Supplementary Information

Rational Design of “Heat Seeking” Drug Loaded Polypeptide Nanoparticles that Thermally Target Solid Tumors

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Materials and Methods

Materials
Restriction enzymes, calf intestinal phosphatase (CIP), and T4 DNA ligase were purchased from New England Biolabs (Ipswich, MA). The pET 24a(+) cloning vector was purchased from Novagen Inc. (Madison, WI), and all custom oligonucleotides were synthesized by Integrated DNA Technologies Inc. (Coralville, IA). The DNA miniprep, gel purification, and PCR purification kits were purchased from Qiagen Inc. (Germantown, MD). EB5α™ and BL21(DE3)™ Escherichia coli cells were obtained from Edge BioSystems (Gaithersburg, MD) and were grown in TBDry™ media (MO BIO Laboratories, Inc; Carlsbad, CA). Chemicals used for the expression and purification of ELPs include: IPTG (Gold Biotechnology; St. Louis, MO), TCEP (Thermo Scientific; Waltham, MA), and kanamycin (CalBioChem; San Diego, CA).

Chimeric Polypeptide Synthesis
The synthetic genes for CPs were assembled from chemically synthesized oligomers (Integrated DNA Technologies Inc.) by plasmid reconstruction recursive directional ligation (PRe-RDL).1 The oligomers used to construct the ELP libraries are described in Figure S1.

Expression of Chimeric Polypeptides
BL21(DE3) cells were transformed with a plasmid that encodes a CP gene and were used to inoculate a 250 mL flask containing 50 mL TBDry media supplemented with 45 µg/mL kanamycin. The Escherichia coli were incubated in a shaker overnight at 200 RPM and 37°C before being used to inoculate six 4-L flasks containing 1 L of TBDry media, supplemented with 45 µg/mL kanamycin. These cultures were incubated in a shaker at 200 RPM at 37°C for 6 h, induced with 0.2 mM IPTG, and grown overnight.

Purification of Chimeric Polypeptides
Escherichia coli expression cultures were centrifuged in 1-L bottles at 4°C for 10 min and 3,000 g to concentrate the cells into a pellet. The supernatant was discarded and the cell pellets were resuspended in 10 mL of PBS. The cells were placed on ice and were lysed by 3 min of sonication (10 s on, 40 s off) (S-4000 Misonix Sonicator; Farmingdale, NY). Polyethyleneamine (PEI; final concentration of 1% w/v) was added to the lysate to precipitate nucleic acid contaminants, and the remaining cell debris was removed from the solution following centrifugation at 14,000 g for 10 min at 4°C. The ELP was then purified by two cycles of inverse transition cycling with minor modifications.2 The supernatant was heated to 60°C for 10 min, thereby triggering the CP phase transition and precipitating contaminant proteins, then immediately placed on ice to redissolve the CP. Insoluble protein was removed by centrifugation (14,000 g, 10 min, 4°C). The supernatant, containing soluble CP, was heated to 37°C, and NaCl crystals were added to a final concentration of 1-3 M to induce the CP phase transition. CP aggregates were precipitated by centrifugation (14,000 g, 10 min, 25°C), and the supernatant was discarded. The CP was then resuspended in 20 mM TCEP (pH 7.4), cooled, and centrifuged (14,000 g, 10 min, 4°C) to remove any remaining insoluble contaminants. This cycle (starting at the 60°C incubation step) was repeated once more to yield the final product. The product was then dialyzed overnight in ddH2O and lyophilized.
Analysis of Chimeric Polypeptides
The purity of the CPs was visually determined by SDS-PAGE, using 4-20% Tris-HCl Ready Gels (Bio-Rad, Hercules, CA) stained with CuCl$_2$ (0.5 M) (Figure S3).

Conjugation of Molecules to Chimeric Polypeptides
15 mg of lyophilized CP was resuspended in 800 µL of 100 mM phosphate buffer, pH 7.4, and spiked with an additional 100 µL of 100 mM TCEP in water, pH 7.4. To generate the CP nanoparticles used to build the model, 100 µL of a 50 mM solution of n-benzyl maleimide in DMSO was added to the CP solution drop-wise, and allowed to mix for 3 h at room temperature. To create fluorescent CP micelles, 0.2 mg of Rhodamine Red-X succinimidyl ester (Life Technologies; Carlsbad, CA) dissolved in 100 µL of DMSO was added to the CP solution drop-wise, and allowed to mix for 3 h at room temperature. 3 mg of n-pyrenyl maleimide in 100 µL of DMSO was then added to the CP-Rhodamine solution dropwise, and allowed to mix for an additional 16 h at room temperature. Following conjugation, the CP conjugate was centrifuged at 13,000 g and 4°C for 10 min and purified by passage through a size exclusion column (PD10, GE Healthcare) and dialysis in ddH$_2$O overnight. The solution was then lyophilized and stored at -20°C for future use.

Thermal Turbidimetry
The optical density at 350 nm (OD$_{350}$) of each CP was measured as a function of temperature on a UV-vis spectrophotometer equipped with a multicell thermoelectric temperature controller (Cary 300, Varian Instruments; Walnut Creek, CA). CP solutions in PBS ranging in concentration from 2 to 100 µM were heated at a rate of 1°C/min. The $T_t$ was defined as the inflection point of the turbidity plot. To measure the transition in 90% fetal bovine serum (FBS), a 500 µM CP solution was diluted 10-fold into the FBS solution.

Conjugation of Doxorubicin
The conjugation of Dox to the CPs was performed as described elsewhere$^3$ with slight modifications. Dox was conjugated to the unique cysteine residues located on the C-terminus of the CP using the heterobifunctional linker n-β-maleimidopropionic acid hydrazide (BMPH; Pierce Biotechnology, Rockford, IL) in a two-step process (Figure S5). First, Dox (215 mg) and BMPH (100 mg) were co-dissolved in 100 mL of anhydrous methanol spiked with 100 µL of trifluoroacetic acid. This solution was stirred for 16 h at 20°C in the dark, dried using rotary evaporation, and then resuspended in 20 mL methanol. In the second step, this solution was immediately added dropwise to a solution containing the CP. Prior to this reaction, the CP was purified as described previously, incubated with a high capacity endotoxin removal resin (Thermo Scientific) for 2 h, and passed through a 0.2 µm filter. The CP solution was then incubated in 50 mM TCEP for 1 h at 20°C to eliminate disulfide crosslinking. Phase separation was triggered by the addition of crystallized NaCl to a final concentration of 1-3 M to remove excess TCEP and concentrate the CP solution. The pellet was then resuspended to a final concentration of 8.6 µmoles of CP in 10 mL of phosphate buffer (100 mM phosphate, 1 mM EDTA, pH 7.4). This solution was stirred for 3 h at 20°C in the dark, spiked with 1 mL of 100 mM TCEP (dissolved in water, pH 7.4), and then stirred for an additional 16 h. To purify the CP-Dox product, the methanol was first evaporated under a constant stream of N$_2$ to a total of 10 mL. A solution of 5 M NaCl was added to the CP-Dox solution to trigger phase separation (a 1:1
The CP-Dox pellet was resuspended in PBS and centrifuged once more to remove any insoluble reactants (14000 g, 10 min, 25°C). Finally, the CP was purified from any remaining free drug reactants by passing the solution through a size exclusion column (PD10, GE Healthcare), dialyzing for 24-48 h (MWCO = 10-12 kDa) in 5 mM ammonium carbonate (pH 8.0), lyophilized, and stored at -20°C for later use. The degree of Dox conjugation to the CP was measured for each sample by resuspending 10–20 mg of lyophilized CP-Dox in 1 mL of PBS, and then dividing the concentration of Dox, determined by UV-vis spectroscopy, by the concentration of CP. The CP concentration was determined gravimetrically from the lyophilized sample by adjusting for the added mass from the attached Dox-linker.

In Vitro Cytotoxicity
C26 murine colon carcinoma cells were maintained in complete media consisting of RPMI-1640 supplemented with 10% FBS, 4.5 g/L D-glucose, 10 mM HEPES, and 1 mM sodium pyruvate. Cells were maintained at 37°C and 5% CO2 and passaged every 3 days. In vitro cytotoxicity was determined through the use of a colorimetric assay. 5x10^3 C26 cells were seeded per 100 µL media on BD Falcon™ 96-well cell culture plates (BD; Franklin Lakes, NJ) and allowed to adhere for 24 h. The cell media was then removed and replaced with 120 µL complete media containing Dox or CP-Dox nanoparticles. The cells were incubated for 72 h at 37°C, after which 20 µL of CellTiter 96 AQueous™ (Promega; Madison, WI) 3-(4,5,6-trimethyl-2-y)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) reagent was added to each well. The cells were incubated for an additional 30 min, and the absorbance of the solution was measured at 490 nm on a Victor3 microplate reader (Perkin Elmer; Waltham, MA). To calculate the IC_{50}, the data were fit to the equation: Viability = 1 / (1 + (C_{Dox}/IC_{50})^p), where C_{Dox} is the effective Dox concentration in the well, the IC_{50} measures the necessary dose to inhibit cell proliferation by 50%, and p represents the slope of the sigmoidal curve.

Pharmacokinetic Analysis
All animal experiments were done in accordance with Duke University's Institutional Animal Care and Use Committee. CP-Dox (15 mg Dox equivalents/kg body weight) was intravenously administered via tail vein into balb/c mice. 10 µL blood was collected at select time points from each mouse via tail vein nick (40 s, 5 min, 15 min, 2 h, 4 h, 8 h, 24 h, 48 h) and diluted into 100 µL of heparin in PBS (1000 U/mL). The samples were centrifuged (1000 g, 5 min, 4°C) to remove the red blood cells, and the Dox was released from the CP by incubating 10 µL of diluted plasma in 490 µL of acidified isopropanol (75 mM HCl, 90% isopropanol) for 16 h at 4°C. The solution was loaded into a 96-well microplate in triplicate (125 µL per well), and Dox fluorescence was determined on a Victor3 microplate reader (485 nm excitation, 590 nm emission). Plasma concentration was fit to a two compartment model to determine the pharmacokinetic parameters using SAAM II software (University of Washington, Seattle, WA).

Biodistribution Analysis
Balb/c mice were inoculated with C26 tumors on their right hind leg (2.5x10^6 cells in 30 µL). Once the tumors reached ~100 mm^3 (approximately 9 days following inoculation), the mice were anaesthetized with 50 mg/kg body weight of nembutol (i.p. administration), and administered CP-Dox (15 mg Dox equivalents/kg body weight) via tail vein injection. The mice were placed in a custom hyperthermia holder with a rectal temperature probe to monitor the core body
temperature and a plastic shield to prevent direct skin contact with the heated water. The mice were placed in a water bath set to 42.5°C, such that the tumor bearing leg was submerged just far enough to completely cover the tumor mass. A fan was used to regulate the body temperature.

To determine whole body biodistribution (Figure S7A), the mice were exposed to 1 h of continuous hyperthermia and were sacrificed 24 h following CP-Dox administration. To elucidate the effect of thermal cycling on tumor accumulation, mice were exposed to various hyperthermia schedules (Figure 5) and were sacrificed 2 h following CP-Dox administration. Tissues were collected (heart, lungs, kidney, spleen, liver, muscle, and tumor), and 75-100 mg of tissue was weighed and suspended in 1 mL of acidified isopropanol (75 mM HCl, 90% isopropanol). The solution was homogenized using 2 mm Zirconia beads and a Beadbeater-16 (Biospec, Bartlesville, OK) for 120 s. The samples were incubated at 4°C for 16 h in the dark to release conjugated Dox. The samples were then centrifuged (4°C, 10 min, 14000 g), and the supernatant was fluorescently assayed as described in the pharmacokinetic analysis. Untreated tissues were similarly extracted and used to create a background curve (fluorescent counts/mg tissue) to compensate for tissue autofluorescence.

**Window Chamber and Microscopy**

Nude mice were anaesthetized with a cocktail of ketamine and xylazine (100 mg/kg ketamine, 10 mg/kg xylazine, *i.p.*) and implanted with a dorsal skin fold window chamber. A titanium chamber was surgically implanted over a 1-cm circular incision in the dorsal skin fold. FaDu human squamous carcinoma cells at a concentration of 2×10⁶ cells in 30 µL of high glucose MEM were injected near the center of the window in the opposing layer of skin. The incision was then covered with a circular glass coverslip. All studies were performed 9-10 days following surgery, once the tumor reached a diameter of 2-3 mm.

To image the tumor vasculature, the mice were anaesthetized with 50 mg/kg body weight of nembutol and placed laterally recumbent upon a custom microscopy stage that enabled heating of the window chamber. The tail vein was cannulated and 100 µL of 5 mg/mL 2 MDa dextran-fluorescein isothiocyanate (Invitrogen; Carlsbad, CA) was injected, followed by 200 µL of 425 µM CP nanoparticles (target plasma concentration of 50 µM) labeled with Rhodamine Red™-X Succinimidyl Ester (Invitrogen). The temperature of the window was maintained at 37°C (physiological temperature) or 42°C (temperature achieved through mild hyperthermia). Images were taken with a Zeiss 780 upright confocal microscope. Data were obtained using two channels, in which dextran-fluorescein isothiocyanate was used to define the vasculature (Ex: 490 nm, Em: 525 nm), and the rhodamine channel was used to view the distribution of CP nanoparticles (Ex 540 nm: Em 625 nm). Upon injection of CPs at 37°C, fluorescent levels were adjusted to provide a yellow overlay.

Movies were obtained using a SX160 HS Canon Powershot held up to the eyepiece of the Zeiss 780 confocal with a 10x objective. The CP nanoparticles tagged with rhodamine were visualized using an X-Cite fluorescent illuminator with a red filter cube (43 Cy3/Rhod/RFP (D) EX BP 545/25, BS FT 565, EM BP 606/70).
**Data Analysis**
Non-linear regression analysis was performed with IBM SPSS Statistics 19.0 (SPSS, Chicago, IL). MATLAB (MathWorks; Natick, MA) was used to generate the 3-dimensional plot of the predicted $T_1$ as a function of composition and chain length.
Leader Sequence

ATG AGC AAA GGG CCG GGC

(M) S K G P G

Drug Conjugation Sequence

TGC GGT GGT TGC GGC GGT GGT CTC TGC GGC GGT TGC GTC GGC CCG TGG CCG TGA TAA

G W P...

\[ f \text{ Alanine } = 1 \]

\[ \text{GCC GGA GTG CCT GGT GGA GTG CCA GGC GGC GGT GTT CCA GGA GCA GGC GTT CCT CCG} \]
\[ A G V P G A G V P G A G V P G A G V P G \]

\[ \text{GCC GGA GTG CCT GGT GCA GGT GTG CCA GGC GGC GGT GTT CCA GGA GCA GGC GTT CCT CCG} \]
\[ A G V P G A G V P G A G V P G A G V P G \]

\[ f \text{ Alanine } = 0.9 \]

\[ \text{GCC GGA GTG CCT GGT GCA GGT GTG CCA GGC GGC GGT GTT CCA GGA GCA GGC GTT CCT CCG} \]
\[ A G V P G A G V P G A G V P G A G V P G \]

\[ \text{GCC GGT GTT CCG GGC GCC GGC GTG CCA GGC GCC GGT GTT CCA GGA GCA GGC GTT CCT CCG} \]
\[ A G V P G A G V P G A G V P G A G V P G \]

\[ f \text{ Alanine } = 0.8 \]

\[ \text{GCC GGA GTG CCT GGT GCA GGT GTG CCA GGC GGC GGT GTT CCA GGA GCA GGC GTT CCT CCG} \]
\[ A G V P G A G V P G A G V P G A G V P G \]

\[ \text{GCC GGA GTG CCT GGT GCA GGT GTG CCA GGC GGC GGT GTT CCA GGA GCA GGC GTT CCT CCG} \]
\[ A G V P G A G V P G A G V P G A G V P G \]

\[ f \text{ Alanine } = 0.7 \]

\[ \text{GCC GGA GTG CCT GGT GCA GGT GTG CCA GGC GGC GGT GTT CCA GGA GCA GGC GTT CCT CCG} \]
\[ A G V P G A G V P G A G V P G A G V P G \]

\[ \text{GCC GGA GTG CCT GGT GCA GGT GTG CCA GGC GGC GGT GTT CCA GGA GCA GGC GTT CCT CCG} \]
\[ A G V P G A G V P G A G V P G A G V P G \]
Figure S1: Gene sequence of CP micelles that assemble through drug conjugation. The CP constructs consist of a leader sequence (MSKGP) followed by an elastin-like polypeptide sequence. The methionine is cleaved during expression. A short cysteine-rich trailer was appended to the C-terminus (CGG)₈WP to both allow site specific drug conjugation to the unique cysteine residues and permit A₂₈₀ nm protein quantification via the tryptophan residue (ε = 5630 mol⁻¹ cm⁻¹). The repeat unit n=4, 8, and 16 represent the 40, 80, and 160 pentamer sequences, respectively.
Figure S2: CP gene libraries. CP genes were run on a 1% agarose gel and stained with Sybr Safe. The left and right lanes represent a size standard ladder with the length (kbp) shown on the left. The remaining lanes represent diagnostic digests of the constructs restricted with BamHI-HF and XbaI (hence appending 66 bp of flanking sequences to each band). The composition of the guest residue ratio and the expected length of the CP constructs (shown in pentamers and basepairs) are displayed on the bottom.
Figure S3: CP micelle expression libraries. Proteins purified by 2-3 cycles of inverse transition cycling were run on an SDS-PAGE gel and stained with CuCl₂. The left lane is the Bio-Rad Kaleidoscope Protein Ladder, with the molecular weights (in kDa) shown on the left. The remaining lanes represent the purified proteins with the guest residue ratio (composition), and length (pentamers and kDa) shown beneath their respective lanes. The dimer and trimer bands that appear above the primary band are indicative of the formation of cysteine-cysteine disulfide bonds between the drug conjugation domains of different ELP chains.
Figure S4: Thermal properties for CP micelle libraries. Each CP construct was conjugated to n-benzylmaleimide and purified, at which point the transition temperature was measured in PBS as a function of CP concentration. The dashed lines represent the predicted transition temperatures derived from the CP micelle model (manuscript Equation 1).
Table S1: Phase transition temperature of three CP nanoparticles of varying molecular weight in PBS in response to conjugation to three compounds

| fAlanine | Length | Model | n-benzyl maleimide | n-pyrenyl maleimide | Doxorubicin |
|----------|--------|-------|--------------------|---------------------|-------------|
| 1        | 160    | 48.7  | 42.7 (0.6)         | 43.2 (0.1)           | 42.9 (1.1)  |
| 0.8      | 80     | 43.6  | 40.5 (0.2)         | 40.7 (0.1)           | 39.8 (0.4)  |
| 0.6      | 40     | 42.0  | 40.5 (0.7)         | 40.8 (0.2)           | 39.8 (0.3)  |

Data reported as estimate (standard error)

Table S2: Phase transition temperature of three CP nanoparticles of varying molecular weight in 90% FBS in response to conjugation to three compounds

| fAlanine | Length | n-benzylmaleimide | n-pyrenylmaleimide | Doxorubicin |
|----------|--------|-------------------|--------------------|-------------|
| 1        | 160    | 42.7 (0.6)        | 43.2 (0.1)         | 42.9 (1.1)  |
| 0.8      | 80     | 40.5 (0.2)        | 40.7 (0.1)         | 39.8 (0.4)  |
| 0.6      | 40     | 40.5 (0.7)        | 40.8 (0.2)         | 39.8 (0.3)  |

Data reported as estimate (standard error)
Figure S5. Schematic to conjugate doxorubicin to an ELP via the acid-labile linker BMPH.
Figure S6: *In vitro* toxicity against a C26 murine colon carcinoma line for (A) free doxorubicin, (B) 40 pentamer construct conjugated to Dox, (C) 80 pentamer construct conjugated to Dox, and (D) the 160 pentamer construct conjugated to Dox. Data shown as mean ± SD averaged over three independent experiments. The dashed line represents the best fit of the equation Viability = 1 / (1 + (C\text{Dox}/IC_{50})^\beta), where C\text{Dox} is the effective Dox concentration in the well, the IC_{50} measures the necessary dose to inhibit cell proliferation by 50%, and \beta represents the slope of the sigmoidal curve.

Table S3: Pharmacokinetic parameters for CP-Dox micelles of various chain lengths

| Parameter                        | Symbol     | Chain Length (Pentamers) |
|----------------------------------|------------|--------------------------|
| initial concentration            | Co [µM]   | 229.1 (13.9) 224.8 (12.4) 293.1 (12.6) |
| distribution half-life           | α t\text{1/2} [min] | 33.6 (9.0) 126.7 (67.2) 71.9 (22.2) |
| elimination half-life            | β t\text{1/2} [hr] | 9.6 (0.4) 12.1 (1.0) 15.5 (0.9) |
| area under the curve             | AUC [µM·hr] | 1202.9 (42.8) 2690.5 (119.6) 3734.6 (112.3) |
| plasma clearance                 | CL [µL·hr⁻¹·g⁻¹] | 0.15 (.01) 0.07 (0.00) 0.05 (0.00) |
| initial volume of distribution   | Vo [µL·g⁻¹] | 0.77 (0.05) 0.78 (0.04) 0.60 (0.03) |
| tissue to plasma rate constant   | k\text{tp} [hr⁻¹] | 0.47 (0.12) 0.23 (0.13) 0.33 (0.11) |
| plasma to tissue rate constant   | k\text{pt} [hr⁻¹] | 0.65 (0.21) 0.08 (0.05) 0.21 (0.07) |
| elimination rate constant        | k\text{e} [hr⁻¹] | 0.19 (0.01) 0.08 (0.01) 0.08 (0.00) |
Figure S7. Distribution of doxorubicin within (A) select organs and (B) within the heart and tumor (zoomed in for clarity) 24 h post administration. Data represent mean ± SD with n=3 mice. Significance (one-way ANOVA, Tukey post-hoc) was calculated for the 40 and 80 pentamer constructs for all time points. *p<0.05 and **p<0.005 against the 160 pentapeptide construct.
Figure S8: *In vivo* visualization of the phase transition in response to heat. (A) 160 pentamer CP construct with X=A ($T_t = 42^\circ C$) held at $42^\circ C$ for 30 min and (B) 160 pentamer construct with X=A$_9$V$_1$ ($T_t = 39^\circ C$) held at $42^\circ C$ for 10 min. Scale bars represent 100 $\mu m$.

Videos S1-3. Phase separation of CP-Rhodamine nanoparticles ($T_t = 39^\circ C$) in vasculature held at (1-2) $42^\circ C$ and (3) $37^\circ C$. The field of view represents 2.5 mm.
Figure S9: Confocal images of tumor vasculature containing 2 MDa dextran (green) and CP-Rhodamine (red) as the vasculature is heated from 37 to 42°C and then cooled back to 37°C. The scale bars represent 100 nm.
References:

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