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Good Manufacturing Practice Strategy for Antibody−Drug Conjugate Synthesis Using Site-Specific Chemical Conjugation: First-Generation AJICAP

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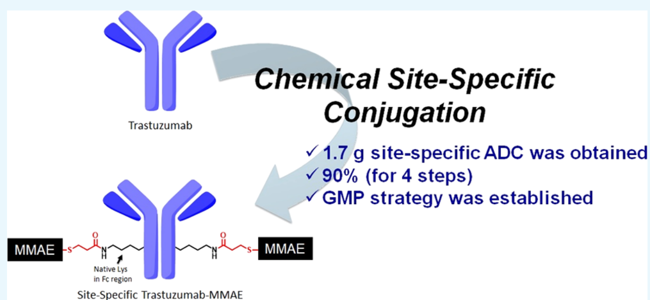
Supporting Information

ABSTRACT: The development of antibody−drug conjugates (ADCs) is in great demand in the oncology field. With the goal of maximizing the therapeutic index, the conjugation technology to produce ADCs has been shifted to a site-specific manner; however, it is still challenging to establish robust and scalable synthetic processes. We have developed a chemical conjugation platform termed AJICAP for site-specific ADC synthesis using IgG Fc-affinity peptides. Here, we report the preparation of site-specific ADCs based on first-generation AJICAP technology for use in good laboratory practice studies. Analysis of the final ADC product was conducted using validated systems and good manufacturing practice. This work may not only prompt further biological studies of AJICAP-ADC but also establish a strategy to provide well-documented manufacturing data to enable new drug application filings (e.g., investigational new drug applications) for site-specific ADCs.



INTRODUCTION

Industrial research and development activity for antibody−drug conjugates (ADCs) has rapidly increased in recent years. Five ADCs have been approved for clinical use by the Food and Drug Administration (FDA): brentuximab vedotin (Adcetris), trastuzumab emtansine (T-DM1, Kadcyla),[1−3] gemtuzumab ozogamicin (Mylotarg), inotuzumab ozogamicin (Besponsa), and very recently, polatuzumab vedotin (Polivy).[4] To date, more than 80 ADCs are in clinical development.[5,6] Current ADCs on the market have a stochastic distribution of cytotoxic drugs linked across several different sites of the antibody.[7−9] This heterogeneous conjugation manner can cause diminished efficacy and/or increased toxicity compared to a homogeneous ADC. Hence, the therapeutic index of heterogeneous ADCs is limited.[10]

The development of site-specific conjugation has become a useful technology in the ADC field to overcome the limitations of traditional ADCs.[11] However, to date, no site-specific ADCs have been commercially approved by the FDA. Thus, the development of scalable and robust ADC processes is still a highly challenging task for drug developers and contract development and manufacturing organizations (CDMOs).[12]

We have developed direct chemical site-specific conjugation technology for intact native antibody modification using Fc-affinity compounds (Scheme 1).[13,14] The first-generation iteration of this technology, termed AJICAP, has already undergone initial process development, including the gram-scale synthesis of site-specific ADCs,[15] biological evaluations,[14,15] and the establishment of appropriate analytical methods.[16] These promising results, showing the scalability and high cytotoxic efficacy of site-specific ADCs produced by AJICAP technology, prompted our group to attempt the application of AJICAP technology to relevant manufacturing production scales. Herein, we report our recent efforts to produce AJICAP-ADCs appropriate for the specific research or preclinical phase for use in good laboratory practice (GLP) studies. The ADC synthetic approach described herein is modeled as a technical transfer from the research and process development stages to clinical and commercial manufacturing scales and methods. To begin, a foundational approach based on a good manufacturing practice (GMP) strategy was used to adapt traditional ADC GMP production for site-specific ADC production. Using this strategy, site-specific ADCs were produced on a gram scale synthesized using AJICAP technology. Analysis of the resulting ADCs was conducted by qualified analysts using validated equipment.

These studies demonstrate the reproducibility and robustness of the AJICAP technology to produce next-generation ADCs, and we believe that our approach to producing site-specific ADCs suitable for use in GLP studies, based on a strategic assessment of appropriate regulatory frameworks, can serve as a model for others as they develop their own approaches to the manufacture of materials for use in studies that support clinical regulatory filings.

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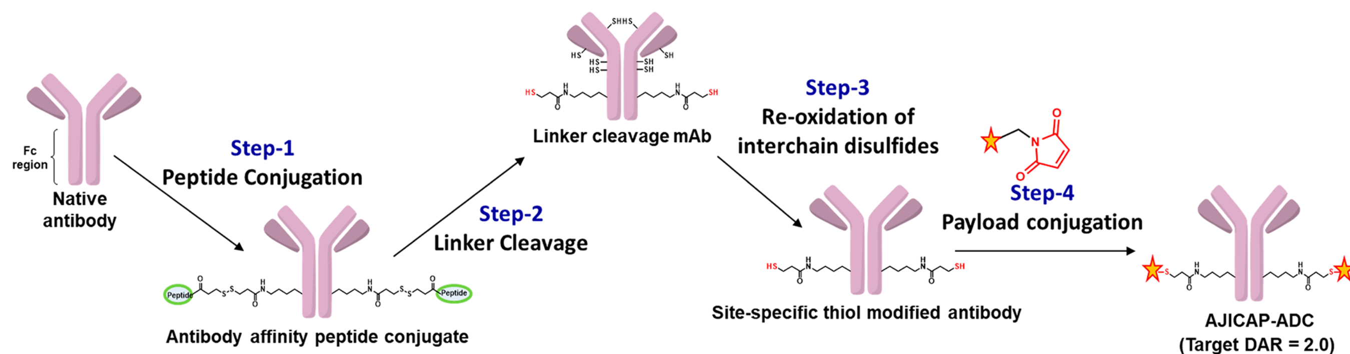
Scheme 1. AJICAP Technology Overview



■ RESULTS AND DISCUSSION

Approaches to the implementation of Current Good Manufacturing Practice (cGMP) principles in early development activities vary widely across the entities conducting these studies. While mature quality systems are in place for larger, established pharmaceutical companies, the majority of the smaller, independent, and research-oriented organizations currently lack these controls. What follows is the development of a process for the production of materials suitable for use in early development and GLP preclinical studies used to support regulatory filings for pharmaceutical products. This process was developed using guidance from multiple regulatory agencies to develop a phase-appropriate cGMP process, which will help ensure acceptance of these filings with regulatory agencies.

Regulations regarding the manufacture of pharmaceutical products for late-phase clinical and commercial use are well established by regulatory agencies, including the FDA cGMP Regulations[17] and European Union GMP Annex 1.[18] Guidance for the manufacture of preclinical materials to support GLP studies,[19] however, is less well defined, which can lead to complications when entities move into early- and late-phase manufacturing where the regulatory requirements ramp up to full cGMP. All activities associated with development of pharmaceutical products are ultimately included in any submission for commercial approval by regulatory agencies, and a lack of early application of a GMP strategy in early product development can be an area of weakness in these filings.

As outlined in the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH), "The aim of pharmaceutical development is to design a quality product and its manufacturing process to consistently deliver the intended performance of the product",[20] and "The goal of manufacturing process development for the drug substance is to establish a commercial manufacturing process capable of consistently producing drug substance of the intended quality."[21] It is critical that the early development work is documented to ensure that the required data is able to be included in submissions to support regulatory approval.

This does not mean that all early development work must be completed according to cGMP standards; as explained by the FDA Guidance Documents for Process Validation, "Generally, early process design experiments do not need to be performed under the cGMP conditions required for drugs intended for commercial distribution. They should, however, be conducted in accordance with sound scientific methods and principles, including good documentation practices."[22] Implementation of cGMP requirements can be resource-prohibitive for early development activities. One of the areas where early development is lacking is in the presence of an independent Quality Management System (QMS) to provide oversight of the project. Per the ICH, "The elements of [a Pharmaceutical Quality Management System] should be applied in a manner that is appropriate and proportionate to each of the product lifecycle stages, recognizing the differences among, and the different goals of each stage."[23] The inclusion of a QMS should be gradually applied to product development to ensure greater efficiency and a seamless transition from early development to late-phase clinical and commercial operations.

Our intent is to use phase-appropriate cGMP processes to produce a material that is suitable for use in GLP studies. Producing these GLP-ready materials with solid documentation packages will enable the filing of investigational new drug applications (INDs) and eventually new drug applications (NDAs). Documentation of early-phase development work is key to the success of any development project.

An emerging area of focus in the current regulatory environment is data integrity, which refers to the completeness, consistency, and accuracy of data. The minimum requirements for data integrity include the fact that all data documented for cGMP activities should be attributable, legible, contemporaneously recorded, original, and accurate (ALCOA).[24] Early implementation of systems to comply with data integrity standards ensures any information included in regulatory submission meets data integrity requirements and will not result in deficiencies in the submitted data.

FDA regulations for GLP studies provide guidance for conducting nonclinical laboratory studies to support new pharmaceutical product applications, such as an IND or NDA.[19] The majority of the guidance is focused on execution of the study itself and does not elaborate on the methods or processes needed to produce the test articles that supply the study. The requirements stated in the regulations for test article characterization require identity, strength, purity, compositions, or other characteristics to be documented and the methods of production to be documented.[19]

Ajinomoto Bio-Pharma Services (Aji Bio-Pharma) has developed an internal procedure to ensure that product development of a client/sponsor material is included within the overall QMS and in a regulatory framework to allow for appropriate data integrity. The requirements of this procedure were developed from regulatory guidance documents and represent the implementation of a regulatory framework to support early product and process development. Materials

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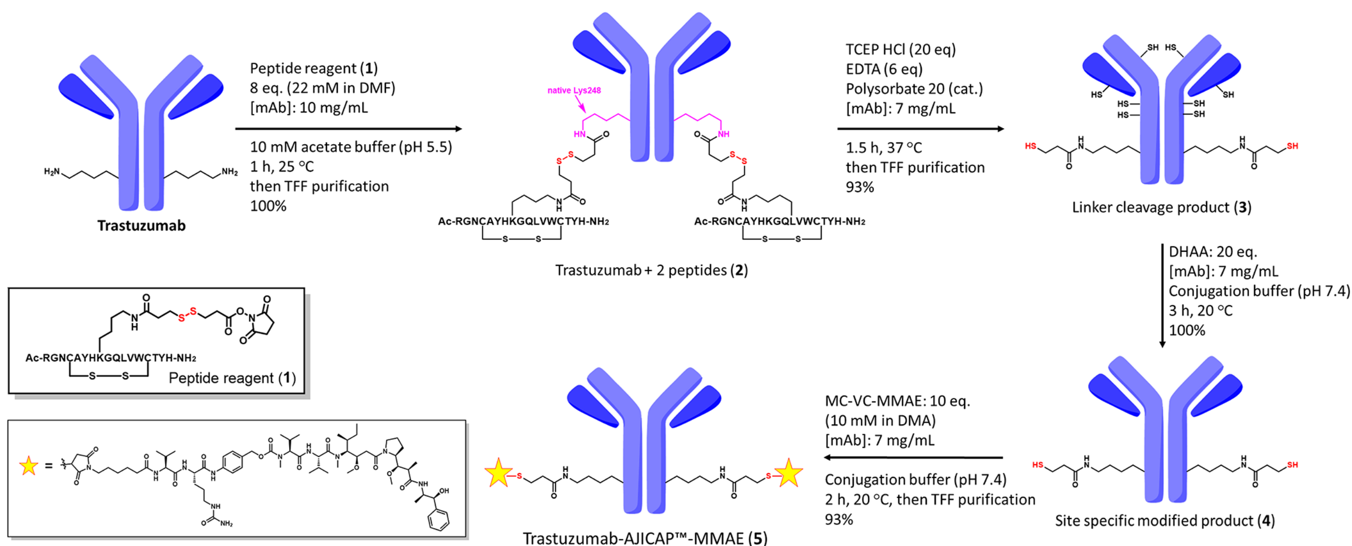
Scheme 2. Gram-Scale Synthesis of Trastuzumab-AJICAP-MMAE (5)



produced using this approach are acceptable for use in GLP preclinical studies used to support pharmaceutical development efforts.

Facility and Equipment. All activities related to development formulations are conducted in the Process Development (PD) laboratory. The PD laboratory is located in a segregated area of a GMP manufacturing facility to ensure no cross-contamination of test articles with other GMP materials. Segregation of the PD laboratory is achieved using dedicated utilities, including heating, ventilation, and air conditioning (HVAC); deionized water; required gases; separate access-controlled entry; and personal protective equipment.

Equipment used in the production of development formulations is not subject to the full requirements of GMP qualification and validation; however, all equipment undergoes routine preventive maintenance (PM) and calibration to ensure adequate operation. Analytical equipment used for the analysis of development formulations is maintained as GMP equipment with supporting qualifications, including installation and operational qualification (IOQ), documented PM and calibration schedules and written procedures for operation and maintenance.

Laboratory manufacturing equipment and materials are single use and disposable wherever possible to eliminate the risk of cross-contamination. Where single use is not possible, materials are product-dedicated.

Control of Raw Materials. All raw materials (RMs) and excipients used in the manufacture of development formulations are classified as GMP materials. GMP materials follow a quarantine, sampling, testing, and quality unit release process to ensure compliance with approved product specifications, which define identity, strength, purity, and quality characteristics.[17] Lot release packets are maintained by the quality unit for a designated record retention timeframe after release. Test articles often do not have pre-established criteria to allow for the creation of product specification to support the GMP RM process described above. Qualification and release of such test articles are at the discretion of the product sponsor, who assumes responsibility for ensuring that the materials meet the requirements of the desired study. At a minimum, an identity test is performed upon receipt of the test article to ensure that it is the correct material for the protocol.

Manufacturing Controls. All activities, including manufacturing data, equipment use, processing steps, and deviations, are documented in an electronic laboratory notebook (ELN) system. All manufacturing-related studies to be executed require a protocol that is subject to pre-approval by the PD owner and quality unit. This protocol describes the intent and purpose of the study and includes a detailed batch record to define the process steps necessary to complete the manufacturing activity. The batch record includes supporting information, including a bill of materials, list of equipment, in-process control testing, and required sampling steps. During execution of the protocol batch record, each step is signed off by the operator performing the activity and verified by a second operator in accordance with GMP principles.[17] Any deviation from the pre-approved batch record is recorded and justified to ensure that it does not have an adverse impact on the formulation.[17] Once execution is complete, the batch record is subject to review by the PD owner and quality unit to address any documentation deficiencies or deviations. Operations and documentation related to these manufacturing processes are subject to an internal audit program administered by the quality unit.

Laboratory Controls. The testing and analysis of the developmental material are executed using analytical methods demonstrated to be suitable for their intended use. Method qualification and validation are generally required for the analysis of GMP material to ensure that the performance of the test method is appropriate for the test article.[25] Analytical methods used to support developmental formulations do not require method qualification or validation; however, the test method should be proceduralized prior to use to ensure that the test execution is as intended.

Samples are taken at defined steps in the process and are entered into a laboratory information management system to track the lifecycle of the sample. All analyses of test materials are performed using GMP-compliant equipment and reagents. Data generated during analysis is reviewed by the laboratory personnel and quality unit to ensure adherence to the study protocols. Given the developmental nature of these studies, most materials do not have predefined acceptance criteria to compare with the analytical results. Data is then assembled into a certificate of testing (COT), which is reviewed by the

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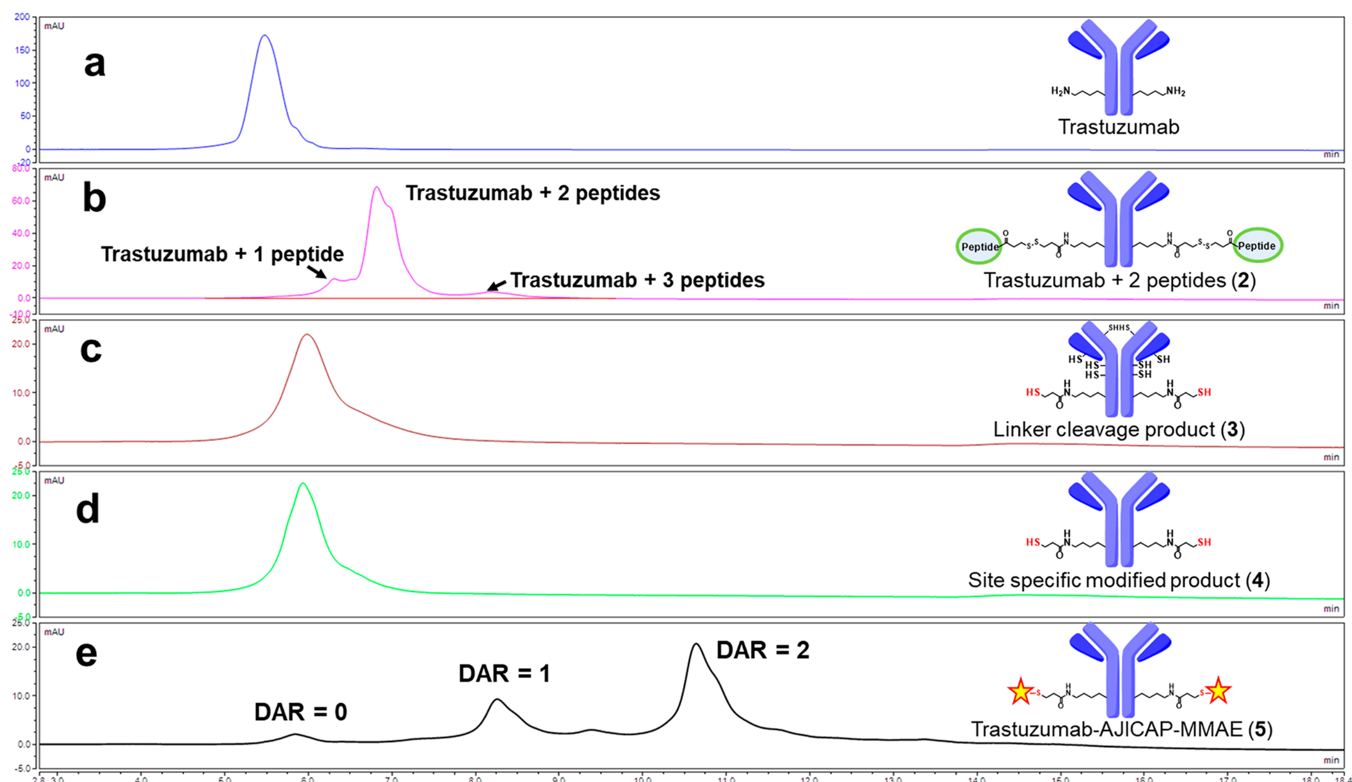

Figure 1. HIC analysis for IPC: (a) trastuzumab; (b) trastuzumab conjugated to peptides (**2**); (c) linker cleavage compounds (**3**); (d) site-specific, thiol-modified product (**4**); and (e) trastuzumab-AJICAP-MMAE (**5**).

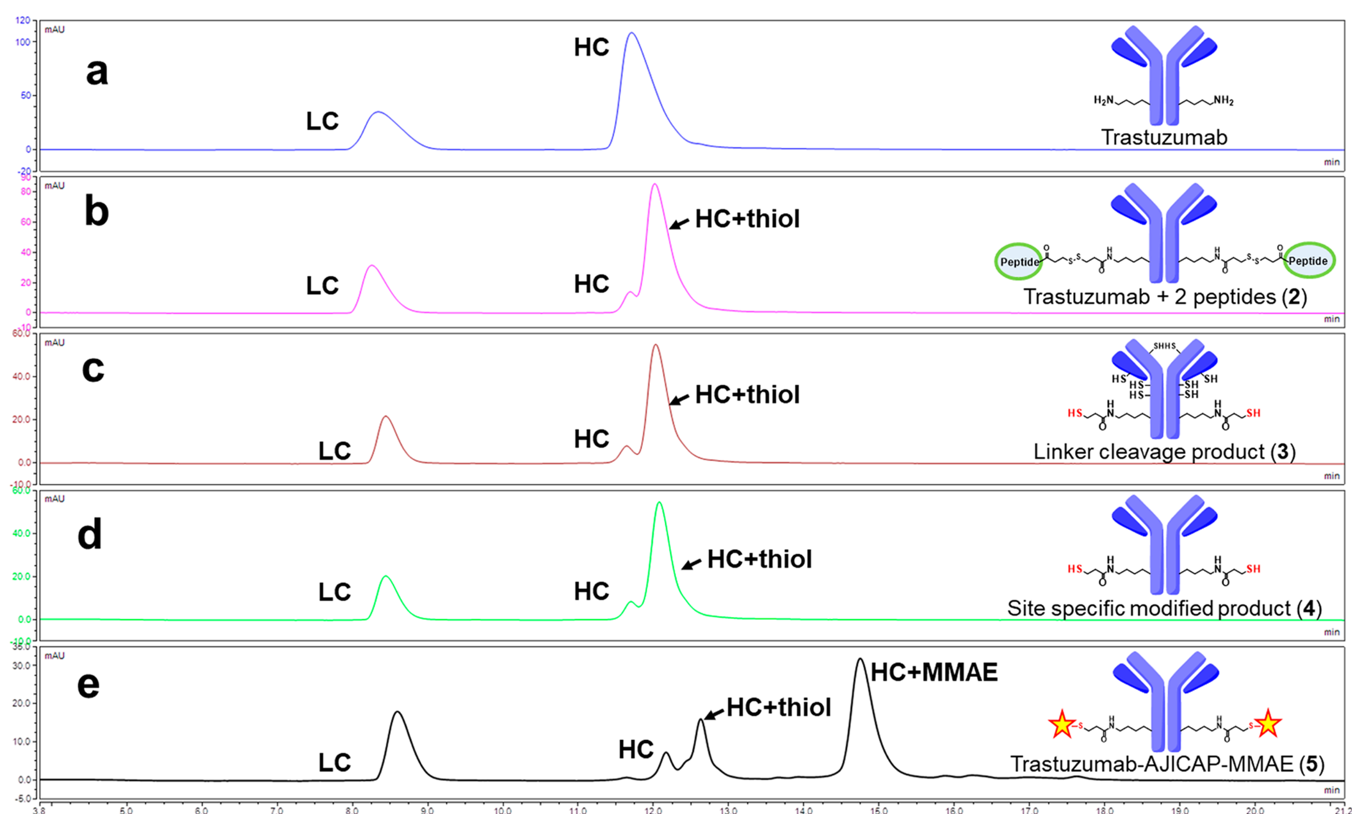

Figure 2. RP-HPLC analysis for IPC: (a) trastuzumab; (b) trastuzumab conjugated to peptides (**2**); (c) linker cleavage compounds (**3**); (**d**) site-specific, thiol-modified product (**4**); and (e) trastuzumab-AJICAP-MMAE (**5**).

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PD owner and quality unit, and used to document acceptance of the material produced in the study and release of the material to the project sponsor.

Packaging, Labeling, and Distribution. Packaging and labeling, including sample aliquoting, container closure, and secondary packaging of the test material, are at the discretion of the sponsor. The minimal labeling requirements include a product description, batch number, date of manufacture, storage conditions, and a "Not for Human Use" statement. Release of the material is documented on the COT, which accompanies the shipment to the sponsor.

Record Keeping. All documentation, including the pre-approved protocol, executed batch record, analytical raw data, and reports and COT associated with the manufacture of the test material, is maintained in the ELN system indefinitely. This system is compliant with 21 CFR Part 11 requirements for electronic records to ensure that the documents are available for further review.[26]

Antibody−Drug Conjugate Synthesis Using Site-Specific Chemical Conjugation. Next, we applied this GMP strategy for AJICAP-ADC synthesis (Scheme 2). From a previous gram-scale ADC synthesis,[15] we optimized several processes aimed at future manufacturing. First, to check the completion of each reaction, characteristic IPC analysis was completed. All reactions, including peptide conjugation, were conducted in glass reactor vessels. This stirring system is a well-known technique, which enables the later application of this synthesis to the larger batch vessels that are often used for kilogram-scale ADC preparation in a manufacturing facility.[27]

To the best of our knowledge, the chemical modification of proteins by an affinity reagent using a glass reactor vial and stirrer has not been reported, and there was concern that this mixing methodology may trigger undesired nonspecific conjugation or reduce the quality of the proteinaceous material produced.

Peptide reagent **1** was added to a trastuzumab solution, and this reaction mixture was stirred using the Chemglass system. IPC analysis of this reaction was conducted using several analytical techniques. Hydrophobic interaction chromatography HPLC (HIC-HPLC), which is well known as the gold-standard method for the analysis of antibody-related products, provided clear visual results (Figure 1a,b). The retention time of the trastuzumab−peptide conjugate (**2**, Figure 1b) was longer compared with that of trastuzumab (Figure 1a), indicating that the peptide conjugation was almost complete within 1 h and trastuzumab modified by two peptides was the major product. However, two additional peaks were also observed in the HIC chromatogram. The smallest peak (retention time, 8.0 min) was attributed to trastuzumab conjugated with one extra peptide, implying that an undesired side reaction had occurred. The other peak, with a retention time of 6.3 min, was assigned as trastuzumab conjugated with only one peptide. This HIC result was identical to the previous results,[14] and quadrupole time-of-flight mass spectrometry (Q-TOF MS) analysis of compound **2** after purification also supported this observation (Figure S16, Supporting Information).

Reverse-phase HPLC (RP-HPLC) is also commonly used for IPC analysis; in particular, the site selectivity of AJICAP conjugation was previously confirmed by this methodology (Figure 2).[16] The RP-HPLC chromatograms of reduced **2** showed three peaks (Figure 2b). The highest peak (retention time, 12.0 min) corresponded to the heavy chain conjugated with one thiol-containing group, which was derived from the peptide reagent. For RP-HPLC, all samples were pretreated with DL-dithiothreitol (DTT) to cleave the disulfide bonds. This reduction also caused cleavage of the linkage between the peptide moiety and trastuzumab in compound **2**. As a result of this cleavage, the heavy chain modified by the newly formed thiol was observed in the chromatogram. Additionally, two peaks (retention times, 8.3 and 11.7 min) matched the retention time for reduced trastuzumab. From these results, the heavy-chain selectivity of the conjugation of peptide **1** to trastuzumab was confirmed, showing the compatibility of this synthesis with the reactor vessel system with stirring.

After tangential flow filtration (TFF) purification, compound **2** was treated with tris(2-carboxyethyl)phosphine hydrochloride (TCEP) under previously optimized conditions using slightly acidic buffer.[15] Compound **3** was eluted with a retention time close to that of the naked antibody in the HIC chromatogram, indicating that the peptide moieties had been removed from compound **2** (Figure 1c). Theoretically, compound **3** should retain the internal noncovalent interactions holding the complex together, even if all the covalent interchain disulfide bonds are cleaved; therefore, the overall surface hydrophobicity of compound **3** should not change significantly, and this is indeed reflected in the retention time in the HIC chromatogram. RP-HPLC was not useful for the IPC analysis of this reaction because the RP-HPLC chromatogram of compound **3** was identical to that of compound **2** (Figure 2c). Therefore, we tested another IPC analytical method to support the HIC result. Ellman's assay of compound **3** showed an average of 10.2 free sulfhydryl groups per antibody, indicating that all the interchain disulfide bonds and the linkage between the peptide and antibody were cleaved to form free thiol groups (Table S2, Supporting Information). Purification by the TFF system gave purified **3**, which was converted to compound **4** by exposure to mild oxidative conditions. For the monitoring of this reaction, Ellman's assay was a useful method of analysis, showing that there was an average of 1.87 free sulfhydryl groups per antibody (Table S3, Supporting Information). HIC and RP-HPLC were not viable options to analyze this reaction step because the retention time of compound **4** did not shift from that of compound **3** in either chromatogram.

Conjugation of MC-VC-MMAE with compound **3** was easily monitored by both HIC and RP-HPLC due to the hydrophobicity of this drug linker. Both chromatograms matched with previous studies [drug antibody ratio (DAR) = 1.6], and 1.7 g of trastuzumab-AJICAP-MMAE (**5**) was obtained after TFF purification.[28,29]

In addition to reaction monitoring, the RP-HPLC results described in Figure 2 indicate the heavy-chain specificity of AJICAP conjugation as no light-chain conjugates were observed in the RP-HPLC chromatogram. In our previous report, a peptide mapping analysis of intermediate **4** (that is corresponding to the precursor of final ADC **5**) indicated that AJICAP technology can perform in a site-specific manner to functionalize a single lysine in the Fc region of an antibody.[14] These results support the conclusion that trastuzumab-AJICAP-MMAE (**5**) retains site-specific conjugation. Further analytical investigations including peptide mapping of **5** are now underway.

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CONCLUSIONS

A GMP strategy, including the regulatory framework and guidance, was established for use in GLP preclinical supply of a material. For the demonstration, AJICAP-ADC synthesis was achieved. All reactions were conducted in appropriate scalable glass reactor vessels, enabling future scale-up in a manufacturing facility. Several IPC analytical methods based on the compound or intermediate characteristics were also conducted. The total product yield for the four steps was increased to 90% from a previous study, and finally, 1.72 g of trastuzumab-AJICAP-MMAE was obtained. These results demonstrated the reproducibility and robustness of AJICAP synthesis, enabling its application for future manufacturing.[30] The establishment of our strategy to produce materials suitable for GLP studies and incorporation in relevant regulatory filings is based on a carefully considered review of relevant regulatory guidance and manufacturing logic and can serve as a model for future biomolecular pharmaceutical development.

EXPERIMENTAL SECTION

Materials. Human IgG1 trastuzumab (Herceptin) was purchased from Roche Pharmaceutical Company (Switzerland). Maleimide-C6-valine-citrulline-monomethyl auristatin E (CAS#: 646502-53-6; MC-VC-MMAE) was purchased from NJ Biopharmaceuticals LLC (USA). Peptide reagent **1** was provided from Ajinomoto Co., Inc. All other chemical reagents were purchased from Sigma-Aldrich (USA).

Experimental Procedure for Peptide Conjugation (Step 1 of AJICAP Technology). To a solution of trastuzumab (10 mg/mL, 1.90 g) in 10 mM AcONa buffer (pH 5.5) was added a 22 mM dimethylformamide (DMF) solution of peptide reagent **1** (9 equiv, 5.15 mL) and stirred at 25 °C using the Chemglass system. After 1 h, a small amount of reaction mixture (0.5 mL) was sampled for IPC analysis. After checking the IPC analysis, the reaction mixture was purified by a TFF system using a Sartocon Slice 200 ECO Hydrosart membrane (30 kDa; Sartorius) and 10 mM AcONa buffer (pH 5.5) as diafiltration (DF) buffer at an antibody concentration of 20 mg/mL. Next, the buffer exchange of this solution was conducted by a TFF system using a Sartocon Slice 200 ECO Hydrosart membrane (30 kDa; Sartorius) and formulation buffer (20 mM histidine containing 5% trehalose, pH 5.2) as DF buffer at an antibody concentration of 6.9 mg/mL to afford trastuzumab−peptide conjugate **2** (1.92 g, 98% yield) in formulation buffer.

Experimental Procedure for Linker Cleavage (Step 2 of AJICAP Technology). To a solution of trastuzumab-peptide conjugate **2** (6.9 mg/mL, 1.92 g) in formulation buffer was added 0.25 M aqueous solution of ethylenediaminetetraacetic acid (EDTA) (12 equiv, 0.572 mL, pH 7.4), 100 mg/mL polysorbate 20 (0.0265 mL), and 0.5 M aqueous solution of TCEP (20 equiv, 0.476 mL) at 37 °C. The resulting mixture was measured by a pH meter, showing that the final pH was 5.2. After 1 h at 37 °C, a small amount of reaction mixture (0.5 mL) was sampled for IPC analysis. After checking the IPC analysis, the reaction mixture was purified by a TFF system using a Sartocon Slice 200 ECO Hydrosart membrane (30 kDa; Sartorius) and 10 mM AcONa buffer (pH 5.5) as DF buffer at an antibody concentration of 20 mg/mL. Next, the buffer exchange of this solution was conducted by a TFF system using a Sartocon Slice 200 ECO Hydrosart membrane (30 kDa; Sartorius) and conjugation buffer (50 mM PBS, 10 mM EDTA, pH 7.4) as DF buffer at an antibody concentration of 6.6 mg/mL to afford linker cleavage product **3** (1.79 g, 96% yield) in conjugation buffer.

Experimental Procedure for Reoxidation (Step 3 of AJICAP Technology). To a solution of linker cleavage product **3** (6.6 mg/mL, 1.79 g) in conjugation buffer was added a 50 mM dimethyl sulfoxide solution of DHAA (40 equiv, 8.60 mL), and the mixture was incubated at room temperature. After 3 h, a small amount of reaction mixture (0.5 mL) was sampled for IPC analysis. After checking the IPC analysis, the reaction mixture was purified by a TFF system using a Sartocon Slice 200 ECO Hydrosart membrane (30 kDa; Sartorius) and conjugation buffer as DF buffer at an antibody concentration of 6.8 mg/mL to afford linker reoxidation product **4** (1.78 g, 100% yield) in conjugation buffer.

Experimental Procedure for Payload Conjugation (Step 4 of AJICAP Technology). To a solution of reoxidation product **4** (6.8 mg/mL, 1.78 g) in conjugation buffer were added dimethylacetamide (DMA) (11.5 mL) and a 10 mM DMA solution of MC-VC-MMAE (10 equiv, 13.3 mL), and the mixture was incubated at 20 °C. After 2 h, a small amount of the reaction mixture (0.5 mL) was sampled for IPC analysis. After checking the IPC analysis, the reaction mixture was quenched by an excess amount of a 50 mM aqueous solution of *N*-acetyl cysteine and incubated at 25 °C for 15 min. This reaction mixture was purified by a TFF system using a Sartocon Slice 200 ECO Hydrosart membrane (30 kDa; Sartorius) and conjugation buffer as DF buffer at an antibody concentration of 20 mg/mL. Next, the buffer exchange of this solution was conducted by a TFF system using a Sartocon Slice 200 ECO Hydrosart membrane (30 kDa; Sartorius) and formulation buffer as DF buffer at an antibody concentration of 6.8 mg/mL to afford trastuzumab-AJICAP-MMAE **5** (1.73 g, 95% yield) in formulation buffer.

General Procedure for IPC. Gel filtration of several reaction mixtures was conducted for removal of impurities to obtain a purified protein, which was used for several analyses.

Instruments/Analytical Method. The concentration of proteins was determined by the Slope Spectroscopy method with a Solo-VPE system.[14]

HIC-HPLC and RP-HPLC analysis were performed as previously reported.[16]

Q-TOF MS analysis was performed as previously reported.[14]

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.9b02419.

Tables comparing research and development, GMP, and our approach; chromatographic figures; and DAR calculation and Ellman's assay results, as described in the text (PDF)

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Notes

The authors declare no competing financial interest.

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(28) In the case of conjugation with maytansinoid-type drug linker, DAR = 1.9 ADC was obtained (see ref 14). This DAR difference between MMAE and maytansinoid was rationalized by their reactivity and hydrophobicity. Typically, conjugation with higher hydrophobic drug linkers gives lower DAR ADCs. See representative example: Xu, Y.; Jiang, G.; Tran, C.; Li, X.; Heibeck, T. H.; Masikat, M. R.; Cai, Q.; Steiner, A. R.; Sato, A. K.; Hallam, T. J.; Yin, G. RP-HPLC DAR Characterization of Site-Specific Antibody Drug Conjugates Produced in a Cell-Free Expression System. *Org. Process Res. Dev.* **2016**, *20*, 1034−1043.

(29) Our research group has completed optimization studies to improve DAR, and these advancements will be discussed in the future literature.

(30) Our research group has reported an initial study of peptide conjugation and labeling for a variety of IgGs including IgG1, IgG2, and IgG4 (see ref 14). ADC synthesis from these intermediates is a current focus of work in our group.