An efficient strategy to bioconjugate disulfide-rich peptides *via* thiol-maleimide chemistry while preserving their bridging pattern

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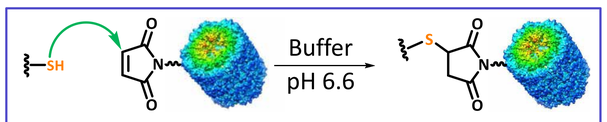
Background and project's aim

The generation of specific antibodies against weakly-immunogenic peptides requires their covalent conjugation to large protein carriers, such as keyhole limpet hemocyanin (KLH). Through such a general strategy, known as "hapten-carrier" approach, the carrier protein component has a dual function: (i) upon being processed by antigen-presenting cells (APCs), it provides a source of $CD4^+$ peptide epitopes that will be essential for the activation of helper T-cells; (ii) it also works as a scaffold for the display of several copies of hapten at once, which promotes B-cell receptor -mediated uptake and antibody production.

Most hapten-carrier approaches exploit the highly-efficient thiol-maleimide ligation, which enables for the attachment of multiple copies of peptides displaying a single cysteine thiol group onto a variety of commercial maleimide-functionalized carriers. Indeed, in addition to its fast kinetics and easy set-up, this benchmark reaction can be carried out under mild conditions (aqueous buffers, nearly-neutral pH, room temperature), compatible with a wide array of biomolecules (**Scheme 1**).

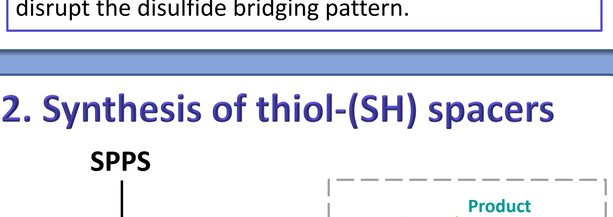


Scheme 1. Thiol-maleimide ligation for the generation of hapten-carrier constructs

While this methodology has been extensively applied for simple linear peptides by introducing a spare thiol residue in their sequence (for example an extra cysteine) the conjugation of disulfide-containing haptens by thiol-maleimide ligation has an inherent limitation (**Scheme 2**): the reducing ability of the free thiol group can disrupt the disulfide bridging pattern of the hapten leading to a mixture of incorrectly folded structures.

To circumvent this limitation, Katayama *et al.* [1,2] introduced a PEG-based spacer (31 atoms) between the thiol function and the disulfide-containing hapten prior to thiol-maleimide conjugation. However, the extent of disulfide scrambling under the conjugation conditions has not been investigated.

With this project, we aim to develop a general strategy for the synthesis of disulfide-containing peptide conjugates that maintains the disulfide pattern unaltered under thiol-maleimide coupling conditions. As model peptide, we focused our attention on the well-structured N-terminal region of the Lingo-1 protein, a 20-mer peptide containing two disulfide bridges, that we coupled to a small battery of thiol-containing spacers (34 to 139 atoms), either flexible or rigid.

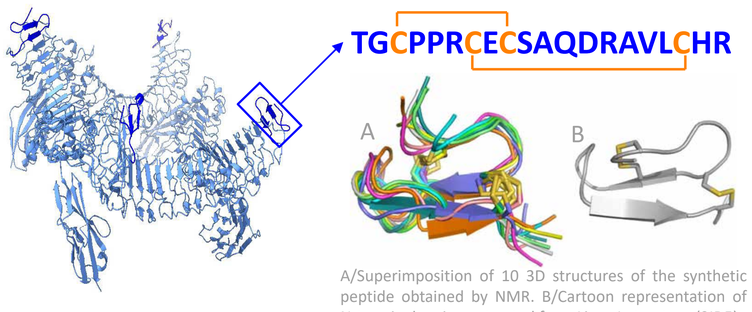


Scheme 2. Introducing an extra thiol group to a disulfide-containing hapten for ligation purposes can disrupt the disulfide bridging pattern.

Model peptide

Lingo-1[1-20]

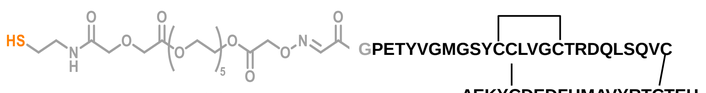
TGCPPRCECSAQDRAVLCHR



A/Superimposition of 10 3D structures of the synthetic peptide obtained by NMR. B/Cartoon representation of N-terminal region extracted from Lingo1 structure (2ID5).

Small disulfide-constrained peptide mimicking the 3D structure of the extracellular N-terminal part of LINGO-1, a membrane protein involved in neurodegenerative diseases.

Spacers to reduce disulfide scrambling



Katayama *et al.* [1] (31 atoms)

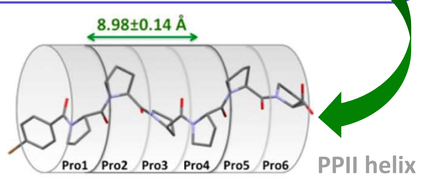
- $(GGGGS)_2GGC(SH)$ ≡ 52 atoms
- $(GGGGS)_4GGGGC(SH)$ ≡ 88 atoms
- $(GGGGS)_8GC(SH)$≡ 139 atoms

Flexible spacers

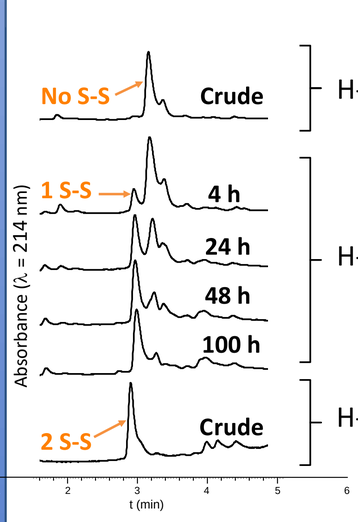
- $P_6C(SH)$ ≡ 34 atoms
- $P_{12}C(SH)$ ≡ 52 atoms

Rigid spacers

Polyproline as rigid "molecular rulers"
Wilhelm *et al.*[3]



1. Synthesis of Lingo-1[1-20]



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*]

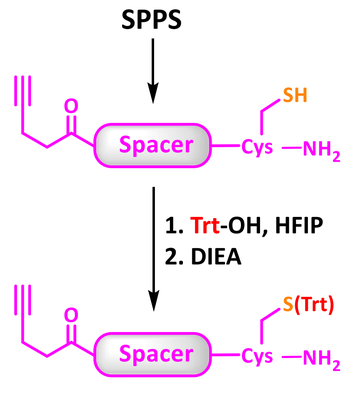
H-TGC(Acm)PPRCEC(Acm)SAQDRAVLCHRGK(N_3)-NH_2

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

H-TGCPPRCECSAQDRAVLCHRGK(N_3)-NH_2

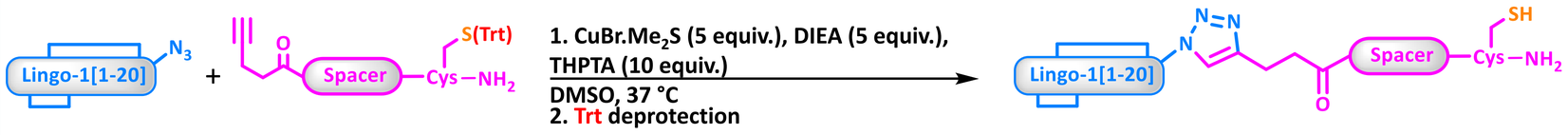
Overall yield after 1 HPLC purification = 20%

2. Synthesis of thiol-(SH) spacers



- **Trityl** protection prevents disulfide scrambling during CuAAC at pH 7
- **DIEA** treatment to reverse the Ser *N*→*O* shift

3. Copper-catalyzed Azide Alkyne Cycloaddition (CuAAC)



Spacer	Yield (%)
$(GGGGS)_2GGC(Trt)-NH_2$	43
$(GGGGS)_4GGGGC(Trt)-NH_2$	35
$(GGGGS)_8GC(Trt)-NH_2$	43
$P_6C(Trt)-NH_2$	78
$P_{12}C(Trt)-NH_2$	42

- **Trityl** deprotection with $TFA/H_2O/Phenol/DODT/iPr_3SiH$: 83/5/5/5/2 followed by precipitation in cold Et_2O.
- HPLC purification after step 1. Lingo-1[1-20]-SPACER-SH **crudes** are used for the scrambling monitoring.

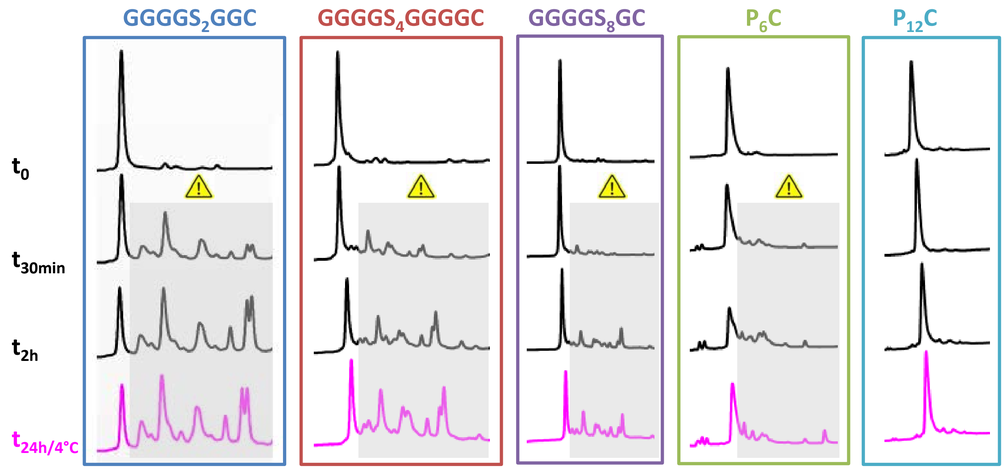
Scrambling evaluation

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 dimer formation (confirmed by MS).*

HPLC monitoring of scrambling at λ = 214 nm



Chromolith, 3 mL/min flow rate. Gradient: 10-30% CH_3CN/H_20 (+0.1% TFA) over 5 min for **GGGGS** spacers, and, 10-45% CH_3CN/H_20 (+0.1% TFA) over 7.5 min for **polyproline** spacers, 25 °C.

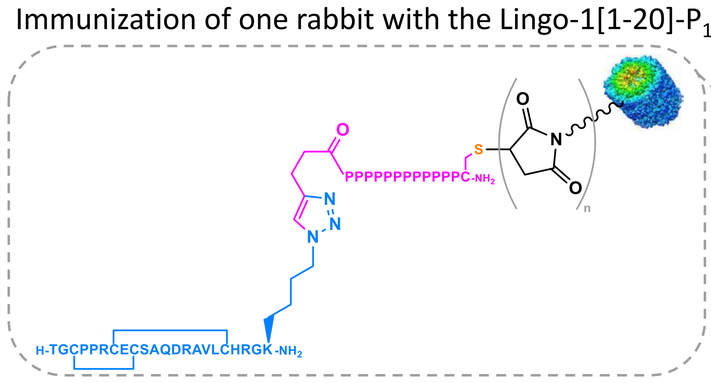
% of intact construct after incubation at 4 °C for 24 h

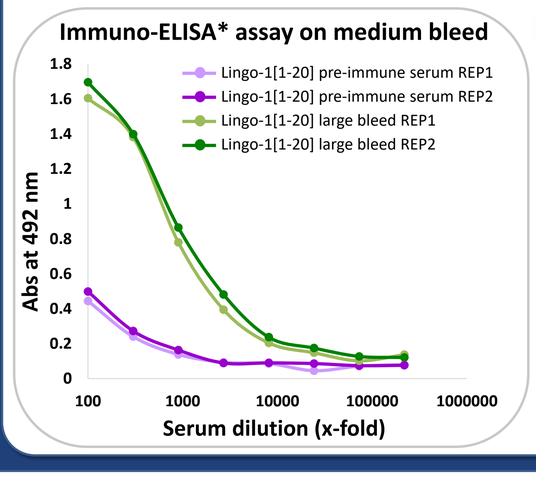
Spacer	%
$(GGGGS)_2$-GGC	11
$(GGGGS)_4$-GGGGC	18
$(GGGGS)_8$-GC	30
P_6C	45
$P_{12}C$	97

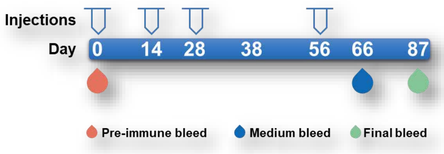
⚠ **Disulfide scrambling**

Immunization studies

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







* Coating: Lingo-1[1-20]-GK(N_3)

✓ ELISA assay on post-immunization serum confirmed the presence of Lingo-1[1-20]-specific IgG antibodies

➢ Final bleed ELISA assay is undergoing.

Conclusions and prospectives

In this study, using the Lingo-1[1-20] model peptide, we systematically evaluated the contribution of different spacer moieties in limiting the extent of disulfide scrambling upon incubation of the "hapten-spacer-thiol" system alone, under standard reaction conditions used for thiol-maleimide coupling (*e.g.* buffered aqueous solution pH 6.6, for 2 hours at room temperature, or 24 hours at 4 °C). Strikingly, our results demonstrated that a **flexible** 52-atom spacer [$(GGGGS)_2GGC$] performed very poorly in maintaining unaltered the hapten disulfide pattern, with ≈20% of the native hapten being present after 2 hours at room temperature, and ≈10% after 24 hours at 4 °C. In sharp contrast, a **rigid**, proline-based 52-atom spacer [$P_{12}C$], allowed to maintain ≈100% of the native disulfide pattern, in both conditions, and it was therefore chosen as optimal spacer to carry out a hapten-carrier conjugation approach.

To further validate the importance of having a rigid linker, we will next perform circular dichroism (CD) on our proline-based compounds to confirm the presence of a PPII helix. At an early stage of the project we validated by NMR that the Lingo-1[1-20] synthetic peptide structure is superimposable with the corresponding N-terminal region from the native Lingo-1 protein. Then, immunization studies involving the corresponding KLH-based conjugate were performed, and immuno-ELISA assays with post-immunization serum confirmed the generation of hapten-specific antibodies. The next important step will be to assess whether the generated anti-Lingo-1[1-20] antibodies are capable to bind the native receptor; this could open interesting avenues to utilize these antibodies for follow-up projects involving the Lingo-1 protein.

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.