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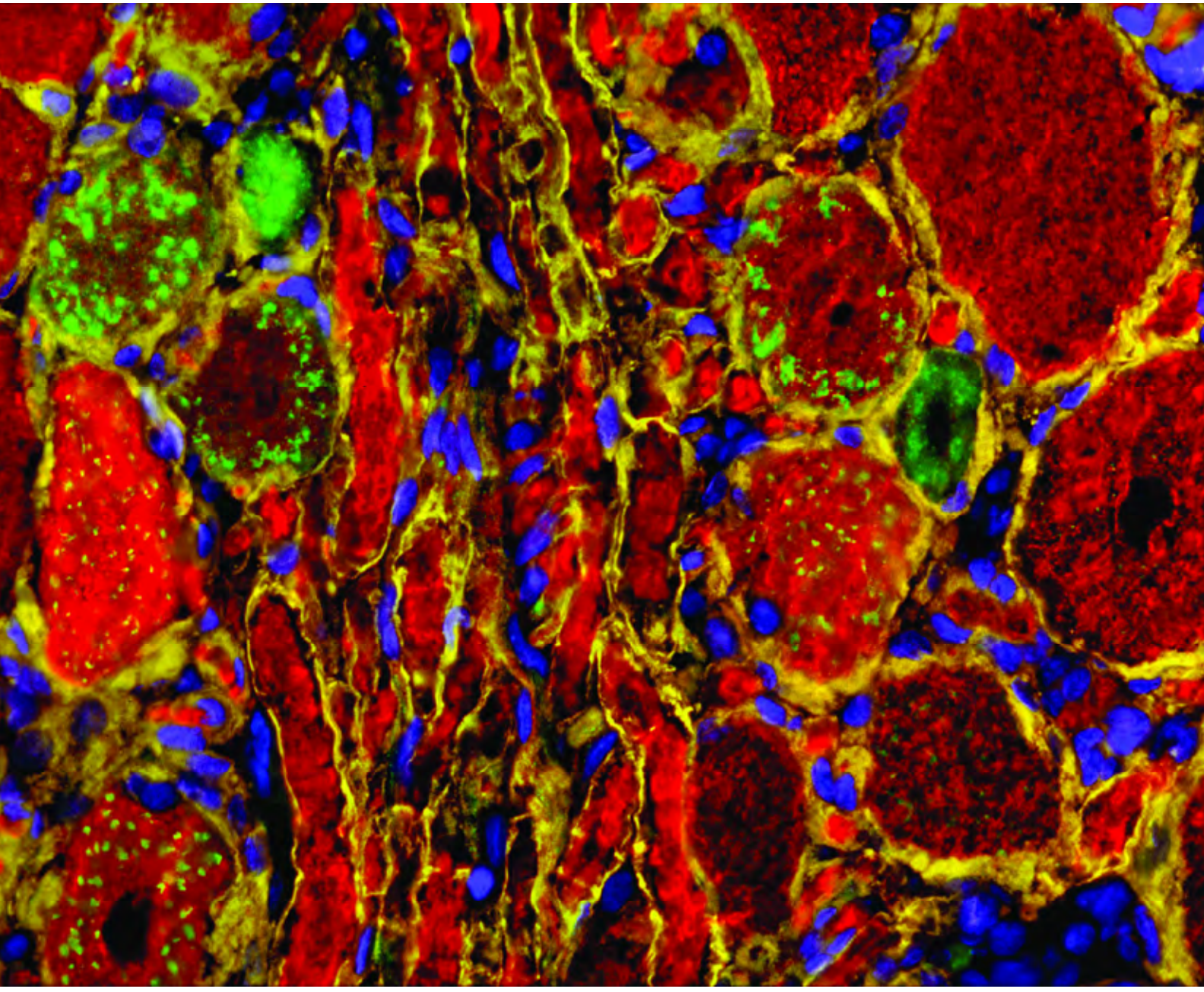

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Structure Characterization of a Recombinant Monoclonal Antibody with One- and Two-Dimensional High Performance Liquid Chromatography with On-line Electrospray Ionization Mass Spectrometry

BY BROOKS R. SUNDAY

Recombinant monoclonal antibodies (rMAbs) are the predominant biotherapeutic protein under development today.[1] FDA requires the structure characterization of rMAbs and other recombinant proteins to grant marketing approval.[2,3] Characterizing such complex, inherently heterogeneous molecules is a significant analytical challenge that requires a broad array of physico-chemical tests. This article reports the use of reversed phase high-performance liquid chromatography (RP-HPLC) with on-line electrospray ionization mass spectrometry (ESI-MS) to rapidly determine the glycoform composition and the heavy chain C-terminal lysine heterogeneity of an intact rMAb. In addition, a novel multidimensional chromatographic platform was developed to investigate the two-dimensional, size exclusion chromatography (HPSEC) separation of the rMAb followed by RP-HPLC (HPSEC-RP-HPLC) with on-line ESI-MS analysis. Such analyses can characterize,identify, and confirm the structure of an intact rMAb.

Recombinant MAbs are complex compounds with a high average molecular weight of ca. 150 kDa, and consist of two heterodimeric disulfide-linked light and heavy chains connected through inter-chain disulfide bridges.[4] They are subject to heavy chain post-translational modifications such as proteolytic clipping of the C-terminal lysine, dehydration of the N-terminal glutamic acid residue, and incomplete oligosaccharide expression.[5] The oligosaccharide structure consists of an asialylated, biantennary, core fucosylated oligosaccharide terminating with galactose on asparagine of the heavy chain.[6,7] A heterogeneous population of oligosaccharides arises from the presence or absence of galactose residues at the oligosaccharide termini to form three distinct glycoforms: G_0 (agalacto), G_1 (monogalacto), and G_2 (digalacto) — 0, 1, or 2 galactose residues, respectively. The C-terminal heavy chain lysine heterogeneity and the oligosaccharide microheterogeneity combine to

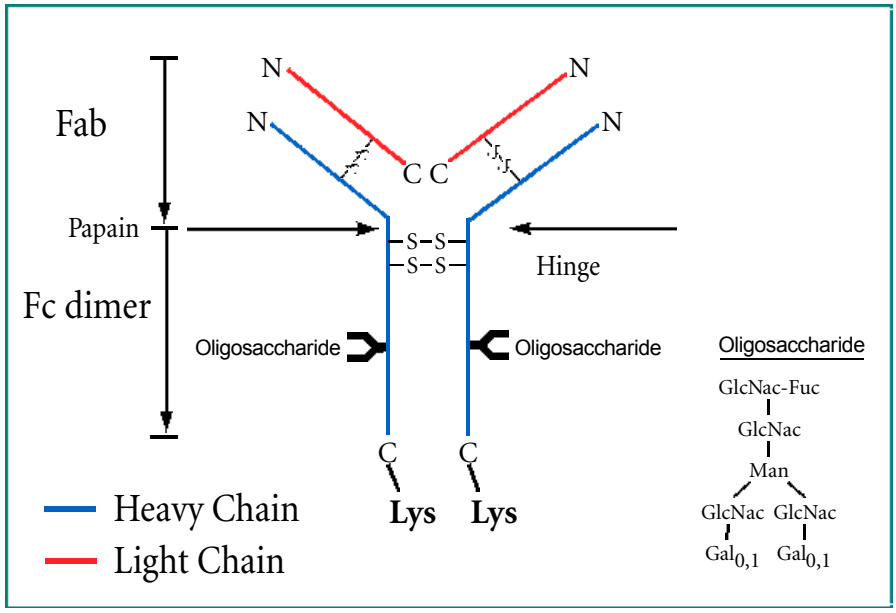

Figure 1.The basic heterodimeric structure of an IgG4 MAb shows the light chains (red), the heavy chains (blue), the site of papain cleavage at the disulfide bridge, and the oligosaccharide microheterogeneity at asparagine 297. The heavy chain may terminate with or without lysine and the asialylated biantennary oligosaccharide chains may terminate with and without galactose (Gal).

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form unique mass isoforms that can be differentiated by mass spectrometry (Fig. 1). The rMAb's glycoform composition may affect its biological activity.[6–12]

Biomolecular mass analysis with mass spectrometry is essential to the structure characterization of rMAb biotherapeutics. Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) and ESI-MS have been used to elucidate the structure of recombinant DNA-derived biotherapeutics.[13–16] However, the high molecular weight, complex structure, and large number of potential mass isoforms present significant challenges to the mass spectrometric analysis of rMAbs. Therefore, to determine rMAbs' structure through mass analysis,they are typically converted to smaller fragments such as heavy chain, Fc, Fc/2 fragments, and peptides.[17–30]

Although MALDI-TOF MS lacks sufficient resolution to identify the intact rMAb glycoform profile, it can report the average molecular mass (M_r).[17–19,31] Early ESI-MS analyses of intact rMAbs by infusion and RP-HPLC-ESI-MS also reported the average M_r.[18–21,32] However, like MALDI-TOF MS, the resolution and/or limited mass range of the mass spectrometer prevented determination of the glycoform profile. Subsequently, the glycoform profiles of intact rMAbs were analyzed via infusion and flow injection analysis.[17,31] Infusion of an intact rMAb has been reported as a quality control release test in which five glycoforms were detected following treatment with carboxypeptidase B to remove C-terminal lysine heterogeneity.[33] An early ESI-TOF mass analysis also reported an average M_r of an intact rMAb with a mass accuracy of ±100 Da without glycoform characterization while a more recent ESI-TOF study determined glycoform heterogeneity.[34,35]

Multidimensional chromatography (MDC) is generally defined for HPLC as the automated separation of complex compounds using sequential and orthogonal modes of chromatography designed to achieve efficient separations. Samain reviewed early MDC applications in biotechnology that included two-dimensional HPSEC-RP-HPLC separations.[36] Numerous reviews of MDC have since been published.[37–43] The two-dimensional separation and analysis of an intact rMAb with HPSEC followed by immunoaffinity separation has also been reported.[44]

We report the application of an on-line RP-HPLC-ESI-MS method that can be used as a one dimensional method or combined with HPSEC in a two-dimensional separation for the analysis and structure characterization of an intact rMAb. The RP-HPLC-ESI-MS methods can be applied to compare experimental lots of the rMAb from different cell culture and purification processes, to confirm identity, and to determine lot-to-lot consistency.

Tools & Techniques

Mass Spectrometry

A Perkin-Elmer SCIEX API 165 single quadrupole ESI-MS (Concord, Ontario, Canada) was used for mass analysis. The mass scan range was 30–3,000 Da. The mass spectrometer was initially equipped with an Ion Spray™ source. Subsequently, a Turbo Ion Spray™ source was installed to increase sensitivity by using heated nitrogen nebulizer gas to increase ion formation and the number of ions entering the mass analyzer.

The mass spectrometer was tuned for maximum mass-to-charge (m/z) resolution using the manufacturer's polypropylene glycol (PPG) standard kit before analysis of the rMAb. Mass analysis was performed in the positive ion mode. The ESI-MS source and Qo RF quadrupole filter were cleaned regularly to ensure optimal m/z signal intensity and accuracy. The step size was 0.2 amu and the dwell time was 1.0 m/second for all analyses reported.

The Ion Spray mass spectrometer scan range was 1,400–2,300 amu with an orifice voltage of 50 volts. The scan time was 4.5 seconds per scan for the intact rMAb. The Turbo Ion Spray scan range was 1,500–2,300 amu (4 second scan time) for analysis of the intact rMAb. The nitrogen nebulizer gas was heated to 120° C with an orifice voltage of 40 volts. A scan range of 400–2,400 amu (10 second scan time) was used for analysis of the reduced and alkylated rMAb heavy chain. The orifice voltage was 50 volts.

Multidimensional Chromatography

A Waters Alliance 2690 HPLC system (Milford, MA) equipped with sample cooler (5° C), column heater, and 996 photodiode array (PDA) detector was used for the chromatographic separations. The column eluant entering the mass spectrometer was split 3:1 with the majority directed to waste or to a fraction collector.

The Alliance 2690 chromatograph was uniquely configured with three Rheodyne LabPro column selectors (Rheodyne, Cotati, CA) to provide a multidimensional chromatographic platform for structure characterization. Two 6-column selectors were used before the PDA detector to select the HPSEC column for the first dimension analysis or to select the RP-HPLC column for the second dimension analysis. A 3-column selector, used after the PDA detector, directed the column effluent to the mass spectrometer or to waste.

The Alliance 2690 chromatograph and Waters Millennium 32™ client/server software can control one column selector

Table 1: RP-HPLC-ESI-MS M_r Glycoform Determination of Intact rMAb with C-terminal Lysine Truncation.

Lot No.	agalacto (G0)	monogalacto (G1)	digalacto (G2)
cDNA Mass	*146451*	*146775*	*147099*
1	146466	146785	147101
2	146465	146790	ND
3	146476	146797	147095
4	146458	146774	147106
5	146466	146801	147116

Note: M_r for five lots of the rMAb is reported in which the C-terminal lysines are truncated. The RP-HPLC mobile phase modifier was 0.1% TFA. The ESI-MS was configured with the Ion Spray source. Lot 5 is the control. ND = not detected.

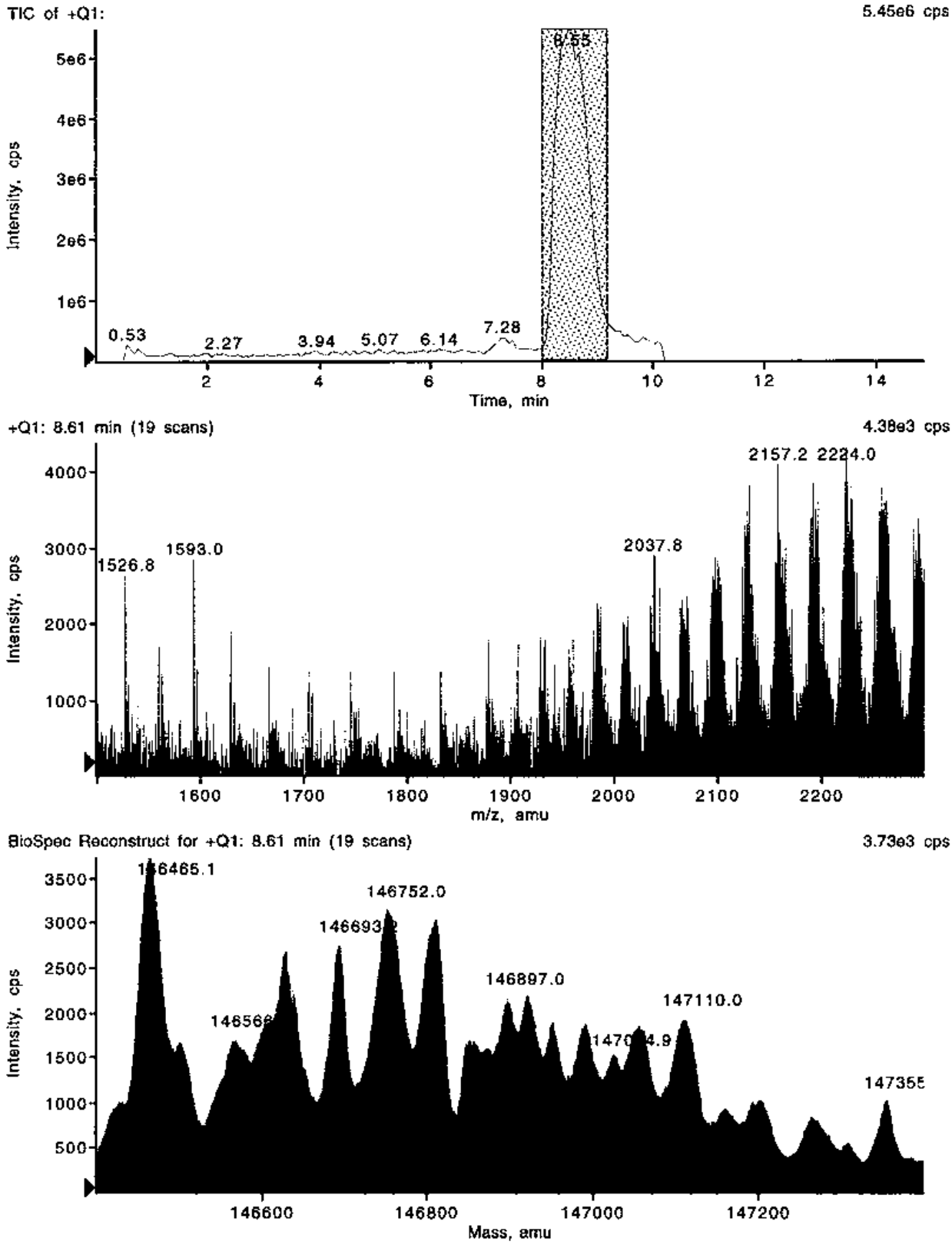

Figure 2.The RP-HPLC-ESI-MS analysis of intact rMAb is presented using formic acid as the RP-HPLC mobile phase modifier. The upper panel shows the ESI-MS TIC. The middle panel shows the ESI-MS mass spectrum. The lower panel shows the deconvoluted ESI-MS mass spectrum of the intact rMAb with the eight glycoforms detected. See Table 2 for the M_r glycoform mass assignments.

Table 2: RP-HPLC-ESI-MS M_r Glycoform Determination.

	0-Lysines			1-Lysine			2-Lysines		
cDNA Mass	146451 (G0)	146775 (G1)	147099 (G2)	146579 (G0)	146903 (G1)	147228 (G2)	146707 (G0)	147031 (G1)	147356 (G2)
1	146465	146752	147110	146566	146897	NF	146693	147024	147354
2	146463	146794	147116	NF	NF	NF	146702	147040	147369
3	146465	146769	147116	NF	146921	147224	146721	147041	NF
4	146466	146775	147119	NF	NF	147232	146711	147027	147361
5	146478	146787	147115	146548	146906	147256	NF	NF	NF
MDC	*146445*	*146777*	*147095*	*146586*	*146903*	*NF*	*146722*	*147019*	*147361*

Note: M_r for five lots of the rMAb is reported using 0.25% formic acid as the RP-HPLC mobile phase modifier. The ESI-MS was configured with a Turbo Ion Spray source. The cDNA-derived mass for G0, G1 and G2 is shown under each of the three C-terminal lysine heavy chain modifications. Lot 5 is the control. NF = not found.MDC reports M_r determined with multidimensional chromatographic analysis of the rMAb.

with its internal switches. The PDA's two contact closures were used to sequentially control the first 6-column selector and the 3-column selector. The second 6-column selector was controlled by the chromatograph's TTL switch to rapidly and non-sequentially select the RP-HPLC column to capture pure components eluting from the HPSEC column for subsequent analysis. A Waters SAT/N module was installed to power the third column selector.

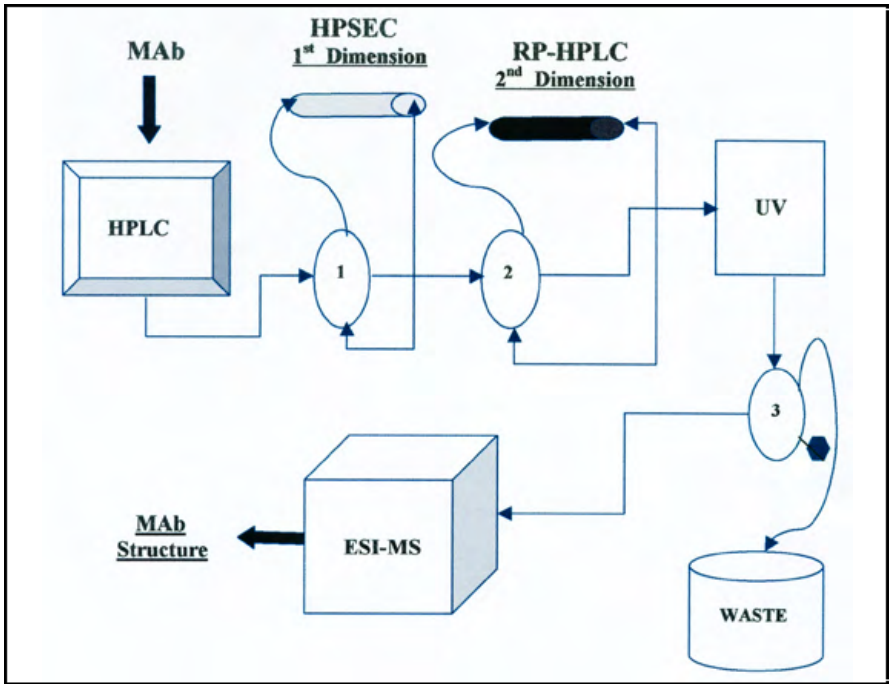

Figure 3.The HPSEC and RP-HPLC two-dimensional separation is depicted with the Alliance HPLC system and the ESI-MS mass spectrometer. HPSEC is used as the first dimension followed by RP-HPLC as the second dimension which is combined on-line with ESI-MS to provide structure analysis of the intact rMAb.

RP-HPLC

Mobile phase A (0.1% Triflouroacetic acid (TFA), Sequanal grade) (Pierce, Rockford, IL), Milli-Q water (Millipore, Bedford,MA), and mobile phase B (90% acetonitrile, Optima grade) (Fisher, Fairlawn, NJ), and 0.1% TFA Milli-Q water were used with the Ion Spray source. A five-minute, 45–55% gradient was used with a Waters Symmetry C-18 column (2.1 x 150 mm, 300 Angstrom, d_p 5 µm) at 45° C to introduce the rMAb into the Ion Spray source for mass analysis.

Mobile phase A (0.25% formic acid) (Sigma, St. Louis, MO), Milli-Q water, and mobile phase B (100% acetonitrile, 0.25% formic acid) were used with the Turbo Ion Spray source. A Poros R2/20 RP-HPLC column (2,000 Angstrom, 2.1 x 30 mm) (PerSeptive BioSystems, Inc., Framingham,MA), was used for the RP-HPLC separation of the intact rMAb at 60° C. A Poros R2/10 RP-HPLC column (1,000 Angstrom, 2.1 x 30 mm) at 60° C was used for the RP-HPLC separation of the reduced and alkylated rMAb. A gradient of 25–50% mobile phase B over 15 minutes was used for the intact rMAb and for the reduced and alkylated rMAb.

A 25–65% aqueous, acetonitrile, 0.25% formic acid gradient over 20 minutes (including an initial five minute hold to remove salts) was used for the RP-HPLC assay in the multidimensional HPSEC-RP-HPLC-ESI-MS analysis of the intact rMAb. All RP-HPLC separations used linear gradients at 0.2 ml/minute,monitored at 214 nm.

Size Exclusion Chromatography (HPSEC)

A Toso Haas G3000SW SEC column (7.5 x 300 mm) (Phenomenex, Torrance, CA), at ambient temperature with a flow rate of 0.5 ml/minute was used for the SEC separations of the rMAb and aggregates. UV detection was at 214 nm. The mobile phase was 0.1M Na_2HPO_4 (Fisher), with pH 7.0, and contained 0.1M NaCl (Fisher) in Milli-Q water.

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***Reductive Alkylation of intact rMAb*[45]**

100 µL (ca. 2 mg) of rMAb was diluted with 500 µL (0.1M) Tris Base (Fisher), 2mM EDTA (Aldrich, Milwaukee, WI), and 6M Sequanal-grade guanidine hydrochloride (Pierce) at pH 8.4 then treated with 10 µL of 1.0M DTT (Sigma) at 65° C for 1.5 hours. The reduced rMAb was cooled to room temperature, alkylated with 50 µL of 0.5M iodoacetamide (Sigma) in the dark with vortexing for 45 minutes, and quenched with 30 µL of 1.0M DTT.

Results

RP-HPLC-ESI-MS analysis of the rMAb control (lot 5) was first investigated with aqueous acetonitrile (0.1% TFA) gradients. The ESI-MS was configured with the Ion Spray source to determine glycoform composition and lot-to-lot consistency.

The acetonitrile RP-HPLC gradient, combined with the ESI-MS analysis of the rMAb control,showed populations of multiple masses indicating heterogeneity. The predominant observed microheterogeneity corresponded to the major glycoforms that result from truncation of the heavy chain C-terminal lysine (G_0, G_1, and G_2). Glycoforms containing the heavy chain C-terminal lysine were not detected nor was the aglycosylated rMAb at 143561 Da. The rMAb peptide map also lacked the corresponding aglycosylated peptide fragment (data not shown) further indicating complete oligosaccharide site occupancy at the heavy chain asparagine.

The accuracy of an ESI mass spectrometer is 0.01% for pure compounds infused into the ESI-MS. Thus, the expected M_r for a rMAb with a calculated molecular mass of 150 kDa would be 150 kDa ±15 Da. The M_r of the rMAbs in this study were therefore capable of distinguishing the glycoforms present, all of which differed by the addition or deletion of a galactose residue with a mass of 162 Da and/or a lysine residue with a mass of 128 Da. However, the mass accuracy is insufficient to determine deamidation of an asparagine with a mass shift of 1 Da. The dehydration of the heavy chain N-terminal glutamic acid and methionine oxidation with mass shifts of 18 Da and 16 Da is potentially within the capabilities of the ESI-MS. However, those mass shifts are near the limit of mass accuracy for the 150 kDa rMAb and are difficult to identify with certainty.

The RP-ESI-MS analysis was used to compare rMAb lots derived from different experimental cell culture and purification processes for product identity and for lot-to-lot consistency. M_rs for the intact rMAb glycoforms detected in the control and the experimental lots of the rMAb were compared to their cDNA-derived masses. The glycoforms present in all lots were $G_0 \geq G_1 > G_2$ with truncation of the heavy chain C-terminal lysine. The cDNA-derived masses of the isoforms and M_r for the intact rMAb resulting from the combined glycoform microheterogeneity and the C-terminal lysine heterogeneity are shown in Table 1.

The deconvoluted mass spectral peak intensity of each glycoform is assumed to derive from the positively charged rMAb structural framework and to be comparable to each other because the rMAb glycans are asialylated and lack charged groups.[23,25] The digalacto glycoform showed the greatest variability in the experimental lots. It was not observed in lot 2.

Thus, this RP-HPLC-ESI-MS investigation successfully determined profiles of the three major glycoforms present in an intact rMAb of mass ca. 150 kDa and was able to compare experimental lots. In addition, RP-HPLC-ESI-MS analysis determined that the rMAb glycoform profile consisted exclusively of the C-terminal lysine truncated heavy chain. However, RP-HPLC-ESI-MS analysis of tryptic digests revealed the presence of two C-terminal heavy chain peptide fragments in which the predominant peptide fragment terminated without lysine while the less-abundant peptide fragment terminated with lysine (data not shown). Therefore, the RP-HPLC-ESI-MS analysis of the intact rMAb lacked the sensitivity required to detect the less abundant C-terminal heavy chain lysine glycoforms.

The ESI mass spectrometer sensitivity is typically low for these large molecular weight biomolecules. This is principally due to limited protonation and nebulization of the ionized species generated,dilution of signal strength resulting from the large number of mass-to-charge (m/z) fragments generated, chemical noise, and the limited number of scans attainable with the RP-HPLC peaks used to introduce the rMAb sample into the ESI-MS source.

The Turbo Ion Spray source was installed on the API 165 mass spectrometer to increase nebulization of the rMAb ion cluster to enhance ESI-MS sensitivity in an attempt to detect the less-abundant heavy chain C-terminal lysine glycoforms. In addition, the RP-HPLC mobile phase modifier was changed from TFA to formic acid to further enhance ion formation and sensitivity because TFA can suppress the ionization of proteins. Huber showed that a 35 to 160-fold improvement in RP-HPLC-ESI-MS detection of proteins with masses of 14–80 kDa can be achieved with the use of formic acid versus TFA.[46]

ESI-MS mass analysis with formic acid as the RP-HPLC mobile phase modifier successfully detected the three major C-terminal heavy chain truncated lysine glycoforms and the three less-abundant glycoforms in which the C-terminal heavy chain terminated with lysine. The glycoform profile was generally observed as: $G_0 > G_1 > G_2$ (Fig. 2).

Recombinant MAbs in which only one of the two C-terminal heavy chains terminated with lysine were also detected

Table 3: Calculated and Experimental (M_r) Masses (Da) of rMAb Alkylated Heavy Chain Glycoforms.

	0-Lysine		1-Lysine	
Glycoform	cDNA Mass	M_r	cDNA Mass	M_r
agalacto (G_0)	50574	50581	50702	50706
monogalacto (G_1)	50737	50733	50865	50866
digalacto (G_2)	50899	50899	51027	51025
aglycosyl	48502	NF	48630	NF

Note: Alkylation of the 11 sulfhydryl groups with iodoacetamide (-CH_2CONH_2, 57 Da) adds mass of 627 to the heavy chain. NF = not found.

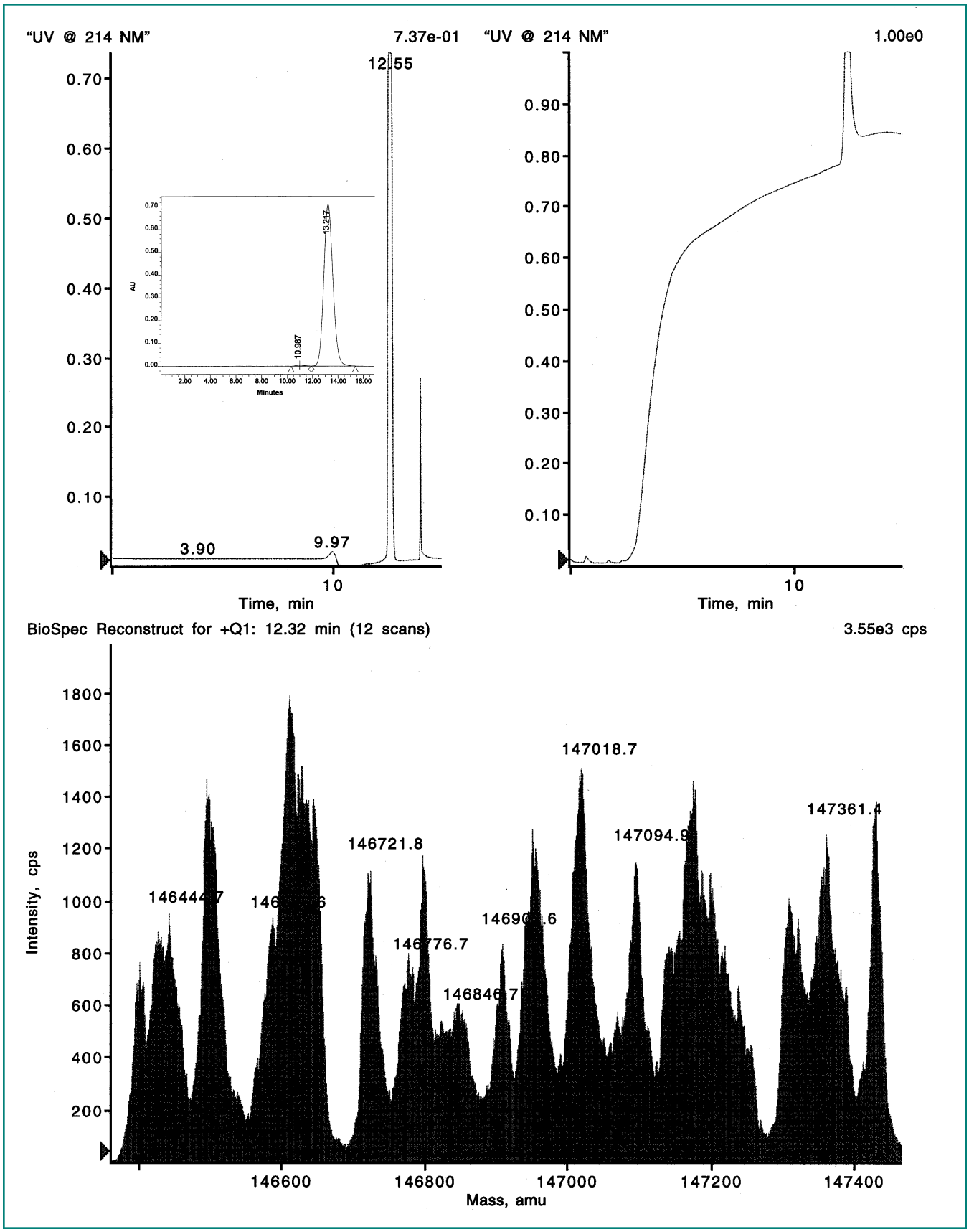

Figure 4. The multidimensional HPSEC-RP-HPLC-ESI-MS analysis of the rMAb is shown. The upper left panel represents the HPSEC UV plot in which the UV signal is lost during the switch to the RP-HPLC column and capture of the rMAb. The two observed peaks are due to refractive index changes in the UV. The inset shows the HPSEC separation without RP-HPLC capture. The upper right panel shows the UV plot of the captured rMAb eluted from the RP-HPLC column. The lower panel is the deconvoluted mass spectrum for the intact rMAb showing the nine glycoforms detected. See Table 2 for the M_r glycoform mass assignments. M_r 146847 corresponds to an isoform with two C-terminal Lysine heavy chains and a single galactose residue (cDNA derived mass = 146869).

in some experimental lots. An intact rMAb expressed with lysine on only one heavy chain can be detected by ESI-MS analysis of the intact rMAb or the Fc fragment derived from papain treatment.[25] The ability to detect those rMAbs in which only one C-terminal heavy chain terminates with lysine is lost when heavy chain rMAb fragments are mass analyzed. Thus, ESI-MS analysis of an intact rMAb can provide more information than analysis of rMAb fragments. A further advantage of analysis of the intact rMAb is that it does not require sample preparation.

The M_r accuracy of the glycoforms reported in Table 2 ranges from less than 0.01% to ca. 0.021% of the cDNA-derived mass. This compares favorably with the theoretical mass accuracy of 0.01% for an ESI-MS system achieved with the infusion of pure compounds. The mass accuracy reported for the RP-HPLC-ESI-MS analysis of the rMAb is a result of the complex m/z envelope, the number of ions formed, ion adduction, the limited number of scans that can be achieved as the eluting rMAb RP-HPLC peak passes through the mass scan range, and the resolution of the mass spectrometer.[17–20] However, careful control of the mass spectrometer and RP-HPLC conditions, and tuning the ESI-MS for maximum resolution can accurately identify C-terminal lysine heterogeneity and the glycoform ensemble in intact rMAbs.

The results of this RP-HPLC-ESI-MS analysis of the intact rMAb were subjected to confirmation by analysis of the smaller mass heavy chain (50 kDA) which is more amenable to accurate mass determination than the larger intact rMAb (150 kDA). The three truncated lysine glycoforms and three less abundant lysine glycoforms were observed in the RP-HPLC analysis of the heavy chain (Table 3). Therefore, the RP-HPLC-ESI-MS determination of lysine and glycoform heterogeneity in the intact rMAb was confirmed to be consistent with the RP-HPLC-ESI-MS analysis of the reduced and alkylated rMAb heavy chain.

MDC performance is dependent on the ability of the HPLC system to use multiple, often incompatible, mobile phases and to control two or more column selectors. MDC separations have been reported using commercially available systems and with chromatographs modified with the addition of column selectors.[46–58]

A Waters Alliance 2690 chromatograph with a 996 PDA detector was combined with three Rheodyne LabPro column selectors under the control of Waters Millennium 32 chromatography software to provide a novel MDC platform to combine with ESI-MS for rMAB characterization. The RP-HPLC-ESI-MS analysis was combined with high-performance size exclusion chromatography (HPSEC) to investigate the feasibility of the two-dimensional chromatographic separation of the rMAb with ESI-MS analysis. The rMAb was separated from its aggregates by HPSEC in the first dimension and captured by the RP-HPLC column beginning at 12.2 minutes with capture ending at 13.8 minutes. The HPSEC separation was terminated after 15 minutes. The RP-HPLC elution of the RP-HPLC captured rMAb began immediately upon completion of the HPSEC separation. Each two-dimensional analysis required 60 minutes for completion.

The two-dimensional HPSEC-RP-HPLC-ESI-MS mass determination of the rMAb glycoforms is shown in Figure 4. The three major C-terminal heavy chain truncated lysine glycoforms and the three less abundant C-terminal lysine glycoforms were determined in this multidimensional HPSEC-RP-HPLC-ESI-MS mass analysis (Table 2). Recombinant MAbs containing 1-lysine were also detected with the one-dimensional RP-HPLC-ESI-MS analysis of the rMAb reported above. A rMAb in which both heavy chains terminate with lysine and in which only one galactose residue is attached to one of the two heavy chain oligosaccharides was also detected in the MDC analysis of the rMAb (M_r 146847, cDNA-derived mass 146869). Additional studies are being conducted to optimize this multidimensional separation for the analysis of rMAbs. Thus, it has been demonstrated that it is feasible to employ two-dimensional HPSEC-RP-HPLC-ESI-MS for the structure characterization of rMAbs.

Summary

This study demonstrates that on-line RP-HPLC-ESI-MS can rapidly identify the mass of intact rMAbs, determine heavy chain C-terminal lysine amino acid heterogeneity, and profile the glycoform composition resulting from oligosaccharide variability. Eight of 15 possible isoforms of the intact rMAb — the three major C-terminal heavy chain truncated lysine glycol-forms, the three less-abundant C-terminal heavy chain lysine glycoforms, and glycoforms in which one of the two C-terminal heavy chain lysines has been proteolytically clipped — were determined with RP-HPLC combined with on-line ESI-MS analysis.

The feasibility of a MDC approach that incorporates HPSEC as the first dimension separation on-line with RP-HPLC-ESI-MS has been demonstrated for the first time. In addition, this is the first report of a Waters Alliance 2690 chromatograph configured to perform MDC and combined with ESI-MS for the structure characterization of a rMAb.

These on-line RP-HPLC-ESI-MS analyses may be used to characterize, identify, and confirm the structure of intact rMAbs for product control and to monitor product quality during development.

ACKNOWLEDGEMENTS

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