Site-directional functionalization of VHH using the C-Direct tag

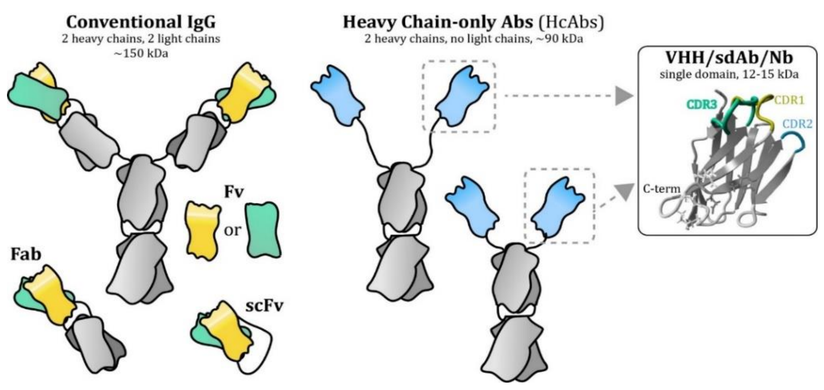
Raimond Heukers[a], Marjolein Kuijpers[a], Daphne van Hoesel[a], Marta Kijanka[a,b], Mohamed El Khattabi[a], Theo Verrips[a] and Edward Dolk[a]

[a] QVQ B.V., Yalelaan 1, Utrecht, The Netherlands

[b] Division of Cell Biology, Science Faculty, Utrecht University, Utrecht, The Netherlands

QVQ Holding BV
Yalelaan 1
3584 CL Utrecht
The Netherlands
www.qvquality.com

Background: Antibodies are popular targeting moieties for imaging probes.[1]
The small size, rapid blood clearance, stability and low immunogenicity, make variable domains of heavy chain-only antibodies found in camelids (**VHH**/sdAb) well suitable as molecular imaging tracers.[2–5]
To functionalize VHHs for imaging, it is essential to conjugate them to imaging probes without affecting its binding characteristics.



Aim:
A stable, flexible and low-immunogenic C-terminal tag for directional labeling of VHH

Methods: C-terminal cys-FLAG-tag was optimized for low immunogenicity, thermo- and enzymatic stability and production yield using bio-informatics and molecular modeling.
VHH were produced in *S. cerevisiae* and purified using the CaptureSelect affinity chromatography.
Purified VHH were site-directionally conjugated to biotin, chelators (e.g NOTA), fluorescent dyes (HiLyte, ATTO, Alexa, IRDyes) using maleimide chemistry. Free label was removed by size exclusion chromatography.
Immunogenicity and toxicity was tested *in vivo* in rats.

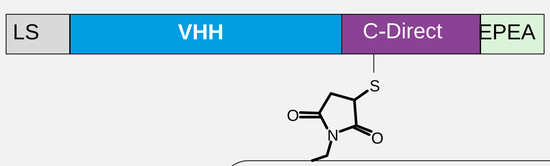
VHH-FLAG-His
Tag sequence: AAA-DYKDDDDK-GAA-HHHHHH
Vector: pMEK222 Host: *E.coli*

Molecular cloning

VHH-C-Direct
Tag sequence: A-**C**-A-XXXXXX-EPEA
Vector: pYQVQ11 Host: *S.cerevisiae*

Sulfhydryl-maleimide reaction

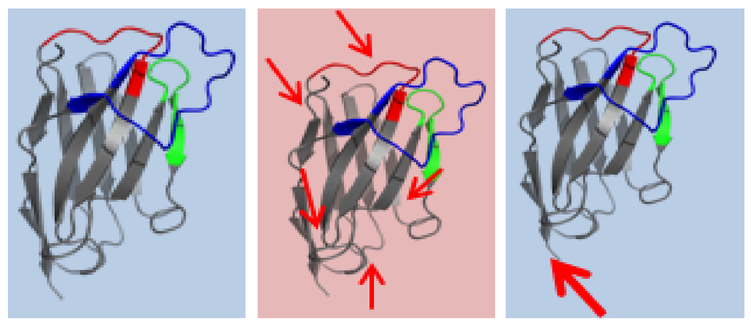
VHH-C-Direct-Labeled



- Biotin
- Chelators (NOTA, DOTA)
- Fluorescent dyes (HiLyte, IRDye)
- Surface or Matrix

Results:

A) Directional conjugation retains VHH integrity



Anti-HER2 VHH 11A4-FLAG-His

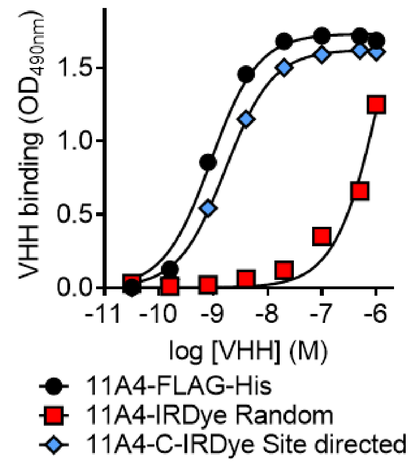
Apparent affinity: **~0.4 nM**

11A4-FLAG-His
Random conjugation to lysines

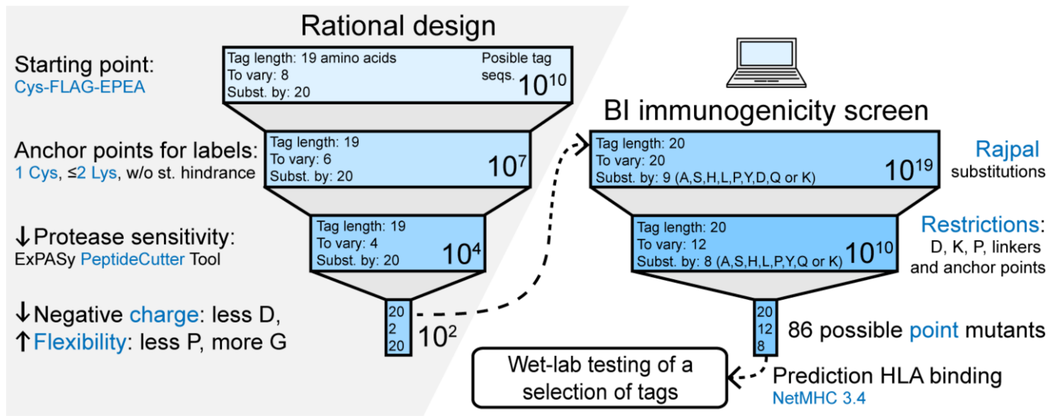
Apparent affinity: **>400 nM**

11A4-Cys-FLAG
Site-directional conjugation to cysteine

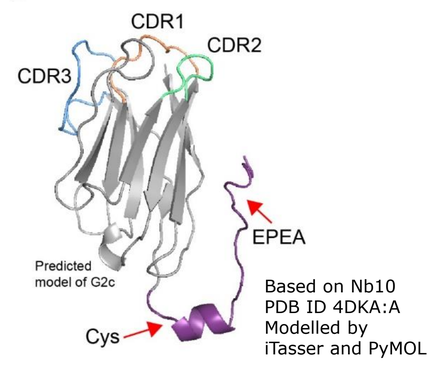
Apparent affinity: **~2 nM**

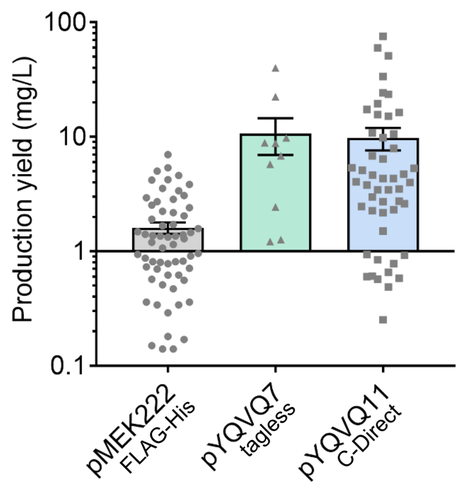


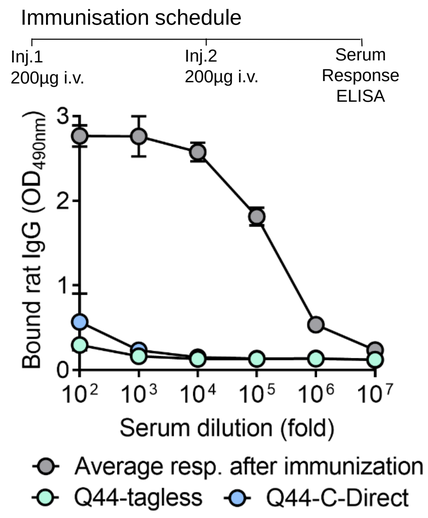
B) Iterations and immunogenicity screen



C) Production of VHH-C-Direct in *S.cerevisiae* and immunogenicity in rat



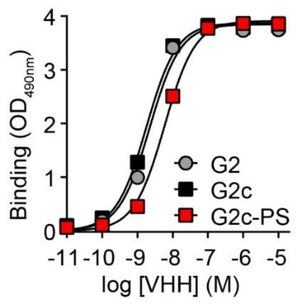


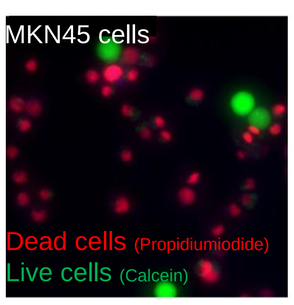


Examples of VHH conjugates in detection and imaging:

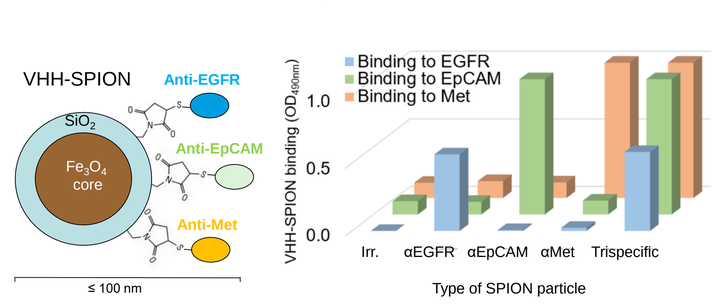
A) Detection of HER2 on different levels [6–8]
Project: CTMM MAMMOTH

B) Met-targeted photodynamic therapy [9]
Project: ERC VHH-targeted PDT





C) Multi-target, VHH-targeted Fe_3O_4 (SPION) particles for MRI- and PA-imaging
Project: EuroStars MetaDetect



Conclusion: VHHs with a C-Direct tag could readily be produced in yeast and purified from yeast supernatant. Functionalization of such VHH via the free thiol in the tag did not significantly affect its binding affinity and enabled its detection using a variety of imaging modalities.

Acknowledgements: VHH-based imaging done within CTMM MAMMOTH (Example A) also involved Aram van Brussel and Paul van Diest (UU and UMC). Met-targeted photodynamic therapy using QME-G2-700DX (Example B) was done with Vida Mashayekhi, Mercedes Ramirez-Escudero, Paul van Bergen en Henegouwen and Sabrina Oliveira (UU) and Hans de Haard (Argenx). The multi-targeted VHH-targeted SPION particles were generated within the MetaDetect program in collaboration with Wei Wan and Lars Dähne of Surflay Nanotec GmbH (Berlin).

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