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Process Analytical Technology for Real-Time Monitoring of Pharmaceutical Bioconjugation Reactions

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ABSTRACT: Process analytical technology (PAT) is increasingly being explored within pharmaceutical production and process development, with a particular emphasis in the vaccine and biologics space. PAT aims to provide increased process understanding and control through real-time monitoring of critical quality attributes and key process parameters as well as detection of process deviations. Downstream purification in pharmaceutical manufacturing processes can be complex and requires copious analytical characterization. Herein, we showcase the successful implementation of PAT for monitoring bioconjugation reactions related to both vaccine and biologic pharmaceutical manufacturing processes. Specifically, we explore a variety of PAT-based techniques and their utility for monitoring polysaccharide−protein and protein−small molecule bioconjugation reactions. PAT applications using at-line multiangle light scattering, *in situ* fluorescence spectroscopy, *in situ* viscosity, and at-line hydrophobic interaction chromatography are shown to each provide distinct, real-time analytical information to enhance the understanding and characterization of bioconjugation reactions.

KEYWORDS: *process analytical technology, biopharmaceuticals, bioconjugation, biologics, vaccines, MALS, fluorescence, viscosity, HPLC*

INTRODUCTION

Bioconjugation generally refers to the combination of two molecules via a stable covalent linkage; one molecule is a biomolecule, and the second molecule depends on the specific purpose of bioconjugation. Various classifications of bioconjugation reactions and frameworks exist, including those which are polymeric, peptide- or protein-based, nanoparticle conjugates, or antibody-drug conjugates (ADCs).[1,2] Bioconjugation can be applied for achieving a variety of important scientific outcomes, including employment as a common framework for modifying ligands in the pharmaceutical sciences. Generally, bioconjugation reactions within the pharmaceutical space have been shown to improve effective targeted drug delivery,[2−4] increase drug efficacy,[5−7] enhance stability,[1,8] and perform other beneficial roles such as reducing clearance and facilitating controlled release systems.[5,9] It has become progressively more important to develop analytical strategies to fully characterize and understand these reactions due to the inherent advantages of such bioconjugation reactions and the increasing popularity of their implementation.

While many technologies exist for supporting bioconjugation reaction monitoring, there exists a need to increase the robustness of characterization and understanding for maximum optimization of these key bioconjugates that only process analytical technology (PAT) can solve. Conjugation reactions can be quite complex, requiring numerous parameters to be considered simultaneously for a successful reaction, including the concentration and ratio of each component, incubation time, residual unconjugated material, and overall yield. PAT presents a distinct opportunity to monitor the formation of bioconjugate molecules in real time. PAT has been defined by the Food and Drug Administration (FDA) as "a system for designing, analyzing, and controlling manufacturing through timely measurements of critical quality and performance attributes of raw and in-process materials and processes, with the goal of ensuring final product quality."[10,11] More specifically, PAT can involve the use of analytical instrumentation in any of the four following formats: offline, at-line, in-line, or online measurements. At-line measurements are typically performed on a sample by operators on the manufacturing floor and can be conducted in real-time, near-real-time, or after processing; offline measurements are similar in concept, but

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the measurements are conducted in a lab external to the manufacturing floor, such as a quality control lab. Online and in-line analytical measurements are made directly from the process in real time, with the difference manifesting in the former requiring a sample to be diverted from the process to the PAT analyzer, whereas in-line measurements can be made directly within the process stream or *in situ*. Because of the broad overview of analytical measurements considered PAT, there is also a large umbrella of analytical instrumentation, which can be considered as a PAT tool. This includes, but is not limited to, spectroscopic techniques such as infrared and Raman spectroscopy, chromatography, mass spectrometry, and particle size measurement tools such as dynamic light scattering and multiangle light scattering. Essentially, any analytical instrument which can be modified to perform at-line or can directly interface with a process can be considered within the scope of PAT.[12]

There are numerous benefits for using PAT that cannot be achieved using typical analytical characterization techniques. The collection of data in real-time or near-real-time allows for a more thorough and complete understanding of the executed process; this can be especially advantageous during analytical method development, when process parameters are being defined, and the impact of process changes can be observed in real time. Process understanding leads to enhancement of process efficiency, selectivity, and output. Once developed, the implementation of PAT on the manufacturing floor results in rapid analytical characterization, driving the process forward while saving time, resources, and energy; it can also reduce waste and increase safety by decreasing or eliminating offline sampling and handling. PAT can be used at a variety of scales, from the lab bench to the largest of manufacturing suites, providing an opportunity to compare data from large-scale reactions to smaller development reactions. When comparing reactions across scales, similar data obtained using PAT offer more confidence and ability to leverage small scale data to inform larger scale processing, while differences among scales offer an avenue to understand the variability. PAT has continuously shown its utility in the pharmaceutical industry[13−16] including specifically for synthesis and reaction monitoring,[17−19] emulsification analysis,[20] protein concentration determination,[21] and cell cultivation and culture process evaluation[22,23] due to its inherent benefits.

Herein, a variety of applications of PAT for monitoring bioconjugation reactions in the pharmaceutical space is described. Specifically, a comprehensive study is executed to investigate the expansive repertoire of PAT tools that can be used for investigating polysaccharide (Ps)-protein bioconjugation, including *in situ* viscosity and fluorescence measurements, as well as at-line multiangle light scattering (MALS). Further, we take a targeted approach to investigate at-line hydrophobic interaction chromatography (HIC) HPLC for monitoring an ADC reaction in real time. When feasible, each PAT tool is compared to the typical offline analysis for the reaction, illustrating the equivalence of the real-time measurements. In total, this work provides a comprehensive and detailed analysis of applications of PAT for monitoring bioconjugation reactions, which are key components of pharmaceutical processing. The advantages of PAT are well shown, enabling real-time process understanding of key quality attributes that are relevant for monitoring and controlling a pharmaceutical process. Each PAT technique provides a unique perspective that can be capitalized upon for understanding, optimizing, and increasing the overall success of pharmaceutical process development and production.

MATERIALS AND METHODS

Polysaccharide−Protein Bioconjugation Process. The protein−polysaccharide (Ps) bioconjugation process (Figure S1) investigated involves ongoing bioconjugation between a specific Ps serotype (ST) and the CRM-197 carrier protein. The studies described herein monitor the bioconjugation of three different STs, referred to as ST-A, ST-B, and ST-C. Further, both a DMSO- and aqueous-based bioconjugation process are studied.

The DMSO-based bioconjugation follows a previously published process.[24] Briefly, the carrier protein CRM-197 was prepared in a solution of DMSO at 2 mg mL^{-1}, and Ps solutions were prepared in a salt buffer at a concentration of 2 mg mL^{-1}. The bioconjugation reaction was initiated by mixing CRM-197 and Ps in a 1:1 ratio, at which point sodium cyanoborohydride was added.[24] The total reaction volume used was approximately 40−60 mL. Samples were removed periodically for offline analysis by manual pipetting followed by a quench of the sample with sodium borohydride. Herein, ST-A and ST-B[25−27] followed the DMSO-based bioconjugation process; the bioconjugation for ST-A and ST-B in DMSO typically takes 4 and 2 h, respectively.

A second Ps-protein bioconjugation was carried out following a similar process as the DMSO-based method[24] but conducted in an aqueous environment. Here, Ps solutions were vortexed and diluted to the desired concentration using a potassium phosphate buffer, pH 7.0. CRM-197 was then added to the Ps solution and vortexed. To this, a mixture of nickel chloride and sodium cyanoborohydride was added using a stock solution. The mixture was then moved to an Innova 44R incubator for the duration of the conjugation reaction. The total reaction volume was about 5 mL, and samples were drawn regularly to monitor the reaction progress using at-line MALS. Herein, ST-B and ST-C followed the aqueous bioconjugation process, which typically takes ∼7 days for each ST.

In Situ Fluorescence Spectroscopy. The *in situ* fluorescence spectroscopy system utilized was constructed in-house from commercially available components. Instrumentation included a QE Pro High Performance portable spectrometer (Ocean Insight, Orlando, FL) coupled to a PX2 transmission probe (Custom Sensors & Technology, Fenton, MO) and utilized an LED light source with an excitation maximum at 295 nm (M285L5, Thorlabs, Inc., Newton, NJ) as the excitation source. Data acquisition was carried out in OceanView software (Ocean Insight). The resultant kinetic fluorescence trends are generated by integrating the fluorescence intensity at 339 nm as a function of time, with no preprocessing applied to the raw data. The R^2 values are calculated by linear regression of the fluorescence signal to the corresponding offline conjugate molecular weight (M_W; see Offline High Performance Size Exclusion Chromatography) data using Microsoft Excel (no forced intercept).

Dynamic Light Scattering, Offline Viscosity, and In Situ Viscosity. Offline dynamic light scattering (DLS) measurements were obtained using a Zetasizer Nano S instrument (Malvern Panalytical, Westborough, MA). A quartz cuvette was used for analysis, and the DMSO dispersant was used for the viscosity input (1.996 cP; RI: 1.479). The Mark−Houwink

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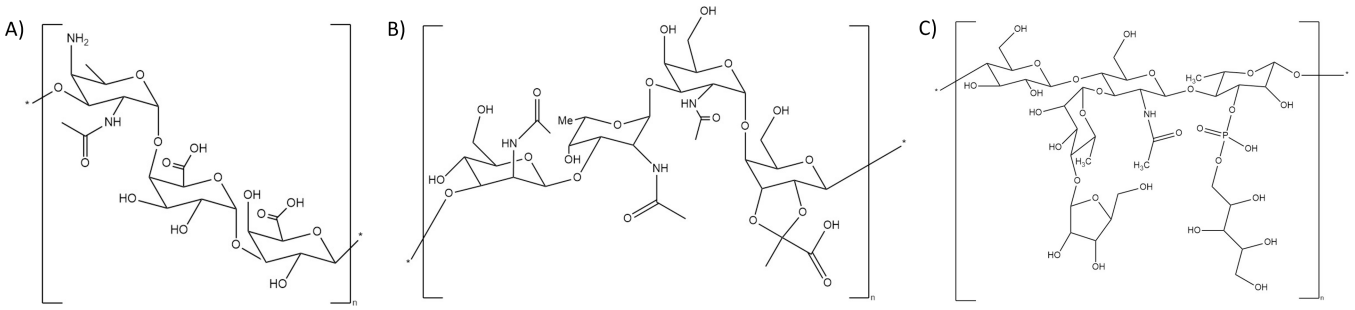

Figure 1. Biochemical structure of the three Ps STs investigated herein, including (A) ST-1, (B) ST-4, and (C) ST-24F.

parameter was used to calculate the molecular weight from backscattered light.[28]

Offline viscosity measurements were collected using a RheocalcT rheometer (AMETEK Brookfield, Middleboro, MA). The *in situ* viscosity measurements were made using a GoVisc 500 Automation viscometer (Hydramotion, Pottstown, PA). An EasyMax 102 thermostat system (Mettler Toledo, Columbus, OH) was used for maintaining a constant temperature during the bioconjugation reaction. The viscometer was inserted into 40 mL of sample, ensuring that the probe was completely submerged. Viscolink4 software (Hydramotion) was used to collect data for 4 h, with measurements made every 20 s and offline samples obtained every hour and submitted for offline HP-SEC analysis. All data was exported to a Microsoft Excel Spreadsheet. Mathematical analysis and investigation of the data was performed by converting the measured dynamic viscosity to kinematic viscosity by using the fluid density,[29,30] which was then compared to the offline M_W measurements.

At-Line Multiangle Light Scattering. Multiangle light scattering (MALS) analysis was performed using an ultraDAWN 18-angle MALS system (Wyatt Technology, Santa Barbara, CA). The sample was delivered using an Agilent pump operating at a flow rate of 2 mL min^{-1}, and ASTRA software ver. 8 (Wyatt Technology) was used to collect and analyze the data. Samples were injected via the batch injection mode. A standard solution of 5 mg mL^{-1} of dextran powder (40 kDa molecular weight) was prepared in water and used to normalize the ultraDAWN detectors. Samples were diluted in Bis-Tris buffer (10 mM Bis-Tris, 150 mM NaCl, pH 6.8) to a final concentration of 0.05 mg mL^{-1} prior to analysis. Data collection and advanced analysis were performed within the ASTRA software to understand the mathematical relationship between the obtained data and the level of conjugation; further, the data was compared to the offline M_W measurements. Data points were collected every 2 s, and the Zimm Model (1st Degree) was used for analysis.[31,32]

Offline High-Performance Size Exclusion Chromatography. Offline analysis of conjugate molecular weight (M_W) was performed using a high-performance size exclusion chromatography (HP-SEC) liquid chromatography method previously reported by Deng et al.[25] System suitability measurements are performed using either a protein (BSA) or a polysaccharide (dextran) standard to ensure accuracy of the M_w measurements and proper functionality of the method.[25]

Protein−Small Molecule Bioconjugation Process. For the protein−small molecule bioconjugation reaction,[33] the linker-payload (LP) was prepared in a solution of DMSO at 20 mg mL^{-1}, and the reduced monoclonal antibody (mAb) solution was prepared in histidine buffer at a concentration of 15 mg mL^{-1}. The LP itself is a small molecule with a maleimide linker, which allows it to attach to the mAb. The bioconjugation reaction was initiated by mixing mAb and LP with varying equivalences (low, medium, and high). The total reaction volume used was approximately 1 mL. Samples were drawn at regular intervals to monitor the reaction progress by using at-line HIC-HPLC.

At-Line Hydrophobic Interaction Chromatography. Minor modifications were made to a previously published hydrophobic interaction chromatography (HIC) method[33] to enable use on a Waters Patrol UPLC system (Waters, Milford, MA). Here, samples were analyzed in an at-line manner using vials with Empower 3 software (Waters) used to acquire data. The HIC methodology was performed on a Proteomix HIC Phenyl-NP5 (p/n: 433NP5−4610) column from Sepax (Newark, DE). Mobile phase A was 3 M ammonium acetate, 50 mM potassium phosphate with 5% acetonitrile at pH 7.0, and mobile phase B was 5% acetonitrile; the column temperature was maintained at 30 °C. The flow rate was set to 0.25 mL min^{-1}, and the gradient was the following: 20% B from 0.0−2.0 min; 95% B from 32.0−37.0 min; re-equilibration back to 20% B from 37.1 to 45.0 min. Additionally, a 5 μL loop was employed, targeting the delivery of 50 μg of the sample. A UV detector was used with the detection wavelength set to 280 nm. The mathematical relationship between the HIC separation of three DAR species formed during conjugation was evaluated to inform on the differing equivalences of mAb to LP.

RESULTS AND DISCUSSION

Protein−Polysaccharide Bioconjugation. The protein−polysaccharide bioconjugation process (Figure S1) investigated herein involves ongoing bioconjugation between a specific Ps serotype (ST) and the CRM-197 (CRM) carrier protein, increasing the size and molecular weight (M_W) of the conjugate until a target M_W is reached. Traditionally, this is achieved by allowing the reaction to proceed for a specific duration of time that varies depending on the Ps ST involved. The opportunity to monitor changes in M_W in real time would enable in-process evaluation of when the process has reached its target M_W, reducing risk for potentially off-target M_W outcomes and allowing forward processing to proceed more rapidly and efficiently without the need for additional offline sample handling and analysis. Real-time *in situ* monitoring of bioconjugation presents an opportunity to both mitigate risk and increase process efficiency. Here, several PAT tools were explored to achieve real-time analysis of three different Ps-CRM bioconjugations. The Ps STs investigated herein are ST-1, ST-4, and ST-24F, as shown in Figure 1[25−27]; however, the STs used in each experiment are blinded.

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In Situ Viscosity. Initial efforts for achieving real-time *in situ* monitoring of the Ps-CRM bioconjugation employed the use of a simple integrated analytics strategy, utilizing commercially available instrumentation. An approach combining dynamic light scattering (DLS) and viscosity measurements was first pursued. The bioconjugation reaction of Ps ST-A following the DMSO-based method was run over the course of 250 min. Offline samples were collected throughout the experiment for submission to DLS, with results indicating no obvious trend was observed via DLS which could be attributed to conjugate formation (Figure 2). This result is somewhat unsurprising

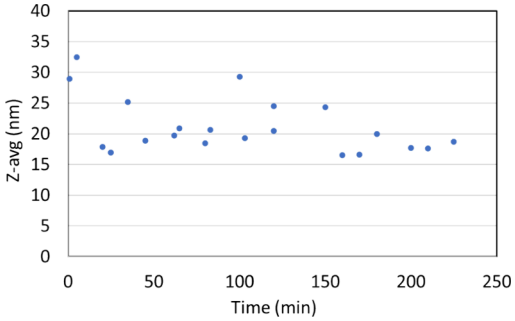

Figure 2. Reported *Z*-average (nm) values measured using dynamic light scattering at various time points during Ps-CRM bioconjugation for ST-A.

because of the similarity in refractive index between the DMSO solvent and the CRM protein used in the bioconjugation reaction. Further, four time point samples were submitted for analysis using offline viscosity, with results indicating a change in viscosity over the course of bioconjugation (Table 1); a change in viscosity during a reaction can make DLS measurements difficult, as viscosity is a key parameter used to ensure accurate DLS measurements; further offline processing and higher density viscosity measurements would be necessary to accurately reflect the size changes observed during bioconjugation. Notably, the values observed, while not illustrating a useful trend during bioconjugation itself, are still similar to what is generally expected for Ps-CRM bioconjugation.[34] Because of the difficulties associated with the DMSO background and viscosity changes requiring additional offline data collection and processing, DLS was not deemed an appropriate PAT technique for investigating Ps-CRM bioconjugation. On the other hand, the viscosity data showed promise for use as a PAT tool and was explored further.

Table 1. Offline Viscosity Measurements Taken at Four Time Points during Ps-CRM Bioconjugation for ST-A

time point (min)	viscosity (cP)
37	6.9
90	8.1
160	9.2
230	9.6

Following the results from the initial experiment, a PAT approach (Figure 3A) was pursued to measure viscosity in line and in real-time during the same ST-A Ps-CRM bioconjugation reaction in DMSO. Reaction conditions typically used for conjugation processing were monitored to determine the potential of this approach for real-time reaction monitoring; in the future, additional studies could focus on varying reaction conditions and the resultant impact on bioconjugation. Here, an *in situ* viscometer with a built-in temperature sensor was used to collect continuous measurements every 20 s. Because the viscosity of a fluid is a function of temperature, parallel monitoring and control of temperature were critical for assessing the validity of the viscosity measurements. Here, the temperature was set to a fixed temperature and controlled using the EasyMax reactor system (Figure 3B). The bioconjugation reaction was again run for 250 min. Offline samples were collected every hour, for a total of four time points during the reaction, to be measured using the offline HP-SEC assay to determine conjugate M_W,[25] the typical method for monitoring the progress of this bioconjugation reaction. The viscosity data for the experiment indicates a steady increase in viscosity from about 5.5 to 6.5 cP over the duration of the reaction while temperature remains constant; the slight variance in viscosity between experiments is a result of the mechanistic differences in measurement technology between the online and at-line tools. The measured dynamic viscosity was then divided by the fluid density to obtain the kinematic viscosity[29,30] (Figure 3C); this was compared to the M_W measurements, with results indicating the increase in viscosity directly trends with the increase in M_W over the

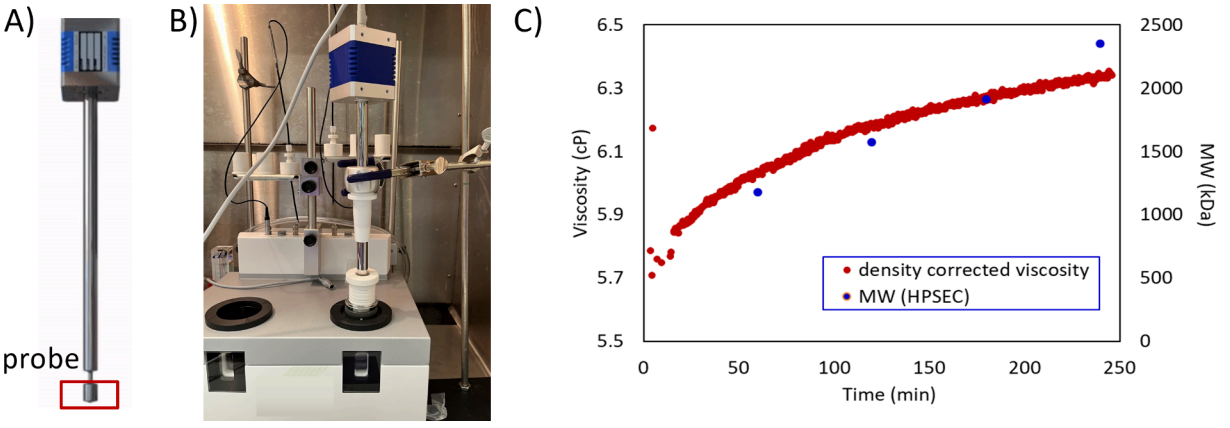

Figure 3. Experimental setup and *in situ* viscosity data obtained during the Ps-CRM bioconjugation reaction for ST-A. (A) In-line viscometer, with probe area indicated; (B) experimental setup indicating the viscometer residing within the EasyMax reactor; and (C) comparison of the density-corrected viscosity with M_W, the traditional offline method for measuring Ps-CRM bioconjugation reaction progress.

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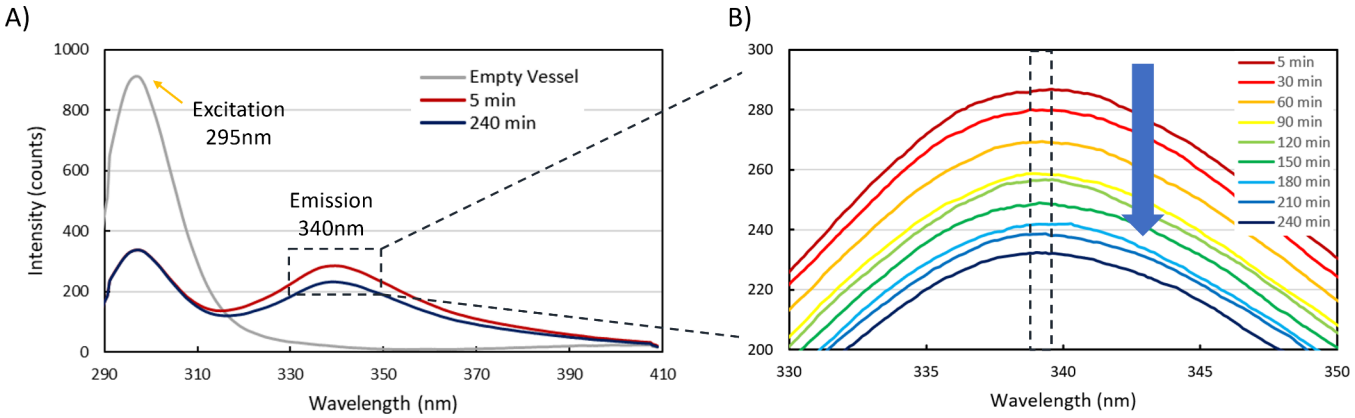

Figure 4. *In situ* measurement of the intrinsic fluorescence (FLR) emission spectrum of the bioconjugation process. (A) Spectral data from 290 to 410 nm detected through the FLR PAT probe in the empty vessel prereaction, 5 min into the conjugation reaction, and 240 min after initiating the conjugation reaction; and (B) zoom-in on the spectral region from 330 to 350 nm focusing on the FLR signal observed and its decrease in magnitude over the course of the conjugation reaction.

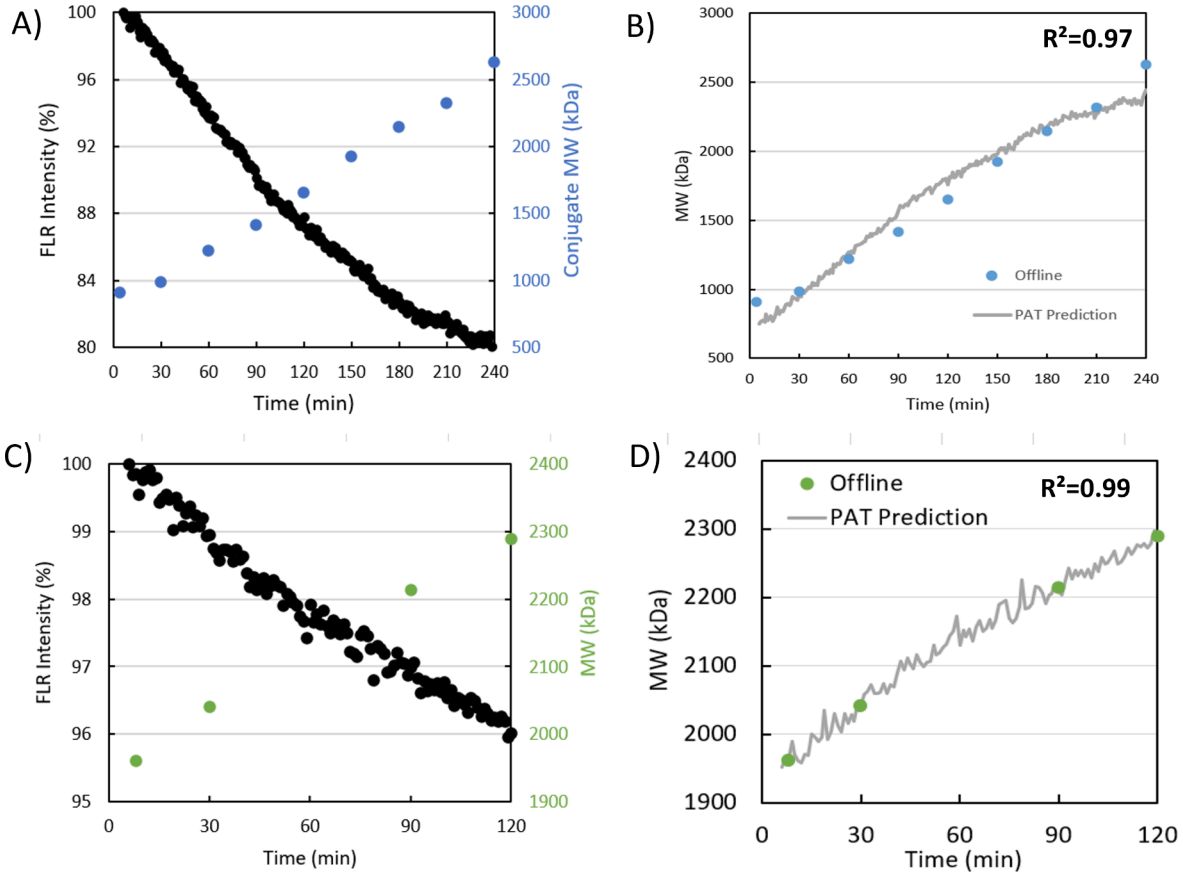

Figure 5. *In situ* fluorescence data from the bioconjugation process of two Ps STs (ST-A and ST-B) to CRM-197, and corresponding offline M_W measurements. (A) Kinetic FLR data for ST-A, normalized by initial intensity, showing decreasing FLR signal over the course of the conjugation reaction (black) overlaid with offline measurements of conjugate M_W (blue); (B) PAT prediction of conjugate M_W for ST-A (gray) obtained by linear regression of the FLR and M_W data for ST-A, overlaid with the offline measurements of conjugate M_W (blue); (C) kinetic FLR data for ST-B, normalized by initial intensity, showing decreasing FLR signal over the course of the conjugation reaction (black) overlaid with offline measurements (green); and (D) PAT prediction of conjugate M_W for ST-B (gray) obtained by linear regression of the FLR and M_W data for ST-B, overlaid with offline measurements of conjugate M_W (green).

course of the reaction (Figure 3C). This data provide a strong case for *in situ* viscosity as a potential route for monitoring conjugation reaction progress and for providing a path forward for determining when the process can proceed to the next step.

In Situ Fluorescence Spectroscopy. Further efforts to achieve real-time *in situ* monitoring of the bioconjugation reaction were pursued, this time using *in situ* fluorescence spectroscopy for real-time continuous monitoring of a bioconjugation process. While fluorescence for conjugation monitoring has been explored previously in an at-line format,[24] an in-line methodology is presented here for the first time for fluorescence conjugation monitoring using an in situ probe-

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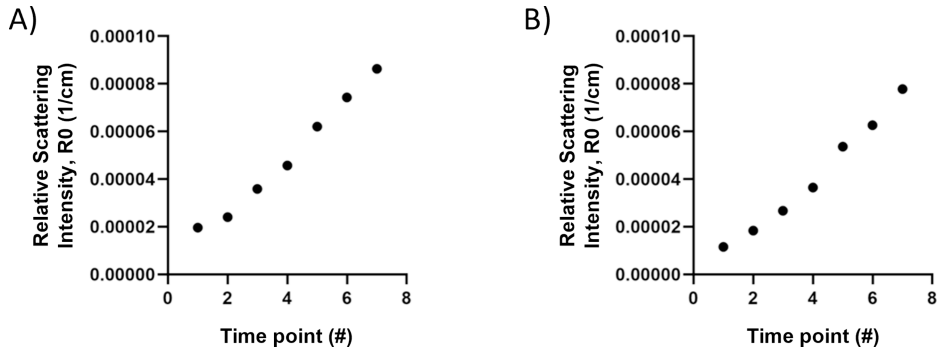

Figure 6. At-line MALS data from the bioconjugation process of Ps ST-B (A) and ST-C (B) to CRM-197 as a function of time. Relative scattering intensity (R_0) measurements for ST-B and ST ST-C increased over the course of the conjugation reaction.

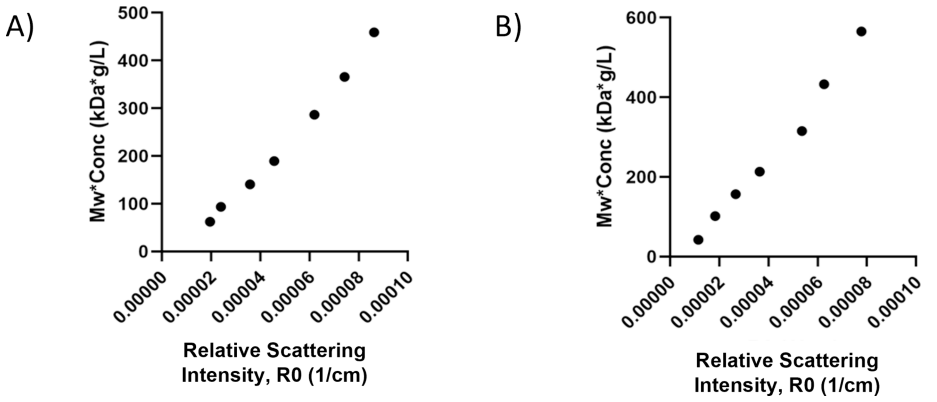

Figure 7. At-line MALS data from the bioconjugation process of Ps ST-B (A) and ST-C (B) to CRM-197, plotted as $M_w \times$ Conc vs R_0 measurements for ST-B and ST-C, each showing an increase over the course of the conjugation reaction.

based fluorescence instrument. Specifically, a home-built, probe-based fluorescence PAT spectroscopy instrument composed of commercially available components (described in Materials and Methods) was designed. This system was then utilized to measure the intrinsic fluorescence emission spectrum of the bioconjugation process for Ps ST-A – following the DMSO-based Ps-protein bioconjugation method – *in situ* in real time (Figure 4) without the addition of fluorescent dyes or labels. Utilizing excitation centered at about 295 nm, an emission profile centered at about 339 nm is observed (Figure 4A). This emission profile is consistent with that of intrinsic fluorescence of tryptophan amino acids in the protein backbone.[35] Over the course of the process, the intensity of the emission maximum gradually decreased (Figure 4B). This decrease is hypothesized to be the result of changes in solvent accessibility of the tryptophan residues and/or other conformational changes associated with the progress of the conjugation reaction. Control measurements, in which a conjugation reaction was not initiated, rule out photobleaching as a cause for the observed decrease in fluorescence intensity. Thus, the decrease in fluorescent emission signal observed at 339 nm may potentially be useful as an indicator of progress of the conjugation reaction.

To assess this further, continuous *in situ* fluorescence data from the bioconjugation process of a single Ps ST-A with the CRM-197 carrier protein was collected, and concurrent offline measurements of conjugate M_W were made at various points throughout the process using the same HP-SEC method as previously mentioned[25] (Figure 5A). Linear regression of the fluorescence PAT and offline M_W data was performed to establish a predictive relationship between the two variables, and the results were applied to generate a fluorescence PAT prediction of the M_W value for each measured fluorescence value; the results of this analysis are overlaid with the offline measured M_W values (Figure 5B). The high observed R^2 (0.97) suggests that the fluorescence results correlate well with conjugate M_W.

To explore the applicability of this technique as a platform tool for Ps-CRM bioconjugation monitoring, the bioconjugation of a different Ps ST-B using the same approach (Figure 5C,D) was monitored. Linear regression of the *in situ* fluorescence and offline M_W measurements again shows a very strong correlation (R^2 = 0.99), indicating that the fluorescence measurements correlate well with conjugate M_W for this ST as well.

Given that for the two serotypes studied different levels of fluorescence signal decrease are observed (Figure 5A,C), an attempt to generalize the total number of tryptophan sites shielded using the data available is not made. However, the differences in the total fluorescence signal decrease in the two data sets presented suggest differing levels of tryptophan shielding across different Ps serotypes, which may also be affected by the total conjugation process duration or other serotype specific process details.

The high correlations observed between fluorescence and conjugate M_W for two independent Ps STs suggest that *in situ* fluorescence spectroscopy as a PAT tool may be a generally useful analytical strategy for real time PAT bioconjugation monitoring. Here, reaction conditions typically used for actual conjugation processing of these PCV serotypes were

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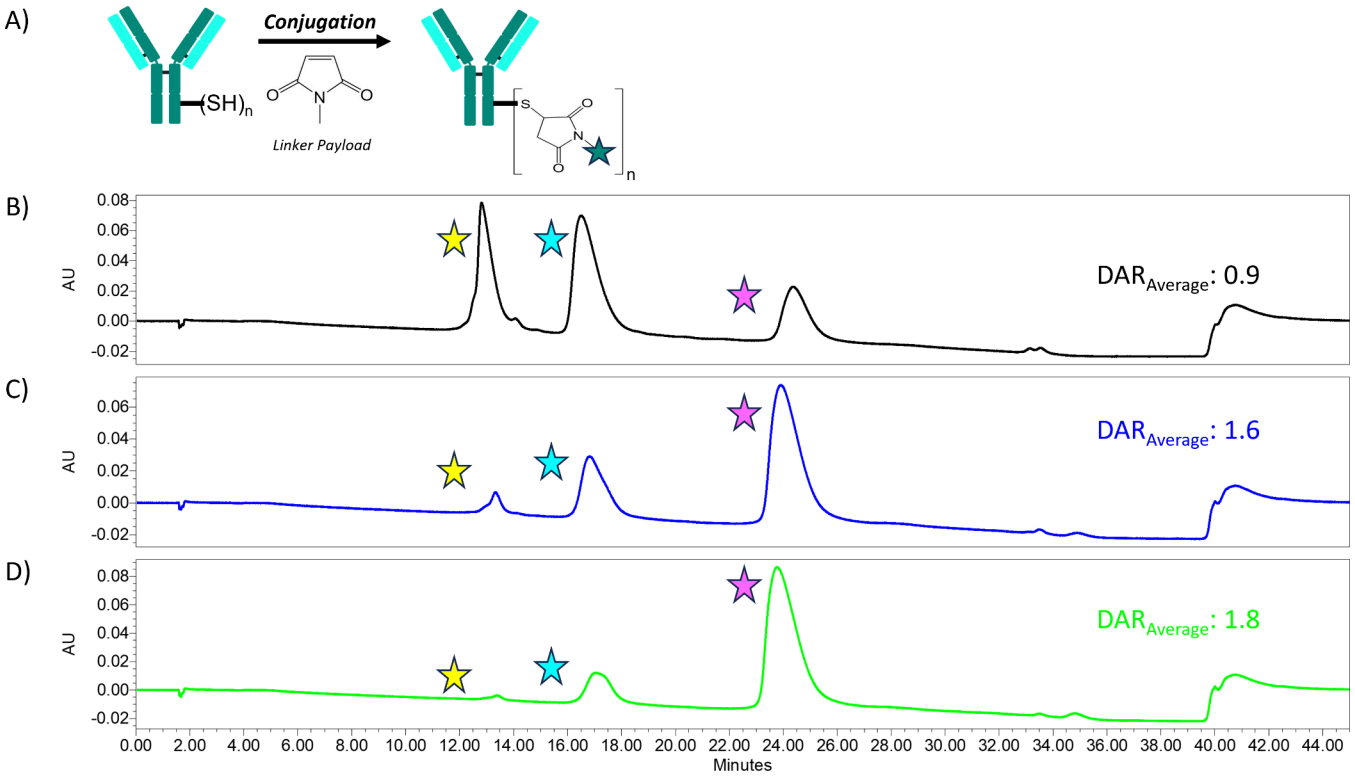

Figure 8. HIC separation of three DAR species formed during conjugation (A) resulting from differing equivalents of mAb to LP at (B) low, (C) medium, and (D) high equivalences. The yellow, cyan, and purple stars denote peaks corresponding to DAR0, DAR1, and DAR2, respectively.

monitored, which serve to explore and demonstrate the potential utility of this PAT for real-time reaction monitoring; however, in the future, additional studies could focus on exploration of varied reaction conditions and the resultant impact on bioconjugation.

At-Line Multiangle Light Scattering (MALS). Further PAT development was conducted using an aqueous-based Ps-protein bioconjugation method. Here, the process was executed in an aqueous environment. Real-time monitoring of Ps-CRM conjugation reactions carried out in DMSO can be challenging to monitor using techniques such as size exclusion chromatography because of the incompatibility of SEC columns with DMSO, as well as poor light scattering effects in a DMSO background.[24] As such, the bioconjugation of two Ps serotypes in an aqueous environment was interrogated using an at-line direct-inject multiangle light scattering (MALS) assay; the configuration of the system is shown in Figure S2. The two STs investigated included ST-B, which was previously studied using the DMSO-based Ps-CRM bioconjugation method, and a new serotype, ST-C. ST-B was investigated for continuity of evaluating PAT techniques, while ST-C was investigated to consider the platform capability of the tool.

The aqueous-based Ps-CRM bioconjugation reaction was carried out for ST-B and ST-C. Samples were drawn regularly to monitor the reaction progress using the at-line MALS PAT technique. The results indicate both ST-B and ST-C bioconjugation reactions show a linear response for the relative scattering intensity (R_0) and molecular weight times concentration ($M_w \times$ Conc) obtained during the PAT at-line MALS experiments (Figures 6 and 7, respectively). The R_0 increase with time indicates the formation of the conjugate for both ST-B and ST-C (Figure 6A, B, respectively). When R_0 is plotted against $M_w \times$ Conc (Figure 7), the trends corroborate strongly with the offline HP-SEC-MALS-RI data (data not shown) for individual time points for the given ST, where M_w is expected to continually increase throughout the conjugation.[34] Notably, a completely linear response is not expected and may not be the most accurate representation of the data generated in real time − at higher $M_w \times$ Conc values, a greater increase in R_0 because of particle to particle interactions is expected, and is observed. Overall, these data suggest that at-line MALS may be used as a viable tool for monitoring the bioconjugation progress of both ST-B and ST-C. This information is valuable as the reaction can be quenched once the desired size of the conjugate is achieved, and the process can proceed to its next step.

The totality of this work, in combination with the previous two studies on Ps-CRM bioconjugation, indicates a robust analytical toolkit that can be used for monitoring and understanding the investigated reaction. Having a mechanism to obtain information in real time is crucial for understanding when the desired M_W conjugate is formed, which allows scientists to move on to the next step in processing. This knowledge will inform key process parameters and allow for overall effective process development. Further, the PAT instruments utilized herein can easily be made compliant and transferred to the manufacturing floor for real use scenarios. Future work will focus on evaluating the robustness of each technique and expanding the feasibility across additional Ps STs.

Protein−Small Molecule Bioconjugation. An effort was made to further expand upon the capabilities of PAT tools for monitoring bioconjugation reactions; to that end, a targeted approach for monitoring protein−small molecule bioconjugation reactions was investigated. Herein, the formation of ADCs was pursued. Several experimental parameters were evaluated

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to determine the optimum drug-to-antibody ratio (DAR) between the monoclonal antibody (mAb) and linker-payload (LP) using PAT. In order to produce an effective ADC, a specific DAR profile must be achieved; this can be accomplished by adding a suitable amount of LP to the mAb to enable conjugation. By employing PAT techniques during the ADC bioconjugation reaction, experimental parameters can be adjusted to ensure that the formation of the correct target DAR profile is accomplished.

At-Line Hydrophobic Interaction Chromatography. Conjugation of hydrophobic LP results in conjugates with varying hydrophobicity. The hydrophobic nature of ADCs allows for HIC to be used to separate various DAR species from the target ADC. HIC works by first retaining the protein analyte in the column; this occurs via a hydrophobic interaction induced by the salting-out effect at high concentrations of kosmotropic salts. Elution of the bound proteins takes place by gradually decreasing the salt concentration, where the least hydrophobic species elute first, and the most hydrophobic species elute last.[36] Because the method operates under mild conditions, HIC analysis of proteins can be conducted while maintaining their native structure and activity.[37] This method provides both a distribution and an average DAR value, which are critical for process development. By utilizing the Patrol UPLC with an HIC HPLC method, the bioconjugation process was monitored in near real time.

In this work, the mAb was conjugated to varying equivalences of LP (Figure 8A), resulting in an ADC with a low, medium, or high DAR distribution; the target DAR was 2.0. The end product of each bioconjugation reaction was studied using the at-line HIC assay, with the results shown in Figure 8. The peaks corresponding to each of the three DAR species are denoted by stars, where the yellow star represents DAR0, the cyan star represents DAR1, and the purple star denotes DAR2; the results are broken down by low (Figure 8B), medium (Figure 8C), and high (Figure 8D) equivalences of LP added. The average DAR for each equivalence added is shown. The results indicate that the high equivalence of LP is required to most closely achieve the desired DAR of 2.0.

The at-line HIC method is shown to successfully monitor the ADC bioconjugation reaction to form three DAR species differing in varying amounts of LP, providing crucial information not only for each DAR species but also allowing for the evaluation of LP equivalency in near real-time to achieve the desired DAR. With the conjugation process being completed in 30 min, this HIC methodology was able to monitor the end point to determine if a desired DAR of 2.0 was obtained; this information can be used to monitor the completeness of bioconjugation to ensure that the DAR and/or DAR distribution specifications are met for the resultant ADC, thereby certifying the efficacy and utility of the desired ADC. Further work will expand on these experiments, including implementation of time-course reactions, to evaluate if an online HIC approach can accomplish similar results in real-time.

CONCLUSIONS

The arena of pharmaceutical process development and manufacturing is complex; process analytical technology (PAT) has increasingly been pursued as an avenue for supporting the process understanding and enabling more efficient manufacturing outcomes. Real-time analytical characterization of pharmaceutical processes can be achieved through monitoring critical quality attributes and key process parameters using PAT. In this work, several applications of PAT are applied to support the monitoring of crucial bioconjugation reactions, a key motif in the pharmaceutical realm. PAT tools ranging from simple to more sophisticated technologies were applied to investigate a polysaccharide-protein bioconjugation; further work then explored the application of advanced PAT for monitoring an antibody–drug conjugation reaction. In each area, the PAT tools were shown to provide direct, real-time information regarding reaction progression, enabling more rapid forward processing while increasing the bioconjugation reaction understanding and overall scientific knowledge. This work shows potential for implementation of PAT in biologics and vaccine pharmaceutical process development and production to increase overall drug substance quality and process productivity, supporting pharmaceutical efforts to save and improve lives through innovative pharmaceutical development.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.oprd.4c00399.

Process information and general schematic of the protein–polysaccharide bioconjugation; experimental information and general schematic of the at-line MALS setup for protein–polysaccharide bioconjugation (PDF)

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Notes

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