

Reinvestigation of the Automated Synthesis of Stoichiometrically Conjugated Antibodies to Access High Molecular Weight Payloads and Multiplexed Conjugation via an In-Solution Trans-Tagging Process

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ABSTRACT: Protein conjugates have found applications in a wide variety of fields, ranging from therapeutics to imaging and detection. However, robust control over the parameters of the conjugation process (such as sites and degree of conjugation) remains challenging. Previously, our group introduced Equimolar NATive Chemical Tagging (ENACT), a method which allows for the monofunctionalization of proteins by combining an iterative low-conversion bioconjugation, an automated process, and a bioorthogonal trans-tagging reaction. However, while the automated ENACT was dimensioned to achieve monoconjugation at the mg scale, in early stage research, because of the rarity and cost of the starting materials, it is often necessary to prepare conjugates at the lower, μg , scale. Here, we introduce modified ENACT protocols, as well as a new ENACT conjugation reagent, which allow for the monofunctionalization of proteins on the micrograms scale, using minimal quantities of payload.

1. INTRODUCTION

Bioconjugation is a chemical process that enables the linkage between biomolecules and synthetically engineered molecules. By providing new functions to biomolecules, conjugation plays a central role in many areas of fundamental research and drug discovery, with prominent applications such as antibody–drug conjugates (ADCs)¹ and high-resolution imaging, but also in analytic biochemistry with recent advances in immuno-PCR,^{2,3} proximity ligation assays,^{4,5} rolling circle amplification,⁶ and DNA paint.⁷ This wide panel of applications underlines the need for generic methods that provide high-quality conjugates from different proteins and a wide variety of payloads. Quality in this regard deals with keeping the integrity of biomolecule function and controlling the outcome of the chemical reaction in terms of molecular composition of the sample and particularly of the homogeneous degree of conjugation. This wide range of applications also implies an extraordinarily wide-scale range in terms of the quantity and concentration at which these chemical bond forming processes have to be implemented, from a μg scale, for microscopy imaging reagent preparation or early drug screening, to a multi kg scale for the marketed ADCs.

In a recent publication,⁸ we introduced Equimolar NATive Chemical Tagging (ENACT, Figure 1) as a new technology allowing access to native antibody (Ab) conjugates with the precise attachment of one single payload per antibody (degree of conjugation DoC = 1). In this automated process, a native Ab is iteratively engaged into cycles of low-conversion chemical modification with an electrophilic biotinylated reagent and subsequently immobilized onto a streptavidin column (loading step). The biotinylated reagent features an iminosydnone

moiety, ultimately allowing for the release of the desired monoconjugated species (i.e., DoC = 1) from streptavidin through a bioorthogonal trans-tagging reaction with a strained alkyne-functionalized payload (trans-tagging step) (Figure 1).⁹

Using this method, the resulting Ab–payload bioconjugates obtained from native mAbs bear the unique characteristics of being prepared from off-the-shelf protein and being stoichiometric adducts while keeping a certain degree of heterogeneity. This DoC specific strategy might have an advantage over lysine or cysteine conjugation for analytic application and overcome the risky selection of a mutation point's location encountered in the development of ADCs from engineered cysteine antibodies such as THIOMAB.

In our first technology platform, the automated ENACT experimental setup was dimensioned in order to enable a mg-scale synthesis of stoichiometric Ab–payload conjugates; starting from 25 mg of Ab, 3 equiv of strained alkyne-functionalized payload resulted in about 12.5–15 mg of pure DoC specific conjugate. While suitable for early *in vivo* testing, this scale is not optimum for early-stage research or for routine preparation of the imaging agent, when it is often necessary to prepare the conjugate at the lower, μg , scale due to the rarity and

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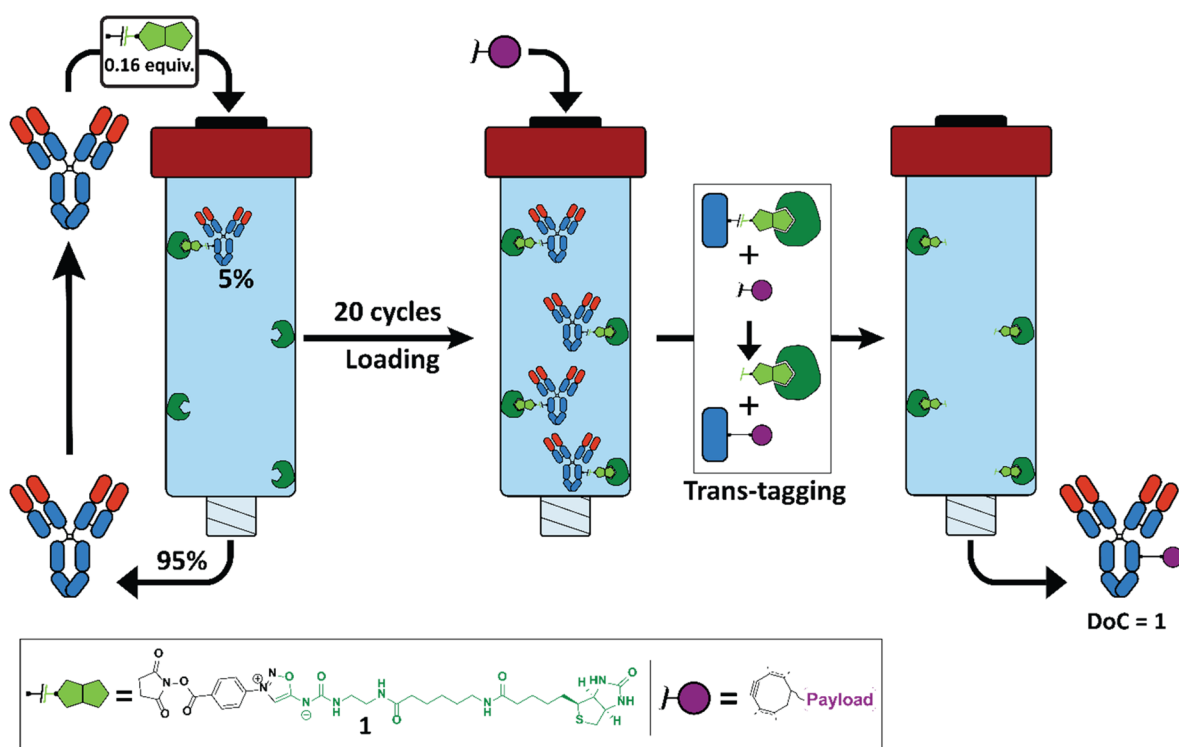


Figure 1. Illustration of the ENACT principle: a native antibody is iteratively engaged into 20 cycles of low-conversion conjugation with reagent **1**, such that at every cycle 5% of antibodies are monobiotinylated and immobilized onto a streptavidin column by filtration of the crude mixture (loading step). The resulting immobilized antibodies are then submitted to a trans-tagging process, thus releasing the monoconjugated antibody.

cost of the biological material and/or the payload. We thus decided to investigate ways to efficiently carry out the ENACT strategy on this smaller scale.

Miniaturizing the whole device would have required a drastic adaptation of the existing setup, while still risking important product loss arising from nonspecific adsorption of proteins on solid surfaces, which often becomes troublesome when working on a small scale.¹⁰ We thus decided to keep the original setup reactor and operating software (which is freely available online) and to adapt the experiment protocol to small-scale conjugation.

In this article, we present modified ENACT protocols allowing DoC selective conjugation for cases where either the payload or the protein is rare and available only at a small scale or when conjugation has to be run in parallel with multiple payloads. To achieve this, we explored two strategies: the first consisted of using a sacrificial nucleophile to artificially keep the total nucleophile concentration high, and the second strategy relied on performing the trans-tagging step in solution, in order to achieve higher kinetics and allowing us to run multiple conjugations in parallel. The latter also allowed us to overcome limitations previously encountered when trying to perform the trans-tagging reaction on the streptavidin column with high molecular weight payloads.

2. MATERIALS AND METHODS

ENACT Conjugation Protocol. Loading Step. A 1 mL HiTrap Streptavidin HP column (Cytiva cat. no. 17511201; Figure S1 and B) was equilibrated with 10 mL of PBS pH 8.5 (adjusted with NaOH). For each experiment, 5 mL of solution of PBS pH 8.5 containing a chosen amount of one or two proteins (see Table 1–Table 4) was prepared and loaded into a 10 mL syringe (Figure S1, C). The protein solution (Figure S1, C), streptavidin column (Figure S1, B), and an open reactor

syringe (Figure S1, A) were installed as pictured in Figure S1. The 5 mL contained in syringe C is pushed through the streptavidin column, into syringe A. This assembly was setup onto the automated ENACT device, as described in the original ENACT article.⁸ Then, the column was equilibrated (by pushing the solution from A to C and then from C to A, twice).

The conjugation reagent, **1** or **2**, was dissolved in DMSO, at the chosen concentration (usually, 500 μ M), and placed into a 1 mL syringe (Figure S1, D). The device then automatically added in portion the reagent solution into the reactor at each step, starting from 54 μ L in the first step and diminishing the volume by 5% after each step. This process is usually performed overnight. The next day, the setup was disassembled, and the streptavidin column was rinsed with 5 mL of PBS pH 7.4, placed at 4 $^{\circ}$ C for at least 12 h, and rinsed a second time to remove the unconjugated proteins and reagent.

Trans-Tagging Step. When the loading step was performed using reagent **1**, the streptavidin column was then equilibrated with a solution of strained alkyne (usually DBCO acid, concentrated at 1 mM in a PBS/DMSO 9:1 solution) and incubated overnight at 25 $^{\circ}$ C. The streptavidin column was then mounted onto the AKTA Pure chromatography system, equipped with a Superdex 200 Increase 10/300 GL column, and the trans-tagged antibody was thus purified and collected.

Elution Step. When the loading step was performed using reagent **2**, the streptavidin column was then equilibrated with a 50 mM D-biotin solution (dissolved in 50 mM potassium phosphate buffer, pH = 6.8) and incubated for 24 h at 25 $^{\circ}$ C. The streptavidin column was then mounted onto the AKTA Pure chromatography system, equipped with a Superdex 200 Increase 10/300 GL column, and the eluted monodesthiobiotinylated antibody was purified and collected.

The trans-tagging step was performed as indicated above.

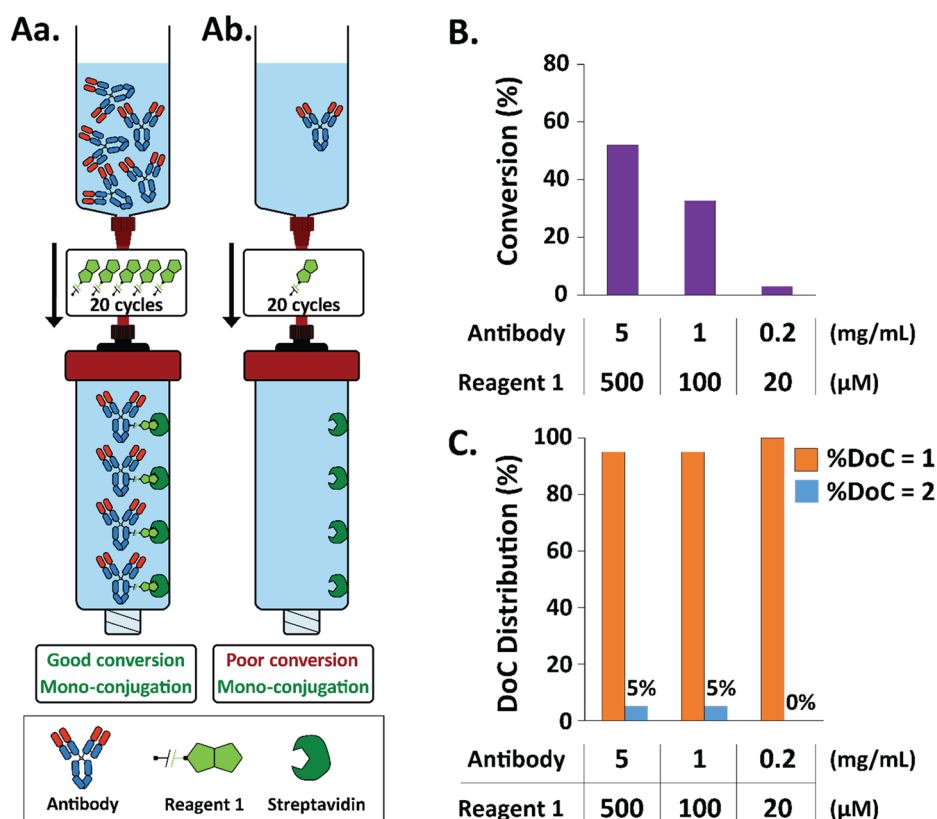


Figure 2. Effect of the concomitant dilution of reagent and Ab on the conversion and DoC distribution: ENACT conjugation was performed either at the original concentrations of antibody (Figure 2Aa) or after 5-fold and 25-fold dilution of both solutions (Figure 2Ab). After the trans-tagging step was performed with an excess of DBCO acid and further size-exclusion chromatography purification (see SI), the conversion was calculated via integration of the UV absorbance at 280 nm from the elution spectrum (Figure 2B), and the DoC distribution was evaluated by native MS (Figure 2C). For experimental details, see SI and Table S1.

Chemical Synthesis Should be same format as ENACT conjugation Protocol. The chemical syntheses of compounds 1–11 are described in SI.

Bioconjugation Chemistry Should be same format as ENACT conjugation Protocol. In-Solution Trans-Tagging Step with DBCO Acid Should be same format as Elution step. Monodesthiobiotinylated trastuzumab (50 μL, 2 mg/mL, in DPBS 1x) was added with DBCO acid (1.5 mM in DMSO, 2, 5, or 10 equiv) and incubated at 25 °C for 24 h. The monoconjugated antibodies were then separated from the excess DBCO acid by gel filtration on Biospin P-30 columns (Bio-Rad, Hercules, U.S.A.) pre-equilibrated with DPBS (1x, pH 7.4).

Strained Alkyne-Functionalized Oligonucleotides Should be same format as Elution step. In a 2 mL Eppendorf tube, 5'-amino-modified oligonucleotide (1 equiv, 50 μL, 1 mM in water) was combined with (1R,8S,9s)-bicyclo[6.1.0]non-4-yn-9-ylmethyl (4-nitrophenyl) carbonate (20 equiv, 50 μL, 20 mM in DMSO) and NaHCO₃ (100 equiv, 5 μL, 1 M in water). The mixture was incubated at 25 °C overnight. The mixture was then diluted with water to a final volume of 300 μL and added to acetone (900 μL) and LiClO₄ (20 μL, 3 M in water) in order to precipitate the oligonucleotide species. The sample was then centrifuged (15000g, 8 min), and the supernatant was discarded. The precipitate was dissolved with water (300 μL) to repeat the precipitation and centrifugation procedure a second time.

It was then dissolved with water (100 μL) and purified by HPLC (detection at 260 nm, mobile phase gradient A/B 9:1 to 6:4 in 30 min; see Figure S4). After lyophilization, the ON conjugate was dissolved in DPBS (1x, pH 7.4) and analyzed by

absorption spectrophotometry (measured at 260 nm using a Nanodrop) to calculate the solution's concentration using Beer–Lambert's law.

Antibody–Oligonucleotide Conjugate Synthesis Should be same format as Elution step. Monodesthiobiotinylated trastuzumab (200 μL, 5 mg/mL, in DPBS 1x) was added with 30 μL of DPBS 10x (to prevent precipitation of the expected antibody–oligonucleotide conjugates) and BCN-functionalized oligonucleotides (1 mM in DPBS 1x, pH = 7.4, 10 equiv). The mixture was incubated at 25 °C for 24 h. The monoconjugated antibodies were then separated from unreacted antibodies and the excess oligonucleotide by size exclusion chromatography using the AKTA Pure System (isocratic elution with DPBS (1x, pH 7.4), 0.5 mL/min; see Figure S5).

Antibody–Polymer Conjugate Synthesis Should be same format as Elution step. Monodesthiobiotinylated rituximab (100 μL, 2 mg/mL, in DPBS 1x) was added to either a DBCO-functionalized 53 kDa linear HPMA polymer (9, 2 mM in DMSO, 10 equiv) or a DBCO-functionalized 260 kDa branched HPMA polymer (11, 2 mM in DMSO, 10 equiv). The mixtures were incubated at 25 °C for 24 h. The monoconjugated antibodies were then separated from unreacted antibodies and the excess of polymers by size exclusion chromatography using an AKTA Pure System (Superdex 200 Increase 10/300 GL column, isocratic elution with DPBS (1x, pH 7.4), 0.5 mL/min; Figure S6).

Native MS Should be same format as Elution step. Representative native MS spectra of conjugated trastuzumab, following trans-tagging with either DBCO acid or 5'BCN-

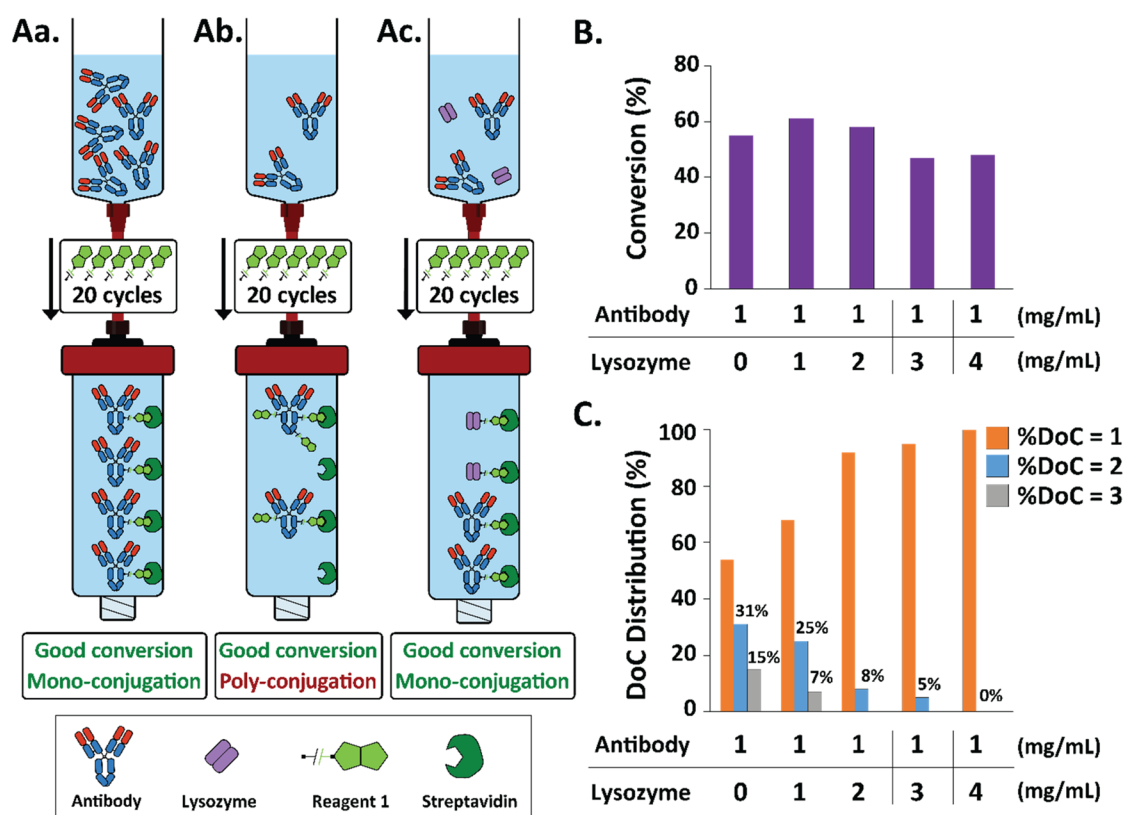


Figure 3. Illustration of different reaction conditions for various Ab/lysozyme ratios. Compared to the original conditions (Figure 3Aa), ENACT conjugation was performed here using a 5-fold diluted trastuzumab solution and the usual conjugation reagent 1 solution (Figure 3Ab). Then, the same experiment was performed four times but with increasing concentrations of lysozymes added to trastuzumab solution (Figure 3Ac). After performing the trans-tagging step with excess of DBCO acid and further size-exclusion chromatography purification (see SI), conversion was calculated via integration of the UV absorbance at 280 nm from the elution spectrum (Figure 3B), and the DoC distribution was evaluated by native MS (Figure 3C). For experimental details, see SI and Table S2.

modified 37mer single-stranded DNA are provided in SI (see Figures S7, S8, S9, and S10).

3. RESULTS AND DISCUSSION

In our first article, 25 mg of antibody was used in a solution of 5 mL of aqueous phosphate buffer saline (PBS) at pH = 7.4 (i.e., 5 mg/mL solution), and the conjugation reagent 1 was dissolved in about 700 μ L of DMSO at a concentration of 500 μ M. In each of the 20 cycles, 0.16 equiv of the conjugation reagent 1 was added to the antibody solution. After the washing and trans-tagging steps, about 12.5–15 mg of antibody–payload conjugate was collected (i.e., 50–60% conversion rate), composed of at least 95% of monoconjugated species.⁸ In order to use the automated ENACT device without modification to the setup itself (Figure S1), we kept the volumes of both the antibody solution (5 mL) and the conjugation reagent (ca. 700 μ L) fixed.

When this conjugation was first performed on a smaller scale, we attempted to simply dilute both antibody and reagent solutions by an identical factor (Figure 2A). Accordingly, ENACT conjugations were performed on the model antibody trastuzumab, at 3 different concentrations: at the reported concentrations of 5 mg/mL for the 5 mL trastuzumab solution and 500 μ M for the activated ester solution (see concentrated solution in Figure 2Aa); at the 5-fold diluted concentrations of 1 mg/mL for the trastuzumab solution and 100 μ M for the reagent solution; and at the 25-fold diluted concentrations of 0.2 mg/

mL for the trastuzumab solution and 20 μ M for the reagent solution (see the diluted solution in Figure 2Ab).

For each experiment, after the standard 20 loading cycles, the streptavidin columns were washed with PBS twice to remove the traces of the remaining unconjugated trastuzumab. The column was then loaded with 1 mL of a 1 mM solution of dibenzocyclooctyne acid (DBCO acid), serving as a model trans-tagging payload, and incubated for 24 h at 25 °C to allow trans-tagging to proceed to completion. The conjugated species released upon trans-tagging were then separated from the excess payload by size exclusion chromatography (SEC). The conversion rates were assessed by measuring the UV absorbance at 280 nm, and the DoC distributions were evaluated using native mass spectrometry (Figure 2B and 2C).

Repeating ENACT at the original concentration conditions afforded as expected a 50–60% conversion evaluated from the initial amount of antibody engaged in the process (Figure 2B first column) and a conjugate composed of more than 95% of the DoC 1 species (Figure 2C first column). When diluting these original conditions 5-fold, the mixture composition, in terms of DoC species, was maintained (Figure 2C second column); however, the conversion rate decreased from 52% to ca. 30% (Figure 2B second column). When further diluting (25-fold), the composition in terms of DoC was still maintained, but the conversion was further dramatically reduced to only a few percent (Figure 2B and C third column).

Hence, it clearly appeared that simple dilution of both antibody and reagent solutions did not yield a viable option to

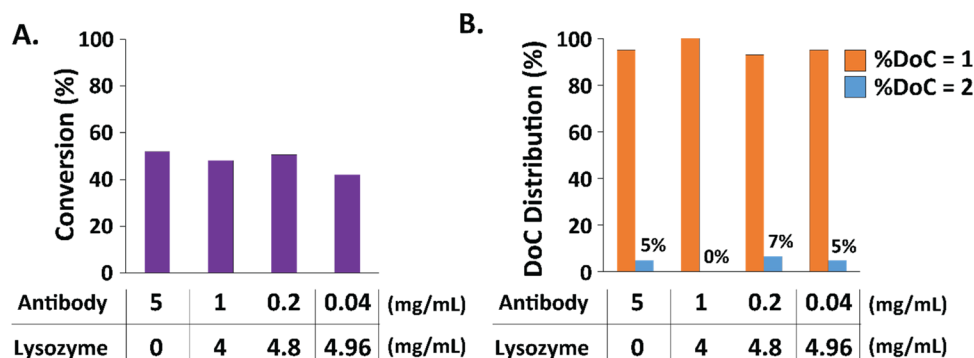


Figure 4. ENACT conjugation was performed using solutions containing both trastuzumab and lysozyme at a total protein concentration of 5 mg/mL. The same experiment was performed with various Ab/lysozyme ratios. After performing the trans-tagging step with an excess of DBCO acid and further size-exclusion chromatography purification (see detailed protocol in SI), conversion was calculated via integration of the UV absorbance at 280 nm from the elution spectrum (Figure 4A), and the DoC distribution was evaluated by native MS (Figure 4B). For experimental details, see SI and Table S3.

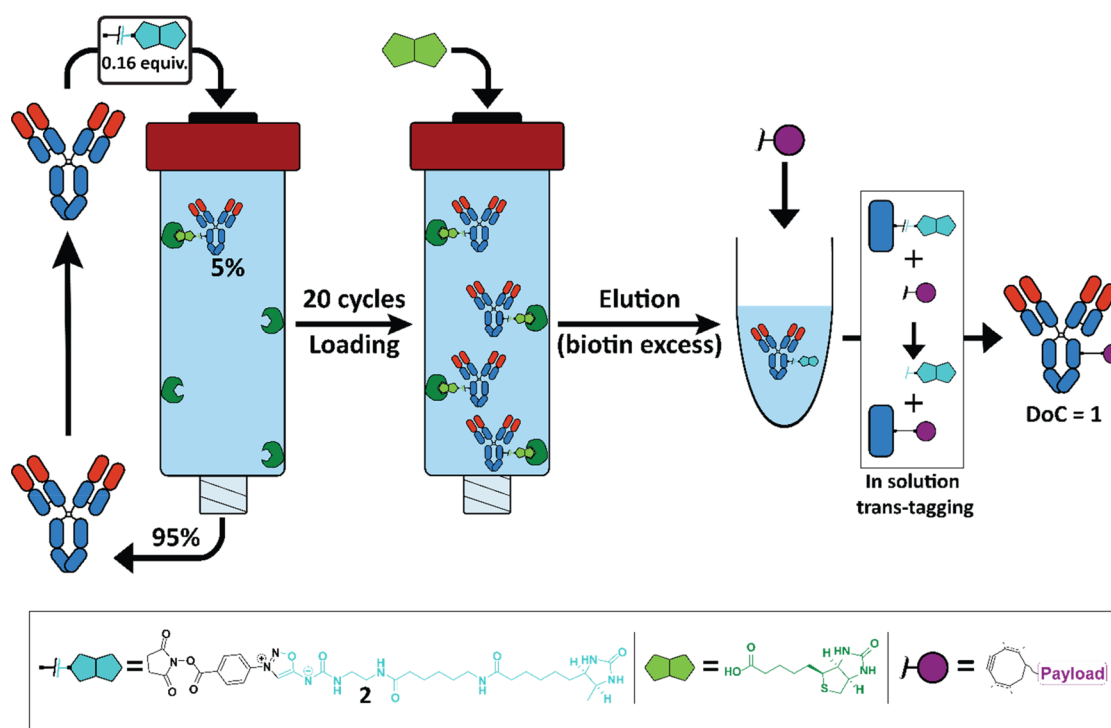


Figure 5. Principle of the ENACT method using the conjugation reagent 2: a native antibody is iteratively engaged into 20 cycles of low conversion with conjugation reagent 2, such that at every cycle 5% of antibodies are monobiotinylated and immobilized onto a streptavidin column (loading step). After washing of the streptavidin column with PBS, it is equilibrated with 1 mL of 50 mM biotin in PBS for 24 h at 25 °C. The monodesthiobiotinylated antibodies are then eluted from the column and isolated by size exclusion chromatography. The trans-tagging reaction is then performed in solution with a strained alkyne-functionalized payload.

perform ENACT on a small protein scale. This drop in conversion is attributed to the conjunction of a slower conjugation reaction and the competitive hydrolysis of the activated ester becoming predominant at low concentrations.

Conjugating a Low Amount of Antibody Using the “Sacrificial Nucleophile” Approach. We decided to maintain the electrophilic reagent solution at its original concentration while diluting 5-fold the concentration of the antibody solution. As with the previous experiments, we performed washings with PBS and trans-tagging with DBCO acid and evaluated the conversion rate by measuring the UV absorbance at 280 nm and the DoC distribution by native MS analysis (Figure 3Ab, parts B and C, first column). As expected, we observed satisfying conversion, superior to 50%. However,

using a diluted antibody solution but nondiluted conjugation reagent resulted in an increased electrophile/trastuzumab ratio at each cycle and, unsurprisingly, resulted in a broadened DoC distribution, with half of DoC 1 contaminated with multi-conjugated DoC 2 and DoC 3 species (Figure 3Ab and C, first column).

In order to keep DoC distribution narrow, we hypothesized that a second protein could be added to the reaction mixture and act as a “sacrificial nucleophile” (SN) which would react with the electrophilic conjugation reagent, 1 (Figure 3Ac). This SN protein should be significantly smaller than the antibody to facilitate its separation via SEC after trans-tagging release. For this purpose, we selected lysozyme, a 14.4 kDa protein featuring 6 lysine residues.

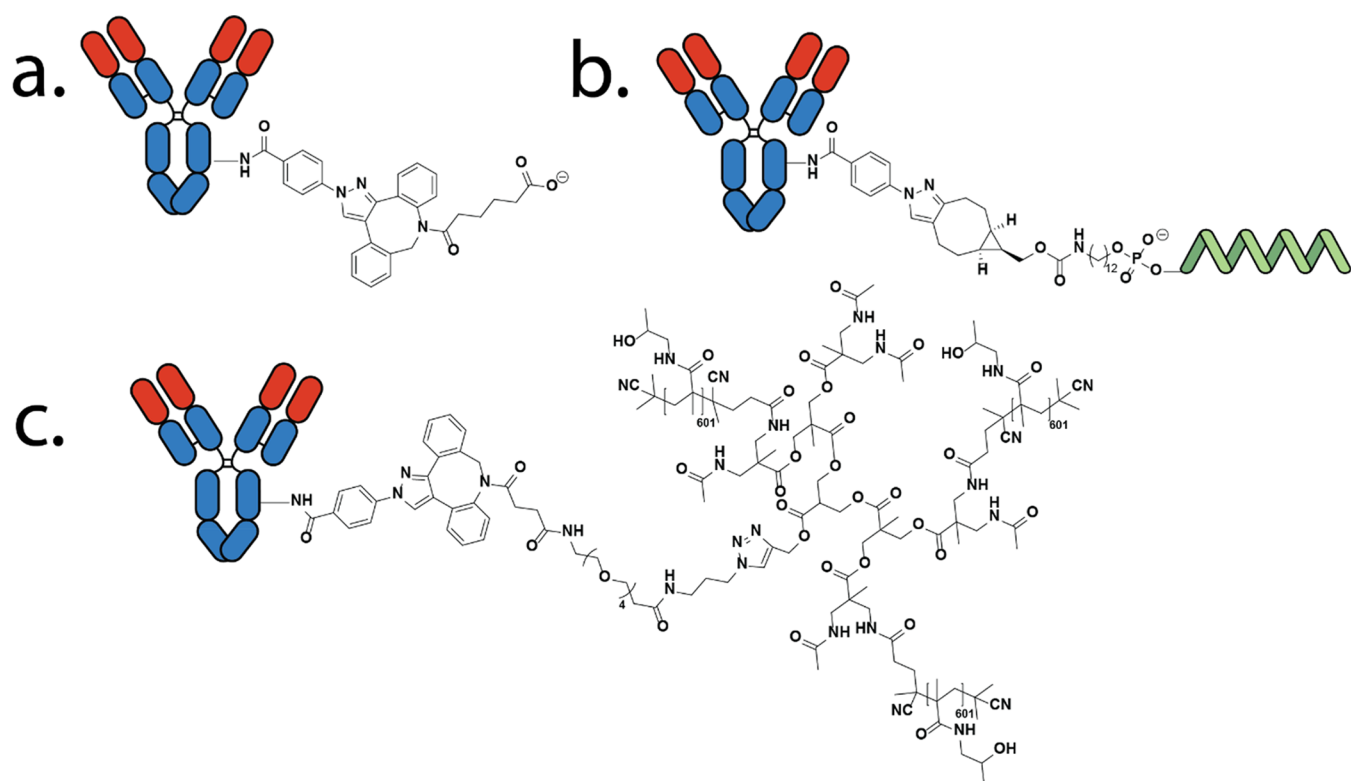


Figure 6. Methods described in this manuscript allowed for the preparation of antibodies monoconjugated with small molecules (a.), oligonucleotides (b.), and large polymers (c.) from DBCO-modified payloads (see structures 9 and 11 in SI).

In order to determine the necessary quantity of lysozyme that would neutralize the excess of reagent and yield monoconjugated antibody, we prepared solutions containing 1 mg/mL of trastuzumab, in a mixture with increasing concentrations of lysozyme (Figure 3Ac). After performing ENACT, using the usual 500 μ M solution of conjugation reagent, we evaluated the conversion rates and DoC distribution obtained for each solution (Figure 3B and C).

As shown in Figure 3B, the conversion rate is consistent, between ca. 50 and 60% for all trastuzumab/lysozyme mixtures. As shown in Figure 3C, the DoC distribution profiles gradually evolve from polyconjugated to monoconjugated upon addition of increasing amounts of lysozyme. Of note, when the solution contains 4 mg/mL of lysozyme, in addition to 1 mg/mL of trastuzumab, the process yields conjugated trastuzumab at a conversion rate and DoC similar to what was obtained in the original protocol with 5 mg/mL of trastuzumab.

This might be explained by a similar lysine density of the two proteins. Lysozyme features 6 lysines out of 129 amino acids, while out of its 1326 amino acids, trastuzumab features 88 lysines in total; however, 69–70 of them are accessible.^{11,12} Thus, for both proteins, about 5% of residues are accessible lysines, which means that 1 mg of either trastuzumab or lysozyme would roughly behave similarly in reaction with an electrophile such as the conjugation reagent 1.

To evaluate the limit of this approach, two additional solutions containing both trastuzumab and lysozyme, at a total protein concentration of 5 mg/mL but a lower concentration of trastuzumab (0.2 and 0.04 mg/mL), were prepared and engaged in ENACT conjugation. Satisfyingly, all experiments resulted in good conversion rates and >95% of Doc 1 species (Figure 4).

It thus appears that the sacrificial protein strategy enables one to perform ENACT bioconjugation very reliably, on as low as

200 μ g of native protein. Trans-tagging is performed easily, and the desired DoC = 1 antibody is conveniently separated from the SN by SEC (see SI).

However, in this protocol, the trans-tagging step required an excess of DBCO payload since both sacrificial protein and the desired antibody were immobilized on the streptavidin column. It is thus not adapted when the payload is scant or when multiple conjugates have to be synthesized from a single tagged protein. To address this, we designed a second process in which the trans-tagging is performed in solution.

Working with a Scarce Quantity of Payload Using a Novel, Desthiobiotin-Based Conjugation Reagent. In the above-described method, the trans-tagging step occurred at the solid support/liquid interface and relied on the favorable kinetics of the click-and-release reaction between the iminosydnone and the strained alkyne.^{8,9} We hypothesized that eluting the biotinylated antibody from the streptavidin column would yield a solution of DoC = 1 conjugates. This solution could then be split into smaller portions on which several trans-tagging reactions could be performed with different payloads in solution, presumably with higher kinetics and using only a minimal amount of payload.

We first attempted to displace the captured biotinylated trastuzumab from the streptavidin column by equilibrating the column with an excess of free biotin. Unfortunately, only a very limited amount of the antibody was recovered. We thus turned to desthiobiotin, a derivative of biotin which also binds streptavidin, though with less affinity (Figure 5).¹³ We synthesized electrophilic reagent 2, analogous to reagent 1 in which desthiobiotin replaces biotin (Figure 5; see detailed synthesis and characterization in SI, Figures S2 and S3).

To evaluate this new reagent, 20 cycles of ENACT were performed in classical conditions (5 mL of solution of

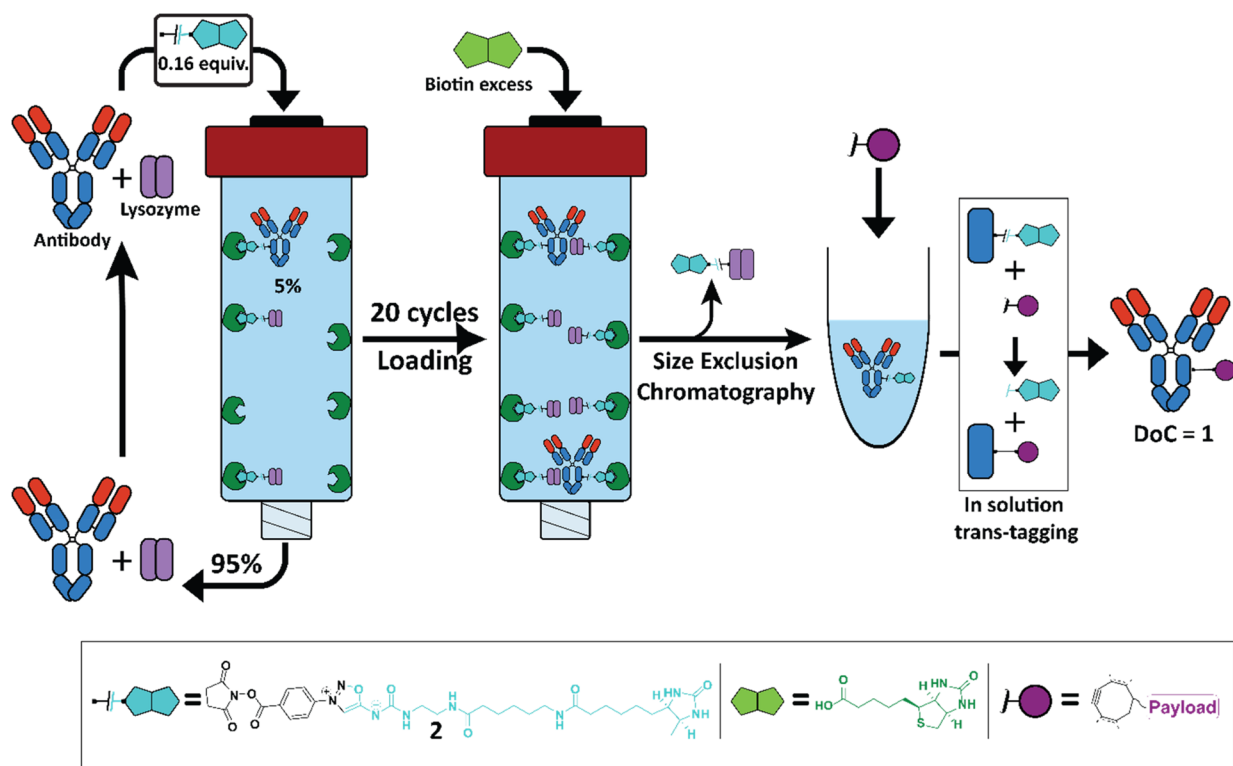


Figure 7. Principle of the ENACT method combining the use of lysozyme as a sacrificial nucleophile and in-solution trans-tagging: A mixture of native antibody and lysozyme is iteratively engaged in 20 cycles of low conversion with conjugation reagent **2** (loading step) after equilibration with biotin for 24 h at 25 °C. The monodesthiobiotinylated antibodies are eluted from the column and separated from desthiobiotinylated lysozyme by size exclusion chromatography. The trans-tagging step is then performed in solution via a click-and-release reaction involving the iminosynone moiety embedded in the linker and a strained alkyne-functionalized payload.

trastuzumab concentrated at 5 mg/mL). After washing with PBS twice to remove unreacted antibody and hydrolyzed conjugation reagent, we equilibrated the streptavidin column with 1 mL of a solution of free biotin, concentrated at 50 mM in PBS, and incubated it at 25 °C for 24 h. The content of the column was eluted and purified in-line via SEC to yield 13.25 mg (53%) of desthiobiotinylated DoC = 1 trastuzumab.

In order to evaluate the efficacy of the *in-solution* trans-tagging, three 50 μ L aliquots of DoC = 1 desthiobiotinylated trastuzumab at 2 mg/mL were mixed with 2, 5, and 10 equiv of DBCO acid (i.e., 1.4, 3.4, and 6.9 nmol, respectively) and incubated at 25 °C for 24 h. Using gel filtration, the conjugates were isolated in ca. 70% yields and analyzed by native MS. In all cases, the monoconjugated product of the click-and-release adduct was observed as the only species, highlighting the efficacy of the click chemistry reaction performed in solution, despite the low amount of DBCO-functionalized payload.⁹

In order to evaluate the advantage of this protocol for difficult to couple payloads, we performed trans-tagging of monodesthiobiotinylated antibody in solution using either 10 equiv of a BCN-functionalized 37mer DNA single strand (see synthesis and characterization, Figure S4) or 10 equiv of either a DBCO-functionalized 53 kDa linear HPMA polymer or a bulkier DBCO-functionalized 260 kDa branched HPMA polymer (see syntheses and characterization of **9** and **11** in SI and Table S5).

Satisfyingly, while trans-tagging of polymer payloads did not work when the antibody was immobilized on the column, performing the trans-tagging in solution on aliquots of monodesthiobiotinylated antibodies allowed the conjugation of a DBCO-modified single-stranded DNA, linear, and branched

HPMA polymer (obtained in 46, 39 and 40% yields, respectively; Figures 6a–c, S5, S6, and S10). This highlights the value of using desthiobiotin-based reagent **2** to undertake the conjugation of macromolecular payloads of high added value from minimal amounts of desthiobiotinylated antibody.

By means of the sacrificial protein method or the desthiobiotin reagent, ENACT can then be performed using either a low amount of protein or a low amount of payload. We next combined the two methods to achieve ENACT from both a low amount of native protein and a low amount of payload.

Working with Both a Low Amount of Antibody and Payload. We first loaded the column with 200 μ g of monoconjugate ramucirumab using the desthiobiotinylated reagent **2** (Figure 7). The conjugation/immobilization were performed as described above, in 20 cycles on a 5 mL solution concentrated at 0.04 mg/mL of ramucirumab and at 4.98 mg/mL of lysozyme, using the conjugation reagent **2** (700 μ L solution, 500 μ M). Then, following the washing/eluting steps with a solution containing biotin, 42 μ g (21%) of monoconjugated ramucirumab was isolated by SEC (see Table S4). As expected, only the DoC = 1 species was observed by native MS analysis. This demonstrates that both methods could be combined, opening the way to prepare a pure DoC = 1 conjugate starting from readily available nonengineered antibody and complex payloads.

4. CONCLUSION

DoC specific conjugation gives access to protein conjugates with close to monodispersity and precise characteristics that may provide advantages for quantitative analysis by imaging and

immuno PCR or for therapeutic applications. The ENACT is very versatile in the way that it enables us to achieve such a narrow distribution from any native off-the-shelf protein with a wide range of payloads. However, the original setup that has been optimized to milligram-scale conjugation was not optimal for conjugation of small quantities. In this paper, we have developed alternative methods suited to the different cases encountered: when the protein is only available in small quantities, when the payload is only available in small quantities, when both are only available in small quantities, or when it is necessary to do a large number of conjugations in parallel. This was achieved by combining the interesting concept of a sacrificial protein, which allowed us to maintain conjugation selectivity while overcoming the fast reagent hydrolysis, and a novel desthiobiotinylated electrophilic reagent that enabled the elution of monoconjugated protein from the streptavidin column.

Combining the two innovations permitted us to perform small-scale parallel DoC 1 conjugation and notably to overcome the kinetic limitations encountered when performing trans-tagging reactions on-column with large MW payloads affording original stoichiometric antibody–polymer conjugates.

■ ASSOCIATED CONTENT

SI Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acsomega.3c05206>.

Figure S1: Schematic representation of the ENACT setup; Figure S2: General synthesis scheme for the conjugation reagent; Figure S3: LCMS analysis of the conjugation reagent; Figure S4: HPLC analysis of modified oligonucleotides; Figure S5: SEC purification of antibody–oligonucleotide conjugates; Figure S6: SEC purification of antibody–polymer conjugates; Figure S7: MS analysis of polyconjugated antibody; Figure S8 an S9: MS analysis of monoconjugated antibody; Figure S10: MS analysis of stoichiometric antibody–oligonucleotide conjugate. Table S1: Exact quantities involved in the experiments of Figure 2; Table S2: Exact quantities involved in the experiments of Figure 3; Table S3: Exact quantities involved in the experiments of Figure 4; Table S4: Exact quantities involved in the experiments of Figure 6; Table S5: SEC-MALS analysis of polymers (PDF)

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Notes

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■ ABBREVIATIONS

ENACT, equimolar native chemical tagging; ADC, antibody–drug conjugate; DoC, degree of conjugation; DBCO, dibenzocyclooctyne; HPMA, *N*-(2-hydroxypropyl) methacrylamide

■ REFERENCES

- Beck, A.; Goetsch, L.; Dumontet, C.; Corvaia, N. Strategies and Challenges for the next Generation of Antibody–Drug Conjugates. *Nat. Rev. Drug Discov* **2017**, *16* (5), 315–337.
- Janssen, K. P. F.; Knez, K.; Spasic, D.; Lammertyn, J. Nucleic Acids for Ultra-Sensitive Protein Detection. *Sensors (Basel, Switzerland)* **2013**, *13* (1), 1353.

- (3) Ryazantsev, D. Y.; Voronina, D. V.; Zavriev, S. K. Immuno-PCR: Achievements and Perspectives. *Biochemistry (Mosc)* **2016**, *81* (13), 1754–1770.
- (4) Dhillon, H. S.; Johnson, G.; Shannon, M.; Greenwood, C.; Roberts, D.; Bustin, S. Homogeneous and Digital Proximity Ligation Assays for the Detection of Clostridium Difficile Toxins A and B. *Biomolecular Detection and Quantification* **2016**, *10*, 2–8.
- (5) Greenwood, C.; Johnson, G.; Dhillon, H. S.; Bustin, S. Recent Progress in Developing Proximity Ligation Assays for Pathogen Detection. *Expert Rev. Mol. Diagn* **2015**, *15* (7), 861–867.
- (6) Schweitzer, B.; Wiltshire, S.; Lambert, J.; O'Malley, S.; Kukanskis, K.; Zhu, Z.; Kingsmore, S. F.; Lizardi, P. M.; Ward, D. C. Immunoassays with Rolling Circle DNA Amplification: A Versatile Platform for Ultrasensitive Antigen Detection. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97* (18), 10113–10119.
- (7) Nieves, D. J.; Gaus, K.; Baker, M. A. B. DNA-Based Super-Resolution Microscopy: DNA-PAINT. *Genes* **2018**, *9* (12), 621.
- (8) Dovgan, I.; Hentz, A.; Koniev, O.; Ehkirch, A.; Hessmann, S.; Ursuegui, S.; Delacroix, S.; Riomet, M.; Taran, F.; Cianfèrani, S.; Kolodych, S.; Wagner, A. Automated Linkage of Proteins and Payloads Producing Monodisperse Conjugates. *Chem. Sci.* **2020**, *11* (5), 1210–1215.
- (9) Bernard, S.; Audisio, D.; Riomet, M.; Bregant, S.; Sallustrau, A.; Plougastel, L.; Decuypere, E.; Gabillet, S.; Kumar, R. A.; Elyian, J.; Trinh, M. N.; Koniev, O.; Wagner, A.; Kolodych, S.; Taran, F. Bioorthogonal Click and Release Reaction of Iminosynones with Cycloalkynes. *Angew. Chem., Int. Ed.* **2017**, *56* (49), 15612–15616.
- (10) Nakanishi, K.; Sakiyama, T.; Imamura, K. On the Adsorption of Proteins on Solid Surfaces, a Common but Very Complicated Phenomenon. *J. Biosci. Bioeng.* **2001**, *91* (3), 233–244.
- (11) Gautier, V.; Boumeester, A. J.; Lössl, P.; Heck, A. J. R. Lysine Conjugation Properties in Human IgGs Studied by Integrating High-Resolution Native Mass Spectrometry and Bottom-up Proteomics. *Proteomics* **2015**, *15* (16), 2756–2765.
- (12) Schumacher, D.; Hackenberger, C. P. R.; Leonhardt, H.; Helma, J. Current Status: Site-Specific Antibody Drug Conjugates. *J. Clin. Immunol.* **2016**, *36*, 100–107.
- (13) Hirsch, J. D.; Eslamizar, L.; Filanoski, B. J.; Malekzadeh, N.; Haugland, R. P.; Beechem, J. M.; Haugland, R. P. Easily Reversible Desthiobiotin Binding to Streptavidin, Avidin, and Other Biotin-Binding Proteins: Uses for Protein Labeling, Detection, and Isolation. *Anal. Biochem.* **2002**, *308* (2), 343–357.