Supporting Information

GALA Peptide Improves Potency of Nanobody-drug Conjugate by Lipid-induced Helix Formation

Ya Jie Chen,[a,#] Qi Wen Deng, [a,#] Wang Li,[a] Xiao Chun Guo,[a] Jian Yuan Yang,[a] Ting Li,[a] Zhengshuang Xu,[a] Hon Cheung Lee,[a] and Yong Juan Zhao [a,b,*]

a State Key Laboratory of Chemical Oncogenomics, Key Laboratory of Chemical Genomics
Peking University Shenzhen Graduate School
Shenzhen University Town, Lishui Road, Shenzhen 518055, China
b Ciechanover Institute of Precision and Regenerative Medicine, School of Life and Health Sciences, The Chinese University of Hong Kong, Shenzhen, China, 518172
* E-mail: zhaoyongjuan@cuhk.edu.cn

Table of Content

1. Experimental section……..……....S2-6
2. Preparation and characterization of NDCs (Figure S1)……..……..…S7
3. The releasing curve of MMAE from the CD38-NDC (Figure S2)……..S8
4. The cytotoxicity of 1053-GALA-MMAE correlates with CD38 expression (Figure S3)……..……..……S9
5. Cytotoxicity of vcMMAE-based NDCs against EGFR$^+$-cells (Figure S4) ……..S10
6. GALA-enhanced internalization can be blocked by the inhibitor of CME (Figure S5) ……..……S11
7. References……....S11

1. Experimental section

Materials. MMAE, mc-Val-Cit-PAB-PNP linker and Mouse anti-MMAE were purchased from Levena Biopharma Co., Ltd. Peptides NH_2-GGGDTDTC-NH_2, NH_2-GGG$(DTDTC)_2$-NH_2 and GALA peptide were synthesized by Sangon Biotech. Mini-Extruder, 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (POPG) and 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) were purchased from Avanti Polar Lipids, Inc. 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[carboxy(polyethylene glycol)-2000, NHS ester] (DSPE-PEG_{2000}-NHS) were purchased from Ponsure Biological. The plasmid pRHSUL2 was a gift from associate professor Dr. Hongmin Zhang (the Department of Biology, Southern University of Science and Technology). The plasmid of pentamutant sortase A, with an improved Kcat, was a gift from Hidde Ploegh (Addgene plasmid # 51140).

Cell Lines. The HEK293T, A431 and HeLa cells were purchased from the American Type Culture Collection. The myeloma cell line LP-1 was kindly provided by Annie An, Peking University, Beijing. To construct cell lines stably expressing protein-of-interest, cDNA fragment was subcloned from another plasmid or amplified by PCR, inserted into corresponding lentivector. The lentiviral particles were prepared by transfecting HEK293T cells with the corresponding lentivector, pMD2.G and psPAX2 with Lipofectamine 2000 (Thermo Fisher Scientific) followed by cell infection and selection. The CD38 knockout LP-1 cell line (CD38-KO/LP-1) was constructed using CRISPR-Cas9 system, described in the previous report.[1] The cells were cultured in DMEM (HEK293T, $CD38^+$/HEK293T, $EGFR^+$/HEK293T, A431 and HeLa) or IMDM (LP-1, CD38-KO/LP-1) supplemented with 10% fetal calf serum and 1% penicillin-streptomycin solution. All the cells were maintained in a standard humidified tissue culture incubator with 5% CO_2.

Recombinant Protein Expression and Purification. Gene fragments were synthesized and inserted into plasmid pRHSUL2[2]. Plasmids were transformed into Rosetta (DE3) cells and cultured at 37°C in LB medium. Protein expression was induced at the cell density of OD_{600}=0.6~0.8 with 0.25 mM IPTG at 16°C for 16~18 hours. The cell pellets were harvested by centrifugation at 5,000 g for 30 min and lysed by sonication in binding buffer (50 mM Tris-HCl pH 8.0, 400 mM NaCl). The cell debris was removed by centrifugation at 50,000 g at 4°C for 1 h and the clarified supernatant was loaded onto a Ni-NTA Agarose (Qiagen) column. The column was sufficiently washed with binding buffer supplemented with 20 mM imidazole, and the protein was eluted with binding buffer supplemented with 300 mM imidazole. The SUMO tag was excised by adding SUMO protease to the eluted protein fraction during dialysis in the binding buffer. And then the protein was re-purified by using the Ni-NTA Agarose

column to remove the cleaved SUMO tag. Finally, the purified protein was concentrated to 4-6 mg/mL (measured by Bradford method) in 50 mM Tris-HCl pH 8.0, 400 mM NaCl and stored at -80°C. Expression and production of pentamutant sortase A were as described before[3].

The Preparation of DyLight 650 NHS Ester Labeled Recombinant Proteins. DyLight 650 NHS Ester (Thermo Fisher Scientific) was diluted in anhydrous DMF to obtain 10 mM stock solution. Before labeling, the buffer of the protein samples was changed to cold 0.2 M $NaHCO_3$, pH 8.2 by using the Amicon Ultra 10K centrifugal filter (Merck Millipore). Then 10 molar equivalents of DyLight 650 NHS Ester were added to the 100 μM protein solution and the reaction was carried out at room temperature for 2 h in the dark. Excess unreacted dye was removed via buffer exchange into cold PBS by using the Amicon Ultra 10K centrifugal filter. 50% (v/v) glycerol (Sigma-Aldrich) was added before storage at -20°C.

Flow Cytometric Analysis. The binding of Nb-1053 or 1053-GALA to the cell surface CD38 was measured by flow cytometry. 5×10^4 cells were incubated with DyLight 650 NHS Ester labeled protein (20 nM) in 200 μL ice-cold PBSB (PBS with 0.1% BSA) for 45 min with rocking at 4°C. After centrifugation at 800 rpm for 5 min at 4°C, the cell pellet was washed once with 1 mL cold PBSB, then applied on CytoFLEX (Beckman Coulter). Data were analyzed by the software FlowJo.

The Synthesis of GGGDTDTC-MC-vc-PAB-MMAE. MC-vc-PAB-MMAE was synthesized as follows: the activated linker mc-Val-Cit-PAB-PNP (57 mg, 77 μmol, 1.1 equivalents), MMAE (50 mg, 70 μmol, 1.0 equivalents), HOBt (1.9 mg, 14 μmol, 0.2 equivalents) and DIPEA (25ul) were dissolved in 2 mL anhydrous DMF. The reactants were stirred at room temperature. The reaction was monitored with high performance liquid chromatography (HPLC). The completion of the reaction was confirmed by the disappearance of original MMAE. The product was purified using preparative HPLC equipped with a C18 column and then lyophilized, providing 57 mg (62%) of amorphous white powder that was >95% pure by HPLC. +ToF MS m/z 1316.7858 $[M+H]^+$.

Peptide NH_2-GGGDTDTC-NH_2 (for 1053-vcMMAE, 1053-GALA-vcMMAE, 1053-mutGALA-vcMMAE, GFPnb-GALA-vcMMAE, 7D12-vcMMAE, 7D12-GALA-vcMMAE, 7D12-mutGALA-vcMMAE) or NH_2-GGG-DTDTC-DTDTC-NH_2 (for 1053-$(vcMMAE)_2$) (5.0 equivalents) and MC-VC-PAB-MMAE (1.0 equivalents) were dissolved in DMF. The contents were stirred at room temperature and monitored with HPLC. The completion of the reaction was confirmed by the disappearance of original MC-VC-PAB-MMAE. The product was purified and lyophilized as described above.

The Site-specific Modification of Recombinant Protein via Sortase A Mediated Transpeptidation Strategy (Sortagging). The reaction mixture contained Tris-HCl (50 mM, pH 8.0), $CaCl_2$ (10 mM), NaCl (400 mM), triglycine-containing toxin (40 eq.), LPETG-containing recombinant protein (150 μM), and pentamutant sortase A (2 μM). After incubation at 4°C with agitation for 4 h, Ni-NTA beads were added to the reaction mixture with gentle shaking for 30 min at 4°C, followed by centrifugation to remove sortase and any remaining unreacted His-tagged substrate. The unconjugated toxin was removed by ultrafiltration using PBS. The labeled proteins were stored at -80°C.

The Characterization of Various Sortase A-Generated NDCs by ESI-ToF MS. Before mass spectrometry characterization, the buffer of in samples was replaced by Milli-Q water supplemented with 0.1% formic acid and 50% acetonitrile by ultrafiltration. The resulting protein solution was concentrated to 1-2 mg/mL, and analyzed on a QStar Elite (Applied Biosystems), which is an electrospray ionization Time-of-Flight mass spectrometry (ESI-ToF MS).

Cell Viability Assays. 4,000 cells were seeded in 96-well plate containing various concentrations of drugs, and incubated for 72 hours at 37°C. Cell viability was determined by resazurin (Sigma-Aldrich), a nonfluorescent substrate that can be reduced to a strongly-fluorescent compound, resorufin by metabolically active cells.[4] In brief, the cells were incubated with 50 μM resazurin for 4 hours, and the fluorescent signals were measured at 530 nm excitation/600 nm emission using an Infinite plate-reader M200 PRO (Tecan). For receptor blocking assay, 10μg/mL Nb-1053 was incubated with cells for 1h before adding drugs. The results were expressed as mean ± SD. Percentage of cell viability was calculated relative to the controls (untreated cells).

Live-cell Imaging. Glass-bottom cell culture dishes (NEST) were pretreated by 0.01% Poly-L-Lysine (Sigma–Aldrich). 4×10^5 cells were seeded in each dish and allowed to grow overnight.

To visualize the spatial and temporal distribution of fluorescently labeled constructs, the $CD38^+$/HEK293T cells were transferred to 4°C for 5 min before treatment. The culture medium was replaced with the cold medium containing 20 nM 1053-DL650, 1053-GALA-DL650 or 1053-mutGALA-DL650. After incubation at 4°C for 45 min, the medium was replaced with fresh medium and the cells were transferred to 37°C to initiate internalization. At different time points (0, 2, 12, 24 h) 1× Hoechst 33342 (MCE) was added to stain cell nuclei and removed 5 min later. Cells were washing twice with Hank's Balanced salt solution (HBSS, Thermo Fisher Scientific), fresh medium was

added, and fluorescence images were acquired using the A1R Confocal Laser microscope (Nikon) with 60× oil lens. To investigate the internalization mechanism of 1053-GALA-DL650, the $CD38^+$/HEK293T cells were pre-incubated with or without a CME inhibitor, PAO(1 μg/mL) for 2 hours at 37°C before treatment of the labeled proteins.

To investigate the subcellular trafficking of 1053-GALA, the $CD38^+$/HEK293T cells were treated with 20 nM 1053-GALA-DL650 for 45 min and incubated in fresh dye-free medium for 8 h at 37°C. After incubation with 50 nM LysoTracker Green DND-26 (Thermo Fisher Scientific) for 1 h at 37°C to stain lysosomal compartments and 1× Hoechst 33342 to stain cell nuclei for 5 min at room temperature, cells were washing twice with HBSS, fresh medium was added, and fluorescence images were acquired using the A1R Confocal Laser microscope with 100× oil lens.

The Preparation of Large Unilamellar Vesicles (LUVs). LUVs were prepared by dissolving POPG or POPC, with or without DSPE-PEG_{2000}-NHS (10 mol% relative to POPG or POPC) in chloroform in a round bottom flask to obtain a homogeneous solution, the solvent was removed by evaporation under high vacuum for 3 h. The resulting dried lipid film was hydrated with 10 mM pH 7.0 potassium phosphate buffer to 10 mM final lipid concentration, and vortexed in the multitube vortex at room temperature for 30 min. Finally, an Avanti Mini-Extruder was used to push the lipid solution 23 times through two polycarbonate filters (100 nm pore size). Stored the LUVs solution at 4°C until use.

CD Spectroscopy. 30 μM of GALA peptide and about 2 mM LUVs were incubated for 1 h, and applied to CD measurements, which were performed in a 1 mm cuvette using a Chirascan circular dichroism spectrometer (Applied Photophysics Ltd.) at 25°C. Data were collected between 190-260 nm at 0.5 nm intervals with bandwidths of 1 nm using 0.5 s time-per-point. Spectra are an average of 5 scans, buffer subtracted, and smoothed.

Quantification of The Free MMAE Released from the NDC by LC-MS. To investigate the releasing kinetics of MMAE from NDC by the lysosomal protease, 5μM of the 1053-MMAE was incubated for different time periods at 37℃ with 100 nM Cathepsin B in the buffer of 10mM MES and 6mM NaCl buffer(pH 6.0). The reaction was quenched with an equal volume of 2% formic acid. Samples were loaded onto a Hypersil Gold™ VANQUISH C18 column (1.9μm, 100×2.1mm, Thermo Scientific) equipped in a Q Executive Focus LC-MS system (Thermo Scientific). The system ran at 0.28 mL/min using 0.1% trifluoroacetic acid (TFA) in water as mobile phase A (MPA) and 0.1% TFA in acetonitrile as mobile phase B (MPB). MMAE (mw: 717.5115) was

eluted with a 16-minute method consisting of equilibration for 1 min at 10% MPB, a 9 min-linear gradient from 10% to 95% MPB, 3 min-holding in 95% MPB, and a 4 min-re-equilibration at 10% MPB.

2. Preparation and characterization of NDCs

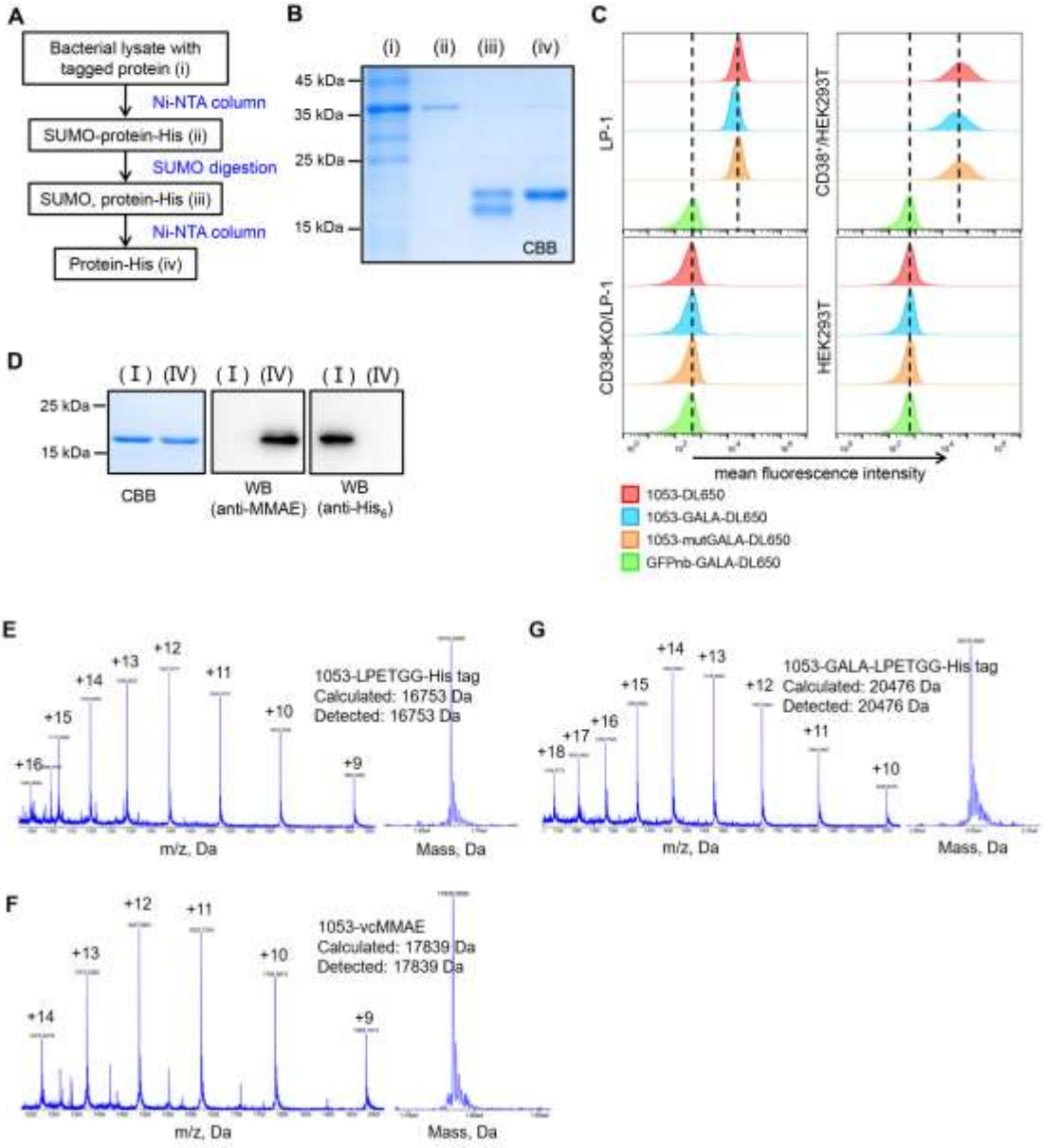

Figure S1. Preparation and characterization of NDCs. (**A**) Flowchart of protein purification and (**B**) representative SDS-PAGE analysis of recombinant proteins, with 1053-GALA as the example, in each step (right). CBB, Coomassie brilliant blue staining. (**C**) Flow cytometry profile of LP-1 cells, $CD38^{+}$/HEK293T cells and their $CD38^{-}$ counterparts, CD38 knockout LP-1 and HEK293T cells. The cells were incubated with 20 nM DyLight 650 NHS Ester labeled recombinant proteins for 45 min at 4°C before flow cytometry. (**D**) SDS-PAGE and Western blots of GALA-1053-vcMMAE before (Ⅰ, Fig. 1B) and after (Ⅳ, Fig. 1B) conjugation. (**E-G**) ESI-ToF MS characterization of 1053-LPETGG-His tag (**E**), 1053-vcMMAE (**F**) and 1053-GALA-LPETGG-His tag (**G**). The left graphs show the total mass spectra, the right graphs show the deconvoluted spectra.

3. MMAE releasing from NDC

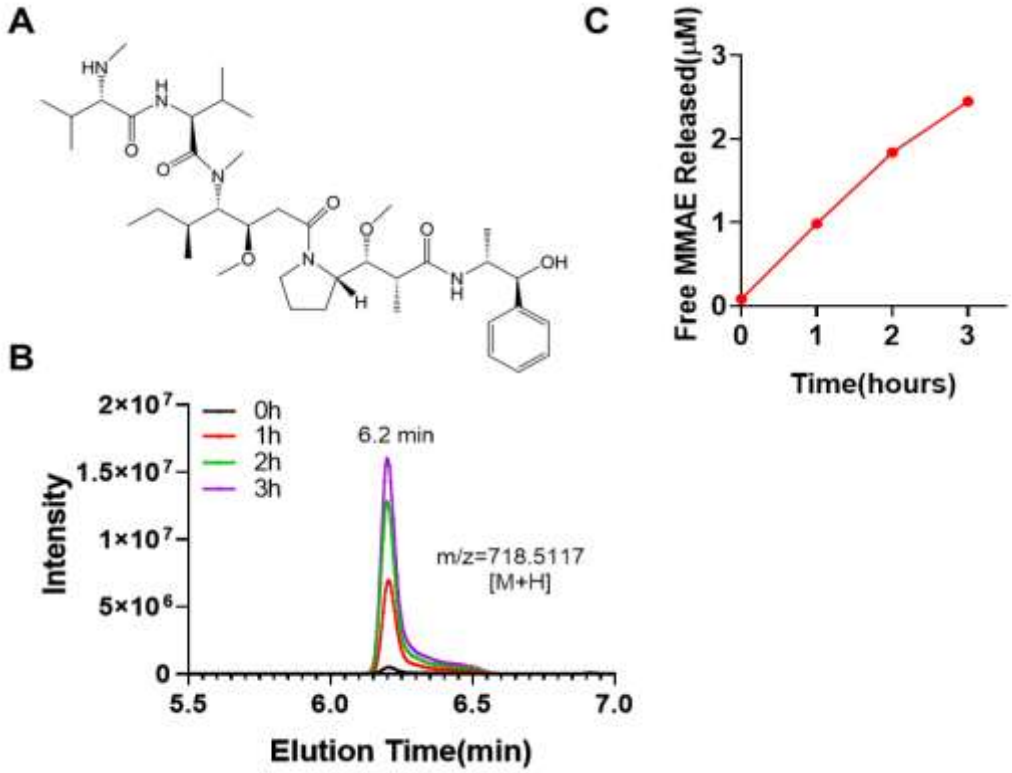

Figure S2. The releasing curve of MMAE from the CD38-NDC. (**A**) Structure of the free MMAE. (**B-C**) Detection (B) and Quantification (C) of free MMAE released from 1053-MMAE upon Cathepsin B proteolysis measured via LC-MS. The experimental details are in the Methods section.

4. The cytotoxicity of 1053-GALA-MMAE correlates with CD38 expression.

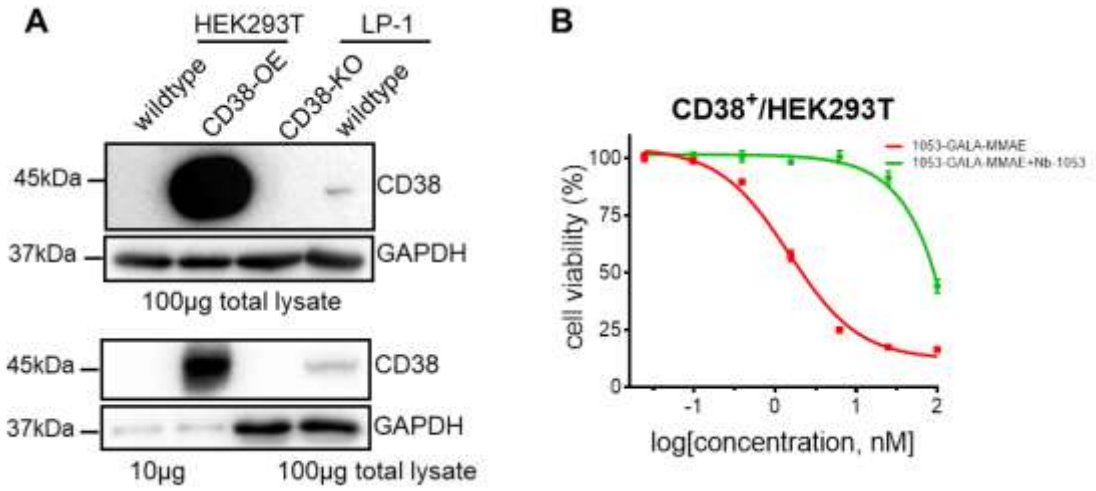

Figure S3. The cytotoxicity of 1053-GALA-MMAE correlates with CD38 expression. (**A**) The expression levels of CD38 were measured by Western Blots in wildtype and CD38-overexpression HEK293T cells; wildtype and CD38-knockout LP-1 cells. $CD38^+$/HEK293T cells express at least 100-fold more CD38 than LP-1 cells. (**B**) The cytotoxicity of 1053-GALA-MMAE was blocked by free Nb-1053. Cell viability of $CD38^+$/HEK293T was quantified by the resazurin assay after incubating with or without 10 μg/mL Nb-1053 and different concentrations of 1053-GALA-MMAE for 72 h at 37°C.

5. Cytotoxicity of vcMMAE-based NDCs against EGFR$^+$-cells.

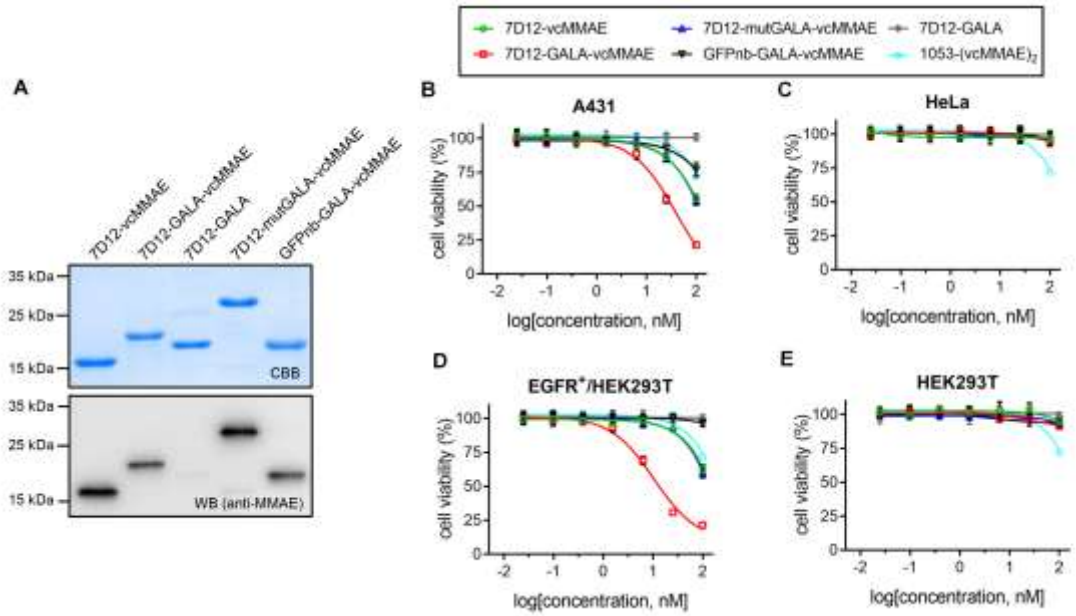

Figure S4. Cytotoxicity of vcMMAE-based NDCs against EGFR$^+$-cells. (**A**) SDS-PAGE analysis of all the NDCs or precursors used in this experiment. Cell viability of A431 (EGFR$^+$) (**B**), HeLa (EGFR$^-$) (**C**), EGFR$^+$/HEK293T (**D**) and HEK293T (**E**) cells were measured by resazurin assay after incubating with different concentrations of NDCs for 72 h at 37°C. The experiments were performed three times and data are presented as mean with SD.

6. GALA-enhanced internalization can be blocked by the inhibitor of clathrin-mediated endocytosis (CME).

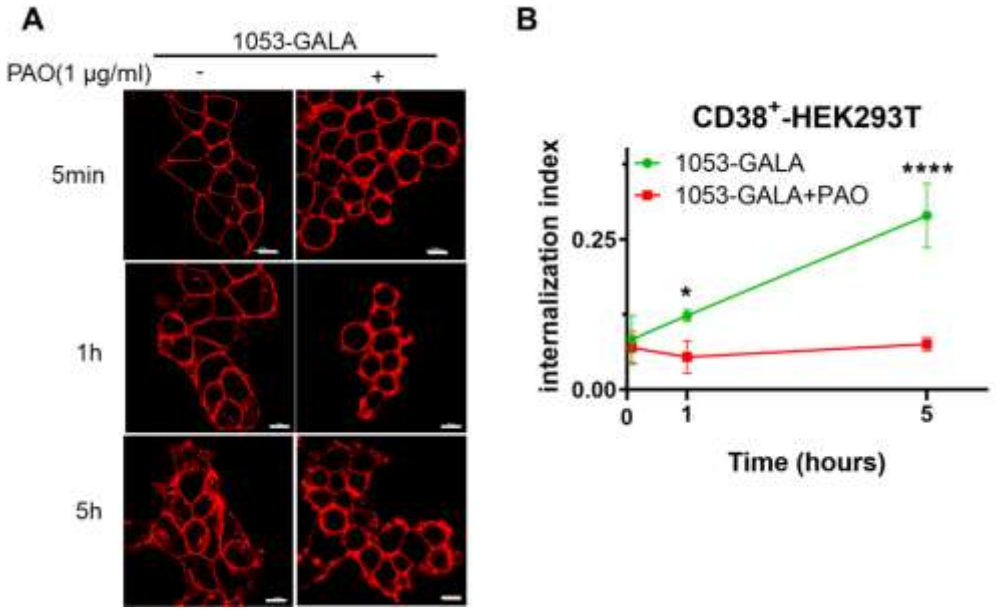

Figure S5. GALA-enhanced internalization can be blocked by the inhibitor of CME. (**A**) CD38$^+$/HEK293T cells were treated or without treated with 1 μg/mL PAO, an inhibitor for CME, for 2 hours and then incubated with 20 nM DyLight 650 NHS Ester labelled 1053-GALA at 4°C allowing binding. After removing the unbound proteins, the cells were transferred to 37°C to initiate internalization and imaged after different periods of time. Confocal images were acquired using the A1R Confocal Laser microscope (Nikon) with a 100× oil lens. Scale bar=10 μm. (**B**) The "internalization index" of fluorescence (around 50 cells) in (A) was calculated and statistically analyzed by Sidak's multiple comparisons test with GraphPad Prism 8.0.

7. References

1. L. Cong and F. Zhang, *Methods Mol Biol*, 2015, **1239**, 197-217.
2. G. Ma, Y. Zhu, Z. Yu, A. Ahmad and H. Zhang, *Sci Rep*, 2016, **6**, 39540.
3. C. P. Guimaraes, M. D. Witte, C. S. Theile, G. Bozkurt, L. Kundrat, A. E. M. Blom and H. L. Ploegh, *Nat Protoc*, 2013, **8**, 1787-1799.
4. S. N. Rampersad, *Sensors-Basel*, 2012, **12**, 12347-12360.