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Rapid report

# Incorporation of the V-ATPase inhibitors concanamycin and indole pentadiene in lipid membranes. Spin-label EPR studies

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**Abstract**

The incorporation of concanamycin A, a potent inhibitor of vacuolar ATPases, into membranes of dimyristoyl phosphatidylcholine has been studied by using EPR of spin-labelled lipid chains. At an inhibitor/lipid ratio of 1:1 mol/mol, concanamycin A broadens the chain-melting transition of the phospholipid bilayer membrane, and effects the lipid chain motion in the fluid phase. The outer hyperfine splitting of a spin label at the C-5 position and the line widths of a spin label at the C-14 position of the lipid chain are increased by concanamycin A. Considerably larger membrane perturbations are caused by equimolar admixture of a designed synthetic 5-(5,6-dichloro-2-indolyl)-2,4-pentadienoyl V-ATPase inhibitor. These results indicate that concanamycin A intercalates readily between the lipid chains in biological membranes, with minimal perturbation of the bilayer structure. Essentially identical results are obtained with concanamycin A added to preformed membranes as a concentrated solution in DMSO, or mixed with lipid in organic solvent prior to membrane formation. Therefore, the common mode of addition in V-ATPase inhibition assays ensures incorporation of concanamycin into the lipid bilayer milieu, which provides an efficient channel of access to the transmembrane domains of the V-ATPase.


*Keywords:* Concanamycin; Bafilomycin; V-ATPase; Lipid bilayer; Spin label; EPR

The plecomacrolides concanamycin A and bafilomycin $A_1$ are potent inhibitors of vacuolar ATPases (V-ATPases), with effectivity in the low nanomolar range (see, e.g., Refs. [1,2]). Blocking the extracellular acidification that is mediated by osteoclast V-ATPases provides a direct means of inhibiting bone resorption, the pathology of which is associated with osteoporosis [3,4]. The mechanism of membrane action of hydrophobic V-ATPase inhibitors, such as concanamycin, which are thought to interact with the transmembrane $V_o$-sector of the ATPase [5,6] is thus of considerable pharmacological interest.

In the present work, we characterise the interaction of concanamycin A (see Scheme 1) with lipid bilayer membranes by using EPR of phospholipids spin-labelled in their *sn*-2 chain. This method is well-suited to studying the interaction of hydrophobic drugs with lipid membranes [7,8]. Comparison is also made with a synthetic 5-(5,6-dichloro-2-indolyl)-2,4-pentadienoyl V-ATPase inhibitor that was designed according to structure–function relationships of concanamycin and bafilomycin derivatives [9]. Intercalation of concanamycin between the lipid chains is demonstrated here by broadening of the chain-melting transition of the lipid membrane and by limited increases in spin-label spectral anisotropy and line widths in the fluid phase. Most significantly, quantitatively similar spectral perturbations are produced by concanamycin added exogeneously (in dimethyl sulfoxide, DMSO), as by direct mixing of concanamycin with lipid prior to membrane formation.

Dimyristoyl phosphatidylcholine (DMPC) was from Avanti Polar Lipids (Alabaster, AL) and concanamycin A ($\geq$90% pure) from Fluka (Buchs, Switzerland). Spin-labelled phosphatidylcholines (1-acyl-2-[*n*-(4,4-dimethyloxazolidine-*N*-oxyl) stearoyl]-*sn*-glycero-3-phosphocholine, *n*-PCSL) were synthesized according to Ref. [10]. The inhibitor 5-(5,6-dichloro-2-indolyl)-2-methoxy-*N*-(1,2,2,6,6-pentam-

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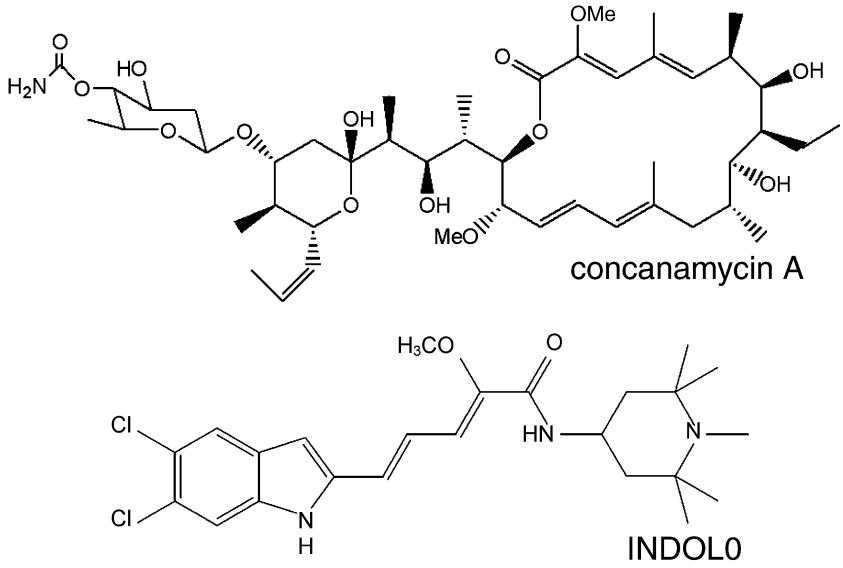


Scheme 1. Chemical structures of concanamycin A and INDOL0.

ethylpiperidin-4-yl)-2,4-pentadienamide (INDOL0—see Scheme 1) was synthesized according to Refs. [11,12].

DMPC (in $CH_2Cl_2$) and concanamycin A (in MeOH) were mixed in equimolar ratio, together with 0.5 mol% *n*-PCSL, and the solution then evaporated with dry nitrogen. After keeping under vacuum overnight, the dry mixture was hydrated in 50 μl of 10 mM Hepes, 10 mM NaCl, 10 mM EDTA, pH 7.8 buffer, with vortex mixing at 37 °C. Samples containing INDOL0 were prepared similarly. Alternatively, concanamycin was added, as a concentrated solution in 15 μl of DMSO, to the spin-labelled lipid dispersion already hydrated in 200 μl of buffer. The dispersion was incubated above the lipid chain-melting temperature and excess DMSO then removed by centrifugation and washing. Addition of an equal volume of DMSO alone was used for a control lipid dispersion. The lipid dispersions were finally transferred to 1-mm diameter glass capillaries and pelleted in a benchtop centrifuge. Excess supernatant was removed and the capillaries were flame-sealed. All sample handling was performed in glass vessels.

Conventional in-phase EPR spectra were recorded either on a Varian Century Line, or on a Bruker EMX, 9 GHz spectrometer that was thermostatted by nitrogen gas-flow. The sample capillary was accommodated in a standard quartz EPR tube that contained light silicone oil for thermal stability. Temperature was measured by a fine-wire thermocouple located in the silicone oil at the top of the microwave cavity. The membrane sample was centred vertically in the rectangular $TE_{012}$ resonator.

Fig. 1 shows EPR spectra of the 5-PCSL phosphatidylcholine spin label in membranes of DMPC, with or without concanamycin A included in the membrane preparation. Incorporation of concanamycin A decreases the mobility (i.e., increases the spectral anisotropy) of 5-PCSL in both gel and fluid phases of DMPC. The largest spectral changes are found in the region of the chain-melting transition at 24 °C.

Fig. 2A shows the temperature dependence of the maximum outer hyperfine splitting, $2A_{max}$, from the 5-PCSL spin label in DMPC membranes, with or without 1:1 mol/mol concanamycin A. The values of $A_{max}$ reflect the amplitude (and in the slow-motion regime, also the rate) of motion of the lipid chain segment at the C-5 position [13]. Incorporation of concanamycin A within the hydrophobic interior of the membrane is indicated by the broadening of the lipid chain-

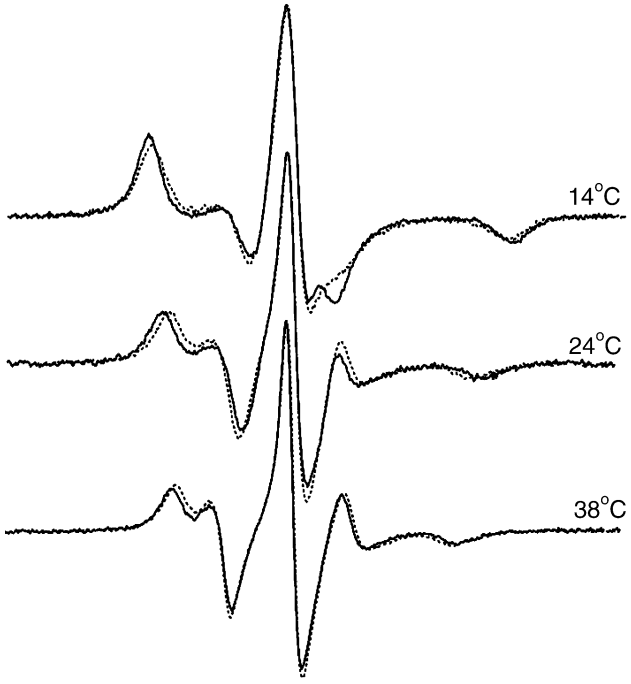


Fig. 1. EPR spectra of 5-PCSL spin label in DMPC membranes with (solid lines) and without (dashed lines) 1:1 mol/mol concanamycin A. Spectra were recorded at the temperatures indicated in the gel (14 °C), transitional (24 °C) and fluid (39 °C) phases. Total scan width = 100 gauss.

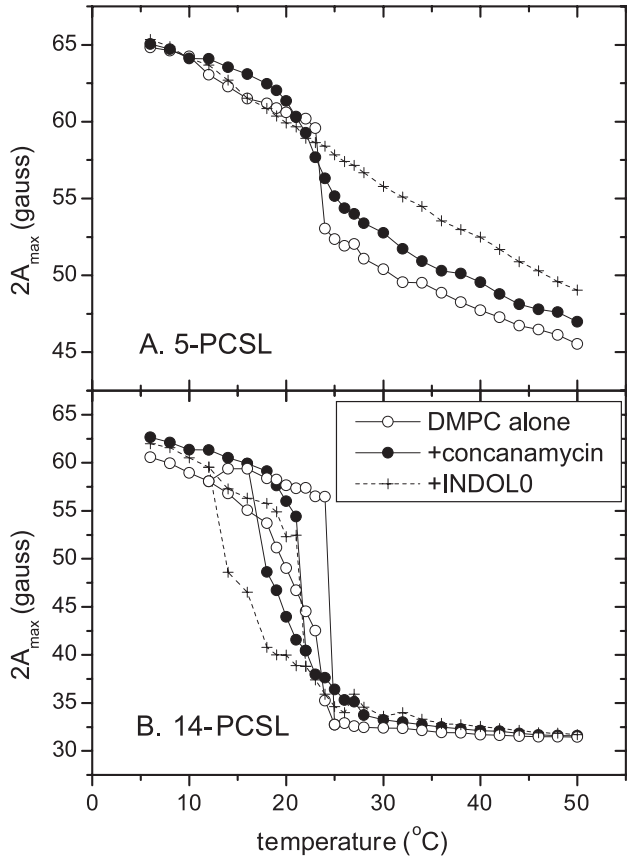


Fig. 2. Temperature dependence of the outer hyperfine splittings, $2A_{max}$, in the EPR spectra of *n*-PCSL spin labels in DMPC membranes that are formed in the presence (solid symbols) and absence (open symbols) of equimolar concanamycin A, or in the presence of equimolar INDOL0 (crosses). (A) 5-PCSL spin label; (B) 14-PCSL spin label. In the region of the transition and the intermediate gel phase, two-component spectra are observed for 14-PCSL.

melting transition. The latter is characterised by the sharp decrease in $A_{max}$ at 23 °C, for DMPC membranes in the absence of the macrolide inhibitor. The increase in transition width corresponds to a reduction in cooperativity of the lipid chain packing by incorporation of concanamycin. The mid-point of the transition is not shifted appreciably, which indicates that concanamycin has no great preference for association with fluid, as opposed to gel, DMPC lipids.

In the fluid $L_\alpha$ phase, the outer hyperfine splitting is greater in the presence of concanamycin than in its absence. Calculation of effective order parameters and isotropic hyperfine splitting constants throughout the fluid phase [14] indicates that this spectral change corresponds to a limited decrease in angular amplitude of chain-segmental motion, rather than to any significant increase in environmental polarity, by concanamycin (see, e.g., Ref. [15]). Below the chain-melting transition, the lipid chain mobility is significantly decreased by concanamycin in the intermediate ripple phase ($P_{\beta'}$), possibly by suppressing the pretransition. No significant difference in chain mobility is detected at $\leq 10$ °C in the $L_{\beta'}$ gel phase, however.

Fig. 2B shows the temperature dependence of the outer hyperfine splitting from 14-PCSL for which the spin-label is situated deeper in the membrane than 5-PCSL, close to the terminal methyl groups of the chains. In this case, there is little difference in the values of $A_{max}$, with and without concanamycin, in the fluid phase. This is because the EPR spectra are essentially isotropic, corresponding to large-amplitude segmental angular fluctuations at this position of chain labelling, and concanamycin does not restrict the amplitude of motion sufficiently to produce a significant increase in $A_{max}$. Effects on spectral line widths are seen, however, as are reflected by very small differences in the effective values of $A_{max}$ in the fluid phase (Fig. 2B). These differences are in the same sequence and direction as the much larger true changes in $A_{max}$ that are recorded for 5-PCSL in Fig. 2A. The line broadening effects for 14-PCSL will be reported further below, in connection with exogeneous addition of concanamycin. In the intermediate gel phase and transition region, the spectra of 14-PCSL consist of two partially resolved components that correspond to lipid populations of differing mobility. This coexistence of spectral components, with varying relative intensity, is indicated by the doubling of the data points at each temperature and complicates the interpretation of the effects of inhibitors in this region. A limited decrease in mobility by concanamycin is observed in the low-temperature $L_{\beta'}$ gel-phase. Whether a significant shift in transition temperature is observed is difficult to decide from the temperature dependence of $A_{max}$, but see further below.

For comparison, similar experiments were conducted with the synthetic 5-(5,6-dichloro-2-indolyl)-2,4-pentadienoyl V-ATPase inhibitor INDOL0. Fig. 2A shows the temperature dependence of the outer hyperfine splitting, $2A_{max}$, from 5-PCSL in DMPC membranes containing equimolar INDOL0. The perturbations of lipid mobility by INDOL0 are considerably larger than those by equimolar concanamycin A, especially in the fluid phase. Fig. 2B shows corresponding data obtained with 14-PCSL. Within the limitations for 14-PCSL that were already stated, these data mirror the effects of INDOL0 relative to concanamycin that are recorded by 5-PCSL. Equimolar INDOL0 attenuates the lipid chain-melting transition (Fig. 2A), rather as does cholesterol (see, e.g., Ref. [16]). Most likely, INDOL0 is anchored at the lipid polar–apolar interface by the dipolar properties of the indole group, as found for other indole-containing amphiphiles [17].

Fig. 3A compares the effects, on the 5-PCSL outer hyperfine splitting, of concanamycin added exogeneously to preformed DMPC membranes with those in which concanamycin is codissolved with DMPC in organic solvent during membrane preparation (i.e., as in Fig. 2). For these experiments, a much smaller amount of lipid (100 μg) was dispersed in a larger volume of buffer than is normal in a spin-label EPR experiment. This was done in order to emulate better the conditions under which inhibition experiments are routinely conducted. The consequent reduction in

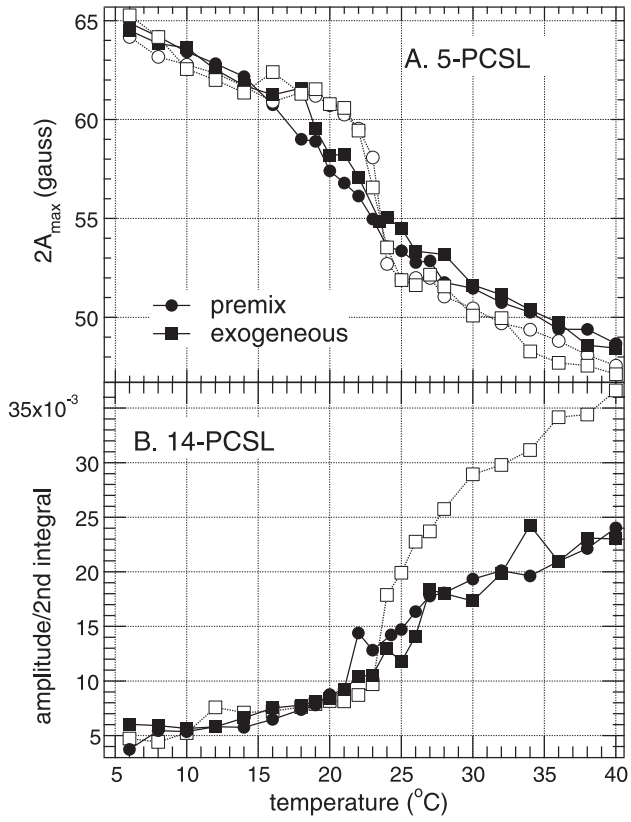


Fig. 3. Effect of concanamycin on the temperature dependence of the EPR spectra from *n*-PCSL spin labels in DMPC membranes. Solid symbols are samples containing concanamycin A at 1:1 mol/mol nominal inhibitor/lipid ratio, and open symbols are control DMPC membranes. (–●–) concanamycin premixed with DMPC in organic solvent (prior to membrane formation); (–■–) concanamycin in DMSO added to preformed DMPC membranes. Control for the latter (···□···) has 15 μl of DMSO, without concanamycin, added. (A) Outer hyperfine splitting, $2A_{max}$, of the 5-PCSL spin label; (B) ratio of maximum spectral amplitude to double-integrated intensity of the (first-derivative) spectrum of the 14-PCSL spin label.

EPR signal-to-noise results in a greater scatter of the experimental measurements, compared with those in Fig. 2. Exogeneously added concanamycin produces a broadening of the chain-melting transition and increase in $2A_{max}$ in the fluid phase that is similar to that produced by concanamycin mixed with lipid prior to membrane formation. To within the experimental scatter, the perturbations produced in the fluid phase by the two methods of addition also agree quantitatively. Addition of DMSO to control membranes produces little significant perturbation throughout the temperature range studied (note that membranes are washed subsequent to exogeneous addition of concanamycin, in order to remove excess DMSO).

Fig. 3B shows the effects of concanamycin on the EPR spectra of the 14-PCSL spin label in DMPC membranes. Again, the two methods of inhibitor addition (exogeneously and prior to membrane formation) are compared, and the amount of lipid used is also that in Fig. 3A. The temperature dependence of the 14-PCSL spectra is characterised by the maximum (central) line height, normalised to the double integral of the first-derivative EPR spectrum. The latter represents the total spin label intensity, which should remain constant. Therefore, the normalised line height is a sensitive measure of spectral line broadening that is inversely proportional to the square of the line width. Both broadening of the chain-melting transition, and an increase in spectral broadening in the fluid phase, are evident in Fig. 3B. In contrast to the outer hyperfine splitting shown in Fig. 2B, perturbation by concanamycin of the lipid mobility at the chain ends is reflected by the increased line widths of the pseudoisotropic 14-PCSL spectra. As for 5-PCSL in Fig. 3A, the extent of perturbation of 14-PCSL by concanamycin is similar for the two methods of addition of the macrolide inhibitor. Taking into account both Figs. 2B and 3B, little shift in the chain-melting transition is evident, in agreement with the measurements using 5-PCSL.

Even when added exogeneously, concanamycin A incorporates readily into the hydrophobic interior of lipid membranes, where it perturbs the acyl chain motion at both the C-5 and C-14 positions. The extent of chain perturbation is limited, suggesting that the macrolide inhibitor is compatible with the lipid bilayer structure. This is supported by the fact that both fluid and gel phases are perturbed by concanamycin, with little preference between the two. The ability of both concanamycin and the indole pentadiene inhibitor to associate avidly with the lipid bilayer component of biological membranes will increase their potency by providing a two-dimensional diffusion channel [18] to the inhibitory site in the transmembrane $V_o$-sector of the vacuolar ATPase.

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## References

[1] S. Dröse, K. Altendorf, Bafilomycins and concanamycins as inhibitors of V-ATPases and P-ATPases, J. Exp. Biol. 200 (1997) 1–8.
[2] S. Dröse, C. Boddien, M. Gassel, G. Ingenhorst, A. Zeeck, K. Altendorf, Semisynthetic derivatives of concanamycin A and C, as inhibitors of V- and P-type ATPases: structure–activity investigations and developments of photoaffinity probes, Biochemistry 40 (2001) 2816–2825.
[3] N. Nelson, Structure and pharmacology of the proton ATPases, Trends Pharmacol. Sci. 12 (1991) 71–75.
[4] D.J. Keeling, M. Herslof, B. Ryberg, S. Sjogren, L. Solvell, Vacuolar $H^+$-ATPases—targets for drug delivery? Ann. N.Y. Acad. Sci. 834 (1997) 600–608.
[5] B.P. Crider, X.S. Xie, D.K. Stone, Bafilomycin inhibits proton flow through the $H^+$ channel of vacuolar proton pumps, J. Biol. Chem. 269 (1994) 17379–17381.
[6] J.P. Mattsson, D.J. Keeling, [$^3$H] bafilomycin as a probe for the transmembrane proton channel of the osteoclast vacuolar $H^+$-ATPase, Biochim. Biophys. Acta 1280 (1996) 98–106.

[7] E. Theodoropoulou, D. Marsh, Interactions of angiotensin II non-peptide $AT_1$ antagonist losartan with phospholipid membranes studied by combined use of differential scanning calorimetry and electron spin resonance spectroscopy, Biochim. Biophys. Acta 1461 (1999) 135–146.
[8] E. Theodoropoulou, D. Marsh, Effect of angiotensin II non-peptide $AT_1$ antagonist losartan on phosphatidylethanolamine membranes, Biochim. Biophys. Acta 1509 (2000) 346–360.
[9] C. Farina, S. Gagliardi, G. Nadler, M. Morvan, C. Parini, P. Belfiore, L. Visentin, M. Gowen, Novel bone antiresorptive agents that selectively inhibit the osteoclast V-$H^+$-ATPase, Il Farmaco 56 (2001) 113–116.
[10] D. Marsh, A. Watts, Spin-labeling and lipid–protein interactions in membranes, in: P.C. Jost, O.H. Griffith (Eds.), Lipid–Protein Interactions, vol. 2, Wiley-Interscience, New York, 1982, pp. 53–126.
[11] G. Nadler, M. Morvan, I. Delimoge, P. Belfiore, A. Zocchetti, I. James, D. Zembryki, E. Lee-Rycakzewski, C. Parini, E. Consolandi, S. Gagliardi, C. Farina, (2*Z*,4*E*)-5-(5,6-dichloro-2-indolyl)-2-methoxy-*N*-(1,2,2,6,6-pentamethylpiperidin-4-yl)-2,4-pentadienamide, a novel, potent and selective inhibitor of the osteoclast V-ATPase, Bioorg. Med. Chem. Lett. 8 (1998) 3621–3626.
[12] S. Gagliardi, G. Nadler, E. Consolandi, C. Parini, M. Morvan, M.N. Legave, P. Belfiore, A. Zocchetti, G.D. Clarke, I. James, P. Nambi, M. Gowen, C. Farina, 5-(5,6-dichloro-2-indolyl)-2-methoxy-2,4-pentadienamides: novel and selective inhibitors of the vacuolar $H^+$-ATPase of osteoclasts with bone antiresorptive activity, J. Med. Chem. 41 (1998) 1568–1573.
[13] D. Marsh, Electron spin resonance: spin labels, in: E. Grell (Ed.), Membrane Spectroscopy. Molecular Biology, Biochemistry and Biophysics, vol. 31, Springer-Verlag, Berlin, 1981, pp. 51–142.
[14] K. Schorn, D. Marsh, Extracting order parameters from powder EPR lineshapes for spin-labelled lipids in membranes, Spectrochim. Acta, A 53 (1997) 2235–2240.
[15] D. Marsh, Polarity and permeation profiles in lipid membranes, Proc. Natl. Acad. Sci. U. S. A. 98 (2001) 7777–7782.
[16] M.J. Swamy, M. Ramakrishnan, B. Angerstein, D. Marsh, Spin-label electron spin resonance studies on the mode of anchoring and vertical location of the *N*-acyl chain in *N*-acylphosphatidylethanolamines, Biochemistry 39 (2000) 12476–12484.
[17] K. Kachel, E. Asuncion-Punzalan, E. London, Anchoring of tryptophan and tyrosine analogs at the hydrocarbon-polar boundary in model membrane vesicles: parallax analysis of fluorescence quenching induced by nitroxide-labeled phospholipids, Biochemistry 34 (1995) 15475–15479.
[18] G. Adam, M. Delbrück, Reduction of dimensionality in biological diffusion processes, in: A. Rich, N. Davidson (Eds.), Structural Chemistry and Molecular Biology, W.H. Freeman, New York, 1968, pp. 198–215.