A Manufacturing Strategy Utilizing a Continuous Mode Reactor toward Homogeneous PEGylated Bioconjugate Production

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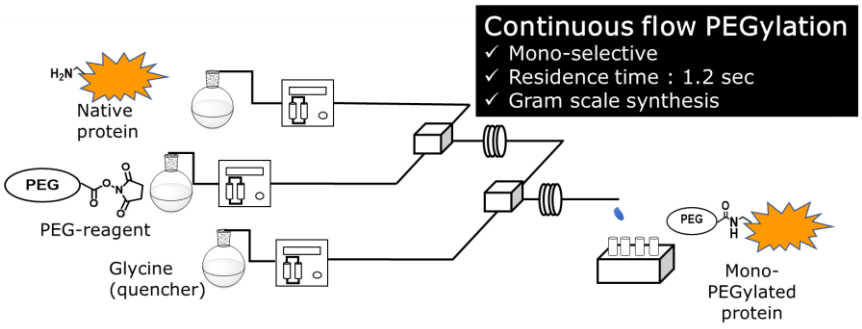

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Abstract Protein PEGylation is a traditional bioconjugation technology that enhances the therapeutic efficacy and *in vivo* half-life of proteins by the formation of covalent bonds with polyethylene glycol (PEG). The most established methodology for PEGylation utilizes activated ester-incorporating PEG reagents; however, the high reactivity of these reagents induces a random reaction with lysine residues on the protein surface, resulting in a heterogeneous mixture of PEGylated proteins. Moreover, the traditional batch-mode reaction has risks relating to scalability and aggregation. To overcome these risks of traditional batch-mode PEGylation, a manufacturing strategy utilizing structural analysis and a continuous-flow-mode reaction was examined. A solvent exposure analysis revealed the most reactive lysine of a protein, and the continuous flow mode modified this lysine to achieve the mono-PEGylation of two different proteins within two seconds. This ultra-rapid modification reaction can be applied to the gram-scale manufacturing of PEGylated bioconjugates without generating aggregates. A similar trend of the exposure level of protein lysine and mono-selectivity performed by continuous-flow PEGylation was observed, which indicated that this manufacturing strategy has the potential to be applied to the production of a wide variety of bioconjugates.

Key words Continuous-flow synthesis, Microreactor, PEGylation, Cytokine, Bioconjugates

Protein PEGylation is a modification technique that forms a covalent link between polyethylene glycol (PEG) and a protein. [1-3] This traditional conjugation approach enhances the therapeutic efficacy and safety profile of protein-based biopharmaceutics due to the hydrophilic nature of PEG molecules. Currently, many PEGylated proteins are approved by the US Food and Drug Administration. PEG reagents possessing activated esters, such as N-hydroxysuccinide (NHS), are widely used in this technique. These reagents modify the lysine residues of proteins to form covalent bonds and create PEGylated conjugates. The conjugation concept is simple; however, most PEGylated proteins currently on the market have wide heterogeneity owing to nonspecific protein modifications, resulting in clinical insufficiencies and chemistry, manufacturing, and control challenges. In addition to the heterogeneous nature of PEGylated proteins, the chemical reaction to install PEG poses a risk of aggregation generation.[4] In particular, several sensitive proteins may have limited compatibility with chemical reactions such as PEGylation; therefore, mild reaction conditions with short reaction times should allow for more reliable conjugations. Furthermore, kinetic reactions, such as amidation by activated ester reagents such as NHS, can cause scalability issues.[5] To overcome the aggregation and scale gap issues in protein PEGylation, a variety of chemical reactions are required during the early stages of manufacturing, including screening of functional groups to react with the amino acid residues of the target protein and/or careful process development. A promising option for achieving homogeneous PEGylation is to utilize a continuous-mode flow reaction. The continuous flow reaction is a rapidly growing manufacturing process in industry.[6,7] This process enables chemical reactions in designated systems consisting of tubes, mixers, and pipes. Flow-mode manufacturing equipment can perform sensitive novel chemical reactions that cannot to be controlled in traditional stirred-batch reactors. Furthermore, this process is environmentally friendly, as it reduces the risk of accidental exposure to toxic chemicals,[8] and is straightforwardness to scale-up.[9] Based on these advantages, a flow microreactor (FMR) system can reduce operating expenditures and facilitate automated manufacturing of industrial materials. PEGylation in continuous mode has been attempted by several groups using enablers such as the on-column counter-current chromatograph,[10] hollow-fiber membrane reactor,[11,12] and coiled flow inverter reactor.[13] These fundamental studies demonstrated the feasibility of protein PEGylation using FMR to achieve bioconjugation with native lysine amino acid side chains. However, this type of lysine conjugation produces a heterogeneous mixture of conjugate molecules. For example, typical IgG1 antibody proteins have

greater than 80 exposed and reactive lysine residues[14] indicating that careful optimization of the reaction conditions is required despite the use of an FMR system. Amino acid nature differs among protein bases; therefore, careful reaction optimization that is dependent on protein characteristics is required. These results prompted us to investigate a versatile manufacturing strategy that supports continuous-mode PEGylation. To establish a practical strategy for homogeneous PEGylated protein manufacturing, we conducted demonstration studies using traditional lysine-based PEGylation with PEG reagents functionalized by an activated ester group. We hypothesized that the exposed lysine groups would have higher reactivity to PEGylation; therefore, we investigated the relationship between the exposure level of each lysine in the protein and the reaction selectivity. Solvent exposure analysis[15] enabled us to predict the reactivity of each lysine in the protein, and the resultant trends were compared with the site selectivity of PEGylation produced using a continuous mode reaction. For this demonstration, a V-shaped mixing system that can be easily applied at the manufacturing scale was selected. Feasibility flow system trials enabled the mono-PEGylation of therapeutic proteins such as lysozyme and interleukin-6 (IL-6) within two seconds (Figure 1). Furthermore, this "ultra-rapid" and mild reaction condition was successfully applied to a gram-scale synthesis of the PEGylated protein without aggregation generation, whereas batch mode synthesis revealed scale-gap issues.

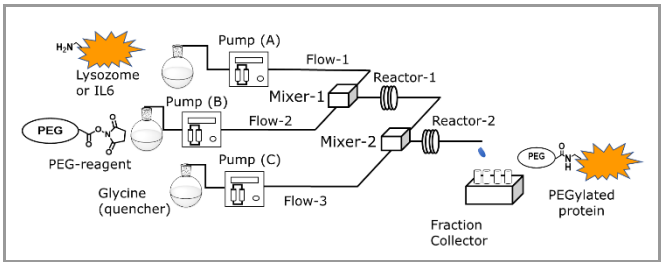
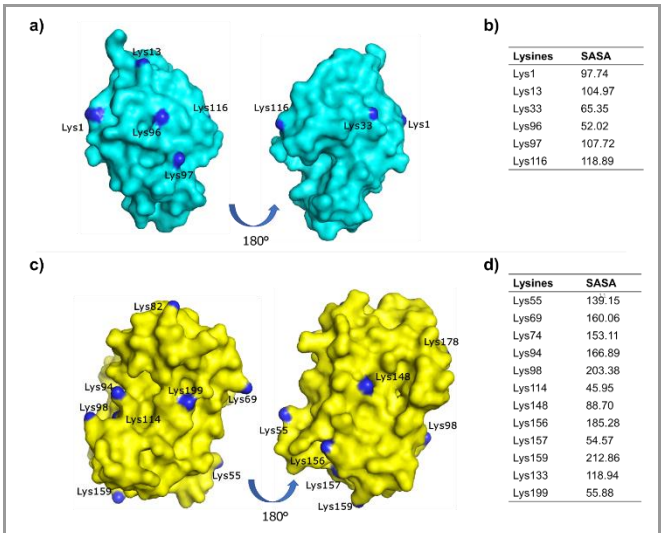


Figure 1 Flow diagram of continuous flow protein PEGylation

Lysozyme has only six lysine groups in its sequence; therefore, the PEGylated product has a relatively low distribution (from mono-to hexa-conjugates). This inexpensive (USD 23 per gram; Millipore Sigma, accessed Jan 29, 2023) protein has been subjected to continuous PEGylation in several previous studies[12]. To predict the reactivity difference of each lysine, we conducted solvent exposure analysis of the lysozyme structure reported in the Protein Data Bank (PDB) (Figure 2).[15,16] The results indicated that although all six lysozyme lysines were well exposed to the solvent, the solvent-accessible surface areas (SASAs) differed slightly. The most exposed lysine (Lys116) showed a 10 % higher SASA level than that of the second most exposed lysine (Lys97).

In addition to lysozyme, IL-6 levels were analyzed. IL-6 has a higher molecular weight and a greater number of lysine residues (14) than lysozyme. PEGylation of IL-6 enhanced the therapeutic efficacy of native IL-6;[17] however, only limited pharmaceutical applications were identified due to the heterogeneous nature of the conjugate First, we attempted to apply the same calculation procedure as that used for lysozyme; however, 52–60 amino acid disorders were observed in the IL-6 structure reported in the PDB (PBD: 1ALU) (Supporting Information (SI), Figure S1).[18] To understand the actual structure, we utilized the AlphaFold-2 database,[19,20] which contains a modified IL-6 monomer structure (AlphaFold Protein Structure Database: AF-P05231-F1-model_v4) that can be used for structural and SASA analyses. These analyses showed that all 14 lysines of IL-6 were well exposed to the solvent; however, the SASA levels of each lysine differed slightly. The most exposed lysine (Lys159) showed an SASA that was only 4 % higher than that of the second most exposed lysine (Lys98). These analyses revealed that IL-6 is a more challenging target than lysozyme for demonstrating this strategy.



Lysines	SASA
Lys1	97.74
Lys13	104.97
Lys33	65.35
Lys96	52.02
Lys97	107.72
Lys116	118.89

Lysines	SASA
Lys55	139.15
Lys69	160.06
Lys74	153.11
Lys94	166.89
Lys98	203.38
Lys114	45.95
Lys148	88.70
Lys156	185.28
Lys157	54.57
Lys159	212.86
Lys133	118.94
Lys199	55.88

Figure 2 Predictions of exposure levels for lysines, a) structural analysis of lysozyme (PBD: 1dpx), b) SASA analysis of lysine residues in lysosome, c) structural analysis of IL-6 (AlphaFold-2: AF-P05231-F1-model_v4.pdb), d) SASA analysis of lysine residues in IL-6

Next, we attempted to determine whether a continuous-flow system could be used to identify small reactivity differences and perform selective lysine modifications. Several studies [9,21] have reported that the FMR reaction is suitable for kinetically controlled reactions; therefore, our first attempt used PEG reagent screening. NOF Corporation, a main PEG reagent company in the bioconjugation field, provides reagents in different PEG units. [22] Batch-mode reaction screening revealed that methoxy-PEG-CH2-COO-NHS (5 kDa, catalog number SUNBRIGHT ME-050AS) showed the highest reactivity. The PEGylation reaction was completed in less than 1 min using SUNBRIGHT ME-050AS, whereas the other PEG reagents continued to react after 3 min (Supporting Information (SI) Figure S2). These reactivity trends were similar to that of the half-life of these PEG reagents[22] and supported the hetero-functional group placing in neighboring positions, which enhanced the reactivity of the NHS group and reduced hydrolysis resistance.

In the development of continuous reactions, several parameters must be considered[23, 24] such as the tube length, time, pH, and temperature of the reaction. However, the appropriate parameter depends on the target protein behavior and this experimental design approach is beyond the scope of the current feasibility study. The purpose of this study was to demonstrate the developability of this modification strategy (SASA analysis

scouting the target lysine followed by a continuous flow reaction). For this purpose, we selected a tentative condition to apply to the PEGylation. The most important factors affecting mixability in flow systems are the geometry and diameter of the mixer unit, and we selected conditions previously reported as effective for early selection. The iodide-iodate reaction, termed the Dushman reaction, is commonly used to evaluate mixing efficiencies[25,26] and we previously confirmed the high reproducibility of this method,[23] whereby V-shaped mixers produced the most efficient mixability for several geometric types (Vortex-shaped, T-shaped, and V-shaped; Figure S3). Therefore, a V-shaped mixer was selected for PEGylation. Diameter is also a critical factor that affects the mixability of the flow system. Based on our previous study[9], a diameter of 0.25 mm was selected for use in the present investigation. Previously, our group succeeded in performing a tandem reaction (reduction of disulfide bonds of an antibody followed by conjugation with a cytotoxic drug) in continuous mode to produce antibody-drug conjugates (ADCs). A 0.25 mm diameter V-shaped mixer sufficiently converted naked antibodies to ADCs, while a mixer with a diameter greater than 0.5 mm did not reach the target drug-to-antibody ratio. We expected that the 0.25 mm diameter V-shaped mixer that was applied to a complicated tandem bioconjugation to produce ADC could also be applied to a single reaction PEGylation. The flow system consisted of two V-shaped mixers (Mixer-1 and Mixer-2 in Figure 1) and two 0.5 m reactors (Reactor-1 and Reactor-2 in Figure 1). The diameter of the reactors was 1.0 mm, and using a high flow rate (8 mL/min for lysozyme), PEGylation was completed in 1.17 s (residence time in Reactor-1). This rapid reaction mode enabled the production of a gram-scale PEGylated lysozyme within 15 min. The conversion yield and mono-PEGylation selectivity were analyzed using RP-HPLC[27] (Figure 3). Additionally, a direct comparison was conducted between the PEGylated lysozyme produced using the batch-mode approach and that synthesized through the continuous flow system.

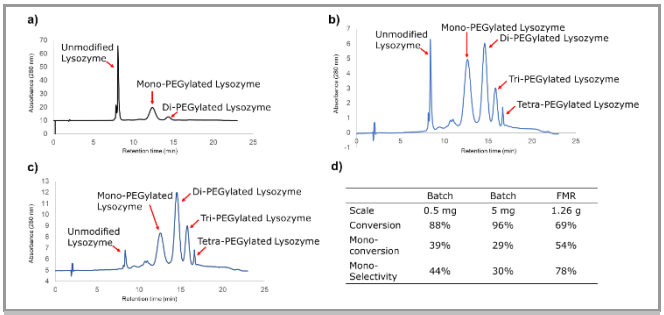


Figure 3 PEGylation of lysozyme a) HPLC analysis of PEGylated lysozyme produced with continuous flow mode (1.26 g scale), b) HPLC analysis of PEGylated lysozyme produced with batch mode (0.5 mg scale), c) HPLC analysis of PEGylated lysozyme produced with batch mode (5 mg scale), d) summary of mono-PEGylation

Continuous flow mode converted 54 % of the lysozyme to mono-PEGylated conjugates, showing a mono-selectivity of 78 %. In contrast, batch mode provided less than 40 % mono-conversion. In addition, the reproducibility and scalability of batch mode were clearly problematic. In larger-scale syntheses, the mono-conversion rate decreased, and an overreaction was observed. Moreover, the ineffective mixability of the batch mode triggered aggregate generation (greater than 44 % by size-exclusion chromatography analysis (SEC),[28] Figure S4 in SI), whereas no aggregates were observed in the gram-scale conjugates produced in continuous mode. These results indicate that the continuous-flow system is a rapid and robust manufacturing process with the potential to achieve mono-selective PEGylation.

Next, continuous mono-PEGlylation was performed to modify IL-6 (Figure 4).

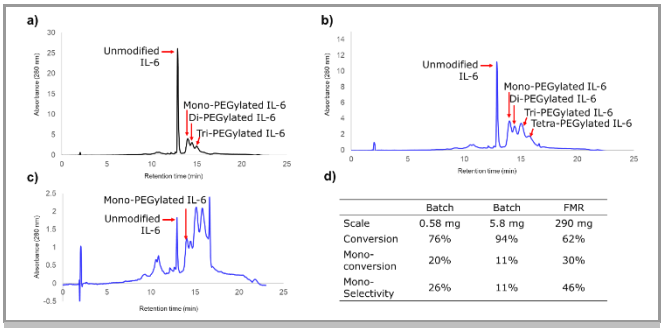


Figure 4 PEGylation of IL-6 a) HPLC analysis of PEGylated IL-6 produced with continuous flow mode (290 mg scale), b) HPLC analysis of PEGylated IL-6 produced with batch mode (0.58 mg scale), c) HPLC analysis of PEGylated IL-6 produced with batch mode (5.8 mg scale), d) summary of mono-PEGylation

Similar comparisons were obtained for the batch and continuous flow modes as those observed with lysozyme. Continuous flow mode converted 30 % of IL-6 to mono-PEGylated conjugates with a mono-selectivity of 46 %, while batch mode presented several issues (low mono-selectivity, reproducibility, and scalability, and high aggregation generation (greater than 54 % by SEC analysis, Figure S4 in SI)). IL-6 has more lysine residues, all of which are more exposed to solvent than those of lysozyme. This exposure level difference caused a relatively lower mono-conversion rate in the IL-6 modification.

In conclusion, a manufacturing strategy utilizing SASA analysis and continuous-flow process-mediated PEGylation was achieved using two proteins that have potential for clinical use. The selected 0.25 mm diameter V-shaped mixer performed rapid (1.17 s) protein modification to achieve mono-selective PEGylation without inducing appreciable aggregation. All flow processes were conducted using a scaled-down manufacturing approach with a sequential mixing system. Furthermore, these early stage (not thoroughly optimized) reaction conditions were able to generate gram-scale PEGylated lysozymes within 15 min. The exposed lysine trend calculated by SASA analysis was similar to that of mono-selective production in the continuous mode. The results described herein indicate that the strategy of using an SASA with continuous-flow chemistry has the potential for application in a wide variety of protein modifications.

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Lysozyme (Chicken egg-white) was purchased from Sigma-Aldrich (USA). IL6 protein was expressed and purified as previously reported.[29] The PEG reagent (Methoxy-PEG-CH_2-COO-NHS, 5 kDa, catalog number SUNBRIGHT ME-050AS) was purchased from NOF Corporation (Japan). All other chemical reagents were purchased from Sigma-Aldrich (USA).

SASA Calculation

The SASA calculations were performed using the Bioluminate software suite (Bioluminate, version 2022-2, Schrödinger, Inc.). The initial

structures of lysozyme (PDB:1DPX)[30] and IL-6 (AlphaFold Protein Structure Database: AF-P05231-F1-model_v4)[19,20] were protonated and minimized, and the SASA score was calculated using the Residue Analysis module.

Molecular modeling

The model structure of the proteins was generated as described previously.[31]

Experimental procedure for PEGylation using batch reactor

PEG reagent in DMSO (10.5 mg/mL, 10 eq) was added to a solution of protein (0.5 mg or 5 mg) in 20 mM Borate buffer (pH 9.0). The mixture was then incubated for 5 min at 20 °C. An excess of 50 mM glycine and 1 M acetate buffer (pH 4.7) was added to adjust the pH of the reaction mixture (to approximately pH 7.0) and the mixture was stirred for an additional 15 min. Subsequently, the reaction mixture was purified using a PD-10 desalting column and eluted with 50 mM acetate buffer (pH 5.5).

Experimental procedure for PEGylation using flow reactor

V- and T-shaped stainless-steel mixers with inner diameters of 0.25 mm (Sankoh-seiki, Tokyo, Japan) were used as Mixer-1 and Mixer-2, respectively, as shown in Figure 1. Reactor-1 (1.0 mm i.d., 0.5 m length) and Reactor-2 (1.0 mm i.d., 0.5 m length) were also made of stainless steel. The PEG reagent in DMSO (3.5 mg/mL, 10 eq) was added to Mixer-1 through Flow-1. The 50m M borate buffer containing the protein (1.05 mg/mL for lysozyme, 1.47 mg/mL for IL-6, pH 9.0) was added to Mixer-1 through Flow-2. The output mixture from Reactor-1 and that delivered from Flow-3 were mixed in Mixer-2. The glycine in the phosphate buffer (50 mM, excess, pH 7.4) was added to Mixer-2 through Flow-3. The output mixture of Reactor-2 was eluted into a fraction collector, to which an excess of 1 M acetate buffer (pH 4.7) was added for neutralization. This elution was combined and purified using large desalting columns as previously reported.[32]

Instruments/analytical methods

The concentration of proteins was determined using the Slope Spectroscopy® method with a Solo-VPE system.[33]

Size exclusion chromatography[28] and RP-HPLC[27] were performed as previously reported.

Author Contributions

The manuscript was written with contributions from all authors. All authors approved the final version of the manuscript. ‡These authors contributed equally (YN and YE).

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The authors declare no competing financial interest.

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Supporting Information

YES (this text will be updated with links prior to publication)

Figures showing schematic diagrams of the mixers, SEC and RP-HPLC chromatograms, as described in the text

Primary Data

NO.

Conflict of Interest

The authors declare no conflict of interest.

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