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ORIGINAL ARTICLE

Evolution-guided design of mini-protein for high-contrast *in vivo* imaging



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Abstract Traditional development of small protein scaffolds has relied on display technologies and mutation-based engineering, which limit sequence and functional diversity, thereby constraining their therapeutic and application potential. Protein design tools have significantly advanced the creation of novel protein sequences, structures, and functions. However, further improvements in design strategies

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HER2-positive breast cancer;
EvoDesign;
In vivo image;
Spatial aggregation properties;
Mini-protein;
Lower liver uptake

are still needed to more efficiently optimize the functional performance of protein-based drugs and enhance their druggability. Here, we extended an evolution-based design protocol to create a novel mini-binder, BindHer, against the human epidermal growth factor receptor 2 (HER2). It not only exhibits super stability and binding selectivity but also demonstrates remarkable properties in tissue specificity. Radiolabeling experiments with ^{99m}Tc , ^{68}Ga , and ^{18}F revealed that BindHer efficiently targets tumors in HER2-positive breast cancer mouse models, with minimal nonspecific liver absorption, outperforming scaffolds designed through traditional engineering. These findings highlight a new rational approach to automated protein design, offering significant potential for large-scale applications in therapeutic mini-protein development.

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1. Introduction

Non-antibody protein scaffolds, small monomeric proteins with stable tertiary structures and high binding specificity, hold the potential to address the limitations of monoclonal antibodies regarding size, stability, and production cost^{1,2}. It has seen a growing number of engineered binding protein scaffolds documented in the SYNBP³. These scaffold proteins are typically engineered by introducing random mutations in surface-exposed residues and are experimentally screened using display technologies to bind with specific targets. Some of the scaffolds include affibody⁴, anticalin⁵, DARPins^{6,7} and monobody⁸, have been successfully engineered for various clinical applications, demonstrating great promise as diagnostic tools and therapeutics for conditions such as cancer^{9,10}, metabolic disease¹¹, and infections^{12,13}. Despite the significant promise and advancements, it remains crucial to develop or discover novel molecules to address critical limitations associated with protein scaffolds, such as patent monopolies and the need for optimization of physicochemical properties¹⁴. Meanwhile, engineering-based approaches often target a limited number of mutation sites on existing nature proteins, which constrains sequence and functional variations of the produced scaffold, thereby limiting their therapeutic potential and utility.

In recent years, significant advancements have been achieved in computer-based protein design, aiming to computationally simulate and generate novel protein sequences and scaffolds with desired physiological functions^{3,15–19}. This approach offers a distinct advantage over traditional protein engineering, allowing the creation of features and functions beyond those found in nature^{20,21}. Successful applications include the design of immune system modulators^{22,23}, viral infection inhibitors^{16,24}, protein-based vaccines^{25,26}, and self-assembling biomaterials^{27,28}. In protein engineering, improving existing functional proteins requires *de novo* design or redesign to more precisely meet practical needs, optimizing function or stability without the need to create entirely new structures or functions. Furthermore, the design process must be more efficient, reducing the workload associated with experimental screening. However, protein design still faces challenges in this application, and further validation is urgently needed to support its broader use in protein engineering.

The advancements in protein design have motivated interest in computationally designing scaffold proteins, such as ABY-

025^{29,30}, known as a second-generation affibody molecule that binds to the human epidermal growth factor receptor 2 (HER2), which was found overexpressed in tumors of several cancers. Due to its low molecular weight (6 kDa), ABY-025 is characterized by rapid blood clearance and favorable biodistribution for molecular imaging applications, as evidenced by an increased tumor-to-organ accumulation ratio compared to the anti-HER2 IgG monoclonal trastuzumab³¹. Clinical studies of ^{68}Ga -ABY-025 have demonstrated exceptional sensitivity and specificity in detecting HER2-expressing metastases in breast cancer patients³². The development of ABY-025 involved an iterative optimization process, wherein substitutions of one or several amino acids were made in the HER2-binding affibody molecule ZHER2:342, resulting in enhanced stability and hydrophilicity²⁸. However, it still exhibits significant hepatic uptake during *in vivo* imaging³³, even with an increased injection dose³⁴ or replacement of the nuclide and chelating sequence^{35,36}. This relatively high background uptake in the liver can obscure signals from smaller liver metastases, particularly for those exhibiting low to moderate levels of HER2-receptor expression, thereby potentially compromising the accuracy of PET-based HER2 scoring³⁷.

In this study, we explore the potential to create novel mini-protein through redesign with the focus on the HER2-targeted Affibodies. We extended the evolutionary profile-based EvoDesign protocol^{38,39} developed in our group to create and optimize sequence decoys through Monte Carlo design simulations. In order to further enhance the therapeutic qualities of the general protein design procedure, we developed a pipeline to simultaneously constrain binding affinity, folding integrity, and spatial aggregation properties (SAP) using EvoEF2⁴⁰, I-TASSER⁴¹, and a novel high-throughput SAP estimation approach⁴² (see Methods). To evaluate the computational results, we conducted multiple experimental assessments on the designed sequences, including binding affinity, thermal stability, enzymatic stability, solubility, tissue permeability, and immunogenicity, following their expression and production. Additionally, we utilized Single-Photon Emission Computed Tomography (SPECT) and Positron Emission Tomography/Computed Tomography (PET/CT) to demonstrate the superior imaging capabilities of our computationally designed mini-proteins, labeled with radionuclides, in comparison to ABY-025, which is currently employed in clinical practice. These results underscore the efficacy of computational protein design as a robust approach for large-scale therapeutic mini-protein development.

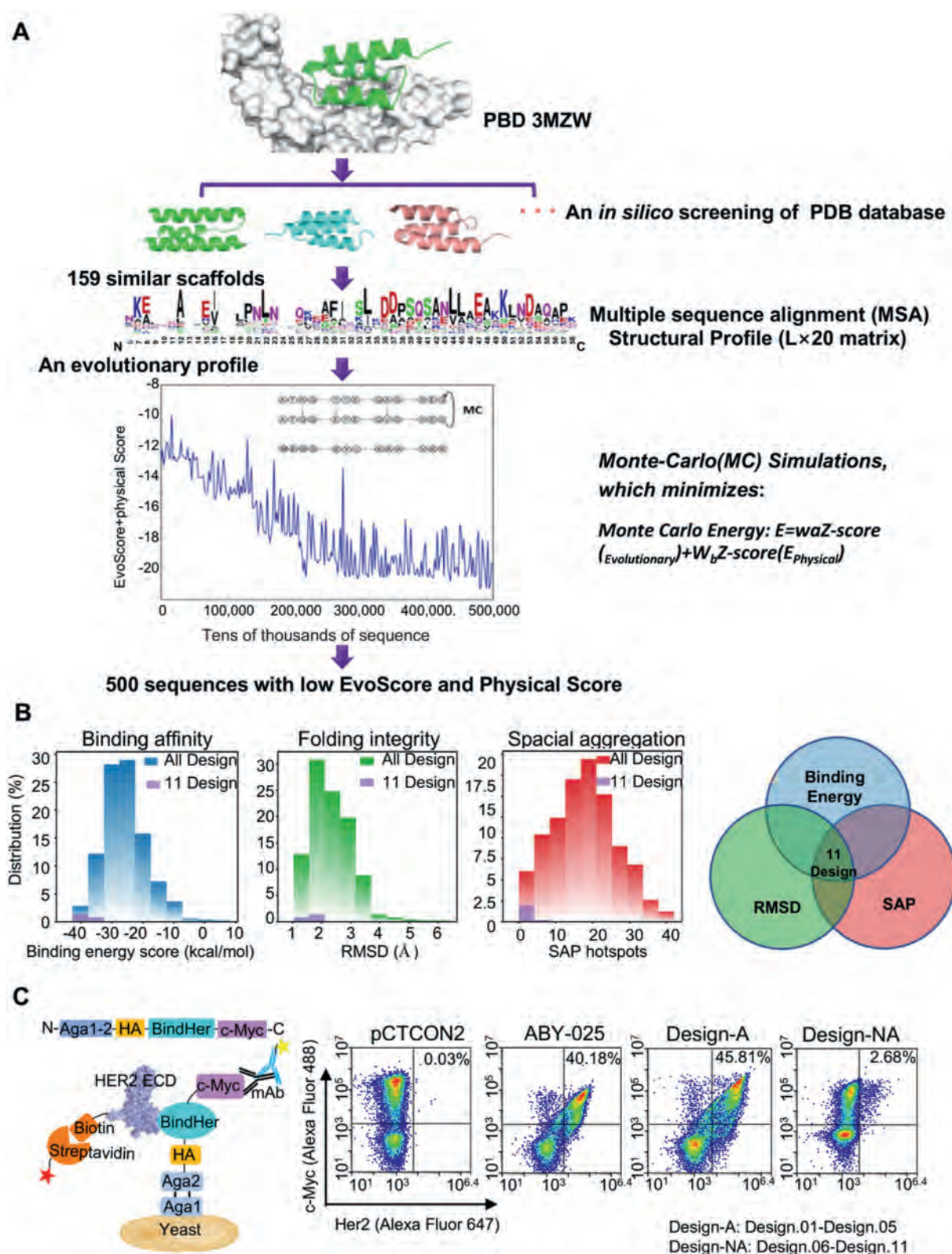


Figure 1 Illustration of *in silico* design and experimental validation of HER2-binding proteins. (A) Structure of ZHER2:342 (green cartoon) in complex with HER2 (gray surface), utilized as a probe for structural profile detection. EvoDesign REMC simulations are performed to create sequence decoys as guided by the structural profile and physical force fields. (B) Left: histogram displaying EvoDesign sequence decoys ranked by EvoEF2 binding energy, folding integrity of I-TASSER model to the probe, and spacial aggregation property, respectively. Eleven top-ranking designs are highlighted in purple. Right: eleven designs are selected based on consensus scoring, with colors in the Venn diagram corresponding to those in left. (C) Left: The pCTCON plasmid vector is engineered to express the designed sequences (BindHer) with N-terminal HA and C-terminal cMyc epitope tags, fused to the yeast mating protein Aga2p on the yeast surface. Right: density plots of protein expression (y-axis, Alexa Fluor 647 detected via anti-cMyc antibody) versus HER2 binding (x-axis, Alexa Fluor 488 via Streptavidin—Alexa assay). Design-A: high HER2 affinity; Design-NA: low HER2 affinity.

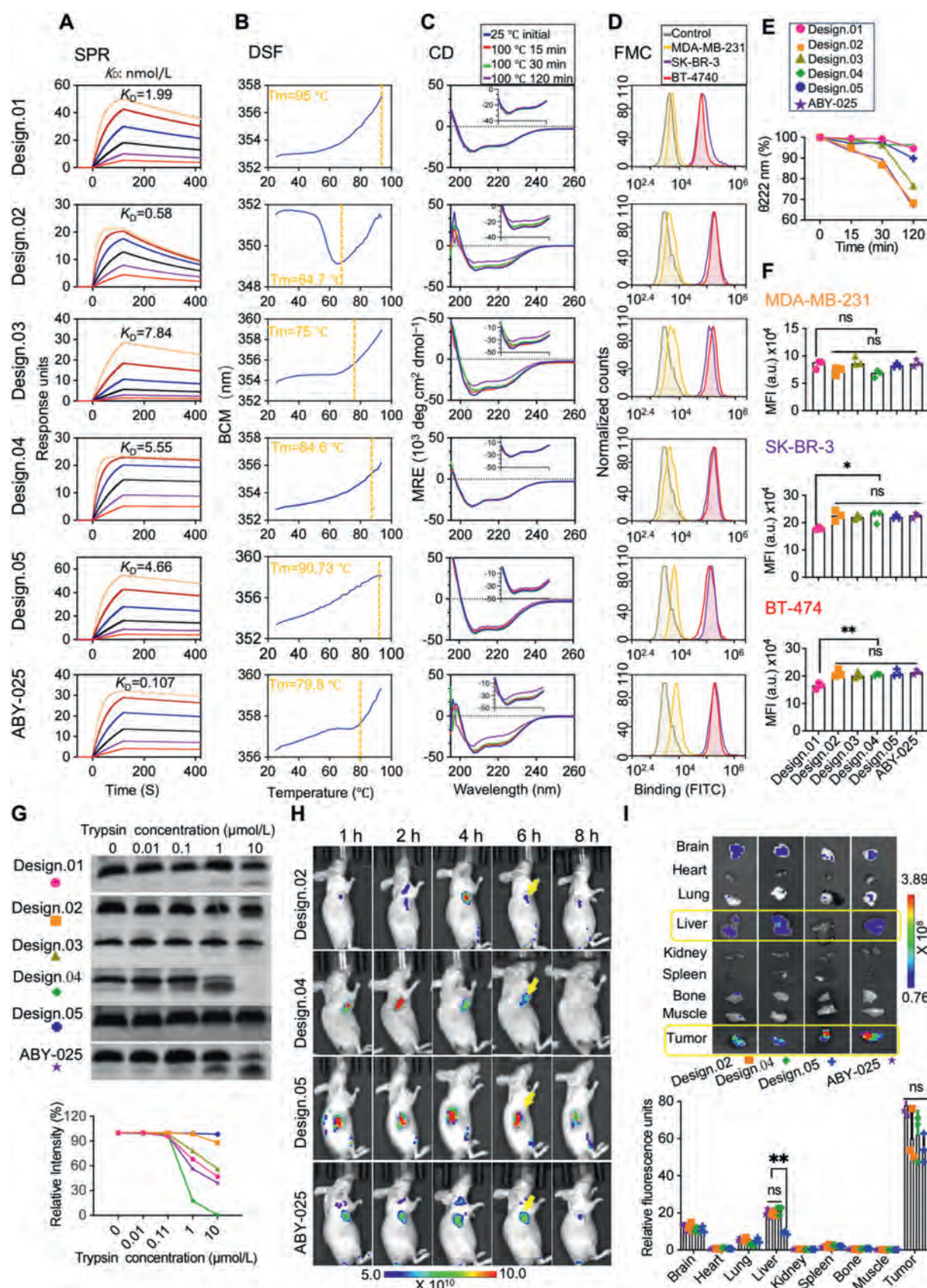


Figure 2 Experimental characterization of designed HER2-binding (BindHer) proteins. (A) Real-time binding profile of computationally designed proteins with the HER2 ECD through surface plasmon resonance (SPR), where designed proteins were injected as analytes in a 2-fold dilution series ranging from 100 to 3.125 nmol/L. K_D is the equilibrium dissociation constant. (B) Denaturation curves obtained using differential

Table 1 The physicochemical characteristics of the optimized binders.

Characteristic	Design candidate					
	Design.01	Design.02	Design.03	Design.04	Design.05	ABY-025
K_D (mol/L)	1.99E-09	5.83E-10	7.84E-09	5.55E-09	4.66E-09	1.07E-10
T_m (°C)	95.00	64.7	75.00	84.60	90.73	79.80
CD spectrum of heated protein	The spectrum does not shift	The spectrum does shift	The spectrum does shift	The spectrum does not shift	The spectrum does not shift	The spectrum does not shift
Anti-digestion (%)	46.86	87.73	56.04	0	98.24	39.18
Binding HER2 positive cells <i>in vitro</i> (%)	<90	>90	>90	>90	>90	>90
Binding HER2 positive tumour <i>in vivo</i>	No binding	Binding	No binding	Binding	Binding	Binding

K_D : The binding affinity (See Fig. 2A and Supporting Information Table S1).

T_m : Melting temperature (See Fig. 2B).

CD spectrum of heated protein: no spectral shift observed compared to the unheated protein (See Fig. 2C and E).

Anti-digestion resistance: Percentage of target protein remaining undigested at a 10 μ mol/L trypsin concentration.

HER2-positive cell Binding *in vitro*: Refer to Fig. 2D and F for binding results.

HER2-positive tumour Binding *in vivo*: Refer to Fig. 2H for *in vivo* binding results.

2. Results

2.1. Computational design of mini-proteins and initial HER2-binding affinity validation

We aim to overcome the limitations of HER2-targeted antibody scaffolds by extending a computational approach to design highly druggable scaffolds for HER2 binding. Our design strategy, illustrated in Fig. 1A, begins with the crystal structure of the HER2 extracellular domain (HER2 ECD) bound to an affinity-matured 3-helix affibody known as ZHER2:342 (PDB ID 3MZW)⁴³. Using 3MZW as a template, we performed an *in silico* structural alignment screen of the Protein Data Bank (PDB) using the TM-align program⁴⁴, identifying 159 structurally analogous scaffolds. Subsequently, an evolutionary profile constructed from multiple sequence alignment (MSA) combined with knowledge-based energy terms guided the replica-exchange Monte Carlo (REMC) simulations⁴⁵, producing a set of 500 low-energy sequence decoys selected by EvoDesign force field (Supporting Information Fig. S1A).

Although the general protein design process can produce a range of protein binders, only a few meet the stringent requirements for therapeutic application⁴⁶. To address this, we developed a pipeline that integrates the optimization of binding affinity, folding integrity, and spatial aggregation properties (SAP) using EvoEF2⁴⁰, I-TASSER⁴¹, and a novel high-throughput SAP estimation method, respectively (see Methods, Fig. 1B). Firstly, we used EvoEF2, a cutting-edge force field for sequence design, to predict binding affinity⁴⁰. Subsequently, we assessed the folding

integrity of the designed sequences with I-TASSER, a highly accurate threading assembly simulation method^{47,48}. Next, in order to minimize nonspecific hepatic absorption by reducing protein hydrophobicity⁴⁹, we utilized SAP for evaluating the hydrophilicity. However, it traditionally requires extensive molecular dynamics simulations to sample protein conformations. To substantially speed up this process, we employed the protein side-chain packing method CIS-RR⁵⁰ which performs conformation sampling by enumerating rotamer pairs near each residue. Finally, only designs excelling in all three druggability metrics to further validation⁴⁶.

We ultimately identified 11 promising designs by using Wynn statistics to determine the overlap in the designed sequences and filtering based on three parameters: binding energy, RMSD, and SAP score (Fig. 1B and Fig. S1B). To evaluate the binding affinity of each HER2-targeting design, we cloned the 11 designs as Design.01 to Design.11, with C-Myc as an expression tag, into a yeast surface-expression vector for display on the yeast surface. The affinity of the biotinylated HER2 ECD to each design protein displayed on yeast cells was evaluated by flow cytometry. Designs 01 to 05 along with the positive control ABY-025, showed strong binding affinity to HER2 (Design-A), while Designs 06 to 11 demonstrated negligible interaction with HER2 (Design-NA) (Fig. 1C).

2.2. Super stability with high HER2-binding affinity

To enable a comprehensive investigation of structural and functional characteristics, we expressed the five highest affinity

scanning fluorimetry (DSF), where designed proteins were subjected to thermal scans from 25 to 95 °C at a heating rate of 1 °C/min with signals recorded at wavelengths of 350/330 nm. Melting temperature (T_m) is marked by vertical lines. (C) Circular dichroism (CD) spectra of designed proteins under controlled temperature and timing conditions. (D) Flow cytometry assessing HER2 binding to MDA-MB-231, BT-474, and SK-BR-3 cells using FITC-conjugated designed proteins. (E) Quantification of the signal transformation in terms of molar residue ellipticity (MRE) at a wavelength of 222 nm for the designed proteins in CD spectra. (F) Quantification of fluorescence intensity performed in Fig. 2D. (G) Evaluation of trypsin resistance, where designed proteins are incubated with trypsin concentrations in 0.01–10 μ mol/L, followed by the analyses using SDS-PAGE gel electrophoresis (top) and gray intensity analysis comparison (bottom). (H) *In vivo* living imaging from 1 to 8 h after FITC-labelled designed proteins administration *via* tail vein in HER2-overexpressing xenograft tumor-bearing mice; where other two designs (Design.01 and Design.03) which failed to target HER2 tumors well *in vivo*, are not shown. (I) Semiquantitative *ex vivo* biodistribution in tumors and organs post-sacrifice, with values expressed as means $\pm$ SD ($n = 3$); where * $P < 0.05$, ** $P < 0.01$, ns non-significant.

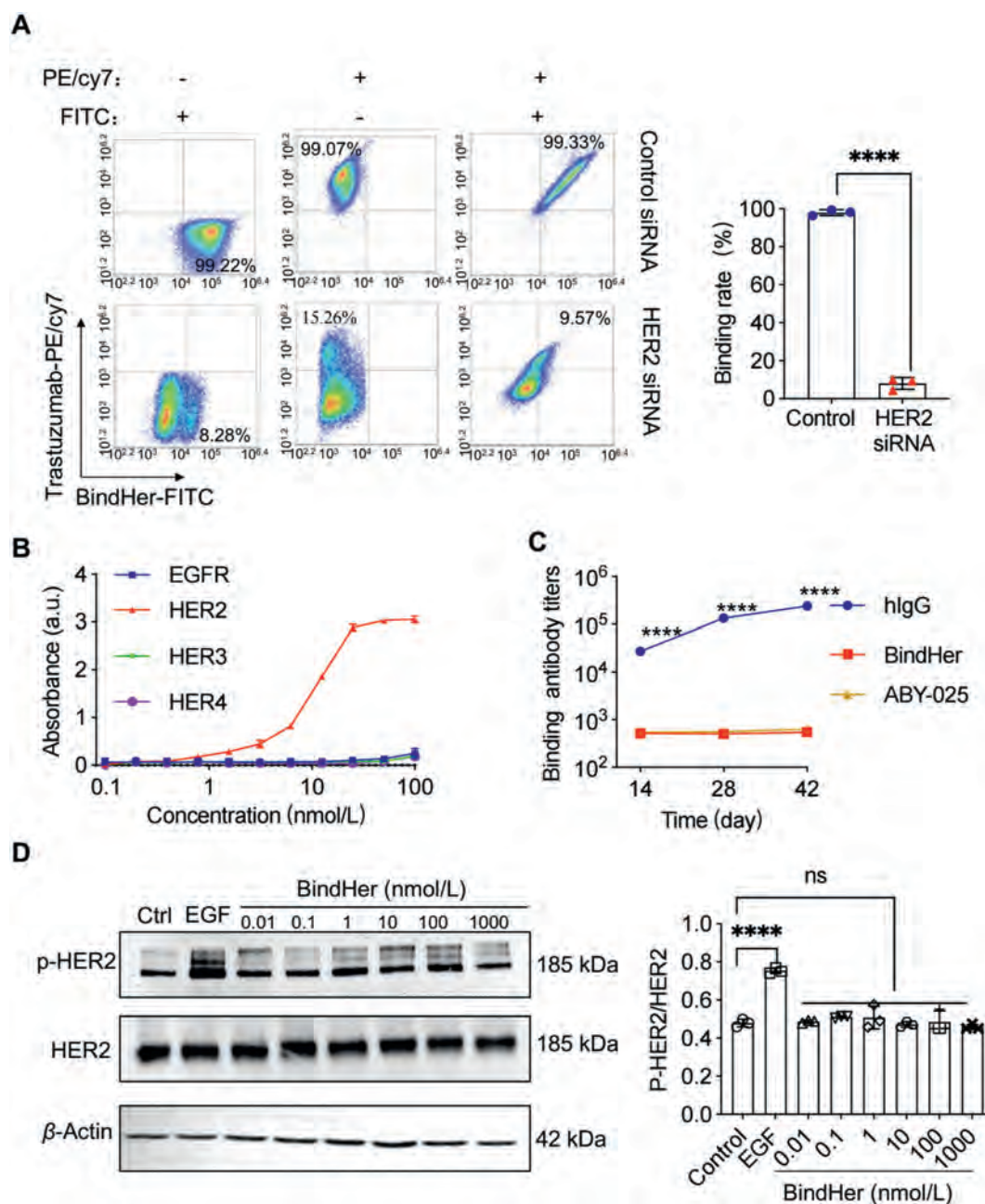


Figure 3 *In vitro* pharmacodynamic characterization of BindHer. (A) Flow cytometry analysis of HER2 levels in SK-BR-3 cells treated with HER2 siRNA, using Trastuzumab-PE/Cy7 to confirm HER2 expression. (B) ELISA confirming BindHer's binding specificity to HER2 across concentrations from 100 to 0.098 nmol/L. (C) *In vivo* immunogenicity of BindHer and ABY-025 with hIgG used as a positive control. (D) Western blot of SK-BR-3 cell lysates following BindHer treatment at various concentrations, with EGF as a positive control. Equal protein amounts were loaded in each lane; SDS-PAGE gels were run, and membranes were sectioned by molecular weight for analysis of total HER2, phospho-HER2, and β -actin. Phospho-HER2/HER2 quantification is depicted in graphs, where **** $P < 0.0001$; ns denotes non-significant results.

optimized binders in *Escherichia coli*. All designs exhibited high levels of soluble expression, exceeding 200 mg/L (Supporting Information Fig. S2A), and were predominantly present in the soluble fraction, allowing for efficient purification through Ni^{2+} -NTA chromatography with a purity levels above 95% (Fig. S2B).

The kinetics of protein binding to the immobilized HER2 ECD were analyzed using surface plasmon resonance (SPR). Dissociation equilibrium constants (K_D) ranging from 0.191 to 1.99 nmol/L, indicating affinity levels comparable to ABY-025 (see Fig. 2A and

Supporting Information Table S1). Protein conformational stability was evaluated by differential scanning fluorimetry (DSF), with melting temperature (T_m) values determined as the peak of the first-order derivative of the fluorescence trace using UNcle software. T_m values of Design.01, Design.04, and Design.05 were observed to be higher than that of ABY-025 (Fig. 2B), indicating their superior thermal stability compared to ABY-025.

Circular dichroism (CD) analysis confirmed that all six proteins predominantly adopted alpha-helical structures. The

structural stability of each design under thermal stress was assessed by examining the changes in CD spectra after exposure to 100 °C for various durations. Designs.01, Design.04, and Design.05 showed minimal peak shifts in their CD spectra (Fig. 2C and E), indicating minor structural alterations and enhanced stability under thermal stress.

To further assess stability, we subjected the proteins to incremental concentrations of trypsin (0.01, 0.1, 1, and 10 $\mu\text{mol/L}$) and compared their degradation profiles to ABY-025. At a trypsin concentration of 10 $\mu\text{mol/L}$, ABY-025 showed significant degradation, whereas Design.05 maintained its structural integrity even at higher trypsin concentrations (Fig. 2G). This resilience suggests that Design.05 exhibits superior resistance to proteolytic degradation, making it a promising candidate for *in vivo* imaging applications.

2.3. BindHer demonstrated high binding specificity for HER2-overexpressing tumor cells

To facilitate subsequent experiments, the optimized proteins were labeled, achieving labeling efficiencies above 0.7 for all samples (Supporting Information Fig. S3). Flow cytometry was conducted to assess the affinity of FITC-labeled designed proteins for HER2-positive breast cancer cell lines (SK-BR-3 and BT-474) and HER2-negative breast cancer cells (MDA-MB-231). The designed proteins exhibited strong specificity for HER2-positive cells, as indicated by significantly lower mean fluorescence intensities in HER2-negative MDA-MB-231 cells (Fig. 2D and F).

Fluorescence imaging of mice injected with FITC-labeled Design.05 and ABY-025 showed substantial fluorescence uptake in transplant tumors for 6 h (Fig. 2H), indicating their ability to target and bind HER2-positive tumors *in vivo*. Next, the mice were sacrificed, and their tumors and main organs were collected for *ex vivo* fluorescence imaging to assess biodistribution. Results indicated that Design.05 and ABY-025 exhibited higher fluorescence signals in tumors compared to other organs, with significantly lower hepatic uptake observed for Design.05 compared to ABY-025 ($P = 0.0018$) (Fig. 2I). Overall, these findings (Table 1) led to the selection of Design.05, designated as “BindHer” for its superior performance.

2.4. In vitro pharmacodynamic characterization of BindHer

The binding specificity of BindHer was evaluated through HER2 knockdown experiments using SK-BR-3 cells treated with HER2/neu-specific siRNA. Trastuzumab-PE/Cy7 was utilized to assess the protein expression level of HER2. The results demonstrated a significant reduction in HER2 protein expression in SK-BR-3 cells treated with HER2 siRNA compared to control siRNA-treated cells. Moreover, FITC-labeled BindHer demonstrated a complete loss of binding affinity to HER2-silenced cells (Fig. 3A). BindHer's binding specificity was further validated by an ELISA binding assay, which detected binding exclusively to HER2, with no binding observed to other receptors within the HER family (Fig. 3B).

The immunogenicity of BindHer was evaluated in naive mice treated at 0, 2, and 4-week intervals. After three intravenous administrations of BindHer every two weeks, a minimal antibody response was observed (Fig. 3C). Antibody levels were similar to those induced by ABY-025 and BindHer but significantly lower than those elicited by human IgG, which served as a positive

control. These results confirm that BindHer elicits a low immunogenic response, comparable to that of nanobodies.

To assess the cytotoxicity of BindHer, an *in vitro* MTT assay was conducted to evaluate its effects on breast cancer cell proliferation. SK-BR-3 cells were incubated with varying concentrations of BindHer for 48 h, showing no inhibition of cell growth or proliferation (Supporting Information Fig. S4). Additionally, to investigate the impact of BindHer binding to HER2 on receptor activation, SK-BR-3 cells were treated with different concentrations of BindHer (0.01–1000 nmol/L) for 2 h, and total and phosphorylated HER2 levels were analyzed by Western blot. Compared to untreated cells, the EGF positive control showed a significant increase in phosphorylated HER2 levels, whereas no significant differences were observed across the various concentrations of BindHer (Fig. 3D). These findings indicate that BindHer does not activate the HER2 signaling pathway, highlighting its potential as an effective tool for *in vivo* targeting.

2.5. $^{99\text{m}}\text{Tc}$ -BindHer accumulated in tumor in vivo with significant uptake over ABY-025

The labeling procedure for $^{99\text{m}}\text{Tc}$ -BindHer with a C-terminal GGGC tag is illustrated in Fig. 4A. After assessing radiochemical purity, $^{99\text{m}}\text{Tc}$ -BindHer was prepared with high radiochemical purity (>95%, as depicted in Supporting Information Fig. S5), thereby eliminating the need for further purification post-radiolabeling. *In vitro* stability results showed that $^{99\text{m}}\text{Tc}$ -BindHer exhibits relatively higher stability compared to $^{99\text{m}}\text{Tc}$ -ABY-025 in both PBS and serum (Fig. 4B).

The effectiveness of $^{99\text{m}}\text{Tc}$ -BindHer for tumor imaging was evaluated in mice bearing HER2-positive SK-BR-3 and HER2-negative MDA-MB-231 tumors. SPECT/CT images were obtained at 1, 2, and 4 h post-administration of $^{99\text{m}}\text{Tc}$ -BindHer. As shown in Fig. 4C–E, uptake of $^{99\text{m}}\text{Tc}$ -BindHer in SK-BR-3 tumors was significantly higher than in the MDA-MB-231 tumor group and the BindHer-blocking group ($P < 0.0001$; $n = 3$), indicating that $^{99\text{m}}\text{Tc}$ -BindHer specifically targets HER2 *in vivo*. In addition to tumor uptake, the biodistribution patterns of $^{99\text{m}}\text{Tc}$ -BindHer across normal organs were consistent among the three groups of mice. Significant renal uptake suggests that the probe is primarily metabolized *via* the urinary system⁵¹.

A comparative SPECT/CT imaging analysis of $^{99\text{m}}\text{Tc}$ -BindHer and $^{99\text{m}}\text{Tc}$ -ABY-025 was conducted in mice with subcutaneously implanted SK-BR-3 breast cancer cells, with imaging at 1, 2, and 4 h (Fig. 4F). Starting at 2 h post-administration, uptake of $^{99\text{m}}\text{Tc}$ -BindHer in SK-BR-3 tumors was significantly higher than that of $^{99\text{m}}\text{Tc}$ -ABY-025 ($P < 0.01$; $n = 3$). Non-specific binding of $^{99\text{m}}\text{Tc}$ -ABY-025 to the liver was observed at all time points, while $^{99\text{m}}\text{Tc}$ -BindHer showed significantly lower liver uptake than $^{99\text{m}}\text{Tc}$ -ABY-025, with statistically significant differences ($P < 0.01$; $n = 3$) (Fig. 4G).

2.6. Micro-PET/CT imaging of ^{68}Ga -NOTA-BindHer in HER2-positive tumor xenografts

The N-terminal sequence of BindHer was modified with an additional cysteine residue to enable incorporation of the NOTA linker, as shown in Fig. 5A. Various protein concentration ratios were tested, demonstrating that a 1:1 ratio enabled efficient coupling of BindHer and NOTA, more easily achieved than with ABY-025 (Fig. 5B and C). Mass spectrometry validation of

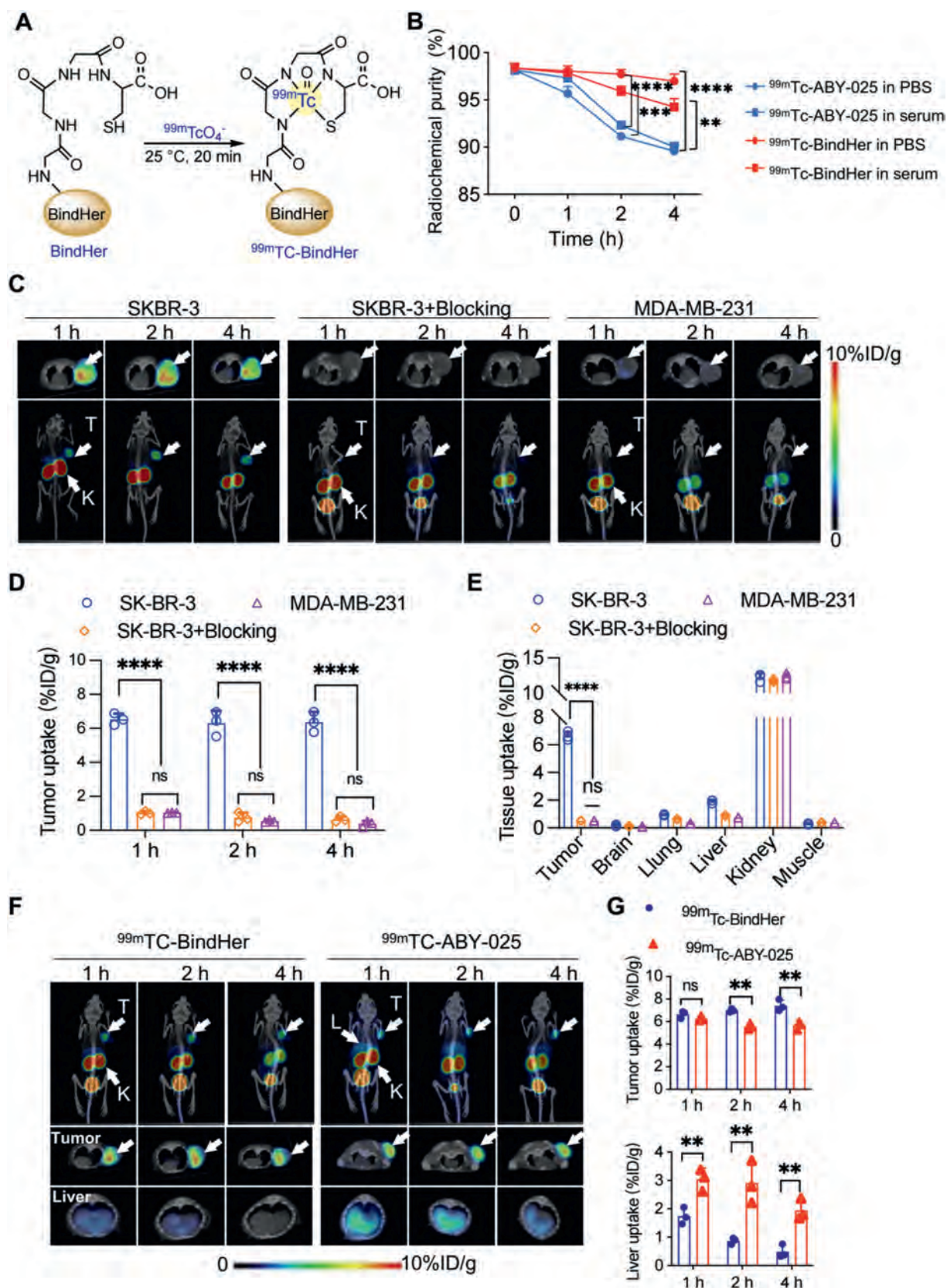


Figure 4 ^{99m}Tc -BindHer noninvasive SPECT imaging of HER2 expression and biodistribution in mice with breast cancer xenografts. (A) Schematic of ^{99m}Tc -labelled BindHer structure, with a GGCC chelator at the C-terminus for ^{99m}Tc complexation. (B) Radiochemical stability of ^{99m}Tc -BindHer (red curve) and ^{99m}Tc -ABY-025 (blue curve) is assessed *in vitro* in PBS (dot) and serum (square) at 37 °C. (C) SPECT/CT imaging of ^{99m}Tc -BindHer in tumour-bearing mice at 1, 2, and 4 h, the arrows indicate the location of the tumor. (D) Data for tumor uptake

NOTA-BindHer showed a ~ 0.5 kDa increase in molecular weight, confirming successful modification (Supporting Information Fig. S6).

SDS-PAGE analysis revealed slight upward shifts in NOTA-ABY-025 and NOTA-BindHer bands relative to their original forms, with no detectable degradation after 1 h at 85 °C or 1 month at 25 °C. Quantitative analysis indicated that over 95% of the primary bands retained their initial intensity (Fig. 5D and E), suggesting comparable thermal stability between NOTA-BindHer and NOTA-ABY-025. This stable interaction with NOTA supports its potential in further radiolabeling experiments. The ^{68}Ga -labeling procedure was completed in under 15 min, yielding a radiochemical purity of over 95% as confirmed by radio-HPLC and iTLC (Supporting Information Fig. S7).

The comparative analysis of tumor uptake between ^{68}Ga -NOTA-BindHer and ^{68}Ga -NOTA-ABY-025 (Fig. 5F) shows that both BindHer and ABY-025 effectively target HER2-positive tumors in tumor-bearing mice. Over time, liver uptake of ^{68}Ga -NOTA-BindHer decreases rapidly, while ^{68}Ga -NOTA-ABY-025 shows progressively higher liver uptake (Fig. 5F), with statistically significant differences between the two proteins (Fig. 5G). Fig. 5H shows that ^{68}Ga -NOTA-BindHer effectively targets HER2-positive tumors in tumor-bearing mice. As seen in the tumor uptake trends in Fig. 5I, when HER2 receptors are pre-blocked with an excess of unlabeled protein, BindHer does not target the tumor and does not bind to tumors formed by HER2-negative cells, confirming that BindHer can specifically bind to HER2-positive receptors for *in vivo* imaging. This finding further validates the specificity of the designed molecule for targeting HER2-positive receptors. The statistical analysis in Fig. 5J shows that in HER2-positive tumor-bearing mice, BindHer uptake was highest in the kidneys, followed by uptake in the tumor, indicating that ^{68}Ga -NOTA-BindHer is excreted through normal metabolic pathways. These results indicate that our protein design approach has successfully produced a protein with improved imaging contrast, targeting specificity, and stability, further addressing clinical diagnostic needs, reducing research workload, and enhancing the efficiency of obtaining optimized proteins.

2.7. Micro-PET/CT imaging of ^{18}F -NOTA-BindHer in HER2 tumor xenografts

The ^{18}F -NOTA-BindHer labeling procedure is depicted in Fig. 6A, with a final radiochemical purity of over 90% after purification (Supporting Information Fig. S8). Micro-PET/CT imaging of SK-BR-3 (HER2-positive) and MDA-MB-231 (HER2-negative) mouse models demonstrated detectable radioactive uptake at tumor sites for both ^{18}F -NOTA-BindHer and ^{18}F -NOTA-ABY-025 at 10, 30, and 60 min post-injection (Fig. 6B and C). Five minutes post-injection, both ^{18}F -NOTA-BindHer and ^{18}F -NOTA-ABY-025 showed liver uptake, which decreased over time. However, from 20 min onwards, a significant difference in liver accumulation between the two was observed ($P < 0.01$) ($n = 3$) (Fig. 6C). These experiments validated that BindHer reduces nonspecific liver accumulation. PET/CT images of mice bearing SK-BR-3 and

MDA-MB-231 tumors at 10, 30, and 60-min post-injection are presented in Fig. 6D, showing notably higher signal intensity in HER2-positive tumors as 30 min post-injection, which remained consistent over time. HER2-positive tumors were clearly visualized, whereas HER2-negative tumors showed minimal detectability. Tumor uptake differences among SK-BR-3 tumor-bearing mice, SK-BR-3 tumor-bearing mice with blocking, and MDA-MB-231 tumor-bearing mice were statistically significant throughout the imaging process (Fig. 6E and F).

2.8. Structural validation of BindHer and ABY-025

Fig. 7A shows the sequence alignments of BindHer, ZHER2:342, and ABY-025, all of which are affibodies with HER2 affinity obtained through library screening and site-directed mutagenesis, originating from the wild-type Z-domain from the immunoglobulin-binding region of staphylococcal protein A^{2,29}. Notably, ZHER2:342 and ABY-025 exhibit typical and one of the largest variations in previous affibody designs², which have 13 and 24 mutations from the wild-type Z-domain, respectively. In contrast, BindHer introduces 38 residue changes, demonstrating the power of protein design in exploring new sequence and structure spaces for novel affibodies.

To examine the structural feature of the designed BindHer, we used two different methods, I-TASSER^{48,52} and AlphaFold2⁵³ which are built on statistics and deep-learning principles respectively, to create structure models of BindHer. Despite the different modeling principles, both programs generate models with high confidence: I-TASSER model has C-score = 0.48 and TM-score = 0.78, AlphaFold2 has pLDDT = 93.4 and TM-score = 0.93 to the scaffold structure (PDB ID 3MZM). Here, C-score for I-TASSER is in the region $[-5, 2]$ with C-score > -1.5 indicating a correct fold⁴¹, while pLDDT is in $[0, 100]$ with pLDDT > 70 indicating a correct fold⁵³, suggesting that the confidence scores of I-TASSER and AlphaFold2 are both significantly beyond the thresholds. The TM-score⁴⁴ between the I-TASSER and AlphaFold2 models is 0.86 (Fig. 7B). Such high-level of modeling confidence and structural consensus from different folding methods show the reliability of the structure models, which also partly reflects the folding stability of the designed BindHer by the EvoDesign.

In Fig. 7C, we present a comparison of the AlphaFold2 model of BindHer with the structure of ABY-025 in complex with HER2 ECD. Although BindHer and ABY-025 have very different sequences (*i.e.*, with 39.6% of residues having different identities, see Fig. 7A), the binding interface with HER2 was highly conserved (Fig. 7C). Our analysis revealed reduction in surface hydrophobic networks and hydrophobic surface area. Specifically, the hydrophobic surface area of BindHer was notably smaller than that of ABY-025 (Fig. 7D). As anticipated, bis-ANS fluorescence assays showed that ABY-025 had 3.66 ± 0.52 times higher fluorescence than BindHer at equivalent protein concentrations (Fig. 7E), indicating less exposed hydrophobic patches in BindHer. These results indicate that rationally reducing surface hydrophobicity through design can effectively lower nonspecific hepatic uptake.

at different time points. (E) Data for tissue uptake at 2 h point. (F) SPECT images of HER2 tumor-bearing mice after tail vein injection of $^{99\text{m}}\text{Tc}$ -BindHer (top) or $^{99\text{m}}\text{Tc}$ -ABY-025 (bottom) at 1, 2, and 4 h. Coronal imaging was performed to visualize the mice (top), while transverse imaging captured the tumor and liver regions (bottom). The arrows indicate significant radionuclide accumulation in various tissues, including liver (L), kidney (K), and tumor (T). (G) Quantification of SPECT signals in HER2 tumors (top) and liver (bottom). Values are expressed as the means $\pm$ SD ($n = 3$), where $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$, ns represents non-significant.

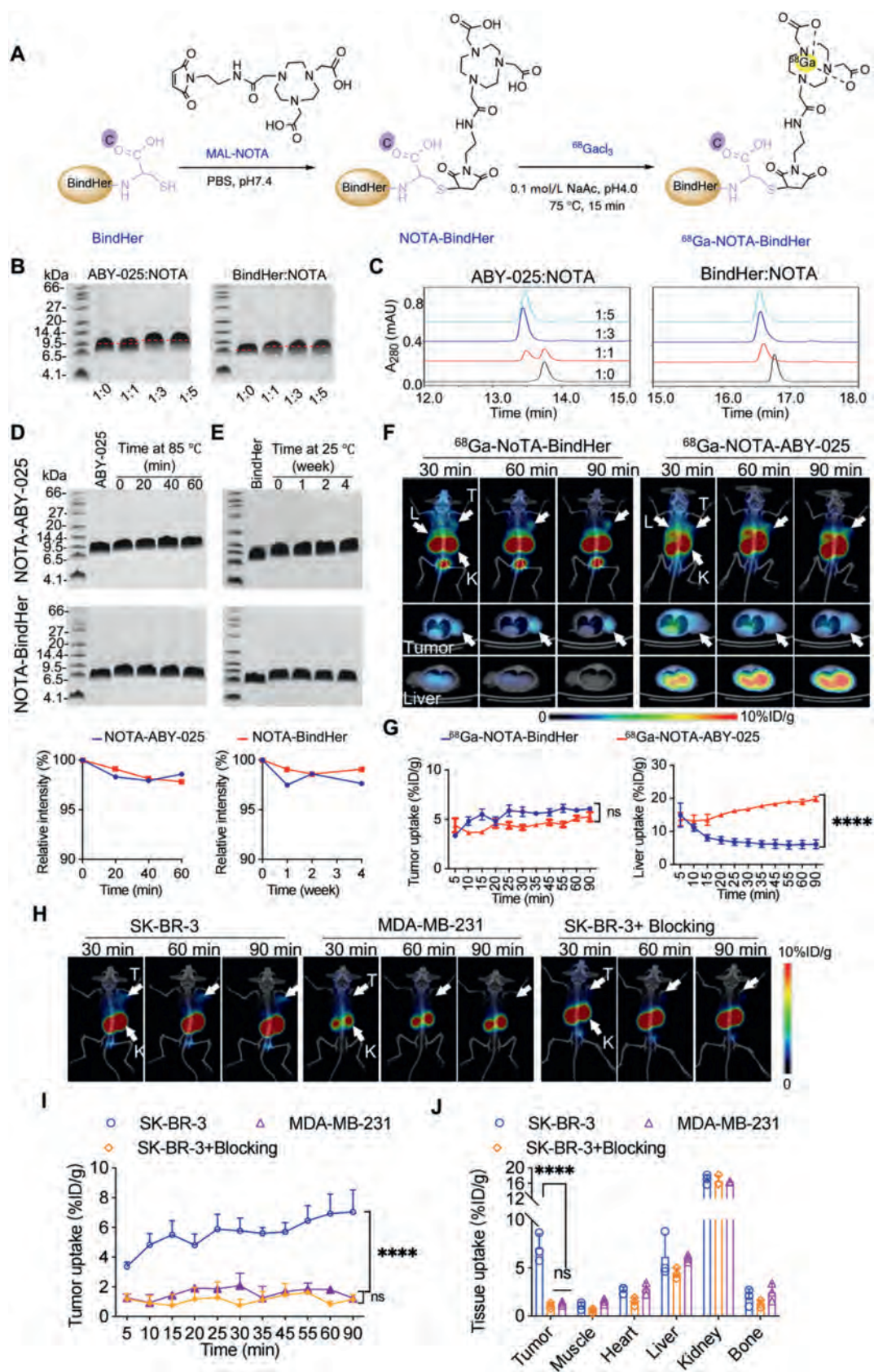


Figure 5 ^{68}Ga -NOTA-BindHer noninvasive PET imaging of HER2 expression and biodistribution in mice with breast cancer xenografts. (A) Synthesis flow chart of ^{68}Ga -NOTA-BindHer. The precursor protein was first formed through a Michael addition reaction at 25 °C overnight using BindHer and MAL-NOTA, followed by chelation of ^{68}Ga and precursors in sodium acetate buffer (pH 4.0) at 75 °C for 15 min which resulted in

Additionally, surface charge analysis reveals that BindHer has significantly reduced surface charge compared to ABY-025 as shown in Fig. 7F. Differences in charged residues were observed, with net charges of -4 for BindHer and $+1$ for ABY-025, respectively (Fig. 7G). This result is consistent with recent studies demonstrating that net negative surface charge reduces nonspecific hepatic uptake^{54,55}. The observed increase in net negative charge in BindHer is linked to the application of the SAP filtering criterion in our design strategy. This outcome substantiates the fundamental principles and effectiveness of our design approach.

3. Discussion

Many existing Affibodies are developed through directed evolution and mutation engineering of wild-type protein scaffolds, such as the B-domain in the immunoglobulin-binding region of staphylococcal protein A^{2,29}. This traditional approach often imposes constraints on sequence and functional variations, thereby limiting their therapeutic potential and application range. Previous studies have shown that non-specific liver uptake during hepatic metastasis diagnosis has adversely affected their use in HER2-specific imaging agents^{34,35,36}. In response, we have enhanced the EvoDesign evolutionary protein design protocol by incorporating a druggability-directed screening process. This innovative approach ranks the designed proteins based on their optimal binding affinity, folding integrity, and spatial aggregation properties, ensuring that only the most promising candidates advance to further development. It finally engineered a novel mini-protein that exhibits exceptional thermal stability and high selectivity in binding to HER2, with a significant reduction in liver uptake. Furthermore, it is anticipated that the pharmacokinetic and pharmacodynamic properties could be further enhanced by fusing it with an immunoglobulin Fc domain or conjugating it with a cytotoxic drug, making it highly suitable for immune-oncology therapeutic applications. Consequently, this computational designed mini-protein holds significant potential as a versatile therapeutic agent, functioning as an antibody-like protein with improved efficacy and broader therapeutic applications^{56,57}.

Conventional protein engineering often involves random mutagenesis and/or site-specific mutation to optimize drug candidates⁵⁸. Such approaches often suffer from a heavy workload and low success rate, especially for small protein molecules that involve extensive negative selections in mutation discrimination. The evolutionary protein design protocol, as demonstrated in this example of BindHer, can effectively integrate homologous-profile guided design simulation with physical force field filtering and structure folding-based design selections. This integrated methodology shows particular promise for optimizing existing therapeutic proteins while maintaining their core functional properties.

Recent machine learning-based protein design tools create innovative folded structures through generative models, enabling the exploration of new folding spaces and the design of complex

and unique proteins. However, the complexity of these structural designs is high, often requiring extensive experimental validation, which affects their rapid application in functional protein optimization. In this study, we chose EvoDesign due to the demonstrated reliability and high success rate by the combination of evolutionary profile with physical energy terms^{59,60}. Compared to other tools, EvoDesign has remarkable advantages in balancing structural innovation with stability and functionality optimization. This high level of sequence changes induced from automated design simulations, while still maintaining functionality and stability, offers a promising potential for broader range of therapeutic applications, compared to the traditional protein engineering approaches.

4. Experimental

4.1. Cell lines and animal models

Human breast adenocarcinoma cell lines SK-BR-3 (ATCC, HTB-30), BT-474 (ATCC, HTB-20) and MDA-MB-231 (ATCC, CRM-HTB-26) cells were obtained from the American Type Culture Collection. The yeast cell EBY100 was donated by Professor Zhang Yang's Lab. All experimental studies were approved by the Experimental Animal Management Committee and the Experimental Animal Ethics Committee of the State Key Laboratory of Biotherapy at Sichuan University. For the xenograft mouse model, female BALB/c nude mice (Beijing Huafukang Biotechnology Co., Ltd., China) at 4–6 weeks of age were injected with 2×10^6 cells. Once palpable tumors were established, tumor size was measured every two days with calipers every two days. Tumor volume was calculated as Eq. (1):

$$\text{Tumor volume} = (A \times B^2) / 2 \quad (1)$$

where A and B represent the tumor length and width, respectively. For *in vivo* evaluation, tumors were monitored until the average size of xenograft tumors reached about 100 mm^3 .

4.2. Computational design of BindHer designed by a modified EvoDesign protocol

The crystal structure of HER2 ECD with affinity matured 3-helix affibody ZHER2:342 (PDB ID 3MZW)⁴³ was refined with the EvoDesign protocol^{38,39} with coordinate constraints, where the ZHER2:342 component was extracted and used as the starting point (probe) for docking and design. EvoDesign first searched for template proteins (templates) with similar structures to the probe in the PDB library through the structural similarity comparison algorithm TM-align⁴⁴, which have similar backbone structures but may not be similar in sequences to the target. By structurally aligning the template and probe proteins, we obtained a MSA based on the structural similarity. Next, a structural profile is created from the MSA, which is specified by an $L \times 20$ matrix, with L being the scaffold length and 20 denoting the number of

the production of ^{68}Ga -NOTA-BindHer. (B) SDA-PAGE analysis of ABY-025 and BindHer reaction products with MAL-NOTA at varying chelation ratios. (C) HPLC analysis of reaction products at various chelation ratios. (D, E) Stability of NOTA-ABY-025 and NOTA-BindHer at 85°C for 60 min or 25°C for 4 weeks, with SDA-PAGE on the left and gray-value analysis on the right. (F) PET/CT imaging of ^{68}Ga -NOTA-BindHer and ^{68}Ga -NOTA-ABY-025 at different time points in HER2-expression tumor bearing mice. L: liver, K: kidneys, and T: tumor. (G) Quantification of PET signal in tumor (left) and liver (right), respectively. (H) The PET/CT imaging of ^{68}Ga -NOTA-BindHer at different time points in tumor bearing mice. (I) Comparison of tumor absorptions of ^{68}Ga -NOTA-BindHer at different time points. (J) Comparison of tissues absorption of ^{68}Ga -NOTA-BindHer at 90 min. Values are means $\pm$ SD ($n = 3$), where **** $P < 0.0001$, ns represents non-significant.

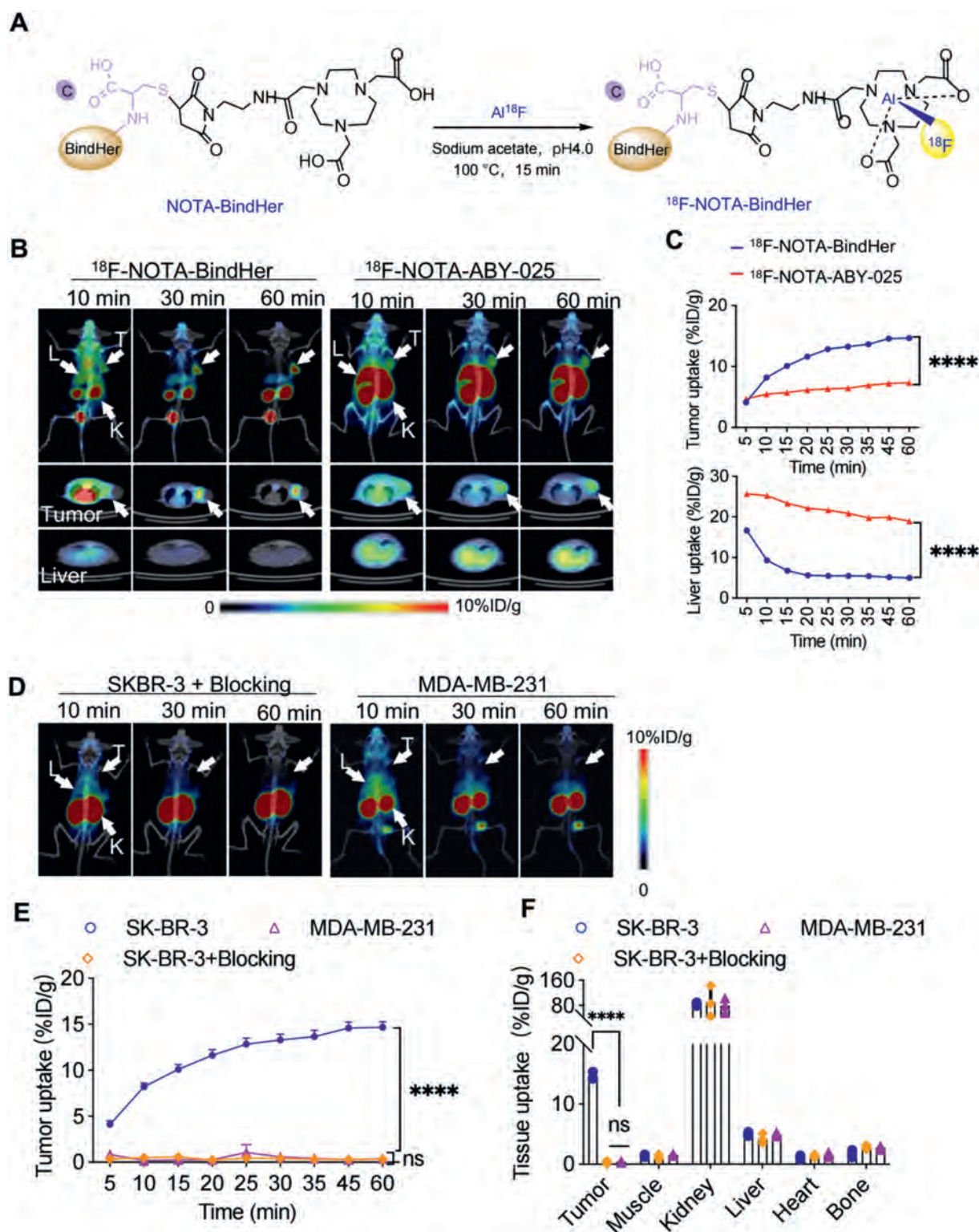


Figure 6 ^{18}F -NOTA-BindHer noninvasive PET imaging of HER2 expression and biodistribution in breast cancer xenograft mice. (A) Synthesis of ^{18}F -NOTA-BindHer through Al^{18}F chelation in sodium acetate buffer (pH 4.0) at 100 °C for 15 min, followed by impurity removal *via* gel filtration chromatography. (B) Coronal and transverse Micro-PET/CT imaging of breast cancer xenograft mice treated with ^{18}F -NOTA-BindHer and ^{18}F -NOTA-ABY-025, showing the presence of HER2 positive breast cancer tumors (T), liver (L) and kidneys (K) at various time points. (C) Quantitative time-radioactivity curves of ^{18}F -NOTA-BindHer (blue circle) and ^{18}F -NOTA-ABY-025 (red triangle) in tumors and liver, based on dynamic PET/CT imaging over 0–1 h. (D) Static Micro-PET/CT scanning of both HER2⁺ SK-BR-3 (blue circle) and MDA-MB-231 (blue circle) tumor-bearing nude mice models at 10, 30, and 60 min. Arrows indicate tumor locations. (E) Tumor absorption of ^{18}F -NOTA-BindHer at different time points. (F) Tissue absorption of ^{18}F -NOTA-BindHer at 60 min. Values are means $\pm$ SD ($n = 3$), where **** $P < 0.0001$, ns represents non-significant.

different amino acid types. The elements of the profile matrix for amino acid a at position p are given by Eq. (2):

$$M(p, a) = \sum_{x=1}^{20} w(p, x) \times B(a, x) \quad (2)$$

where $B(a, x)$ is the BLOSUM62 substitution matrix⁶¹ with x varying for 20 amino acids. $w(p, x)$ is the weighted frequency of the amino acid x appearing at the p th position of the MSA by the Henikoff weight⁶² $H(p, x)$, i.e., $w(p, x) = \sum H(p, x)$.

The sequence design search was performed by REMC simulations⁴⁵ under guidance of a composite energy function which combines the evolutionary structure profile with multiple knowledge- and physics-based energy terms, including secondary structure prediction⁶³, solvent accessibility prediction⁶⁴, backbone torsion angle prediction⁶⁵, and the FoldX force field⁶⁶. While the original EvoDesign protocol selects sequence designs by sequence clustering⁵⁹, we extended the protocol and made the selection based on the consensus of therapeutic qualities of protein binders. It includes the constraints of binding affinity, folding integrity, and spatial aggregation property (SAP). For this, we first collected 500 sequence decoys from the EvoDesign trajectory sorted by the EvoDesign energy score. Next, we ranked the decoys by the binding and folding energy terms of EvoEF2⁴⁰, RMSD of the I-TASSER^{48,52} to the target scaffold ZHER2:342, and the residue number of SAP hotspots. SAP of residue i is calculated as Eq. (3):

$$SAP_i = \sum_{\text{Simulation Average}} \left\{ \sum_{\text{Residue within R from i}} \left(\frac{\text{SAA of side chain atoms within R}}{\text{SAA of side chain atoms of fully exposed residue}} \times \text{Residue Hydrophobicity} \right) \right\} \quad (3)$$

It is important to note that traditional calculation of SAP relies on averaging conformations from molecular dynamics simulations, which is prohibitively time-consuming for assessing a large number of designed protein structures. To overcome this, we introduced a protein side-chain conformation sampling method that identifies the most probable side-chain rotamers around each residue. This approach, named Clash-Detection Guided Iterative Search (CIS-RR)⁵⁰, significantly accelerates the SAP calculation, completing it in approximately 10 s for proteins with fewer than 100 residues. This is over 100 times faster than traditional molecular dynamics simulations. Benchmarking demonstrated that our enhanced SAP method produces results highly consistent with molecular dynamics-based SAP calculations (Supporting Information Fig. S9). Our enhanced SAP program can be obtained at <http://cao.labshare.cn/SAP-cisrr/>. Finally, a set of 11 designs are selected based on the consensus of selected designed which all filtering based on each of scoring functions.

4.3. Yeast display

Proteins were synthesized from synthetic DNA (GeneWiz). Yeast cells were then transformed with genes encoding the proteins for display, along with a linearized pCTcon2 vector (41843, Addgene). The sequences of the expression vectors pCTcon2 were inserted between HA and Myc using *NdeI* and *BamHI* restriction enzyme digestion sites, and then transformed into yeast cells for surface display. Yeast cells were cultured in SDCAA medium before induction in SGCAA medium. Following 18–24 h of induction, the cells were washed with chilled PBS buffer and

incubated with Anti-cmyc (p01106, Gallus Immunotech) and biotinylated HER2, while being agitated at 4 °C. After a 30-min incubation, cells were washed again in chilled PBS buffer and then incubated on ice for 15 min with Goat Anti-Chicken IgG Antibody-Alexa Fluor 647 (A-21449, Thermo Scientific, 1 µL per 100 µL yeast suspension) and Streptavidin-Alexa Fluor 488 (S32345, Thermo Scientific, 1 µL per 100 µL yeast suspension). Yeast cells were then counted by flow cytometry (ACEA NovoCyte, Agilent).

4.4. Protein expression and purification

The genes encoding the designed protein sequences were synthesized and cloned into the pET-29a(+) expression vector (Novagen). Each designed protein incorporated an N-terminal tag sequence (MHHHHHH), immediately followed by the designed protein sequence and a cysteine residue at the C-terminus for labeling purposes. The plasmids were transformed into chemically competent *E. coli* BL21 (DE3) cells. Protein expression was carried out in LB broth at 37 °C with 50 µg/mL kanamycin until the optical density at 600 nm (OD600) reached approximately 0.8. Expression was induced with 1 mmol/L isopropyl β-D-thiogalactopyranoside (IPTG), and the temperature was lowered to 18 °C for approximately 18 h. Cultures were pelleted and resuspended in lysis buffer (20 mmol/L Tris pH 8.0, 500 mmol/L

NaCl) using an ultrasonic disintegrator. The soluble fraction was clarified by centrifugation at 15,000×*g* for 30 min. Purification of the soluble fraction was performed using Ni Sepharose 6 Fast Flow resin (17531803, Cytiva), followed by size-exclusion chromatography on a Superdex 75 prep grade column (17104402, Cytiva) in 20 mmol/L sodium phosphate buffer pH 8 with 150 mmol/L NaCl. The purified proteins were characterized by SDS-PAGE and mass spectrometry to verify their monomeric state and molecular weight. Purity was confirmed by reverse-phase high-performance liquid chromatography.

4.5. Surface plasmon resonance (SPR) interaction analyses

Recombinant Human HER2 ECD (1004-HCCH, Sino Biological) was immobilized (~2000 resonance units) on a CM5 sensor chip (BR-1000-12, GE Life Sciences) and analyzed using a Biacore instrument (Biacore X100, GE Life Sciences). Two-fold serial dilutions of proteins were allowed to flow over the immobilized ligands for 180 s, with dissociation measured for 420 s. Surface regeneration for all interactions was conducted using a 120-s exposure to 10 mmol/L HCl, pH 2. The SPR experiments were carried out in 10 mmol/L HEPES, pH 7.4, with 150 mmol/L NaCl at 25 °C, with a flow rate of 10 µL/min to prevent analyte rebinding. Data visualization and processing were done using Biacore evaluation software (Version 2.0, GE Life Sciences). Equilibrium titration curve fitting and equilibrium binding dissociation values were determined using GraphPad Prism (Version 8.0, GraphPad Software Inc.).

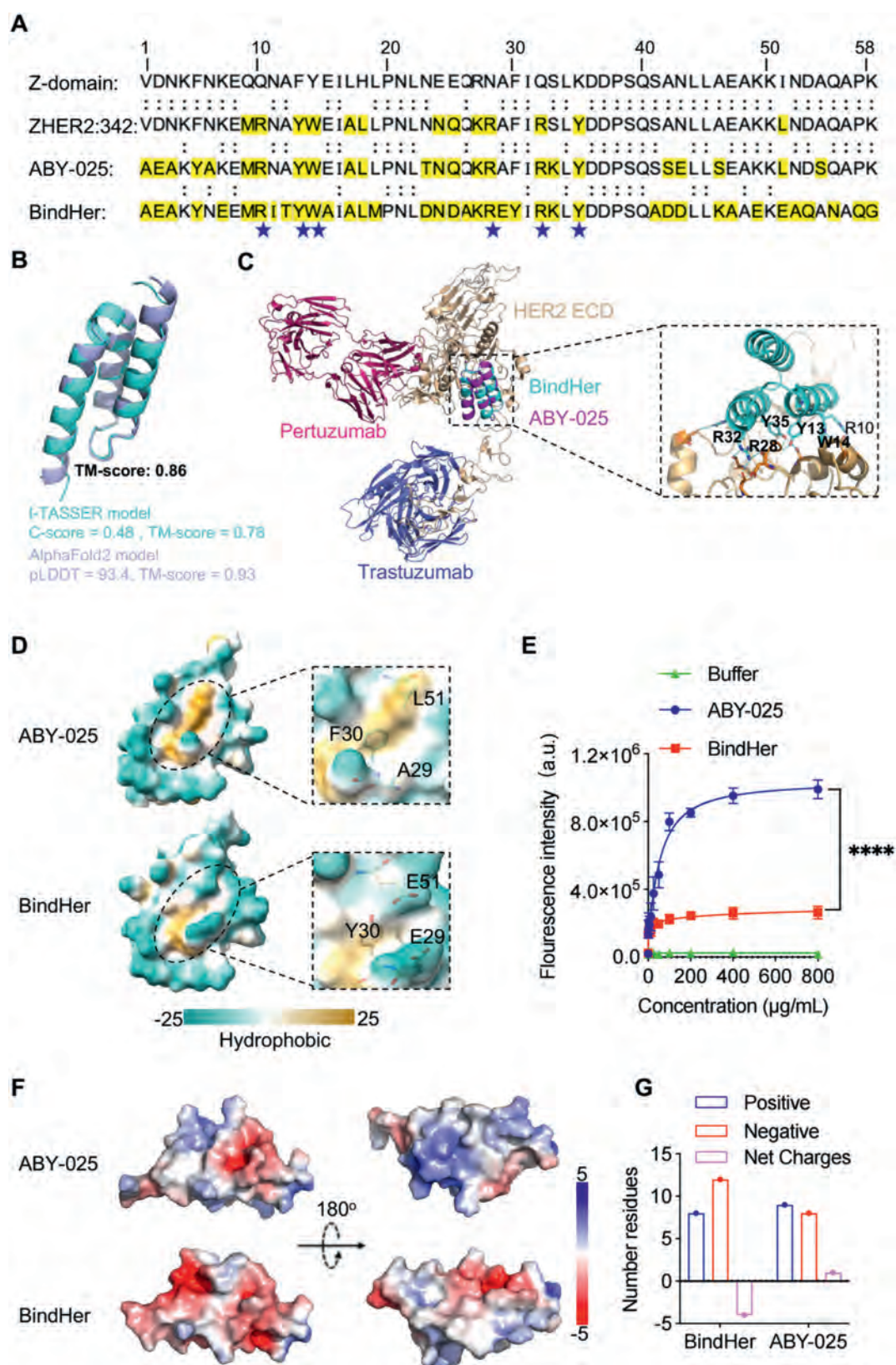


Figure 7 Characteristics of designed BindHer protein. (A) Sequence alignments of BindHer (Design.05) with ZHER2:342, ABY-025, and wild-type Z-domain. Conserved residues are indicated by “:”, yellow highlights mutated residues against Z-domain, and blue stars mark the core interface binding residues (R10, Y13, W14, R28, R32, Y35) with HER2. (B) Structural alignment of the I-TASSER (green) and AlphaFold2 (orange) prediction of BindHer. (C) Structure overlay of AlphaFold2 model of BindHer on ABY-025 in complex with HER2. The right panel shows the zoom-in of the ABY-025 structure, where the core interface residues to HER2 ECD are displayed in sticks. For reference, the Fab

4.6. Differential scanning fluorometry

All proteins were prepared in the same buffer at a concentration of 1 mg/mL prior to the experiment. Each 9 μ L sample was loaded in triplicate into test tubes and heated from 25 to 95 °C at a rate of 1 °C per minute, using a high-throughput protein stabilization analyzer (Uncle, Unchained Labs).

4.7. Circular dichroism

Purified proteins were dialyzed overnight at 4 °C in 20 mmol/L sodium phosphate buffer, pH 7.4, with the addition of 500 μ mol/L TCEP for cysteine-containing proteins. All circular dichroism data were collected using an AVIV Model 400 spectrometer (Aviv Biomedical Inc.). Far UV spectra and temperature melts were measured with 10 μ mol/L protein in a quartz cuvette with a path length of 10 mm. Wavelength spectra were recorded between 195 and 260 nm at both 25 and 100 °C.

4.8. Trypsin digestion

Trypsin was diluted into various concentration gradients and added to equal amounts of protein (1 mg/mL) to achieve final reaction concentrations of 10, 1, 0.1, 0.01 μ mol/L. The mixtures were incubated for 60 min at 25 °C subsequently, the reaction was terminated by adding an equal volume of 1% BSA. Samples were then analyzed by non-reducing SDS-PAGE, and the grayscale of the bands was examined for analysis.

4.9. Fluorescence conjugation

BindHer molecules contain a unique C-terminal cysteine residue that allows for site-specific labeling. To reduce oxidized cysteine levels prior to conjugation, the BindHer molecules were incubated with 5 mmol/L neutral TCEP (pH 7.4) for 20 min at 25 °C. Immediately after reduction, Fluorescein-5-maleimide (62245, Thermo Fisher Scientific) dissolved in DMSO was added, and conjugation was completed following the manufacturer's protocol. Unreacted Alexa Fluor-maleimide dyes were removed using a NAP-5 desalting column (17-0853-02, GE Life Sciences). All procedures were performed under dimmed light. The protein labeled with fluorescent markers and the protein from which free fluorescein was removed were analyzed using paper chromatography. Imaging was conducted using the IVIS LUMINA III system (PerkinElmer) to ensure complete removal of free fluorescein. The degree of labeling was calculated according to the manufacturer's instructions.

4.10. Flow cytometry

Cell suspensions of SK-BR-3, BT-474, and MDA-MB-231 were adjusted to a concentration of 2×10^6 cells/mL in phosphate-

buffered saline (PBS). These cells were stained with 100 nmol/L FITC-labeled protein and incubated for 2 h at 25 °C. After incubation, cells were washed once with PBS before flow cytometry analysis. Flow cytometry assays were conducted using a NovoCyte Flow Cytometer (Agilent).

4.11. In vivo fluorescence imaging

The imaging agent was administered *via* tail vein injection into breast cancer-bearing mice. Small animal live imaging was then performed using the IVIS LUMINA III system (PerkinElmer). Tumor-bearing mice were anesthetized with isoflurane before imaging. Imaging settings included auto exposure and an absorption value of 488 nm for static acquisition. Four hours post-injection, the mice were euthanized *via* spinal dislocation. The brain, heart, lungs, liver, kidneys, spleen, muscles, bones, and tumor tissues were immediately harvested and collected in a weighing dish, arranged on a dark board, and imaged using the small animal live imaging system to measure the fluorescence intensity of each tissue.

4.12. Immunogenicity

BALB/c mice (female, 6–8 weeks old, three mice per group) received tail vein injections of BindHer, human immunoglobulin (hIgG), or ABY-025 every two weeks, for a total of three doses at 3 mg/kg. Blood samples were collected bi-weekly to determine antigen-specific IgG titers. 96-well plates were coated with proteins at a concentration of 1 μ g/mL in carbonate coating buffer and incubated overnight at 4 °C. After three washes with PBS containing 0.1% Tween-20 (PBST), the plates were blocked with 200 μ L/well of 1% bovine serum albumin for 1 h at 37 °C. The immune serum was thawed slowly at 4 °C, serially diluted, added to the plates, and incubated at 37 °C for 1 h. Following three additional washes with PBST, 100 μ L/well of Goat anti-mouse IgG Secondary Antibody HRP (31430, Thermo Fisher Scientific) was added and incubated for 1 h at 37 °C. The plates were then washed five times before adding 100 μ L/well of TMB reagent for color development. After 10–15 min, 100 μ L/well of ELISA stop solution was added to stop the reaction. Finally, a microplate reader (Spectramax i3x, Molecular Devices) was used to measure the absorbance.

4.13. ^{99m}Tc radiolabelling

BindHer radiolabeling was performed using an optimized two-vial kit method as described by Ahlgren⁶⁷. Each kit contained 5 mg of sodium α -D-gluconate dihydrate, 100 μ g of edetate disodium (Na_2EDTA), and 75 μ g of tin (II) chloride dihydrate ($\text{SnCl}_2 \times 2\text{H}_2\text{O}$). To perform the labeling, the kit contents were dissolved in 100 μ L of degassed PBS and added to 100 μ g of the BindHer molecule. Subsequently, 100 μ L (~ 37 MBq) of the

fragments of therapeutic monoclonal antibodies trastuzumab (red) and pertuzumab (cyan) are shown binding HER2 epitopes, which are distant from the binding regions of BindHer and ABY-025. Right showed that the core interface binding residues (R10, Y13, W14, R28, R32, Y35) with HER2. (D) Distribution of surface hydrophobic networks, with orange indicating hydrophobic residues of ABY-025 and BindHer. (E) Bis-ANS fluorescence of ABY-025 and BindHer at different concentration, with error bar representing 95% confidence interval (CI) from three technical replicates, $****P < 0.0001$. (F) The ABY-025 and BindHer structures are depicted in the electrostatic surface view, with red representing potentials of -5 kTe^{-1} and blue representing potentials of 5 kTe^{-1} . The electrostatic potentials were computed using PyMol's APBS module. (G) Comparison of positive, negative and net charges of ABY-025 and BindHer. The secondary structure, surface hydrophobicity, and surface electrostatic potentials of each protein in the plot were visualized using PyMol and UCSF ChimeraX.

^{99m}Tc -pertechnetate-containing generator eluate (DRN4329, Curium Netherlands B.V.) was added to the reaction mixture. The vial was then filled with argon gas to prevent oxidation. The reaction vials were vortexed thoroughly and incubated at 25 °C for 20 min. The radiochemical purity (RCP) was assessed using instant thin-layer chromatography (iTLC) after the reaction. Chromatography was conducted with a mobile phase consisting of PBS and a pyridine: glacial acetic acid: water mixture in a ratio of 10:6:3. Stability tests were performed by incubating the labeled molecules in either PBS or serum for 4 h, followed by iTLC analysis.

4.14. *NOTA-BindHer-conjugation*

Proteins were conjugated with MAL-NOTA (56491-86-2, Chematech) under standard reaction conditions⁶⁸. Specifically, a solution of 6 μmol BindHer was mixed with 18 μmol MAL-NOTA in 0.2 mol/L ammonium acetate solution. The reaction was carried out at 25 °C for 12 h. After the reaction, excess MAL-NOTA was removed using a NAP-5 desalting column. The chelated protein was then analyzed by RP-HPLC, SDS-PAGE and mass spectrometry.

4.15. *Preparing ^{68}Ga -NOTA-BindHer*

The concentration of NOTA-BindHer was adjusted to 1.5 mg/mL in 0.1 mol/L sodium acetate solution at pH 6.0. This was mixed with lysed ^{68}Ga at a volume ratio of 1:1. Specifically, 100 μL of pre-buffered ^{68}Ga (20–40 MBq) solution was added to 100 μL of 1 mg/mL NOTA-BindHer solution, mixed thoroughly, and allowed to react at 75 °C for 15 min. As a control, 100 μL of pre-buffered ^{68}Ga (20–40 MBq) solution was also added to 100 μL of 0.1 mol/L pH 4.0 buffer, mixed thoroughly, and reacted at 75 °C for 15 min. The resulting solution was diluted with 10 mL of saline and passed through a 0.22 μm Millipore filter into a sterile vial. Quality control was performed using radio-HPLC and thin-layer chromatography (TLC).

4.16. *^{18}F radiolabelling*

A solution of NOTA-BindHer molecule (200 μg) in sodium acetate buffer (20 μL , pH 4.0, 0.2 mol/L) was mixed with a solution of AlCl_3 (6 μL , 2 mmol/L in sodium acetate buffer, pH 4.0, 0.5 mol/L) and Fluoride-18 (~ 370 MBq) in 20 μL of target water. This mixture was heated at 100 °C for 15 min. Following the reaction, the solution was diluted with 10 mL of water and purified using a NAP-5 column, which had been equilibrated with 10 column volumes of PBS. The purified product was reconstituted in saline and passed through a 0.22 μm Millipore filter into a sterile vial.

4.17. *In vivo SPECT/CT imaging*

For small-animal SPECT/CT imaging, mice bearing SK-BR-3 or MDA-MB-231 tumors were scanned at 1, 2, and 4 h post-injection of approximately 3.7 MBq ^{99m}Tc -BindHer (approximately 10 μg BindHer). In the blocking group, mice were subcutaneously injected with 500 μg of unlabeled recombinant BindHer in 250 μL PBS 1 h before the injection of the radioactive compound. The mice were anesthetized with 2% isoflurane in O_2 . SPECT/CT scans were performed using a nanoScan-SPECT/CT (MILabs) with the following parameters: peak at 140 keV, 20% width, and a

frame time of 25 s, for a total acquisition time of 13.5 min. Subsequently, CT images were acquired at 50 kVp, 0.67 mA, with a rotation of 210 and an exposure time of 300 ms. All SPECT images were reconstructed and analyzed by delineating volumes of interest on the tumor and major organs, and tissue uptake values are presented as the percent injected dose per gram (%ID/g).

4.18. *In vivo PET/CT imaging*

For small-animal PET/CT imaging, mice bearing SK-BR-3 or MDA-MB-231 tumors were given a ~ 3.7 MBq injection (in 100 μL PBS) of ^{68}Ga -NOTA-BindHer or ^{18}F -NOTA-BindHer. A dynamic PET scan with using the micro PET/CT (SIEMENS). As in the previous protocol, the blocking group received a subcutaneous injection of 500 μg of unlabeled recombinant BindHer in 250 μL PBS 1 h before the radioactive injection. Dynamic PET/CT imaging was performed using the PET imaging instrument immediately after the injection of the imaging agent. All mice were anesthetized with isoflurane and placed in the prone position on the examination bed before imaging. Acquisition parameters included a magnification of $3 \times$, an acquisition matrix of 256×256 , and an acquisition time of 1.5 h. All PET/CT images were reconstructed and analyzed by delineating volumes of interest on the tumor and major organs, and tissue uptake values are presented as the percent injected dose per gram (%ID/g).

4.19. *ANS fluorescence detection*

The protein was initially incubated at concentrations ranging from 0 to 0.8 mg/mL with a solution of 25 $\mu\text{mol/L}$ bis-ANS in PBS buffer (pH 7.2) for 5 min at room temperature to ensure equilibration. The fluorescence emitted by bis-ANS was then measured using a spectrofluorometer (SpectraMax i3x, Molecular Devices). The excitation wavelength was set to 385 nm, and emission was recorded at 475 nm, with both excitation and emission band passes adjusted to a width of 2 nm. A negative control experiment (blank) was established by omitting the protein.

4.20. *Cell proliferation assay*

The MTT assay was employed to assess the toxicity of BindHer. Briefly, cells were seeded at a density of 1×10^4 cells per well in 96-well plates. Following exposure to varying concentrations of the protein under specific treatment conditions, the cells were incubated with MTT at a final concentration of 0.5 mg/mL for 4 h at 37 °C. Subsequently, the medium was removed, and the formazan crystals were dissolved by adding 150 μL of dimethyl sulfoxide (DMSO). The absorbance was measured at 570 nm using a multiwell scanning spectrophotometer.

4.21. *Knockdown of HER2*

SK-BR-3 cells were plated in 6-well plates at a density of 10^6 cells per well and incubated for 24 h. HER2 siRNA I (#6282, Cell Signaling Technology) and control siRNA (#6568, Cell Signaling Technology) were transfected into the cells using Lipofectamine™ 3000 (Invitrogen) according to the manufacturer's instructions. Cells were harvested 72 h post-transfection, and HER2-silenced cells were subjected to an aptamer-binding assay analyzed by flow cytometry.

4.22. Immunoblotting analyses for protein phosphorylation detection

SK-BR-3 cells were treated with BindHer at concentrations of 0.01, 0.1, 1, 10, 100 and 1000 nmol/L for 2 h, followed by EGF (10 ng/mL, Sino Biological) for 10 min. The cells were then washed once with PBS and solubilized at 4 °C for 30 min in RIPA lysis buffer. The samples were pelleted by centrifugation at 13,000×g for 15 min at 4 °C, mixed with 4 × sample buffer, and heated to 100 °C for 10 min. Proteins were fractionated by 10% SDS–PAGE and transferred to nitrocellulose membranes (Cell Signaling Technology). The membranes were blocked in TBST buffer containing 5% skim milk for 1 h at room temperature, followed by overnight incubation at 4 °C with specific primary antibodies, including β -actin (4970, 1:1000, Cell Signaling Technology), HER2/ErbB2 (D8F12) XP® Rabbit mAb (4290, 1:1000, Cell Signaling Technology), and phospho-HER2 (Y1221 + Y1222) (2243, 1:1000, Cell Signaling Technology). After three 5-min washes in TBST, the membranes were incubated with the appropriate HRP-conjugated secondary antibody (7074, 1:10,000, Cell Signaling Technology) at room temperature for 1 h. Following three additional 5-min washes in TBST, the membranes were incubated with SignalFire™ ECL Reagent (Cell Signaling Technology) and imaged using the chemiluminescent imaging system (iBright 1500, Invitrogen).

4.23. Figures and statistical analysis

Figures were created using Inkscape, GraphPad Prism, PyMOL, UCSF ChimeraX, BioRender, Python, InteractiVenn, and FlowJo. Statistical analyses were conducted in GraphPad Prism 9. Detailed information about sample sizes and the specific statistical tests used is provided in the Figure legends. Differences between two groups were analyzed using Student's paired *t*-test. For multiple comparisons, two-way analysis of variance (ANOVA) followed by a Bonferroni *post hoc* test was performed. Values of $P < 0.05$ were considered statistically significant, with * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$ indicating increasing levels of significance.

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Author contributions

Nongyu Huang, Yang Cao, Suwen Chen, and Juan Chen designed and performed most of the experiments and analyzed the data. Xiaoqiong Wei and Chengxin Zhang optimized the protein design strategy. Guangjun Xiong, Yifan Zhou, and Wenling Wu were responsible for protein purification. Yawen Hu, Pei Zhou, Guolin Li, Fulei Zhao, Fanlian Zeng, Xiaoyan Wang, and Xinai Cui conducted animal immunization and ELISA. Nongyu Huang, Suwen Chen, Juan Chen, Jiadong Yu, Huawei Cai and Chengcheng Yue carried out nuclide labeling and *in vivo* evaluations.

Nongyu Huang and Yang Cao drafted the manuscript. Kaijun Cui, Yuquan Wei, Yang Cao, Yang Zhang, and Jiong Li designed the experiments, interpreted the data, revised the manuscript, and supervised the project. All authors have read and approved the manuscript.

Conflicts of interest

The authors declare no conflicts of interest.

Appendix A. Supporting information

Supporting information to this article can be found online at <https://doi.org/10.1016/j.apsb.2025.07.015>.

References

1. Binz HK, Amstutz P, Pluckthun A. Engineering novel binding proteins from nonimmunoglobulin domains. *Nat Biotechnol* 2005;**23**:1257–68.
2. Lofblom J, Feldwisch J, Tolmachev V, Carlsson J, Stahl S, Frejd FY. Affibody molecules: engineered proteins for therapeutic, diagnostic and biotechnological applications. *FEBS Lett* 2010;**584**:2670–80.
3. Li Y, Li F, Duan Z, Liu R, Jiao W, Wu H, et al. SYNBIIP 2.0: epitopes mapping, sequence expansion and scaffolds discovery for synthetic binding protein innovation. *Nucleic Acids Res* 2025;**53**:D595–603.
4. Stahl S, Graslund T, Eriksson Karlstrom A, Frejd FY, Nygren PA, Lofblom J. Affibody molecules in biotechnological and medical applications. *Trends Biotechnol* 2017;**35**:691–712.
5. Peper-Gabriel JK, Pavlidou M, Pattarini L, Morales-Kastresana A, Jaquin TJ, Gallou C, et al. The PD-L1/4-1BB bispecific antibody-anticalin fusion protein PRS-344/S095012 elicits strong t-cell stimulation in a tumor-localized manner. *Clin Cancer Res* 2022;**28**:3387–99.
6. Rothenberger S, Hurdiss DL, Walser M, Malvezzi F, Mayor J, Ryter S, et al. The trispecific DARPin ensovibep inhibits diverse SARS-CoV-2 variants. *Nat Biotechnol* 2022;**40**:1845–54.
7. Leka O, Wu Y, Zanetti G, Furler S, Reinberg T, Marinho J, et al. A DARPin promotes faster onset of botulinum neurotoxin A1 action. *Nat Commun* 2023;**14**:8317.
8. Albert C, Bracaglia L, Koide A, DiRito J, Lysy T, Harkins L, et al. Monobody adapter for functional antibody display on nanoparticles for adaptable targeted delivery applications. *Nat Commun* 2022;**13**:5998.
9. Yang EY, Shah K. Nanobodies: next generation of cancer diagnostics and therapeutics. *Front Oncol* 2020;**10**:1182.
10. Luo R, Liu H, Cheng Z. Protein scaffolds: antibody alternatives for cancer diagnosis and therapy. *RSC Chem Biol* 2022;**3**:830–47.
11. Oroujeni M, Bezverkhniaia EA, Xu T, Liu Y, Plotnikov EV, Klint S, et al. Evaluation of affinity matured affibody molecules for imaging of the immune checkpoint protein B7-H3. *Nucl Med Biol* 2023;**124–125**:108384.
12. Yang J, Zhang Z, Yang F, Zhang H, Wu H, Zhu F, et al. Computational design and modeling of nanobodies toward SARS-CoV-2 receptor binding domain. *Chem Biol Drug Des* 2021;**98**:1–18.
13. Koenig PA, Das H, Liu H, Kummerer BM, Gohr FN, Jenster LM, et al. Structure-guided multivalent nanobodies block SARS-CoV-2 infection and suppress mutational escape. *Science* 2021;**371**:eabe6230.
14. Veliky I, Wennborg A, Feldwisch J, Lindman H, Carlsson J, Sorensen J. Good manufacturing practice production of [(68)Ga]Ga-ABY-025 for HER₂ specific breast cancer imaging. *Am J Nucl Med Mol Imaging* 2016;**6**:135–53.
15. Zhang JZ, Nguyen WH, Greenwood N, Rose JC, Ong SE, Maly DJ, et al. Computationally designed sensors detect endogenous Ras activity and signaling effectors at subcellular resolution. *Nat Biotechnol* 2024;**42**:1888–98.

16. Watson JL, Juergens D, Bennett NR, Trippe BL, Yim J, Eisenach HE, et al. *De novo* design of protein structure and function with RFdiffusion. *Nature* 2023;**620**:1089–100.
17. Cao L, Coventry B, Goresnik I, Huang B, Sheffler W, Park JS, et al. Design of protein-binding proteins from the target structure alone. *Nature* 2022;**605**:551–60.
18. Huang B, Xu Y, Hu X, Liu Y, Liao S, Zhang J, et al. A backbone-centred energy function of neural networks for protein design. *Nature* 2022;**602**:523–8.
19. DARPins score against COVID-19. *Nat Biotechnol* 2022;**40**(3):285.
20. Pearce R, Huang X, Omenn GS, Zhang Y. *De novo* protein fold design through sequence-independent fragment assembly simulations. *Proc Natl Acad Sci U S A* 2023;**120**:e2208275120.
21. Kuhlman B, Dantas G, Ireton GC, Varani G, Stoddard BL, Baker D. Design of a novel globular protein fold with atomic-level accuracy. *Science* 2003;**302**:1364–8.
22. Silva DA, Yu S, Ulge UY, Spangler JB, Jude KM, Labao-Almeida C, et al. *De novo* design of potent and selective mimics of IL-2 and IL-15. *Nature* 2019;**565**:186–91.
23. Berger S, Seeger F, Yu TY, Aydin M, Yang H, Rosenblum D, et al. Preclinical proof of principle for orally delivered Th17 antagonist miniproteins. *Cell* 2024;**187**:4305–17. e18.
24. Cao L, Goresnik I, Coventry B, Case JB, Miller L, Kozodoy L, et al. *De novo* design of picomolar SARS-CoV-2 miniprotein inhibitors. *Science* 2020;**370**:426–31.
25. Sesterhenn F, Yang C, Bonet J, Cramer JT, Wen X, Wang Y, et al. *De novo* protein design enables the precise induction of RSV-neutralizing antibodies. *Science* 2020;**368**:eaay5051.
26. Arunachalam PS, Walls AC, Golden N, Atyeo C, Fischinger S, Li C, et al. Adjuvanting a subunit COVID-19 vaccine to induce protective immunity. *Nature* 2021;**594**:253–8.
27. Chen Z, Johnson MC, Chen J, Bick MJ, Boyken SE, Lin B, et al. Self-assembling 2D arrays with *de novo* protein building blocks. *J Am Chem Soc* 2019;**141**:8891–5.
28. Shen H, Fallas JA, Lynch E, Sheffler W, Parry B, Jannetty N, et al. *De novo* design of self-assembling helical protein filaments. *Science* 2018;**362**:705–9.
29. Feldwisch J, Tolmachev V, Lendel C, Herne N, Sjoberg A, Larsson B, et al. Design of an optimized scaffold for affibody molecules. *J Mol Biol* 2010;**398**:232–47.
30. Tolmachev V, Orlova A. Affibody molecules as targeting vectors for PET imaging. *Cancers (Basel)* 2020;**12**:651.
31. Cho HS, Mason K, Ramyar KX, Stanley AM, Gabelli SB, Denney Jr DW, et al. Structure of the extracellular region of HER2 alone and in complex with the Herceptin Fab. *Nature* 2003;**421**:756–60.
32. Alhuseinikhudhur A, Lubberink M, Lindman H, Tolmachev V, Frejd FY, Feldwisch J, et al. Kinetic analysis of HER2-binding ABY-025 affibody molecule using dynamic PET in patients with metastatic breast cancer. *EJNMMI Res* 2020;**10**:21.
33. Sorensen J, Sandberg D, Sandstrom M, Wennborg A, Feldwisch J, Tolmachev V, et al. First-in-human molecular imaging of HER2 expression in breast cancer metastases using the ¹¹¹In-ABY-025 affibody molecule. *J Nucl Med* 2014;**55**:730–5.
34. Sorensen J, Velikyan I, Sandberg D, Wennborg A, Feldwisch J, Tolmachev V, et al. Measuring HER2-receptor expression in metastatic breast cancer using [68Ga]ABY-025 affibody PET/CT. *Theranostics* 2016;**6**:262–71.
35. Oroujeni M, Rinne SS, Vorobyeva A, Loftenius A, Feldwisch J, Jonasson P, et al. Preclinical evaluation of ^{99m}Tc-ZHER2:41071, a second-generation affibody-based HER2-visualizing imaging probe with a low renal uptake. *Int J Mol Sci* 2021;**22**:2770.
36. Bragina O, Chernov V, Larkina M, Rybina A, Zelchan R, Garbukov E, et al. Phase I clinical evaluation of ^{99m}Tc-labeled affibody molecule for imaging HER2 expression in breast cancer. *Theranostics* 2023;**13**:4858–71.
37. Li Y, Wang S, Yang W, Liu H. Prognostic significance of molecular subtype, metastatic site and primary tumor surgery for survival in primary metastatic breast cancer: a SEER-based study. *Medicine (Baltim)* 2021;**100**:e26619.
38. Pearce R, Huang X, Setiawan D, Zhang Y. EvoDesign: designing protein-protein binding interactions using evolutionary interface profiles in conjunction with an optimized physical energy function. *J Mol Biol* 2019;**431**:2467–76.
39. Huang X, Pearce R, Zhang Y. *De novo* design of protein peptides to block association of the SARS-CoV-2 spike protein with human ACE2. *Aging (Albany NY)* 2020;**12**:11263–76.
40. Huang X, Pearce R, Zhang Y. EvoEF2: accurate and fast energy function for computational protein design. *Bioinformatics* 2020;**36**:1135–42.
41. Zhang Y. I-TASSER server for protein 3D structure prediction. *BMC Bioinf* 2008;**9**:40.
42. Chennamsetty N, Voynov V, Kayser V, Helk B, Trout BL. Design of therapeutic proteins with enhanced stability. *Proc Natl Acad Sci U S A* 2009;**106**:11937–42.
43. Eigenbrot C, Ultsch M, Dubnovitsky A, Abrahmsen L, Hard T. Structural basis for high-affinity HER2 receptor binding by an engineered protein. *Proc Natl Acad Sci U S A* 2010;**107**:15039–44.
44. Zhang Y, Skolnick J. TM-align: a protein structure alignment algorithm based on the TM-score. *Nucleic Acids Res* 2005;**33**:2302–9.
45. Swendsen RH, Wang JS. Replica Monte Carlo simulation of spin glasses. *Phys Rev Lett* 1986;**57**:2607–9.
46. Ebrahimi SB, Samanta D. Engineering protein-based therapeutics through structural and chemical design. *Nat Commun* 2023;**14**:2411.
47. Zheng W, Zhang C, Li Y, Pearce R, Bell EW, Zhang Y. Folding non-homologous proteins by coupling deep-learning contact maps with I-TASSER assembly simulations. *Cell Rep Methods* 2021;**1**:100014.
48. Zhou X, Zheng W, Li Y, Pearce R, Zhang C, Bell EW, et al. I-TASSER-MTD: a deep-learning-based platform for multi-domain protein structure and function prediction. *Nat Protoc* 2022;**17**:2326–53.
49. Ding H, Altai M, Rinne SS, Vorobyeva A, Tolmachev V, Graslund T, et al. Incorporation of a hydrophilic spacer reduces hepatic uptake of HER2-targeting affibody-DM1 drug conjugates. *Cancers (Basel)* 2019;**11**:1168.
50. Cao Y, Song L, Miao Z, Hu Y, Tian L, Jiang T. Improved side-chain modeling by coupling clash-detection guided iterative search with rotamer relaxation. *Bioinformatics* 2011;**27**:785–90.
51. Lawrence MG, Altenburg MK, Sanford R, Willett JD, Bleasdale B, Ballou B, et al. Permeation of macromolecules into the renal glomerular basement membrane and capture by the tubules. *Proc Natl Acad Sci U S A* 2017;**114**:2958–63.
52. Zheng W, Zhang C, Li Y, Pearce R, Bell EW, Zhang Y. Folding non-homology proteins by coupling deep-learning contact maps with I-TASSER assembly simulations. *Cell Rep Methods* 2021;**1**:100014.
53. Jumper J, Evans R, Pritzel A, Green T, Figurnov M, Ronneberger O, et al. Highly accurate protein structure prediction with AlphaFold. *Nature* 2021;**596**:583–9.
54. Rinne SS, Dahlsson Leitao C, Gentry J, Mitran B, Abouzayed A, Tolmachev V, et al. Increase in negative charge of ⁶⁸Ga/chelator complex reduces unspecific hepatic uptake but does not improve imaging properties of HER3-targeting affibody molecules. *Sci Rep* 2019;**9**:17710.
55. Hosseinimehr SJ, Tolmachev V, Orlova A. Liver uptake of radio-labeled targeting proteins and peptides: considerations for targeting peptide conjugate design. *Drug Discov Today* 2012;**17**:1224–32.
56. Xu T, Ding H, Vorobyeva A, Oroujeni M, Orlova A, Tolmachev V, et al. Drug conjugates based on a monovalent affibody targeting vector can efficiently eradicate HER2 positive human tumors in an experimental mouse model. *Cancers (Basel)* 2020;**13**:85.
57. Nosrati M, Housaindokht MR. New insights into the effect of mutations on affibody-Fc interaction, a molecular dynamics simulation approach. *J Struct Biol* 2023;**215**:107925.
58. Engqvist MKM, Rabe KS. Applications of protein engineering and directed evolution in plant research. *Plant Physiol* 2019;**179**:907–17.

59. Mitra P, Shultis D, Brender JR, Czajka J, Marsh D, Gray F, et al. An evolution-based approach to *De novo* protein design and case study on *Mycobacterium tuberculosis*. *PLoS Comput Biol* 2013;**9**: e1003298.
60. Shultis D, Mitra P, Huang X, Johnson J, Khattak NA, Gray F, et al. Changing the apoptosis pathway through evolutionary protein design. *J Mol Biol* 2019;**431**:825–41.
61. Henikoff S, Henikoff JG. Amino acid substitution matrices from protein blocks. *Proc Natl Acad Sci U S A* 1992;**89**:10915–9.
62. Henikoff S, Henikoff JG. Position-based sequence weights. *J Mol Biol* 1994;**243**:574–8.
63. Yan R, Xu D, Yang J, Walker S, Zhang Y. A comparative assessment and analysis of 20 representative sequence alignment methods for protein structure prediction. *Sci Rep* 2013;**3**:2619.
64. Wu S, Skolnick J, Zhang Y. Ab initio modeling of small proteins by iterative TASSER simulations. *BMC Biol* 2007;**5**:17.
65. Wu S, Zhang Y. ANGLOR: a composite machine-learning algorithm for protein backbone torsion angle prediction. *PLoS One* 2008;**3**: e3400.
66. Schymkowitz J, Borg J, Stricher F, Nys R, Rousseau F, Serrano L. The FoldX web server: an online force field. *Nucleic Acids Res* 2005;**33**: W382–8.
67. Ahlgren S, Andersson K, Tolmachev V. Kit formulation for ^{99m}Tc-labeling of recombinant anti-HER2 affibody molecules with a C-terminally engineered cysteine. *Nucl Med Biol* 2010;**37**:539–46.
68. Xu Q, Zhu C, Xu Y, Pan D, Liu P, Yang R, et al. Preliminary evaluation of [¹⁸F]AIF-NOTA-MAL-Cys39-exendin-4 in insulinoma with PET. *J Drug Target* 2015;**23**:813–20.