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Properties of erythrocyte light refraction in diabetic patients

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Abstract. Since hyperglycaemia changes the erythrocyte cell membrane fluidity and impairs cell deformity, our goal was to characterize hemoglobin and red blood cell (RBC) light refractive property changes in diabetic patients. Microscopic investigation was carried out on intact and fixed RBCs. To determine the refractive index (RI): smears of peripheral blood were air dried and fixed for 3 min in methanol. Mixtures of polyvinylpyrolidine and buffer of different *p*H (1:1) were used as embedding media. Intact RBCs were mixed with a buffered embedding medium, placed on a slide and overlaid with a coverslip. Interference microscopy was used for RI measurements at 18 different *p*H (*p*H=2–13). The results showed that curves of the RI of diabetic patients and of a control group were of similar configuration, with one branch in the acidic portion of the *p*H scale, a maximum and two minima in the neutral (middle) portion, and one branch in the alkaline portion. The curves of the individuals from the control group overlapped each other. To the contrary, the curves of the diabetic patients were not uniform in the neutral portion and the alkaline portion. The curves of the diabetic patients in the neutral zone were shifted towards the alkaline end of the *p*H scale, and the RBC RI curves were lower in comparison to the control curves. The center maximum of the curves of diabetic patients corresponded to *p*H=6.6 whereas the central maximum of the control group curves was at *p*H=6.2–6.8. Contrary to in the diabetic group, intact RBC RI curves in the control group revealed only one significantly different minimum at *p*H of 7.2 in the neutral zone. Using this method it is possible to show phenotypic differences between uniform type intact and fixed cells, erythrocytes of diabetic patients and of healthy donors. *© 2002 Society of Photo-Optical Instrumentation Engineers.* [DOI: 10.1117/1.1463043]

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1 Introduction

It is known that hyperglycaemia changes red blood cell membrane fluidity[1–5] and impairs red cell deformability.[6,7] Evaluation of the amount of glycated hemoglobin in erythrocytes could be an early diagnostic marker of hyperglycemia. An easily accessible red blood cell (RBC) characteristic is its refractive index,[8–10] which is due to the amount of hemoglobin it contains, since it is about 95% of the erythrocyte weight. Using polarizing-interference microscopy it is possible to measure the light refractive index in separate erythrocytes. Additional information about the hemoglobin amino acid content can be obtained by measuring the refractive index of erythrocytes at different *p*H levels.[11] Each amino acid molecule contains an amino group (NH_2) at one end and a carboxyl group (COOH) at the other end, and different side groups (R). R groups are classified according to their polarity: (1) apolar or hydrophobe R groups, (2) uncharged polar R groups, (3) "+" charged R groups, (4) "−" charged R groups.

In neutral solutions amino acids are doubly charged, but overall are neutral. In these circumstances molecules have high dielectric permeability and large dipole moments. These amino acid qualities are similar in protein solutions. Moreover, each protein has only one amino group and one carboxyl group, but several different R groups.

Classical theory of light dispersion in fluids can be applied to a cell. According to this theory, the light refractive index depends on the light's wavelength or on the frequency of light waves. There is a relationship between the polarizability of an atom or a molecule and the light refractive index. In general, if material consists of molecules of different polarizability, the total polarizability can be obtained as the sum of all individual molecules' polarizability. It is necessary to take into account that the molecule is affected by the external electric field and by electric fields induced by neighboring molecules.[12] According to light dispersion theory, the light refractive index formula is as follows:

$$n = \Re(\sqrt{\varepsilon}) = 1 + \frac{N}{\varepsilon_0}\,\frac{q^2}{m}\,\frac{\omega_o^2 - \omega^2}{(\omega_o^2 - \omega^2) + 4\gamma^2\omega^2}, \qquad (1)$$

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where ε is material permeability, N the number of molecules, m the molecule's mass, q the molecule's charge, ω the oscillation frequency, ω_o the oscillation self-frequency, and $2\gamma = g/m$ the force index, similar to friction force. This formula suggests that protein R group charge can affect the value of the refractive index regardless of its charge sign. Dissociation of R group protein molecules describes each protein and the refractive index (RI) depends on the content of charged amino acids in the molecule.

The aim of this study was the qualitative and quantitative evaluation of the hemoglobin of separate erythrocytes in diabetic patients and in healthy donors. To do this we have elaborated on a new optical method for hemoglobin characterization in intact and fixed cell erythrocytes.[13,14]

2 Material and Methods

2.1 Polarizing-interference Microscopy of RBC

Two kinds of RBCs, fixed and intact, were investigated. Smears of PB were prepared on slides, air dried, fixed for 3 min in methanol and air dried again. Mixtures of polyvinylpyrolidine and buffers of different pH (1:1) were used as embedding media. The RI of the embedding media was stable ($n = 1.5133 \pm 0.0001$) and independent of the type and the pH of the buffer. It was used as a standard value for the evaluation of the RI at different pH levels. Smears were embedded in the buffered medium and the refractive index for 35 RBCs was measured at each of the subsequent 18 different pH levels in the range of $p\text{H} = 2-13$. Intact PB was mixed with buffered embedding media and overlaid with a coverslip just before being investigated. Intact RBC refractive index measurements were carried out in a manner analagous to those with fixed cells. A Nomarski polarizing-interference microscope MPI-5 (Poland) was used for the measurements of light phase retardation.

Using a Wollaston prism mounted on an objective the erythrocyte images for ordinary and extraordinary light beams were completely separated. In the thickest erythrocyte region the first interference maxima were visually adjusted to the eye sensitive purple color for ordinary and extraordinary images by shifting the second Wollaston prism placed on the objective's rear focus. For each erythrocyte measured the Wollaston prism displacement rendered a second value. From the whole interference band width h and measured Wollastone prism displacement $2d$ the phase retardation Φ was calculated for each erythrocyte. Measurements were carried out at white light with an arbitrary wavelength of $\lambda = 0.550\ \mu$m. The value of the erythrocyte refractive index was calculated from the light phase retardation using

$$n = n_v + \Phi/t = n_v + \frac{d\lambda}{ht}, \tag{2}$$

where $n_v = 1.5133 \pm 0.0001$ refractive index of the embedding media. Deviation d and interference band width $h = 2.71$ were obtained from the Wollaston prism shifting in millimeters. In the first step the RBC thickness was estimated using two embedding media with different n (n_{v1} and n_{v2}) and the measuring phase deviation for fully divided RBC images. Full RBC image duplication was acquired using both Wollaston prisms

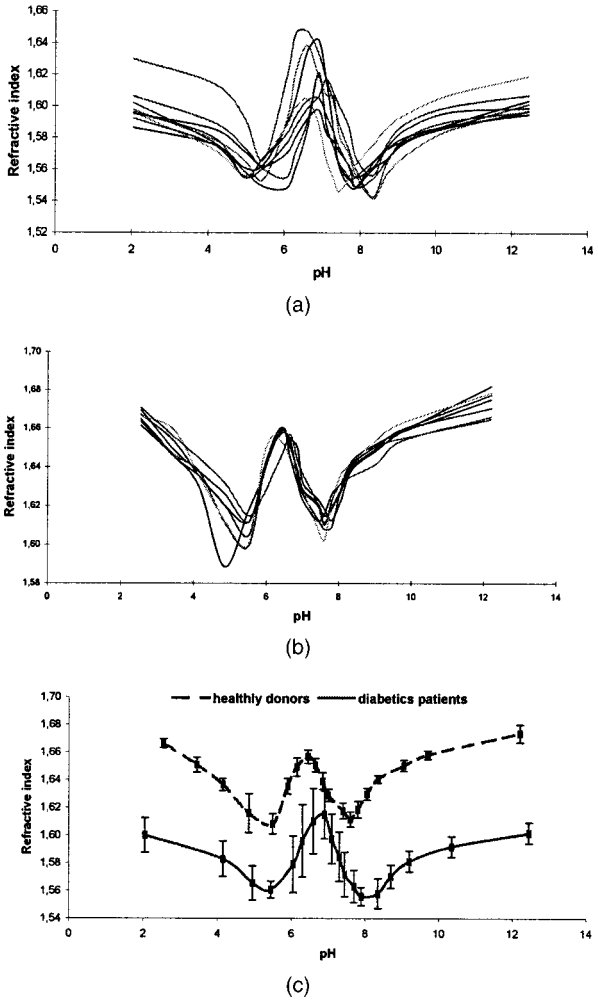

Fig. 1 Fixed RBC refractive index curves measured at 18 different levels of pH. (a) Individual curves of diabetic patients. (b) Individual curves of healthy donors. (c) Mean curves with standard errors for diabetic and healthy donor groups.

of the microscope objective and the prism between the objective and the ocular. The thickness of the erythrocyte was calculated as

$$t = \frac{\Phi_1 - \Phi_2}{n_{v1} - n_{v2}} = 0.89\ \mu\text{m}. \tag{3}$$

Using the RBC thickness measured, the refractive index was calculated for each pH value with a standard error of $n \pm 0.0005$ and $p\text{H} \pm 0.05$.

3 Results

3.1 Refractive Index of RBCs

3.1.1 *Refractive Index of Fixed Cells*

Curves of the refractive index were obtained for nine diabetic patients and seven healthy donors. For each individual, the refractive index values determined at 18 different pH levels are depicted, in Figure 1(a) for the diabetics patients and in Figure 1(b) for the control group. The RI curves of both groups were of similar configuration, with one branch in the

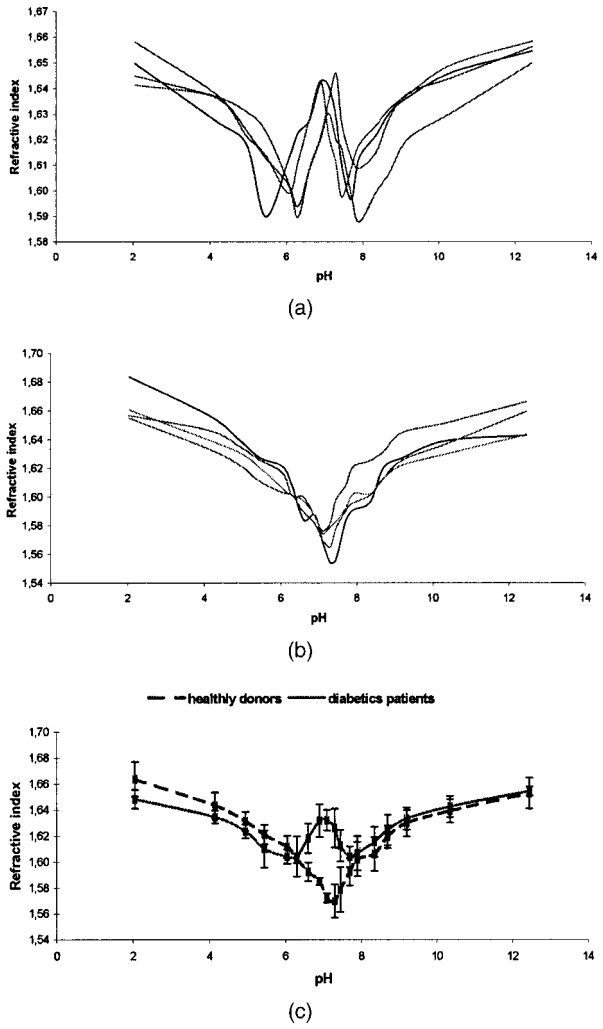

Fig. 2 Intact RBC refractive index curves measured at 18 different levels of *p*H. (a) Individual curves of diabetic patients. (b) Individual curves of healthy donors. (c) Mean curves with standard errors for diabetic and healthy donor groups.

acid portion of the *p*H scale (the acidic branch), a maximum (the isoelectric point) and two minima in the neutral portion of the *p*H scale, and one branch in the alkaline portion of the scale (the alkaline branch). The curves of the individuals from the control group overlap each other, especially the isoelectric point and the alkaline branches [Figure 1(b)]. To the contrary, the curves of the diabetic patients revealed a distinct dispersion of their middle parts in comparison to one another within this group [Figure 1(a)]. Moreover, the middle portion (in the neutral *p*H zone) of the curves of the diabetic patients was shifted towards the alkaline end of the *p*H scale in comparison with the control curves. The isoelectric point in diabetic patients corresponded to $p\mathrm{H}=7.1$, whereas the isoelectric point in the control group was $p\mathrm{H}=6.5$. In healthy donors the refractive index was in the range of $n=1.58-1.68$, but the refractive index values in diabetic patients were lower ($n=1.54-1.66$).

3.1.2 *Refractive Index of Intact Cells*

Intact RBC refractive index curves were obtained for four diabetic patients and four healthy donors. We observed differently shaped refractive index curves for intact RBCs of healthy donors [Figure 2(b)], with one deep minimum in the middle part (at $p\mathrm{H}=7.4$) of the RI curve. However, the shape of the refractive index curve for intact RBCs in diabetic patients [Figure 2(a)] is similar to the RI curve for fixed RBCs in diabetics.

Table 1

Patient No.	Blood glucose concentration (%)
1	14, 0
2	8, 7
3	10, 6
4	13, 0
5	12, 8
6	8, 1
7	8, 5
8	10, 1
9	10, 4

3.2 RBC Refractive Index Relationship with Hyperglycaemia

Pearson correlation coefficients were calculated at each *p*H between the RI values and the blood glucose concentration (Table 1) for nine diabetic patients (Table 2). A negative correlation tendency between the RI and the blood glucose concentration at the acidic and neutral zones of the RI curve was obtained, but a positive correlation tendency was observed between the RI and the blood glucose concentration in the alkaline zone of the RI curve.

4 Discussion

The data clearly demonstrate that the refractive properties of the RBCs in diabetic patients differ greatly from those of healthy donors. Among members of the control group, the refractive index maxima are located close together, with small deviations, which strongly suggests a uniform fixed RBC protein content [Figure 1(b)]. Significant deviation and depression of the RI curves for fixed RBCs were observed in the diabetic patients [Figure 1(a)]. The relationship between the refractive index and *p*H level can be explained by classic dispersion theory.[12] We observed three maximum values of the refractive indices of RBCs along the *p*H scale, where the protein charge is larger due to the dissociation of basic (arginine or lysine) or acid (aspargine or glutamic acid) R groups. This suggests that the influence of amino groups and carboxyl groups on the refractive index is relatively small. The concentration of charged R groups that interfere with light (without reference to the sign) is an important factor in determining the refractive index. The significantly decreased RI value for the diabetic group can be explained by the strong interaction of

Table 2 (*p*H-RBC embedding medium *p*H; *r*-Pearson coefficient of correlation.)

*p*H	2, 05	4, 15	4, 95	5, 45	6, 05	6, 30	6, 60	6, 90	7, 10
r	–0, 492	–0, 631	–0, 611	–0, 168	–0, 041	–0, 084	0, 098	0, 153	–0, 057
*p*H	7, 30	7, 45	7, 70	7, 90	8, 35	8, 70	9, 20	10, 35	12, 45
r	–0, 346	–0, 526	–0, 492	–0, 031	0, 406	0, 234	0, 274	0, 430	0, 521

glucose with hemoglobin. In addition, from the light refractive index formula, Eq. (1), we can see that RI value is inversely proportional to the molecule's mass, and since the molecular mass of glycated hemoglobin is now increased, the RI value is smaller [Figure 1(c)]. The negative correlation between the blood glucose concentration and RI in the acidic portion of the RI curves supports this point of view (Table 2). The absence of a correlation in the alkaline end of the *p*H scale could be due to partial dissociation of glycated hemoglobin, leading to greater exposure of the hemoglobin molecule's aspargine or glutamic acid charged R groups to the light electromagnetic field. Although the increasing charge of R groups increases RI [see Eq. (1)], at the same time the increased mass of glycated hemoglobin molecules decreases the RI. Since the influence of R-group charge is stronger than the mass of glycated hemoglobin molecules, the overall effect is to increase the refractive index. The intact RBC curve minimum in healthy donors [Figure 2(b)] could be associated with RBC membrane resistance to the buffered embedding medium in an attempt to maintain an intracellular *p*H and salt balance. Under such conditions RBC hemoglobin preserves the native zero-sum charged state. As a result the diabetic patients' glycated hemoglobin RI value in the neutral *p*H region is the same as that in the fixed RBC case. In an acidic or alkaline environment the RBCs may lose membrane integrity, and hemoglobin may show similar erythrocytes RI properties upon glucose dissociation.

5 Conclusion

Using this method it is possible to show differences in RBCs between healthy donors and diabetic patients. After clinical approbation this approach could be a good early diabetes diagnostic method.

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