

# TECHNICAL NOTES

## Evaluation of Ketone-Oxime Method for Developing Therapeutic On-Demand Cleavable Immunoconjugates

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The use of antibody molecules in immunoassay, molecular targeting, or detection techniques encompasses a broad variety of applications affecting nearly every field of medical science. In cancer therapy, monoclonal antibodies (mAb) have been used as vehicles to deliver radionuclides, toxins, or drugs to the target cancer cells. New conjugation methods are most needed to conjugate a wide variety of targeting small molecules and peptidomimetic compounds. Here, we exploited a keto-oxime method for conjugation of protease susceptible linkers to an antibody. This modified method involves two steps: (i) introduction of methyl ketone linkers (referred to as linker moiety) to the primary amines present in the antibody and (ii) conjugation of ketone linkers to aminoxy functional group present in the conjugated moiety (referred to as functional moiety). We have optimized this conjugation method and shown that ~10 functional moieties can be conjugated to antibody. Conjugation was verified by MALDI-TOF MS and Western blot analysis. The acidic pH conditions used in this method did not change the immune reactivity of the Ab. In addition, *in vitro* protease susceptibility assay was performed to validate this method for prodrug release assay as well as to remove excess radioimmune conjugates from circulation. This orthogonal method is compatible with peptides containing a thiol, amino, or carboxyl groups in the conjugation moiety.

### 1. INTRODUCTION

Ketones selectively react with amino-oxo groups and form oxime bonds at slightly acidic pH. This type of chemoselective ligation is more preferable for making bioconjugates because it does not require anhydrous conditions, excessive heat, or any catalyst (1, 2). It is also tolerant of a wide range of functional groups that are found in proteins and oligonucleotides. This ligation has been successfully used in various applications such as making small molecule drug conjugates (3), peptide and oligonucleotide conjugates (4), protein conjugates (5), glycoconjugates (2), polymer conjugates (6, 7), and coating biomolecules on planar microarray slides (8) and sialoglycoproteins present on cell wall (9, 10). Except for N-terminal labeling (11), this ligation method has not been used to make antibody drug conjugates especially in the area of cancer targeted therapy. Antibodies are important biomolecules developed by nature to protect hosts by eliminating disease-causing pathogens. Several conjugation methods have been developed to conjugate radionuclides, toxins, or drugs to antibody for cancer therapy (12–17). Here, we report conjugation of tissue plasminogen activator (t-PA) substrate rYKYkf (18) to ChL6 antibody by the keto-oxime method. Chemical synthesis of methylketo linkers, aminoxy-functional peptides and optimization of conjugation method and its bioapplications are discussed.

### 2. MATERIALS AND METHODS

Rink amide MBHA resin (0.45 mmol/g) was purchased from Tianjin Nankai Hecheng (Tianjin, China). Levulinate *N*-suc-

cinimidyl ester was prepared using our previously published procedure (8). All the amino acid derivatives and HOBt (1-hydroxybenzotriazole) were purchased from CPC (San Jose, CA). DIC (diisopropylcarbodiimide) was purchased from Advanced ChemTech (Louisville, KY). DMF (*N,N*-dimethylformamide) was purchased from VWR (Brisbane, CA). DCM (dichloromethane), MeOH (methanol), diethyl ether, and acetonitrile were purchased from Fisher (Houston, TX). All other chemical reagents were purchased from Aldrich (Milwaukee, WI). Analytical HPLC analyses (Vydac column, 4.6 mm × 250 mm, 5 μm, 300 Å, C<sub>18</sub>, 1.0 mL/min, 25 min gradient from 100% aqueous media (0.1% TFA) to 100% CH<sub>3</sub>CN (0.1% TFA), 214, 220, 254, and 280 nm) and preparative HPLC purification (Vydac column, 20 mm × 250 mm, 5 μm, 300 Å, C<sub>18</sub>, 7.0 mL/min, 45 min gradient from 100% aqueous media (0.1% TFA) to 100% CH<sub>3</sub>CN (0.1% TFA), 254 nm) were performed on a Beckman System Gold HPLC system (Fullerton, CA). MALDI-TOF MS analysis was performed on a Bruker BIFLEX III mass spectrometer (Billerica, MA). All of the experiments are carried out at room temperature unless otherwise noted. ChL6 (Bristol-Myers Squibb Pharmaceutical Research Institute, Seattle, WA) was a chimera consisting of human IgG1-constant regions and the murine L6 mAb antigen-binding site.

**2.1. Synthesis of Biotin-rYKYkf-spacer-K(Aoa)-NH<sub>2</sub> Peptide.** Solid-phase synthesis of biotin-rYKYkf was carried out using Rink amide MBHA resin after the treatment of resin with 20% piperidine solution in DMF (2 mL) for 15 min to remove Fmoc protecting group. Onto the Rink resin (200 mg, 0.09 mmol) with free amino groups, Fmoc-Lys(Alloc)-OH (122.2 mg, 0.27 mmol) was coupled following the standard Fmoc chemistry using HOBt (36.5 mg, 0.27 mmol) and DIC (42.3 μL, 0.27 mmol) as coupling agents. The coupling was monitored using ninhydrin test agents until a negative result was obtained. The resin was washed with DMF, MeOH, and DCM 3 times,

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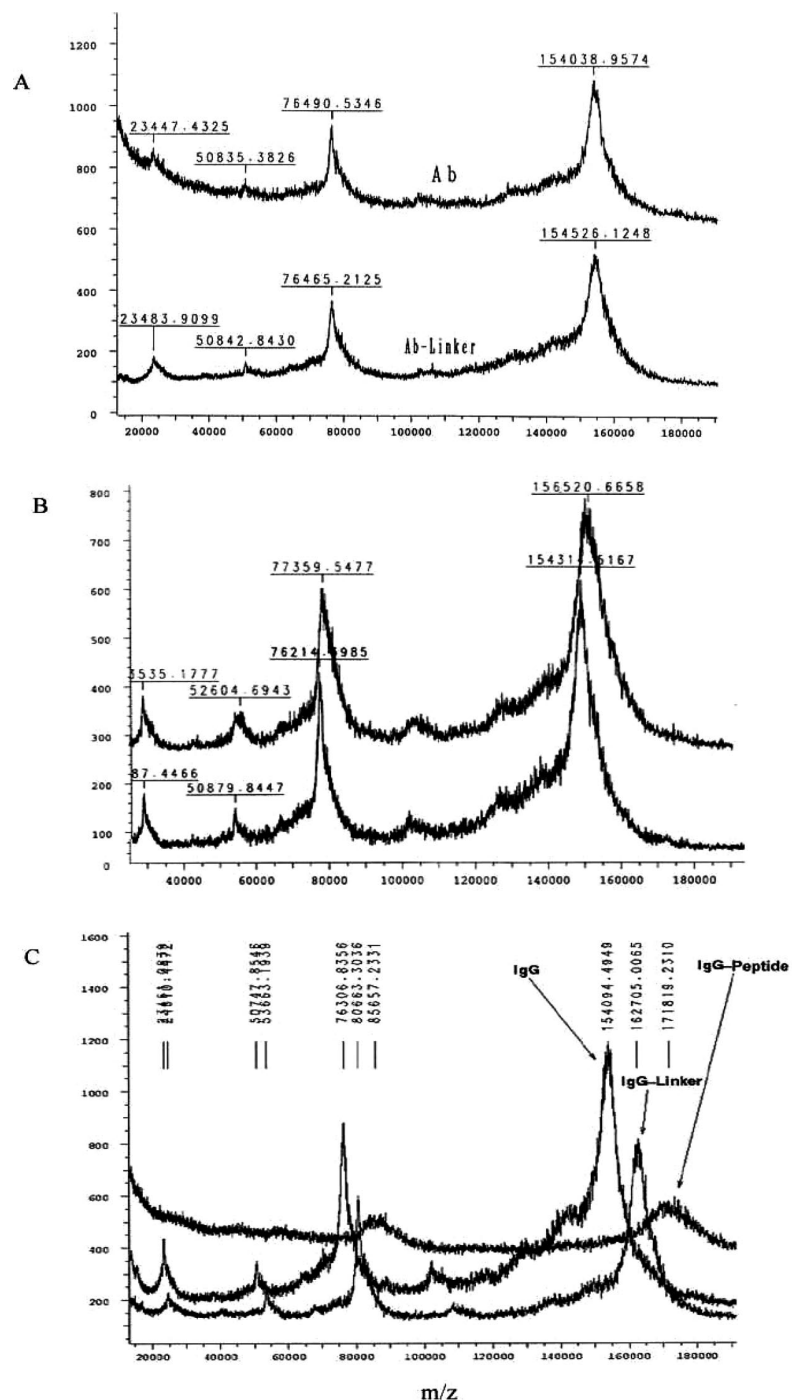


**Figure 3.** MALDI-MS analysis of ChL6 Ab, ChL6-linker moiety, and ChL6-functional moiety: (A) At 30:1 molar ratio of linker to Ab, ~5–6 linker moiety was conjugated to ChL6 Ab. (B) At 30:1 molar ratio, ~1–3 functional moiety (MW 1688) was conjugated to ChL6. (C) At 300:1 molar ratio, ~7–11 functional moieties were conjugated. The average incorporated values are shown. At this ratio, ChL6 with linker moiety showed a distinct peak suggesting that most of the primary amines in ChL6 are conjugated with linker moiety.

products are separated, transferred to membrane, and stained with avidin-HRP as described above.

**2.4. Immunohistochemistry.** Breast adenocarcinoma cancer cells were seeded on a six-well plate one day before the experiments were performed. 1  $\mu$ g of ChL6 and biotin-rqYKYkf-conjugated ChL6 were added directly into 1 mL of growth media in duplicate and kept for 1 h on ice. Unbound antibodies were removed by washing three times with PBS containing 1% BSA. FITC-conjugated streptavidin was added to each well and kept on ice for 30 min, followed by washing three times with PBS containing 1% BSA to remove the

unbound streptavidin. Fluorescent staining was observed under the fluorescence microscope in green filter (U-M51004 F/R-V2; Olympus).

### 3. RESULTS AND DISCUSSION

**3.1. Characterization of Biotin-rqYKYkf-spacer-K [Aminoxy (Aoa)]-NH<sub>2</sub> Peptide.** The methyl ketone linker (Levulinate *N*-succinimidyl ester) was synthesized according to the reported procedure using levulinic acid and *N*-hydroxyl succinimide as starting materials and 85% yield was obtained (8, 22). Synthetic

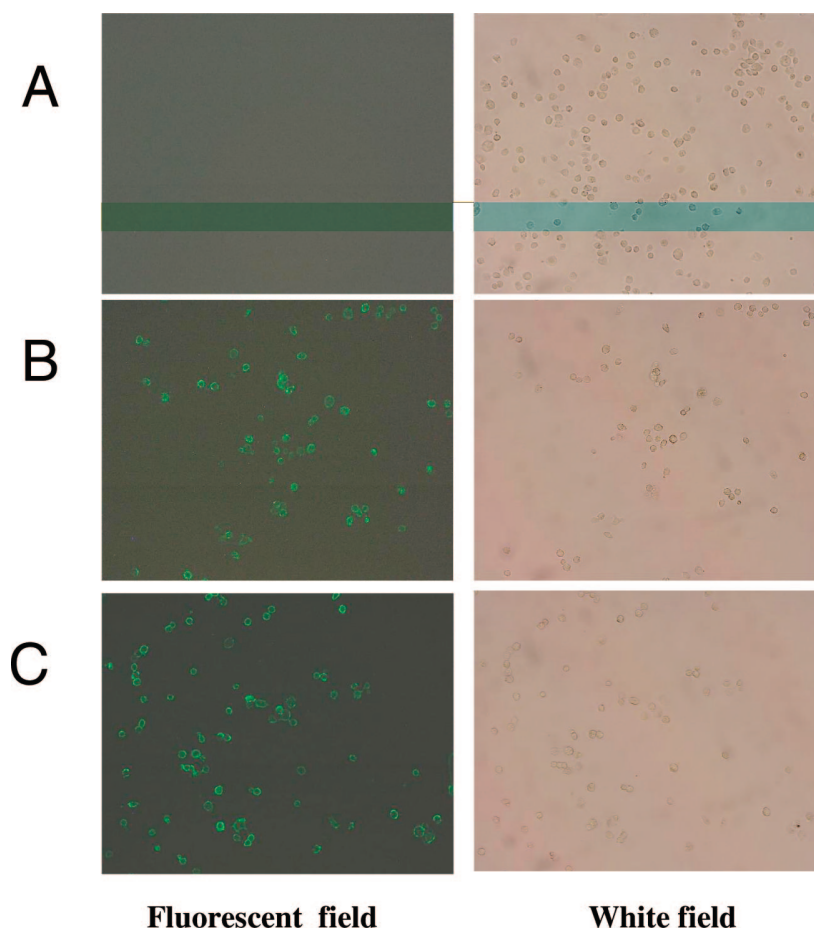


**Figure 4.** Immunoreactivity assay: Breast cancer cells (HBT) were incubated with ChL6 (B) and ChL6 conjugated to functional moiety (C) followed by FITC conjugated murine antihuman Ab. ChL6 conjugated with functional moiety (C) showed binding as good as native unaltered ChL6 (B). FITC conjugated murine antihuman IgG did not show any binding (A).

scheme of biotin-rqYKYkf-spacer-K(Aoa)-NH<sub>2</sub> peptide was shown in Figure 1. A total of 25 mg of biotin-rqYKYkf-spacer-K(Aoa)-NH<sub>2</sub> was obtained from 200 mg of resin (yield: 16%). The synthetic product was verified by MALDI-TOF MS ( $M+H$ )<sup>+</sup> calculated: 1688.9; found: 1688.5.

**3.2. Optimization of Ketone-Oxime Conjugation.** This modified ketone-oxime method has two steps. (i) introduction of the NHS-ketone linkers (referred as linker moiety) to the primary amines present in the antibody and (ii) conjugation of ketone group to aminoxy functional group present in the conjugated moiety (referred to as functional moiety). Steps involved in this conjugation are shown in Figure 2.

First, ketone linkers were introduced by reacting the NHS group with primary amines of the antibody at various molar ratio (L/Ab 5:1, 10:1, 30:1, and 300:1). MALDI-MS results revealed that antibody derivatives occurred when a 20-fold excess or more of ketone linker was used in the initial antibody modification step. No conjugation was observed when 5:1 or 10:1 molar ratio of L/Ab was used. Table 1 summarizes the level of antibody derivatives under various L/Ab ratios. At 30:1 (L/Ab) ratio, ~5–6 linker moieties were conjugated to each Ab molecule (Figure 3A). The same preparation will yield 1–3 functional conjugates (Figure 3B). At (L/Ab) ratio of 300:1, mass spectrometry analysis of ChL6 Ab showed a distinct peak (Figure 3C) suggesting that most of the primary amines present in the Ab were conjugated. When functional moiety is conjugated at 30 molar ratio to an antibody, ~3 functional moieties were generated from this preparation. Previous reports have shown that, in order to retain the biological function of

antibodies, conjugation of a functional moiety should not exceed 8. Therefore, we have selected 30:1 L/Ab ratio to perform our conjugation studies.

**3.3. Immunoreactivity Assay.** CHL6, a chimeric antibody that targets a breast cancer antigen, was used for conjugation studies (21). To verify that this conjugation method does not alter antigen binding properties of CHL6 antibody, it was conjugated with biotin-tagged peptide at 30:1 L/Ab ratio, and immunoreactivity assay was performed on breast cancer cells (HBT). Figure 3 shows that both conjugated and unconjugated ChL6 antibody were binding HBT breast cancer cells with similar intensity, confirming that the conjugation method did not alter the antigen binding property of CHL6 (Figure 4).

**3.4. Protease Susceptibility Assay.** In clinics, antibodies have been used for targeting as well as delivering therapeutic drugs, especially prodrugs to eliminate infected and transformed cells. These nontoxic prodrugs get converted to a toxic metabolite when it is cleaved by a specific protease such as Cathepsin-B at the target site (23). A similar strategy has been used in radioimmunotherapy (RIT), to remove circulating radioimmunoconjugates after tumor targeting. This will boost the therapeutic potential of RIT which was greatly limited by exposure of radiation to vital organs (liver, bone marrow) during the treatment. These linkers can be cleaved either internally by proteases expressed in vital organs such as the liver (e.g., Cathepsin-D) (24) or externally by administering enzymes such as  $\beta$ -lactamase (25) and TNKase (18). We have selected TNKase as an on-demand cleavable enzyme because it is already in clinical use to dissolve fibrin clots (26, 27). Protease susceptibil-

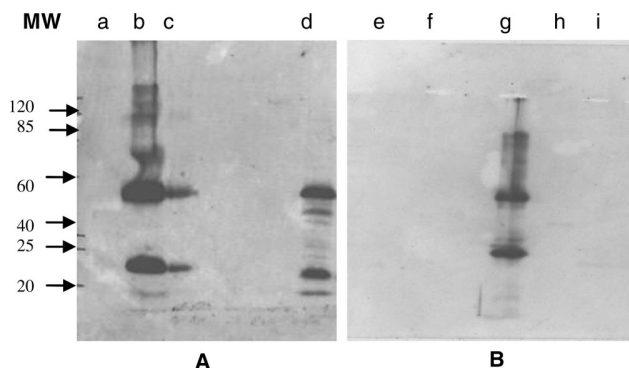


**Figure 5.** Western blot and protease susceptibility analysis: (A) ChL6–10 $\mu$ g (a), ChL6-conjugated with biotin-rqYKYkf–10 $\mu$ g (b), ChL6-conjugated with biotin-rqYKYkf–1 $\mu$ g (c), biotinylated anti-insulin Ab (d) was electrophoresed in 10% SDS-PAGE gel and developed using ECL after transferring to a nylon membrane. Positive bands appeared only on conjugated ChL6 (b,c,d) and not the native ChL6 (a). (B) To evaluate protease susceptibility of the functional moiety (t-PA substrate), conjugated ChL6 was incubated with various concentrations of TNKase, 0  $\mu$ g/mL (g), 10  $\mu$ g/mL (h), and 25  $\mu$ g/mL (i). The band has disappeared in TNKase-treated lanes (h,i) and not in untreated lanes (g). Native ChL6 and TNKase were run together as a negative control. Standard MW markers are shown on the left side of the figure.

ity was evaluated by incorporating t-PA substrate “rqYKYkf”, identified by screening one-bead–one-compound combinatorial chemistry libraries in linker moiety with biotin tag (18). After conjugation with ChL6 at 30:1 molar ratio, 5  $\mu$ g/mL samples were taken in triplicate and digested with 0, 10, and 25  $\mu$ g/mL of recombinant t-PA (TNKase) for 2 h at RT. Digested samples were identified by Western blot analysis as described in the Materials and Methods section. ECL showed bands only in the conjugated ChL6 and not with ChL-6 alone or in TNKase-digested conjugated ChL6 samples, suggesting that biotin was removed by protease digestion. This confirms that this method can be used as an alternative method to conjugate therapeutic drugs, especially prodrugs (Figure 5).

## CONCLUSION

Previously, we used the methods described here to print peptides and small molecules in microarrays over a planar surface (8). Previously, *in vivo* small molecule drug conjugate studies have demonstrated oxime bond stability at the physiological pH of body fluids (28, 29). Here, we have demonstrated this method to conjugate protease (TNKase) cleavable peptides to antibodies without affecting their antigen binding properties. We also showed that conjugated substrates were susceptible to TNKase at the clinical dosage. We conclude that the ketone-oxime method can be substituted in place of other methods like maleimide (12) and click chemistry conjugation (30) for making therapeutic antibody conjugates. This method is compatible with peptides containing a thiol, amino, or carboxyl group in the conjugation moiety.

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