Maleimide scavenging enhances determination of protein S-palmitoylation state in acyl-exchange methods

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S-palmitoylation (S-acylation) is emerging as an important dynamic post-translational modification of cysteine residues within proteins. Current assays for protein S-palmitoylation involve either *in vivo* labeling or chemical cleavage of S-palmitoyl groups to reveal a free cysteine sulfhydryl that can be subsequently labeled with an affinity handle (acyl-exchange). Assays for protein S-palmitoylation using acyl-exchange chemistry therefore require blocking of non-S-palmitoylated cysteines, typically using N-ethylmaleimide (NEM), to prevent non-specific detection. This in turn necessitates multiple precipitation-based clean-up steps to remove reagents between stages, often leading to variable sample loss, reduced signal, or protein aggregation. These combine to reduce the sensitivity, reliability, and accuracy of these assays, which also require a substantial amount of time to perform. By substituting these precipitation steps with chemical scavenging of NEM by 2,3-dimethyl-1,3-butadiene in an aqueous Diels-Alder 4+2 cyclo-addition reaction, it is possible to greatly improve sensitivity and accuracy while reducing the hands-on time and overall time required for the assay.

Protein S-palmitoylation (S-acylation) involves the addition of long-chain fatty acids to cysteine residues within proteins through a thioester bond. Recent work has demonstrated that, far from being a passive means of anchoring proteins to membranes, S-palmitoylation is a dynamic regulator of protein function (1,2). In particular, S-palmitoylation has been shown to affect many integral membrane proteins, with nearly 40% of the membrane proteome estimated to be S-palmitoylated (3–5). Given the importance of membrane proteins as drug targets and regulators of trans-membrane transport, as well as their roles in signal transduction across membranes, understanding membrane protein function, and in particular their regulation, is a high priority. As a result, rapid, reproducible, and accurate methods for examining S-palmitoylation are much in demand.

Two complementary approaches are currently used for assaying protein S-palmitoylation: acyl-exchange (6) and alkyne fatty acid labeling (7). Both methods for studying S-palmitoylation have allowed researchers to achieve in days what previously took weeks or months using traditional ³H-palmitate labeling methods (6,7). The alkyne fatty acid labeling method uses *in vivo* feeding of cells with alkyne-derivatized fatty acids (generally stearate and palmitate mimics) followed by extraction and azide-alkyne click chemistry to tag and subsequently detect proteins of interest (7). This method is, however, restricted to tissue culture of cells that are able to take up the applied label and is therefore of limited use in the context of studying whole organisms or tissue-specific *in vivo* processes. Acyl-exchange assays on the other hand can be used to assess levels of protein S-palmitoylation from

samples of any origin. Subsequent refinement of acyl-exchange assays for use in a variety of species, at proteomic scales (3,4) or using more convenient reagents (acyl-resin assisted capture; acyl-RAC) (8), have improved matters further. Nonetheless, there are still improvements to be made. The existing protocols require a lot of time and are less than ideal for quantitative analysis, particularly of large or aggregation-prone proteins. The many handling and precipitation steps required to remove reagents from samples before progressing to the next stage also introduce sample-to-sample variation and, therefore, impact accuracy and reproducibility. Here, we present an optimized protocol that reduces sample handling and eliminates all precipitation steps for acyl-RAC style assays and requires just one precipitation step for acyl-biotin exchange (ABE) assays.

METHOD SUMMARY

Here, we describe a precipitation-free method to remove maleimides from aqueous solutions, allowing for rapid, reproducible, and quantitative analysis of protein S-palmitoylation states.

Materials and methods

All standard reagents and chemicals were purchased from Sigma-Aldrich (Gillingham, UK) or unless noted otherwise.

Organism growth conditions

Arabidopsis thaliana (Col-0 accession) was grown on 0.5× Murashige and Skoog (MS) medium, 0.8% phytagar under 16:8 h light:dark cycles at 20°C in MLR-350 growth chambers (Panasonic, Loughborough, UK). Ten days post-germination, seedlings were harvested and flash frozen in liquid nitrogen before processing. Rat hearts were briefly perfused with Krebs–Henseleit solution in the Langendorff mode to remove contaminating blood, then flash frozen in liquid nitrogen and ground to a fine powder.

Western blotting

SDS-PAGE of ABE samples was performed using 7.5% gels run under standard Laemmli conditions and proteins transferred to PVDF using Towbin buffer (65V, 2.5 h, 4°C). Membranes were blocked with 5% skim milk powder in TBS with 0.05% Tween20 (TBS-T) for 1 h at room temperature (RT). Rabbit polyclonal anti-FLS2 antiserum was diluted 1:5000 in blocking solution, and membranes were incubated 1 h at RT. Membranes were washed 3 times for 5 min each with TBS-T before incubation for 45 min with anti-rabbit HRP conjugate (sc-2004; Insight Biotechnology, Wembley, UK) diluted 1:5000 in blocking buffer. Membranes were washed 3 times with TBS-T and twice with TBS before being exposed to a G:box storm XT4 imaging system (Syngene, Cambridge, UK) using a mixture of Supersignal West Femto and Pico substrates (Thermo Fisher Scientific, Paisley, UK) in a 1:3 ratio. SDS-PAGE of acyl-RAC samples was performed on 6%–18% gradient gels and transferred to PVDF using a semi-dry transfer system. Membranes were blocked for 1 h in 5% skimmed milk powder in PBS 0.1% Tween20 (PBS-T) and probed overnight at 4°C with anti-NCX1 (P 11–13; Swant, Marly, Switzerland) at 1:1000 or anti-flotillin 2 (610383; BD Biosciences, Oxford, UK) at 1:5000. After 6 washes with PBS-T, membranes were incubated with HRP-linked anti-mouse (315–035–045;

Jackson ImmunoResearch, West Grove, PA) at 1:2000 for 1 h in blocking buffer, washed 6 more times, and images were acquired with a ChemiDoc XRS imaging system (BioRad, Hemel Hempstead, UK) using Immobilon HRP substrate (EMD Millipore, Watford, UK).

Acyl-biotin exchange procedure

ABE assays were performed essentially as described previously (4,9,10). Briefly, samples were ground to a fine powder in liquid nitrogen and resuspended in 2 ml lysis buffer [50 mM Tris-HCl pH 7.2, 5 mM EDTA, 150 mM NaCl, 2.5% SDS, 25 mM N-ethylmaleimide (NEM) (AC156100100; Fisher Scientific), 5 µl/mL protease inhibitor (P9599; Sigma-Aldrich), 100 mg polyvinylpolypyrrolidone (PVPP)]. Samples were solubilized at RT for 10 min with gentle mixing, centrifuged at 3000 × *g* for 1 min, filtered through 2 layers of miracloth (EMD Millipore), and re-centrifuged at 16,000 × *g* for 10 min. The clarified supernatant was retained, and the protein concentration was determined using a BCA assay (23225; Thermo Fisher Scientific). Protein (2 mg) was combined with lysis buffer (no PVPP) to a final volume of 1 mL and incubated with gentle mixing for 1 h. Samples were chloroform/methanol precipitated, briefly air-dried, and resuspended in 1 ml 50 mM Tris-HCl pH 7.2, 2% SDS, 8 M urea, 5 mM EDTA by gentle mixing. Samples were then split in 2; 1 aliquot was combined with 0.5 ml 1 M hydroxylamine (159417; Sigma-Aldrich), while the other was combined with 0.5 ml 1 M NaCl (negative control). Fifty micro-

liters was removed from each sample as a loading control and incubated at RT for 1 h before being chloroform/methanol precipitated. One hundred microliters of 2 mM biotin HPDP (21341; Thermo Fisher Scientific) in DMSO was added to the remaining sample, which was then gently mixed for 1 h before being chloroform/methanol precipitated, briefly air-dried, and resuspended in 150 µl 50 mM Tris-HCl pH 7.2, 2% SDS, 8 M urea, 5 mM EDTA by gentle mixing. Samples were diluted 1:10 with PBS and mixed with 20 µl of a PBS-washed 50% slurry of high capacity neutravidin beads (29202; Thermo Fisher Scientific) for 1 h. Beads were washed 3 times with PBS containing 1% SDS and dried by aspiration. Bead-bound samples and loading control precipitates were resuspended in 20 µl 2× reducing SDS-PAGE sample buffer containing 6 M urea at 37°C for 30 min with frequent mixing.

Acyl-resin assisted capture procedure

Acyl-RAC was performed essentially as described previously (10–12). Powdered left ventricle was lysed directly in 2.5% SDS, 1 mM EDTA, 100 mM HEPES, 25 mM NEM pH 7.5, and incubated at 40°C for 4 h to block free thiols. NEM was removed by acetone precipitation followed by multiple washes with 70% acetone and resolubilization in 1% SDS, 1 mM EDTA, 100 mM HEPES pH 7.5 at 40°C. Samples were split in 2 and S-palmitoylated proteins were captured on thiopropyl Sepharose 6b (T8387; Sigma-Aldrich) in the presence of 250 mM neutral hydroxylamine or water

Figure 1. FLS2 aggregation prevents quantitative acyl-exchange assay-based assessment of S-palmitoylation state. (A) FLS2 aggregates and smears on 7.5% SDS-PAGE gels after multiple precipitations. Fifty micrograms of total protein was loaded per lane. FLS2 appears heavier than the predicted 130 kDa due to extensive glycosylation of the leucine-rich repeats. (B) Minimum concentrations of N-ethylmaleimide (NEM) required for elimination of false positive identification of FLS2 S-palmitoylation levels in acyl-biotin exchange (ABE) assays. ABE assays were performed using the indicated concentrations of NEM during the blocking stage. All other steps in the protocol were as described in the methods. HYD indicates presence (+) or absence (-) of hydroxylamine required for acyl group cleavage during the biotin labeling step. Samples were bound to neutravidin beads following biotin labeling; enriched proteins were eluted and represent S-palmitoylated proteins (EX). Prior to neutravidin binding, a sample was removed as an input loading control (LC). All samples were run on 7.5% SDS-PAGE gels and blotted for FLS2.

Figure 2. 2,3-dimethyl-1,3-butadiene efficiently removes N-ethylmaleimide from aqueous solution. (A) Reaction scheme of N-ethylmaleimide (NEM) combining with 2,3-dimethyl-1,3-butadiene. (B) Determination of 2,3-dimethyl-1,3-butadiene concentration required for elimination of 10 mM NEM from lysis buffer in 1 h. A standard curve of NEM was prepared in lysis buffer and absorbance at 300 nm was measured. Solutions containing 10 mM NEM were treated with a range of 2,3-dimethyl-1,3-butadiene concentrations for 1 h at 25°C, and the remaining NEM concentration (i.e., unreacted NEM) in each sample was determined from the standard curve by spectrophotometric (filled circles) or fluorometric (open circles) methods. Data points are averages of three technical replicates. (C) Time required for elimination of 10 mM NEM by 100 mM 2,3-dimethyl-1,3-butadiene and chloroform extraction in lysis buffer. A standard curve of NEM was prepared in lysis buffer, and absorbance at 300 nm was measured. A 10 mM solution of NEM was treated with 100 mM 2,3-dimethyl-1,3-butadiene for the times indicated in the graph at 25°C and then phase partitioned using chloroform. The remaining NEM concentration in each sample was determined from the standard curve by spectrophotometric (filled circles) or fluorometric (open circles) methods. Data points are averages of three technical replicates.

(negative control) by agitating for 2.5 h at RT. Loading controls were collected before addition of thiopropyl Sepharose 6b beads. Following 5 washes with 1% SDS, 1 mM EDTA, 100 mM HEPES pH 7.5, proteins were eluted from thiopropyl Sepharose 6b beads in 50 µl SDS-PAGE sample buffer supplemented with 100 mM DTT.

Removal of N-ethylmaleimide from solutions during acyl-RAC and ABE procedures

This step replaces the first precipitation step to remove NEM in the acyl-RAC and ABE protocols: Following the NEM blocking stages of acyl-RAC or ABE, 2,3-dimethyl-1,3-butadiene (145491; Sigma-Aldrich) was added to 100 mM and incubated with vigorous mixing for 1 h at 25°C. Subsequently, chloroform (1/10th of the sample volume) was added, and the sample was vortexed for 1 min, then centrifuged at maximum speed. The supernatant was retained and used in subsequent hydroxylamine treatment

steps (continuing after resolubilization steps of standard protocols).

Quantification of N-ethylmaleimide

NEM concentration in solution was measured by spectrophotometry at 300 nm using an Ultrospec 2100 pro (GE Healthcare, Little Chalfont, UK). The limit of detection by spectrophotometry in our buffer system was determined to be ~40 µM. To calculate NEM concentrations in the range of 0.1–100 µM, a fluorometric maleimide quantification kit (ab112141; Abcam, Cambridge, UK) was used in conjunction with a Varioskan LUX plate reader (Thermo Fisher Scientific).

Results and discussion

Established acyl switch methods for assaying protein palmitoylation state rely on using hydroxylamine to cleave away S-acyl groups to reveal a cysteine that can then be labeled with biotin (6,9) or bound directly by sulfhydryl reactive resin (8). This requires that all other cysteines

in the protein are blocked to prevent false positives. Traditionally NEM, a sulfhydryl reactive reagent, has been the favored reagent to irreversibly block all free cysteines in a protein (6). NEM subsequently has to be removed by one or more precipitation steps to avoid downstream competition with reagents required to label the previously S-acylated cysteine.

While working with a number of difficult, low abundance or aggregation-prone plant proteins, we noticed that protein precipitation led to smearing on western blots or protein remaining trapped in the gel wells. This is likely due to precipitated proteins forming insoluble aggregates or not fully unfolding during resolubilization. As a result, trying to perform quantitative analyses to examine changes in S-palmitoylation is almost impossible under these conditions. The *Arabidopsis* receptor-like kinase FLS2 typifies many of these problems and is particularly prone to aggregation and smearing after precipitation (Figure 1A) and during sample processing for ABE (Figure 1B). Despite using different detergents, chaotropes, and resolubilization regimes, we were never able to fully eliminate this problem. We therefore sought ways to eliminate the precipitation and resuspension steps in our protocol without interfering with downstream processes.

Maleimides such as NEM that are used as the blocking reagent in ABE and acyl-RAC S-palmitoylation assays are electrophiles. Maleimides also form one of the classic components of Diels-Alder 4+2 cyclo-addition reactions, where a substituted alkene known as a dienophile, in this case NEM, reacts with a conjugated diene (Figure 2A). Interestingly, although classically thought of as organic chemistry, many Diels-Alder reactions not only work (13) but appear to proceed faster in aqueous reaction buffers (14). We therefore speculated that a suitable diene would be able to scavenge NEM that had not reacted with sulfhydryls and remove the need for precipitation clean up. We have been unable to find reports of conjugated dienes reacting with protein functional groups, making it unlikely that proteins would be modified by this treatment.

To evaluate this method, we first optimized the minimum NEM concentration required for the full blocking of

sulfhydryls in protein extracts. FLS2 contains 16 cysteines, 4 of which are likely involved in disulfide bonds (15), and 2 of which are S-acylated (4), making it an excellent test case for examining thiol blocking efficiency in S-palmitoylation assays. Using *Arabidopsis* seedlings, a range of NEM concentrations were tested for efficient blocking of FLS2 cysteines in ABE assays. We determined that 10 mM was sufficient to eliminate false positives while still maintaining rapid and reproducible blocking of cysteines (Figure 1B).

We then tested the ability of 2,3-dimethyl-1,3-butadiene, as a classic Diels-Alder diene, to react with NEM in our typical assay buffer. We found that a 10-fold molar excess of 2,3-dimethyl-1,3-butadiene was able to reduce NEM concentration from 10 mM to undetectable levels ($<50 \mu\text{M}$ NEM in our assay buffer as determined by spectrophotometry at 300 nm) after 1 h of vigorous mixing at 25°C (Figure 2B). Using a fluorescence assay to detect NEM, we were able to determine that NEM concentrations were reduced to $\sim 35 \mu\text{M}$ using a 10-fold excess of 2,3-dimethyl-1,3-butadiene. A 20-fold

molar excess reduced NEM concentrations to $\sim 15 \mu\text{M}$.

2,3-dimethyl-1,3-butadiene is volatile, flammable, and has an unpleasant odor; therefore, removal of excess 2,3-dimethyl-1,3-butadiene after reaction is a sensible precaution. As 2,3-dimethyl-1,3-butadiene is immiscible with water, it can be separated from aqueous components by centrifugation after reaction. However, 2,3-dimethyl-1,3-butadiene is less dense than water and floats on top of the aqueous phase. Careful pipetting or allowing 2,3-dimethyl-1,3-butadiene to evaporate in a fume hood is required to collect sample without disturbing the upper organic phase. Neither of these options are desirable for a number of practical reasons, so we investigated alternatives. As chloroform is denser than water and is a good water-immiscible organic solvent, we speculated that it would be able to remove unreacted 2,3-dimethyl-1,3-butadiene from aqueous solutions by phase partitioning. Adding 1/10th of the aqueous reaction volume of chloroform efficiently extracted all visible 2,3-dimethyl-1,3-butadiene into the chloroform layer that formed at the

bottom of the tube after centrifugation. A diffuse white layer formed at the interphase, but further analysis indicated that this layer did not contain any detectable protein. As this interphase layer was also formed when using buffer only, it is likely formed by detergent in the sample buffer. Chloroform is also an effective solvent for NEM, and 1/10th of the sample volume of chloroform alone was capable of reducing the NEM concentration in the aqueous phase by nearly 70% (69.8%, SD = 0.021, $n = 3$); therefore addition of chloroform is a worthwhile procedure in its own right.

Next, the time required for removal of NEM from samples by 2,3-dimethyl-1,3-butadiene and chloroform was optimized. We found that 10 mM NEM was depleted by $\sim 90\%$ after a 15 min treatment with 100 mM 2,3-dimethyl-1,3-butadiene at 25°C with vigorous shaking. NEM was at the limit of detection using absorbance at 300 nm after 30 min and was not detectable in samples after 45 and 60 min of treatment (Figure 2C). Using the more sensitive fluorescence assay, we determined that NEM concentrations were $\sim 50 \mu\text{M}$ (45 min)

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Figure 3. 2,3-dimethyl-1,3-butadiene scavenging of N-ethylmaleimide enhances sensitivity and accuracy of acyl-exchange-based assays of protein S-palmitoylation. (A) Use of 2,3-dimethyl-1,3-butadiene in place of precipitation to remove N-ethylmaleimide (NEM) from solution greatly improves detection of FLS2 S-palmitoylation by acyl-biotin exchange (ABE). The ABE protocol presented in this article was followed using either chloroform/methanol precipitation or 2,3-dimethyl-1,3-butadiene (DMB) treatment for NEM removal. The same initial extract was split into four and processed in parallel. HYD indicates presence (+) or absence (-) of hydroxylamine, which is required for acyl group cleavage during the biotin labeling step. Samples were bound to neutravidin beads following biotin labeling, and the enriched proteins that were eluted represent S-palmitoylated proteins (EX). Prior to neutravidin binding, a sample was removed as an input loading control (LC). All samples were run on 7.5% SDS-PAGE gels and blotted for FLS2. (B) Use of 2,3-dimethyl-1,3-butadiene in place of precipitation to remove NEM from solution is compatible with acyl-RAC procedures. The acyl-RAC protocol presented in the methods was followed using either acetone precipitation or 2,3-dimethyl-1,3-butadiene treatment for NEM removal. The same initial extract was split into two and processed in parallel. HYD indicates presence (+) or absence (-) of hydroxylamine required for acyl group cleavage during the thiopropyl Sepharose 6b capture step. Thiopropyl Sepharose 6b-enriched proteins were eluted and represent S-palmitoylated proteins (EX). Prior to Thiopropyl Sepharose 6b capture, a sample was removed as an input loading control (LC).

and ~15 μ M (60 min). These levels of NEM are well below the concentrations of sulfhydryl capture reagents typically used in acyl-RAC and ABE assays and are therefore unlikely to interfere in subsequent steps. While use of 200 mM 2,3-dimethyl-1,3-butadiene would arguably be most effective, it is difficult to completely remove using chloroform at 1/10th of the sample volume, meaning that it is carried over into subsequent steps and increased chloroform volume led to protein precipitation. Further refinements of the method, potentially using different dienes or water immiscible solvents, may be able to circumvent this limitation. To ensure maximal removal of NEM from solutions and prevent interference with downstream reactions, we decided to use a 1-h treatment with 100 mM 2,3-dimethyl-1,3-butadiene followed by chloroform phase separation for further experiments.

Using these new procedures we examined the ability to reproduce and improve upon our previous data on FLS2 palmitoylation using ABE and 2,3-dimethyl-1,3-butadiene treatment instead of protein precipitation for NEM removal. As shown in Figure 3, our new procedure appears to be completely compatible with the pyridyl disulfide chemistry used in the acyl-RAC and ABE procedures. The new method produced stronger, sharper, and more repro-

ducible bands of FLS2 on western blots, with reduced smearing, thereby making future quantification and interpretation of data easier and more accurate. To ensure that the new protocol was applicable to other proteins, organisms, and variations in procedure, we assessed the palmitoylation state of the NCX1 sodium/calcium exchanger (16) and FLOT2 (17) in rat hearts using the original and new acyl-RAC procedures. No difference was observed in the assessment of palmitoylation state of either protein between protocols. However, with the new protocol, hands-on and total time required were reduced. These data together indicate that this method will be useful for the assessment of palmitoylation of all proteins, not just aggregation-prone or large proteins. This will allow faster or higher-throughput analyses to be performed, including automation, and benefit large-scale proteomic analyses of S-palmitoylation, where sample handling errors can impact quantification. With the increasing interest in dynamic S-palmitoylation, sensitive, accurate and reproducible assays capable of dealing with multiple samples are required. The refinements presented here to NEM-based acyl-exchange assays fulfill these requirements.

Other sulfhydryl-reactive reagents commonly used for analysis of cysteine modifications include pyridyl disulfides,

haloacetamides, haloacetic acids, methyl methanethiosulfonate (MMTS), vinyl sulfones, and 4-vinyl pyridine. With the exception of some vinyl group-containing compounds, none of these compounds are reported to undergo reactions with (conjugated) dienes such as 2,3-dimethyl-1,3-butadiene. 2,3-dimethyl-1,3-butadiene is therefore unlikely to be able to remove these other chemical species from reaction mixtures. The suitability of the reported procedure for removal of vinyl sulfones and 4-vinyl pyridine needs to be empirically determined. Using maleimides alongside these alternative cysteine labeling reagents along with 2,3-dimethyl-1,3-butadiene as a scavenger for maleimides, it should be feasible to perform one-pot differential labeling reactions for the identification of modified cysteines in a similar manner to what is described here for ABE and acyl-RAC assays. For example, PEG-shift/APE assays for protein palmitoylation state, which currently require more than three precipitation clean-up steps (18), could be performed using maleimides to block unmodified cysteines and 2,3-dimethyl-1,3-butadiene to scavenge maleimides, followed by hydroxylamine and iodoacetamide-derivatized PEG treatment to label previously S-palmitoylated cysteines, all without any precipitation steps. Although developed for the analysis of S-palmitoylation, our procedure should also be compatible with methods that require the removal of maleimides such as NEM from aqueous solution under mild conditions. This includes analysis of other cysteine post-translational modifications such as S-nitrosylation, S-glutathionylation, sulfenylation, sulfinylation, sulfonylation, and disulfide mapping.

Author contributions

P.A.H. conceived and developed the work. C.H.H., D.T., F.P., W.F., and P.A.H. performed the experiments and analyzed the results. P.A.H. and W.F. wrote the manuscript with input from D.T., C.H.H., and F.P.

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Competing interests

The authors declare no competing interests.

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