Measurement of Mammalian Erythrocyte Indices from Light Scattering with Scaning Flow Cytometer

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ABSTRACT

The technique of scanning flow cytometery (SFC) was adopted for measurement of erythrocyte indices: volume, hemoglobin concentration and surface area. The method has been verified on two types of mammalian red cells: human and murine. In order to access distribution of values, precision and stability of inverse algorithm of reconstitution of size and refractive index of microsphere from light scattering angular dependency (indicatrix) have been increased performing analysis of Fourier spectrum of indicatrix. Volume - hemoglobin concentration (*V-HbC*) map was obtained following well-known procedure of isovolumetric sphering, presented by Technicon Instruments. Additionally new method for measurement of paired distribution of erythrocyte surface area (*S*) and hemoglobin content (*Hb*) was implemented via registration of spherical stage in the course of colloid-osmotic hemolysis. Approach to characterization with SFC of native red cells in nonspherical stage is demonstrated.

Keywords: erythrocyte indices, hemoglobin, cell volume, surface area, light scattering, inverse light-scattering problem, flow cytometry.

1. INTRODUCTION

The measurement of absolute values of two-angular light scattering proved to be powerful method for morphologic cell discrimination in flow cytometry. In addition to the ordinary flow cytometer the scanning flow cytometery SFC has enhancement for measurement of the differential cross section of light scattering indicatrix in the angular range of 10-70 degrees on a single cell level. This technique is very simple and fast in comparison to other approaches to indicatrix registration. The SFC setup was described in details elsewhere [2]. Having in hand indicatrix pattern of the object one should expect that it contains complete information on particle morphology. The problem of extracting characteristics of particle from light scattering data is called the inverse light scattering problem. It has not been solved analytically even for the case of simple homogeneous sphere. We proposed the parametric approach to inverse problem, which, in turn, begins from analysis of direct problem, from the optical model of particle. The parameters of indicatrix are function of shape and refractive index of the particle. It is important to select the parameters correctly. Supported by parametric solution of inverse light scattering problem SFC permits direct measurement of the diameter and refractive index for homogenous spherical particles, covering the size region significant for cell biology ~(0.5 -18 μm).

In clinical hematology there is a need for accurate and precise measurements of the volume and hemoglobin concentration of individual erythrocytes. Whereas the instruments based on Coulter principle determine only individual erythrocyte volume, the new generation of flow cytometric instruments uses forward light scattering at two angular intervals to measure both cell parameters. The method utilizing two angular intervals was introduced by Tycko et al [1] and theoretically analyzed by Maltsev [2]. Since two-angle light-scattering method (2ALS) was intensively verified by Mohandas et al [3], the modern hematological instruments use one for accurate and independent measurement of the volume and hemoglobin concentration of erythrocytes.

In general, intensity of light scattered by a particle depends not only on the size and refractive index of the particle but also on its shape and orientation. These factors complicate interpretation of light scattering data limiting an accuracy and

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precision of absolute particle analysis. As a native red blood cell has a biconcave discoid shape the 2ALS method eliminates the shape and orientation dependence by sphering cells isovolumetrically. The final particles are essentially homogeneous dielectric spheres. Mie scattering theory [4] accurately describes scattering by these sphered cells. The 2ALS method can produce absolute measurements of particle volume and refractive index that relates to hemoglobin concentration if a suitable calibration sample is available [1,5]. Most of commonly available instruments require a calibration of light-scattering channel by certified particles with known size and refractive index.

The flying light-scattering indicatrix (FLSI) method of inverse parameterization was introduced by authors [6,7] and first applied SFC data. The SFC acquisition rate is 600 particles per second. In order to provide a real-time analysis of the particles, the FLSI method was theoretically supported with a parametric solution of the inverse light-scattering problem [8]. In our previous work [9] the parametric solution of the inverse light-scattering problem (the FLSI method) has been expanded to be applicable for measurement of red blood cells introducing absorption of hemoglobin, i.e. complex part of the refractive index. The parametric solution consists in construction of approximating equations those relate the indicatrix parameters to particle characteristics. In contrast to our previous work [9] where we have used the following indicatrix parameters: fringe pitch $\Delta_2(\varphi)$ and forward visibility V_f (15) for determination of sphered erythrocytes diameter and hemoglobin concentration in this study we define another parameters: peak frequency index P_f and integral J, which allow us to increase the accuracy of the method. The approximating equations relate these parameters to the following particle characteristics: the size parameter $\alpha = \frac{\pi \cdot d}{\lambda} m_0$ and the phase-shift parameter $\rho = 2\alpha\beta \times HbC$, where, d is the particle diameter, m_0 is the refractive index of the medium, HbC is the hemoglobin concentration expressed in g/dl, β is the polarizability of hemoglobin in water. The produced approximating equations were verified measuring indicatrices of sphered erythrocytes. The concentration of hemoglobin in erythrocytes and cell volume were determined from the indicatrices measured with SFC.

2. THEORY

2.1 Optical properties of Red Blood Cell

From the point of view of optical properties, a RBC could be modeled as a homogeneous aqueous solution of hemoglobin (~34 g/dl), salts (~0.7 g/dl) and other organic compounds (~0.2 g/dl) [1] contained in a transparent cell membrane of negligible thickness. Therefore, a sphered RBC can be characterized by a size and a complex index of refraction $n' = n'_R - i \cdot n'_I$. Since the interior of the cell is almost completely occupied by water and hemoglobin, variations in n' from cell to cell can be attributed solely to variations in hemoglobin by following empirical equation:

$$n'_R - n_0 = \beta \cdot HbC, \qquad (1)$$

where β is the coefficient expressed in dl/g, n_0 is the refractive index of surrounded medium. The radiation wavelength interval of interest in this investigation is between 0.5 and 1.2 μm; in this range β has a typical value of 0.0019 dl/g . In this same range, hemoglobin is the only cell constituent exhibiting a significant amount of absorption. Consequently, assuming Beer's law applies, [4] the imaginary part of n is given by

$$n'_I = \frac{1}{4\pi} \lambda \sigma \cdot HbC \frac{N_A}{M} 10^{-4}, \qquad (2)$$

where σ is the absorption cross-section of hemoglobin ($8.1\ 10^{-18}$ cm^2 for λ = 632.8 nm), M is the molecular weight of hemoglobin (66,500), N_A is the Avagadro's number ($6.02\ 10^{23}$). Thus for a given wavelength, the complex index of refraction of RBC is determined through Eqs. (1) and (2) by a single physical variable, the cell hemoglobin concentration.

2.2 Light scattering theory of the method

To calculate the intensities of the indicatrices we have used a computer program that is based on the algorithm of Bohren and Huffman [4]. The exact solution of Mie scattering theory was applied to calculate the indicatrix for absorbing spheres and unpolarized incident light. In our calculations the particle size d was varied from 3.5 μm to 7.5 μm with a step of 0.1 μm (size parameter α from 26 to 60), hemoglobin concentration of the red blood cells HbC was varied from 5

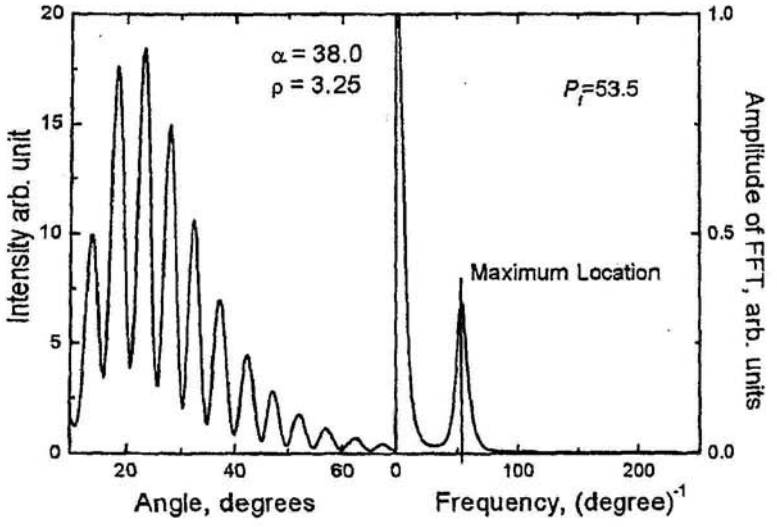

Fig. 1. Indicatrix of spherical particle and its spectrum

g/dl to 60 g/dl with a step of 0.5 g/dl (phase-shift parameter $\rho = 2\alpha(n_R - 1) = 2\alpha\beta \times HbC$ from 0.4 to 8, the absorption parameter $\varepsilon = 2\alpha n_I = d\sigma \cdot HbC \frac{N_A}{M} 10^{-4}$ from 1.5 10^{-5} to 2.5 10^{-4}), where $n_R = n'_R/n_0$, $n_I = n'_I/n_0$. The indicatrices were calculated for the following parameters: wavelength of the incident light λ=0.6328 μm, refractive index of the medium n_0=1.333. The phase-shift parameter of a particle means a change in a phase between waves passing through the particle substance, refractive index n_R and length d, and surrounded medium, refractive index n_0 and length d. The absorption parameter means attenuation of the amplitude of the wave passing through particle substance, refractive index n_I and length d.

We developed method for simultaneous determination of volume V and hemoglobin concentration HbC from light scattering indicatrix of individual spherical cells. The indicatrix structure of spherical particle has dominant frequency which is proportional to diameter d of particle. The dominant frequency can be received from procedure of fast Fourier transform (FFT) of indicatrix. Indicatrix of spherical particle and corresponding Fourier spectrum are shown in Fig. 1. Spectrum has sharply distinguished maximum. Simple linear equation connects size parameter α of sphere and location of spectrum maximum P_f i.e. peak frequency index. In order to obtain the approximating equation that relates peak frequency index P_f to the size parameter α, a nonlinear fitting procedure was applied to both the initial size parameters and those calculated from the fitted equations. A χ^2 test has been used to minimize the residual standard error between size parameters the initial and calculated. The following functional form for the approximating equation has been chosen:

$$\alpha = 0.71326 \cdot P_f + 0.35974, \qquad (3)$$

In order to determine hemoglobin concentration we use integral of indicatrix in range from 10 to 70 degrees

$$J = \int_{10}^{70} I(\theta) d\theta. \qquad (4)$$

The integral J basically depends on the phase-shift parameter. We have produced an equation with a set of coefficients that relate the indicatrix parameters to phase-shift ρ. The same nonlinear fitting procedure was applied to both the phase-shift parameters that were evaluated for the indicatrix set, and the phase-shift parameters calculated from the fitted equations. A χ^2 test has been chosen to minimize the residual standard error of the phase-shift parameters. The main equation for the phase-shift parameter calculation is as follows:

$$\rho=0.49176\cdot J^{0.423}, \qquad (5)$$

The hemoglobin concentration of the RBC is calculated from the determined size and phase-shift parameters using the definition of the phase-shift parameter $\rho=2\alpha\beta\cdot HbC$.

The size and hemoglobin concentration of the erythrocyte can be determined from the following algorithm:

(1) calculate the indicatrix parameters P_f, J from the indicatrix measured;

(2) calculate the size parameter α and the phase-shift parameter ρ from; eq. (3), (5);

(3) the size d and hemoglobin concentration HbC of RBC are found from definition of α and ρ.

Peak frequency index P_f is relative parameter and does not depend on absolute intensity. Unfortunately, integral of indicatrix is absolute parameter and requires calibration procedure. We use immersion oil microdroplets with known refractive index for calibration.

In addition to peak frequency index we can define another relative parameter which is defined as spectral visibility $V_S=A_f/A_0$, where A_f – amplitude of spectral maximum and A_0 – amplitude of zero frequency i.e. mean value of indicatrix. Spectral visibility V_S like as forward visibility $V_f(15)$ is a function of phase shift parameter ρ for spherical particle (see e.g. [8]). For particle to be not spherical visibilities V_S and $V_f(15)$ do not depend only on ρ, they depend on particle shape and orientation relative to incident illumination 10. In our measuring system of SFC all of non spherical erythrocytes are oriented along direction of illuminating laser beam. In this case indicatrices of biconcave erythrocytes have lower visibility parameter then indicatrices of spherical cells. We have calculate spectral visibilities V_S of indicatrices for spherical erythrocytes. It was found that for spherical erythrocytes value of spectral visibilities V_S is larger then 0.3.

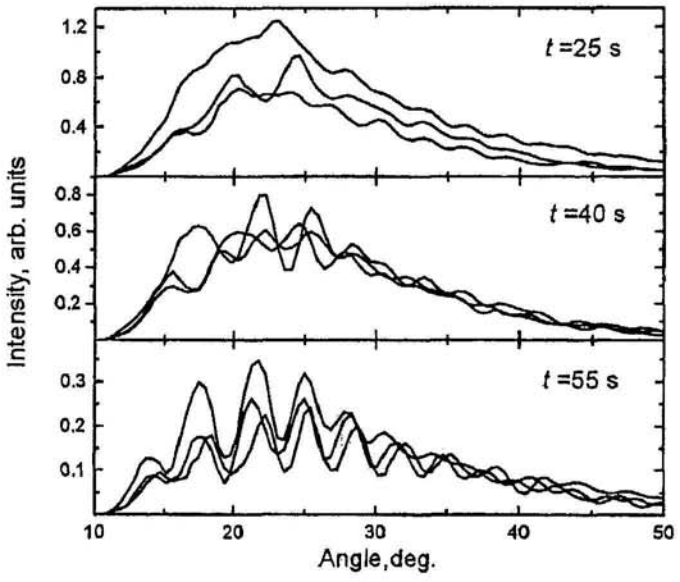

Fig. 2. Transformations of indicatrices of human erythrocytes in the process of hemolysis

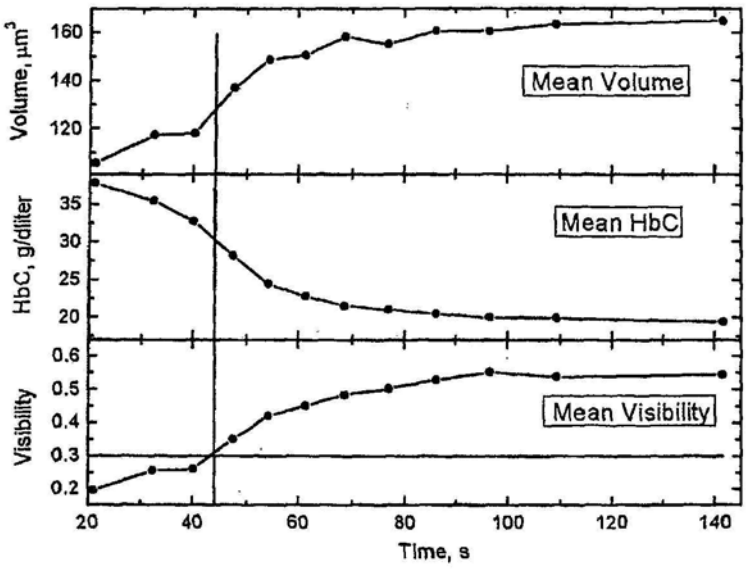

Fig. 3. Kinetics of mean cell parameters of human erythrocytes

3. MATERIALS AND METHODS

Blood samples were obtained from healthy volunteer donors. All measurements were performed within three hours. Since the Mie light scattering theory can be directly applied only to particles with spherical geometry, it is necessary to sphere the erythrocytes before the measurement. There are methods for sphering red cells prior to flow-cytometric analysis [11]. We had selected the method of diluted whole blood (1/5000) treatment with 0.04% sodium dodecyl sulfate (SDS) and 0.1% bovine serum albumin (BSA) in isotonic phosphate buffer saline. The method of sphering is

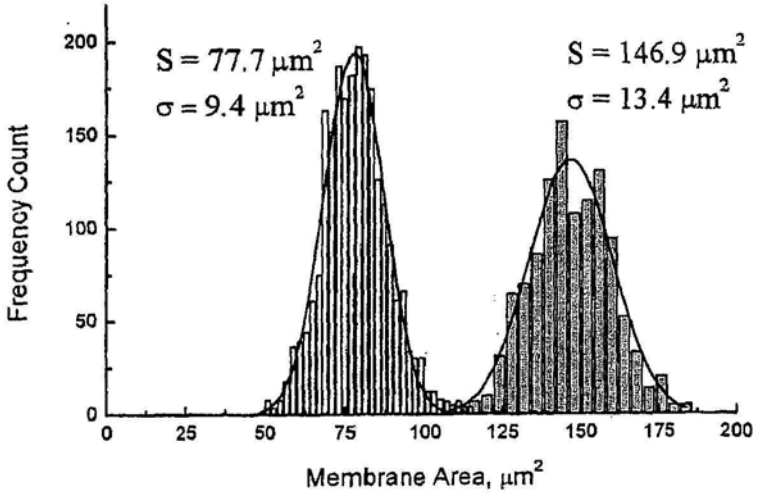

Fig. 4. Membrane area distributions of murine erythrocytes (to the left) and human erythrocytes (to the right)

isovolumetric and based on the membrane area change whereas preserving the osmolarity of erythrocyte interior.

Because the red cell membrane is practically unstretchable, osmotic swelling is convenient method to measure the surface area of erythrocyte. An easy way to do this is to drop the osmolarity of the external solution to about 1/2 of normal. Part of the discoid RBC's will swell. Any fragile RBC will most likely burst. Recently it has been shown theoretically that for any sample there is no preferred osmolarity when all cells are sphered and not lysed [12].

We have chosen sphering in the course of colloid osmotic hemolysis that lasts several minutes. Isotonic ammonium chloride is one of the most widespread erythrocyte lysing solution [13]. Its operation is based on transport properties of band 3 protein, which present on mammalian red cells. Resulting process has purely osmotic nature making all mature erythrocytes to swell gradually and to pass spherical stage before membrane rupture and cell death. Calculating surface area of sphered cells, one, therefore measures their membrane area at this stage. Fortunately, erythrocyte membrane is practically unstretchable and surface dilatation occurring in the spherical pre-hemolytic stage is less than 4% [14] constituting major error in the determination of membrane area.

4. RESULTS AND DISCUSSION

Indicatrices of individual erythrocytes were measured. About 10,000 erythrocytes were per sample. Transformations of indicatrices of human erythrocytes in the process of colloid-osmotic hemolysis in 0.84% (isotonic) NH_4Cl is shown in Fig. 2. Three typical types of indicatrices are exposed in three points in time: 25 seconds (upper patterns), 40 seconds (middle patterns) and 55 seconds (lower patterns). How one can see indicatrices changes their form with increasing visibility and making them more similar. Due to measuring of spectral visibility of indicatrices one can easily register spherical stage of erythrocytes. We can determine diameter and hemoglobin concentration of RBC not only for spherical stage but also for slightly nonspherical stage. The kinetics of RBC's colloid-osmotic hemolysis was also recorded as a demonstration of direct extraction of spherical particles. MCHC decreases, MCV and mean spectral visibility increases in the time course (Fig. 3). Erythrocytes can be regarded as spheres when spectral visibility exceeds the value of 0.3.

We have measured the membrane area distributions in course of colloid-osmotic hemolysis for two sorts of mammalian erythrocytes: human and murine. The membrane area is measured when erythrocyte was in spherical stage. The membrane area distributions are shown in Fig. 4. Volume and hemoglobin concentration of murine and human

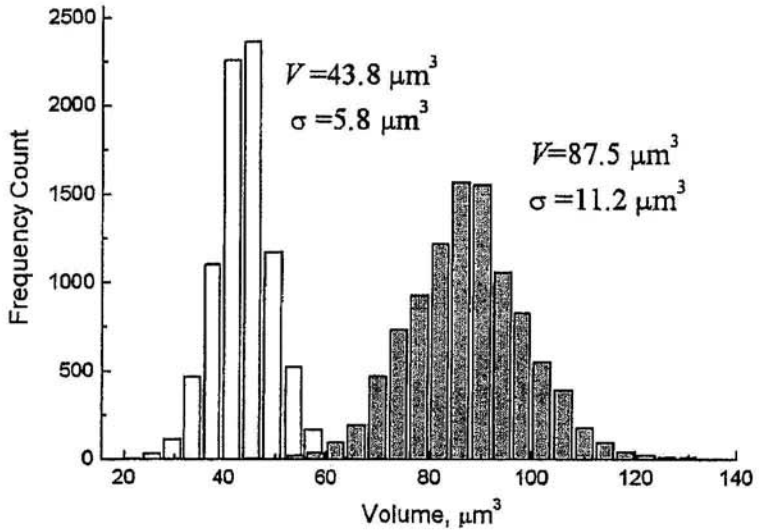

Fig. 5. Volume distributions of murine erythrocytes (left) and human erythrocytes (right)

distributions (see Fig. 5 and Fig. 6) were obtained following well-known procedure of isovolumetric sphering.

5. CONCLUSION

Erythrocyte population could analyzed on the SFC be paired data of (*V*, *HbC*) or (*S*, *Hb*) distribution depending of the sphering method. The automatic measurement of red cell membrane area distribution is reported for the first time. Solution of the inverse problem does specific for the given class of cells and can be applied to cells of wide range of sizes as illustrated on murine and human erythrocytes.

This method imposes intensity calibration requirements for precise determination of *HbC*. In exchange for this restriction the algorithm appeared to be sensitive to volume and hemoglobin content independently to cells shape even in the pre-spherical stages, practically up to native biconcave shape of erythrocyte. This phenomenon is unexplained at present and is subject to additional study.

Kinetics of colloid-osmotic hemolysis in combination with sphering kinetics is apparently informative because all

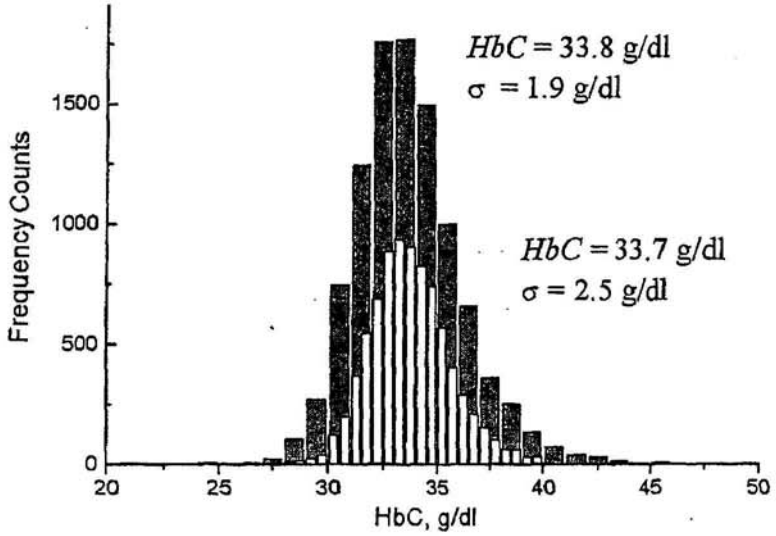

Fig. 6. Hemoglobin concentration distributions of murine erythrocytes (white colored) and human erythrocytes (gray colored)

subpopulations of the sample bypass the spherical stage and could be recorded. But its complete interpretation in the terms of initial cells properties requires an additional examination.

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