{D}FFV as seen by the strain sweep (below). Moreover, it appears that sonication has also a minor effect on the crossover point between G' and G" in the strain sweep experiments; this effect becomes apparent only after the first 24h. In particular, sonicated ^{D}FFV samples display a crossover at higher strain values than non-sonicated samples. The opposite is true for ^{D}VFF.

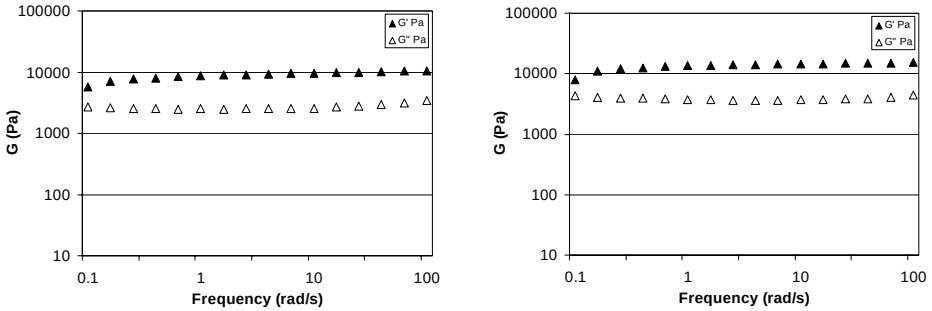
Dynamic frequency sweep rheology data (2% strain) for ^{D}FFV without (left) or with (right) sonication after 24h.



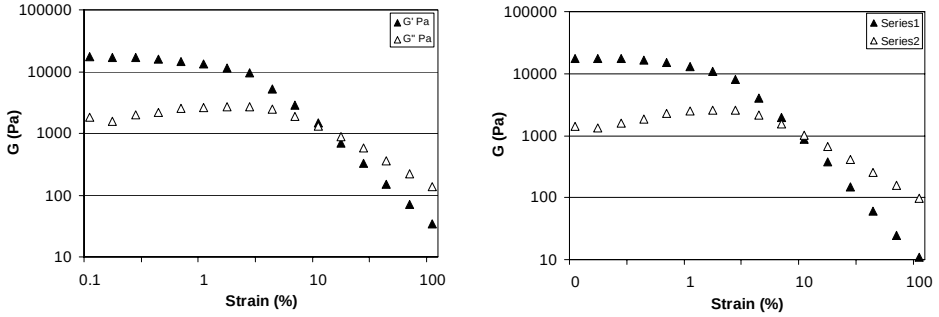
Dynamic strain sweep rheology data (10rad/s frequency) for ^{D}FFV without (left) or with (right) sonication after 24h.



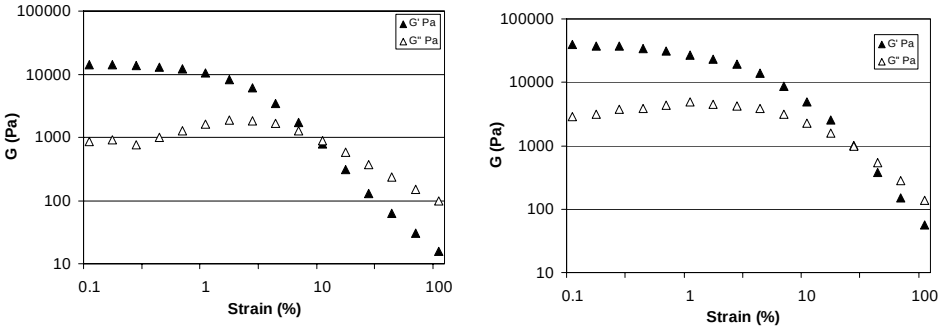
Dynamic frequency sweep rheology data (2% strain) for ^{D}VFF without (left) or with (right) sonication after 24h.



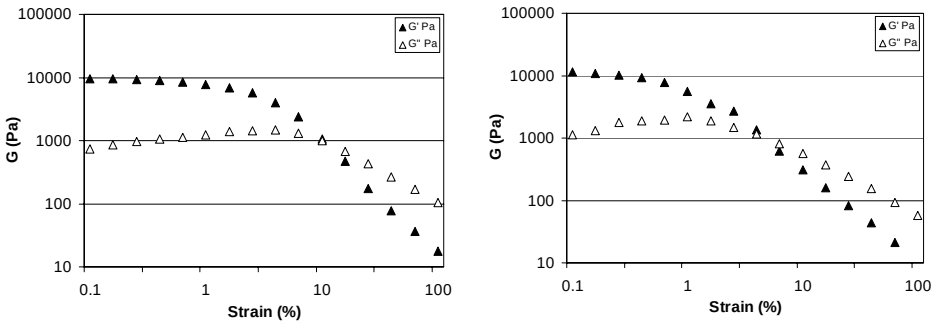
Dynamic strain sweep rheology data (10rad/s frequency) for ^{D}VFF without (left) or with (right) sonication after 24h.



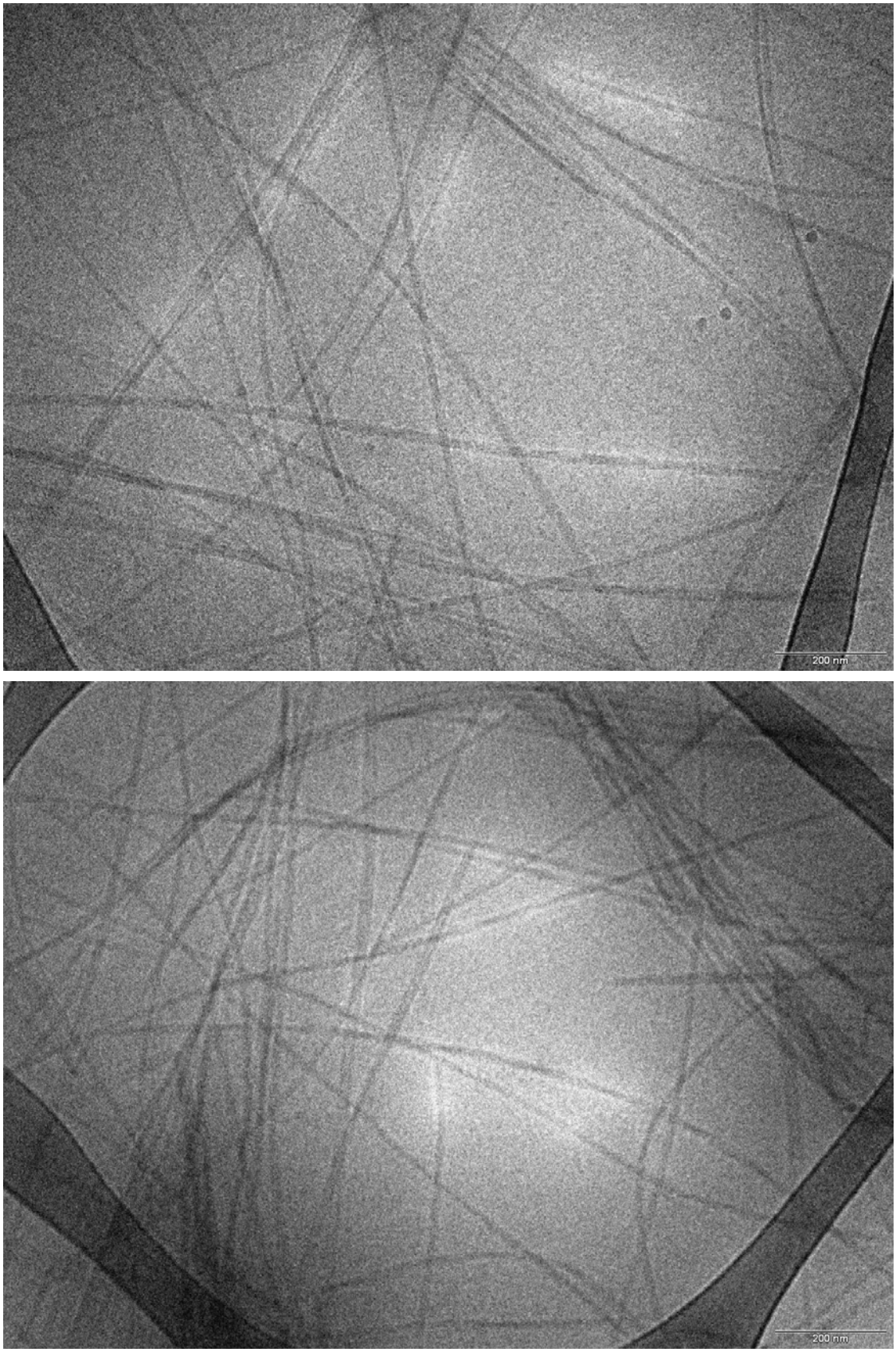
Dynamic strain sweep rheology data (10rad/s frequency) for ^{D}FFV without (left) or with (right) sonication after 3dd.



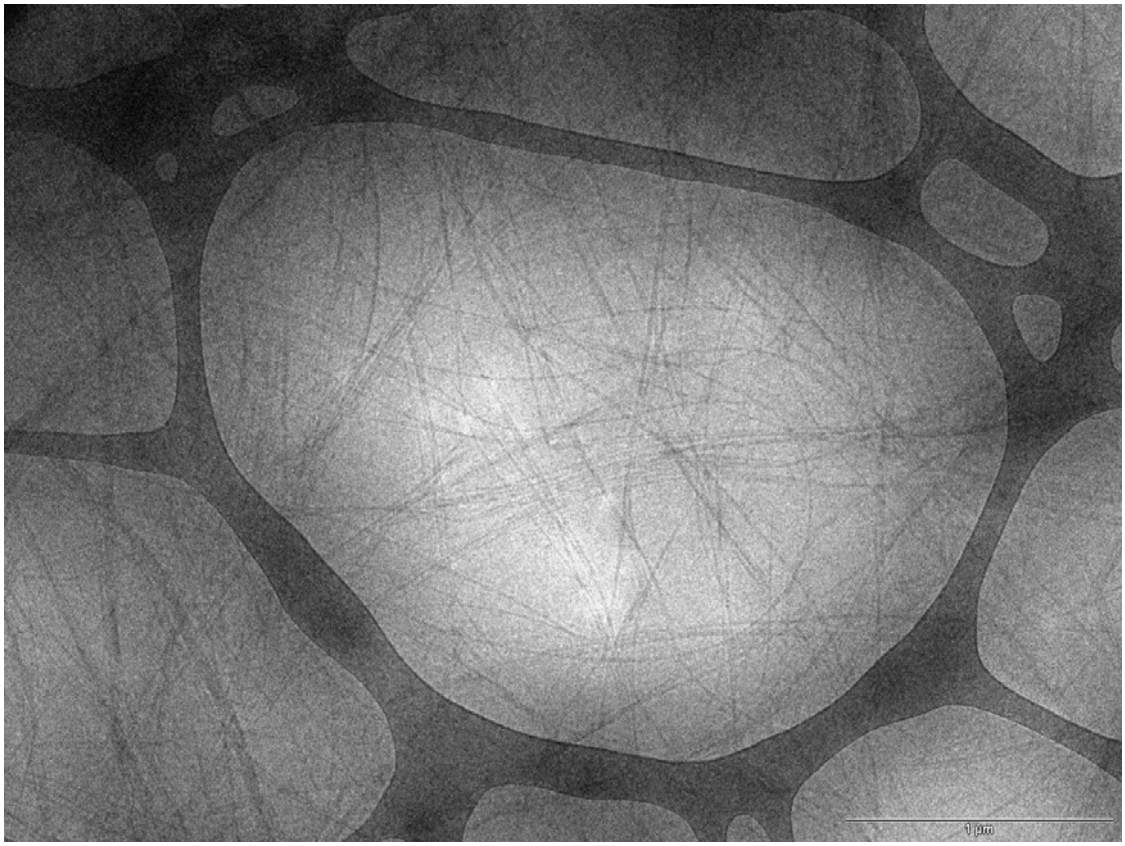
Dynamic strain sweep rheology data (10rad/s frequency) for ^{D}VFF without (left) or with (right) sonication after 3dd.



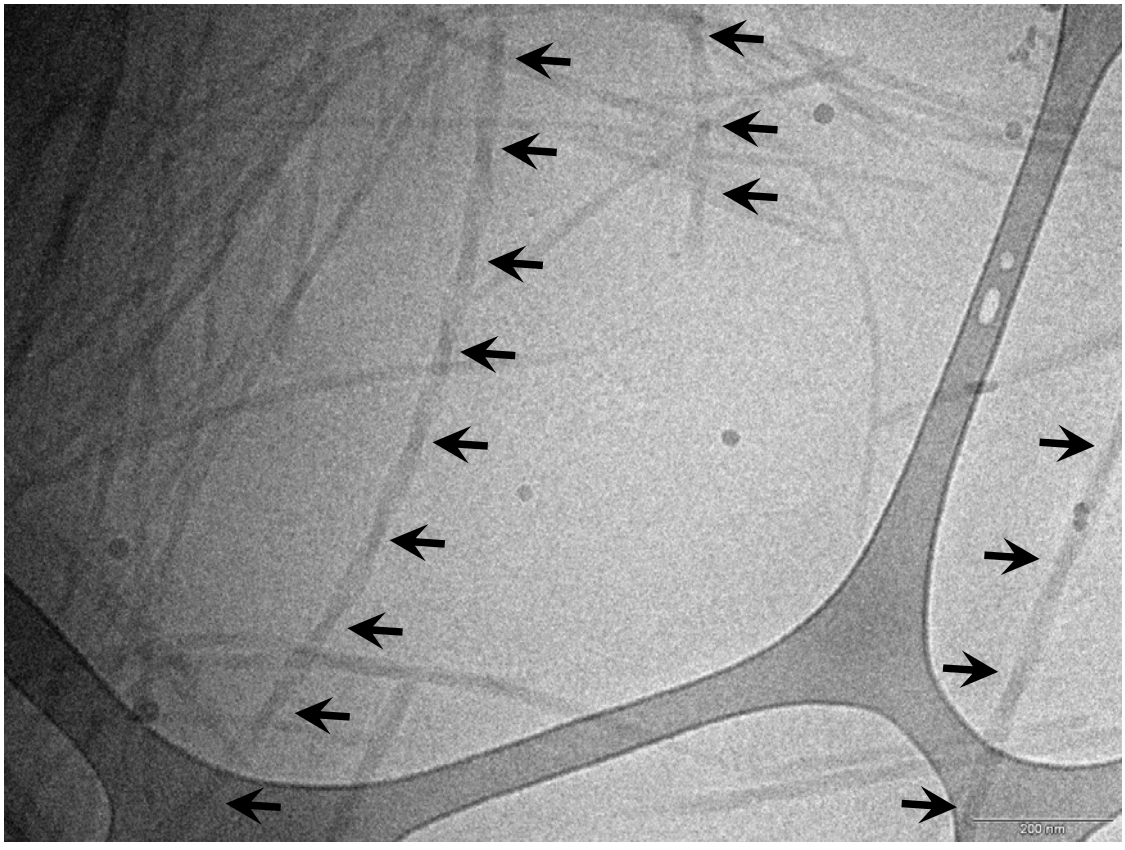
IV- Results- Cryo-TEM images for ^{D}VFF. Scale bar = 200 nm.

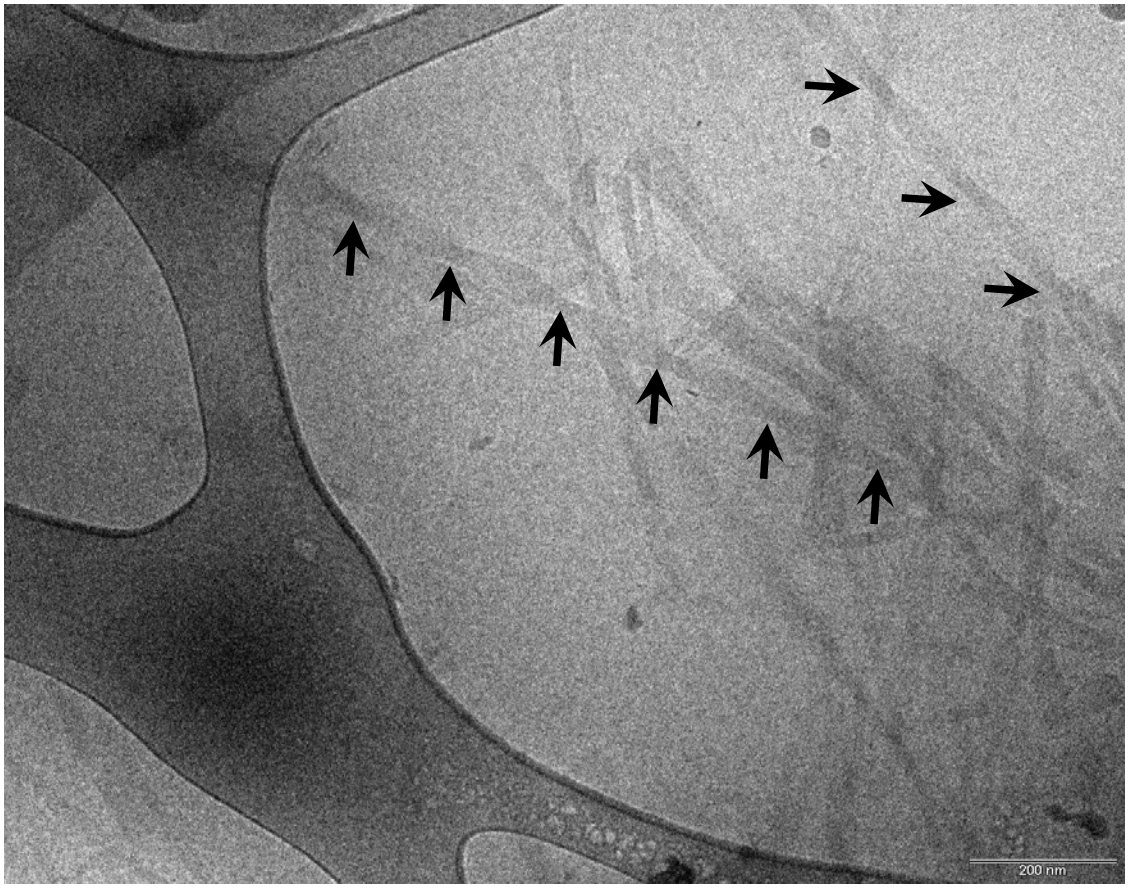


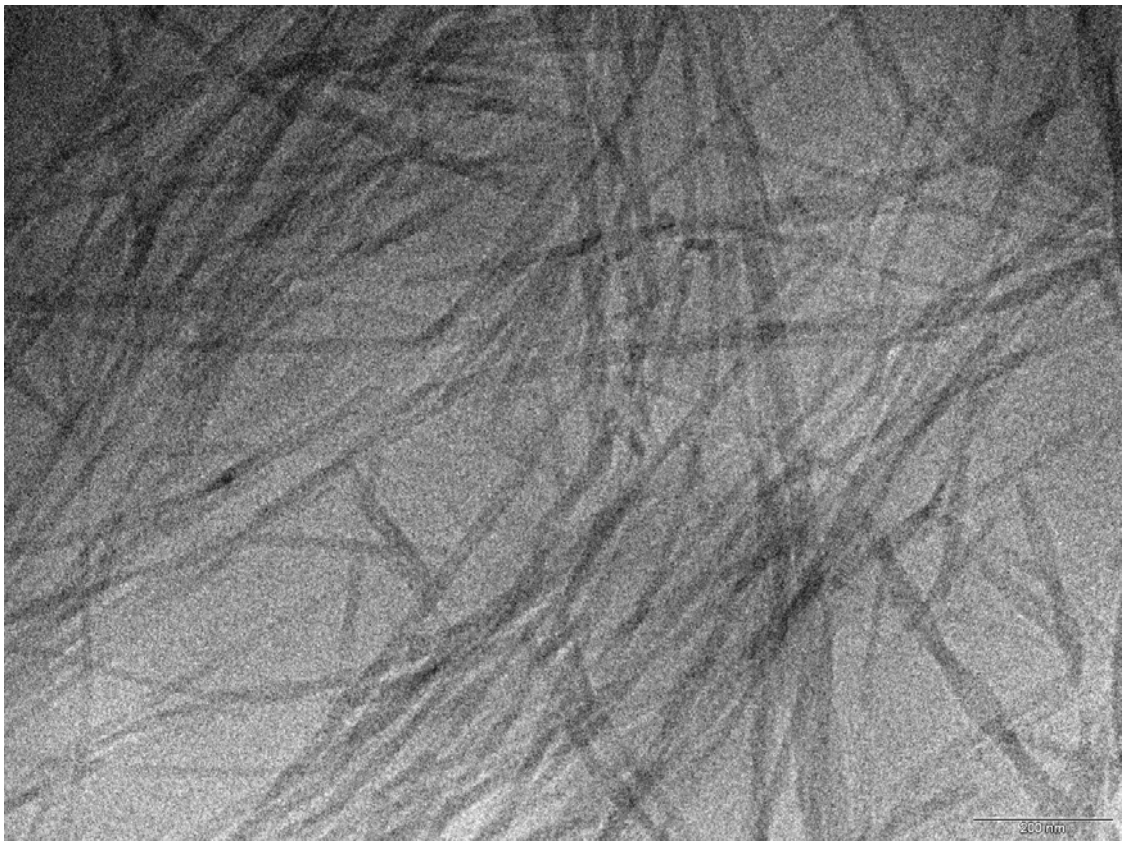
Cryo-TEM images for ^{D}VFF. Scale bar = 1 micron.



Cryo-TEM images for ^{D}FFV (black arrows indicate helix period). Scale bar = 200 nm.

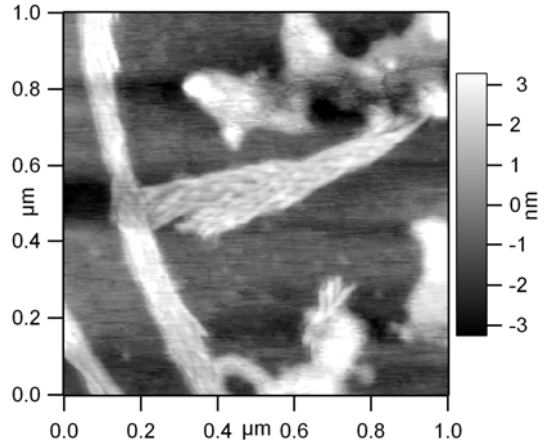






V- Results- AFM images.

AFM image of twisted fibers for ^{D}FFV



AFM images for VFF (left) and FFV (right)