

RHEOLOGICAL CHARACTERIZATION OF THE DISSOCIATION OF ERYTHROCYTE AGGLUTINATES INDUCED BY LECTINS

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Abstract: Energy evolved from hemagglutination reaction or spent in dissociating erythrocyte agglutinates has been proved to be an excellent parameter for analyzing cell-cell interactions mediated by bridging molecules such as antibodies or lectins. We developed a new rheo-optical method to estimate the energy of dissociation of red blood cell agglutinates. In a Couette shear field agglutinates can be dissociated until a suspension of monodispersed cell is obtained. Intensity of light backscattered by suspended agglutinates increases during their mechanical dissociation. Variation of backscattered light intensity correlates with the energy spent in the process. The adhesive energy of erythrocyte agglutination induced by lectins has been estimated by applying this method. Two specific lectins (Dolichus Biflorus agglutinin and Ulex Europaeus agglutinin) and a new lectin obtained from Amaranthus Gruentus seeds which specificity is unknown were studied. Results obtained in this work for Dolichus Biflorus lectin are comparable with values published by other authors. An asymptotic decrease of adhesive energy was observed when the mechanical dissociation was applied several times on the same sample. Our results suggest that the cell detachment is accompanied by the extraction of membrane receptors. This finding is consistent with results obtained by other authors.

Key words: agglutinate, lectin, dissociation, energy, blood group

Introduction

Lectins are carbohydrate-binding proteins which can be extracted from plants or animals and are capable of recognizing specific glycolipids and glycoproteins whose specificities are linked to glycosyl residues. Some lectins can recognize carbohydrate receptor located on the human red blood cell (RBC) membrane leading to cell agglutination. Such lectins are called "*specific*". Other ones can agglutinate RBC of any blood group so they are called "*panagglutinating*" lectins. Other effects can be expected from these proteins on their activity on the cells as lysis or swelling of treated cells.

It is well known that erythrocyte agglutination results from molecular bridging between adjacent cell surfaces. Such cell adhesion is carried out with a little spent of energy. Separation of agglutinated cells can be attained if the work done by external stresses overcome the net agglutinating energy. In a previous paper [1] the authors reported a new rheo-optical method to analyze immunological erythrocyte agglutination by shear dissociation of suspended agglutinates and backscattering light measurement [2].

Backscattered light intensity correlates very well with the specific area (per volume unit) of suspended particles (isolated cells and agglutinates). Differences between backscattered intensities corresponding to agglutinates and to completely dissociated cells are used to estimate the specific area of membrane adhered cell. Hence the energy of agglutination can be expressed in term of contact surface in the agglutinate state. A parameter estimating the specific energy of cellular adhesion can be derived from data of backscattering light variation obtained while agglutinated cells become dissociated in a Couette shear field.

Theoretical approach

Rheological analysis of erythrocyte suspension require the knowledge of aggregate formation and dissociation processes [3]. It is very important to emphasize at the outset that our calculations are approximate because an idealized condition of agglutination equilibrium has been assumed and some components of the energy in the equation of conservation of energy have been neglected. When shear stress generated in a Couette flow is applied to suspended agglutinates, they can be dissociated into smaller particles until only isolated cells remain in suspension.

Agglutinate dissociation increases the total scattering area of specific free surface increasing the diffuse reflectivity r of suspended particles [4]. The diffuse reflectivity can be related to the specific scattering area σ [5]

$$\frac{\sigma}{\sigma_0} = \frac{r}{r_0} \cdot \left[\frac{1-r_0}{1-r} \right]^2 \cong \frac{r}{r_0} \quad (1)$$

where r_0 and σ_0 are the diffuse reflectivity and the specific scattering area of completely dispersed red blood cells in suspension (monodispersed RBC). The macroscopic reflectometric relationship can be related to the specific bridging area average over the flow cross section [2]

$$\frac{r_C}{r_0} = 1 - \frac{r}{r_0} \cong \frac{\langle \sigma_C \rangle}{\sigma_0} \quad (2)$$

During dissociation process carried out under constant shear stress, backscattered light intensity increases with time. This variation can be fitted by an exponential expression

$$r(t) = r_0 \cdot (1 - e^{-t/\beta}) \quad (3)$$

that is, the velocity of dissociation is depending on β . This parameter characterizes the dissociation process. By combining eqs. (2) and (3)

$$r_C(t) = r_0 \cdot e^{-t/\beta} \quad \text{and} \quad \sigma_C(t) = \sigma_0 \cdot e^{t/\beta} \quad (4)$$

Specific energy of adhesion (φ) have been defined as the energy required to separate an unit area of contact surface of adhered cells, starting from equilibrium distance [6]. Considering two self adhered cells (doublet) suspended in a viscous fluid, the balance of energy at the equilibrium state of adhesion can be expressed in a global form

$$\frac{dw_F}{dt} = -\varphi \cdot \frac{d\sigma_C}{dt} \quad (5)$$

where w_F is the specific work produced by external stresses. In expression (5) we have neglected variations of elastic and kinetic energies in order to simplify next deduction. By deriving expression (4) and introducing it in eq. (5)

$$\frac{dw_F}{dt} = -\varphi \cdot \left[\frac{\sigma_0}{\beta} \cdot e^{-t/\beta} \right] \quad (6)$$

integrating eq.(6)

$$w_F = \varphi \cdot \frac{\sigma_0}{\beta} \cdot \int e^{-t/\beta} dt = \varphi \cdot \sigma_0 \cdot \frac{t}{\beta} \cdot \left[\frac{S_0 - S_1}{S_0} \right] = \left(\varphi \cdot \sigma_0 \cdot \frac{t}{\beta} \right) \cdot E_d \quad (7)$$

where E_d can be defied as the dissociating fraction of the total work done by the instrument during the dissociation process. Surfaces S_0 and S_1 are indicated in Fig. 1, being $S_0 = r_0 \cdot t$.

The force F required to dissociate the cell doublet attains its maximum value at the moment when the doublet is completely dissociated [7]. Its varies as the specific adhesive energy does. In a Couette flow the doublet will be separated if the critical shear stress acting on the exposed cell surface overcomes the force F .

The cell rotation in a Couette flow reduces the effectiveness of shear stress by a factor $\Gamma=10$ [2]. This equilibrium condition leads to the calculation of the specific adhesive energy

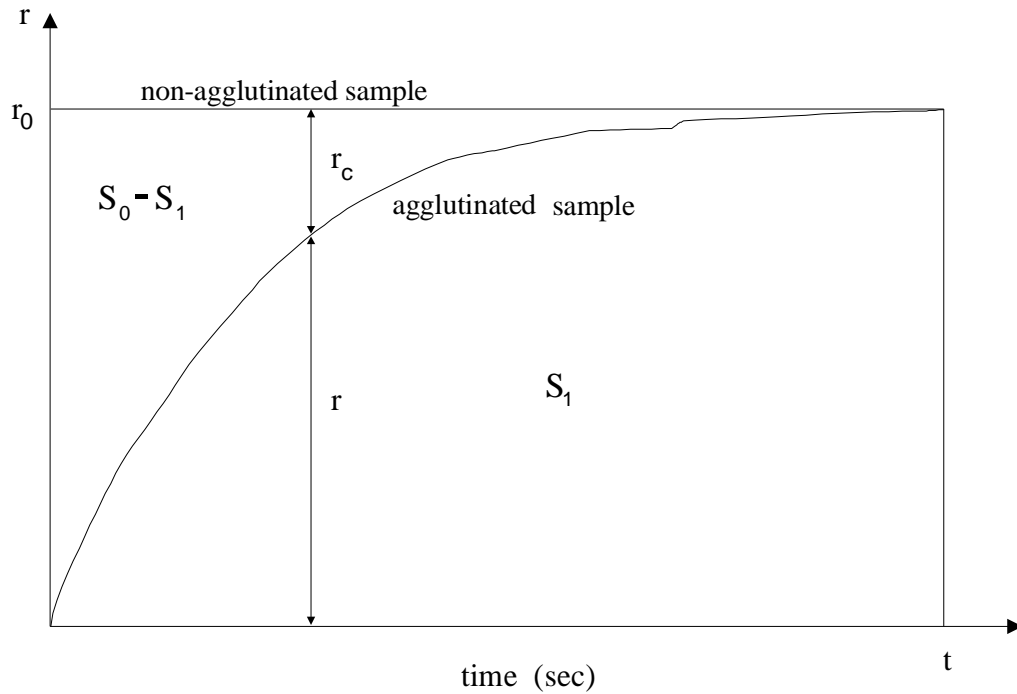


Fig. 1. Principle of determination of the parameter of energy E_d .

$$\varphi = \frac{\dot{\gamma} \cdot \eta \cdot \sigma_0 \cdot E_d}{2 \cdot \pi \cdot R_p \cdot \Gamma} \quad (8)$$

where $R_p = 10^{-4}$ is the curvature radius of the bridged surface at the moment of the complete separation of cells, $\dot{\gamma}$ is the shear rate and η is the viscosity of the suspending medium.

Materials and methods

Lectins: DBA (Dolichus Biflorus Agglutinin) and UEA (Ulex Europaeus Agglutinin) were provided by the ETS of Lorraine (CHU - Nancy - France). ACA (Amarantus Cruentus Agglutinin) was obtained from seeds provided by the "Grupo de Energia Solar - Instituto de Fisica Rosario - Argentine", according to the technique as described by Moore et al [8].

Suspending Medium (SM): was prepared by dissolving Dextran 70 (D.1537 Sigma Chemical Co.) at 4.1% (w/v) in phosphate buffered saline (PBS). Its viscosity was adjusted to 1.8 mPa.sec at 36°C, measured in a Contraves LS 30 viscometer.

Fresh Human Erythrocytes of A1, A2, O and B blood groups were provided and classified by the ETS of Lorraine (France). Red blood cells (RBC) were washed three times in PBS and resuspended in SM at 40% hematocrit.

Sample preparation: Serial two fold dilutions (from 1/4 to 1/64) of each lectin was prepared in suspension and gently homogenized. Control samples were also prepared by suspending washed erythrocytes in SM containing no lectin in solution.

Method: Experimental works were carried out using an Erythroaggregometer® (constructed by Regulest-Florange-France) which was described in detail in elsewhere [9]. An infrared (780 nm) laser beam incites laterally on the sample contained in the gap between two concentric cylinders. Particle suspensions are sheared between the inner static and the outer transparent rotating cylinder under constant shear rate (596 sec^{-1}). Laser backscattered intensities are measured every 10 msec by a solid state photodetector, during the total dissociation process (60 seconds). Read data are continuously transferred and stored in a PC while the curve of backscattered intensity vs. Time displayed in the PC monitor. This curve is called the "dissociation curve"[1].

A volume of 1.5 ml of the sample is poured into the gap between both concentric cylinders of the instrument. Cylinder rotation starts and the maximum shear rate is applied during 10 sec. Rotation is then stopped and the sample is maintained at rest during 6 minutes to complete the erythrocyte agglutination. At the end of this period the outer cylinder is put again in rotation at maximum speed during 60 seconds. Six thousand readings are taken during this period, transferred and stored. Calculations performed on stored data leads to the obtention of five dynamic parameters with are displayed simultaneously with the dissociation curve.

Dissociation test was repeated five times on each agglutinated sample without remove it from the instrument. Sample was maintained at rest six minutes between repetitions in order to reestablish the agglutinated state. These repetitions were carried out to investigate if the energy spent during mechanical dissociation varied with successive repetitions.

Results and discussion

Several papers concerning cell-cell interaction mediated by lectin recognizing RBC surface glycoproteins and glycolipids and inducing erythrocyte agglutination have been published [10-18]. Only few of these papers analyze the energy evolved from the reaction. The technique applied in the present work can provide a useful parameter (E_d) to estimate the specific energy of dissociation of agglutinates (φ), which correlates wit the energy evolved from the agglutination reaction. By measuring the intensity of light backscattered by suspended agglutinates under shear, the specific energy of dissociation has been approached.

To further validate the laser backscattering method for approaching the agglutination energy by dissociation of agglutinates we analyze by this method the binding reaction induced on human erythrocytes by two specific lectins and one lectin which specificity was unknown.

The macroscopic reflectivity relationship r/r_0 is suitable for interpreting the variation of adhered cell surfaces when agglutinates become dissociated by shear stresses in a Couette flow system. The quantitation of contact surface of agglutinates and its variation during shear separation provide a parameter of energy (E_d) and then a way for estimating the surface adhesive energy of bridged membranes in dynes per cm.

Erythrocytes of A₁,A₂,B and O groups have been tested against three vegetal lectins (DBA, UEA and ACA). Mean values (and SD) of φ (10^{-4} dyn/cm) are presented in table 1 for two lectin dilutions (1/4 and 1/32). Between these two dilutions linear regression of specific energy on the logarithm of dilution has been observed. The lowest dilution (1/4) becomes a threshold of saturation while the highest dilution (1/32) appears as limit for detectable energy.

Specificity of each lectin is also indicated in table 1, expected for ACA this agglutinin appears as a panagglutinating lectin. However ACA reacts differently with each tested blood group.

Studies carried out by Evans et al [12,13] on erythrocytes agglutinated by a monoclonal antibody specific to glycophorin A and also by a lectin extracted from Helix Pomatia (anti-A specific) demonstrated that mechanical separation of bonded cells was accompanied by the extraction of membrane receptors. This finding suggested that the agglutination energy is better estimated during agglutination rather than dissociation of agglutinates, hence, a repeated dissociation of agglutinates could lead to a decrease of spent energy with the number of repetitions. To test this assumption five repetitions of the mechanical dissociation were carried out on each agglutinated sample which was maintained six minutes at rest between two consecutive repetitions. The reduced value of E_d (R_d) was calculated at each repeated process

$$R_d = \frac{E_d^{(n)}}{E_d^{(0)}}$$

where $E_d^{(n)}$ is the energy parameter corresponding to the nth. Repetition and $E_d^{(0)}$ is the energy parameter corresponding to the first dissociation.

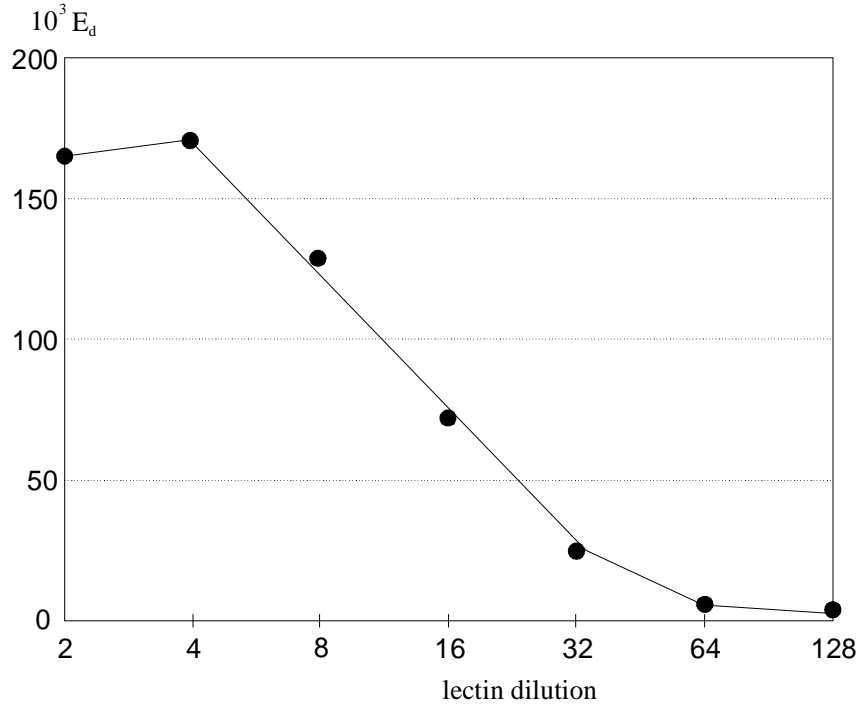


Fig. 2. Parameter of energy E_d plotted against lectin dilution.

Fig. 3 shows the plot of R_d values vs. the number of repetitions. R_d decreases asymptotically with repetitions of mechanical dissociations.

The dissociation of agglutinates in a shear field is a very complex phenomenon. Some work must be done to overcome the energy evolved during the agglutination reaction and stored. The behavior results from the ability of erythrocytes to store energy during agglutination and restore this energy during separation. However, as it was demonstrated by Evans et al. [12-13] adhesive energy should be better estimated during agglutination rather than during dissociation.

In our study we used an Erythroaggregometer® to measure and store backscattered light data from RBC agglutinates under shear dissociation in a Couette flow system. From those data we calculated the specific adhesive energy corresponding to different concentrations of lectin molecules on erythrocytes of A₁, A₂, O and B groups.

Table 1.
Mean Values and (Standard Deviations) of Specific Energy (φ) Required to Dissociate RBC Agglutinates Induced by DBA, UEA and ACA Lectins.

RBC group	Sample number	Lectin	Specificity	φ [10^{-4} dyn./cm]	
				1/4	1/32
A ₁	8	DBA	anti-A	1.23 (0.21)	0.53 (0.04)
		UEA	anti-H	0.03 (--)	0.03 (--)
		ACA	unknown	1.12 (0.28)	0.52 (0.06)
A ₂	5	DBA	anti-A	0.08 (--)	0.05 (--)
		UEA	anti-H	0.37 (0.05)	0.17 (0.03)
		ACA	unknown	1.63 (0.22)	0.74 (0.06)
		DBA	anti-A	0.02 (--)	0.02 (--)

O	8	UEA	anti-H	0.46 (0.04)	0.21 (0.03)
		ACA	unknown	1.85 (0.21)	0.85 (0.09)
B	4	DBA	anti-A	0.04 (--)	0.03 (--)
		UEA	anti-H	0.21 (0.01)	0.10 (0.01)
		ACA	unknown	0.89 (0.11)	0.42 (0.05)

As shown in Table 1 for DBA at maximal concentration on RBC of A₁ group the specific energy is 1.23x10⁻⁴ dyn./cm. This result appears very near of the energy calculated by Sung et al. [17] who determined the specific energy of 1.15 dyn./cm for DBA and A₁ erythrocytes having a density of 365 molecules of lectin per μm².

Values of R_d decreases asymptotically as increases the number of mechanical dissociations applied to the sample as it is shown in Fig. 3. This finding suggests that the number of molecular bridges between bonded cell surface decreases by mechanical extraction of antigen receptors from the cell membrane. Hence the strength of molecular bridges to mechanical separation could be greater than membrane strength to mechanical extraction.

The results reported in this paper can be summarized as follows:

- The Erythroaggragmeter® enables the specific energy of RBC agglutinate dissociation to be estimate.
- A linear regression of E_d on the logarithm of lectin dilution has been observed between 1/4 and 1/32 dilutions.
- Agglutinate dissociation energy decreases as the mechanical dissociation process is repeatedly applied on the same agglutinated sample. This behavior suggest that the some membrane

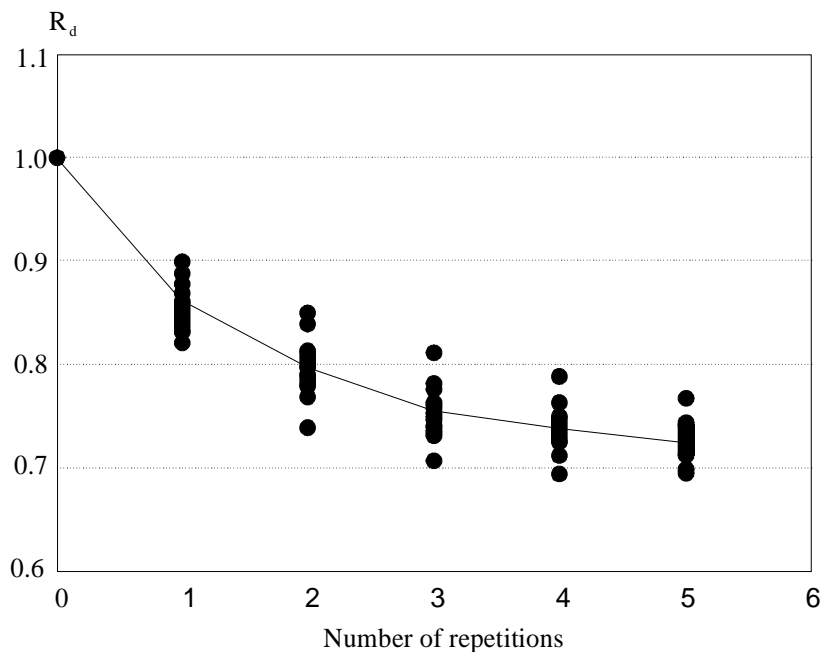


Fig. 3. Variation of E_d with the number of repetitions of mechanical dissociation.

receptors are extracted during shear detachment of bonded cells.

- The lectin extracted from Amaranthus Cruentus seeds appears as a panagglutinating agglutinin. However it shows some specificity evidentiated by differences between dissociation energies corresponding to different blood groups.

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Реологическое описание распада агглютинаций эритроцитов, вызванных лектинами

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Энергия, выделяющаяся при реакции слипания эритроцитов или затрачиваемая в процессах распада агглютинаций (склеенных групп) эритроцитов, оказывается прекрасным параметром для описания взаимодействия клеток, вызванных такими молекулами как антитела и лектины. В данной работе развит новый рео-оптический метод для оценки энергии распада агглютинаций красных кровяных клеток. Распад агглютинаций происходит в сдвиговом течении Куэтта вплоть до образования суспензии монодисперсных клеток. Интенсивность отраженного света после распада агглютинаций увеличивается из-за увеличения площади, отражающей свет. Энергия адгезии агглютинаций, вызванных лектинами, оценена теоретически с помощью этого метода. Два ранее известных лектина (*Dolichus Biflorus agglutinin* и *Ulex Europaeus agglutinin*) и новый лектин, полученный из семян тропического амарантового дерева, были изучены. Результаты, полученные для нового лектина, были сравнены с данными других авторов. Асимптотическое уменьшение энергии адгезии было отмечено, если механическая диссоциация проводилась несколько раз на одном и том же образце. Полученные результаты подтверждают, что разделение клеток сопровождается извлечением мембранных рецепторов. Это наблюдение согласуется с результатами других авторов. Библ. 18.

Ключевые слова: гемодинамика, агглютинация эритроцитов, лектин, диссоциация, энергия, отражение света.

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