AJICAP-M: Traceless Affinity Peptide Mediated Conjugation Technology for Site-Specific Antibody-Drug Conjugate Synthesis

AUTHOR NAMES

Yutaka Matsuda*†‡, Natsuki Shikida†, Noriko Hatada†, Kei Yamada†, Takuya Seki†, Yuichi Nakahara†, Yuta Endo†, Kazutaka Shimbo†, Kazutoshi Takahashi†, Akira Nakayama†, Brian A. Mendelsohn‡§, Tomohiro Fujii†*, Tatsuya Okuzumi†, Shigeo Hirasawa†

* Corresponding authors

TF: tomohiro.fujii.3m2@asv.ajinomoto.com

YM: Yutaka.Matsuda@US.AjiBio-Pharma.com

†Ajinomoto Co., Inc., 1-1, Suzuki-Cho, Kawasaki-Ku, Kawasaki-Shi, Kanagawa 210-8681, Japan.

‡Ajinomoto Bio-Pharma Services, 11040 Roselle Street, San Diego, CA 92121, United States.

§Present addresses: Exelixis Inc, 1851 Harbor Bay Pkwy, Alameda, CA 94502, United States.

Abstract

A traceless site-selective conjugation method, "AJICAP-M," was developed for native antibodies at specific sites using Fc-affinity peptides, focusing on Lys248 or Lys288. It produces antibody-drug conjugates (ADCs) with consistent drug-to-antibody ratios, enhanced stability, and simplified manufacturing. Comparative in vivo assessment demonstrated AJICAP-M's superior stability over traditional ADCs. This technology has been successfully applied to continuous-flow manufacturing, marking the first achievement in site-specific ADC production. This manuscript outlines AJICAP-M's methodology and its effectiveness in ADC production.

Keywords: antibody-drug conjugates, AJICAP, drug-to-antibody ratio, site-specific conjugation technology, flow microreactor

In oncology therapeutics, antibody-drug conjugates (ADCs) are becoming increasingly significant, with 14 ADCs approved for cancer treatment and more than 100 approved in global clinical trials. [1] [2] Traditional ADCs, produced through random conjugation to lysine or cysteine residues, often exhibit structural heterogeneity. This has led to a shift towards next-generation ADCs, which utilize specific conjugation chemistry for precise payload-linker attachment at designated antibody sites, aiming to enhance therapeutic indices[3] [4]. Affinity peptide-mediated chemical conjugation, which is becoming the standard method for site-specific ADC production, is typically challenged by peptide release and cleavage reagent removal (Figure 1a). [5] [6] The conjugation method developed by Zeng et al. in 2022, which utilizes optimized Fc-affinity peptides, marks a considerable advancement, notable for its single-step process [7]. However, areas could still benefit from further refinement, such as the in vivo stability and developability of ADC. To ensure these qualities, additional pharmacokinetic studies are required to better understand the behavior of ADCs. In addition, the current method poses challenges for good manufacturing practices (GMP) ADC production. The synthesis of peptide reagents linked to the payload-linker is complex and raises concerns in the context of GMP, particularly given the necessity to minimize steps involving high-potency payloads for environmental, health, and safety reasons[8]. This traceless affinity approach requires multiple steps that involve payload handling. Therefore, we advocate a simpler manufacturing process.

An ideal approach involves using commercially available payload-likers (Figure S1 in supporting information, SI). ready for conjugation at a later stage, although this requires a two-step conversion process from native antibodies. This can streamline production while maintaining adherence to GMP standards and ensuring safety. The "AJICAP-M" method, described herein, utilizes an optimized affinity reagent with an internal N-hydroxysuccinimide ester (Figure 1b). This method

efficiently produces site-specific ADCs using readily available payload linkers in two steps (antibody modification followed by payload-linker conjugation by click chemistry). AJICAP-M can modify two distinct sites (Lys248 and Lys288), and the resulting site-specific ADC was subjected to *in vivo* efficacy and pharmacokinetic evaluations. Furthermore, this technology has been applied to continuous-mode ADC production, achieving rapid site-specific conjugation within 5 min, thus addressing the limitations of the existing traceless conjugation techniques and marking significant advancements in ADC development.
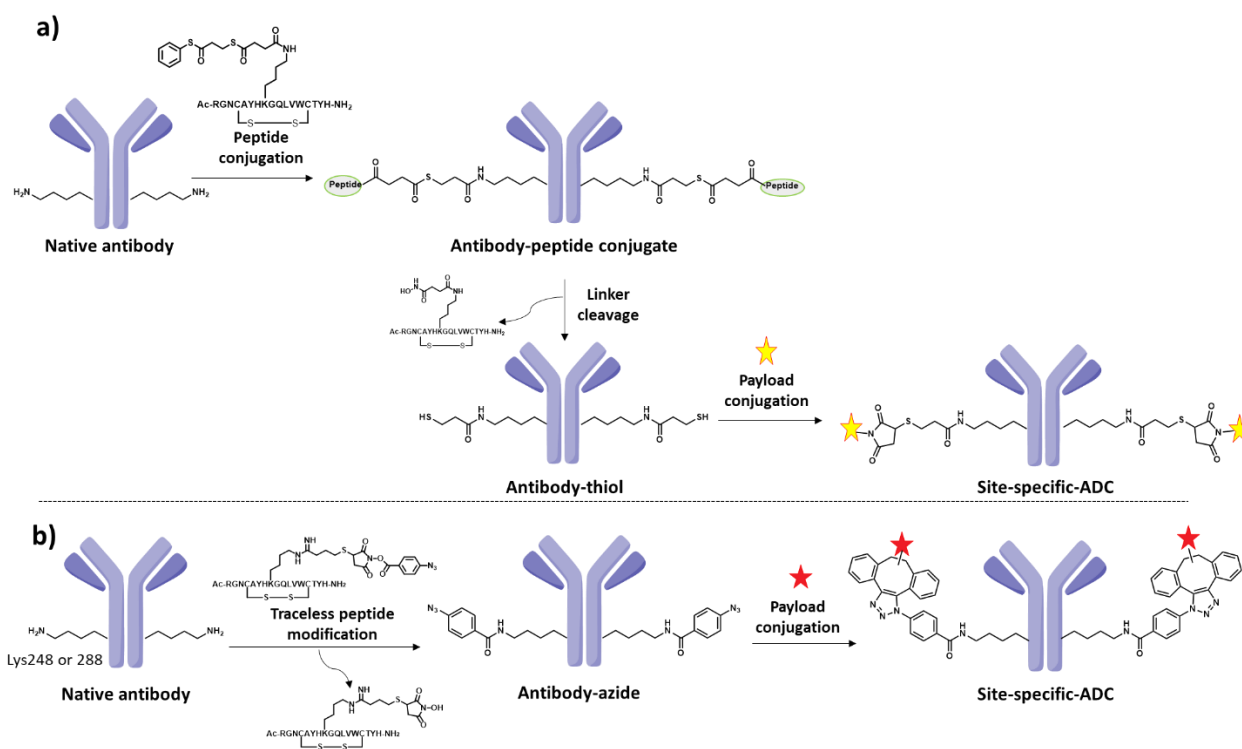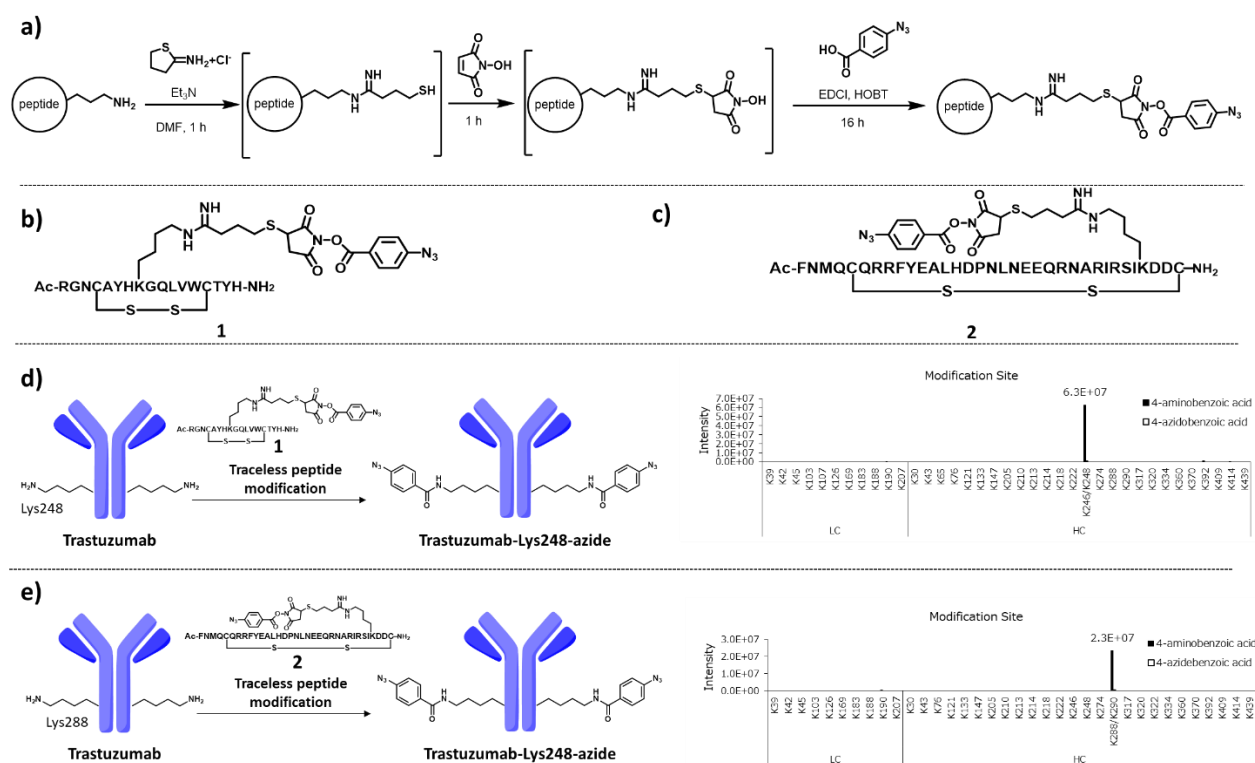

Figure 1. Overview of affinity peptide-mediated conjugation. a) traditional affinity peptide-mediated conjugation strategy; b) AJICAP-M traceless affinity peptide-mediated conjugation strategy

68 Traceless affinity conjugation targets lysine residues in native antibodies using acyl transfer

69 reagents linked to self-releasing peptides. The primary challenge is to maintain stable reactivity at

70 room temperature while avoiding heating, particularly with commonly used functional groups such

71 as thioesters and carbonates. [7] [9] [10-11] [12] To address the reactivity and stability issues in antibody

72 modification, we developed a highly reactive reagent using N-hydroxysuccinimide (NHS) ester,

73 capable of facilitating modification under mild conditions. We incorporated an NHS moiety

74 internally and introduced an azidophenyl group [13]. This modification was designed to increase

75 steric hindrance around the carbonyl group of NHS. Consequently, the modified NHS moiety

76 exhibits increased resistance to hydrolysis [14]. This balance between enhanced stability against

77 hydrolysis and preserved reactive capability is significant, as it ensures the effectiveness of the

78 NHS moiety in forming stable amide bonds with lysine residues. The reagent, synthesized for

79 Lys248 modification, exhibited exceptional stability through multiple freeze-thaw cycles in DMF

80 solution and showed efficient reactivity with native antibodies, completing the modification

81 process within one hour at ambient temperature. This is detailed for the optimized AJICAP-M

82 reagent (**1**) (Figure 2a). Furthermore, we developed an alternative AJICAP-M peptide reagent (**2**)

83 that targets Lys288 on antibodies using a different affinity peptide sequence (Figure 2b). In our

84 recent study, we streamlined the synthesis of peptide reagents, which traditionally involved a

85 multi-step procedure [15]. Using Traut's reagent, we developed an efficient one-pot method that

86 encompasses three distinct reactions (Figure 2a). This approach employs a versatile base peptide

87 compatible with various conjugation methodologies, including AJICAP and AJICAP-M. The

88 adaptability of this base peptide is pivotal because it enables the synthesis to be finely tuned to

89 meet the specific requirements of each antibody and the corresponding mechanism of action of the

90 ADC. AJICAP-M conjugation compatibility was evaluated through using two different antibodies.

91 Trastuzumab and rituximab underwent assessment, as demonstrated in Table 1. In every instance,

92 sufficient conversion was observed between both AJICAP-M reagents (**1** and **2**) and these mAbs,

93 facilitating the site-specific installation of azide groups. The conjugation sites for the resultant

94 trastuzumab-Lys248 or 288 azides were identified using peptide mapping analysis (Figure S4 and

95 S5 in the SI). A comparison of the azide adducts (and the corresponding amines reduced during

96 pretreatment for peptide mapping, figure 2 d and e) and naked trastuzumab underscored the target-

97 specific modification of the AJICAP-antibody-azides at Lys248 and Lys288.

99 Figure 2. Overview of AJICAP-M reaction, a) Synthetic scheme for AJICAP-M peptide reagents,

100 b) Lys248 conjugation reagent (**1**), c) Lys288 conjugation reagent (**2**), d) Lys248 conjugation and

101 Peptide mapping summary of Lys248-azide modification, e) Lys288 conjugation and Peptide

102 mapping summary of Lys288-azide modification

103

Subsequently, the effectiveness of newly installed azides was assessed using two commercially available payload linkers (DBCO-maytansinoid and DBCO-VC-PAB-MMAE) (Table 1). Site-specific ADCs synthesized from trastuzumab-Lys248-azide, trastuzumab-Lys288-azide, or rituximab-Lys248-azide exhibited drug-to-antibody ratios (DARs) exceeding 1.8. The DAR was determined using two analytical methods: quadrupole time-of-flight mass spectrometry (Q-TOF MS) and hydrophobic interaction chromatography (HIC)-HPLC (SI, Figures S4–S11 and S18–S23). [16] Analysis via size exclusion chromatography (SEC) [17] revealed that the aggregation percentages were maintained post-modification in all reaction scenarios (SI, Figures S12–S17).

Table 1. Site-specific ADC synthesis
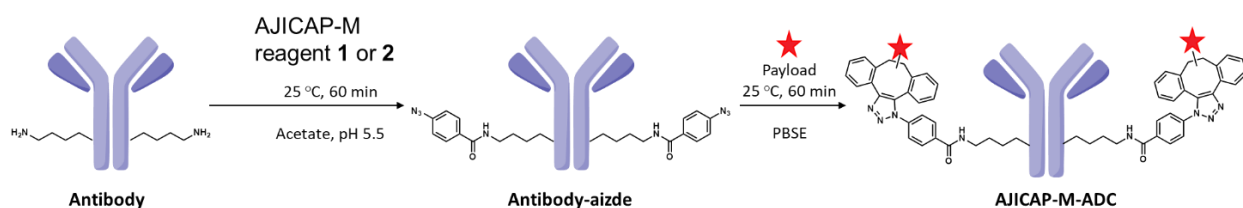
| mAb	Conjugation site	Payload-linker	DAR by Q-TOF	Aggregation by SEC
Trastuzumab	Lys 248	DM1	1.8	1.6%
Trastuzumab	Lys 248	MMAE	1.8	1.8%
Trastuzumab	Lys 288	MMAE	1.8	1.9%
Rituximab	Lys 248	DM1	1.7	2.0%
Rituximab	Lys 248	MMAE	1.6	2.0%

115

Subsequently, the biological profiles of the synthesized ADCs were evaluated. To elucidate the stability of the novel AJICAP-M linkage against traditional cysteine-haloamide conjugation, ADCs were synthesized using the latter method to serve as a control group. Considering the known risks associated with the thiol-maleimide linkage in commonly employed maleimide-based compounds—principally the potential for retro Michael reactions leading to inadvertent payload release[18]—alternative linkers to maleimide were incorporated in the control ADCs. Haloamides, frequently utilized as substitutes within the maleimide group[19], were selected for this purpose, thereby directly comparing the stability between the AJICAP-M ADCs and controls. A series of cell-based assays were performed to assess the in vitro cytotoxicity. These assays involved the use of cell lines with varying levels of HER2 antigen expression: NCI-N87 and HCC1954 for high HER2 expression and PC-3 for low HER2 expression, as illustrated in Figure 3a. The results indicated that AJICAP-M and control ADC demonstrated significant antitumor efficacy against cell lines with high HER2 antigen expression. Conversely, negligible activity was observed in cell lines with low HER2 antigen expression, highlighting the specificity of ADCs.

In earlier investigations, our team demonstrated that ADCs utilizing Fc affinity-mediated conjugation exhibited superior stability and efficacy compared to those assembled using stochastic methods. Based on these findings, we further explored the in vivo efficacy of AJICAP-M-ADCs (Figure 3b). In this study, trastuzumab-AJICAP-M-MMAE and control ADC based on haloamide chemistry were evaluated using a HER2-positive NCI-N87 gastric cancer xenograft model. Notably, trastuzumab-AJICAP-M-MMAE administered at a dose of 5 mg/kg showed significant tumor regression. In prior studies, the minimum effective dose of stochastically conjugated ADCs with a DAR of 4, achieved through native cysteine conjugation, was established at 2.5 mg/kg[15]. Intriguingly, despite the DAR discrepancy (stochastic ADC = 4, AJICAP-M-ADC = 2), AJICAP-

139   M ADC demonstrated in vivo efficacy on par with its stochastic counterparts. Conversely, the

140   control ADC, which was synthesized via thiol-haloamide conjugation and utilized the same

141   conjugation site (Lys248) as the AJICAP-M ADC, exhibited inferior activity. This differential

142   performance suggests potential variance in the local environment of the Val-Cit linker in each

143   ADC. The Val-Cit linker is susceptible to premature cleavage by carboxylesterase Ces1C, which

144   leads to early payload detachment in vivo[20]. This phenomenon is particularly pronounced in

145   murine models and could lead to underestimation of the in vivo efficacy of ADC. The control

146   ADC, with a marginally longer bond from Lys248 to the Val-Cit moiety, may be more exposed to

147   plasma, rendering it more vulnerable to Ces1C cleavage. This observation suggests that the

148   compact linker configuration enabled by AJICAP-M technology could be conducive to the

149   creation of efficacious and stable ADCs[21].

150   To elucidate the different stability characteristics of the two site-specific ADCs, an in vivo

151   pharmacokinetic study was performed using a rat model (Figure 3c). For the control ADC,

152   discernible detachment of the payload was observed a few days post-administration. In contrast,

153   the AJICAP-M-ADC exhibited a considerably lower rate of payload detachment, as demonstrated

154   by a comparative analysis of the concentration profiles of the total antibody mAb and the total

155   ADC. The present data suggest that the improved stability and minimized payload release of

156   AJICAP-ADCs could significantly enhance their therapeutic efficacy and safety profile, making

157   them advantageous candidates for further exploration of ADC-based treatments.
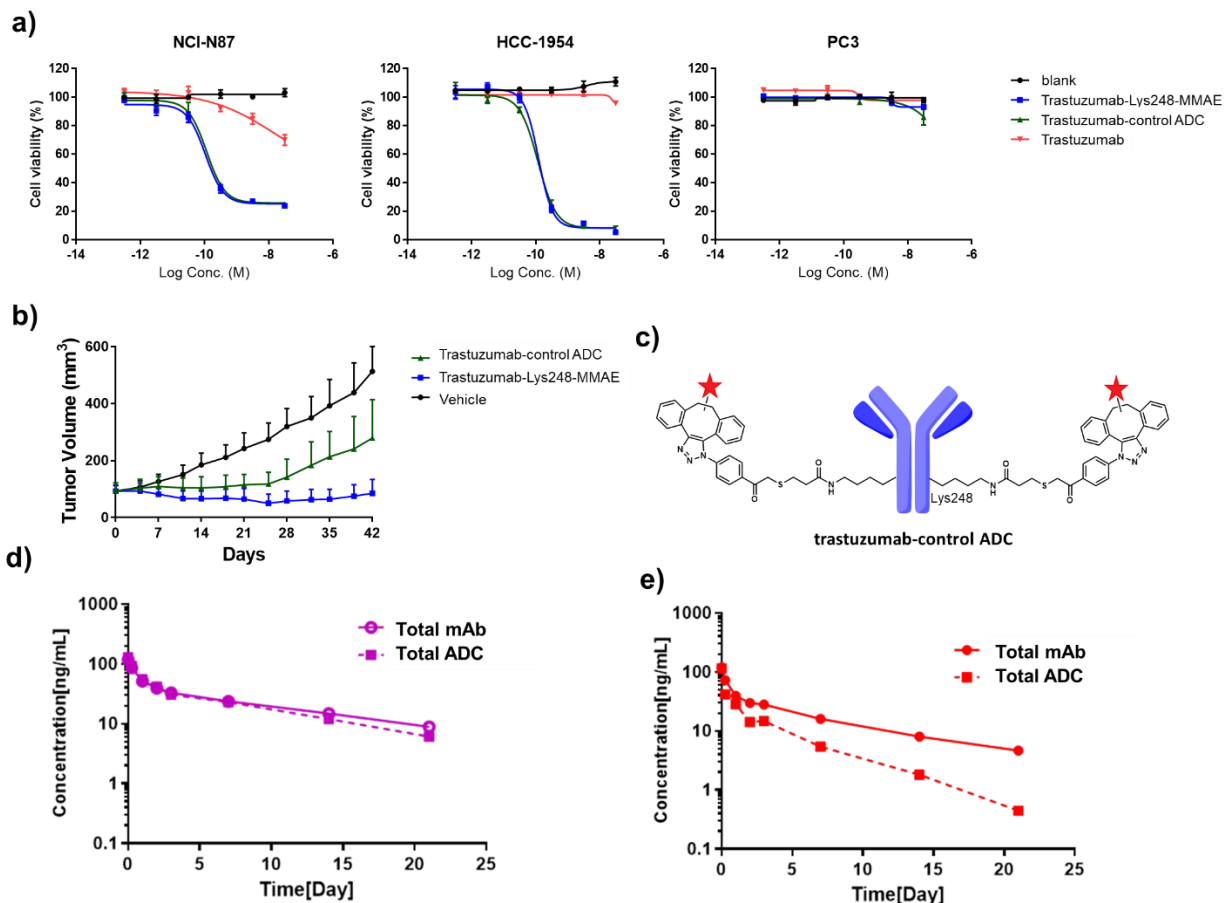
Figure 3. Biological evaluation of trastuzumab-Lys248-MMAE produced by AJICAP-M, a) in-vitro cell-based assay, b) in vivo xenograft, c) illustration of control ADC, d) Plasma concentration of total mAb (flat linear) and total ADC (hash linear) in rats measured by ELISA (trastuzumab-Lys248-MMAE produced by AJICAP-M), d) Plasma concentration of total mAb (flat linear) and total ADC (hash linear) in rats measured by ELISA (control ADC)

Finally, we explored the AJICAP-M technology in a continuous flow microreactor (FMR) synthesis for ADC production. FMR manufacturing using pipes and tubes for chemical reactions surpasses traditional batch reactors in terms of environmental safety and scalability and enables new reactions [22]. This shift is significant in the pharmaceutical and fine chemical sectors. A key aspect of FMR in ADC production is the safe handling of highly potent payloads. FMRs, designed

169   with safety in mind, markedly decrease the risk of exposure to hazardous substances such as

170   payload linkers in ADC manufacturing. In 2022, our group successfully produced cysteine-

171   conjugated ADCs using FMR, marking the first report of FMR-based ADC synthesis[23]. This

172   achievement entailed using clinically relevant antibodies and drug linkers and optimizing mixer

173   types, reaction times, and diameters to produce ADCs without significant aggregation. However,

174   there is a report wherein site-specific ADC production or affinity-guided protein modification

175   could not be achieved in continuous mode. There were initial concerns that the rapid mixing

176   approach could lead to unintended nonspecific conjugation or compromise the quality of the

177   protein product. However, given its notably high affinity for antibodies at the nanomolar level, we

178   anticipated that the AJICAP-M conjugation method would be compatible with the flow kinetic

179   mixing mode. This high affinity is expected to mitigate the risk of nonspecific reactions and

180   maintain the integrity of the protein. We used a V-shaped stainless-steel mixer with an inner

181   diameter of 0.25 mm and a cascade-dual-mode mixing system (Figure 4). The critical step in the

182   AJICAP-M conjugation process was identified as the initial antibody modification by the peptide

183   reagent, which occurred primarily in mixer-1 and reactor-1. To facilitate this, we employed an 8-

184   meter reactor tube for a reaction time of 3 min. Our design focused on flash chemistry to optimize

185   mixing efficiency, characterized by rapid mixing at high flow rates. This strategy led to the

186   successful replication of site-specific ADC production, comparable to that achieved in the batch

187   mode. Notably, this method enables rapid site-specific ADC synthesis within 5 min, presenting a

188   significant advantage for efficient future ADC manufacturing.
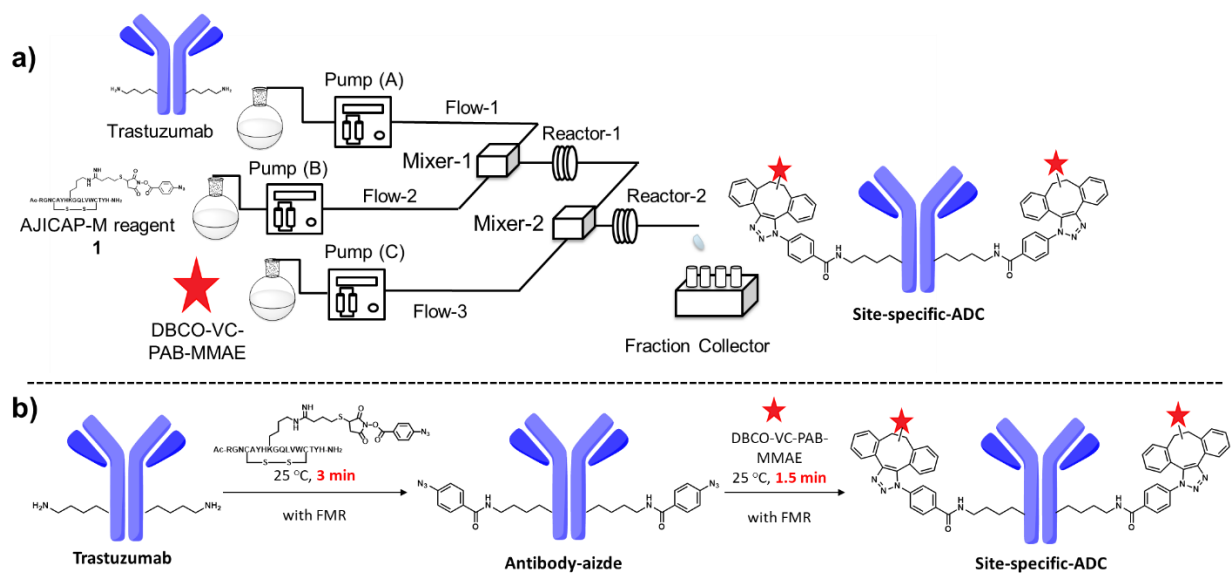
Figure 4. FMR-mode manufacturing of trastuzumab-Lys248-MMAE, a) Illustration of FMR system, b) reaction scheme of FMR-mediated AJICAP-M conjugation

In summary, our research presents a substantial advancement in oncology therapeutics through the development and application of ADCs using AJICAP-M technology. This method addresses the structural heterogeneity issues inherent in traditional ADCs by enabling precise payload-linker attachment at specific antibody sites, thus improving the therapeutic indices. The AJICAP-M technology, characterized by an optimized affinity reagent for an NHS ester, facilitates efficient site-specific ADC production. It effectively modified two key sites on antibodies (Lys248 and Lys288), validated by comprehensive in vivo efficacy and pharmacokinetic studies. These studies demonstrated the enhanced stability and reduced payload detachment of AJICAP-M-ADCs, indicating their potential for broader therapeutic applications and efficacy compared with traditional stochastic ADCs. A notable aspect of our research is the successful integration of the AJICAP-M technology into continuous-flow ADC manufacturing. This approach streamlines the production process, offers benefits in terms of environmental impact and scalability, and marks a

12

significant innovation in ADC production by overcoming the challenges associated with traditional

methods. These advancements have streamlined the ADC modification process and enhanced the

stability and efficiency of the synthesis.

1.   Sasso, J. M.; Tenchov, R.; Bird, R.; Iyer, K. A.; Ralhan, K.; Rodriguez, Y.; Zhou, Q. A., The Evolving Landscape of Antibody-Drug Conjugates: In Depth Analysis of Recent Research Progress. *Bioconjug Chem* **2023,** *34* (11), 1951-2000.
2.   Qian, L.; Lin, X.; Gao, X.; Khan, R. U.; Liao, J. Y.; Du, S.; Ge, J.; Zeng, S.; Yao, S. Q., The Dawn of a New Era: Targeting the "Undruggables" with Antibody-Based Therapeutics. *Chem Rev* **2023**.
3.   Yamazaki, S.; Matsuda, Y., Tag‐Free Enzymatic Modification for Antibody−Drug Conjugate Production. *ChemistrySelect* **2022,** *7* (48).
4.   Zhou, Q., Site-Specific Antibody Conjugation with Payloads beyond Cytotoxins. *Molecules* **2023,** *28* (3).

5.	Yang, C.; He, B.; Zhang, H.; Wang, X.; Zhang, Q.; Dai, W., IgG Fc Affinity Ligands and Their Applications in Antibody-Involved Drug Delivery: A Brief Review. *Pharmaceutics* **2023,** *15* (1).

6.	Kim, S.; Kim, S.; Kim, S.; Kim, N.; Lee, S. W.; Yi, H.; Lee, S.; Sim, T.; Kwon, Y.; Lee, H. S., Affinity-Directed Site-Specific Protein Labeling and Its Application to Antibody-Drug Conjugates. *Adv Sci (Weinh)* **2023**, e2306401.

7.	Zeng, Y.; Shi, W.; Dong, Q.; Li, W.; Zhang, J.; Ren, X.; Tang, C.; Liu, B.; Song, Y.; Wu, Y.; Diao, X.; Zhou, H.; Huang, H.; Tang, F.; Huang, W., A Traceless Site-Specific Conjugation on Native Antibodies Enables Efficient One-Step Payload Assembly. *Angew Chem Int Ed Engl* **2022,** *61* (36), e202204132.

8.	Bulger, P. G.; Conlon, D. A.; Cink, R. D.; Fernandez-Cerezo, L.; Zhang, Q.; Thirumalairajan, S.; Raglione, T.; Liang, R.; Zhou, J.; Chalgeri, A., Drug-Linkers in Antibody– Drug Conjugates: Perspective on Current Industry Practices. *Organic Process Research & Development* **2023,** *27* (7), 1248-1257.

9.	Lee, T.; Kim, J. H.; Kwon, S. J.; Seo, J. W.; Park, S. H.; Kim, J.; Jin, J.; Hong, J. H.; Kang, H. J.; Sharma, C.; Choi, J. H.; Chung, S. J., Site-Selective Antibody-Drug Conjugation by a Proximity-Driven S to N Acyl Transfer Reaction on a Therapeutic Antibody. *J Med Chem* **2022,** *65* (7), 5751-5759.

10.	Yuan, D.; Zhang, Y.; Lim, K. H.; Leung, S. K. P.; Yang, X.; Liang, Y.; Lau, W. C. Y.; Chow, K. T.; Xia, J., Site-Selective Lysine Acetylation of Human Immunoglobulin G for Immunoliposomes and Bispecific Antibody Complexes. *J Am Chem Soc* **2022,** *144* (40), 18494- 18503.

11.	Postupalenko, V.; Marx, L.; Viertl, D.; Gsponer, N.; Gasilova, N.; Denoel, T.; Schaefer, N.; Prior, J. O.; Hagens, G.; Levy, F.; Garrouste, P.; Segura, J. M.; Nyanguile, O., Template directed synthesis of antibody Fc conjugates with concomitant ligand release. *Chem Sci* **2022,** *13* (14), 3965-3976.

12.	Postupalenko, V.; Marx, L.; Pantin, M.; Viertl, D.; Gsponer, N.; Giudice, G.; Gasilova, N.; Schottelius, M.; Lévy, F.; Garrouste, P.; Segura, J.-M.; Nyanguile, O., Site-selective template-directed synthesis of antibody Fc conjugates with concomitant ligand release. *Chemical Science* **2024**.

13.	Meng, W.; Muscat, R. A.; McKee, M. L.; Milnes, P. J.; El-Sagheer, A. H.; Bath, J.; Davis, B. G.; Brown, T.; O'Reilly, R. K.; Turberfield, A. J., An autonomous molecular assembler for programmable chemical synthesis. *Nature Chemistry* **2016,** *8* (6), 542-548.

14.	Takaoka, Y.; Nishikawa, Y.; Hashimoto, Y.; Sasaki, K.; Hamachi, I., Ligand-directed dibromophenyl benzoate chemistry for rapid and selective acylation of intracellular natural proteins. *Chem Sci* **2015,** *6* (5), 3217-3224.

15.	Fujii, T.; Matsuda, Y.; Seki, T.; Shikida, N.; Iwai, Y.; Ooba, Y.; Takahashi, K.; Isokawa, M.; Kawaguchi, S.; Hatada, N.; Watanabe, T.; Takasugi, R.; Nakayama, A.; Shimbo, K.; Mendelsohn, B. A.; Okuzumi, T.; Yamada, K., AJICAP Second Generation: Improved Chemical Site-Specific Conjugation Technology for Antibody-Drug Conjugate Production. *Bioconjug Chem* **2023,** *34* (4), 728-38.

16.	Yamazaki, S.; Shikida, N.; Takahashi, K.; Matsuda, Y.; Inoue, K.; Shimbo, K.; Mihara, Y., Lipoate-acid ligase a modification of native antibody: Synthesis and conjugation site analysis. *Bioorg Med Chem Lett* **2021,** *51*, 128360.

17.	Matsuda, Y.; Leung, M.; Tawfiq, Z.; Fujii, T.; Mendelsohn, B. A., In-situ Reverse Phased HPLC Analysis of Intact Antibody-Drug Conjugates. *Anal Sci* **2021,** *37* (8), 1171-1176.

18.   Szijj, P. A.; Bahou, C.; Chudasama, V., Minireview: Addressing the retro-Michael instability of maleimide bioconjugates. *Drug Discov Today Technol* **2018,** *30*, 27-34.

19.   Erian, A.; Sherif, S.; Gaber, H., The Chemistry of α-Haloketones and Their Utility in Heterocyclic Synthesis. *Molecules* **2003,** *8* (11), 793-865.

20.   Dorywalska, M.; Dushin, R.; Moine, L.; Farias, S. E.; Zhou, D.; Navaratnam, T.; Lui, V.; Hasa-Moreno, A.; Casas, M. G.; Tran, T. T.; Delaria, K.; Liu, S. H.; Foletti, D.; O'Donnell, C. J.; Pons, J.; Shelton, D. L.; Rajpal, A.; Strop, P., Molecular Basis of Valine-Citrulline-PABC Linker Instability in Site-Specific ADCs and Its Mitigation by Linker Design. *Mol Cancer Ther* **2016,** *15* (5), 958-70.

21.   Giese, M.; Davis, P. D.; Woodman, R. H.; Hermanson, G.; Pokora, A.; Vermillion, M., Linker Architectures as Steric Auxiliaries for Altering Enzyme-Mediated Payload Release from Bioconjugates. *Bioconjug Chem* **2021,** *32* (10), 2257-2267.

22.   Capaldo, L.; Wen, Z.; Noel, T., A field guide to flow chemistry for synthetic organic chemists. *Chem Sci* **2023,** *14* (16), 4230-4247.

23.   Nakahara, Y.; Mendelsohn, B. A.; Matsuda, Y., Antibody–Drug Conjugate Synthesis Using Continuous Flow Microreactor Technology. *Organic Process Research & Development* **2022,** *26* (9), 2766-2770.