Characterization of Expected and Scrambled Disulfide Bonds Using BPV Flex Software

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The role of disulfide bonds is to provide stability for tertiary and/or quaternary structure in proteins. Since the overall structure of proteins is related to their function, ensuring disulfide bond arrangements are as expected is critical for the safety and efficacy of biotherapeutics. Currently, the largest class of biotherapeutics is monoclonal antibodies, which contain a number of cysteine residues that are commonly involved in disulfide bonds (32 cysteines involved in 16 disulfides bonds for IgG1 mAb, for example). While the cellular expression of antibody-based therapeutics typically produces the expected and efficacious arrangement of disulfide bonds, in some cases their arrangement can be changed during the process from expression to final formulation. These dislocated linkages are often referred to as being “scrambled”.

Characterization of disulfide bond arrangements is commonly executed using high resolution mass spectrometry which allows for detection of both expected and scrambled disulfide bonds. While detection of the peptides linked via disulfide bonds has become routine with modern mass spectrometers, interpretation

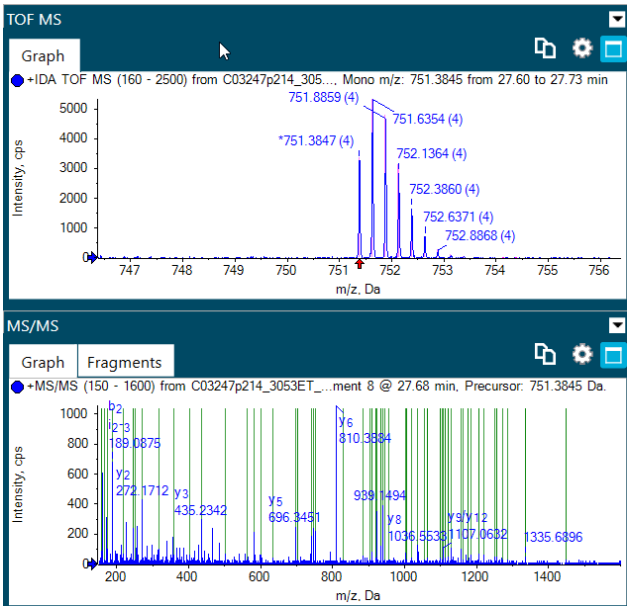

Figure 1. MS (top) and MS/MS (bottom) spectra for a spiked scrambled disulfide containing peptide detected by BPV Flex Software.

SCIEX X500B QTOF System

of data to confidently identify disulfide bonds has remained challenging. Most of the time, corresponding peptides are large and difficult to fragment. Moreover, taking into account the possibility of scrambling events considerably enlarges the search space, due to combinatorial explosion, which often results in lengthy processing times. While there are approaches to limit the search space, this results in the need to perform multiple searches to ensure identification of all potential scrambling events.

Presented in this technical note is the use of BPV Flex Software for comprehensive and rapid characterization of disulfide bonds in an IgG1. To ensure that the software is able to detect both expected and scrambled disulfides, synthetic peptides mimicking a scrambling event were spiked in a mAb digest. The spiked scrambled peptides were obtained by starting from synthetic cysteine-containing tryptic peptides normally present in the mAb sequence and treating them to form disulfide bonds representative of a scrambling event. The scrambled peptides were spiked at different levels in the mAb digest to test the sensitivity of the whole assay. The workflow for detection of disulfide bonds as well as the results of the scrambling search will be discussed.

Key Features of BPV Flex Software Scrambled Disulfide Searching

- Rapid processing times for scrambled disulfides
- Easy sorting and filtering of data to focus on expected and scrambled disulfide bonds
- Viewing of expected disulfide bonds and scrambled disulfides using an interactive sequence viewer
- Peptide auto-validation that guides data review for greater confidence in results
- A customizable view for streamlined data analysis

Methods

Sample Preparation:

mAb1 digest preparation in non-reducing conditions:

20 µL of mAb1 (500 µg) were subjected to denaturation using 75 µL of RapiGest solution, and 1.5 µL of a 100 mM N-ethylmaleimide (NEM) solution was added to label potential free cysteines present in the sample and to prevent any artificial disulfide bonds scrambling that could occur during trypsin digest preparation. This solution was incubated for 2 hours at 37°C. The mAb1 sample was then submitted to trypsin digestion using a 1:20 protein/enzyme ratio and incubating overnight at 37°C. The reaction was quenched and RapiGest precipitated by adding 20 µL of HCl 0.5N and the mixture was centrifuged for 4 min at 14000g.

Spiking of scrambled disulfide peptide in mAb1 digest:

The known scrambled disulfide species was prepared using synthetic peptides corresponding to light chain T10 and heavy chain T11. Each of the peptides was received with a purity of >96% and used without further purification. To form the disulfide bonds the peptides were incubated with $CuCl_2$ at a ratio of 2:1 for 2.5 hours at 85°C. The reaction mixture was then spiked into the antibody digest at 10%, 5%, 1%, 0.5%, and 0.1%. The percentage of spiking corresponded to the molar abundance and are given according to the initial amount of synthetic peptides reacted with $CuCl_2$.

Chromatography:

20 µg of mAb1 tryptic digest, spiked or not spiked with scrambled disulfide peptides, was separated using a CSH C18 column (1.7 µm particle size, 130 Å, 2.1x150 mm, Waters) using an ExionLC™ System. The aqueous mobile phase (A) consisted of HPLC-grade water with 0.1% FA, while the organic phase (B) was acetonitrile 0.1% FA. A gradient profile was used at a flow rate of 200 µL/min, Table 1. The column temperature was maintained at 50°C.

Mass Spectrometry:

Mass spectrometry experiments were completed using a SCIEX X500B QTOF system fitted with a Turbo V™ Ion Source with a twin spray probe. Data was acquired using information dependent acquisition (IDA) for the top 10 candidates. MS conditions are listed in Table 2.

Data Processing:

Data processing was accomplished using BPV Flex Software 2.0 with and without the scrambled disulfide option enabled as dictated by the study in question.

Table 1. Chromatographic Gradient.

Time (min)	%A	%B	Flow Rate (ml/min)
0	*100*	*0*	*0.3*
50	*55*	*45*	*0.3*
60	*55*	*45*	*0.3*
65	*20*	*80*	*0.3*
65.1	*100*	*0*	*0.3*
70	*100*	*0*	*0.3*

Table 2. Mass Spectrometer Parameters.

Parameter	Setting
Scan Mode	*Positive*
GS1	*40 psi*
GS2	*40 psi*
Curtain Gas	*35*
Temperature	*450 °C*
Ion Spray Voltage	*5500 V*
Time Bins to Sum	*4*
Accumulation Time	*0.15 sec*
TOF Start Mass	*160*
TOF Stop Mass	*2500*
Declustering Potential	*80 V*
Collision Energy	*Dynamic*
IDA Candidates	*10*
Intensity Threshold	*100 cps*

Expected Disulfide Bonds

Non-reduced peptide map data was collected using the ExionLC System connected to an X500B QTOF system. The data was searched using BPV Flex Software for the expected disulfide bonds which were entered on the Define Molecule page. For the search, additional modifications were not considered to reduce complexity in data interrogation. As shown in Figure 2, the data was grouped by peptide sequence and then filtered to show only the expected disulfide bonds. All of the peptides corresponding to expected disulfide bonds were detected in multiple charge states. For many of them at least one charge state was automatically validated by the software. Auto-validation refers to peptides which passed a user-defined MS/MS score threshold. Additional charge states were manually reviewed and those that time aligned with auto-validated species or were determined to match expected disulfide bonds were selected for use in the assay. As shown in Figure 3, overall sequence coverage was near 100% for the control sample with each of the expected disulfide bond pairs found. The peptide sequences linked by expected disulfide bonds are highlighted and the defined linkages are clearly visible.

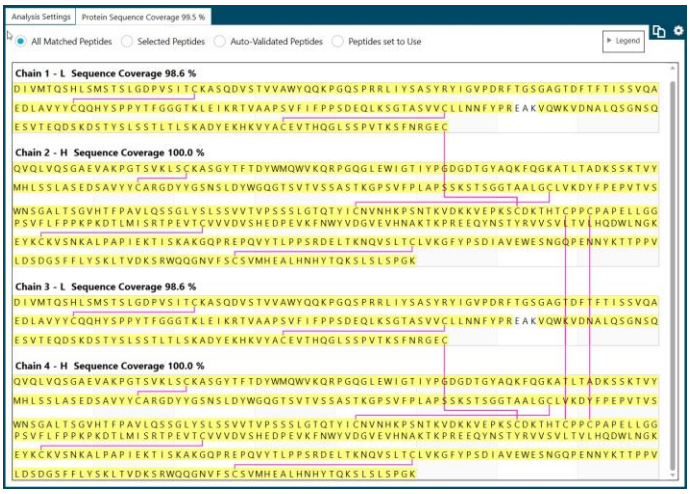

Figure 3. Sequence coverage map for BPV Flex Software. Result of control disulfide sample searching only for expected disulfide bonds. Yellow highlighted sequence reflects observed peptides, while lines connecting Cys residues indicate expected linkages observed in the sample. Observed scrambling linkages may also be displayed as well.

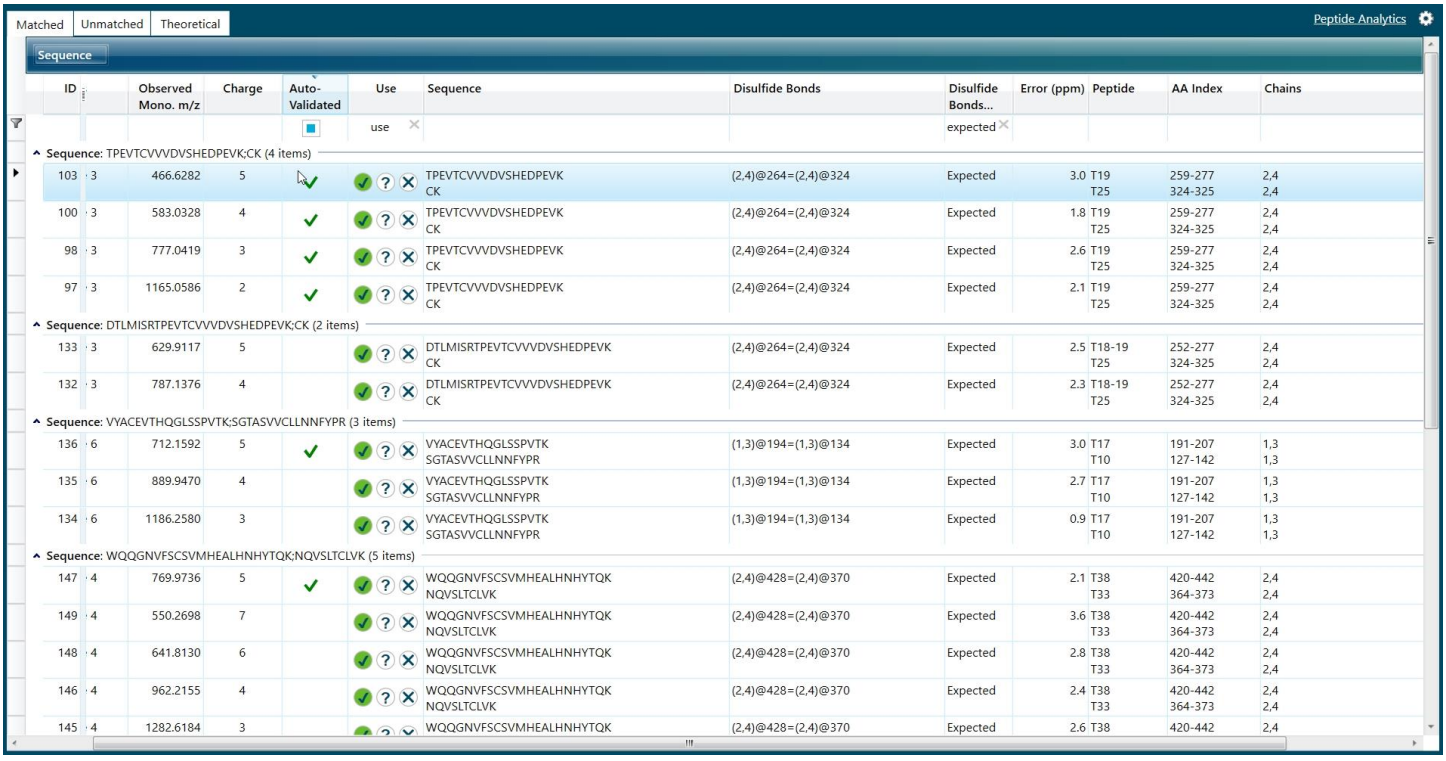

Figure 2. Grouped and filtered results for expected disulfides. The peptides and the linkages involved are clearly defined for each bond. In many cases more than one charge state are detected and these are aggregated under each peptide sequence entry.

Scrambled Disulfides

As previously described, two cysteines containing tryptic peptide sequences of mAb1, T10 from LC and T11 from HC were selected, purchased under a synthetic form and incubated with $CuCl_2$ to generate disulfide peptides. The conversion of these two peptides into disulfide bonded species is not complete. The content of the reaction mixture is illustrated in Figure 4. The scrambled disulfide peptide mix generated was first spiked into digested samples at a level of 10%. For the remainder of this work we refer to the spiked levels based on the assumption of quantitative conversion, however actual spiked levels are roughly half of those quoted for T11 (HC)-T10 (LC) scrambled disulfide peptides and less for homodimers of T10 and T11 peptides which represent respectively 13% and 16% of total MS signal as illustrated.

Data generated with the mAb1 digest spiked with 10% of scrambled disulfide bond peptides were searched using the scrambled feature in BPV Flex Software. To enable the search for scrambled disulfides the option must be selected within the advanced settings in the peptide mapping workflow. As shown in Figure 5, the feature is activated by checking the “Search for Scrambled Disulfide Bonds” option. Once selected, the number of peptides involved as well as the number of free cysteines can be defined. The detection criteria for all of the components in the search space are also defined within the advanced settings and are applied to all components in the sample.

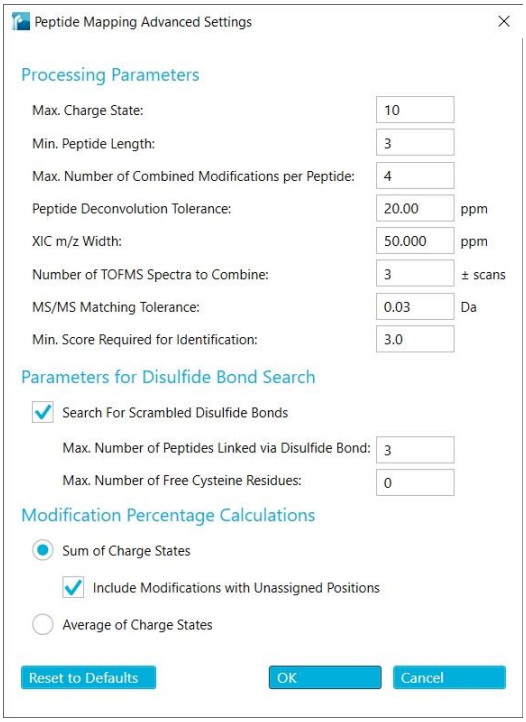

Figure 5. Peptide mapping advanced settings are used to activate scrambled disulfide search.

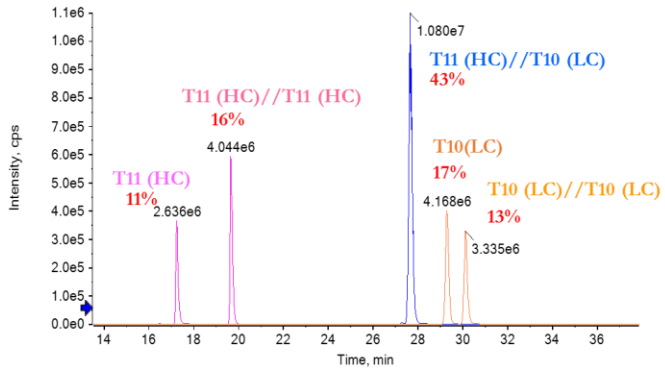

Figure 4. Extracted Ion Chromatograms for the different species observed in the reaction mixture of T10 (LC) and T11 (HC) peptide reacted with $CuCl_2$. (pink traces correspond to unreacted T11 (HC) and to T11(HC) disulfide peptide, blue trace corresponds to the T11(HC)-T10 (LC) disulfide peptide, oranges traces correspond to T10(LC) and T10(LC)-T10(LC) disulfide peptide

After applying the search options, the highest level spiked sample was processed using the same parameters used for the expected disulfide study. After processing, the results were filtered to focus on the components that were identified as being scrambled. As shown in Figure 6, the expected scrambled disulfide species were observed in the data set with high quality MS and MS/MS data. For each of the identified charge states the identification was auto-validated by the software. In addition to the expected scrambling event, the homo-dimer of each of the spiked peptides was observed as shown by the top and bottom entries in Figure 6. These species would be expected based on the preparation of the scrambled peptides.

The sensitivity of detection of the scrambled disulfide bonds was then investigated using samples spiked with different levels of scrambled species. Spiked levels of 10%, 5%, 1%, 0.5%, and 0.1% were prepared and analyzed using the same parameters applied for searching the highest spiked level of 10%. As shown in Figure 6, for all spiked levels down to 0.5% the scrambled disulfide spiked peptide was detected. In addition, in each case the identified peptide was auto-validated based on the underlying MS/MS data. The homo-dimers of each of the peptides were also detected in the same analyses, however they were not auto-validated due to their low intensity.

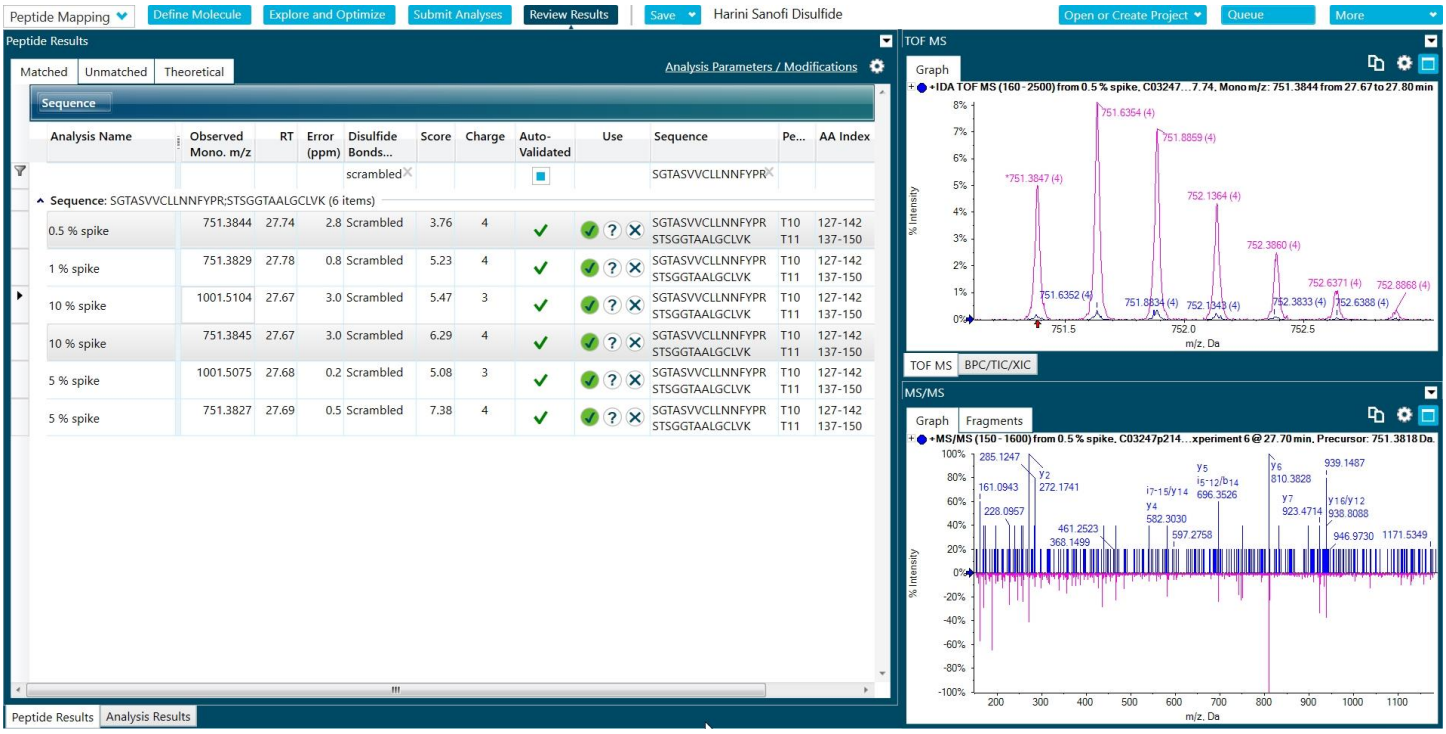

Figure 6. Observed scrambled disulfide events of spiked peptides from 10% to 0.5%. Top right panel shows overlaid MS data for the same charge state at 10% (pink) and 0.5% (blue). Lower right shows a mirror plot of MS/MS spectra for 10% (pink) and 0.5% (blue) spiked levels with good agreement between spectra. Each shown spiked level was auto-validated.

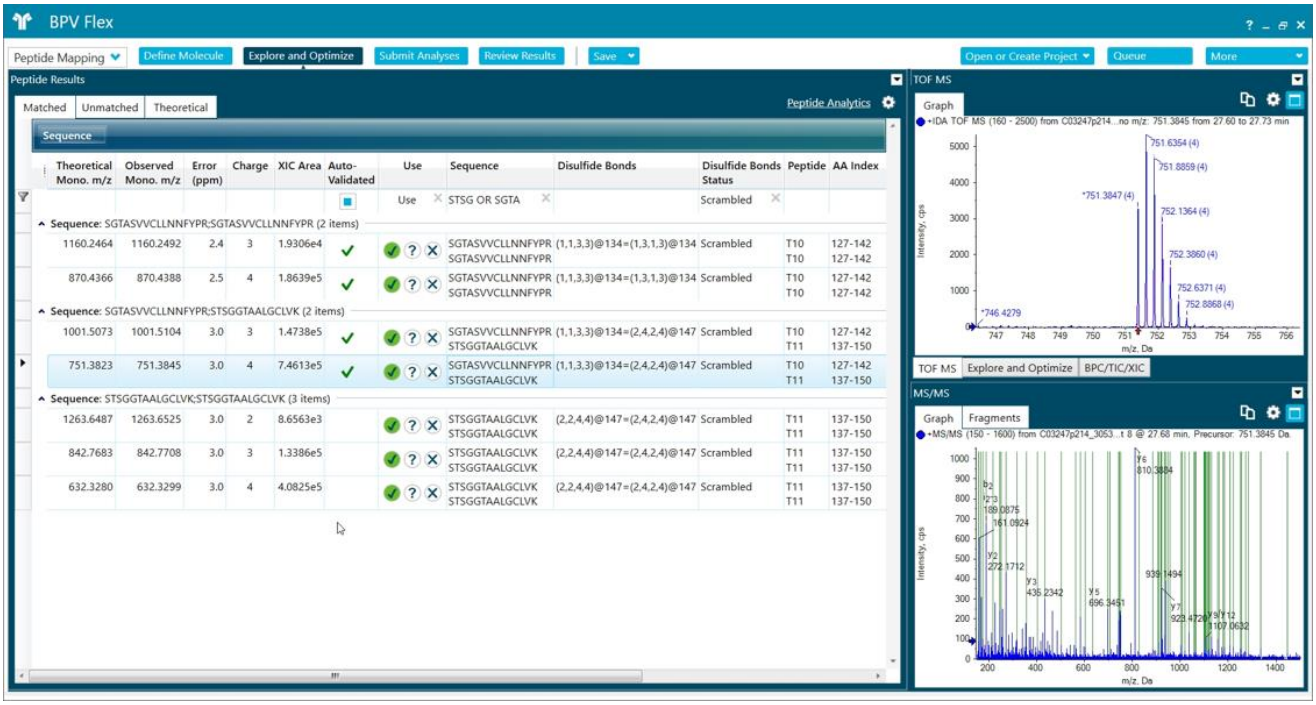

Figure 6. Anticipated scrambled disulfide search. Bonds observed based on spiked synthetic peptides treated to form disulfides.

Conclusions

- Data processing time for scrambled disulfides is rapid without the need to limit search space.
- It is possible to rapidly review results with powerful sorting and filtering capabilities to focus on the expected and scrambled disulfide bonds of interest.
- It is possible to easily view expected and scrambled disulfides using the interactive sequence viewer.
- The peptide auto-validation guides data review for greater confidence in results.
- The highly customizable view allows for streamlined data analysis that fits user needs.

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