Supporting Information

for

Clustered Nanobody-Drug Conjugates for Targeted Cancer Therapy

Experimental Procedures

Expression and purification of the nanobody.

The plasmid Nb-C_3-Q was constructed for the expression of the anti-EGFR nanobody 7D12 containing an N-terminal His_6-tag and a C-terminal -$(GGGGS)_2$-CCC-GSGSGS-LLQS sequence. The culture and purification steps were followed as described.[1] The recombinant protein Nb-C_3-Q was purified using a Ni-NTA column (His-Trap HP column 5 mL, GE Healthcare) with a linear gradient of 40-250 mM imidazole in 20 mM Tris-HCl, 150 mM NaCl, pH 8.0. The concentration of Nb-C_3-Q protein was measured with Brandford assay and the purity was analyzed by SDS polyacrylamide gel electrophoresis (SDS-PAGE).

The protein sequence is shown below. The TEV cleavage site is underlined, and the His-tag, C_3-tag, Q-tag and linker sequence were shown in red.

MHHHHHHSDKIIHLTDDSFDTDVLKADGAILVDFWAEWCGPCKMIAPILDEIADEYQGKLTVAKLNID
QNPGTAPKYGIRGIPTLLLFKNGEVAATKVGALSKGQLKEFLDANLAGSGSGSGSENLYFQSMQVKLEE
SGGGSVQTGGSLRLTCAASGRTSRSYGMGWFRQAPGKEREFVSGISWRGDSTGYADSVKGRFTISRD
NAKNTVDLQMNSLKPEDTAIYYCAAAAGSAWYGTLYEYDYWGQGTQVTVSSGGGGSGGGGSCCCG
SGSGSLLQS

Synthesis of PEG-DPK.

Solid-phase peptide synthesis (SPPS) was used to prepare dendrimeric polylysine (dendrimeric poly(l-lysine), DPK). All reactions were carried out with N_2 flowing at room temperature (25 °C) unless specified. 600 mg 2-chlorotrityl chloride resin was dispersed in DMF and swelled for 30 min. After the resin is fully swollen, Fomc-L-Lys(Fomc) (957.1 mg, 2 eq. relative to the functional group of the resin) and ethylisopropylamine (DIEA) (129.24 mg, 4 eq. relative to the functional group of resin) were added. After reaction for 3 h, the resin was washed with DMF (5×5 mL). The resin was treated with a solution of DMF/piperidine (4:1, v/v, 5 mL) for 20 min and washed with DMF (5×3 mL) for deprotection of amino. Then Fomc-L-Lys(Fomc) and DIEA were doubled and mixed with HBTU (1 eq. relative to Fomc-L-Lys(Fomc)) and HOBt (1 eq. relative to Fomc-L-Lys(Fomc)) in DMF, then the mixture was transferred into the reactor. The DPK2-Fomc was synthesized after 2 h reaction at room temperature. The product was cleaved by adding a mixture of trifluoroacetic acid (TFA): methanol (1:99, v/v). The combined solutions were concentrated under a stream of nitrogen.

DPK2-Fomc powder (211.8 mg, 1.25 eq.), mPEG-NH_2 (500 mg, 1 eq.), HBTU (38 mg, 1 eq.), and DIEA (25.8 mg, 2 eq) were dissolved in DMF. The reaction was conducted at room temperature till the ninhydrin test showed negative. Piperidine (4:1, v/v) was added for the deprotection of the Fomc group. The solvent was removed by rotary evaporation. The viscous liquid as a crude product was dialyzed against water. The final product was freeze-drying to powder.

Synthesis of Maleimido-platin(IV).

The maleimide platinum(IV) complex (Mal-Pt(IV)) was prepared following the procedure as reported with several changes.[2] Hydrogen peroxide (3.5 mL 30% w/v, 0.03 mol) was added in a suspension of cisplatin (300 mg, 0.6 mmol) in 40 ml acetic acid, the mixture was stirred for 4 h at 35 °C. Then, the mixture was preliminary evaporated of the acetic acid by rotary evaporation and followed by lyophilization. Meanwhile, 6-maleimidohexanoic acid (100 mg, 0.46 mmol) and oxalyl chloride (380 mg, 3 mmol) were mixed in the ice bath under nitrogen in anhydrous dichloromethane (2 mL) for at least 3 h. The solution was concentrated under nitrogen to get a viscous oil of 6-maleimidohexanoic acid chloride.

For the preparation of maleimide platinum, 200 mg Ac-Pt(IV) (0.6 mmol) was solved in 15 mL anhydrous N, N-dimethylformamide (DMF) and mixed with 4-methylmorpholine (NMM). The mixture was added dropwisely with 6-maleimidohexanoic acid (260 mg, 1.2 mmol) in an ice-water bath. The mixture was stirred for 6 h at room temperature in dark. The solution was condensed by rotary evaporation and the resultant was washed with acetone and diethyl ether to yield a pale yellow powder.

Preparation and characterization of NDC, NDC@PEG, and cNDC@PEG.

The PEGylation of nanobody was conducted followed the standard procedures as described.[1] Nb-C_3-Q (3 mg) was mixed with mPEG-NH_2 (1 mg) or PEG-DPK (0.27 mg) in PBS buffer containing 1 mM ascorbic acid. The PEGylation was initiated by adding mTGase (1 U/mL). After incubation for 1 h at room temperature, the PEGylated nanobody (Nb-PEG/Nb_4-PEG) was purified using gel filtration through a ÄKTA purifier system equipped with a HiLoad 10/60 Superdex 200 column.

Nb, Nb-PEG, and Nb_4-PEG were pretreated with 5 molar equivalents (based on Nb) of β-aminoethanethiol hydrochloride at 37 °C for 3 h, then the products were purified using desalting columns (HiTrap™ Desalting) on ÄKTA purifier system (GE Healthcare). The purified Nbs were mixed with the Mal-Pt(IV) in a ratio of 3:5 based on the thiol groups on Nbs, generating nanobody-drug conjugates, namely NDC, NDC@PEG, or cNDC@PEG. After 6 h incubation at room temperature, the unreacted Mal-Pt(IV) was removed by desalting columns. The concentration of NDC, NDC@PEG, and cNDC@PEG was measured with Bradford assay based on Nb. The platinum content was measured with inductively coupled plasma mass spectrometry (ICP-MS).

Stability of nanobody drug conjugates.

NDC, NDC@PEG, and cNDC@PEG were concentrated to different concentration by ultrafiltration at 4 °C. After incubation at 25 °C for different time, samples were centrifuged at 4 °C (15000 rpm × 15 min), and the protein concentration was measured by OD_{280}.

DNA binding assay. The platination of DNA was analyzed using fluorescence spectroscopy with EtBr probe. Herring sperm DNA was used to react with cisplatin, Mal-Pt(IV), NDC, NDC@PEG, or

cNDC@PEG at 37 °C. Platinum drugs were dissolved in 10 mM phosphate buffer (pH 7.4) containing 10 mM $NaClO_4$ with the concentration of 50 μM (based on Pt) in the dark. 0.04 mg mL^{-1} herring sperm DNA was added into the mixture. Then 0.025 mg mL^{-1} EtBr and 0.4 M NaCl were added to samples with or without 10 mM reducing agent (AsA or GSH). The fluorescence spectra were recorded after different reaction time at an excitation wavelength of 530 nm and an emission wavelength of 615 nm.

Cellular binding and internalization of NDCs. The cellular binding and intracellular distribution of NDC, NDC@PEG, and cNDC@PEG were investigated with a confocal laser scanning microscope. Nb was labeled with a fluorescent probe FITC in advance, which could be detected under the excitation at 488 nm. The labeled ratio of FITC to Nb is 1:1. The FITC-labeled Nb was used to produce FITC-Nb-PEG, FITC-Nb_4-PEG, FITC-NDC, FITC-NDC@PEG, and FITC-cNDC@PEG. 1×10^6 cells were seeded in 24-well plates for 12 hours for cell adhesion. Then the culture medium was replaced with 1 mL fresh medium containing different formulations with a concentration of 5 μM based on FITC-Nb. The cells were further incubated for 3 h at 37 °C. After incubation, the cells were washed with PBS for 3 times and fixed by 4% paraformaldehyde. The nuclei were stained with DAPI (1 μg/mL). After that, the cell binding and internalization of the NDCs were analyzed using confocal laser scanning microscopy (LSM 710 CLSM, Carl Zeiss, Jena, Germany).

Cell based binding assay. To prevent internalization of NDCs during the measurement, the cell binding assays were performed at 4 °C. A431(++) cells were incubated with NDCs at the desired concentration. After the culture with NDCs in different time, the drugs were removed and cells were analyzed immediately on flow cytometry. The calculation of the K_d, K_{on}, and K_{off} was followed the procedure as reported.[3]

Drug penetration in multicellular spheroids (MCSs). The MCSs were cultured according to the literature method.[4] The A431(++) cells were seeded and cultured overnight. The T75 flask was pre-covered by 10 mL of hot agarose (1 w/v %) and cooled to completely solidified. 1×10^6 A431(++) cells were seeded in a flask and incubated for 4 days. The MCSs was treated with 15 mL of FITC-Nb, FITC-NDC, FITC-NDC@PEG, or FITC-cNDC@PEG. 5 μM FITC-NDCs was used based on the concentration of Nb. After 4 h of incubation at 37 °C, the spheroids were collected and washed with PBS for three times. The spheroids were fixed with PFA 4% (w/v) in PBS for 1 h in room temperature and observed with confocal laser scanning microscopy (LSM 710 CLSM, Carl Zeiss, Jena, Germany).

Cellular uptake of platinum complex and NDCs. The cellular platinum accumulation was measured by ICP-MS. A431(++) cells and A2780(-) cells were seeded in 6-well plates and cultured overnight. After adhesion, cells were incubated with cisplatin, NDC, NDC@PEG, and cNDC@PEG at a final concentration of 50 μM Pt at 37 °C. Then the cells were washed with PBS for 3 times and harvested by trypsinization. After cell count, the harvested cells were digested with nitric acid for the ICP-MS measurement.

***In vitro* cytotoxicity assays.** Cells were seeded in 96-well plate at 5×10^3 cells per well and incubated in a 5% CO_2 incubator at 37 °C for 12 h for cell adhesion. The medium was replaced by 100 μL fresh medium containing cisplatin, NDC, NDC@PEG, or cNDC@PEG at different concentrations ranging from 0 to 100 μM. The cells were further incubated for 72 h at 37 °C. Then the medium was replaced by fresh culture medium and MTT was added. The cells were incubated for another 4 h to allow viable cells to reduce the yellow tetrazolium salt (MTT) into dark blue formazan crystals. Finally, 100 μL of lysis buffer was added and cells were incubated for another 4 h at 37 °C. The absorbance at 490 nm was measured using a Bio-Rad 680 microplate reader. The IC_{50} values were calculated using GraphPad Prism software (version 6.01) based on data from three parallel experiments.

***In vivo* pharmacokinetic studies.** Pharmacokinetic studies of platinum formulations (cisplatin, Ac-Pt(IV), NDC, NDC@PEG, and cNDC@PEG) were conducted on nude mice after a single-dose injection. The mice were randomly assigned to 5 groups (3 mice per group). Mice were injected with platinum drug (2 mg/kg, at equivalent platinum doses) via the tail vein intravenously. Blood was collected from the retro-orbital plexus of the mouse at 5 min, 30 min, 1 h, 2 h, 4 h, 6 h, 8 h, 12 h, and 24 h. The blood samples were then centrifuged at 4 °C (3000 g, 10 min) to collect the plasma. Then the plasma was digested with a mixture of HCl and HNO_3 in a ratio of 3:1, samples were heated on heating plate in 270 °C to remove the solution. Then diluted samples with deionized water to 4ml. The content of platinum in plasma was measured with ICP-MS.

Tissue distribution of drugs. Cisplatin, NDC@PEG, and cNDC@PEG were administered intravenously at an equivalent dose of 40 μg of platinum per mouse bearing an A431(++) xenograft tumor (n = 3 per group). The mice were sacrificed at 3, 6, 9, 12, and 24 h after a single injection. Then the tumors were excised, washed with cold PBS, dried on filter paper, and weighed. The excised tumors were cut into small pieces, digested and the content of platinum was determined by ICP-MS. For the biodistribution measurement, cisplatin, Ac-Pt(IV), NDC, NDC@PEG, and cNDC@PEG were administered at an equivalent dose of 40 μg of platinum per mouse and the mice were sacrificed at 12 h post injection. The major organs, including heart, liver, spleen, lung, and kidney, were excised, washed, dried, weighed, and digested. The amounts of platinum in these organs were determined by ICP-MS.

***In vivo* drug penetration study.** The nude mice bearing A431(++) xenografts tumor were injected with FITC-Nb-PEG, FITC-Nb4-PEG, FITC-NDC, FITC-NDC@PEG, and FITC-cNDC@PEG at a dose of 2 μg of Nb. Six hours after injection, the mice were sacrificed and the tumors were excised and fixed in 4% paraformaldehyde overnight at room temperature. The tumors were immersed overnight in 30% sucrose solution. The samples were sectioned into slides of 8 μm thickness in a cryostat, incubated with Cy5 labeled anti-CD31 antibody followed the instruction manual. The fluorescence signals of the samples were observed under a Zeiss LSM710 Meta confocal microscope with a 10× or 20× objective.

Tumor growth inhibition assay. Mice bearing A431(++) xenografts tumor were randomly divided into 5 groups (5 mice per group). After tumor volumes reached 100-200 mm^3, mice were treated with cisplatin, NDC@PEG, cNDC@PEG (1 or 2 mg Pt/kg body weight) or PBS. All drugs were injected intravenously on days 0 and 4 and 8. The body weight and tumor volume were measured every day. Tumor volume was calculated as $V = lw^2/2$ (l represents the larger diameter, w represents the smaller diameter). The inhibition rate of each treatment was calculated according to the equation $[1-(V_{tf} -V_{ti})/(V_{pf} -V_{pi})] \times 100\%$, where V_{tf} and V_{ti} represent the final and initial tumor volume of the treatment group, while V_{pf} and V_{pi} represent the final and initial tumor volume of the PBS group, respectively. Twenty-four hours after the last measurement of tumor volume, mice were sacrificed, and tumor and organs were excised. The tumors were weighted after excision.

Immunohistochemical analysis. Excised tumors and main organs were fixed in 4% formaldehyde and embedded in paraffin. Then these tissues were cut into slides in 5 μm thickness for immunohistochemical analysis. TUNEL and EGFR staining were performed on tumor tissues and TUNEL on other main organs. Apoptotic cells were identified using a terminal transferase dUTP nick-end labeling (TUNEL) following the manufacturer's protocol. The EGFR level was analyzed using an EGFR assay kit (Roche, USA). DAPI solution was added to the tissue and incubated for 2 min at room temperature. After PBS washing, the tissues were mounted with anti-fade mounting solution (Sigma-Aldrich, USA) to reduce fluorescence photo bleaching. Then the samples were analyzed using a Zeiss LSM 710 inverted laser confocal scanning microscope imaging system (Germany).

Analysis of EGFR expression. The EGFR expression of tumor cells was measured by western blot. Tumors were excised and washed with PBS for three times. The tumor tissue was cut into small block and grinded to fully lyse. The supernatants were collected by centrifugation at 15000 g for 10 min at 4 °C. Total tissue protein concentration was determined by the BCA method according to manufacturer's protocol (Thermo Fisher Scientific, USA). Approximately 50 μg of total protein was used for SDS-PAGE and transferred onto a polyvinylidene fluoride (PVDF) membrane. Immunoblotting using a rabbit polyclonal anti-EGFR antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Anti-β tubulin antibody was used as an internal control. An enhanced ECL Western Blotting Detection kit (Thermo Fisher Scientific, USA) was used to detect the luminescence.

References:

1. T. Wu, H. Huang, Y. Sheng, H. Shi, Y. Min and Y. Liu, *J. Mater. Chem. B*, 2018, **6**, 1011-1017.
2. V. Pichler, J. Mayr, P. Heffeter, O. Domotor, E. A. Enyedy, G. Hermann, D. Groza, G. Kollensperger, M. Galanksi, W. Berger, B. K. Keppler and C. R. Kowol, *Chem. Commun.*, 2013, **49**, 2249-2251.
3. K. Yong, D. Yuen, M. Z. Chen and A. P. R. Johnston. *ACS Appl. Mater. Inter.* 2020, **12**, 5593-5600.
4. J. Friedrich, C. Seidel, R. Ebner, L. A. Kunz-Schughart, *Nat. Protoc.* 2009, **4**, 309.

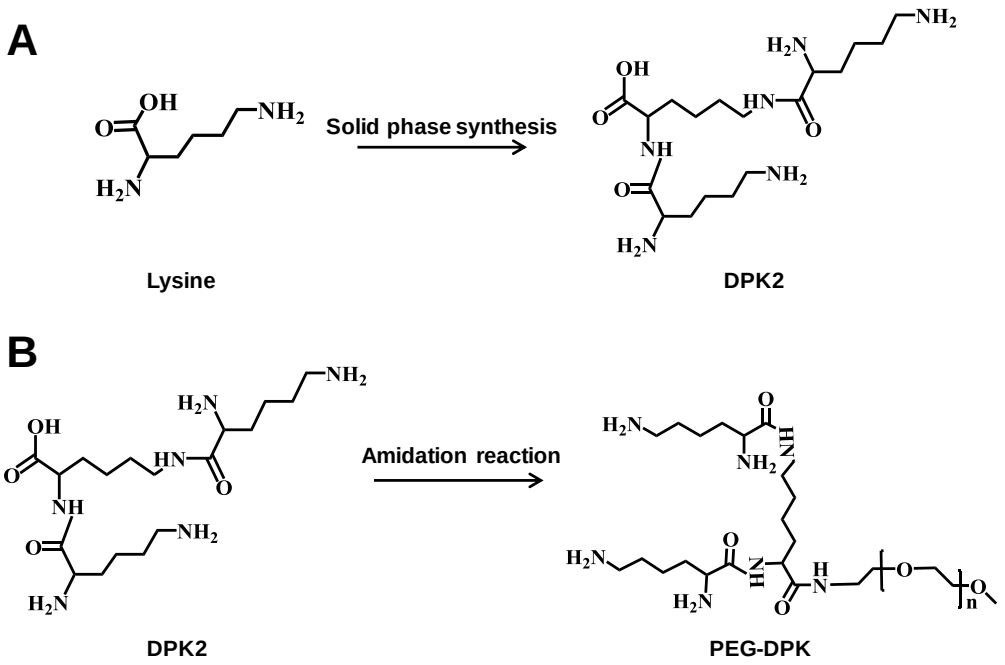

Figure S1 Schematic illustration of the synthesis of PEG-DPK. A) Synthesis of DPK2 by solid phase synthesis. B) Conjunction of mPEG to DPK2 by the amidation reaction.

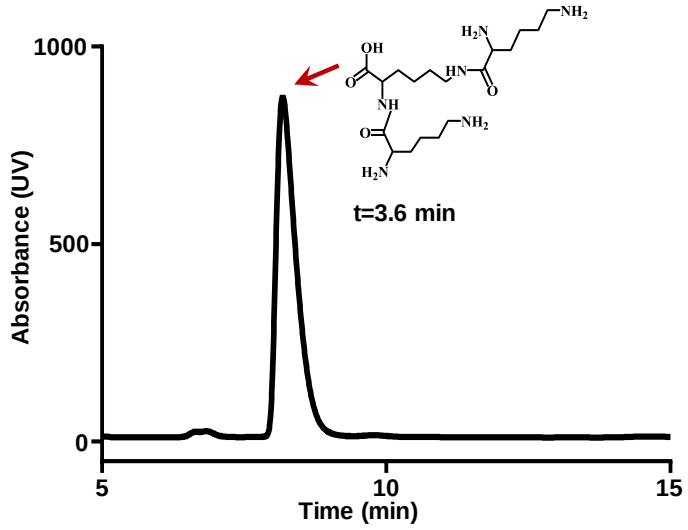

Figure S2 Characterization of DPK2 by HPLC. (0 to 60% B over 30 min, 0.1% TFA)

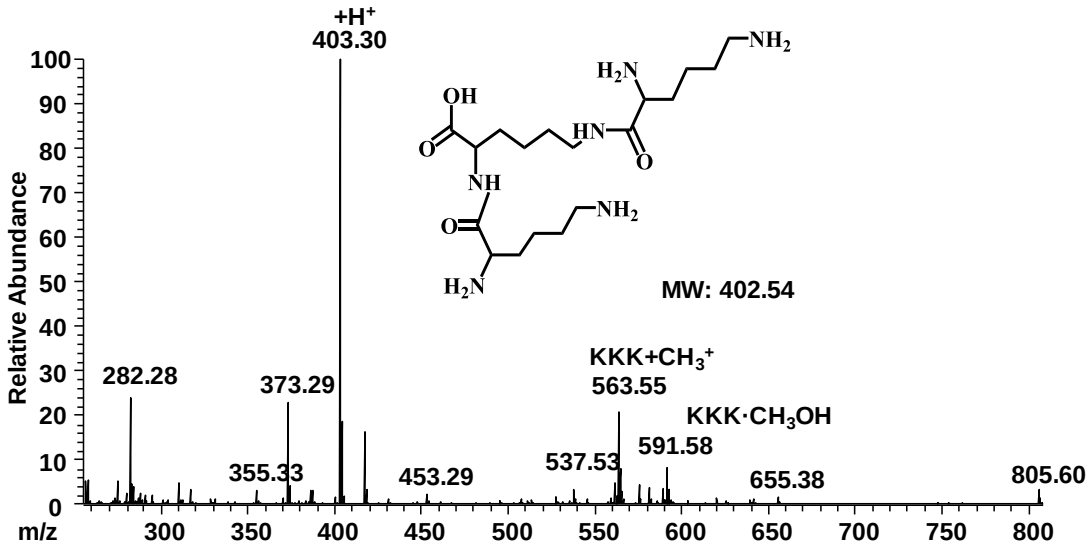

Figure S3 Characterization of DPK2 by ESI MS.

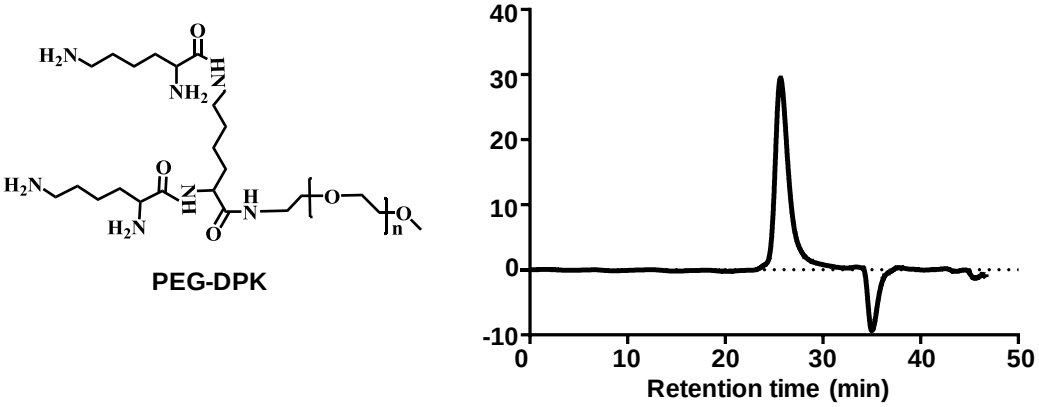

Figure S4 Characterization of PEG-DPK by gel permeation chromatography (GPC).

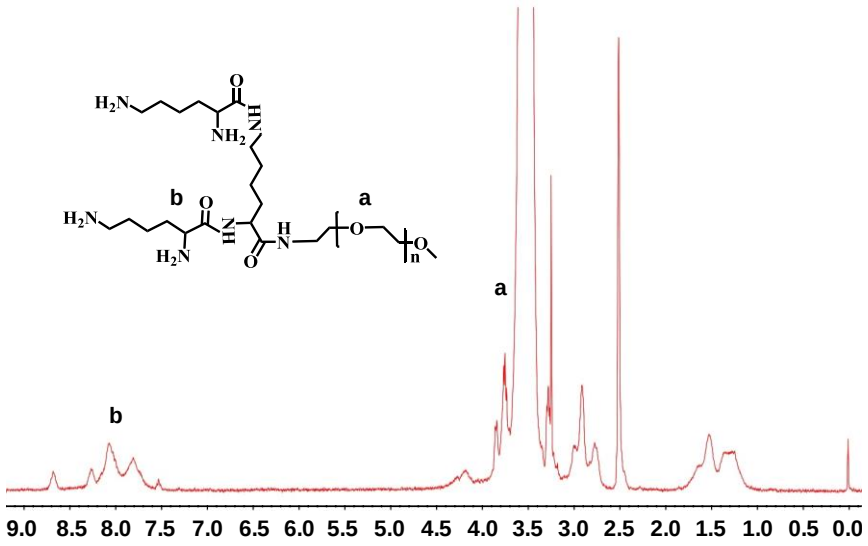

Figure S5 Characterization of PEG-DPK by ^{1}H NMR in DMF.

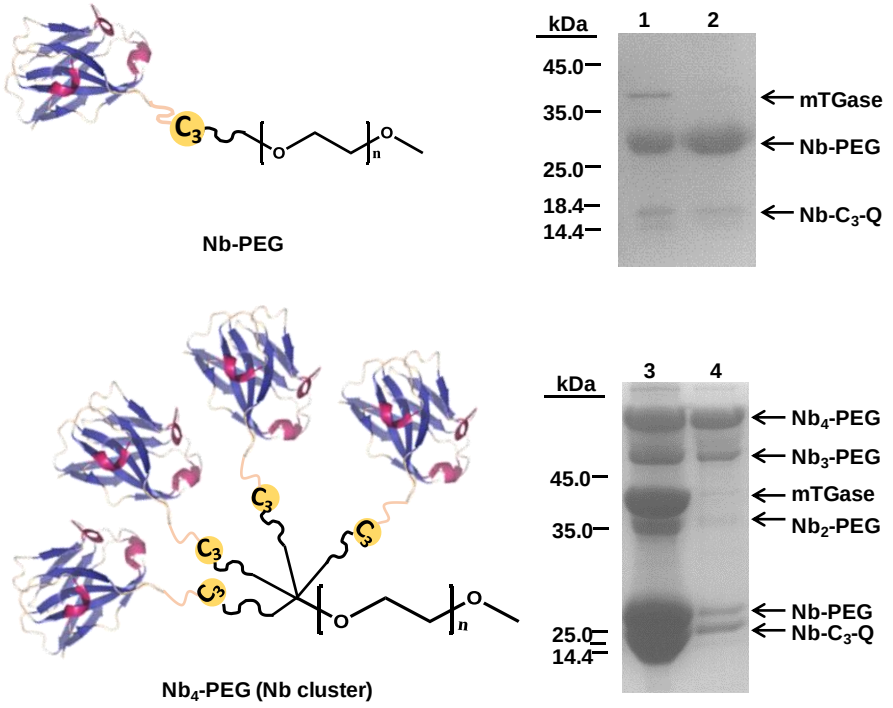

Figure S6 Conceptual strategy to achieve site-selective nanobody-modification using mTGase catalysis reactions. Lane 1: PEGylation of Nb-C_3-Q conducted by mTGase (substrate: mPEG-NH_2), Lane 2: Nb-PEG purified with size exclusion column, Lane 3: Tetramerization of Nb-C_3-Q conducted by mTGase (substrate: PEG-DPK), Lane 4: Nb_4-PEG cluster purified with size exclusion column. (Samples were purified with HiLoad 10/60 Superdex 200 column in PBS)

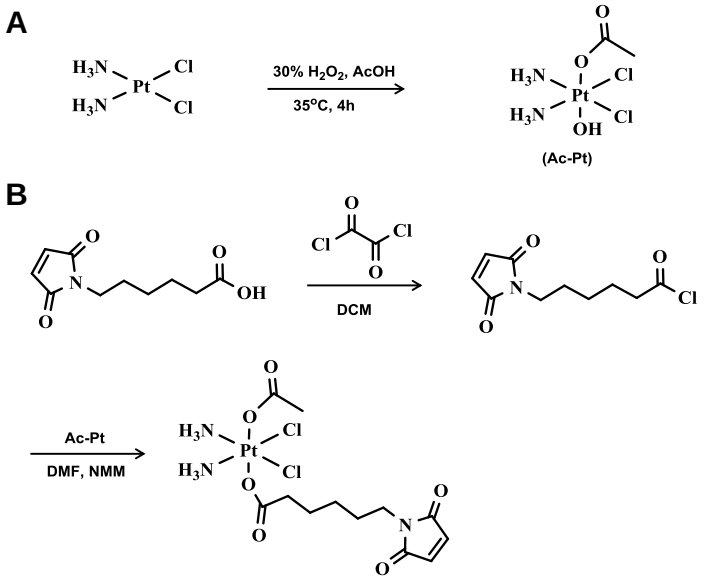

Figure S7 Schematic illustration of the synthesis of Mal-Pt(IV). A) Oxidation of cisplatin to Ac-Pt(IV). B) Adding maleimido to Ac-Pt(IV).

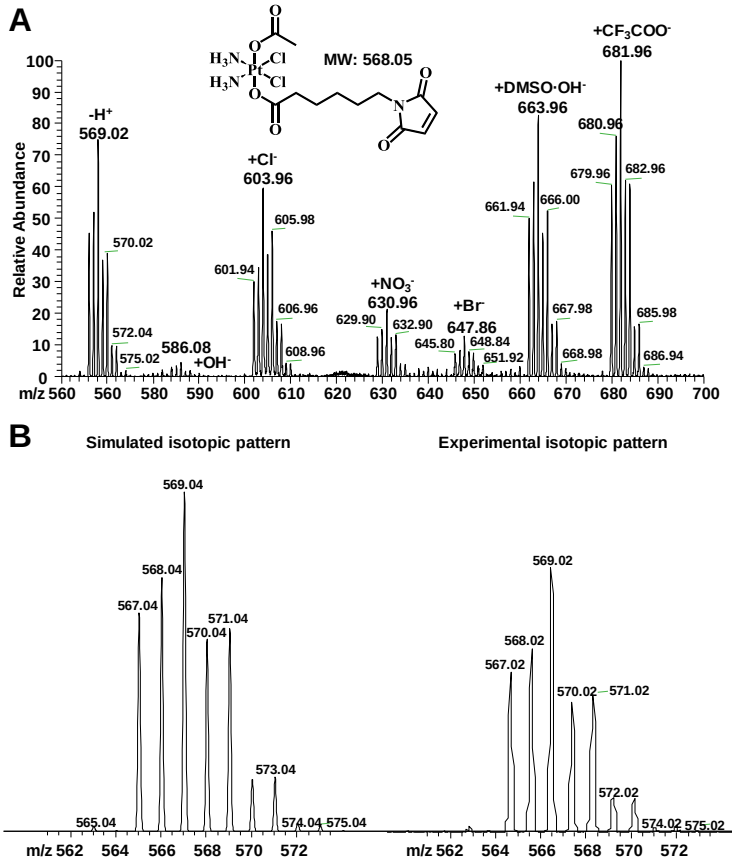

Figure S8 Characterization of Mal-Pt(IV) by ESI-MS.

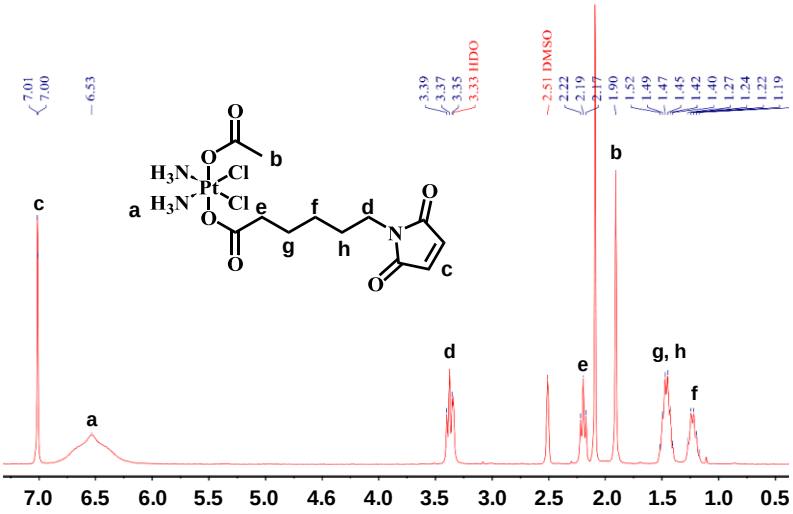

Figure S9 The characterization of Mal-Pt(IV) by ^{1}HNMR in d_6-DMSO. ^{1}H NMR (d_6-DMSO): δ (ppm) = 7.02 (s, 2H), 7.03-6.6.7 (m, 6H), 3.44 (t,J=7.3Hz, 2H), 2.22 (t, J=7.3Hz,2H), 1.90 (s, 3H),1.57-1.46 (m, 4H), 1.31-1.23 (m, 2H).

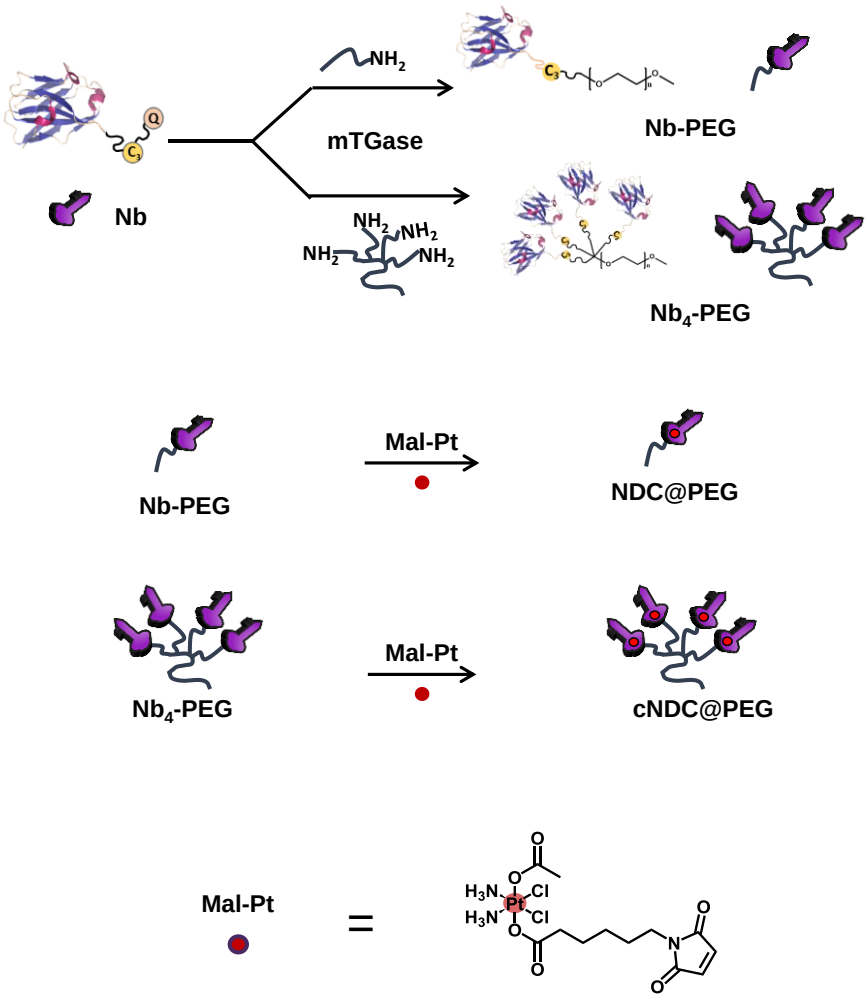

Figure S10 Schematic illustration of the preparation of the NDCs.

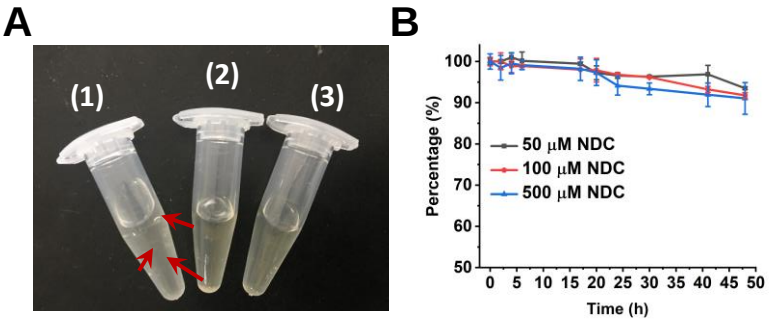

Figure S11 Stability of NDCs. A) Picture of NDC (1), NDC@PEG (2), and cNDC@PEG (3), showing that the protein aggregation occurred on NDC in a high concentration of 1.0 mg Pt/mL (equivalent to 5 mM Pt), while NDC@PEG and cNDC@PEG are still stable at this concentration. B) Stability of NDC in different concentration. The samples were incubated at 25 °C for different time. The protein in solution was measured after centrifugation at 4 °C (15000 rpm × 15 min), and the protein concentration was measured by OD_{280}. The color of curves indicates the concentration of NDC used in the measurement: 50 μM (black); 100 μM (red); 500 μM (blue).

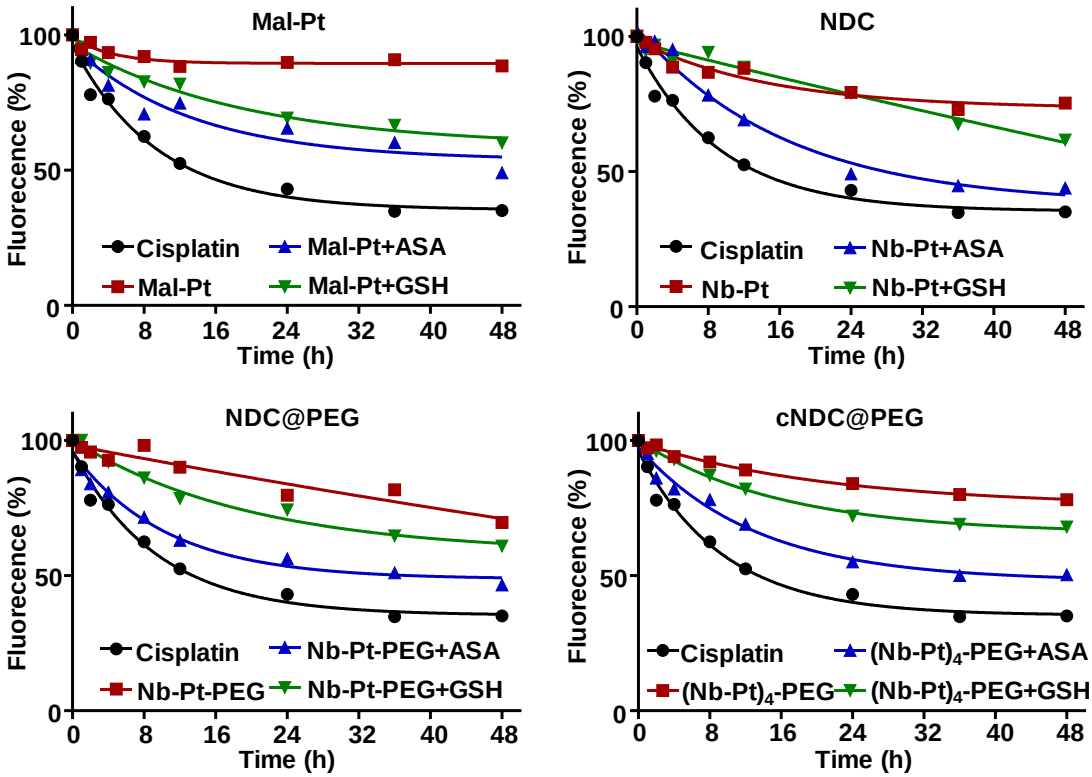

Figure S12 Fluorescence measurement of the reaction between DNA and platinum drugs. The reaction was taking use of 10 mM ascorbic acid (AsA) or 10 mM glutathione (GSH) as the reducing agent. The reaction was carried out at 37 °C in 10 mM phosphate buffer (pH 7.4) containing 10 mM $NaClO_4$ in the dark. The fluorescence spectra were recorded with EtBr as a probe at an excitation wavelength of 530 nm and an emission wavelength of 615 nm.

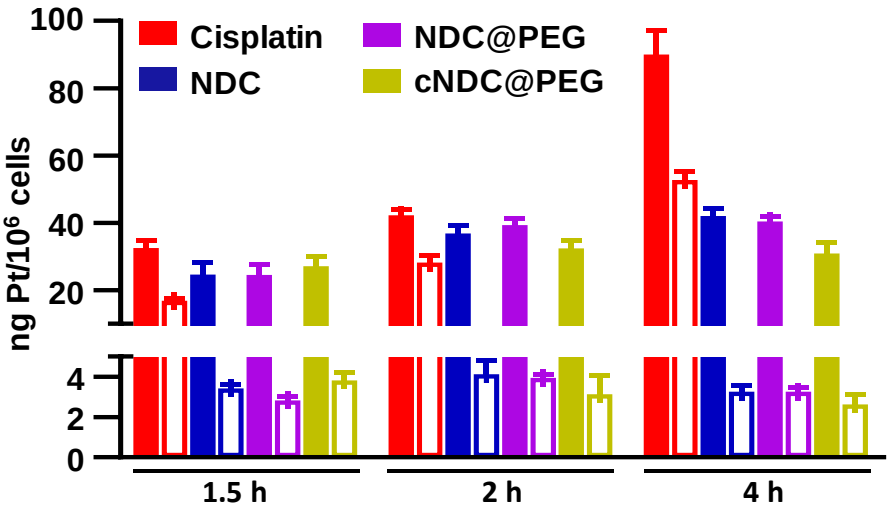

Figure S13 Drug accumulation of cisplatin and NDCs on EGFR overexpressed A431 cells (■ solid) and EGFR negative A2780 cells (□ hollow).

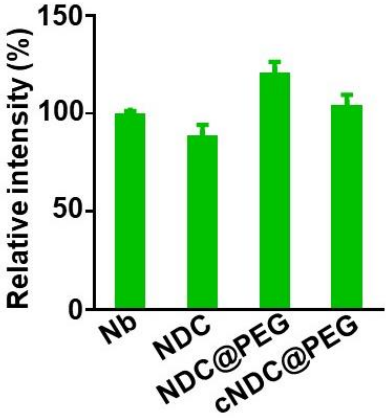

Figure S14 The relative intensity of the relative fluorescence of multicellular spheroids.

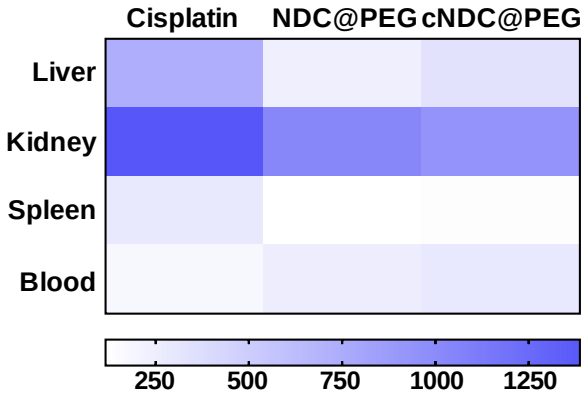

Figure S15 Platinum content of main organs after administered with platinum drugs. The mice were sacrificed at 12 h after a single injection.

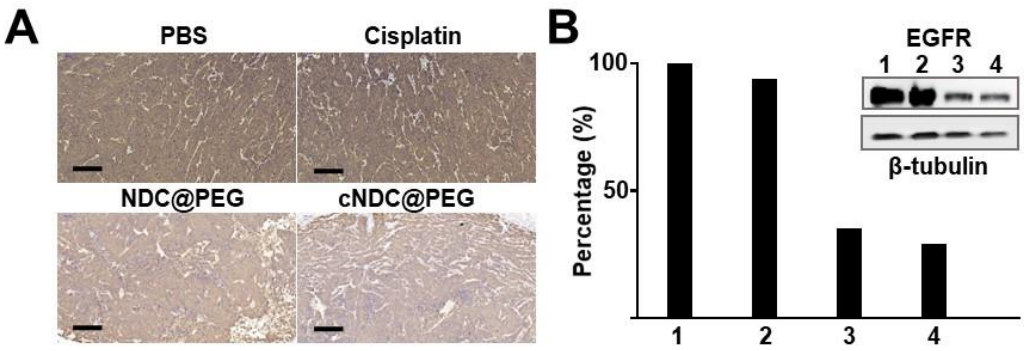

Figure S16 The EGFR level of the tumor after the treatment. A) Immunohistochemical analysis of EGFR of A431(++) tumor (Scale bar: 200 μm). Positive cells were stained brown. B) Quantification of EGFR of the tumor by western blot at the end of treatment. The tumor samples were taken from mice after treatment with PBS (1), cisplatin (2), NDC@PEG (3), or cNDC@PEG (4).

Table S1. Binding affinity and Kinetic dissociation rate constants of nanobody conjugates

Group	K_d (nM)	K_{on} (μM^{-1} min^{-1})	K_{off} (s^{-1}) $\times 10^{-3}$
Nb	23.1	0.31	7.16
NDC	24.2	0.36	8.71
NDC@PEG	22.9	0.40	9.16
cDNC@PEG	3.4	0.28	0.95