Supporting Information

Channel and non-channel forms of spin-labeled gramicidin in membranes and their equilibria.

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Contents

Synthesis of new spin-labeled gramicidin derivatives:

Double-labeled gramicidin A (GADL):

Desformyl gramicidin A was synthesized in a procedure similar to described in [43]. 84 mg of the product without further purification was mixed dry with 12 mg DMAP and then a mixture of 129 mg of 2,2,5,5 – tetramethyl – 3 – pyrrolin – 1 - oxyl – 3 – carboxylic acid in 1 ml DMF with 0.4 ml of 1M DCC in CH_2Cl_2 was added. After 96 hours of stirring at room temperature DMF was removed in vacuum overnight, the reaction mixture was initially washed by suspending/spinning down with several portions of water until the triplet ESR signal of the supernatant disappeared, then dried and applied to a column. The column was packed with 230-400 mesh silica gel 60. Chromatography was carried out under nitrogen pressure in 95:5 $CHCl_3$/MetOH. The fractions were collected; each fraction was checked by ESR and TLC. 61 mg of a double labeled product was obtained. . R_f = 0.51 in $CHCl_3$/MetOH 9:1 ESI MS: M/z =2187.4. The C- terminal label, as in GASL, is attached via ester bond; the other spin label forms an amide bond with the desformylated N-terminus.

N-terminal labeld gramicidin A (GALN):

To 10.3 mg GADL 2 ml saturated solution of Cs_2CO_3 in EtOH was added. The mixture was left stirring overnight at room temperature. The organic solvent was initially removed in the flow of nitrogen and then in vacuum. After thorough washing on the glass filter with excess water ~8 mg of dry product was collected. R_f = 0.45 in $CHCl_3$/MetOH 9:1; ESI MS: 2021.2 M/z

Tyr[11] - labeld gramicidin C (TyrLGC):

10 mg of GC was mixed dry with 2.6 mg of 3-(2-Iodoacetamido)-PROXYL free radical (~ 1.5 excess) and 3mg Cs_2CO_3. 0.56 ml of dry DMF was added. The mixture was stirred at room temperature for 2 hrs and then solvent was removed with the rotor evaporator. A MALD mass spectrum of the reaction mixture showed in the range 1800-3000 M/z only two significant peaks at 1991.0 and 2189.1 M/z at a ratio of 2:3. The peaks apparently correspond to cesium adducts of unlabeled GC and a monolabeled product. Further purification by TLC (silica, Chl/Met 9:1) gave 5 mg of pure product, R_f = 0.37 in $CHCl_3$/MetOH 9:1; ESI MS: 2057.4 M/z.

N, C-dibenzoyl-(desformyl)-gramicidin:

To a dry mixture of 82 mg of raw desformyl GA with 10 mg DMAP 93 mg of benzoic acid in 0.9 ml DMF was added, followed then by 0.4 ml 1N DCC in CH_2Cl_2. After 92 hrs of stirring at room temperature the solvent was removed on the rotor evaporator. The dry mass was stirred then for 16 hrs with a solution of 90 mg Na_2CO_3 in 10 ml water. The precipitate was washed on a glass filter, dried and separated on a silica column in a $CHCl_3$/MetOH 95:5 system. Fractions were collected and analyzed by TLC on silica in $CHCl_3$/MetOH 9:1. Fractions giving a single fluorescent spot with R_f = 0.58 were combined. 55 mg of white product was obtained. ESI MS: 2062.3 and 2085.3 M/z corresponding double acylated product and its sodium adduct.

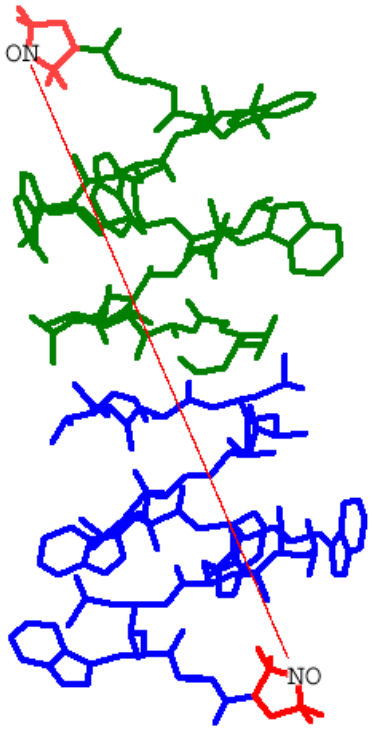
N-benzoyl-(desformyl)-gramicidin was obtained from N, C-dibenzoyl-(desformyl)-gramicidin by Cs_2CO_3- promoted hydrolysis (See obtaining GALN from GADL). The

substance, which shows R_f = 0.45 in $CHCl_3$/MetOH 9:1 and ESI MS of 2090.9 M/z for its cesium adduct, was further introduced into DCC – promoted coupling with 2,2,5,5 – tetramethyl – 3 – pyrrolin – 1 - oxyl – 3 – carboxylic acid to obtain **N-benzoyl-(desformyl)-GASL** which was then purified by preparative TLC in $CHCl_3$/MetOH 9:1 (R_f=0.56) and characterized by its ESI MS peak of 2125.4 M/z and the more intense peak of its sodium adduct at 2147.4.

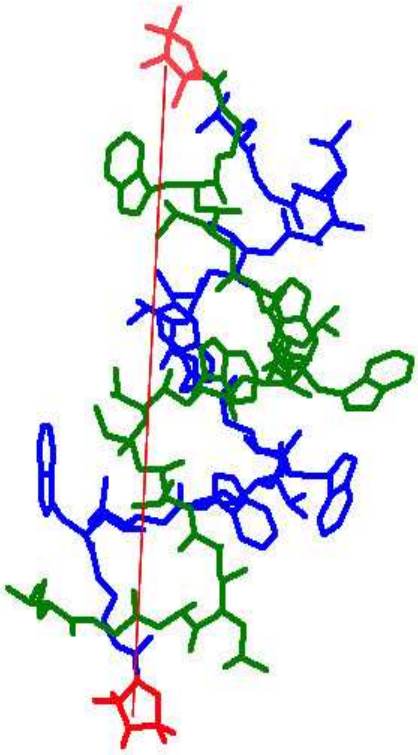
GASL in HHD and $\pi\pi^{5.6}$ left-handed antiparallel DH structures.

Supplement Fig.1. ***(a)*** GASL in the HHD (PDB:1MAG) and the ***(b)***$\pi\pi^{5.6}$ left-handed antiparallel DH (PDB:1ALZ) structures. Note that the interspin distances for these structures are very similar, 29.5Å and 31.2Å correspondingly.

a

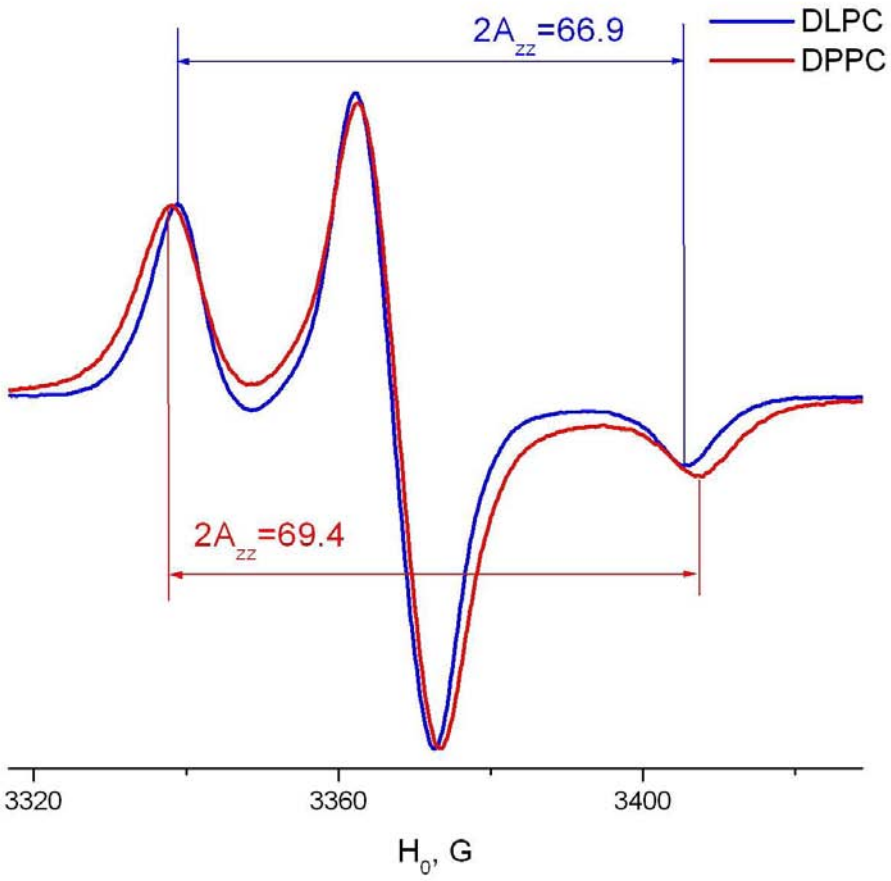


b



TyrLGC in DLPC and DPPC. Polarity of the local environment for the nitroxide group

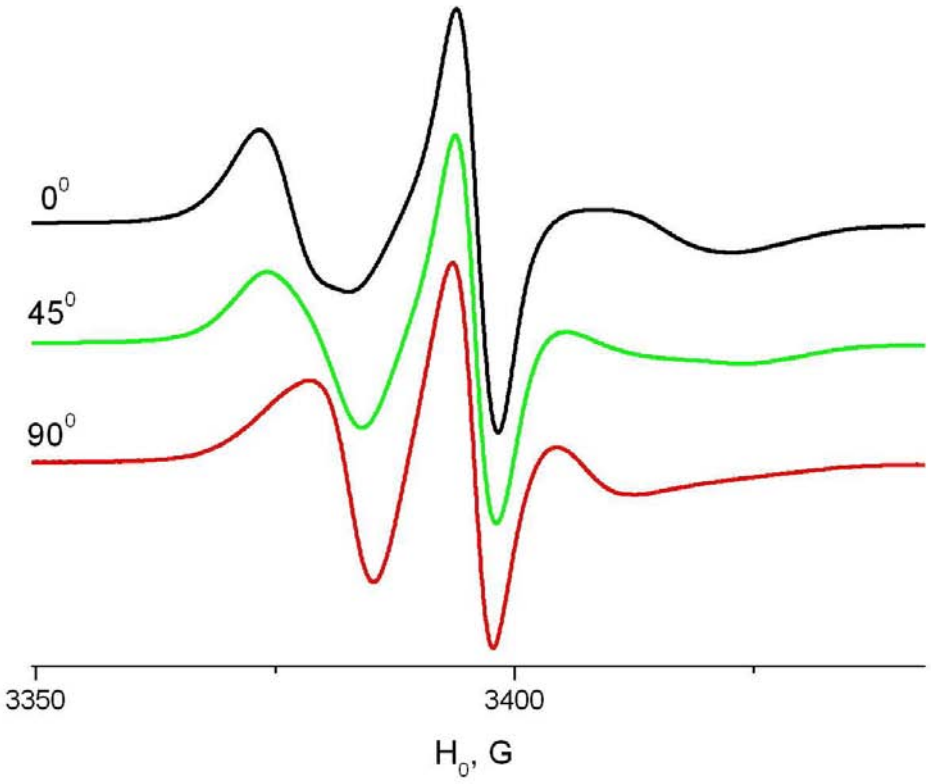
Supplement Fig.2. TyrLGC shows, similarly to GASL, higher polarity in the lipid environment favoring channel formation [25]. The spectra of are taken at 77K the TyrLGC/Lipid ratio is 0.5% mol.



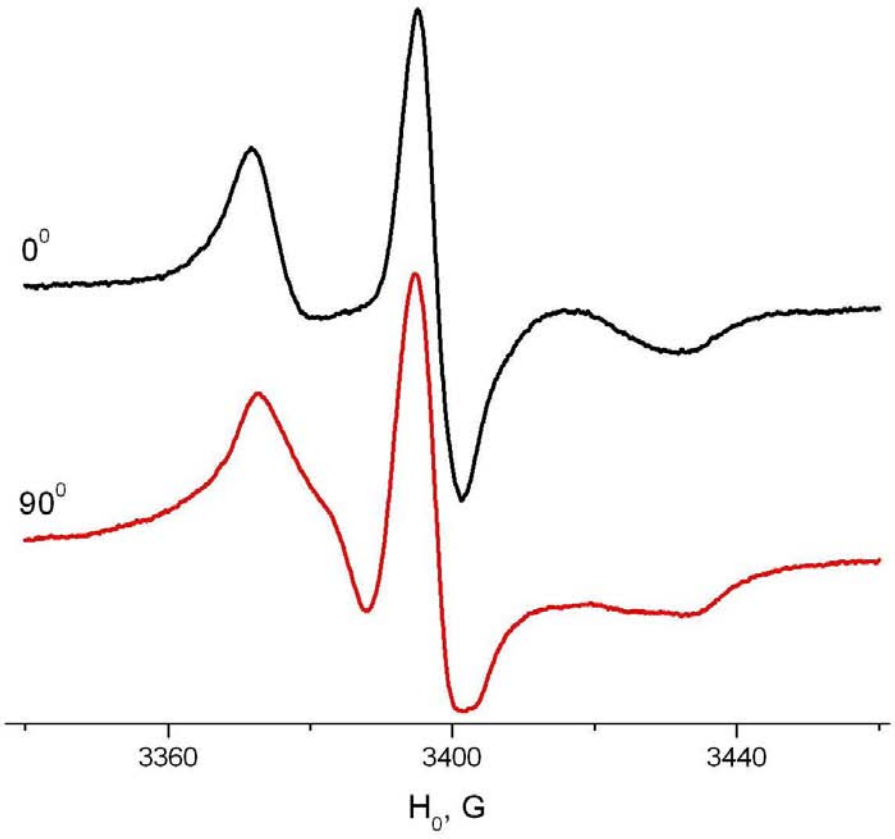
TyrLGC in aligned membranes

Supplement Fig.3. Spectra of TyrLGC in ISDU aligned membranes of ***a)*** DPPC, 20^0C ***b)*** DMPC, 4^0C

a)



b)



Effect of N-terminal substitution on HHD channel formation

Estimates of the dimerization constant

The dimerization constants for gramicidin $\boldsymbol{K_D} = \frac{[\boldsymbol{D}]}{[\boldsymbol{M}]^2}$, where [D] and [M] are the surface concentrations of dimers and monomers respectively expressed in mol/cm^2, determined for a variety of membranes are in the range 2×10^{13}-4×10^{16} cm^2/mol [28, 29, 33, 37]. Hydrophobic mismatch has a dramatic effect on GA channel. In the gel phase an increase in the bilayer thickness by ~ 3Å (from30.3 Å in DMPC to 34.4 Å in DPPC) completely blocks channel formation [25] and causes dissociation of existing channels [26]. Since at the peptide/lipid molar ratio of 1% no pairs can be detected in a well equilibrated DPPC bilayer, the dimerization constant at these conditions can be estimated as $<< 10^{10}$ cm^2/mol. On the other hand, GASL in DMPC at the peptide/lipid molar ratios down to 0.001%, within the sensitivity range ±5%, pulse dipolar ESR could not reliably detect any decrease in the fraction of spins in HHD pairs. It gives as the lowest estimate for $\boldsymbol{K_D} \geq 2\times10^{15}$ cm^2/mol, higher than 9.6×10^{13} determined for a thicker fluid DPhPC bilayer [29]. However, this value may correspond to channels formed in the fluid phase and kinetically trapped in the gel phase. Since in DPPC it takes hours and possibly days for the dimers to dissociate [26], it could take even longer time to reach equilibrium in the softer and less mismatching DMPC bilayer.

As mentioned above, N-terminal substitution in the gramicidin molecule affects channel formation and decreases the dimerization constant. Channels formed by N-terminally substituted derivatives of gramicidin in matching lipids also disappear with increasing

bilayer thickness, but for bulky substitutes it may require less hydrophobic mismatch to disrupt the channel. As seen in fig.5, fig.6 in DLPC, despite impaired channel-forming capacity due to a bulky nitroxide group attached to the N-terminus, GALN still forms HHDs. On the contrary, in the gel phase of DMPC, where GASL exists predominantly as HHD [25], GALN shows at 77 K a rigid limit spectrum without any conspicuous broad features, which is indicative of a much smaller, if any, fraction of HHD. In the aligned DMPC membrane at 4C, however, one can see a spectral feature (see arrows fig.6) which is consistent with a small fraction of HHDs. The field positions of these satellite lines in aligned DMPC match well to the corresponding features for GALN/DLPC. Double integration gives an approximate estimate of 2-4% for the biradical fraction in DMPC. If one assumes a 53 $Å^2$ area per lipid [66] this ratio corresponds to a dimerization constant $\boldsymbol{K_D}$ of ~ $2 \cdot 10^{10} cm^2/mol$, at least 5 orders of magnitude less than the lower estimate of $\boldsymbol{K_D}$ in GASL and 3-4 orders of magnitude lower than was determined for GA in DiphytanoylPC [29] or DOPC/n-decane [28]. A less bulky benzoyl group at the N-terminus has less effect on channel formation compared with the spin label. N-benzoyl-(desformyl)-GASL behaves in the DMPC membrane very similar to GASL. It forms channels in DMPC with the same interspin distance as GASL and does not show any non-channel fraction which would be detectable by pulse dipolar ESR at peptide/lipid ratios down to 0.01%, indicative of $\boldsymbol{K_D} > 10^{14} cm^2/mol$. However, the difference between In aligned EYL, GALN do not show strong narrow lines with resolved splittings, which could be attributed to rigid biradicals. As seen in fig 6, the ordering is relatively good and some broad features are still present. The most likely explanation is a lower fraction of HHD due to the higher thickness of the EYL bilayer.

Homodimers vs heterodimers

Interestingly, the broad signal of GALN in DLPC does not disappear upon 20 fold magnetic dilution with unlabeled GD, though its fraction substantially decreases (fig. 6). It indicates that a mixture of labeled and unlabeled GA molecules has a tendency to form homodimers rather than heterodimers. If instead of GA for the magnetic dilution we used N-benzoyl-(desformyl)-gramicidin with a benzoyl group at the N-terminus the broad signal disappears. This observation seems counterintuitive at the first glance. Unlabeled GA which should better bind to GALN monomers and prevent formation of close spin pairs provides less efficient magnetic dilution than N-benzoyl-(desformyl)-gramicidin. However, this behavior can be easily explained by the interplay of homo- and hetero-dimerization constants in the GA/GALN mixture. Apparently, the dimerization constant for unlabeled GA is much greater than the heterodimerization constant with GALN. Almost all unsubstituted GA monomers form GA homodimers. Even though the heterodimerization constant should be likely somehow larger than the homodimerization constant for GALN due to sterical factors, the equilibrium concentration of GA monomers is very low compared to GALN monomers. Eventually, GALN monomers find each other and form homodimers. Benzoyl substitution increases the concentration of unlabeled monomers; they bind more monomers of GALN and more interfere with spin pair formation than unsubstituted GA monomers. Thus, although dimerization constants for unlabeled GA derivatives cannot be directly measured by ESR, the difference in their $\boldsymbol{K_D}$ values manifests itself in the ESR spectra of GALN.

Another, more quantitative, example of this preferential homodimer formation is N-benzoyl – (desformyl)-GASL. Similar to GASL, in DMPC it forms HHD. For a 1:19 mixture with N,C-dibenzoyl-(desformyl)-gramicidin in DMPC DEER detects a ~ 5% fraction of spins in radical pairs, which corresponds to equal probabilities of homo- or heterodimer formation. If, however, N,C-dibenzoyl-(desformyl)-gramicidin is replaced by natural GD the fraction of radical pairs increases to ~32% .

Qualitatively, the increase in the fraction of spin labeled homodimers can be explained by the equilibrium of labeled (B) and unlabeled gramicidin species (A) including two homodimers (AA and BB) and a hetorodimer (AB):

$[\boldsymbol{AA}] = \boldsymbol{K_{AA}[A][A]}$

$[\boldsymbol{BB}] = \boldsymbol{K_{BB}[B][B]}$

$[\boldsymbol{AB}] = \boldsymbol{K_{AB}[A][B]}$

Excluding concentrations of free monomers gives following relation between the concentrations of dimeric species and corresponding dimerization constants:

$$\frac{[\boldsymbol{AA}][\boldsymbol{BB}]}{[\boldsymbol{AB}]^2} = \frac{\boldsymbol{K_{AA}K_{BB}}}{\boldsymbol{K_{AB}^2}}$$

In the case of equal probability for formation of ***AA***, ***BB*** and ***AB:*** $\boldsymbol{K_{AA} = K_{BB} = ½K_{AB}}$ (because ***AB*** means also indiscernible ***BA***). Then, assuming for simplicity that the constants are high enough to keep the concentration of free monomers much lower than dimers, consistent with DEER data for GASL and N-benzoyl – (desformyl)-GASL, for an 1:19 mixture of B and A one gets [AB]=38[BB] with 1/20 of spins in the BB homodimers.

From the experiment we directly determine the fraction of spins in pairs:

$\boldsymbol{f} = \frac{2[\boldsymbol{BB}]}{[\boldsymbol{AB}] + 2[\boldsymbol{BB}] + [\boldsymbol{B}]} = \frac{2[\boldsymbol{BB}]}{\langle \boldsymbol{B} \rangle}$, where ‹B› is the total concentration of B and,

neglecting the monomer fraction [B], the ratio $\alpha = \frac{2[\boldsymbol{BB}]}{[\boldsymbol{AB}]} = \frac{\boldsymbol{f}}{1-\boldsymbol{f}}$ and $\boldsymbol{f} = \frac{\alpha}{1+\alpha}$.

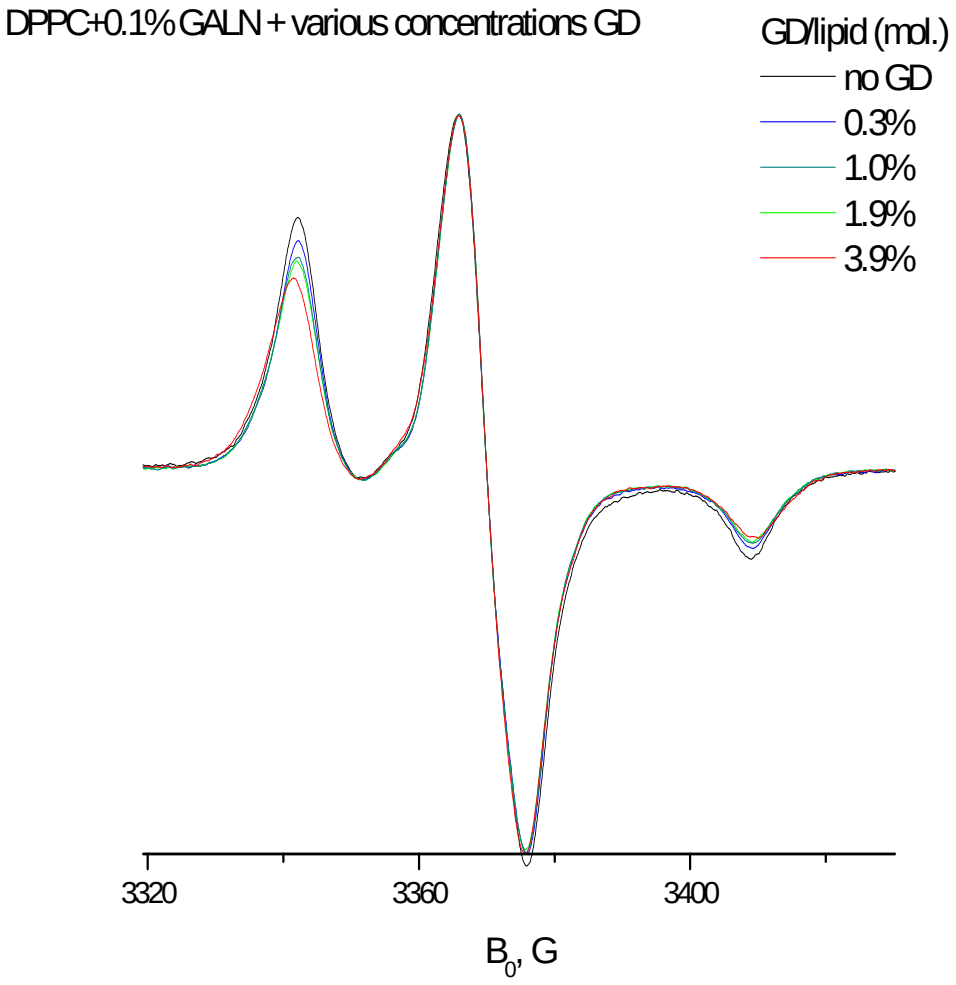
Assuming ‹A›=2[AA]+[AB] is the total concentration of A, we get

$$\frac{\alpha(\alpha+1)}{4}\frac{\langle \boldsymbol{A} \rangle}{\langle \boldsymbol{B} \rangle} - \frac{\alpha}{4} = \frac{\boldsymbol{K_{AA}K_{BB}}}{\boldsymbol{K_{AB}^2}}$$

With the observed increase in $\boldsymbol{f}$ = 0.05 to 0. 32, which corresponds to an increase in α from 0.053 to 0.47, we have a ~13-fold increase in the value of $\frac{\boldsymbol{K_{AA}K_{BB}}}{\boldsymbol{K_{AB}^2}}$. Since $\boldsymbol{K_{BB}}$ remains the same in this expression, the change indicates more increase in $\boldsymbol{K_{AA}}$ compared to heterodimer formation $\boldsymbol{K_{AB}}$ upon removal of the N-benzoyl group from the unlabeled molecule. For example, if $\boldsymbol{K_{AB}}$ increases 10fold, $\boldsymbol{K_{AA}}$ needs an increase by more than 3 orders of magnitude to provide the observed change in the fraction of pairs.

CW spectra of GALN and GASL, indications of DHD formation

Supplement Fig.4. Dependence of CW spectra in GALN/GD/DPPC on the concentration of GD.



The DHD dimer is long enough to traverse the hydrophobic part of the DPPC membrane (Table 1) so that the spin label reaches the part of the membrane with higher polarity. On the other hand, for GALN monomers the spin label should be located close to the least polar area of the membrane. As seen in the fig, with an increase in the concentration of

unlabeled GD the central (I=0) component of the spectrum shows little change, whereas the low-field component shows substantial gradual broadening. One also can see some conspicuous increase in the outer extreme splitting at higher concentrations of GD. This trend is consistent with a growing fraction of an ESR signal with higher local polarity. A low fraction of the signal manifests itself in the low- and high- field components as a "shoulder" on the background of a signal with lower polarity. This A-strain broadens these components and affects their intensity compared to the central component, which shape is not affected by the A-tensor. A larger fraction of the signal eventually starts to increase the apparent value of A_{zz}.

A similar change in the apparent value of A_{zz} is observed for the system GASL/GD/DPPC. In this case, the increase in the total gramicidin concentration decreases the apparent A_{zz} value (not shown). These observations are also consistent with our assumption of an increasing DHD fraction, since monomers in DPPC pop up to the surface and expose their nitroxide moieties into the polar area. On the other hand, for DHD, with the interspin distance (Table.1) insufficient to traverse the DPPC membrane, the nitroxides should locate in less polar environment. However, for GASL one should also consider a possibility that higher total gramicidin concentrations may somehow perturb the membrane structure and enable some HHD formation. And HHD formation also manifests itself as a decrease in observed local polarity [25]. For GALN its impaired propensity to form HHD and totally different spectrum expected for this conformation completely rule out the possibility and support the explanation through DHD's.