Reliability of Parameters obtained from Time-resolved Fluorescence Polarization in Macroscopically Isotropic Liquid Crystals and Membrane Vesicles

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The practical possibility of extracting static and dynamic parameters from fluorescence anisotropy decay of rod-like probes in membrane vesicles and other macroscopically isotropic samples with a locally ordered structure, such as cubic liquid crystals or unoriented polymer liquid crystals is discussed. Particular attention is paid to the obtaining of the fourth-rank order parameter ($P_4$). For monodomain samples, global target analysis of samples at different temperatures have been prepared using a mean-field model and then analysed both individually and globally by means of target analysis. The results show that there is little hope of obtaining ($P_4$) from macroscopically unoriented systems, except for some limiting cases. On the other hand, the recovery of $<P_2>$ and of the tumbling diffusion coefficient, $D_1$, seems to be quite insensitive to the selected model for the anisotropic potential.

1. Introduction

The application of the time-resolved fluorescence polarization technique to membranes and to other ordered systems can, in principle, provide a wealth of information at molecular level, ranging from order parameters and molecular mobility to photophysical quantities such as transition-moment orientations.1-3 Nevertheless, these potentialities are not easy to exploit because of the relatively large number of parameters determining the anisotropy decay as well as the problems associated with deconvolution of experimental intensities. In a series of papers4-7 we have studied the possibility of obtaining these molecular parameters in monodomain samples. In that case the situation is reasonably optimistic and we have shown both theoretically and experimentally how the global target analysis (GTA) approach, consisting of a suitable simultaneous analysis of decays at different temperatures, can remove the strong correlations often present between adjustable parameters and help in assessing the correct molecular-ordering model. The preparation of monodomain samples is, on the other hand, not always possible or easy, e.g. it is not practical in the case of a routine examination of specimens for diagnostically applications.8 In this paper we wish to consider the problem of recovering molecular parameters in those locally anisotropic systems which are macroscopically isotropic, such as membrane bilayers suspensions (see, for example, ref. 9) and thermotropic cubic liquid crystals.10 More simply, we can have the case of a bulk unoriented liquid crystal or liquid-crystal polymer with local orientational order. It is known that fluorescence depolarization can provide not only the second-rank but also the fourth-rank order parameter for the emitting chromophore. This constitutes a precious piece of structural information about the molecular arrangement in the bilayer.9 For instance, knowledge of ($P_4$) can help in discriminating between distributions with maxima at the bilayer normal (Gaussian models) or at an angle (cone models). In the last few years some groups have reported the independent determination of ($P_4$) using the fluorescent probe 1,6-diphenylhexatriene and other rod-like molecules11-19 in phosphatidylcholine model membranes. In one particular case14 it was found that two models involving different values of ($P_4$) were both consistent with experimental data, indicating the difficulty in obtaining this parameter for those systems and that the possibility of getting ($P_4$) should certainly not be taken for granted. The question we try to address is whether this difficulty can be alleviated using the GTA procedure. It should be said that even in macroscopically aligned samples the determination of ($P_4$), although possible, is far from simple, since it depends on several molecular and instrumental factors.4 In macroscopically isotropic samples we certainly expect the situation to be less favourable because of the partial averaging of the microscopic properties over angular variables. This should result in a reduced sensitivity of the experimental observables to molecular parameters, possibly requiring an instrumental or a data analysis improvement. As already mentioned, here we wish to investigate systematically the error sources and the precision limits in recovering static as well as dynamic molecular quantities from the time-resolved fluorescence anisotropy of rod-like probes dissolved in a system with locally ordered, though macroscopically isotropic, environments. It is important to realize that there is no a priori guarantee that the molecular parameters of interest can be recovered, even if the experiments are, now routinely performed and even if more or less meaningful numbers are obtained by a deconvolution treatment. For the only way to test the feasibility of the analysis problem is to act on simulated data, so that the true molecular parameters are known. To do so we first set out to generate simulated single-photon-counting fluorescence intensities20 with a plausible temperature dependence and with some of the artifacts always present in real experiments. Clearly we cannot test all possible temperature dependences so we have concentrated on some cases that we have already shown5,6 to be analysable in monodomain samples. We then analyse the decay curves as if they were true experimental ones and judge the results by comparing the recovered parameters with the input ones.

2. Theory

Here we wish to recall briefly the theory for fluorescence depolarization in macroscopically isotropic samples, referring to specific papers9,21 for more details. In a time-dependent fluorescence polarization experiment, a pulse of exciting light, plane polarized along a direction $e_x$, is absorbed by a fluorescent probe molecule dissolved in the sample at a concentration sufficiently low to avoid depolarization mechanisms other than molecular reorientation. Its fluorescence is then
observed, as a function of time, through a second polarizer, set at a direction $\varepsilon_f$. Owing to the macroscopically spherical distribution of the local director in the sample, only two independent fluorescence intensities can be observed, namely with polarizers parallel, $I_p(0)$, or perpendicular, $I_s(0)$, to the polarization of the excitation. The analytic expression of these intensities for a probe having effective cylindrical symmetry and transition moments $\mu_p$ and $\mu_s$, respectively, for the absorption and the emission are

$$I_p(0) = \left(\frac{1}{9} + \frac{2}{15}\right) \sum_{+} A_{2s}^{+} A_{2p}^{+} \phi_{p}(0)$$

$$I_s(0) = \left(\frac{1}{9} + \frac{2}{15}\right) \sum_{+} A_{2s}^{+} A_{2p}^{+} \phi_{s}(0)$$

(1)

where $F(0)$ is the intrinsic fluorescence decay, $\phi_{p,s}(t)$ are second-order correlation functions of the Wigner rotation matrices.$^{22}$

$$\phi_{p,s}(t) = (D_{2s}^{+}(0)D_{2p}^{+}(t))$$

(2)

where $\omega \equiv (\alpha, \beta, \gamma)$ is the set of Euler angles relating the laboratory to the chosen molecular frame. $A_{2s}^{+}$ and $A_{2p}^{+}$ are spherical components of the absorption and emission tensors,$^{12}$ $A = \mu \times \mu/|\mu|$ and $A = \mu \times \mu$, direct products of the transition moments, $\mu_p$ and $\mu_s$. For these systems the polarization ratio, defined as

$$r(0) = \frac{I_p(0)}{I_s(0)} = \frac{I_s(0)}{2I_s(0)}$$

(3)

has a particularly simple expression:

$$r(0) = 3 \sum_{\pm} A_{2s}^{+} A_{2p}^{+} \phi_{p,s}(0)$$

(4)

In the present paper we shall limit the investigations to the case of a rod-like molecule for which the transition moments can be assumed parallel to the principal symmetry axis, even if an arbitrary orientation can be considered. The fluorescent dye 1,6-diphenylhexatriene$^{23}$ is one of the most popular probes approximately possessing these characteristics, although the true value of the angle between the transition moments is still open to question.$^{12,14}$ With the above limitations $A_{2s}^{+} = A_{2p}^{+} = \sqrt{3}/\lambda_{\phi}$ and eqn. (5) reduces to the sum:

$$r(0) = \frac{1}{3} \phi_{p}(0) + 2 \phi_{p}(0) + 2 \phi_{p}(0)$$

(6)

The two limiting values of $r(0)$ are$^{4,27}$

$$r(0) = \frac{1}{2}$$

(7)

$$r(0) = \frac{1}{2}$$

(8)

Notice that $r(0)$ does not yield any hint about the order parameters and that $r(0)$ depends only on the squared value of $(P_2)$. Therefore the information about $(P_2)$ is contained only in the decay of $r(0)$, which is also affected by the dynamics. For a probe with effective cylindrical symmetry, the least biased orientational distribution function $f(0)$ we can gather, at a certain temperature, from a fluorescence polarization experiment, is$^{4,29}$

$$f(0) = \exp[a_2+ a_4(T)]P_2[\cos \beta] + a_4(T)P_2[\cos \beta]$$

(9)

where $\beta$ is the angle between the axis of the molecule and the director. The normalization condition of $f(0)$ determines $a_2$, while $a_4$ and $a_6$ are temperature-dependent maximum entropy parameters which should give back the experimental $(P_2)$ and $(P_2)$ when introduced into the expressions for the order parameters.$^{30}$ A practical way to employ the same concepts is to introduce an orientational anisotropic $P_2 - P_2$ potential for the probe

$$U_{probe}(\beta, T) = k_4 T[a_4(T)]P_2[\cos \beta] + a_4(T)P_2[\cos \beta]$$

(10)

from which order parameters can be evaluated.$^{30}$ As a special case we can have a pure $P_2$ or a pure $P_2$ potential if the first or the second item of the expression in eqn. (10) is present. The correlation functions $\phi_{p,s}(t)$ can be calculated as a sum of exponentials from the same potential, using the small-step diffusion theory$^{31}$ and solving the proper equation of motion, once the diffusion tensor, $D$, is known. In this way only a few parameters are sufficient to describe completely the static and dynamic molecular properties. These parameters would be the natural "target" in an analysis of fluorescence anisotropy data. Notice that these few target parameters determine the whole multieponential decay of the time-dependent anisotropies. It would be a rather bad choice to fit the decays to a sum of free exponentials. Indeed, apart from the difficulties in fitting more than a few exponentials, the pre-exponential coefficients and the decay times cannot be considered as independent quantities since they are all determined by the same set of target parameters.

We do not use the approximate short-time expressions for the diffusion correlation functions in terms of $(P_2)$ and $(P_2)$ and diffusion coefficients$^{12-21}$ sometimes employed. These approximations, called model independent since they do not depend explicitly on a previously assumed model potential, present a slight numerical convenience. However, as we have shown in ref. 6 they are drastically in error when the potential has more than one minimum as for the pure $P_2$ potential.$^{12}$ Thus it is tempting to consider whether they may be involved when surprising results are obtained, e.g. increase of the order parameters with temperature in some oriented membrane systems.$^{15}$

3. Simulation of Temperature-dependent Decay Curves

The starting point for preparing a realistic set of simulated intensities at a number of temperatures is to assign a reasonable temperature dependence to the parameters $a_2(T)$, $a_4(T)$ in the orientational potential, eqn. (10), and to the diffusion tensor, $D$. Then the correlation functions $\phi_{p,s}(t)$ are calculated by solving the rotational diffusion equation and the theoretical time-dependent fluorescence intensities are evaluated from eqns. (1) and (2). The final step is to convolute numerically these intensities with an assumed excitation pulse profile, and then add Poisson noise according to single-photon-counting statistics.

The temperature dependence for $a_2(T)$ and $a_4(T)$ has been simulated assuming a mean-field interaction between the probe and the surrounding solvent molecules. The mean-field theory for molecules interacting with second- and fourth-rank potential has been developed a long time ago by Humpty, James and Luckhurst (HJL potential).$^{24}$ In the frame of this theory the expressions for the two coefficients are

$$a_2(T) = \frac{a_2}{k_B T} \langle P_2 \rangle_{\text{D}}$$

(11)

$$a_4(T) = \frac{a_4}{k_B T} \langle P_2 \rangle_{\text{D}}$$

(12)

where $a_2$ and $a_4$ are, respectively, second- and fourth-rank solute-solvent interaction coefficients and $\langle P_2 \rangle_{\text{D}}$ are the order parameters for the pure solvent. These can be calcu-
lated applying the same HJL theory to the solvent. \( \langle P_{2} \rangle_{\text{sol}} \) and \( \langle P_{2} \rangle_{\text{air}} \) are then determined by solving a set of equations which have to be self-consistent with the anisotropic potential of the solvent \( V_{\text{sol}}(\theta) \):

\[
V_{\text{sol}}(\theta) = c_{1} \langle P_{2} \rangle_{\text{sol}} \cos^{2} \theta + \lambda_{\text{air}} \langle P_{4} \rangle_{\text{sol}} \cos^{4} \theta.
\]

Here \( c_{1} \) is an average solvent–solvent interaction energy and \( \lambda_{\text{air}} \) is the ratio between the fourth- and second-rank coefficients. As a special case, the Maier–Saupe potential would be obtained by putting \( \lambda_{\text{air}} = 0 \). In the frame of mean-field theory, the solvent order parameters are determined at a certain temperature, \( T \), once \( \lambda_{\text{air}} \) and \( \phi_{\text{rot}} \), the nematic-isotropic transition temperature, have been fixed. It is clear from eqn. (10) that the temperature dependence of the potential \( V_{\text{sol}}(\theta) \) explicitly reflects that of the solvent order parameters. Eqn. (13) strictly applies only to thermotropic nematic mesophases, so that its application in the simulation of the behaviour of a membrane system may seem inappropriate. However, as will be seen later on, the temperature dependence obtained for the order parameters is a rather plausible one.

4.1 Individual Target Analysis (ITA)

Each decay is analysed independently using the previously proposed target analysis. In this way the parameters \( D_{\perp}(T) \), \( a_{\|}(T) \) and/or \( a_{\perp}(T) \) (depending on the potential assumed, namely pure \( P_{2} \), pure \( P_{3} \) or \( P_{2} - P_{4} \) type) are fitted separately at each temperature. Scaling factor, time shift and scattered light are optimized together with the anisotropy parameters. The results from ITA were also employed to evaluate the trial starting parameters for the global analyses.

4.2 Husneps–James–Lutkher Globalization

This approach is simply a test of the actual possibility of recovering for all the parameters the same values used in the simulations. However, the fitted parameters are \( D_{\perp}, E_{\theta} \) [eqn. (14)], \( \lambda_{\text{air}} \) [eqn. (13)], \( u_{\alpha_{1}} \) and/or \( u_{\alpha_{2}} \) [eqn. (11) and (12)], depending on the potential used. The failure of this test would indeed exclude the possibility of recovering all the parameters involved in the problem; in other words the fitting would be meaningless because of the intrinsically scarce information contained in the data.

4.3 Interpolating Global Target Analysis

From what we have discussed until now it is clear that the analysis of polarized intensities relies on a complex nonlinear least-squares deconvolution procedure. Moreover, the problem might be "ill conditioned" in the sense that it could admit more than one statistically acceptable solution corresponding to different molecular target parameters. Indeed, it is dubious if the solution corresponding to the smallest sum of squared residuals \( \chi^{2} \) is by necessity the most meaningful one. We have thus introduced a global target analysis\(^{6,7} \) that tries to limit the parameter space available to the fitting by linking various sets of experimental data at different temperatures and analysing them simultaneously. The simplest cases are when a parameter is constant for various measurements,\(^{42,44} \) or when a physical law for the temperature dependence of a certain parameter can be established (for example, an Arrhenius behaviour for the diffusion coefficients). However, we believe that linking can be carried out in a much more general way by implementing the constraint that the data fall on a continuous and differentiable curve of a rather general nature. Thus a spline or polynomial interpolating function could be used. Elsewhere\(^{45} \) we have seen that even a simple polynomial relationship can be very
useful in obtaining reliable data from monodomain samples. In the present paper a quadratic temperature dependence has been assumed for \(a_2(T)\) and \(a_4(T)\):

\[
a_2(T) = \lambda_2, 2 + \lambda_2, 1(T - T_{\text{min}}) + \lambda_2, 0(T - T_{\text{min}})^2 \tag{16}
\]

\[
a_4(T) = \lambda_4, 4 + \lambda_4, 2(T - T_{\text{min}}) + \lambda_4, 0(T - T_{\text{min}})^2 \tag{17}
\]

where \(\lambda_2, 1, \lambda_2, 2, \lambda_2, 4, \lambda_4, 2, \lambda_4, 4\) and \(\lambda_4, 0\) have to be globally optimized together with \(D_{ij}^2\) and \(E_{ij}\). \(T_{\text{min}}\) is the lowest temperature introduced in the analysis. Obviously some of these coefficients have to be fixed to zero when a pure \(P_2\) or pure \(P_4\) potential is assumed. The goodness of the fit is assessed by simply using the \(\chi^2\) test,\(^4\) i.e. we retain all those models which give a reduced \(\chi^2(\nu)\) below a certain critical value. We have also applied the \(F\)-test\(^4\) to verify if two models with different numbers of parameters and both giving a statistically significant \(\chi^2\) can be discriminated. Although these tests are designed for least-squares procedures linear in the fitting parameters, they can also be used in fluorescence decay deconvolutions (which are strictly non-linear) because of their near-linearity in the proximity of the minimum of the \(\chi^2\). Finally, we wish to stress that we have used only statistical tests to reject or accept a particular model, thus treating simulated data as if they were true experimental ones.

5. Results and Discussion

Four data series, each made up of ten simulations, have been generated using the scheme described in Section 3. Every series was set up to study a particular type of potential. The results will be discussed in turn.

5.1 Pure \(P_2\) Potential

At first we studied the case of a probe subjected to a pure \(P_2\) potential with parameters \(a_2/\Delta k_{\text{max}} = 1600\) K, \(a_4/\Delta k_{\text{max}} = 0\) K and diffusional coefficients \(D_{ij}^2 = 800\) nm\(^2\) s\(^{-1}\) and \(E_{ij} = 6\) kcal mol\(^{-1}\). The theoretical polarization ratios corresponding to the studied temperature are shown in Fig. 1, while in Fig. 2 we report the order parameters and the perpendicular diffusion coefficient as a function of temperature.

The decays have been analysed using the three different strategies reported in Section 4, and assuming both a pure \(P_2\) and a \(P_2 - P_4\) potential. The results, expressed as differences between the recovered and the simulated (true) values are reported in Fig. 2B-D. In Tables 1 and 2 (entry 1) we also report the fitted global parameters.

The analyses with a pure \(P_2\) potential (labelled (a) and (b) in Fig. 2B-D) do not exhibit any particular difficulty in recovering the simulated values. In fact both individual and global analyses give very good results. Moreover, the recovered parameters are independent of the trial ones, showing that the \(\chi^2\) surface has a single minimum.

Analysing the same curves with the ITA method and a \(P_2 - P_4\) potential gives results rather contradictory and strongly dependent on starting values. In particular, we found two distinct solutions with about the same \(\chi^2\). A similar behaviour was also reported by Levine and co-workers in real systems.\(^4\) In Fig. 3 we show the parameters corresponding to these two solutions together with the associated \(\chi^2\). In this case the spurious solution has a negative \(\langle P_2\rangle\), with an anomalous temperature dependence.

### Table 1

Results from parabolic global target analysis obtained assuming a pure \(P_2\) (A), a \(P_2 - P_4\) (B) and a pure \(P_4\) model (C) to analyse the various series of simulations generated, respectively, with a pure \(P_2\) potential (1), a \(P_2 - P_4\) potential with contributions of opposite sign (2), a \(P_2 - P_4\) potential with contributions of the same sign (3) and a pure \(P_4\) potential (4).

<table>
<thead>
<tr>
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<th>1. pure (P_2)</th>
<th>2. (P_2 - P_4) opposite sign</th>
<th>3. (P_2 - P_4) same sign</th>
<th>4. pure (P_4)</th>
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<tr>
<td>(\lambda_{a, 1})</td>
<td>3.8364</td>
<td>5.7387</td>
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<td>1.8026</td>
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<td>(\lambda_{a, 2}/\Delta k_{\text{max}})</td>
<td>-0.0266</td>
<td>-0.0565</td>
<td>-0.0118</td>
<td>-0.0509</td>
</tr>
<tr>
<td>(\lambda_{a, 4}/\Delta k_{\text{max}})</td>
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<td>-0.0000</td>
<td>-0.0001</td>
<td>0.0002</td>
</tr>
<tr>
<td>(\lambda_{a, 4}/\Delta k_{\text{max}})</td>
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<td>-0.0131</td>
<td>-0.0131</td>
<td>-0.0131</td>
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<tr>
<td>(D_{ij}^2/\text{nm}^2\text{s}^{-1})</td>
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<td>883</td>
<td>830</td>
<td>733</td>
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<tr>
<td>(E_{ij}/\text{kcal mol}^{-1})</td>
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<td>6.05</td>
<td>6.02</td>
<td>5.93</td>
</tr>
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<td>(\chi^2)</td>
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<td>5279</td>
<td>5198</td>
<td>5173</td>
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</table>

* Value of \(\chi^2\) corresponding to the 5% probability of exceeding \(\chi^2\) with the appropriate degrees of freedom.

Fig. 1 Simulated anisotropies, \(I(ab)\), for a rod-like probe with transition moments parallel to the long axis in a pure \(P_2\) potential with coefficients \(a_2/\Delta k_{\text{max}} = 1600\) K, \(a_4/\Delta k_{\text{max}} = 0\) K in a macroscopically isotropic monolayer governed by a Maier-Saupe anisotropic potential \(\Delta k_{\text{max}} = 0\) in eqns. (13). Here \(D_{ij}^2 = 800\) nm\(^2\) s\(^{-1}\) and \(E_{ij} = 6\) kcal mol\(^{-1}\). Curves (a)-(c) correspond to temperatures from 5 to 30°C in steps of 5°C. The transition temperature of the system has been fixed to 52°C, channel width 0.1 ns.

\(F\)-test, \(\chi^2\) for a rod-like probe with transition moments parallel to the long axis in a pure \(P_2\) potential with coefficients \(a_2/\Delta k_{\text{max}} = 1600\) K, \(a_4/\Delta k_{\text{max}} = 0\) K in a macroscopically isotropic monolayer governed by a Maier-Saupe anisotropic potential \(\Delta k_{\text{max}} = 0\) in eqns. (13). Here \(D_{ij}^2 = 800\) nm\(^2\) s\(^{-1}\) and \(E_{ij} = 6\) kcal mol\(^{-1}\). Curves (a)-(c) correspond to temperatures from 5 to 30°C in steps of 5°C. The transition temperature of the system has been fixed to 52°C, channel width 0.1 ns.
Fig. 2 Results of the analyses of the curves simulated with a pure \( P_2 \) potential. A, Values of \( \langle P_2 \rangle \) (\( P_2 \)) (a) and \( D_2 \) (b) used in the simulations for the pure \( P_2 \) potential: the corresponding coefficients are reported in the caption of Fig. 1. The continuous line shows the complete temperature dependence. B, Results from individual target analyses assuming both a pure \( P_2 \) (a), (b) and a \( P_3 - P_4 \) (c), (d) potential. The symbols correspond to the difference between recovered and true values for \( \langle P_2 \rangle \) (\( \bigcirc \)), \( P_2 \) (\( \bullet \)) and \( D_2 \) (\( \bigotimes \)). Here the continuous line is only a guide. C, Results from parabolic global target analysis assuming both a pure \( P_2 \) (a), (b) and \( P_3 - P_4 \) (c), (d) potential. Symbols have the same meaning as in B. D, Results from HJL global target analysis assuming both a pure \( P_2 \) (a), (b) and a \( P_3 - P_4 \) (c), (d) potential. Symbols as in B. \( z^2 = 1.039 \)

Table 2 Results from HJL global target analysis obtained assuming a pure \( P_2 \) (A), a \( P_3 - P_4 \) (B) and a pure \( P_a \) model (C) to analyse the various series of simulations generated, respectively, with a pure \( P_2 \) potential (1), a \( P_3 - P_4 \) potential with contributions of opposite sign (2), a \( P_3 - P_4 \) potential with contributions of the same sign (3) and a pure \( P_a \) potential (4).

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<th>4, pure ( P_a )</th>
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</thead>
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<td>B</td>
<td>A</td>
<td>B</td>
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<td>( F \text{-test} )</td>
<td>1.17</td>
<td>1.17</td>
<td>1.17</td>
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<tr>
<td>( F \text{crit} )</td>
<td>3.84</td>
<td>3.84</td>
<td>3.84</td>
<td>3.84</td>
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</tbody>
</table>

* Limiting value imposed in the fitting. * Value of \( z^2 \) corresponding to the 5% probability of exceeding \( z^2 \) with the appropriate degree of freedom. * LOF (lack of fit) means that the F-test is not applicable because of the failure of the \( z^2 \) test. * Value of \( F \) corresponding to the 5% probability of exceeding \( F \) with the appropriate degrees of freedom.
would do in monodomain samples. In other words, the strong correlation between $\langle P_D \rangle$ and $D_s$, present in those systems, seems to be removed in membranes. This may be explained noticing that the initial value of the polarization ratio eqn. (7) does not depend on the order parameters.

The results from global analyses with a $P_3 - P_4$ potential are apparently contradictory. In the parabolic global analysis the introduction of the fourth-rank parameters [Fig. 2C (c) and (d)] gives a statistically significant reduction of $\chi^2$ according to the F-test (see Table 1), but in the HJL global analysis [Fig. 2D (c) and (d)] it does not (see Table 2). Therefore, it is clear that the parabolic approximation eqn. (1b) is only partially sufficient to reproduce all the features of the $a_5(T)$ temperature dependence. So the introduction of additional parameters, even of different rank, can give apparently significant reductions of $\chi^2$ leading to erroneous conclusions about the potential involved. Obviously, the values recovered for $\langle P_D \rangle$ are incorrect [see Fig. 2C (c)].

In the HJL global analysis we use in the fit the same equations adopted for the simulations, so that is possible to demonstrate the redundancy of the fourth-rank parameter. Unfortunately, in real systems we can make a reasonable hypothesis only about the temperature dependence of the parameter $a_5(T)$, then we believe that the introduction of such complex potentials as $P_3 - P_4$ is correct only when simpler potentials such as pure $P_2$ or pure $P_3$ have to be rejected on the basis of the $\chi^2$-test.

5.2 $P_3 - P_4$ Potential with Coefficients of Different Sign

We examined the case of a probe subjected to a $P_3 - P_4$ potential, with second- and fourth-rank contributions of opposite sign, $a_3 = 800$ K, $a_4 = -800$ K), and the same temperature dependence of the diffusion coefficient as in the previous case. For this potential, the values of $\langle P_D \rangle$ are close to zero and negative. The theoretical polarization ratios are shown in Fig. 5B, while in Fig. 7 we report the order and diffusional parameters corresponding to the studied temperatures.

As with the previous case, the decays have been analysed individually with ITA and globally, assuming both a pure $P_2$ and a $P_3 - P_4$ potential. The results are reported in Fig. 5B-D and in Tables 1 and 2 (entry 2).

The individual target analyses with a pure $P_3$ potential are not able to point out the inadequacy of the assumed model.

This kind of behaviour was also detected in the other series of simulations corresponding to the potentials we shall discuss in more detail later. In the worst cases, four different solutions with a statistically acceptable $\chi^2$ were found. We then decided to adopt a general approach in ITA. We have fitted each curve starting from four different sets of trial parameters and choosing the solution with the smallest $\chi^2$.

Afterwards, these solutions were used to evaluate the trial parameters in the global analyses.

In Fig. 2B (c) and (d) we report the results from individual target analyses, selected according to this criterion. In spite of its simplicity it leads to good results for $\langle P_D \rangle$ and $D_s(T)$. On the other hand, the recovered values of $\langle P_D \rangle$ are rather rough, but this does not affect the precision on $D_s$, as it

Fig. 3 Spurious results obtained from individual target analyses assuming a general $P_3 - P_4$ model for the curves generated with a pure $P_2$ potential. Here we report the $\langle P_D \rangle$ (a), $\langle P_D \rangle$ (b), $D_s$ (c) and $\chi^2$ (d) values corresponding to the physically correct (C) and the spurious (X) solution. The continuous lines in (a), (b) and (c) correspond to the full temperature dependence of the exact parameters, while in (d) the line is only a guide

Fig. 4 Simulated anisotropies, $r(t)$, for a rod-like probe with transition moments parallel to the long axis in a $P_3 - P_4$ potential with coefficients $a_3 = 800$ K, $a_4 = -800$ K, in a macroscopically isotropic mesophase governed by a Maier-Saupe anisotropic potential and with $T_m = 32^\circ$ C. Here $D_s^0 = 0.9$ nm$^{-1}$ and $E_s = 6$ kcal mol$^{-1}$. The labelling of the curves is the same as in Fig. 1.
In fact, they all lead to acceptable \( x^2 \) values, but the recovered \( \langle P_{2s} \rangle \) values are wrong. Rather surprisingly, the assumption of an incorrect model does not affect the determination of \( D_1 \), which is indeed recovered with good precision [see Fig. 5B (a) and (b)]. Even with a \( P_2 - P_4 \) potential the ITA strategy shows the impossibility of recovering the true values of the order parameters and, in particular, of \( \langle P_{2s} \rangle \), as we can see from Fig. 5B (c) and (d). We have also analyzed the curves globally, but this just confirmed the intrinsic problems of the simulated potential. Using both the parabolic and the HJL global target analysis with a pure \( P_2 \) potential we obtain an acceptable \( x^2 \). With a \( P_2 - P_4 \) potential \( x^2 \) decreases significantly, on the basis of the F-test (see Tables 1 and 2), but even in this case the results are completely wrong. On the other hand, the recovered \( D_1 \) are always very good (see Fig. 5C and D). So it is clear that for this potential one can only obtain some scarce information about \( \langle P_{2s} \rangle \) and no information about \( \langle P_{2s} \rangle \). Therefore, on the basis of the previous considerations, the pure \( P_2 \) is the only model that is reasonably reliable.

5.3 \( P_2 - P_4 \) Potential with Coefficients of the Same Sign

In the third case we considered a \( P_2 - P_4 \) potential with second- and fourth-rank contributions of the same sign (\( u_4/k = 800 \text{ K}, u_6/k = 800 \text{ K} \)). The dynamical coefficients were fixed to \( D_2 = 1200 \text{ ns}^{-1} \) and \( E_2 = 6 \text{ kcal mol}^{-1} \). In Fig. 6 we report the theoretical polarization ratios and in Fig. 7A the order and dihedral parameters. At first glance they are quite similar to those of the first case. The curves have been analysed as previously described and the results are reported in Tables 1 and 2 (entry 3), and plotted in Fig. 7B-D.

On adopting a pure \( P_2 \) model, both the ITA and the global analyses converge to a good \( x^2 \); recovering well the order parameter \( \langle P_{2s} \rangle \) and the diffusional coefficient \( D_1 \), but leading to a completely incorrect determination of \( \langle P_{2s} \rangle \) [see plots (a) and (b) in Fig. 7B-D]. Using the \( P_2 - P_4 \) model, \( x^2 \) generally decreases slightly for the individual target analyses, even if in some cases \( \langle P_{2s} \rangle \) is still badly recovered [see Fig. 7B (c) and (d)]. A similar improvement has been found for \( x^2 \) in the parabolic and, more markedly, in the HJL global target analysis. Moreover, the globalization has, in both cases, the effect of considerably enhancing the precision in the determination of \( \langle P_{2s} \rangle \) [see (c) and (d) in Fig. 7C and D].

In this case the F-test would confirm the reliability of the fourth-rank parameters (see Tables 1 and 2), but in our opinion they have anyway to be rejected. In fact, the previous cases show how the assumption of a \( P_2 - P_4 \) potential, for systems giving good results just on the basis of the \( x^2 \)-test with the simple pure \( P_2 \) model, can easily lead to wrong.
results, in particular if the parabolic global target analysis is applied.

5.4 Pure $P_4$ Potential

The second limiting case of the $P_3 - P_4$ potential is the pure $P_4$ model. Its most interesting feature is that the value of the order parameter $P_4$ is larger than that of $P_3$ from zero to nearly complete order. This behaviour has been found to be adequate to analyse the fluorescence intensities of the probe 1,6-diphenylhexatriene in single-component lecithin vesicles. The parameters used to simulate the curves for this model were $u_d/k_B = 0$ K, $u_4/k_B = 3000$ K, $D_0^d = 1200$ ns$^{-1}$ and $E_4 = 6$ kcal mol$^{-1}$. The labeling of the curves is the same as in Fig. 1.

Fig. 6 Simulated anisotropies, (a), for a rod-like probe with transition moments parallel to the long axis in a $P_3 - P_4$ potential with coefficients $u_d/k_B = 800$ K, $u_4/k_B = 800$ K in a macroscopically isotropic mesophase governed by a Maier-Saupe anisotropic potential and with $T_m = 52$ C. Here $D_0^d = 1200$ ns$^{-1}$ and $E_4 = 6$ kcal mol$^{-1}$. The labeling of the curves is the same as in Fig. 1.

Fig. 7 Results of the analyses of the curves simulated with a $P_3 - P_4$ potential: the corresponding coefficients are reported in the caption of Fig. 6. The labeling of the curves and the meaning of the symbols are the same as in Fig. 2. Note $\chi^2 = 1.039$ [C (a), (b)], 1.031 [C (c), (d)], 1.105 [D (a), (b)], 1.031 [D (c), (d)].
second-rank contribution one order of magnitude smaller than the fourth-rank one. Nevertheless, order parameters and diffusional coefficients are affected by large errors, as shown in Fig. 9B (c) and (d). Using the pure $P_x$ potential generally leads to an acceptable $x^2$ and to good results for the order parameters, but at the highest temperatures the values of $D_x$ are still wrong (see Fig. 9B (a) and (b)). This is mainly due to the fast decay of $r(1)$ (Fig. 8), which prevents good precision being achieved in the determination of the dynamical parameters.

Global analyses were carried out using all three of the models and the numerical results are reported in Tables 1 and 2 (entry 4). In Fig. 9C and D we show only those corresponding to the pure $P_x$ and $P_2 - P_x$ potential. For this case, the F-test applied to the parabolic and to the Hjl global target analysis indicates that the second-rank parameters are statistically meaningless, therefore firmly assigning the pure $P_x$ model.

Conclusions

It is evident that the possibility of obtaining order parameters and diffusional coefficients from time-dependent fluorescence

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Fig. 8 Simulated anisotropies, $r(1)$, for a rod-like probe with transition moments parallel to the long axis in a pure $P_x$ potential with coefficients $u_x/b_x = 0$ K, $u_y/b_y = 3000$ K in a macroscopically isotropic mesophase governed by a Maier-Saupe anisotropic potential and with $T_m = 52$ C. Here $D_1 = 3000$ ns$^{-1}$ and $E_x = 6$ kcal mol$^{-1}$. The labelling of the curves is the same as in Fig. 1

Fig. 9 Results of the analyses of the curves simulated with a pure $P_x$ potential. A. Values of $\langle P_x \rangle$, $\langle P_y \rangle$ (a) and $D_x$ (b) used in the simulations for the pure $P_x$ potential; the corresponding coefficients are reported in the caption of Fig. 8. The continuous line shows the complete temperature dependence. B. Results from individual target analyses assuming both a pure $P_x$ (a), (b) and a $P_2 - P_x$ (c), (d) potential. The symbols correspond to the difference between recovered and true values for $\langle P_x \rangle$ (C), $\langle P_y \rangle$ (D) and $D_x$ (E). The continuous line is only a guide. C. Results from parabolic global target analysis assuming both a pure $P_x$ (a), (b) and a $P_2 - P_x$ (c), (d) potential, $x^2 = 1.067$. Symbols have the same meaning as in B. D. Results from Hjl global target analysis assuming both a pure $P_x$ (a), (b) and a $P_2 - P_x$ (c), (d) potential. Symbols as in B, $x^2 = 1.067$. 
anisotropy data in membrane vesicles should not be taken for
granted. In general, we have verified that the information one
can extract from these systems is reduced compared with that
obtainable for monodomain samples. In particular, recov-
ering the fourth-rank order parameter and discriminating be-
between different orienting potentials is very difficult even
when transition moments are known. These structural data
are, in fact, extracted simply from the decay of the aniso-
tropy. Indeed we have found that often the simple pure \( P_2 \)
potential is sufficient to obtain apparently good and sta-
tistically significant results. This deceivingly simple good fit
may occur even if the second and the fourth-rank contribu-
tions to the true anisotropic potential are equally important.
In our experience the statistical F-test cannot resolve the
ambiguities about the model because the anisotropy curves
are in many cases scarcely sensitive to the fourth-rank order
parameter.

We have also seen that, for certain anisotropic potentials
(see Section 5.2), even adopting in the fitting the same expres-
sion used to generate the data is not sufficient to warrant
that the correct parameters will be obtained. Thus, in certain situa-
tions the problem is mathematically 'ill posed'.

On the other hand, some of the parameters, and notably
\( P_2 \) and \( D_1 \), can be reliably obtained. Also the order parameter
\( P_2 \) can be accurately extracted in the case of a
dominant fourth-rank anisotropic potential or a pure \( P_2 \)
potential.

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