Reviews

Estimation of morphological characteristics of single particles from light scattering data in flow cytometry

V. P. Maltsev

Institute of Chemical Kinetics and Combustion, Siberian Branch of the Russian Academy of Sciences, 3 ul. Institutskaya, 630090 Novosibirsk, Russian Federation.
Fax: +7 (383) 235 2350

Numerical methods for solving the reverse problem of light scattering for single particles and a variety of experimental results have been reviewed. In particular, the two-dimensional Mie scattering method for the estimation of sizes and refractive indices of single particles has been developed. The method of triple two-dimensional Mie scattering has been elaborated, which extends the range of particle sizes with correct solutions of the reverse light scattering problem. The flying light scattering indicatrix method, which allows the estimation of the parameters of spherical particles, is presented. The results of the use of a flow cytometer of standard design for the anaysis of milk microflora and for an AIDS immunoassay by latex agglutination are given. The design of a scanning flow cytometer is considered. The results of measuring the light scattering from polystyrene latex particles are given.

Key words: analysis of single particles; light scattering; flow cytometry.

Introduction

In recent years, due to rapid development of laser technology and the means for automating measurements and data processing and the appearance of diagnostic equipment on the world market, optical analyzers of single particles have acquired wide use in scientific research and technological control. Light scattering is one of the parameters that is measured in these analyzers.

Flow cytometry is the most promising tecnique for analyzing single particles (see, for example, reviews 1—3). The development and application of flow cytometric systems for the automatic analysis and separation of particles in hydrosols have opened up new opportunities for investigations in biology and medicine. Flow cytometry is a great step forward from conventional microscopic methods in which analysis of several particles takes several hours. Flow cytometric systems allow one to analyze up to 300 000 particles per minute. The measurement of light-scattering properties of particles with these systems makes it possible to gain information on their morphological characteristics (*viz.*, size, shape, peculiarities of the internal structure, absorption coefficient, *etc.*). The unique character of the flow cytometry procedure lies in the fact that the measurements are carried out for separate particles at a very high rate. This ensures high statistical accuracy and allows small populations to be reliably revealed. In addition, analyzers of this type provide relatively high productivity (duration of the analysis is 2 min) and reliable results.

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More and more stringent requirements have been imposed on the duration of the processing of the light scattering data measured for single particles while maintaining the accuracy of the measurements.

A variety of studies have demonstrated the possibilities of using light scattering for determining the morphological characteristics of single particles. However, the method of adjustment of the theoretical calculations to the experimental results used in most of these works is time-consuming, and it is unlikely that it will find extensive application in the high-speed analysis of particles. Therefore, it is important to further improve the methods for calculating the parameters of particles from light scattering data (the reverse problem of light scattering). The requirements imposed on the newly developed methods are that the time required for the evaluation of the parameters be short (in flow cytometry, it is 1—10 ms) and that they have sufficient accuracy. With the advances in cytometric systems these express methods may find especially wide application in medicine (for immunoassay and analysis of blood elements), in the quality control of agricultural products (for example, to determine the fat content of milk and to detect milk bacteria), in ecological control, *etc.*

In the present work we have considered numerical methods for solving the reverse light scattering problem for single particles and experimental results obtained at the Institute of Chemical Kinetics and Combustion of the Siberian Branch of the RAS (IKhKiG). In particular, the two-dimensional Mie scattering method for determining the size and refractive index of spherical particles has been further developed. The optimal angles (from the viewpoint of the accuracy of the solution of the reverse light scattering problem) of the collection of radiation scattered by a single particle have been determined. The method of triple two-dimensional Mie scattering, which makes it possible to extend the particle size region with correct solutions of the reverse light scattering problem, and a method using the flying indicatrix of light scattering from a single particle for determining the parameters of spherical particles using the measured indicatrix have been elaborated. The results of the application of a standard flow cytometer for analyzing milk microflora and for AIDS immunoassay by latex agglutination are presented. The design of a scanning flow cytometer is described and the experimental results of measuring light scattering by polystyrene latex particles are given.

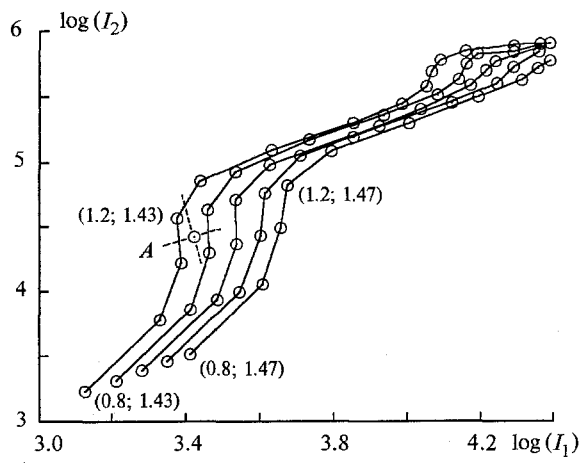

Fig. 1. The scheme of two-dimensional Mie scattering. I_1 and I_2 are intensities of light scattering from a single spherical particle within solid angles Ω_1 (20—60°) and Ω_2 (5—10°). The nodes are denoted by (d; n), where d and n are the size in μm and the refractive index of a particle, respectively.

All of the numerical results in this work were obtained with the following invariable parameters (unless stated otherwise): radiation wavelength 632.8 nm; refractive index of the medium 1.333. The incident light was unpolarized.

Methods for the numerical solution of the reverse light scattering problem for single particles

Two-Dimensional Mie Scattering, 2DMS

The results obtained in a series of studies[4—7] have demonstrated that the size and the refractive index of spherical particles can be calculated from light scattering signals recorded on a standard flow cytometer. The characteristics of the particles were calculated by the two-dimensional Mie scattering method. The use of this method made it possible to determine the volume and content of hemoglobin in erythrocytes,[4] to measure the size distribution of polystyrene particles and particles in milk,[5] and to analyze phytoplankton and other species in sea water.[6]

The two-dimensional Mie scattering method is based on the nodes method of the direct scattering problem in a two-dimensional plane. A typical scheme of 2DMS is shown in Fig. 1. Intensities at the nodes are calculated according to the Mie theory (see, for example, Ref. 8). The values for d (size) and n (refractive index) at points located between the nodes (point A in Fig. 1) are calculated to the required approximation from the nodal values. The 2DMS method allows one to determine the d and n values for spherical particles rather quickly and accurately, provided that the intensities at the nodal points have been preliminarily calculated.

However, the 2DMS method has serious drawbacks: a) the existence of regions with multiple solutions of the reverse light scattering problem (the twisting domains of the 2DMS grid); b) the necessity of calibrating the intensity values calculated for the nodes of the grid using data for particles with known sizes and refractive indices. The former restriction requires a detailed analysis, while the latter will not be discussed here, since it is associated with the convenience of using the method.

Twisting domains result from the choice of the collection angles of the scattered light for particular ranges of sizes and refractive indices of particles. The existence

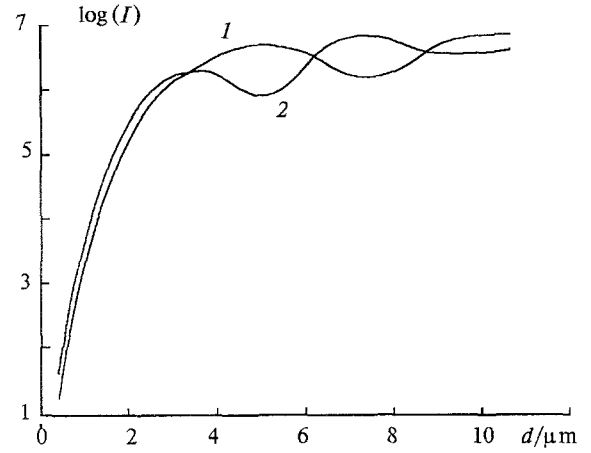

Fig. 2. The logarithm of the intensity of light scattered into a solid angle (vectorial angles 2—5°, azimuthal angles 0—360°) as a function of the size of a particle for two refractive indices: 1.43 (*1*) and 1.48 (*2*).

of these domains has been reported previously.[4] The size of the region with correct solutions of the reverse light scattering problem is affected[4] by the magnitudes of the collection angles of the scattered light. The size and the refractive index of erythrocytes were calculated with sufficient accuracy at d = 3.5 to 6.2 μm ($n \approx 1.40$) by measuring the intensity of light scattering in the ranges 3—5.5° and 5.5—9.0°. Figure 2 presents the calculation of the logarithm of the intensity of light scattered at angles of 2—5° as a function of the size of a particle for two values of the refraction index. Twisting domains can be clearly seen at d = 2.5 to 3.5, ~6, and 8.8 μm. A two-dimensional representation of these twisting domains is shown in Fig. 3 (Ω_1 = 120 to 170° and Ω_2 = 20 to 60°).

To extend the region of correct solutions, we calculated[7] the average accuracy of the solution of the reverse light scattering problem by the 2DMS method. The

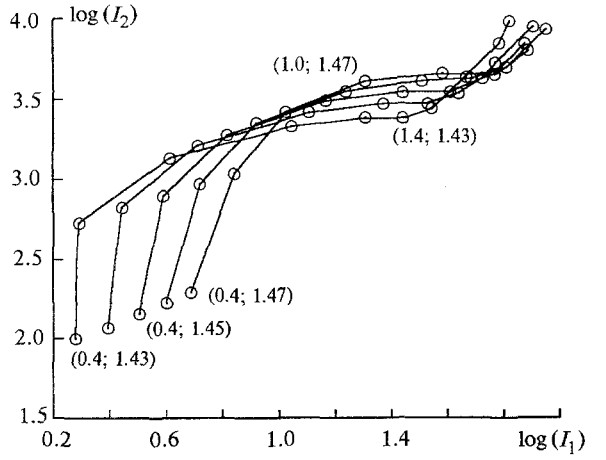

Fig. 3. The scheme of two-dimensional Mie scattering. I_1 and I_2 are intensities of light scattering from a single spherical particle within solid angles Ω_1 (120—170°) and Ω_2 (20—60°).

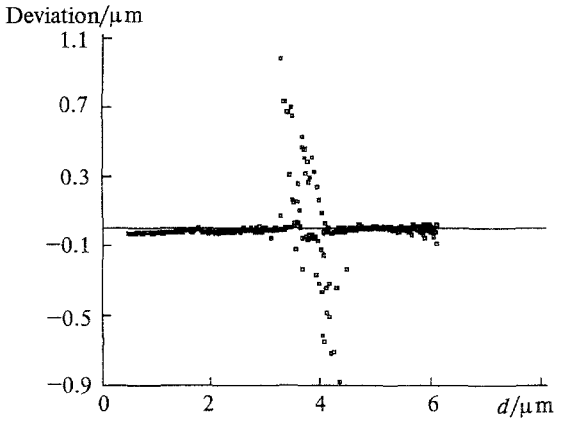

Fig. 4. The systematic error of the calculation of the size of a particle by the 2DMS method.

2DMS grid was constructed for d = 0.4 to 20 μm with a spacing of 0.2 μm and for n = 1.41 to 1.49 with a spacing of 0.01 at various collection angles of the scattered radiation (2—5°; 5—10°; 5—15°; 10—20°; 20—40°; 20—60°; 75—115°; 120—170°; 140—175°). To calculate the average error over these ranges of sizes and refractive indices, we plotted dependences similar to those presented in Fig. 2 for n 1.435, 1.453, 1.455, 1.458, and 1.475 with a size spacing of 0.05 μm. From the calculated intensities we reproduced the sizes and refractive indices of particles using the 2DMS method. Statistical processing of the values thus obtained allowed us to determine the optimal collection angles of the scattered radiation, based on a comparison of the average errors in the calculation of sizes by the 2DMS method. Optimal angles of light collection are: Ω_1 = 20 to 60° and Ω_2 = 5 to 10°, and the average error of the size in the range of sizes 0.5—6 μm is equal to 0.16 μm, while the error of the estimation of the refraction index is 0.003. The results of the statistical treatment are shown in Fig. 4.

It can be seen that the main error of the method falls within the range 3.3—4.7 μm (the twisting domain of the 2DMS grid). When the construction of 2DMS grid and the calculation of the average error were carried out with other collection angles, this range and the average error of the calculation of the size of a particle increased. These results indicate that the 2DMS method can be used for processing light scattering data in the analysis of a large class of particles. One may safely assume that the range of refraction indices can be substantially extended without noticeable loss in the accuracy of the method. The essential advantages of the 2DMS method are the ease of calculations (linear or quadratic approximations) and fast processing (most time is spent for searching for the nodal point nearest the experimental value).

Triple Two-Dimensional Mie Scattering, 3×2DMS

The 2DMS method allows one to quickly obtain rather accurate values for the size and refraction index

of single particles by processing the data on the intensity of light scattered at two angles. The operating range of the method is limited to sizes of particles in the range 0.4—6 μm. For particles larger than 6 μm, the 2DMS method gives substantial errors in the determination of both the size and refraction index. To extend the operating range of the method with respect to sizes and to decrease the error we suggest that the light scattering data be processed according to the triple two-dimensional Mie scattering method (3×2DMS).[7] This consists of the simultaneous processing of three 2DMS schemes with a preliminarily specified priority for each of these schemes. The data obtained in the study of the 2DMS method were used for determining the potentialities of the 3×2DMS method. Optimization with respect to the third collection angle of the scattered radiation was carried out. The average error of the estimation over the range of sizes was minimum when the following collection angles of the scattered radiation were used: Ω_1 = 5 to 10°; Ω_2 = 20 to 60°; Ω_3 = 120 to 170°. The results of the statistical processing of the calculated data are shown in Fig. 5.

The average errors of the estimation were 0.24 μm and 0.003 over the whole ranges of sizes and refraction indices (1.41—1.49), respectively. It is important that the 3×2DMS method provides a lower error of the calculation of the size of a particle in the range 3.3—4.7 μm than 2DMS. The use of the 3×2DMS allowed the operating range with respect to size to be increased to 15 μm, and the relative error of the calculation was no more than 10 %. This was achieved at the cost of a threefold increase in the duration of the processing of the light scattering data.

However, it should be noted that the accuracy of the 3×2DMS method substantially increases, when the overall characteristics of polydisperse systems, such as the total volume of particles and the mean refractive index, are determined. In this case, the accuracy of the calculations depends on the aggregate error over the whole range, which is close to zero due to the regular character of its dependence on the size (see Fig. 5). The total volume and the mean refractive index of the particles were calculated assuming a log-normal size distribution of particles with a definite refractive index. The results of these calculations are listed in Table 1.

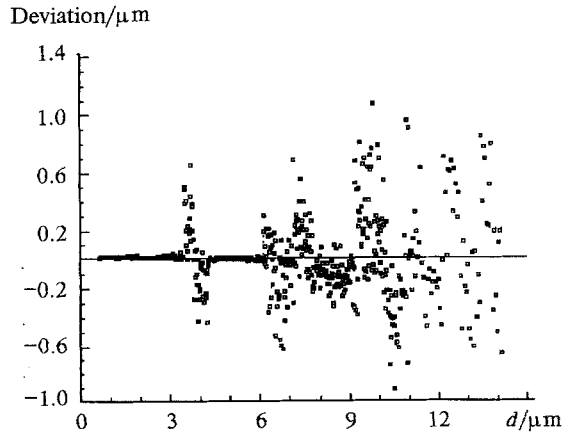

Fig. 5. The systematic error of the calculation of the size of a particle by the 3×2DMS method.

It can be seen from these data that the mean refractive index and the total volume of particles can be calculated with fair accuracy. The interest in this region of sizes and refractive indices is due to the fact that the 3×2DMS method can be used for calculating the weight concentration of fat particles in milk, while simultaneously checking the fat quality on the basis of the mean refractive index (fat spheres in milk: size 1—15 μm, refractive index 1.45—1.47). Another characteristic of the 3×2DMS method is that the error of the determination of the overall parameters decreases as the width of the size distribution function of particles increases (see Table 1). The actual size distribution function for milk fat is rather broad. The use of this fact will make it

Table 1. The results of the calculations according to the 3×2DMS method

σ /μm	d /μm	V_{real} (rel. units)	n_{real}	n_{calc}	V_{calc} (rel. units)	ΔV (%)
1.3	2.0	0.15	1.4530	1.4532	0.15	0
	3.0	0.76		± 0.0005	0.76	0
	4.0	2.39			2.40	−0.2
1.5	2.0	0.34			0.34	0
	3.0	1.66			1.67	−0.4
	4.0	4.62			4.64	−0.5
1.8	2.0	0.86			0.86	−0.4
	3.0	3.27			3.28	−0.2
	4.0	7.23			7.22	0.1
1.3	2.0	0.15	1.4560	1.4564	0.15	0
	3.0	0.77		± 0.0005	0.76	1.3
	4.0	2.48			2.41	3.0
1.5	2.0	0.34			0.34	0
	3.0	1.74			1.72	0.9
	4.0	5.33			5.32	0.2
1.8	2.0	0.97			0.97	0
	3.0	4.19			4.20	−0.1
	4.0	10.63			10.66	−0.3
1.3	2.0	0.15	1.4580	1.4582	0.15	0
	3.0	0.76		± 0.0004	0.76	0
	4.0	2.41			2.40	0.1
1.5	2.0	0.34			0.34	0
	3.0	1.69			1.69	0
	4.0	4.93			4.92	0.1
1.8	2.0	0.89			0.89	0
	3.0	3.50			3.50	0
	4.0	8.00			8.00	0

Note. The following designations have been used: n_{real} and V_{real} are the starting refractive index and total volume of the particles in the calculation of scattering intensities; σ and *d* are the width and the position of the maximum of the starting log-normal function of the size distribution of the particles; n_{calc} is the calculated mean refractive index of the particles; V_{calc} is the calculated total volume of the particles; ΔV is the relative error of the calculation of the total volume of the particles.

possible to omit standardization of a milk sample (homogenization), which is carried out in modern analyzers.

Flying Light Scattering Indicatrix, FLSI

The most comprehensive information on the properties of a scattering particle can be gained from the angular dependence of the intensity of light scattering (the indicatrix).[9] Its significance for the determination of the morphological characteristics of a particle is similar to that of fingerprints in criminal law. The main method allowing one to evaluate the parameters of scattering particles is the adjustment technique. The d and n values for spherical particles are selected in such a way that they most closely approximate the experimental results. In a number of studies,[10–15] indicatrices measured for various d and n have been compared with those calculated according to the Mie theory.

The method of adjusting the calculated indicatrices to fit the measured indicatrices has been used in experiments dealing with the indicatrices of single detected particles. A technique involving the retention of a particle in a laser beam by an electric field (Differential II, Science Spectrum, Inc.) has been reported.[10] This technique was applied to the scattering indicatrix of a particle of polystyrene latex, 1099±6 nm in diameter. For this latex, the adjustment afforded d = 1200±10 nm and n = 1.59±0.01. Later, the possibilities of the adjustment method were enhanced.[11] Indicatrices of single biological cells have been measured using the Differential II technique. The adjustment method was applied to a model of a spherical particle with a coating. As a result, the parameters of *Staphylococcus epidermidis* bacteria were determined to be the following: radius 353±5 nm; thickness of the membrane 25±5 nm; refractive index of the membrane 1.54±0.01.

Calculations[12] based on the positions of the minima on the indicatrix of single *Staphylococcus epidermidis* bacteria gave d = 350±5 nm.

In flow cytometry, the method of the adjustment of the calculated indicatrices to fit the experimental indicatrices has been used in a series of studies.[13,14] For example, using the light scattering data measured on a flow cytometer, the composition of a five-component mixture containing particles of sizes 1.1, 5.0, 10.0, 15.6, and 19.5 μm was determined.[13] Rather good agreement between the measured scattering function and that calculated according to the Mie theory was obtained for polystyrene latex particles. By comparing scattering indicatrices calculated for homogeneous spheres according to the Mie theory, the size distribution and the mean refractive index of spores were determined.[14]

The adjustment of the experimentally measured indicatrices to fit the calculated indicatrices is the most accurate method for the solution of the reverse light scattering problem for single particles. However, this method requires prolonged calculations and the rather precise specification of the initial parameters for the adjustment. The flying light scattering indicatrix method considered in this work (described in more detail previously[9]) allows one to determine the characteristics of a particle using only some parameters of the indicatrix, rather than the whole indicatrix. The term "flying" reflects a principal feature of the method: the characteristics of a particle are calculated during the specific time of the flight of the particle through the measuring system.

The indicatrix of a single particle is a complex interference pattern, which reflects the maximum and the minimum magnitudes of the scattering intensity at various angles of observation. Some parameters of the indicatrix (the number of minima, their angular location, degree of contrast, *etc.*), whose magnitudes are related to the morphologiocal characteristics of a scattering particle (size, refraction index, shape, *etc.*), may be distinguished. In this case, the solution of the reverse light scattering problem involves: a) the selection of the parameters of the indicatrix that would provide the most accurate evaluation of the characteristics of the particle; b) the establishment of the relationships between the characteristics of the particle and these parameters; and c) the estimation of the calculation error.

Calculation of the size and the refraction index of nonabsorbing spherical particles requires the knowledge of only two parameters of the indicatrix. The following parameters may be used: the distance between the first and the j-th minima, $\Delta\theta_j(\varphi_d)$, selected after the boundary angle φ_d, and the degree of contrast of the indicatrix, $V(\varphi_v) = (I_{max}-I_{min})/(I_{max}+I_{min})$, where I_{max} and I_{min} are scattering intensities at the maximum and minimum points selected after boundary angle φ_v. For the indicatrix shown in Fig. 6, the following parameters are presented: j = 3; φ_d = 20°; $\Delta\theta_3(20)$ = 24.92; φ_v = 40°; $V(40)$ = 0.605. The values for $\Delta\theta_3(20)$, I_{max}, and I_{min} were determined from the graphical analysis of this

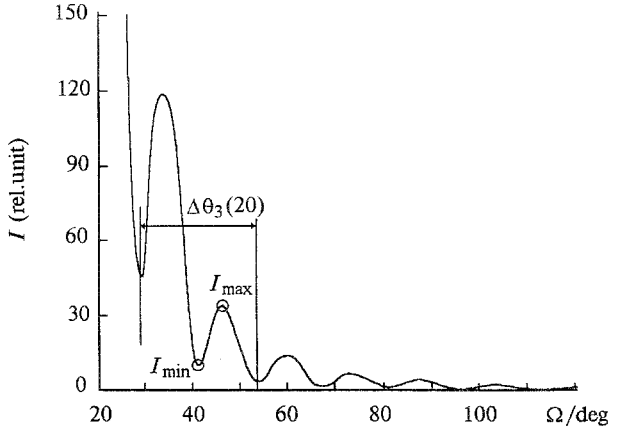

Fig. 6. The scattering indicatrix of a single particle. The calculated parameters: λ 480 μm; refractive index of the medium 1.333; diameter of the particle 1.7 μm; refractive index of the particle 1.45.

dependence. An equation that relates the size of a particle d (in μm) to the distance between the minima, $\Delta\theta_3(20)$, has been derived:[9]

$$d = \alpha_1 + \alpha_2[\Delta\theta_3(20)]^{-1} + \alpha_3[\Delta\theta_3(20)]^{-3} + \alpha_4[\Delta\theta_3(20)]^{-4}, \quad (1)$$

where $\alpha_1 = 0.156$ ($s = 0.026$), $\alpha_2 = 51.5$ ($s = 0.6$), $\alpha_3 = 330$ ($s = 50$), $\alpha_4 = 1310$ ($s = 230$). The plot of this function is presented in Fig. 7. The absolute average error of the calculations of the particle size amounts to 0.090 μm (the relative error does not exceed 5 %). It should be emphasized that Eq. (1) was obtained for λ 632.8 nm and a refractive index of the medium of 1.333. The parameters of a particle must lie in the following ranges: $d = 1$ to 12 μm, $n = 1.40$ to 1.50. The scattering parameter, $\alpha = \pi n_0 d/\lambda$, and the relative refractive index, $m = n/n_0$, lie in the ranges 6.6—80 and 1.050—1.125, respectively.

For arbitrary values of the radiation wavelength λ' and the refractive index of the medium n'_0, the size of a particle may be calculated from the following equation:

$$d' = d \frac{n_0}{n'_0} \frac{\lambda'}{\lambda} = 2.11\, d \frac{\lambda'}{n'_0}, \quad (1a)$$

where d is the value calculated from Eq. (1). In order to use Eqs. (1) and (1a) one must confirm that α and m lie within the above-specified ranges.

To calculate the refractive index of a particle we have used two parameters of the indicatrix, $\Delta\theta_3(20)$ and $V(40)$.

$$n = \beta_1 + \beta_2 V(40) + \beta_3 V(40)[\Delta\theta_3(20)]^{-3} + \beta_4[V(40)]^2[\Delta\theta_3(20)]^{-1} + \beta_5[\Delta\theta_3(20)]^{-3} + \beta_6[\Delta\theta_3(20)]^{-4}, \quad (2)$$

where $\beta_1 = 1.581$ ($s = 0.006$), $\beta_2 = -0.173$ ($s = 0.014$), $\beta_3 = 37$ ($s = 7$), $\beta_4 = -1.99$ ($s = 0.20$), $\beta_5 = 13$ ($s = 4$); $\beta_6 = -123$ ($s = 27$). The average error in the calculation of the refractive index of a particle according to Eq. (2) is equal to 0.014 in the given ranges of sizes and refractive indices with the refractive index of the medium equal to 1.333.

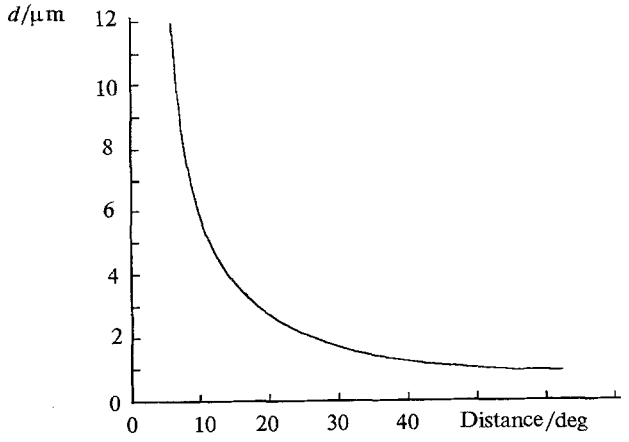

Fig. 7. The size of a particle as a function of the distance between the first and the third minima, selected after the boundary angle 20° for the scattering indicatrix of a single particle.

For an arbitrary refractive index of the medium, n'_0, the refractive index of a particle may be calculated from the following equation:

$$n' = n \frac{n'_0}{n_0} = 0.75\, n\, n'_0, \quad (2a)$$

where n is the refractive index of a particle determined from Eq. (2). To apply Eqs. (2) and (2a) one must confirm that the relative refractive index m falls within the above-mentioned interval.

Using Eqs. (1), (1a), and (2) with the indicatrix parameters (see Fig. 6) $\Delta\theta_3(20) = 24.92°$ and $V(40) = 0.605$, we calculated the size and the refractive index of a particle to be the following: $d = 1.708$ μm, $n = 1.449$. The scattering parameter, α, is 14.9 and the relative refractive index, m, is 1.087 (*i.e.*, both values fall within the ranges specified).

Thus, the use of such parameters of an indicatrix as the degree of contrast and the distance between the minima makes it possible to determine the size and the refractive index of particles with sufficiently high accuracy. It should be noted that the FLSI method for determining the parameters of a particle offers an advantage over the 3×2DMS method, since the absolute values of light scattering are not used in the calculations (*i.e.*, no calibration is required). Simultaneously, high accuracy of the calculation of the mean characteristics of polydisperse systems (mean size, total volume, mean refractive index, *etc.*) is retained. Another feature of the FLSI method is a high speed of the estimation of the parameters of a particle with the aid of simple equations (Eqs. (1) and (2)), which is important in flow cytometry, because the result can be obtained prior to the sorting of the particles.

Flow cytometric measurements of light-scattering characteristics of single particles

Flow cytometer of standard design

At present, light scattering measurements in flow cytometers are widely used for sorting particles.[1,2] For this purpose, the intensities of scattering in a forward direction, scattering at an angle of 90°, and attenuation are usually determined. The effect of the angles of the collection of the scattered radiation on the efficiency of the selection has been examined.[4,16—18] Light scattering signals over solid angles formed by vectorial angles of 3.0—5.5° and 5.5—9.0° and azimuthal angles of 0—360° have been recorded to determine the volume of erythrocytes and the content of hemoglobin (its concentration is related to the refractive index).[4] The radiation from a

semiconductor laser with a power of 2.8 mW at λ 842 nm was used. The volume and the refractive index of erythrocytes were determined by the 2DMS method using drops of heptane, nonane, and dodecane in water for the callibration of the 2DMS grid. The size distribution of phytoplankton has been determined[6] on the basis of the intensity of light scattered into vectorial angles of 1.5—19° and 73—107°. An argon laser (λ 514 nm, 100 mW) was used. The determination of the size and the refractive index of the particles was also carried out by the 2DMS method with a similar calibration. To decrease the region of incorrect solutions for the 2DMS method, the scattered radiation was measured at two wavelengths (a He—Ne laser, 632.8 nm, 10 mW and a He—Cd laser, 441.6 nm, 17 mW) at vectorial angles of 17 and 75° (the numerical aperture of lenses was 0.29).[5] The 2DMS grid was adjusted using polystyrene particles. Size distributions of particles of polystyrene and milk were determined.

A flow cytometer with a standard design has been developed at the laboratory of laser photochemistry of the IKhKiG. To optimize the technique of conducting cytometric measurements, a procedure for the simultaneous determination of the concentrations of bacteria and somatic cells in milk has been developed,[19] and the process of the agglutination of latex particles used in immunoassay has been investigated.[20]

A two-component polydisperse system (milk after preliminary treatment) was studied[19] on a flow cytometric unit where radiation scattered from a single particle was measured. The procedure developed for the preparation of samples made it possible to dissolve the protein micelles and fat globules of a milk sample. After that, the sample became a two-component polydisperse system consisting of *E.coli* bacteria and somatic cells (CV-1).

To record the signals of the scattering of laser radiation (632.8 nm) from a single particle, the sample was passed through a hydrofocusing system. This allowed us to operate with a sample volume of $\sim 10^{-8}$ mL. The scattered radiation was recorded over angles of 5 to 20°. With the aid of a function calculated by the Mie theory (d ~0.4 to 20 μm; n ~1.38), each of the light scattering amplitudes is matched to an efficient size of the particle, d. The resulting function of the distribution over efficient sizes was processed in two ranges (0.4—5 μm and 5—20 μm).

An equation that relates the concentration of *E.coli* ($N_{E.coli}$) to the number of particles with $d < 5$ μm ($n_<$) has been obtained: $N_{E.coli} = 790n_< - 570000$. The sensitivity of the procedure for determining the concentration of *E.coli* in the two-component system was $(9.0\pm0.6)10^5$ mL^{-1} in these experiments. A similar equation for the concentration of CV-1 cells has been derived: $N_{CV\text{-}1} = 1020n_< - 120\ n_> + 12000$. The sensitivity of this procedure for determining the concentration of somatic cells in the two-component system may be estimated in these experiments to be $(2.5\pm0.2)10^5$ mL^{-1}.

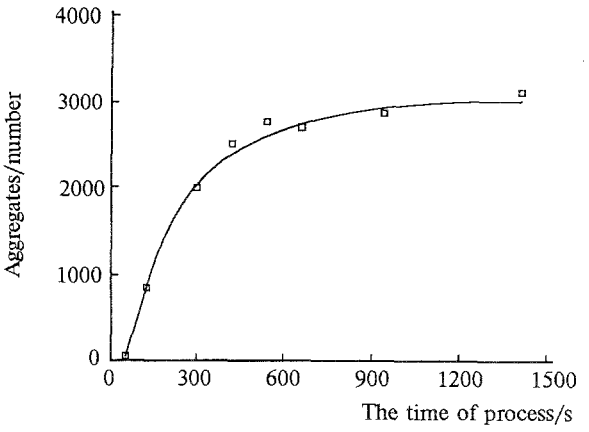

Fig. 8. Kinetics of the agglutination of latex particles with antigens adsorbed on the surface in the presence of antibodies.

In the study of latex agglutination, polypeptides of HIV-1 (the 601—606 and 593—604 fragments from the sequence of the GP41 transmembrane protein) and HIV-2 (the 587—605 fragments from the sequence of the GP36 transmembrane protein) viruses were adsorbed on the surface of latex particles of size 0.8 μm. The agglutination of the suspended latex particles was initiated by adding sera containing antibodies to HIV-1 and HIV-2 from the control panel of Gemini Bioproducts Inc. (USA). The number of latex complexes formed during agglutination was measured by recording the scattering signals of laser radiation (632.8 nm). The use of the hydrofocusing device and focused laser radiation made it possible to record the light scattering signals from each of the resulting complexes of latex particles. The scattered radiation was measured over angles of 5 to 20°. The time dependence of the number of latex complexes formed during agglutination in the presence of various sera was measured. The kinetic curves (Fig. 8) were approximated by the function $N = N_\infty(1 - \exp[-k(t - t_0)])$, where t and t_0 are the current and the initial time of the agglutination process, respectively, N is the number of complexes formed up to point t, N_∞ is the final number of complexes; and k is the characteristic reciprocal time of the agglutination process. The characteristic time of the process in these experiments was 170—300 s for various positive sera. The number of complexes formed with diluted (1 : 10 to 1 : 3200) sera was determined. The optimal concentrations of antibodies in the sera for the formation of agglutinate were determined.

Scanning flow cytometer

Several types of hydrofocusing heads for flow cytometers, which allow indicatrices of single particles to be measured, have been described previously (see, for example, Ref. 2). Indicatrices of single particles allow the most comprehensive determination of morphologi-

cal characteristics of scattering particles. Therefore, to measure the indicatrix is of prime importance. In particular, from the analysis of an indicatrix, such parameters of a particle as size, refractive index, absorption coefficient, shape, *etc.*, may be inferred.

A differential scattering photometer made it possible to measure the indicatrix of a single particle at the highest rate (several μs).[13] An ingenious ellipsoidal mirror was used, which directed the light scattered at vectorial angles of 2.5—177.5° and azimuthal angles of 0—360° to a circular matrix consisting of 120 photodiodes. This optical scheme of a flow cytometer allows one to measure the scattering indicatrix and thus to analyze particles at the highest rate.

The indicatrices of polystyrene particles and spores were measured using a scanning diffractometer with one photomultiplier.[14] A rotating disc and 174 optical light guides used in the system made it possible to measure indicatrices of single particles in vectorial angles of 3 to 177° over a period of 2.8 ms. To take into account the difference between the optical characteristics of the light guides, a digital-analog converter operating in the mode of correction of analog signals was used.

An approach that makes use of the movement of a particle in a flow has been described previously.[18] The optical system of a flow cytometer made it possible to measure indicatrices of single particles over vectorial angles of 1 to 49° with one photomultiplier. To take into account the variation in the intensity of the radiation incident upon a particle during its movement, and in the solid angle of the collection of scattered light, a correcting signal of fluorescence from dye-colored particles was recorded.

Let us consider briefly the optical system of a flow cytometer in which the light scattered from a single particle is scanned over the aperture of a photodetector in the course of the movement of the particle in a flow (see Ref. 15). This optical scheme is an additional tool for the FLSI method. The main characteristics of the scanning flow cytometer are the following: 1) the indicatrix of a single particle is measured over vectorial angles of 5 to 120° and azimuthal angles of 0 to 360°; 2) the measurements are carried out with one photodetector; 3) during the measurement the particle moves in an area of constant intensity of illumination.

The basic distinction of a scanning flow cytometer from a flow cytometer of the standard design is the presence of a special optical system. The scheme of the hydrofocusing head and optical system of the scanning flow cytometer is shown in Fig. 9. The hydrofocusing head creates two concentric flows: the inner flow (10—30 μm in diameter) in which the particles being analyzed move, and the outer flow freed from the particles with the aid of a membrane filter. The main radiation is focused by an alignment device (not shown in Fig. 9) and a lens into a cell, through an optical window located at the upper part of the hydrofocusing head. Focusing the beam into the optical cell provides constant intensity of illumination of a moving particle during the measurement of the indicatrix. Another lens focuses the scattered radiation that emerges from the cell (through a prism) onto a diaphragm located at the entrance window of a photomultiplier. The scheme of the cell is presented in Fig. 10. The flows created by the hydrofocusing head are directed to a quartz capillary located inside the cell, which consists of a prism, a quartz cylindrical ring, and a spherical mirror. The inner

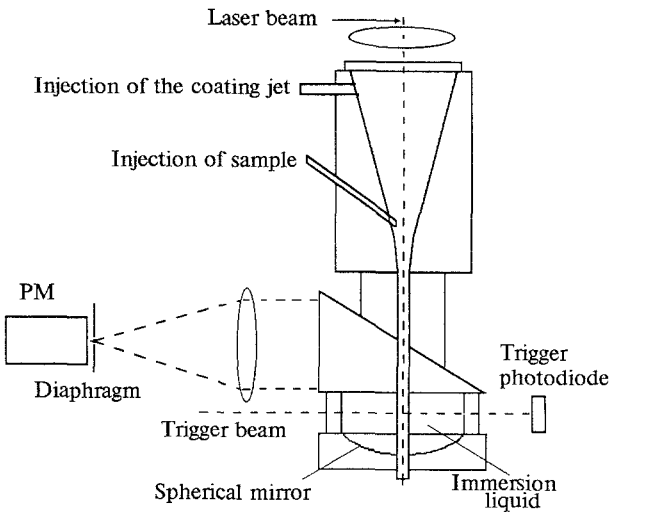

Fig. 9. The scheme of the hydrofocusing head with an optical cell of a scanning flow cytometer.

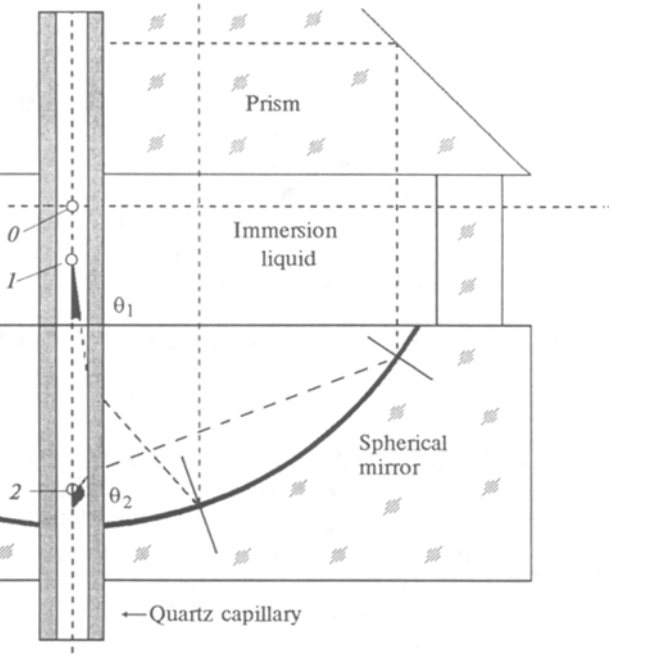

Fig. 10. The optical scheme of the scanning system of a flow cytometer. The dashed line denotes the basic radiation, the trigger beam, and scattering beams. A particle that passes through points *0*, *1*, and *2* triggers the electronic system and scatters light at angles θ_1 and θ_2.

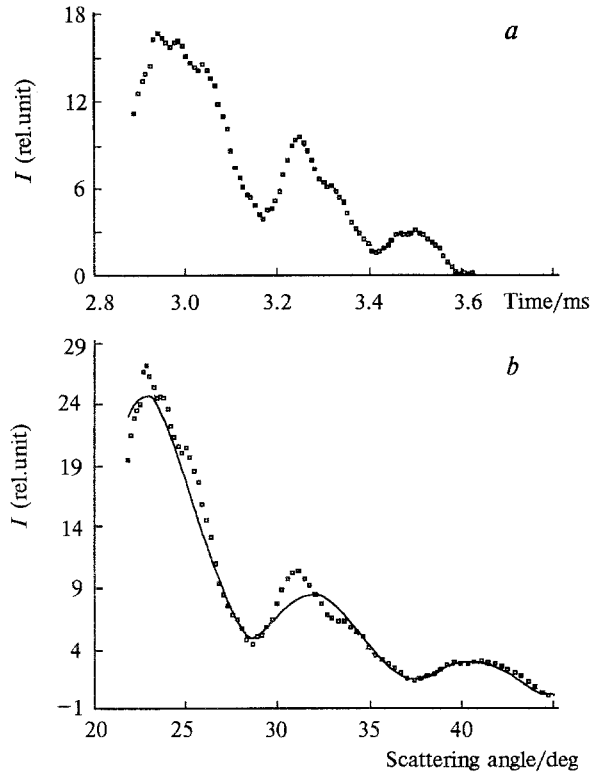

Fig. 11. Flying light scattering indicatrix of a latex particle *vs* the time (*a*) and the angle (*b*). The indicatrix obtained using the adjustment method (solid line) was calculated for a sphere of diameter 3 μm and a refractive index of 1.58.

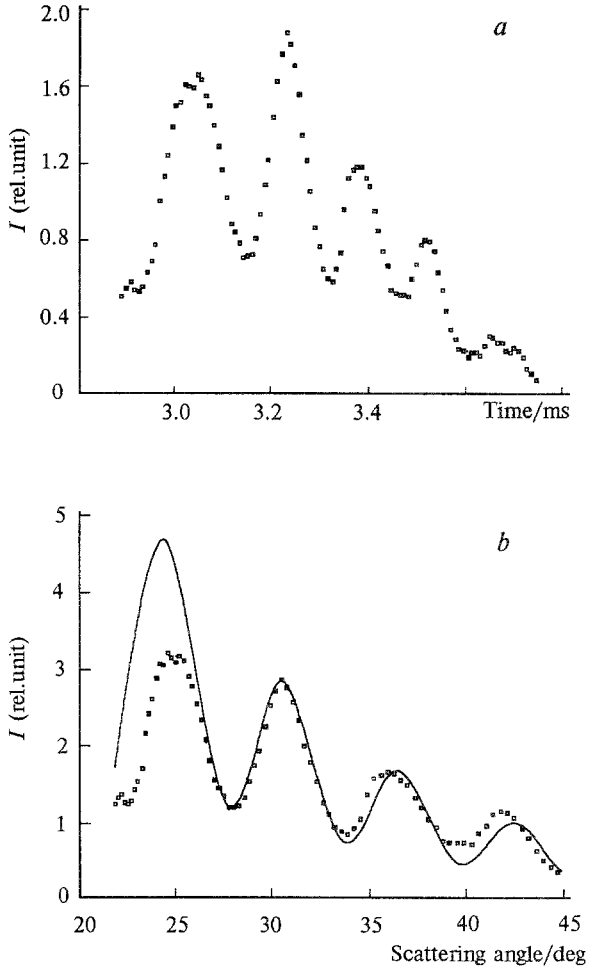

Fig. 12. Flying light scattering indicatrix of a latex particle *vs* the time (*a*) and the angle (*b*). The indicatrix obtained using the adjustment method (solid line) was calculated for a sphere of diameter 4.68 μm and a refractive index of 1.58.

space of the cell is filled with an immersion liquid. The laser beam, which passes through a cylindrical ring (a trigger beam), serves for actuating the electronic system of the cytometer. For any point within the area of measurement, the light scattered only at a particular angle is reflected from a spherical mirror in a direction parallel to the axis of the flow; then it is reflected from the surface of the prism and leaves the optical cell. This optical cell allows one to measure indicatrices of single particles over angles of 5 to 120°.

The effectiveness of the scanning flow cytometer was checked using latex particles with unknown parameters. Figures 11, *a* and 12, *a* present the flying indicatrices of latex particles recorded directly from the photomultiplier, and Figs. 11, *b* and 12, *b* show the indicatrices transformed with due regard for the variation in the solid angle of the collection of the scattered radiation. The size and the refractive index of the latex particles were estimated by the FLSI method with modified Eqs. (1) and (2). The following parameters of the indicatrix were used: $\Delta\theta_2(20)$ and $V(30)$. The resulting values for the sizes and refractive indices of the latex particles were 3 μm and 1.576 (for the indicatrix shown in Fig. 11, *b*) and 4.95 μ and 1.57 (for Fig. 12, *b*). The adjustment method afforded the following results: 3 μm and 1.58 and 4.68 μm and 1.58, respectively. Microscopic analysis made it possible to determine the mean sizes of latex particles in the two suspensions measured. The average diameter of particles in one of the suspensions was 3 μm, that in the other suspension was 4.7 μm. According to the literature data,[21] the refractive index of latex particles lies in the range 1.57 to 1.59.

The experiments with latex particles on the scanning flow cytometer demonstrated the possibility of using an indicatrix for determining the parameters of particles. The use of this type of cytometer and the FLSI method for processing light scattering data allowed us to estimate the sizes and refractive indices of spherical particles without the preliminary calibration of the optical and electrical tracts of the cytometer. In order to obtain more precise values for the parameters of a particle, results obtained by the FLSI method may be used as the starting data for the adjustment technique.

The design features of the scanning flow cytometer ensure further development of flow cytometry-based methods for the analysis of single particles. In particular, integration of the scattered light over the azimuthal

angle makes it possible to obtain a high signal-to-noise ratio for rather small particles, which, in turn, allows cheaper and more reliable He—Ne lasers to be used, instead of the conventionally applied argon lasers. This is very significant for the development of a clinical flow cytometer, since the existing flow cytometers of the Bacton Dickinson and Epic Division companies are rather expensive[3] ($50,000 to $500,000). The scanning flow cytometer also allows one to measure the fluorescence of single particles in real time and to develop procedures for the analysis of particles labeled with a dye with a long fluorescence time and, thus, to eliminate the nonspecific autofluorescence signal arising during the irradiation of macromolecules.[22] Recently, the corresponding preliminary results concerning fluorescence measurements have been obtained at the IKhKiG. A pulsed nitrogen laser was used in these experiments.

The potential of the FLSI method can be demonstrated, when multivariant analysis of macromolecules is carried out using latex particles (for the use of latex particles in the analysis of macromolecules see, for example, Refs. 2, 23). In particular, to determine six types of macromolecules in one run using a flow cytometer of the standard design, three types of dyes are required as labels and two lasers are required for the excitation of fluorescence. The FLSI method makes it possible to use latex particles of different sizes (for example, 1 to 10 μm with spacing of 0.5 μm) and one fluorochrom. A sensor sensitive to molecules of a particular structure is adsorbed onto particles of a given size. When this system is investigated on a scanning flow cytometer, the type of a molecule (the size of a particle) can be determined from the indicatrix, and the concentration of this type of molecules in the solution is estimated from the fluorescence intensity.

* * *

The calculation methods and experimental results considered in this paper allow one to estimate the situation in a rapidly developing field of research: diagnostics of single particles using light scattering. The data presented imply that light scattering may serve as a basis for developing diagnostic methods for the analysis of moving particles. According to the latest review on flow cytometry,[3] further development of fluorescent methods may be in the direction of the sophistication of the methods for recording signals: the combination of a flow cytometer with a Fourier spectrometer, the application of phase-sensitive methods involving the determination of the fluorescence time, *etc.* Unfortunately, the possibilities of flow cytometry with the use of light scattering have not been discussed. In our opinion, the FLSI processing of light scattering data obtained on a scanning flow cytometer, and recording the fluorescence in a time-resolved mode allow flow cytometry to be updated without substantial sophistication of the recording equipment. At present, it is expedient to start with the development of convenient procedures based on FLSI and relaxation fluorometry that can be used in the food industry, in medicine, and in biology.

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