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Comparative analysis of fusion tags used to functionalize recombinant antibodies

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ABSTRACT

Recombinant antibodies can be expressed as fusion constructs in combination with tags which simplify their engineering into reliable and homogeneous immunoreagents by allowing site-specific, 1:1 functionalization. Several tags and corresponding reagents for recombinant protein derivatization have been proposed but benchmarking surveys for the evaluation of their effect on the characteristics of recombinant antibodies have not been reported. In this work we evaluated the impact on expression yields, shelf-stability, thermostability and binding affinity of a set of C-terminal tags fused to the same anti-Her2 nanobody. Furthermore, we assessed the efficiency of the derivatization process. The constructs always bore a 6xHis tag plus either the controls (EGFP and C-tag) or CLIP, HALO, AviTag, the LEPTG sequence recognized by Sortase A (Sortase tag), or a free cysteine. The advantages and drawbacks of the different systems were analyzed and discussed.

1. Introduction

Antibodies represent the most important class of diagnostic molecules for basic research and clinical and technological applications. Their functionalization with active chemical groups and/or reporter tags is largely used to broaden and simplify their applicability as highly versatile bioreagents. The strategies to obtain such ready-to-use constructs starting from the original binders can critically vary between conventional and recombinant antibodies. Conventional antibodies rely on post-production chemical modifications [1] that usually have limited efficiency and are not easily reproducible. This implies cumbersome procedures to improve the site-specificity of the modifications [2] that leads to the generation of heterogeneous populations of labeled antibodies varying for number and position of the involved amino acid residues. Due to these shortcomings, conventional chemical modifications seem inappropriate when alternatives are available. Antibody fragments such as nanobodies show a growing interest because they preserve the selectivity and affinity of a conventional IgG but are simpler to recover from pre-immune libraries [3,4], to engineer and produce [5], and even to model *in silico* [6,7]. Recently, their reduced mass has been exploited to optimize the performance of super-resolution microscopy and *in vivo* imaging [8,9]. As recombinant proteins, nanobodies can be easily expressed as fusion proteins together with one or more polypeptidic tags suitable for affinity purification and controlled, 1:1 functionalization. The resulting constructs are reagents which simplify and render more reproducible the downstream applications [10,11].

Nevertheless and despite the large number of tags described in literature as fusion partners of recombinant antibodies [12–15], comparative surveys studying the effect of such tags on the stability, yields, and functionality of the final constructs [5,16] are still insufficient for enabling their rational selection. In the attempt to fill in this knowledge gap, we compared the effect of different tag combinations on the biophysical characteristics of the same nanobody and at the same time the efficiency of the derivatization process was evaluated. Specifically, all the analyzed constructs contain a 6xHis tag designed for purification by immobilized metal chromatography (IMAC) and differ for the second tag. This second tag was selected to directly confer fluorescence to the fused antibody, as in the case of Enhanced Green Fluorescent Protein (EGFP), or to enable site-specific modifications. For instance, the Sortase tag (LPETG sequence) located at the antibody C-terminal allows for the Sortase A-dependent covalent binding to N-terminal poly-glycine probes [17], while the C-terminal Avi-tag [18] can be biotinylated *in vivo, ex vivo* or *in vitro* by biotin ligase BirA and consequently used for

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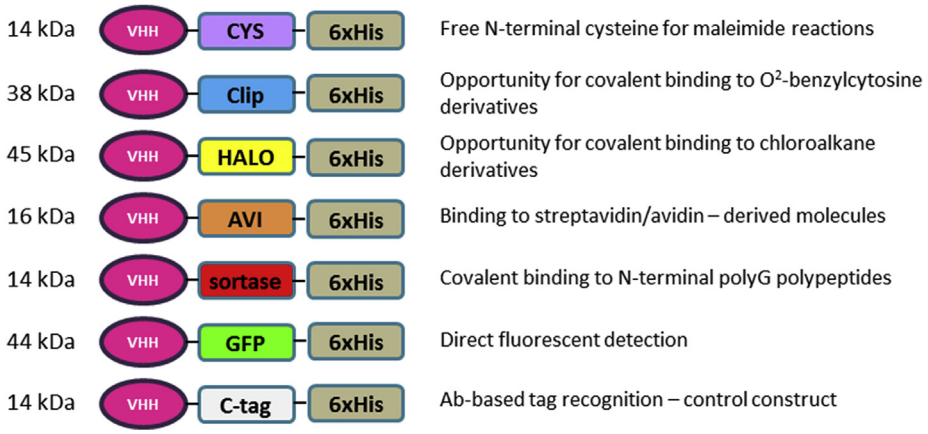

Fig. 1. Schematic descriptions of the constructs. The constructs evaluated in this paper possess a double fusion tag. The 6xHis tag is common to all and enables affinity purification by IMAC. The second varies and allows for different strategies of nanobody functionalization/visualization: i) free N-terminal cys; ii) CLIP; iii) HALO; iv) Avi; v) Sortase; vi) GFP.

selective and strong binding to any avidin/streptavidin reagent [19]. The insertion of a C-terminal free cysteine opens to several chemical derivatization opportunities (i.e. maleimide, iodoacetamide, 2-thiopyridine) for site-specific antibody modifications [20]. Finally, autocatalytic modified enzymes such as SNAP, CLIP and HALO [5,21] are tags suitable for being derivatized with a large variety of commercially available chemicals such as chromophores, PEG or biotin. A schematic representation of the constructs assessed in this work is reported in Fig. 1.

2. Material and methods

2.1. Nanobody expression plasmids

The vectors for the expression of nanobodies fused to 6xHis and GFP, C-tag, CLIP or HALO were described previously [5,22]. By substituting the GFP sequence in the pET14b-GFP vector (Suppl. Fig. 1) the remaining double-tag vectors were generated. Nanobodies were expressed fused to 6xHis plus: i) a free cysteine; ii) the peptide LEPTG (Sortase tag); iii) the peptide GLNDIFEAQKIEWHE (Avi-tag) (Suppl. Fig. 2-4). The base sequences corresponding to the tags were inserted at the C-terminal site of the VHH using the restriction sites *NotI/BamH*I, whereas a stuffer sequence was cloned between *Nco*I and *Not*I. The nanobody A10 was used as a standard in all the experiments, the nanobody C8 as a further control in the experiments with the Sortase tag [5]. Both wt nanobodies possess only the two standard cysteines common in llama heavy-chain variable region.

2.2. Expression and purification of the nanobody-tag fusion constructs

Nanobodies were purified as described previously [5,23] with minor modifications. The SOX bacteria [*E.coli* BL21 (DE3) cells, pre-transformed with a chloramphenicol resistant plasmid carrying the sequences coding for DsbC and sulfhydryl oxidase] were first transformed with the VHH-tag expression vectors and then grown at 37 °C under constant shaking at 220 rpm in either Studier autoinduction media or LB supplemented with ampicillin (100 μg/ml) and chloramphenicol (34 μg/ml). In the case of double-tagged His/Avi recombinant antibodies, SOX bacteria were co-transformed with the plasmid carrying the sequences for the biotin ligase BirA and the kanamycin resistance gene. Kanamycin (30 μg/ml) and D-biotin (100 μM) were added to the growth medium. When LB medium was used, the temperature was lowered to 20 °C and arabinose was added at the concentration of 5 mg/ml when the OD_{600} reached the value of 0.4 to induce the expression of sulfhydryl oxidase and DsbC isomerase. Isopropyl β-D-1-thiogalactopyranoside (IPTG, 0.2 mM) was provided after further 30 min and bacteria were pelleted following overnight incubation. In the case of autoinduced media, bacteria were initially grown 4 h at 37 °C before arabinose addition, then 1 h at 30 °C, and finally overnight at 17 °C.

Pellets (5 g) were first washed in 20 ml of PBS + 10% glycerol and then resuspended in four volumes of lysis buffer (50 mM TrisHCl, pH 8, 500 mM NaCl, 10 mM imidazole, 0.5 mM 4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF), 0.25 mg/ml lysozyme, 50 μg/ml DNAse, 5 mM $MgCl_2$), incubated 30 min on ice, sonicated, and finally centrifuged 1 h at 30,000 × *g*. The supernatant was loaded on an IMAC column (1-ml Hi-Trap NiNTA column, Qiagen) and washing buffer (50 mM TrisHCl, pH 8, 500 mM NaCl, 20 mM imidazole) was added until no more protein was eluted. His-tagged proteins were recovered in 50 mM TrisHCl, pH 8, 500 mM NaCl, 300 mM imidazole and buffer exchanged into 50 mM MES, pH 6, 50 mM NaCl, 1 mM DTT before undergoing ion exchange chromatography (IEX) using a 1-ml HiTrap SP column (GE Healthcare) and a 20 ml linear 0–1 M NaCl gradient. Final eluates were stored in 30 mM HEPES, pH 7.2, 100 mM NaCl, 1 mM DTT, 20% glycerol.

Ex vivo biotinylation was performed by incubating the supernatant of bacteria used to express VHH-HisAvi in the presence of 100 μM biotin for 30 min at 37 °C before double affinity purification (IMAC plus streptavidin) of the tagged nanobody. MyOne Streptavidin T1 magnetic beads (Life Technologies) were used according to manufacturer's instructions. The VHH-HisAvi construct was biotinylated also *in vitro* by incubating 25 μM of antibody with 150 μM biotin + 2 mM ATP + 5 μM $MgCl_2$ + 1 μM BirA for 1 h at 30 °C and its reactivity for streptavidin was evaluated by ELISA assay. After overnight incubation on immunoplate (NUNC) at 4 °C in the presence of 0.2 M sodium carbonate, pH 9.4, the plate was washed 4 times in PBS before being blocked 1 h with 0.5% BSA in PBS. After another washing cycle at the same conditions, the samples were incubated 2 h at 21 °C with 100 μl of 1:1000 HRP conjugated-streptavidin (GE Healthcare) in PBS. Samples were washed once again before the addition of tetramethylbenzidine. The reaction was stopped after 15 min and quantified by measuring the absorbance at 450 nm with an Infinite200Pro TECAN plate reader.

2.3. Preparation of the sortase experiment reagents

The Δ59 Sortase A from *S. aureus* cloned in pET23a was kindly provided by Dr. Kirill Piotukh (Leibniz-Institut für Molekulare Pharmakologie, Berlin) and expressed in BL21 (DE3) *E. coli* cells. Its purification was performed according to the protocol described in Piotukh et al. [24]. The GGG-EGFP sequence was cloned *Nco*I-*Not*I into a pETM14 vector [25], expressed in BL21 (DE3) cells and purified by IMAC as described above. Sortase A-dependent reactions were performed in 50 mM Tris-HCl, pH 8.2, 150 mM NaCl, 0–10 mM $CaCl_2$ for 2–4 h at 41 °C and stopped on ice plus 10 mM EDTA. Reagents (A10_6xHis_Sort-tag, GGG-GFP, Sortase A) were combined at different concentrations in the range of 1–25 μM.

2.4. Biophysical characterization of the fusion constructs

Melting temperature was assessed by NanoDSF label-free technology using a Prometheus NT.48 instrument (Nanotemper) [26]. The melting scan of the samples was performed between 20 °C and 95 °C at 1 °C/min.

Construct shelf stability was assessed preparing, for each sample, three aliquots of 20 µl in PCR tubes that were incubated for 15 days at −20 °C, 4 °C, 21 °C, respectively. Later the samples were processed by SDS-PAGE and band intensity/presence of degradation products was evaluated.

Affinity was measured by SPR in a Biacore T200 instrument (GE Healthcare). The experiments were performed at 25 °C, the complete kinetic set was collected in a single run and results fitted with either 1:1 Langmuir or steady-state interaction models. HER2 ectodomain-Fc produced in mammalian cells [5] was diluted to 50 µg/ml in sodium acetate buffer (pH 5.0) and immobilized by amine-coupling on a CM5 chip (GE Healthcare) at 900–1200 RU. Monovalent single-domain antibodies were used as analytes and injected at 30 µl/min at concentrations between 1000 and 3 nM (120 s injection, 600 s dissociation), the running buffer was 20 mM HEPES, pH 7.3, 150 mM NaCl, 2 mM EDTA, 0.005% P20. Surface regeneration (10 mM glycine HCl, pH 2.5, for 30 s at 30 µl/min) took place only between two successive series.

3. Results and discussion

The possibility of producing the same antibody fused to different tags can be of great advantage provided that the tags do not interfere negatively with the biophysical characteristics of the antibody. Following this approach, optimal tags can be selected according to the applications to shorten the experimental time and increase the sensitivity. We already demonstrated the advantage of having a free cysteine available for maleimide reactions to decorate nanoparticles and biosensors with nanobodies [27,28], of inserting a fluorescent protein to use in microscopy and flow-cytometry, as well as of using tags such as C-tag, SNAP, CLIP and HALO for labelling with customized and complementary reagents [5,15]. In all these cases, we noticed only limited effects due to the tag, as for instance the slight modification of the binding kinetics to the antigen [5]. In the past, recombinant antibodies were expressed also as fusions with sequences suitable for biotinylation (Avi tag) and Sortase-dependent conjugation [16,29,30], but a comparison with the other tags to show their advantages and drawbacks was never reported.

In this bench-marking work, aimed at comparing fusion immunoreagents, we inserted the sequence coding for a specific anti-HER2 VHH (A10) in a set of vectors for the expression of differentially C-terminally double-tagged constructs (Fig. 1). Some of the constructs have been described previously [5,22] and part have been designed specifically for this study. All immune-constructs were expressed in *E. coli* cytoplasm in the presence of the recombinant sulfhydryl oxidase [23] and were purified by two chromatographic steps (IMAC and ion-exchange chromatography - IEX). The purification outputs (purity above 90%) are reported in Fig. 2 and Suppl. Fig. 5, the yields were in the range of 2–10 mg per L of culture, corresponding to 0.5–2.5 mg per gram of wet weight cell pellet.

The reliable functionalization of CLIP, HALO, and free-cys VHH-constructs was demonstrated previously [5,27,28] and constituted the reference to evaluate the performance of Avi and Sortase tags. The interest for Avi tag is due to the availability of several commercial streptavidin-based reagents that can be used in combination with biotinylated proteins. The simplest way to prepare reactive VHH-HisAvi constructs would be their co-expression in the same host together with the enzyme BirA. This approach has been reported being not very efficient in conventional *E. coli* strains, no matter whether BirA and antibody are co-localized in the cytoplasm (inhibited antibody folding in reducing environment), in the periplasm (low BirA activity), or in different subcellular compartments (too short time for cytoplasmic biotinylation before antibody secretion) [16,29]. In our expression system the nanobodies are effectively folded in the bacterial cytoplasm thanks to the activity of the sulfhydryl oxidase [5,23] and are co-localized with the functional BirA that accumulates in the same cell compartment. We therefore expected to observe an increased biotinylation rate *in vivo* and to obtain highly pure VHH-HisAvi by tandem affinity purification exploiting sequentially the 6xHis and Avi tags. Nevertheless, this strategy failed because the *in vivo* biotinylation was insufficient and the VHH-HisAvi construct did not bind effectively to streptavidin beads (Fig. 3A). As reported before [29], biotinylation was much more effective when VHH-HisAvi, BirA and biotin were let react *ex vivo* (after bacterial lysis) or *in vitro*. In general, the sample construct was first purified by IMAC to eliminate the excess of free biotin, and then underwent a second affinity step using streptavidin. The overall procedure is cumbersome but the biotinylated VHH-HisAvi construct purified by this protocol could efficiently bind to streptavidin-peroxidase and enabled the enzymatically catalyzed reaction (Fig. 3B).

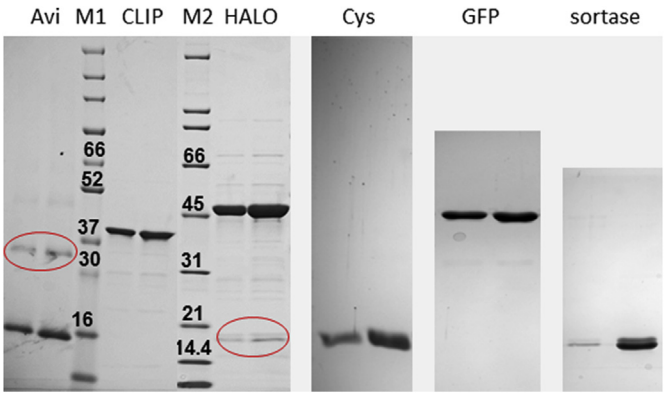

Fig. 2. Assessment of the purified constructs. SDS-PAGE was used to assess the contamination/degradation products of the purified fusion constructs (VHH plus tags). TGX precast gradient (4–20%) gels (BioRad) were used to increase the separation by possible contaminants. One and two µg of protein were loaded, M1 and M2 correspond to Sharpmass VII (Euroclone) and Broad Range (BioRad) MW protein markers, respectively.

Another, theoretically appealing, site-specific functionalization opportunity is represented by Sortase-dependent conjugation that requires nanobodies provided with the opportune pentapeptide LEPTG (Sortase) C-terminal tag and a partner possessing an N-terminal poly-glycine sequence. The method is not intended for adding polypeptides, because in this case the direct expression of a fusion construct would be simpler. However, poly-glycine sequences can be synthetized and added as “links” to conjugate other classes of molecules –such as DNA or imaging probes [30]- to antibodies. The feasibility of the approach has been demonstrated by proof-of-principle reports [31,32] and some accurate optimization protocols [33,34]. At the same time, it was pointed out that the Sortase-mediated reaction has a dynamic equilibrium that critically limits its efficacy. This can be increased by using large excess of both the enzyme and the substrate [35] or by exploiting depsipeptides [36]. This last solution is very difficult to implement as companies usually do not offer their synthesis. Consequently, to test the approach we adopted the first option since mutant Sortase A Δ59 [24] as well as recombinant nanobodies with a C-terminal LEPTG sequence can be expressed at high yields in bacteria. As a substrate, we produced recombinant GGG-GFP. We systematically tested different experimental combinations, according to the indications proposed by Levary et al. [34], and some representative results are reported in Suppl. Fig. 6. Summarizing, it is possible to functionalize 50% of the nanobody sample, but only in the presence of large excess of the GGG-GFP substrate (Fig. 4). We also noticed differences of reaction efficiency when different VHHs (A10 and C8) were used (Suppl. Fig. 6). In conclusion,

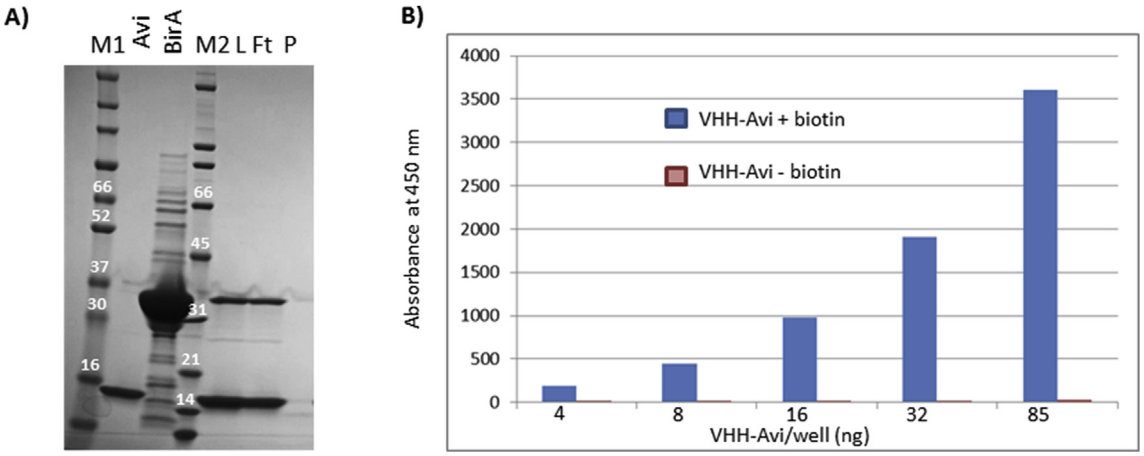

Fig. 3. Purification and functional assessment of the VHH-HisAvi tag construct. A) SDS-PAGE performed with TGX precast gradient (4–20%) gels (BioRad). The first two loaded samples were: (Avi) IMAC + IEX-purified double-tagged VHH-HisAvi construct biotinylated *in vivo*; and (BirA) IMAC-purified His-tagged BirA. VHH-HisAvi and BirA were incubated together and finally mixed with streptavidin beads. Samples corresponded to: the total mix (L), the flow-through after bead washing (Ft), the fraction corresponding to the boiled beads (P). M1 and M2 correspond to Sharpmass VII (Euroclone) and Broad Range (BioRad) MW protein markers, respectively. B) Increasing amounts of IMAC-purified VHH-HisAvi construct biotinylated *in vitro* were used to coat a microplate. After incubation with streptavidin-peroxidase, antibody concentration was estimated by TMB-based color reaction. The reaction was possible due to the interaction between biotinylated nanobodies and streptavidin-peroxidase.

the antibody functionalization based on Sortase A required a particularly time-consuming optimization, to reach high efficiency relied on excess of the GGG-substrate and the functionalization yields still remained lower than in available alternative methods. Consequently, we did not consider Sortase tag as a suitable functionalization method and did not proceed with to the biophysical characterization of the corresponding construct together with the other samples.

These were compared for their biophysical properties such as integrity, homogeneity, shelf-stability and affinity for their common antigen (Table 1). Concerning the oligomerization state, all the studied constructs proved to be monomeric, with the exception of the Avi tag construct that showed a propensity to dimerize (Fig. 2) and to form insoluble aggregates (Table 1). We observed previously that VHH progressive polymerization can lead to aggregation [37]. Also the formation of truncated constructs has been reported when tags are fused to the C-terminal of target proteins [16,23]. In our experiments, we noticed such by-products in the IMAC eluates but, with the exception of HALO, they could be removed efficiently during the IEX step and the resulting final purified immunoreagents were stable when stored at 4 °C and −20 °C (Table 1 and Fig. 2). The effect of the tags on nanobody shelf-stability was evaluated by storing the purified constructs for 2 weeks at room temperature (21 °C). This test showed that fusions of the nanobody with GFP and Avi were more sensitive than fusions with short tags such as free-cys and C-tag (Table 1). Intriguingly, the time dependent instability did not correlate with the measured thermal stability since all constructs had comparable melting temperatures, except for VHH-EGFP (Table 1). We cannot anticipate the reasons for the observed construct variable stability, since it could result from factors as different as the sensitivity to protease contaminations or to

Table 1
Biophysical effects induced by the fusion tags.

Fusion Tag	Stability test			KD (nM)	Tm (°C)
	−20 °C	4 °C	21 °C		
Control (C-tag)	stable	stable	stable	4.4 ± 1.9	51.4
Free-cys	stable	stable	stable	6.0 ± 1.6	53.7
HALO	stable	stable	stable	20.8 ± 4.3	59.8
CLIP	stable	stable	stable	26.2 ± 2.2	52.7
AVI	stable	stable	**precipitation**	15.4 ± 3.1	51.8
EGFP	stable	stable	**degradation**	10 ± 1.2	80.0

The same nanobody A10 was produced as a fusion with tags varying for length and chemical features. The effect of such tags on the stability (15 days at −20 °C, 4 °C, 21 °C), affinity and melting temperature of the fusion constructs was evaluated. *Precipitation* indicates the formation of insoluble aggregates, *degradation* the appearance of proteins with reduced mass.

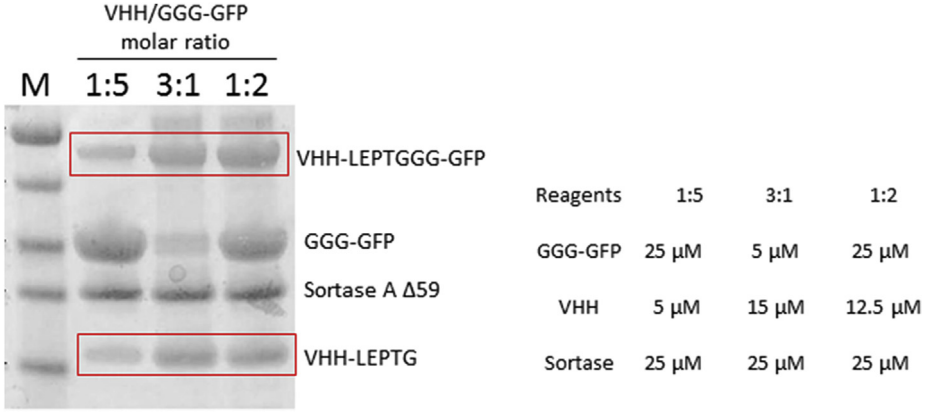

Fig. 4. Example of reaction optimization for Sortase A-dependent nanobody functionalization. Sortase A-mediated conjugation of GGG-GFP to VHH-LEPTG (Sortase tag) was performed varying the reagent concentration.

storage buffer components. Nevertheless, the data indicate that the tag moiety can affect such parameter.

In a previous work [5], we noticed that nanobodies fused to some large tags had decreased K_D for their antigen. The more systematic analysis performed in this work (Table 1) confirmed that large tags have a moderate negative effect on the K_D of the fused nanobody but we did not observe a direct correlation between tag mass and binding inhibition. For instance, the effect of EGFP (26 kDa) was lower than the effect of the smaller tags CLIP (20 kDa) and Avi (2 kDa), suggesting that further steric features might be involved in the mechanism leading to decreased antigen-antibody binding.

4. Conclusions

Recombinant antibodies have the possibility to be expressed fused to different proteins and tags. Immunoreagents composed by an antibody and a fluorescent protein or an enzyme are reagents ready-to-use in the appropriate applications. When non-peptidic molecules must be attached to fused tags, such it is the case for free-cys, CLIP or Avi, the resulting functionalization will be site-specific and 1:1. Nevertheless, it was necessary to verify the effect of different tags on the antibody reactivity and stability. Altogether, the biophysical characterization of the constructs indicated that the tags did not affect substantially the nanobody stability and binding capacity. We noticed that EGFP can undergo partial degradation during storage at room temperature but otherwise it is suitable for applications such as flow cytometry, ELISA, and fluorescence microscopy [38], whereas Avi-tag, despite the short sequence, increased the aggregation propensity of the fusion construct and required a supplementary *in vitro* or *ex vivo* biotinylation step because the *in vivo* BirA activity was insufficient.

The only functionalization strategy we clearly advise against is that based on Sortase activity because of its several drawbacks: i) the reaction depends on the availability of Sortase A that must be previously purified, added to the reaction mixture and then removed post-reaction; ii) unreacted antibody must be removed as well; iii) it is necessary to buy expensive customized synthetic substrates with N-terminal GGG sequence; iv) to increase the reaction efficiency, large excess of enzyme and GGG-x substrate must be added and this drastically increases the reaction costs; v) the reaction is reversible and therefore the efficiency is low in comparison to other enzymatic reactions; vi) the optimization of the reaction conditions is cumbersome and Sortase Δ59 activity requires high $CaCl_2$ concentrations, a condition that is not compatible with the stability of every protein. After these considerations, the use of the Sortase platform seems by far less convenient than the use of highly efficient autocatalytic tags such as SNAP/CLIP/HALO or even of a free C-terminal cysteine that enables similar conversion yields but requires less expenses reagents and simpler removal of unreacted substrates [28]. More recently proposed tags (SpyTag, SnoopyTag, VirD2) have the potential for becoming convenient alternatives for protein functionalization [11] and should be tested in combination with recombinant antibodies.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.pep.2019.105505.

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