Application Note

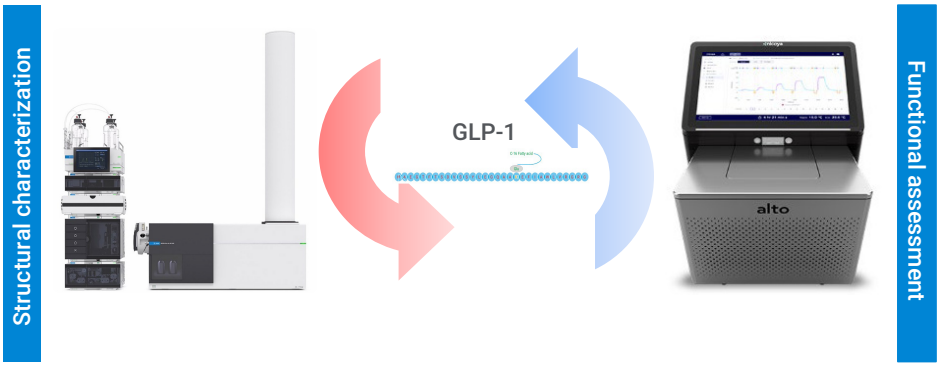
Biopharma/Pharma

Agilent

Trusted Answers

Suitable for Agilent
1290 Infinity III LC

Integrated Structural and Functional Characterization of a GLP-1 Analogue Using LC/MS and SPR



Authors

Beatrice Sacripanti and
Kyle Piccolo
Nicoya Lifesciences, Inc.

Ravindra Gudihal and
Soleil Grise
Agilent Technologies, Inc.

Abstract

Glucagon-like peptide 1 receptor agonists (GLP-1 RAs) are a rapidly expanding class of peptide therapeutics, transforming diabetes and obesity management and now recognized for their growing list of health benefits. As new chemical modifications are introduced into these peptides, early structural and functional characterization becomes essential. This application note demonstrates an integrated workflow using an Agilent 1290 Infinity II bio LC system, an Agilent 6545XT AdvanceBio LC/Q-TOF, and a Nicoya digital surface plasmon resonance (digital SPR) system to link structural integrity with functional receptor binding (in some cases with unexpected results), demonstrating the importance of performing both assays. By combining orthogonal techniques, biopharma developers can correlate chemical modifications and degradation with biological activity, supporting stability, comparability, and impurity profiling studies. This workflow aligns with industry trends toward more comprehensive characterization strategies that accelerate development timelines.

Introduction

GLP-1 RAs have emerged as one of the most impactful classes of peptide therapeutics in modern medicine. Initially developed for glucose control in type 2 diabetes, GLP1 RAs are now widely recognized for their substantial benefits in weight management, cardiometabolic health, and organ-protective effects.[1] Their rapid adoption has accelerated innovative efforts, driving the development of next-generation analogs with extended half-lives, enhanced potency, and improved stability. As manufacturers explore new sequence variants, chemical modifications, and formulation strategies, the need for early and comprehensive characterization becomes essential. These peptides often incorporate lipidation, PEGylation, amino acid substitutions, or other structural changes intended to modulate pharmacokinetics and receptor engagement.[2] However, such modifications can also influence peptide folding, degradation pathways, aggregation, or target binding in ways that are not always intuitive. Understanding how structural integrity translates into functional activity is therefore critical for guiding design decisions, optimizing stability, and ensuring product comparability throughout development.

This application note presents an integrated analytical workflow combining an Agilent 1290 Infinity II bio LC system, an Agilent 6545XT AdvanceBio LC/Q-TOF, and a Nicoya Digital Surface Plasmon Resonance (digital SPR) system to provide a holistic assessment of GLP-1 RA structure and function. LC/MS enables detailed structural profiling—including mass confirmation, impurity analysis, and degradation monitoring—while digital SPR delivers real-time, label-free measurements of GLP-1 receptor binding kinetics. When used together, these orthogonal techniques allow developers to directly correlate chemical modifications or degradation products with changes in biological activity. In several cases, this combined approach reveals unexpected disconnects between structural homogeneity and receptor binding behavior, underscoring the value of functional assays alongside structural characterization. By integrating LC/MS and digital SPR, biopharmaceutical scientists can streamline early-stage screening, support stability and forced degradation studies, improve comparability assessments, and gain deeper insight into structure-activity relationships. This workflow aligns with current industry trends toward faster development timelines and more robust characterization strategies, enabling confident decision-making from early discoveries through development and quality control.

Experimental

Reagents and chemicals

Liraglutide was purchased from MedChemExpress (Monmouth Junction, NJ, USA) and stored according to the manufacturer's instructions. Difluoroacetic acid (DFA), Trizma base, Tris-HCl, and 30% (v:v) hydrogen peroxide (H_2O_2), calcium chloride, and formic acid was procured from Sigma-Aldrich (St. Louis, MO, USA). Sequencing-grade chymotrypsin were obtained from Promega (Madison, WI, USA). All other chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA).

LC/MS grade acetonitrile (ACN) was obtained from Fisher (Waltham, MA. USA). Ultrapure water was collected from an in-house Millipore Sigma Milli-Q system (Billerica, MA. USA).

GLP-1R for SPR experiments was procured from Sino Biological (Paoli, PA, USA). All other SPR reagents used were prepared by Nicoya (Kitchener, ON, USA): PBS-T (0.1% Tween 20), pH 7.4 (DSPR-PBST), Carboxyl Surfacing Kit (DSPR-CBX-SURF), 10 mM Sodium Acetate Buffer, pH 4.0 (DSPR-IMB-4.0) and 10 mM Glycine-HCl, pH 1.5 (DSPR-GLYHCl-1.5).

Analytical equipment

- An Agilent 1290 Infinity II bio LC system included the following modules:
  - Agilent 1290 Infinity II Bio high-speed pump (G7120A)
  - Agilent 1290 Infinity II Bio multisampler (G7137A)
  - Agilent 1290 Infinity II multicolumn thermostat (G7116B)
- Agilent 6545XT AdvanceBio LC/Q-TOF (G6549AA)
- Digital SPR 16-Channel Instrument with Nicosystem Pro Software (DSPR16-PRO)
  - 16-Channel Carboxyl Cartridge (KC-CBX-CMD-16)

Software and data processing

- Agilent MassHunter data acquisition software, version 11.0
- Agilent MassHunter BioConfirm software, version 12.1
- Agilent MassHunter Qualitative Analysis, version 12.0
- Digital SPR Nicosystem User Portal

Sample preparation

Oxidation protocol: Liraglutide was dissolved to 2.0 mg/mL in 30% ACN. Sonication was applied to aid solubilization until a homogeneous, visually clear solution was obtained. The resulting stock was used immediately for oxidation studies. For oxidative stress, stock solutions were diluted to 0.5 mg/mL and incubated at 2.5% concentrations of the oxidizing agent H_2O_2 overnight at room temperature.

Chymotrypsin digestion protocol: Liraglutide peptide samples (200 µg/mL) were dissolved in 100 mM Tris buffer (pH 8.0) containing 10 mM $CaCl_2$. Peptide digestion was performed at 37 °C for 2 hours with a chymotrypsin enzyme to peptide ratio of 1:30 (w/w). The reaction was stopped by acidifying the mixture with formic acid, and LC/MS experiments were then performed.

LC/MS analysis

For the oxidized samples, the LC separation was performed on an Agilent AdvanceBio Peptide Mapping column (part number 653750-902) using a 30-minute gradient as described earlier.[3] The raw data were acquired by a 6545XT AdvanceBio LC/Q-TOF and data analysis was performed in MassHunter BioConfirm version 12.1 and MassHunter Qualitative Analysis version 12.0.

For the chymotrypsin digested liraglutide, the LC separation was performed on an Agilent Altura Peptide Plus column, 2.1 × 150 mm, 2.7 µm (part number 227215-903) using an 8-minute gradient (Table 1). The raw data were acquired by a 6545XT AdvanceBio LC/Q-TOF (Table 2) and data analysis was performed in MassHunter BioConfirm version 12.1 and MassHunter Qualitative Analysis version 12.0.

Table 1. Liquid chromatography parameters.

Parameter	Value
Column	Altura Peptide Plus, 2.1 × 150 mm, 2.7 µm (p/n 227215-903)
Sample Thermostat	10 °C
Mobile Phase A	0.1% formic acid in water
Mobile Phase B	0.1% formic acid in ACN
Gradient	Time (min) %A %B 0 95.00 5.00 1.00 95.00 5.00 5.00 65.00 35.00 6.00 30.00 70.00 7.00 30.00 70.00 8.00 95.00 5.00
Stop Time	As pump/injector
Column Temperature	50 °C
Injection Volume	3.00 µL
Flow Rate	0.3 mL/min

Table 2. MS data acquisition parameters.

Parameter	Value
Ion Mode	Positive mode, dual AJS ESI
Gas Temperature	325 °C
Gas Flow	13 L/min
Nebulizer	35 psig
Sheath Gas Temperature	350 °C
Sheath Gas Flow	12 L/min
Capillary Voltage	4,000 V
Nozzle Voltage	1,000 V
Fragmentor	175 V
Skimmer1	65 V
OctopoleRFPeak	750 V
MS Minimum Range	*m/z* 100
MS Maximum Range	*m/z* 1,700
MS Scan Rate	3.00 spectra/sec
MS/MS Minimum Range	*m/z* 50
MS/MS Maximum Range	*m/z* 1,700
MS/MS Scan Rate	3.00 spectra/sec

SPR methods

Binding kinetics were measured using a capture-based assay on an Alto 16-channel Digital SPR instrument with Nicosystem Pro software (DSPR16-PRO). Liraglutide samples were immobilized on a 16-Channel Carboxyl Cartridge (KC-CBX-CMD-16) by amine coupling and were interrogated by GLP-1R in solution for kinetic analysis.

1. **Reconstitution:** The lyophilized proteins were reconstituted in sterile water according to the manufacturer's instructions to generate a stock solution.
2. **Normalization:** Carboxyl sensors were normalized with normalization solutions.
3. **Immobilization:** Carboxyl sensors were activated with 200 mM EDC/NHS, followed by immobilization of 10 µg/mL liraglutide diluted in sodium acetate, pH 4.5 onto all even sensors. All sensors were blocked with 1 M ethanolamine to quench the remaining active carboxyl groups.
4. **Conditioning:** All sensors were conditioned with 10 mM Gly HCl, pH 1.5.
5. **Automated serial dilution:** The Alto Digital SPR automatically executed a five-step, threefold serial dilution on the cartridge with running buffer. A 900 nM stock of GLP-1R yielded 3.7, 11.1, 33, 100, and 300 nM solutions.

6. **Kinetics:** All sensors were exposed to the lowest GLP-1R concentration, allowed to dissociate in running buffer, and regenerated with 10 mM Gly HCl, pH 1.5. This step was repeated for the remaining four liraglutide concentrations, which then constituted a full round of multi-cycle kinetics (MCK).

Kinetic analysis

All interactions were automatically referenced in the Nicosystem by subtracting the signal from reference channels (no liraglutide immobilized). The resulting sensorgrams were fitted to a 1:1 Langmuir binding model to extract the association rate constant k_a, dissociation rate constant k_d, and equilibrium dissociation constant K_D.

Results and discussion

Structural characterization by LCMS

Liraglutide peptide characterization was performed using the 1290 Infinity II bio LC system with an AdvanceBio Peptide Mapping column, coupled to a 6545XT AdvanceBio LC/QTOF and processed in MassHunter BioConfirm 12.1. The bioinert configuration minimizes peptide-metal interactions, ensuring accurate chromatography and sensitive detection of low-level modifications.

This setup was used to study H_2O_2-induced oxidative degradation of liraglutide. Methionine and tryptophan are the primary oxidation prone residues in peptide therapeutics, and the GLP-1 agonist examined contains a single tryptophan. H_2O_2 is commonly applied in stress testing to generate oxidative variants, which may alter receptor binding.

Figure 1 shows the LC/MS profiles after overnight H_2O_2 exposure. The resulting TICs reveal multiple peaks, indicating formation of several oxidized species that are well resolved on the AdvanceBio column.

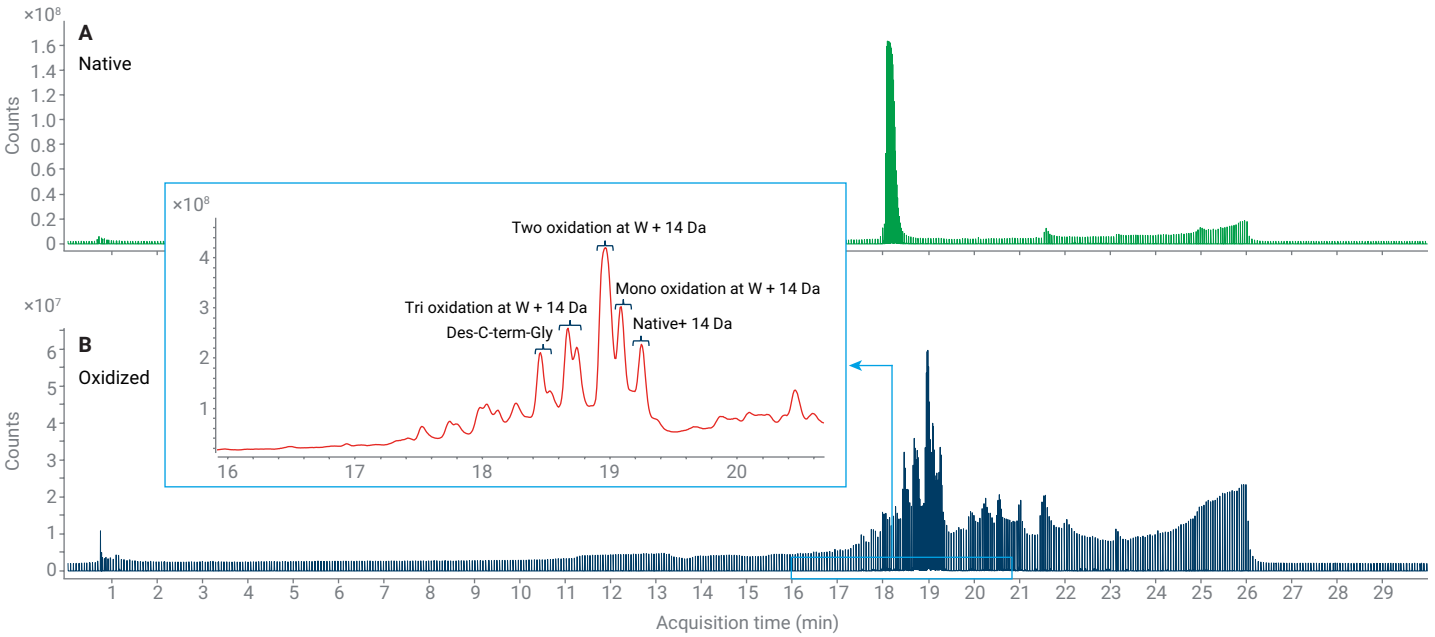


Figure 1. Total ion chromatogram: TIC of native (A) and oxidized liraglutide (B). The inset in B shows the assignment of different oxidized species.

Table 3 lists experimental monoisotopic masses obtained through resolved isotope deconvolution in MassHunter Qualitative Analysis 12.0. Deconvolution of the multiply charged ions confirms multiple oxidation states. Mass increases of +16, +32, and +48 Da correspond to mono, di, and trioxidized products, respectively. A +14 Da shift is also observed and attributed to carbonylation.[4]

Tryptophan oxidation commonly yields isomeric products[5], and the deconvolved masses allow assignment of these species as summarized in the Figure 1 inset. Reversed-phase LC provided partial separation of the singly, doubly, and triply oxidized isomers.

To further investigate the structural stability of liraglutide, chymotrypsin digestion was carried out. Chymotrypsin preferentially cleaves peptide chains at the carboxyl side of aromatic amino acids (Tyr, Phe, and Trp) and may also cleave—though less frequently—at selected aliphatic residues. In this study, six fragments were generated from the chymotrypsin digestion of liraglutide, as shown in the TIC in Figure 2. These fragments correspond to C1, C2, C3, C4, and C5 in order of their retention times (Table 4). The fragment designated C4* is attributed to a secondary cleavage site at a leucine residue. Figure 2 inset also shows the assignments of the buffer-related peaks.

Table 3. Experimental monoisotopic masses of liraglutide.

Peptide	Sequence	Molecular Weight (Monoisotopic)					
		Native	Des-C-term-Gly	Two Oxidation at W + 14 Da	Mono Oxidation at W + 14 Da	Tri Oxidation at W + 14 Da	Native +14/16 Da
Liraglutide	HAEGTFTSDVSSYLEGQAA-{Lys-N6-[N-(1-oxohexadecyl)-L-g-glutamyl]}-EFIAWLVRGRG	3,748.94	3,691.88	3,794.93	3,778.93	3,811.93	3,764.92

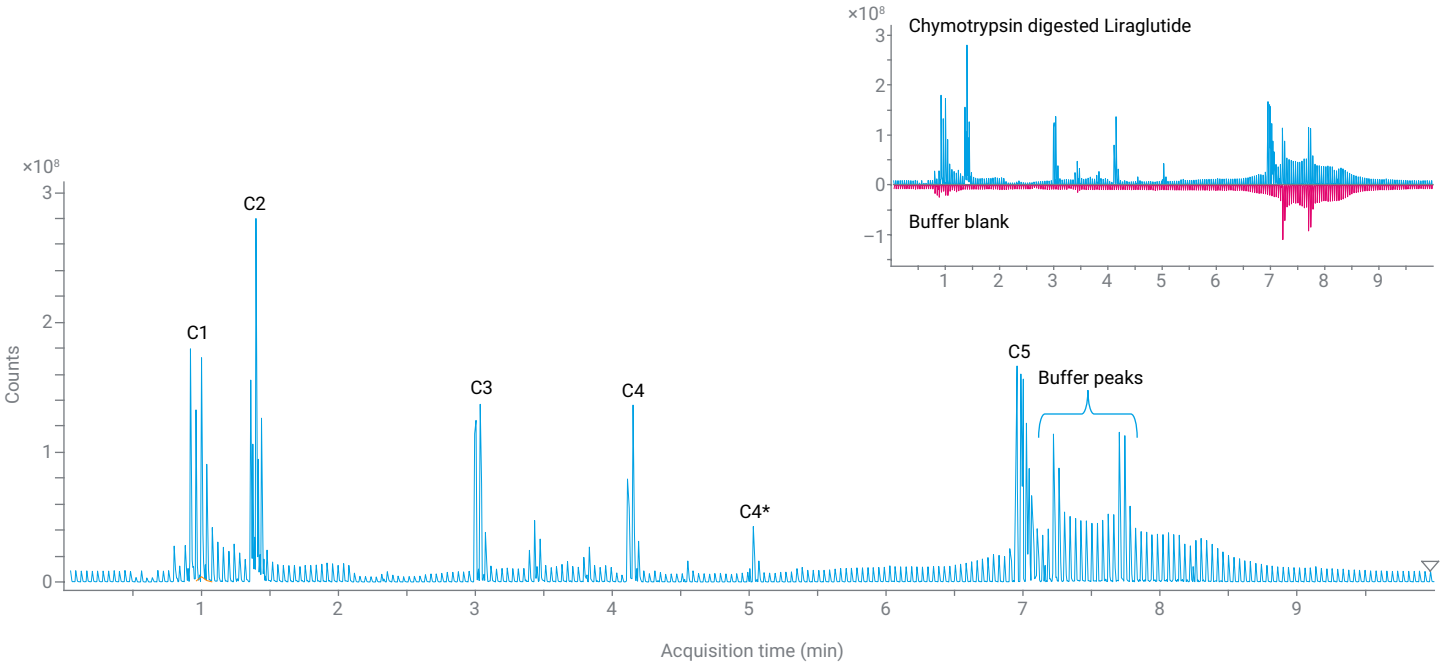


Figure 2. Total ion chromatogram (TIC) of chymotrypsin digested liraglutide. Inset shows the mirror plot of chymotrypsin-digested liraglutide with buffer blank.

Table 4. Fragments of chymotrypsin-digested liraglutide.

Fragment	Retention Time (min)	Peptide Fragment	Observed Mass (Da)	Target Mass (Da)	Mass Error (ppm)
C1	0.968	LVRGRG	656.4044	656.4082	−5.78
C2	1.349	HAEGTF	660.2834	660.2867	−5.03
C3	2.995	TSDVSSY	757.31	757.313	−3.98
C4	4.123	IAW	388.2089	388.2111	−5.67
C4*	5.028	IAWL	501.2926	501.2951	−5.13
C5	6.981	LEGQAA**K**EF	1,358.764	1,358.7697	−4.51

* Indicates secondary cleavage site of chymotrypsin
K Indicates lipidated Lys

The identities of the peptide fragments were confirmed through MS/MS analysis, with representative spectra shown in Figure 3. LC/MS analysis of liraglutide under two different treatment conditions further confirmed structural changes induced by these treatments. Together, these structural characterizations were used in conjunction with SPR studies to establish correlations between structural alterations and functional behavior.

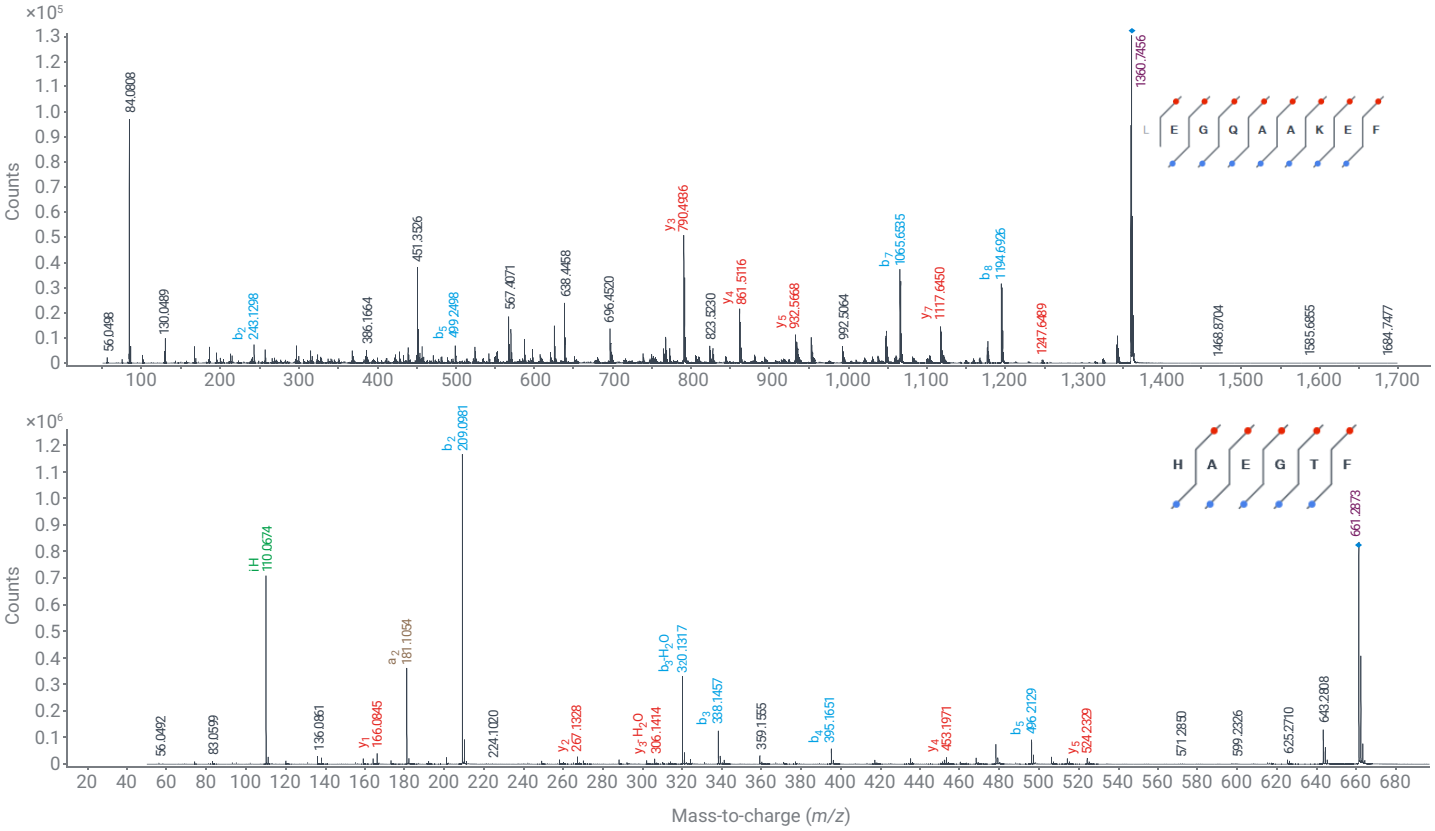


Figure 3. Representative MS/MS spectra of fragments of liraglutide following chymotrypsin digestion.

Functional assessment by SPR

In this study, digital SPR was used to compare the binding kinetics of liraglutide against GLP-1R using direct amine coupling. The immobilization of ligands on the carboxyl and streptavidin surfaces was optimized to obtain a low ligand density while allowing for the detection of all analyte concentrations sampled. Sensors were regenerated fully using 10 mM Gly-HCl, pH 1.5. Kinetic parameters for each liraglutide sample were calculated using a 1:1 Langmuir fit (Figures 4A and 4B).

SPR requires sample concentration as an input to determine kinetic parameters. The Agilent Cary 3500 UV-Vis enables rapid quantitation of biomolecules. In addition to quantitation, its high wavelength resolution enables detection of subtle structural changes for peptide characterization and stability assessments.[9]

The kinetics parameters calculated for native and oxidized liraglutide are summarized in Table 5. The K_D values measured were nearly identical, 3.51 and 3.68 nM for native and oxidized liraglutide, respectively. The k_a and k_d for each liraglutide sample are also the same, indicating there is no change in liraglutide function upon oxidation. Modification of the tryptophan residue to anchor the lipid moiety, instead of the lysine residue, has been reported to produce no change in binding affinity compared with the lysine-anchored lipid species. These findings suggest that the tryptophan residue does not make a measurable contribution to GLP-1 receptor engagement.[6] Digesting liraglutide with chymotrypsin abolished binding affinity for the GLP-1R completely, as shown in Figure 4C. Chymotrypsin cleaves peptide bonds following aromatic amino acids (such as phenylalanine, tyrosine, and tryptophan). Liraglutide contains several such residues, and therefore chymotrypsin breaks the backbone in multiple places, resulting in small fragments that cannot maintain the necessary structure to fit into the receptor's binding pocket. Previous studies have established that GLP-1 receptor binding is primarily mediated by the N-terminal residues, as reported in earlier publications.[7,8] These results demonstrate that some changes in peptide structure result in a change or inhibition of receptor binding, while other structural changes may have no impact on receptor binding. Therefore, Nicoya's Digital SPR is a powerful functional complement to Agilent's dependable LC/MS platforms, enabling scientists to link biological function back to structural integrity.

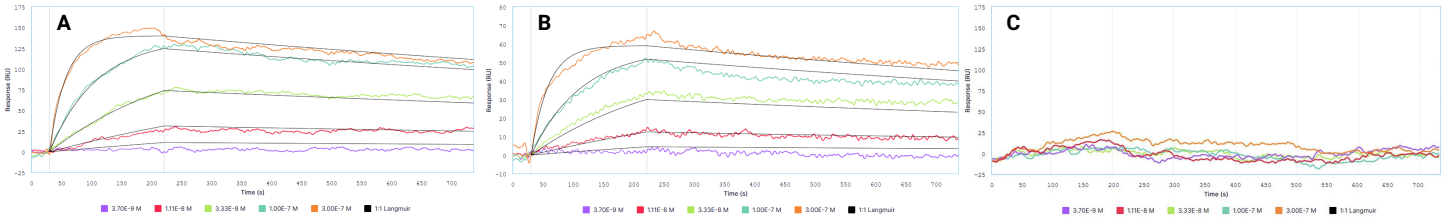


Figure 4. Representative corrected binding data for GLP-1R binding to (A) native liraglutide, (B) oxidized liraglutide, and (C) liraglutide digested with chymotrypsin using a multi-cycle kinetics (MCK) assay format. The analyte concentrations and associated colors are shown in the figure legends, while the Langmuir 1:1 binding fit is shown in black. A binding fit was not applied to (C) as no analyte binding was observed.

Table 5. Binding kinetics of native and oxidized liraglutide with GLP-1R.

Sample	k_a (M^{-1})	k_d (M^{-1} s^{-1})	K_D (nM)
Native Liraglutide	$1.79 \times 10^5 \pm 4.28 \times 10^4$	$6.26 \times 10^{-4} \pm 2.02 \times 10^{-4}$	3.51 ± 0.77
Oxidized Liraglutide	$1.57 \times 10^5 \pm 2.98 \times 10^4$	$5.64 \times 10^{-4} \pm 8.48 \times 10^{-5}$	3.68 ± 0.77

Conclusion

The integrated workflow described in this study—combining LC/MS for structural analysis with SPR for ligand binding assessment—provides an important framework for understanding structure-function relationships in GLP-1 analogues and their receptor.

The results generated using this integrated approach contribute valuable insights to the existing knowledge base for these molecules. The study exposed that cleaving of the peptide caused it to completely lose its function, highlighting the risk of degraded species in the final product. Meanwhile, we showed that oxidation, a common degradation pathway for peptides, had no impact on the function of liraglutide, perhaps reducing risk in some manufacture and storage approaches.

Beyond supporting fundamental characterization, this workflow offers meaningful advantages to the biopharmaceutical industry, helping optimize candidate selection and refinement during both discovery and development.

As GLP-1 RAs continue to expand in therapeutic scope and market impact, advanced analytical solutions will be essential for ensuring patient safety and accelerating access to innovative therapies.

References

1. Müller, T. D.; *et al.* Glucagon-Like Peptide 1 (GLP-1). *Mol. Metab*. **2019**, *30*, 72–130.
2. Přáda Brichtová, E.; Edu. I. A.; Li, X.; Becher, F.; Gomes Dos Santos, A. L.; Jackson, S. E. Effect of Lipidation on the Structure, Oligomerization, and Aggregation of Glucagon-like Peptide 1. *Bioconjug. Chem*. **2025**, *36(3)*, 401–414.
3. Suresh Babu, C. V. Characterization of Forced Degradation Impurities of Glucagon-Like Peptide-1 Agonists by LC/Q-TOF Mass Spectrometry. *Agilent Technologies application note*, publication number 5994-7794EN, **2025**.
4. Minkoff, B. B.; Bruckbauer, S. T.; Sabat, G.; Cox, M. M.; Sussman, M. R. Covalent Modification of Amino Acids and Peptides Induced by Ionizing Radiation from an Electron Beam Linear Accelerator Used in Radiotherapy. *Radiat. Res.* **2019**, *191(5)*, 447–459.
5. Bellmaine, S.; Schnellbaecher, A.; Zimmer, A. Reactivity and Degradation Products of Tryptophan in Solution and Proteins. *Free Radic. Biol. Med.* **2020**, *160*, 696–718.
6. Lu, X.; *et al.* Effects of Tryptophan-Selective Lipidated Glucagon-Like Peptide 1 (GLP-1) Peptides on the GLP-1 Receptor. *J. Endocrinol.* **2025**, *264(3)*, e240026.
7. Donnelly, D. The Structure and Function of the Glucagon-Like Peptide-1 Receptor and Its Ligands. *Br. J. Pharmacol.* **2012**, *166(1)*, 27–41.
8. Adelhorst, K.; Hedegaard, B. B.; Knudsen, L. B.; Kirk, O. Structure-Activity Studies of Glucagon-Like Peptide-1. *J. Biol. Chem*. **1994**, *269(9)*, 6275–6278.
9. Babu, S. C. V. Enhanced Peptide Characterization and Stability Assessment Using UV-Visible Second-Derivative Spectroscopy on an Agilent Cary 3500 Multicell UV-Vis Spectrophotometer. *Agilent Technologies application note*, publication number 5994-8551EN, **2025**.

www.agilent.com

RA260415.695

Printed in the USA, May 27, 2026
5994-9176EN

Agilent
Trusted Answers