

SUPPLEMENTARY METHODS

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Five micrograms of each mAb were analyzed under non-reducing and reducing conditions by SDS-PAGE using NuPAGE 4%–12% Bis-Tris gels (Life Technologies) and MES running buffer (Life Technologies). The protein samples were mixed 1:4 with 4 × NuPAGE-LDS sample buffer (Life Technologies) and 15 mM iodoacetamide (IAA) (Life Technologies, USA). For reduced samples, 50 mM dithiothreitol (DTT) (Life Technologies) was added. All sample mixtures were then heated at 70 °C for 10 min. A constant voltage of 150 V was applied for 60 min. Gels were then stained using Coomassie blue R-250 (Teknova) and destained with a mixture of 40% v/v methanol, 10% v/v acetic acid, and 50% v/v ultrapure water. Gel pictures were obtained using an AlphaImager gel imaging system (Protein Simple), and molecular mass was estimated based on migration of Novex Sharp Pre-Stained Protein Standards (Life Technologies).

Size-exclusion chromatography (SEC-HPLC)

SEC experiments were performed using a Shimadzu Prominence high-performance liquid chromatography (HPLC) system equipped with a photodiode array detector and a temperature controlled autosampler (Shimadzu). Twenty micrograms of each mAb diluted in PBS were injected onto a TSKgel SWXL guard column (6.0 mm × 40 mm, TOSOH Biosciences) followed by a TSK-Gel G3000 SWXL column (7.8 mm ID × 30.0 cm, TOSOH Biosciences). The flow rate was 0.7 mL/min for a run-time of 30 min with the column held at 30 °C. A mobile phase of 0.2 M sodium phosphate, pH 6.8, was used. Protein peaks were monitored by the absorbance at 214 nm, and data analysis was performed using LC Solutions software (Shimadzu).

Liquid chromatography/mass spectrometry (LC/MS) peptide mapping

The mAb samples were washed from the original formulation in an Amicon® filter (Millipore, Burlington, MA), with a 10 kDa cutoff, with 50 mM ammonium bicarbonate buffer, pH 7.8, and centrifuged at 14,000 rpm ($16873 \times g$), for 12 min at 4 °C. The washes were done in triplicate. The innovator mAb (Repatha®, Amgen, USA) was digested by four enzymes separately: trypsin (Promega, RefV511A, 20 µg), trypsin/LysC (Promega, RefV507, 20 µg), GluC (Promega, RefV165A, 10 µg \times 2), and trypsin/chymotrypsin (Sigma, T7168, 1 mg tablet). The biosimilar candidate was enzymatically digested by trypsin/chymotrypsin. For trypsin/chymotrypsin digestion, the protein was placed in a microtube containing 400 µL of 8 M guanidinium hydrochloride and 5 mM dithiothreitol in 50 mM ammonium bicarbonate buffer, pH 7.8. The reduction was performed in a water bath with a temperature ramp from 37 to 70 °C in 40 min. The alkylation was performed by the addition of 25 mM iodoacetamide, and the samples were incubated at 45 °C for 45 min. Finally, the samples were washed in Amicon® filters with 10 kDa cutoff as described above. The digestion was performed by adding 20 µg of a mixture of trypsin and chymotrypsin, and samples were incubated overnight at 37 °C. Subsequently, the proteases and peptides were separated by centrifugation using Amicon® filters at 14,000 rpm for 12 min at 4 °C. The digested protein was diluted in ultrapure water containing 0.1% formic acid to a final concentration of 5 µM. For trypsin, trypsin/LysC, and GluC samples, Repatha® solution was first concentrated eight times using Amicon® filters. The concentrated protein solution was denatured and reduced in 50 mM ammonium bicarbonate buffer pH 7.8, containing 6.2 M guanidine hydrochloride, 10 mM dithiothreitol, and 10 mM tris(2-carboxyethyl)phosphine, with a total volume of 500 µL. The mixture was incubated in the water bath, with the temperature ramping up from 37 °C to 60 °C, and continuously holding at 60 °C for 10 min. Each reduced sample was then alkylated by adding 100 mM iodoacetamide and incubating in the water bath at 45 °C for 1 h. Each alkylated sample was transferred into Amicon® filters and washed twice with 50 mM ammonium bicarbonate buffer pH 7.8 as described above. The solution (50 µL) was transferred to a microtube, and the digestion was performed

subsequently by adding 10 µg of each enzyme and incubating the mixture at 37 °C in the water bath for 2 h. Finally, another 10 µg of enzyme was added, and samples were incubated overnight at 37 °C.

The peptides were separated on a nanoAcquity ultra-performance liquid chromatography system (UPLC; Waters Corporation, Milford, MA) coupled to a Waters Xevo G2 Q-TOF mass spectrometer (Waters Corporation, Milford, MA). Mobile phase A was 0.1% formic acid in water (Optima, Fisher Chemical, Fair Lawn, NJ), and mobile phase B was acetonitrile containing 0.1% formic acid (Optima, Fisher Chemical, Fair Lawn, NJ). A volume of 2 µL of each enzymatic digested sample was injected onto the CSH C18 analytical column (ACQUITY UPLC M-Class Peptide, 130 Å, 1.7 µm, 75 µm×250 mm; Waters Cooperation, Milford, MA) and separated with a linear gradient, with an increase of mobile phase B from 3 to 60% within 80 min. The MSE data were acquired within a mass range between m/z 200 and m/z 2000; the ramp collision energy was set from 18 to 45 V, and the cone voltage was 30 V. Analysis of the digested peptides was performed with the MassLynx™ software (Waters Inc., Milford, CA), Protein Prospector (<http://prospector.ucsf.edu/>), and a trial version of the PeaksX® Studio software (Bioinformatics Solutions Inc., Ontario, Canada).

N-glycosylation profiling by hydrophilic interaction liquid chromatography (HILIC-HPLC)

The following steps were based on the experimental protocol developed by Dr. José Cremata (*in memoriam*, CIGB, Cuba), as detailed elsewhere [41]. For N-linked glycans analysis, samples containing 35 µg of mAbs in PBS were incubated with 1 µL of PNGase F at 37 °C for 16 h [42]. N-linked glycans were purified by cold ethanol precipitation, dried, and fluorescently labeled with a 2-aminobenzamide (2-AB) kit (#GKK-804, Prozyme). The 2-AB labeled oligosaccharides were further cleaned using GlycoClean™ S cartridges (#GKI-4726, Prozyme), once more dried and resuspended in 200 µL water. Samples were analyzed in a Shimadzu Prominence HPLC system equipped with a TSKgel Amide-80 column (5 µm, 25.0 cm x 4.6 mm, TOSOH BIOSCIENCE) and a fluorescence detector. Labeled N-glycans were resolved at 30 °C in a linear gradient with a flow rate of 0.4 mL/min with mobile phase A (50 mM ammonium formate, pH 4.4) and

mobile phase B (100% acetonitrile). The fluorescent signal was detected at an excitation wavelength of 330 nm and an emission wavelength of 420 nm. The peak assignment was done by comparison with the 2-AB glucose homopolymer standard (#GKSB-503, Prozyme) to convert retention time into glucose units (GU). Predictions of probable N-glycan structures based on their GU were made using the GlycoStore database (<https://glycostore.org>). The individual major peaks were integrated, and the relative amounts of N-glycan species were determined.

Imaged capillary isoelectric focusing (icIEF)

Imaged capillary isoelectric focusing was performed using an ICE3 system (Protein-Simple) equipped with a D2 lamp (280 nm), an autosampler, and a FC Cartridge fluorocarbon-coated capillary (Protein-Simple). For focusing, 20 µg of each mAb in PBS was mixed with Pharmalyte® 3.0-10.0 (GE Healthcare), acidic and basic isoelectric point (pI) markers of 4.65 and 9.46 (Protein-Simple), and 1% methylcellulose (Protein-Simple). The mixtures were loaded into 300-µL vials and injected in duplicate. The samples were resolved using the prefocusing condition of 1 min at 1500 V and the focusing condition of 4.5 min at 3000 V at room temperature (~ 23 °C). A hemoglobin analytical standard was injected before and after focusing of samples as quality control material. Data analysis was performed using Chromperfect software (Protein-Simple).

Micro-polyethylene glycol (PEG) precipitation assay

The following experimental protocol was adapted from Toprani et al [43]. Briefly, mAb stock solutions in PBS (at 0.7 mg/mL) and PEG-10,000 stock solutions in PBS were mixed in a 1:4 ratio to prepare various concentrations of PEG solutions ranging from 0% to 30% (m/v). The mixtures prepared in microtubes were incubated overnight (~ 16 h) at room temperature (~ 23 °C). A volume of 30 µL of each sample was loaded in triplicate into a 384-well polystyrene filter plate (Corning). The filter plate was then placed upon a 384-well UV-transparent microplate (Greiner) and centrifuged at 1610 × g for 15 min to

recover the filtrate. The protein concentration in the filtrates was determined using a SpectraMax M5 UV-Visible plate reader (Molecular Devices) at 214 nm. The %PEG midpoint (%PEG_{midpt}) was determined by plotting the mAb concentration versus the PEG-10,000 concentration and fitted to a Sigmoidal Boltzmann function ($y = A_2 + (A_1 - A_2) / (1 + \exp((x - x_0) / dx))$). The x_0 point was defined as the %PEG_{midpt}. To determine the apparent solubility, the same data were plotted on a logarithmic scale for the transition region (9.5–13.0% PEG) and fitted to a linear function. The extrapolated intercept was taken as the apparent solubility.⁴ Data analysis was performed using Origin software (OriginLab Corporation).

Invitro antibody binding kinetics

The binding kinetics of mAb samples to the target protein PCSK9 were characterized by bio-layer interferometry (BLI) optical detection using an Octet Red 96 system (Pall ForteBio) and employing anti-human IgG-Fc (AHQ) capture biosensors (Pall ForteBio). Prior to the binding measurements, the sensor tips were hydrated for 20 min in kinetic buffer (10 mM HEPES, 3 mM EDTA, 150 mM NaCl, 1 mg/mL BSA, 0.02% Tween-20, pH 7.4). Buffers, mAbs (at 2.5 nM), and 7 titrated concentrations (100 nM–1.56 nM, in 2-fold serial dilutions) of recombinant human PCSK9 (#592502, BioLegend) were loaded into a black bottom 96-well plate (Corning) with a volume of 200 μ L in each well. The kinetic assay had five steps: (1) Equilibration: 120 s with kinetic buffer to get a stable baseline; (2) Loading: 300 s of antibody loading to capture anti-PCSK9 mAbs onto the biosensors; (3) Equilibration/Wash: 120 s equilibration with the kinetic buffer to remove unbound mAb; (4) Association: 600 s of human PCSK9 binding to anti-PCSK9; mAbs and (5) Dissociation: 600 s dissociation with kinetic buffer. After that, the AHQ biosensor tips were regenerated with 10 cycles of 10 s dips in regeneration buffer (10 mM glycine, pH 1.7) followed by 10 s dips in kinetic buffer. Data analysis was performed using Octet Data Analysis (v 8.2) software. The kinetic rate constants for association (k_a) and dissociation (k_{dis}), as well as the equilibrium dissociation constant (K_D), were obtained by fitting the resulting curves to a 1:1 binding model.

Far-UV circular dichroism (CD) spectroscopy

Far-UV CD spectroscopy was performed using a Chirascan-plus CD spectrometer (Applied Photophysics) equipped with a Peltier temperature controller with a 6-cuvette position holder (Quantum Northwest). The spectra of mAb samples (at 0.2 mg/mL) were collected from 200 to 260 nm using a 1 nm step and a 2.0 s integration time. Thermal melts were performed over a temperature range of 10–90 °C at 1.0 °C intervals with a 2 min equilibration time at each temperature. Measurements were made in duplicate using quartz cuvettes (1-mm path length) sealed with a Teflon stopper (Starna Cells). The raw data were converted into mean residual molar ellipticity (MRME), and the CD at 217 nm (an indicator of changes in β -sheet structure) was plotted as a function of temperature. The thermal unfolding midpoint temperature (melting temperature, T_m) was determined by the local maximum of first derivative curves as previously described employing Origin software (OriginLab Corporation) [44].

Raman spectroscopy

Raman spectroscopy was performed at 25 °C using a Zetasizer Helix (Malvern Instruments) instrument equipped with a 785-nm laser. mAb samples (20 μ L at 10 mg/mL) were loaded into a metal microcuvette (Malvern, USA). Raman spectra were recorded in triplicate measurements of 10 acquisitions of 40 s each. Raw spectra were buffer subtracted and normalized according to the phenylalanine peak (1003 cm^{-1}). Amide I (1600–1700 cm^{-1}), tryptophan (1520–1580 cm^{-1}), and tyrosine (780–860 cm^{-1}) bands were analyzed and the secondary structure content predicted using the Zetasizer Helix Analyze software (Malvern Instruments). After measurement, the samples were recovered and used for the FTIR experiments.

Intrinsic and extrinsic fluorescence spectroscopy

Fluorescence spectroscopy experiments were performed using a fluorescence plate reader (Fluorescence Innovations) [27]. A volume of 20 μ L (at 0.2 mg/mL) of each mAb sample was loaded in duplicate into a 384-well plate (Bio-Rad). The microplate was centrifuged at 1610 $\times g$ for 1 min, and two

microliters of silicon oil were pipetted onto the sample to avoid evaporation. For intrinsic tryptophan (Trp) fluorescence, mAb samples were excited at 295 nm (> 95% Trp emission), and the emission spectra were collected at 300–405 nm with an acquisition time of 5 s. For extrinsic fluorescence, a SYPRO® Orange stock solution (5000x, Invitrogen) was added to mAb samples at a dilution factor of 1000. The samples were excited at 532-nm. A 532-nm long-pass filter and a 585-nm band-pass filter with 40-nm band were used, and the emission signal was collected and quantified by a photomultiplier. Thermal melts were set from 10-90 °C at 2.5 °C (for intrinsic fluorescence) or 1.0 °C (for extrinsic fluorescence) intervals with a 2-min equilibration time at each temperature. The total fluorescence intensity or the moment (the mean spectral center of mass, MSM) was plotted as a function of temperature as previously described [27]. The T_m values were calculated as described above for CD measurements using Origin software (OriginLab Corporation).

Dynamic light scattering (DLS)

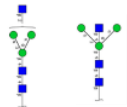
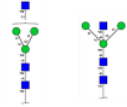



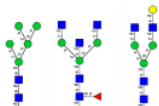
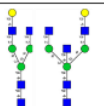
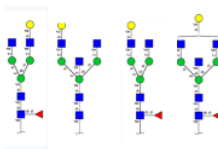
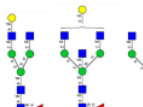
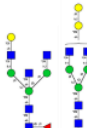
Dynamic light scattering was performed using a DynaPro DLS plate reader (Wyatt Technology). Twenty microliters of samples (at 0.2 mg/mL) were loaded in duplicate into a 384-well black plate (Corning). The plate was centrifuged at 1610 g for 1 min, and 5 μ L of silicon oil were added to avoid sample evaporation. Thermal melts were set as a linear ramp from 10 to 80 °C (0.5 °C/min), and five measurements of 30 s per sample were recorded. A cumulant analysis was performed, and intensity-averaged hydrodynamic diameters were reported. Raw data were interpolated using 29 points (10-80 °C, using 2.5 °C increments) using Origin software (OriginLab Corporation). The hydrodynamic diameter was plotted as a function of temperature.

Differential scanning calorimetry (DSC)

Differential scanning calorimetry was performed with a Microcal VP-DSC capillary microcalorimeter (GE Healthcare). Four hundred microliters of samples (0.2 mg/mL) and respective buffers were loaded in duplicate into a 96-well plate and held at 4 °C. Thermograms were recorded from 10 to 110 °C at a scanning rate of 1 °C/min using a filtering period of 16 s. Reference buffer subtraction, concentration

normalization to molar heat capacity, and data fitting to calculate onset temperature (T_{onset}) and T_m values were performed using the MicroCal LLC DSC plug-in for the Origin 7.0 software (OriginLab Corporation).

Supplementary Table S1. Possible N-glycans for innovator and biosimilar candidate mAbs. The main peaks were assigned according to the GlycoStore database. The standard deviation was calculated from five experimental replicates. ND: not detected. *: De Leoz et al [45].

Peaks	Innovator		Biosimilar		Relative abundance (%)		Possible structures (GlycoStore)	Oxford notation (GlycoStore)	Short name used with IgG glycans*
	Retention time (min)	GU	Retention time (min)	GU	Innovator	Biosimilar			
1	62.09 ± 0.07	4.93 ± 0.01	ND		0.5 ± 0.0	ND		A1; M3B	G0-N; Man3B
2	62.86 ± 0.07	5.00 ± 0.01	62.86 ± 0.05	5.00 ± 0.01	1.7 ± 0.1	0.4 ± 0.1		A1; M3B	G0-N; Man3B
3	67.11 ± 0.07	5.49 ± 0.01	67.24 ± 0.03	5.50 ± 0.00	14.0 ± 0.3	0.7 ± 0.1		A2	G0
4	ND		67.62 ± 0.04	5.55 ± 0.00	ND	2.4 ± 0.2		A1G1	G1-N
5	71.27 ± 0.07	5.97 ± 0.01	71.27 ± 0.04	5.96 ± 0.00	62.6 ± 0.8	56.6 ± 1.2		F(6)A2	G0F
6	73.32 ± 0.07	6.23 ± 0.01	73.31 ± 0.04	6.23 ± 0.01	12.5 ± 0.2	2.0 ± 0.1		M5; F(6)A2B; A2[6]G(4)1	Man5; G0FB; G1[6]
7	74.78 ± 0.07	6.42 ± 0.01	74.83 ± 0.04	6.43 ± 0.01	0.8 ± 0.1	1.1 ± 0.1		A2[3]G(4)1; A2[6]BG1	G1[3]; G1B
8	77.29 ± 0.07	6.76 ± 0.01	77.27 ± 0.04	6.75 ± 0.01	2.3 ± 0.1	14.4 ± 0.5		F(6)A2[6]G(4)1; A2[3]BG(4)1; F(6)A2[3]G(4)1; F(6)A2BG(4)1	G1F[6]; G1B[3]; G1F[3]; G1FB
9	78.13 ± 0.08	6.87 ± 0.01	78.13 ± 0.04	6.87 ± 0.01	2.7 ± 0.1	16.8 ± 0.4		F(6)A2[3]G(4)1; F(6)A2BG(4)1; F(6)A2[6]BG(4)1	G1F[3]; G1FB; G1FB [6]
10	79.87 ± 0.07	7.11 ± 0.01	ND		1.2 ± 0.2	ND		F(6)A2[3]BG(4)1; A2G(4)1Ga(3)1	G1FB [3]; G1 + 1aGal

A

- HC: 371/441 covered (84.1%)

EVQLVQSGAEVKKPGASVKVSCKASGYTLTSYGISWVRQAPGQGLEWMGWVSFYNGNTNYAQKLQGRGTMITDPSTSTAY
MELRSLRSDDTAVYYCARGYGMDVWVGQGTITVSSASTKGPSVFPLAPCSRSTSESTAALGCLVKDYFPEPVTVSWNSGALTS
GVHTFPAVLQSSGLYSLSSVTVPSNFGTQYTCNVDHKPSNTKVDKTVRKCCECPPCAPPVAGPSVFLFPPKPKDTLMIS
RTPEVTCVVVDVSHEDPEVQFNWYVDGVEVHNAKTKPREEQFNSTFRVSVLTVVHQDWLNGKEYKCKVSNKGLPAPIEKTI
SKTKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTPPMLDSGDSFFLYSKLTVDKSRWQQ
GNVFSCSVMEALHNHYTQKSLSLSPGK

- LC: 196/215 covered (91.2%)

ESALTQPASVSGSPGQSITISCTGTSSDVGGYNSVSWYQQHPGKAPKLMIEVSNRPSGVSNRFGSKSGNTASLTISGL
QAEDEADYYCNSYTSTSMVFGGGTKLTVLGQPKAAPSVTLFPPSSEELQANKATLVCLISDFYPGAVTVAWKADSSPVK
AGVETTPSKQSNNKYAASSYLSLTPEQWKSHRSYSCQVTHEGSTVEKTVAPTECS

- Total: 567/656 covered (86.4%)

B

- HC: 267/441 covered (60.5%)

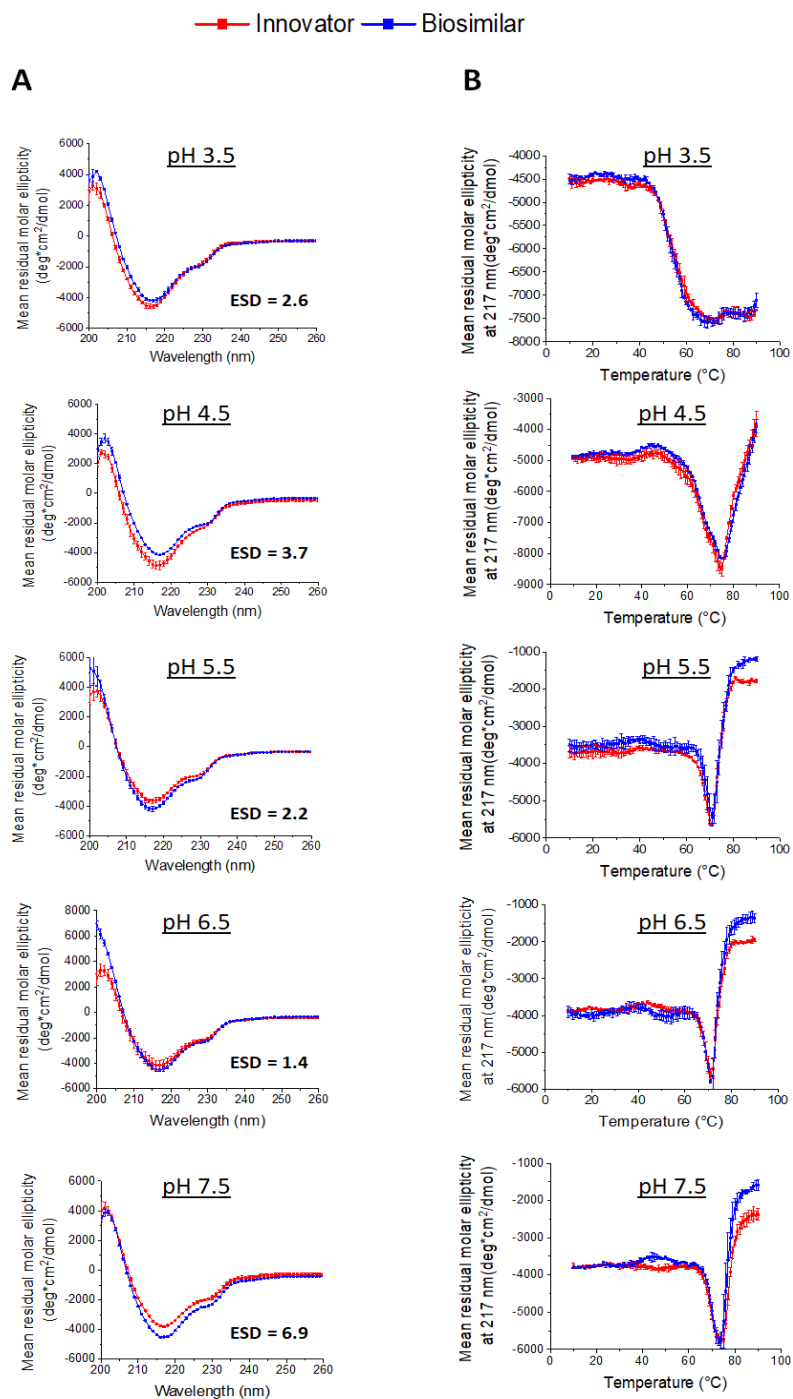
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MELRSLRSDDTAVYYCARGYGMDVWVGQGTITVSSASTKGPSVFPLAPCSRSTSESTAALGCLVKDYFPEPVTVSWNSGALTS
GVHTFPAVLQSSGLYSLSSVTVPSNFGTQYTCNVDHKPSNTKVDKTVRKCCECPPCAPPVAGPSVFLFPPKPKDTLMIS
RTPEVTCVVVDVSHEDPEVQFNWYVDGVEVHNAKTKPREEQFNSTFRVSVLTVVHQDWLNGKEYKCKVSNKGLPAPIEKTI
SKTKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTPPMLDSGDSFFLYSKLTVDKSRWQQ
GNVFSCSVMEALHNHYTQKSLSLSPGK

- LC: 150/215 covered (69.8%)

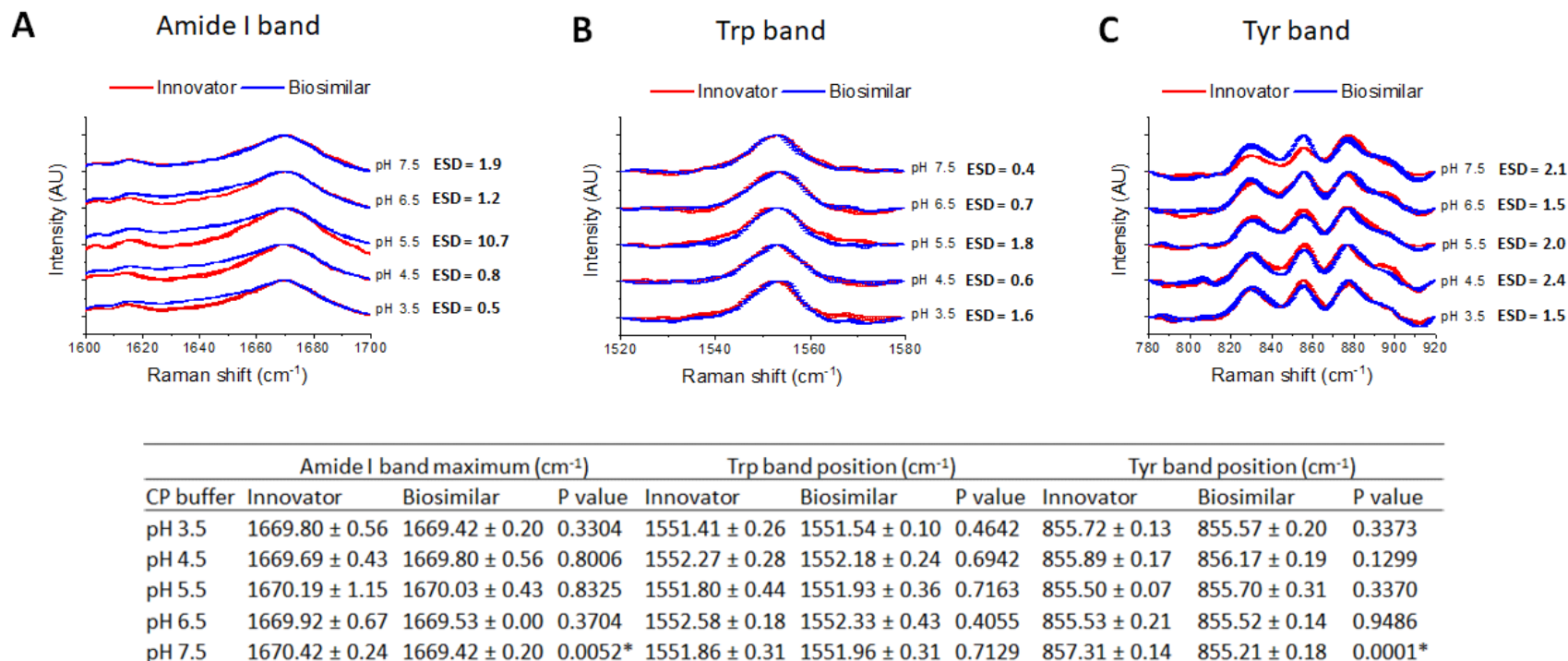
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ADYYCNSYTSTSMVFGGGTKLTVLGQPKAAPSVTLFPPSSEELQANKATLVCLISDFYPGAVTVAWKADSSPVKAGVETTPS
KQSNNKYAASSYLSLTPEQWKSHRSYSCQVTHEGSTVEKTVAPTECS

- Total: 417/656 covered (63.6%)

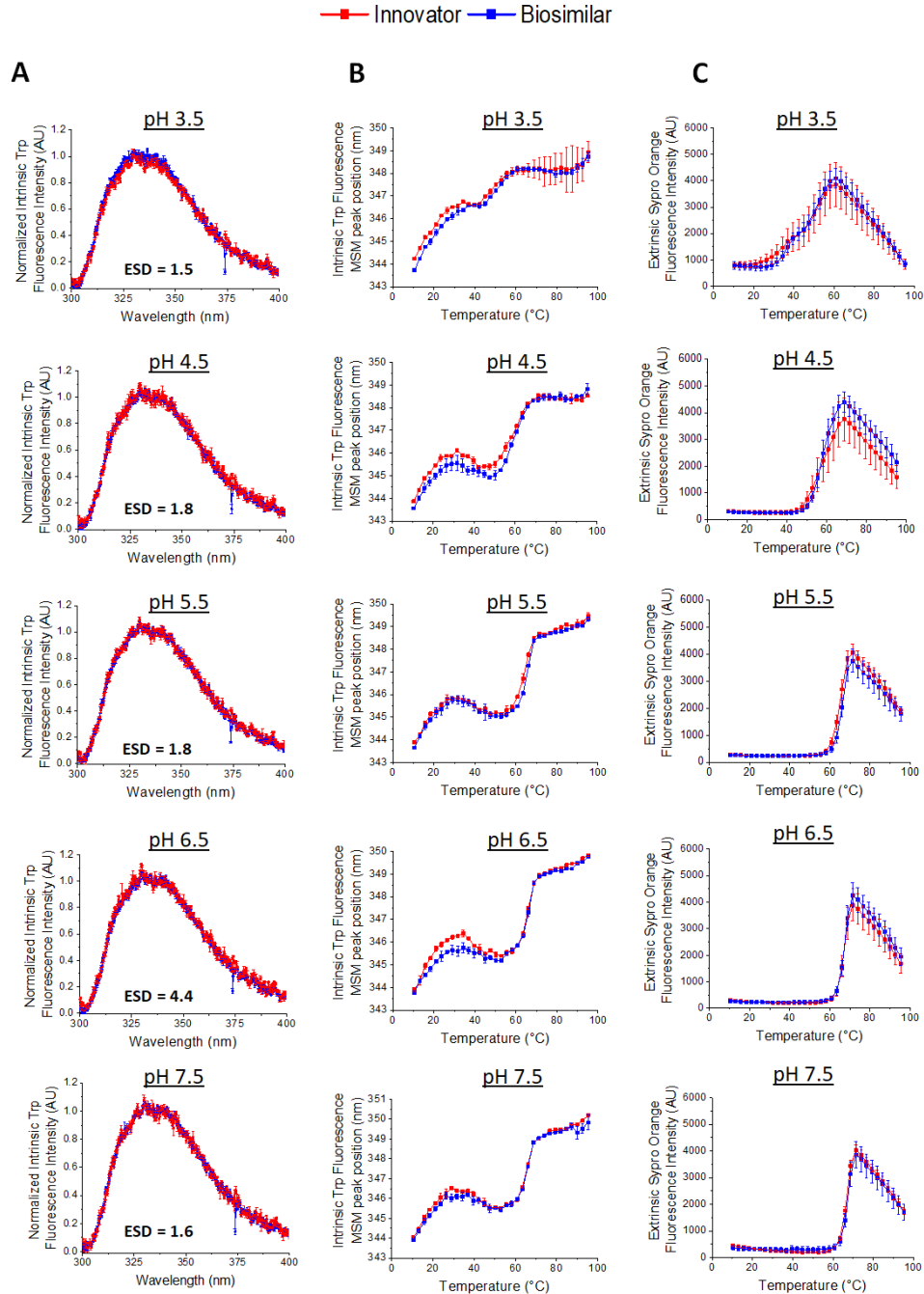
Supplementary Figure S1. Primary structure analysis. (A) Amino acid coverage of innovator mAb (combined results from individual trypsin, trypsin/LysC, GluC and, trypsin/chymotrypsin digestions). (B) Amino acid coverage of biosimilar candidate mAb (trypsin/chymotrypsin digestion). Peptides confirmed by MS-MS are highlighted in yellow. HC: heavy chain. LC: light chain.



Supplementary Figure S2. Far-UV circular dichroism spectra. (A) Comparison of far-UV CD spectra at 10 °C of innovator and biosimilar candidate mAbs at different pH values. The ESD values are reported for each pH. (B) Comparison of melting curves showing the effect of temperature and pH on the average ellipticity at 217 nm. The error bars represent the standard deviation of duplicate measurements.

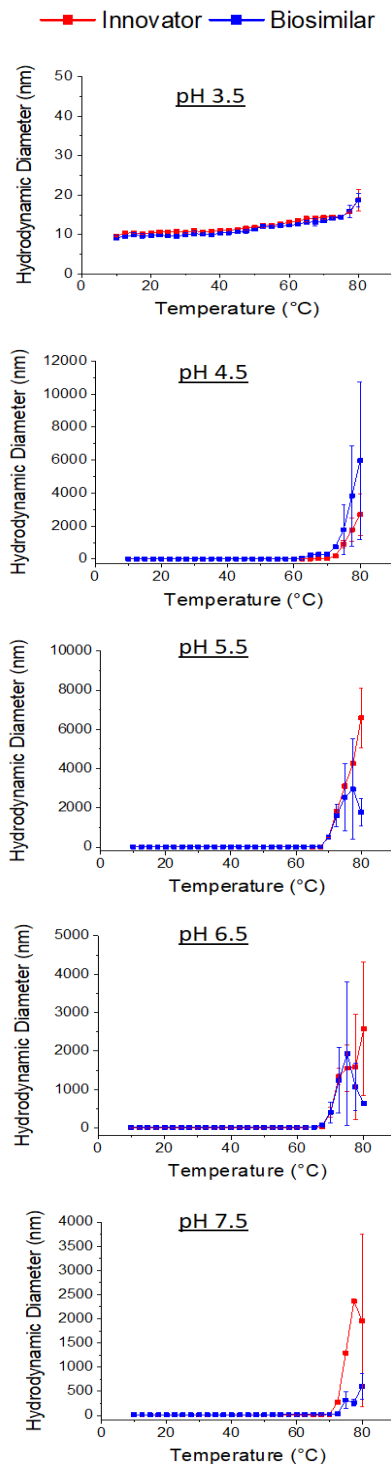


Supplementary Figure S3. Raman spectroscopy analysis at 25 °C. Comparative Raman spectra for: (A) Amide I; (B) tryptophan; (C) tyrosine regions of innovator and biosimilar candidate mAbs in different pH values. The error bars and standard deviation refer to three analytical measurements. ESD and peak positions values are reported for each pH.

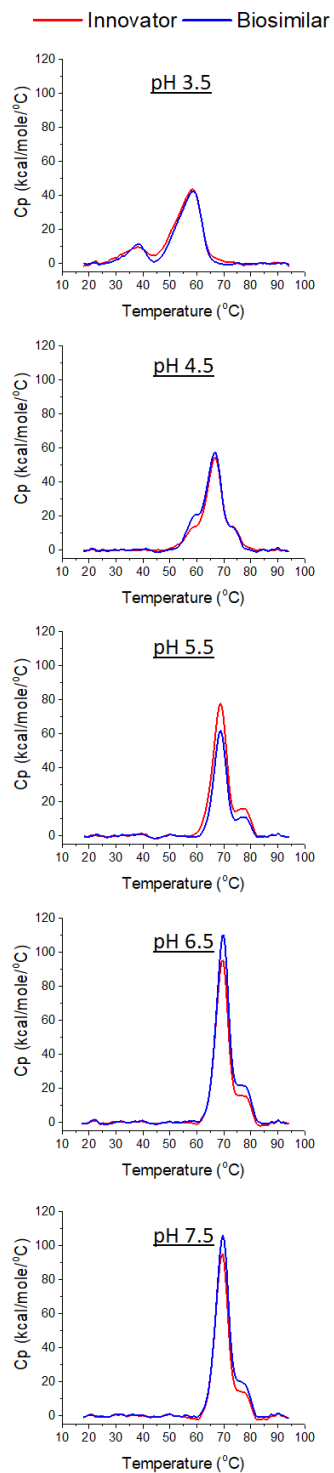


Supplementary Figure S4. Fluorescence spectroscopy analysis. (A) Comparison of intrinsic Trp fluorescence spectra at 10 °C for innovator and biosimilar candidate mAbs at different pH values. The ESD values are reported for each pH. (B) Comparison of melting curves showing the effect of temperature and pH on the intrinsic Trp fluorescence MSM. (C) Comparison of melting curves showing the effect of

temperature and pH on the extrinsic SYPRO Orange fluorescence intensity. The error bars represent the standard deviation of duplicate measurements.



Supplementary Figure S5. Dynamic light scattering analysis. The effect of pH and temperature on the aggregation behavior of innovator and biosimilar candidate mAbs as measured by DLS. The error bars represent the standard deviation of duplicate measurements.



Supplementary Figure S6. Differential scanning calorimetry analysis. Representative thermograms showing the effect of pH and temperature on the overall conformational stability of innovator and biosimilar candidate mAbs.