Supporting Information

Site-specific Antibody-polymer Conjugates for siRNA Delivery

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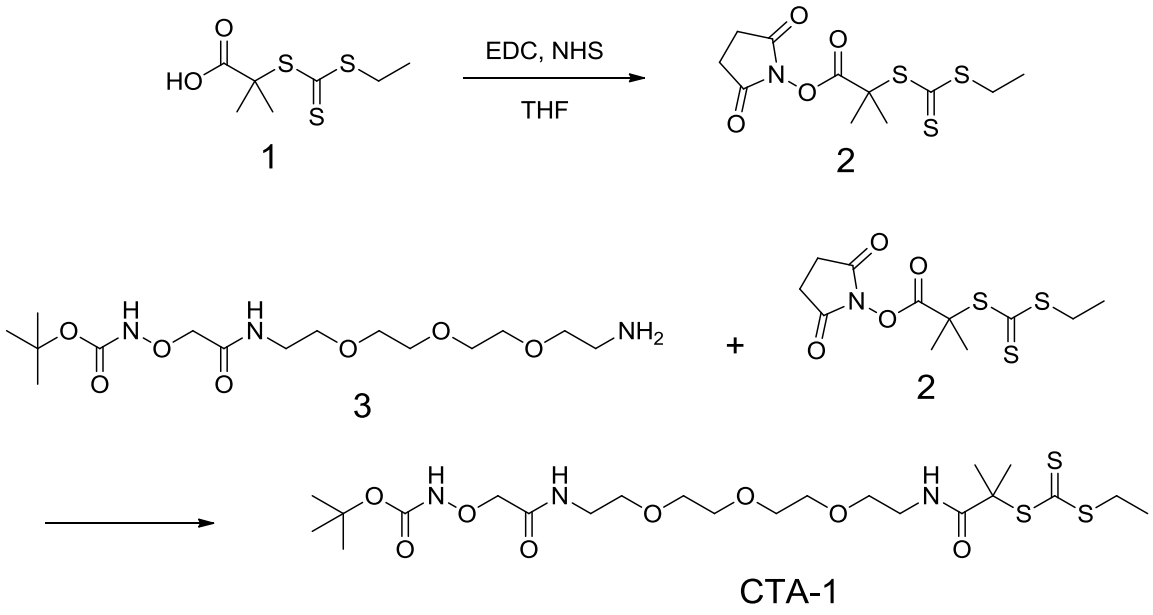
Protocols and Methods

Materials: All chemicals were purchased from common commercial sources and used as received unless otherwise specified. *N*-isopropyl acrylamide (NIPAAm) was recrystallized three times from hexane prior to use. Azobisisobutyronitrile (AIBN) was recrystallized from methanol. GAPDH-siRNA (human, mouse, rat) was purchased from Life Technologies (Carlsbad, CA). FITC-siRNA, scramble-siRNA negative control, and DNAJB11-siRNA were obtained from Dharmacom|Thermo Scientific (Pittsburgh, PA). DNA primers, MDM2-siRNA and GFP-siRNA, were ordered from IDT Inc. (San Diego, CA). The sequences (from 5' to 3') of MDM2-siRNA, antisense: GCU UCU CAU CAU AUA AUC GdTdT; sense: CGA UUA UAU GAU GAG AAG CdTdT. The sequences of GFP-siRNA, antisense: UUG AAG UUC ACC UUG AUG CdTdT; sense: GCA UCA

AGG UGA ACU UCA AdTdT. Anti-HER2 S202-*p*AcF Fab and A121-*p*AcF IgG were obtained using protocols previously reported.[1]

*Instrumentation***:** NMR spectra were recorded on a Varian 400 MHz, a Bruker DRX-500 MHz or a DRX-600 with CryoProbe spectrometer. Gel permeation chromatography (GPC) experiments were performed on a Shimadzu Prominence System equipped with an LC-20-AD pump, a RID-10A Refractive Index detector and a SPD-M20A Diode Array Detector; samples were separated by a tandem Waters Styragel HR4 and HR3 columns at 40 ^{o}C in 0.1% LiBr DMF mobile phase; the system was calibrated with standard PMMA samples for molecular weight determination. Dynamic light scattering (DLS) measurements were recorded on a Dynapro plate reader (Wyatt Technology, Santa Barbara, CA) fitted with a glass-bottomed optical 96-well plate. Confocal microscopy study was performed on a Leica LSM 710 confocal microscope. qRT-PCR was conducted on a 7900 fast real time PCR system (Applied Biosystems, Carlsbad, CA).

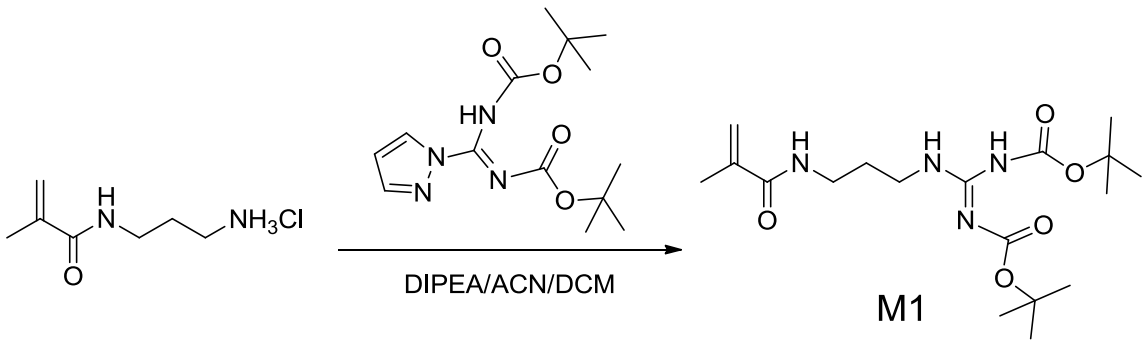
Cell culture: SK-BR-3, HeLa, CHO and NIH-3T3 cells were purchased from ATCC (Rockville, MD). The 293T-GFP stable cell line was obtained from Cell BioLabs (San Diego, CA). Cells were grown in DMEM (Cellgro, Manassas, VA) supplemented with 100 IU penicillin and 100 μg/mL streptomycin and 10% fetal bovine serum (FBS) at 37 ^{o}C in a humidified atmosphere containing 5% CO_2. Suspension CHO cells were grown in Freestyle CHO expression medium (Life technologies, Carlsbad, CA).



Scheme S1. Synthesis of **CTA-1**

Synthesis of 2: **1** was synthesized following a protocol from literature.[2] **1** (3.5 g, 15.6 mmol) and 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC, 3.5 g, 18.5 mmol) were dissolved in THF (40 mL) in an ice bath and stirred for 30 min followed by addition of *N*-hydroxysuccinimide (NHS, 2.16 g, 18.8 mmol) and *N*,*N*-dimethyl 4-amino pyridine (DMAP, 30 mg, 0.2 mmol). The mixture was gradually warmed to ambient temperature and stirred overnight. The resulting solution was washed with brine (20 mL × 2) twice, dried over Na_2SO_4 and concentrated to dryness by rotavapor. The crude yellow oil was purified by silica gel column eluting with hexane: ethyl acetate (2:1). The yellow fractions were combined and evaporated to afford 4.3 g product as a yellow powder (yield 86 %). ^{1}H NMR ($CDCl_3$, 500 MHz) δ 3.30 (q, J = 7.5 Hz, 2H), 2.80 (s, 4H), 1.86 (s, 6H), 1.34 (t, J = 7.5 Hz, 3 H); ^{13}C NMR ($CDCl_3$, 500 MHz) δ 218.5, 169.0, 168.7, 54.2, 31.4, 25.6, and 12.8; MS (ESI+): calc M/Z = 321, found 225 (M-NHS+H_3O).

*Synthesis of **CTA-1**:* **3** was synthesized following a reported protocol.[3] **3** (170 mg, 0.45 mmol), **2** (193 mg, 0.6 mmol) and DMAP (3 mg, 0.02 mmol) were dissolved in dichloromethane (DCM, 5 mL) at room temperature. Triethylamine (0.1 mL) was added to the solution in one portion and the mixture was stirred overnight. Solvent was removed under vacuum and the residue was purified by silica column chromatography eluting with hexane: ethyl acetate (2:1). Pure product was obtained as a yellow oil (185 mg, yield 72 %). ^{1}H NMR ($CDCl_3$, 400 MHz) 8.09 (s, 1H), 7.86 (s, 1H), 6.94 (s, 1H), 4.34 (s, 2H), 3.64 (s, broad, 4H,),), 3.61(m, 4H), 3.56 (m, 2H,), 3.51 (m, 4H), 3.43 (m, 2H), 3.28 (q, *J* =7.6 Hz, 2H), 1.70 (s, 6H), 1.48 (s, 9H), 1.32 (t, *J* =7.6 Hz, 3H); ^{13}C NMR ($CDCl_3$, 500 MHz) δ 219.9, 172.5, 168.9, 157.4, 104.7, 82.5, 75.8, 70.4, 70.3, 70.2, 69.6, 69.5, 57.0, 39.8, 38.8, 31.2, 28.1, 25.8, and 12.8; MS (ESI+): calc M/Z = 571, found 572 (M+H), 594 (M+Na), 472 (M+H-Boc)



Scheme S2. Synthesis of monomer **M1**

*Synthesis of **M1**:* 3-aminopropyl methacrylamide hydrochloride (400 mg, 2.2 mmol) and *N*,*N*'-Di-Boc-1H-pyrazole-1-carboxamidine (588 mg, 1.90 mmol) were dissolved in a

mixture of DCM/acetonitrile (3 mL/5 mL) at room temperature (rt). With stirring, diisopropylethylamine (DIPEA, 0.7 mL) was added to the solution in one portion. The mixture was stirred overnight at rt and quenched by adding DCM (10 mL) and DI water (15 mL). The aqueous phase was discarded and the organic phase was collected and washed with brine twice (15 mL each). DCM was dried over Na_2SO_4 and concentrated to dryness by rotavapor. The crude product was purified by silica gel chromatography eluting with hexane: ethyl acetate (4:1) and pure product was obtained as a white solid (643 mg, yield 88 %). ^{1}H NMR ($CDCl_3$, 400 MHz) 11.54 (s, 1H), 8.50 (t, J =6.0 Hz, 1H), 7.40 (t, J =6.0 Hz, 1H), 5.81 (s, 1H), 5.82 (s, 1H), 3.49 (dt, , J_1 =6.0 Hz, , J_2 =6.0 Hz, 2H), 3.33 (dt, , J_1 =6.0 Hz, , J_2 =6.0 Hz, 2H), 2.00 (s, 3H,),), 1.71(m, 2H), 1.51 (s, 9H), 1.47 (s, 9H). ^{13}C NMR ($CDCl_3$, 500 MHz) δ 168.6, 163.3, 157.2, 153.1, 140.0, 119.3, 83.3, 79.3, 37.0, 35.3, 29.8, 28.2, 28.0, and 18.8. MS (ESI+): calc M/Z = 384, found 385 (M+H).

ELISA assay: HER2 antigen (R&D Biosystems, Minneapolis, MN) was dissolved in PBS, coated to a 96-well ELISA plate (50 ng/well) and incubated at rt for 1 h (~50 ng of antigen/well). The antigen was then blocked with PBST (PBS + 0.1 % tween-20) containing 3% blotting grade milk and incubated at rt for 1 h. Various concentrations of **S202-Fab-P1** and the S202-*p*AcF mutant in 100 μL 3% milk-PBST were then added in triplicate to the plate and rocked at rt for 1 h. After washing with PBST three times (200 μL × 5 min each), secondary antibody (5 μL), an anti-human kappa HRP conjugate in 100 μL 3% milk-PBST, was added to the plate and rocked for another 1 h. The washing step was repeated three times (200 μL × 5 min each), and the fluorescent signal was then

detected by QuantaBlu Fluorogenic Peroxidase Substrate (Pierce, Rockford, Illinois) following the manufacture's instruction.

Gel retardation assay: Both the scramble-siRNA and **S202-Fab-P1** were freshly diluted to 4 μM with PBS before the assay. Various volumes of **S202-Fab-P1** were added to the scramble-siRNA solutions (4 μM × 5 μL) and incubated at rt for 1 min. The mixtures were then loaded onto a 1% agarose gel containing ethidium bromide and resolved by electrophoresis in 1× TAE buffer.

Quantitative real time-PCR: Total mRNAs were purified from RNeasy kit (Qiagen, Valencia, CA) and cDNAs were reverse-transcribed using Superscript® III First-strand Synthesis System (Life Technologies, Carlsbad, CA) following the manufacturer's procedures. Real time PCR was performed on a 7900 fast real time PCR system (Applied Biosystems, Carlsbad, CA) using SYBR Green qPCR Master Mix (Clontech, Mountain View, CA). All reactions were done in a 10 μL volume in triplicate. PCR conditions were as follow: Stage 1, 50 ^{o}C for 2 min; Stage 2, 95 ^{o}C for 10 sec; Stage 3, 40 repeats of cycle of (95 ^{o}C for 15 sec, 60 ^{o}C for 60 sec). Data were processed using $2^{-\Delta\Delta C(T)}$ method. Primer sequences (from 5' to 3') are described as below. β-actin (human): forward, CCT GGC ACC CAG CAC AAT, reverse, GGG CCG GAC TCG TCA TAC; GAPDH (human): forward, GAA ATC CCA TCA CCA TCT TCC AGG, reverse, GAG CCC CAG CCT TCT CCA TG; DNAJB11: forward, GAT CTG GGT GCT GCT TAT GAG, reverse GAC GAG GGG TTC CTC CAA AC; MDM2: forward, TGC CAA GCT TCT CTG TGA AAG, reverse, TCC TTT TGA TCA CTC CCA CC; β-actin (mouse): forward, ATC TTC

CGC CTT AAT ACT, reverse, GCC TTC ATA CAT CAA GTT; GAPDH (mouse): forward, AGT GGC AAA GTG GAG ATT, reverse, GTG GAG TCA TAC TGG AAC A.

Western Blot: 48-72 h after treatment with APC/siRNA complexes, cells were lysed in 1× RIPA buffer containing 1× protease inhibitor cocktail tablet (Roche, Indianapolis, IN). The total protein concentrations of the cell lysates were determined by BCA assay (Pierce, Rockford, IL). The cell lysates were then boiled in SDS loading buffer with DTT (10 mM) for 10 min. 10-20 μg of the lysates in SDS buffer of were loaded to the Novex® NuPAGE® gel (Life Technologies, Carlsbad, CA), resolved by electrophoresis, and transferred to a PVDF-membrane (Life Technologies, Carlsbad, CA). The membrane was blocked with 5% blotting grade milk in PBST (PBS with 0.1 % tween-20) at room temperature for 1 h and incubated with appropriate primary antibodies in 5% milk-PBST overnight at 4 °C. The following primary antibodies were used: anti-MDM2, ab-1 (Millipore); anti-γ-tubulin (Sigma), anti-β-actin, 8H10D10, (Cell Signaling Technology); anti-GAPDH, H-12 (Santa cruz Biotech.), anti-GFP, ab290 (abcam. Cambridge, MA). The membrane was washed with PBST three times for 15 min each at room temperature, and then incubated with 1:20000 diluted anti-mouse (anti-rabbit for the anti-GFP antibody) IgG-horse radish peroxidase conjugated secondary antibody (Sigma) in 5% milk-PBST for 1 h at room temperature. After washing, the proteins were detected with ECL Western Blotting Substrate (Pierce, Rockford, IL) for GAPDH, γ-tubulin, β-actin and GFP, or SuperSignal West Femto Chemiluminescent Substrate (Pierce, Rockford, IL) for MDM2.

Cell viability assay: In a 96-cell plate, SKBR3 cells were seeded at 10,000/well and incubated at 37 ^{o}C for 24 h. Medium was removed and cells were supplied with Opti-MEM (80 μL). The cells were then treated with 20 μL of **S202-Fab-P1**/scramble-siRNA complexes in triplicate at various concentrations for 4 h. The complexes were aspirated and cells were grown in complete medium without antibiotics for 24 h. 20 μL Cell Titer Blue reagent (Promega, Madison, WI) was then added to each well and the plate was incubated at 37 ^{o}C for 4 h. The relative cell viability of each treatment group was assessed by reading the fluorescence at 590 nm (excitation 560 nm) and normalized to the untreated group.

References:

(1) Axup, J. Y.; Bajjuri, K. M.; Ritland, M.; Hutchins, B. M.; Kim, C. H.; Kazane, S. A.; Halder, R.; Forsyth, J. S.; Santidrian, A. F.; Stafin, K.; Lu, Y. C.; Tran, H.; Seller, A. J.; Biroce, S. L.; Szydlik, A.; Pinkstaff, J. K.; Tian, F.; Sinha, S. C.; Felding-Habermann, B.; Smider, V. V.; Schultz, P. G. *.P Natl. Acad. Sci. USA* **2012**, *109*, 16101-16106.

(2) Schmidt, B. V. K. J.; Hetzer, M.; Ritter, H.; Barner-Kowollik, C. *Macromolecules* **2011**, *44*, 7220-7232.

(3) Kim, C. H.; Axup, J. Y.; Dubrovska, A.; Kazane, S. A.; Hutchins, B. A.; Wold, E. D.; Smider, V. V.; Schultz, P. G. *J. Am. Chem. Soc*. **2012**, *134*, 9918-9921.

Supplementary Figures

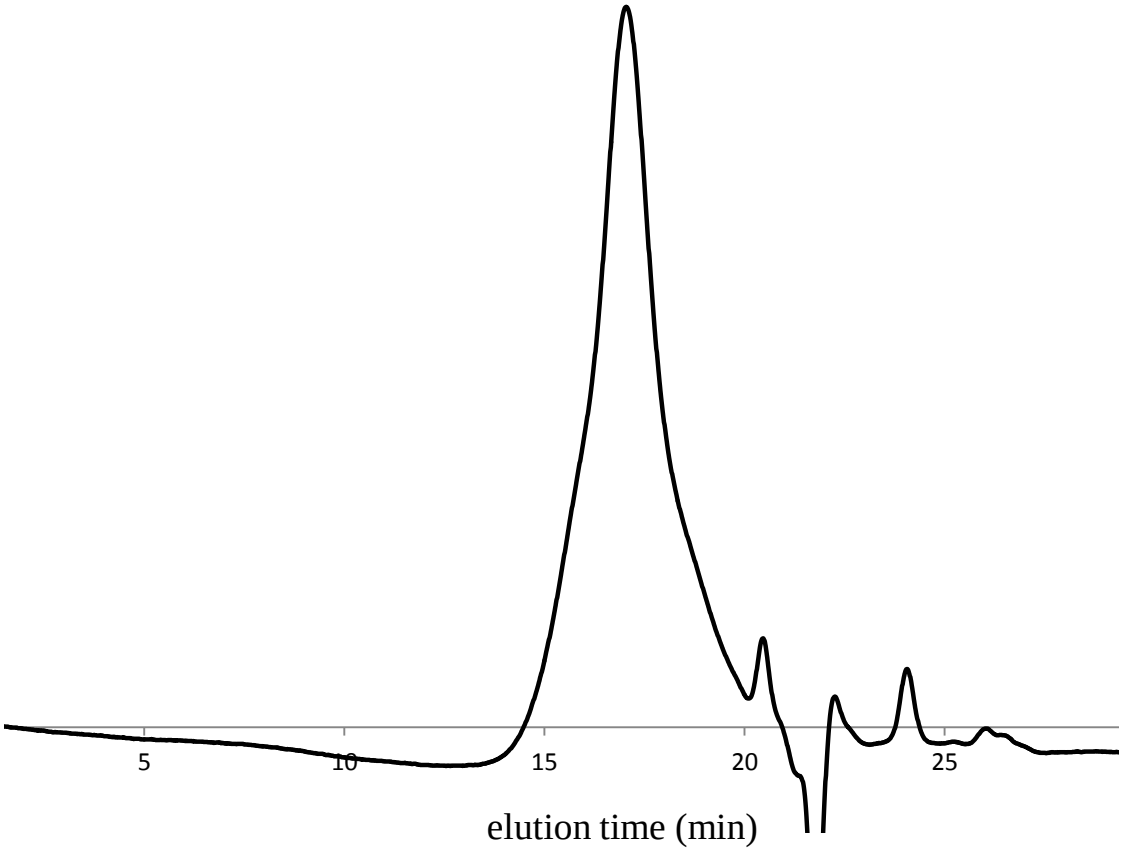


Figure S1. GPC curve of the polymer **P1-Boc**. The crude polymerization solution was directly injected; the GPC system was calibrated using standard PMMA samples; the MW of **P1**-Boc was calculated as 11.2 kDa and the PDI is 1.29.

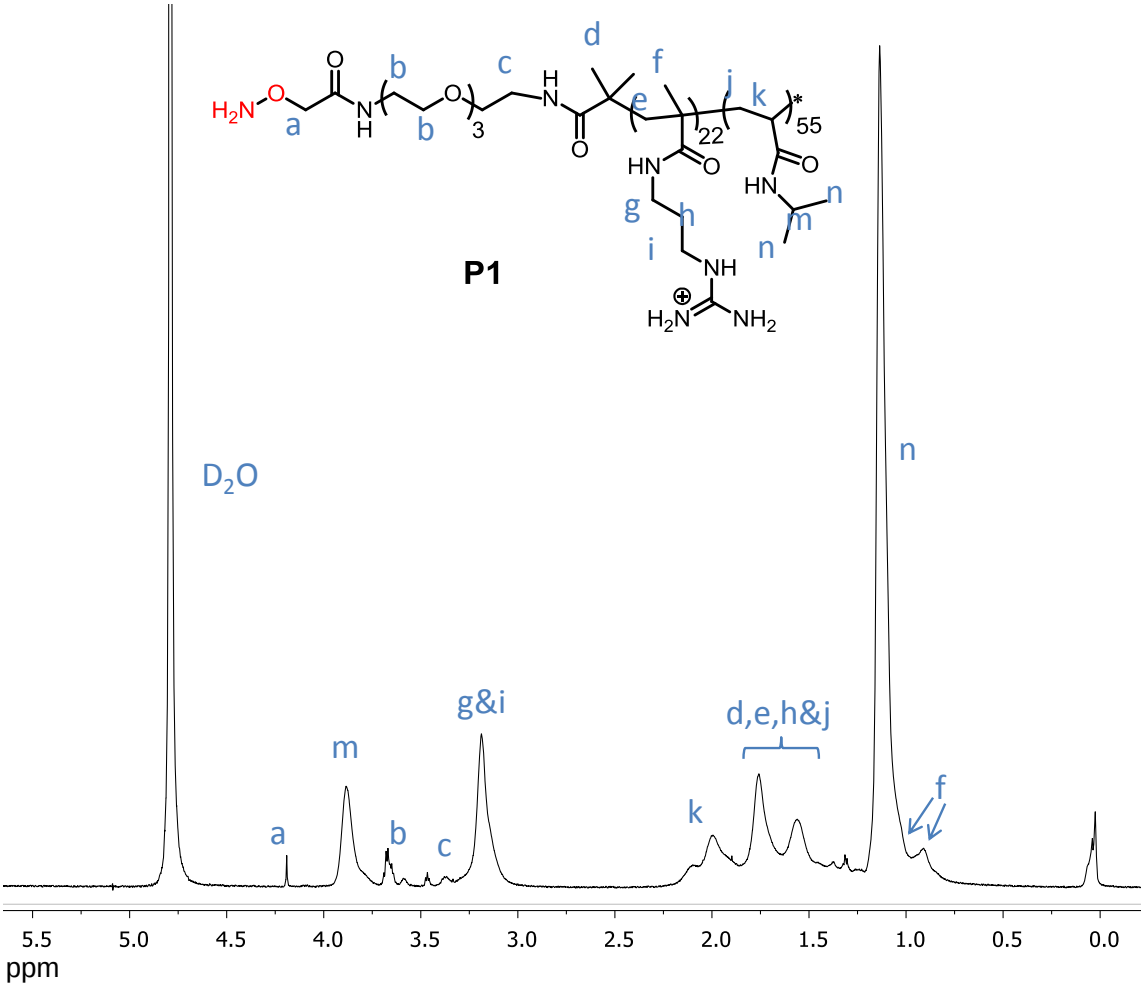


Figure S2. ^{1}H NMR spectrum of **P1** in D_2O.

4 μM scramble-siRNA (μL)	5	5	5	5	5
4 μM **S202-Fab-P1** (μL)	0	2	5	7.5	10

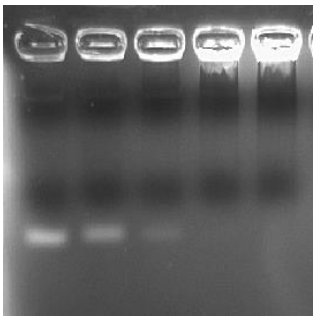


Figure S3. Gel retardation assay of the **S202-Fab-P1**/siRNA complexes in PBS buffer. Both the siRNA and **S202-Fab-P1** were diluted to 4 μM in PBS before analysis. Various volumes of **S202-Fab-P1** were added to the siRNA solutions (5 μL) and incubated at rt for 1 min; the solutions were then loaded to 1% agarose gel containing ethidium bromide and resolved by electrophoresis in 1× TAE buffer; complete binding of the siRNA to the **S202-Fab-P1** conjugate was detected starting from lane 4, which is at **S202-Fab-P1**/siRNA molar ratio of 1.5/1

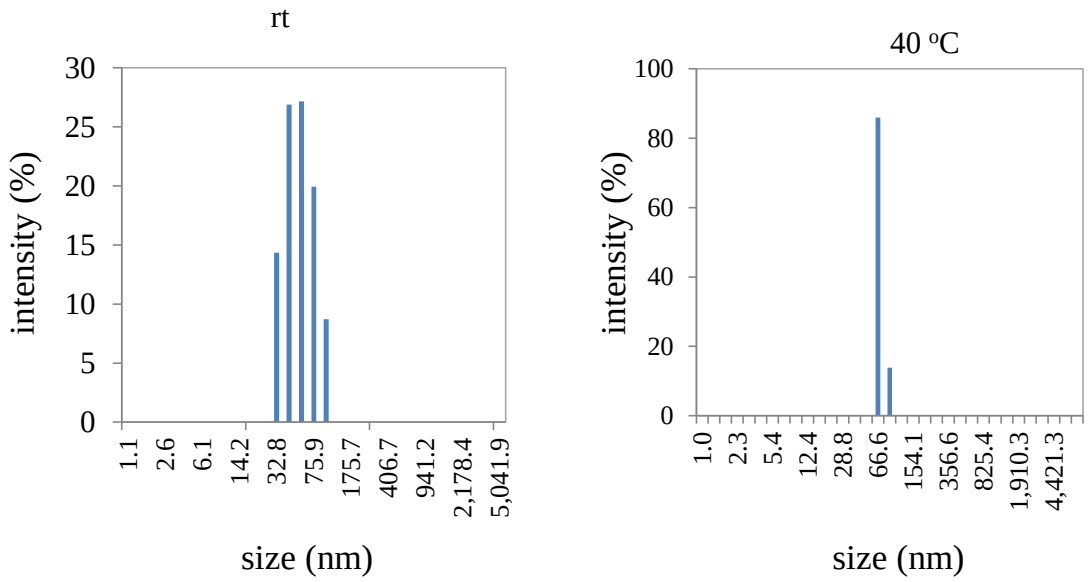


Figure S4. DLS analysis of the sizes of the **S202-Fab-P1**/siRNA complex in PBS buffer at rt or 40 °C. **S202-Fab-P1 (**10 μM × 25 μL**)** and siRNA **(**20 μM × 25 μL**)** in PBS were mixed and incubated at rt for 10 min. For DLS analysis at 40 °C, samples were placed in the isothermal chamber and equilibrated at 40 °C for 30 min. The average size of the **S202-Fab-P1**/siRNA complex at rt is about 53 nm and increased slightly to about 66 nm at 40 °C likely due to the low critical solution temperature (LCST) of the PNIPAAm block in the **P1**.

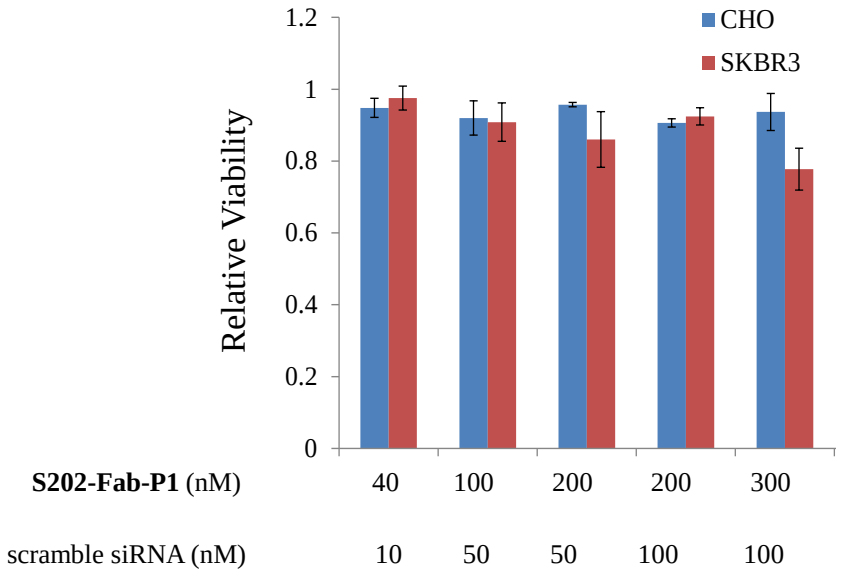


Figure S5. The **S202-Fab-P1**/scramble siRNA complex is not toxic to either HER2 positive or negative cells. In a 96-cell plate, SK-BR-3 or CHO cells were seeded at 10,000 cells/well and incubated at 37 ^{o}C for 24 h. Medium was removed and cells were supplied with Opti-MEM (80 µL); cells were then treated with 20 µL of **S202-Fab-P1**/scramble-siRNA complexes in triplicate at various concentrations for 4 h. The complexes were aspirated and cells were grown in complete medium without antibiotics for 24 h. 20 µL Cell Titer Blue reagent (Promega, Madison, WI) was then added to each well and the plate was incubated at 37 ^{o}C for 4 h. The relative cell viability of each treatment group was assessed by reading the fluorescence at 590 nm (excitation 560 nm) and normalized to the untreated group.

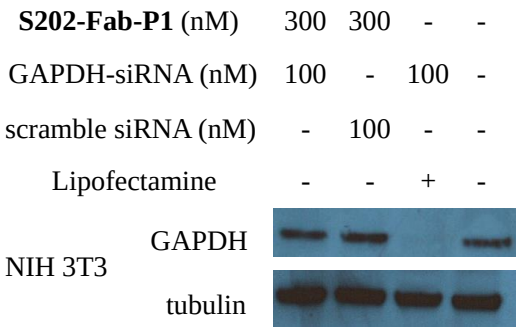


Figure S6. Western blot results showed that **S202-Fab-P1**/GAPDH-siRNA complexes did not silence GAPDH protein expression in NIH-3T3 cells. Cells were treated with the **S202-Fab-P1**/GAPDH-siRNA complexes or appropriate controls at the designated concentrations in Opti-MEM for 4 h; 48 h after treatment, cells were lysed for western blot analysis.

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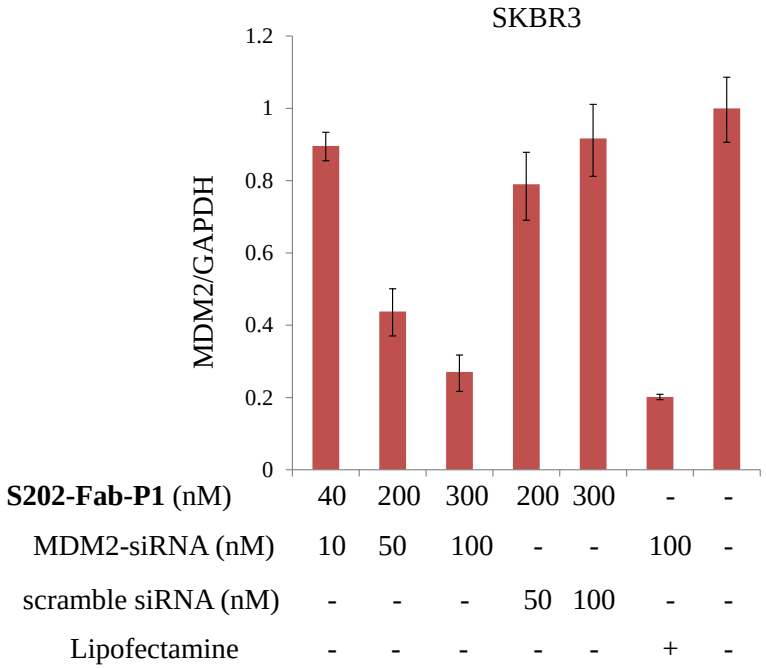

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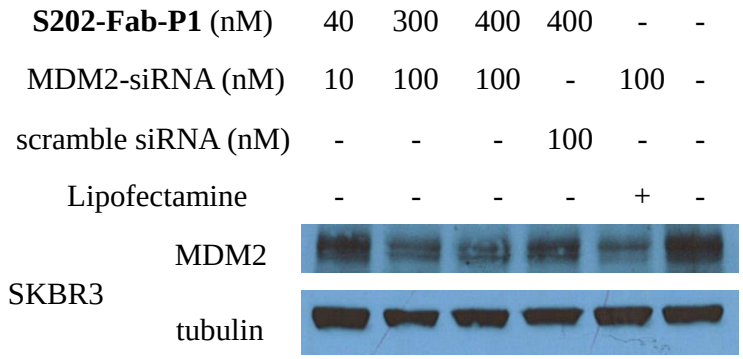


Figure S7. The **S202-Fab-P1**/siRNA complexes reduce the mRNA and protein levels of MDM2 in SKBR-3 cells, as determined by qRT PCR (A) and western blot (B). Cells were treated with the **S202-Fab-P1**/GAPDH-siRNA complexes or appropriate controls at the designated concentrations in Opti-MEM for 4 h; 48 h after treatment, cells were harvested for qRT PCR and western blot analysis.

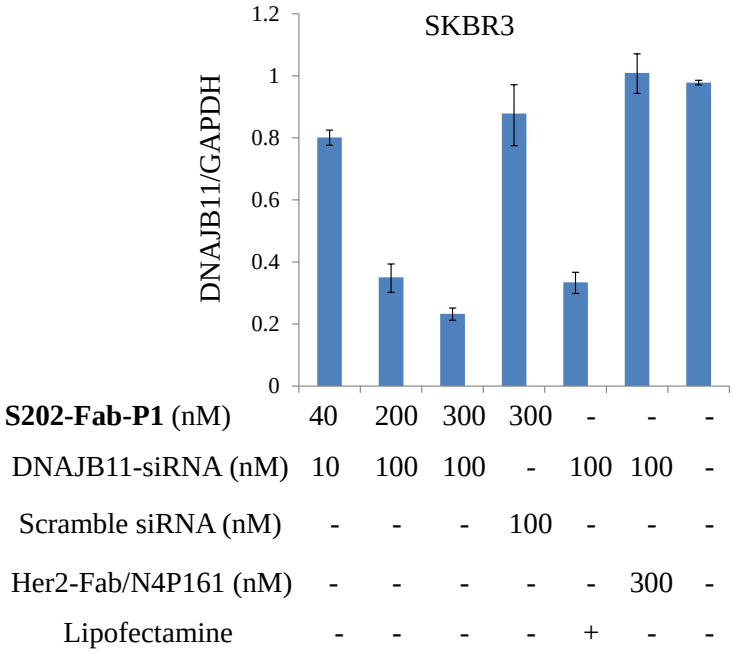


Figure S8. The **S202-Fab-P1**/siRNA complexes reduce the mRNA and protein levels of DNAJB11 in SKBR-3 cells, as determined by qRT PCR. Cells were treated with the **S202-Fab-P1**/GAPDH-siRNA complexes or appropriate controls at the designated concentrations in Opti-MEM for 4 h; 48 h after treatment, cells were harvested for qRT PCR analysis.

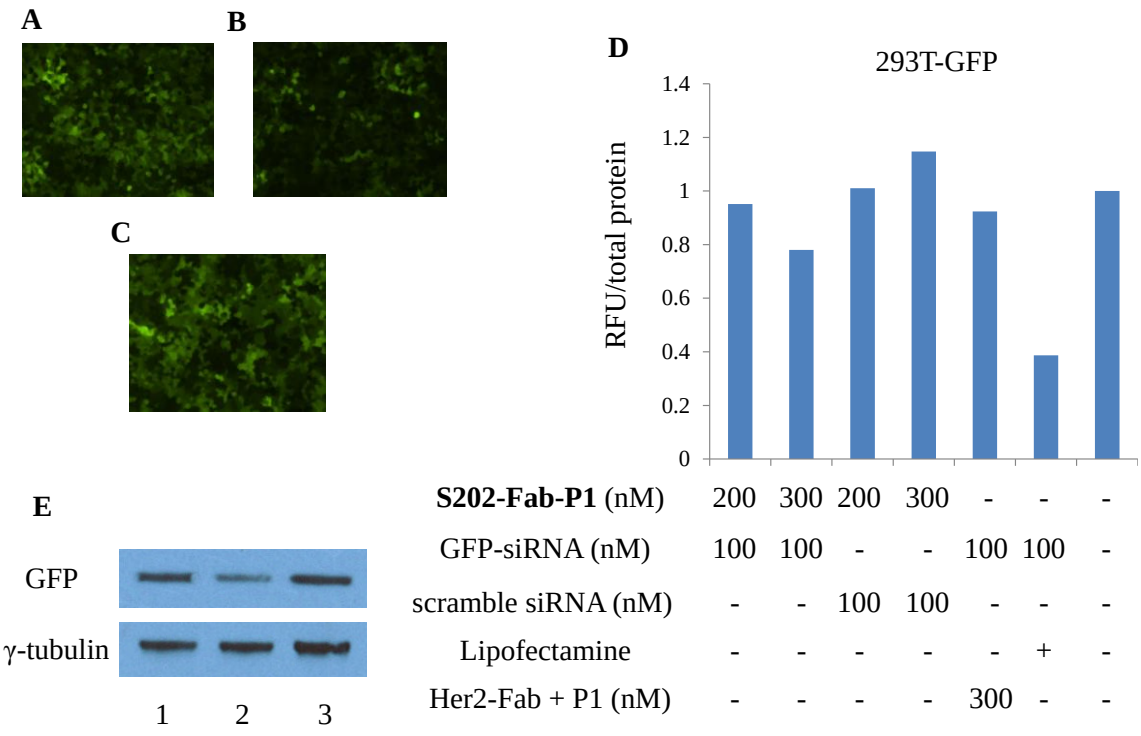
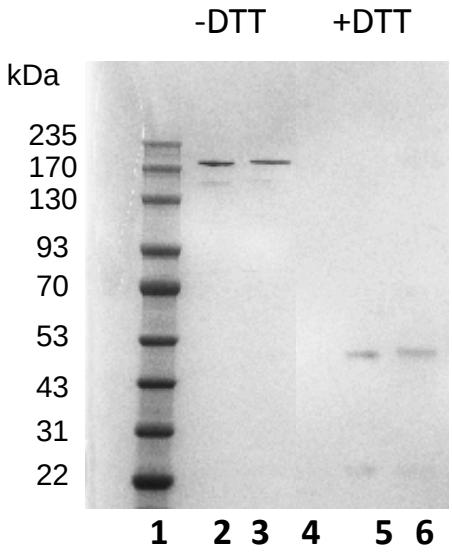


Figure S9. The **S202-Fab-P1**/GFP-siRNA complexes did not silence GFP protein expression in a HER2$^-$ 293T-GFP cell line. Stable 293T-GFP cells were seeded and treated with the **S202-Fab-P1**/siRNA complexes or appropriate controls at the designated concentrations in Opti-MEM for 4 h. 24 h after treatment, cells were lysed by 1× RIPA and the EGFP fluorescence emission at 509 nm was recorded by excitation at 488 nm. Total protein level was measured by BCA assay; the lysates were separated by SDS-PAGE gel and analyzed by western blot. (**A-C**) Fluorescence microscopy of 293T-GFP cells treated with the **S202-Fab-P1** /GFP-siRNA complex at 300 nM/100 nM (**A**), Lipofectamine/GFP-siRNA at 100 nM (**B**), and buffer (**C**); (**D**) Plot of relative fluorescence units (RFU) normalized to total protein levels (mg/mL, determined by BCA assay) of each group and compared to the untreated group; (**E**) western blot analysis of 3 selected samples: the **S202-Fab-P1**/GFP-siRNA complex at 300 nM/100 nM (lane 1), Lipofectamine/GFP-siRNA at 100 nM (lane 2); untreated (lane 3);



Lane **1**: protein ladder
Lane **2**: anti-Her2 A121-*p*AcF IgG (-DTT)
Lane **3**: anti-Her2 Q389-*p*Ac IgG (-DTT)
Lane **4**: Blank
Lane **5**: anti-Her2 A121-*p*AcF IgG (+DTT)
Lane **6**: anti-Her2 Q389-*p*Ac IgG (+DTT)

Figure S10. SDS-PAGE gel of the mutant A121-*p*AcF IgG and Q389-*p*AcF IgG under non-reducing and reducing conditions (with 10 mM DTT)

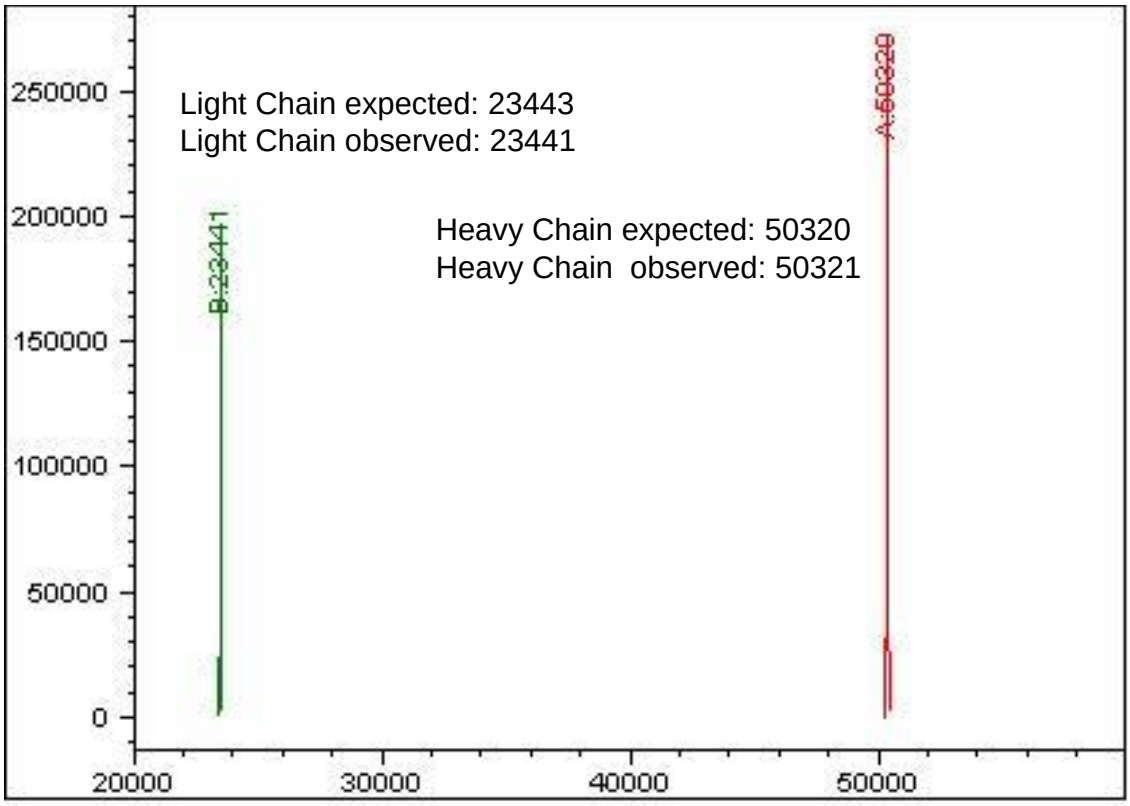
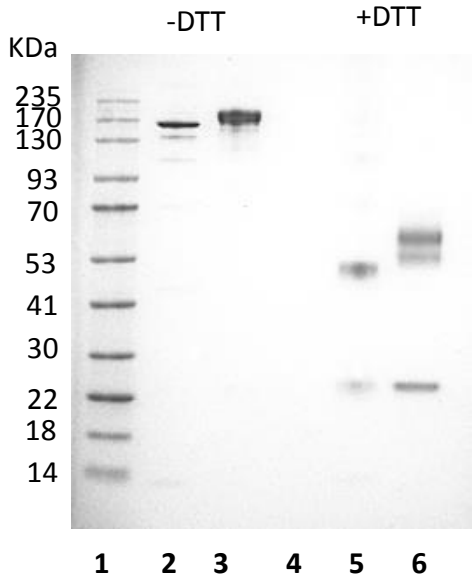
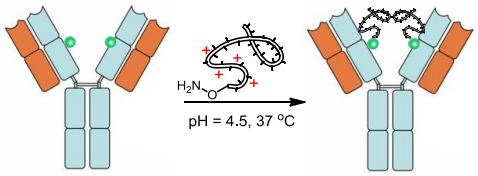


Figure S11. ESI-MS analysis of the mutant Q389-*p*AcF IgG under reducing conditions. The mutant Q389-*p*AcF IgG (0.1 mg/mL × 15 μL) in PBS was treated with 1 μL of PNGase (New England BioLabs) and incubated at 37 ^{o}C overnight to remove the glycols on the Fc-region. The mixture was then treated with DTT (50 mM × 1 μL), incubated at 37 ^{o}C for 20 min, and subjected to ESI-MS analysis

A

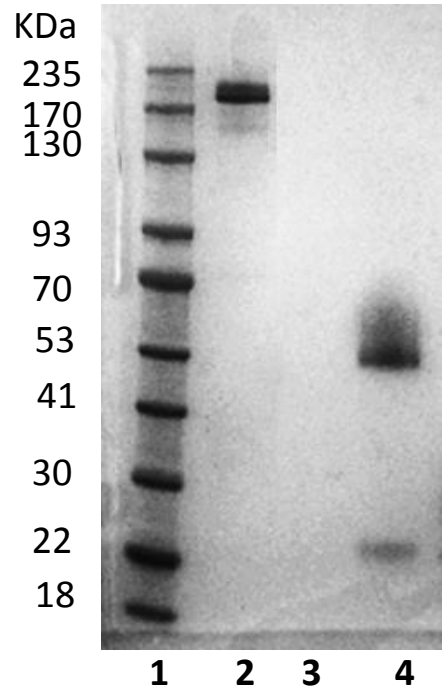


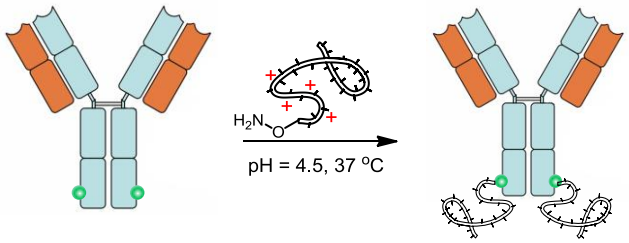


A121-*p*AcF IgG **A121-IgG-P1**

Lane **1**: protein ladder
Lane **2**: anti-Her2 A121-*p*AcF IgG (-DTT)
Lane **3**: **A121-IgG-P1** conjugate (-DTT)
Lane **4**: Blank
Lane **5**: anti-Her2 A121-*p*AcF IgG (+DTT)
Lane **6**: **A121-IgG-P1** conjugate (+DTT)

B





Q389-*p*AcF IgG **Q389-IgG-P1**

Lane **1**: protein ladder
Lane **2**: **Q389-IgG-P1** conjugate (-DTT)
Lane **3**: Blank
Lane **4**: **Q389-IgG-P1** conjugate (+DTT)

Figure S12. SDS-PAGE gel of **A121-IgG-P1** (A) and **Q389-IgG-P1** (B) under non-reducing and reducing conditions

	EC_{50} (nM)
Q389-*p*AcF IgG	1.1 ± 0.2
Q389-IgG-P1	0.9 ± 0.1
A121-*p*AcF IgG	0.2 ± 0.1
A121-IgG-P1	0.4 ± 0.1

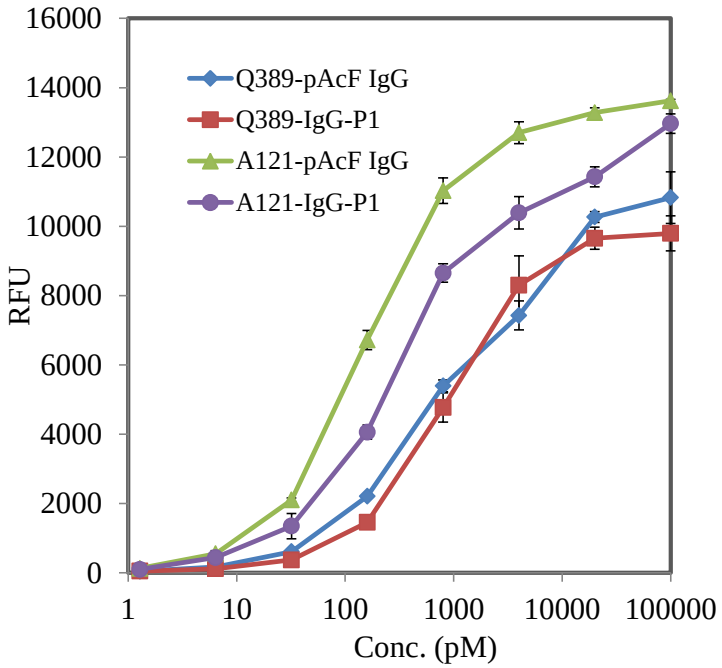


Figure S13. ELISA assay of the mutant A121-*p*AcF IgG, **A121-IgG-P1**, Q389-*p*AcF IgG, and **Q389-IgG-P1** with HER2 antigen. HER2 antigen was coated onto a 96-well ELISA plate at 50 ng/well. IgGs and APCs at various concentrations were added to the plate in triplicate and incubated at rt for 1 h; an anti-human Ig kappa chain-HRP conjugate was used as the secondary antibody and the binding affinity was assayed using fluorescence by QuantaBlu Fluorogenic Peroxidase Substrate (Pierce, Rockford, Illinois) following the manufacture's instruction.

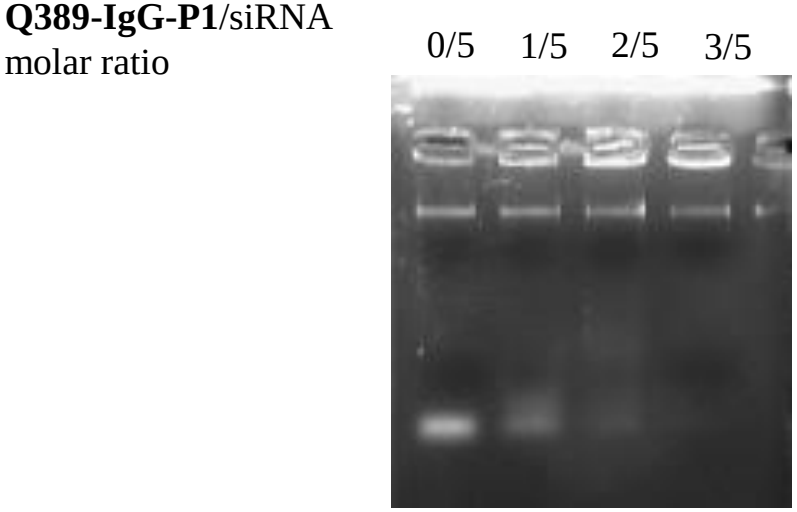


Figure S14. Gel retardation assay of the **Q389-IgG-P1**/scramble-siRNA complex in PBS buffer. Both the scramble-siRNA and **Q389-IgG-P1** were diluted to 2 μM in PBS before analysis; various volumes of **Q389-IgG-P1** were added to the siRNA solutions (5 μL) and incubated at rt for 1 min. The solutions were then loaded to a 1% agarose gel containing ethidium bromide and resolved by electrophoresis in 1× TAE buffer; complete binding of the siRNA to **Q389-IgG-P1** was detected starting from lane 3, which is at **Q389-IgG-P1**/siRNA molar ratio of 2/5.

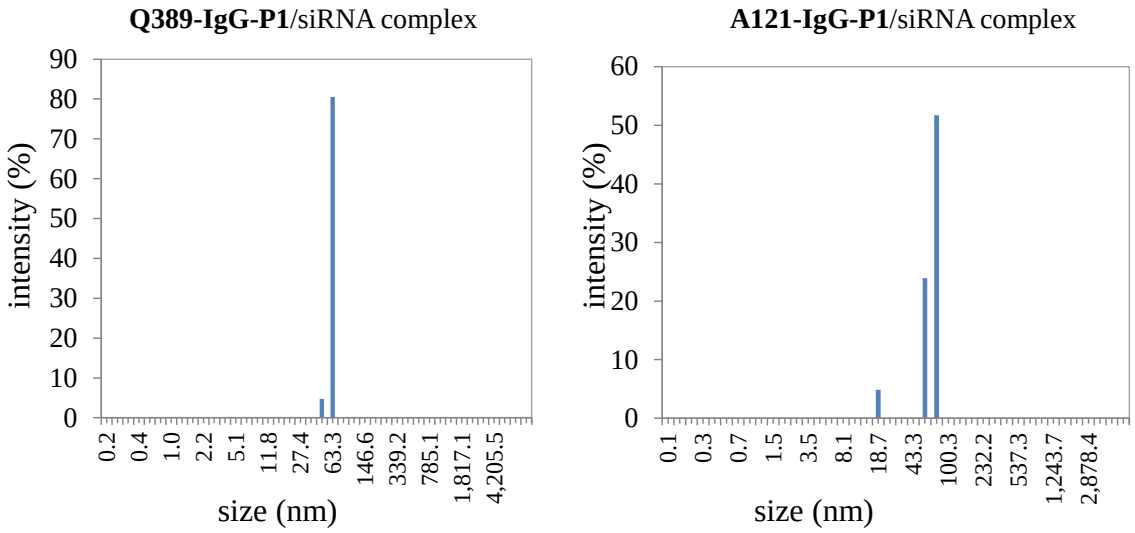


Figure S15. DLS analysis of the sizes of the **Q389-IgG-P1**/siRNA (left) and the **A121-IgG-P1**/siRNA (right) complexes in PBS buffer at rt. The APC/siRNA ratio were 1/1 for both complexes

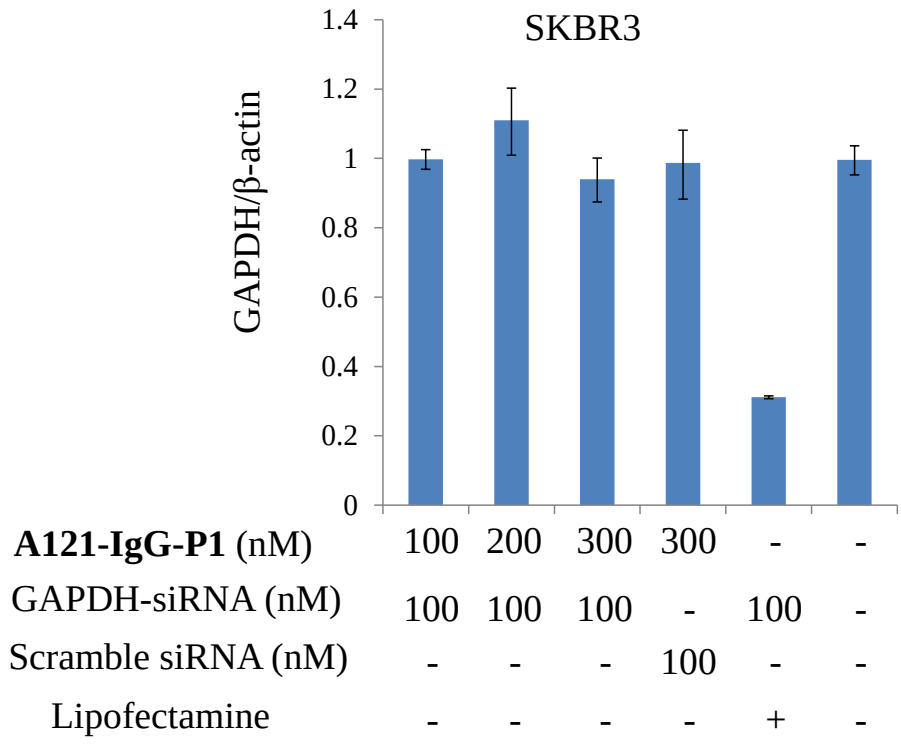


Figure S16. The **A121-IgG-P1**/GAPDH-siRNA complexes did not reduce mRNA levels of GAPDH in SKBR-3 cells. Cells were treated with the **A121-IgG-P1**/GAPDH-siRNA and controls at the designed concentrations in Opti-MEM for 4 h; 24 h after treatment, cells were harvested for qRT PCR analysis.

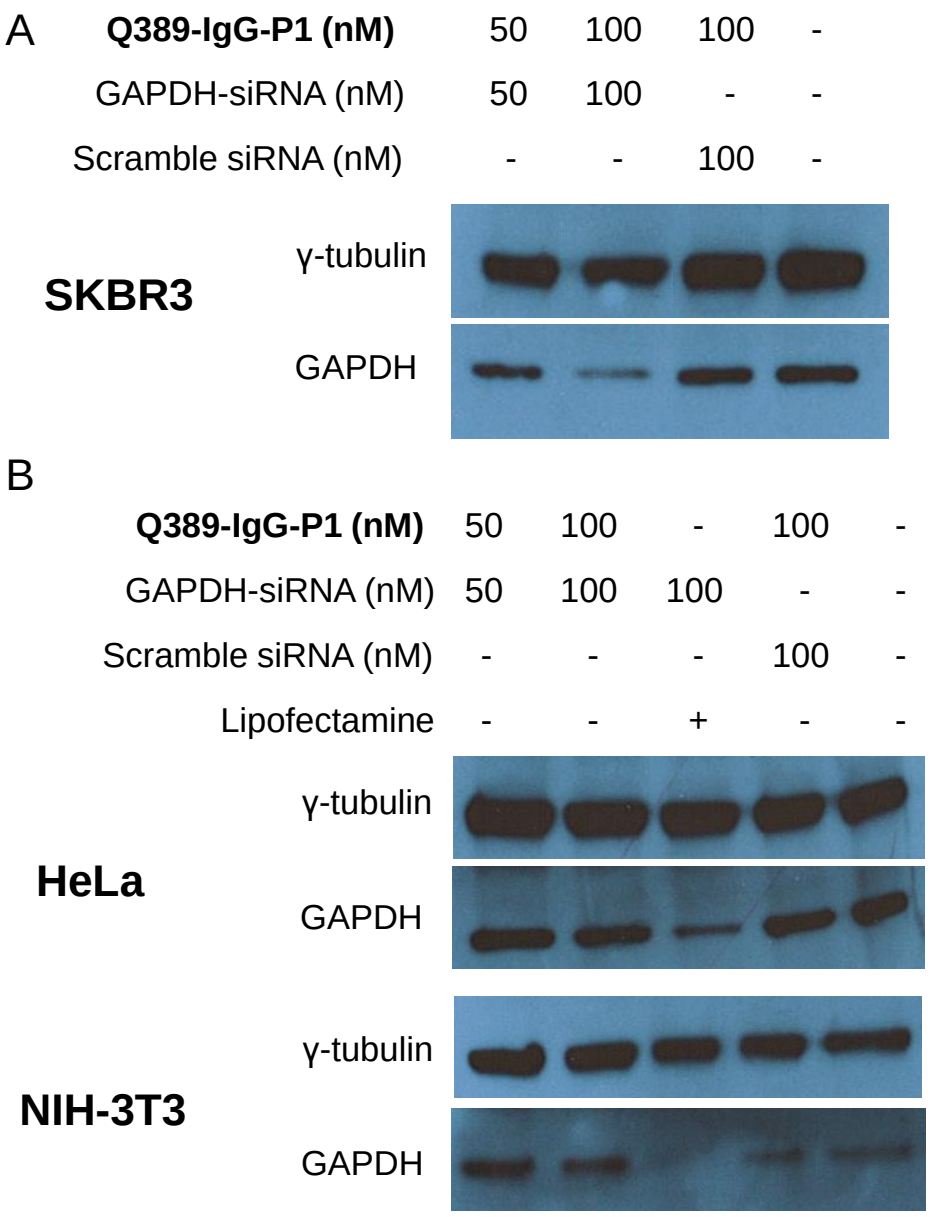


Figure S17. The **Q389-IgG-P1**/GAPDH-siRNA complexes induce potent RNA silencing and reduce GAPDH protein levels in SKBR-3 cell **(A)** but not in HeLa and NIH-3T3 cells **(B)**. Cells were treated with the **Q389-IgG-P1**/GAPDH-siRNA and controls at the designated concentrations in Opti-MEM for 4 h; 72 h after treatment, cells were harvested and analyzed by western blot.

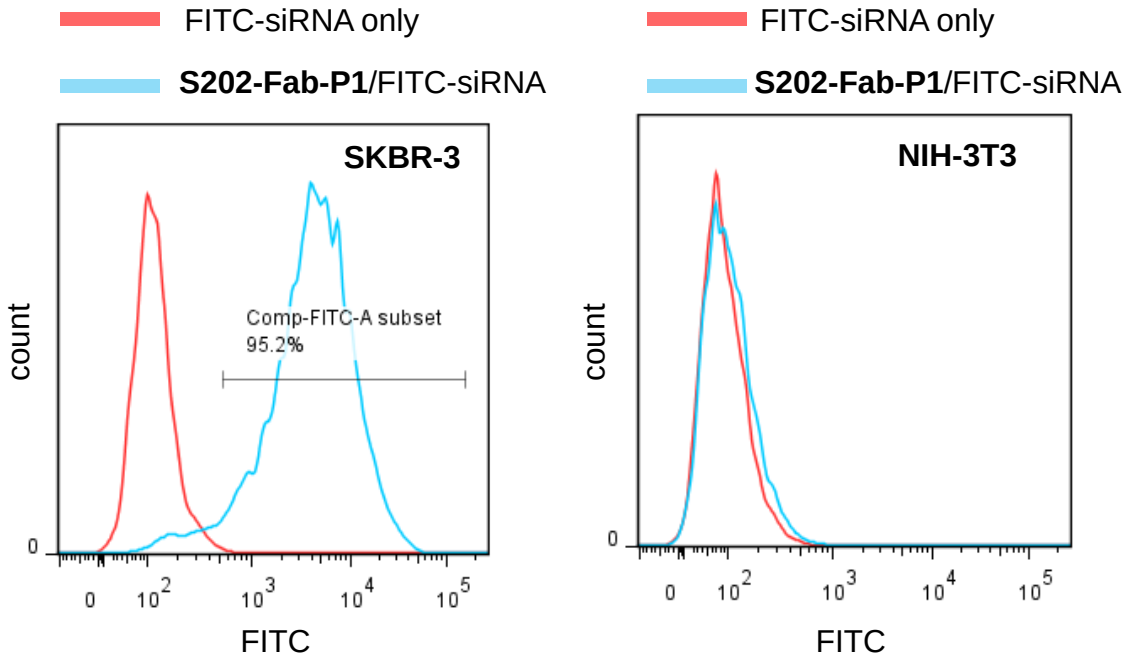


Figure S18. FACS analysis of the delivery of FITC-siRNA by **S202-Fab-P1** in SKBR-3 cells **(A)** and in NIH-3T3 cells **(B)**. Cells were treated with 100 nM FITC-siRNA and the **S202-Fab-P1**/FITC-siRNA complexes at 100 nM in Opti-MEM for 3 h; cells were washed with PBS (with 1 μg/mL heparin) once, trypsinized, spinned down, and resuspended in PBS for FACS analysis.