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Tribody: Robust Self-assembled Trimeric Targeting Ligands with High Stability and Significantly Improved Target-binding Strength

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Abstract

The C-terminal coiled-coil region of mouse and human cartilage matrix protein (CMP) self-assembles into a parallel trimeric complex. Here, we report a general strategy for the development of highly stable trimeric targeting ligands (tribody), against epidermal growth factor receptor (EGFR) and prostate-membrane specific antigen (PSMA) as examples, by fusing a specific target-binding moiety with a trimerization domain derived from CMP. The resulting fusion proteins can efficiently self-assemble into a well-defined parallel homotrimer with high stability. Surface plasmon resonance (SPR) analysis of the trimeric targeting ligands demonstrated significantly enhanced target binding strength compared with the corresponding monomers. Cellular binding studies confirmed that the trimeric targeting ligands have superior binding strength towards their respective receptors. Significantly, EGFR-binding tribody was considerably accumulated in tumor in xenograft mice bearing EGFR positive tumors, indicating its effective cancer targeting feature under *in vivo* conditions. Our results demonstrate that CMP-based self-assembly of tribody can be a general strategy for the facile and robust generation of trivalent targeting ligands for a wide variety of *in vitro* and *in vivo* applications.

Keywords

Self-assembly; Targeting ligand; Trimerization; Trivalent; High stability; High affinity; EGFR; PSMA

Introduction

Targeting ligands play a pivotal role in all the *in vitro* and *in vivo* targeting applications.¹ Depending on the nature of the application and the molecules to be targeted, some basic properties of the target ligand, including molecular weight, surface charge, target-binding specificity, affinity, and valency, should be optimized. It is now clear that one of the most

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critical parameters for satisfactory *in vivo* targeting is the valency of the targeting ligand. Most monovalent targeting ligands, even those with very high binding affinities, have fast dissociation rates and provide only modest retention time on the target antigen under nonequilibrium physiological conditions.¹ Impressively, nature addresses this problem by extensively using multivalent interactions, as observed in almost all types of antibodies and numerous multimeric interactive proteins. Currently, there is an unmet need for a technology platform that allows for facile and robust development of desired panels of multivalent targeting ligands that possess significantly increased target-binding strength, decreased dissociation from target and thus longer and more accumulation on diseased tissues.

Monoclonal antibody is the major class of targeting ligands that has been widely used in many biomedical fields.² More than 20 monoclonal antibodies are being clinically used as therapeutic agents and many more are under preclinical development.³ However, immunoglobulin scaffold-based antibodies have intrinsic limitations, including large size (~150 kDa), the presence of disulfide bonds, complex tetrameric structure and high cost of production, that complicate their many applications.³ Substantial efforts have been made to develop targeting ligands that can be quickly tuned to mimic antibodies with multivalent features.^{2–5} The protein domains capable of forming multimeric complex have been extensively investigated to generate recombinant proteins to achieve avidity effect through multivalency.^{1,6} To develop a robust system that allows for facile generation of targeting ligands with multivalent features, the multimerization domains should be of small sizes and possess favorable biophysical properties, including thermal stability, resistance to protease, and cost effectiveness in its production, while still able to generate highly stable multimeric complex that can display multiple target-binding moieties in parallel. Different scaffolds that allow for enhanced avidity have been reported.^{1,2,5–7} These scaffolds include the bacteriophage T4 foldon domain, collagen like peptide (Gly-Pro-Pro)₁₀, NC1 domain of collagen XV and XVIII domain, and GCN leucine zipper domain for trimers,^{1,2,8,9,10} streptavidin and transcription factor p53 for tetramers,¹¹ the B-subunit of bacterial verotoxin and cartilage oligomeric matrix protein (COMP) for pentamers,^{12,13} and recently the hyperthermophilic Sm protein for heptamers.⁶ However, most of these scaffolds are derived from non-human proteins and have limited clinical application due to immunogenicity. Ideally, the multimerization domain should be a highly conserved extracellular protein that is abundant in mouse and human proteomes, which could result in less immunogenicity and allow for smooth transition from animal studies to translational and clinical investigations. While protein domains forming trimeric structures are widely used in nature, few possess desired features that allow for effective development of trivalent ligands that are clinically amenable. To address this unmet need, we chose the C-terminal domain from mouse CMP-1 (matrilin), which is highly homologous to human CMP-1, for the development of self-assembly trivalent targeting ligands based on its remarkable property of forming stable trimeric structures.^{14–16} As a major component of various cartilages, CMP-1, a 148 kDa extracellular matrix glycoprotein serves as an adaptor in the assembly of the extracellular matrix structure. The oligomerization characteristic of CMP or matrilin has been extensively studied.^{14–16} There are four members of matrilins, namely, matrilin-1, -2, -3 and -4, whose domain structures are highly conserved among species from chicken to human. It has been reported that the matrilin-1 and -4 form trimeric, whereas matrilin-2 and -3 form tetrameric complexes.¹⁶ The last 40 amino acids at the C-terminus of CMP-1 play an important role in the formation of the trimeric structure.^{14,16} This C-terminal coiled-coil domain has a heptad repeat on which hydrophobic residues reside in the first and the fourth positions, while polar residues are common in the fifth and the last positions.¹⁴ The strong hydrophobic and ionic interactions within this α -helical coiled-coil result in a parallel, disulfide-linked, and three-stranded domain. The resulting rod-shaped trimeric structure, with a length of about 5.1 nm and a diameter of 3 nm, has a high degree of co-operativity and high stability, with a T_m higher than 60 °C at neutral pH, which can be attributed to the hydrophobic packing and a

salt bridge between R27 and E32 residues.¹⁴ It appears that the trimeric structure was still well maintained when the disulfide bonds that flank the N-terminus of the coiled-coil were abolished.¹⁴

Overexpression of epidermal growth factor receptor (EGFR) and prostate-membrane specific antigen (PSMA) receptor is frequently associated with various cancers including lung, breast, prostate, and glioblastoma.^{3,17} Selective targeting towards EGFR or PSMA holds the promise of early diagnosis and improved treatment.^{18,19} In this report, we describe the use of the trimerization domain derived from the cartilage matrix protein that is abundant and highly homologous in mouse and human to develop trivalent targeting molecules (tribodies) with high stability and significantly enhanced avidity against EGFR and PSMA. The resulting tribodies are extensively characterized and used for *in vivo* tumor targeting in xenograft mouse models.

Materials and Methods

Plasmid Construction of Tribodies

Mouse cDNA library was used for the amplification of the gene encoding the mouse CMP-1 trimerization domain. Sequences coding EGFR-binding Z domain (Z1907) or PSMA binding homing peptide were constructed as we reported previously.⁶ Each PCR fragment was ligated into pET28b between NcoI and XhoI sites followed by sequencing to ensure that each affinity molecule was generated correctly.

Expression and Purification of Tribodies

The plasmid containing a gene of interest was transformed into *E. coli* BL21 (DE3) Rosetta cells and the positive clones were selected on LB plates containing kanamycin (50 µg/mL) and chloramphenicol (34 µg/mL). A single colony was picked and grown in 5 mL of LB overnight at 37 °C. The overnight grown cell culture was added to 500 mL of LB containing kanamycin and chloramphenicol. The culture was grown at 37 °C till the OD600 reached between 0.5 and 1.0, and 1 mM of IPTG was added to induce protein expression. After induction at 22 °C for 16 h, the cells were spun down at 3000× g for 10 min at 4 °C, and the pellet was stored at -20 °C prior to purification. To purify each monomeric and trimeric molecules, each cell pellet was resuspended in buffer A (25 mM HEPES pH 7.4 and 50 mM NaCl) and sonicated for 1 min for a total of 3 to 4 times. After cell lysis, the soluble fraction was recovered by centrifugation at 12,000× g for 10 min at 4 °C. The resulting soluble fraction was loaded into a TALON metal affinity column (Clontech, Mountainview, CA) pre-equilibrated with buffer B (25 mM HEPES pH 7.4 and 300 mM NaCl). The column was washed with 20 column volumes of buffer B, followed by extensive washing with buffer C (buffer B and 20 mM imidazole). The targeting molecules were eluted with buffer D (buffer B and 200 mM imidazole). The quality of purified proteins was examined by SDS-PAGE.

Native Gel Electrophoresis

An 8% discontinuous native gel was prepared in the absence of SDS and reducing agent based on the standard Laemmli SDS-PAGE protocol. About 5 µg of highly purified monomer or tribody was loaded for separation. Proteins were stained with Coomassie brilliant blue R-250.

Circular Dichroism Spectroscopy

Highly purified monomeric and trimeric proteins were prepared in 10 mM phosphate buffer (pH 7.5) and used for circular dichroism (CD) scanning with an AVIV model 202-01 Spectropolarimeter at the UNC Macromolecular interaction facility. To determine thermal

stability, the CD spectra at 220 nm were measured from 25 °C to 94 °C, and the ramp was cool down to 25 °C followed by measuring the CD spectra at 220 nm again from 25 °C to 94 °C. The melting temperature (T_m) was calculated as a temperature which has the midpoint CD spectra between the lowest and the highest CD spectra with reversible melting curve.

Protease Resistance

Thermolysin, papain, and MMP-9 were used for the protease resistance assays. Thermolysin and papain were purchased from Sigma-Aldrich, MO. MMP-9 was purchased from EMD Millipore (Billerica, MA). Approximately 2 µg of monomeric or trimeric targeting ligands were incubated with thermolysin (10 ng of thermolysin per µg of protein). The protease digestion was performed in an HBS buffer (10 mM HEPES, pH 7.4 and 150 mM NaCl) for 30 min at 37 °C. The reaction mixtures were applied to SDS-PAGE to examine protein degradation. Approximately 2 µg of monomeric or trimeric targeting ligands were incubated with 20 ng of papain for 30 min at 37 °C. The reaction was performed in a papain digestion buffer (50 mM phosphate buffer pH 7.0, 0.5 mM EDTA). Papain was activated by 5 mM of L-cysteine, followed by removing L-cysteine using a MWCO 5 kDa spin column through centrifugation. The reaction mixtures were applied to SDS-PAGE to examine protein degradation. Approximately 2 µg of monomeric or trimeric targeting ligands were incubated with 100 ng of MMP-9 for 4 h at 37 °C. The reaction was performed in a MMP-9 digestion buffer (50 mM Hepes pH 7.5, 10 mM CaCl₂, 0.02% BRIJ-35). The reaction mixtures were applied to SDS-PAGE to examine protein degradation.

Target-binding Analysis Using Biacore

BIAcore 2000 (BIAcore AB, Uppsala, Sweden) was used for surface plasmon resonance analysis. The extracellular domain of recombinant human EGFR or PSMA (R&D Systems, Minneapolis, MN) was diluted in 10 mM sodium acetate pH 5.0 and immobilized on CM5 sensor chip (GE healthcare, Piscataway, NJ) to achieve ~2,500 resonance units by amine coupling according to the manufacturer's instruction. Various concentrations of monomeric and trimeric ligands were injected into the flow cell in a HBS-P buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, and 0.005% surfactant P20) at a flow rate of 20 µl/min. The association and dissociation constants were calculated using BIA evaluation software by fitting data on one-to-one Langmuir binding model.

Cell Culture

All cell lines including EGFR positive A431, EGFR negative Jurkat, PSMA positive LNCaP and PSMA negative PC3 cancer cells were obtained from the Tissue Culture Facility at UNC-Chapel Hill. All cell lines were maintained by serial passage in the appropriate media containing 10% fetal bovine serum in a 5% CO₂ incubator at 37 °C.

Confocal Microscopy

Each cell type (about 2×10⁴) was seeded onto coverslips and allowed to grow in an appropriate media for 16 h. The resulting coverslips were washed twice with 1× PBS, followed by incubation with different concentrations of FITC-labeled monomeric and trimeric ligands for 30 min at room temperature. After incubation, the coverslips were washed three times with 1× PBS. The resulting samples were examined with a Zeiss LSM 510 confocal microscope at the UNC microscopy core facility.

Flow Cytometry

Cell binding of the monomeric and trimeric ligands was evaluated by using flow cytometry. Approximately 2×10⁴ cells were grown on a 24-well plate for 16 h. After washing with 1× PBS, the cells were incubated with an appropriate amount of FITC-labeled monomeric or

trimeric ligand for 30 min at room temperature, followed by washing twice with 1× PBS. The resulting samples were analyzed by flow cytometer (BD FACS Canto flow cytometry) and the data analyzed by the Flow Jo System (Tree star, Inc. Ashland, OR). FITC-labeled anti-PSMA antibody (MBL, Nagoya, Japan) was used as a positive control.

Serum Stability

Purified tribody (5 µg) was incubated at 37 °C with 100 µL of mouse serum (50 mg/mL, Sigma-Aldrich, MO) for 6 and 24 h, respectively. After incubation, 10 volumes of buffer A were added and the protein was recovered by using Co²⁺-NTA column as described above.

Cell Proliferation MTS Assay

The CellTiter96 Aqueous Non-Radioactive Cell Proliferation Assay kit from Promega (Madison, WI) was used for the MTS cell proliferation assays. Approximately 1×10⁴ cells were seeded in each well of a 96-well plate and grown for 16 h at 37 °C. Appropriate amount of each targeting ligand was added and incubated with the cells for 36 h. 10 µM Cisplatin (II) diamine dichloride (Sigma-Aldrich, St Louis, MO) was used as a positive control. After incubation, approximately 20 µl of MTS/PMS solution was added to each well followed by incubation for 4 h at 37 °C. The absorbance at 490 nm was recorded using an ELISA plate reader.

PEGylation and Fluorescent Labeling with Alexa 750

Highly purified monomeric or trimeric ligand was incubated with 30 kDa mPEG-NHS ester (Creative PEGWorks, Winston Salem, NC) in 1× PBS (1:15 molar ratio) at room temperature for 4 h. The reaction mixture was purified by Co²⁺-NTA column chromatography to remove unreacted PEG. After PEGylation, Alexa 750 succinimidyl ester (Life technology, Grand Island, NY) was added (10:1 molar ratio) and incubation for 2 h at room temperature. Un-reacted Alexa 750 was removed through extensive dialysis against 1× PBS using a dialysis membrane with 5 kDa MWCO.

Animal Study

Female athymic nude mice of 6–8 weeks were purchased from Charles River Laboratories (Wilmington, MA). All work performed on animals were in accordance with and approved by UNC Institutional Animal Care and Use Committee. Specifically, A431 cells (5×10⁶) were subcutaneously injected onto the lower back of female nude mice (about 20 g), and were allowed to grow to tumor size of about 200 mm³. Approximately 0.5 nmol of PEGylated, Alexa750-labeled Z^{EGFR} monomer or trimer in DPBS was applied to the mice by intravenous injection. Six hours post-injection, mice were sacrificed followed by organ isolation through cervical dislocation. The images of tumor and each organ were taken by the Kodak In-Vivo Imaging System FX-PRO (Carestream Health, Inc). Densitometric analysis of tumor signal was performed by Image J (NIH, Bethesda, MD).

Results

Design and Generation of Tribodies

In order to generate trimeric targeting ligands, we started with the 43-residue trimerization domain from mouse CMP-1:
EEDPCACESILKFEAKVEGLLQALTRKLEAVSGRLAVLENRII. This sequence is highly homologous to the same trimerization domain of human CMP-1:
EEDPCACESLVKFQAKVEGLLQALTRKLEAVSKRLAILENTVV, making it easy to switch to the human version if translational application is desired. In principle, many target-binding moieties can be fused with this trimerization domain for the facile generation and

conversion into the trimeric form. These targeting moieties include V_H or V_L domains from the natural antibodies, non-immunoglobulin protein domains that mimic natural antibodies,¹ and various short homing peptides isolated from phage display or other selection strategies.¹ To demonstrate that this tribody approach can be generally applied to fuse with targeting moieties with different 3D structures from non-structural homing peptides to well-folded protein domains, and with target-binding affinities from low to high, we chose a PSMA-binding short homing peptide HP^{PSMA} (WQPDTAHHWATL) as an example for a low affinity target-binder,¹⁷ and an EGFR-binding Z domain as an example for a high affinity target-binder (Figure 1A).⁴ To compare the tribody with its monomeric form, the monomeric Z^{EGFR} targeting molecule was constructed without the trimerization domain. We also truncated the trimerization domain by deleting the last 14 residues that are critical for intermolecular interaction and mutating Cys to Ala to disrupt the disulfide bond (Table 1). The resulting domain is not possible to trimerize, and was used for the generation of monomeric PSMA binding protein. The coding sequence for each recombinant fusion protein was synthesized and cloned into expression vector pET-28b for over-expression in *E. coli* (Table 1). A C-terminal His-tag was introduced to facilitate affinity purification. The expression of each tribody and its corresponding monomer was induced by the addition of 1 mM IPTG. One of the key advantages of using the trimerization domain of CMP-1 is that its self-assembly considerably favors a trimeric form compared to other trimeric and pentameric scaffolds.^{20,21} The CMP based recombinant proteins were highly expressed and purified to nearly homogeneity through Co²⁺-NTA purification. Indeed, more than 99% of the molecules exist in the trimeric form following purification with only a trace amount of monomer and dimer (Figure 1B). In order to verify the prominence of the trimer, native gel electrophoresis was performed. As shown in Figure 1C, both EGFR targeting and PSMA targeting tribodies existed only as a trimer under native and non-denaturing conditions. These results indicated that the tribodies are self-assembled highly efficiently, and can be purified as homogeneous trimeric complexes by a one-step Co²⁺-NTA chromatography procedure.

Thermal Stability and Protease Resistance of Tribodies

In order to use the tribodies under *in vivo* conditions, the biophysical properties such as solubility, thermal stability and resistance against protease degradation should be investigated. The CMP-based tribodies were highly expressed in *E. coli* as soluble recombinant proteins. To determine the thermal stability of the tribodies, we first performed Circular Dichroism (CD) analysis using highly purified monomeric and trimeric molecules by gradually increasing temperature from 25 °C to 94 °C to examine the secondary structure changes (Figures 2A to 2F). It was found that the melting temperature (T_m) of the monomeric HP^{PSMA} was about 65 ± 1 °C (Figure 2B). The HP^{PSMA} trimer showed remarkable stability when temperature was increased from 25 °C to 94 °C with a reversible melting curve (Figure 2B). Significantly, trimeric HP^{PSMA} appears to be more stable than the monomeric HP^{PSMA} at higher temperatures (Figure 2C). Since both the targeting moiety and the flexible linker in trimeric HP^{PSMA} are short peptides that are presumably non-structural, the observed remarkable thermal stability of HPPSMA tribody can be attributed to the highly stable trimerization domain. The T_m of the Z^{EGFR} monomer was approximately 60 ± 2 °C (Figure 2E). The Z^{EGFR} trimer also showed high stability when temperature was increased from 25 °C to 94 °C (Figure 2E). Although the melting curve of the Z^{EGFR} trimer was reversible only in the range from 25 °C to 60 °C (Figure 2F), the second and third melting curves showed similar pattern as their corresponding monomer (Figure 2E), suggesting a T_m around 61 ± 1 °C for the Z^{EGFR} trimer. Since Z^{EGFR} tribody was very stable when temperature was increased to 60 °C, it is likely that T_m of Z^{EGFR} tribody is much higher than 61 °C and that the first heating to 94 °C causes irreversible changes that result in reduction of the T_m of subsequent measurements. It appears that another transition

above 80 °C in Z^{EGFR} monomer and trimer melting curves was evident. While the results were reproducible, the nature of this transition is not clear.

Protein degradation by serum proteases is another obstacle that limits *in vivo* applications of many targeting ligands. To investigate this issue, we performed protease-mediated digestion of the targeting ligands by using thermostable metallopeptidase thermolysin, cysteine protease papain, and matrix metallopeptidase-9 (MMP-9). Significantly, all the monomeric and trimeric molecules were resistant to thermolysin digestion (Figure 3). Figure 4A shows that both the monomeric and trimeric molecules were resistant to papain. MMP-9 is one of the best characterized extracellular proteases that are overexpressed in numerous tumors. Figure 4B shows that both the monomeric and trimeric molecules were resistant to MMP-9 digestion (Figure 4B), whereas the control protein that contains a MMP-9 cleavage site was readily cleaved. These results suggest that these tribodies can be used under *in vivo* conditions when biologically active proteases are highly abundant.

In Vitro Target-binding Towards Immobilized and Cell Surface Targets

To examine whether these tribodies have desired targeting feature, we first used surface plasmon resonance (SPR) to compare the target-binding properties of the monomeric and the corresponding trimeric targeting ligands. Each purified protein target (the extracellular domain of EGFR or PSMA) was immobilized on CM5 sensor chip through amine coupling. Various concentrations of monomeric or trimeric targeting ligands were injected into the flow cell for binding strength analysis. To examine the multivalent effect, relatively low concentrations of tribodies were used in the flow phase. As shown in Figure 5 and Table 2, the monomeric Z^{EGFR} rapidly dissociated from EGFR immobilized on chip surface ($k_d \sim 6.78 \times 10^{-4} \text{ 1/s}$) with a binding affinity around $2.06 \pm 0.51 \text{ nM}$. Significantly, the Z^{EGFR} tribody dissociated very slowly compared to the monomer. The multiple target-binding sites involved in the interaction between tribody and EGFR significantly increase the local concentration of the affinity unit, thus effectively providing a decreased overall off-rate. This multivalent avidity or functional affinity is distinct from the intrinsic affinity used to describe the single antibody-antigen interaction, and can be orders of magnitude higher than the conventional affinity of a monovalent interaction. As shown in Figure 5B, the dissociation rate of the tribody observed from the SPR experiments was indeed significantly decreased. However, it is difficult to fit the data on any BIAevaluation model for multivalent kinetic analysis, presumably due to the complexity of the multivalent interaction between tribody and dimeric EGFR receptor. Therefore, the kinetic parameters of this EGFR-binding tribody are not calculated. In the case of PSMA tribody, we found that PSMA immobilized on CM5 sensor chip was not functional when examined with widely used anti-PSMA antibody, making it impossible to measure the PSMA-binding of HP^{PSMA} tribody. To address this issue, flow cytometry was adopted to estimate the binding of FITC-labeled HP^{PSMA} monomer and tribody with native PSMA present on the surface of cancer cells (Figure 6), while FITC-labeled anti-PSMA antibody was used as a positive control. As shown in Figure 6A, anti-PSMA antibody bound to LNCaP cells but not PC3 cells when used at 100 nM. HP^{PSMA} monomer showed no obvious binding with both PSMA positive LNCaP cells and PSMA negative PC3 cells (Figure 6B). However, as shown in Figure 6B, HP^{PSMA} tribody showed selective binding toward LNCaP but not PC3 cells, with a K_d of $455 \pm 24 \text{ nM}$.

We further confirmed the significantly enhanced target-binding of the tribodies by using biomarker-expressing cell lines. A431 cells have a high expression level of EGFR and were used as positive cells, while Jurkat cells were used as negative cells. As shown in Figure 7A, the trimeric targeting ligands demonstrated much stronger cell-binding compared to the monomeric forms. In the negative control experiments, both FITC-labeled Z^{EGFR} monomer

and trimer failed to bind to Jurkat cells even at high concentration (100 nM). In contrast, Z^{EGFR} tribody can recognize A431 cells with a concentration as low as 1 nM, whereas more than 10 fold higher concentration of Z^{EGFR} monomer was required to observe similar cell-binding extent. In the case of HP^{PSMA}, a FITC-labeled HPPSMA tribody can bind to PSMA positive LNCaP cells with a concentration as low as 100 nM (Figure 7B). We found that the monomeric PSMA homing peptide did not result in specific cell-binding signal even used at high μ M concentrations (Figure 7B and unpublished data). Since the target-binding affinity of monomeric HP^{PSMA} was too weak to measure, we were not able to determine the exact fold of increase in target-binding strength for HP^{PSMA}. Nevertheless, the results clearly indicate that the CMP-based trimerization approach allows for a quick conversion of a short homing peptide with undetectable binding affinity to a trivalent form with target-binding strength close to many monoclonal antibodies. Taken together, both Z^{EGFR} and HP^{PSMA} tribodies recognize their target receptors with greatly enhanced binding strength without sacrificing specificity compared with their corresponding monomers.

Serum Stability and Toxicity

Successful *in vivo* application of tribodies including imaging or therapy requires excellent serum stability and non-toxicity. To address the stability of the tribody in serum, trimeric Z^{EGFR} was incubated with mouse serum at 37 °C for up to 24 h. After incubation, the protein was recovered by Co²⁺-NTA chromatography. As shown in Figure 8A, Z^{EGFR} tribody displayed unusually high stability in mouse serum without detectable degradation even after 24 h at 37 °C. The toxicity of the Z^{EGFR} tribody was also examined by using the cell proliferation assay. As illustrated in Figure 8B, no cell growth inhibition was observed when the Z^{EGFR} tribody was used at 500 nM, a concentration that results in very strong cell-binding signals. Taken together, these results demonstrated that Z^{EGFR} tribody showed high serum stability and non-toxicity, and held the great potential for various *in vivo* and translational applications.

In Vivo Tumor Targeting

To examine the *in vivo* application of the trimeric targeting ligands, we used the EGFR-binding tribody to target tumor in xenograft mouse models. The molecular weight of the tribody is in the range of 30 kDa (for HP^{PSMA}) to 58 kDa (for Z^{EGFR}). To increase the circulation time, both the monomeric and trimeric Z^{EGFR} were PEGylated using 30 kDa PEG-NHS ester. To examine whether Z^{EGFR} tribody can efficiently target tumor on which EGFR was highly expressed, Alexa 750-labeled, PEGylated Z^{EGFR} monomer and tribody were intravenously injected into A431 xenograft nude mice. Six hours after injection, mice were sacrificed and the organs were isolated. The images of each organ and tumor were taken by an In-Vivo Imaging System FX-PRO. As shown in Figure 9, when the PEGylated tribody was used, the strongest accumulation was found in tumor with no or minimal accumulation in the heart, lungs, spleen, and liver. There was modest accumulation in the kidney, presumably due to the well-known renal elimination of the animal. Approximately 54% of the fluorescent signal from Z^{EGFR} tribody was accumulated in the tumor, compared to that of 7.7% from the monomer. These results clearly indicate that the tribody holds great promise for *in vivo* tumor targeting applications.

Discussion

In this study, we report the successful development and application of a novel class of trimeric targeting ligands based on the highly conserved trimerization domain of mammalian CMP-1. Compared with other trimeric scaffolds that have been reported,^{8–10,21–22} this tribody approach possesses several key advantages. First, this trimerization domain is derived from a highly conserved extracellular protein that is abundant in both mouse and

human. Presumably, the tribodies that are used for animal studies and translational applications can be readily generated with similar targeting features and low immunogenicity. This will greatly facilitate smooth transition from *in vitro* investigation to *in vivo* application. Second, the tribody has remarkable biophysical features with regard to thermal stability and protease resistance (Figures 2, 3 and 4). They were very stable when the temperature was up to 90 °C (Figure 2). Also, the tribodies were resistant to the degradation mediated by different proteases, including physiologically relevant papain and MMP-9 (Figures 3 and 4). While these tribodies existed as a trimer predominantly under denaturation condition such as boiling and in the presence of SDS (Figure 2), the bacteriophage T4 foldon derived trimer dissociated into a monomeric form after boiling.⁸ The human collagen XVIII NC1 domain, another trimerization domain that has been used for the generation of multimeric antibody, was sensitive to heat-induced denaturation and protease degradation.⁹ Third, the trimerization of tribody is a convenient and highly efficient self-assembling process (Figure 1). Compared to most other multimerization domains such as mucin 1-based trimer and COMP-based pentamer in which intermolecular disulfide bond linkages are indispensable for the formation of multimeric complexes,^{20,21} it was found that the tribody efficiently self-assembled into a stable trimeric form in the absence of any disulfide bonds, presumably due to the strong and extensive intermolecular hydrophobic and ionic interactions among the trimeric complex. This feature makes the tribody very useful in many *in vitro* and *in vivo* applications under reducing conditions. The trimer predominance of this system also provides an advantage on cost effectiveness over other disulfide bond-mediated trimers and pentamers, which are often generated with a significant amount of intermediate forms that are extremely difficult and time-consuming to purify.^{13,23,24} For example, the GCN4 isoleucine zipper based-trimer as well as the chicken tenascin C-mediated trimer often co-exist with the corresponding monomer and dimer,^{23,24} making it critical to remove the intermediate forms through often inefficient gel filtration chromatography. Fourth, the self-assembly multimerization process results in significant increase in molecular weights for short homing peptides and small target-binding domains, which otherwise display much shorter half-life *in vivo* due to rapid proteolytic cleavage and renal clearance.

The enhancement of the binding strength of a tribody against a cell surface receptor was demonstrated by SPR experiments (Figure 5), supporting the avidity effect of the tribody and further making it an attractive reagent for *in vivo* tumor detection and targeting. Cellular binding analysis using biomarker-expressing cell lines demonstrated that the tribodies recognized their corresponding receptors much more tightly than the monomer counterparts (Figure 7). It is worth mentioning that the length of the flexible linker could be critical to achieve desired multivalency and should be tuned depending on the nature of the targeting moiety and the receptor. The experimental conditions for SPR are artificial. The density of the receptor used in SPR is different from the native biomarker on the cell surface. It should be noted that unlike the surface of cancer cells where a biomarker of interest is present with thousands of other receptors and membrane-bound proteins, only purified biomarker was present on the surface of SPR biosensor. It is expected that this absence of competition during *in vitro* assays facilitates binding of tribody through multivalent interaction with more than one receptor. While the results from such *in vitro* analysis do not necessarily correlate well with what observed by using living cells, they reveal more precisely the biochemical and biophysical interactions (i.e., k_{on} and k_{off}) between a trimeric targeting ligand and a purified receptor of interest, which facilitate the rational design of targeting ligands with desired features.

Tribodies showed remarkable stability against protease degradation and serum proteases (Figures 3,4 and 8A). Also, they did not show detectable cell toxicity when applied up to 500 nM (Figure 8B), suggesting that they have the potential of being utilized for *in vivo*

tumor targeting. Compared to full-length monoclonal antibodies and small protein domain-based antibody mimics, the modest size and slower off-rate of tribody (i.e. 58 kDa for Z^{EGFR} tribody) could presumably provide rapid tissue penetration and longer retention time on a target receptor on diseased tissues.^{9,19} To demonstrate the *in vivo* application of the tribody, we performed *in vivo* tumor targeting using PEGylated tribody in mice bearing EGFR-positive tumor. It is well known that PEGylation of a targeting ligand aids in the reduction of nonspecific binding and increase of circulatory half-life.²⁵ Indeed, effective targeting to the EGFR-positive A431 tumor was demonstrated 6 h post-injection (Figure 9). The tribody was accumulated most in the tumor, with no or minimal accumulation in the heart, lungs, spleen, and liver. It was not surprising that modest accumulation was found in kidney, due to its well-known function in clearance of macromolecules. It has been reported that Z^{EGFR} monomer also bound murine EGFR.²⁶ This probably explained why there was some accumulation in mouse liver, which is known to have relatively more expression of EGFR compared to other organs.

Despite the advantages we have demonstrated in this work, the CMP-based tribody system does have its limitations. First, it is unrealistic to fuse with a very large protein domain, particular those with complex structures or multiple cysteine residues, while still expect to achieve the dramatic multivalent effect as we demonstrated for short homing peptides and target-binding small protein domains. Presumably, the close proximity of the three large domains in the tribody will greatly complicate the intermolecular interaction and disulfide bond exchange, resulting in incorrect disulfide bond formation and misfolding of the protein, despite the use of a long flexible linker. This will greatly reduce the multivalent effects, barely less than 10-fold increase as recently reported by Saha and co-workers,²⁷ even under *in vitro* conditions such as in SPR experiments. Second, one critical question to ask is which valency is enough and better to achieve the desired avidity effect? We have reported previously the pentameric and heptameric targeting ligands by using distinctive self-assembly approaches.^{6,19} Based on the *in vitro* biochemical and biophysical characterization that can be quantified, it appears that the trimeric targeting ligands perform as well as, and sometimes even better than, the pentameric and heptameric targeting ligands.^{6,19} However, caution should be taken to draw a general conclusion, because the interaction between a multivalent ligand with a cell surface receptor is highly dependent on the molecular nature, oligomeric status, surface density, correct conformation, post-translational modification, and other biochemical and biophysical properties of the receptor. Our studies shed light on the further development of multivalent targeting ligands against numerous cell surface receptors that are implicated in different human diseases.

In summary, we have developed trimeric targeting ligands based on the highly conserved CMP trimerization domain in mouse and human with remarkable stability against heat and proteolysis as well as significantly improved target binding affinity and effective *in vivo* tumor targeting feature in animals. Facile and tunable production of tribody suggests its great potential in specific tumor imaging and targeted delivery of numerous therapeutic agents.

Acknowledgments

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Abbreviations used

CMP	cartilage matrix protein
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Tribody	trimeric targeting ligand
EGFR	epidermal growth factor receptor
PSMA	prostate-membrane specific antigen
SPR	surface plasmon resonance
Z^{EGFR}	EGFR binding affibody
HP^{PSMA}	PSMA binding homing peptide

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Figure 1A



Target binding domain: Z^{EGFR1907}

Target binding homing peptide: HP^{PSMA} (WQPDTAHHWATL)

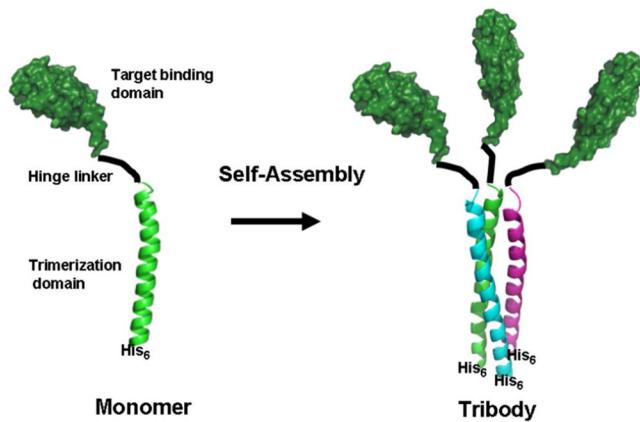


Figure 1B

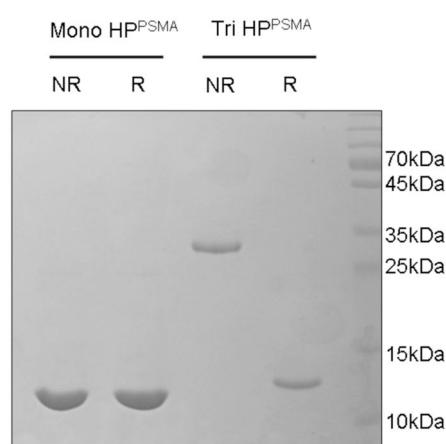
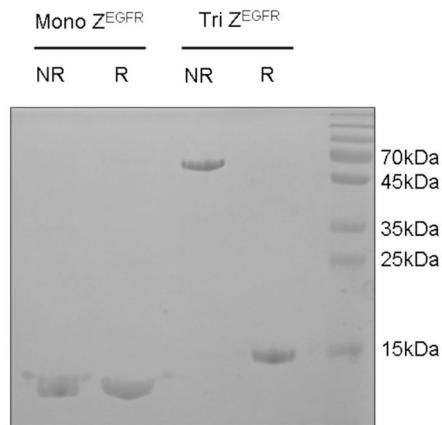


Figure 1C

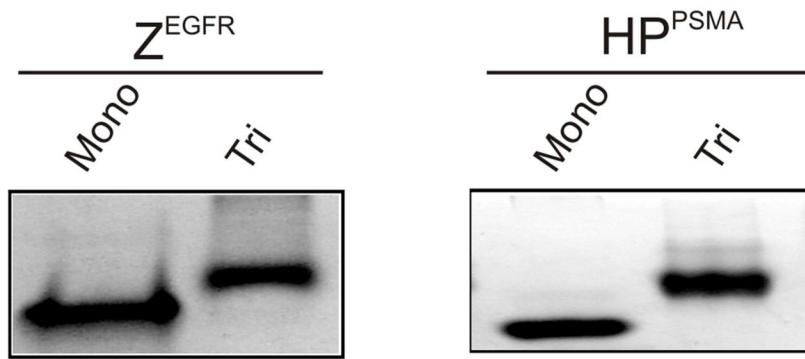


Figure 1. Schematic diagram and the purification of the tribody

A) a self-assembled trimeric targeting ligand consists of a target binding domain (Z^{EGFR}) or homing peptide (HP^{PSMA}), a flexible hinge linker, and a trimerization domain derived from the C-terminus of mouse CMP-1. His \times 6 tag was engineered at the C-terminus of each ligand to facilitate affinity purification. B) Purification of the monomeric and trimeric targeting ligands on SDS-PAGE. Z^{EGFR} monomer, Z^{EGFR} trimer, HP^{PSMA} monomer, and HP^{PSMA} trimer were purified by metal affinity column chromatography and loaded on 12% SDS-PAGE for analysis. NR stands for nonreducing condition, and R stands for reducing condition with 10 mM DTT. C) Native gel electrophoresis. Purified monomeric and trimeric proteins (5 μg) were loading on an 8% native gel to analyze the oligomeric status. Proteins were stained by Coomassie brilliant blue.

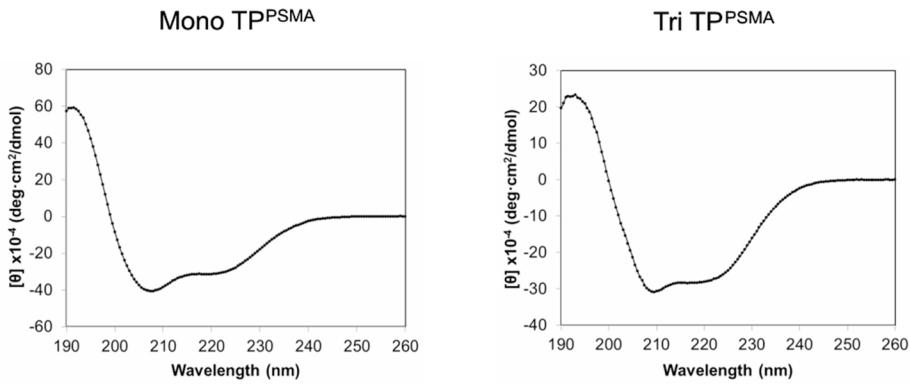
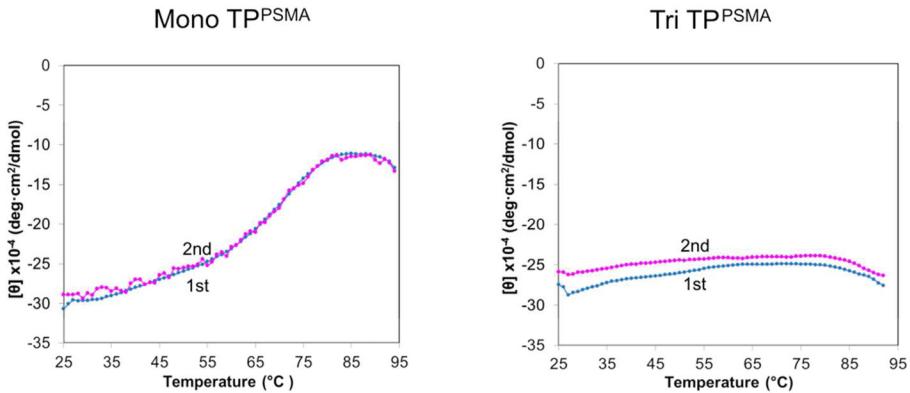
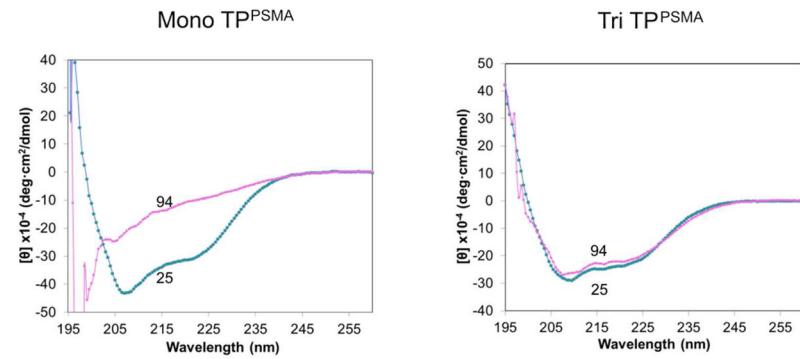
Figure 2A**Figure 2B****Figure 2C**

Figure 2D

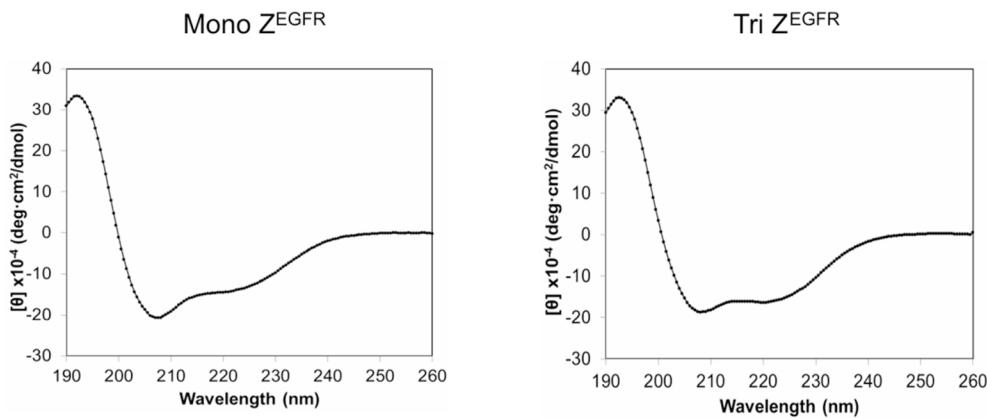


Figure 2E

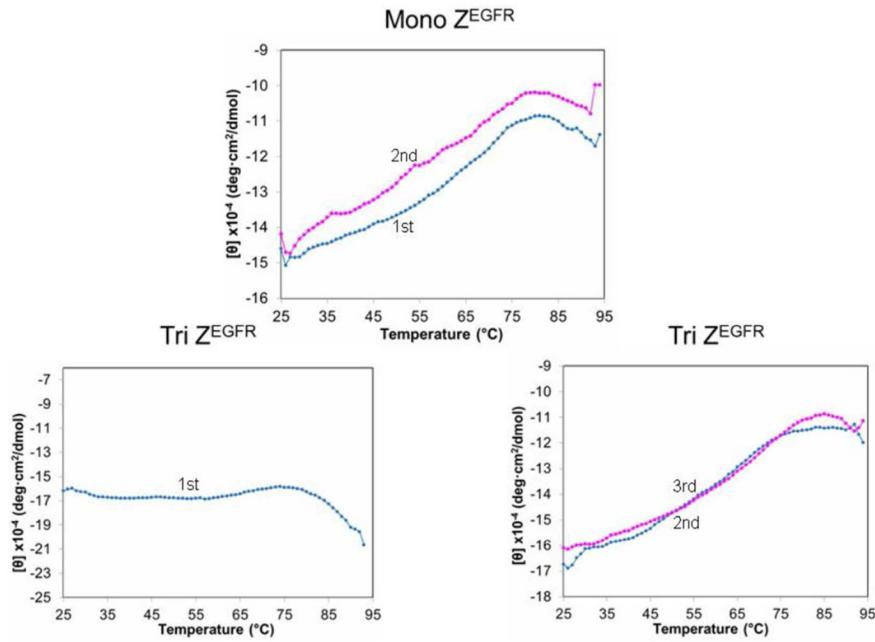


Figure 2F

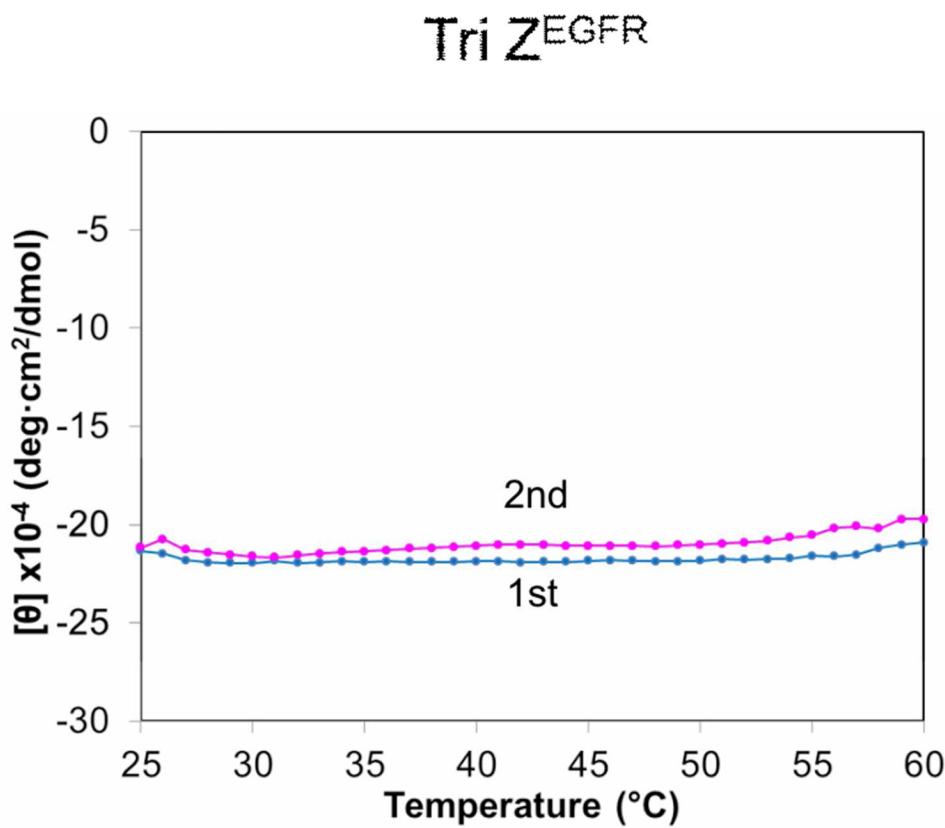


Figure 2. Analysis of heat induced denaturation using Circular Dichroism

A) CD spectra of HP^{SMA} monomer and HP^{SMA} trimer were recorded from 190 nm to 260 nm at 25 °C, respectively. B) CD spectra were recorded at various temperatures from 25 °C to 94 °C. The ellipticity at 220 nm was used for analysis. C) CD spectra of HP^{SMA} monomer and HP^{SMA} trimer was recorded from 190 nm to 260 nm at 25 °C and 94 °C, respectively. D) CD spectra of Z^{EGFR} monomer and Z^{EGFR} trimer were recorded from 190 nm to 260 nm at 25 °C, respectively. E) CD spectra of Z^{EGFR} monomer and Z^{EGFR} trimer were recorded at various temperatures from 25 °C to 94 °C. The ellipticity at 220 nm was used for analysis. F) CD spectra of Z^{EGFR} trimer were recorded at various temperatures from 25 °C to 60 °C. The ellipticity at 220 nm was used for analysis.

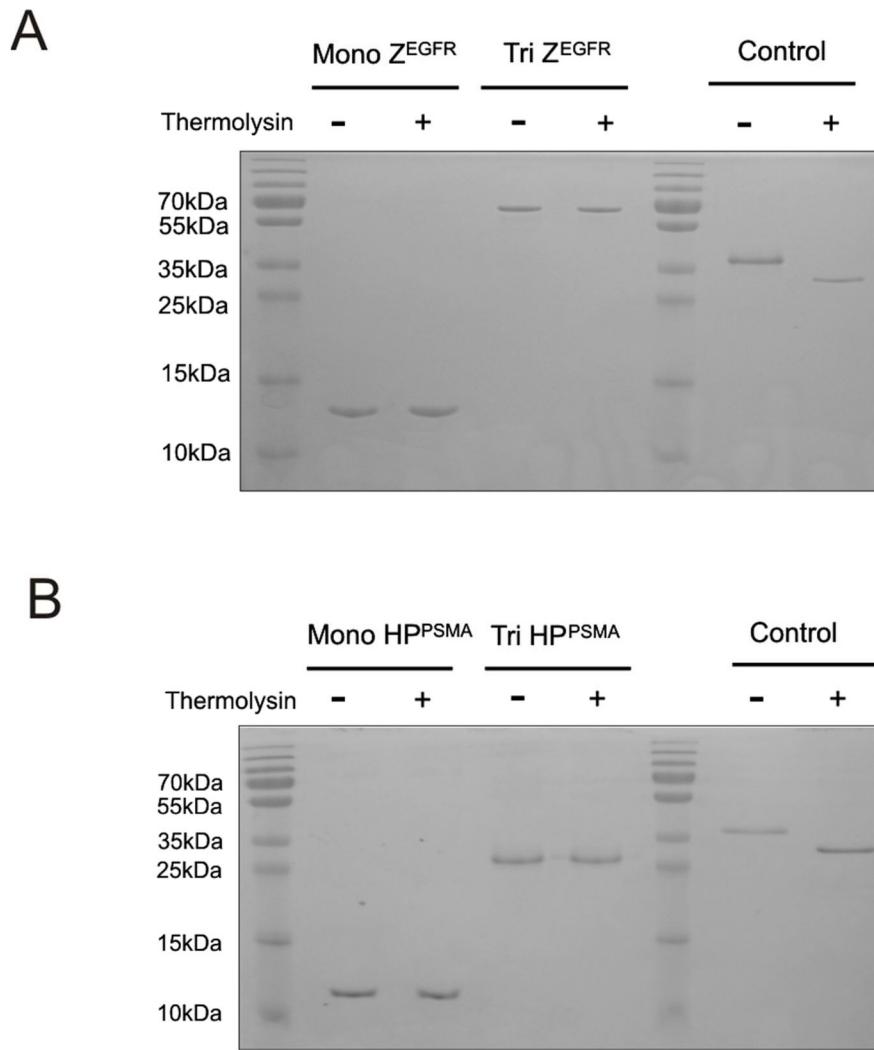
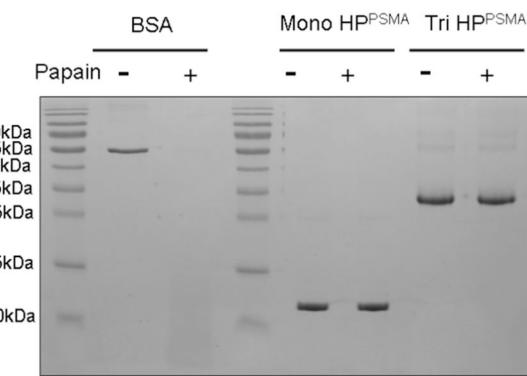
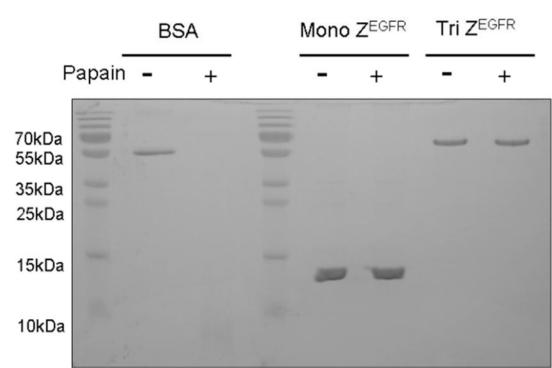


Figure 3. Analysis of protease resistance using thermolysin

Each purified protein (A: Z^{EGFR} monomer and trimer, B: HPPSMA monomer and trimer) was incubated with thermolysin at 37 °C for 30 min. The reaction mixture were loaded on a 12 % SDS-PAGE for analysis.

A

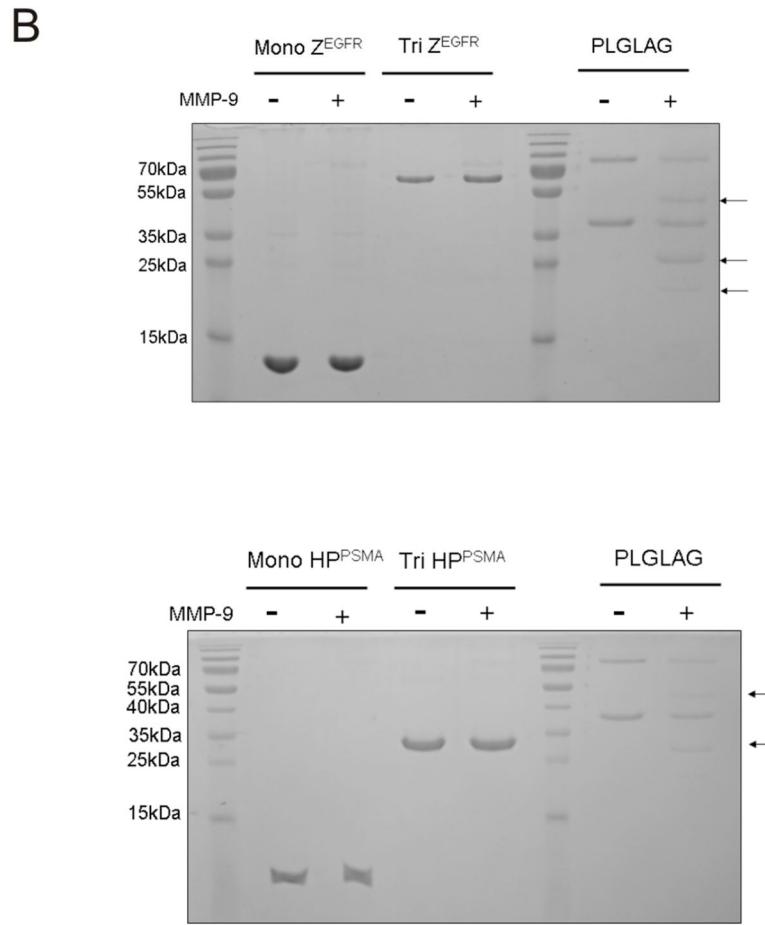


Figure 4. Protease resistance assay using papain and MMP-9

A) Each purified protein (Z^{EGFR} monomer and trimer, HP^{PSMA} monomer and trimer) was incubated with papain at 37 °C for 30 min with BSA as a positive control. The reaction mixtures were loaded on 12 % SDS-PAGE. B) MMP-9 was incubated with each purified protein (Z^{EGFR} monomer and trimer, HP^{PSMA} monomer and trimer) as well as an engineered protein which contains MMP-9 cleavage site (PLGLAG). The reactions were performed at 37 °C for 2 h. The reaction mixtures were loaded on 12 % SDS-PAGE.

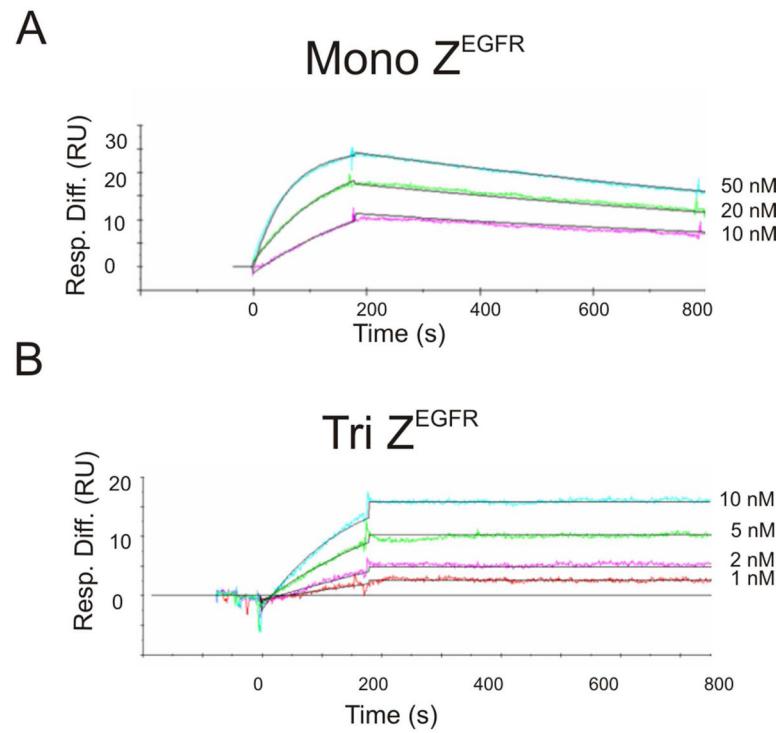
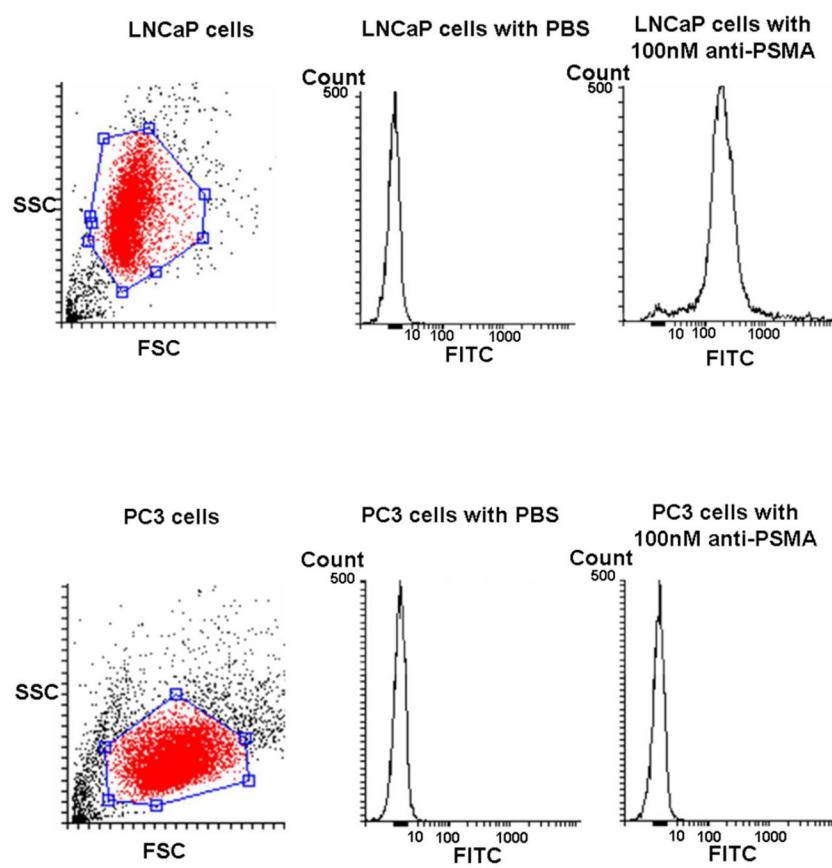


Figure 5. Biacore analysis of target binding properties

Recombinant human EGFR-Fc was immobilized on a CM5 biosensor chip via amine coupling. Different concentrations of targeting ligands (A. Z^{EGFR} monomer, and B. Z^{EGFR} trimer) were injected onto the biosensor channels and the analysis was performed at a flow rate at 20 μ l/min at room temperature.

A

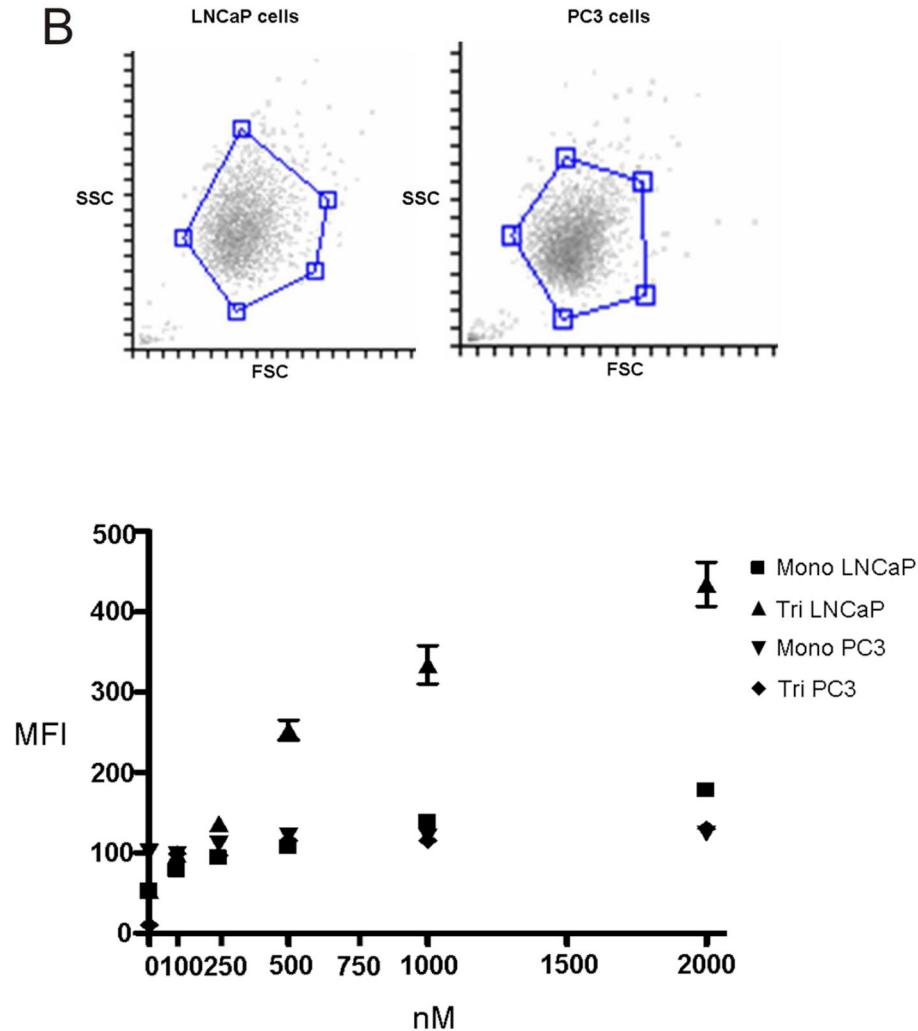


Figure 6. Flow cytometry for cell-binding

A) PSMA positive LNCaP cells and PSMA negative PC3 cells were grown on a 6-well plate for overnight. 100 nM of FITC labeled anti-PSMA was incubated with LNCaP or PC3 cells at 25 °C for 30 min. The cell-binding signals were analyzed by flow cytometry. MFI: mean fluorescent intensity. B) PSMA positive LNCaP cells and PSMA negative PC3 cells were grown on a 24-well plate for overnight. FITC-labeled HPPSMA monomer and HP^{PSMA} trimer at various concentrations were incubated with LNCaP or PC3 cells at 25 °C for 30 min. The cell-binding signals were analyzed by flow cytometry. MFI: mean fluorescent intensity.

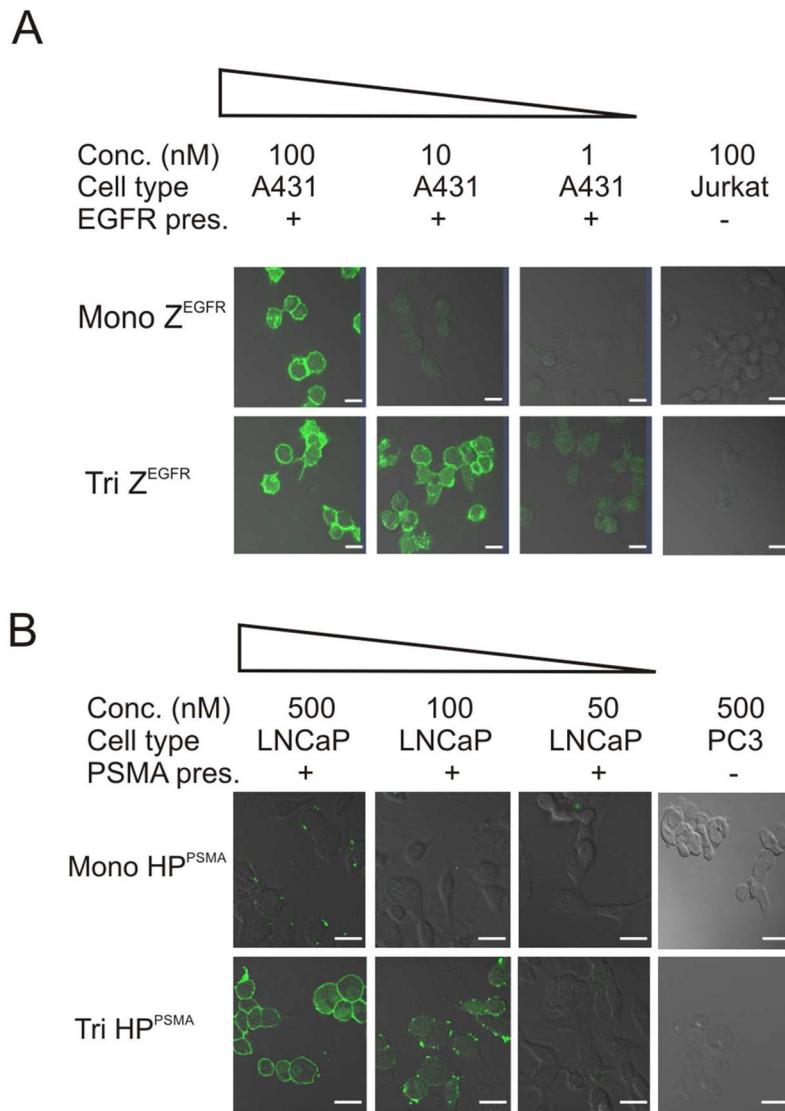
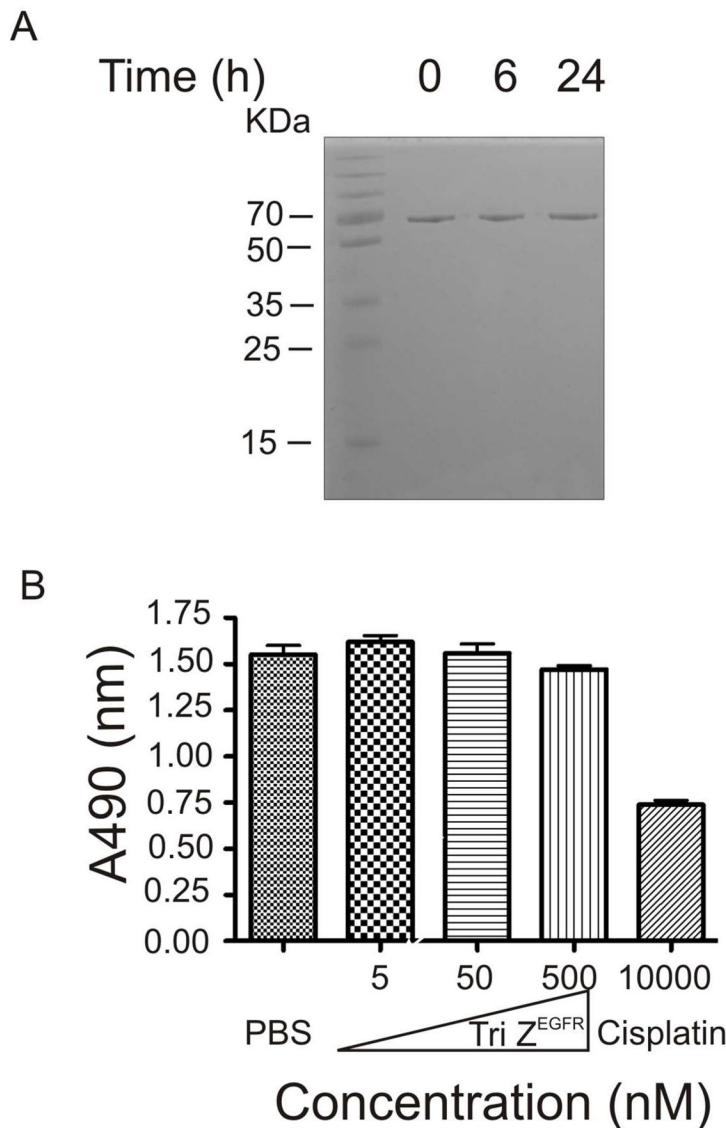


Figure 7. Comparison of the binding of monomeric and trimeric targeting ligands with targets on cell surface

A) EGFR positive A431 cells and EGFR negative Jurkat cells were grown on coverslides. Different concentrations of FITC-labeled Z^{EGFR} monomer and Z^{EGFR} trimer were incubated with cells for 30 min at 25 °C. B) PSMA positive LNCaP cells and PSMA negative PC3 cells were grown on coverslides. Different concentrations of FITC-labeled HP^{PSMA} monomer and HP^{PSMA} trimer were incubated with cells for 30 min at 25 °C. The cell-binding signals were visualized by confocal microscopy. The scale bars indicate 20 μm.

**Figure 8. Serum stability and toxicity**

A) Serum stability: purified Z^{EGFR} tribody (5 µg) was incubated with mouse serum (5 mg) for 0, 6, and 24 h, respectively. After incubation, the proteins in the reaction mixture were recovered with Co²⁺-NTA resin. The recovered tribodies were loaded on a 12% SDS-PAGE to examine stability. Lane 1: 0 h; lane 2: 6 h; lane 3: 24 h. B) Toxicity: A431 cells were incubated with various concentrations of Z^{EGFR} tribody for 36 h at 37 °C. Cisplatin (10 µM) was used as a positive control. Cell proliferation was determined by using absorbance at 490 nm (A490). Three repeated assays were performed.

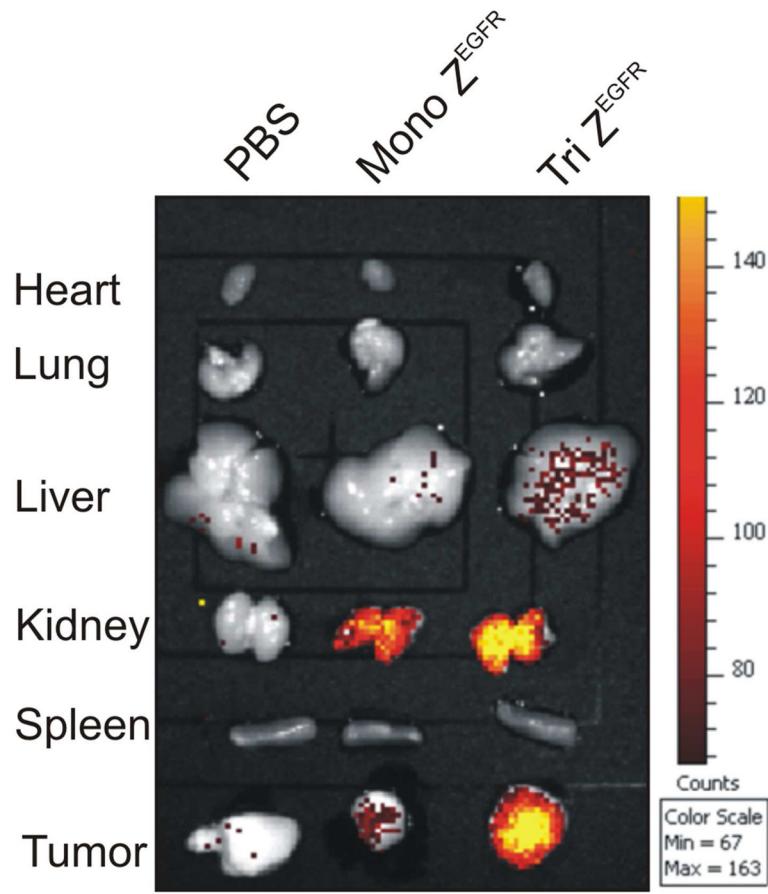


Figure 9. *In vivo* tumor targeting using PEGylated monomeric and trimeric Z^{EGFR}
PBS (1), 0.5 nmol of PEGylated, Alexa750-labeled monomeric Z^{EGFR} (2), and 0.5 nmol of PEGylated, Alexa750-labeled trimeric Z^{EGFR} (3) in PBS were injected to A-431 xenograft nude mice. 6 h after injection, mice were sacrificed and the images of tumor and each tissue were taken by Kodak *In-Vivo* Imaging System FX-PRO.

Table 1

Amino acid sequences of tribody component

	Amino acids
ZEGFR	MVDNKFNKEM WAAWEEIRNL PNLngWQMTA FIASLVDDPS QSANLLAEAK KLNDAQAPK
TP ^{PSMA}	WQPDTAHHWATL
Hinge Linker	GPQPQPQPKPQPK PEPEPQQPGG
Trimerization domain	EEDPCACESILKFE AKVEGLLQALTRK LEAVSGRLAVLENRII
Truncated Trimerization domain	EEDPAAAESILKFE AKVEGLLQALTRKLE

Table 2
Binding constants of Z^{EGFR} monomer

The association and dissociation constants were calculated using BIA evaluation software by fitting data on one to one Langmuir binding model.

	k _a (1/Ms)	k _d (1/s)	K _D (M)
Z ^{EGFR} Monomer	3.29 ± 0.82 × 10 ⁵	6.78 ± 1.69 × 10 ⁻⁴	2.06 ± 0.51 × 10 ⁻⁹