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ORIGINAL RESEARCH ARTICLE

Stability Characterization of a Polysorbate 80-Dimethyl Trisulfide Formulation, a Cyanide Antidote Candidate

Craig M. Bartling[1] · Jon C. Andre[1] · Carrie A. Howland[1] · Mark E. Hester[1] · Jeffrey T. Cafmeyer[1] · Andrew Kerr[1] · Trevor Petrel[1] · Ilona Petrikovics[2] · Gary A. Rockwood[3]

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Abstract Novel cyanide countermeasures are needed for cases of a mass-exposure cyanide emergency. A lead candidate compound is dimethyl trisulfide (DMTS), which acts as a sulfur donor for rhodanese, thereby assisting the conversion of cyanide into thiocyanate. DMTS is a safe compound for consumption and, in a 15 % polysorbate 80 (DMTS-PS80) formulation, has demonstrated good efficacy against cyanide poisoning in several animal models. We performed a stability study that investigated the effect of temperature, location of formulation preparation, and pH under buffered conditions. We found that while the stability of the DMTS component was fairly independent of which laboratory prepared the formulation, the concentration of DMTS in the formulation was reduced 36–58 % over the course of 29 weeks when stored at room temperature. This loss typically increased with increasing temperatures, although we did not find statistical differences between the stability at different storage temperatures in all formulations. Further, we found that addition of a light buffer negatively impacted the stability, whereas the pH of that buffer did not impact stability. We investigated the factors behind the reduction of DMTS over time using various techniques, and we suggest that the instability of the formulation is governed at least partially by precipitation and evaporation, although a combination of factors is likely involved.

Key Points

Dimethyl trisulfide (DMTS) is a lead candidate cyanide countermeasures that is safe for consumption and has demonstrated good efficacy against cyanide poisoning in several animal models.

We performed a stability study of a 15 % polysorbate 80-DMTS formulation and found that a reduction in concentration of DMTS in the formulation (e.g., 36–58 % over the course of 29 weeks when stored at room temperature) could not be explained by formulation storag temperature, preparative laboratory, or formulation pH.

Based on various other analytical techniques performed, we suggest that the instability of the formulation is governed at least partially by precipitation and evaporation, although a combination of factors is likely involved.

1 Introduction

Current FDA-approved cyanide countermeasures (CyanoKit® and Nithiodote®) are constrained by a requirement for intravenous administration, thereby severely limiting their usefulness in a mass-exposure cyanide emergency. Thus, alternatives to these countermeasures are needed. Recently, compounds with organo-sulfur donor

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✉ Craig M. Bartling
bartlingc@battelle.org

[1] Battelle Memorial Institute, 505 King Ave, Columbus, OH 43201, USA

[2] Sam Houston State University, Huntsville, TX, USA

[3] US Army Medical Research Institute of Chemical Defense (USAMRICD), Aberdeen Proving Ground, MD, USA

scaffolds have been explored [1]. A detailed discussion of the current cyanide antidote research is reviewed elsewhere [2]. Mechanistically, these compounds act as sulfur donors for rhodanese, thereby assisting the conversion of cyanide into thiocyanate. One of the lead candidate compounds in this group is dimethyl trisulfide (DMTS), an acyclic aliphatic trisulfide found naturally in garlic, onions, cabbage, and similar vegetables. DMTS is used in the USA and Europe as a flavor enhancer, is considered by the FDA to be 'Generally Recognized as Safe' (GRAS) under conditions of intended use, and is listed on the FDA's "Everything Added to Food in the United States" list. Human DMTS intake estimates in the USA have been reported as 0.0003 μg/kg B/day and 0.02 μg/capita/day [3].

In previous studies, neat DMTS was administered to mice via the intramuscular route. When muscle necrosis was observed at the injection site in a subset of these animals, a new formulation was developed (DMTS in 15 % polysorbate 80 [DMTS-PS80]). This formulation was rationalized based on the improvement of the solubility, stability, and efficacy of similar cyanide antidote compounds [1]. In this new formulation, DMTS has demonstrated efficacy against cyanide poisoning in several animal models at a concentration of 50 mg/ml and at doses up to 100 mg/kg. Preliminary stability studies indicated that DMTS-PS80 is relatively stable (90–95 % retention of DMTS) out to 31 days, at various pHs at refrigerated temperatures (Petrikovics, 2014, unpublished data).

In this study, we performed a longer-term (29 weeks), comprehensive stability study that investigated the effect of temperature, location of formulation preparation, and pH under buffered conditions. Storage conditions, times, and buffer compositions were selected to cover a range of conditions in an exploratory and comprehensive way, and were not meant to be a final stability testing of the formulation. We used a gas chromatography-mass spectrometry (GC-MS) assay to determine the concentration of DMTS over time within the liquid formulation as well as to identify any chemical degradation products in selected samples. This GC-MS assay was also used to determine the presence of evaporated DMTS and DMTS adsorbed to vial caps during the stability study. As we noted loss in DMTS over time, we also performed several other assays to characterize the entire formulation. These analyses included nuclear magnetic resonance (NMR) to characterize both the DMTS and PS80 components of the formulation; size-exclusion chromatography high-performance liquid chromatography (SEC-HPLC) and dynamic light scattering (DLS) to characterize changes in the PS80 micellar concentration and size; critical micelle concentration (CMC) assays to characterize changes in the bulk micellar concentration; and transmission electron microscopy (TEM) to characterize microscopic structural changes in the formulation. Here, we describe the results of these analyses and suggest potential destabilization mechanisms.

2 Materials and Methods

2.1 Test Articles

DMTS-PS80 formulations were prepared by three different laboratories, but all tests were performed by Battelle (Columbus, OH, USA) (Table 1). Those formulations prepared outside of Battelle were shipped overnight to Battelle for testing.

Table 1 Description of formulations

Formulation name	Laboratory that prepared samples	Components	DMTS			PS80		
			DMTS source	Cat. no.	Lot no.	PS80 source	Cat. no.	Lot no.
A	Sam Houston State	50 mg/mL DMTS in 15 % PS80	Sigma Aldrich	W327506-250G-K	MKBJ8038V	VWR (manufactured by Alfa Aesar)	AAAL13315-AP	L15X051
B	Battelle			W327506-100G-K	MKBN7414V	Sigma Aldrich	P1754-500 ml	MKBQ4985V
C	USAMRICD			W237506-100-K	MKBH8110V	Sigma Aldrich	W291706-1KG-K	MKBJ0197V
B5	Battelle	50 mg/mL DMTS in 15 % PS80/ 30 mM three component pH 5.0, 7.0, or 9.0 buffer[a]		151882-100G	MKBC8878	Sigma Aldrich	P175-500 mL	MKBQ4985V
B7	Battelle							
B9	Battelle							

[a] These formulations contained 15 mM bis-tris (MP, catalog 194546, lot MR29626), 7.5 mM EPPS (Sigma, catalog E1894, lot SUBB3608 V), and 7.5 mM CHES (Sigma, Catalog 29311, lot BCBG3190 V)

In general, the following procedure was followed unless otherwise noted. A 15 % (w/w) PS80 solution was made by mixing the appropriate amount of PS80 with deionized water or a three-component buffer consisting of 15 mM bis-tris, 7.5 mM EPPS, and 7.5 mM CHES (pH 5.0, 7.0, or 9.0). The pH of the final water formulations was determined to be 7.0 at the start of the stability study. The mixture was vortexed and manually shaken until the solution was clear (typically overnight, but at least 1 h was required for dissolution). DMTS 5 g was added to a 100-ml volumetric flask, and 15 % PS80 was added until the total volume reached 100 ml. This solution was vigorously mixed via vortexing and inversion for at least 1 h until the DMTS was completely dissolved (as judged by visual inspection). We placed 2-ml aliquots of this 50-mg/ml DMTS solution into 2-ml glass screw-top vials (Agilent part no. 5182-0715 and 5182-0725). The screw-cap vial was then placed in a secondary 5-mL glass crimp vial (Wheaton part no. 223685 and 224100-180). The outer crimp vial was then purged with a stream of nitrogen gas and stoppered with a rubber stopper, and the cap was crimped. The secondary container void volumes (volume of secondary container minus primary container) from the formulation A setup and the formulation B and C preparations were different. The void volumes in the outer vial of the A samples and B samples were measured to be 9.06 and 6.34 ml, respectively. Following preparation, samples were placed at room temperature (20 °C), 2–8 °C, or 40 °C in controlled and dark environments. For each formulation, initial concentrations were determined 1 day following sample preparation (for consistency, as some of the samples were shipped overnight to Battelle, while others were prepared at Battelle).

2.2 Transmission Electron Microscopy (TEM)

Both negative-stain TEM and cryo-TEM were used to obtain high-resolution images of micellar structures in selected samples. All sample processing and imaging was performed at the Electron Microscopy Core Facility at the University of Massachusetts Medical School. Negative-stain TEM was initially performed for ease of sample preparation and to provide images with high contrast to easily differentiate micellar structures. In brief, 5-ml samples were spread on freshly prepared carbon-stabilized Formvar support films (either copper or gold grids) within an enclosed chamber with a relative humidity of 60 %. After 30 s, the excess sample droplet was wicked away with a wedge of filter paper. Finally, the grids were negatively stained with 1 % uranyl acetate in water to contrast the spread particle samples. The freshly prepared specimens were then imaged on a FEI Tecnai 12, Spirit BioTwin, transmission electron microscope at 80 kV accelerating voltage, and images were recorded at 87,000× using a Gatan Erlangshen CCD 2K camera system.

Additionally, cryo-TEM was performed to observe micellar structures more closely associated with their native environment since this method does not utilize chemical preparative steps, which greatly minimizes the level of sample artifacts. Selected samples were prepared on Quanta foil grids obtained from Electron Microscopy Sciences (EMS), and the entire procedure was carried out in an enclosed chamber at a relative humidity of 60 %. A 5-ml aliquot of sample was loaded onto a Quanta foil 200 mesh grid and secured into the cryo-plunging apparatus. Liquid ethane was then loaded into the reserve well and frozen in a bath of liquid nitrogen at –196 °C. Using a copper rod, the ethane bath was thawed to –155 °C (the melting point of ethane). The droplet was then wicked away with a small wedge of number 50 Whatman filter paper, and then the grid was immediately pneumatically plunged into the liquid ethane. Once frozen, the grid with the frozen sample was removed from the plunging apparatus and stored in a cryo-grid box in liquid nitrogen for transport to the cryo-electron microscope. The freshly prepared cryo-specimens were then imaged on a Philips CM120, Cryo-transmission electron microscope at 120 kV accelerating voltage, with the specimen stage cooled with liquid nitrogen to –196 °C. All images were recorded at various magnifications using a Gatan Orius optically coupled CCD 2K camera system.

2.3 Gas Chromatography-Mass Spectrometry (GC-MS) Analysis of Liquid and Headspace Samples

2.3.1 Preparation of Dimethyl Trisulfide (DMTS)-Polysorbate 80 Samples for GC-MS Analysis

An internal standard solution was prepared by mixing 41 mg of dibutyl disulfide (Sigma-Aldrich, catalog number B93989-25ML) with 25 ml of methanol (Fisher Scientific, catalog number A456-4). DMTS-PS80 liquid samples were prepared for GC-MS analysis by mixing 25 µl of each liquid sample (gently inverted several times prior to sampling) with 75 µl dibutyl disulfide internal standard solution and 2.9 ml of methanol.

The headspace above select DMTS-PS80 samples and the headspace within select secondary containers were also evaluated using GC-MS. A Hamilton gas-tight syringe was used to collect and inject 10 µl of each headspace sample.

Isolated precipitate samples were prepared by separating the precipitated material from the supernatant by centrifugation at 17,530×*g* for 15 min at room temperature and dissolving the isolated precipitate in methanol.

The caps of select DMTS-PS80-containing glass vials were flash frozen with liquid nitrogen, pulverized (mortar

and pestle), and extracted for 1 h with one ml of methanol. Each methanolic extract was analyzed using GC-MS.

2.3.2 Preparation of DMTS and Dimethyl Disulfide (DMDS) GC-MS Calibration Standards

DMTS GC-MS calibration standard solutions, ranging from 10 to 600 μg/ml, were prepared by mixing varying volumes of a stock DMTS solution (5.03 mg DMTS/ml methanol), an internal standard solution (100 μl), and varying volumes of methanol. The following standard concentrations were prepared: 10, 40, 80, 160, 320, 480, and 600 μg/ml. DMDS GC-MS calibration standard solutions, ranging from 10 to 600 μg/ml, were prepared in a similar manner.

2.4 GC-MS Analyses

The GC-MS instrumentation used consisted of an Agilent Model 6890 chromatograph, Agilent Model 7683 injector, and Agilent Model 5973 mass selective detector. The GC column was an Agilent HP-5MS, 30 m × 0.25 mm with 0.25 μm film. We used Agilent ChemStation Version D.01.02.16 software to process the data. Chromatography analysis was conducted at 45 °C for 3 min, 3 °C/min to 250 °C, and 250 °C for 5 min with an He flow rate of 1 ml/min. The inlet temperature was 250 °C, and the injection volume was 1 μl for liquid samples and 10 μl for headspace samples. The MS parameters were as follows: source temperature 230 °C, quadrupole temperature 150 °C, and scan range of 45–400 *m*/*z*. The ions used for quantification included 94 *m*/*z* (DMDS) and 126 *m*/*z* (DMTS).

2.5 Statistical Analyses of GC-MS Data

All statistical analyses were performed with SAS version 9.3. Summary statistics were produced for the mean concentration and 95 % confidence interval of each formulation at each temperature and time point. The percent change in DMTS concentration was calculated for each sample of each formulation at each time point and temperature combination relative to the average baseline value for that formulation. Separate time point and temperature analysis of variance (ANOVA) statistical models were fit to the percent change data for all of the formulations. Least squares means and 95 % confidence intervals were generated for each combination.

Pairwise comparisons for each time point were performed using Dunnett's procedure to generate simultaneous 95 % confidence intervals. The rate at which concentration decreased as a function of time was compared between formulations using a linear model. The linear model was fit to the data such that a separate slope and intercept was used for each of the 18 formulations and storage temperature combinations. The linear model is of the form

$$Y_i = b_{0i} + b_{1i}x + \varepsilon_i$$

where i ranges from 1 to 18 for each of the formulations and storage temperature combinations, Y_i represents the concentration level (in mg/ml), b_{0i} is the y-intercept for combination i, b_{1i} is the slope for combination i, x is the number of weeks past, and ε_i represents the error term.

Once the slopes were generated, the rates of linear decrease between different storage temperatures within a given formulation as well as between different formulations for a given storage temperature were compared. For these comparisons, a two-sample test was performed for each of the 45 combinations, and the resulting p values were adjusted via a Bonferroni–Holm adjustment factor. Adjusted p values are used to assure that the overall error rate for all comparisons is no more than 0.05.

2.6 Nuclear Magnetic Resonance (NMR)

^{1}H and ^{13}C NMR spectra were collected on a Bruker AVANCE 500 spectrometer equipped with a 5 mm high-resolution broadband inverse (BBI) probe. Initially, fresh samples were prepared in D_2O, but subsequent testing revealed that samples from the stability study described above (non-pH samples prepared in H_2O) could be directly analyzed via ^{1}H NMR using a solvent suppression pulse program. For this analysis, samples were prepared by diluting 9 parts sample with 1 part D_2O to facilitate field locking and shimming of the samples. Spectra were manually interrogated for the presence of signals not derived from the DMTS or PS80 in the formulation. For comparisons at different time points during the stability study, fresh formulation controls were prepared by Battelle. Further, DMTS concentrations from NMR spectra were determined according to Eq. 1 under the assumption that the PS80 signals were not diminishing over time. Concentration of DMTS determined by ^{1}H NMR:

$$[\text{DMTS, mg ml}^{-1}] = 50\ \text{mg ml}^{-1} \times \left\{ \frac{\frac{(\text{DMTS Integral Area})}{(\#\ \text{protons in DMTS Signal})}}{\frac{(\text{PS80 Integral Area})}{(\#\text{protons in PS80 signal})}} \right\} / 3.6087 \quad (1)$$

Where 3.6087 = the ratio of the starting molar concentration of DMTS to the starting molar concentration of PS80 (i.e., 0.396 M/0.109 M) based on the assumption of molecular weights of DMTS and PS80 of 126.26 and 1310 Da, respectively.

NMR analysis of a sample containing precipitate was accomplished by separating the precipitated material from the supernatant by centrifugation at 17,530×*g* for 15 min at room temperature. This was followed by dissolution of the precipitate in deuterated methanol. The supernatant was diluted twofold in D_2O prior to analysis.

2.7 Critical Micelle Concentration (CMC) Assay

CMCs for selected formulations were determined using the fluorescence polarization method reported by Held et al. [4]. This method utilizes the lipophilic probe 5-dodecanoylaminofluorecenein (DAF), which contains a fluorescent head group connected to an aliphatic tail that becomes incorporated into detergent micelles at polysorbate concentrations above the CMC. When inserted into a micelle, the fluorescence polarization increases as a result of the inverse relationship between the emission of polarized light and the probe's rotational speed.

To perform the fluorescence polarization assays, DAF (Life Technologies) was prepared fresh as a 5 mM stock solution in 0.2 M KOH/methanol. In addition, a 125 mM stock solution of HEPES buffer (pH 8) was prepared and used as a diluent for the samples. Serial dilutions of stock solutions were performed in Milli-Q water to achieve 14 dilutions ranging from 1 to 0.0001 % of PS80, PS20, or PS80/DMTS formulations. Final concentrations of assay reagents consisted of 25 mM HEPES buffer and 1 µM of DAF in Milli-Q water. Dilutions of samples and assay reagents were added to black Corning Costar 96-well assay plates with clear well bottoms and mixed and then incubated in the dark for approximately 30 min. Fluorescence polarization of samples was measured in triplicate at 20 °C; measurements were acquired on a Molecular Devices SpectraMax M5 plate reader using a 485 nm excitation and a 535 nm emission filter. Fluorescence polarization (*P*) values were plotted as a function of the detergent concentration *x*, and a 4-parameter logistic (4-PL) curve was fit to the data according to Eq. 2. 4PL Curve Relating Fluorescence Polarization to Detergent Concentration:

$$P = \frac{A - D}{1 + (x/C)^B} + 1 \tag{2}$$

where *A* is the minimum response, *B* is the Hill slope, *C* is the half effective dose (i.e., the concentration of the detergent at the inflection point of the curve), and *D* is the maximal response. The CMC was then calculated as the intersection (Ix) of the rapidly changing portion of the curve and the horizontal portion at the minimal point of the curve using the series of equations below where LS is LogSlope, LSb is the *y*-intercept of the tangent line, and Ix is the CMC. Slope of tangent line of the 4-PL Curve:

$$P = \mathrm{LS} \times \mathrm{Log}(x) + \mathrm{LSb} \tag{3}$$

Definition of LogSlope (LS):

$$\mathrm{LS} = B \times (D - A) \times \mathrm{Ln}(10)/4 \tag{4}$$

Definition of LogSlope intercept (LSb):

$$LSb = (A + D)/2 - LS \times Log(C) \tag{5}$$

Relationship between CMC (Ix), minimum response (*A*), LS, and LSb:

$$A = \mathrm{LS} \times \mathrm{Log}(Ix) + \mathrm{LSb} \tag{6}$$

CMC (Ix) Calculation based on rearrangement of Eq. 6:

$$\mathrm{Ix} = 10^{(A - \mathrm{LSb})/\mathrm{LS}} \tag{7}$$

2.8 Size-Exclusion Chromatography High-Performance Liquid Chromatography (SEC-HPLC)

SEC-HPLC was performed to determine the concentration and integrity of PS80 in the formulation over time according to the method reported by Tani et al. [5]. Briefly, a standard curve that ranged from 0.5 % PS80 plus 1.67 mg/ml DMTS to 10 % PS80 plus 33 mg/ml DMTS was used to measure micelle concentrations in samples diluted 1.5-fold (from 15 % PS80 plus 50 mg/ml DMTS) using a Tosoh TSKgel G2000SWXL (7.8 mm × 30 cm, 5 µm) column and Waters 2695 HPLC with a 2487 detector. The mobile phase consisted of 10 mM sodium phosphate, 150 mM sodium chloride, and 0.1 % PS80, pH 7.0. The flow rate, injection volume, and detection wavelength was 1 ml/min, 2.5 µl, and 235 nm, respectively, for this analysis. Samples and standards were filtered through a 0.2 µm PVDF filter prior to loading. For concentration determinations of unknowns, samples were diluted 1.5-fold to ensure concentrations were within the linear range of the assay.

2.9 Dynamic Light Scattering (DLS)

DLS was used to measure the hydrodynamic diameter of micelles at concentrations above the CMC for PS80 using a BI-200SM motorized goniometer and BI-9000AT Digital Autocorrelation (Brookhaven Instruments Corporation). The particle size distribution data were based on weighting of the intensity of the scattered light. The values of effective diameter or average hydrodynamic radius and polydispersity index were determined. Polydispersity index is the intensity-weighted relative variance of the diffusion coefficient and is a measure of the size distribution width. Samples were prepared at ~3 % PS80 and were not filtered or vortexed prior to analysis.

Fig. 1 Images of DMTS-PS80 water formulations prepared by each laboratory at the start of the stability study show some evidence of instability. Shown are representative samples from each of the three PS80 DMTS formulations prepared by each laboratory from a *bottom view* (B formulation, far *left*; A formulation, middle; C formulation, far *right*). The A samples (*middle*) were prepared in a slightly different outer vial that allowed additional headspace between the inner and outer vial. This is more clearly illustrated in Figure S2 in the Electronic Supplementary Material. The *arrow* points to a white solid precipitate apparent in a C sample. *DMTS* dimethyl trisulfide

3 Results

3.1 Formulation Preparation and Visual Observations

Because of the volatility of DMTS, formulations were prepared in a double-vial system to reduce evaporation (Fig. 1, and Figures S1 and S2 in the Electronic Supplementary Material [ESM]). DMTS-PS80 formulations were prepared in bulk, and 2 ml of each formulation was aliquoted into individual 2-ml glass vials. Following sealing, each 2-ml filled vial was placed into an outer glass vial (approximately 5-ml vials), purged with a stream of nitrogen, and sealed with a rubber septum and crimp cap. Individually sealed vials were placed at room temperature (20 °C), 4 °C, or 40 °C. Six different formulations were prepared as described in Table 1. Samples were prepared by three different laboratories using identical protocols and formulations. Samples were prepared either without a buffering agent (samples A, B, and C) or with a light three-component buffer to control pH (B5, B7, and B9) as described in the methods section.

Upon initiation of the stability study, the C formulations had a slightly yellow appearance, with some white precipitate material in the bottom of the vials. The other formulations were clear without any precipitate. However, following a few days of incubation at all temperatures, haziness and/or at least some white precipitate that settled to the bottom of the vial was noticed in at least one sample from each triplicate set of samples. The precipitate and haziness increased with increasing temperature, as apparent from the pictures of the A samples after 2 weeks of storage at 40 °C (Figure S1 in the ESM). No additional precipitate was noticed for the A samples stored at 4 and 20 °C after 2 weeks. Similarly, sample haziness was noticed in the C samples stored at 40 °C for 2 weeks, with no additional precipitate noticed in the 4 and 20 °C samples. In contrast, the B samples were not hazy at any temperature after 2 weeks of incubation.

Again at 10 weeks, detailed visual inspections were performed on all samples. Table 2 summarizes these observations, and Figure S2 in the ESM shows digital images of representative samples from each formulation. In all but a few cases, at least some precipitate that settled to the bottom of the vial upon storage was noticed. The only exception to this observation was the C and B5 samples incubated at room temperature; however, both of these formulations were hazy, suggesting that a precipitate was formed but was small enough in particle size to stay suspended. Except for the C samples, the trend of increasing precipitate with increasing temperature was a consistent observation. Based on preliminary tests, this precipitate was soluble in methanol and water, but not chloroform. Similar observations were seen after 29 weeks, although the amount of precipitate increased over time.

3.2 GC-MS Liquid Phase Analysis of DMTS Formulation (Stability Study)

During the course of the stability study, GC-MS was used to quantify DMTS and DMDS (a potential degradation product) in liquid, headspace, and vial cap extracts from DMTS-PS80 formulations stored in glass vials within sealed secondary glass vials. DMDS levels, when detected, were below the limit of quantitation of 10 µg/ml. It was not possible to quantitate dimethyl tetrasulfide in samples because this compound was not commercially available. The initial concentrations of DMTS in each of the DMTS-PS80 formulations measured by GC-MS are summarized in Fig. 2. The DMTS concentration in each formulation was below the nominal 50 mg/ml concentration, suggesting that some of the DMTS was lost during preparation.

To determine the impact of laboratory, temperature, presence of buffer, and pH, the stability of each

Table 2 Sample appearance after 10 weeks of incubation

Sample ID	Temperature (°C)	Precipitate at bottom of vial (qualitative relative amount)	Solution appearance[a]
B	2–8	+	Clear
	20	+	Clear
	40	++	Clear
A	2–8	+	Clear
	20	+	Clear
	40	+++	Clear
C	2–8	+++	Very hazy
	20	−	Hazy
	40	+++	Clear
B5	2–8	+	Clear
	20	−	Hazy
	40	+++	Clear
B7	2–8	+	Clear
	20	+	Hazy
	40	++	Clear
B9	2–8	+	Hazy
	20	+	Hazy
	40	++	Hazy, yellow tint

[a] Appearance above any precipitate at the bottom of the vial

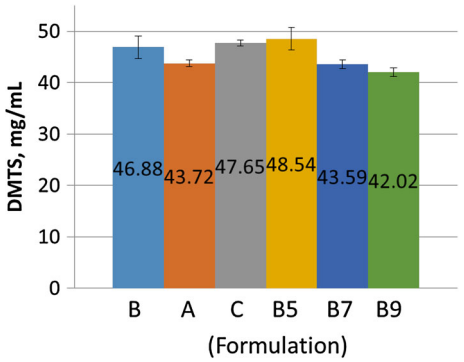


Fig. 2 Initial formulation concentrations are slightly below 50 mg/ml. Shown is the initial concentration of DMTS for each formulation as determined by GC-MS after preparation of the formulation. *DMTS* dimethyl trisulfide, *GC-MS* gas chromatography-mass spectrometry

formulation was followed over time. At each time point, a fresh sample was unsealed, and the concentration of DMTS in the liquid portion of the formulation was determined as described in the Methods section. We then performed a series of statistical tests to determine the impact of each of these parameters. Table S1 in the ESM lists means and 95 % confidence intervals for the recorded concentration levels, and Table S2 lists the means and 95 % confidence intervals for the percent change for each week and storage temperature combination for each formulation. Figure 3 displays the percent change in concentration for each of the formulations. A general trend of DMTS concentration reduction in all formulations over time is evident from these plots. However, there are some anomalous patterns (e.g., B formulation at week 4 and week 6) where concentration reduction is not monotonically decreasing. There is some visual evidence of greater loss at the high temperature conditions on later days, but this is not universal for all formulations and time points.

To determine the impact of the formulation variables (buffer and pH) and condition variables (laboratory and temperature) on the concentration change of DMTS in the liquid phase over time, two general statistical approaches were performed on the data illustrated in Fig. 3. First, pairwise comparisons were performed at each time point (Table S3 and S4 in the ESM). Second, the rate at which the DMTS concentration decreased as a function of time was compared between formulations (Tables 3, 4, 5). This allowed us to follow the overall trend of the data over time as opposed to treating each time point individually. For these comparisons, a linear model was fit based on visual inspection of the data and confirmed with residual plots.

In all cases, the statistical tests included the use of the simultaneous confidence intervals, which reduces the likelihood of identifying results as significant based solely on the number of comparisons being made. For the set of comparisons at each time point/temperature combination, the interpretation of the results should be that there is no more than a 1 in 20 chance that the statistically significant results observed could have occurred by random chance. While results observed as statistically significant are meaningful in the context described above, lack of significance cannot be interpreted as proof of no difference. It is possible that true differences exist that have low probability of appearing as statistically significant because of the limited number of replicate tests per time point/temperature combination, and the level of variability in the recovery process.

In general, we saw an increased rate of DMTS loss with increasing temperature for all formulations (Table 3). However, after adjusting for multiple comparisons, most differences in slopes between formulations stored at different temperatures were not found to be significant (Table 4), likely due to the spread in the slope predictions from the model. However, there were a few exceptions to these findings. For example, the A samples showed statistical differences between the 2–8 °C and 40 °C storage temperatures (Table 4). Further, when the data at week 29 for the room temperature storage for B, A, and C are included in model fits, the differences in slopes between A at room temperature and 40 °C, C at 2–8 °C and room temperature, and C at room temperature and 40 °C all become statistically significant. In contrast, because of the

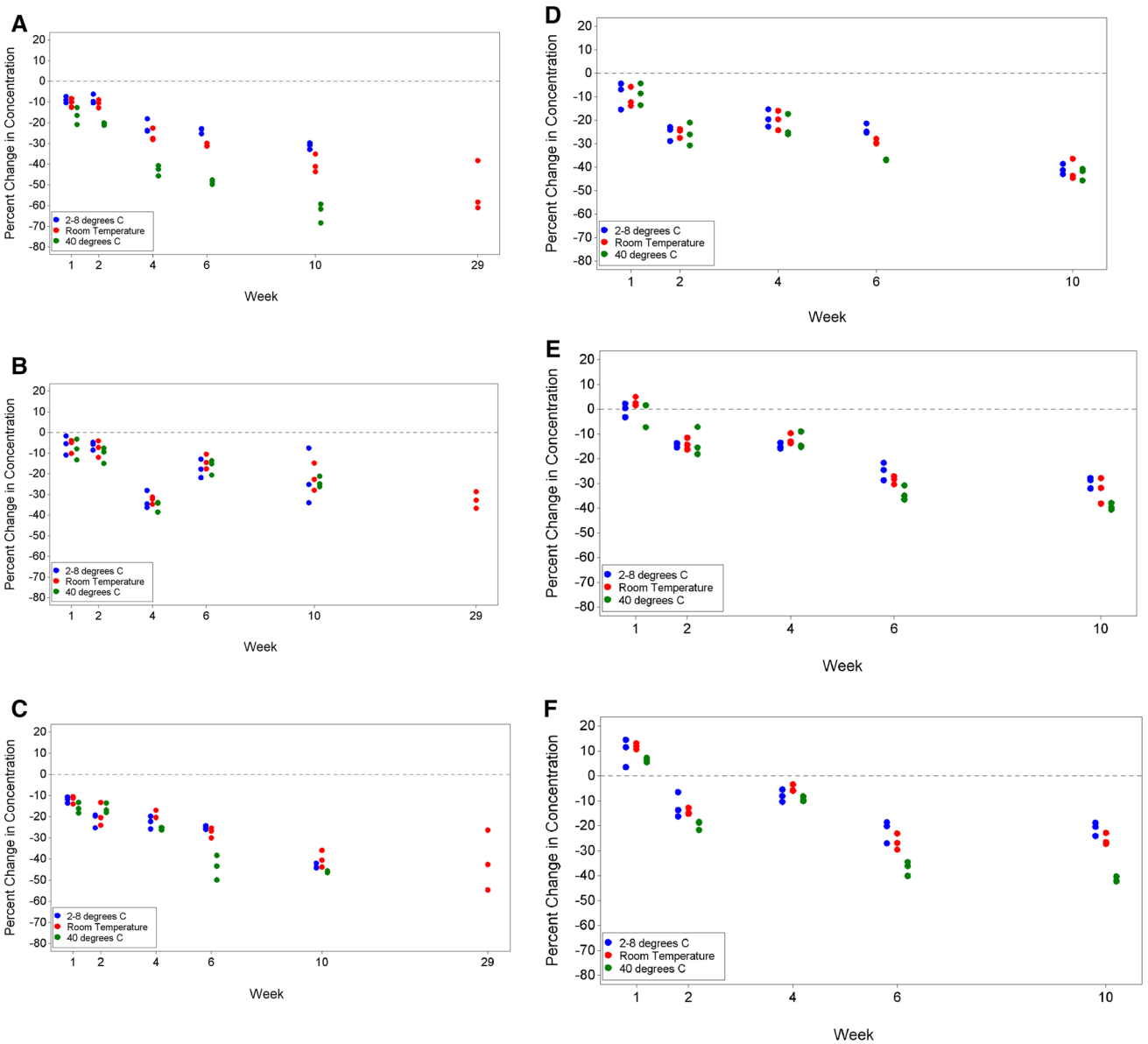


Fig. 3 The concentration of DMTS in the liquid phase declines over time. Shown is a graph of the percent change in DMTS concentration over time as determined by GC-MS for each formulation: formulation A (*panel* **a**), formulation B (*panel* **b**), formulation C (*panel* **c**), formulation B5 (*panel* **d**), formulation B7 (*panel* **e**), and formulation B9 (*panel* **f**). For each formation, sample percent changes from triplicate samples for each week at each temperature condition are plotted. *DMTS* dimethyl trisulfide

adjustment method of *p* values, the difference in slopes between A at the 2–8 °C and 40 °C storage temperatures is no longer classified as significant when the week 29 room temperature data are included for B, A, and B. This is because the addition of the week 29 room temperature data causes the slopes to change so drastically that the newly found significant slope differences are so large.

We found variable results for the impact of laboratory preparation. Based on pairwise comparisons (Table S3), we found significant differences in percent change in concentration between C and B samples for week 2 at the 2–8 °C and room temperature storage and at all storage temperatures for weeks 4, 6, and 10. The direction of difference was a greater reduction for C samples at weeks 2, 6, and 10, and less of a reduction for C samples at week 4. There were also significant differences in percent change in concentrations between A and B samples for the 40 °C storage temperature for week 2, for all storage temperatures for week 4, and for the room temperature and 40 °C storage temperature for weeks 6 and 10. The direction of difference was a greater reduction for A samples in all cases except for week 2 at the 2–8 °C and room temperature

Table 3 Linear coefficient for each formulation and temperature combination with 95 % confidence intervals

Formulation	Temperature	Linear coefficient with 95 % CI
B5	2–8 °C	–1.37 (–1.86 to –0.87)
	Room temperature	–1.41 (–1.91 to –0.92)
	40 °C	–1.60 (–2.09 to –1.11)
B7	2–8 °C	–1.25 (–1.75 to –0.76)
	Room temperature	–1.56 (–2.05 to –1.07)
	40 °C	–1.81 (–2.30 to –1.32)
B9	2–8 °C	–1.18 (–1.67 to –0.68)
	Room temperature	–1.46 (–1.95 to –0.97)
	40 °C	–1.99 (–2.48 to –1.50)
B	2–8 °C	–0.81 (–1.30 to –0.32)
	Room temperature	–0.69 (–1.19 to –0.20)
	40 °C	–0.68 (–1.17 to –0.19)
A	2–8 °C	–1.13 (–1.63 to –0.64)
	Room temperature	–1.52 (–2.02 to –1.03)
	40 °C	–2.30 (–2.79 to –1.81)
C	2–8 °C	–1.46 (–1.95 to –0.97)
	Room temperature	–1.40 (–1.8915 to –0.906)
	40 °C	–1.82 (–2.31 to –1.33)

CI confidence interval

Table 4 *p* values for differences in slope estimates between storage temperature by formulation

Treatment combination comparison	Adjusted *p* value
B5 2–8 °C vs. B5 RT	1.000
B5 2–8 °C vs. B5 40 °C	1.000
B5 RT vs. B5 40 °C	1.000
B7 2–8 °C vs. B7 RT	1.000
B7 2–8 °C vs. B7 40 °C	1.000
B7 RT vs. B7 40 °C	1.000
B9 2–8 °C vs. B9 RT	1.000
B9 2–8 °C vs. B9 40 °C	0.842
B9 RT vs. B9 40 °C	1.000
B 2–8 °C vs. B RT	1.000
B 2–8 °C vs. B 40 °C	1.000
B RT vs. B 40 °C	1.000
A 2–8 °C vs. A RT	1.000
A 2–8 °C vs. A 40 °C	0.048[a,b]
A RT vs. A 40 °C	1.000[b]
C 2–8 °C vs. C RT	1.000[b]
C 2–8 °C vs. C 40 °C	1.000
C RT vs. C 40 °C	1.000[b]

RT room temperature

[a] The difference in slopes is significant at the overall 0.05 significance level

[b] The difference in slopes changes significance when week 29 for B, A, and C at the room temperature storage are included in the model fits

conditions. When we compared the overall trends (Table 5) over time, only the rates from the A and B samples at 40 °C were significantly different. Thus, we did find some evidence that where the formulation was prepared could have a significant impact on its stability. However, this trend was not uniform at each time point and temperature, and the overall linear rates were not significantly different in most cases.

While the laboratory that prepared the formulation did not show a dramatic impact on the stability of the formulation, the presence of buffer did seem to have a negative impact on the stability of the formulation. Pairwise comparisons for B5, B7, and B9 formulations compared with the B formulation were all observed to be reduced at week 2 to a greater extent than B samples for all temperatures, with almost all comparisons statistically significant (Table S4). During week 4, the differences between percent changes in concentration in the buffered formulations and unbuffered B formulations were all positive (less degradation for B5, B7, and B9 than for B) as a result of a very large drop in B concentration. We do not know the reason for this drop in concentration for B, although the DMTS concentration would be unlikely to drop then rise again over time, suggesting that the week 4 data for B samples may not be reliable. After week 4, the buffered formulations returned to having a greater reduction than for B in almost every case, with 11 of the 18 differences statistically significant (Table S4 in the ESM). When we considered the overall rate (Table 5), the difference in slopes between B9 and B samples at 40 °C and the difference in slopes between B and A samples at 40 °C were found to be statistically significant. Further, when the data at week 29 for the room temperature storage for A, B, and C are included in model fits, the differences between slopes of B5 and B formulations, B7 and B formulations, and B9 and B formulations at the room temperature storage become significant. Thus, in general, addition of buffer to the formulation (at any pH) seems to have a negative impact on the stability of the formulation.

In contrast to the finding that the presence of a light buffer in the formulation possibly has a negative impact on stability, the actual pH of the formulation did not seem to impact the stability. In general, the average rates of DMTS loss increased with increasing pH at high temperatures and slightly decreased at lower temperatures (Table 3), but the confidence intervals in these rate predictions were wide enough that this trend was not statistically significant. This lack of statistical difference is demonstrated in Table 5, in which no significant differences in rate were observed at

Table 5 *p* values for differences in slope estimates between formulations by storage temperature

Treatment combination comparison	Adjusted *p* value
B5 2–8 °C vs. B7 2–8 °C	1.000
B5 2–8 °C vs. B9 2–8 °C	1.000
B5 2–8 °C vs. B 2–8 °C	1.000
B7 2–8 °C vs. B9 2–8 °C	1.000
B7 2–8 °C vs. B 2–8 °C	1.000
B9 2–8 °C vs. B 2–8 °C	1.000
B 2–8 °C vs. A 2–8 °C	1.000
B 2–8 °C vs. C 2–8 °C	1.000
C 2–8 °C vs. A 2–8 °C	1.000
B5 RT vs. B7 RT	1.000
B5 RT vs. B9 RT	1.000
B5 RT vs. B RT	1.000[a]
B7 RT vs. B9 RT	1.000
B7 RT vs. B RT	0.578[a]
B9 RT vs. B RT	1.000[a]
B RT vs. A RT	0.747
B RT vs. C RT	1.000
C RT vs. A RT	1.000
B5 40 °C vs. B7 40 °C	1.000
B5 40 °C vs. B9 40 °C	1.000
B5 40 °C vs. B 40 °C	0.390
B7 40 °C vs. B9 40 °C	1.000
B7 40 °C vs. B 40 °C	0.064
B9 40 °C vs. B 40 °C	0.012[b]
B 40 °C vs. A 40 °C	0.0003[b]
B 40 °C vs. C 40 °C	0.059
C 40 °C vs. A 40 °C	1.000

Note: the calculations for the slopes did not include data from week 0 or week 29 for the B, A, and C formulations

RT room temperature

[a] The difference in slopes changes significance when week 29 for B, A, and C at the room temperature storage are included in the model fits

[b] The difference in slopes is significant at the overall 0.05 significance level

any temperatures when the B5, B7, and B9 formulations were compared. Thus, the pH of the formulation did not seem to have an impact on stability. This finding is consistent with preliminary data of the stability of this DMTS formulation in unbuffered formulations varying from pH 2 to 11 (Petrikovics, 2014, unpublished data).

3.3 Vapor Phase Analysis of DMTS formulations

The DMTS-PS80 test samples for the stability analysis were prepared with very little headspace in order to minimize potential DMTS and DMDS vapor losses; therefore, evaluating the concentration of DMTS and DMDS vapor above these liquid formulations was not possible. It should be noted that the vapor pressure of DMDS, DMTS, and dimethyl tetrasulfide at 25 °C is 3.8, 0.14, and 0.007 kPascals (kPa), respectively; the vapor pressure of DMDS is nearly 27 times the vapor pressure of DMTS and nearly 560 times the vapor pressure of dimethyl tetrasulfide at 25 °C. To determine whether evaporation into the headspace was causing loss of DTMS, vials of DMTS-PS80 (50 mg/ml DMTS) were prepared with 0.4 ml headspace (1.6 ml liquid volume) and analyzed using GC-MS with the same composition of formulation B. Little, if any, DMDS (well below the limit of quantitation, 10 μg/ml) was found in any of the headspace samples collected from the vials containing formulated DMTS. Levels of DMTS following 24–48 h of incubation detected in headspace samples were variable, ranging from 45 to 91 μg DMTS/ml.

Thus, with 80 mg of DMTS initially in the liquid phase (50 mg/ml × 1.6 ml) and only 18–36 μg in the headspace (45–91 μg/ml × 0.4 ml), approximately only 0.3 % of the DMTS evaporated into the headspace of the inner vial. Although we suspect 24–48 h is long enough for the system to reach equilibrium (in a perfectly sealed system), we currently do not know the impact of a longer incubation time. However, Table 3 suggests that the loss of DMTS in the liquid phase is approximately 1 % per day (depending on time and temperature), so the loss of DMTS from evaporation may be significant. Further, the headspace within the secondary vials of the B samples was analyzed at the end of the stability study using GC-MS to determine whether any leaking occurred from the inner vial to the outer vial. DMDS and DMTS were not detected in any of the headspace samples collected from the secondary vials, although a detailed study and time course were not performed.

In addition to measuring the vapor phase concentration of selected samples, the remaining volume of the A, B, and C samples that had been stored at room temperature was measured at the end of the stability study (29 weeks) (Table S5 in the ESM). On average, 3.4 % of the volume (0.068 ml) was lost (possibly from evaporation), with a slightly higher volume loss in A samples than in the other samples. The potential ramifications of this are presented in the discussion section.

3.4 Analysis of Precipitate

A white precipitate formed over time in the vials containing DMTS-PS80 formulations. The precipitate was isolated after approximately 30 weeks from three A samples that were stored at 40 °C, dissolved in 1 ml of methanol, and then analyzed using GC-MS. Trace levels of DMDS (not quantifiable) were detected in the solubilized

Table 6 Characterization of DMTS-PS80 formulation via dynamic light scattering

Formulation	Effective diameter (nm)	Polydispersity Index
PS80 alone	8.7	0.17
DMTS-PS80 (formulation B)	11.6	0.19

precipitate. Moderate levels of DMTS, 385 ± 36 μg (mean ± standard deviation), were detected in the isolated precipitate samples. Thus, assuming an initial DMTS mass of 100 mg in the vial (50 mg/ml × 2 ml), the amount of DMTS adsorbed to the cap was found to be only approximately 0.4 %.

3.5 Adsorption Tests

The caps of the vials containing formulated DMTS likely came into contact with the formulation during sample preparation or prior to removal of aliquots for analysis since the vials were filled nearly to the top and some vials were shipped. Additionally, volatile compounds could have been adsorbed to the cap. To determine the amount of DMTS loss due to adsorption, the caps of three of the B7 samples stored at room temperature were separately flash frozen, pulverized, and then extracted with methanol. Each methanol extract was analyzed using GC-MS. DMDS was not detected in any cap extracts. Low levels of DMTS, 31 ± 2 μg total (mean ± standard deviation), were detected in the vial caps.

3.6 Characterization of the Formulation via DLS

As the GC-MS analyses did not reveal any chemical degradation products and insufficient amounts to completely account for liquid phase concentration losses over time (Fig. 3), we sought to characterize the formulation in other ways to determine potential destabilization mechanisms. To generally characterize the micellar size of PS80 micelles containing DMTS, DLS was performed. For DLS analysis, formulation B samples were diluted to ~3 % w/v with water (based on the initial concentration of 15 % PS80) and analyzed as described in the methods section. Consistent with what others have reported [6], we found the average diameter of a PS80 micelle (without addition of DMTS) to be approximately 8.7 nm (Table 6). When DMTS was added to the formulation, a slight change in the effective diameter and polydispersity index was apparent, although no major changes were apparent. After 6 weeks, samples were again analyzed; however, because of precipitate within the samples as well as drastic differences in measurements between replicate samples, data were uninterpretable.

3.7 TEM Analysis

In addition to DLS measurements, TEM was performed to provide a more comprehensive analysis of the micellar structures within the samples. In general, macroscopic and DLS observations were consistent with TEM analyses. Initial attempts included negative stain TEM of neat (undiluted) samples as well as samples diluted 1:1 with distilled water. Unfortunately, these samples were too viscous for negative stain TEM and cryo-TEM because the high viscosity of the formulation did not allow proper wicking of the samples to produce a consistent thin film on the grid prior to vitrification. Thus, although images were obtained (not shown), they were not analyzed because of the high likelihood of artifacts being present. A third attempt involved diluting samples 1:10 with distilled water prior to analysis. During this dilution, we noticed that the precipitate material dissolved. Cryo-TEM images showed a large number of crystalline structures from these samples, which were thought to be artifacts attributed to the freezing process. In contrast, negative stain TEM images revealed that DMTS-PS80 formulations contained larger aggregates in addition to micelle-like structures, which were absent in samples containing 15 % PS80 alone (Fig. 4).

Negative stain images of PS80 alone revealed micelles less than approximately 30 nm (Fig. 4, panel A), which is consistent with the DLS data (Table 6). In contrast, DMTS-PS80 formulations showed larger vesicles, suggesting some agglomeration of micelles due to time or the presence of DMTS. The rate of formation of these vesicles is unknown, but in the case of the A samples, uniform vesicles of approximately 500 nm in size formed at least within 6 weeks of storage (Fig. 4, panel B). Larger structures were also observed in B and C samples, but the aggregated structures were less ordered (Fig. 4, panel C and D, respectively).

3.8 NMR Analysis

NMR analysis was also performed on selected samples to provide overall structural information on the entire formulation, including DMTS, PS80, and any degradation products that may be present. Initial ^{1}H and ^{13}C NMR spectra were obtained by dissolving DMTS alone, PS80 alone, or DMTS and PS80 in combination in D_2O. These

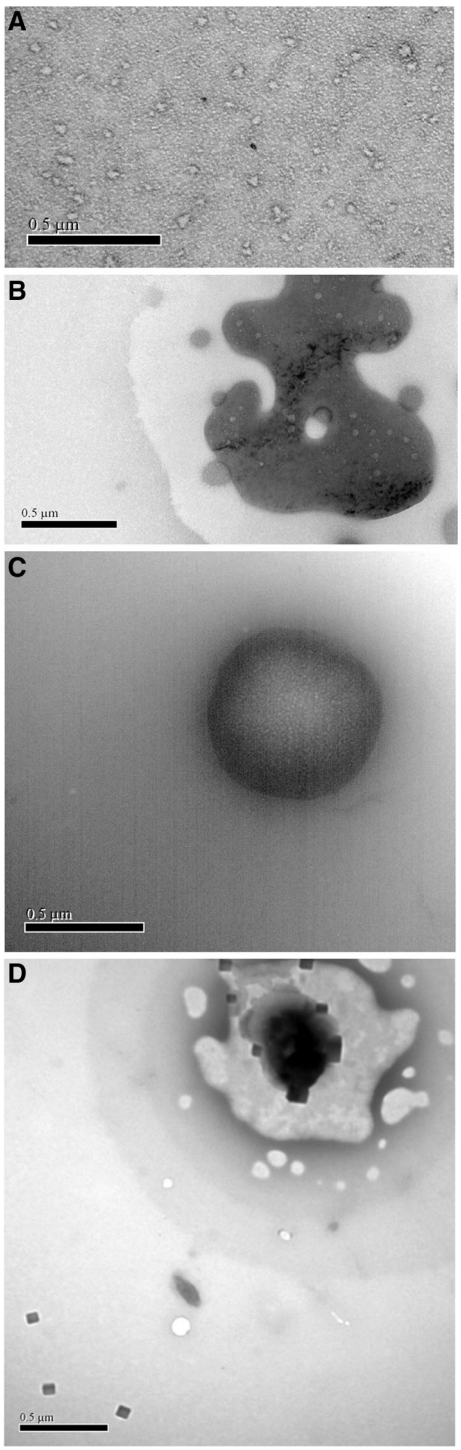
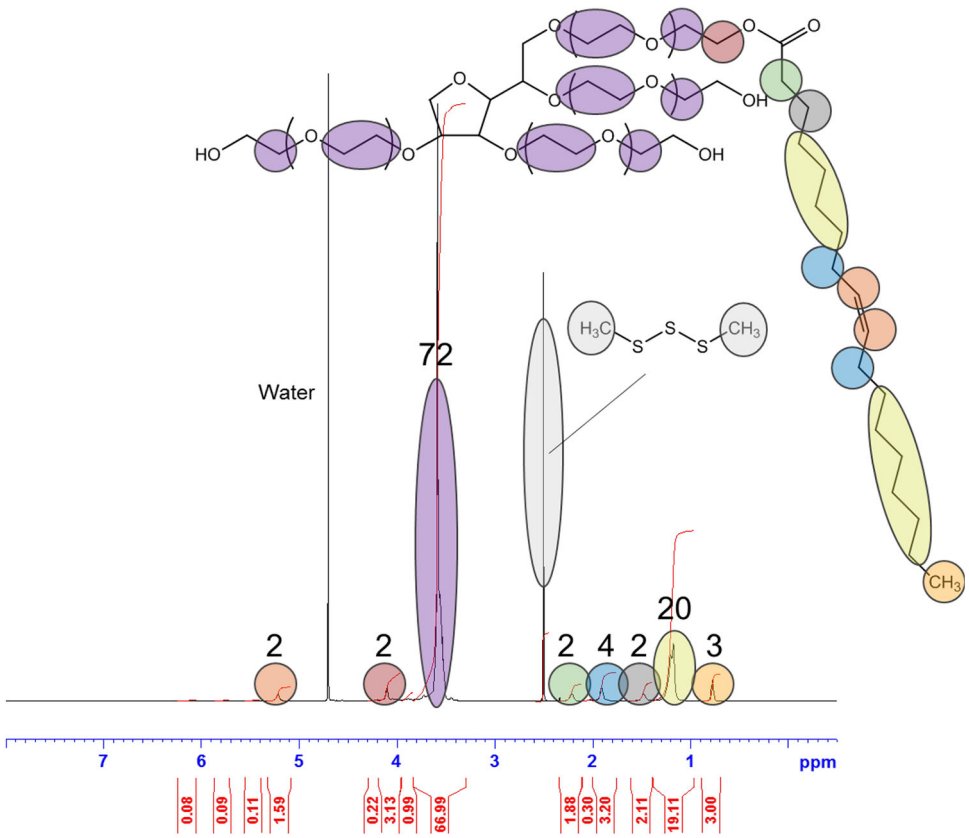


Fig. 4 DMTS-PS80 formulations form vesicles. Shown are representative negative stain TEM images from freshly prepared PS80 (*panel* **a**), as well as B samples (*panel* **b**), formulation A samples (*panel* **c**), and formulation C samples (*panel* d) that were stored at 2–8 °C for approximately 6 weeks. The *black line* in each image is 500 nm in size. *TEM* transmission electron microscopy

spectra showed that the DMTS signals for both proton and carbon spectra (Figure S3 in the ESM) were clearly resolved and identifiable in the formulation. The proton spectra revealed that direct analysis of the formulations (in H_2O) was feasible using solvent suppression techniques (to prevent dominance of the water signal and obscuring of other signals of interest), since the water signal (~4.7 ppm) was resolved from the rest of the peaks in the formulation (Figure S4 in the ESM). The solvent suppression technique allowed direct analysis of the formulations with resolution gained in the water region of the spectra (3.4–6 ppm), thus allowing NMR to be used for direct interrogation of the DMTS and PS80 present.

Figure 5 shows the peak assignments for DMTS and PS80 based on previously reported spectra [7, 8]. DMTS resonates as a single peak since all protons are equivalent within the molecule. PS80 commercial preparations are actual mixtures of related compounds, with inexact ethoxylation. The majority of the hydrophobic tail is composed of oleate (~72 %), but linoleate, linolenate, and sterate can also comprise a portion of this moiety. Although PS80 is mixture of several different structures, 107 protons of the theoretical 118 protons could be assigned. The unassigned proton signals are likely within the water region (e.g., hydroxyl protons) or less distinctly convoluted with other proton resonances.

To determine whether degradation products of DMTS and/or PS80 appeared following incubation, several samples were analyzed by ^{1}H NMR after 6 weeks and 10 weeks. Triplicate samples from the 6-week stability time point prepared by each lab as well as a freshly prepared formulation were compared, and all replicates showed very consistent spectra (Figure S5 in the ESM). The 6-week B sample is shown; data for others are not shown. This consistency suggests that single samples were representative of each of the triplicate samples and could be used for comparative purposes. Comparison of ^{1}H spectra of the various formulations (between labs) revealed only slight differences between A, B, and C samples (following 6 weeks of incubation at room temperature, Fig. 6). In general, the B samples and C samples appeared to be more similar to the freshly made controls, but only very minor differences were apparent. A close inspection of the DMTS region revealed a very small amount of dimethyl tetrasulfide (2.571 ppm) and DMDS (2.325 ppm) (based on resonance reported by Argyropoulos et al. [7], as shown in Fig. 7. Since the satellite peaks at 2.332 and 2.613 ppm, which are due to the natural abundance of ^{13}C, are ~1 % of the main DMTS peak, the overall concentrations of the tetrasulfide and disulfide are ~0.1 to 0.2 % of the DMTS peak. These data are consistent with the GC-MS data, which showed very little accumulation of these two degradation products. In fact, small amounts of the tetrasulfide (2.57 ppm) and the disulfide (2.325 ppm) were present in freshly made controls as well (not shown) and again are consistent with the GC-MS data.



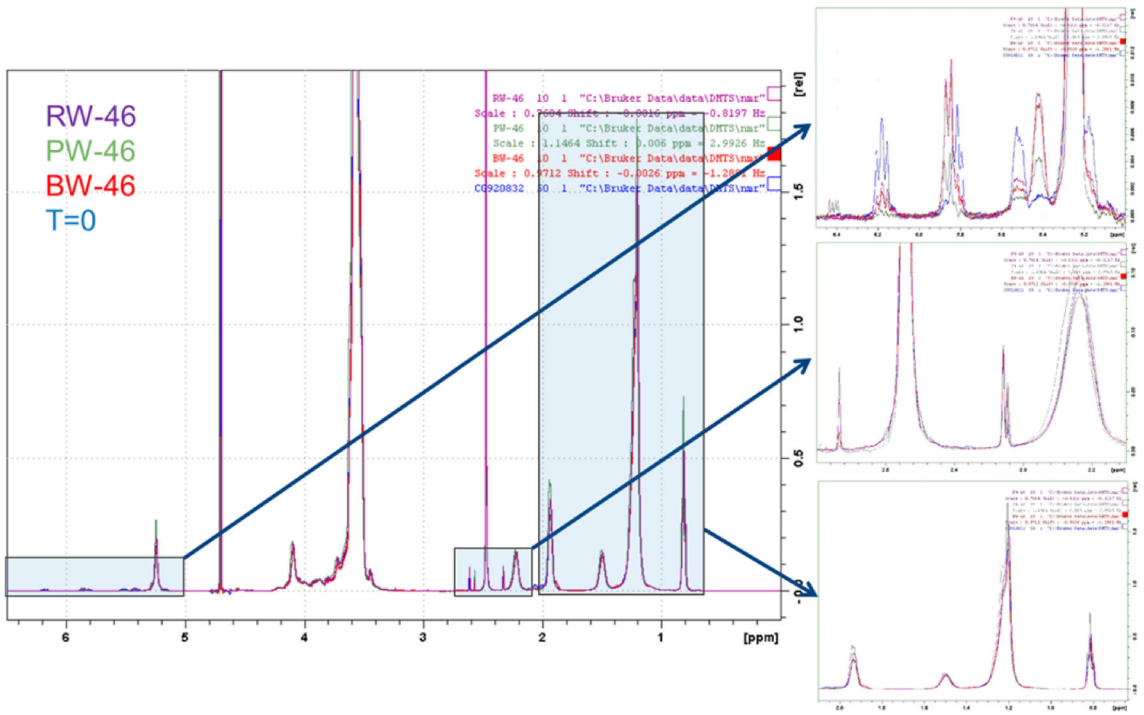


Fig. 5 DMTS-PS80 formulation can be analyzed via NMR without D_2O exchange. Shown is a representative spectra of a DMTS-PS80 formulation (in H_2O with ^1H NMR peak assignments for DMTS and PS80). The *numbers* above the colored identifiers indicate theoretical integration values, and *red numbers* below represent actual normalized to the expected value of 3 for the terminal methyl group of the oleate moiety of PS80. *DMTS* dimethyl trisulfide, *NMR* nuclear magnetic resonance

Fig. 6 NMR Spectra of DMTS-PS80 formulations prepared by different laboratories are similar before and after 6 weeks of storage. Shown is the ^1H NMR spectral comparison of A samples (*green trace*), B samples (*red trace*), and C samples (*purple trace*) following 6 weeks of storage compared with a freshly made control (*blue trace*). The insets show slight variations in minor peaks between formulations. *DMTS* dimethyl trisulfide, *NMR* nuclear magnetic resonance

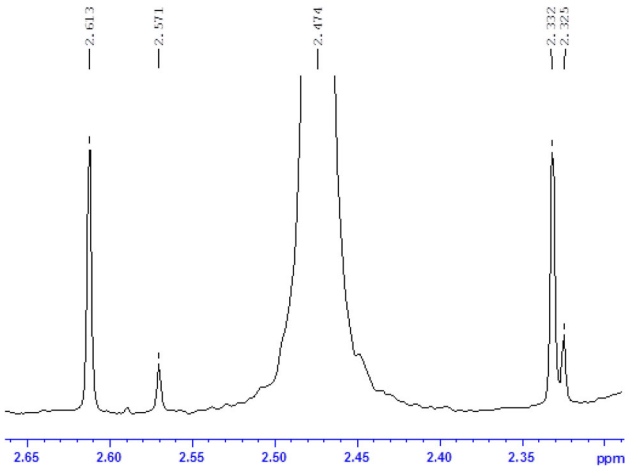


Fig. 7 The disulfide and tetrasulfide derivatives of DMTS represent minor components of the formulation following 6 weeks of incubation. Shown is the ^{1}H NMR spectra of the DMTS region of a B formulation following 6 weeks of storage at room temperature. Based on comparison with the DMTS peak, the overall concentrations of the tetrasulfide and disulfide are ~0.1 to 0.2 % of the DMTS concentration. *DMTS* dimethyl trisulfide, *NMR* nuclear magnetic resonance

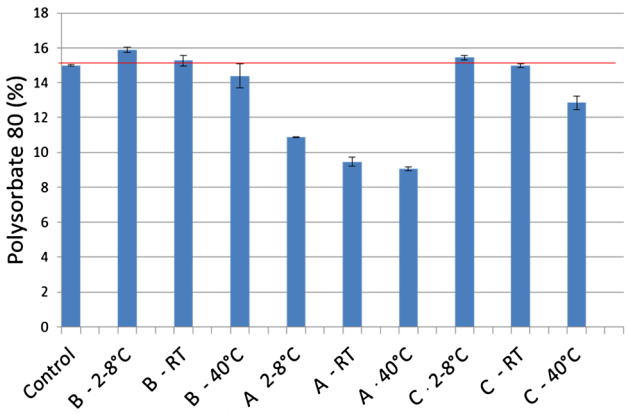


Fig. 8 The solution stability of PS80 within the DMTS PS80 formulation is laboratory and temperature dependent. Shown is the concentration of PS80 in triplicate samples of DMTS PS80 formulations prepared by three different laboratories after 10 weeks of storage at various temperatures. The *red line* indicates the starting concentration of 15 v/w. Values are ± standard deviation. *DMTS* dimethyl trisulfide

As with the GC-MS analyses, the NMR did not reveal any striking accumulation of chemical degradation products. Additionally, using the assumption that the PS80 was not significantly degrading over time compared with DTMS, the NMR spectra could also be used to determine the DMTS concentration using selected signals as described in the methods section. As shown in Figure S6 in the ESM, the GC-MS and NMR data generally correlated with each other.

In addition to soluble material, the precipitate was analyzed by NMR by dissolving the precipitate (separated from the bulk liquid by centrifugation) from an A sample (after 10 weeks of storage at 40 °C) in deuterated methanol. This sample was compared with the soluble material diluted into D_2O. As shown in Figure S7 in the ESM, both the soluble material and isolated solid contained similar ratios of DMTS and PS80, and an overall similar composition (with minor shifts due to solvent effects of D_2O and d4-methanol). However, the insoluble material was not rinsed of residual soluble material (since a suitable washing solvent could not be determined). Thus, the NMR signal may have in fact been merely due to the residual liquid remaining, and the precipitate may have contained compounds without protons. Preparation of a larger amount of bulk precipitate would be needed to analyze this material further from an NMR standpoint.

3.9 SEC-HPLC Analysis

To determine whether degradation to the PS80 component of the formulation occurs over time, selected samples were also analyzed by SEC-HPLC and CMC analysis. The simple SEC-HPLC method, originally reported by Tani et al. [5], allows the determination of PS80 concentration in the formulation through the use of a mobile phase containing PS80 above its CMC. Our data revealed a linear response of absorbance of the main PS80 peaks as a function of PS80 concentration in the range of 0.5–10 % w/v, with an R^2 value of 0.994 (Figure S8 in the ESM). To determine whether the PS80 was degrading over time, SEC-HPLC data were collected for the samples prepared by each lab following 6 and 10 weeks of incubation at room temperature and compared with a freshly made control. Figure 8 shows the concentration of PS80 remaining in each formulation analyzed. We found similar patterns between 6-week-old (not shown) and 10-week-old samples, with no additional loss in PS80 from 6 to 10 weeks. In all cases, a slight loss in PS80 is apparent with increasing temperature (Fig. 8). Further, the A samples showed a much lower amount of PS80 remaining than with the other laboratory preparations. While the reason for this loss is unknown, it may be due to disintegration of the micelle or to loss of vesicles upon filtration prior to SEC-HPLC analysis. We filtered samples with a 0.2-µm filter prior to SEC-HPLC analysis, and TEM analysis suggests that the A, B, and C samples formed vesicles of nearly 0.5 µm in size, with the A samples having more ordered structures. Thus, the possibility exists that the loss seen with the B samples was due to loss during filtration. Nonetheless, these results suggest that different PS80 sources and/or laboratory manipulation can lead to differences in formulation stability over time.

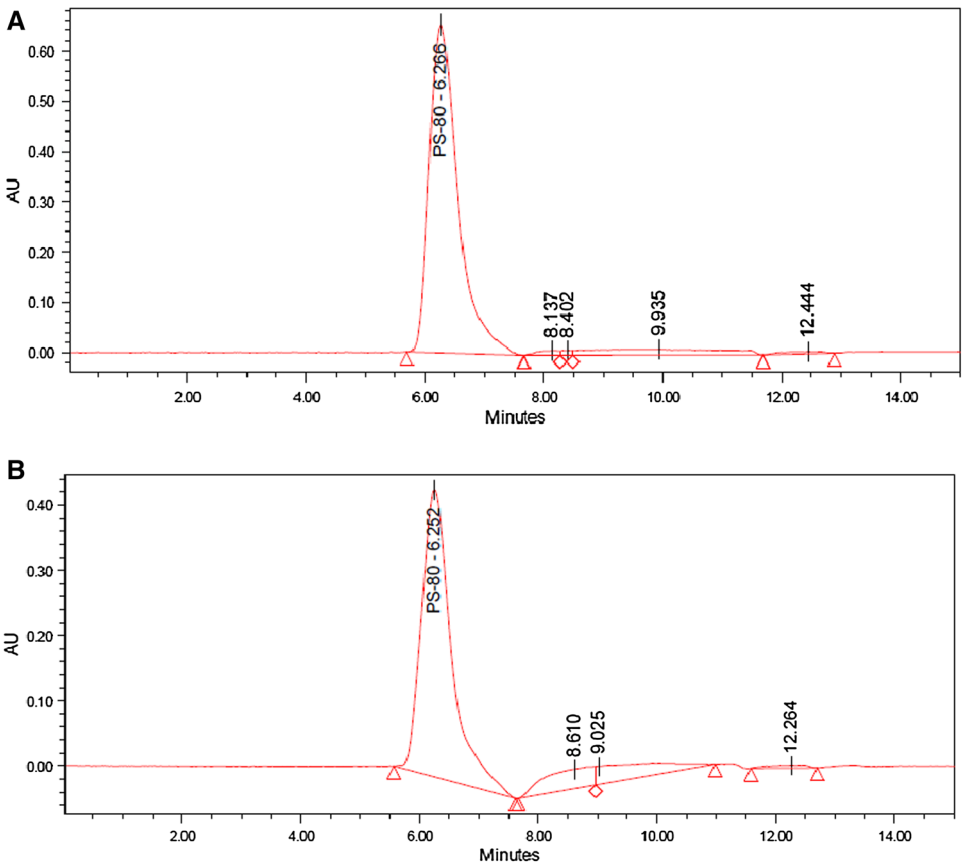


Fig. 9 The HPLC trace of DMTS formulations after 10 weeks of storage is laboratory dependent. Shown are representative HPLC traces from 10-week-old B (*panel* **a**) and A samples (*panel* **b**) stored at 2–8 °C. *DMTS* dimethyl trisulfide, *HPLC* high-performance liquid chromatography

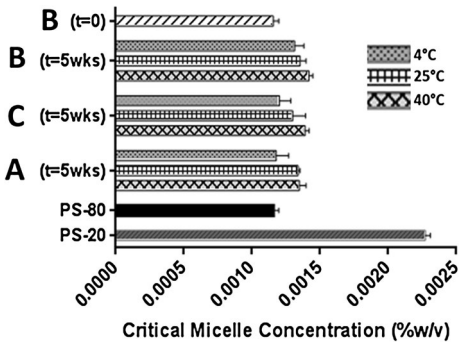


Fig. 10 Slight increases in CMC of the DMTS-PS80 formulation are due to time and temperature. Shown are the calculated CMC concentrations for various DMTS-PS80 formulations (A, B, or C at various temperatures) in comparison with PS80 or PS20 alone. CMC concentrations were calculated as described in the Sect. "2". *CMC* critical micelle concentration, *DMTS* dimethyl trisulfide

A closer investigation of the chromatograms between the A and B samples demonstrated that following the main peak, a small valley followed by a slight increase in absorbance was apparent in the A samples that was not apparent in the other samples (Fig. 9). This pattern can be indicative of PS80 degradation [5], as smaller micelles of PS80 will migrate more slowly than larger ones, and when the PS80 concentration reaches the level below the mobile phase concentration (0.1 %), a negative peak will occur.

3.10 CMC Analysis

In addition to SEC-HPLC analysis, CMC assays were performed to determine whether the CMC of the PS80 component of the formulations changed as a function of time, temperature, or pH. For these analyses, the CMC is determined using fluorescence polarization. The formulation is diluted in water in the presence of a fluorescent probe that includes an aliphatic tail that is inserted into the micelle upon formation. Because fluorescence polarization is indirectly proportional to a molecule's rotation speed, a sharp increase in fluorescence polarization is apparent at its CMC (Figure S9 in the ESM). Figure 10 demonstrates no significant difference between a freshly prepared DMTS-PS80 formulation and one of PS80 alone, suggesting that DMTS does not change the CMC based on this assay. The calculated concentration of PS80 of 0.12 % is in good agreement with published values of 0.07–0.2 % [5, 9, 10]. However, DMTS was diluted during these analyses to concentrations below 1 mg/ml. This dilution was needed because of the low solubility of DMTS in water. Further, upon dilution, any precipitated material was fully

Table 7 Potential mechanisms for reduction of DMTS concentration in DMTS-PS80 formulations

General mechanism	Evidence for	Evidence against
Chemical degradation	No substantial evidence	Very little formation of dimethyl disulfide or dimethyl tetrasulfide over time
		No major chemical degradation products identified by NMR or GC-MS
		Addition of buffer did not stabilize formulation
		Stability did not change with pH
Evaporation	When outer vial is opened, DMTS smell is apparent, suggesting leaking into outer vial	Large amounts of DMTS not detected in headspace measurements
	Some A samples, which were contained in slightly larger outer vials, contained condensate and showed larger reduction in DMTS concentration over time	
	Some DMTS detected in headspace measurements	
	~2 to 5 % volume loss after 29 weeks	
Precipitation	Precipitation increased over time and with increasing temperature	Only a small amount of DMTS (385 ± 36 μg DMTS, $N = 3$) found in precipitate material
	Large vesicle formation demonstrated in TEM images	
	Precipitate composed of DMTS and PS80	
Adsorption	Some DMTS found in vial caps	Only 31 ± 2 μg DMTS ($N = 3$) extracted from vial caps

DMTS dimethyl trisulfide, *GC-MS* gas chromatography-mass spectrometry, *NMR* nuclear magnetic resonance, *TEM* transmission electron microscopy

dissolved, so any increases in CMC could be interpreted as an actual change to the CMC value and not due to loss of PS80 from precipitation. A slight increase in CMC was apparent when samples were stored at any temperature for a period of 5 weeks (Fig. 10). Further, this slight increase continued with rising temperatures, suggesting that some change to the PS80 molecule or micelle was occurring over time. Similar results were seen with the buffered formulations, and no significant difference was apparent between buffered conditions (Figure S10 in the ESM).

4 Discussion

Here, we have reported the stability of a DMTS-PS80 formulation over the course of 29 weeks. While the stability of the DMTS component was fairly independent of which laboratory prepared the formulation (and potentially the chemicals used to prepare the formulation), the liquid concentration of DMTS in the formulation was reduced 36–58 % over the course of 29 weeks in all cases when stored at room temperature. This loss typically increased with increasing temperatures, although we did not find statistical differences between the stability at different storage temperatures in all cases. Further, we found that addition of a light buffer negatively impacted the stability, whereas the pH of that buffer did not significantly impact stability. Additionally, initial concentrations of DMTS were less than the target concentration of 50 mg/ml (empirically determined concentration, Fig. 2), suggesting difficulties in the accurate preparation of DMTS formulations.

The reduction of DMTS concentration could be due to a number of factors, including chemical degradation, evaporation, precipitation, and/or adsorption. To determine a potential destabilization mechanism, we used a number of analytical methods to characterize the formulations throughout the stability study. Table 7 summarizes the evidence for each of the mechanisms based on the data collected during this study, and each of these mechanisms are described in more detail following the table.

4.1 Chemical Degradation

Previous studies have revealed that the major chemical degradation components of a self-assembled micellar formulation of diallyl trisulfide (DATS) are diallyl disulfide (DADS) and diallyl tetrasulfide [11]. This formulation was prepared with PS80 and co-solvents and heated to 100 °C for 15 min. While we identified a small amount of the analogous compounds DMDS and (potentially) dimethyl tetrasulfide, the concentration of these degradation products did not substantially increase over time, suggesting that a similar chemical degradation mechanism to DATS was not an overriding factor for the decrease in DMTS solution concentration over time. Further, no other

degradation products were revealed by GC-MS or NMR, and pH did not seem to impact the stability of the formulation. However, unknown chemical degradation products or reactive species may be highly volatile or undetectable by GC-MS or NMR. For example, dimethyl disulfide (DMDS) has been shown to react with sulfur to form dimethyl polysulfides under high pressure and high temperature conditions [12]. Thus, a deeper investigation into the presence of elemental sulfur and/ or polysulfides in the formulation may be warranted. Although we see no gross evidence of this based on NMR, other methods such as LC-MS may be needed to fully characterize any other chemical species present in the system.

The presence of oxidizing compounds could potentially react with DMTS. Ju et al. [11] showed that addition of antioxidants to micellar preparations of diallyl trisulfide does not elicit stabilization, suggesting that the presence of oxidants did not contribute to degradation. However, Kruger et al. [13] demonstrated that an aqueous solution of dimethyl disulfide could be partially degraded in the presence of hydrogen peroxide, but degradation conditions were fairly extreme (low pH, 60 °C, and high concentrations of hydrogen peroxide). Similarly, Adewuyi and Carmichael [14] showed oxidation of dimethyl sulfide by hydrogen peroxide under acidic conditions.

Oxidizing compounds such as peroxides may contaminate PS80 during preparation [15] and may form during storage [16], but only at low levels. Further, PS80 can be degraded via autoxidation and hydrolysis, but typically higher temperatures and extreme pHs are needed for substantial degradation [17, 18]. In our analysis, we see no gross evidence of loss of ^{1}H NMR signal of protons from the fatty ester moiety in signal over time (Fig. 6), although a more detailed analysis would be needed to look for minor changes in NMR profiles within this region. In addition, we see very little drop in the CMC of PS80 over time, suggesting that hydrolysis is not occurring to significant levels. Although SEC-HPLC analysis suggests that the A samples had a substantial reduction in PS80 concentration, this may be due to precipitation as discussed further below.

4.2 Evaporation

Although chemical degradation of DMTS does not seem to be an overriding factor for the instability of the formulation, evaporation may be a concern because of the relatively high volatility of DMTS in aqueous solutions. While DMTS is a volatile compound, assembly into micelles should dramatically lower its volatility. PS80 increases the solubility of DMTS in aqueous solution, and our DLS results indicate a slight increase in micelle size upon addition of DMTS, which suggests that DMTS is in fact partitioning into micelles. However, the fraction of DMTS that partitions into micelles is unknown, although it can be inferred that the fraction that partitions into micelles is likely high since solubility is increased approximately an order of magnitude or more. Nonetheless, the small fraction of DMTS in the bulk water phase could evaporate over time, and as the system reaches equilibrium, DMTS within the micelle is displaced to replenish evaporated DMTS. Thus, even though the formulation is micellar, volatilization of DMTS is still a concern.

Despite taking steps to reduce or eliminate evaporation, we found some evidence that evaporation was occurring. Physical observations included a DMTS smell when the outer vials were opened for analysis. However, the odor threshold for DMTS is very low, approximately 5–10 ng/l [19, 20]. Further, we noticed the buildup of a small amount of condensate in the outer vials of some samples. Overall, we measured a 2–5 % drop in the total volume of the formulation after 29 weeks at room temperature. Since the vapor pressure of DMTS is higher than water, the DMTS in the bulk phase would likely preferentially evaporate over water. Further, the initial DMTS fraction of the formulation is only approximately 4 %, so a small drop in volume could contain a significant proportion of DMTS.

The most substantial evidence is the fact that GC-MS measurements revealed small amounts of DMTS contained in the headspace of the DMTS-PS80 formulation-containing vials. If the inner and outer vial system is completely sealed, equilibrium should be reached between gas and liquid phase. However, if the outer vial is not completely sealed, equilibrium may never be reached. Jin [21] demonstrated that solubilization of volatile organic compounds in aqueous PS80 solutions reach equilibrium between the aqueous phase (including equilibrium between micellar concentration and bulk aqueous concentration) and gaseous phase after a few hours of shaking, and addition of co-solvents had little to no effect on aqueous–gas partitioning.

Given the micellar arrangement of the formulation, future characterization should include determination of Henry's law constants and micelle–water partition coefficients. Henry's law constant would measure the ratio of DMTS in the vapor and liquid phase, and could be measured in the presence and absence of PS80 to determine whether micelle preparation reduces evaporation in a similar manner as reported by Vane and Giroux [22]. Further, such measurements allow determination of the fraction of DMTS that partitions into micelles (f_m). Vane and Giroux [22] reported that 23 % of benzene partitioned into micelles in the presence of 1.3 % Tween 20, and 49 % of toluene partitioned into micelles. Similar results were demonstrated for trichloroethylene, and as the detergent concentration increases, f_m increases. Determination of these parameters in the current formulation and any future

developed formulations would allow for optimization of volatility reduction.

In general, alternative surfactants and/or addition of co-solvents could be used to both increase solubility and partitioning into micelles. However, to rule out evaporation as a source of formulation instability, completely air tight, fully sealed vials should be tested, or heavier gases, such as argon, could be used in place of nitrogen. Alternatively, DMTS could be encapsulated to reduce volatility. Encapsulation of volatile compounds into cyclodextrins [23] or gum arabic/chitosan [24] capsules has proven useful for volatility reduction.

4.3 Precipitation

Since the solubility of DMTS in water is low, the addition of a detergent such as PS80 is critical to increase solubility. The current formulation increases the solubility to approximately 50 mg/ml, but precipitation is still a concern. The noticeable haziness in each sample is likely due to the formation of small particles that stay suspended. According to several reports, the cloud point of PS80 is 65 °C [10] or higher, with one report suggesting that the cloud point is >100 °C for neat PS80 in water [25]. The impact that DMTS has on the cloud point is unknown. Thus, future characterization of this and other DMTS-PS80 formulations should include cloud point determination to ensure that DMTS does not substantially lower the cloud point.

TEM analyses demonstrated that larger particles can form within the formulation. Unfortunately, reliable data were not obtained immediately following formulation preparation. Thus, the rate of formation of these vesicles is unknown, although precipitated material could be due to large vesicles being formed and eventually falling out of solution. Similar vesicles were observed by Won et al. [26] for poly(ethylene oxide)-water-based formulations and Rai and Yasir [27] for drug-loaded oleic acid-Tween-80 emulsifications. Thus, while the addition of DMTS may initially cause only a minor increase in micelle size (Table 6), these micelles may aggregate into larger structures over time. However, to substantiate this claim, a more rigorous analysis of the formulation is needed.

Based on NMR analysis, the precipitate that forms contains both DMTS and PS80, although we cannot rule out the possibility that we were merely analyzing the residual liquid. Further, only moderate levels of DMTS, 385 ± 36 µg (mean ± standard deviation), were detected in the isolated precipitate via GC-MS. In general, addition of co-solvents, such as those used with compounds similar to DMTS [1, 11], may be beneficial to enhance the solubility of DMTS and reduce precipitate formation, and should thus be considered in future formulations.

4.4 Adsorption

In addition to evaporation and precipitation, we considered the possibility that the reduction of DMTS concentration in the bulk phase could be due to adsorption. The presence of PS80 should reduce adsorption of DMTS, but to investigate this possibility, we studied the adsorption of DMTS in the formulation to the inner vial caps. DMDS was not detected in any cap extracts, and only low levels of DMTS were detected in the vial caps, suggesting that adsorption to the caps may be a minor contributor to the loss of DMTS. We did not investigate the adsorption to the glass walls of the vial, although we assume that this is unlikely because of the presence of a high amount of surfactant in the formulation. However, we cannot rule this out as a possibility, and future tests could focus on determining adsorption. A detailed investigation of different tubes may be advantageous to select the best container that results in the lowest possible adsorption of DMTS and/or whether adsorption is leading to loss of DMTS from the solution.

5 Conclusions

Based on the results of this study, we have shown that storage temperature and potentially laboratory preparation may have some impact on the stability of the DMTS-PS80 formulation. Therefore, care must be taken during formulation preparation. Further, while pH does not seem to impact degradation, the addition of a light buffer does seem to have a negative impact on stability. Based on the data collected, we suggest that the instability of the formulation is governed at least partially by precipitation and evaporation, although a combination of factors is likely involved. Further, we were not able to completely track (i.e., mass balance) the loss in DMTS over time. Thus, while a combination of factors is likely, the fate of DMTS within the current system is yet to be completely understood.

Compliance with Ethical Standards

Dr. Bartling, Dr. Andre, Ms. Howland, Mr. Kerr, Dr. Cafmeyer, Dr. Hester, and Dr. Petrel have no known conflicts of interest. Dr. Petrikovics and Dr. Rockwood declare that two patents have been submitted, both relating to DMTS use as a novel cyanide countermeasure, and, depending on the submission outcome, there may be royalties associated with the use of DMTS as a cyanide countermeasure.

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