Fluorescence Resonance Energy Transfer on Fractals

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In 1949 Theodor Foerster measured the quenching of trypaflavine fluorescence by rhodamine B in a methanol solution.[1] Although by today's standards this was not a particularly remarkable experiment, the theory presented in this work served as a guidepost for the extensive theoretical and experimental literature to follow. The fluorescence quenching observed in these types of experiments results from long-range, nonradiative transfer of electronic excitation energy. This transfer occurs when transition dipoles are close enough (<100 Å) to interact strongly and when the energies of the transition are identical (the resonance condition). Foerster derived an expression which predicts the fluorescence decay when dipolar energy transfer occurs from a population of donors to a population of acceptors or traps. The fluorescence intensity, ϕ, as a function of time t is given by

$$\phi(t) = e^{-(t/\tau)}e^{-A(t/\tau)^{D/6}} \tag{1}$$

where τ is the fluorescence lifetime and A is a constant which depends on a number of parameters such as the acceptor concentration and the "characteristic Foerster distance". The parameter D is the spatial dimension of the system and for Foerster's experiment is simply 3. The factor of 6 dividing the dimension results from the dipole–dipole interactions which lead to energy transfer. In a slightly different experiment, Tweet, Bellamy, and Gaines[2] measured excitation energy transfer between chlorophylls in monolayer films. In this case D is equal to 2.

In Foerster's original experiment the steady-state fluorescence was observed, rather than the time course of fluorescence decay. For steady-state measurements, it is common to define an efficiency of energy transfer, E, by

$$E = 1 - F_{DA}/F_D \tag{2}$$

where F_{DA} is the fluorescence intensity of a donor in the presence of acceptors and F_D is the donor's fluorescence intensity in the absence of acceptors. The parameter E varies from 0 (no fluorescence quenching) to 1 (total quenching). It can be related to eq 1 because F_{DA}/F_D is simply the integral over all time of $\phi(t)$. Although steady-state fluorescence has been measured in numerous simple applications, in general the time-resolved data are more convenient for detailed analysis. This is because a wide temporal range can be observed, providing an additional variable for establishing scaling laws. Nevertheless, the concentration dependence of

acceptors can still be used to determine D from steady-state data.

One might naturally assume that D would only take on the integer values of 1, 2, or 3. However, with the advent of fractal geometry it has become clear that D need not be an integer. Benoit Mandelbrot wrote, "Now that fractal dimension is injected into the study of coastlines and other natural phenomena, even if specific reasons come to be challenged, I think we shall never return to the stage when $D = 1, 2, 3$ was accepted thoughtlessly and naively. He who continues to think that $D = 1, 2, 3$ has to argue his case."[3] There has been a growing awareness of the fractal dimensionality of complex media and an increased effort to probe these microdomains. Thus, eq 1 has been elevated from a concise explanation of simple experimental data to a unique probe of the fractal geometry of complex systems. Fluorescence resonance energy transfer (FRET) and excitation transfer (self-transfer) have been used to determine fractal dimensions in such diverse systems as silicas,[4–8] polymer films,[9] lipid vesicles,[10–12] and membrane protein aggregates.[13,14]

An obvious response to the possibility of structures in which D is not an integer is, "What do they look like?" These structures often appear quite natural and are now well documented in a number of "picture books" on fractals.[3,15] In this Account two of the simplest fractal structures are considered, and they are illustrated in Figure 1. The first case is a fractal aggregate (Figure 1A) in which the density, ρ, of the units making up the structure decreases with the radius, r, from the center of the structure according to $\rho(r) = Ar^{D-E}$ where A is a constant, D is the fractal dimension, and E is the Euclidean dimension in which the structure is embedded. Thus, in this case a fractal is a structure whose density distribution is not uniform but rather follows a fractional power law. Diffusion-limited ag-

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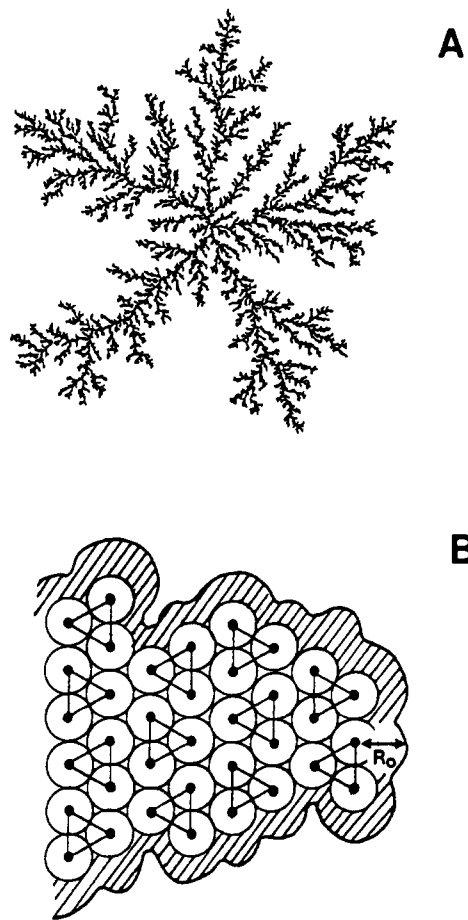
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Figure 1. Examples of fractal structures. Example A is a cluster aggregate with dilation symmetry. A common example is a diffusion-limited aggregate, which has been observed in electrodeposition. Fluorescence resonance energy transfer models consider transport of excitation energy from donors to acceptors adsorbed onto the lattice structure. Example B is the coastline of an aggregate. The aggregate is made up of trimeric units, which result in a more contoured coastline than monomers. FRET is measured from donors in the lipid void volume to acceptors on the protein. The bulk of the transfer occurs from a strip whose width is equal to the Foerster distance, R_0.

gregates are a well-studied example of this type of fractal and have been used to model a wide range of physical structures. For instance, these structures have been observed in studies on electrodeposition and colloid formation. Their occurrence may be even more pervasive, but because the extended, dendritic structures lack mechanical stability, they may be difficult to observe. For such materials the resulting morphology could represent packings of the "broken" arms of these structures.

The second case of interest is the fractal structure formed by the coastline of an aggregate (Figure 1B). The coastline problem is by now classic in fractal geometry. The contoured path of a coastline will fill more space than a line and less space than a plane, and therefore the fractal dimension, D, will be $1 < D < 2$. Highly corrugated coastlines fill more space and have fractal dimensions close to 2. Smooth coastlines have dimensions closer to 1. The geometry of boundary regions often affects the physicochemical properties of the interface.[16] Therefore, it is important to develop

techniques for characterizing these boundaries. The present review discusses how fluorescence resonance energy transfer can be used to determine the fractal characteristics of density distributions and of boundaries. A specific, biophysical application of the boundary determination is discussed.

Fluorescence Resonance Energy Transfer in Fractal Aggregates

Molecular/surface interactions are key to a range of processes such as heterogeneous catalysis, chemisorption, and adsorption.[18] The nature of such interactions is dictated to a large extent by the geometry of the environment. Thus, there has been continued interest in developing techniques to characterize surface geometries and environments. This field has benefited from the use of fractals as a convenient mathematical formalism to describe these complex structures. Nevertheless, the problem of relating chemical and physical properties to surface geometry remains formidable. A limited number of studies have shown correlations between the fractal dimension of the surface and chemical reactivities.[17,18] Such correlations are occasionally problematic because reactivities may correlate with subsets of the entire surface, such as edge effects. To fully utilize the fractal description of surface morphology, new techniques must be developed to address such specific questions of surface geometry.

Fluorescence resonance energy transfer has shown considerable promise as a technique for probing microdomains in complex media. In a typical experiment, fluorescence donors and acceptors are adsorbed onto a surface and either the time-resolved or the steady-state fluorescence is observed. Because the fluorescence lifetimes and steady-state intensities are related to the microscopic surface density of acceptors, FRET provides a probe of the molecularly accessible surface morphology. The time-resolved fluorescence for systems involving FRET between multiple donors and acceptors is nonexponential and is quite often accurately fit by eq 1. Equation 1 can be used to establish "fractal" dimension for virtually any system, whether it is truly fractal or not, merely from fitted parameters. This is not an artifact of the fitting procedure but rather reflects the complexity of excitation energy transport. For example, with self-transport there are typically two temporal regimes. The first occurs at short time or low concentrations and is characterized by time-dependent diffusion constants. At long time or high concentrations the behavior often switches over to a normal diffusional process.[19,20] This crossover can be exploited in situations of donors and acceptors confined to a specific geometry (cylindrical pores, for instance) to provide additional information and model discrimination.[21] In these instances eq 1 will be obeyed in both regimes except that the value of D will change. The analysis of these situations has been reviewed recently,[21] and one can potentially discriminate fractal and nonfractal models in this way. The key point for the experimen-

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talist is that the fractal dimensions and the crossover region can be determined accurately and are insensitive to uncertainties in parameters such as the Foerster distance (contained in the parameter A in eq 1). This avoids much of the criticism which has surrounded biochemical applications of FRET.

The question remains as to the source of the apparent fractal transport at short times for nonfractal systems. There have been a number of experimental and theoretical reports on this effect for donor–donor transfer.[22,23] Transport of excitation energy like transport of particles on a fractal lattice gives the appearance of a time-dependent diffusion "constant". In the familiar Einstein formula, the mean squared displacement of a diffusing particle, $\langle r^2 \rangle$, follows the relation $\langle r^2 \rangle \propto t$, and the diffusion constant will be time independent. For energy transport, $\langle r^2 \rangle \propto t^{(8-D)/6}$ where, depending on the problem, D can be either the fractal dimension or the normal spatial dimension.[24] It is seen then that, even for integer dimensionalities, the Einstein relationship shows a fractional scaling with time. Diffusion on a fractal lattice also does this, but in a somewhat different manner. This time-dependent diffusion in both cases is a result of the unusual diffusion equations associated with each problem. The energy-transport problem uses a complicated, "generalized" diffusion equation[25] while the fractal problem can be approximated using a position-dependent diffusion constant.[26] In these cases the diffusion equations are not equivalent and energy transport cannot be considered the same as fractal diffusion. Thus, the apparent fractal behavior of energy transport in a nonfractal system is really a misnomer and simply results from a complicated diffusion equation rather than from some underlying fractal nature of the system.

FRET can provide a wealth of information on surface morphologies in complex media. It is particularly useful when combined with complementary techniques such as low-angle X-ray scattering. Although the detailed, model-dependent analysis of FRET data may at first appear difficult, there are a number of well-established tests. Results for the scaling behavior and crossover regimes for a variety of specific restricted geometries are available.[21] Restricted geometries can often be easily distinguished from fractal geometries because the fractal behavior is contained in a single scaling regime.[27] Thus, the suitability of the fractional exponent in eq 1 over an entire temporal range and with a variety of acceptor concentrations provides strong support for a fractal model. A good example is the extensive study on porous silica gels.[4–8] Consistent and highly accurate fits are obtained using eq 1 for three different donor–acceptor pairs over a range of acceptor concentrations.[5] Additionally, fractal dimensions from these experiments coincide with those obtained using self-transfer of excitation energy.[6] The fractal dimension determined with these techniques is in agreement with those obtained with adsorption methods and with small-angle X-ray scattering.[7] This example demonstrates the

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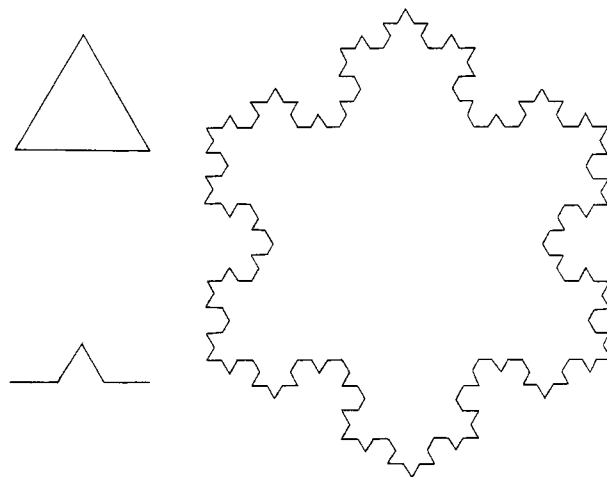

Figure 2. Construction of a triadic Koch island. The triangle (upper left) is the initiator, and the curve underneath (lower left) is the generator. Repeated replacement of line segments in the initiator with the generator results in the island (right). The triadic Koch island is used as a model of aggregate boundaries.

utility of FRET techniques in dealing with difficult problems of surface geometry in materials science and will undoubtedly encourage additional efforts.

Measuring Coastlines Using FRET

Knowledge of the extent and nature of boundary regions is crucial for an understanding of surface phenomena.[16] In the analysis presented here, it is shown how FRET can be used to determine the fractal dimension of such boundaries. We consider energy transfer between separated domains of donors and acceptors (see Figure 1). For simplicity the two-dimensional case will be discussed. The general approach can also be applied to three-dimensional structures. The fractal dimension, D, of the boundary can be established from area–perimeter relationships[28] such as

$$P(\epsilon)^{1/D} = \alpha A(\epsilon)^{1/2} \qquad (3)$$

where P is the perimeter measured with a "yardstick" of length ϵ, A is the area, and α is a numerical constant of proportionality. For the simple case of a circle, $D = 1$ and $\alpha = 2\pi^{1/2}$. However, monomeric units will not pack into a perfect circle but rather the coastline will be slightly more contoured. In a cluster theory of phase transitions, Fisher developed a simple model to account for boundary forces.[29] This model used the scaling laws derived from the Ising model on a two-dimensional trigonal lattice. The value of D expected for this model is 1.06 and reflects the slight contour of the boundary.

It is often possible to model domains by a simple, fractal structure known as a Koch island. These structures have been used in previous models of the fracture of materials.[28] An example of a Koch island is shown in Figure 2. The first curve on the lower left of the figure is the generator. Application of this generator to each line segment of the initiator (the triangle) results in a prefractal. Higher order structures are formed by repeated application of the generator to line segments in the prefractal. A true fractal structure is achieved by an infinite number of generations. Physical structures, of course, are more random in nature. They also have a lower limit cutoff which occurs when the

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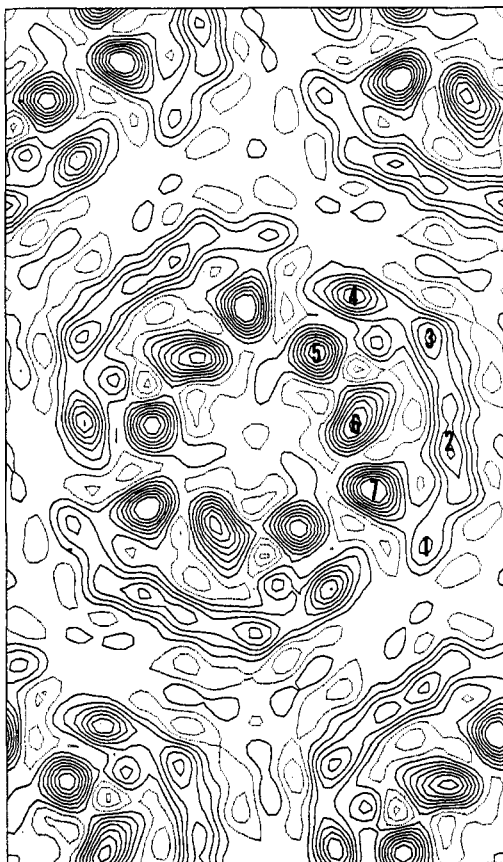198 *Acc. Chem. Res., Vol. 25, No. 4, 1992*

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Figure 3. Electron density map showing the trimeric structure of the membrane protein, bacteriorhodopsin. Electron density is projected in the plane of the membrane and shows the seven α-helical regions in each protein. (Courtesy of Dr. Robert M. Stroud.)

Table I
Perimeter–Area Fractal Dimensions

system	dimension
2-D Ising model	1.07
triadic Koch island	1.26
quadratic Koch island	1.5
hexadic Koch island	1.67
bacteriorhodopsin aggregates (exptl)	1.3 ± 0.1

distance scale is comparable to the diameter of the unit structure. Thus, any physical structure must by necessity be a prefractal. In practice, fractals can be good structural models over a limited range of distances. The advantage of the Koch island model is that mathematical relationships between the perimeter and the area can be readily derived for the prefractals[28] and, therefore, are representative of finite structures. Although the fractal dimension will be dependent on the generation number of the prefractal, this dimension rapidly approaches that for the infinite structure. In practice it is usually reasonable to use the fractal dimension in eq 3 derived for the infinite case. Fractal dimensions for a number of different structures are listed in Table I. As can be seen, these dimensions are very sensitive to the geometry of the generator.

In a simple analysis one assumes that all the quenching is due to boundary acceptors and that, on the average, each acceptor quenches identically. Given these assumptions, the efficiency of energy transfer, E, is given by
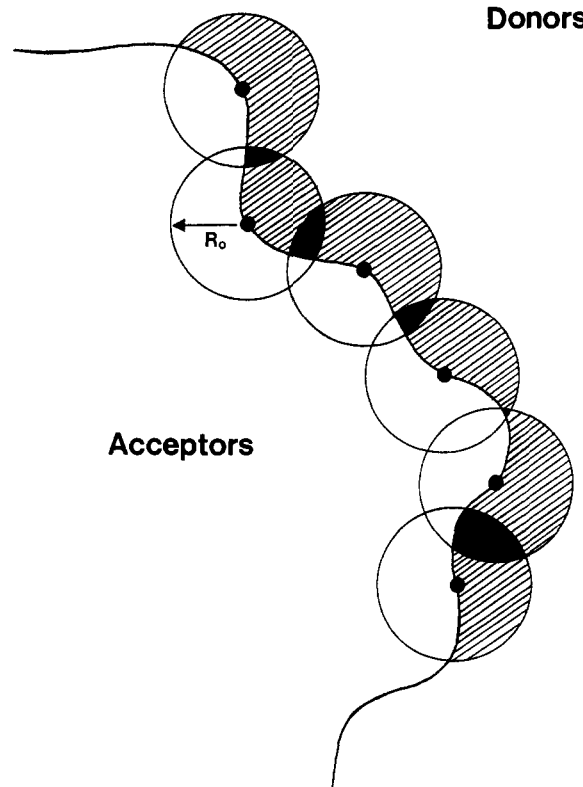
$$E = \bar{E}N_{A,B} \qquad (4)$$

Figure 4. Model of fluorescence resonance energy transfer from the void volume of donors to acceptors on the boundary of an aggregate or separate domain. Most of the energy transfer occurs in a region of radius R_0 from the donor (hatched areas). Overlapping regions (dark areas) will have different quenching efficiencies. A formal analogy exists between this situation and the excluded-volume problem of polymer physics.

where $N_{A,B}$ is the number of acceptors in the boundary layer of the domain and $\bar{E}$ is the average efficiency per acceptor. Typically, the experimentally controlled parameter is the surface density of acceptors, σ, which is directly proportional to N_A, the total number of acceptors in the domain. It is well established that $N_{A,B}$ is proportional to N_A,[30] so the slope of a log E versus log σ plot should be unity.

It is readily seen that the above analysis is an oversimplification. In general, each acceptor will not quench in an identical fashion. For simplicity, we can consider that each acceptor quenches, to some extent, all donors within a radius of R_0. Quenching by acceptors is not additive so that when an acceptor sphere of radius R_0 intersects a second acceptor sphere, the donors in the intersected region will be quenched differently. Thus, acceptors close together will quench differently than those far apart. This effect is illustrated in Figure 4, where the darkened regions will have different quenching efficiencies than the other regions. Figure 4 may also be viewed as an analogue to the well-known excluded volume problem of polymer physics.[31] In this case, the coastline becomes the polymer and the spheres of quenching become the van der Waals radii of the units in the polymer. The problem of calculating the efficiency of energy transfer from the boundary then has a formal analogy with that of calculating the root mean square distance, R, for a polymer. This problem

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represents one of the successes of renormalization group theory. In these approaches the details of the molecular interactions (or in this case energy transfer) are not so important as establishing the universality of the exponents. This is why we can use the simplistic view of energy-transfer quenching presented in Figure 4.

For the polymer problem the root mean square distance is a function of the number of units, N, and the reduced parameter, u:[31]

$$R = af(N,u) \qquad (5)$$

where a is the diameter of the unit and u is v/a^d with v being the excluded volume characterizing the interactions between units and d being the dimension of space. The renormalization result gives

$$R \propto N^\nu \qquad (6)$$

with ν being the critical exponent. In the energy-transfer problem a similar relationship is obtained with N being replaced by $N_{B,A}$. In this case the efficiency of energy transfer, E, will be proportional to the area of a strip of width R_0 surrounding the boundary. If the variable a is now taken to be the length associated with each acceptor and u is a "reduced" efficiency of energy transfer, then E is a function of the number of boundary acceptors, $N_{A,B}$, and u:

$$E = R_0 af(N_{A,B}, u) \qquad (7)$$

Renormalization as in the polymer problem then gives

$$E \propto (N_{A,B})^\nu \propto (N_A)^\nu \qquad (8)$$

where ν is the critical exponent for the problem. The goal is then to relate this critical exponent to the fractal dimension of the coastline. The value for ν in the two-dimensional polymer problem is $3/4$. However, this is not the appropriate value for the energy-transfer problem. In the latter problem the contour of the boundary is fixed by the forces within the aggregate and is independent of the interactions between donors and acceptors. Thus, the value of the variable, a, has a different physical meaning. It represents the step size required to renormalize the energy-transfer problem so that "units" of this size do not have overlapping zones of transfer efficiency. We assert that the proper exponent for the scaling law in eq 8 is the one associated with the root mean square distance of the Koch curve, R_{KC}. This is readily shown to be given by $R_{KC} \propto N^{1/D}$. The Koch curve will have the same fractal dimension associated with its length as appears in the perimeter–area relationship. Thus, the final scaling relationship is

$$E \propto N^\nu \propto N^{1/D} \qquad (9)$$

Equation 9 can be arrived at independently using nonrenormalization methods. This result indicates that the slope of the log E versus log σ plot is equal to $1/D$. Thus, it establishes the connection between the experimental data and the fractal dimension of the theoretical model.

A Biophysical Example

An important and difficult problem in membrane biochemistry is the determination of the subunit structure of membrane-bound proteins. Multimeric proteins typically have a range of cooperative and regulatory phenomena not accessible to monomeric proteins. It has been notoriously difficult to determine the functional unit of membrane-bound proteins. This is illustrated by the problem plaguing electron microscopic studies. Typically electron micrographs of biomembranes show phase-separated structures in which the protein is segregated in large spatial domains. When examining such images one must distinguish between protein–protein contacts resulting from specific, stable structures and those contacts which are a result of packing in the phase domain. Typically, the nearest neighbor distance can be comparable in both cases. Unfortunately phase-separated domains are experimentally difficult to disperse, and this hampers efforts to visualize the underlying subunit structure.

When the protein aggregates consist of multisubunit complexes, a more contoured boundary is formed. This is because the boundary cannot "cut through" the multimeric units and consequently the coastline is more extended. This effect is illustrated in Figure 1 for trimeric units. The forces holding the trimer together are stronger than the surface forces, and the aggregate cannot rearrange to minimize its surface energy. The resulting jagged coastlines are similar to those observed in the fracture of materials. As mentioned previously, this latter case has been successfully modeled using Koch islands.[28] It is anticipated that monomeric proteins packed in a domain will have a fractal boundary dimension of 1.07 (see Table I). Proteins with an underlying subunit structure and, therefore, specific packing geometry will have dimensions greater than 1.07. Thus, FRET of the boundary of protein aggregates provides a diagnostic test for multimeric units.

As an initial investigation to assess the utility of this approach, FRET experiments were performed on bacteriorhodopsin (bR).[13,32] This membrane-bound protein has been extensively studied and, for a number of experimental reasons, is particularly convenient. Electron diffraction studies have shown that this protein in the native membrane forms extensive semicrystalline arrays consisting of trimers which are hexagonally packed.[33] The trimeric structure is shown in the electron density map in Figure 3. Circular dichroism,[34] calorimetric,[35] and rotational anisotropy[35] measurements indicate that aggregates of trimers are formed in reconstituted artificial membrane systems as well. Thus, this established subunit structure provides a good "test case" for the FRET analysis.

In our experiments, retinal, the single, intrinsic chromophore of bacteriorhodopsin, is used as the energy acceptor. The protein is reconstituted into artificial phospholipid vesicles, providing a well-defined system in which the surface density, σ, of protein is readily varied. A fluorescent lipid donor is incorporated into the vesicle membrane. The protein aggregate will then be uniformly labeled with acceptors, and the lipid donors will occupy the membrane surrounding the aggregate (the void volume).[13,32] Typically, the width of a protein will be roughly the same as the characteristic Foerster distance, R_0. Because the efficiency of quenching falls off sharply at distances greater than R_0, the quenching of the lipid donor will be caused pre-

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dominantly by the acceptors in the boundary layer of the aggregate. This provides the basis for the measurement of the coastline of the aggregate.

The efficiency of energy transfer (E) was determined as a function of protein surface density (σ). Since σ is proportional to N_A, a plot of log E versus log σ has a slope of ν (see eq 9). The fractal dimension determined from our experimental results on bR[13,32] are shown in Table I along with the fractal dimensions of different theoretical models. As can be seen, the experimental dimension of 1.25 agrees within error with the value for the triadic Koch island (1.26). This agreement is anticipated for a membrane aggregate that forms stable units. Because the trimeric structure has been determined independently, these initial results are especially encouraging. We would clearly like to establish the generality of the model using other protein systems. As in the case for the silica gels, the biophysical FRET studies will be most useful when applied in conjunction with other structural techniques. We are currently developing methods for processing electron micrographs to determine fractal dimensions. Ideally these methods can be used hand-in-hand to establish the fractal dimensions of other systems with known subunit structures. A particularly convenient system for this might be dimers created by using an antibody to cross-link membrane proteins at specific sites.

This use of FRET offers distinct advantages over previous applications. The goal is to establish a scaling law and not to calculate specific distances. Thus, we require very little knowledge of the Foerster distance, R_0. All that is necessary is that this distance be shorter than the width of the boundary layer. This is an important consideration as uncertainties in R_0 have hampered conventional applications of FRET in biological systems.

Summary and Perspectives

Historically, the bulk of the applications of fluorescence resonance energy transfer have been on biological systems. This technique has found its greatest utility in situations that are not amenable to traditional structural approaches. Examples include multienzyme complexes[36] and biological membranes.[37] Despite these successes, the technique has been criticized for its low spatial resolution. This is a result of uncertainties in the Foerster distance and is due in part to the inability to determine the orientation of donor and acceptor dipoles.

In the present work, a new generation of energy-transfer techniques have been discussed which does not have some of the pitfalls of earlier methods. These new methods, which have found application to materials science, polymers, and biomembranes, focus on the scaling behavior of energy transfer. Exponents for scaling of energy transfer with respect to time and/or acceptor concentration lead to unique interpretations of the structure of microdomains in complex media. The scaling results are insensitive to the uncertainties in the Foerster distance which have plagued previous applications. The ability to experimentally determine scaling exponents and crossover points for a range of temporal/concentration conditions provides a rich phenomenology which enables the discrimination of an array of models. With the increased emphasis on the scaling behavior, new theoretical models will be developed, and these will doubtless incorporate ideas from the theory of polymers and critical phenomena. These new models should in turn stimulate further new experimental applications over a broad range of disciplines.

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