Technical report

Engineering of a bispecific affibody molecule towards HER2 and HER3 by addition of an albumin-binding domain allows for affinity purification and *in vivo* half-life extension

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Abbreviations:

ABD	Albumin Binding Domain
Her	Human Epidermal Growth Factor Receptor 1 – 4
HSA	Human Serum Albumin
IPTG	β -D-1-thiogalactopyranoside
Z	Affibody molecule

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Abstract

Emerging strategies in cancer biotherapy include the generation and application of bispecific antibodies, targeting two tumor-associated antigens for improved tumor selectivity and potency. Here, an alternative format for bispecific molecules was designed and investigated, in which two Affibody molecules were linked by an albumin-binding domain (ABD). Affibody molecules are small (6 kDa) affinity proteins and this new format allows for engineering of molecules with similar function as for full-length bispecific antibodies, but in a dramatically smaller size (around 8-fold smaller). The ABD was intended to function both as a tag for affinity purification as well as for in vivo half-life extension in future preclinical and clinical investigations. Affinity-purified bispecific Affibody molecules, targeting HER2 and HER3, showed simultaneous binding to the three target proteins (HER2, HER3 and albumin) when investigated in biosensor assays. Moreover, simultaneous interactions with the receptors and albumin were demonstrated using flow cytometry on cancer cells. The bispecific Affibody molecules were also able to block ligand-induced phosphorylation of the HER receptors, indicating an anti-proliferative effect. We believe that this compact and flexible format has great potential for developing new potent bispecific affinity proteins in the future, as it combines the benefits of a small size (e.g. improved tissue penetration and reduced cost of goods) with a long circulatory half-life.

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Introduction

Simultaneous targeting of several disease-related proteins using bi- or multispecific affinity proteins is considered to be a promising emerging strategy for increasing potency and decreasing potential problems of drug resistance in cancer therapy [1, 2]. The epidermal growth factor receptor family is comprised of four structurally related members (EGFR (ErbB1/HER1), HER2 (ErbB2/Neu), HER3 (ErbB3) and HER4 (ErbB4)) with extensive crosstalk within the family and has therefore been a rational target for some of the first bispecific agents [1, 2]. Although the significance of HER2 in breast cancer is well established, a considerable number of patients become resistant to HER2-targeted treatments [3, 4]. The mechanisms of resistance are not completely understood but several theories involve activation of compensatory signaling by other tyrosine kinase receptors. In breast cancer, HER2 and HER3 are more often co-expressed than other HER receptor pairs and the function of HER3 in HER2-positive breast cancers has been revealed as critical for tumor progression [5, 6].

A few monoclonal antibodies and tyrosine kinase inhibitors are today approved for targeting HER2 in cancer patients, and additional drug candidates are currently under clinical investigations, including several HER3-specific monoclonal antibodies (e.g. MM-121 and AMG 888) [7]. More recently, bispecific antibodies have emerged as candidate drugs, comprising HER2 and HER3 targeting in one molecule [8, 9]. Several potential benefits with bispecific binders have been suggested [1, 2]. One example is bispecifics, in which one arm of the molecule has affinity for an overamplified receptor (e.g. HER2) for targeting of the drug to the tumor, while the function of the other arm is to interfere with a signaling pathway for a therapeutic effect (e.g. ligand-induced activation of HER3). Bispecific antibodies are however large, multidomain, glycosylated proteins with the binding moieties at a relatively fixed distance, which may limit optimal efficacy.

A promising alternative to antibodies for development of bispecific binders is to use non-immunoglobulin based alternative scaffolds [10], such as Affibody molecules [11]. Affibody molecules are small (58 amino acids) cysteine-free three-helical affinity proteins (~6 kDa), which are generated by combinatorial protein engineering. The small size combined with a disulfide-independent and rapid folding, makes Affibody molecules ideal candidates for engineering of bi- and multispecific affinity

proteins. It is important to note that the overall size of multispecific constructs based on Affibody molecules will remain small. As an example, the multispecific Affibody molecules that were investigated in this study are based on three binding domains, but have still a molecular weight comparable to a single scFv antibody fragment.

We have previously engineered Affibody molecules for specific recognition of HER2 and HER3, respectively [12, 13]. The HER2-targeting Affibody molecule has been extensively investigated, both in preclinical as well as in exploratory clinical studies [11]. The anti-HER3 Affibody molecule was developed more recently using a new bacterial display technology [14, 15] and we have shown anti-proliferative effects on cancer cells by blocking the natural ligand heregulin from inducing HER3 signaling [13, 16]. Due to the small size, Affibody molecules have generally a short plasma half-life, even in most multimeric formats.

Here, we have designed and explored (using HER2 and HER3-specific binders) a new format for bispecific Affibody molecules intended to have a long plasma half-life. In this new design, two Affibody molecules with distinct specificities are linked by a 46 amino-acid albumin-binding domain (ABD) [17], resulting in an Affibody-ABD-Affibody arrangement. ABD has been engineered to femtomolar affinity for HSA [17] and it has previously been demonstrated that fusing ABD to Affibody molecules results in a dramatic extension of the circulatory half-life [18-20]. In addition, here we also evaluated ABD as an affinity tag for efficient protein purification. We engineered a panel of nine bispecific Affibody molecules in the new format with the domains in different orientations. The constructs were produced and successfully purified by affinity chromatography using albumin as ligand. The bispecific Affibody molecules were thereafter investigated in biosensor assays, showing retained function of all three domains. In cell assays, the different constructs demonstrated binding to both the HER2 and HER3 receptor on the surface of cancer cells as well as inhibition of ligand-induced receptor phosphorylation. Importantly, flow-cytometric analysis revealed that the bispecific Affibody molecules were able to target the receptors on cancer cell lines, while simultaneously interacting with HSA, suggesting that the format is promising for the intended purposes. We therefore believe that the Affibody-ABD-Affibody design has potential for future development and application of minimized bispecific affinity proteins with prolonged circulatory half-life.

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Materials and Methods

Design and genetic production of bispecific Affibody molecules

The genes encoding ZHER2:02891 [12, 21], ZHER3:05417 [13], ZTaq:03638 [22] and ABD035 [17] were amplified by PCR using Phusion® High-Fidelity DNA polymerase (New England Biolabs, Beverly, MA). The bispecific Affibody constructs were generated by ligation of each fragment into the expression vector pET26b(+) (Novagen, Madison, WI) in three subsequent cloning steps using T4 DNA ligase (New England Biolabs). The three domains were separated by $(S_4G)_4$ linkers. DNA sequence verification of each construct was performed using BigDye Thermo Cycle Sequencing reactions with an ABI Prism 3700 instrument (Applied Biosystems, Foster City, CA). The verified vectors were prepared from overnight cultures using JETSTAR 2.0 Plasmid Maxiprep Kit (Genomed, Bad Oeynhausen, Germany) or QIAprep Miniprep Spin Kit (Qiagen GmbH, Hilden, Germany).

Protein expression and HSA affinity purification

Isolated plasmids were transformed into the *E. coli* strain Rosetta pLysS by heat shock treatment. A single colony was inoculated into TSB+Y and incubated at 37 °C until a final OD_{600} of ~ 1 was reached. Protein expression was induced by addition of Isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 1 mM followed by overnight cultivation at 25°C. Cells were subsequently harvested by centrifugation, resuspended in 20 mL TST buffer and disrupted by sonication on ice. The Affibody constructs were purified by affinity chromatography on an ÄKTAexplorer (GE Healthcare, Uppsala, Sweden) using a 1 mL HiTrap sepharose column (GE Healthcare) immobilized with HSA, TST buffer (pH 7.5) as running buffer, 5 mM NH4Ac (pH 5.5) for washing steps and 0.5 M HAc (pH 2.8) for elution of bound proteins. The eluted proteins were buffer-exchanged to PBS using NAP-10 columns (GE Healthcare) and concentrations were determined by absorbance measurements at 280 nm. The purity of the purified proteins was evaluated by SDS-PAGE and the molecular weight was verified by LC/MS on an Agilent Technologies 6520 ESI-Q-TOF.

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Biosensor interaction analysis

Real time interaction analysis was performed on a ProteOn XPR36 Protein Interaction Array System (Bio-Rad Laboratories, CA, USA). In total, three different surfaces on ProteOn GLM chips were immobilized with the following ligands: I) human HER2-Fc (R&D systems, Minneapolis, MN); II) human HER3-Fc (R&D systems);III) human HER3 (R&D systems); IV) HSA (Sigma-Aldrich, St. Louis, MO). Ligands were diluted in 10 mM sodium acetate pH 4.5 to a concentration of 5 μg/mL, and immobilized by standard amine-coupling chemistry at a flow rate of 30 μL/min. Immobilization levels reached approximately 3000-4000 RU for all surfaces. All experiments were performed with PBST (pH 7.4) as running buffer and 50 nM NaOH for surface regeneration. All data was subtracted with the signals from a blank reference surface on the same chip. In order to evaluate retained binding of each affinity domain of the Affibody constructs, 50 nM of each purified protein was initially injected in a volume of 100 μL over all of the surfaces at a flow rate of 50 μL/min. Bispecific simultaneous binding of the Affibody fusion proteins was analyzed by two succeeding injections (co-inject) of 1) 100 μL ZHER2-ABD-ZHER3 or ZHER3-ABD-ZHER2, 2) 100 μL of HER3-His or 100 μL of HER2-His or PBST at an analyte concentration of 50 nM and a flow rate of 100 μL/min. Trispecific simultaneous binding of the Affibody fusion proteins was analyzed on the HER2-immobilized surface by three succeeding injections (co-inject) of 1) 100 μL ZHER2-ABD-ZHER3 or ZHER3-ABD-ZHER2, 2) 100 μL of HER3-His or PBST and 3) 25 μL of HSA at an analyte concentration of 50 nM and a flow rate of 100 μL/min. In addition, triple-injections, where ZHER2-ABD-ZHER3 and ZHER3-ABD-ZHER2 were preincubated with 500 nM HSA for 40 minutes at RT, was performed in order to verify simultaneous binding under HSA-saturated conditions. Kinetic data was obtained by injections of three concentrations ranging between 2.5 and 25 nM of respective construct over HER2 and HER3-immobilized surfaces. The flow rate was set to 100 μL/min and association and dissociation was followed for 180 s and 1200 s, respectively. Obtained sensorgrams were fitted by non-linear regression to a 1:1 Langmuir binding model. All biosensor experiments were performed in duplicate.

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Cell binding

The AU565 breast cancer cell line (ATCC) was cultivated in RPMI1640 cell culture medium (Sigma-Aldrich) containing 10 % FBS (Gibco, Life Technologies, Carlsbad, CA) at 37 °C 5 % CO2. Cells were harvested and preincubated with 1 μM of ZHER2, ZHER3, a combination of both or PBS for 30 minutes at room temperature. The nine different bispecific Affibody molecules were then added to a final concentration of 20 nM, respectively, and incubated for 1 hour at room temperature. Cells incubated in PBS were included as negative control. After washing, HSA-Alexa Fluor 488 conjugate was added to the cells and incubated for 30 minutes before analysis using flow cytometry (Gallios, Beckman Coulter). The flow-cytometric analysis was performed in duplicate.

Receptor phosphorylation

MCF7 breast cancer cell line (ATCC) was cultivated at 37°C, 5% CO_2 in RPMI1640 medium (Sigma-Aldrich) containing 10% FBS. Cells were washed with PBS and transferred to medium containing dialyzed FBS (Gibco) without heregulin for 4 h. Cells were thereafter incubated with 20 and 200 nM of respective construct for 30 minutes. Heregulin was added to a concentration of 4 nM for 10 minutes before removing all medium from the cells on ice. Cold PBS containing Sodium orthovanadate (200 mM) and Phosphostop (Roche, Basel, Switzerland) was added and the cells were collected. Cells were centrifuged at 400 *g* for 3 minutes. The supernatant was removed and cell lysis buffer (diluent #12 R&D systems) was added to the pellet and the samples were incubated on ice for 30 minutes. The lysed cells were centrifuged at 400 *g* for 15 minutes. Supernatants were collected and phosphorylation of HER2 and HER3 receptors was determined in duplicate using HER2 and HER3 phosphorylation kits according to the supplier's recommendations (Human Phospho-ErbB3/Her3 Cell-Based ELISA and Human Phospho-ErbB2/Her2 Cell-Based ELISA; R&D systems).

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Results and Discussion

Design and engineering of the new format for bispecific Affibody molecules

The different HER2 and HER3-targeting bispecific Affibody molecules were designed and subcloned for overexpression in *E. coli*. In all constructs, an engineered albumin-binding domain (ABD) was incorporated in between the Affibody molecules (Fig. 1A). The ABD has several intended functions, the most important of which is to provide prolonged circulatory half-life in future preclinical and clinical studies, as previously demonstrated with other ABD-fusions [18-20]. We positioned the Affibody molecules on each side of the ABD to increase the distance between the domains with the ABD hence acting as a linker (Fig. 1A). We also exploited the albumin-binding domain as a biotechnological tool to simplify production and purification of bispecific molecules in this format. Affinity chromatography is generally efficient for recombinant protein purification. However, purification tags (e.g. hexa-histidine peptides) are often proteolytically removed after purification in order to avoid unpredicted influences on the properties of the recombinant protein. Histidine tags might for example significantly alter the in vivo biodistribution profile of recombinant proteins, likely due to its lipophilic properties, resulting in an elevated uptake in the liver [23]. We therefore evaluated the use of ABD as an affinity tag in the bispecific constructs, thereby eliminating the need for a dedicated purification tag. Two orientations of the bispecific fusion proteins were engineered: ZHER2-ABD-ZHER3 and ZHER3-ABD-ZHER2. Additionally, we included five constructs with one or both HER-targeting arms substituted with an Affibody molecule of irrelevant specificity (ZTaq, specific for Taq polymerase), as well as two monospecific bivalent Affibody molecules (ZHER2-ABD-ZHER2 and ZHER3-ABD-ZHER3). Taken together, nine different fusion proteins were designed (Fig. 1B) and subcloned to an *E. coli* expression vector.

Production and purification of bispecific Affibody molecules

The fusion proteins were produced in *E. coli* and purified using affinity chromatography with human serum albumin (HSA) immobilized as ligand on the sepharose matrix. All nine purified proteins were observed as single bands of correct size on a SDS-PAGE gel, demonstrating high sample purity and hence confirming

successful affinity purification via ABD (Fig. 1B), and consequently validating the retained affinity of the domain for HSA in all different constructs. The molecular weight of each fusion protein was verified by ESI-LC/MS (data not shown).

Biosensor analysis

A biosensor-based assay was used to analyze binding of each individual domain of the fusion proteins to HER2, HER3 and HSA. To investigate whether some orientations were incompatible with binding to any of the targets, all nine constructs were injected over HER2-, HER3- and HSA-immobilized surfaces, respectively. The results showed that in all nine constructs each domain had retained binding to its respective target (Fig. 2A-D). The analysis also showed that the affinities for respective receptor (HER2 and HER3) were similar for ZHER2-ABD-ZHER3 and ZHER3-ABD-ZHER2. The equilibrium dissociation constants (K_D) were estimated to around 0.6 (± 0.01) nM for HER2 and 0.6 (± 0.05) nM for HER3 for ZHER2-ABD-ZHER3 and around 0.7 (± 0.1) nM for HER2 and 0.8 (± 0.1) nM for HER3 for ZHER3-ABD-ZHER2. The on-rate for the interaction with HER2 was approximately 8-fold lower compared to previously reported values obtained in studies with monomeric ZHER2 [12], which might be due to a relatively slower diffusion because of the increase in molecular weight for the trimeric constructs. As expected, the ZTaq (negative control) did not interact with any surface (Fig. 2D).

In a second assay, we investigated whether the new format with ABD as a linker allowed for simultaneous interaction of the two Affibody molecules with respective targets (Fig. 2E). The bispecific proteins were first injected over the HER2 and HER3 surfaces, followed by a second injection with soluble HER2 and HER3, respectively (Fig. 2E-F). The results showed that the bispecific format enabled the Affibody molecules to bind to one receptor on the sensor chip surface while simultaneously capturing the other receptor in the solution (Fig. 2E-F). A second injection of buffer or a second injection of the same receptor as on the sensor surface, did not show an increase in signal, hence demonstrating that the interactions were specific (Fig. 2F). Next, we performed a similar assay complemented with a third injection of HSA (Fig. 2G). This experiment was analyzed on the HER2-immobilized surface in order to mimic the *in vivo* situation where overexpression of HER2 could potentially facilitate initial tumor targeting. As in the previous experiment, the bispecific Affibody

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molecule was able to simultaneously bind to HER3 in solution and HER2 immobilized on the biosensor chip surface (Fig. 2H). In addition, a third injection of albumin also resulted in an increase in signal, indicating that the bispecific format allowed for simultaneous interactions with both targets as well as albumin (Fig. 2H). In a control experiment, the bispecific Affibody molecule was preincubated with an excess of HSA before injection over the surface. Nonetheless, the complex of the bispecific construct and albumin could bind to the receptor on the surface and again also capture free receptor that was injected in solution (Fig. 2H). The experiment also showed that the bispecific binder that was preincubated with albumin was not able to bind additional albumin in the third injection, hence suggesting that the bispecific Affibody molecules that bound the receptors on the sensor surface were saturated with albumin (Fig. 2H).

Cell binding

The biosensor experiments demonstrated that the Affibody molecules in this bispecific format were able to bind to recombinantly produced HER2 and HER3. The next step was to investigate whether they had also retained the binding capacity to native receptors on cancer cells. All nine fusion proteins were therefore analyzed by flow cytometry for their binding to the breast cancer cell line AU565. AU565 is HER3 positive and overexpresses HER2. The cell assay was designed to somewhat mimic the conditions in vivo, thus measuring the binding in presence of HSA. Moreover, we used fluorescently labeled HSA for detection, with the consequence that potential cell-binding signals in the flow cytometer should be the result of bispecific Affibody molecules binding simultaneously to the receptor and to albumin (Fig. 3A).

Since HER2 is overexpressed on AU565 cells, we expected a substantial shift in fluorescence for the samples incubated with fusion proteins comprising the HER2-specific Affibody molecule. Indeed, the five variants with ZHER2 demonstrated at least 200-fold higher fluorescence compared to the negative control (ZTaq-ABD-ZTaq) (Fig. 3B). Moreover, although the expression level of HER3 on AU565 is considerably lower compared to HER2, the ZHER3-ABD-ZTaq and ZHER3-ABD-ZHER3 showed a clear shift in the flow cytometer (Fig. 3B). To assess the specificity of the cell binding, we blocked the cells with unlabeled monomeric Affibody

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molecules prior to incubation with the bispecific proteins. Blocking with the HER2-specific Affibody molecule, resulted in around 50-fold lower fluorescence, which suggested that the signals were HER2-specific (Fig. 3C). Simultaneous blocking with both HER2 as well as HER3-specific Affibody molecules further decreased the fluorescence signals, indicating that the bispecific molecules interacted with both receptors (Fig. 3D). However, blocking with only the HER3-specific Affibody molecules did not result in any significant shift in fluorescence, most likely due to the substantial difference in HER2 and HER3 expression on AU565 (Fig. 3E).

Inhibition of ligand-induced phosphorylation

The flow-cytometric analysis showed that the bispecific Affibody molecules were able to target the breast cancer cell line, AU565. Due to the relatively low HER3 expression level, we reasoned that it might be more straightforward to analyze a potential influence on HER3-signaling rather than only the HER3-binding that was difficult to detect. We have previously demonstrated that the HER3-specific Affibody molecule can inhibit signaling by blocking the natural ligand heregulin from binding [16], which we now exploited to confirm binding to the receptor in a receptor phosphorylation assay. The previously investigated monomeric HER3-specific Affibody molecule (denoted 3A) was included as a positive control, and ZTaq-ABD-ZTaq was included as a negative control. MCF7 breast cancer cells were incubated with the different fusion proteins prior to stimulation with heregulin. After stimulation, ligand-induced phosphorylation of HER2 and HER3 was measured in an ELISA and compared to non-stimulated cells. Both bispecific Affibody molecules as well as the bivalent variant (ZHER3-ABD-ZHER3) were more efficient in blocking ligand-induced phosphorylation of HER2 compared to the monospecific control (ZHER3-ABD) (Fig. 3F). Interestingly, the effects on phosphorylation of HER3 were more diverse. One of the bispecific Affibody molecules (ZHER2-ABD-ZHER3) was for example less efficient in decreasing HER3 phosphorylation than the monospecific control (Fig. 3F). Moreover, the results from the unstimulated control indicated that the same bispecific Affibody molecule had a significant agonistic effect on HER3 phosphorylation, showing the importance of investigating bispecific affinity proteins in different orientations (Fig. 3F). For bispecific targeting of HER2 and HER3, the results from the phosphorylation assay indicated that the ZHER3-ABD-ZHER2 would

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be the preferred orientation. The observed difference in inhibition of receptor signaling was probably not due to different affinities for the receptors, since the biosensor assay indicated that the two orientations bound both HER2 and HER3 with similar binding strengths. Furthermore, the bivalent HER3-specific Affibody molecule (3A3) was significantly more efficient in blocking phosphorylation of both HER2 and HER3 compared to the monovalent control (Fig. 3F). In summary, the assay demonstrated that the HER3-specific Affibody molecule in the new bispecific format was able to bind to HER3 on cancer cells and thereby inhibit ligand-induced phosphorylation.

Conclusions

In this study, we have designed and investigated a new format for bispecific Affibody molecules. Including an ABD molecule linking the two Affibody molecules enabled efficient purification using affinity chromatography on an HSA-column and allowed simultaneous interaction with both HER2 and HER3 in biosensor assays. Since our long-term intention with the bispecific format is to proceed to *in vivo* studies, tumor-targeting capacity in presence of albumin was essential. Consequently, we devised a flow-cytometric assay that ensured that we could detect potential simultaneous binding to cell surface receptors and albumin in solution. The resulting experiments demonstrated that the bispecific proteins were indeed able to target cancer cells while simultaneously binding to albumin. In addition, a receptor phosphorylation assay indicated that the HER3-binding domain had retained capacity to inhibit ligand-induced signaling. One of the bispecific molecules was also more efficient than the monospecific control in inhibiting both HER2 and HER3 phosphorylation. In parallel to the studies described here, we have recently improved the affinity of the HER3-specific Affibody molecule even further (21 pM monovalent affinity) [24, 25]. We hence propose that future preclinical studies should be performed using the ZHER3-ABD-ZHER2 format, comprising the Affibody molecules with low picomolar affinity for both HER2 and HER3 as well as the engineered ABD with femtomolar affinity for albumin.

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FF and LG are employees at Affibody AB. SS and JL are members of the Technical advisory board at Affibody AB. All other authors declare no financial or commercial conflict of interest.

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Figure legends

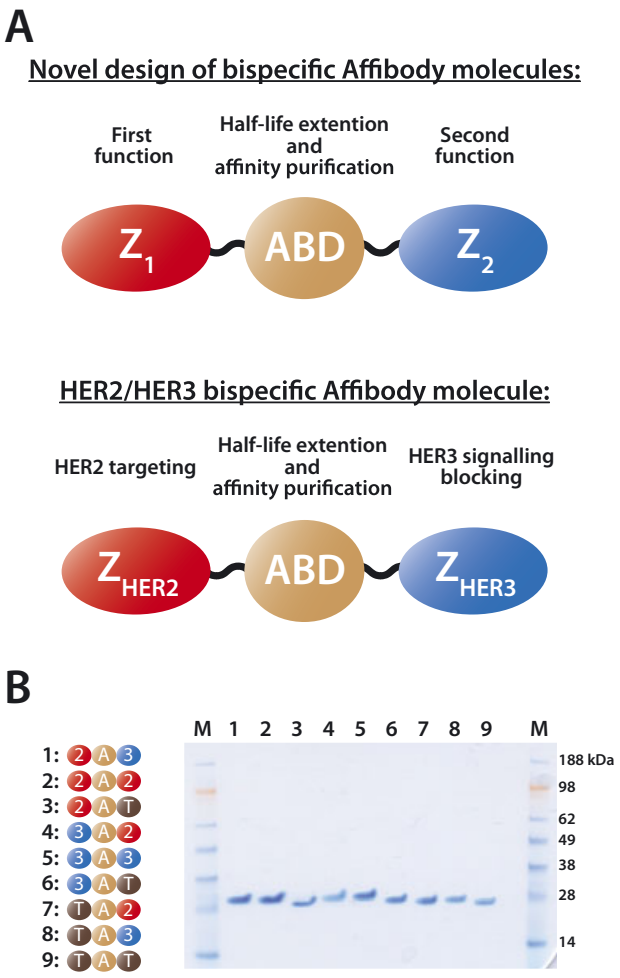

Figure 1. (A) Schematic representation of the new modular format for bispecific Affibody molecules (upper panel) and an exemplified orientation of one particular construct (lower panel). Abbreviations: Z = Affibody molecule, ABD = Albumin-binding domain, ZHER2 = HER2-specific Affibody molecule, ZHER3 = HER3-specific Affibody molecule. **(B)** Nine different bispecific constructs were produced (1-9) and purified on an HSA-coupled sepharose matrix. Purified proteins analyzed using SDS-PAGE with identical denomination. Abbreviations: 2 = ZHER2, 3 = ZHER3, T = ZTaq (negative control with irrelevant specificity), A = ABD. The orientation of the domains in the fusion proteins is shown in the schematic illustration to the left of the gel. The molecular weights of the protein ladder are indicated to the right of the gel.

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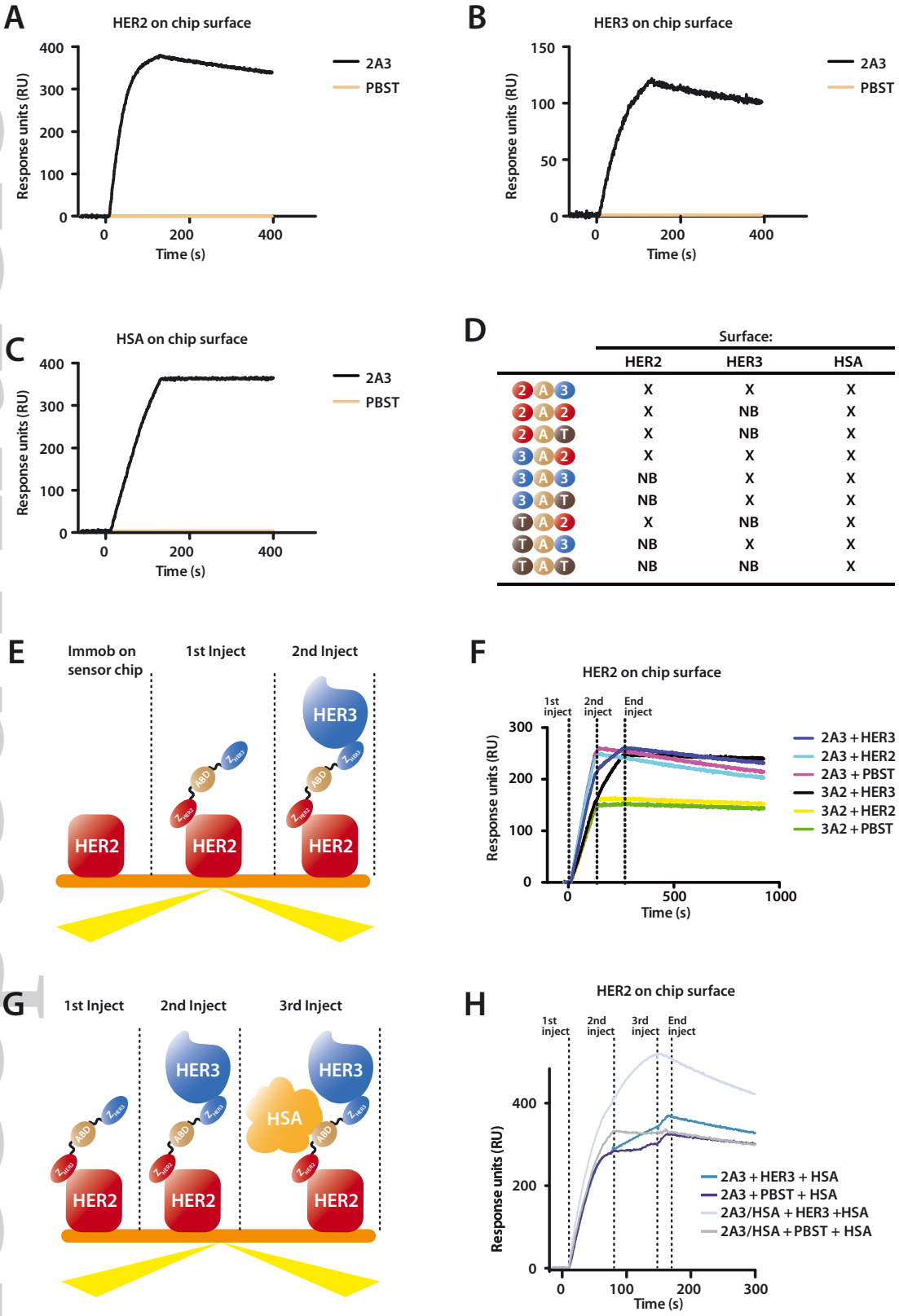

Figure 2. Biosensor assays. Representative sensorgrams from SPR biosensor experiments showing the response from bispecific Affibody molecules targeting HER2 and HER3 injected over and interacting with immobilized **(A)** HER2, **(B)**

HER3 and **(C)** HSA. PBST represents buffer injection (negative control). **(D)** Matrix that summarizes the results from the biosensor assays. The different fusion proteins are illustrated to the left. *X* in the matrix represents *binding* and *NB* represents *no binding*. **(E)** Schematic representation of the co-inject biosensor assay, illustrating HER2 immobilized on the biosensor surface, followed by bispecific Affibody molecule in the first injection and HER3 in the second injection. **(F)** Representative sensorgrams showing the results from the co-inject assay. Dotted lines illustrate the start and end points for the two injections. The labels to the right show the order of injections in each sample, where for example 2A3 + HER3 represents first an injection of ZHER2-ABD-ZHER3 followed by an injection of HER3. **(G)** Schematic representation of the triple co-inject biosensor assay, illustrating bispecific Affibody molecule in the first injection, HER3 in the second injection and HSA in the third injection. **(F)** Representative sensorgrams showing the results from the triple co-inject assay. Dotted lines illustrate the start and end points for the three injections. The labels to the right show the order of injections in each sample, where for example 2A3 + HER3 +HSA represents first an injection of ZHER2-ABD-ZHER3 followed by an injection of HER3 and finally an injection of HSA. 2A3/HSA represents bispecific Affibody molecule that was preincubated with HSA prior to injection. All experiments were performed in duplicates.

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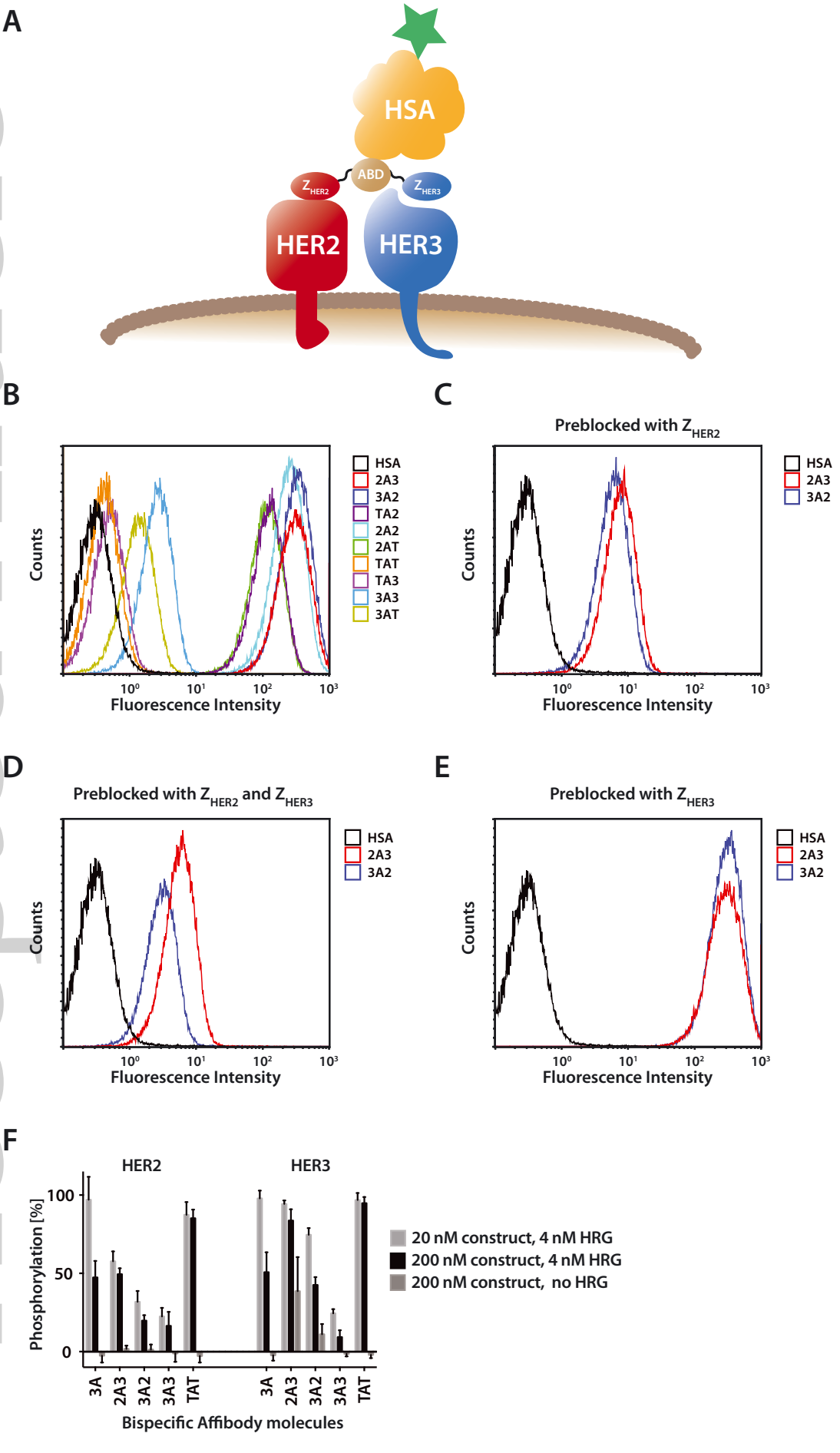

Figure 3. (A) Schematic representation of the flow-cytometric assay for analysis of the ability of the bispecific constructs to bind HER2 and HER3 on cancer cells, while simultaneously interacting with fluorescently labeled HSA. All flow cytometry data were gated in forward and side scatter plots to only include the single cell populations. All flow cytometry experiments were performed in duplicates. **(B)** Histograms showing the results from flow cytometry assay of bispecific Affibody molecules binding to AU565 breast cancer cells (HER2 overexpressing and HER3 positive). Labels to the right of the histograms show the different fusion proteins. Fluorescently labeled HSA was used as secondary detection reagent in all experiments. HSA = negative control where cells were only incubated with fluorescently labeled HSA. **(C)** Histograms showing the results from flow cytometric analysis of AU565 cells preincubated with monomeric and unlabeled ZHER2 before being treated as in (B). **(D)** Histograms showing the results from flow cytometric analysis of AU565 cells preincubated with monomeric and unlabeled ZHER3 before being treated as in (B). **(E)** Histograms showing the results from flow cytometric analysis of AU565 cells preincubated with monomeric and unlabeled ZHER2 and ZHER3 before being treated as in (B). **(F)** *In vitro* receptor phosphorylation assay on breast cancer cell line MCF7 (HER2 overexpressing and HER3 positive). Cells were incubated with the Affibody constructs followed by stimulation with heregulin (HRG). Receptor phosphorylation was measured by ELISA. 3A: ZHER3-ABD (positive control). The columns are mean values with error bars representing one standard deviation calculated from duplicate experiments.

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