

Supporting Information

Brushed polyethylene glycol and phosphorylcholine for grafting nanoparticles against protein binding

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Supplementary Methods

Materials

All chemicals were obtained from Sigma-Aldrich and used as received unless otherwise specified. Dimethyl(2-hydroxyethyl) phosphonate was purchased from Apollo Scientific and used to synthesize RAFT agents. 2-2'-azobisisobutyronitrile (AIBN) and 4-4'-azobis(4-cyanovaleric acid) (ACVA) were recrystallized twice from methanol prior to use. Oligo(ethylene glycol) methyl ether acrylate with average M_n of 480 g.mol⁻¹ (OEGA) was filtered through an alumina column to remove the inhibitors prior to use. 2-(methacryloyloxy)ethyl phosphorylcholine (MPC) was used as received. Cyanine5 (Cy5) amine was purchased from Lumiprobe.

Analytical Instruments

NMR spectroscopy. Nuclear Magnetic Resonance (NMR) spectra were recorded using Bruker UltraShield 400 spectrometer running Bruker Topspin, version 1.3 and operating at 400.13 MHz for ¹H, 161.96 MHz for ³¹P and 100.62 MHz for ¹³C. Deuterated chloroform (CDCl₃) and deuterated water (D₂O) were used as solvents. All chemical shifts are quoted in parts per million (ppm), referenced to residual solvent frequencies ¹H NMR: CDCl₃ = 7.26, D₂O = 4.79 and ¹³C NMR: CDCl₃ = 77.16. ³¹P NMR spectra were proton decoupled.

Size exclusion chromatography. DMAc Size exclusion chromatography (SEC) was implemented using a Shimadzu modular system comprising a SIL20AD automatic injector, a DGU12A degasser, a CTO10A column oven, a LC10AT pump, a RID10A differential refractive-index detector, and a SPD10A Shimadzu UV/Vis detector. A 50×7.8 mm guard column followed by three KF-805L columns in series (300 × 8 mm linear columns, bead size: 10 μm, pore size maximum: 5000 Å) were used for the analyses. *N,N'*-Dimethylacetamide (DMAc, HPLC grade, 0.03% w/v LiBr) with a flow rate of 1 mL.min⁻¹ and a constant temperature of 50 °C was used as the mobile phase. The samples were filtered through 0.45 μm filters prior to injection. The unit was calibrated using commercially available linear poly(styrene) standards (0.5~2000 kDa, Polymer Laboratories). Chromatograms were processed using Cirrus 2.0 software (Polymer Laboratories). Aqueous GPC analyses were performed on a Shimadzu liquid chromatography system fitted with a Wyatt DAWN Treos LS detector (λ = 658 nm), a Shimadzu RID-10 refractometer (λ = 633 nm) and a Shimadzu SPD-20A UV-Vis detector, using three identical PL aquagel-OH MIXED-M columns in series. Water (80%)/acetonitrile (20%) containing 0.1% of trifluoroacetic acid (TFA) with a flow rate of 1 mL.min⁻¹ and a constant temperature of 40 °C was used as the mobile phase. NovaMALS software (PostNova Analytics) was used to determine the molecular weight characteristics using calculated dn/dc values. The samples were filtered through 0.45 μm filters prior to injection. The unit was calibrated using commercially available linear PEG standards.

Infrared spectroscopy. Attenuated Total Reflectance-Fourier Transform Infrared Spectroscopy (ATR-FTIR) spectra were recorded using a Shimadzu IRTracer 100 Fourier transform infrared spectrometer with a GladiATR 10 single reflection ATR accessory. Spectra were obtained in the mid infrared region of 4000 – 600 cm⁻¹ at a resolution of 8 cm⁻¹ (512 scans) and analyzed using the LabSolution IR software.

Synthesis

RAFT. We synthesized IONPs grafted with bPEG, bPC, and their mixture to determine their protein antifouling properties. Briefly, phosphonic acid terminated poly(OEGA) and poly(MPC) were synthesized by RAFT polymerization¹. Then, the polymers were attached to IONPs utilizing the strong affinity of the phosphonic acid group for the iron oxide surfaces. To facilitate the tracking of NPs in applications, we also attached Cy5 fluorophores to bPEG (Fig. 2A, main text). Specifically, the RAFT polymerization of a first block of OEGA was followed by a second block of PFP-*stat*-OEGA comprising an activated ester monomer (pentafluorophenyl acrylate PFPA), leading to a poly(OEGA-*b*-(PFP-*stat*-OEGA)) block copolymer. Then, the substitution of pentafluorophenyl groups by fluorophore Cy5 was realized to render a Cy5-PEG polymer. Finally, the phosphonic ester end group was deprotected by following the same procedure as for poly(OEGA) and poly(MPC) and the final step of grafting of IONPs was accomplished (Fig. 2C, main text). ATR-FTIR spectra (Fig. S3) of Cy5-IONP-PEG confirmed the ability of the labelled polymer to graft IONPs by the spectral presence of the functionalized IONPs of ester and ether bonds at 1730 and 1150 cm⁻¹, respectively. The FTIR spectra of grafted IONPs were consistent with the functionality of the surface confined polymer, together with an additional absorbance at 600 cm⁻¹ attributed to iron oxide. Transmission electron microscopy (TEM) imaging was performed in order to determine the primary size distributions of the IONPs (Fig. 2B, main text). The IONPs were spherical in shape and 11.4 ± 3.6 nm in diameter in all cases. No coating layers were visible under TEM due to the low electron densities of PC and PEG.

Nanoparticles. IONP-PEG and IONP-PC were synthesized as our recent publication². The synthesis of fluorescent Cy5-PEG polymer was carried out by the following method. A first block of poly(OEGA) was synthesized using a stoichiometry of [OEGA]₀: [AIBN]₀: [RA]₀ = 10:0.1:1. Briefly, in a glass vial equipped with a magnetic stirrer bar and capped with a rubber septum were added 0.1 g of RA (0.27 mmol), 1.28 g OEGA (2.67 mmol), 4.4 mg of AIBN (0.025 mmol) and 6.4 mL of toluene. After deoxygenated for 30 min at 0 °C by sparging with N₂, the polymerization was run with stirring for 4 h at 65 °C. The resulting mixture was then allowed to cool to 0 °C and exposed to air to stop the polymerization. The monomer conversion of 76% was determined by ¹H NMR spectroscopy. Then, without any purification, 0.49 g of pentafluorophenol acrylate (1.71 mmol) was added to 6.0 g of the previous solution for the polymerization of the second block using a stoichiometry of [OEGA]₀: [PFP]₀: [AIBN]₀: [poly(OEGA)]₀ = 2:10:0.1:1. After 30 min of sparging with N₂ at 0 °C, the polymerization was run for 5 h at 65 °C under constant stirring. The resulting mixture was then allowed to cool to 0 °C and exposed to air to stop the polymerization. The monomer conversion of 50% for PFP and 47% for OEGA were determined by ¹H NMR spectroscopy. The polymer was purified of unreacted monomer by 5 precipitation/centrifugation cycles into a large quantity of a 3:2 (v:v) mixture of diethyl ether and petroleum benzine (bp 60~80 °C) and then placed in a vacuum oven overnight at 35 °C to remove remaining solvent. The final product was analyzed by ¹H and ¹⁹F NMR in CDCl₃ to determine the composition by following the method used in the literature³.

The grafting of Cy5 to the previously synthesized poly(OEGA-*b*-(PFP-*stat*-OEGA)) was carried out using the following procedure. 225 mg of copolymer (Mn~5885 g.mol⁻¹, 0.191 mmol of PFP units) was dissolved in 3 mL of dry DMF in a vial equipped with a magnetic stirrer bar and covered with aluminium foil. Then, 25 mg of Cy5 amine (0.038 mmol) and 5.4 µL of triethylamine (0.038 mmol) were added and the solution was degassed with N₂ for 15 min and the stirring was continued for 24 h. After reaction, the Cy5 grafted copolymer was precipitated using a large quantity of a 3:2 (v:v) mixture of diethyl ether and petroleum benzine (bp 60-80 °C). The grafting was confirmed using UV detection at 648 nm of the DMAc GPC (Fig. S4).

The deprotection of the phosphonate group was carried out as follows: in a glass vial equipped with a magnetic stirrer bar, nitrogen inlet and covered with aluminium foil was added 0.1 mmol of Cy5-PEG polymer dissolved in 3 mL of DCM. The solution was stirred at 0 °C under nitrogen and a solution of trimethylsilyl bromide (TMSBr, 0.1725 g, 147.5 µL, 1 mmol) in 2 mL of DCM was added dropwise. The reaction mixture was stirred for 24 h at room temperature. At the end of the reaction, the large excesses of bromosilane and DCM were removed by evaporation under low pressure. After the total elimination of bromosilane, ethanol (5 mL) was added to the flask and the stirring was continued for 24 h at room temperature. Then, the final deprotected Cy5-PEG polymer was precipitated into a large quantity of petroleum benzine (bp 60~80 °C). The remaining solvent was removed in a vacuum oven overnight at 35 °C and the final product was analyzed by ¹H NMR and ³¹P NMR (Fig. S5).

Experimental characterizations

Fluorescence quenching. A typical procedure for the grafting of fluorescent polymer Cy5-PEG to IONPs is described as follows. 1 mL of an aqueous dispersion of 10 mg/mL of IONPs (10 mg) was diluted with methanol (5 mL). Then, a solution of 15 mg of phosphonic acid terminated

Cy5-PEG and 15 mg of phosphonic acid terminated poly(MPC) polymers in methanol (5 mL) was added slowly to the IONP dispersion. The resulting dispersion was sonicated for 15 min (power = 25 W), followed by incubation in a shaker overnight at 50 °C. The mixture was centrifuged using an Eppendorf Centrifuge 5804 for 30 min (14,000 rpm/min) to isolate the functionalized IONPs from the base of the centrifuge tube. The supernatant was removed, and the particles were redispersed in methanol using sonication for 10 min. This washing process was repeated 5 times. Then, the same process of washing was used to replace methanol by Milli-Q water to give a final concentration of 10 mg/mL of functionalized IONPs. Absorption spectroscopy (Fig. 3A, main text) was used to analyse the fluorescence of Cy5-labelled IONPs. After grafting of 50% Cy5-PEG and 50% PC polymers, the total absence of a peak at 660 nm was attributed to the quenching of Cy5.

Thermogravimetric analysis. TGA analysis of the synthesized IONPs was performed using a PerkinElmer Pyris 1 TGA and Pyris 1 software. IONPs, or IONPs grafted with PEG, PC or PEG-PC (50:50) (1 mg/mL) were incubated overnight with HSA (1 mg/mL, Sigma-Aldrich) at 37 °C on a rotator at 100 rpm. The suspensions were centrifuged for 10 min at 16,000 g and the supernatants were removed and pellets freeze dried. The obtained powders (2-2.5 mg) were then analyzed by TGA. The weight losses of the samples were measured from 30 °C to 600 °C at a rate of 20 °C min⁻¹. The percentages of weight losses were calculated by the differences between the sample weights at 600 °C and 30 °C. The percentages of HSA adsorption onto the IONPs were then derived according to the differences in weight loss between the particles with and without HSA.

Transmission electron microscopy. The primary sizes and morphologies of the IONPs were examined by TEM imaging. 5 µL of IONPs, either bare, coated with PEG, PC or PEG-PC (50:50) (at 0.1 mg mL⁻¹) were pipetted onto 100 mesh copper grids (Formvar film, ProSciTech) and allowed 60 s of adsorption. Excess samples were then drawn off using filter paper and the grids were washed twice using 10 µL Milli-Q water, with excess drawn off as previously described. The grids were air-dried as needed. The samples were examined using a Tecnai G2 F20 Transmission Electron Microscope (FEI, Eindhoven, The Netherlands), operating at a voltage of 200 kV. Images were recorded using a Gatan UltraScan 1000 P 2k CCD camera (Gatan, California, USA) and Gatan Digital Micrograph 3.9.5 software. The primary sizes of the IONPs were analyzed by ImageJ software.

Computational modelling

Discrete molecular dynamics simulations. DMD is a special type of molecular dynamics algorithm where we applied approximate step-wise potential functions instead of continuous potentials. A detailed description of the DMD algorithm can be found elsewhere.^{4,5} Briefly, we used a united atom representation and an implicit solvent to model the ligand and proteins, explicitly modelling all polar hydrogen and heavy atoms. The interatomic interactions included *Van der Waals*, solvation, electrostatic interactions and hydrogen bond.⁶ The solvation energy was estimated with the Lazaridis-Karplus implicit solvent model, EEF1.⁷ The force field parameters for non-protein molecules were tabulated from the binding affinities between small-molecule ligand and proteins.⁸ The distance- and angular-dependent hydrogen bond interaction was modelled using a reaction-like algorithm.⁹ Screened electrostatic interactions were modelled by the Debye-Hückel approximation. A Debye length of 1 nm was used by assuming a water

dielectric constant of 80 and a monovalent electrolyte concentration of 0.1 M. The Anderson's thermostat was used to for the constant temperature simulations.¹⁰

The autocorrelation of a polymer is calculated by averaging the DMD trajectories with the following equation:

$$C_i = \frac{1}{T(N-i)} \sum_T \sum_{j=0}^{N-i-1} \mathbf{r}_j(T) \cdot \mathbf{r}_{j+i}(T), \quad (1)$$

where $\mathbf{r}_i(T)$ denotes the unit vector between monomer unit $i+1$ and i along the polymer backbone at time T .

We used three structural parameters, including end-to-end distance, radius of gyration Rg , and ellipticity e , to compare the shapes of linear PEG, bPEG and bPC polymers. The end-to-end distance was defined between atoms locating at the two ends of the polymer (e.g., Fig. 1A, atoms a&b). Both Rg and ellipticity for a polymer conformation can be obtained from diagonalizing the 3×3 inertia tensor, which results into three eigenvalues $I_x < I_y < I_z$:

$$Rg = \sqrt{(I_x + I_y + I_z)/m}$$

$$e = \sqrt{1 - (I_x/I_z)}, \quad (2)$$

where m is the mass of the polymer. All the polymers were quickly relaxed, followed by the production simulation for a total of 50 ns. The averaged Rg for the linear PEG were computed from 5,000 snapshots evenly picked from the last 25 ns. Error bars were represented by the standard deviations.

We performed DMD simulations of all three grafted NP systems that each comprised of 50 ligands. Thus, the ligand density was ~ 1.14 chains/nm² for the 7 nm NP and ~ 0.57 chains/nm² for the 10 nm NP. As in the modelling of bSAM in Ref.¹¹, the NP was modelled by a single coarse-grained atom and the phosphate atom of each ligand (Fig. 1A, atom b) was constrained to the surface with its distance to the NP center within the range of [35.0, 35.5] for the small NP and [50, 50.5] for the larger NP. Initially, each ligand was oriented outward with the head-tail vector along the radial direction. We used the spherical coordinate (θ, ϕ) to position a ligand. In terms of the ideal mixed state, for each ligand added to the NP surface, we randomly generated θ within $[0, \pi]$ and ϕ within $[0, 2\pi]$. Ligands with atomic clashes (< 2.5 Å) to previous ligands were discarded until all ligands were positioned to the surface. In terms of the separated state, we constrained $\theta < \pi$ for PC and $\theta \geq \pi$ for PEG respectively while ϕ was still within $[0, 2\pi]$. Once all the ligands were “grafted”, a relaxation step was performed for the system energy minimization.

To ensure sufficient sampling, we performed 6 independent simulations for each of the three types of IONPs. Each independent simulation lasted 5 ns with an accumulative 30 ns simulation of the molecular system under study. 6,000 snapshots were evenly picked from the 30 ns for the calculation of ligand height.

Umbrella sampling of HSA binding with bPC and bPEG grafted NPs. The structural coordinates of HSA were obtained from the protein data bank (PDB code 1AO6). Basic and acidic amino acids of the HSA were assigned charges corresponding to their titration states at physiological condition (pH=7.4). Arg and Lys residues were assigned +1, Asp and Glu assigned -1, and His neutral. Counter ions (Na) were added to maintain the net charge of the systems zero and accounted for possible counter-ion condensation¹².

In order to reduce the system complexity, all residues of HSA were kept static while the IONPs were free to move. The intermolecular distance between a center residue of HSA and the center of an IONP, r_{center} , was chosen as the reaction coordinate. IONP were assigned with 6 different orientations corresponding to 6 replicas of DMD simulations. Each replica had an infinite square-well bias potential as a function of r_{center} . All the bias potential were assigned to overlap with each other. For the 7 nm IONP system, the first bias potential had a wider range {0, 90.75 Å} because of hard sphere repulsion. The rest bias potentials were centered by 91, 92, 93 ... 125 Å with a square width of 1.5 Å (i.e. {90.25 Å, 91.75 Å}, {91.25 Å, 92.75 Å}, {92.25 Å, 93.75 Å}..., and {119.25 Å, 120.75 Å}). Similarly, for the 10 nm IONP system, the first bias potential had a wider range {0, 108.75 Å} and the rest bias potentials were centered by 109, 110, 111 ..., and 130 Å with a square width of 1.5 Å. Each independent simulation was carried for 5 ns at T=300 K with an accumulative 30 ns simulation. We utilized 6,000 snapshots evenly distributed throughout the 30 ns simulations to compute the intermolecular distances, r_{center} . We applied the WHAM analysis¹³ to estimate the 1D-PMF with respect to the intermolecular distance r_{center} .

For the 10 nm IONP where the bound and unbound state can be separated by a single barrier (Fig. 4B), the binding free energy ΔG can be estimated by integrating the 1D-PMF:

$$\begin{aligned} G^b &= -k_B T \ln \int_0^{d^\ddagger} \exp[-\beta PMF(r)] dr \\ G^u &= -k_B T \ln \int_{d^\ddagger}^{\infty} \exp[-\beta PMF(r)] dr \\ \Delta G &= G^b - G^u = k_B T \ln \int_{d^\ddagger}^{\infty} e^{-\beta PMF(r)} dr - k_B T \ln \int_0^{d^\ddagger} e^{-\beta PMF(r)} dr \end{aligned} \quad (3)$$

where the distance $d^\ddagger$ corresponds to the free energy barrier separating bound and unbound states in the PMF plot, K_B is the Boltzmann constant, and β denotes $1/K_B T$. Assuming the same free energy for the unbound states of both IONPs with HSA, which corresponds to the integration of PMF beyond the barrier $d^\ddagger$, the difference in the HSA binding free energy between bPEG-IONP and bPC-IONP, $\Delta\Delta G$, corresponds to

$$\begin{aligned} \Delta\Delta G &= -k_B T \left\{ \ln \int_0^{d_{bPEG}^\ddagger} e^{-\beta PMF_{bPEG}(r)} dr + \ln \int_0^{d_{bPC}^\ddagger} e^{-\beta PMF_{bPC}(r)} dr \right\} \\ &+ [PMF_{bPEG}(d_{bPEG}^\ddagger) - PMF_{bPC}(d_{bPC}^\ddagger)] \end{aligned} \quad (4)$$

Here, the second term corresponds to the free energy correction of the unbound state, where two molecules are not interacting with each other (both ligands are neutrally charged).

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Table S1. TGA results for IONPs in the absence and presence of HSA.

Sample	Weight loss (%)	HSA (%)
IONPs	4.0 ± 0.9	
IONPs + HSA	18.5 ± 1.3	14.6 ± 0.5
IONP-PEG	8.7 ± 0.5	
IONP-PEG + HSA	14.4 ± 0.8	5.7 ± 0.2
IONP-PC	8.3 ± 1.3	
IONP-PC + HSA	13.2 ± 1.4	4.9 ± 0.1
IONP-PEG-PC (50:50)	8.9 ± 1.3	
IONP-PEG-PC (50:50) + HSA	15.0 ± 1.3	6.1 ± 0.1

Supplementary Figures

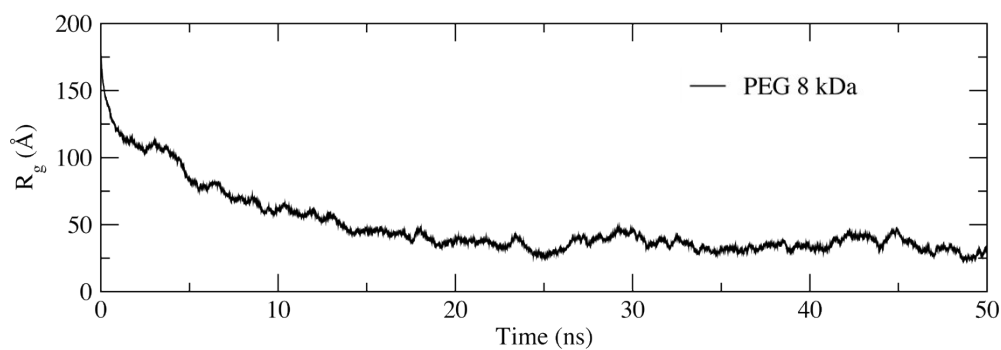


Figure S1. Radius of gyration as a function of time for linear PEG of 8 kDa.

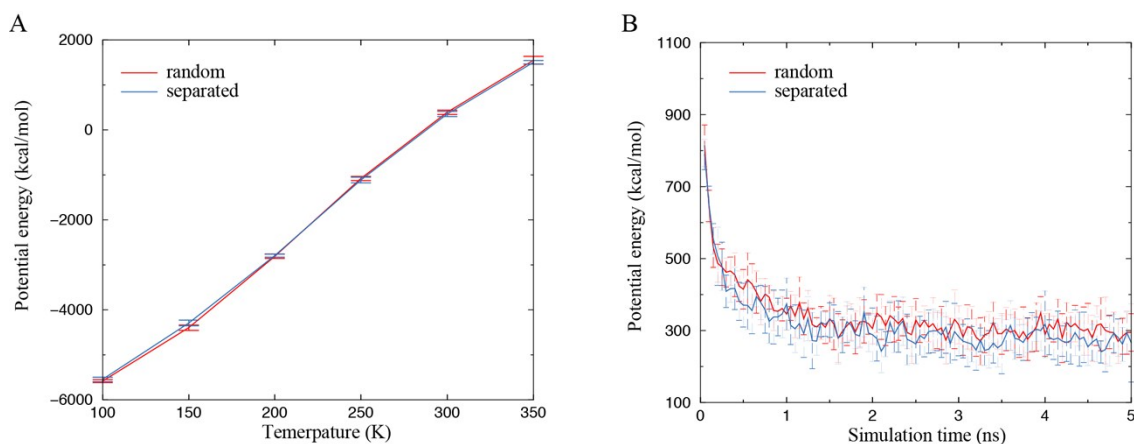


Figure S2. Potential energies at (A) different temperatures and (B) simulation times (300 K). Error bars correspond to standard deviations.

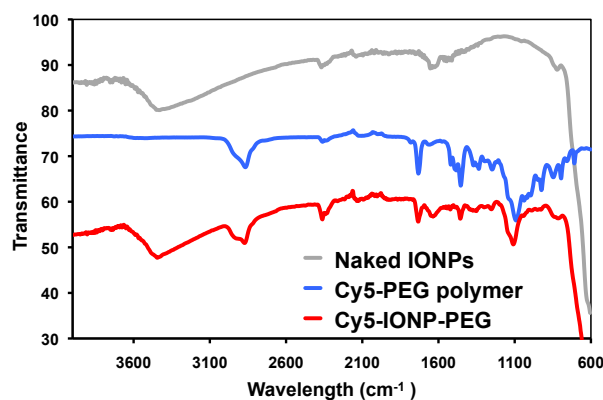


Figure S3. Infrared spectra of naked IONPs (grey), Cy5-PEG (blue) polymer and Cy5-IONP-PEG (red).

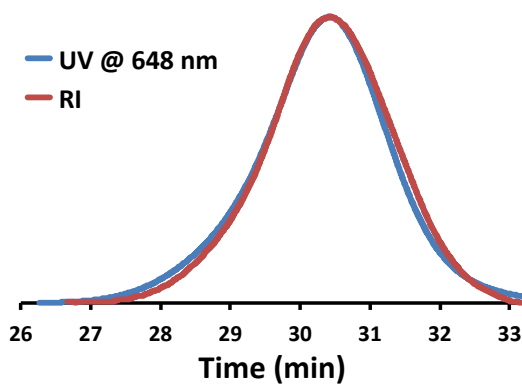


Figure S4. UV (blue) and RI (red) traces of the Cy5 modified *poly(OEGA-b-(PFP-stat-OEGA))*.

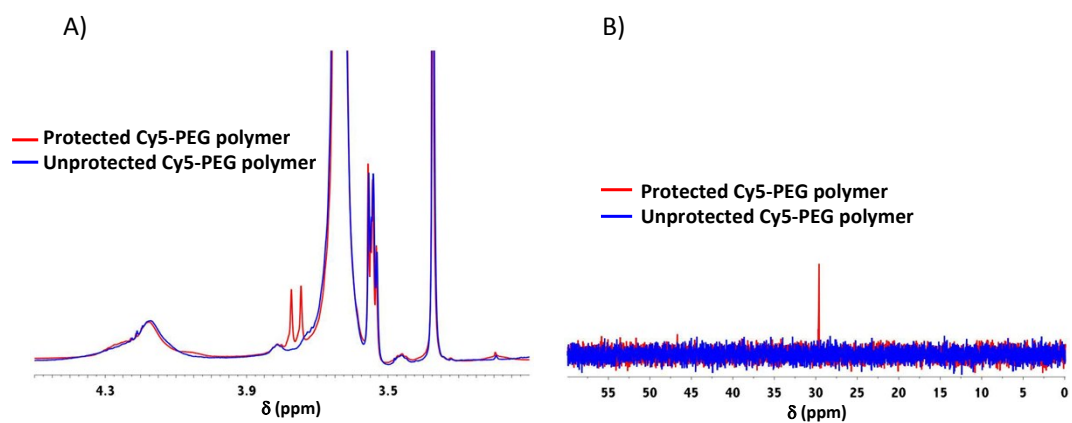


Figure S5. NMR spectra of protected (red) and unprotected (blue) Cy5-PEG polymer. (A) ^1H NMR, (B) ^{31}P NMR.