Gram-Scale Synthesis of Site-Specific Antibody-Drug Conjugates Using AJICAP Second-Generation Technology

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Abstract

Chemical site-specific conjugation technology utilizing immunoglobulin-G (IgG) Fc-affinity reagents is a versatile and promising tool for producing next-generation antibody-drug conjugates (ADCs). Our research group recently reported a novel Fc-affinity peptide-mediated conjugation method, termed AJICAP second-generation. This technology, based on thioester chemistry, produces site-specific ADCs without aggregation. Herein, we report further investigations into the AJICAP second-generation technology. By varying the parameters of the peptide conjugation step, it was found that this reaction is feasible under a wide range of reaction conditions. All synthetic intermediates of the AJICAP-ADCs were sufficiently stable, indicating that each synthetic step is a possible holding point in ADC manufacturing. The Lys248- and Lys288-conjugated ADCs were prepared on a gram-scale using two different Fc-affinity peptide reagents, employing a scale-down manufacturing approach involving tangential flow filtration (TFF). The total product yield was > 80%, and ultimately, 13.2 g of trastuzumab-Lys248-MMAE and 1.26 g of trastuzumab-Lys288-MMAE were obtained with high drug to antibody ratios (DARs). The results strongly indicate that the AJICAP second-generation method is a robust and practical approach for the manufacture of ADCs.

Keywords: antibody-drug conjugates, AJICAP, ADC manufacturing, site-specific conjugation technology, scale-up
Introduction

Chemical conjugation technology has emerged as a principal approach for the production of oncology therapeutics, especially antibody-drug conjugates (ADCs), owing to the advantages of straightforward chemistry, manufacturing and control (CMC).1,2 All ADCs currently on the market are manufactured using this technology. However, most ADCs display heterogeneity, such as cytotoxic drug distribution and variable drug-to-antibody ratios (DARs). This stochastic conjugation technology is the current gold standard for generating ADCs.3 Such heterogeneity impacts negatively on the drug efficacy and toxicology profile of ADCs, thereby diminishing the therapeutic window.4

To overcome the heterogeneity drawback of current ADCs, several site-specific and chemical conjugation methodologies have emerged in recent decades.5,6 Utilizing Fc-affinity binders is a promising approach for the construction of site-specific ADCs.6 However, as this technology is mainly used at the research stage, the challenge of developing scalable and robust manufacturing processes using Fc-affinity reagents remains. The limited scope of Fc-affinity technologies for site-specific ADC generation prompted us to develop a versatile and facile direct antibody modification technology that circumvents antibody engineering.7 Optimization of the reaction sequence led to the present improved technology, termed “AJICAP second generation.” The technology provides a promising chemical platform, which is potentially applicable to the production of a wide variety of site-specific ADCs (Figure 1). Unique affinity peptide-based reagents modify the Fc region of the antibodies and subsequent linker cleavage results in site-specific thiol-modified antibodies (Figure 1b). Two thioester groups of the AJICAP peptide reagent (1) allow selective amidation of target lysines via the amine side chain due to the proximity effect. Hydroxylamine can cleave the alky thioester bond between antibody and peptide moiety to form thiol groups. This newly formed
antibody thiol reacts with maleimide-containing drug linkers to complete the ADC synthesis. This affinity-based strategy uses two different AJICAP peptide reagents to access two distinct conjugation sites (Lys248 and Lys288). Early biological studies, including in vivo efficacy and toxicology studies, showed that site-specific AJICAP-ADCs (both Lys248- and Lys288-based ADCs) resulted in an enhanced therapeutic window.

These promising data acquired from site-specific ADCs produced via the AJICAP second generation approach necessitate the manufacture of large-scale ADCs to support further biological investigations, such as safety studies in non-human primates. Thus, process development aimed at scale-up is required to adapt this affinity peptide strategy for large-scale ADC production.

In this study, we disclose further investigations regarding AJICAP second-generation technology to demonstrate its scalability and robustness. The parameters of the peptide conjugation step, which is unique to the AJICAP strategy, were probed, and it was concluded that the reaction can operate under a wide range of conditions. Even when an excess amount of AJICAP peptide reagent was used, the peptide-antibody ratio (PAR) was 2.0, suggesting no overreaction. Both synthetic intermediates, AAPC (2) and antibody-thiol (3), were sufficiently stable under appropriate storage conditions, indicating that every synthetic intermediate can be a holding point compound during the manufacturing process. Lys248- and Lys288-conjugated ADCs were synthesized on gram-scale using two different Fc-affinity peptide reagents employing a scale-down manufacturing approach using tangential flow filtration (TFF). The residual peptide reagents (for Lys248 and Lys288 modifications) were removed to acceptable levels via TFF purification. The total manufacturing yield was over 80%, and ultimately, 13.2 g of trastuzumab-Lys248-MMAE and 1.26 g of trastuzumab-Lys288-MMAE with a high DAR was obtained. These results
strongly indicate that the AJICAP second-generation strategy is a robust and practical approach for the manufacture of ADCs.

Figure 1. Overview of AJICAP second-generation technology: a) reaction sequence and the structures of AJICAP reagents for Lys248 and Lys288 conjugation, b) reaction mechanism of AJICAP second-generation technology
**Experimental procedure**

**Materials**

Human IgG1 trastuzumab (Herceptin®) was purchased from the Roche Pharmaceutical Company (Switzerland). MC-VC-MMAE (CAS#646502-53-6) was purchased from NJ Biopharmaceuticals, LLC (USA). Peptide reagents (1a and 1b) were prepared according to a published method. All other chemical reagents were purchased from Sigma-Aldrich (USA).

**Instruments and analytical methods**

The ADC concentration and recovery were measured using the Slope Spectroscopy® method and a Solo-VPE system. Q-TOF MS analysis was performed as previously reported.

Hydrophobic interaction chromatography (HIC)-HPLC analysis was performed as previously reported.

Size-exclusion chromatography (SEC)-HPLC analyses of AAPC (2) and antibody thiols (3) were performed as previously reported.

RP-HPLC analysis of antibody thiols (4) was performed as previously reported.

**Experimental procedure for small-scale peptide conjugation (step-1 of AJICAP Lys248 conjugation method)**

To a solution of trastuzumab (concentration range of 0.3 – 20 mg/mL, 1 mg) in 20 mM AcONa buffer (pH 5.5) was added a 20 mM solution of peptide reagent 1a (equivalent range 0.8 – 20 equiv.) in dimethylformamide (DMF) and the mixture was incubated at 20 ºC. After 1 h, the reaction mixture was purified by gel filtration using 20 mM AcONa buffer (pH 5.5). PAR was assessed using HIC-HPLC.
Experimental procedure for repeat peptide conjugation (step-1 of AJICAP Lys248 conjugation method)

To a solution of AAPC (10 mg/mL, 1 mg, PAR = 0.8) in 20 mM AcONa buffer (pH 5.5) was added 20 mM solution of peptide reagent 1 (2.0 eq.) in DMF and the mixture was incubated at 20 °C. After 1 h, the reaction mixture was purified by gel filtration using 20 mM AcONa buffer (pH 5.5). PAR was assessed using HIC-HPLC.

Experimental procedure for gram-scale peptide conjugation/linker cleavage (step-1 and step-2 of AJICAP Lys248 method)

To a solution of trastuzumab (10 mg/mL, 15.0 g) in 10 mM AcONa buffer (pH 5.5) was added a 20 mM solution of peptide reagent 1a (4.0 eq.) in DMF and the mixture was incubated at 20 °C. After 1 h, excess NH₂OH·HCl was added, and the mixture was stirred for an additional 1 h. The reaction mixture was purified using a TFF system with a Sartocon Slice 200 Eco Hydrosart membrane (30 kDa, Sartorius) and 10 diavolumes of 20 mM AcONa buffer (pH 5.5) as the diafiltration (DF) buffer at an antibody concentration of 20 mg/mL. Next, the TFF system performed a buffer exchange of this solution using a Sartocon Slice 200 Eco Hydrosart membrane (30 kDa, Sartorius) and PBSE buffer (50 mM PBS, 10 mM EDTA, pH 7.4) as the DF buffer at an antibody concentration of 7.5 mg/mL to afford the antibody-thiol (3a) (13.7 g, 91% yield) in PBSE buffer.

Experimental procedure for payload conjugation (step 3 of AJICAP Lys248 method)

To a solution of antibody-thiol (3a) (13.7 g, 7.5 mg/mL) in PBSE buffer was added dimethylacetamide (DMA) and a 20 mM DMA solution of MC-VC-MMAE (2.7 eq.). This solution was incubated at 20 °C. After 1 h, the reaction mixture was quenched by the excess addition of a 50 mM aqueous solution of N-acetyl cysteine and incubated at 25 °C for 15 min. This
reaction mixture was purified by the TFF system using a Sartocon Slice 200 Eco Hydrosart membrane (30 kDa, Sartorius) and histidine buffer (20 mM histidine containing 5% trehalose, pH 5.2) as the DF buffer at an antibody concentration of 10 mg/mL to afford trastuzumab-Lys248-MMAE (4a) (13.2 g, 95% yield) in the formulation buffer.

Experimental procedure for gram-scale peptide conjugation/linker cleavage (step-1 and step-2 of AJICAP Lys288 method)

To a solution of trastuzumab (10 mg/mL, 1.5 g) in 10 mM HEPES buffer (pH 8.2) was added a 20 mM solution of peptide reagent 1b (6.0 eq.) in DMF and the mixture was incubated at 20 °C. After 1 h, excess NH₂OH·HCl and 1 M acetate buffer (pH 4.7) were added to adjust the pH of the reaction mixture to below pH 6.0 and the mixture was stirred for an additional 1 h. The reaction mixture was purified using a TFF system with a Sartocon Slice 200 Eco Hydrosart membrane (30 kDa, Sartorius) and 15 diavolumes of 20 mM AcONa buffer (pH 4.5) as the diafiltration (DF) buffer at an antibody concentration of 10 mg/mL. Next, buffer exchange of this solution was conducted using the TFF system with a Sartocon Slice 200 Eco Hydrosart membrane (30 kDa, Sartorius) and PBSE buffer (50 mM PBS, 10 mM EDTA, pH 7.4) as the DF buffer at an antibody concentration of 5.0 mg/mL to afford antibody-thiol (3b) (1.35 g, 90% yield) in PBSE buffer.

Experimental procedure for payload conjugation (step 3 of AJICAP Lys288 method)

To a solution of antibody-thiol (3b) (1.35 g, 5.0 mg/mL) in PBSE buffer was added a 20 mM DMA solution of MC-VC-MMAE (2.7 eq.) This solution was incubated at 20 °C. After 1 h, the reaction mixture was quenched by the excess addition of a 50 mM aqueous solution of N-acetyl cysteine and incubated at 25 °C for 15 min. This reaction mixture was purified using a TFF system with a Sartocon Slice 200 Eco Hydrosart membrane (30 kDa, Sartorius) and histidine buffer (20 mM histidine containing 5% trehalose, pH 5.2) as the DF buffer at an antibody concentration of
10 mg/mL to afford trastuzumab-Lys248-MMAE (4b) (1.26 g, 92% yield) in the formulation buffer.

*General procedure for stability studies*

Both Lys248- and Lys-288 AAPCs (2a and 2b) and antibody-thiols (3a and 3b) were subjected to stress conditions based on previously reported methods.\textsuperscript{12} Aggregation was determined using SEC.

*Effect of storage temperature on stability*

Three sets of standard solutions of AAPCs (2a and 2b) and antibody thiols (3a and 3b) were prepared in histidine buffer (5 mg/mL). The samples were stored at two different temperatures (-80, and 4 °C) for a period of 4 weeks. Aggregation was analyzed using SEC.

*Effect of repeated freeze-thaw cycles on stability*

Three sets of standard solutions of AAPCs (2a and 2b) and antibody thiols (3a and 3b) were prepared in histidine buffer (5 mg/mL). All samples underwent five cycles of storage at -80 °C for 24 h followed by thawing at room temperature. Aggregation was analyzed using SEC.

*Molecular structure analysis*

Computational analysis was performed using the Bioluminate software suite (Bioluminate, version 2022-3, Schrödinger, Inc.). The mercaptopropionic acid group was attached to the amine side chain of each target lysine (Lys248 or Lys288) by amidation\textsuperscript{13}. The initial Fc proteins were deposited in the Protein Data Bank as 3D6G after protonation and minimization. The amino acid surface of the resulting proteins was colored according to hydrophobicity using the PyMOL molecular graphics system (version 2.3.0, Schrödinger, LLC)\textsuperscript{4}.

*Results and discussion*
Recently, several peer-reviewed publications have reported "traceless" antibody labeling using unique Fc-affinity peptide reagents.\textsuperscript{14-19} From a scientific perspective, the traceless modification method has the advantage of requiring fewer reactions. However, this approach carries the potential risk of overreaction, as relatively weak non-covalent binding of the existing peptide reagent prevents other affinity reagents from reaching the conjugation site of the Fc region. On the other hand, the AJICAP technology installs a covalent bond between the antibody and Fc peptide reagent; therefore, the existing peptide portion, which is covalently bound to antibodies, can interfere with antibody modification. To test this hypothesis, we probed the parameters of the peptide conjugation reaction (Table-1).

\textbf{Table 1.} Study of site-specific peptide conjugation parameters

<table>
<thead>
<tr>
<th>Entry</th>
<th>mAb conc. (mg/mL)</th>
<th>Conjugation site</th>
<th>AJICAP reagent (eq.)</th>
<th>Reaction time (h)</th>
<th>Reaction temp (°C)</th>
<th>Average PAR</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10</td>
<td>Lys 248</td>
<td>1a (4.0)</td>
<td>1</td>
<td>25</td>
<td>2.0</td>
</tr>
<tr>
<td>2</td>
<td>15</td>
<td>Lys 248</td>
<td>1a (4.0)</td>
<td>1</td>
<td>25</td>
<td>2.0</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>Lys 248</td>
<td>1a (4.0)</td>
<td>1</td>
<td>25</td>
<td>2.0</td>
</tr>
<tr>
<td>4</td>
<td>0.3</td>
<td>Lys 248</td>
<td>1a (4.0)</td>
<td>1</td>
<td>25</td>
<td>1.7</td>
</tr>
<tr>
<td>5</td>
<td>10</td>
<td>Lys 248</td>
<td>1a (4.0)</td>
<td>6</td>
<td>25</td>
<td>2.0</td>
</tr>
</tbody>
</table>
Peptide conjugation to Lys248 showed consistent results across the tested range of concentrations (entries 1–4). Even at a low antibody conc (1 mg/mL), this reaction was completed with a PAR of 2.0. Prolonged reaction time (>16 h) did not result in an overreaction, and a PAR of 3.0 was not observed in the HIC-HPLC analysis (entries 5 and 6). This antibody modification reaction was found to be highly compatible with a wide range of reaction temperatures (4–37 °C) (entries 10 and 11). This wide tolerability may allow for the application of this reaction to the production of unusual mAbs with unfavorable solubility. A similar observation was provided in the case of Lys288 conjugation reactions (entries 12–17). Furthermore, intentionally defective PAR AAPC (PAR=0.8, entries 8 and 14) was modified with newly added peptide reagents (1a or 1b) to complete the AAPC with PAR=2.0. These results indicate that the AJICAP peptide conjugation reaction was compatible with a range of conditions, demonstrating robustness.

Next, the conjugation study was applied to the gram-scale synthesis of ADCs (Figure 2).
Figure 2. Flow diagram for gram-scale syntheses of a) trastuzumab-Lys248-MMAE and b) trastuzumab-Lys288-MMAE

The reaction of affinity reagent 1a with trastuzumab, followed by linker cleavage, afforded the antibody-Lys248-thiol (3a) in high yield (91%). To remove residual impurities (residual hydroxylamine and peptide-related compounds), TFF purification was conducted using a 30 kDa membrane. Ten consecutive DF buffer treatments reduced the residual amount of 1a to acceptable
levels (>95%). The clearance rate was analyzed using RP-HPLC at a wavelength of 280 nm (Figure S1 in Supporting Information: SI). Conjugation of a small excess of the MC-VC-MMAE solution with antibody-Lys248-thiol (3a) afforded trastuzumab-Lys248-MMAE (4a). N-acetyl cysteine (NAC)-quenched MMAE was removed by TFF purification. In a previous study, this TFF purification step required careful monitoring, as the removal of MMAE-related compounds using a simple TFF step was challenging due to the hydrophobicity of MMAE. However, in the present study, the MMAE-related compounds were completely removed in a single TFF operation using an acidic buffer (histidine, pH 5.2). Compared with our previous synthesis, AJICAP second-generation technology enabled the reduction of MC-VC-MMAE using fewer equivalents (present study: 2.7 equiv. of MC-VC-MMAE, previous study: 10 equiv. of MC-VC-MMAE); therefore, a single TFF was sufficient to clear the residual payload linkers. Overall, 13.2 g of trastuzumab-Lys248-MMAE (4a) was obtained, and the total manufacturing yield was 87%.23

A similar process was used for the production of the Lys288 ADC (Figure 2b). After peptide conjugation, slightly acidic DF buffer was used to remove excess peptide reagents. Because the basic peptide of 1b, termed the Z34C peptide, is known to lose its affinity under acidic condition, we used an acetate buffer (pH 4.5) for the efficient clearance of Z34C-related peptide impurities. The clearance rate was calculated using RP-HPLC at a wavelength of 280 nm (Figure S2). Eventually, 1.26 g of trastuzumab-Lys288-MMAE (4b) was obtained, and the total manufacturing yield was 83%. To improve the overall manufacturing yield, further process development studies, including the application of a continuous mode, are currently underway.

Finally, the stabilities of the synthetic intermediates were assessed (Figure 3). In manufacturing, stable synthetic intermediates are considered holding points. To establish an operator-friendly manufacturing process, storage conditions and freeze-thaw cycles were assessed
for two different AAPCs (Lys248: 2a and Lys288: 2b) and antibody thiols (Lys248: 3a and Lys288: 3b).

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**Figure 3.** Stability assessment of synthetic intermediates. a) Lys248-related intermediates b) Lys288-related intermediates

A previous in vivo efficacy and pharmacokinetic study revealed that the biological profile of Lys248 ADC was similar to that of Lys288 ADC. However, in the present stability study, the intermediates exhibited dissimilar behavior. Lys248-related compounds were found to be more...
stable than Lys288-related compounds. The aggregation percentage of trastuzumab-Lys248 thiol did not change even after the samples were stored at 4 °C for 4 weeks. Lys248-AAPC (2a) was more stable than antibody-Lys248-thiol (3a). This observation is reasonable considering that free thiol groups in antibody thiols can potentially aggregate. Lys288-related derivatives, even if less stable than Lys248, were sufficiently stable at -80 °C. The freeze-thaw test results indicated no significant increase in the aggregation levels of either Lys248 or Lys288 intermediates. This difference between Lys248-relatives and Lys288-relatives can be explained by the exposure level of each lysine. Structural analysis of these thiol-modified lysines indicated that the Lys288 thiol was exposed to the solvent to a greater extent than the Lys248 thiol (Figure S3 and S4 in SI). The solvent-accessible surface area (SASA) analysis results for each corresponding lysine supported this observation. According to a previous report, the SASA level of Lys248 is 44.0, whereas that of Lys288 is 171.1.29 These initial stability studies support the reasoning that site-specific modified intermediates along the AJICAP conjugation route can be considered holding points in ADC manufacturing.

Conclusion

Gram-scale ADC manufacture was achieved employing Fc affinity reagents. A detailed study of the parameters involved in the peptide conjugation step indicated the robustness of this unique reaction. The purification processes in each manufacturing step toward gram-scale ADC preparation were conducted according to a scale-down approach utilizing a well-established TFF to remove > 95% of small-molecule impurities. The recovery yield for each step was > 90%, and over 10 g of trastuzumab-Lys248-MMAE with a high DAR was obtained. Similarly, an Lys288-ADC manufacturing route was established after fine-tuning the TFF conditions. Furthermore, an
initial stability assessment of the synthetic intermediates indicated that the site-specific modified intermediates could be used as holding points under appropriate storage conditions for future ADC manufacture. The results indicate that the second-generation AJICAP system provides a robust and reliable process for site-specific ADC production.

References


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![Link](https://doi.org/10.1021/acs.bioconjugchem.3c00040)


(23) DAR of trastuzumab-Lys248-MMAE was determined by two different analyses (HIC-HPLC and Q-TOF MS). See figure S5 and S6 in SI.


(25) DAR of trastuzumab-Lys288-MMAE was determined by two different analyses (HIC-HPLC and Q-TOF MS). See figure S5 and S6 in SI.


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