Self-Assembling Properties of Membrane-Modifying Peptides Studied by PELDOR and CW-ESR Spectroscopies

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Abstract: A new technique is described that is suitable to determine the formation of aggregates from monomeric biomolecules. This technique has been tested in the study of the self-assembling properties of the antibiotic trichogin GA IV which belongs to the class of peptaibols. We have investigated the self-assembling properties of three trichogin analogues by pulsed double resonance in electron spin−echo (PELDOR) spectroscopy combined with conventional continuous wave ESR spectroscopy. In the peptides examined Aib has been substituted by its spin-labeled analogue TOAC at three specific positions of the sequence. More specifically, the magnetic dipole−dipole relaxation of the spin-labeled peptides is measured in glassy polar and apolar solvents at 77 K. Specific assemblies of trichogin molecules are formed in an apolar solvent but addition of a more polar solvent leads to dissociation of the aggregates. The estimates based on experimental data show that each aggregate cluster contains four peptide molecules. Some of the distances between spin labels in the cluster have been determined. In addition, CW-ESR data suggest the occurrence of aggregated species in the same solutions at room temperature. The experimental results are consistent with a model wherein four amphiphilic helical peptide molecules form a vesicular system with the polar amino acid side chains pointing to the interior and the apolar side chains to the exterior of the cluster.

Introduction

In the past few years methods of continuous wave ESR spectroscopy (CW-ESR) have been exploited to analyze peptide secondary structures by using information about the distance between two nitroxide spin labels in a single molecule on the basis of measurements of dipole−dipole and exchange interaction parameters in liquids[1,2] and in solids.[3] In the solid phase the dipole−dipole and exchange interactions of labels are usually masked by a strong inhomogeneous broadening of CW-ESR spectra determined by the anisotropy of the *g* and hyperfine interaction tensors. This broadening substantially limits the application of CW-ESR methods, as in this case only rather strong interactions can be studied.[4]

The pulsed ESR methods, such as electron spin−echo (ESE) spectroscopy and in particular the pulsed double resonance in electron spin−echo (PELDOR) technique, make it possible to study the weak dipole−dipole couplings of spins in solids hidden by inhomogeneous broadening of the spectra. These methods permit one to extend the range of distances under investigation up to several tens of Ångströms and thus to considerably expand the potentialities of ESR spectroscopy in this area. The PELDOR technique has been successfully used to examine the peculiarities of a mutual spatial distribution of spins in the solid phase by analyzing the phase relaxation of spins due to their dipole−dipole coupling.[4,5] Recently, the PELDOR technique was employed to determine dipole−dipole couplings of single and double spin-labeled trichogin GA IV molecules in frozen glassy alcohols and chloroform−dimethyl sulfoxide mixtures.[6,7] These papers demonstrate the potentialities of PELDOR in the study of the secondary structure of spin-labeled peptides in polar glassy solvents.

The PELDOR method, which has been described in detail,[5,6] is a modification of the ESE method. The PELDOR signal, $V(T)$ in Figure 1, is the usual ESE signal measured in the presence of an additional pumping microwave pulse which changes the dipole−dipole coupling of spins and thus the ESE decay. Figure 1 shows a sequence of mw pulses in PELDOR (a) and the CW-ESR spectrum of a nitroxide in the solid state in a frequency domain (b). Two mw pulses, 1 and 2, induce the ESE signal 3 at frequency ω_A in a spin system. The spin−echo signal arises at time 2τ after the first mw pulse. Between pulses 1 and 2 a pumping pulse 4 is applied at frequency ω_B at time T after the first pulse. The spins are labeled as spins A (at ω_A) and spins B excited by the pumping pulse at ω_B. The pumping pulse induces transitions between the Zeeman levels of spins B and thus changes local magnetic fields at spins A. This, in turn,

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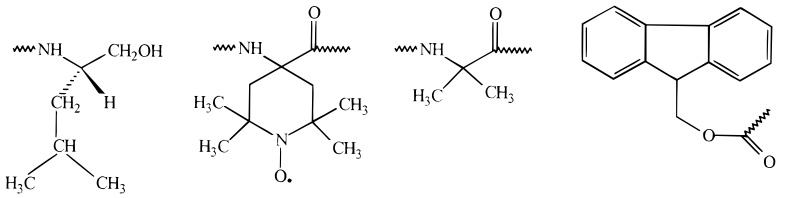
Table 1. Amino Acid Sequence of Trichogin GA IV and the Single and Double Spin-Labeled Analogues **I**, **II**, and **III**[a]

trichogin GA IV	*n*-Oct-Aib-Gly-Leu-Aib-Gly-Gly-Leu-Aib-Gly-Ile-Lol
I	Fmoc-Aib-Gly-Leu-**TOAC**-Gly-Gly-Leu-Aib-Gly-Ile-Leu-OMe
II	*n*-Oct-**TOAC**-Gly-Leu-Aib-Gly-Gly-Leu-Aib-Gly-Ile-Leu-OMe
III	Fmoc-**TOAC**-Gly-Leu-Aib-Gly-Gly-Leu-**TOAC**-Gly-Ile-Leu-OMe



Lol **TOAC** **Aib** **Fmoc**

[a] In addition, the structures of the 1,2-amino alcohol leucinol (Lol), the amino acids α-aminoisobutyriclid (Aib) and 2,2,6,6-tetramethylpiperidine-1-oxyl-4-amino-4-carboxylic acid (TOAC), and the protecting group *N*-(9-fluorenylmethoxycarbonyl) (Fmoc) are shown.

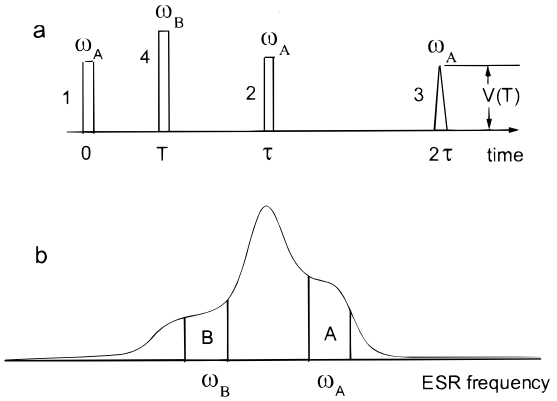


Figure 1. A sequence of mw pulses (a) and their schematic position in the ESR absorption spectrum (b); 1 and 2 are ESE-producting pulses at ω_A, 3 is the ESE signal at ω_A, and 4 is the pumping pulse at ω_B. The areas A and B in part b schematically present the section of the ESR spectrum excited by the two different pulses.

results in additional dephasing of spins A and hence in a decrease of the PELDOR signal. This decrease depends on the value of dipole−dipole spin coupling, time position, and intensity of the pumping pulse. The main decay of the PELDOR signal occurs within time $T \sim 1/D$, where D is a typical value of the dipole−dipole spin coupling between A and B spins. In the usual experiments, time interval τ between the pulses 1 and 2 is fixed and an analysis is made of the signal decay on time T.

Theoretical analysis and experimental results show that upon a random distribution of spins in solids the relaxation due to spin−spin dipole interactions, which can be determined from PELDOR experiments, is described by a simple exponential decay of signal amplitude with time T. The characteristic relaxation time is determined in this case by the concentration of spins. Deviations from a random distribution of spins is manifested in a more complicated relaxation behavior. For example, when the local concentration of spins exceeds the mean one (spin pairs, groups, clusters, etc.), there is a more effective relaxation at short times owing to the strong dipole−dipole coupling of spins which results in a fast decay of PELDOR signal. As time T increases, the relaxation efficiency becomes weaker and approaches that for the average concentration of spins. These deviations from simple exponential curves were repeatedly observed in systems with a high local concentration of spins in solids.[5]

Herein, the PELDOR method has been employed to investigate the spin-labeled peptaibol trichogin GA IV analogues **I**, **II**, and **III** whose primary structures are given in Table 1. Peptaibols are peptides of fungal origin characterized by an acylated N-terminal residue, a C-terminal 1,2-amino alcohol, and the presence of several α-aminoisobutyric acid (Aib) residues. It is generally assumed that the membrane modifying properties of peptaibols are due to formation of amphiphilic helix bundles with polar groups pointing to the inside of the bundle and hydrophobic groups projecting toward the hydrophobic membrane. However, despite a variety of investigations, experimental data on the formation of peptide clusters in the phospholipid bilayer or even in membrane mimicking hydrophobic solvents are still lacking. In polar glassy solvents, like chloroform−dimethyl sulfoxide, trichogin, does not tend to aggregate.[6,7] It is the aim of the present study to investigate the effects of less polar solvents on the self-assembling properties of this particular antibiotic peptide by using the PELDOR technique. Independent studies have shown that the 2,2,6,6-tetramethylpiperidine-1-oxyl-4-amino-4-carboxylic acid (TOAC) can replace Aib in the peptide sequence without causing any significant change either in conformation or in biological activity.[8,9]

Dipole−dipole couplings of spin labels have been determined for frozen solutions of **I** and **II** in chloroform−toluene (7:3). This solvent mixture is appropriate because it contains components of low polarity, the peptides are readily solubilized and it forms a transparent glass upon freezing to 77 K. An additional solvent system, chloroform−toluene−ethanol (3.5:1.5:5) was also examined to reveal the effect of a polar solvent on the intermolecular interaction between peptides. We present also PELDOR data of the double-labeled peptide **III** in a frozen methanol solution, which will be used for the determination of experimental parameters in the analysis of the PELDOR results for peptide, **I** and **II**. In addition, CW-ESR spectra of the above spin-labeled peptides were obtained both at 77 K and at room temperature.

Experimental Section

Three synthetic TOAC single and double spin-labeled peptides[8,9] (Table 1) were used in the experiments. In peptides **I**−**III** the C-terminal leucinol of trichogin GA IV has been replaced by leucine methyl ester (Leu-OMe), and in peptide **I** and **III**, the *n*-octanoyl group has been substituted by the Fmoc-protecting group. It has been shown before that the antibiotic activity as well as the membrane-modifying properties of trichogin are not significantly changed by these substitutions.[8,9]

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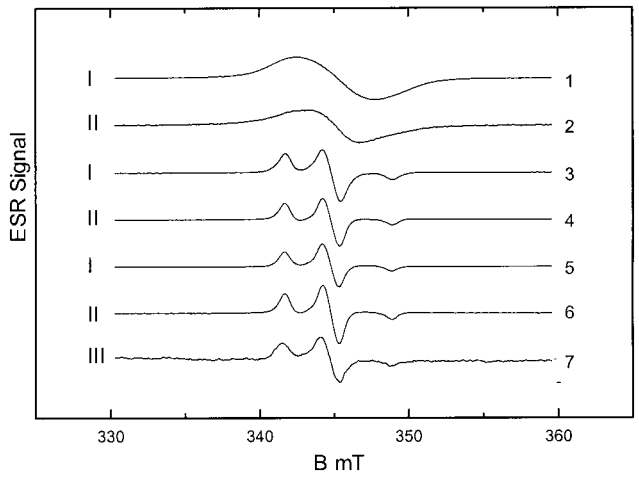

Figure 2. CW-ESR spectra of peptides **I**−**III** in the solid phase at 77 K: 1,2, dry powder of peptides **I** and **II**; 3,4, peptides **I** and **II** in a chloroform−toluene mixture; 5,6, peptides **I** and **II** in a chloroform−toluene−ethanol mixture; and 7, peptide **III** in methanol.

CW-ESR spectra of spin-labeled peptides were recorded on an X-band 380 ESP Bruker spectrometer at a modulation frequency of 100 kHz and modulation amplitude of 0.1 mT in the absence of spectrum saturation.

The PELDOR experiments were performed using a homemade X-band ESE spectrometer supplied with a bimodal resonator and a source for the additional pumping mw pulse.[10] The difference between the recording ω_A and pumping ω_B frequencies was about 100 MHz. Durations of the pulses 1 and 2 (Figure 1) were 40 and 70 ns, respectively. Durations of the pumping pulses were about 40 ns.

The samples (glass ampules, 5.0 mm in diameter) contained about 0.1 mL of the solution studied, e.g. volume mixtures of chloroform−toluene (7:3) and chloroform−toluene−ethanol (3.5:1.5:5). The samples were frozen by inserting ampules into liquid nitrogen. The mixtures turned into transparent glass after freezing. The chemically pure solvents were used without additional purification.

In the CW-ESR and PELDOR experiments at 77 K the samples were placed inside the nitrogen-cooled finger of a Dewar flask, which was located in the cavity of the spectrometer. The number of spin labels in the sample was controlled by comparing the double integrals of the CW-ESR spectra of the sample with that of $CuCl_2 \cdot 2H_2O$ crystals containing a known number of paramagnetic centers. The concentration of spin labels in the samples amounted to about 2×10^{18} cm^{-3}.

Molecular models were constructed by using the program Sybyl, version 6.5, Tripos Associates 1999. The following respective ϕ and ψ values were used to build models for secondary structures: −57°, −30° (3_{10}-helix), −63°, −42° (α-helix). For building the alternating residues of the π-helix two different sets of ϕ and ψ values were taken: −153°, 144° and 125°, −124°.

Results and Discussion

Qualitative Peculiarities. CW-ESR spectra of the spin-labeled peptides **I**−**III**, studied at 77 K, are shown in Figure 2. The microcrystalline powder spectra (curves 1 and 2) are simple singlets, whose widths are caused by strong dipole−dipole and exchange interactions between spin labels at short distances in crystals. The spectra of frozen peptide solutions (curves 3−7) are typical for nitroxide radicals under these conditions. The spectral shapes indicate that freezing of solutions does not cause peptides to pass into a separate phase.

Curves 1 and 2 in Figure 3 illustrate the PELDOR signal decays, $V(T)$, for the two single-labeled peptides **I** and **II** in glassy chloroform−toluene. Two unusual features are striking. The first peculiarity is a fast decrease in the amplitude of $V(T)$ at the time region between $T = 0$ and 150 ns. A fast decrease of the decay in this time region indicates the existence of compact groups of spin labels in the system under study.[5] A strong dipole−dipole coupling of spin labels in groups leads to a fast dephasing at short times. This type of dependency was repeatedly observed for glassy solutions of biradicals[5] and double-labeled peptides.[6,7] The last mentioned systems represent a simple case of groups consisting of two spins, which is exemplified by curve 5 in Figure 3 obtained for a frozen solution of double-labeled peptide **III** in methanol. By comparing curves 1, 2, and 5 one sees that, within the initial time region, the depth of the fast PELDOR signal decay of peptides **I** and **II** is much greater than that of peptide **III** whose structure contains only two spin labels. According to our previous study,[5] a substantially greater depth for peptides **I** and **II**, relative to the corresponding value for the biradical, indicates that these compounds form aggregates which contain more than two peptide molecules.

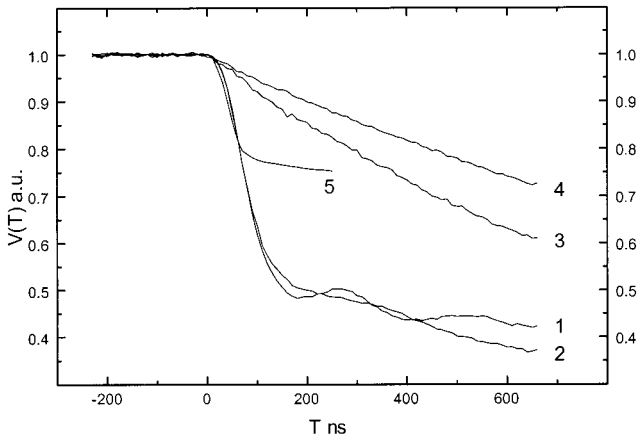

Figure 3. PELDOR signal decay for glassy peptide solutions at 77 K: 1 and 2, peptides **I** and **II**, respectively, in a chloroform−toluene−ethanol mixture; and 5, peptide **III** in methanol.

The second unusual feature is the observation of a slower decrease in the time region at $T > 150$ ns, which is accompanied by signal oscillations. The slow decrease of $V(T)$ is probably related to the dipole−dipole coupling of spin labels belonging to different aggregates. Since this coupling is almost independent of the interaction between spin labels within the aggregate, the total decay of the PELDOR signal is usually considered as the product of two time dependencies: $V = V_{inter}.V_{intra}$, where V_{intra} is the PELDOR signal decay function due to the interaction of labels within the aggregate and V_{inter} is the same parameter for the interaction of labels between different aggregates.[5] The amplitude oscillations observed for curves 1 and 2 are related to the interaction of labels within aggregates (V_{intra}) and are of the same origin as the previously observed oscillations of biradicals and double-labeled peptides which have been measured in frozen glassy solution.[5−7] They arise in the case of fixed distances between spin labels. In this case the dipole−dipole coupling between the labels is averaged only by angles, due to random orientations of aggregates, and not by distances. The averaging of dipole−dipole coupling by angles is not sufficient for complete smoothing of the $V(T)$ function. A spread in distances between spin labels would lead to both averaging of oscillations in the time interval studied and a decrease in their efficiency. Oscillations observed for the experimental dependencies 1 and 2 in Figure 3 indicate that the aggregates of peptides **I** and **II** have fragments with a fixed structure in which the distances between spin labels are determined with a minor spread. These distances can be estimated from the period of oscillations.

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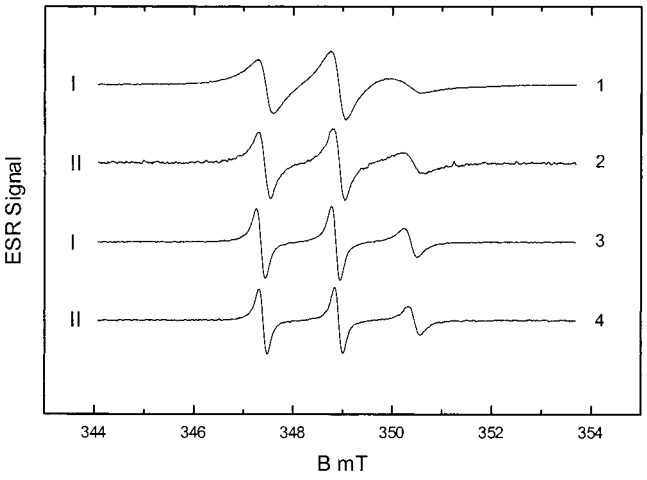

Figure 4. CW-ESR spectra of peptides **I** and **II** in solution at room temperature: 1,2, peptides **I** and **II** in a chloroform−toluene mixture; and 3,4, peptides **I** and **II** in a chloroform−toluene−ethanol mixture.

Addition of ethanol to the mixture of low polarity (chloroform−toluene) sharply changes the behavior of the PELDOR signal decay. Thus, experimental $V(T)$ functions 3 and 4 in Figure 3 were obtained from solutions of peptides **I** and **II** in a frozen mixture of chloroform−toluene−ethanol. As compared with curves 1 and 2, in these cases the signal decays have no oscillations and can be described by a simple exponential decay, typical for a random distribution of spin labels in the bulk. Transitions from dependencies 1 and 2 in Figure 3 to dependencies 3 and 4 after addition of ethanol to the chloroform−toluene mixture indicate dissociation of peptide aggregates into their monomeric constituents. Previously, an exponential behavior of the PELDOR decay similar to those of curves 3 and 4 has been recorded for the same single-labeled, randomly distributed peptides dissolved in the relatively polar frozen chloroform−dimethyl sulfoxide mixture.[6]

Figure 4 shows the CW-ESR spectra of peptides **I** and **II** in the same solvent mixtures at room temperature. Additional broadening of spectra 1 and 2 as compared with those of spectra 3 and 4 allows one to conclude that peptide aggregates do also exist in liquid solutions at room temperature. This latter observation is consistent with the results of a previous FTIR absorption study in $CDCl_3$.[11] The broadening effort can be caused by additional spin relaxation in the aggregates due to dipole−dipole or exchange interactions of spin labels. Similar CW-ESR spectra were observed for some double-labeled peptides in other solutions at room temperature.[2] An additional contribution to the line width from the anisotropy of hyperfine interaction and g tensor is also possible in the case of a slow rotational mobility of aggregates. A detailed analysis of these effects is currently in progress.

Quantitative Estimates. The number of peptide molecules in the aggregate can be estimated from curves 1 and 2 in Figure 3 on the basis of a theoretical consideration about the decay of the PELDOR signal for the case where spins are distributed in the bulk as separate groups.[5,12] For simplicity, it is assumed that all peptide molecules in the frozen solution under investigation are combined into identical aggregates and each cluster contains N peptide molecules. To neglect the probability that more than one spin B flips in the aggregate caused by a pumping microwave-pulse at ω_B, the value of N has to be small. In this case the correlation between the positions of spins B in the aggregates can be neglected. As a result, eqs 1 and 2 in ref 12 are reduced, and because of the coupling of spin labels within the aggregates the echo signal behavior will have the form

$$V(T)_{\text{intra}} = \frac{1}{N}\sum_{j=1}^{N}\{\prod_{\substack{k=1\\k\neq j}}^{N}(1 - p_b(1 - \langle\cos(D_{jk}T)\rangle))\} \qquad (1)$$

$$D_{jk} = \gamma^2\hbar(1 - 3\cos^2(\theta_{jk}))/r_{jk}^3 \qquad (2)$$

where $V(T)_{\text{intra}}$ is the PELDOR signal decay function normalized to unity at $T = 0$, T is the time separation of the pumping mw pulse after the first pulse, N is the number of spin labels in the aggregate, j is the index of spins A in the aggregate, k is the index of spins B, p_b is the probability of spin B flip induced by the pumping pulse, γ is the gyromagnetic ratio for an electron, $\hbar$ is the Planck constant, r_{jk} is the distance between spins j and k, θ_{jk} is the angle between vector r_{jk} and the external magnetic field, and $\langle ... \rangle$ is the averaging over values θ_{jk} and r_{jk}.

Averaging of (1) over angles θ_{jk} due to a random orientation of aggregates provides a fast decay of the echo signal at time T corresponding to the value of the mean dipole−dipole coupling of spins followed by a slow decay with oscillations at frequencies ω_{jk}:

$$\omega_{jk} = \gamma^2\hbar/r_{jk}^3 \qquad (3)$$

According to eq 1 the oscillations are attenuated with time T and $V(T)_{\text{intra}}$ tends to its limit V_p. The V_p value can be obtained from eq 1 at $\langle\cos(D_{jk}T)\rangle = 0$:

$$V_p = (1 - p_b)^{N-1} \qquad (4)$$

Equation 4 makes it possible to estimate the number of spin labels in the aggregate (N). To this end it is necessary to know the V_p and p_b values.

It is difficult to make a reliable determination of V_p directly from the experimental decay $V(T)$ (Figure 3), as $V(T)$ is the product of $V(T)_{\text{intra}}$ and $V(T)_{\text{inter}}$. According to (1), the$V(T)_{\text{intra}}$ function, after the fast decay at short T, has oscillations around the V_p value. $V(T)_{\text{inter}}$ smoothly depends on T in a similar way as appears in curves 3 and 4 (Figure 3). This means, unfortunately, that the increasing influence of the $V(T)_{\text{inter}}$ part of the decay and the not completed oscillation attenuation do not permit us to get the precise value of V_p from the experimental curves 1 and 2 at long T values. However, at short T values the contribution to the total $V(T)$ decay by $V(T)_{\text{inter}}$ is relatively small and we can assume that $V(T) \sim V(T)_{\text{intra}}$. This means that the depth of the initial decay of the experimental $V(T)$ function at short T corresponds to the V_p value with the accuracy that is determined by the oscillation amplitude. The V_p value was taken from the mean $V(T)$ at $T = 150$ ns, for curves 1 and 2 (Figure 3). The fast decay is by then over and the slow decay of $V(T)_{\text{inter}}$ is not significant, due to the low interaction between spin labels in different aggregates. This estimate gives the mean value of $V_p = 0.51 \pm 0.01$ for both peptides **I** and **II**.

To determine the p_b value for peptides **I** and **II**, we used the $V(T)$ function of the double-labeled peptide **III** (curve 5 in Figure 3). This approach is possible as the p_b value is only a function of the ESR line shape and pumping mw pulse parameters.[5] Therefore, the value of p_b is the same for all peptides under investigation because the ESR spectra and the experimental PELDOR parameters are the same for all peptides. We chose the double-labeled peptide **III** in methanol as in this

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case we have no oscillations in the $V(T)$ decay function. This property gives us the opportunity to determine p_b from the depth of the initial decay at short T. For double-labeled peptide **III** $N = 2$ and the V_p value obtained is 0.8 ± 0.01. After substituting $N = 2$ and $V_p = 0.8$ into (4), we obtain $p_b = 0.2$, which can be used in the calculation of N for peptides **I** and **II**.

It is necessary to mention here that it has been previously shown for some double-labeled peptides that it is possible to observe oscillations in $V(T)$, but the frequency of oscillations and their amplitude depend on the nature of both the solute and the positions of the labels in the peptide chain.[6,7] One other reason for the absence of oscillation in the case of curve 5 (Figure 3) may be also the spread in distances between the labels.[7] Nevertheless, this absence makes this specimen very suitable for the correct determination of p_b.

By substitution of $V_p = 0.51 \pm 0.01$ and $p_b = 0.2 \pm 0.01$ into (4), we obtain $N = 4 \pm 0.3$ for both peptides. Thus, the quantitative estimate shows that in a chloroform−toluene mixture peptides **I** and **II** form aggregates consisting of four molecules. Note that the assumption we have made above about the absence of a spread in N needs additional verification. If N varies for different aggregates, only a mean effective value can be determined using the method that has been developed.[12,13]

It is possible to roughly estimate a mean value for distances between the labels within the aggregate from the time T_f of the fast decay function $V(T)$ (Figure 3, curves 1 and 2). This time corresponds to the mean value of dipole−dipole interaction parameter D: $T_f \approx 1/D$, $D = \gamma^2\hbar/r^3$, wherein r is the mean distance between the spin labels in the aggregate. For $T \approx 100$−150 ns this will give $r \approx 30$−36 Å. This value gave us the estimation of the aggregate dimension.

More correct distance values between spin labels in aggregates can be calculated from the frequency of oscillations of the PELDOR signal. The experimental values of oscillation frequencies are 2.51×10^7 rad·s^{-1} for peptide **I** and 1.85×10^7 rad·s^{-1} for peptide **II**. Using eq 3 for the oscillation frequency, we obtain a distance of 23.5 Å for peptide **I** and 26.0 Å for peptide **II**. The error in the determination of distance (r) depends on the error in the determination of the oscillation frequency (ω) of the PELDOR signal. From the experiment we can determine ω with the accuracy of about 4−5% (Figure 3). According to (3)

$$\delta r/r = {}^1/_3(\delta\omega/\omega) \qquad (5)$$

and the accuracy for r is about 1.5%.

Although the position of the spin label in the primary structures of peptides **I** and **II** is different (Table 1), the difference between the observed distances of the respective peptides is not large. As mentioned above, oscillations indicate the existence of rigid structural fragments in the aggregates without any spread in distances between spin labels. Not all possible distances between spin labels are manifested in our experiments. We are likely to observe oscillations due to pairs of spin labels with relatively small distances, because these pairs are located in rigid fragments of the aggregate. Therefore, the values of distances obtained from oscillations can only characterize the size of the *rigid part* of the aggregate structure.

Molecular Model of Trichogin Aggregates. The values of distances found for aggregates of two different trichogin analogues, one labeled at the first and the other at the fourth position of the peptide chain, establish a specific set of constraints that may be used to build a molecular model. X-ray diffraction analysis of [TOAC-4,8]-trichogin revealed two independent molecules in the $P2_1$ asymmetric unit.[8,14] The N-terminal region of each molecule folds in a 3_{10}-helical conformation, while the central and C-terminal regions are mainly α-helical. From CW-ESR studies of three different double TOAC-labeled trichogin analogues it was concluded that the overall secondary structure of these lipopeptaibol analogues in solution remains essentially unchanged.[8] The strict similarity in the conformational propensities of Aib and TOAC residues motivated us to build a tetrameric cluster of [TOAC-1,4]-

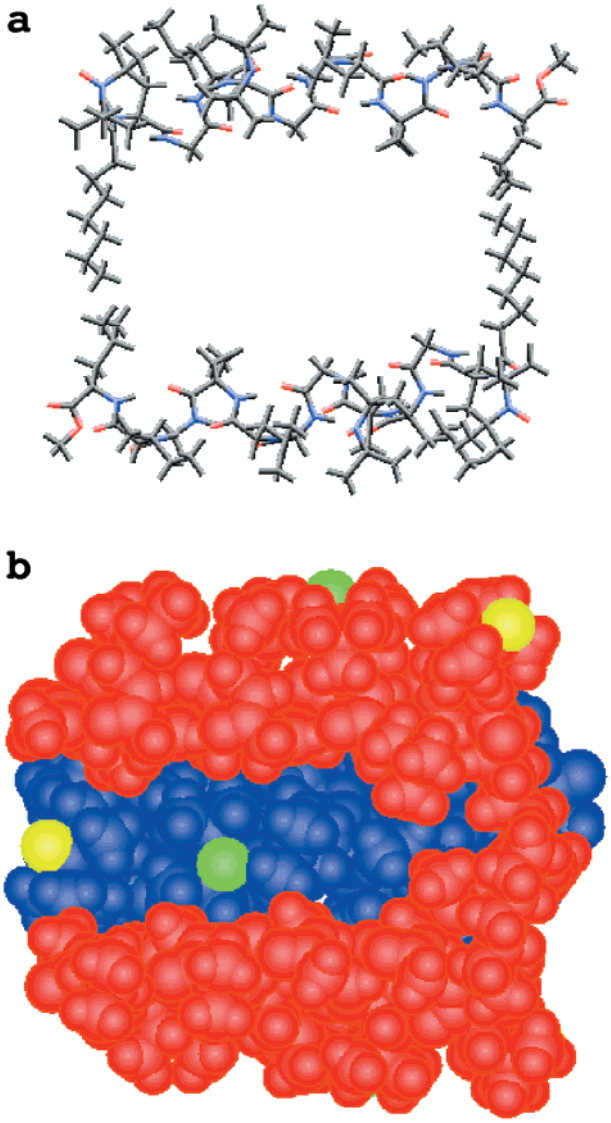

Figure 5. A molecular model is shown for the following: (a) two antiparallel [TOAC-1,4]-trichogin helices with polar groups oriented to the inside of the cavity which is formed by two *n*-octanoyl groups and two side chains of Leu-11; and (b) a tetramer that has been constructed from four [TOAC-1,4]-trichogin helices with the polar sides pointing to the center. The axes of the four antiparallel 3_{10}-helices are positioned at the corners of an imaginary beam (looking along the diagonal the helices are oriented in a parallel fashion). The yellow and green colored atoms correspond to the nitroxide oxygens of the respective 1 and 4 Toac positions of the peptide chain. The following interresidue distances were calculated: TOAC-1···TOAC-1, 29 (antiparallel) and 23 Å (parallel) and TOAC-4···TOAC-4, 26 (antiparallel) and 18 Å (parallel).

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trichogin molecules starting from the crystal structure of [TOAC-4,8]-trichogin. Aib at position 1 was substituted by TOAC and TOAC-8 was replaced by Aib. After applying a set of $P2_1$ transformations, four different tetramers were selected which are in agreement with the experimental data. However, because of the "roof tiles" like packings of these nanocrystals, it is difficult to understand why crystallization would stop at the tetramer.

In the next attempt to model the aggregate we investigated first a set of dimers with head-to-head, tail-to-tail, and head-to-tail ordered monomers with different kinds of secondary structures, e.g. α-, 3_{10}-, or π-helices. On the basis of the amphiphilic nature of the peptaibol helices, tetramers were built from the dimers by adjusting helix orientations with polar groups oriented to the inside of the cavity. However, none of these models gave a satisfying account of our experimental PELDOR observations.

From X-ray studies it is known that transition between α- and 3_{10}-helix types is often readily achieved, for instance by changing the crystallization medium.[15] In contrast to the amphiphatic α-helical structure of trichogin, the 3_{10}-helical structure shows the hydrophobic side chain of Leu-11 at the hydrophilic side of the helix. Because of this bulky group, the intermolecular distance of the dimer, constructed by docking two antiparallel helices together, is significantly larger than for a dimer consisting of two α-helical molecules (Figure 5a).

Next, a model was constructed from four 3_{10}-helices by adjusting the helical axes in pairs with the polar sides pointing to the center of the tetrameric peptide cluster (Figure 5b). Four helices were arranged in an antiparallel manner. After several steps of energy minimizations a model was produced with the following average *inter*-residue distances: TOAC-1···TOAC-1 26 Å and TOAC-4···TOAC-4 22 Å. These values are consistent with the distances obtained from the PELDOR experiments: 26.0 (peptide **II**) and 23.5 Å (peptide **I**). The exterior of the aggregate appears to be highly hydrophobic. The interior of the peptide cluster leaves room for several solvent molecules. At each end, the polar cavity is closed by four different hydrophobic groups, i.e. the two side chains of Leu-11 and the two *n*-octanoyl groups (or the two Fmoc groups in peptide **I**).

The question on how solvent molecules are interacting to stabilize these aggregates has to be examined in further detail, but in this respect it is worth recalling a recent X-ray study of another amphiphilic peptaibol molecule, antiamoebin, that was cocrystallized with *n*-octanol.[16] One solvent molecule mimics a membrane segment along the hydrophobic exterior of a channel-like assembly. The other solvent molecules fill the polar pocket of the channel in such a way that their −OH termini satisfy the carbonyl moieties pointing to the interior of the channel.

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Conclusions

The dipole−dipole coupling of spin labels has been studied in frozen glassy solutions of trichogin GA IV spin-labeled peptide analogues by PELDOR spectroscopy. Peptide aggregates have been found in a chloroform−toluene mixture at 77 K. Results from CW-ESR experiments indicate that the peptide clusters are also conserved to some extent in liquid solution. It has also been shown that addition of ethanol to the chloroform−toluene mixture leads to dissociation of the aggregates.

Using the data of dipole−dipole relaxation in frozen glassy solutions, the number of peptide molecules in the aggregates has been estimated. Despite the different structure of the N-terminal group and the position of the spin label in the sequence, all aggregates consist of four peptide molecules. It has been established that the aggregates include fragments with a rigid structure in which the distances between spin labels have been determined to be in the range of 23.5−26 Å. These distances provide a characteristic size of rigid fragments in the aggregate structure. A model of four 3_{10}-helical trichogin molecules has been proposed that is consistent with the experimental data.

In conclusion, we believe that the new observations made by using the PELDOR technique reported in this work open a promising avenue to determine not only peptide conformations but the details of self-assembling peptide structures as well.

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