Electronic Supporting Information for:

Antiviral Supramolecular Polymeric Hydrogels by Self-Assembly of Tenofovir-Bearing Peptide Amphiphiles

Maya K Monroe,[a,b] Han Wang, [a,b] Caleb F Anderson,[a,b] Meng Qin,[a,b] Chloe L Thio,[c] Charles Flexner,[d] and Honggang Cui*[a,b,e,f,g]

[a.] *Department of Chemical and Biomolecular Engineering, The Johns Hopkins University, Baltimore, MD 21218, USA.*
[b.] *Institute for NanoBioTechnology, The Johns Hopkins University, Baltimore, MD 21218, USA.*
[c.] *Department of Medicine, The Johns Hopkins University School of Medicine, Baltimore, MD 21205, USA.*
[d.] *Divisions of Clinical Pharmacology and Infectious Diseases, The Johns Hopkins University School of Medicine and Bloomberg School of Public Health, Baltimore, MD 21205, USA.*
[e.] *Department of Materials Science and Engineering, The Johns Hopkins University, Baltimore, MD 21218, USA.*
[f.] *Department of Oncology and Sidney Kimmel Comprehensive Cancer Center, The Johns Hopkins University School of Medicine, Baltimore, MD 21205, USA.*
[g.] *Center for Nanomedicine, The Wilmer Eye Institute, The Johns Hopkins University School of Medicine, Baltimore, MD 21231, USA.*
* Corresponding Author, email: hcui6@jhu.edu

Contents

S1 Materials and Equipment

S1.1 Materials

Fmoc amino acids and resins for peptide synthesis were obtained from Advanced Automated Peptide Protein Technologies (AAPPTEC, Louisville, KY). Tenofovir was purchased from Combi-Blocks (San Diego, CA). All other solvents and reagents were sourced from VWR (Radnor, PA), Thermo Fisher Scientific (Pittsburgh, PA), or MilliporeSigma (St. Louis, MO). HepAD38 cells were provided by Prof. Chloe Thio's lab of the Johns Hopkins University School of Medicine (Infectious Diseases Department, Baltimore, MD).

S1.2 Equipment

Reverse phase high performance liquid chromatography (RP-HPLC) was performed using a Varian ProStar Model 325 HPLC (Agilent Technologies, Santa Clara, CA) equipped with a fraction collector. Preparative separations used a Varian PLRP-S column (100 Å, 10 µm, 150 × 25 mm) at a flow rate of 20 mL/min, with 10 mL injection volumes. Analytical HPLC used an Agilent Zorbax Extend-C18 RP column (5 µm, 150 × 4.6 mm) at a flow rate of 1 mL/min, with 25 µL injection volumes. Select analytical HPLC experiments (as denoted in protocol subsequent protocols) were carried out using a 1260 Infinity II LC system (Agilent Technologies, Santa Clara, CA) and the Agilent Zorbax Extend-C18 RP column (5 µm, 150 × 4.6 mm) at a flow rate of 1 mL/min, with 25 µL injection volumes. Acidic (0.1% v/v trifluoroacetic acid) and basic (0.1% v/v ammonium hydroxide) water and acetonitrile were used as the mobile phase. 220 nm and 260 nm wavelengths were monitored for all molecules during RP-HPLC. Molecules were lyophilized using a FreeZone -50 °C 4.5L freeze dryer (Labconco, Kansas City, MO). Mass spectrometry data was obtained using either a BrukerAutoflex III Smartbeam (Bruker, Billerica, MA) instrument for matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) MS using sinapic acid as the matrix, or a Finnigan LCQ ion trap mass spectrometer (Thermo-Finnigan, Waltham, MA) for electron spray ionization (ESI) MS. Bruker Avance 300 or 400 MHz FT-NMR spectrometers were used to acquire ^{1}H and ^{13}C NMR spectra. Circular dichroism measurements were performed using a Jasco J-710 spectropolarimeter (JASCO, Easton, MD). Electron microscopy was performed on a FEI Tecnai 12 TWIN transmission electron microscope operating at an acceleration voltage of 100 kV or a FEI Talos 200SC FEG transmission electron microscope operating at an acceleration voltage of 200 kV. Negatively stained images obtained by the Tecnai were recorded using a SIS Megaview III wide-angle CCD camera and cryogenic images obtained by the Tecnai were recorded 16-bit 2K FEI Eagle bottom mount camera, all images obtained by the Talos were recorded using CETA direct detection CMOS camera. Fluorescence spectroscopy data was obtained using a Duetta fluorescence and absorbance spectrometer (Horiba Scientific, Irvine, CA). Quantitative polymerase chain reaction (qPCR) assays were conducted using a LightCycler 480 Real-Time PCR System (Roche Diagnostics, Rotkreuz, Switzerland).

S2 Molecular Synthesis and Characterization

S2.1 Peptide and Linker Synthesis and Purification

Peptide amphiphiles (PAs) were synthesized using the standard 9- fluorenylmethoxycarbonyl (Fmoc) solid phase peptide synthesis technique. All peptides were synthesized on an Fmoc-Glu(OtBu)-Wang Resin. Fmoc deprotection was performed using 20% piperidine in N,N-dimethylformamide (DMF) for two fifteen-minute intervals. Following Fmoc removal, amino acids were coupled to the peptide chain at the molar ratio of 4:4:10 ratio of resin to Fmoc amino acid, hexafluorosphate benzotriazole

tetramethyl uranium (HBTU), and N,N-diisopropylethylamine (DIEA) in DMF, the reaction was allowed to proceed for at least two hours. Palmitic acid was conjugated to the Fmoc-deprotected, N-terminus of the peptides at a ratio of 1:4:4:10 resin:palmitic acid:HBTU:DIEA in DMF and allowed to react for 12 hours. Completed peptide amphiphiles were cleaved from the resin and their side chains were deprotected through reaction in a solution of 92.5% trifluoroacetic acid (TFA), 5% triisopropylsilane (TIS), and 2.5% water for 3 hours. Following cleavage, the PA containing TFA was collected and the PA was precipitated through the addition of cold diethyl ether and centrifugation (repeated 2 more times). The precipitated PA was then allowed to dry in the fume hood overnight.

Following PA synthesis, PAs were dissolved in 0.1% v/v ammonium hydroxide (NH_4OH) containing water and 0.1% v/v NH_4OH containing acetonitrile (ACN). Molecules below 95% purity (as determined by analytical RP-HPLC) were purified using RP-HPLC in mobile phases of basic H_2O and ACN. Collected fractions were subjected to matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) or electron spray ionization (ESI) mass spectrometry to determine the fraction with the desired product. Correct fractions were combined and ACN was removed using rotary evaporation. PAs were then lyophilized and the purified powders were stored in a -20 °C freezer for future use.

Synthesis of **2-(Pyridyl-disulfanyl)ethanol** (etpSS-Pyr) was adapted from a previously reported procedure.[1] Briefly, 2-Aldrithiol (2.4 g, 10.89 mmol) was dissolved in MeOH (7 ml) and 2-mercaptoethanol (334 µl, 370 mg, 4.74 mmol) was added and allowed to react for three hours. The solution was diluted with 0.1% aq. TFA and purified by RP-HPLC. Product fractions were combined and solvents removed using rotary evaporation to give a pale yellow oil.

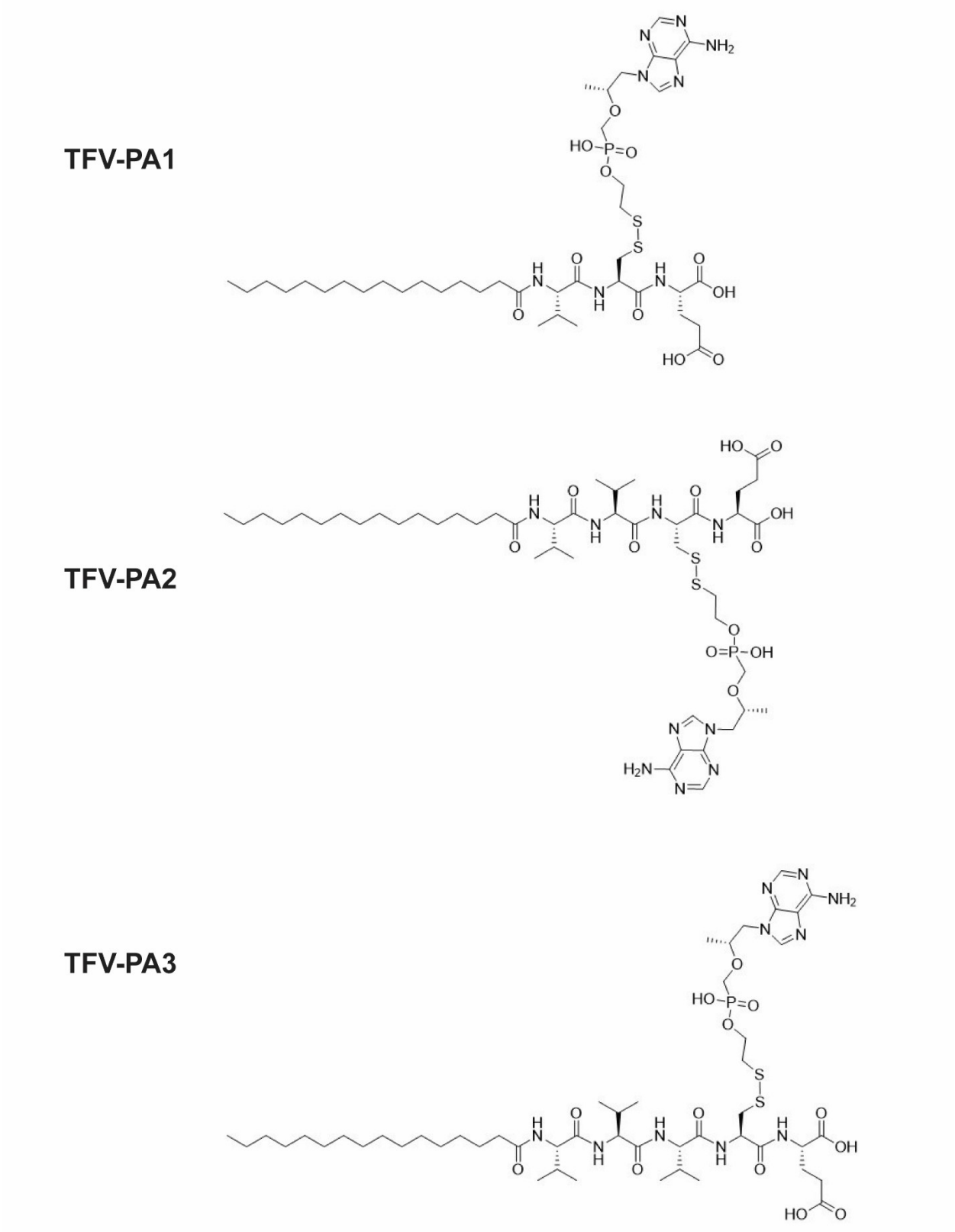

Figure S1. Chemical structures of the three TFV-PAs studied in this work.

S2.2 Electron Spray Ionization (ESI) MS

Molecular weights of synthesized etpSS-Pyr and TFV-etpSS-Pyr were determined using ESI mass spectrometry. Molecular weights of synthesized PAs and TFV-PAs were also determined using ESI MS when MALDI-TOF MS analysis was unavailable. Samples were prepared for analysis by diluting 20 µL of aqueous sample solution (containing 0.1% v/v formic acid) with 200 µL methanol. Diluted samples were then diluted another 5 to 20 times using methanol within a 500 µL glass syringe to attain a final sample concentration of 0.1-1 µM. The samples were analyzed in positive ion mode. A representative mass spectrum of TFV-etpSS-Pyr is presented in **Figure S2**.

S2.3 Matrix-Assisted Laser Desorption/Ionization Time of Flight (MALDI-TOF) MS

Molecular weights of synthesized PAs and TFV-PAs were determined using MALDI-TOF mass spectrometry whenever possible. Samples were prepared for analysis by depositing 2 µL of sinapic acid matrix (10 mg/mL in 1:1 v/v water/ACN with 0.05% v/v S4 TFA; Sigma-Aldrich, St. Louis, MO) onto a room temperature MTP 384 ground steel target plate (Bruker, Billerica, MA) and allowing the matrix to dry for 10 minutes. 1 µL of aqueous PA solution was then added to the spot of dried matrix followed by the immediate addition of 1 µL of sinapic acid matrix, which was mixed with the PA solution. The samples were then allowed to dry for 10-20 minutes. During MS analysis, the samples were irradiated with a 355 nm UV laser and analyzed in the reflectron mode. Representative mass spectra of the TFV-PAs are presented in **Figures S3-5**.

S2.4 Purity and Concentration Assessment

Purity and concentration of TFV-PAs and intermediate products were determined using analytical RP-HPLC, monitoring the 220 and 260 nm wavelengths. To assess purity, 25 µL of solution was injected into the HPLC and subjected to a gradient of 5% to 95% ACN over 15 minutes, in basic phase. Area under the curve (AUC) of the desired molecule's peak was compared to the sum of the AUCs for all peaks in the 220 and 260 nm RP-HPLC chromatographs to confirm product purity ≥ 95%. The purity data for TFV-etpSS-Pyr and all TFV-PAs is presented in **Figures S2-5**. A calibration curve of TFV concentration was built by running different concentrations of TFV (ranging from 50 µM to 1 mM) on the HPLC in acidic phase at a gradient of 5% to 95% ACN over 15 minutes. The AUC of the peaks in the 260 nm (the absorbance of TFV) chromatograph were plotted against TFV concentration and the data was fit with a linear regression to give a TFV calibration curve. To determine the concentration of TFV-PA aliquots, a previously reported TCEP reduction assay was modified and applied as follows.[1] 25 µL of solution was diluted with 25 µL of ACN and added to 3 mg of TCEP, the mixture was then vortexed and sonicated at 37 °C for twenty minutes to attain complete cleavage of the TFV from the peptide. 25 µL of the vortexed solution was subjected to HPLC analysis under acidic conditions and the 260 nm AUC of the TFV peak was used to calculate the concentration of the aliquot from the TFV calibration curve.

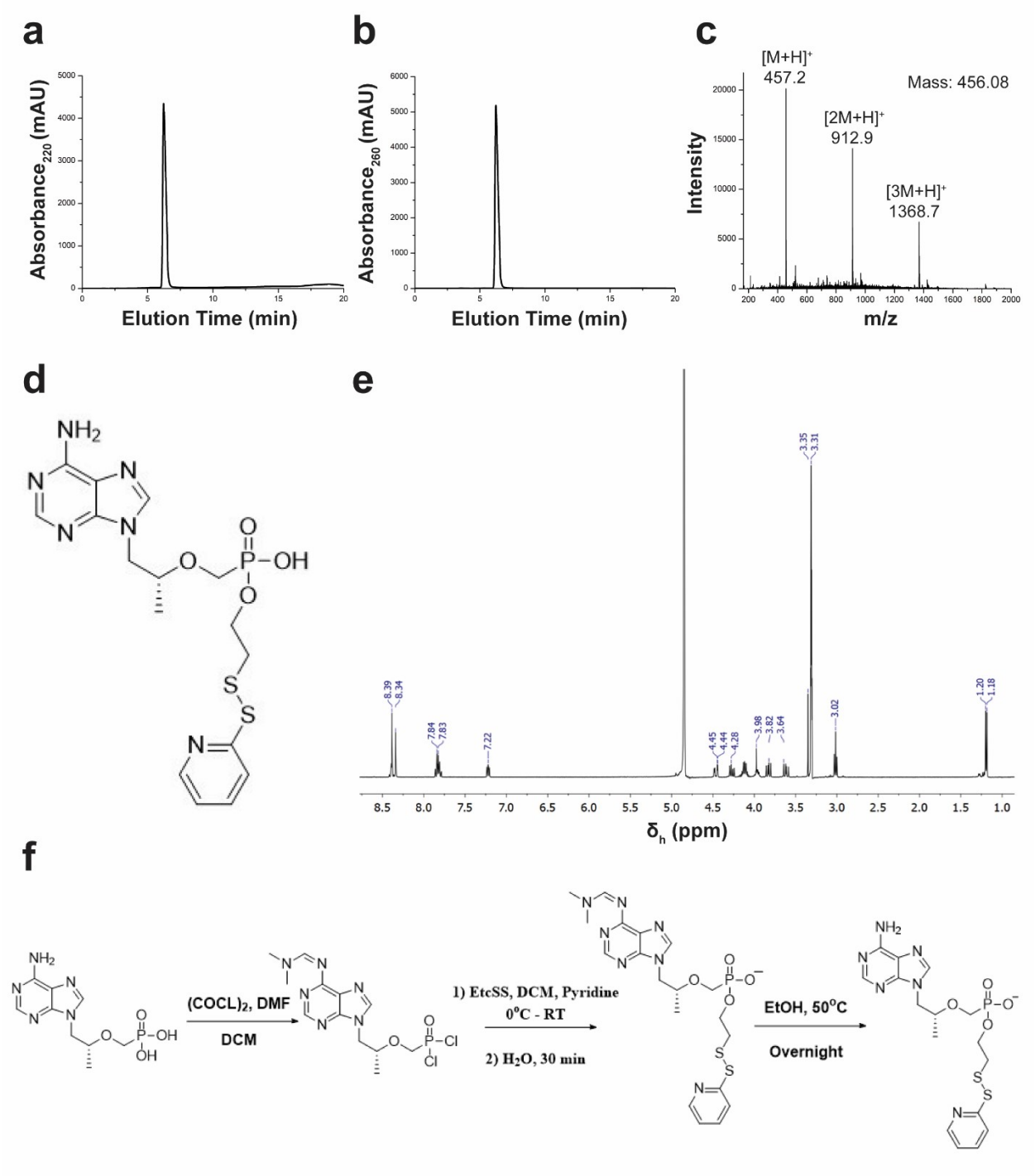

Figure S2. Analytical RP-HPLC chromatographs of the a) 220 nm wavelength and b) 260 nm wavelength, c) the ESI mass spectrum, d) chemical structure, e) the NMR spectrum: ^{1}H-NMR (400 MHz, CD_3OD, 25 °C, δ ppm) 8.39 (m, 2H), 8.34 (s, 1H), 7.84 (m, 2H), 7.22 (t, 1H), 4.28- 4.48 (m, 2H), 4.12 (m, 2H), 3.98 (dd, 1H), 3.57-3.82 (m, 2H), 3.02 (t, 2H), 1.18 (s, 3H), and f) the reaction scheme for synthesis of TFV-etpSS-Pyr.

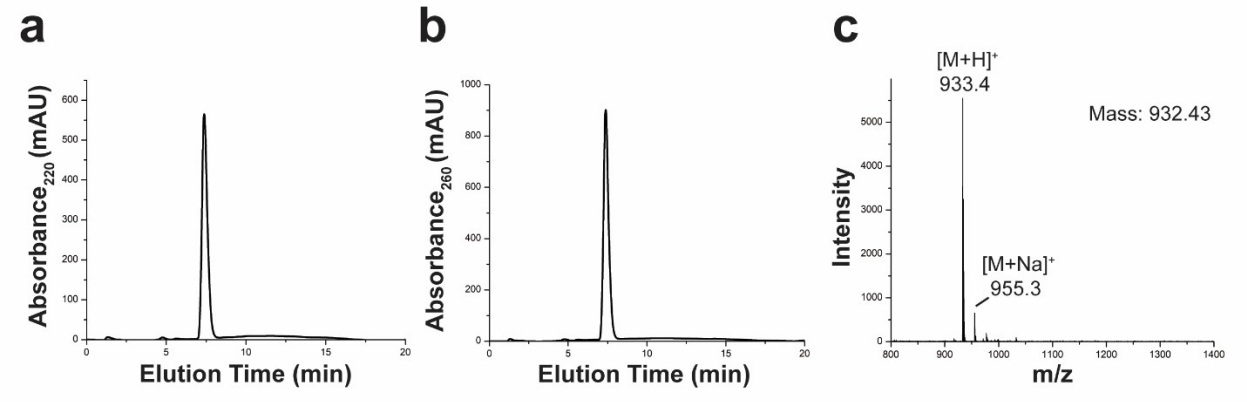

Figure S3. Analytical RP-HPLC chromatographs of the a) 220 nm wavelength and b) 260 nm wavelength and c) the MALDI-TOF mass spectrum of TFV-PA1.

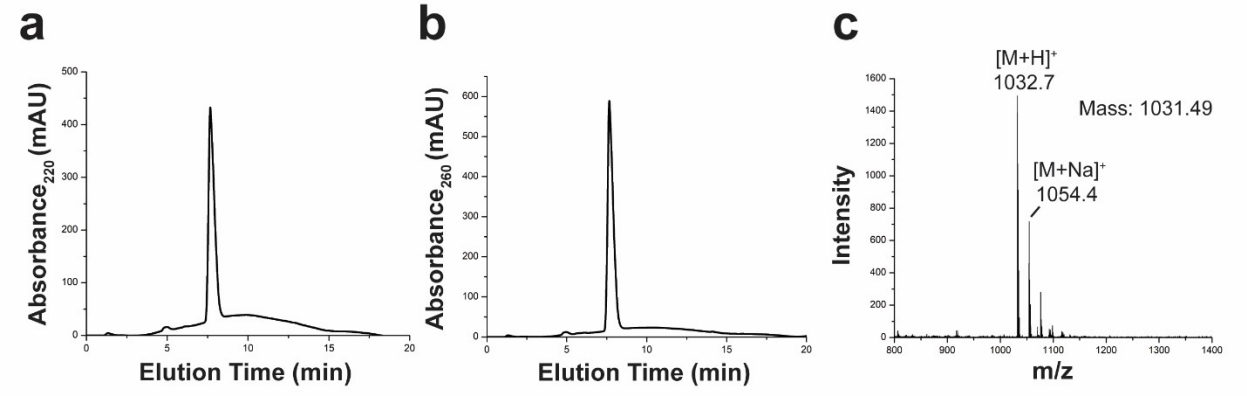

Figure S4. Analytical RP-HPLC chromatographs of the a) 220 nm wavelength and b) 260 nm wavelength and c) the MALDI-TOF mass spectrum of TFV-PA2.

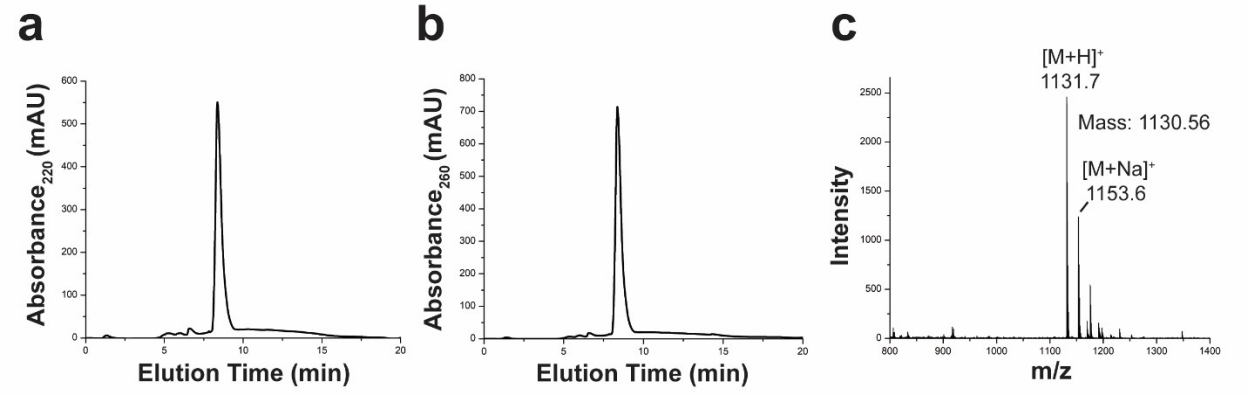

Figure S5. Analytical RP-HPLC chromatographs of the a) 220 nm wavelength and b) 260 nm wavelength and c) the MALDI-TOF mass spectrum of TFV-PA3.

S3 Self-Assembly Characterization

S3.1 Transmission Electron Microscopy

1 mM aqueous TFV-PA solutions were prepared using deionized water and allowed to age for 48 hours at RT. Negative stained transmission electron microscopy (TEM) samples were prepared by depositing 5 µL of sample onto a 400 square mesh, carbon film copper grid (Electron Microscopy Services, Hatfield, PA). After 1 minute, excess sample was wicked away using filter paper, leaving a thin film of sample on the grid, which was allowed to dry for 2 hours. 7 µL of 2 wt % aqueous uranyl acetate was deposited on the grid and allowed to sit for 30 seconds before excess stain was wicked away with filter paper. The girds were allowed to dry for at least three hours before being imaged using the Tecnai 12 TEM. Nanobelt widths were measured using ImageJ software, 35 distinct structures were measured for each image. For cryogenic TEM, plasma cleaned 300-mesh lacey carbon-coated grids (Electron Microscopy Services, Hatfield, PA, USA) were mounted by the forceps on Vitrobot Mark IV (Thermo Fisher, Waltham, MA). 6 µL of 5 mM TFV-PA3 aqueous solutions were applied onto the grid, then blotted for 1 s at a blot force of 0. The grid was plunge-frozen into the liquid ethane reservoir in a Dewar precooled by liquid nitrogen. The vitrified samples were then transferred to a Gatan 626 cryo-holder on the cryo-transfer. The temperature of the cryo holder was maintained below -170 °C to prevent vitreous ice formation. The cryo-holder was transferred to the FEI Talos 200SC FEG transmission electron microscope for imaging.

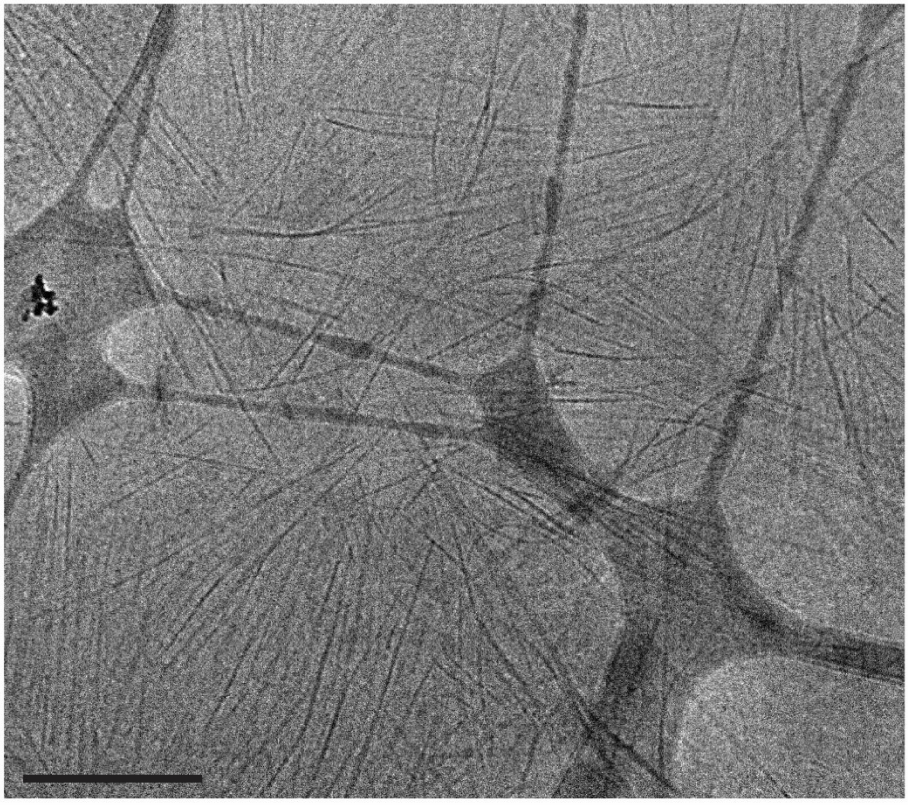

Figure S6. A cryogenic TEM image of TFV-PA3 (5 mM solution, aged for 48 hours at RT), scale bar represents 500 nm.

S3.2 Critical Micellular Concentration Fluorescence Spectroscopy

A Nile Red encapsulation assay was used to determine the critical micellization concentration (CMC) of each design. TFV-PAs were treated with hexafluoroisopropanol (HFIP) and then excess HFIP was fully removed by evaporation. Aqueous solutions of the HFIP-treated TFV-PAs were prepared at various concentrations ranging from 0.1 to 500 µM. 10 µL of a 500 µM acetone-based solution of Nile Red was added to 1.5 mL microcentrifuge tubes and the acetone was allowed to evaporate off in the dark. 500 µL of each concentration for each TFV-PA was added to a tube with Nile Red. Solutions were aged for 2 days in the dark at RT. Samples were then run on the spectrofluorometer at an excitation wavelength of 550 nm, with three parallel emission spectra recorded from 580 to 720 nm. The emission intensity ratio of encapsulated versus free Nile red (635 nm/660 nm) was then plotted versus concentration and the CMC value was calculated using the point of transition from 660 nm to 635 nm emission (by calculating the intersection of the linear regressions fit to the two emission phases).

S3.3 Circular Dichroism

Circular dichroism was employed to determine the extent of intermolecular hydrogen bonding within the supramolecular assemblies. 1 mM concentration samples were prepared and allowed to age at room temperature for 48 hours. Samples were then diluted to 100 µM concentration using deionized H_2O and 200 µL of sample was immediately loaded into a 0.1 cm path length quartz UV–vis absorption cell (Thermo Fisher Scientific, Pittsburgh, PA). Samples were analyzed using three repeated scans from 300 to 190 nm and high tension values for each spectrum were monitored to ensure they remained between 200 and 600 V. A background spectra of deionized water was acquired using the same run parameters. The three runs were averaged for each sample and the water background spectra was subtracted from the sample spectra. The averaged spectra was then normalized with respect to sample concentration and path length in order to convert from ellipticity (mdeg) to molar ellipticity ($deg \cdot cm^2 \cdot dmol^{-1}$) and mean residue ellipticity ($deg \cdot cm^2 \cdot dmol^{-1} \cdot n_{residues}^{-1}$).

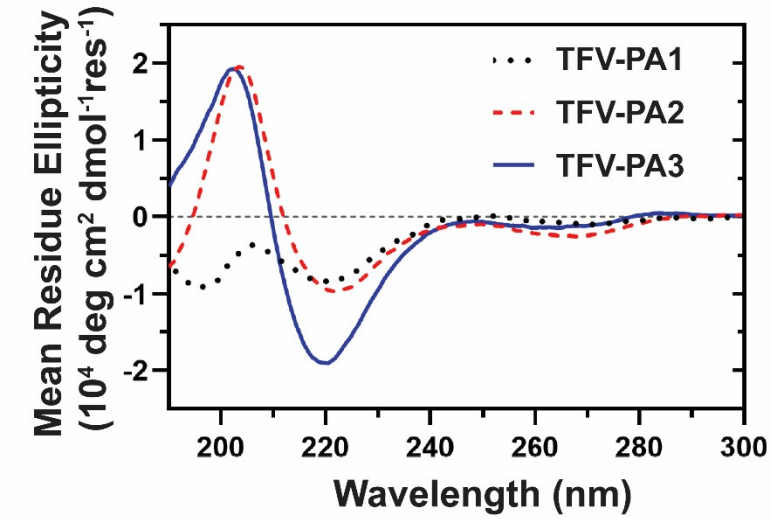

Figure S7. Circular Dichroism spectra of assembled solutions for TFV-PA1-3 designs (100 µM in H_2O), plotted as mean residue ellipticity.

S4 Gelation and Release Studies

S4.1 Critical Gelation Concentration

The critical gelation concentrations of the different TFV-PAs were assessed by preparing aqueous DA solutions at different concentrations (1, 2, 5, and 10 mM) and allowing them to age for 48 hours at RT. 100 µL of each solution was then transferred to a half dram glass vial and 10 µL of 10x phosphate-buffered saline (PBS) solution was added to trigger gelation via salt-screening and to give a final concentration of 1x PBS. Samples were subjected to an inversion test to assess formation of a self-supporting hydrogel.

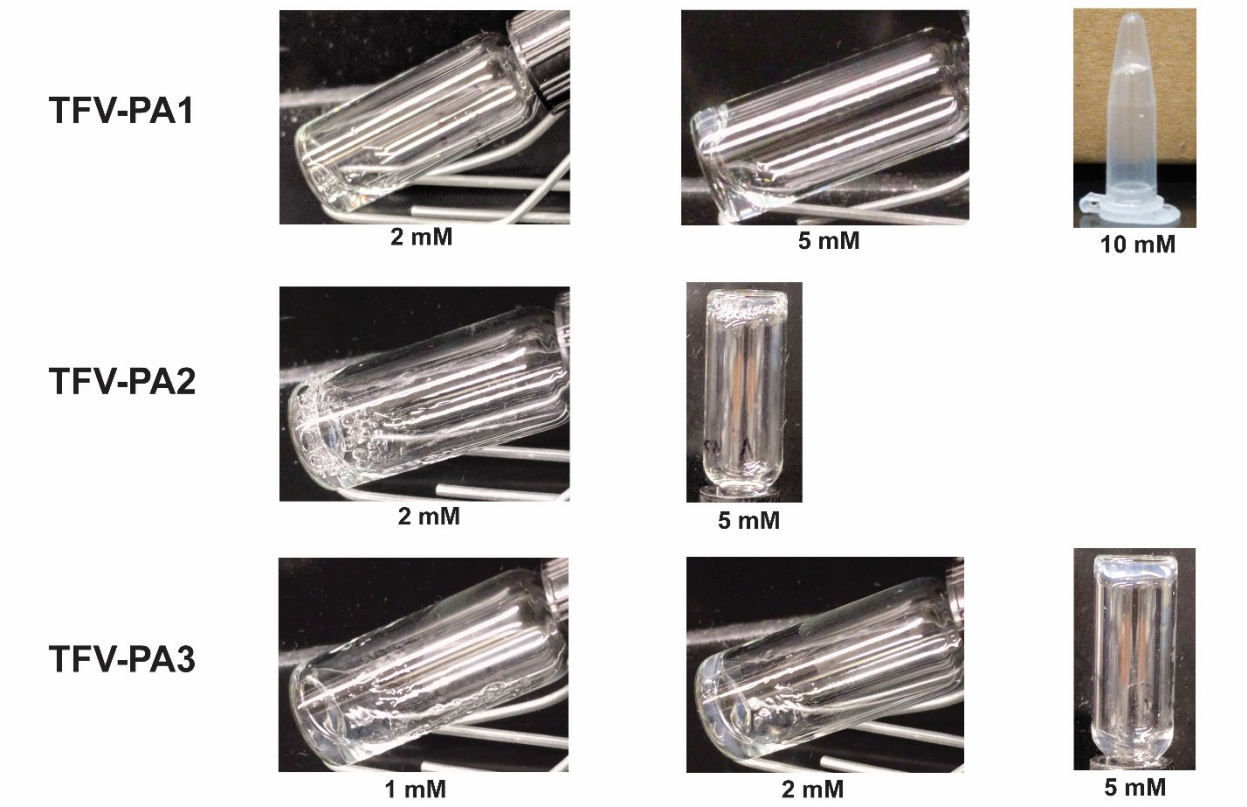

Figure S8. Images of TFV-PA solutions and gels at different concentrations following the addition of 10x PBS, vials were inverted to assess gel formation in order to determine the CGC.

S4.2 Free Drug Release Studies

Stability of the TFV-PAs and redox triggered release of free TFV was assessed by aging TFV-PA solutions in the presence and absence of 10 mM dithiothreitol (DTT). 200 µM solutions of TFV-PA2 and TFV-PA3 solutions were prepared using deionized H_2O and allowed to age at RT for 24 hours. A 20 mM solution of DTT was prepared by diluting a stock 1 M DTT solution with 2x phosphate buffered solution (PBS). 500 µL of 2x PBS was added to an equal volume of each TFV-PA solution to give the DTT negative samples with a final TFV-PA concentration of 100 µM in 1x PBS. 500 µL of 20 mM DTT solution was added to an equal volume of each TFV-PA solution to give the DTT positive samples with a final TFV-PA concentration of 100 µM and 10 mM DTT in 1x PBS. Each experimental condition was prepared in triplicate. Solutions were allowed to age at 37 °C and 50 µL samples were collected at predetermined

time points (0, 1, 2, 4, 8, 12, 24, 48, and 72 h), flash frozen in liquid nitrogen, and then stored in a -20 °C freezer. 2 µL of hydrochloric acid was added to the DTT positive samples upon collection to quench the reaction. Samples were thawed and immediately analyzed using the Agilent Infinity II RP-HPLC. The relative proportions of remaining TFV-PA were determined using the ratio of the AUC of the TFV-PA peak in the 260 nm chromatograph to that of the 0 hour samples. For select samples, 1 µL of solution was diluted in 900 µL MeOH and 100 µL 0.1% aq. formic acid and subjected to ESI MS to identify the molecules in the sample (**Figure S9)**

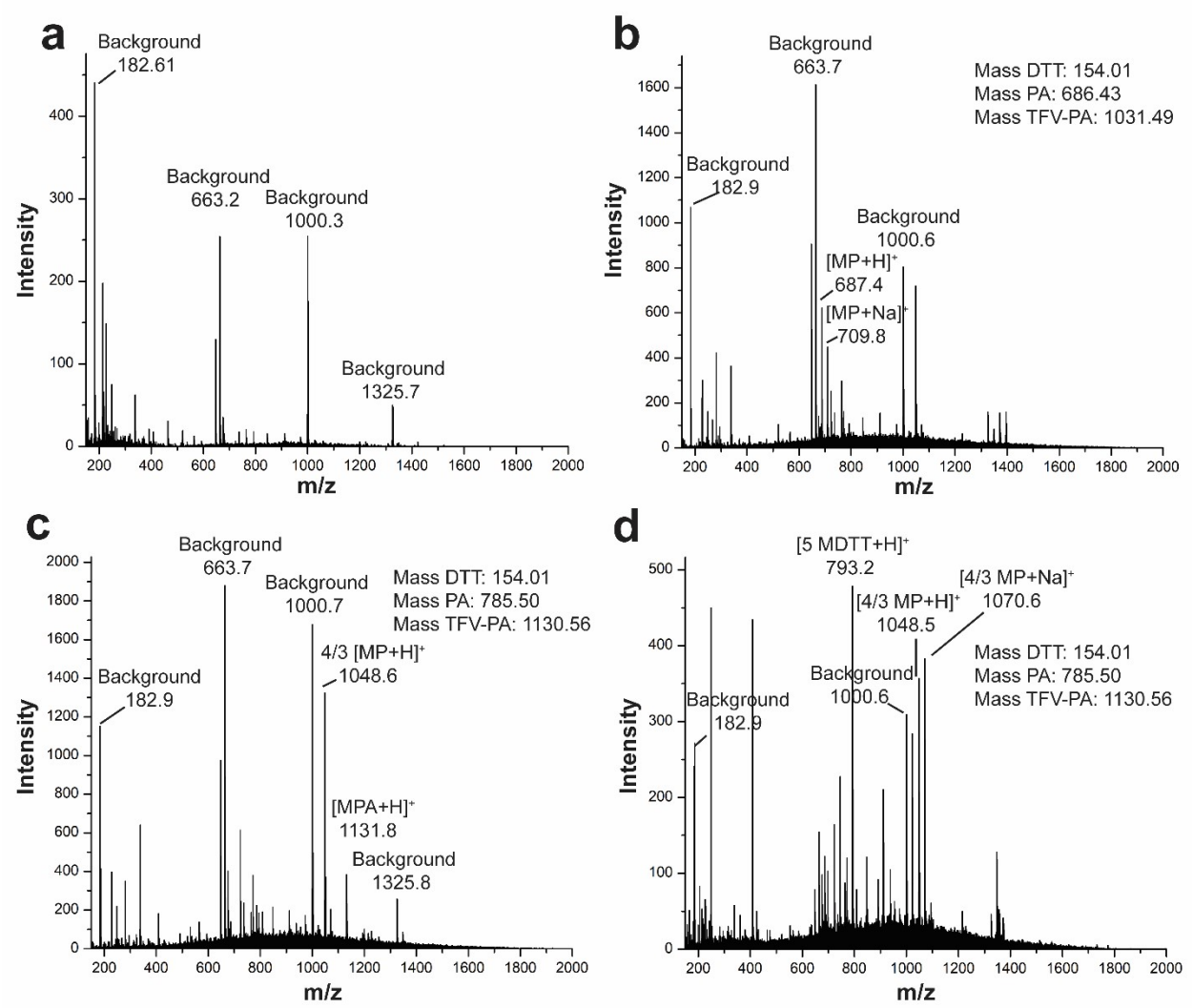

Figure S9. ESI mass spectra for a) 0.1% aq. formic acid and MeOH Blank, DTT positive release samples of b) TFV-PA2 at 24 hours, c) TFV-PA3 at 2 hours, and d) TFV-PA3 at 24 hours.

S4.3 Gel Release Studies

5 mM and 10 mM solutions of TFV-PA2 and TFV-PA3 were prepared using deionized water and allowed to age at RT for 48 hours. 45 µL of each solution was aliquoted into three 0.5 mL microcentrifuge tubes and 5 µL of 10x PBS was added to each vial to form a hydrogel with a final concentration of 1x PBS. Gels were allowed to set for 5-10 minutes before 60 µL of 1x PBS was added to the surface as the release media. The vials were then allowed to age at 37 °C. Every 2 days, 50 µL of gel

release supernatant was collected and replaced with an equal volume of fresh 1x PBS. Samples were frozen and stored at -20 °C for future analysis by RP-HPLC. On select days, 5 µL of release supernatant was collected 24 hours after supernatant exchange and used to prepare a negatively stained transmission electron microscope grid for analysis with the Talos transmission electron microscope. The grid was prepared as described in section S3.1 of the Supporting Information, except the sample was blotted with 5 µL deionized water (wicked immediately) after the sample was wicked away to remove excess salts from the PBS. Once the release experiment was complete, samples were thawed and 1-2 mg of TCEP was added to 25 µL sample solution and an equal volume of acetonitrile, then vortexed and sonicated at 37 °C for 10 minutes to ensure complete cleavage of the TFV moiety from the TFV-PAs. 25 µL of the TCEP treated solution was run in acidic phase through the Varian ProStar analytical HPLC and the 260 nm AUC of the TFV peak was used to calculate the concentration of the aliquot from the TFV calibration curve.

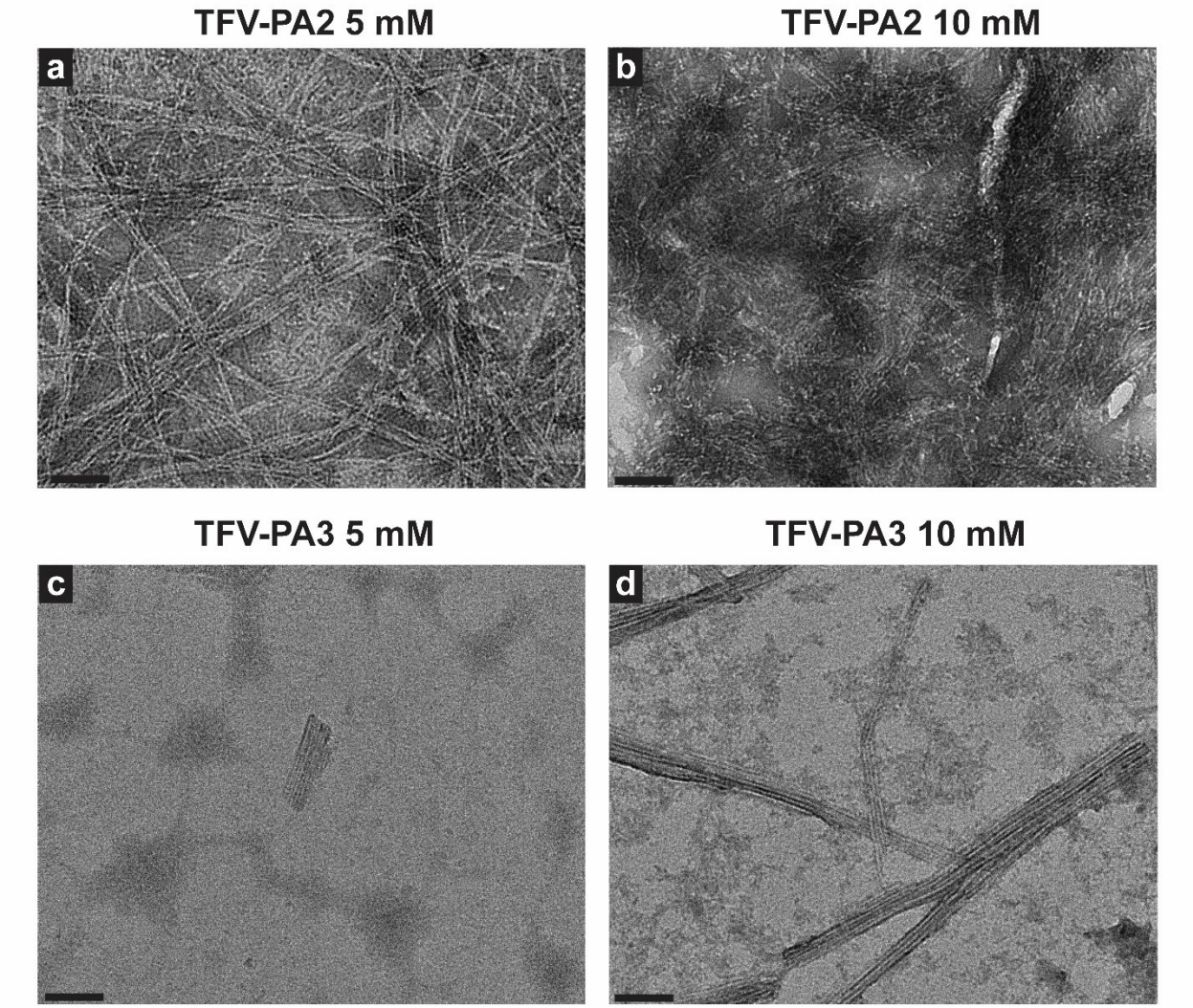

Figure S10. Negatively stained transmission electron microscopy images of hydrogel release media 24 hrs after exchange for a) TFV-PA2 5 mM, b) TFV-PA2 10 mM, c) TFV-PA3 5 mM, and d) TFV-PA3 10 mM, scale bars represent 100 nm.

S5 References

1 A. G. Cheetham, Y. C. Ou, P. Zhang and H. Cui, *Chem. Commun.*, 2014, **50**, 6039–6042.