Activity-Based Protein Profiling

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Total Chemical Synthesis of SUMO and SUMO-Based Probes for Profiling the Activity of SUMO-Specific Proteases

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Abstract: *SUMO is a post-translational modifier critical for cell cycle progression and genome stability that plays a role in tumorigenesis, thus rendering SUMO-specific enzymes potential pharmacological targets. However, the systematic generation of tools for the activity profiling of SUMO-specific enzymes has proven challenging. We developed a diversifiable synthetic platform for SUMO-based probes by using a direct linear synthesis method, which permits N- and C-terminal labelling to incorporate dyes and reactive warheads, respectively. In this manner, activity-based probes (ABPs) for SUMO-1, SUMO-2, and SUMO-3-specific proteases were generated and validated in cells using gel-based assays and confocal microscopy. We further expanded our toolbox with the synthesis of a K11-linked diSUMO-2 probe to study the proteolytic cleavage of SUMO chains. Together, these ABPs demonstrate the versatility and specificity of our synthetic SUMO platform for in vitro and in vivo characterization of the SUMO protease family.*

Post-translational modifications (PTMs) are reversible chemical additions to a protein following translation. Phosphorylation and ubiquitination are prominent examples of important and well-studied PTMs that regulate a plethora of cellular processes.[1] Similarly, SUMOylation is another PTM that is known to apply to large number of proteins.[1,2] Despite the name small ubiquitin-like modifier (SUMO), ubiquitin (Ub) and SUMO share less than 20% amino acid sequence identity, but they possess comparable three dimensional structures.[3] Unlike Ub (8 kDa), SUMO proteins have an N-terminal flexible extension, which makes them significantly larger (11 kDa). SUMOylation is directed by an enzymatic cascade analogous to ubiquitination and SUMO is generally conjugated onto lysine residues of target proteins.[4] SUMO has 3 active isoforms, SUMO-1, -2 and -3, with mature SUMO-2 and -3 being virtually identical.[5] Importantly, SUMO-1–3 themselves can be further modified by additional PTMs, including acetylation, phosphorylation, methylation, ubiquitination, or even SUMOylation, with the latter resulting in polySUMO chains.[6] SUMOylation can then be removed by specific SUMO proteases, namely the cysteine proteases USP-L1, the Sentrin-specific proteases (SENP1, SENP2, SENP3, SENP5, SENP6, SENP7), and Desi-1 and Desi-2, thus rendering this PTM reversible and dynamic. SUMO proteases also mediate the maturation of SUMO precursors.[7]

SUMO is mainly found in the nucleus and plays indispensable roles in the DNA damage response, gene expression, and cell-cycle progression. It has been established that many tumours rely on a functional SUMO pathway, which makes SUMO-related processes potential anti-cancer drug targets.[8] However, the molecular mechanisms underlying the role of SUMO modification in these processes remain poorly understood, in part due to the slow development of activity-based reagents.[9] In contrast, research in the ubiquitin field has accelerated due to the convenient chemical synthesis of ubiquitin,[10] which has given access to assay reagents and probes[11–14] Therefore, a reliable route towards SUMO-based conjugates is urgently needed to provide the reagents required to visualize and understand the biology of reversible SUMOylation.

SUMO conjugates have been prepared before by semi-synthesis using intein chemistry[15] or by synthesis and ligation of peptide fragments.[16–19] In this report, we present a fully synthetic linear solid-phase peptide synthesis (SPPS)

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approach towards full-length SUMO conjugates, which enables facile parallel synthesis procedures. Since SUMO is substantially larger than ubiquitin (SUMO-1 96 AA versus ubiquitin 76 AA), it was considered to be beyond the limits of linear SPPS. However, we explored the use of "aggregation breakers" such as pseudoproline[20] and dimethoxybenzyl (DMB)[21] residues at strategic positions and devised a synthetic route that takes advantage of the specific building blocks depicted in Figure 1 A. This procedure allowed us to make synthetic SUMO-1, -2, and -3 efficiently in a linear fashion. Next, we used the linear SUMO synthesis to prepare N-terminally fluorescently labelled SUMO-propargyl probes (Figure 2) for monitoring SUMO-specific protease activities in cell-based assays and visualizing their action in live cells by fluorescence microscopy (Figures 3 and 4). Our parallel methodology not only allowed us to make synthetic SUMO and SUMO-based probes, but also enabled the synthesis of a diSUMO covalent probe (Figure 2).

In approaching the challenge of a fully synthetic approach, we identified two positions in the sequence of SUMO-1 and five positions in the sequences of SUMO-2 and -3 where dipeptide pseudoproline and DMB building blocks could be incorporated (Figure 1 A). Using conventional

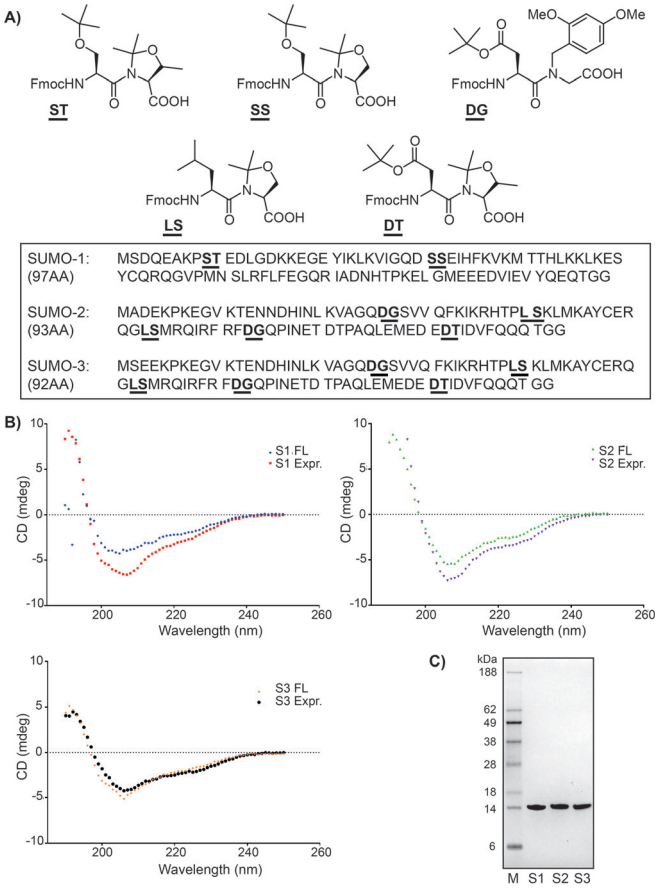


Figure 1. A) SUMO sequences and the position of the dipeptides (pseudoprolines and dimethoxybenzyl) used during SUMO synthesis. B) CD spectra of expressed SUMO proteins (Expr.) versus synthetic SUMO proteins (FL). C) SDS-PAGE analysis of synthetic SUMO polypeptides. M = marker, S1 = SUMO-1, S2 = SUMO-2, S3 = SUMO-3.

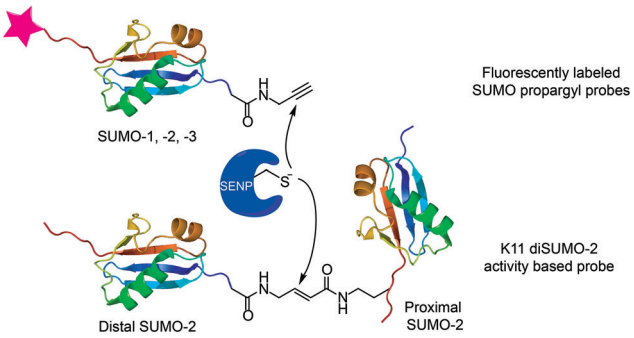


Figure 2. Design of the SUMO-propargyl and covalent diSUMO-2 activity-based probes for studying proteolytic cleavage, preference, and selectivity by capturing active SENPs.

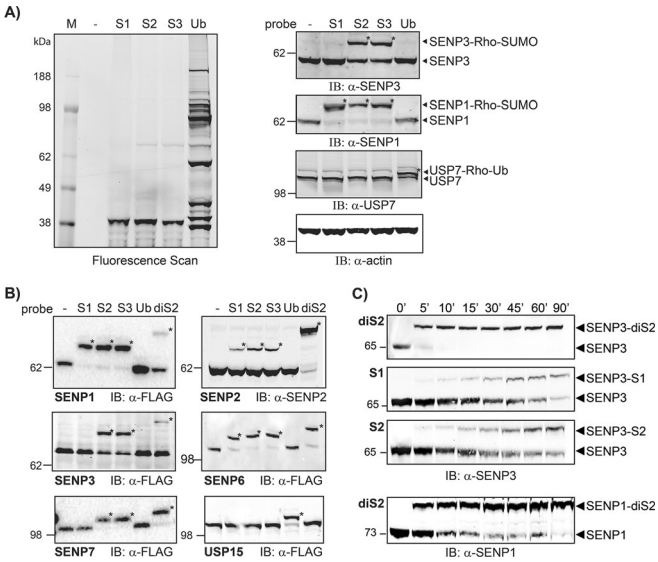


Figure 3. Activity-based profiling with SUMO ABPs. A) Labelling of endogenous enzymes in HeLa cell lysates. Fluorescence scan and immunoblot analyses for endogenous SENP1, SENP3, and USP7 are shown; B) Labelling of ectopically expressed FLAG-SENPs 1–7 and FLAG-USP15 (negative control) in HeLa cell lysates, visualized by immunoblot. For fluorescence scans see Figure S3. C) Time-dependent labelling of endogenous SENP3 and SENP1. For blots of other SENPs see Figure S7. M = marker, S1 = Rho-SUMO-1-PA, S2 = Rho-SUMO-2-PA, S3 = Rho-SUMO-3-PA, Ub = Rho-Ub-PA (negative control), diS2 = K11 diSUMO-2-VA. Asterisks (*) indicate probe-labelled enzymes.

Fmoc-based SPPS coupling conditions (4 equiv Fmoc protected amino acid, 4 equiv PyBOP, and 8 equiv DIPEA) and double couplings, simultaneous incorporation of selected building blocks led to successful synthesis of SUMO-1, -2, and -3 in high purity. Correct folding of purified synthetic SUMO proteins was verified by circular dichroism (CD) spectroscopy (Figure 1 B).

With a productive synthesis approach in hand, we focussed on the generation of SUMO-based ABPs applicable to profiling SUMO-specific proteases and visualizing proteolytic activity in cells (Figure 2). For this purpose, we synthesized SUMOΔG proteins on a hyper-acid-labile chlorotrityl

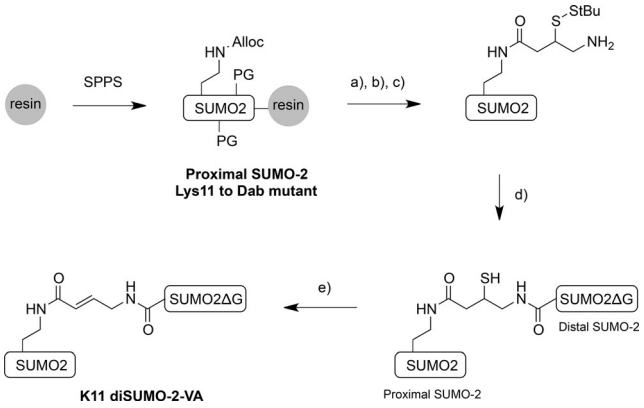
resin (Scheme S1 in the Supporting Information) and functionalized their N-termini with N,N′-Boc-protected 5-carboxyrhodamine.[22] Upon cleavage of the protected peptide from the resin with hexafluoroisopropanol (HFIP), the propargylamide (PA) electrophile[15,23] was introduced by selective coupling of propargylamine to the C-termini of SUMO using standard coupling procedures. After global deprotection and HPLC purification, the desired Rho-SUMO-PA probes were obtained with an average overall yield of 10%.

To validate reactivity and specificity of our SUMO-based probes, purified catalytic domains of SENP1 and SENP6 were incubated for 30 min at RT with Rho-SUMO-PA or Rho-Ub-PA probes (Figure S2). SDS-PAGE analysis (Figure S2) showed SENP1 reactivity toward probes of all three SUMO isoforms, while SENP6 showed a clear preference for SUMO-2/3, thus demonstrating the ability of these probes to selectively react with active SENPs.

With a set of functional SUMO-based ABPs in hand, we tested their binding for all known SUMO proteases in cell lysates (Figure 3). Labelling of endogenous enzymes (Figure 3A) in HeLa cell lysates showed low expression of endogenous SENP enzymes. Although reactivity for SENP1 and SENP3 could be visualized by immunoblotting (Figure 3A), we choose to transfect HeLa cells with plasmid cDNAs for full-length, FLAG-tagged human SENP1-7 to access the reactivity of the probes towards the full SENP panel. Whole-cell lysates of transfected cells were incubated with the Rho-SUMO-PA probes for 15 min at RT. Fluorescent scan and western blot analysis (Figures 3B and S3) showed SUMO-bound SENP1 and SENP2 for all three SUMO isoforms and a small preference of SENP6 for SUMO-2/3 (longer exposure Figure S4), while SENP3 and SENP7 showed a clear preference for SUMO-2/3 probes.

Next, we investigated whether we could generate a diSUMO probe. Di-SUMO is known to be the natural substrate for the two SUMO-chain-processing human SUMO protease family members SENP6 and SENP7.[24] This new type of reagent would allow us to study proteolytic cleavage of SUMO chains.

In contrast to ubiquitin chains, where linkages through all seven lysine residues have been observed, one type of SUMO chain—linked through lysine 11 of SUMO-2/3—appears to predominate.[4] Since SUMO-2/3 show a high degree of sequence similarity, we chose to focus on a K11 diSUMO-2 vinylamide (VA) suicide version that can bind covalently. To achieve this, we chemically mutated lysine K11 in the proximal SUMO-2 to a diaminobutyric acid residue (Dab) to preserve the length of the native isopeptide linkage. A SUMO-2 K11Dab(Alloc) mutant (Scheme 1) was synthesized using our linear Fmoc-based SPPS method, and subsequent selective deprotection of the Dab(Alloc) residue was achieved through treatment with $Pd(PPh_3)_4$ and Ph_3SiH, followed by on-resin coupling of a previously reported ligation handle.[25] Global deprotection and purification with HPLC gave the desired proximal SUMO-2 K11 mutant. Then, SUMO-2-Δ*G*-SEt thioester, corresponding to the distal part of our probe design, was synthesized in a similar fashion by Fmoc SPPS (see the Supporting Information). Native chemical ligation of the (proximal) SUMO mutant and (distal)



Scheme 1. Synthesis of K11 diSUMO-2-VA. Reagents and conditions: a) Ph_3SiH, $Pd(PPh_3)_4$, NMP; b) 4-((tert-butoxycarbonyl)amino)-3-(tert-butyl disulfaneyl)butanoic acid, PyBOP, DIPEA, NMP; c) TFA/iPr_3SiH/phenol/H_2O (90:2.5:2.5:5), 3 h, RT; d) 6 M Gnd·HCl, 0.15 M sodium phosphate buffer pH 7, 250 mM MPAA, SUMO2ΔGSEt, overnight; e) 50 mM sodium phosphate buffer pH 8, 2,5-dibromohexanediamide, 37 °C, overnight. NMP = *N*-methylpyrrolidon, PyBOP = (benzotriazol-1-yl-oxy)tripyrrolidinophosphonium hexafluorophosphate, DIPEA = di-isopropylethylamine, TFA = trifluoroacetic acid, Gnd = Guanidine.

SUMO-2-Δ*G*-SEt thioester was performed under denaturing conditions in 6 M Gdn·HCl, 0.15 M sodium phosphate (pH 7.2) with mercaptophenylacetic acid (MPAA, 250 mM) as a ligation catalyst. Overnight incubation at 37 °C, monitored by LC–MS, resulted in full consumption of the proximal SUMO mutant and formation of the ligation product as an MPAA disulfide. A short incubation with TCEP, followed by preparative HPLC, afforded the K11 diSUMO-2 precursor. Final thiol elimination of the warhead was achieved by overnight incubation of the K11 diSUMO-2 precursor with 100 equiv of 2,5-dibromohexanediamide at 37 °C in 50 mM sodium phosphate buffer (pH 8) to yield K11 diSUMO-2-VA (Scheme 1, Figure S5).

To validate the reactivity of K11 diSUMO-2-VA, purified catalytic domains of SENP1-8 were incubated for 1 h at 37 °C with the K11 diSUMO-2 probe. As expected, SDS-PAGE analysis showed diSUMO-reacted bands for all SENPs except SENP8, which is reported to be a NEDD8-specific protease[26] (Figure S6). Next, we tested the reactivity of the probe towards known SUMO proteases in cell lysates. Both endogenous and ectopically expressed SENPs readily and specifically reacted with our mono- and di-SUMO ABPs, but not with Ub-PA (Figure 3, Figure S3, S7) or mono-K11 γ-amino-VA-SUMO-2 control probes (Figure S8). Conversely, the deubiquitinating enzymes (DUBs) USP7 and USP15, both active against Ub-PA, displayed no reactivity against any of the SUMO ABPs (Figure 3A and B). Interestingly, labelling of endogenous SENPs with our K11 diSUMO-2-VA (Figures 3C and S7) revealed for the first time that SENP3 possesses a preference for diSUMO-2. SUMO-2 chain-processing capacity has been previously demonstrated for SENP1,[27] SENP2, SENP6, and SENP7.[28] Unlike SENP1, SENP3 appears to prefer diSUMO-2-VA to SUMO-1/2-PA (Figure 3C and Figure S7), thus illustrating the potential of diverse SUMO ABP reagents for comprehensively interrogating the cellular machinery of SUMO deconjugation.

Subsequently, we used the fluorescent SUMO-PA reagents to detect relevant enzymatic activities occurring inside the cell. Fluorescent Rho-SUMO-2-PA, introduced into HeLa cells by electroporation according to previously devised methods,[29] was detected throughout the cell, but it was concentrated in the nucleoplasm (N), nucleolus (NC), and nuclear puncta (NP), likely corresponding to PML bodies[30] (Figure 4A). To evaluate whether specific cellular

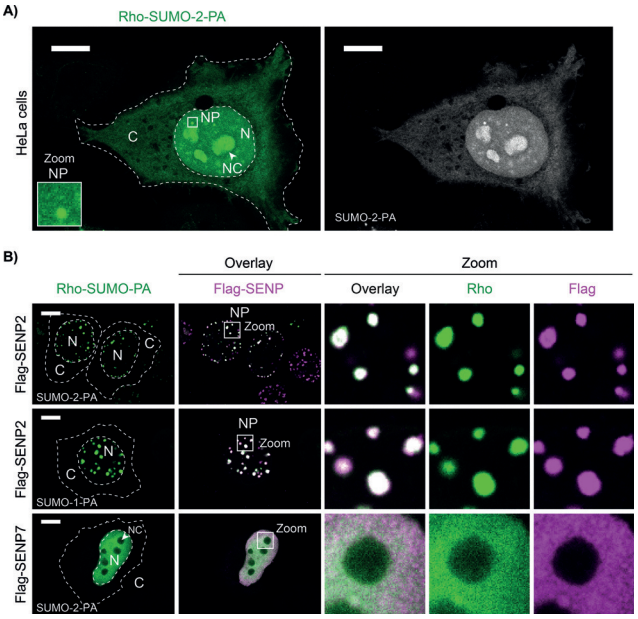


Figure 4. Fluorescence micrographs of HeLa cells electroporated with Rho-SUMO-PA probes (green) in the absence (A) or presence (B) of ectopically overexpressed Flag-tagged SENP enzymes as indicated. Cells were fixed 2.5 h after electroporation, stained with an anti-Flag antibody (magenta), and imaged by confocal microscopy. Cell boundaries and nuclei are demarcated with dashed lines. N = Nucleoplasm, C = Cytosol, NC = Nucleolus, NP = nuclear puncta. Scale bars: 10 μm. Quantification of the concentration of Rho-SUMO probes in cells after electroporation can be found in Figure S9.

SENP activities could be visualized using this reagent, we observed changes in the distribution of Rho-SUMO-2-PA in response to ectopic overexpression of Flag-tagged SENP enzymes (Figure 4B). Strikingly, in cells overexpressing Flag-SENP2, Rho-SUMO-2-PA accumulated predominantly at Flag-positive nuclear puncta (Figure 4B, top panels) and was largely undetectable in either cytosol or nucleoli, thus suggesting that the overexpressed Flag-SENP2 bound and sequestered the probe. In agreement with gel-based activity assays in Figure 3, the same observation was made for Rho-SUMO-1-PA (Figure 4B, middle panels). By contrast, in cells overexpressing Flag-SENP7, Rho-SUMO-2-PA was distributed throughout the nucleoplasm (but not nucleoli), mirroring the dispersed nuclear localization of this enzyme (Figure 4B, bottom panels). Collectively, these experiments demonstrate that the cellular distribution of our fluorescent SUMO probes is responsive to modulation of cellular SENP activities and can thus be used to interrogate SUMO protease activity in cellular contexts.

Taken together, these experiments highlight a variety of assays utilizing our SUMO ABPs for the interrogation of SENP activities both in vitro and in their cellular context. The straightforward nature of the experimental setups is expected to make them readily adaptable to comparative profiling of SUMO protease activity as a function of various biological or chemical perturbations, such as starvation, infection, or treatment with small-molecule inhibitors. The responsiveness of our SUMO ABP tools to changes in SENP activities as a result of heat shock[31] is illustrated in Figure S10.

In conclusion, we present a direct linear chemical synthesis of SUMO proteins for the fast and scalable production of mutants and conjugates with full control over the incorporation of (unnatural) building blocks that enables virtually unlimited structural modifications. In contrast to extensive studies on ubiquitin chains, SUMO chains remain relatively unexplored. The strategies presented here expand the SUMO toolbox and open novel avenues for interrogation of the SUMO-specific deconjugation machinery as well as drug discovery efforts aimed at the development of SENP inhibitors.[32]

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Conflict of interest

The authors declare no conflict of interest.

Keywords: activity-based protein profiling · post-translational modifications · proteolysis · solid-phase peptide synthesis · SUMO

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