NMR Spectroscopy

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Dynamic Nuclear Polarization NMR Spectroscopy: Revealing Multiple Conformations in Lipid-Anchored Peptide Vaccines**

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Many pharmacological properties of conventional drugs can be improved using drug delivery systems, which are composed primarily of polymers or lipids.[1] Liposomes have become versatile delivery systems for induction of antibody and T-lymphocyte responses to associated subunit antigens.[2] The resulting immune response depends on the structural and physicochemical properties of liposomal vaccines. Biophysical methods, including circular dichroism (CD) or attenuated total reflectance IR spectroscopy (ATR-IR), have been used to investigate liposomal vaccines or other drug delivery systems (see, for example Ref. [3]). Solid-state NMR (ssNMR) allows studying proteoliposomes at atomic level.[4] However, the requirement of high polypeptide concentrations may not be compatible with the formulation of liposomal vaccines.

We investigated the use of DNP-ssNMR (DNP, dynamic nuclear polarization[5]) to study liposomal vaccines designed to target Alzheimer's disease. Previous work[3,6] has shown that tetrapalmitoylated β-amyloid 1–15 peptide (Palm1–15) embedded into liposomes along with monophosphoryl lipid A (MPLA) can elicit an immune response that restores the cognitive impairment of APP (amyloid precursor protein) transgenic mice. Biophysical and one-dimensional ssNMR experiments on Palm1–15 uniformly labeled with $^{13}C/^{15}N$ at Ala2, Ser8, and Gly9 (Palm1–15(ASG); Figure 1) suggested that the peptide adopts an extended backbone conformation that may be influenced by the lipid environment.[3] However, further in-depth structural ssNMR studies were precluded by limited spectroscopic sensitivity. For our DNP studies, we treated liposomes containing Palm1-15 with DMPC/DMPG/cholesterol/MPLA (9:1:7:0.06 molar ratio) (Figure 1 a,c) or DMTAP/cholesterol/MPLA (10:7:0.06) (Figure 1 b,d) with the polarizing reagent TOTAPOL[7] and [D_8]glycerol (DMPC = 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine,

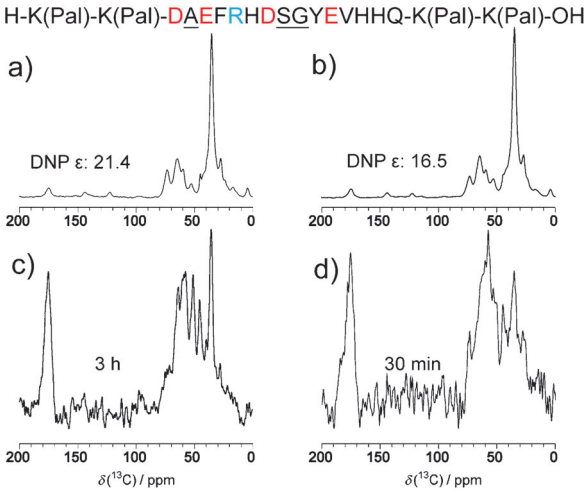

Figure 1. Top: Sequence of Palm1-15(ASG) with $^{13}C/^{15}N$ labeled residues underlined and charged residues (at pH 7) colored: blue = positively charged, red = negatively charged. 1D NMR spectra of liposomal Palm1-15 in DMPC/DMPG/Chol (a,c) or DMTAP/Chol (b,d). CP-MAS spectra at 100 K are depicted in (a) and (b); the 1D 2QF spectra (with an 1 ms excitation 2Q time) in (c) and (d). All samples were washed in 5 mM TOTAPOL in (2:6:2) [D_8]glycerol/D_2O/H_2O. Signal enhancements are given relative to the case with microwave irradiation off.

DMPG = 1,2-dimyristoyl-*sn*-glycero-3-(phospho-*rac*-(1-glycerol), DMTAP = 1,2-dimyristoyl-3-trimethylammoniumpropane). These preparations exhibited strong signal enhancements in 1D CP and double-quantum filtered (2QF) ^{13}C ssNMR experiments for both lipid compositions (Figure 1 a–d).

Further experiments suggested that low concentrations of TOTAPOL provide a good compromise between signal enhancement and paramagnetic relaxation enabling us to conduct two-dimensional (2Q,1Q) experiments (Figure 2). Because of the low peptide concentration ((1:200) peptide/lipid ratio), the resulting ssNMR spectra contained signals of ^{13}C-labeled peptide residues as well as correlations originating from natural abundance lipids. Compared to the labeled peptide, lipid signals should be attenuated by at least a factor of 10 000, suggesting that the effective lipid to peptide ratio is higher than 500:1, thus strongly limiting the use of conventional ssNMR methods.[3]

We could readily distinguish peptide and lipid signals owing to their distinct chemical-shift pattern. For the three labeled residues (Ala2, Ser8, and Gly9), we observed two signal sets for the DMPC/DMPG/Chol sample (Figure 2 a). In the DMTAP/Chol case, four sets of ssNMR correlations could

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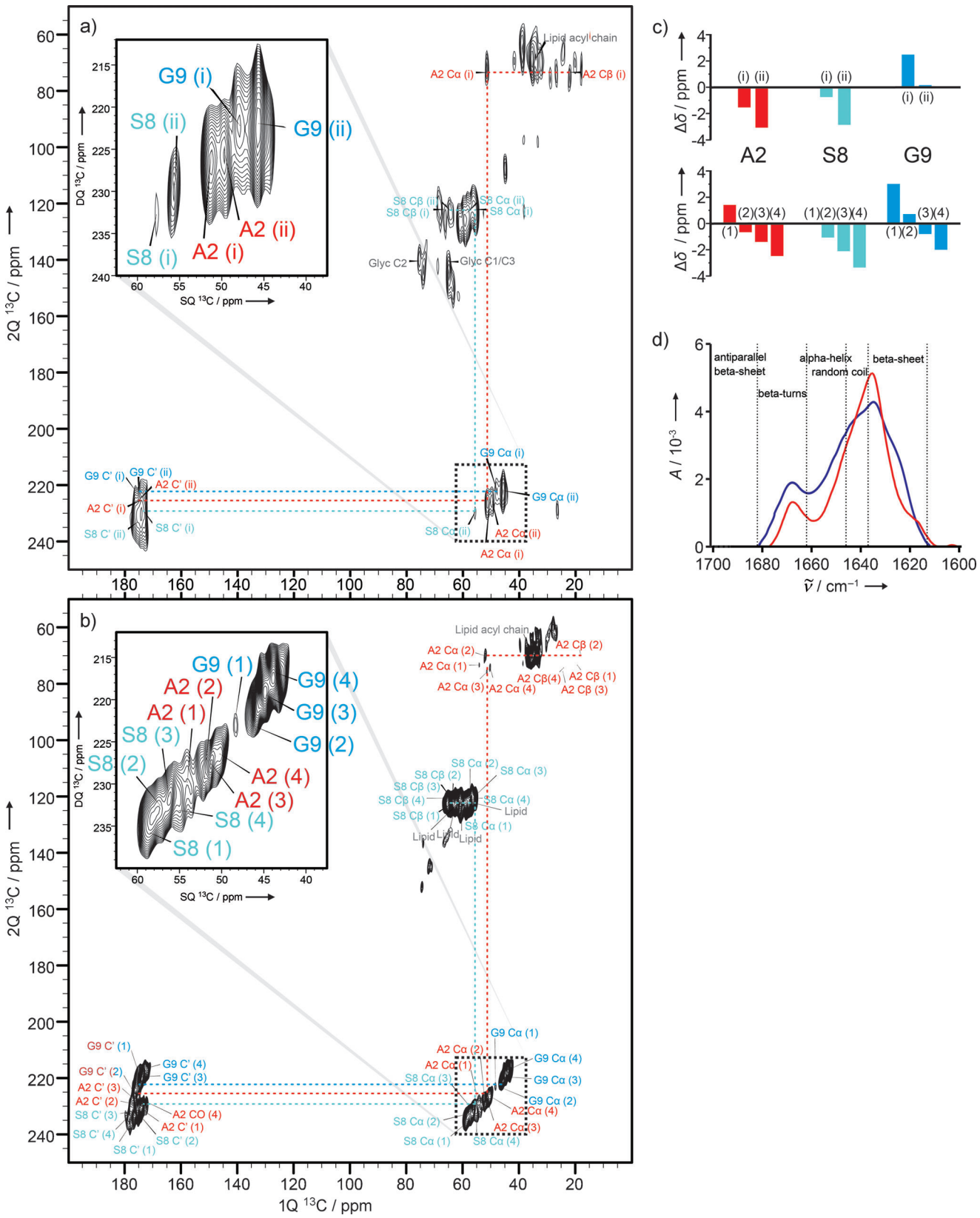

Figure 2. ^{13}C (2Q/1Q) 2D experiments on liposomal Palm1-15 (ASG). Liposomes consisted of a) DMPC/DMPG/Chol or b) DMTAP/Chol. c) NMR difference chemical shifts $\Delta\delta = \delta C\alpha - \delta C\alpha(rc)$ for liposomal Palm1-15 vaccine in DMPC/DMPG/Chol (top) and in DMTAP/Chol (bottom); d) ATR-IR amide I band of liposomal Palm1-15 spectra in anionic liposomes (DMPC/DMPG/Chol, red) and cationic liposomes (DMTAP/Chol, blue).

be identified (Figure 2b). As shown before,[8] we subsequently computed secondary chemical shifts $\Delta\delta$ that represent established parameters to infer polypeptide conformation in solution and solid-state NMR.[9] In Figure 2c, $\Delta\delta$ were determined for Cα resonances for both lipid compositions. In the DMPC/DMPG/Chol case (Figure 2c, top graph), the more dominant correlations (denoted by ii) for Ala2 and Ser8 are strongly negative. This is consistent with an extended backbone conformation. In this arrangement, $\Delta\delta$ values for Gly residues are close to random coil values,[10] in line with the more dominant signals (ii) in Figure 2a. A second weaker population (i) exhibited secondary chemical shifts close to random coil arrangements. In the case of the cationic DMTAP/Chol (Figure 2c, bottom graph), we also observed secondary chemical shifts that were compatible with extended conformations (denoted by species 3 and 4). However, their spectroscopic intensities were comparable to additional signals that exhibited small or even positive $\Delta\delta$ values and are referred to as populations 1 and 2 (Figure 2b,c). Note that in the absence of sequential assignments, the numbering of the populations may not correspond to the same peptide unit.

ATR-IR experiments (Figure 2d) confirmed the presence of additional peptide folds in liposomal Palm1-15. Curve-fitting procedures suggest a prominent β-sheet conformation (72% in total) in the case of DMPC/DMPG/Chol (Figure 2d, red line) in qualitative agreement with 60:40 intensity ratio between populations (ii) and (i) seen in ssNMR. In line with our earlier findings[3] (Supporting Information, Figure S1), the use of cationic DMTAP/Chol leads to a significant reduction of β-sheet conformation and an increase in conformations with other peptide folds (Figure 2d, blue line).

For further investigation, we resorted to mesoscopic coarse-grained molecular dynamics (CGMD) simulations (Figure 3). Peptides in the aqueous phase partitioned at the membrane surface within less than a microsecond. Here, extended peptide conformations formed in line with our ssNMR data obtained on the free peptide that suggest the β-strand formation is more pronounced at the membrane compared to the free case (Supporting Information, Figure S2).

We observed spontaneous aggregation of peptides into β-sheet-like aggregates in both lipid mixtures. Further analysis of the surface-bound peptides revealed significantly broader density profiles of the peptide backbone in DMTAP/Chol in comparison to DMPC/DMPG/Chol (Figure 3a,b). In DMPC/DMPG/Chol, peptide backbones can approach each other with limited steric or electrostatic hindrance (Figure 3c), because negatively charged side chains predominately orient orthogonal to the membrane surface (Figure 3d). In cationic DMTAP lipids, anionic side chains orient almost parallel to the bilayer surface, suggesting that membrane charge modulates peptide aggregation behavior.[3] Note that another modulator of peptide aggregation may be the reduced thickness and head group size of DMTAP lipids, which led to a slight positive curvature of the peptide-containing leaflet in the simulations.

Taken together, our results show that DNP-enhanced ssNMR provides a powerful method to structurally examine surface-associated biomolecules at low molecular concentrations. In the case of Palm1–15, our studies indicate that the active vaccine contains antigen moieties for which β-strands are, at least in part, triggered by peptide–peptide interactions that subsequently associate from dimers to larger oligomers to the membrane. Our work suggests that larger β-sheets only form in the DMPC/DMPG/Chol case and that additional conformations that are particularly relevant for the DMTAP case may reduce in vivo target specificity. These findings open the way for rational structure-based design of liposome-bound peptide immunogens with defined conformations to generate optimized vaccines against a range of protein misfolding diseases. Future studies may involve cellular settings in which DNP-ssNMR studies have been demonstrated.[11] Moreover, DNP-supported ssNMR studies may help to elucidate the role of lipid surfaces for protein aggregation, toxicity and pore formation in general or aid the study of amyloid folding intermediates.

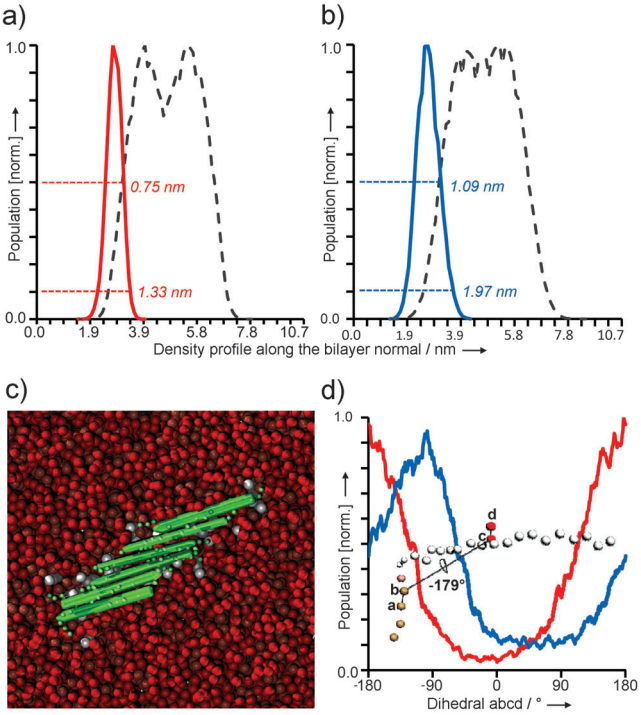

Figure 3. Density profile of the peptide backbone in a) a 9:1:7 DMPC/DMPG/Chol and b) a 10:7 DMTAP/Chol mixture. Continuous and dashed lines stand for peptide and membrane profiles, respectively. c) Example of an ordered hexameric β-sheet of Palm1–15 in DMPC/DMPG/Chol after 25 μs of simulation. DMPC/DMPG lipids and cholesterol are colored in light and dark red, respectively. Palmitoyl anchors and peptide backbones are given as gray and green beads, respectively. d) While negatively charged side chains such as Asp7 predominately orient along the bilayer normal in DMPC/DMPG/Chol (red), side chains in DMTAP/Chol orient almost parallel to the surface (blue). Dihedral angles were averaged over all peptides and 25 μs of simulation.

Experimental Section

Liposomal Palm1–15 was prepared as described before.[3] For the NMR measurements, 666 μL of the liposomes were spun down

(100000 g, 1 h) to a volume of approximately 25 μL. The pellet was washed with 50 μL of 5 mM TOTAPOL (DyNuPol, MA) in 2:6:2 glycerol-d8/D_2O/H_2O. Liposomes were pelleted for 1–3 hrs at 100000 g, followed by the removal of the supernatant. The washing procedure was repeated twice. NMR experiments were performed using a 400 MHz/263 GHz DNP system (Bruker Biospin). The sample was cooled to approximately 100 K in a 3.2 mm sapphire rotor spinning at 8 kHz. DNP enhancements were measured by overlaying HC CP/MAS spectra recorded with and without microwave irradiation. (2Q/1Q) experiments were performed using the SPC5 sequence[12] with a 1 ms double-quantum excitation time. CGMD simulations[13] were conducted for each lipid composition using eighteen Palm1–15 copies over 25 μs (see also the Supporting Information).

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