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A (poly)Pro tip for preserving native disulfide connectivity during thiol-maleimide bioconjugation of disulfide-rich peptides

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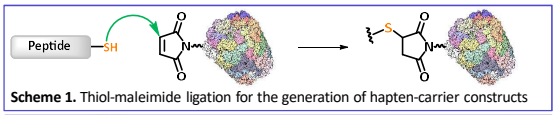
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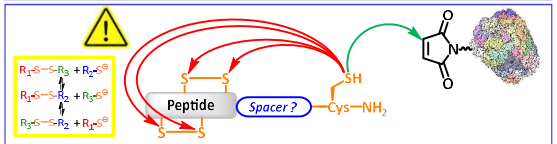
Background and project's aim

Generating specific antibodies against weakly immunogenic peptides requires their covalent conjugation to large protein carriers, such as keyhole limpet hemocyanin (KLH). This "hapten-carrier" approach exploits the carrier protein in two ways: (i) it provides, upon processing by antigen-presenting cells (APCs), CD4+ peptide epitopes essential for helper T-cell activation; and (ii) it displays multiple hapten copies, promoting B-cell receptor-mediated uptake and antibody production.

Most hapten-carrier approaches exploit highly efficient thiol-maleimide ligation, which enables the attachment of multiple copies of cysteine-containing peptides to commercial maleimide-functionalized carriers. Besides its fast kinetics and simple setup, this benchmark reaction proceeds under mild conditions (aqueous buffers, near-neutral pH, room temperature), compatible with a wide range of biomolecules (Scheme 1). While widely used for linear peptides by introducing a spare thiol (e.g., an extra cysteine), **thiol-maleimide conjugation of disulfide-containing haptens has an inherent limitation: the free thiol can attack disulfide bonds, disrupting the native hapten pattern and generating incorrectly folded, disulfide-scrambled structures** (Scheme 2). To address this issue, Katayama et al. [1,2] introduced **a long spacer between the thiol and disulfide-containing hapten to reduce the effective molarity of the thiol/disulfide reaction and slow it down**. While conceptually convincing, their approach was validated only by demonstrating the generation of native disulfide-rich hapten-specific antibodies, which was not possible using classical approaches. However, the possibility and extent of disulfide scrambling under conjugation conditions were not investigated, prompting us to explore this issue and develop a potentially more general strategy. Here, **we aimed to develop a general strategy for synthesizing disulfide-containing peptide conjugates while preserving their native disulfide pattern under thiol-maleimide coupling conditions**. We selected the well-structured N-terminal region of LINGO-1 as a model: a 20-mer containing two disulfide bridges. This peptide was coupled to a small set of thiol-containing spacers, and disulfide scrambling was rigorously monitored under maleimide conjugation conditions.



Scheme 1. Thiol-maleimide ligation for the generation of hapten-carrier constructs

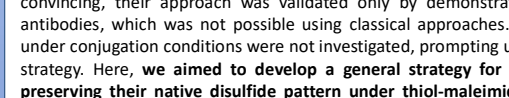


Scheme 2. Introducing **an additional thiol group** into a disulfide-containing hapten may **compromise its native disulfide connectivity** through an undesired intramolecular thiolate attack on disulfide bonds, competing with the intended thiol–maleimide conjugation. Incorporating an appropriate **spacer between the free thiol and the disulfide-rich core could suppress this side reaction** by decreasing the effective molarity of the reactive thiol near the disulfide bonds.

Spacers aimed to reduce disulfide scrambling

Previous work: the advent of the concept

Absence of disulfide pattern disruption during thiol / maleimide conjugation to a carrier protein was expected, but not checked. Indirect proof through the production of selective antibodies.

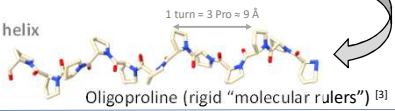


This work: validating and generalizing the approach

Peptide-based spacers, readily synthesized through SPPS

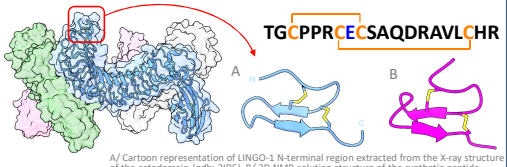
Flexible spacers:
- $(GGGGS)_2GGC(SH)$ ≡ 54 atoms
- $(GGGGS)_4GGGGC(SH)$ ≡ 90 atoms
- $(GGGGS)_8GC(SH)$ ≡ 141 atoms

Rigid spacers:
- $P_6C(SH)$ ≡ 36 atoms
- $P_{12}C(SH)$ ≡ 54 atoms



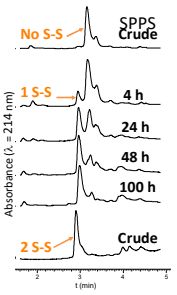
A model disulfide-rich peptide: LINGO-1[1-20]

Small disulfide-constrained peptide mimicking the 3D structure of the extracellular N-terminal part of LINGO-1, a trans-membrane protein involved in neurodegenerative diseases. Valuable model because **super sensitive to disulfide scrambling.**



A/ Cartoon representation of LINGO-1 N-terminal region extracted from the X-ray structure of the ectodomain (pdb: 2ID5). B/ 3D NMR solution structure of the synthetic peptide.

1. Synthesis of an azide derivative of LINGO-1[1-20]



H-TGC(Acm)PPRCEC(Acm)SAQDRAVLCHRGK(N_3)-NH_2

380 µM peptide
10% aq. DMSO,
RT, 100 h [*then lyophilization*]

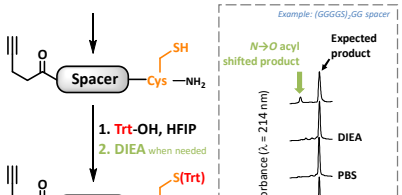
H-TGC(Acm)PPRCEC(Acm)SAQDRAVLCHRGK(N_3)-NH_2

1 mM peptide, 10 mM I_2
6.25% aq. HCl (160 mM) in AcOH, RT, 2 h
[*then precipitation with* Et_2O]

H-TGCPPRCECSAQDRAVLCHRGK(N_3)-NH_2

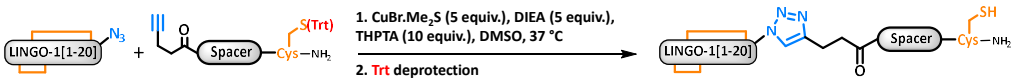
Overall yield after HPLC purification = 20%

2. Synthesis of spacers



- ***S*-Trityl** protection to prevent thiolate and thus disulfide scrambling during CuAAC
- **DIEA** treatment to reverse serine *N→O* shift (occurring upon TFA cleavage, for GGGGS-containing spacers).

3. Assembly through copper-catalyzed azide/alkyne Cycloaddition (CuAAC)



Spacer	Isolated yield (%)
$(GGGGS)_2GG$	43
$(GGGGS)_4GGGG$	35
$(GGGGS)_8G$	43
P_6	78
P_{12}	42

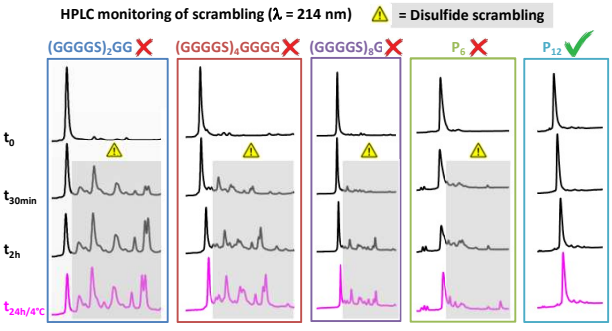
Yields not optimized.

- One single HPLC purification after step 1 (CuAAC).
- Non-standard CuAAC conditions because low water solubility of GGGGS linkers.
- ***S*-trityl** deprotection with TFA/H_2O/phenol/DODT/iPr_3SiH : 83/5/5/5/2 followed by precipitation in cold Et_2O. Very clean, quantitative reactions.
- LINGO-1[1-20]-SPACER-SH crude conjugates used for scrambling monitoring, and for KLH conjugation.

Evaluation of disulfide scrambling under thiol-maleimide conjugation conditions

Incubation of the LINGO-1[1-20]-SPACER-SH constructs under standard thiol-maleimide conjugation conditions: 20 mM phosphate buffer pH 6.6, 30 mM EDTA and 150 mM NaCl.

➢ HPLC and LC-MS monitoring at t = 0 and up to t = 2 h at RT and at t = 24 h at 4 °C (recommended conditions for conjugation to maleimide activated proteins).

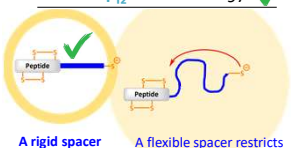


All solutions were thoroughly degassed and flushed with Ar to avoid SS dimer formation that would compromise the chromatograms (confirmed by MS).

The RIGID $(Pro)_{12}$ spacer very efficiently prevents disulfide scrambling

% of intact construct after incubation at 4 °C for 24 h

Spacer	% intact
$(GGGGS)_2GG$	11
$(GGGGS)_4GGGG$	18
$(GGGGS)_8G$	30
P_6	45
P_{12}	97 ✓

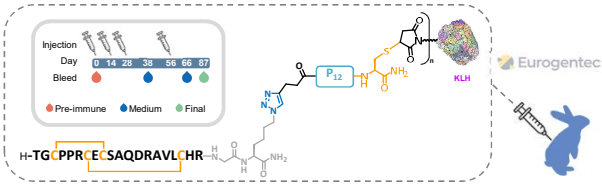


A rigid spacer restricts the accessible space for the thiolate to a sphere surface. => NO SS ATTACK (if spacer long enough)

A flexible spacer restricts the accessible space for the thiolate to a sphere volume => longer spacers slow down but do not abolish the SS attacks. Plus potential preferential folding issues.

Biological validation of the synthetic methodology

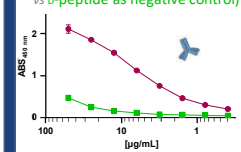
Immunization of a rabbit with the Lingo-1[1-20]-P_{12}-KLH conjugate



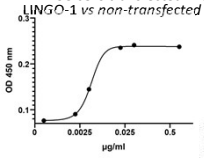
Immuno-ELISA and Western Blot assays of obtained polyclonal IgGs

Purification of IgGs from serum through protein-A affinity chromatography – similar results obtained using non-purified serum

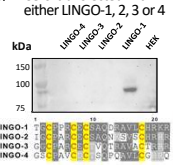
ELISA : *coating* = LINGO-1[1-20]-GK(N_3) *vs* D-peptide as negative control)



Live cells ELISA : HEK293 cells transfected with LINGO-1 *vs non-transfected*



Western blot : Cells transfected with either LINGO-1, 2, 3 or 4



WB shown = standard SDS PAGE, 1/5000 Ab dilution. Behaves equally well under (unboiled) native conditions.

=> Obtention of specific LINGO-1 antibodies

Validated for ELISA assays, cell assays and Western blot (either denaturating or not SDS-PAGE)
Large amounts (418 mg from the immunisation of only one animal)
Much superior than the best commercially available Ab against LINGO-1 (470$ for 100 µg !)

Conclusions and perspectives

Small disulfide-rich peptides (DRPs) require conjugation to large carrier proteins to elicit an effective immune response because of their low immunogenicity. Thiol–maleimide chemistry is widely used for such peptide–protein conjugates, facilitated by commercial maleimide-functionalized carriers such as KLH. However, introducing a spare thiol into a DRP can disrupt its native disulfide pattern, generating heterogeneous immunogens that fail to accurately mimic the intended epitope. Here, **we established a robust strategy for the late-stage introduction of thiol-containing linkers onto DRPs, generating "DRP–spacer–thiol" constructs that preserve their native disulfide pattern under thiol–maleimide coupling conditions** and enable conjugation to protein carriers. Using LINGO-1[1–20] as a model DRP, whose solvent-exposed disulfides are particularly prone to nucleophilic attack, we generated an immunogen that elicited an IgG response following standard immunization. ELISA showed that protein A-purified polyclonal IgG selectively bound LINGO-1 on transfected cells. Western blot analysis demonstrated superior sensitivity to a commercial IgG and strong specificity for LINGO-1 over homologs LINGO-2, -3, and -4, which share the same cysteine pattern and 45–75% sequence similarity. Overall, our results establish a robust strategy for generating DRP-specific antibodies while preserving native disulfide patterns, readily adaptable to diverse DRPs.

Check out for the full story ! link to the published article:

References: [1] Katayama, H. and Mita, M. Bioorg. Med. Chem., **2016**, *24*, 3596. [2] Katayama, H.; Mizuno, R. and Mita, M. *Biosci. Biotechnol. Biochem.*, **2019**, *83*, 1791. [3] Wilhelm, P.; Lewandowski, B.; Trapp, N.; Wennemers, H. *J. Am. Chem. Soc.*, **2014**, *136*, 15829.