Supporting Information

Site-Specific and Covalent Immobilization of His-Tagged Proteins *via* Surface Vinyl Sulfone-Imidazole Coupling

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Experimental Section

1. Expression and Purification of HaloTag-6His

1.1. Plasmid Construction

The HaloTag-6His gene sequence was acquired from pH6HTC plasmid (Addgene, pENTR4-HaloTag (w876-1)) by PCR experiment with the sense primer 5`-AAGGAGATATACATATGGGATCCG-3` containing a Nde I restriction site and the anti-sense primer 5`-GTGCGGCCGCAAGCTTTTATTAGTGGTGATGGTGATGATGACCGGAAATCTCC-3` containing a Hind III restriction site and a 6His-Tag site. The Nde I / Hind III digested PCR product was subsequently ligated into a Nde I and Hind III linearized PET 21a expression vector resulting in PET21aHT6H.

The amino acid sequence of HaloTag-6His is as below:

MGSEIGTGFPFDPHYVEVLGERMHYVDVGPRDGTPVLFLHGNPTSSYVWRNI
IPHVAPTHRCIAPDLIGMGKSDKPDLGYFFDDHVRFMDAFIEALGLEEVVLVIH
DWGSALGFHWAKRNPERVKGIAFMEFIRPIPTWDEWPEFARETFQAFRTTDV
GRKLIIDQNVFIEGTLPMGVVRPLTEVEMDHYREPFLNPVDREPLWRFPNELPI
AGEPANIVALVEEYMDWLHQSPVPKLLFWGTPGVLIPPAEAARLAKSLPNCKA
VDIGPGLNLLQEDNPDLIGSEIARWLSTLEISGHHHHHH

1.2. Protein Expression

The constructed plasmid was transfected into *E.coli* BL21 (ED3) for protein expression. The colony was cultured in LB medium at 37℃ into OD_{600}nm 0.6 and 1 mM IPTG was added for 4 hours at 37℃ to induce the protein expression and mature. The cells were centrifuged for 15 min at 5000 rpm under 4℃. Cell pellets were suspended in cold PBS buffer (10 ml for 1 g wet cells). Sonication was followed with 60% power amplitude and 3 sec/7 sec pulse for 30 minutes. The sample was centrifuged for 30 min at 11000 rpm under 4℃. The supernatant was purified by Ni sepharose Resin (GE Healthcare, Sweden).

1.3. Protein Purification

The purification of HaloTag-6His was evaluated with the ÄKTA Purifier 10 system. 1 mL Ni sepharose Resin was loaded into a D801-C column. 10 CV equilibrium buffer (20 mM phosphate, 0.5 M NaCl, 20 mM imidazole, pH 7.4) was loaded to equilibrate the column. 5 CV clear supernatant was loaded at the flow rate of 0.5 mL/min (about 75 cm/h). After sample loading, the column was washed with 5 CV equilibrium buffer (20 mM phosphate, 0.5 M NaCl, 0.5 M imidazole, pH 7.4). Then, the elution solution was dialyzed with ultrapure water for 3 times. At last, the HaloTag-6His solution was lyophilized and store in the dark at 4℃.

2. Preparation of Cl-linked HRP

2.1. Synthesis of ligand

6-chloro-hexanoicacid (2mmol) and NHS/EDC (4 mmol/8 mmol) were dissolved in 15 ml dichloromethane and reacted for 12 h at 25℃. Then the reagent was extracted 3 times with saturated saline and dried with sodium sulfate. After dried with rotary evaporator, the product was dissolved in 15 mL acetonitrile and NH_2-EG_3-COOH (2 mmol) was added. The reaction was carried out at 25℃ for 12 h and the product was purified with silica-gel column. Finally, the purified chlorinated ligand (the synthesis route and chemical structures of compound were illustrated in Figure S9) was characterized by ^{1}H NMR (Figure S10). Spectra were acquired on an Avance II400 MHz spectrometer (Bruker, Swizerland).

2.2. Preparation of Cl-linked HRP

The mixture of ligand (5 μmol) and NHS/EDC (10 μmol/20 μmol) were added into 1 mL of HRP solution (0.2 μM, 20 mM PB, pH 7.0) drop wisely, and incubated for 6 h in an ice bath. The reagent was dialyzed (molecular weight cut-off: 3500 D) in 1 L of ultrapure water for at least 3 times at 4℃ to remove the free chemical compounds. Finally, the Cl-linked HRP was lyophilized and stored at 4℃.

2.3. Solution-based competitive fluorescence assay

HaloTag-6His and Cl-linked HRP (1:2) were dissolved in PB buffer (20 mM, pH 7.0), and incubated at 25℃ for 1 h. HaloTag-6His and the unlabeled HRP (1:2) were set as a control. Then the excess HaloTag® coumarin ligand was added into the solution and reacted for 1 h. The HaloTag® coumarin ligand solution without HaloTag-6His was set as blank control. The free HaloTag® coumarin ligand was then removed by

ultrafiltration. Finally, the fluorescence intensity at $353_{Ex}/434_{Em}$ was quantified using a microplate reader.

3. Expression and Purification of Anti-HER2 Fab-6His (Fab-6His)

3.1. Plasmid construction

The target sequence of Fab light chain was synthesized and inserted into the pcDNA3.3 expression vector by TA cloning to construct the target plasmid of HSP006-02L. The amino acid sequence of light chain is as below:

DIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQQKPGKAPKLLIYSASF
LYSGVPSRFSGSRSGTDFTLTISSLQPEDFATYYCQQHYTTPPTFGQGTKVEIK
RTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNS
QESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNR
GEC

The target sequence of Fab heavy chain was synthesized and inserted into the pcDNA3.3 expression vector by TA cloning to construct the target plasmid of HSP006-02H. The amino acid sequence of truncated heavy chain is as below:

EVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYIHWVRQAPGKGLEWVARIY
PTNGYTRYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRWGGDG
FYAMDYWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPE
PVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHK
PSNTKVDKKVEPKSCHHHHHH

The synthesized plasmids of interest were subjected to TOP10 *E.coli* competent transformation, and the successfully transformed monoclonal was amplified in LB liquid medium containing 100 μg/mL ampicillin. The plasmids were extracted with MN endotoxin-free plasmid DNA purification and sequenced.

3.2. CHO culture conditions

CHO cells were maintained in a proprietary medium with 4 mM of L-Glutamine in a shake flask at 36.5 °C, 6% CO_2 and 110 rpm. Cells were passaged at a seeding density of 0.3 to 1.0 x 10^5 cells/mL, every 3-4 days. 3 or 4 days prior to transfection, CHO cells were seeded at 1.5-4.0 x 10^5 cells/mL in a proprietary medium with 4 mM of L-glutamine. For transfection, the cells were recovered by centrifugation at 200 g for 5 minutes and re-suspended in proprietary medium. Polyethyleneimine (PEI)-mediated transfections were performed: PEI and DNA for both chains were sequentially added to the cells. The CHO cell culture supernatant was harvested after 10 days of incubation. All scale transfections (culture volume from 0.25 to 1.2 L), were performed in appropriately sized shake flasks on a Kuhner shaker.

3.3. Purification of Anti-HER2 Fab-6His

The purification of Fab-6His from CHO cell culture supernatant was evaluated with the ÄKTA Purifier 150 system. 25 mL Kappa Select (GE, USA) was loaded into a 16/20 column. 10 CV equilibrium buffer A (20 mM PB, 20 mM Arg, pH 7.0) was loaded to equilibrate the column. 1 L cell culture supernatant was loaded at the flow rate of 5 mL/min. After sample loading, the column was washed with 10 CV

equilibrium buffer. Then, the Fab-6His was eluted with 10 CV buffer B (20 mM CA, 20 mM Arg, pH 3.0).

The polishing step of Fab-6His after affinity chromatography was evaluated with the ÄKTA Purifier 150 system. 30 mL SP-HP (GE, USA) was loaded into a 16/20 column. 10 CV equilibrium buffer A (20 mM NaAC, pH 5.5) was loaded to equilibrate the column. The sample needs to be adjusted to the appropriate conductivity and pH before loading. Then the sample was loaded at the flow rate of 5 mL/min. After sample loading, the column was washed with 10 CV equilibrium buffer. Then, the Fab-6His was eluted with 20 CV buffer B (20 mM sodium acetate, 500 mM NaCl, pH 5.5).

4. Protein Immobilization Experiments by Surface Plasmon Resonance (SPR)

Protein immobilization experiments were conducted on a Biosuplar-400T SPR spectrometer (Analytical μ-Systems, Germany) with a light-emitting diode light source (λ = 670 nm), high-refractive-index prism (n = 1.61), and two-channel flow cell. The flow rate was set at 50μl/min. Before the protein immobilization, Carboxy-EG_6 chips require an activation step with NHS/EDC solution (20 mM NHS, 40 mM EDC) for 30 min, and NTA- EG_6 chips need pumped with $NiCl_2$ solution (0.1 M in water) for 10 min. The resulting chips were rinsed with copies of ultrapure water and dried with nitrogen.

For protein immobilization, the following protocol was used: a equilibrate step with PB buffer (20 mM, pH 7.0) for 10 min, followed by the protein immobilization step with 0.5 mg/mL HaloTag-6His or BSA solutions (20 mM PB buffer, pH 7.0), and a

washing step with PB buffer (20 mM, pH 7.0). Finally, 0.01 M NaOH solution was flowed for 10 min and washed with PB buffer (20 mM, pH 7.0). The NTA- EG_6 chips add an elution step with 0.5 M imidazole buffer (20 mM PB buffer, pH 7.0) for 15 min and then washed with PB buffer (20 mM, pH 7.0).

5. Preparation of Cell Membrane-derived Liposome

5.1. Cell Cultures

SK-BR-3 cells were a generous gift from Professor Wu H.J. group (School of Bioengineering, Dalian University of Technology, Dalian, China). SK-BR-3 cells were grown at 37℃/5% CO_2 in RPMI medium 1640 basic with L-glutamine and HEPES (25 mM) containing 10% FBS and 1% Penicillin-Streptomycin solution. The cells were harvested by digested with 0.25% trypsin (Gibco) for 2 min and washed with PBS buffer for 2 times.

5.2. Preparation of FITC-labeled anti-HER antibody[1]

Anti-HER2 antibody was dissolved in the reaction solution (9 volumes of PBS buffer (20 mM, pH 7.4) and 1 volume of sodium bicarbonate buffer (1 M, pH 9.0)) to a concentration of 1 mg/ml. 0.25 mL FITC solution (1 mg/mL in DMSO) was mixed with 10 mL anti-HER2 antibody solution and reacted for 1 h at 25℃. The mixture was dialyzed in PBS at least 3 times at 4 ℃ in dark.

5.3. Detection of HER2 receptors on breast cancer cells

The harvested cells were dispersed at a density of 2×10^6 cells/mL in the tube. Cells were washed once with PBS buffer (20 mM, pH 7.4). 10 μL of 1 mg/mL FITC-labeled anti-HER antibody was incubated with 1 mL cell suspension for 30 min at 4℃ in dark. Then, cells were washed with PBS buffer and resuspended in 300 μL PBS buffer. Cells were subjected to flow cytometry analysis (BD FACSCanto flow cytometer, BD Biosciences, USA) using 488 nm excitation and 525 nm emission. Approximately, 10,000 cells were measured for each sample.

5.4. Liposome Preparation

The preparation method of liposomes refers to the work of Xinyi Wang et al[2]. Briefly, the collected cells were washed twice with HEPES buffer (10 mM, pH 7.4), then resuspended in HEPES buffer (10 mM, pH 7.4) at a concentration of 1×10^6 cells/mL. Liposomes were obtained by repeatedly extruding cells for 11 times using the Mini-Extruder (Avanti Polar Lipids Inc., USA) with a filter membrane (0.8 μm). The size of cell-derived liposomes was measured using a particle size analyzer (NanoBrook Omni, Brookhaven instruments, USA). The concentrations of cell-derived liposomes were estimated by the total mass after lyophilization.

Figure S1. XPS spectra of survey scans (a) and S 2p (b) for the VS-EG_6 surfaces before and after reaction with Boc-Lys and Boc-His at pH 7.0.

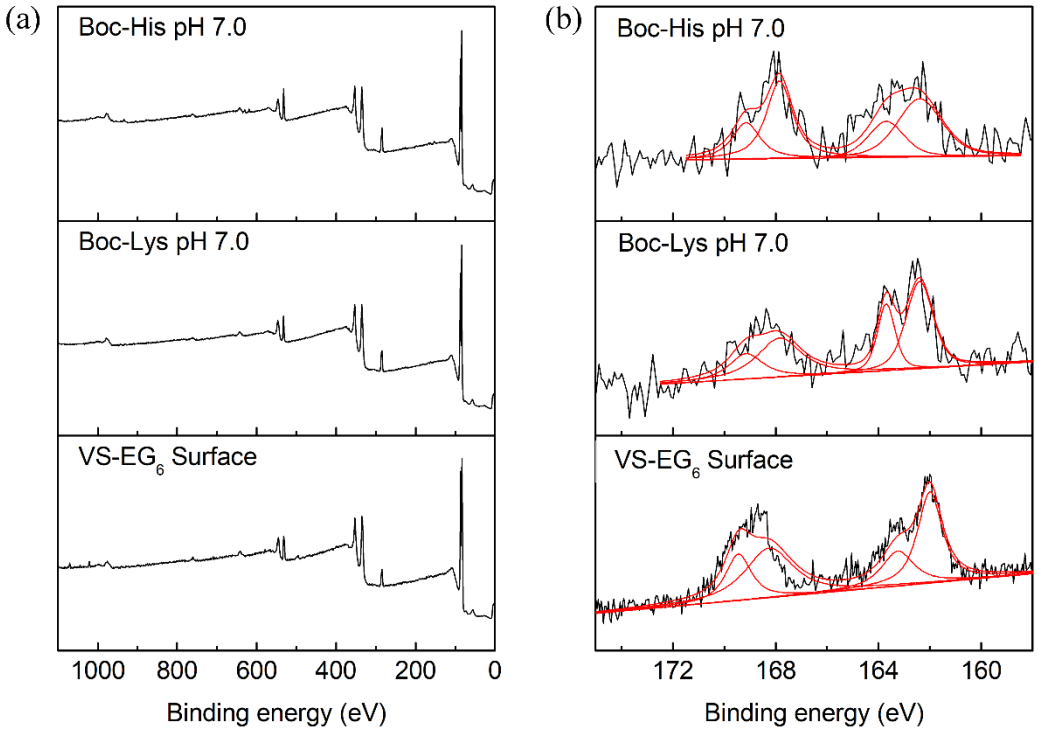


Table S1. Atomic percentage of C, S, O and N in the VS-EG_6 surfaces before and after reaction with Boc-His and Boc-Lys at different solution pH.

	EG_6-OH	VS-EG_6	Boc-His pH 6.5	Boc-His pH 7.0	Boc-His pH 8.0	Boc-Lys pH 6.5	Boc-Lys pH 7.0	Boc-Lys pH 8.0
C 1s	71.3	64.9	71.1	68.6	71.0	71.1	69.1	71.3
S 2p	3.7	5.7	5.3	4.1	4.0	4.7	5.3	3.1
O 1s	25.0	29.4	23.6	26.5	24.1	24.2	25.6	24.5
N 1s	N/D	N/D	N/D	0.8	0.9	N/D	N/D	1.1

*N/D is not detected.

Figure S2. XPS spectra of survey scans (a), C 1s (b) and N 1s (c) for VS-EG_6 surfaces before (1) and after reaction with the peptides of RGD (2), GGGRGDS (3), and Boc-HHHHHH (4) and at pH 7.0

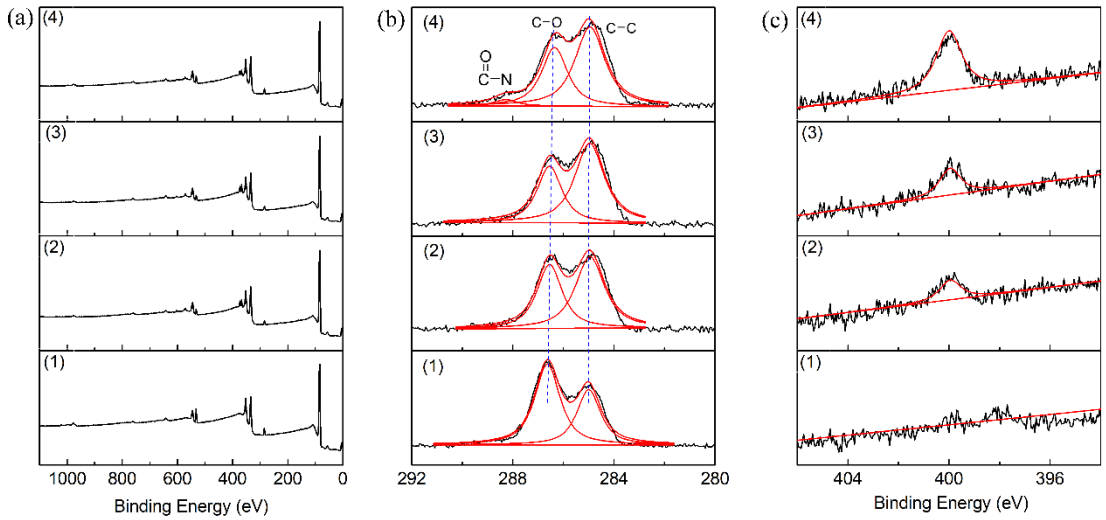


Figure S3. Atomic percentages of nitrogen on VS-EG_6 surfaces before and after reaction with the peptides of RGD, GGGRGDS, and Boc-HHHHHH at pH 7.0.

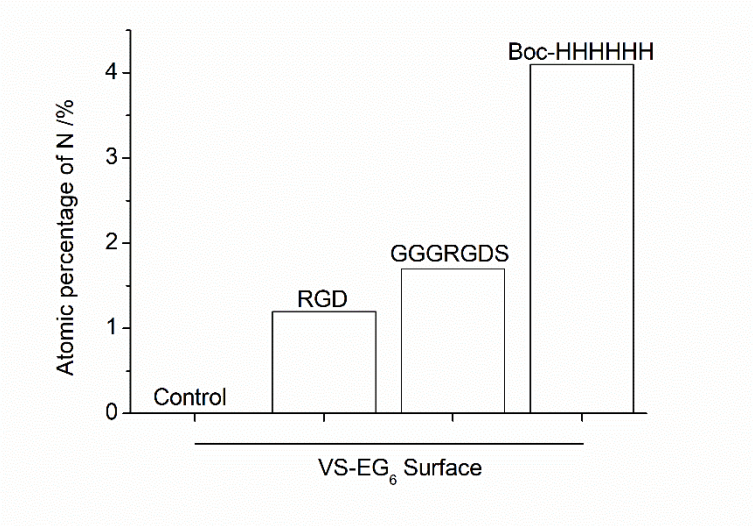
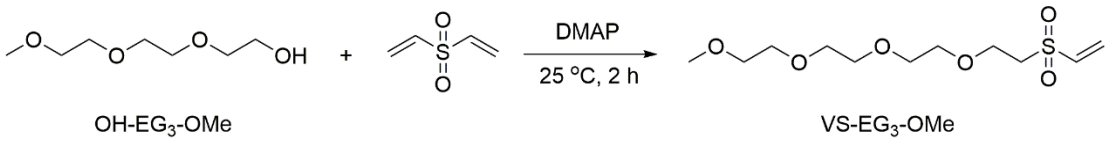


Figure S4. The synthesis and chemical structure of VS-EG_3-OMe.



OH-EG_3-OMe VS-EG_3-OMe

Briefly, 330 mg of OH-EG_3-OMe (2 mmol) was mixed with 950 mg of DVS (8 mmol), followed by the addition of 24 mg of DMAP (0.2 mmol) as catalyst. The mixture was stirred for 2 h at 25℃. The product was purified with a silica gel column.

Figure S5. ^{13}C NMR spectra of VS-EG_3-OMe (1), Boc-His (2), Boc-Lys (3) and the resulting products after reaction (4).

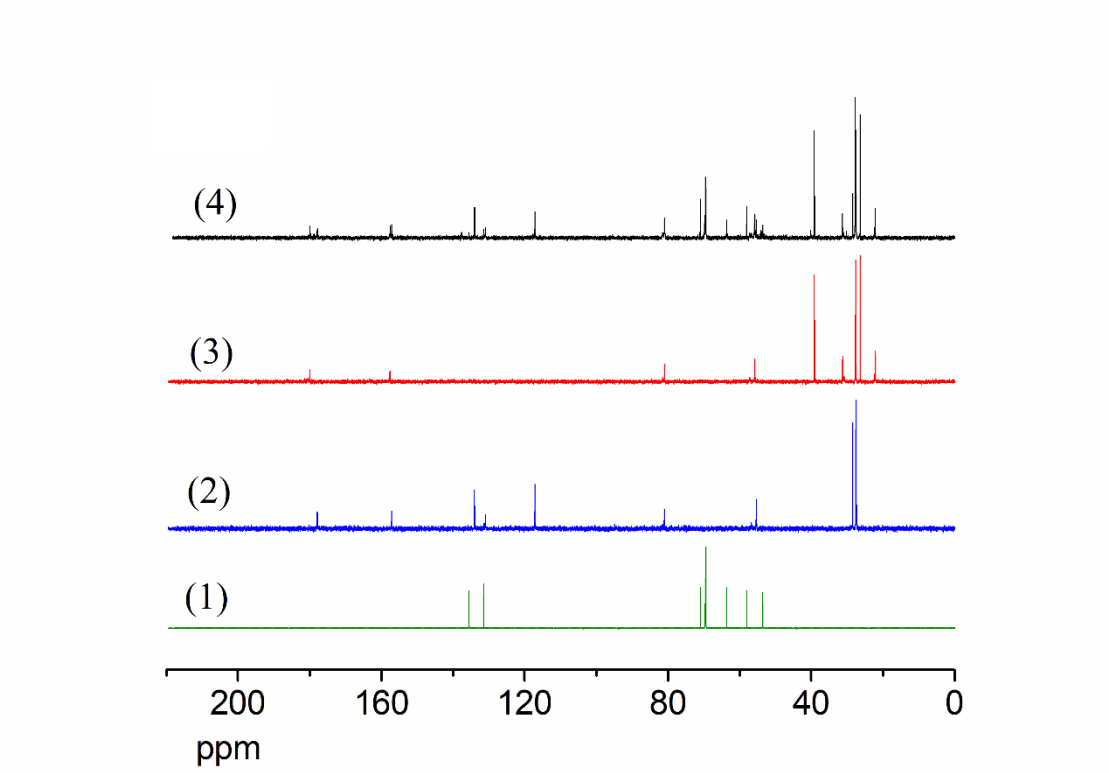


Figure S6. The real-time sensorgrams of HaloTag-6His immobilization onto VS-EG_6 (a), Carboxyl-EG_6 (b) and NTA-EG_6 (c)surfaces using SPR.

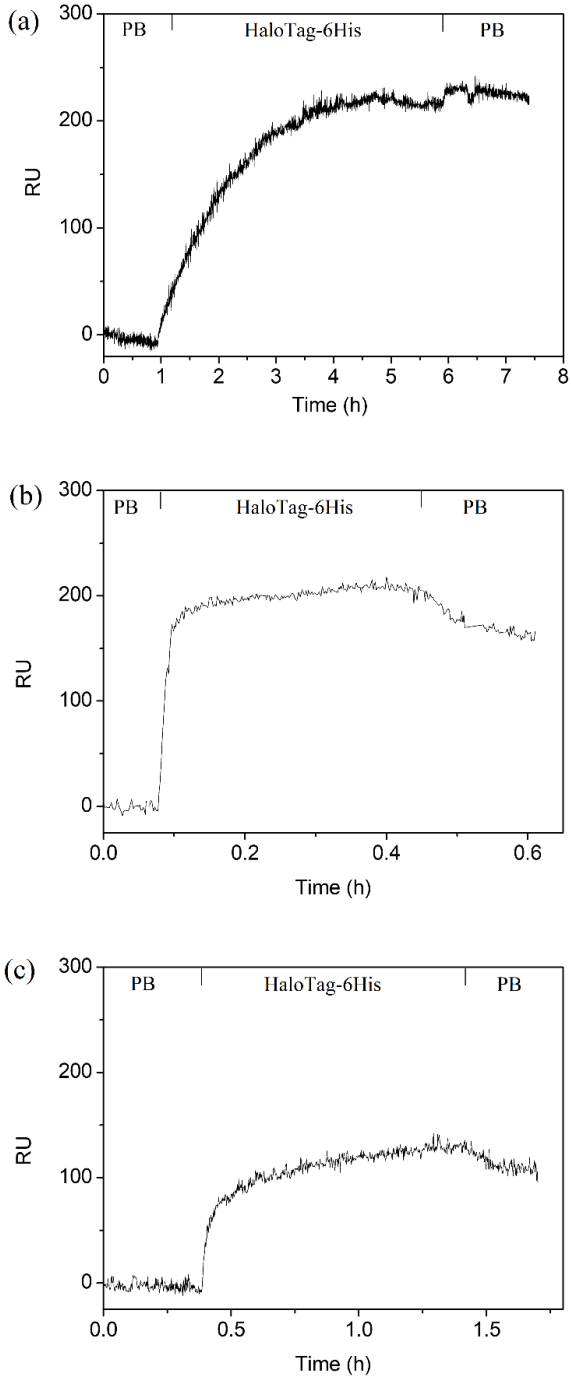


Figure S7. The amounts of immobilized HaloTag-6His on VS-EG_6, Carboxyl- EG_6 and NTA- EG_6 surface by SPR.

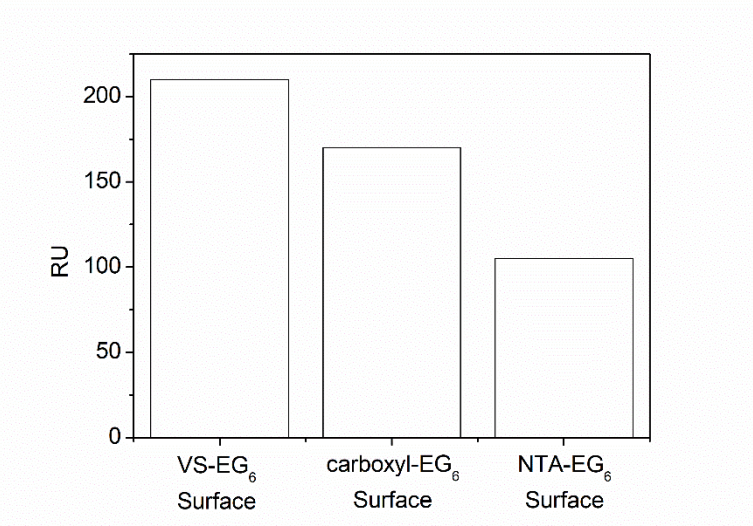


Figure S8. The nonspecific adsorption of HaloTag-6His on EG_6-OH SAMs by QCM measurement. The concentration of HaloTag-6His was 0.5 mg/ml and the flow rate was 50 μL/min.

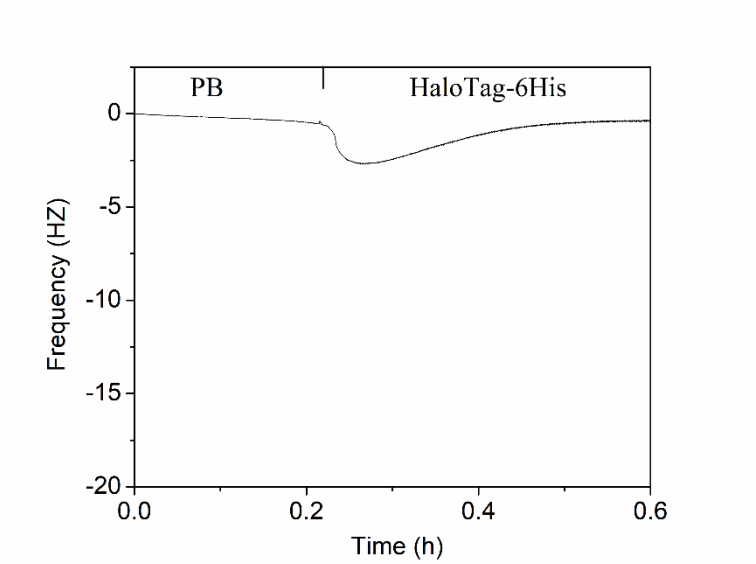


Figure S9. The nonspecific adsorption of HaloTag-6His on the Boc-His blocked VS-EG_6 surface by QCM measurement. The VS-EG_6 surface was treated with 20 mM Boc-His solution (pH 7.0, PB buffer) at 25℃ for 12 h before protein adsorption. The concentration of HaloTag-6His was 0.5 mg/ml and the flow rate was 50 μL/min.

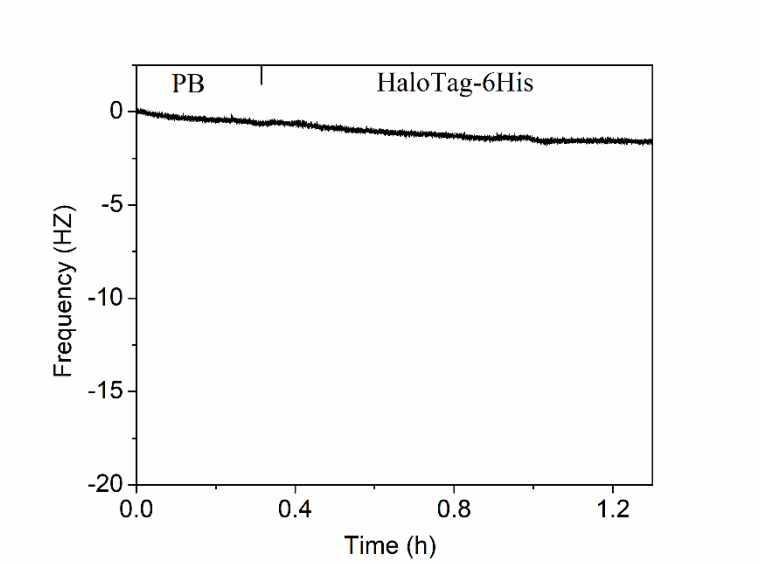


Figure S10. Real-time QCM sensorgrams for BSA immobilization onto VS-EG_6, Carboxyl-EG_6 and NTA-EG_6 surfaces using QCM experiments.

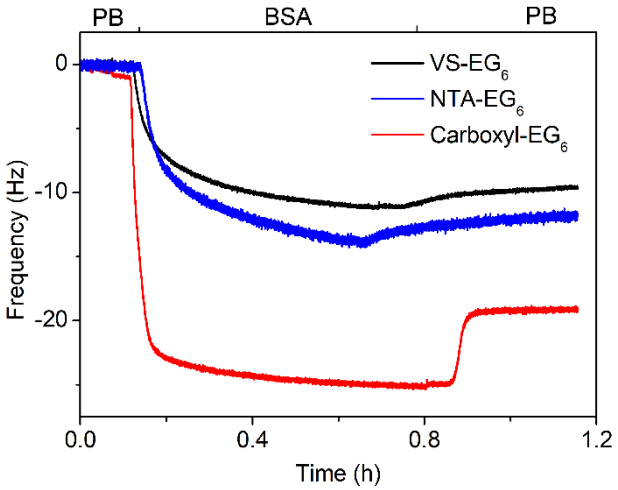


Figure S11. XPS spectra of survey scans (a), C 1s (b) and N 1s (c) for the VS-EG_6 surfaces after reaction with streptavidin (1) and streptavidin-6His (2), and Carboxyl-EG_6 surfaces before (3) and after (4) reaction with streptavidin-6His at pH 7.0.

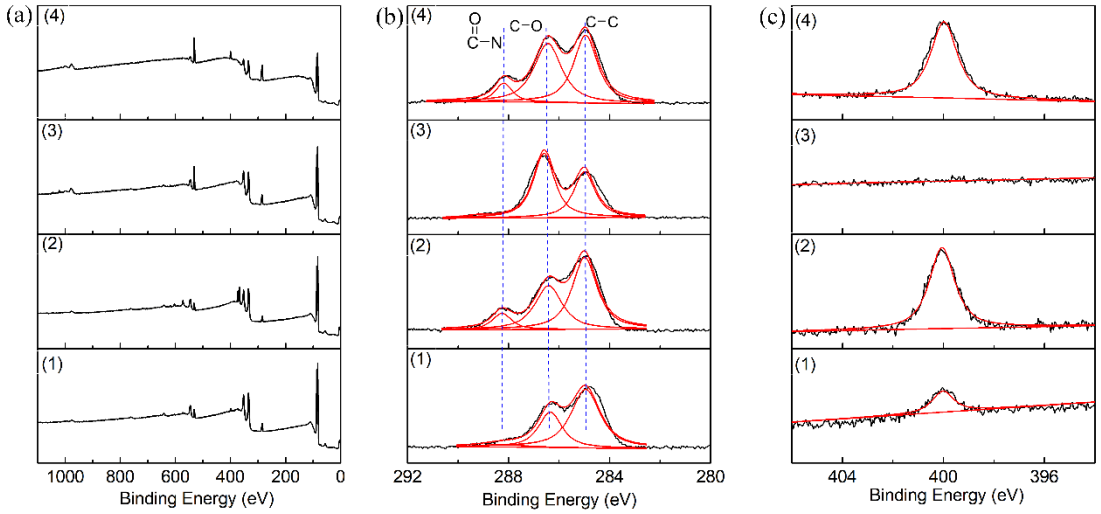


Figure S12. Atomic percentages of nitrogen detected by XPS on VS-EG_6 surfaces before and after reaction with streptavidin and streptavidin-6His, and Carboxyl-EG_6 surfaces before and after reaction with streptavidin-6His at pH 7.0.

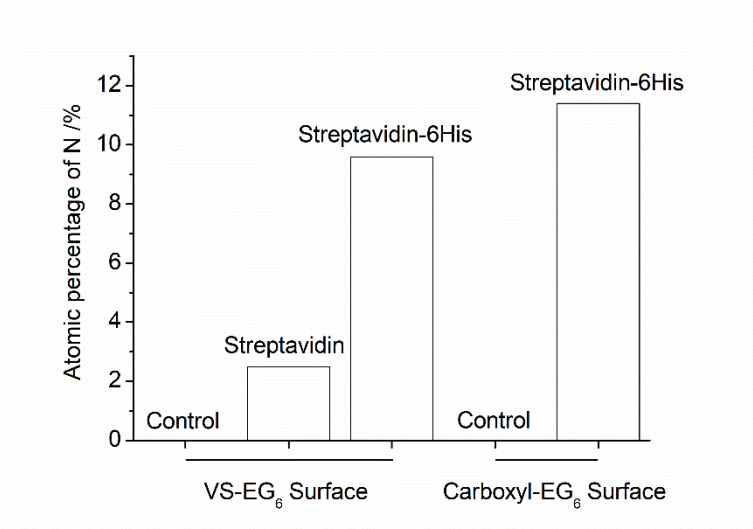


Figure S13. Protein structure of HaloTag (PDB: 4KAA, ~34 kDa). HaloTag is a mutant dehalogenase. This enzyme possesses a hydrophobic pocket, in which a chlorinated ligand can covalently bind inside. The His-Tag was located at the C-terminus of HaloTag, which is in the opposite position to the binding pocket.

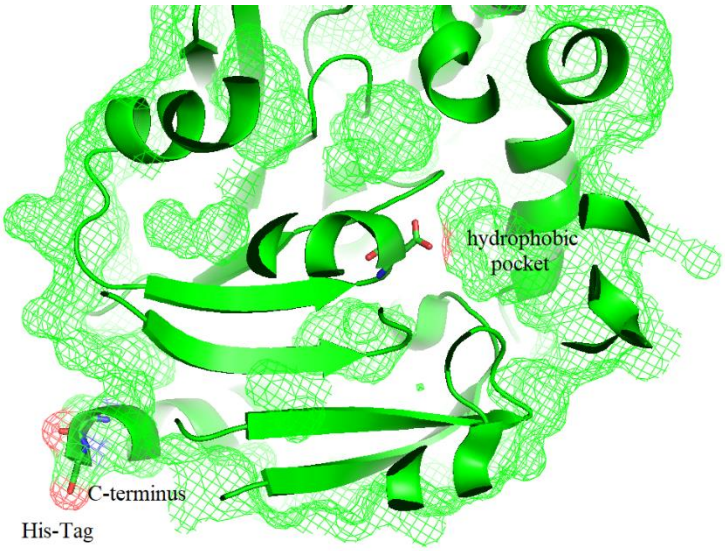


Figure S14. The synthetic route of carboxyl-terminated chlorinated ligand (compound 1). The ligand consists of the following two moieties: (1) a hydrocarbon chain, which could generate hydrophobic interaction within the binding pocket; (2) an oligo (ethylene oxide) chain, which could extend the length of the ligand and increase the solubility as well.

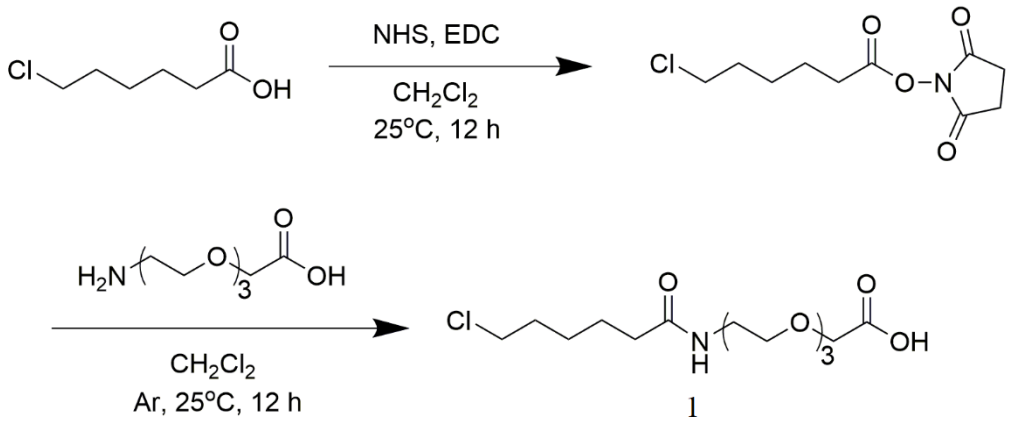


Figure S15. ^{1}H NMR spectra of the carboxy-terminated chlorinated ligand.

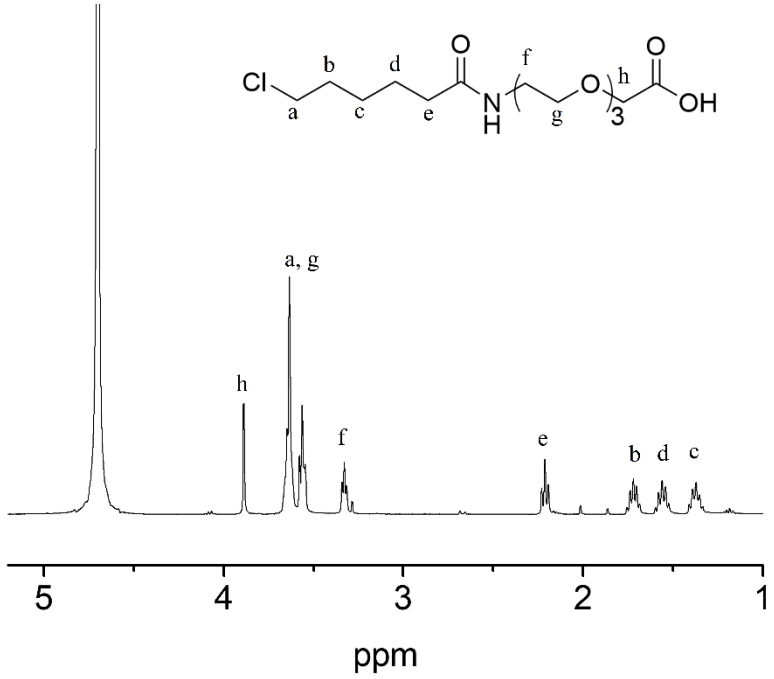


Figure S16. Illustration (a) and fluorescence assay (b) of the solution-based competitive binding of Cl-linked HRP with HaloTag. The fluorescence intensity was detected at $353_{Ex}/434_{Em}$.

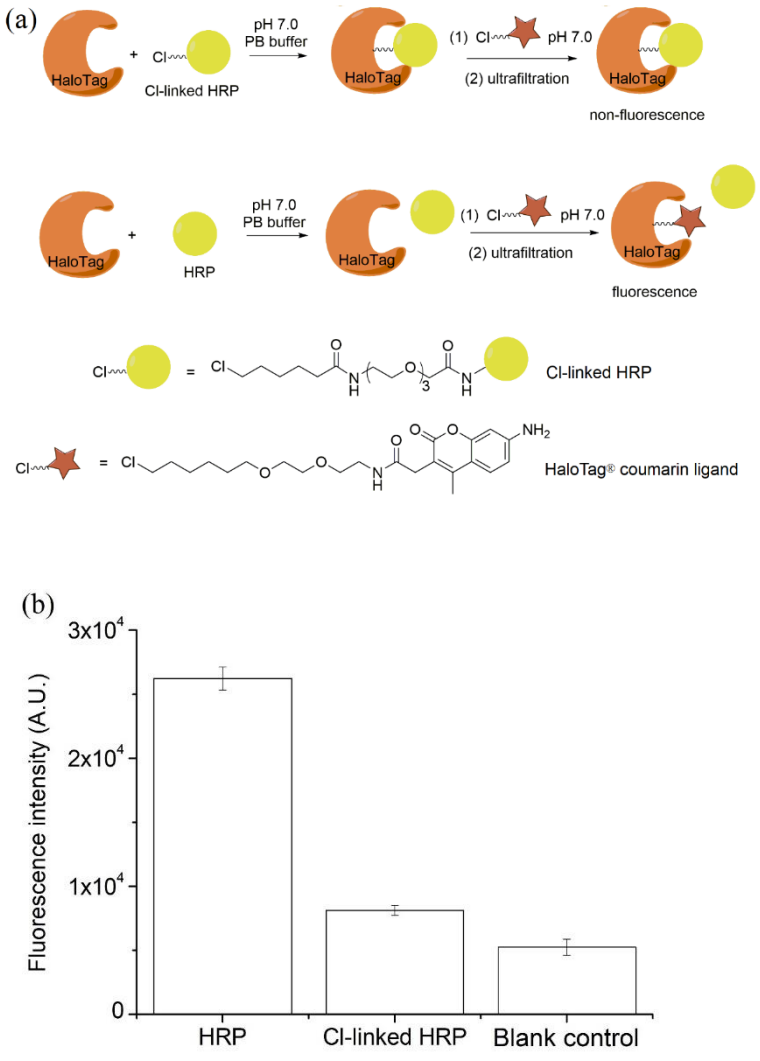


Figure S17. The SDS-PAGE analysis of anti-HER2 Fab-6His and anti-HER2 antibody. Lane 1 was protein marker, Lane 2 was anti-HER2 Fab-6His and Lane 3 was anti-HER2 antibody. Anti-HER2 Fab-6His composes of a light chain and a heavy chain.

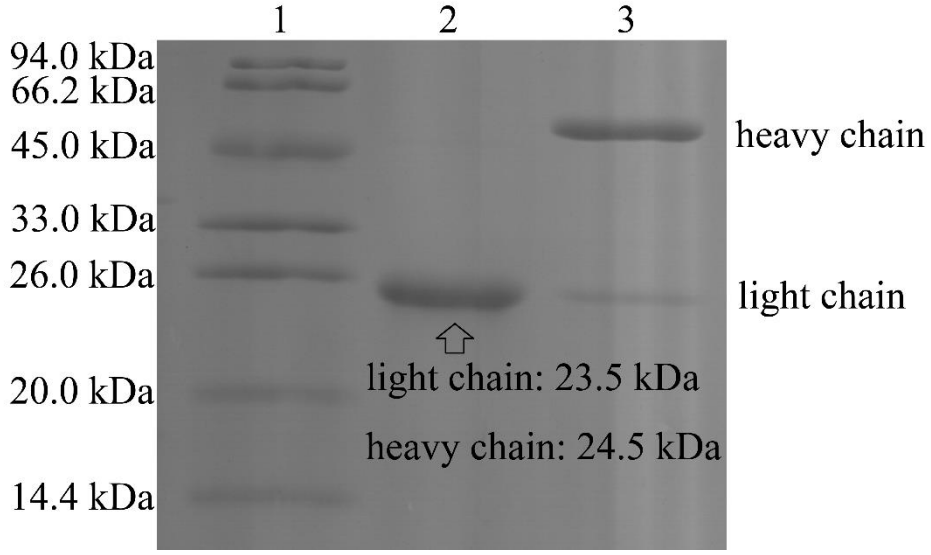


Figure S18. The flow cytometry analysis of SK-BR-3 cells with or without FITC-labeled anti-HER2 antibody incubation.

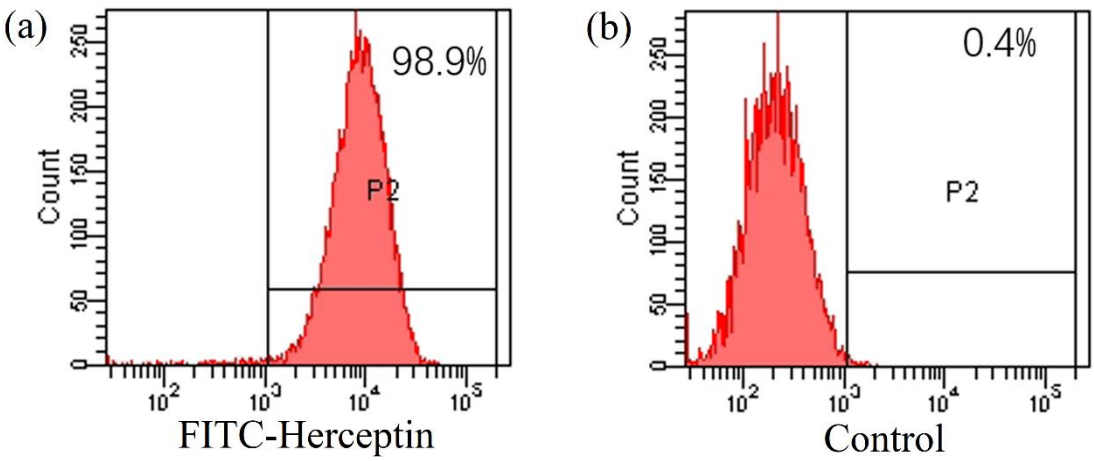


Figure S19. The hydrodynamic diameter of cell-derived liposomes. The liposomes were obtained by repeatedly extruding SK-BR-3 cells for 11 times through a filter membrane with 0.8 μm pore diameter.

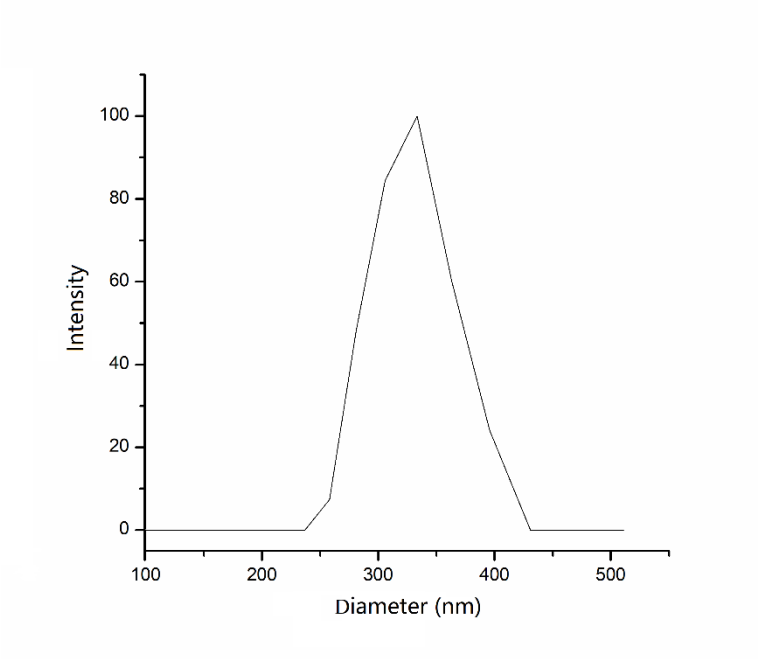


Figure S20. The real-time binding experiments of synthesized liposome ($HER2^-$) onto anti-HER2 Fab-6His surface prepared by VS-EG_6 method.

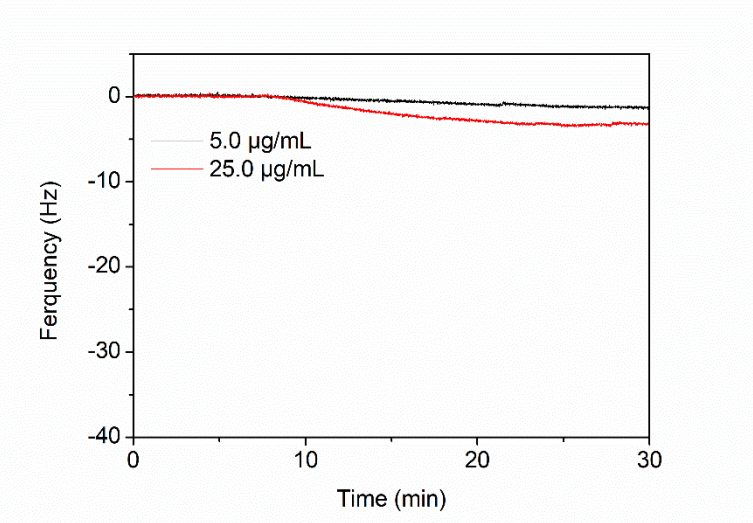


Figure S21. The calibration plot of frequency versus liposomes concentration. The correlation equation of VS-EG_6, carboxyl-EG_6 and NTA-EG_6 surfaces are $F=7.56516lgC+6.40122$ ($R^2=0.96$), $F=15.85933lgC-3.75695$ ($R^2=0.98$) and $F=17.94616lgC-12.19228$ ($R^2=0.99$), respectively.

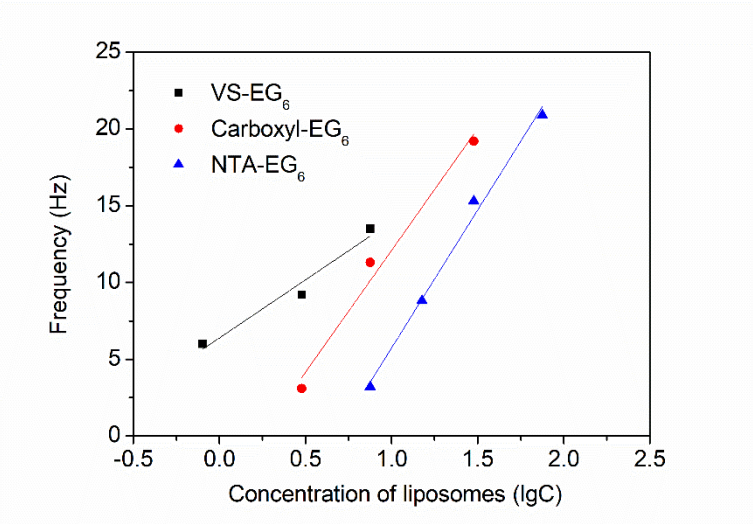


Table S2. LODs of $HER2^{+}$ liposome binding on anti-HER2 Fab-6His surfaces that prepared by VS-EG_6, Carboxyl-EG_6 and NTA-EG_6 methods. According to 3σ (3*0.09 Hz) rules, LOD values were calculated by correlation equation[3].

Sample surfaces	LOD (μg/mL)
VS-EG_6	0.15
Carboxyl-EG_6	1.79
NTA-EG_6	4.95

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