Protein molecular weight standards can compensate systematic errors in diffusion-ordered spectroscopy

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Diffusion-ordered spectroscopy (DOSY) 1 is an NMR method that reports translational diffusion coefficients for the molecule of interest [1,2]. DOSY is prone to a number of systematic errors related to gradient calibration, temperature convection inside the NMR tube, and sample-induced changes in viscosity [3]. Due to these problems, it is often perceived as difficult to obtain accurate results from DOSY. However, a number of studies have shown that DOSY can be a powerful tool in the study of intermolecular interactions, for example, in a binding study [4]. In this Note, we use molecular weight standards to circumvent systematic errors by converting the diffusion coefficients obtained by DOSY into effective molecular weights (EMW). The concept is similar to referencing to molecular weight standards in size-exclusion chromatography (SEC) analyses. For ideal, spherical molecules, theory predicts reciprocal-cube-root dependence of the diffusion coefficient on molecular weight [5]. In practice, experimental calibration curves using similarly shaped molecules may be sufficient for EMW measurements of molecules belonging to the same class of molecules.

\[ I(q) = I_0 e^{-Dq^2\Lambda} \]  

(1)

The DOSY-derived diffusion coefficient \( D \) is obtained from a gradient-based experiment, processed according to Eq. (1) [6], where \( D \) is the diffusion coefficient \( (cm^2 s^{-1}) \); \( q = \gamma g \delta \) \( G \); gyromagnetic ratio \( \text{rad s}^{-1} G^{-1} \); \( g \), amplitude of the applied gradient \( (G \text{ cm}^{-1}) \); and \( \delta \), duration of the applied gradient \( (s) \); \( \Lambda' = \Lambda - \delta / 3 [\Lambda', \text{experimental diffusion period} (s)] \).

The value of \( D \) is related by Eq. (1) to signal intensity and applied gradient strength. The main disadvantage with reporting values of \( D \) is that it depends on the sample conditions (temperature, viscosity, gradient calibration, and buffer conditions) rather than a fixed parameter (molecular weight, hydrodynamic radius) of the analyzed molecule. This makes the treatment of systematic errors important and hence it is difficult to compare values of \( D \) determined for the same molecule in two different laboratories.

Biochemical methods such as SEC and analytical ultracentrifugation (AUC) report effective molecular weight, or hydrodynamic radius, rather than \( D \). Molecular weight standards are used to replace systematic errors related to SEC and AUC with a random error related to the calibration curve. The nature of the standards depends on what molecules are to be analyzed. For example, chitooligosaccharides are used to calibrate SEC analyses of related oligosaccharides and we recently showed that they are suitable standards for DOSY [7].

Proteins such as aprotinin and bovine serum albumin are typically used in the SEC and AUC analysis of proteins. Fig. 1 illustrates the DOSY data of a series of
protein molecular weight standards as one-dimensional diffusion traces derived from two-dimensional DOSY spectra (Fig. 1A) and as a calibration curve (Fig. 1B). The diffusion traces indicate the diffusion coefficients of residual water (HDO) and the proteins themselves. It is clear from the traces that the diffusion coefficients of the proteins are dependent on their molecular weight. A double-log plot of diffusion coefficient against molecular weight provides a clear correlation (Fig. 1B). We use this calibration curve to determine the EMW of unknown samples.

Table 1 provides the determined values of log $D$ and EMW for a number of proteins of interest in our laboratories. The monomeric EF-hand domain of rat calretinin (CR I–II) is of particular note, having previously been studied by DOSY and three other methods [8]. The EMW of CR I–II is somewhat larger than the nominal monomer MW, since in the calcium-bound form, this protein may adopt an extended conformation to offer hydrophobic patches for binding, as reported for the related protein, calmodulin [9]. The EMW determined by DOSY can be compared to that determined by SEC (14 ± 2 kDa) [10]. The DOSY and SEC values of EMW are consistent and slightly elevated compared to the real molecular weight.

A second point to be made on the basis of Table 1 is that the EMW can be more easily compared with the expected values for the oligomeric forms of the proteins. Usually, log $D$ values are reported as “consistent with monomer/dimer” [6,11–13]. The DOSY-derived EMW is more easily interpreted than reported log $D$ values with respect to oligomer or complex stoichiometry. Table 1 reports the galectin 7 complex as a dimer. This result is consistent with other data supporting the tendency of galectins to occur as dimers [14]. Hevein, a small, 43 amino acid, carbohydrate-binding protein heavily studied in our laboratory [5,15], is clearly monomeric according to the DOSY data (Table 1). Oligomeric state affects the quality of NMR data that can be collected and the strategy to solve the structures of homodimers is different from the strategy for monomers and heterodimers. The oligomeric state of a protein can be different at
NMR concentrations compared to the micromolar concentrations used in other biochemical characterizations. As DOSY data are quickly acquired and do not require $^{15}$N- or $^{13}$C-labeled material, it is an efficient method for confirming the oligomeric state of proteins and determining the most efficient strategy for solving the NMR structure of proteins.

To summarize, we advocate the use of protein molecular weight standards to overcome the systematic errors associated with DOSY techniques in the determination of protein oligomeric states and the size of protein complexes. The reporting of EMW, rather than $D$, will allow better comparison of data originating from different research groups.

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