Palmitoylated Proteins in *Plasmodium falciparum*-Infected Erythrocytes: Investigation with Click Chemistry and Metabolic Labeling

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Dedicated to Prof. R. Heiner Schirmer, whose knowledge and kindness continue to inspire

The examination of the complex cell biology of the human malaria parasite *Plasmodium falciparum* usually relies on the time-consuming generation of transgenic parasites. Here, metabolic labeling and click chemistry are employed as a fast transfection-independent method for the microscopic examination of protein S-palmitoylation, an important post-translational modification during the asexual intraerythrocytic replication of *P. falciparum*. Applying various microscopy approaches such as confocal, single-molecule switching, and electron microscopy, differences in the extent of labeling within the different asexual developmental stages of *P. falciparum* and the host erythrocytes over time are observed.

1. Introduction

1.1. Malaria Tropica and *Plasmodium falciparum*: An Ancient Burden

Malaria, a mosquito-borne disease caused by protozoan parasites of the genus *Plasmodium*, is responsible for ≈228 million clinical cases and 405,000 deaths per year.[1] Morbidity and mortality mostly affect pregnant women and young children living in the World Health Organization African Region.[1]

*Plasmodium falciparum* (*P. falciparum*), the causative agent of malaria tropica, is the most dangerous of the six human-pathogenic *Plasmodium* species and responsible for the vast majority of deaths.[1,2] To decrease the burden in endemic areas, malaria researchers worldwide strive to identify and test drug targets as well as vaccine candidates against *P. falciparum* and other *Plasmodium* species. Multiple vaccine candidates are in clinical development at the moment.[3,4] RTS,S/AS01 (or Mosquirix), which targets the *P. falciparum* circumsporozoite protein, is currently being tested in a pilot study roll-out in Kenya, Ghana, and Malawi.[3,4]

1.2. Erythrocytic Schizogony and parasite-Driven Host Erythrocyte Modifications Can Lead to Severe Complications

*P. falciparum* has a complex life cycle that involves a warm-blooded intermediate human host and a cold-blooded mosquito of the genus *Anopheles* as final host.[5,6] During its life cycle, *P. falciparum* infects and inhabits multiple cell types and niches and progresses from one developmental stage to the next, culminating in cell division and production of daughter parasites.[5,6] The erythrocytic schizogony that occurs in the erythrocytes of the human host requires an exceptional reorganization of the terminally differentiated and metabolically reduced host erythrocyte to ensure nutrient supply, life cycle progression, and protection against the immune response of the human host (Figure 1a; Figure S1a, Supporting Information).[5,7-9] The parasite, embedded in a parasitophorous vacuole, actively remodels the host erythrocyte to facilitate the de novo generation of a Golgi-like secretory organelle, the Maurer’s clefts.[10] The organelle was discovered in 1902 in Medan-Deli (Sumatra) by Georg Maurer, who used light microscopy to study the blood of malaria patients.[11,12]
Figure 1. S-Palmitoylation in erythrocytes infected with different developmental stages of *P. falciparum*. a) Schematic representation of different stages of *P. falciparum* development: free merozoite during erythrocyte infection, ring, trophozoite, and schizont stage at ≈12, 24, and 36 h postinvasion, respectively. P, parasite; E, erythrocyte; K, knobs; MC, Maurer’s clefts. Adapted with permission. 

b) Chemical structures of clickable palmitic acid alkyne and Alexa Fluor 647 azide. c) Representative confocal microscopy images showing the localization of Alexa Fluor 647 azide fluorescence within erythrocytes parasitized with different developmental stages of *P. falciparum*. Arrowheads indicate discrete fluorescent structures within the cytoplasm of the infected erythrocytes. Scale bar: 3 µm. d) Representative SMS microscopy images showing the localization of Alexa Fluor 647 azide fluorescence within the infected erythrocyte and within the different developmental stages of *P. falciparum*. Infected erythrocytes were pulsed with 50 µm clickable palmitic acid alkyne for 30 min. Fluorescence hotspots could be detected within the parasite. P, parasite; ECP, erythrocyte cytoplasm; EPM, erythrocyte plasma membrane. Scale bar: 3 µm. e) Representative transmission electron micrograph of an erythrocyte infected with a young trophozoite after a 30-min pulse with 50 µm clickable palmitic acid alkyne. Gold particles indicate the potential localization of S-palmitoylated proteins within the parasite and the host erythrocyte. P, parasite; ECP, erythrocyte cytoplasm; EPM, erythrocyte plasma membrane; K, knobs; MC, Maurer’s clefts. Scale bar: 1 µm. f) Quantification of the distribution of gold particles in erythrocytes infected with different developmental stages of *P. falciparum*. Statistical analysis of the proportion of gold particles within the erythrocytes for each sample revealed strongly significant differences between the different developmental stages (*p* = 8.06e-5). Number of infected erythrocytes analyzed: 46.
The Maurer’s clefts facilitate the transport of immunovariant adhesins for presentation on knob-like protrusions at the erythrocyte plasma membrane (EPM) for cytoadherence to vascular endothelial cells, autoagglutination to other infected erythrocytes, and rosetting of infected with uninfected erythrocytes (Figure 1a; Figure S1b–e, Supporting Information). These erythrocyte modifications mainly evolved to generate immune escape mechanisms of the parasites, but they can also contribute to the pathogenesis of severe or complicated malaria tropica, for example, by causing the blockage of the postcapillary venules. It is therefore crucial to properly examine and understand the cell biology of the parasite to successfully target and hopefully eradicate the disease.

1.3. Fast Microscopic Examination of the Complex Parasite Cell Biology Can Be Achieved with Click Chemistry and Metabolic Labeling

The examination of the complex cell biology of *P. falciparum* is highly challenging and time-consuming because it typically requires genetic manipulation of the parasite, for example, via establishment of stable transfectants in concert with protein overexpression or the application of different genome editing techniques. This in turn limits the scope of cell biological analyses because only one or a few proteins can be examined at any given time. Strategies that allow the microscopic examination of a large set of proteins are therefore critical to understanding the global protein behavior in a single infected erythrocyte. Recently, we established metabolic labeling and click chemistry in mammalian cells as a tool to monitor the trafficking of *S*-palmitoylated anterograde cargo proteins from the Golgi apparatus and beyond. [17]

We propose that the extension of these imaging approaches to *P. falciparum*-infected erythrocytes may simplify the fast examination of a subset of the variety of *S*-palmitoylated proteins produced by this parasite during its intraerythrocytic life cycle. Protein S-palmitoylation is a tightly regulated and reversible post-translational modification (PTM) (acylation) that involves the linkage of a fatty acid chain to cysteine residues of the target protein. [18,19] This lipid modification has been suggested to play a crucial role in cytoadherence, drug resistance, cell signaling, organelle positioning, cytoskeletal function, development, maturation, and even host cell invasion during the complex life cycle of *P. falciparum*. [16,17,20–25] In silico analyses identified an estimated 400 palmitoylated proteins produced in the schizont stages and 12 putative protein acyl-transferase enzymes (DHHC enzymes, PfDHHC1-12) that contain the conserved DHHC motif ([Asp-His-His-Cys] within a cysteine-rich domain). PfDHHC enzymes catalyze S-palmitoylation within the secretory pathway (endoplasmic reticulum and Golgi apparatus) or at unique apicomplexan organelles, such as the inner membrane complex and the rhoptries. [17,20–22,24,26] Palmitoylation represents the second most prominent PTM after phosphorylation in *Plasmodium* parasites, which suggests an important role for palmitoylated proteins in parasite development. [6]

Here we present a transfection-independent, fast, and easy approach that capitalizes on metabolic labeling and click chemistry to visualize the localization of S-palmitoylated proteins produced by *P. falciparum*, revealing marked differences in the extent of labeling as well as localization during its intraerythrocytic life cycle.

2. Results

2.1. Visualization of S-Palmitoylated Proteins within Parasites and Erythrocytes

To monitor S-palmitoylated proteins of *P. falciparum* by confocal microscopy, in vitro-cultured human erythrocytes (blood type A+), infected with different developmental stages of the parasite (trophozoites, and schizonts) (Figure 1a; Figure S1a, Supporting Information), were incubated with 50 µm of a clickable palmitic acid alkyne in delipidated RPMI medium for 30 min. The incubation in delipidated medium ensures that the clickable palmitic acid alkyne is the only source of available palmitate for the parasite. After fixation, a copper-dependent azide-alkyne clickodidition (CuAAC; click reaction) to 1.25 µm Alexa Fluor 647 azide triethylammonium salt (Alexa Fluor 647 azide) was performed (Figure 1b). After additional immunolabeling of the anion transport protein Band 3 with monoclonal antibodies against human Band 3 (secondary antibodies coupled to Alexa Fluor 488) in the EPM as well as labeling of parasite DNA with DAPI (4',6-diamidino-2-phenylindole), confocal images were acquired at the Leica TCS SP8 STED 3X microscope (SP8 microscope). After a 30-min exposure, all developmental stages of the parasite appeared to have successfully incorporated the palmitic acid analogue (Figure 1c). The fluorescence signal of the probe was detected within the parasite and discrete dot-like palmitate-positive structures were also present within the cytoplasm of the infected erythrocytes (see selected arrowheads, Figure 1c).

We next applied single-molecule switching super-resolution microscopy (SMS microscopy) to examine the nanoscale distribution of S-palmitoylated proteins. [17] Strikingly, we observed well-defined fluorescent structures within the cytoplasm of ring-, trophozoite-, and schizont-infected erythrocytes (Figure 1d). However, only a weak fluorescence signal could be observed within the cytoplasm of the host erythrocyte.

To further characterize the localization and distribution of the S-palmitoylated proteins within the parasites and the host erythrocytes, a click reaction with proteins that were metabolically labeled with alkynyl-palmitate for 30 min to biotin azide (PEG4 carboxamido-6-azidohexanyl biotin) was conducted. This was followed by electron microscopy (EM) analysis using streptavidin-fluoronoranogold (representative image of an erythrocyte parasitized with a young trophozoite, Figure 1e). The gold particles in the electron micrographs of erythrocytes infected with different developmental stages of *P. falciparum* were quantified and their distribution in the parasite and erythrocyte cytoplasm (ECP) was determined (Figure 1f). Our quantitative analysis showed that the cytoplasm of ring stage-infected erythrocytes contained the majority (≈94%) of gold particles, whereas only ≈6% of gold particles were found in the parasite cytoplasm. Interestingly, this distribution dramatically changed as the parasite progressed to trophozoite and schizont stage development with 45% versus 55% and 27% versus 73% distributions in the ECP versus parasite cytoplasm in trophozoites and schizonts, respectively.
2.2. Accessing the Triacsin C and Hydroxylamine Sensitivity of S-Palmitoylated Proteins

Coenzyme A (CoA) activation, catalyzed by the long chain fatty acid CoA synthetase, is required for S-palmitoylation of substrate proteins.\textsuperscript{[28]} The microbial metabolite Triacsin C is a specific and potent inhibitor of the long fatty acyl CoA synthetase and therefore further inhibits the S-palmitoylation of proteins.\textsuperscript{[28,29]} To determine if the previously observed Alexa Fluor 647 azide fluorescence signal is indeed caused by S-palmitoylated proteins, we pretreated the infected and uninfected erythrocytes for 30 min with 100 μM Triacsin C (diluted in delipidated medium). Afterwards they were subjected to a pulse with 50 μM clickable palmitic acid alkyn, which should result in a fluorescence decrease.\textsuperscript{[17]} In parallel, we incubated the infected and uninfected erythrocytes for 30 min in delipidated medium prior to the pulse with 50 μM clickable palmitic acid alkyn as a control. Examination of the recorded confocal Z-stacks showed the expected decrease in Alexa Fluor 647 azide fluorescence of ≈50–84% in the infected erythrocytes as a response to the pretreatment with Triacsin C compared to the untreated infected erythrocytes (Figure 2a and Table 1, see Statistical Methods section in Supporting Information). The pretreatment of uninfected erythrocytes with Triacsin C showed a similar response to the pulse with clickable palmitic acid alkyn when compared to the control uninfected erythrocytes that were pretreated with delipidated medium (Figure 2a and Table 1, see Statistical Methods section in Supporting Information). This result suggests that no or only a very low incorporation of palmitate took place in uninfected erythrocytes.

Hydroxylamine cleaves the S-linked acyl bonds of S-palmitoylated proteins rather than the O-linked acyl bonds.\textsuperscript{[17,30]} Therefore, it was important to test whether the CoA-activated palmitate analogue was indeed incorporated into proteins, rather than into membrane lipids. To that end, we treated the infected and uninfected erythrocytes after the completion of the 50 μM pulse and click reaction with 1.5 mM hydroxylamine (pH 7.0) for 3 h. After the click reaction with 500 nM Alexa Fluor 647 azide, we added SDS protein sample buffer and treated one half of the samples with 1.5 μM hydroxylamine (pH 7.0) for 5 min at 98 °C. Proteins were separated on a 10–20% tricine gel (Novex) and analyzed via in-gel fluorescence. Probe and dye were provided in excess to avoid the limitation of functionalized palmitate and ensure the click reaction efficiency. This resulted in an intense fluorescence signal at a low molecular weight that may either represent untreated dye or free clickable palmitate in the extracts that has been modified with Alexa Fluor 647 azide (Figure S3, Supporting Information). However, whereas no distinct protein bands were visible in the samples that contained only uninfected erythrocytes (Figure S2a, Supporting Information), the samples containing trophozoite stage-infected erythrocytes showed distinct protein bands that were further sensitive to the treatment with hydroxylamine (Figure S2b, Supporting Information). Our results thus demonstrate that it is possible to visualize S-palmitoylated proteins of P. falciparum by in-gel fluorescence and further suggest that the previously measured fluorescence signals (Figure 2) may indeed stem from the S-palmitoylated proteins. Our in-gel fluorescence results further suggest that the metabolically reduced uninfected erythrocytes may exhibit only negligible levels of S-palmitoylation activity.

2.3. Pulse/Chase Experiments to Investigate the Trafficking Behavior of S-Palmitoylated Proteins in P. falciparum-Infected Erythrocytes

To identify a putative trafficking behavior of the S-palmitoylated proteins within parasite and host erythrocytes, we conducted both pulse and pulse/chase experiments. For the pulse experiments, we subjected erythrocytes infected with different developmental stages of P. falciparum to pulses of increasing durations with 50 μM clickable palmitic acid alkyn. The samples were taken at specific time points and subsequently clicked to Alexa Fluor 647 azide. The fluorescence in the parasite and in the ECP over time was quantified from confocal Z-stacks acquired at the SP8 microscope (Figure 3a,b). In both the parasite and the ECP, we observed an increase in fluorescence over time (Figure 3a,b). The strongest fluorescence increase could be observed in the schizont stages (Figure 3a) and in the cytoplasm of erythrocytes that were infected with schizont stages (Figure 3b). The increase of fluorescence within trophozoite and ring stages was similar. These results suggest that the decrease of the fluorescence signal in the experiments can mostly be attributed to the hydroxylamine-derived S-linked acyl bond cleavage of S-palmitoylated proteins (Figure 2b and Table 2). Interestingly, we also observed that the treatment of the infected erythrocytes with hydroxylamine and Triacsin C did not result in the complete disappearance of the Alexa Fluor 647 azide fluorescence signal. A similar effect could be observed with hydroxylamine-treated uninfected erythrocytes.
Figure 2. The sensitivity of S-palmitoylated proteins to Triacsin C and hydroxylamine treatment. a) Pretreatment of infected erythrocytes with the long chain fatty acyl-CoA synthetase inhibitor Triacsin C (100 µm in delipidated medium) leads to the subsequent decrease of Alexa Fluor 647 azide fluorescence when the infected erythrocytes are treated with 50 µm clickable palmitic acid alkyne. Uninfected erythrocytes on the other hand did not show a response to the pretreatment with Triacsin C when compared to the control cells. A.U., arbitrary units. Number of experiments: 4. Number of cells analyzed per time point, stage, and experiment: ≥4. Mean ± SEM. b) Treatment of infected and uninfected erythrocytes with 1.5 M hydroxylamine (HX) after a 50 µm clickable palmitic acid alkyne pulse results in fluorescence decrease. A.U., arbitrary units. Number of conducted experiments: 4. Number of cells analyzed per time point, stage, treatment, and experiment: ≥4. Mean ± SEM. See Tables 1 and 2 for p-values.
Table 1. Decrease (d) and p-values for infected erythrocytes treated with Triacsin C.

| Stage/Time   | 5 min | 15 min | 30 min |
|--------------|-------|--------|--------|
| Ring         | d = 0.75, p = 0.03 | d = 1.28, p = 0.0041 | d = 1.12, p = 0.0076 |
| Troph        | d = 0.89, p = 0.02 | d = 1.23, p = 0.0049 | d = 1.37, p = 0.0029 |
| Schizont     | d = 1.12, p = 0.01 | d = 1.57, p = 0.0015 | d = 1.44, p = 0.0023 |
| Uninfected   | d = -0.2, p = 0.56 | d = 0.80, p = 0.0424 | d = 0.36, p = 0.3046 |

Table 2. Decrease (d) and p-values for infected erythrocytes treated with hydroxylamine.

| Stage/Time   | 5 min | 15 min | 30 min |
|--------------|-------|--------|--------|
| Ring         | d = 0.55, p = 6.42e-4 | d = 0.64, p = 1.47e-4 | d = 0.75, p = 2.97e-5 |
| Troph        | d = 0.81, p = 1.35e-5 | d = 0.83, p = 1.05e-5 | d = 0.82, p = 1.20e-5 |
| Schizont     | d = 0.86, p = 6.75e-6 | d = 0.5, p = 0.0011 | d = 0.61, p = 2.45e-4 |
| Uninfected   | d = 0.76, p = 0.0001 | d = 0.87, p = 2.13e-5 | d = 0.53, p = 0.004 |

but significantly differed when compared to the schizont stages (Figure 3a, see Statistical Methods section in Supporting Information). Meanwhile, the increase of fluorescence in the cytoplasm of erythrocytes infected with ring or trophozoite stages only slightly differed (Figure 3b, see Statistical Methods section in Supporting Information). The rates of increase for parasites and infected erythrocytes were also significantly larger than those for uninfected erythrocytes (Figure 3a,b, see Statistical Methods section in Supporting Information). On the other hand, uninfected erythrocytes exhibited only low residual S-acylation activity.

In order to test whether the observed signals within the infected ECP represented S-palmitoylated proteins exported from the parasite or whether the proteins were directly modified in the cytoplasm of the host erythrocyte, we conducted pulse/chase experiments (Figure 3c,d). Here, the parasitized erythrocytes were subjected to a brief 10-min pulse with 50 μM clickable palmitic acid alkyn. Afterwards, the infected erythrocytes were washed with delipidated medium and the chase was performed with a 10X molar excess of 500 μM palmitic acid alkyn for up to 1 h. We observed an overall decrease in the Alexa Fluor 647 azide fluorescence in both the parasite (Figure 3c) and the ECP during the various chosen time points of the 60-min chase (Figure 3d). The largest absolute decrease in fluorescence was observed in schizont stages and in erythrocytes parasitized with schizonts, which suggests a high rate of turnover of the acylation/acylated proteins (Figure 3c,d). Less absolute decrease in fluorescence was observed in ring and trophozoite stages, including the cytoplasm of erythrocytes parasitized with ring and trophozoite stages (Figure 3c,d). However, we did not observe a significant difference in the rates of decay on the log scale (0.5% min\(^{-1}\)) between the different developmental stages, suggesting a similar rate of deacylation across all stages (see Statistical Methods section in Supporting Information).

The fluorescence signal within uninfect erythrocytes showed only a slight decrease as a result of the chase. This suggests that the signal detected in the cytoplasm of infected erythrocytes i) could stem from a residual S-palmitoyltransferase activity within the host erythrocyte, and ii) that S-palmitoylated parasitic proteins may not be exported from the parasite into the ECP in their modified state, but could potentially be acylated outside of the parasite. Our pulse and pulse/chase experiments further indicate that schizonts are the developmental stage of \(P. falciparum\) that exhibit the highest rate of S-palmitoylation of proteins followed by trophozoites and rings. Our results also suggest that the S-palmitoyltransferase activity might be present in both the parasites and the cytoplasm of infected erythrocytes. In contrast, only residual levels are detected within the uninfected erythrocytes (see Statistical Methods section in Supporting Information).

3. Discussion

Driven by our recent work in which we applied metabolic labeling and click chemistry to examine the trafficking of anerograde, S-palmitoylated cargo in mammalian cells, we explored the application of this method to study S-palmitoylated proteins in a human malaria-based model. The investigation of \(P. falciparum\) proteins usually requires a genetic manipulation of the parasite that is difficult and time-consuming compared to standard in vitro-cultured mammalian cell lines such as HeLa or COS7 cells that can be transiently transfected and examined the next day\(^{[32]}\). The usage of metabolic labeling and click chemistry allowed us to investigate S-palmitoylation in \(P. falciparum\)-infected erythrocytes in less than 2 days, a process that holds further potential for other model systems that are difficult to stably transfect and genetically engineer.

3.1. S-Palmitoylated Proteins Can be Visualized and Localized within \(P. falciparum\) and the Host Erythrocyte

We showed that metabolic labeling of the parasites with clickable palmitic acid alkyn and the subsequent application of click chemistry allowed the localization of S-palmitoylated proteins within erythrocytes infected with different developmental stages of \(P. falciparum\) (Figure 1c–f). The observed intensity of the Alexa 467 azide fluorescence signal is supported by the fact that palmitoylation represents the second most prominent PTM after phosphorylation and is therefore a major regulator of the asexual-stage biology of the parasite\(^{[6]}\). Certain fluorescence hotspots observed within schizonts via the application of SMS microscopy (Figure 1d) might correlate with the site of one of the various PDHHHC enzymes that were described in late stage parasites and recently investigated with \(Escherichia coli\) bacteria by Wetzel et al. and Yadav et al., respectively\(^{[21,26]}\). Our EM approach further showed the distribution of gold particles, representing palmitoylated proteins, between the parasite and the cytoplasm of the infected erythrocyte (Figure 1e,f). Interestingly, the cytoplasm of erythrocytes infected with ring stage parasites appeared to contain more gold particles than the cytoplasm of erythrocytes infected with trophozoite and schizont stages (Figure 1e). This could be due to an early reorganization of the host erythrocyte and the usage of more S-palmitoylated proteins involved in this process\(^{[13]}\). In the later developmental stages, the reorganization of the host erythrocyte is completed and the sophisticated trafficking network is established\(^{[15]}\). During this developmental
Figure 3. Pulse/chase analysis to examine the behavior of S-palmitoylated proteins within the parasite and the host erythrocyte cytoplasm. Behavior of S-palmitoylated proteins within a) the parasite and b) the erythrocyte cytoplasm of the infected erythrocyte after a 30-min pulse with clickable palmitic acid alkyne. Fluorescence values of clickable palmitic acid alkyne-treated uninfected and infected erythrocytes were normalized to fluorescence values of uninfected or infected erythrocytes treated only with delipidated medium, respectively. A.U., arbitrary units. Number of conducted experiments: 4. Number of cells analyzed per time point, stage and experiment: ≥4. Mean ± SEM. See Statistical Methods section in Supporting Information for p-values.

Treatment of erythrocytes with a 10-min pulse with clickable palmitic acid alkyne followed by a 60-min chase with palmitic acid at 10x molar excess shows the behavior of S-palmitoylated proteins c) within the parasite and d) within the erythrocyte cytoplasm and in comparison, to uninfected erythrocytes. A.U., arbitrary units. Number of conducted experiments: 3. Number of cells analyzed per time point, stage and experiment: ≥9. Mean ± SEM. See Statistical Methods section in Supporting Information for p-values.
phase of the parasite, S-palmitoylated proteins may more important in progeny production and in future erythrocyte infection after merozoite egress, and may thereby be found in abundance within the parasite instead of the ECP.\textsuperscript{[20]}

Differences in the number of gold particles in our EM micrographs (Figure 1e,f), in comparison to the fluorescence signal that we observed in our confocal images (Figure 1c) after a 30-min pulse with clickable palmitic acid alkyne, can be explained by the thickness of the sample. Namely, for our EM approach, we only analyzed a 60-nm section of the parasite–host cell complex, whereas the acquisition of confocal fluorescence images in general achieved a better signal depth, even when only a single focal plane is imaged (500 nm). Another factor may be that the click reaction in the EM approach involves a biotin–streptavidin click, whereas a direct click reaction is performed in the fluorescence approach. We compared the distribution of the gold particles in the EM micrographs (Figure 1f) with the distribution of the fluorescence signal in our confocal images after a 30-min pulse (data not shown). We observed the same pattern with gold particles versus fluorescence signal distribution for all developmental stages, hence indicating that the same conclusions can be drawn from two different experimental approaches.

### 3.2. Visualized S-Palmitoylated Proteins Show Sensitivity to the Treatment with Hydroxylamine and Triacsin C

We further investigated whether the observed Alexa 647 azide fluorescence signal indeed originated from S-palmitoylated proteins. This would be the case if the signal was sensitive to treatment with neutral hydroxylamine (cleaving the lipid analogue off the proteins) and sensitive to Triacsin C (activation of the probe with CoA). We did observe a decrease in fluorescence in both experimental approaches (Figure 2). We also noticed that some of the fluorescence signal remained in the infected erythrocytes after the treatment with hydroxylamine and Triacsin C. Uninfected erythrocytes, on the other hand, did not respond to Triacsin C treatment, but also showed a decrease in fluorescence after the treatment with hydroxylamine. The fact that we did not see the fluorescence disappear completely (Figure 2) could possibly be attributed to the clickable palmitic acid alkyne, which might to some extent contribute to the labeling of other fatty acylated proteins and maybe even lipids, an observation that has been made before.\textsuperscript{[19,35]} However, a significant fraction of the overall labeled proteins in our approach does represent S-acylated proteins.

To further support this result, we conducted an in-gel analysis with erythrocytes infected with trophozoite stages of \textit{P. falciparum} and uninfected erythrocytes as control (Figure S2, Supporting Information). We observed defined protein bands that showed a distinct sensitivity to the treatment with hydroxylamine, which indicates that the observed signal represents S-palmitoylated proteins. Furthermore, we observed that the metabolically reduced host erythrocytes did not show a residual S-palmitoylation activity in this experimental approach (Figure S2a, Supporting Information). However, a noticeable S-palmitoylation occurred after the infection of the erythrocyte (Figure S2b, Supporting Information).

S-Palmitoylation is used extensively to modify the abundance of produced parasite proteins, a process that further promotes successful establishment of the infection. The earliest known use of S-palmitoylation as a PTM in the erythrocytic schizogony of \textit{P. falciparum} occurs when the freshly released merozoites infect a new host erythrocyte.\textsuperscript{[20]} We also observed that the protein S-palmitoylation machinery showed early postinfection activity already in the juvenile ring stages (Figures 1c–f, 2, and 3). This suggests a putative function of S-palmitoylated proteins during the early development of the parasite and the reorganization of the host erythrocyte.

Jones et al. reported that S-palmitoylated proteins are crucial for the development of the parasite and future pathogenesis of merozoites that are formed by late schizont stages.\textsuperscript{[20]} We also saw an excessive production of S-palmitoylated proteins in trophozoite and in schizont stages, the latter stage being the developmental stage producing S-palmitoylated proteins at the highest rate (Figures 1c–f and 3). In parallel, we were able to observe discrete fluorescent dots in the cytoplasm of erythrocytes infected with the different developmental stages of \textit{P. falciparum} (especially the trophozoite and schizont stages) (Figure 1c). This result suggests that the palmitoylated proteins may be located in close proximity to the Maurer’s clefts. This is not surprising because the Maurer’s clefts receive and harbor numerous proteins that were synthesized and trafficked by the parasite. Therefore, this organelle represents the most crucial transport hub involved in the reorganization of the host erythrocyte, cytoadherence of the infected erythrocyte, and general host–parasite interaction.\textsuperscript{[7]}

### 3.3. Pulse/Chase Experiments Suggest Putative Trafficking Behavior of S-Palmitoylated Proteins in \textit{P. falciparum}-Infected Erythrocytes

Recent in silico analyses annotated about one tenth of the 400 identified putative palmitoylated proteins in the mature schizont stages as secreted proteins.\textsuperscript{[6,20]} We therefore conducted pulse and pulse/chase experiments involving all the asexual developmental stages of \textit{P. falciparum} to determine whether S-palmitoylated proteins might be destined for anterograde secretory transport (Figure 3).\textsuperscript{[23]}

The pulse experiments revealed that the fluorescence signal increases within the parasite and the cytoplasm of the infected erythrocyte over time when the erythrocyte is exposed to 50 µM clickable palmitic acid alkyne (Figure 3a,b). We again observed that schizont stages appear to perform the majority of S-palmitoylation reactions followed by trophozoite and ring stages (Figure 3a,b). However, we did also notice a significant but low extent of fluorescence increase within uninfected erythrocytes during our pulse experiments (Figure 3a,b), suggesting that a residual S-palmitoylation activity may be present in the mature erythrocytes. The fact that we did not observe any signal in the uninfected erythrocytes during our in-gel fluorescence analysis (Figure S2a, Supporting Information) could be attributed to the low amount of palmitoylated proteins produced within uninfected erythrocytes and the general lower sensitivity of the method when compared to the confocal imaging approach.

Our pulse/chase experiments revealed that the fluorescence signal within the different developmental stages of the parasite decreased over the 60-min chase time, indicating S-palmitoylated
proteins may either be destined for anterograde transport or for a fast turnover within the parasite (Figure 3c). Interestingly, we did not observe an increase in fluorescence in the ECP during the 60-min chase time (Figure 3d). This would have suggested anterograde trafficking of proteins from the parasite to the host cell in their S-palmitoylated state. Instead, we observed a decrease of fluorescence in the ECP as well, suggesting that no proteins from the parasite were exported into the host erythrocyte in their acylated state. The acylation of these proteins might occur at their final destination within the ECP.

There are two possibilities regarding how this acylation could be conducted by the parasite. It has been postulated by Ruff and colleagues that erythrocytes are able to acylate membrane proteins in vitro.[36] Different studies published in the same decade have further reported activity and isolation of palmitoyl acyl-transferases from mature erythrocytes, whereas more recent studies could not identify DHHC enzyme presence within mature erythrocytes.[37–39] A defined palmitate turnover on a selected subset of EPM proteins has been reported recently, but the enzyme machinery that maintains this turnover remains unidentified to date.[40] The fact that we observed a low, but significant response of uninfected erythrocytes to metabolic labeling with clickable palmitic acid alkyne suggests that the uninfected erythrocytes may have a residual S-acylation activity that could contribute to the acylation of parasitic proteins (Figure 3, see Statistical Methods section in Supporting Information).

It is therefore possible that *P. falciparum* may be able to exploit a currently unidentified palmitoylation machinery that is already present in the host erythrocyte to facilitate the S-palmitoylation of its secreted proteins within the ECP or at the EPM. The other possibility could be a de novo establishment of an S-palmitoylation machinery during the excessive reorganization of the host erythrocyte occurring in infection and progression of development.[41] Exploiting an existing machinery and establishing a parasitic S-palmitoylation machinery in the host erythrocyte might not be mutually exclusive.

The organelle of parasitic origin that seems likely to host this unknown S-palmitoylation machinery may be the Maurer’s clefts.[41,42] The morphology and putative function of this secretory organelle within the ECP appears to resemble the structure and function of the Golgi apparatus. The Golgi represents the center of S-palmitoylation activity in mammalian cells and hosts the majority of DHHC enzymes,[43] and we therefore envisage that outside of the parasitic Golgi, the Maurer’s clefts may be able to harbor PifDHHC enzymes as well.

**4. Conclusions and Outlook**

Click chemistry and metabolic labeling can be applied to investigate S-palmitoylated proteins in erythrocytes infected with the human malaria parasite *P. falciparum*, a model organism that cannot be transiently transfected, but instead needs to be stably transfected to allow the investigation of a few proteins at a time. Our work provides a fast and simple high-throughput approach for the microscopic examination of this crucial group of PTM proteins within infected erythrocytes at any given time during the erythrocytic schizogony of the parasite life cycle.

Our data further suggest that *P. falciparum* may be able to employ S-palmitoylation for different tasks within and beyond its own cellular boundaries. A machinery for PTM might even be established in the host erythrocytes or an already existing S-palmitoylation machinery may be used by the parasite instead.

Future studies should address the potential contribution of other types of fatty acylation and how these modifications are applied in the different developmental stages of *P. falciparum* during the erythrocytic schizogony. This would allow the specific mapping of time and place of protein and even lipid modifications in concert with the effect of defined inhibitor treatment on the developmental progression of the erythrocytic schizogony. Future studies will further lead to the identification and thorough classification of the S-palmitoylated proteome as a function of the developmental stage of the parasite. Such approaches may even hold the potential to localize and to identify putative S-palmitoylation machinery components as novel drug targets to further dissect the infection biology of *P. falciparum*.

**Supporting Information**

Supporting Information is available from the Wiley Online Library or from the author.

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**Conflict of Interest**

The authors declare no conflict of interest.

**Keywords**

click chemistry/metabolic labeling, in-gel fluorescence, malaria, microscopy, *Plasmodium falciparum*, S-palmitoylation

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