Salient features of medical nanoparticles in biological fluids from an analytical ultracentrifuge

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1. Experimental procedures

1.1. Chemicals and materials
PLGA NPs were received as research samples from SmartDyeDelivery GmbH (Jena, Germany). These contain an anti-inflammatory drug relevant for treatment purposes and a liver-targeting by a cell-specific dye unit (DY-635) with an absorbance maximum in water located at λ = 635 nm. Human serum (HS) was purchased from Sigma Aldrich. HS proteins (human serum albumin (HSA) and γ-globulin) and phosphate buffered saline (PBS, pH=7.4) were received from Merck KGaA. Ultrapure water was freshly acquired from a Thermo ScientificTM BarnstedTM GenPureTM x CAD Plus water purification system (Thermo Electron LED GmbH, Langenselbold, Germany).

1.2. AUC and experimental operation
In-situ analysis using sedimentation velocity experiments were performed by an Optima AUC (Beckman Coulter Instruments, Brea, CA) with an An-50 Ti eight-hole rotor and double-sector epon centerpieces of a 12 mm optical solution path length. AUC studies were performed in a temperature range of 6 to 40 °C. The cells were assembled by utilizing sapphire windows. The corresponding sector chambers were filled with ca. 440 µL solvent in the reference sector and ca. 420 µL diluted NP sample solution in the sample sector. Rotor position eight was used as the counterbalance and to enable optical radial distance instrument detection module calibration. All experiments were performed at a rotor velocity of 7500 rpm.

1.3. Density and viscosity measurements
Density measurements of the solutions of the 55 wt.% HS were performed with a DMA4100 densimeter (Anton Paar, Graz, Austria) at 40 °C, 37 °C, 20 °C, and 6 °C, respectively. Additionally, viscosities of the solutions of the 55 wt.% HS were determined at the very same temperatures with an Automated Microviscometer (AMVn, Anton Paar, Graz, Austria) via a capillary / ball combination.

1.4. Data analysis
Acquired sedimentation velocity data were analyzed with SEDFIT and the $\text{ls-g}^*(s)$ model by numerically approximating sedimentation velocity data with the assumption of non-diffusing species. The resultant differential distributions of sedimentation coefficients, $\text{ls-g}^*(s)$, were inspected in accordance with the residuals. Integration of the distributions enables the estimation of signal (weight) averages of sedimentation coefficients, $s$, and measures of concentration from interference fringes or optical densities (ODs). Under conditions where materials apparently degraded, care was taken to model sedimentation velocity data over appropriate timescales and carefully chosen boundaries by considering, e.g. the utilized 55 wt.% HS, and its behavior under identical experimental conditions.

1.5. NP studies at 37 °C and a variety of solvents
The AUC was preheated for several hours at 37 °C. The rotor was stored in an incubator at 37 °C. A solution of each protein was prepared by dissolving the respective protein in 0.01 M PBS (pH = 7.4). After that, the NP stock in aqueous solution was added to the protein solutions in PBS or HS resulting in the desired NP concentration (1.4 mg mL$^{-1}$) and protein concentrations (i) 7 mg mL$^{-1}$ γ-globulin, (ii) 22 mg mL$^{-1}$ HSA, (iii) 55 wt% HS). The reference centrifuge cell sectors were filled with the same PBS solution mixture as the samples (i, ii) or simply with water (iii). For the control, samples of NPs diluted with water or diluted with 0.01 M PBS, the reference cells were filled with the corresponding solvent mixture. After the cells were filled, they were inserted in the preheated rotor and then in the preheated AUC.

The AUC needed to reach temperature equilibrium under vacuum at 37 °C again. The respective 4 h incubation time of the samples at 37 °C was followed by the experimental setup time of 0.5 h and temperature re-equilibration of the AUC (1.5 h) which in total led to a contact time of the NPs with the fluids of 6 h before starting the rotor.

After the respective incubation and equilibration time, the sedimentation velocity AUC measurements were performed using the refractive index and absorbance detection at a wavelength of λ = 635 nm in terms of optical density (OD) at a radial distance data resolution of 50 µm and utilizing a rotor speed of 7500 rpm for 20 h. The time between scans was 6 min. Every scan was used for data evaluation. For samples containing HSA and HS, beginning with the fifth scan, every scan was used for the data evaluation.

The same procedure was applied for another run with the same NPs and human serum proteins with the same experimental setup. Additionally, HS was diluted by adding aqueous NPs resulting in a NP concentration of 1.4 mg mL$^{-1}$ and 55 wt.% HS. All mentioned HS proteins were dissolved in 0.01 M PBS solution, NP solutions were added to the protein solutions, resulting in the desired concentration of 1.4 mg mL$^{-1}$. In this case, the incubation time was skipped, the reheat of the AUC to reach a temperature of 37 °C lasted for a timescale of 1.5 h. The experimental setup at room temperature took 0.5 h, so that the contact time of NDDS in the biofluids was in total 2 h before starting the rotor.

In all cases, the data range for the evaluation of the radial sedimentation velocity profile scans was set 0.25 cm ahead from the modeling boundary located close to the meniscus.

1.6. NP studies in HS at 6 °C, 37 °C, and 40 °C
The measurements in HS at 40 °C, 37 °C, and 6 °C were performed by using the absorbance detector at λ = 635 nm in terms of OD and utilizing a rotor speed of 7500 rpm for 20 hours. The time between scans was 3 min. Also, in this case, the AUC was temperature-conditioned for several hours to reach and equilibrate for the desired measurement temperature. The rotor speed data resolution of 50 µm and utilizing a rotor speed of 7500 rpm for 20 h. The time between scans was 6 min. Every scan was used for data evaluation. For samples containing HSA and HS, beginning with the fifth scan, every scan was used for the data evaluation.

The same procedure was applied for another run with the same NPs and human serum proteins with the same experimental setup. Additionally, HS was diluted by adding aqueous NPs resulting in a NP concentration of 1.4 mg mL$^{-1}$ and 55 wt.% HS. All mentioned HS proteins were dissolved in 0.01 M PBS solution, NP solutions were added to the protein solutions, resulting in the desired concentration of 1.4 mg mL$^{-1}$. In this case, the incubation time was skipped, the reheat of the AUC to reach a temperature of 37 °C lasted for a timescale of 1.5 h. The experimental setup at room temperature took 0.5 h, so that the contact time of NDDS in the biofluids was in total 2 h before starting the rotor.

In all cases, the data range for the evaluation of the radial sedimentation velocity profile scans was set 0.25 cm ahead from the modeling boundary located close to the meniscus.
was stored at the desired measurement temperature (fridge or incubator). The experimental setup lasted 0.5 h and the time of the AUC to reach the desired measurement temperature was 1.5 h, so that the time of contact of NPs in the HS was in total the practical incubation times and an additional 2 h. At certain time intervals, the dissolved NPs were added to 100% HS, resulting in a NP concentration of 1.4 mg mL\(^{-1}\) and 55 wt.% HS. These successively prepared samples were then incubated at 40 °C, 37 °C or 6 °C for 0 h, 0.5 h, 1 h, 2 h, 4 h, 8 h, and 16 h. After that, these samples were filled in the corresponding sector of the centrifuge cells. The reference cells were filled with water. This experimental setup at room temperature took 0.5 h. The filled AUC cells were inserted in the pre-tempered rotor, which was then inserted in the pre-tempered AUC. The re-tempering of the AUC under vacuum with the inserted rotor and samples to reach the desired measurement temperature took 1.5 h. Starting from the tenth scan, every fifth scan was used for data evaluation.

The data range for the evaluation of the radial sedimentation velocity profile scans was set approximately 0.25 cm from the modeling boundary close to the meniscus.

1.7. NPs incubated in HS at 37 °C and investigated at 22 °C
The presumable degradation of samples for measurements of NPs in 55 wt.% HS for shorter incubation timescales was measured at 22 °C and a rotor speed of 7500 rpm. The time between scans was 3 min. The dissolved NP solutions were added to 100% HS, resulting in a NP concentration of 1.4 mg mL\(^{-1}\) and 55 wt.% HS. Samples were prepared this way at once, stored at 6 °C and then subsequently stored in the incubator for 0 min, 10 min, 20 min, 30 min, 60 min, and 90 min at 37 °C. After the mentioned incubation times, the samples were cooled at a temperature of 6 °C pending the AUC measurement procedure.

For example, the sample incubated for 60 min at 37 °C, was stored 30 min at 6 °C before being incubated at 37 °C for 60 min. The control sample of 1.4 mg mL\(^{-1}\) NPs only in water was stored for 90 min at 6 °C. For these experiments, the equilibration time of the AUC was possible to be maximally reduced, the experiment setup (filling the centrifuge cells and inserting the rotor) took again an additional 0.5 h. Beginning with the tenth scan, every fifth scan was used for the data evaluation.

The data range for the evaluation of the radial sedimentation velocity scans was set approximately 0.25 cm from the modeling boundary close to the meniscus.
2. Additional results

2.1. Figures S1 to S5

**Fig. S1** Differential distributions of sedimentation coefficients, $l_s$-$g^*(s)$, of NPs at a concentration of 1.4 mg mL$^{-1}$ monitored at a rotor speed at 7500 rpm and a temperature of 37 °C. (a) Overlay of $l_s$-$g^*(s)$ obtained by absorbance detection at a wavelength of $\lambda = 635$ nm in terms of OD and RI detection of NPs in PBS solution. (b) $l_s$-$g^*(s)$ of NPs in different fluids obtained by the absorbance detection in terms of OD at a wavelength of $\lambda = 635$ nm. In all cases the samples setup took 0.5 h, the samples were then placed in the centrifuge that re-equilibrated at 37 °C for 1.5 h. Overall, the NDDS had a contact time with the biofluids of 2 h before starting the rotor (see Supporting Information, section 1.5.).

**Fig. S2** Radially resolved scans at $\lambda = 635$ nm in terms of OD at a rotor speed of 7500 rpm and at 37 °C. (a) $\gamma$-globulin at a concentration of 7 mg mL$^{-1}$, (b) HSA at a concentration of 21 mg mL$^{-1}$, and (c) 55 wt.% HS. The samples setup took 0.5 h, the samples were then placed in the centrifuge that re-equilibrated at 37 °C for 1.5 h. Overall, the biofluids spend 2 h before starting the rotor (see Supporting Information, section 1.5.).
The control sample of NPs in water was immediately cooled at 6 °C until the measurement. Measurements were performed at a rotor speed of 7500 rpm. The initial NP concentration was 1.4 mg mL\(^{-1}\) in 55 wt.% HS and were incubated for the given times.

Fig. S3 Differential distributions of sedimentation coefficients, \(l_s g^*(s)\), of NPs observed with the absorbance detector at \(\lambda = 635\) nm in terms of OD and at a rotor speed of 7500 rpm. The initial NP concentration was 1.4 mg mL\(^{-1}\) in 55 wt.% HS and were incubated for the given times. All samples had a setup time at room temperature for 0.5 h and 1.5 h for reaching AUC temperature re-equilibrium at the desired measurement temperature. See Supporting Information, section 1.6. for further experimental details. (a) Incubation and measurement at 40 °C, and (b) incubation and measurement at 6 °C.

Fig. S4 Normalized differential distributions of sedimentation coefficients, \(l_s g^*(s)\), of NPs monitored by absorbance detection at a wavelength of \(\lambda = 635\) nm in terms of OD and at a rotor speed of 7500 rpm. NPs were incubated in 55 wt.% HS for different timescales at 37 °C and subsequently cooled at 6 °C. The control sample of NPs in water was immediately cooled at 6 °C until the measurement. Measurements were performed at 22 °C to maximally reduce the temperature re-equilibration time of the AUC. These samples experienced only a further 0.5 h of experimental setup time at room temperature. See Supporting Information, section 1.7. for further experimental details.

Fig. S5 Normalized differential distributions of sedimentation coefficients, \(l_s g^*(s)\), or intrinsic sedimentation coefficients, \(l_s g^*([s])\), of NPs determined with the absorbance detector at \(\lambda = 635\) nm in terms of OD and at a rotor speed of 7500 rpm. NPs had a concentration of 1.4 mg mL\(^{-1}\) in 55 wt.% HS. Measurements were performed at different temperatures as indicated. See section 1.5. for details. Samples were not incubated but were in contact with HS during the experimental setup at room temperature for 0.5 h and the AUC temperature re-equilibration time of 1.5 h. Overall, the NPs had a contact time with the biofluids of 2 h before starting the rotor. (a) Normalized \(l_s g^*(s)\) distributions and (b) Normalized \(l_s g^*([s])\) distributions with 
\[
[s]=\frac{1}{1-n_0 \rho_0},
\]
where \(n_0\) is the 55 wt.% HS viscosity and \(\rho_0\) the density at the different temperatures (Table S1).
Normalized differential distributions of intrinsic sedimentation coefficients, \( \text{ls} \cdot g^*(\text{[s]}) \), of NPs determined with the absorbance detector at \( \lambda = 635 \text{ nm} \) in terms of OD and at a rotor speed of 7500 rpm. NPs had a concentration of 1.4 mg mL\(^{-1}\). NPs were incubated at a temperature of 37 °C for 4 h and spent subsequently further time during experimental setup (0.5 h) and temperature re-equilibration (1.5 h) of the AUC. For detailed incubation and experimental conditions, the reader is referred to Section 1.5. The \( \text{ls} \cdot g^*(\text{[s]}) \) distributions were established using the relation \( \text{[s]} = \text{s} \eta_0 / (1 - \nu_{NP} \rho_0) \), where \( \eta_0 \) is the water or PBS viscosity and \( \rho_0 \) the respective density of the solvents (Table S2).

### 2.2. Tables S1 & S2

**Table S1.** Measured viscosities, \( \eta_0 \), and densities, \( \rho_0 \), of 55 wt.% HS in aqueous dilution at different temperatures

<table>
<thead>
<tr>
<th>T, °C</th>
<th>( \eta_0 ), mPas</th>
<th>( \rho_0 ), g cm(^{-3})</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>1.8156</td>
<td>1.0118</td>
</tr>
<tr>
<td>20</td>
<td>1.2321</td>
<td>1.0097</td>
</tr>
<tr>
<td>37</td>
<td>0.8554</td>
<td>1.0046</td>
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<tr>
<td>40</td>
<td>0.8099</td>
<td>1.0034</td>
</tr>
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</table>

**Table S2.** Measured viscosities, \( \eta_0 \), and densities, \( \rho_0 \), of utilized solvents at 37 °C for Fig. 1.

<table>
<thead>
<tr>
<th>Solvent</th>
<th>T, °C</th>
<th>( \eta_0 ), mPas</th>
<th>( \rho_0 ), g cm(^{-3})</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \text{H}_2\text{O} )</td>
<td>37</td>
<td>0.7035</td>
<td>0.9911</td>
</tr>
<tr>
<td>( \text{H}_2\text{O} / 0.01 \text{ M PBS} )</td>
<td>37</td>
<td>0.7172</td>
<td>0.9979</td>
</tr>
</tbody>
</table>

### 3. References