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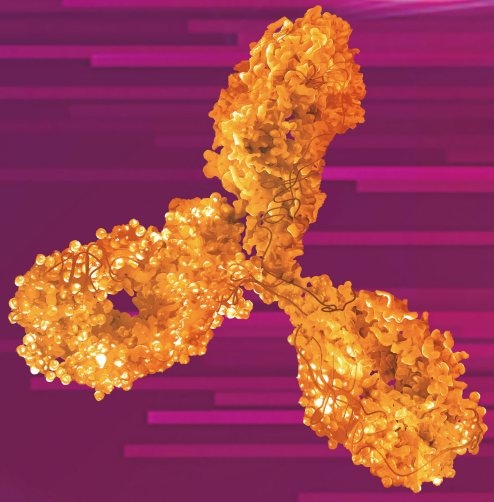
Orbitrap 高分辨质谱在抗体偶联药物质量分析中的应用



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前言

抗体偶联药物（ADC）是近年来针对肿瘤治疗增长最快的药物种类之一，经过近半个世纪的研究，已有 4 种商品化的 ADC 药物，其中 FDA 分别在 2011 和 2013 年批准 Brentuximab Vedotin 和 Trastuzumab Emtansine 上市，大大促进 ADC 药物的研发数量 [1]。与抗体药物相比，ADC 的设计更加复杂，需综合考虑抗体、连接子（linker）、小分子药物三个组成成分。ADC 药物质量控制策略基于对关键原料的质量评价（单抗、连接子、小分子药物等）、对终产品关键质量属性的理解、对工艺认识的积累，结合风险评估手段综合指定 [2]，简而言之 ADC 药物质量要求有表征、规格和质量控制策略。针对 ADC 药物质量研究指导方针有 ICH Q5B, ICH Q5D, ICH Q5A, ICH Q6B, USA(Points to Consider in the Manufacture and Testing of Monoclonal Antibody products for Human Use, February 1997)，主要分为生物学表征(如 ADCC, CDC 功能) 和物理化学性质表征（如 N、C 端异质性，氨基酸修饰，聚体，碎片，糖基化，二硫键，氨基酸突变等）。其中高分辨质谱是物理化学表征中的重要工具，根据样品的类型选择适合的分析方法。

根据抗体偶联药物的偶联方式可分为非靶向共价偶联（如赖氨酸的氨基偶联）、半胱氨酸巯基偶联（重链间靠非共价键结合）、定点氨基酸共价偶联、N- 糖基化偶联。利用高分辨质谱仪可对 ADC 药物每一成分进行充分表征如图 1，将 LC-MS/MS 的方法分为完整分子量测定（确证分子量、DAR 值）、亚基分子量测定、肽图解析（确证氨基酸序列、氨基酸突变、翻译后修饰定性和定量、二硫键连接、药物偶联位点的鉴定）、糖型分析、游离小分子药物定量、宿主细胞残留蛋白定性和定量、药物体内稳定性的监测、药物代谢分析、蛋白质空间结构、表位分析（氢氘交换质谱解析）。本应用文集针对以上应用做全面展示，服务于每位分析人员。

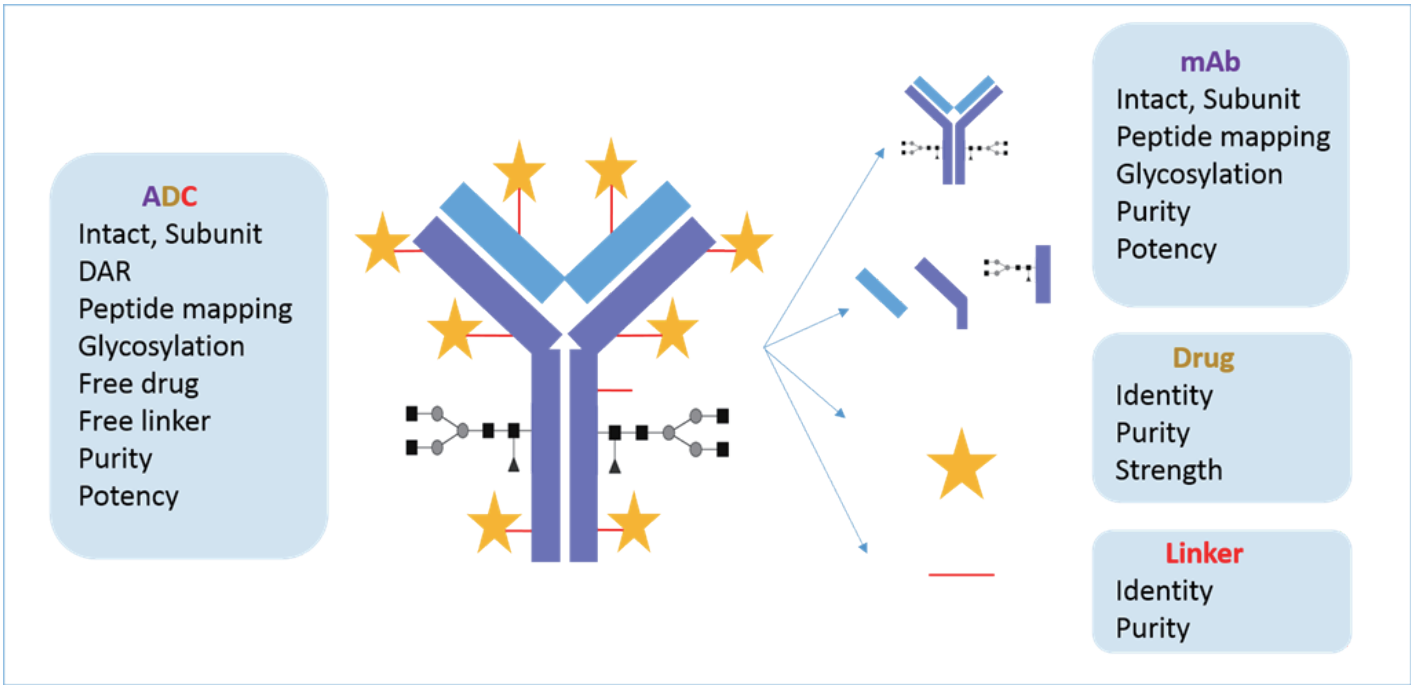


图 1. ADC 药物的成分表征

完整分子量测定：基于 Orbitrap 超高分辨率，高灵敏度，高质量精度的性能，在蛋白质完整分子量层面，确认其分子量、DAR 值分布、定性和定量其组成成分，如对抗体混合物、抗体的降解产物、抗体的不同修饰状态（糖基化修饰，小分子药物偶联修饰等）、非共价相互作用和抗体 - 抗原间的相互作用进行初步鉴定和定量 [4]。

完整分子量 LC-MS/MS 的测定方法可分为变性质谱和非变性质谱（native MS）方法。变性质谱法指蛋白质在进入质谱前其空间结构（如氢键、非共价键）已被破坏，如蛋白质缓冲液、液相条件（流动相为 0.1%FA-ACN，柱温 70 度），此外蛋白质在酸性流动相中结合更多的氢质子，电喷雾离子化时带上更多的电荷并产生更多的电荷态，分子量 150KDa 左右的蛋白质其质荷比的分布位于 m/z 2000-4000，电荷数分布为 30 到 70 价。

在蛋白质变性条件下解析其分子量，有三种样品前处理方法供选择，原则是禁止不挥发性和腐蚀性的盐成分进入质谱中。

1. 质谱级 0.1%FA-H2O 将样品稀释成 1mg/mL，经色谱分离后通过质谱前端六通阀进行脱盐，此方法适用于样品中不含有去垢剂且没有加和离子出现。

2. 离线使用凝胶色谱小柱（Micro Bio-Spin™ P-6 Gel Columns, Tris Buffer）或超滤管（10 KD or 30 KD MWCO Centrifugal Filters, Amicon® Ultra-0.5 mL）对样品进行脱盐后经过液相分离进行质谱鉴定，此方法可去除去垢剂和其他盐成分，避免与样品加和，影响分子量测定。

3. 离线使用凝胶色谱小柱（Micro Bio-Spin™ P-6 Gel Columns, Tris Buffer）或超滤管（10 KD or 30 KD MWCO Centrifugal Filters, Amicon® Ultra-0.5 mL）对样品进行脱盐后，使用含有 0.1%FA 的 50%ACN-H2O 稀释后，经注射器直接进入质谱分析。

非变性质谱（native MS）法指在整个分析过程中，蛋白质始终维持其三级或四级结构，即蛋白质在非变性状态下被分析，因此非共价键、氢键被完整保留，除适用于重链间靠非共价连接的抗体药物成分定性以及定量或半定量外，同样适用于蛋白质结构生物学研究，如蛋白质复合物的结构学、拓扑学，非共价的蛋白低聚和多聚体，蛋白抗体 - 抗原相互结合来评估结构特性、动态变化和相互作用的强弱 [3]。流动相为乙酸铵体系，在 ESI 离子化条件下电荷数和电荷态较变性条件下低，150KD 的单抗在此条件下其电荷数为 23 到 27 价，大大降低样品中不同成分、不同电荷态之间信号的重叠 [4][5]。

以商品化异质性程度较高的 TDM-1 样品为例，且未脱糖情况下，分别在变性和非变性的 LC-MS/MS 条件下进行分析，详细实验方法如图 2A 和 2B，从原始谱图 2C 可看出变性条件下谱图的复杂程度远远的高于非变性状态，但依旧能够获得高信噪比的原始数据，经 BioPharma Finder 软件去卷积后分子量、DAR 值及相对含量分布如图 2D、2E。

LC Condition	
Column: Thermo MAbPac RP (5 um; 2.1mm x 5 cm)	
Mobile Phase A: 0.1% Formic acid in water ; B: 0.1% Formic acid in Acetonitrile	
Flow Rate: 0.3mL/min	
Column Temperature: 70°C	
Gradient:	Time B% 0 20 2 20 6 50 7 90 8 90 8.1 20 10 20

MS Condition_Tune File Parameters for Ion Max Source with HESI-II Probe Setting	
Sheath Gas	30
Auxiliary Gas	10
Probe Heater Temperature	200°C
Source Voltage	3.8kV
Capillary Temperature	300°C
S-Lens RF Voltage	60
Mode	Stand Mode
Trapping Gas Pressure	1.0 arbitrary units
Method File_Full MS Parameters	
Full MS Mass Range	*m/z* 2000–4000
Resolution Setting	17,500 (FWHM at *m/z* 200)
Microscans	10
AGC Target Value	3e6
Max Injection Time	100ms
In-source CID	80eV

图 2A. TDM-1 在变性条件下液相和质谱条件

LC Condition	
Column: Thermo MAbPac SEC-1(5um; 4mm x 15 cm)	
Mobile Phase: 50 mM ammonium acetate in water	
Flow Rate: 0.3mL/min	
Column Temperature: 30°C	
Isocratic Elution: 100% mobile phase A for 10 min	Time A% 0 100 10 100

MS Condition_Tune File Parameters for Ion Max Source with HESI-II Probe Setting	QE Plus with Biopharma Finder
Sheath Gas	30
Auxiliary Gas	10
Probe Heater Temperature	2000C
Source Voltage	3.8kV
Capillary Temperature	3000C
S-Lens RF Voltage	200
Mode	High Mass Range Mode
Trapping Gas Pressure	1.5 arbitrary units
Method File_Full MS Parameters	
Full MS Mass Range	m/z 2000–8000
Resolution Setting	70,000 (FWHM at m/z 200)
Microscans	10
AGC Target Value	3e6
Max Injection Time	100ms
In-source CID	80eV

图 2B. TDM-1 在非变性条件下液相和质谱条件

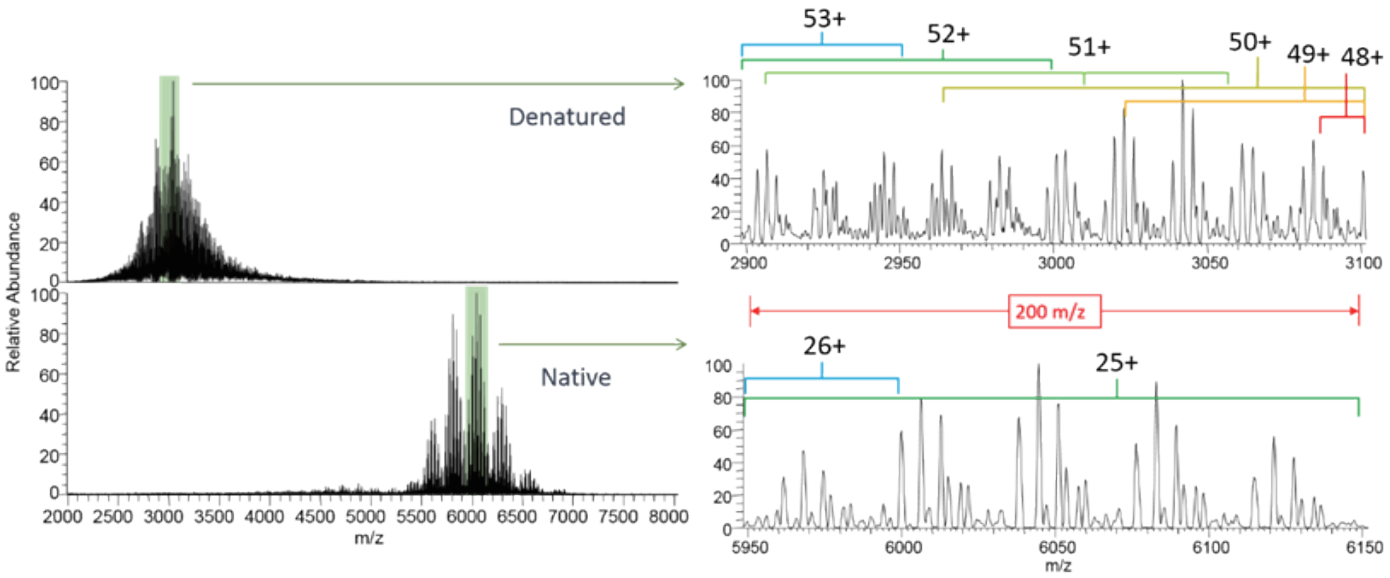


图 2C. 未脱糖的 TDM-1 在变性和非变性条件下分子量测定的原始谱图

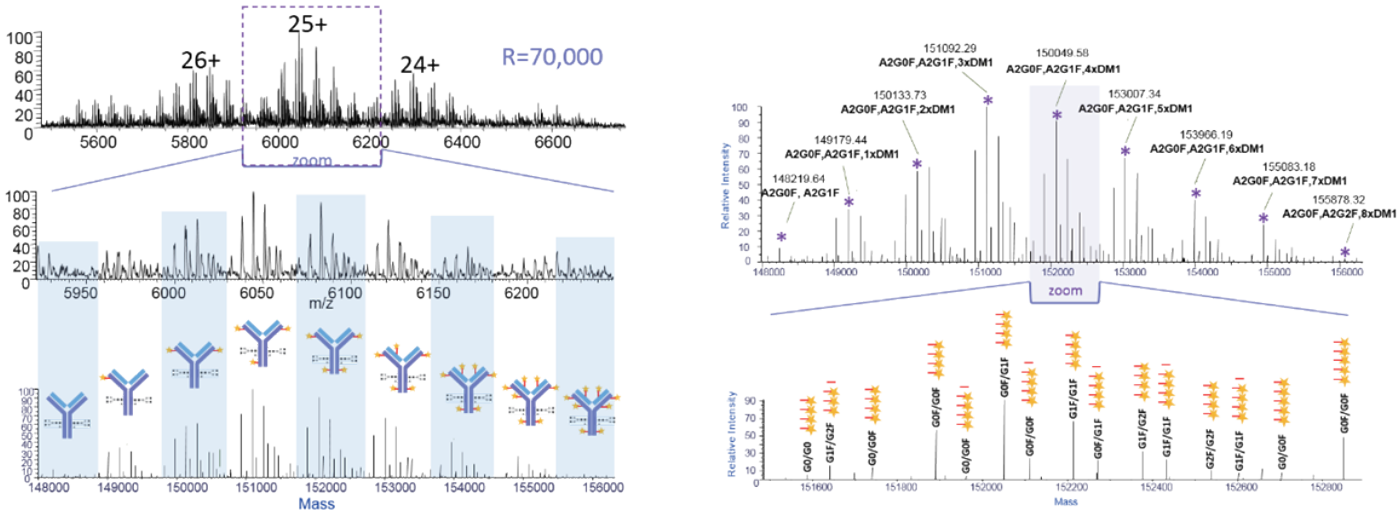
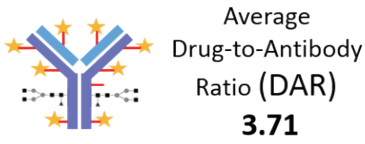


图 2D. 原始数据经 BioPharma Finder 软件去卷积后分子量的分布



* G0F/G1F DAR	Mass Accuracy (ppm)	Relative Abundance
DAR0	6.49	9.19
DAR1	21.69	34.26
DAR2	0.05	59.03
DAR3	6.81	100.00
DAR4	5.17	91.16
DAR5	6.69	67.42
DAR6	15.20	40.46
DAR7	6.28	24.28
DAR8	3.78	3.84

图 2E. 经 BioPharma Finder 软件计算 TDM-1 的 DAR 值分布

肽图解析：通过肽图解析，确证氨基酸序列、翻译后修饰定性和定量、二硫键连接、药物偶联位点。

样品前处理流程包括蛋白质变性、还原、酶解。常见问题有漏切、非特异性酶切和修饰的引入，为避免以上问题需对样品前处理步骤进行优化。常用的变性条件有 6-7.5M 盐酸胍 +DTT 室温 30min（注：胍与 Asp189 形成氢键，阻碍酶解）；6-8M 尿素 +DTT 室温 1h（注：尿素加热生成异氰酸，长时间酶解后与自由氨基反应出现 +43Da 氨甲酰化修饰）；商品化的 RapiGest 是兼容蛋白酶的变性剂。蛋白还原常用 DTT 或 TCEP，浓度为 10-50mM 取决于样品性质，还原温度取决于变性剂的类型；烷基化试剂为 IAA 或 IAM，加入量是还原剂的 2 倍；烷基化后再使用 DTT 终止烷基化。酶解条件优化的目标是确保酶解完全，不引入其他修饰 (deamidation, N-terminal cycization) ，耗时最短。影响酶解完全因素有酶解缓冲液（PH，种类），酶解时间，酶用量，温度。常见的酶解缓冲液有 Tris-HCl（pH=7.5, 0.1M）、NH4HCO3（0.1M，pH=8.0），但 NH4HCO3 更易引起脱酰胺化尤其对于 NG 位点，无论在哪种酶解体系中，随着酶解时间的延长，NG 位点的脱酰胺化比例都会增加，由于 37 度条件下 NH4HCO3 体系的 pH 随时间延长而增大，因此脱酰胺化比例的增加较为明显 [6]。同时酶解时间的延长导致焦谷氨酸环化、天冬氨酸异构化比例增加。胰蛋白酶的常用量为 1:30（胰蛋白酶：抗体）37℃酶切 4h。另一种快速酶解的试剂盒 SMART Digest（ThermoFisher）相比于传统的酶解方法具有操作简单（1h 即可完成抗体酶解）、酶解效率高、重现性好等优点，适用于大规模的样品制备。

多肽的鉴定原理为实际采集到的碎片离子谱图与预测的多肽碎片离子谱图（未修饰、修饰）进行匹配的过程，提取离子流色谱图用于肽段的相对定量 [7]， BioPharma Finder 软件针对肽段鉴定和定量流程如图 3。

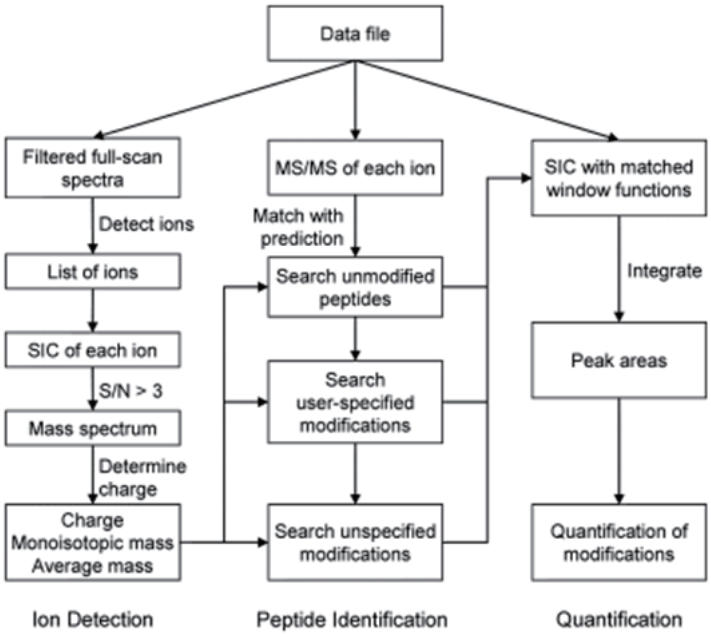


图 3. BioPharma Finder 软件针对多肽鉴定和定量的原理图

如对 TDM-1 样品进行肽图分析，使用双蒸水溶解后 SMART Digest 70℃酶解 1h（如图 4 SMART Digest 酶解试剂盒操作流程），随后使用 DTT+IAM 进行还原烷基化处理，酶解后肽段终浓度为 0.1μg/μL。LC-MS/MS 实验条件如图 4A，由于样品中含有不挥发性盐，因此将梯度的前 5 分钟通过质谱前端六通阀切到废液中。数据经过 BioPharma Finder 软件解析，结果如图 4B，TDM-1 氨基酸序列覆盖度为 100%，其中共有 44 个 Lysine，加上 N 端修饰，共有 46 个可能的修饰位点，当采用 Trypsin 一种酶切时，在含有药物偶联的 K 处发生漏切，在 Q Exactive 系列质谱仪的 HCD 碎裂模式下共鉴定到 30 个高可信度的药物偶联位点，其一级质量偏差小于 1ppm（如图 4C），且含有丰富的肽段碎片和药物特征性碎片离子（m/z 547.2211, 1010.5232, 853.4493），由于药物非对映异构体的性质，因此偶联药物的肽段在色谱图中出现两个色谱峰如图 4D。利用化学反应的方式将抗体，linker，药物偶联，因此存在只连接 linker 的肽段和裸肽形式（如图 4E）。

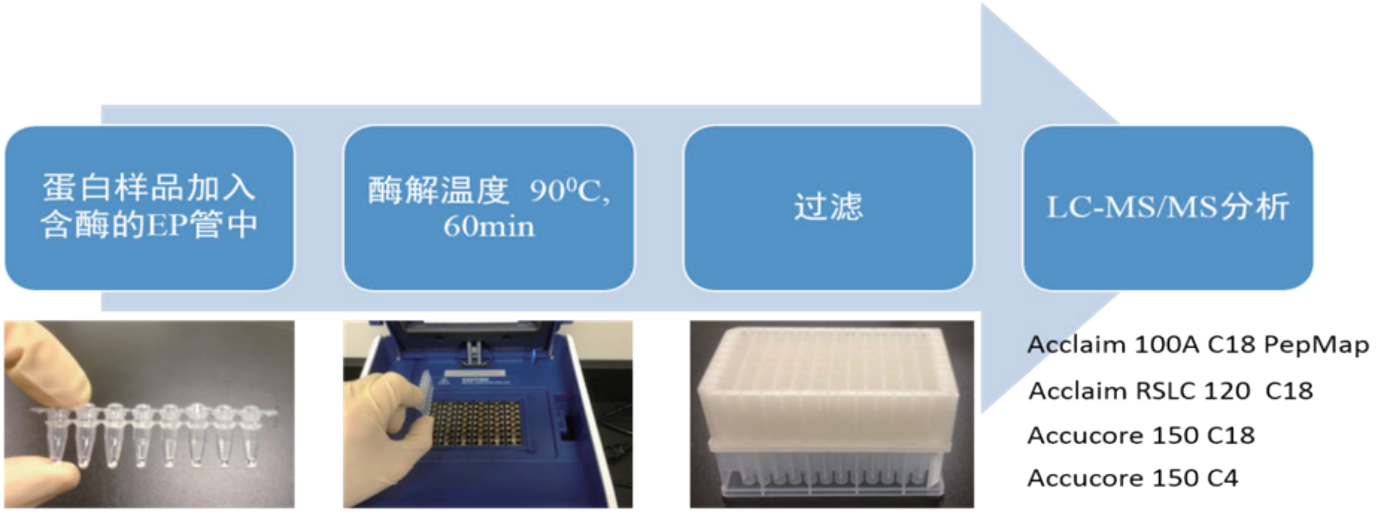


图 4. Thermo SMART Digest 酶解试剂盒操作流程

LC Condition	
Column: Acclaim RSLC 120 C18 (2.1 × 150 mm)	
Mobile Phase A: 0.1% Formic acid in water ; B: 0.1% Formic acid in Acetonitrile	
Flow Rate: 0.25mL/min	
Column Temperature: 45°C	
Gradient:	Time B% 0 2 5 2 43 35 53 80 55 80 58 2 60 2

MS Condition_Tune File Parameters for Ion Max Source with HESI-II Probe Setting	Q Exactive系列质谱仪
Sheath Gas	30
Auxiliary Gas	10
Probe Heater Temperature	200°C
Source Voltage	3.8kV
Capillary Temperature	300°C
S-Lens RF Voltage	50
Mode	Stand Mode
Trapping Gas Pressure	1.0 arbitrary units
Method File_Full MS Parameters	
Full MS Mass Range	m/z 200–2000
Resolution Setting	70,000 (FWHM at m/z 200)
Microscans	1
AGC Target Value	3e6
Max Injection Time	50ms
In-source CID	0eV
Default Charge State	2
MS/MS Parameters	
Resolution	17,500 (FWHM at m/z 200)
AGC Target Value	1e5
Max Injection Time	200ms
Loop Count	10
Isolation Window	1.8
Collision Energy （NCE）	27%
Intensity Threshold	2.0e4
Charge Exclusion	Unassigned， >8
Dynamic Exclusion	10 s

图 4A. 肽图分析时液相和质谱条件

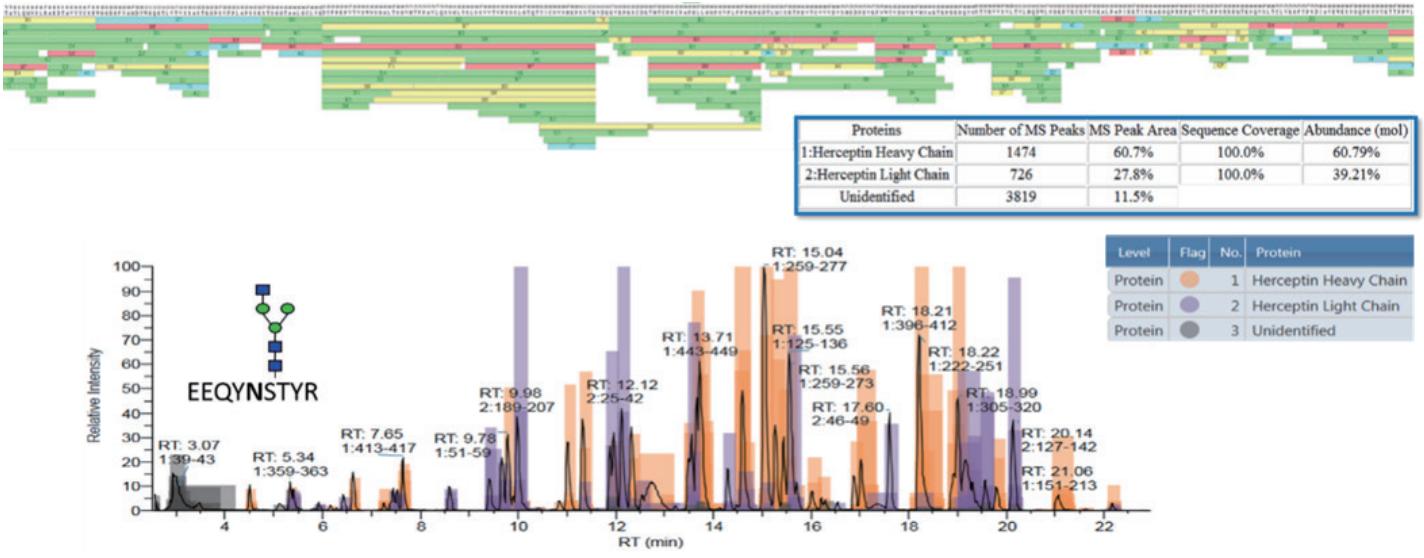


图 4B. TDM-1 氨基酸序列覆盖度为 100%

Peptide Sequence	Modification	Protein	Site	Delta Mass (ppm)	RT
ASQDVNTAVAWYQQKPGK	DM1	Light Chain	K39	-0.25	67.47
PGKAPK	DM1	Light Chain	K42	-0.31	63.21
VEIKR	DM1	Light Chain	K107	-0.46	65.96
EAKVQWK	DM1	Light Chain	K145	0.09	67.23
VQWKVDNALQSGNSQESVTEQDSK	DM1	Light Chain	K149	0.09	68.57
VDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSK	DM1	Light Chain	K169	0.2	68.77
DSTYSLSSTLTLSKADYEK	DM1	Light Chain	K183	1.07	73.01
ADYEKHK	DM1	Light Chain	K188	-0.77	59.14
HKVYACEVTHQGLSSPVTK	DM1	Light Chain	K190	0.01	58.54
VYACEVTHQGLSSPVTKSFNR	DM1	Light Chain	K207	0.15	64.92
LSCAASGFNIKDTYIHWVR	DM1	Heavy Chain	K30	-2.37	73.12
QAPGKGLEWVAR	DM1	Heavy Chain	K43	-0.62	71.01
YADSVKGR	DM1	Heavy Chain	K65	-0.67	65.35
FTISADTSKNTAYLQMNSLR	DM1	Heavy Chain	K76	0.39	70.82
VDKK	DM1	Heavy Chain	K216	-0.74	63.99
KVEPK	DM1	Heavy Chain	K217	0.46	63.38
SCDKTHTCPPCPAPELLGGPSVFLFPPKPK	DM1	Heavy Chain	K225	-1.61	72.91
THTCPPCPAPELLGGPSVFLFPPKPK	DM1	Heavy Chain	K249	-0.4	73.5
PKDTLMISR	DM1	Heavy Chain	K251	0.01	70.58
TPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAK	DM1	Heavy Chain	K277	-0.05	71.84
FNWYVDGVEVHNAKTK	DM1	Heavy Chain	K291	0.26	67.17
TKPR	DM1	Heavy Chain	K293	0.19	64.16
EYKCK	DM1	Heavy Chain	K323	-0.04	64.44
CKVSNK	DM1	Heavy Chain	K325	-0.47	62.93
VSNKALPAPIEK	DM1	Heavy Chain	K329	-0.38	68.18
ALPAPIEKTISK	DM1	Heavy Chain	K337	-0.66	71.07
TISKAK	DM1	Heavy Chain	K341	-0.25	64.06
AKGQPR	DM1	Heavy Chain	K343	-0.07	62.99
EEMTKNQVSLTCLVK	DM1	Heavy Chain	K363	-0.07	72.43
LTVDKSR	DM1	Heavy Chain	K417	-0.6	66.04

图 4C. 高可信度偶联位点汇总表

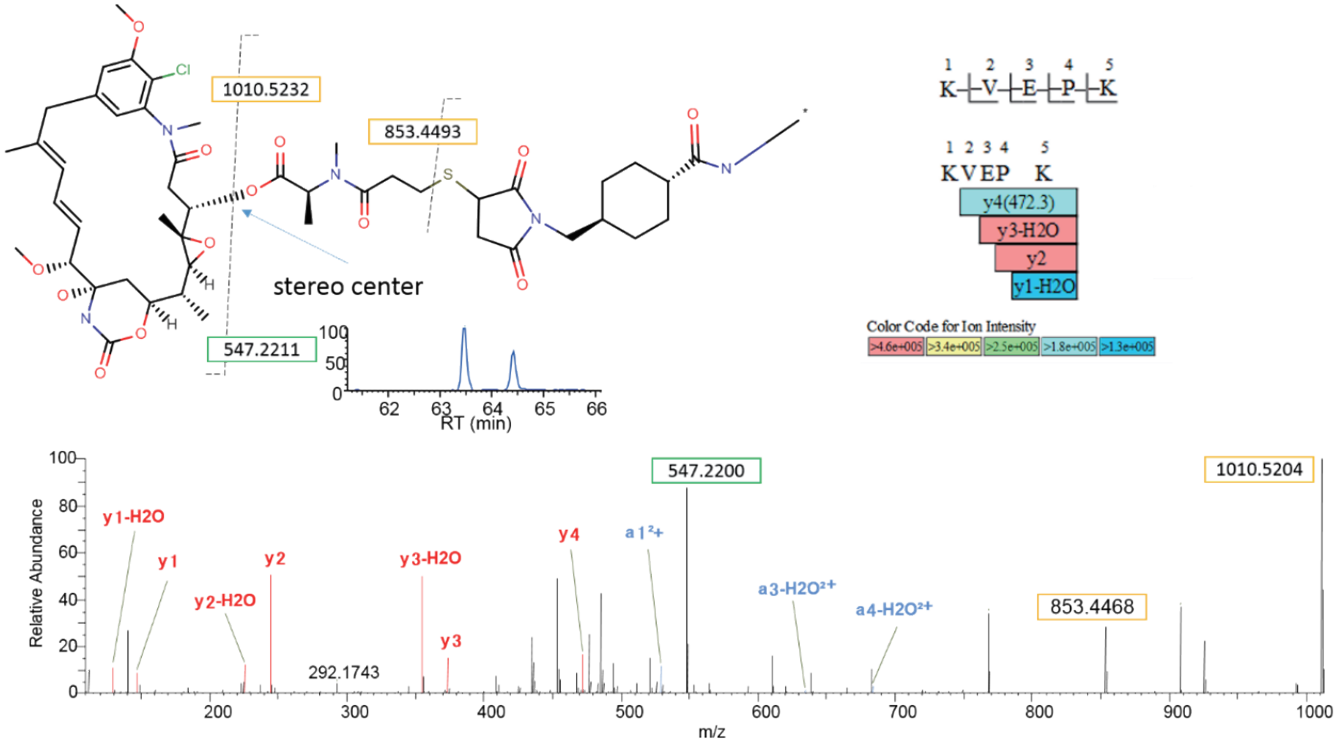


图 4D. 药物偶联肽段 KVEPK 的一级色谱图和高质量丰富的二级碎片离子谱图

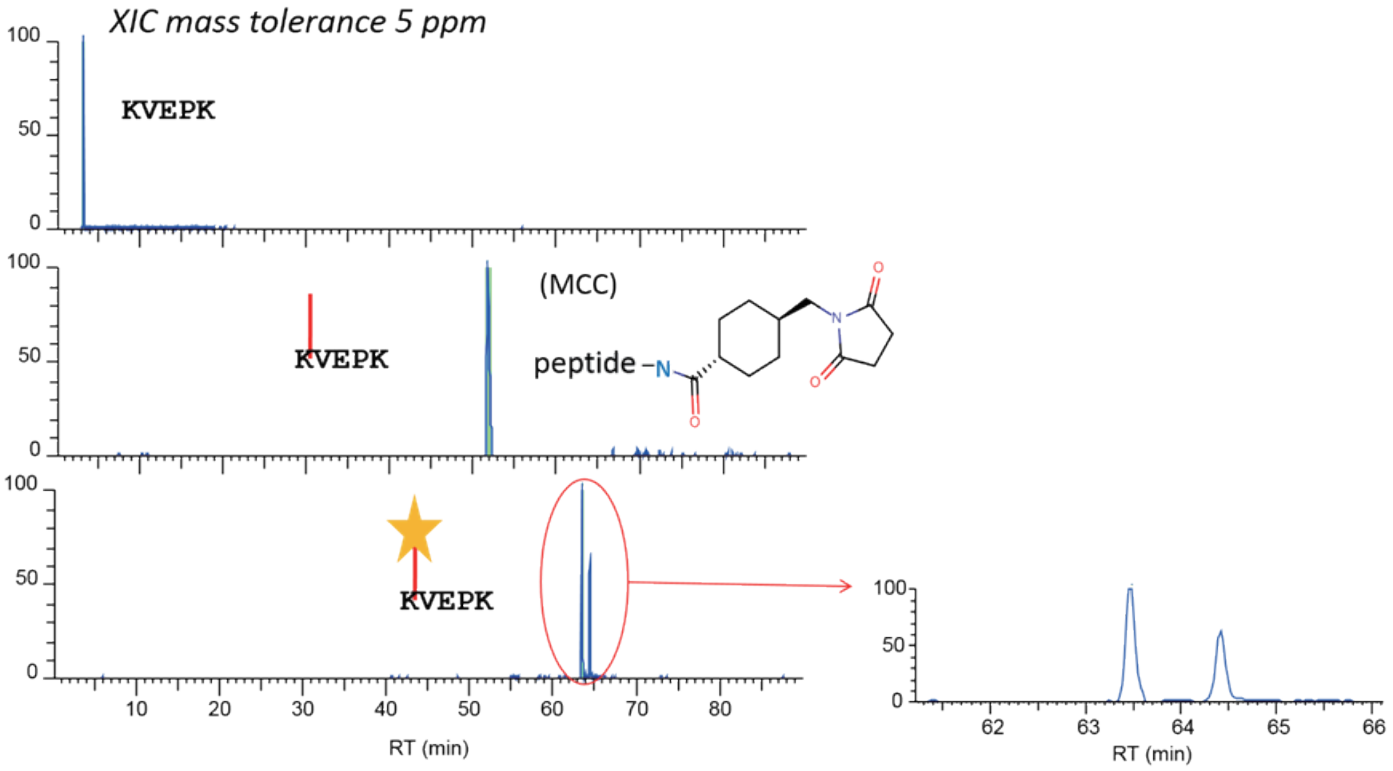


图 4E. 抗体偶联药物中同一肽段的不同存在形式

当肽段中含有 2 个或以上的 K 漏切位点时，使用常规的 CID，HCD 碎裂方式无法准确鉴定具体的偶联位点，ETD 碎裂方式则可将药物保留在 K 上，根据其丰富的 c, z 碎片离子判定具体的偶联位点如图 5。

	No.	Peptide Sequence	# K	Modification	Site	Delta (ppm)	ID Type	RT (min)	M/Z	Charge State	Mono Mass Exp.	Mono Mass Theo.	MS Area	Protein
1	21049	ADYEKHK	2	DM1	K188	0.60	MS2	50.16	616.272	3	1845.7949	1845.7938	20,170,934.00	LC_Trastu_Emta
2	21419	ADYEKHK		DM1	K188	-0.13	MS2	50.94	616.272	3	1845.7936	1845.7938	14,392,560.00	LC_Trastu_Emta
3	22848	AKGQPR	1	DM1	K343	-0.09	MS2	53.88	806.878	2	1611.7408	1611.7410	1,016,677.25	HC_trastu_Emta
4	23430	AKGQPR		DM1	K343	-0.17	MS2	54.75	806.878	2	1611.7407	1611.7410	790,552.56	HC_trastu_Emta
5	26843	ALPAPIEKTISK	2	DM1	K337	0.64	MS2	64.62	742.381	3	2223.1206	2223.1192	462,441.38	HC_trastu_Emta
6	26950	ALPAPIEKTISK		DM1	K337	0.42	MS2	65.11	742.381	3	2223.1201	2223.1192	353,500.62	HC_trastu_Emta
7	25889	ASQDVNTAVAWYQQKPGK	2	DM1	K39	0.40	MS2	60.66	983.794	3	2946.3564	2946.3553	99,419.80	LC_Trastu_Emta
8	25971	ASQDVNTAVAWYQQKPGK		DM1	K39	0.56	MS2	61.18	983.794	3	2946.3569	2946.3553	78,092.43	LC_Trastu_Emta
9	23503	ASQDVNTAVAWYQQKPGKAPK	3	DM1	~K42	0.58	MS2	54.93	1082.521	3	3242.5420	3242.5401	3,348,808.00	LC_Trastu_Emta
10	23773	ASQDVNTAVAWYQQKPGKAPK		DM1	~K42	0.20	MS2	55.50	1082.521	3	3242.5408	3242.5401	2,925,363.50	LC_Trastu_Emta
11	27147	DSTYSLSSTLTLSKADYEK	2	DM1	K183	0.50	MS2	66.91	1023.135	3	3064.3821	3064.3806	72,910.94	LC_Trastu_Emta
12	27210	DSTYSLSSTLTLSKADYEK		DM1	K183	0.10	MS2	67.35	1023.136	3	3064.3809	3064.3806	42,332.44	LC_Trastu_Emta
13	22964	TKPR	1	DM1	K293	-1.18	MS2	54.16	729.342	2	1456.6698	1456.6715	1,648,967.12	HC_trastu_Emta
14	23556	TKPR		DM1	K293	-0.01	MS2	55.02	729.343	2	1456.6715	1456.6715	1,267,997.38	HC_trastu_Emta

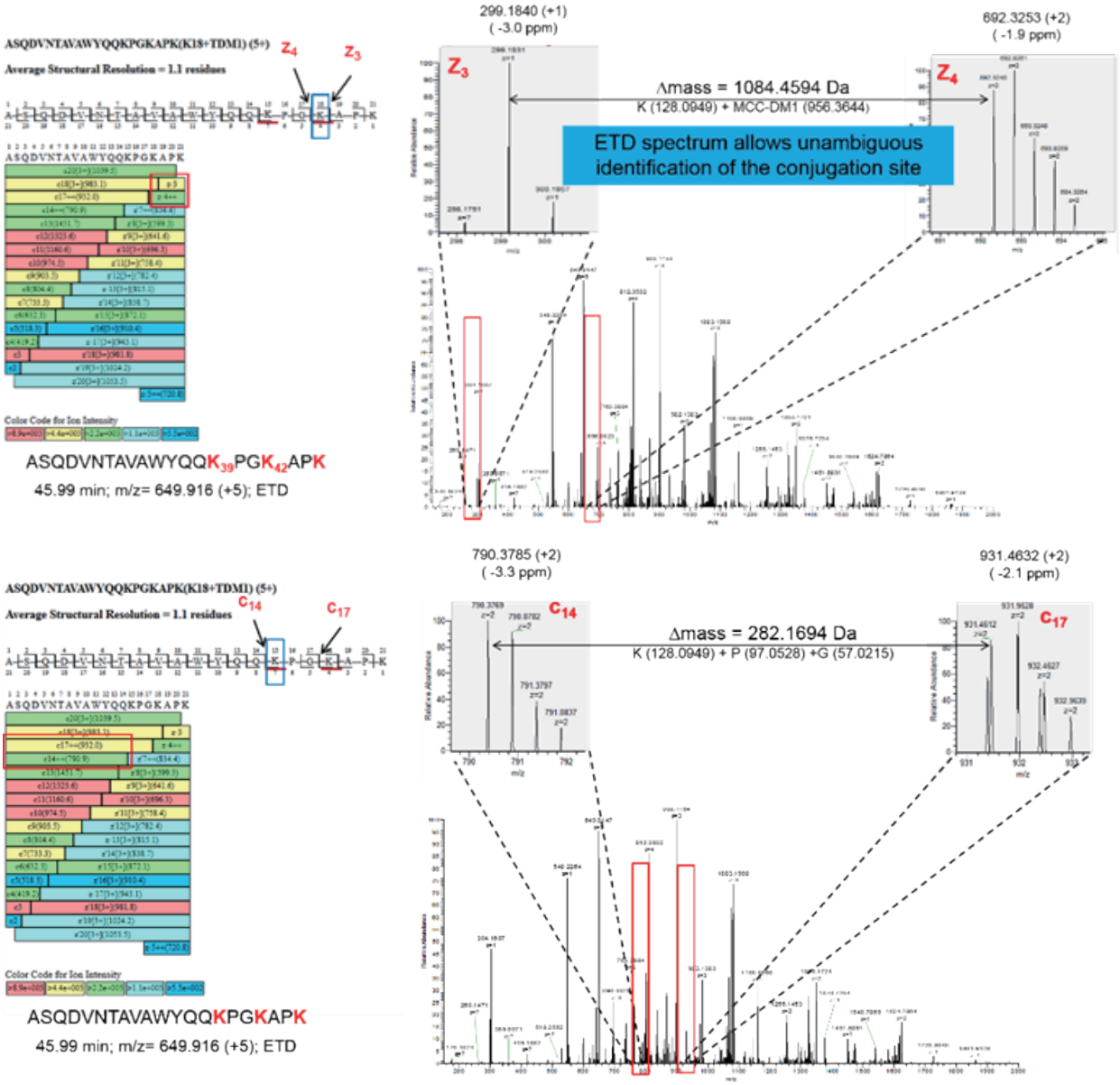


图 5. 该肽段含有 3 个 K，一个偶联位点，通过碎片离子 z3, z4 和 c14, c17 判定出药物偶联在该肽段的 K18 位

糖型分析

糖基化对蛋白药物的疗效，稳定性，免疫原性具有重要的影响。以单克隆抗体为例，常见的 N- 糖型主要为 G0F、G1F、G2F、G1F-GlcNAc、G0F-GlcNAc 等；如果出现 OligoMan、Gal-a-1,3-Gal、NGNA、Xyl-1,2 Fuc-1,3 等糖型，则会影响免疫原性或半衰期，需要避免；此外，利用细胞工程及分子生物学技术，提高 A-Fuc，GlcNAc bisecting，G2F，Hyper Sialyation(NANA) 的含量，以增强 ADCC，CDC，Anti-inflammatory 等效能。因此对糖蛋白药物和生物类似药糖基化的理解、检测和控制非常重要，然而糖为非模板合成，并呈树状结构，其结构极其复杂，仅由 6 种单糖组成的寡糖链，其理论结构达到惊人的 1012 种。

LC-MS 联用分析 N- 糖链（由天冬酰胺连接寡糖）结构，包括寡糖链释放、标记、液质联用分析和数据处理四个步骤（图 6A）。寡糖链释放根据不同的糖基化类型选择不同的方法，N- 糖链主要使用 PNGase F 酶。衍生化标记步骤能够增强寡糖的质谱响应，其中，还原末端标记 2-AB 等苯胺或杂环化合物能够使寡糖链通过荧光检测，全甲基化标记增强寡糖链的疏水性，实现反相分离检测。以 Q Exactive 为代表的 Orbitrap 超高分辨率质谱具有高灵敏度的优势，用于糖链的分析能取得满意的结果。

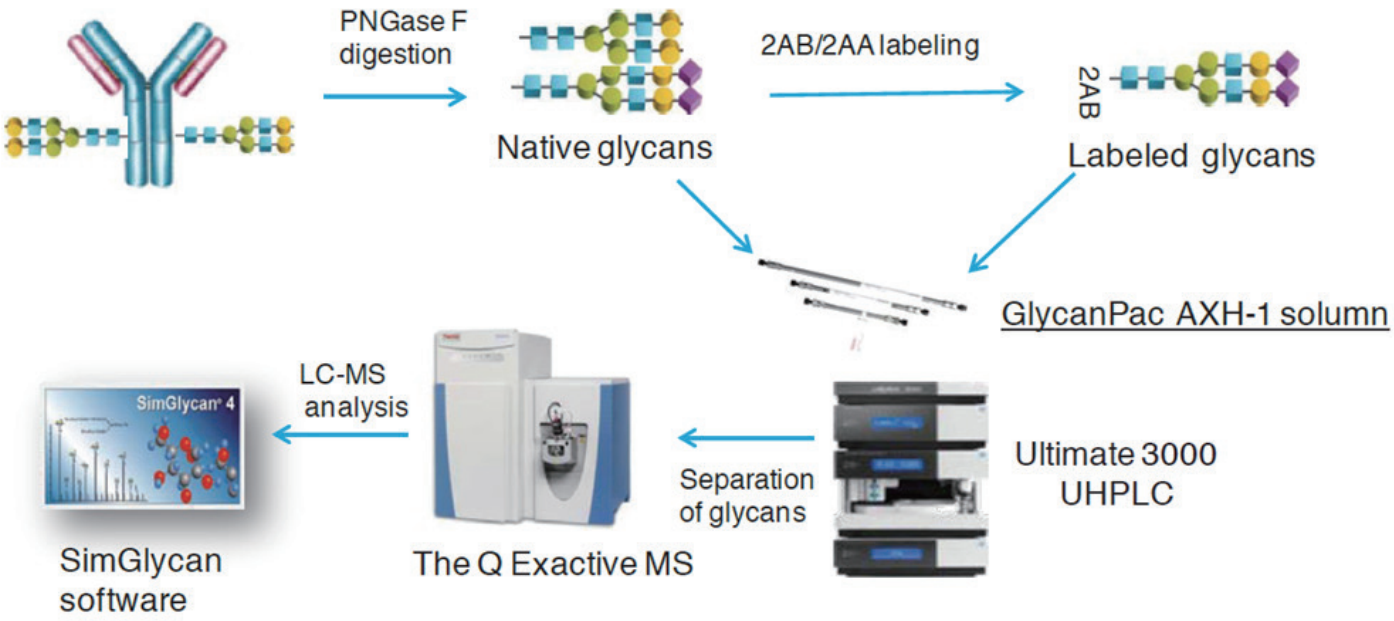


图 6A. LC-MS/MS 对单克隆抗体 N- 糖链表征的分析流程

在寡糖色谱分析中，专为寡糖分离设计的 GlycanPac AXH-1/AXR-1 色谱柱，结合了弱阴离子交换 WAX 和亲水相互作用 HILIC (AXH-1)/ 反相 RP (AXR-1) 两种保留机理，能够高效率地分析复杂寡糖链混合样品。经典的 HILIC/RP 机理根据电荷数、极性、大小实现寡糖链分离，而 WAX 机理保留和选择带负电荷的寡糖链，对含唾液酸的寡糖链具有很好的分离效果。GlycanPac AXH-1 对 2AB 标记的单克隆抗体 N 糖分离，提取其中主要的中性糖 G0-GlcNAc、G0F、G1F 和 G2F 质谱峰如图 6B 所示。实际应用案列见下文 AN。

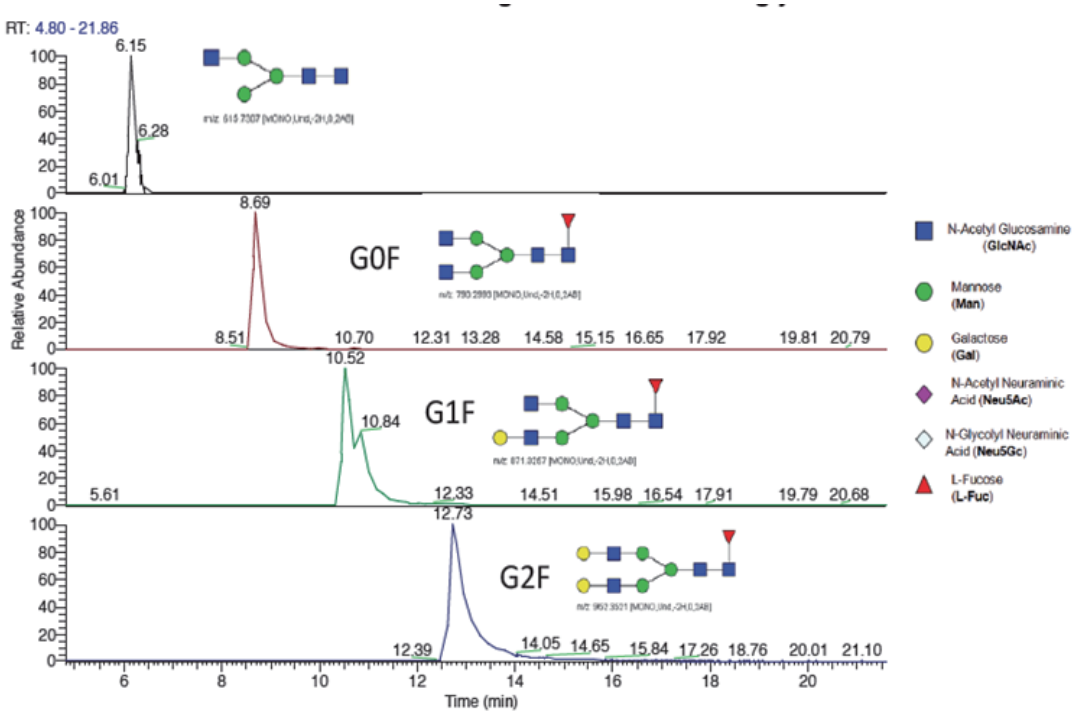


图 6B. GlycanPac AXH-1 色谱柱分离单克隆抗体中主要的中性 N- 糖

终产品中游离小分子毒素药物的定量

终产品中痕量的游离小分子毒素药物的含量影响抗体偶联药物安全性、有效性及治疗窗口，目前定量分析方法主要有 ELISA、RPLC-UV 或者高专属性和高灵敏度的 RPLC-MS/MS。不同的方法有相应的优势和其局限性，ELISA 方法具有高的专属性、灵敏度和高通量，然而开发时间长，与相关药物杂质有交叉反应，对降解产物的测定缺乏特异性，以及由于基质的干扰会导致结合效率降低 [8]。RPLC-UV 和 RPLC-MS/MS 分析方法则具有开发速度快，专属性高等特点，但需要进行样品前处理和色谱柱平衡等维护工作，Orbitrap 静电场轨道阱类高分辨质谱仪能够胜任定性和定量工作，其定量性能可媲美高端的三重四级杆。样品前处理通常采用蛋白沉淀、离线或在线固相萃取，保证前处理具有较高的回收率。RPLC-MS/MS 定量方法同小分子定量流程，可采用高分辨率、高灵敏度的 SIM 模式进行一级定量，PRM 采集方式进行高分辨率的二级碎裂离子定量，定量能力可媲美高端的三重四级杆。

如对 ADC 产品中小分子毒素 MMAE（单甲基奥瑞他汀）进行定量，地塞米松作为内标，5 倍体积的乙腈沉淀抗体主成分，配置标准曲线。LC-MS/MS 方法如图 7A，检出限达 1ppt，定量限为 5ppt，线性范围为 5ppt-400ppb（其中由于样品原因，上限未继续向上做）如图 7B，足以满足定量需求。

A. UHPLC 条件

LC系统: Thermo Dionex Ultimate 3000

色谱柱：Hypersil GOLD (2.1×100mm,1.9μm)

柱温: 30 °C

进样体积：5 μL

流速: 0. 3mL/min

流动相：

A: H2O

B: ACN

Time(min)	A%	B%
0.0	95	5
0.5	95	5
3.5	5	95
4.5	5	95
4.6	95	5
6.0	95	5

B. MS 条件

MS系统: Q Exactive Plus

离子化模式: ESI+

喷雾电压: 3800

蒸发温度: 300°C

鞘气:35 Arb

辅助气:10 Arb

离子传输管温度: 400°C

扫描模式：Targeted-SIM

分辨率:70,000FWHM

图 7A. MMAE 小分子毒素定量的 LC-MS/MS 方法

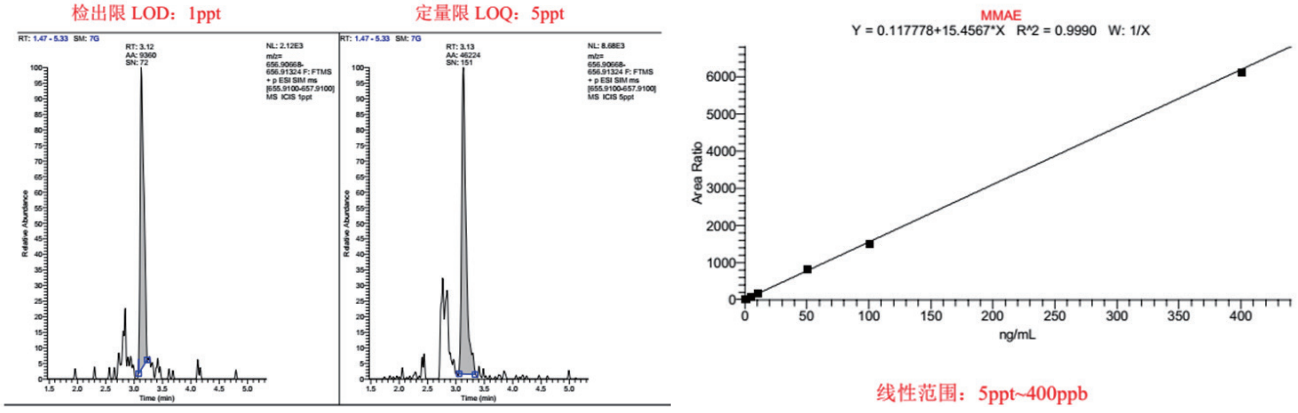


图 7B. LC-MS/MS 方法的检出限、定量限及线性范围

抗体偶联药物的生物分析

ADCs 相比于抗体类药物是具有高度复杂异质性的混合物，主要来源抗体本身的糖型异质性、小分子药物偶联位点的异质性、小分子偶联药物数量的异质性。在体内代谢过程中，由于 DAR 值的不同，小分子的释放和清除率存在差异，其成分更为复杂，使得 ADCs 类药物的生物分析更具有挑战性。生物分析主要集中在 ADC 的研发阶段，早期的研发需要评估 ADC 候选药物体内的稳定性，主要检测平均 DAR 值变化或特异偶联位点处小分子药物的完整性。在临床开发阶段，重要的是建立 ADC 药物成分与安全性和有效性指标的相关性。经典的生物分析方法 LBAs（Ligand Binding Assays）能够评估总抗体的药代动力学行为，但不能完全满足药代动力学的需求和生物转化研究如 DAR 值变化，而 LC-MS/MS 能够鉴定并定量每一成分。基于 LBAs 方法的灵敏度、LC-MS/MS 方法的专属性，将两者结合越来越多的用于 ADCs 药物生物分析中 [9]。He, Jintang, et al.[10] 利用亲和捕获的方式将血浆中的 ADCs 富集，联合 Orbitrap 高分辨质谱评估几种 ADCs 候选药物在体内的稳定性，结合活性测定从而选出稳定性更高、活性更好的药物。ThermoFisher 提供针对 ADCs 定量生物分析的完整解决方案，即 LB-MSIA（Ligand Binding Mass Spectrometric Immunoassay）Workflow 如图 8A

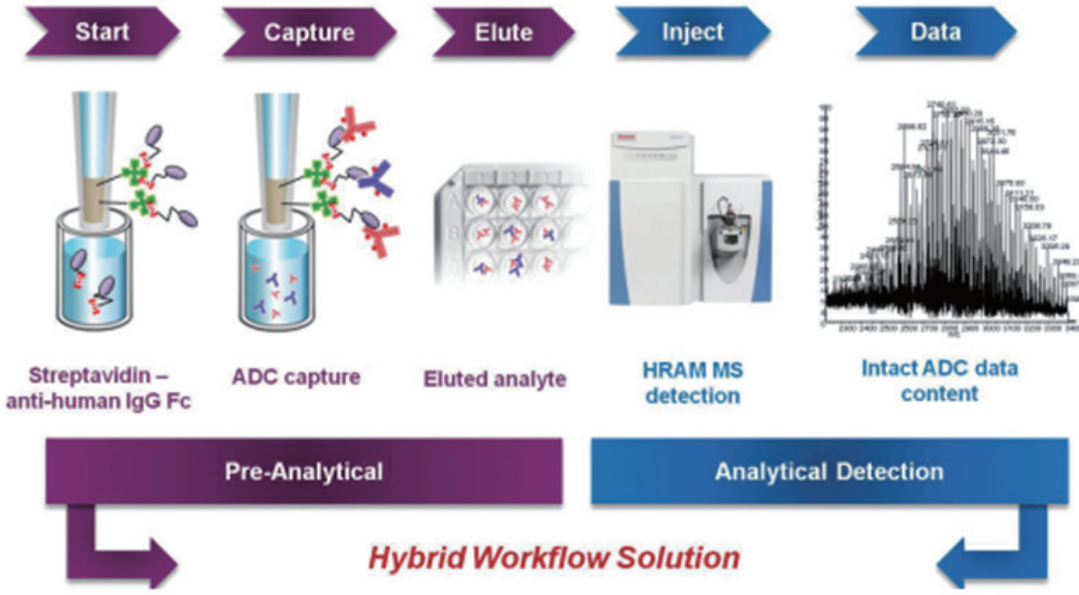


图 8A. LB-MSIA 工作流程的主要操作步骤

抗体药物高级结构解析 -- 氢氘交换质谱（HDX-MS）

HDX-MS 的主要应用于结构生物学研究和生物制药行业中，提供有关蛋白质的高级结构信息，用以判断样品是否因聚合、蛋白 - 蛋白或者蛋白 - 受体相互作用、或翻译后修饰等发生了构象变化，以及蛋白质相互结合时的结合界面、抗原表位分析等 [11]。氢氘交换质谱的全流程包括：交换反应、终止反应、将蛋白快速酶切为多肽、液相分离、质谱检测、数据解析如图 9，实际应用案例见下文 AN。

图 9A. 氢氘交换质谱的全流程示意图

其原理为将目的蛋白或者蛋白复合物放置到氘水中，设计不同的氢氘交换时间，终止交换，在蛋白水平或者酶解后在肽段水平上进行液相质谱分析，通过观测氢氘交换速率的变化来确定蛋白高级结构的变化，详细的实验条件如图 9B。

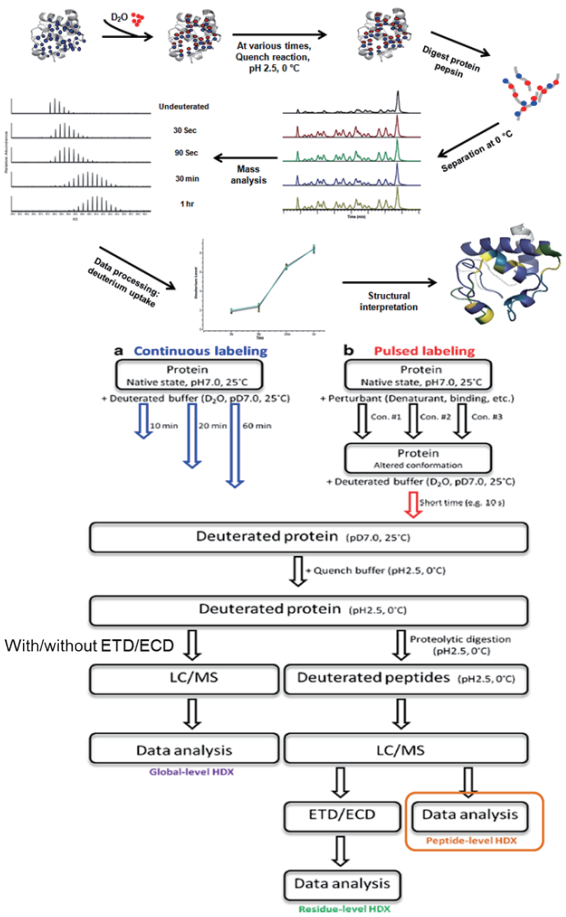


图 9B. 具体实验条件示意图

宿主细胞残留蛋白的鉴定

重组蛋白类药物是由遗传修饰的原核或真核宿主细胞培养 / 发酵产生，在此过程中，宿主细胞也共同产生与正常细胞功能相关的蛋白质，由于细胞凋亡 / 死亡 / 裂解，其他非必需蛋白质也可能释放到细胞培养基 / 发酵液中，因此残留在终产品中的其他蛋白质即为宿主细胞残留蛋白（HCPs）。HCPs 的存在主要影响药物的安全性和有效性，如引起机体免疫反应，作为佐剂以增强对药物产品的免疫应答，以及具有蛋白水解活性的 HCPs 影响药物产品的稳定性 [13, 14, 15]。ICH 的 Q6B 指导方针指出需采用具有高灵敏度和宽动态范围的方法监测 HCPs，要求其含量尽可能的低，甚至低于检测限，不同产品要求不同，一般在 1-100ng/mg（1-100ppm）范围内，然而并未规定其检测的具体分析方法。目前 Enzyme-Linked Immunosorbent Assay (ELISA) 因其具有高灵敏度、高通量和易操作优势成为工业界的金标准，但其存在很多限制，包括不能对 HCPs 进行精准鉴定，低丰度 HCPs 的检测易受限于高丰度蛋白的干扰，定量动态范围（3 个数量级）较低，抗体受限，开发周期长，无法改变已有的方法，缺少校正标准限制定量的准确性，因此需多种检测方法相结合 [16]。随着高分辨质谱技术性能的不断提升，已具有高灵敏度和宽的定量动态范围，方法开发周期短等特点，以及蛋白质组学技术的飞速发展，可实现对未知蛋白质的高通量定性和定量。LC-MS/MS 方法对 HCPs 检测的灵敏度可达 1ppm，动态范围为 6 个数量级，此方案足以满足生物药客户的需求，具体实验见下文 AN。

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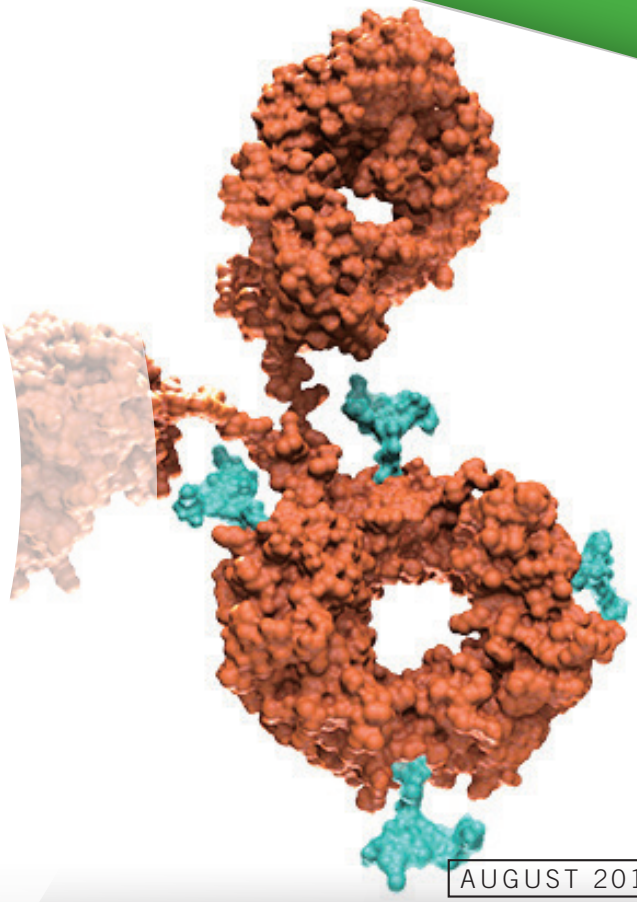
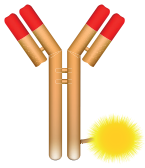
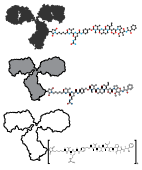


Advances in Biopharmaceutical Characterization: Antibody–Drug Conjugates

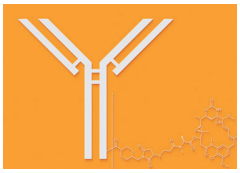
AUGUST 2018



Antibody–Drug Conjugates: Perspectives and Characterization
Rowan E. Moore, Kelly Broster, Ken Cook, Kyle D'Silva, Eric Niederkofler, Aaron O. Bailey, Jonathan Bones, and Michael W. Dong



Solving the Analytical Challenges of ADC Characterization
An interview with Krisztina Radi



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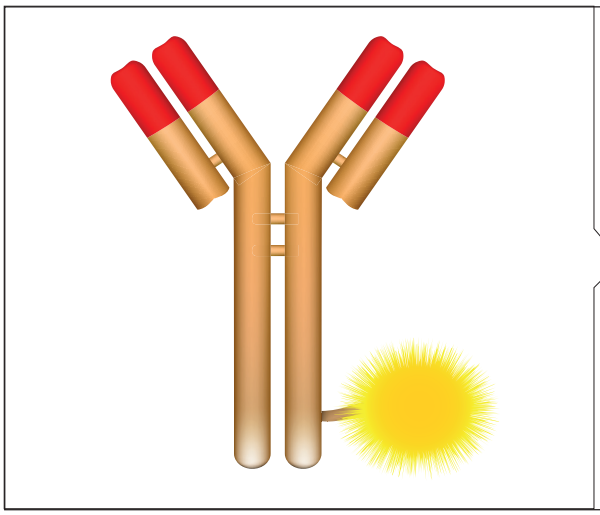


Antibody–Drug Conjugates: Perspectives and Characterization

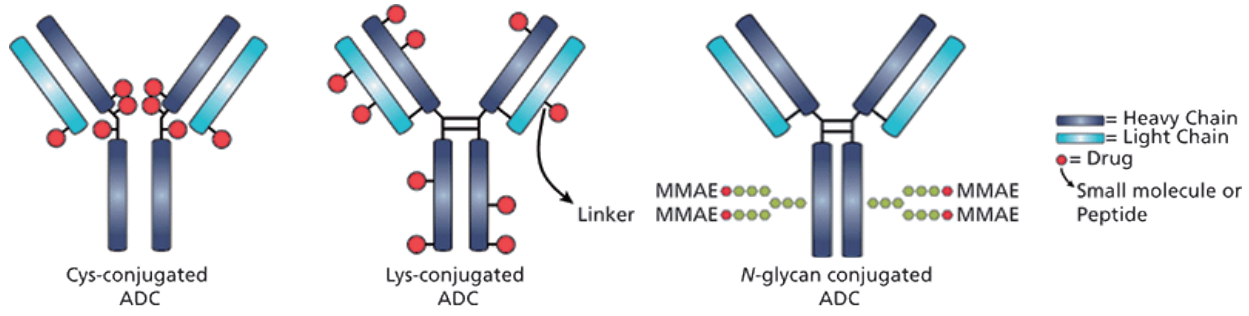
Rowan E. Moore, Kelly Broster, Ken Cook, Kyle D'Silva, Eric Niederkofler, Aaron O. Bailey, Jonathan Bones, and Michael W. Dong

This installment of "Perspectives in Modern HPLC" provides an overview of antibody–drug conjugates (ADCs) as a new class of biotherapeutics and describes their analytical characterization for quality assessment with examples from extensive applications libraries.

Chemotherapeutic agents have been the mainstay of anticancer therapy since the early 1940s. Chemotherapy, or the use of cytotoxic agents in medical oncology to inhibit the process of mitotic cell division, is routinely administered with curative intent, to prolong life or as part of palliative care. Although the use of chemotherapy can result in a significant response—for example, in the treatment of testicular cancer—its use is associated with a range of adverse effects. Many of the adverse effects of chemotherapy are the result of damage to healthy cells that divide rapidly and are thus sensitive to antimitotic drugs.

Antibody–drug conjugates (ADCs) are an increasingly important class of biotherapeutics that utilize the specificity of monoclonal antibodies (mAbs) and the cytotoxicity of a potent anticancer payload (1–3). The two molecules are connected via chemical linkers, and the result is a therapy that is able to provide sensitive discrimination between healthy and diseased tissues. The antibody targets and binds to a selected antigenic cell-surface receptor that is, ideally, only expressed on the target cancer cell. After an ADC binds to its target cell, the cell internalizes the ADC through receptor-mediated endocytosis, and the cytotoxic payload is then released inside the lysosomal cellular compartment to provide precise, selective delivery to the cancerous cells. Payload conjugation typically takes place on the amino groups of lysine residues or the sulfhydryl groups of interchain cysteine residues as is the case in ado-trastuzumab emtansine (Kadcyla, Genentech/Roche) and brentuximab vedotin (Adcetris, Seattle Genetics/Millennium Pharmaceuticals),

Figure 1: ADC structures showing different sites of attachment to mAb of the drug with the linker. MMAE = monomethyl auristatin E, an extremely potent synthetic antineoplastic agent.



respectively. With 80–100 lysine residues and only eight interchain cysteine residues available in each mAb molecule, lysine conjugation yields a more heterogeneous mixture of species compared to cysteine-conjugated ADCs. **Figure 1** depicts examples of common payload conjugation types, namely lysine, cysteine, and glyco-conjugates (4).

In addition to the described primary amino acid structure, mAbs and ADCs also have distinct higher order structures that dictate their function and immunogenicity. They may be influenced by the above-described modifications and can appear as dimers or aggregates that also have the potential to induce immune responses and affect clearance rates.

For an ADC to demonstrate efficacy, it must incorporate a mAb that recognizes a specific tumor-associated antigen, a linker that has systemic stability but is specifically released at the target cell, and a cytotoxic agent that exhibits toxicity to the tumor cell as a stand-alone modality.

ADC Regulations

Whether submitting to the United States Food and Drug Administration (U.S. FDA), European Medicines Agency (EMA), or other regulatory bodies, ADC developers are covering new territory. Since ADCs incorporate both biologics and small-molecule moieties, these complex therapeutics are difficult to characterize, and multiple health authority experts are required to evaluate different aspects of the end product.

An ADC may be based on a previously approved mAb. For example, trastuzumab (Herceptin) is the mAb portion of the ADC Kadcyla. In such instances, new analytical technologies that have emerged since the development of the original mAb drug product should be evaluated for use in characterizing the related ADC. Consistent with the principles of quality by design (QbD), regulators expect sponsors to use the most current and effective technologies available to build product and process knowledge into controlling product quality.

With the approvals of Kadcyla, Adcetris, and more recently inotuzumab ozogamicin (Besponsa, Pfizer), gemtuzumab ozogamicin

(Mylotarg, Pfizer), and more than 50 ADCs in clinical trial pipelines, the clinical application of ADCs is accelerating rapidly (5).

It is important to have a clear understanding of the relationship between the conjugation and manufacturing process, and the resulting product quality and heterogeneity of the ADC. The potency of an ADC is due, in part, to the extent of drug-linker incorporation on the mAb. Methods that can structurally characterize the drug load and distribution have been developed and proven to be critically important for understanding ADC product quality. Wakankar and colleagues have summarized several considerations for the development of analytical methods that measure quality attributes unique to ADCs, such as drug load and drug distribution (6). In addition, several articles documenting the analytical strategies (7) as well as chromatographic and electrophoretic techniques for the characterization of ADCs have been published (8–10).

Characterization and Quality Control Requirements

Quality control (QC) testing of an ADC needs to account for its identity, purity, concentration, and activity (potency or strength)—the same as for any other biopharmaceutical product. Because of the inherent structural complexity of mAbs along with the covalently linked cytotoxic agents, several QC tests are required (8–10). A full understanding of the manufacturing process and its effect on the physicochemical and biological attributes of an ADC must be ascertained. However, in the case of ADCs, even the well-established QC terminology is not straightforward—for instance, the terms potency and strength have different meanings depending on whether the molecule being developed is large or small. The International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) Q6A for small molecules lists strength (or assay) as a measure of the amount of an active pharmaceutical ingredient (API) (11). ICH Q6B for large molecules uses the term potency as a quantitative measure of biological activity (12). For an ADC that includes both of these components, total function (or potency) would need to be measured with a cell-based assay that assesses overall structure, antigen binding, drug loading, and drug delivery.

Unlike their pharmaceutical predecessors and more straightforward protein-based therapeutics, there is limited availability of certified standards for ADC test method development or comparison. Recently, Merck launched SigmaMAb Antibody-Drug Conjugate Mimic for use as a standard for mass spectrometry (MS) and high performance liquid chromatography (HPLC). SigmaMAb is an "ADC mimic" that conjugates SigmaMab (MSQC4), an IgG1 mAb, to dansylcadaverine fluorophores via a succinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate (SMCC) crosslinker (13). At this time, the onus is completely on the developers to devise and implement a set of critical tests for identity and purity, involving the most appropriate analytical technologies. Each intermediate (mAb, linker, and drug) should have a reference standard in addition to an ADC reference standard, to be used in designated release and stability tests. These standards are critical reagents used for analytical

method system suitability and in characterization, stability, and bridging studies, as is currently expected for all pharmaceutical and biopharmaceutical products. The cohort of tests would be performed as part of chemistry, manufacturing, and control (CMC) efforts during drug development. Many of these tests would become assays for critical quality attributes (CQA) or analytical methods for specification testing in lot release.

Small-molecule conjugation to mAbs, using any type of strategy, has enormous potential to produce several variant isoforms. Appropriate tests are needed to measure heterogeneity and ensure product consistency. Routine QC testing and characterization may measure the following characteristics:

- Aggregates and fragments
- Charge variants
- Free drug
- Average drug-to-antibody ratio (DAR)
- Drug load distribution, including unconjugated mAb
- Endotoxins or bioburden

Because of the heterogeneity of ADCs, isoforms derived from mAb glycosylation and other post-translational modifications (PTMs) are often controlled at the point of mAb release. The inclusion in the certificate of analysis (CoA) for routine testing of other product-related impurities—such as aggregates, fragments, charge variants, and unconjugated antibodies—discussed above should be assessed product by product. For example, data could be generated to show that an unconjugated antibody is adequately monitored and controlled as part of DAR testing.

Chemical impurities other than free drug or drug-related substances may be evaluated with both ICH Q3B (R2) limits and pharmacology or toxicology input for the specific product (14). Some process-related impurities might be omitted from release testing with sufficient data and process experience over multiple ADC lots or multiple ADC products using the same conjugation platform.

Regulators consider compendial monographs, which exist for small-molecule intermediates, to be the minimum standard for chemical components when used in ADCs.

Drug and Linker: Approaches and Chemistries

The conjugation of anticancer payloads to lysine or cysteine residues found in mAbs results in the generation of ADCs that exhibit significant heterogeneity, with some of the ADC potentially having altered antigen-binding properties leading to suboptimal potency, solubility, stability, and pharmacokinetics. To reduce heterogeneity, expand payload options, and prolong circulating stability, novel site-specific conjugation approaches are actively being pursued within the field (15).

The hydrophobic nature of the payloads used in current ADCs leads to the creation of conjugates of increasing hydrophobicity versus their starting mAb scaffolds. The hydrophobicity of ADCs can promote aggregation, which in turn can lead to hepatotoxicity (16) or increased immunogenicity (17). The hydrophobicity of ADCs can also promote drug resistance via increased affinity for multidrug resistance transports, with the incorporation of hydrophilic linker chemistries shown to bypass multidrug resistance (18).

ADCs use three main tumor-specific microenvironmental factors to selectively release their cytotoxic payloads: cleavable linkers ex-

hibiting protease-sensitivity, pH-sensitivity, and glutathione-sensitivity. Within each of these main linker release mechanisms, significant linker technology advancements are ongoing.

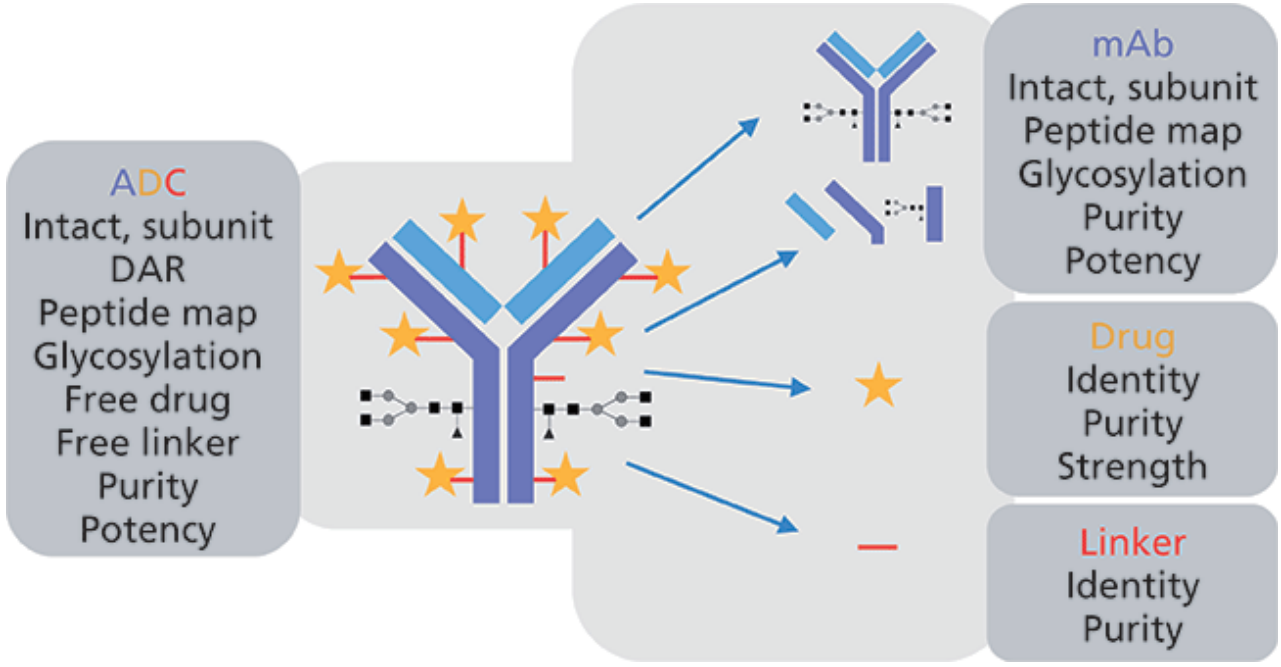
Among the types of conjugation chemistries, enzyme-based site-specific modification shows great potential by eliminating the potential interruption of an antibody–antigen interaction and providing a highly reproducible and modular conjugation system when compared to standard lysine and cysteine conjugation.

Developments in linker chemistries also provide a greater opportunity to incorporate increasingly potent cytotoxic payloads. Quaternary ammonium linkers now enable stable conjugation of payloads with tertiary amine residues (19); the extremely potent synthetic antineoplastic agent monomethyl auristatin E (MMAE) has been linked to mAbs via a linker that is selectively cleaved by cathepsin (for example, in Adcetris) upon entrance into the tumor cell (20). A conjugate with the potent maytansinoid DM1 has been approved (for example, Kadcyla), and Seattle Genetics recently published work on a novel methylene alkoxy carbamate (MAC) self-immolative unit for hydroxyl-containing payloads within ADCs (21). The latter compound enables direct conjugation of drugs through alcohol functional groups that are present on a diverse range of synthetic drugs as well as natural cytotoxic products. Most recently, Spirogen (now part of the AstraZeneca Group) developed a potent and flexible class of ADC payload based on a proprietary pyrrolobenzodiazepine (PBD) technology. PBDs are a family of sequence-selective DNA minor-groove binding agents and are among the most cytotoxic agents known. They are ideally suited for antibody–drug conjugation because of their unique mechanism of action that retains activity against cancer stem cells and is compatible with multiple linker and conjugation technologies. There are two ADCs currently undergoing clinical trial from the collaborative efforts of Spirogen and Seattle Genetics (22), and many more are in the pipeline. As previously mentioned, most of the payload and linker technologies used or studied today impart increasing levels of hydrophobicity on the mAb scaffold (10); for example, DM1 has an estimated LogP value of 3.95 per molecule incorporated. PBDs are even more hydrophobic, with an estimated LogP value of 5.08 per incorporated molecule. To address this issue, hydrophilic spacers (for example, para-aminobenzyl alcohol [PAB]) and linkers (such as polyethylene glycol [PEG]) are often incorporated as part of the bioconjugation chemistry to balance out the increased hydrophobicity introduced by the conjugation of the payload.

Chromatography for mAb, Drug, Linker, and ADC

Various ultrahigh-pressure liquid chromatography (UHPLC) techniques have proved to be useful for analyzing ADC heterogeneity at the intact level, including hydrophobic-interaction chromatography (HIC), ion-exchange chromatography (IEC), size-exclusion chromatography (SEC), and reversed-phase chromatography. Where appropriate, the coupling of these separation techniques with high-resolution accurate mass spectrometry (HRAM MS) presents a powerful characterization tool. Further structural details can

Figure 2: Typical characterization approaches performed on ADC therapeutics.



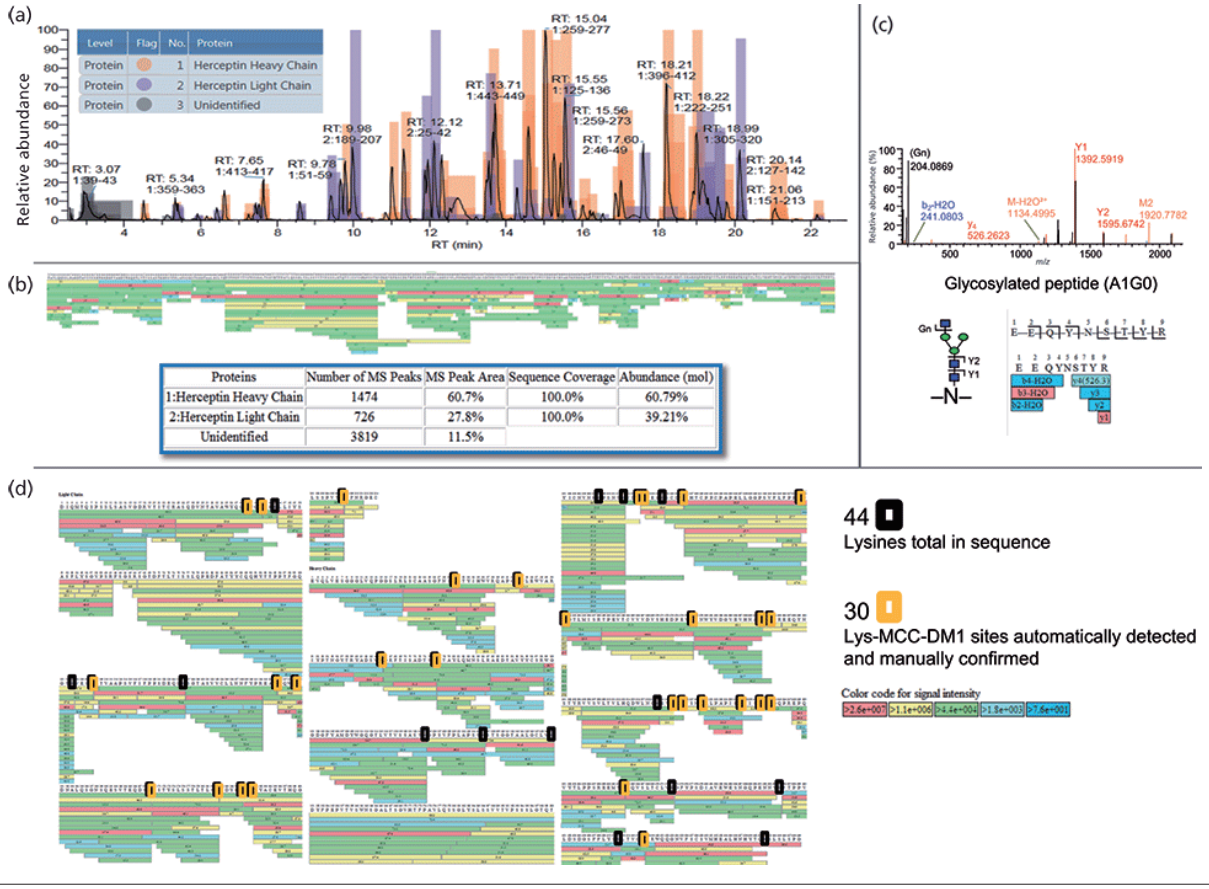
be ascertained by breaking down the intact ADC; both peptide mapping using reversed-phase chromatography and released glycan analysis with hydrophilic-interaction chromatography (HILIC) are deemed essential tools. Each of these analytical approaches reveals different CQAs of the ADC—from primary amino acid sequence and associated modifications (peptide mapping) to the presence of higher order aggregated structures (SEC) that could impact product efficacy and safety. In addition to the standard cohort of small molecule and large biomolecule characterization methodologies, a whole set of tests must be performed to interrogate the level of drug conjugation and the levels of unconjugated mAb, payload, and linker (as shown in **Figure 2**).

Monoclonal Antibody Primary Sequence Analysis

As a technique, peptide mapping is well established in the biotechnology industry with roots lying in protein characterization, proteomics, and de novo peptide sequencing. In recent years, advances in sample preparation (protein digestion), peptide separation, HRAM MS capabilities, and bioinformatics have enabled the biotech industry to confidently apply peptide mapping workflows in routine, high-throughput environments.

Peptide mapping can reveal many CQAs of a protein. In the case of ADCs, peptide mapping is fundamental in confirming not only the sequence of the mAb, but also the site and level of drug

Figure 3: Trastuzumab emtansine lysine-conjugation mapping (26). (a) Color-coded base peak ion chromatogram (BPI) showing heavy and light chain peptides. (b) Coverage map showing 100% sequence coverage, number of MS peaks, and relative abundance of heavy and light chain peptides detected. (c) Example higher energy collisional dissociation (HCD) MS/MS spectrum of a glycopeptide showing fragmentation of both peptide and glycan. (d) Identification of lysine conjugated MCC-DM1 at the peptide level.



conjugation (**Figure 3**). The accuracy with which this information can be determined is based on the method of protein digestion and fidelity of the subsequent UHPLC and MS analysis. The type of fragmentation used within the MS system should also be carefully considered because standard collision-induced dissociation (CID) experiments often fail to reveal the precise site of drug conjugation or glycosylation. Alternative or additive fragmentation techniques such as higher energy collisional dissociation (HCD), electron transfer dissociation (ETD), and ultraviolet photodissociation (UVPD) are becoming increasingly important in the elucidation of site-specific modifications and can generate informative fragmentation patterns, even at the subunit level (23–25).

Chromatographic Techniques for the Determination of Antibody Variants, Fragments, DAR, and Payload Mapping

Hydrophobic Interaction Chromatography

HIC separates proteins by the interactions between hydrophobic pockets present on the surface of the protein and the hydropho-

bic ligands on the HIC resin. Proteins are loaded onto the column in relatively high salt concentrations to induce hydrophobic interactions and are eluted by reducing the salt concentration of the mobile phase during the chromatographic separation. The binding of the proteins is dependent on the inherent surface hydrophobicity, which is influenced by the conformation of the protein. Changes in protein conformation can be characterized by this mode of chromatography, and several publications exist that indicate that common modifications of mAbs, such as oxidation and deamidation, can be seen using HIC (27). With the conjugation of hydrophobic payloads to the mAb to form ADCs, the use of HIC for DAR analysis has become increasingly popular (6,28).

With each additional linkage of the drug to the mAb the retention of the ADC species on the column increases, thus allowing quantification of drug load on the ADC and resolution of isomeric configurations of the same DAR (**Figure 4**).

Figure 4: Comparison of synthesized Cys-conjugated ADC mimics with different drug load (29): (a) unconjugated mAb (5 mg/mL), (b) Cys-conjugated ADC mimic (low load, 5 mg/mL), (c) Cys-conjugated ADC mimic (moderate load, 5 mg/mL), (d) Cys-conjugated ADC mimic (high load, 5 mg/mL). Column: 100 mm × 4.6 mm, 5-µm dp Thermo Scientific™ MAbPac™ HIC-Butyl; mobile-phase A: 95:5 (v/v) 1.5 M ammonium phosphate (pH 7.0)–isopropanol; mobile-phase B: 80:20 (v/v) 50 mM sodium phosphate (pH 7.0)–isopropanol; gradient: 0% B for 6 min, 0–100% B in 14 min, hold at 100% B for 5 min; temperature: 25 °C; flow rate: 1.0 mL/min; injection volume: 5 µL (5 mg/mL); detection: UV absorbance at 280 nm.

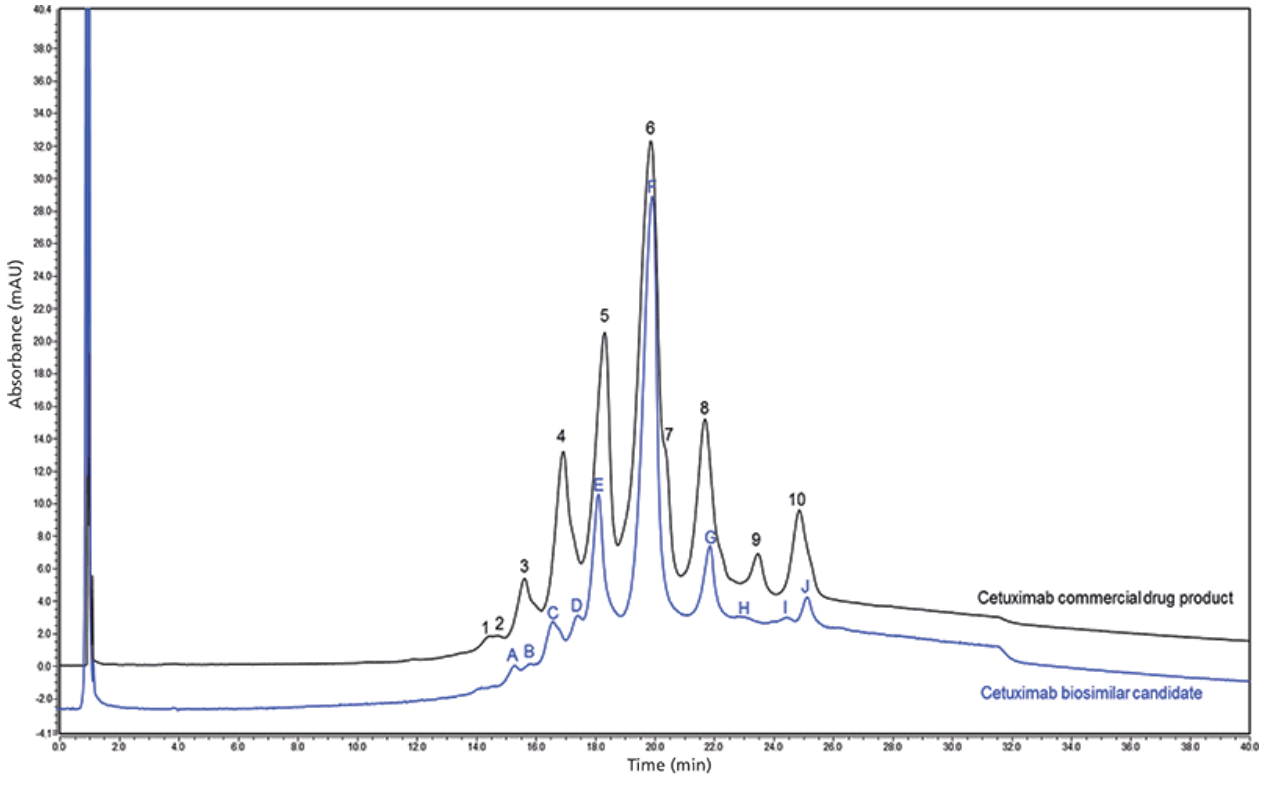
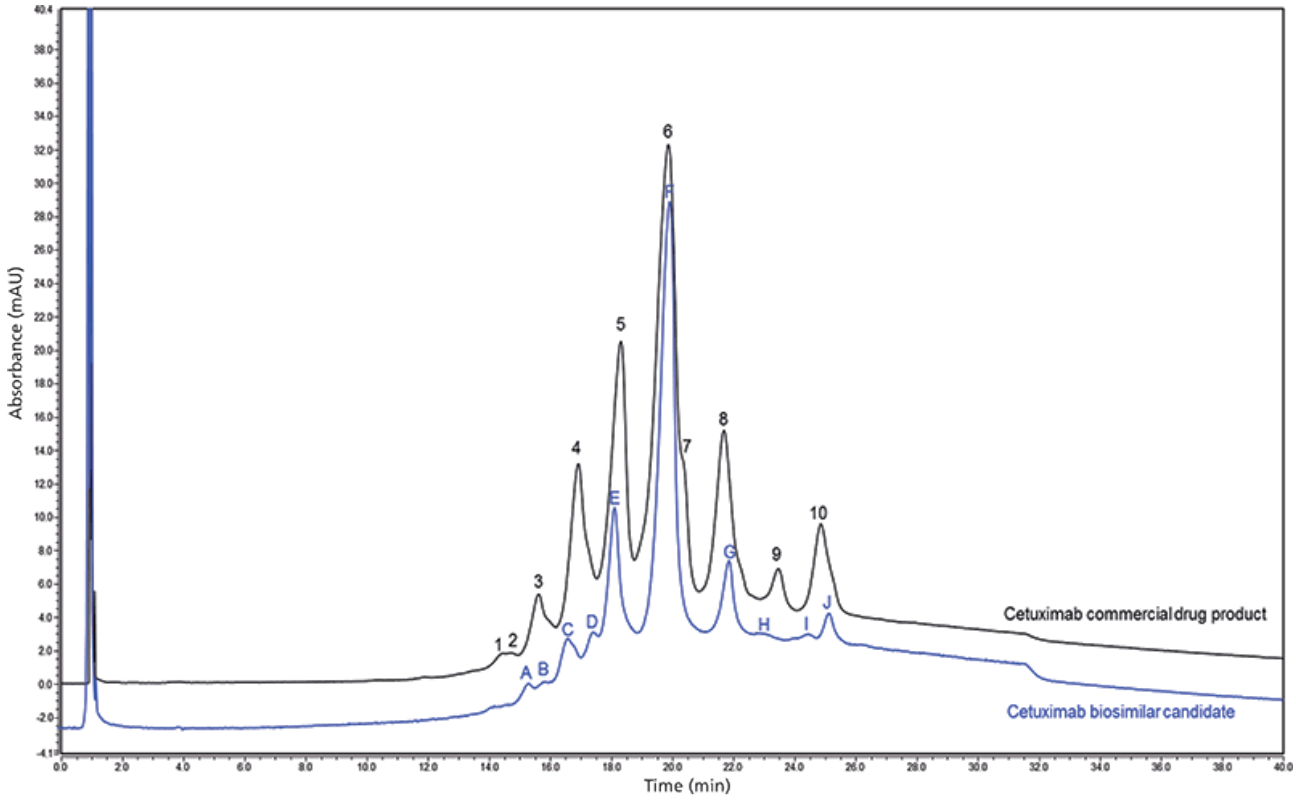


Figure 5: Charge variant chromatographic profile comparison of commercial chimeric IgG1 mAb (black trace) and cetuximab biosimilar candidate (blue trace) obtained with cation-exchange chromatography in pH-based gradient mode (31). Peak labeling corresponds to the number of peaks in each trace and does not indicate peak identification.



Ion-Exchange Chromatography

IEC involving cation-exchange column chemistries is a standard method used to separate and monitor the charge-variant profile of mAb-based therapeutics (30). Charge-variant separations have been further developed with the use of pH gradients that provide ease of use and a more global approach to the method development process (**Figure 5**) (31). There are several PTMs that can alter the charge or conformation of a protein and can, therefore, be characterized using IEC. Glycan variants, deamidation, oxidation, and even aggregation are among them.

The specific charge-variant profile that is obtained from a mAb is closely monitored at each stage in the production to ensure the product quality remains the same. In the case of ADCs, mAbs may not provide an informative charge-variant profile—if the drug or linker is charged, or linkage occurs through a charged amino acid (such as lysine), the underlying mAb charge heterogeneity is difficult to assess because conjugation affects the overall charge of the conjugated molecule. In such cases, the "charge profile" is often more of a "conjugation profile." Despite this, measuring the distribution

of charged species can be a good way to demonstrate process consistency and thus should be included in an ADC comparability toolkit.

Reversed-Phase Chromatography-MS

MS analysis of ADC drug distribution provides insights into the relative concentration of different drug-linked forms, which may elicit distinct pharmacokinetic and toxicological properties. MS analysis of ADC drug distribution is particularly advantageous for conjugates produced using linkage through surface-accessible lysine residues, which are not easily separated by chromatography alone because of their high degree of heterogeneity.

Reversed-phase LC–MS can be used to elucidate the positional isomers of ADCs. Reversed-phase LC–MS following IdeS proteolytic digestion facilitates the subunit analysis of ADCs and enables rapid comparison of the ADC samples, for instance for batch assessment (**Figure 6**). Indeed, IdeS proteolytic digestion has been proposed as an analytical reference method at all stages of ADC discovery, preclinical and clinical development, for routine comparability assays, formulation, process scale-up and transfer, and to define CQAs in a QbD approach (32).

Chromatography and Native Mass Spectrometry

The ADCs currently approved for use utilize naturally occurring lysine side chain amino groups or the cysteine thiol groups, which are formed upon partial reduction of IgG intramolecular disulfide bonds, for conjugation of the drug load (34).

Cysteine-linked ADCs present a unique challenge for characterization because proper intact analysis requires native MS conditions to preserve structurally critical noncovalent binding between antibody chains.

ADCs exhibit significant heterogeneity resulting from the number and distribution of drug molecules across the antibody. This level of molecular complexity and heterogeneity presents a considerable challenge for current analytical techniques.

Native MS of intact proteins allows direct observation of molecules that rely on noncovalent interactions to preserve critical structural features, such as interchain associations that hold together cysteine-linked ADCs. The use of 100% aqueous and physiological pH buffers in native MS analysis produces decreased charge states (increased *m/z*) and improves mass separation of heterogeneous mixtures.

An orbital trap native MS workflow has recently been developed that is compatible with SEC, allowing online desalting and sample delivery, to observe intact proteins at high m/z ranges. This strategy reduces mass interference in complex protein spectra by increasing peak capacity in the m/z space. This workflow has recently been applied to the analysis of Adcetris and Kadcyla, cysteine-linked and lysine-linked ADCs,

Figure 6: Denaturing LC–MS analysis of the ADC brentuximab vedotin (Adcetris) (33). (a) Unmodified sample (1 µg) was analyzed by reversed-phase chromatography coupled to an orbital trap MS system produced several peaks. (b) The resulting averaged MS spectrum is a complex mixture of charge state envelopes as well as a vcM-MAE-specific reporter fragment ion at m/z 718. (c) Data analysis with ReSpect deconvolution and Sliding Window integration show roughly six covalently-structured forms of unraveled cysteine-linked ADC.

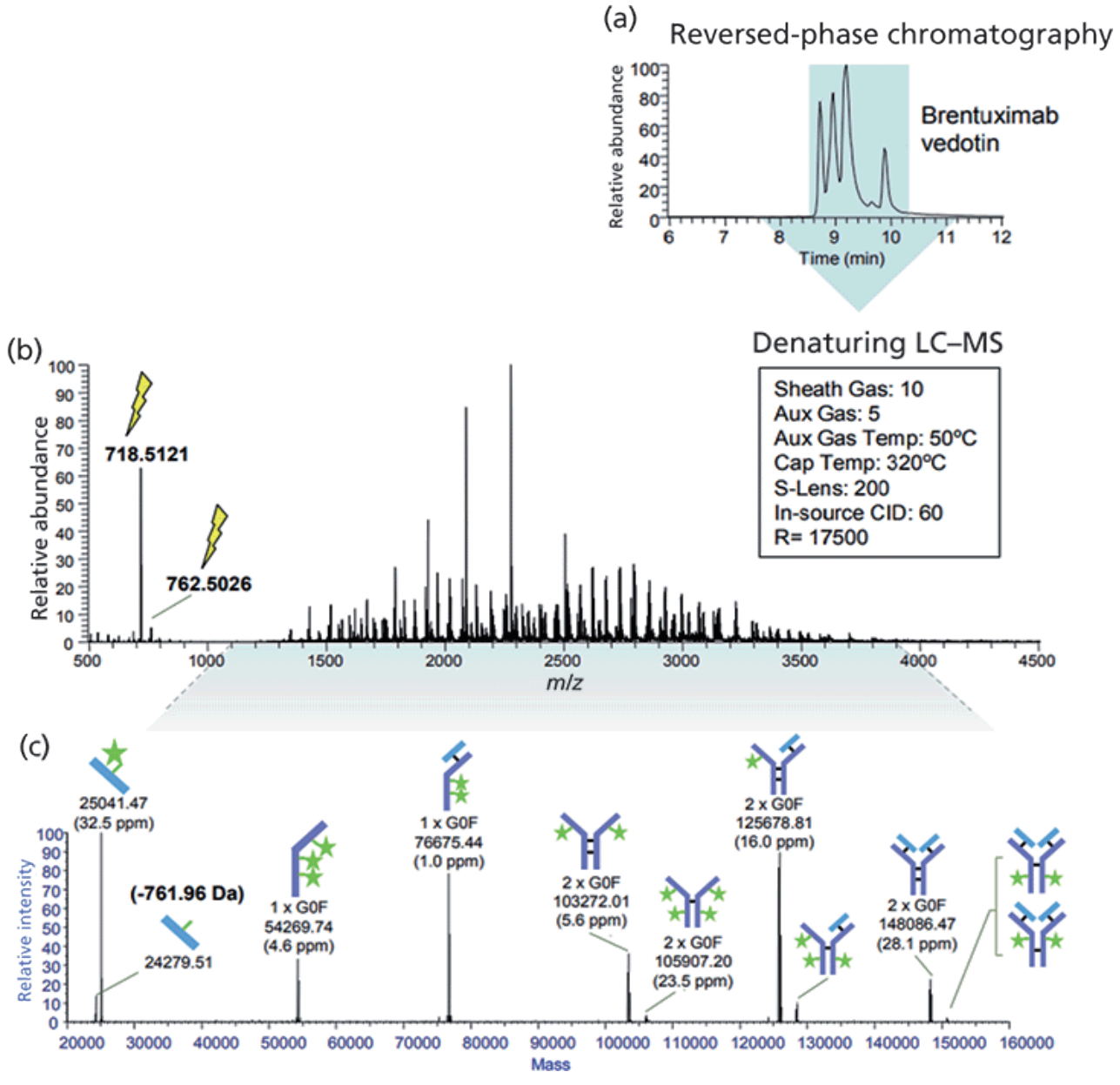
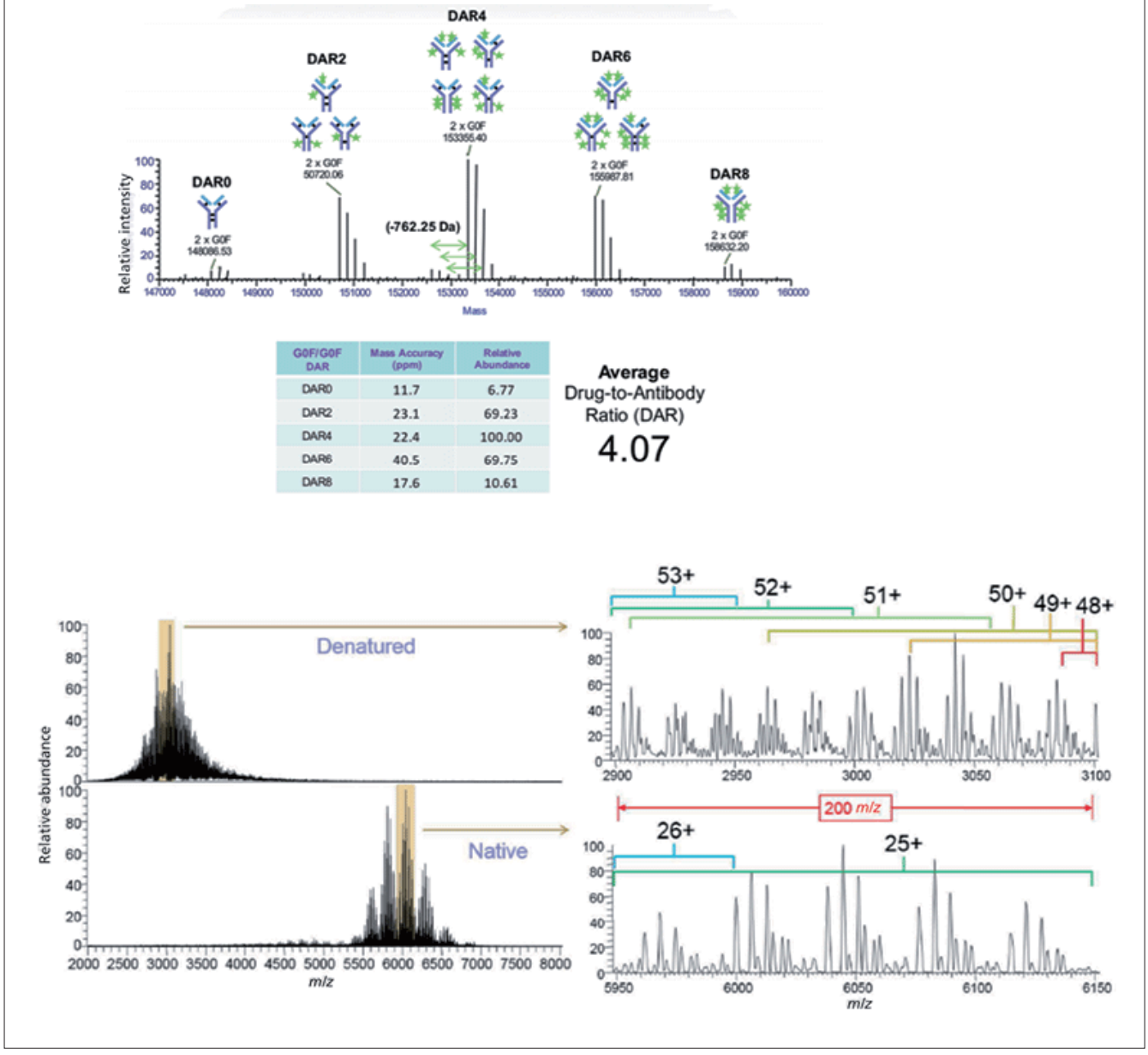


Figure 7: Desalting SEC–MS DAR of Adcetris and Kadcyla (35). (a) Desalting SEC is compatible with a native MS approach and allows preservation of noncovalent interactions which support the structure of cysteine-linked ADCs. Based on the individual deconvoluted abundances of the G0F/G0F glycoform, the authors calculated an average DAR value of 4.07 (32). (b) Denaturing MS spectra (from reversed-phase LC) are observed at lower *m/z* ranges while native MS spectra from online SEC are observed at higher m/z ranges. A detailed view shows that 2–3 sequential charge state envelopes overlap compared to an overlap of 0– charge state envelopes in the native MS spectrum.



G0F/G0F DAR	Mass Accuracy (ppm)	Relative Abundance
DAR0	11.7	6.77
DAR2	23.1	69.23
DAR4	22.4	100.00
DAR6	40.5	69.75
DAR8	17.6	10.61

respectively, and the accurate calculation of DAR (**Figure 7**).

This work built on a similar approach that was first applied to the study of Adcetris using an orbital trap mass spectrometer equipped with a high-mass quadrupole mass selector (36).

Higher Order Structural Analysis

Hydrogen–deuterium exchange (HDX)-MS is a powerful tool for studying the dynamics of higher-order structure of protein-based therapeutics. The rate of hydrogen-to-deuterium exchange within the amide hydrogen on the backbone of biotherapeutics provides solvent accessibility information, and thus protein structure and conformation can be inferred.

Although HDX-MS cannot be used to define an absolute structure in the manner of X-ray crystallography, it can be used to directly assess the native structure in a comparative fashion. Proteins in solution are highly dynamic, and the stability and functionality of any protein therapeutic are closely associated to a specific conformation.

The manufacturing of ADCs involves additional processing steps during conjugation, and it is important to evaluate how the drug conjugation process impacts the conformation and dynamics of the mAb intermediate. The ability of HDX-MS to monitor conformational changes at the peptide level makes the technique well-suited for providing detailed insights into the impact of drug conjugation processes on the higher-order structure of mAbs.

Orbital trap–based HDX-MS has previously been used to probe the conformation and dynamics of interchain cysteine-linked ADCs (37). In this publication, a side-by-side HDX comparison of ADCs, mAbs, reduced mAbs, and partially reduced mAbs was used to identify minor local conformational changes and confirm that these were because of the partial loss of interchain disulfide bonds in ADCs. These findings were used to indicate that ADC manufacturing processes that involve partial reduction of mAb interchain cysteine residues followed by conjugation with drug linkers do not significantly impact the conformational integrity of the mAb. A similar approach has been used to study the antibody structural integrity of site-specific ADCs (38). Together these results highlight the utility of HDX-MS for interrogating the higher-order structure of ADCs and other protein therapeutics.

TABLE I: Impurity dose based on the level of conjugatable impurities in the linker-drug intermediate (table adapted with permission from reference (39)

Impurity Level in Linker-Drug	Maximum Impurity Level in DS (wt/wt%)	ADC 5 mg Dose		ADC 50 mg Dose		ADC 50 mg Dose	
		Maximum Impurity Level	Maximum Daily Impurity Level	Maximum Impurity Level	Maximum Daily Impurity Level	Maximum Impurity Level	Maximum Daily Impurity Level
3%	1.5 µg/mg DS (0.15%)	7.5 µg/dose	0.36 µg/day	75 µg/dose	3.6 µg/day	0.75 mg/dose	36.0 µg/day
1%	0.5 µg/mg DS (0.05%)	2.5 µg/dose	0.1 µg/day	25 µg/dose	1.2 µg/day	0.25 mg/dose	12.0 µg/day
0.5%	0.25 µg/mg DS (0.025%)	1.25 µg/dose	0.06 µg/day	12.5 µg/dose	0.6 µg/day	0.125 mg/dose	6.0 µg/day
0.1%	0.05 µg/mg DS (0.005%)	0.25 µg/dose	0.01 µg/day	2.5 µg/dose	0.12 µg/day	0.025 mg/dose	1.2 µg/day

Residual Free Drug Analysis and Control Strategy for Small-Molecule Impurities in ADCs

On the analytical front, one approach to conducting free-drug analysis for ADC drug substance and drug-product preparations is to precipitate the proteins (along with protein-bound drug) and analyze the resulting supernatant using a method that is effective for detecting the small molecule such as those using UHPLC–MS or UHPLC with ultraviolet (UV) detection.

Residual Solvents and Volatile Organic Impurities in ADCs

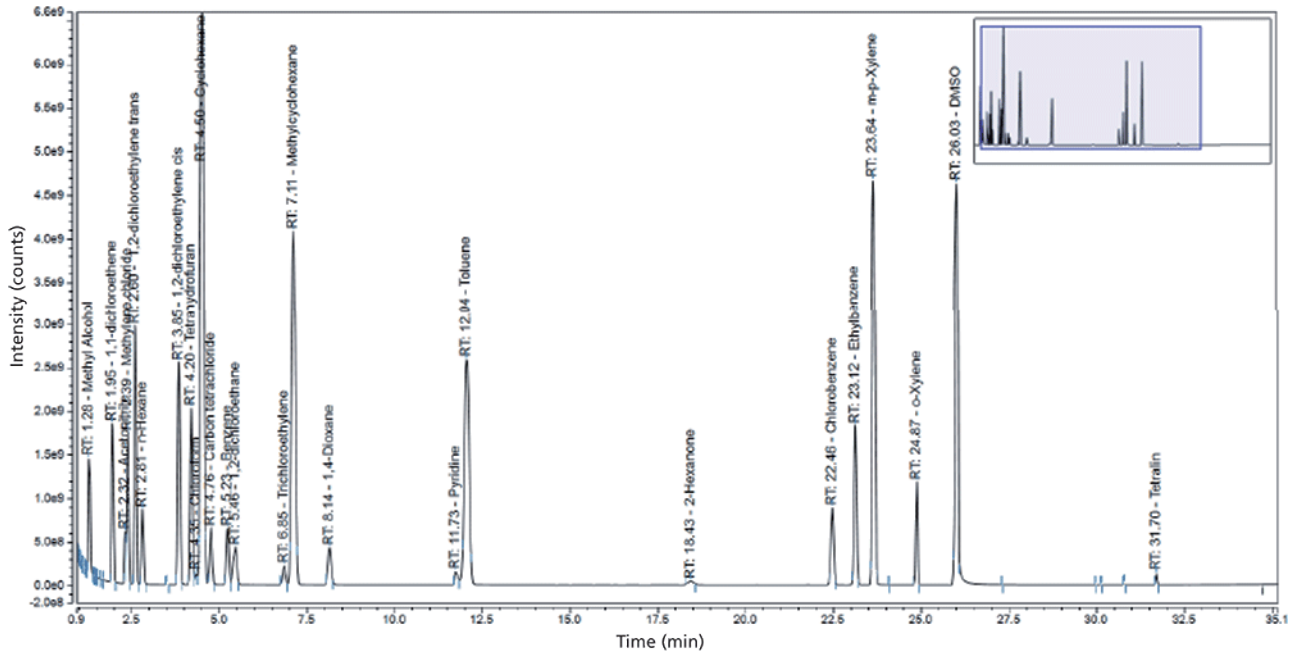
It is uncommon that residual solvent analysis is conducted for post-production quality assurance of conventional protein-based biopharmaceuticals such as mAbs. Organic solvents are not typically used in cultured cell trains and seldom form part of the risk profile of the drug.

In contrast, the conjugation reaction to form ADCs generally involves a site-selective enzymatic or chemical reaction of antibody to linker to small-molecule drug warhead, where the hydrophobic warhead and linker are solubilized in solvents such as N,N-dimethylacetamide (DMA), N,N-dimethylformamide (DMF), dimethyl sulfoxide (DMSO), or propylene glycol (PG). The conjugation process is followed by protein purification techniques to remove process-related contaminants (unconjugated toxin and residual solvents). However, strategies must be in place to monitor for such impurities. For the analysis of these residual solvents, one possible approach is to use a direct gas chromatography (GC) technique (43) after removal of the proteins rather than the traditional headspace GC approach in *USP* <467> (44). Because of the low levels expected for residual solvents in ADC samples, an alternative GC–MS method (particularly using the selected ion monitoring mode) is likely to yield higher sensitivity as well as provide identification information on unknown peaks, as shown in the example in **Figure 8.**

Bioanalysis of ADCs

ADCs are complex heterogeneous mixtures resulting from differences in glycosylation of the antibody, the number of small-molecule drug moieties attached to the antibody, and the location of the conjugation sites. This situation is further complicated as the drug undergoes in vivo changes such as spontaneous deconjugation of the small-molecule drug and differential clearance rates of

Figure 8: GC–MS of residual solvents following analytical headspace GC conditions similar to those in *USP* <467> that may provide higher sensitivity under single ion monitoring mode as well as information for unknown peak identification.



ADC components as a result of their different DARs. These changes, as well as other attributes of ADCs, contribute to the unique challenges in their bioanalysis. Furthermore, it is becoming clearer that the data required by the bioanalytical scientist is also dependent on the phase of the ADC development. The early discovery phase requires in vivo stability of ADC candidates based on monitoring average DAR or presence and integrity of the drug moiety at a specific conjugation site, while in the clinical development phase, it is important to establish a correlative relationship between one or more components of the ADC and various safety and efficacy indicators. Therefore, to address these bioanalytical challenges both ligand binding assays (LBAs) and LC–MS have been used. For instance, measurement of total antibody to assess antibody pharmacokinetic (PK) behavior and measurement of conjugated antibody (DAR ≥ 1) is typically performed using LBAs, with unconjugated drug monitored by LC–MS. However, a hybrid of the two approaches, referred to as hybrid LC–MS, is becoming more actively developed and applied in ADC bioanalysis. This platform uses the affinity capture of the LBA to retain sensitivity and LC–MS for detection to provide greater specificity and improved characterization of the ADC component being monitored. Therefore, the hybrid LC–MS approach provides benefits of both the LBA and LC–MS, enabling scientists to better address some

of the unique challenges of ADC bioanalysis and to allow for the use of a single platform to generate the data required for ADC bioanalysis (45).

Summary

ADCs are an increasingly important class of biotherapeutics. As the list of the first-generation ADCs entering the clinic grows, new generations of ADCs will benefit from their insights. The future looks set to see ADCs that have higher levels of cytotoxic drug conjugation, lower levels of unconjugated antibodies, more-stable linkers between the drug and the antibody, and increasing analytical challenges. The stability of linkers in circulation is critical to ensure patient safety and to mitigate the side effects caused by the off-target release of toxic payloads.

Today's ADCs pose unique analytical challenges requiring increasingly powerful approaches, consisting of small- and large-molecule techniques for their comprehensive characterization. The complexity of their analysis is matched only with their potential to become the "magic bullet" of anticancer treatment.

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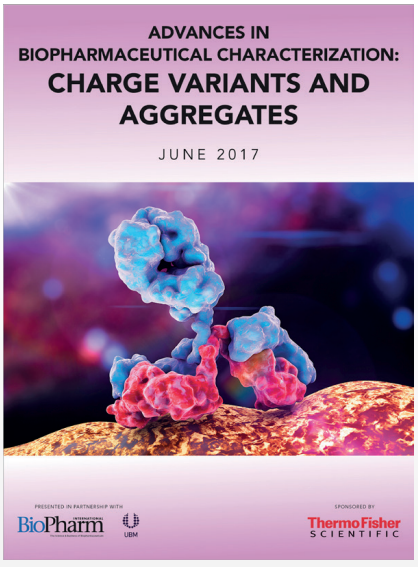

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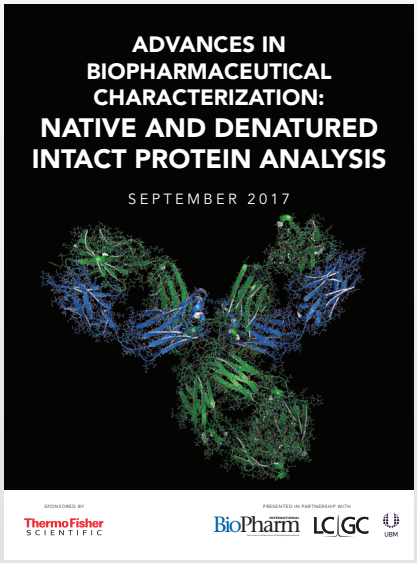

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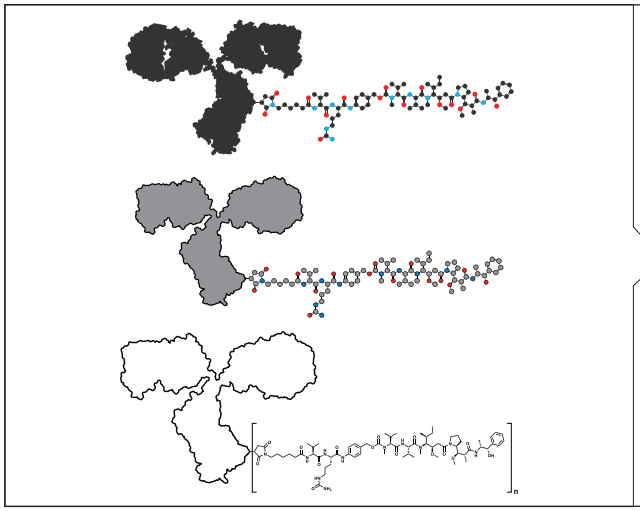

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Update: Decomplexifying ADCs with Native Intact Mass Analysis

An interview with Dr. Krisztina Radi

The complexities of therapeutic proteins present numerous challenges to analytical laboratories. Analysts are increasingly finding that native mass spectrometry provides several benefits over traditional techniques, including faster, more complete information. Here, Dr. Krisztina Radi, BioPharma Manager at Thermo Fisher Scientific, and previously an antibody-drug conjugate (ADC) Analytical Scientist with MedImmune, describes the current state of this important and growing area of mass spectrometry.

Biopharmaceuticals, and in particular ADCs, are very complex. What are some challenges and pain points that laboratories face when characterizing these molecules?

Radi: Whereas traditional small molecules are synthesized chemically, larger biopharmaceutical molecules are produced through complex bioprocesses. Numerous possible structural modifications can be created during the production of biologics, which can lead to product impurities and variants in the drug profile. All of these factors can influence the safety and the efficacy of the drug product.

Confirmation of the primary structure and post-translational modifications (PTMs) of large molecules are necessary quality control mechanisms. For instance, charge heterogeneity must be analyzed for monoclonal antibodies, their aggregates and fragments (which might cause immunogenic responses) should be controlled, and higher order structure must be investigated (which can affect the biological function). Other areas that must be looked at include biological functions, bioassays, binding assays, and other multi-level analyses.

One must also look at process-related impurities, which are not as straightforward to control as they are for chemical synthesis small molecule drug products. Biopharmaceuticals may contain host cell proteins, which may be retained. Thus, process-related impurities should be eliminated during process development.

Antibody–drug conjugates (ADCs) are even more complex than monoclonal antibodies, due to the additional heterogeneity resulting from conjugating a cytotoxic drug with a chemical linker to the protein. This necessitates analysts to look at additional unique quality attributes related to drug load/drug distribution and drug-to-antibody ratio. Several orthogonal analytical techniques—heavily based on liquid separation techniques and mass spectrometry (MS) techniques—are often applied for the quality control of biologic molecules.

What have you seen in terms of developments in LC–MS instrumentation or techniques to keep up with some of these challenges?

Radi: Mass spectrometry is the one technique that is constantly evolving and seems to be developing quicker than any other technique and with more impressive updates.

Thermo Fisher Scientific has focused the development of its recent high-resolution accurate mass MS system to the needs of the analysts in the biopharmaceutical characterization laboratory. The Thermo Scientific™ Q Exactive™ Biopharma platform of mass spectrometers have recently been updated to include the Thermo Scientific™ Q Exactive™ HF-X mass spectrometer, capable of 10 times greater sensitivity under native conditions than previous models. The biopharma option available on these instruments offers three operational modes to cover different level protein characterization (peptide mapping, intact, and native MS characterization). Orbitrap-based MS instruments have long been the technique of choice for peptide mapping; but now they are offering exceptional intact and native protein analysis capabilities too.

Intact level subunit analysis (LC, HC and conjugate chains for ADCs, IdeS digested mAb subunits) is the first level strategy for ADC laboratories, is readily available in Protein mode, and intact mass analysis under native and denaturing conditions in the High mass range mode.

What is really new and exciting in the field of MS is that native mass spectrometers are becoming a readily accessible and suitable tool for looking at extremely complex biopharmaceuticals. These instruments provide the opportunity to look at large molecules in their own true, native structure, which is very useful. For instance, this direct view allows analysts to assess heterogeneity instantly, like drug load distribution varieties for ADCs. Legacy analytical methods required the use of several orthogonal analytical techniques

to answer key characterization questions. Now, with native MS capabilities on the Q Exactive BioPharma platform, which allows mass-to-charge ratios of up to 8000 *m/z*, the antibody samples can be analyzed in non-denatured form with the charge envelope shifted to a mass range of *m/z* 5000-7000. This simplifies spectral interpretation and allows small features in the data to be identified, that would not be detectable under denaturing conditions.

"With native MS capabilities on the Q Exactive BioPharma platform, which allows mass-to-charge ratios of up to 8000 *m/z*, the antibody samples can be analyzed in non-denatured form with the charge envelope shifted to a mass range of *m/z*, 5000–7000."

This feature rich data demands flexible and powerful software tools to support intact analysis; Thermo Scientific™ BioPharma Finder™ software has two deconvolution algorithms for intact protein analysis to turn a charge state series into a molecular mass. One algorithm deconvolves the isotopically resolved high-resolution spectra to provide monoisotopic masses while another deconvolves the unresolved lower resolution mass spectra to provide average masses to help analysts look at the native state and immediately acquire important information. So, you can get a much more straightforward view of the native state, and a higher spatial separation of your ions in reduced charge states.

Native MS started as a research or academic kind of tool under static flow conditions using gold-coated borosilicate capillaries. Now, we have chromatographic techniques coupled to the mass spectrometer, which allows us to look at more native-like structures as these chromatographic techniques allow to maintain that kind of structure. For example, size-exclusion chromatography separation is now readily coupled to the native MS instrument. This provides a very quick analysis without major sample preparation because the buffer exchange is readily done through the chromatographic step, which saves time. This analytical process can be used as a quick screening method.

Another key thing about native MS is that it maintains non-covalent interactions, which are very helpful to look at. For example,

some ADCs are conjugated through cysteines after reduction of interchain disulphide bonds. Usually with an intact denatured MS analysis these molecules would simply fall apart into their subunits, not maintaining non-covalent binding between antibody chains and then you would never really be able to look at its true form. Here, you can maintain non-covalent interactions, and assess the drug-to-antibody ratio on the whole antibody scaffold, which is very much a critical quality attribute for these types of conjugated biomolecules.

You mentioned that this technique started out as a research tool. Do you see drug companies now using this equipment in the discovery stage or are they carrying it through the development process and checking for quality throughout their processes?

Radi: I think this could become a screening tool for both. In both discovery and development phases, you need really good analytical tools that can give you good structural insight as quickly as possible. Scientists at LifeArc are using the Q Exactive BioPharma platform as they look at the conjugation chemistry to help screen and select molecules to take into the next development steps.

This technique is really good for getting a quick answer during the discovery phase. For instance, you can quickly assess how a conjugation condition affects the drug-to-antibody ratio or the drug load with the native MS.

It is also a good technique when you get to development or later phases and you need a quick snapshot overview of the structure for QC purposes. While it is developing area for native MS, having a good method for delivering good results very quickly is a key tool in biopharmaceutical development because it's important to ensure process consistency with various analytical assessments in a timely manner.

So overall, native MS definitely has potential for moving into later phases of the development.

Is the technique well understood by analysts? Is it something that they can use without a lot of training?

Radi: Definitely. Customers like LifeArc were up and running with this equipment in days and can confidently use the instrument to routinely analyze their complex biological molecules in the different characterization workflows.

Our equipment has several pre-optimized tune settings and the Thermo Scientific™ Chromeleon™ Chromatography Data System (CDS) Software helps laboratories get into this kind of easily simplified workflow setting, in a fully GMP compliance-ready environment. These molecules are very complex and although the analytical tools have great complexity behind them, end users can easily use them with the characterization workflows following an initial familiarization training. Users really like that kind of integrated software and workflows to simplify their processes and get answers as soon as possible.

What other advances have you seen in native MS?

Radi: We recently released the Thermo Scientific™ Q Exactive™ UHMR ultra-high mass range MS instrument. This very

unique instrument gives structural biologists and biotherapy companies the opportunity to look at complex protein–protein interactions. For instance, you could look at ADCs or antibodies actually bind to their antigens while you are still maintaining the very high resolution. It has a mass range up to 80,000 *m/z* allowing the study of ever-larger molecules, as well as protein-protein interactions, such as aggregation.

The combination of high mass range and high resolution is a key advantage and is a really good way of helping to get more structural insight about complex molecules, which increases product knowledge and improves analytical method development.

What might the future hold for analytical technologies with regard to ADC characterization?

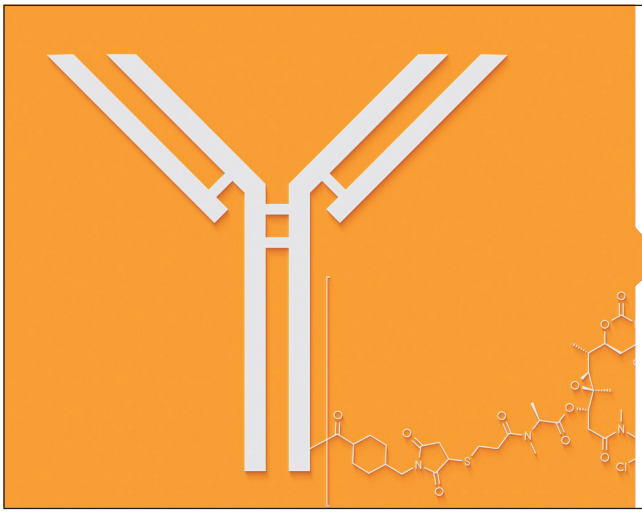
Radi: Equipment suppliers want to make analytical technology easier for analysts to use, such as providing really good workflows, developing excellent instruments, and supporting the whole process with the necessary software.

There is a lot of effort going into software development. We have integrated software solutions for analytical process like the Chromeleon software, which can be compliance ready. People really appreciate having compliance-ready data integrity as well as tools to look at the data with the Biopharma Finder. The two together provide a ready data control and interpretation tool for users, which is key. Years ago, Native MS was a technique for specialist mass spectrometrists. Today, native mass spectrometry is becoming ever more democratized; putting deep structural insights into the hands of drug developers, from discovery through to manufacturing.

That's one direction we definitely go in the analytical lab—not just concentrating on the technical developments, but also supporting the method development with integrated software and data interpretation tools. For example, we can use the sliding window algorithm, which eliminates the need to look at individual chromatographic peaks. This algorithm in the software makes it possible not just to combine certain regions, but also to produce a list of "component peaks" for individual retention times. It identifies the components directly, rather than looking at the peaks. That might give you an additional level of information that you might not otherwise get, for example low abundance DAR species or ADC variants with drug related impurities being conjugated to the mAb.

In addition, we are expanding the boundaries of Native MS by coupling it with ion-exchange chromatography and other chromatographic techniques that are traditionally not coupled with mass spectrometers because they use high concentrations of non-volatile salts. These chromatographic techniques may better separate the different DAR-species further decomplexifying the MS analysis of ADCs.

In summary, instrument suppliers are working to support hardworking biopharmaceutical scientists by helping them get the answers as soon as possible as best as possible, and to provide reliable, high-quality data.



Tackling Analytical Method Development for ADCs

Cynthia A. Challener

The targeted therapy possible with antibody-drug conjugates (ADCs) makes them an attractive class of drugs. Their composition–a biologic linked to a small-molecule cytotoxic, which enables targeted delivery—also creates challenges for analytical method development, both for characterization and lot release purposes. Expertise in both chemical and biological analysis is required, and techniques for both types of molecules must be used. In addition to performing typical characterization studies, the linker chemistry and its impact on heterogeneity and applicable analytical techniques must be understood. The influence of conjugation on antibody binding must also be evaluated, and a wide range of stability studies must be conducted. Fortunately, both improvements in analytical technology and increased experience with ADCs are leading to improved strategies for analytical method development and validation.

Both Small and Large

As for any drug, analytical methods for the characterization of ADCs must be developed, and separate quality control (QC) tests must be established for lot release of the final drug substance. Because ADCs are both small drug and biologic compounds, characterization and validation need to be appropriate for both types of products. "Testing requirements will still be identity, purity, impurities, activity, concentration, and stability as outlined in the International Conference on Harmonization's ICH Q5C (1) and ICH Q6B (2) biologics guidelines; however, the testing must cover both functional and physiochemical properties, including process control methods and release and stability-indicating assays for both the large and small molecule," says Lisa McDermott, principal scientist at SAFC.

Because ADCs are made of three different components, McDermott believes it is crucial to have an advanced control strategy for each of the intermediates, with testing profiles determined as if each of

the components is being developed as a stand-alone drug substance. "With this approach, many of the quality parameters can be controlled in the release of these intermediates and allow the final release strategy to focus on the quality of the ADC."

Main Methods

"Conjugation usually results in a mixture of ADCs with different drug-to-antibody ratios (DARs), free drug, and naked antibody concentrations," says Harpreet Kaur, synthetic chemistry team leader with Dalton Pharmaceutical Services. This increased heterogeneity associated with all ADCs, even site-specifically linked ones, requires the development of robust methods with sufficient resolution to characterize and measure the diversity of product-related species and potential impurities, according to Fred Jacobson, staff scientist and Kadcyla technical development leader with Genentech.

The critical properties for ADC characterization include target site-specificity and binding properties, stability of the linker and drug species, drug potency and free drug, site(s) of conjugation, DAR, heterogeneity, and solubility. In general, Jacobson notes that most modern chromatographic, electrophoretic, and spectroscopic (ultraviolet-visible [UV-vis] and mass spectrometry [MS]) methods have proven adequate to the task.

UV-Vis spectrophotometry has traditionally been used to measure ADC and free drug concentrations and average DAR. The challenge with this method, according to Kaur, is that the extinction coefficient (λ_{max}) of the drug may change when conjugated to the antibody or in a different buffer. In addiiton, the drug and antibody should have different λ_{max} values.

Chromatographic methods based on hydrophobicity (hydrophobic interaction chromatography [HIC]) and size (size-exclusion chromatography [SEC], SEC-multi-angle laser light scattering (SEC-MALLS) can provide information about the number and location of conjugation sites, average DAR, and free drug content, although these methods are not suitable for purification and characterization of ADCs produced using linkage through lysine residues due to their high degree of heterogeneity, according to Kaur.

Mass spectrometry (MS) methods are often introduced early in the development process to better understand the product and conjugation or to act as an orthogonal tool to standard chromatographic techniques that will ultimately be required for product release, according to Allan Davidson, analytical development manager for Piramal Healthcare. "While processes can be developed to provide a consistent and accurate drug load, most products remain highly heterogeneous and as such there is the need to better understand the complex picture of antibody structure and drug distribution," he says.

Electrospray ionization (ESI)-MS, liquid chromatography (LC)-MS/MS, and matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) have been used extensively to analyze the DAR, free drug

and metabolite concentrations, and linker stability. The validation of MS methods can be a challenge, however, due to differences in ionization of ADCs with different DAR values and ADC linker hydrolysis under acidic LC-MS conditions or by the acidic matrices used for MALDI, according to Kaur.

Finally, bioanalytical immunoassays, such as enzyme-linked immunosorbent assays (ELISA) are used for quantitation of the ADC, naked antibody, and free drug content, determination of the extent of ADC binding to the target antigen, and to establish the stability of linker and drug and the immunogenicity, while cell-based mechanism of action assays are used to assess the target-killing ability of the drug. "The biggest challenge with these methods is that the binding of the antibody to the target antigen can be altered by the site and degree of conjugation," says Kaur.

Analysis of the small-molecule components (linker and drug) is relatively straightforward with well-defined expectations in line with what would be required for an API, according to Jacobson. "One important difference, however, is the requirement for understanding the impact of impurities in the small molecule on the process and quality of the ADC," he observes.

In addition, bioanalytical methods are required to determine ADC potency, both with respect to and target binding (ELISA-based). Characterization should also cover the impact of conjugation on antibody binding properties towards specific Fc-receptors, because they may affect pharmacokinetic (PK) or secondary mechanisms of action, according to Jacobson.

Priority assays, according to McDermott, include determination of the potency, drug load and distribution, and size variants. Early development of cytotoxicity assays and chromatographic assays such as HIC, reduced reverse phase high-performance liquid chromatography (RPHPLC), and SEC is therefore important. She adds that additional analytics are often used to underpin the accuracy of these methods in early development stages. LC-MS, for example, is often used to assign structural information to the individual components of a mixture for confirmation of drug load and distribution.The presence of hydrophobic linkers and drugs occasionally leads to problems, however.

As an example, Jacobson points to the difficulty associated with charge-based assays such as ion exchange HPLC (IEX). "Although several new stationary phases have appeared, none have proven great for ADCs due either to nonspecific interactions or inadequate resolution. Capillary isoelectric focusing (cIEF), such as imaged cIEF, has been a reasonable substitute, but experience with monoclonal antibodies (mAbs) suggests that there may be differences in the information obtainable by each method," he comments. Jacobson also notes that it is difficult to do preparative collection of charge variant species from capillary electrophoresis (CE) for characterization.

Bioanalysis in Biological Fluids

From an immunoassay perspective, the development and validation of methods for the bioanalysis of ADCs require the

consistent availability of high affinity, high specificity, anti-toxin antibody reagents to ensure appropriate selectivity in the analysis, according to Michael Brown, director of ICON's Bioanalytical Laboratories. "Generation of these types of antibodies to small molecular weight toxins is considered an art, and often times their availability can be limited," he observes.

Given the potency of the toxins, it can be a challenge getting an MS method developed and validated with the required lower limit of quantitation (LLOQ). Identifying the metabolites of the toxin can be even more difficult because they are generally present in even lower concentrations than the parent. In addition to the determination of very low concentrations of released drug in physiological fluids in the presence of relatively high ADC levels, interaction of reactive intermediates with albumin or other biomolecules, changing DAR values, and the inability to use assays developed for the parent antibody for the corresponding ADC with a different architecture are also issues that must be considered when evaluating the systemic exposure of ADCs as part of drug pharmacodynamic and pharmacokinetic analyses, according to Kaur from Dalton.

One approach is to use hybrid techniques such as affinity capture LC-MS/MS, but that adds complexity to the analysis. A combination of immunoassay and LC-MS/MS techniques can also be used in addition to the hybrid techniques. "There is also increasing interest in the use of high resolution accurate mass spectrometry to support various facets of ADC bioanalysis given the complex and dynamic nature of these molecules," says Mario Rocci, senior vice-president of ICON Bioanalytical Laboratories.

Generally, five different analyses are required to characterize the *in-vivo* performance of an ADC, according to Paula Jardieu, senior vice-president and general manager of ICON's Bioanalytical Laboratories, including quantitation of the time course of the intact ADC, the total antibody, and the toxin with and without linker, plus the immunogenicity of the ADC. Changes in the DAR over time in vivo, as well as the stability of the ADC in the matrix, also must be evaluated. It is also important to establish the stability of the ADC through the analytical procedure, because instability could introduce artifacts, according to Jardieu.

Free drug issues are typically monitored using reverse-phase chromatography with UV detection, or if the levels of detection must be very low, using LC-MS/MS against a drug linear curve, according to Dan Peckman, biochemistry manager with Eurofins Lancaster Laboratories. He also notes that confirmation that the DAR does not change with time as a function of molecular stability is typically achieved using LC or UV approaches. Mass spec analysis of the intact ADC and the naked antibody can give relevant information about the stability of the ADC.

The Platform Approach

For many small-molecule drugs, a platform of generic screens is often used for drugs

based on their structures and mechanism of action. For ADCs, however, it is difficult to generate "platform" methods that are suitable to a variety of ADCs because there are many non-specific interactions with stationary phases post-conjugation, according to Davidson. McDermott adds that while many of the analytical methods for ADCs are based on similar techniques, each construct is unique and requires an understanding of the basic chemical or physical properties that must be assessed. "Asking the right question is as important as getting an answer," she says.

Even given these challenges, SAFC has been able to develop screens for drug load using HIC and reverse-phase chromatography, and a screen for monomer purity using SEC. "These platform methods allow us to move quickly through the development phase and focus on more challenging assays early in the project," she explains. Other techniques such as iCE, CGE, ELISA, cytotoxicity and methods for residuals are developed by subject matter experts and optimized for each product. Testing for safety and quality attributes (bioburden, endotoxin, pH, osmolality, excipients, and appearance) are either verified using compendial-based platform methods or developed per product.

Analysis through the Development Process

As ADCs move through the drug-development process, different analytical methods are often required. Much of the early-stage development is driven by methods required to support development of the conjugation process, according to Davidson, and only a few methods may be required to support early conjugation process development, for example, SEC for aggregation, HIC for DAR, and RPC for free drug. Once the conjugation process has been established, additional analytical methods are required to ensure that the functionality of the antibody and potency are effective, and other characterization methods are then introduced (e.g., cytotoxicity, binding, charge heterogeneity, residual solvents, excipient testing, etc.).

For the production of early-phase clinical supplies, in fact, McDermott notes that multiple orthogonal methods are required to ensure method accuracy and process consistency. Then, as the phase of development advances further and multiple lots are produced, the number of methods can be reduced to the ones determined to provide information around critical quality attributes. Methods are often optimized as the project progresses, and further understanding of the chemistry is achieved. "A good example for ADCs is the assay for monitoring any residual-free drug equivalents," she comments. "Typi-

cally, this method is first developed for either the drug or drug linker used in production. As stability information or multiple lot information is available, further compounds that are drug-related may be detected, and the method will be modified to track these impurities. Continual monitoring of release and stability data is necessary to ensure adequate methods are available for validation."

It is important to realize, Jacobson adds, that sometimes knowledge from characterization studies will result in the need for a method for lot-release or may be used to justify its omission. "The availability of analytical methods and product-specific knowledge that may lead to the addition of new assays (or replacement of older technologies) as a clinical candidate approaches licensure," he says.

Building a Bridge

ADCs present a complex bioanalytical problem that requires leveraging large- as well as small-molecule analytics. Recent developments in LC-MS/MS technology and the use of orthogonal analytical and bioanalytical methods combined with process knowledge support the idea that manufacturing and analysis of ADCs with consistent quality and efficacy is achievable, according to Kaur. He also notes that with the move to adopt a quality-by-design (QbD) approach for defining critical quality attributes for ADC, it is becoming easier to demonstrate the reproducibility of conjugation and ADC analysis. Meanwhile, McDermott asserts that "Advances in method development require subject matter experts in both fields to forge integrated techniques that will bridge the knowledge gap and blend pharma and biotech platforms, and the interface of these analytical techniques allows real understanding of the ADC construct to be achieved."

Importantly, sharing of information is taking place not only between different groups within pharmaceutical manufacturers, but also between companies. "Cross-fertilization within the biopharmaceutical industry, particularly driven by the proliferation of ADC-focused conferences, workshops, instrument company webinars, etc., has lead to an increased convergence in the methods being used," observes Jacobson. "While there are clearly some differences, for example those that might result from the specific requirements imposed by a particular company's unique conjugation technology, in general good ideas are being picked up and incorporated widely as they are introduced," he continues. Peckman agrees. "I think the most helpful resource is the sharing of information from technical presentations and

technical papers. We are at a point where many groups are experiencing the same challenges with characterizing ADCs, and evaluating the research results obtained by others inspires new ideas for method changes in the future."

Evolving ADC Chemistry

Adding to the complexity of ADC method development and validation is the growing diversity of bioconjugates being advanced into the clinic. "Traditional cysteine and lysine chemistry is still a significant part of the ADC regime, but we are seeing a greater breadth of linkers, toxins, mAbs, and classes of conjugation chemistry. New analytical methods are needed for each of these new classes, both for characterization of the chemistry and release and stability testing," says McDermott.

In addition, as more products make it into the clinic and then into the market, Jacobson expects that regulators will become more familiar, and more comfortable, with the ability of current analytical tools to demonstrate the robustness of ADC manufacturing. "One consequence may be a better definition of what really needs to be controlled by product testing. In such a rapidly evolving area, the regulatory requirements are clearly evolving as more knowledge makes it into health authority submissions and into the peer-reviewed literature," he says.

On the other hand, as the ADC industry continues to expand at pace and current understanding of the complex interactions between drug payloads and antibodies increases, Davidson notes that new problems are constantly being uncovered that often require analytical solutions. "It can therefore be expected that more issues will be identified and there will be more challenges to come," he concludes.

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Cynthia A. Challener, PhD, is a contributing editor to BioPharm International.

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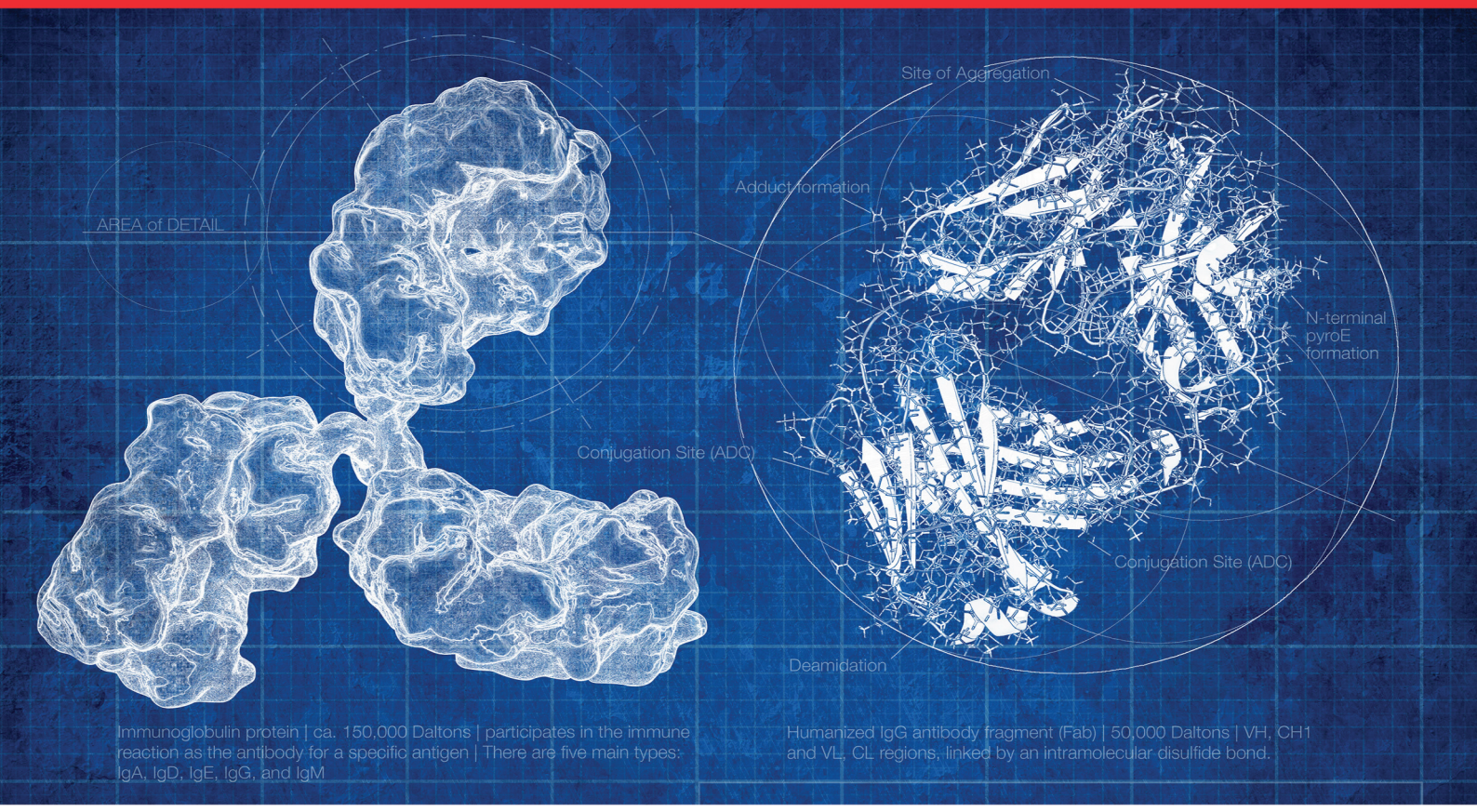

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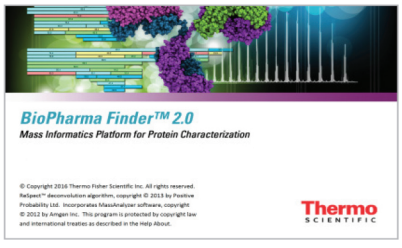

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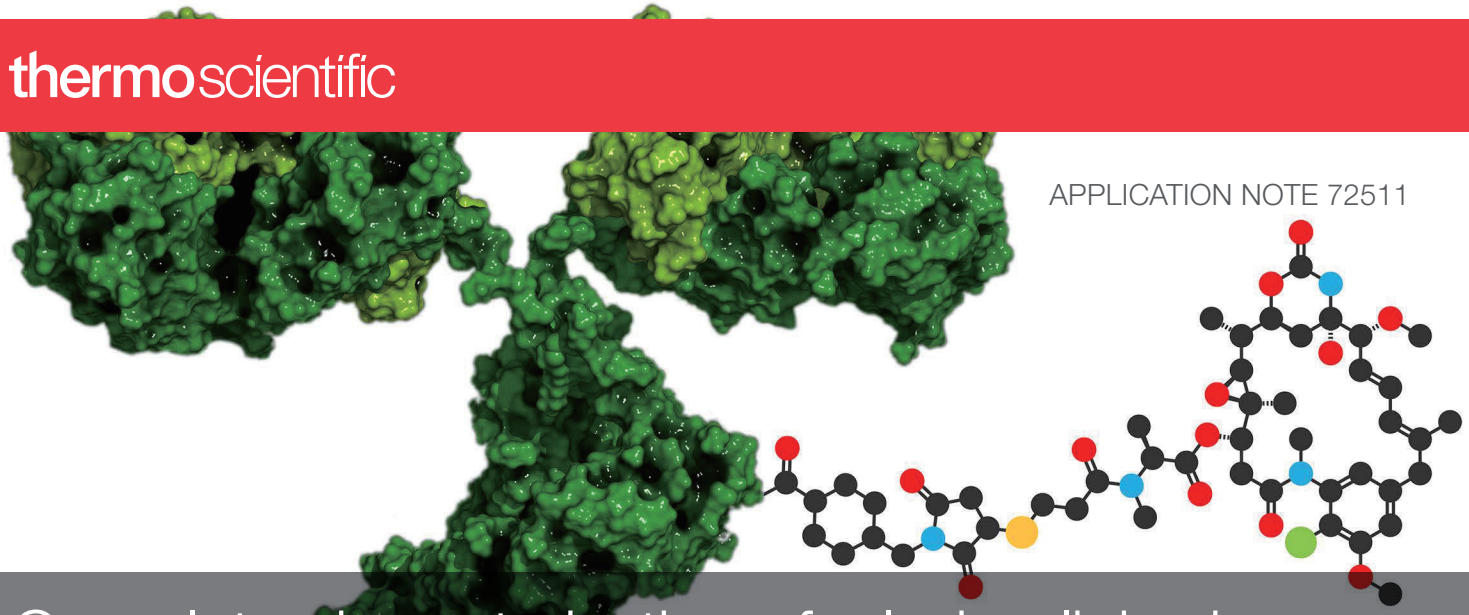

Complete characterization of a lysine-linked antibody drug conjugate by native LC/MS intact mass analysis and peptide mapping

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Keywords

Native MS, intact mass, peptide mapping, characterization, antibody drug conjugate, ADC, drug-to-antibody ratio, DAR, size exclusion, reversed phase, chromatography, mass spectrometry, Q Exactive, Orbitrap, biopharmaceutical, biomolecules, High Mass Range mode, BioPharma Option

Goal

To perform complete characterization of the lysine-linked antibody drug conjugate (ADC) trastuzumab emtansine using both intact mass and peptide mapping approaches with a single benchtop mass spectrometer. Critical quality attribute (CQA) measurements include drug-to-antibody ratio (DAR) and glycosylation pattern via intact mass analysis, and conjugation site localization via peptide mapping.

Introduction

Biotherapeutic compounds, also known as biologics, have been produced for almost four decades to provide new treatments for a variety of disease classes, including heart disease, arthritis, and several types of cancer. Monoclonal antibody (mAb)-based drugs have proven to be a powerful means for highly specific targeting of a therapeutic chemical activity to the site of a disease epitope. Such a strategy is employed in the design of antibody-drug conjugates (ADCs). ADCs are comprised of a disease-specific

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(usually cancer-specific) mAb that is decorated with one or more cytotoxic small molecules. Utilizing antibody-based targeting to deliver highly toxic compounds directly to cancer cells minimizes off-target activity and patient morbidity.

ADCs can be highly complex chemical structures. Trastuzumab emtansine is a lysine-linked ADC sold commercially as Kadcyla® (Genentech, Inc.). The manufacturing strategy for first generation lysine-linked ADCs involves conjugation of a linker moiety to free side chains of lysines on a mAb, followed by an additional round of conjugation of the mAb-linker to a cytotoxic drug. Two-stage conjugation results in a complex mixture of mAb-linker-drug compounds, which varies in terms of the number of conjugated drugs and/or linkers, and is further complicated by any chemical complexity already presented by the mAb. Such a high level of chemical complexity poses significant challenges for the analytical characterization of lysine-linked ADCs.

Complementary MS-based approaches of peptide mapping and intact mass analysis are needed to facilitate complete characterization of all complex biologics including ADCs. The use of 100% aqueous mobile phases at neutral physiological pH is known as native MS, or native LC/MS when coupled to separations. Native LC/MS analysis will allow proteins to retain physiologically similar structure. Compared to denaturing approaches such as reversed-phase liquid chromatography (RP LC) coupled to MS, performing size exclusion chromatography (SEC) using native, MS-friendly mobile phases produces spectra at higher mass-to-charge (*m/z*) and with reduced charge state values. Using native MS to improve mass separation of heterogeneous mixtures is a powerful strategy when considering the analysis of complex biotherapeutics such as random-lysine-linked ADCs. Here, a benchtop quadrupole-Orbitrap™ mass spectrometer has been utilized to perform both peptide mapping and high-resolution native LC/MS intact mass analysis. In this study, integrated characterization of a lysine-linked ADC, utilizing high mass range for native intact mass analysis and standard mode for peptide mapping, is demonstrated.

Experimental

Sample preparation

Consumables

- Thermo Scientific™ MAbPac™ SEC-1 column, 4.0 × 300 mm (P/N 075592)
- Thermo Scientific™ Acclaim™ Vanquish™ C18 column, 2.2 µm, 2.1 × 250 mm (P/N 074812-V)
- Thermo Scientific SMART Digest™ Kit (P/N 60109-101)
- Fisher Scientific™ LC/MS grade water (P/N W/011217)
- Fisher Scientific™ LC/MS grade acetonitrile (P/N A/0638/17)
- Thermo Scientific™ Pierce™ formic acid LC/MS grade (P/N 28905)
- Thermo Scientific™ Pierce™ DTT (Dithiothreitol), No-Weigh™ format (P/N 20291)
- Thermo Scientific™ Invitrogen™ UltraPure™ Tris hydrochloride (P/N 15506017)
- Honeywell Fluka™ TraceSELECT™ ammonium acetate, 99.9995% purity (metals basis)

Sample preparation for intact mass analysis

Powdered trastuzumab emtansine in formulation buffer was resuspended in water, yielding a final concentration of 5 mg/mL. A volume of 10 µL (50 µg total amount) sample in formulation buffer was autosampler injected on to a MAbPac SEC-1 column for LC/MS analysis.

Sample preparation for peptide mapping

Trastuzumab emtansine (50 µL) in formulation buffer (5 mg/mL) was added to a volume of 150 µL SMART Digest buffer (SMART Digest Kit) and vortexed for 10 s. The mixture was then transferred into a SMART Digest vial and incubated for 45 min at 70 °C with agitation (1,400 RPM, Eppendorf™ ThermoMixer™). Supernatant was transferred to a fresh 0.5 mL microcentrifuge tube. To reduce disulfide bonds, 50 µL of a 100 mM solution of DTT in water (final concentration of 20 mM) was added to the digested peptides and the solution was incubated for 45 minutes at 57 °C. The resulting peptide digest concentration was 0.5 µg/µL. A volume of 20 µL (10 µg total amount) was injected on to an Acclaim Vanquish C18 RP column.

Liquid chromatography and mass spectrometry

LC system configuration

- Thermo Scientific™ Vanquish™ Binary Pump H (P/N VH-P10-A)
- Split Sampler FT (P/N VF-A10-A)
- Column Compartment H (P/N VH-C10-A)

MS system configuration

- Thermo Scientific™ Q Exactive™ Plus hybrid quadrupole-Orbitrap mass spectrometer (P/N 0726030)
- BioPharma Option (P/N 0726055)

Native intact mass analysis LC/MS

SEC-LC conditions are described in Table 1. Settings for HMR mode MS tuning and method parameters are described in Table 2.

Table 1. Size exclusion LC conditions for native intact mass analysis.

LC Conditions	
Column:	MAbPac SEC-1 (4.0 × 150 mm)
Mobile Phase A:	50 mM ammonium acetate in water
Flow Rate:	0.300 mL/min
Column Temperature:	30 °C, forced air mode
Isocratic Elution:	100% mobile phase A for 10 min

Time	*% B*
0	0
10	0

Table 2. HMR mode settings for intact mass analysis.

Tune File	
Parameters for Ion Max Source with HESI-II Probe	**Setting**
Sheath Gas Pressure	20 psi
Auxiliary Gas Flow	5 arbitrary units
Probe Heater Temperature	225 °C
Source Voltage	4.0 kV
Capillary Temperature	325 °C
S-Lens RF Voltage	200 V
Mode	High Mass Range Mode
Trapping Gas Pressure	1.5 arbitrary units
Method File	
Full MS Parameters	**Setting**
Full MS Mass Range	*m/z* 2000–8000
Resolution Setting	70,000 (FWHM at *m/z* 200)
Microscans	10
AGC Target Value	3e6
Max Injection Time	500 ms
In-source CID	100 eV

Peptide mapping LC/MS

Reversed-phase LC gradient conditions are described in Table 3. Settings for Standard mode MS tuning and method parameters are described in Table 4. LC and MS parameters were not extensively optimized specifically for trastuzumab entansine peptide mapping.

Table 3. Reversed-phase LC conditions for peptide mapping analysis.

Column:	Acclaim RSLC 120 C18 (2.1 × 250 mm)
Mobile Phase A:	0.1 % formic acid in water
Mobile phase B:	0.1 % formic acid in acetonitrile
Flow Rate:	0.250 mL/min
Column Temperature:	60 °C, forced air mode
Gradient:	2–35% mobile phase B in 60 min

Time	*% B*
0	2
10	2
70	35
75	80
77	80
80	2
90	2

Table 4. Q Exactive Plus MS settings for peptide mapping.

Tune File	
Parameters for Ion Max Source with HESI-II Probe	**Setting**
Sheath Gas Pressure	40 psi
Auxiliary Gas Flow	10 arbitrary units
Probe Heater Temperature	320 °C
Source Voltage	3.6 kV
Capillary Temperature	150 °C
S-Lens RF Voltage	60 V
Mode	Standard Mode
Trapping Gas Pressure	1.0 arbitrary units
Method File	
Full MS Parameters	**Setting**
Full MS Mass Range	*m/z* 200–2000
Resolution Setting	70,000 (FWHM at *m/z* 200)
Microscans	1
Target Value	1e6
Max Injection Time	100 ms
Default Charge State	2
In-source CID	0 eV
MS/MS Parameters	**Setting**
Resolution Setting	17,500 (FWHM at *m/z* 200)
Target Value	1e5
Max Injection Time	200 ms
Loop Count	5
Isolation Window	100 eV
Collision Energy	27% NCE
Intensity Threshold	2.0e4
Charge Exclusion	Unassigned
Dynamic Exclusion	10 s

Data analysis

Native intact LC/MS data analysis

Intact mass spectra were analyzed using the Intact Protein workflow in Thermo Scientific™ BioPharma Finder™ 2.0 integrated software, performing time-resolved deconvolution using the ReSpect™ algorithm in combination with Sliding Window integration. Detailed deconvolution method parameter settings are described in Table 5. Deconvolution spectra were annotated by entering four individual sequences into Protein Sequence Manager. To make each sequence, the amino acid sequence of trastuzumab, with a total of 16 disulfide bonds, fixed modifications (at amino acid position N300 on each heavy chain) corresponding to four possible glycan combinations (G0F/G0F, G0F/G1F, G1F/G1F, G1F/G2F), and a variable modification of MCC-DM1 (8 maximum) was used. An example of creating a protein sequence for intact mass analysis is shown in Figure 1. Masses for fixed and variable modifications are described in Table 6. DAR values were calculated by activating 'Enable Drug-To-Antibody Ratio' (in Identifications tab) and selecting MCC-DM1 as the variable modification.

Peptide mapping data analysis

Peptide mapping data were searched using the Peptide Mapping workflow in BioPharma Finder 2.0 software. Detailed peptide mapping method parameter settings are described in Table 7. Variable modifications included N-glycan (CHO-derived), deamidation (N, Q), oxidation (M, W), and MCC-DM1. An example of creating a protein sequence for peptide mapping is shown in Figure 2, where there is only entry for the light and heavy chains.

Specific masses for fixed and variable modifications are described in Table 8. MCC-DM1 peptide identifications were manually validated by monitoring for the presence of a chromatographic doublet in an extracted ion chromatogram (5 ppm window) and a specific HCD fragmentation signature ion with a theoretical monoisotopic mass of 547.2211 Da, corresponding to fragmentation of DM1.

Table 5A. Intact protein analysis parameter settings in BioPharma Finder software.

Component Detection	
Chromatogram Parameters	**Setting**
m/z Range	4,000–8,000 *m/z*
Chromatogram Trace Type	BPC
Sensitivity	High
Rel. Intensity Threshold	1%
Source Spectra Method	**Setting**
Sliding Window	*On*
RT Range	3.0 to 5.5 min
Target Avg Spectrum Width	0.5 min
Target Avg Spectrum Offset	17%
Merge Tolerance	12 ppm
Max RT Gap	0.5 min
Min. Number of Detected Intervals	3

Table 5B. Intact protein analysis parameter settings in BioPharma Finder software.

Component Detection	
Deconvolution Parameters	**Setting**
Algorithm	ReSpect
Model Mass Range	148,000.00 to 157,000.00 Da
Mass Tolerance	12 ppm
Charge State Range	20 to 30
Minimum Adjacent Charges	3 to 3
Choice of Peak Model	Intact Protein
Target Mass	157,000.00 Da
Resolution at 400 *m/z*	Raw File Specific
Noise Rejection	95% Confidence
Rel. Abundance Threshold	1%
Identification	
Sequence Matching Mass Tolerance	20 ppm
Enable Drug-to-Antibody Ratio	On
Variable Modification	“MCC-DM1”

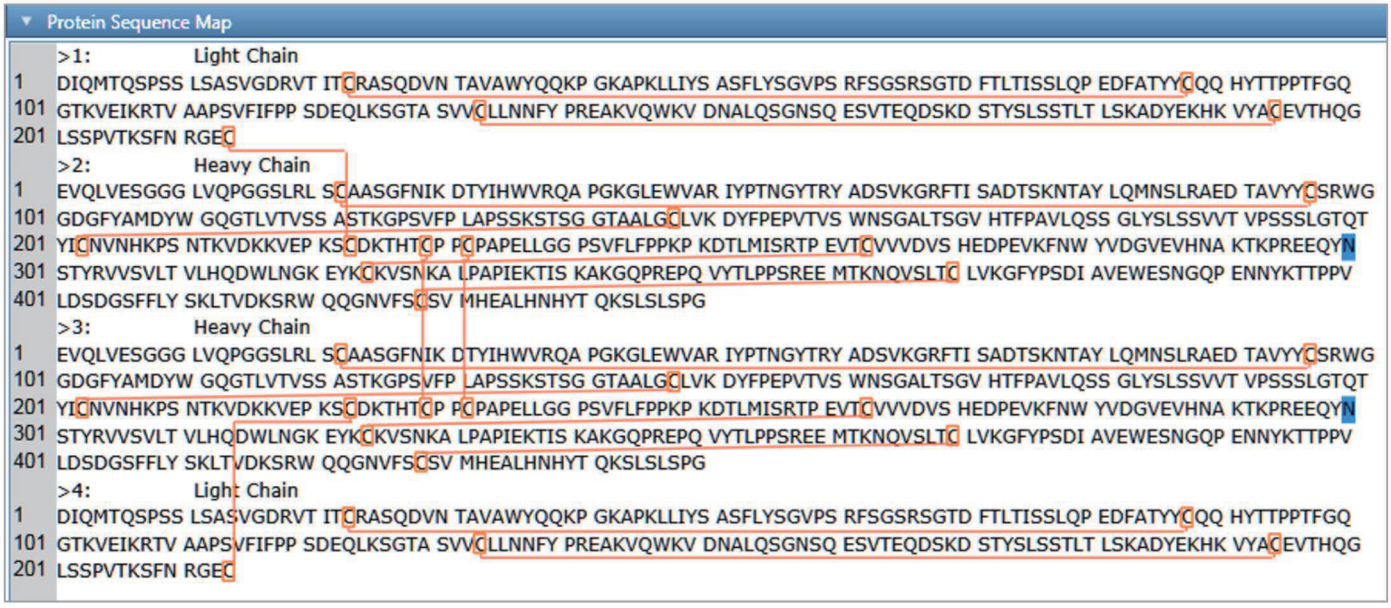


Figure 1. Setting up the Protein Sequence Map for intact protein analysis. For intact mass analysis, trastuzumab amino acid sequence is added as four separate polypeptide chains, with two copies each of light and heavy chains. Disulfide bonds were added by right-clicking at the site of a cysteine residue to create a linkage, then right-clicking at the site of a second cysteine residue to complete the linkage. The blue highlighted asparagine residues are N-glycan consensus sites (Nx[S/T]), which are known to become glycosylated in trastuzumab. A fixed modification at these sites was added by left double-clicking and selecting a specific glycoform for attachment.

Table 6. Modifications for intact protein analysis workflow.

Variable Modifications				
Name	**Type**	**Mono. Mass**	**Avg. Mass**	**Residue(s)**
DM1	Side Chain	956.3644	957.53	K
Possible Static Modifications on Sequence				
Name	**Mono. Mass**	**Avg. Mass**	**Residue(s)**	**Location**
G0F	1,444.533	1,445.32	N	Heavy Chain N300
G1F	1,606.586	1,607.46	N	Heavy Chain N300
G2F	1,768.639	1,769.60	N	Heavy Chain N300

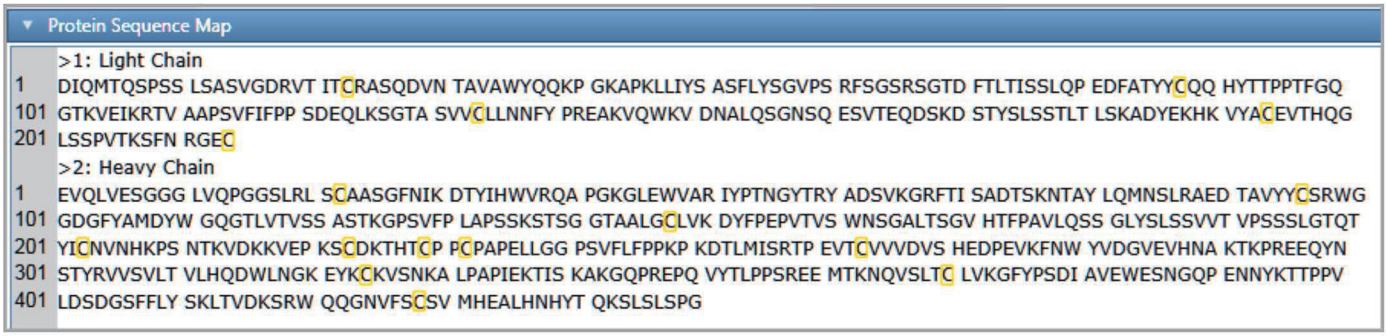


Figure 2. Setting up the Protein Sequence Map for peptide mapping analysis. For peptide mapping only one copy of each trastuzumab polypeptide chains is added.

Table 7. Peptide mapping analysis parameter settings in BioPharma Finder software.

Component Detection	
Task	Find All Ions in the Run
Peak Detection	**Setting**
Absolute MS Signal Threshold	4.00e4
MS Noise Level	2000.00
S/N Threshold	20.00
Typical Chromatographic Peak Width	0.40 min
Use Restricted Time	Off
Relative MS Signal Threshold	1.00%
Width of Gaussian Filter	3
Minimum Valley to be Considered	80%
Minimum MS Peak Width	1.20 Da
Maximum MS Peak Width	4.20 Da
Mass Tolerance	4 ppm
Ion Alignment	**Setting**
Maximum Retention Time Shift	2.33 min
Mass Measurement	Setting
Maximum Mass	30,000 Da
Mass Centroiding Cutoff	15%
Identification	
Peptide Identification	**Setting**
Search by Full MS Only	No
Use MS/MS	Use All MS/MS
Maximum Peptide Mass	15,000 Da
Mass Accuracy	5 ppm
Maximum Number of Modifications for a Peptide	1
Advanced Search	Setting
Enable Mass Search for Unspecified Modifications	Off
N-Glycosylation	CHO
Search for Amino-Acid Substitutions	None

Results and discussion

Native LC/MS analysis improves spectral separation of components in complex mixtures of intact proteins

The diversity of protein isoforms in an ADC sample is contingent upon the enzymatic or spontaneous chemical modifications that arise during the manufacturing process of the core antibody as well as the drug conjugation chemistry (Figure 3). Major forms of lysine-linked ADCs differ in N-glycan composition, the number of linker-drugs attached, as well as potential linker-only attachments.

The lysine-linked ADC, trastuzumab emtansine, is observed in intact mass analysis as a complex mixture. The broad mass range of components in ADC samples causes extensive *m/z* interferences when analyzed using denaturing approaches such as reversed-phase LC/MS. Compared to denaturing conditions, native LC/MS allows greater *m/z* separation of sequential charge state envelopes, and is ideal for resolving complexity of co-eluting protein species (Figure 4).[1]

The powerful Orbitrap high-resolution mass analyzer is ideal for studying very complex mixtures of intact proteins in great depth. Compared to conventional technologies, Orbitrap mass spectrometers can achieve much higher effective resolution of individual intact protein species, which allows an increased capacity to distinguish near-isobaric protein isoforms.[2] This characteristic of Orbitrap mass spectrometry allows users to approach ADC intact protein characterization in a manner which avoids sample pretreatment, such as spectral simplification methods like deglycosylation and digestion to subunits. This strategy precludes the potential of artifacts due to sample handling and adds the convenience of omitting the sample handling step. By analyzing trastuzumab emtansine ADC without any sample pretreatment, the LC/MS experiment reflects a true intact mass analysis.

Trastuzumab emtansine (5 mg/mL in formulation buffer) was desalted online using native SEC coupled directly to the mass spectrometer. Native SEC-MS was performed using a MAbPac SEC-1 size exclusion column with a sample injection volume of 10 µL (50 µg total amount). Mobile phase A (50 mM ammonium acetate) was delivered isocratically at a flow rate of 300 µL/min. The column eluent was coupled directly to an electrospray

Table 8. Modifications for peptide mapping workflow.

Variable Modifications				
Name	**Type**	**Mono. Mass**	**Avg. Mass**	**Residue(s)**
N-glycan	Side Chain	CHO library	CHO library	Nx(S/T)
Oxidation	Side Chain	15.9949	16.00	M, W
Deamidation	Side Chain	0.9840	0.99	N, Q
MCC-DM1	Side Chain	956.3644	957.53	K

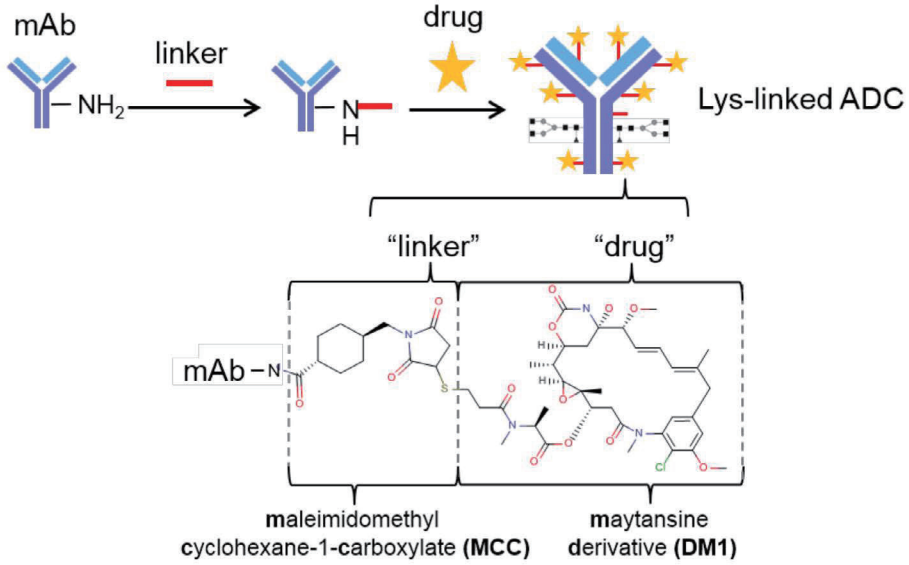


Figure 3. Multi-stage assembly of lysine-linked ADCs can result in a highly complex mixture. Trastuzumab emtansine is a lysine-linked ADC that is constructed using trastuzumab monoclonal antibody as a starting platform. ADC construction is a two-step conjugation process. The first step involves attachment of a hetero-bifunctional linker at the site of lysine side chains. In a second step, the remaining active site of the MCC linker becomes a conjugation site for a DM1 payload. Total ADC heterogeneity owes to both antibody growth and manufacturing as well as the linker-drug conjugation process.

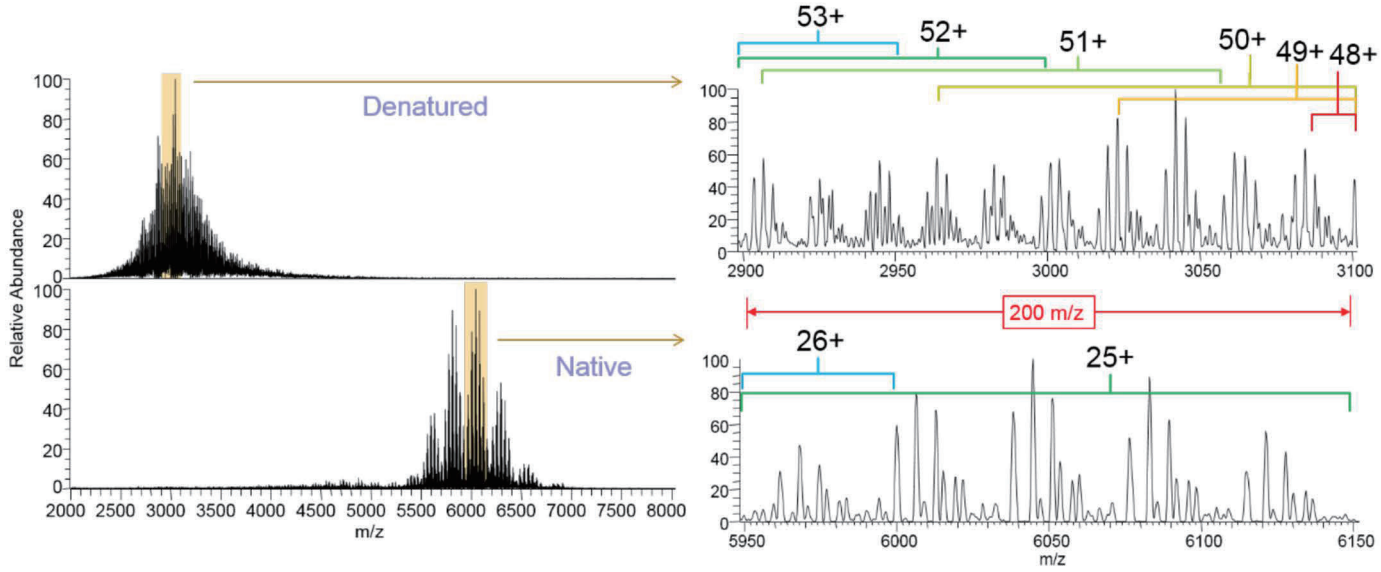


Figure 4. Native LC/MS analysis of intact proteins allows improved separation of mass peaks at higher *m/z* range. MS spectra acquired under denaturing conditions by reversed-phase LC are observed at lower *m/z* ranges while native MS spectra from online size exclusion LC are observed at higher *m/z* ranges. A detailed view (right side) shows that 2–3 sequential charge state envelopes overlap compared to an overlap of 0–1 charge state envelopes in the native MS spectrum.

ionization source on the inlet of a commercially available Q Exactive Plus mass spectrometer. The Q Exactive Plus MS was outfitted with the BioPharma Option and was operated in High Mass Range (HMR) mode to allow improved high mass transmission and scanning up to *m/z* 8000 for native intact analysis. MS1 spectra were collected using 10 microscans and a resolution setting of 70,000 FWHM.

The resulting base peak chromatogram showed one chromatographic peak (eluting 3.7–4.7 min), which corresponds to the desalted ADC and a later eluting chromatographic peak (eluting 4.8–5.7 min) corresponding to the formulation buffer salts (Figure 5A). The raw mass spectrum shows a distribution of protein ions in the range of approximately 5,500–7,000 *m/z*, corresponding to a charge state distribution of 23+ to 27+ (Figure 6A). Here, the baseline separation of the buffer salt peak was critical for preventing ion suppression during electrospray ionization of the ADC protein species. A major advantage of using SEC is efficient desalting, which is attained as a result of the size-based separation itself. This is analogous to the mandatory offline desalting step(s) performed in conventional native MS approaches that use centrifugal buffer exchange columns upstream of static nanospray infusion.[3]

Partial chromatographic resolution of the different drug loads was observed, with higher drug load isoforms eluting at slightly later retention times (Figure 5B-C). This phenomenon is presumably due to some weak secondary interactions of the chromatographic media with the linker-drug conjugations. In cases where secondary interactions appear to be a major influence on separations of a particular compound class, alternative choices for the SEC column should be considered. The Thermo Scientific™ Acclaim™ SEC-300 column has a hydrophilic surface and is thus an excellent alternative to the MAbPAC SEC-1 column. The partial separation of ADC isoforms can be accommodated by analyzing the raw data from the LC/MS intact mass analysis using the Sliding Window feature in BioPharma Finder software 2.0. Sliding Window

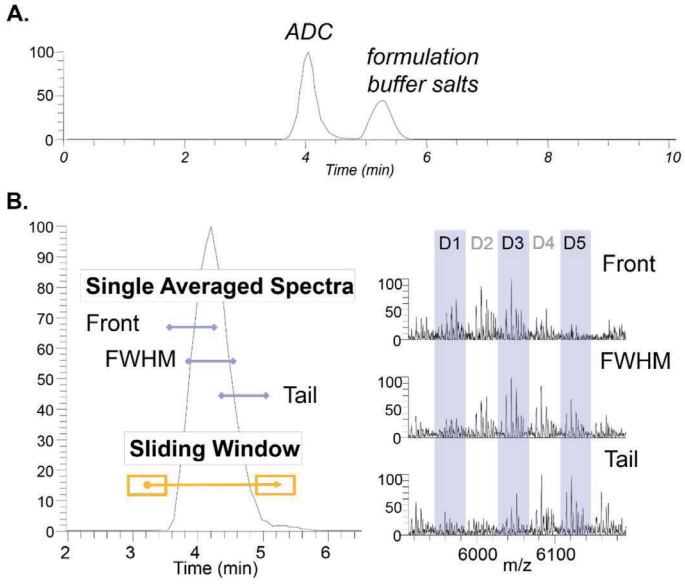


Figure 5. Native size exclusion chromatography (SEC)-MS of trastuzumab emtansine shows partial chromatographic separation of drug load isoforms. (A) Online SEC-MS allows automatic desalting of ADC by baseline separation of buffer salts that interfere with electrospray ionization. (B) A detailed analysis of the raw LC/MS data show that higher drug load isoforms (e.g., D1 compared to D5) are eluting at slightly later retention times. The deconvolution data analysis was performed using Sliding Window integration to accommodate this partial chromatographic separation to obtain good relative abundances necessary for correctly calculating a drug-to-antibody ratio.

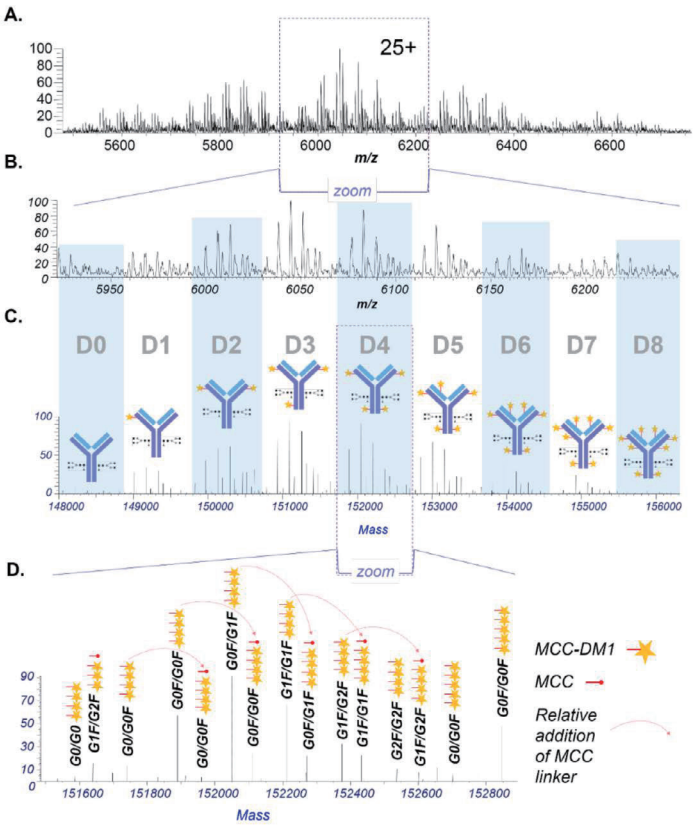


Figure 6. Native MS conditions allow excellent spectral separation of trastuzumab emtansine isoforms and enable high quality deconvolution. (A) Native intact Orbitrap MS spectra of trastuzumab emtansine acquired at R=70,000 setting. (B) A detailed view of the 25+ charge state. (C) The ReSpect / Sliding Window deconvolution results show a distribution of 0–8 MCC-DM1 linker-drug attachments. Native MS conditions yield excellent spectral separation of the ADC isoform masses, which can be easily comparted to deconvolution results for manual confirmation. (D) A detailed view of the D4 cluster shows the relative addition of an MCC linker-only attachment.

allows use of a deconvolution algorithm (e.g., ReSpect) continuously throughout a chromatographic run, or in this case through the entire SEC peak. Sliding Window is critical to the data analysis platform for correctly measuring the accurate relative quantities of the different drug load isoforms that become partially separated in chromatography. Good relative quantitation is a requirement for correctly calculating drug-to-antibody ratio.

Measuring a drug-to-antibody ratio (DAR) is a primary objective of ADC characterization, which seeks to estimate the drug potency. The true intact mass analysis of the untreated ADC showed a distribution of 0–8 linker-drug conjugations with each drug load 'cluster' showing an expected profile of N-glycoform masses (Figure 6B). A close inspection of the raw data compared to the deconvolved result showed a very high degree of similarity, which gave confidence in the accuracy of the deconvolution results (Figure 6C).

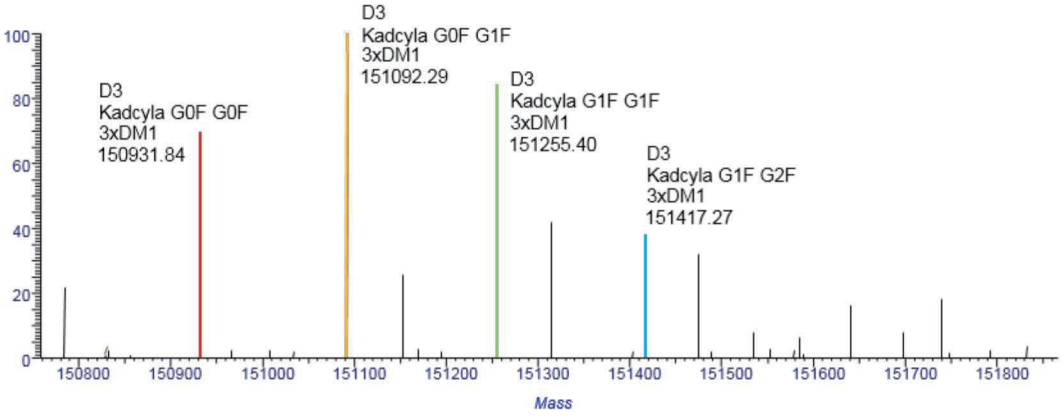
As an additional 'layer' of heterogeneity in the ADC sample, a low abundance distribution of isoforms which had become conjugated at lysines with MCC linker, but did not have a DM1 payload attached were observed (Figure 5E). These species, with a delta mass of approximately 219 Da, were recently reported to be a side product where during the step-wise conjugation process the active lysine-conjugated MCC linker becomes conjugated to another lysine on the ADC instead of the DM1 payload as intended. This results in an internally-crosslinked species.[4] These low-level linker-only species could be accurately identified within 20 ppm.

At each DAR cluster, it was observed that the profile of N-glycoforms was consistent (Figure 7A). The automatic DAR calculator feature on BioPharma Finder software 2.0 was used to calculate an average DAR value based on the four most abundant glycoforms. Using the G0F/G0F, G0F/G1F, G1F/G1F, and G1F/G2F N-glycoform species, an average DAR value of 3.65 was calculated (Figure 7B). This value is consistent with the previously observed values of using static nanospray and native intact analysis.[1]

Peptide mapping of trastuzumab emtansine allows localization of conjugation sites

Peptide mapping was performed in triplicate using 90 min reversed-phase gradients for separation. The Q Exactive Plus mass spectrometer with BioPharma Option was operated in Standard mode at a resolution setting of 70,000 FWHM for MS and 17,500 FWHM for MS/MS. Tryptic peptide mapping analysis resulted in 100% sequence coverage (Figure 8).

A.



B.

Glycoform	G0F/G0F	G0F/G1F	G1F/G1F	G1F/G2F	Top 4 avg
DAR	3.58	3.71	3.64	3.65	3.65

Figure 7. Automatic calculation of Drug-to-Antibody (DAR) ratio using multiple glycoforms. (A) Detail of glycoform distribution shown with three conjugated drugs. (B) An average DAR of 3.65 was calculated using the average of values for four independently measured glycoforms.

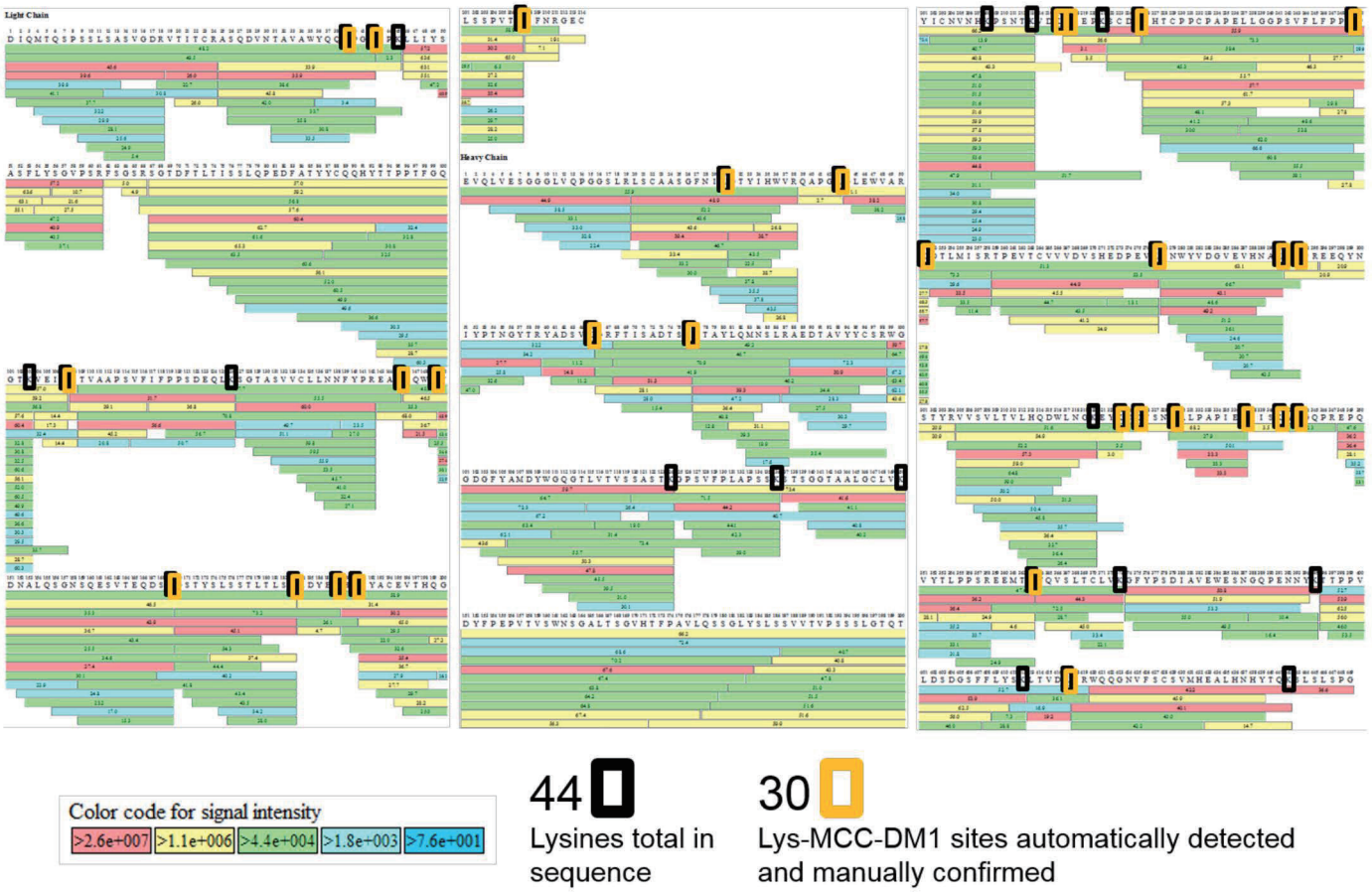


Figure 8. Trastuzumab emtansine peptide mapping results and lysine of MCC-DM1 conjugation sites. Peptides were automatically identified using BioPharma Finder software. MCC-DM1 linker-drug conjugated peptide identifications were manually confirmed and mapped onto the sequence coverage diagram.

BioPharma Finder software automatically identified 30 out of 44 lysine sites of MCC-DM1 linker-drug conjugation (Figure 8) (Table 9). The unique properties of the DM1 payload allowed for manual confirmation of the automatically identified conjugated peptides. The hydrophobic and stereocentric nature of the MCC-DM1 linker-drug moiety causes conjugated peptides to elute with increased retention times and as a doublet (Figure 9). Additionally, HCD spectra showed fragmentation of both the peptide as well as the linker-drug moiety. In this case, a signature fragment ion of 547 *m/z* was utilized to confirm the automatic identifications.[5]

Table 9. MCC-DM1 conjugated peptides identified in trastuzumab emtansine.

Peptide Sequence	Modification	Protein	Site	Delta Mass (ppm)	RT
ASQDVNTAVAWYQQKPGK	DM1	Light Chain	K39	-0.25	67.47
PGKAPK	DM1	Light Chain	K42	-0.31	63.21
VEIKR	DM1	Light Chain	K107	-0.46	65.96
EAKVQWK	DM1	Light Chain	K145	0.09	67.23
VQWKVDNALQSGNSQESVTEQDSK	DM1	Light Chain	K149	0.09	68.57
VDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSK	DM1	Light Chain	K169	0.2	68.77
DSTYSLSSTLTLSKADYEK	DM1	Light Chain	K183	1.07	73.01
ADYEKHK	DM1	Light Chain	K188	-0.77	59.14
HKVYACEVTHQGLSSPVTK	DM1	Light Chain	K190	0.01	58.54
VYACEVTHQGLSSPVTKSFNR	DM1	Light Chain	K207	0.15	64.92
LSCAASGFNIKDTYIHWVR	DM1	Heavy Chain	K30	-2.37	73.12
QAPGKGLEWVAR	DM1	Heavy Chain	K43	-0.62	71.01
YADSVKGR	DM1	Heavy Chain	K65	-0.67	65.35
FTISADTSKNTAYLQMNSLR	DM1	Heavy Chain	K76	0.39	70.82
VDKK	DM1	Heavy Chain	K216	-0.74	63.99
KVEPK	DM1	Heavy Chain	K217	0.46	63.38
SCDKTHTCPPCPAPELLGGPSVFLFPPKPK	DM1	Heavy Chain	K225	-1.61	72.91
THTCPPCPAPELLGGPSVFLFPPKPK	DM1	Heavy Chain	K249	-0.4	73.5
PKDTLMISR	DM1	Heavy Chain	K251	0.01	70.58
TPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAK	DM1	Heavy Chain	K277	-0.05	71.84
FNWYVDGVEVHNAKTK	DM1	Heavy Chain	K291	0.26	67.17
TKPR	DM1	Heavy Chain	K293	0.19	64.16
EYKCK	DM1	Heavy Chain	K323	-0.04	64.44
CKVSNK	DM1	Heavy Chain	K325	-0.47	62.93
VSNKALPAPIEK	DM1	Heavy Chain	K329	-0.38	68.18
ALPAPIEKTISK	DM1	Heavy Chain	K337	-0.66	71.07
TISKAK	DM1	Heavy Chain	K341	-0.25	64.06
AKGQPR	DM1	Heavy Chain	K343	-0.07	62.99
EEMTKNQVSLTCLVK	DM1	Heavy Chain	K363	-0.07	72.43
LTVDKSR	DM1	Heavy Chain	K417	-0.6	66.04

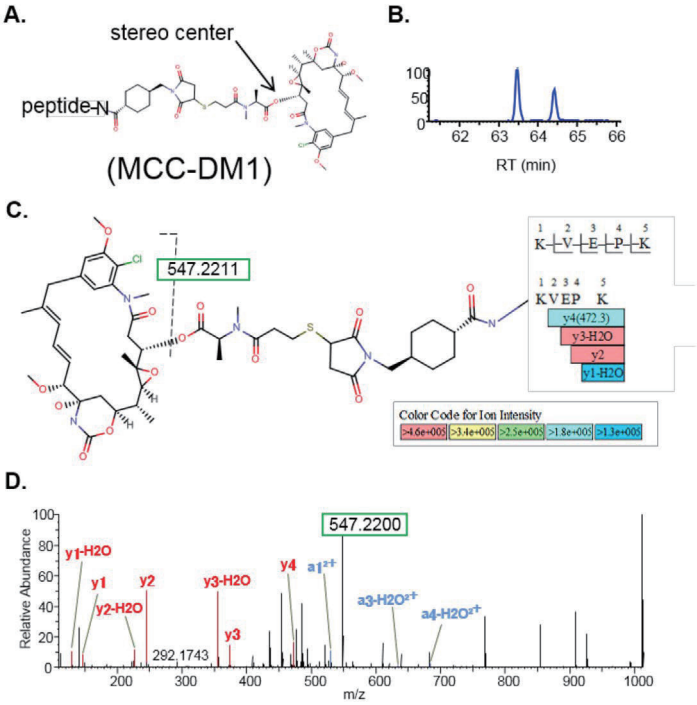


Figure 9. MCC-DM1 conjugation causes peptides to elute as a doublet and can be verified using HCD signature ion *m/z* 547. (A) The structure of DM1 contains a stereocenter. (B) MCC-DM1 conjugation imparts a stereocenter and causes peptides to elute as a doublet in C18 reversed-phase LC. A peptide KVEPK is shown as an extracted ion chromatogram (5 ppm window) with doublet behavior. (C) The DM1 stereocenter is labile in HCD conditions and produces a fragment with a calculated mass of 547.2211 Da. (D) The HCD fragmentation spectrum of KVEPK shows a high abundance ion at *m/z* = 547.2200, which matches the calculated signature ion with an accuracy of 2 ppm.

Conclusions

- Native LC/MS improves *m/z* separation of complex ADC spectra.
- Lysine-linked ADC mixtures may also include linker-only forms.
- Drug conjugation may change peptide behavior in peptide mapping.
- HCD fragmentation generates conjugate-specific signature ions.
- The Q Exactive BioPharma Option allows pre-optimized modes for peptide mapping and native intact mass analysis.

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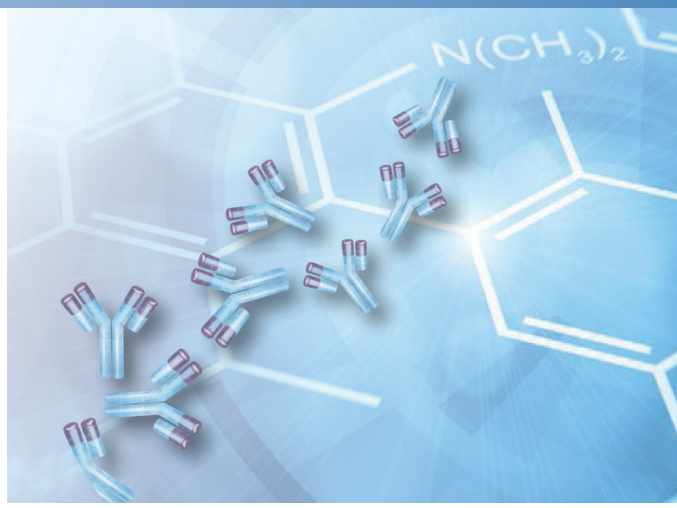
ADC 单抗药物的分子质量、氨基酸序列、糖基化位点以及 ADC 药物结合位点鉴定

聂爱英
赛默飞世尔科技（中国）有限公司



1. 前言

一直以来，单克隆抗体被认为是具有高度特异性的靶向药物，其对肿瘤细胞的靶向性非常高，被誉为治疗恶性肿瘤的“生物导弹”。ADC 技术，就是在抗体蛋白的天然氨基酸上非定点偶联具有抗肿瘤作用的化疗药物（或称小分子药物），以增加单克隆抗体的疗效、并降低小分子药物的毒性。相比于传统 ADC 技术，ADC 抗体药则是通过定点嵌入非天然氨基酸，实现在单克隆抗体上定点、定量接入抗肿瘤的小分子药物，以此获得单一的 ADC 纯品。对此，研究人士表示，这相当于在“生物导弹”上精确地装上了“核弹头”，使得治疗更加安全、有效、定向。目前，在整个肿瘤药研究领域，ADC 抗体药物已得到了业内的普遍认可。不少跨国药企相继投身到 ADC 药物的研究开发中。为了保证 ADC 单抗药物的安全性和有效性，以及对单抗药物生产过程进行实时监控，对于 ADC 单抗药物的整体分子质量的测定、氨基酸序列的鉴定、糖基化位点的确认、ADC 药物结合位点的确认、二硫键的定位，糖链结构的解析等重要的 ADC 药物分析指标的测试和确认，越来越受到各大制药公司的关注。目前，随着生物质谱技术的不断发展和推广，基于生物质谱的单抗分析技术也开始全面、快速的建立起来。

目前，Q-Exactive 串联质谱仪结合了高选择性的四级杆质谱检测器和高分辨、高灵敏度的新型高场 Orbitrap™ 质量分析器，大大提高了离子的选择性、灵敏度和质量精度。该系统具有 140,000 的分辨率（FWHM）、更高的灵敏度、更快的扫描速度和更大的动态范围，同时结合了 HCD 碎裂技术，可以实现串级质谱碎裂和鉴定，为深度复杂样品分析，包括蛋白质组学、代谢组学、脂类组学等样品的鉴定，翻译后修饰和定量分析，提供了全面、高效和快速的质谱分析平台。同样，Q-Exactive 质谱可以有效地应用到 ADC 单抗药物的研发和生产监控中，基本的实验分析流程如下所示。

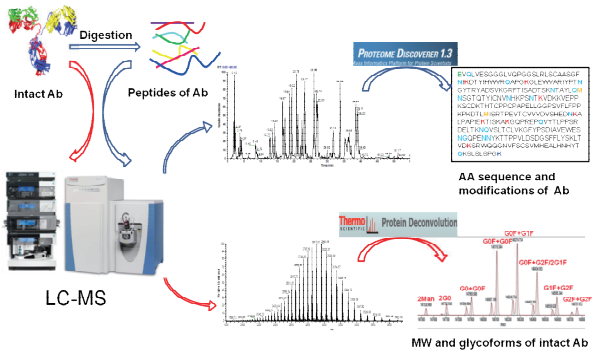


图 1. 单抗分析基本流程图

2. 实验部分

2.1 分子质量测定

2.1.1 仪器和试剂

质谱仪器：Q-Exactive（赛默飞世尔科技，美国）；

色谱仪器：Accela 液相色谱系统（赛默飞世尔科技，美国）；

色谱柱：Agilent Zorbax 300, SB-C8, 3 μ m，2.1 × 50 mm ；

试剂：二次去离子水，色谱级乙腈，色谱级甲酸。

2.1.2 仪器方法

色谱分析条件：具体见表 1；

质谱分析条件：具体见表 2；

2.1.3 数据分析方法

采用 Protein Deconvolution 2.0 软件对原始质谱图进行去卷积处理，得到完整的蛋白质分子质量信息。

表 1. 单抗分子质量测定的色谱分析条件

流动相	A：0.1% 甲酸水溶液；	B：0.1% 甲酸乙腈溶液
流速	500μL/min	
柱温	75℃	
色谱梯度	时间 /min	B 相浓度 /%
	0	5
	4	5
	12	90
	12.1	90
	16	90
	16.5	5
	20	5

表 2. 单抗分子质量测定的质谱分析条件

喷雾电压	4 kV
毛细管加热温度	275℃
S-lens	60 %;
鞘气流速	30（arb）
辅助气流速	5（arb）
质量扫描范围	m/z 2000-4000
分辨率	15000（m/z 400）

2.2 氨基酸序列、糖基化位点和 ADC 药物结合位点测定

2.2.1 仪器和试剂

质谱仪器：Q-Exactive（赛默飞世尔科技，美国）；

色谱仪器：Accela 液相色谱系统（赛默飞世尔科技，美国）；

色谱柱：Thermo, C18, 100Å ,1.9 μm, 2.1 × 100 mm

试剂：二次去离子水，色谱级乙腈，色谱级甲酸。

2.2.2 仪器方法

色谱分析条件：具体见表 3；

质谱分析条件：具体见表 4；

2.2.3 数据分析方法

采用 Proteome Discoverer 1.3 软件对原始谱图进行数据库搜索，具体搜库参数为：包含单抗氨基酸序列的数据库；半胱氨酸 (C) 烷基化（+57.021Da）设置为固定修饰；甲硫氨酸 (M) 氧化（+15.995Da）、天冬酰胺 (N) 和谷氨酰胺 (Q) 脱氨基化 (+0.984Da)、赖氨酸（K）ADC 结合（+956.3644Da）、天冬酰胺（N）G0F 糖基化（+1444.5300Da）设置为可变修饰；trypsin 设置为酶；酶漏切位点为 2。

表 3. 氨基酸序列测定的色谱分析条件

流动相	A：0.1% 甲酸水溶液；	B：0.1% 甲酸乙腈溶液
流速	200 μL/min	
柱温	35℃	
色谱梯度	时间 /min	B 相浓度 /%
	0	5
	4	5
	45	35
	50	90
	52	90
	52.5	5
	60	5

表 4. 氨基酸序列测定的质谱分析条件

喷雾电压	3.5 kV
毛细管加热温度	275℃
S-lens	60 %;
碰撞能量	27% 归一化能量
碎裂方式	HCD
质量扫描范围	m/z 300-1800
分辨率	一级 30,000（m/z 400）， 二级 15,000（m/z 400）

3. 结果与讨论

3.1 蛋白质分子质量测定

基于高分辨质谱 Q-Exactive，为了保证采集谱图的质量和数量，我们设定高场 Orbitrap 检测器的采集分辨率为 17,500。经过 Q-Exactive 质谱采集的 ADC 单抗药物的原始色谱质谱流出图和原始质谱图如图 2 和 3 所示，我们可以观察到 ADC 抗体药物在整个质量范围内表现出均匀的电荷分布，不同电荷的质谱峰之间可以实现基线分离，从而可以判断质谱的灵敏度、分辨率和信噪比都很高。同时选取强度较高的几组质谱峰进行放大，放大之后的几组质谱峰显示相似的分布模式。

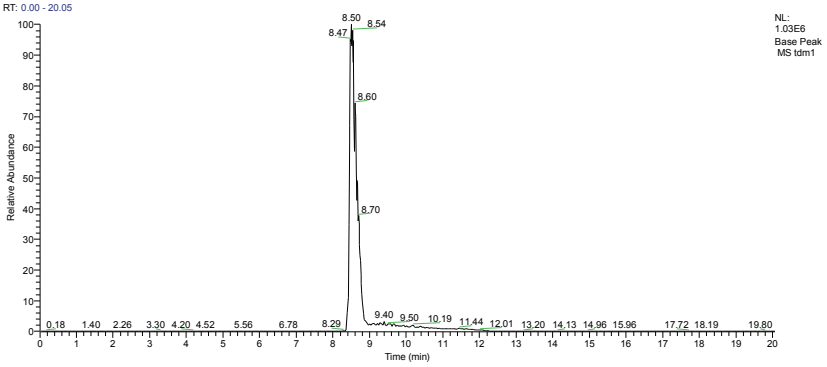


图 2. ADC 抗体药物色谱质谱流出图

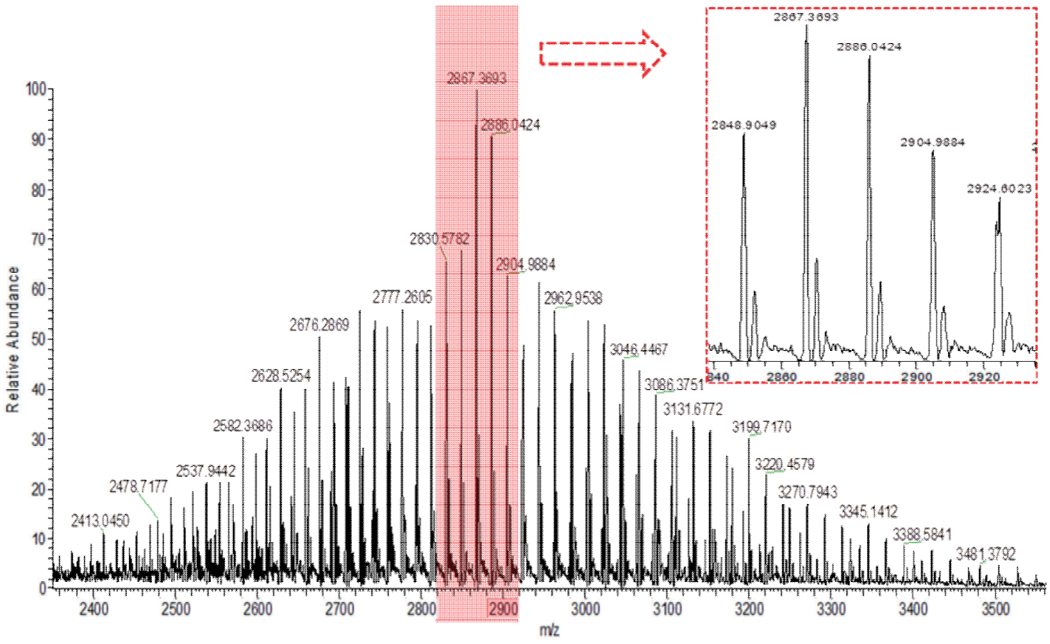


图 3. ADC 抗体药物原始质谱图（红色虚线框内为中间几组峰的放大图）

经过 Protein Deconvolution 2.0 软件去卷积处理之后的单抗分子质量分布图如下所示（图 4），根据单抗的氨基酸序列理论分子质量进行计算，将观察到的质谱峰进行归属，从图中我们观察到，该 ADC 单抗分子之间存在 956Da 左右的质量增加，由此推断该单抗分子结合了不同数目的分子质量在 956Da 左右的药物小分子，并且最多可以检测到 6 个药物小分子的结合。同时还观察到 160 Da 左右的质量增加，这主要是由于连接基团的存在引起的。从而初步推断该 ADC 单抗药物主要结合了 6 个 ADC 小分子药物，该结果与理论期待基本一致，同时根据质谱峰的峰强度信息，我们还可以计算得到该药物的 ADR 值，该数值对于 ADC 药物的有效性评估至关重要。

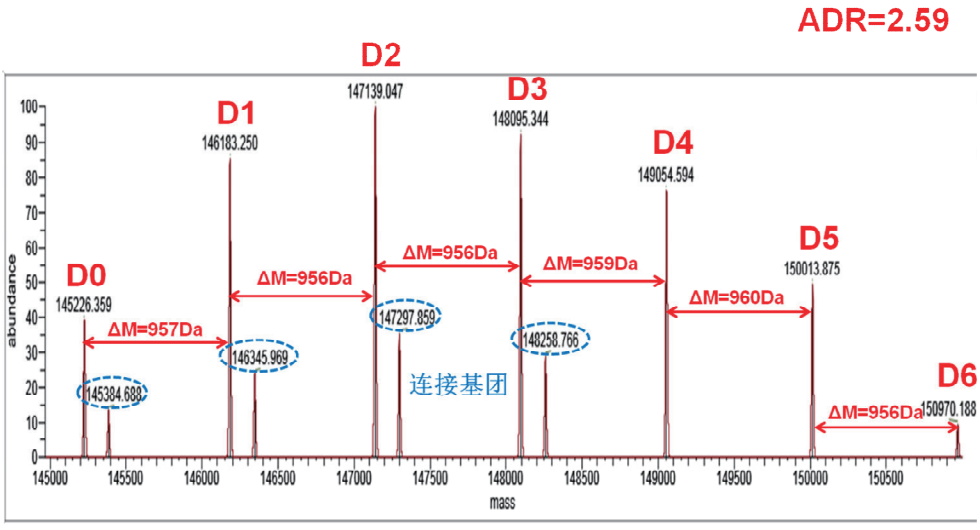


图 4. Q-Exactive 质谱采集的 ADC 单抗原始质谱图经过 Protein Deconvolution 2.0 软件去卷积处理之后的单抗分子质量分布图（D0-D6 表示不同 ADC 药物小分子结合的单抗变体，蓝色圆圈表示连接基团对应的质谱峰。）

3.2 氨基酸序列、糖基化位点和 ADC 药物结合位点鉴定

3.2.1 氨基酸序列覆盖

通过 trypsin 酶解后的肽段，在 Q-Exactive 质谱上采集到的色谱质谱流出图如图 5 所示，再经过 SEQUEST 数据库搜索后，ADC 单抗重链的蛋白序列覆盖度为 90.24 %，如图 6（A）所示，轻链的蛋白序列覆盖度为 100 %，如图 6（B）所示，其中未鉴定到的氨基酸序列主要是由于缺乏酶切位点和酶切后肽段较短造成的，为了实现 100% 的氨基酸序列覆盖，可以通过选择其它内切蛋白酶来实现。

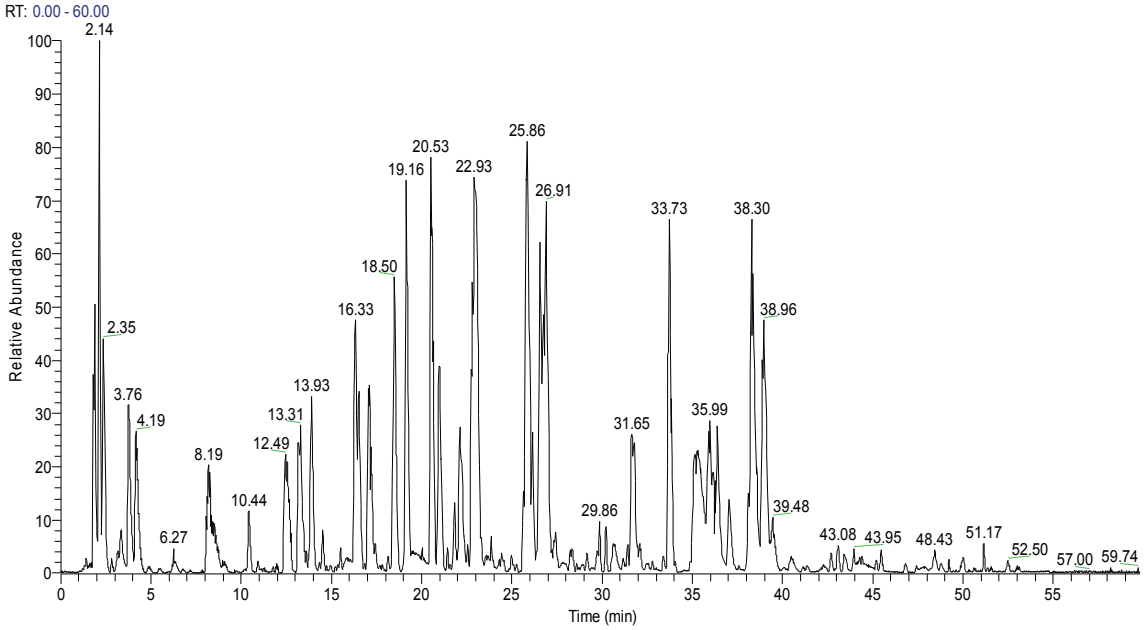


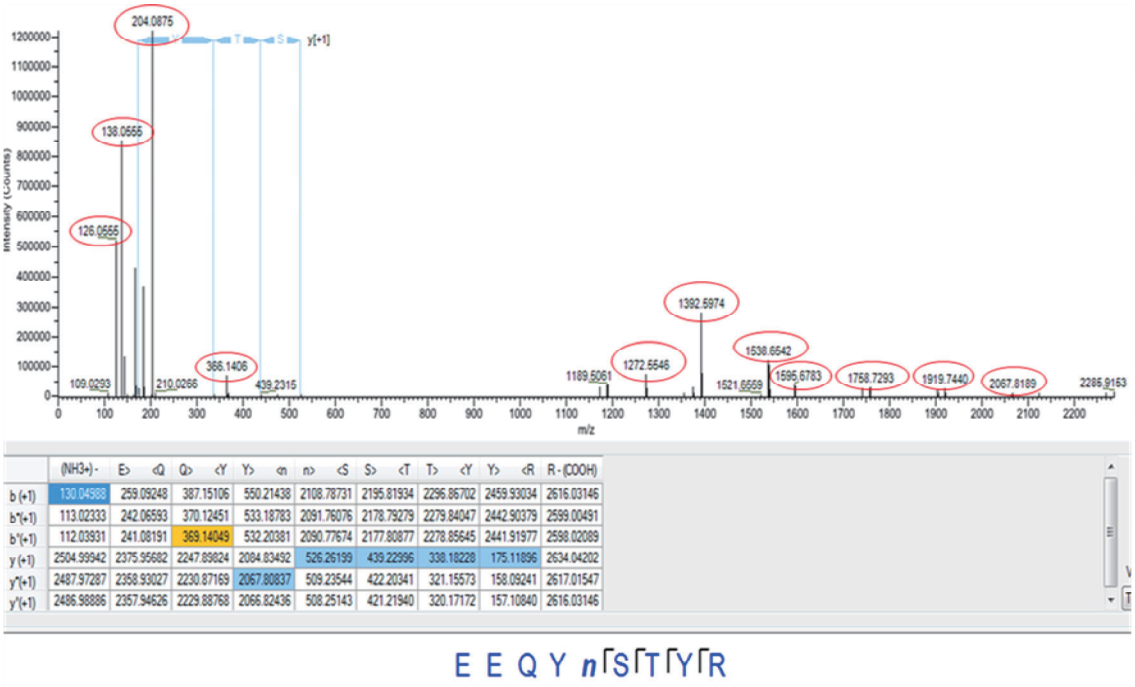
图 5. ADC 单抗药物的 Trypsin 酶解肽段的色谱质谱流出图

图 6. ADC 抗体药物经过 trypsin 酶解和 Q-Exactive 质谱实现的氨基酸序列覆盖度示意图。

（A 为重链氨基酸序列覆盖示意图，B 为轻链氨基酸序列覆盖示意图，其中绿色标记的氨基酸序列表示该段序列鉴定的可靠性非常高，达到 99% 以上，红色标记的氨基酸序列表示该段序列鉴定的可靠性为 95% 以下，无颜色标记的氨基酸序列表示该段序列没有被鉴定到，氨基酸序列上方的数字代表氨基酸的位置。）

3.2.2 糖基化位点确定

ADC 单抗蛋白与其他单抗蛋白相同，也都包含 N- 糖基化修饰，一般发生在保守序列 NXS 或 NXT 中（X 为除脯氨酸外的任意氨基酸）。在糖链完整的情况下，直接进行 trypsin 酶解，我们在搜库时进行 GOF 糖基化可变修饰设定，可以直接获得该 N 糖基化位点：重链氨基酸序列 EEQY N_{261}STYR 中的 N_{261} 位点，下图为该肽段的 GOF 串级质谱图，除了图中黄色和蓝色代表的该肽段的 b, y 碎片离子外，红色圆圈所示的为该糖肽的糖特征碎片离子（低 m/z 端）和糖肽碎片离子（高 m/z 端），从而可以准确确证该糖肽和糖基化位点的存在。



	(NH3+) -	E>	<Q	Q>	<Y	Y>	<n	n>	<S	S>	<T	T>	<Y	Y>	<R	R - (COOH)
b (+1)	130.04988	259.09248		387.15106		550.21438		2108.78731		2195.81934		2296.86702		2459.93034		2616.03146
b*(+1)	113.02333	242.06593		370.12451		533.18783		2091.76076		2178.79279		2279.84047		2442.90379		2599.00491
b°(+1)	112.03931	241.08191		369.14049		532.20381		2090.77674		2177.80877		2278.85645		2441.91977		2598.02089
y (+1)	2504.99942	2375.95682		2247.89824		2084.83432		526.26199		439.22996		338.18228		175.11896		2634.04202
y*(+1)	2487.97287	2358.93027		2230.87169		2067.80837		509.23544		422.20341		321.15573		158.09241		2617.01547
y°(+1)	2486.98886	2357.94626		2229.88768		2066.82436		508.25143		421.21940		320.17172		157.10840		2616.03146

图 7. 重链氨基酸序列 EEQYN261STYR 的串级质谱图

3.2.3 ADC 药物结合位点确定

根据 Proteome Discovery 软件搜库结果显示，我们发现在赖氨酸结合药物分子的肽段的串级质谱图中存在特征碎片离子 453.20、485.23 和 547.21，因此我们通过这些特征碎片对所有的可能发生 ADC 结合的肽段的串级质谱图进行人工确认，最终准确确认了轻链中 2 个赖氨酸位点和重链中 7 个赖氨酸位点发生了 ADC 小分子药物结合，具体位点如下图（图 8）所示，并且以其中一条肽段为例，给出了 ADC 结合肽段的串级质谱图（图 9），红色圈表示 ADC 特征碎片离子。同时我们也观察到 ADC 药物结合后的肽段的疏水性比较强，色谱流出比较靠后，这一现象完全符合理论推测。

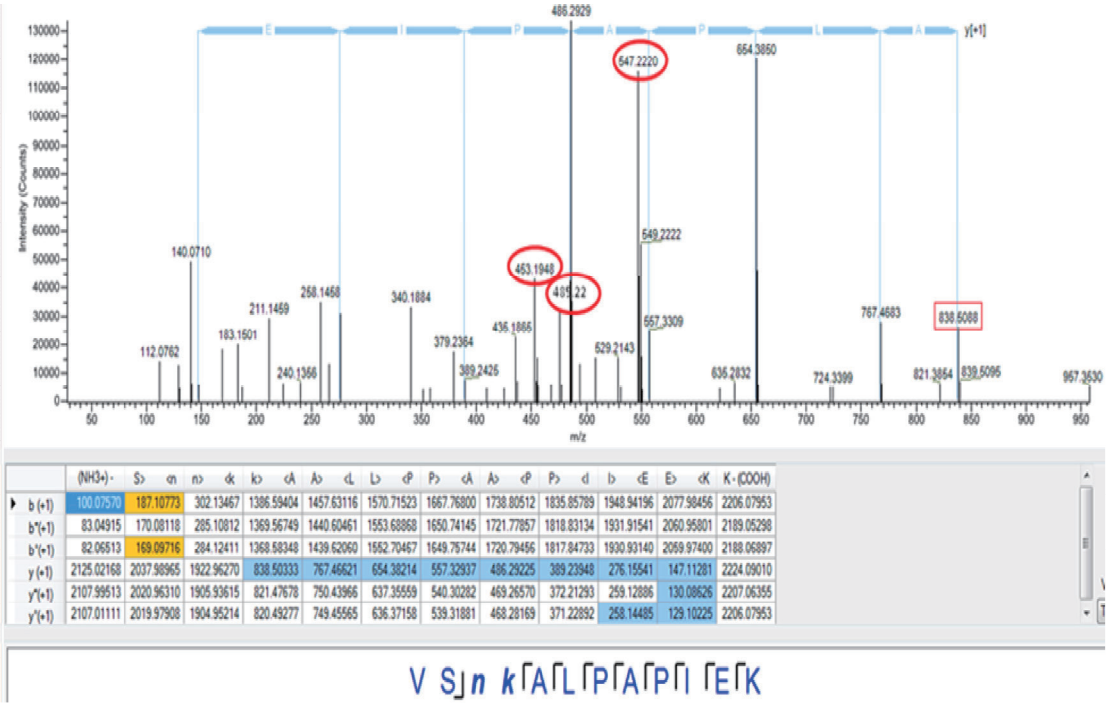
>Light chain

XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
XXXXXXXXXXXXXXXXXXKXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
XKXXXXXXXXXXXXXXXXXXXXXXXXXXXX

>Heavy chain

XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXKXXXXXXXXXXXXXKXXXXXXXXXX
XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXKXXXXXXXXXXXXXXXX
XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXxKXXXXXXKX
XXXXXKXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
XXXXXXXXXXXXXXXKXXXXXXXXXXXXXXXXXXXXXxx

图 8. ADC 药物小分子结合位点示意图



	(NH3+)-	S>	n>	k>	k>	A>	A>	L>	P>	A>	A>	P>	d	b	E>	K>	K-(COOH)
b (+1)	100.07570	187.10773	302.13467	1386.59404	1457.63116	1570.71523	1667.76800	1738.80512	1835.85789	1948.94196	2077.98456	2206.07953					
b*(+1)	83.04915	170.08118	285.10812	1369.56749	1440.60461	1553.68868	1650.74145	1721.77857	1818.83134	1931.91541	2060.95801	2189.05298					
b°(+1)	82.06513	169.09716	284.12411	1368.58348	1439.62060	1552.70467	1649.76744	1720.79456	1817.84733	1930.93140	2059.97400	2188.06897					
y (+1)	2125.02168	2037.98965	1922.96270	838.50333	767.46621	654.38214	557.32937	486.29225	389.23948	276.15541	147.11281	2224.09010					
y*(+1)	2107.99513	2020.96310	1905.93615	821.47678	750.43966	637.35559	540.30282	469.26570	372.21293	259.12886	130.08626	2207.06355					
y°(+1)	2107.01111	2019.97908	1904.95214	820.49277	749.45565	636.37158	539.31881	468.28169	371.22892	258.14485	129.10225	2206.07953					

图 9. ADC 结合肽段 VSNKALPAPIEK 的串级质谱图

4. 结论

本文通过 Q-Exactive 质谱建立了 ADC 单抗药物的整体分子质量测定、氨基酸序列鉴定、糖基化位点和 ADC 药物结合位点确认的分析方法，为 ADC 单抗药物研发分析和实时生产检测提供了高效、快速的分析平台。实验结果表明 Q-Exactive 串联质谱仪，凭借其超高的分辨率，超快的扫描速度，超高的质量精度、超低的灵敏度和超大的动态范围，极大地完善和推动了 ADC 单抗药物的鉴定分析。

POSTER NOTE

Versatile data processing software leverages Orbitrap data for intact and sub-unit mass analysis of protein biotherapeutics

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ABSTRACT

Intact protein analysis workflows benefit greatly from Orbitrap measurements, both in low resolution (average mass) spectra and high resolution (monoisotopic mass) spectra. A redesigned set of intact protein analysis tools for BioPharma Finder ™ 2.0 software allows new analytical capabilities for sub-unit and intact mAb mass analysis workflows.

INTRODUCTION

The increasing requirements in characterizing complex biotherapeutics for safety and efficacy place ever-increasing demands on analytical technologies and scientists. Therefore, high quality raw data must be converted into meaningful information. Data processing software is the key interface between high resolution accurate mass data and product knowledge. Intact and sub-unit mass analyses, routine assays in the biopharmaceutical industry, are important because these assays provide critical quality attributes (CQAs) and are usually among the first steps of biotherapeutic characterization. Here, we present three different examples on how data processing software can provide insight not only on product CQAs for antibody drug conjugates and monoclonal antibodies but also on how this software platform can be used to optimize raw data quality.

MATERIALS AND METHODS

The intact protein analysis side of BioPharma Finder software was redesigned to incorporate new functions such as automatic average drug-to-antibody ratio (DAR) and multiconsensus analysis (Figure 1). Raw files of Trastuzumab Emtansine were acquired under native conditions without removing the N-glycans. After ReSpect ™ deconvolution, masses corresponding to the combination of different DAR values and glycoforms were identified and a global average DAR of 3.65 was automatically calculated. The software offers the flexibility for users to calculate the average DAR ratio for specific glycoforms. Several raw files were acquired by varying the source settings. Raw files were processed in BioPharma Finder 2.0 using the multiconsensus option. A master table was created automatically and the mass accuracy and intensity of components can be compared between conditions. This table allows the user to quickly find the optimum source settings. Finally, a commercially available murine mAb test standard was stressed in an ammonium bicarbonate buffer (pH7.8) for either zero or four days at an elevated 37ºC temperature. Each sample was split in two. One half was subjected to trypsin proteolysis for peptide mapping purposes. The other was denatured and reduced for subunit analysis. All raw files obtained following HRAM LC-MS were processed using the sliding window feature combined with the Xtract ™ deconvolution algorithm. We found that high resolution accurate mass data showed deamidation at the sub-unit level, with approximately 1 ppm mass accuracy for both unmodified and deamidated species. Reconstructed chromatograms of the deconvolved components also showed a slight shift in the elution profile for deamidated vs. unmodified light chain subunits.

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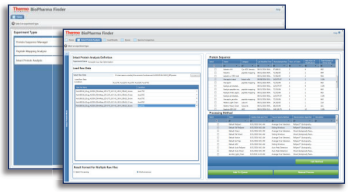
RESULTS

Figure 1. Intact Protein Analysis in BioPharma Finder 2.0 software



BioPharma Finder 2.0 software was redesigned and it now incorporates new functions such automatic average drug-to-antibody ratio (DAR) and multi consensus analysis.

Multiconsensus raw file comparisons

Five raw files were processed using the multiconsensus option to optimize the auxiliary gas temperature. The auxiliary gas temp was varied from 175ºC to 375ºC in individual SEC-MS runs (300 µL/min, 50 mM $NH_4CH_3CO_2$ (Figure 2). By looking at the raw data, the auxiliary gas at 375°C is probably too high but to differentiate the other conditions, the processed data will be have to be reviewed carefully.

Figure 2. Multiconsensus results table allows simultaneous visualization of both raw and deconvolution data. (A) Chromatogram, (B) raw spectrum. and (C) detail of m/z range of charge 24+ for different source auxiliary gas temperatures.

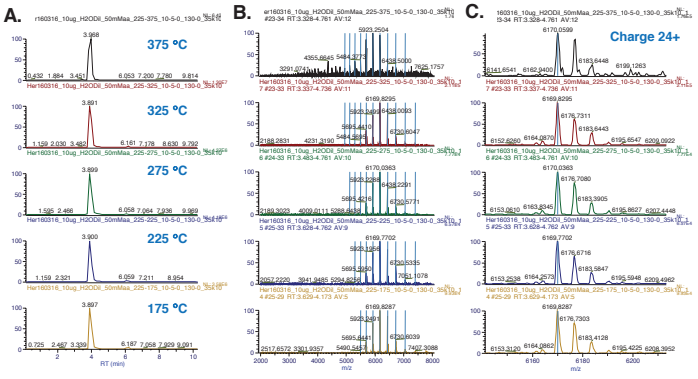


Figure 3. ReSpect score comparison of known glycoforms allows optimization.

Relative ReSpect score was calculated by dividing each ReSpect score by the average of all runs (each temperature). The overall best auxiliary gas temperature is around 325°C.

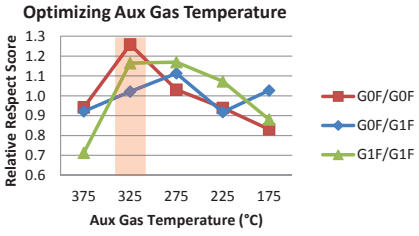
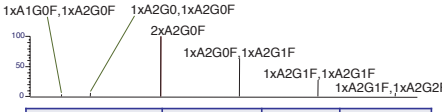


Figure 4. ReSpect deconvolution results of optimized native SEC-MS intact mAb analysis (A) Deconvolution spectrum. (B) Table of deconvolution results.



B.

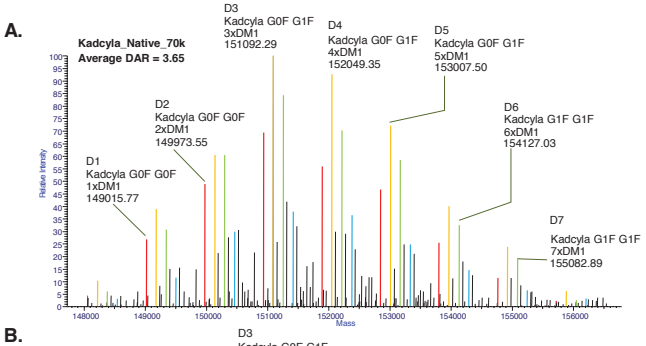
Glycoform	Mass Measured (Da)	Mass Accuracy (ppm)	Relative Abundance (% Intensity)
1xA1G0,1xA2G0F	147708.93	11.7	0.59
1xA2G0,1xA2G0F	147912.60	14.9	5.41
2xA2G0F	148057.19	4.5	100.00
1xA2G0F,1xA2G1F	148219.37	4.7	72.03
1xA2G1F,1xA2G1F	148381.74	6.2	23.36
1xA2G1F,1xA2G2F	148543.29	2.2	4.92

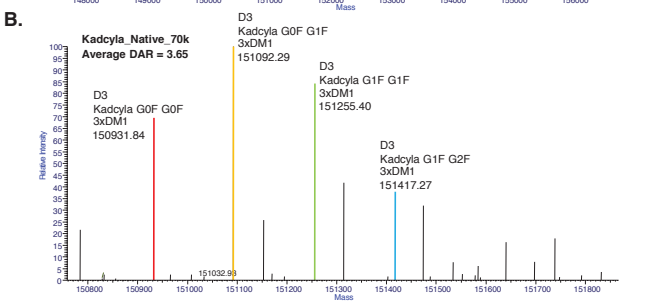
Automatic drug-to-antibody ratio calculation

After ReSpect deconvolution using the Sliding Window algorithm, the resulting masses were searched against the Trastuzumab amino acid sequence with the different glycoforms as fixed modification and the linker-drug mass (+957.53) as a variable modification. When a component is highlighted (ex: G0F/G1F +2 linker-drugs) in the master table, an abundance trace is created on the chromatogram window and data used for deconvolution are identified with blue bars in the raw spectrum. A blue bar is also present in the deconvoluted spectrum to mark the selected component. Average DAR value is calculated automatically and the software offers the flexibility for users to calculate the average DAR ratio for specific glycoforms.

Figure 5. Data Vizualization in BioPharma Finder 2.0. (A) Comparison of raw data and deconvolution results. (B) Setting refercence modification for DAR measurement. (C) DAR measurement results panel.

Figure 6. Automated DAR calculation allows use of multiple reference masses to generate high confidence. (A) Full spectrum of deconvoluted masses of Trastuzumab Emtansine show a broad distribution of linker-drug additions. (B) A detail of D3 forms shows 4 abundant glycoforms. (C) Average DAR value calculated using 4 glycoforms after processing a raw file of Trastuzumab Emtansine is in agreement with previous measurements **(Ref 1)**.





C.

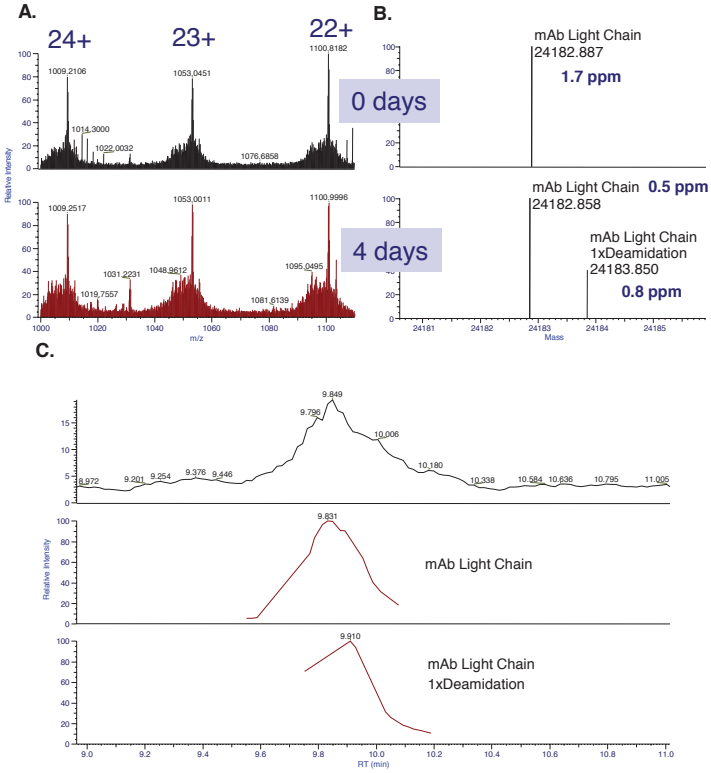
Glycoform	G0F/G0F	G0F/G1F	G1F/G1F	G1F/G2F	Top 4 avg
DAR	3.58	3.71	3.64	3.65	3.65

True intact analysis using a native MS approach allows measurement of DAR ratio without need for deglycosylation. This approach offers a faster, more direct route to analysis, in which we remove the possibility of artifacts due to sample preparation. Also with one LC-MS run, four independent average DAR values are calculated, increasing the confidence level of the average DAR value.

Sub-unit deamidation analysis using Sliding Window deconvolution

Deamidation can be measured using isotopic resolution of proteins or protein sub-units **(Ref 2)**. We incubated a monoclonal antibody sample in alkaline conditions for 4 days and analyzed the sample using a shallow reverse phase gradient (23-30% ACN in 10 min) and a selected ion monitoring (SIM) MS method (R=280,000). Xtract deconvolution showed that sample treatment resulted in observation of a new mass corresponding to deamidation of light chain. These data are easily visualized in multiconsensus, where the 0 and 4 day time points can be compared.

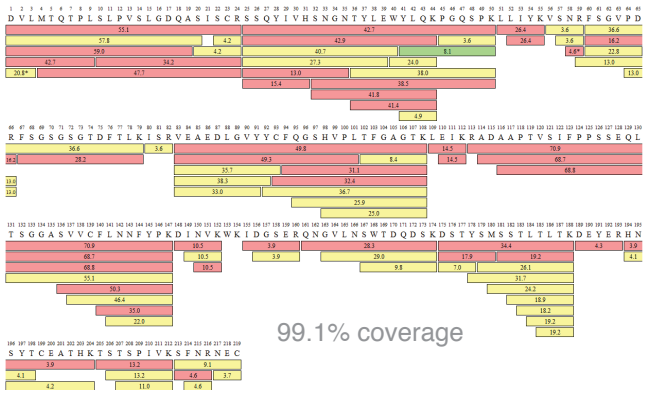
Figure 7. Xtract deconvolution of deamidated mAb Light Chain. (A) Data were collected using a SIM method which acquired data on three charge states of the mAb light chain. (B) Xtract deconvolution using the Sliding Window feature detected a new species corresponding to deamidation of light chain at ~40% intensity. (C) Abundance traces of the deconvolved components showed a slightly shifted elution profile of the deamidated species.



To confirm our sub-unit analysis results we performed a trypsin peptide mapping analysis of the mAb light chain. Triplicate analysis of each sample type resulted in coverage of 99.1% of the sequence. We found 2 sites of deamidation; N33 and N162. Deamidation was present at a low level (~1%) at site N33 and in moderate amounts (~23%) at site N162 in the untreated sample (Figure 8). After 4 days of treatment deamidation levels increased a total of approximately 50% (~5% at N33 and 68% at N162). As we did not measure any deamidation in the untreated sub-unit analysis we suspect that our trypsin digestion protocol is responsible for the moderate amounts of deamidation present at site N162 in the peptide mapping results.

Figure 8. Peptide mapping confirmation of subunit-level deamidation results

A.



B.

	0 days			4 days		
Light Chain deamidation site	Run 1	Run 2	Run 3	Run 1	Run 2	Run 3
N33	0.80%	0.92%	0.84%	6.22%	5.44%	5.20%
N162	23.23%	23.02%	23.74%	67.65%	68.25%	68.79%

CONCLUSIONS

- BioPharma Finder 2.0 allows new analytical capabilities for intact protein analysis workflows
- Multiconsensus view allows cross-comparison of deconvolution results from multiple raw files
- Automatic DAR calculation allows use of multiple reference species to confidently measure ADCs
- Sliding Window algorithm produces abundance trace of deconvolved components which helps sensitive determination of new species.

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PN64888-EN 11/16S

Monoclonal Antibody and Related Product Characterization Under Native Conditions Using a Benchtop Mass Spectrometer

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Key Words
Orbitrap native MS, extended mass range MS, monoclonal antibody (mAb), monoclonal antibody-drug-conjugate (ADC), monoclonal antibody antigen complexes (mAb/Ag)

Goal
Demonstrate the characterization of mAbs, antibody-drug conjugates (ADC), mAb/antigen (mAb/Ag) complexes, and a mixture of mAbs under their native conditions by using a high-resolution, accurate-mass (HRAM) benchtop mass spectrometer with extended mass range (EMR) in combination with a chip-based electrospray ionization interface.

Introduction
Native mass spectrometry (MS) has emerged as a valuable technique for characterization of intact noncovalent protein complexes, reaching a high level of reliability within the last ten years.[1] For the analysis of intact monoclonal antibodies (mAbs), native MS yields accurate mass measurements of the molecules, glycoform identification, and assessment of higher-order structures (dimer, trimer, tetramer), thus providing a robust, fast, and reliable first-line analytical characterization tool.[2,3] This approach can now be applied to the routine characterization of heterogeneous therapeutic monoclonal antibodies. Native MS has gained interest not only for analysis of intact mAb, but also for analysis of antibody-drug conjugates (ADCs), bispecific mAbs, antibody-antigen complexes, and characterization of antibody mixtures. It benefits from simplified data interpretation due to the presence of fewer charge states compared to classical denaturing MS.

This application note describes the use of a new Orbitrap mass spectrometer with an extended mass range of up to *m/z* 20,000 and improved detection of high-mass ions for the characterization of mAbs, ADCs, mAb/Ag, and mAb mixtures under native conditions.

Figure 1. Exactive Plus EMR mass spectrometer equipped with a TriVersa NanoMate chip-based electrospray ionization interface

Experimental
Sample Preparation
The intact trastuzumab (Herceptin®, Roche), the monoclonal antibody-drug conjugate brentuximab vedotin (ADC, Adcetris®, Seattle Genetics), the mAb/antigen complexes of J10.4 mAb/JAM-A, and one mixture of eleven distinct IgG antibodies were introduced using the TriVersa NanoMate® (Advion, USA) onto the Thermo Scientific™ Exactive™ Plus EMR Orbitrap™ mass spectrometer.

Brentuximab vedotin was deglycosylated using EndoS endoglycosidase (IgGZERO™, Genovis). Titration experiments involving J10.4 mAb and JAM-A were monitored by native MS in order to determine the binding stoichiometry. The fixed amount of J10.4 (5 µM) was incubated with increasing amounts (1:1, 1:2, 1:4, 1:8) of JAM-A up to 40 µM. The mixture of eleven distinct deglycosylated humanized IgG antibodies included two marketed therapeutic mAbs (rituximab and trastuzumab) and nine point mutation variants of the Hz6F4-2 mAb [4, 5]. They were mixed together prior to PNGase-F deglycosylation.

Finally, all the samples were buffer exchanged against 150 mM ammonium acetate (AcONH4) pH 7.5. Trastuzumab, deglycosylated Brentuximab vedotin, and the mAb/antigen complexes of J10.4 mAb/JAM-A were injected at 5 µM, and the deglycosylated IgG mixture was injected at 1 µM on the Exactive Plus EMR Orbitrap mass spectrometer.

Direct-Infusion Native MS Conditions

Chip-based infusion conditions	
Instrumentation	TriVersa NanoMate® (Advion, USA) system
Ionization voltage (kV)	1.6–1.8
Gas pressure (psi)	0.3–0.6

The ESI Chip® consists of an array of 400 nanoelectrospray emitters with 5 µm inner diameters.

MS conditions	
Instrumentation	Exactive Plus EMR Orbitrap MS system (Figure 1)
EMR mode	ON
Mass range *(m/z)*	350–20,000
Resolution	17,500 to 140,000, depending on spectral complexity
Target value	3×10^6
Microscans	10
Max injection time (ms)	300
Insource CID energy (eV)	60 to 150, manually tuned for optimized desolvation
S-lens level (%)	100 to 200, manually tuned for optimized transmission and avoiding in-source fragmentation
Injection flatapole DC (V)	8
Inter flatapole lens (V)	7
Bent flatapole DC (V)	6
C-Trap entrance lens tune offset (V) EMR	0
Trapping gas pressure setting factor	4
Spectra average	Enabled (10 to 50 scans are averaged to achieve S/N ratio of >100)

Data Processing

Software	Thermo Scientific™ Protein Deconvolution software version 2.0 SP2 and version 3.0
Deconvolution parameters	
Number of iterations	4
Noise compensation	On
Minimum adjacent charges	1 to 3

Results and Discussion

High-Resolution Native MS Analysis of Intact Monoclonal Antibody Trastuzumab

Trastuzumab (Herceptin®) is a humanized IgG1 mAb, approved for HER2-overexpressing breast cancer treatment since 1998. Several mechanisms of action are thought to contribute to trigger the tumor-inhibitory effect of this protein therapeutic. Among them, trastuzumab can mediate the effector functions of immune cells through its constant region (Fc) by binding to the Fc gamma receptor III (FcγRIII) and triggering antibody-dependent, cell-mediated cytotoxicity (ADCC).

Based on the published amino acid sequence of both the light and heavy chain of trastuzumab, the calculated mass of this protein is $C_{6560}H_{10132}O_{2090}N_{1728}S_{44}$ = 148,057 Da. This calculation includes 16 disulfide bridges (–32 Da), two main glycoforms (G0F; +1445 Da), and near 99% cleavage of two heavy chain C-terminal lysines (–128 × 2 Da). Partial cyclization of one or two N-terminal glutamic acids (–18 Da) may also occur as well as methionine oxidations (+16 Da). Three Asn deamidation/Asp isomerization hot spots have also been described in the CDRs and shown to negatively impact HER2 antigen binding when degraded (Figure 2A).

Trastuzumab was analyzed on the Exactive Plus EMR MS with resolution set at both 17,500 and 35,000. The deconvoluted mass spectrum calculated using Protein Deconvolution software version 2.0 SP2 represents the classical glycosylation pattern of a mAb with baseline-resolved glycan peaks. Figure 2B shows the complete mass spectrum at resolution of 35,000 and a zoom of the corresponding 23^+ charge state of trastuzumab acquired with the resolution set at both 17,500 and 35,000 in native conditions. Compared to the raw spectrum acquired at 17,500 resolution, an interference peak can be resolved by using a resolution of 35,000 or higher. The high resolution can resolve the analyte from the interferences, therefore, ensuring the low ppm mass accuracy. Molecular weights of each trastuzumab glycoform were measured with good mass accuracy in the low ppm range, as shown in Figure 2C. The mass differences between species are +146 Da and +162 Da, corresponding to a fucose or to the addition of multiple hexose units, respectively.

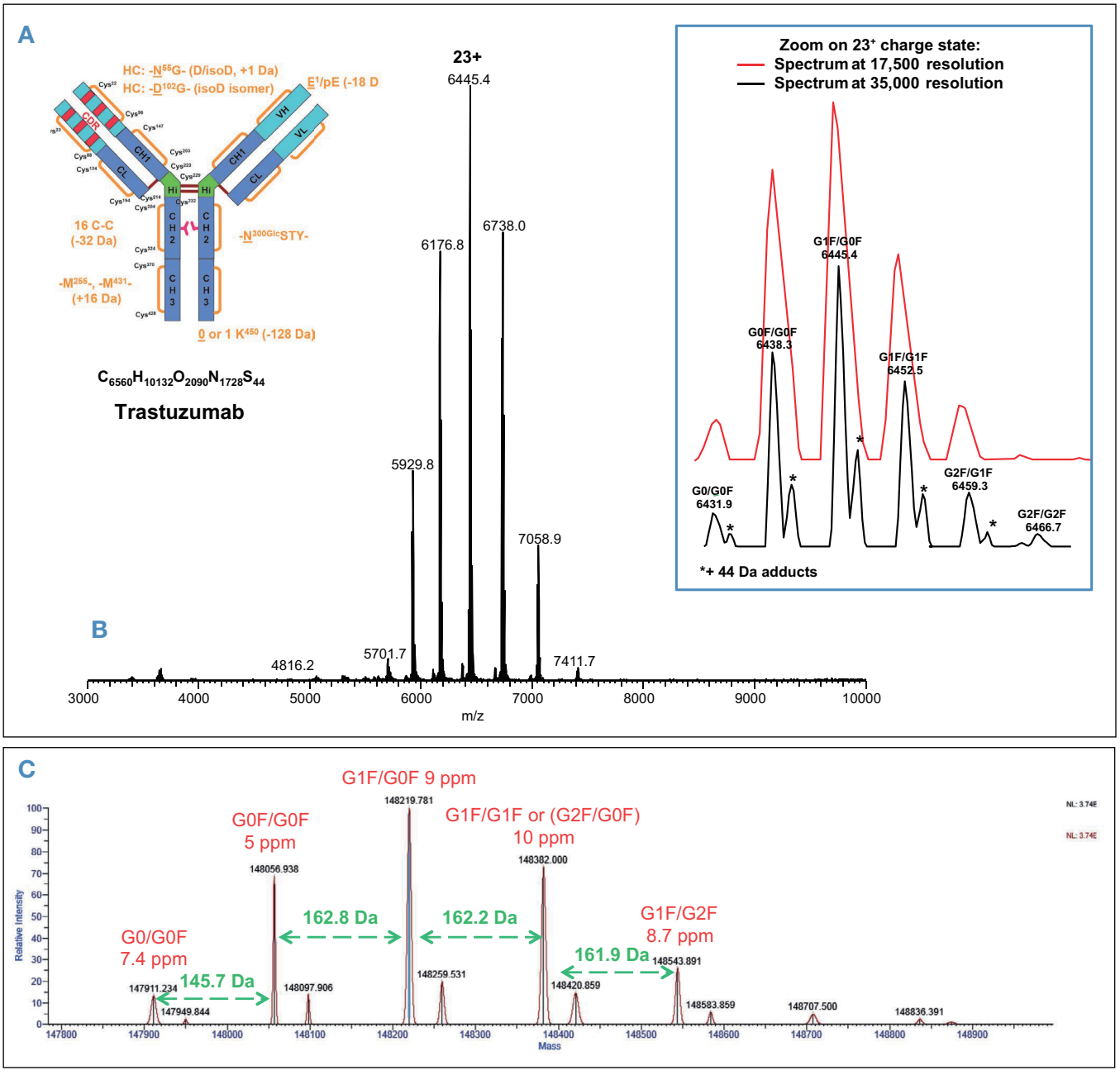

Figure 2. Orbitrap native MS detection of intact monoclonal antibody trastuzumab. A. Intact mAb trastuzumab. B. High-resolution, native MS showing complete mass spectrum and zoom of corresponding 23^{+} charge state. C. Deconvoluted spectrum showing molecular weights of each trastuzumab glycoform with low ppm mass accuracy.

Orbitrap Native MS Analysis of a Monoclonal Antibody-Drug-Conjugate (ADC) Brentuximab Vedotin

Antibody-drug conjugates (ADCs) are an increasingly important modality for treating several types of cancer. The impact of ADCs in this field is due to the exquisite specificity of antibodies that deliver the conjugated cytotoxic agent to targeted tumor cells preferentially, thus reducing the systemic toxicity associated with traditional chemotherapeutic treatments. ADCs are differentiable on the basis of the drug, linker, and also the amino acid residue of attachment on the antibody. Recently, two ADCs were approved by the FDA (Adcetris®, brentuximab vedotin, and Kadcyla®, trastuzumab emtansine) and 35 more are being investigated in clinical trials.

The brentuximab vedotin mass spectrum was recorded at a resolution of 35,000 and in-source CID voltage was set to 75 eV. Figure 3A shows the native deconvoluted mass spectrum of the deglycosylated ADC. Populations with zero (grey), two (black), four (blue), six (red), and eight (green) molecules loaded onto the antibody (payloads) were detected with a mass difference between peaks corresponding to the addition of two payloads (+2,634 Da). The drug loading clearly increases in steps of two, which corresponds to binding of one payload to the two accessible cysteine amino acids after disulfide bridge reduction. For each set of peaks, the drug-to-antibody ratio (DAR) can be determined. Relative ratios of each detected compound were determined using MS peak intensities and served to estimate the mean DAR (4.2), which is in agreement with hydrophobic chromatography data (data not shown). Figure 3B shows the corresponding raw mass spectrum with the entire charge state distribution of brentuximab vedotin under native conditions.

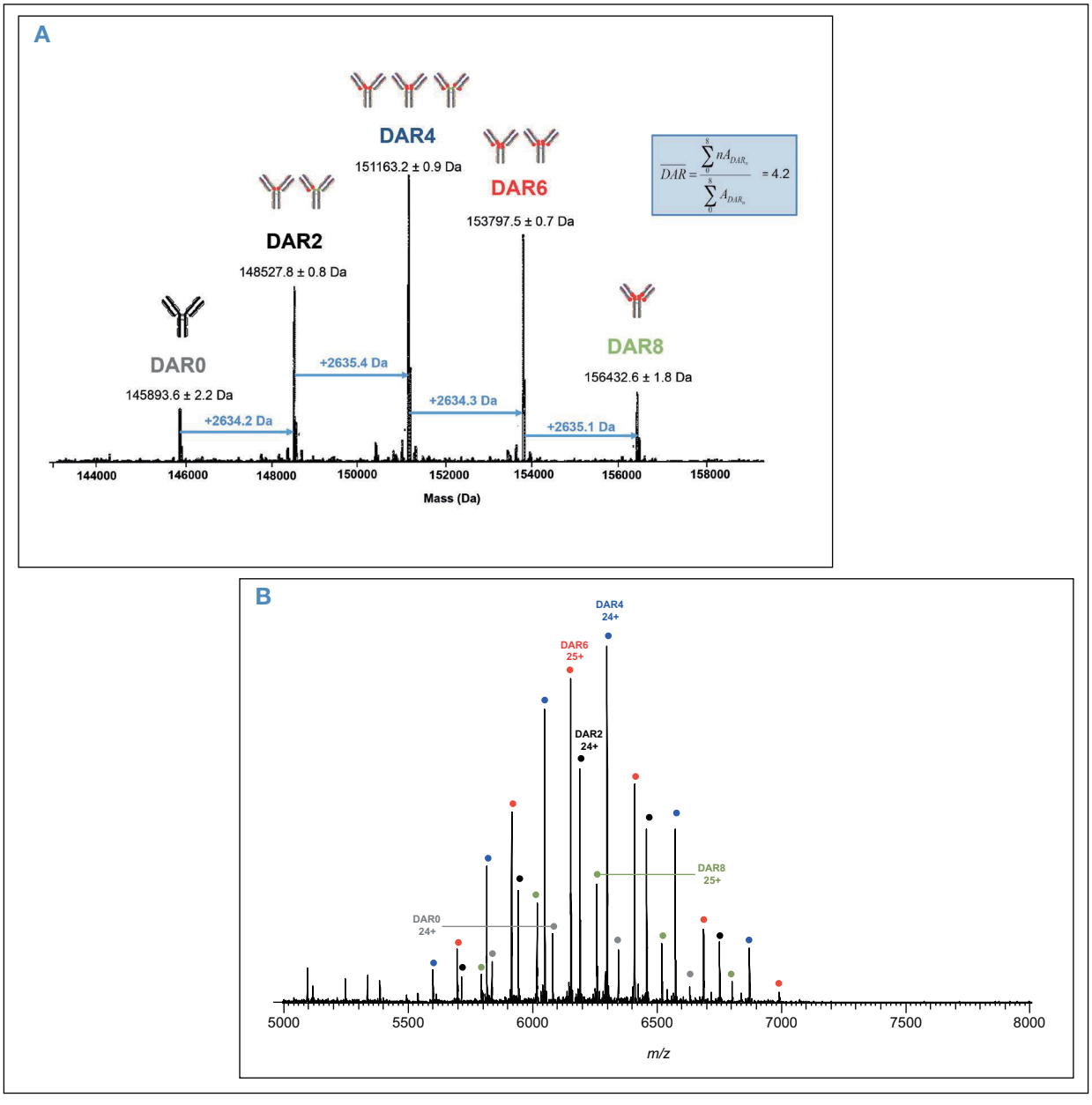

Figure 3. Orbitrap Native MS analysis of a monoclonal Antibody-Drug-Conjugate (ADC). A. Native deconvoluted mass spectrum showing the determination of drug-to-antibody ratio (DAR). B. Raw mass spectrum with the entire charge state distribution of ADC under native conditions.

Orbitrap Native MS Analysis of Immune mAb/Antigen Complexes

Native MS can also be used to analyze mAb/antigen (mAb/Ag) complexes, providing additional information including mAb/antigen binding stoichiometries, specificities and affinities.[4] These properties are essential for originator and biosimilar candidates comparison studies. ESI-MS presents the advantage to allow the direct observation of noncovalent immune complexes without any chemical modification. J10.4 is a commercial mouse monoclonal IgG1 raised against recombinant JAM fusion protein of human origin that is recommended for detection of JAM-A by western blotting and immunopurification techniques. JAM-A, used here as antigen, is a single transmembrane protein belonging to the immunoglobulin superfamily. JAM-A localizes in tight junctions in normal epithelial and endothelial cells where homophilic JAM-A interactions have been shown to be important for regulation of epithelial barrier function.[4,5] This newly identified target is over-expressed in many tumor tissues and therefore is of prime interest as a target in oncology. Two JAM-A molecules are expected to bind to one J10.4 mAb.

The native mass spectrum of mAb/antigen complexes was recorded at a resolution of 35,000 with the in-source CID voltage set to 150 eV. As shown in Figure 4A, when an 4-fold excess of JAM-A (20 µM) is added to J10.4 mAb (5 µM), three species are detected: the intact free mAb (MW 150237.1 ± 1.1 Da, black), 1:1 (MW 174304.4 ± 2.0 Da, blue) and 1:2 (MW 198369.6 ± 2.3 Da, red) mAb:JAM-A complexes. Native MS thus confirmed that two JAM-A molecules can bind to J10.4 mAb. MWs correspond to the main G0F/G0F glycoforms. Relative abundances were estimated from MS peak intensities and proportions of mAb:Ag complexes at 1:1 and 1:2 stoichiometries were observed to be 37% and 30%, respectively, while free mAb represents 33%. Figure 4B shows the corresponding mass spectrum with the entire charge state distribution in native conditions.

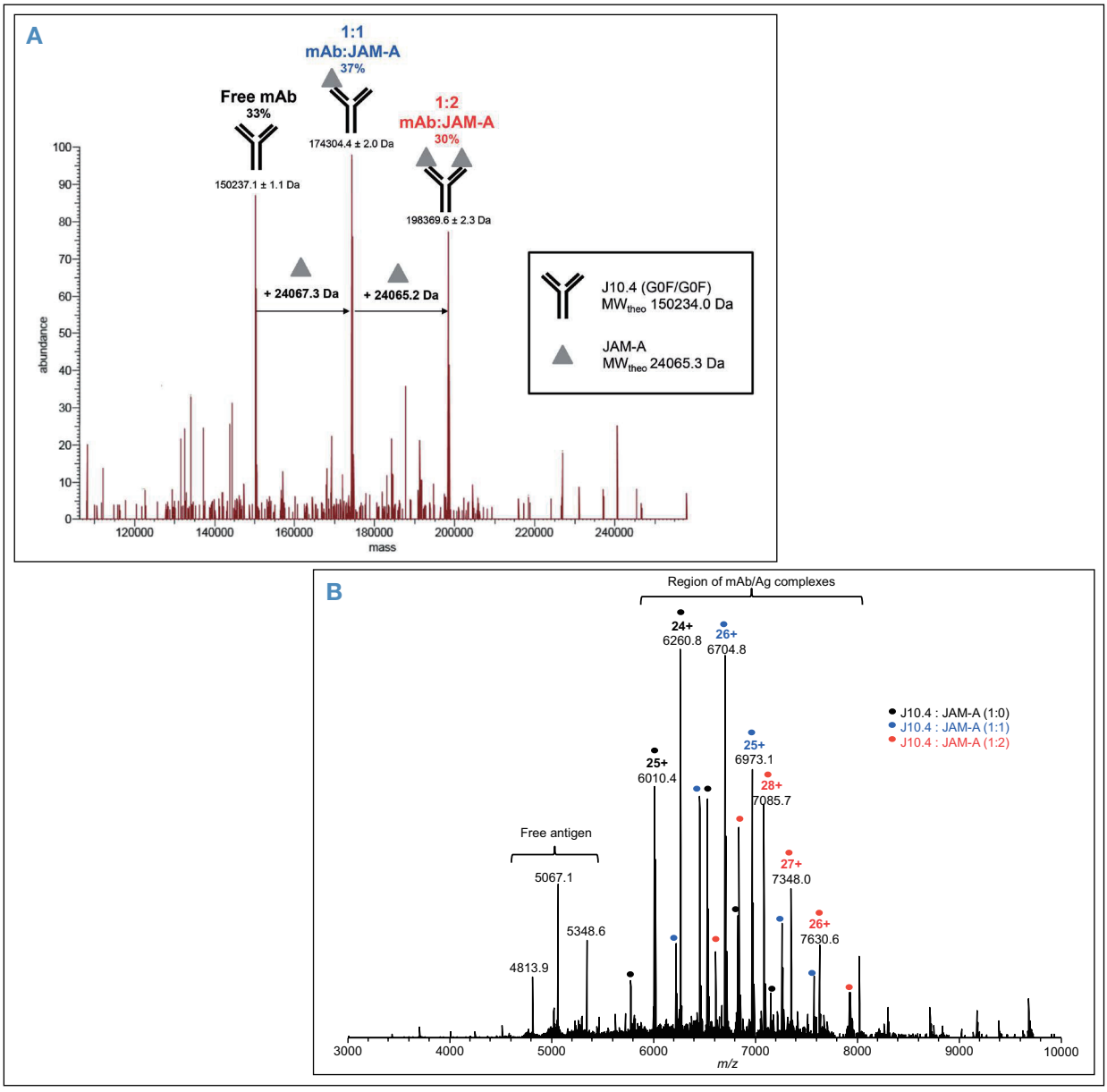

Figure 4. Orbitrap native MS detection of immune mAb/antigen complexes. A. Deconvoluted mass spectrum showing mAb/antigen binding stoichiometries. B. Charge state distribution in native conditions.

Native MS Analysis of a Mixture of Eleven N-deglycosylated Humanized Antibodies

Analysis of mAb mixtures is of utmost interest for high-throughput screening purposes and for therapeutic use to block simultaneously multiple epitopes. Indeed cocktails of mAbs with additive or synergic effects are increasingly foreseen as potential new therapeutic entities.

Figure 5A presents a convoluted mass spectrum of a mixture of eleven distinct deglycosylated humanized IgG antibodies. This mix includes two marketed therapeutic mAbs (rituximab and trastuzumab) and nine point mutation variants of the Hz6F4-2 mAb.[4,5] Figure 5B shows a full native mass spectrum of the mAb mix with an in-source CID energy set to 100 eV.

The well-resolved ion signals at a detection resolution of 140,000 and accurately measured masses enable the unambiguous assignment of ten out of the eleven compounds. Trastuzumab and Hz6F4-2v6 could not be differentiated due to very close molecular weights (2 Da). Peaks corresponding to Hz6F4-2 and Hz6F4-2v3, which differ by only 21 Da in mass, are clearly distinguished on the mass spectrum. However, they are not baseline resolved, and when combined with the low signal-to-noise (S/N) ratio (S/N < 20), that causes a relatively low mass accuracy for Hz6F4-2. However, with a good signal-to-noise ratio (S/N > 50), even without baseline-resolved peaks, for example, peaks of Hz6F4-2v9 and 6F4-2v10, the mass accuracies are achieved in the low ppm range for both species. The measured and theoretical masses for the mixture of eleven N-deglycoslated humanized antibodies are listed in Table 1.

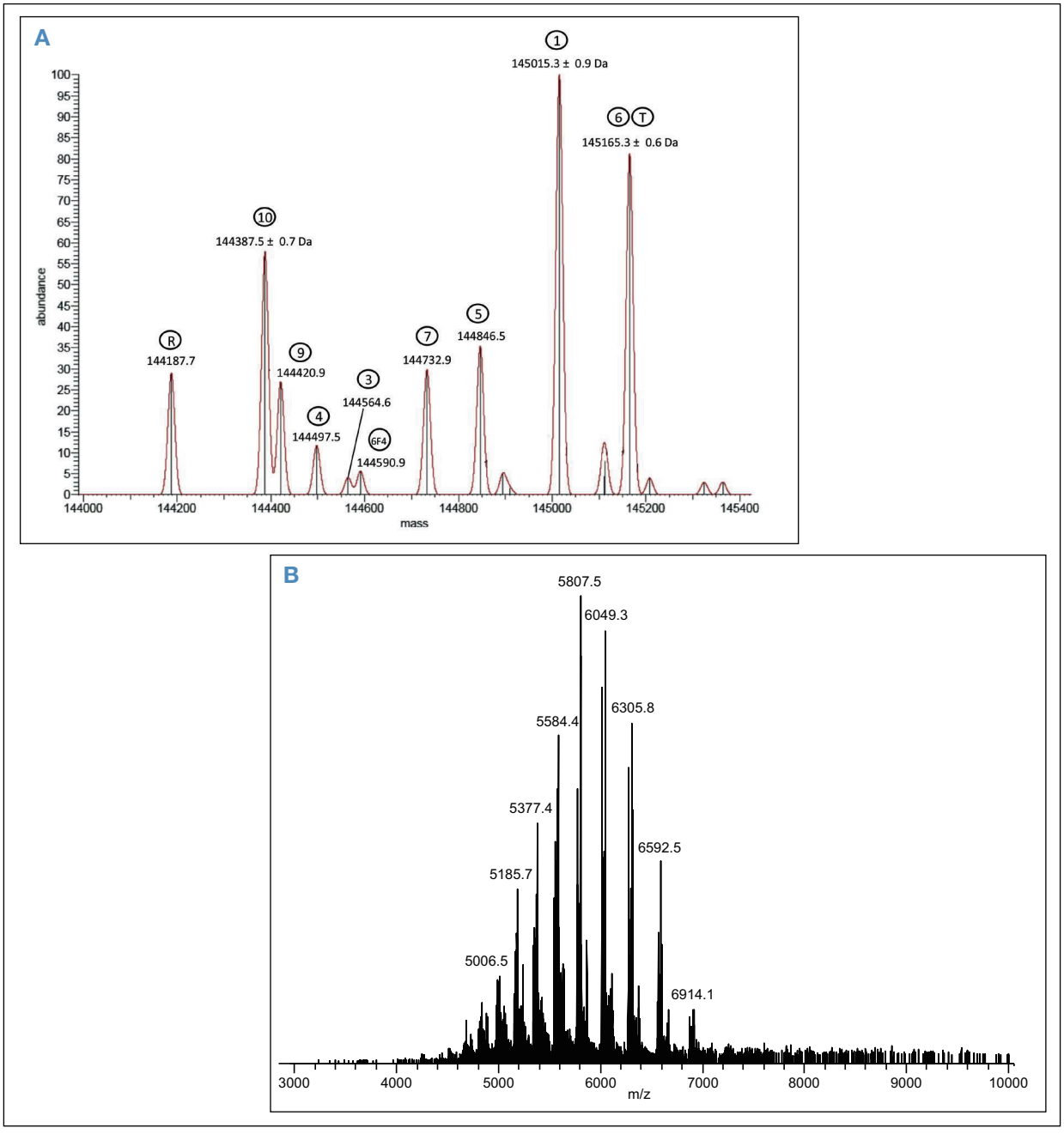

Figure 5. Native MS analysis of a mixture of eleven N-deglycosylated humanized antibodies. A. Deconvoluted mass spectrum. B. Charge state distribution in native conditions.

Table 1. Measured and theoretical masses for the mixture of eleven N-deglycosylated humanized antibodies at an Orbitrap detection resolution of 140,000

	Species	Theoretical Masses (Da)	Measured Masses (Da)	Mass Accuracy (ppm)
R	Rituximab	144186.3	144187.7	9.7
10	6F4-2 v10	144388.3	144387.5	5.5
9	6F4-2 v9	144420.5	144420.9	2.8
4	6F4-2 v 4	144498.4	144497.5	6.2
3	6F4-2 v3	144564.4	144564.6	1.4
6F4	6F4-2	144585.5	144590.9	37.3
7	6F4-2 v7	144732.5	144732.9	2.8
5	6F4-2 v5	144846.9	144846.5	2.8
1	6F4-2 v1	145015.3	145015.3	0
6	6F4-2 v6	145163.3	N.D	N.D
T	Trastuzumab	145165.5	145165.3	1.4

Conclusion

In the analysis (0.3–5 min) using the Exactive Plus EMR MS, molecular weight measurements of mAb and related products in the low ppm mass deviation range allowed the identification of all species simultaneously present in solution. The number of DAR and relative abundance of mAb/Ag complexes was also assessed with the peaks intensities serving for relative quantification of the detected species.

- The high resolving power of the Orbitrap mass analyzer can baseline resolve a native mAb's glycan peaks, as well as the interference peaks, ensuring an excellent mass accuracy in the low ppm range.
- The Exactive Plus EMR MS is able to sensitively characterize ADC complexes with mass differences between peaks corresponding to different additional number of payloads/drugs. For each set of peaks, the drug-to-antibody ratio (DAR) can be determined as well as the relative ratio of each detected compound in order to assess the mean DAR value.
- Native Orbitrap MS can reveal the number of antigens bound to mAbs. Relative abundances of mAb/Ag complexes at different stoichiometries can be achieved from MS peak intesities.
- The Exactive Plus EMR MS enables the high throughput screening of mAb mixtures, ensuring a excellent mass accuracy for each individual mAb.

Acknowledgements

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APPLICATION NOTE 72358

Quick screening of intact antibody and antibody-drug conjugates with integrated microfluidic capillary electrophoresis and mass spectrometry

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Keywords

CE-MS, ZipChip, Q Exactive BioPharma mass spectrometer, BioPharma Finder software, Monoclonal antibody, Antibody-drug conjugates

Goal

Rapid screening of the heterogeneity of monoclonal antibodies and antibody-drug conjugates by an integrated microfluidic capillary electrophoresis (CE) and mass spectrometry (MS) workflow

Introduction

Monoclonal antibodies (mAb) and antibody-drug conjugates (ADC) constitute two of the most important biopharmaceuticals within the class of biotherapeutic drugs.[1] During drug development and manufacturing, undesired mutations and *in vitro* modifications may introduce sample heterogeneity, causing changes to the protein structure that may lead to the loss of drug efficacy.[2,3] Therefore, the availability of a quick screening method at the intact protein level to detect and assess any variability that might occur during drug development is attractive. Multiple analytical methods such as high-resolution liquid chromatography (HPLC), capillary electrophoresis (CE), and mass spectrometry (MS) have been used separately or together to characterize monoclonal antibodies.

Introduced here is a CE-MS solution achieved by coupling the ZipChip™ (908 Devices) system and the Thermo Scientific™ Q Exactive™ Orbitrap™ MS with the BioPharma option to rapidly assess the heterogeneity of mAb and

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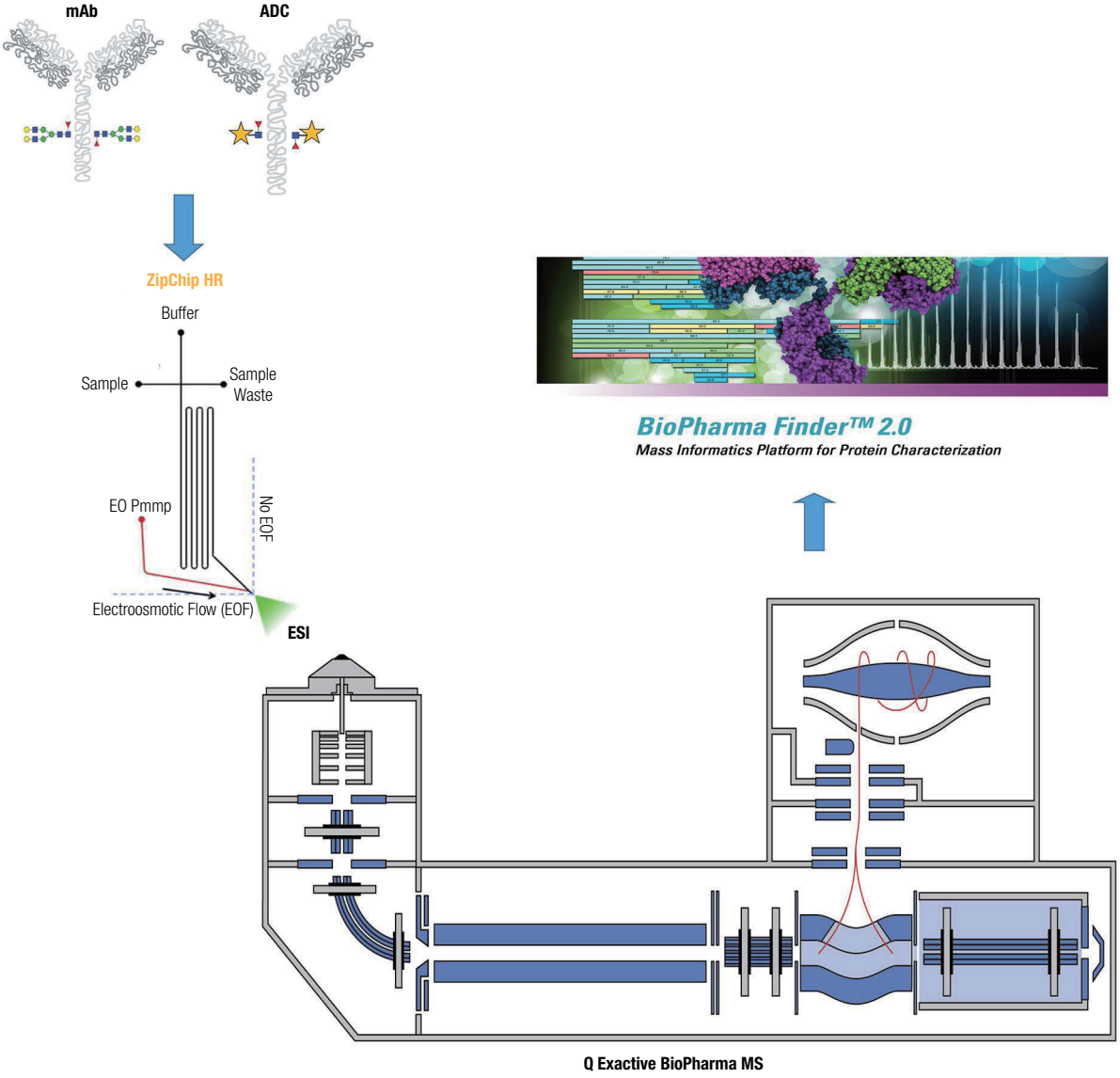
ADC as a complementary approach to existing analytical methods. CE has been previously described as a high-resolution separation method for intact proteins.[5–7] The implementation of charge-based separations as a microfluidic device, the ZipChip provides shorter analysis time due to its high electrical field strength.[8] The Q Exactive MS with BioPharma option is quickly becoming popular for biopharmaceutical characterization due to its high-resolution, accurate-mass (HRAM) performance for the detection of very large biomolecules with a mass range up to *m/z* 8000. This application describes the combination of these two techniques in one platform and demonstrates its ability for quick screening of biopharmaceutical drug heterogeneity.

Experimental

Materials

NIST mAb (reference material 8671)[9] is a recombinant, humanized IgG1 κ and its structure and post-translational modifications have been well characterized. The antibody sample is known to have a relative abundance of C-terminal lysine clipping, N-terminal pyroglutamination, glycosylation, as well as a lower abundance of oxidation, deamidation, and glycation modifications. An NIST mAb ADC analogue was made from the NIST mAb reference material using enzyme-based N-glycan labeling and SiteClick™[10] chemistry, resulting in covalent binding of two molecules of DIBO-Biotin per mAb.



Scheme 1. The workflow of intact mAb/ADC characterization. Characterization of mAb/ADC is accomplished by the separation of CE ZipChip, online analysis of Q Exactive BioPharma MS, and deconvolution/identification with BioPharma Finder 2.0 software.

Sample preparation

NIST mAb was stored in 12.5 mM of L-histidine and 12.5 mM of L-histidine HCl (pH 6.0). NIST mAb ADC was stored in 1× of Tris-buffered saline (TBS). Both samples were diluted with deionized water to 0.5 µg/µL and ready for analysis.

Capillary electrophoresis

A ZipChip HR chip (P/N 00950-01-00499) designed with a 22 cm separation channel and a built-in ESI emitter was used in the ZipChip system (P/N 00950-01-00492) (Scheme 1). A premixed background electrolyte solution (ZipChip Intact Antibody Kit, P/N 00950-01-00502) was used. Samples were injected into the CE separation channel by pressure injection of 2 psi for 5 s, which corresponds to an injection volume of 0.65 nL. An electrical field strength of 770 V/cm with 2 psi pressure assistance was used during CE separation, resulting in an average analysis time of 3 min (Table 1).

Mass spectrometry

A Q Exactive Plus MS with BioPharma option (P/N 0726055) was used in this study in high mass range (HMR) mode for the analysis of intact mAb and ADC (Scheme 1). The HMR mode extends the detection capability of the Orbitrap™ mass spectrometry up to *m/z* 8000. In this work, a mass range between *m/z* 2500 and 6000 was sufficient to detect the mildly-denatured mAb at a MS scan rate of 12 scans/s. In-source CID (100 eV) was applied to assist desolvation (Table 1).

Data analysis

The raw data from the mAb and ADC measurements were analyzed with Thermo Scientific™ BioPharma Finder™ 2.0 software (P/N OPTON-30592). For the characterization of NIST mAb, a protein sequence of 1326 amino acids with 16 disulfide bonds was created in the protein sequence manager, which included a fixed modification for N-terminal pyroglutamination and variable modifications for glycosylation, C-terminal lysine clipping, and glycation. For the study of NIST mAb ADC, in which the glycosylation moiety had been removed with endoglycosidase S (Endo S) and replaced by DIBO-Biotin groups through azide activation (Scheme 2), the DIBO-Biotin was considered as the variable modification. The average mass of the NIST mAb and NIST mAb ADC were calculated with the sliding-window deconvolution method using the ReSpect™ algorithm and the identification automatically aligned with the structural information in protein sequence manager (Table 1).

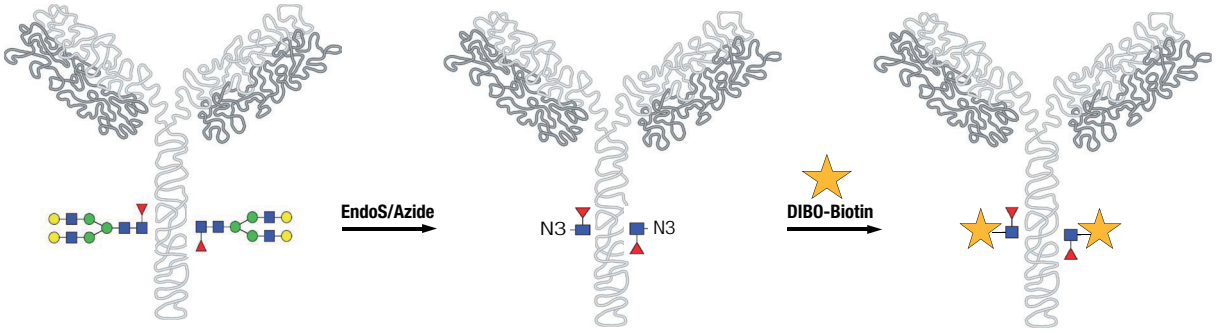
Table 1. Experimental conditions on the CE ZipChip, Q Exactive BioPharma MS, and BioPharma Finder 2.0 software.

CE ZipChip	
Chip:	HR chip
CE Electrical Field Strength:	770 V/cm
Pressure Assistance:	Enabled
Background Electrolyte Solution (BGE):	ZipChip Intact Antibody Kit
Sample Injection:	2 psi, 5 second
Q Exactive BioPharma MS	
Mode:	HMR mode
Scan Type:	Full MS
m/z Range:	2500 to 6000
Fragmentation:	In-source CID 100 eV
Resolution:	17,500
Sheath Gas Flow Rate:	2
Capillary Temp:	200 °C
S-lens RF Level:	100
BioPharma Finder 2.0 Software	
Disulfide Bond:	12 inter-chain; 4 intra-chain
Fixed Modification:	N-terminal pyroglutamination
Variable Modification:	C-terminal lysine clipping glycosylation
Source Spectra Method:	Sliding windows
Deconvolution Algorithm:	ReSpect™

Results and discussion

NIST mAb analysis

Recombinant therapeutic antibodies are large and complex molecules with a variety of modifications such as glycosylation, terminal clipping, oxidation, deamination, and glycation. These NIST mAb modifications have to be confidently identified through mass accuracy and the site of modification has to be localized through MS/MS analysis. Presented here is a powerful analysis method based on the ZipChip technology for fast separation of mAb samples in combination with HRAM Orbitrap MS for confident identification of the mAb and its variants. Near baseline separation of the lysine charge variants of intact NIST mAb sample was achieved within 3 minutes. (Figure 1A). Using the sliding deconvolution method, baseline-resolved mass spectra were obtained from the very low abundant 2-lysine variant whose relative abundance is only about 1.3% of the base peak. The charge distribution of the intact mAb mass spectrum remained within the mass range of *m/z* 4600–5800 (Figure 1A), which is close to the distribution of the mAb under native conditions.



Scheme 2. Synthesis of site-specific ADC from the NIST mAb with click chemistry.

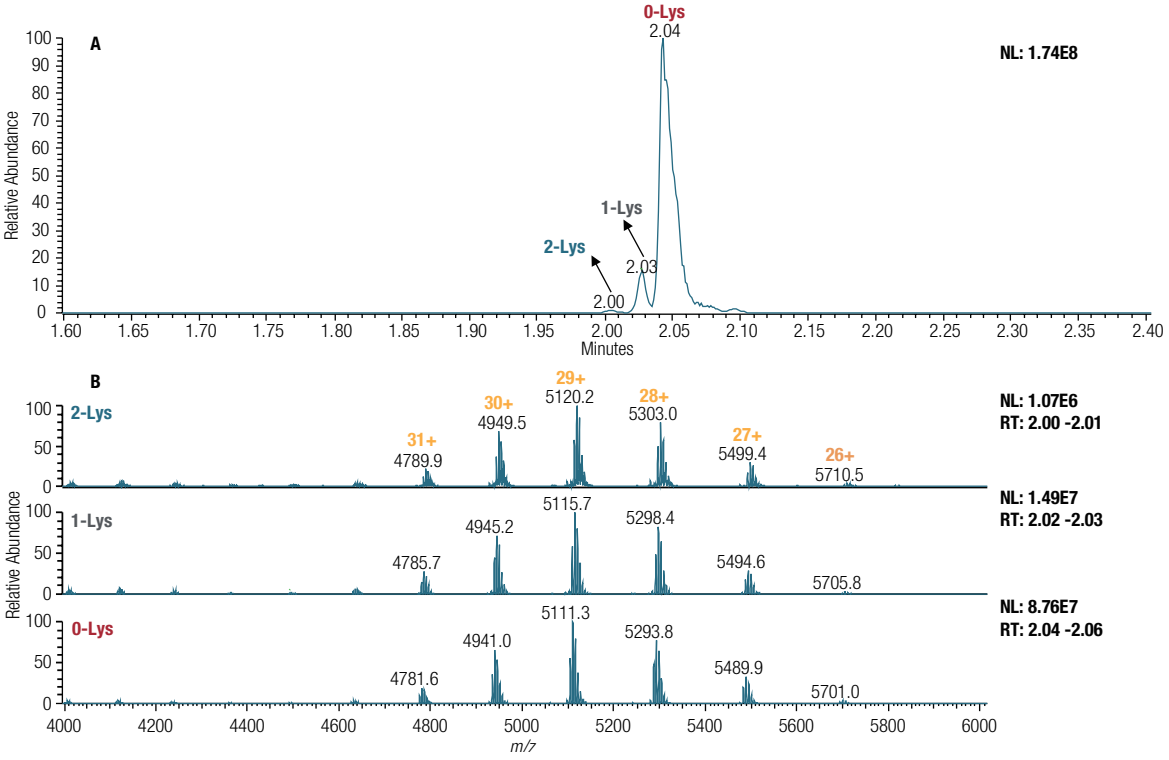


Figure 1. A) Electropherograms of NIST mAb in a 3 min run on a ZipChip CE device hyphenated to Q Exactive BioPharma MS. Three different lysine variants were conducted in a baseline separation B) Three mass spectra were averaged over the widths from the three peaks on the electropherograms from left to right.

The deconvoluted mass spectra of the 2-lysine, 1-lysine, and 0-lysine variants of the NIST mAb (Figure 2) show five major glycoforms. Each variant was separated by a mass difference of 162 Da indicative of the presence of hexoses. The same distribution of mAb glycoforms were observed for all three N-terminal lysine variants. Altogether, 17 variants were identified with mass error below 20 ppm at the intact protein level by BioPharma Finder 2.0 software (Table 2), as determined from the analysis of three injection replicates. Sum intensity of all NIST mAb variants peaks was determined and relative quantification of the 17 variants was assessed to span over three orders of magnitude in dynamic range (Table 2). The mAb variant containing two additional N-terminal lysines and the glycoforms G2F/G2F was detected at 0.19% signal intensity (row 17 in Table 2). Relative quantification for 17 variants was enabled through the combination of high resolution CE separation together with the high mass accuracy and long-term mass stability provided by the Orbitrap mass analyzer.

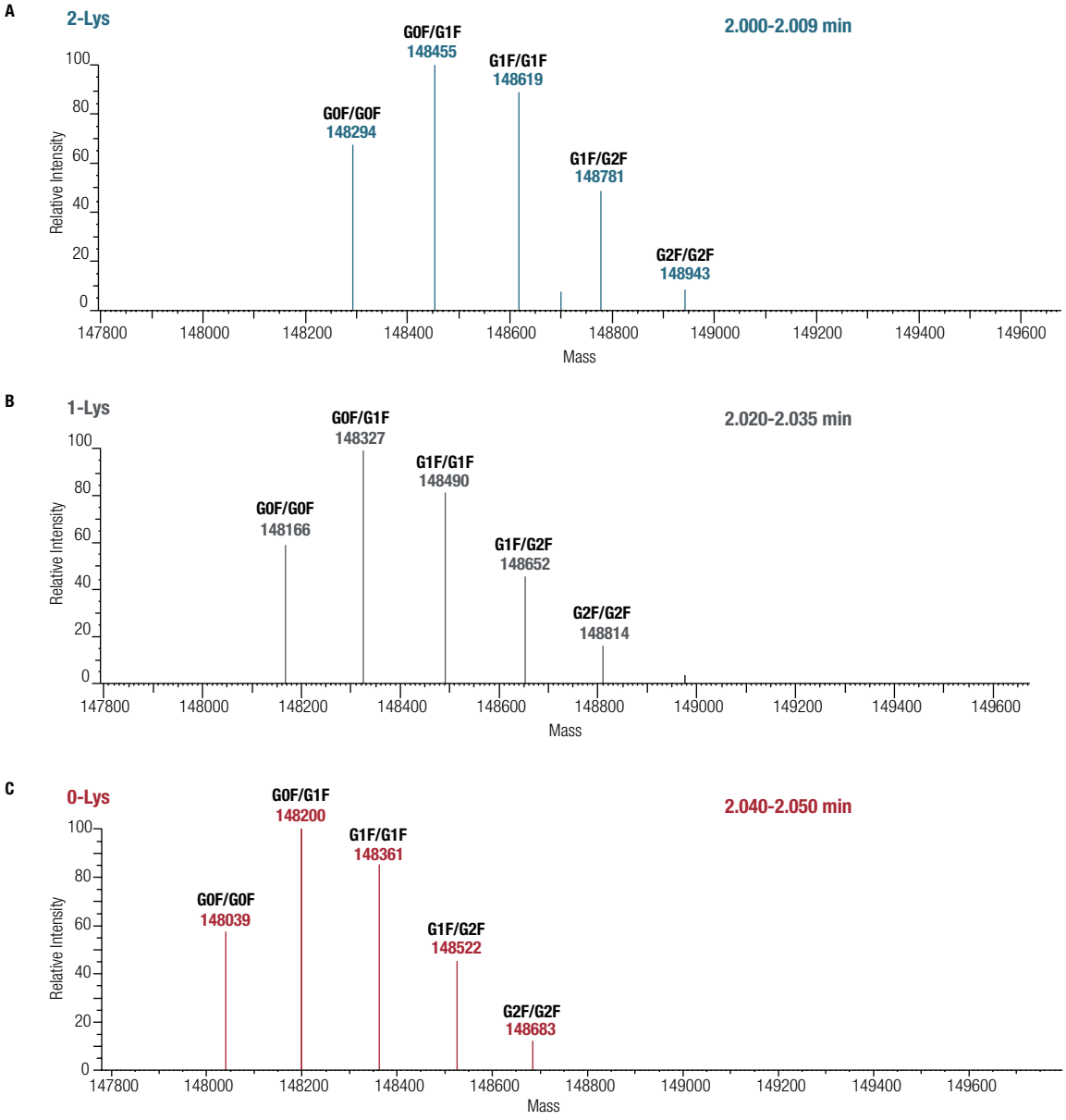


Figure 2. Three deconvoluted mass spectra from different migration time intervals, which correspond to different lysine variants. A) NIST mAb with two lysine adducts including five glycoforms was detected B) NIST mAb with one lysine adduct including five glycoforms was detected C) NIST mAb without lysine adduct including five glycoforms was detected.

Table 2. Seventeen identified variants of NIST mAb from three replicates of run by using BioPharma Finder 2.0 software for data analysis.

Protein Name	Modification	Average Mass(Da)	Theoretical Mass (Da)	Mass Error (ppm)	Sum Intensity	Relative Abundance
NIST mAb	1xG0FG1F	148199.66	148199.21	3.1	8.54E+09	100.00
NIST mAb	1xG1FG1F	148361.30	148361.35	0.3	7.30E+09	85.52
NIST mAb	1xG1FG2F	148523.57	148523.49	0.6	3.33E+09	38.98
NIST mAb	1xG0FG0F	148037.72	148037.07	4.4	3.18E+09	37.29
NIST mAb	1xG2FG2F	148685.06	148685.63	3.8	1.23E+09	14.35
NIST mAb_Hexose	1xG2FG2F	148845.21	148847.77	17.2	1.61E+08	1.89
NIST mAb minus 1GlcNac	1xG0FG0F	147831.30	147833.88	17.4	9.78E+07	1.15
NIST mAb plus 1K	1xG0FG1F	148327.72	148327.41	2.1	1.67E+09	19.57
NIST mAb plus 1K	1xG1FG1F	148489.69	148489.55	1.0	1.44E+09	16.81
NIST mAb plus 1K	1xG0FG0F	148166.13	148165.27	5.8	1.07E+09	12.56
NIST mAb plus 1K	1xG1FG2F	148651.44	148651.69	1.7	6.99E+08	8.18
NIST mAb plus 1K	1xG2FG2F	148811.79	148813.83	13.7	2.67E+08	3.13
NIST mAb plus 2K	1xG0FG1F	148456.98	148455.61	9.3	1.13E+08	1.32
NIST mAb plus 2K	1xG1FG1F	148618.90	148617.75	7.7	1.09E+08	1.28
NIST mAb plus 2K	1xG0FG0F	148294.86	148293.47	9.4	8.65E+07	1.01
NIST mAb plus 2K	1xG1FG2F	148780.66	148779.89	5.2	5.81E+07	0.68
NIST mAb plus 2K	1xG2FG2F	148942.67	148942.03	4.3	1.61E+07	0.19

NIST mAb ADC analysis

Recombinant therapeutic antibodies have been used to treat a variety of diseases. This successful therapeutic approach has been extended to a new class of biopharmaceutical drugs: antibody-drug conjugates, where the highly potent drug payload is covalently linked to the antibody to specifically target and affect the site of disease. To date, only two ADCs are marketed while more than 60 ADC molecules are currently undergoing clinical evaluation.[11] Historically, the introduction of a drug molecule to the mAb through statistical conjugation brings a heterogeneous drug load distribution to the ADC, which could result in altered therapeutic effects with various pharmacokinetic implications and potentially a narrower therapeutic window. Site-specific conjugation methods with a more homogenuous drug distribution are now considered a standard method in ADC development.[12,13] The site-specific ADC analogue analyzed here was synthesized from the NIST mAb with a known payload of two at the site of glycan modifications to provide a reference for other ADC molecules.

The same experimental setting used for the NIST mAb analysis was applied to the NIST mAb ADC analysis. Similar to the NIST mAb analysis, near-baseline separation of the lysine variants of the ADC was achieved within 3 minutes (Figure 3A). The mass spectrum is less complex due to the removal of the complex glycosylation modification and replacement with a simple biotin moiety (Figure 3B). Five major ADC variants were identified by BioPharma Finder software (Table 3). We observed high abundance of 0-lysine, 1-lysine, and 2-lysine NIST mAb ADC variants each carrying a payload of two. Variants with zero or one payload were not observed. The drug-to-antibody ratio (DAR) was calculated as 2.0 in this site-specific NIST mAb ADC. Two additional peaks with the molecular weight of ADC + 162 Da and ADC + 324 Da were detected and identified as ADC+1 hexose and ADC+2 hexose molecules. These Hexose adducts were also observed in the NIST mAb on the glycoforms of G2F/G2F (Figure 4). They have also been reported as the glycation adducts from the NIST mAb[9] and were not removed during the the PNGase F digestion.

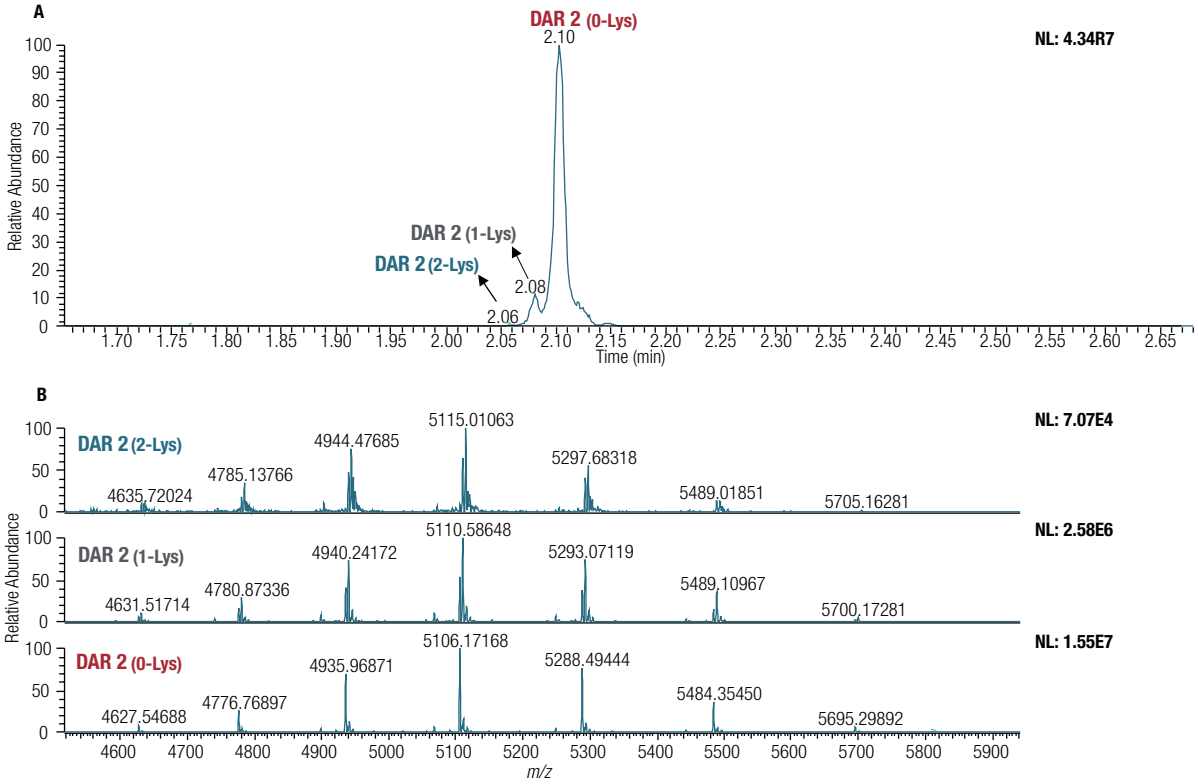


Figure 3. A) Electropherograms of NIST mAb ADC in a 3 min run on a CE ZipChip hyphenated to Q Exactive BioPharma MS. Three different forms of lysine variants were separated B) three mass spectra were averaged from the three peaks on the electropherograms over their widths from left to right.

Table 3. Five identified variants of NIST mAb ADC using the BioPharma Finder software.

Protein Name	Modification	Average Mass(Da)	Theoretical Mass (Da)	Mass Error (ppm)	Relative Abundance
NIST mAb_Endo_Azide	1xtwo_DIBO_Biotin	148049.09	148049.67	3.9	100.00
NIST mAb_Endo_Azide	1xtwo_DIBO_Biotin_1 Hexose	148213.40	148211.81	10.8	14.13
NIST mAb_Endo_Azide	1xtwo_DIBO_Biotin_2 Hexose	148373.95	148373.95	0.0	2.10
NIST mAb plus 1K_Endo_Azide	1xtwo_DIBO_Biotin	148177.60	148177.87	1.8	8.68
NIST mAb plus 2K_Endo_Azide	1xtwo_DIBO_Biotin	148306.59	148306.07	3.5	0.12

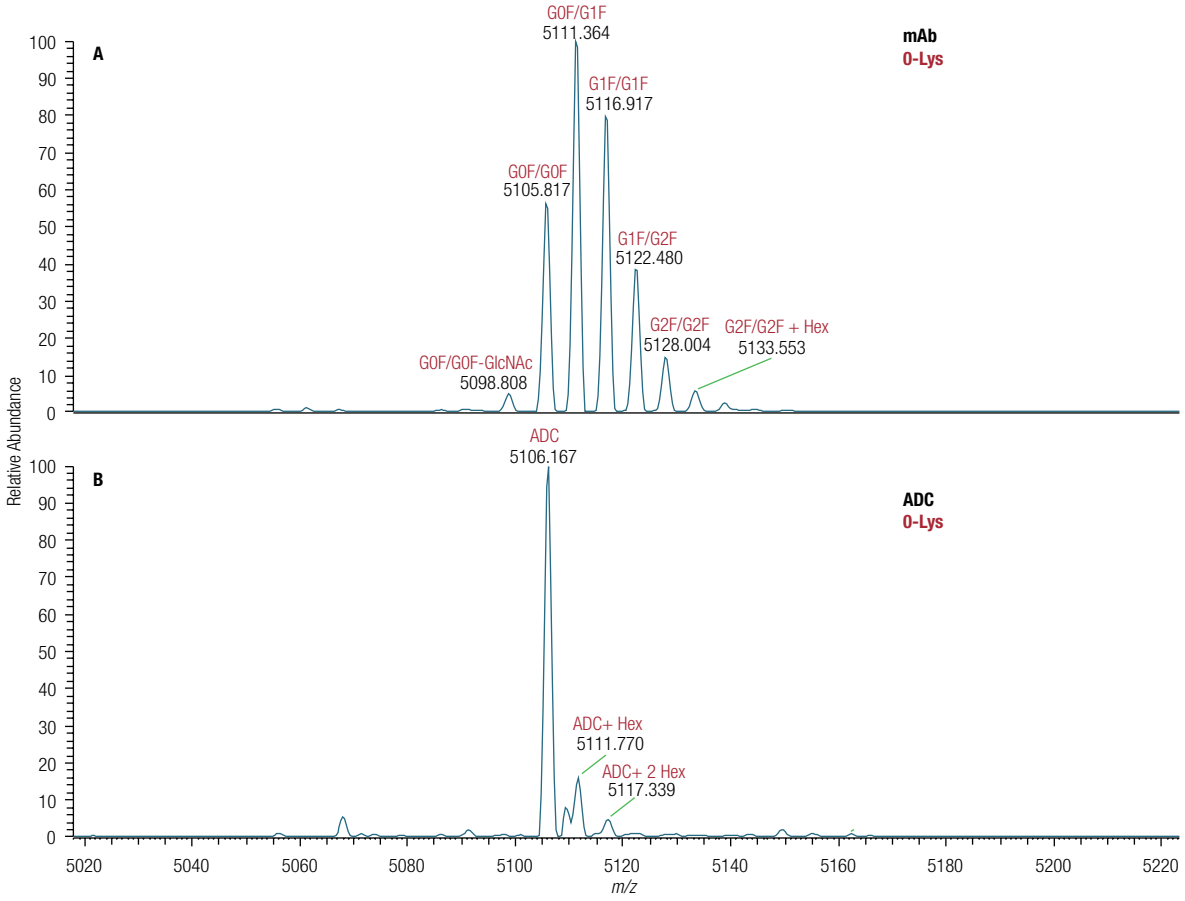


Figure 4. Identification of peaks from raw mass spectra acquired by CE-MS from A) NIST mAb and B) NIST mAb ADC.

Conclusion

In this study, fast analysis of the heterogeneity of the intact NIST mAb and ADC samples was achieved within 3 min using a CE-MS workflow. Identification of highly differently abundant sample components was accomplished by automatic peak identification through sliding-window deconvolution. Samples were directly injected on the chip, eliminating the need for desalting or additional sample preparation before analysis. The minimal sample preparation and online desalting capability make this approach even more amenable to quick screening and characterization of mAbs and ADCs during various phases of drug development and production. Compared to infusion-based MS methods, the separation of charge variants by capillary electrophoresis reduces sample complexity for MS detection and allows a more complete sample profiling. In this study, 17 NIST mAb variants with signal intensities spanning over four orders of magnitude were identified. The high sensitivity and dynamic range of the Q Exactive Orbitrap mass spectrometer were able to detect and relatively quantify variants with abundance as low as 0.19% of the base peak intensity. One of the additional benefits of this platform is its low sample consumption. Only about 0.2 ng of mAb or ADC sample was used for the successful analysis, which makes it particularly well suited during early stage drug development where sample might be limited.

For mAb characterization, near isobaric masses in heterogeneous mAb preparations pose a substantial analytical challenge. In this study, the ZipChip coupled to a Q Exactive MS was able to identify and quantify all known variants with a high dynamic range in a few minutes, making the workflow the method of choice for quick screening of the heterogeneous mAb and ADC samples during biopharmaceutical drug development.

Abbreviations:

ADC: antibody-drug conjugate
CE: capillary electrophoresis
cIEF: capillary isoelectric focusing
ESI: electrospray ionization
HILIC: hydrophilic interaction liquid chromatography
HMR mode: high mass range mode
HRAM: high-resolution accurate-mass
IgG: immunoglobulin G
mAb: monoclonal antibody
MS: mass spectrometer

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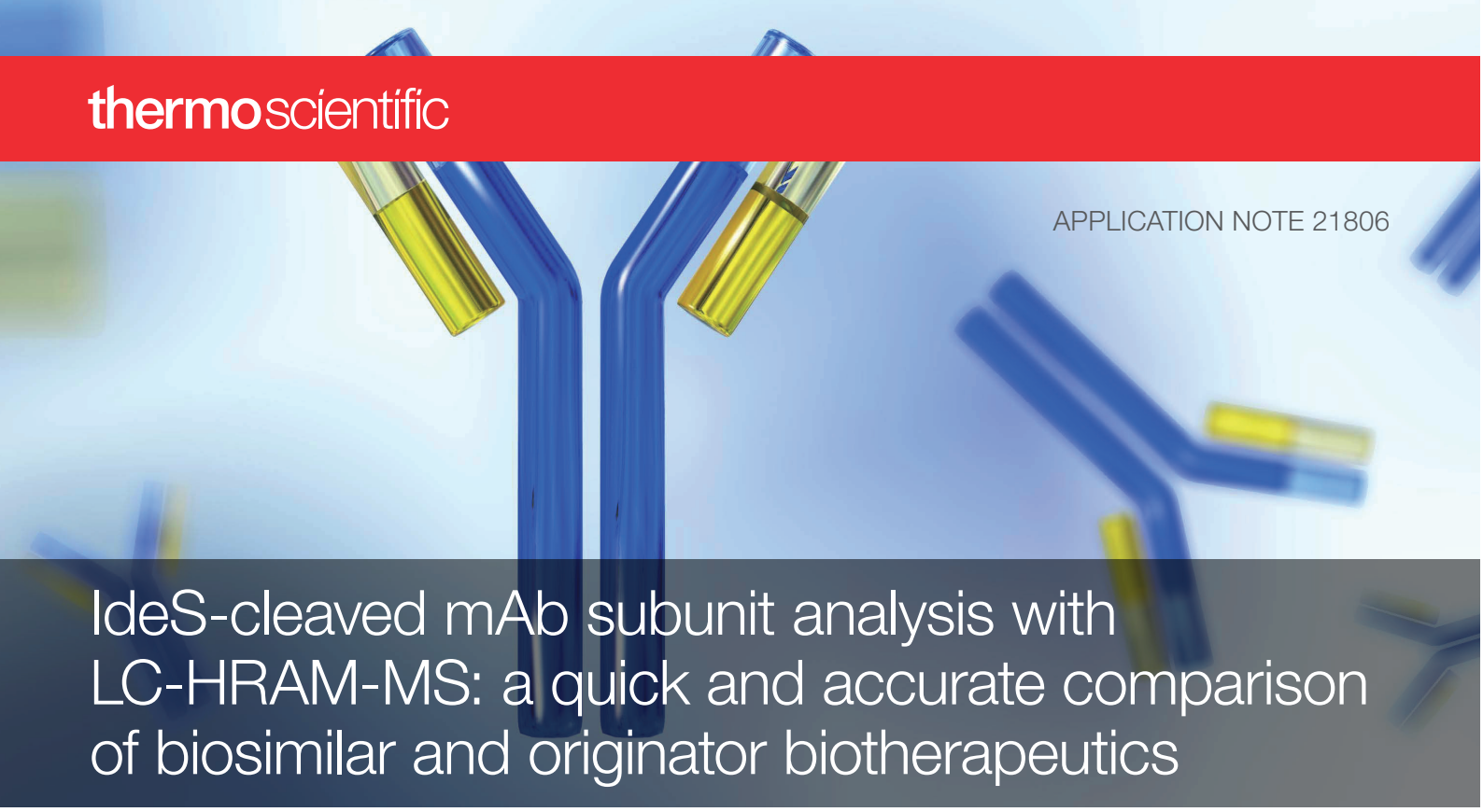

IdeS-cleaved mAb subunit analysis with LC-HRAM-MS: a quick and accurate comparison of biosimilar and originator biotherapeutics

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Keywords

NIBRT, biopharmaceutical, biotherapeutic, monoclonal antibody (mAb), IgG, middle-up, glycan analysis, MAbPac RP columns, Vanquish Flex Binary UHPLC system, Virtuoso Vial Identification System, Q Exactive Plus Hybrid Quadrupole-Orbitrap mass spectrometer, IdeS, rituximab, trastuzumab, bevacizumab, BioPharma Finder

Application benefits

- Demonstrate the benefits of using a middle-up approach for biotherapeutics characterization
- Demonstrate the applicability of Thermo Scientific™ MAbPac™ RP columns for subunit analysis
- Highlight the benefits of using the Thermo Scientific™ Q Exactive™ Plus Hybrid Quadrupole-Orbitrap™ mass spectrometer for high-resolution, accurate-mass MS analysis on different subunits of monoclonal antibodies to evaluate glycans and other micro-variants

Goal

To highlight the use of middle-up techniques for biotherapeutics characterization and biosimilar evaluation. To show the importance of high-resolution, accurate-mass MS techniques to characterize mAbs variants at middle-up level, obtaining structural data on glycoforms, lysine truncation and other micro-variants present on the product. To demonstrate the support that subunit analysis can give during product development. To demonstrate that the method is easy to optimize, fast, and reproducible.

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Introduction

Recombinant monoclonal antibodies (mAbs) are the fastest growing class of human therapeutics. Their success in the biopharmaceutical industry and the introduction of a growing number of biosimilar therapeutics go along with the need for reliable and fast characterization methods to establish drug quality and safety. Indeed, a biosimilar is a drug presenting minimal variations from its originator due to several factors like the expression system and growing conditions, the purification steps, or the final formulations. It is of critical importance to monitor and quantify these variations to correlate them to any potentially different *in vivo* activity, as different clearance time or other interactions within the patient.

To spot these differences, full sequence coverage and post-translational modifications (PTMs) are usually obtained with a bottom-up approach employing a combination of several LC-MS/MS datasets derived from different and orthogonal enzymatic digestions of the protein. Top-down or middle-up approaches have the potential to minimize sample handling and artefacts and to give quicker or complementary information.

In this study, liquid chromatography hyphenated with high-resolution, accurate-mass spectrometry (LC-HRAM-MS) was used in a middle-up approach for the comparison of three commercially available mAb drug substances (DS) and their respective biosimilar obtained in house (BS). Samples were digested with IdeS enzyme, cleaving the monoclonal antibody in the hinge region and generating, after reduction of disulfide bonds, two pairs of polypeptides from the heavy chain along with the light chain portion (Figure 1).[1]

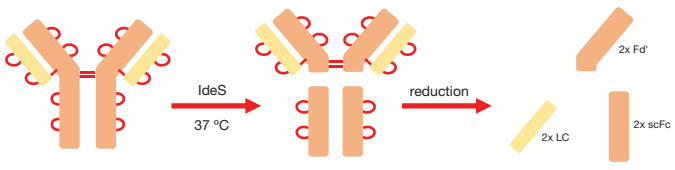

Figure 1. IdeS digestion scheme. IdeS enzyme cleaves the monoclonal antibody below the hinge region allowing the separation of the Fc region and F(ab')$_2$ region. Following treatment with a reducing agent, *intra-* as well as *inter*-molecular disulfide bonds are reduced, generating 2× scFc, 2× LC and 2× Fd' polypeptides.

Analysis was performed on a high-resolution analytical platform consisting of a Thermo Scientific™ Vanquish™ Flex Binary UHPLC and Thermo Scientific™ Q Exactive™ Plus Hybrid Quadrupole-Orbitrap™ mass spectrometer. Data were analyzed using the platform for intact mass analysis within Thermo Scientific™ BioPharma Finder™ 3.0 software. The quality of the data together with the quick analysis allowed a confident identification and sequence verification of light chain and Fd' region and a rapid analysis of Fc region variants, including glycoform and N-terminal lysine loss. Moreover, BioPharma Finder 3.0 software is able to rapidly provide a comparison with previously acquired data used as reference; this provides a rapid and confident way for the determination of batch-to-batch variations in analytical laboratories.

Experimental

Recommended consumables

- Deionized water, 18.2 MΩ·cm resistivity
- Water, Fisher Chemical™ Optima™ LC/MS grade (P/N 10505904)
- Water with 0.1% formic acid (v/v), Fisher Chemical™ Optima™ LC/MS grade (P/N 10188164)
- Acetonitrile with 0.1% formic acid (v/v), Fisher Chemical™ Optima™ LC/MS grade (P/N 10118464)
- HiTrap® Protein A (GE Healthcare)
- Tris(2-carboxyethyl)phosphine hydrochloride (TCEP), Thermo Scientific™ Pierce™ (P/N 20490)
- Ammonium hydrogen carbonate, Acros Organics™ (P/N 393212500)
- FabRICATOR® (Genovis) (P/N A0-FR1-020)
- Amicon® spin filter units, 10 KDa MWCO
- 8M Guanidine-HCl, Thermo Scientific™ Pierce™ (P/N 10167783)
- Thermo Scientific™ MAbPac™ RP column, 4 µm, 2.1 × 50 mm (P/N 088648)
- Thermo Scientific™ Virtuoso™ vial, clear 2 mL kit with septa and cap (P/N 60180-VT405)
- Thermo Scientific™ Virtuoso™ Vial Identification System (P/N 60180-VT100)

Sample handling equipment

- Thermo Scientific™ Vanquish™ Flex Binary UHPLC System including:
  - Binary Pump F (P/N VF-P10-A)
  - Column Compartment H (P/N VH-C10-A)
  - Split Sampler FT (P/N VF-A10-A)
  - System Base Vanquish Horizon (P/N VH-S01-A)
- Thermo Scientific™ Q Exactive™ Plus hybrid quadrupole- Orbitrap™ mass spectrometer (P/N IQLAAEGAAPFALGMBDK)
- Thermo Scientific™ Nanodrop™ 2000 Spectrophotometer (P/N ND-2000)

Sample preparation

ExpiCHO-S™ Cells (Gibco, #A29127) were derived from a non-engineered subclone that has been screened and isolated from Chinese hamster ovary (CHO) cells. Cells were cultured in suspension in serum-free, chemically defined media (Gibco), and transiently transfected with plasmid DNA encoding particular monoclonal antibody using a lipid-based transfection system (Gibco). The vectors (pFUSEss-CHIg-hG1 and pFUSE2ss-CLIg-hk) were purchased from Invivogen. Following transfection, the cells were harvested, and samples of clarified media were passed through a HiTrap Protein A column (GE Healthcare), then washed with phosphate buffered saline before elution of mAb from the Protein A column using 100 mM citric acid, pH 3.2. MAbs solutions were buffer exchanged in PBS and protein concentration was evaluated with the Nanodrop 2000 Spectrophotometer.

Middle-up analysis of IdeS-digested mAb

For middle-up analysis, 160 µg of each mAb in phosphate buffered saline (PBS) or formulation buffer (2 µg/µL solution) were combined with 2 µL of the IdeS digestion solution (67 units IdeS/µL in Optima grade water) and incubated at 37 °C for 2 hours at 500 rpm. For the reduction of disulfide bonds, 4M guanidine hydrochloride and 50 mM TCEP for 45 minutes at 56 °C were used. Following incubation, samples were reduced to dryness via vacuum centrifugation and reconstituted in 0.1% formic acid prior to LC-MS analysis.

LC conditions

Mobile phase A:	Water with 0.1% formic acid (v/v)
Mobile phase B:	Acetonitrile with 0.1% formic acid (v/v)
Flow rate:	0.3 mL/min
Column:	MabPac RP, 4 µm, 2.1 x 50 mm
Column temperature:	80 °C (active pre-heater) (Still air)
Autosampler temperature:	5 °C
Injection volume:	1 µL
Injection wash solvent:	Methanol/water, 10:90
Needle wash:	Enabled pre-injection
Gradient:	See Table 1 for details
Valve:	First two minutes to waste
Intact Protein Mode:	On
Trapping gas:	0.2

Table 1. Mobile phase separation for middle-up analysis

Time (min)	%A	%B	Curve
0	75	25	5
1	75	25	5
16	68	32	5
17	20	80	5
18	20	80	5
18.5	75	25	5
28.0	75	25	5

MS conditions

Table 2. Summary of used tune parameters

MS Source Parameters	Setting for Middle-up Analysis
Source	HESI
Sheath gas flow rate	25
Auxiliary gas flow	10
Probe heater temperature	150 °C
Source voltage	3.8 kV
Capillary temperature	320 °C
S-lens RF voltage	60.0

Table 3. Summary of used MS parameters

General	Setting Middle-up Analysis
Runtime	0 to 28 min
Polarity	Positive
Full MS Parameters	
Full MS mass range	600–2400 *m/z*
Resolution settings	240,000
Protein mode	On
AGC target value	3e6
Max injection time	200 ms
SID	0.0 eV
Microscans	5

MS data processing

Detailed parameter settings are shown in Table 4.

Table 4. Biopharma Finder 3.0 software parameter settings for analysis of IdeS subunits. Default Xtract™ – Average over selected region method used.

Component Detection	
Output mass range	1,000 to 60,000
Output mass	M
S/N threshold	3.00
Rel. abundance threshold	0.00
Charge range	5 to 50
Min. num. detected charge	3
Isotope table	Protein
Fit factor	80%
Remainder threshold	25%
Consider overlap	Yes
Resolution at 400 *m/z*	Raw File Specific
Charge carrier	H+ (1.00727663)
Minimum intensity	1
Expected intensity error	3
m/z range	600.00 to 2400.00
Chromatogram trace type	TIC
Sensitivity	High
Rel. intensity threshold (%)	1
Identification	
Sequence matching mass tolerance	20.00 ppm
Mass tolerance	10.00 ppm
RT tolerance	1.000 min
Min. number of required occurrences	1

Results and discussion

The importance of quick and reliable analytical methods to characterize monoclonal antibody variants and modifications has been highlighted already. In an analytical laboratory it is important to evaluate lot-to-lot consistency or to investigate potential problems present along the production pipeline. In addition, reliable analytical methods are relevant to R&D laboratories where biosimilar products are developed to establish comparability with their innovator.

To investigate structural differences of our biosimilar products with commercially available monoclonal antibodies, a subunit analysis was performed. All monoclonal antibodies were expressed in Chinese Hamster Ovary (CHO) cell lines. The mAbs were digested with IdeS (FabRICATOR, Genovis). IdeS is a cysteine protease with high specificity for the hinge region of IgG1. After disulfide bond reduction and alkylation, three polypeptide populations are generated: the intact light chain (LC), a heavy chain fragment containing the N-terminus (Fd'), and the remaining heavy chain region containing the glycosylation site and the C-terminus (scFc). For LC separation, a MabPac RP, 4 µm 2.1 × 50 mm, column was used. Following LC-MS analysis, data was processed using the Xtract™ algorithm in BioPharma Finder 3.0 software, which is specific for the isotopically resolved data obtained with 25 kDa proteins at 240,000 high resolution. In the TIC trace three species corresponding to the different subunits were present. Figure 2 shows the TIC traces and charge envelope profiles for each peak for the in-house expressed biosimilar of bevacizumab. In Table 5 the experimental values for the three subunits of each DS and BS are compared with theoretical values for LC, Fd', and scFc, taking into account the most prominent variants. All variants were identified within an error of ±3 ppm. All the analyses were performed in triplicate.

The great advantage of using the Q Exactive platform is clearly visible from the spectra obtained for the three subunits (Figure 2); using "protein mode" and high-resolution settings for the MS analysis, it is possible to observe isotopically resolved signals that distinguish between protein variants with very close masses.

Table 5. Experimental and theoretical masses (Da) obtained for the three investigated drug products and their biosimilars

Chain (modifications)	Experimental Mass	Theoretical Monoisotopic Mass	Mass Δ (ppm)	Experimental Mass	Theoretical Monoisotopic Mass	Mass Δ (ppm)
	Bevacizumab Drug Substance			Bevacizumab Biosimilar		
LC	23436.4647	23436.4337	1.3	23436.4787	23436.4337	1.9
Fd'	25929.7278	25929.6590	2.7	25929.7166	25929.6590	2.2
scFc-Man5 No C-term Lys	–	–	–	24992.3997	24992.3524	1.9
scFc-G0F No C-term Lys	25220.4842	25220.4634	0.8	25220.5239	25220.4634	2.4
scFc-G0 No C-term Lys	25074.4234	25074.4055	0.7	–	–	–
scFc-G1F No C-term Lys	25382.5245	25382.5162	0.3	25382.5552	25382.5162	1.5
scFc-G2F No C-term Lys	25544.5858	25544.5690	0.7	–	–	–
	Rituximab Drug Substance			Rituximab Biosimilar		
LC	23042.3317	23042.3437	-0.5	23104.2915	23104.3052	-0.6
LC Pyro-Glu Q1	23025.3486	23025.3171	1.4	23087.3344	23087.2786	2.4
Fd'	25329.3602	25329.3663	-0.2	25357.4164	25357.3976	0.7
Fd' Pyro-Glu Q1	25312.3939	25312.3397	2.1	25340.4451	25340.3710	2.9
scFc-Man5 No C-term Lys	–	–	–	24992.3947	24992.3524	1.7
scFc-G0F No C-term Lys	25188.5186	25188.4913	1.1	25220.5144	25220.4634	2.0
scFc-G1F No C-term Lys	25350.5776	35350.5441	1.3	25382.5446	25382.5162	1.1
scFc-G2 No C-term Lys	25366.5267	25366.5390	-0.5	–	–	–
scFc-G2F No C-term Lys	25512.6200	25512.5970	0.9	–	–	–
	Trastuzumab Drug Substance			Trastuzumab Biosimilar		
LC	23428.5659	23428.5238	1.8	23428.5729	23428.5238	2.1
Fd'	25367.5717	25367.5174	2.1	25367.5752	25367.5174	2.3
scFc-Man5 No C-term Lys	24992.4013	24992.3524	2.0	24992.3892	24992.3524	1.5
scFc-G0F No C-term Lys	25220.5049	25220.4634	1.6	25220.5093	25220.4634	1.8
scFc-G0 No C-term Lys	25074.4149	25074.4055	0.4	–	–	–
scFc-G1F No C-term Lys	25382.5514	25382.5162	1.4	25382.5179	25382.5162	0.1
scFc-G2F No C-term Lys	25544.6259	25544.5690	2.2	–	–	–

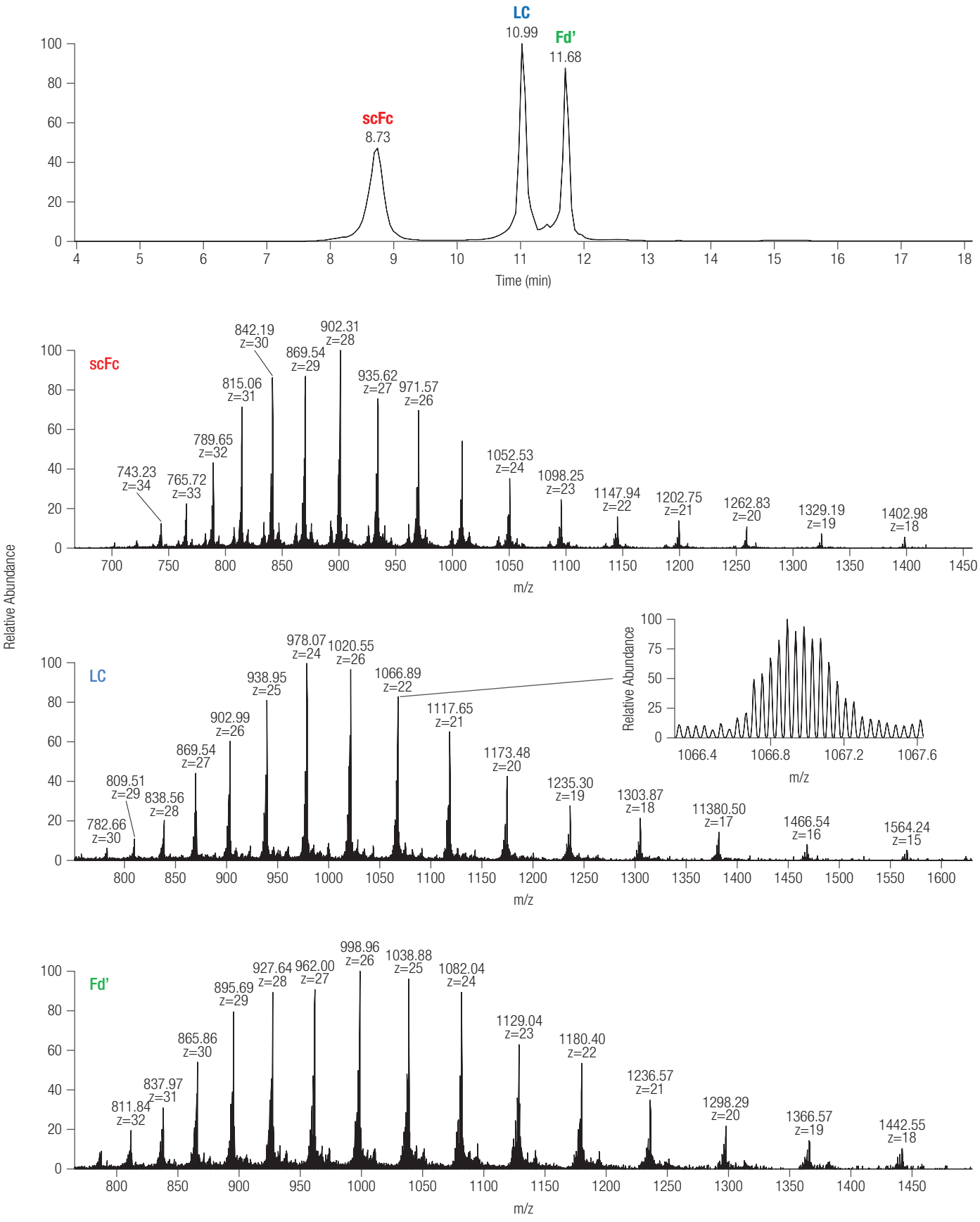

Figure 2. LC-MS analysis of IdeS digested bevacizumab biosimilar. In the top panel the TIC trace shows the three subunits obtained after enzymatic digestion and separated on a MabPac RP, 2.1 × 50 mm column. Bottom panels show the charge envelope profiles obtained for the three subunits using the protein mode on the Q Exactive Plus mass spectrometer; in the zoom it is possible to observe fully isotopically resolved peaks for +22 charge state of the LC region.

As an example, thanks to high resolution and mass accuracy obtained on the Q Exactive Plus Hybrid Quadrupole Platform, it was possible to monitor pyroglutamic acid formation on the Q1 residue for both heavy and light chain of rituximab drug product, prove that the same modification was present on the biosimilar, and quantify the two variants (Figure 3). As well, BioPharma Finder 3.0 software returns a table containing identified variants after deconvolution together with their total signal intensities; using this data a quick comparison of variants abundances and ratio can be performed.

Figure 4 shows the data related to the scFc region for the three investigated mAbs. Quantitative analysis was performed on all the samples using three distinct sample preparations. The data revealed an overall similarity between the complex *N*-glycan profiles of bevacizumab drug product and the in-house produced biosimilar. In the latter, high-mannose glycan with five hexoses residues (Man5) was expressed only in the biosimilar product (9.3%) and the abundancies of the remaining glycoforms were uniformly lower in BS, accordingly. As for rituximab and trastuzumab, a lower level of terminal galactose was obtained in the biosimilar products. Indeed, in all the samples G0F glycoform is the most abundant but its abundance has values around 50% in both drug products but it goes up to 80–90% in the biosimilars. Glycoforms containing one terminal galactose are strongly expressed in the drug products with values of 42.3% and 35.2% for rituximab and trastuzumab, respectively. Fully galactosylated glycans are also present in these biotherapeutics with percentages of 10.6% for rituximab (accounting for both G2 and G2F glycoforms) and 4.6% for trastuzumab. Their corresponding biosimilars show only one glycoform containing galactose (G1F) and their abundance is as low as 7.2% for rituximab and 6.4% for trastuzumab. In an opposite way, the abundance of Man5 glycoform is high in biosimilars (15.0% and 6.4% for rituximab and trastuzumab, respectively) and close to zero in drug products. All these data can be potentially related to the mAbs *in vivo* and *in vitro* activity; it has been shown that afucosylated glycans can enhance the antibody-dependent cellular cytotoxicity (ADCC) as the binding affinity of the Fc region of mAbs to the Fcγ receptor increases.[2] As well, higher levels of galactosylation can impact mAb effector function[3], while a different type of activity is attributed to high mannose glycans.[4] It is clear that glycan analysis is of crucial importance in biotherapeutic analysis for both quality control and product development to ensure product safety and maximize desired effects.

Other peaks in the deconvoluted spectra for the three subunits are present in low abundance; these can be associated with the presence of lysine on the C-terminus (+128 Da), glycation (+162 Da), or minor glycoforms with only one N-acetylglucosamine on the core (-203 Da).

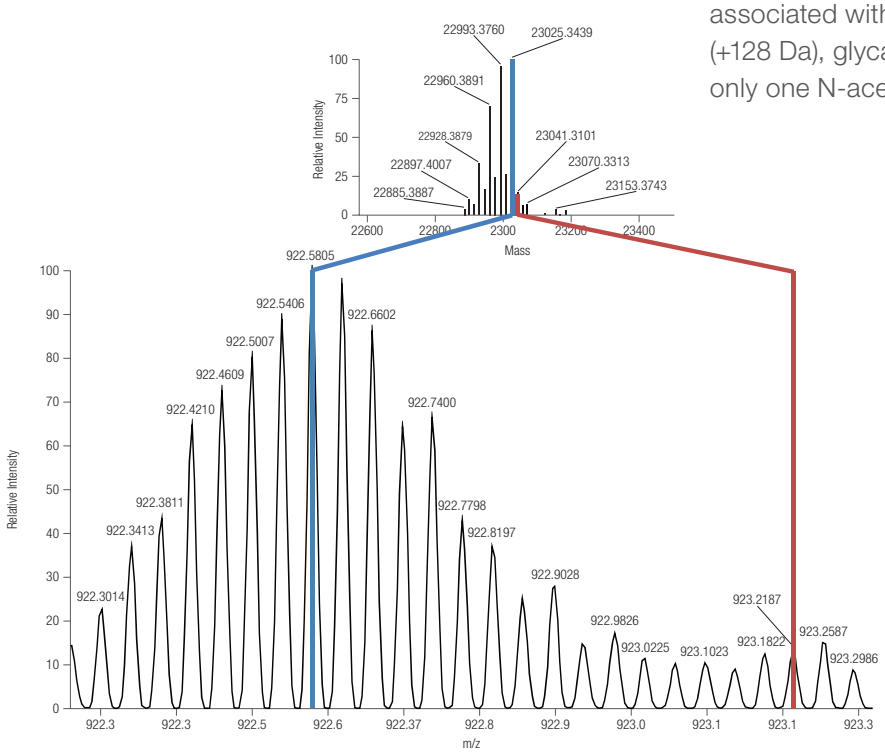

Figure 3. Light chain analysis of rituximab drug product. The MS and deconvoluted spectra show the peaks processed to obtain light chain (red) and light chain modified at position Q1 with a pyroglutamic acid (blue).

To have a quick overview of the differences between samples, a mirror plot can be used. By storing data from a reference sample in the BioPharma Finder library it is possible to perform a direct comparison with new samples, without the need to reprocess old data files. As an example, in Figure 4 the mirror plot for the scFc regions of the drug product vs. the corresponding biosimilar is shown. The most intense variations are soon visible as well as differences in the primary sequence of amino acids that can cause a shift of the mass values in the deconvoluted spectra. This is possible to observe in the mirror plot associated with rituximab in Figure 4; the primary sequence of the rituximab BS presents two modifications at D360 and L362 that are substituted by a glutamic acid and a methionine, respectively, confirmed by peptide mapping analysis [not shown]. This shifts the deconvoluted mass spectra of 32 Da (Figure 4).

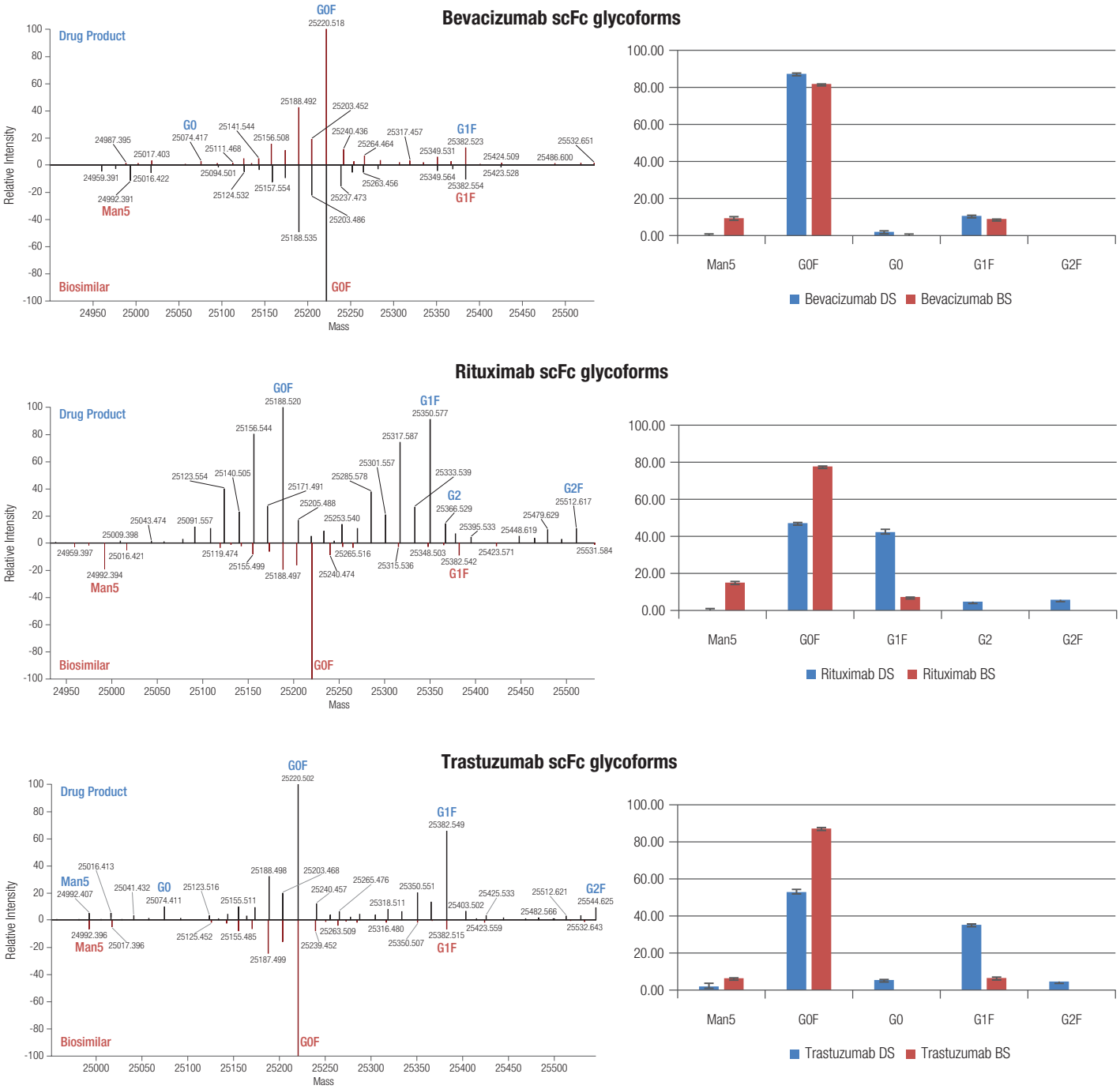

Figure 4. On the left, a mirror plot of deconvoluted mass spectra of scFc regions from drug products and their respective biosimilars. On the right, abundancies of the most abundant glycoforms were compared for drug products and biosimilars. All data were obtained in triplicate and standard deviation values are represented on the error bars.

During product development it is also important to monitor correct expression of the biotherapeutic, e.g. correct cleavage of the signal peptide. Indeed, with the subunit analysis performed on the in-house produced biosimilars, it was possible to monitor the presence of five additional amino acids on the light and heavy chains. These correspond to the first five aminoacids (LVTNS) belonging to the signal peptide on the N-terminus. This signal is present in all samples with a very low percentage (<1%) except for trastuzumab biosimilar, where the presence of this peptide reaches 10% on the heavy chain and represents the most abundant species on the light chain, while the correctly expressed light chain has an intensity around 75% of the most abundant peak (Figure 5). The high-resolution, accurate-mass spectrometry data allowed to unambiguously identify this variant with a mass deviation (Δ = 1.6 ppm).

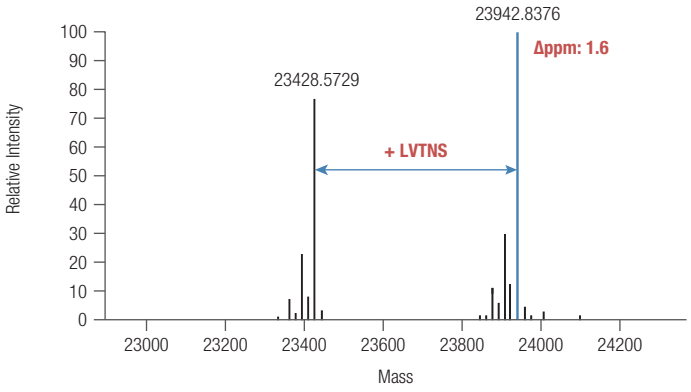

Figure 5. Deconvoluted mass spectrum of trastuzumab biosimilar light chain region. An intense signal at 23942.8376 Da is visible; this corresponds to the addition of five amino acids belonging to the signal peptide only partially cleaved from the antibody. Accurate mass (Δ < 2 ppm) allowed the unambiguous identification of this variant.

Conclusions

- High quality data was obtained for IdeS subunit analysis using the Q Exactive Biopharma platform and MabPac RP columns.
- The combination of the IdeS digestion with the high resolution of LC-HRAM-MS analysis allows fast and efficient data generation of important details on mAbs structure, like glycosylation profile, lysine truncation, and primary sequence modifications.
- BioPharma Finder 3.0 software allows quick deconvolution of the spectra and confident identification of the subunits and their variations. The integrated tool for data comparison can give an overview of differences between samples.
- The new and complete workflow for IdeS subunits analysis is fast and efficient with a simple sample preparation. The LC-MS method is easily optimized and can be used for routine data analysis.

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Complete Characterization of a Cysteine-linked Antibody-Drug Conjugate Performed on a Hybrid Quadrupole-Orbitrap Mass Spectrometer with High Mass Range

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Poster Note 64802

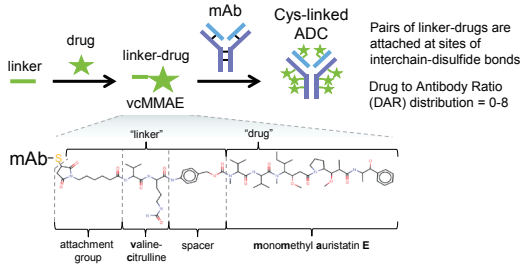
ABSTRACT

We have modified the instrument control software of a benchtop quadrupole-Orbitrap mass spectrometer to add native MS capability. In this study we demonstrate complete characterization of *Brentuximab vedotin*, a cysteine-linked ADC, which requires native MS conditions for intact analysis. We demonstrate preservation of non-covalent bonding of antibody subunits during electrospray ionization. HMR mode can be turned off for peptide mapping. We use trypsin peptide mapping approach with HCD fragmentation to achieve 99% coverage of the *Brentuximab vedotin* sequence using a single LC-MS analysis of a 90 min reverse phase gradient. Finally, we demonstrate that signature ions specific for HCD fragmentation of Brentuximab vedotin can be utilized to increase MS/MS assignment confidence.

INTRODUCTION

The complexity of modern therapeutic proteins, such as antibody-drug conjugates (ADCs), present a great analytical challenge which requires high resolution chromatography combined with high resolution mass spectrometry. Complementary MS approaches such as peptide mapping and intact mass analysis are needed for complete characterization of therapeutic proteins. Cysteine-linked ADCs present a unique challenge for characterization as proper intact analysis requires native MS conditions to preserve structurally-critical non-covalent binding between antibody chains. We have modified commercially-available Thermo Scientific™ Q Exactive™ Plus and Q Exactive™ HF Orbitrap™ mass spectrometers to perform native LC-MS experiments. In the present study, we demonstrate this capability with intact analysis of *Brentuximab vedotin*, a cysteine-linked ADC **(Figure 1)**. Additionally, we have performed denaturing LC-MS and peptide mapping on these same instruments to generate complementary datasets for complete characterization.

Figure 1. Schematic for Constructing Cysteine-Linked ADC
Brentuximab vedotin is a cysteine-linked ADC which is constructed by modifying an antibody with vcMMAE, a preformed linker-drug comprised of a valine-cirtuline-based linker and a monomethyl auristatin E toxic drug. Saturatured (8 drugs) cys-linked ADCs are held intact with only non-covalent binding.



MATERIALS AND METHODS

Brentuximab vedotin was prepared for peptide mapping (reduction, alkylation, and trypsin digestion) or intact analysis (no treatment). For denaturing LC-MS intact analysis 1 µg of protein samples were separated using a 10 min gradient of 10-90% ACN in H_2O and 0.1% formic acid (Thermo MAb-Pac RP; flow rate 250 µL/min). For native LC-MS intact analysis 10 µg of sample was desalted online using size exclusion chromatography (Waters™ BEH SEC 4.6x150mm; 50 mM NH_4OAc isocratic elution, flow rate 300 µL/min) and directly presented to the mass spectrometer via electrospray ionization. Peptide mapping was performed using 2.5 µg of sample separated using a 90 min gradient of 2-90% ACN in H_2O and 0.1% formic acid (Acclaim RSLC 120 C18; flow rate 250 µL/min). Commercially-available Orbitrap mass spectrometers (Q Exactive HF and Q Exactive Plus) which were modified to include High Mass Range (HMR) mode to allow improved high mass transmission and scanning up to m/z 8000. Native intact and denaturing MS data were acquired in HMR mode at setting of R=15k or 17.5k and deconvolved using the ReSpect™ algorithm and Sliding Window integration in Thermo Scientific™ BioPharma Finder™ 1.0 SP1 software. Deconvolution species were identified automatically using the publicly-available FASTA sequence for Brentuximab vedotin, a mass tolerance of 50 ppm, and a static modification of Gln>Pyro-Glu for the heavy chain. Peptide mapping data were acquired by data dependent selection with R=60k or 70k for Full MS and R=15k or 17.5k for MS/MS. Peptide mapping data were searched using the MassAnalyzer algorithm in BioPharma Finder software with a tolerance of 5 ppm.

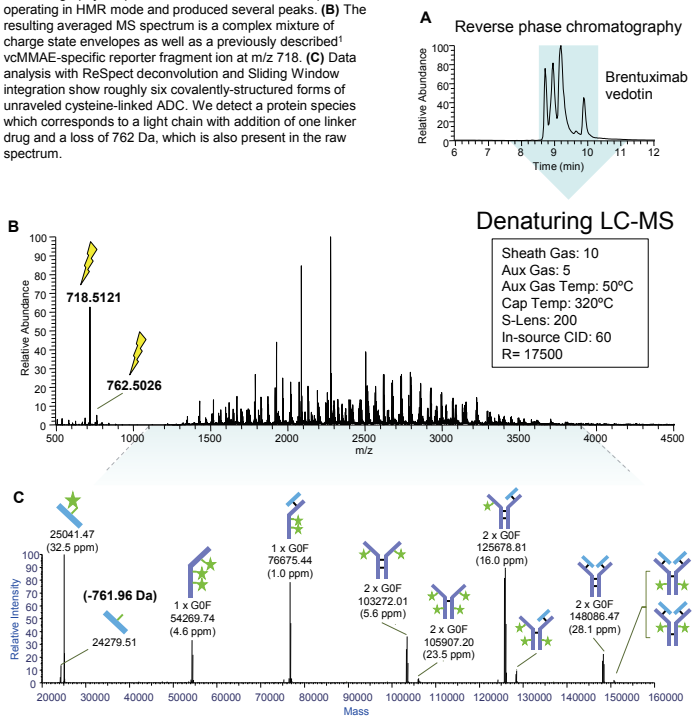
Figure 2. LC-MS Instrumentation for Complete ADC Characterization
All experiments were performed using a Vanquish UHPLC connected to a Exactive HF or Q Exactive Plus with High Mass Range (HMR) mode.

RESULTS

DENATURING LC-MS, CYSTEINE-LINKED ADC

Intact protein LC/MS analysis conventionally involves using mobile phases which are comprised of organic and acidic/basic pH, often suited specifically for reverse phase chromatography. This strategy can be useful for achieving high resolution protein separations. Conditions such as these, however, are not compatible for performing intact analysis on certain classes of compounds which require preservation of non-covalent bonds to maintain structural integrity, such as cysteine-linked ADCs. We demonstrate this phenomenon using the cysteine-linked ADC *Brentuximab vendotin*. Denaturing (reverse phase) LC/MS analysis of *Brentuximab vedotin* results in detection of roughly six unraveled forms **(Figure 3A-C)**. We observed a previously-reported[1] collisionally-induced m/z 718 fragment of the fragile vcMMAE linker-drug **(Figure 3B)**. Upon deconvolution we also observed a mass corresponding to light chain with addition of one vcMMAE and a loss of approximately 762 Da.

Figure 3. Denaturing LC-MS analysis
(A) Unmodified sample (1 ug) was analyzed by reverse phase chromatography coupled to a Q Exactive Plus Orbitrap MS operating in HMR mode and produced several peaks. **(B)** The resulting averaged MS spectrum is a complex mixture of charge state envelopes as well as a previously described[1] vcMMAE-specific reporter fragment ion at m/z 718. **(C)** Data analysis with ReSpect deconvolution and Sliding Window integration show roughly six covalently-structured forms of unraveled cysteine-linked ADC. We detect a protein species which corresponds to a light chain with addition of one linker drug and a loss of 762 Da, which is also present in the raw spectrum.



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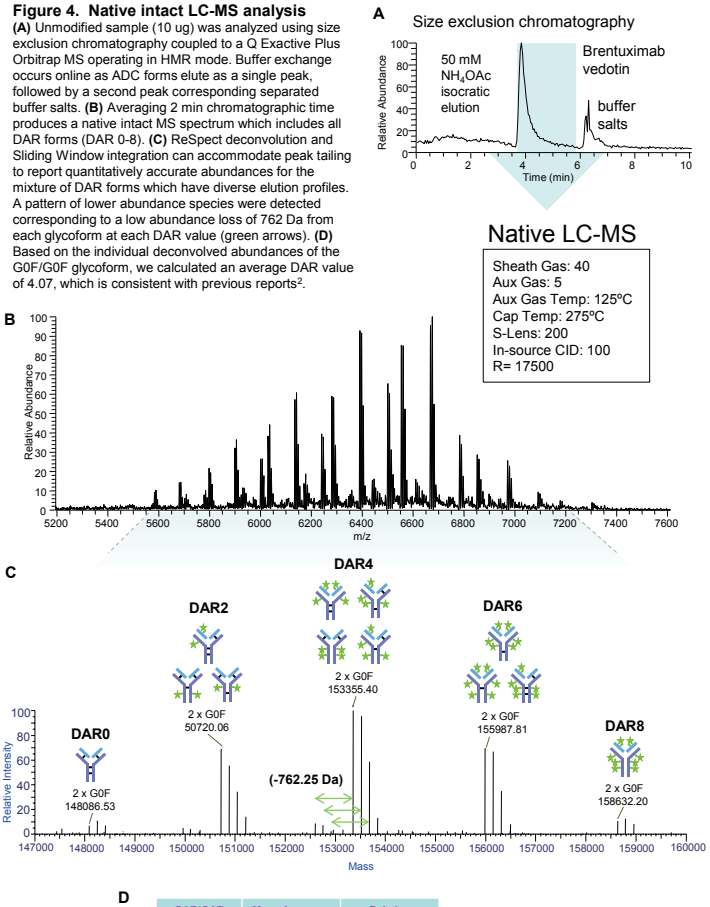
NATIVE INTACT LC-MS, CYSTEINE-LINKED ADC

Native MS intact protein analysis allows direct observation of molecules which rely on non-covalent interactions to preserve critical structural features, such as maintaining interchain associations which hold together cysteine-linked ADCs. The use of 100% aqueous physiological pH buffers in native MS analysis produces decreased charge states (increased m/z) and improves mass separation of heterogeneous mixtures. We performed native size exclusion LC-MS and observed 5 distinct species corresponding to intact *Brentuximab vedotin* with 0, 2, 4, 6, or 8 vcMMAE linker-drugs **(Figure 4)**. We measured an average drug-to-antibody ratio of 4.07, which is consistent with a previously published studies reporting 3.9-4.2 drugs per antibody[2] .

Figure 4. Native intact LC-MS analysis
(A) Unmodified sample (10 ug) was analyzed using size exclusion chromatography coupled to a Q Exactive Plus Orbitrap MS operating in HMR mode. Buffer exchange occurs online as ADC forms elute as a single peak, followed by a second peak corresponding separated buffer salts. **(B)** Averaging 2 min chromatographic time produces a native intact MS spectrum which includes all DAR forms (DAR 0-8). **(C)** ReSpect deconvolution and Sliding Window integration can accommodate peak tailing to report quantitatively accurate abundances for the mixture of DAR forms which have diverse elution profiles. A pattern of lower abundance species were detected corresponding to a low abundance loss of 762 Da from each glycoform at each DAR value (green arrows). **(D)** Based on the individual deconvolved abundances of the G0F/G0F glycoform, we calculated an average DAR value of 4.07, which is consistent with previous reports[2].



D

G0F/G0F DAR	Mass Accuracy (ppm)	Relative Abundance
DAR0	11.7	6.77
DAR2	23.1	69.23
DAR4	22.4	100.00
DAR6	40.5	69.75
DAR8	17.6	10.61

Average Drug-to-Antibody Ratio (DAR)
4.07

Figure 5. Peptide Mapping of Cysteine-Linked ADC *Brentuximab vedotin*
Reduced, alkylated, and trypsin-digested sample (2.5 ug) was separated using a 90 min gradient and eluted into a Q Exactive HF (equipped with HMR mode) operating in Standard mode. Using a mass accuracy cut off of 5 ppm, we achieved 99% sequence coverage of both light and heavy chains. We detected known glycopeptides and were able to detect MMAE-conjugated peptides at all four cysteines (red circles) which are normally involved in interchain disulfide pairs in naked antibodies. A trypsin peptide sequence at the hinge region of the heavy chain (red asterisk) was present in forms ranging from 0-2 vcMMAE conjugations. A missed cleavage peptide contained up to 3 conjugations.

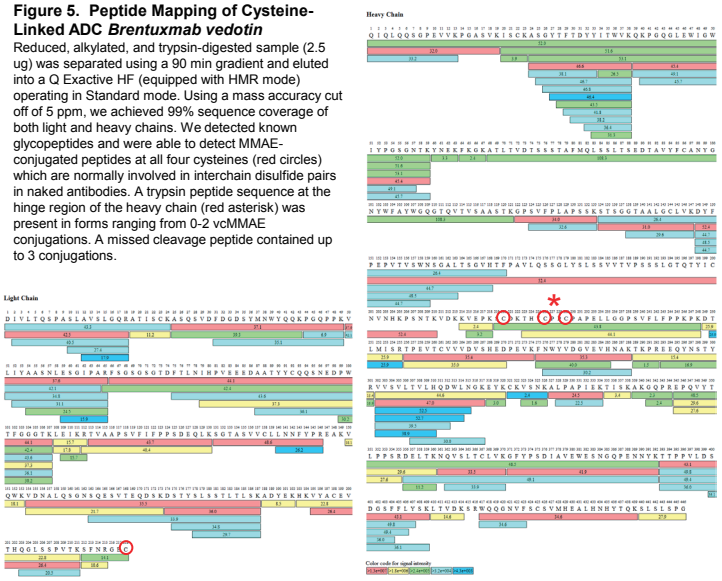
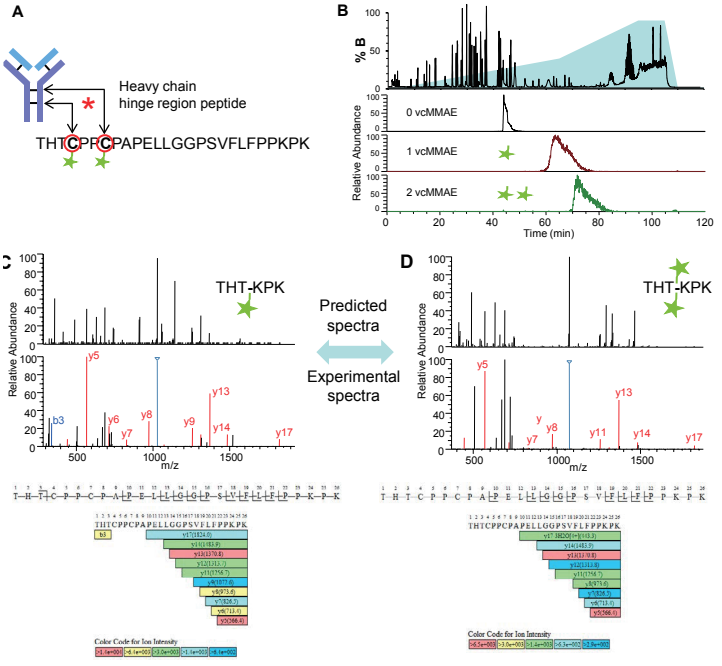


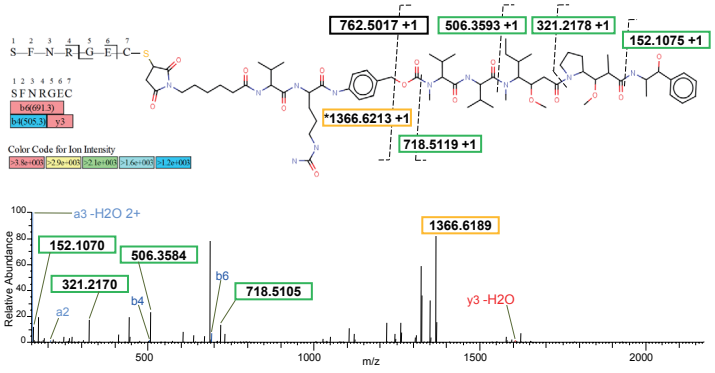
Figure 6. Hinge Region Peptide of Cysteine-linked ADC is Site of Multiple Conjugations
(A) Our data analysis in BioPharma Finder software resulted in detection of peptides which covered the hinge region of the heavy chain (red asterisk). A faithfully-trypsin-cleaved THT-KPK peptide was detected with 0-2 vcMMAE conjugations at cysteines (red circles) normally involved in interchain disulfide pairs. **(B)** Addition of vcMMAE to peptides dramatically increases hyphobicity which results in poor elution and increased retention time. MS/MS analysis of the **(C)** 1 linker-drug (both positional isomers) and **(D)** two linker-drug forms in BioPharma Finder allowed clear sequencing of y-ions in the hinge peptides, and thus facilitated automatic detection.



PEPTIDE MAPPING, CYSTEINE-LINKED ADC

A fundamental component of biotherapeutic protein characterization is peptide mapping. Whereas intact mass analysis aims to detect the abundances and distributions of mass deviation combinations, peptide mapping allows highly sensitive analysis of site-specific sequence features. The vcMMAE linker-drug on *Brentuximab vedotin* poses particular challenges when attempting to identify drug conjugation sites. We prepared a sample for peptide mapping using reduction and alkylation to block non-drug-conjugated cysteines, followed by trypsin digestion. In one 90 min LC-MS gradient we were able to achieve 99% sequence coverage for both light and heavy chains and detect peptides spanning all four drug conjugation sites. HCD fragmentation allowed detection of a peptide in the hinge region of the heavy chain that is differentially modified with 0-2 vcMMAE drugs. As a result efficient elution requires sustained delivery of high organic mobile phase.

Figure 7. HCD Signature Fragment Ions for vcMMAE Linker-Drug
The light chain C-terminal peptide SFN-GEC is a conjugation site for vcMMAE. This modified peptide was automatically identified by BioPharma Finder (left side top panel). Further manual inspection produced additional fragment assignments for vcMMAE signature ions (right side top panel). Theoretical masses (top panel) were calculated manually and matched to experimental masses (bottom panel) within 5ppm (green boxes). A cleavage site for the loss of 762 Da is shown (black box; theoretical monoisotopic mass = 762.5017). We observed a high abundance ion at m/z 1366.6189 (orange box, asterisk) which corresponds to the peptide-retaining fragment pair of a 762 Da loss with an additional loss of 2 protons, presumably due to formation of a seven-membered aromatic ring.



CONCLUSIONS

•We have modified the control software in Q Exactive Plus and Q Exactive HF mass spectrometers to add native MS capability.

•Native LC/MS intact analysis of Brentuximab vedotin resulted in detection of intact ADC forms, DAR0-8. ReSpect deconvolution and Sliding Window integration showed an average DAR of 4.07, consistent with previous studies.

•Acquisition of MS/MS spectra with HCD fragmentation on Q Exactive Plus and Q Exactive HF Orbitrap mass spectrometers followed by data analysis with BioPharma Finder resulted in 99% sequence coverage from a single 90 min gradient using 5 ppm mass tolerance.

•Addition of vcMMAE linker drug dramatically increases peptide hydrophobicity and retention time.

•Signature HCD fragment ions of linker-drug may allow additional means for identifying drug-conjugated peptides.

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Exactive Plus EMR 高分辨质谱仪实现非变性状态 ADCs 药物的分析

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关键词

Exactive Plus EMR；ADCs；cysteine -linked ADCs；Native MS

1 前言

单克隆抗体被认为是具有高度特异性的靶向药物，其对肿瘤细胞的靶向性非常高。而抗体 - 药物偶联物（Antibody-drug conjugates，以下简称 ADCs）技术，就是在抗体蛋白的特定氨基酸上偶联具有抗肿瘤作用的高效应化疗药物（或称小分子药物），以增加单克隆抗体的疗效、并降低小分子药物的毒性。相比单克隆抗体，ADCs 药物的生产工艺更为复杂，因此为了保证 ADCs 药物的安全性和有效性，需对 ADCs 药物的质量进行监控。药物抗体比（drug to antibody ratio，以下简称 DAR）是评价 ADCs 药物的生产工艺和产品质量的一个重要参数。目前大部分上市和在研 ADCs 药物主要包括基于抗体自身的赖氨酸进行偶联的 ADCs 药物（lysine-linked ADCs）和基于抗体自身链间二硫键经还原后的半胱氨酸进行偶联的 ADCs 药物（cysteine -linked ADCs）（图 1）。两类 ADCs 的 DAR 测定通常有 UV、HIC 和 MS 三种方法，其中 MS 检测方法因其快速、灵敏度高和强大的定性功能等优点，愈来愈广泛地被用于测定 ADCs 的 DAR。目前 MS 用于测定 ADCs 的 DAR 通常是基于传统的 RPLC/MS 平台，该平台下 RPLC 采用的流动相呈酸性，且含有较高浓度的乙腈，蛋白在此条件下大多发生变性，因此测定结果严格意义上讲反映的是 ADCs 变性后的 DAR。对于 lysine-linked ADCs 而言，上述变性条件不影响其抗体结构的完整性，故 RPLC/MS 平台不影响其 DAR 测定。然而，对于 cysteine-linked ADCs 而言，上述变性条件破坏了维持抗体空间结构的非共价作用，ADCs 部分解离其轻链或重链，故 RPLC/MS 平台无法用于其 DAR 测定。随着非变性质谱（Native MS）技术的不断发展和推广，采用非变性质谱进行 cysteine-linked ADCs 分析显示了强大的应用潜力。

Thermo Scientific™ Exactive™ Plus EMR 质谱仪保持了 Orbitrap 高分辨率、高质量精度的优势，同时扩展质量数范围至 m/z 20000，并且在硬件设计上提升高质量端离子的传输效率，改进了 HCD 压力，使其更加适用于完整蛋白质的分析。适用于保留有三级和四级结构的非变性蛋白质和蛋白复合物的结构学、拓扑学研究以及生物制药中非变性状态下 cysteine-linked ADCs 的分析。

本实验建立了基于 Exactive™ Plus EMR 和 Protein Deconvolution 3.0 软件的非变性状态下蛋白质的分析流程（图 2），并成功用于 cysteine-linked ADCs 的分析，测定载有不同药物分子数的 ADCs 混合物的精确分子量，并测定其 DAR 值。

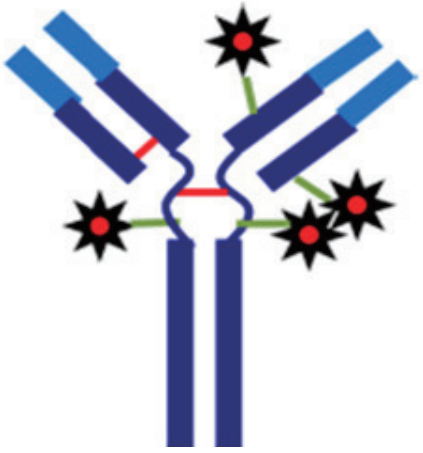

图 1 Cysteine-linked ADCs 示意图

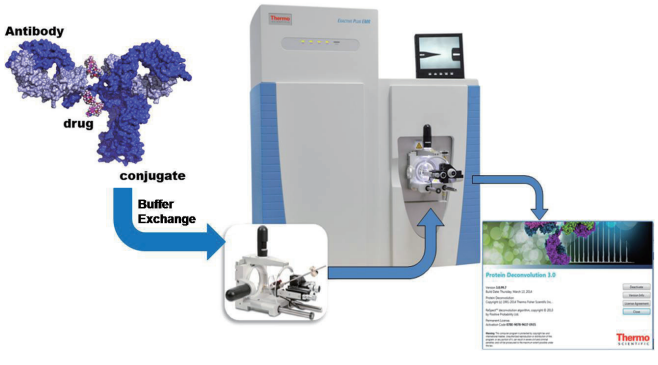

图 2 非变性状态 Cysteine-linked ADCs 的分析流程

2 实验条件

2.1 材料与方法

样品 PCT 为 Cysteine-linked ADCs，PNGase F 去糖基化后，采用 Micro Bio-Spin® Chromatography Columns 进行缓冲溶液交换，保存于 100 mM 乙酸铵（pH 7.0）中，配制成浓度为 5.5 μM 的溶液备用。

2.2 质谱分析

质谱仪	Thermo Scientific Exactive Plus EMR
离子源	NanoFlex Source with emitter
质谱数据采集模式	Direct Infusion
离子模式	正离子
喷雾电压	1.8 KV
毛细管温度	275 ℃
毛细管温度	ON
质量范围	m/z 3000–10000
分辨率	17500、35000、70000
S-lens (%)	200
In-souce CID	175
HCD	25
MicroScan	10
Spectra average	50

2.3 数据处理

采用 Thermo Scientific™ Protein Deconvolution 3.0 对原始质谱图进行去卷积。

参数如下

Noise compensation	ON
Minimum adjacent charges	1 to 3
Noise Rejection	95% confidence

3 实验结果

本实验通过直接进样方式，分别设置 Orbitrap 分辨率为 17500，35000，70000， ADCs 药物的原始质谱图如图 3，ADCs 药物的质谱峰主要分布在 m/z 5000-7000 范围内，具有理想的信噪比。选取两组质谱峰进行放大（图 4 所示）可以发现，当逐渐提高质谱分辨率时，主峰逐渐与加合离子峰分离。分辨率越高，分离效果越明显。

经 Protein Deconvolution 3.0 软件去卷积处理之后的不同分辨率下的 ADCs 药物分子质量分布如图 5 所示，根据 ADCs 药物单抗的氨基酸序列和小分子药物的理论分子量进行计算，将观察到的质谱峰进行归属，从图中我们观察到，五个主峰呈现等质量间隔（约 2635Da），由此可推断该单抗分子结合了不同数目的药物小分子。结合 cysteine-linked ADCs 的特点，判断主峰依次为结合了 0、2、4、6、8 个小分子药物的 ADCs 混合物，该结果与理论预期一致。

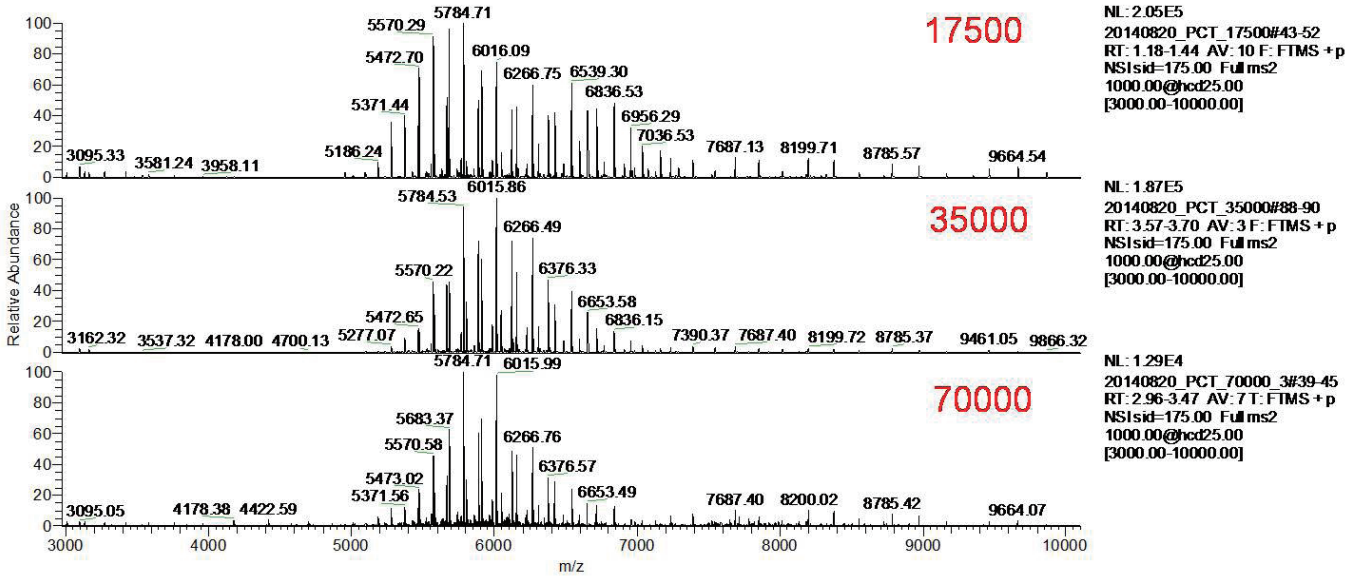

图 3 不同分辨率设置下（17500，35000，70000）ADCs 药物的原始质谱图

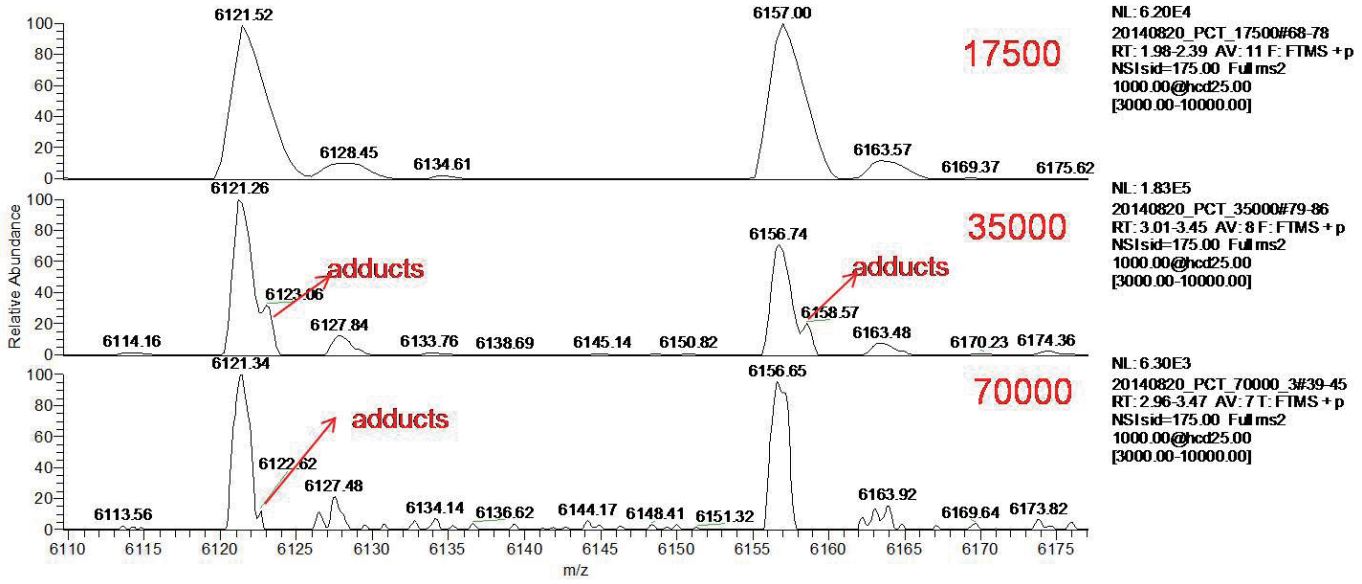

图 4 不同分辨率设置下（17500，35000，70000）ADCs 药物局部放大质谱图

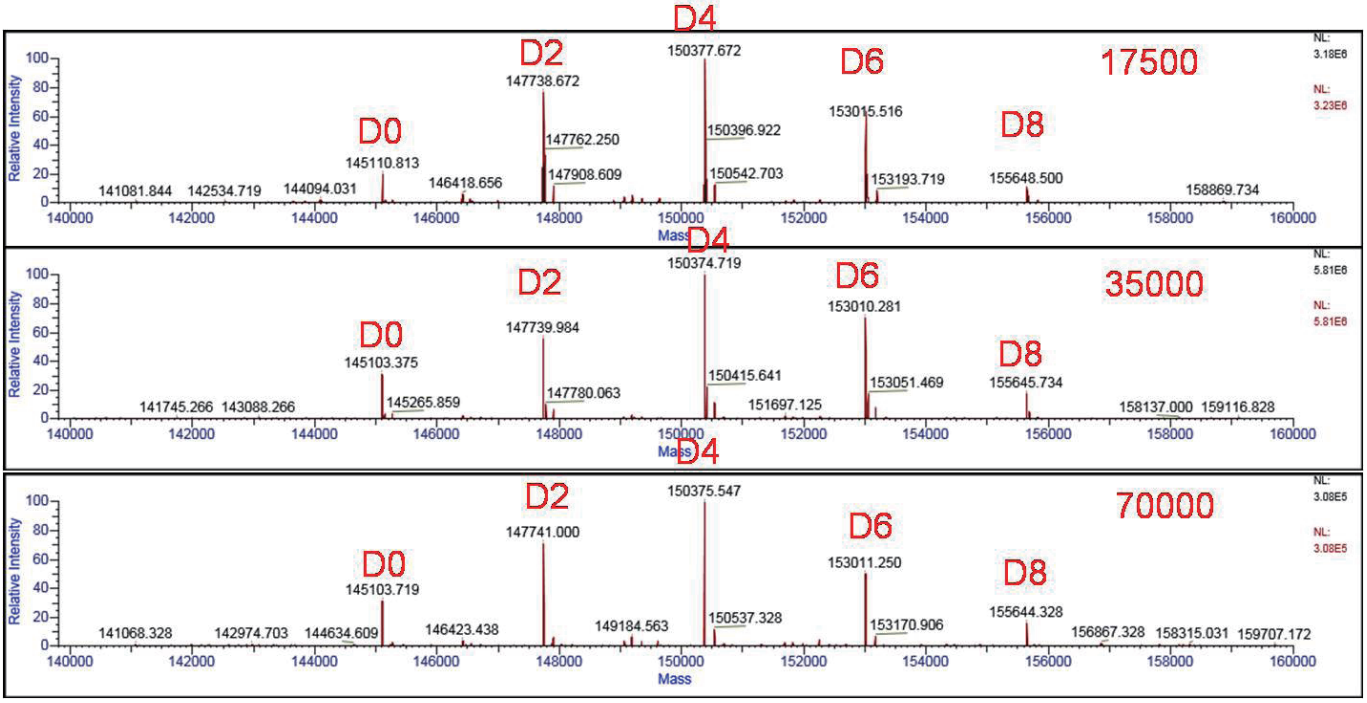

图 5 不同分辨率设置下（17500，35000，70000）ADCs 药物去卷积后结果

Resolution	Drug Load	Measured M_w (Da)	Theoretical M_w (Da)	Delta M (Da)
17500	D0	145111	145103	7.7
	D2	147739	147738	0.3
	D4	150378	150374	4.1
	D6	153016	153009	6.6
	D8	155649	155644	4.4
35000	D0	145103	145103	0.3
	D2	147740	147738	1.6
	D4	150375	150374	1.1
	D6	153010	153009	1.4
	D8	155646	155644	1.6
70000	D0	145104	145103	0.6
	D2	147741	147738	2.7
	D4	150376	150374	1.9
	D6	153011	153009	2.4
	D8	155644	155644	0.2

表 1 不同分辨率（17500，35000，70000）下去卷积后的精确分子量和质量偏差

不同分辨率（17500，35000，70000）下去卷积后的精确分子量如表 1 所示，比较发现，当分辨率设置为 17500 时，由于无法实现主峰和加合离子峰的有效分离，质量与理论值偏差较大，最大偏差达到 7.7Da。当分辨率逐渐提升到 35000，主峰逐渐与加合离子峰分离，质量偏差控制在 0.3-1.6Da，具有极佳的质量准确度。当分辨率逐渐进一步提升到 70000 时，主峰与加合离子峰分离度进一步提高，同样可获得理想的质量准确度（0.2-2.7Da），满足测定需求。

根据质谱峰的峰强度信息，可计算该药物的 DAR 值，该数值对于 ADCs 药物的有效性评估至关重要。以分辨率设置 35000 下质谱图去卷积结果为例（如图 6 所示），按照

DAR = Σ(relative peak area × number of loaded drugs)/100 计算 DAR 值，获知该 ADCs 药物 DAR=3.9。

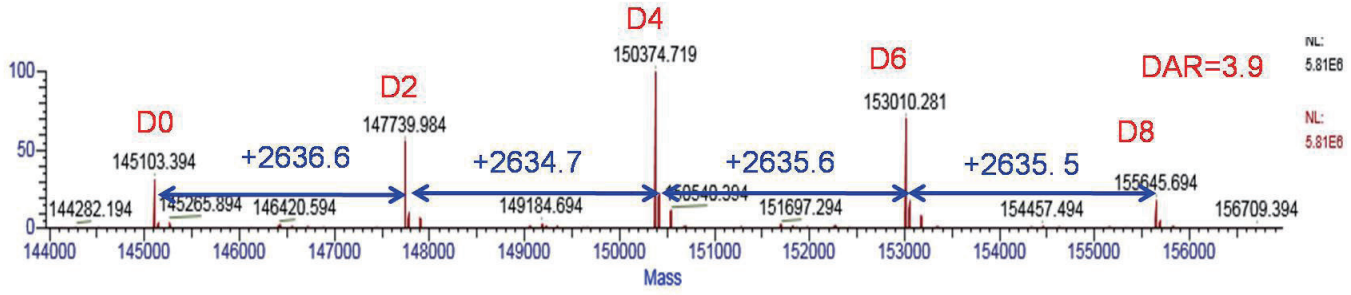

图 6 分辨率设置 35000 下质谱图去卷积后 ADCs 药物质量分布图（D0-D8 表示载有不同药物分子数的 ADCs 混合物）

4 结论

本文采用 Exactive Plus EMR 质谱仪，直接进样方式，突破了传统的 RPLC/MS 平台无法进行 cysteine-linked ADCs 分析的瓶颈，建立了 cysteine-linked ADCs 的精确分子量测定方法，为 cysteine-linked ADCs 单抗药物研发和生产检测提供了高效、快速的分析平台。实验结果表明 Exactive Plus EMR 质谱仪凭借其超高的分辨率、超快的扫描速度、超高的质量精度、超高的灵敏度以及拓展的质量范围，极大地完善和推动了 ADCs 药物的鉴定分析。

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Enabling Mass Spectrometric Analysis of Intact Proteins in Native Conditions on A Hybrid Quadrupole-Orbitrap Mass Spectrometer

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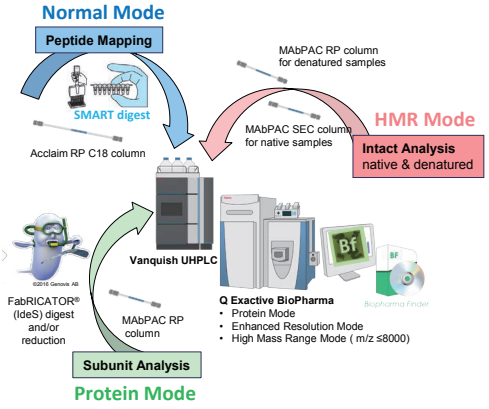
Poster Note 64804

INTRODUCTION

Analysis of proteins in native-like conditions free of organic solvents can allow proteins to preserve non-covalent interactions and retain high degrees of folding. This effect has analytical benefits: greater protein folding leads to reduced charge states leading to increased mass separation and increased signal at higher m/z. This strategy has been utilized for analysis of antibodies and antibody drug conjugates present in highly complex mixtures of different antibody/drug combinations [1]. Requirements for performing native MS on antibody samples include scanning towards 8000 m/z and increased transmission optimization for large compounds. Such features are not compatible with current commercially available quadrupole-Orbitrap instruments. Here we show results obtained after successful implementation of modifications aimed at adding the capability to perform native MS analysis without compromising performance of normal operation modes.

The analysis of intact proteins under native conditions is more challenging than under denaturing conditions since the buffers used don't contain any organic solvents. Performing electrospray from aqueous buffer solutions produces larger solvent droplets size and desolvation is less efficient. Moreover, for large proteins such as intact antibodies the required mass range for analysis under native conditions requires a mass range of more than 6000 m/z due to a smaller number of accepted charges. The increase of the upper mass range on the mass spectrometer was achieved by implementing instrument control software changes. The analysis of molecules across the full mass range including the detection of proteins under native conditions required the use of optimized parameter settings to ensure efficient desolvation in the front region of the instrument, the efficient transfer via multipoles, efficient trapping in the C-trap/HCD region and the sensitive injection and detection in the Orbitrap mass analyzer. Critical parameters are the optimization of in-source fragmentation that strongly influences the support of the desolvation process. Also, for the transmission efficiency specific voltages have been evaluated and optimized to ensure robust and sensitive performance in the higher mass range when performing analyses under native conditions, experiments that have not been possible so far on this type of mass spectrometer. Also, the standard calibration routine previously used was modified and adapted to ensure high mass accuracy across the full mass range.
With the data collected on different types of samples and presented in this study we demonstrate the successful analysis after implementation of the High Mass Range (HMR) mode, successful desolvation and optimized critical hardware operation settings. This makes the instrument an ideal platform to cover the three major workflows in BioPharma: intact mass analysis under denaturing and under native conditions in HMR mode, subunit analysis (reduced mAb and or IdeS digested mAb) in protein mode and peptide mapping in standard mode (see Figure 1).

Figure 1: Operating modes for the three major BioPharma workflows: Normal Mode, Protein Mode and HMR mode



MATERIALS AND METHODS

Samples:
Samples used in this study are ammonium hexafluorophosphate (AHFP, Fisher Scientific, part number A0368370), and Trastuzumab (tradename Herceptin, Roche, UK).

Chromatography:
A Thermo Scientific™ Vanquish™ UHPLC system was used for all LC/MS experiments. For native analysis, 50 mM ammonium acetate buffer (99.99%, Sigma Aldrich) was used. Reversed phase chromatography was performed with water/0.1% formic acid and acetonitrile/0.1% formic acid on a Thermo Scientific™ MAbPac™ RP 2.1x50 mm column.

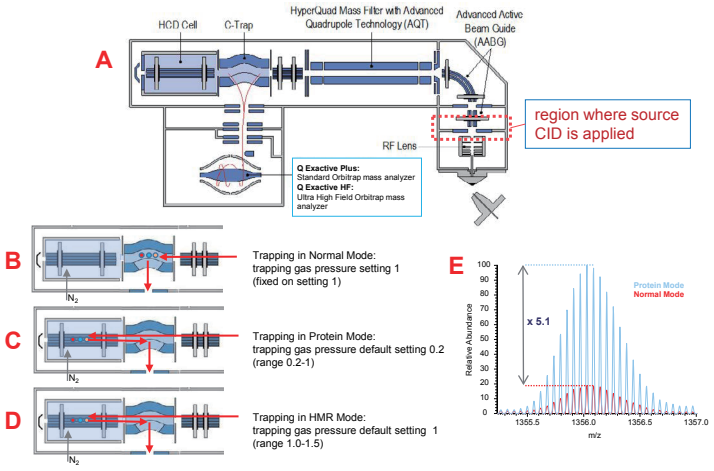
Mass Spectrometry:
Mass spectrometers used in this study are the Thermo Scientific™ Q Exactive™ Plus and Q Exactive™ HF systems with BioPharma Option. The instruments were operated under Tune 2.8 instrument control software in HMR mode, in which the RF applied to the C-trap was increased from 2,400 V p-p to 2,900 Vp-p for better trapping of the high m/z ions. Also, to ensure better capture of high-*m/z* ions in the Orbitrap analyzer, the initial central electrode voltage was adjusted from −3.7 kV to −3.4 kV, while the setting during detection remained unchanged (−5 kV). The S-lens RF level was allowed to be increased to a setting of 200 in HMR mode and set to that level for all experiments shown here.

Data Analysis:
Data analysis was performed with Thermo Scientific™ BioPharma Finder™ 1.0 SP1 software.

RESULTS

There are many factors that play a key role in the analysis of proteins, some of which relate to sample preparation (buffers, solvents, additives) while others relate to the mass spectrometer's source conditions as well as the physical environment inside the instrument [2,3]. The Q Exactive Plus and Q Exactive HF mass spectrometers (Figure 2A) have previously been introduced with the Protein Mode option, which was one of many advancements for intact protein analysis on the Orbitrap platform. For these two instruments an automated HCD gas control was introduced by using an electronically controlled valve for nitrogen gas in the HCD cell for easier optimization of experimental conditions required for different types of analyses wished to run on a single platform.
In Normal Mode pressure settings are factory-optimized, suitable for most analyses and ions are cooled in the C-trap (Fig. 2B). The trapping gas pressure setting is 1 which corresponds to a high vacuum pressure delta (ΔHV) of ~3.1 e-5mbar. The ΔHV is defined as the difference between HV with HCD gas on minus HV with HCD gas off.
In Protein Mode the default trapping gas pressure setting is 0.2 and that corresponds to a ΔHV which is 5x lower than in Normal Mode. Additionally, ions are transferred and cooled in the HCD cell and thus have a longer flight path (Fig. 2C).
The combination of reduced C-trap and HCD cell gas pressures, and trapping ions in the HCD cell prior to mass analysis extends the life time of protein ions resulting in increased signal intensities of isotopically resolved species (Fig. 2E).

Figure 2. A) Schematic of the Q Exactive Plus/HF mass spectrometers and differences in the trapping path in the three different operating modes available: B) Normal Mode, C) Protein Mode and D) HMR Mode. E) Illustration of improvement in signal intensity for +17 charge state of a mAb light chain comparing Protein Mode and Normal Mode.



For higher gas pressures, high charge states of the same protein decay faster than lower charge states. This is because center-of-mass collision energy

$$K_{ce}=E*m/(M/z)$$

with
M/z: the mass-to-charge ratio for a given charge state
m: mass of residual gas, nitrogen
E: ion energy inside the Orbitrap

resulting in: **K_{ce} is proportional to the charge state z.**

This explains observations of charge envelope shifts on the m/z scale when comparing data acquired in different modes with different pressure regimes in the HCD cell and C-trap region, as shown in one example in Figure 6.

Here we have investigated and implemented the new High Mass Range (HMR) Mode that is especially required for the analysis of proteins under native conditions when samples are kept in aqueous buffers with no organic solvents involved at near neutral pH.

For HMR mode the default trapping gas pressure setting is 1 but it can be slightly increased up to 1.5 for even improved trapping of certain species such as protein complexes and heterogeneous large proteins (e.g. antibody drug conjugates). The trapping path in HMR mode is the same as in Protein Mode with ion cooling taking place in the HCD cell. And also, mass detection is enabled ranging up to m/z 8000 compared to m/z 6000 in the two other modes.

The trapping gas pressure in all modes is set and saved in the tune files and since a method allows for segmentation using different tune files different pressure settings can be used within one LC-MS run. In contrast the mass range setting is set in the method and the method editor allows for several nodes with different experiment types using different mass ranges within one LC-MS run.

Figure 3. Full MS spectra of ammonium hexafluorophosphate direct infusion experiment in Normal and HMR modes.

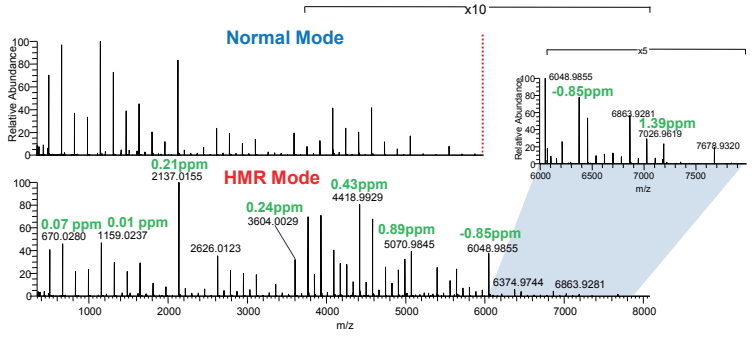


Figure 4. HMR Mode spectral mass accuracy test using ammonium hexafluorophosphate. The displayed test results in rms = 0.5 ppm.

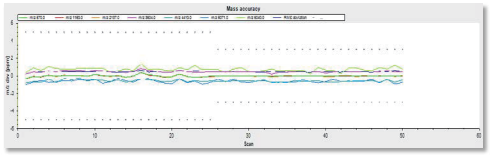


Table 1. Theoretical and measured masses (Figure 3) of ammonium hexafluorophosphate for calibration of HMR mode

theoretical m/z	experimental m/z	Δ Mass (ppm)
670.02805	670.0280	0.07
1159.02371	1159.0237	0.01
2137.01504	2137.0155	-0.21
3604.00204	3604.0029	-0.24
4418.99481	4418.9929	0.43
5070.98903	5070.9845	0.89
6048.98036	6048.9855	-0.85
7026.97170	7026.9619	1.39

hexafluorophosphate (AHFP) in direct infusion mode. Figure 3 displays the spectra obtained in Normal versus HMR mode with excellent mass accuracy (Table 1) even for masses detected with low abundance at m/z higher than 6000, also shown in the mass accuracy test in Figure 4.

Figure 5 displays the spectra obtained for intact Trastuzumab analyzed in native and denaturing conditions resulting in different charge envelopes inherent to the solvent and pH conditions (aqueous, near neutral pH for native; organic solvent content and low pH for denaturing). Both types of spectra result in nearly identical results after spectra deconvolution (Fig. 5C).

Figure 5. Trastuzumab analyzed under native (A) and denaturing (B) conditions resulting in highly similar deconvolution results (C).

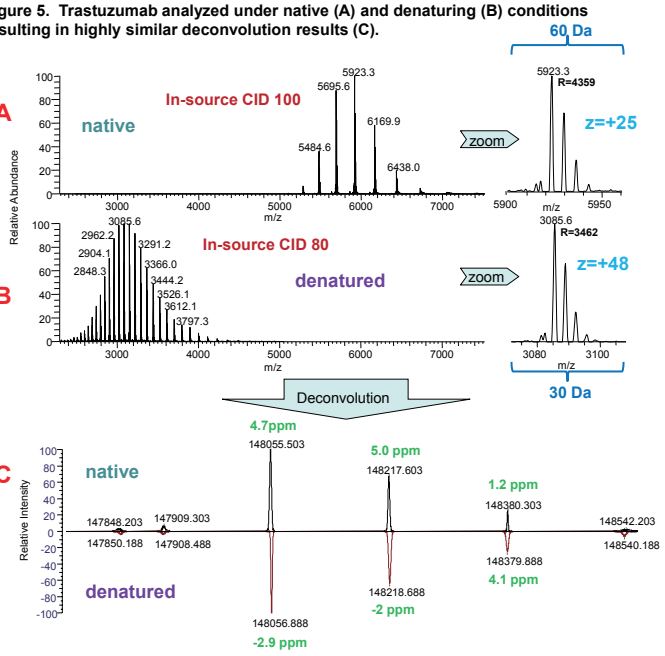
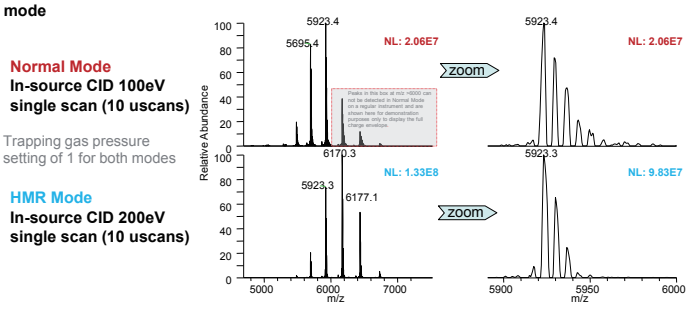
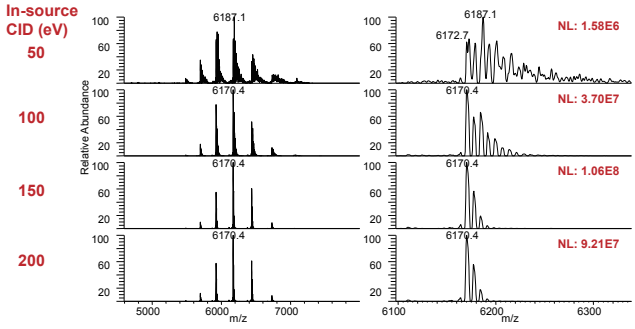


Figure 6. Effect of in-source CID supporting desolvation/ declustering in Normal vs. HMR mode



Figures 6 and 7 depict the influence and importance of source fragmentation supporting desolvation and declustering required to obtain the correct pattern of glycoforms. The data in these two figures were acquired in nanospray mode, requiring significantly higher in-source CID settings for native analyses. Due to neither gases available in the nanospray source nor a heated probe, the desolvation process needs to be supported with source fragmentation taking place inside the mass spectrometer, a potential step implemented between the S-lens and the Q00 (Figure 2A).

Figure 7. Intact Trastuzumab analysis under native conditions with increasing in-source CID settings highlighting the importance of this parameter on desolvation/ declustering.



Further parameters that were found critical in optimizing source conditions for native analysis using size exclusion chromatography (SEC) were the capillary temperature (also referred to as transfer tube) and the probe heater temperature (also referred to as Aux heater temperature). Figure 8 shows one example of two different temperature settings resulting in differences in charge state distribution.

Figure 8. Intact Trastuzumab analysis under native conditions with different probe heater and capillary temperatures.

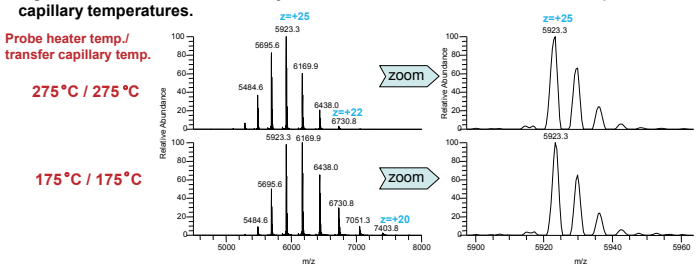


Figure 9. Intact Trastuzumab SEC-MS analysis under native conditions with different resolution settings.

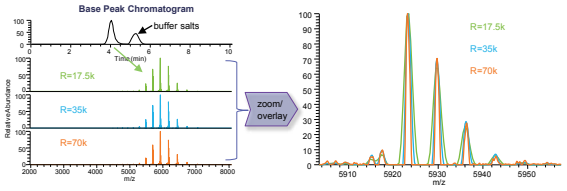
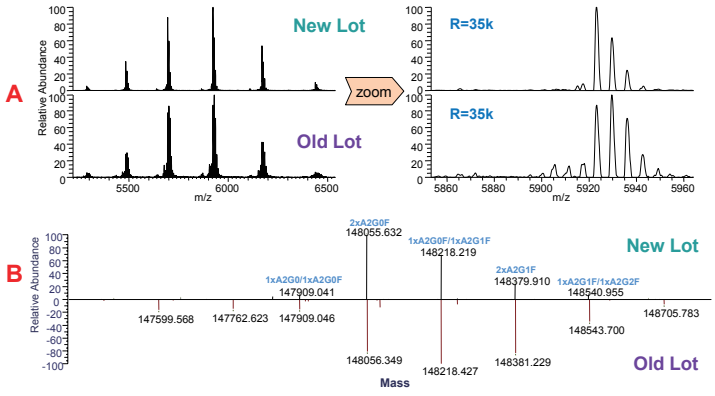


Figure 9 shows the SEC-LC/MS analysis of intact Trastuzumab under native conditions acquired on the Q Exactive Plus in HMR mode at different resolution settings of 17.5k, 35k and 70k. Increasing the resolution setting in this case is not aiming at achieving isotopic resolution but allowing to resolve possible sodium and potassium adducts [1] arising e.g. from salts and/or formulation buffer and thus significantly improving mass accuracy after deconvolution.

Figure 10 displays glycoform pattern variability from two different lots of Trastuzumab analyzed with SEC-LC/MS, which is not desired but has been observed and reported previously. The possibility for such a variation is inherent to the production process of biopharmaceuticals and shows the capabilities of mass spectrometry to easily pick up these variations since the analyses of glycoform patterns on the intact antibody level are very reproducible and reliable, considering the use of consistent optimized parameter settings for all experiments in a study.

Figure 10. Comparison of two different lots of Trastuzumab showing significant variation in the pattern of the glycoforms, a known issue in production of biopharmaceuticals.



CONCLUSIONS

- We have successfully implemented the High Mass Range Mode on the Q Exactive Plus and Q Exactive HF mass spectrometers allowing for mass detection up to m/z 8000.
- This new operating mode extends the instrument's capabilities to cover all three major workflows for BioPharma characterization.
- Desolvation/declustering conditions, ions transfer and trapping have been optimized to allow for improved sensitivity in HMR mode for resolution settings as high as 70k.
- Critical parameters for online LC-MS analyses under native conditions are capillary and probe heater temperatures as key factors in supporting desolvation/declustering.

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Quantitative Analysis of an Antibody Drug Conjugate using MSIA D.A.R.T.'S Technology, an Integral Part of the Universal Ligand Binding Mass Spectrometric Immunoassay (LB-MSIA) Workflow

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Application Note

Key Words
Q Exactive™ Plus, Antibody-Drug Conjugate, ADC, Deconvolution, HRAM, High Resolution/Accurate Mass, MSIA™, Mass Spectrometric Immunoassay,, High -Throughput, Versette™, Streptavidin MSIA™ D.A.R.T.'S

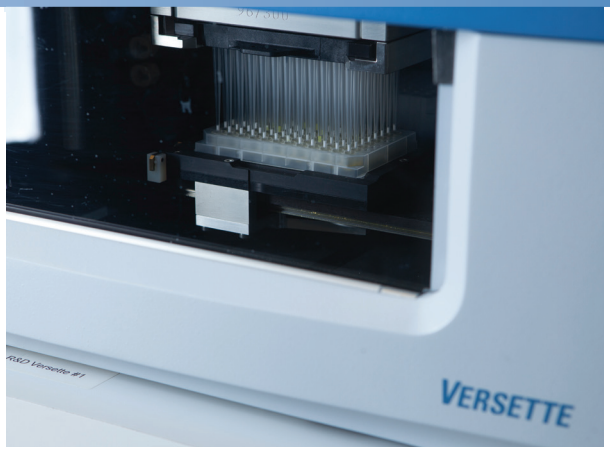
Goal

To demonstrate the quantitative analyses of an ADC (antibody drug conjugate) using Thermo Scientific™ MSIA™ D.A.R.T.'S with the LB-MSIA™ workflow; a pre-clinical bioanalytical solution, based on mass spectrometric detection of an intact biotherapeutic.

Introduction

With the increase of biotherapeutic innovation and biological complexity, classic bioanalytical techniques for proteins such as LBAs (Ligand Binding Assays) are unable to meet the data needs for pharmacokinetics, biotransformation assessment, and antibody functional determination studies. The development of novel antibody therapeutics requires structural information such as variants, sites of glycosylation, and PTMs (post-translational modifications); necessary for drug safety, efficacy and stability. For example, an LBA would not provide the unique data requirements necessary for the establishment of DARs (Drug-Antibody Ratios) for Antibody-Drug Conjugates (ADCs). Many of these data requirements are met by the use of mass spectrometric (MS) based assays; an analytically robust and sensitive detection method that adds the specificity of identifying biomolecules by their intrinsic property of molecular mass.

The Ligand Binding-Mass Spectrometric Immunoassay (LB-MSIA) is a universal workflow solution for targeted pre-clinical analysis of biotherapeutics, such as ADCs, that combines the robust nature of traditional ligand binding assays with HRAM (High Resolution/Accurate Mass) mass spectrometric detection. By focusing on the enablement of preclinical discovery and development research the resultant automated and high throughput LB-MISA provides characterization data necessary to keep pace with new biotherapeutic innovation and increased biological complexity. This hybrid Immunoaffinity-LC/MS (IA-LC/MS) workflow is specifically enabled by Streptavidin MSIA D.A.R.T.'S; a unique pipette tip that contains molecular trapping microcolumns, covalently derivatized with streptavidin.



By mounting the Streptavidin MSIA D.A.R.T.'S onto a Thermo Scientific™ Versette™ automated liquid handler or a Thermo Scientific Finnpipette® Novus i Multichannel Electronic Pipette, the LB-MSIA workflow can be applied to high throughput sample processing for standardized routine applications. The functional design of the Streptavidin MSIA D.A.R.T.'S combined with the consistency of the Versette automated liquid handler or the Thermo Scientific Finnpipette Novus i Multichannel Electronic Pipette provides a high factor of ease-of-use and reproducibility that is not present in beads-based methodologies. When the Streptavidin MSIA D.A.R.T.'S are paired with a high affinity reagent, such as biotinylated anti-human IgG Fc affinity ligands, the workflow is enabled to selectively analyze for mAbs of a human IgG subclass.

Presented here is a study that utilized LB-MSIA to quantitatively measure the concentration of an ADC that was spiked into rodent plasma. While this study focuses on applications of intact analysis of an ADC, the LB-MSIA workflow has the potential to perform bottom up, middle down as well as intact analyses for comprehensive characterization of a biotherapeutic, as demonstrated in our previous application, "MSIA Workflow for Therapeutic Antibodies: Qualitative, Quantitative, and Functional Verification Data from HR/AM Detection of Intact, Reduced, and Peptide-level Forms of Adalimumab".

ThermoFisher SCIENTIFIC

The high biological complexity of an ADC mass spectra comes from the heterogeneous mixture of glycans and drug conjugates chemically linked to the monoclonal antibody. Two methods of data analysis were applied to the LB-MSIA performed in this study in order to demonstrate how the complexity of the MS data generated from the analysis of an ADC may be reduced in order to quantitate.

The first method is to deconvolve the MS data, reducing several peaks resulting from several charge states for each of the DAR species to a single peak representing each of the DAR species, which may then be summed to give the total ADC. The second method relies on the summation of the three most abundant charge states of each DAR species from the mass spectrum in order to achieve the AUC (area under the curve) for each DAR species. Similar to the deconvolved method, the summation of the AUCs for each of the DAR species was used to provide the AUC value for the total ADC. Both methods of data analysis when applied to this LB-MSIA were able to show reproducibility with CVs of less than 15% and within 20% accuracy.

Also shown is quantitation of a single DAR species of the ADC. By qualitatively profiling the ADC it is possible to determine the percentage each DAR species that is representative of the total ADC. Applying the appropriate percentage to the calibration curve resulted in an equation of the line that was then used to quantitate deconvolved peak intensities of the DAR3 species.

Materials

- Thermo Scientific™ Streptavidin MSIA™ D.A.R.T.'S, PN: 991STR12
- Thermo Scientific™ Versette™ Automated Liquid Handler
- Thermo Scientific™ Finnpipette™ F1 Adjustable-Volume Pipettes, PN: 4700850
- CaptureSelect™ Biotin Anti-IgG-Fc (Human) Conjugate, PN: 7103262100
- Antibody-Drug Conjugate (ADC) (Custom Made)
- Sigma-Aldrich® SILu™Lite SigmaMAb Universal Antibody Standard human, PN: MSQC4
- Mouse Plasma (K2 EDTA)
- Thermo Scientific™ BupH™ Modified Dulbecco's Phosphate Buffered Saline (PBS) Packs, PN: 28374
- MSIA™ Elution Buffer
- Fisher Chemical™ Optima™ LC/MS Grade Water, PN: W6
- Fisher Chemical™ Optima™ LC/MS Grade Formic Acid, PN: A117
- Fisher Chemical™ Optima™ LC/MS Grade Acetonitrile, PN: A955
- Thermo Scientific™ Nunc™ 500µL 96-Well Plates, Polypropylene, PN: 12-565-368
- Thermo Scientific™ ProSwift™ RP-4H Monolith Column, 1.0 x 250 mm, PN: 066640
- Thermo Scientific™ Vanquish™ UHPLC System
- Thermo Scientific™ Q Exactive™ Plus Hybrid Quadrupole-Orbitrap™ Mass Spectrometer
- Thermo Scientific™ Xcalibur™ Software, Version 3.0
- Thermo Scientific™ Protein Deconvolution Software, Version 4.0 with the ReSpect™ algorithm
- Thermo Scientific™ Protein Deconvolution Software, Version 4.0 with the ReSpect™ algorithm

Method

The LB-MSIA workflow for the bioanalysis of an ADC may be broken down into five major steps as illustrated in Figure 1. A Thermo Scientific™ Versette™ Automated Liquid Handler was used to provide the repetitive bi-directional pipetting (aspirating and dispensing cycles) necessary to pass solutions through the microcolumn housed within each of the Streptavidin MSIA D.A.R.T.S. The Streptavidin MSIA D.A.R.T.S are first derivatized with a biotin-conjugated anti-IgG Fc, an affinity ligand that specifically binds to the Fc portion of all four human IgG subclasses.

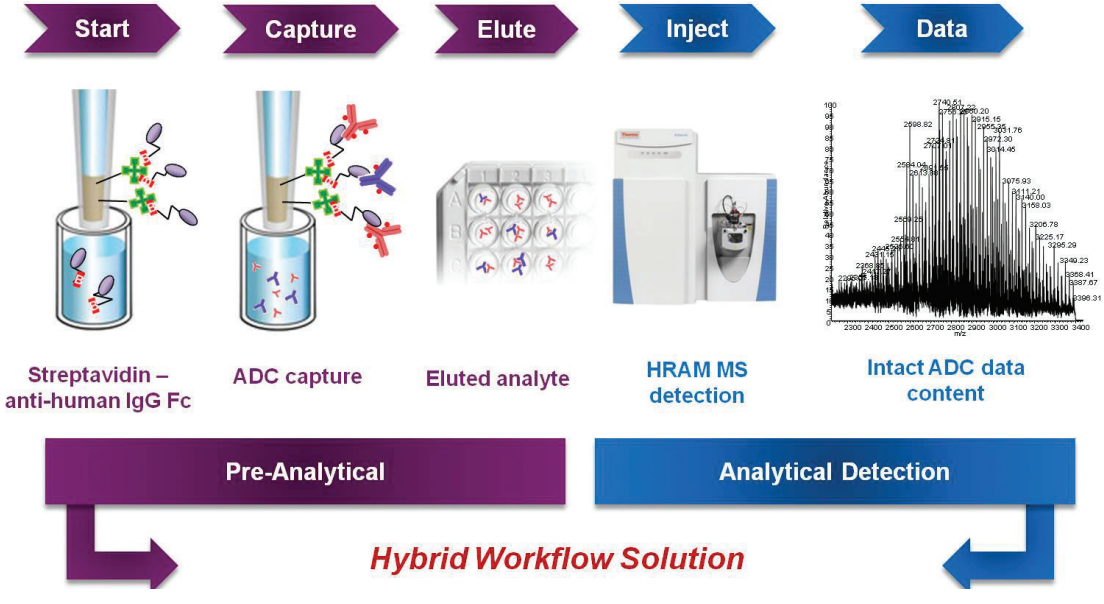


Figure 1. A schematic showing the five major steps of the LB-MSIA Workflow

The next step is to assay for the ADC from rodent plasma samples by incubating the samples with the anti-IgG-Fc-derivatized Streptavidin MSIA D.A.R.T.S. The affinity bound ADC is subsequently released from the D.A.R.T.S by treatment with the elution buffer. The ensuing eluate containing the ADC is then analyzed using LC-MS (HRAM). Utilizing Thermo Scientific's™ Xcalibur™ (Version 2.2) and Protein Deconvolution (Version 4.0) Software the resulting raw HRAM MS data is processed to provide high content quantitative data.

Pre-Analytical

Derivatization of Streptavidin MSIA D.A.R.T.'S with Affinity Ligand

To enable the Streptavidin MSIA D.A.R.T.'S to have a specific affinity for the Fc region of human IgG subclasses, each of the streptavidin derivatized microcolumns were loaded with 125 µL of 4 µg/mL CaptureSelect™ biotin anti-IgG-Fc (Human) conjugate, a single domain antibody (Life Technologies), prepared in PBS (BupH™ Modified Dulbecco's PBS). This was accomplished by following the steps provided in Table 1 utilizing a Thermo Scientific™ Versette™ Automated Liquid Handler equipped with Streptavidin MSIA D.A.R.T.'S.

	Assay Step	Assay Solution	Total Well Volume (µL)	Asp/Disp Volume (µL)	Asp/Disp Cycles	Syringe Speed (%)
1	Buffer Pre-Rinse	PBS	200	150	10x	100
2	Immobilization of anti-human IgG-Fc	Biotin anti-IgG Fc conjugate antibody	125	70	500x	100
3	Buffer Rinse	PBS	200	150	10x	100
4	Buffer Rinse	PBS	200	150	10x	100

Table 1 – Derivatization of Streptavidin MSIA D.A.R.T.'S with biotinylated anti-human IgG-Fc; Versette protocol in descending order

Sample Preparation

All samples prepared consisted of 20 µL of mouse plasma supplemented with varying concentrations of the ADC as referenced in Table 2. The ADC control sets were each prepared in replicates of five and the calibration curve was prepared in duplicate. Then, the curve and control sets were performed three times over three non-consecutive days.

ADC Samples	Concentration (µg/mL)
Dynamic Range	2.5-320
Control Set 1	5.0
Control Set 2	13.5
Control Set 3	53.5
Control Set 4	213.5

Table 2 – ADC Sample Concentrations. Each control set consisted of 5 replicate samples and each calibration curve was run in duplicate.

Prior to incubation of the samples with the anti-IgG-Fc-derivatized Streptavidin MSIA D.A.R.T.'S, each sample was further diluted with 80 µL of PBS supplemented with 0.7 µg of SILu™Lite SigmaMAb as an internal standard. Using the Versette Liquid Handler, the following steps outlined in Table 3 were performed to capture the ADC and internal standard from the samples.

	Assay Step	Assay Solution	Total Well Volume (µL)	Asp/Disp Volume (µL)	Asp/Disp Cycles	Syringe Speed (%)
1	ADC Capture*	Sample Solution	100	70	700x	50
2	Buffer Rinse	PBS	200	150	10x	100
3	Buffer Rinse	PBS	200	150	10x	100
4	Water Rinse	Water	200	150	10x	100
5	Water Rinse	Water	200	150	10x	100

**ADC Capture performed using Anti-human IgG-FC MSIA D.A.R.T.'S*

Table 3 – ADC Capture; Versette protocol in descending order

Sample Elution

Following the selective capture of the ADC with the anti-IgG-Fc-derivatized Streptavidin MSIA D.A.R.T.'S, each device was treated with 100 µL of the MSIA™ Elution Buffer liberating the ADC and internal standard. Reference Table 4 for the specifics of the repetitive pipetting used to elute the captured ADC from the D.A.R.T.'S. The intact ADC and internal standard were detected by LC-MS (HRAM).

	Assay Step	Assay Solution	Total Well Volume (µL)	Asp/Disp Volume (µL)	Asp/Disp Cycles	Syringe Speed (%)
1	Elution	Elution Buffer	100	30	20x	100

Table 4 - Versette protocol for elution of affinity-captured ADC from anti-IgG-Fc-derivatized D.A.R.T.'S. .

Analytical-Detection

Liquid Chromatography

The affinity-purified ADC eluates were separated on a Thermo Scientific™ Vanquish™ UHPLC system utilizing a Thermo Scientific™ ProSwift™ RP-4H (1 x 250 mm) column heated to 60 °C. Separation was performed utilizing a gradient of 10% - 48% of 0.2% formic acid in acetonitrile over 12 minutes at a flow rate of 200 µL/min.

Mass Spectrometry

For all samples, full-scan MS data were acquired over the range of m/z 2000-3400 m/z in positive-ion mode on a Thermo Scientific™ Q Exactive™ Plus Hybrid Quadrupole-Orbitrap mass spectrometer with a resolving power of 17,500 (FWHM) at m/z 200 and the AGC (Automatic Gain Control) set to a target value of 3.00E6.

Data Analysis

During this study, a comparison of two methods of data analysis was demonstrated for the quantitative analysis of the ADC. The LC-MS raw data utilized by both methodologies was collected using Thermo Scientific's Xcalibur™ Software, Version 3.0. Thermo Scientific's™ Protein Deconvolution™ Software Version 4.0 utilizing the Sliding-Window feature in the ReSpect™ algorithm was used to process the MS raw data as the first method of quantitation. The intensities of the deconvolved peaks were used to determine the peak intensity ratio (Sum of ADC peaks/SILuLite peaks) for each sample analyzed. The second method of data analysis, described here as the XIC Method, utilizes the raw MS data to generate an extracted ion chromatogram for the three most abundant charge states of each intact ADC DAR species, which were then integrated to obtain the AUC (Area Under the Curve) value for each sample analyzed. The same method was applied to the corresponding SILuLite internal standard (IS) allowing for the area ratio to be determined between each DAR species and the internal standard.

Results and Discussion

Normalization of the Samples

Quantification of the total ADC present in each sample requires the summation of the heterogeneous mixture of DAR (Drug to Antibody Ratio) species that is normalized by the use of the SILuLite IS (Figure 2). Referring back to the sample preparation of the pre-analytical section of the method, it should be noted that the SILuLite is added to each sample prior to the affinity capture. Upfront normalization of the affinity capture helps to increase sample analysis accuracy and reproducibility.

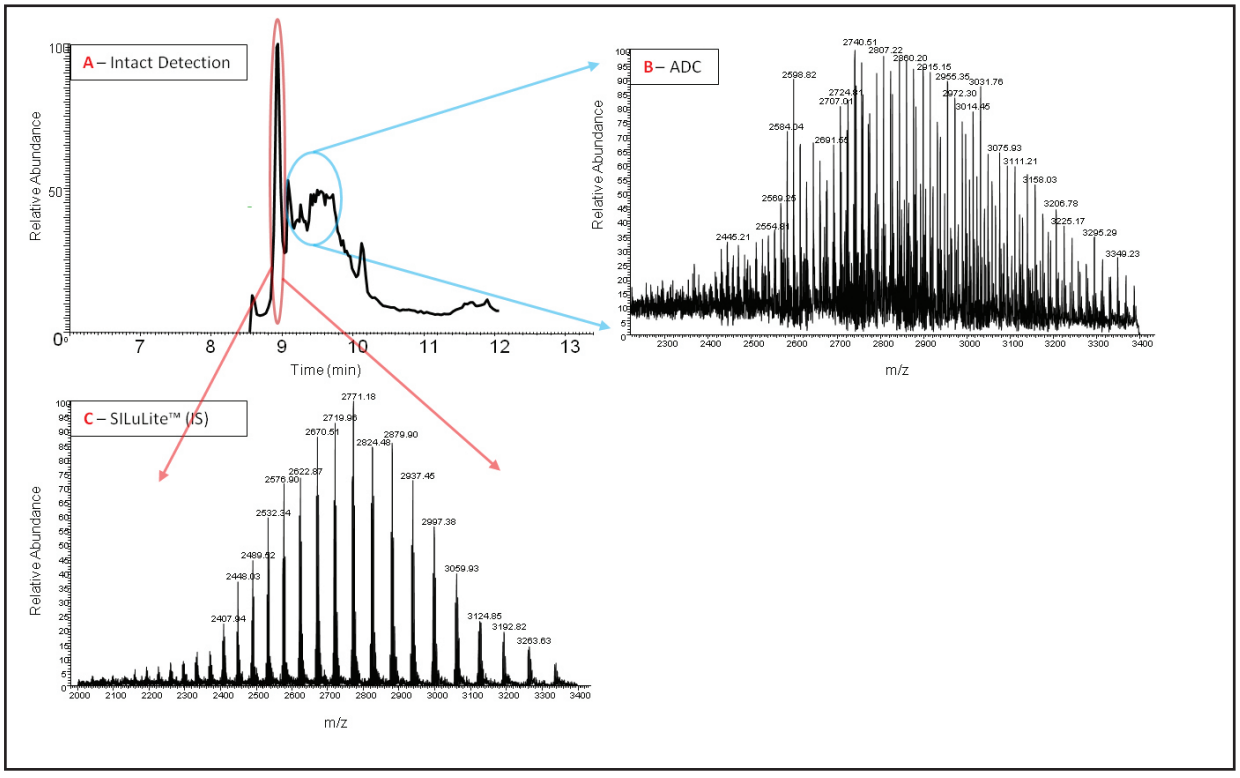


Figure 2. The results of the LB-MSIA workflow performed on a 20µL sample containing 53.5 µg/mL of the ADC and 35 µg/mL SILuLite™ from rodent plasma. A) Total ion chromatogram of the ADC sample showing the elution profile of the intact ADC and SILuLite IS. B) Mass spectrum of the several DAR species from the ADC sample. C) Mass Spectrum of the SILuLite IS from the ADC sample.

Deconvolution Method for the Quantitation of the ADC – Data Analysis Method 1

In this application, peak intensities obtained from deconvolved mass spectra were used for plotting the calibration curves for the ADC. The deconvolution software was applied to reduce the complexity in the raw MS data for the ADC (Figure 3A). The deconvolution data in Figure 3B shows multiple variants of the ADC ranging from DAR0 to DAR8. To generate the ADC calibration curve, a ratio between peak intensities of ADC and SILuLite IS was determined for each sample (that is, sum of DAR peak intensities/sum of SILuLite peak intensities) and plotted against the concentration of ADC as shown in Figure 4.

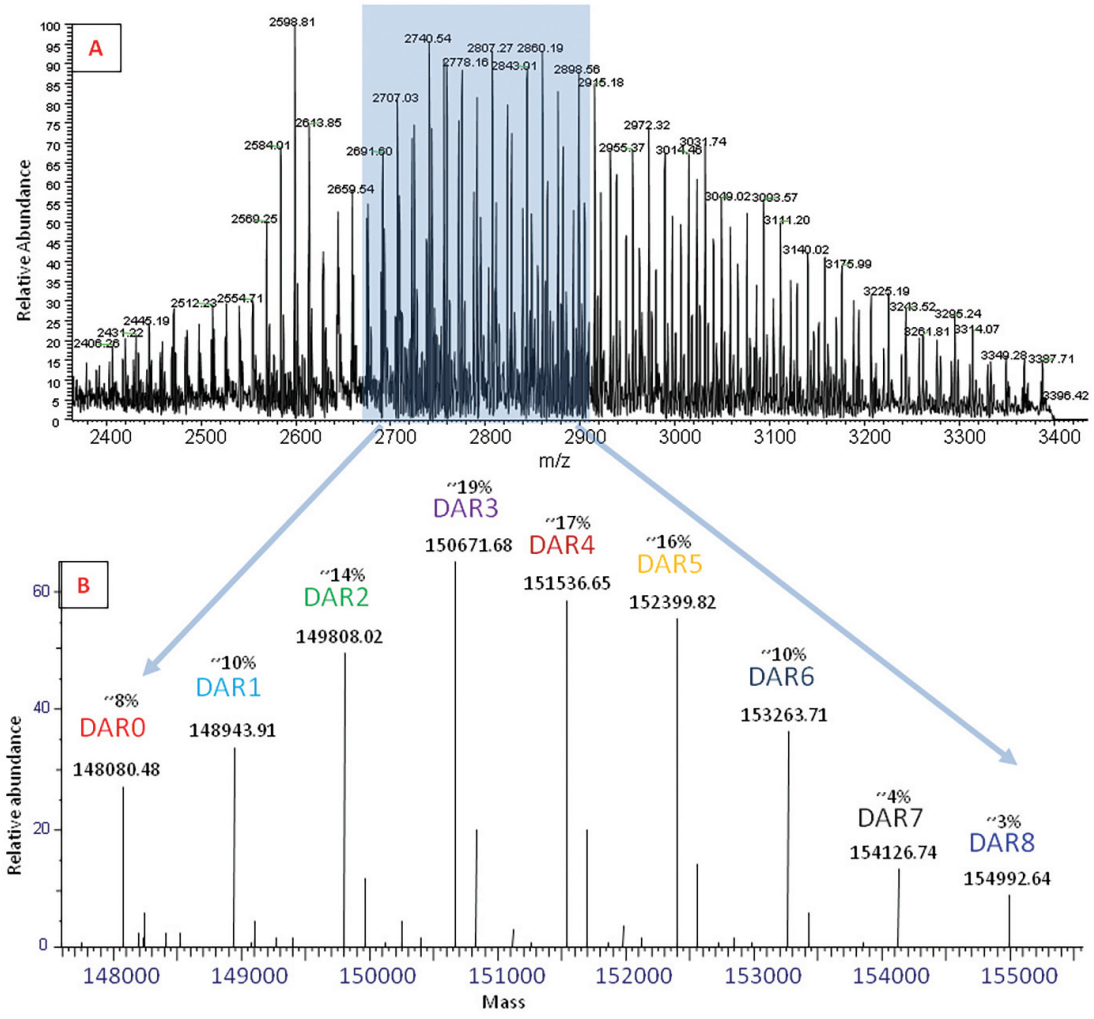
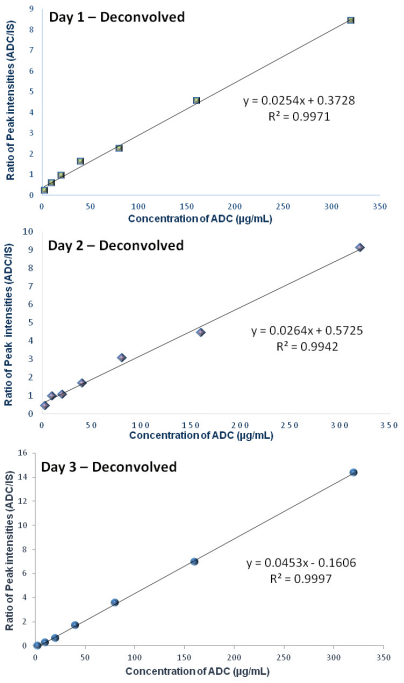


Figure 3. The results of the LB-MSIA workflow performed on a 20µL sample containing 53.5 µg/mL of the ADC from rodent plasma. A) Mass spectrum of numerous DAR species from the ADC sample. Each DAR species represents a different number of attached drug conjugates to the carrier antibody. B) Deconvolution of the mass spectrum from A resulting in the identification of a total of 9 DAR species. The intensities of all the DAR species were summed and the percentage of each DAR species was calculated based off of the total.

Following the calculation of the peak intensity ratio an average of duplicate samples was then used to generate the plots in Figure 4. The assay achieved a reproducible linear calibration curve ranging from 2.5-320 µg/mL for three curve sets generated on three separate non-consecutive days. The assay also showed CVs for most of the quintuplicate control samples below 15% and accuracy within 12%.



Controls

Sample Concentration (µg/mL)	Average concentration (µg/mL) (n=5)	% CV (n=5)	% Accuracy (n=5)
Day 1			
5.0	4.5	7.2	-10.7
13.5	14.2	5.9	5.2
53.5	53.4	5.0	-0.1
213.5	225.6	4.9	5.4
Day 2			
5.0	5.3	23.2	5.6
13.5	13.2	15.3	-2.6
53.5	52.4	8.3	-2
213.5	237.6	3.8	11.3
Day 3			
5.0	5.4	9.4	8.5
13.5	12.5	13.1	-7.5
53.5	59.5	13.7	11.2
213.5	238	8.0	11.5

Figure 4. Deconvolved Quantitation of an ADC analyzed with LB-MSIA from rodent plasma over a discontinuous period of 3 days: Calicbration Curve Range of 2.5-320 µg/mL.

XIC Method for the Quantitation of the ADC – Data Analysis Method 2

A comparative data analysis method, the XIC method, was also used to process the raw MS data obtained from each sample. In this method, the m/z values of the three most abundant charge states (+53, +54 and +55) for each ADC DAR species (Figure 5B) were combined to generate an extracted ion chromatogram. The ADC peak in the extracted ion chromatogram was then integrated to obtain AUC (area under the curve) value for each ADC sample. The same approach was also used to obtain the AUC value for the IS (SILuLite) in each sample. The ratios between the AUC values for ADC and IS (SILuLite) were determined and plotted against the concentrations of ADC as shown in Figure 6. Each calibration plot represents an average of two curves (n=2). The assay achieved a reproducible linear working curve ranging from 2.5-320 µg/mL for three curve sets. The assay also showed CVs of the quintuplicate below 15.8% and accuracy within 20%.

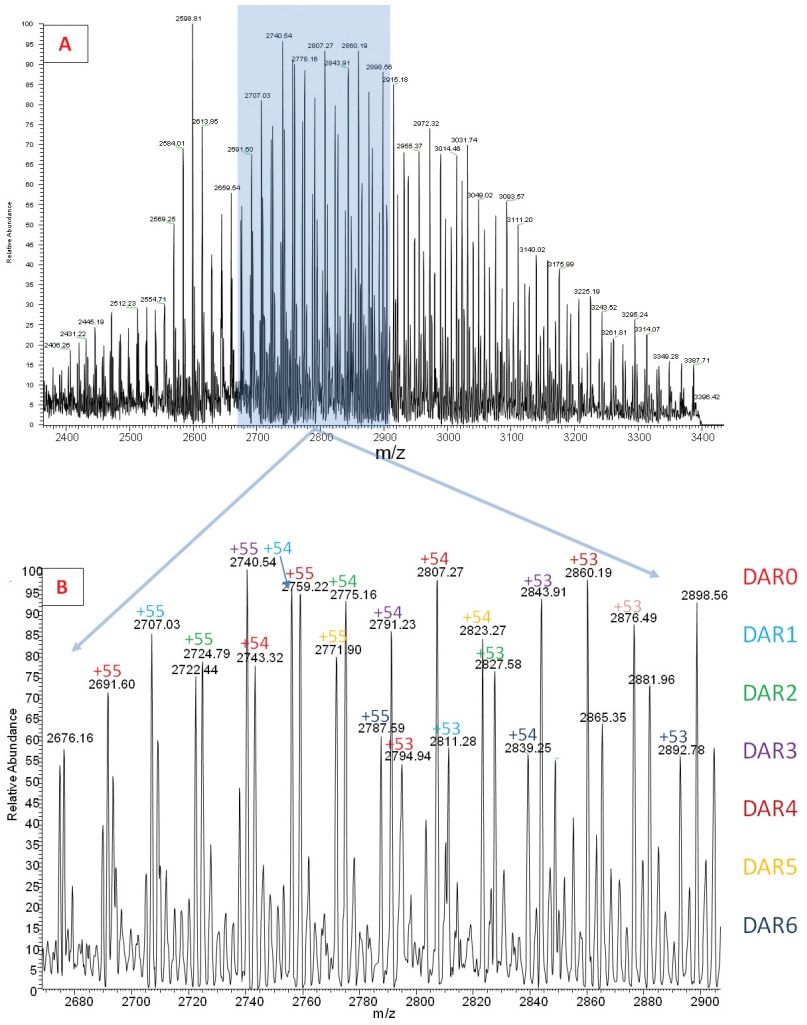
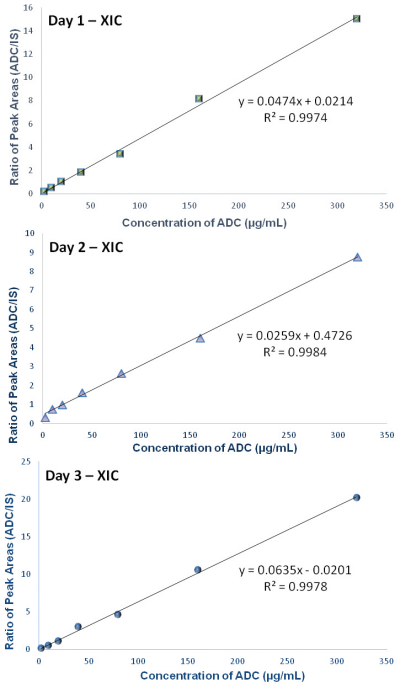


Figure 5. The results of the LB-MSIA workflow performed on a 20µL sample containing 53.5 µg/mL of the ADC from rodent plasma. A) Mass spectrum of numerous DAR species from the ADC sample. Each DAR species represents a different number of attached drug conjugates to the carrier antibody. B) Zoomed in image of A showing multiple charge states of DARo-DAR6 species.



Controls

Sample Concentration (µg/mL)	Average concentration (µg/mL) (n=5)	% CV (n=5)	% Accuracy (n=5)
Day 1			
5.0	5.1	15.0	2.9
13.5	14.2	15.8	6.9
53.5	53.4	8.2	7.2
213.5	219.4	7.1	2.8
Day 2			
5.0	6	12.4	19.3
13.5	10.8	10.7	-19.9
53.5	49.4	7.2	-7.7
213.5	208.7	8.2	11.3
Day 3			
5.0	4.5	7.2	-10.7
13.5	14.2	5.9	5.2
53.5	53.4	5.0	-0.1
213.5	225.6	4.9	5.4

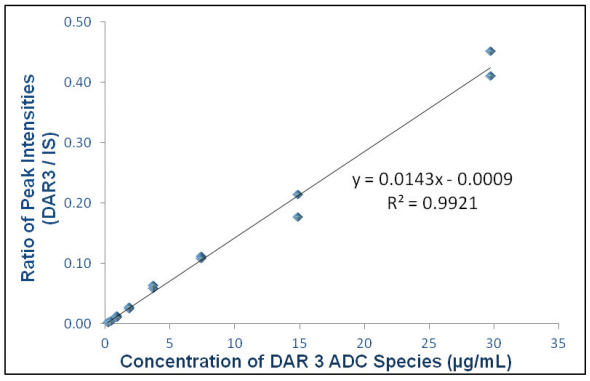
Figure 6 – XIC Quantitation of an ADC analyzed with LB-MSIA from rodent plasma over a discontinuous period of 3 days: Working Curve Range of 2.5-320 µg/mL.

Quantitation of an Individual DAR species of the ADC

A new study was performed in order to quantitate a single DAR species. The total ADC peak intensity was calculated by taking the summation of the deconvolved peak intensities from the nine ADC DAR species. The intensities for each individual DAR species were used to determine the percentage each contributed to the sum of their intensities listed in Table 5. Multiplying the percentage of each DAR species with the known concentration of the ADC stock, 225.6 µg/mL, the concentration for each DAR species was calculated and are listed in Table 5.

DAR Species	% Total	Concentration (µg/mL)
0	7.81	17.62
1	9.62	21.70
2	14.20	32.03
3	18.58	41.92
4	16.81	37.93
5	16.04	36.19
6	10.44	23.56
7	3.95	8.90
8	2.55	5.75

Table 5 -DAR Species Profile: The percentages of each DAR species were calculated by dividing the DAR species individual peak intensity by the total ADC. Then the percent contribution of each DAR species was multiplied by the stock concentration of the ADC, 225.6 µg/mL.



Controls

Total ADC Theoretical Sample Conc. (µg/mL)	DAR 3 Species % of Total ADC	DAR 3 Species Theoretical Conc. (µg/mL)	DAR 3 Species Peak Intensity	Avg. Dar 3 Species Experimental Conc. (µg/mL) (n = 5)	% CV (n=5)	% Accuracy (n=5)
2.50	18.58	0.47	5.249E-03	0.43	15.50	-8.3
5.00		0.93	1.369E-02	1.02	13.50	10.0
40.00		7.43	9.134E-02	6.45	3.35	-13.2
160.00		29.76	3.966E-01	27.80	4.80	-6.6

Figure 7 - A standard curve was created using the intensities of the DAR3 ADC species. The Total ADC dilution curve spanned a dynamic range from 1.25-160 µg/mL. In order to apply the dynamic range to the DAR3 ADC species the total ADC concentrations were multiplied by the DAR3 percentage listed in Table 5 (18.58%), reducing the dynamic range for the DAR3 ADC species to 0.23-29.73 µg/mL. The resultant equation of the line was within 15% accuracy for the controls (DAR3 Control Concentrations: 0.47, 0.93, 7.43, 29.76 µg/mL).

A control curve was generated using the DAR3 ADC peak intensity ratios (Figure 7). As seen in Table 5, the DAR 3 species represents 18.58% of the total ADC. By applying the same percentage to the curve concentrations for the Total ADC standard, the dynamic range is reduced from 1.25-160 ug/mL (Total ADC) to 0.23-29.73 µg/mL (DAR 3 ADC Species) resulting in an equation of the line that is specific for the DAR 3 ADC species (Figure 7). The MS data for the control samples were deconvolved and the peak intensities for the DAR 3 species were normalized by the SILuLite IS. The concentrations of the controls were then calculated from the equation of the line as represented in Figure 7.

Conclusion

The universal LB-MSIA utilizing Streptavidin MSIA D.A.R.T.'S demonstrated here, provided a sensitive, robust, and reproducible method for the quantitative analysis of an ADC. The high selectivity of the CaptureSelect™ biotin anti-IgG-Fc (human) conjugate combined with the molecular trapping technology of the MSIA D.A.R.T.'S created an ideal scenario to quantitatively assay a low abundant (µg/mL) intact ADC from rodent plasma with a dynamic range spanning two orders of magnitude. Furthermore, the workflow supported a high throughput application by performing the pre-analytical steps on the Thermo Scientific™ Versette™ automated liquid handler. As a hybrid approach, the use of the Q Exactive Plus for HRAM detection helped provide additional analytical flexibility and data content over other developing triple quadruple methods that are reliant on peptide analysis. The two methods of data analysis demonstrated here each help to reduce the biological complexity of the ADC MS. As shown, the combined benefits of the LB-MSIA enabled the quantitation of the total ADC and a single DAR species of the ADC with CVs that were less than 15% and accuracy that was within 20%.

Ordering Information

MSIA D.A.R.T.'S for Immunoaffinity Capture		
Compatible with the Thermo Scientific Versette Automated Liquid Handler and Thermo Scientific Finnpipette® Novus i Multichannel Electronic Pipette		
Cat. No.	**Description**	**Packaging**
991CUS02	300µl MSIA D.A.R.T.'S, Custom	Pack of 96 units
991PRT11	300µl MSIA D.A.R.T.'S, Protein A	Pack of 96 units
991PRT12	300µl MSIA D.A.R.T.'S, Protein A	Pack of 24 units
991PRT13	300µl MSIA D.A.R.T.'S, Protein G	Pack of 96 units
991PRT14	300µl MSIA D.A.R.T.'S, Protein G	Pack of 24 units
991PRT15	300µl MSIA D.A.R.T.'S, Protein A/G	Pack of 96 units
991PRT16	300µl MSIA D.A.R.T.'S, Protein A/G	Pack of 24 units
991STR11	300µl MSIA D.A.R.T.'S, Streptavidin	Pack of 96 units
991STR12	300µl MSIA D.A.R.T.'S, Streptavidin	Pack of 24 units
991001096	300µl MSIA D.A.R.T.'S, Insulin	Pack of 96 units
991001024	300µl MSIA D.A.R.T.'S, Insulin	Pack of 24 units
991R	300 µL MSIA D.A.R.T.'S, Reloadable Rack	1 reloadable rack, D.A.R.T.'S are not included
MSIA Streptavidin-EVO for Immunoaffinity Capture		
Compatible with the Tecan™ Freedom EVO® Liquid Handling Robotic Platform equipped with a MCA96 option		
Cat. No.	**Description**	**Packaging**
992STR96	500µl MSIA Streptavidin EVO microcolumns	Pack of 96 units
Automated Liquid Handling Platform		
Cat. No.	**Description**	
650-MSIA	MSIA Versette Automated Liquid Handler	
Multichannel Pipettes and Pipette Stand		
Cat. No.	**Description**	**Packaging**
991S	Finnpipette Novus i Adjustable Pipette Stand	1 pipette stand
991SP12	Finnpipette Novus i Electronic 12-Channel Pipette, 30-300µl and Pipette Stand	1 pipette and 1 pipette stand
Liquid Chromatography		
Cat. No.	**Description**	
	Thermo Scientific™ Dionex™ UltiMate® 3000 UHPLC System	
	Thermo Scientific™ Vanquish™ UHPLC System	
066640	ProSwift™ RP-4H Monolith Column, 1.0 x 250 mm	
Mass Spectrometry and Software		
Description		
Thermo Scientific™ Q Exactive™ Hybrid Quadrupole-Orbitrap Mass Spectrometer		
Thermo Scientific™ Q Exactive™ Plus Hybrid Quadrupole-Orbitrap™ Mass Spectrometer		
Thermo Scientific™ Pinpoint Software		
Thermo Scientific™ XCalibur™ Software		
Thermo Scientific™ Protein Deconvolution Software, Version 4.0 with the ReSpect™ algorithm		

Poster Note 64771

Characterization of Conformation of Therapeutic Antibody Aggregation with Optimized Hydrogen/Deuterium Exchange Mass Spectrometer

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[1]Thermo Fisher Scientific, San Jose, CA; [2]Thermo Fisher Scientific, Sunnyvale, CA

ABSTRACT

Protein aggregation is one of the major critical quality attributes (CQA). To determine the structural change of antibody in connection with aggregation is important. In this study, a therapeutic antibody, Herceptin®, was treated with different acidic solutions (pH 0.5, 1.5 and 2.5) to induce aggregation. The aggregation was quantified by UV 280 absorption. The conformation of Herceptin and its aggregation was characterized by an optimized hydrogen/deuterium exchange mass spectrometer (HDX). The HDX results revealed the more significant conformation change regions when aggregation was induced.

INTRODUCTION

Monoclonal antibodies (mAbs) have been increasingly used for detection and treatment of cancer and other diseases. Characterization of critical quality attributes (CQA) of mAb-based drugs is a primary concern for biopharmaceutical development. Structural characterization is used to assess the CQAs of biopharmaceutical products. Protein aggregation is a major CQA during monoclonal antibody (mAb) production. The formation of aggregates may impact safety and efficacy of mAbs. It is thus important to understand the mechanism of aggregation and the conformational changes of the aggregates. Hydrogen/deuterium exchange mass spectrometry (HDX) has emerged as a powerful tool to investigate the conformation of intact proteins, including mAbs. In this study, an optimized HDX workflow was used to probe the conformation of Herceptin and its aggregation.

MATERIALS AND METHODS

Instrument and Materials

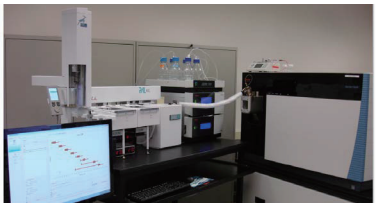
A fully automated HDX platform, based on the H/D-X PAL™ system (LEAP Technologies) and the Thermo Scientific™ UltiMate 3000 pump system coupled online with an Thermo Scientific™ Orbitrap Fusion™ Tribrid™ mass spectrometer, was used. Figure 1 shows the HDX work station. It is composed of an autosampler equipped with individually temperature-controlled sample plate and labeling plate. The syringe head and pepsin column can also be independently temperature-controlled. Chronos™ control software is fully integrated with the Thermo Scientific™ Xcalibur™ platform and the user interface is shown in Figure 2. Figure 3a) describes the three valve configurations at various stages of the experiment inside the cooling chamber. The flexibility allows for maximized productivity. Figure 3 b) shows the dual heads high pressure mixing pump on the left and ternary loading pump on the right. Flow rates and solvents can be changed on the fly during the experiment. The execution of the whole experiment was completely automated and controlled by Chronos™ software.

Therapeutic antibody (Herceptin) aggregation was induced by adjusting the pH to 0.5, 1.5 and 2.5 with HCl and by incubating each sample at room temperature for 30 min. The pH of the samples was subsequently adjusted to pH 9. Both non aggregated and aggregated mAbs were diluted with labeling buffer and incubated for multiple time points. The samples were then quenched and digested online with a pepsin column in a fully automated manner using the H/D-X PAL system. The digested peptides were injected into a Thermo Scientific™ Acclaim™ Pepmap™ C18 reverse phase column with a 7 min gradient. MS analysis was performed with the Orbitrap Fusion mass spectrometer.

Data Analysis

Peptide identification was performed with Thermo Scientific™ Proteome Discoverer™ 1.4 software . Peptide mapping and PTM analysis was performed with Thermo Scientific ™ BioPharma Finder™ 1.0 software. HDX experimental data were analyzed with HDExaminer™ software (Sierra Analytics) and the HDX tool in Mass Analyzer (licensed from Amgen).[1,2].

FIGURE 1. HDX work station coupled online to the Orbitrap Fusion mass spectrometer



RESULTS

Herceptin and Herceptin Aggregation

Herceptin and its pH stressed samples were quantified by measuring UV 280 absorption. Figure 4 is the overlay absorption spectra of four samples. Aggregations around 25% for pH 0.5, 6% for pH 1.5 and 0% for pH 2.5 were observed.

Figure 2. Chronos software interface, integrated with Xcalibur software

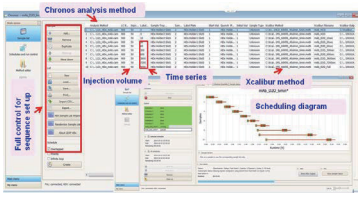


Figure 3. a). Flexible three valve configuration for carrying out different experimental tasks simultaneously in the cooling chamber, b). NCS-3500RS with binary rapid separation micro flow pump with ternary loading pump

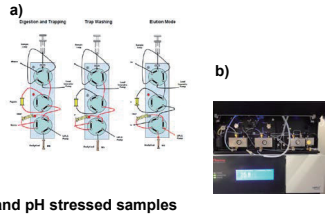


Figure 4. UV absorption spectrum of Herceptin and pH stressed samples

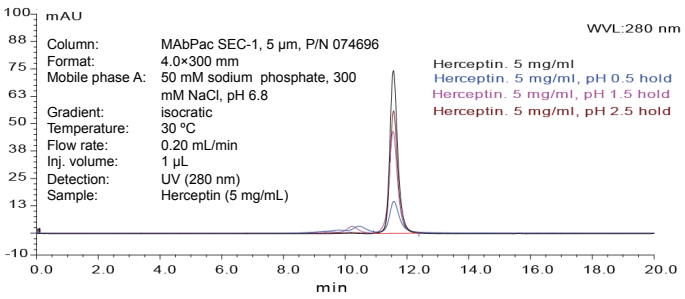
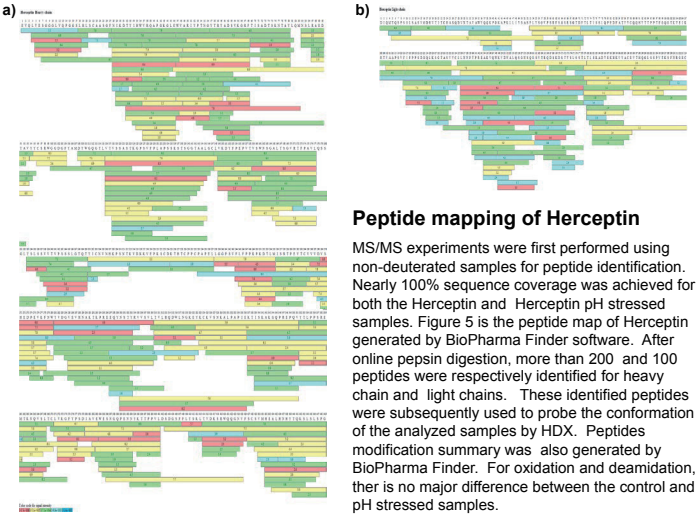


Figure 5. Peptide map of Herceptin by BioPharma Finder a) Heavy chain b) Light Chain



Peptide mapping of Herceptin

MS/MS experiments were first performed using non-deuterated samples for peptide identification. Nearly 100% sequence coverage was achieved for both the Herceptin and Herceptin pH stressed samples. Figure 5 is the peptide map of Herceptin generated by BioPharma Finder software. After online pepsin digestion, more than 200 and 100 peptides were respectively identified for heavy chain and light chains. These identified peptides were subsequently used to probe the conformation of the analyzed samples by HDX. Peptides modification summary was also generated by BioPharma Finder. For oxidation and deamidation, ther is no major difference between the control and pH stressed samples.

Herceptin and its Aggregation conformation characterization

Multiple time point HDX experiments were performed for both Herceptin and pH stressed Herceptin samples. Highly reproducible chromatograms were obtained for the various experimental time points. MS full scan spectra were collected to measure the deuterium uptake to probe the conformation.

Overall, the control and pH stressed samples had similar deuterium uptake profile but for most of the pH stressed sample peptides showed slightly more deuterium uptake, around 2 to 5%, than for the non-stressed sample. The differences could reach up to 10 to 15% for different regions for the light chain and could be even higher for the heavy chain. The sample stressed at pH = 0.5 showed more deuterium uptake than the pH = 1.5 sample. Finally, there was no significant deuterium uptake change between Herceptin and the stressed sample pH=1.5 (data not shown).

Figure 6. a): Herceptin and pH 0.5 stressed sample light chain deuterium uptake light chain residual plot. b), c): Example of specific peptides deuterium uptake plots of Herceptin and its pH 0.5 stressed sample

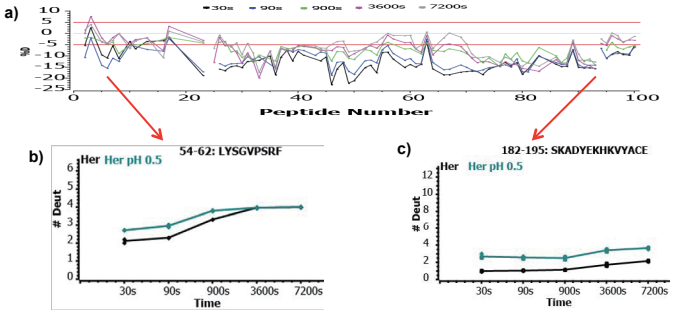


Figure 6 a) is the light chain deuterium uptake residual plot whereas figure b) and , c) are peptides uptake plots. In figure 6 b) peptide showed more differences for short labeling time but would reach to similar levels at longer time points, while in figure 6 c) peptide showed the significant differences persistent along all the time points. Protection factors were calculated and shown in figure 7. Certain regions have similar protection factors while other regions have significant differences, i.e., between amino acids160 and 190 for the heavy chain. These results were consistent with HDExaminer's analysis.

Figure 7. Herceptin and pH 0.5 stressed sample light chain protection factor comparison

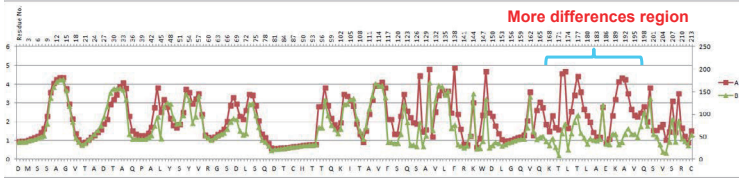
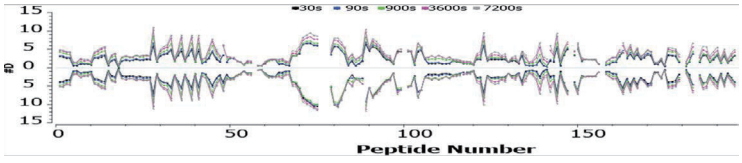


Figure 8. Herceptin and pH 0.5 stressed sample heavy chain deuterium uptake mirror plots



Similar deuterium uptake profiles of Herceptin and its pH stressed sample were observed for heavy chain and are shown in figure 8. In general, the stressed sample showed more deuterium uptake, and the amount of uptake increased with degree of aggregation. This implies that aggregation would impact the 3D structure of Herceptin and more exposed to solvents, i.e., more deuterium uptake. Furthermore, the increase in deuterium uptake was not uniform across all the peptides. Example deuterium uptake plots were shown in figure 9. In these examples, different peptides showed different deuterium uptake dynamics and levels. With mass spectrometry, the regions with such increase can be pinpointed. A pronounced deuterium uptake increase (up to 40%, see left panel of figure 10) was observed from amino acid 120 to 200 and 240 to 255 of the heavy chain for the sample stressed at pH=0.5. The differences could be up to 5 daltons, see figure 10, right panel. These results indicated that aggregation would cause more conformational change for the Fab region and smaller changes for the Fc region.

Figure 9. Herceptin and pH 0.5 stressed sample heavy chain peptides deuterium uptake plots

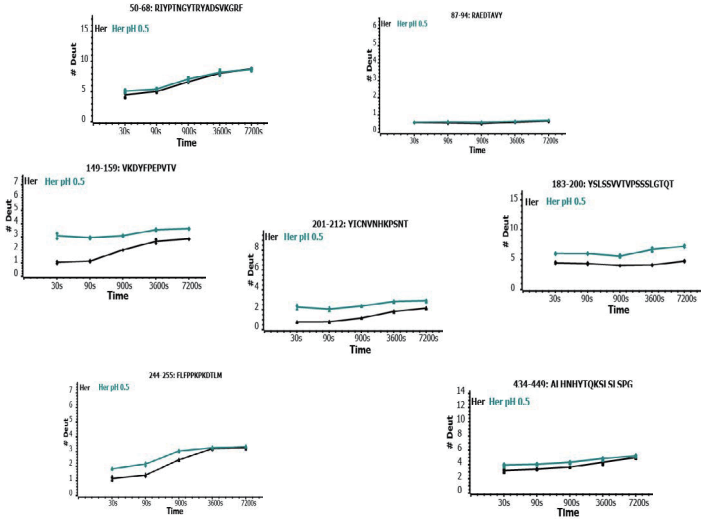


Figure 10. Herceptin and pH 0.5 stressed sample heavy chain deuterium uptake residual plots, Left: %D Right: #D

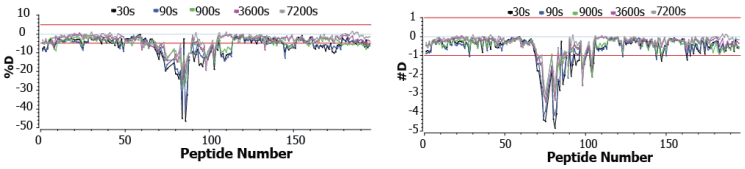
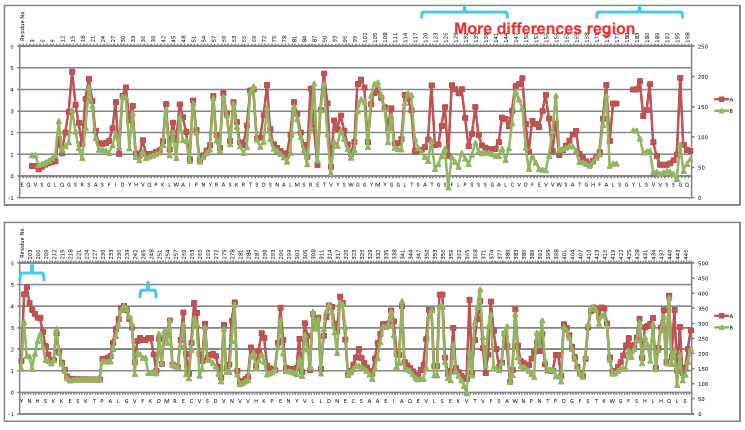
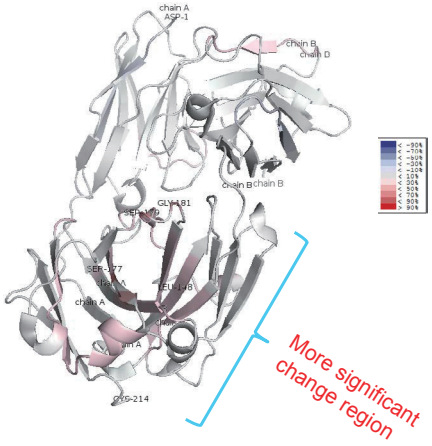


Figure 11. Herceptin and pH 0.5 stressed sample heavy chain protection factor comparison. Top: residual 1-200 Bottom: residual 201-449



To further understand the conformational dynamics, single amino acid residue level protection factors were calculated using Mass Analyzer HDX, (see figure 11). In most of the regions Herceptin showed slightly higher protection factor than the pH 0.5 stressed sample. From amino acid 110 to 210 and 240 to 255, Herceptin had a much higher protection factor, which is consistent with the deuterium uptake residual plots obtained from HDExaminer.

Figure 12. Herceptin and pH 0.5 stressed sample 1hr deuterium labeling Fab differential exchange map on crystal structure (PDB: 1N8Z)



Antibody aggregation is a complex, multistep process[3]. The conformation change with more deuterium uptake observed for the sample stressed at pH 0.5 agreed with the proposed aggregation mechanism[3,4] and the reported results[5] in the literature. The differential deuterium uptake of Herceptin and the sample stressed at pH 0.5 for1 hour labeling was mapped with Herceptin Fab crystal structure (PDB:1N8Z) and shown in figure 12. Most of the significant change regions were in the C_L, C_H and part of Fc C_H2 domains.

CONCLUSIONS

•A fully automated HDX workflow was successfully applied to the study of conformational changes of Herceptin upon aggregation.

•In this study, we were able to pinpoint the subtle but significant changes around the C_L, C_H and C_H2 regions.

•The MS data were analyzed by two independent software packages (HDExaminer and Mass Analyzer) and the conclusions were consistent.

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Characterization of the Conformation of Therapeutic Antibody Oxidation Variants with Optimized Hydrogen/Deuterium Exchange Mass Spectrometry

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Poster Note 64465

Overview

Purpose: Probe the conformation of herceptin and its oxidation variants.

Methods: Fully automatic hydrogen/deuterium exchange mass spectrometry

Results: There are no significant conformational changes for most regions of herceptin and its oxidation variants. However, local solvent exposure differences in the vicinity of the peptides containing methionine oxidation were observed.

Introduction

Monoclonal antibodies (mAbs) have been increasingly used for detection and treatment of diseases. Characterization of chemical degradation of mAb-based drugs is a primary concern for biopharmaceutical development due to the subtle but critical local conformational changes that may impact safety and efficacy.[1,2] It is thus important to have an analytical tool that can detect these minor conformational changes. Hydrogen/deuterium exchange mass spectrometry (HDX) has emerged as a powerful tool to investigate the conformation of intact proteins, including mAbs. In this study, an optimized HDX workflow was developed and used to probe the conformation of Herceptin and its oxidation variants.

Methods

Therapeutic antibody, Herceptin, was partially oxidized with 0.01% H_2O_2 overnight. Both non-oxidized and oxidized mAb were diluted (1 to 9 ratio) with labeling buffer and incubated for multiple time points. The samples were then quenched with 4M guanidine, 200mM citric acid (pH 2.7) at 0.5 °C and subject to online pepsin digest at 8 °C for three minutes at 50 µL/min flow rate in a fully automated manner using H/D-X PAL™ (LEAP Technology). The digested peptides were injected into a Thermo Scientific™ PepMap™ trapping column washed for one minute and eluted to a Thermo Scientific™ Hypersil™ Gold C18 reverse phase column. A Thermo Scientific™ Ultimate™ 3000 nano pump system was employed to separate the digested peptides with 5% to 40% mobile phase B in 6 minutes gradient at flow rate of 40 µL/min. The separated peptides MS analysis was performed with Thermo Scientific™ Orbitrap Fusion™ Tribrid™ mass spectrometer. The data dependent MS/MS HCD spectra were collected using undeuterated protein for peptides identification first. And MS full scan at 60K was collected for HDX analysis. Figure 1 is the HDX work station set up. Figure 2 is HDX experimental workflow.

Liquid Chromatography

Thermo Scientific™ online pepsin Column: 2.1 x 3 mm

Thermo Scientific™ Dionex™ trapping Column: 500 µm x 15mm, C18 PepMap300, 5µm

Hypersil Gold analytical Column: 0.5 mm x 100mm, 3µm

Data Analysis

Data was processed with Proteome Discoverer 1.4™ software for peptide identification. Peptide mapping and PTM analysis was performed with PepFinder 2.0™ software. HDX experimental data were analyzed with HDExaminer and the Mass Analyzer HDX algorithm.[3,4]

FIGURE 1. HDX Work Station

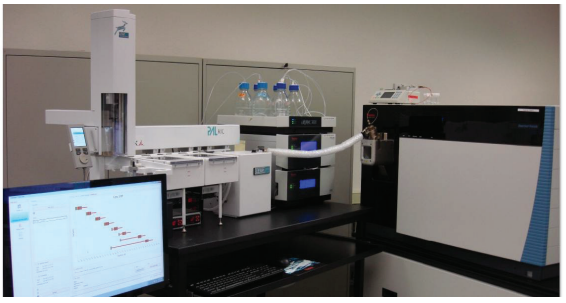
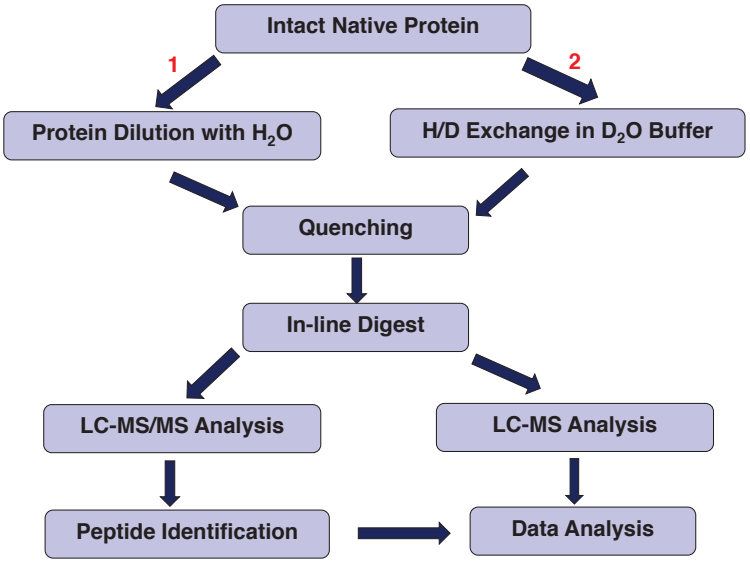


FIGURE 2. HDX experimental workflow



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Results

Peptide mapping of Hercetin

MS/MS experiments were first performed using non-deuterated Herceptin for peptide identification. Nearly 100% sequence coverage was achieved for both the Herceptin and oxidized Herceptin samples. Figure 3 is the peptide map of Herceptin generated by the Pepfinder software. More than 200 peptides generated by online pepsin digestion from the optimized HDX workflow were identified. These were subsequently used to probe the conformation of the two samples by HDX.

FIGURE 3. Peptide map of Herceptin

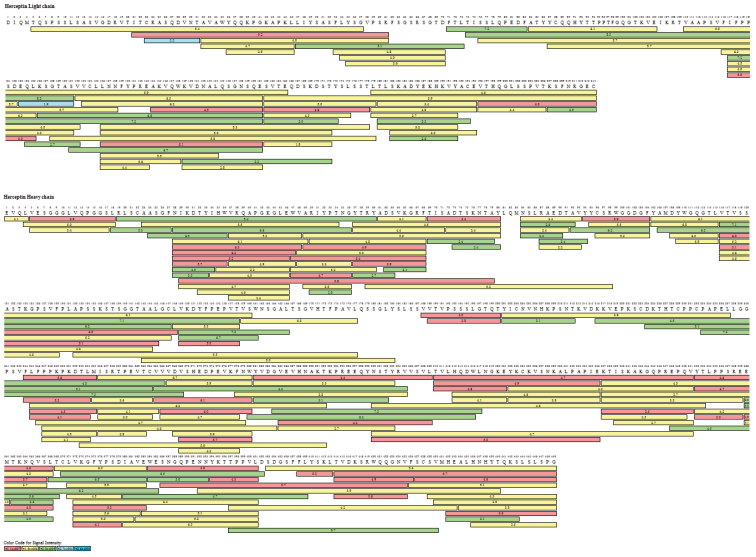
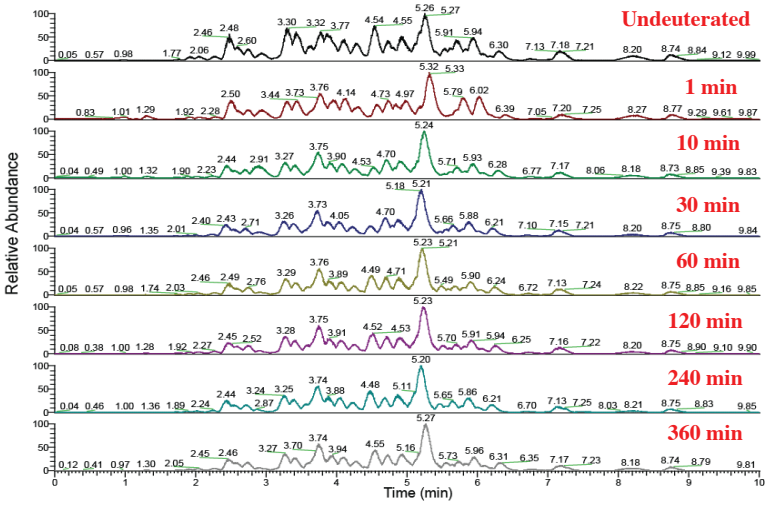


FIGURE 4. Herceptin HDX experimental base peak chromatogram



Conformation of Herceptin and its oxidized variants

Multiple time points of HDX experiment were performed for both Herceptin and oxidized Herceptin samples. Highly reproducible chromatograms were obtained for the various experimental time points (Figure 4). MS full scan spectra were collected to measure the deuterium uptake to probe the conformation of the therapeutic antibody and its variants. The deuterium uptake information were processed by HDExaminer.

FIGURE 5. a) Light chain deuterium uptake mirror plot of Herceptin and Herceptin variants. b) Deuterium uptake information modeled to Herceptin light chain crystal structure (PDB 1N8Z). The relative percent deuterium incorporation is shown at 30, 600, 3600 and 7200 seconds respectively.

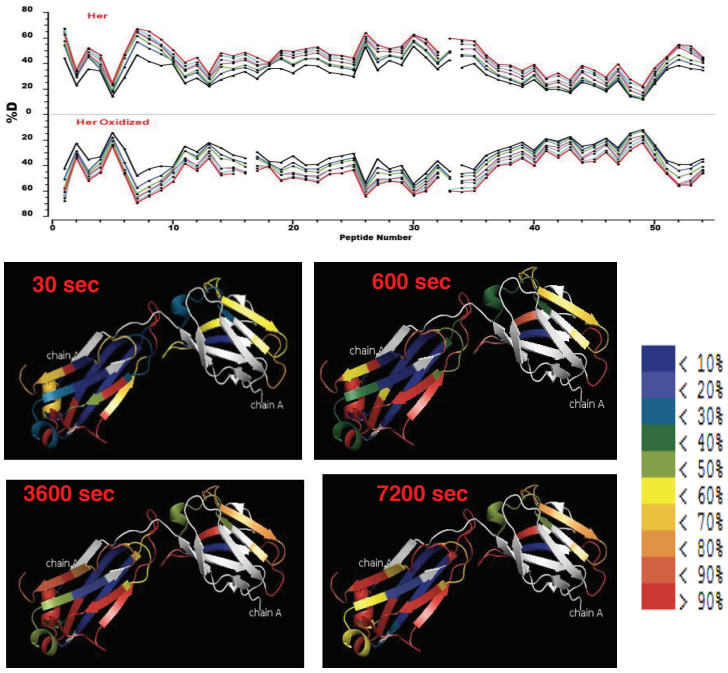
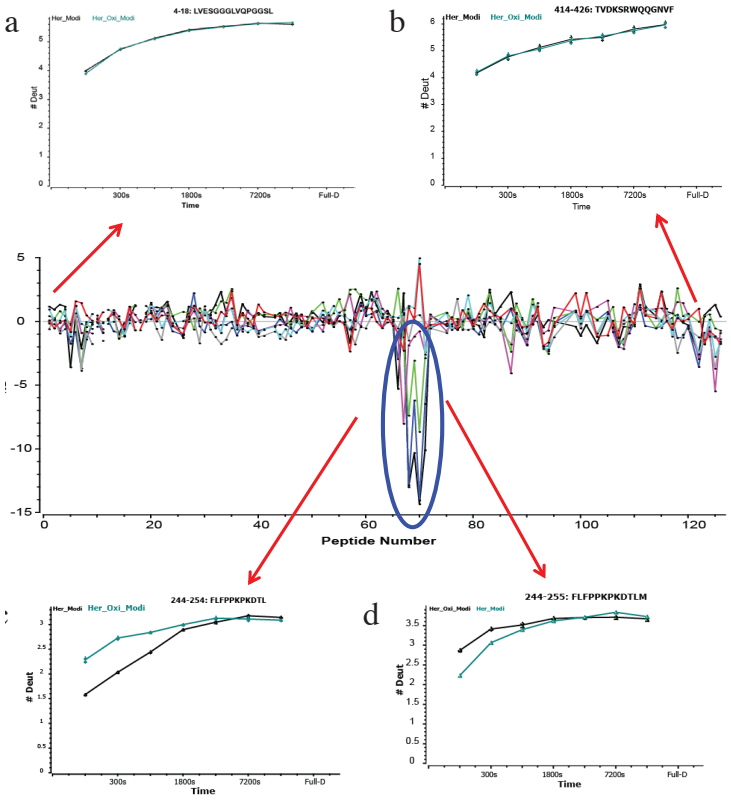


Figure 5 shows the light chain deuterium uptake measurement information. The very similar deuterium uptake patterns of the two samples indicate that there is no significant light chain conformational differences between Herceptin and Herceptin variants samples as shown in Figure 5a). The deuterium uptake measurements were exported to PMOL software and incorporated with the available Herceptin crystal structure as shown in Figure 5 b). The deuterium incorporation difference provides the information understanding the conformation dynamics of the light chain.

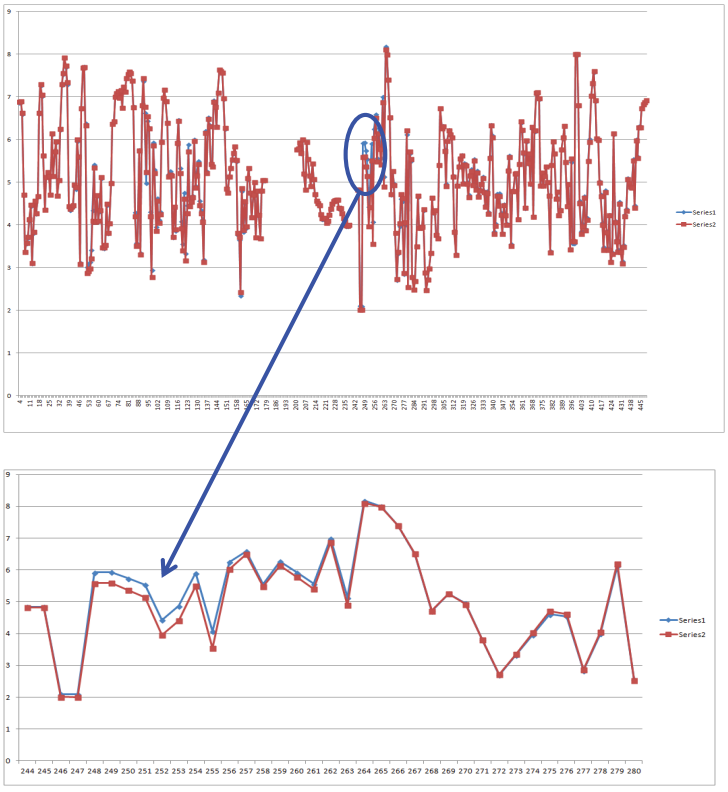
FIGURE 6. Herceptin versus Herceptin variants heavy chain deuterium uptake residual plot. Inserts a, b, c d are the specific peptides deuterium uptake plots of Herceptin and its variants



In Figure 6, heavy chain deuterium uptake difference between Herceptin and its oxidized variants is plotted vs. peptide number. The data were obtained with HDExaminer from MS full scan at various deuterium exchange time points. At most regions the difference is minimal (inserts a, b), except at the specific region where in the vicinity of methionine (residue 255), which is the amino acid that is oxidized. The inserts (c, d) of Figure 6, deuterium uptake plots of peptide FLFPPKPKDTL and FLFPPKPKDTLM, show the different kinetic behavior of deuterium uptake of Herceptin and its oxidized forms; after oxidation, the deuterium uptake is faster. Structurally, it is more sterically accessible for solvent exchange when methionine's SCH_3 terminal is oxidized to $SOCH_3$ or SO_2CH_3.

In Figure 7 protection factors for each residue in heavy chain of Herceptin and its variants were plotted. Mass Analyzer HDX algorithm was used to calculate the protection factor at the amino acid level. HDX model is built to simulate the whole deuterium labeling and back exchange processed during the digestion and analysis. The HDX model utilized the maximal information of the entire HDX MS data set (both the HDX kinetics and the labeling information from all overlapping peptides)[4]. 400 simulation was employed for this data set. Similar to findings shown in Figure 6, the protection factors are identical for most of the residues except in the region where methionine (residue 255) is involved. The oxidized variants have lower values compared to the original form, consistent with results obtained with HDExaminer.

FIGURE 7. (top): the average value of protection factors for each residue in heavy chain of Herceptin and its variants. (bottom): zoom in of the specific region with significant protection factor change



Conclusion

•A fully automated HDX workflow was developed and successfully applied to the study of conformational changes of Herceptin upon oxidation.

•The workflow was reliable and able to pinpoint the subtle but significant changes in the methionine region.

•The MS data were analyzed by two independent packages HDExaminer and Mass Analyzer and the conclusions are consistent.

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Acknowledgements

We would like to thank Professor Mark Avdalovic from the University of California Davis for supplying the monoclonal antibodies.

POSTER NOTE

No. 64890

Evaluation of different Hydrogen/Deuterium Exchange data processing software by looking at conformational changes induced by mAb aggregation

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ABSTRACT

Hydrogen/deuterium exchange mass spectrometry (HDX) has emerged as a powerful tool to investigate the conformation of intact proteins, including mAbs. One of the biggest challenges in HDX experiment is data processing. In this study, three HDX processing software, HDExaminer (Sierra Analytics), HDX WorkBench (Scripps) and Thermo Scientific ™ BioPharma Finder 2.0 were compared and used to characterize the conformation of Trastuzumab and its aggregation.

INTRODUCTION

Monoclonal antibodies (mAbs) have been increasingly used for detection and treatment of cancer and other diseases. Characterization of critical quality attributes (CQA) of mAb-based drugs is a primary concern for biopharmaceutical development. Structural characterization is used to assess the CQAs of biopharmaceutical products. Protein aggregation is a CQA for monoclonal antibody (mAb) product. The formation of aggregates may impact safety and efficacy of mAbs. Antibody aggregation is a complex, multistep process[1]. Thus, it is important to understand the mechanism of aggregation and the conformational changes of the aggregates. Hydrogen/deuterium exchange mass spectrometry (HDX) has emerged as a powerful tool to investigate the conformation of intact proteins, including mAbs. One of the biggest challenges in HDX is data processing. Accurately measuring the deuterium uptake would yield more precise information of the protein conformation and conformation dynamics. In this study, three HDX process software, HDExaminer (Sierra Analytics), HDX WorkBench (Scripps) and Thermo Scientific ™ BioPharma Finder 2.0 were compared and used to characterize the conformation of Trastuzumab and its aggregation.

MATERIALS AND METHODS

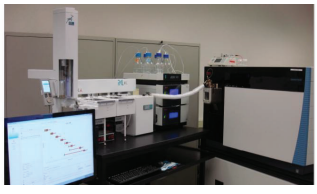
A fully automated HDX platform, based on the H/D-X PAL™ system (LEAP Technologies) and the Thermo Scientific™ UltiMate 3000 pump system coupled online with an Thermo Scientific™ Orbitrap Fusion™ Tribrid™ mass spectrometer, was used. Figure 1 shows the HDX work station. It is composed of an autosampler equipped with individually temperature-controlled sample plate and labeling plate. The syringe head and pepsin column can also be independently temperature-controlled. Chronos™ control software is fully integrated with the Thermo Scientific™ Xcalibur™ platform and the user. The execution of the whole experiment is completely automated and controlled by Chronos™ software.

Therapeutic antibody Trastuzumab (Herceptin) aggregation was induced by adjusting the pH to 0.5, 1.5 and 2.5 with HCl and by incubating each sample at room temperature for 30 min. The pH of the samples was subsequently adjusted to pH 9. Both non aggregated and aggregated mAbs were diluted with labeling buffer and incubated for multiple time points. The samples were then quenched and digested online with a pepsin column in a fully automated manner using the H/D-X PAL system. The digested peptides were injected into a Thermo Scientific™ Acclaim™ Pepmap™ C18 reverse phase column with a 7 min gradient. MS analysis was performed with the Orbitrap Fusion mass spectrometer.

Data Analysis

Peptide identification, mapping, PTM analysis and HDX data analysis were performed with Thermo Scientific ™ BioPharma Finder™ 2.0 software. HDX experimental data were also analyzed with HDExaminer™ software (Sierra Analytics) and HDX WorkBench (Scripps, Florida)

FIGURE 1. HDX work station coupled online to the Orbitrap Fusion mass spectrometer



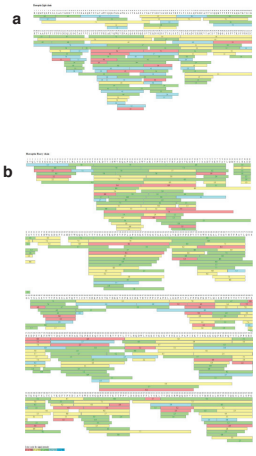


Figure 2. Peptide map of Herceptin by BioPharma Finder a) Light chain b) Heavy Chain

RESULTS

Herceptin and Herceptin Aggregation

Herceptin (Her) and its pH (Her pH0.5) stressed samples were quantified by measuring UV 280 absorption. Aggregations around 25% for pH 0.5, 6% for pH 1.5 and 0% for pH 2.5 were observed. The HDX data from the control Her and Her stressed at pH 0.5 samples were used for this study.

MS/MS experiments were first performed using non-deuterated samples for peptide identification. Nearly 100% sequence coverage was achieved for both the Herceptin and Herceptin pH stressed samples. Figure 2 is the peptide map of Herceptin generated by BioPharma Finder software. . After online pepsin digestion, around 200 and 100 peptides were respectively identified for heavy and light chains. These identified peptides were subsequently used to probe the conformation of the analyzed samples by HDX.

Overall, the control and pH stressed samples showed similar deuterium uptake. However, most of the pH stressed sample peptides showed slightly more deuterium uptake, between 2 and 5%, than for the control sample. The differences could reach up to 15% for different regions of the light chain and could be even higher for the heavy chain. The sample stressed at pH = 0.5 showed more deuterium uptake than the pH = 1.5 sample. Finally, there was no significant deuterium uptake change between Herceptin and the stressed sample pH=1.5,as reported previously[2].

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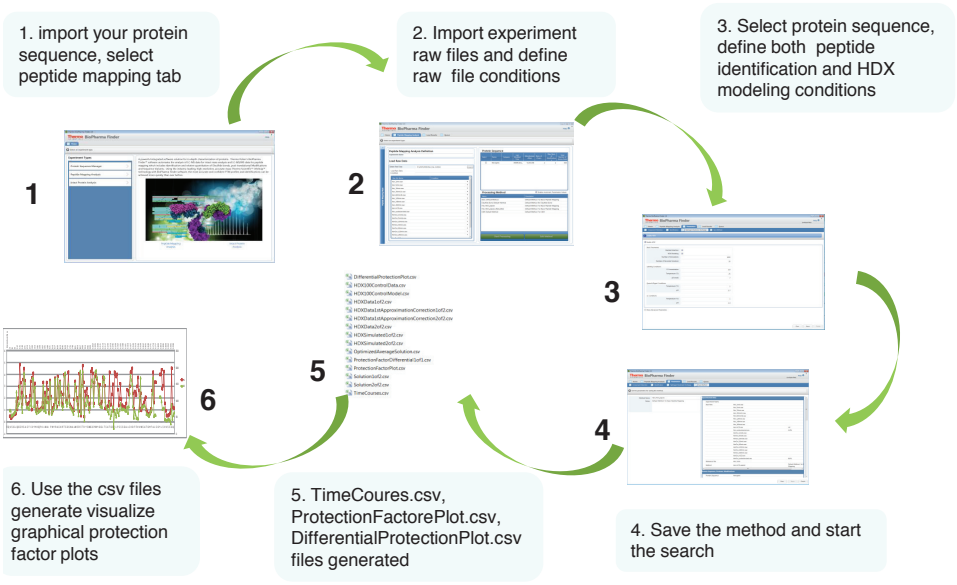
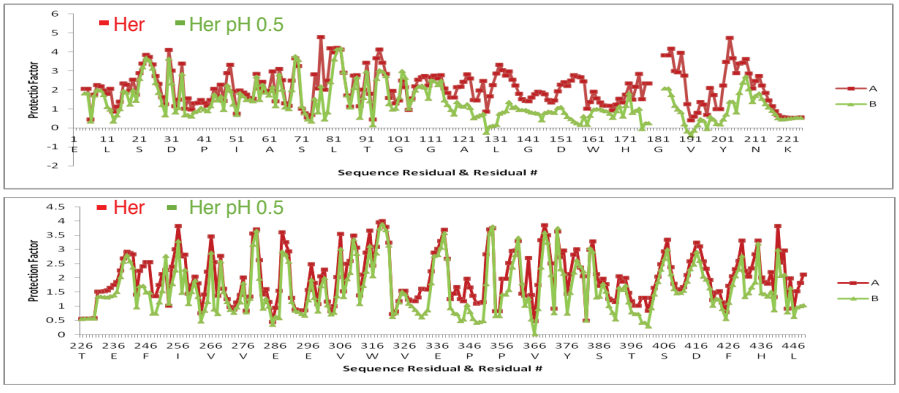
Figure 3. Biopharma Finder HDX data analysis workflow



The HDX tool in mass analyzer[3] has been implemented into BioPharma Finder 2.0. The HDX MS data can be processed in an automated fashion by BioPharma Finder 2.0. The unlabeled protein samples must be analyzed with data-dependent MS/MS for peptide identification. Deuterium labeling is performed at different time intervals to obtain time courses for each condition. The software uses a 5-parameter equation to fit the curves to calculate the variance in the data[3]. Ideally, a 0% and 100% deuteration control should be performed for appropriate back exchange modeling. Figure 3 shows the HDX data process workflow of BioPharma Finder. First, import the protein sequence in fasta format, including all chains of the protein into the protein sequence manager, then select peptide mapping analysis tab. Browse in all the raw files and define the experiment name, the file conditions and search parameters. There are two major steps involved once the data analysis process started, peptide detection and HDX modeling. The peptide detection including feature exaction, retention time alignment, gap-fillling and peptide identification. The HDX modeling including deuterium level calculation, back exchange modeling and full HDX modeling. HDXData, TimeCourses, HDXSilulation, ProtectionFactorPlot, Differential Protection Plot csv result files were created for analyzed protein including all the chains, ie, light and heavy chains for mAb. To evaluate the overall protein conformation properties at different conditions, visualized deuterium uptake and protection factor plots can be generated from the csv files.

Figure 4. Herceptin and pH 0.5 stressed sample heavy chain protection factor comparison Top: residual 1-225 Bottom: residual 226-449



The HDX data from Her and stressed Her samples were processed by Biopharma Finder. The heavy chain protection factor plots of Her and stressed Her samples were generated from the BioPharma Finder searched csv file as shown in figure 4. In most of the regions Herceptin showed slightly higher protection factor than the stressed sample. The differences were not uniform along the sequence. From amino acid 110 to 210 and 240 to 255, Herceptin had a much higher protection factor than Herceptin pH stressed sample which would reflect in figure 5 the deuterium uptake profile. One peptide with less deuterium uptake difference, peptide 49-60, and two peptides with more significant deuterium uptake changes , 149-159 and, 244-255, were selected to evaluate the deuterium uptake profiles as shown in figure 5. The deuterium uptake differences range from 0.1 to around 2 Daltons. These results indicated that aggregation would induce more conformational changes in the Fab region and less changes for the Fc region.

Figure 5. Example deuterium uptake plots generated by Biopharma Finder

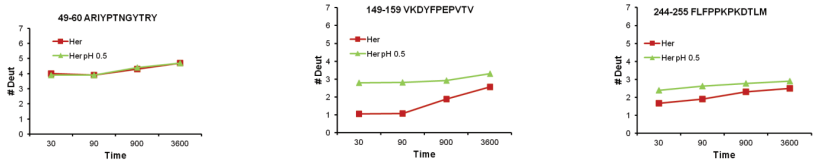


Figure 6. HDExaminer HDX data analysis workflow

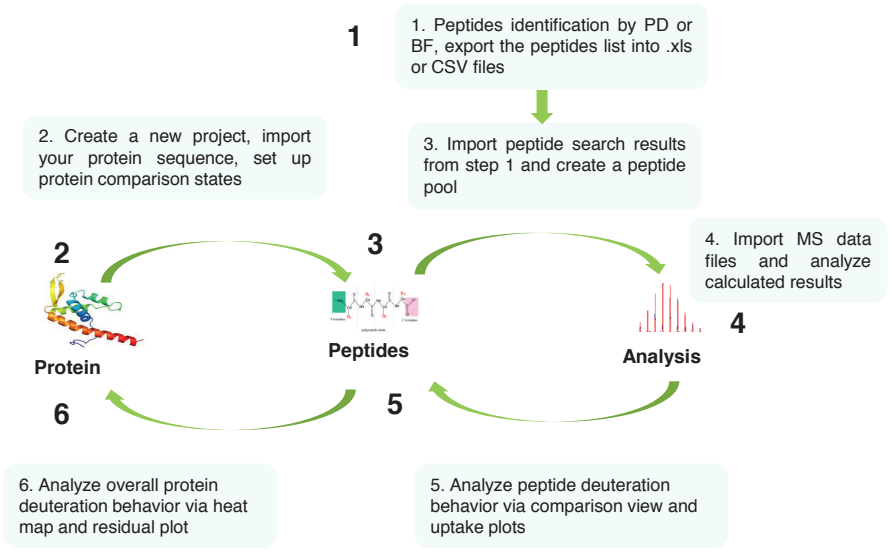


Figure 6 is the HDX data process workflow of HDExaminer. To process the HDX data with HDExaminer, a .xsl or csv file had to be generated by exporting the peptide identification list from the peptide identification search results. Similar to BioPharma Finder, the protein sequence had to be defined first in the protein tab (the software would only process one protein at a time, the different chains of the same protein would be treated as different protein and would be processed separately), the .xsl or csv peptide list would be directly imported into a peptide pool for peptide deuterium uptake measurement in the peptides tab. The undeuterated and deuterated raw files at different time points would be loaded in the analysis tab to start the data analysis. The deuterium uptake heat map of the protein, the peptide deuterium uptake plots, different protein states deuterium uptake mirror and residual plots would be created to probe the protein conformation and conformational changes.

The peptide identification list csv file of Her and Her stressed samples from the BioPharma Finder was exported and directly used to process the HDX data by HDExaminer. The heavy chain peptides deuterium uptake residual plots in both %D (top left) and absolute #D (top right) were shown in figure 7. In general, the Herceptin had less deuterium uptake compare to stressed sample and the differences were not uniform. Peptides with more significant change, were identified between amino acides 70 and 100 and this observation was consistent with the BioPharma Finder protection factor prediction shown in figure 4. Figure 7 a), b), c) were the deuterium uptake plots from the same three peptides shown in figure 5. The deuterium uptake of the three selected peptides was very similar to the plots shown in figure 5.

Figure 7. Herceptin and pH 0.5 stressed sample heavy chain deuterium uptake residual plots, Top left: %D Top right: #D Bottom: peptide deuterium uptake plots a), b), c)

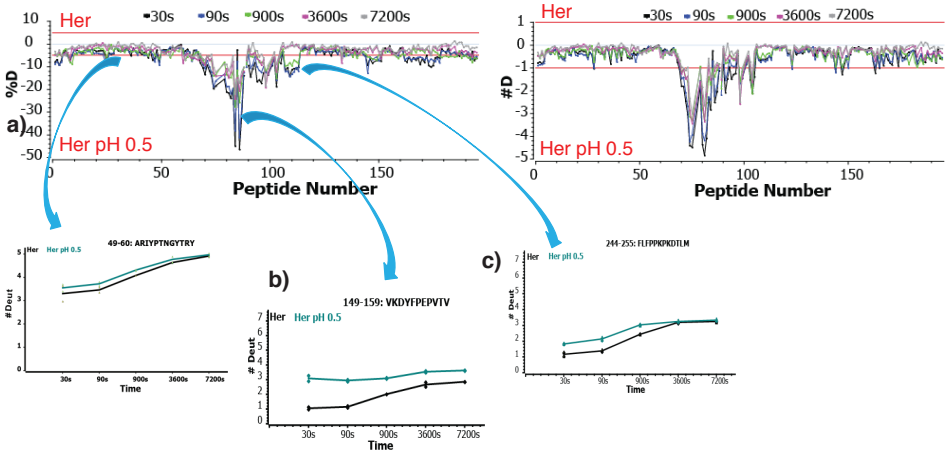


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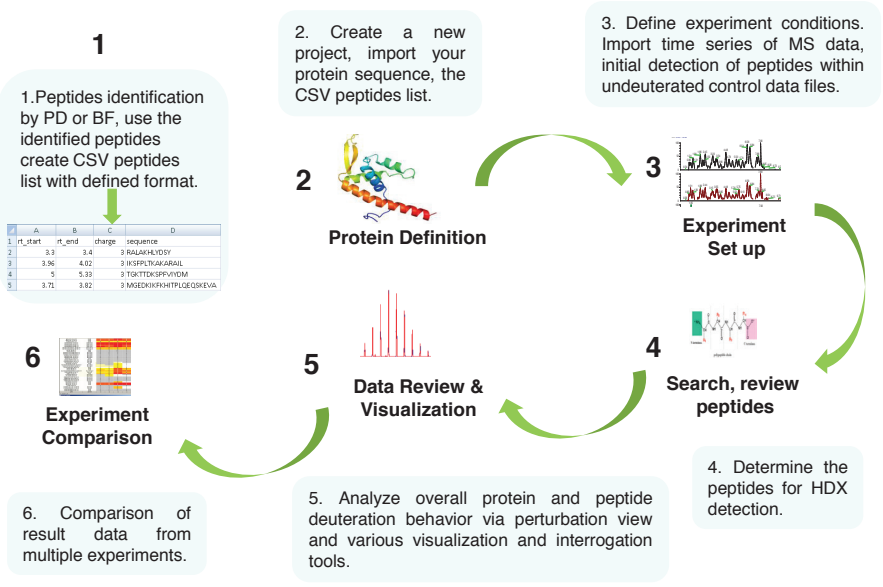
Figure 8. HDX WorkBench data analysis workflow



HDX WorkBench from Scripps is an integrated software platform for analysis of HDX mass spectrometry data. Figure 8 was the HDX WorkBench HDX data analysis workflow. The first step was to build a specific format csv peptide list based on the peptide identification results (the list from BioPharma Finder) as indicated in step 1. The next step was to define the protein and to import the csv peptides list . The protein could be edited to add secondary structure features. The experimental conditions and labeling time series of the identified peptide in the undeuterated sample would be defined in the experimental set up. The different chains of the protein, heavy and light chain of mAb would be treated as two proteins under the same experiment. Only the identified peptides from the undeuterated sample would be used to start the HDX data analysis.The software would create a very comprehensive results dashboard including: peptide list, chromatogram, peptide deuterium update plot, peptide spectra, and time points statistics as shown in figure 9.

A csv file with the specific format was generated by using the BioPharma Finder search results and used in HDX WorkBench to process the data from the Her and Her stressed samples. Similar results were obtained with HDX Workbench and the two previous software packages. The same three peptide deuterium uptake plots were shown in figure 10. Figure 11 was the single residual consolidated perturbation sequence coverage of the heavy chain.

Figure 9. HDX WorkBench Results Dashboard

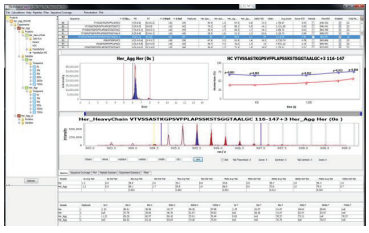


Figure 10. Her and Her pH 0.5 stressed samples heavy chain peptides deuterium uptake plots

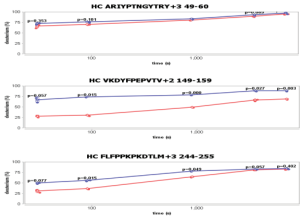
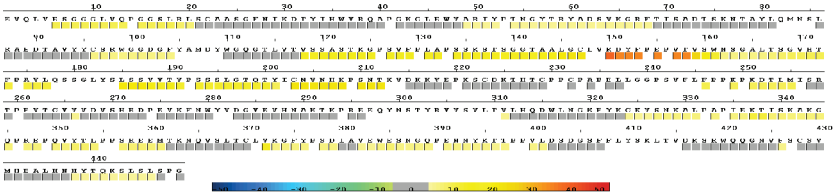


Figure 11. Herceptin and pH 0.5 stressed sample heavy chain consolidated perturbation sequence coverage



CONCLUSIONS

•The HDX data from Her and Her pH stressed samples were successfully processed by all three software packages and the results were consistent.
•The BioPharma Finder is a comprehensive software package that can perform both peptide identification and HDX data analysis. HDX model simulates the H/D exchange and back exchange process at the single residue level. The protection factor plot can be used to evaluate the whole protein conformational property.
•HDExaminer can directly import the .xsl or csv files from a peptides identification results. The HDX data process is straight forward and results are easy to implement. The peptides deuterium uptake residual plots can be used to probe the protein deuterium uptake behavior.
•HDX WorkBench generates a very comprehensive results dashboard. It can yield a consolidated amino acid level perturbation sequence coverage map.

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PN64890-EN 11/16S

A Complete Workflow Solution for Monoclonal Antibody Glycoform Characterization Combining a Novel Glycan Column Technology and Bench-Top Orbitrap LC-MS/MS

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Overview

Purpose: To develop a complete workflow solution for monoclonal antibody (mAb) glycoform characterization using a unique glycan column technology and a Thermo Scientific™ bench-top Orbitrap™ LC-MS/MS.

Methods: Glycans are separated using a recently developed high-performance HPLC/UHPLC column, a Thermo Scientific™ GlycanPac™ AXH-1 column. A data-dependent high-energy collision dissociation (HCD) method was performed in negative ion mode to analyze the glycans.

Results: The GlycanPac AXH-1 column separates glycans with unique selectivity based on charge, size and polarity. A complete workflow solution was developed for glycan profiling combining the unique column technology and a bench-top Orbitrap LC-MS/MS (Figure 1). This workflow was applied to antibody glycoform characterization. Confident identification and structural confirmation were achieved for released glycans from a standard glycoprotein and a monoclonal antibody.

Introduction

Because glycosylation is critical to the efficacy of antibody therapeutics, the FDA requires that a consistent human-type glycosylation be maintained for recombinant monoclonal antibodies (mAb), irrespective of the system in which they are produced. The complex branching and isomeric nature of glycans pose significant analytical challenges for their identification and characterization. Liquid chromatography (LC) coupled to mass spectrometry (MS) has emerged as one of the most powerful tools for the structural characterization of glycans.

The recently developed GlycanPac AXH-1 column is a high-performance HPLC/UHPLC column specifically designed for structural, qualitative and quantitative analysis of glycans. It has a unique selectivity for biologically relevant glycans including glycans from antibodies, either labeled or native and is designed for high-resolution, high-throughput analysis by LC-fluorescence or LC-MS methods. Because glycans are very hydrophilic and polar, hydrophilic interaction liquid chromatography (HILIC) columns based on amide, amine or zwitterionic packing materials are often used for their analysis. HILIC columns separate glycans mainly by hydrogen bonding, resulting in size and composition-based separation. Identification of the glycan charge state is not possible by HILIC. The GlycanPac AXH-1 column overcomes these limitations and can separate glycans based on charge, size and polarity configuration. It provides both greater selectivity and higher resolution. In this study, we characterized N-linked glycans released from a glycoprotein standard and a monoclonal antibody by LC-MS/MS methods using the new column technology and high-resolution Orbitrap mass spectrometry.

Methods

Sample preparation

Native glycans are released from glycoproteins or mAb with PNGase F enzyme. The released glycans are conjugated with 2-amino benzamide (2-AB) label group with reported procedure of Bigge *et. al.*[1]

Liquid chromatography

All the glycans are separated using a recently developed high-performance HPLC/UHPLC column, GlycanPac AXH-1, on a Thermo Scientific™ Dionex™ Ultimate 3000 UHPLC with either s fluorescence or MS detector.
For intact antibody, a Thermo Scientific™ ProSwift RP-10R monolithic column (1 x 50 mm) was used for desalting. LC solvents are 0.1% formic acid in H_2O (Solvent A) and 0.1% formic acid in acetonitrile (Solvent B). Column was heated to 80 °C during analysis. Flow rate was 60 μL/min. After injection of 1 μg mAb, a 15 min gradient was used to elute mAbs from the column (0.0 min, 20%B; 1.0 min, 35%B; 3.0 min, 55%B; 4.0 min, 98%B; 7.0 min, 98% B; 7.1 min, 20%B; 15.0 min, 20%B).

Mass spectrometry

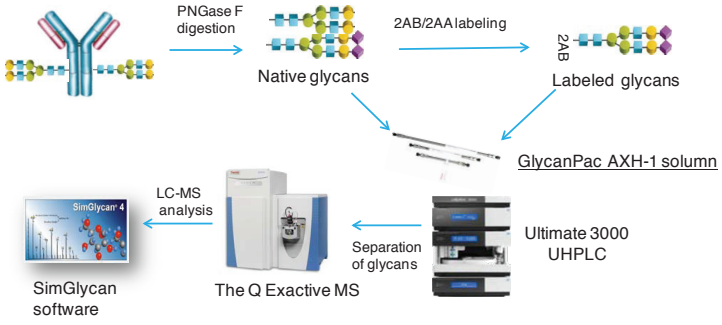
A data-dependent high-energy collision dissociation (HCD) method was performed in negative ion mode to analyze the glycans. The following MS and MS/MS settings were used: MS scan range 380-2000 *m/z.* FT-MS was acquired at 70,000 resolution at *m/z* 200 with AGC target of $1x10^6$ and DDA MS2 acquired at 17,500 resolution at *m/z* 200 with AGC target of $2x10^5$. Intact mAbs were analyzed by ESI-MS for intact molecular mass. The spray voltage was 4kV. Sheath gas flow rate was set at 10. Auxiliary gas flow rate was set at 5. Capillary temperature was 275 °C. S-lens level was set at 55. In-source CID was set at 45 eV. For full MS, resolution was 17,500 for intact mAb. The AGC target was set at $3x10^6$. Maximum IT was set at 250 ms.

Data analysis

SimGlycan® software from PREMIER Biosoft was used for glycan identification and structural elucidation[2]. SimGlycan software accepts raw data files from Thermo Scientific mass spectrometers and elucidates the associated glycan structure by database searching and scoring techniques.

Full MS spectra of mAb were analyzed using Thermo Scientific™ Protein Deconvolution™ 2.0 software. Mass spectra for deconvolution were produced by averaging spectra across the most abundant portion of the elution profile for the mAb. A minimum of at least 8 consecutive charge states from the input *m/z* spectrum were used to produce a deconvoluted peak. To identify glycoforms, the masses were compared to the expected masses of various combinations of commonly found glycoforms

Figure 1. A complete LC-MS/MS workflow solution for monoclonal antibody glycan profiling

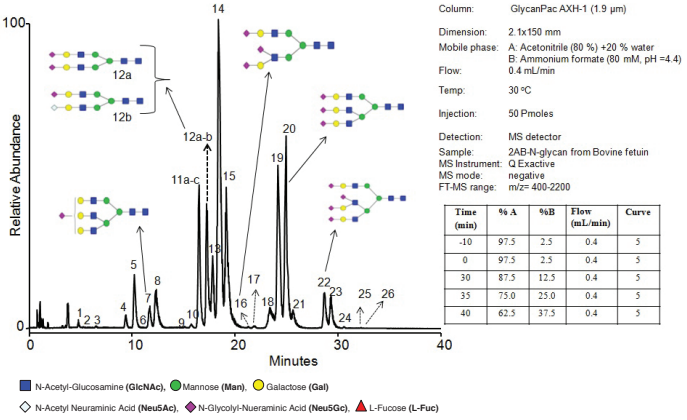


Results

Separation of Glycans Based on Charge, Size and Polarity

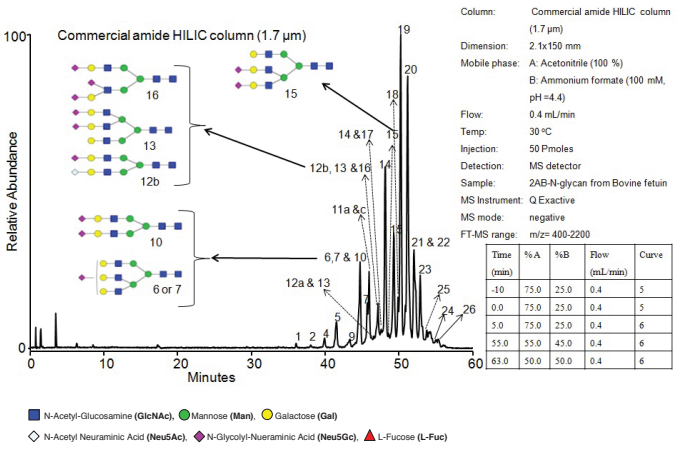
The GlycanPac AXH-1 column can be used for qualitative, quantitative, structural analysis and characterization of uncharged (neutral) and charged glycans present in proteins. The separation and elution of glycans are based on charge; the neutral glycans elute first, followed by the separation of acidic glycans from mono-sialylated, di-sialylated, tri-sialylated, tetra-sialylated and finally penta-sialylated species. Glycans of each charge state are further separated based on their size and polarity. In this study, the structure of glycans present in each peak was determined using high resolution LC-MS/MS. As shown in Figure 2, the detailed structural information obtained from the MS/MS data validated the ability of GlycanPac AXH-1 column to separate labeled N-glycans based on charge, size and polarity. However, co-elution of different charge state glycans is common with other commercially available HILIC column as shown in Figure 3.

Figure 2. LC-MS analysis of 2-AB labeled N-glycans from bovine fetuin by GlycanPac AXH-1 (1.9 *µ*m) column with MS detection.



Time (min)	%A	%B	Flow (mL/min)	Curve
-10	97.5	2.5	0.4	5
0	97.5	2.5	0.4	5
30	87.5	12.5	0.4	5
35	75.0	25.0	0.4	5
40	62.5	37.5	0.4	5

Figure 3. LC-MS analysis of 2-AB labeled N-glycans from bovine fetuin by a commercial amide HILIC column (1.7 μm) with MS detection.



The GlycanPac AXH-1 column is also well suited for high performance LC/MS separation and analysis of native glycans from proteins (data not shown). Analyzing unlabeled glycans not only eliminates the extra reaction step and cumbersome cleanup methods during labeling, but also retains the original glycan profile without adding further ambiguity imposed by the labeling reaction.

Monoclonal antibody (mAb) glycan profiling using GlycanPac AXH-1 column and high resolution LC-MS/MS

Intact mass measurement of a monoclonal antibody identified glycoforms derived from the combination of any two of the three N-glycans, G0F, G1F and G2F. However, the mass errors for some of the intact glycoforms of this antibody ranged from 20-60 ppm (Figure 4A) which is larger than the <10 ppm observed for other samples (data not shown). Furthermore, the intact mass error for the deglycosylated form of this antibody was within 10 ppm (Figure 4B), suggesting that some minor glycosylation forms of this molecule that were not detected at the intact level had interfered with the observed intact mass of the major glycoforms. To further characterize this antibody, released glycans from this protein were separated using the GlycanPac AXH-1column. The separation and elution of glycans from GlycanPac AXH-1 column are based on charge with neutral glycans eluting first, followed by the acidic sialylated species. Glycans of each charge state are further separated based on their size and polarity (**Figure 5**).

Figure 4. Observed molecular mass of glycosylated (A) and deglycosylated (B) forms of a intact monoclonal antibody. Some of the intact antibody major glycoforms have an observed mass error larger than expected. There are also two potentially double fucosylated peaks that need to be confirmed.

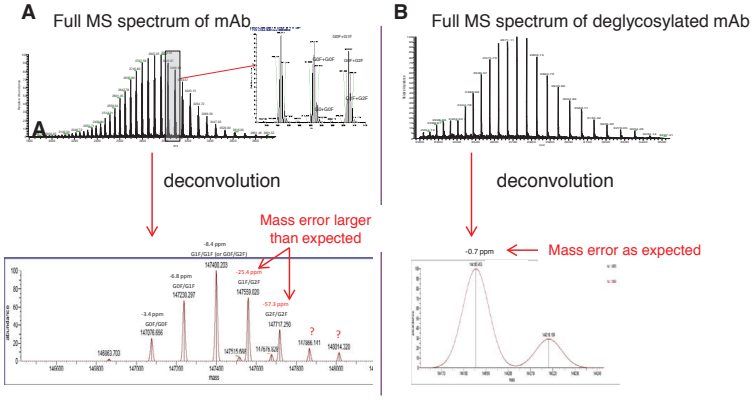
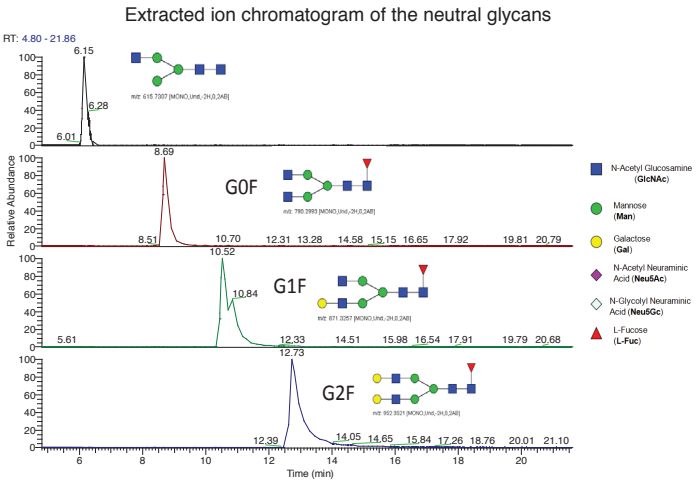
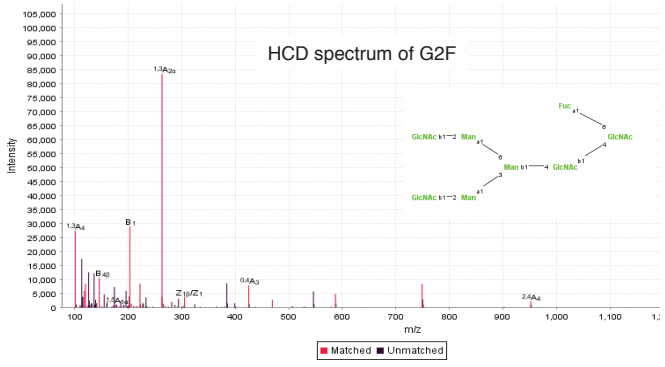


Figure 5. Separation of the major, neutral N-glycans on GlycanPac AXH-1 column



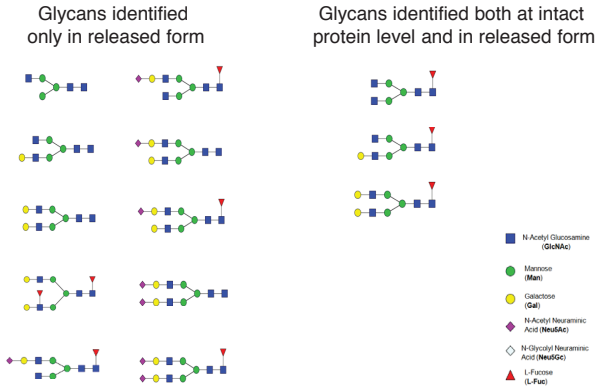
Characterization of glycans in each peak was performed by Full MS and data dependent MS/MS using HCD. The information-rich HCD spectra contain fragment ions that were generated from both cross-ring and glycosidic bond fragmentations (Figure 6). Three different types of glycans were found from this monoclonal antibody, the majority of glycans identified were neutral, including G0F, G1F and G2F which were also the major glycoforms identified at the intact protein level for this antibody (Figure 4A). Also identified were less abundant, non-fucosylated forms of G1 and G2, minor amounts of mono-sialylated and di-sialylated species with and without fucosylation, as well as double fucosylated species that were not identified at the intact protein level (Figure 7).

Figure 6. Identification and structural confirmation of released glycan using high resolution HCD MS/MS



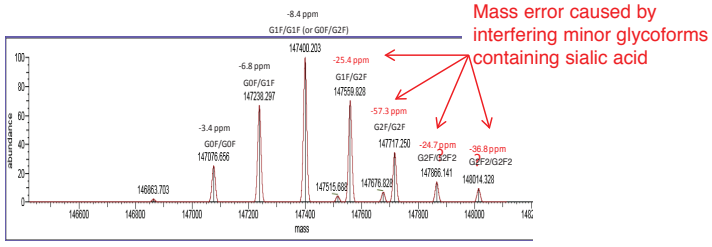
Fragment ion type	Percentage match (%) of theoretical fragments
Single glycosidic	32.14
Glycosidic/glycosidic	30.95
Single cross ring	20.21
Cross ring/glycosidic	14.95

Figure 7. Identified glycans from monoclonal antibody



These results explain that the unexpected mass error observed previously is due to the interfering minor glycoforms that have a molecular mass close to the major ones. In the deconvoluted MS spectrum, the base of the antibody major glycoform peaks covers a mass range of about 40 Da due to the distribution of the unresolved isotopic peaks of a large protein of this size. Therefore any interfering species within 20 Da of mass difference would cause a mass shift of the major glycoform peaks, rather than forming a separate peak. For example, in this case, the replacement of a Fuc and a Gal by Neu5Ac, which would have a mass difference of -17Da, could cause the negative mass shift observed in this study, especially when the interfering species is relatively low in abundance (Figure 8). Results in this study indicate that rapid and sensitive antibody glycan profiling can be achieved using GlycanPac AXH-1 column and HR/AM Orbitrap LC-MS/MS.

Figure 8 Annotated glycoforms of a monoclonal antibody



Conclusion

- GlycanPac AXH-1 column separates glycans with unique selectivity based on charge, size and polarity.
- The GlycanPac AXH-1 columns are compatible with MS instruments. LC-ESI-FTMS or FT-MS/MS analysis of both native and labeled glycans from proteins and antibodies were carried out successfully using GlycanPac AXH-1 columns.
- Confident identification and structural confirmation of glycans can be achieved using high-resolution HCD MS/MS which produces an informative spectrum containing glycosidic and cross ring fragment ions.
- A complete workflow solution was developed for glycan profiling combining the unique GlycanPac AXH-1 column technology and a bench-top Orbitrap LC-MS/MS.
- This workflow was applied to characterize a monoclonal antibody glycoforms. Confident identification and structural confirmation was achieved for released glycans from the monoclonal antibody.

References

1. Bigge, J. C. et al., Non-selective and efficient fluroscent labeling of glycans using 2-amino benzamide and anthranilic acid. Analytical Biochemistry, **1995**, *230*, 229-238.
2. Apte, A and Meitei, N.S., Bioinformatics in glycomics: glycan characterization with mass spectrometric data using SimGlycan, **2010**, *600*, 269-81.

基于nanoLC-MS/MS建立对重组蛋白药物中宿主细胞残留蛋白分析方法

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一、前言

重组蛋白类药物是由遗传修饰的原核或真核宿主细胞培养/发酵产生，在此过程中，宿主细胞也共同产生与正常细胞功能相关的蛋白质，由于细胞凋亡/死亡/裂解，其他非必需蛋白质也可能释放到细胞培养基/发酵液中，因此残留在终产品中的其他蛋白质即为宿主细胞残留蛋白（HCPs）。HCPs的存在主要影响药物的安全性和有效性，如引起机体免疫反应，作为佐剂以增强对药物产品的免疫应答，以及具有蛋白水解活性的HCPs影响药物产品的稳定性[1][2][3]，因此生物医药公司不断优化其纯化工艺，从而控制终产品中HCPs的种类和含量。ICH 的Q6B指导方针指出需采用具有高灵敏度和宽动态范围的方法监测HCPs，要求其含量尽可能的低，甚至低于检测限，不同产品要求不同，一般在1-100ng/mg（1-100ppm）范围内，然而并未规定其检测的具体分析方法。目前Enzyme-Linked Immunosorbent Assay (ELISA)因其具有高灵敏度、高通量和易操作优势成为工业界的金标准，但其存在很多限制，包括不能对HCPs进行精准鉴定，低丰度HCPs的检测易受限于高丰度蛋白的干扰，定量动态范围（3个数量级）较低，抗体受限，开发周期长，无法改变已有的方法，缺少校正标准限制定量的准确性，因此需多种检测方法相结合[4]。随着高分辨质谱技术性能的不断提升，已具有高灵敏度和宽的定量动态范围，以及蛋白质组学技术的飞速发展，可实现对未知蛋白质的高通量定性和定量。但由于HCPs含量极低，且酶切后与高含量的主成分存在共洗脱，对低含量HCPs的鉴定仍存在挑战，为进一步提升质谱检测方法的动态范围，需对样品前处理及液相方法进行优化。

本篇以NIST（National Institute of Standards and Technology）标准品Humanized IgG1 κ Monoclonal Antibody（P/N RM 8671）为例，建立基于nanoLC-MS/MS高灵敏平台的方法对HCPs进行准确定性和相对定量。

二、样品前处理、仪器、实验条件及数据分析

2.1 样品前处理

高通量未知蛋白质的质谱鉴定通常采用bottom-up分析流程，即蛋白质变性、还原（打开二硫键）、烷基化、酶切、LC-MS/MS分析，而对于HCPs鉴定而言，采用此流程时蛋白药物主成分同时被酶切产生高含量的肽段，降低共流出HCPs肽段的鉴定可能性，从而影响此方法的灵敏度和动态范围。2017年礼来制药公司Huang, Lihua, et al.在Analytical Chemistry杂志上发表题为“A Novel Sample Preparation for Shotgun Proteomics Characterization of HCPs in Antibodies”，以NIST抗体标准品为例，在蛋白质非变性的条件下进行酶切，抗体主成分被部分酶切，酶切后经90度高温变性，未被酶切的部分将被沉淀去除，从而提高HCPs的鉴定几率和方法的动态范围，如图1[4]。本篇应用文集在此基础上进一步优化，缩短前处理时间提高工作效率，同时为考察此套流程对1-100ppm的HCPs的鉴定和相对定量能力，加入内标蛋白UPS2（48种蛋白，6个不同浓度）进行定性和相对定量。

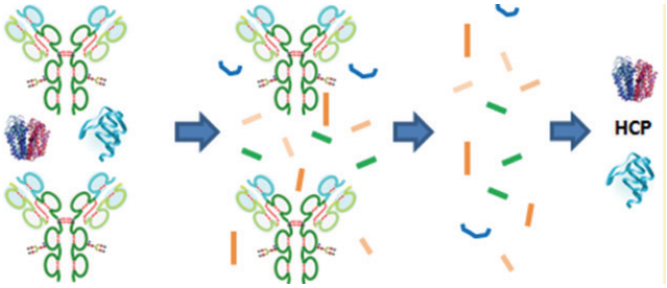


图1. 样品前处理流程示意图（来自Huang, Lihua, et al.）

2.1.1试剂及样品信息

Trizma base（Sigma-Aldrich），浓盐酸（Sigma-Aldrich），二硫苏糖醇（Sigma-Aldrich），Sequencing Grade Modified Trypsin（Promega），质谱级水（ThermoFisher），甲酸（ThermoFisher），乙腈（ThermoFisher），Humanized IgG1 κ Monoclonal Antibody（NIST，P/N RM 8671），UPS2内标蛋白（Sigma-Aldrich）

2.1.2 前处理步骤

1. 取300μg 抗体标准品，50mM Tris-HCl（pH=7.8）将样品稀释成1μg/μL，加入2pmol内标蛋白（UPS2中浓度最高的一组蛋白质总含量为2pmol ）；

2. 加入1.5μg Trypsin （500ng/μL），37°C酶切2h；

3. 加入终浓度5mM DTT，90°C变性10min；

4. 14,000g 离心20min，取上清；

5. 真空浓缩仪进行样品旋干；

6. 18μL 0.1%FA-H_2O复溶；

7. 每针进样5μL；

2.2 仪器、色谱柱、数据分析软件

目前根据液相色谱的流速可分为纳升液相色谱（nanoLC, <1μL/min）、毛细管液相色谱（capillaryLC,1-10μL/min）、微流速液相色谱（microLC,10-100μL/min）、分析流速液相色谱（LC, >100μL/min），由于样品单位浓度和离子化效率依次下降，因此其灵敏度逐渐下降，本次实验采用灵敏度最高的纳升液相系统。

纳升液相色谱仪：EASY nLC 1200

高分辨质谱仪：Q Exactive Plus

色谱柱：Thermo Scientific™ Acclaim™ PepMap™ 100 C18（P/N 164941）；Trap column （P/N164946），柱温45°C

数据分析软件：Thermo Proteome Discoverer2.2

2.2.1 色谱条件

流动相A: 0.1%FA-H2O 流动相 B: 0.1%FA- 80%ACN-20%H2O

流速：300nL/min

梯度：见下表

time	A(%)	B(%)
0	97	3
8	92	8
106	70	30
115	10	90
120	10	90

2.2.2 质谱条件

扫描方式：数据依赖型扫描方法（Data Dependent Acquisition）

一级扫描参数：分辨率 70K，AGC target 3e6， 扫描范围 m/z 300-1700

二级扫描参数：分辨率 35K，AGC target 1e5，Top15，First Mass: 120 m/z，四极杆隔离窗口1.8 m/z， 碎裂能量NCE 28

2.2.3软件参数设置

蛋白质数据库：Swiss-Prot Mus musculus，UPS2理论序列，NIST抗体标准品序列，污染蛋白库

酶切方式：Trypsin全酶切； 最大漏切个数：2个

一级质量偏差：10ppm 二级质量偏差：0.02Da

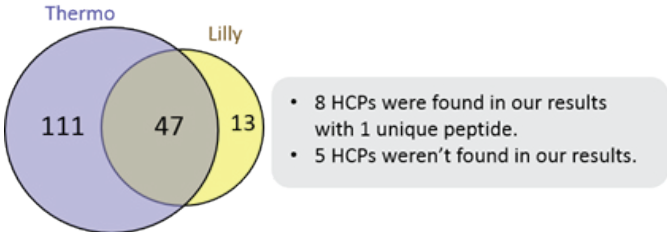
可变修饰：氧化（M），蛋白N端乙酰化

FDR控制：PSM FDR<1%，Peptide FDR<1%，Protein FDR<1%

三、实验结果与讨论

3.1 定性结果

连续进样3针，排除污染蛋白和空白对照中存在的蛋白质，且筛选特异性肽段大于等于2的蛋白质，共鉴定HCPs 有158种，具体蛋白列表见链接。Huang, Lihua,et al.文章鉴定到60种HCPs，我们的数据覆盖其中47种，其余13种鉴定到8种（特异性肽段等于1），5种未鉴定，如图2。这5种的相对含量除了一种近10ppm，其余均近1ppm，表明LC-MS/MS方法在不同平台之间的高度重现性和可靠性。



	Accesion	Description	Unique Pep	Inj.no.1(ppm)	Inj.no.2(ppm)	Inj.no.3(ppm)
5种HCPs在本次实验中未被鉴定到	Q99KN9	clathrin interactor 1	4	10	12	11
	P11680	properdin	3	1	2	1
	P34902	cytokine receptor common subunit gamma	2	1	1	<0.5
	P53996	cellular nucleic acid-binding protein	2	1	<0.5	1
	P19157	glutathione S-transferase P 1	2	<0.5	<0.5	<0.5
8种HCPs在本次实验中鉴定到1个Unique Peptide	P09041	phosphoglycerate kinase 2	2	1	1	1
	P99029	peroxiredoxin-5, mitochondrial	4	1	1	1
	P26928	hepatocyte growth factor-like protein	5	14	11	16
	P40124	adenylyl cyclase-associated protein 1	4	0.8	0.8	0.8
	P45878	peptidyl-prolyl cis-trans isomerase FKBP2	3	2	3	2
	Q8BND5	sulfhydryl oxidase 1	3	2	2	2
	P35700	peroxiredoxin-1	3	1	1	1
	P18242	cathepsin D	2	1	1	1

图2.与Lilly结果进行比较（Huang, Lihua, et al.）

UPS2中含有48种内标蛋白6个不同浓度，共鉴定其中24种，其实际含量结果如表1，含量范围为0.05-444.88ppm，实际含量>1ng（10ppm）的16种内标蛋白全部鉴定到，实际含量在0.06-0.38ng（0.6-3.8ppm）范围内共鉴定到6种，此方案的灵敏度足以满足生物药领域客户的需求。

表1.本次实验中鉴定到UPS2内标蛋白的种类及其实际含量

Accession	Amount(pmol)	MW (kDa)	Actual weight(ng)	Ratio(ppm)
P00918ups	0.67	29.2	19.564	195.64
P41159ups	0.67	16.1	10.787	107.87
P69905ups	0.67	15.1	10.117	101.17
P00915ups	0.67	28.7	19.229	192.29
P68871ups	0.67	15.9	10.653	106.53
P62988ups	0.67	10.7	7.169	71.69
P02768ups	0.67	66.4	44.488	444.88
P02768ups	0.67	8.3	5.561	55.61
P15559ups	0.067	30.8	2.0636	20.636
P15559ups	0.067	22	1.474	14.74
P63165ups	0.067	38.8	2.5996	25.996
P00167ups	0.067	16.1	1.0787	10.787
P62937ups	0.067	20.2	1.3534	13.534
P01133ups	0.067	6.35	0.42545	4.2545
P01133ups	0.067	59.6	3.9932	39.932
P02144ups	0.067	17	1.139	11.39
P12081ups	0.0067	58.2	0.38994	3.8994
P16083ups	0.0067	25.8	0.17286	1.7286
P06732ups	0.0067	43.1	0.28877	2.8877
P61626ups	0.0067	14.7	0.09849	0.9849
P02753ups	0.0067	20.6	0.13802	1.3802
P63279ups	0.0067	18	0.1206	1.206
P01008ups	0.00067	49	0.03283	0.3283
P02787ups	0.000067	75.1	0.0050317	0.050317

3.2蛋白相对定量结果

目前蛋白质的质谱相对定量计算方法有多种，如iBAQ、TOP3 peptides[5]法，可对不同条件下同一蛋白质进行准确的相对定量。由于不同肽段离子化效率、受共流出肽段干扰程度不同，因此准确的绝对定量需合成同位素标记内标肽段。本次实验进行HCPs的相对定量，采用蛋白TOP3的特异性肽段峰面积的平均值算法，得出每种蛋白的峰面积，经过三次技术重复，对特异性肽段大于等于1的蛋白峰面积进行统计，平均峰面积分布如图3A，峰面积大于1e9的蛋白为此样品的主抗体，可看出本实验定量范围达6个数量级。UPS2内标蛋白的峰面积与绝对含量的分布如图3B，峰面积与绝对含量存在一定的线性关系，HCPs的相对含量可根据其峰面积进行初步判断，进而优化纯化工艺。

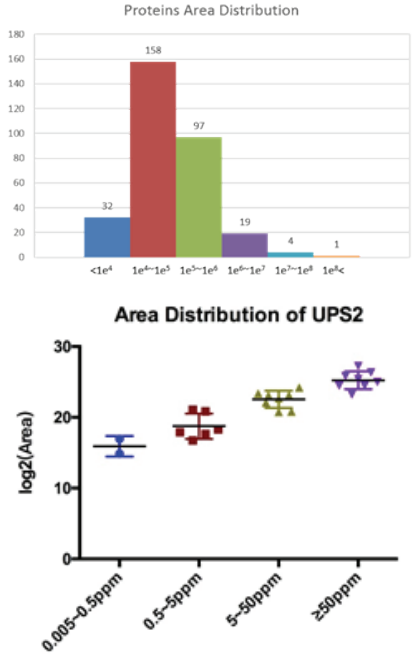


图3A.特异性肽段大于等于1的蛋白峰面积分布图；图3B.　内标蛋白UPS2的峰面积（log2）与其绝对含量的分布图

讨论

本次实验基于优化的样品前处理、纳升液相系统与Orbitrap高分辨质谱，建立了迄今为止最高灵敏度的质谱HCPs检测方法，并利用内标蛋白进行准确的相对定量。由于我们没有查到NIST抗体标准品ELISA定量结果，所以未进行进一步对比质谱与ELISA的一致性。接下来将利用此方法对商品化的抗体药物进行HCPs的定性和相对定量，以及考察方法的重现性。

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