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► To cite this version:

Eric Chabrol, Johann Stojko, Alexandre Nicolas, Thomas Botzanowski, Benjamin Fould, et al.. VHH characterization.Recombinant VHHs: Production, characterization and affinity. *Analytical Biochemistry*, 2020, 589, pp.113491. 10.1016/j.ab.2019.113491 . hal-02566741

HAL Id: hal-02566741

<https://hal.science/hal-02566741>

Submitted on 15 Dec 2020

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VHH characterization.

Recombinant VHHs: production, characterization and affinity

Eric Chabrol^{1&}, Johann Stojko^{1&}, Alexandre Nicolas¹, Thomas Botzanowski², Benjamin Fould¹, Mathias Antoine¹, Sarah Cianférani², Gilles Ferry¹, Jean A. Boutin^{1,3,*}

¹ PEX Biotechnologies, Chimie, Biologie, Institut de Recherches Servier, 125 chemin de Ronde, 78290 Croissy-sur-Seine, France

² Laboratoire de Spectrométrie de Masse BioOrganique, Université de Strasbourg, CNRS, IPHC, UMR 7178, 67000 Strasbourg, France

³ Institut de Recherches Internationales Servier, 50 rue Carnot, 92284 Suresnes Cedex, France

& These two authors contributed equally to the work

* To whom correspondence should be addressed: Dr. JA Boutin, Institut de Recherches Internationales Servier, 50 rue Carnot, 92284 Suresnes Cedex, France. Email : jean.boutin@servier.com; phone : +33155724400

Running title: VHH expression and characterization

Total number of manuscript pages: 41

Tables: 8

Figures: 10

25 **Highlights:**

26

- 27 • VHH are small antibody-like proteins. They are entering the therapeutic arsenal.
- 28 • VHH purity, homogeneity, structure and affinity towards their targets are described.
- 29 • An analytical workflow to qualify those proteins is presented to validate the molecules.

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31

32 **Abstract:** Among the biological approaches to therapeutics, are the cells, such as CAR-T cells engineered
33 or not, the antibodies armed or not, and the smaller protein scaffolds that can be modified to render them
34 specific of other proteins, *à la façon* of antibodies. For several years, we explored ways to substitute
35 antibodies by nanobodies (also known as VHHs), the smallest recognizing part of camelids' heavy-chain
36 antibodies: production of those small proteins in host microorganisms, minute analyses, characterization,
37 and qualification of their affinity towards designed targets. Here, we present three standard VHHs
38 described in the literature: anti-albumin, anti-EGF receptor and anti-HER2, a typical cancer cell surface -
39 associated protein. Because they differ slightly in global structure, they are good models to assess our
40 body of analytical methodologies. The VHHs were expressed in several bacteria strains in order to
41 identify and overcome the bottlenecks to obtain homogeneous preparations of this protein. A large panel
42 of biophysical tools, ranging from spectroscopy to mass spectrometry, was here combined to assess VHH
43 structural features and the impact of the disulfide bond. The routes are now ready to move to more
44 complex VHHs raised against specific targets in numerous areas including oncology.

45

46 **Keywords:** VHH, expression, characterization, structure, affinity, CD, differential scanning fluorimetry,
47 mass spectrometry, ion-mobility, hydrogen/deuterium exchange, surface plasmon resonance.

48

50 1. INTRODUCTION:

51 Antibody therapy has taken a new boost these last years, due to the generalization of recombinant protein
52 production techniques, on one hand and to the use of armed antibodies (Ab) on the other [1,2]. Indeed,
53 several huge successes were recorded in many therapeutic areas, including, but not limited to cancer [3],
54 diabetes [4], poisoning [5], immune-inflammation [6], and infectious diseases [7].

55 Anti-cancer therapy has been one of the main sources of progress [8] in this area, especially by pioneering
56 and using in clinic armed antibodies also known as antibody-drug conjugates (ADCs) [9]. Basically, this
57 consists to load a cytotoxic compound on an antibody and use it as a cargo to selectively deliver the
58 conjugated drug on its target: a cell expressing the protein against which it has been raised [10]. The goal
59 is to deliver the payload to the cancer cells with a higher efficiency thanks to the selectivity of the
60 antibody, in order to potentializing its efficacy while diminishing its off-target effects (see Thomas *et al*
61 for review [11]). Although this has been a breakthrough in the anticancer armada, with numerous
62 successful examples [12–15], this approach essentially suffers from practical obstacles: the recombinant
63 synthesis of those antibodies is not only tricky but also quite expensive, even if the amount of this product
64 injected to patients remains, in some cases but not all, remarkably small due to the high affinity of the
65 antibody for its target [16]. Furthermore, once purified to apparent homogeneity, antibodies usually
66 include an intrinsic micro-heterogeneity, essentially due to many possible minute modifications -
67 including glycosylations [17,18] - of some amino acids in the Ab heavy chain (see for example Husson *et al*
68 [19]). This heterogeneity can be further increased by chemical modifications induced upon conjugation
69 of the antibody with a cytotoxic compound, leading to new chemical species containing the monoclonal
70 antibody (150 kDa) as well as a defined number of molecules of the cytotoxic compound [16]. Despite
71 those difficulties and the obvious benefit brought to the patients, many progresses should and will be done
72 in the future.

73 Alternative approaches are scrutinized, and particularly those concerning smaller protein molecules such
74 as ankyrin (see review by Skerra [20]). The original idea was to take advantage of the particular single
75 heavy chain antibody from camelids and sharks [21]. Indeed, the smallest intact antigen-binding part of
76 those huge proteins is the variable domain of the heavy chain [22,23]. Those protein parts were called
77 nanobodies or VHHs. Their sequences retain the antibody specificity for the targets they were raised
78 against. Therefore, a smaller and less complex protein recognizing a given target could be used as a
79 substitute to monoclonal antibodies. VHHs have been reported against many proteins that are supposedly
80 preferentially expressed in cancer cells, such as PD-1 [24], EGFR [25], HER2 [26], HLA-DR [27],

81 GFRA1 [28], HSA [29] and many others. Needless to stress that the beauty of the approach relies on the
82 specific nature of the protein expressed at the surface of the targeted cells (cancerous or others) and
83 recognized by the VHHs. An anti-HER2 VHHs have been shown to be valuable tools for breast cancer
84 cells detection [30–32]. Aside from oncology, those tools could also be used in many different application
85 areas, including diagnosis [33].

86 To date, however, there is a clear deficit in peptide (and protein) characterization, particularly regarding
87 their homogeneity [34]. **Despite clear warnings in excellent reviews on the subject [35,36], as well as**
88 **rapid progress in analytical methodologies, it still felt that many more characterization approaches should**
89 **be systematically developed, in particular concerning therapeutic biologics [37] (as opposed to research**
90 **biologics).** Indeed, whenever a polypeptide is produced, whether from recombinant or synthetic
91 approaches, it is too often assumed, on the unique basis of a SDS-PAGE electrophoresis and a liquid
92 chromatography coupled to mass spectrometry analysis (LC-MS) that the peptide is pure and
93 homogeneous, while the expected biological activity is at the *rendezvous*. A recent discussion on this
94 subject has been put forward for synthetic peptide [34], while examples have been reported for
95 recombinant proteins [19]. In fact, it is far from clear that such a limited characterization guarantees the
96 homogeneity of the preparation, leading to a false interpretation about the amount of such polypeptide
97 active in a given preparation. **Led by this idea, we wanted to thoroughly describe the characteristics of**
98 **VHHs, as they turned out to be promising alternatives to bigger biologics on the threshold of therapeutic**
99 **use. While VHHs have been so far produced against many different targets, and very well characterized in**
100 **terms of antigen recognition, their structure and overall biophysical characteristics yet remain scarcely**
101 **documented.**

102 In the present paper, we aimed at benchmarking the production and characterization workflows of VHH,
103 using an anti-HER2 VHH as a model. This exploratory study, combining solution-based as well as gas-
104 phase cutting edge approaches, enabled to broaden our functional and structural knowledge of this
105 emerging class of proteins and laid the ground for future investigations. As we entered this new era during
106 which small biologics, and particularly VHHs will take over as first intention drugs against cancers (see
107 discussions on the various advantages of these solutions [38,39]), it will be crucial that the biochemical
108 and analytical methods for a complete characterization of those entities become available with published
109 backgrounds to help promoting and developing the rise of such molecules in the therapeutic arsenal.

110

111

112 2. Materials and methods

113 *2.1 Cloning, expression and purification.* Coding DNA fragments of the various VHHs have been *E.coli*
114 codon optimized, synthesized and cloned into high copy expression vectors with a poly-His-tag at the C-
115 terminal end of each protein. We used two different vectors, pET-15b and pET-20b, respectively for a
116 cytoplasmic and periplasmic expression of the anti-HER2 VHH and only pET-15b for a cytoplasmic
117 expression of both anti-HSA and anti-EGFR1 VHHs. The genetic constructs were transformed into two
118 different *E.coli* strains for the anti-HER2 VHH: *Shuffle T7 express* and *BL21(DE3)* for the cytoplasmic
119 and periplasmic expression respectively and only *Shuffle T7 express* strain for both anti-HSA and anti-
120 EGFR1 VHHs. Bacteria were cultured in a LB medium with ampicillin (100 µg/mL final concentration)
121 and the expression was induced with 0.1 mM IPTG when the OD at 600 nm reached 0.6. The temperature
122 was decreased from 37°C to 22°C during expression that was carried on overnight. Cells were harvested
123 by centrifugation (5000 g, 20 min, 4°C) and lysed by lysozyme membrane digestion (1 mg/mL) into a
124 20 mM Tris-HCl pH 8, 300 mM NaCl buffer supplemented with 10 µg/mL DNaseI and protease
125 inhibitor cocktail during 30 min at room temperature. Cell fragments were harvested by
126 ultracentrifugation (100000 x g, 30 min, 4°C). The supernatant containing the his-tagged VHHs was then
127 subjected to a three-steps purification. The supernatant was first injected onto an equilibrated Ni-NTA
128 agarose resin (Qiagen) with a 20 mM Tris-HCl pH 8, 300 mM NaCl, 10 mM imidazole buffer. The
129 column was washed with 20 mM imidazole and the proteins were eluted with 250 mM imidazole. The
130 second purification step was performed by cation exchange chromatography (IEX, SourceS, GE
131 healthcare) after dialysis of the sample against a buffer composed of 50 mM Tris-HCl pH 7.5, 50 mM
132 NaCl. The sample was injected onto the cation exchange column previously equilibrated with the dialysis
133 buffer. Elution was then achieved with a linear gradient of 1 M NaCl in 50 mM Tris-HCl pH 7.5. The
134 eluted proteins were then concentrated on a 3 kDa MWCO Amicon device (Millipore). The concentrated
135 sample was finally purified by sized exclusion chromatography (SEC) using a HiLoad 26/600 Superdex
136 200 column (GE Healthcare) equilibrated with a 50 mM Tris-HCl pH 7.5, 150 mM NaCl buffer.

137 *2.2 Inclusion bodies (IB) expression and refolding of anti-HER2 VHH.* The *BL21(DE3)* *E.coli* strain
138 transformed with the pET15b vector containing the anti-HER2 VHH DNA was used to express the VHH
139 in IB. Bacteria were cultured in a MagicMedia™ *E. coli* Expression Medium (ThermoFisher Scientific,
140 Villebon, France) with ampicillin (final concentration at 100 µg/mL). The culture was performed
141 overnight at 37°C following the supplier recommendation. Bacteria were then harvested by centrifugation
142 (5000 x g, 20 min, 4°C) and lysed as previously described. After an ultracentrifugation step (100 000 x g,
143 20 min, 4°C), the supernatant was removed and stored for further use. The pellet was washed once with
144 30 mL/L of culture in a buffer composed of 20 mM Tris-HCl pH 8, 150 mM NaCl, 1 M Urea and 1%

145 Triton X-100 and resuspended using a 45 mL Potter-Elvehjem homogeneizer (Sigma). This preparation
146 was then pelleted by ultracentrifugation (100000 x g, 30 min) at 4°C. Again, the supernatant was removed
147 and stored. The resulting pellet was washed a second time using the same method but with a 20 mM Tris-
148 HCl pH 8, 150 mM NaCl buffer. The wash solution was removed and stored, while the pellet was finally
149 solubilized in 30 mL of 20 mM Tris-HCl pH 8, 6 M guanidium (Gdn) buffer using a Potter-Elvehjem
150 homogeneizer. After an ultimate ultracentrifugation (100 000 x g, 30 min, 4 °C), all supernatant and wash
151 fractions collected during the process and containing the denatured VHHs were mixed together in order to
152 proceed to the refolding step. Therefore, the resulting sample was supplemented with 3 mM of reduced
153 glutathione and 0.3 mM of oxidized glutathione then incubated at 4°C at a final concentration of 1 mg
154 protein/mL for 48 h. The refolded proteins were then dialysed overnight at 4 °C against 100 volumes of a
155 buffer containing 50 mM TAPS pH 8, 50 mM NaCl, 0.6 M Gdn and 0.8 M L-Arginine. The dialysis
156 process was repeated using a 20 mM Tris-HCl pH 8 and 150 mM NaCl buffer until a one million final
157 dialysis factor was reached. Purification was first performed by affinity chromatography as previously
158 described and then by hydrophobic chromatography (HIC) to separate the oxidized from the reduced
159 forms of the anti-HER2 VHH using a 5 mL butyl High Performance column (GE Healthcare) for 10 mg of
160 injected proteins. Briefly, the refolded and dialyzed sample were diluted in a buffer comprising 100 mM
161 phosphate pH 7.4 and 1.5 M ammonium sulphate, and injected on the column connected to an Akta FPLC
162 instrument. The proteins were eluted using a gradient of the equilibrium buffer (100 mM NaH₂PO₄
163 pH 7.4). The fractions of interest were finally concentrated and dialysed in a 20 mM Tris-HCl pH 8,
164 150 mM NaCl buffer, before being used for further characterizations.

165 *2.3 Electrophoresis.* SDS-Page experiments were performed using a 4-12 % Nu-Page® gel in MES buffer
166 (Invitrogen, ThermoFisher) with a migration time of 90 minutes and a constant voltage of 120 V. The
167 non-reduced samples (NR) were diluted in a LDS® buffer (Invitrogen) supplemented with 30 mM
168 iodoacetamine and heated 2 min at 55°C. The reduced samples (R) diluted in a LDS® buffer
169 supplemented with 50 mM DTT (Thermo Scientific Pierce) were boiled 5 min at 95°C. For each sample,
170 about 2 µg of proteins were loaded onto the gels. The molecular weight reference was a SeeBlue Plus 2
171 Prestained Standard® (Invitrogen) and the Coomassie blue was a Quick Coomassie Stain® from Generon
172 (Slough, UK). Images were captured with a Gel Doc™ EZ Gel.

173 *2.4 Analytical light scattering.* Analytical light scattering (ALS) analyses were performed using a 1260
174 Infinity HPLC (Agilent) coupled to a light scattering detector Dawn8+ HELEOS II® and a refractive
175 index detector Optilab T-rex™ (Wyatt Technology, Santa Barbara, CA). The size exclusion column was a
176 BioSec3, 3 µm, 100 Å, 4.6 x 300 mm (Agilent). The buffer used for the column equilibration and the
177 sample dilution was composed of 50 mM Tris-HCl pH 7.5, 150 mM NaCl. All samples were diluted in the

178 running buffer to contain 20 µg of proteins for an injection of 30 µL with a flow rate of 0.2 mL/min. The
179 data were collected and analyzed using the Astra® 6.1 software.

180 *2.5 Circular dichroism (CD).* Circular dichroism experiments were performed using a JASCO J-815 CD
181 spectrometer coupled to a JASCO MCB-100 mini-circulation bath and a JASCO MPTC-490S/15 6-
182 position Peltier Type Turret Cell Changer. The cuvettes used were Starna Scientific type 21/Q/1 quartz
183 spectrophotometer cells with a path length of 1 mm. CD, high tension voltage (HT) and absorbance
184 signals were collected from 190 to 260 nm with standard sensitivity and data pitch of 0.1 nm, a DIT of 1
185 sec, a bandwidth of 1 nm, a scanning speed of 50 nm/min, a temperature gradient of 0.5°C/min, a
186 temperature interval of 5°C. Data were accumulated 5 times for each sample which was dialyzed against a
187 100 mM NaH₂PO₄ pH 7.5 buffer with a final concentration of 0.5 mg/mL. The formula used to calculate
188 the ellipticity was the following: $(33 \times (A \times 100)) / (l \times \text{Protein concentration} / \text{MW} \times (\text{number of residues}$
189 $- 1))$ where A, l and MW correspond to the absorbance, the measure cell length and the molecular weight
190 of the protein, respectively. The CD data were recorded at 25°C for each VHH and analysed using
191 BeStSel, an online software enabling to calculate the proportion of secondary structures ^{44,43}.

192 *2.6 Differential scanning fluorimetry and light scattering.* Differential scanning fluorimetry and light
193 scattering experiments were performed on an OPTIM 2® instrument (AVACTA Analytical, Wetherby ,
194 UK). Briefly, intrinsic fluorescence and CD signals from each VHH sample were measured through a
195 temperature ramp ranging from 25 °C to 90°C with an interval of 0.5°C/min and a starting incubation time
196 of 120 s and the data collected was treated with the AVACTA Analysis software. Each experiment was
197 performed in triplicate at a concentration of 2 mg/mL in a 50 mM Tris-HCl pH 7.5, 150 mM NaCl buffer.
198 Data were finally processed using the AVACTA Analysis software v2.0.

199 *2.7 Surface plasmon resonance.* All experiments were performed on a Biacore T200 (GE Healthcare,
200 Uppsala, Sweden) using a functionalized CM5 chip and the corresponding reagents from Biacore. Four
201 flow-cells were activated by 50 µL of an EDC/NHS mixture. Flow-cell one was blocked with 50 µL of
202 ethanolamine and used as a control surface. Flow-cell two three and four were functionalised up to a level
203 of about 1000 resonance units (RU) with a 20 µg/mL solution of a recombinant human ErbB2 antigen
204 (Sino Biological, Wayne, PA, USA, ref: 10004-H08H), a recombinant human EGFR1 antigen (Sino
205 biological, ref: 10001-H08H) or a human serum albumin antigen (Sigma) in sodium acetate pH 5.0 buffer
206 respectively. The immobilization procedure was performed in a PBS buffer (Biacore) supplemented with
207 0.005 % of P20 surfactant. Increasing amounts of each VHH were injected at a 50 µL/min flow rate in
208 triplicate. The running buffer comprised 50 mM Tris-HCl pH 7.5, 150 mM NaCl supplemented with
209 0.005% P20 surfactant. Each protein was diluted twice into the running buffer with concentrations ranging

210 from 0.122 to 125 nM for anti-HER2 VHH, and from 0.195 to 100 nM for anti-EGFR1 VHH and anti-
211 HSA VHH, respectively. The chips were regenerated with two cycles of 10-sec injections of 3 M MgCl₂
212 separated by a 900-sec delay. The association phase and dissociation phase were run during 4 or 6 min and
213 15 min respectively. Sensorgrams were fitted with Langmuir equation using the BIAeval software.

214 *2.8 Liquid chromatography-mass spectrometry characterization of VHHs.* UHPLC-MS analyses of VHH
215 samples were performed on an Acquity UPLC H-Class Bio system (Waters, Manchester, UK) coupled to a
216 Q-TOF mass spectrometer (Xevo G2-XS, Waters, Manchester, UK). A volume corresponding to 140 pmol
217 of each sample was injected onto a reverse phase liquid chromatography column (BEH C4, 150 x 2.1 mm,
218 300Å, 1.7 µm, Waters). Sample elution was carried-out at 40°C from mobile phases A (water, 0.1% FA)
219 and B (acetonitrile, 0.1% FA) using a flow rate of 0.2 mL/min and a 38-min gradient applied as follows:
220 0% B during 1 min, 0 to 10% B in 0.1 min, 10% to 40% B in 29.9 min, 40% to 90% in 2 min, 90% to 0%
221 B in 0.1 min, 0% B during 4.9 min. Signal acquisition was realized by both UV absorbance measurement
222 at 280 nm and mass spectrometry. The instrument was set in the positive ion mode with a capillary
223 voltage, a desolvation gas flow, a sampling cone voltage and a source temperature of 1.5 kV, 800 L/h, 80
224 V and 120 °C respectively. MS spectra were recorded in the mass range of 500–4000 m/z with a scan time
225 of 1 s. Calibration was performed using the singly charged ions produced by sodium iodide clusters
226 diluted to 2 g/L in a 1:1 (v/v) water:isopropanol solution, and an online mass correction with Leu-
227 enkephalin peptide as the lock mass. Data processing was carried out on Mass Lynx V4.1 (Waters,
228 Manchester, UK). Relative quantification of chromatographically resolved species was performed by
229 integrating their corresponding UV peak area.

230 *2.9 Denaturing and native mass spectrometry.* Anti-HER2 VHH samples were first buffer exchanged
231 against a 100 mM ammonium acetate (NH₄Ac) solution at pH 7.5 using gel filtration (Zeba 0.5 ml,
232 Thermo Scientific, Rockford, IL, USA). Protein concentration was determined spectrophotometrically.
233 NanoESI-MS characterization of these samples was next generated on a hybrid Q-TOF mass spectrometer
234 with an ion mobility cell (Synapt G2-S HDMS, Waters, Manchester, UK) equipped with an automated
235 chip-based nanoESI source (Triversa Nanomate, Advion Biosciences, Ithaca, NY, USA) operating in the
236 positive ion mode. External calibrations were generated from the multiple charged ions produced by a
237 2µM horse heart myoglobin solution diluted in a 1:1 (v/v) water:acetonitrile mixture acidified with 1%
238 (v/v) formic acid, and caesium iodide clusters diluted to 2 g/L in a 1:1 (v/v) water:isopropanol solution.
239 Intact masses of anti-HER2 VHHs were first determined in denaturing conditions by diluting the samples
240 to 2µM in a 1:1(v/v) water/acetonitrile mixture acidified with 1% (v/v) formic acid. Native protein
241 analysis in non-denaturing conditions was then performed by diluting samples to 5µM in 100 mM NH₄Ac
242 buffer at pH 7.5. Fine-tuning of instrumental parameters was notably applied to preserve native 3D

243 structures in the gas phase and ensure efficient ion desolvation and transmission. Particularly, the Synapt
244 G2-S source back-pressure was equal to 4.5 mbar while the acceleration voltages applied on the sample
245 cone as well as in the trap cell were set to 40 and 4 V, respectively. Data processing was finally carried out
246 on MassLynx V4.1 (Waters, Manchester, UK).

247 *2.10 Native HDX-MS.* The protocol was similar to those previously described in native MS, except that
248 D₂O incubations were triggered by a 20-fold dilution of NH₄Ac buffer-exchanged anti-HER2 VHH
249 samples in the same but deuterated 100 mM NH₄Ac buffer at pD 7.5 before infusion (95% final deuterium
250 content). Mixtures were prepared in triplicates and analyzed by native MS in rigorously identical
251 conditions, across an incubation time window ranging from 1 minute to 1 hour. Instrumental conditions
252 were slightly refined (sampling cone and trap acceleration voltages respectively raised to 150 and 20V) to
253 achieve a complete ion desolvation required for accurate deuterium uptake measurements.

254 *2.11 Ion-mobility mass spectrometry (IM-MS).* IM-MS experiments were performed on the
255 abovementioned mass spectrometer. Fine-tuning of the instrument enabled to achieve a good trade-off
256 between native ion preservation, desolvation, separation and TWIMS resolution. Low accelerating
257 voltages were used, especially before IM separation, to guide the ions through the mobility cell to the TOF
258 analyzer without ion activation. Specifically, the Synapt G2-S source back pressure and sampling cone
259 voltage were equal to 5.1 mbar and 20 V, respectively. The argon flow rate and the collision energy inside
260 the ion trap cell were fixed to 2.5 mL/min and 5V, respectively. Prior to IM separation, ions were
261 thermalized in the helium cell (140 mL/min). Ion separation was performed in the pressurized ion mobility
262 cell using a constant N₂ flow rate of 30 mL/min. The IM wave height and velocity were respectively set to
263 35 V and 1334 m/s. Transfer collision energy was fixed to 2 V to extract the ions from the IM cell to the
264 TOF analyzer. Arrival time distributions (ATDs) and collisional cross sections (^{TW}CCS_{N₂→He}) of oxidized
265 and reduced 8⁺ ions were extracted and determined in their native (Trap CE = 5 V) and gas-phase
266 activated conformations (Trap CE = 15 and 60 V) using an appropriate IM calibration (cytochrome-c and
267 β-lactoglobulin native ions used as external calibrants [40,41]). Drift time (t_D) and ^{TW}CCS_{N₂→He} values for
268 each conformer were finally expressed as an average measured in triplicate on the 8⁺ charge state.

269 *2.12 Collision induced unfolding (CIU) experiments.* ATDs were recorded in triplicate by gradually
270 increasing the trap collision energy in the traveling-wave-based ion trap before the IM cell. All the ions
271 were accelerated into the ion trap without any previous selection. However, only ion mobility data
272 corresponding to the 8⁺ charge state of studied samples were compiled to create the averaged CIU
273 fingerprints. The trap collision energy was ramped from 0 to 120 V using an energy interval of 5 V. This
274 voltage interval corresponds to the best trade-off between CIU fingerprint resolution and acquisition time.
275 2D CIU fingerprint plots were analysed using the open source CIUSuite software, especially the

276 CIUSuite_stats and CIUSuite_compare modules [42]. Briefly, these modules allow for drift time data
277 extraction at each trap collision energy (trap CE). Ion intensities were normalized to a maximum value of
278 1 and the ATDs were smoothed using a Savitzky-Golay filter with a window length of 3 and a polynomial
279 order of 2. The individual IM data were gathered to create the CIU data shown. Drift time (ms) is shown
280 in the y-axis versus trap collision energy (V) in the x-axis. The relative intensities of the ATD
281 distributions are denoted by a color-coded axis. CIU fingerprint plots and standard deviations were
282 generated for three replicates using CIUSuite_stats function to quantify the reproducibility of the
283 experiment. Comparison of 2 different CIU fingerprint plots was achieved using the CIUSuite_compare
284 module.

285

287 **3. Results:**

288 *3.1 Production of VHHs, purity and disulfide bridge homogeneity assessment.* The model used in this
289 study is the anti-HER2 VHH 11A4 to which we have added a poly-His tag at the C-terminus. This VHH
290 has been widely described and produced in bacteria system [32]. The periplasmic localization peptide
291 signal sequences, such as *OmpA* or *PelB*, were systematically used to direct the produced VHH in an
292 oxidative environment for the formation of the expected disulfide bridge (see **Figure 1**, for detailed
293 sequences of the different VHHs used). We produced the anti-HER2 VHH in *E. coli BL21(DE3)* strain
294 with or without the alternative periplasmic peptide signal sequence, *PelB*, to evaluate its impact on the
295 production yield compared to the classically used *OmpA* sequence, as well as on the disulfide bridge
296 formation. The results are presented in **Figure 2**. First, we obtained about 1 to 10 mg/L of purified
297 proteins from periplasmic (*OmpA* or *PelB*) and cytosolic expressions, respectively (**Table 1**), suggesting
298 that the periplasmic signal sequence *PelB* from the pET20b vector does not affect the expression of
299 VHHs. The SDS-PAGE analysis of the final products demonstrates the purity of the anti-HER2 VHH and
300 gives some information on the disulfide bridge presence. First of all, a well-defined band with an apparent
301 molecular weight of 14kDa is commonly observed for each preparation, confirming sample purity. As
302 expected, the production in the periplasmic space (PS) results in a clear shift of the migration distance
303 between non-reducing and reducing conditions and thus, indicates the presence of the disulfide bridge
304 upon periplasmic expression, while not formed in the cytosolic space (**Cyto, Figure 2A**). To maintain
305 both of these favourable conditions (high production yield and disulfide bridge formation), we chose to
306 produce this VHH in the bacterial strain *Shuffle T7* (Shu) capable of protein disulfide bond formation in its
307 cytoplasm as described for other proteins [43]. As shown in **Table 1**, the production yield of the *Shuffle*
308 *T7* strain remained expectedly high (≈ 10 mg/L) compared to the cytosolic expression in the *E.coli*
309 *BL21(DE3)* strain. In **Figure 2A**, however, the “Shu” gel lane does not show a significant band shift
310 between non-reducing and reducing conditions. This NR band is more diffuse than in the cytosolic and
311 periplasmic samples, indicating a potential mixture of disulfide bonded and non-bonded species.
312 Alternatively, VHHs can also be expressed in inclusion bodies (IB) to increase their production yield [44].
313 The main downside of this approach is that this production mode necessitates subsequent refolding steps,
314 which can affect the protein ternary structure and potentially induce undesired chemical modifications.
315 However, these constraints are counterbalanced by a gain in production yield. We thus produced another
316 batch of anti-HER2 VHH in *BL21(DE3)* using high expression conditions (Magic Media®). The protein
317 was directly purified under denaturing condition (6 M guanidine) and refolded with an optional oxidation
318 step in the presence of the redox GSSG/GSH couple. We refined the refolding process described by Dagar

319 *et al* [45] for the screen of buffers and added a preliminary oxidation step by bringing a reduced and
320 oxidized glutathione mixture (10:1 ratio) to form the disulfide bridge. We adapted the refolding process
321 described by Tsumoto *et al* [46] by adding high concentration of L-Arginine (1 M) during the refolding
322 steps. Subsequent purification enabled us to isolate two refolded fractions (**Figure 2A**, lines RO and RR
323 respectively). Markedly, these conditions led to a 10 fold production yield increase compared to the
324 reference cytosolic conditions and thus represent a beneficial solution for the production of large amounts
325 of the desired protein (see **Table 1** for further details). We obtained a high refolding yield of nearly 90%
326 and 75% for the oxidized and reduced conditions, respectively. The band shift upon reduction was
327 observed on the SDS-PAGE gel for the RO sample but not for the RR sample, indicating respectively the
328 presence and absence of the disulfide bridge (**Figure 2A**, lines RO and RR).

329 Based on these trials, we also produced two other VHHs in the *Shuffle T7 E. coli* strain, namely the anti-
330 HSA [29] and the anti-EGFR1 [25] which share a high framework sequence homology with the anti-
331 HER2 (see **Figure 1** for sequence details). The main difference lies in the length of their CDR regions,
332 particularly the CDR3. Contrarily to the anti-HER2 VHH, the clear migration shift observed between
333 reducing and non-reducing gel analyses of the anti-EGFR1 VHH strongly suggested the formation of the
334 disulfide bond (**Figure 2B**). Conversely, such a shift was not observed for the anti-HSA VHH,
335 hypothesizing that the S-S bond was not formed under these *ad hoc* conditions (**Figure 2C**).

336 *3.2 Size exclusion chromatography with multi-angle light scattering analysis (SEC-MALS)*. The disulfide
337 bridge is an important key lock for the β -sheet stabilization of VHH structures. It can then impact the
338 VHH recognition and affinity properties for its target. Before looking more precisely into this matter, the
339 VHHs of the present work were all characterized by SEC-MALS in order to assess their homogeneity and
340 size distribution under non-denaturing conditions (**Figure 3**). SEC chromatograms of anti-EGFR1 and
341 anti-HSA VHHs displayed a unique symmetrical peak confirming sample purity and homogeneity (**Figure**
342 **3G**). All the anti-HER2 chromatograms except the RR sample also showed a unique and symmetrical
343 elution peak (**Figures 3A, 3B, 3C, 3D and 3F**). The RR sample evidenced a small shoulder preceding the
344 monomeric elution peak that might be due to a dimerization of the protein (**Figure 3E**). MALS molecular
345 weight calculations based on RI and UV signals give similar results, as summarized in **Table 2**. Although
346 slightly over-estimated, the molecular weights calculated are in the range of the theoretical molecular
347 weights determined from the primary sequence of each VHH.

348 *3.3 Liquid chromatography coupled to ultraviolet absorption and to mass spectrometry (LC-UV-MS)*
349 *analysis*. To get a more comprehensive picture of the samples' homogeneity and purity, LC-UV-MS
350 experiments were performed. This conventional technique affords to separate and quantify
351 hydrophobically resolved species, before identifying them from their denatured intact mass measurements.

352 Anti-HER2 VHH samples were first characterized (**Figures 4A to 4F** and **Table 3**). Periplasmic and
353 cytoplasmic VHHs were shown to be the most homogeneous samples, LC-UV chromatograms revealing a
354 predominant species eluted at 20.1 min (97 % abundance) or 21.3 min (86 %) which corresponds to the
355 oxidized (14153.1 ± 0.3 Da) or the reduced form (14155.0 ± 0.1 Da) of the anti-HER2 VHH (theoretical
356 average mass: 14155.8 Da, assuming no SS bridge and N-terminal Met), respectively (**Figures 4A** and
357 **4B**). Interestingly, the disulfide state could also be addressed by LC because the SS bridge led to a
358 significant hydrophobicity decrease in denatured and oxidized anti-HER2 VHH fractions (-1.2 min of
359 retention time), potentially due to additional constraints induced in their solvent-accessible surface. This
360 enables to separate them from denatured and reduced fractions under the baseline. Furthermore, a
361 significant proportion of oxidized forms (13 % abundance) was also detected in the cytoplasmic sample,
362 while reduced forms appeared to be negligible in the periplasmic sample (2 %). Finally, traces of extra-
363 oxidation (1 %) occurring on both redox and main forms were concomitantly detected in the cytoplasmic
364 (14170.6 ± 0.3 Da, 21.1 min) and the periplasmic sample (14169.2 ± 0.3 Da, 19.9 min). *Shu* sample
365 appeared to be much more heterogeneous than the two previous ones (**Figures 4C** and **4F**). Indeed, a
366 higher mixture of the abovementioned oxidized (14152.9 ± 0.2 Da, 23 % abundance) and reduced
367 (14155.1 ± 0.2 Da, 56 %) fractions was detected at the same retention times. More preoccupying,
368 additional variants were unexpectedly observed and associated to formylated forms of reduced ($14183.0 \pm$
369 0.1 Da, 15 % abundance, 22.5 min retention time) and oxidized anti-HER2 VHHs (14181.0 ± 0.3 Da, 5 %
370 abundance, 21.0 min), and to a hypothetical GSH adduct form of the reduced VHH (14284.1 ± 0.2 Da, 1
371 % abundance, 22.1 min). Such modifications were also found in RO and RR samples of similar
372 heterogeneity (**Figures 4D** and **4E**). First of all, and similarly to periplasmic and cytoplasmic batches, a
373 high redox homogeneity was found in each sample. Indeed, RO is mainly composed of oxidized anti-
374 HER2 VHHs (14153.0 ± 0.2 Da, 66 % abundance) with only traces of reduced fractions (14154.9 ± 0.2
375 Da, 2 %) (**Figure 4D** and **Table 3**). Conversely, RR displayed a high proportion of reduced anti-HER2
376 VHHs (14155.1 ± 0.2 Da, 74 %) and only traces of oxidized fractions (14153.9 ± 0.1 Da, 4 %) (**Figure 4E**
377 and **Table 3**). However, these main redox states also included modified anti-HER2 VHH forms: extra-
378 oxidation and formylation from RO (14168.8 ± 0.1 Da, 2% abundance and 14181.0 ± 0.3 Da, 30 %), but
379 also from RR (14170.7 ± 0.2 Da, 2 % and 14183.1 ± 0.2 Da, 20 %), respectively. Anti-HSA and EGFR1
380 VHHs were next LC-UV-MS probed to be compared to anti-HER2 VHH also produced in *Shuffle T7 E.*
381 *coli* (**Figures 4F, 4G** and **4H** and **Table 3**). Contrarily to anti-HER2, anti-HSA and anti-EGFR1 VHH
382 samples appeared highly homogeneous, both from the disulfide state and the post-translational
383 modifications (PTM) side. Indeed, LC-UV chromatogram from anti-HSA VHH sample depicted a main
384 (89 % abundance) and a minor species (11%), eluted at 21.2 and 21.8 min, and corresponding to the free
385 oxidized form (12990.0 ± 0.4 Da) and a hypothetical GSH adduct form (13119.2 ± 0.3 Da) of the anti-

386 HSA VHH (theoretical average mass: 12992.5 Da, assuming no SS bridge and a N-terminal Met),
387 respectively (**Figure 4G** and **Table 3**). Besides behaving as a fully oxidized sample, anti-EGFR1 also
388 showed to be PTM free, as suggested by the unique LC-UV species detected at 22.4 min and associated to
389 the oxidized form (15295.2 ± 0.1 Da) of the anti-EGFR1 VHH (theoretical average mass: 15297.9 Da,
390 assuming no SS bridge and N-terminal Met) (**Figure 4H** and **Table 3**). Interestingly here, hydrophobicity
391 of oxidized and unmodified forms from these 3 denatured VHHs (HER2 < HSA < EGFR1) did not exactly
392 follow their measured average masses (HSA < HER2 < EGFR1), suggesting a strong hydrophobic
393 contribution of the less-conserved CDR3 domain notably between HER2 and HSA.

394 *3.4 Binding properties analysed by surface plasmon resonance (SPR).* Once the homogeneity and purity
395 of these VHHs has been assessed, we wanted to measure the affinity of these proteins for their respective
396 “antigens” and to compare it to already available data: anti-HER2 (1.9 nM) [30]; anti-HSA (17 nM) [29];
397 anti-EGFR (14 nM on cells) [47]. Surface plasmon resonance (SPR) measurements revealed that all VHHs
398 presented a good affinity for their corresponding target with respective Kds of 11.3 nM, 4.8 nM and about
399 1 nM for anti-HSA, anti-EGFR1 and anti-HER2 VHHs (see **Table 4** for details, and **Figure 5** for
400 sensorgram examples). SPR analyses also enabled to determine binding kinetics, notably the association
401 (k_{on}) and dissociation rates (k_{off}) for each VHH. Herein, the anti-EGFR1 VHH associates more slowly with
402 its target ($k_{on} \approx 2.10^5 \text{ M}^{-1} \text{ s}^{-1}$) than anti-HER2 VHH ($k_{on} \approx 4.10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$) and anti-HSA VHH ($k_{on} \approx 8.10^5$
403 $\text{M}^{-1} \cdot \text{s}^{-1}$), but also dissociated more slowly ($k_{off} \approx 9.10^{-4} \text{ s}^{-1}$) than the two others ($k_{off} \approx 4.10^{-3}$ and 9.10^{-3} s^{-1} ,
404 respectively). Interestingly, all the anti-HER2 VHHs exhibit similar k_{on} and k_{off} parameters, suggesting
405 that in-mixture micro-heterogeneous forms previously depicted (disulfide bridge, PTMs), would not
406 significantly modify binding properties.

407 *3.5 Secondary structure analysis by circular dichroism.* The high conservation of the immunoglobulin Vh
408 domain scaffold is mainly based on a β -sheet stabilized by the canonical disulfide bridge, which can be
409 easily observed by circular dichroism (CD). CD spectra were thus recorded for each VHH (**Figures 6A**
410 and **6B**). As expected, CD spectra of the different anti-HER2 formats (**Figure 6A**), anti-HSA and anti-
411 EGFR1 VHHs (**Figure 6B**) shared a common spectral feature characterized by a negative ellipticity signal
412 around 210-220 nm and corresponding to a β -sheet motif. However, all anti-HER2 VHHs also displayed
413 an additional negative signal around 230 nm, reported as a distorted β -sheet signature and representing a
414 singular particularity of this VHH compare to the others [48]. CD data sets were next processed using
415 BeStSel (Beta Structure Selection), an online software which enables to calculate the proportion of each
416 secondary structure [49,50]. The results are summarized in **Table 5**. Accordingly, the anti-HSA VHH
417 would be composed of antiparallel β -sheet (64.5 %), turn (13.5 %) and other structures (22 %).
418 Antiparallel β -sheets would thus mostly contribute to the structure of this VHH comprising short CDR

419 domains (22 % of other structures) and would be subdivided into left-twisted (16 % of antiparallel β -sheet
420 content), relaxed (35 %) and right-twisted β -sheets (49 %). Secondary structures would be more complex
421 for anti-EGFR1 VHH due to longer CDR3 domains, but still dominated by antiparallel β -sheets (47.6 %)
422 followed by other structures (45.6 %), turns (6 %) and α -helices (0.8 %). These results are in agreement
423 with the crystal structure of this protein (PDB entry: 4KRN), which notably reveals a distorted α -helix
424 inside the CDR3 loop [51] and a simulated CD spectrum matching our experimental data (data not
425 shown). Again, the antiparallel β -sheets of the anti-EGFR1 VHH would be made of left-twisted (17.6 % of
426 the antiparallel β -sheet structure), right-twisted (28.8 %) and relaxed β -strains (53.6 %). Anti-HER2
427 VHHs CD spectra clearly differed from the two others by a distorted β -sheet contribution at 230 nm.
428 BeStSel analysis suggested that these anti-HER2 VHHs would include 58.3% of antiparallel β -sheets, 1.7
429 % of parallel β -sheets, 10.1 % of turns and 29.9 % of other structures. Contrary to the two other VHHs,
430 this predicted parallel β -sheet part could explain the distortion of the β -sheet structure leading to the
431 additional CD signal at 230 nm. The antiparallel β -sheet content would herein split into left-twisted β -
432 strain (21.6 %), relaxed (32 %) and right twisted β -strains (46.7 %). Interestingly, anti-HSA and anti-
433 HER2 VHHs would rather be composed of right-twisted antiparallel β -strains, while the anti-EGFR1
434 VHH would essentially be made of relaxed antiparallel β -strains.

435 *3.6 Thermal stability analysis.* Two orthogonal techniques were here applied to determine the thermal
436 stability of native 3D structures from each VHH: circular dichroism and differential scanning fluorimetry
437 (DSF), which enables to track any change of secondary structure (**Figures 6C to 6H**) or intrinsic
438 tryptophan fluorescence (**Figure 7**). CD spectral signatures of each VHH (signals at 217 and 232 nm for
439 anti-HER2, 217 nm for anti-EGFR1 and 220nm for anti-HSA) were first monitored to calculate their half-
440 denaturation temperatures (T_m) (**Table 6**). As expected, anti-HER2 VHHs enriched with the SS-forms
441 (PS and RO, **Figures 6C, 6F and 6I**) displayed a higher T_m (around 66 °C) than SH-enriched forms
442 (around 55 °C for Cyto and Shu, **Figures 6D, 6E and 6I**). This structural stability, improved by the
443 presence of the disulfide bridge in anti-HER2 VHHs, was equivalent in the SS-enriched anti-EGFR1 VHH
444 ($T_m \approx 66$ °C, **Figure 6G and 6I**). Unexpectedly, such stability was not found in the fully oxidized anti-
445 HSA VHH, showing a T_m similar to those of anti-HER2 reduced forms ($T_m \approx 55$ °C, **Figure 6H and 6I**).

446 DSF-based stability studies evidenced two kinds of fluorescence profiles, depending on the type of VHH
447 considered. On the one hand, the anti-HSA VHH experienced an expected fluorescence increase as
448 function of the temperature. It translates a growing tryptophan solvent exposition due to protein
449 denaturation (**Figure 7G**). On the other hand, for both anti-EGFR1 (**Figure 7F**) and anti-HER2 VHHs
450 (**Figures 7A to 7E**), a fluorescence decrease was observed as a function of the temperature. This was
451 atypical but could be here explained by the presence of a tryptophan in the CDR2 or CDR3 loop of anti-

452 EGFR1 and anti-HER2 VHHs, respectively. Following this hypothesis, solvent-exposed tryptophans from
453 CDR loops would first result in a high fluorescence signal at low temperature which would then decrease
454 with protein aggregation upon heating. Thermal stability data thereby obtained from DSF analyses of anti-
455 HSA and anti-EGFR1 VHHs were in line with CD results and respectively showed a T_m of about 59 °C
456 and 68 °C (**Table 6, Figures 7G and 7F**). A two-step curve of fluorescence decrease was then measured
457 for Cyto, *Shu* and RR samples of the anti-HER2 VHH (**Figures 7B, 7C and 7E**). The first curve was
458 associated with the highest fluorescence drop and displayed an inflection point or T_m of around 58 °C,
459 close to those obtained by CD on these same samples (55°C, **Figures 6D and 6E, Table 6**). The inflection
460 point of the second curve revealed another T_m of around 73 °C similar to those deduced from the CD
461 analyses of fully oxidized anti-HER2 and anti-EGFR1 samples (70 and 66 °C, **Figures 6C, 6F and 6G,**
462 **Table 6**). Identification of two concomitant T_m values from the above-mentioned DSF studies was
463 however consistent since these samples have been shown to contain a significant mixture of oxidized and
464 reduced VHH forms (**Figure 4 and Table 3**). Taking their respective UV proportions into account, DSF
465 proved to be sensitive enough to unravel such redox mixtures. In contrast, PS and RO samples of the anti-
466 HER2 VHH presented a single-step curve of fluorescence decrease, corresponding to a unique T_m of
467 around 73 °C (**Figures 7A and 7D**). Again, DSF data corroborated CD values (70 °C, **Table 6**) and
468 confirmed the redox homogeneity of these two samples, mostly detected under disulfide bridged forms by
469 LC-MS (**Figure 4 and Table 3**).

470 *3.7 Native mass spectrometry investigation of anti-HER2 VHH intact structures.* In order to get structural
471 insights into anti-HER2 VHHs and to better understand how the disulfide bridge can affect their native 3D
472 structures, native MS was performed. This approach, suited to study non-covalent complexes of
473 biomolecules in the gas phase, has also proved to be sensitive to the solvent accessible surface area
474 (SASA) of in-solution native proteins, enabling to probe their overall folding or induced conformational
475 changes through the charge state distribution (CSD) of their native ions [52–55]. Native MS experiments
476 were here focused on periplasmic and cytoplasmic anti-HER2 VHH samples, previously shown to be the
477 most homogeneous representatives of disulfide bridged or unbridged forms, respectively. MS spectra
478 obtained in non-denaturing conditions were thus compared to those generated in denaturing conditions
479 from these two samples (**Figure 8**). Denaturing MS analyses of infused periplasmic and cytoplasmic
480 samples first corroborated LC-UV-MS data, leading to the single detection of the most abundant oxidized
481 (14153.5 ± 0.1 Da) or reduced (14155.4 ± 0.1 Da) forms, respectively (**Figure 8A**). In addition to the
482 average mass measurements, the redox state of these two denatured species could also be deduced from
483 the isotopic distribution of their related ions (**Figure 8A inset**). Indeed, comparing the isotopic
484 distribution of similarly charged ions observed in denaturing MS analyses of both oxidized and reduced
485 forms (11^+ herein), enabled to systematically evidence a 2 Da mass shift resulting from the disulfide state.

486 Moreover, such observations also led to notice that CSDs resulting from these two denatured redox
487 species strongly differed: the oxidized form displayed a broad CSD shifted towards higher m/z ratios (i.e.
488 lower charge states z, ranging from 6⁺ to 12⁺) and centered on the 8⁺ charge state, while the reduced form
489 exhibited a broad CSD shifted towards lower m/z ratios (i.e. higher charge states z, ranging from 6⁺ to
490 16⁺) and centered on the 13⁺ charge state. These CSD differences suggest that solvent accessibility of
491 basic residues exposed at the surface of these denatured VHHs and prone to ionisation, that is the SASA,
492 would decrease upon SS bridging. In other words, denatured and oxidized species would display a more
493 compact conformation (lower SASA) than denatured and reduced species, in agreement with their
494 differences of hydrophobicity observed by LC-UV-MS. Periplasmic and cytoplasmic samples were then
495 analysed under non-denaturing conditions by native MS (**Figure 8B**). Except from additional species
496 linked to non-covalent and unspecific acetate adducts (+ 62 Da), oxidized and reduced forms were anew
497 detected under the same masses (14153.4 ± 0.1 Da and 14155.3 ± 0.1 Da, respectively) and could still be
498 differentiated using isotopic distributions of their native ions (**Figure 8B inset**). However, CSD
499 differences pinpointed in denaturing MS were no longer observed in native MS. Indeed, each redox
500 species provided a similar and narrower CSD, shifted towards higher m/z ratios (i.e. lower charge states z,
501 only ranging from 6⁺ to 8⁺) and centred on the 7⁺ charge state. Altogether, these data first confirm that
502 SASA of both oxidized and reduced species decrease from denaturing to native conditions, in agreement
503 with the preservation of native-like structures [52,53,55]. But more importantly, the fact that oxidized and
504 reduced forms share an identical CSD in native MS would mean that these two species adopt a similar
505 native structure. Accordingly, the disulfide bridge would not significantly impact anti-HER2 VHH native
506 folding, as suggested by the SEC-MALS and CD data.

507 *3.8 Structural impact of the disulfide bridge in anti-HER2 VHHs assessed by native HDX-MS.* An
508 alternative method to promptly and globally probe the structural impact of the disulfide bridge in anti-
509 HER2 VHHs was to combine native MS with hydrogen/deuterium exchange (HDX). In brief, the ability
510 of amide hydrogens from native protein backbones to exchange with ambient deuterium typically serves
511 as a sensor to track structural and dynamic features of protein complexes [56–58]. This property, linked to
512 solvent accessibility and secondary structures, is commonly addressed from protein to peptide level by MS
513 and has been widely used to decipher folding state and partner binding modes of various protein classes
514 [59–63]. Despite less resolutive, native HDX-MS constitutes an original quench and LC-free approach to
515 additionally probe H/D exchange of side chain labile hydrogens while circumventing potential online
516 digestion and subsequent peptide fractionation bottlenecks [64–66]. In this context, anti-HER2 VHH
517 represents a 127-residue protein containing a total of 243 labile hydrogens (120 on the side chains and
518 termini, 123 on the amide backbone) in its neutral form (**Figure 1**). Since a disulfide bridge implies the
519 loss of two exchangeable hydrogens in both cysteines side chains involved in the bridge, only deuterium

520 uptake differences significantly higher than 4 Da could be interpreted as the result of structural and/or
521 dynamic changes between the two redox forms. Due to the depletion of PS sample stock, such
522 experiments were thus performed between RO and Cyto batches, respectively shown to be mostly
523 oxidized and reduced in LC-UV-MS (**Figure 9** and **Table 7**). Native MS analyses of unlabeled oxidized
524 (RO, **Figure 9A**) and reduced forms (Cyto, **Figure 9D**) confirmed the detection of two single species
525 sharing the same 7⁺-centered CSD but differing from each other by 2 Da (**Table 7**, 14153.8 ± 0.3 and
526 14155.4 ± 0.1 Da, respectively). Extra-formylated forms were also observed in the oxidized VHH sample
527 (**Figure 9A inset**, 14181.7 ± 0.3 Da), increasing MS spectrum heterogeneity and corroborating LC-UV-
528 MS data. Incubating each redox form in D₂O from 1 min (**Figure 9B** and **9E**) to 1h (**Figure 9C** and **9F**)
529 led to a significant but constant mass uptake over time (**Table 7**): unlabeled and oxidized VHH signals
530 were shifted towards higher average masses of 14301.8 ± 2.1 (1min, **Figure 9B**) and 14302.9 ± 1.2 Da
531 (1h, **Figure 9C**), while unlabeled and reduced VHH signals were displaced towards higher average
532 masses of 14304.6 ± 1.0 (1 min, **Figure 9E**) and 14305.0 ± 1.3 Da (1 h, **Figure 9F**). Interestingly, this
533 global deuterium uptake of around 150 Da, appeared limited regarding the number of available labile
534 hydrogens (243, i.e. 62%) and did not markedly progress along the labeling time, suggesting either a rigid
535 and tightly folded anti-HER2 VHH scaffold or in-source back exchange [65]. More importantly, no
536 significant differences could be depicted here between average deuterium uptake curves of oxidized and
537 reduced forms of anti-HER2 VHH (**Table 7**). **Note however that any potential in-source back exchange**
538 **could be here non-reproducible (since occurring on non-denatured ion species) and hide existing uptake**
539 **differences, which is very unlikely and not considered here.** Further comparison of isotopic distributions
540 behind charge states of each redox form was unfortunately hampered by overlapping signals from extra-
541 formylated forms. Altogether, these data also support a negligible effect of the disulfide bridge in the anti-
542 HER2 VHH structure.

543 *3.9 Gas-phase structure and stability of anti-HER2 VHHs probed by native IM-MS and CIU experiments.*
544 Another way to explore anti-HER2 VHH intact structures and investigate to which extent the disulfide
545 bridge can affect them, consisted into coupling native MS with ion mobility (IM). This additional gas-
546 phase dimension, enabling to separate native ions according to their charge but also to their shape, was
547 shown to be a valuable way to assess intact conformations of proteins and their non-covalent assemblies
548 [67–69]. As already described, native IM-MS experiments were carried out on periplasmic and
549 cytoplasmic samples. Arrival time distributions (ATDs), corresponding here to the time spent by oxidized
550 and reduced native ions to drift across the ion mobility cell, were pairwise compared on each common
551 charge state (namely from 6⁺ to 8⁺, see **Figure 8B**) and thus used as a direct mean to compare their
552 respective gas phase conformations (**Figure 10 A**). Indeed, for a given charge state, the more extended the
553 native ion shape is, the more frequent its collisions with IM gas molecules are and the longer its drift time

554 will be. Accordingly, injecting oxidized (PS) and reduced (cyto) native ions of similar charge states in the
555 IM cell under soft energy conditions (trap collision energy = 5 V), systematically provided similar ATDs,
556 as shown on the 8⁺ charge state with average drift times (t_D) centered on 5.62 ± 0.00 ms and 5.69 ± 0.06
557 ms, respectively (**Figure 10A top panel** and **Table 8**). Using an appropriate IM-MS calibration [40], these
558 drift time values could be converted into collision cross sections ($^{TW}CCS_{N_2 \rightarrow He}$, as referred to in Gabelica
559 *et al* [41]) which also showed to be similar for each charge state, as depicted between 8⁺ oxidized ($14.56 \pm$
560 0.04 nm²) and reduced (14.62 ± 0.09 nm²) native ions. Brought together, native IM-MS analyses of
561 oxidized and reduced native ions suggest here that these two redox species would yet display a similar
562 native conformation, in agreement with previous native MS, SEC-MALS and CD studies.

563 Native IM-MS can hence provide access to the global conformation of biological systems in the gas-phase
564 but generally suffers from a limited resolution, rendering detection of subtle conformational changes
565 tricky [70–72]. However, this instrumental bottleneck can sometimes be circumvented by monitoring ion
566 conformations from their native state(s) to their activated state(s) through a collision induced unfolding
567 (CIU) step performed in the gas-phase [73]. Besides giving insights into ion conformational stability, this
568 alternative can also help to resolve two undistinguishable native conformations evolving however towards
569 IM-distinguishable conformational intermediates and has already been applied with success to various
570 protein complexes [74–76]. Such studies are typically led by first submitting ions to growing collisional
571 energies in the trap cell before separating them in the IM cell. CIU experiments were thus performed on
572 the neighbouring oxidized and reduced conformations of anti-HER2 VHH, and more particularly on their
573 8⁺ charge state, selected on the basis of its intensity and its propensity to unfold in the gas phase (**Figures**
574 **10A middle and bottom panels, 10B** and **Table 8**). To that end, oxidized and reduced 8⁺ native ions from
575 periplasmic and cytoplasmic samples were conformationally pre-activated through a ramp of trap collision
576 energy prior to be injected in the IM cell. Remarkably, their conformational stability profiles showed a
577 significant divergence. Indeed, ATD of 8⁺ reduced native ions quickly reacted to collisional energy (from
578 15 V), experiencing a fast and large transition toward higher drift times to ultimately reach (from 20 V) a
579 t_D of 7.06 ± 0.00 ms and a corresponding $^{TW}CCS_{N_2 \rightarrow He}$ of 15.69 ± 0.02 nm² (**Figure 10A middle and**
580 **bottom panels** and **Table 8**). Such a CIU-based conformational change (+7.3 % $^{TW}CCS_{N_2 \rightarrow He}$) was
581 however not observed from the native conformation of 8⁺ oxidized ions. Despite being shifted towards
582 higher drift times over growing collisional energies, ATD of these ions underwent a much more limited
583 transition. Indeed, collisional induced unfolding also started to occur at 15 V but led to an ultimate less
584 extended conformation after 20V, as suggested by a t_D of 6.61 ± 0.00 ms and a $^{TW}CCS_{N_2 \rightarrow He}$ of $15.36 \pm$
585 0.02 nm² (+5.5 % $^{TW}CCS_{N_2 \rightarrow He}$) (**Figure 10A middle and bottom panel** and **Table 8**). Tridimensional
586 representation and comparison of CIU fingerprints rendered these two distinct behaviours more concrete
587 (**Figure 10B**). As described, 8⁺ oxidized native ions appeared conformationally more stable than the

588 reduced ones, unfolding at the same collisional energy threshold (Trap CE = 15 V) but able to retain a
589 much more compact conformation ($t_D \approx 6.6$ ms) than activated reduced forms ($t_D \approx 7.1$ ms). To conclude,
590 8^+ native and PTM free ions extracted from native IM-MS and CIU analyses of RO and RR samples,
591 revealed identical native conformations and CIU fingerprints between RR and Cyto, as well as between
592 RO and PS samples (**Figure 10C**). The same native conformation was also found for 8^+ native and PTM
593 free ions generated from Shu sample while their CIU fingerprint proved here to be a perfect convolution
594 of oxidized and reduced CIU signatures (**Figure 10D**). Interestingly, relative quantifications based on
595 ATD peak areas extracted from activated conformations of oxidized and reduced species (respectively 33
596 and 67%, data not shown) in the transition CE window (20-40 V), match those deduced from LC-UV-MS
597 data (respectively 29 and 71% by only considering these two species).

598

4. Discussion:

600 As previously stated, VHH, the smallest functional part of camelid heavy-chain antibodies, was
601 transformed from a Mother Nature exception into an ingenious way to address diseases [77]. As often in
602 Research, the first steps towards new technologies, beyond the visions nourished by those discoveries on
603 what they can be used for, are the practical considerations that are too often neglected in the first place.
604 Because the therapeutic potential of the antibody area is immense, but also because the technical
605 practicability forms a major obstacle, any extension of that domain towards routes that will simplify the
606 approaches should be welcomed. The general view of the antibody therapy in oncology has been that the
607 proteins *per se* were only a way to characterize the cancer cells in a physio-pathological environment. It
608 was next suggested that adding one or several cytotoxic compounds to those antibodies would turn them
609 into missile-launching cargos far more effective than previous addressing systems, using a series of
610 proteins that might cover cancer-specific targets, such as kit [78], TIM-1 [79] or guanylyl cyclase C [80]
611 to name only but a few. Then the complexity to obtain such large proteins in industrial quantity, to
612 manipulate and load them, specifically and homogeneously, with cytotoxic compounds turned out to be
613 major problems, sometimes to a point where the project was killed because of practical considerations.
614 The rise of VHH proteins, with molecular weights in the 12 to 15 kDa range – as opposed to the 150 kDa
615 of antibodies – renders those alternative strategies far more attractive. The benefits of those VHHs have
616 been nicely discussed by van Bockstaele *et al* [77]. Furthermore, one can also imagine, in view of recent
617 progresses in the chemical synthesis of proteins [81,82], that those proteins can be chemically synthesized.
618 This opportunity would also facilitate the introduction of exotic amino acids that could be linked to
619 cytotoxic compounds using alternative chemistry routes. **We recently obtained the first synthetic anti-
620 HER2 VHH [83], opening a route towards the possibility of using this approach to introduce orthogonal
621 new chemistry to build armed VHHs.** Too often as well, the practical evaluation of the characteristics of a
622 given compound, such as innovative (bio)molecules is at first neglected. In the present work, we wanted to
623 assess the technical steps required to understand the way those small proteins were produced, purified and
624 characterized. For this, we designed a workflow that we dissected and benchmarked on three relevant
625 VHHs raised against potential biomarkers in numerous pathologies, namely HSA, HER2 and EGFR1.
626 This pilot study challenged here different modes of bacterial production and showed how these can impair
627 VHH key attributes such as yield, homogeneity, structure and function. It is somewhat surprising that an
628 area of such great potential be too often poorly characterized in terms of biophysical properties, despite
629 beautiful biological data obtained with those objects [84–89]. Therefore, we embarked in a multi-stage
630 characterization process during which model functional VHHs were thoroughly analyzed for their

631 homogeneity and structures. Attempts were even made to crystalize the anti-HER2 VHH without success
632 despite the 167 VHH structures deposited in the protein Data Bank.

633 Taking advantage of our passed knowledge in protein expression, we addressed the question of the
634 bacteria production of anti-HER2 VHH and set our experiments to compare its behavior with those of
635 other similar VHHs (anti-HSA and anti-EGFR1). After having shown that VHHs could be expressed at
636 high purity and quality, it seemed essential to rigorously characterize these recombinant VHHs. Indeed,
637 one should keep in mind that boosting the yield in bacterial production systems enhances the risk to
638 increase the sample heterogeneity. Despite those drawbacks, the whole scheme is amenable to an
639 industrial process, broadening the perspective to commercially propose these proteins. However, it would
640 be advised to avoid refolding strategies for big scale productions, due to potential modifications that can
641 result from such processes. LC-UV-MS experiments have revealed the heterogeneity of VHHs produced
642 in bacteria system in terms of disulfide bridge formation (anti-HER2 VHH: Shu, Cyto) and post-
643 translational modifications performed in the *Shuffle T7* strain and in IB expression system. The
644 periplasmic expression system shows one way to promote VHH production even if the yield is low
645 comparing to cytoplasmic or expression in IB.

646 As in many other domains, a rigorous quality control is required for the production of proteins aimed at
647 human treatments. As evidenced in the present study, SDS-PAGE appeared insufficient to extensively
648 assess sample purity and homogeneity. Indeed, this approach clearly confirmed the disulfide bridge
649 formation in anti-EGFR1 VHH but led to ambiguous or erroneous interpretations for anti-HER2 and anti-
650 HSA VHHs. Such information requires to be investigated with more sophisticated techniques. Among
651 those techniques, LC-UV-MS was proved to be a valuable tool not only to identify but also to quantify
652 hydrophobically fractionated species such as reduced, oxidized and modified forms of anti-HER2 VHH.
653 Thus, the main technique to observe the adequate formation of this bridge would be LC-MS. Furthermore,
654 MS offers the advantage to validate the presence of the desired product, and to assess its purity, while
655 documenting the nature of possible amino acid modifications that could have occurred along the process.

656 Many efforts have notably been made here to characterize and understand the importance of the disulfide
657 bridge in the overall VHH structure and affinity for its target. The covalent bond was proved to be a key
658 point not only to contribute to VHH folding but also to maintain its structure. Indeed, the SDS-PAGE
659 clearly indicates the presence of the disulfide bridge as it has been shown for the anti-EGFR1 VHH but it
660 could be more uncertain as for the anti-HER2 VHH or totally false as for the anti-HSA VHH and, by
661 consequence, high resolution techniques should be used to clearly characterize the sample composition.
662 This first part of the data obtained on samples produced in the *Shuffle T7* strain indicates that this bacteria
663 strain could be useful for VHH expression in a SS state (anti-HSA and anti-EGFR1 VHHs), although this

664 competence seems also to depend on the amino-acid sequence of the protein and could lead to additional
665 protein modifications (anti-HER2 VHH). Altogether, these results suggest that the formation of the
666 disulfide bridge is not only related to the oxidative environment during the expression but also to the
667 sequence of the VHH CDRs, despite the close similarity between the three VHH frameworks presented
668 herein. Interestingly, no significant K_d differences were observed between the different anti-HER2
669 VHHs, suggesting that the presence of disulfide bridge has a negligible impact on the protein binding
670 ability. Furthermore, the affinity constants of those VHHs were in the range of the corresponding
671 antibodies. VHHs possess a canonical structure comprising a relatively standard scheme, with common
672 features separated by usually 3 variable domains: CDR1, 2 and 3. Briefly, the three VHHs studied here
673 are all composed of 8 residues for the CDR1 with a common GXTFXXF/YX pattern, a CDR2 of 7 or 8
674 residues with very closely related sequences and a highly variable CDR3 (**Figure 1**). Indeed, the CDR3 of
675 the anti-HSA is only composed of 9 residues while the CDR3 of anti-HER2 and anti-EGFR1 VHHs
676 contain 14 and 20 residues, respectively. It is obvious that those CDRs are responsible for the recognition
677 of the “antigen”, with a particular contribution of the larger and less conserved one, CDR3. Altogether,
678 data reported in the present work suggest that these VHHs would share an overall antiparallel β -sheet
679 structure, but that small differences in their primary sequences and notably in their CDRs, could strongly
680 affect the torsion/orientation state of their respective antiparallel β -strain. Thus, we believe that these
681 different contributions could be directly linked to the difference of affinity observed between the 3 VHHs
682 and to their ability to form the framework canonical disulfide bridge in the *Shuffle T7 E.Coli* strain,
683 especially in the case of the anti-HER2 VHH which exhibits an additional distorted β -sheet signature in its
684 CD spectrum

685 In conclusion, we believe that the quality control of VHHs, whenever they are produced for research or
686 therapeutic use, **must** be implemented by such means, to ensure their structural and stability properties.
687 Altogether, native MS, HDX-MS, IM-MS and CIU studies first suggest than the disulfide bridge would
688 not alter but strongly stabilize native conformations of anti-HER2 VHHs by covalently locking them, in
689 line with CD and DSF-based stability data. These results also support that the production mode of
690 oxidized and reduced anti-HER2 VHHs would not significantly hamper here their overall structures and
691 stabilities. Finally, native IM-MS and CIU approaches demonstrated to be sensitive enough and with a
692 high resolution capacity to semi-quantitatively address such structural properties inside mixtures of redox
693 forms. To industrialize a VHH as a research or a therapeutic tool, it is really important to identify the
694 contribution of the CDR sequences on the ability to produce high quantity at high quality in an
695 industrialized process and not be only focused on the affinity of this object **towards its “antigen”**.

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5. References

- 698 [1] S. Singh, N.K. Kumar, P. Dwiwedi, J. Charan, R. Kaur, P. Sidhu, V.K. Chugh, Monoclonal
699 Antibodies, *Curr Clin Pharmacol* 13 (2018) 85–99.
- 700 [2] A. Mullard, 2018 FDA drug approvals, *Nat Rev Drug Discov* 18 (2019) 85–89.
- 701 [3] S. Parakh, A.C. Parslow, H.K. Gan, A.M. Scott, Antibody-mediated delivery of therapeutics for
702 cancer therapy, *Expert Opin Drug Deliv* 13 (2016) 401–419.
- 703 [4] M. Chodorge, A.J. Celeste, J. Grimsby, A. Konkar, P. Davidsson, D. Fairman, L. Jenkinson, J. Naylor,
704 N. White, J.C. Seaman, K. Dickson, B. Kemp, J. Spooner, E. Rossy, D.C. Hornigold, J.L. Trevaskis,
705 N.J. Bond, T.B. London, A. Buchanan, T. Vaughan, C.M. Rondinone, J.K. Osbourn, Engineering of a
706 GLP-1 analogue peptide/anti-PCSK9 antibody fusion for type 2 diabetes treatment, *Scientific Reports*
707 8 (2018) 17545.
- 708 [5] A.H. Laustsen, A. Karatt-Vellatt, E.W. Masters, A.S. Arias, U. Pus, C. Knudsen, S. Oscoz, P. Slavny,
709 D.T. Griffiths, A.M. Luther, R.A. Leah, M. Lindholm, B. Lomonte, J.M. Gutiérrez, J. McCafferty, In
710 vivo neutralization of dendrotoxin-mediated neurotoxicity of black mamba venom by oligoclonal
711 human IgG antibodies, *Nature Communications* 9 (2018) 3928.
- 712 [6] A.C. Chan, P.J. Carter, Therapeutic antibodies for autoimmunity and inflammation, *Nat Rev Immunol*
713 10 (2010) 301–316.
- 714 [7] A. Markham, Bezlotoxumab: First Global Approval, *Drugs* 76 (2016) 1793–1798.
- 715 [8] A.M. Scott, J.D. Wolchok, L.J. Old, Antibody therapy of cancer, *Nat Rev Cancer* 12 (2012) 278–287.
- 716 [9] G. Elia, T. Fugmann, D. Neri, From target discovery to clinical trials with armed antibody products, *J*
717 *Proteomics* 107 (2014) 50–55.
- 718 [10] A. Beck, L. Goetsch, C. Dumontet, N. Corvaia, Strategies and challenges for the next generation
719 of antibody-drug conjugates, *Nat Rev Drug Discov* 16 (2017) 315–337.
- 720 [11] A. Thomas, B.A. Teicher, R. Hassan, Antibody–drug conjugates for cancer therapy, *The Lancet*
721 *Oncology* 17 (2016) e254–e262.
- 722 [12] T.N. Iwata, C. Ishii, S. Ishida, Y. Ogitani, T. Wada, T. Agatsuma, A HER2-Targeting Antibody-
723 Drug Conjugate, Trastuzumab Deruxtecan (DS-8201a), Enhances Antitumor Immunity in a Mouse
724 Model, *Mol Cancer Ther* 17 (2018) 1494–1503.
- 725 [13] C. Szot, S. Saha, X.M. Zhang, Z. Zhu, M.B. Hilton, K. Morris, S. Seaman, J.M. Dunleavy, K.-S.
726 Hsu, G.-J. Yu, H. Morris, D.A. Swing, D.C. Haines, Y. Wang, J. Hwang, Y. Feng, D. Welsch, G.
727 DeCrescenzo, A. Chaudhary, E. Zudaire, D.S. Dimitrov, B. St Croix, Tumor stroma-targeted
728 antibody-drug conjugate triggers localized anticancer drug release, *J Clin Invest* 128 (2018) 2927–
729 2943.
- 730 [14] S. Cho, F. Zammarchi, D.G. Williams, C.E.G. Havenith, N.R. Monks, P. Tyrer, F. D’Hooge, R.
731 Fleming, K. Vashisht, N. Dimasi, F. Bertelli, S. Corbett, L. Adams, H.W. Reinert, S. Dissanayake,
732 C.E. Britten, W. King, K. Dacosta, R. Tammali, K. Schifferli, P. Strout, M. Korade, M.J. Masson
733 Hinrichs, S. Chivers, E. Corey, H. Liu, S. Kim, N.H. Bander, P.W. Howard, J.A. Hartley, S. Coats,
734 D.A. Tice, R. Herbst, P.H. van Berkel, Antitumor Activity of MEDI3726 (ADCT-401), a
735 Pyrrolobenzodiazepine Antibody-Drug Conjugate Targeting PSMA, in Preclinical Models of Prostate
736 Cancer, *Mol Cancer Ther* 17 (2018) 2176–2186.
- 737 [15] P. Strop, T.-T. Tran, M. Dorywalska, K. Delaria, R. Dushin, O.K. Wong, W.-H. Ho, D. Zhou, A.
738 Wu, E. Kraynov, L. Aschenbrenner, B. Han, C.J. O’Donnell, J. Pons, A. Rajpal, D.L. Shelton, S.-H.

739 Liu, RN927C, a Site-Specific Trop-2 Antibody-Drug Conjugate (ADC) with Enhanced Stability, Is
740 Highly Efficacious in Preclinical Solid Tumor Models, *Mol Cancer Ther* 15 (2016) 2698–2708.

741 [16] P. Polakis, Antibody Drug Conjugates for Cancer Therapy, *Pharmacol Rev* 68 (2016) 3–19.

742 [17] Y. Mimura, T. Katoh, R. Saldoval, R. O’Flaherty, T. Izumi, Y. Mimura-Kimura, T. Utsunomiya,
743 Y. Mizukami, K. Yamamoto, T. Matsumoto, P.M. Rudd, Glycosylation engineering of therapeutic IgG
744 antibodies: challenges for the safety, functionality and efficacy, *Protein Cell* 9 (2018) 47–62.

745 [18] F.S. van de Bovenkamp, N.I.L. Derksen, P. Ooijevaar-de Heer, K.A. van Schie, S. Kruithof, M.A.
746 Berkowska, C.E. van der Schoot, H. IJspeert, M. van der Burg, A. Gils, L. Hafkenscheid, R.E.M.
747 Toes, Y. Rombouts, R. Plomp, M. Wuhler, S.M. van Ham, G. Vidarsson, T. Rispens, Adaptive
748 antibody diversification through N-linked glycosylation of the immunoglobulin variable region, *Proc*
749 *Natl Acad Sci U S A* 115 (2018) 1901–1906.

750 [19] G. Husson, A. Delangle, J. O’Hara, S. Cianferani, A. Gervais, A. van Dorsselaer, D. Bracewell, C.
751 Carapito, Dual Data-Independent Acquisition Approach Combining Global HCP Profiling and
752 Absolute Quantification of Key Impurities during Bioprocess Development, *Anal. Chem.* 90 (2018)
753 1241–1247.

754 [20] A. Skerra, Alternative non-antibody scaffolds for molecular recognition, *Curr Opin Biotechnol* 18
755 (2007) 295–304.

756 [21] E.R. Goldman, J.L. Liu, D. Zabetakis, G.P. Anderson, Enhancing Stability of Camelid and Shark
757 Single Domain Antibodies: An Overview, *Front. Immunol.* 8 (2017) 865.

758 [22] C. Vincke, S. Muyldermans, Introduction to Heavy Chain Antibodies and Derived Nanobodies,
759 in: D. Saerens, S. Muyldermans (Eds.) *Single Domain Antibodies*, Humana Press, Totowa, NJ, 2012
760 pp. 15–26.

761 [23] S. Muyldermans, Nanobodies, *Annu. Rev. Biochem.* 82 (2013) 775–797.

762 [24] J. Rios-Doria, J. Harper, R. Rothstein, L. Wetzel, J. Chesebrough, A. Marrero, C. Chen, P. Strout,
763 K. Mulgrew, K. McGlinchey, R. Fleming, B. Bezabeh, J. Meekin, D. Stewart, M. Kennedy, P. Martin,
764 A. Buchanan, N. Dimasi, E. Michelotti, R. Hollingsworth, Antibody–Drug Conjugates Bearing
765 Pyrrolobenzodiazepine or Tubulysin Payloads Are Immunomodulatory and Synergize with Multiple
766 Immunotherapies, *Cancer Res* 77 (2017) 2686–2698.

767 [25] A.C. Phillips, E.R. Boghaert, K.S. Vaidya, H.D. Falls, M.J. Mitten, P.J. DeVries, L. Benatuil, C.-
768 M. Hsieh, J.A. Meulbroek, S.C. Panchal, F.G. Buchanan, K.R. Durbin, M.J. Voorbach, D.R. Reuter,
769 S.R. Mudd, L.I. Loberg, S.L. Ralston, D. Cao, H.K. Gan, A.M. Scott, E.B. Reilly, Characterization of
770 ABBV-221, a Tumor-Selective EGFR-Targeting Antibody Drug Conjugate, *Mol Cancer Ther* 17
771 (2018) 795–805.

772 [26] D. Zhang, S.-F. Yu, S.C. Khojasteh, Y. Ma, T.H. Pillow, J.D. Sadowsky, D. Su, K.R. Kozak, K.
773 Xu, A.G. Polson, P.S. Dragovich, C.E.C.A. Hop, Intratumoral Payload Concentration Correlates with
774 the Activity of Antibody–Drug Conjugates, *Mol Cancer Ther* 17 (2018) 677–685.

775 [27] T.M. Cardillo, S.V. Govindan, M.B. Zalath, D.L. Rossi, Y. Wang, C.-H. Chang, D.M.
776 Goldenberg, IMMU-140, a Novel SN-38 Antibody–Drug Conjugate Targeting HLA-DR, Mediates
777 Dual Cytotoxic Effects in Hematologic Cancers and Malignant Melanoma, *Mol Cancer Ther* 17
778 (2018) 150–160.

779 [28] S. Bhakta, L.M. Crocker, Y. Chen, M. Hazen, M.M. Schutten, D. Li, C. Kuijl, R. Ohri, F. Zhong,
780 K.A. Poon, M.A.T. Go, E. Cheng, R. Piskol, R. Firestein, A. Fourie-O’Donohue, K.R. Kozak, H.
781 Raab, J.-A. Hongo, D. Sampath, M.S. Dennis, R.H. Scheller, P. Polakis, J.R. Junutula, An Anti-GDNF

- 782 Family Receptor Alpha 1 (GFRA1) Antibody–Drug Conjugate for the Treatment of Hormone
783 Receptor–Positive Breast Cancer, *Mol Cancer Ther* 17 (2018) 638–649.
- 784 [29] R. Klooster, B.T.H. Maassen, J.C. Stam, P.W. Hermans, M.R. ten Haaft, F.J.M. Detmers, H.J. de
785 Haard, J.A. Post, C. Theo Verrips, Improved anti-IgG and HSA affinity ligands: clinical application of
786 VHH antibody technology, *J Immunol Methods* 324 (2007) 1–12.
- 787 [30] M. Kijanka, F.-J. Warnders, M. El Khattabi, M. Lub-de Hooge, G.M. van Dam, V. Ntziachristos,
788 L. de Vries, S. Oliveira, P.M.P. van Bergen en Henegouwen, Rapid optical imaging of human breast
789 tumour xenografts using anti-HER2 VHHs site-directly conjugated to IRDye 800CW for image-
790 guided surgery, *Eur J Nucl Med Mol Imaging* 40 (2013) 1718–1729.
- 791 [31] M.M. Kijanka, A.S.A. van Brussel, E. van der Wall, W.P.T.M. Mali, P.J. van Diest, P.M.P. van
792 Bergen en Henegouwen, S. Oliveira, Optical imaging of pre-invasive breast cancer with a combination
793 of VHHs targeting CAIX and HER2 increases contrast and facilitates tumour characterization,
794 *EJNMMI Res* 6 (2016) 109.
- 795 [32] M. Kijanka, E.G. van Donselaar, W.H. Müller, B. Dorresteyn, D. Popov-Čeleketić, M. el
796 Khattabi, C.T. Verrips, P.M.P. van Bergen en Henegouwen, J.A. Post, A novel immuno-gold labeling
797 protocol for nanobody-based detection of HER2 in breast cancer cells using immuno-electron
798 microscopy, *Journal of Structural Biology* 199 (2017) 1–11.
- 799 [33] M. Vandesquille, T. Li, C. Po, C. Ganneau, P. Lenormand, C. Duffeffant, C. Czech, F.
800 Grueninger, C. Duyckaerts, B. Delatour, M. Dhenain, P. Lafaye, S. Bay, Chemically-defined camelid
801 antibody bioconjugate for the magnetic resonance imaging of Alzheimer’s disease, *MAbs* 9 (2017)
802 1016–1027.
- 803 [34] J.A. Boutin, A.L. Tartar, A. van Dorselaer, H. Vaudry, General lack of structural characterization
804 of chemically synthesized long peptides, *Protein Sci* 28 (2019) 857–867.
- 805 [35] I.A. Kaltashov, C.E. Bobst, R.R. Abzalimov, G. Wang, B. Baykal, S. Wang, Advances and
806 challenges in analytical characterization of biotechnology products: mass spectrometry-based
807 approaches to study properties and behavior of protein therapeutics, *Biotechnol Adv* 30 (2012) 210–
808 222.
- 809 [36] U. Leurs, U.H. Mistarz, K.D. Rand, Getting to the core of protein pharmaceuticals—
810 Comprehensive structure analysis by mass spectrometry, *Eur J Pharm Biopharm* 93 (2015) 95–109.
- 811 [37] M. Tassi, J. de Vos, S. Chatterjee, F. Sobott, J. Bones, S. Eeltink, Advances in native high-
812 performance liquid chromatography and intact mass spectrometry for the characterization of
813 biopharmaceutical products, *J Sep Sci* 41 (2018) 125–144.
- 814 [38] P. Bannas, J. Hambach, F. Koch-Nolte, Nanobodies and Nanobody-Based Human Heavy Chain
815 Antibodies As Antitumor Therapeutics, *Front. Immunol.* 8 (2017) 1603.
- 816 [39] P. Bannas, F. Koch-Nolte, Perspectives for the Development of CD38-Specific Heavy Chain
817 Antibodies as Therapeutics for Multiple Myeloma, *Front. Immunol.* 9 (2018) 2559.
- 818 [40] M.F. Bush, Z. Hall, K. Giles, J. Hoyes, C.V. Robinson, B.T. Ruotolo, Collision cross sections of
819 proteins and their complexes: a calibration framework and database for gas-phase structural biology,
820 *Anal Chem* 82 (2010) 9557–9565.
- 821 [41] V. Gabelica, A.A. Shvartsburg, C. Afonso, P. Barran, J.L.P. Benesch, C. Bleiholder, M.T.
822 Bowers, A. Bilbao, M.F. Bush, J.L. Campbell, I.D.G. Campuzano, T. Causon, B.H. Clowers, C.S.
823 Creaser, E. de Pauw, J. Far, F. Fernandez-Lima, J.C. Fjeldsted, K. Giles, M. Groessl, C.J. Hogan, S.
824 Hann, H.I. Kim, R.T. Kurulugama, J.C. May, J.A. McLean, K. Pagel, K. Richardson, M.E. Ridgeway,

825 F. Rosu, F. Sobott, K. Thalassinou, S.J. Valentine, T. Wyttenbach, Recommendations for reporting ion
826 mobility Mass Spectrometry measurements, *Mass Spectrom Rev* 38 (2019) 291–320.

827 [42] J.D. Eschweiler, J.N. Rabuck-Gibbons, Y. Tian, B.T. Ruotolo, CIUSuite, *Anal. Chem.* 87 (2015)
828 11516–11522.

829 [43] J. Lobstein, C.A. Emrich, C. Jeans, M. Faulkner, P. Riggs, M. Berkmen, SHuffle, a novel
830 *Escherichia coli* protein expression strain capable of correctly folding disulfide bonded proteins in its
831 cytoplasm, *Microb Cell Fact* 11 (2012) 327.

832 [44] M. Maggi, C. Scotti, Enhanced expression and purification of camelid single domain VHH
833 antibodies from classical inclusion bodies, *Protein Expression and Purification* 136 (2017) 39–44.

834 [45] V.K. Dagar, Adivitiya, Y.P. Khosa, High-level expression and efficient refolding of
835 therapeutically important recombinant human Interleukin-3 (hIL-3) in *E. coli*, *Protein Expression and*
836 *Purification* 131 (2017) 51–59.

837 [46] K. Tsumoto, M. Umetsu, I. Kumagai, D. Ejima, J.S. Philo, T. Arakawa, Role of arginine in
838 protein refolding, solubilization, and purification, *Biotechnol Prog* 20 (2004) 1301–1308.

839 [47] R.C. Roovers, M.J.W.D. Vosjan, T. Laeremans, R. el Khoulati, R.C.G. de Bruin, K.M. Ferguson,
840 A.J. Verkleij, G.A.M.S. van Dongen, P.M.P. van Bergen en Henegouwen, A biparatopic anti-EGFR
841 nanobody efficiently inhibits solid tumour growth, *Int J Cancer* 129 (2011) 2013–2024.

842 [48] P. Manavalan, W.C. Johnson, Sensitivity of circular dichroism to protein tertiary structure class,
843 *Nature* 305 (1983) 831–832.

844 [49] A. Micsonai, F. Wien, É. Bulyáki, J. Kun, É. Moussong, Y.-H. Lee, Y. Goto, M. Réfrégiers, J.
845 Kardos, *BeStSel*, *Nucleic Acids Res* 46 (2018) W315–W322.

846 [50] A. Micsonai, F. Wien, L. Kernya, Y.-H. Lee, Y. Goto, M. Réfrégiers, J. Kardos, Accurate
847 secondary structure prediction and fold recognition for circular dichroism spectroscopy, *Proc Natl*
848 *Acad Sci U S A* 112 (2015) E3095–103.

849 [51] K.R. Schmitz, A. Bagchi, R.C. Roovers, P.M.P. van Bergen en Henegouwen, K.M. Ferguson,
850 Structural Evaluation of EGFR Inhibition Mechanisms for Nanobodies/VHH Domains, *Structure* 21
851 (2013) 1214–1224.

852 [52] Z. Hall, C.V. Robinson, Do charge state signatures guarantee protein conformations?, *J Am Soc*
853 *Mass Spectrom* 23 (2012) 1161–1168.

854 [53] I.A. Kaltashov, R.R. Abzalimov, Do ionic charges in ESI MS provide useful information on
855 macromolecular structure?, *J Am Soc Mass Spectrom* 19 (2008) 1239–1246.

856 [54] K. Lorenzen, E. van Duijn, Native mass spectrometry as a tool in structural biology, *Curr Protoc*
857 *Protein Sci* Chapter 17 (2010) Unit17.12.

858 [55] A. Natalello, C. Santambrogio, R. Grandori, Are Charge-State Distributions a Reliable Tool
859 Describing Molecular Ensembles of Intrinsically Disordered Proteins by Native MS?, *J Am Soc Mass*
860 *Spectrom* 28 (2017) 21–28.

861 [56] G.R. Masson, M.L. Jenkins, J.E. Burke, An overview of hydrogen deuterium exchange mass
862 spectrometry (HDX-MS) in drug discovery, *Expert Opin Drug Discov* 12 (2017) 981–994.

863 [57] A.J. Percy, M. Rey, K.M. Burns, D.C. Schriemer, Probing protein interactions with
864 hydrogen/deuterium exchange and mass spectrometry—a review, *Anal Chim Acta* 721 (2012) 7–21.

865 [58] M.J. Chalmers, S.A. Busby, B.D. Pascal, G.M. West, P.R. Griffin, Differential
866 hydrogen/deuterium exchange mass spectrometry analysis of protein-ligand interactions, *Expert Rev*
867 *Proteomics* 8 (2011) 43–59.

- 868 [59] G. Terral, T. Champion, F. Debaene, O. Colas, M. Bourguet, E. Wagner-Rousset, N. Corvaia, A.
869 Beck, S. Cianferani, Epitope characterization of anti-JAM-A antibodies using orthogonal mass
870 spectrometry and surface plasmon resonance approaches, *MAbs* 9 (2017) 1317–1326.
- 871 [60] N.M. Duc, Y. Du, C. Zhang, S.Y. Lee, T.S. Thorsen, B.K. Kobilka, K.Y. Chung, Effective
872 application of bicelles for conformational analysis of G protein-coupled receptors by
873 hydrogen/deuterium exchange mass spectrometry, *J Am Soc Mass Spectrom* 26 (2015) 808–817.
- 874 [61] D.P. O'Brien, B. Hernandez, D. Durand, V. Hourdel, A.-C. Sotomayor-Pérez, P. Vachette, M.
875 Ghomi, J. Chamot-Rooke, D. Ladant, S. Brier, A. Chenal, Structural models of intrinsically disordered
876 and calcium-bound folded states of a protein adapted for secretion, *Scientific Reports* 5 (2015) 14223.
- 877 [62] J. Lu, R.A. Harrison, L. Li, M. Zeng, S. Gondi, D. Scott, N.S. Gray, J.R. Engen, K.D. Westover,
878 KRAS G12C Drug Development: Discrimination between Switch II Pocket Configurations Using
879 Hydrogen/Deuterium-Exchange Mass Spectrometry, *Structure* 25 (2017) 1442-1448.e3.
- 880 [63] M.B. Trelle, D.M. Dupont, J.B. Madsen, P.A. Andreasen, T.J.D. Jørgensen, Dissecting the effect
881 of RNA aptamer binding on the dynamics of plasminogen activator inhibitor 1 using
882 hydrogen/deuterium exchange mass spectrometry, *ACS Chem Biol* 9 (2014) 174–182.
- 883 [64] V. Katta, B.T. Chait, Conformational changes in proteins probed by hydrogen-exchange
884 electrospray-ionization mass spectrometry, *Rapid Commun Mass Spectrom* 5 (1991) 214–217.
- 885 [65] C.C. Going, Z. Xia, E.R. Williams, Real-time HD Exchange Kinetics of Proteins from Buffered
886 Aqueous Solution with Electrothermal Supercharging and Top-Down Tandem Mass Spectrometry, *J*
887 *Am Soc Mass Spectrom* 27 (2016) 1019–1027.
- 888 [66] H. Xiao, I.A. Kaltashov, Transient structural disorder as a facilitator of protein-ligand binding:
889 native H/D exchange-mass spectrometry study of cellular retinoic acid binding protein I, *J Am Soc*
890 *Mass Spectrom* 16 (2005) 869–879.
- 891 [67] F. Lanucara, S.W. Holman, C.J. Gray, C.E. Eyers, The power of ion mobility-mass spectrometry
892 for structural characterization and the study of conformational dynamics, *Nat Chem* 6 (2014) 281–294.
- 893 [68] C. Uetrecht, R.J. Rose, E. van Duijn, K. Lorenzen, A.J.R. Heck, Ion mobility mass spectrometry
894 of proteins and protein assemblies, *Chem Soc Rev* 39 (2010) 1633–1655.
- 895 [69] J. Seo, W. Hoffmann, S. Warnke, M.T. Bowers, K. Pagel, G. von Helden, Retention of Native
896 Protein Structures in the Absence of Solvent: A Coupled Ion Mobility and Spectroscopic Study,
897 *Angew Chem Int Ed Engl* 55 (2016) 14173–14176.
- 898 [70] C. Atmanene, D. Chaix, Y. Bessin, N. Declerck, A. van Dorsselaer, S. Sanglier-Cianferani,
899 Combination of noncovalent mass spectrometry and traveling wave ion mobility spectrometry reveals
900 sugar-induced conformational changes of central glycolytic genes repressor/DNA complex, *Anal.*
901 *Chem.* 82 (2010) 3597–3605.
- 902 [71] C. Atmanene, S. Petiot-Bécard, D. Zeyer, A. van Dorsselaer, V. Vivat Hannah, S. Sanglier-
903 Cianférani, Exploring key parameters to detect subtle ligand-induced protein conformational changes
904 using traveling wave ion mobility mass spectrometry, *Anal Chem* 84 (2012) 4703–4710.
- 905 [72] J. Stojko, S. Fieulaine, S. Petiot-Bécard, A. van Dorsselaer, T. Meinel, C. Giglione, S.
906 Cianférani, Ion mobility coupled to native mass spectrometry as a relevant tool to investigate
907 extremely small ligand-induced conformational changes, *Analyst* 140 (2015) 7234–7245.
- 908 [73] S.M. Dixit, D.A. Polasky, B.T. Ruotolo, Collision induced unfolding of isolated proteins in the
909 gas phase: past, present, and future, *Curr Opin Chem Biol* 42 (2018) 93–100.

- 910 [74] J.N. Rabuck-Gibbons, J.E. Keating, B.T. Ruotolo, Collision induced unfolding and dissociation
911 differentiates ATP-competitive from allosteric protein tyrosine kinase inhibitors, *International Journal*
912 *of Mass Spectrometry* 427 (2018) 151–156.
- 913 [75] T. Botzanowski, S. Erb, O. Hernandez-Alba, A. Ehkirch, O. Colas, E. Wagner-Rousset, D.
914 Rabuka, A. Beck, P.M. Drake, S. Cianfèrani, Insights from native mass spectrometry approaches for
915 top- and middle- level characterization of site-specific antibody-drug conjugates, *MAbs* 9 (2017) 801–
916 811.
- 917 [76] O. Hernandez-Alba, E. Wagner-Rousset, A. Beck, S. Cianfèrani, Native Mass Spectrometry, Ion
918 Mobility, and Collision-Induced Unfolding for Conformational Characterization of IgG4 Monoclonal
919 Antibodies, *Anal Chem* 90 (2018) 8865–8872.
- 920 [77] F. van Bockstaele, J.-B. Holz, H. Revets, The development of nanobodies for therapeutic
921 applications, *Curr Opin Investig Drugs* 10 (2009) 1212–1224.
- 922 [78] T. Abrams, A. Connor, C. Fanton, S.B. Cohen, T. Huber, K. Miller, E.E. Hong, X. Niu, J. Kline,
923 M. Ison-Dugenny, S. Harris, D. Walker, K. Krauser, F. Galimi, Z. Wang, M. Ghoddusi, K. Mansfield,
924 S.T. Lee-Hoeflich, J. Holash, N. Pryer, W. Kluwe, S.A. Ettenberg, W.R. Sellers, E. Lees, P. Kwon,
925 J.A. Abraham, S.C. Schleyer, Preclinical Antitumor Activity of a Novel Anti-c-KIT Antibody-Drug
926 Conjugate against Mutant and Wild-type c-KIT-Positive Solid Tumors, *Clin Cancer Res* 24 (2018)
927 4297–4308.
- 928 [79] L.J. Thomas, L. Vitale, T. O’Neill, R.Y. Dolnick, P.K. Wallace, H. Minderman, L.E. Gergel, E.M.
929 Forsberg, J.M. Boyer, J.R. Storey, C.D. Pilsmaier, R.A. Hammond, J. Widger, K. Sundarapandian,
930 A. Crocker, H.C. Marsh, T. Keler, Development of a Novel Antibody-Drug Conjugate for the
931 Potential Treatment of Ovarian, Lung, and Renal Cell Carcinoma Expressing TIM-1, *Mol Cancer Ther*
932 15 (2016) 2946–2954.
- 933 [80] K. Almhanna, T. Kalebic, C. Cruz, J.E. Faris, D.P. Ryan, J. Jung, T. Wyant, A.A. Fasanmade, W.
934 Messersmith, J. Rodon, Phase I Study of the Investigational Anti-Guanylyl Cyclase Antibody-Drug
935 Conjugate TAK-264 (MLN0264) in Adult Patients with Advanced Gastrointestinal Malignancies, *Clin*
936 *Cancer Res* 22 (2016) 5049–5057.
- 937 [81] M. Bacchi, B. Fould, M. Jullian, A. Kreiter, A. Maurras, O. Nosjean, T. Coursindel, K. Puget, G.
938 Ferry, J.A. Boutin, Screening ubiquitin specific protease activities using chemically synthesized
939 ubiquitin and ubiquitinated peptides, *Anal Biochem* 519 (2017) 57–70.
- 940 [82] M. Bacchi, M. Jullian, S. Sirigu, B. Fould, T. Huet, L. Bruyand, M. Antoine, L. Vuillard, L.
941 Ronga, L.M.G. Chavas, O. Nosjean, G. Ferry, K. Puget, J.A. Boutin, Total chemical synthesis,
942 refolding, and crystallographic structure of fully active immunophilin calstabin 2 (FKBP12.6), *Protein*
943 *Sci* 25 (2016) 2225–2242.
- 944 [83] L. Hartmann, T. Botzanowski, M. Galibert, M. Jullian, E. Chabrol, G. Zeder-Lutz, V. Kugler, J.
945 Stojko, J.-M. Strub, G. Ferry, L. Frankiewicz, K. Puget, R. Wagner, S. Cianfèrani, J.A. Boutin, VHH
946 characterization. Comparison of recombinant with chemically synthesized anti-HER2 VHH, *Protein*
947 *Sci* (2019).
- 948 [84] H. Nakayama, A. Murakami, M. Yoshida, J. Muraoka, J. Wakai, N. Kenjyou, Y. Ito,
949 Characterization and Selection of 3-(1-Naphthoyl)-Indole Derivative-Specific Alpaca VHH
950 Antibodies Using a Phage Display Library, *Monoclon Antib Immunodiagn Immunother* 35 (2016)
951 231–234.

- 952 [85] J.L. Liu, L.C. Shriver-Lake, D. Zabetakis, G.P. Anderson, E.R. Goldman, Selection and
953 characterization of protective anti-chikungunya virus single domain antibodies, *Mol Immunol* 105
954 (2019) 190–197.
- 955 [86] G. Hussack, S. Raphael, M.J. Lowden, K.A. Henry, Isolation and characterization of camelid
956 single-domain antibodies against HER2, *BMC Res Notes* 11 (2018) 866.
- 957 [87] S. Wöll, C. Bachran, S. Schiller, M. Schröder, L. Conrad, R. Scherließ, L.K. Swee, Sortagging of
958 liposomes with a murine CD11b-specific VHH increases in vitro and in vivo targeting specificity of
959 myeloid cells, *Eur J Pharm Biopharm* 134 (2019) 190–198.
- 960 [88] F. Faraji, N. Tajik, M. Behdani, M.A. Shokrgozar, A.H. Zarnani, F. Shahhosseini, M. Habibi-
961 Anbouhi, Development and characterization of a camelid single-domain antibody directed to human
962 CD22 biomarker, *Biotechnology and Applied Biochemistry* 65 (2018) 718–725.
- 963 [89] L. Schoonaert, L. Rué, B. Roucourt, M. Timmers, S. Little, L. Chávez-Gutiérrez, M. Dewilde, P.
964 Joyce, A. Curnock, P. Weber, J. Haustraete, G. Hassanzadeh-Ghassabeh, B. de Strooper, L. van den
965 Bosch, P. van Damme, R. Lemmens, W. Robberecht, Identification and characterization of
966 Nanobodies targeting the EphA4 receptor, *J Biol Chem* 292 (2017) 11452–11465.
- 967

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969 **Table 1: VHH production and refolding yields from the different bacterial expression systems**
970 **evaluated.**

971

Sample name	Bacteria strain/ Production localization	Production Yield (mg/L of culture)	Refolding process yield (%)
Anti-HER2 PS	<i>E.coli</i> <i>BL21(DE3)/Periplasmic</i>	≈1	ND
Anti-HER2 Cyto	<i>E.coli BL21(DE3)/Cytosolic</i>	≈10	ND
Anti-HER2 Shu	<i>E.coli Shuffle T7/Cytosolic</i>	≈10	ND
Anti-HER2 RO	<i>E.coli BL21(DE3)/Cytosolic</i>	≈100	≈90
Anti-HER2 RR	<i>E.coli BL21(DE3)/Cytosolic</i>	≈100	≈75
Anti-EGFR	<i>E.coli Shuffle T7/Cytosolic</i>	≈10	ND
Anti-HSA	<i>E.coli Shuffle T7/Cytosolic</i>	≈10	ND

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973

974 **Note:** ND: not determined

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Table 2: SEC-MALS analysis

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Sample name	Injected mass (μg)	Mass recovery (%)	Mw (Da)
Anti-HER2 PS	21.60	88.8	14500 \pm 195
Anti-HER2 Cyto	18.80	103.9	14730 \pm 334
Anti-HER2 Shu	21.60	95.6	14670 \pm 223
Anti-HER2 RO	17.40	101.5	14930 \pm 397
Anti-HER2 RR	21.60	80.1	14630 \pm 182
Anti-EGFR	19.00	104.1	15450 \pm 329
Anti-HSA	21.60	92.3	13730 \pm 547

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984 **Table 3: Relative UV quantification and MS assignment of VHH species revealed by LC-UV-MS.**

Species	▲	▲‡	●	●†	●‡	○	○†	○‡	○‡	○‡	■
Measured average mass (Da)	12990.0 ± 0.4	13119.2 ± 0.3	14153.1 ± 0.3	14169.2 ± 0.3	14181.0 ± 0.3	14155.0 ± 0.1	14170.6 ± 0.3	14183.0 ± 0.1	14284.1 ± 0.2	15295.2 ± 0.1	
Assignment	oxidized VHH (HSA)	GSH linked forms?	oxidized VHH (HER2)	extra oxidized forms	formylated forms	reduced VHH (HER2)	extra oxidized forms	formylated forms	GSH linked forms?	oxidized VHH (EGFR1)	
Retention time (min)	21.2	21.8	20.1	19.9	21.0	21.3	21.1	22.5	22.1	22.4	
Relative UV abundance (%)	PS		97	1		2					
	Cyto		13			86	1				
	Shu		23			56		15	1		
	RO		66	2	30	2					
	RR		4			74	2	20			
	HSA	89	11								
	EGFR1									100	

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Table 4: Binding kinetics analysed by Surface Plasmon Resonance

990

	kon ± SD (M⁻¹ s⁻¹) E⁺⁶	koff ± SD (s⁻¹) E⁻³	Kd ± SD (nM)
Anti-HER2 PS	5.39 ± 0.13	4.85 ± 0.13	0.9 ± 0.00
Anti-HER2 Cyto	4.54 ± 0.05	2.77 ± 0.01	0.6 ± 0.00
Anti-HER2 Shu	3.83 ± 0.03	4.01 ± 0.00	1.05 ± 0.01
Anti-HER2 RO	3.05 ± 0.01	4.29 ± 0.12	1.4 ± 0.04
Anti-HER2 RR	3.35 ± 0.06	2.5 ± 0.03	0.7 ± 0.00
Anti-EGFR1	0.20 ± 0.01	0.98 ± 0.00	4.8 ± 0.03
Anti-HSA	0.81 ± 0.03	9.22 ± 0.22	11.3 ± 0.2

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Table 5: CD spectra-derived secondary structures of the studied VHHs.

VHHs	Helix	Helix1 (regular)	Helix2 (distorted)	Antiparallel	Anti1 (left twisted)	Anti2 (relaxed)	Anti3 (right twisted)	Parallel	Turn	Others
Anti-HSA	0	0	0	64.5	10	22.8	31.7	0	13.5	22
Anti-HER2	0	0	0	58.3	12.6	18.6	27.2	1.7	10.1	29.9
Anti-EGFR1	0.8	0	0.8	47.6	8.4	25.5	13.7	0	6	45.6

995

996 **Note:** Proportions of each secondary structure were determined from the CD spectra using the BeStSel online software. The values in brackets
997 correspond to the percentage of the anti1, anti2 and anti3 structure normalized to 100% of antiparallel structure.

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999

1000 **Table 6: Tm calculation by CD and OPTIM.**

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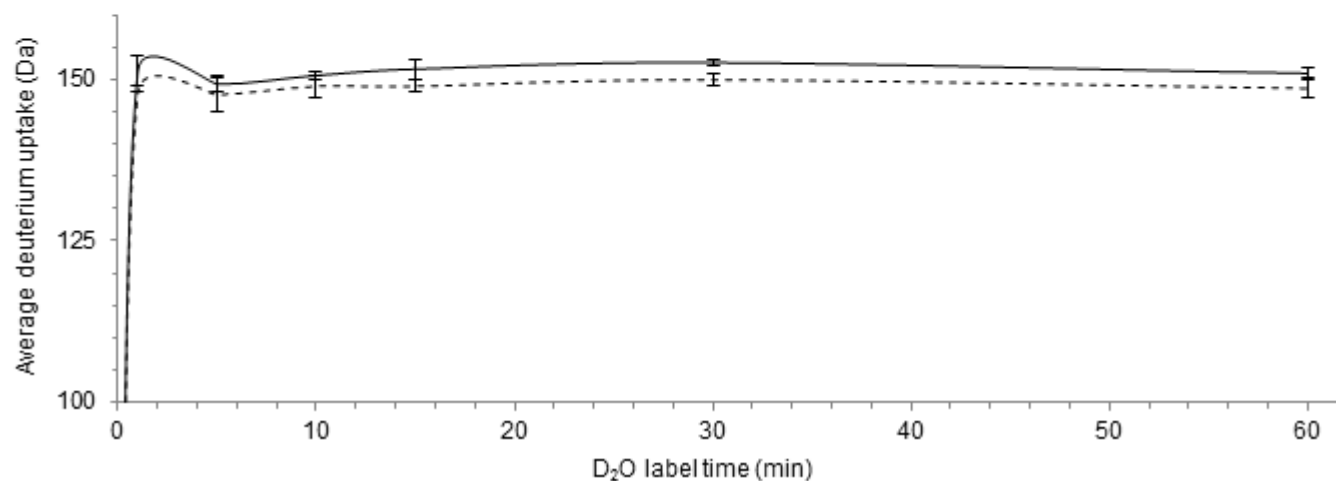
	Tm by CD (°C)	Tm1 by OPTIM with ratio 350/330 nm (°C)	Tm2 by OPTIM with ratio 350/330 nm (°C)
Anti-HER2 PS	70.10 ± 0.21	73.22 ± 0.3	*
Anti-HER2 Cyto	55.09 ± 0.42	56.04 ± 0.17	72.82 ± 0.62
Anti-HER2 Shu	54.93 ± 0.58	56.09 ± 0.16	73.24 ± 1.29
Anti-HER2 RO	70.04 ± 0.18	72.15 ± 0.2	*
Anti-HER2 RR	ND	54.06 ± 0.23	*
Anti-EGFR1	65.50 ± 0.46	67.63 ± 0.32	*
Anti-HSA	54.63 ± 0.3	59.01 ± 0.43	*

1003

1004 **Note:** * No second Tm was observed. ND: not determined

1005 **Table 7: Deuterium uptake of oxidized and reduced anti-HER2 VHHs monitored by native HDX-MS.**

Samples		RO		Cyto	
Native species		●	●	○	○
Assignment		Undeuterated oxidized VHH	Deuterated oxidized VHH	Undeuterated reduced VHH	Deuterated reduced VHH
Measured average mass (Da)	t_0 (unlabeled)	14153.8 ± 0.3		14155.4 ± 0.1	
	t_{1min} (D ₂ O label)		14301.8 ± 2.1		14304.6 ± 1.0
	t_{1hr} (D ₂ O label)		14302.9 ± 1.2		14305.0 ± 1.3



1006

1007 Average deuterium uptakes of oxidized (RO sample) and reduced (Cyto sample) native forms of anti-HER2 VHHs are plotted over label time as
 1008 dashed and solid curves, respectively. Standard deviations displayed represent the experimental variability from sample triplicates for each label
 1009 time.





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1012 **Table 8: Conformational characterization of oxidized and reduced 8⁺ ions under native and activated states by IM-MS.**

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8⁺ charge state	Reduced VHH		Oxidized VHH	
Conformer				
t_D (ms)	5.69 ± 0.06	7.06 ± 0.00	5.62 ± 0.00	6.61 ± 0.00
^{TW}CCS_{N₂→He} (nm²)	14.62 ± 0.09	15.69 ± 0.02	14.56 ± 0.04	15.36 ± 0.02
Δ^{TW}CCS_{N₂→He} (%)	ref.	+7.3	ref.	+5.5

1015

1016

1017 **Note:** ^{TW}CCS_{N₂→He} values of native reduced and oxidized conformations (open and filled circles, respectively) serve here as references (ref.) to
 1018 express corresponding ^{TW}CCS_{N₂→He} increases (%) upon gas-phase activation.

1019

1020 **Legends to the figures:**

1021 **Figure 1: Sequence alignment of anti-HSA, anti-HER2 and anti-EGFR1 VHHs.**

1022 **Figure 2: SDS-Page 4-12% in MES buffer of anti-HER2 VHH samples (A), anti-EGFR1 VHH**
1023 **sample (B) and anti-HSA VHH sample (C) in non-reducing (NR) and reducing (R) conditions.** NR
1024 conditions result in the addition of idoacetamine in the LDS buffer (Invitrogen) while the sample was
1025 boiled 2 minutes at 55°C. R conditions correspond to the addition of 50 mM DTT in the LDS buffer while
1026 the sample was boiled 5 minutes at 95°C.

1027 **Figure 3: SEC-MALS chromatograms of the anti-HER2 VHH samples (A, B, C, D and E), anti-**
1028 **EGFR1 and anti-HSA VHH (F, G) samples.** Chromatograms of the different anti-HER2 VHH samples
1029 are showed in the panel A, B, C, D and E for the PS, Cyto, Shu, RO and RR samples respectively. The
1030 panel F shows the alignment of all anti-HER2 VHH sample chromatograms with in black line the PS, in
1031 dot line the Cyto, in blue line the Shu, in red line the RO and in green line the RR sample. In the panel G,
1032 the anti-EGFR1 chromatogram is represented in black line and the anti-HSA chromatogram in dot line.
1033 The X axis represents the elution time and the Y axis is expressed in a relative scale of the UV signal at
1034 280 nm.

1035 **Figure 4: VHHs redox state and microheterogeneity assessed by LC-UV-MS.** RPLC-UV
1036 chromatograms at 280nm of denatured anti-HER2 VHH samples (A-F), anti-HSA (G) and anti-EGFR1
1037 VHH samples (H). A: PS; B: Cyto; C and F: Shu; D: RO; E: RR. Filled and open symbols respectively
1038 indicate oxidized (SS) and reduced (SH) forms of anti-HER2 (circle), anti-HSA (triangle) and anti-EGFR1
1039 (square) VHHs. Simple, double and triple crosses correspond to extra-oxidized, formylated and GSH-
1040 linked forms, respectively.

1041 **Figure 5: SPR sensorgrams of anti-HER2, anti-EGFR1 and anti-HSA VHH samples.** Anti-HER2
1042 VHH sensorgrams of the PS, Cyto, Shu, RO and RR samples are showed in the panel A, B, C, D and E,

1043 respectively. Anti-EGFR1 and anti-HSA VHH sensorgrams are represented in the panel F and G,
1044 respectively. Each curve corresponds to the kinetic of one concentration from 0.122 nM to 62.5 nM for the
1045 PS, Cyto, RO and RR samples; from 0.17 nM to 40 nM for the Shu sample; from 0.195 nM to 100 nM for
1046 the anti-EGFR1 VHH sample and from 0.39 nM to 200 nM for the anti-HSA VHH sample. The PS, Cyto,
1047 RO and RR samples were injected during 4 min while the Shu, anti-EGFR1 and anti-HSA VHH samples
1048 were injected during 6 min. The dissociation time was 15 minutes for all samples excepted for the anti-
1049 EGFR1 where the dissociation time was done during 30 minutes. Experiments were performed in three
1050 concentration series starting from the lowest to the highest concentration but two series are showed in the
1051 figure. The X axis represents the time and the Y axis the response in RU.

1052 **Figure 6: Circular dichroism spectra of the VHHs. Effect of the temperature.** A: PS sample in black,
1053 Cyto in red, Shu in green and RO in blue. B: anti-EGFR1 and anti-HSA VHH samples are represented in
1054 black line and in dot line, respectively. CD spectra as a function of the temperature are showed in the
1055 panel C, D, E, F, G and H for the PS, Cyto, Shu, RO, anti-EGFR1 and anti-HSA VHH samples,
1056 respectively. Each spectrum corresponds to one temperature from 25°C to 85°C. The panel I represents
1057 the CD value in mdeg at 217 nm for the anti-HER2 (PS in black, Cyto in red, Shu in green and RO in
1058 blue) and anti-EGFR1 VHHs (in orange) and at 220 nm for the anti-HSA VHH (in cyan). From these
1059 curves, the T_m of each sample was calculated using the following equation with Prism software:
1060 $Y = \text{Bottom} + (\text{Top} - \text{Bottom}) / (1 + 10^{-(X - T_m)})$.

1061 **Figure 7: Intrinsic fluorescence of the VHHs as a function of the temperature.** DSF experiments of
1062 anti-HER2 VHH samples are showed in the panel A, B, C, D and E for the PS, Cyto, Shu, RO and RR
1063 samples, respectively. DSF experiments of anti-EGFR1 and anti-HSA VHHs are represented in the panel
1064 F and G, respectively. The X-axis represents the temperature and the Y-axis the fluorescence ratio at 350
1065 and 330 nm. Each experiment was performed in triplicate and the bars represent the error of the
1066 measurement for each point.

1067

1068 **Figure 8: Impact of the disulfide bridge on the anti-HER2 VHH SASA probed by denaturing and**
1069 **native MS.** Denaturing (A) and native ESI-MS spectra (B) obtained from SS (top panel, PS sample) and
1070 SH-enriched forms (bottom panel, Cyto sample) of anti-HER2 VHH under infusion. Filled and open
1071 circles respectively mark oxidized (SS) and reduced (SH) charge state distributions of anti-HER2 VHH.
1072 Insets provide a zoom on a common charge state observed in denaturing (11⁺) and native (7⁺) conditions.
1073 * indicate non-covalent acetate adducts (+ 62 Da).

1074 **Figure 9: Impact of the disulfide bridge on the anti-HER2 VHH SASA probed by native HDX-MS.**
1075 Native ESI-MS spectra obtained from oxidized (A-C, RO sample) and reduced (D-F, Cyto sample) anti-
1076 HER2 VHHs in absence of D₂O (A,D) and after a D₂O incubation of 1 minute (B,E) and 1 hour (C,F).
1077 Filled and open circles respectively indicate oxidized (SS) and reduced (SH) forms of anti-HER2 VHH.
1078 Black and orange coloured circles mark undeuterated and deuterated species. Double crosses correspond
1079 to extra-formylated forms. Insets provide a zoom on the common native charge state 7⁺ observed in
1080 undeuterated and deuterated conditions.

1081 **Figure 10: Impact of the disulfide bridge on the anti-HER2 VHH conformational stability probed**
1082 **by native IM-MS and collision-induced unfolding.** A: Arrival time distributions of oxidized (dashed
1083 curves and filled symbols, PS sample) and reduced (solid curves and open symbols, Cyto sample) 8⁺
1084 charge states extracted from native IM-MS analyses upon various Trap collisional energies: 5V (top
1085 panel), 15 V (middle panel) and 60V (bottom panel). Symbols shape conceptualizes changes occurring in
1086 oxidized and reduced 8⁺ ions conformation along gas-phase activation. B, C: Comparison of CIU
1087 fingerprints obtained between 8⁺ native charge states from B Cyto (left, mostly reduced) and PS (right,
1088 mostly oxidized) samples, C RR (left, mostly reduced) and RO (right, mostly oxidized) samples. d) CIU
1089 fingerprint obtained from 8⁺ native charge state of Shu sample (mixture of reduced and oxidized forms).
1090

HSA	EVQLLESGGGLVQPGGSLRLS	CAAS	GFTFRSFGMSWVRQAPGKGPEWVSSISGSGSDTLY
HER2	EVQLVESGGGLVQAGGSLRLS	CATSGITFMRYALGWYRQSPGKQREMVASIN-SGGTTNY	
EGFR1	EVQLVESGGGLVQAGGSLRLS	CAAS	GRTFSSYAMGWFRQAPGKEREFVVAINWSSGSTYY
	:***:*****	**	*****:*** * : : . * ** :*** * * :* . . . * *
	FR1	CDR1	FR2 CDR2
HSA	ADSVKGRFTISRDNK-TLYLQMNSLRPEDTAVYY	CTIGGSLRS	-----SQG
HER2	ADSVKGRFTISRDNKNTVYLQMNSLKPEDTAVYY	CNARWVKP	-----QFIDNNYWGQG
EGFR1	ADSVKGRFTISRDNKNTMYLQMNSLKPEDTAVYY	CAAGYQINSGNYNFKDYEYDYWGQG	
	*****	. : * * :***** : * :*** **	. **
	FR3	CDR3	
HSA	TLVTVSS---		
HER2	TQVTVSS---		
EGFR1	TQVTVSSALE		
	* *****		
	FR4		

Figure 1

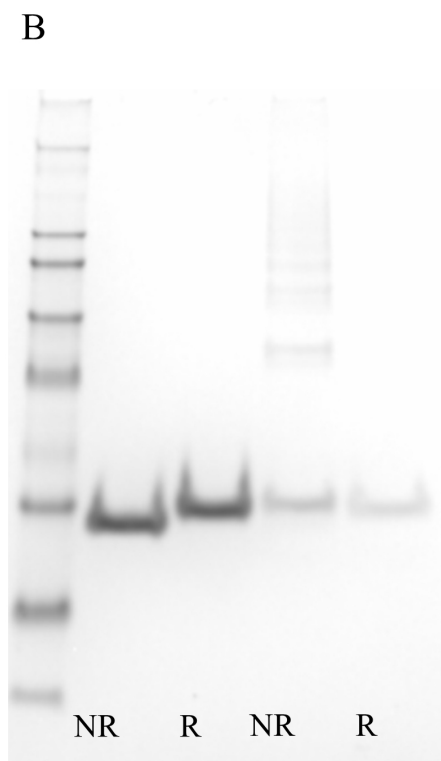
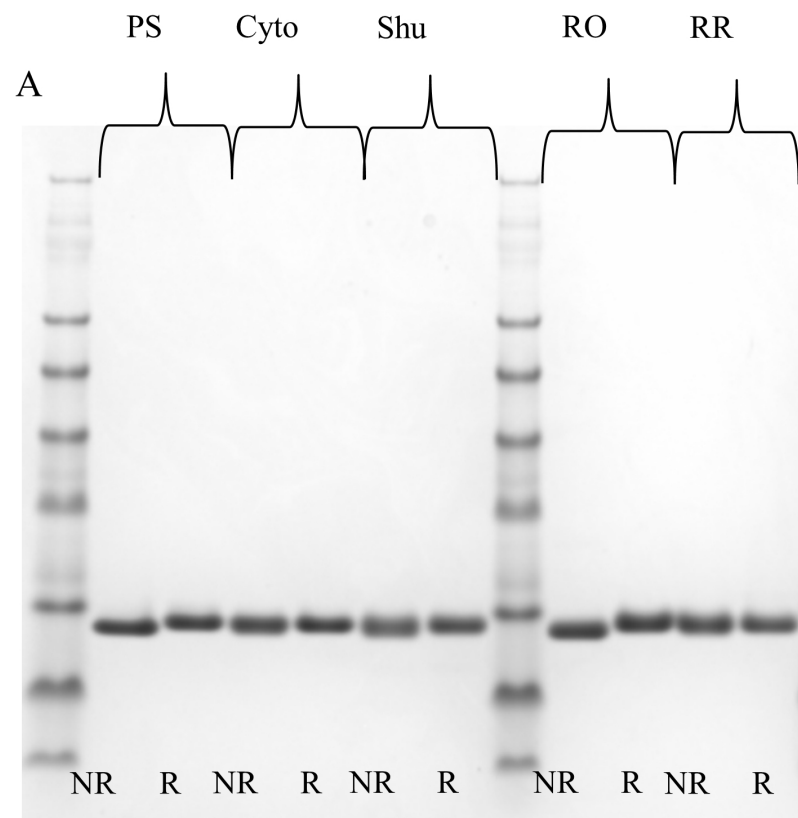


Figure 2

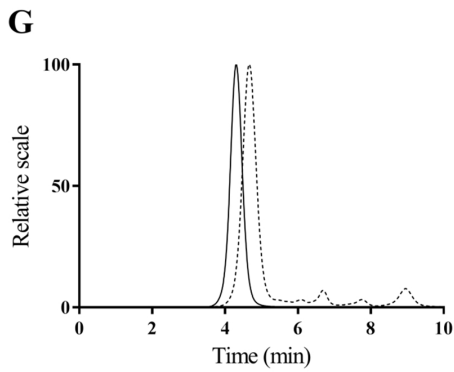
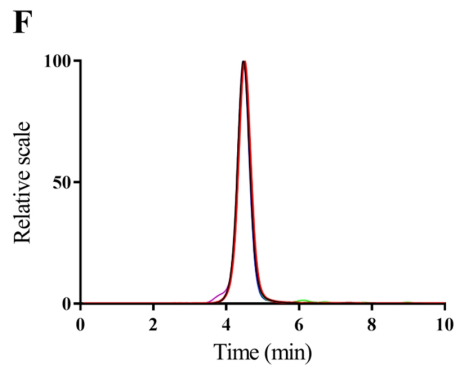
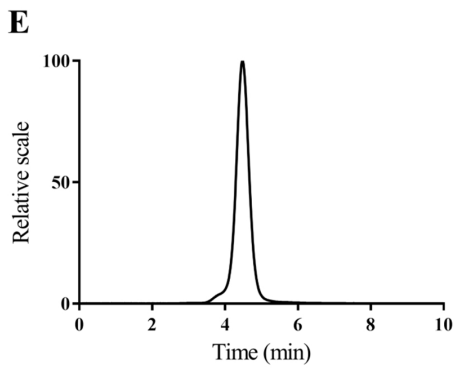
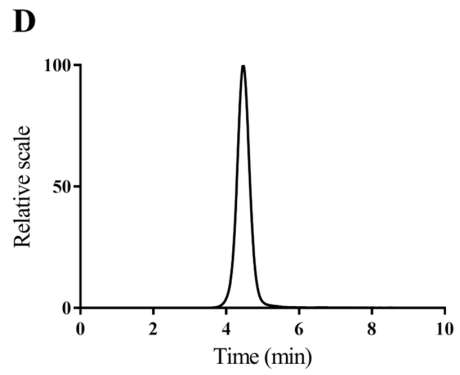
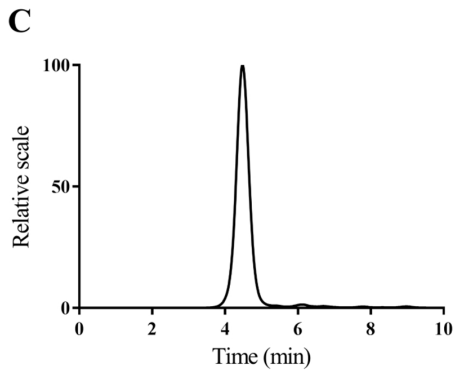
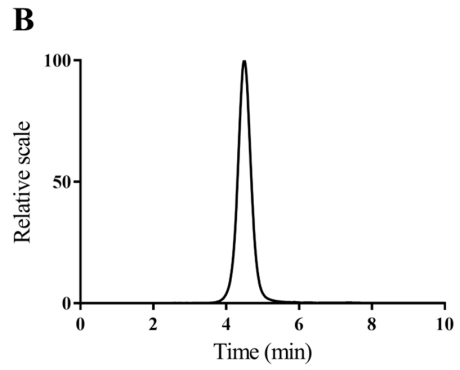
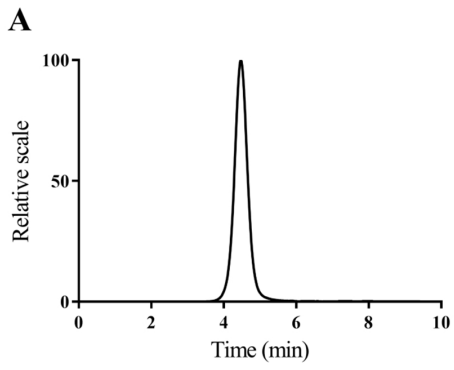
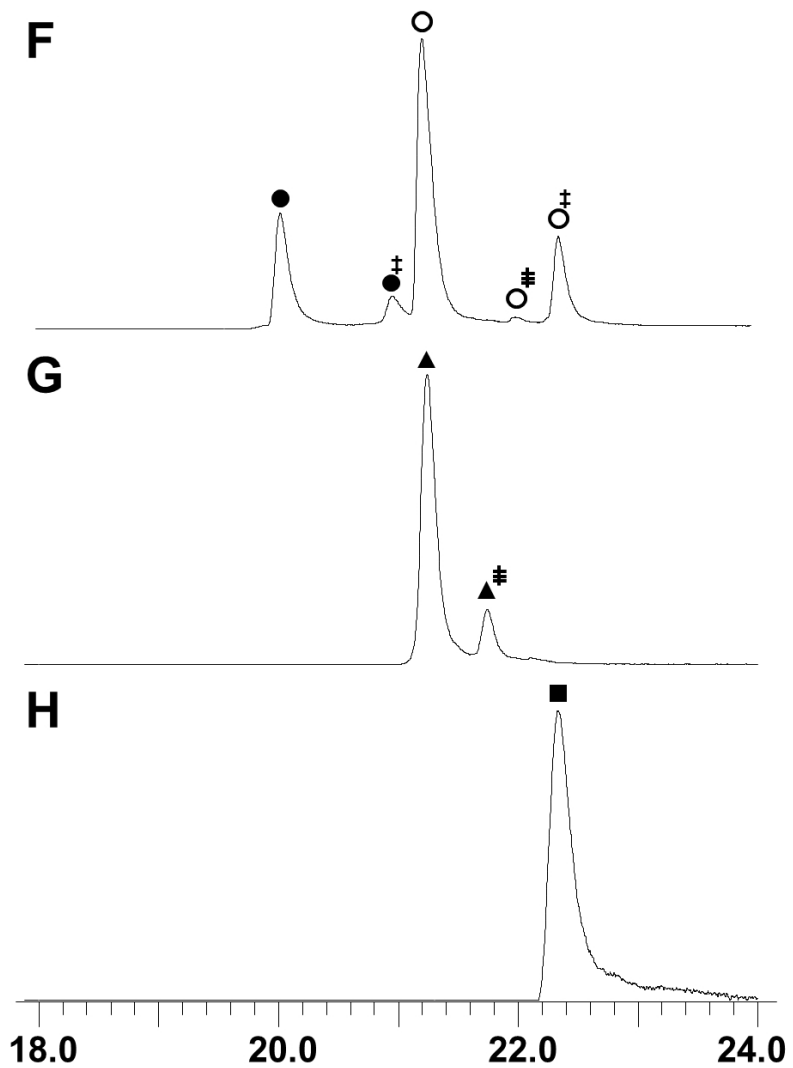
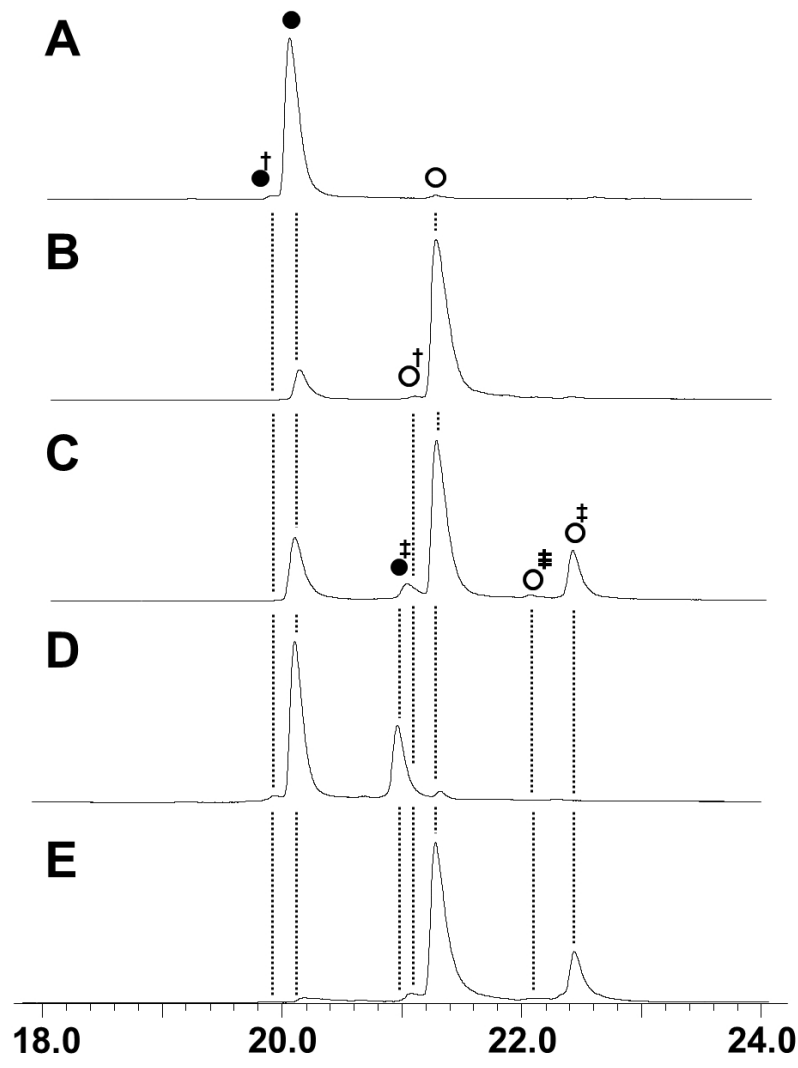


Figure 3



Time (minutes)

Figure 4

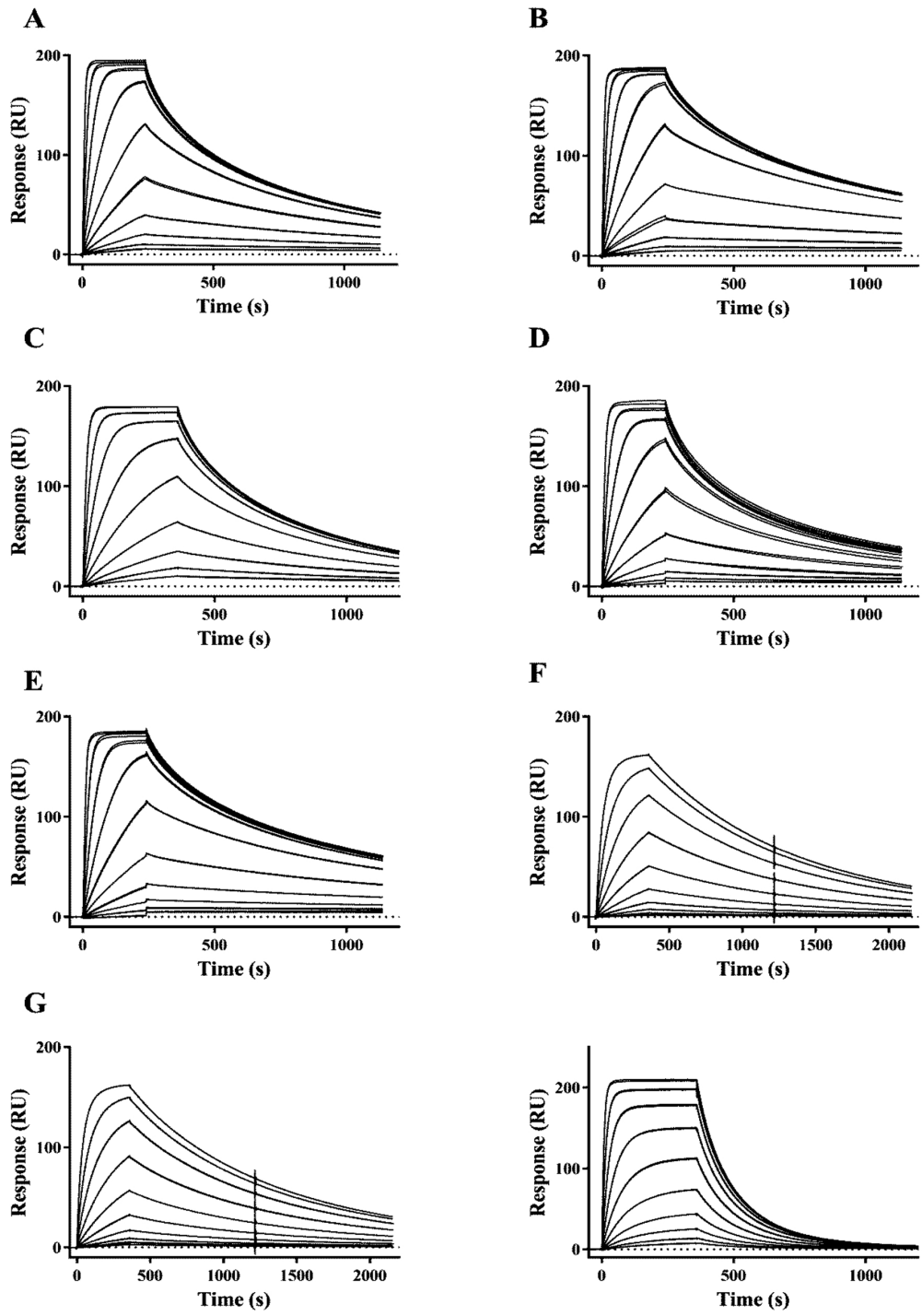


Figure 5

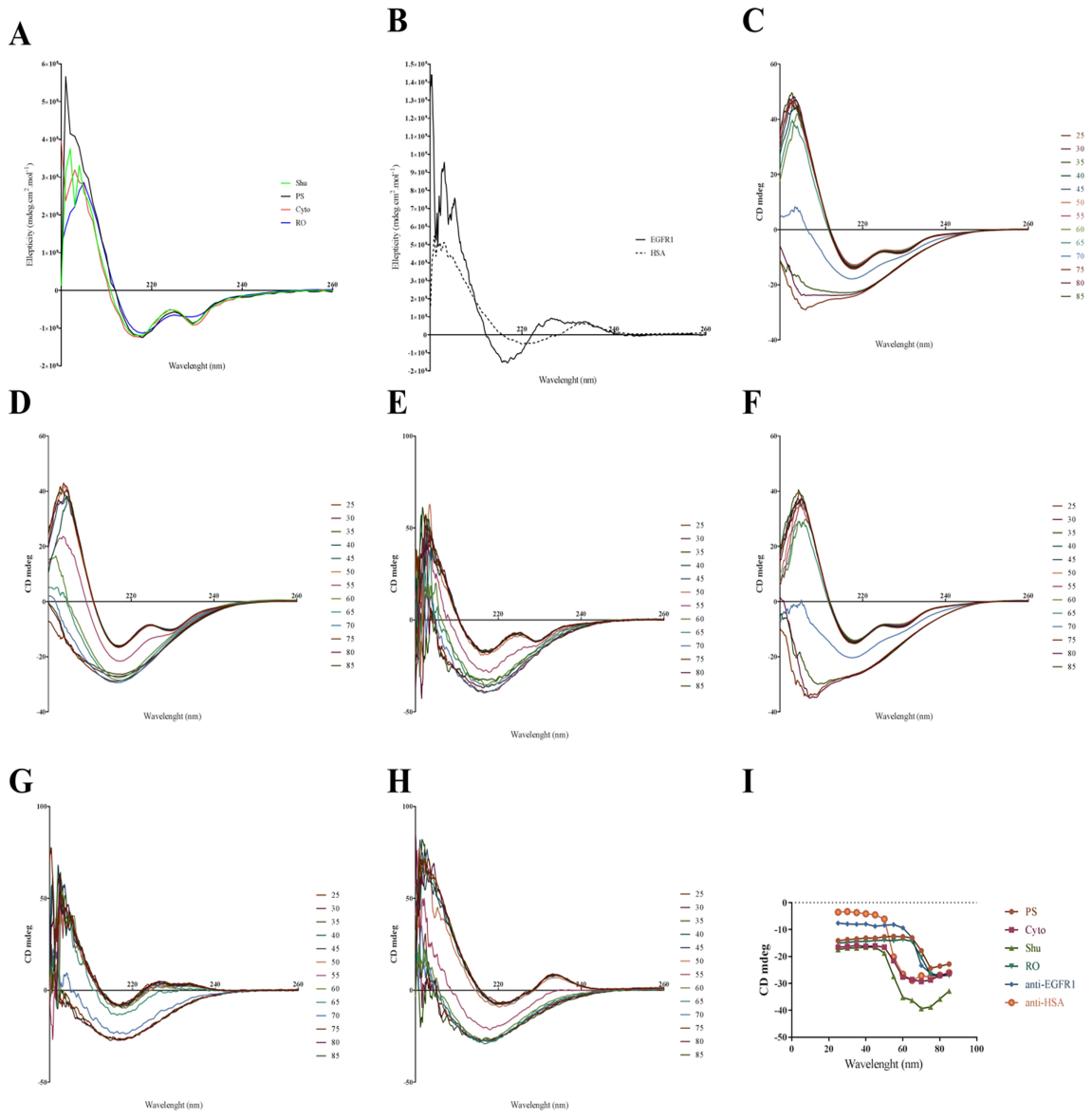


Figure 6

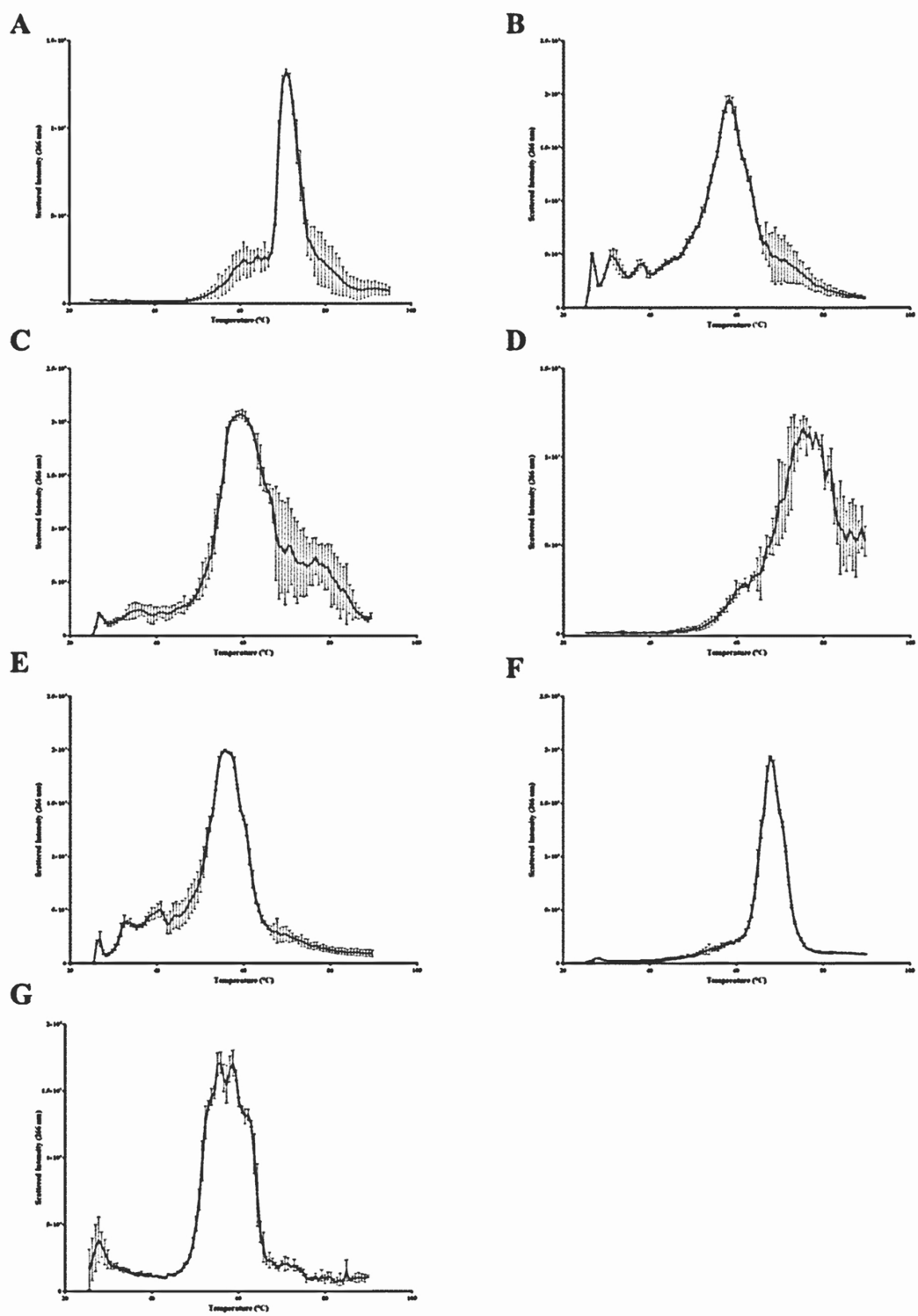


Figure 7

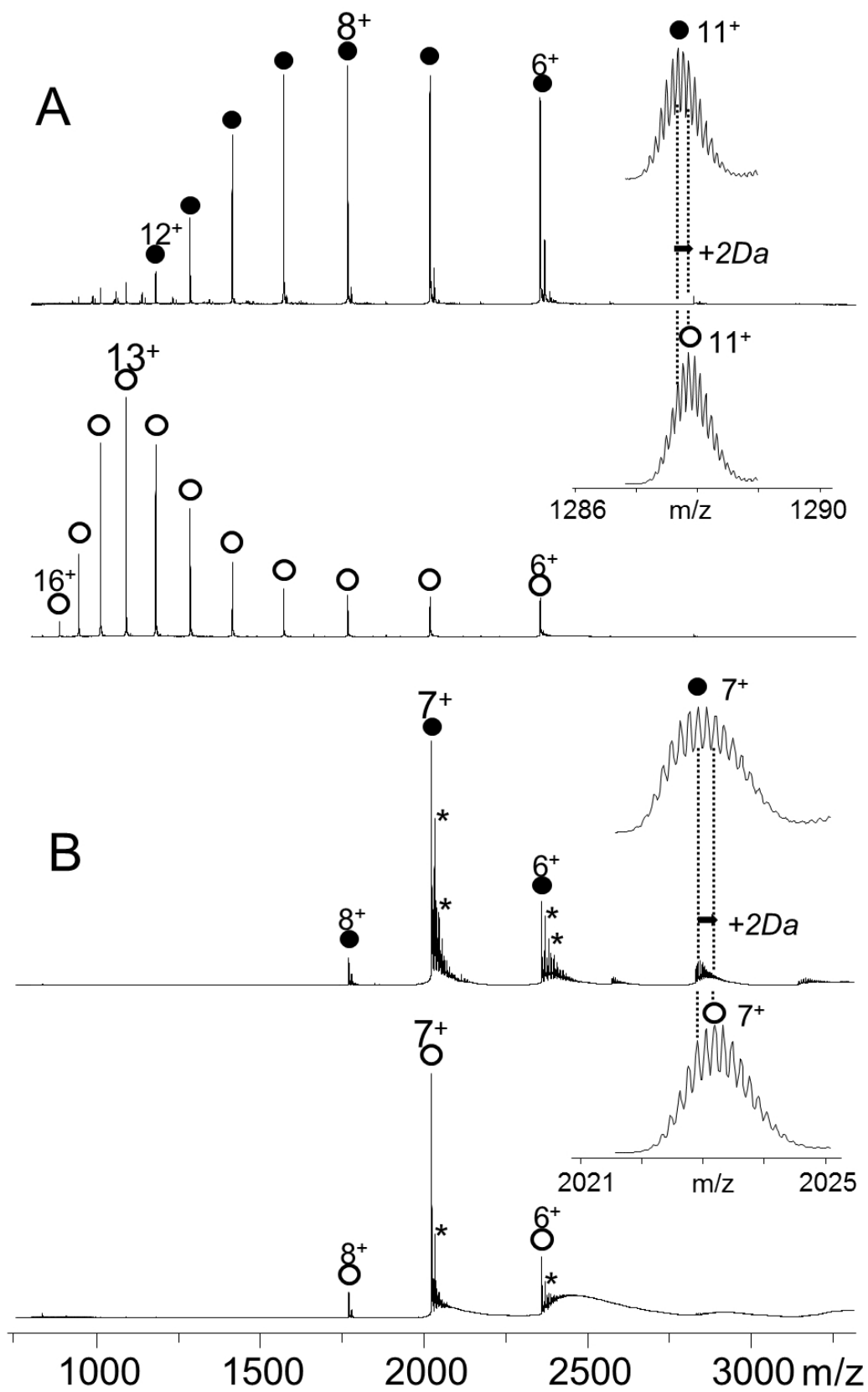


Figure 8

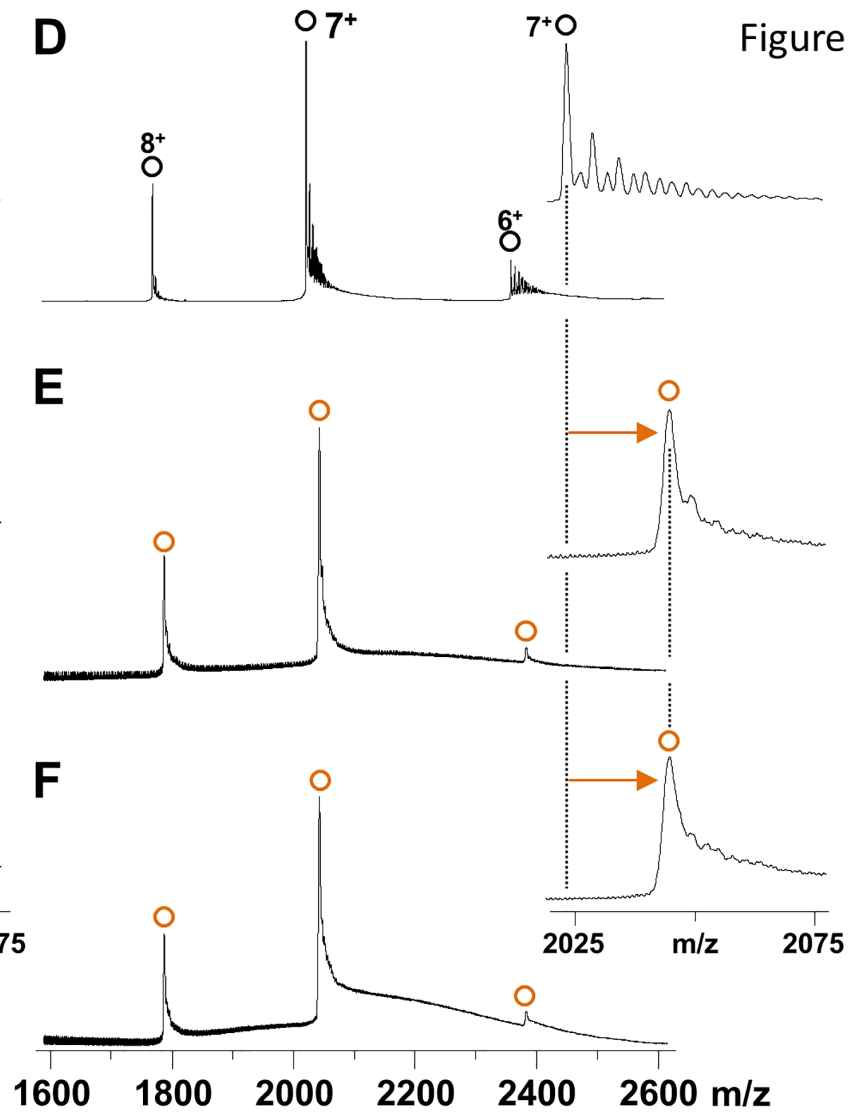
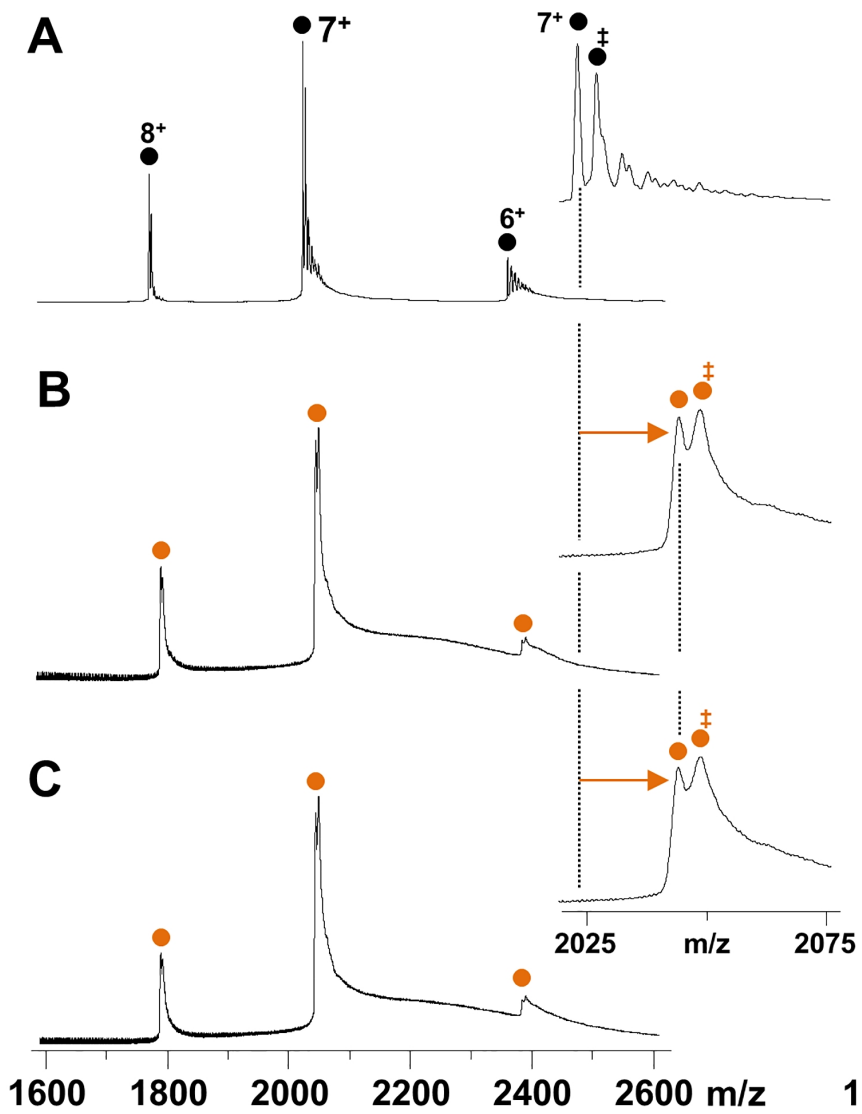


Figure 9

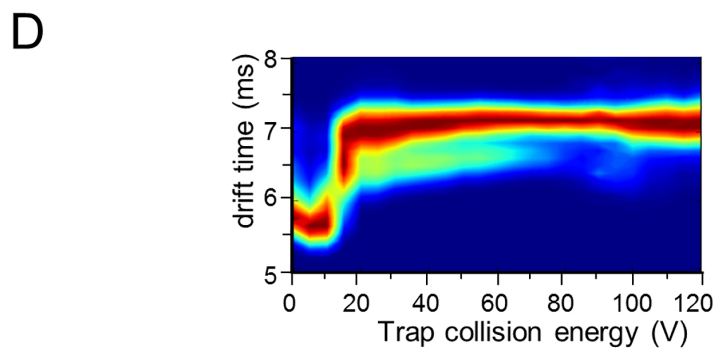
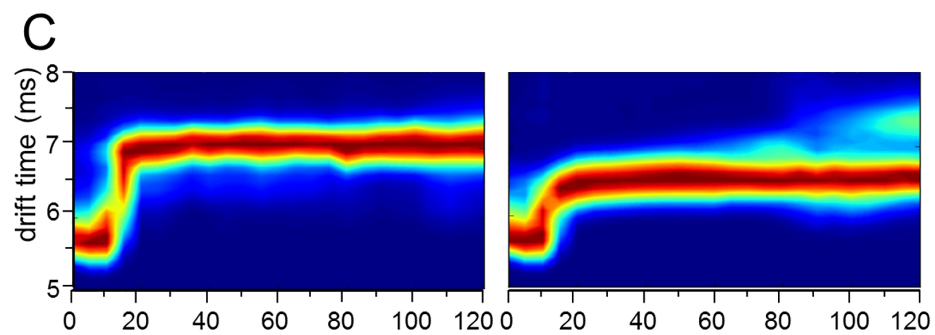
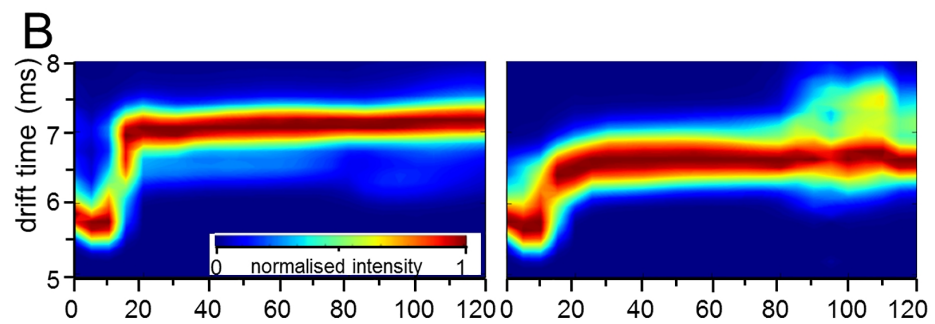
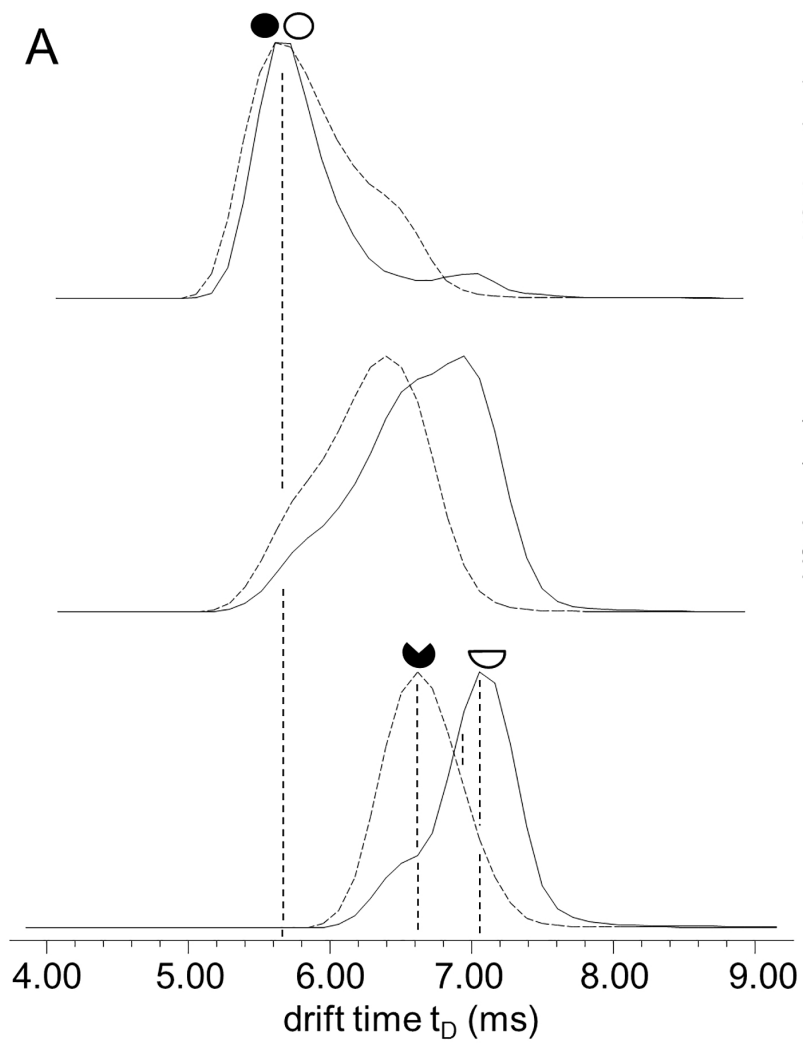


Figure 10