Supporting Information

for

Systematic studies into uniform synthetic protein nanoparticles

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Additional details
1 Instrumentation

1.1 Scanning electron microscopy

The SEM micrographs presented in this article are cropped and magnified from larger fields of view for clarity and ease of particle observation. Figure S1 provides full field of view of the typical images utilized to build population statistics throughout the study.

1.2 Dynamic light scattering

The particle size distributions were measured with dynamic light scattering (Malvern ZSP ZEN-5600). The protein material was chosen with refractive index of 1.45 and absorbance of 0.001. Phosphate-buffered saline was used as the dispersant with refractive index of 1.332 and viscosity of 0.9074. Measurements were done at 25 °C, at 3.00 mm position in a disposable microcuvette. (ZEN0040).

1.3 Analysis software

FIJI (a distribution of ImageJ v1.53c) was used for all image analysis. OfficeLibre Calc was used for all data manipulation and for the generation of summary statistics. Graphpad Prism 9.0 was used for presentation of distributions, scatter plots, and violin plots. Origin 9 was used for peak extraction.

2 SEM Analysis via ImageJ/FIJI

SEM images are collected as lossless TIFF files and were processed via ImageJ.

3 Data processing via open source spreadsheet software

In order to obtain results that will allow for comparison of the data from imaging with DLS-based PDI results for the systems, histograms and individual data were calculated as volume-based values in order to generate a calculated SEM-based PDI value (denoted here as PDI_{SEM}) and to present an intensity-based analog (iSEM) for comparison to intensity-based DLS. iSEM distributions, which were calculated from nSEM × (d/2)^3 and then normalized. This was done to provide dry particle (as manufactured) analysis that is comparable to the nDLS and iDLS results (as used in solution after post-processing). The entirety of the analysis for this step was done utilizing OfficeLibre Calc. The various expressions of diameter populations were evaluated using statistical analysis including t-test, IQR, and ANOVA.
4 Two-dimensional analysis

To understand how the geometric characteristics for blended SPNPs are influenced by their constituents, we quantitatively and qualitatively described the SPNPs. The diameter was compared to other geometric attributes (min. diameter, anisotropy, circularity, and roundness). The linear regressions of the paired \( x-y \) data sets were created and a scoring factor was used (from 0 to 10) to describe the extent of similarity to the monospecies SPNPs. For example, to understand how a blend of HSA and transferrin SPNP resemble their constituents, this scoring system can be applied. The scoring factor represents a convolution of the relative agreement of the blended regression slope and the agreement of the regression strength (\( \langle r^2 \rangle \)) when compared to the monospecies SPNPs. This is done by treating the slope of the blend regression as a linear combination of the slopes of the constituent regressions, scaling based on the extent of agreement between the strengths of the regressions.
Figure S1: Large-area FOV SEM images of single-protein and blended SPNPs. (A) HSA, (B) HEM/HSA, (C) HEM, (D) TF/HSA, (E) TF, (F) MUC/HSA, (G) MUC, (H) INS/HSA, and (I) INS. Scale bars: 2000 nm
Table S1: SPNPs size and secondary geometric factors data. Average diameters are presented based on nSEM, nDLS, iSEM, and iDLS results. Minimum diameter, anisotropy, circularity, and roundness results are provided as secondary geometric factors.

| Formulation Name | Diameter, nSEM (nm) | Diameter, nDLS (nm) | Diameter, iSEM (nm) | Diameter, iDLS (nm) | PDI SEM | PDI iDLS | Min. Dia. SEM (nm) | Min. Dia. iDLS (nm) | Anisotropy SEM (a.u.) | Anisotropy iDLS (a.u.) | Circularity SEM (a.u.) | Circularity iDLS (a.u.) | Roundness SEM (a.u.) | Roundness iDLS (a.u.) |
|------------------|---------------------|---------------------|---------------------|---------------------|---------|---------|-------------------|---------------------|----------------------|----------------------|----------------------|----------------------|----------------------|----------------------|
| HEM              | 65 ± 25             | 254 ± 157           | 64 ± 12             | 382 ± 106           | 0.11    | 0.38    | 53 ± 19           | 1.20 ± 0.23          | 0.85 ± 0.08           | 0.86 ± 0.12           |                     |                     |                     |
| HEM/HSA          | 51 ± 20             | 127 ± 108           | 50 ± 15             | 475 ± 138           | 0.15    | 0.47    | 43 ± 17           | 1.14 ± 0.15          | 0.90 ± 0.09           | 0.89 ± 0.09           |                     |                     |                     |
| TF               | 81 ± 36             | 170 ± 84            | 109 ± 36            | 284 ± 124           | 0.19    | 0.22    | 65 ± 29           | 1.21 ± 0.24          | 0.85 ± 0.08           | 0.85 ± 0.12           |                     |                     |                     |
| TF/HSA           | 59 ± 23             | 86 ± 48             | 92 ± 36             | 328 ± 87            | 0.16    | 0.41    | 49 ± 17           | 1.17 ± 0.23          | 0.89 ± 0.11           | 0.87 ± 0.11           |                     |                     |                     |
| MUC              | 73 ± 45             | 39 ± 17             | 168 ± 79            | 262 ± 108           | 0.16    | 0.349   | 50 ± 25           | 1.45 ± 0.59          | 0.82 ± 0.15           | 0.76 ± 0.19           |                     |                     |                     |
| MUC/HSA          | 72 ± 42             | 55 ± 34             | 138 ± 61            | 270 ± 169           | 0.16    | 0.38    | 55 ± 30           | 1.28 ± 0.33          | 0.85 ± 0.09           | 0.81 ± 0.14           |                     |                     |                     |
| INS              | 60 ± 22             | 37 ± 15             | 49 ± 9              | 220 ± 82            | 0.17    | 0.468   | 43 ± 14           | 1.36 ± 0.35          | 0.85 ± 0.13           | 0.77 ± 0.16           |                     |                     |                     |
| INS/HSA          | 61 ± 23             | 70 ± 41             | 83 ± 38             | 269 ± 130           | 0.13    | 0.5     | 49 ± 17           | 1.20 ± 0.25          | 0.87 ± 0.07           | 0.86 ± 0.12           |                     |                     |                     |
| HSA              | 77 ± 37             | 97 ± 86             | 116 ± 44            | 283 ± 115           | 0.18    | 0.44    | 60 ± 27           | 1.25 ± 0.27          | 0.83 ± 0.09           | 0.82 ± 0.13           |                     |                     |                     |
Figure S2: Two-factor individual analysis for the HEM series. Scatter plots of minimum diameter, anisotropy, circularity, and roundness vs diameter.
Table S2: HEM series statistical analysis results of secondary geometric factors and scoring data for two-factor analysis. Minimum diameter, anisotropy, circularity and roundness results are provided as secondary geometric factors.

| SPNPs series | T-Test   | Min. Dia | Anisotropy | Circularity | Roundness | Min. Dia | R^2 | Score | Anisotropy | R^2 | Score | Circularity | R^2 | Score | Roundness | R^2 | Score |
|--------------|----------|----------|------------|-------------|-----------|----------|-----|-------|------------|-----|-------|-------------|-----|-------|-----------|-----|-------|
| HEM          | vs.      | p < 0.0001 | 0.7008 | 0.8265 | - | 0.002715 | 0.08659 | - | -0.001226 | 0.162 | - | -0.001393 | 0.08027 | - |
| HEM/hsa      | vs.      | p < 0.0001 | 0.7939 | 0.8191 | - | 0.001965 | 0.06754 | - | -0.001788 | 0.1645 | - | -0.0009889 | 0.04516 | - |
| Hsa          | vs.      | p < 0.0001 | 0.6736 | 0.8973 | - | 0.002611 | 0.1308 | - | -0.001203 | 0.1308 | - | -0.001203 | 0.1308 | - |
| three-way    | p < 0.0001 | 0.71602039 | - | - | 0 | - | 0 | - | - | - | 1.62334542 | - | - | - | - | - |

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Figure S3: Two-factor individual analysis for the TF series. Scatter plots of minimum diameter, anisotropy, circularity, and roundness vs diameter.
Table S3: TF series statistical analysis results of secondary geometric factors and scoring data for two-factor analysis. Minimum diameter, anisotropy, circularity and roundness results are provided as secondary geometric factors.

| SPNPs series | T-Test | Min. Dia | Anisotropy | Circularity | Roundness | Min. Dia | Anisotropy | Circularity | Roundness | Min. Dia | Anisotropy | Circularity | Roundness | Min. Dia | Anisotropy | Circularity | Roundness | Min. Dia | Anisotropy | Circularity | Roundness | Min. Dia | Anisotropy | Circularity | Roundness |
|--------------|--------|----------|------------|-------------|-----------|----------|------------|-------------|-----------|----------|------------|-------------|-----------|----------|------------|-------------|-----------|----------|------------|-------------|-----------|----------|------------|-------------|-----------|
|              |        | Diameter |            |             |           | Slope    | R^2        | Score       | Slope    | R^2        | Score       | Slope    | R^2        | Score       | Slope    | R^2        | Score       | Slope    | R^2        | Score       | Slope    | R^2        | Score       | Slope    | R^2        | Score       | Slope    | R^2        | Score       |
| TF           |        |          |            |             |           | -        | -          | -           | 0.7296   | 0.8393     | -           | -0.001863| 0.07776    | -           | 0.0007748| 0.1298     | -           | -0.0008078| 0.05926    | -           |          |            |             |          |            |             |          |
| vs.          | p < 0.0001 | * *** | p < 0.0001 | * *** | p < 0.0001 | * *** | p < 0.0001 | * *** | -        | -          | 0          | -           | 0          | -        | -          | 0           | -        | -          | 0           | -        | -          | 0           | -        | -          | 0           | -        | -          | 0           | -        |
| TF/HSA       |        |          |            |             |           | 0.6694   | 0.795      | -           | 0.004341| 0.1888     | -           | -0.002779| 0.361      | -           | -0.001994| 0.1613     | -           |          |            |             |          |            |             |          |            |             |          |
| vs.          | p < 0.0001 | * *** | p < 0.0001 | * *** | p < 0.0001 | * *** | p < 0.0001 | * *** | -        | -          | 8.2417796 | -           | -          | 2.09125046| 0.77411618| -           | -0.001203| 0.1308     | -           | -0.001203| 0.1308     | -           | -0.001203| 0.1308     | -           | -0.001203| 0.1308     | -           |
| HSA          |        |          |            |             |           | 0.6736   | 0.8973     | -           | 0.002611| 0.1308     | -           | -0.001203| 0.1308     | -           | -0.001203| 0.1308     | -           |          |            |             |          |            |             |          |            |             |          |
| vs.          | p < 0.0001 | * *** | p < 0.0001 | * *** | p < 0.0001 | * *** | p < 0.0001 | * *** | -        | -          | -          | -           | -          | -        | -          | -           | -        | -          | -           | -        | -          | -           | -        | -          | -           | -        | -          | -           | -        |

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Figure S4: Two-factor individual analysis for the MUC series. Scatter plots of minimum diameter, anisotropy, circularity, and roundness vs diameter.
Table S4: MUC series statistical analysis results of secondary geometric factors and scoring data for two-factor analysis. Minimum diameter, anisotropy, circularity and roundness results are provided as secondary geometric factors.

| SPNPs   | T-Test | Min. Dia | Anisotropy | Circularity | Roundness | Min. Dia | Anisotropy | Circularity | Roundness | Min. Dia | Anisotropy | Circularity | Roundness | Min. Dia | Anisotropy | Circularity | Roundness | Min. Dia | Anisotropy | Circularity | Roundness |
|---------|--------|----------|------------|-------------|-----------|----------|------------|-------------|-----------|----------|------------|-------------|-----------|----------|------------|-------------|-----------|----------|------------|-------------|-----------|----------|
| MUC     | -      | -        | -          | -           | -         | 0.434    | 0.6186     | -           | 0.006931  | 0.2886   | -         | -0.001442  | 0.2886     | -        | -0.002028  | 0.2308      | -         |
| vs.     | ns     | p < 0.001| ****       | ****        | ****      | -        | -          | 0.55790396 | -         | 0.51046559| -         | -         | -         | -         | -         | -         | -         | -         | -         | -         |
| MUC/HSA | -      | -        | -          | -           | -         | 0.6559   | 0.8191     | -           | 0.003035  | 0.1501   | -        | -0.0009956 | 0.1501     | -        | -0.001086  | 0.1073      | -         |
| vs.     | p < 0.01| p < 0.001| ****       | ****        | ****      | -        | -          | 8.91934775 | -         | 7.85890888| -         | 4.64471797 | -         | -        | 7.18447477 | -          | -         | -         | -         | -         | -         |
| HSA     | -      | -        | -          | -           | -         | 0.6736   | 0.8505     | -           | 0.002611  | 0.1308   | -        | -0.001203  | 0.1308     | -        | -0.001203  | 0.1308      | -         |
| Three-  | p < 0.01| p < 0.001| ****       | ****        | ****      | -        | -          | -           | -         | -        | -         | -          | -         | -        | -          | -          | -         | -         | -         | -         |
| Way     | **     | ****     | ****       | ****        | ****      | -        | -          | -           | -         | -        | -         | -          | -         | -        | -          | -          | -         | -         | -         | -         |
Figure S5: Two-factor individual analysis for the INS series. Scatter plots of minimum diameter, anisotropy, circularity, and roundness vs diameter.
Table S5: INS series statistical analysis results of secondary geometric factors and scoring data for two-factor analysis. Minimum diameter, anisotropy, circularity and roundness results are provided as secondary geometric factors.

| SPNPs series | T-Test | Min. Dia | Anisotropy | Circularity | Roundness | Min. Dia | R^2 | Score | Min. Dia | R^2 | Score | Min. Dia | R^2 | Score | Min. Dia | R^2 | Score |
|--------------|--------|----------|------------|-------------|-----------|----------|-----|-------|----------|-----|-------|----------|-----|-------|----------|-----|-------|
| INS          | -      | -        | -          | -           | -         | 0.5347   | 0.7088 | -     | 0.008143 | 0.2598 | -     | -0.003668 | 0.3622 | -     | -0.003629 | 0.2598 | -     |
| vs.          | ns     | p < 0.0001 | p < 0.0001 | p < 0.0001 | -         | -        | 1.84428555 | -     | -     | 2.54632088 | -     | -     | 0.13286172 | -     | -     | 3.52396288 | -     | -     |
| INS/HSA      | -      | -        | -          | -           | -         | 0.6449   | 0.7941 | -     | 0.004634 | 0.1809 | -     | -0.001277 | 0.1603 | -     | -0.002358 | 0.1923 | -     |
| vs.          | p < 0.0001 | p < 0.0001 | p < 0.0001 | p < 0.0001 | -         | -        | 7.02128945 | -     | -     | 4.58638358 | -     | -     | 7.91474463 | -     | -     | 3.56355293 | -     | -     |
| HSA          | -      | -        | -          | -           | -         | 0.6736   | 0.8973 | -     | 0.002611 | 0.1308 | -     | -0.001203 | 0.1308 | -     | -0.001203 | 0.1308 | -     |
| Three-Way    | p < 0.0001 | p < 0.0001 | p < 0.0001 | p < 0.0001 | -         | -        | -        | -     | -     | -        | -     | -     | -        | -     | -     | -        | -     | -     |
Table S6: nDLS results after multipeak deconvolution for SPNPs series. A multipeak (LogNormal) deconvolution was utilized to extract the average sizes ($d_1$, $d_2$), distribution breadth ($\sigma_1$, $\sigma_2$), and population fraction ($\alpha_1$, $\alpha_2$) for both the individual particles (population 1) and the transient clusters (population 2). (In the main text, for all SPNPs, $d_1$ refers to the average for the smallest diameter distribution and $d_2$ refers to the average of any larger diameter distribution.)

| SPNPs   | Population 1 |          | Population 2 |          |
|---------|--------------|----------|--------------|----------|
|         | Dia. ($\text{nm}$) & $\sigma$ ($\text{nm}$) & $\alpha$ (%) | Dia. ($\text{nm}$) & $\sigma$ ($\text{nm}$) & $\alpha$ (%) |
| HEM     | 91 & 15 & 17 | 347 & 119 & 83 |
| HEMHSA  | 97 & 19 & 65 | 455 & 159 & 35 |
| TF      | 125 & 29 & 29 | 233 & 98 & 71 |
| TFHSA   | 80 & 16 & 83 | 326 & 136 & 17 |
| MUC     | 39 & 8 & 95 | 180 & 93 & 5 |
| MUCHSA  | 30 & 4 & 7 | 68 & 21 & 93 |
| INS     | 35 & 6 & 92 | 144 & 70 & 8 |
| INSHSA  | 64 & 15 & 79 | 138 & 65 & 21 |
| HSA     | 46 & 10 & 16 | 222 & 121 & 84 |

Table S7: Physiochemical properties of proteins.

| Protein | Molecular weight | Ratio of hydrophilic residues / total number of residues [12] | Isoelectric point | References |
|---------|------------------|---------------------------------------------------------------|------------------|------------|
| HSA     | 66.5 kda [1]     | 41%                                                           | 4.7 [2]          | [1,2]      |
| Transferrin | 79 kDa [3]     | 38%                                                           | 5.6 [4]          | [3,4]      |
| Mucin   | 4000-5,500 kDa [5,6] | 19%                                                        | 2.75 [7]         | [5,6,7]    |
| Hemoglobin | 64.5 kDa [8]   | 30% - alpha subunit 29% - beta subunit | 7.0 [9]          | [8,9]      |
| Insulin | 5.808 kDa [10]  | 29%                                                           | 5.5 [11]         | [10,11]    |
**Table S8: SPNP jetting solution formulations.**

| Formulation | Protein (mg) | Crosslinker (mg) | Water (mL) | Ethanol (mL) | Acetic Acid 10% (mL) |
|-------------|--------------|-----------------|-----------|-------------|---------------------|
| HSA         | 50           | 5               | 400       | 50          |                     |
| TF          | 50           | 5               | 400       | 50          |                     |
| HEM         | 50           | 5               | 400       | 50          |                     |
| INS         | 50           | 5               | 405       | 50          | 45                  |
| MUC         | 10           | 1               | 400       | 50          |                     |
| TF/HSA      | 25/25        | 5               | 400       | 50          |                     |
| HEM/HSA     | 25/25        | 5               | 400       | 50          |                     |
| INS/HSA     | 25/25        | 5               | 427.7     | 50          | 22.5                |
| MUC/HSA     | 10/10        | 2               | 430       | 50          |                     |

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