Supplementary Information

Light-responsive self-assembled microstructures of branched polyethyleneimine at low pH

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A. *Materials and Methods*:

(i) Materials:

Branched Poly(ethylenimine) (PEI) having molecular weight M_w ~ 750 kDa (M_n ~ 60 kDa; 50 %w/w in water, 181978); Fluorescein Isothiocyanate Isomer 1, F7250, Rhodamine B isothiocyanate (mixed isomers), R1755, Fluorescein sodium salt, 46960, were purchased from Sigma Aldrich, Dimethyl sulfoxide (66,120, anhydrous ≥ 99.5 %), Hydrochloric acid (about 35 %), Nitric acid (about 69 %), were purchased from Merck chemicals, and were used as received. Glass coverslips of thickness 0.22 μm and Microscope glass slides (75 mm × 25 mm × 1.35 mm) were bought from Blue Star (Mumbai, India). Snake Skin dialysis tubing (MWCO; 10 kDa I.D.; 35 mm) was purchased from Thermo Fischer Scientific. Milli-Q water with a resistivity of 18.2 MΩ cm at 25 °C, and a TOC value <5 ppb was utilized for all the experiments.

(ii) Methods

(a) Preparation of dilute aqueous solution of PEI and its tagging with dyes:

Fluorescent dye stock solution was prepared by dissolving required amount of dye (FITC/RITC) to 50 μL of dried DMSO (purged with N_2 gas). Concentration of stock solution was obtained using absorbance measurements and 0.1 μM to 10 μM solutions of each FITC and RITC were added in 65 μM of 750 kDa PEI dissolved in 40 mL water. Before dye addition, the PEI aqueous solution was kept on magnetic stirring for 6-8 hours at room temperature to ensure the complete dissolution of polymer before measurement. The native pH of the PEI solution was ~ 10.8. The dye addition to this PEI solution was done slowly in a dark room and the solution was kept on stirring for 24 hours at room temperature. This will result in a covalent thio-urea linkage between isothiocyanate groups of FITC/RITC and primary amines of PEI. To remove the excess dye, the solution was then dialyzed against Milli Q water for 3-4 days by changing the dialysis water after every 12 hours.[1] The concentration of dye left in the solution

was then calculated by absorbance measurements performed before and after dialysis, taking the dilution factor into account. The pH of dye labelled and unlabelled PEI was then measured using a calibrated Mettler Toledo pH meter. 6 N HCl solution was used to change the pH of solution to 3. The vials containing dye labelled PEI solutions were properly covered with aluminium foils and were kept under dark conditions at all times. The samples were then characterized using different microscopy, scattering and spectroscopy techniques which are described in details are given below and in the supplementary information. For control experiments involving co-incubation with fluorescein dye (which does not covalently couple with PEI) similar procedure as mentioned was followed, however dialysis was not performed in this case as there is no covalent linkage of the dye to PEI in this case. Another control experiment was also performed involving the FITC addition to PEI at pH 3 and observing the sample over a period of time.

While passing, it is important to mention here that with the concentration of dyes used the PEI chains are highly unsaturated in dye concentration. We have used PEI with M_w = 750 kDa and M_n= 60 kDa. Assuming M_w for the calculation purposes, PEI chain to FITC/RITC dyes molar ratio is in the range 130:1 to 130:10. This has been mentioned in the manuscript on Page 2. The RITC/FITC are known to covalently couple with primary amine groups of PEI chains as shown in the literature.[2]

Further, the ratio of primary to secondary and tertiary amines can be investigated using a combination of ^{1}H-NMR and ^{13}C-NMR studies as shown in the reference no. 23 cited in the main manuscript. It has been shown that approximately 20% primary amines are present in a similarly branched PEI system of Mw 2000 Da. According to this assumption, in our case of Mw = 750 kDa, the number of primary amines per chain are ~ 3488 (using Mw for the calculations), and are, therefore, much higher than the amount of FITC/RITC required for complete saturation.

(b) Polarized Optical Microscopy (POM) and Epifluorescence Microscopy:

For optical microscopy, white translucent fluffy structures floating in the solution were extracted using 200 µL pipette and were drop casted on a glass slide. The samples were covered using a 24 mm coverslip and were imaged at 4x, 10x, 20x and 40x magnifications using Olympus inverted fluorescence microscope, IX53. Mercury arc lamp was used as the light source for the fluorescence study, and Olympus exciter filter optics (BP 540–550 nm), (BP 418–442 nm), dichroic mirrors (DM 570 nm), (DM 495 nm), and emission barrier filters (BA 575–625 nm), (LP 542 nm) were used to characterize the RITC and FITC labelled PEI samples, respectively. QImaging MicroPublisher 5.0-RTV color camera (MP5.0-RTV-RCLR10CA) was used to acquire the images and Olympus cellSens (CS-ST-V1) imaging software was used for image analysis.

(c) Environmental Scanning Electron Microscopy (ESEM):

For ESEM studies, 200 µL of sample containing microstructures was put in 10 mL water to remove the excess PEI on its surface. The tiny suspended structures were pipetted out of the vial by observing them against light and were drop-casted on a copper grid. The sample was properly dried using IR lamp and then sputter coated with Platinum up to 5-10 nm thickness. The sample was then observed in high vacuum mode on FEI Quanta 200 scanning electron microscope using Tungsten Electron source.

(d) Laser Scanning Confocal microscopy:

100 µL of sample was drop casted on a 24 × 24 mm glass coverslip and viewed using Laser Scanning Confocal microscope (LSM 780, Carl Zeiss, Germany) using Plan-Apochromat 10x/0.45 NA (air) Plan-Apochromat 40x/1.3 NA (oil) iPlan-Apochromat 63x/1.4 NA (oil), iPlan-Apochromat 100x/1.4 (oil) objectives at room temperature. Photomultiplier tube detector was used for capturing Z-stack images and Zen software was used for obtaining maximum intensity projection wherever needed.

(e) ^1H NMR experiments:

For NMR experiments, small amount of dye was added using a 0.5 mm glass capillary to 100 µL Dimethyl Sulphoxide D6 (99.80 %D) to prepare the stock dye solution. The stock dye was sonicated for 5 minutes and then 2 µL of it was added to 800 µL of water with stirring and absorbance of the sample was noted down through UV spectroscopy to measure stock dye concentration. 3 mL of 50 µM PEI solution was prepared in D_2O and then calculated amount of stock dye was added slowly to this solution so that PEI: dye molar ratio becomes 10: 1. The solution was kept on stirring for 5-6 hours. D_20+HCl solution was used to change the pH of solution to 3 whenever required. Afterwards, 500 µL of each solution was taken in a NMR tube. Bruker 400 and 500MHz instrument was used to record the NMR spectra. NMR spectra were measured relative to the signal of deuterated solvent (D_2O) and are reported in ppm.

(f) Dynamic Light Scattering (DLS):

65 µM PEI solution was prepared in Milli Q water (filter size 0.2 µM). Stock solution of RITC dissolved in dry DMSO, purged with N2 gas was prepared and added to 65µM PEI solution in dark conditions so that the RITC: PEI molar ratio in the solution becomes 130:10. The solution kept on stirring for 24 hours. The solutions were then centrifuged for 10 minutes at 10,000 rpm and the filtrate was used for DLS measurements. 3 mL disposable cuvettes were used for DLS measurements at 630 nm wavelength in Anton Paar Litesizer 500 instrument. Samples were equilibrated for 3 minutes before measurements and same sample timing; positioning and laser attenuation was kept same for all measurements.

(g) UV-Visible spectroscopy-based turbidity measurement:

Turbidity measurements were performed using Cary 100 UV-Visible spectrophotometer to trace the nucleation process in freshly prepared unlabeled and dye-labeled samples.700 nm wavelength was chosen to measure change in % transmittance of different pH samples over time as neither FITC nor RITC absorbs at this wavelength. Fresh PEI solutions of 65 µM

concentration (750 kDa) were prepared. Samples were centrifuged at 12000 rpm for 10 mins and filtrate was analyzed. Samples were measured in 3 mL Quartz cuvettes, 1 cm path length with continuous stirring at 25℃. Time dependent transmittance of both dyes labelled and unlabeled 65 μM PEI at its original pH =11 and pH=3 for 60 minutes was recorded.

(h) Steady state spectra:

Horiba Fluoromax 4 spectrofluorimeter and Shimadzu UV-NIR-3600 spectrophotometer was used to record the steady-state spectra of the species.

(i) Temperature dependent microscopy studies:

To study the effect of temperature on films, 100ml of RITC-PEI sample aged for 72 hours at pH=3 was put in a 0.4 X 8.00 mm rectangle boro tubing. The tubing was sealed from both the sides after sample loading and Linkam T96-P stage was used for heating the sample.

B) Figures:

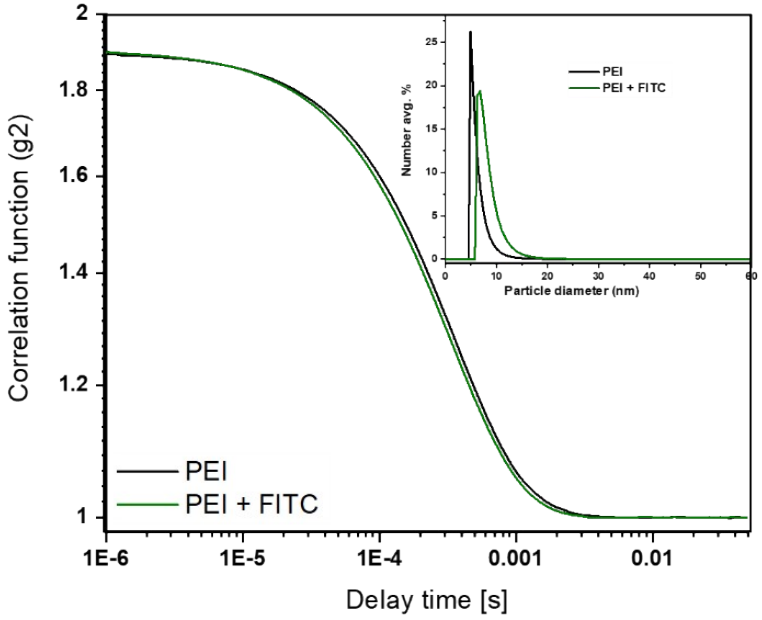

Figure S1: Dynamic Light Scattering (DLS) plot of decay of autocorrelation function (g_2) with respect to delay time and the inset shows number average size distribution vs size for unlabelled PEI (6.1± 1.1nm) and FITC labelled PEI (8.0 ± 1.5nm) at pH 10.

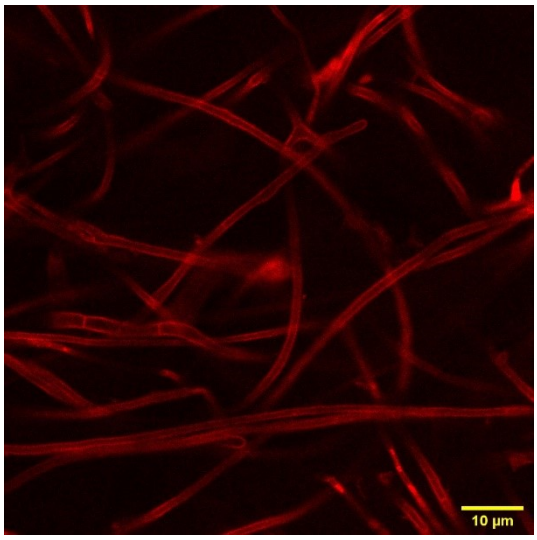

Figure S2: Representative optical section of z-series laser scanning confocal micrograph with X= 85.02µM, Y= 85.02µM and Z=1µM showing hollowness of PEI-RITC (130:1) based fibres. PEI-FITC fibrils also showed similar hollow fibril structures for 130:1 ratio.

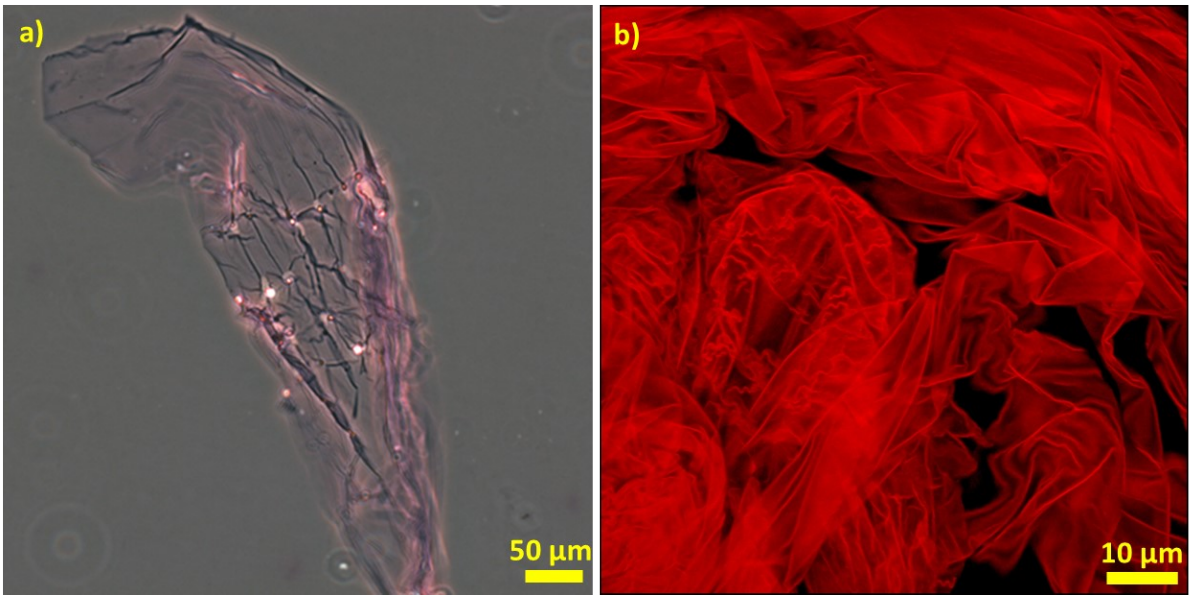

Figure S3: Phase contrast optical micrograph and confocal images of highly flexible structure PEI-RITC sheets (130:10 molar ratio).

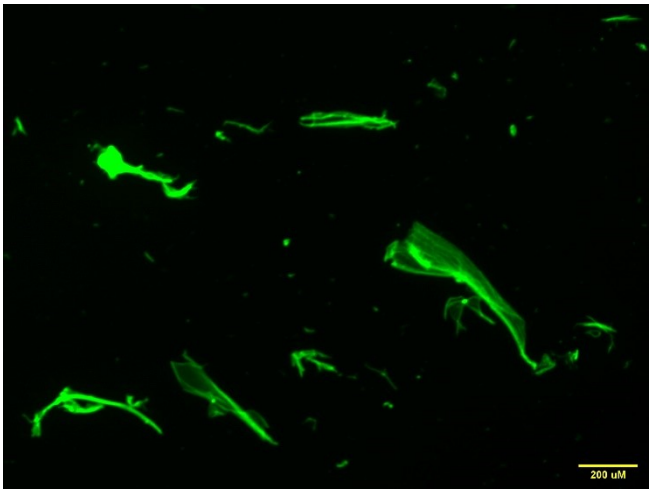

Figure S4: Representative fluorescence micrograph showing PEI-FITC sample (130:10 molar ratio) with many micron sized-sheets.

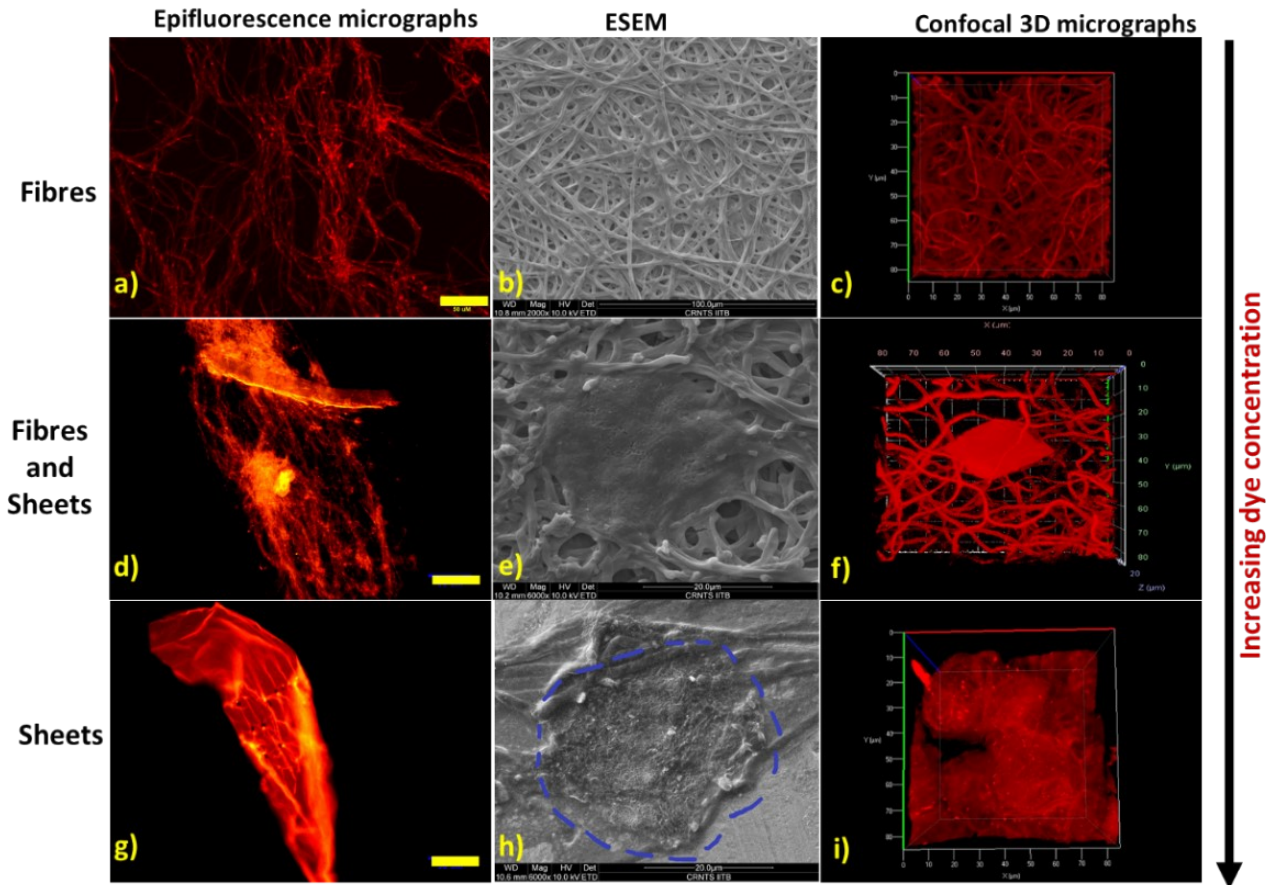

Figure S5: Imaging of fibres and sheets formed in solutions containing PEI labelled with RITC using fluorescence microscopy (a, d, g), environmental scanning electron microscopy (ESEM) (b, e, h) and confocal microscopy (c, f, i) of distinct kinds of self-assembled structures formed from 65 µM PEI at pH=3 samples kept for 7 days at room temperature. *Note*: b, e, and h images have been taken after drying the sample. (a-c) Micrographs showing presence of only fibres in a sample with an average PEI: RITC concentration ratio as 130:1 (d-f) With ratio 130:3, a coexistence of fibres and sheets is seen. (g-i) With 130:10 or higher number of PEI:RITC ratio, predominantly sheets are seen. Scale bars in all optical micrographs are 50 µm (a, d and g) ESEM are 100 µm ,20 µm and 20 µm for b, e and h, respectively.

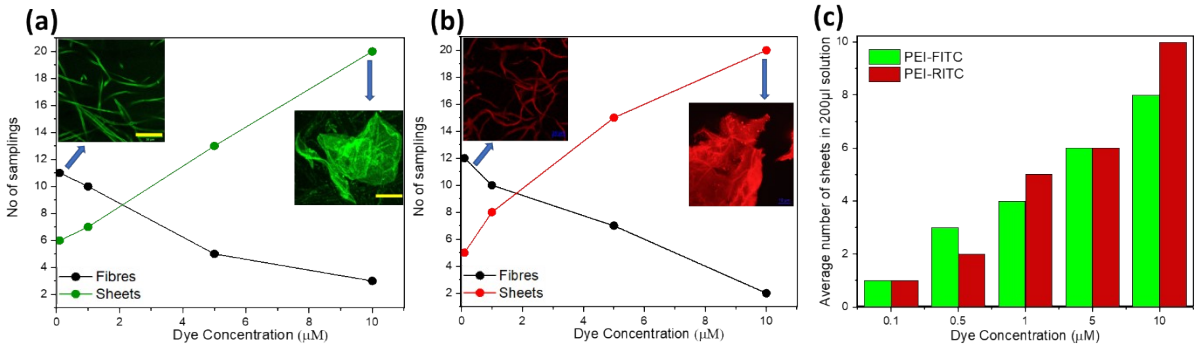

Figure S6: **Quantification of sheets and fibres**: (a,b) Dependence of formation of fibres or sheets on the FITC/RITC dye concentration (from 0.1 to 10 μM) as evaluated using fluorescence micrographs with a 488/555nm laser. (c) Increase in the number of sheets present in FITC/FITC labelled PEI solution as a function of dye concentration observed after 72 hours.

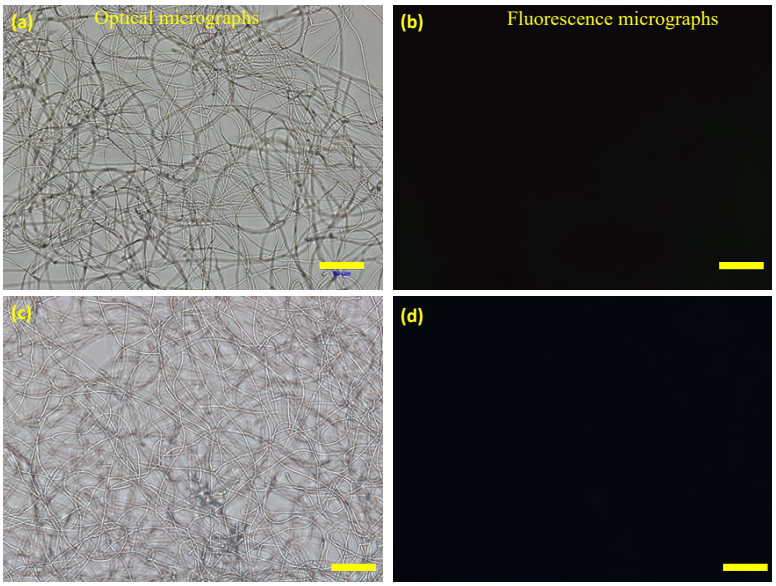

Figure S7: Microscopy images after 6 days from: (a, b) co-incubation of PEI with FITC at pH 3, and (c, d) co-incubation of PEI with sodium salt of fluorescein to PEI at pH 11 for 4-6 hrs, and reducing the pH of the solution to 3. The images show non-fluorescent fibres formed in both cases and sheet formation was not observed in any of these two cases. Scale bar in all the images is 50 μm. Also, the 1H- NMR of the sample for case (a) did not show any FITC proton peak on dye addition at pH 3 (Si, Figure S16). This suggested that the covalent linkage of dye to PEI at pH ≥ 9 was an important step for the Π-Π stacking of FITC-PEI chains leading to formation of sheets. FITC covalently binds only to primary amino groups to form a thiourea bond. Therefore, the dye labelling is always done at pH 9 when free amino groups are present in the solution.[3,4]

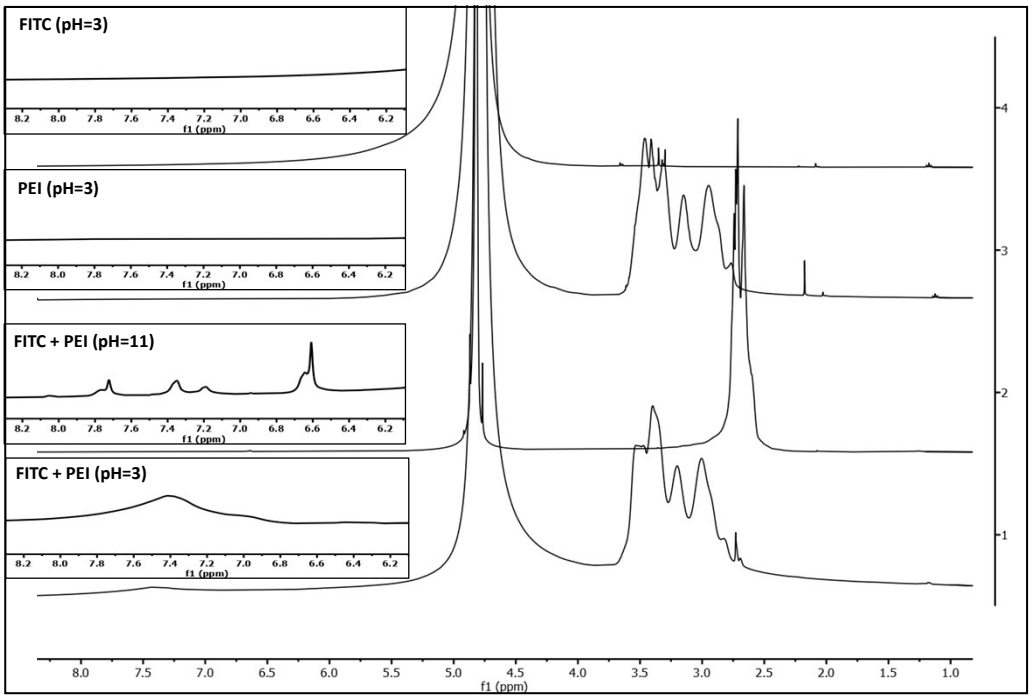

Figure S8: ^{1}H-NMR spectra of FITC in D2O at pH=3 , unlabelled PEI in D_2O at pH 11 and PEI labelled with FITC in 130:10 molar ratio at pH=11 and 3.

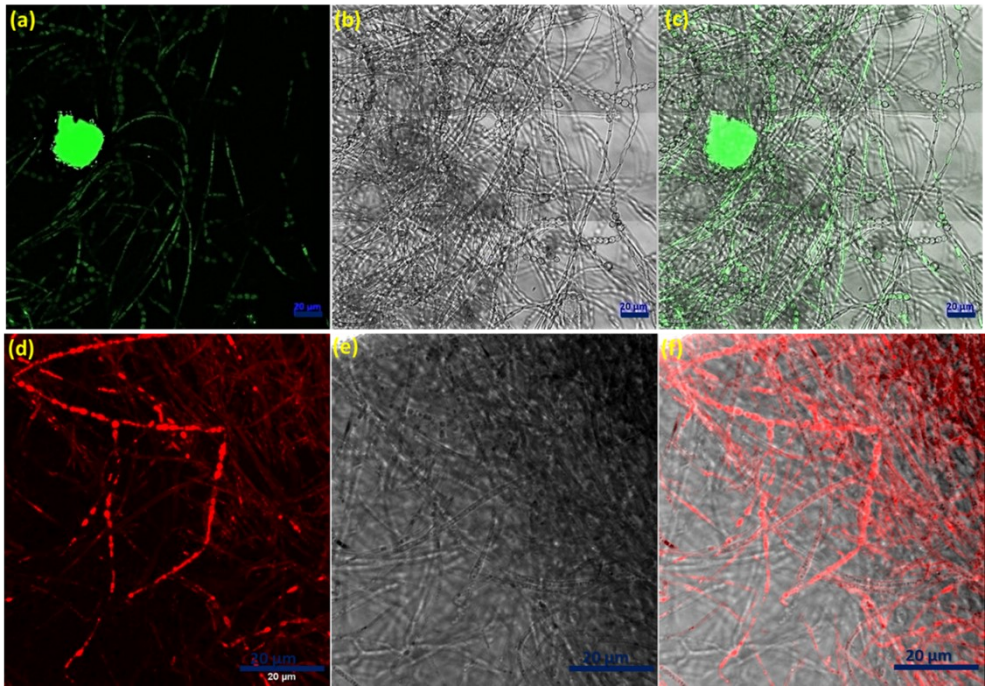

Figure S9: Laser scanning confocal microscopy images of PEI-FITC (a-c) and PEI-RITC (d-f) fibres irradiated with 488nm/555 nm respectively for 10 minutes showing bead like structures; fluorescent image (a,d), DIC image (b,e) and overlapping (c,f). Such constrictions are observed only in certain regions of fibres and indicate heterogeneity in dye labelling as the primary amine: dye ratio is extremely high as shown in the methods.

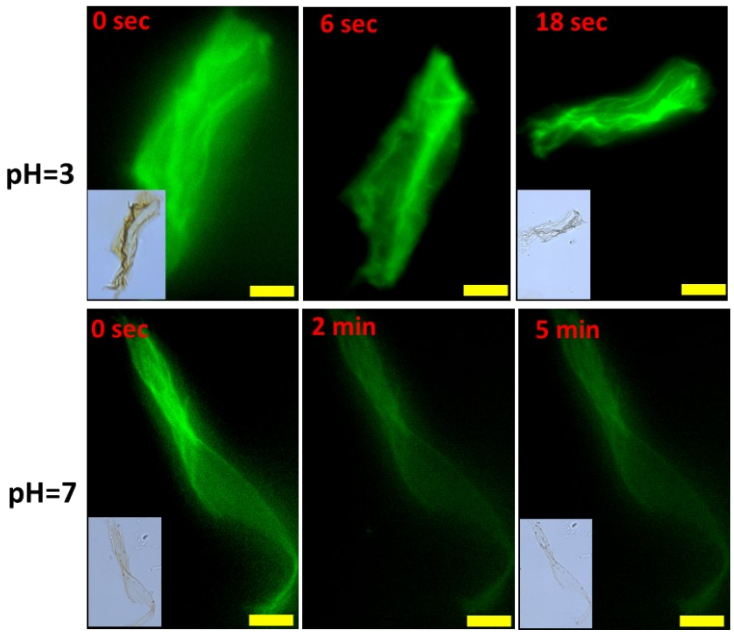

Figure S10: Fluorescence images showing effect of light excitation on sheets at pH=3 and pH=7. Scale bar is 20 µM.

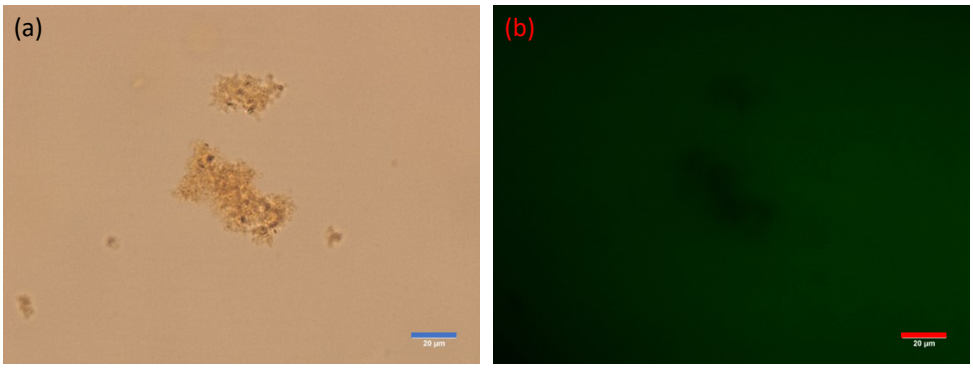

Figure S11: FITC aggregates formed in water (a) optical micrograph (b) fluorescent microscopy image using 488nm excitation light. FITC concentration used is 10 µm.

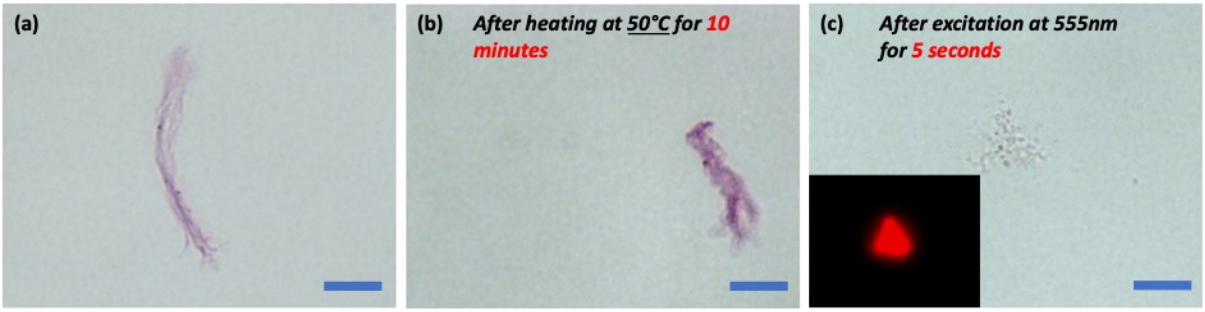

Figure S12: Optical micrographs showing the effect of heating the sheet at 50 °C for 10 minutes (a) before heating (b) after heating , and (c) shows the effect of 5 seconds of excitation with 488nm light on the same RITC-PEI sheet. Scale bar is 50µm. Inset of (c) shows the fluorescence micrograph taken while excitation.

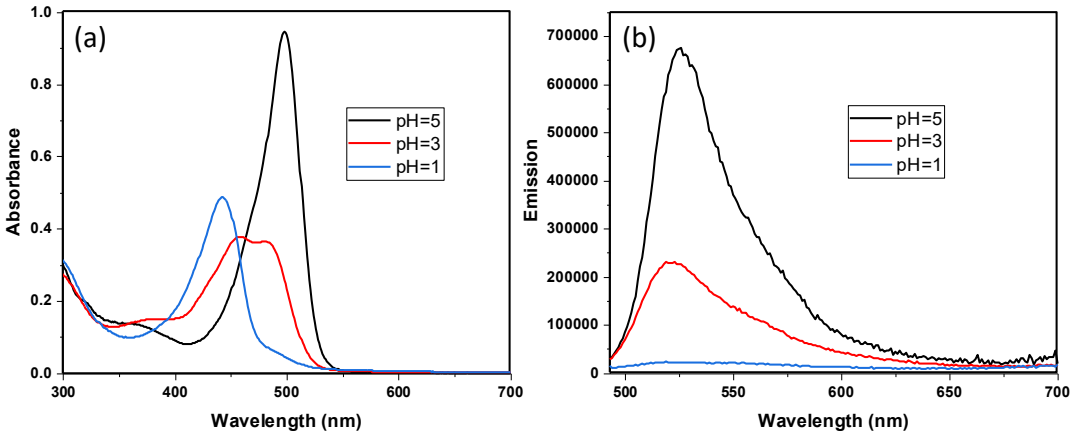

Figure S13: Absorbance (a) and emission at 488 nm excitation (b) studies done on FITC-PEI sample at pH=5,3 and 1. PEI and FITC concentrations used are 65µm and 5µm, respectively.

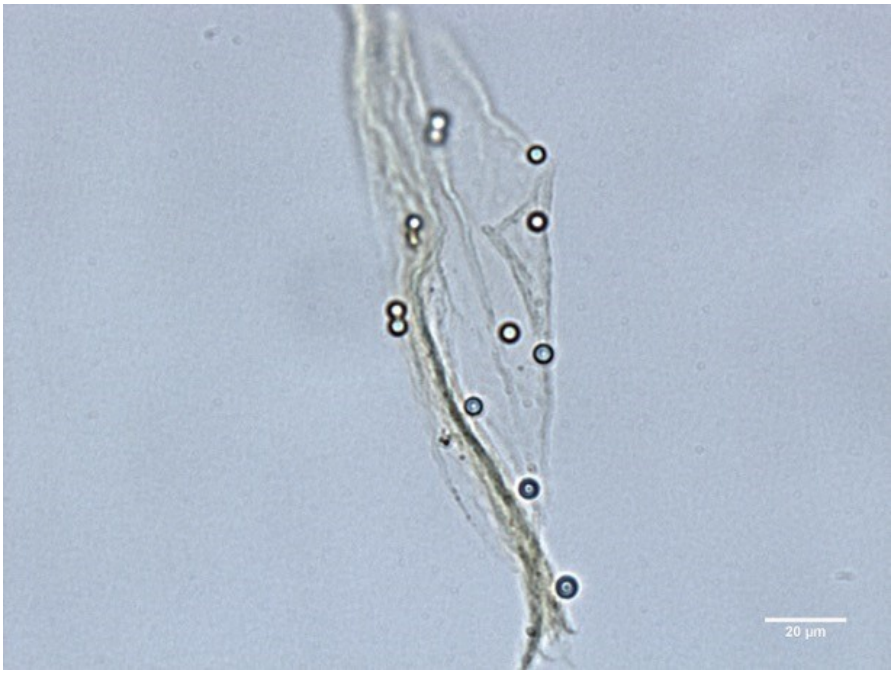

Figure S14: Figure showing negatively charged microparticles adhering to the surface of PEI-FITC films.

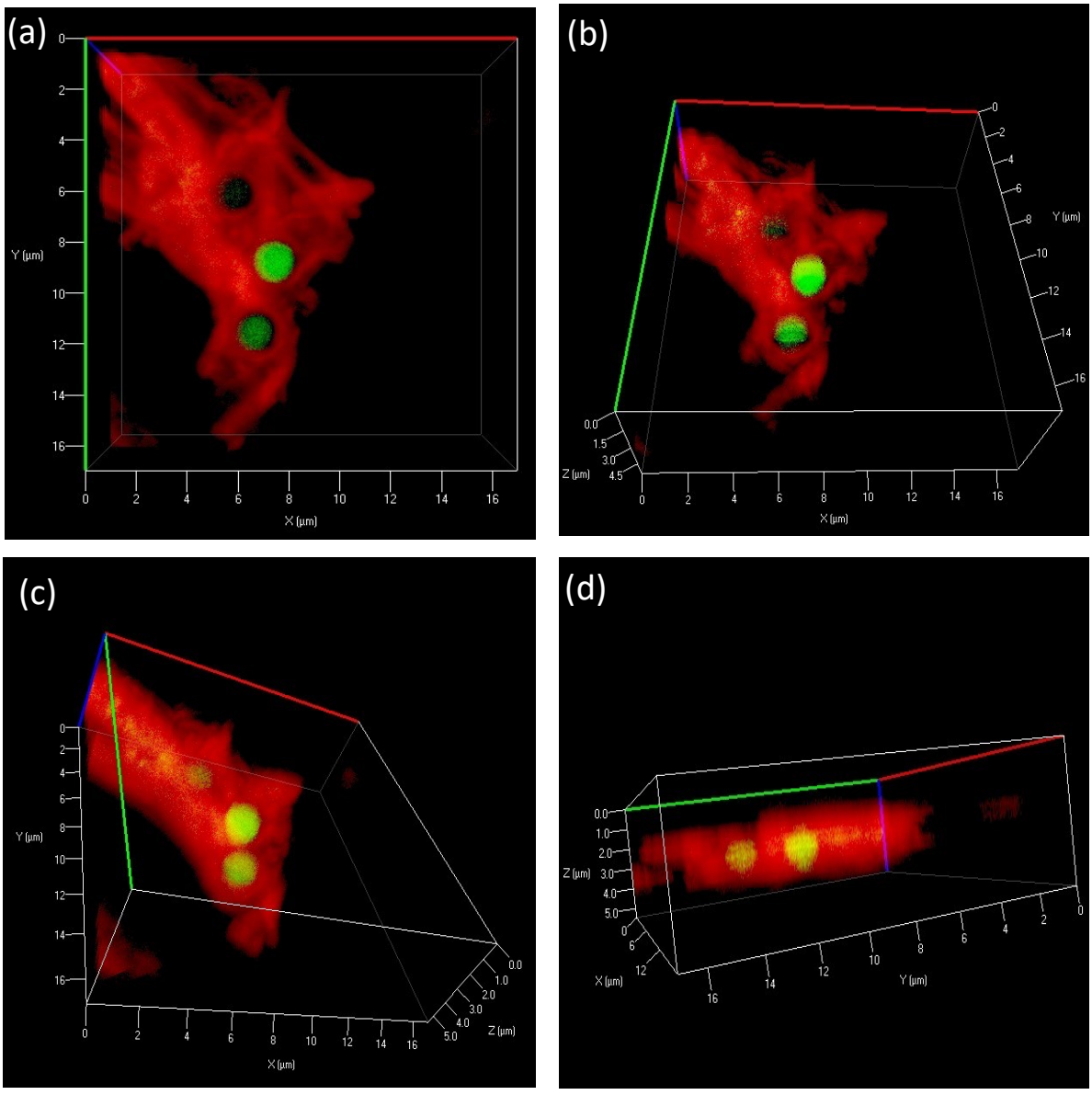

Figure S15: Snapshots of 3D projection of PEI-RITC sheets onto latex beads (carboxylate modified polystyrene fluorescent particles) at pH 3. Sequence of events: In an aqueous solution (at pH 3) comprising positively charged PEI-RITC sheets, negatively charged latex particles were added. This led to the latter getting adhered on the surface of sheets due to electrostatic interactions. On irradiation with 555 nm light (i.e. excitation wavelength of RITC), excited state proton loss occurred in RITC, with the lost proton being taken up by the PEI chains due to its proton-sponge like buffering capacity. This led to electrostatic attraction between the dye and PEI chains, thus collapsing of the film, which ultimately resulted in the encapsulation of the microparticles within the compact structure.

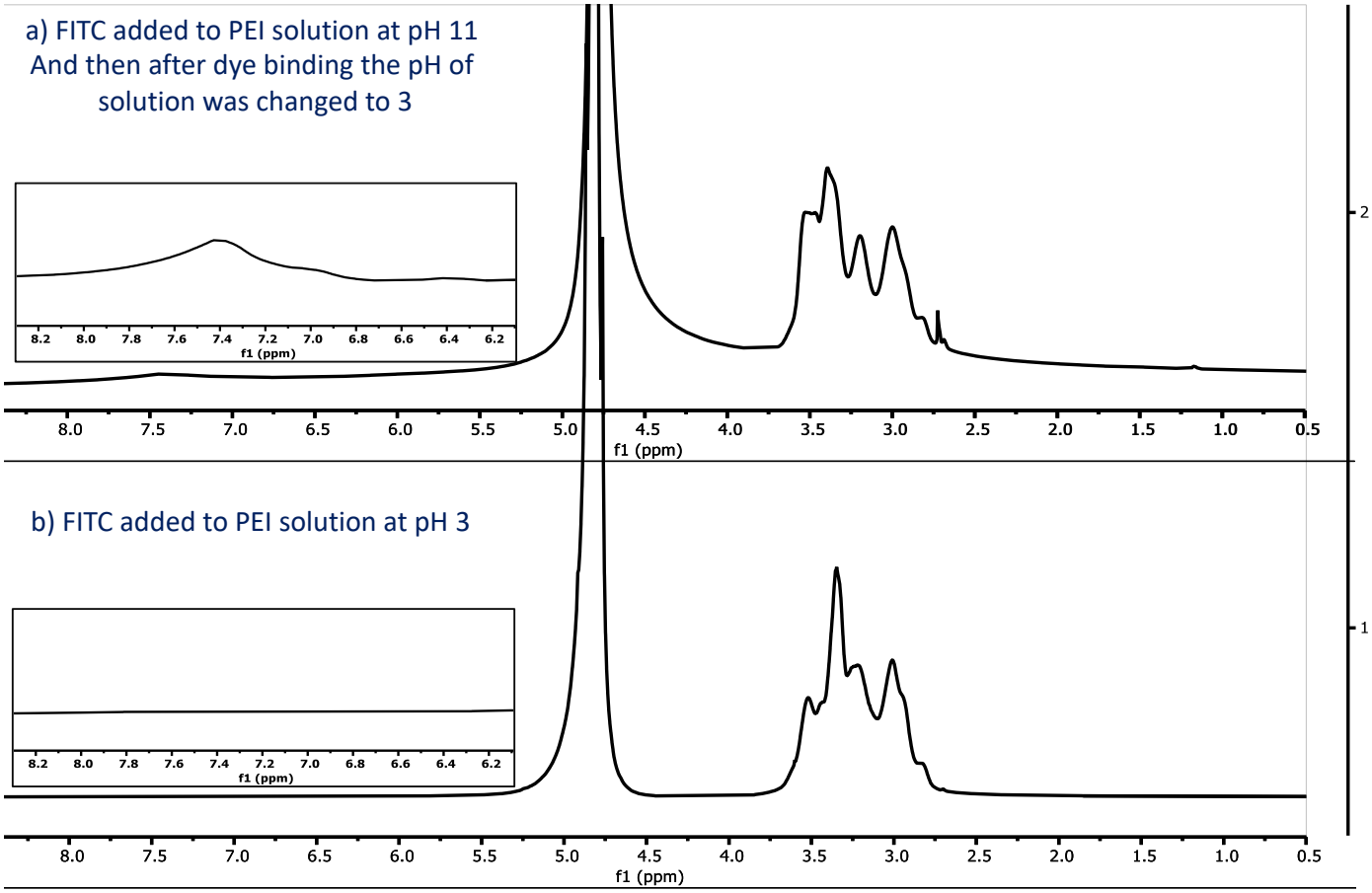

Figure S16: ^{1}H-NMR spectra of pH=3 solution where FITC was added to PEI solution at pH 11 (a) and pH 3(b).

References:

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4) K. F. Geoghegan Modification of amino groups. *Curr. Protoc. Protein Sci.*, **2016**, 86, 1-200. https://doi: 10.1002/cpps.17