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ARTICLE

Concentration-dependent changes to diffusion and chemical shift of internal standard molecules in aqueous and micellar solutions

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Abstract
Sodium 4,4-dimethyl-4-silapentane-1-sulfonate (DSS) is the most widely accepted internal standard for protein NMR studies in aqueous conditions. Since its introduction as a reference standard, however, concerns have been raised surrounding its propensity to interact with biological molecules through electrostatic and hydrophobic interactions. While DSS has been shown to interact with certain proteins, membrane protein studies by solution-state NMR require use of membrane mimetics such as detergent micelles and, to date, no study has explicitly examined the potential for interaction between membrane mimetics and DSS. Consistent with its amphipathic character, we show DSS to self-associate at elevated concentrations using pulsed field gradient-based diffusion NMR measurements. More critically, DSS diffusion is significantly attenuated in the presence of either like-charged sodium dodecyl sulfate or zwitterionic dodecylphosphocholine micelles, the two most commonly used detergent-based membrane mimetic systems used in solution-state NMR. Binding to oppositely charged dodecyltrimethylammonium bromide micelles is also highly favourable. DSS-micelle interactions are accompanied by a systematic, concentration- and binding propensity-dependent change in the chemical shift of the DSS reference signal by up to 60 ppb. The alternative reference compound 4,4-dimethyl-4-silapentane-1-ammonium trifluoroacetate (DSA) exhibits highly similar behaviour, with reversal of the relative magnitude of chemical shift perturbation and proportion bound in comparison to DSS. Both DSS and DSA, thus, interact with micelles, and self-assemble at high concentration. Chemical shift perturbation of and modulation of micellar properties by these molecules has clear implications for their use as reference standards.

Keywords Internal chemical shift standard · Aqueous detergent micelle solutions · Solution-state NMR spectroscopy · Membrane-mimetic environments

Introduction

Accurate chemical shifts relative to a standardized reference frequency are critical for the comparison of NMR data between studies, and for assignment of signals to nuclei within a molecule of interest. In protein NMR, quantification of deviation of chemical shifts from either random coil values (Wishart et al. 1992; Wishart and Sykes 1994) or relative to database values (Cornilescu et al. 1999) is a major source of structural restraint. Any bias introduced by even a slight change in the reference frequency might, therefore, affect structural accuracy. The IUPAC–IUBMB–IUPAB-endorsed secondary chemical shift reference molecule for biomolecular NMR in aqueous environments is sodium 4,4-dimethyl-4-silapentane-1-sulfonate (DSS, Fig. 1) (Wishart et al. 1995; Markley et al. 1998).

DSS has been shown to interact with a variety of biomolecules. Reported examples include interactions with adenosine triphosphate (Lam and Kotowycz 1977), β- and γ-cyclodextrins (Li et al. 1993), peptides (Laurents et al. 2005; Nowick et al. 2003), and partially unfolded proteins (Shimizu et al. 1994). To circumvent the issue that DSS may not be inert to a biomolecular system under investigation, other derivatives of tetramethylsilane (TMS) have been prepared with different polar groups to substitute for DSS. As an example,

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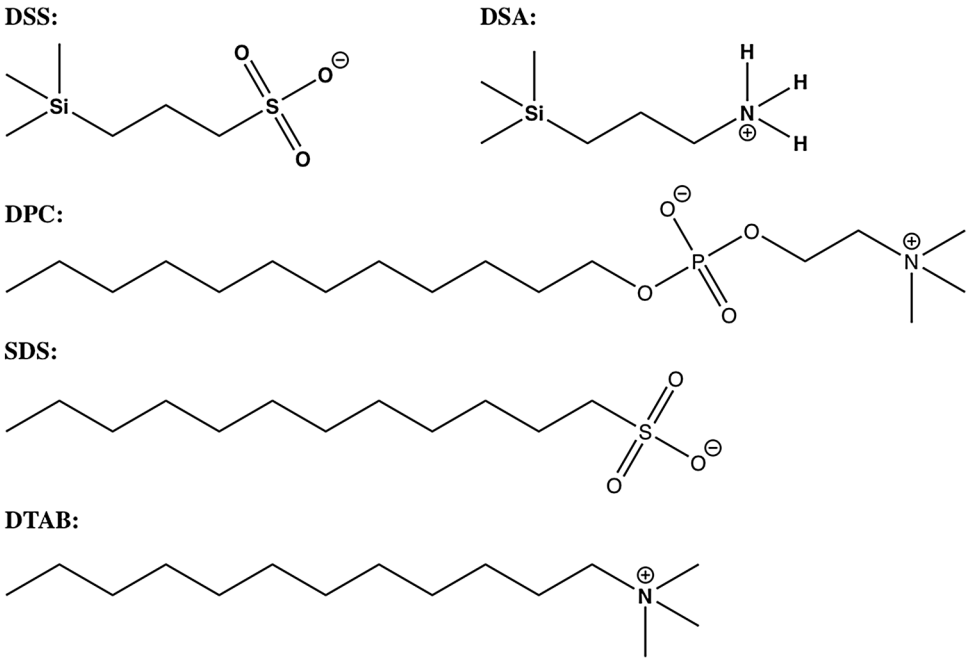

Fig. 1 Chemical structures of the chemical shift internal standard molecules, 4,4-dimethyl-4-silapentane-1-sulfonate (DSS) and 4,4-dimethyl-4-silapentane-1-ammonium trifluoroacetate (DSA), and the three detergents, dodecylphosphocholine (DPC), sodium dodecyl sulfate (SDS), and dodecyltrimethylammonium bromide (DTAB), employed herein. Note that all molecules are shown without counterions

4,4-dimethyl-4-silapentane-1-ammonium trifluoroacetate (DSA, Fig. 1) was introduced as a cationic alternative to the anionic DSS to avoid interaction with a set of engineered β-turn peptides that interact with DSS (Nowick et al. 2003). The chemical shifts of DSA have also been shown to be less affected by complex biofluids compared to other reference standards (Alum et al. 2008).

While DSS is not an inert internal reference for certain systems, its perturbations in many cases are not so obvious unless studied in isolation. In solution-state NMR studies of membrane proteins, membrane mimetics such as detergent micelles or phospholipid bicelles are typically employed (Opella 2013; Pandey et al. 2016). Since DSS, DSA and other soluble TMS derivatives are, by necessity, amphipathic molecules, they have the potential to interact with other amphipathic molecules to form mixed supramolecular systems. These derivatives of TMS also have the potential to form self-micelles or other aggregates in solution at sufficiently high concentrations.

Here, the potential of both DSS and DSA to interact with various micelles is examined through systematic analysis of chemical shift and pulsed field gradient-based diffusion ordered spectroscopy (DOSY) NMR experiments. Three detergents with identical dodecyl tailgroup but different headgroup/charge profile have been employed: zwitterionic dodecylphosphocholine (DPC), anionic sodium dodecyl sulfate (SDS) and cationic dodecyltrimethylammonium bromide (DTAB) detergents (Fig. 1). The potential for DSS to aggregate at high concentrations has also been investigated using these methods.

Materials and methods

All chemicals were used as purchased or received without further purification. DSS was either diluted from a commercially-prepared 50.6 mM stock in D_2O (C/D/N Isotopes, Pointe-Claire, QC) or prepared from powder (Wilmad, Vineland, NJ) in D_2O (Sigma-Aldrich, Mississauga, ON). DSA was a gift from James Nowick (University of California, Irvine). Deuterated SDS-d_{25} (Cambridge Isotope Laboratories, Tewksbury, MA), DPC-d_{38} (Cambridge Isotope Laboratories), and DTAB-d_{34} (C/D/N Isotopes) were used. All NMR experiments were performed at 11.74 T (500 MHz ^{1}H frequency; Avance, Bruker Canada, Milton, ON) using a double-resonance BBFO SmartProbe (Bruker Canada). Unless otherwise noted, experiments were performed at 37 °C in D_2O and the pH was adjusted to 5.0 ± 0.1 using NaOD and DCl (neglecting deuterium isotope effects).

NMR experiments at DSS or DSA concentrations of 0.05–50 mM were performed in pure D_2O, and in the presence of 100 mM DPC-d_{38}, 100 mM SDS-d_{25}, or 100 mM DTAB-d_{34} (only at 0.1, 0.5 and 1 mM DSS or DSA). Additional experiments were performed on DSS up to saturation in D_2O. Samples containing 0.5 mM DSS and 25–200 mM DPC-d_{38} or SDS-d_{25} were also studied. Experiments at 0.5 mM DSS in 100 mM DPC-d_{38} or SDS-d_{25}, pH 5.0 ± 0.1 were performed at 22 and 52 °C in addition to 37 °C. Finally, samples containing 0.5 mM DSS in 100 mM DPC-d_{38} or SDS-d_{25} at 37 °C were also prepared at pH 7.0 ± 0.1.

1D ^{1}H NMR experiments were performed using presaturation to suppress the residual HOD signal (1–2 s

relaxation delay; 16–128 scans; 2 s acquisition time with ~ 28,000 acquired points). ^{1}H DOSY (Morris and Johnson 1992) experiments were performed using a stimulated echo pulse program that includes presaturation during the relaxation delay, bipolar pulse gradients and eddy current delays (Wu et al. 1995). Sixteen spectra were collected at increasing gradient strengths of 2–95% calibrated such that the final increment attenuated all signals by at least 95%. A diffusion time (Δ) of 100 ms was consistently employed, with gradient pulse lengths (δ) of 1000 µs (HOD), 1250 µs (DSS or DSA in D_2O), or 3000–4000 µs (DSA or DSS in detergent) as required to achieve 95% or greater attenuation. In the case of DOSY experiments observing diffusion of HOD, presaturation was not applied during the relaxation delay (Wu et al. 1995).

NMR spectra were processed using Bruker TopSpin 3.1. Referencing in all instances at pH 5.0 and 37 °C was based upon a 0.000 ppm reference methyl peak of the corresponding chemical shift standard in D_2O at 0.1 mM. For experiments where temperature or pH were varied, the methylene peak of a given detergent at pH 5.0 and 37 °C was employed as a reference (i.e., 1.2372 ppm for DPC, 1.2427 ppm for SDS, and 1.2541 ppm for DTAB) for experiments in that detergent under different temperature or pH conditions. Translational diffusion coefficients (D) were calculated using the TopSpin Relaxation Module for the strongest peaks corresponding to DSS (0 ppm), DSA (0 ppm), DPC (~ 1.24 ppm), SDS (~ 1.24 ppm), DTAB (~ 1.25 ppm), and HOD (~ 4.62 ppm) by non-linear least squares fitting of the observed signal attenuation (I) under a given gradient strength (g) relative to the unattenuated signal (I_0) to the Stejskal–Tanner equation (Stejskal and Tanner 1965) in a form appropriate for the DOSY experiment in question (Wu et al. 1995):

$$I = I_0 e^{-\left[1000 \times D(2\pi\gamma g\delta)^2\left(\Delta-\frac{\delta}{3}\right)\right]} \quad (1)$$

where δ is the length of time the gradient is applied, Δ is the delay between the two gradient pulses (the diffusion time), and γ is the gyromagnetic ratio of the nucleus. Experiments were performed twice and D values reported are the average ± average deviation of these trials.

The bound fraction of chemical shift standard in a given micellar condition was estimated on the basis of the following four assumptions: (i) the observed diffusion coefficient ($D_{observed}$) is a linear combination of the diffusion of free (D_{free}) and bound (D_{bound}) species:

$$D_{observed} = f_{bound}\left(D_{bound}\right) + \left(1 - f_{bound}\right)\left(D_{free}\right) \quad (2)$$

where f_{bound} is the fraction of the observed species bound; (ii) far above the critical micelle concentration of the detergent, almost the entire population is bound in micelles and the diffusion coefficient of the detergent ($D_{detergent}$) corresponds to the micellar diffusion coefficient; (iii) a micelle-bound species of DSS or DSA diffuses at the same rate as the micelles; and, (iv) free DSS or DSA diffuses at the same rate at the same concentration in the absence of detergent. These assumptions give rise to the relationship:

$$f_{bound} = \frac{D_{observed} - D_{free}}{D_{detergent} - D_{free}} \quad (3)$$

It should be explicitly noted that $D_{detergent}$ will actually have a non-zero contribution from free detergent (in a linear combination as in Eq. (2), ~ predictable if the critical micelle concentration and aggregation number are known under the given conditions). The fractions of DSS or DSA bound estimated by Eq. (3) will, thus, consistently be expected to be underestimates given that the expected increase in the observed $D_{detergent}$ would increase the magnitude of the denominator.

Results and discussion

DSS self-association

Given its amphipathic character (Fig. 1), micelle formation by DSS is a plausible phenomenon. To test for this, ^{1}H 1D NMR and DOSY experiments were performed for DSS in aqueous solution at concentrations of 0.05–300 mM. Each ^{1}H resonance exhibits a concentration dependent chemical shift perturbation (CSP) at concentrations of 50 mM and above, relative to the chemical shift at 0.05 mM concentration, with the perturbation being greatest for the Si-($C\underline{H}_3$)$_3$ singlet and least for the SO_3^-–$C\underline{H}_2$ multiplet (Fig. 2a, with inset provided to clearly illustrate lack of perturbation at low concentrations). Correspondingly, DSS exhibits a concentration-dependent decrease in diffusion coefficient over the entire regime examined, with translational diffusion at higher concentrations being hindered relative to lower concentrations (Fig. 2b; Table 1). The diffusion coefficient of $\underline{H}$OD also decreased over this DSS concentration regime, but to a lesser extent (Table 1), implying that viscosity and/or crowding are not the primary source(s) of the observed decreased translational diffusion by DSS.

Amphipathic molecules below their critical micelle concentration are known to transiently form dimers or other small structures termed pre-micellar aggregates (Bakshi et al. 2002), which would be consistent with the decrease in the diffusion coefficient of DSS over the 0.1–5 mM regime. The lack of a corresponding CSP correlates with the finding that DSS incorporated into β-ball structures overlaps completely with DSS isolated from the rest of the sample within a capillary (Laurents et al. 2005).

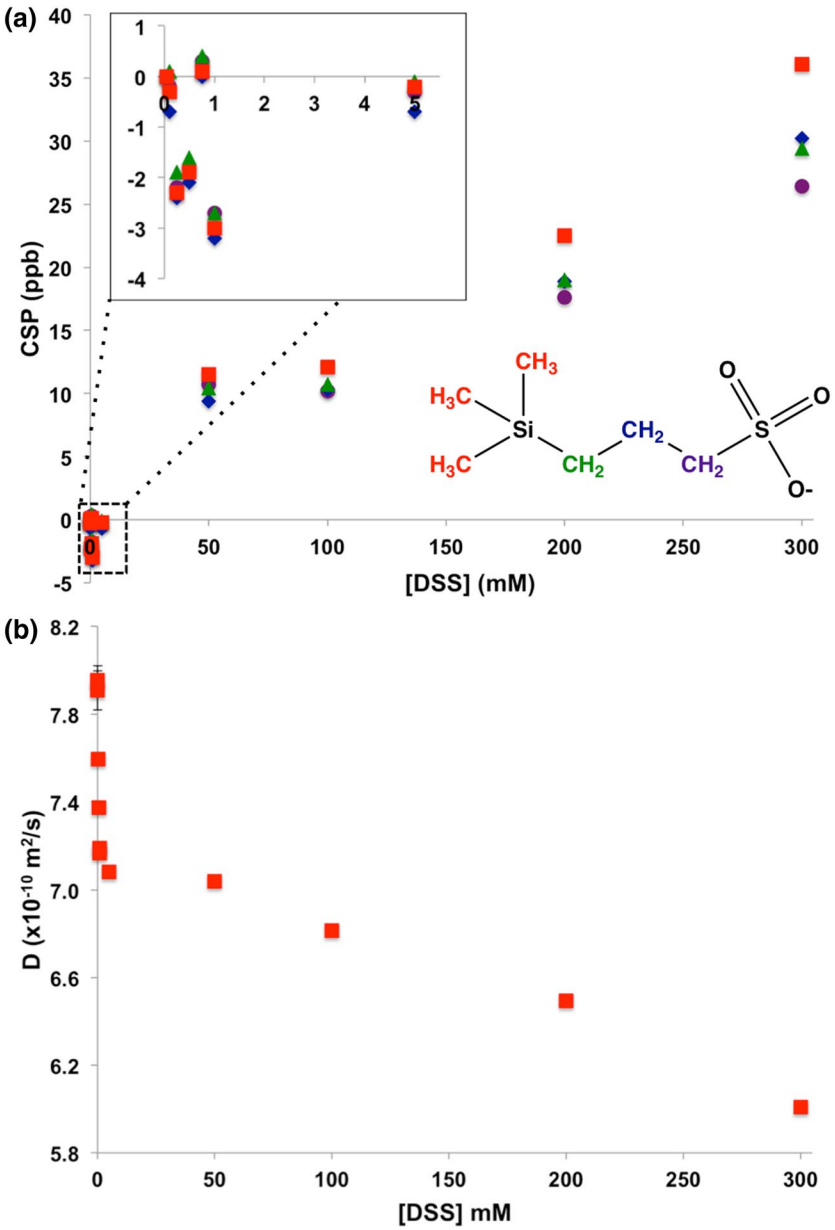

Fig. 2 Concentration dependent **a** ^{1}H chemical shift perturbation (CSP) and **b** translational diffusion coefficient (D) of DSS in D_2O at pH ~ 5 and 37 °C. In **a**, the CSP of methylene groups at positions 1–3 are represented as circles, diamonds, and triangles, respectively. In **a, b** the CSP and D of methyl groups at position 5 are represented as squares. An inset is provided to clarify behaviour in low concentration regime in (**a**)

Further decrease in DSS diffusion coefficient at elevated concentrations implies that it self-assembles at sufficiently high concentrations. Observation of greater CSP for the hydrophobic trimethylsilyl moiety relative to the methylene group attached to the sulfonate would be consistent with micellar-type behaviour. Over the concentration regime studied, however, the decrease in diffusion coefficient is not as large as might be expected for the formation of a perfect micelle. The diffusion coefficient of SDS, for example, decreases by a factor of ten above its critical micelle concentration (Misselyn-Bauduin et al. 2000), though the aggregation number of SDS is likely much higher than that of DSS based upon the relative size of these amphiphiles. While the critical micelle concentration of DSS is not known, amphipathic molecules with branched hydrocarbon chains are known to have critical micelle concentrations higher than related straight-chain compounds (Jalali-Heravi and Konouz 2000). For reference, heptane-1-sulfonate, a related straight-chain sulfonate that lacks the trimethylsilyl moiety of DSS, has a reported critical micelle concentration of 130 mM (Calhoun and King 2007). Assuming fast exchange of DSS between the self-assembled and monomeric states, an estimated critical micelle concentration of > 130 mM means that even at a DSS concentration of 300 mM, the observed diffusion coefficient would contain a large contribution from free DSS (e.g., Eq. (2)). Therefore, the pure micellar

Table 1 Translational diffusion coefficients (D; × 10^{-10} m^2 s^{-1}) of indicated species (average ± average deviation for two trials; ± 1 for last significant figure, where deviation is smaller) under given conditions at pH ~ 5 and 37 °C as determined using ^{1}H DOSY

Condition	D_{HOD}	D_{DSS}	$D_{Detergent}$
D_2O at pH 5	23.5 ± 0.2	–	–
100 mM DPC	22.3 ± 0.1	–	0.980 ± 0.004
100 mM SDS	22.7 ± 0.1	–	0.948 ± 0.001
100 mM DTAB	21.9 ± 0.1	–	1.16 ± 0.01
0.05 mM DSS	24.0 ± 0.1	7.91 ± 0.09	–
0.10 mM DSS	24.2 ± 0.1	7.96 ± 0.06	–
0.25 mM DSS	23.3 ± 0.1	7.59 ± 0.01	–
0.50 mM DSS	23.9 ± 0.1	7.38 ± 0.03	–
0.75 mM DSS	23.8 ± 0.2	7.19 ± 0.01	–
1.0 mM DSS	22.7 ± 0.1	7.16 ± 0.02	–
5.0 mM DSS	22.9 ± 0.1	7.08 ± 0.02	–
50 mM DSS	22.6 ± 0.1	7.04 ± 0.01	–
100 mM DSS	22.1 ± 0.1	6.81 ± 0.01	–
200 mM DSS	21.8 ± 0.1	6.50 ± 0.02	–
300 mM DSS	20.8 ± 0.1	6.01 ± 0.01	–
0.050 mM DSS + 100 mM DPC	22.6 ± 0.2	2.79 ± 0.01	0.978 ± 0.001
0.10 mM DSS + 100 mM DPC	22.9 ± 0.2	2.36 ± 0.04	0.956 ± 0.001
0.50 mM DSS + 100 mM DPC	22.0 ± 0.1	2.23 ± 0.01	0.964 ± 0.003
1.0 mM DSS + 100 mM DPC	22.4 ± 0.1	2.24 ± 0.01	0.974 ± 0.001
50 mM DSS + 100 mM DPC	23.4 ± 0.1	4.06 ± 0.01	1.01 ± 0.01
0.050 mM DSS + 100 mM SDS	24.0 ± 0.1	4.54 ± 0.07	0.937 ± 0.002
0.10 mM DSS + 100 mM SDS	23.0 ± 0.1	5.82 ± 0.09	0.917 ± 0.002
0.50 mM DSS + 100 mM SDS	22.8 ± 0.1	5.58 ± 0.05	0.915 ± 0.008
1.0 mM DSS + 100 mM SDS	22.6 ± 0.1	6.09 ± 0.02	0.944 ± 0.007
50 mM DSS + 100 mM SDS	22.5 ± 0.1	5.67 ± 0.01	0.973 ± 0.003
0.1 mM DSS + 100 mM DTAB	22.5 ± 0.1	1.27 ± 0.03	1.76 ± 0.01
0.50 mM DSS + 100 mM DTAB	22.0 ± 0.1	1.06 ± 0.01	1.40 ± 0.01
1.0 mM DSS + 100 mM DTAB	22.6 ± 0.1	1.11 ± 0.01	1.37 ± 0.01

species of DSS is likely to diffuse much more slowly than observed in these experiments. Given that typically employed DSS concentrations in biomolecular NMR studies are on the order of 0.5–1 mM (with 10 mM or less originally suggested (Wishart et al. 1995)), we opted not to further investigate this self-assembly process.

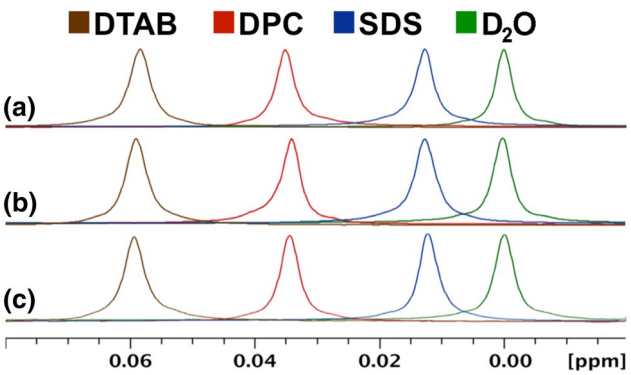

Fig. 3 Overlaid ^{1}H NMR spectra showing Si-C$\underline{H}_3$ resonances of DSS observed in D_2O (most shielded) relative to those observed in 100 mM of SDS, DPC or DTAB (from second-most to least shielded, respectively). DSS concentrations of **a** 1.0 mM, **b** 0.5 mM, and **c** 0.1 mM are illustrated. In all cases, experiments were performed at pH ~ 5 and 37 °C, with spectral referencing relative to the DSS Si-C$\underline{H}_3$ resonance at 0.000 ppm observed at 0.1 mM in D_2O

DSS interactions with micellar species

To test for and characterize DSS interactions with common micellar species, 1D ^{1}H NMR and DOSY experiments were performed for samples containing 0.05–1 mM DSS and either 100 mM DPC or 100 mM SDS. For direct comparison, samples containing 0.1–1 mM DSS were also examined in DTAB at 100 mM. This cationic detergent was not extensively investigated as it is not a common choice for membrane protein NMR studies given the general low abundance of cationic lipids in biological systems (as reviewed, e.g., by Khakbaz and Klauda (2015)). When referenced to DSS in buffer, the presence of all three detergents caused a systematic deshielding from 0 ppm for the DSS trimethylsilyl moiety reference peak (Fig. 3). At all DSS concentrations tested, the same trend is observed, with DTAB samples exhibiting the greatest degree of deshielding, DPC an intermediate level, and SDS the least deshielding. The chemical shift of DSS was deshielded by up to 60 ppb in 100 mM DTAB, 40 ppb in 100 mM DPC, and 20 ppb in 100 mM SDS. Systematic perturbation of DSS methyl group chemical shifts is indicative of an interaction with each class of micelle, and implies that referencing based upon the DSS chemical shift will be systematically, and differently, offset from the "true" 0 ppm value in each of these classes of micelle.

Corresponding to the observed chemical shift perturbation, the diffusion coefficient of DSS also decreased in the presence of 100 mM DTAB, DPC or SDS (Fig. 4a). HOD diffusion did not change significantly in any of the DPC/SDS/DTAB mixtures relative to DSS in D_2O (Table 1), implying that the restriction in DSS diffusion is due to binding rather than a change in solvent viscosity. Following from Eq. (3), the fraction of DSS binding in each micellar

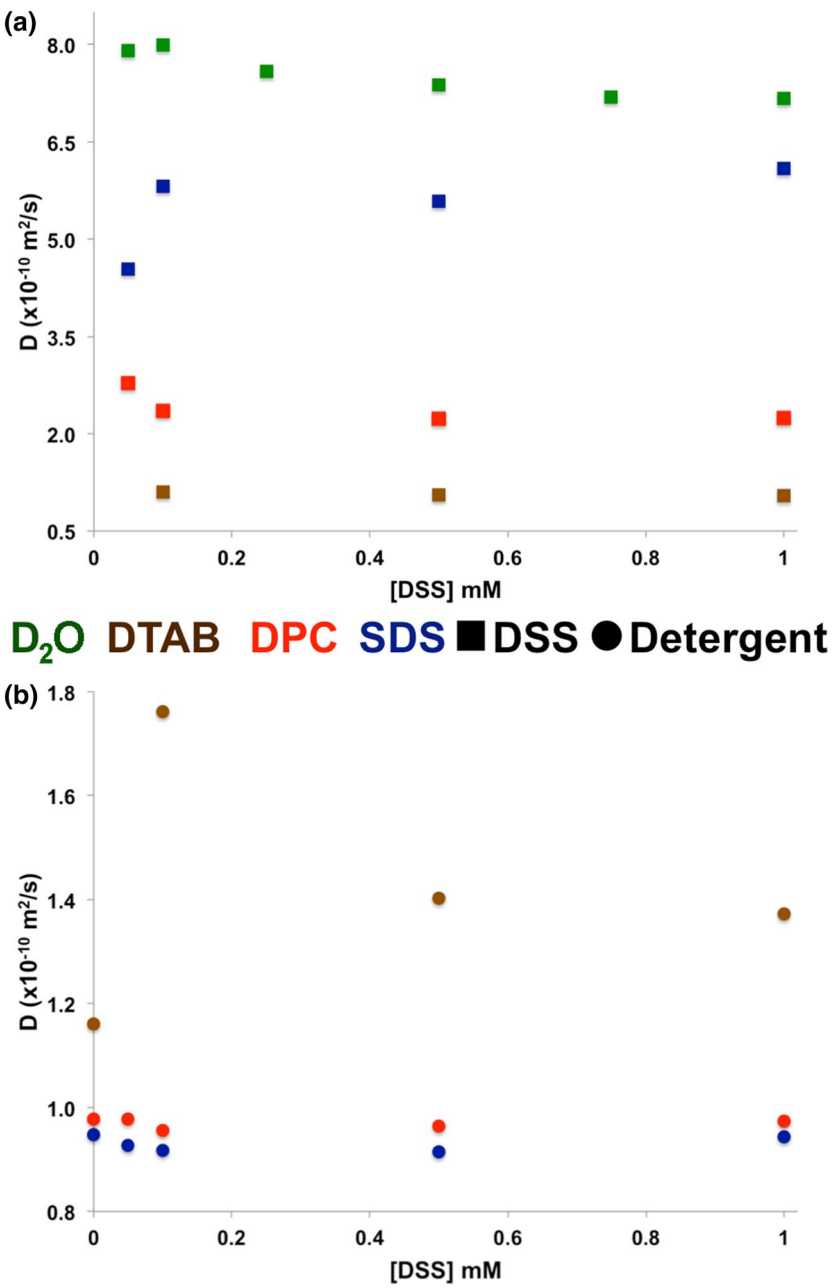

Fig. 4 D for **a** DSS and **b** detergents observed at indicated DSS concentration and 100 mM detergent. For reference, D of DSS in D_2O is also plotted (highest values of D in **a**), with lower values of D observed, uniformly at any given DSS concentration, for DSS in SDS, DPC, and DTAB. Detergent D values mirror this, with the highest values of D observed for DTAB, intermediate values for DPC, and the lowest values for SDS. Experiments were performed at pH ~ 5 and 37 °C, with DOSY-based (Morris and Johnson 1992) attenuation of the Si-CH_3 resonance at ~ 0 ppm employed to determine D of DSS and that of the detergent methylene resonances at ~ 1.24–1.25 ppm used to determine D of each detergent. Values are shown as average of two replicate experiments with error bars indicating average deviation

Table 2 Estimated micelle-bound DSS fraction (±0.1) as a function of concentration in the presence of 100 mM of a given detergent inferred from relative diffusion coefficients of DSS and detergent (Eq. 3)

Micelle type	SDS	DPC	DTAB
(DSS) = 0.1 mM	0.3	0.8	1.1
(DSS) = 0.5 mM	0.3	0.8	1.1
(DSS) = 1.0 mM	0.2	0.8	1.0

system has been estimated (Table 2). Interestingly, the fraction bound to a given micelle type follows the observed trend of chemical shift perturbation.

DSS-micelle interactions, in turn, modulate the observed diffusion coefficient (a population-weighted linear combination of the D for the micelle and the free detergent, Eq. (2)) for each detergent (Fig. 4b). In the case of DTAB, this is most pronounced, with an elevated D upon DSS incorporation. Conversely, for both SDS and DPC, the observed modulation of D is minimal at low (0.05–1 mM) DSS concentrations, ultimately leading to a slight increase in the D observed at high (50 mM) DSS concentration (Table 1).

Considering the hydrodynamic and chemical shift behaviour together, the following trends are apparent. Mixture of the anionic DSS species with cationic (DTAB) micellar species increases the D for the detergent and decreases D for

the DSS, with the greatest CSP to DSS of the three detergents, consistent with mixed-micelle formation leading to an increase in population of free detergent species (i.e., considering the linear combination in Eq. (2) as it would apply to free vs. micellar detergent species). The mixture of anionic DSS with anionic (SDS) micellar species, conversely, exhibits the least perturbation in chemical shift, consistent with the least drive for DSS partitioning into the micellar species. Finally, mixing DSS with zwitterionic (DPC) micelles shows, as anticipated, intermediate behaviour.

For the more commonly employed DPC and SDS micelles, changes in DSS chemical shift were examined as a function of detergent concentration. DSS was kept constant at a typically employed concentration of 0.5 mM and titrated with either DPC or SDS. Titration with both detergents led to increased CSP of the DSS trimethylsilyl ^{1}H resonance as a function of detergent concentration (Fig. 5), following the degree of perturbation observed at fixed detergent concentration (Fig. 4). The chemical shift of the DSS methyl peak in micellar solution is also somewhat sensitive to temperature and pH (Fig. 6), implying that development of empirical correction factors is not trivial through disparate environmental influences.

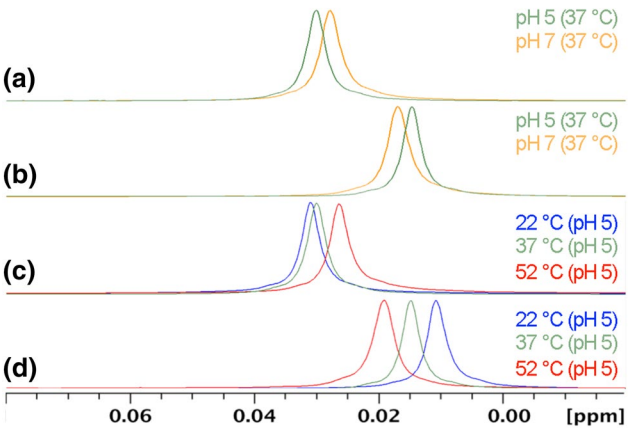

Fig. 6 Overlaid ^{1}H NMR spectra showing Si-C$\underline{H}_3$ resonances of DSS observed in D_2O with 100 mM DPC (*a*) and (*c*) or SDS (*b*) and (*d*). In *a, b*, pH ~ 5 (more deshielded in DPC, more shielded in SDS) is compared to pH ~ 7 (less deshielded in DPC, less shielded in SDS) at 37 °C. In *c, d*, temperatures of 22, 37 and 52 °C are compared at pH ~ 5, with lower temperature leading to greater deshielding in DPC versus higher temperature leading to greater deshielding in SDS. In all cases, the chemical shift of the methylene peak of the detergent at ~ 1.25 ppm was maintained at a consistent value to that observed at pH ~ 5 and 37 °C

DSA follows similar trends to DSS with opposite binding affinity

^{1}H 1D NMR and DOSY spectra were acquired over the range of 0.05–50 mM DSA in D_2O and, for 0.05–1 mM DSA, in the presence of DPC, SDS, and DTAB. As with DSS, all DSA concentrations employed are below the critical micelle concentration of related straight chain amphiphiles such as octylammonium chloride (175 mM) and hexylammonium hydrochloride (900 mM) (Mukerjee and Mysels 1971). The DSA concentrations studied therefore likely also fall in the pre-micellar concentration regime, consistent with a minor concentration-dependent decrease in the diffusion coefficient of DSA in D_2O (Fig. 7a; Table 3).

Like its structural analogue DSS, the behaviour of DSA changes dramatically in micellar solutions. Distinct from

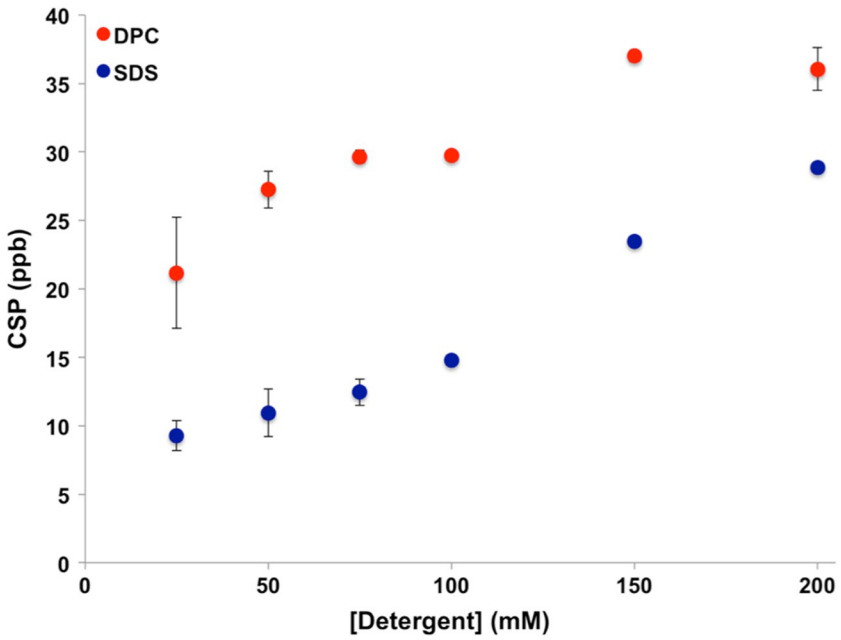

Fig. 5 CSP of DSS (0.5 mM) Si-C$\underline{H}_3$ resonance as a function of SDS (lower perturbation at a given detergent concentration) or DPC (higher perturbation) concentration. DSS is deshielded (e.g., Fig. 3) in the presence of detergent in all instances. All experiments were performed at pH ~ 5 and 37 °C, with averages ± average deviation of two independent replicates shown

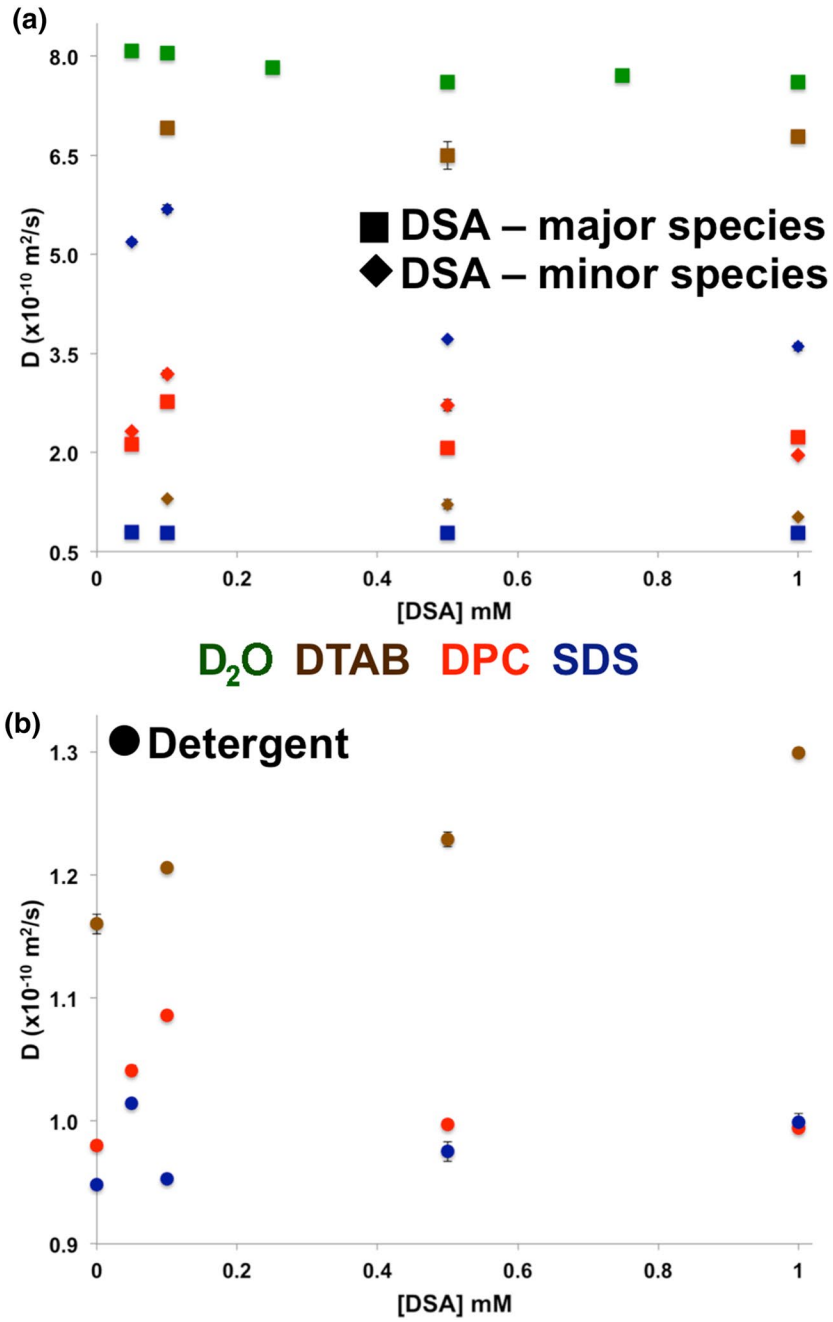

Fig. 7 D for **a** DSA (major and minor species observed in detergents) and **b** detergents observed at indicated DSA concentration and 100 mM detergent. D of DSA in D_2O is also plotted (highest values of D in **a**), with lower values of D observed, uniformly at any given DSA concentration, for DSA in: DTAB (major), SDS (minor), DPC (minor), DPC (major), and DTAB (minor) and SDS (major). Detergent D values are highest for DTAB, intermediate values for DPC, and lowest values for SDS. Experiments were performed at pH ~ 5 and 37 °C, with DOSY-based (Morris and Johnson 1992) attenuation of the Si-$C\underline{H}_3$ resonance at ~ 0 ppm employed to determine D of DSA and that of the detergent methylene resonances at ~ 1.24–1.25 ppm used to determine D of each detergent. Values are shown as average of two replicate experiments with error bars indicating average deviation

DSS, DSA surprisingly exhibits two peaks in the ~ 0 ppm region (one major, one minor) in the presence any of the three detergents employed but not in D_2O, implying two states in slow exchange on the NMR timescale when micelles are present (Fig. 8). While the identities of the two states are not known, they could reflect two different binding modes of DSA, perhaps differing in degree of solvent or micellar hydrophobic core exposure. The relative intensities of the DSA peaks observed in a given micelle type do not show a simple concentration dependent ratio, although the minor peak generally becomes less intense with respect to the major peak as a function of increasing DSA concentration at a fixed detergent concentration. Diffusion coefficients were calculated for each peak separately when possible, but the low intensity of the minor peaks tended to lead to poor fits to the Stejskal–Tanner equation resulting in large error in the associated diffusion coefficients.

The chemical shifts of both the major and minor DSA trimethylsilyl peaks in each micellar environment were deshielded relative to that observed in D_2O (Fig. 8). Mirroring the behaviour of DSS, the major peak was perturbed most in the presence of the oppositely charged

Table 3 Translational diffusion coefficients (D; $\times 10^{-10}$ m^2 s^{-1}) of indicated species (average ± average deviation for two trials; ± 1 for last sig. figure, where deviation is smaller) under given conditions at pH ~ 5 and 37 °C as determined using ^{1}H DOSY

Condition	D_{HOD}	D_{DSA}	$D_{Detergent}$
0.05 mM DSA	22.9 ± 0.1	8.08 ± 0.06	–
0.10 mM DSA	23.2 ± 0.1	8.05 ± 0.02	–
0.25 mM DSA	23.2 ± 0.1	7.83 ± 0.06	–
0.50 mM DSA	23.0 ± 0.1	7.60 ± 0.05	–
0.75 mM DSA	23.1 ± 0.1	7.71 ± 0.07	–
1.0 mM DSA	22.9 ± 0.1	7.61 ± 0.03	–
5.0 mM DSA	23.0 ± 0.1	7.62 ± 0.01	–
50 mM DSA	22.4 ± 0.1	7.43 ± 0.02	–
0.050 mM DSA + 100 mM DPC	22.3 ± 0.1	2.13 ± 0.01	1.04 ± 0.01
Minor DSA peak		2.32 ± 0.02	
0.10 mM DSA + 100 mM DPC	22.0 ± 0.2	2.78 ± 0.02	1.09 ± 0.01
Minor DSA peak		3.19 ± 0.05	
0.50 mM DSA + 100 mM DPC	22.2 ± 0.1	2.07 ± 0.01	0.997 ± 0.001
Minor DSA peak		2.72 ± 0.08	
1.0 mM DSA + 100 mM DPC	22.3 ± 0.1	2.24 ± 0.01	0.994 ± 0.001
Minor DSA peak		1.96 ± 0.02	
50 mM DSA + 100 mM DPC	22.0 ± 0.1	3.48 ± 0.01	0.860 ± 0.028
Minor DSA peak		3.48 ± 0.01	
0.050 mM DSA + 100 mM SDS	22.4 ± 0.1	0.794 ± 0.008	1.01 ± 0.01
Minor DSA peak		5.19 ± 0.04	
0.10 mM DSA + 100 mM SDS	22.4 ± 0.1	0.781 ± 0.003	0.953 ± 0.001
Minor DSA peak		5.69 ± 0.06	
0.50 mM DSA + 100 mM SDS	22.3 ± 0.2	0.787 ± 0.001	0.975 ± 0.002
Minor DSA peak		3.71 ± 0.01	
1.0 mM DSA + 100 mM SDS	22.1 ± 0.1	0.783 ± 0.001	0.999 ± 0.001
Minor DSA peak		3.60 ± 0.06	
0.1 mM DSA + 100 mM DTAB	21.7 ± 0.1	6.92 ± 0.21	1.21 ± 0.01
Minor DSA peak		1.03 ± 0.02	
0.50 mM DSA + 100 mM DTAB	22.1 ± 0.1	6.50 ± 0.10	1.23 ± 0.01
Minor DSA peak		1.22 ± 0.02	
1.0 mM DSA + 100 mM DTAB	22.6 ± 0.1	6.78 ± 0.01	1.30 ± 0.01
Minor DSA peak		1.30 ± 0.07	

SDS (~ 60 ppb), to an intermediate degree in zwitterionic DPC (~ 40 ppb), and least in like-charged DTAB micelles (~ 20 ppb) at 0.5 and 1 mM DSA. For SDS and DPC, the minor peak was more shielded relative to the major peak; for DTAB, it was less shielded.

As with DSS, the diffusion coefficient of DSA was decreased in the presence of each type of micelle without a corresponding decrease in the diffusion of HOD (Table 3). This was true for either the major or minor peaks (Fig. 7a squares vs. diamonds; Table 3). The observed detergent diffusion rates (Fig. 7b; Table 3) in the presence of DSA exhibited some clear differences relative to in the presence of DSS. As with SDS–DSS mixtures, like-charged DTAB–DSA mixtures exhibited minimal change to detergent D. With DPC, however, slightly enhanced detergent diffusion was apparent at low DSA concentrations (e.g., perhaps due to an increased free detergent concentration), stabilizing at higher concentrations and becoming attenuated at 50 mM DSA. In the case of SDS, the detergent diffusion coefficient was generally observed to increase, contrary to that of DSA itself which decreased to a value below SDS itself. This implies a fairly strong perturbation to the SDS micelle-free detergent equilibrium in the presence of DSA, with a slight turbidity also apparent in these mixtures. Increasing DSA to 2 mM or above led to precipitate formation, consistent with other studies of mixed-micelles formed with anionic and cationic species, especially when the charged groups are not sterically hindered (Bakshi et al. 2002). The perturbation of SDS behaviour by DSA even at concentrations typical for chemical shift standard application implies that this would be a non-ideal combination.

Following the same approach as for DSS, DSA and detergent diffusion rates under each condition were used to estimate DSA binding to each micelle type (Table 4). In almost

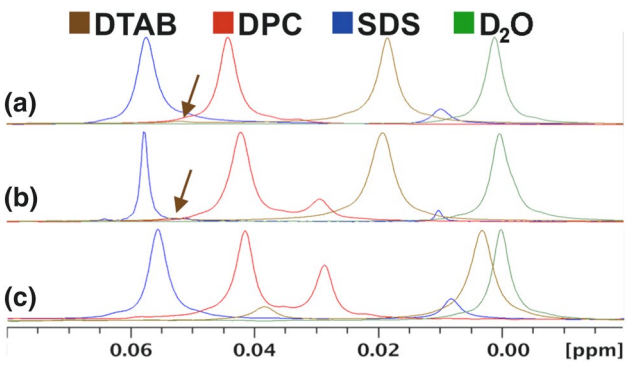

Fig. 8 Overlaid ^{1}H NMR spectra showing Si-C$\underline{H}_3$ resonances of DSA observed in D_2O (most shielded) relative to those observed in 100 mM of DTAB, DPC or SDS (from second-most to least shielded, respectively, for the major peak). Minor DSA Si-C$\underline{H}_3$ peaks mirror this, with SDS being most shielded, DPC being intermediate, and DTAB being least shielded; arrows indicate the positions of the minor DTAB peaks in (*a, b*). DSA concentrations of *a* 1.0 mM, *b* 0.5 mM, and *c* 0.1 mM are illustrated. In all cases, experiments were performed at pH ~ 5 and 37 °C, with spectral referencing relative to the DSA Si-C$\underline{H}_3$ resonance at 0.000 ppm observed at 0.1 mM in D_2O

all cases (with the exceptions being ~ equal), the more deshielded peak exhibited a greater attenuation of diffusion than the less deshielded peak, with a correspondingly greater proportion of DSA estimated to be bound. DSA-SDS micelle binding was ~ 100%, with DPC binding being intermediate and similar for both the major and minor species in slow exchange, and the major species of DSA in DTAB consistent with ~ 20% binding and the minor species being strongly micelle associated.

Concluding remarks

The propensity for DSS to self-associate at high concentrations and to interact with the commonly used detergents DPC and SDS, as well as DTAB, above their critical micelle concentrations was investigated by both 1D ^{1}H and DOSY NMR experiments. The latter phenomenon was also investigated for the DSS analogue DSA. The decrease in the diffusion coefficient of DSS as a function of concentration increase implies the potential for self-assembly of this relatively small amphiphile. However, this process appears to take place only at concentrations that are orders of magnitude higher than the typical range of DSS used as an internal standard in aqueous conditions and should not affect its use as a reference molecule.

This amphipathy has ramifications in that both DSS and DSA exhibit interactions with DPC, SDS and DTAB micelles at typical experimental concentrations of 0.1–1 mM DSS/DSA and 25–200 mM detergent. In each case, the chemical shift standard interacted most favourably with the micellar species of opposite charge, at an intermediate level with the zwitterionic DPC, and least with the like-charged species. The largest chemical shift perturbations observed herein (~ 60 ppb) are equivalent in magnitude to the secondary chemical shift threshold that we previously found to be appropriate under some circumstances for secondary structure prediction (Tremblay et al. 2010), implying that this is not an insignificant degree of modulation to the 0 ppm reference. Both reference compounds also appear to perturb the hydrodynamics of SDS and DPC micelles, or at least the equilibrium between micellar and non-micellar detergent species, over the typical concentration regime of 0.1–1 mM. Hence, DSS and DSA may both impact and be perturbed by membrane-mimetic systems even at low concentrations.

One potential solution for the issue of a reference standard molecule interacting with a given membrane mimetic is to employ the C$\underline{H}_2$ or C$\underline{H}_3$ chemical shift from the tail-group of a detergent or lipid species as an internal reference (e.g., the referencing method employed for Fig. 6). These nuclei should be protected from solution and experience a relatively consistent environment given a high stoichiometry relative to the membrane-associated or -spanning species in question and stable self-assembly state. This, in turn, avoids issues with CSP by, e.g., changes in dielectric constant (Tremblay et al. 2010) or pH (De Marco 1977). Care, of course, must then be taken in the value assigned to this reference chemical shift; however, given an accurate reference chemical shift value for the conditions in question, this would provide a rigorous and robust referencing method for membrane protein NMR spectroscopy.

Table 4 Estimated micelle-bound DSA fraction (± 0.1 for major species; ± 0.3 for minor species) as a function of concentration in the presence of 100 mM of a given detergent inferred from relative diffusion coefficients of DSA and detergent (Eq. 3)

Micelle type	SDS		DPC		DTAB	
DSA species	Major	Minor	Major	Minor	Major	Minor
(DSA) = 0.1 mM	1.0	0.4	0.8	0.7	0.2	1.0
(DSA) = 0.5 mM	1.0	0.3	0.8	0.7	0.2	1.0
(DSA) = 1.0 mM	1.0	0.5	0.8	0.9	0.2	–[a]

[a]Insufficient signal for determination

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