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HDX-MS based epitope mapping of antibody targeting the cysteine-rich region of IGF1R enabled by electrochemical reduction

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Introduction

Hydrogen Deuterium Exchange mass spectrometry is a powerful and flexible method for epitope mapping [1]. As therapeutic targets increase in complexity, sophisticated analytical methods are necessary for detailed and complete insights with HDX-MS. Online electrochemical reduction has emerged as a versatile tool for enhancing sequence coverage in heavily-disulfide bonded antigens. This technology has proven invaluable for epitope mapping in previously inaccessible targets [3]. The insulin-like growth factor receptor (IGF1R) is a promising target for receptor-mediated delivery of antibodies across the blood brain barrier [4]. This large and dynamic receptor possesses a complex disulfide-stabilized architecture that is challenging to analyze with conventional structural methods. We employed EC-HDX-MS to map a difficult-to-access epitope in the cysteine-rich region of a BBB-crossing single-domain antibody, VHH-IR4.

Methods

Two separate HDX-MS workflows were optimized to ensure comprehensive and redundant sequence coverage of the rhesus IGF1R ectodomain (eIGF1R) (Fig. 1).

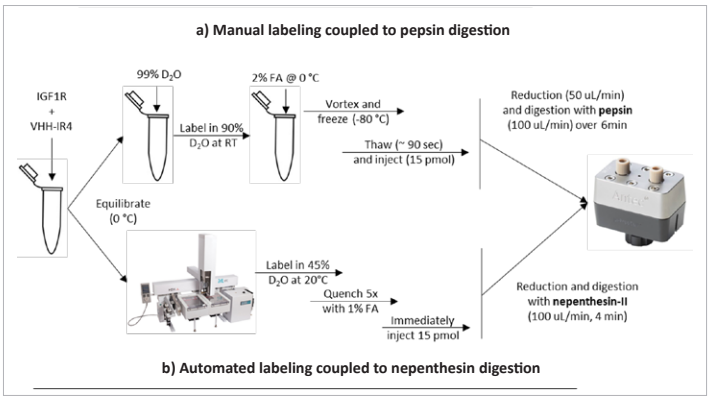

Figure 1: *(a) Manual and automated (b) HDX-MS workflows used to map VHH-IR4 binding profiles against eIGF1R. Two labelling time points (0.5 and 3min) were collected in all workflows, and data was collected in triplicate using a Synapt G2Si with ion mobility enabled.*

In both cases, the online electrochemical reduction was performed using a µ-PrepCell™ SS flow cell (Fig. 2a) controlled by a Roxy™ Exceed Potentiostat (Antec Scientific, The Netherlands). EC Pulse settings are shown in Fig. 2b.

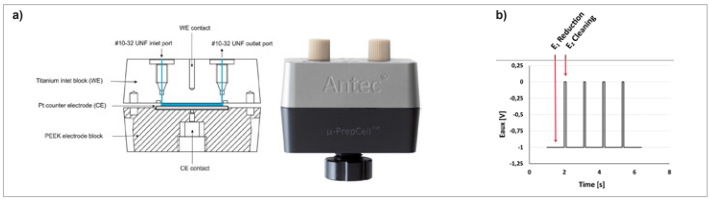

Figure 2: *(a) Dual electrode µ-PrepCell SS consisting of a Titanium inlet block and a Platinum counter electrode. (b) Applied pulse settings.*

EC-HDX-MS with multi-protesase approach enables near-complete sequence coverage of fully glycosylated eIGF1R

A summary of each individual HDX-MS workflow is shown in Table 1. The complementarity of the individual peptide maps is shown in Fig 3.

Table 1: *HDX summary*

	Pepsin		Nepenthesin	
Data Set (Pepsin)	**Control**	**IR4**	**Control**	**IR4**
HDX reaction details	20 mM Tris, 90% D2O, pD 7.0		10 mM Tris, 45% D2O, pD 7.0	
HDX time course (min)	0.5, 3	0.5, 3	0.5, 3	0.5, 3
# of Peptides	239	238	226	225
Sequence coverage	85.43%	85.43%	69.22%	69.22%
Average peptide length / Redundancy	15.42 / 3.7764	15.42 / 3.7764	11.73 / 2.6373	11.73 / 2.6373
Replicates (biological or technical)	3 technical	3 technical	3 technical	3 technical
Repeatability (average SD)	0.0597	0.0605	0.0471	0.0468
Significant differences (Δ HDX > X % D)	1.80%		4.70%	

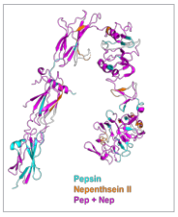

Figure 3: *Contributions of each protease to the overall coverage of eIGF1R.*

The combined eIGF1R coverage map (93%, 465 peptide features, redundancy of 6.9) is shown in Fig 4. This marks a significant improvement relative to our previous chemical reduction workflows where coverage ranged from 47-78% [5]. Electrochemical reduction allowed for the identification of 99 cysteine-containing peptides, covering of 36 out of 39 cysteines. Importantly, 21/22 cysteine residues in the 147-residue long cysteine-rich region (CRR), were at least partially reduced (Fig 4, dashed rectangle). Remaining gaps in coverage are attributed to the extensive N-linked glycosylation of eIGF1R.

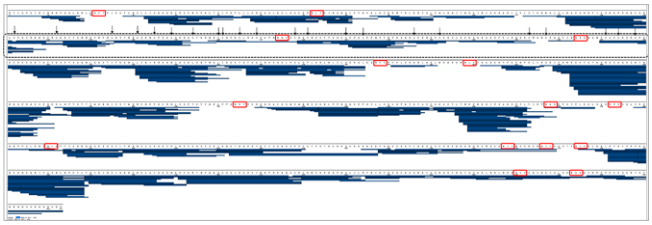

Figure 4: *Combined coverage map for peptides generated by pepsin and nepenthesin II. Peptides are shown as blue rectangles along the primary sequence of eIGF1R, N-linked glycans are highlighted by solid red squares. The cysteine rich-region is demarcated with a dashed black box, and individual cysteines in the CRR are highlighted with black arrows.*

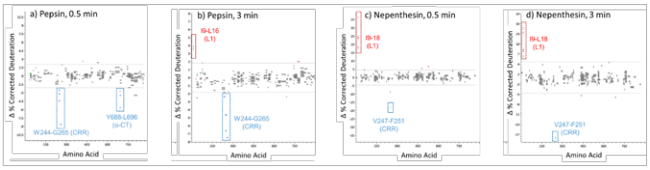

Figure 5: *Woods plots for each HDX timepoint. Differential deuteration (ΔD = DVHH-IR4 – DIGF1R) for pepsin (a-b) and nepenthesin-II (c-d) datasets plotted versus eIGF1R sequence. Each peptide feature is represented as a rectangle, with significant decreases in deuteration shown in blue, increases in red, and no significant changes in grey. Dashed grey lines correspond to 3 x SD values, and significance assigned based on a 1 – p value of 0.98.*

Insights into the VHH-IR4 binding profile from HDX-MS kinetics

Complementary binding profiles were observed for VHH-IR4 peptide sets stemming from both workflows (Fig 5)
These include both stabilizations and destabilizations in key structural features (CRR, L1 and α-CT helix)

VHH-IR4 binds epitope in the cysteine rich region

Given that VHH-IR4 does not compete with binding of a known α-CT helix binder (VHH-IR5, data not shown) [5], we assigned residues 244-265 as the linear epitope of VHH-IR4. This epitope overlaps with that of the monoclonal antibody BIIB4 [6]. Conformational changes in the L1 and α-CT helix overlap the binding site of IGF-1, the endogenous ligand of IGF1R, and may play a key role in the inhibitory activity VHH-IR4.

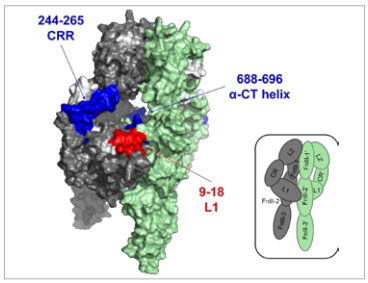

Figure 6: *Projection of combined HDX-MS datasets in three dimensions. Significant stabilization is shown in blue, while destabilization is shown in red, along with associated sequence numbering. No significant change in deuteration is shown in grey and green for the αB and (α6)' monomers respectively, and missing coverage is highlighted in white. For reference, the respective domains of each monomer as described in the subset BOX.*

Conclusions

- To our knowledge, this is the first known single-domain antibody targeting the cysteine rich-region.
- This data demonstrates the utility online electrochemical reduction in large protein systems in both manual and automated operating modes.
- This platform will enable the selection of anti-IGF1R nanobodies with improved functionality, and will ultimately open up new research opportunities in the area of protein trafficking and transcytosis.

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