Electronic Supplementary Material (ESI) for Molecular BioSystems.

SUPPORTING INFORMATION

Engineering chemically modified viruses for prostate cancer cell recognition

Kritika Mohan[a] and Gregory A. Weiss[*,a,b]

[a]Department of Chemistry, [b]Department of Molecular Biology and Biochemistry, University of California, 1102 Natural Sciences 2, Irvine, California 92697-2025 USA

Table of Contents:

Materials and Methods

All chemicals and reagents were purchased from Sigma-Aldrich, and used as received unless otherwise noted. PSMA and the cell lines LNCaP and PC3 were generous gifts from Drs. William Ernst and Gary Fuji (Molecular Express). Maleimide-PEG100-amine and 15-azido-4,7,10,13-tetraoxapentadecanoic acid (azido-PEG4-carboxylic acid) were purchased from Alfa Aesar. N,N-Diisopropylethylamine (DIPEA) and azide-functionalized PEG7, 22 and 45 were purchased from Sigma, and 4-Azidobutanoic acid was purchased from Synthonix. 4-Pentynoic acid (GFS Chemicals, Inc.), O-benzotriazole-N,N,N',N'-tetramethyl-uronium-hexafluoro-phosphate, HBTU (GL Biochem Ltd.), triethyammonium acetate buffer (Fluka Biochemika) and Tween-20 (EMD Science) were used as received. HPLC-grade water was used for the preparation of solutions.

M13 bacteriophage propagation

Phage propagation and isolation was performed as previously described.[1,2] Briefly, the phagemid DNA was transformed into $CaCl_2$ competent *E. coli* XL-1 Blue cells. The cells were grown at 37 °C in 2 mL 2YT media supplemented with carbenicillin and tetracycline until the culture reached log-phase growth. The culture was then infected with KO7 helper phage with a multiplicity of infection of 4.5:1. The starting culture was then transferred to 75 mL 2YT media supplemented with carbenicillin and kanamycin. The phage culture was incubated for 16 h at 37 °C with shaking. The phage were isolated from the culture supernatant by centrifugation at 10 krpm after precipitation through addition of 1/5th volume of PEG-NaCl (2.5 M NaCl, 20% PEG-8000). After second precipitation, the phage were resuspended in phosphate-buffered saline (PBS, 135 mM NaCl, 2.50 mM KCl, 8.00 mM Na_2HPO_4, 30.0 mM KH_2PO_4, pH 7.2). Phage concentration was determined by UV absorbance at 268 nm (OD_{268} of 1.0 = 8.31 nM phage).

Solid Phase Peptide Synthesis

The peptides were synthesized by conventional solid-phase peptide synthesis with Fmoc-protected amino acids on Rink-amide resin (Novabiochem), as previously described.[1,3,4] The peptide *N*-terminus was coupled to 4-azido butanoic acid or 4-pentynoic acid to yield the azide- or alkyne-functionalized peptides, respectively. For incorporation of the PEG4 linker, the last coupling step was performed with 15-azido-4,7,10,13-tetraoxapentadecanoic acid. The reported peptides were purified by reverse-phase HPLC purification with a C_{18} column. Fractions containing the purified peptides were combined and concentrated using rotary evaporation, followed by lyophilization and characterization by MALDI-TOF mass spectrometry.

The calculated *m*/*z* for peptide-**1** [M^+] 1349.67, found 1349.77. The calculated *m*/*z* for peptide-**2** [M^+] 2040.28, found 2040.23. The calculated *m*/*z* for peptide-**1** fused to the azido-PEG4 linker is [M^+] 1510.80, found 1511.78. The calculated *m*/*z* for peptide-**2** fused to the azido-PEG4 linker is [M^+] 2201.02, found 2202.06. The calculated *m*/*z* for alkyne-functionalized K_{14} peptide $[M+Na]^+$ 1914.37, found 1914.18. The calculated *m*/*z* for K_{14}-Cys peptide [M^{3+}] 638.79, found 638.79.

Click chemistry reaction for the synthesis of PEGylated oligolysines

The protocol for the synthesis of PEGylated oligolysines was adapted from Lumiprobe Corporation's protocol, as described previously.[1,2,5,6] Briefly, the reaction was performed at a final concentration of 100 µM azide-functionalized PEG. The product obtained was purified using reverse-phase analytical HPLC and characterized by MALDI-TOF mass spectrometry. The calculated *m*/*z* for alkyne-functionalized K_{14} fused to azide-functionalized PEG7 [M^+] 2285.61, found 2286.93.The mass spectrometry data obtained for PEGylated oligolysine showed a shift in the characteristically polydispersed PEG spectra by the expected mass of K_{14}-alkyne, Figure S3 and S4.

Synthesis of PEGylated ligands – specific attachment mode

The protocol for the synthesis of the PEGylated ligands was adapted from the solid phase peptide synthesis and click chemistry reaction described above, and also described in Figures S7 and S9. In a glass test tube, 40 µL of 1 mM Mal-PEG100-NH_2 (commercially purchased from Alfa Aesar) in water, 12 µL of 10 mM pentynoic acid (in water), 12 µL of 10 mM HBTU (in NMP), 40 µL of DIPEA and 296 µL of HPLC grade water were combined and stirred at

room temperature for 2 h, yielding alkyne-functionalized-PEG100-Mal. To obtain more quantities of the product, multiple reactions at this volume were run in parallel. To remove unreacted starting material and to concentrate the product, the reaction mixtures were diluted with an equal volume HPLC grade water before concentration to 1/5th volume using 2K MWCO concentrators (Sartorius).

For the next step of the synthesis, ~50 µL of the alkyne-functionalized-PEG100-Mal, obtained as described above, was conjugated to azide-functionalized peptide ligands (final concentration of 40 µM) by click chemistry as before.[1,5] The reaction mixture was stirred overnight at room temperature. Four identical reactions were run in parallel. To remove unreacted starting materials, the four reaction mixtures were then combined and concentrated to 1/4th volume using 3K MWCO concentrators. Next, the resultant solution was diluted with an equal volume of HPLC grade water before concentrating to ~1/2 volume using 5K MWCO concentrators. The concentrated reaction mixture was purified using reverse-phase analytical HPLC (Figures S11 and S12) and fractions were identified by MALDI-TOF mass spectrometry. The high polydispersity of the high MW PEG polymer prevents accurate mass determination by this technique, and instead Gel Permeation Chromatography (GPC) was used as described in the next section.

For the non-specific attachment mode described in the text and Figure S9, the PEGylated ligands were synthesized in the reverse order. The azide-functionalized peptides were first conjugated to pentynoic acid using click chemistry. The resultant peptide was then coupled to Mal-PEG100-NH_2 using HBTU and DIPEA as described above.

Gel Permeation Chromatography (GPC)

GPC was used to characterize the MW's of the PEGylated peptides. The molecular weights of the polymers, calibrated with PEG MW standards, were obtained with an Agilent 1100 series GPC system (Agilent Technologies, Santa Clara, CA) using 0.1% (v/v) LiBr/DMF solution (1.0 mL/min) as the eluent. The commercially purchased, unmodified Mal-PEG100-NH_2 was found to have an average molecular weight of Mn = 3690, and this polymer eluted as a broad peak consistent with its size distribution. The calculated molecular weights for PEGylated-**1** and -**2** with the PEG4 linker, based on the molecular weight of Mal-PEG100-NH_2, were estimated as 5300 and 5990, respectively. The corresponding molecular weights observed by GPC were 5310 and 6060, respectively.

Dynamic Light Scattering (DLS)

To demonstrate wrapping by PEGylated ligands and other materials on the phage surface, DLS measurements were obtained using Nano ZetaSizer ZS series. For determination of size, 1 mL of each sample was measured at the same concentration as used for the biological assay. Each sample was measured at least three times at 25 °C, with each individual size measurement being the average of 10 runs.

Cell growth

The cell lines were grown as monolayers in media supplemented with 10% fetal bovine serum (Cellgro), 1 mM sodium pyruvate[7] and 1% penicillin-streptomycin-glutamine in a 5% CO_2 and 95% air-humidified atmosphere at 37 °C. LNCaP cells were cultured in RPMI 1640 media. For studies with LNCaP cells cultured in charcoal-stripped serum (LNCaP CSS cells), LNCaP cells

were washed with PBS and then incubated with phenol free RPMI 1640 media supplemented with 10% charcoal-stripped serum (Cellgro) for five minutes. The cells were again washed with PBS, and provided fresh media.[8] PC3 cells were grown in Ham's F-12 media.

Cell-based phage Enzyme-Linked Immunosorbent Assay (ELISA)

Day 1: The cell-based ELISA was performed as previously described by Watanabe et. al. with the following modifications.[9] Cells were detached with Trypsin-EDTA, resuspended in PBS, and then collected by centrifuging at 1200 rpm for 5 min. The cells were further washed with PBS and then concentrated as in the previous step. The concentration of the cells was adjusted to 4.5×10^6 cells/mL in PBS using a hemocytometer, and 100 µL was aliquoted to specific wells of a 96-well microtiter plate (Maxisorp plates from Nunc). The Maxisorp plates used here have a high protein-binding capacity. Thus, the plates can be used to run assays with either the cells or the phage immobilized in the wells. Next, 50 µL of a 0.15% glutaraldehyde in PBS solution was added to the wells at 4 °C, and the solution was gently mixed by pipetting. The ELISA plate was then centrifuged at 1200 rpm for 10 min at 4 °C, followed by overnight incubation at 4 °C.

Day 2: The cell solution was gently removed, and the wells were blocked with 200 µL/well of blocking buffer containing 100 mM glycine, 1% gelatin and 0.1% w/v BSA (bovine serum albumin) in PBS. The plate was incubated overnight (~20-22 h) at room temperature.

Separately, phage were prepared before attachment to PEG and PEGylated ligands. Phage (10 nM in 100 µL of PBS) and 1 µL of K_{14}-alkyne (525 µM in water) were thoroughly mixed by pipetting ~25 times. For phage wrapped with PEGylated ligands, phage were mixed with 0.75 µL of K_{14}-Cys (525 µM in water). For mixed wrapping on the phage surface, 0.5 µL of

K_{14}-alkyne was pre-mixed with 0.75 µL of K_{14}-Cys, and then mixed with 100 µL of 10 nM phage. The solution was shaken at room temperature for 15 min on an orbital shaker. Next, 2 µL of PEGylated ligand (625 µM in water) was added to the appropriate wells. For the dual ligand combinations, the PEGylated ligands were pre-mixed in the desired ratio (a 2:1 molar ratio for example), and then 2 µL of the mixture was added to the appropriate wells. The solutions were gently mixed by pipetting, and incubated overnight at 4 °C.

Day 3: Next, the click reaction was performed, as previously described, but with the following modifications.[1,2,5] To buffer the pH, triethylammonium acetate was added to a final concentration of 50 mM, followed by the addition of 1.5 µL of 1 mM azide-functionalized PEG. The solutions were mixed by pipetting. Next, ascorbic acid was added to a final concentration of 1 mM and the solutions were mixed by gently pipetting. Then, copper sulfate was added to a final concentration of 1.5 mM, followed by pipetting to mix the solutions. Water was added to the other wells to maintain consistent phage concentrations. The plate was incubated at room temperature for 30 min.

The wells of the ELISA plate were then incubated with the phage samples. The blocking buffer was removed and the wells were gently washed two times with PBS. Next, the phage solution was added to the respective wells and incubated for 45 min. The phage solution was removed, and the wells were washed three times with 300 µL/well of wash buffer PT (0.05% Tween-20 in PBS), once with PBS, and then incubated with horseradish peroxidase-conjugated anti-M13 antibody (100 µL/well, 1:5000 dilution in PBS) for 40 min. The wells were washed three times with PT and once with PBS. The plate was then developed by incubating with HRP substrate solution (100 µL/well; 1 mg/mL *o*-phenylenediamine dihydrochloride and 0.02% w/v

H_2O_2) in citric acid buffer (50 mM citric acid, 50 mM Na_2HPO_4, pH 5.0). The HRP activity was measured spectrophotometrically at 450 nm using a microtiter plate reader (Bio-Tek). The absorbance at 630 nm was subtracted from the absorbance at 450 nm to eliminate background.

Phage-based sandwich ELISA for cell capture.

To demonstrate cell capture by the PEGylated-ligand phage, the phage were coated on the plate, and cells added before quantifying binding. This assay setup inverts other cell-based phage ELISAs reported here. This experiment is a significant step towards establishing the relevance of this phage architecture for biosensing assays planned in the future. In this assay, the PEGylated phage architecture is immobilized on the plate as demonstrated in Figure S10. Next, a cell suspension is added to the wells, and the amount of cells captured are then measured spectrophotometrically as detailed here and in the text. The protocol here focuses on experimental details altered from the above-described ELISA; all other conditions remained unchanged.

Day 1: In this phage capture ELISA, specific wells of a 96-well microtiter plate were coated with 100 µL/well of a solution of 10 nM phage pre-wrapped with oligolysine wrappers, as described above. The plate was incubated for 1 h on a shaker at room temperature. The coating solution was removed, and the wells were blocked with 200 µL/well of 0.2% w/v solution of BSA in PBS for 30 min, and washed two times with PT. Next, 98 µL PBS was added per well, followed by PEGylated ligands and incubated overnight at 4 °C.

Day 2: Azide-functionalized PEG variants were then conjugated as described above. Separately, the cells were collected and the concentration adjusted as described above; the

ELISA plate was then incubated with 100 µL/well of the cell solution or media for 1 h. The wells were washed with PBS and incubated with 100 µL/well of the anti-PSMA antibody, YPSMA antibody (Abcam) at 1:1000 dilution. The wells were then washed with PBS, followed by incubation with horseradish-peroxidase-conjugated anti-mouse antibody (Sigma) at a 1:1000 dilution. The levels of phage binding were quantified as described above.

Supplementary Figures

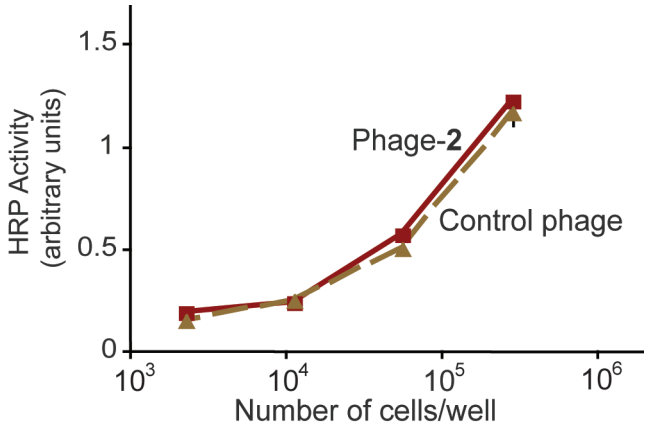

Fig. S1 Phage-based ELISA demonstrating unacceptably high non-specific adhesion of phage to the surface of LNCaP cells. Phage-**2** display the PSMA ligand **2**. Control phage provides a negative control with no ligand displayed on the phage. Throughout this report, error bars for ELISA data represent standard error (n = 3). All experimental data points include such error bars, though often these are quite small.

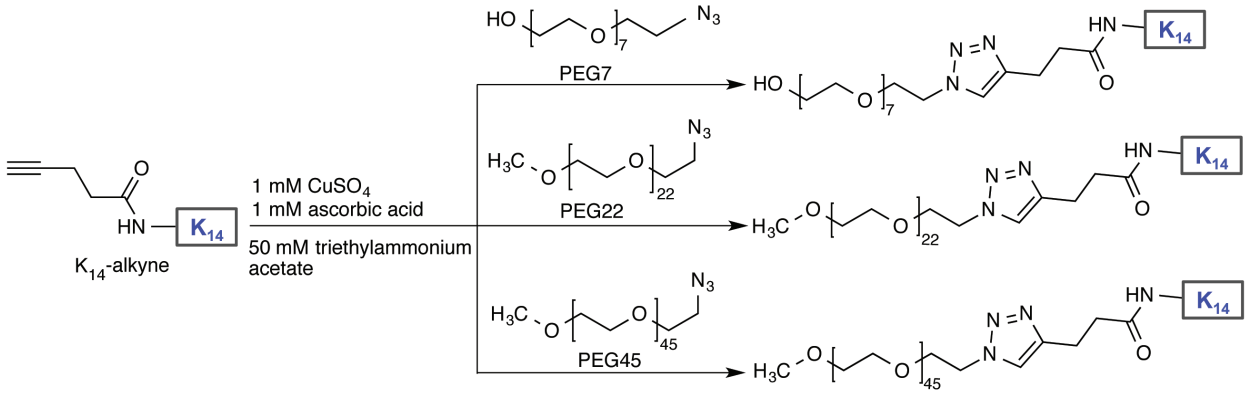

Fig. S2 Cu^{I}-catalyzed azide-alkyne cycloaddition reaction for the generation of oligolysine-PEG wrappers.

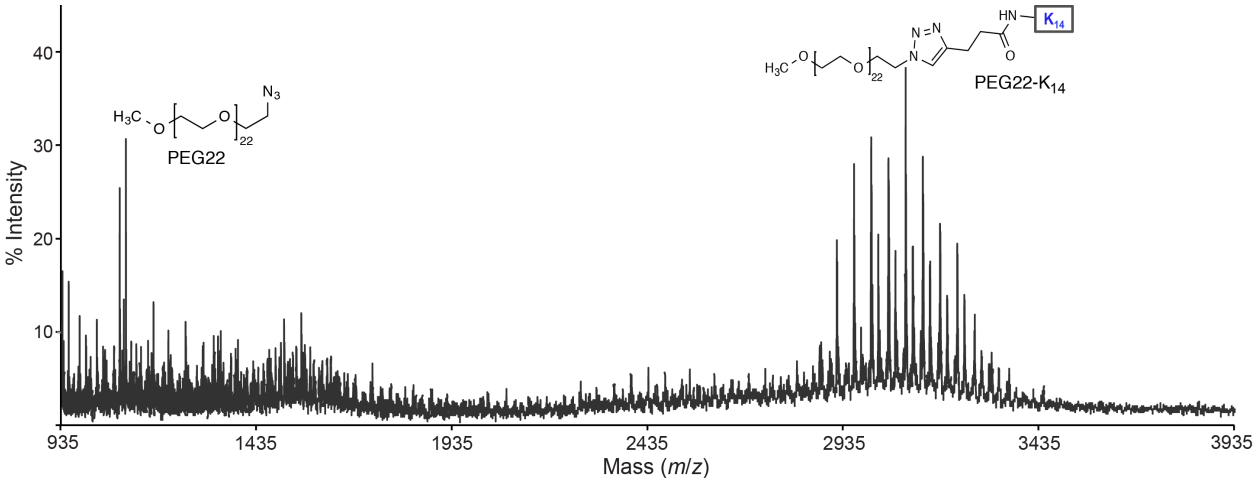

Fig. S3 MALDI-TOF characterization of K_{14}-alkyne fused to azide-functionalized PEG22. The data obtained for PEGylated oligolysine showed a characteristic shift in the polydispersed PEG spectra by the expected mass of K_{14}-alkyne (1891.57).

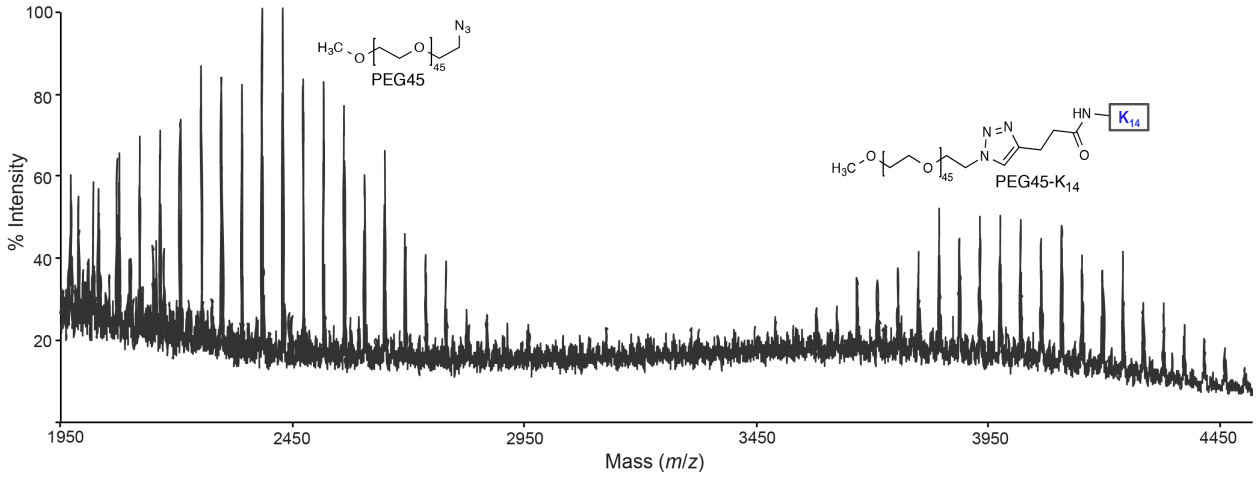

Fig. S4 MALDI-TOF characterization of K_{14}-alkyne fused to azide-functionalized PEG22. The data obtained for PEGylated oligolysine showed a characteristic shift in the polydispersed PEG spectra by the expected mass of K_{14}-alkyne (1891.57).

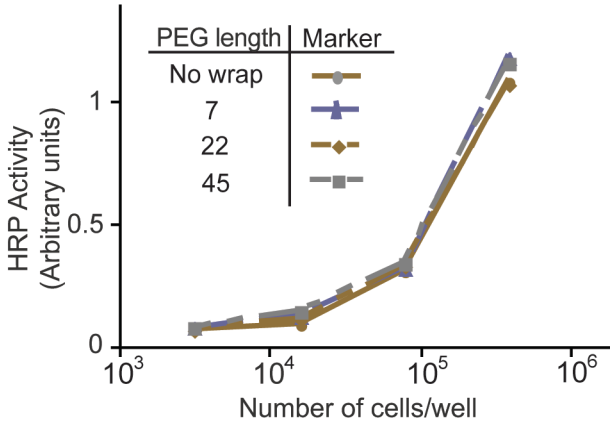

Fig. S5 Phage-based ELISA demonstrating the ineffectiveness of wrapping phage with PEG polymers due to the encapsulation of oligolysine side chains. In this experiment, phage without wrapper and phage wrapped with PEG7, 22 or 45 at 5 µM concentrations were compared.

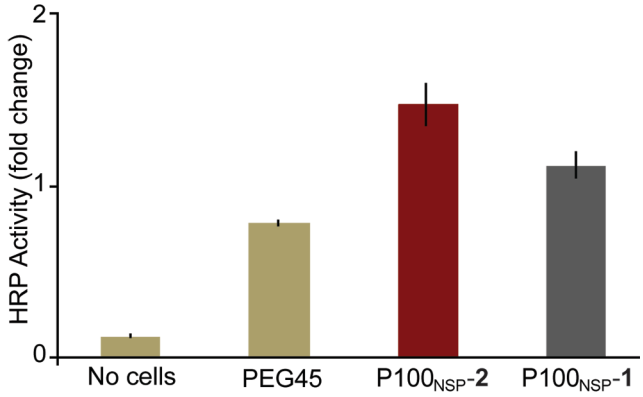

Fig. S6 Phage-based ELISA illustrates a modest increase in binding affinity using PEGylated ligands on phage targeting LNCaP cells. PEGylated ligands on phage were further engineered for higher affinity recognition.

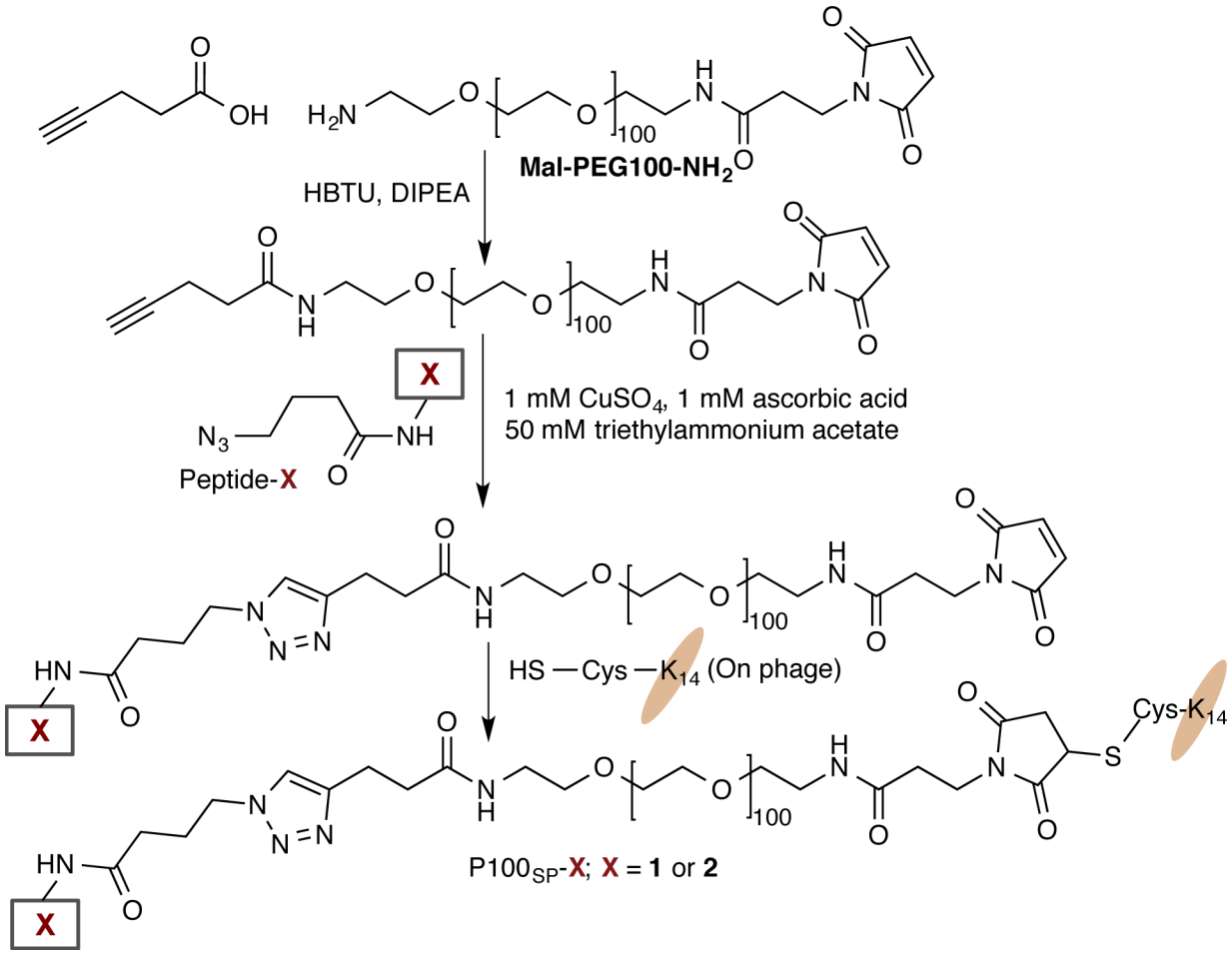

Fig. S7 Synthesis scheme for the generation of PEGylated ligands on phage through the specific attachment mode.

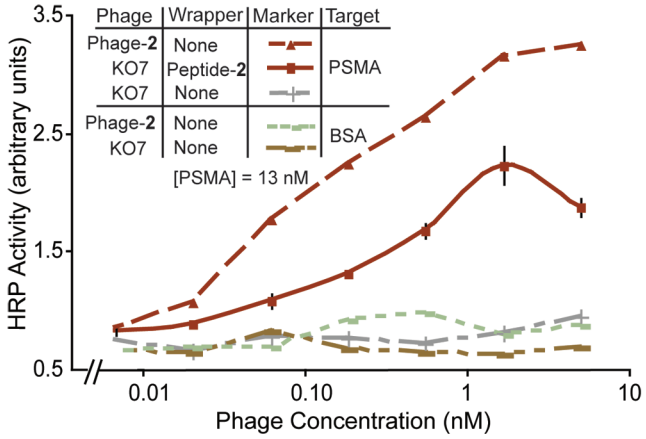

Fig. S8 Phage-based ELISA demonstrating the significance of the free *N*-terminus of peptide-**2** for PSMA binding as shown by the higher affinity of phage-displayed peptide-**2**. Synthesis and

wrapping of oligolysine-peptide-**2** leads to the inversion of geometry providing a free *C*-terminus. The solid line and two of the controls are being reprinted with permission from Mohan, K., Donavan, K. C., Arter, J. A., Penner, R.M., and Weiss, G. A. 2013. Sub-nanomolar Detection of Prostate-Specific Membrane Antigen in Synthetic Urine by Synergistic, Dual-Ligand Phage. J. Am. Chem. Soc. 135:7761–7767. Copyright (2013) American Chemical Society.

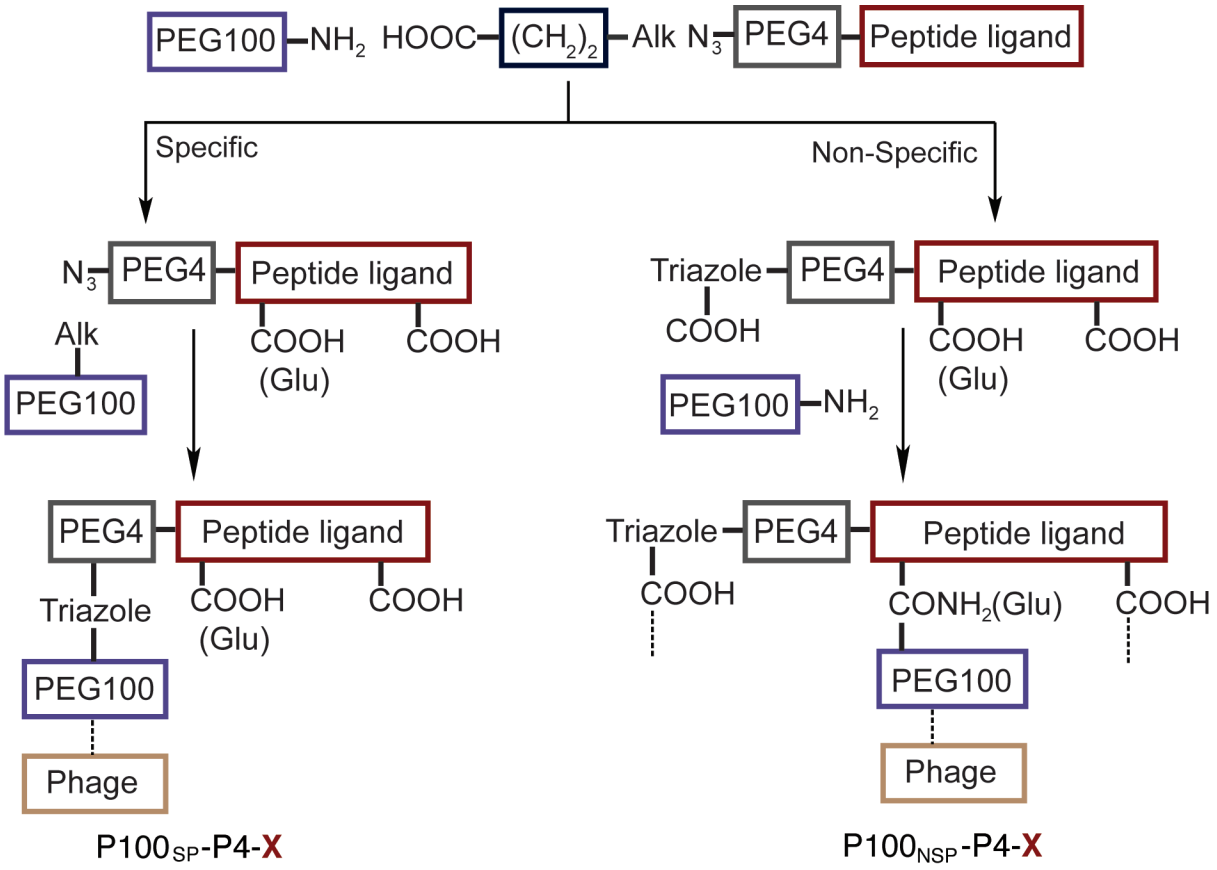

Fig. S9 Schematic representation of the two constitutional isomers of P100-P4-**X**. The differences between the products obtained through the specific attachment mode (left) and the non-specific attachment mode (right) are illustrated. The non-specific attachment mode leads to partially modified Glu sidechains, along with a flexible PEG4 linker. In contrast, the specific attachment mode provides the free Glu side chain with a sandwiched PEG4 linker. The abbreviation 'Alk' in this schematic represents the alkyne group.

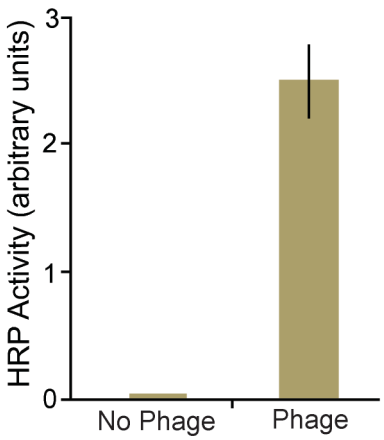

Fig. S10 Phage-based ELISA demonstrating immobilization of phage in the wells of a microtiter plate, as used in the sandwich ELISA assay (Figure 8). The wells were incubated with 100 mL/well of 10 nM phage, and then a BSA blocking solution was used. Levels of bound phage wre quantified using a horseradish peroxidase-conjugated anti-M13 antibody. The negative control has identical conditions without the addition of phage.

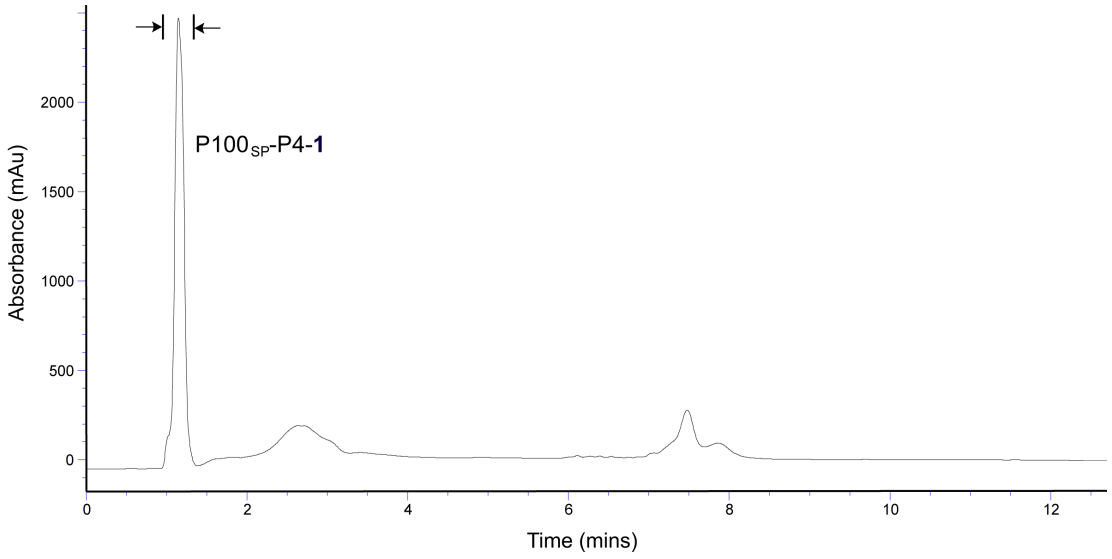

Fig. S11 Representative reverse-phase HPLC for purification of the concentrated reaction mixture to form $P100_{SP}$-P4-**1**. To obtain pure $P100_{SP}$-P4-**1**, the peak designated in the trace was collected and accurate mass determination was performed using gel permeation chromatography. As shown here, the use of 3K and 5K MW cut off concentrators and extensive washing removed a majority of the unreacted starting materials from the reaction mixture.

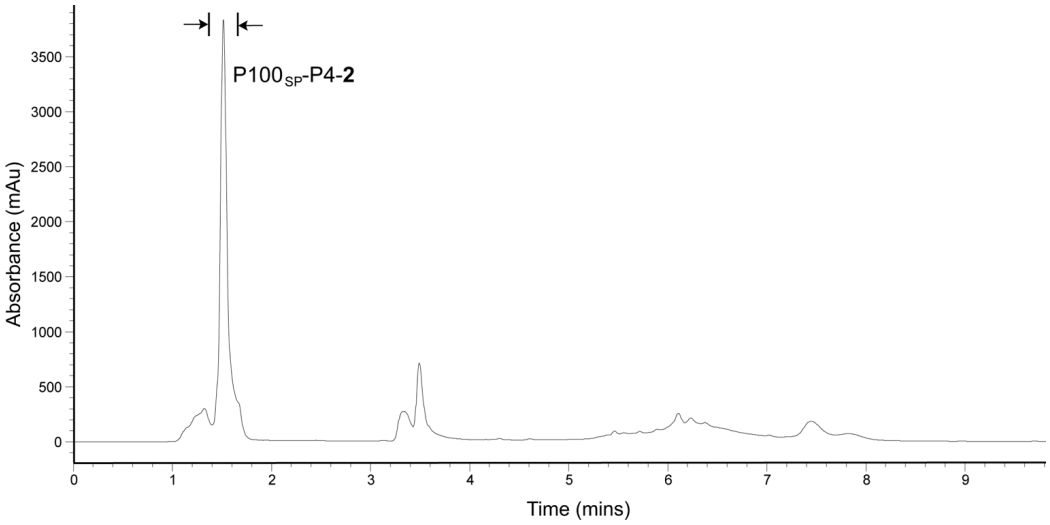

Fig. S12 Representative reverse-phase HPLC for purification of the concentrated reaction mixture for $P100_{SP}$-P4-**2**. As described above, gel permeation chromatography characterized the mass of this PEGylated ligand. As shown here, the use of 3K and 5K MW cut off concentrators and extensive washing removed a majority of the unreacted starting materials from the reaction mixture.

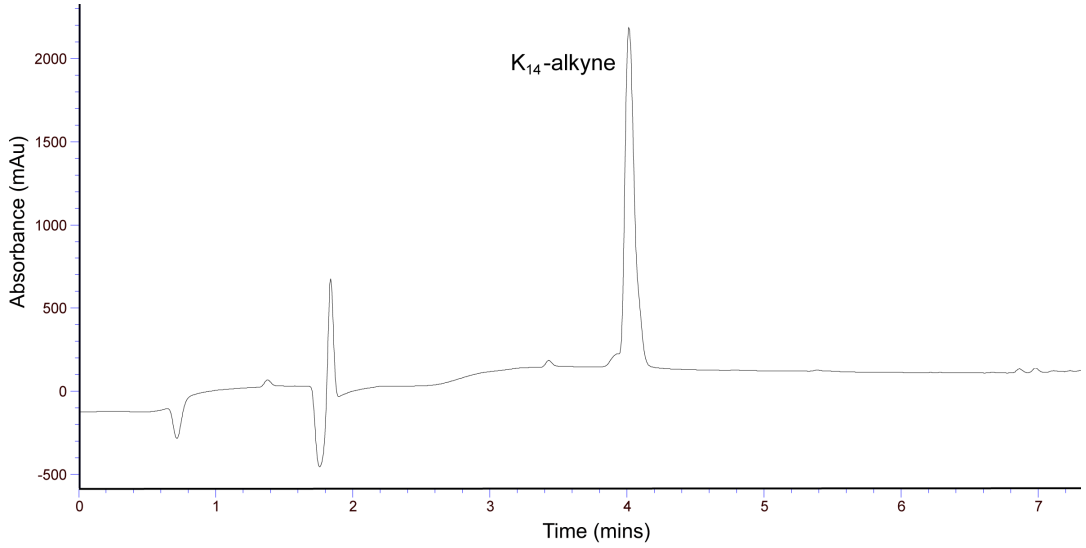

Fig. S13 Representative reverse-phase HPLC analysis of the purified K_{14}-alkyne. Accurate mass determination was performed using MALDI-TOF mass spectrometry. The features in the HPLC chromatogram before the two minute mark are also observed when only water is injected onto the HPLC column; thus, these features do not reflect the purity of the peptide.

Additional References:

1 K. Mohan, K. C. Donavan, J. A. Arter, R. M. Penner and G. A. Weiss, *J. Am. Chem. Soc.*, 2013, **135**, 7761–7767.

2 K. Mohan, R. M. Penner and G. A. Weiss, *Curr. Protoc. Chem. Biol.*, 2015, **7**, 53–72.
3 R. B. Merrifield, *J. Am. Chem. Soc.*, 1963, **85**, 2149–2154.
4 M. Amblard, J.-A. Fehrentz, J. Martinez and G. Subra, *Mol. Biotechnol.*, 2006, **33**, 239–254.
5 Lumiprobe http://www.lumiprobe.com/protocols/click-chemistry-dna-labeling (accessed Sep 7, 2011).
6 V. V Rostovtsev, L. G. Green, V. V Fokin and K. B. Sharpless, *Angew. Chem., Int. Ed.*, 2002, **41**, 2596–2599.
7 S. A. Kularatne, K. Wang, H. R. Santhapuram and P. S. Low, *Mol. Pharm.*, 2009, **6**, 780–789.
8 C. Tovar, B. Higgins, K. Kolinsky, M. Xia, K. Packman, D. C. Heimbrook and L. T. Vassilev, *Mol. Cancer*, 2011, **10**, 49–59.
9 K. Watanabe, T. Joh, K. Seno, M. Sasaki, I. Todoroki, M. Miyashita, K. Tochikubo and M. Itoh, *Clin. Biochem.*, 2001, **34**, 291–295.