Electronic Supplementary Material (ESI) for Lab on a Chip.

Supplementary Information

Tumor Cell Targeting Peptide Ligands Distinguishing through Color-Encoding Microarray

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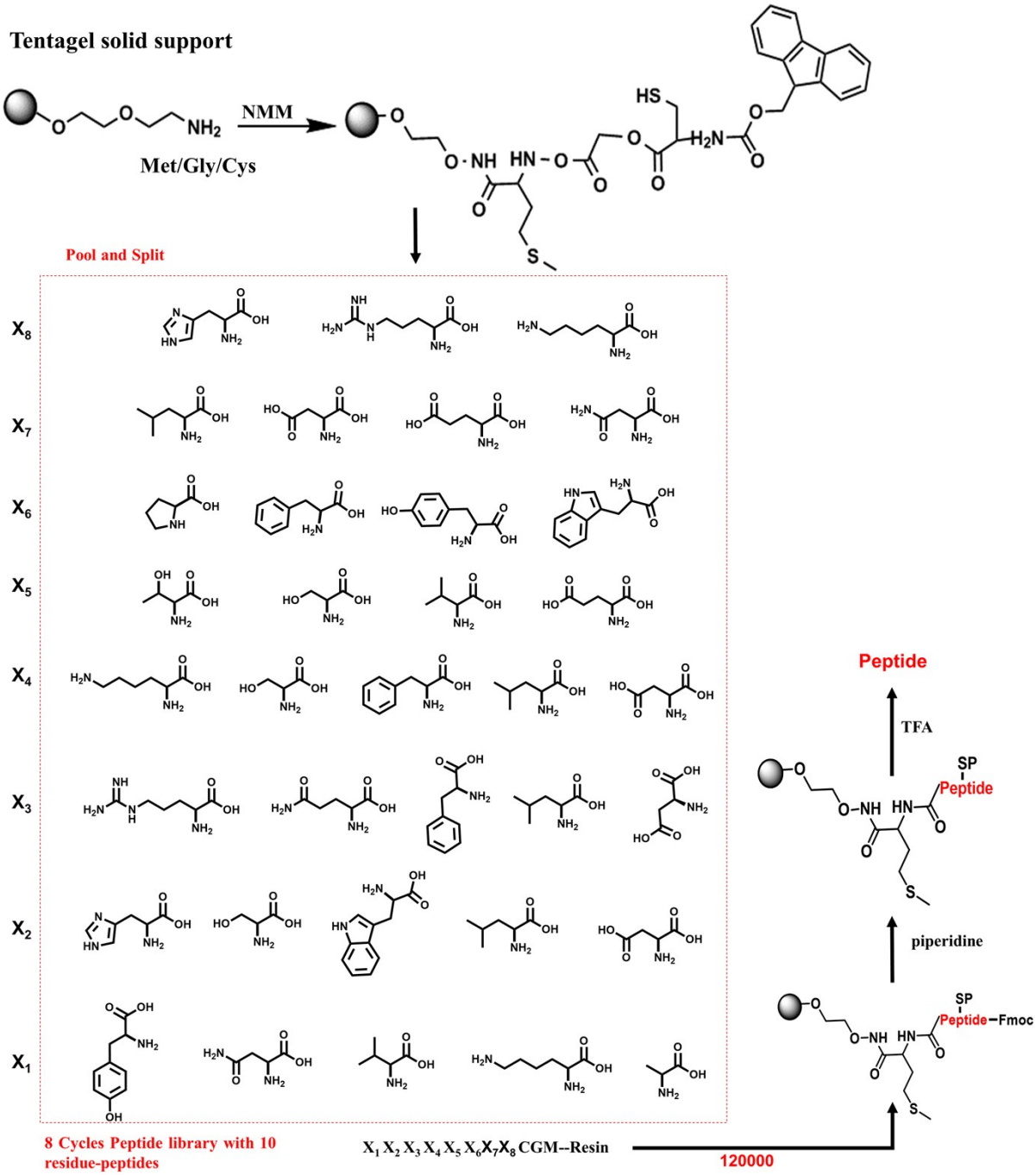
1. Design and synthesis of the OBOC peptide library

The library was served as X_1 X_2 X_3 X_4 X_5 X_6 X_7 X_8 CGM in which X_1 represent either Y, N, V, K or A residues. X_2 represent either H, S, W, L or D residues. X_3 represent either R, Q, F, L or D residues. X_4 represent either K, S, F, L or D residues. X_5 represent either T, S, V or E residues. X_6 represent either P, F, Y or W residues. X_7 represent either L, D, E or N residues. For X_3-X_7, different characters of amino acids were chosen to form various mutations. X_8 represent either H, R or K residues at C-terminal involving hydrophilic and electrostatic interactions may improve ligands interaction with HER family. Met was designed in the C-terminal as the cyanogen bromide cleavage site. The peptide sequences on each bead would be randomly distributed result from the "split and pool" approach. The diversity of the peptide library was $3\times4\times4\times4\times5\times5\times5\times5=1.2\times10^5$ and the redundancy was four. Therefore, the high-throughput screening was performed from approximately 4.8 $\times10^5$ candidate peptide beads. Scheme S1 shows the synthesis process.

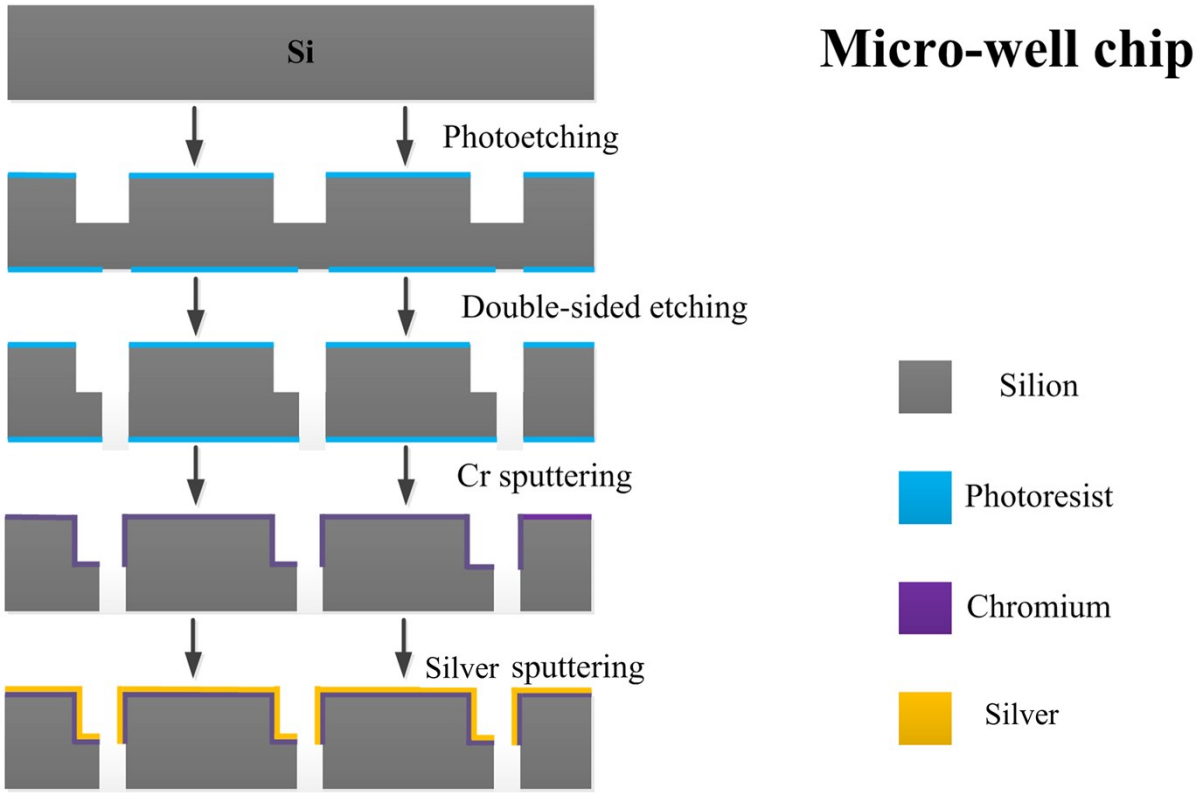


Scheme S1. Synthesis process of the OBOC peptide library

2. Fabrication processes of bi-functional microchip

To fabricate the micro-well array chip, a 4-inch (100) silicon wafer was used as the substrate. The thickness of silicon wafer was 300 μm. The photoresist (the front thickness is 6 μm; the back thickness is 5 μm, SUM-115P, Suntific) was used as mask in the both side and the pattern was transferred onto the silicon wafer. The micro-wells array was formed by ICP (induced couple plasma) etching in both side. The geometric parameters of micro-wells were 230 μm×230 μm. The transparent half through the diagonal of the

microwell was etched through the silicon wafer. After removing the photoresist by acetone, a chrome layer (30 nm) and a silver layer (200 nm) were sequentially coated on the silicon wafer as the conducting layer. Chrome was served as adhesive materials. Silver was used because it has high conductivity. The fabrication details were shown in Scheme S2.



Scheme S2. Fabrication processes of bi-functional microchip

3. *In situ* MALDI-TOF sequencing of the three types of model peptides

The following procedures were carried out: a. matrix, α-cyano-4-hydroxycinnamic acid (CCA), was dissolved in Solvent TA30 (30:70 [v/v] Acetonitrile: TFA 0.1% in water; b. peptide beads was suspended in matrix solution; c. load the peptide beads; d. exposed to the UV light (365 nm, 9 w) for 30 min and then incubated at 4^{o}C overnight in water box. MALDI-TOF MS analysis was performed on a Bruker ULTRAFLEXTREME mass spectrometer (Bruker Daltonics, Germany) equipped with a nitrogen laser with reflectron and positive-ion modes. The laser power energy was adjusted between 0% and 100% to provide laser pulse energy between 0 and 100 μJ per pulse. Each mass spectrum was acquired as an average of 500 laser shots. For *in situ* "one well one bead" analysis, TOF-TOF MS spectra were characterized by searching the MASCOT server after uploading the

sequences list into the Swissport database. Figure S1 show the representative ms/ms spectra of the three model peptides from the *in situ* single beads sequencing.

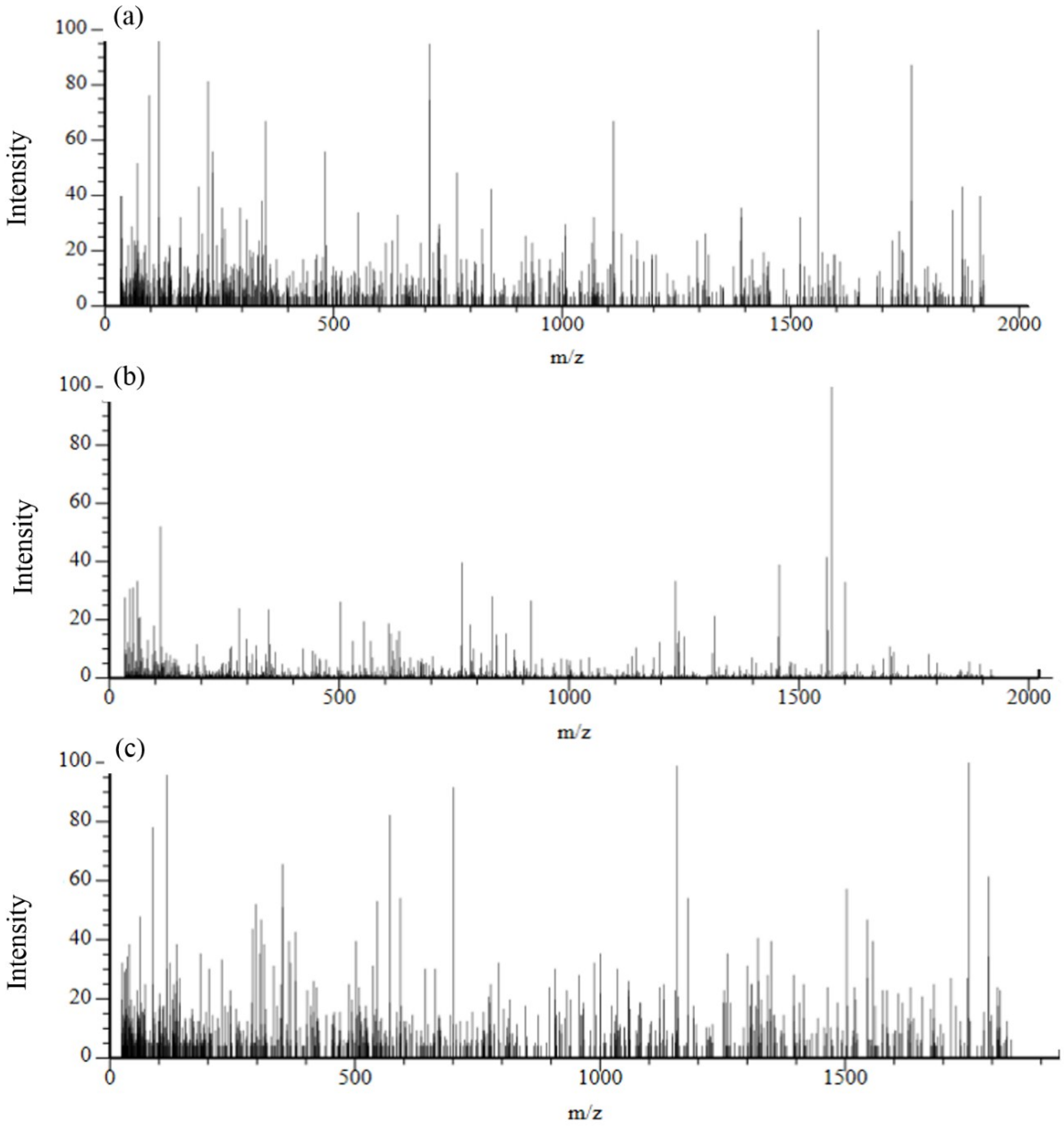


Figure S1. Representative ms/ms spectra for the single peptide beads sequencing after *in situ* photocleavage (a) ms/ms spectrum of the HMP (a) ms/ms spectrum of the H1P (c) ms/ms spectrum of the H2P.

4. Confocal fluorescence imaging of 293A cells

To check the specificity of the H1P, H2P and HMP, human embryonic kidney cell lines 293A (HEK) with no expression of HER2 and HER1 was served as negative cells. As illustrated in Figure S2, none of the peptides showed binding to the cell surface. It was

revealed that the three peptides bind to HER2 and HER1 protein in a specific manner. The human embryonic kidney cell lines 293A were cultured in Hyclone DMEM/high glucose with 10% FBS, penicillin and streptomycin at 37°C containing 5% CO_2. Approximately 1×10^5 mL^{-1} 293A cells were seeded into culture dishes and cultured overnight for cell adherence. FITC-labeled peptide was dissolved in cold PBS at a concentration of 5.0×10^{-5} M. The cells was incubated with FITC-labeled peptide solution (200 μL, with Hoechest 33342 (1 mM) in) in dark for 20 min at 4°C. Finally, the cells were washed three times with cold PBS. Confocal fluorescence imaging was performed on an ZEISS LSM 710 confocal-laser scanning microscope. A 488 nm laser was the excitation source for FITC throughout the experiment and emission was collected between 520-620 nm. Hoechest 33342 was excited at 405 nm. The objective lens used for imaging was a 63×oil-immersion objective (ZEISS).

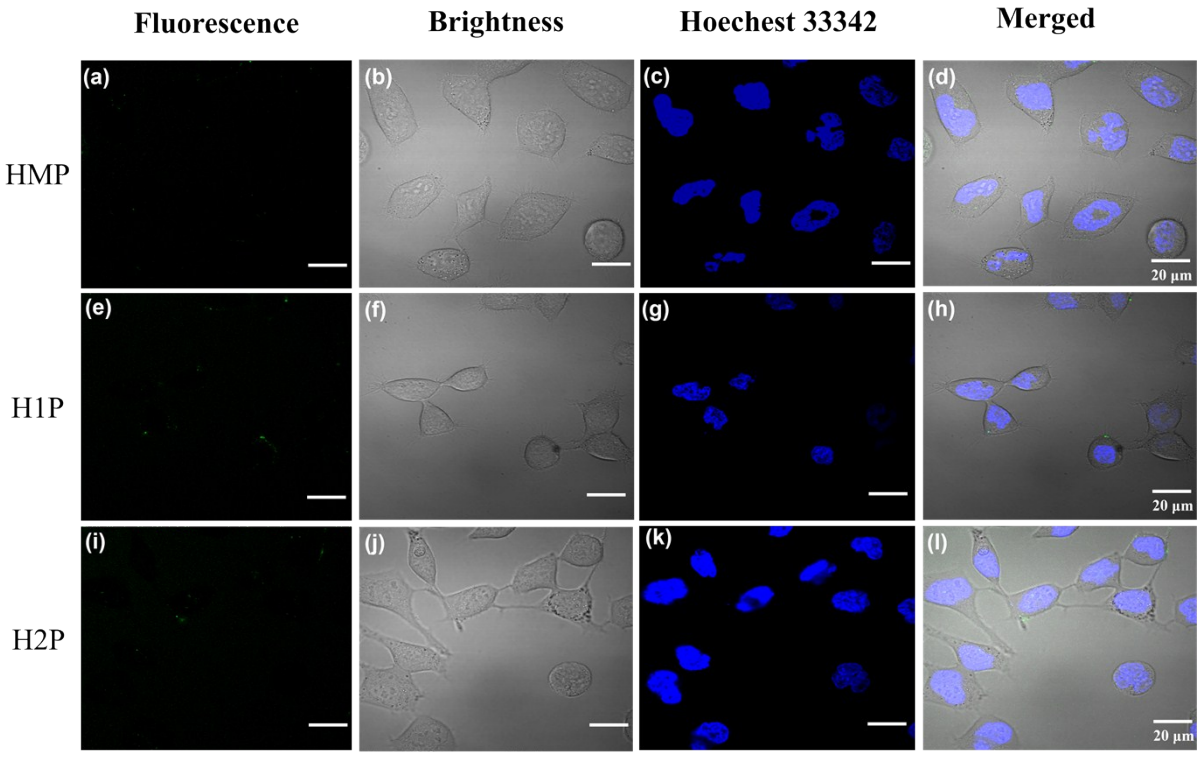


Figure S2. Confocal image of FITC labeled peptides binding towards 293A cells. (a-d) confocal images of the FITC labeled HMP peptides binding towards 293A cells. (e-h) confocal images of the FITC labeled H1P peptides binding towards 293A cells. (i-l) confocal images of the FITC labeled H2P peptides binding towards 293A cells.

5. MTT assay of the two peptides towards cancer cells and normal cells

Cell growth or inhibition was measured by MTT assay, 5×10^3 SKBR-3, MCF-7 and 293A cells were seeded overnight in each well of a 96-well plate in DMEM and RPMI-1640

medium. Various concentrations of peptide were added in each well for 48 hours followed by the adding of MTT solution for 4 hours at 37°C. The solution was removed and 200 μL of dimethyl sulfoxide (DMSO) was added to each well. After 10 minutes of vibration mixing, the optical density (OD) at 570 nm was measured using an ELISA reader. The survival rates of three cells exposed to H1P, H2P and HMP at concentrations ranging from 0.1 μM to 100 μM were close to 100% (Figure S3). It is indicated that peptides show the good biocompatibilities and without significantly stimulate growth or inhibit cell viability.

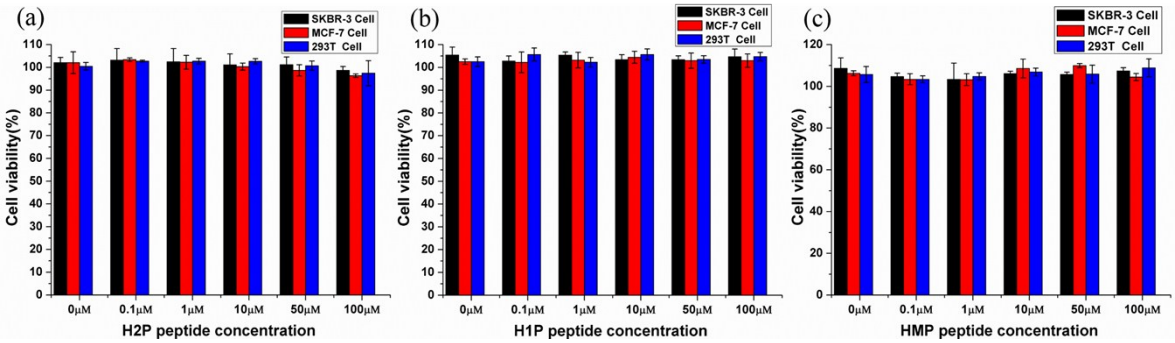


Figure S3. Activity of peptides on the growth of SKBR-3 cells, MCF-7 cells and 293A cells was measured by MTT assay. (Standard error of the mean were obtained from three independent experiments, n=3)

6. Influence detection of the H2P peptide on cell growth process

To further explore the influence of the H2P peptide on cell growth process, we draw cell growth curves by MTT assay. SKBR-3 cells (1×10^4 cells/mL) were grown in 96-well plates with 100 μL RPMI-1640 culture medium (with 10% FBS) in each well. After 24 hours, culture medium was removed and fresh medium containing H2P (50 μmol/L) was added. Control cells were treated with RPMI-1640 culture medium (with 10% FBS) only. In the subsequent seven days, detection was carried out every day. 20 μL MTT solution was added in each well for 4 hrs (37 ^{o}C), then the supernatant was removed and 100 μL DMSO was added in each well. After 10 minutes of vibration mixing, the optical density (OD) at 570 nm was measured using an ELISA (enzyme-linked immuno sorbent assay) reader. Compared with untreated group, there is no obvious difference in cells treated with H2P, the results were shown in Figure S4 (a).

We also check the expression level changes of HER1 and HER2 protein during the cell growth process in the presence of H2P. Cells were treated with H2P (50 μmol/L in 1640 culture medium) for 48 hrs and the control cells were treated with culture medium only. After washing the cells once with cold PBS, they were lysed with protein lysates (5%

SDS) for 5 min and the supplemented were treated with protease inhibitors. Protein lysates were removed by centrifugation at 12,000 rpm for 10 min at 4 °C. Protein concentration was determined using the Pierce BCA Protein Assay kit (Thermo scientific). Equivalent amounts of protein (8 µg) from each sample were resolved by SDS-PAGE and transferred by electroblotting onto PVDF (Polyvinylidene Fluoride, Invitrogen) membranes. Membranes were then blocked with milk and immunoblotted with the HER1-antibody, HER2-antibody and β-Actin antibody overnight at 4 °C. After washing three times in PBST buffer, the membranes were then incubated for 1 h at room temperature with anti-mouse horseradish peroxidase (HRP)-conjugated secondary antibodies (1:1000, Invitrogen). Antibody-protein complexes were visualized by x-ray film method. It also reveaed that there is no obvious difference in cells treated with H2P compared with untreated ones, Figure S4 (b).

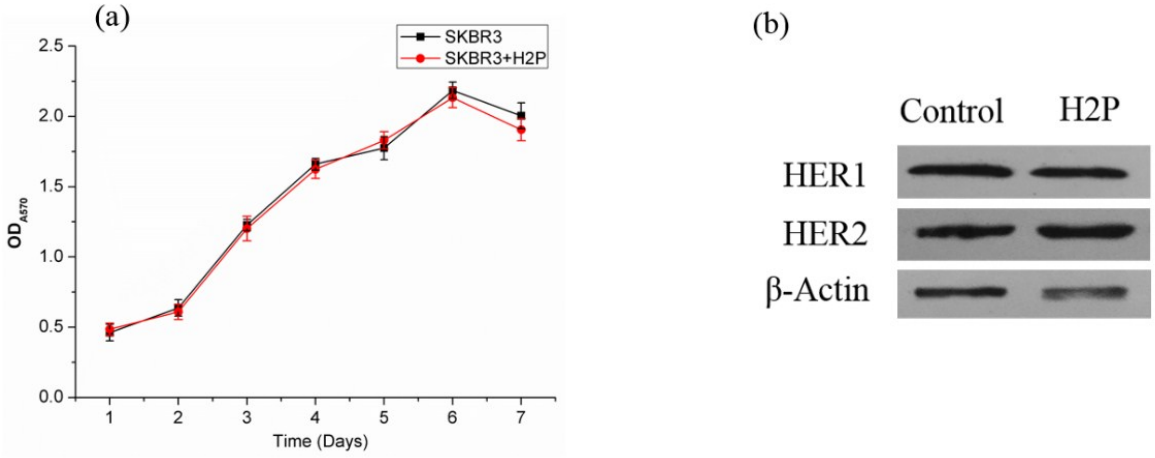


Figure S4. Determination of SKBR-3 cell growth in the presence of H2P. (a) Growth curves for SKBR-3 cell line in the presence of H2P. The results are shown as mean ± SD of three different experiments. (b) Western Blot detection of the total protein (HER1/HER2) in SKBR-3 cells in the presence of H2P.

7. Computer simulation of the interaction energy between peptides and proteins

For a thorough understanding of ligand docking and binding site of protein/ligand complex, a combination protocol of molecular dynamics modelling was carried out. MM/GBSA binding free energy calculations and PyMOL binding free energy decomposition analysis were applied to study the interactions of HER protein/peptide complex. Details of the simulation and were calculated based on 500 snap-shots from 7 to 10 ns simulation trajectories of each complex by using the mm pbsa program in AMBER12. For all four

complexes, our calculation of the binding free energies of HER1/H1P, HER2/H2P, HER1/HMP and HER2/HMP are -40.12, -43.56,-54.87 and -44.17 kcal/mol, respectively (Table S1). The trend of free energy change is consistent with the experimentally measured dissociation constant (K_D). The binding free energy was then decomposed to study the contribution of each residue in the receptor and ligand interactions. Each residue's contribution was shown in Figure S5. The four individual energy components (ΔDele, ΔDvdw, ΔG_{GB}, ΔG_{SA}) are compared through a computational methods MM/GBSA to confirm that which energy term has more impact on the binding affinities (Table S1).

Table S1. Binding free energies and individual energy terms of complexes of protein and peptides calculated by MM/GBSA (kcal/mol)

	ΔE_{vdw}	ΔE_{ele}	ΔG_{GB}	ΔG_{SA}	ΔG_{pred}
HER2/HMP	-48.72 ± 7.99	-72.81 ± 12.28	85.40 ± 11.38	-121.53± 13.99	-44.17 ± 6.45
HER1/HMP	-61.39± 4.46	-59.73± 16.53	77.15 ± 14.23	-121.12±18.65	-54.87 ± 5.79
HER2/H2P	-49.99 ± 4.75	-153.61 ± 12.73	169.30±13.04	-203.60± 14.32	-43.56 ± 3.53
HER1/H1P	-48.82±4.18	-53.38±13.90	70.46 ± 13.34	-102.21± 14.32	-40.12± 4.00

ΔE_{vdw}, van der Waals contribution; ΔE_{ele}, electrostatic contribution; ΔG_{GB}, the polar contribution of desolvation; ΔG_{SA}, nonpolar contribution of desolvation; ΔG_{pred}, the total binding free energy without conformational entropy.

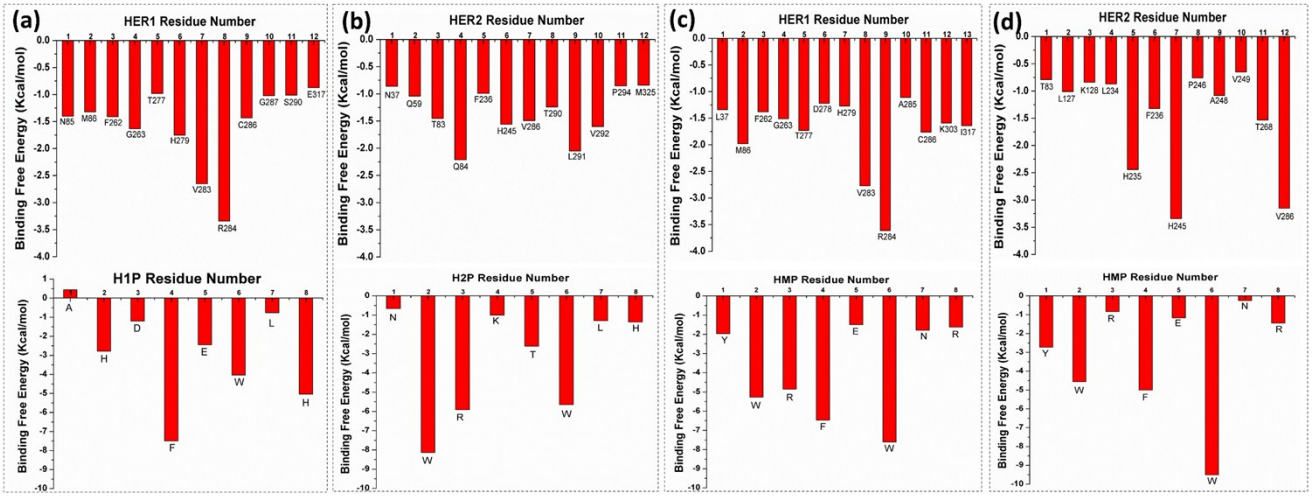


Figure S5. The comparisons of Binding free energy decomposition for key residues of the ligands in the four complexes. **(a)** HER1/H1P complex. **(b)** HER2/H2P complex. **(c)** HER1/HMP complex. **(d)** HER2/HMP complex. It is the N-terminal to C-terminal direction from the left to the right.

Some Additional Experiment Details

1. Materials and reagents

Tentagel Resin (loading 0.26 mmol/g) was from Rapp Polymere (Germany), 9-Fluorenylmethoxy carbonyl (Fmoc)-amino acids were purchased from GL Biochem (China). Trifluoroacetic acid (TFA), streptavidin coated magnetic beads (1 μm) and CHCA (α-Cyano-4-hydroxycinnamic acid) were from Sigma (USA). N-methylmorpholine (NMM), piperidine and N, N'-dimethylformamide (DMF) were all from Beijing Chemical Plant (China). Fmoc-3-Amino-3-(2-nitrophenyl) propionic acid (ANP) was from Advanced ChemTech (USA). Silicon wafer (N/1-0-0, 500 μm) was from KYKY Tech. (China) and the gold surface SPRi chip was Plexera Nanocapture bare gold chip with a gold layer of 47.5 nm thickness and size of 25 cm×75 cm. biotin-labelling kit was from Solulink (USA). SKBR-3, MDA-MB-468, 293A and MCF-7 cells were from China Infrastructure of Cell Line Resources. Cy5 Streptavidin , PE (Phycoerythrin) anti-human HER1 and HER2 antibody was from Biolegend (CA, USA) and FITC Streptavidin was from the eBioscience (CA, USA). HER2 and EGFR protein was from Sino Biological Inc (Beijing, China). Bio-Adembeads Streptavidin was from Ademtech (Paris).

2. Apparatus

MALDI-TOF-MS (ULTRAFLEXTREME mass spectrometer, Bruker Daltonics, Germany); ICP etching system (Plasmalab System100 ICP180); ZEISS LSM 710 Confocal-laser scanning microscope; Surface plasmon resonance imager (Plexera PlexArray® HT system (Plexera LLC, Bothell, WA))；Waters e2695 Alliance HPLC (France).

3. Solid phase synthesis of the model peptides and the OBOC peptide library

Synthesis of the three model peptides: Fmoc strategy SPPS (solid phase peptide synthesis) was employed for synthesis of the three peptides. Tentagel Resin is used as the solid phase support.All the synthesis process was carried out in dehydrous DMF. In the coupling step, the Fmoc-amino acid reagent was dissolved in 0.4 mol/L NMM in DMF and the coupling time was 40 min. In the deprotection step, 20% (v%) piperidine was used to remove the Fmoc group and the deprotection time was 10 min. Synthesis of the OBOC library: Fmoc SPPS was also employed. For the elongation for each peptide in the library, ten types of amino acid reagents were employed in each synthesis cycle except the fixed amino acid position. All the above experiments were carried out in the solid phase peptide synthesis vessels with sieves in it.

4. SPRi detection of the microarray

The peptide H1P, H2P and HMP sample were printed on the gold coated chip at the concentration of 1 mg/mL. The gold coated chip was blocked by 5% (m/v) non-fat milk. The SPRi analysis procedure follows the following cycle of injections: running buffer (PBST, baseline stabilization); sample (five concentration of the protein, binding); running buffer (PBST, washing); and 0.5% (vol/vol) H_3PO_4 in deionized water (regeneration). Protein HER2 and EGFR were diluted into 10 μg/mL with PBST and multiple proportion diluted into 5 μg/mL, 2.5 μg/mL, 1.25 μg/mL and 0.625 μg/mL. Real-time binding signal were recorded and analysed by SPRi system. The PlexArray® Analyzer was used to process the data.

5. The process of microfluidic separation, *in situ* trapping and fluorescent analyses

Biotinylated HER1/HER2 was incubated with the streptavidin-magnetic beads to prepare a "magneitic-HER1/HER2"(in 1×PBS, pH7.4, 37°C, shake for 1 h). In addition, biotinylated HER2 was incubated with the streptavidin-coated Cy5 (Cy5-HER2) while biotinylated HER1 was incubated with the streptavidin-coated FITC (FITC-HER1) in 1×PBS(pH7.4), 37°C shake for 1 h. After washed with 1×PBS 3 times, and then mixed "magneitic-HER1/HER2" and equimolar FITC-HER1/Cy5-HER2 with the OBOC library peptide beads (in 1×PBS, pH7.4, 37°C, shake for 2h). Positive peptide beads would be wrapped up by magnetic beads, whereas fewer or no magnetic beads bound to negative peptide beads. The HER1/HER2 coated magnetic beads will give the positive beads magnetic surfaces through which they could be isolated out of the library using magnetic separation approaches in a continuous-flow microfluidic process (Scheme 1a). All the peptide beads were suspended in a tube and pumped into the microfluidic channel at a flow rate of 600 μ L/min. The positive beads were trapped with a magnet closely to the outer wall of the channel, so that the negative beads exit through negative outlet. When the magnet was removed, the positive beads were flushed out and trapped in the microwell array. In this way, about 800 positive beads were isolated from the library. The *in situ* detection was performed on an ZEISS LSM 710 confocal-laser scanning microscope. A 488 nm laser was the excitation source for FITC-HER1 throughout the experiment and emission was collected 520 nm. Cy5-HER2 was excited at 649 nm and collected 670 nm. Herein, magneitic-HER1/HER2 was acted as a fishing element and the dye-proteins were acted as color encoding detection elements.

6. Confocal Fluorescence Imaging of Living Cancer Cells

Human breast cancer cell line MDA-MB-468 with high HER1 expression and human breast cancer cell line SKBR-3 with high HER2 expression were respectively cultured in Hyclone DMEM/High glucose medium and RPMI 1640 with 10% FBS, penicillin (100 U/mL) and streptomycin (100 U/mL) at 37°C containing 5% CO_2. For SKBR-3 and MDA-MB-468 cells, approximately 1×10^5 mL^{-1} cells were seeded into culture dishes and cultured overnight for the adhesion of cells. Meanwhile, FITC-labeled peptides (H1P, H2P and HMP) were added (1mg/mL in PBS, 200 μL) with Hoechest33342 (10 μg/mL) and PE labelled antibody (5 μg/mL). Binding was achieved in dark by incubating the cells in the solution for 30 min at 4 ^oC. Finally, the cells were washed three times with cold PBS. ZEISS LSM 710 confocal-laser scanning microscope was used. The detection was carried out in the excitation wavelength of 405 nm (Hoechest33342), 488 nm (FITC) and the 565 nm (PE). The fluorescent images were obtained at the emission wave length of 425 nm, 525 nm and 670 nm, respectively. The objective lens used for imaging was a 63×oil-immersion objective (ZEISS). During the characterization of different samples, all of the parameters of the microscope were set to be the same for the comparison of the different peptides.