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抗体类药物非聚糖翻译后修饰的LC-MS表征分析

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摘　要：抗体类药物往往存在复杂多样的翻译后修饰 (post-translational modifications, PTMs)，由此产生高度的异质性。PTMs 表征是抗体类药物研发的重要组成部分。尤其在早期研发阶段，高质量的结构表征可以为药物筛选、药物发现、工艺开发和优化提供指引和依据。基于液相色谱 - 质谱联用 (LC-MS) 技术的表征分析手段可快速、准确地识别 PTMs，已成为抗体类药物 PTMs 分析及结构表征的有力工具。该文综述了抗体类药物非聚糖 PTMs 的鉴定成果，内容包括修饰类型、修饰位点、修饰所在区域、表达系统信息及潜在影响，并对 LC-MS 表征分析策略进行了一定的探讨，希望为抗体类药物早期研发阶段的表征分析提供参考。综述的非聚糖 PTMs 均通过 LC-MS 或 LC-MS/MS 技术得到了鉴定。

关键词：抗体类药物；LC-MS；表征；翻译后修饰
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LC-MS characterization of non-glycan PTMs of antibody-based drugs

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Abstract: Antibody-based drugs often show high heterogeneity due to complex and diverse post-translational modifications (PTMs). As an important support for drug development, characterization of PTMs can provide guidance and reference for drug screening, drug discovery, process development and optimization. LC-MS has become a powerful tool for identification and characterization of PTMs, due to the advantages of rapidness and high accuracy. Here we provide an overview of identification results of non-glycan PTMs of antibody-based drugs, including modification types, modified sites, located regions, expression systems and the resultant potential effects. Also, LC-MS based analytical strategies for characterization are discussed. We hope this paper can provide references for characterization of antibody-based drugs in the early development. PTMs reviewed in this paper were all identified by LC-MS or LC-MS/MS.

Key words: antibody-based drugs; LC-MS; characterization; post-translational modifications

抗体类药物已成为新疗法中的代表药物，能有效治疗癌症、自身免疫性疾病、神经系统疾病及传染病 [1]，为患者提供了许多新的药物治疗选择。抗体类药物研发也成为目前生物医药行业发展最快的领域之一 [2]。

抗体类药物在研制阶段往往会产生多种不同形式的异质体，其中，电荷异质体主要由翻译后修饰 (post-translational modifications, PTMs) 产生 [3-5]。PTMs 会对药物的稳定性、疗效和安全性产生影响 [1]，其类型和水平是评价抗体类药物质量属性时需要关注的重点。在早期研发阶段，需要对异质体进行快速准确的表征分析，评估候选药物的质量属性，为药物筛选、工艺开发提供依据。

传统的抗体药物异质体 (相关物质和相关杂质) 表征主要包括分子大小变异体表征、电荷变异体表

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征及聚糖 (糖基化) 修饰表征。分子大小变异体的表征方法有聚丙烯酰胺凝胶电泳 (sodium dodecyl sulfate-polyacrylamide gel electrophoresis, SDS-PAGE) [6]、毛细管电泳 (capillary electrophoresis-sodium dodecyl sulfate, CE-SDS) [6-7] 及体积排阻色谱 (size exclusion chromatography, SEC) [8]。其中，SDS-PAGE 既可用于纯度测定，也可通过使用蛋白质 Marker 测定抗体类药物的分子量，但准确度较低 [6, 9]；CE-SDS 和 SEC 是测定抗体类药物纯度的常用方法，也用于单体、聚体及片段的定量分析 [10-12]。电荷异质体的表征方法有平板等电聚焦 (isoelectric focusing, IEF) [13]、毛细管等电聚焦 (capillary isoelectric focusing, CIEF) [14]、全柱成像毛细管等电聚焦 (imaged CIEF, iCIEF) [15]、毛细管区带电泳 (capillary zone electrophoresis, CZE) [16]、阳离子交换色谱 (cation exchange chromatography, CEC) [17] 及阴离子交换色谱 (anion exchange chromatography, AEC) [18]。其中，IEF、CIEF 与 iCIEF 是基于等电点不同的分离技术，也用于测定等电点；CZE 是基于质量与电荷比值差异的分离技术；CEC 与 AEC 是基于电荷差异及电荷异质体与色谱柱介质之间作用力差异的分离技术。电荷异质性的表征方法均是基于电荷差异分离出主峰、酸性峰 (酸性变体) 和碱性峰 (碱性变体)。电荷异质体主要由 PTMs 产生，不同的 PTMs 会形成不同的电荷异质体，例如，脱酰胺会形成酸性变体，C- 端赖氨酸未完全切除可形成碱性变体 [19]，通过分离出酸性峰和碱性峰对 PTMs 进行表征分析。此外，反相高效液相色谱 (reverse phase liquid chromatography, RPLC) 也用于抗体类药物的纯度分析及杂质分析 [20]。聚糖修饰表征常用的技术是亲水作用色谱 (hydrophilic interaction chromatography, HILIC) [21]。

传统的异质体表征方法以液相色谱和电泳分离技术为主，无法获得各异质体的准确分子量。质谱技术能够精确地测定药物分子的质量，实现抗体类药物异质体的识别和鉴定。与传统表征技术相比，基于质谱的表征技术具有高分辨率、高通量、高精度的绝对优势，已成为药物研发不可缺少的分析手段，是抗体类药物结构表征及 PTMs 分析最有力的工具 [22-23]。尤其是液相色谱 - 质谱联用 (LC-MS) 技术，可在完整抗体、脱糖抗体、亚基及脱糖亚基水平上对异质体进行全面系统的表征，通过高分辨率质谱准确测定质量偏移值来识别、分析 PTMs。此法简便、准确、直接、快速，10 min 即可完成一个样品的测定 [24]。LC/MS/MS 肽图分析虽然能够在氨基酸残基水平上精确地鉴定 PTMs 的类型和修饰位点，也能对 PTMs 进行定量分析 [25]，但会因酶切处理引入修饰 [26]，且肽图数据处理与分析都是非常耗时的工作。因此，在早期研发阶段，快速、直接的 LC-MS 表征分析尤为实用，通过测定分子量，检测分子量是否符合设计，对分子大小变体和 PTMs 进行表征分析。而由于聚糖修饰的复杂性，通常使用糖苷酶 PNGase-F 脱去 N- 糖 [24, 27]，再进行脱糖抗体和经还原处理得到的脱糖亚基的分子量测定 [24]，由此得到的解卷积质谱图更易于分子大小变体及非聚糖 PTMs 的表征分析。如果抗体类药物含有复杂多样的非聚糖 PTMs，LC-MS 数据的解析难度会随之增大。因此，不断更新抗体类药物的 PTMs 信息，有利于更有效地使用质谱分析软件对 LC-MS 数据进行解析，提高表征分析的质量。

本文综述了抗体类药物非聚糖 PTMs 的质谱鉴定成果，涉及到的抗体类药物包括 IgG 单抗、双特异性抗体、融合蛋白及抗体偶联 (antibody-drug conjugates, ADC) 药物，并对表征分析策略进行了一定的探讨，希望为抗体类药物早期研发阶段的表征分析提供参考。

1 抗体类药物非聚糖PTMs

目前已知的蛋白质 PTMs 有 400 多种 [22]。随着表征分析技术的不断发展，越来越多的新型 PTMs 被发现 [28]，质谱分析软件也在不断的升级，为飞速发展的抗体类药物研发提供支撑。已报道的由 LC-MS 及 LC-MS/MS 确证的抗体类药物非聚糖 PTMs 主要包括末端修饰、脱酰胺 (deamidation)、氧化 (oxidation)、羟基化 (hydroxylation)、糖化 (glycation)、硫酸化 (sulfation)、磷酸化 (phosphorylation) 及半胱氨酸相关修饰。

1.1 脱酰胺修饰

脱酰胺修饰是抗体类药物常见的修饰，天冬酰胺和谷氨酰胺都可以发生脱酰胺修饰。相关研究结果显示抗体类药物的脱酰胺修饰主要发生在天冬酰胺，产物为天冬氨酸 (+1 Da)、异天冬氨酸 (+1 Da) 或琥珀酰亚胺 (succinimide) 中间体 (−17 Da) [29-30]。需要注意的是，现阶段的高分辨率质谱仪测定完整抗体分子量的准确度在 ±2 Da 之内 [26]。因此，在 LC-MS 表征分析抗体类药物时，无法仅依据 +1 Da 的质量偏移就判断可能存在脱酰胺修饰 [31]。

互补决定区 (complementarity determining region, CDR) 的脱酰胺修饰可能导致抗体亲和力与药效的

降低。2009 年，Vlasak 等 [29] 报道，轻链 CDR 区的天冬酰胺发生脱酰胺修饰转变成天冬氨酸和异天冬氨酸时会导致抗体亲和力的降低。同年，Yan 等 [32] 报道，重链 CDR 区的天冬酰胺发生脱酰胺修饰转变成琥珀酰亚胺时，Fab 的亲和力降低了约 50%，药效降低了约 70%。

1.2　氧化与羟基化修饰

抗体类药物最容易发生的氧化修饰是甲硫氨酸的氧化，产生 +16 Da 的质量偏移 [33-35]；此外，色氨酸也会发生氧化修饰，经不同的氧化途径，可生成犬尿氨酸 (+4 Da)、羟基色氨酸 (+16 Da)、羟基吲哚丙氨酸 (+16 Da)、二羟基吲哚丙氨酸 (+32 Da) 及 N-甲酰犬尿氨酸 (+32 Da) [34, 36]。

研究表明，Fc 区域的甲硫氨酸氧化修饰对抗体亲和力没有影响 [37]，但会导致 CH2 结构域的构象改变 [38]，与蛋白 A、蛋白 G 及 FcRn 的亲和力也会降低 [39-40]。2011 年，Wang 等 [41] 报道，当 FcRn 结合位点周围存在高丰度的氧化修饰时，Fc 对 FcRn 的亲和力会明显降低，从而导致半衰期的缩短。CDR 区的氧化修饰具有潜在降低药效的作用。2007 年，Wei 等 [42] 报道，重链 CDR 区的色氨酸氧化修饰可导致抗体亲和力和药效的显著降低，也会导致热稳定性的降低。2016 年，Dashivets 等 [43] 发现重链 CDR 区的色氨酸氧化修饰还会导致聚集倾向的增加。

羟基化修饰产生与氧化修饰相等的质量偏移，即 +16 Da。已报道的羟基化修饰发生在天冬氨酸 [44-45]、赖氨酸 [46] 和脯氨酸 [47-48]。天冬氨酸的羟基化修饰多见于凝血因子中 [49]，特定结构域中的天冬氨酸羟基化是其与 Ca^{2+} 结合并保持活性所必需的 [50-51]。根据相关报道，羟基脯氨酸不太可能影响免疫原性 [48]，但是修饰产生的羟基可能导致非期望氢键的形成，具有潜在的导致药物活性改变的可能性 [48, 52]。赖氨酸的羟基化是胶原蛋白中常见的修饰，2012 年，Sricholpech 等 [53] 报道，羟基赖氨酸既可参与交联反应，也可发生 O- 聚糖修饰。此修饰在哺乳动物细胞表达的治疗性蛋白中也有报道，例如，Molony 等 [54] 在 CHO 细胞表达的 CD4 受体及 CD4-IgG 中均发现了羟基赖氨酸，为非期望 PTMs，同时发现治疗性蛋白中的赖氨酸羟基化修饰与胶原蛋白中的赖氨酸羟基化修饰具有同样的共有序列 XKG [46]。

1.3　赖氨酸糖化与丝氨酸岩藻糖化修饰

不同于天冬酰胺、丝氨酸、苏氨酸上复杂的聚糖修饰，赖氨酸会与环境中的葡萄糖反应发生糖化修饰 (glycation)，产生 +162 Da 的质量偏移，赖氨酸糖化是抗体类药物常见的修饰 [55]。丝氨酸可发生岩藻糖化修饰 (O-fucosylation)，产生 +146 Da 的质量偏移 [56]。

研究表明，赖氨酸的糖化修饰一般会增加抗体药物的聚集倾向 [57-58]，并具有潜在封闭生物学功能位点的作用，赖氨酸糖化修饰对生物活性的影响取决于修饰的位置 [59-61]。丝氨酸的岩藻糖化修饰主要存在于表皮生长因子重复序列和血小板反应蛋白 1 重复序列中，表皮生长因子重复序列丝氨酸岩藻糖化的功能是调节癌症相关的 Nortch 信号通路，血小板反应蛋白 1 重复序列中的丝氨酸岩藻糖化可影响蛋白质的分泌 [62-63]。由于尚不明确丝氨酸岩藻糖化修饰对抗体类药物安全性和有效性的影响，早期研发阶段倾向于选择没有此修饰的候选药物 [64]。

1.4　半胱氨酸相关修饰

未参与二硫键形成的自由巯基可发生半胱氨酸化修饰 (cysteinylation)，产生 +119 Da 的质量偏移 [65-69]，也可发生谷胱甘肽化修饰 (glutathionylation)，产生 +305 Da 的质量偏移 [65-66, 69]。此外，还可发生 β- 消除反应 (β-elimination)，使半胱氨酸转变成脱氢丙氨酸 (-34 Da) [66, 70]。

半胱氨酸发生半胱氨酸化或谷胱甘肽化修饰后不能形成链内二硫键，筛选药物时一般尽可能避免选择含有半胱氨酸相关修饰的候选药物，以防引入未知的风险。2016 年，McSherry 等 [71] 报道，CDR 区的半胱氨酸化修饰会干扰抗体与抗原结合，从而导致药物失效。另外，未配对的半胱氨酸残基可导致二硫键错配或形成分子间二硫键，继而产生低生物活性的单体变体、二聚体或聚集体。

1.5　硫酸化与磷酸化修饰

硫酸化修饰和磷酸化修饰都会产生 +80 Da 的质量偏移，两种修饰产生的质量偏移精确差值只有 9.6 mDa [72]，可以通过高分辨率质谱的准确质量测定及同位素相对丰度分析，鉴定出是磷酸化修饰还是硫酸化修饰 [47]。丝氨酸、苏氨酸和酪氨酸都可以发生硫酸化和磷酸化修饰 [73]，研究显示，抗体类药物最常见的硫酸化修饰发生在酪氨酸 [44-45, 47, 74]，常见的磷酸化修饰发生在丝氨酸 [44-45, 75]，且 CHO 细胞与 HEK 细胞表达系统中产生的磷酸化修饰水平存在显著差异 [75]。

研究发现，硫酸化修饰会使抗体类药物的亲和力增强 [74]，磷酸化修饰则通过变构调节影响抗体类

药物的活性[75-76]，早期研发阶段需确定磷酸化修饰是否为期望修饰，其对药物的影响需要进一步的评价和研究。

1.6 N-端与C-端修饰

焦谷氨酸 (pyroGlu) 的形成是抗体类药物重链和轻链最常见的 N- 端修饰，可由谷氨酰胺脱氨环化形成 (−17 Da)[77-78]，也可由谷氨酸脱水环化形成 (−18 Da)[79-80]。抗体类药物 C- 端修饰主要是 C- 端特定氨基酸的切除 (clipping)，最常见的是重链 C- 端赖氨酸的切除 (−128 Da)[78, 81-82]，其次是重链 C- 端甘氨酸切除 (−57 Da)[83] 及 C- 端脯氨酸酰胺化 (−1 Da)[83-84]。2019 年，Jin 等[83] 报道，当重链 C- 端序列为 XPG 时，重链 C- 端的甘氨酸可发生切除修饰，随即末端的脯氨酸可发生酰胺化修饰，C- 端的这 2 种修饰产生 −58 Da 的质量偏移。

研究表明，N- 端的焦谷氨酸修饰、C- 端的赖氨酸切除与脯氨酸酰胺化修饰不会对药效和安全性产生实质性的影响，只会增加电荷异质性[84-87]。

1.7 其他修饰

表 1 总结了上述已报道的抗体类药物常见非聚糖 PTMs 的类型、修饰位点、修饰所在区域及表达系统信息。除了上述修饰，抗体肽链断裂水解会产生 +18 Da 的质量偏移[82]。而 ADC 药物还可能发生连接子开环水解反应，如 2021 年 Yang 等[88] 报道，

表1 抗体类药物的非聚糖PTMs

位点	质量偏移/Da	修饰类型	修饰区域	样品类型	表达系统	文献
Asn	−17	Succinimide	CDR	IgG	CHO	[32]
	+1	Deamidation	CDR	IgG	−	[29-30]
Asp	+16	β-Hydroxylation	Factor IX	Clotting factor IX-Fc fusion	HEK	[45]
Cys	−34	β-elimination	C-terminal of Lc	IgG	−	[66]
	+119	Cysteinylation	Lc	IgG	−	[65-66]
			Hc	IgG	*E.coli*	[67]
			CDR	IgG	CHO	[68]
			scFv	scFv-IgG BsAb	CHO	[69]
	+305	Glutathionylation	Lc	IgG	−	[65-66]
			scFv	scFv-IgG BsAb	CHO	[69]
Gln	−17	Gln→pyroGlu	N-terminal	IgG	−	[78]
Glu	−18	Glu→pyroGlu	N-terminal	IgG	CHO	[80]
	+44	γ-Carboxylation	Factor IX	Clotting factor IX-Fc fusion	HEK	[45]
Gly	−57	Gly clipping	C-terminal of Hc	IgG	CHO	[83]
Lys	−128	Lys clipping	C-terminal of Hc	IgG	CHO	[82]
	+16	Hydroxylation	XKG on Hc Fab	IgG	CHO	[46]
	+162	Glycation	CDR	IgG	CHO	[55]
Met	+16	Oxidation	Fc	IgG	CHO	[33-34]
			CDR	IgG	CHO	[35]
Pro	−1	Amidation	C-terminal of Hc	IgG	CHO	[83-84]
	+16	Hydroxylation	Ligand domain	BsAb	CHO	[47]
			G4P-linker	Growth factor-Fc fusion	CHO	[48]
Ser	+80	Phosphorylation	Factor IX	Clotting factor IX-Fc fusion	HEK	[45]
			$(G4S)_2$-linker	IgG-based fusion	CHO, HEK	[75]
	+146	O-Fucosylation	CDR	IgG	CHO	[56]
Trp	+4	Trp→Kyn	CDR	IgG	CHO	[34]
	+16	Trp→OH-Trp, Oia	CDR	IgG	CHO	[34]
	+32	Trp→DiOia, NFK	CDR	IgG	CHO	[34]
Tyr	+80	Sulfation	CDR	IgG	CHO	[47, 74]
			Factor IX	Clotting factor IX-Fc fusion	HEK	[45]

Lc：轻链；Hc：重链；scFv：单链抗体(single-chain variable fragment)；BsAb：双特异性抗体(bispecific antibody)；pyroGlu：焦谷氨酸；Kyn：犬尿氨酸(kynurenine)；Oia：羟基吲哚丙氨酸(oxindolylalanine)；DiOia：二羟基吲哚丙氨酸(dioxindolylalanine)；NFK：N-甲酰犬尿氨酸(N-formylkynurenine)；*E.coli*：大肠杆菌(*Escherichia coli*)；CHO：中国仓鼠卵巢细胞(Chinese hamster ovary cells)；HEK：人胚肾细胞(human embryonic kidney cells)。

ADC 药物中的马来酰亚胺结构可发生开环水解，产生 +18 Da 的质量偏移。另外，可能存在与抗体类药物非共价结合的加合物和中性丢失。已报道的加合物有 Na^+ (+23 Da) [89]、K^+ (+39 Da) [89-90] 及 H_3PO_4 (+98 Da) [90]，常见的中性丢失主要是脱羧 (−44 Da) [91] 和脱水 (−18 Da) [83, 92]。

2　讨论

对抗体类药物的药效及安全性产生较大影响的 PTMs 是研发人员关注的重点，尤其是位于 CDR 区的 PTMs。相关研究显示，需重点关注的非聚糖 PTMs 主要包括脱酰胺、甲硫氨酸氧化、色氨酸氧化及赖氨酸糖化。另外，需要关注抗体功能所必需的 PTMs，分析潜在引入非期望 PTMs 的可能性，对影响尚不确定的非期望 PTMs 需要进行进一步的研究和评价。

不同表达系统产生的 PTMs 水平可能会出现显著的差异，与功能关系密切的 PTMs 在不同表达系统中的丰度也是研发人员重点关注的问题。2010 年，Peter 等 [45] 对 HEK-293 细胞表达的凝血因子 IX-Fc 融合蛋白 (clotting factor IX-Fc fusion) 进行了表征分析，并对谷氨酸羧化 (γ-carboxylation) 修饰进行了评估。谷氨酸羧化修饰是凝血因子 IX 的关键 PTMs，相关结构域中的谷氨酸羧化修饰有助于凝血因子 IX 与磷脂膜结合，对其功能至关重要。研究者选择 HEK-293 细胞表达系统的原因在于，HEK-293 细胞能够高水平表达凝血因子 IX-Fc 融合蛋白，且相比于 CHO 细胞，HEK-293 细胞表达系统更利于产生谷氨酸羧化修饰。研究表明，HEK-293 细胞表达凝血因子 IX-Fc 融合蛋白产生的谷氨酸羧化、丝氨酸磷酸化、酪氨酸硫酸化及聚糖修饰水平都与凝血因子 IX 相当。另一方面，需要注意非期望 PTMs。如 2017 年，Tyshchuk 等 [75] 发现融合蛋白中的甘氨酸 - 丝氨酸连接子会发生磷酸化修饰，为非期望 PTMs。HEK 细胞表达与 CHO 细胞表达系统产生的磷酸化水平存在显著差异，相对丰度分别为 11.3% 和 0.4%。在抗体类药物开发中，应仔细评估连接子序列，避免由连接子引入非期望 PTMs。

在药物发现、药物筛选及工艺开发阶段，准确、快速的 PTMs 表征分析可起到早期预警的作用，也为预测 PTMs 序列标识提供依据，助力新药研发。如同 N- 糖基化 (即 N- 聚糖修饰) 位点具有共有序列 NXT 或 NXS (其中 X 为除脯氨酸外的任意氨基酸) [93-94]，其他 PTMs 潜在的共有序列也有待发现。2016 年，Xie 等 [46] 发现赖氨酸羟基化修饰存在一个共有序列 XKG。在早期研发阶段，这样的共有序列可作为标识来预测可能产生的 PTMs，从而最大限度地减少耗时分析，避免因表征不及时造成新药研发的延迟。

LC-MS 表征分析脱糖抗体类药物样品时 , 除了主要的脱 N- 糖处理，根据样品异质性的复杂情况，可考虑脱唾液酸和 N- 糖、脱唾液酸和 O- 糖处理，再进行 LC-MS 测定。对于脱糖不易成功的样品，可以考虑采用非变性质谱法 (native MS) [95] 分析 PTMs。由于质谱技术是测定带电粒子质荷比 (质量与电荷的比值) 的分析技术，聚糖修饰造成的高度电荷异质性会导致分析软件的无效解卷积，非变性质谱可以实现电荷异质体的有效分离 [96-99]，大大降低了高度异质性造成的解析难度，通过质谱检测获得电荷异质体的分子量信息。目前典型的非变性分离技术是 ZipChip 系统 [97-98]，一种基于微流控芯片技术的区带电泳装置，可与 Q-Exactive 系列高分辨率质谱仪实现在线联用，形成微流控毛细管电泳 - 质谱联用 (CE-MS) 系统，灵敏度高，所需样品量可低至 1 ng，可实现抗体类药物的深度表征 [97]，成为 LC-MS 的补充手段。2020 年，Carillo 等 [97] 采用 ZipChip CE-MS 对利妥昔单抗 (rituximab)、曲妥珠单抗 (trastuzumab) 和贝伐单抗 (bevacizumab) 药物进行了表征分析，结果显示，此技术可实现对脱酰胺修饰变异体、N- 聚糖 - 唾液酸修饰变异体、N- 末端修饰变异体及 C- 末端修饰变异体的有效分离和鉴定。此外，研究者们还探索了其他类型的非变性质谱技术。2021 年，Haberger 等 [96] 开发了一种阳离子交换色谱 - 非变性质谱 (native CEC-MS) 技术，对大约 200 kDa 双特异性单抗电荷异质体进行了表征分析，使脱酰胺修饰变异体、Fab 区域同时存在聚糖修饰和唾液酸修饰的变异体与 Fab 区域存在聚糖修饰但不存在唾液酸修饰的变异体得到了有效的分离和鉴定。2022 年，Liu 等 [99] 开发并优化了一种基于盐梯度的阴离子交换色谱 - 非变性质谱 (native AEC-MS) 技术，结果显示，AEC-MS 适用于等电点相对偏低的 IgG4 单抗的电荷异质体表征，不适用于等电点相对偏高的 IgG1 单抗的电荷异质体表征。由于 CEC 不能很好的分离相对偏酸性的单抗，AEC-MS 可作为 CEC-MS 的潜在替补技术。非变性质谱的优点是可以保持抗体类药物的天然结构，这使得其在 ADC 药物的 DAR 值 (药物抗体比值) 分析方面具有明显的优势。2020 年，Jones 等 [100]

采用体积排阻色谱 - 非变性质谱 (native SEC-MS) 测定了半胱氨酸连接的 ADC 药物的 DAR 值，测定结果与疏水作用色谱 (hydrophobic interaction chromatography, HIC) 测定结果一致，具有可比性。此外，CIEF-MS 也是颇具潜力的质谱联用技术，电荷异质体在各自的等电点聚集得到有效的分离，随即进入质谱被检测。但此技术需要解决的问题比较多 [101]，例如，蛋白质在等电点条件下的溶解度最低，容易沉淀析出造成毛细管堵塞，若选择添加增溶剂则需要慎重考虑增溶剂的影响 [102]，维持等电聚焦的 pH 梯度容易在聚焦区带迁移时遭到破坏 [103-105] 等等。此技术还在持续开发和优化中，且已取得了一定的成果 [106-108]。

3 小结

LC-MS 能够快速、精确地测定蛋白质和多肽的相对分子质量。2020 年，Sokolowska 等 [109] 证实了亚基水平的 LC-MS 具备与 LC-MS/MS 肽图分析同等的准确度，可取代肽图监测单克隆抗体 Fc 的氧化水平。在抗体类药物的研发阶段，LC-MS 已被广泛应用于抗体类药物分子大小变体和 PTMs 的表征分析。抗体类药物往往因含有复杂多样的 PTMs 而具有高度的异质性，由此造成 LC-MS 数据解析困难。高质量的表征分析结果既需要有效的 LC-MS 表征分析策略，也需要质谱分析软件的有效使用。

本文对抗体类药物非聚糖 PTMs 的类型、修饰位点及潜在风险和价值进行了综述，并对 LC-MS 表征分析策略、需注意的问题及难点解决方案进行了探讨。基于质量源于设计 (quality by design, QbD) 的理念，通过序列分析可以初步预测抗体类药物可能存在的 PTMs、需重点关注的 PTMs (潜在高风险 PTMs)、功能必需 PTMs、潜在非期望 PTMs 及表达系统影响，有利于根据样品特点和表征目的选择合适的表征分析策略，在质谱数据解析时也可以做到有的放矢，合理设置软件参数，能够更有效地使用质谱分析软件，提高表征分析的质量，为药物研发提供明确的方向。

随着新型抗体类药物开发热潮对表征分析水平的要求越来越高，专用质谱分析软件在不断开发和升级，相关表征难点的解决方案也在不断开发，新技术不断涌现成为 LC-MS 的补充手段，共同促进表征分析越来越准确、快速。

[参 考 文 献]

[1] Wei B, Han G, Tang J, et al. Native hydrophobic interaction chromatography hyphenated to mass spectrometry for characterization of monoclonal antibody minor variants. Anal Chem, 2019, 91: 15360-4

[2] Mullard A. FDA approves 100th monoclonal antibody product. Nat Rev Drug Discov, 2021, 20: 491-5

[3] He X, ElNaggar M, Ostrowski MA, et al. Evaluation of an icIEF-MS system for comparable charge variant analysis of biotherapeutics with rapid peak identification by mass spectrometry. Electrophoresis, 2022, 43: 1215-22

[4] Dai J, Lamp J, Xia Q, et al. Capillary isoelectric focusing-mass spectrometry method for the separation and online characterization of intact monoclonal antibody charge variants. Anal Chem, 2018, 90: 2246-54

[5] Yan Y, Liu AP, Wang S, et al. Ultrasensitive characterization of charge heterogeneity of therapeutic monoclonal antibodies using strong cation exchange chromatography coupled to native mass spectrometry. Anal Chem, 2018, 90: 13013-20

[6] Shi Y, Li Z, Lin J. Advantages of CE-SDS over SDS-PAGE in mAb purity analysis. Anal Methods, 2012, 4: 1637-42

[7] Wagner E, Colas O, Chenu S, et al. Determination of size variants by CE-SDS for approved therapeutic antibodies: key implications of subclasses and light chain specificities. J Pharm Biomed Anal, 2020, 184: 113166

[8] Goyon A, Fekete S, Beck A, et al. Unraveling the mysteries of modern size exclusion chromatography-the way to achieve confident characterization of therapeutic proteins. J Chromatogr B Analyt Technol Biomed Life Sci, 2018, 1092: 368-78

[9] Mahler HC, Friess W, Grauschopf U, et al. Protein aggregation: pathways, induction factors and analysis. J Pharm Sci, 2009, 98: 2909-34

[10] Rustandi RR, Washabaugh MW, Wang Y. Applications of CE SDS gel in development of biopharmaceutical antibody-based products. Electrophoresis, 2008, 29: 3612-20

[11] Lu C, Liu D, Liu H, et al. Characterization of monoclonal antibody size variants containing extra light chains. MAbs, 2013, 5: 102-13

[12] Dada OO, Rao R, Jones N, et al. Comparison of SEC and CE-SDS methods for monitoring hinge fragmentation in IgG1 monoclonal antibodies. J Pharm Biomed Anal, 2017, 145: 91-7

[13] Gangopadhyay A, Petrick AT, Thomas P. Modification of antibody isoelectric point affects biodistribution of 111-indium-labeled antibody. Nucl Med Biol, 1996, 23: 257-61

[14] Suba D, Urbányi Z, Salgó A. Capillary isoelectric focusing method development and validation for investigation of recombinant therapeutic monoclonal antibody. J Pharm Biomed Anal, 2015, 114: 53-61

[15] Goyon A, Excoffier M, Janin-Bussat MC, et al. Determination of isoelectric points and relative charge variants of 23 therapeutic monoclonal antibodies. J Chromatogr B Analyt Technol Biomed Life Sci, 2017, 1065-1066: 119-28

[16] Moritz B, Schnaible V, Kiessig S, et al. Evaluation of

capillary zone electrophoresis for charge heterogeneity testing of monoclonal antibodies. J Chromatogr B Analyt Technol Biomed Life Sci, 2015, 983-984: 101-10

[17] Jing SY, Gou JX, Gao D, et al. Separation of monoclonal antibody charge variants using cation exchange chromatography: resins and separation conditions optimization. Sep Purif Technol, 2020, 235: 116136

[18] Teshima G, Li MX, Danishmand R, et al. Separation of oxidized variants of a monoclonal antibody by anion-exchange. J Chromatogr A, 2011, 1218: 2091-7

[19] Khawli LA, Goswami S, Hutchinson R, et al. Charge variants in IgG1: isolation, characterization, *in vitro* binding properties and pharmacokinetics in rats. MAbs, 2010, 2: 613-24

[20] Gilardoni E, Regazzoni L. Liquid phase separation techniques for the characterization of monoclonal antibodies and bioconjugates. J Chromatogr Open, 2022, 2: 100034

[21] Ikegami T. Hydrophilic interaction chromatography for the analysis of biopharmaceutical drugs and therapeutic peptides: a review based on the separation characteristics of the hydrophilic interaction chromatography phases. J Sep Sci, 2019, 42: 130-213

[22] Zhang Y, Fang C, Bao H, et al. Discover the post-translational modification proteome using mass spectrometry. Chin J Chem, 2021, 39: 550-8

[23] He J, Su D, Ng C, et al. High-resolution accurate-mass mass spectrometry enabling in-depth characterization of *in vivo* biotransformations for intact antibody-drug conjugates. Anal Chem, 2017, 89: 5476-83

[24] 张红梅, 张峰, 武刚, 等. 抗EpCAM+CD3双特异性抗体关键表征解析. 药物分析杂志, 2020, 40: 620-32

[25] Dong Q, Liang Y, Yan X, et al. The NISTmAb tryptic peptide spectral library for monoclonal antibody characterization. MAbs, 2018, 10: 354-69

[26] Bondarenko PV, Second TP, Zabrouskov V, et al. Mass measurement and top-down HPLC/MS analysis of intact monoclonal antibodies on a hybrid linear quadrupole ion trap-orbitrap mass spectrometer. J Am Soc Mass Spectrom, 2009, 20: 1415-24

[27] Wohlschlager T, Scheffler K, Forstenlehner IC, et al. Native mass spectrometry combined with enzymatic dissection unravels glycoform heterogeneity of biopharmaceuticals. Nat Commun, 2018, 9: 1713-21

[28] 李义晓, 蒋瑀涵, 张宏权. 新型蛋白质酰基化修饰与肿瘤的发生发展. 中国细胞生物学学报, 2022, 44: 723-36

[29] Vlasak J, Bussat MC, Wang S, et al. Identification and characterization of asparagine deamidation in the light chain CDR1 of a humanized IgG1 antibody. Anal Biochem, 2009, 392: 145-54

[30] Ouellette D, Chumsae C, Clabbers A, et al. Comparison of the *in vitro* and *in vivo* stability of a succinimide intermediate observed on a therapeutic IgG1 molecule. MAbs, 2013, 5: 432-44

[31] Macht M. Top-down characterization of biopharmaceuticals. Trends Analyt Chem, 2013, 48: 62-71

[32] Yan B, Steen S, Hambly D, et al. Succinimide formation at Asn 55 in the complementarity determining region of a recombinant monoclonal antibody IgG1 heavy chain. J Pharm Sci, 2009, 98: 3509-21

[33] Liu H, Gaza-Bulseco G, Zhou L. Mass spectrometry analysis of photo-induced methionine oxidation of a recombinant human monoclonal antibody. J Am Soc Mass Spectrom, 2009, 20: 525-8

[34] Pavon JA, Xiao L, Li X, et al. Selective tryptophan oxidation of monoclonal antibodies: oxidative stress and modeling prediction. Anal Chem, 2019, 91: 1141-200

[35] An Y, Zhang Y, Mueller HM, et al. A new tool for monoclonal antibody analysis: application of IdeS proteolysis in IgG domain-specific characterization. MAbs, 2014, 6: 879-93

[36] Li Y, Polozova A, Gruia F, et al. Characterization of the degradation products of a color-changed monoclonal antibody: tryptophan-derived chromophores. Anal Chem, 2014, 86: 6850-7

[37] Lam XM, Yang JY, Cleland JL. Antioxidants for prevention of methionine oxidation in recombinant monoclonal antibody HER2. J Pharm Sci, 1997, 86: 1250-5

[38] Liu D, Ren D, Huang H, et al. Structure and stability changes of human IgG1 Fc as a consequence of methionine oxidation. Biochemistry, 2008, 47: 5088-100

[39] Gaza-Bulseco G, Faldu S, Hurkmans K, et al. Effect of methionine oxidation of a recombinant monoclonal antibody on the binding affinity to protein A and protein G. J Chromatogr B Analyt Technol Biomed Life Sci, 2008, 870: 55-62

[40] Pan H, Chen K, Chu L, et al. Methionine oxidation in human IgG2 Fc decreases binding affinities to protein A and FcRn. Protein Sci, 2009, 18: 424-33

[41] Wang W, Vlasak J, Li Y, et al. Impact of methionine oxidation in human IgG1 Fc on serum half-life of monoclonal antibodies. Mol Immunol, 2011, 48: 860-6

[42] Wei Z, Feng J, Lin HY, et al. Identification of a single tryptophan residue as critical for binding activity in a humanized monoclonal antibody against respiratory syncytial virus. Anal Chem, 2007, 79: 2797-805

[43] Dashivets T, Stracke J, Dengl S, et al. Oxidation in the complementarity-determining regions differentially influences the properties of therapeutic antibodies. MAbs, 2016, 8: 1525-35

[44] Duivelshof BL, Murisier A, Camperi J, et al. Therapeutic Fc-fusion proteins: current analytical strategies. J Sep Sci, 2021, 44: 35-62

[45] Peters RT, Low SC, Kamphaus GD, et al. Prolonged activity of factor IX as a monomeric Fc fusion protein. Blood, 2010, 115: 2057-64

[46] Xie Q, Moore B, Beardsley RL. Discovery and characterization of hydroxylysine in recombinant monoclonal antibodies. MAbs, 2016, 8: 371-8

[47] Tyshchuk O, Gstöttner C, Funk D, et al. Characterization and prediction of positional 4-hydroxyproline and sulfotyrosine, two posttranslational modifications that can occur at substantial levels in CHO cells-expressed biotherapeutics. MAbs, 2019, 11: 1219-32

[48] Spahr C, Gunasekaran K, Walker KW, et al. High-resolution mass spectrometry confirms the presence of a hydroxyproline (Hyp) posttranslational modification in the GGGGP linker of an Fc-fusion protein. MAbs, 2017, 9: 812-9
[49] Kumar SR. Industrial production of clotting factors: challenges of expression, and choice of host cells. Biotechnol J, 2015, 10: 995-1004
[50] Stenflo J, Stenberg Y, Muranyi A. Calcium-binding EGF-like modules in coagulation proteinases: function of the calcium ion in module interactions. Biochim Biophys Acta, 2000, 1477: 51-63
[51] Griffin JH, Fernández JA, Gale AJ, et al. Activated protein C. J Thromb Haemost, 2007, 5: 73-80
[52] Berisio R, Vitagliano L, Mazzarella L, et al. Crystal structure of a collagen-like polypeptide with repeating sequence Pro-Hyp-Gly at 1.4 Å resolution: implications for collagen hydration. Biopolymers, 2000, 56: 8-13
[53] Sricholpech M, Perdivara I, Yokoyama M, et al. Lysyl hydroxylase 3-mediated glucosylation in type I collagen: molecular loci and biological significance. J Biol Chem, 2012, 287: 22998-3009
[54] Molony MS, Wu SL, Keyt LK, et al. The unexpected presence of hydroxylysine in non-collagenous proteins. Techn Protein Chem, 1995, 6: 91-8
[55] Miller AK, Hambly DM, Kerwin BA, et al. Characterization of site-specific glycation during process development of a human therapeutic monoclonal antibody. J Pharm Sci, 2011, 100: 2543-50
[56] Valliere-Douglass JF, Brady LJ, Farnsworth C, et al. O-Fucosylation of an antibody light chain: characterization of a modification occurring on an IgG1 molecule. Glycobiology, 2009, 19: 144-52
[57] Banks DD, Hambly DM, Scavezze JL, et al. The effect of sucrose hydrolysis on the stability of protein therapeutics during accelerated formulation studies. J Pharm Sci, 2009, 98: 4501-10
[58] Adrover M, Marino L, Sanchis P, et al. Mechanistic insights in glycation-induced protein aggregation. Biomacromolecules, 2014, 15: 3449-62
[59] Wei B, Berning K, Quan C, et al. Glycation of antibodies: modification, methods and potential effects on biological functions. MAbs, 2017, 9: 586-94
[60] Quan C, Alcala E, Petkovska I, et al. A study in glycation of a therapeutic recombinant humanized monoclonal antibody: where it is, how it got there, and how it affects charge-based behavior. Anal Biochem, 2008, 373: 179-91
[61] Mo J, Jin R, Yan Q, et al. Quantitative analysis of glycation and its impact on antigen binding. MAbs, 2018, 10: 406-15
[62] Holdener BC, Haltiwanger RS. Protein O-fucosylation: structure and function. Curr Opin Struct Biol, 2019, 56: 78-86
[63] Mormann M, Maček B, de Peredo AG, et al. Structural studies on protein O-fucosylation by electron capture dissociation. Int J Mass Spectrom, 2004, 234: 11-21
[64] Xu Y, Wang D, Masonc B, et al. Structure, heterogeneity and developability assessment of therapeutic antibodies. MAbs, 2019, 11: 239-64
[65] Alvarez M, Tremintin G, Wang J, et al. On-line characterization of monoclonal antibody variants by liquid chromatography-mass spectrometry operating in a two-dimensional format. Anal Biochem, 2011, 419: 17-25
[66] Wang S, Liu AP, Yan Y, et al. Characterization of product-related low molecular weight impurities in therapeutic monoclonal antibodies using hydrophilic interaction chromatography coupled with mass spectrometry. J Pharm Biomed Anal, 2018, 154: 468-75
[67] Nainwal N, Chirmade T, Gani K, et al. Understanding unfolding and refolding of the antibody fragments (Fab). II. Mapping intra and inter-chain disulfide bonds using mass spectrometry. Biochem Eng J, 2022, 182: 108439
[68] Banks DD, Gadgil HS, Pipes GD, et al. Removal of cysteinylation from an unpaired sulfhydryl in the variable region of a recombinant monoclonal IgG1 antibody improves homogeneity, stability, and biological activity. J Pharm Sci, 2008, 97: 775-90
[69] Cao M, Wang C, Chung WK, et al. Characterization and analysis of scFv-IgG bispecific antibody size variants. MAbs, 2018, 10: 1236-47
[70] Cohen SL, Price C, Vlasak J. β-Elimination and peptide bond hydrolysis: two distinct mechanisms of human IgG1 hinge fragmentation upon storage. J Am Chem Soc, 2007, 129: 6976-7
[71] McSherry T, McSherry J, Ozaeta P, et al. Cysteinylation of a monoclonal antibody leads to its inactivation. MAbs, 2016, 8: 718-25
[72] Chen G, Zhang Y, Trinidad JC, et al. Distinguishing sulfotyrosine containing peptides from their phosphotyrosine counterparts using mass spectrometry. J Am Soc Mass Spectrom, 2018, 29: 455-62
[73] Maitre P, Scuderi D, Corinti D, et al. Applications of infrared multiple photon dissociation (IRMPD) to the detection of posttranslational modifications. Chem Rev, 2020, 120: 3261-95
[74] Zhao J, Saunders J, Schussler SD, et al. Characterization of a novel modification of a CHO-produced mAb: evidence for the presence of tyrosine sulfation. MAbs, 2017, 9: 985-95
[75] Tyshchuk O, Völger HR, Ferrara C, et al. Detection of a phosphorylated glycine-serine linker in an IgG-based fusion protein. MAbs, 2017, 9: 94-103
[76] Hou Y, Su H, Luo Z, et al. Nutrient optimization reduces phosphorylation and hydroxylation level on an Fc-fusion protein in a CHO fed-batch process. Biotechnol J, 2019, 14: 1700706
[77] Dick LW Jr, Kim C, Qiu D, et al. Determination of the origin of the N-terminal pyro-glutamate variation in monoclonal antibodies using model peptides. Biotechnol Bioeng, 2007, 97: 544-53
[78] 武刚, 王文波, 于传飞, 等. 利用高分辨液相质谱综合表征抗CTLA4单克隆抗体的研究. 中国药学杂志, 2020, 55: 457-64
[79] Yu L, Vizel A, Huff MB, et al. Investigation of N-terminal

glutamate cyclization of recombinant monoclonal antibody in formulation development. J Pharm Biomed Anal, 2006, 42: 455-63

[80] Chelius D, Jing K, Lueras A, et al. Formation of pyroglutamic acid from N-terminal glutamic acid in immunoglobulin γ antibodies. Anal Chem, 2006, 78: 2370-6

[81] Bults P, Spanov B, Olaleye O, et al. Intact protein bioanalysis by liquid chromatography-high-resolution mass spectrometry. J Chromatogr B Analyt Technol Biomed Life Sci, 2019, 1110-1111: 155-67

[82] Li X, Xiao L, Kochert B, et al. Extended characterization of unpaired cysteines in an IgG1 monoclonal antibody by LC-MS analysis. Anal Biochem, 2021, 622: 114172

[83] Jin Y, Lin Z, Xu Q, et al. Comprehensive characterization of monoclonal antibody by Fourier transform ion cyclotron resonance mass spectrometry. MAbs, 2019, 11: 106-15

[84] Johnson KA, Paisley-Flango K, Tangarone BS, et al. Cation exchange-HPLC and mass spectrometry reveal C-terminal amidation of an IgG1 heavy chain. Anal Biochem, 2007, 360: 75-83

[85] Lyubarskaya Y, Houde D, Woodard J, et al. Analysis of recombinant monoclonal antibody isoforms by electrospray ionization mass spectrometry as a strategy for streamlining characterization of recombinant monoclonal antibody charge heterogeneity. Anal Biochem, 2006, 348: 24-39

[86] Antes B, Amon S, Rizzi A, et al. Analysis of lysine clipping of a humanized Lewis-Y specific IgG antibody and its relation to Fc-mediated effector function. J Chromatogr B Analyt Technol Biomed Life Sci, 2007, 852: 250-6

[87] Jiang G, Yu C, Yadav DB, et al. Evaluation of heavy-chain C-terminal deletion on product quality and pharmacokinetics of monoclonal antibodies. J Pharm Sci, 2016, 105: 2066-72

[88] Yang X, Seol H, Lin W, et al. Site-specific quantitation of drug conjugations on antibody-drug conjugates (ADCs) using a protease-assisted drug deconjugation and linker-like labeling (PADDLL) method. Anal Chem, 2021, 93: 9549-58

[89] Nguyen JM, Smith J, Rzewuski S, et al. High sensitivity LC-MS profiling of antibody-drug conjugates with difluoroacetic acid ion pairing. MAbs, 2019, 11: 1358-66

[90] Nicolardi S, Deelder AM, Palmblad M, et al. Structural analysis of an intact monoclonal antibody by online electrochemical reduction of disulfide bonds and Fourier transform ion cyclotron resonance mass spectrometry. Anal Chem, 2014, 86: 5376-82

[91] Wang J, Zhang W, Salter R, et al. Reductive desulfuration as an important tool in detection of small molecule modifications to payload of antibody drug conjugates. Anal Chem, 2019, 91: 2368-75

[92] Wagner-Rousset E, Janin-Bussat MC, Colas O, et al. Antibody-drug conjugate model fast characterization by LC-MS following IdeS proteolytic digestion. MAbs, 2014, 6: 173-84

[93] Courtois F, Agrawal NJ, Lauer TM, et al. Rational design of therapeutic mAbs against aggregation through protein engineering and incorporation of glycosylation motifs applied to bevacizumab. MAbs, 2016, 8: 99-112

[94] Wang Q, Wang T, Wu WW, et al. Comprehensive N- and O-glycoproteomic analysis of multiple Chinese hamster ovary host cell lines. J Proteome Res, 2022, 21: 2341-55

[95] Tamara S, Den Boer MA, Heck AJR. High-resolution native mass spectrometry. Chem Rev, 2022, 122: 7269-326

[96] Haberger M, Heidenreich AK, Hook M, et al. Multiattribute monitoring of antibody charge variants by cation-exchange chromatography coupled to native mass spectrometry. J Am Soc Mass Spectrom, 2021, 32: 2062-71

[97] Carillo S, Jakes C, Bones J. In-depth analysis of monoclonal antibodies using microfluidic capillary electrophoresis and native mass spectrometry. J Pharm Biomed Anal, 2020, 185: 113218

[98] Dykstra AB, Flick TG, Lee B, et al. Chip-based capillary zone electrophoresis mass spectrometry for rapid resolution and quantitation of critical quality attributes in protein biotherapeutics. J Am Soc Mass Spectrom, 2021, 32: 1952-63

[99] Liu AP, Yan Y, Wang S, et al. Coupling anion exchange chromatography with native mass spectrometry for charge heterogeneity characterization of monoclonal antibodies. Anal Chem, 2022, 94: 6355-62

[100] Jones J, Pack L, Hunter JH, et al. Native size-exclusion chromatography-mass spectrometry: suitability for antibody-drug conjugate drug-to-antibody ratio quantitation across a range of chemotypes and drug-loading levels. MAbs, 2020, 12: 1682895

[101] Hühner J, Lämmerhofer M, Neusüß C. Capillary isoelectric focusing-mass spectrometry: coupling strategies and applications. Electrophoresis, 2015, 36: 2670-86

[102] Mack S, Cruzado-Park I, Chapman J, et al. A systematic study in CIEF: defifining and optimizing experimental parameters critical to method reproducibility and robustness. Electrophoresis, 2009, 30: 4049-58

[103] Tang Q, Harrata AK, Lee CS. Capillary isoelectric focusing-electrospray mass spectrometry for protein analysis. Anal Chem, 1995, 67: 3515-9

[104] Liu R, Cheddah S, Xu X, et al. Capillary isoelectric focusing with free or immobilized pH gradient in silica particles packed column. Anal Chim Acta, 2019, 1079: 230-6

[105] Xu T, Shen X, Yang Z, et al. Automated capillary isoelectric focusing-tandem mass spectrometry for qualitative and quantitative top-down proteomics. Anal Chem, 2020, 92: 15890-8

[106] Xu T, Han L, Sun L. Automated capillary isoelectric focusing-mass spectrometry with ultrahigh resolution for characterizing microheterogeneity and isoelectric points of intact protein complexes. Anal Chem, 2022, 94: 9674-82

[107] Li M, Zhao X, Shen D, et al. Identification of a monoclonal antibody clipping variant by cross-validation using

capillary electrophoresis-sodium dodecyl sulfate, capillary zone electrophoresis-mass spectrometry and capillary isoelectric focusing-mass spectrometry. J Chromatogr A, 2022, 1684: 463560

[108] Zhang X, Kwok T, Zhou M, et al. Imaged capillary isoelectric focusing (icIEF) tandem high resolution mass spectrometry for charged heterogeneity of protein drugs in biopharmaceutical discovery. J Pharm Biomed Anal, 2023, 224: 115178

[109] Sokolowska I, Mo J, Pirkolachahi FR, et al. Implementation of a high-resolution liquid chromatography-mass spectrometry method in quality control laboratories for release and stability testing of a commercial antibody product. Anal Chem, 2020, 92: 2369-73