brief communication

On the measurement of particle number and mobility in nonideal solutions by fluorescence correlation spectroscopy

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ABSTRACT Interparticle interactions are incorporated into the theoretical description of the initial amplitude, $G(0)$, of the normalized fluorescence correlation spectroscopy autocorrelation function. Measurements of particle number, aggregate size, and interaction-dependent diffusion are then analyzed in the context of this generalized theory. It is shown that the neglect of interactions can introduce order-of-magnitude errors into estimates of particle number and aggregate size. It is also shown that measurement of $G(0)$ provides an essentially unique method for testing the validity of theories of interaction-dependent membrane protein diffusion.

INTRODUCTION

Fluorescence correlation spectroscopy (FCS) remains, 15 years after its introduction, a powerful though difficult method of monitoring molecular aggregation and mobility. The power of the FCS technique arises from the unique information it can provide. For example, FCS monitors number fluctuations, and so, unlike fluorescence recovery after photobleaching, it gives a direct measure of particle number and particle aggregation. FCS also employs specific labels, and so, unlike light scattering, it can be used to monitor individual species in complex systems. Finally, data obtained from FCS, and the other techniques mentioned above, can be used to characterize intermolecular interactions, although, to date, FCS has been primarily applied to systems that are modeled as ideal.

Here we show how the theory of FCS can be generalized to describe nonideal systems of interacting particles. FCS-based measurements of particle number and particle aggregation (Petersen, 1986; Petersen et al., 1986; Palmer and Thompson, 1987, 1989*a*; Meyer and Schindler, 1988; Qian and Elson, 1990) and studies of interaction-dependent diffusion (Pink, 1985; Saxton, 1987; Scalettar et al., 1988; Minton, 1989; Abney et al., 1989*a*, *b*) are then discussed within the context of this generalized theory. For clarity, coefficients describing the fluorophores, the beam profile, and detection efficiency are suppressed in this discussion; these may be incorporated into the analysis without affecting the conclusions obtained here.

THEORY

The starting point for FCS is the normalized fluorescence fluctuation autocorrelation function, $G(\tau)$, defined as

$$G(\tau) = \langle \delta F(t + \tau)\delta F(t)\rangle / \langle F(t)\rangle^2, \quad (1)$$

where $\langle\ \rangle$ denotes an ensemble average, F is the fluorescence intensity from an illuminated region of a labeled sample, $\delta F \equiv F - \langle F\rangle$ is the fluctuation in this intensity, and t and τ are times. In previous work on FCS of interacting systems, Phillies (1975) and Andries et al. (1983) showed how the time dependence of $G(\tau)$ can be used to determine mutual- and self-diffusion coefficients (Scalettar et al., 1988) if the solute species are all labeled or mostly unlabeled, respectively. Here we would like briefly to address a complementary problem: the effects of interparticle interactions on the initial amplitude, $G(0)$, of the correlation function.

FCS theories can be generalized as follows. The total fluorescence emitted by a sample is proportional to the number of fluorophores under illumination. Fluctuations in the total fluorescence give rise to the FCS signal and reflect variations in the number of fluorophores in the open, finite observation volume. For a system in which only one species is labeled, Eq. 1 implies

$$G(0) = (\langle N^2\rangle - \langle N\rangle^2)/\langle N\rangle^2, \quad (2)$$

where $\tau = 0$ and $N \equiv N(t)$ is the number of illuminated labeled particles. The initial amplitude of $G(\tau)$ is thus a direct measure of the equilibrium fluctuations in the number of labeled particles under observation.

The averages in Eq. 2 can be computed for arbitrary interactions in the grand canonical ensemble using the grand canonical partition function, Ξ (Hill, 1956; McQuarrie, 1976). For a one-component system comprising only labeled particles, Ξ is given by

$$\Xi(V, T, \mu) = \sum_N Q(N, V, T)e^{-\mu N/k_B T}, \quad (3)$$

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where Q is the canonical partition function, V is the volume of the system, T is the temperature, μ is the chemical potential, and k_B is Boltzmann's constant. Starting from the definition

$$\langle N^2 \rangle = (1/\Xi) \sum_N N^2 Q(N, V, T) e^{-\mu N/k_B T}, \quad (4a)$$

it can be shown (Hill, 1956; McQuarrie, 1976) that

$$\langle N^2 \rangle = \langle N \rangle^2 k_B T \kappa / V + \langle N \rangle^2, \quad (4b)$$

where κ is the isothermal compressibility. Eq. 2 may thus be rewritten as

$$G(0) = k_B T \kappa / V. \quad (5)$$

Eq. 5 is strictly valid only for a one-component system. For a solution consisting of a fluorescently labeled solute and an unlabeled solvent, fluctuations in solute number are related to the isothermal osmotic compressibility rather than the total compressibility (Friedman, 1985). The appropriate generalization of Eq. 5 is then

$$G(0) = k_B T / [\langle N \rangle (\partial \Pi / \partial \rho)_T], \quad (6)$$

where Π is the osmotic pressure, $\rho \equiv \langle N \rangle / V$ is the particle number density, and $\partial \Pi / \partial \rho$ is the isothermal osmotic compressibility.

Finally, note that although we have presented a derivation based on statistical mechanics, similar results can be obtained from purely thermodynamic considerations (Magde, 1977).

RESULTS AND DISCUSSION

Effects of nonidealities on $G(0)$

Eq. 6 is completely general and applies to both ideal and nonideal solutions. For an ideal (i.e., noninteracting) system, the osmotic pressure is given by the van't Hoff equation, $\Pi = \rho k_B T$. Eq. 6 then yields the familiar relationship

$$G(0) = 1/\langle N \rangle, \quad (7)$$

which is more commonly derived directly from Eq. 2 by assuming that the number of particles in the observation volume obeys Poisson statistics.

For a nonideal system, the formalism is more complex. The osmotic pressure is given by the pressure equation, which in two dimensions states

$$\Pi = \rho k_B T + (\rho^2/4) \int_0^\infty r f(r) g(r)\, 2\pi r dr, \quad (8)$$

where r is the separation between solute particles, $f(r)$ is the effective solute–solute force, and $g(r)$ is the radial distribution function, which characterizes solute order in the fluid (Braun et al., 1987). This expression shows that $G(0)$ will in general depend on temperature, particle density, and the effective interaction between solute particles. We emphasize the word effective because the solute–solute force appearing in Eq. 8 includes both direct interactions between solute molecules and indirect interactions mediated (in a MacMillan-Mayer sense) by the solvent (Braun et al., 1987). Solvent-mediated interactions are predicted theoretically to occur in membrane systems (Abney and Owicki, 1985) but have never been directly measured in a membrane. In the remainder of this paper, we focus our attention on the density and interaction dependence of $\partial \Pi / \partial \rho$, although the temperature dependence may also be interesting.

A detailed theoretical analysis of two-dimensional osmotic compressibilities was recently given (in the context of the mutual-diffusion coefficient) by Abney et al. (1989*b*). There it was found that a purely repulsive effective interaction causes the osmotic compressibility to increase with density, whereas a mixed interaction consisting of this same repulsive force superimposed on a long-range attraction causes the compressibility to decrease at low densities, but increase at high densities. Intuitively, in repulsive systems particles tend toward a maximal separation to minimize interactions with neighbors, making the system more homogeneous and reducing fluctuations. In attractive systems particles tend to aggregate or clump, leading to greater heterogeneity and enhancing fluctuations (see Fig. 1). These same effects are expected in three-dimensional systems.

Nonidealities are manifest even in the correlation function of a hard-disk fluid; see Fig. 2. This example is

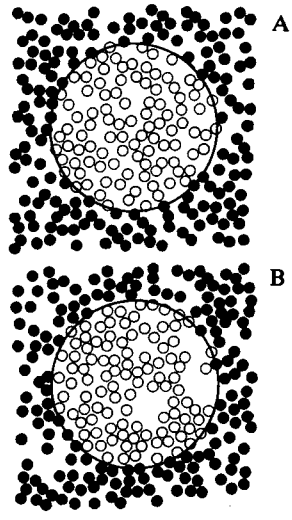

FIGURE 1 Schematic representation of an experimental observation volume and the distribution of mutually repulsive (*A*) and attractive plus repulsive (*B*) membrane proteins within that volume. Particle positions were generated by Monte Carlo simulation using potentials *R* and *A*, respectively, from Abney et al. (1989*a*, *b*). (Simulation conditions correspond to a reduced temperature of $T^* = 1$ and a reduced density of $\rho^* = 0.5$.) The "beam" size has been chosen to correspond to experimental conditions that give an average of 100 illuminated (*unshaded*) particles. Following the logic given in Results and Discussion, the particles in *A* will exhibit weaker fluctuations than ideal particles, whereas the particles in *B* will exhibit stronger fluctuations.

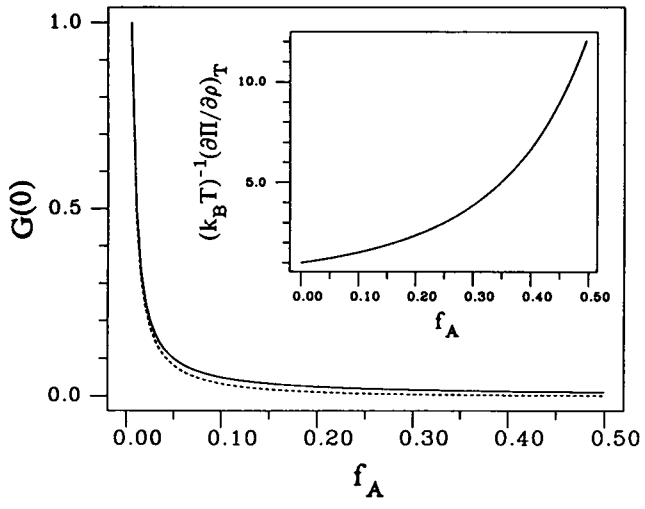

FIGURE 2 The FCS amplitude $G(0)$ vs. area fraction f_A in ideal (*solid line*) and hard-disk (*dashed line*) two-dimensional fluids and their ratio (*solid line in inset*). Here the particle size was set by the (arbitrary) requirement that 100 particles in the observation volume corresponds to $f_A = 0.50$; a different choice merely rescales the axes without changing the functional form of the curves. The ideal curve was calculated from Eq. 7; the hard-disk curve was generated from Eq. 6 using Eqs. 11 and 12 in Abney et al. (1989*b*) to compute $\partial\Pi/\partial\rho$. The inset shows the ratio of the ideal to the hard-disk results. This curve represents the density dependence of the direct contributions to the hard-disk mutual-diffusion coefficient, Eq. 10, and is identical to Fig. 4 in Abney et al. (1989*b*). This ratio is independent of particle size, as indicated in the text.

especially illustrative because the magnitude of the effects does not depend on particle number or particle size, but only on the area fraction occupied by the solute. For this fluid, at very low densities, $G(0)$ is essentially given by the ideal expression, Eq. 7. At higher densities, $G(0)$ is given only by Eq. 6, and the deviation from ideality can be pronounced. For example, when the disks occupy 50% of the system's area, $G(0)$ is <8% of the ideal value.

How important are these effects in real fluids? Osmotic compressibilities have not been measured in membrane systems, but the osmotic pressure has been calculated using Eq. 8, for the mouse liver gap junction (Abney et al., 1987). There it was found that when ~30% of the membrane area is occupied by gap junction proteins, nonideal contributions to the osmotic pressure are ~10 times greater than ideal contributions, suggesting that the compressibility may also deviate significantly from ideality. Osmotic compressibilities in aqueous solution have been measured for bovine serum albumin (Phillies et al., 1976) and phage lambda DNA (Scalettar et al., 1989), among others, and show that nonidealities can have at least a fewfold effect on the compressibility. Finally, the compressibility of spherical silica beads in nonpolar solvents has been measured as a function of volume fraction (Vrij et al., 1983). The data are in excellent agreement with predictions that result when the three-dimensional analogue of Eq. 8 is used to compute $\partial\Pi/\partial\rho$. In particular, at volume fractions of 30–40% the compressibility is enhanced 10–15-fold (Vrij et al., 1983).

Implications for particle counting and aggregation measurements

Much recent interest in FCS has centered on the use of $G(0)$ to measure particle number and the extent of particle aggregation in biological samples. To measure particle number in a single-solute system, $G(0)$ is measured and typically $\langle N \rangle$ is calculated from the ideal relationship $\langle N \rangle = 1/G(0)$. However, if this procedure is followed in a nonideal system, interactions (primarily repulsions) that lead to an increase in $\partial\Pi/\partial\rho$ and thus a decrease in $G(0)$ will lead to an overestimate of the number of particles. Conversely, interactions (primarily attractions at low density) that decrease $\partial\Pi/\partial\rho$ will lead to an underestimate of particle number. For example, an ideal analysis of the fluctuations in the (repulsive) hard-disk system in Fig. 2 would lead us to conclude that there were over 1200 particles in the observation region at 50% area fraction, rather than 100. When particle concentrations are high, neglect of interactions could thus lead to order-of-magnitude errors.

Similarly, interactions between identical particle aggregates can lead to errors in estimated aggregate size. For example, the analysis presented above shows that repulsive interactions between aggregates will cause an overestimate of the number of aggregates. For systems containing a fixed number of monomeric units, an overestimate of aggregate number will lead to an underestimate of aggregate size.

These observations suggest that a straightforward application of the ideal theories will be most appropriate when interactions are minimized. This condition obtains in dilute systems but not in concentrated systems, such as biological membranes or the cytosol. Perhaps more importantly, a nonlinear (i.e., nonideal) relationship between the amplitude of the correlation function and the inverse of concentration in a single-solute system can be taken as evidence for the existence of interactions between the solute particles. The nature and extent of the deviations from linearity can then be used to characterize the interparticle interactions.

These arguments can be extended to systems containing different types of fluorescent particles or different-sized aggregates of the same particle. Ideal analyses of multifluorescent-component systems have been presented in the context of conventional, as well as scanning and high-order, FCS (Petersen, 1986; Palmer and Thompson, 1987; Meyer and Schindler, 1988; Qian and Elson, 1990).

The most complicated analyses involve moments in particle number such as $\langle N_i^m \ldots N_j^n \rangle$, where N_i and N_j are the number of particles of species i and j in the observation volume, respectively, and m and n are integer exponents. Such moments can be evaluated for interacting systems by direct analogy with the calculation preceding Eq. 6, using an appropriate form for the multicomponent partition function. For a multisolute system in which interactions are neglected, the predicted relationship between FCS amplitudes and the inverse of concentration is no longer linear. When interactions are included, the results may involve too many variables to be of direct use experimentally. However, they can illustrate physically important features of the problem and clarify the approximations leading up to the more tractable ideal expressions. More importantly, they form the starting point for numerical analysis of the effects of interactions.

It is not clear to what extent previous FCS measurements of particle number and aggregate size are affected by the approximations inherent in an ideal analysis. However, Vrij et al. (1983) have recently shown that when light scattering is used to monitor number fluctuations in three-dimensional systems consisting of spherical beads, the compressibility affects the number fluctuations in exactly the manner predicted by the light scattering analogue of Eq. 6. Moreover, in a recent review, Palmer and Thompson (1989*b*) stated that concentrations measured using the FCS technique are usually accurate only to within a factor of two. Here we suggest the possibility that nonideal effects, in addition to the factors cited by Palmer and Thompson, contribute to error when FCS is used as a particle counting method.

Implications for diffusion (in membranes)

The magnitude of $G(0)$ can also be related to the mutual-diffusion coefficient. Recent work has suggested that diffusion coefficients in membranes, like those in aqueous solutions, are influenced by direct and hydrodynamic interactions (Abney et al., 1989*a*, *b*). (Diffusion coefficients in membranes may also manifest the effects of solute [protein]-induced changes in solvent [lipid] viscosity [Saxton, 1987; Abney et al., 1989 *a*, *b*].) Published theories of membrane protein diffusion have explicitly considered only the effects that direct interactions have on diffusion coefficients. Unfortunately, an *experimental* method for isolating the contribution that direct interactions make to either diffusion coefficient in membranes has not, to date, been described or implemented, and the theories remain only partially tested.

The ideas discussed here can be used to develop an experimental method for testing theories of mutual diffusion in membranes. The mutual-diffusion coefficient, D^m, is given by the generalized Stokes–Einstein relation (Pusey and Tough, 1985)

$$D^m(\rho) = [1/f^m(\rho)](\partial\Pi/\partial\rho)_T, \quad (9)$$

where f^m is the mutual-friction coefficient. Direct interactions contribute to both the osmotic compressibility and the friction coefficient, whereas hydrodynamic interactions contribute only to f^m. To date, theories of membrane protein diffusion have predicted precisely the interaction dependence that is embodied in the osmotic compressibility, and it therefore follows that these theories can be subject to experimental verification by computing

$$(\partial\Pi/\partial\rho)_T = k_B T/[\langle N \rangle G(0)] \quad (10)$$

using values of $G(0)$ measured at a range of protein densities; see the inset in Fig. 2. $\langle N \rangle$ can be calculated from the laser beam waist and known concentrations (e.g., in a model system), from direct microscopic visualization, or other methods.

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