Title
Improving TCO-Conjugated Antibody Reactivity for Bioorthogonal Pretargeting

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Improving TCO-Conjugated Antibody Reactivity for Bioorthogonal Pretargeting

THESIS

submitted in partial satisfaction of the requirements for the degree of

MASTER OF SCIENCE

in Biomedical Engineering

by

Tina Tingyi Chu

Thesis Committee:
Assistant Professor Jered Haun, Chair
Assistant Professor Chang Liu
Assistant Professor Wendy Liu

2016
DEDICATION

To

my parents, sister, and friends

In recognition of their unconditional love and support
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ACKNOWLEDGMENTS

I would like to express my deepest appreciation to my committee chair, Professor Jered Haun, who continually provided knowledge and encouragement throughout my undergraduate and graduate career. His enthusiasm and passion in teaching and research showed me that failure is not an option. He was always open to idea and suggestion, and gave direction whenever I ran into obstacles. This thesis would not have been possible without his guidance and support.

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ABSTRACT OF THE THESIS

Improving TCO-Conjugated Antibody Reactivity for Bioorthogonal Pretargeting

By

Tina Tingyi Chu

Master of Science in Biomedical Engineering

University of California, Irvine, 2016

Professor Jered Haun, Chair

Cancer remains a major cause of death because of its unpredictable progression. Utilizing bioorthogonal chemistry between trans-cyclooctene (TCO) and tetrazine to target imaging agents to tumors in two subsequent steps offers a more versatile platform for molecular imaging. This is accomplished by pretargeting TCO-modified primary antibody to cell surface biomarkers, followed by delivery of tetrazine-modified imaging probes. In previous work, it has been established that TCO-tetrazine chemistry can be applied to in vivo imaging, resulting in precise tumor detection. However, most TCO modifications on an antibody are not reactive because they are buried within hydrophobic domains. To expose and improve the reactivity, Rahim et al. incorporated a polyethylene glycol (PEG) linker through a two-step reaction with DBCO-azide, which successfully maintained 100% TCO functionality. In this project, various types of linkers were studied to improve the reactivity in a single step. Three primary types of linkers were studied: hydrophilic PEG chains, hydrophobic short linkers, and amphiphilic linkers. Our results show that PEG chain alone can only maintain 40% TCO reactivity. Unexpectedly, a short alkyl chain (valeric acid) provided superior results, with 60% TCO reactivity. Lengthening the alkyl chain did not improve results further. Finally, an amphiphilic linker containing valeric acid and PEG
performed worse than either linker type alone, at ~30% functionality. We conclude that our previous 100% functional TCO result obtained with the two-step coupling may have stemmed from generation of the DBCO/azide cycloaddition product. Future work will explore factors such as rigidity of linker structure, polarity, or charges.
CHAPTER 1: Introduction

There has been a rise of interest in nanomaterials for biomedical applications in disease targeting due to their inherent advantages of multivalency and coupling efficiency. Studies have shown that nanoparticles can not only act as controlled release drug carriers, but also exhibit stable signals due to their tunable physical properties [1]. To make use of their full diagnostic and therapeutic potentials, studies were conducted to develop a sensitive and rapid solution to overcome current diagnostic limitations in specificity and sensitivity.

1.1 Bioconjugation in Direct Targeting

![Figure 1: Schematic of a two-step bioorthogonal nanoparticle detection with pretargeting [1].](image)

The human system is regulated by intricate cell interactions to control its function and development. The balance between cell growth and programmable death maintains normal system activities. When these signals are disrupted, cells begin to proliferate uncontrollably, invade the surrounding normal tissues and cause diseases such as cancer [2]. Cancer is a metastatic disease that can spread throughout the body by penetrating vessel walls and into the circulation. Due to their unpredictable behavior, cancer remains among the leading causes of death worldwide [3]. Current cancer diagnostics and
treatments lack selectivity, destroying normal cells while eliminating the diseased ones. In order to provide efficient cancer therapy and accurate disease quantification, molecular probes have been developed to detect abnormal cell expression through pretargeting and bioorthogonal chemistry (Figure 1).

1.2 Development of Bioorthogonal Reaction Schemes

Staudinger ligation was an extensively used biomolecule labeling technique that can be performed in live animals without causing perturbation due to the small size of the azide molecule [4]. Reaction between azide and phosphine forms a stable amide bond after hydrolysis, creating potential in imaging and labeling application. Since azide does not exist in biological systems, the process is chemoselective. However, despite its popularity in labeling, phosphine has drawbacks in reaction kinetics, solubility, and susceptibility to oxidation [1, 4].

To address the kinetic limitation of Staudinger Ligation, copper-catalyzed azide-alkyne cycloaddition (CuAAC), or “click” chemistry, was developed. Utilizing the original advantages of azide, Huisgen cycloaddition reacts azides and alkynes to form triazoles to provide faster reaction speed [5]. Yet, because cycloaddition requires unfavorable high temperature or pressure, copper(I) is added as a catalyst. CuAAC is valued for its fast reaction speed and stability in aqueous solution under wide pH range from 4 to 12, but remains limited in application since Cu(I) is cytotoxic [5] and found to irreversibly quench the fluorescence of quantum dots (QD) if used as a nanoprobe [6]. To drive cycloaddition under catalyst-free condition, the Bertozzi group introduced higher ring strain to the alkyne moiety (cyclooctene) to increase the reactivity of alkyne. Other strained dienophiles
tested by other groups include norbornene, cyclooctyne, cyclopropene, and \textit{trans}-cyclooctene [1]. A faster kinetic rate is achieved comparing to the unstrained alkynes with no apparent cellular toxicity, but a slower rate than that of CuAAC and its aqueous solubility still prove limitations in applications [4].

\textbf{1.3 Inverse Electron-Demand Diels-Alder Cycloaddition}

Inverse electron-demand Diels-Alder cycloaddition is a labeling strategy that utilizes the unique cycloaddition chemistry between \textit{trans}-cyclooctene (TCO) and tetrazine (Tz) to prevent biological interference in a cellular environment [7]. Unlike other current methods used in clinics such as biotin-streptavidin or antibody-hapten, TCO-tetrazine conjugation produces little to no immunogenicity for its rapid and selective interaction in biological environment [8]. Along with other mutually bioorthogonal elements, this chemistry has the potential to allow simultaneous labeling of different probes within one system [7].

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{Figure2.png}
\caption{Bioorthogonal nanoparticle detection (BOND) [1].}
\end{figure}

Using TCO-modified antibodies as a scaffold, Tz-modified nanoparticles can be covalently coupled for direct targeting, providing selectivity and sensitivity within a
complex in vivo system [9]. Direct targeting of bioorthogonal nanoparticle detection (BOND) is scalable and can be done in a one-step (BOND-1) or two-step (BOND-2) reaction (Figure 2). In a one-step reaction, nanoprobes are coupled to affinity molecules first before exposing to cellular environment. This scheme allows straightforward detection, but with variability depending on the immunoconjugate antibodies selected (Figure 3). It is less consistent and provides minimal signal amplification. To address these, a two-step model was implemented, which has shown to be more specific, modular, and yield better signal amplification compared to BOND-1. Different from one-step, BOND-2 allows multiple nanoparticles binding to one antibody after the latter has attached onto cell surface markers. BOND-2 scheme is more stable and provides consistent signal amplification across different molecular determinants [9, 10, 11].

Figure 3: Comparison of BOND-1, BOND-2, and maleimide/thiol chemistry targeting schemes on three different antibodies [9].

TCO-Tz reaction is often used in studies for bioorthogonal conjugation for its advantages of exceptionally fast kinetics, chemoselectivity, and biocompatibility. Through Diels-Alder cycloaddition, reactions can run under catalyst-free condition even with low
protein concentration. Avoiding the use of Cu(I) catalysts, this non-cytotoxic application is suitable for in vivo imaging. Because of its fast ligation process, the chemistry enables intracellular assembly of complex molecular structures under dilute condition [12]. This is exceptionally useful because protein conjugation usually has concentration constraints due to antibody availability and cost.

1.4 Pretargeting Application

![Figure 4: In situ pretargeting on live A431 cells using QD-EGF (left two) and tetrazine-modified EGF antibody following by norbornene-QD coupling (right two) [14].](image)

Tetrazine-dienophile cycloaddition had been used in live cell labeling by Devaraj et al. to demonstrate the utility of such reaction for in vivo pretargeting imaging. Using a synthesized benzylamine-modified tetrazine, inverse Diels-Alder norbornene-Tz cycloaddition was found to be selective, stable, and reactive in aqueous solution and resulted in high signal yield. Two QD coupling methods were experimented. In a one-step reaction, norbornene-labeled QD was first coupled with Tz-modified EGF, and the QD-EGF complex was directly labeled onto A431, a human squamous carcinoma cell line that overexpressed EGFR. The group also developed a two-step reaction where Tz-modified
EGF was first pretargeted onto A431, following by coupling of norbornene-labeled QD under cycloaddition. Inverse Diels-Alder cycloaddition in both methods proved to be efficient in in situ cell targeting (Figure 4) [13, 14]. Haun et al. had also applied the bioorthogonal labeling technique on in situ clinical application, which showed a highly sensitive and accurate measurement. It was identified that bioorthogonal chemistry resulted in a higher accuracy of 96% than conventional cytology biopsy method (74%) or pathologist examination (84%) [9, 11, 15].

1.5 Project Overview

![Schematic diagram of TCO and DBCO conjugation](image)

**Figure 5:** Direct TCO conjugation vs. hydrophilic PEG linker introduction [10].

The purpose of this thesis project was to study the reactivity of conjugated TCO linkers and properties that attributed to preserving optimal functionality for bioorthogonal pretargeting. The goal was to develop an affinity molecule that could maintain cell binding efficiency and fluorophore emission of the conjugated probe while improving its reactivity to achieve maximum signal amplification. Many strained alkyne moieties had been studied...
to create biomolecular determinants that could not only amplify signals, but also be biocompatible and sensitive to low expression biomarkers. Although inverse Diels-Alder cycloaddition chemistry has advantages of rapid reaction rate without the use of cytotoxic reagents, poor solubility of dienophiles posts concerns on high-density modification of nanoparticles [1].

Figure 6: Confocal images of live A431 cancer cells. (i) and (iii) are controls with no antibody and unmodified anti-EGFR. Cells were labeled with anti-EGFR modified with (ii) azide, (iv) TCO, (v) PEG4-TCO, and (vi) PEG24-TCO, and probed with DIBO-Alexa Fluor 488 or tetrazine-Oregon Green 488 [10].

Rahim et al. had developed antibody conjugates and studied their coupling efficiencies through the reaction between TCO dienophiles and Tz-modified probes. Upon finding that not all modified NHS–TCO were reactive, which suggested the possibility of buried linkers due to hydrophobic interaction with the antibody, polyethylene glycol (PEG) chain was incorporated to study the effect of reactivity when a hydrophilic polymer was added (Figure 5). PEG–TCO of 4 and 24 PEG units were attached to dibenzylcyclooctyne (DBCO) and azide-modified antibody, a reaction mutually orthogonal to tetrazine–TCO chemistry. It was believed that PEG chain prevented TCOs from burying inside the
antibody, thus improved the functional reactivity by more than 5-folds without interfering with cell binding (Figure 6) [10].

Although azide/DBCO–PEG–TCO cycloaddition showed a promising result, a two-step modification process was not favorable. It was questioned whether improvement in reactivity could be due to the incorporation of a hydrophilic PEG linker, a hydrophobic DBCO moiety, or an amphiphilic condition. Thus, various hydrophobic molecules such as decanoic acid (DA), valeric acid (VA), and benzoic acid (BA) were used to replace the DBCO-Azide chemistry with or without the PEG linker.
CHAPTER 2: Improving TCO-Conjugated Antibody for Bioorthogonal Pretargeting

Monoclonal Immunoglobulin G (IgG) antibodies are often used as scaffolds for nanoprobe attachment for its potential after affinity ligand modification. Antibodies specifically for extracellular cancer biomarkers such as HER2 and EGFR can be modified at their reactive groups, including primary amine, thiol, carboxylic acid, and hydroxyl groups, to allow greater flexibility [1]. In this study, primary amine modifications were assessed.

2.1 Background

I. Functional Targets for Immunoconjugates

![Figure 7: Size comparison of mouse IgG antibody, lysine residues (yellow), TCO, and avidin protein [9].](image)

Antibody labeling techniques can be done by targeting three major groups: the primary amines (-NH2), sulfhydryl groups (-SH), and carbohydrates. It is important to not block the antigen binding sites when performing conjugation to prevent reduction in antibody binding ability as well as fluorophore reaction. Sulfhydryl groups, or thiols, can undergo oxidation to form disulfide bonds or alkylation with Michael addition to form thioethers. To prevent damages in binding sites and retain biological activities, cysteine
sulfur atoms can be selectively cleaved at hinge region only, leaving the connections at light and heavy chains intact [16, 17]. Primary amine is the most common labeling site not only because of its accessibility on antibody surface, but also its reactivity; trastuzumab, or herceptin, has approximately 90 lysine residues where the primary amines are located [9]. Amines can react with activated esters, sulfonyl chlorides, isocyanates, or isothiocyanates to form products of amides, sulfonamides, ureas, and thioureas respectively [16]. However, because lysine residues also exist in antigen binding sites, high amount of modification may interfere with the protein binding activity [17].

II. NHS-Ester Reaction Chemistry

![Figure 8](figure8.png)

**Figure 8**: NHS-ester reaction chemistry [17].

N-hydroxysuccinimide (NHS) ester is the most common activated ester used to incorporate TCO moiety into biomolecules. During protein modification, NHS ester of TCO linkers react with the primary amine nucleophiles in aqueous solution to form a stable amide bond with NHS being the leaving group of the reaction. However, such amine reaction competes with hydrolysis of the NHS ester reagent in aqueous environment, which decreases the modification yield. Studies had shown that hydrolysis of the ester bonds occurs at higher pH; half-life of NHS ester decreased from 4-5 hours to 10 minutes when pH was increased from pH 7.0 (at 0°C) to pH 8.6 (at 4°C). However, low pH protonated the
amine groups. Since both hydrolysis and amine reactivity increase with increasing pH, high protein concentration is preferred with excess reacting compounds to account for the loss. Adjusting molar ratio of antibodies to amine-reactive ligands allows control of modification reaction. Also, since NHS ester is relatively insoluble in water, organic solvents such as dimethylformamide or dimethyl sulfoxide can be added [17].

2.2 Materials and Methods

1. TCO-Antibody Conjugation

Antibodies were buffer exchanged into phosphate-buffered saline (PBS, 1x; Lonza) solution prior to modification using Zeba spin desalting columns (40K MWCO, Thermo Fisher Scientific) following product instruction. 0.25 mg of primary antibodies (anti-HER2) was modified with TCO linkers at standard 10, 30, and 100 molar excess. The following TCO linkers were studied: NHS–TCO, NHS–PEG₄–TCO, NHS–DA–TCO, NHS–VA–TCO, NHS–VA–PEG₄–TCO, and NHS–BA–PEG₄–TCO (Figure 9). 1x PBS was used as buffer solution with 10% sodium bicarbonate (0.1M, pH 8.4; EMD Millipore) to achieve alkaline condition without causing protein folding.

Reaction mixture was vortexed and incubated at room temperature for 3 hours. At the end of reaction, the mixture was transferred to Zeba column for purification. Sample concentration was measured using NanoDrop 2000 UV-Vis Spectrophotometer (Fisher Scientific).
Figure 9: Chemical structure of TCO model presented in this project. All linkers were synthesized to be amine reactive.


III. Total Modification Analysis

Matrix assisted laser desorption/ionization time-of-flight (MALDI-TOF; AB SCIEX TOF/TOF 5800 MALDI-TOF spectrometer) mass spectrometer was used to analyze the total TCO density. MALDI matrix was prepared by dissolving 1 mg of sinapinic acid (3, 5-Dimethoxy-4-hydroxycinnamic acid, Sigma Aldrich) in 100 μl of solvent containing 0.1% trifluoroacetic acid (Sigma Aldrich), 30% acetonitrile (Fisher Scientific) and 70% water (Fisher Scientific). Sample was buffer-exchanged into water using Amicon Ultra-0.5 ml Centrifugal Filters (Millipore Sigma), centrifuging for 30 minutes at 14,000 rcf to obtain a final concentration of at least 1 mg/ml. Concentrated samples were combined with MALDI matrix at 1:2 ratio, where 1 μl of the mixture was transferred and dried on MALDI plate.

Data acquired from MALDI was exported to MATLAB (MathWorks) to calculate the number of total modification per antibody based on the difference in molecular weight compared to the unmodified antibody. Direct TCO modification had expected mass of 152.2 g/mol; PEG−TCO had expected mass of 399.15 g/mol; DA−TCO had expected mass of 321.44 g/mol; VA−TCO and VA−PEG−TCO had expected mass of 251.31 g/mol and 498.6 g/mol respectively; and BA−PEG−TCO had expected mass of 518.59 g/mol.

IV. TCO-Loading Characterization

TCO loading was characterized using tetrazine-Oregon Green 488 (Tz-OG). To saturate the amount of dye given total modification, 0.1 mg of modified antibody was reacted with 250 molar excess of Tz-OG with fixed final molar concentration of 500 μM across all linker reactions. Reaction mixture was vortexed and incubated at room temperature for 3 hours, then purified using Zeba columns. Sample absorbance was
measured using NanoDrop and the amount of dye per antibody was calculated. To study the functionality of modified antibodies, percent yield was calculated by dividing the functional modification over total.

2.3 Results and Discussion

1. Reactivity of TCO-Conjugates

Rahim et al. had established a 100% TCO reactivity recovery through the cycloaddition chemistry between DBCO–PEG–TCO and azide-modified antibody. It was suggested that incorporation of PEG chains could improve the functionality. With this hypothesis, NHS–PEG–TCO was reacted with amine groups on the Herceptin and compared to the TCO data collected. We expected an increase in total functional yield because PEG could make the linker less hydrophobic and more soluble in the aqueous environment. It was predicted that addition of PEG linker would prevent TCO from burying inside the antibody due to its hydrophilic property. To test its reactivity, Tz-OG was used as the conjugated probe. We discovered that TCO reactivity increased by more than 4-fold with the incorporation of a 4-unit PEG chain. Amount of active TCOs improved from 10.95% to 46.65% of the total linker modified. Through this study, we confirmed the effect of PEG chain in improving targeting reactivity. Yet, this improvement was far from that of DBCO–PEG–TCO. From here, we decided to test if the enhancement was due to the hydrophobicity of DBCO instead.
Figure 10: Functionality comparison between TCO and PEG-TCO modified anti-HER2.

Short alkyl chains of DA–TCO and VA–TCO were conjugated to Herceptin. We discovered that exceptionally more TCO reactivity was maintained, especially for VA–TCO, which preserved a 66% of the TCO reactivity. Observing the improvement resulted from PEG and short hydrophobic chains, our group now predicted a close to 100% TCO functionality with the combination of both linkers. We hypothesized that introducing a PEG linker to VA–TCO would result in a greater reactivity enhancement.

Interestingly, VA–PEG–TCO had fewer active sites than that of VA–TCO, dropping its reactivity to ~30%, with BA–PEG–TCO performed even worse. Incorporation of a PEG linker to the alkyl chains not only did not improve its TCO reactivity, but also decreased it by about half. Although all TCO linkers presented a better yield than NHS–TCO, our intent was to push the reactivity closer to 100%. We concluded that adding a PEG or short hydrophobic alkyl chain separately increased the percent TCO reactivity, but an integration of both was not ideal.
Figure 11: Functionality of anti-HER2 conjugated with hydrophobic short linkers (top), valeric acid-TCO with and without PEG linker (middle), and amphiphilic linkers (bottom).
A summary of conjugated TCO reactivity is shown below (Figure 12). While the amphiphilic linkers did not react as we expected, it narrowed down factors that could contribute to the increase in TCO functionality. An assumption was that since PEG polymer was not rigid, TCOs could loop back to have hydrophobic interaction with the antibody as it did for the NHS–TCO. We concluded that the 100% functionality observed in the DBCO–PEG–TCO modification could be the product of cycloaddition chemistry.

![Graph showing TCO reactivity](image)

**Figure 12:** All TCO linkers conducted in this study. Trend lines show consistent percent yield as the total modifications increase.
Figure 13: Percent reactivity of all linkers reported in this study.

<table>
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<th>Linker</th>
<th>% Functional</th>
<th>+/- fold to TCO</th>
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<tr>
<td>TCO</td>
<td>10.95</td>
<td>n/a</td>
</tr>
<tr>
<td>PEG-TCO</td>
<td>46.65</td>
<td>4.26</td>
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<td>DBCO-PEG-TCO</td>
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<td>DA-TCO</td>
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<td>VA-PEG-TCO</td>
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<td>3.15</td>
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<tr>
<td>BA-PEG-TCO</td>
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Table 1: Summary of all TCO data with reactivity comparison.
CHAPTER 3: Summary and Conclusions

Bioorthogonal conjugation has significant potential in direct targeting for diagnostic and therapeutic purposes. It offers a more selective, sensitive and rapid solution to current imaging limitations. Through the use of inverse electron-demand Diels-Alder cycloaddition chemistry between a strained dienophile, trans-cyclooctene, and tetrazine-modified nanoprobes, the reaction can be done without cytotoxicity or disturbing biological functions while providing high sensitivity and signal amplification.

In this study, we sought to develop a TCO-conjugated antibody that can improve the reactivity of modified TCOs to optimize pretargeting signal amplification. Herceptin was modified with the following TCO linkers: TCO, PEG−TCO, DA−TCO, VA−TCO, VA−PEG−TCO, and BA−PEG−TCO. All the ligands were amine reactive, which simplified the process of modification comparing to that of azide-DBCO cycloaddition chemistry. It was identified that PEG chain can improve the reactivity of TCO moiety by adding a more hydrophilic chain to prevent reactive site burying. However, although PEG−TCO performed better than TCO, it was not 100% active. To test the hypothesis of DBCO hydrophobicity in improving functionality, short alkyl chains (valeric and decanoic acid) of TCO were conjugated to anti-HER2. VA−TCO managed to maintain approximately 60% of the TCO functionality although not saturating. We predicted that linker reactivity might have been affected by the integration of both hydrophobic and hydrophilic region. Therefore, amphiphilic linkers were examined. Interestingly, reactivity dropped when an additional PEG chain was introduced to VA−TCO.

From this project, we concluded that incorporation of either a hydrophilic PEG chain or hydrophobic linker can preserve reactivity of the TCO-conjugated antibody, but the
chemistry behind it was more complex. Amphiphilic linkers could have caused the TCO moiety to undergo hydrophobic interaction with the antibody, which buried and made the TCOs unavailable for imaging probe binding. Many reasons could be suggested to affect the linker behaviors. Future work will explore other factors such as the rigidity of linker structure, polarity, or charges. Discovering the methods of maintaining TCO reactivity will help us develop a pretargeting molecule that can push signal amplification to a higher level.
REFERENCES


APPENDIX A

Antibody Modification and Characterization

A1. Buffer Exchange for Antibody Modification using Zeba Desalting Columns

1. Centrifuge desalting column at 1000x g for two minutes to remove storage solution.
2. Exchange buffer by adding 1ml PBS slowly over column bed.
3. Centrifuge at 1000 x g for two minutes.
4. Repeat step 2 and 3 two times, with the last centrifugation time being 3 minutes.
5. Transfer desalting column to a fresh 15ml conical tube.
6. Apply Herceptin slowly over the column bed.
7. Centrifuge at 1000 x g for three minutes.
8. Transfer flow-through to a 1.5 ml centrifuge tube.
10. Dilute sample concentration to 1 mg/ml with PBS if needed.

A2. Primary Antibody Modification

Calculation

1. Antibody (Ab) molar equivalence:

\[ \text{Antibody (\mu mol)} = \text{Ab (mg)} \times \frac{g}{1000 \text{ mg}} \times \frac{1}{\text{Ab MW (g/mol)}} \]

2. 1:1 TCO linker equivalence:

\[ \text{TCO (mg)} = \# \text{Ab (\mu mol)} \times \frac{\text{mol}}{10^6 \text{ \mu mol}} \times \text{TCO linker MW (g/mol)} \times \frac{1000 \text{ mg}}{g} \]
3. Amount excess calculation:

\[ TCO \, (\mu l) = \text{amount excess} \times 1:1 \, TCO \, equivalence \, (\mu l) \]

4. Total Volume:

\[ \text{Total Volume} \, (\mu l) = \frac{\text{Amount Ab modified (mg)}}{\text{Expected conc (mg/ml)}} \times \frac{\mu l}{1000 \, ml} \]

5. 10% Sodium bicarbonate by volume

**Procedure**

1. In a 1.5 ml centrifuge tube, add 1x PBS, 10% Sodium Bicarbonate (0.1 M, pH 8.4), and primary antibody. Vortex the mixture.

2. Add TCO linker to reaction mixture from step 1. Vortex.

3. Incubate at room temperature for 3 hours on GyroMini.

**A3. Protein Recovery**

1. Centrifuge desalting column at 1000x g for two minutes to remove storage solution.

2. Exchange buffer by adding 1ml PBS slowly over column bed.

3. Centrifuge at 1000 x g for two minutes.

4. Repeat step 2 and 3 two times, with the last centrifugation time being 3 minutes.

5. Transfer desalting column to a fresh 15ml conical tube.

6. Drip reaction mixture over the column bed slowly.

7. Centrifuge at 1000 x g for three minutes.

8. Transfer flow-through to a 1.5 ml centrifuge tube.

9. Obtain sample concentration using NanoDrop. Store at 4° C.
A4. Oregon Green Tetrazine Reactivity Assay: TCO Loading Characterization

Calculation

1. TCO-modified antibody (Ab) molar equivalence:

\[
Antibody \ (\mu mol) = Ab \ (mg) \times \frac{g}{1000 \ mg} \times \frac{1}{Ab \ MW \ (g/mol)}
\]

2. Tz-OG equivalence:

\[
Dye \ (mg) = \# \ Ab \ (\mu mol) \times 250 \times dye \ conc \ (g/mol) \times \frac{mol}{10^6 \ \mu mol} \times \frac{1000 \ mg}{g}
\]

3. Total Volume:

\[
Total \ Volume \ (\mu l) = Ab \ molar \ equivalence \ (\mu mol) \times 250 \times \frac{1}{500 \ \mu M} \times \frac{10^6 \ \mu l}{L}
\]

Procedure

1. In a 1.5 ml centrifuge tube, add 1x PBS and TCO-modified primary antibody. Vortex.

2. Add Oregon Green tetrazine to reaction mixture. Vortex.

3. Wrap centrifuge tube in aluminum foil to prevent light exposure.

4. Incubate at room temperature for 3 hours on GyroMini.