SUPPLEMENTARY INFORMATION

Determination of methemoglobin in hemoglobin based oxygen carriers by spectral extinction measurements

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### Abbreviations

| Abbreviation | Description |
|--------------|-------------|
| AC           | Analytical centrifugation |
| AHD          | Alkaline hematin and detergent method for total Hb determination |
| AFM          | Atomic force microscopy |
| CCD          | Charge coupled device |
| deoxyHb      | Deoxygenated variant of hemoglobin |
| deoxyHbMP    | Suspension of HbMPs prepared to deoxyHb |
| DLS          | Dynamic light scattering |
| DCHb         | Diaspirin cross-linked hemoglobin |
| EDTA         | Ethylenediaminetetraacetic acid |
| FCM          | Flow cytometric measurement |
| Gl           | Heme-free globin |
| Hb           | Hemoglobin |
| HbMP(s)      | Hemoglobin microparticle(s) |
| HBOC         | Hemoglobin based oxygen carrier |
| HiCN         | Cyanhemoglobin |
| HSA          | Humans serum albumin |
| metHb        | Methemoglobin with Fe in the ferric (Fe(III)) state |
| metHbMP      | Suspension of HbMPs treated to convert Hb into metHb |
| NEXAFS       | Near edge X-ray absorption fine structure |
| oxyHb        | Oxygenated variant of hemoglobin |
| oxyHbMP      | Suspension of HbMPs prepared to obtain oxyHb, i.e. oxygen saturation |
| PGM          | Plane grating monochromator |
| PLO          | Poly-L-Ornithine |
PSD Particle size distribution
RI Refractive Index
SDD Silicon drift detector
SEM Scanning electron microscopy
SE2 Low energy secondary electrons in SEM experiments emerging from the sample surface
SEMPA Spectral extinction measurement in particle suspension and analysis
VSECS Volume specific extinction cross section

Symbols

\( \alpha_{\text{Hb}}(\lambda) \) Increment of the real part of the RI as function of the total Hb concentration
\( \alpha_{\text{HSA}}(\lambda) \) Increment of the real part of the RI as function of the concentration of HSA
\( \alpha_x(\lambda) \) Increment of the real part of the RI as function of the concentration of the Hb variants \( x \in \{ \text{oxyHb, deoxyHb, metHb} \} \)
\( \beta_{\text{Gl/HSA}} \) Mass concentration of Gl/HSA in the HbMPs
\( \beta_{\text{Hb}} \) Mass concentration of Hb in the HbMPs
\( \beta_{\text{Gl/HSA}}^{\text{sup}} \) Mass concentration of Gl / HSA in the supernatant of the HbMP stock suspension
\( \beta_{\text{Hb}}^{\text{sup}} \) Mass concentration of Hb in the supernatant of the HbMP stock suspension
\( \beta_{\text{Gl/HSA}}^{\text{sus}} \) Mass concentration of Gl / HSA in the HbMP stock suspension
\( \beta_{\text{Hb}}^{\text{sus}} \) Mass concentration of Hb in the HbMP stock suspension
\( C_0 \) Concentration of HbMPs in the stock suspension
\( C_{\text{ext}}(\lambda) \) Spectral extinction cross section
\( \overline{C}_{\text{ext}}(\lambda) \) Ensemble averaged spectral extinction cross section
\( D \) Diameter of HbMPs
\( D_{\text{median}} \) Median diameter of the PSD of HbMPs
\( \text{DI}_{\text{Hb}} \) Density increment of Hb
\( \text{DI}_{\text{Gl/HSA}} \) Density increment of Gl/HSA
\( \epsilon_x(\lambda) \) Molar extinction cross sections of the Hb variants \( x \in \{ \text{oxyHb, deoxyHb, metHb} \} \)
\( \varphi \) Volume fraction of stock suspension in the measurement suspension
\( \phi_x \) Mass fractions of the Hb variants \( x \in \{ \text{oxyHb, deoxyHb, metHb} \} \)
\( \gamma_{\text{Hb}}(\lambda) \) Increment of the imaginary part of the RI as function of the total Hb concentration
\( \gamma_{\text{HSA}}(\lambda) \) Increment of the imaginary part of the RI as function of the HSA concentration
\( \gamma_x(\lambda) \) Increment of the imaginary part of the RI as function of the concentration of the Hb variants \( x \in \{ \text{oxyHb, deoxyHb, metHb} \} \)
\( \kappa(\lambda) \) Imaginary part of the RI
\( \ell \) Absorbance length of the cuvette
\( \lambda \) Wavelength
\( M_{\text{Hb}} \) Molar mass of the hemoglobin tetramer
\( n(\lambda) \) Real part of the RI
\( \text{PPV} \) Packed particle volume
\( \rho^{\text{HbMP}} \) Density of HbMPs
\( \rho^{\text{Pro}} \) Density of Pronase solution (0.9982g in 100.2617g H\(_2\)O)
\( \rho^{\text{RAc}} \) Density of acetated Ringer’s solution
\( \rho^{\text{sup}} \) Density of supernatant of HbMP stock suspension
\( \rho^{\text{sus}} \) Density of HbMP stock suspension
\( T(\lambda) \) Spectral transmittance
\( \overline{T}(\lambda) \) Ensemble averaged spectral transmittance
\( V \) Median volume of the HbMPs
\( Z(\lambda) \) Volume specific extinction cross section
Figure S1. Optical Setup for collimated transmittance measurements. Mirrors to increase the length of light path as well as the apertures ensure a low divergence of both the incident and detected beams.
Figure S2. Influence of median particle size on the volume-specific extinction cross section for HbMPs of given relative size distribution width $\sigma_D = 25.8\%$ and composition. For the calculations we used the mass concentrations of $120 \text{ g L}^{-1}$ Hb and $130 \text{ g L}^{-1}$ Gl/HSA) and an RI corresponding to 100% metHb.
Figure S3. Influence of distribution width parameter $\sigma_D$ of the log-normal particle size distribution on the volume-specific extinction cross section of HbMPs at fixed median $D_{\text{median}} = 760$ nm.
Figure S4. Packed particle volume (PPV) or solid fraction of the HbMP stock suspension as function of time. The relative centrifugation force RCF amounted to 6250 g, the centrifugation time was 21 min. The three symbols (filled circle, diamond and cross) characterize the three different persons involved in the measurements. Data were fitted by a dose response function.
Figure S5. Oxygenation – deoxygenation and effect of particle loss. “air 1” denotes the measurement before bubbling with argon, “argon” denotes the measurement directly after and “air 2” the measurement after the sample was re-exposed to air. The latter two curves were re-scaled to have the lowest deviation from curve “air 1”.

RI determination of HSA. Literature values are available for the real RI increment of HSA in aqueous solutions. However, these were either determined using white light or a single wavelength (typically the sodium D-line at 589 nm). Hence, we determined the wavelength-dependence of the real part of the RI increment by own measurements. We used a HSA infusion solution (Human Albumin 200 g L\(^{-1}\) Baxalta Infusionslösung) as the sample, which contains proteins from human plasma, of which at least 95% are albumin (HSA).

Firstly, absorption spectra were recorded with a Cary6000i UV-Vis-NIR spectrophotometer (Agilent) using multiple dilutions with ultrapure water in pair-matched 10mm cuvettes. This yields the imaginary part \(\gamma_{\text{HSA}}(\lambda)\) of the RI increment. At wavelengths higher than 700 nm, the absorbance is too low to be measured for the undiluted 200 g / L solution in a 10 mm cuvette. Here, we set \(\gamma_{\text{HSA}}(\lambda) = 0\) in the simulations (compare Figure 5). Furthermore, \(\gamma_{\text{HSA}}(\lambda)\) is significantly lower than \(\gamma_{\text{Hb}}(\lambda)\) everywhere in the spectral range of 300 nm to 800 nm. The \(\gamma(\lambda)\) curves come close to each other in the UV below 300 nm, where the spectrum of the HSA infusion solution also exhibits a significant absorbance contribution due to its content of 16 mmol/L sodium acetyltryptophanate, making up an estimated 50% of the total absorbance at 279 nm (the absorbance peak of the amino acid tryptophan). However, this is outside the spectral range under consideration. Hence, the error due to the presence of sodium acetyltryptophanate has no significant effect on the simulation results.

The real part of the spectral RI increment \(\alpha_{\text{HSA}}(\lambda)\) was determined using the same measurement setup as for the extinction measurements for the HbMPs. To this end, quasi-monodisperse polystyrene beads with 2.539 µm nominal diameter (Lot. PS-ST-L2552, microparticles GmbH, Germany) were suspended in the 200 g L\(^{-1}\) HSA solution at different
particle concentrations and the collimated transmittance was measured. Given the spectral RI of the particles, obtained from an extinction measurement in pure water\textsuperscript{S1}, the real part of the spectral RI of the suspending fluid (i.e., the HSA solution) can be obtained from these measurements. For this purpose, the method described in Ref. S1 was modified\textsuperscript{S2}. The fact that the HSA solution absorbs light in UV limits the wavelength range available for transmittance measurements to $\lambda \geq 300$ nm, but otherwise does not hamper the data analysis which is based on the assumption of a non-absorbing fluid\textsuperscript{S2}. Hence, the RI of the aqueous HSA solution is

$$n(\lambda) = n_{H_2O}(\lambda) + \beta_{HSA} \alpha_{HSA}(\lambda).$$ \hspace{1cm} (S1)

For the model fit of the measured $\bar{C}_{\text{ext}}(\lambda)$ the spectral dependence of the RI increment $\alpha_{HSA}(\lambda)$ was expressed by a 2-term Cauchy equation, i.e.

$$\alpha_{HSA}(\lambda) = B + \frac{C}{\lambda^2}.$$

\hspace{1cm} (S2)

The result for the coefficients is $B = 0.180$ mL g\textsuperscript{-1} and $C = 3.2 \times 10^{-3}$ mL g\textsuperscript{-1} \textmu m\textsuperscript{-2} for $300$ nm $\leq \lambda \leq 1100$ nm. The estimated uncertainty of $\alpha_{HSA}(\lambda)$ is $u(\alpha_{HSA}(\lambda)) = 0.008$ mL g\textsuperscript{-1}. For verification purposes, the RI was also determined with an Abbe refractometer at 590 nm, which resulted in $0.190(4)$ mL g\textsuperscript{-1} compared to $0.188(8)$ mL g\textsuperscript{-1} from the Cauchy equation. Both values agree with each other as well as with literature values\textsuperscript{S3}.

As already stated in Section Model for Optical Properties of HbMPs of the paper, we take the optical properties of HSA for both, HSA and (heme-free) globin, since the increments of the real and imaginary parts $\alpha(\lambda)$ and $\gamma(\lambda)$ for globin are unknown but expected to be very similar to HSA. Compared to Hb, globins do not have a heme group causing a strong absorption associated
with strong variations of the complex RI. In addition, the molecular masses of the two globular proteins are similar.

(S1) Gienger, J.; Bär, M.; Neukammer, J. Extinction spectra of suspensions of microspheres: determination of the spectral refractive index and particle size distribution with nanometer accuracy. *Applied Optics* **2018**, Vol. 57, 344-355.

(S2) Gienger, Jonas. Determination of optical and geometrical properties of blood cells and microparticles from light scattering measurements. *PhD thesis* **2019**, Technische Universität Berlin.

(S3) Barer, R. and Joseph, S. Refractometry of Living Cells: Part I. Basic Principles. *Quarterly Journal of Microscopical Science* **1954**, Vols. s3-95, 32, pp. 399-423.
**Figure S6.** Particle diameter of HbMPs measured by DLS for different concentrations using back scatter (blue dots) and side scatter (red triangles). The mean value is plotted as black straight line and the standard deviation is included as dashed lines.
**Figure S7.** Correlation of the results for the particle size calculated from AC sedimentation measurements and the density of the HbMPs in the region of the values derived by the mechanical oscillator method. The line is interpolated by a polynomial equation.
**Figure S8.** Flow cytometric measurements of HbMPs and polystyrene microspheres. Dot plots of signal intensities for forward light scatter versus side scatter for HbMPs (a) and polystyrene microparticles (b). The cluster of HbMPs is gated to show only the population of HbMPs in the (c) SSC histogram together with signals originating from monodisperse polystyrene particles with diameters of 220 nm and 500 nm, respectively.
**Figure S9.** Absorption spectra of the HbMP suspension resulting from different dilutions. The respective volume fractions $\varphi_i$ of the HbMP suspension in the measurement sample are included in the Figure. HbMPs were digested by a Pronase solution and converted by AHD reagents in a stable end product, i.e. hematin.