Electronic Supplementary Information for "The effect of protein concentration on the viscosity of a recombinant albumin solution formulation"

1. Supplementary Methods

1.1. Microfluidic chip sodium-dodecylsulfate polyacrylamide gel electrophoresis (μ-SDS-PAGE)

An Agilent 2100 Bioanalyzer system and its corresponding Protein 230 kit were used (Agilent Technologies, Germany) for protein SDS-PAGE analysis. The kit reagents were: gel matrix solution, protein dye concentrate, a marker protein sample buffer solution (containing both a high and low molecular weight fluorescent marker, also present in the protein ladder, as well as SDS) and a protein molecular mass ladder solution.

The gel matrix and destain solutions were prepared following the manufacturer's protocol with only slight changes. To prepare a 1 M DTT solution (dithiothreitol; reducing agent), the contents of one tube of 'No-weigh' DTT (Pierce Labs, Thermo Fisher Scientific, UK) were added to 50 μL of ultrapure water. A reducing sample buffer was prepared by adding and vortexing 3.6 μL of 1 M DTT solution to a sample buffer 60 μL aliquot. 1 M Non-reducing NEM (N-ethylmaleidemide, Fluka Biochemika, UK) solution was prepared by weighing 12.5 mg of NEM into 100 μL of ultrapure water. This sample was vortexed and left for 2 min at 80 ºC for full dissolution. The non-reducing sample buffer was prepared by adding 3.6 μL of 1 M NEM solution into 60 μL of sample buffer. Protein samples to be analysed were individually diluted to 4 mg/mL in phosphate buffered saline (PBS) (constituting individual protein sample stock solutions). From each of these sample stock solutions, 5 μL were taken and added to 5 μL of either non-reducing or reducing sample buffers in small tubes. The protein ladder was prepared by aliquoting (6 μL) separately into a new tube. The protein samples and protein ladder were heated at 80 ºC for 5 minutes (reduced samples and protein ladder) and for 1 minute (non-reduced samples). After heating, all tubes were cooled down by centrifuging at 13 000 rpm for 1 minute (Haereus Pico, Thermo Fisher Scientific, UK). In new tubes, 84 μL of ultrapure water was added to 6 μL of each reduced and non-reduced samples, and to the protein ladder. All protein samples, protein ladder, reducing and non-reducing sample buffers were freshly prepared, thoroughly mixed and used only within 24 hours.

After priming the chip with gel-dye mix, each of the 4 wells was filled with more gel-dye mix (12 μL each). The destain solution (12 μL), protein ladder (6 μL) and protein samples (6 μL each) were then aliquoted to their corresponding well. Detection of protein was made by laser-induced fluorescence. After reading the chip, the software used a method for automated integration for the peaks detected in each sample well.

The rAlbumin samples that were analysed were at 200, 250, 300, 350, 400, 450 and 500 mg/mL.

2. Supplementary Results

2.1. Viscosity versus concentration table

Table SI-1 shows the measured concentrations of samples in comparison to the target concentrations. It was difficult to achieve targeted concentration values with higher concentrations (≥ 300 mg/mL), due to the difficulty of accurately achieving such values using centrifugal concentrators. This was mostly relevant with the sample targeted at 500 mg/mL, where its higher viscosity yielded difficulties in further concentrating the sample.

Table SI-1 Measured concentrations and respective viscosities corresponding to target concentrations of rAlbumin samples. All values reported are an average of 3 measurements, with corresponding standard deviations.

Target Concentration (mg/mL)	Measured Concentration (mg/mL)	Viscosity (Pa.s)
0.1	0.10 ± 0.001	$1.06 \times 10^{-3} \pm 1.2 \times 10^{-5}$
0.5	0.36 ± 0.01	$1.09 \times 10^{-3} \pm 6 \times 10^{-6}$
1	0.96 ± 0.01	$1.08 \times 10^{-3} \pm 6 \times 10^{-6}$
5	5.01 ± 0.02	$1.09 \times 10^{-3} \pm 1.7 \times 10^{-5}$
10	9.84 ± 0.18	$1.11 \times 10^{-3} \pm 6 \times 10^{-6}$
50	50.2 ± 0.27	$1.37 \times 10^{-3} \pm 6 \times 10^{-6}$
100	96.0 ± 0.08	$1.78 \times 10^{-3} \pm 0.0$
150	149.6 ± 0.41	$2.59 \times 10^{-3} \pm 6 \times 10^{-6}$
200	190.8 ± 0.26	$3.89 \times 10^{-3} \pm 1.2 \times 10^{-5}$
250	253.1 ± 0.49	$9.74 \times 10^{-3} \pm 2.2 \times 10^{-4}$
300	287.4 ± 2.03	$1.36 \times 10^{-2} \pm 3.5 \times 10^{-4}$
350	354.2 ± 0.40	$9.35 \times 10^{-2} \pm 4.3 \times 10^{-3}$
400	398.0 ± 0.71	0.59 ± 0.11
450	440.2 ± 0.51	2.35 ± 0.21
500	506.8 ± 0.95	9.29 ± 0.39

2.2. SEC chromatograms

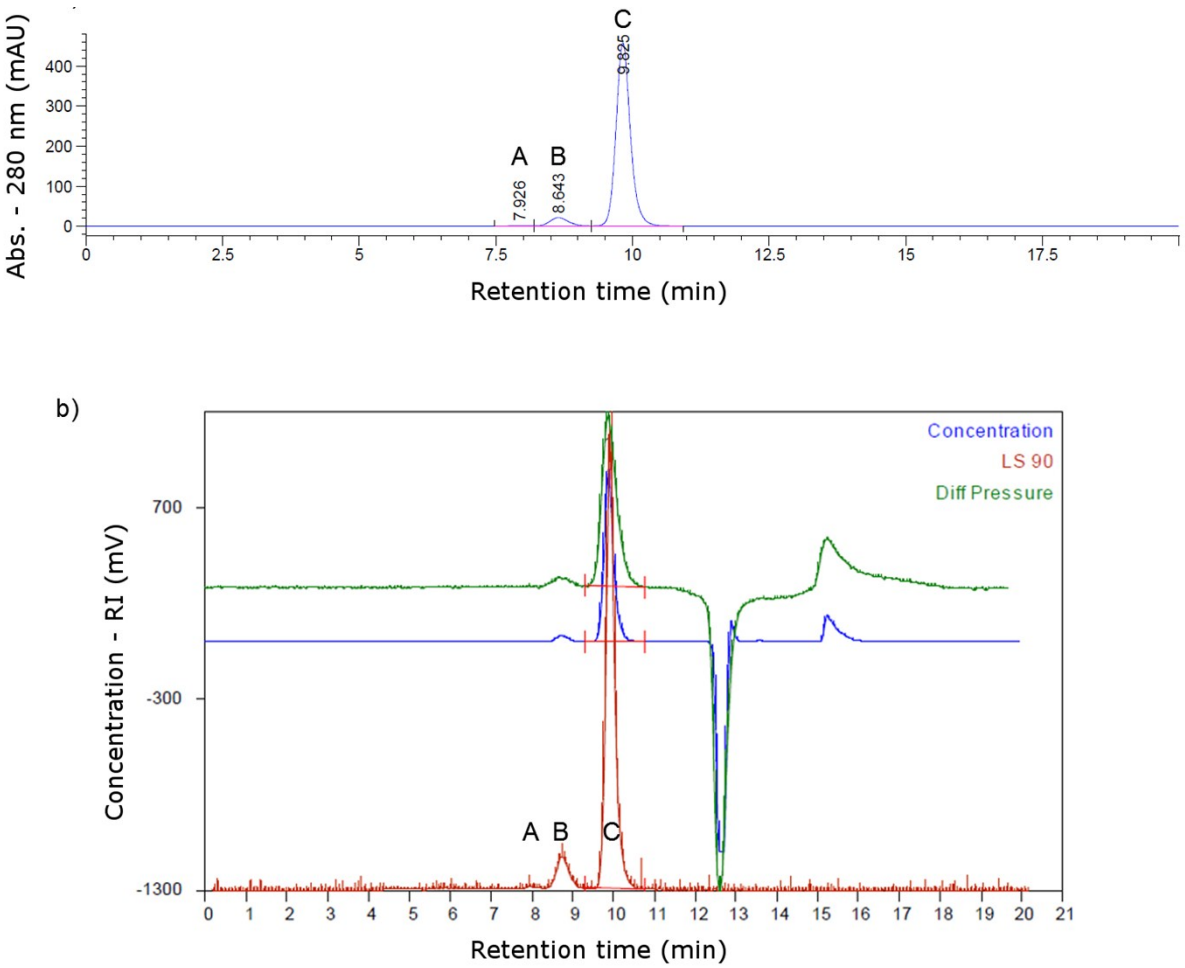

Fig. SI-1 Size exclusion chromatograms for a 200 mg/mL rAlbumin solution diluted to 10 mg/mL and analysed on **A)** HPSEC for level of aggregation and **B)** HPSEC with triple detection to determine bulk intrinsic viscosity and bulk molecular weight. In the chromatograms, the letters correspond to: A - trimer; B - dimer and C - monomer.

2.3. Additional data on stability at storage conditions of rAlbumin

A stability study of four weeks was done for fresh dilutions of rAlbumin (10 and 50 mg/mL) stored at 5 ºC. The aim was to confirm that rAlbumin solutions would not aggregate when stored at 2-8 ºC. It was found that within four weeks, a decrease of approximately 1 % of dimer occurred, corresponding to a similar increase of monomer. This change attained a plateau after the first two weeks (Figure SI-2). It has been found that it is possible that dimerisation of albumin can occur due to self-association, not involving the free cysteine[1]. Therefore, in this particular case, it could be possible that a low percentage of dimers may have formed by self-association during manufacture, and remained while the stock was kept at 200 mg/mL and only dissociated with time after dilution. However, this effect was only

observed with time. As stated in the main text, there was a negligible difference in the relative peak areas between low and high concentrations of rAlbumin at each time point (less than 1 %).

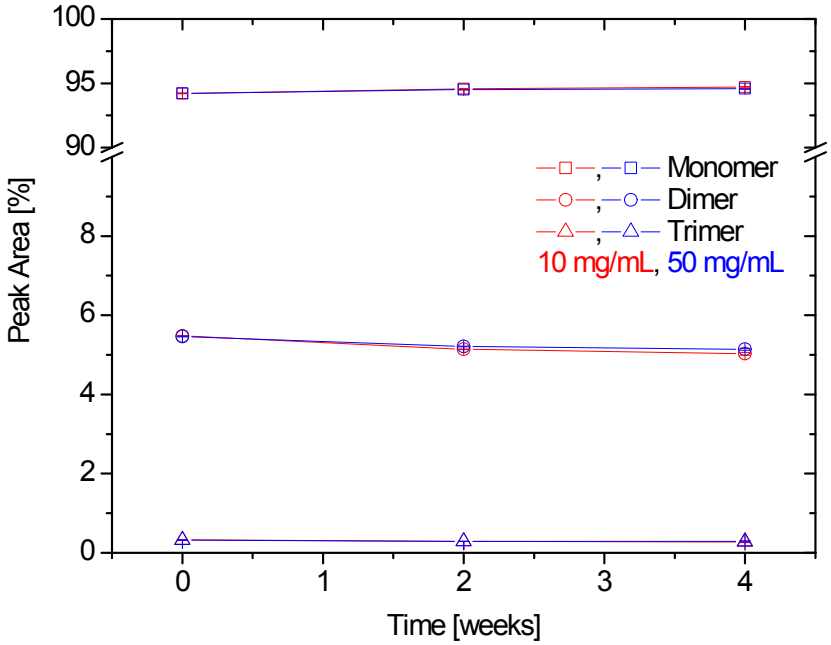

Fig. SI-2 HPSEC conventional method comparing neat injections of 10 and 50 mg/mL rAlbumin solutions along 4 weeks with storage at 5 °C. Average and standard deviation (error bars represented) were reported from 3 separate readings. Line is for eye guidance only.

2.3. microSDS-PAGE

Analysis with microfluidics SDS PAGE system allowed for further characterisation of rAlbumin solutions of higher concentrations. The samples chosen for comparison were from 200 mg/mL to 500 mg/mL, since all prepared rAlbumin samples with concentration below 200 mg/mL would have been diluted from the same stock solution. For all analysed samples and in both reducing and non-reducing conditions, the major peak/ band detected at approximately 63 kDa was attributed to rAlbumin's monomer, in agreement to literature[2] (Fig. SI-3a and SI-3b). The purity of rAlbumin solutions via SDS PAGE showed consistency with the level of aggregation measured via HPSEC as it did not detect any protein fragments or other protein species besides the expected dimerisation of monomers (peak/band present in reducing and non-reducing conditions, between 95 and 150 kDa markers). The absent

detection of trimers, which are expected to have a molecular weight of approximately 200 kDa [2], could be due to their low quantity observed in the HPSEC results (Fig. 2b in main text) and/or due to dissociation upon dilution.

Dimerisation of human serum albumin or bovine serum albumin is mostly due to formation of a covalent disulfide bridge between the free cysteine present in the monomer[3]. It was expected that dimers in rAlbumin solutions would be reduced by dithriothreitol at reducing conditions, although it has been reported that HSA's oligomeric species can remain present even at similar reducing conditions[2].

To allow for system suitability evaluation, a stable and high purity (> 98 % by HPSEC) mAb (IgG_1) sample was added as a control to each of the microchips (reducing and non-reducing). The peak/band detected on the non-reducing gel could be attributed to a mAb monomer of approximately 150 kDa. Upon reducing conditions the mAb sample yielded two peaks/bands at approximately 50 and 25 kDa, corresponding respectively to the heavy and light chains present in a IgG_1 [4,5] (Fig. SI-3a and SI-3b).

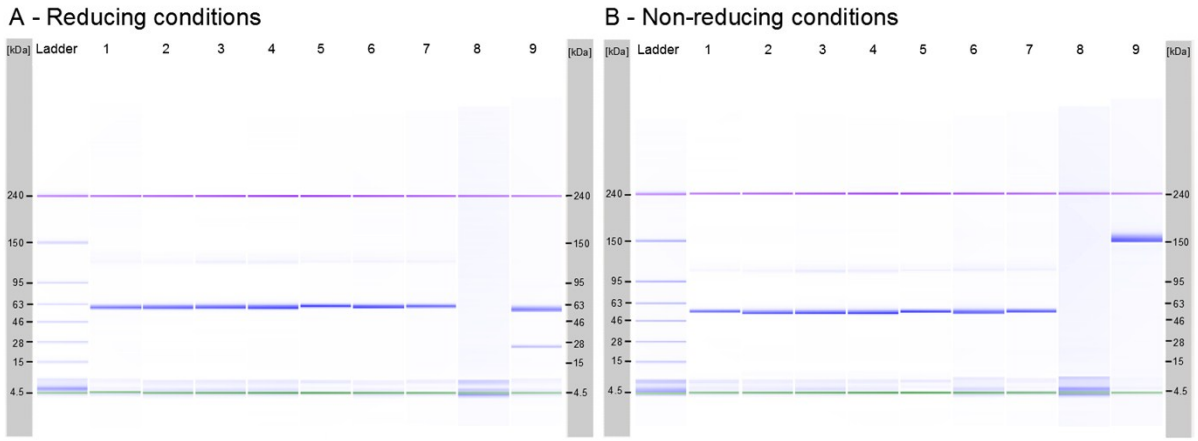

Fig. SI-3 Microfluidics protein electrophoresis gel image for rAlbumin solutions originally at high concentration. **(A)** Gel in reducing conditions and **(B)** gel in non-reducing conditions. For both gels: concentration of rAlbumin = 200 (lane 1), 250 (lane 2), 300 (lane 3), 350 (lane 4), 400 (lane 5), 450 (lane 6) and 500 mg/mL (lane 7); formulation bu_er (lane 8) and monoclonal antibody control (lane 9).

2.4. Additional control experiment

To demonstrate that using centrifugal concentrators would not affect the rheological behaviour nor increase the level of aggregation, an aliquot of the original rAlbumin solution at 200 mg/mL was diluted in formulation buffer and further concentrated back to 200 mg/mL. The centrifugal concentrators used would allow smaller molecules to pass through, such as

water, salts and polysorbate-80. The comparison of the rheological behaviour between this control sample and the original formulation showed that both had superimposable profiles (Fig. SI-4). This prepared control sample at ~200 mg/mL showed similar HPSEC and DLS results to those observed with the original 200 mg/mL sample.

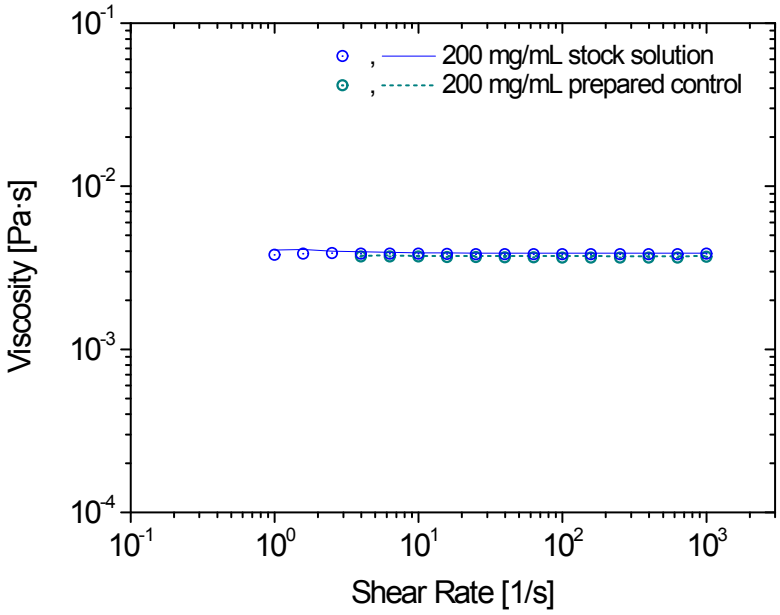

Fig. SI-4 Comparison of the viscosity curve between 200 mg/mL control sample prepared via centrifugal concentration (crossed circles) and the originally formulated 200 mg/mL sample (full circles). Respective lines correspond to ramping up shear rates, while the circles correspond to ramping down data.

References

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