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An Automated and Qualified Platform Method for Site-Specific Succinimide and Deamidation Quantitation Using Low-pH Peptide Mapping

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ABSTRACT

mAbs undergo several post-translational modifications, including the formation of succinimide from the deamidation of asparagine or the isomerization of aspartic acid. Because of the potential impact of succinimide formation on the biological activity of mAbs, detection and quantification of this species is a key area of interest for the pharmaceutical industry. However, studies assessing succinimide stability have been limited, and methods developed to monitor succinimide are either product specific or not robust. Here, we report the development of a platform low-pH peptide-mapping method using a combination of low-pH-resistant Lys-C and modified trypsin to maintain succinimide stability, eliminate deamidation assay artifact, and achieve efficient mAb digestion equivalent to conventional tryptic peptide-mapping method under alkaline condition. Using this method, succinimide stability in serum was accurately assessed *in vitro* study and the half-life was determined to be 1.5 days. With potential patient exposure to succinimide intermediate, a reliable method was developed to measure site-specific deamidation and succinimide intermediate. Coupled with a single quadrupole mass detector, our method was automated from digestion to data processing and applicable in a good manufacturing practice environment. The method was fully qualified to demonstrate accuracy, precision, linearity, and robustness.

Introduction

Various post-translational modifications (PTMs) such as deamidation, isomerization, and succinimide formation, have been reported extensively in therapeutic proteins, especially in mAbs.[1-10] Succinimide is the common product intermediate of 2 important protein degradation pathways, asparagine deamidation and aspartic acid isomerization, both *in vitro*[1,2,11-15] and *in vivo*.[2,3,16,17] In asparagine deamidation, the amide backbone nitrogen engages in a nucleophilic attack on the side chain carbonyl carbon and subsequently loses an ammonia, resulting in the formation of a succinimide intermediate. Similarly, in aspartic acid isomerization, the amide backbone nitrogen engages in a nucleophilic attack on the side chain carbonyl carbon, resulting in the loss of a water molecule and the formation of a succinimide intermediate. The succinimide intermediate product can be hydrolyzed further into isoaspartic acid and aspartic acid.[9]

Under mildly acidic conditions such as at pH 6.0, succinimide can accumulate to a significant amount, especially under elevated temperature.[2,18-21] Conversely, under neutral to basic conditions,

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List of Abbreviations

CDR	complementarity-determining region
cIEF	capillary isoelectric focusing
Fc	crystallizable fragment (in antibody)
GMP	good manufacturing practice
HIC	hydrophobic interaction chromatography
IEC	ion exchange chromatography
IgG	immunoglobulin
LC	liquid chromatography
MS	mass spectrometry
MS/MS	tandem mass spectrometry
PENNY	Peptide contains sequence of PENNY: GFYPSDIAVEWESNGQPENNYK
PIMT	protein L-isoaspartyl methyltransferase
PTM	post-translational modification
QC	quality control
QDa	quadrupole Dalton mass detector
SEC	size exclusion chromatography
SIR	single ion recording
TFA	trifluoroacetic acid
UPLC	ultra-performance liquid chromatography
GuHCl	guanidine hydrochloride

the succinimide hydrolysis occurs more rapidly than succinimide formation, resulting in the formation of aspartic acid and isoaspartic acid and a low amount of succinimide remaining.[22,23] mAbs often are formulated at a pH range of 5.0 to 6.5 to minimize degradations such as deamidation, oxidation, fragmentation, and aggregation. However, these conditions may induce the formation of succinimide.

Studies showed that succinimide formation in the complementary determining region (CDR) of mAbs can alter protein structure and function, resulting in a significant decrease in the binding affinity and potency compared with the unmodified antibody.[1-3,7,9,10] In one study, succinimide formation at residue 105 of CDR3 in the heavy chain was stable at pH 7 or lower and increased over time at 25°C and 40°C. The formation of CDR succinimide on one of the heavy chains affected binding affinity only moderately, but the formation of CDR succinimide on both the heavy chains decreased binding affinity by a log factor.[2] In another study, however, succinimide was indispensable for structural stability and enzymatic activity of MjGATase at elevated temperature.[24] Because of the wide occurrence of succinimide formation and its potential impact on the biological activity of therapeutic proteins, the detection and quantitation of this variant is a key area of interest in the pharmaceutical industry.[13,25]

Methods to separate succinimide-containing variants from native protein include hydrophobic interaction chromatography,[1,2,7,12,15,26] ion exchange chromatography (IEC),[4,9,10,26,27] size-exclusion chromatography,[2,27] and capillary isoelectric focusing (cIEF).[2,27] With hydrophobic interaction chromatography method, succinimide variants elute later than the main peak because of their higher hydrophobicity compared with the native protein.[7,12,15] With IEC and cIEF, succinimide formed by aspartic acid isomerization is detected as a basic peak due to the loss of a negative charge when forming a cyclic imide. Interestingly, succinimide formed by asparagine deamidation was also reported to appear as an IEC basic peak, although there is no net charge difference between asparagine and succinimide.[9] This detection indicates that physicochemical microenvironment changes due to succinimide modification may alter the p*K*a of neighboring ionizable groups, which in turn allow succinimide variants to interact with and be retained longer by the IEC column. In size-exclusion chromatography, the separation of succinimide-containing variants from unmodified species is hypothesized to be a consequence of its smaller hydrodynamic size.[2,27] All these methods are project specific and may not apply to other molecules. Moreover, these methods alone cannot be used for succinimide identification and characterization.

For succinimide identification and characterization, the most commonly used method is enzymatic digested peptide mapping followed by liquid chromatography and mass spectrometry (MS) because MS and tandem mass (MS/MS) information from succinimide-containing peptides provide mass-based site-specific identifications. In addition, this approach generally can be applied to most molecules. However, the instability of succinimide at the neutral or alkaline pH used in conventional peptide mapping presents a major challenge in succinimide identification and quantification, and this variant can therefore be significantly underrepresented or missed entirely. To overcome this, digestions were commonly performed under neutral or slightly acidic conditions to preserve succinimide.[3-5,16,19,21,22] In an *in vivo* study of the deamidation of asparagine 55 in the heavy chain of trastuzumab,[3] digestion for 3 h at pH 7.0°C and 37°C resulted in greater than 80% digestion efficiency and less than 1% digestion-induced deamidation. Although pH 7.0 improved the preservation of succinimide, however, the stability of succinimide-containing peptides at this neutral pH was still questionable; only 2% of the succinimide intermediate was detected in samples stored for 56 days at pH 7.0. In another study, surfactant RapiGest helped solubilizing protein samples and made them more susceptible to enzymatic cleavage at pH 6.0.[5] However, this method requires the degraded surfactant to be precipitated, whereas highly hydrophobic peptides coprecipitate with the degraded surfactant, leading to lower sequence coverage. An alternative approach minimized succinimide hydrolysis by denaturing and reducing the protein sample at pH 5.0, eliminating the alkylation step and shortening the digestion time to 1 h at pH 7.0.[10] This method, however, has its own pitfalls. Efficient digestion is difficult to achieve for proteolytically resistant proteins, and the absence of the alkylation step can lead to random cross-linking between cysteine-containing peptides, which can complicate the analysis significantly.

Another low-pH peptide-mapping method that used 8 M urea as a denaturing buffer and a mixture of 50 mM sodium acetate and 50 mM sodium phosphate at pH 5.0 as a digestion buffer has been developed using lysozyme as a model protein for digestion optimization.[27] With this method, however, up to 13% of preexisting succinimide was lost owing to hydrolysis at the optimal condition of 4 h trypsin digestion at pH 5.0. Any impurities in urea used at high concentration may have shifted the pH of the digestion buffered at pH 5.0 by a relatively lower concentration of the buffering agent and resulted in a higher pH. In addition, digestion with regular trypsin alone at acidic pH leads to the accumulation of abnormally high levels of missed cleavages at lysine residues. Moreover, efficient digestion of proteolytically resistant proteins is difficult to achieve by using urea as the sole denaturing reagent.

Endoprotease Glu-C that shows activity at low pH was reported to be used in peptide mapping to reduce artificial deamidation.[28] This study used a 17,000 Dalton small protein, calmodulin, and digestion was at pH 4.5 under native condition. This condition is highly unlikely work for antibody molecules and this paper did not show any data on succinimide formation.

For protein structure and function studies, correlating the level of a specific attribute to the results from functional assays is essential for assessing the attribute's potential impact upon efficacy. Hence, an assay that accurately measures succinimide is

needed. Here we present a new low-pH peptide-mapping method for succinimide and deamidation identification and quantitation. All procedural steps of this method are performed at pH 5.5. The method is thoroughly optimized using immunoglobulin (IgG) as a model molecule to match a conventional peptide-mapping sample preparation in terms of reduction, alkylation, and proteolytic efficiency. Method-induced succinimide hydrolysis and deamidation are virtually eliminated during sample preparation steps. Efficient digestion at acidic conditions is achieved by using a combination of low pH resistant Lys-C and modified trypsin along with a specialized low pH buffer.

We developed a streamlined, 2-step analytical approach to identify and quantitate succinimide and deamidation in biotherapeutic proteins with ready implementation in GMP environment. In the first step, succinimide and deamidation sites are identified using low-pH peptide-mapping sample preparation method followed by a high-end mass spectrometer that provides both MS and MS/MS information. This information was then used to validate the high-sensitivity, single-quadrupole Dalton mass detector (QDa)-based, focused peptide-mapping workflow, providing a rapid and robust quality control (QC) method that can monitor succinimide formation and deamidation in a GMP environment. We also report its successful automation.

Results

Protein Degradation in Heat Stress, Phosphate-Buffered Saline, and Serum Incubation Studies

A purified mAb drug substance (mAb-A) was incubated under various conditions to identify protein degradation pathways and to evaluate quality attributes. As measured by cIEF, acidic variants increased following incubation in formulation buffer at pH 5.5 under heat stress (40°C), in IgG-depleted serum, or in phosphate-buffered saline (PBS) incubation at 37°C (Fig. 1). Conventional peptide-mapping data showed that the deamidation of an asparagine residue in the light-chain CDR region is the major modification contributing to acidic variants under these conditions. Deamidation in the crystallizable fragment (Fc) region was minor. Because the deamidation percentages detected by peptide mapping is calculated using ion intensities of deamidated peptides divided by total ion intensities of deamidated and unmodified peptides generated from 2 reduced light chains, in theory, the deamidated IgG percentage could be twice as measured if deamidation only occurred on one CDR. In reality, the level of acidic species in cIEF, which is 2-fold higher as compared to the level of deamidation in CDR determined by peptide mapping, is attributable to charge variation due to other modifications, such as deamidation in Fc, glycation, or sialylation, although it is possible that some molecules may contain deamidation in both CDRs. For instance, mole ratios of sialic acid, glucose, and Fc-deamidated IgG are 0.03, 0.02, and 0.1 moles per a mole of IgG, respectively, in mAb-A. Therefore, it is not surprising that the levels of acidic variants by cIEF were more than 2 folds of the levels of light chain CDR asparagine deamidation from peptide mapping in serum and PBS studies. However, the difference between deamidation and acidic species levels was less prominent in heat stress study at pH 5.5 (Fig. 1). After 1-, 2-, and 3-month incubation at 40°C, observed CDR deamidation levels from peptide mapping were 18%, 29%, and 38%, respectively. The acidic variant levels in cIEF were 30%, 43%, and 52%, respectively, all of which were less than 2 folds of the levels of corresponding light chain CDR asparagine deamidation from peptide mapping. The substantial discrepancy between peptide mapping and cIEF results in different experimental conditions suggested the accumulation of an unstable succinimide intermediate as another asparagine degradation form in mAb-A and that the stability of succinimide varied with sample stress conditions, as well as assay conditions, particularly during sample preparation.

At the mild alkaline pH conditions in serum and PBS incubation, succinimide was not stable—it hydrolyzed gradually to the deamidated forms and were detected by both cIEF (as acidic variants) and peptide mapping. Thus, the increase in asparagine deamidation detected by peptide mapping correlated strongly with the increase in acidic variants detected by cIEF under these conditions. Under heat-stress conditions, however, samples were maintained in formulation buffer at pH 5.5, which stabilized succinimide. Because succinimide and unmodified asparagine have the same net charge and the nondenaturing cIEF environment prevented succinimide conversion in the capillary, both species comigrated in the main peak in cIEF, resulting in lower cIEF acidic peak percentages than those predicted by the total deamidation detected by conventional peptide-mapping method in which the succinimide have been hydrolyzed to deamidated species in alkaline digestion condition. These observations were consistent with the understanding that succinimide was the first detectable degradation product upon heat stress.

Succinimide Identification With the Low-pH Peptide Mapping

Succinimide in mAb-A was relatively stable under native conditions as an intact molecule and presented in the main peak in charge-based assays such as cIEF and IEC, despite the substantially alkaline local pH environment in cIEF capillary during focusing. However, it was unstable in conventional peptide mapping at the denatured and alkaline conditions and converted to a deamidated species. To stabilize the succinimide intermediate product, a low-

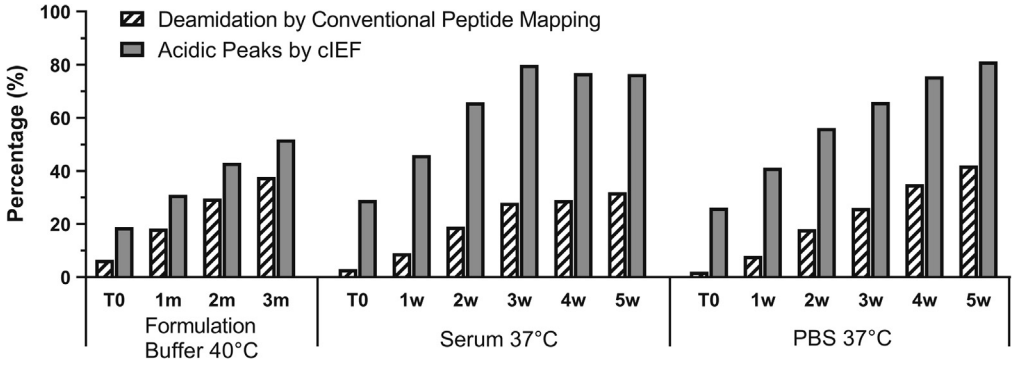

Figure 1. CDR deamidation (total aspartic acid and isoaspartic acid) measured by conventional peptide mapping versus acidic variants measured by cIEF in heat stress, serum, and PBS incubation studies.

pH peptide-mapping sample preparation method was developed. This method used a combination of new low pH-resistant Lys-C and modified trypsin along with an optimized buffer to enable efficient protein digestion at acidic conditions and maintain the stability of succinimide. All sample preparation steps including reduction, alkylation, and digestion were performed at mildly acidic conditions (pH 5.5). Using the low-pH peptide-mapping method, unmodified CDR asparagine and its 3 degraded species (deamidated aspartic acid, isoaspartic acid, and succinimide) could be differentiated and quantified (Fig. 2a). The peaks were verified by MS and MS/MS spectra (Figs. 2b-2d). As iso-Asp and Asp have identical MS and MS/MS spectra by collision-induced dissociation, their identities were verified by chromatography of synthetic peptides (Supplemental Fig. S1) and MS/MS spectra using electron transfer dissociation (Supplemental Fig. S2).

Analysis of Succinimide Half-Life in Human Serum Using Low-pH Peptide Mapping

To determine the potential impact of the succinimide product upon drug administration in humans, we assessed the conversion rate of succinimide to aspartic acid or isoaspartic acid in an *in vitro* serum incubation study. A mAb-A sample containing 31.4% CDR succinimide (as determined by low-pH peptide mapping) was spiked into human IgG-depleted serum and incubated at 37°C for up to 24 h. Two-thirds of the succinimide remained after this incubation as determined by low-pH peptide mapping. In a subsequent 4-day serum incubation study, we observed the gradual conversion of succinimide to aspartic acid (Fig. 3) and estimated the half-life for CDR-associated succinimide is 1.5 days. Interestingly, isoaspartic acid also converted to aspartic acid, although slowly. Besides succinimide hydrolysis to aspartic acid, about 3% of asparagine was also deamidated to aspartic acid after 4 days in serum.

Determining succinimide's stability in serum is very important in assessing its potential impact on drug efficacy. For example, if succinimide is converted quickly to deamidated forms in serum, patients would likely be exposed to the deamidated forms, in which case succinimide formation is not as critical, thus monitoring succinimide in the drug product is of little significance. Furthermore, pretreating mAb samples and hydrolyzing succinimide to deamidated forms could be considered before testing. Conversely, if succinimide is slowly converted to deamidated forms in serum,

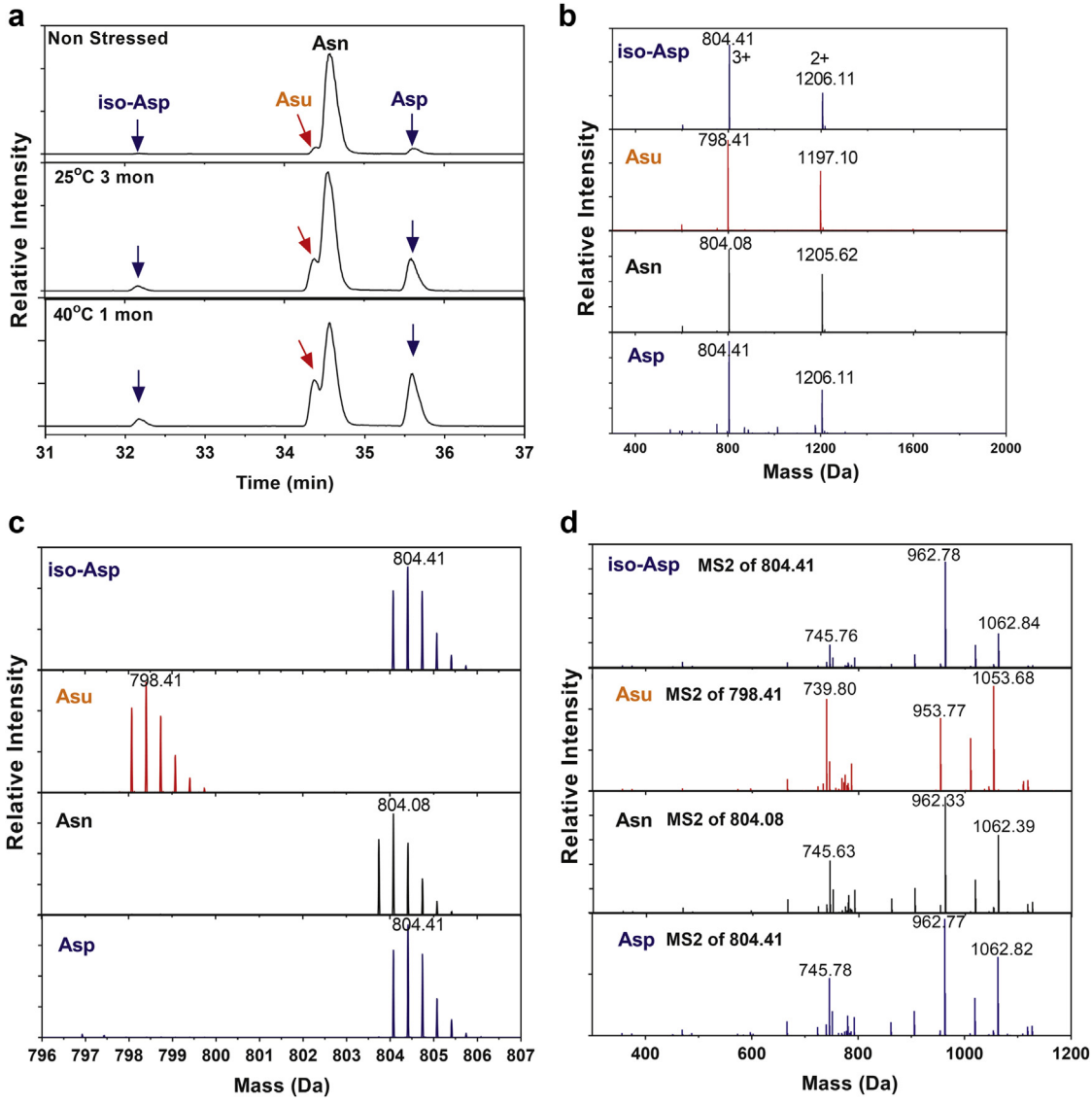

Figure 2. Selected ion chromatograms and mass spectra of the light-chain CDR asparagine degradation species identified by low-pH digestion peptide mapping. Asn, asparagine; Asp, aspartic acid; Asu, succinimide; iso-Asp, isoaspartic acid. (a) Selected ion chromatograms; (b) mass spectra; (c) zoomed-in mass spectra; and (d) tandem mass spectra (MS/MS).

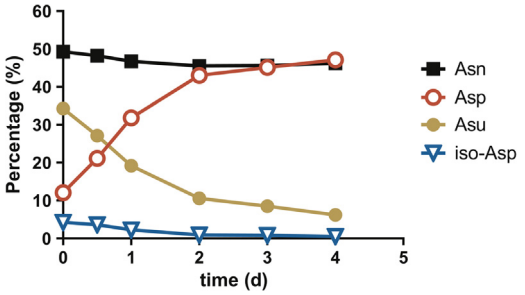

Figure 3. Percentages of LC CDR asparagine, succinimide, isoaspartic acid, and aspartic acid during 4-d incubation in serum. Asn, asparagine; Asp, aspartic acid; Asu, succinimide; iso-Asp, isoaspartic acid.

patients would expose to all or most of the succinimide form of drug, in which case it would be important to assess the impact of succinimide on efficacy. Based on serum incubation results, succinimide would be expected to have minimum change in cell culture medium over the 14- to 16-h incubation time required in cell-based bioassays, and the relative potency measured by bioassay would reflect the impact of succinimide on biological activity. In the heat-stressed mAb-A samples, the presence of succinimide in one of the light chains in the CDR resulted in a moderate loss in biological activity, but a more drastic loss with the presence of CDR succinimide on both light chains. This result resembled the impact of a succinimide formation at CDR position 105 had on the activity of an immunoglobulin G1 (IgG1).[2]

Pathways of Succinimide Conversion in Native Condition Versus in Denatured Peptide Mapping Digestion Condition

The conversion of isoaspartic acid to aspartic acid has been reported in the presence of protein L-isoaspartyl methyltransferase which facilitates the conversion of isoaspartic acid to aspartic acid.[29,30] To evaluate succinimide stability under high pH conditions and to determine whether isoaspartic acid could be converted to aspartic acid in the absence of the human serum matrix, heat-stressed mAb-A sample containing high succinimide content and nonstressed mAb-A sample with low succinimide content were incubated in buffer at pH 8.5 for up to 72 h and then subjected to low-pH digestion peptide mapping (Figs. 4a and 4b). Similar to the results observed in the serum incubation study, succinimide steadily converted to aspartic acid exclusively, and isoaspartic acid also converted slowly to aspartic acid, in both nonstressed and heat-stressed samples at pH 8.5. The half-life for succinimide at an alkaline buffer was approximately 24 h, which was just a little shorter than the 1.5 days half-life stability in serum. Faster rate of the conversion could be attributed to higher pH in the buffer (pH 8.5) than in serum (pH 7.4-7.6).

For comparison, the samples were also analyzed with a conventional peptide mapping at pH 7.6 (Figs. 4c and 4d). The data confirmed that succinimide underwent hydrolysis in alkaline buffer, resulting in minimally detectable levels of succinimide at all time points. Unlike succinimide-containing antibody, succinimide-containing peptide hydrolyzed to both isoaspartic acid and aspartic acid, with isoaspartic acid as the dominant (Fig. 4). Similar results from succinimide-containing peptide hydrolysis have been observed in other studies,[10] with an isoaspartic acid to aspartic acid ratio of 3:1. The levels of isoaspartic acid and aspartic acid detected by conventional peptide mapping (Figs. 4c and 4d) were higher than those detected by low-pH digestion peptide mapping (Figs. 4a and 4b). These differences became smaller over time as the amount of succinimide-containing antibody was gradually hydrolyzed to antibody containing aspartic acid over the 72 h time course in pH 8.5 buffer.

Comparison of PTMs Measured by Low-pH Peptide-Mapping and Conventional Peptide-Mapping Methods

In addition to comparing CDR deamidation and succinimide levels between the 2 peptide-mapping methods, we also examined differences in other sites and PTMs, such as one common Fc tryptic peptide GFYPSDIAVEWESNGQPENNYK (PENNY) deamidation and Met-252 oxidation, from nonstressed and heat-stressed mAb-A samples using these 2 peptide-mapping methods (Fig. 5). Consistent to what we have observed that disparities in the CDR deamidation level detected by these methods in the stressed sample were driven largely by the amount of succinimide at this site, differences in the deamidation of the PENNY peptide, which has low succinimide content, were small. Methionine oxidation levels detected by the 2 methods were comparable.

Impact of Digestion Time and Digestion Completeness for Succinimide and Deamidation Quantitation

The instability of succinimide makes the measurement of its true level by peptide mapping difficult. Low-level conversion of succinimide to deamidation species could still occur even at low pH condition, particularly if digestion is allowed for extended hours. To assess any change in the level of succinimide during the low-pH peptide mapping of mAb-A, we performed a time-course study with digestion time spanning from 1 h to 24 h. The level of CDR asparagine remained the same over the 24 h period (Fig. 6a) and some conversion of CDR succinimide to isoaspartic acid and aspartic acid was, indeed, observed (Fig. 6a insert), indicating that the increased deamidation over the entire time course arose solely from succinimide hydrolysis, rather than from asparagine deamidation. Thus, unlike conventional peptide mapping, low-pH digestion peptide mapping did not yield the artifact of asparagine deamidation during digestion. The association between succinimide levels and digestion time was linear, and the true value of succinimide in the original sample could be estimated by extrapolating the linear regression line to time zero. Based on this extrapolation, the true level of succinimide in mAb-A was estimated to be 8.2% at time zero. We calculated the amount of succinimide lost to hydrolysis during digestion by subtracting the succinimide level at time 0 from the value measured at a specified digestion time. Decrease in succinimide level was negligible after 4 h digestion (0.4% decrease). However, after 24 h digestion, succinimide level decreased for 3.2%. Over the same 24 h digestion at low pH, the deamidation level of the PENNY peptide remained the same (Fig. 6b). The ion intensities of the peptides used for the quantitation of CDR deamidation, succinimide and PENNY deamidation reached plateau after 1 h digestion and remained at similar levels over 24 h under low pH digestion conditions (Fig. 6c).

To ensure sufficient digestion for the peptides used for the quantitation of deamidation and succinimide and to evaluate digestion completeness for the mAb-A at different time points, we calculated and plotted the percentages of completely digested peptides and peptides with varying numbers of miscleavages. We observed, as digestion proceeds, the total abundance of completely digested peptides increases, whereas that of peptides with one or more miscleavage decreases (Fig. 6d), indicating convincingly that the overall digestion completeness is proportional to the digestion time. After a 4 h digestion at low pH, 68% of the peptides were completely digested, and 27% of the peptides had only one miscleavage. Missed cleavages in the low pH digests were more abundant than in conventional digest (Fig. 6e). However, this is not

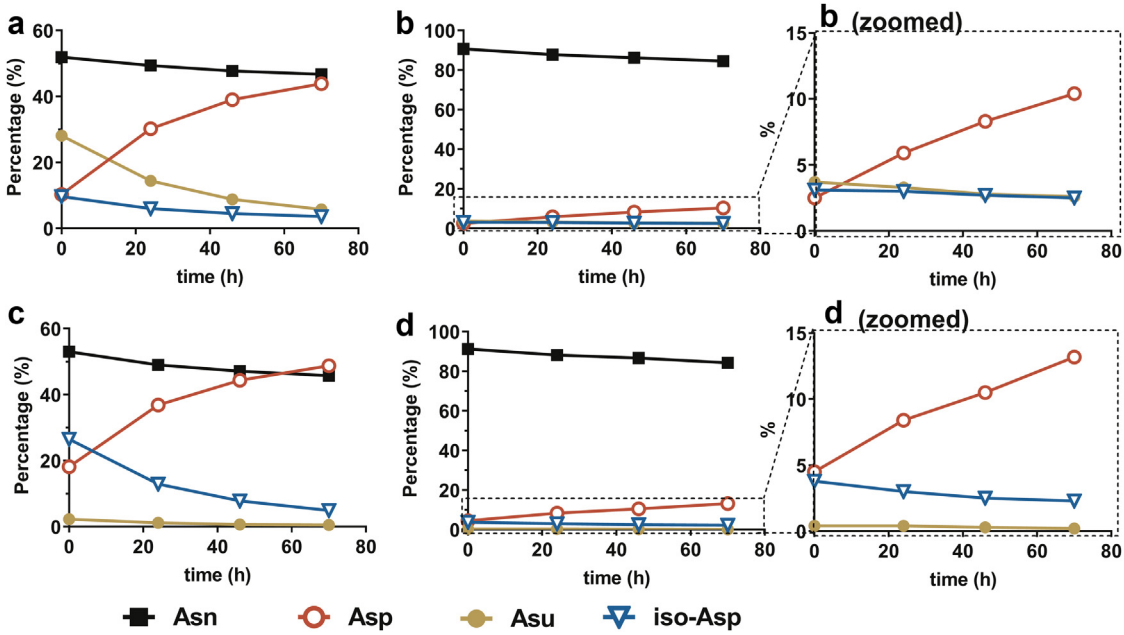

Figure 4. Percentages of LC CDR asparagine, succinimide, isoaspartic acid, and aspartic acid in nonstressed and heat-stressed mAb-A samples during 3-d incubation at pH 8.5. (a and b) Percentages of species detected by low-pH peptide mapping at pH 5.5; (c and d) Percentages of species detected by conventional peptide mapping at pH 7.6. (a and c) Heat-stressed mAb-A; (b and d) nonstressed mAb-A; (b zoomed and d zoomed) showing only aspartic acid, succinimide, and aspartic acid in nonstressed mAb-A by low pH and conventional peptide mapping, respectively. Asn, asparagine; Asp, aspartic acid; Asu, succinimide; iso-Asp, isoaspartic acid.

because succinimide decreases proteolysis in the local digestion site. Succinimide level is comparable between the miscleaved peptides and its fully cleaved counterpart peptides, as seen in Supplementary Table S1. Higher missed cleavage was caused by relatively high GuHCl concentration in the low pH digestion procedure as compared to the conventional digest (1M vs. 0.26M GuHCl, respectively). GuHCl is a strong trypsin inhibitor and it causes significant trypsin inhibition even at relatively low concentration. Missed cleavages in the low pH digest can be minimized by extra dilution of the reaction before digestion if needed (data not shown). We examined the kinetics of all peptide digestions and found that most of the differences in miscleavage peptides at the early time points compared to late time points are short peptides in IgG CH3 domains (Fig. 6f). For example, the abundances of CH3 domain peptides HC339-360, HC339-355, HC341-360, which contain multiple miscleavage sites, decrease significantly from 4 h digestion to 24 h digestion. These slow cleavage sites are consistent with Siepen's rule[31] where an Arg residue followed by Trp or Glu residues is not susceptible to trypsin cleavage. Two other predominant slow cleavage sites involve the kappa light chain peptide 108-126 (RTVAAPSVFIFPPSDEQLK) and 208-214 (C-terminal peptide SFNRGEC). These peptides signature a missed cleavage site that is close to the termini of the peptide. In fact, certain degree of incomplete digestion is beneficial for better sequence coverage in peptide-mapping method. Most peptides used in PTM quantitation will not be affected after 4 h digestion. Actually, for mAb-A, the intensities of peptides used for the quantitation of CDR asparagine deamidation and succinimide formation reached plateau after 1 h digestion in low pH—presence of missed cleavage sites at 1 h and after was irrelevant to the CDR asparagine-containing peptides and therefore would not impact quantitation.

Our data demonstrated that the low-pH peptide-mapping procedure provided efficient digestion of biotherapeutic proteins for peptide-mapping analysis. We select 4 h as the optimal low pH digestion time, taking into consideration the plateaued ion intensities of monitored CDR peptides, less than 5% of peptides with 2 or more miscleavages, and minimum succinimide hydrolysis (0.4%) under this condition.

QDa-Based Focused Peptide Mapping to Quantify CDR Asparagine Deamidation and Succinimide

Traditional high-end mass spectrometer was used for initial succinimide identification and characterization for mAb-A. However, for routine lot release and stability testing in GMP environment, a rapid and robust quality control method with validated instrument is needed. A high-sensitivity, single QDa is an ideal

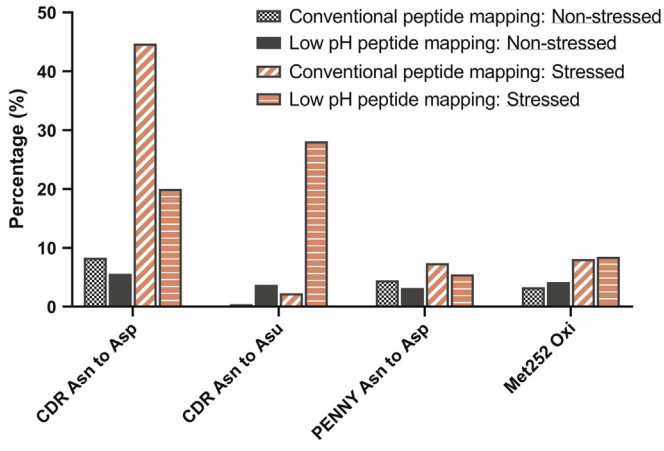

Figure 5. Comparison of post-translational modifications of nonstressed and stressed mAb-A subjected to conventional peptide mapping or low-pH peptide mapping. For conventional mapping, samples were digested for 4 h at pH 7.6. For low-pH peptide mapping, samples were digested overnight at pH 5.5. CDR asparagine deamidation, CDR succinimide, Fc PENNY peptide deamidation, and Fc methionine 252 oxidation were the post-translational modifications measured. Asn, asparagine; Asp, aspartic acid; Asu, succinimide; Met, methionine; Oxi, oxidation.

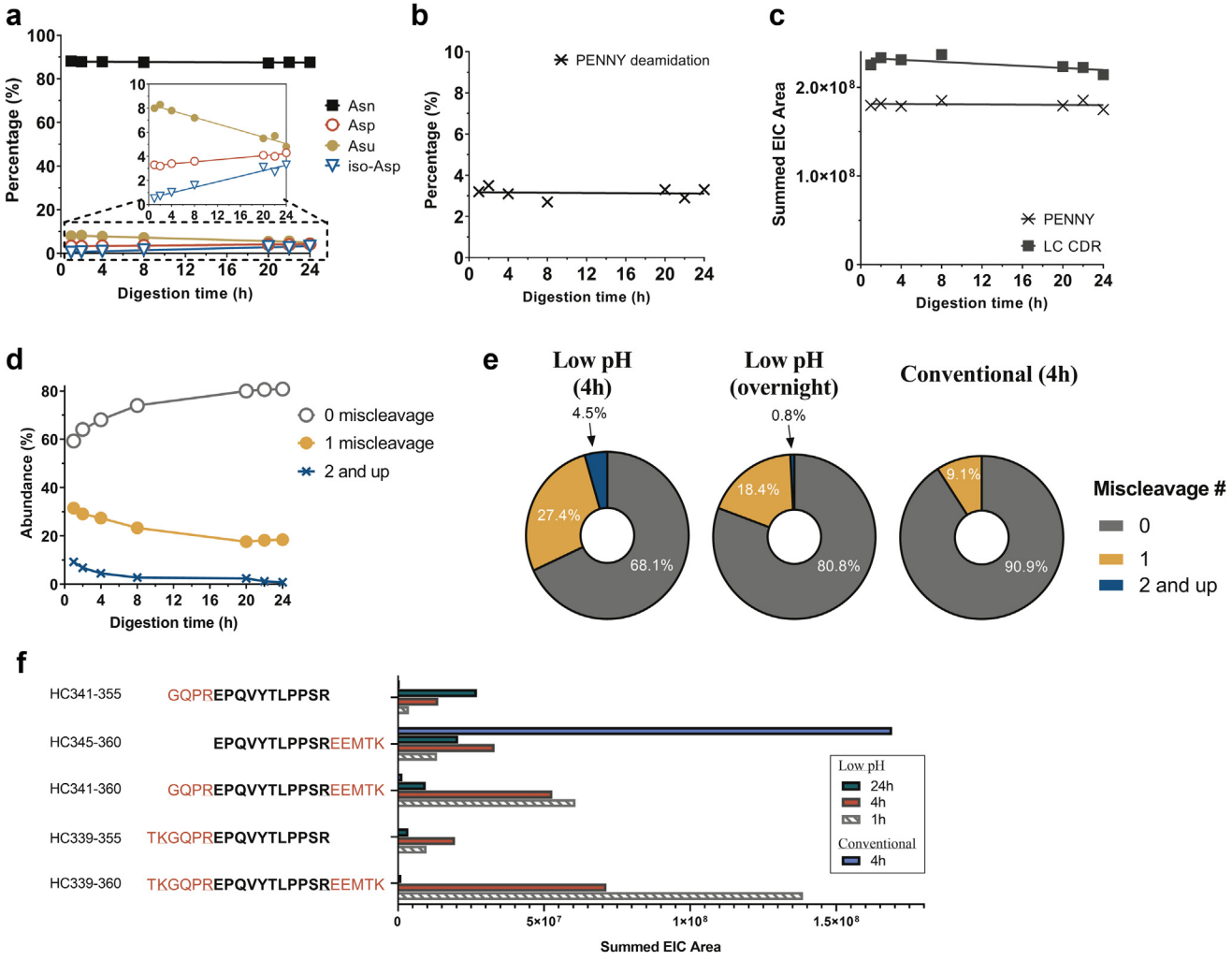

Figure 6. Succinimide and deamidation profiles and digestion completeness over 1 to 24 h digestion time. (a) Percentages of CDR asparagine, aspartic acid, succinimide, and isoaspartic acid in nonstressed mAb-A samples. (b) Percentages of PENNY deamidation in nonstressed mAb-A samples. (c) Total peak areas of extracted ion chromatograms for penny peptides and for LC CDR peptides. (d) Percentages of completely digested peptides and peptides with varying numbers of miscleavages. (e) Comparison of digestion efficiency of low pH digestion at 4 h and 24 h with conventional peptide-mapping method (4 h digestion). (f) Examples of miscleaved peptides in IgG CH3 domains at various digestion time in low pH digestion method compared with conventional peptide-mapping method.

platform for building QC method in this environment. Therefore, we adapted QDa as an analytical segment of the low-pH peptide-mapping method. QDa-based focused peptide mapping with ultra-performance liquid chromatography (UPLC) was used to monitor tryptic peptides containing the CDR deamidation and succinimide sites in mAb-A (Fig. 7a). The mass-charge ratios were selected for each peptide and used in specific single ion recording (SIR) channels, and ions with these mass-charge ratios were detected and quantified by the QDa mass detector in specified retention-time windows. To maximize detection sensitivity, the highest-intensity monoisotopic masses of each peptide were selected for the SIR channel. A range of cone voltages, capillary voltages, and probe temperatures were screened in multiple injections to achieve optimal peak intensities. SIR profiles using trifluoroacetic acid (TFA) or formic acid (FA) as an ion-pairing reagent in mobile phases were compared, and TFA was selected because of its lower peak interference from the sample matrix. To ensure correct peak assignment during method development, we confirmed all peaks monitored by QDa using a high-resolution, ion-trap mass spectrometer that provided both MS and MS/MS information for peak identification. This high-end mass spectrometer was also used to verify the quantitation detected by QDa-based focused peptide mapping (Fig. 7b).

Qualification of the Low-pH Peptide-Mapping Method

The ability of the low-pH digestion peptide-mapping assay with QDa detector to quantify CDR succinimide and deamidation was evaluated by determining the assay's specificity, linearity, accuracy, precision, and limit of quantitation and limit of detection, using both the stressed and the nonstressed mAb-A. Qualification results are summarized in Table 1, and details are discussed in Supplementary Materials.

Automation of the Low-pH Peptide-Mapping Method

Our low-pH reagents reliably and efficiently reduced, alkylated, and digested mAb-A and other therapeutic antibodies during manual sample preparation. However, this positive experience did not necessarily guarantee similarly efficient performance of the reagents in an automated procedure. Particularly, the 2-step proteolytic digestion performed at low pH may contribute to

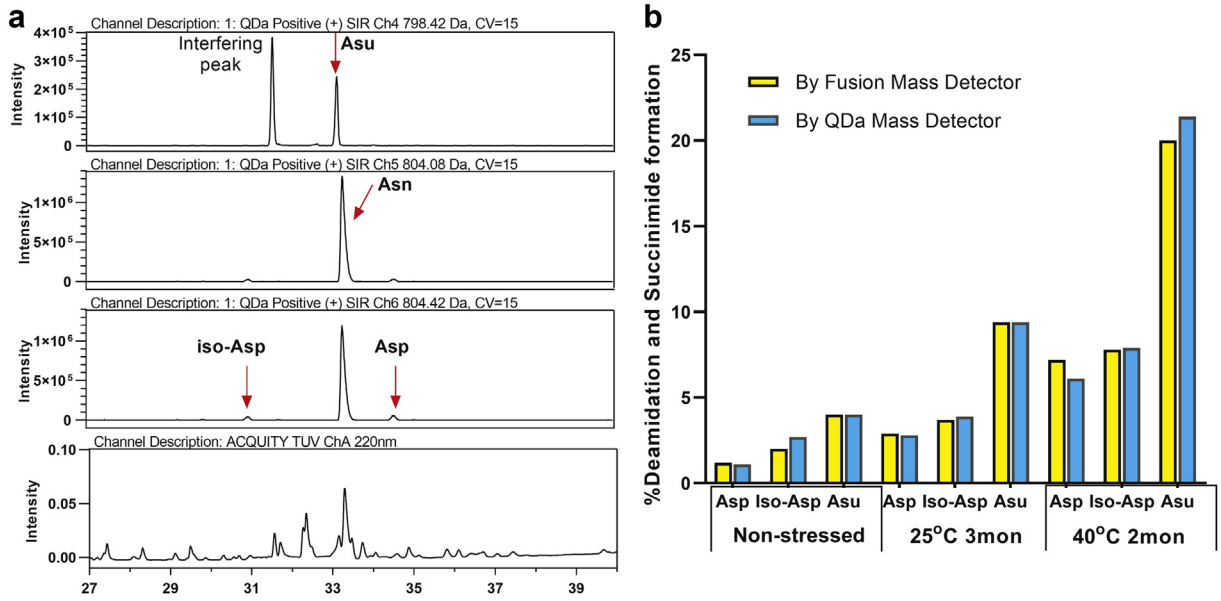

Figure 7. (a) Selected SIR-channel ion chromatograms of CDR deamidation species and ultraviolet chromatogram from low-pH digestion QDa-based focused peptide mapping. Top panel: peptide containing CDR succinimide Asu. Second top panel: native CDR peptide Asn. Third top panel: deamidated CDR peptides iso-Asp and Asp. Bottom panel: ultraviolet chromatogram; (b) Comparison of CDR succinimide, CDR deamidation measured by fusion mass detector versus by QDa mass detector.

experimental variability the most. It requires the maintenance of denaturing agent (guanidine hydrochloride or urea) within defined concentration range, which could be changed in an automated procedure by water evaporation, poor reagent mixing, and other factors. Stability of the low pH reagents in an automated procedure was another concern—the reagents needed to maintain full stability at room temperature for the several hours required to complete an automated sample preparation procedure. To address these concerns, mAb-A control sample and heat-stressed samples at 25°C and 40°C for 1, 2, and 3 months were digested with low-pH reagents manually or by an automated preparation using the Tecan Evo 200 robotic system. Digests were analyzed by reverse-phase liquid chromatography and a QDa mass detector. The succinimide and deamidation results from manual preparation were comparable with those from automated digestion, both for the control and heat-stressed samples (Supplemental Fig. S3). Thus, our low-pH peptide-mapping sample preparation procedure was fully compatible with automation, and our reagents met the stability requirements for automated sample preparation.

Discussion

Incubation of mAb-A under heat-stressed conditions led to an increase in succinimide from an asparagine residue in the light-chain CDR. Succinimide variant impacts potency of mAb-A and is therefore a CQA which needs routine monitoring.

A new method using low-pH-resistant Lys-C combined with modified trypsin was optimized to measure succinimide and suppress sample preparation–induced deamidation artifact during sample preparation. As demonstrated with the model IgG mAb-A,

Table 1
Summary of Method Qualification Results

Parameter	Result					
Specificity	No Response for Blank and Formulation Buffer					
	Positive Response for Known Sample					
	% Total CDR Succinimide			% Total CDR Deamidation		
	Mean		% CV	Mean		% CV
Precision						
Repeatability (6 preps × 1 injection)	10.2		1.6	6.5		1.8
Intermediate precision						
Day-to-day (3 preps × 1 injection/day, 2 d)	9.3		10.2	6.8		4.6
Analyst-to-analyst (3 preps × 1 injection/analyst, 2 analysts)	9.2		8.2	6.7		7.2
Column-to-column (3 preps × 1 injection/column, 2 columns)	8.5		3.2	7.1		1.1
Linearity and range						
R^2	0.9998			0.9984		
Range	4.7-33.3			2.6-10.9		
LOQ and LOD						
LOQ, from calibration curve, 10 (SD/S)	1.1			1.1		
LOD, from calibration curve, 3.3 (SD/S)	0.4			0.3		
Accuracy						
Level	low	medium	high	low	medium	high
% Recovery	102	99	100	103	99	100
% CV	2.7	0.9	2.9	1.6	2.4	0.6

CV, coefficient of variation; LOD, limit of detection; LOQ, limit of quantitation; S, slope.

digestion completeness under low-pH conditions was comparable with that achieved under conventional peptide-mapping conditions with an exception for certain cleavage sites that were particularly resistant to digestion and which digestion was slower at low pH conditions. Succinimide hydrolysis was low, only 0.4% over a 4 h digestion, and deamidation levels remained constant over 24 h. Therefore, low-pH digestion-based peptide mapping could measure the original amount of succinimide and deamidation in samples, making this method ideal for succinimide and deamidation quantitation.

A special advantage of our method is the use of UPLC QDa instrument in the analysis. This instrument is affordable, is easy to operate, and provides means for rapid and robust analysis of PTMs in biotherapeutic proteins. UPLC QDa showed a sensitivity comparable to that of the ion-trap peptide-mapping method used in development laboratories. However, because its mass detector and software are designed for a QC environment, UPLC QDa-based peptide mapping can be implemented in QC laboratories. Our 2-step process also offers the advantages of site-specific succinimide information with minimal method development because of the high sensitivity and resolving power of the mass detector, compared with commonly used methods for succinimide detection and quantitation. A simple linear gradient and standard peptide-mapping mobile phases are sufficient for chromatographic separation. Interferences from other peptides in the sample matrix are not a concern as these peptides tend to have different mass to charge ratio (m/z) than the targeted peptides, and these differences in m/z are easily resolved by the QDa mass detector.

We applied our method to determine stability of succinimide in serum. Our data indicated that half-life of succinimide in serum is 1.5 days and succinimide conversion is processed by chemical mechanism (alkaline pH). Relatively long stability of succinimide in serum warrants further studies focused on succinimide role in drug efficacy.

Because sample preparation is the most labor-intensive step in peptide-mapping and mAb analysis overall, an increasing number of biopharmaceutical companies are exploring automation for this step. Not only does automation reduce the labor associated with sample preparation, it also provides analytical reproducibility and eliminates human error. During automating the low pH digestion protocol, we encountered certain challenges including the optimization of sample volumes for automation and complicated sample dilution in which a wide range of concentrations across samples was involved. Another challenge was the treatment of the temperature-sensitive enzymes, which cannot be sitting in room temperature during the sample preparation process. We addressed these challenges by scaling up sample volumes, programming the robotic system to choose the best pipet tip volume, and including user prompts to place the enzymes on deck and remove them at the right time.

In summary, we developed a streamlined analytical method using low-pH digestion for sample preparation followed by UPLC-QDa-based focused peptide mapping. This method is fully qualified and automatable for the routine monitoring of succinimide in proteins, and this QC-friendly method is ready for the implementation in Good Manufacturing Practice laboratories.

Materials and Methods

Materials

mAb-A was a purified mAb drug substance and formulated at pH 5.5. The AccuMAP low pH digestion kit and trypsin were obtained from Promega (Madison, WI). Urea (OmniPur®), OmniSolve water, and acetonitrile were obtained from Millipore Sigma (Billerica, MA). Sodium phosphate dibasic, sodium phosphate monobasic monohydrate, sodium chloride, FA, TFA were from Sigma (St. Louis, MO). Guanidine hydrochloride, iodoacetamide, dithiothreitol, and tris(2-carboxyethyl) phosphine were obtained from Thermo Fisher Scientific (Waltham, MA). Human serum was provided by BioIVT.

Methods

Imaged Capillary Isoelectric Focusing and Conventional Peptide-Mapping Methods

These two methods are the same as described in previous publication.[32]

mAb-A Serum Incubation

Human serum was flowed through a protein A column (MabSelect SuRe) twice to fully deplete the endogenous protein-A-bound IgGs. mAb-A was then spiked into the IgG-depleted serum to a final concentration of 1 mg/mL. After incubating at 37°C for desired time, the spiked mAb-A was recovered by protein A chromatography purification.

Low-pH Protein Digestion Method for Peptide Mapping

An AccuMAP low-pH protein digestion kit (Promega) was used to prepare peptide-mapping samples under low-pH conditions (pH 5.4 to 5.8) according to the manufacturer's instructions. Briefly, the antibody sample was first reduced with a mixture of denaturing buffer containing 8M guanidine hydrochloride, a specifically formulated proprietary low-pH reaction buffer, and TCEP at 37°C for 30 min. The final concentrations of guanidine chloride and TCEP were 5M and 3mM, respectively, at this step. After reduction, 2-iodoacetamide was added to the sample to the final concentration of 18 mM, and the mixture was incubated in the dark at 37°C for 30 min. The antibody was then digested with a specifically formulated low-pH resistant LysC at 37°C for 1 h. Final guanidine concentration was 2.7M at this step. The reaction was diluted with water to bring the guanidine concentration to 1M, and the digestion was completed by adding modified trypsin and an additional quantity of low-pH resistant Lys-C and incubating the mixture at 37°C for 14 to 16 h (AccuMAP kit technical manual specifies 3 h). The reaction used a protease to protein ratio of 1:5. After digestion was complete, the reaction was quenched with 2% FA or TFA to allow for >99.8% alkylation efficiency and high digestion reproducibility. The digest was analyzed with LC-MS as described earlier (see "Conventional Peptide Mapping").

UPLC-QDa Method for Succinimide and Deamidation Monitoring

Digested peptide mixtures were analyzed by using a Waters I-class UPLC system coupled with a QDa mass detector. Mobile phase A contained 0.02% TFA in water, and mobile phase B contained 0.02% TFA in acetonitrile. The Waters ACQUITY UPLC BEH300 C18 column (1.7 μm, 2.1 × 150 mm) was maintained at a temperature of 55°C. Flow rate was 0.2 mL/min. Mobile phase B started at 0% for 4 min, then increased to 5% at 7 min and 20% at 31 min. This was followed by a shallow increase to 21% mobile phase B over 5.25 min, then a gradient increase to 24% at 40.5 min and a slow increase to 26% at 47 min. The column was then washed with 95% mobile phase B from 47.5 to 49.9 min and equilibrated at 0% mobile phase B from 50 to 60 min. The total run time per sample was 60 min. Peptides containing CDR deamidation sites eluted between 30 and 35 min.

Automated Low-pH Digestion Assay

To address special automation considerations such as low-volume pipetting, the ability to incubate small volumes at different temperatures, and the ability to handle light-sensitive reagents, the Tecan Evo 200 robotic system was chosen. The versatility and peripheral capabilities of this system included a liquid-handling arm that can pipet volumes from 5 μL to 990 μL, a 6-position incubator where samples can be incubated in a 96-well PCR plate in the dark, the ability to move that plate to different positions in the deck, and the ability to heat samples to 37°C. Sample volumes were scaled up to accommodate the automation script without using too much reagent. To optimize the initial dilution and sampling step, and to accommodate the wide concentration range of mAb-A samples, the automation script was written to allow the robot to choose the proper volume pipet tip. To accommodate the temperature sensitivity of trypsin and Lys-C, the automation script included user prompts to tell the analyst when to place the enzymes on the deck with controlled temperature and when to remove them.

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