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Review

Kinetics of bimolecular reactions in model bilayers and biological membranes. A critical review

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Abstract

The quantitative study of the probability of molecular encounters giving rise to a reaction in membranes is a challenging discipline. Model systems, model in the sense that they use model bilayers and model reactants, have been widely used for this purpose, but the methodologies employed for the analysis of the results obtained in experiments, and for experimental design, are so disparate that a concerned experimentalist has difficulty in deciding about the value of each approach. This review intends to examine the several approaches that can be found in the literature showing, when feasible, the weakness, strengths and limits of application of each of them. There is not, so far, a full experimental validation of the most promising theories for the analysis of reactions in two dimensions, what leaves open a large field for new research. The major challenge resides in the time range in which the processes take place, but the possibilities of the existing techniques for these studies are far from exhausted. We review also the attempts of several authors to quantitatively analyze the kinetics of reactions in biological membranes. Especially in this field, the recently developed microspectroscopies enclose a still unexplored potential.

Keywords: Reactions in bilayers; Reactions in biological membranes; Reaction kinetics in 2D; Bimolecular reaction in membranes; Diffusion controlled reactions

Contents

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1. Introduction

By the end of the 1960s it was clear that the reduced spatial dimensionality imposed by interfaces existing in cells restricts the diffusion of solutes, and the limited molecular movements impart a specific kinetics to the bimolecular reactions taking place in these media. A few years before the proposal of the continuous fluid mosaic model for biomembrane structure by Singer and Nicolson in 1972 [1], there was already a evidence that some biochemical reactions could take place at the interfaces already known to exist in the cell protoplasm [2]. These structures were believed to be responsible for a rate enhancement of reactions in the cell [3]. With the model of Singer and Nicolson, and the discovery that several biochemically active molecules reside in the cell membrane much attention has been paid to the molecular diffusion in biological and synthetic bilayers, and to the channels and transporters responsible for their selective permeability, but, in comparison, a relatively small effort was made to study the kinetics of the reactions that take place between membrane components. It is also noteworthy that, with few exceptions, whenever reaction kinetics is studied in biological or fluid synthetic bilayers, the primary objective enunciated is to indirectly measure the diffusion coefficient of the reactants, not to study the characteristics of the kinetics itself. Nonetheless, reactions between components of biological membranes are, since long, widely recognized to play dominant roles in cell physiology and metabolism [4]. True that many bimolecular reactions that take place in biological membranes do not necessarily involve reactant diffusion. In the present work we will only address the case of diffusion-influenced reactions.

Nearly all of the presently existing theoretical studies and model experiments that attempt to unravel how a reduction in the spatial dimensionality of the reaction medium, from three to two dimensions, affects biochemical or biological processes explicitly assume that the concentration of one of the reactants far exceeds that of the other. In those works the reagent in excess quenches an activated species in a diffusion-controlled pseudo first-order reaction, and in most cases the activated species is an electronically excited state. While not negating that those studies contribute to a better understanding of membrane reactions, we have to recognize that the treated models can be quite far from reality. One criticism may be raised regarding the number of reactant molecules used, large in comparison with those generally found in cell compartments. The scarcity of reactants in relatively small compartments may, in many cases, lead to a kinetics essentially dominated by stochastic effects. A second criticism arises from the fact that, even if there is no unquestionable proof of the existence of diffusion-controlled biochemical reactions in membranes, the considered model reactions are, in most cases, diffusion-controlled (for a classification of reactions see, for example, the appendix of Axelrod's paper [5]). This last condition is probably not so critical, because the existing experimental data for some sequential biological reactions involving molecules concentrated in the plane of the membrane may indicate rates that approach the diffusion-controlled limit [6,7]. We can also fault the existing studies based on electronically excited species for the short lifetime of the activated form, which is not the case of the reactions that they intend to model. It may be further commented that reports using molecules involved in real biological reactions are scarce, and that biological membranes are quite different from model ones particularly in what concerns the freedom of lateral diffusion [8]. In particular, mainly continuous model bilayers have been so far used in experimental studies of bimolecular reactions. In these bilayers the molecular lateral diffusion is limited by the hydrodynamic drag caused by the viscosity of the lipid matrix in which the reactants are imbedded with an additional small contribution of the external viscosity [9]. In a biological membrane, even if we only consider the Singer and Nicolson simplified model, diffusion takes place in a crowded two-dimensional (2D) space because of the integral proteins that may occupy up to 60% of the membrane area. More recently, there is a conviction that in some biological membranes, namely in the plasma membrane, there are micro-disperse lipid domains due to lipid lateral phase separation [10–12]. In such systems the reactants that are soluble in the continuous phase will have to percolate in two dimensions before collision, but the existence of lateral heterogeneity may bring many more variables into the arena – reagents may have different solubility in the coexisting phases and cross, or not, the barrier between lipid domains resulting in an enhanced or hindered reactivity depending on the relative importance of the roles played by diffusion/percolation [13] and stochastic effects [14].

From what is said above it is clear that much work needs still to be done before convenient theoretical and experimental tools exist to confidently approach the much more difficult scenario of the kinetics of bimolecular reactions in biological systems. As it will be shown, there is still a deficit in which concerns simple diffusion-controlled and diffusion-influenced reactions, and nearly no work exists involving, for example, the effect of Coulombic interactions, reactant recollision or geminate recombination, which are certainly relevant for biological reactions in membranes. More specific characteristics of biological membranes involve percolation and hindered diffusion of reactant molecules, subjects for which the experimental progress is far behind the already existing theoretical advances.

2. Theoretical approaches to the kinetics of reactions in membranes

2.1. Bimolecular reactions in homogeneous bilayers

Several approaches to the theory of diffusion-influenced reactions in a three-dimensional space (3D) have a counterpart in the studies in two dimensions. As indicated previously luminescence quenching, due to its theoretical and experimental simplicity and good mimesis of the biological reactions, has been widely used as a model reaction for the bilayer resident reactants. In this approach a number of works follow the hydrodynamic theory for diffusion-controlled reactions, others, less numerous, are based on less common theories such as the non-equilibrium thermodynamics, fractal kinetics, or mean-field theory. In many cases with practical relevance, analytic expressions describing the physical process are not encountered, and the alternative is the use of Monte Carlo techniques to simulate the molecular random walk leading to the encounter of reagents.

2.1.1. Diffusion-controlled bimolecular reactions in 2D

2.1.1.1. The specificity of diffusion-controlled reactions in 2D. In the more widely studied case of 3D pseudo first-order diffusion-controlled reactions between molecules E (the excited or activated species) and Q (the quencher), there is an initial time regime (time zero is defined as the time of creation of E) during which the originally random distribution of Q, the more abundant reagent, around E is lost due to the higher probability of disappearance of E molecules originally having a Q in its immediate neighborhood. As a consequence, the immediate average vicinity of E is depleted of Q, what can be also interpreted as the formation of a gradient of Q concentration around the average E molecule. This gradient may be considered the driving force for the prosecution of the reaction [15]. However, as a result of the Brownian motion of Q in fluid media, a steady-state is attained within a sub nanosecond time interval after the creation of E and, for most practical cases, the time-dependent rate coefficient, predicted by Smoluchowski [16,17], can be replaced by a steady-state rate constant. Once the spherical geometry is replaced by a circular geometry, the area at a given distance around the molecular sink, E, increases linearly, instead of quadratically, with the distance, and the same happens to the number of Q around E. Under these conditions, the rate at which the reaction takes place is not compensated by the flux of Q towards E, which means that the steady-state is never attained. The first reference to this particularity of diffusion-controlled reactions in reduced dimensionality was made by Emeis and Fehder in 1970 [18]. These authors, in the course of their molecular modeling of diffusion-controlled reactions, concluded: "diffusion in two dimensions does not provide a sufficient supply of inflowing molecules to sustain a steady-state concentration gradient". In a work almost simultaneous to that of Emeis and Fehder, Suna attained a formally identical conclusion for the kinetics of exciton–exciton annihilation in crystals, in the case the exciton migration takes place in crystal planes [19].

2.1.1.2. Hydrodynamic theory. The hydrodynamic approach to diffusion controlled bimolecular reactions makes use of the integration of the Fick's law of diffusion, Eq. (1), as originally proposed by Smoluchowski to model the kinetics of colloid coagulation [16,17],

$$\frac{\partial c(R,t)}{\partial t} = D\nabla^2 c(R,t) \tag{1}$$

where D is the diffusion coefficient of the moving particles relative to the sink, and the concentration of particles, c, is a function of distance, R, and time, t. For the integration, Smoluchowski used the initial condition of uniform concentration, $c(R,0)=c_0$, and the boundary conditions of infinite reservoir, $c(\infty,t)=c_0$, and immediate reaction at R_C, the collisional distance, $c(R_C,t)=0$. Under these constraints the time-dependent rate coefficient is given by Eq. (2) where D_{eff} is the mutual diffusion coefficient (sum of the diffusion coefficients of the particle and trap) and N the Avogadro constant.

$$k_{3diff}(t) = 4\pi N D_{eff} R_C \left(1 + \frac{R_C}{\sqrt{\pi D_{eff} t}}\right) \tag{2}$$

Later on, 1935, Sveshnikoff [20] recognized the value of this formalism to model the kinetics of luminescence quenching in solution. As previously commented, for large t values $k_{3diff}(t)$; $4\pi N D_{eff} R_C$ is practically time independent, and the reaction velocity proportional to the reagent concentrations.

In 1974 Naqvi [21] presented an analytical solution for the time-dependent rate coefficient of diffusion-controlled quenching of excited molecules in 2D, expression (3),

$$k_{2diff}(t) = \frac{8 D_{eff} N}{\pi} \int_0^\infty \exp(-\alpha u^2) \frac{du}{u[J_0^2(u) + Y_0^2(u)]}. \tag{3}$$

In the equation, α is a function of D_{eff} and R_C, $\alpha = D_{eff}/R_C^2$, $J_0(u)$ and $Y_0(u)$ are the zero-order Bessel functions of the first and second kind, and u a dummy integration variable.

Eq. (3) was obtained from the integration of Fick's law of diffusion with the Smoluchowski's initial and boundary conditions, and is the equivalent in two dimensions to the Smoluchowski–Sveshnikoff formalism for 3D fluorescence quenching. In Fig. 1, the time dependence of reaction velocity for 2D, Fig. 1a, is compared with that for 3D, Fig. 1b, in

identical time range and for equivalent physical parameters for diffusion and collisions. To build the lines the rate constant for 2D is calculated with Eq. (3), and that for 3D with Eq. (2). It is noticeable that in 2D there is not a tendency towards a steady state, contrarily to what happens in 3D.

Shortly after the publication of Naqvi, Owen [22] published the same equation for the same physical situation, pure 2D diffusion-controlled quenching. It is worth noting that, probably because the Fig. 1b from Owen's paper is misleading, showing a finite cylinder (trap) surrounded by small spherical diffusing particles, or because in many places the text itself is confusing, many readers have misunderstood the real physical situation modeled in the paper of Owen. To be fair, the author expresses himself very clearly saying that "the net motion of the quencher molecules is planar..." and calculates the concentration of quencher as a true 2D (mol cm^{-2}) concentration, leaving the responsibility of extra interpretations to the readers. An originality of Owen's paper is the calling of attention that, for small free molecules, when the mean diffusion path is small when compared with the bilayer thickness, the use of a pure 2D formalism is questionable and he recommends the use of the usual formalism for 3D, a recommendation that is not without weakness due to the non-isotropy of the diffusion tensors in the bilayer palisade and the vicinity of the possibly reflective interface.

The previous expressions (2) and (3), based on the Smoluchowski's initial and boundary conditions, are only valid for diffusion-controlled reactions. In the case of diffusion influenced reactions, where not all collisions are effective, Collins and Kimball radiation boundary condition, $\beta(\partial c/\partial R)_{R=R_C}=c(R_C,t)$, should be used instead [15]. For relatively efficient reactions, the integrated form of $k_3(t)$ relates with that of $k_{3\mathrm{diff}}(t)$ as follows [15]

$$k_3(t) = k_{3\mathrm{diff}}(t)\left(1-\frac{\beta}{R_C}\right), \tag{4}$$

where β is related to the probability of diffusion without reaction.

The extension of this formalism to diffusion-influenced bimolecular reactions in 2D was also made by Naqvi [21]. The author uses the Collins and Kimball radiation boundary condition in the integration of the diffusion equation, obtaining Eq. (5).

$$k_2(t) = \frac{2\pi D_{\mathrm{eff}} R_C}{\beta} \times \int_0^\infty \frac{\exp(-\alpha u^2)}{\left[\frac{\pi u J_1(u)}{2\sqrt{\frac{D_{\mathrm{eff}}}{\beta}}} + \frac{\pi\sqrt{\frac{D_{\mathrm{eff}}}{\beta}} J_0(u)}{2}\right]^2 + \left[\frac{\pi u Y_1(u)}{2\sqrt{\frac{D_{\mathrm{eff}}}{\beta}}} + \frac{\pi\sqrt{\frac{D_{\mathrm{eff}}}{\beta}} Y_0(u)}{2}\right]^2} \frac{\mathrm{d}u}{u} \tag{5}$$

The theoretical validity and molecular significance of such methodology has been largely discussed for the 3D case and the same criticisms may be raised for two dimensions, for a review see Naqvi [23]. However, while some authors claim that their experimental data can only be explained if not all the encounters are effective [24], we are only aware of one research group that applied Eq. (5) for data analysis [25,26].

The use of Eq. (3) involves one practical difficulty. For small values of u (initial times) the two Bessel functions in the denominator of the integral are nearly symmetrical, leading to unstable values of their sum (this is a problem even with double precision computer calculations). However, the substitution of the Bessel functions by their series definition is a rather straightforward mathematical stratagem that easily solves this computing problem.

Both Naqvi and Owen introduced approximate mathematical expressions to Eq. (3), based on estimations valid for some ranges of the parameters and time. Besides them, several other authors have proposed simplified formulas to use in the analysis of time-resolved and steady-state diffusion-controlled reactions in 2D [27–31]. Some of these simplified formalisms recognize that, within certain time limits, depending on D_{eff} and R_C, the time decay of the excited species concentration, $I_2(t)$, may be approximated by a square root law formally identical to that of 3D but in which A_2, coefficient of t, and B_2, of $\sqrt{t}$, are adapted to the 2D condition as shown in Eq. (6), in which $[Q]_2$ is the 2D molecular concentration [22,27,28].

$$I_2(t) \simeq I_2(0)\exp\left[-\left(A_2 t + B_2\sqrt{t}\right)\right]$$

$$A_2 = \frac{1}{\tau_E} + a[Q]_2 D_{\mathrm{eff}}$$

$$B_2 = bR_C[Q]_2\sqrt{D_{\mathrm{eff}}} \tag{6}$$

Different authors give non-identical values for the best fitting parameters a and b, those proposed in 1991 by Caruso et al. and in 2000 by Naqvi et al. giving the best approximations.

The objective of these simplifications was enunciated as being a help to those that wanted to make such studies without the hassle of the mathematic delicacies involved in the calculation of the original equation. The formulas presented are, indeed, very convenient and, within the applicability range, sufficient for most practical cases. However, not many researchers in the field took advantage of those recipes except

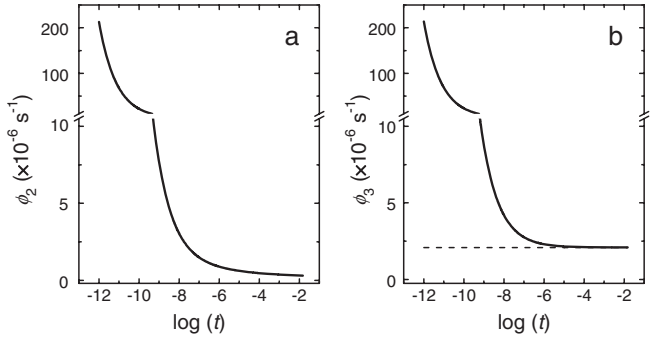


Fig. 1. Comparison of the time dependence of the reactive flux, ϕ_n (n=2 for 2D flux, and n=3 for 3D flux), number of diffusion-controlled encounters per second and per unit area or volume, in two (a) and three (b) dimensions. Parameters used for the calculation in 2D are: R_C=1 nm, $D_{\mathrm{eff}}=10^{-7}$ cm^2 s^{-1} and $[Q]_2=3\times10^{-12}$ mol cm^{-2}; and in 3D: R_C=1 nm, $D_{\mathrm{eff}}=10^{-6}$ cm^2 s^{-1} and $[Q]_3=1.5\times10^{-3}$ mol dm^{-3}. The dashed line shows ϕ_3 for the 3D rate constant.

the authors themselves. Another formula, Eq. (7), was proposed by Szabo and coworkers [30], is very accurate in a large time range and also simple to employ but, to our knowledge, has never been used in the analysis of reactions in two dimensions.

$$k_{2\mathrm{diff}}(t) \simeq \frac{\exp(-\sqrt{\pi\alpha}/10)}{\sqrt{\pi\alpha}} + \frac{1}{\ln\left[\sqrt{4\exp(-\gamma\alpha)} + \exp(5/3)\right]} \tag{7}$$

where $\gamma = 0.5772156\ldots$.

2.1.1.3. Other theories based on hydrodynamic concepts. Another theory applicable to reactions in 2D is the nonlocal fluctuation theory of chemical reactions of Keizer [32,33], later on criticized in its theoretical foundations by Garst [34]. Despite the advantages enunciated by the author, namely for the analysis of the quenching of short excited lifetime species and for crowded media, it has never been used in the 2D context. The fact is that the theory predicts a single-exponential decay of the quenched excited species and, as Vanderkooi and Callis have found [35] early on this sort of work, this is not the general observation in membranes.

The mean field theory (also known as self-consistent field theory) replaces all interactions to any one body with an average or effective interaction [36]. The concept is roughly similar to that worked out by Smoluchowski and the result for steady-state fluorescence quenching does not differ from what is obtained from the direct application of hydrodynamic theory [37], but it has never been used for the analysis of experimental data. This approach has been explored for the first time by Waite [38] that pointed out its advantage when the reaction cannot be considered pseudo first-order. Less successful in predicting the kinetics is the mean first passage time formalism developed by Berg and Purcell [39]. A quite comprehensive comparison of different theoretical approaches, discussing which are the limitations involved in the existing derivations, was published by Torney and McConnell in 1983 [40].

2.1.1.4. Non-hydrodynamic approach. Still other models have been developed, namely those based on diffusion in fractal spaces. Reaction in fractal spaces seems very convenient to describe reactions in gel bilayer phases or highly restricted conditions in which percolation of reagents towards each other is the essential feature that limits the kinetics [41]. In fluid continuous bilayers the concept of fractals applied to chemical kinetics with diffusion is more difficult to understand [42,43]. The analysis of the quenching of excited molecules in membranes [44] is based on a formalism developed to analyze energy migration [45] and adapted to diffusion-controlled bimolecular reactions. The decay being approximately described by the law [46]

$$I(t) \simeq I_{t=0}\exp(-k_0 t)\exp\left(-c_1 t^{f} + c_2 t^{2f}\right) \tag{8}$$

In this equation f is proportional to the dimensionality of the reactional space and c_1 and c_2 are constants, and hence, the decay law is a non-integer power of time.

Eq. (8) is efficient in the simulation of experimental data; however, we should note that, applied to the well-documented case of pseudo first-order fluorescence quenching in homogeneous 3D viscous media, Eq. (8) adapts the fractal dimension to justify the non-exponential behavior of reactant disappearance, Eq. (2). In this case, there is no doubt that the reaction takes place in a physically perfect Euclidean space and is obvious that the fractal dimension obtained lacks physical meaning. This outcome is legitimated by the theory, because the reduction of reaction speed with time results from the increase of the average distance a molecule has to travel before reaction (commonly called "transient effects"). However, to our knowledge, it is not possible to separate this effect, from that resulting from physical constraints to molecular diffusion in a non-Euclidean space. As a hypothesis, this formalism may be of value when the hindrance to molecular encounter results essentially from a percolative diffusion, but is not adequate to interpret situations where the main constraint is created by viscosity driven vicinal reagent depletion as is the case in homogeneous bilayers and viscous three dimensional media that are, intrinsically, Euclidean spaces.

2.1.2. Random-walk simulations

Monte Carlo techniques have been largely used for studying lateral diffusion as well as diffusion–reaction processes in simulated biological membranes. Some authors judge the outcome of simulations by comparing them with the predictions of diffusion theory i.e., by evaluating how well the simulation complies with the hydrodynamic model. However, many workers use the simulations to interpret experimental data, and the "goodness" of the result can only be commented in terms of the comparison of the retrieved parameters, namely the diffusion coefficient, with those obtained by other techniques. Most of these studies make use of mathematical planes, with triangular or square lattice where the particles or tracers are placed in regular positions, either as lattice points or as regular geometric figures such as hexagons. Some also explored the alternative of a continuous plane with randomly positioned particles.

In an early study Galla et al. [47] compared the outcome of a Montroll type random-walk [48] with that of Förster prediction for steady-state pyrene excimer formation. A very complete analysis of the creation of free volume in a membrane and mechanism of diffusion is presented in this interesting paper. Torney and McConnell [40,49] used a square lattice also with a Montroll type diagonal random-walk and the simulated decay was noticeably slower than that predicted by the diffusion theory, a discrepancy that became more pronounced when N_E/N_Q increased. A square lattice was also used by Kozak et al. [50] and they find that, for the same mutual diffusion coefficient, the efficiency of reaction between two diffusing molecules is not identical to that obtained when one of them is fixed, a conclusion that disagrees with the prediction from the diffusion equation. The finding that the kinetics depends on the initial position of the particles in the plane despite the use of periodic boundary conditions is also unexpected.

A random-walk procedure, denominated by the authors the "milling crowd model", was developed by Eisinger et al. [51]. It consists of a triangular plane lattice where the nodes are either lipid or solute molecules in a real crystalline space, real in the

sense that the nodes are separated by a distance that is twice the van der Waals radius of the lipids forming the matrix. Diffusion is simulated by the exchange of the reagent with an adjacent lipid. Reaction takes place, with a pre-defined probability, when reagents occupy contiguous lattice points. The authors compared the outcome of the simulation for a particular situation with the prediction of the Förster formalism for fluorescence quenching in low viscosity 3D medium (rate constant formalism) and, as could be expected, found significant differences.

Besides Eisinger, many other authors used triangular lattices for simulating diffusion [52,53] and reactions [54] in membranes. Shea and collaborators [54], in particular, simulated the reaction of cross-linking of receptors in a membrane and the simulations are compared with several other methods including Keizer's, but not with kinetics involving time dependent reaction rate coefficients.

It is not per se evident that lattice models can accurately simulate chemical kinetics mediated by random walks in continuous media because the continuum implied in the diffusion equation and the Smoluchowski boundary condition can be attained only by letting the step length tend to zero [23,55–58]. Riley, using a continuous random walk with exponential distribution of step length studied the trapping of a particle in several trap conformations [59] but made no comparison with other theoretical or experimental data. Martins et al. [60] used a random walk in which the length of each step, l, remains constant but the direction changes randomly at each jump (Pearson-type random walk). Using very small steps ($l \leqslant R_C/5$) the simulation of the reaction kinetics for the diffusion-controlled interaction between circular rigid particles, follows what is theoretically predicted by the diffusion equation in a time range covering from nanoseconds to milliseconds. The simulations were also successfully compared with the experimentally observed decays for self-quenched fluorescence of pyrene labeled lipids in fluid membranes (sub-microsecond range). The coherence between theory/experiment with the random walk simulations may be, in part, due to the algorithm used, that allowed a full coverage of the plane, but, most probably, the main reason is to be found in the small steps used. When the step length, l, equaled the encounter radius, R_C, the authors found large deviations from the predictions of hydrodynamic theory.

2.2. Reactions in crowded and phase-separated bilayers

Biological membranes are crowded due to the integral proteins some of which being immobilized, perturb the homogeneity of the diffusing environment. The free diffusion of reactants may be further hindered if the lipid matrix is phase-separated. In the case of in-plane phase-separation three different situations may be expected for reactions: (i) both reagents are exclusively soluble in the continuous phase, (ii) both are confined to the discontinuous phase, or (iii) there is a partition between phases of at least one of the reagents. When the continuous phase is the reactional vessel, case (i), the hindrance of diffusion results from obstacles that are not theoretically distinct from proteins, and it may be analyzed with any model for diffusion-mediated reactions in 2D with obstacles. If both reagents are compartmentalized in non-communicating micro-reaction vessels, case (ii), the statistics of the distribution of the reagent molecules by the compartments may influence the kinetics if the number of reactants per compartment is small. To this stochastic effect we should add the increase of collisions resulting from the reactant reflection at the 2D vessel wall. The third case is still more difficult to model because it involves the knowledge of the kinetics and thermodynamics of inter-compartment exchange, besides all the problems already present in case (ii), and, to our knowledge, has never been considered.

2.2.1. Reactions in crowded 2D space

Using the already mentioned "milling crowd model" [51], the authors added obstructions to the diffusion of probes in a triangular lattice in an attempt to simulate the crowding effect produced by proteins in an erythrocyte membrane. Saxton used a triangular lattice for random walk simulation of diffusion and of chemically limited reactions in clustered planes [13]. A very interesting conclusion of this last study is that, particularly in the case of inefficient reactions, reagent recollision, promoted by the nearby barriers to diffusion, might result in an enhancement of the reaction rate, meaning that the intuitive decrease in reaction efficiency due to percolation may be misleading. Besides the cited work and its application to the case of "lipid rafts" [61], Saxton published a series of papers under the main title "Lateral diffusion in an archipelago" that deeply explore the different aspects of diffusion with percolation and are indirectly related to reaction kinetics in crowded media [52,62–64]. Of particular interest is the publication from 1989 [52] and that from 1994 [65] where the distance dependence of the diffusion coefficient in percolative diffusion is analyzed. These works are based in random-walk simulations and, to our knowledge, have never resulted in analytical descriptions of molecular percolation in a 2D medium.

2.2.2. Reactions in non-continuous 2D phases

Below the percolation threshold the reactants become isolated in microreactors and, if the number of reactants per reactor is small and exchange between reactors slow in comparison with reaction frequency, the effects of statistical distribution of the reactants by the reaction vessels contribute to the overall kinetics. This may be the case in phase-separated bilayers for reagents only soluble in the discontinuous phase, but in a biological membrane other situations may give rise to isolation of a small number of molecules in a restricted space. The simulations already referred in the previous case, when extended to non-percolative systems, take care of the distribution of reagents by the non-contiguous regions, but to our knowledge they have never been used with this specific objective. Numerous studies exist, dedicated to the kinetics of reactions in small compartments, namely micelles, taking into account the stochastic distribution of reagent molecules by the microreactors [66,67]. However, these studies imply a uniform size of the reagent containers and are not adapted for diffusion

in a plane. Thus, any extrapolation to bilayer domains is not straightforward. The yield of reactions in phase-separated membranes, considering monodisperse domains, has been modeled, and large variations in efficiency relative to homogeneous bilayers predicted [14], so we can anticipate that there is also a strong perturbation of the kinetics.

3. Experiments with reactions in membranes

3.1. Reactions in model membranes

3.1.1. The choice of technique

Bimolecular diffusion-mediated reactions between components of the biological membrane are expected to take place in the μs to few ms time range that limits the choice of techniques available for their study. An overview of the techniques useful for the study of the kinetics of molecular encounters in this time range was presented by Eigen in 1963 [68]. The predicted useful techniques are pressure or temperature jumps, NMR, ESR, and absorption or emission of long-lived electronically excited states. With a few variations, and the addition of continuous flow [69,70] and fluorescence correlation microscopy [71], Eigen's list is still up-to-date. Whatever the method, the sensitivity should be enough to measure the variation of reagent concentration, or that of added probes acting as reagents. It is also a requirement that the probes can be considered as solutes that do not perturb the system. The limiting concentration at which it is safe to work is obviously dependent upon the composition of the lipid matrix and molecular characteristics of reactants, but from a general survey of the literature this may became a problem only when the reactant to phospholipid ratio is above 1:50. Sensitivity and low concentration led to luminescence emission as the most adequate method for such studies, and, in fact, it is the more widely used [72,73].

3.1.2. Early studies

Early studies using spin exchange interaction obtained a diffusion coefficient of 1.8×10^{-8} cm^2 s^{-1} for phosphocholine with the headgroup labeled with a spin probe in egg lecithin bilayers at 25 °C [74] or a similar diffusion coefficient for a labeled steroid in monolayers [75]. Of the same period are also the measurements using NMR [76–78], which indicate values around or of same order of magnitude of ESR. In 1972 Radda and Vanderkooi [79] proposed the use of steady-state Stern-Volmer plots of fluorescence quenching of *n*-9-anthroyloxy stearic acid (*n*-AS) to estimate the diffusion coefficient of probe molecules in membranes. Exploratory experiments [80] showed that ubiquinone quenched the fluorescence of 12-AS incorporated in DPPC bilayers. They found that neither dynamic nor static quenching were adequate to analyze their results. This failure is in part related to the short fluorescence lifetime of *n*-AS probes not allowing for significant lateral diffusion and collision of reactants during its excited lifetime.

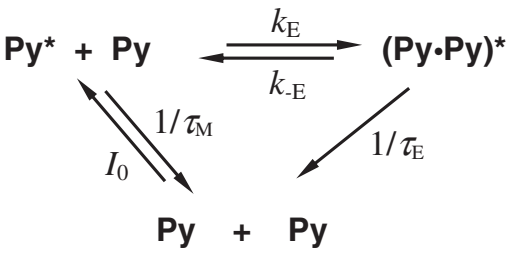
Fluorescent probes emitting in the μs to ms time scale are not abundant especially if we add the requirements that they should have high quantum efficiency, given by the product $\varepsilon_{abs}\times\Phi_{fluo}$ (molar absorption coefficient times fluorescence quantum yield), and that they must be efficiently quenched by contact. The only common chromophores that comply with all these conditions are pyrene, Py, coronene, and some of their derivatives. Recently an alternative appeared with a new $n\pi*$ fluorescent probe, having long fluorescence lifetime and reasonable quantum efficiency, but very low ε_{abs}, developed by the group of Nau [81].

It seems logical that for truly mimicking biological reactions in membranes by photophysical methods, a long-lived probe is needed, and most of the authors of the first experimental studies were aware of it. To our knowledge, the only early attempt to study a model reaction in synthetic bilayers using a really long-lived species ($\tau_0>1$ μs) was made in 1974 by monitoring by flash photolysis the triplet–triplet annihilation of a 9-anthracenamide labeled phospholipid in DPPC at a quite low probe to lipid ratio (ca. 1:250) [82,83]. The diffusion coefficient obtained with this method was of 2.3×10^{-7} cm^2 s^{-1} at 323 K, to be compared with the value from fluorescence recovery after photobleaching, FRAP, at the same temperature for a fluorescent-labeled lipid, 1.24×10^{-7} cm^2 s^{-1} [84] (concerning FRAP *D* values used for comparison, see note [85]). The long-lived triplet state, as used in this work, is the obvious answer to the short mean diffusion length, but due to several technical complications has not been, so far, thoroughly explored for this purpose.

3.1.3. Quenching of pyrene fluorescence

Because of this paucity of probe molecules for the study of the kinetics of reactions in 2D, the quenching of pyrene fluorescence, in particular, the self-quenching of the singlet-excited state of pyrene, Py*, yielding an emissive excimer, (PyPy)*, Scheme 1, has been very popular since, in 1974, Vanderkooi and coworkers [35,86] and Galla and Sackmann [87] proposed its use as a model reaction for the study of bimolecular reactions in bilayers, namely to study lateral diffusion in membranes.

In Scheme 1 k_E and k_{-E} are, respectively, the rate constants of excimer formation and dissociation, τ_M and τ_E the fluorescence lifetimes of pyrene monomer and excimer, and I_0 is the intensity of excitation radiation absorbed by the monomer. In fact, the long lifetime of the forbidden S_1-S_0 transition of Py, about 200 ns in deaerated aqueous suspensions containing phospholipid bilayers and the quite high $\varepsilon_{abs}\times\Phi_{fluo}$ when excited in S_2, allows the use of relatively low probe concentration. Galla and Sackmann employed the Förster formalism for the analysis of fluorescence emission data (rate



Scheme 1.

constant type formalism) and obtained diffusion coefficients for free pyrene in DPPC bilayers at several temperatures, for example $D = 1.4 \times 10^{-7}$ cm^2 s^{-1} at 50 °C, remarkably accurate if we take into account the methods used (see above the value from FRAP for a labeled phospholipid). The Förster–Birks [88–90] formalism for diffusional quenching of an excited state is a good approximation in the case of non-viscous 3D continuous media in which the time-resolved decay of the quenched excited species obeys a single-exponential law as pointed by Birks. However, already in 1974, and before the formalisms specific to 2D were published, Vanderkooi noticed that the fluorescence decay of quenched pyrene is far better fitted by a law of Smoluchowski type, Eq. (2), then by a single exponential [35]. The author attributed this to the viscosity of the medium but we presently know that a law in square root of t fits adequately the reagent concentration decay in membranes, see Eq. (6), in spite of the incorrect evaluation of the molecular parameter D.

It can be argued that pyrene is not the most adequate model system for the study of 2D reactions, firstly because a free pyrene molecule is able to move transversely across the bilayer resulting in a non ideal 2D system [91], secondly due to the relatively short mean displacement of pyrene into the membrane during its excited lifetime (mean square displacement ca. 2500 Å^2 in 400 ns). On the other hand, Py, being quite small, minimizes membrane perturbation; it can be easily attached to a phospholipid or fatty acid chain, solving the transverse movement problem, and its self-quenching has been thoroughly studied in homogeneous solution, ensuring that below 50 °C the reversibility of the excimer is negligible, and the excimer formation reaction is diffusion-controlled in homogeneous solvents [92].

Prior to 1974, many authors analyze experimental diffusion-controlled reactions in membranes, taking hydrodynamic theory as a starting point but not recognizing the specificities resulting from the dimensional limitation for the diffusion and the low molecular diffusion coefficients both leading to inherently non-exponential pseudo-unimolecular reaction kinetics as early found by Vanderkooi. These works use the Förster–Birks formalism for isotropic diffusion, with or without excimer reversibility.

Some of the authors following this approach gave no notice of having difficulty in the fitting of this oversimplified model to their experimental data [93]. There are also authors who, comparing the fit obtained with a sum of exponentials to that with a square root law, conclude that the sum of exponentials is a better approach and that there is no sign of transient effects (time dependence of the rate coefficient), consequently they use the Birks formalism [94,95]. The diffusion coefficients obtained for 1-methylpyrene in DMPC at 50 °C is 10×10^{-7} cm^2 s^{-1} and at 30 °C is 5×10^{-7} cm^2 s^{-1} [95], about one order of magnitude higher than those for phospholipid diffusion in the same conditions [96]. A similar conclusion was reached by Liu and coworkers [97] that analyzed pyrene in fluid DPPC bilayers in concentrations not explicitly given. These last authors postulate the formation of two different pyrene excimers. Two pyrene excimers are, in fact, described in the literature to exist in homogeneous solvents, but it only happens for severely constrained dipyrenyl molecules [92].

However, many authors agree that a single or multiple exponential law is not adequate to describe the decay of pyrene or pyrene-labeled lipids in bilayers as early found by Vanderkooi and Callis [35]. Whatever the case, the use of the 3D-specific Eq. (2) for experimental analysis, considering or not transient effects, results in diffusion coefficients larger than those predicted by Eq. (3). For this or other reasons, several authors that obtained diffusion coefficients they considered too high for their expectations explained the result invoking extraneous physical reasons. Between those, the more common are the proto-association of the pyrene molecules [24,98–100] and "medium range diffusion" [101,102].

While rooted in the wrong reasons, the use of a non-adequate formalism, ground state proto-aggregates correspond to a real physical situation in the case of concentrations equal or higher than 1 : 50 (probe / lipid), as demonstrated by several works of Somerharju et al. [103,104]. According to these studies, there is a molecular distribution with a defined stoichiometry of 1-palmitoyl-2-pyrenedecanoyl-*sn*-glycero derivatives in the host lipid [103]. At these high concentrations, however, it is not only the formation of non-true solutions that affect the kinetics but it also collides with a fundamental assumption of the hydrodynamic theory that other vicinal sinks do not disturb the concentration gradient that surrounds them.

As said, some authors have put forward the concept of "medium range diffusion" coefficient as a possible explanation of their results [101]. The concept spread in the literature and deserves a clear comment. While it is simple to ascribe a physical meaning to both, long- and short-range diffusion, there is no support for the concept of medium range diffusion besides the mentioned overevaluated molecular diffusion coefficients [105].

The analysis of the kinetics of pyrene excimer formation in membranes was also attempted using the fractal theory. The polydispersity of MLV (multilamellar vesicles) has been invoked as one reason for non-Euclidean characteristics of the space in which quenching in vesicles takes place [44,106], and, according to the authors, this could validate a fractal analysis. But, comparison of literature data seems to indicate identical behavior in multilamellar and unilamellar vesicles, either small or large [94], and one author claims that no difference can be observed between pyrene self-quenching in MLV or in extruded LUV (large unilamellar vesicles) [107].

The first attempt to experimentally verify the integral form of the 2D kinetic law dating from 1994 was performed in Langmuir–Blodget films using the pyrene excimer reaction in 2D in diluted samples [108]. The authors used a modified form of Naqvi's equation for the analysis of the quenched pyrene fluorescence that considered only the long time regime. However, the authors battled against the low intensity of the fluorescence, and the measurements, already difficult to perform with enough precision in vesicle suspensions, became nearly impossible in monolayers.

The first complete proof of the suitability of Eq. (3) for the analysis of diffusion-controlled bimolecular reactions in fluid model bilayers was presented in the work of Martins et

al. in 1996 [107]. In this work the authors study the kinetics of the excimer formation reaction between an excited and a ground state pyrene moiety of 1-palmitoyl-2-(1-pyrenedecanoyl)-*sn*-glycero-3-phosphocholine, $py_{10}PC$, in a fluid POPC bilayer at concentrations lower than 1:75 probe to phospholipid ratio. The analysis of the time resolved emission of the monomer and excimer give diffusion coefficients that are in accordance with the results obtained from FRAP or pulsed-field-gradient NMR, e.g. $D=3.1\times10^{-8}$ cm^2 s^{-1} at 25 °C and an activation energy for diffusion, $E(a)=35$ kJ mol^{-1}, while the value for the lipid diffusion obtained from FRAP at the same temperature, 4.2×10^{-8} cm^2 s^{-1} [96], is a little higher [85], and the activation energy, 28.0 kJ mol^{-1}, is lower due to the larger cavity needed for diffusion steps. Coherence between monomer and excimer kinetics, and with the steady-state fluorescence data was also verified. The work seems quite conclusive but it may be argued that the mean square displacement of the reactant during its lifetime (ca. 200 ns) is not more than 5 nm, of the order of the membrane thickness.

More recently, the possibility of performing global analysis of the monomer and excimer time-resolved data was exploited by Novikov et al. [25,26]. The authors used the formalism for 2D but with the partially reflecting boundary condition of Collins and Kimball, Eq. (5), allowing for collisions that are not 100% efficient, and concluded that in DOPC liposomes some of the collisions between $py_{10}PC$ molecules are not effective, obtaining a diffusion coefficient of $py_{10}PC$ in this lipid matrix at 20 °C of 7.2×10^{-8} cm^2 s^{-1}. The non-diffusion control seems to point to hindered encounters between the pyrene moieties in this system, something that is not found in homogeneous media for free pyrene or pyrene attached to an aliphatic chain. The diffusion coefficient obtained, while high relative to other lipid matrixes at the same temperature (D is supposed to be relatively independent from the chain length and saturation of probe and lipid matrix [84]), agrees with that obtained independently for lipids in DOPC obtained by pulse-field gradient NMR [109], about 8×10^{-8} cm^2 s^{-1} for DOPC at 25 °C, and fluorescence correlation spectroscopy, 6.3×10^{-8} cm^2 s^{-1} at room temperature [110].

Eisinger and collaborators used the "milling crowd model" to simulate the steady-state fluorescence of monomer and excimer of $py_{10}PC$ in DMPC SUV (small unilamellar vesicles) and MLV above the phase transition [101]. In principle, the method should give nearly correct parameters, but the diffusion coefficients retrieved are about 4 times larger than those obtained with independent techniques. In default of a better explanation, our opinion is that the authors extended their analysis to concentrations of pyrene for which no randomness is expected, attaining 1 probe for 10 lipids, which may justify the difference in the diffusion coefficients obtained, and the need of postulating a reaction probability of 0.25.

Quenching of pyrene and pyrene-labeled lipids by molecules acting as intersystem crossing enhancers, electron donors, etc., was also used to model reactions in membranes. The charge-transfer interaction with diethylaniline and heavy atom effect of bromobenzene and iodide were studied for a series of pyrene derivatives with different locations relative to the membrane interface [111]. The work gives an interesting insight concerning the mechanisms of quenching, namely a discussion of charge separation and recombination in bilayers. The quenching by tetracyanoquinodimethane of excited $py_{10}PC$ and its excimer in liposomes was studied by Lemmetyinen et al. [112], and conclude that both forms are quenched. The authors consider formation of excimers originating in pyrene moieties aggregated in such a way that they require only a small rotational motion to attain excimer configuration, what is to be expected at the probe concentrations used. As with the excimer reaction, these and other works treat the kinetics neglecting the difference resulting from the reduction of dimensionality [113,114]. In 1987 Blackwell et al. [115] obtained diffusion coefficients for plastoquinone and plastoquinol from steady-state and time-resolved studies of pyrene fluorescence quenching. The data analysis was performed using the approximated formula proposed by Owen in 1975 as point of departure, but non-single exponential decay was found for pyrene in the absence of quencher and the fit of theory to experiment was bad.

Pyrene can also label the headgroup of phospholipids, a case in which it will contact the bilayer interface and water, resulting in much shorter fluorescence lifetime. The quenching by iodide and an electron donor of pyrene-DPPE (lifetime of about 10 ns) in monolayers was analyzed using Eq. (3) [27,116,117]. However, as the authors remark, the comparison of steady state with time-resolved results is not coherent. This may possibly be due to a large contribution of static quenching and other effects unavoidable with short-lived probes and/or to deviations of a true two-dimensional geometry.

3.1.4. The non-pyrene experiments

Besides pyrene, the other well-known long-lived $\pi\pi^*$ singlet excited state is that of coronene with an excited-state lifetime $\tau_0=165$ ns in DMPC bilayers above phase transition [118], but to our knowledge, it has never been used for the study of reaction kinetics in membranes.

A few studies involving short-lived species followed the original work of Radda et al. with the *n*-AS probes already mentioned [79,80] and that of Bieri and Wallach who used perylene fluorescence quenched by paramagnetic species [119]. Some authors question the use of short-lived fluorescent probes such as anthroyloxy derivatives, perylene, diphenylhexatriene, *p*-terphenyl and tryptophan, for dynamic quenching studies [120]. In fact, if an estimate is made of the quencher concentration theoretically needed to reduce by 5% the emission intensity of a probe with unquenched lifetime of 10 ns as a result of dynamic quenching, the conclusion is that around 20 mol% of the membrane has to be constituted by the quencher (for the calculations, we used the approximated formula of Szabo [37]). Under these conditions, it is obvious that a careful inspection of the thermotropic phase diagram of the lipid/quencher mixture is necessary, and in no way the mixture may be treated as a dilute solution. Despite the

obvious inefficiency of diffusion-mediated quenching of fast decaying states, experiments trying to extract diffusion coefficients from such systems abound in the literature. Besides the short mean molecular displacement during its lifetime, short-lived probes have another common drawback, their complex decay in the absence of quencher. The origin of this behavior is in most cases uncertain and is usually arbitrarily dealt with by the use of sums of exponentials. The multiexponential decay has been ascribed to different environment of probe molecules inside the bilayer, but it can also be the consequence from an intrinsic photochemistry; in both cases, the retrieved parameters do not have a clear physical significance. Whatever the case, no fluorescence inhibition is observed except for extremely high quencher concentrations [121–123]. When these experiments are analyzed with a simple Stern–Volmer type kinetics the diffusion coefficients retrieved are, in general, overestimated, not only due to neglecting static and transient effects and dimensionality constrictions but mainly as a consequence of the non-ideal mixing of quencher with solvent (lipid). The analysis of the fluorescence quenching under these conditions is often better made with formalisms involving both static and 3D type dynamic quenching with a hopping mechanism [124,125]. Within this context, a peculiar analysis of very high quencher concentration, discussing the distribution of quencher and its interactions with the fluorophore was presented by Yeager and Feigenson [126].

An alternative to large concentrations is to follow a photochemical reaction under steady-state irradiation that will take place with very low quantum yield due to the rare collision between reactants. For example, parinaric acid (9,11,13,15-octadecatetraenoic acid) in the singlet excited state undergoes a second-order photodimerization in phospholipid bilayers [127]. The authors used a 3D based formalism for data analysis and obtained meaningful fluorescence lifetimes for predefined diffusion coefficients in fluid DMPC. Using the same method, Moreno and Melo [128] studied the kinetics of photodimerization, of a short-lived excited state species, 6-(9-anthroyloxy) stearic acid, 6-AS, in fluid POPC bilayers. As predicted, within the experimental error the small lifetime of the probe in fluid POPC (<11 ns) is not modified by self-quenching for concentrations ranging from 1:1000 to 1:50 6-AS to lipid molar ratio. At these concentrations, no static quenching is measurable but a slow dimerization kinetics is observed. The authors used the formalisms for 3D with and without transient effects and also that for 2D in the analysis of the rate of dimerization. Credible, but different, parameters were obtained with both formalisms, 3D with transient effects and 2D.

A new long-lived fluorescent membrane probe based on the azo chromophore 2,3-diazabicyclo[2.2.2]oct-2-ene functionalized in palmitoyl acid, named by the authors Fluorazophore-L, has a relatively long fluorescence lifetime in POPC liposomes, τ_0=125 ns at 25 °C not much affected by the presence of oxygen [129]. Fluorazophore-L $n\pi^*$ excited state is efficiently quenched through hydrogen abstraction. This quenching reaction was used to measure a mutual diffusion coefficient of 1.8×10^{-7} cm^2 s^{-1} for α-tocopherol in POPC bilayers at 27 °C and, based on their findings, the authors propose a possible molecular mechanism for lipid peroxidation and its inhibition [133,131]. The analysis was done using the approximated formula proposed by Naqvi et al. [31] for the analysis of time-resolved fluorescence quenching in membranes.

In the line of the original study of triplet–triplet annihilation in bilayers [82,83], Baranyai et al. [132] used porphyrin derivatives as well as anthracene and claimed to have found rough estimate of mutual diffusion coefficient in small and large unilamellar vesicles. The work uses classical Förster kinetics approach.

In a recent study, Medvedeva et al. [133] analyzed the *cis–trans* isomerization of a stilbene probe incorporated in liposomes when sensitized by erythrosine B triplet excited state, in competition with the quenching of erythrosine B triplet by a nitroxide radical added in variable amounts. The kinetics of formation of the *trans* isomer is followed and the data analyzed using the formalism proposed by Naqvi et al. [31] for steady-state measurements.

Other kind of long-lived probes are those based on the luminescence of metal complexes of rhenium(I) [134], ruthenium(III) [135] and lanthanides [136]. The problem with these probes, so useful for resonance energy transfer experiments, is that collisional quenching does not easily deactivate their luminescence, what makes them inadequate for the purpose. With the exception of one application using FRET [137], we are not aware of other diffusion studies in model membranes using this kind of probes.

3.1.5. ESR experiments

While the spin exchange detected in ESR experiments is not a bimolecular reaction in the sense discussed in this review, it results from molecular encounters and is valuable as an alternative method for bimolecular reaction simulation. The first quantitative attempts to study the lateral diffusion in model membranes were carried out in the early 70s by Traüble and Sackmann [74] and Devaux and McConnell [75], using ESR methods based on nitroxide radical spin-labels. Both studies used very high concentrations of the spin-label (molar probe to lipid ratio, P/L, higher than 1:29) which raised the question of the homogeneity of the probe–phospholipid mixture and the risk of structural perturbation of the model membrane by the probes. Popp and Hyde [138] propose an alternative method using continuous wave electron–electron double resonance spectroscopy (ELDOR) to measure the bimolecular collision frequency between stearic acid spin-labels in DMPC bilayers. More recently, several authors are still refining this technical approach by using Heisenberg spin exchange [139] and dissipation of electron spin resonance signal gradient [101,140]. A further development is FT-ESR (Fourier transform ESR), enhances its relatively low sensitivity and is able to separate the different contributions to the line broadening [141]. However, ESR techniques suffer from low sensitivity, a problem that is difficult to overcome.

3.1.6. Kinetics in phase-separated membranes

Not much experimental work involving bimolecular reactions mediated by diffusion in phase-separated membranes has been, until now, done. Piknová et al. studied the thermotropic behavior of the system DMPC–DSPC [142] monitoring the quenching of fluorescence of a phospholipid labeled with DPH by a spin-labeled DMPC, both insoluble in the gel phase. The short lifetime of the fluorophore (ca. 6 ns) only allowed for static quenching and the quencher concentrations attained 1 : 12.5 (quencher/lipid). Data was analyzed using Monte Carlo simulation with diffusion in a triangular lattice. The authors conclude that the number of lipid molecules forming a fluid phase domain is between 5 and 480 depending on the temperature and composition. The small number of molecules obtained may be attributed to the small lifetime of DPH, is inconsistent with the definition of a separated thermodynamic phase [143] and not coherent with other experimental data for the same system [144].

3.1.7. The new microspectroscopic methods

Recent technical advances allow time-resolved spectroscopic measurements with high spatial resolution in biological and model systems. We refer in special to the recent developments in fluorescence correlation spectroscopy (FCS) and single molecule fluorescence microscopy (SMF). In which concerns the study of bimolecular reactions, these techniques have been successful in the clarification of reaction mechanisms by direct observation of molecules and molecular interactions [145], but we are not aware of its use for the analysis of reaction kinetics in two dimensions. However, FCS has inherent potential to be used in the field of chemical kinetics [146], but its use for this purpose has not been much explored, not even in 3D. In fact, the measurement of kinetics by FCS is made in equilibrium (it is not a perturbation technique) and its temporal range, between 1 μs and 1 s, is well suited for the study of bimolecular kinetics in membranes (for a review see the paper of Hess et al. [147]). The fact that the system is in equilibrium results in a situation much different, but not less important, from what we have been discussing until now, but we must be aware that it is subject to a quite different formalism. The possibilities opened by these techniques are difficult to predict without further experimental work and theoretical development, and the reader is referred to an inspiring kinetic analysis of equilibrium reactions in 3D by Hom and Verkman [71]. The small number of molecules observed in SMF has limited, until now, the possibility of an accurate kinetic modeling of the reactions (not forgetting that kinetic parameters are intrinsically ensemble properties), and the crude kinetic models used for the analysis of the kinetics of bimolecular reactions in 3D detected by FCS, are certainly not the maximum quantitative refinement allowed by the technique. In conclusion, the study of kinetics in equilibrated systems, the convenience of time range for the analysis, and the relatively high spatial resolution are reasons that make these methods worth to explore in particular for the study of reactions in biological membranes.

3.2. Reactions in biological membranes

During the last 35 years or so, the kinetics of bimolecular reactions in bilayers has been experimentally examined mostly with the objective of obtaining molecular diffusion coefficients of lipoid membrane components. This pursuit results from the evidence that a variety of cellular processes involving the interface or occurring in the plane of the membrane depend on the lateral diffusion of biomembrane components. The most fundamental cellular functions rely on membranes at some point, including such diverse processes as: prokaryotic DNA replication, lipid and protein biosynthesis, protein secretion, bioenergetics and transduction of physical and chemical signals. The Human Genome Project stresses this point by finding that roughly one-half of genes code for membrane-associated proteins. Nevertheless, the kinetics of bimolecular reactions that occur under the constraints imposed by a biological membrane has not received due attention, particularly from the experimental point of view.

Despite the emphasis given to diffusion in biological systems, it is difficult to find out if a given biochemical reaction is diffusion limited [148]. In homogeneous solution, around nine enzymes have been found to have kinetic parameters that could be interpreted to involve diffusion-controlled limited reactions [149]. However, for bulk biochemical reactions, especially metabolic processes, diffusion is not a determinant consideration when compared to the mass action effects. It is only in reactions or processes that involve small number of reactants or restricted geometries that diffusion may play a key role. To what extent reactions that occur in biological membranes are diffusion-controlled processes is still an open question that can only be solved by using the appropriate 2D formalism in the analysis. Until the mid-1990s, it had been established that lateral diffusion of membrane components is essential to a variety of cellular processes occurring in the plane of the membrane [150,151], some of them believed to depend at some stage on diffusion-controlled reactions. Among those are the following examples of membrane enzymology and chemoreception: electron transport chains; enzymes that utilize membrane-bound substrates; enzymes that shuttle between the cytosol and the membrane and whose activities are modulated by membrane binding; and sensory and regulatory processes at the membrane interface.

3.2.1. The microsomal electron transfer chains

The electron transfer chains present on the cytoplasmic surface of the endoplasmic reticulum are composed of two interacting systems. In one of them, NADH is oxidized by a flavoprotein, cytochrome b_5 reductase, which reduces cytochrome b_5, which in turn reduces an acyl-CoA desaturase. The second system involves the oxidation of NADPH by another flavoprotein, cytochrome *P*450 reductase, which then provides reducing equivalents to a family of cytochrome *P*450. In a series of influential papers, Strittmatter and Rogers argued that cytochrome b_5 was randomly distributed and diffusion-coupled [152,153]. However, later studies strongly suggest that a portion of the cytochrome b_5 in the microsomes is not freely diffusing,

being, at least transiently, involved in stoichiometric complexes with cytochrome *P*450 species, what may be expected to reduce the mobility of both interacting species. Cytochrome *P*450 is known to form oligomers [154], and molecular complexes with cytochrome *P*450 reductase and cytochrome b_5 [155] that could potentially result in formation of large networks or in aggregation. The immobilization of the redox proteins makes it difficult to understand their role in the mechanism of electron transfer for which, possibly, both immobile and free cytochrome *P*450 molecules contribute [156]. The hypothesis may not be ignored that, since the microsomal membranes are densely packed with integral proteins [157], electron transfer may be controlled by local lateral collisions and not require long range lateral diffusion.

3.2.2. The mitochondrial respiratory chain

The components of the mitochondrial electron transport system, originally conceived as being fixed in the inner membrane [158], are, since the early 1980s, known to move freely and interact by random collisions [7]. UQ_{10}, moving more rapidly within the membrane, act as a shuttle transferring electrons from complexes I and II to complex III. Similarly, cytochrome *c*, also moving more rapidly because of its relatively small size and loose binding to the outer surface of the inner mitochondrial membrane, transports electrons from complex III to complex IV. Some of the evidence supporting the random collision model come from extensive electron microscopy studies and lateral diffusion measurements in the inner mitochondrial membrane by Hackenbrock and cow-orkers. Measurements of lateral diffusion coefficients of the complexes [159,160] were interpreted as the chain components being completely mobile, e.g. the lateral diffusion coefficients of complexes I, III and IV, are of the order of 10^{-10} cm^2 s^{-1} and increase with decreasing protein concentration. The proteins were found to diffuse somewhat slower, being more sensitive to the protein content than the lipid components, e.g. fluorescently labeled UQ_{10} displays a lateral diffusion coefficient around 3×10^{-9} cm^2 s^{-1} [6,161]. Also the apparent activation energies for diI (3,3′-dioctyldecylindocarbocyanin) lateral diffusion and mitochondrial electron transfer vary in a parallel manner with changes in protein content. Moreover, the apparent activation energy associated with the lateral diffusion of UQ_{10} analogues accounts for most of the total activation energy in the electron transfer process, indicating that this process, as a whole, is limited by diffusion. However, the meaning of activation energies in such complex membranes is certainly not straightforward and the interpretation presented may be too simplistic. What may be said is that there is strong evidence that the overall kinetics of this electron transfer chain is largely diffusion-controlled in vivo, occurring between randomly distributed components of the electron transport chain [158].

Based on studies performed in SUV and submitochondrial particles, Lenaz [162] argued that the lateral diffusion of ubiquinone is not the rate-limiting step in electron transfer and proposed that the chain components would be associated to form still larger complexes, of variable lifetime [163]. However, the studies are based on the analysis of the collisional quenching of fluorescence labeled fatty acids, *n*-AS, using the classical Stern–Volmer formalism for homogeneous solutions with low viscosity. More recently, the respiratory chain has been described as a system that is partially organized as super-complex assemblies [164]. Schägger [165] reviewed the evidences concerning the occurrence of stoichiometric assemblies of individual complexes, in bacteria, in yeast and in mammalian mitochondria, and suggested a model for the respiratory chain (the respirosome) based on the direct electron channeling between complexes.

3.2.3. The photosynthetic electron transport system in thylakoids

The mechanism of absorption of light and its conversion into chemical energy in eukaryotic plants involving photosystem I (PSI) and photosystem II (PSII) is mediated by the transfer of reducing equivalents between these two complexes. This transfer is accomplished by water-soluble plastocyanin and membrane-associated plastoquinone. The distribution of PSI and PSII is non-random – PSII is more abundant in the appressed regions (the granal stacks) than in the non-appressed regions (the stromal lamellae), the reverse holding for PSI. The two photosystems are kinetically linked by an electron transport chain that conducts reducing equivalents extracted from water by PSII to PSI, and delivers electrons to $NADP^+$ at the end of the pathway.

It is now well established that diffusion in the thylakoid membrane is highly restricted. Given the heterogeneous distribution of the involved components and the crowding of the medium, theories considering domain-limited reactivity [13,14,166] and/or anomalous lateral diffusion [148] are necessary. While Milano et al. [167] used the classical Smoluchowski 3D equation to examine the kinetics of electron transfer between a photosystem and plastoquinone, Tremmel et al. [168] recognized the limitations of a homogeneous treatment, and used Monte Carlo simulation methods for the study of plastoquinol diffusion. Joliot, Lavergne and collaborators [169,170] used statistical and percolation arguments, considering only the mobility of plastoquinone diffusing in an "archipelago" of randomly distributed immobile protein complexes. In more recent studies, the obstruction of plastoquinone lateral diffusion was analyzed considering the mobility of both, plastoquinones and protein complexes [168,171,172].

3.2.4. Membrane lipid peroxidation

Lipid peroxidation is mediated by free radicals (non-enzymatic lipid peroxidation) or catalyzed by enzymes (enzymatic lipid peroxidation) [173], and involves propagation steps that can probably be modeled using a 2D reactions kinetics formalism [174]. The same happens with the action of the antioxidant α-tocopherol (vitamin E), inhibiting lipid peroxidation by scavenging the lipid peroxyl radicals [175]. Subsequent regeneration of the α-tocopheroxyl radical may also proceed via an analogous termination reaction with ubiquinone [176]. However, the relatively long lifetime of lipidoid radicals observed in a native biological membrane [175] is probably

indicative that the scavenging process is not necessarily mediated by the diffusion of the molecular radical. Until recently these reactions were analyzed using formalisms for kinetics in homogeneous media (concentrations and rate constants) [177]. Gramlich et al. [130], studied the quenching of excited Fluorazophore-L (the lipid probe) by α-tocopherol using an adequate 2D formalism, and determined a dependable mutual diffusion coefficient for the two species, but the applicability of these findings to the kinetics of scavenging of lipid peroxyl radicals in a native biological membrane is still an open question.

3.2.5. Enzymes for lipid membrane substrates

A wide range of metabolic and control processes mediated by membrane-associated enzymes occur at membrane interfaces and many of these trigger transmembrane signals transduced across membranes by other regulatory metabolic, biosynthetic and transport events [178].

Pancreatic phospholipase A_2 was used as a prototype of an interfacial enzyme for the characterization of their kinetics [179]. Generally, the kinetics of exchange of the substrate and product molecules between the coexisting microscopically dispersed interfaces of the lipidic aggregates and the intervening aqueous phase is not rapid on the time scale of the catalytic turnover. The authors of this study proposed a general kinetic scheme that is an extension of the classical Henry–Michaëlis–Menten formalism, including the catalytic step for independent turnover cycles in the aqueous compartments and in the interface. The diffusional effects in the interface, namely the peculiarities of the 2D diffusion-controlled reactions, have been taken into account [179], but probably because the concentrations used are calculated as 3D concentrations, there is a discrepancy of about three orders of magnitude between the experimental and calculated 2D diffusion-controlled rates [179]. A related work referring to the lipase catalysis on monolayers at the air–water interface uses the 2D formalism for the analysis of the in-membrane steps. The equation seems to adequately fit the data, despite the dispersion inherent to the experimental system and setup [180,181]. Probably, many mechanisms concerning other membrane-associated enzymes involve 2D diffusional steps, as proposed for the catalysis of lathosterol 5-desaturase [182] and recently for ACAT (acyl-CoA : cholesterol acyltransferase) [183].

3.2.6. Blood coagulation

The reactions that result in thrombin formation are sequentially ordered in a reactional cascade, where an active serine protease activates the next zymogen in the cascade, an arrangement that ensures the necessary amplification of the small initial stimulus. Blood clotting factors are prothrombin (factor II), factor VII, factor IX and factor X (collectively referred as the "vitamin K-dependent coagulation factors") [184].

To be biologically active, factor IXa must form a complex with factor VIIIa (the active form of cofactor, factor VIII) on a cell membrane. The active membrane-bound macromolecular complex activates factor X to factor Xa, which forms another macromolecular complex (prothrombinase) with membrane bound factor Va [185]. Binding studies have indicated that factors Va and Xa interact on the membrane surface with a dissociation constant of approximately 10^{-9} M [186], whereas the dissociation constant for this interaction in solution has been estimated to be 10^{-6} M [187]. The increased affinity on the membrane surface could be accounted for by phospholipid-mediated conformational changes in the proteins, but it may also happen that the formation of binary protein–phospholipid complexes and translational diffusion of the proteins in the plane of membrane may suffice to enhance the frequency of productive collisions between membrane-bound proteins and account for the increase in binding affinity [186,188]. Moreover, arguments have been presented that invoke effects derived from reduction of dimensionality [184,189] that are considered crucial for the effective assembly of enzymatically active macromolecular complexes.

3.2.7. Sensory and regulatory processes at membrane interface

Precise control of physiological phenomena is performed by various kinds of receptor-mediated signaling. The vast majority of receptors belong to a superfamily of G-protein-coupled receptors (GPCR), which form one of the largest protein families found in nature. A diverse array of external stimuli including neurotransmitters, hormones, lipids, photons, odorants, taste ligands, nucleotides and calcium ions can act through GPCR giving rise to remarkably diverse physiological functions [190]. In these processes G proteins are the molecular mediators that link GPCR to their effectors proteins – enzymes or ion channels.

The classical view of GPCR signaling is based on random collisions between proteins that diffuse freely in the plasma membrane [191,192], and the specificity of molecular interactions is dependent entirely on information encoded in the three-dimensional structures and recognition surfaces of the individual moieties. However, recent evidence suggests that receptors, G proteins and effectors are less mobile than previously appreciated [193]. Moreover, the specificity of signaling in intact cells appears to be significantly greater than in reconstituted systems. Thus, a more organized system, rather than a pure freely diffusible arrangement, has been suggested to enable both rapid and specific propagation of extracellular stimuli to intracellular signaling molecules [194]. G proteins tend to exist not randomly on the intracellular surface of the plasma membrane, but rather concentrated in specialized distinct microdomains. Shea and Linderman [195], taking into account this scenario, simulated the effects of in-plane compartmentalization in signal transduction, but their calculations do not adequately account for the dimensionality of the system. An experimental approach to the kinetics of the signaling reaction cascade can be found in the study of dynamic confinement of neurokinin (NK2) receptor expressed in HEK293 cells [196].

One particular signaling process for which diffusion mediated steps have been proposed is phototransduction. Rhodopsin, the primary membrane protein containing the chromophore retinal [197], is activated by a single photon and induces subunit dissociation of transducin (rod photoreceptor

G protein), amplifying the light signal [198]. A unique aspect of this transduction process is the rapid rate of this G protein activation: recent measurements established that transducin activation by rhodopsin occurs about 100 times faster than the activation of other G proteins [199]. This observation relies on a collision coupling mechanism, in which photoexcited rhodopsin and transducin diffuse on the disk membrane, collide and then bind [200]. Recent experiments with whole rod cells from transgenic mice that expressed half the normal amount of rhodopsin establish that the rate of phototransduction in the rod cell is determined by protein lateral diffusion in the disk membrane [201]. Despite the awareness of the authors of the specific kinetics imparted by the existing dimensional constrains, the calculation of the frequency of diffusion-controlled encounters was done based solely on the sum of the molecular diffusion coefficients (of rhodopsin and transducin) and on the packing density of transducin in the disk membrane.

4. Conclusions

We may conclude that all needed theoretical background for the study of diffusion-controlled bimolecular reactions in biological membranes exists. At least the theoretical description is complete and ready for experimental test. However, until now, the experimental validation of such theories and formalisms using model systems under well-controlled conditions has been disperse and scarce. The use of Naqvi's equation, or its mathematically simplified versions, for the study of model reactions is limited by the short lifetime of the activated reactants used, in other words, the relatively small mean square displacement during its lifetime. Some confidence may result from random-walk simulations that seem to validate the 2D hydrodynamic formalism for the case of long-lived species, but it lacks the convincing power of a true experimental verification. From a pure physical–chemical standpoint, the beauty, or strangeness, of diffusion-controlled reactions in 2D is the absence of a steady state. With the probes until now used it is the lifetime of the reactants that cancels the prosecution of the reaction, not the depletion of reactants around them. So, the more striking consequences of the dimensional reduction of the reactional space have never been experimentally observed to their full extent. Once achieved a full experimental validation of the existing theories, refinements relevant for biological reactions such as charge effects, recombination, or complex collision geometries, can be considered.

When going to crowded or phase-separated systems, where the processes are mediated by percolative diffusion, the absence of experimental studies is almost complete. The reason may be attributed to an increased difficulty relative to homogeneous diffusion spaces, but some probes and experimental methods already existing can certainly be used, at least for preliminary studies.

In the section on Reactions in biological membranes we made an effort to enumerate the immense variety of biological reactions and processes for which steps involving diffusion/percolation prior to reaction are possible, predictable, or have been invoked. The interest for the kinetics of reactions in bilayers, homogeneous or not, has to be renewed if a more quantitative view of the membrane biochemistry is aimed.

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