

Influence of osmolarity on the optical properties of human erythrocytes

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Abstract. Plasma osmolarity influences the volume and shape of red blood cells (RBCs). The volume change is inversely related to the hemoglobin concentration and as a consequence to the complex refractive index within the cell. These morphological changes can be linked to changes in the optical behavior of the cells. The optical parameters, absorption coefficient μ_a , scattering coefficient μ_s , and effective scattering phase function of red blood cells are investigated in dependence on osmolarity in the spectral range from 250 to 1100 nm. Integrating sphere measurements of light transmittance and reflectance in combination with inverse Monte-Carlo simulations are carried out for osmolarities from 225 to 400 mosmol/L. Osmolarity changes have a significant influence on the optical parameters, which can in part be explained by changes in the complex refractive index, cell shape, and cell volume. Spherical forms of RBCs induced by low osmolarity show reduced scattering effects compared to the normal RBC biconcave disk shape. Spinocytes, which are crenated erythrocytes induced by high osmolarity, show the highest scattering effects. Even only a 10% change in osmolarity has a drastic influence on the optical parameters, which appears to be of the same order as for 10% hematocrit and oxygen saturation changes. © 2010 Society of Photo-Optical Instrumentation Engineers. [DOI: 10.1117/1.3486542]

Keywords: optical parameters; red blood cells; osmolarity; Monte-Carlo simulation; scattering coefficient; absorption coefficient; anisotropy factor; effective scattering phase function.

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1 Introduction

It is known that the optical parameters μ_a , μ_s , and g of blood depend on various physiological parameters. The frequently investigated parameters are oxygen saturation and hematocrit.¹⁻⁶ These parameters change, among other things, the concentration of the red blood cells or the absorption behavior of the hemoglobin. If these two main parameters remain constant, the parameters cell shape and volume of physiological red blood cells can cause changes in the optical properties.

The variations in osmolarity not only change the shape and the volume of the cells but also the inner cell hemoglobin concentration. Due to an increase in the hemoglobin concentration, the complex refractive index increases.⁷ A hyperosmolar medium causes a shrinking of the cells due to water outflow, resulting in characteristically shaped cells called spinocytes. In low-osmolarity media, the cells start to swell by diffusion of water into the cell, leading to spherically formed cells, which become full to the point of bursting.

Osmolarity changes can be caused as the result of diagnostic measurements in extracorporeal blood circulations and dur-

ing *in vitro* investigations on blood, e.g., immersion optical clearing.⁸⁻¹⁰ Data concerning the influence of osmolarity on the optical properties are not readily available.

In this study, the influence of the osmolarity was investigated on the optical parameters of red blood cells in physiological saline solution. Using the integrating sphere technique combined with an inverse Monte Carlo simulation¹¹ (iMCS), three optical parameters (absorption coefficient μ_a , scattering coefficient μ_s , and anisotropy factor g) of undiluted, flowing red blood cells (RBCs) could be determined, dependent on osmolarity in the spectral range of 250 to 1100 nm, including the spectral areas of high hemoglobin absorption.

2 Materials and Methods

2.1 Blood Preparation

To measure native RBCs in saline solution, fresh human erythrocytes from healthy blood donors were centrifuged three times and washed with isotonic phosphate buffer (300 mosmol/L, pH 7.4) to remove the blood plasma and free hemoglobin. RBC suspensions with a hematocrit of 42.1% were investigated.

The hematocrit was determined using an RBC counter (Micros 60 OT 18, ABX Diagnostics, Montpellier, France).

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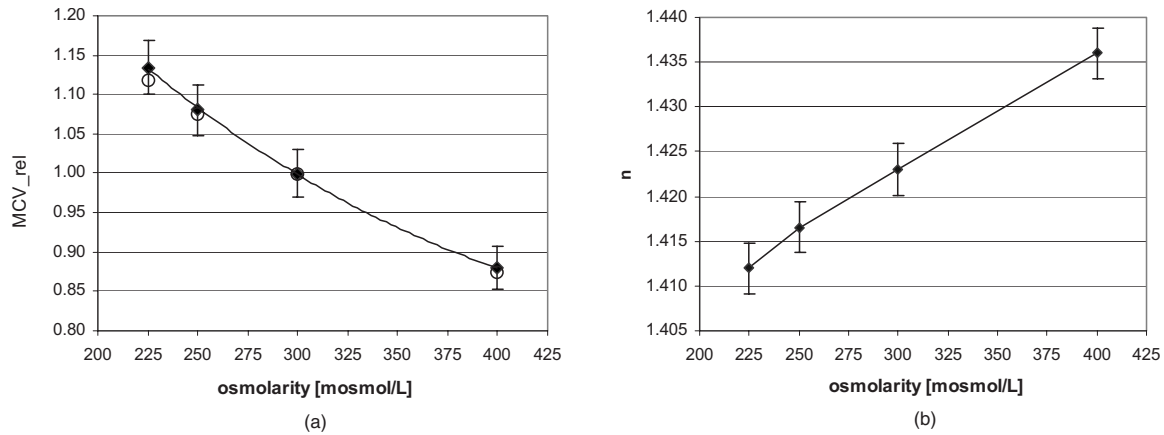


Fig. 1 (a) Osmolarity dependent relative mean cell volume (MCV_{rel}) measured and calculated according to the empirical formula of Zhestkov et al.¹⁰ (circles), and (b) the mean refractive index (n) at 600 nm of the RBC dependent on osmolarity, calculated according to Ref. 14.

Osmolarity-induced changes in cell volume were measured by centrifugation of hematocrit capillaries. All samples were oxygenated in excess of 98%. The oxygen saturation was determined with a blood gas analyzer (OPTI Care, AVL Medizintechnik GmbH, Bad Homburg). A miniaturized blood circulation setup was used with a roller pump (Sorin Group, Germany) and a blood reservoir, which was continually aerated with a gas mixture of O₂, N₂, and CO₂. The temperature was kept constant at 20°C. The blood was gently stirred to avoid uncontrolled sedimentation or cell aggregation within the reservoir. The blood was kept flowing with a wall shear rate of 600 s⁻¹ at the cuvette windows by a customized turbulence-free cuvette with laminar flow and a sample thickness of 116 μm. To investigate the influence of osmolarity, six different buffer solutions were prepared with osmolarities between 225 and 400 mosmol/L.

2.2 Spectral Measurements

The diffuse reflectance R_d , the total transmission T_t , and the diffuse transmission T_d of all blood samples were measured using an integrating sphere spectrometer (Perkin Elmer, Lambda 900) in the spectral range of 250 to 1100 nm at data intervals¹² of 5 nm. The experimental setup was identical to that described by Friebel et al.¹¹ and enabled the measurement of macroscopic radiation distribution with an error of less than 0.1%. For calculating intrinsic optical parameters, a special high-precision iMCS program was used that takes into consideration various radiation losses.⁵ Hematocrit (Hct)-dependent effective phase functions for RBCs flowing with a shear rate of 600 s⁻¹ were evaluated previously using the double integrating sphere technique.⁴ No significant osmolarity dependency of the phase function, other than changes in the anisotropy factor g , could be observed. Therefore, the iMCS was carried out using the Reynolds-McCormick phase function¹³ for Hct 42% with $\alpha=1.7$ for all osmolarity values from 225 to 400 mosmol/L. An error threshold of 0.1%, i.e., the difference between measured and simulated macroscopic radiation distribution, was used for the simulation of the intrinsic optical parameters of blood. A total of three independent measurement series was carried out for all different osmolarities. They were independently simulated, using about

10⁷ photons for each simulation. The standard deviation of the three measurements was 2 to 5% for μ_a , μ_s , (1-g), and μ'_s as is shown in the diagrams.

3 Results

The optical properties of red blood cells depend on the biological variability of the size and shape of the RBCs and the hemoglobin (Hb) content. To exclude the optical influence of this biological variability of RBC samples with identical Hcts taken from different donors, all intrinsic optical parameters given in this paper are relative values related to those determined at an osmolarity of 300 mosmol/L.

The investigated RBC samples with RBC concentration (RBCc) of $5 \times 10^6/\mu\text{l}$ had an Hct of 42.1%, a mean corpuscular volume (MCV) of 89.3 μm³, and a mean corpuscular hemoglobin concentration (MCHC) of 34.4 g/dL. An increase in osmolarity to 400 mosmol/L resulted, via cell shrinking, in a reduced Hct of 37%; a decrease to 225 mosmol/L resulted, via cell swelling, in an increased Hct of 45.5%. The MCV can be calculated by $\text{Hct} [\%] = \text{MCV} \times \text{RBCc} \times 100\%$. Changes in cell volume lead to a reciprocal change in hemoglobin concentration within the cell (MCHC) and therefore also in the refractive index.¹⁴ The changes in the relative cell volume (MCV) and the refractive index with different osmolarities are shown in Fig. 1.

The measured MCV changes are in agreement with calculated values, based on a formula presented by Zhestkov et al.¹⁰ and measurements of Roggan et al.⁵ The swelling of the cells resulted in a higher MCV and a lower refractive index and vice versa. The refractive index was estimated using the formulas of Friebel and Meinke.¹⁴ At 600 nm, for example, a refractive index of $n_{300} = 1.423 \pm 0.003$ can be calculated under isotonic conditions (300 mosmol/L). According to the reciprocal changes in the MCHC the refractive index decreases to $n_{225} = 1.416 \pm 0.003$ at 225 mosmol/L and increases to $n_{400} = 1.436 \pm 0.003$ at 400 mosmol/L. For comparison, effects on the absorption and scattering cross sections and the anisotropy factor were estimated from these refractive index changes via Mie theory for spheres of the same volume, which is used as a simplified RBC model. In special cases, Mie theory can also be used^{4,11} to calculate absorption and

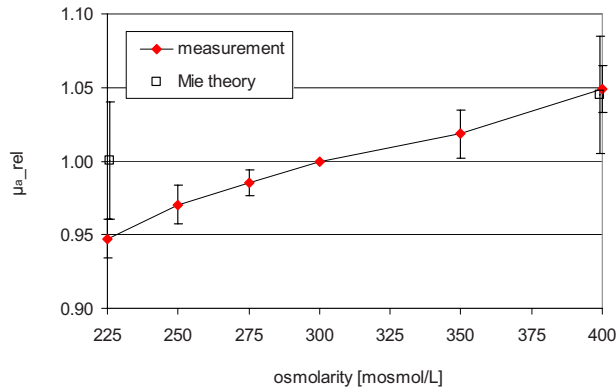


Fig. 2 Mean values of μ_{a_rel} of the spectral range 250 to 1100 nm of RBCs in saline solution dependent on osmolarity and values for 225 and 400 mosmol/L calculated by Mie theory relative to 300 mosmol/L.

scattering cross section of RBCs. It is known from recent investigations that calculation of the absorption coefficient is possible in the wavelength range 600 to 1100 nm, where μ_a is low, μ_s may only be estimated for highly diluted blood (\ll Hct 1%), and the anisotropy factor can only be predicted for diluted blood (Hct \approx 1%) in the spectral range of low absorption. Nevertheless, Mie theory may be useful for calculating effects on the optical parameters induced by small relative volume and refractive index changes. These expected relative changes of the optical parameters at 225 and 400 mosmol/L relative to 300 mosmol/L were determined and averaged over all 171 wavelengths in the range 250 to 1100 nm.

3.1 Absorption Coefficient

The relative absorption spectra show no significant dependence on wavelength. Therefore, mean values over the whole spectrum could be established. The influence of the osmolarity on the relative absorption coefficient is shown in Fig. 2.

The absorption coefficient increases with increasing osmolarity. This means shrinking of the cells increases and swelling decreases μ_a . At 225 mosmol/L, the value of μ_a is 1.95 ± 0.01 , and at 400 mosmol/L, it is 1.05 ± 0.02 . The values calculated with Mie theory show a comparable increase to 1.04 ± 0.04 (the error describes the statistical distribution of cell volume and refractive index within the cell) at 400 mosmol/L, whereas the decrease of the osmolarity to 225 mosmol/L does not lead to a significant change with 1.00 ± 0.04 .

3.2 Scattering Coefficient

As observed for the absorption, μ_{s_rel} of RBC solutions show no significant dependence on wavelength but are significantly influenced by changes in osmolarity. Figure 3 shows the mean relative values of μ_s dependent on osmolarity.

As found for μ_{a_rel} , the relative scattering coefficient also increases with increasing osmolarity with values of 0.87 ± 0.04 at 225 mosmol/L and 1.11 ± 0.04 at 400 mosmol/L. Induced by the change in volume and refrac-

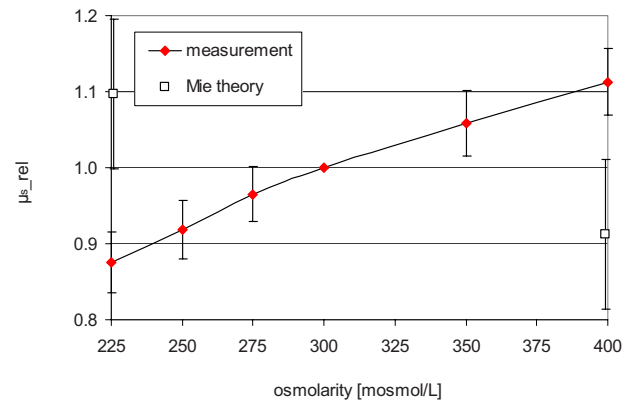


Fig. 3 Mean values of μ_{s_rel} in the spectral range 250 to 1100 nm of RBCs in saline solution dependent on osmolarity and values for 225 and 400 mosmol/L calculated by Mie theory relative to 300 mosmol/L.

tive index, Mie theory shows an inverse change in the scattering cross section of 1.1 at 225 mosmol/L and 0.9 at 400 mosmol/L.

3.3 Anisotropy

In contrast to μ_{a_rel} and μ_{s_rel} , g_rel shows distinct wavelength dependence below 600 nm. Figure 4 shows the relative values of the anisotropy factor g of RBCs suspended in saline solution for different osmolarity values, dependent on wavelength. Therefore, a mean g_rel only averaged over the spectral range 600 to 1100 nm was calculated for each osmolarity. In addition, the osmolarity dependence at 415 nm was determined to characterize the wavelength dependence in the range of high absorption. The averaged relative changes of g are depicted versus the osmolarity in Fig. 5, including g_rel at 415 nm. The decrease with increasing osmolarity is more pronounced in the high absorption region of hemoglobin and has its maximum at 415 nm.

In the wavelength range above 600 nm, the mean relative g value is linearly dependent on osmolarity. At 225 mosmol/L, g_rel has the value 1.007 ± 0.001 , and at

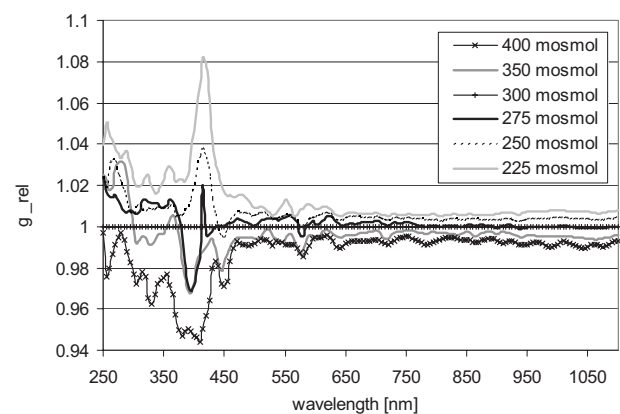


Fig. 4 Mean values of g_rel dependent on wavelength for six different osmolarity values (relative to 300 mosmol/L).

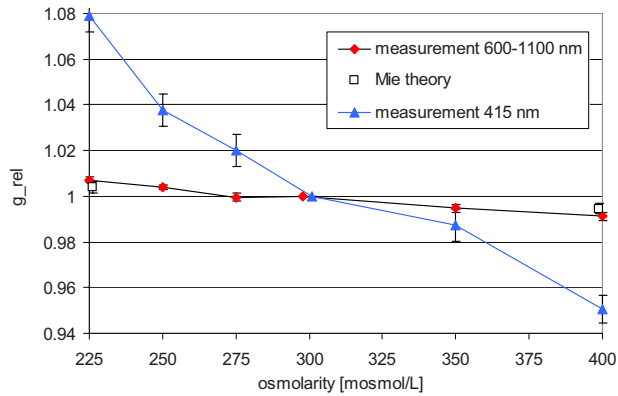


Fig. 5 Mean values of g_{rel} at 415 nm and of the spectral range 600 to 1100 nm of RBCs in saline solution dependent on osmolarity and values for 225 and 400 mosmol/L calculated by Mie theory relative to 300 mosmol/L.

400 mosmol/L, only 0.991 ± 0.002 . This is within the error tolerance in the range of the values calculated with the Mie theory.

As already could be observed in Fig. 4, the influence of the osmolarity on g is significantly more pronounced at 415 nm, where the hemoglobin absorption is high than in the wavelength region above 600 nm where there is low absorption. The g_{rel} values for 415 nm results in 1.080 ± 0.007 at 225 mosmol/L and 0.950 ± 0.008 at 400 mosmol/L.

3.4 Effective Scattering Coefficient

Corresponding to the anisotropy factor, μ'_s shows slightly different behavior below and above 600 nm. Therefore mean values of μ'_s in the wavelength range 600 to 1100 nm were calculated for the osmolarity dependence as well as for 415 nm to characterize the wavelength dependence in the range of high absorption (Fig. 6).

In the spectral range 600 to 1100 nm μ'_s increases linearly with increasing osmolarity. At 400 mosmol/L μ'_s is 1.39 ± 0.06 , twice as high as at 225 mosmol/L with 0.65 ± 0.06 . At 415 nm, μ'_s shows a lower increase with values of 0.73 ± 0.06 at 225 mosmol/L and 1.27 ± 0.05 at

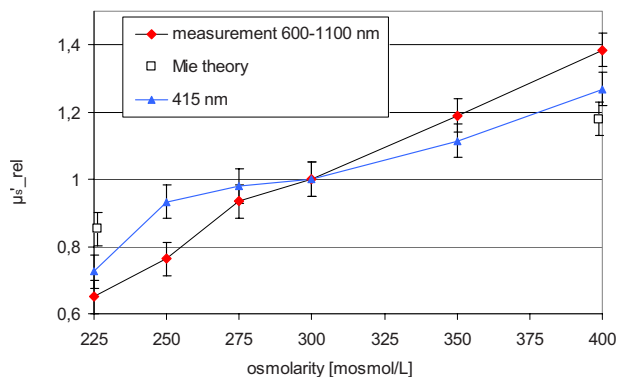


Fig. 6 Mean values of μ'_s of RBCs in saline solution in the spectral range 600 to 1100 nm and at 415 nm dependent on osmolarity and values for 225 and 400 mosmol/L calculated by Mie theory relative to 300 mosmol/L.

400 mosmol/L. The Mie theory agrees with an increase with the osmolarity but the extent of the increase is only half of that in the range 600 to 1100 nm with values of 0.85 ± 0.03 at 225 mosmol/L and 1.18 ± 0.03 at 400 mosmol/L.

4 Discussion

All optical parameters are influenced by osmolarity changes. Moreover, dependence was found to exist between the osmolarity-dependent effects and the Hb absorption. An osmotically induced decrease in the cell volume by approx. 10% with an identical increase in Hb concentration within the cell leads to an increase in the absorption coefficient by approx. 5% and vice versa. This is, in principle, in agreement with the results of Roggan et al.,⁵ who also found a μ_a increase with increasing osmolarity at 633 nm and Hct 7.5% but with a higher steepness. This may be explained by the very low Hb absorption at this wavelength and the relatively high blood dilution. At a constant RBCc, the absorption coefficient, and therefore the absorption cross section, increases continuously with the osmolarity, i.e., cell shrinking leads to an increase of μ_a , cell swelling to a decrease. According to this, the absorption cross section is mainly determined by the Hb concentration, which changes inversely to the cell volume and determines the complex refractive index within the cell, whereas the geometric cross section of the cells is of minor importance. This is partly confirmed by Mie theory, predicting a comparable increase of the absorption cross section at the increasing imaginary and real parts of the refractive index within the cell by decreasing volume. However, the Mie theory does not show a decrease of the absorption cross section for a decreasing refractive index.

Averaged over the investigated spectral range, the increase in the refractive index induced by osmotic cell shrinking leads to an increase of the scattering coefficient for increasing osmolarity, in spite of the decreased geometric cross section and vice versa. In principle, this dependence is in agreement with the theoretical results of Bashkatov et al.⁸ using Mie theory and quantifier of the scattering cross section by the often used^{9,15,16} "packing factor" $1 - (\text{Hct}/100\%)$. He showed that increasing the osmolarity from 300 to 580 mosmol/L resulted in an increase of μ_s for seven investigated wavelengths in the range 415 to 900 nm, with the exception of 415 nm, where μ_s decreased slightly. A linear interpolation of the relative μ_s increase at an osmolarity increase from 300 to 400 mosmol/L averaged over all seven wavelengths resulted in a relative μ_s increase of approximately 3%. This indicates an attenuated dependence and is in contradiction to the presented Mie calculations based on our own refractive index measurements.^{7,14} Following this theory, the scattering cross section of a sphere is determined mainly by the sphere size and the difference in the refractive indices and predicts a decrease of the scattering cross section with increasing osmolarity and vice versa. As shown in experiments for the hematocrit dependence of the scattering coefficient,^{4,11} the scattering cross section per cell is not constant at increasing Hct, but continuously decreasing. The cause of this phenomenon is the decreasing distance between the cells, leading to an increasing superposition and interference of the single scattering events¹⁷ ("collective scattering"). The osmolarity-induced change in Hct influences analogously the distance between the cells, not

by changing the cell concentration, but the cell volume.

Based on these data, an interpolation of the relative scattering cross section per cell for the osmotically changed Hct values 37 and 45.5% leads, without consideration of the refractive index change within the cell, to a scattering cross section decrease to 0.92 at 225 mosmol/L and an increase to 1.12 at 400 mosmol/L. The correction of the calculated relative μ_s changes with values of 1.01 ± 0.09 at 225 mosmol/L and 1.02 ± 0.1 at 400 mosmol/L leads to an almost complete compensation of the μ_s changes predicted by Mie theory without correction. Within the error tolerances, the corrected values are comparable to the results of Bashkatov but still disagree with the experimentally determined distinct increase of μ_s with increasing osmolarity. Therefore, the change of the cell shape must be considered, which is not considered by the Mie theory. It is possibly the change in cell shape from the sphere via the normal discoid shape to the star-shaped spinocyte shape that leads to an additional increase in the scattering cross section.

The inverse dependence of μ_s on osmolarity presented by Roggan et al.⁵ can be explained by the use of another scattering phase function in the MCS used, since in combination with the lower increase of g with osmolarity, the changes in the resulting effective scattering coefficient μ'_s are comparable to the osmotically induced changes already presented.

In spectral ranges where the absorption is low, the anisotropy factor seems to be mainly determined by the difference between the real parts of the refractive index within the cell and in the surrounding medium. An osmolarity-induced decrease in volume by approx. 12 to 13% leads¹¹ via refractive index increase to a decrease in g of approx. 0.01 at wavelengths >600 nm. In principle, this is in agreement with the theoretical results of Bashkatov et al.,⁸ who also found that g decreased with increasing osmolarity from 300 to 580 mosmol/L, but by much lower relative values between 0.001 and 0.002. Similar to these values, our own calculated theoretical values of 0.005 ± 0.003 at 400 mosmol/L in the wavelength range above 600 nm are lower than the measured values but with overlapping error tolerances. However, Mie theory fails to describe the strong osmolarity-dependent decrease of g at 415 nm. This osmolarity dependence seems to increase significantly with the absorption. Induced by this phenomenon of swelling of the RBCs to a spherical shape, the marked decrease of g with increasing absorption, which was shown to be not explainable by Mie theory,^{4,11} will be diminished. Possibly, this absorption-dependent decrease of g is connected to the non-spherical shape of the erythrocyte.

The relative changes in the effective scattering coefficient for the wavelength range 250 to 1100 nm are qualitatively in agreement with the predictions of Mie theory. However, the μ'_s increase when the osmolarity increases is underestimated by Mie theory. When correcting the μ'_s values with the Hct-dependent scattering cross section, as was already done for the scattering coefficient, the predicted relative osmolarity-induced changes in μ'_s are with values of 0.75 at 250 mosmol/L and 1.3 at 400 mosmol/L within the error tolerances of the measured values. This may be also connected with the changes in the geometric shape. According to this, the ideal spherical shape may result in a lower effective

scattering effect compared to the discoid erythrocyte shape; deformation in the direction of a spinocyte would increase the effective scattering effect.

5 Conclusion

The investigation on osmolarity-dependent optical effects of RBCs showed that changes in cell volume and shape and the inversely related Hb concentration lead to significant changes in all the optical parameters. The maximal absorption and scattering cross sections were found for shrunken RBCs. For the anisotropy as well as for the effective scattering coefficient it was found that the osmolarity-dependent effects are different in wavelength ranges of high and low Hb absorption.

Apart from concentration- and absorption-induced changes in optical parameters due to Hct and oxygen saturation changes, the optical parameters can be drastically influenced by cell volume and cell shape changes due to osmolarity variations. Therefore, osmolarity-induced changes must be considered when optical measurements for the determination of blood properties are used.¹⁸

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