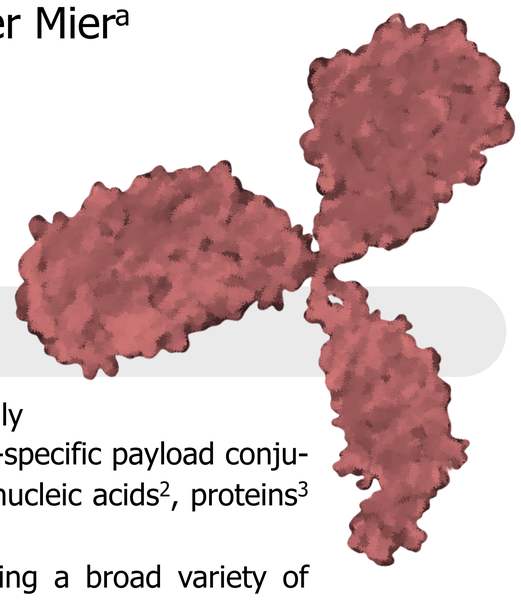
Comprehensive Chromatographic and Mass Spectrometric Approaches for in Vitro Analysis of Site-Specific Antibody–Peptide Conjugates

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Background

Biologics are indispensable compounds in today's pharmaceutical landscape. Since the field of antibody–drug conjugates (ADCs) is rapidly expanding and in continuous development, new technologies and concepts evolve day by day. While many recent studies focus on site-specific payload conjugation[1], the spectrum of cargos used in research expands as well. Given this gaining significance of more complex payloads, such as nucleic acids[2], proteins[3] or peptides[4], universal strategies for a thorough analysis are strongly required.

Here, we synthesized 15 site-specific Trastuzumab–peptide conjugates with a peptide-to-antibody ratio (PAR) of two, while covering a broad variety of structural features and therefore divers chemical properties of the payload. As part of that, we validated comprehensive yet readily adjustable mass spectrometric and chromatographic methods towards systematic *in vitro* characterization of antibody–peptide conjugates and re-evaluated existing methods for their reliability in this field.

Scope and Results

Conjugate Synthesis

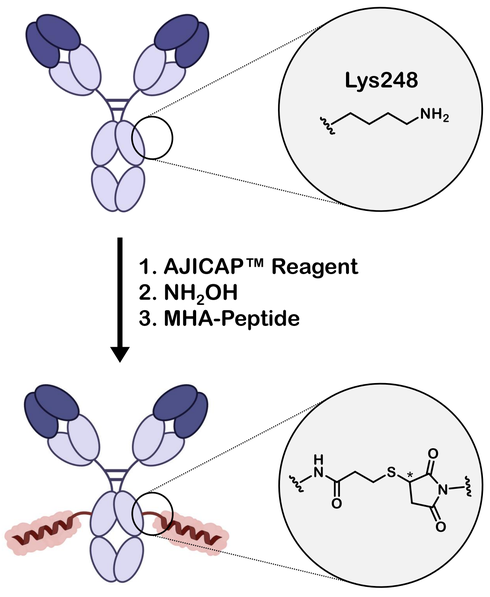


Table 1. Sequences of MHA-peptides used for protocol validation. Unless *N*-terminally attached, brackets indicate the coupling of maleimidohexanoic acid (MHA) to the ε-amino group of the lysine side chain.

Peptide	Class	Sequence
NLS	Hydrophilic	PKKKRKVK(MHA)
TAT		MHA-GRKKRRQRRRPPQ
R_9		MHA-RRRRRRRRR
D_6		MHA-DDDDDD
iRGD		MHA-CRGDKGPDC (cyclic disulfide)
$SynB_1$		MHA-RGGRLSYSRRRFSTSTGR
Ang-2		MHA-TFFYGGSRGKRNNFKTEEY
LPETG		MHA-GGGGSGGGGSGGGGSLPETGG
Pen		MHA-RQIKIWFQNRRMKWKK
MAP	Amphipathic	MHA-KLALKLALKALKAALKLA
TP_{10}		AGYLLGK(MHA)INLKALAALAKKIL
L17E		IWLTALKFLGKHAAKHDAKQQLSKLK(MHA)
pVEC		MHA-LLIILRRRIRKQAHAHSK
$PreS_2$		MHA-PLSSIFSRIGDP
MTS	Hydrophobic	AAVALLPAVLLALLAPK(MHA)

Scheme 1. Synthesis of site-specific Trastuzumab–peptide conjugates *via* the second generation AJICAP™ method. Using hydroxylamine the covalently bound AJICAP™ affinity peptide is cleaved resulting in 3-mercaptopropionyl modified Lys248 followed by maleimide–thiol conjugation.[5]

Hydrophobic Interaction Chromatography (HIC)

While HIC is a common tool used for drug-to-antibody ratio determination in ADCs, its utility in antibody–peptide conjugates is hardly elucidated yet. Here, the examined intact conjugates showed distinct retention time shifts strongly correlating with the hydrophilic properties of their amino acid sequences (Fig. 1, top).

Notably, for some of the conjugates significant peak broadening occurred. In particular, late-eluting conjugates (Fig. 1, bottom) were prone to this phenomenon. The presence of more than one signal opens up the thought of conformational heterogeneity of the payload. This may lead to structurally varying conjugate species restricting reliable PAR analysis.

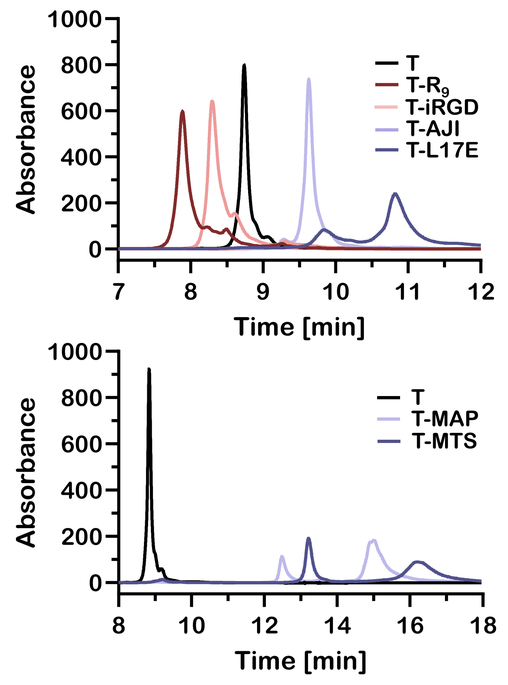
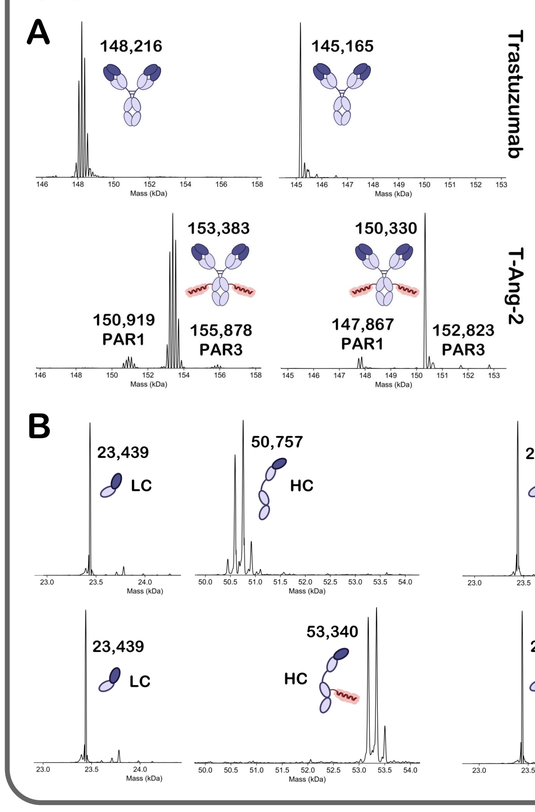


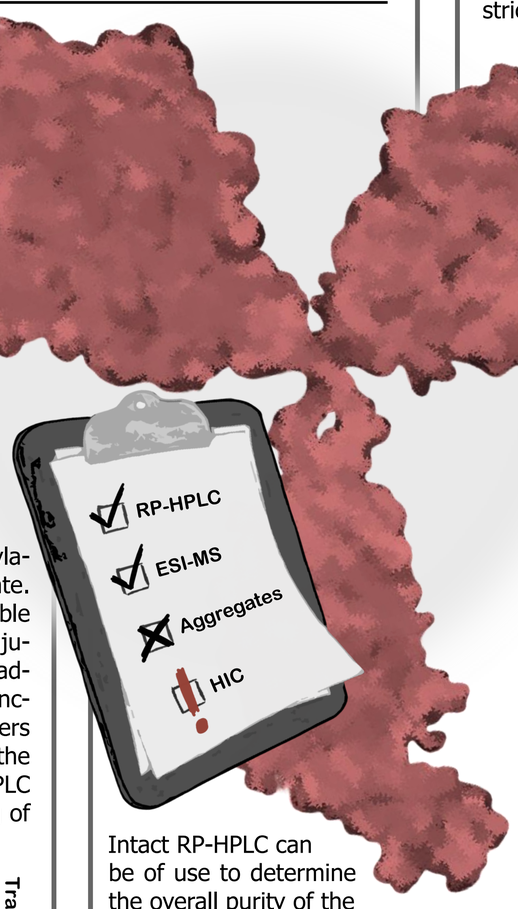
Figure 1. HIC chromatograms of early- (top) and late-eluting (bottom) antibody–peptide conjugates. T-AJI: Synthetic intermediate comprised of two AJICAP™ affinity peptides covalently attached to Trastuzumab.

ESI-Mass Spectrometry (ESI-MS)

Figure 4. Deconvoluted ESI-MS spectra of (A) intact and (B) TCEP-reduced antibody samples in the glycosylated (left) and deglycosylated (right) forms.



Mass spectrometric analysis of antibody conjugates was performed both for intact (Fig. 4A) and reduced (Fig. 4B) samples. Maximum mass accuracy was achieved through deglycosylation of the reduced conjugate. Sample preparation was feasible for all antibody–peptide conjugates (Tab. 1) and readily adjustable for more sensitive functional groups, such as thioesters found in T-AJI. Supporting the findings from reduced RP-HPLC analysis, site-specific labelling of the heavy chain was verified.



Intact RP-HPLC can be of use to determine the overall purity of the conjugate sample. Highly hydrophobic peptide payloads even showed baseline separation of PAR species (Fig. 3, top). Upon sample reduction retention time shifts of the modified heavy chain (HC) changed significantly whereas light chain (LC) signals remained identical (Fig. 3, bottom). While reduction can be performed using both DTT and TCEP, the latter resulted in broadened signals.

RP-HPLC

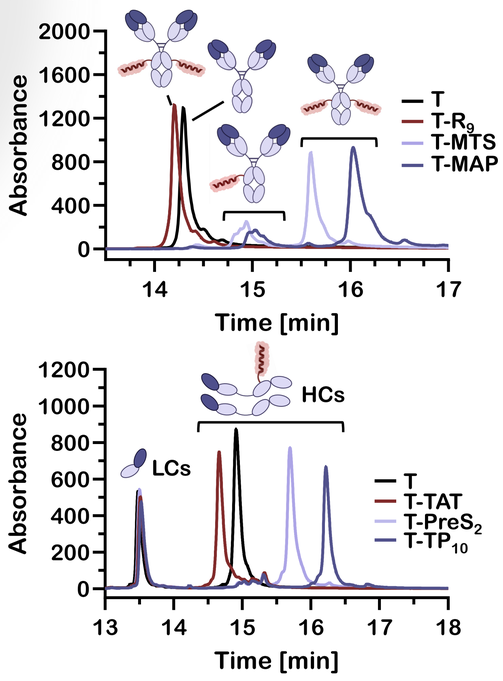


Figure 3. RP-HPLC chromatograms of intact (top) and DTT-reduced (bottom) Trastuzumab–peptide conjugates. Both intact samples and reduced heavy chains show distinct hydrophilic or hydrophobic retention time shifts depending on the peptide payload.

DLS

Particle size measurements did not show formation of aggregates but suggest monomeric species with a diameter of around 10 nm, which is typical for mAbs.[6]

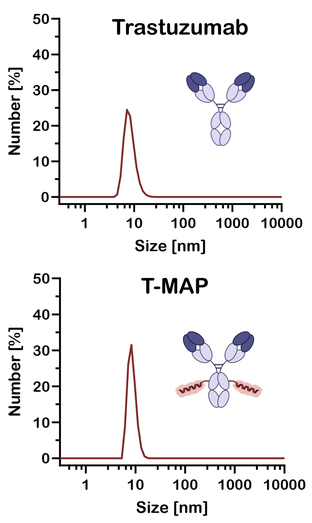


Figure 2. Particle size distribution measured in PBS (0.3 g/L) after storage in PBS (10 g/L) at 4 °C for 14 days.

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Summary and Outlook

We validated and standardized mass spectrometric and chromatographic approaches towards a more systematic analysis of site-specific antibody–peptide conjugates. We found that by a combination of both ESI-MS and RP-HPLC, a reasonable and thorough quality control of such conjugates can be achieved. Moreover, we performed HIC for all of the prepared conjugates and would like to put forward that it does not seem to generally qualify for a reliable PAR determination tool without the support of other aforementioned methods.

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