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Amplification of a FRET Probe by Lipid–Water Partition for the Detection of Acid Sphingomyelinase in Live Cells

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Abstract: *Real-time monitoring of acid sphingomyelinase (ASM) activity is crucial for investigating its role in lipid-mediated signaling processes. In this study, we synthesized fluorescent phosphosphingolipids capable of FRET by phosphorodichloridate chemistry. These sphingomyelin analogues are substrates for recombinant human ASM and can be used to monitor ASM activity by fluorescence spectroscopy. Incubation with cell lysates from wild-type and knock-out mice further confirmed probe cleavage to be exclusive to ASM. We also systematically exploited the environmental sensitivity of the fluorophores to achieve significant increases in responsiveness. This concept may be transferred to other lipid probes in the future. The ASM activity in live cells was imaged by two-photon-excitation microscopy.*

Monitoring chemical events in complex matrices, such as cells, with high spatial and temporal resolution remains a key challenge for biomolecular research.[1] In particular, reactions of membrane-bound substrates elude characterization in homogenous solution because they require a complex cellular environment that cannot be fully mimicked in a test tube. The analysis of reactions occurring on membranes by ex situ techniques (HPLC, MS) is limited by the loss of crucial topological and temporal information. Non-destructive spectroscopic methods based on fluorescence, such as Förster resonance energy transfer (FRET), have been developed to address these issues.[2] Fluorescence spectroscopy is very sensitive and can be used to monitor processes in cells in real time over several hours as the cells are sufficiently stable under the measurement conditions. FRET is frequently used to determine the proximity and orientation of two fluorophores, such as a fluorescent fusion protein and a ligand. Unfortunately, the bulkiness of fluorescent proteins permits only small ratiometric changes. In contrast, small-molecule FRET probes possess shorter inter-fluorophore distances owing to increased FRET efficiency and response. The main advantage of FRET probes over simple turn-on or turn-off probes lies in the possibility of ratio-imaging, enabling the simultaneous quantification of cleaved and non-cleaved probes. To date, only few enzyme-probing systems have been reported, mainly because their synthesis requires expertise that most bioimaging facilities do not provide.[1] Most examples published thus far deal with the detection of small analytes (e.g., sulfides, metal ions)[3] despite the fact that these small molecules can be tailored to meet the requirements of considerably more complex objects of investigation. Given the clear need for a biochemical system to investigate acid sphingomyelinase (ASM) activity in vivo, we decided to design a tailor-made small-molecule ASM FRET probe.

Acid sphingomyelinase (ASM), which is present in all eukaryotes, is a soluble lipid phosphodiesterase essential for lipid homeostasis.[4] Inherited lack of ASM activity leads to the Niemann–Pick disease, which causes severe health issues and leads to early death.[5] Furthermore, ASM is involved in lipid-mediated signaling processes.[6] Upon activation by different stimuli such as cytokines, irradiation, or serum deprivation, ASM generates antiproliferative ceramides from the membrane constituent sphingomyelin (Figure 1 A), and

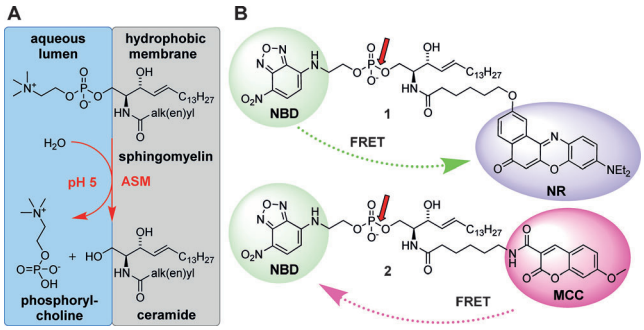

Figure 1. A) Sphingomyelin hydrolysis at the lipid–water interface catalyzed by ASM. B) Synthesized ASM FRET probe **1** (NBD/NR) and probe **2** (MCC/NBD). The red arrow indicates the ASM hydrolysis site.

has hence been attributed a crucial role in cell fate. The enzyme resides primarily in lysosomes but can be secreted extracellularly following stimulation. Indeed, 200-fold ASM enrichment was observed in the urine of peritonitis patients more than two decades ago by Sandhoff and co-workers.[7] More recent data strongly emphasize the role of ASM in a variety of diseases ranging from acute lung injury (ALI),[8] cancer,[9] and tumor metastasis[10] to severe depression.[11] Notably, the majority of reports suggest different physiological functions for the lysosomal and secreted fractions of the enzyme.[12] Most disease-related functions of ASM have been attributed to its secreted form, which may act to remodel the outer leaflet of the plasma membrane. However, a conclusive

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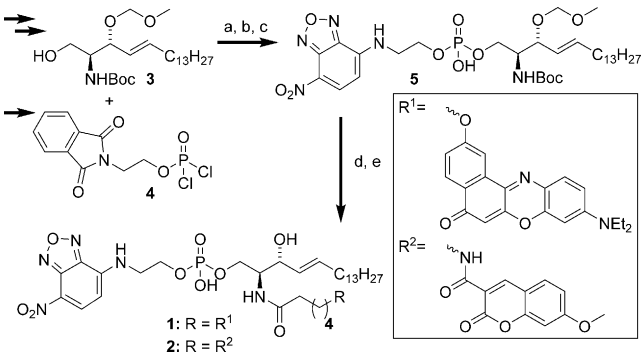
model of how the enzyme could be active at the neutral pH of the cell surface has not been reported.[13] A more recent report connects lysosomal ASM to the mediation of lysosomal stability and thus the survival of cancer cells.[9,14] In the past, we have developed several types of inhibitors with the aim to clarify the role of ASM in biology and human diseases.[15] Very recently, the X-ray structure of the enzyme in complex with one of our inhibitors has been published, enabling rational drug design.[16] Consequently, screening and evaluation of newly synthesized potential ASM inhibitors will soon demand for an efficient (in vivo) assay. Herein, we have developed FRET probes that enable the in situ non-destructive detection of enzymatic cleavage by fluorescence imaging.

Encouraged by previously developed lipid probes, where the combination of Nile Red (NR) and nitrobenzoxadiazole (NBD) was shown to provide efficient FRET in a lipid environment,[17] we designed a first sphingomyelin analogue with these two fluorophores (Figure 1B). NBD is commonly used as a covalent label for lipids despite the fact that it can cause loop formation with acyl chains in phospholipid membranes owing to its significant polarity.[18] To mimic natural sphingomyelin in terms of its polarity, conformation, and steric bulk, small and polar NBD was chosen for head group labeling,[19] and the less polar NR moiety was used to label the acyl chain of the envisioned sphingomyelin analogue. The synthesis consisted of three steps: 1) stereoselective synthesis of the sphingosine core, 2) asymmetric phosphodiester formation, and 3) attachment of the fluorophores.

Starting from L-serine, a decorated sphingosine **3** with natural stereochemistry was synthesized in eight steps in analogy to previous reports.[20] The subsequent introduction of the aminoethyl phosphate moiety proved to be challenging as the use of hydrogen phosphonates and phosphoramidites as well as phosphorylation with $POCl_3$ seemed to be incompatible with the present functional groups. However, upon employing the reactive phosphorodichloridate reagent **4**,[21] the desired unsymmetric phosphodiester **5** was isolated in good yield (Scheme 1). This inexpensive reagent has received little attention since its development,[21] but proved to be an efficient precursor for phosphosphingolipid synthesis in our hands. The synthesis was completed by hydrazinolysis of the phthalimide and by nucleophilic aromatic substitution of NBD−Cl with the liberated primary amine. In parallel, a hexanoic acid bearing an NR (R^1) substituent was obtained following a procedure reported previously.[17b,22] Alternatively, 6-aminohexanoic acid was coupled to 7-methoxycoumarin-3-carboxylate (MCC, R^2) using a commercially available MCC active ester. The two acids were then activated as *N*-hydroxysuccinimide (HOSu) esters under Steglich conditions. Following global deprotection of **5**, the two labeled acids were coupled to the NBD-substituted phosphosphingosine to furnish probes **1** and **2** (Scheme 1). Using similar methods, the respective monolabeled ceramides (NR-Cer and MCC-Cer), which represent the expected ASM cleavage products from probes **1** and **2**, were synthesized, too (see the Supporting Information for details).

Fluorometric characterization of probe **1** containing the NBD/NR FRET pair was performed in aqueous buffer after the probe had been embedded into Triton X-100 micelles. As



Scheme 1. Synthesis of ASM probes. a) 1) Pyridine, CH_2Cl_2, 0°C→RT, overnight, 2) aq. $NaHCO_3$, RT, 30 min, 94%; b) $H_4N_2{\cdot}H_2O$, MeOH, 96%; c) NBDCl, DIPEA, MeOH, RT, 150 min; d) HCl, MeOH, 70°C, 3 h, quant.; e) $R(CH_2)_5COOSu$, DIPEA, CH_2Cl_2, 0°C→RT, overnight, 34–80%. Boc = *tert*-butoxycarbonyl, DIPEA = diisopropylethylamine, NBD = 7-nitrobenzo-2-oxa-1,3-diazol-4-yl, Su = *N*-succinimidyl.

expected, the probe exhibited exclusively acceptor (NR) fluorescence at 624 nm upon excitation of the donor dye (NBD) at 430 nm while the absence of donor (NBD) emission suggested the probe to have high FRET efficiency. Subsequently, recombinant human ASM was added to the micellar solution of the probe, and spectra were recorded after different time intervals. We expected that the emission of the acceptor (NR) would decrease owing to enzymatic cleavage of the FRET pair, with a concomitant increase in the fluorescence of the donor dye (NBD). Indeed, upon incubation with ASM, we measured a rapid decrease in acceptor fluorescence. However, we did not observe the anticipated rise in donor (NBD) fluorescence. Further characterization of the expected cleavage product, NBD-substituted aminoethyl phosphate, revealed its weak fluorescence in aqueous environments. In contrast, the NBD fluorescence of the intact probe was much stronger when embedded in more hydrophobic micelles. Therefore, we concluded that the expected increase in donor (NBD) fluorescence upon cleavage of the probe was not observed owing to quenching of the polar NBD in aqueous solution upon cleavage and liberation into the aqueous environment. Ultimately, a ratio change (RC) of 2.6 was determined as the responsiveness of the probe, a moderate result when compared to other lipid FRET probes.[17a]

Next, we wondered whether we could exploit the phenomena described above. The inherently disadvantageous quenching of the donor upon cleavage might be turned into an asset if the NBD moiety was turned into the FRET acceptor. Based on recent publications,[23] 7-methoxycoumarin-3-carboxylate (MCC) was chosen as a suitable FRET donor for NBD, and incorporated into the fatty acid as a replacement of the NR fatty acid. In contrast with the first probe, the MCC-containing probe **2** indeed exhibited a superior performance upon incubation with recombinant human ASM. A pronounced decrease (−87%) in the acceptor fluorescence was accompanied by the appearance of intense donor fluorescence (+900%), leading to an 80-fold ratio change (Figure 2A). Organic extracts[24] of the reaction mixture were analyzed by TLC and UPLC-MS and further

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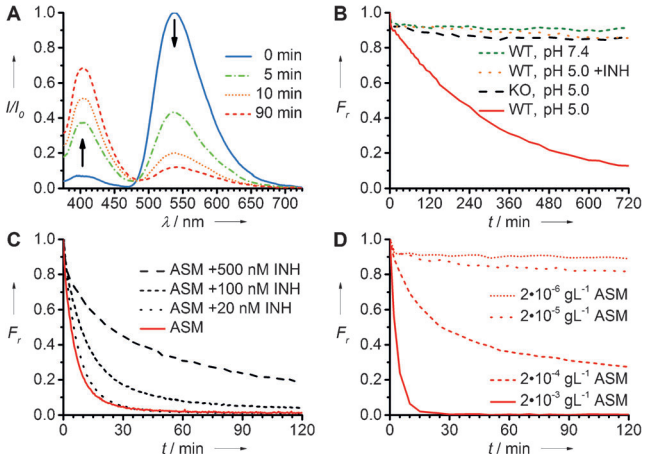

Figure 2. A) Normalized fluorescence change of probe **2** upon incubation with recombinant ASM. B) FRET decrease of probe **2** upon incubation with MEF lysates (100 μg mL^{-1}) under ASM (pH 5.0) or NSM conditions (pH 7.4) at 37 °C. C) FRET decrease of probe **2** upon incubation with recombinant ASM at different inhibitor concentrations. D) FRET decrease of probe **2** upon incubation with different amounts of recombinant ASM. λ_{ex} = 347 nm, λ_{em} = 536 nm. F_r = residual FRET, INH = ASM inhibitor 1-*O*-hexadecylsulfonyl-*myo*-inositol-3,5-bisphosphate.[15b].

confirmed the complete cleavage of the probe, giving material identical to synthetic MCC-Cer (see the Supporting Information, Figures S4–S7). Using the latter compound as a donor-only reference, the FRET efficiency of the intact probe **2** was calculated to be 90 % (Figure S2). Probe **2** indeed turned out to be a biochemical tool well suited for monitoring ASM activity.

Next, we wanted to assess the specificity of the probe for ASM over other enzymes present in cell lysates. The probe was readily cleaved by crude HeLa cell lysates at pH 5 whereas under neutral conditions, hardly any hydrolysis occurred (Figures S3 and S5). This suggests that other enzymes such as the neutral sphingomyelinases are either not able to cleave the probe or that the respective activities in the cell lysates are comparatively low. To rule out probe cleavage at pH 5 by enzymes other than ASM, probe **2** was incubated with lysates from mouse embryonic fibroblasts (MEFs). Whereas lysates from wild-type (WT) fibroblasts promoted efficient cleavage of the probe, hardly any signal change was observed when lysates from ASM knock-out (KO) cells were used (Figures 2 B and S4). The same result was obtained when an inositol-based ASM inhibitor[15b] was added to the WT lysates (Figures 2 B, C and S4). With this probe, relative ASM activities for different ASM concentrations as well as for different ASM inhibitor concentrations could be determined (Figure 2 C, D). These results suggest that even in complex matrices such as cell lysates, the responsiveness of the probe can be mainly or even fully attributed to the activity of ASM. This feature enables facile assessment of ASM activity in cell lysates of various cell types and may prove useful for screening compound libraries for potential ASM inhibitors.

Encouraged by these findings, we applied probe **2** to monitor ASM activity in live cells. Owing to the MCC excitation optimum of 355 nm, we chose two-photon excitation (2PE) for non-destructive bioimaging. Thus we incubated probe **2** with intact unstimulated WT or KO MEFs, respectively. Irradiation at 488 nm or 730 nm ($2\lambda_{ex,MCC}$) yielded NBD acceptor fluorescence. This indicates efficient FRET from MCC to NBD in the intact probe also under cell-culture conditions, as further confirmed by photobleaching experiments. Upon probe incubation, the KO cells showed increased lysosomal accumulation of intact probe as confirmed by LysoTracker colocalization experiments (Figures S9 and S10). In the WT cells, such an accumulation did not occur, and they showed continuously decreasing NBD fluorescence owing to hydrolysis and concomitant quenching of the FRET acceptor. A simultaneous rise in MCC fluorescence further indicates probe cleavage in live cells, as previously observed with cell lysates (Figure S11). Remarkably, unlike in the KO cell lysates, some probe cleavage was also detectable in live KO cells, which was confirmed by detection of MCC-Cer by HPLC analysis. This suggests that one of the neutral sphingomyelinases, for example, was able to cleave the probe in intact cells. This obvious difference to cell lysates further highlights the need for in situ measurements.

After using the probe for the qualitative detection of ASM, we wanted to test whether the probe was also useful to discriminate between cells with different ASM expression levels. Towards this end, overexpressing (OE) cells were obtained by transient lipofection of WT cells with an ASM-encoding plasmid 20 h before treatment with probe **2**. As shown in Figure 3, OE cells have a significantly different appearance compared with the WT cells, especially 1 h and 6 h after probe loading, while this difference appears to be lower after 24 h and is almost absent after 48 h. The markedly increased MCC fluorescence (magenta) in OE cells suggests increased probe cleavage compared to the WT cells, also leading to increased colocalization of MCC and NBD fluorescence (white). In parallel to these experiments, WT and OE cells were incubated with probe **2** for the indicated

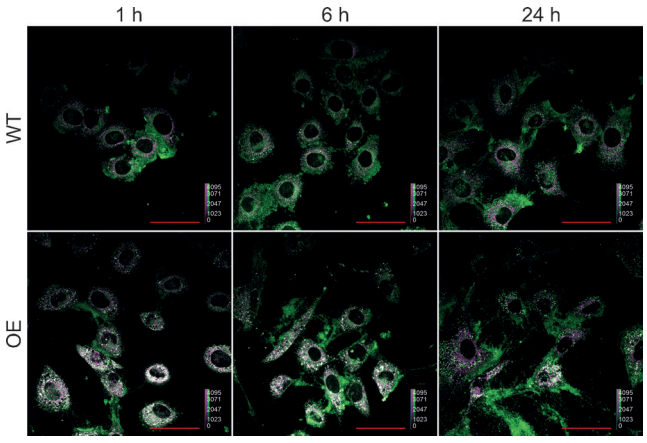

Figure 3. Microscopy image overlays of WT (top) and OE (bottom) MEF cells incubated with 1 μM **2** for the indicated periods of time showing NBD (green) and MCC fluorescence (magenta) after two-photon excitation (730 nm). White pixels indicate colocalization. All scales are equal. Red scale bar: 50 μm.

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periods of time, and the lipids were extracted. HPLC analysis of the extracts confirmed a higher probe turnover in OE cells, corroborating the hypothesis that the different appearance of the cells was due to higher ASM activity in the OE cells (Figure S8).

Moreover, the HPLC analysis revealed that even after 48 h, only an estimated 80% of the incorporated probe had been cleaved. Such a slow probe turnover is not surprising as these values represent the constitutive activity of ASM, and even appears favorable as it should allow for the observation of stimulated ASM activity even in the presence of constitutive lysosomal sphingomyelin degradation. To test this hypothesis, we treated WT cells with a second portion of probe **2** 24 h after the first treatment. Another 30 min later, sphingomyelinase (0.5 U mL^{-1}) from *Staphylococcus aureus* (bacSM) was added, which is a common experiment to mimic the activity of the secreted form of ASM.[25] Indeed, after 20 h, no NBD fluorescence was observed while the MCC fluorescence dramatically increased. The virtually equal appearance of cells treated with synthetic MCC-Cer confirmed that bacSM furnished complete probe turnover (Figure S12). The relevance of this exogenous sphingomyelinase treatment was recently highlighted by a report that melanoma cells stimulate platelets to secrete ASM, which in turn triggers rapid sphingomyelin turnover in melanoma cells, an event essential for their metastasis into the lung and other tissues in mouse models.[10]

In conclusion, we have described the synthesis and application of a novel small-molecule FRET-based probe to measure ASM activity in micelles, liposomes, and live cells. With a high FRET efficiency of 90% and an 80-fold ratio change upon complete conversion, the performance of probe **2** is, to the best of our knowledge, unprecedented for enzyme-probing FRET probes. Comparison of probe **1** and **2** reveals that this feature is not only due to simple FRET turn-off, but also a result of our efforts to reduce FRET channel background by exploiting the sensitivity of the dye to changes in its microenvironment. We believe that this principle may provide a general concept to ameliorate signal-to-noise ratios and thus the performance of related FRET probes in vitro and in vivo.

Probe **2** can be imaged with two-photon excitation microscopy with good spectral discrimination of donor and acceptor fluorescence. With this compound in hands, we want to further elucidate the triggers of ASM secretion and its downstream events. As two-photon excitation usually provides deep-tissue penetration, we intend to study ASM activity in inflamed tissues in the future. Notably, ASM KO cells mimic the phenotype of Niemann–Pick patient cells. Thus probe **2** may be applied to detect a lack of functional ASM in patient biopsies in the future.

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Conflict of interest

The authors declare no conflict of interest.

Keywords: enzymes · fluorescence spectroscopy · FRET probes · membranes · sphingolipids

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Communications

Bioanalytical Chemistry

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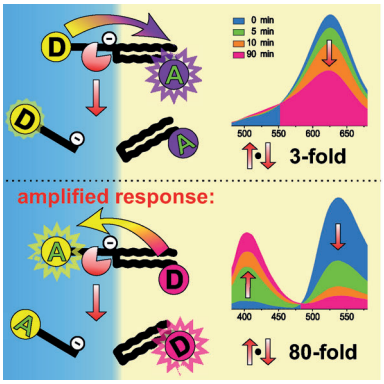
Amplification of a FRET Probe by Lipid–Water Partition for the Detection of Acid Sphingomyelinase in Live Cells



A FRET probe for acid sphingomyelinase was designed. Whereas a first probe suffered from quenching by the environment (top), a second-generation probe made use of the same phenomenon to suppress background fluorescence (bottom). The probe was used to detect the activity of acid sphingomyelinase in vitro as well as in live cells by two-photon-excitation microscopy.

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