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Heteronuclear NMR As a 4-in-1 Analytical Platform for Detecting Modification-Specific Signatures of Therapeutic Insulin Formulations

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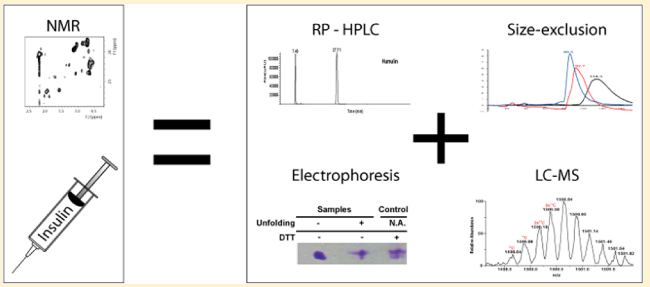
Supporting Information

ABSTRACT: Detecting possible modifications of therapeutic proteins is a critical element of the quality control of protein drugs. Typically, a number of techniques are used to evaluate different modifications of therapeutic protein formulations. Using heteronuclear NMR spectroscopy, we show that the difference between various insulin formulations can be detected "as is" with little pretreatment and quickly. As an application to the quality control of insulin formulations, the NMR approach was compared with four different analytical methods: with reverse phase high pressure liquid chromatography (HPLC) (for mutations), with size exclusion chromatography (for oligomerization), with electrophoresis (for denaturation), and with mass spectrometry (for deamidation). All of the results showed that this single NMR method can provide the specific signatures for each modification and information that is at least equivalent to that offered by the conventional analytical methods. Importantly, NMR could yield information at each amino acid residue level which no other technique provided. The suggested NMR method, then, can be considered to be a facile and effective means of evaluating therapeutic protein formulations in a multifaceted way.



Therapeutic proteins constitute the most important class among biological drugs and include hormones, growth factors, and antibodies. They are increasingly widely used in clinics, as they tend to target specific molecules with fewer side effects than conventional small molecular drugs. The expected share of biological drugs among the top 100 drugs in 2014 is about 50%, compared with 28% in 2008, and 11% in 2000,[1] and research and development efforts in protein therapeutics are increasing. In addition, the patents of the early developed therapeutic protein drugs expired recently or will expire soon, and the so-called biosimilar drugs are of high interest to pharmaceutical companies worldwide.[2,3] However, unlike small molecular drugs, the properties of protein therapeutics can be strongly affected by the manufacturing and even postproduction storage/distribution steps,[4] and the quality assurance of the biosimilar proteins is a challenge to their development.[3] For example, protein denaturation during the manufacturing and storage steps can affect the drug's efficacy.[4]

Currently, a number of analytical techniques are employed to characterize various modifications of therapeutic protein products, as protein molecules have higher order structural features than simple chemical structures. Among the most widely used techniques are SDS-PAGE for basic identity testing, HPLC for mutation, size-exclusion chromatography for oligomer formation, and mass spectrometry for post-translational modifications.[5] However, the use of these different approaches entails different sample handling procedures for each, which in fact can introduce additional variables into quality assurance processes. Also, the different data they produce need to be interpreted as a whole to determine if a particular batch of samples meets the specified quality criteria. Therefore, there is a need for a biophysical approach that can address multiple modifications for therapeutic proteins.

Among the various therapeutic proteins, insulin and related biosimilar drugs are expected to yield the most revenue for the pharmaceutical industry.[6] It can be seen from the sales of therapeutic proteins in 2010, in which year the combined sales for insulin and its analogues were about 15 billion USD compared with 7.2 billion USD for Etanercept, the top-selling protein drug.[7] This might be due to their large patient base relative to other specialized therapeutic proteins, absence of glycosylation, and low production cost. Therefore, many different formulations of biosimilar human insulins are expected in the near future. For industries and regulatory agencies, this necessitates highly efficient analytical techniques to analyze the integrity of each product and standardize the quality control and assurance processes for insulin formulations. Insulin products can serve as good model systems, except for glycosylation, for studies on the applicability of analytical methods for quality control, because their composition and structure can change greatly according to solution conditions.

NMR, particularly the one-dimensional approach, has long been used to study overall folding of proteins, but low resolution and overlap of peaks has limited its general use in the quality control of therapeutic proteins. Very recently, however,

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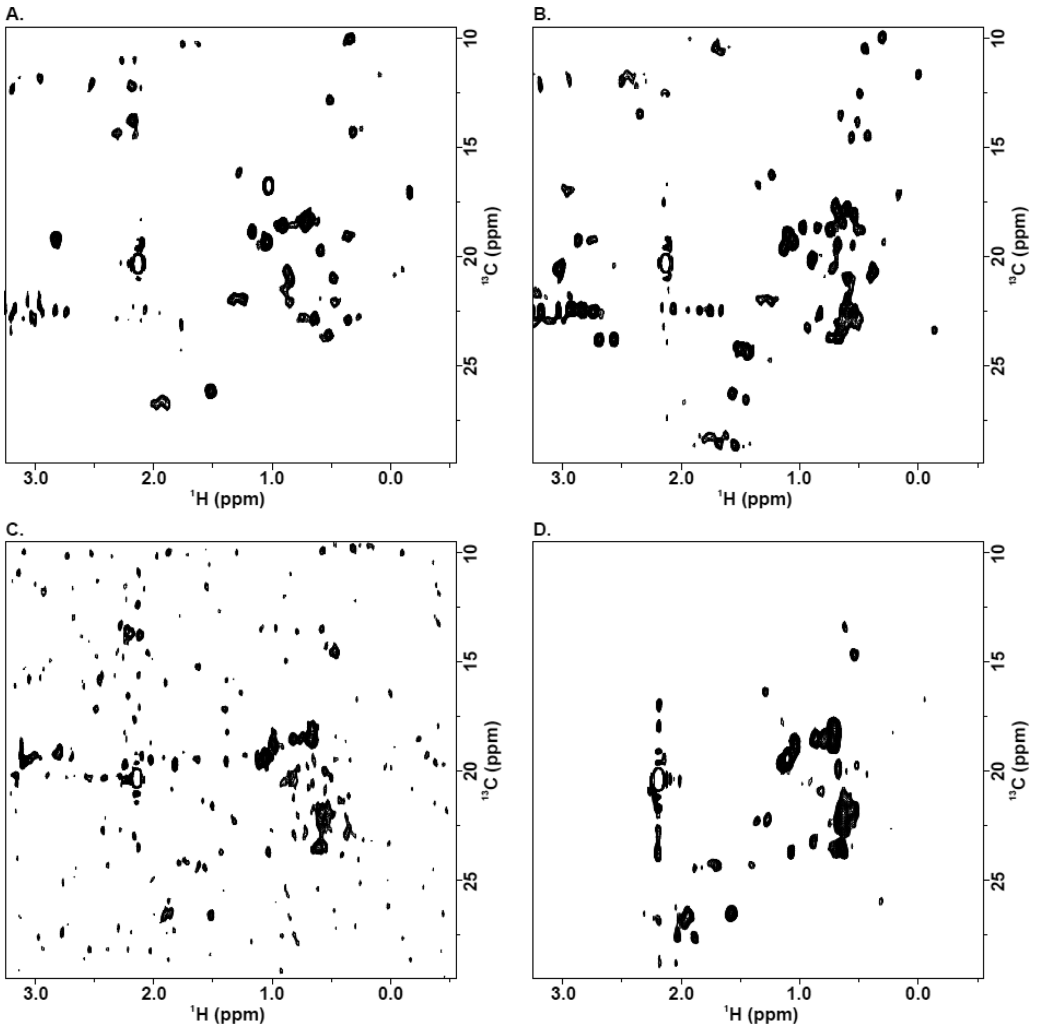

Figure 1. Natural abundance ^{1}H−^{13}C HSQC spectra for various therapeutic insulin products in their native formulation. Commercial products were directly put in the NMR tube with the trivial addition of 10% D_2O and the spectra were obtained "as is". (A) IHUM, (B) IGLAR, and (C) IGLU. The spectra were obtained with a 600 MHz NMR spectrometer with 1 h total acquisition time. The center frequency and the spectral width were optimized for the methyl groups. The spectral contours were normalized with the known concentrations of *meta*-cresol, so that the *meta*-cresol peaks have proportional intensity contours according to the concentrations (IGLAR, 2.7 mg/mL; IGLU, 3.15 mg/mL; IHUM, 2.5 mg/mL). (D) The IGLU spectrum was obtained at 900 MHz NMR spectrometer for 9 h to demonstrate the difference of peak intensities of IGLU.

one-dimensional ^{1}H NMR was demonstrated to provide hydrodynamic profiles of antibody therapeutics.[8] Two-dimensional NMR was also used with either natural abundance[9,10] or isotope labeled proteins[11] to show its relevance to the detection of differences resulting from formulations or mutations. With these developments, there is a need to compare NMR with conventional techniques for several protein drug properties to assess its specific merits in the quality control of therapeutic proteins.

Here, using insulin and its analogues, we demonstrate that the natural abundance two-dimensional NMR method can provide modification-specific information that usually requires multiple analytical techniques. The NMR method could also offer residue-specific information upon each modification, which no other technique can provide. The NMR approach shown here, with its multifaceted features, can be applied for fast and convenient analysis of therapeutic protein drugs.

METHODS

Samples and Specific Modifications. Insulin glulisine (Apidra, Sanofi-Aventis, IGLU), insulin glargine (Lantus, Sanofi-Aventis, IGLAR), and human insulin (Humulin, Eli Lilly and Company, IHUM) were purchased from local distributors. These samples were treated differently to meet the requirements for the particular modifications. For deamidation, each sample was incubated at 45 °C overnight in 50 mM sodium citrate (25 mM Tris, pH 9.0). For partial unfolding, the sample was incubated at 55 °C for 30 min in the presence of DTT (3 mM). Complete unfolding for the control sample was achieved by boiling with 100 mM DTT. Oligomerization was performed as described in the Size Exclusion Chromatography section.

NMR. Two-dimensional ^{1}H−^{13}C heteronuclear single quantum coherence (HSQC) spectra were obtained with a 600 or 900 MHz NMR spectrometer (Bruker Biospin, Germany) equipped with cryogenic probes. Complex data matrices of 1024 (t2) by 64 (t1) were collected for the total acquisition time of 1 h (for IGLU experiments, 12 h). Only the methyl and aliphatic regions were collected for the ^{13}C domain (spectral width of 60 ppm and center frequency of 32 ppm) to increase the digital resolution of the most informative region. The spectra were acquired using Echo/Antiecho-TPPI gradient

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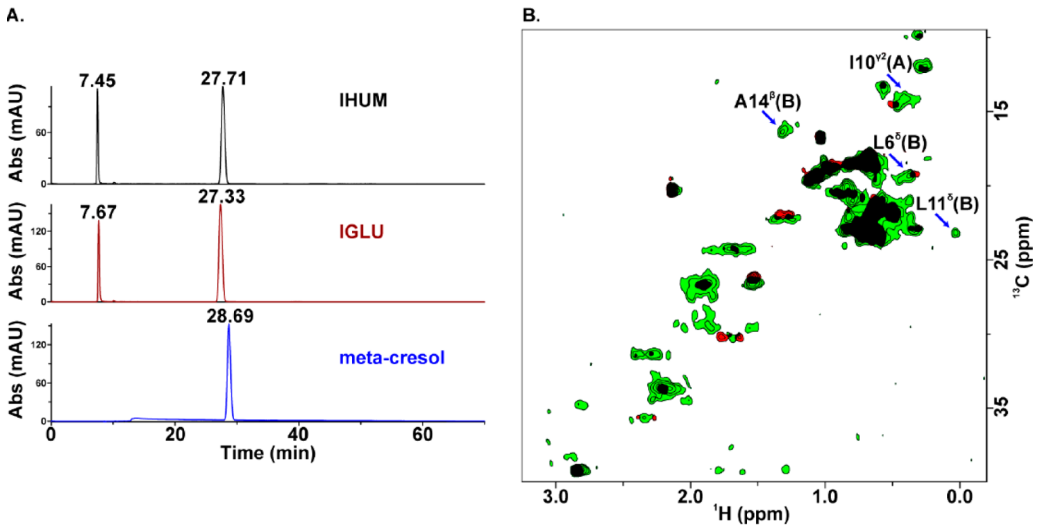

Figure 2. Primary sequence difference (mutation) assessed by reverse phase-HPLC and HSQC spectra. (A) The reverse phase-HPLC was performed as described in the Methods section. The peaks at 7.45 and 7.67 min correspond to the insulin species for IHUM (upper) and IGLU (middle), respectively. The peaks at 27.71 and 27.33 min for IHUM and IGLU, respectively, are from *meta*-cresol, as evidenced by the chromatogram of the *meta*-cresol standard (lower). (B) NMR spectral overlay of IHUM and IGLU. IHUM spectrum is represented in red, IGLU in green, and the overlapping contour in black. The two insulin products were dialyzed in one dialysis bucket (10 mM Tris, pH 7.0), and the spectra were normalized so that *meta*-cresol has the same intensities in the two spectra. The blue arrows indicate the readily identifiable modification-specific changes and the corresponding amino acid residues. The spectra were taken with the 600 MHz spectrometer (12 h) due to the exceptional line broadening of IGLU. The IHUM spectrum could be readily obtained in 1 h but acquired for 12 h for the comparison purpose.

selection for the indirect dimension frequency discrimination. Buffer exchange was performed to remove either the effects of additives in different formulations or the buffer species added to perform the modifications. It should be noted that the buffer-exchange step is unnecessary in actual quality control situations where one particular insulin formulation, hence with identical additives, is tested for modification. For primary sequence difference and deamidation, both control and modified samples were buffer-exchanged with the same buffer (10 mM Tris, pH 7.0). Dialysis was used for the primary sequence difference, and the Zebra spin desalting column (Thermo, MA) was used for the deamidation. For oligomerization, different concentrations of NaCl were achieved by adding small amounts of concentrated stock solutions of NaCl.

Reverse Phase-HPLC (RP-HPLC). IHUM and IGLU were analyzed using a Gemini 3 μm C6-Phenyl column (Phenomenex, CA) connected to an Agilent 1100 series HPLC system (Agilent Technologies, CA). Solvent A contained ammonium sulfate 0.1 M (pH 2.3), and solvent B, acetonitrile. The analysis used a 0.3 mL/min flow rate with isocratic elution of 40% B solvent for 70 min. The peaks were monitored by UV absorbance at 214 nm. The samples were injected (3 μL) after 1/20 dilution with deionized water.

Size Exclusion Chromatography. IHUM was analyzed using a Superdex 16/60 column connected to an AKTA prime Plus FPLC system (GE Healthcare, Uppsala, Sweden). Glycerol was added to deionized water (16 mg/mL, as in the original formulation) which was used as a running solvent. For individual experiments, additional NaCl was added to the running solvent at a final concentration of 20 mM, 75 mM, or 150 mM. The flow rate was 1 mL/min, and the total running time was 150 min. The peaks were detected by absorbance of UV light at 280 nm.

Mass Spectrometry. Samples were injected by syringe pump infusion after 10-fold dilution with methanol. The flow rate was 10 μL/min. The data were obtained in the normal or zoom mode, as needed, with an LTQ-XL linear ion trap mass spectrometer (Thermo, MA). The sheath gas was set to 15 (arbitrary units), the auxiliary gas to 6 (arbitrary units), and the capillary temperature to 275 °C. The following voltage values were used: ion spray, 5 kV; capillary, 5 V; tube lens, 175 V.

Electrophoresis. Original and unfolded samples were analyzed using SDS-PAGE. Samples were prepared by the addition of SDS sample buffer without dithiothreitol (DTT). They were then loaded onto 18% SDS-PAGE gel (8.6 cm × 6.8 cm) and run for 45 min at 200 V.

RESULTS AND DISCUSSION

To test the applicability of the $^{1}H-^{13}C$ HSQC approach to assessment of therapeutic insulin product differences, we compared the NMR spectra of human insulin (IHUM), insulin glargine (IGLAR), and insulin glulisine (IGLU), three widely used insulin formulations (Figure 1). We used the commercial products "as is" (i.e., as obtained on the market) rather than laboratory-prepared or reagent-grade samples, in order to demonstrate the real-life practicability of the NMR approach. The spectra showed that the overall properties of each insulin formulation are very different. Compared with IHUM (Figure 1A), IGLAR (Figure 1B) exhibited about twice as many peaks, which are readily visible in the region for isoleucine methyl groups (^{1}H, 0.0−0.8 ppm and ^{13}C, 10−15 ppm). This suggests that IGLAR has slowly exchanging multiple conformations in its formulation. By contrast, IGLU (Figure 1C) exhibited very broad peaks in its native formulation to the extent that peaks are barely visible. For example, for IGLU, only one isoleucine methyl peak could be observed, compared with 4 (or 5) for IHUM and 8 for IGLAR. The spectrum for the IGLU at a higher field with longer time confirmed that there are actually peaks from IGLU but that they are much smaller than those from IGLAR and IHUM (Figure 1D). Another important feature of our result is that all of the differences could be identified in just 1 h of spectral acquisition with the commercial

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formulation. Therefore, our results showed that NMR could be an effective approach for detection of differences sensitively and quickly.

To demonstrate the utility of the NMR approach for quality control of insulin formulations, we chose four common modifications for therapeutic proteins: primary structure modification (mutation), oligomerization, denaturation, and deamidation. We wanted to see if $^{1}H-^{13}C$ HSQC can detect specific signatures for each of them and can provide information equivalent to that obtained with conventional biophysical modalities.

First, we addressed primary structure modification, for which IHUM and IGLU were compared. IHUM differs from IGLU in primary structure at two positions. In IGLU, asparagine 3 and lysine 29 of the B chain of IHUM are replaced by lysine and glutamate, respectively. Therefore, IGLU can be considered a mutation product of IHUM. HPLC is commonly used to detect mutations in protein drugs, and thus, we performed the HPLC experiment first. The HPLC result (Figure 2A) showed that IHUM and IGLU have very similar retention times and cannot easily be differentiated in this particular setting. It should be noted that the separation condition completely separated an additive, *meta*-cresol, from the insulin species with a retention time difference of ~20 min. In comparison, the changes in the sequence caused noticeable peak changes in the NMR spectra (Figure 2B). For example, the peaks for the methyl groups of A14$^{\beta}$(B) and L11$^{\delta}$(B) disappeared and those of I10$^{\gamma 2}$(A) and L6$^{\delta}$(B) shifted by about 0.1 ppm (Table 1). The results suggest that mutations in the B chain also affect the chemical environments of the amino acids in the A chain. Furthermore, there were noticeable differences in the line-widths. NMR has several advantages over HPLC. NMR data are transferrable between laboratories, whereas HPLC data are not, due to the fact that they can vary widely according to the columns and solution conditions used. In addition, HPLC columns can be contaminated or deteriorate over time, resulting in different retention times, whereas NMR spectrometers typically provide consistent results for more than 20 years.

Table 1. Modification-Specific Signatures of Insulin Products Detected by the NMR Approach

		affected peaks and the corresponding residues		
modifications	insulin formulations	^{1}H (ppm)	^{13}C (ppm)	residues
mutation	Humulin (IHUM) Apidra (IGLU)	0.03	23.18	L11$^{\delta}$(B)[a]
		0.36	19.23	L6$^{\delta}$(B)
		0.42	14.21	I10$^{\gamma 2}$(A)
		1.31	16.37	A14$^{\beta}$(B)
oligomerization	Humulin (IHUM)	−0.02	20.80	L11$^{\delta}$(B)
		0.06	11.66	I2$^{\delta 1}$(A)
		0.27	22.89	L15$^{\delta}$(B)
		0.48	22.11	L15$^{\delta}$(B)
		1.29	16.14	A14$^{\beta}$(B)
denaturation	Lantus (IGLAR)	1.21	16.37	
		1.45	37.24	
		1.46	39.59	
		1.52	23.09	
		1.74	26.77	NA[b]
		1.84	39.54	
		2.63	40.63	
		2.84	35.00	
deamidation	Apidra (IGLU)	0.27	11.94	I2$^{\delta 1}$(A)
		0.34	22.94	L15$^{\delta}$(B)
		0.37	19.36	V12$^{\gamma}$(B)
		0.56	13.52	I2$^{\gamma 2}$(A)

[a]L11$^{\delta}$(B) denotes the delta methyl group of Leucine 11 in the B chain. [b]Not applicable due to the conformational exchange.

For the second modification, we measured the effects of oligomerization status most likely to be affected by protein formulations. Many proteins can have different oligomerization statuses according to solution conditions, and this property is quite difficult to characterize. Still, it is a critical property of any therapeutic protein drug. In fact, insulin oligomerization is a well-known phenomenon, and stringent combinations of salts and buffers are used to meet particular requirements.[12] To mimic different formulations, we added different amounts of NaCl directly to the IHUM solutions. We then analyzed the samples with size exclusion chromatography, a conventional method for characterization of protein oligomerization. The chromatograms of IHUM in 20, 75, and 150 mM NaCl exhibited salt concentration-dependent decreases with elution time. This shows that IHUM tends to oligomerize under higher salt conditions (Figure 3A). These results were compared with $^{1}H-^{13}C$ HSQC to assess the oligomerization analysis by NMR. The NMR data obtained with the same samples showed that the peak intensities decreased according to the salt concentration without significant shifts in the peak positions (Figure 3B). It should be noted that the decrease of the insulin peak intensities was not due simply to the sensitivity of the cryogenic probe to high salt concentrations, because they were normalized with *meta*-cresol, and the *meta*-cresol peak intensities were the same across all of the spectra. In addition, some peaks were more prominent in their peak intensity decrease, as indicated by the arrows (those for L11$^{\delta}$(B), I2$^{\delta 1}$(A), L15$^{\delta}$(B), and A14$^{\beta}$(B), see Table 1). Generally, larger molecular species such as protein oligomeric forms have broader peak line-widths, effectively decreasing both the intensities and the number of detectable peaks.[13] This is due mainly to the more efficient transverse relaxation of nuclear spins in such species. Therefore, the concentration-dependent peak intensity decreases without significant peak shifts can be an oligomerization status-specific signature detected by NMR. Although size exclusion chromatography is widely used for protein oligomerization analysis, care should be taken in the interpretation. The retention time depends not only on the size of the analytes, but also on the shape. It is well-known that an unfolded protein elutes much faster, just like a large protein, in size exclusion chromatography.[14] Therefore, with the size exclusion technique, it is difficult to discriminate the effects of unfolding from oligomerization. In comparison, unfolded proteins often show narrower line-shapes with different peak positions in $^{1}H-^{13}C$ HSQC. This was actually shown, in the present study, with chemical unfolding, where DTT-unfolded insulin exhibited generally narrower line-widths (see Figure 4B). There is another important advantage of NMR over size exclusion chromatography. The latter requires "exactly" the same running buffer as the formulation, which often is impossible to achieve where product batches are different. NMR does not have this requirement, since it analyzes a sample "as is."

For the third modification, we tested if denaturation can also be assessed by NMR. We denatured IGLAR with DTT by reducing the disulfide bonds and monitored the denaturation

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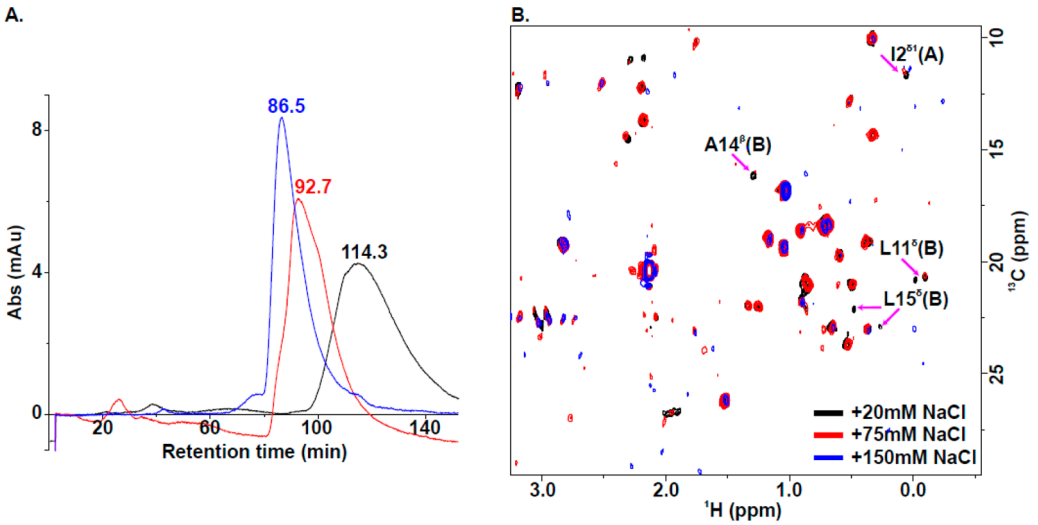

Figure 3. Oligomerization of IHUM assessed by size exclusion chromatography and HSQC spectra. (A) The size exclusion chromatogram of IHUM at three different NaCl concentrations: 20 mM (black), 75 mM (red), and 150 mM (blue). The running solvent contained 16 mg/mL of glycerol as in the original formulation. (B) NMR spectral overlay of IHUM at the same conditions as part A. The spectra were normalized with the *meta*-cresol peaks so that the *meta*-cresol peaks have the same intensity levels. The spectra were taken with a 600 MHz spectrometer (1 h). The arrows indicate the identifiable modification-specific changes and the corresponding amino acid residues.

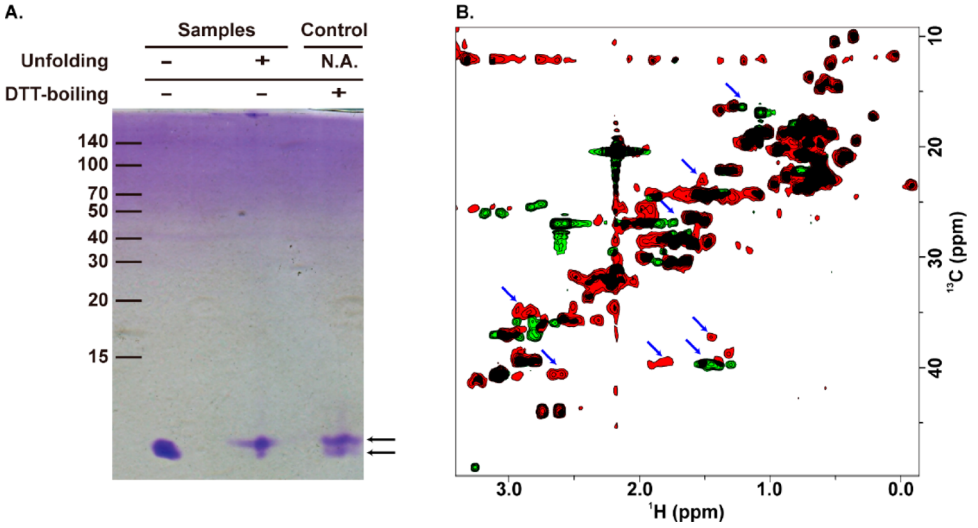

Figure 4. Denaturation by DTT assessed by SDS-PAGE and HSQC spectra. (A) The nonreducing SDS-PAGE gel for the DTT-denatured IGLAR. For the sample lanes, the first lane denotes no addition of DTT and the second lane 3 mM DTT treatment at 55 °C for 30 min. The control lane sample was boiled with 100 mM DTT for 5 min to ensure complete denaturation. Molecular weight standards are indicated on the left side. The two arrows on the right indicate the individual chain separated by the denaturation. (B) The HSQC spectral overlay of the DTT-denatured (green) and nontreated (red) IGLAR samples with the overlapping peaks in black. The spectra were acquired at 900 MHz for 9 h, but equivalent information could be obtained with 1 h acquisition at 600 MHz as shown in Figure 1.

initially with conventional SDS-PAGE (nonreducing gel). IGLAR was selected, as other insulins exhibited undesirable properties under the accelerated denaturation conditions used (3 mM DTT at 55 °C for 30 min). As insulin A and B chains are connected by two disulfide bonds, reduction of these would lead to separation of the individual chains. We observed that unfolding by DTT led to the appearance of a second band, while there was just one band for the untreated sample (Figure 4A). Although the electrophoretic mobility change upon DTT-reduction was much smaller than expected by the separation of the two peptides linked by disulfide bonds, it is sometimes observed that proteins with very small molecular weights (i.e., MW < 10 kDa) may not migrate according to their molecular weight.[15] As a new, weaker-intensity band appeared on the gel, the denaturation seemed to be partially completed. This was confirmed with reference to the control sample, which was completely reduced by boiling with DTT-containing sample buffer (control lane). To determine if even this partial denaturation, which could be more relevant to a real situation, can be detected by NMR, we compared the $^{1}H-^{13}C$ HSQC spectra of the native and DTT-denatured IGLAR. The overall characteristics of the spectra were similar in that the methyl peaks upfield to 0.5 ppm still existed. This indicated that the protein did not undergo complete denaturation but retained the three-dimensional fold, as consistent with the partial denaturation observed with SDS-PAGE analysis. Actually, the partial denaturation is not surprising, considering the high concentration of the disulfide bonds (1.8 mM, 3 times that of

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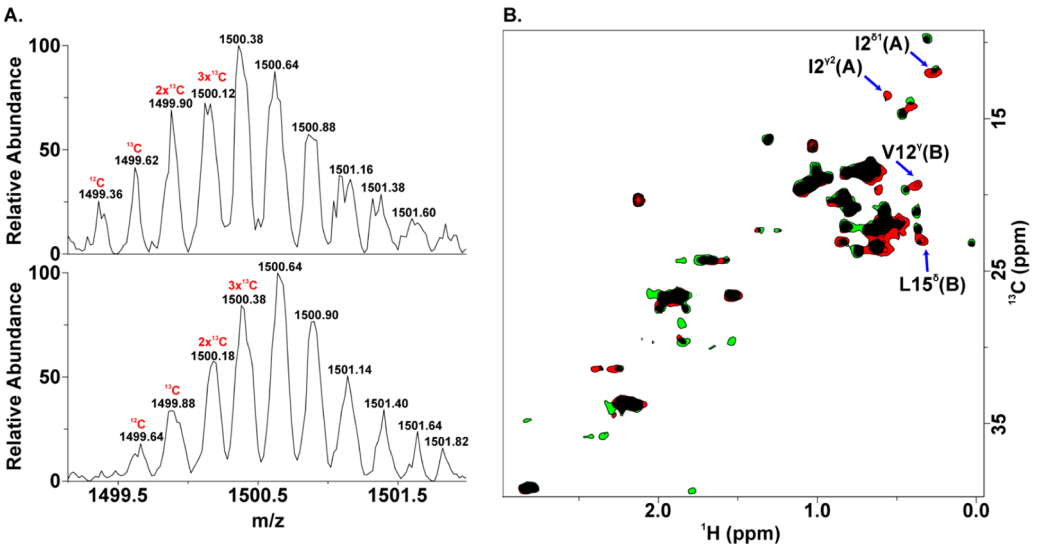

Figure 5. Deamidation assessed by mass spectrometry and HSQC spectra. (A) Mass spectroscopic analysis of deamidation of IGLU. The upper spectrum is for the nontreated sample and the lower for the deamidated sample. The deamidation was achieved by incubating the sample at 45 °C for overnight in 50 mM sodium citrate (25 mM Tris, pH 9.0). The clusters are for the monoisotopic peaks of quadruply charged ions with 8 Na ions ($[M + 8Na]^{4+}$). The ^{13}C compositions of the peaks are shown for the first four peaks. (B) The HSQC spectral overlay of the deamidated (green) and nontreated (red) IGLU samples with the overlapping peaks in black. The arrows indicate the readily identifiable modification-specific changes and the corresponding amino acid residues. Both deamidated and nontreated samples were buffer-exchanged to 10 mM Tris (pH 7.0) using desalting columns. The spectra were acquired at 600 MHz for 12 h due to the exceptional line broadening of IGLU.

the insulin concentration) and the brief reduction condition used (3 mM DTT at 55 °C, 30 min). The retention of the general fold might also have been due to noncovalent interactions, typically the major player in protein folding, which can hold the polypeptide chains together even after reduction. For IGLAR in particular, the presence of multiple conformations, as evidenced by the 8 isoleucine methyl peaks instead of 4 seen in IHUM, strongly suggests that noncovalent interactions are important for IGLAR conformation and folding. Despite the similarity in the methyl region, specific changes were readily visible in the mostly −CH or −CH2 regions, as indicated by the arrows (Figure 4B). Although the identity of the residues for these peaks could not be established, due to the exchanging multiple conformations of IGLAR mentioned above, this group of peaks could serve as the denaturation-specific signature of IGLAR. The chemical shift values of these peaks are listed in Table 1. While we showed the results for DTT-induced denaturation, denaturation can also occur by other conditions, most notably by heat. Heat-induced denaturation cannot be detected by SDS-PAGE but readily by NMR. This should be an important advantage of NMR, as it would be able to determine if a particular batch was exposed to heat during processing or storage.

For the fourth modification, we compared the deamidation results as assessed by the conventional mass spectrometric approach[16] and the NMR method. We prepared the deamidated form of IGLU by incubating it with sodium citrate. We chose IGLU, because other insulins precipitated during this artificial deamidation condition and/or the subsequent buffer-exchange step. The deamidation modification was tested by mass spectrometric analysis of the monoisotopic peaks of quadruply charged ions (Figure 5A). The pattern of the monoisotopic peaks shifted by the deamidation process. The peak with three ^{13}C, for example, had m/z = 1500.12 ($[M + 8Na]^{4+}$, Supporting Information S1) in the control sample, whereas the corresponding peak in the deamidated sample had m/z = 1500.38. The difference in m/z (0.26) multiplied by the number of charges (4) is very close to a mass of 1, indicating an increase of 1 Da in the deamidated sample. This is what would be expected from the conversion of CONH to COOH during the deamidation process. A similar mass spectrometric approach has been used in barstar deamidation analysis.[16] The $^{1}H-^{13}C$ HSQC spectrum could also detect the deamidation, as shown in Figure 5B. Compared with the unmodified sample, the deamidated samples exhibited much weaker intensities for the methyl groups of $I2^{\delta1,\gamma2}$(A) and $V12^{\gamma}$(B) and $L15^{\delta}$(B), which can serve as the deamidation-specific signature for IGLU (see the arrows in Figure 5B and Table 1).

In the present study, we established that the $^{1}H-^{13}C$ HSQC-based NMR approach can detect modification-specific signatures for frequently occurring modifications such as mutation, oligomerization, deamidation, and denaturation. For each of these modifications, we compared the results from conventional approaches with those from the $^{1}H-^{13}C$ HSQC-based NMR approach. Significantly, the NMR also provided the identities of the amino acid residues most affected by the modifications (see Table 1). Therefore, this single approach, advantageously, can be used in lieu of four different techniques. As noted above, this is desirable not only in terms of the data interpretation but also with respect to the reduced number of sample preparation steps. This is especially important in protein quality assurance, as "process is product" in therapeutic protein manufacturing. Another merit of the NMR approach is the ability to analyze the sample "as is" without separation. Other commonly used methods frequently involve separation of protein molecules from other stabilizing additives in the formulation during the actual analysis. This can greatly affect the properties of the analyzed proteins. HPLC and mass spectrometry, for example, can result in the extraction of zinc ions from insulin or interferon alpha-2B, which are required for the formation of particular oligomeric forms in some

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formulations.[17] In comparison, the $^{1}H-^{13}C$ HSQC approach can be straightforwardly applied to the quality assurance of final commercial samples of therapeutic proteins. As requirements of the natural abundance $^{1}H-^{13}C$ HSQC approach, the protein concentration should be high (i.e., >500 μM). In addition, very large proteins, such as whole antibodies, may not be analyzed with $^{1}H-^{13}C$ HSQC at natural abundance, due to the fast transverse relaxation.

Isotope-edited two-dimensional NMR can solve many of the problems of one-dimensional NMR. Still, the two-dimensional NMR suffers from the sensitivity issue due to the low natural abundance of ^{13}C and ^{15}N. One possible solution is isotopic enrichment of proteins using recombinant protein technology, which has been applied to the detection of detailed structural changes in proteins.[11,18] Isotopic enrichment, however, cannot be used for actual protein drugs. With the general availability of highly sensitive cryogenic probes and high-field NMR magnets, it now seems relevant to test the practical applicability of two-dimensional NMR with unlabeled (natural abundance) protein drugs.[19] Actually, ^{13}C-based natural abundance HSQC was recently applied, but mainly to show the similarity between human insulin products from different makers and differences in primary structures.[9] Our main purpose was the actual demonstration of its applicability to the detection of the various modification-specific differences commonly found among insulin products in the manufacturing processes.

CONCLUSIONS

Overall, we showed the utility of $^{1}H-^{13}C$ HSQC-based heteronuclear NMR as a multifaceted, 4-in-1 analytical approach for therapeutic proteins that can provide information at least equivalent to that which otherwise requires four different conventional methods. One obvious advantage of NMR is that it can report the structural status of proteins at individual amino acid level, which, in turn, is critically related to the bioactivities of therapeutic proteins. Another important merit of the NMR approach is the ability to analyze the formulation "as is", without any separation of protein molecules from stabilizing excipients. Although we tested just four common modifications of therapeutic proteins, the method can be straightforwardly extended to address other modifications. If approved by the relevant administrative agencies, our method could augment, or serve as an orthogonal tool for, current biophysical techniques employed in the quality control of therapeutic proteins.

ASSOCIATED CONTENT

Supporting Information

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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