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Optimization and Validation of the Cationization Method for the Fab' Fragment of Antibody Rituximab

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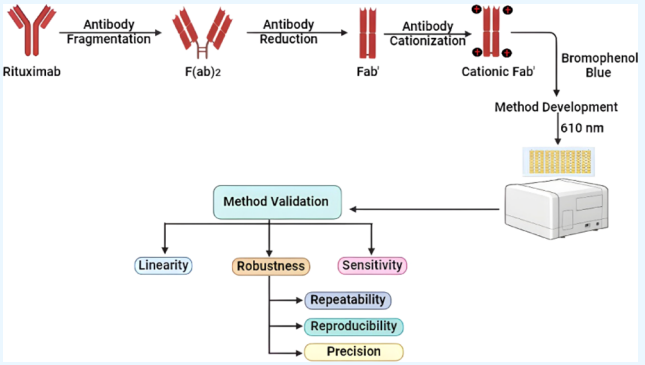
ABSTRACT: This study aimed to optimize and validate a cationization method for the Fab' fragment of antibody rituximab, following the guidelines set by the International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use (ICH). The optimization process involved fragmentation of antibody rituximab into an $F(ab)_2$ fragment using pepsin, followed by reduction into the Fab' fragment, and subsequent cationization. Various parameters, such as time intervals, enzyme ratios, reducing agent concentrations, pH levels, and reaction durations, were systematically investigated to achieve optimal cationization efficiency. The developed method was validated through spectrophotometry using the bromophenol blue (BPB) dye assay method. The validation process included assessment of linearity, robustness, and sensitivity. Results demonstrated the efficacy of the optimized cationization method, providing a reliable approach for analyzing rituximab antibody cationization of Fab'.



INTRODUCTION

Immunoglobulins, or antibodies, represent pivotal components of the immune system, meticulously crafted by B-cells to recognize and neutralize foreign pathogens.[1] Their multifaceted nature has led to their extensive utilization across diverse domains, including biotherapeutics and biotechnology. Notably, monoclonal antibodies have emerged as formidable weapons in the arsenal against cancer, lauded for their precision in immunotherapy with a commendable safety profile. However, their applicability in targeting the central nervous system (CNS) is hindered by their considerable size, necessitating innovative strategies for effective delivery.[2] Fragmentation into smaller, more maneuverable units, such as Fab' fragments of the chimeric antibody rituximab, stands out as a promising solution to this challenge.[3−5] The present study is motivated by the overarching goal of optimizing and validating a cationization method tailored for the Fab' fragment of the monoclonal antibody rituximab, adhering rigorously to the guidelines outlined by the International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use (ICH).[6,7] To systematically achieve this objective, our approach encompasses the optimization of key processes—fragmentation, reduction, and cationization—followed by meticulous validation of the developed methodology. Commencing with the optimization process, our efforts focus on fragmenting rituximab into $F(ab)_2$ fragments, employing pepsin as the enzyme catalyst.[8,9] Through systematic exploration of various time intervals and antibody-to-enzyme ratios, we endeavor to maximize the fragmentation efficiency. Pepsin, operating optimally in acidic pH conditions, facilitates cleavage below the two disulfide bridges, yielding $F(ab)_2$ fragments (with a molecular weight of approximately 100 kDa) and the Fc region.[10,11] Subsequently, the reduction process is fine-tuned to convert the $F(ab)_2$ fragment into the desired Fab' fragment (with a molecular weight of approximately 50 kDa), with modulation of 2-mercaptoethylamine (2-ME) concentration and reaction duration yielding optimal conditions.[12−15] Upon successful optimization of fragmentation and reduction processes, our attention turns to optimizing the cationization process—a critical step in enhancing the biotechnological utility of the Fab' fragment. Leveraging established protocols and insights from relevant literature, cationization is achieved using hexamethylene diamine in conjunction with *N*-(3-(dimethylamino)propyl)-*N*′-ethyl carbodiimide hydrochloride (EDC) as the catalytic agent.[16] Systematic variations in pH levels and reaction durations enable the fine-tuning of cationization efficiency, with optimal results observed at specific pH levels and time intervals.[17−19]

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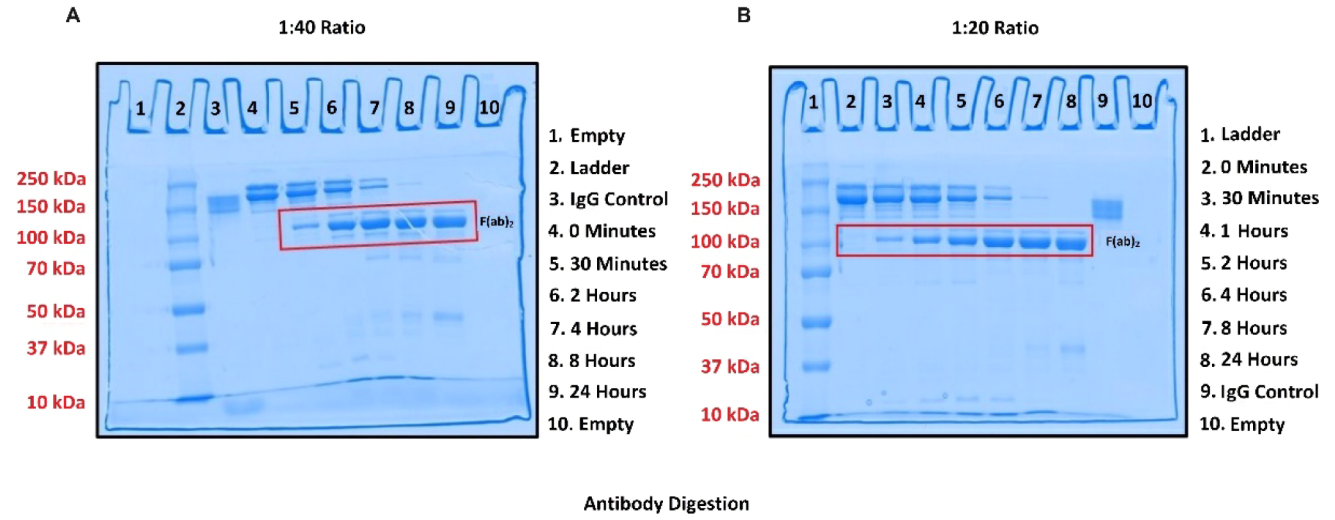

Figure 1. Figure depicts the digestion process of rituximab analyzed through SDS-PAGE and Coomassie blue staining. In part (A), lane 2 exhibits the molecular weight standard, while lanes 3−9 present rituximab samples digested for varying durations (0 min, 30 min, 2, 4, 8, and 24 h). Lane 3 represents the intact antibody without treatment, at a 1:40 antibody to enzyme ratio. Part (B) mirrors this setup, with lanes 2−8 containing samples digested for different durations and a 1:20 antibody to enzyme ratio. Lane 9 also displays untreated rituximab under the experimental conditions. Lanes 1 and 10 remain vacant in both parts, offering a comparative assessment of the enzyme concentration's influence on digestion.

Despite the pivotal role of cationization in various biotechnological applications, the optimization and validation of cationization methods for the Fab' fragment remain formidable challenges, necessitating meticulous experimental design and validation. Addressing the absence of methods for detecting antibody cationization through spectroscopy, we introduce a novel approach that utilizes the bromophenol blue dye assay method. This method (BPB dye) enables the quantification of cationic Fab' fragments of rituximab under acidic conditions, providing a reliable means of validating the level of the cationization process through spectrophotometry.[20] The validation process aims to rigorously assess the reliability and accuracy of the developed cationization method using the BPB dye method. Through comprehensive evaluations of linearity, robustness, and sensitivity, we demonstrate the method's efficacy and reproducibility. Results demonstrated significant linearity within the tested concentration range, with %CV values consistently below 10%. Moreover, robustness experiments confirmed compliance with acceptance criteria across varying pH and time intervals in all tested variables, endorsing the efficacy of the developed BPB dye assay method.[21] Additionally, assessment of sensitivity (LLOQ) indicated the method's ability to accurately quantify the analyte within a specific concentration range. The successful optimization and validation of the cationization method for the Fab' fragment of rituximab underscore its potential applications in diverse biotechnological and therapeutic endeavors, paving the way for further refinements and exploration in specific biomedical contexts.

Briefly, the development of a cationization method for Fab' fragments using HMD, along with its validation via spectrophotometry, represents a significant advancement over conventional techniques. Cationization increases the fragment's positive charge, potentially enhancing cell-binding properties and tissue penetration. Traditional cationization methods, such as chemical modification with amine-reactive reagents, enzymatic conjugation, and genetic engineering, often involve time-consuming, complex procedures, incur high costs, and require specialized equipment, limiting their routine applicability.[38] Furthermore, conventional validation techniques like isoelectric focusing (IEF), mass spectrometry, and capillary electrophoresis are time-consuming and also require specialized instrumentation. In contrast, the HMD-based approach, combined with spectrophotometric validation using the BPB dye assay, provides a simple process under mild conditions, enabling real-time monitoring of Fab' cationization. This approach simplifies the workflow and also ensures reproducibility, offering a more efficient, cost-effective, and accessible solution for producing cationized Fab' fragments, which is particularly advantageous for therapeutic and diagnostic applications.[16,20]

In conclusion, by overcoming limitations associated with antibody size and targeting, our methodology opens doors to enhanced therapeutic strategies and diagnostic applications. Through systematic experimentation and validation, the developed method provided a reliable approach for analyzing the level of antibody cationization of the Fab' fragment of rituximab. However, further refinements and explorations are warranted to fully harness their potential across diverse biomedical contexts. Future research may focus on further refining the method and exploring its applications in specific biomedical contexts. The optimized method offers potential applications in various biotechnological and therapeutic endeavors requiring modified antibody fragments. In this study, the fragmentation, reduction, and cationization processes of the Fab' fragment of the chimeric monoclonal antibody rituximab were demonstrated. A novel bromophenol blue (BPB) dye method was developed to determine the level of cationization of the Fab' fragment. The data were validated using the BPB assay methods and absorption spectroscopy for the Fab' fragment of rituximab. The study suggested that the smaller size of the Fab' fragment, along with its cationization, reduced the barrier to antibody absorption into the CNS for various diseases.[22] The developed method was simple, precise, and rapid. Data validation was carried out through linearity, robustness (repeatability and reproducibility), and sensitivity for the level of cationization in accordance with ICH guidelines.

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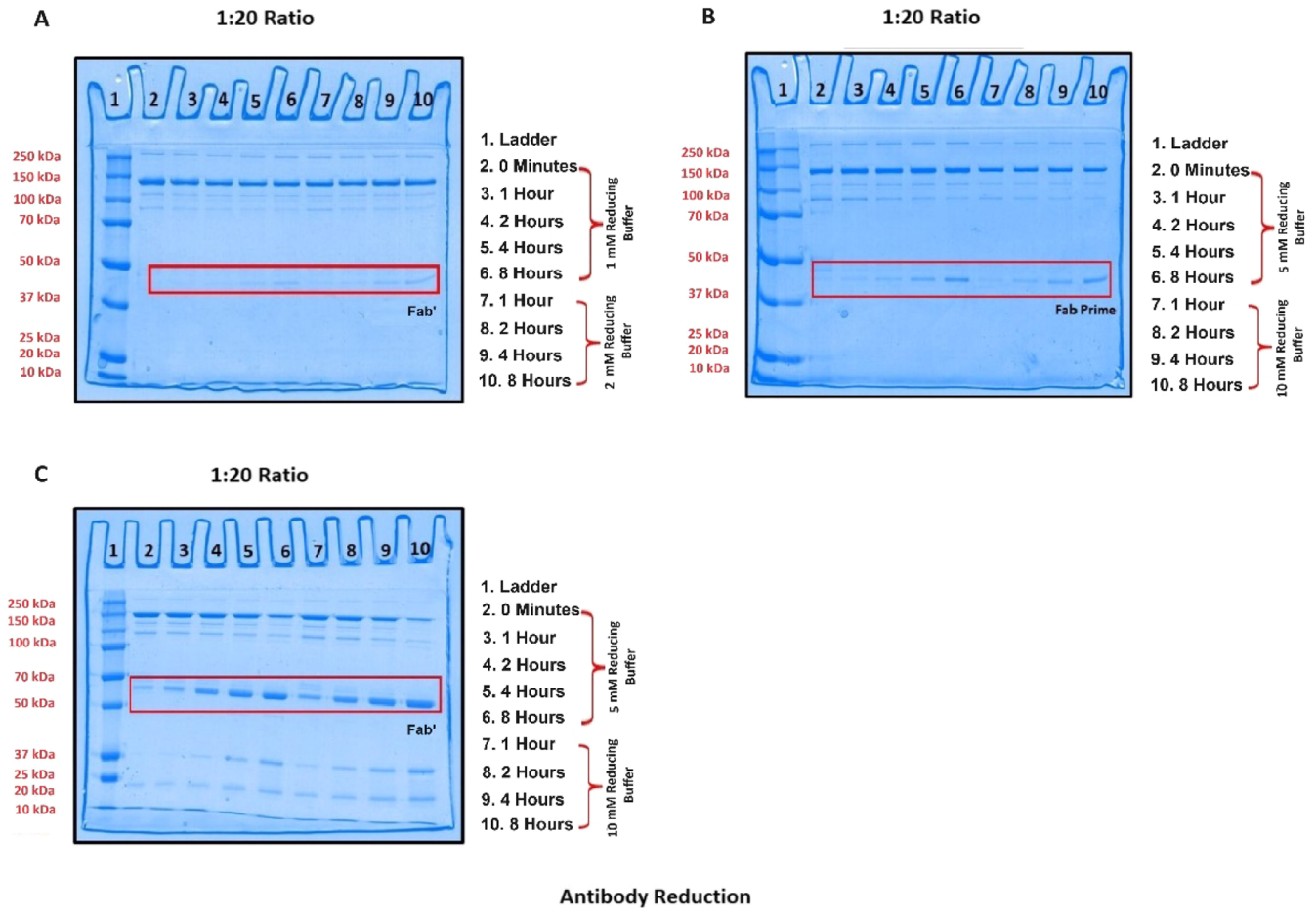

Figure 2. Reduction process was conducted via sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with Coomassie blue staining. Lane 1 contained a molecular weight standard/ladder, while lanes 2−10 featured rituximab ($F(ab)_2$). Rituximab was reduced using β-mercaptoethanol (2-ME) across four different reducing buffer concentrations: 1 mM, 2 mM (A), 5 mM, and 10 mM (B), at five time intervals: 0 min, 1 h, 2 h, 4 h, and 8 h, with a 1:2000 antibody to 2-ME ratio. The optimized reduction process is depicted in (C), where a distinct band pattern at 5 and 10 mM confirmed the successful reduction.

RESULTS

Optimization of Fragmentation of Antibody into $F(ab)_2$ Fragments. The digestion of the monoclonal antibody rituximab was performed by using the enzyme pepsin, leading to the production of F(ab)2 fragments and several smaller peptides from the Fc portion of the antibody. A series of experiments were conducted to optimize the production of $F(ab)_2$ fragments. Initially, preparations were made with 1 mg per milliliter of monoclonal antibody rituximab and a stock of pepsin enzyme. Various ratios of antibody to enzyme, approximately 1:20 and 1:40, were employed to optimize the digestion of the antibody (Figure 1A,B). These reactions were carried out at different time intervals, as outlined in the methodology section.

For the enzyme digestion reactions, specific ratios were utilized for the chimeric monoclonal antibody rituximab and the enzyme pepsin (1:20). The experiments were conducted across different time intervals, as outlined in the study's methodology. The optimal results for fragmenting the antibody rituximab into $F(ab)_2$ were achieved using the 1:20 antibody-to-enzyme ratio, where the reaction mixture was incubated in a citrate buffer at pH 3.5 for a period of 8 h.

The findings revealed that efficient digestion of the antibody rituximab into $F(ab)_2$ fragments, with a molecular weight of approximately 100 kDa, was accomplished within this 8 h time frame at a pH of 3.5. This condition proved to be more effective compared to other tested reactions. The outcome underscores the importance of carefully selected antibody-to-enzyme ratios and pH levels in enhancing the efficacy of the digestion process, suggesting that these factors play a critical role in achieving the desired fragmentation of the antibody.

Optimization of the Reduction Process from $F(ab)_2$ to Fab' Fragments. The reduction experiment of the antibody rituximab involved using the reducing agent 2-mercaptoethylamine (2-ME) at various concentrations, including 1 mM, 2 mM, 5 mM, and 10 mM (Figure 2A−C). The reaction's efficiency increased with higher molar concentrations of the reducing agent and longer durations of exposure. Notably, the most significant reduction was observed when $(Fab)_2$ was treated with 2-ME at a 10 mM concentration for 8 h (Figure 2B). This condition demonstrated superior efficacy in reducing $(Fab)_2$ compared to lower concentrations, highlighting the critical impact of the reaction parameters on the efficiency of the reduction process. To confirm these findings, an additional experiment was conducted using the same conditions but focusing on 5 mM and 10 mM concentrations. The results confirmed that the 10 mM concentration provided a more effective reduction than the 5 mM concentration.

The outcome of the reduction of the antibody rituximab revealed the successful conversion of $F(ab)_2$ into the Fab'

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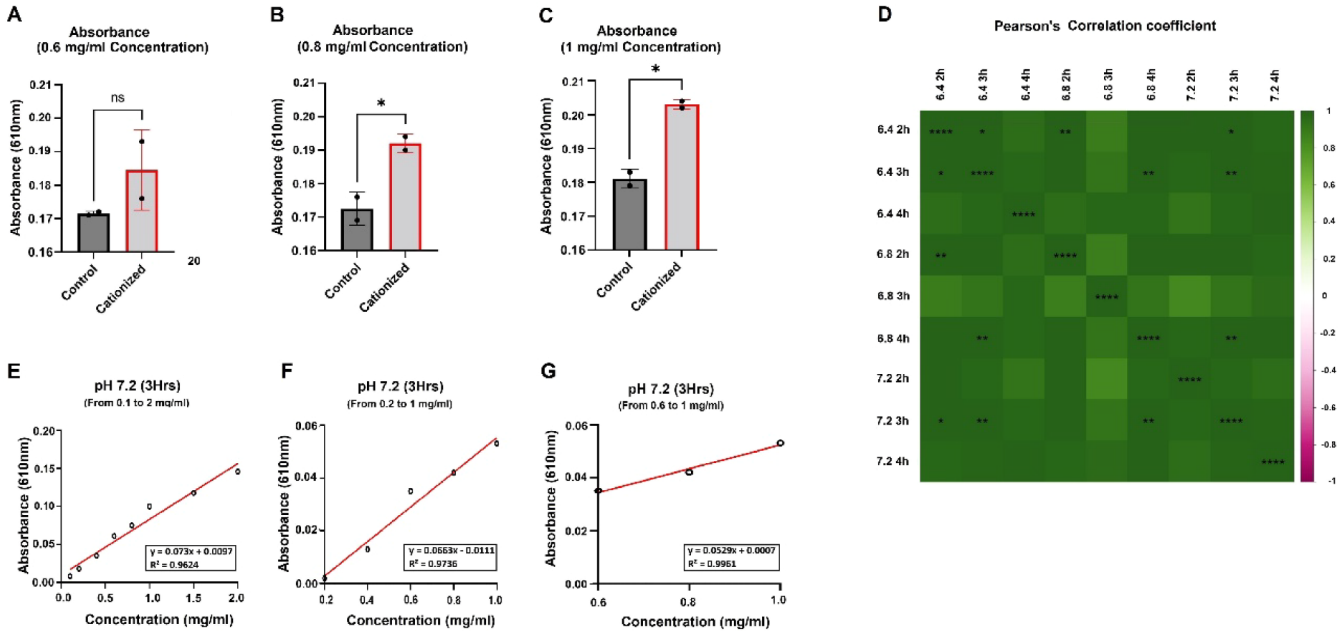

Figure 3. Figure presents confirmation of rituximab Fab' cationization through a concentration-dependent significant increase ($p < 0.05$, denoted by asterisk) in BPB dye absorbance observed across concentrations ranging from 0.6 to 1 mg/mL (A−C). Pearson correlation analysis (D) showcases the correlation of chimeric monoclonal antibody rituximab Fab' cationization profiles across different time intervals and pH conditions. Linearity across concentration ranges is illustrated in panels (E−G), where concentrations of 0.1−2 mg/mL (E) show a notable increase in linearity ($y = 0.073x + 0.0097$, $r^2 = 0.9624$), 0.2−1 mg/mL (F) exhibit a slight increase in linearity ($y = 0.0663x - 0.0111$, $r^2 = 0.9736$), and 0.6−1 mg/mL (G) display a significant increase in linearity ($y = 0.0529x + 0.0007$, $r^2 = 0.9961$), demonstrating higher linearity compared to other concentration ranges."

segment, as indicated by the presence of a 50-kDa band on the SDS-PAGE gel. This finding suggests that the selected conditions, including the ratio of fragmented antibody $(Fab)_2$ to 2-ME, the concentration of the reducing agent, and the duration of the reaction, significantly influenced the efficiency of the process. Specifically, the utilization of 2-ME at a 10 mM reducing concentration for 8 h resulted in optimal reduction, emphasizing the importance of meticulous optimization in achieving desired outcomes in antibody reduction reactions (Figure 2C).

Method Development for the Detection of Cationized Fab' Fragments. *Linearity.* In the method proposed for linearity assessment, concentrations ranging from 0.1 to 2, 0.2 to 1, and 0.6 to 1 mg/mL were selected. Concentrations of 0.1, 0.2, 0.4, 0.6, 0.8, 1, 1.5, and 2 mg/mL were chosen for the first range, followed by concentrations of 0.2, 0.4, 0.6, 0.8, and 1 mg/mL for the second range, and finally 0.6−1 mg/mL for the third range. These concentrations were drawn from the stock solutions and dispensed into a 96-well microplate after thorough verification. Milli-Q water and 190 μL of BPB dye were added to each well. Subsequently, readings were taken using a spectrophotometer, and calibration plots were constructed by correlating concentration with absorbance for the cationized Fab' of rituximab. The coefficient of determination (r^2), slope, and intercept values were then determined and statistically evaluated.

The cationization of the Fab' portion of the rituximab antibody was confirmed using a developed bromophenol dye assay. Various concentrations ranging from 0.6 to 1 mg per ml were employed initially to confirm cationization in a spectrophotometer. The results indicated a significant concentration-dependent increase in the absorbance of the cationized Fab' segment compared to the noncationized segment in this assay ($p < 0.05$, denoted by asterisks in the graph) (Figure 3A−C).

Following confirmation of cationization, the cationization reaction was optimized by varying pH and time. pH levels of 6.4, 6.8, and 7.2, and time intervals of 2, 3, and 4 h were investigated for this purpose. The data obtained from these experiments were then correlated using the Pearson method (Corr plot package), as illustrated in Figure 3D. These findings collectively suggest that the pH 7.2 and 3-h reaction time conditions are conducive to achieving the highest level of cationization. Therefore, all of our further experiments were conducted under these optimized conditions. This optimization provides valuable insights for future research endeavors aimed at exploring the functional implications and therapeutic potential of cationized Fab' fragments of rituximab.

It was observed that the detector's response fell within the linear range, spanning concentrations from 0.1 to 2 mg/mL. The linear regression equations derived for the cationized Fab' fragment of rituximab were expressed as $y = 0.073x + 0.0097$, with an r^2 value of 0.9624 (Figure 3E). Further investigation within the concentration range of 0.2−1 mg/mL revealed a linear regression equation of $y = 0.0663x - 0.0111$, with an r^2 value of 0.9736. Interestingly, a notable improvement in linearity was observed when concentrations ranging from 0.6 to 1 mg/mL were utilized, extending the linear range significantly (Figure 3F).

Consequently, a concentration-dependent increase in BPB dye absorbance was observed with escalating concentrations of cationized Fab' of rituximab (0.6, 0.8, and 1 mg/mL). The measured absorbance values were 0.035, 0.042, and 0.053 for cationized Fab' of rituximab, respectively. Linear regression equations derived for these concentrations were expressed as $y = 0.0529x + 0.0007$, with an r^2 value of 0.9961 (Figure 3G).

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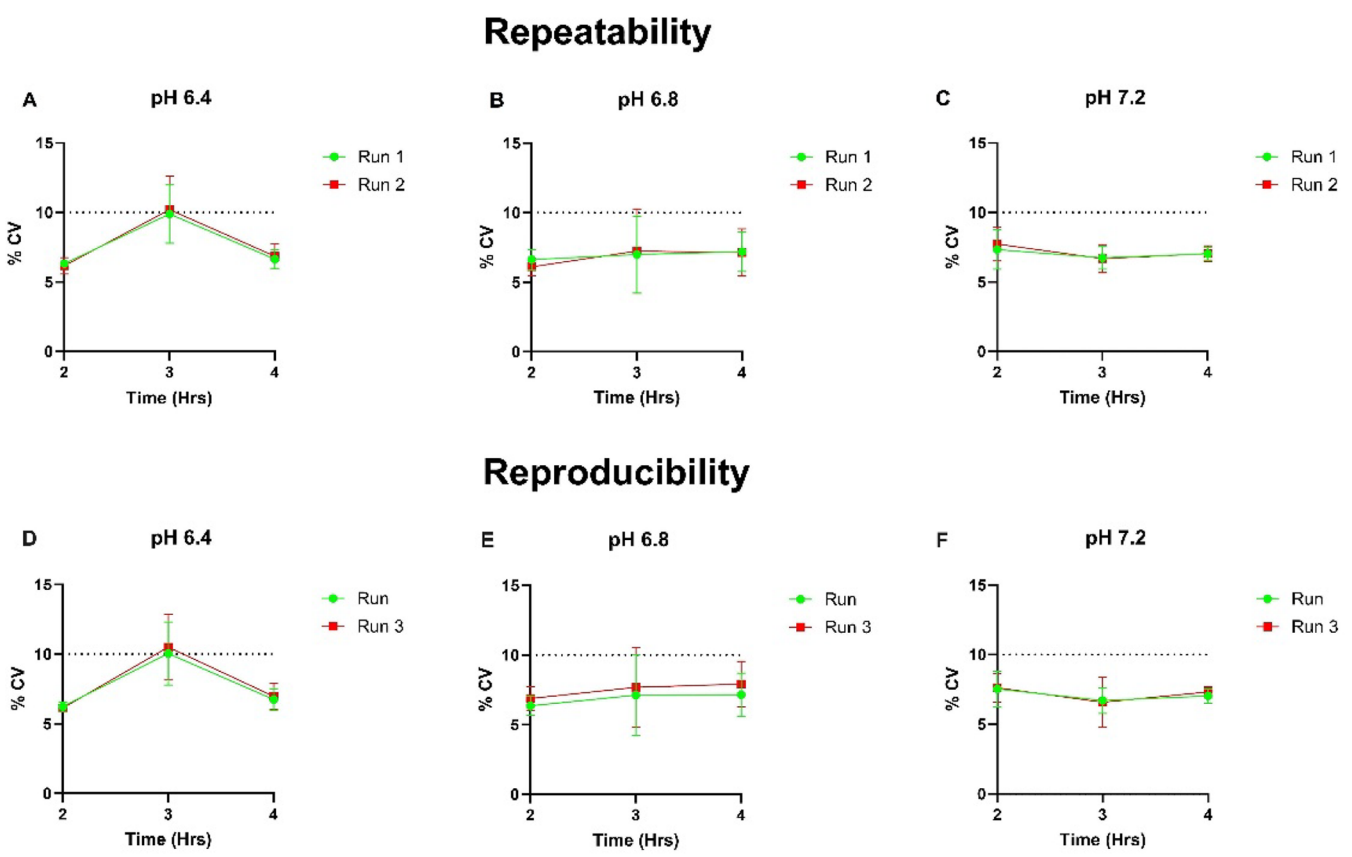

Figure 4. In this figure, the robustness of rituximab's Fab' segment cationization is assessed through repeatability and reproducibility validations. Repeatability comparisons between Run1 and Run2 in (A−C), with (C) meeting the 10% acceptance criterion, while (A) exceeded it. Reproducibility was evaluated between R (averaging Run1 and Run2) and Run3 in (D−F), with (F) meeting the criterion and (D) exceeding it. Notably, parts (C) and (F) show significant decreases in %CV, ensuring adherence to acceptance criteria. Dotted lines represent the 10% acceptance criterion.

These findings suggested an enhanced interaction between the cationized Fab' fragment of rituximab and the negatively charged BPB dye, confirming the process of antibody cationization. The slope and intercept obtained from the regression equation ($Y = mx + c$) were utilized to validate the linearity of the developed BPB method.

Robustness. Repeatability. To validate repeatability, experiments were conducted on three samples (in triplicate for each condition) under varying conditions, including pH levels of 6.4, 6.8, and 7.2, and time intervals of 2, 3, and 4 h, respectively. Nine conditions in total were employed for method validation, with samples labeled as Run1 and Run2. Repeatability was confirmed by assessing the consistency of analytical results within two analytical runs. Analysis of repeatability data across different pH levels and time intervals provided insights into sample stability and revealed random errors inherent in the method.

In this experimental study, the influence of pH on the percentage coefficient of variation (%CV) and standard deviation over time was systematically evaluated. At pH 6.4, initial observations at the 2-h interval revealed that the %CV was below the set acceptance threshold, accompanied by a reduced standard deviation. Progressing to the 3-h interval, a slight elevation in %CV was noted, exceeding the acceptance criteria, alongside an increase in standard deviation. Nevertheless, by the 4-h mark, %CV realigned with acceptance standards, despite a modest rise in standard deviation (Figure 4A).

Upon adjusting the pH to 6.8, it was observed that from the 2−4-h intervals, the %CV experienced an increment yet remained within the prescribed acceptance criteria. Interestingly, this period exhibited a lower standard deviation relative to the initial and final time points, with a notable deviation in standard deviation observed at the 3-h mark (Figure 4B).

Further adjustments to a pH of 7.2 demonstrated a marginal increase in %CV at both the 2 and 4-h marks, maintaining adherence to acceptance limits. A distinct improvement in both %CV and standard deviation was recorded at the 3-h interval, surpassing the performance observed at other pH levels and time points (Figure 4C).

Therefore, based on the findings of this study, a pH of 7.2 at the 3-h time point emerges as the optimal setting for analytical assays requiring high precision and stability. This conclusion is drawn from the consistent and significant enhancements in % CV and standard deviation observed, which surpass the analytical performance under varying pH conditions and different time points.

Reproducibility. Experimental procedures were undertaken to validate reproducibility, involving three samples subjected to triplicate testing under various conditions. These conditions included pH levels of 6.4, 6.8, and 7.2, as well as time intervals of 2, 3, and 4 h, resulting in a total of nine conditions for method validation (with samples in triplicate). For reproducibility, we calculated R1 and R2 averages for R and Run3, compared them with each other, and utilized the value of the

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cationization determination to calculate the mean, standard deviation (SD), and percentage coefficient of variation (%CV).

The results found that at pH 6.4, the %CV remained below the acceptable criteria, but at 3 h, both the %CV and standard deviation exceeded acceptable thresholds (Figure 4D).

At pH 6.8, CV fluctuated within acceptable limits, with the standard deviation varying. The standard deviation increased at 2 h but decreased at 3 h, indicating a potential stabilization effect over time (Figure 4E). Our experimental results found that pH 7.2 significantly influenced the acceptance of %CV at all time points, with a notable improvement at 3 h. This highlights the significant impact of pH variations on observed outcomes, especially at 3 h intervals (Figure 4F).

In brief, the experiment showed varying stability and reproducibility across pH levels and time intervals. pH 6.4 showed potential instability, while pH 6.8 maintained acceptable %CV values. Notably, pH 7.2 showed significant improvements in %CV at 3 h, indicating a favorable condition. These results emphasize the importance of pH control for reliable experimental outcomes and suggest that further research could improve optimization and data quality assurance.

Sensitivity. During the assessment of the lower limit of quantification, concentrations spanning from 0.1 to 2 mg/mL were examined, revealing a %CV exceeding 10% (0.2−0.4 mg/mL). However, concentrations ranging from 0.6 to 1 mg/mL maintained a coefficient of variation (%CV) below 10%. This observation indicates a consistent improvement in the assay's precision within this specified concentration range, as evidenced by the persistent %CV values below 10% (Table 1).

Table 1. Lower Limit of Quantification

Concentration	OD1	OD2	Avg (A)	Blank-Avg
0	0.141	0.136	0.1385	0
0.1	0.147	0.146	0.1465	0.008
0.2	0.156	0.157	0.1565	0.018
0.4	0.172	0.175	0.1735	0.035
0.6	0.191	0.208	0.1995	0.061
0.8	0.214	0.213	0.2135	0.075
1	0.223	0.253	0.238	0.100
1.5	0.244	0.268	0.256	0.118
2	0.29	0.278	0.284	0.146

Discussion. The present study was conducted with the overarching aim of optimizing and validating a cationization method for the Fab' fragment of the antibody rituximab in accordance with the rigorous guidelines outlined by the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH). To systematically achieve this objective, we embarked on a multifaceted approach involving the optimization of the fragmentation, reduction, and cationization processes, followed by a meticulous validation of the developed methodology.[21]

Initially, our efforts were directed toward optimizing the fragmentation of the antibody rituximab into an $F(ab)_2$ fragment utilizing pepsin as the enzymatic catalyst. Through a comprehensive series of experiments that involved manipulating various parameters such as time intervals and antibody-to-enzyme ratios, we identified conditions yielding maximal fragmentation efficacy.[10] Notably, optimum outcomes of fragmentation were observed at 8 h and a specific 1:20 ratio, signifying a critical milestone in the process (Figure 1A,B).

Building upon the successful optimization of the antibody rituximab fragmentation process, our focus shifted toward refining the reduction process to convert the rituximab $F(ab)_2$ fragment into the desired Fab' fragment.[14] By systematically varying the concentration of the reducing agent 2-mercaptoethanol (2-ME) and the reaction duration, we successfully determined the optimal conditions, achieving maximum outcomes at a specific molar ratio $F(ab)_2$:2-ME of 1:2000, with a 10 mM concentration of reducing agent 2-mercaptoethanol and a reaction duration of 8 h (Figure 2A−C). The stepwise processing of rituximab to purified Fab' fragments, including the digestion, reduction, and purification steps, provides a clear overview of the procedure (Figure S3).

Subsequently, attention was directed toward optimizing the cationization process. This involved conducting experiments under conditions previously outlined in the pertinent literature, with careful consideration given to pH levels and time intervals. Leveraging the cationic agent hexamethylene diamine in conjunction with the catalytic agent 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), successful cationization of the Fab' fragment was achieved in the pH range of 6.4 to 7.2.[35,36] Recognizing the absence of a spectrophotometric method for determining antibody cationization, we innovatively developed a novel approach utilizing the bromophenol (BPB) dye assay method.[37]

Prior to initiating our method validation experiments, we developed the bromophenol dye method. For this purpose, we first focused on optimizing experiments to determine the concentration of bromophenol dye for the cationization reaction, followed by the optimization of a 0.0075% BPB dye. These experiments were conducted using both noncationic and cationic Fab' fragments of rituximab, resulting in significant increases in absorption of the cationic Fab' fragment compared to the noncationic counterpart. Therefore, these results suggested an increased interaction of the cationized antibody with the negatively charged BPB dye, confirming the antibody cationization and validating the efficacy of the developed methodology[12] (Figure 3A−C).

Further optimization of the cationization process ensued with experiments exploring different pH levels and time intervals. Noteworthy variations in the degree of cationization were observed within the pH range of 6.4−7.2 and 2−4-h time intervals, underscoring the importance of methodological refinement. Utilizing the BPB dye assay method facilitated accurate estimation and determination of the degree of cationization, with validation conducted via spectrophotometry at the 610-nM range.

For this purpose, we conducted an experiment to investigate the relationship between pH and time intervals. During the experiment, we made several observations under different conditions:

pH 6.4 and 2 h
pH 6.4 and 3 h
pH 7.2 and 3 h

Each condition was correlated, ranging from pH 6.4 to pH 7.2 and from 2 to 3 h. Subsequently, we identified that at pH 7.2 and 3 h, there was a significant increase in cationization output (Figure 3D). To confirm this, we compared the R^2 values of different replicates (R1, R2, and R3) by correlating their pH levels and time durations. This analysis revealed that the highest R^2 value was consistently observed at pH 7.2, aiding us in selecting the optimal conditions for cationization

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(Figure S4). Based on this observation, we suggest that conducting all experiments within this pH range and time interval could maximize cationization.[14]

In our first validation experiment, initially, we conducted a linearity experiment to assess the increase in the cationic Fab' fragment of rituximab within the 610 nm range of the BPB dye method. The concentration range tested spanned from 0.1 to 2 mg/mL at pH 7.2. However, we observed that the linearity significantly improved within the concentration range of 0.6−1 mg/mL at pH 7.2. Consequently, we chose to perform all subsequent experiments within this narrowed range, where significant linearity was achieved with a %CV of less than 10%. We proceeded with all our experiments at this concentration range and pH (Figure 3E−G).

In the second experiment, we addressed the robustness of the method. Specifically, we conducted experiments to assess two parameters: repeatability and reproducibility. Significant differences were observed at pH 7.2 and after 3 h, where we met the acceptance criteria at all time points, albeit with varying standard deviation values. Notably, at the 3-h mark, despite a significant improvement in %CV compared to other pH levels (6.4 and 6.8) and time points (2 and 4 h), the standard deviation remained elevated, indicating potential variability in the data (Figure 4A−C). During the reproducibility experiment conducted at pH 7.2 and after 3 h, %CV values fell within the acceptance criteria, being less than 10% (Figure 4D−F). Moreover, we compared the concentrations of groups R1 and R2 at pH 6.4 to 7.2 over 2−4 h for repeatability, using concentrations ranging from 0.6 to 1 mg/mL (Figure S1A−J). Similarly, we compared groups R2 and R3 under the same conditions for reproducibility (Figure S2A−J). In both repeatability and reproducibility experiments, we observed increased concentrations and levels of cationization as the pH was adjusted from 6.4 to 7.2, particularly at the 3-h interval. These results were notably higher compared to the non-cationized antibody. Following these experiments, we confirmed and optimized the conditions to pH 7.2 for 3 h, achieving the maximum level of cationization.

In our next experiment focusing on the sensitivity or lower limit of quantification (LLOQ), it was determined that the lower limit fell within acceptable criteria when experiments were conducted within the 0.6−1 mg/mL concentration range. Below this concentration range, the %CV was found to be insignificant. This study indicates that by utilizing concentrations within the 0.6−1 mg/mL range, the assay's accuracy in quantifying the analyte improved, as evidenced by consistent % CV values below 10%.

In our study, we rigorously evaluated the robustness of the developed BPB assay method by assessing the repeatability and reproducibility across various parameters. The consistently low %CV values, all below 10%, underscored the method's reliability and stability, making it well-suited for precise analytical applications. Notably, the observed consistency in %CV values across different conditions further validated the method's efficacy, highlighting its potential for widespread use in analytical settings. Despite encountering some variations, particularly noticeable at the 3-h mark during validation experiments, all time points met acceptance criteria, reaffirming the method's robustness. Additionally, the sensitivity assessment demonstrated compliance within the designated concentration range, further bolstering confidence in the method's accuracy.

In summary, our findings identified pH 7.2 and a 3-h interval as optimal conditions for the cationization of rituximab Fab' fragments, guiding further experimentation. Thorough validation of the developed method ensured the reliability of the obtained data, positioning our approach as a robust method for cationizing antibodies. This study marks a significant advancement in the field of antibody cationization, offering a validated methodology for analyzing the cationized Fab' fragment of rituximab. The developed approach holds promise for a wide array of biotechnological and therapeutic applications, with implications for drug delivery, diagnostic assays, and beyond. Moving forward, future research endeavors may focus on expanding the scope of applications for the optimized methodology. Additionally, the exploration of alternative approaches and techniques could further enrich our understanding of antibody cationization processes and pave the way for innovative advancements in the field of biotechnology.

Limitations of the Study. Despite successfully validating the cationized antibodies by addressing specificity, stability, and reproducibility, challenges inherent to the process persist. It is crucial that the antibody modification does not adversely affect target binding, necessitating careful optimization throughout the digestion, reduction, and cationization steps. Furthermore, the purification process plays a vital role; effective purification can enhance the overall yield of reduced and cationized fragments, thereby improving the consistency of their binding properties. Inadequate purification may introduce contaminants or residual fragments that compromise the integrity of the final product. Therefore, meticulous attention to purification protocols is essential to maintain the functional efficacy of the modified antibodies.

EXPERIMENTAL PROCEDURES

Materials. The chimeric monoclonal antibody rituximab (cat. no. MSQC17), along with essential reagents including pepsin (cat. no. 9001-75-6), mercepto-ethylamine (MEA) (cat. no. 641022), and dithiothreitol (DTT) (cat. no. 12-3-3483), was procured from Sigma-Aldrich without requiring additional purification. Chemicals such as monosodium phosphate monohydrate (cat# 10049-21-5), disodium phosphate (dibasic) (cat#13472-35-0), citric acid monohydrate (cat# 5949-29-1), trisodium citrate dihydrate (cat# 4-3-6132), bovine serum albumin (cat# 9048-46-8), sodium chloride (NaCl) (cat# 7647-14-5), glacial acetic acid (cat# 64-19-7), methanol (cat#67-56-1, ethylenediaminetetraacetic acid (EDTA) (cat# 6381-92-6), sodium carbonate (cat#497-19-8), trisma base (cat# 77-86-1), glycine (cat# 56-40-6), glycerol (cat# 56-81-5), sodium dodecyl sulfate (SDS) (cat# 151-21-3), and iodoacetamide (cat#144-48-9) were also sourced from Sigma-Aldrich. Additionally, hexamethylenediamine (HMD) (cat. no. 124-09-4) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) (cat. no. 1892-57-5) were obtained from Tokyo Chemical Industries. Supporting information such as bromophenol blue (cat. no. 115-39-9), Coomassie Brilliant Blue *R*-250 (cat. no. 6104-59-2), Micro BCA Protein Assay Kit (cat. no. 23235), Ammonium Persulfate (APS) (cat. no. 7727-54-0), tetramethylethylenediamine (TEMED) (cat. no. 110-18-9), and hydrochloric acid (HCl) (cat. no. 7647-01-0) were purchased from Sigma-Aldrich. Benchmark Precision Plus Protein Dual Color Standards (cat. no. 1610374) were procured from Bio-Rad. The water used in the experiments was obtained from a Milli-Q purification system with a resistance of 18.2 MΩ. UV−vis absorbance measurements

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were conducted using a BioTek Epoch 2 microplate spectrophotometer equipped with a Xenon lamp. Gel images were captured by using a ChemiDoc Bio-Rad GS Image Lab 900 densitometer. The physiological temperature conditions for the antibody were maintained using a Hera Cell 150i.

Methods. *SDS PAGE Analysis.* All specimens prepared for SDS-PAGE analysis underwent meticulous handling under nonreducing conditions. Analysis was carried out utilizing 10% SDS-PAGE gels derived from a 30% acrylamide solution, with a precise mass ratio of 29:1 between acrylamide and bis-acrylamide. Hand-cast polyacrylamide gels were inserted into the Bio-Rad Gel Tank apparatus, and a 1X phosphate running buffer was introduced. A nonreducing loading dye, containing 1.0 M tris-HCl at pH 6.8, 10% SDS solution, glycerol, and bromophenol blue, was prepared and applied to the samples before loading onto the gel apparatus, along with "precision plus protein" unstained protein standards (10−250 kDa) for molecular weight determination. Electrophoresis was performed at a constant voltage of 150 V for 1 h and 5 min.

After electrophoresis, Coomassie staining was conducted by using a solution containing 0.05% (w/v) Coomassie Blue stain (CBB *R*-250) in a 40:50:10 ratio of methanol, water, and glacial acetic acid. The gels were incubated in a shaker for approximately 30 min for staining, followed by destaining with a solution of methanol, water, and glacial acetic acid in the same ratio. This destaining process was repeated twice for approximately 30 min each until the antibody bands became visibly distinct.[23−26]

Optimization of the Fragmentation of Antibody into F(ab)$_2$ Fragments. The enzymatic digestion of the chimeric monoclonal antibody rituximab using pepsin was tested with a 20-mM sodium citrate buffer (diluting buffer) at pH 3.5. The experiment involved testing various incubation times, including 0 min, 30 min, 1, 2, 4, 8, and 24 h. The antibody sample was divided into eight portions, with seven undergoing pepsin treatment and one serving as a control with only diluting buffer added. The pepsin solution, containing 1 mg per ml, was prepared using Milli-Q water.[27] Two different ratios of pepsin to antibody mass, namely, 1:20 and 1:40, were employed to minimize antibody binding site by pepsin. The solutions containing added pepsin were then incubated at 37 °C.[8,9,28]

The reactions, initiated at various time intervals, were stopped by the addition of 2M Trizma base buffer, resulting in a solution buffer with a pH of 8. Following this, aliquots from each reaction were prepared in SDS-PAGE loading dye and subsequently analyzed using SDS-PAGE.[10,29]

Optimization of the Reduction Process from F(ab)$_2$ to Fab′ Fragments. For the efficient reduction reaction of F(ab)$_2$ to Fab' prime fragments of the chimeric antibody rituximab using the reducing agent β-mercaptoethanol (2-ME), four different reducing buffer conditions (1 mM, 2 mM, 5 mM, and 10 mM) and six incubation time intervals (0 min, 30 min, 1, 2, 4, and 8 h) were employed to obtain the Fab' fragments (MW: 50 kDa).[14] In brief, in our next process, the reduced monoclonal antibody (Fab)$_2$ (MW: 100 kDa) at a concentration of 1 mg/mL was reacted with the 2-ME reducing buffer solution in the presence of 5 mM EDTA and Milli-Q water, where the molar ratio of F(ab)$_2$ to 2-ME was maintained at 1:2000 for the Fab' fragment of monoclonal antibody rituximab. The reaction was carried out at 37 °C for the specified time mentioned above. Finally, the reaction was quenched with 2 mM Iodoacetamide at the various incubation time intervals and allowed to incubate for at least 30 min before being stored at −20 °C.[11,14]

Cationization of Fab′ Fragments. Chimeric monoclonal antibody rituximab Fab' segment was cationized according to the method by Pardridge et al. (June 1989) with slight modifications.[16] The cationization reaction was conducted using cationic agent hexamethyleniamine at three different pH and time points. In brief, three reactions were designed to achieve varying levels of cationization. Initially, 125 μL of noncationized monoclonal antibody Fab prime segment was mixed with 375 μL of a mixture containing hexamethylenediamine and *N*-ethyl-*N*- [3- (dimethyl amino) propyl] carbodiimide (EDC) buffer solution. The mixture was then stirred for 3 h at room temperature. Subsequently, the reaction was quenched by adding 500 μL of 2 M glycine, followed by incubation for 60 min at room temperature.[6,12,30]

After the completion of the incubation reaction, the reaction mixture underwent desalting and concentration via diafiltration using an Amicon Ultra-15 centrifugal filter with a 30-kDa molecular weight cutoff (MWCO) from Millipore. This process was carried out at 4000 rcf and 4 °C for 15 min, resulting in the isolation of the cationized antibody, which was then stored at −20 °C for further use.[11,18,19]

Protein Estimation Using the Bicinchoninic Acid (BCA Method) Assay. Initially, BCA reagents A, B, and C were utilized in a ratio of 50 parts A, 48 parts B, and 2 parts C to prepare the BCA working solution. The mixture was thoroughly combined. Additionally, for the preparation of standard solutions, 1 mg of BSA was dissolved in 1 mL of Milli-Q water to achieve a concentration of 1 mg/mL.

Subsequently, BSA standards, along with the noncationized (n-Ab) and cationized (c-Ab) Fab' fragments of the antibody rituximab, were dispensed into separate wells of a 96-well microplate for the quantification of both cationized and noncationized Fab' fragments of the antibody rituximab. Following this, the BCA working reagent was added to each well, and the 96-well microplate was incubated for 30 min at 37 °C. The concentrations of noncationized (n-Ab) and cationized (c-Ab) Fab' fragments of the antibody rituximab were then determined at 562 nm using a Biotech Epoch microplate spectrophotometer, with the assistance of a standard curve.[31,32]

Optimization of the Bromophenol Blue (BPB) Dye-Based Assay to Confirm Antibody Cationization. The degree or level of cationization of Fab' fragments of antibody rituximab was determined using a bromophenol blue (BPB) dye-based assay.[33] Initially, the bromophenol dye solution was prepared by dissolving 0.0075% bromophenol blue in a solution containing 15 mL of 95% ethanol, 2.5 mL of glacial acetic acid, and 82.5 mL of Milli-Q water. Furthermore, 1 mg/mL solutions of noncationized (n-Ab) and cationized (c-Ab) antibody stock solutions were prepared using a sample diluting buffer, along with a 1 mg/mL standard BSA solution prepared using Milli-Q water.[20]

Subsequent to the preparation steps, BSA standards, noncationized (n-Ab), and cationized (c-Ab) Fab' fragments of antibody rituximab were aliquoted into separate wells of a 96-well microplate. The microplate was then incubated for 30 min at 37 °C in the dark to facilitate the reactions. The confirmation of cationization of the antibody Fab' fragment was conducted by measuring the absorbance at 610 nm using a BioTek Epoch microplate spectrophotometer, with reference to the standard curve.[20,34]

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Method Development for Cationized Fab' fragmnents. Linearity. A spectroscopic method was used for the linearity. A calibration curve was constructed to correlate the concentration of cationized Fab' of rituximab with its absorbance. The effectiveness of this method was evaluated by analyzing the coefficient of determination (r^2) and intercept values obtained through statistical analysis. Initially, stock solutions at 1 mg/mL concentrations of cationized Fab' of rituximab were prepared using a sample dilution solution to achieve different concentrations within these ranges: 0.1 to 2 mg/mL, 0.2 to 1 mg/mL, and 0.6 to 1 mg/mL and evaluate the linearity of c-Fab' samples.

Robustness. The robustness experiments demonstrated the method's reliability and stability by assessing its repeatability and reproducibility following the incorporation of various optimization variables. These results affirm the method's suitability for precise analytical applications.

Repeatability. Acceptance criteria for repeatability were established. Repeatability was assessed by utilizing all values of the cationization determination to calculate the mean, standard deviation (SD), and percentage coefficient of variation (%CV). Specifically, the acceptance criteria for cationization of the Fab prime segment of the antibody were set at a percentage CV of 10%.

Initially, the Fab fragment antibody samples (triplicates for each condition) were aliquoted into the wells of a 96-well microplate with a sample concentration of 1 mg/mL. Subsequently, 190 μL of bromophenol dye was added to each sample. After mixing, the samples were incubated for a minimum of 30 min in darkness. Readings were then taken at 610 nm by using a spectrophotometer to determine the degree of cationization.

Reproducibility. Reproducibility (interassay) was established by scrutinizing the consistency of analytical outcomes within three analytical runs. Reproducibility can be defined as obtaining consistent results using the same input variables, methodological and computational steps, and analysis conditions. For example, examination of reproducibility data across distinct pH and time intervals yielded insights into the sample validity and robustness of the method. The precision between measurement findings acquired at different laboratories is expressed using the term "between-lab reproducibility".

Acceptance criteria for reproducibility were established. Reproducibility was assessed by utilizing all values of cationization determination to calculate the mean, standard deviation (SD), and percentage coefficient of variation (%CV). Specifically, the acceptance criteria for cationization of the Fab prime segment of the antibody were set at a percentage CV of 10%.

Initially, the Fab' fragments of rituximab were aliquoted into the wells of a 96-well microplate at a concentration of 1 mg/mL. Subsequently, 190 μL of bromophenol blue dye was added to each sample, and the samples were incubated for a minimum of 30 min in darkness. Readings were then taken at 610 nm by using a spectrophotometer to determine the degree of cationization. The first and second readings of the plate were denoted as Run1 and Run2. A different experiment was performed under the same conditions as those for Run3.

Sensitivity. The lower limit of quantification (LLOQ) indicates the smallest amount of an analyte that can be reliably identified. LLOQ, alternatively termed analytical sensitivity, signifies the lowest measurable level of analyte that can be statistically distinguished from a blank sample. The determination of the LLOQ relies on both the variability of the blank sample and the sensitivity of the assay.

The lower limit of detection (LLOQ) was assessed by utilizing all values to calculate the mean, standard deviation (SD), and percentage coefficient of variation (%CV). Specifically, the acceptance criteria for the LLOQ of Fab' of rituximab were set at a percentage CV of 10%.

$$\text{Formula for LLOQ} = \%\ \text{CV} \leq 10\% + \text{blank absorbance}$$

Statistical Analysis. Statistical analysis was performed using GraphPad Prism (version 10.0.3), and a graph was prepared to illustrate the findings. A paired *t*-test was conducted to compare noncationized and cationized antibodies, revealing statistical significance ($p < 0.05$) and emphasizing the importance of the results.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.4c07727.

The repeatability and reproducibility of cationization levels across different pH conditions, the stepwise processing of rituximab to Fab' fragments, and statistical analysis of R^2 values at varying time points and pH levels; Figure S1: repeatability of cationization; comparative analysis of noncationized (A) and cationized (B−J) antibodies using BPB dye; Figure S2: reproducibility of cationization; Figure S3: processing of rituximab to Fab' fragments; Figure S4: R^2 values for cationization at different pH (PDF)

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Author Contributions

Both authors participated in drafting and reviewing the manuscript. S.V. conceptualized, designed, and performed experiments; optimized digestion, reduction, and cationization; developed the confirmation methodology; interpreted and validated data for the cationization of the Fab' antibody; and wrote the manuscript. S.R. reviewed the manuscript and supervised the project.

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■ ABBREVIATIONS

BPB, bromophenol blue; 2-ME, 2-mercaptoethylamine; SDS, sodium dodecyl sulfate; CBB R-250, coomassie Blue stain; EDC, *N*-ethyl-*N*- [3-(dimethyl amino)propyl] carbodiimide; n-Ab, noncationized antibody; c-Ab, cationized antibody; LLOQ, lower limit of quantification

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