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MORPHOLOGICAL CHARACTERISTICS OF NUCLEATED BLOODCELLS STUDIES BY ELASTIC LIGHT SCATTERING

Peter M.A. Sloot[*,**], Alfons G. Hoekstra[*,**] and Carl G. Figdor.[*]

[*] Division of Biophysics
The Netherlands Cancer Institute
Plesmanlaan 121
1066 CX Amsterdam
The Netherlands.

[**] Department of Computer Systems
University of Amsterdam
Kruislaan 409
1098 SJ Amsterdam
The Netherlands.

ABSTRACT

Light scattered from nucleated blood cells contains information on both the cell size and the morphology of the cell. This nondestructive sensing of a single cell has led to numerous analytical and preparative applications in cell biology. Recent data provide solid experimental evidence that increase in cell size of human lymphocytes, due to osmotic stress, is not directly proportional to forward scattering intensity but is rather inversely proportional. In this presentation , we provide arguments that this phenomenon can be quantitatively explained by means of a model based on a modified Rayleigh-Debye-Gans theory. As a consequence the current view that forward scattering intensities can be used as a measure of gross cell size, needs to be reconsidered. In addition, we postulate that structural changes of the cytoplasm, imposed by an osmotic stress, reflects corresponding changes in the nucleus. Recent slitscan measurements and (visco-elastic) calculations confirm this predicted behavior of nuclear dynamics.

INTRODUCTION

Regulation of cell volume following the exposure to anisotonic media, has been extensively studied to improve our understanding of the mechanisms employed by a cell to adapt to anisotonic situations. Recently it was shown unambiguously that Forward Scattering (FS) intensities vary inversely with cell volume.[1] This paradoxical result could not be explained, since low-angle light scattering is assumed to be proportional to cellular size.[2] These results prompted us to investigate theoretically in detail the light scattering phenomena of human lymphocytes measured under various osmotic conditions.

The number of theories describing light scattering from biological cells is limited. Rayleigh scattering cannot be applied to mammalian cells, since this theory is only valid for particles with a size comparable to the wavelength of the incident light, whereas the size of nucleated blood cells is much larger (3-40 μm diameter). Various other theories have been developed to describe elastic light scattering beyond the Rayleigh domain.[3] Most models proposed so far, such as Mie theory and other theories regard light scattering as a boundary value problem. These models have a number of serious disadvantages. The computational schemes resulting from Mie theory are complicated and time-consuming, although improvement of the speed of calculation is sometimes possible.[4] The theories are difficult to interpret and provide information which is far beyond experimental accuracy.[5] In addition, the Mie theory describes elastic light scattering based on a homogeneous spherical body of arbitrary size and refractive index, whereas nucleated blood cells are inhomogeneous (cytoplasm, nucleus) and may be irregularly shaped. Coated sphere models are much more realistic since they can account for intracellular heterogeneities. However, these models result in even more complex algorithms.[6] In this study we apply a spherical shell model to describe elastic light scattering from a single particle with dimensions of a nucleated blood cell in a modified Rayleigh-Debye-Gans approximation. The relevant light scattering parameters (cellular - and nuclear size and refractive indices) are derived from the Lorenz-Lorentz relation and the Boyle van 't Hoff relation.

THEORY

mRDG Theory for Concentric Spheres

The model proposed here is based on the Rayleigh-Debye-Gans approximation (or Born approximation), where the scatterer is considered as a particle constructed by Rayleigh scattering volumes and the phase shift is added for each volume dV. Only single scattering and elastic scattering are studied. The angular intensity of the scattered radiation is given by

$$I(\theta) = I_0 \frac{i_1(\theta) + i_2(\theta)}{2k_0^2 R^2} \qquad [1]$$

where

$$i_n(\theta) = | S_n(\theta) |^2$$

and θ is the angle of detection. The scattering functions $S_n(\theta)$ result from perpendicular (n=1) or parallel (n=2) polarized incident light relative to the plane of scattering. I_0 represents the intensity of the incident light. The distance from the particle to the point of observation is given by R, and k_0 is the propagation constant of the applied field outside the particle and is

defined by $k_0=(2\pi/\lambda_0)$, where λ_0 is the wavelength of the incident light in vacuum. The form factor P(θ) contains all the information on cellular size, shape and refractive index (m(r)) and is defined by

$$\left.\begin{matrix} S_1(\theta) \\ S_2(\theta) \end{matrix}\right\} = ik_0^3 \alpha(r) P(\theta) \begin{cases} 1 \\ \cos(\theta) \end{cases} \qquad [2]$$

The polarizability α(r) describes the changes in the charge distribution of the particle induced by the oscillating electromagnetic field. Interference from the waves emerging from the total volume V is described by P(θ):

$$P(\theta) = \frac{1}{V} \int_V | m^2(r) - 1 | \exp\left[ik_0 r \, 2\sin(\tfrac{\theta}{2}) \right] dv \qquad [3]$$

The internal field can be replaced by the incident field if the relative refractive index is ~1 and the phase shift is sufficiently small.[3] Shimizu[7] showed that modification of the propagation constant of the electromagnetic field in the particle, for waves to (applied field) and from (induced field) the scattering volume dV, can be accomplished by insertion of the relative refractive index into the Fourier kernel of Equation [3]. In this case the limitations on the phase shift and on the relative refractive index are less stringent, which makes it possible to define light scattering of a large entity such as a nucleated blood cell. Consequently in Equation [3] k_0 is replaced by $k_0.m(r)$.

A collection of concentric spheres can now be described by

$$P(\theta) = \frac{1}{\alpha(r)} \int_0 4\pi r^2 \alpha'(r) \frac{\sin\left[2k_0 m(r)\, r \sin(\frac{\theta}{2})\right]}{2k_0 m(r)\, r \sin(\frac{\theta}{2})} dr \qquad [4a]$$

where the volume polarizability α'(r) is defined by

$$\alpha(r) = \int_V \alpha'(r) dV \qquad [4b]$$

The volume polarizability inside the structured sphere may be approximated by the volume weighted average of the polarizability of a homogeneous sphere[6]:

$$\frac{\bar{m}^2-1}{\bar{m}^2+2}=\frac{1}{V}\int_V \frac{m^2(r)-1}{m^2(r)+2}\,dV = M_0 \qquad [5]$$

A nucleated blood cell can be mimicked by two concentric spheres where the nucleus represents the inner sphere (radius a; relative refraction index m_a) and the cytoplasm represents the outer sphere (radius b; relative refraction index m_b) (Figure 1).

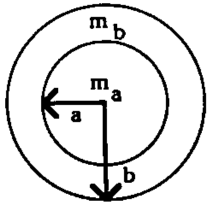

Figure 1: Concentric sphere model for nucleated cells: m_a and m_b are the relative refractive indices, a and b are the radii of the inner and outer spheres, respectively.

Integration of Equation [5] with respect to r regarding the boundary values of the relative refractive indices,

$$m(r)=\begin{cases} m_a & 0<r\le a \\ m_b & a<r\le b \end{cases}$$

results in

$$M_0=\frac{1}{V_b}\left[M_a V_a + M_b (V_b - V_a)\right] \qquad [6]$$

where $M_a = (m^2_a-1)/(m^2_a+2)$ and the volume of the nucleus $V_a = (4/3)\pi a^3$. M_b and V_b are defined similarly. The mean polarizability for the inhomogeneous sphere is given by b^3M_0 and the polarizability of the nucleus equals $\alpha_a = a^3M_a$. Therefore the volume polarizability of the cytoplasm and the nucleus can be derived by integration of Equation [4b], which results in

$$\alpha'_a=\frac{3}{4}M_a \quad\text{and}\quad \alpha'_b=\frac{b^3M_0-a^3M_a}{V_b-V_a} \qquad [7]$$

Subsequently the form factor $P(\theta)$ is calculated by integration of Equation [4a]:

$$P(\theta)=\frac{(2\pi)^{3/2}}{\alpha(r)}\left\{\alpha'_a\frac{a^3}{U_1^{3/2}}J_{3/2}(U_1) + \alpha'_b\left[\frac{b^3}{U_2^{3/2}}J_{3/2}(U_2)-\frac{a^3}{U_3^{3/2}}J_{3/2}(U_3)\right]\right\} \qquad [8]$$

where $U_1 = 2ak_0m_a.\sin(\theta/2)$,
$U_2 = 2bk_0m_b.\sin(\theta/2)$,
$U_3 = 2ak_0m_b.\sin(\theta/2)$.

TABLE I Influence of changes in scattering parameters on the normalized forward scattering intensities calculated from Sloot et al.,[8]

							
$b = 3.5\ \mu m$ $m_a = 1.10$ $m_b = 1.08$		$a/b = 3.0/3.5$ $m_a = 1.10$ $m_b = 1.08$		$a/b = 3.0/3.5$ $m_b = 1.02$		$a/b = 3.0/3.5$ $m_a = 1.10$	
a (μm)	Norm. FS	b(μm)	Norm. FS	m_a	Norm. FS	m_b	Norm. FS
0.0	1.00	2.0	1.00	1.09	1.00	1.0500	1.00
1.5	1.12	2.5	2.76	1.07	0.65	1.0050	0.72
3.0	1.41	3.0	5.78	1.05	0.34	1.0005	0.69

These equations can be applied to calculate the complete scattering spectrum of lymphocytes given the various parameters. In Table I we summarize the influence of small variations in size and relative refractive indices (a, b, m_a and m_b)

on the forward light scattering intensities. The incident light is a He/Ne laser (λ= 632.8 nm) and the cells are irradiated in an aqueous solution (refractive index = 1.333). The calculations apply for parallel polarized incident light. The Forward Scattering (FS) intensities were integrated from 1^0 to 7^0 . Further details concerning these calculations are described elsewhere[8].

Low Angle Light Scattering from Osmotically Stressed Lymphocytes

It can be derived from Table I that FS is proportional to changes in the nuclear size or the gross cell size. This result is consistent with the idea that FS increases with increasing cellular size. However, a decrease in refractive index of the nucleus or the cytoplasm results in a corresponding decrease of the FS signal. These data suggest that the influence of changes in refractive index, of both the cytoplasm and the nucleus should be considered when FS of nucleated blood cells is concerned.

In this section we shall discuss the physical consequences of the osmotic response of cells in an anisotonic environment. Basically, there are three diffusion mechanisms that allow a cell to adjust to anisotonic media: free diffusion, facilitated diffusion and active diffusion.[9] It has been shown that, to a first approximation, the dynamics of diffusion are well described by free diffusion.[10] In the case of free (or passive) diffusion, the condition for equilibrium requires that the chemical potential in the cell equals the chemical potential in the medium. The volume of the cell V_{cell} equals the volume of the intracellular water (V) plus the volume occupied by the endogenous cell solutes V'.[10] Therefore, the part (V_{cell} - V') = V contributes dynamically to the equilibrium condition. It can be shown from thermodynamical considerations that the response of a lymphocyte to an osmotic pressure Π is given by:

$$V_{cell} = \frac{\Pi^o}{\Pi}\left(V^o_{cell} - V'\right) + V' \qquad [9]$$

where the isotonic situation is labeled by Π^o and V^o_{cell}.

We modeled a lymphocyte by two concentric spheres where it is assumed that both the inner and the outer sphere act like an ideal osmometer. Eventually, the changes in cellular/nuclear volume can be derived from Equation 9. Next, the influence of changes in refractive index of the two compartments involved are investigated. It is assumed that solutes neither enter nor leave the cell.[10] Since the refractive indices are related to the number of dipoles per volume, changes in volume will induce changes in refractive index. Quantification of this phenomenon can be obtained from the Lorenz-Lorentz equation:[3]

$$\frac{m^2-1}{m^2+2} = C\,\frac{1}{V} \qquad [10]$$

where C [m^3] is a constant. Taylor expansion results in (assuming m-1 --> 0)

$$m - 1 = \frac{3}{2}\frac{C}{V} \qquad [11]$$

The volume V can be calculated from Equation 9

Consequently, the influence of the application of an osmotic shock on the scattering parameters m_a, m_b, a and b can be obtained from:

$$m = \frac{m_o - 1}{\Pi^o/\Pi} + 1$$

$$b = b^o\left(\frac{\Pi^o}{\Pi}\right)^{1/3} \qquad [12]$$

$$a = b\left(\frac{3}{3.5}\right)$$

where it is assumed that V' << V^o_{cell}. This simultaneous increase in volume and decrease in refractive index is illustrated in Figure 2. The ratio nucleus/cytoplasm, 3/3.5, is used as a typical value for human peripheral lymphocytes.[3]

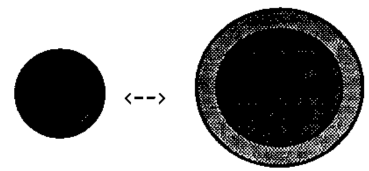

Figure 2 : Illustration of an osmotic shock on the scattering parameters a, b, m_a and m_b of a nucleated bloodcell. The darker areas denote a relative higher refractive index.

Finally we evaluate this model to calculate the changes in the FS intensities of light scattered from a lymphocyte in various anisotonic solutions.

RESULTS

Measurement of cell volumes in osmotically stressed and frozen-thawed cells were recently reported by McGann et al.[1]. The light scattering experiments were performed by means of an EPICS V flowcytometer with an incident wavelength of 488 nm and an effective acceptance angle of 2.2° to 6.2°. Changes in osmolarity from $\Pi^o/\Pi = 0.4$ to $\Pi^o/\Pi = 1.6$ (1.0 = isotonic) were recorded. We calculated the corresponding changes in scattering parameters, for this range of osmolarities, in accordance with the equations derived in the previous section. The resulting values are tabulated in Table II .

TABLE II : Changes in light scattering parameters for a lymphocyte modelled by two concentric spheres, in anisotonic solutions.

		Hypertonic environment	Isotonic environment	Hypotonic environment
$V/V^o = \Pi^o/\Pi$	:	0.40	1.00	1.60
a (µm)	:	1.90	2.57	3.01
b (µm)	:	2.21	3.00	3.51
m_a	:	1.25	1.10	1.06
m_b	:	1.13	1.05	1.03

Next, Equation [8] is integrated numerically with the defined acceptance angle, by means of the trapezoidal rule:

$$FS = \int_{2^\circ}^{6^\circ} P(\theta)\sin(\theta)\,d\theta$$

(Note that the range of integration corresponds to the experimental set up of McGann et al. [14] and differs from that applied in reference 7).

The variables of Table II are inserted in the form factor $P(\theta)$ (step-size $\Delta(V/V^o) = 0.06$), the result of which is shown in Figure 3. The experimental values from McGann et al., are shown in figure 4.
Finally, the data of both figures were fit to an exponential curve. This resulted in : Norm. $FS = 2.16\ 10^{(-0.33\ \Pi^o/\Pi)}$ for the theoretical calculations (Fig. 3) and Norm. $FS = 2.31\ 10^{(-0.38\ \Pi^o/\Pi)}$ for the experimentally observed intensities (Fig. 4). The correlation coefficients are 0.99 and 0.97 respectively.

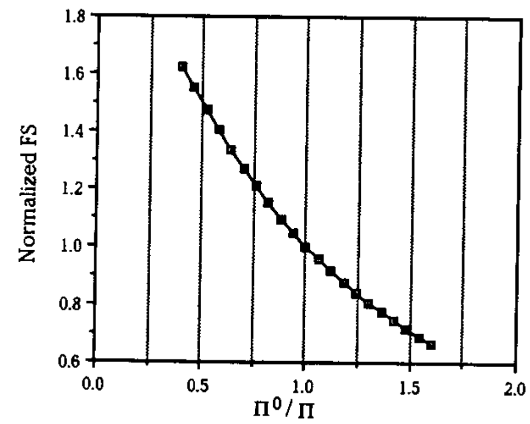

Figure 3: Implementation of the proposed model and numerical integration of the form factor $P(\theta)$. Π = Osmotic pressure; Π^o = Osmotic pressure for the isotonic situation. The Forward Scattering (FS) intensities are normalized to unity for cells in an isotonic environment.

DISCUSSION

Interpretation of low-angle light scattering from particles as large as biological cells, is usually based on the Fraunhofer diffraction theory [2]. Here it is assumed that light passing along a particle forms a plane wavefront which can be divided into a number of individual rays, following the Huygens principle.[11] As a consequence, FS can be regarded as a projected area response and no information on cellular transparency is present. It was shown by Steinkamp et al.,[12] that for homogeneous particles, varying in size, but not in refractive index, a proportionality exists between FS and electronically sized uniform plastic microspheres. The influence of beamshaping optics on the linearity of the FS signal versus the volume was discussed both theoretically and experimentally[12]. From these observations it can be concluded that, to a first approximation, FS is proportional to d^x where $1 \le x \le 3$ and d is the diameter of the particle. The exact value of x is a complex function of cellular size, optics, detection system and range of detection angles. However, the data from table I clearly indicate that changes in refractive index for nucleated blood cells (i.e. lymphocytes) introduce an inverse effect on the FS intensities. Therefore, to investigate the phenomenon of FS decrease with increasing cellular size under hypotonic stress, we suggest the model described in the preceding sections.

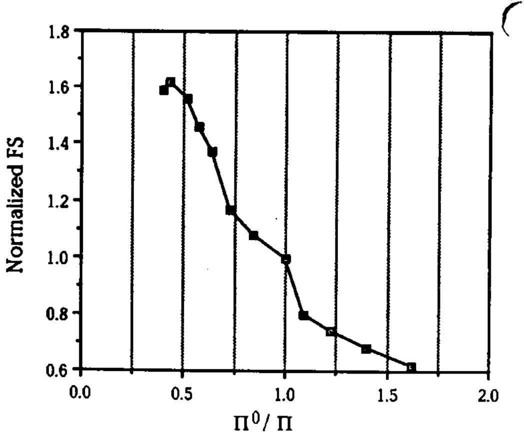

Figure 4: Measurement of forward light scattering from osmotically stressed lymphocytes (ref. 1). Π = Osmotic pressure; Π^0 = Osmotic pressure for the isotonic situation. The Forward Scattering (FS) intensities are normalized to unity for cells in an isotonic environment.

A number of assumptions are implicit here: First the assumption that the relative refractive index changes can be derived from Equations 11 and 12, is true only if m --> 1. Table II shows that for extreme hypertonic situations, this approximation is not valid anymore. However, direct insertion of the Lorenz-Lorentz equation (Eq. 10) instead of the expansion (Eq. 11) into the calculations for hypertonically stressed lymphocytes, showed a close resemblance to the data obtained in the previous section. It was concluded therefore, that a refinement of equation 10 within the range of observed osmolarities does not contribute to our model. In addition, small changes in the ratio nucleus/cytoplasm (3/3.5) or changes in the range of size parameters or refractive index, or small changes in the span of integration angles, did not affect our results significantly (data not shown).

Secondly it is assumed that both the gross cell size and the size of the nucleus progressively change when the cells are osmotically stressed. From the Coulter volume data reported by McGann et al.[1] it is obvious that the cytoplasm swells in hypotonic media and shrinks in hypertonic media. It has been reported however that, due to the large pores in the nuclear membrane, isolated nuclei do not swell in hypotonic media[13]. We postulate, as a possible explanation to this antinomy, that the fibrillar networks inside the cell regulate the changes in the size of the nuclei. We designed dedicated experiments to confirm these predicted conformational changes of the nuclei. In the appendix we describe these (time resolved) measurements of the nuclear volume response. An extension to the standard solid viscoelastic model to interprete the observed dynamical behavior of the nuclei is proposed. Experimental and theoretical evidence, shown in the appendix, indicate a passive nuclear response merely induced by a mechanical link between the cell membrane and the nuclear envelope.

CONCLUSIONS

Recent interest in the light scattering form osmoticaly active lymphocytes, prompted us to investigate in detail a new elastic light scattering model for this type of biological particles.

We presented a theoretical basis to explain the apparent forward light scattering anomalies of osmotically stressed lymphocytes measured in a flow-through system, where it was shown that a simple light scattering algorithm can be applied to monitor changes in cellular morphology.

The proposed model describes the changes in refractive index of the cellular compartments and, simultaneously, changes in the size of the nucleus and the cytoplasm for lymphocytes in anisotonic solutions. The theoretical data correspond quantitatively with the experimentally obtained data.

Up till now the hypothesis that size changes in the cytoplasmatic region of the cell reflect corresponding changes in the nucleus had not been verified. Therefore we studied in detail the response of the nucleus by means of fluorescent probes. These experiments are discussed in the appendix.

In addition to previous work, where the lateral and back scattering directions for concentric spheres were investigated,[8] the results presented here legitimate a further detailed study of the light scattering phenomena of biological cells that can be described by the form factor $P(\theta)$. For instance, extrapolation of the form factor to describe more complex cellular structures will be explored. Especially detailed cellular texture such as granules and segmented nuclei may be studied numerically.

In conclusion, this presentation illustrates for the first time that quantitative interpretation of light scattering measurements of realistic biological particles is possible. Moreover, the results presented here demonstrate that such calculations may contribute to new biophysical insights.

APPENDIX

Relying on the light scattering model described above, we predicted a nuclear response after an osmotic shock, although it is known that isolated nuclei, due to large pores in the nuclear membrane, don't behave as osmometers.[13] We demonstrate in figure 5 that the nucleus of intact lymphocytes *does* respond to anisotonic conditions (for details of the work described in this appendix, see reference 14).

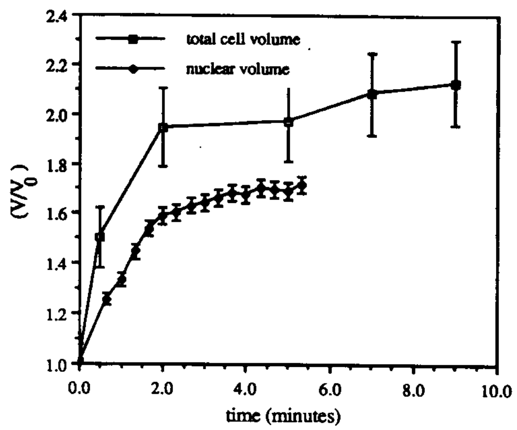

figure figure 5:The total cell volume response and the nuclear volume response, as a function of time, after an osmotic shock at t = 0 minutes; $\Pi/\Pi_0 = 1.5$; V_o is the volume at t = 0.

The measurement of the nuclear volume was performed with a specially designed flow cytometer. Staining of the nucleus with Hoechst 33342 and measuring the width of the fluorescence signal in the flow cytometer yields the nuclear diameter. The total cell volume was obtained by electronic sizing.

The relaxation of leukocytes after small deformations can be modelled with the standard solid viscoelastic model We have extended this model with a Voigt element to describe the retardation of the nucleus. The nuclear response data can be fitted to this extended model.[14] This clearly shows that the nuclear response is a passive viscoelastic response. induced by a mechanical link between nuclear- and cellular membrane.

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