

CELL BIOLOGY

Comment on “Evidence that the ProPerDP method is inadequate for protein persulfidation detection due to lack of specificity”

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The recent report by Fan *et al.* alleged that the ProPerDP method is inadequate for the detection of protein persulfidation. Upon careful evaluation of their work, we conclude that the claim made by Fan *et al.* is not supported by their data, rather founded in methodological shortcomings. It is understood that the ProPerDP method generates a mixture of cysteine-containing and non-cysteine-containing peptides. Instead, Fan *et al.* suggested that the detection of non-cysteine-containing peptides indicates nonspecific alkylation at noncysteine residues. However, if true, then such peptides would not be released by reduction and therefore not appear as products in the reported workflow. Moreover, the authors' biological assessment of ProPerDP using *Escherichia coli* mutants was based on assumptions that have not been confirmed by other methods. We conclude that Fan *et al.* did not rigorously assess the method and that ProPerDP remains a reliable approach for analyses of protein per/polysulfidation.

INTRODUCTION

Persulfidation, i.e., the formation of -SSH groups in protein cysteine residues, is gaining increasing attention due to regulated modulation of protein function by persulfidation events and their importance for hydrogen sulfide-related signaling (1, 2). The newly recognized biological relevance of protein per/polysulfidation (polysulfides are longer polysulfur chains on Cys thiol side chains) called for the development of selective and sensitive methodologies for monitoring these species in biological matrices. The study of

per/polypersulfidation is a young and rapidly evolving field that relies, to a notable extent, on ongoing methodological innovation (3, 4). Therefore, careful cross-validation of analytical techniques and critical assessment of the underlying principles applied is essential to separate hype from hope and for science to progress into the right direction.

RESULTS AND DISCUSSION

Fan *et al.* (5) studied the effects of deletion of OxyR, a redox-regulated transcription factor, on the global persulfidation levels in *Escherichia coli*. They had previously found that OxyR deletion promoted accumulation of total polysulfide levels in *E. coli* (6) and hence expected protein persulfidation to increase concomitantly in the mutant strain. The authors, thus, wished to apply the Protein Persulfide Detection Protocol (ProPerDP) assay that we had previously developed (depicted as workflow 1 in their article) to assess this system. A detailed experimental protocol, which typically might include the number of cells, growth phase, treatment conditions, sample processing after alkylation, imaging, and data processing, is notably missing from their report. Nonetheless, the authors reported in their Figure 2 that 66 or 118 persulfidated proteins were identified from the OxyR-deficient (Δ OxyR) or wild-type (wt) strains, respectively. The apparent higher global persulfidation in the wt versus the Δ OxyR strain differed from their expectations, which led them to conduct further experiments. The design of those experiments, however, raises substantial methodological concerns. First, in their Figure 2A, the persulfidation level is shown as percentages, but it is unclear from their study how quantification of the total versus the persulfidated proteome had been carried out. Moreover, they do not report the number of replicate samples analyzed nor do they provide an assessment of variance either within or between experimental groups.

The authors used the unusual assumption that “only” or “more” (not clear from their wording) Cys-containing peptides should have been detected from the proteins as obtained using workflow 1 (see Fig. 1). Workflow 1 yields Cys-containing proteins that are per- or

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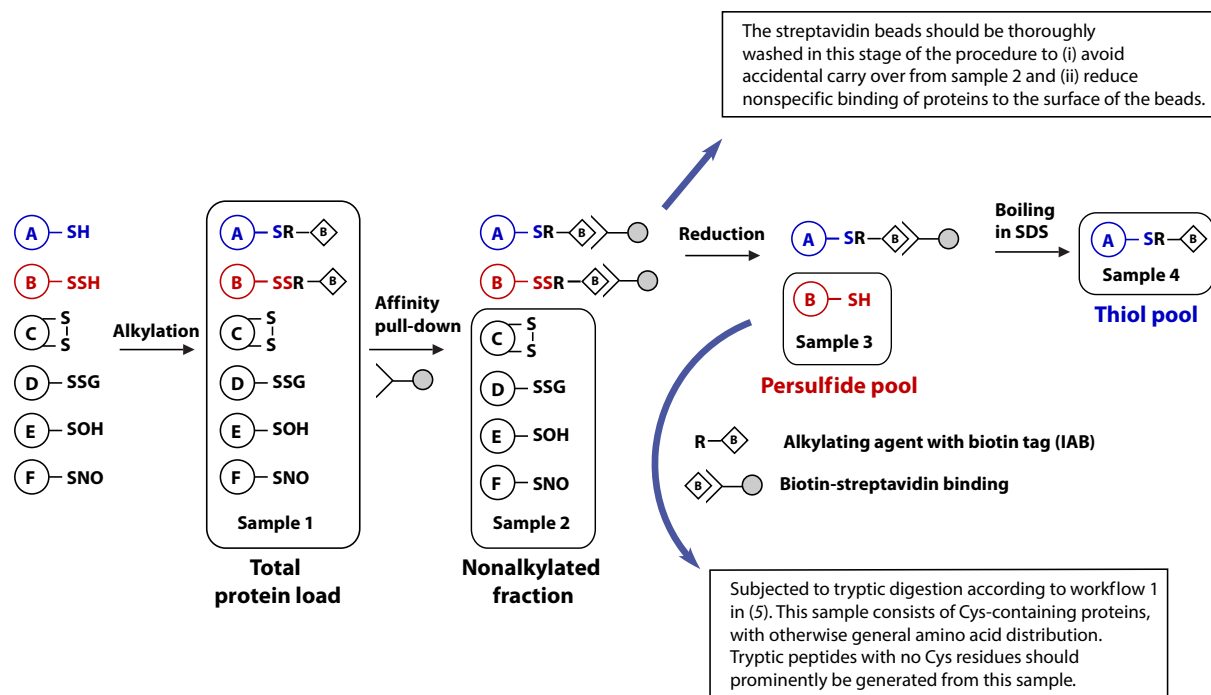


Fig. 1. Extended experimental scheme of the ProPerDP method (workflow 1). The figure emphasizes the importance of intermittent washes to control nonspecific binding. The tryptic digestion of sample 3 obtained from a whole-cell lysate is expected to naturally contain a high number of peptides without Cys residues. The figure was adapted from (10) (<https://creativecommons.org/licenses/by-nc/4.0/>), with permission.

polysulfidated on one or more Cys residues. Subsequent tryptic digestion of these Cys-containing proteins will release the Cys-containing peptide(s) that allowed affinity purification in workflow 1, along with a much larger number of peptides from each protein that contain no Cys residues. The occurrence of Cys in the *E. coli* proteome is only ~1% (7). Furthermore, a fraction of Cys residues in the proteome might have irreversibly modified Cys residues (e.g., Cys-SO₂H, Cys-SO₃H, or alkylation) and thereby remain undetected in the protocol. Therefore, contrary to the conclusions by Fan *et al.* (5), there is nothing unexpected in their finding that only a low percentage of the peptides they analyzed contained Cys.

Nonetheless, we also note that the list of proteins reported by Fan *et al.* (5) that were detected as persulfidated and identified by liquid chromatography–tandem mass spectrometry [data file S1 in (5)] revealed that 36 of the 132 proteins (27%) lack Cys residues in their native sequence, implying that there was at least some nonspecific capture on the streptavidin matrix they used. In their paper, Fan *et al.* (5) used streptavidin agarose resin for the affinity pull-down, whereas the original ProPerDP method uses magnetic streptavidin beads (8). A technical report on how to perform immunoprecipitation suggests using magnetic beads rather than agarose resin for protein separations (9). For example, separation of the supernatant from solid phase is more problematic in the case of agarose, which requires centrifugation, as compared with magnetic beads, which are instead pulled to the tube wall by a magnet. This issue is important in the ProPerDP method because the unbound “S2 fraction” represents the nonalkylated proteins used for later calculations. For this to work, the streptavidin-containing solid phase has to be thoroughly washed and separated (as indicated in Fig. 1) after pull-down and before tris(2-carboxyethyl)phosphine (TCEP) reduction, because residual proteins in this fraction will otherwise be

misannotated as “nonspecific binding.” As emphasized in our detailed method paper (8), we recommend minimizing nonspecific protein binding to the magnetic beads by multiple washing steps in a mild detergent buffer (tris-buffered saline with 0.05% Tween 20). Furthermore, we have performed several additional control experiments, which implicated the importance of negative controls using cell- or protein-free samples for background signals in the S3 fraction, because the reduction step can eliminate unknown contaminating proteins from the magnetic beads with major differences among vendors. From the experimental information provided, it remains unclear whether Fan *et al.* (5) validated their deviation from the established protocol with a known system before applying these conditions to the *E. coli* system in their paper or not.

In the next stage of their study, Fan *et al.* (5) analyzed their *E. coli* samples with a different procedure (their workflow 2), in which the biotin-streptavidin pull-down step was preceded by tryptic digestion. This approach is what we had proposed in Figure 8C of the original ProPerDP paper as a means to decrease potentially false-negative and false-positive hits (10). Unfortunately, once again, the specific conditions used by Fan *et al.* (5) in their analyses are not described in sufficient detail to evaluate this. Moreover, Fan *et al.* (5) applied this method only in *E. coli* and yeast, which makes it impossible to compare the performance of their detection system with our own studies on mammalian samples, which are likely to differ in extent of persulfidation. Since Fan *et al.* (5) reported only a single measurement for each experimental condition, it is also impossible to assess the extent of either experimental or biological variance.

We agree with the authors (5) that, in the case of workflow 2, only Cys-containing peptides would be expected in the product fractions because the pull-down follows the tryptic digestion step. Thus, the low percentage of peptides containing Cys residues that

they found in their workflow 2 is unexpected. The authors offered no plausible explanation as to how these non-Cys-containing peptides were recovered in their final elution step. Contrary to their conclusions, we believe that this likely resulted from an insufficient optimization of their workflow 2 rather than from a problem with the validity of the ProPerDP method. Their speculation that apparent nonspecificity in the method is an alkylation-related issue is supported neither by the data they presented nor by rigorous experimental analyses (i.e., mass spectra of the suspected nonspecifically alkylated peptides). For example, their final sample may still have included nonspecifically bound peptides if the preceding washing step was not quantitative. A parallel control, in which a “mock reduction” wash step (i.e., without TCEP) is analyzed in a similar fashion, would help confirm or rule out this possibility.

In their Discussion, the authors hypothesize that the alkylation reagent we used for the ProPerDP method (10), *N*-iodoacetyl-*N*-biotinylhexylene-diamine (IAB), might have resulted in artifactual *O*- or *N*-alkylation of cellular proteins (5). Chemically, however, such reactions are not likely to prevail under the experimental conditions of the original method because (i) thiolates and, especially, per/polythiolates are much better nucleophiles than are proteinous hydroxy or amino residues, and (ii) *O*- or *N*-alkylated products would not be cleaved off from the streptavidin matrix by TCEP reduction and therefore would not appear in the final analyzed samples (10). The primary rationale behind ProPerDP is to liberate only TCEP-reducible moieties of proteins that had earlier been labeled with IAB, thereby specifically allowing detection only of physiologically persulfidated Cys residues (10). On the other hand, if the IAB tag had reacted with, e.g., histidine and lysine residues to a large extent in the first step, then the appearance of Cys-containing peptides in their final TCEP elution step might also have originated from peptides that were bound by mixed disulfides, and these recovered peptides would be misidentified as reflecting polysulfide species. Without more rigorous examination or assurance that IAB tag binding was specific for cysteines, this possibility cannot be completely ruled out.

Note that the Cys-SOH and Cys-SNO groups were found susceptible to iodoacetamide-based alkylation in the case of a single Cys-mutated bacterial peroxiredoxin isoform, generating dithiothreitol- or TCEP-cleavable products, which might potentially be misidentified in our method (11).

Last, Fan *et al.* (5) used IAB labeling by workflow 1 to compare three *E. coli* strains. Cultures were harvested in mid-log phase, a very dynamic phase in which cells might be expected to exhibit varying Cys-per/polysulfide profiles. For this experiment, Fan *et al.* (5) show no error bars or assessment of variance either within or between strains, making it impossible to evaluate the significance of strain-specific differences. We are aware of no reports, nor are any cited by Fan *et al.* (5), for what the relative levels of protein per/polysulfidation should be in these strains. Rather, Fan *et al.* (5) concluded the ProPerDP method provided inaccurate results because the data did not meet intuitively expected outcomes.

Note that reliable assessment of persulfidation patterns in biological samples by ProPerDP is critically dependent on rigorous optimization of pull-down efficiency conditions for the samples to be analyzed, faithful utilization of replicate samples, and reliable persulfidated and nonpersulfidated control standards for calibration and validation (10). We previously calculated the minimal amount of magnetic streptavidin beads needed to ensure that the pull-down

efficiency is optimal for a given total protein content of mammalian cell/tissue extract (8). Moreover, to achieve optimal pull-down conditions, we reported that removal of excess IAB by desalting before the pull-down step is critical (8). One to 2% remaining unbound IAB could easily saturate the streptavidin binding sites on the beads, especially since the low-molecular weight biotinylated compound will bind the beads with more rapid kinetics than will proteins (8). By contrast, Fan *et al.* (5) reported no assessment or optimization of pull-down efficiency under their modified conditions.

In conclusion, we readily acknowledge that the ProPerDP method, like any newly developed experimental technique, comes with caveats, as discussed at length in our original publication (10). When assimilating a new technology like this, it is important to include rigorous critical assessment and validation, in particular for “off-label” use in areas beyond the original validation space. However, to state that the ProPerDP yields 90% false-negative results, with very little data to support such claim (5), we consider an unfortunate overstatement that has the potential to sow confusion and hinder progress in this field. We have continued active research on protein per/polysulfidation since reporting the ProPerDP method, and we continually assess the reliability of this method in diverse experimental conditions (1, 12–14). Although we have not assessed ProPerDP for work with bacterial or yeast cultures, we have no reason to question its reliability in these systems, if applied as originally reported. We suspect the problems Fan *et al.* (5) encountered during their studies arose from methodological shortcomings. In our experience, ProPerDP remains a reliable method for the assessment of protein persulfidation, and we believe the method helps advance the emerging and rapidly evolving insight into the function and significance of protein per/polysulfidation in biology.

MATERIALS AND METHODS

This perspective is aimed to reflect on the criticisms of the ProPerDP method reported earlier by Fan *et al.* (5). All methodological information supporting the arguments presented herein can be found in our previous studies developing and characterizing the ProPerDP method (8, 10).

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Acknowledgments: We acknowledge the late J. R. Mitchell from ETH Zürich for the valuable intellectual input to this commentary. **Funding:** P.N. acknowledges support from the Hungarian Thematic Excellence Programme under grant no. TKP2020-NKA-26. This project was funded in part with Federal funds from the National Cancer Institute, NIH, under Contract HHSN26120080001E (D.A.W.). **Author contributions:** All authors contributed to the preparation of the manuscript. **Competing interests:** The authors declare that they have no competing interests. **Data and materials availability:** All data needed to evaluate the conclusions in the paper are presented in the paper and as referred to in the text in our previous studies (8, 10). Additional data related to this paper may be requested from the authors.

Submitted 9 September 2020

Accepted 3 March 2021

Published 21 April 2021

10.1126/sciadv.abe7006

Citation: É. Dóka, E. S. J. Arnér, E. E. Schmidt, T. P. Dick, A. van der Vliet, J. Yang, R. Szatmári, T. Ditrói, J. L. Wallace, G. Cirino, K. Olson, H. Motohashi, J. M. Fukuto, M. D. Pluth, M. Feelisch, T. Akaike, D. A. Wink, L. J. Ignarro, P. Nagy, Comment on “Evidence that the ProPerDP method is inadequate for protein persulfidation detection due to lack of specificity”. *Sci. Adv.* **7**, eabe7006 (2021).