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The influence of alkylating agents on sulfur–sulfur bonds in per- and polysulfides

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Abstract

Per- and polysulfides are sulfane sulfur species produced inside living cells, in organisms as diverse as bacteria, plants and humans, but their biological roles remain to be fully understood. Unfortunately, due to their reactivity, per- and polysulfides are easily altered, interconverted or lost during the processing and analysis of biological material. Thus, all current analytical methods make use of alkylating agents, to quench reactivity of hydropersulfides and hydropolysulfides and also to prevent free thiols from attacking sulfur chains in hydropolysulfides and dialkyl polysulfides. However, recent findings reveal that alkylating agents can also destroy per- and polysulfides, to varying degrees, depending on the choice of alkylating agent. Here, we discuss the challenges associated with the alkylation of per- and polysulfides, the single most important step for their preservation and detection in biological samples.

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Hydropersulfides, Hydropolysulfides, Dialkyl polysulfides, Alkylating agents.

Introduction

Sulfane sulfur species are increasingly recognized as biologically important metabolites [1–3]. Major representatives are hydropersulfides (e.g., GSSH, Cys-SSH), hydropolysulfides (e.g., GSSSH, Cys-SSSH), dialkyl polysulfides (e.g., GSSSSG), and their protein analogues (e.g., P-SSH, P-SSSH, P-SSS-P). These species appear to be produced both enzymatically and non-enzymatically [4] and can be found across all three domains of life, i.e., in bacteria, archaea, and eukaryota [5,6]. However, our understanding of the physiological functions of per- and polysulfides is still limited. Several observations now implicate small molecule persulfides as antioxidants and cytoprotective agents [7,8]. Indeed, the latest results show them to be excellent radical scavengers which inhibit lipid peroxidation and protect cells against ferroptotic cell death [9–11]. Moreover, it emerges that the persulfidation (and polysulfidation) of protein thiols serves protective and adaptive functions. Persulfidated protein thiols are shielded from irreversible thiol oxidation and electrophile conjugation because an oxidized or conjugated outer sulfur atom can be removed by reduction [3,12]. In addition, there is evidence that protein persulfidation regulates protein function in an adaptive manner [13,14]. Despite these advances, the understanding of persulfide and polysulfide biology is still in its infancy. Further progress depends on the availability of robust methods that allow the assessment of the quality and quantity of per/polysulfide species in biological samples. Unfortunately, per/polysulfides are labile molecules and easily lost during the processing of biological material. Therefore, all existing analytical methods include the alkylation of per- and polysulfides, to preserve them for analysis during sample processing. However, as recognized only recently, the alkylation of per- and polysulfides can destroy them, depending on the choice of alkylating agent. In this review we summarize and discuss the challenges associated with the alkylation of per- and polysulfides, the single most important step for their preservation and detection.

Per- and polysulfides are highly reactive and therefore labile

In general, per- and polysulfides can act both as nucleophiles and electrophiles, and also engage in radical reactions. Importantly, the chemical properties of hydropersulfides (R-SSH), hydropolysulfides (R-SSS_nH; $n \geq 1$) and dialkyl polysulfides (R-SSS_n-R; $n \geq 1$) differ substantially from those of the corresponding thiols (R-SH) and disulfides (R-SS-R) [4].

Compared to thiols, the S—H bond in hydroper/polysulfides is substantially weaker, as the negative charge resulting from deprotonation can be delocalized over (at least) two adjacent sulfur atoms. Thus, at physiological pH the reactive form of hydroper/polysulfides (R-SS_n^-) is much more available for reactions with electrophiles than the reactive form of thiols (R-S^-). In addition, hydroper/polysulfides are superior nucleophiles, as the lone pair electrons of the penultimate sulfur atom increase the electron density at the terminal sulfur atom (the so-called "alpha effect") [15—17]. Because of their enhanced nucleophilicity relative to thiols, hydroper/polysulfides are sometimes called "hyperactivated thiols" [4]. Together, lower pK_a and higher nucleophilicity make hydroper/polysulfides very efficient electrophile scavengers at physiological pH. For example, while uncatalyzed thiol-disulfide exchange reactions are relatively slow [18], the attack of hydropersulfides on disulfide species is much faster [19,20].

Importantly, when in their protonated state, hydroper/polysulfides are also potent electrophiles. The same is true for dialkyl polysulfides. Compared to dialkyl disulfides, per- and polysulfides are superior electrophiles. This is because per/polysulfides provide good leaving groups. For example, a hydropersulfide acting as an electrophile can release H_2S/HS^-, which is an excellent leaving group (R-SSH + Nuc^- → R-S-Nuc + HS^-), and a dialkyl trisulfide can release a persulfide, which is an even better leaving group (R-SSS—R + Nuc^- → R-S-Nuc + RSS^-) [4].

Finally, hydroper/polysulfides are much better H atom donors than thiols, because the resulting perthiyl (or polythiyl) radicals can delocalize the unpaired electron between two (or more) sulfur atoms [21]. In contrast to dialkyl disulfides, dialkyl polysulfides engage in radical exchange reactions because they can release the low energy perthiyl radical (e.g., R-SSS-R + X• → R-SX + R-SS•) [22,23].

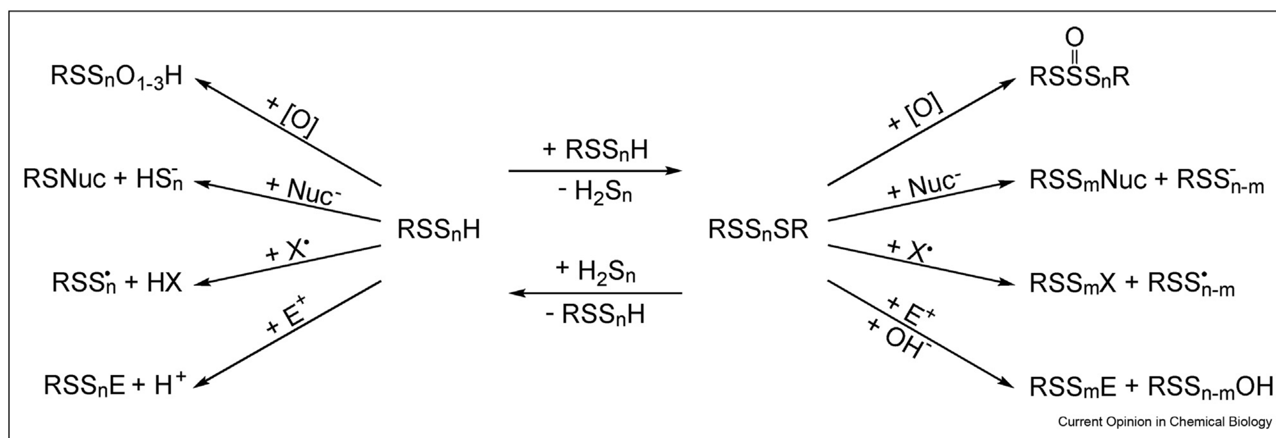
Overall, it is interesting to note that the conversion of a thiol (oxidation number −2) into a hydroper/polysulfide (oxidation numbers −1 or 0) is an oxidative process. Therefore, oxidation converts thiols into groups that are much more reactive towards oxidants, electrophiles and radicals. In other words, conditions of oxidative stress, by modifying thiols, promote the formation of a powerful antioxidative system.

As a result of their nucleophilic, electrophilic and radical reactivity, per/polysulfides readily engage in disproportionation and comproportionation reactions (e.g., $2RS^{-I}S^{-I}H \rightleftarrows RS^{-I}S^{0}S^{-I}R + H_2S^{-II}$). This leads to a dynamic equilibrium of species with a distribution of sulfur chain lengths [19] (Figure 1). Moreover, the intracellular per/polysulfide pool is shaped by enzymes catalyzing their formation, interconversion and reduction [10,24—29]. Given both enzymatic and non-enzymatic interconversions, cell disruption necessarily interrupts these reactions and/or enables new reactions by breaking subcellular compartments and other barriers. Thus, upon cell lysis both enzymatic and non-enzymatic reactions will start to converge towards a new, artificial equilibrium, unless these interconversions are rapidly and efficiently quenched.

The idea of using alkylating agents to stabilize per- and polysulfides for isolation and analysis

Considering the instability of per- and polysulfides, as detailed above, researchers typically treat cells with alkylating agents just prior to lysis and/or include an alkylating agent in the lysis buffer. The desired and expected effect of adding a membrane-permeable electrophilic alkylating agent at high concentration is twofold: First, alkylation should directly trap hydroper/polysulfides (R-SS-Alk, R-SSS_n-Alk), as well as inorganic polysulfides (Alk-SSS_n-Alk), thus preventing them from acting as nucleophiles on other molecules or on themselves. In particular, alkylation should prevent oxidation of terminal sulfur atoms to sulfenic, sulfinic or sulfonic acids (e.g., R-SSOH, R-SSO_2H, R-SSO_3H). Second, alkylation should also trap free thiols (R-S-Alk), in particular the highly abundant GSH, which may otherwise attack hydroper/polysulfides (e.g. GSH + R-SSH → R-SSG + H_2S), or organic polysulfides (e.g. GSH + R-SSS-R → R-SSG + R-SSH). Thus, by adding alkylating agents prior to and/or during cell lysis, hydroper/polysulfides should be conserved for further analysis as alkylated species (Figure 2). Ideally, treatment with an alkylating agent would instantly freeze all relevant reactions, thus providing a snapshot of sulfane sulfur species at the very time point of alkylation. In reality, however, it can be expected that the kinetics of alkylation will differ between different species. This means that equilibria between interconverting species may be shifted (according to the Curtin-Hammett principle) before everything is alkylated [19,30,31]. Nevertheless, it is generally assumed that treatment with high concentrations of alkylating agents can conserve per/polysulfides well enough to allow meaningful comparisons between samples. More recently, however, it has been realized that

Figure 1



Characteristic reactions of hydroper/polysulfides and dialkyl polysulfides. Hydroper/polysulfides (RSS_nH) and dialkyl polysulfides (RSS_nSR) interconvert by exchanging sulfide or inorganic polysulfides (H_2S_n) (middle). Hydroper/polysulfides are subject to oxidation, electrophile conjugation, nucleophile and radical attack (left side). Dialkyl polysulfides are subject to similar reactions (right side).

the use of alkylating agents poses additional unexpected challenges with regard to the preservation of per- and polysulfides, as will be explained in the next sections.

Alkylation of hydropersulfides: Observations & interpretations

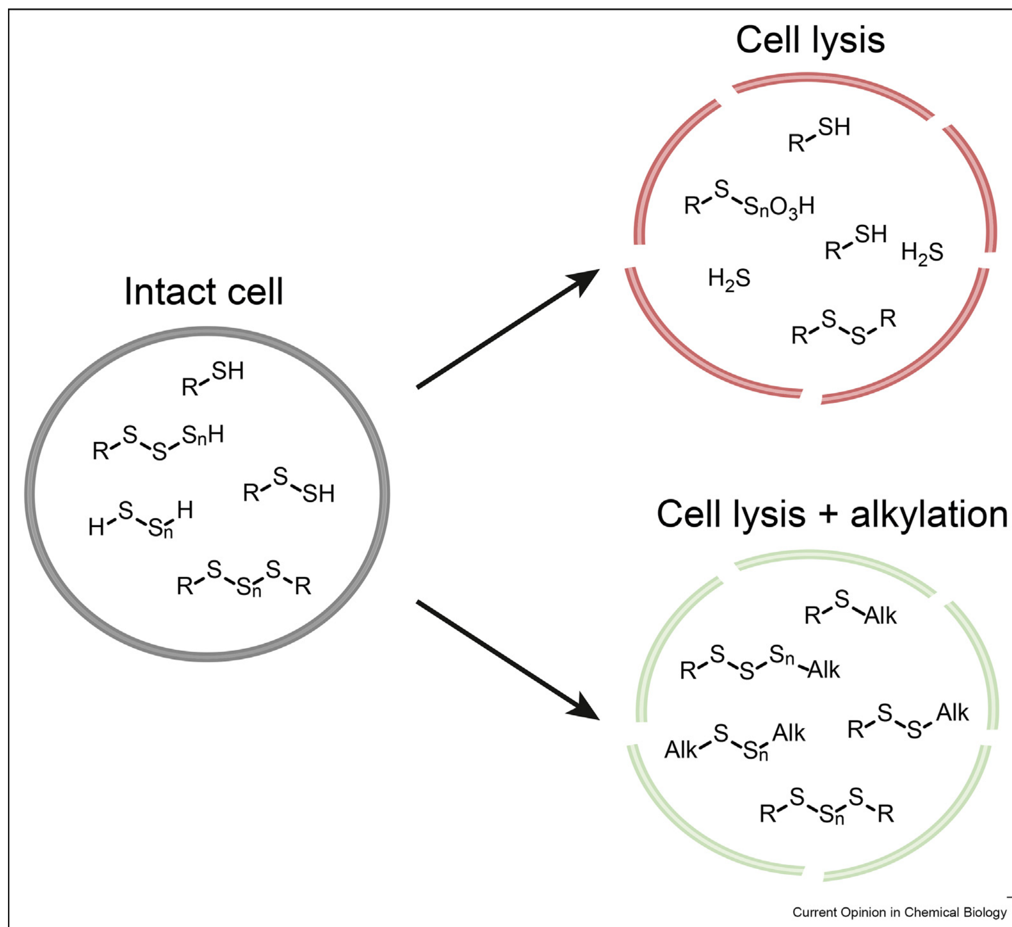
Until recently, it has been generally assumed that all alkylating agents typically used for blocking free thiols, including N-ethyl maleimide (NEM), monobromobimane (MBB) and iodoacetamide (IAM) (depicted in Figure 3a), can also be used for blocking hydropersulfides. However, a few years ago it was revealed that successful trapping of hydropersulfides as R-SS-Alk species critically depends on the choice of alkylating agent. Specifically, it was found that, depending on the alkylating agent, hydropersulfides may yield an alkylated thiol (R-S-Alk) instead of the expected alkylated persulfide (R-SS-Alk) [19,27,32]. Thus, it appears that hydropersulfides can lose sulfur when reacting with certain alkylating agents.

Akaike et al. were the first to report on this phenomenon. They observed that the hydropersulfide of cysteine (Cys-SSH) loses sulfur when reacted with NEM (Cys-SSH + NEM → Cys-S-NES; NES: N-ethylsuccinimide), but not when reacted with the IAM derivative (4-hydroxyphenyl)ethyl iodoacetamide (HPE-IAM; depicted in Figure 3b) (Cys-SSH + HPE-IAM → Cys-SS-HPE-CAM; CAM: carbamidomethyl) [27]. Similar observations were subsequently reported by Bogdandi et al. [19], who also found that treatment of Cys-SSH with NEM led to the corresponding thioether (Cys-S-NES), while HPE-IAM largely preserved Cys persulfides as Cys−SS−Alk species. Additionally, they observed that MBB efficiently preserved the persulfide (Cys-SSH + MBB → Cys-SS-Bim; Bim: Bimane). Most recently, Schilling et al. [32] again showed that the S−S bond in Cys-SSH is preserved by MBB, but not by NEM. Taken together, these three studies agree on the differential influence of NEM, HPE-IAM and MBB on the recovery of cysteine hydropersulfide (Figure 3c).

Schilling et al. then showed that a specific protein persulfide (Trx1-SSH) was best preserved by MBB, largely preserved by HPE-IAM, but destroyed by NEM [32]. They also found IAM to destroy the protein persulfide. Testing a series of IAM derivatives, smaller molecules were found to favor desulfurization, while bulkier ones, in particular tert-butyl-IAM, disfavored desulfurization of the persulfide (Figure 3d). Moreover, the presence of a β-carbonyl group was found to be a major destabilizing factor. A disulfide with β-carbonyl groups lost more sulfur to a nucleophile than a corresponding disulfide lacking β-carbonyl groups (Figure 3e). Of note, another recent study observed that a β-carbonyl adjacent to a disulfide promotes sulfur extrusion and thioether generation [33]. Thus, the tendency of alkylated persulfides to convert into alkylated thiols is found to depend on structural features of the alkylating agent.

While the above-mentioned studies agree on the differential influence of NEM, HPE-IAM and MBB on the recovery of hydropersulfides, it remains unclear how to explain these observations. The first two studies [19,27] did not attempt to explain the observed differences. The third study [32] hypothesized that certain alkylating agents (but not others) may promote tautomerization of the alkylated persulfide to the thiosulfoxide (R-SS-Alk ⇄ R-S(=S)-Alk) which is then desulfurized by ambient nucleophiles (R-S (=S)-Alk + Nuc → R-S-Alk + Nuc-S) (Figure 3f).

Figure 2



Alkylation is expected to preserve hydroper/polysulfides during cell lysis and sample preparation. In the absence of alkylating agents, cell rupture is likely to promote oxidation, reduction and interconversion reactions, leading to modifications and altered distributions (upper right). In the presence of alkylating agents, hydroper/polysulfides should be preserved for further analysis as alkylated species, at least in theory (lower right).

In sum, it has become clear that the choice of alkylating agent is critical for the detection and quantitation of hydropersulfides. The use of persulfide-destabilizing alkylating agents (first and foremost NEM) can lead to a substantial underestimation of actual persulfide levels.

Alkylation of hydropolysulfides and dialkyl polysulfides: Observations & interpretations

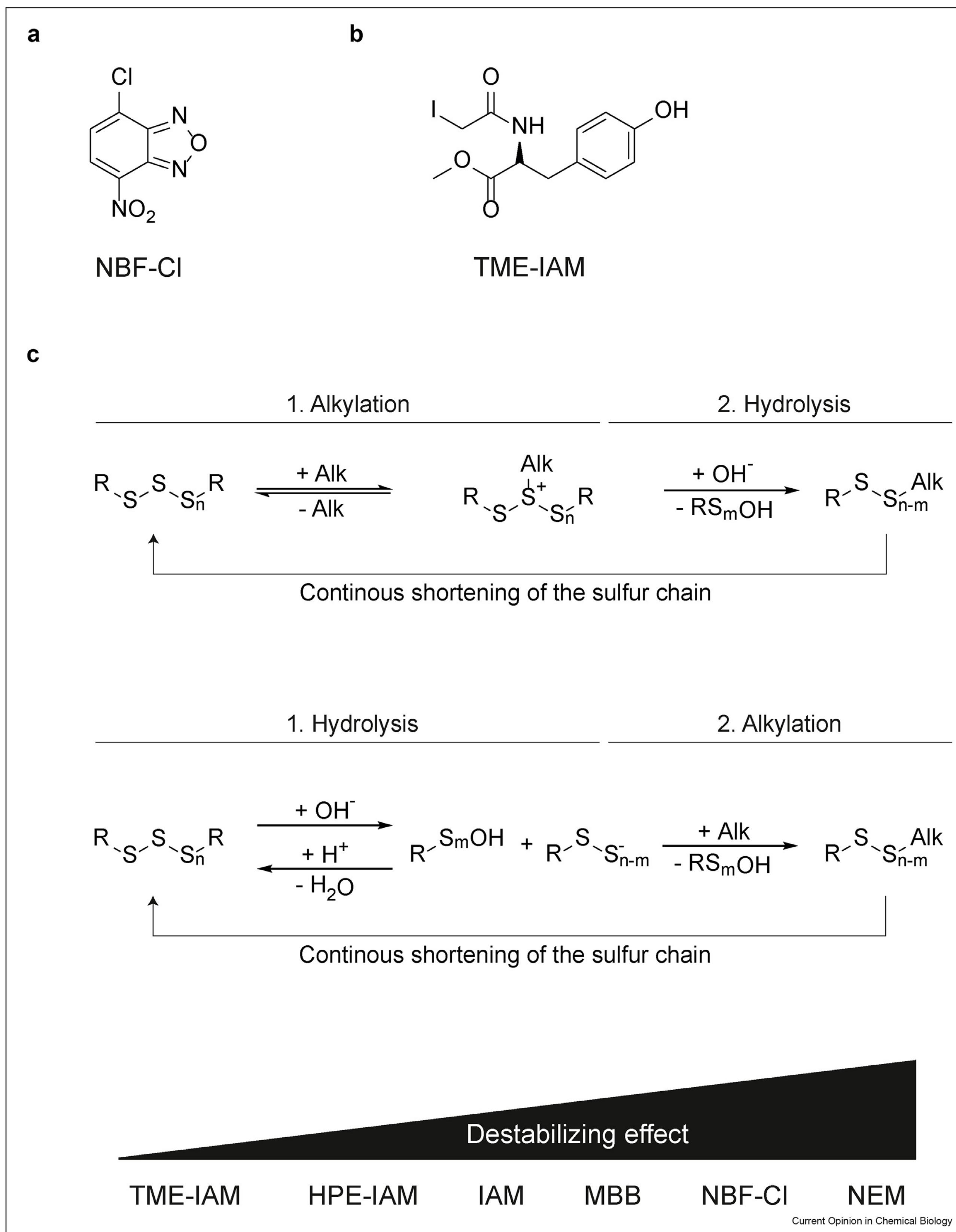
Similar to hydropersulfides, both hydropolysulfides (R-SSS_nH) and dialkyl polysulfides (R-SSS_n-R) were observed to lose sulfur in the presence of alkylating agents, as evidenced by the formation of shortened and cleaved polysulfide chains. Again, this effect is highly dependent on the choice of alkylating agent [19,27,31]. Similar to hydropersulfides, NEM destroyed hydropolysulfides and HPE-IAM largely preserved them. However, in contrast to hydropersulfides, MBB was not efficient in preserving the integrity of polysulfur chains [19].

Regarding dialkyl polysulfides, several research groups investigated the in vitro stability of glutathione tetrasulfide (GSSSSG) in the presence of alkylating agents. Incubation of GSSSSG with MBB, NEM or 4-chloro-7-nitrobenzofurazan (NBF—Cl; depicted in Figure 4a) led to the loss of GSSSSG over time. The formation of short chain products (GS-Alk > GSS-Alk) suggested that the tetrasulfide chain underwent repeated cleavage events. In contrast, GSSSSG was substantially more stable in the presence of either HPE-IAM or *N*-iodoacetyl L-tyrosine methyl ester (TME-IAM, depicted in Figure 4b) [19,34—36]. Hydropolysulfides also suffer from polysulfur chain cleavage, as alkylation converts them into asymmetrical dialkyl polysulfides, which are then subject to cleavage [19].

Figure 3

Destabilization of hydropersulfides by alkylating agents. (**a**) Alkylating agents most commonly used for blocking thiols are NEM, MBB and IAM. (**b**) Structure of the bulky IAM derivative HPE-IAM. (**c**) NEM reacts with cysteine persulfide (Cys-SSH) to yield the alkylated thiol (left panel), while MBB or HPE-IAM conserve the persulfide (right panel). (**d**) The bulkiness of IAM derivatives correlates with their ability to preserve a protein persulfide (figure reproduced from ref. 32). (**e**) The presence of β-carbonyl groups next to a disulfide bond promotes desulfuration in the presence of nucleophiles. (**f**) A hypothesis of how alkylated persulfides may convert into thioesters: a labile thiosulfoxide intermediate is formed and promotes desulfurization by ambient nucleophiles.

Figure 4



Hypothetical mechanism of electrophile-assisted hydrolysis of dialkyl polysulfides. (**a**) Structure of NBF−Cl, a polysulfide destabilizing alkylating agent. (**b**) Structure of TME-IAM, a polysulfide stabilizing alkylating agent. (**c**) The previously observed cleavage of polysulfide chains in the presence of alkylating agents has been proposed to be connected to hydrolysis. Two possibilities are considered: Either the alkylating agent is first conjugated by a sulfur chain free electron pair, thus promoting subsequent hydrolytic cleavage (upper part). Or hydrolytic cleavage is the first step, to be “pulled” by subsequent alkylation of the hydrolysis product (lower part).

The mechanism by which alkylating agents cleave S—S bonds in polysulfide chains remains unknown. One possibility is electrophile-assisted hydrolysis: In this case, the alkylating agent would be attacked by a mid-chain lone electron pair of the polysulfide chain. The resulting conjugate would then be susceptible to hydrolysis, yielding fragments R-S_n-Alk and R-S_n-OH (Figure 4c) [19,27]. This hypothesis is supported by the observation that in the additional presence of dimedone R-S_n-dimedone adducts are formed, implying the formation of R-S_n-OH [19,27]. In theory, the reverse order of events, hydrolysis followed by alkylation of hydrolytic fragments, may also drive the decomposition of polysulfide chains. The observation of R-S_n-dimedone adducts in the absence of other alkylating agents may support this idea [19]. It is also conceivable in principle that polysulfides fragment by homolytic S—S bond dissociation, considering the relative weakness of these bonds and the relative stability of the resulting per/polythiyl radicals.

As mentioned above, HPE-IAM and TME-IAM, both of which contain a phenolic side chain, are reported to have a polysulfur chain preserving effect, and are now used in improved polysulfide detection protocols [34,36]. Along these lines, the presence of tyrosine, but not of phenylalanine, was reported to protect dialkyl polysulfides against decomposition by alkylating agents, suggesting that phenolic groups have a polysulfide protecting or stabilizing effect [34]. However, the mechanism behind this phenomenon remains unknown.

Conclusions

It is increasingly clear that alkylating agents exhibit complicated and varied chemistry with regard to per- and polysulfides. Recent studies demonstrate that numerous alkylating agents, including the most commonly used ones, have a negative influence on the stability of these species. Some alkylating agents, NEM in particular, destroy both hydropersulfides and polysulfides. The alkylating agent MBB stands out as a special case, as it appears to have different effects on hydropersulfides and polysulfides: it conserves hydropersulfides very efficiently, but at the same time seems to destabilize longer sulfur chains. It is therefore possible that hydropersulfides trapped by MBB are partially derived from hydropolysulfides. Importantly, some alkylating agents appear to be a reasonable choice for the trapping of both hydropersulfides and polysulfides, in particular HPE-IAM.

A mechanistic understanding of the observed phenomena, namely desulfuration of alkylated persulfides and cleavage of longer sulfur chains, is still lacking. At this point, all interpretations have to be considered preliminary. A concern relating to the above-mentioned hypothesis (Figure 3f) is that it postulates the involvement of a thiosulfoxide. Thiosulfoxides are high energy structures, and it remains unclear if and under which conditions they can be formed. Likewise, the proposed model of electrophile-driven hydrolysis of polysulfides (Figure 4c) raises questions that remain to be answered. For example, it remains unclear why and how phenolic groups should inhibit the hydrolysis of polysulfides.

In sum, there is growing awareness that the choice of alkylating agents can have a substantial impact on the quality and quantity of observed thiol redox modifications, especially in the context of sulfane sulfur chemistry. Acknowledging and addressing these complications is certain to lead to improved methods and new insights.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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