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Gradual disintegration of protein lumps contained in thin emulsion films: Role of the surface diffusion

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Abstract We study thin aqueous films immersed in oil phase. The system is stabilized by globular protein (BSA), adsorbed on the liquid interfaces. Reversible surface aggregation of protein is observed. Big lumps remain entrapped within the film, where they are squashed by the pressing action of the curved wrapping menisci. The lumps gradually disintegrate and finally disappear. We propose a theoretical explanation of this phenomenon. Molecular migration by the mechanism of surface diffusion is found to be responsible for removing the material from the aggregates. The latter shrink isotropically (at least in a certain range of sizes). The particle radius was measured by interference microscopy, as a function of time. The data were fitted with theoretical computations. The partial differential equation of non-stationary surface diffusion was solved numerically, with boundary condition imposed on the moving circumference of the big protein cluster. Very good agreement between theory and experiment is obtained. From the best fit we find one parameter, whose value gives the maximum adsorption along the rim where the lump is attached to the interface. The results suggest existence of protein multilayers on the liquid surface.

Key words Surface diffusion – Protein aggregation – Thin liquid films – Emulsion-type interfaces

Introduction

Here we present a theoretical explanation of the experimentally observed phenomenon of progressive squashing and disappearance of protein aggregates pressed between the surfaces of thin liquid films. We have previously studied aqueous films, sandwiched between styrene phases, in the presence of bovine serum albumin (BSA) [1]. Reversible surface aggregation of the protein was discovered: Films made in aged systems, more than about 30 min after loading of the two phases in the cell, comprised large protein clusters. No aggregation in the bulk of the solution was detected. Therefore, we could conclude that the slow process of lump formation was promoted by the conformational changes and partial denaturation of the protein molecules on the interface [1].

The aggregation is seen to be reversible. The excess material, entrapped in Newton black films of emulsion type, is gradually squeezing out, and the particles are destroyed. Finally, the whole film becomes uniformly thin and black, as the lumps disappear. This process takes a relatively short period of time – usually about 2–3 min. A question can be raised about the kind of physical mechanism which is responsible for removing the protein from the clusters. Dissolution of molecules into the bulk aqueous phase that fills up the film interior should be ruled out: the Newton black films virtually do not thin, and there is

very little liquid (if any) between the two opposing interfacial layers of protein. Moreover, the process of protein redispersion to the bulk is likely to be very slow. The physical state of the molecules in the lump (partially denatured and entangled with their neighbours) is quite different from that in the water solution.

In the present work we shall investigate the possibility that the macromolecules migrate by surface diffusion, and in this way they are removed from the big particles. It is well known that proteins, similarly to lipids and low molecular weight surfactants, are involved in surface diffusion on liquid boundaries [2]. The process was studied by the method of fluorescent recovery after photobleaching (FRAP), applied to thin foam films. Systems stabilized by proteins [2–4] and phospholipids [5] were investigated. The surface diffusion coefficient, D_s, was determined to be about 1×10^{-7} cm^2/s for BSA [2]. We shall use this value in our analysis below. In the frames of a simple model, the lateral distribution of the surface concentration of protein will be calculated as a function of position and time, for a lump whose size gradually diminishes. The time dependence of the particle diameter will be computed and the results will be compared with direct microscopic observations in thin emulsion films containing BSA.

Theoretical model

Let us consider the idealised geometry depicted in Fig. 1. The protein lump is represented as a cylinder with radius a and height H (functions of time). For the sake of simplicity, only one film surface, S, is shown. In reality, the particles captured within a thin liquid film are subjected to increased internal pressure, Δp, due to the squeezing action of the curved wrapping menisci. Explicit calculation of Δp will not be attempted here. Instead, we shall focus on the changes in the adsorption.

When there is no film, the aggregates attached to the interface would be resting in equilibrium with the surrounding surface layer of protein. One can write the condition for equality of the chemical potentials:

$$\mu_{\text{lump}}(T, p) = \mu^0_{\text{surf}}(T, p) + kT \ln \Gamma_0 . \tag{1}$$

The expression in the right-hand side of Eq. (1) represents the surface chemical potential in the form used by Gurkov *et al.* [6], where Γ_0 is the equilibrium adsorption. $\mu_{\text{lump}}(T, p)$ refers to the cluster. When the latter is entrapped in the film, *local* equilibrium is maintained between the material inside the particle and the nearest points on the interface, in close contact with the lump (i.e., along the circumference $r = a$ in Fig. 1). The physical state of the protein in the aggregate is very close to that on the surface.

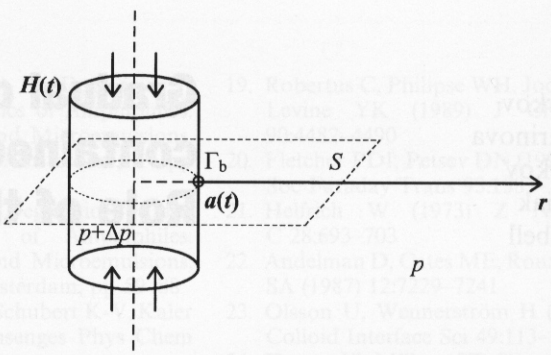

Fig. 1 Simplified geometric model used for solution of the diffusion problem. The cylindrical lump has radius $a(t)$ and height $H(t)$

The counterpart of Eq. (1) for a pressed lump reads

$$\mu_{\text{lump}}(T, p + \Delta p) = \mu^0_{\text{surf}}(T, p) + kT \ln \Gamma_b , \tag{2}$$

at $r = a$. Here we have by definition $\Gamma_b \equiv \Gamma(r = a)$, and this quantity will be supposed to remain constant during the process of squashing of the cluster. Indeed, experimental observations have shown that the particle shrinks isotropically, its shape does not change with time. Hence, Δp will stay more or less the same, at least for not very small sizes.

The mass balance of protein at the interface, for $r > a$ (in cylindrical symmetry, see Fig. 1), is expressed by the following equation [7]:

$$\frac{\partial \Gamma}{\partial t} = D_s \left[\frac{\partial^2 \Gamma}{\partial r^2} + \frac{1}{r} \frac{\partial \Gamma}{\partial r} \right], \tag{3}$$

where Γ is the adsorption (number of molecules per unit area). The respective boundary conditions are

$$\Gamma(r = a(t), t) = \Gamma_b = \text{const.}, \quad \Gamma(r \to \infty, t) = \Gamma_0 = \text{const.}, \tag{4}$$

and the initial condition (at the moment of film formation) is

$$\begin{aligned} &\Gamma(r, t = 0) = \Gamma_0 \quad \text{for } r > a_0 , \\ &\Gamma(a_0, t = 0) = \Gamma_b \quad \text{for } r = a_0 , \end{aligned} \tag{5}$$

where a_0 denotes the initial value of the lump radius, $a_0 \equiv a(t = 0)$.

Equation (3) implies absence of surface convection (zero macroscopic velocity on the fluid boundary). Experimentally, one does not notice any motion within the interfaces, and moreover, the films practically do not thin, so it is legitimate to restrict our considerations to surface diffusion only.

The main mathematical complication in this problem arises from the fact that the first condition (4) is imposed

on a moving circumference, $a(t)$. We introduce new dimensionless radial coordinate

$$y \equiv \frac{a(t)}{r}, \quad 0 \leqslant y \leqslant 1. \tag{6}$$

Then, Eq. (3) acquires the form

$$a^2 \frac{\partial G}{\partial t} + a \frac{\mathrm{d}a}{\mathrm{d}t} y \frac{\partial G}{\partial y} = D_S y^3 \left[y \frac{\partial^2 G}{\partial y^2} + \frac{\partial G}{\partial y} \right], \tag{7}$$

where the dimensionless adsorption is

$$G(y, t) \equiv \frac{\Gamma - \Gamma_0}{\Gamma_b - \Gamma_0}, \quad 0 \leqslant G \leqslant 1. \tag{8}$$

Now, it is convenient to define

$$b(t) \equiv \frac{a(t)}{a_0}, \quad 0 \leqslant b \leqslant 1, \qquad \tau \equiv \frac{D_S}{a_0^2} t, \quad 0 \leqslant \tau < \infty. \tag{9}$$

Transforming Eq. (7), we finally arrive at the following differential equation for the two unknown functions, $G(y, \tau)$ and $b(\tau)$:

$$b^2 \frac{\partial G}{\partial \tau} + b \frac{\mathrm{d}b}{\mathrm{d}\tau} y \frac{\partial G}{\partial y} = y^3 \left[y \frac{\partial^2 G}{\partial y^2} + \frac{\partial G}{\partial y} \right], \tag{10}$$

with the corresponding boundary and initial conditions:

$$G(y = 1, \tau) = 1; \qquad G(y = 0, \tau) = 0; \tag{11}$$

$$\left.\begin{array}{l} G(y, \tau = 0) = 0 \quad \text{for } y < 1 \\ G(1, \tau = 0) = 1 \quad \text{for } y = 1 \end{array}\right\}, \qquad b(\tau = 0) = 1. \tag{12}$$

A second equation, required for solution of this problem, can be derived from the mass balance at the rim of the shrinking lump. There is a surface diffusion flux through the circumference $r = a$: $j_a = -D_S(\partial\Gamma/\partial r)|_{r=a}$. It carries away the material from the particle, whose volume, V, decreases

$$\frac{\mathrm{d}V}{\mathrm{d}t} = -2 \times 2\pi a v_1 j_a = 4\pi a v_1 D_s \left. \frac{\partial \Gamma}{\partial r} \right|_{r=a}. \tag{13}$$

Here v_1 represents the volume of one protein molecule. The multiplier 2 in Eq. (13) accounts for the presence of two film surfaces attached to the cluster, and two rims through which molecules are leaving.

We can find a connection between $\mathrm{d}V/\mathrm{d}t$ and $\mathrm{d}a/\mathrm{d}t$, using the experimentally established fact that the aggregates contract isotropically. Let us introduce a shape factor

$$c \equiv \frac{H(t)}{a(t)} = \text{const}. \tag{14}$$

(Fig. 1). Then, simple geometric considerations yield

$$\frac{\mathrm{d}V}{\mathrm{d}t} = 3\pi a^2 c \frac{\mathrm{d}a}{\mathrm{d}t}. \tag{15}$$

Combining Eqs. (13) and (15), one obtains

$$\frac{\mathrm{d}b}{\mathrm{d}\tau} = -\frac{A}{b^2} \left. \frac{\partial G}{\partial y} \right|_{y=1}, \quad \text{where } A = \frac{4}{3} \frac{v_1}{a_0 c} (\Gamma_b - \Gamma_0). \tag{16}$$

The system of two equations, (10) and (16), together with the conditions (11), (12), represent the complete mathematical formulation of the problem.

Results and discussion

We performed numerical solution of the set of differential equations (10), (16). Standard procedure was used, based on an implicit scheme [8]. The spatial and time intervals were discretised with increments Δy, $\Delta\tau$. The derivatives with respect to y in Eq. (10) were expressed at the time moment $(\tau + \Delta\tau)$, with central differences of accuracy $(\Delta y)^2$. The resulting linear set of equations was solved by Gauss–Jordan elimination with full pivoting [8]. For the time derivatives we used forward finite differences. In this way we computed the distribution of the adsorption throughout the interface at any moment, $G(y, \tau)$, and the lump radius as a function of time, $b(\tau)$. In dimensionless variables only one parameter, A, is needed for the calculation.

Our goal will be to compare the theoretical results with experimentally measured size of diminishing protein aggregates entrapped in emulsion films. For that purpose we carried out microscopic observations in reflected monochromatic light (wavelength 546 nm). The films were formed in a glass capillary immersed into the oil. The experimental method is described in details elsewhere [1, 9]. The interference picture (see Fig. 2) was recorded by videocamera, and the images were processed with the help of Targa + 16/32P grabbing board.

We investigated aqueous solution of 0.015 wt% lyophilised BSA (fatty acid free, purchased from Sigma), in the presence of 0.15 M NaCl, at pH = 6.4. This value of pH is reached when BSA is dissolved in water, without any addition of acid or base. The oil phase was soybean oil, preliminarily purified by passing it through a column packed with chromatographic adsorbent (Florisil, see Ref. [10] for details).

Figure 2 shows a Newton black film which contains big protein lump. The latter gradually diminishes and finally disappears. We measured the lump diameter, $2a$, accounted at the outer bright ring. Initially, just after formation of the film, the protein cluster contains some liquid. At that stage the boundary between the aggregate and the surrounding thin black film is not sharp, but is diffuse, and the diameter cannot be determined with good precision. For this reason, we do not start measuring $a(t)$ from time

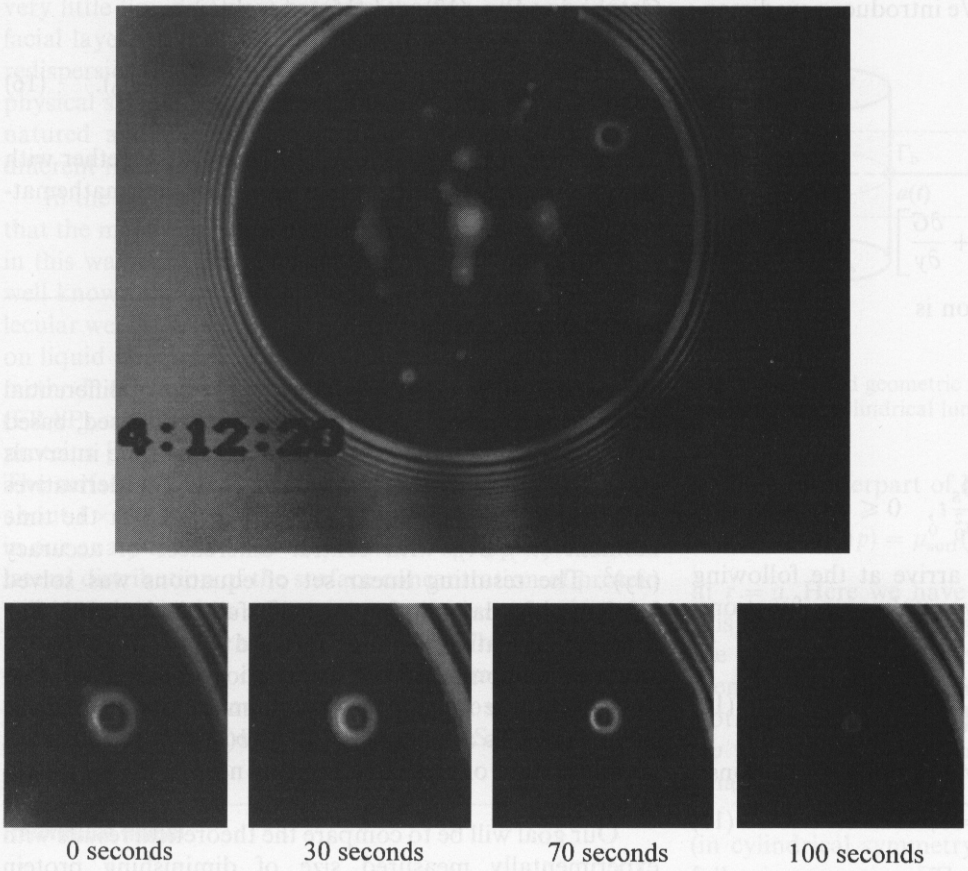

Fig. 2 Interference picture of a Newton black film containing big protein aggregate (above); images of the shrinking lump in the upper part of the film, at four consecutive time moments (below). The observation was carried out in reflected monochromatic light. Black and white zones correspond to thicknesses in multiples of $\lambda/(4n)$ (λ is the wavelength of the light, n is the refractive index of the aqueous phase). The reference distance between the two vertical bars is equal to 50 μm

zero (i.e., from the moment of film formation). Instead, we begin at a later time, t^*, when the solvent has already drained from the lump. (t^* is accounted from $t = 0$; at the moment $t = 0$ the hypothetical initial adsorption distribution, Eq. (5), is assumed to hold.) If the lump had been compact all the time, then at $t = 0$ there would have been $a = a_0$ (but a_0 and t^* are unknown quantities). The experimental running time can be denoted by $(t - t^*)$; the first measured point is $a = a^*$ at $t = t^*$.

The height of the particle, H, is measured at its center, using the conditions for interference and the intensity of the reflected light. The shape factor, c (Eq. (14)), remains almost constant, which means that the protein cluster contracts isotropically.

Figure 3 presents experimental data for the radius, a, as a function of time, for three different lumps. We shall fit these points with our theory. However, as discussed above, the parameter a_0 is unknown and therefore, conversion to dimensionless variables is impossible. In order to overcome this difficulty, we developed the following procedure: A portion of the experimental dependence (Fig. 3) is fitted with a straight line. One writes

$$a = a^* - u(t - t^*), \tag{17}$$

where u is found from the slope. Switching over to the variables b and τ in Eq. (17) yields

$$b = \frac{1}{a_0}(a^* + ut^*) - \frac{ua_0}{D_s}\tau. \tag{18}$$

Next, arbitrary number is assigned to A (Eq. (16)), and the computation is run, providing the whole curve $b(\tau)$. This theoretical curve also has a linear portion, whose slope and intercept are determined. a_0 and t^* are then calculated according to Eq. (18), for the given A (with $D_s = 1 \times 10^{-7}$ cm^2/s, Ref. [2]). Using a_0 and t^* we convert the

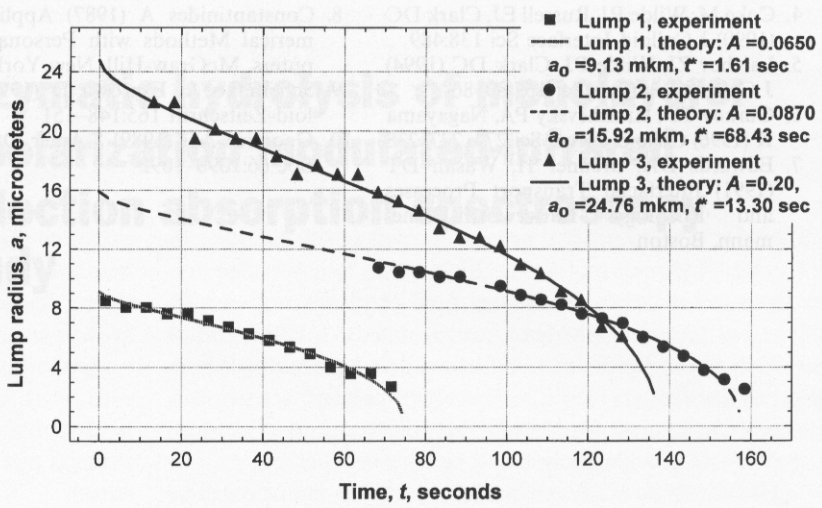

Fig. 3 Experimentally measured (symbols) and theoretically calculated (lines) values of the radii of three different protein aggregates whose size gradually diminishes during the process of squashing

computational results to physical variables and compare with the experimental data. The procedure is repeated iterating for A, until the best fit through the points is finally achieved (Fig. 3). The values of A, a_0 and t^* for the three particular aggregates are indicated on Fig. 3. Very good agreement between theory and experiment is observed. Actually, there is only one free parameter, A, to be varied independently. Although a_0 and t^* are also unknown a priori, they are explicitly calculated for the respective values of A at each step of the fitting procedure.

Thus, we can conclude that our simple model, which accounts for the surface diffusion of protein molecules, describes adequately the process of lump decomposition in thin films. From Eq. (16) it is possible to estimate the excess adsorption, Γ_b. The dimensions of one BSA molecule are $4.16 \times 4.16 \times 14.09$ nm [11], so that the volume is $v_1 = 243.84\,\text{nm}^3$. Let us take as an example the lump #1 from Fig. 3. We have $c = 0.033 \pm 0.004$ and $A = 0.065$. Then, from Eq. (16) one deduces $\Gamma_b - \Gamma_0 = 6.02 \times 10^{12}\,\text{cm}^{-2}$. Measurements of protein adsorption on oil/water interfaces were carried out by other authors [12]. As an order of magnitude, Γ_0 is about $4\,\text{mg/m}^2$ at bulk concentrations close to 0.01 wt% [12]. This value of Γ_0 is equivalent to $3.62 \times 10^{12}\,\text{cm}^{-2}$ (for the molecular weight of BSA we take ~ 66500 g/mol from literature data [13]). Hence, it turns out that $\Gamma_b \approx 2.6\Gamma_0$. In much the same way one obtains $\Gamma_b \approx 5.1\Gamma_0$ for the lump #2, and $\Gamma_b \approx 9.8\Gamma_0$ for #3 (Fig. 3). These results demonstrate that (i) BSA forms multilayers on the liquid boundary (which is a well known fact [12]); (ii) bigger aggregates are more compressed, and consequently, the adsorption along the periphery, Γ_b, acquires higher values — cf. Fig. 3.

The calculation of $\Gamma(r, t)$ reveals that the disturbance $(\Gamma - \Gamma_0)$ becomes more far-reaching (i.e., extends to larger distances), as time goes by. This is in accord with the expectations.

Conclusions

We propose a theoretical model which describes the process of gradual decomposition of big protein aggregates pressed between the two interfaces of a thin liquid film. The molecular migration by the mechanism of surface diffusion is found to be responsible for removing the material from the lumps. The particle radius was measured experimentally as a function of time. The data are fitted with the theoretical computations, and very good agreement is observed. In order to achieve the best fit we vary one parameter, whose value gives the maximum adsorption (Γ_b) along the rim where the lump is attached to the interface. Bigger clusters are more compressed, which leads to higher values of Γ_b. Protein multilayers form on the liquid surface.

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