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A Reagent for Amine-Directed Conjugation to IgG1 Antibodies

*Anders Märcher, Johan Palmfeldt, Marija Nisavic, Kurt V. Gothelf**

Abstract: *Functionalized antibodies are an indispensable resource for diagnosis, therapy and as a research tool for chemical biology. However, simpler and better methodologies are often required to improve the labeling of antibodies in terms of selectivity and scalability. Herein, we report the development of an easily available chemical reagent that allows site-directed labeling of native human IgG1 antibodies in good yield and mono-labeling selectivity. The salicylaldehyde moiety of the reagent reacts with surface exposed lysine residues to transiently form an iminium ion, and this positions a semi-reactive ester into proximity of a second lysine residue that reacts with the ester to form an amide. Interestingly, it appears that the formation of the iminium ion also has a significant activating effect of the ester. We use flow cytometry and bio-layer interferometry to confirm that the labeled antibodies retain antigen binding.*

Introduction

By conjugating synthetic molecules to proteins their functional and therapeutic properties can be changed and optimized. This inspires the development of methods for precise engineering of native proteins. Antibody conjugates are especially interesting, since functionalized antibodies have been used extensively for diagnostics, *in vivo* imaging, therapy and as a tool for molecular biology.[1–5] Conjugation to antibodies is, however, far from trivial. Full size antibodies are large proteins, around 150 kDa, with more than 80 lysine residues,[6] and they are dimeric structures that consist of two HC and two LC. The two HCs, and LCs, have the same amino acid sequence, respectively, meaning that multiple different residues that are chemically identical are found on each antibody. Conventionally, functionalized antibodies are prepared by a random global lysine labelling with *N*-hydroxysuccinimide (NHS) esters[7,8] or by cysteine conjugation via reduction of solvent accessible disulfide bonds and reaction with maleimides.[9] For both methods there is limited control of attachment sites and stoichiometry, which means the methods produce nonhomogeneous conjugates. It has been shown for antibody drug conjugates (ADC) that heterogeneous conjugates means poorer therapeutic indices and higher clearance rates.[10–12] Another conjugation method is the use of site-directed mutagenesis for incorporation of cysteines. While this strategy produces more homogeneous conjugates, it is also more work intensive and often gives rise to poor expression yields.[13]

Site-directed labelling is a strategy that overcomes some of the obstacles mentioned above.[14,15] Here, a directing reagent forms a complex with a chosen protein of interest (POI). The complex increases the local concentration of a reactive part of the reagent enabling reaction with the POI. The size of the probe restricts the labelling, such that it preferentially occurs in vicinity of the complexation site. Hamachi and coworkers have pioneered this method for protein labeling, primarily with focus on ligand directed approaches. Over the last decade they have developed several probes for site-directed labeling for a variety of proteins.[16–18] The high specificity of the ligand directed approach, allows for protein labeling *in vivo*, such as visualization of live neurons.[19]

Targeting more universal sites such as metal binding sites or disulfides, allows for the utilization of the same reagent or strategy on different proteins.[15] Recently, we, and Forte *et al*., published two related approaches for disulfide directed labeling of proteins.[20,21] The metal chelator tris-nitrilotriacetic acid has been used in different approaches for targeting metal-binding sites.[22,23] In one case, a metal-targeting strategy was used to prepare deoxyribonucleic acid (DNA) functionalized antibodies with a yield of around 23%.[24]

In 2018 and 2020 Rai and co-workers reported on a new method for lysine-directed labeling of histidine or lysine residues respectively.[25,26] The probes entail a salicylaldehyde directing group, a linker of different lengths and a reactive moiety for either histidine or lysine. In both cases they report the modification of one native immunoglobulin G1 (IgG1), in which both heavy chain (HC) and light chain (LC) is labeled. In one case a Trastuzumab FAB is modified with a conversion of 30-41%, no yields are given for native IgGs.

Results and Discussion

Inspired by the above described work we have designed a new, smaller type of lysine directed labelling reagent (LDLR) **1a** for labeling of IgG1 antibodies (Figure 1). In addition to the proximity directing effect the reagent is also activated by interaction with the directing group. The LDLR reagent is envisioned for straight forward labeling with low time demand and to produce antibody conjugates with higher control of labeling amount than for common NHS labeling strategies. We focus on IgG1 antibodies, since it is by far the therapeutically most relevant subtype, both in development and in the clinic.[27] The LDLR **1a** is easily prepared in three steps from readily available starting materials. It entails a salicylaldehyde that can in a rapid and reversible reaction form an iminium ion with surface exposed lysine residues.[26,28] It has been reported that the reaction of salicylaldehyde with amines under aqueous conditions leads to the preferred formation of the iminium/phenolate (Figure 1b) rather than the imine/phenol structure.[29] The main hypothesis of this work is that the *p*-phenol ester is activated by formation of the iminium ion through

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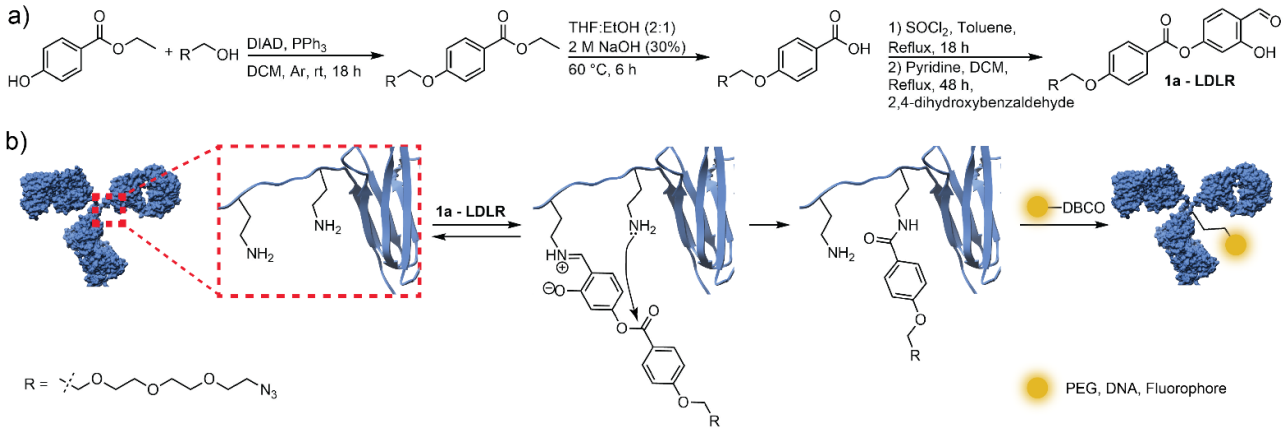

Figure 1. a) Synthetic route used to prepare the LDLR **1a**. The LDLR is prepared in 27% yield in three synthetic steps, starting from mono azide functionalized TEG. b) Illustration of the concept of the LDLR. A lysine residue on the surface of a protein of interest reacts in a rapid and reversible reaction to form an iminium ion. A nucleophilic lysine residue in proximity to the formed iminium ion attacks the now labile intramolecular ester in the LDLR and is labeled with the small azide tag. The tag can be further functionalized with a DBCO reagents of interest, we have used a 20 K Peg, DNA and a fluorophore.

stabilization of the negative charge of the leaving group, and thereby increases the reactivity towards nucleophilic lysines in proximity of the formed iminium ion. The salicylaldehyde iminium ion leaving group will in time be hydrolyzed. We further imagined that **1a** would only react when the iminium ion is formed, as the reactivity and concentration of the ester is too low for the reaction to take place if the labeling is not facilitated by proximity and thereby an increased local concentration.

The LDLR **1a** is designed with an azide tag on the amide forming part of the reagent. The azide tag is used for further conjugation in strain-promoted alkyne azide cycloadditions (SPAAC) with a functionalized dibenzocyclooctyne (DBCO). Importantly numerous DBCO reagents with functionalities of interest are commercially available.

With the LDLR **1a** at hand we wanted to test and optimize labeling conditions for IgG1s. Cetuximab, one of the first therapeutic IgG1s, was chosen as the test substrate. In a first attempt 10 µM Cetuximab was treated with 4 eq. of LDLR **1a** overnight (ON), at pH 8.5 and rt. Excess **1a** was removed by molecular weight cut off (MWCO) filtration and the azide-tag now attached to the protein was reacted with 10 eq. 20 K DBCO-PEG ON in a SPAAC. The product was then analyzed by reducing SDS-PAGE (Figure 2a). Unless otherwise noted, the same procedure of conjugation with 20 K DBCO-PEG and SDS-PAGE gel analysis was used in the following experiments. We were excited to see that labeling did occur using such low number of equivalents. In other studies, mentioned above, where the same guiding moiety is used, much harsher conditions were required with up to 25 eq. of reagent and in some cases 20% DMSO.[25,26] Furthermore, the labeling from the LDLR **1a** occurred predominantly once on the heavy chain. Diminutive amounts of LC labeling and double labeled HC was also observed (Figure 2a).

Next the labeling conditions were optimized to achieve the highest degree of double and mono labeled antibody, while keeping the degree of higher labeled antibody constructs low. Mono and double labeled antibody was targeted to produce conjugates with high homogeneity.

Labeling with the LDLR **1a** was investigated at pH 7.5 and 8.5 as the labeling is dependent upon the protonation state of the lysines. Different equivalents (1, 2.5, 5 or 10 eq.) of **1a** were incubated with the protein ON. Excess reagent **1a** was removed by MWCO filtration, and the conjugates were subjected to ON reaction with a 20 K DBCO-PEG. The products were analyzed by non-reducing and reducing SDS-PAGE (Supporting information, Fig S2 and S3). The best conditions were determined to be 2.5 eq. LDLR **1a** at pH 8.5. These conditions provided predominately mono labeled HC and the highest degree of mono and double labeled Cetuximab (approximately 39% and 15%, respectively).

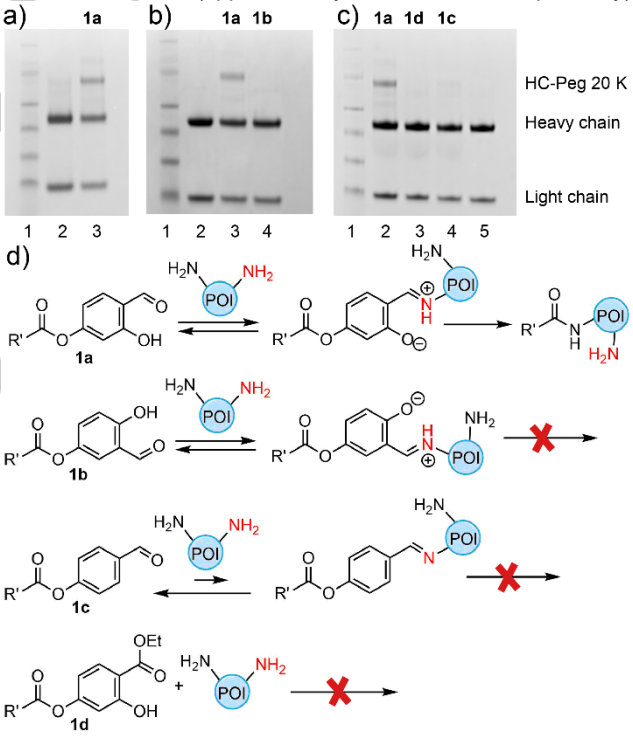

Figure 2. a) Initial labeling of Cetuximab with **1a**, analyzed with reducing SDS-PAGE. Lane 1: Protein ladder, Lane 2: Native Cetuximab, Lane 3: Cetuximab labeled with the **1a**, reacted with 10 eq. 20 K PEG-DBCO. Labeling conditions: Cetuximab (10 µM), pH 8.5, 100 mM NaCl, 4 eq. **1a**, ON. b) Investigation of the activation of the LDLR **1a** analyzed with reducing SDS-PAGE. Lane 1: Protein Ladder, Lane 2: Native Cetuximab, Lane 3: Cetuximab labeled with the **1a**, Lane 4: Cetuximab labeled with **1b**. c) Confirmation of site-directed labeling of **1a**, analyzed with reducing SDS-PAGE. Lane 1: Protein ladder, Lane 2: Cetuximab labeled with the **1a**, Lane 3: Cetuximab labeled with **1d**, Lane 4: Cetuximab labeled with **1c**, Lane 5: Native Cetuximab. Labeling conditions: Cetuximab (10 µM), pH 8.5, 100 mM NaCl 2.5 eq. reagent **1a/1b/1c/1d**, ON. d) Structure and reactions of reagents **1a, b, c and d** prepared to investigate the mechanism of the LDLR labeling. Reagents are shown with their proposed reaction with a protein except for **1d**, which is not expected to react in any way. For full gel image see Fig. S1, S4 and S5 in the Supporting information.

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Encouraged by the promising results we wanted to investigate if the LDLR **1a** is indeed both directed and activated by a neighboring amine. To explore this three new reference reagents **1b**, **1c** and **1d** were synthesized. In reagent **1b** the salicylaldehyde moiety is conserved, but the positions of the aldehyde and phenol hydroxyl group are swapped (Figure 2b and 2d). As the aldehyde is no longer placed in the *para* position to the intramolecular ester, this reagent should be much less activated upon iminium formation. For this reagent no labeling of Cetuximab is observed. Since the salicylaldehyde is conserved in **1b** it is expected that the directing effect is conserved through formation of the iminium ion. Therefore, the lack of reactivity must be explained by a less reactive ester moiety, which may be explained by the lack of activation upon iminium ion formation. But it is also likely that the ester in reagent **1b** is to some degree deactivated by the iminium ion formation, since this positions the electron donating phenolate in conjugation with the leaving group of the ester. In reagent **1c** the hydroxyl group is removed, which, as described by Neri and coworkers,[26] shifts the equilibrium towards the aldehyde. Furthermore, the species that is formed with the first lysine is most likely the neutral imine, which is not activated relative to the aldehyde. In agreement with this assumption, no coupling to Cetuximab is observed for reagent **1c**. The same observation is made for reagent **1d** which is prepared with an ethyl ester instead of an aldehyde and thus there can be no proximity guiding. It has been shown by computational studies that the electron withdrawing capacity of an ethyl ester was within the same range as an aldehyde,[30,31] and we rationalized that if probe **1d** did not label unguided, then neither would reagents **1a-c**. Based on these reference experiments we conclude that both the directing and activating effect of the salicylaldehyde is of importance for the successful labeling with reagent **1a.**

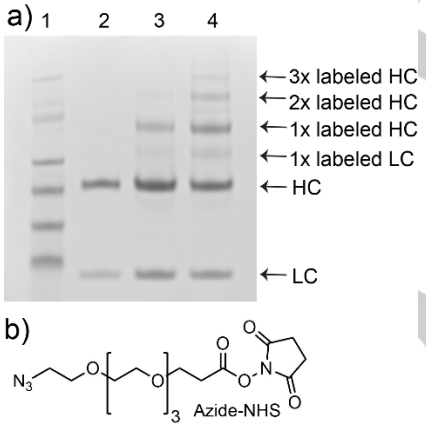
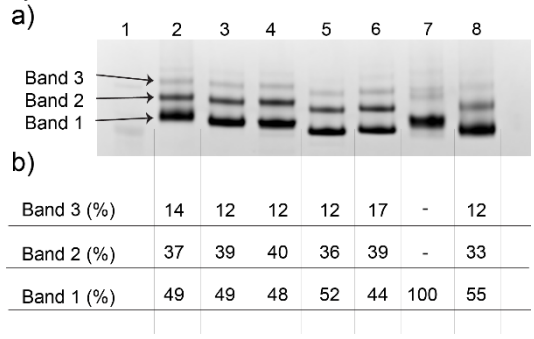


Figure 3. Labeling comparison of an NHS-ester and the LDLR **1a** analyzed by reducing SDS-PAGE. Lane 1: Protein ladder, Lane 2: Native Cetuximab, Lane 3: Cetuximab labeled with **1a**, Lane 4: Cetuximab Labeled with Azide-NHS. Labeling conditions: Cetuximab (10 µM), pH 8.5, 100 mM NaCl, 2.5 eq. reagent, ON. For full gel image see Fig. S6 in the Supporting information.

Next the LDLR labeling was compared to the conventional NHS labeling using the azide-NHS ester shown in Figure 3. Both reagents were used to label Cetuximab, and the antibodies were analyzed by reducing SDS-PAGE (Figure 3a). The overall conversion of antibody was higher for the NHS-ester. However, the LDLR **1a** produces conjugates with considerable higher homogeneity, as bands with more than three or more labels attached are observed for the NHS labeling as well as a more prominent light chain labeled band. As antibodies are dimeric structures, HC with multiple labels will equal even greater complexity of number of labels on the native protein. Other studies have also shown that producing antibody conjugates with high homogeneity and yield is very difficult with NHS-ester based labeling methods.[32,33]

We next challenged the methodology by other IgG antibodies. Four other IgG1 antibodies (Rituximab, Trastuzumab, Belimumab and Pertuzumab), one IgG2 (Panitumumab) and one IgG4 (Nivolumab) were chosen for further studies. The antibodies were all subjected to the LDLR **1a** using the optimized protocol and were then analyzed by non-reducing SDS-PAGE (Fig 4a). Reference non-reducing and reducing SDS-page of the native antibodies are shown in the Supporting information (Fig. S10-S11). As noticed all IgG1s and Nivolumab (IgG4) were successfully labeled, whereas Panitumumab (IgG2) showed no labeling. Still, the methodology allowed for similar labeling of five IgG1s requiring no further optimization for each protein. The optimized LDLR conditions labeled all IgG1 antibodies with similar conversion for mono and double labeled conjugates (36-40% and 12-17% respectively). Also, it was found by reductive SDS-PAGE that the label was almost exclusively attached to the HC of the antibodies (Supporting information, Fig. S12).

The observation that no labeling occurs on Panitumumab is interesting. We additionally attempted labeling IgG2 Kappa using the LDLR, which resulted in trace labeling (Supporting information, Fig. S13). The primary difference between IgG1 and IgG2 antibodies is in the hinge region. The IgG2 hinge region is shorter and much more rigid than the IgG1 hinge region, consisting of twelve amino acids residues with four disulfide linkages contrary to the fifteen amino acids with two disulfide linkages in IgG1s.[34] A lysine residue in the hinge region of Cetuximab was the first modifications sites identified with MS (data not shown), which might explain why the tested IgG2 antibodies labels poorly. The IgG4 hinge region is much more comparable to the IgG1 in terms of rigidity.



Band 3 (%)	14	12	12	12	17	-	12
Band 2 (%)	37	39	40	36	39	-	33
Band 1 (%)	49	49	48	52	44	100	55

Figure 4. a) Different IgGs labeled with the LDLR **1a** and analyzed by non-reducing SDS-PAGE. Labeling conditions: Antibody (10 µM), pH 8.5, 100 mM NaCl, 2.5 eq. **1a**. Full antibodies are seen in each lane. Band 1 is native antibody, band 2 is antibody with one modification and band 3 is antibody with two modifications. Lane 1: Protein ladder, Lane 2: **1a** labeled Cetuximab, Lane 3: **1a** labeled Trastuzumab, Lane 4: **1a** labeled Rituximab, Lane 5: **1a** labeled Pertuzumab, Lane 6: **1a** labeled Belimumab, Lane 7: Panitumumab (IgG2) attempted labeled with **1a**, Lane 8: **1a** labeled Nivolumab (IgG4). Lane 1-5 are IgG1s. Control with native unlabeled antibodies is found in Fig. S10 in the Supporting information. For a full gel image see Fig. S7 in the Supporting information. b) Quantification of band 1, 2 and 3 in each lane of. Each band is the conversion of each antibody to that amount of labels. Band percentage is an average of the shown gel and two more replicates found in the supporting information Fig. S8-S9.

All the LDLR **1a** labeled antibodies, without the 20k PEG DBCO modification, were then prepared for MS studies by in gel

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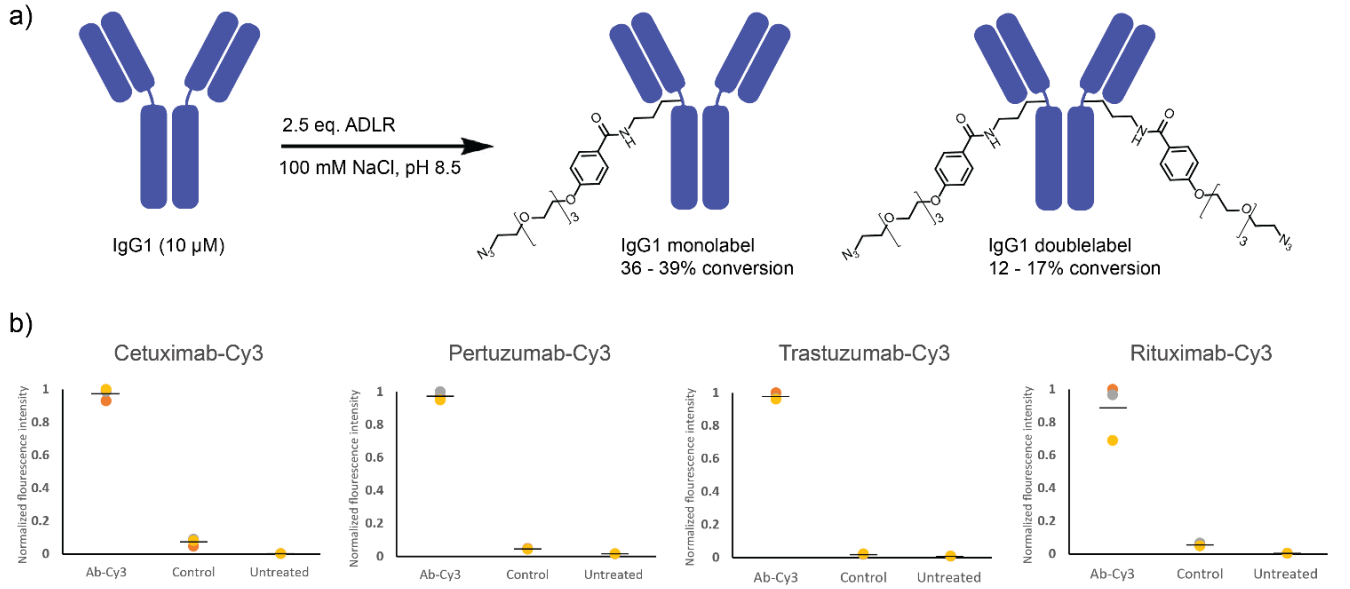

Figure 5. Illustration of the labeling of IgG1 antibodies using the LDLR **1a**. General optimized conditions are given, which provide 36-39% and 12-17% mono labeled and double labeled antibody respectively. b) Flow cytometry data using Cy3-antibody conjugates prepared using the LDLR reagent. Control is in all cases pretreatment of the cells with native unconjugated antibody. Cell types used: Cetuximab (A431, EGFR+), Pertuzumab and Trastuzumab (SKBR3, HER2+), Rituximab (Ramos, CD20+).

trypsin digestion. Labeling sites were identified and major labeling sites were located. In general 3-4 sites were found on each antibody (Data shown in Supporting information, pages S14-16). Multiple lysine residues in the same small peptide sequence were found to have been modified across different IgG1 antibodies. Two such examples are the hinge region lysine residue found in the sequence CD**K**TH and a lysine residue found in the top of the heavy chain in the sequence T**K**NQ. They were both found in three of the antibodies. In general, most of the labeling sites identified were found on at least two of the antibodies. Common for most labeling sites is that they have another lysine residue in close proximity. However, in some cases, no lysine residue can be found in proximity in the sequence, but here it is likely that a lysine residue is still closely positioned in the 3D structure of the protein. Analysis of crystal structures of Fab domains of Rituximab, Cetuximab and Belimumab showed that these labelling sites indeed have a nearby lysine residue in the 3D structure (Supporting information Fig. S19-S21). We know from earlier studies, using the same salicylaldehyde guiding moiety for protein labeling, that labeling of the LC was also observed.[26] We were however, pleased to see that for the LDLR **1a** reagent most sites were located on the HC (Data shown in Supporting information pages S14-16).

After establishing the efficacy of the LDLR **1a** for site-directed labeling of IgG1s we wanted to investigate if the antibodies retained affinity for their native antigen. Flow cytometry was used to investigate this, for four of the LDLR-antibody conjugates (Cetuximab, Rituximab, Pertuzumab and Trastuzumab). For this purpose, Cy-3 conjugates were prepared of these antibodies. The standard LDLR protocol was used for each antibody, followed by reaction with Cy3-DBCO in a SPAAC reaction overnight. Excess Cy3-DBCO is removed by molecular weight cut off (MWCO) filtration, using an Amicon™ 30 K filter with PBS. The Cy3-antibody conjugates were then analyzed by reducing SDS-PAGE, to ensure that Cy3 was attached to the antibodies (Supporting information, Fig S14). As expected, predominantly HC labeling had occurred. A cell type that overexpress the target epitope for each antibody was then selected. Each cell type was treated with the respective antibody-Cy3 conjugate and analyzed by flow cytometry. As a control in all cases a cell population was pretreated with native antibody to block the epitopes. Control samples were used to ensure that binding, to the cells, of the antibody-Cy3 conjugates remained through the original epitope. In all cases the antibody-Cy3 conjugates retained binding, and binding could be minimized by pre-blocking the expressed epitopes (Figure 5).

Bio-layer interferometry (BLI) was used to verify whether or not Belimumab retained binding to its epitope B-cell activating factor (BAFF). However, BLI requires clean conjugate without residues of the native unmodified antibody. To achieve pure antibody-LDLR conjugate, a DNA-antibody construct was prepared using the LDLR methodology. Belimumab was labeled with LDLR **1a** using optimized conditions. A DBCO-DNA strand was then reacted with labeled Belimumab ON and the Belimumab-DNA conjugate was purified by anion exchange HPLC. Impressively, DNA-antibody conjugate prepared in 10 nmol scale could be purified in a 36 and 14% yield for the mono and double labeled antibody respectively, when using this strategy (Supporting information, Fig. S15-S16). Belimumab-DNA (monolabeled) and native Belimumab was then used for BLI. The size of the DNA labeled Belimumab has changed because of the attached DNA, which will affect the readout of BLI, meaning that the binding cannot be directly compared to unlabeled Belimumab. However, it is clear that, as expected, LDLR **1a** labeled Belimumab retains affinity for BAFF (Supporting information, Fig. S17-S18)

Conclusion

A new reagent **1a** was designed, which is not only directed, but also activated by a neighboring lysine through iminium ion formation for reaction with the first lysine. Three reference reagents were prepared and their lack of reactivity support that labeling with LDLR **1a** is both directed and activated upon iminium ion formation. The LDLR **1a** is efficient for insertion of a reactive

azide handle onto native human IgG1s. This handle was applied for conjugation to DBCO functionalized cargos. This allowed for fabrication of antibody PEG, fluorophore and DNA conjugates, but it could in theory be used for most functionalities with a DBCO handle. The methodology is simple and mild, since it only requires the addition of one component in low stoichiometry at standard conditions and requires no elaborate laboratory techniques. Also, it produces conjugates with better homogeneity than the commonly used NHS-ester. Generally, the conversions of mono and double labeled antibodies are around 38% and 14%, respectively. The methodology exhibited robustness as five different IgG1s and one IgG4 antibody was labeled with similar conversion. Reducing SDS-PAGE and MS studies confirm that labeling occurs primarily on the HC. From MS studies it was also found that generally 3-4 major labeling sites were found for each antibody. All five labeled IgG1s were found to retain epitope binding with either flow cytometry or BLI.

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Conflict of interest

The authors declare no conflict of interest.

Keywords: IgG1 • antibody • Site-directed • labeling • fluorophore

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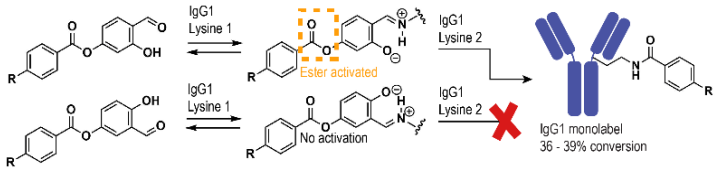

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A Reagent for Amine-Directed Conjugation to IgG1 Antibodies

Double up: A method for site-directed conjugation to antibodies is developed, in which a first lysine both directs and activates the reagent for reaction with a neighboring lysine.