Supporting Information

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Multifunctional Synthetic Protein Nanoparticles via Reactive Electrojetting

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Experimental Section

Materials: Recombinant Human Serum Album (Cellastim S) was purchased from InVitria. Human Transferrin, Human Hemoglobin, Lysozyme, Human Recombinant Insulin, 2KDa O,O′-Bis[2-(N-Succinimidyl-succinylamino)ethyl]polyethylene glycol (PEG-NHS) or 4,7,10,13,16,19,22,25,32,35,38,41,44,47,50,53-Hexadecaoxa-28,29-dithiahexapentacontanedioic acid di-N-succinimidyl ester (PEG-NHS-S) were acquired from Sigma Aldrich. All buffers, purchased in solution form, and all other reagents used were of lab grade and acquired from Sigma Aldrich or Thermo Fisher.

Electrohydrodynamic Co-jetting: All protein nanoparticles were synthesized using EHD jetting, with differences in the protein solution used and subsequent processing resulting in different copolymer SPNPs. In general, the EHD jetting method was done as previously described[1–4], where a protein solution is pressure driven through a 25G blunt tip needle at a flow rate of 0.1 mL h^{-1}, and a sufficient voltage applied between the needle and a collecting surface to produce a stable Taylor cone. The voltage causes the droplet to be pulled towards the collecting substrate, and the stream subsequently breaks up into nanometer sized spheres. In mid-flight, the solvents rapidly evaporate to form solid nanoparticles. For fluorescent tagging, BSA-Alexa Fluor dyes were incorporated into the protein solution at a concentration of 0.8% w/w of the total mass of protein unless otherwise noted.

SPNP Synthesis Using PEG-NHS and PEG-NHS-S: For SPNPs made with ester-based macromers, a protein solution was made by fully dissolving a protein of interest at 10% w/v in a 90:10 (Ultra-Pure H_2O:EtOH) solution. Depending on the method, PEG-NHS or PEG-NHS-S was added at 10% (w/w of protein mass) to the solution. After EHD jetting, the nanoparticles were placed in a dry 37 °C oven for 7 days, and subsequently collected by scraping them off the collection surface using a solution of DPBS supplemented with 0.01% Tween 20.

SPNP Synthesis Using GA: To synthesize SPNPs polymerized with Vapor-Phase Glutaraldehyde (GA), a protein solution was made by fully dissolving a protein of interest at 10% w/v in a 90:10 (Ultra-Pure H_2O:EtOH) solution. After EHD co-jetting the resulting particles were incubated at room temperature in a closed container, which contained 2.5mL of 20% Glutaraldehyde in a plastic reservoir, for 30 minutes. The unreacted glutaraldehyde was quenched by collecting the particles by scraping them off the collecting surface using Ultra-Pure H_2O supplemented with glycine (100 mM) and 0.01% Tween 20.

SPNP Synthesis Using S-S: SPNPs synthesized through macromer-free disulfide bonds (S-S) were made by dissolving the protein of interest at 2.5% w/v in a 90:10 (2,2,2-Trifluoroethanol: Ultra-pure H_2O) solution, and the protein allowed to denature for 2 hours in order to fully break all disulfide bonds. 2-Mercaptoethanol was then added at a 10x molar excess to the number of disulfide bonds in the protein and allowed to incubate for 30 minutes at room temperature. The solution was then jetted and the resulting particles collected as previously described.

Nanoparticle Collection: After collection, the collected solution was sonicated on ice, run through a 40um cell filter, and then centrifuged at 3200 rcf for 5 minutes to remove large particles. The resulting supernatant was then centrifuged at 21130 rcf for 40 minutes to collect the desired particles. The final particles were washed at least 5 times through centrifugation using DPBS supplemented with 0.01% Tween 20.

Nanoparticle Characterization: Particles, prior to collection, were imaged using Scanning Electron Microscopy (Thermo Fisher Nova 200 Nanolab Dualbeam FIB). Particle diameters were measured using the protocol outlined below. To determine their hydrodynamic size distribution after isolation, the particles were suspended in 0.22 μm filtered DPBS supplemented with 0.01% Tween 20, sonicated on ice, and measured using dynamic light scattering (Malvern ZSP ZEN-5600). Standard settings were used and an average of 3 measurements are reported. Particle zeta potential was measured on the same instrument using a disposable folded capillary cell (DTS1070, Malvern) and using standard settings. Particle concentration was measured using a BCA assay, using a BSA standard for a standard curve. Particle number concentrations were measured using Nanoparticle Tracking Analysis on a Malvern Nanosight.

CD Spectroscopy: Circular Dichroism (CD) spectroscopy was used to study the effects of EHD jetting on the proteins that compose SPNPs prior to polymerization. Fully synthesized SPNPs were not studied using CD spectroscopy due to the method's inability to obtain measurements that can be analyzed with deconvolution based secondary structure analysis from aggregated protein complexes.[150] The different methods used to polymerize the SPNPs were carried out, without the corresponding macromers, for the NHS-PEG, NHS-PEG-S and GA Macromers. As S-S crosslinking occurs immediately during the jetting process, it was not possible to quantitatively study the effect of jetting on the proteins, but since the process for

S-S includes purposeful denaturation of the proteins with a strong organic solvent and a reducing agent, it stands to reason that little to no of the original secondary structure would be maintained after the jetting process.

Particles were jetted, but no macromers were included in the jetting formulations or post-jetting. The particles were then treated identically as they would have been if macromers were added, with incubation at 37°C for 7 days for PEG-NHS based macromers, and storage at 4°C overnight for GA treated SPNPs. Further information about experimental design can be found in **SI 1**. After treatment, the particles were collected and treated following protocols for secondary structure analysis based on deconvolution of CD signals.[151] Briefly, the particles were collected using a 10 mM Potassium Phosphate, 100 mM potassium fluoride (pH: 7.4) buffer. The collected solution was filtered using a 0.22 um syringe filter, and the protein concentration measured using a Nanodrop 2000c Spectrophotometer (Thermo Fisher Scientific), with the absorption at 280nm measured and converted to mass concentration using an extinction coefficient of 85.1 $M^{-1}cm^{-1}$.[152]

The samples were then diluted to a concentration of 0.15 mg/mL, and measured in a 0.1 cm pathlength Hellma quartz cuvette in a Jasco J-815 CD Spectrometer. Temperatures were controlled using a Peltier stage. Spectra were acquired at a stage temperature of 20°C from 185-260 nm, using a data pitch of 0.2 nm, D.I.T. of 1 sec, bandwidth of 1 nm, and a scan speed of 50 nm/min. Each sample was measured for a total of 10 accumulations and was smoothed using Savitzky-Golay algorithm (Convolution Width of 21) and normalized to the buffer. Native and denatured proteins were measured by dissolving undisturbed protein in the same buffer at the nanoparticle samples. A thin layer of mineral oil was placed atop the sample to reduce evaporation, and the sample was measured at 20°C for the native protein control measurement. The sample was then heated to 90°C and subsequently allowed to equilibrate for 5 min prior to measurement for the denatured sample. The smoothed signals were analyzed for secondary structure using DichroWeb.[153–157]

SEM Particle Analysis: The SEM image presented and analyzed post-jetting and prior to resuspension. The images were assessed sequentially for different regions of the samples until the total number of particles assessed were greater than 200 for each formulation (uncrosslinked and crosslinked). The images were then assessed *via* ImageJ (FIJI distribution ImageJ 1.53c). Briefly, the methodology employed is as follows.

1. Intensity histograms of the raw 16-bit images were assessed.
2. Image balancing was performed such that rescaling of the intensity distribution per pixel was set to span the entire intensity range.
3. Images were converted to 8-bit and a single gaussian average was taken (0.5 pixel sigma).
4. Thresholding was performed such that the kept binary image represented all pixels that were more intense than the trailing inflection point on the histogram associated with the background (dark) pixels.
5. Watershed separation of overlapping particles was then performed, with manual separation of unseparated particles based on observations in the raw image.
6. Particle analysis was performed for all systems utilizing an area threshold of >500 nm^2 and a circularity of >0.50, with collection of the Feret diameter, area, and circularity being paramount.
7. Resulting data sets were compiled and untilized for bulk number average statistics (average diameter and sample distribution presented as the standard deviation of diameter).
8. A calculated PDI (polydispersity index), denoted by PDI*, was generated via the following method in order to relate dry state (SEM) data to hydrodynamic state (DLS) data:
    a. The data sets for diameter were binned in 10 nm bins centered on increments of 10 nm.

b. These bins were then converted to arbitrary mass units, using half of the diameter as the radius while converting to volume.

c. Number average, weight average, and z-average molecular weights were determined for each data set.

d. PDI* was then calculated using the number average standard deviation divided by the z-average molecular weight derived diameter (in a accordance with the PDI reported by light scattering methods; PDI = S/dz) allowing direct comparison to the DLS data.

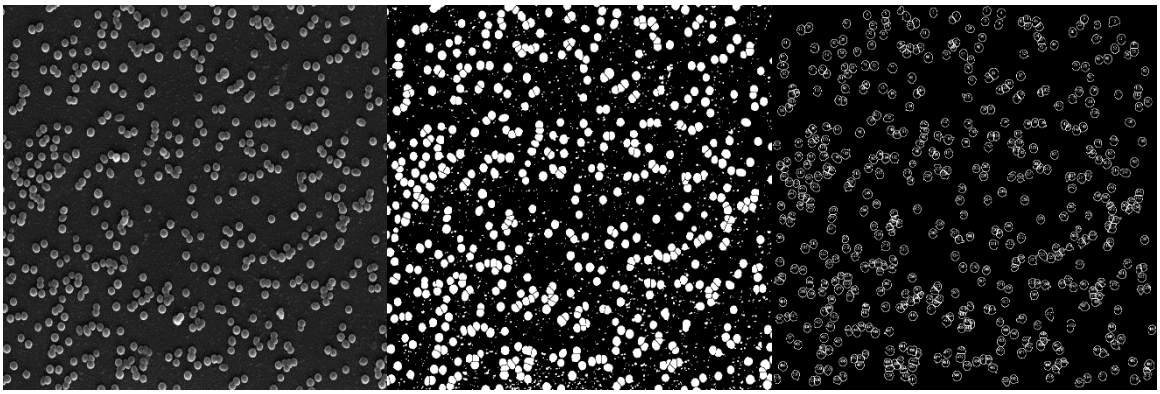


Figure S1. Example of inputs and outputs for particle characterization. Left: well contrasted and calibrated SEM image. Center: Binary representation of thresholded image. Right annotated skeletonized plot of particles for which data was extracted.

Assessment of the resulting SEM-based data sets was performed using GraphPad Prism software (v. 8.4.3), to include all summary statistics and the fitting of diameter histograms for the purpose of comparisons to DLS results. Fitting of the histograms was performed by using the lognormal distribution fuction and the least-square method. All fits were reasonably well correlated to the distributions, having R^2 values ranging from 0.89 to 0.98 and a 95% confidence interval og the geometric mean being less than ±4 nm in all cases. Graphical presentation of the comparison of the SEM particle diameter distribution fitted lognormal equations to the DLS intensity-based results exist for ease of communication; direct comparison between an intensity-based and count-based system via statistical methods would be inappropriate. Instead, result summary statistics (diameter-SEM v. diameter-DLS, PDI* v. PDI) should be compared.

Assessment of the SEM diameter was performed using one-way ANOVA, with Tukey's post-test, between raw data sets. Presentation of the ANOVA results are presented as embedded tables in the figures where this was performed. P-values for failing to accept statistical equivalency between distributions are presented with conventional denotation: np ($p > 0.05$), * ($p \leq 0.05$), ** ($p \leq 0.01$), **** ($p \leq 0.001$), and **** ($p \leq 0.0001$). Assessment of the profiles for DLS results, which are intensity derived and not based on counts, was instead performed with conversion of the intensity data into whole counts. These counts were normalized to n = 200 in order to have statistical significance similar to the SEM data (which was performed until n > 200 was reached).

Cell Culture: HeLa cells (ATCC) were grown at 37C in a humidified environment at 5% CO_2. Cells were cultured in DMEM media supplemented with 10% fetal bovine serum (FBS), 1% Non-essential Amino Acids (NEAA), D-glucose (25 mM), and Sodium Pyruvate (1 mM). Cells were passaged at 70-80% confluence and media changed as needed.

The immortalized human cerebral microvascular endothelial cell line hCMEC/D3 (Millipore Sigma) was grown at 37C in a humidified environment at 5% CO_2. Cells were maintained using the EndoGRO-MV Complete Culture Media Kit supplemented with 1 ng/mL human animal-free basic fibroblast growth factor (bFGF-AF) and 1% Penicillin–Streptomycin. Cells were cultured on collagen-coated tissue culture flasks, which was prepared using a 1:20 dilution of Collagen Type I, Rat Tail, and allowed to coat in the incubator for 1 hr prior to use. Cells were passaged at 70-80% confluence, between passage 27 and 36, and media was changed as needed.

Confocal Microscopy: HeLa cells were seeded in 8 well chamber slides at a concentration of 50000 cells per well and allowed to adhere overnight. SPNPs were sonicated in ice, and immediately added at a concentration of 10 μg mL^{-1} to cell media supplemented with penicillin (100 Units mL^{-1}), streptomycin (100 μg mL^{-1}), and Amphotericin B (250 ng mL^{-1}). 200 μL of SPNP solution was incubated with the particles for 1h. Particle media was removed following incubation, and the cells thoroughly washed with DPBS. Cells were fixed with 4% paraformaldehyde, washed with DPBS, and then stained with Alexa Fluor 488 Phalloidin following manufacturer recommendations. The samples were then air dried, mounted using ProLong Diamond Antifade Mountant with DAPI, and allowed to cure for 24h prior to imaging. Confocal micrographs were obtained using a Nikon A1si inverted confocal microscope. A 60X water objective with excitation at 401, 488, and 641nm for the cell nuclei, actin fibers, and SPNPs, respectively was used for image acquisition. NIS-Elements and ImageJ software was used for image acquisition and processing.

Flow Cytometry: HeLa cells were seeded in a 96-well plate at a density of 30000 cells per well. After overnight incubation at 37°C and 5% CO2, the media was then removed from the

wells, and fresh media containing each SPNPs group at 10 μg mL^{-1} was added to the wells. The cells were incubated with SPNPs for 24h. The cells were washed with DPBS three times and then trypsinized. The cells were washed two more times and stained with DAPI before analyzing them with Cytoflex (Beckman Coulter) cell analyzer located at the Flow Cytometry Core of the University of Michigan. FlowJo software was used for data analysis. Statistical analysis was conducted using a one-way ANOVA, followed by Tukey's post-test, using GraphPad software. A P-value of <0.05 was considered statistically significant (*P < 0.05, **P < 0.01, ***P < 0.001; ****P < 0.0001).

Blood Brain Barrier Transwell Assay: Transwell inserts (6.5 mm, 3.0 μm Pore Polyester Membrane) were coated with 50 uL of diluted human fibronectin to achieve 10 ug/cm^2 and allowed to coat for at least 1hr at 37°C. Upon removing excess coating solution, inserts were washed with Dulbecco's phosphate-buffered saline (DPBS) before immediate use. hCMEC/D3 cells were seeded into the apical compartment at a density of 330,000 cells/mL in 100 uL of complete media. The basolateral compartment was filled with 600 uL of complete media. Inserts were incubated at 37°C, 95% humidity and 5% CO_2. Media was changed every other day. Transendothelial Electrical Resistance (TEER) was monitored throughout the growth. Models were used for particle transport studies after 7 days of culture.

For particle transport studies, 5E10 NP/mL hTF- or HSA-SPNPs were prepared in complete cell culture media and added to the apical compartment of the blood brain barrier transwell insert after the 7 days of culture. The companion plate in the basolateral compartment contained only complete culture media. At each hour timepoint for 6hr, a 100 uL aliquot was sampled from the basolateral compartment and pipetted into a 96-well plate. 100 uL of fresh complete cell culture media was immediately added to the basolateral compartment upon removal. Fluorescence intensities of samples obtained from the basolateral

medium upon 1hr, 2hr, 3hr, 4hr, 5hr and 6hr intervals were measured in triplicate using the BioTek Synergy H1 Hybrid Multi-Mode Microplate Reader. Solute concentration was determined based on calibration curves of standard fluorescence intensities created in triplicate. Percent transport (%) of SPNS were then calculated.

Bicompartmental SPNP Synthesis: Bicompartmental SPNPs were synthesized using GA but using a parallel capillary EHD co-jetting setup previously described.[1–4]

SIM Microscopy: Anisotropic SPNPs with two compartments were synthesized as described above using HSA and hTf. To facilitate imaging of the resulting particles, BSA Alexa 488 and hTf Alexa 647 were incorporated, at 0.08% of the total protein mass, into the albumin and transferrin protein jetting solutions, respectively. GA was used to crosslink the resulting ASPNPs. Albumin-transferrin ASPNPs were collected and purified as previously described and finally suspended directly in Prolong Diamond before being deposited onto glass slides. Samples were allowed to cure for at least 24h prior to imaging. Structured Illumination Microscopy (SIM) imaging was conducted using a Nikon N-SIM +A1R confocal microscope equipped with a 100x objective oil objective. Excitation using the 488 and 647 lasers were used for image acquisition. Three dimensional z-stacks of multiple regions were collected and deconvoluted using the Nikon Elements software. The resulting z-stacks were analyzed to confirm bicompartmental particle architecture.

Statistical analysis of biological studies: Statistical analyses were performed using Graphpad, Prism 8.3.0, (GraphPad Software, LaJolla, CA). One-way analysis of variance (one-way ANOVA), followed by Tukey's post-test was used in the Hela cell SPNP uptake experiment and non-paired, two-tailed *t*-test was used in the blood brain barrier SPNP transport assay to determine significance among groups. A P-value of < 0.05 was considered statistically

significant (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$; ****$P < 0.0001$); P-values of >0.05 were considered not significant (ns).

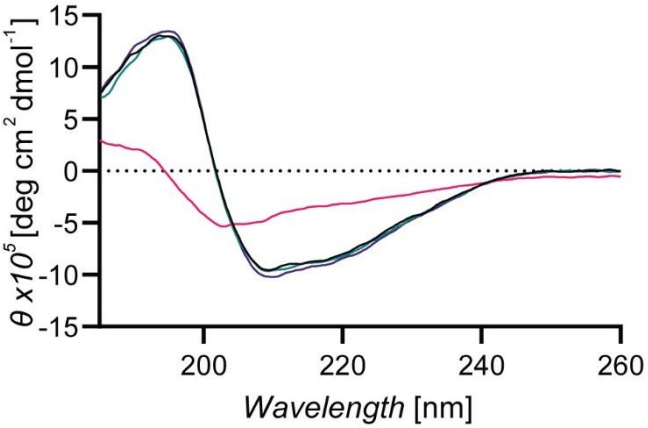


Figure S2. The effect of EHD jetting on proteins was analyzed using Circular Dichroism (CD) Spectroscopy. Transferrin was jetted and treated as described in the text but did not include macromer for NHS-PEG and NHS-PEG-S (purple) or GA (green). Native (black) and heat denatured transferrin (pink) were measured as controls.

Table S1. The measured CD spectra for free transferrin controls and analogues for methods 1-3 were deconvoluted using the different algorithms and neural networks available in Dichroweb. For all but the denatured transferrin, most of the algorithms were able to fit the measured signals within high degrees of certainty, and the ratios of secondary structures were similar to those found using X-Ray crystallography (from PDB). Additionally, the ratios found for all algorithms matched almost identically for the different polymerization methods studied.

Sample	Sample Treatment	Structure	X-Ray	Selcon3	Contin-LL	CDSSTR	K2d
Free Transferrin	Native	α-Helix	0.34	0.29	0.25	0.24	0.29
		β-Sheet	0.19	0.24	0.27	0.30	0.27
		Turn	0.22	0.23	0.23	0.22	ND
		Unordered	0.25	0.28	0.26	0.26	0.43
	Denatured	α-Helix	NA	0.07	0.15	0.06	0.09
		β-Sheet		0.32	0.32	0.33	0.39
		Turn		0.19	0.23	0.27	ND
		Unordered		0.30	0.30	0.33	0.52
Jetted Transferrin	Methods 1 and 2	α-Helix	NA	0.25	0.25	0.24	0.29
		β-Sheet		0.24	0.27	0.28	0.30
		Turn		0.23	0.22	0.22	ND
		Unordered		0.28	0.26	0.26	0.41
	Method 3	α-Helix	NA	0.25	0.25	0.24	0.29
		β-Sheet		0.24	0.27	0.28	0.30
		Turn		0.23	0.22	0.22	ND
		Unordered		0.28	0.26	0.26	0.41

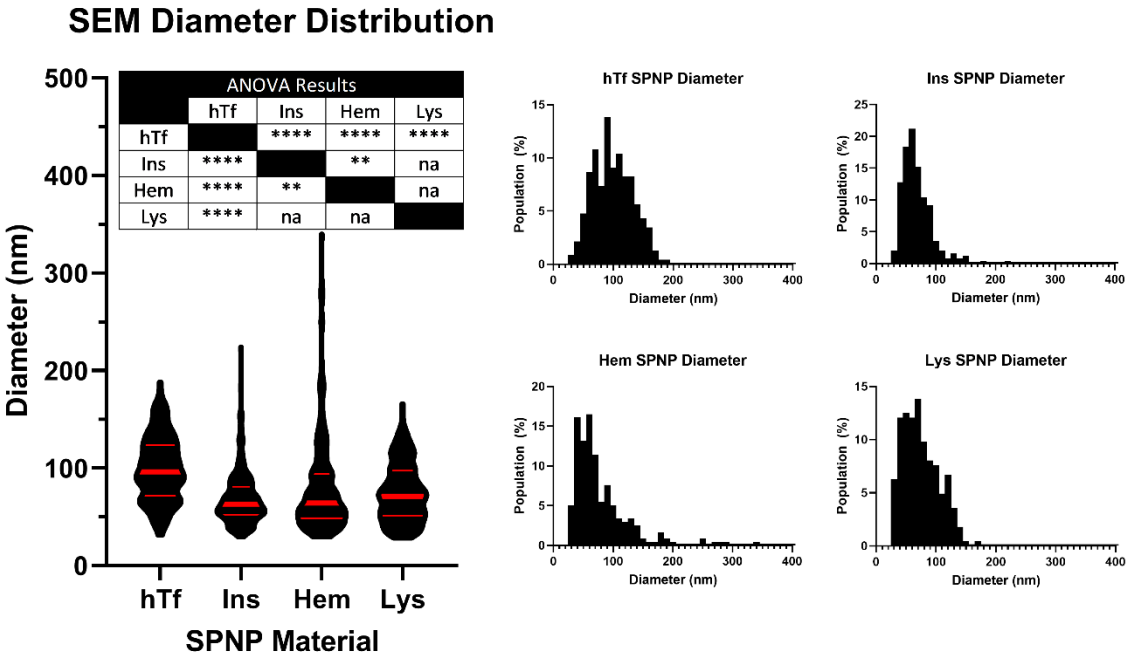


Figure S3. Diameters measured by SEM. Left: Diameter distributions for the SEM micrographs of SPNPs presenting as a count distribution violin graph (with mean and quartile markers in red) and the associated ANOVA results. Right: diameter histograms of SPNP made with Transferrin (upper left) , Insulin (upper right), Hemoglobin (lower left), and Lysozyme (lower right). This data was obtained from the samples presented in Figure 2.

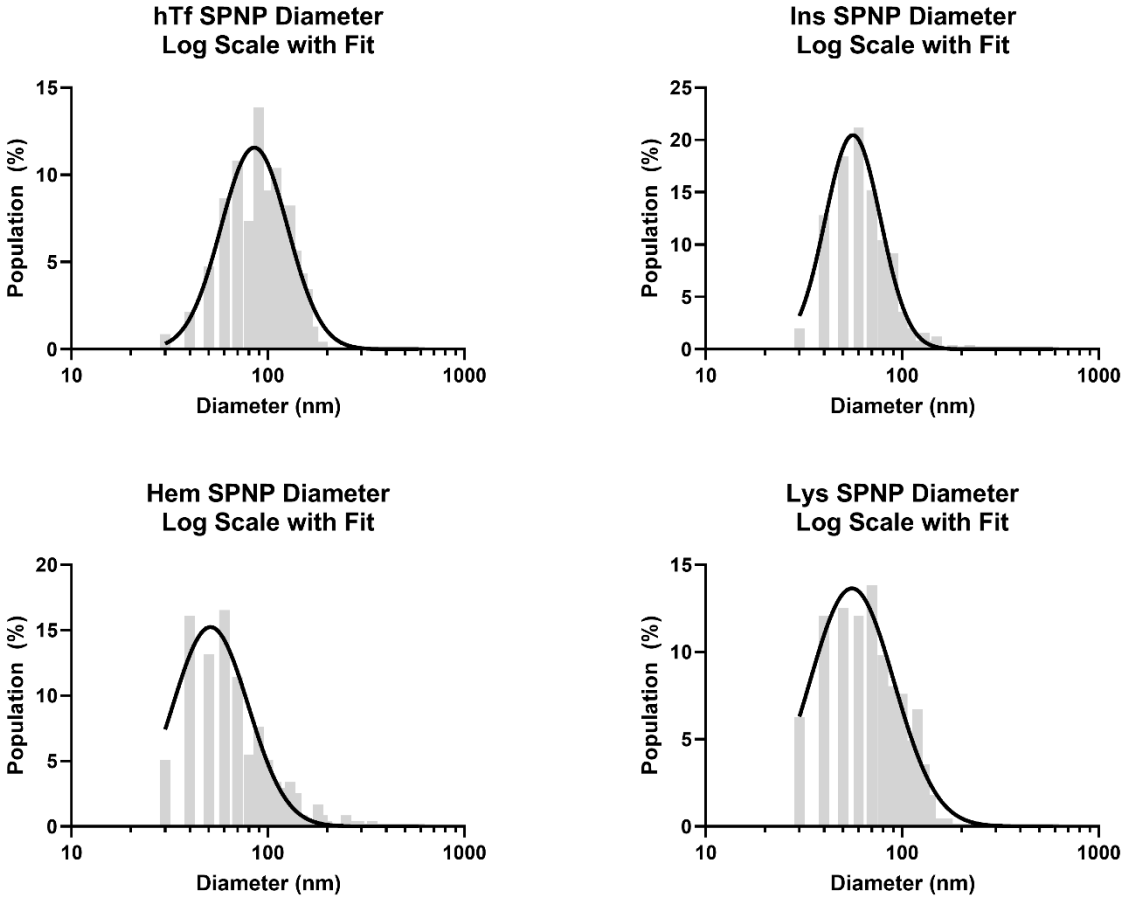


Figure S4. Lognormal fitting of diameter histograms measured by SEM. Fitted diameter histograms of SPNP made with Transferrin (upper left), Insulin (upper right), Hemoglobin (lower left), and Lysozyme (lower right). This data was obtained from the samples presented in Figure 2; for an explanation of the methodology refer to the experimental section.

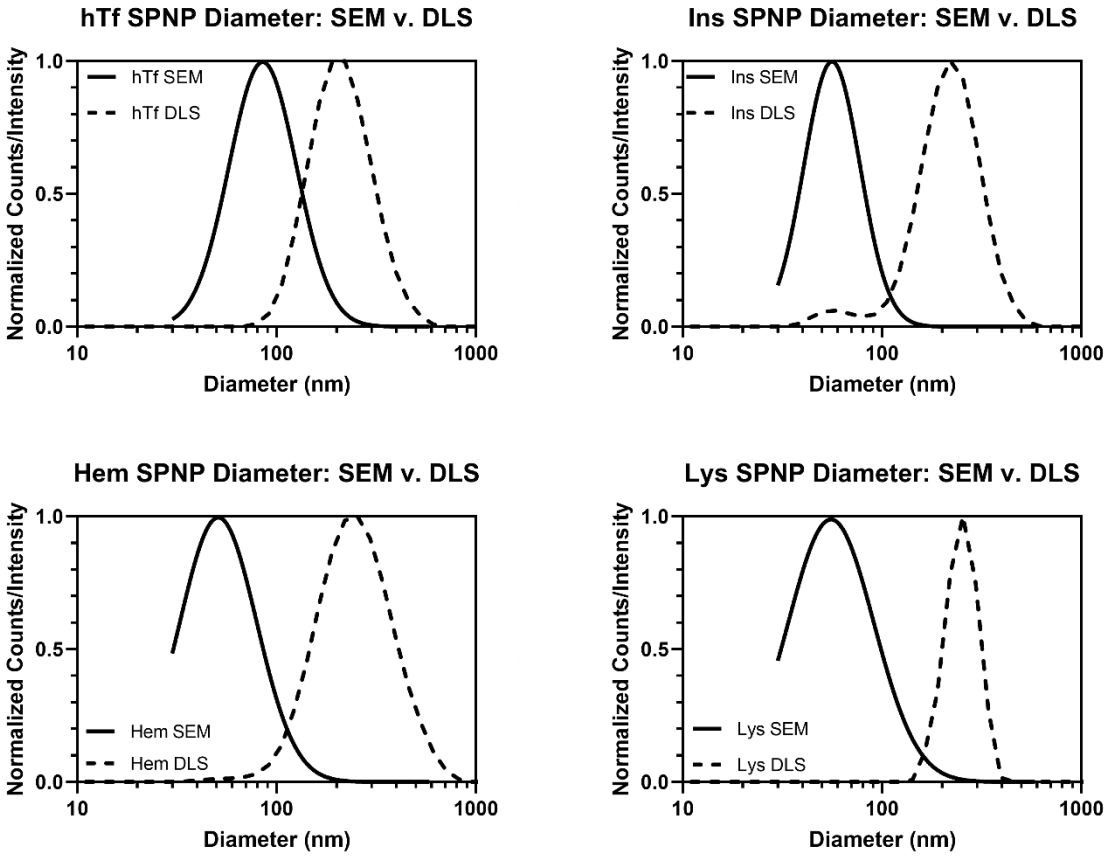


Figure S5. Comparison of fitted SEM diameter data from Figure S4 and the DLS results for SPNP made with Transferrin (upper left), Insulin (upper right), Hemoglobin (lower left), and Lysozyme (lower right). This data were obtained from the samples presented in Figure 2.

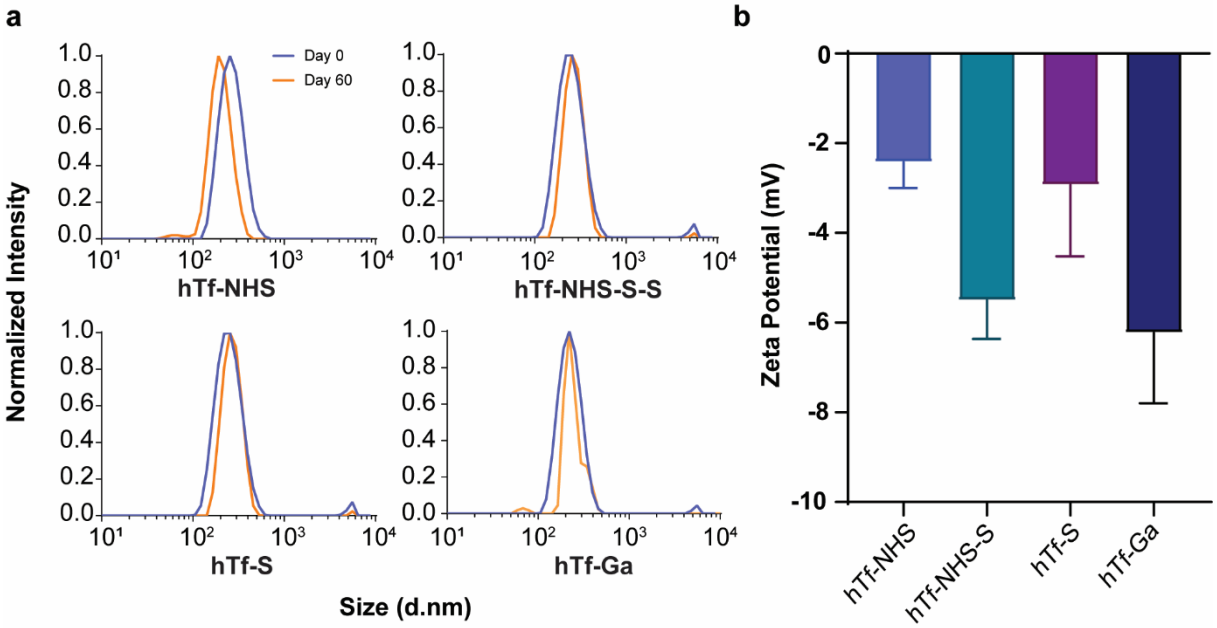
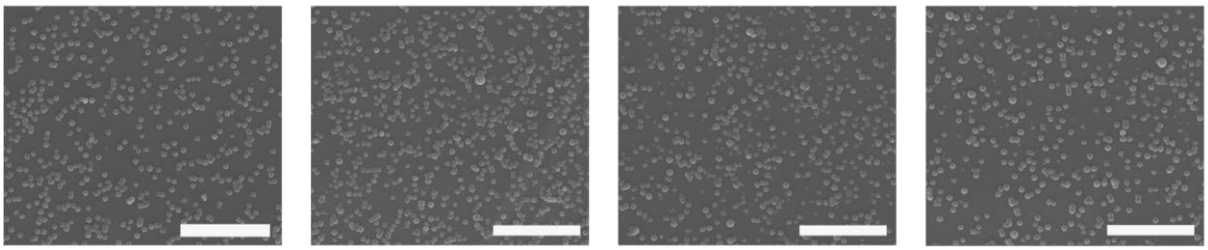


Figure S6. Different macromers do not significantly change size or zeta potential, and are stable over a 1 month period. hTf SPNPs were made with all 4 different macromers, and (A) their sizes after synthesis and 60 days later were measured using DLS. Macromers were found to not affect particle size, and the particles maintained stability over the time period. (B)The same SPNPs were measured using ELS and found to not have significantly different zeta potentials.



10% 20% 30% 40%

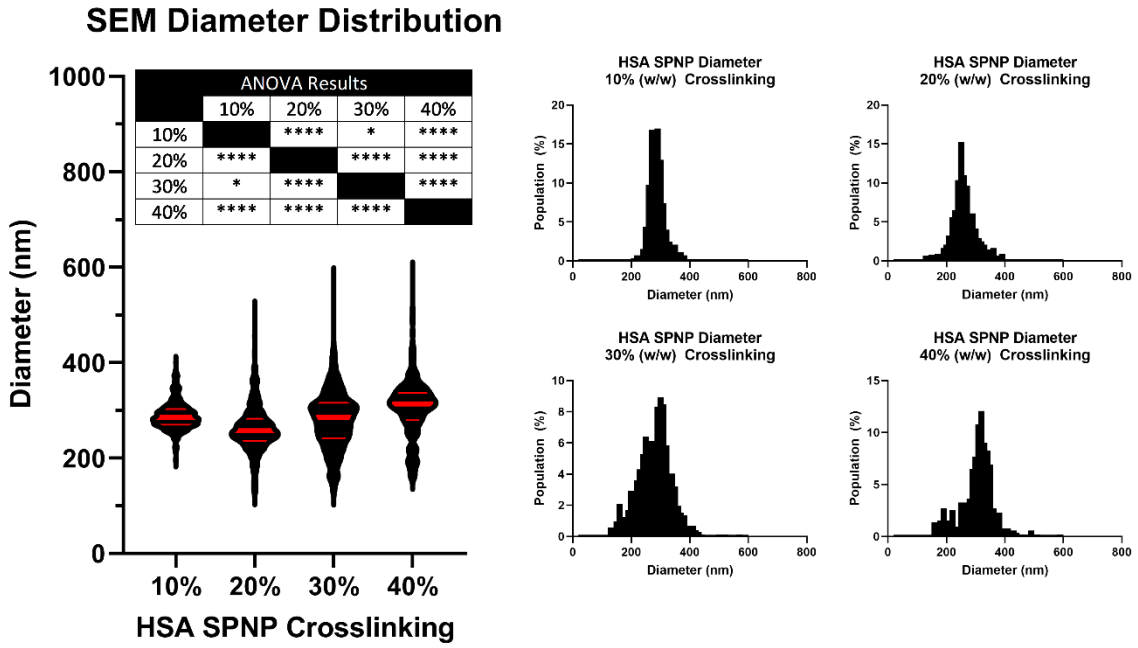


Figure S7. SEM diameters for varied content of crosslinking macromer in HSA SPNPs. Images (left to right): SEM images of SPNPs synthesized with 10%, 20%, 30%, and 40% (w/w%) of crosslinking macromer relative to Human Serum Albumin; scale bars are 4 µm. Lower Left: Diameter distributions for the SEM micrographs of SPNPs presenting as a count distribution violin graph (with mean and quartile markers in red) and the associated ANOVA results. Lower Right: diameter histograms of SPNP made with 10% (upper left inset), 20% (upper right inset), 30% (lower left inset), and 40% (lower right) crosslinking macromer relative to HSA content.

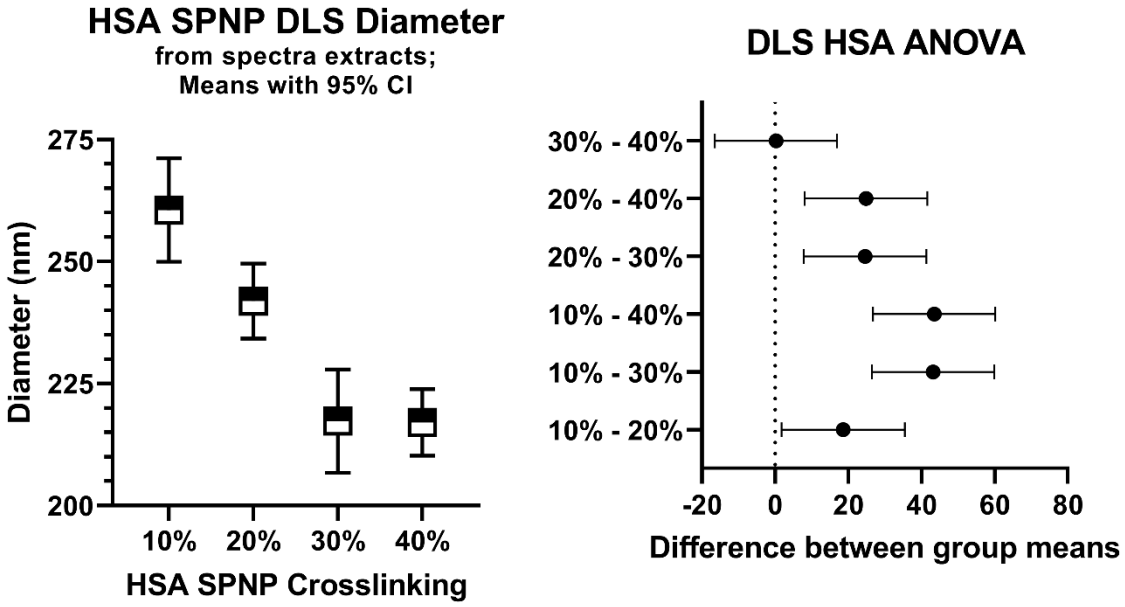


Figure S8. HSA SPNP diameter by degree of crosslinking. Left: statistical means with 95% confidence interval of the error in the mean. Right: Graphical presentation of the ANOVA results indicating that 10% < 20% < 30% ≈ 40% in terms of hydrodynamic diameter. Data were obtained from the samples presented in Figure S7 with explanation of methodology above in the experimental section.

Table S2. Summary dimensional data for all SPNPs presented in this work, to include aging studies.

	SEM Diameters (dry)		DLS Diameters (hydrodynamic)	
	Diameter (nm)	PDI*	Diameter (nm)	PDI
hTf-PEG-NHS	99 ± 33	0.20	223 ± 12	0.23
Ins-PEG-NHS	68 ± 27	0.15	224 ± 25	0.47
Hem-PEG-NHS	79 ± 49	0.16	269 ± 21	0.26
Lys-PEG-NHS	75 ± 30	0.23	264 ± 10	0.42
HSA (10%)	288 ± 29	0.10	273 ± 75	0.29
HSA (20%)	261 ± 48	0.14	237 ± 76	0.38
HSA (30%)	280 ± 60	0.16	212 ± 55	0.36
HSA (40%)	305 ± 60	0.15	182 ± 58	0.28
hTf-PEG-NHS (day 7)	-	-	204 ± 10	0.28
Ins-PEG-NHS (day 7)	-	-	243 ± 11	0.41
Hem-PEG-NHS (day 7)	-	-	253 ± 18	0.28
Lys-PEG-NHS (day7)	-	-	278 ± 19	0.37
hTf-NHS (Day 0)	-	-	263 ± 32	0.497
hTf-NHS-S (Day 0)	-	-	220 ± 28	0.478
hTf-S (Day 0)	-	-	262 ± 16	0.31
hTf-Ga (day 0)	-	-	256 ± 9	0.577
hTf-NHS (Day 60)	-	-	207 ± 16	0.43
hTf-NHS-S (Day 60)	-	-	209 ± 35	0.52
hTf-S (Day 60)	-	-	272 ± 6	0.35
hTf-Ga (day 60)	-	-	215 ± 39	0.57

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