Communication

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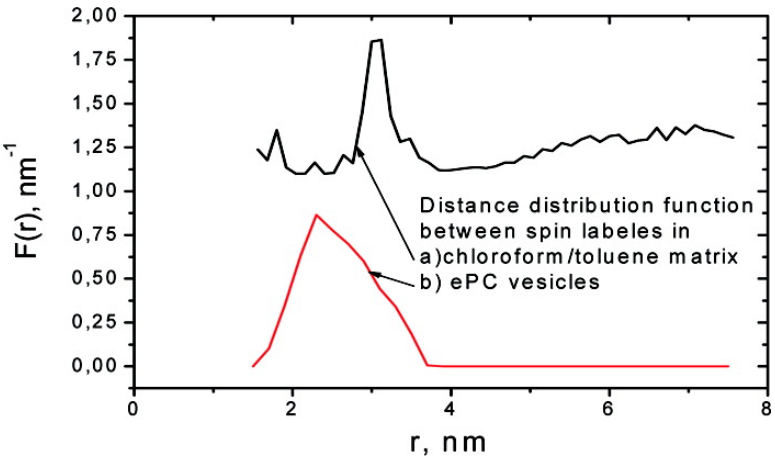

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Self-Aggregation of Spin-Labeled Alamethicin in ePC Vesicles Studied by Pulsed Electron−Electron Double Resonance

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In this communication we demonstrate the use of the pulsed electron−electron double resonance (PELDOR) technique to determine long-range distances among transmembrane helical peptide molecules. To shed light on the mechanism of channel formation, we studied the fundamental process of self-aggregation generated by a spin-labeled synthetic alamethicin analogue in phospholipid bilayers. Alamethicin is a membrane active peptaibol[1] antibiotic of fungal origin that is able to change the permeability of biological membranes by forming conductive ion channels.[2] It is generally believed that these channels are formed by self-assembling of a variable number of amphipathic molecules upon insertion into the phospholipid bilayer.[3] In ref 4 the advantages offered by the PELDOR technique were highlighted in the investigation of the aggregation of a spin mono-labeled alamethicin F50/5 analogue[5] in a membrane mimicking environment, that is, a frozen glass formed by a mixture of chloroform and toluene. In this medium the aggregation of the amphiphatic molecules was examined at 77 K and a 3D-structure for the supramolecular aggregate was proposed. More specifically, the experimental data on the magnetic dipole−dipole interactions of the spin labels allowed us to estimate the number of peptide molecules forming the aggregate and to derive a function for the distance distribution between labels. The goal of the present work is to elucidate the self-aggregation phenomenon of the alamethicin in egg phosphocholine (ePC) large multilamellar vesicles (LMV).

The PELDOR technique that allows one to determine distances in the range of 1.5−7.5 nm is applied with the usual two-pulse electron spin-echo ($\pi/2-\tau-\pi$) technique, at the observing frequency ν_A.[6] A pumping pulse was added at frequency ν_B occurring at time position T after the first $\pi/2$ echo pulse. This pumping pulse changes the dipole−dipole interaction and, as a result, the spin- echo amplitude starts to depend on both the magnitude of the dipole−dipole interaction between the spins and the delay T intensity of the pumping pulse.

Samples of spin-labeled alamethicin bound to the ePC LMV suspension were frozen to 77 K. These experimental conditions, as compared to liquid solutions at room temperature, are expected to have a marginal influence on the alamethicin secondary structure. The primary structure of the spin-monolabeled alamethicin F50/5 analogue (**A16**) examined is Ac-Aib-Pro-Aib-Ala-Aib-Ala-Glu-(OMe)-Aib-Val-Aib-Gly-Leu-Aib-Pro-Val-**TOAC**-Aib-Glu(OMe)-Glu(OMe)-Phl, where the nitroxide spin-labeled, C^{α}-tetrasubstituted, α-amino acid **TOAC** is 2,2,6,6-tetramethylpiperidine-1-oxyl-4-amino-4-carboxylic acid, which replaces the equally helicogenic Aib (α-aminoisobutyric acid) at position 16 of the peptide chain. Phl is the 1,2-amino alcohol phenylalaninol. The synthesis and the conformational preferences of **A16** in solution and in the crystal state were described elsewhere.[7]

Samples of peptide bound LMV were prepared as described in ref 8. PELDOR studies were carried out using a modified PELDOR spectrometer.[6] The durations of the ν_A pulses were 40 and 70 ns and the duration of the ν_B pumping pulse was 30 ns. The frequency difference $\nu_A - \nu_B$ was 65 MHz. The other details of sample preparation and PELDOR experiment are given in the Supporting Information.

The experimental kinetics of the PELDOR signal decay, $V(T)$, were normalized to the value of the spin-echo signal in the absence of the pumping pulse. Figure 1a shows the $V(T)$ dependence for **A16** in multilamellar ePC vesicles. Curves 1, 2, and 3 were obtained for different peptide/lipid (P/L) molar ratios (1/160, 1/70, 1/50, respectively). In this concentration range, we can distinguish two characteristic regions: (1) at $T < 100$ ns, a fast decay of the $V(T)$ value is observed, which is actually independent of the P/L; (2) at $T > 100$ ns, a relatively slow decrease of $V(T)$ is evident, which, however, is dependent on the P/L. This behavior of the PELDOR signal is that typically generated by aggregates of spin-labeled molecules.[6,9,10] In this case, the contribution to the PELDOR signal decay is given by both the dipole−dipole interactions between labels inside the aggregates at $T < 100$ ns and by other intermolecular interactions between labels at $T > 100$ ns. The depth of the initial decay depends on the number of labels in the aggregate and the parameters of the pumping pulse.[6,9,10]

To separate the contributions of the intra- and interaggregate interactions of spin labels in the PELDOR signal decay, we used the method based on the assumption of the independence of these contributions to the general $V(T)$ plot. In this case $V(T) = V_{INTRA}V_{INTER}$, where V_{INTRA} and V_{INTER} are determined by the intra- and interaggregate interactions of spin labels.[6,9−11] To derive the dependence of V_{INTRA}, we used the method described in refs 9−11, that is based on the extraction of V_{INTRA} from the experimental $V(T)$ curves obtained at different concentrations of **A16**. The $V_{INTRA}(T)$ decay obtained by this method, using the curves 1−3 in Figure 1a, is shown by dots in Figure 1b. The solid curve in this latter Figure was calculated using the spin-label distance distribution function in the aggregates (see below).

Free of interaggregate interactions, PELDOR decay $V_{INTRA}(T)$ can be used now both to derive a spin-label distance distribution function and to estimate the number of spin labels in the aggregate.[12] The spin-label distribution function is denoted as $F(r) = dn(r)/dr$, where $dn(r)$ is the fraction of spin-label pairs in aggregates with a distance between labels of a pair over the range of r to $r + dr$. To derive a distribution function, the distance range of 1.4 to 7.4 nm was divided into equal intervals with a step of 0.2 nm and the system of linear algebraic equations obtained was solved by the Tikhonov regularization method for ill-posed problems.[12d]

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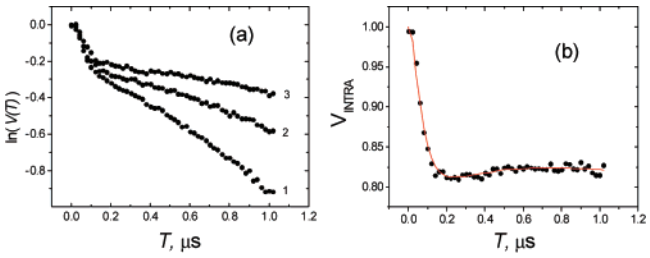

Figure 1. (a) PELDOR signal decays for frozen **A16** in ePC vesicles using the **A16** concentrations of 3.6×10^{-3} (3), 7×10^{-3} (2), and 1×10^{-2} M (1) (molar P/L ratios of 1/160, 1/70, 1/50, respectively). (b) The dots show the experimental V_{INTRA} decay for **A16** in ePC vesicles. The dependence was obtained from curves 1−3 in panel a, as described in the text. The solid line was calculated using the distance distribution function 1 in Figure 2.

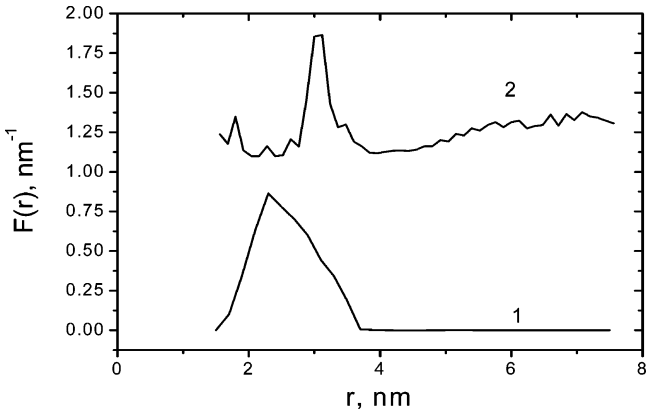

Figure 2. Distance distribution functions $F(r)$: (Curve 1) **A16** in ePC vesicles. This dependence was obtained from the experimental V_{INTRA} decay shown in Figure 1a, as described in the text. (Curve 2) **A16** in a chloroform-toluene mixture. This curve is shifted upward to the ordinate value of 1.1.

Curve 1 of Figure 2, which illustrates the distribution function $F(r)$ highlights a maximum at a distance of 2.3 nm and a half-height width of about 1.3 nm. The PELDOR signal decay calculated from this function, shown by the solid line in Figure 1b, indicates that the distribution function describes the experimental V_{INTRA} decay well. The experimental data obtained clearly demonstrate that, over the concentration range studied, the alamethicin molecules self-aggregate in large multilamellar ePC vesicles. The structure of the aggregates in ePC differs greatly from those which were previously found in chloroform−toluene solutions in terms of both the number of alamethicin molecules in the aggregate and the distance between the spin labels.[4] The aggregates of **A16** in ePC contain about four molecules, whereas the number of molecules in the aggregates in a chloroform−toluene mixture exceeds six. For comparison, curve 2 of Figure 2 shows the distribution function for **A16** in chloroform−toluene. Compared to the latter conditions, the maximum of the distribution is shifted from 3.0 to 2.3 nm and the distribution width increases from 0.5 to 1.3 nm. In this case, no pairs are observed at the longer distance $r \approx 6$ nm. As compared with the chloroform−toluene environment, the aggregates in ePC vesicles display a "looser" structure consisting of four peptide molecules. It is worth noting that continuous wave ESR measurements made for similar systems at lower P/L molar ratios did not reveal any peptide aggregation.[13,14]

We hope that the ongoing experiments on other mono- and bis-spin-labeled peptides will not only allow us to propose a detailed 3D-structure of the alamethicin aggregate, but might also provide a better understanding for the self-association mode of transmembrane helices and the voltage-gated ion conduction mechanism for this class of biologically active ionophores.

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Supporting Information Available: Details of experimental procedure, sample preparation, and PELDOR measurements. This material is available free of charge via the Internet at http://pubs.acs.org.

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