Characterization of new modalities: nanobodies and their modifications

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INTRODUCTION

- Nanobodies (VHH) are single-domain antibodies of a size of 12-15 kDaabout 1/10 the size of an IgG, containing one disulfide bond .
- Nanobodies have similar selectivity and potency compared to IgGs, but improved stability and penetration profile[1,2].
- Nanobodies have found increasing use in diagnostic and therapeutic procedures, as well as research products.
- We present here the detailed product quality characterization of four recombinantly produced nanobodies and their labeled counterparts.
- Purity, molecular weight, disulfide bond status and degree of labeling were all successfully assessed.

MATERIALS AND METHODS

- Four nanobodies were produced in *E.coli* and subsequently purified via orthogonal means. These nanobodies were then conjugated to either biotin via N-hydroxysuccinimide (NHS) or the dye Alexa Fluor® 488 via maleimide. The former is directed towards free amines at the protein N-terminus or lysine side chains, while the latter targets the thiol groups of free cysteines.
- In a further conjugation experiment, the ratio of NHS-biotin to protein was increased step-wise to identify an optimal ratio.
- Protein digestion was performed under non-reducing conditions for one hour at a trypsin to protein ratio of 1:10.
- A benchtop X500B QTOF System (SCIEX) was used for the intact mass measurement as well as peptide mapping. A Phenomenex bioZen Intact XB-C8 2.1x50mm column was used for intact protein and bioZen Peptide XB-C18 2.1x150mm column for peptide separation. The Analytics module and the Bio Tool Kit within SCIEX OS Software were used for mass deconvolution. BPV Flex Software 2.1 was used for peptide mapping analysis.

RESULTS

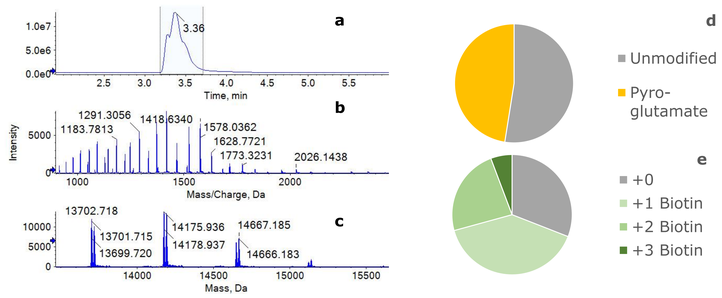

Fig 1 LC-MS characterization of the biotinylated VHH 1 nanobody via NHS. Slight separation of different labeled forms could be seen (a). Measured mass distribution (b) and reconstructed masses (c) over the retention time window. Notice the four clusters of doublets corresponding to increasing degrees of biotin labeling with or without pyro-glutamate modification. The percentages of pyro-glutamate and degrees of biotin labeling can also be extracted and are shown in (d) and (e) respectively.

Tab 1 Molecular weight of the most abundant species in each sample

Sample	VHH1	VHH1 +Biotin	VHH2	VHH2 +AF488	VHH3	VHH3 +Biotin	VHH4	VHH4 +AF488
Average MW, Da	13720.2	14666.6	14246.5	15643.9	14169.7	15116.7	14695.1	16093.3
Observed MW, Da	13719.7	14666.9	14245.7	15643.1	14168.9	15116.3	14695.2	16092.5
Δ mass, Da	-0.54	0.32	-0.81	-0.78	-0.78	-0.38	0.12	-0.75
Labeling*		69%		98%		69%		100%

*Degree of labeling is calculated as percentages of proteins carrying at least one label.

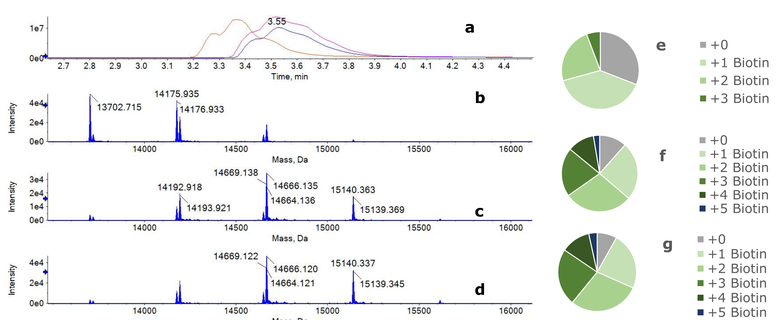

Fig 2 Optimization of biotinylation of capture nanobodies using three different reagent-to-protein ratios (a). Reconstructed intact masses of the differentially labeled proteins (b-d). The exact percentages of each biotinylation form are also calculated (e-g). Notice the increase of biotinylation with increasing amount of labeling reagent from (e) to (g).

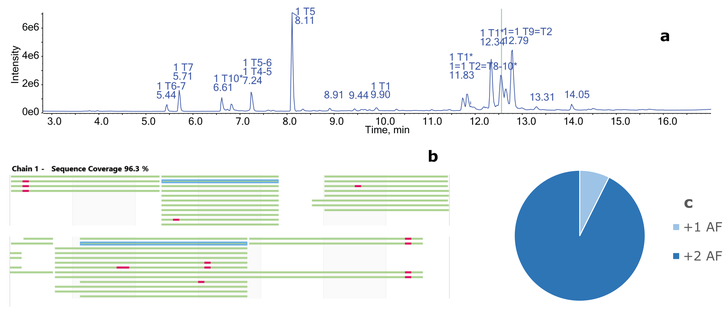

Fig 3 Characterization of VHH4 labelled with Alexa Fluor 488 (AF) via maleimide linkage at peptide level after tryptic digest. Chromatogram displaying the identified peptides (a). Sequence coverage analysis with a disulfide-bond linked peptide highlighted in blue. Sequence not shown (b). Green bars represent individual peptides identified. The magenta mark denotes a modification identified on this amino acid residue. The disulfide bond is calculated to be 97.9% intact based on summed intensities of respective peptides. Maleimide labeling is highly efficient with 93% having both cysteine labeled and 7% having one labeled (c).

CONCLUSIONS

- Nanobodies and nanobody-conjugates represent a high-value class of next generation biologics.
- A simple method was developed for its rapid characterization at both intact and peptide level including disulfide bonds using an X500B QTOF System being applicable to a variety of nanobody products.
- NHS chemistry results in a more heterogenous labelling efficiency than maleimide does, but allowed for fine tuning using different reagent concentrations.

REFERENCES

1 Jovčevska, Ivana and Muyldermans, Serge: The Therapeutic Potential of Nanobodies; BioDrugs 2020 34(1), pp. 11-26
2 Chanier, Timothée and Chames, Patrick: Nanobody Engineering: Toward Next Generation Immunotherapies and Immunoimaging of Cancer, Antibodies 2019 8(1), 13