Offeddu et al.:

Microphysiological Endothelial Models to Characterize Subcutaneous Drug Absorption

Supplementary Data

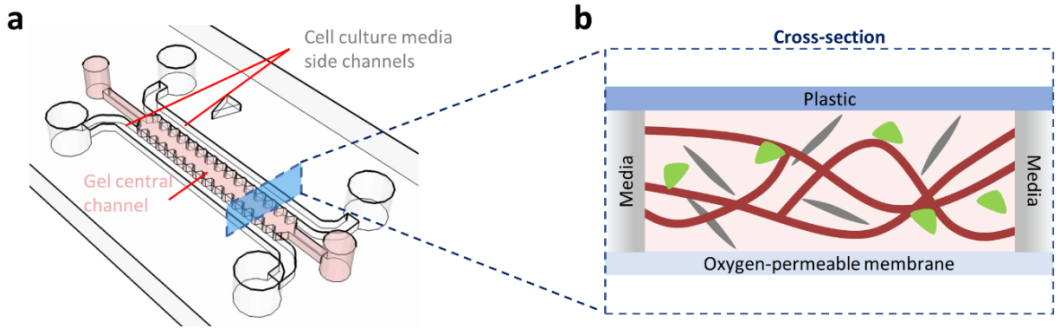

Fig. S1: Diagram of the AIM microfluidic chip used in the present study to form the dMVNs
(a) The device is comprised of 3 channels: the central one is filled with the gel solution incorporating cells, while the side channels are perfused daily with fresh cell culture medium. Triangular plastic posts separate the three channels. The central gel channel is 1.3 mm wide, 0.25 mm tall, and 10.5 mm long. (b) Cross-section of the AIM microfluidic chip showing the dMVN microvessels in the central gel channel connecting the two side channels of the chip filled with culture media (not to scale).

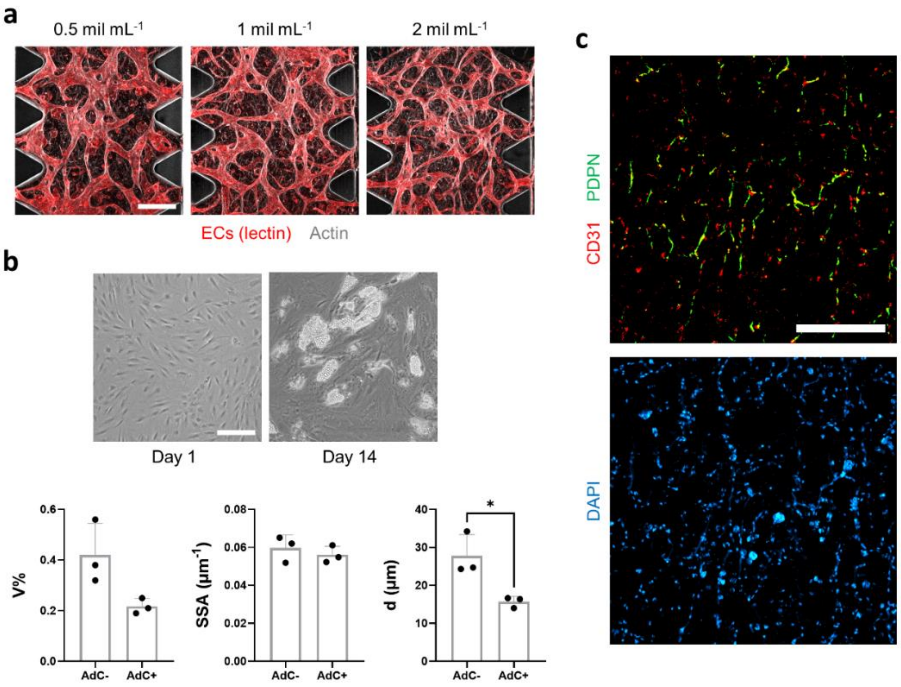

Fig. S2: Images of fibroblasts, adipocytes, and microvasculature in dMVNs
(a) Projected confocal images of dMVNs with increasing concentrations of dermal fibroblasts. (b) Brightfield images (top) of adipocytes (right, recognizable by the presence of lipid vacuoles) differentiated from adipose-derived mesenchymal stem cells (left), and vascular morphology comparison between dMVNs with or without the inclusion of adipocytes (AdC, bottom). Significance assessed by Student's t-test; *, $p < 0.05$. (c) Enlarged image of hypodermal microvasculature showing blood (CD31) and lymphatic (PDPN) microvessel staining (top) and nuclear (DAPI) counterstaining (bottom). The scale bar is 200 µm.

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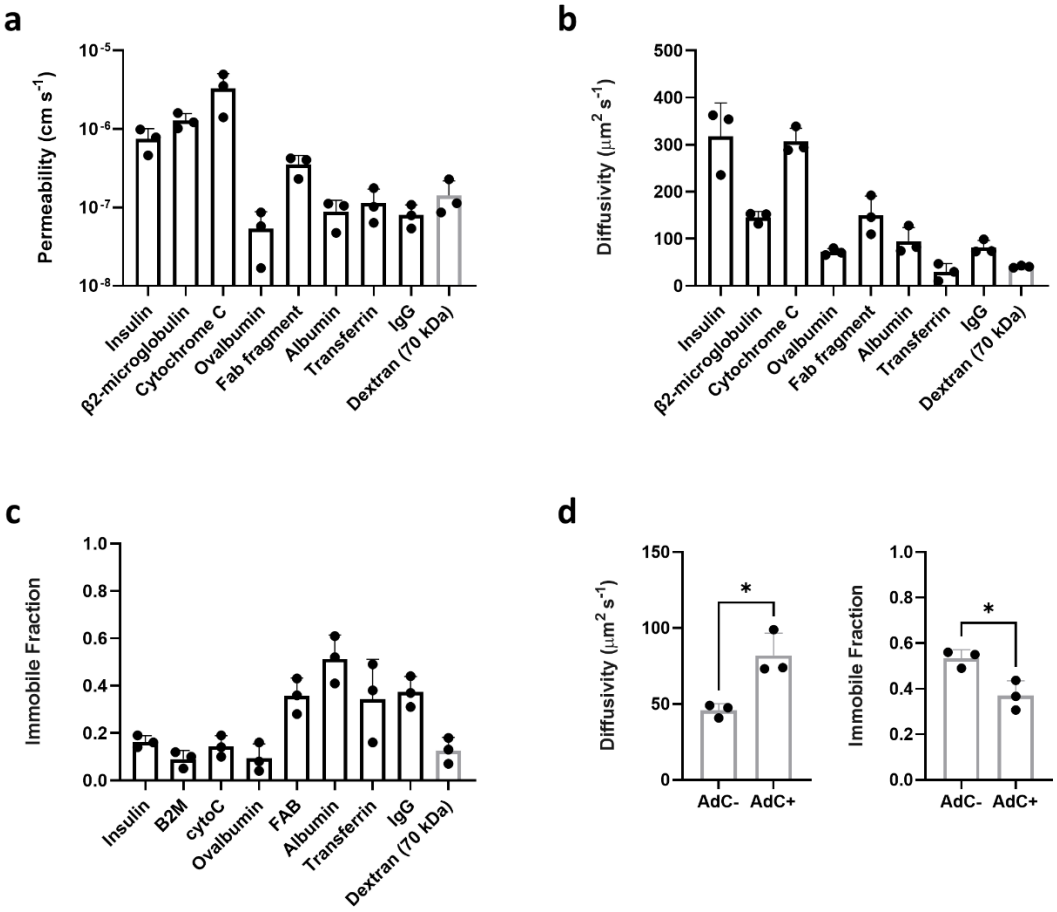

Fig. S3: Single data point values for (a) endothelial permeability, (b) matrix diffusivity, and (c) matrix immobile fraction of various proteins and dextran in the dMVN model. (d) Matrix diffusivity (left) and immobile fraction (right) of plasma IgG in dMVN models with or without inclusion of adipocytes (AdC)
Significance assessed by Student's t-test; *, $p < 0.05$.

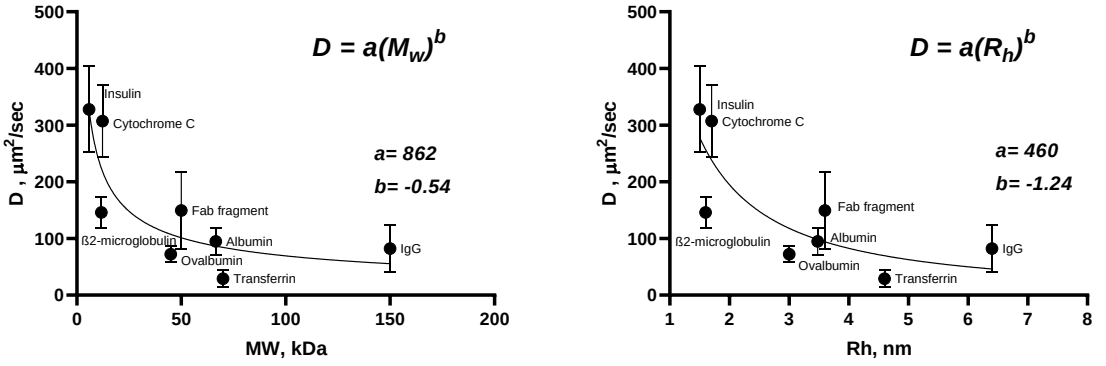

Fig. S4: Diffusivity size dependence in dMVN as a function of molecular weight (left) or hydrodynamic radius (right)
Hydrodynamic radii for the tested proteins were obtained from the scientific literature (Enns and Sussman, 1981; Edwards et al., 1983; Atmeh, Arafa and Al-Khateeb, 2007; Jensen et al., 2014; Stetefeld, McKenna and Patel, 2016; Ghosh et al., 2017). Power law fitting of the data was performed with Prism (GraphPad, version 9).

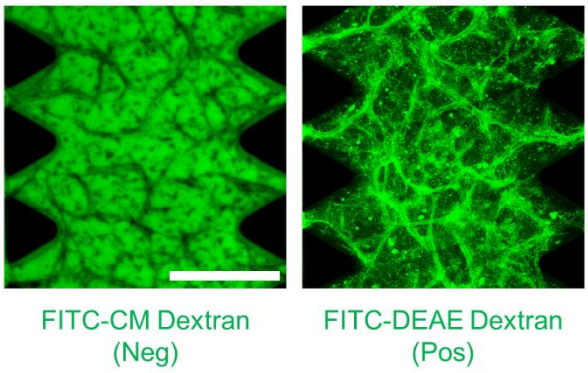

Fig. S5: Confocal microscopy images of dMVNs permeated with negatively-charged dextran (left) and positively-charged dextran (right) for approximately 30 min
The scale bar is 500 µm.

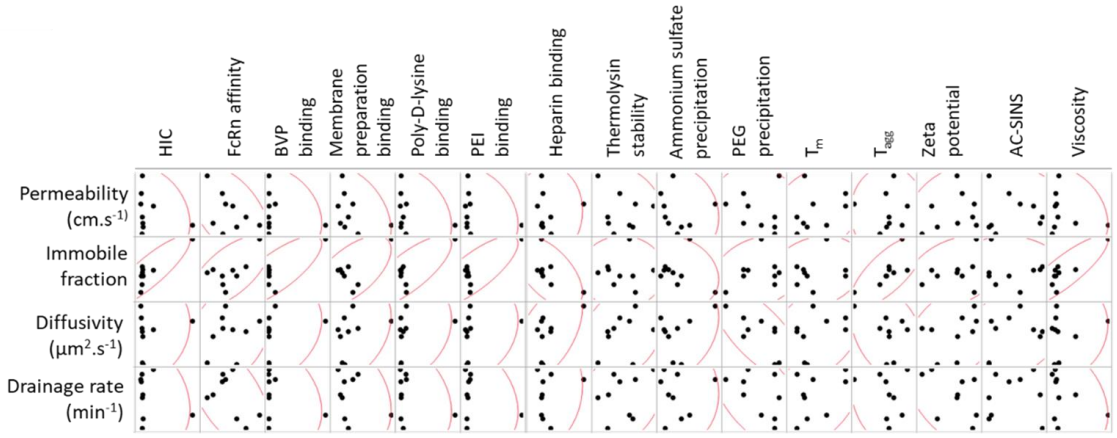

Fig. S6: Scatterplot matrix comparing model output parameters and experimentally measured physiochemical properties
Red line indicates a density ellipse showing the extent of data, the center of mass, the least squares fit line, and the degree of correlation between a selected dependent variable and a selected independent variable.

Overview of assays to assess mAb attributes

Attribute	Method	Conditions
colloidal stability	PEG precipitation	[mAb] = 1 mg/mL, 0-40% w/v PEG
	ammonium sulfate precipitation	[mAb] = 1 mg/mL, 0.5-1.8 M $(NH_4)_2SO_4$
self-interaction	AC-SINS	[mAb] = 0.1 mg/mL 10 mM NaOAc, 150 mM NaCl, pH 5.2
	viscosity – cone and plate	[mAb] = 70 mg/mL
	T_m	[mAb] = 1 mg/mL
	T_{agg}	[mAb] = 1 mg/mL
	zeta potential – DLS	10 mM NaOAc, 9% sucrose, pH 5.2
cross interaction	BVP ELISA	[mAb] =33 nM
	membrane prep ELISA	[mAb] = 33 nM
hydrophobic interaction	mAb-Pac HIC chromatography	[mAb] = 1 mg/mL
charged interaction	heparin chromatography	[mAb] = 0.4 mg/mL, 5-400 mM NaCl gradient
	PEI ELISA	[mAb] = 33 nM
	poly-D-Lys ELISA	[mAb] = 33 nM
FcRn binding	FcRn chromatography	[mAb] = 0.4 mg/mL, pH 5.5 - pH 8.0 gradient
protease sensitivity	thermolysin challenge	[mAb] = 2.89 nM, thermolysin = 28.9 nM

Methods for each assay (alphabetical order)

AC-SINS assay

Immobilization of capture antibody

The anti-human IgG Fc capture antibody was desalted with a 7 kDa MWCO Zeba Spin desalting column (Thermo Fisher, #89891) after 3 column washes with 20 mM sodium acetate, pH 4.3 (Sigma). Column washes were discarded, and the sample eluent concentration was measured by UV absorbance at 280nm. When necessary, the capture antibody concentration was adjusted to 0.4 mg/mL with 20 mM sodium acetate, pH 4.3. The capture antibody was then mixed in a 1:10 ratio with citrate-stabilized unconjugated gold colloids (7×10^{11} particles/mL) (Ted Pella Inc, #15701-1) and incubated for 1 h at ambient temperature (RT). Then 10 mM polyethylene glycol methyl ether thiol (thiolated PEG, Sigma) in 20 mM sodium acetate, pH 4.3, was added to the conjugates and incubated for an additional hour to prevent nonspecific interactions. When the assay was not run within 12 h, the mixture was stored at 4°C for up to 72 h following the addition of thiolated PEG.

Concentrating the immobilized capture antibody-bead complex

The solution was then passed through a 13-mm 0.22 µm PVDF membrane (Millex-GV, # SLGV013SL) using a Luer-Lock sterile syringe (BD, # BD302995). Here the capture antibody-conjugated nanoparticles (purple) are retained on the PVDF membrane, producing a clear filtrate. To elute the antibody-conjugated nanoparticles, phosphate-buffered saline (PBS, Gibco) was added at 1/10 the starting volume to the syringe-filter apparatus and pushed into an Eppendorf tube. The total volume at this final step should be about 1 mL, and the resulting solution should be purple as it should contain the antibody-conjugated nanoparticles.

Immobilization of monoclonal antibodies

All monoclonal antibodies were formulated in 10 mM sodium acetate, 9% sucrose, pH 5.2 (A52Su), and adjusted to a concentration of 1.0 mg/mL and 0.1 mg/mL. To immobilize the mAbs onto the conjugated nanoparticles, 10 µL of the 10X concentrated capture antibody-conjugated nanoparticles was added to 80 µL of 10 mM sodium acetate, 150 mM sodium chloride, pH 5.2 (A52NaCl), and incubated at RT for 1 h with 10 µL of each mAb solution in a UV transparent 384 well plate.

Plasmon wavelength analysis of nanoparticle conjugates

Absorption spectra were collected from 450 nm to 750 nm at an increment of 2 nm using a SpectraMax M5. Raw data was processed and analyzed by Amgen's internal software for peak smoothing and fitting to determine the plasmon wavelength's λ_{max}. Risk binning criteria were based on the difference between the low risk mAb control's λ_{max} from each sample's λ_{max}. $\Delta\ \lambda_{max}$ ranged from 0 to 35.

Dynamic light scattering measurements of nanoparticle conjugates

Dynamic light scattering measurements of the antibody-conjugated gold nanoparticles were made at 25 ± 0.1°C using a DynaPro Plate Reader (Wyatt Technology Corporation, Santa Barbara, California) with a 384-well Aurora microplate (1012-00110 or BA2-00110). The sample volume used for analysis was 30 µL. Triplicates of wells were filled for each sample. The software used was Dynamics 7.6.0.48. Ten acquisitions of 2-second measurements were collected from each well.

Ammonium sulfate precipitation assay

Ammonium sulfate precipitation of each mAb sample was achieved by mixing of ammonium sulfate from a 4 M stock solution of 10 mM acetate, 9% sucrose at pH 5.2 to final concentrations ranging from 0.5 to 1.8 M. The final protein concentration in each sample was 1 mg/mL. The samples were added to a 96-well transparent Sensoplate Microplate, and the measurements of turbidity were performed similarly to the procedure for PEG precipitation described above.

Baculovirus particle (BVP) non-specific binding assay

Baculovirus particles (LakePharma, #25690) were diluted in 50 mM sodium carbonate buffer, pH 9.6, to 1.25 µg/mL. 96-well ELISA plates (Costar, #3590) were coated with 100 µL/well of BVP solution at 4°C overnight. The plates were blocked with 200 µL/well of SuperBlock™ T20 (PBS) Blocking Buffer (Thermo Scientific, #37516) for 1 h at room temperature (RT) and then washed three times with PBS. Antibodies to be tested were diluted to 33 nM with SuperBlock™ T20 (PBS) Blocking Buffer. 100 µL of antibodies were added in triplicate to the wells and incubated for 1 h at RT. The plates were washed 6 times with PBS. 100 µL/well of 10ng/mL goat anti-human IgG conjugated to horseradish peroxidase (Jackson ImmunoResearch, # 109-035-008) were added to each well. The plates were incubated for 30 min at RT, then the plates were washed 6 times with PBS. 100 µL/well of TMB substrate (Thermo Scientific, # 34021) were added to each well. After incubation for 15 min, reactions were stopped by adding 100 µL/well of 2M sulfuric acid to each well. Absorbance at 450 nm was read by AquaMax 4000 Spectra (Molecular Devices). Data was analyzed by BVP score: OD450 with BVP divided by OD450 without BVP.

FcRn chromatography assay

Materials

FcRn affinity columns were purchased from Roche Custom Biotech (# 08128057 001). Fresh HPLC elution buffers were prepared 20 mM MES/HCl, 140 mM NaCl, pH = 5.5 (Buffer A); 20 mM Tris/HCl, 140 mM NaCl, pH = 8.8 (Buffer B).

Methods

FcRn – human IgG interaction affinity was characterized by comparing the relative elution characteristics between molecules under standard gradient elution assay conditions. Briefly, each panel member was diluted 5-fold into Buffer A to a final mAb concentration of 0.40 mg/mL prior to injection (8 µg) onto the column. A gradient elution method was applied after equilibration of the column in 20% Buffer B @ 0.5 mL/min (pressure ≤ 5Bar): 20% Buffer B (0-10 min), 100% Buffer B (80 min), 100% Buffer B (90 min), 20% Buffer B (93 min), 20% Buffer B (103 min). MAb elution was monitored using both absorbance (280 nm) and intrinsic fluorescence (280 nm ex., 350 nm em.). Analyte injections were performed only after verifying reproducible peak retention characteristics of standard FcRn affinity (AMG 655) and high affinity (huIgG1-Y/T/E, 'YTE') calibration standards (AMG 655). AMG 655 and YTE calibrants were injected between every 12 analyte runs to verify column performance. The 83 member BTI panel was analyzed using a single column, where column performance was graded by assessment of retention time drift of the calibration standards (≤ 0.1min) as well as maintenance of system pressure (≤ 20% increase over all injections).

Analysis

The data were exported into .csv format as a text file for manipulation in Origin Pro (OriginLab). First, the data were reduced by a factor of 10 by way of filtering. Next, for each affinity chromatogram a curve-fitting routine was applied: asymmetric baseline fitting and subtraction, normalization of the chromatograms based on peak intensity, followed by fitting to a Gaussian peak model. Peak retention time (centroid) and FWHM statistics were tabulated from resultant chromatogram transforms. For conversion of retention time to elution pH, the pH was calculated in RStudio[2] from the known HPLC gradient composition over time via the methods of Nguyen et al. (2009) and verified by measuring the pH at select time points throughout a typical run.

Heparin chromatography assay

The interaction of the tested molecules with heparin was characterized by chromatography using a HiTrap Heparin high-performance 1 mL column (Cytiva, catalog #17040601). 0.4mg of the protein under evaluation was diluted in buffer A (50 mM Tris pH 7.6 and 5 mM NaCl) up to a final volume of 1 mL and then loaded onto a heparin column pre-equilibrated in buffer A. After loading, the column was washed for 5 cv with buffer A, and then a linear gradient of 5-400 mM M NaCl over 20 cv was performed. The conductivity at which the protein was eluted (at the center of the elution peak) was used to characterize its heparin-binding affinity. Molecules showing no interaction eluted at buffer A conductivity while molecules with higher affinity eluted at higher conductivity values. At the end of each run the column was washed with 5 cv of 50 mM Tris pH 7.6 and 2 M NaCl and re-equilibrated in buffer A before the next protein was tested.

Hydrophobic interaction chromatography assay

Materials

MAb analyte stocks (2 mg/mL) were diluted 1:1 in mobile phase A to a final concentration of 1 mg/mL.

- *pH 6.0 assay:* Mobile phase A (1.8 M ammonium sulfate, 100 mM potassium phosphate, pH 6.0), mobile phase B (100 mM potassium phosphate, pH 6.0).
- *pH 7.4 assay:* Mobile phase A (1.8 M ammonium sulfate, 100 mM potassium phosphate, pH 7.4), mobile phase B (100 mM potassium phosphate, pH 7.4).

Method

HPLC was performed using an Agilent 1200 (Santa Clara, CA) system equipped with a binary pump (G1312B), an autosampler, a heated column compartment, and a diode array detector (G1315C). System management and data acquisition were performed by Chemstation software (B.04.03 v 16). Chromatographic separation was achieved at 30°C on a MAbPac™ HIC-20 HPLC column (250×4.6 mm, 5 µm particle size; ThermoFisher Scientific, #088554). Ten microliters of analyte were injected at a 0.5 mL/min flow rate and eluted over a 35-min gradient from 20% B to 100% B. Effluent was monitored at λ_{ex} = 290 nm, λ_{em} 320 nm. Column drift was monitored using early- and late-eluting control mAbs injected approximately every 10 injections and shown to be negligible (0.03 min retention time standard deviation at pH 6.0 (n = 6 per mAb); 0.2-0.3 min retention time standard deviation at pH 7.4 (n = 6 per mAb)).

Analysis

Retention time and full-width half-max (FWHM) were determined using the OriginPro 2019 software package by OriginLab. Retention time was recorded by identifying the time at maximum peak height, and FWHM was determined by normalizing all of the peaks to have a maximum height of 1 and fitting the normalized peak with a Gaussian function.

PEG precipitation
We modified the common procedure of precipitation measurements in order to increase throughput. Specifically, stock solutions of 10 mM acetate, 9% sucrose, and 0.01% polysorbate 80 at pH 5.2 and containing 40% (w/v) PEG-6000 and 10 mM acetate, 9% sucrose, at pH 5.2, were mixed to prepare solutions of varying final PEG levels (from 0% to 40% w/v PEG). These solutions were added (100 µL) to wells of a 96-well transparent Sensoplate Microplate (Greiner Bio-One). A stock solution of mAb at 70 mg/mL was spiked into the wells containing varying levels of PEG to a final protein concentration of 1 mg/mL. The 96-well plate(s) were incubated overnight at RT. The wells on the plates were then thoroughly mixed and analyzed at 350 nm for turbidity by SpectraMaxPlus (Molecular Devices) plate reader spectrophotometer. The precipitation concentration was defined as PEG concentration when turbidity level increased two-fold over background.

PEI assay
Materials: PEI Max, Polysciences, 24765-2
Methods: Same as BVP Assay except the ELISA plates were coated with PEI Max (0.1 µg/well) instead of BVP.

Poly-D-lysine assay
Same as BVP assay except the ELISA plates were coated with Poly-D-lysine (0.1 µg/well, Millipore, Cat# A-003-E) instead of BVP.

Membrane prep assay
Same as BVP assay except the ELISA plates were coated with membrane prep (0.1 µg/well, Millipore, Cat#HTS000MC1) instead of BVP.

T_m/T_{agg} assay
Thermal melting (T_m) temperatures were measured for each molecule at 1 mg/mL and at 70 mg/mL in formulation buffer (A52Su). Samples were concentrated using 30K MWCO Millipore protein concentrators. T_m were determined by running a TrpShift Study on the Prometheus, NT.48. Thermal ramp was applied at 1.0°C/min with start temperature 25°C and stop temperature 95°C. Unfolding was measured by the fluorescence ratio 350 nm/330 nm. Onset of aggregation (T_{agg}) was determined by measuring the difference between the initial light intensity and the back-reflected intensity. Data analysis and T_m/T_{agg} determination was preformed using PR.ThermControl v2.0.4.

Viscosity assay
Viscosity was determined using an Anton Paar MCR Rheometer by measuring the flow resistance due to the frictional forces between molecules. A flow sweep procedure was applied from 10 to 1000 s^{-1} using a 20 mm 1.988° cone plate and Peltier plate Steel –990918. Viscosity was measured in Pa s, where 1 m Pa s = 1 cP at 1000 s^{-1}. 80 µL was loaded onto the plate for each measurement. Viscosity was measured for each molecule at approximately 150 mg/mL with 0.01% surfactant added to the formulation buffer (A52Su).

Zeta potential
To perform the zeta potential measurement, a protein sample of approximately 25 µL was loaded into the center of a disposable capillary cell (DTS 1070) prefilled with the A52Su formulation and was measured on a Zetasizer (Malvern Inc.) with default settings for the alternative voltage and frequency. DLS measurements was also performed before and after the zeta potential measurement to ensure no appearance of proteinaceous aggregates. The procedure was repeated for all BAT mAb samples, and the zeta potential values were directly reported.

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