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Synthesis and Proton NMR Spectroscopy of Intra-Vesicular Gamma-Aminobutyric Acid (GABA)*

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Abstract— **We report the synthesis of vesicles containing gamma-aminobutyric acid (GABA), and their proton nuclear magnetic resonance (^{1}H NMR) spectra. These vesicles were constructed to more closely mimic the intracellular environment wherein GABA exists. For this study, these GABA-containing vesicles were examined under ^{1}H NMR as a potential platform for future studies on the differences between aqueous phantoms, *ex vivo* brain extracts, and *in vivo* magnetic resonance spectroscopy results. We found that intra-vesicular GABA faithfully yielded the chemical shifts and *J*-coupling constants of free aqueous GABA, alongside the chemical shift signals of the vesicle wall.**

I. Introduction

While phantoms made in aqueous solution are widely used to corroborate the spectroscopic signals of brain metabolites, it has not been substantively studied how accurately such phantoms approximate their *in vivo* counterparts. For example, it recently has been determined that the T_2 relaxation time for gamma-aminobutyric acid (GABA) is 88 ms *in vivo* [1]. This is much shorter than a measured value of 260 ms within an aqueous phantom control, and also shorter than other detected metabolites, which range between 150 and 300 ms [2].

It is postulated that the reason stems from the cellular environment within which GABA exists [1]. Decreased rotational motion from cooler temperatures or higher viscosity is known to reduce T_2; but whether such an environment exists within an intracellular vesicle is unknown. If it does, its characterization would not only help more accurately quantify GABA *in vivo*, but also shed light on the intra-vesicular behavior of this inhibitory neurotransmitter.

Secondly, *in vivo* spectra will necessarily contain overlapping signals from lipids and other metabolites. As a means to distinguish these contents by proton nuclear magnetic resonance (^{1}H NMR) spectroscopy, *ex vivo* preparations have been studied [3]. However, synaptic vesicle fractionation requires differential centrifugation steps; and the extent to which extraction techniques alter or preserve intracellular vesicles cannot be easily corroborated.

A completely synthetic construct mimicking cellular structures of interest would be a helpful comparator. In the case of GABA (Fig. 1), the amount of neurotransmitter detected *in vivo* likely represents the intracellular content of GABAergic neurons [3,4], where GABA exists packaged within vesicles reported to measure from 29 to 36 nm in diameter [5]. A non-invasive and reliable way of measuring, and calibrating, intracellular GABA would be of great clinical interest.

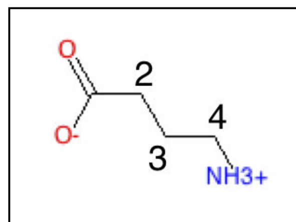

Figure 1. Structure of gamma-aminobutyric acid (GABA). The numbers correspond to the carbons in the molecule, ordered by standard nomenclature.

To our knowledge, there are no prior reports in which synthetic GABA-containing vesicles have been characterized by NMR. Such a vesicle would be an important model for *in vivo* GABA measurements. Therefore, as a first step, we asked whether a synthesized vesicle containing GABA near physiological concentrations could, in fact, produce similar spectra to that of free GABA in aqueous solution.

II. Methods

A. Vesicle preparation

A thin film of dioctyl sodium sulfosuccinate (DSS) was prepared inside a 100-mL round-bottom flask by dissolving 0.178 g (0.4 mmol) DSS in 5 mL chloroform, with subsequent evaporation by nitrogen gas. The film was dried overnight under high vacuum, and then rehydrated with 30 mL of a 5-mM GABA solution in phosphate-buffered saline (PBS). The obtained DSS-GABA suspension was sonicated for 5 min in a sonication bath (Branson 2510 ultrasonic cleaner), followed by tip sonication for another 3 minutes at room temperature (Vibracell ultrasonicator). The resulting vesicle suspension was repetitively washed by PBS (50 mL × 3) to remove any extra-vesicular GABA using ultra-centrifugation (Amicon Ultra centrifugal filter, cut-off pore size 10 kDa), and concentrated to desired volume. Vesicles remained stable for up to 4 weeks at 4 °C.

B. Intra-vesicular GABA concentration confirmation using high-performance liquid chromatography (HPLC)

The vesicle suspension was pretreated with dansyl chloride (Sigma Aldrich) at 80 °C in the dark and centrifuged. The supernatant was subjected for analysis (HPLC UV detector wavelength at 285 nm) [6]. Vesicle sizes were determined by dynamic light scattering (Malvern Zetasizer Nano).

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C. ^{1}H NMR spectroscopy

Samples were placed in a standard 5-mm NMR tube, and high-resolution spectra were acquired on a Varian 400 MHz spectrometer using water suppression (WET). Parameters were: flip angle 45°, relaxation delay 10 s, spectral width 8021.8 Hz, and 32 repetitions. Spectra were post-processed with a 1.0-Hz line- broadening function, and referenced to water at 4.7 ppm. The process was repeated for a 1-mM GABA solution in PBS alone.

III. Results

The final internal concentration of GABA by HPLC was 2.44 ± 0.29 mM. The mean vesicle diameter was 37 nm (Fig. 2), close to the sizes reported in tissue [5].

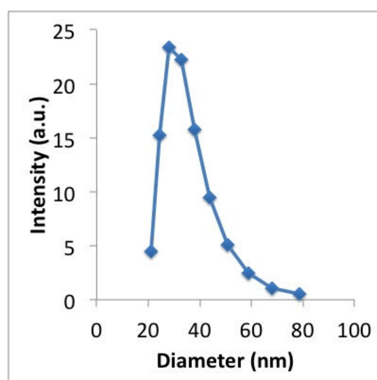

Figure 2. Distribution of vesicle diameters (nm).

Fig. 3 displays the near-identical spectra acquired from free aqueous (*top*) and intra-vesicular (*bottom*) GABA. For aqueous GABA, the GABA C2 protons resonated as a triplet, centered at 1.839 ppm; the C3 protons resonated as a multiplet, centered at 1.442 ppm; and the C4 protons resonated as a triplet, centered at 2.559 ppm.

For intra-vesicular GABA, the C2 protons resonated as a triplet, centered at 1.839 ppm; the C3 protons resonated as a multiplet, centered at 1.442 ppm; and the C4 protons resonated as a triplet, centered at 2.560 ppm. The *J*-coupling values were identical, and are summarized in Table 1.

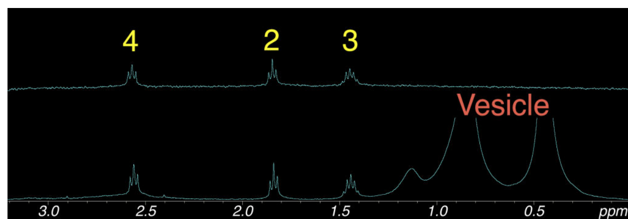

Figure 3. Spectra from aqueous gamma-aminobutyric acid (GABA; *top*) and GABA-containing vesicles (*bottom*). The yellow numbers correspond to protons on the carbons identified in Fig. 1.

TABLE I. Coupling Constants Between Aqueous and Intra-Vesicular GABA

Carbon #	Coupling constant *J* (Hz)	
	Aqueous GABA	*Intra-vesicular GABA*
2-3	7.3	7.3
3-4	7.4	7.4

IV. Discussion

There were no chemical shift or *J*-coupling differences between aqueous and intra-vesicular GABA. Additionally, the greater signal-to-noise ratio within the vesicular GABA sample appeared to agree with the higher GABA concentration (2.44 mM) measured within the synthesized vesicles by HPLC.

These results indicate GABA-containing vesicles successfully replicate the chemical shifts characteristic of GABA. The lack of chemical shift and line-width changes also confirms that the diamagnetic susceptibility of the vesicle wall has an extremely small effect, and does not appreciably alter the B_0 field inside.

From X-ray diffraction studies, vesicles synthesized from DSS have a lipid bilayer thickness of 19.5 Å [7-9]. Molecular dynamics simulations of a closely related surfactant, sodium bis-3,5,5-methyl-1-hexyl sulfosuccinate, result in a similar thickness of 19.2 Å [10]. Due to packing differences and a shorter carbon chain length, DSS vesicles possess a shallower wall than those constituted from the unsaturated phospholipid 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (38 Å) typical of cell membranes [11,12]. Nevertheless, packaging GABA within vesicles represents a step toward the synthesis of more advanced and realistic phantoms. Given the intricacies of synaptic vesicle fractionation *ex vivo* [13], such phantoms would offer more robust comparisons to *in vivo* ^{1}H NMR data.

With the close reproduction of expected chemical shifts, further experiments on optimizing these vesicles are certainly warranted. Once optimized, these synthesized vesicles should serve as a useful substrate in T_2 experiments. In particular, this intermediate environment will provide a promising platform to study in further detail the intra-vesicular behavior of GABA, and help define the mechanisms underlying the discrepancy in T_2 relaxation times between *in vitro* and *in vivo* conditions.

Acknowledgment

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