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VHH characterization.

Recombinant VHHs: production, characterization and affinity

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Running title: VHH expression and characterization

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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],

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

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

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

2. Materials and methods

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

3.9 Gas-phase structure and stability of anti-HER2 VHHs probed by native IM-MS and CIU experiments.

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

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

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

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

599 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

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

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

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

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

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

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

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

Sample name	Bacteria strain/ Production localization	Production Yield (mg/L of culture)	Refolding process yield (%)
Anti-HER2 PS	<i>E.coli 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

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 ± 195
Anti-HER2 Cyto	18.80	103.9	14730 ± 334
Anti-HER2 Shu	21.60	95.6	14670 ± 223
Anti-HER2 RO	17.40	101.5	14930 ± 397
Anti-HER2 RR	21.60	80.1	14630 ± 182
Anti-EGFR	19.00	104.1	15450 ± 329
Anti-HSA	21.60	92.3	13730 ± 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		5	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

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

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

VHHs	Helix	Helix1	Helix2	Antiparallel	Anti1	Anti2	Anti3	Parallel	Turn	Others
		(regular)	(distorted)		(left twisted)	(relaxed)	(right twisted)			
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

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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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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	*

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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

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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} (nm ²)	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

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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.

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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,

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

Figure 6: Circular dichroism spectra of the VHHs. Effect of the temperature. A: PS sample in black, Cyto in red, Shu in green and RO in blue. B: anti-EGFR1 and anti-HSA VHH samples are represented in black line and in dot line, respectively. CD spectra as a function of the temperature are showed in the panel C, D, E, F, G and H for the PS, Cyto, Shu, RO, anti-EGFR1 and anti-HSA VHH samples, respectively. Each spectrum corresponds to one temperature from 25°C to 85°C. The panel I represents the CD value in mdeg at 217 nm for the anti-HER2 (PS in black, Cyto in red, Shu in green and RO in blue) and anti-EGFR1 VHHs (in orange) and at 220 nm for the anti-HSA VHH (in cyan). From these curves, the T_m of each sample was calculated using the following equation with Prism software:

$$Y = \text{Bottom} + (\text{Top} - \text{Bottom}) / (1 + 10^{-(X - T_m)})$$

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

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

Figure 9: Impact of the disulfide bridge on the anti-HER2 VHH SASA probed by native HDX-MS. Native ESI-MS spectra obtained from oxidized (A-C, RO sample) and reduced (D-F, Cyto sample) anti-HER2 VHHs in absence of D_2O (A,D) and after a D_2O incubation of 1 minute (B,E) and 1 hour (C,F). Filled and open circles respectively indicate oxidized (SS) and reduced (SH) forms of anti-HER2 VHH. Black and orange coloured circles mark undeuterated and deuterated species. Double crosses correspond to extra-formylated forms. Insets provide a zoom on the common native charge state 7^+ observed in undeuterated and deuterated conditions.

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

HSA	EVQLLES GGGLVQ PGGSLRLS CA ASGFTFRSFGMSWVRQAPGKGPEWVSSISGSGSDTLY
HER2	EVQLVESGGGLVQAGGSLRLS CAT SGITFMRYALGWYRQSPGKQREMVASIN-SGGTTNY
EGFR1	EVQLVESGGGLVQAGGSLRLS CA ASGRTFSSYAMGWFRQAPGKEREFVVAIN W SSGSTYY
	:***:***** ** *****:*** *: : :.* **:* ** * * :*. ... * *
	FR1 CDR1 FR2 CDR2
HSA	ADSVKGRFTISRDNK-TLYLQMNSLRPEDTAVYY CT TIGGSLRS-----SQG
HER2	ADSVKGRFTISRDNKNTVYLQMNSLKPEDTAVYY CNAR W VKP-----QFIDNNYWGGG
EGFR1	ADSVKGRFTISRDNKNTMYLQMNSLKPEDTAVYY CA AGYQINSGNYNFKDYEYDYWGQG
	*****. : * *:*****: *:*** **
	FR3 CDR3
HSA	TLVTVSS---
HER2	TQVTVSS---
EGFR1	<u>TQVTVSSALE</u>
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Figure 1

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