brief communication

Determination of the fractal dimension of membrane protein aggregates using fluorescence energy transfer

T. G. Dewey and M. M. Datta
Department of Chemistry, University of Denver, Denver, Colorado 80208

ABSTRACT It is demonstrated that fluorescence resonance energy transfer may be used to determine the fractal dimension of aggregates of membrane-bound proteins. Theoretical and experimental results are presented for two different experimental designs: energy transfer between proteins and energy transfer from lipids to proteins. For energy transfer between proteins the lattice spacing must be known independently for a fractal dimension to be uniquely determined, and this represents a disadvantage to this experimental design. Results are presented for the calcium ATPase and a fractal dimension of 1.9 is estimated for ATPase aggregates by assuming a lattice spacing of 50 Å. Energy transfer from lipids to protein provides a means of estimating the length of the "coastline" of the aggregate. In this case the fractal dimension is uniquely determined from a log-log plot. An analysis of data for bacteriorhodopsin reconstituted in phospholipid vesicles gives a fractal dimension of 1.6. The structural basis of the value for the fractal dimension is discussed for these two systems. These techniques provide a means of assessing the nature of protein-protein interactions in membranous systems.

I. INTRODUCTION

Fractional geometry has found application to a diversity of natural phenomena (1). Colloid and polymer science has particularly benefited from fractal analysis (2, 3). In this field new insights into the structure and growth of colloidal aggregates have been gained. In particular it has been possible to distinguish between irreversible, diffusion-limited aggregates and chemical equilibrium aggregates. In principle many of the analyses and concepts developed for colloids may carry over to investigations of the physical chemistry of biological macromolecules. However, to date there have been limited applications (4, 5). Of particular interest is the aggregation of proteins in a membrane. In many cases this aggregation has a functional significance. Surprisingly little is known concerning protein-protein interactions in biomembranes. A key problem is distinguishing specific stable oligomeric structures from phase-separated structures. Determination and interpretation of the fractal dimension of protein aggregates could provide a better understanding of these different types of protein-protein interactions. This will in turn allow a better assessment of their functional role.

Unfortunately there is a limited number of general techniques for determining the fractal dimension of membrane aggregates. Electron micrographs may be analyzed with simple geometric methods, i.e., measuring the length of the aggregate "coast-line" by "walking a divider" or measuring number densities within concentric circles (1). The major concerns with these techniques are the effect of fixation on the structure of the aggregates and the large number of micrographs needed to generate good statistics. A second method involving low-angle scattering (6) avoids these problems. The scattering intensity from a fractal structure is proportional to the wave vector to a fractional power. The fractal dimension, d, may then be determined from a log-log plot. For a good determination of d, experimental data spanning several orders of magnitude of the wave vector is needed. For aggregates consisting of proteins in biomembranes, this would require data from both low angle x-ray and light scattering experiments. These scattering intensities will contain contributions from other sources (e.g., multiple scattering from vesicles) and analysis may not be straightforward.

Here, the possibility of using fluorescence energy transfer to measure the fractal dimension of membrane protein aggregates is explored. Two experimental designs are considered and these are illustrated in Fig. 1. In the first the fluorescence donors and acceptors are located on separate proteins in the aggregate. In this case it is assumed that the donors and acceptors are uniformly distributed on the fractal lattice formed by the aggregated protein. The second design has the donors (or acceptors) in the surrounding lipid domain and the acceptors (or donors) are located on the aggregate. Under appropriate experimental conditions, the layer of protein at the edge of the aggregate will be responsible for most of the energy transfer, this method is another means of measuring the coastline of the aggregate.

Please send correspondence to T. G. Dewey.

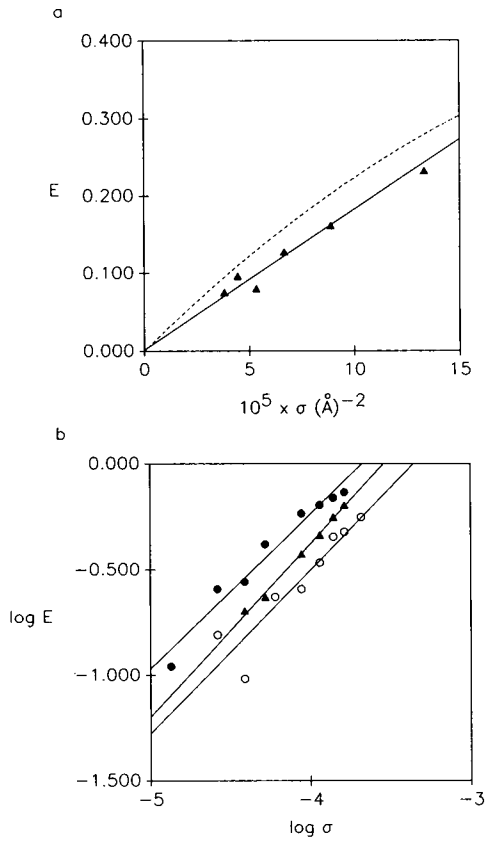

FIGURE 1 Experimental designs for using fluorescence energy transfer to measure the fractal dimension of membrane-protein aggregates. (*a*) Separate protein units are measured with donors and acceptors. Acceptors are uniformly distributed on a fractal lattice. (*b*) Lipid donors transfer energy to protein acceptors. When protein diameter is greater than the Foerster distance, quenching is dominated by the boundary protein. Energy transfer then measures the coastline of the aggregate.

In the next section a theoretical analysis of these two types of energy transfer experiments is presented. This analysis is then applied to experimental data for aggregates of bacteriorhodopsin and aggregates of the sarcoplasmic reticulum calcium ATPase. The final section discusses the advantages and disadvantages of this technique. The interpretation of the numerical values determined for the fractal dimension is considered as well.

II. THEORETICAL ANALYSIS

Energy transfer within an aggregate

General expressions for energy transfer from a donor to acceptors randomly distributed on a fractal structure have been derived previously (7). These expressions yield the following familiar form for the efficiency of energy transfer E:

$$E = 1 - \frac{1}{\tau_0}\int_0^\infty e^{-t/\tau_0} e^{-\sigma\pi[1-e^{-t/\tau_0(R_0/R)^6}]2\pi R^{d-1}dR}\,dt, \qquad (1)$$

where σ is the surface density of acceptors, R_0 is the characteristic Foerster distance, τ_0 is the fluorescence lifetime of the donor, and R is the distance between donor and acceptor. The fractal dimension, d, and the distance of closest approach or lattice spacing, L, may be treated as parameters to be determined from a curve-fitting procedure. In previous applications in membranes, $d = 2$, and L is the only parameter that is determined (8, 9). Eq. 1 does not provide a convenient form for data analysis. Consequently, an approximate expression is derived which provides an explicit analytic form. In applications to membrane proteins, the lattice spacing, L, will be approximately equal to the protein diameter. In most cases this distance will be comparable with R_0. The advantage of this situation is that accurate, simple approximations to Eq. 1 may be derived (10). The disadvantage is that a limited range of transfer efficiencies can be achieved experimentally. In previous work a general method was developed for deriving continued fraction approximants to expressions of the form of Eq. 1 (10). The first approximant is:

$$E/(1 - E) = \sigma\int_L^\infty (R_0/R)^6\, 2\pi R^{d-1}\,dR \qquad (2)$$

$$= \frac{2\pi\sigma R_0^6}{(6 - d)L^{6-d}}, \qquad (3)$$

where σ is now the surface density of acceptor-labeled protein in the protein lattice. Eq. 3 will be highly accurate when $L \gg R_0$ or when $\sigma R_0^2 \ll 1$. Eq. 3 may also be directly obtained from Eq. 1 when $(R_0/R)^6 t/\tau \ll 1$. In many instances d and L will not be known. The functional form of Eq. 2 prohibits the separate determination of these two parameters. This will severely restrict the utility of this experimental approach. However, if L can be estimated independently using methods for determining the diameter of the protein, then fluorescence energy transfer may be used to determine the fractal dimension.

Energy transfer from the lipid domain to the aggregate

For multiple donors and multiple acceptors, the ratio of quantum yields of donor in the presence, Q_{DA}, and absence, Q_D, of acceptor is given by:

$$\frac{Q_{DA}}{Q_D} = \frac{1}{N_D}\sum_j \left(1 + \sum_i (R_0/R_{ij})^6\right)^{-1}, \qquad (4)$$

where N_D is the number of donors and R_{ij} is the distance

between the jth donor and the ith acceptor. The summation j runs over all donors and i runs over all acceptors (note that $E = 1 - Q_{DA}/Q_D$).

The case is now considered where the donors are located in the lipid domain and the acceptor is uniformly distributed on a fractal lattice. Previously, approximate expressions were derived for energy transfer from the lipid domain to a circular disk (11). An extension of these general approximations to the fractal problem requires the evaluation of extremely complicated integrals. Instead of considering the more general case, we restrict ourselves to the case where R_0 is less than the diameter of the protein. This condition will usually be experimentally accessible. It ensures that the boundary layer of protein in the aggregate is responsible for the bulk of the quenching. Thus, fluorescence energy transfer will be a monitor of the length of the coastline of the aggregate. When R_0 is small relative to the protein width, Eq. 4 may be approximated by:

$$\frac{Q_{DA}}{Q_D} \approx 1 - \frac{1}{N_D}\sum_j \sum_i (R_0/R_{ij})^6 \approx 1 - \frac{1}{N_D}\sum_j \sum_i^{N_{A,B}} (R_0/R_{ij})^6. \quad (5)$$

The second approximation is that the summation of acceptors need consider only the ones on the boundary. In the situation where aggregates are too small to distinguish between boundary and inner layers of the protein, the second equality in Eq. 5 will be exact. The total number of boundary acceptors is $N_{A,B}$. Using the mean value theorem

$$\sum_j \sum_i^{N_{A,B}} \frac{R_0}{R_{ij}}^6 = N_D N_{A,B} \left\langle \left(\frac{R_0}{R_{ij}}\right)^6 \right\rangle, \quad (6)$$

where the braces represent the mean value of the enclosed quantity. Thus

$$Q_{DA}/Q_D = 1 - N_{A,B}\langle (R_0/R)^6 \rangle. \quad (7)$$

Unfortunately $N_{A,B}$ is not an experimentally controlled parameter. Rather it must be related to N_A, the total number of acceptor molecules. This may be done using basic relationships of fractal geometry (1). First,

$$S^{1/2} \propto P^{1/d}, \quad (8)$$

where P is the length of the perimeter of the fractal lattice and d is the fractal dimension that must be >1. It is assumed that the surface area, S, will be directly proportional to N_A and σ. Similarly,

$$N_{A,B} = P/\delta_0, \quad (9)$$

where δ_0 is the width of the protein. Thus,

$$N_{A,B} \propto N_A^{d/2}. \quad (10)$$

Using Eq. 10 and 7 one may obtain the relationship:

$$\frac{d \log E}{d \log \sigma} = d/2. \quad (11)$$

This allows the fractal dimension to be determined directly using the slope of a log-log plot.

III. ANALYSIS OF EXPERIMENTAL DATA

In the first set of experiments, fluorescence energy transfer was measured from IAF[1] (iodoacetomido-fluorescein)-labeled ATPase (acceptor) to IAEDANS (N-iodoacetyl-N'-(5-sulfo-1-naphthyl)-labeled ATPase (donor). This donor-acceptor pair was used previously by Vanderkooi et al. (12), and their deoxycholate-solubilization procedure was followed for the preparation of mixed aggregates. Here, purified Ca^{++}ATPase was used (13) instead of sarcoplasmic reticulum vesicles. This preparation is still in vesicular form as calcium transport activity is retained (14). Fluorescence energy transfer was measured using steady state methods as described previously (14). The characteristic Foerster distance, R_0, was determined to be 52 Å. Total protein concentration was varied at fixed levels of donor and acceptor-labeled ATPase by introducing unlabeled protein into the solubilization mixture. For the application of Eq. 2, the surface density of acceptor in the protein aggregate is calculated using Eq. 12:

$$\sigma = N_A/S = N_A/(N_{TOT} \times 3{,}750\ \text{Å}^2) = C_A/(C_{TOT} \times 3{,}750\ \text{Å}^2), \quad (12)$$

where N_A and C_A are the number and concentration of acceptor labeled ATPases and N_{TOT} and C_{TOT} are the corresponding values for total protein. The surface area per protein was taken to be 3,750 Å^2 as determined by dividing the area of the unit cell for crystalline ATPase (15) by the number of proteins in it. Fig. 2 *a* shows the efficiency of energy transfer versus surface density of acceptor. This data could be analyzed using Eq. 2. However, L and d could not be determined independently. Because IAEDAN labeling is not specific to a single site (12), a value for L can only be estimated. From electron microscopy the diameter of the ATPase is estimated to be 40 Å (16, 17), and this gives a rough estimate of L. Assuming L is 40 Å the data in Fig. 2 *a* is best fit (*solid line*) with a fractal dimension, d, of 1.7. If L is taken as 50 Å then d must be 1.9. Thus, d is fairly sensitive to the value assumed for L. For comparison the theoretical values (calculated using Eq. 17 of reference 31) for a uniform distribution are also plotted (*dotted line*) for L equal to 50 Å. This distance is approximately the longest realistic distance of closest approach possible for labels on

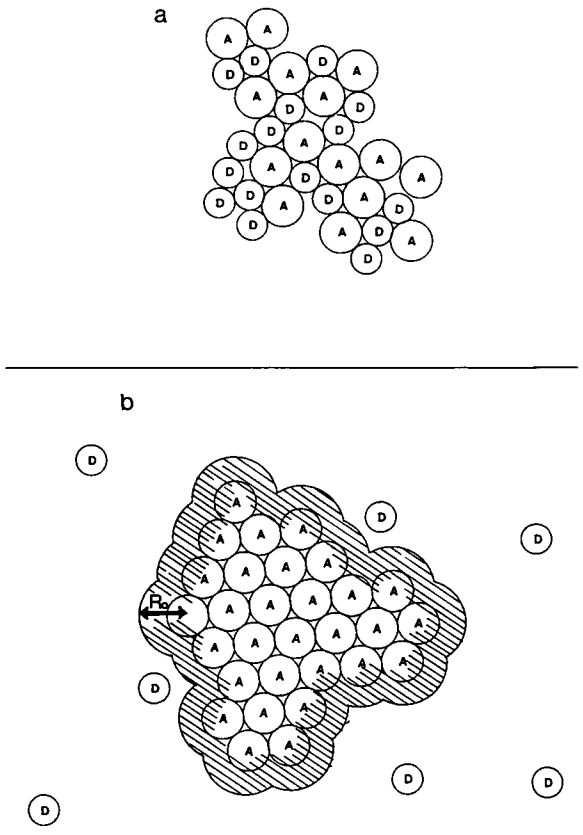

FIGURE 2 Experimental data used to determine the fractal dimension on membrane aggregates. (*a*) The efficiency of energy transfer, E, versus surface density of acceptor-labeled calcium ATPase, σ. Energy transfer was measured from separately labeled ATPase. IAF (iodoacetamidofluorescein) labeled ATPase is the acceptor and IAEDANS (*N*-iodoacetyl-*N'*-(5-sulfo-1-naphthyl) ethylene naphthalene) is the donor. Surface density was varied by varying the amount of unlabeled protein. Solid line is fit to fractal model. Dotted line is calculated for uniformly distributed donors and acceptors, using the model of Wolber and Hudson (reference 31) and assuming closest approach of two proteins is 50 Å. (*b*) A log-log plot of the efficiency of energy transfer versus surface density acceptor. Energy transfer is from lipid donors to the retinal of bacteriorhodopsin. Data are from reference 11 and all results are for dimyristolylphosphotidylcholine vesicles. Fractal dimension, d, of bacteriorhodopsin aggregates is determined from the reciprocal of the slope. Symbols and results are as follows: Δ, donor is octadecylrhodamine *B* chloride (OR) at 10°C, $d = 1.5$; O, donor is OR at 20°C, $d = 1.5$; O, donor is 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate at 10°C, $d = 1.6$.

two separate proteins. As can be seen, the fit is very poor. Shorter distances of closest approach give even poorer fits. This strongly suggests that the data cannot be fit to a uniform distribution.

For the second approach, the previously obtained data (11) has been analyzed to determine the fractal dimension of bacteriorhodopsin aggregates when it is reconstituted into phospholipid vesicles. This data consists of separate quenching experiments on two different fluorescence lipid probes at various surface densities of bacteriorhodopsin. The acceptor was bacteriorhodopsin's intrinsic chromophore, retinal. Using a number of physical techniques, bacteriorhodopsin has been shown to aggregate at temperatures below the lipid phase transition (18, 19). These aggregates are structures that are spectroscopically similar to purple membrane, the triagonal lattice of native protein. This data was originally analyzed with the circular disk model (11) and the aggregate radius was estimated. This analysis gave the physically unrealistic result that the aggregate radius was independent of the surface density of bacteriorhodopsin. This was interpreted as indicating that as the surface density increased multiple patches of comparable sizes were formed. Using Eq. 11 the fractal dimension of the aggregates is determined from the reciprocal of the slope of the log-log plot shown in Fig 1 *b*. For DMPC vesicles under three different sets of conditions (see Fig 2 *b*), d was shown to be 1.6 ± 0.1. The data for DPPC vesicles showed significantly less quenching, and larger experimental error. This data could not be analyzed to give a consistent fractal dimension.

IV. DISCUSSION

Theoretical and experimental results have been presented to show that fluorescence energy transfer may be used to determine the fractal dimension of membrane protein aggregates. The advantage of this approach is that it is experimentally accessible to a wide range of systems and can be used under physiological conditions. The disadvantage is that it is difficult to achieve high levels of fluorescence energy transfer. Experimental conditions are restricted to measuring parameters that vary by less than a decade. This makes it virtually impossible to measure multiple fractal dimensions. However, in physiological setting it is unlikely that the surface density of a given component varies widely enough to cause a transformation from one scaling law to another. Of the two experimental approaches (Fig. 1, *a* and *b*), energy transfer from lipid to aggregate is preferable to transfer within an aggregate. With transfer within the aggregate the fractal dimension cannot be independently determined. Instead the lattice spacing, L, must be estimated by other techniques. Depending on the probe location, there is considerable ambiguity in using hydrodynamic data to determine L. This, combined with the sensitivity of d to the specific value of L, makes this approach less attractive.

Knowledge of the value for the fractal dimension of a given aggregate will hopefully shed light on the structural basis of the aggregation process. Table 1 shows fractal

TABLE 1 Fractal dimension of two-dimensional structures

Fractal dimension d	Structure	Reference
1.26	Rivers on triagonal lattices; fracture boundaries	(1, 21, 22)
1.44	Brownian cluster-cluster aggregates	(23)
1.51	Ballistic cluster-cluster aggregates	(24)
1.55	Chemical cluster-cluster aggregates	(25, 26, 27)
1.56	Lattice animals	(28)
1.70	Diffusion-limited aggregates	(29)
1.89	Percolation clusters	(28)
1.95	Ballistic aggregates	(30)

dimension values for a variety of two dimensional structures. The d value of 1.6 for bacteriorhodopsin is comparable with the value of 1.56 for lattice animals and of 1.55 for chemical cluster-cluster aggregates. In native purple membrane, bacteriorhodopsin forms trimers that are hexagonally packed. This structure will uniformly fill a two-dimensional space. The fractal dimension of the coastline, however, differs from 1 and is consistent with favorable and relatively nonspecific protein-protein interaction. These aggregates are probably extended structures resulting from such interactions.

The calcium ATPase provides an interesting contrast. Its membraneous quarternary structure is not as well defined, although a dimer on tetrameric structure seems to be favored by most workers in the field (20). The high fractal dimension, ~1.8, obtained in this case, may not be related to the underlying unit structure. Rather the purified ATPase vesicles may have such high protein-lipid ratios that a totally contacted network is formed. This could give values close to those for percolation clusters. Unfortunately, the error in d due to the uncertainty in L precludes a detailed analysis at this time. Nevertheless, these preliminary results demonstrate the utility of fluorescence energy transfer for determining the fractal dimension of membrane aggregates. Additionally, with the increased sophistication of aggregation models the value of the fractal dimension can give insight into the structural basis for protein-protein interactions in the membrane.

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REFERENCES

1. Mandelbrot, B. B. 1983. The Fractal Geometry of Nature. W. H. Freeman & Co., New York, 109.
2. Sander, L. M. 1986. Fractal growth processes. *Nature (Lond.).* 322:789–793.
3. Pietronero, L., and E. Tosatti. 1986. Fractals in Physics. Elsevier North-Holland Biomedical Press, Amsterdam. 189.
4. Lewis, M., and D. C. Rees. 1985. Fractal surfaces of proteins. *Science (Wash. DC).* 230:1163–1165.
5. Stapleton, H. B., J. P. Allen, L. P. Flynn, D. G. Stinson, and S. R. Kurtz. 1980. Fractal forms of proteins. *Phys. Rev. Lett.* 45:1456–1459.
6. Schaefer, D. W., and J. E. Martin. 1984. Fractal geometry of colloidal aggregates. *Physiol. Rev. Lett.* 52:2371–2374.
7. Klafter, J., and A. Blumen. 1984. Fractal behavior in trapping and reaction. *J. Chem. Phys.* 80:875–877.
8. Shaklai, N., J. Ygueribide, and H. M. Ranney. 1977. Interaction of hemoglobin with red blood cell membranes as shown by a fluorescent chromophore. *Biochemistry.* 16:5585–5592.
9. Fung, B. K., and L. Stryer. 1978. Surface density determination in membranes by fluorescence energy transfer. *Biochemistry.* 17:5241–5248.
10. Dewey, T. G., and G. G. Hammes. 1980. Calculation of fluorescence resonance energy transfer on surfaces. *Biophys. J.* 32:1023–1036.
11. Hasselbacher, C. A., T. L. Street, and T. G. Dewey. 1984. Resonance energy transfer as a monitor of membrane protein domain segregation: application to the aggregation of bacteriorhodopsin reconstituted into phospholipid vesicles. *Biochemistry.* 23:6445–6452.
12. Vanderkooi, J. M., A. Ierokamas, H. Nakamura, and A. Martonosi. 1977. Fluorescence transfer between Ca^{2+} transport ATPase molecules in artificial membranes. *Biochemistry.* 16:1262–1267.
13. Banerjee, R., M. Epstein, M. Kandrack, R. Zimniak, and E. Racker. 1979. New method of preparing Ca^{2+}-ATPase from sarcoplasmic reticulum-extraction with octylglucoside. *Membr. Biochem.* 2:283–296.
14. Fagan, M. H., and T. G. Dewey. 1985. Steady state kinetics of ATP synthesis and hydrolysis coupled calcium transport catalyzed by the reconstituted sarcoplasmic reticulum ATPase. *J. Biol. Chem.* 260:6147–6152.
15. Taylor, K., L. Dux, and A. Martonosi. 1984. Structure of the vanadate-induced crystals of sarcoplasmic reticulum Ca^{2+}-ATPase. *J. Mol. Biol.* 174:193–204.
16. Scales, D., and G. Inesi. 1976. Assembly of ATPase proteins in SR membranes. *Biophys. J.* 16:735–751.
17. Jilka, R. L., A. N. Martonosi, and T. W. Tillack. 1975. Effect of the purified [Mg^{2+} + Ca^{2+}]-activated ATPase of sarcoplasmic reticulum upon the passive Ca^{2+} permeability and ultrastructure of phospholipid vesicles. *J. Biol. Chem.* 250:7511–7524.
18. Heyn, M. P., A. Blume, M. Rehorek, and N. A. Dencher. 1981. Calorimetric and fluorescence depolarization studies on the lipid phase transition of bacteriorhodopsin-dimyristoylphosphatidylcholine vesicles. *Biochemistry.* 20:7109–7115.
19. Heyn, M. P., R. J. Cherry, and N. A. Dencher. 1981. Lipid-protein interactions in bacteriorhodopsin-dimyristoylphosphatidylcholine vesicles. *Biochemistry.* 20:840–847.
20. Moller, J. V., J. P. Andersen, and M. LeMaire. 1982. The Sarco-

plasmic Reticulum Ca^{2+}-ATPase. *Mol. Cell. Biochem.* 42:83–107.

21. Mandelbrot, B. B., D. C. Passoja, and A. J. Paullay. 1984. Fractal character of fracture surfaces of metals. *Nature (Lond.).* 308:721.
22. Lung, C. W. 1986. Fractals in Physics. L. Pietromero, and E. Tosatti, editors. Elsevier North-Holland Biomedical Press, Amsterdam. 189–192.
23. Jullien, R., M. Kolb, and R. Botet. 1984. Aggregation by kinetic clustering of clusters in dimensions d > 2. *J. Physique Lett. (France).* 45:L211–L216.
24. Ball, R., and R. Jullien. 1984. Finite size effects in cluster-cluster aggregation. *J. Physique Lett. (France).* 45:L1031–L1035.
25. Jullien, R., and M. Kolb. 1984. Hierarchical model for chemically limited cluster-cluster aggregation. *J. Physiol. (Lond.).* A17:L639–L643.
26. Kolb, M., and R. Jullien. 1984. Chemically limited versus diffusion limited aggregation. *J. Physique Lett. (France).* 45:L977–L981.
27. Brown, W., and R. Ball. 1985. Computer-simulation of chemically limited aggregation. *J. Physiol. (Lond.).* A18:L517–L521.
28. Stauffer, D. 1979. Scaling theory of percolation clusters. *Physical Report.* 54:1.
29. Witten, T., and L. Sander. 1981. Diffusion-limited aggregation, a kinetic critical phenomenon. *Physiol. Rev. Lett.* 47:1400–1403.
30. Meakin, P. 1983. The Vold-Sutherland and Eden models of cluster formation. *J. Colloid Interface Sci.* 96:415–424.
31. Wolber, P. K., and B. S. Hudson. 1979. An analytic solution to the Foerster energy transfer problem in two dimensions. *Biophys. J.* 28:197–210.