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Encapsulation and NMR on an Aggregating Peptide before Fibrillogenesis

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In amyloidosis, peptides aggregate first into soluble oligomers, and then into insoluble protofibrils and fibrils.[1] Soluble aggregates are cytotoxic[2] but difficult to observe directly because they are short-lived and prone to form insoluble fibrils. One approach for studying oligomers structurally would be to encapsulate peptides such that they are prevented from further aggregation into fibrils. Wand and co-workers reported methods for encapsulating an aggregating protein in the aqueous core of reverse micelles in liquid hexane.[3] A low-viscosity solvent is used on the exterior of the reverse micelles to increase the tumbling rate of the protein. While these techniques preserved the structural integrity of the proteins they examined, it is also of interest to examine protein aggregation in aqueous media under conditions that more closely approximate physiological conditions.

We encapsulated a fibril-forming peptide, Peptide **1**: NH_2-SDDYYYGFGSNKFGRPRDD-COOH, in 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine single bilayer vesicles (POPC SBVs). As a control, we also encapsulated a smaller, nonaggregating peptide, Peptide **2**: NH_2-EEWEE-COOH. In initial experiments, we verified that Peptides **1** and **2** do not bind appreciably to POPC SBVs, using a modification of a published method (see Supporting Information).[4] We have also shown, by the same method, that β-amyloid(1−40) does not appreciably associate with POPC SBVs, despite its amphiphilicity.[5]

POPC SBVs were prepared using a modification of the ethanol injection method of Batzri and Korn.[6] To encapsulate Peptide **1**, an ethanolic solution of POPC was injected into a solution containing peptide dissolved in 10 mM sodium phosphate, pH 7.40, with 7.5% DMSO and 6M urea to solubilize the aggregating peptide. Vesicles were concentrated using Amicon ultrafiltration and chromatographed using Sephacryl S-500 to remove multi bilayer vesicles (MBVs) and any residual DMSO and urea that had not been removed by ultrafiltration. From calibration of the column using polystyrene and protein standards, we calculated a diameter of 40 nm for the SBVs, which was confirmed by electron microscopy. An NMR sample was prepared by concentrating the SBVs using Amicon ultrafiltration in 10 mM sodium phosphate, pH 7.40, with 5% D_2O. Phospholipid and protein concentrations of these samples were determined using the Phospholipids B Assay (Wako) and the BCA Protein Assay (Pierce), respectively. In two vesicle preparations with Peptide **1**, average lipid and peptide concentrations were 16.5 ± 1.1 mM and 31.0 ± 3.5 μM, respectively. For SBVs of 40 nm diameter at this lipid concentration, this corresponds to 2.4 ± 0.1 mM peptide in the SBV interior, or 24.6 ± 1.1 molecules of Peptide **1** per SBV.

To demonstrate that Peptide **1** had been encapsulated, we added a paramagnetic substance, Gd-EDTA, to the solution surrounding the vesicles, and measured longitudinal relaxation times (T_1) by NMR.[7] Peptide encapsulated in vesicles would not be in direct contact with Gd-EDTA, and so would be shielded from T_1 shortening shown by peptides in direct contact with Gd-EDTA. In control experiments, we performed one-dimensional proton (1D-^{1}H) NMR on 100 μM Peptide **1** or Peptide **2** in 10 mM sodium phosphate, pH 7.40 with 5% D_2O, in the presence or absence of 1 mM Gd-EDTA (Figure 1). Without Gd-EDTA, NMR lines for Peptide **1** were slightly broader than those for Peptide **2**, as expected for an aggregating peptide. For peptides free in solution, the T_1 values decrease markedly in the presence of 1 mM Gd-EDTA (Table 1). In addition, the line widths were significantly broadened by 1 mM Gd-EDTA. (Figure 1C,D).

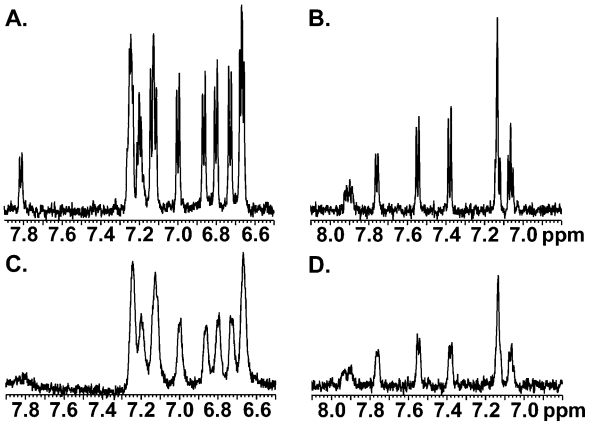

Figure 1. 1D-^{1}H NMR spectra showing the aromatic/amide region of 100 μM Peptides **1** (A) and **2** (B) in 10 mM sodium phosphate, pH 7.40 with 5% D_2O. Significant line broadening was observed after the addition of 1 mM Gd-EDTA to the solution containing Peptides **1** (C) and **2** (D). 1D-^{1}H spectra were obtained at 25 °C at 600 MHz. Identical window functions were applied to all spectra in the figure.

1D-^{1}H NMR spectra of the vesicles after encapsulating Peptide **1** (Figure 2A) showed several peaks in the aromatic/amide region indicating the presence of peptide. These peaks are somewhat broader than corresponding peaks for peptide free in solution. In contrast to peptides in solution, addition of Gd-EDTA to the solvent surrounding the vesicles did not decrease T_1 values for the peptide (Table 1). Similarly, whereas 1 mM Gd-EDTA broadened NMR lines of Peptide **1** or **2** free in solution, no such broadening was observed for encapsulated peptide upon addition of Gd-EDTA (Figure 2B). After adding Gd-EDTA to the bulk solvent surrounding the vesicles containing Peptide **1**, we added Peptide **2** to the outside of the vesicles so that it was equimolar with Peptide **1**. Peptide **2** had shortened T_1 values, similar to the values obtained with this peptide alone in solution (Table S1, Supporting Information). These experiments show that Peptide **1** was indeed encapsulated within vesicles since it, and not peptide free in solution, was protected from T_1 shortening due to Gd-EDTA in the bulk solvent. Peptide

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Table 1. T_1 Values for Peptide **1**, in Solution or Encapsulated in POPC SBVs and in the Absence or Presence of Gd-EDTA

Peptide 1 in solution				Encapsulated Peptide 1					
		1mM Gd-EDTA				1mM Gd-EDTA		1mM Gd-EDTA and Peptide 2 outside	
δ (ppm)	T_1 (s)	δ (ppm)	T_1 (s)	δ (ppm)	T_1 (s)	δ (ppm)	T_1 (s)	δ (ppm)	T_1 (s)
7.81	0.62	7.80	0.014	7.82	0.38	7.82	0.86		
7.23	1.93	7.24	0.19	7.23	1.66	7.23	1.62	7.23	1.51
7.20	1.96	7.20	0.20	7.18	1.92	7.18	2.04	7.18	2.00
7.13	1.47	7.12	0.21	7.12	1.36	7.12	1.43		
7.00	1.44	7.00	0.21	6.99	1.23	6.99	1.25	6.99	1.03
6.87	1.40	6.86	0.18	6.85	1.38	6.85	1.23	6.85	1.15
6.80	1.40	6.80	0.18	6.79	1.23	6.79	1.13	6.79	1.14
6.73	1.77	6.73	0.17	6.72	1.24	6.72	1.28	6.72	1.10
6.67	1.73	6.66	0.20	6.66	1.24	6.66	1.16	6.66	1.13

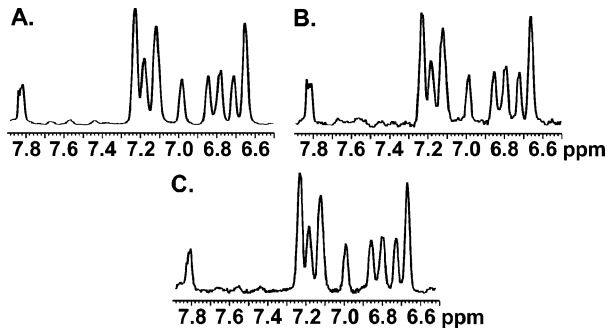

Figure 2. 1D-^{1}H NMR spectra showing the aromatic/amide region of 31.0 $\pm$ 3.5 μM Peptide **1** encapsulated in POPC SBVs, without (A) or with (B) 1 mM Gd-EDTA in the surrounding bulk solvent (10 mM sodium phosphate, pH 7.40 with 5% D_2O). (C) Aromatic/amide portion of the 1D-^{1}H spectrum of Peptide **1** encapsulated in POPC SBVs 4 days earlier. 1D-^{1}H spectra were obtained at 25 °C at 600 MHz. Identical window functions were applied to these three spectra to improve signal-to-noise.

2 could also be encapsulated and showed similar preservation of line widths and T_1 values when Gd-EDTA was added to the solvent surrounding the vesicles (Figure S7 and Table S1, Supporting Information). No NMR signals were observed from DMSO or urea, confirming that the purification had removed most of these additives.

It was also important to show that encapsulation inhibits fibril formation. Since Peptide **1** fibrillizes within 1 day, we obtained NMR spectra of samples encapsulated 4 days earlier. The 1D-^{1}H spectrum of Peptide **1** encapsulated for 4 days is essentially identical to that of the freshly encapsulated peptide, showing the same line widths (Figure 2A,C). There is also a slight loss of peak intensity (Figure S8, Supporting Information). Fibrillization is typically associated with loss of NMR signal, as shown for a 2.8 mM solution of Peptide **1** over 14 h (Figure S9, Supporting Information). The preservation of the spectral properties in the encapsulated samples indicates that for up to 4 days, i.e., well within the time frame of most high-resolution NMR experiments, the peptide remains within vesicles and soluble in the included solvent.

In this paper, we have shown encapsulation of an aggregating peptide at concentrations much higher than those necessary for fibril formation of peptide free in solution. NMR line broadening for encapsulated Peptide **1** may be due to oligomerization, or vesicle wall effects. Both peptide concentration in the SBV interior and vesicle size were chosen somewhat arbitrarily, and we have encapsulated peptide at a wide range of initial concentrations and SBV sizes. Additional specifications of SBVs with encapsulated Peptide **1** are given in Table S2, Supporting Information.

The technique reported herein will facilitate comparisons with theoretical studies of aggregation, which often start with a small number of peptide molecules enclosed in an explicit or implicit solvent "box". Our overall goal is to obtain high-resolution NMR data on aggregation intermediates of proteins implicated in amyloidoses such as Alzheimer's Disease, and to provide the first detailed structural information about cytotoxic, soluble oligomers. This method may also be used to study the folding of aggregation-prone proteins in a chaperone-like protected environment.

Future development of this technique could include the use of photocrosslinkable phospholipids to minimize vesicle fusion;[8] the use of deuterated lipids or multiple frequency presaturation techniques to suppress the lipid signals;[9] and the use of ^{13}C and ^{15}N labeled peptides for the use of two- and higher dimensional NMR techniques. Even without these refinements, these results show that sufficient quantities of proteins can be encapsulated to obtain structural information. With such developments, it may be possible to obtain detailed structural data on temporally unstable protein aggregates that hitherto have been inaccessible to analysis.

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Supporting Information Available: Electron micrographs of Peptide **1** fibrils and 1 mM POPC SBVs; POPC SBV binding assays for Peptides **1** and **2**, and β-amyloid(1−40); Sephacryl S-500 chromatogram of POPC SBVs with encapsulated Peptide **1**; 1D-^{1}H NMR of Peptide **2** encapsulated in POPC SBVs; 1D-^{1}H NMR of Peptide **1** encapsulated in POPC SBVs over time; 1D-^{1}H NMR spectra of 2.8 mM solution of Peptide **1**; fibrils formed around POPC SBVs. Tables of T_1 values of Peptide **2** and properties of POPC SBVs with encapsulated Peptide **1**. This material is available free of charge via the Internet at http://pubs.acs.org.

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