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The Weak Link: Optimization of the Ligand-Nanoparticle Interface To Enhance Cancer Cell Targeting by Polymer Micelles

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Abstract

Many promising targeting ligands are hydrophobic peptides, and these ligands often show limited accessibility to receptors, resulting in suboptimal targeting. A systematic study to elucidate the rules for the design of linkers that optimize their presentation on nanoparticles has not been carried out to date. In this study, we recombinantly synthesized an elastin-like polypeptide diblock copolymer (ELP_{BC}) that self-assembles into monodisperse micelles. AHP and EC1, two hydrophobic ErbB2-targeted peptide ligands, were incorporated at the C-terminus of the ELP_{BC} with an intervening peptide linker. We tested more than 20 designs of peptide linkers, where the linker could be precisely engineered at the gene level to systematically investigate the molecular parameters—sequence, length, and charge—of the peptide linker that optimally assist ligands in targeting the ErbB2 receptor on cancer cells. We found that peptide linkers with a minimal length of 12 hydrophilic amino acids and an overall cationic charge—and that impart a zeta potential of the micelle that is close to neutral—were necessary to enhance the uptake of peptide-modified ELP_{BC} micelles by cancer cells that overexpress the ErbB2 receptor. This work advances our understanding of the optimal presentation of hydrophobic ligands by nanoparticles and suggests design rules for peptide linkers for targeted delivery by polymer micelles, an emerging class of nanoparticle carriers for drugs and imaging agents.

Graphical Abstract

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Supporting Information

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Notes

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Keywords

Linker optimization; ErbB2; hydrophobic ligand; elastin-like polypeptide; affinity targeting; nanoparticle

Many solid tumors overexpress specific receptors or tumor-associated antigens on their cell surface.¹ Ligands that bind these targets on the tumor cell surface with high affinity and specificity can be harnessed for the tumor specific delivery of drugs and imaging agents, an approach that is termed “affinity targeting”.^{2,3} Affinity targeting has been extensively explored for decades in the context of cancer imaging and therapy, with some, albeit, modest success.^{4,5} The most important class of affinity targeting agents that have been developed, especially for therapy, are monoclonal antibodies.⁶ Peptide ligands have also been designed that provide an alternative to antibodies for targeting, motivated by the fact that peptides are often more convenient to incorporate, than an entire antibody, into multicomponent delivery systems for drugs and imaging agents.^{7,8}

However, peptide ligands for targeting suffer from three problems for drug delivery.⁹ The first problem, and one that is common to all peptides, is their fast elimination from systemic circulation in vivo because of their low molecular weight and corresponding fast renal clearance. The second problem is that they typically have a lower affinity for their target receptor than antibodies. The third problem of many peptides is their hydrophobicity, which limits their utility as ligands for targeted drug delivery systems. The incorporation of peptide ligands into nanoparticle drug delivery systems can easily solve the first two problems because they increase the apparent size of the peptide by presentation on a nanoparticle and can hence retard the clearance of the peptide from systemic circulation.¹⁰ The presentation of multiple copies of the peptide on the surface of a nanoparticle can also increase its “avidity” because of multivalent binding.¹¹ Peptide-functionalized nanoparticles also enable two stage targeting of tumors: passive targeting of the tumor tissue via the enhanced permeability and retention (EPR) effect that enhances the accumulation of the nano-particle in the tumor,¹² and a second stage of targeting wherein the peptide ligand that is presented by the nanoparticle can target tumor cells via ligand-receptor binding, which can promote cell uptake of nanoparticles into tumor cells via receptor-mediated endocytosis.¹³

The third problem—hydrophobicity of the peptide—is perhaps the most refractory to an easy solution. In principle, presenting a hydrophobic ligand on the surface of a nanoparticle should enable presentation of the ligand to its cognate receptor on the cell surface. This is an important issue, because many peptide ligands isolated by phage display techniques¹⁴ or designed computationally¹⁵ have high affinity and specificity for their cognate receptor but are hydrophobic, which limits their utility as targeting agents.^{7,8} This is because hydrophobic ligands preferentially avoid interactions with water, and the precise stratagems they choose to avoid water depend upon their interfacial context. For example, hydro-phobic ligands that are tethered at the hydrophilic end of an amphiphilic diblock copolymer can bury themselves into the micelle during self-assembly of amphiphilic copolymers, thereby limiting their accessibility to their cognate receptor. It has been reported that only 20% of hydrophobic folate ligands are accessible at the surface of a poly(lactide-*co*-glycolide)-*b*-polyethylene

glycol (PLGA-PEG) micelle, with the rest presumably buried in the micelle core due to the hydrophobic interaction between the folate ligand and the micelle.¹⁶ Another study reported that without incorporating a hydrophilic linker and optimizing the PEG length, liposomes and phosphoethanolamine-polyethylene glycol (PE-PEG) micelles whose exterior is functionalized with AHNP, a hydrophobic ErbB2-targeted peptide ligand, did not exhibit enhanced cell uptake on ErbB2 overexpressing cancer cells compared to their unfunctionalized controls, presumably because the hydrophobic AHNP ligands were not accessible at the surface of the liposomes and PE-PEG micelles.^{17,18}

The introduction of a hydrophilic linker can optimize the presentation of a hydrophobic ligand to maximize its accessibility to its receptor by reducing the overall hydrophobicity at the micelle-water interface, which can promote colloidal stability.^{17,18} However, a comprehensive and systematic study of the molecular properties of peptide linkers that can optimize the presentation of hydrophobic peptide ligands on a nanoparticle has not been carried out to date. Motivated by this lacuna in the field, herein we systematically investigate the molecular design of peptide linkers that optimally target the ErbB2 receptor on cancer cells by two hydrophobic peptide ligands.

To investigate this problem, we selected a nanoparticle system that is self-assembled from a single, genetically encoded polypeptide chain that consists of three components from the N- to C-terminus: (1) a self-assembling polypeptide segment that is attached to (2) a peptide linker, and which, in turn, is attached to (3) a peptide ligand (Scheme 1A). Because the polypeptide polymer is genetically encodable, it yields perfectly monodisperse polymers, and the ligand, linker, and polymer sequence can be all independently specified and modified at the gene level. The self-assembling segment that drives the formation of micelles consists of an elastin-like polypeptide diblock copolymer (ELP_{BC}), a temperature-triggered amphiphilic copolymer that self-assembles into micelles, and that can sequester drugs or imaging agents which are chemically conjugated to the ELP in the core of the micelle.^{19–22} ELPs are a class of peptide polymers composed of repeats of the XGVPG pentapeptide, where X is a guest residue that can be any amino acid except proline.²³ In an appropriately designed ELP_{BC} that consists of two ELP segments with different guest residues (X) and hence hydrophobicity, an increase in the solution temperature selectively desolvates the more hydrophobic block, and at a specific temperature, defined as the critical micelle temperature (CMT), the ELP_{BC} gains sufficient amphiphilicity to self-assemble into monodisperse sub-100 nm diameter spherical micelles in aqueous solution.^{24–27} The ELP_{BC} is especially useful for this study because it is genetically encodable, so that it is perfectly monodisperse, and the ligand, linker, and ELP sequence can all be independently specified and modified at the gene level, which allows different linker designs to be easily screened. Furthermore, the structure of ELP micelles have been extensively characterized by light and neutron scattering and cryogenic transmission electron microscopy (cryo-TEM),^{22,28} as well as their in vitro stability and in vivo pharmacokinetics,^{19,21,22} making them among the best characterized nanoparticle systems that are currently available, especially for in vivo applications.

The receptor that we chose for this study, ErbB2, is a protooncogene cell surface receptor that is useful for tumor targeting. ErbB2 is overexpressed in ~30% of breast, ovarian, and

colon cancers, and up-regulated ErbB2 correlates with increased cell proliferation, reduced apoptosis, progressive metastasis, and poor patient prognosis.^{29,30} In the clinic, to predict patient response to different types of treatment and predict long-term patient prognosis, the ErbB2 status of breast cancers is characterized by immunohistochemistry by scoring tumor biopsies on a scale of 0 to +3 prior to treatment. Scores of 0 and +1 are considered indicative of a ErbB2 negative status, while +2 and +3 are ErbB2 positive.³¹ Patients with ErbB2 positive breast cancer are usually offered treatment with a monoclonal antibody, such as trastuzumab that binds to ErbB2 and inhibits the proliferation and differentiation of ErbB2-overexpressing cancer cells.³²

We selected two breast cancer cell lines to investigate ligand/receptor binding and ligand-triggered uptake: SK-BR-3 human breast cancer cells that are known to overexpress ErbB2, and MCF-7 human breast cancer cells that show low, baseline level of expression of the ErbB2 receptor, and which hence served as a negative control.³³ We confirmed the expression level and the cellular localization of the ErbB2 receptor on SK-BR-3 breast cancer cells and the lack of expression on MCF-7 breast cancer cells by immunofluorescence. Cells were fixed, permeabilized, and stained with a PE-conjugated anti-ErbB2 antibody, and the cellular ErbB2 expression level was then quantified by flow cytometry, and the cellular localization of ErbB2 was also independently visualized by confocal fluorescence microscopy (Figure S1). As expected, ErbB2-positive SK-BR-3 cancer cells show much higher ErbB2 expression than the ErbB2-negative MCF-7 cancer cells that only express a low, baseline level of ErbB2 (Figure S1). Confocal fluorescence microscopy of cells incubated with fluorescently labeled antibody for the ErbB2 receptor showed that the cell membrane of SK-BR-3 cancer cells are clearly delineated because of the binding of the antibody to the overexpressed receptors on the cell membrane of SK-BR-3 cancer cells. In contrast, there was no visible binding of the antibody to the cell membrane of MCF-7 cancer cells (Figure S1).

ErbB2-targeted peptides are an attractive alternative to antibodies for use as targeting ligands on nanoparticles. Of the several available ErbB2-targeted peptide ligands, we chose AHNP and EC1 peptides. AHNP (amino acid sequence: FCDGFYACYMDV) was designed based on the heavy-chain CDR3 loop of trastuzumab antibody^{17,18,34–38} while the EC1 peptide (amino acid sequence: WTGWCLNPEESTWGFCTGSF) was discovered by in vitro phage display.^{39–41} Both AHNP and EC1 have submicromolar binding affinity for ErbB2, and their binding accelerates cellular internalization of these peptides,^{17,18,34–41} so that they can be used as targeting ligands to enhance the intracellular delivery of drug cargo carried by nanoparticles decorated with these peptides. However, both peptides are quite hydrophobic as they are abundant in tryptophan (W) and tyrosine (Y) residues; they are insoluble in aqueous buffer and are likely to be buried into the hydrophobic layer/core of the nanoparticle,^{17,18} which limits their utility as targeting moieties in nanoparticle drug delivery systems. For these reasons, it also makes them ideal ligands to test the central hypothesis of this study that engineering the ligand-nanoparticle interface can optimize their presentation and rescue their function.

Building upon the genetically encoded ELP_{BC} micelle system, we carried out what may possibly be the most comprehensive study of the peptide ligand-nanoparticle interface to

date, by testing more than 20 different designs of peptide linkers between the ErbB2-targeting peptide ligand and the ELP_{BC} micelle, where the sequence, length, and charge—and thereby the physicochemical properties—of the linker could be precisely engineered at the molecular level, to investigate their impact on the binding of peptide ligand-decorated micelles to ErbB2 receptors and ligand-triggered cell uptake by ErbB2-positive SK-BR-3 breast cancer cells.

The parent ELP_{BC} used in this study consists of 40 repeats of the hydrophobic pentapeptide XGVPG (X = W:V = 1:4) at the N-terminus and 40 repeats of a hydrophilic pentapeptide SGVPG at the C-terminus (Scheme 1A). Various peptide linkers were encoded C-terminal to the parent ELP_{BC} in a modified pET-24a vector using recursive directional ligation by plasmid reconstruction (Pre-RDL) method, as described previously.⁴² The peptide ligand was encoded at the C-terminus of the linker, yielding a set of gene constructs encoding ELP_{BC}-linker-ligand from the N- to C-terminus so that the peptide ligand is presented at the hydrophilic terminus of the ELP_{BC} (Scheme 1A). Each gene was overexpressed in BL21(DE3) *E. coli* cells that were transformed with a modified pET-24a plasmid that encodes the constructs. Purified ELP_{BC}-linker or ELP_{BC}-linker-ligand fusions were obtained after multiple rounds of inverse transition cycling (ITC) purification.⁴³ Because the AHP or EC1 peptide ligand includes two cysteine residues, ELP_{BC}-linker-ligand proteins purified by ITC have monomer, dimer, and higher oligomers due to the formation of intramolecular and intermolecular disulfide bonds. To obtain a pure monomer population of ELP_{BC}-linker-ligand, the ITC product was further purified by size-exclusion chromatography (SEC) to separate monomer from dimer and higher oligomers. As an example, Figure S2 shows the SEC elution curve of ELP_{BC}-K₈D₄-EC1, and a SDS-PAGE image of purified ELP_{BC}-K₈D₄-EC1 and ELP_{BC}-K₈D₄ which, as expected, shows a single band.

Using pyrene as a fluorescence probe, we determined that the critical micelle concentration (CMC) of the parent ELP_{BC} at 37 °C was ~6 μ M (Figure S3), and adding a linker at the hydrophilic end of the parent ELP_{BC} did not change the CMC (Table S1). We next characterized the thermal behavior, size, and zeta potential of a 20 μ M concentration solution of ELP_{BC} micelles at 37 °C. Their thermal phase behavior was characterized by turbidity measurements on a UV-vis spectrophotometer as a function of solution temperature; their size was determined by dynamic light scattering (DLS) and cryo-TEM, and their zeta potential was measured on a Zetasizer. These data for all ELP_{BC}-linker combinations studied herein are summarized in Table S1.

Temperature-programmed turbidity of a 20 μ M solution of the parent ELP_{BC} was measured by monitoring the optical density of the ELP_{BC} at 350 nm (OD₃₅₀ nm) from 10 to 80 °C. Figure 1A shows that the parent ELP_{BC}, which has no peptide linker or ligand at its hydrophilic C-terminus, exhibits two inflection points in its turbidity versus temperature profile at 24 °C (T_i -1) and 66 °C (T_i -2). The temperature at the first inflection point, defined as T_i -1, corresponds to the collapse of the more hydrophobic block [(XGVPG)₄₀, X = W:V = 1:4] in the ELP_{BC}, which drives self-assembly of the block copolymer into micelles, consistent with previous observations for this class of block copolymers,^{24–27} and this temperature is defined as the critical micelle temperature (CMT) (Figure 1A). The second

inflection point at 66 °C (T_i-2) corresponds to the temperature at which the hydrophilic segment ((SGVPG)₄₀) of the diblock ELP that forms the corona of the micelle desolvates sufficiently to trigger aggregation of the micelles into larger micron size aggregates and corresponds to the cloud point temperature of the polymer. Incorporating a hydrophilic peptide linker at the C-terminus of the parent ELP_{BC}, such as eight lysine residues and four aspartic acid residues (K₈D₄), did not affect the CMT (Figure 1A). However, the T_i-2 of the ELP_{BC}-K₈D₄ increases significantly to >80 °C, indicating that appending a hydrophilic linker only impacts the thermal behavior of the hydrophilic block but not the hydrophobic block, and the increased T_i-2 indicates greater solvation, and hence increased hydrophilicity of the corona of the micelle. This observation of an invariant T_i-1 (or CMT) but an increase in T_i-2 is true for all the other ELP_{BC}-linker constructs as well, that present different hydrophilic and charged peptide linkers, as well as mixed micelles that present two different linkers on their corona (Table S1). This is important as it shows that modifying the corona of the micelle by swapping ligands or linkers does not impact self-assembly, and that at 37 °C, all constructs are micelles (Figure S4, Table S1).

DLS as a function of solution temperature confirms these findings, as the parent ELP_{BC} in aqueous buffer has a hydrodynamic radius (R_h) of ~5 nm that is consistent with soluble polymer chains (Figure 1A). When the temperature is between 24 and 66 °C, the parent ELP_{BC} self-assembles into micelles with a R_h of ~20 nm in aqueous buffer that is driven by the intermolecular interaction between the desolvated hydrophobic blocks. Cryo-TEM images shows spherical particles of ~14 nm in radius (Figure 1B); although this size is somewhat smaller than the ~20 nm R_h measured by DLS, cryo-TEM only visualizes the hydrophobic and desolvated component of the ELP_{BC} micelles, as the solvated corona has poor electron contrast in cryo-TEM. These results are also consistent with our previous studies of this class of ELP_{BC} by static light scattering and cryo-TEM which have shown that these micelles are spherical.^{24–27} The DLS data are corroborated by the slight increase in the OD_{350nm}, that is consistent with the greater light scattering of the micelles compared to soluble ELP_{BC} unimers in this temperature range (Figure 1A). At 37 °C in PBS, the parent ELP_{BC} micelles have a very narrow size distribution centered at a R_h of ~20 nm (Figure S4, Table S1). The incorporation of a K₈D₄ peptide at the hydrophilic C-terminus does not impact self-assembly, as the ELP_{BC}-K₈D₄ fusion also has a R_h of ~20 nm (Figure 1A) and cryo-TEM shows particles with a radius of ~14 nm (Figure 1B). The R_h of all the other constructs at 37 °C are summarized in Table S1, and the size distribution for each construct is shown in Figure S4. Despite the different peptides that were presented by these constructs, there is no difference in their R_h , as all values are close to the 20 nm R_h of the parent ELP_{BC}, indicating that presentation of a short peptide on the micelle surface has no appreciable impact on self-assembly into spherical micelles or the size of the micelle.

The parent ELP_{BC} micelle has a negative zeta potential of -6.9 ± 0.4 mV at 37 °C (Table S1), consistent with the fact that the corona of the micelles is terminated by C-terminal carboxyl groups—contributed by each ELP chain in the micelle—that are charged at physiological pH (Scheme 1). Unlike the R_h or CMT or CMC that is insensitive to structural modifications at the solvent exposed C-terminus of the micelles, the zeta potential values are different between constructs depending on the linkers (Table S1). Incorporating positively charged peptide linkers at the corona of the micelle, such as K_m, increases the zeta potential

of ELP_{BC} micelles to close to neutrality, while attaching negatively charged peptide linkers, such as D_n, decreases the zeta potential of ELP_{BC} micelles to more negative values (Table S1).

We first prepared ELP_{BC}-AHNP micelles—that do not contain a peptide linker between the AHNP ligand and the ELP_{BC}—and compared their uptake by SK-BR-3 cancer cells with parent ELP_{BC} micelles. To track the micelles, 2 mol % Cy5-labeled ELP_{BC} was mixed with unlabeled ELP_{BC} and heated in aqueous buffer to 37 °C to drive self-assembly. This procedure yielded ~20 nm *R_h* micelles as measured by DLS (Figure S4). Next, the micelles were incubated with SK-BR-3 cancer cells at 37 °C for 1 h, and the cell fluorescence of Cy5 was quantified by flow cytometry. Fluorescently labeled parent ELP_{BC} micelles were prepared similarly and were used as a negative control in cell uptake studies. However, no difference in internalization was observed between ELP_{BC}-AHNP micelles and parent ELP_{BC} micelles on SK-BR-3 cancer cells at 37 °C (Figure 2). This result suggests that presentation of the hydrophobic AHNP ligand directly on the hydrophilic end of the ELP_{BC} micelle does not allow steric access of the AHNP ligand to the ErbB2 receptor. We hypothesized that the failure of the AHNP ligand to be effectively presented on the corona of ELP_{BC} micelles is caused by the hydrophobic interactions of the AHNP ligand with the hydrophobic block of the ELP_{BC}, which presumably buries the AHNP ligand within the core of the ELP_{BC} micelle during micelle self-assembly (Scheme 1B, top).

To display the AHNP ligand at the ELP_{BC} micelle surface in an orientation that makes it sterically accessible to its receptor, we next introduced a hydrophilic peptide linker between the ELP_{BC} carrier and the AHNP ligand (Scheme 1B, bottom). We began with two groups of peptide linkers that have been previously used in other studies: (1) hydrophilic flexible (GGSG)_n peptide linkers of varying lengths, which provide high mobility between the functional domains that they connect;⁴⁴ and (2) rigid α -helix forming A(EAAAK)_nA peptide linkers, that are typically chosen when the spatial separation of the protein domains is critical to preserve the stability or bioactivity of the fusion proteins.⁴⁴

We synthesized ELP_{BC}-AHNP with (GGSG)₁, (GGSG)₃, A(EAAAK)₂A, and A(EAAAK)₄A linkers appended at the C-terminus of the parent ELP_{BC}, as well as negative controls that present the linker but not the peptide ligand. All constructs formed micelles with a *R_h* of ~20 nm in aqueous solution at 37 °C (Figure S4), and the incorporation of the linkers did not significantly change the zeta potential of the micelles compared with the parent ELP_{BC} micelle (Table S1). Next, we quantified the uptake of these eight micelles by SK-BR-3 cancer cells at 37 °C by flow cytometry. Compared to their negative controls that present the corresponding linker at the corona of the micelle but do not present a peptide ligand, none of the ELP_{BC}-AHNP micelles with any of these linkers showed any difference in uptake by SK-BR-3 cancer cells (Figure 2).

To test more hydrophilic linkers, we next chose aspartic acid (D) and lysine (K)—two of the most hydrophilic and charged amino acids—to construct a set of charged peptide linkers. These charged peptide linkers can be classified into three groups: cationic K_n, anionic D_n, and zwitterionic (KD)_n. As AHNP is a cyclic peptide, we also tried distributing the charged K or D residues on both sides of the AHNP ligand, and these constructs are labeled as

ELP_{BC}-K₄-AHNP-K₄ and ELP_{BC}-D₄-AHNP-D₄. The cationic linker group included ELP_{BC}-K₄-AHNP, ELP_{BC}-K₈-AHNP, and ELP_{BC}-K₄-AHNP-K₄. The anionic linker group included ELP_{BC}-D₄-AHNP, ELP_{BC}-D₈-AHNP and ELP_{BC}-D₄-AHNP-D₄. The zwitterionic linker group included ELP_{BC}-(KD)₂-AHNP and ELP_{BC}-(KD)₄-AHNP.

All constructs, as well as negative control constructs which only present the linkers, formed micelles with a R_h of ~20 nm in aqueous solution at 37 °C (Figure S4). ELP_{BC}-K₄ micelles and ELP_{BC}-K₈ micelles had a zeta potential of -3.2 ± 0.3 mV and 1.4 ± 0.2 mV, respectively (Table S1); which is greater than the zeta potential of -6.9 mV of the parent ELP_{BC} micelles, consistent with the cationic residues presented by these linkers at the micelle-water interface. Conversely, the ELP_{BC}-D₄ micelles and ELP_{BC}-D₈ micelles had a zeta potential of -8.0 ± 0.2 mV and -8.8 ± 0.2 mV, respectively (Table S1), which is lower than that of the parent ELP_{BC} micelles, consistent with the incorporation of negatively charged residues at the corona of the micelle. ELP_{BC}-(KD)₂ micelles and ELP_{BC}-(KD)₄ micelles had a zeta potential close to that of parent ELP_{BC} micelles (Table S1). The cell uptake by SK-BR-3 cells for each construct normalized to the parent ELP_{BC} micelles are summarized in Figure 2. None of these ELP_{BC}-linker-AHNP micelles showed enhanced uptake by SK-BR-3 cancer cells at 37 °C as compared to their negative control micelles with only the linker.

In our search to identify the linkers that could promote receptor-mediated cell uptake, we next incorporated K_{*n*}D_{*m*} and D_{*m*}K_{*n*} linkers between the AHNP ligand and the ELP_{BC} and systematically varied *n* and *m* to examine the impact of linker length and charge distribution on AHNP-triggered cell uptake. To do so, the following constructs were recombinantly synthesized: ELP_{BC}-K₄D₄-AHNP, ELP_{BC}-D₄K₄-AHNP, ELP_{BC}-K₄D₈-AHNP, ELP_{BC}-D₈K₄-AHNP, ELP_{BC}-K₈D₄-AHNP, and ELP_{BC}-D₄K₈-AHNP. All of these constructs and their respective negative controls—ELP_{BC}-linker fusions—form micelles at 37 °C with a R_h of ~20 nm (Figure S4), and their zeta potential values relative to the parent ELP_{BC} micelles depend on the overall charge of the linker (Table S1). As shown in Table S1, ELP_{BC}-K₄D₈ micelles and ELP_{BC}-D₈K₄ micelles had a zeta potential of -8.2 ± 0.4 mV and -8.0 ± 0.3 mV, respectively, while ELP_{BC}-K₈D₄ micelles and ELP_{BC}-D₄K₈ micelles had a zeta potential of -2.0 ± 0.6 mV and -2.1 ± 0.4 mV, respectively. ELP_{BC}-K₄D₄ micelles and ELP_{BC}-D₄K₄ micelles had a zeta potential close to that of the parent ELP_{BC} micelle. These results show that, compared to the parent ELP_{BC} micelles, K₄D₈ and D₈K₄ linkers with an overall negative charge had a lower (more negative) zeta potential, while K₈D₄ and D₄K₈ linkers with an overall positive charge had a greater zeta potential (less negative) than the parent ELP_{BC} micelle. The introduction of the AHNP ligand at the C-terminus had no impact on micelle formation, as all AHNP-functionalized ELP_{BC} constructs formed stable micelles at 37 °C with a R_h ~ 20 nm (Figure S4) and the size of ELP_{BC}-K₈D₄-AHNP micelles measured by cryo-TEM (13.7 ± 1.9 nm in radius) is close to the size of ELP_{BC}-K₈D₄ micelles (14.0 ± 1.8 nm in radius) and the parent ELP_{BC} micelles ($13.0 \text{ nm} \pm 1.9 \text{ nm}$ in radius) measured by the same technique (Figure 1B). We also measured the zeta potential of ELP_{BC}-K₈D₄-AHNP, which was -2.5 ± 0.5 mV, and is experimentally indistinguishable from the -2.0 ± 0.6 mV zeta potential of the linker only—ELP_{BC}-K₈D₄—control micelles.

The cell uptake by SK-BR-3 cells for each construct normalized to the parent ELP_{BC} micelles are summarized in Figure 2. Only two constructs—ELP_{BC}-K₈D₄-AHNP micelles and ELP_{BC}-D₄K₈-AHNP micelles—showed ~5.8-fold and ~4.5-fold increases, respectively, in internalization over their corresponding controls—ELP_{BC}-K₈D₄ micelles and ELP_{BC}-D₄K₈ micelles that only present the linker—by SK-BR-3 cancer cells at 37 °C (Figure 2). We note that their overall uptake is ~8.2-fold and ~6.3-fold relative to the parent ELP_{BC} micelles, but the uptake is somewhat lower when compared to their linker only controls because of the ~1.4-fold increase in the cell uptake of the linker only ELP_{BC} micelles relative to the parent ELP_{BC} micelles, which can be attributed to the nonspecific cell uptake mediated by the cationic nature of these linkers. These results strongly indicate that the increased uptake of ELP_{BC}-K₈D₄-AHNP micelles and ELP_{BC}-D₄K₈-AHNP micelles compared to their linker only micelle controls is due to binding of the AHNP ligands to ErbB2 receptors on SK-BR-3 cancer cells, with subsequent receptor-mediated endocytosis.

This conclusion was further confirmed by confocal fluorescence microscopy, as SK-BR-3 cancer cells incubated with Cy5-labeled ELP_{BC}-K₈D₄-AHNP micelles showed significantly more green fluorescence than cells incubated with the linker only negative control, Cy5-labeled ELP_{BC}-K₈D₄ micelles (Figure 3A). The Cy5-labeled ELP_{BC}-K₈D₄-AHNP micelles (green fluorescence) triggered the internalization of cell membrane ErbB2 receptors and colocalized with ErbB2 receptors, which were stained with PE-conjugated anti-ErbB2 antibody (red fluorescence), in the endocytosis vesicles (Figure 3A). Preincubating SK-BR-3 cancer cells with 500 μ M of free AHNP peptide decreased the internalization of ELP_{BC}-K₈D₄-AHNP micelles on SK-BR-3 cancer cells by ~50% (Figure 3B). We were unable to totally block the ErbB2 receptor by coinubation with free peptide, as the AHNP peptide has limited solubility in aqueous solution, and the monovalent free ligands cannot totally block multivalent ligands presented by a spherical micelle that have much higher avidity for the receptor.^{17,18} In contrast, no difference in internalization was observed between ELP_{BC}-K₈D₄-AHNP micelles and the control ELP_{BC}-K₈D₄ micelles at 37 °C by ErbB2-negative MCF-7 cancer cells (Figure 3C). Together, these data clearly demonstrate that AHNP-triggered internalization of ELP_{BC}-K₈D₄-AHNP micelles by SK-BR-3 cancer cells is largely due to receptor-mediated endocytosis, rather than nonspecific endocytosis.

These results also suggest that charged linkers with a length of four or eight hydrophilic amino acid residues fail to display the AHNP ligand on the surface of ELP_{BC} micelles in an orientation such that AHNP is accessible to the ErbB2 receptor. When the linker's length was extended to 12 amino acids, however, the K₈D₄ linker succeeded in enhancing cell uptake of AHNP-modified ELP_{BC} micelle on SK-BR-3 cancer cells, while switching the positions of K and D in K₈D₄ had no significant impact on cell uptake (Figure 2). However, the negatively charged K₄D₈ and D₈K₄ linkers failed to assist the AHNP ligand to be displayed on the micelle surface in a receptor accessible orientation (Figure 2). Together, these results suggest that linkers with an overall positive charge and a length of 12 residues promote cell uptake of the ELP_{BC} micelles decorated with the AHNP ligand by receptor-mediated endocytosis via binding to the ErbB2 receptor.

We next created ELP_{BC}-K₄-AHNP/ELP_{BC}-D_{*n*} (*n* = 4, 8, 12) and ELP_{BC}-D₄-AHNP/ELP_{BC}-K_{*n*} (*n* = 4, 8, 12) mixed micelles. Here, we distributed the K and/or D residues in two

separate ELP_{BC} polymers to examine the effect of decoupling linker length from the overall cationic compositional bias at the interface of the micelle corona and the peptide ligand, as these two parameters appeared to be important to promote ligand-triggered cell uptake, based on the results presented in Figure 2. In the first group, ELP_{BC}-K₄-AHNP was mixed with ELP_{BC}-D₄, ELP_{BC}-D₈, and ELP_{BC}-D₁₂, respectively, at a 1:1 molar ratio. At 37 °C, all binary mixtures, including AHNP-functionalized and control unfunctionalized mixtures, formed ~20 nm R_h spherical micelles in aqueous solution (Figure S4). The zeta potential of ELP_{BC}-K₄/ELP_{BC}-D₄ mixed micelles, ELP_{BC}-K₄/ELP_{BC}-D₈ mixed micelles, and ELP_{BC}-K₄/ELP_{BC}-D₁₂ mixed micelles at 37 °C were -6.6 ± 0.5 mV, -7.4 ± 0.5 mV, and -8.3 ± 0.3 mV, respectively (Table S1). To study their internalization, 20 μ M of Cy5-labeled mixed micelles were incubated with SK-BR-3 cancer cells at 37 °C for 1 h, and then the cell fluorescence intensity was quantified by flow cytometry. As shown in Figure 2, none of these three types of AHNP-functionalized mixed micelles showed enhanced internalization compared to their corresponding control micelles.

In the second group, ELP_{BC}-D₄-AHNP was mixed with ELP_{BC}-K₄, ELP_{BC}-K₈, and ELP_{BC}-K₁₂, respectively, at a 1:1 molar ratio. At 37 °C, all of the AHNP-functionalized and unfunctionalized mixtures formed ~20 nm R_h spherical micelles in aqueous solution (Figure S4), and the zeta potential of ELP_{BC}-D₄/ELP_{BC}-K₄ mixed micelles, ELP_{BC}-D₄/ELP_{BC}-K₈ mixed micelles, and ELP_{BC}-D₄/ELP_{BC}-K₁₂ mixed micelles at 37 °C were -6.6 ± 0.5 mV, -2.2 ± 0.3 mV, and -0.2 ± 0.2 mV, respectively (Table S1). As shown in Figure 2, the internalization of ELP_{BC}-D₄-AHNP/ELP_{BC}-K_{*n*} mixed micelles by SK-BR-3 cancer cells were 1.1-fold ($n = 4$), 6.2-fold ($n = 8$), and 11-fold ($n = 12$) greater than the parent ELP_{BC} micelles. However, increasing the positive charge in the linker also increases the nonspecific cell uptake, as seen by the fact that the linker only controls show a monotonic increase in cell uptake as the number of cationic lysine (K) residues increases from 4 to 12. Taking this into account, the fold increase in cell uptake that can be attributed to the effect of the peptide ligand is 1.1-fold ($n = 4$), 4.5-fold ($n = 8$), and 6.4-fold ($n = 12$) of the uptake exhibited by their corresponding control micelles that lack the AHNP ligand.

We next carried out three additional experiments to confirm these findings. First, confocal microscopy study confirmed the enhanced cell uptake of ELP_{BC}-D₄-AHNP/ELP_{BC}-K₁₂ mixed micelles by SK-BR-3 cancer cells and showed their colocalization with ErbB2 receptors (Figure 3A). Second, in a free peptide competition experiment, free AHNP peptide was added prior to incubation with the micelles. Flow cytometry showed that the free peptide blocked the internalization of ELP_{BC}-D₄-AHNP/ELP_{BC}-K₁₂ mixed micelles by ~45% (Figure 3B). Next, we carried out cell uptake studies with an ErbB2-negative MCF-7 cell line. The internalization of ELP_{BC}-D₄-AHNP/ELP_{BC}-K₁₂ mixed micelles by MCF-7 was similar to that the negative control, the linker only micelle (Figure 3C). Taken together, these results confirm that ligand/receptor interactions are responsible for the bulk of the increase in cell uptake of AHNP-functionalized micelles compared to the linker only micelles and parent ELP micelles.

By using mixed micelles, we further studied whether the ErbB2-targeted ELP_{BC}-D₄-AHNP/ELP_{BC}-K₈ mixed micelle can still enhance the cell uptake of micelles by ErbB2-positive SK BR-3 cancer cells when lysine (K, $pK_a = 10.67$, $\log P = -3.21$) residues are

replaced with more cationic arginine (R, $pK_a = 12.10$, $\log P = -3.16$) residues, or when anionic aspartic acid (D, $pK_a = 3.71$, $\log P = -3.50$) residues are replaced with glutamic acid (E, $pK_a = 4.15$, $\log P = -3.24$) residues. We prepared ELP_{BC}-D₄-AHNP/ELP_{BC}-R₈ mixed micelles and ELP_{BC}-E₄-AHNP/ELP_{BC}-K₈ mixed micelles, as well as their negative controls lacking AHNP ligands. All of these four kinds of mixed micelles have a R_h of ~20 nm at 37 °C (Figure S4). Because of their pK_a differences, the zeta potential of the ELP_{BC}-D₄/ELP_{BC}-K₈ micelles (-2.2 ± 0.3 mV) is close to that of the ELP_{BC}-E₄/ELP_{BC}-K₈ micelles (-2.4 ± 0.4 mV). In contrast, the ELP_{BC}-D₄/ELP_{BC}-R₈ micelles have a significantly larger and positive zeta potential of 2.2 ± 0.6 mV (Table S1). We then labeled these micelles with Cy5 and studied their cell uptake by SK-BR-3 cancer cells. We found that the cell uptake of the ELP_{BC}-D₄/ELP_{BC}-R₈ mixed micelles is ~8 fold greater than that of the parent ELP_{BC} micelles because of the strong electrostatic attraction of R₈ with negatively charged cell membrane followed by macropinocytosis, consistent with the fact that R is a cell penetrating peptide.^{26,27} Previous studies have also demonstrated that R₈-modified ELP_{BC} micelles bind to membrane-associated heparin sulfate proteoglycan (HSPG) and then initiate macropinocytosis, thereby increasing the cell uptake of R₈-modified ELP_{BC} micelles compared to the parent ELP micelles.⁴⁵ However, the cell uptake of ELP_{BC}-D₄-AHNP/ELP_{BC}-R₈ mixed micelles was slightly lower compared to the control ELP_{BC}-D₄/ELP_{BC}-R₈ micelles (Figure 2). These results suggest that the binding of R₈ to the negatively charged cell membrane and to HSPG competes with the AHNP/ErbB2 binding. In contrast, the ELP_{BC}-E₄-AHNP/ELP_{BC}-K₈ micelles showed ~5.4 fold greater uptake compared to the parent ELP_{BC} micelles in SK-BR-3 cells, and these results are similar to those obtained for the ELP_{BC}-D₄-AHNP/ELP_{BC}-K₈ micelles. These results are consistent with fact that the E₄ linker has a similar hydrophilicity and charge to D₄ linker, so that the uptake of both micelles is very similar. (Figure 2).

To summarize the major findings of this section, K₈D₄ or D₄K₈ peptide linkers in ELP_{BC}-linker-AHNP micelles or mixed micelles of ELP_{BC}-D₄-AHNP/ELP_{BC}-K_{*n*} ($n = 8, 12$) enable the AHNP ligand to be displayed on the surface of ELP_{BC} micelle such that AHNP can bind to the ErbB2 receptor and lead to enhanced internalization by SK-BR-3 cancer cells by ErbB2 receptor-mediated endocytosis. In contrast, K₄D₈ or D₈K₄ linkers or ELP_{BC}-K_{*n*}-AHNP/ELP_{BC}-D_{*n*} ($n = 8, 12$) mixed micelles do not exhibit enhanced cell uptake compared to the negative controls without the ligand. Furthermore, replacing K with R did not enhance cell uptake but replacing D with E in the linker successfully assisted the proper display of AHNP ligands. These results suggest that (1) a hydrophilic linker with (2) a minimum length of 12 residues, and (3) an overall excess of cationic residues at the interface between the ligand and micelle to balance the anionic nature of the ligand and the terminal carboxyl residue presented on the surface of micelles—and hence impart a zeta potential close to neutrality—is necessary for receptor-mediated internalization. While the R₈ linker in the ELP_{BC}-D₄/ELP_{BC}-R₈ mixed micelles imparts an overall positive charge, the charge imparted at the micelle–aqueous interface is so large that it results in a zeta potential to ~2.2 mV, which biases the uptake largely toward the electrostatic interaction between the micelle and the cell surface and the subsequent R₈-mediated macropinocytosis rather than ErbB2 receptor-mediated uptake.

To verify the generality of these results, we next examined EC1, another hydrophobic and anionic peptide ligand that targets the ErbB2 receptor.^{39–41} All the ELP_{BC} constructs with EC1 ligand self-assembled into ~20 nm R_h micelles in aqueous buffer at 37 °C (Figure S4). The internalization of ELP_{BC}-EC1 micelles on SK-BR-3 cancer cells at 37 °C showed no difference from parent ELP_{BC} micelles, similar to AHNP, presumably because of its hydrophobicity (Figure 4A). When the K₈D₄ peptide linker was inserted between the ELP_{BC} and the EC1 ligand, the internalization of ELP_{BC}-K₈D₄-EC1 micelles by SKBR-3 cancer cells was ~12-fold greater than the control ELP_{BC}-K₈D₄ micelles (Figure 4A), similar to results obtained for AHNP, though the absolute uptake of EC1-functionalized micelles by SK-BR-3 cells is somewhat greater than AHNP-decorated micelles.

We also looked at the effect of distributing the charged residues on different polymer chains. Similar to the results obtained for the AHNP peptide, the internalization of ELP_{BC}-D₄-EC1/ELP_{BC}-K_{*n*} mixed micelles showed ~8-fold ($n = 8$) and ~16.7-fold ($n = 12$) greater uptake over the corresponding control micelles that lack the EC1 ligand (Figure 4A). Confocal fluorescence microscopy visually confirmed the enhanced cell uptake of the ELP_{BC}-K₈D₄-EC1 micelles and ELP_{BC}-D₄-EC1/ELP_{BC}-K₁₂ mixed micelles by SK-BR-3 cancer cells, and showed their colocalization with ErbB2 receptors in the endocytosis vesicles (Figure 4B). Furthermore, 500 μ M of free EC1 peptide that was coincubated with these two types of EC1-functionalized micelles inhibited their internalization by SK-BR-3 cancer cells by ~40% (Figure 4C). In contrast, neither ELP_{BC}-K₈D₄-EC1 micelles nor ELP_{BC}-D₄-EC1/ELP_{BC}-K₁₂ mixed micelles showed enhanced cell uptake by MCF-7 cancer cells that have low expression of ErbB2 receptor, as compared to the control micelles that are missing the EC1 ligand (Figure 4D).

In this study, we used ELP_{BC} micelles as a model polymer nanoparticle system to study the design of peptide linkers that can optimize the presentation of hydrophobic peptide ligands on a micelle surface to maximize cell uptake. We hypothesized that a possible reason that hydrophobic peptide ligands lose their targeting ability in many instances when they are presented on the surface of a nanoparticle is because these ligands are not optimally presented on the nanoparticle surface to engage their target receptor. This issue is especially acute in polymer micelles, an important class of nanoparticles for drug delivery,⁴⁶ because the constituent polymer chains can exhibit significant mobility, which can bury a hydrophobic ligand within the micelle core to minimize its interfacial free energy. To solve this problem, we—as have other investigators in prior studies^{16–18}—hypothesized that a hydrophilic linker might reduce this hydrophobic interaction and enable the presentation of the ligand in an orientation that would engage its receptor on the cell membrane. To systematically study this problem, we carried out the largest study to date of the impact of the sequence, length, and charge of hydrophilic peptide linkers that connect a peptide ligand to the micelle on cell uptake.

We first tried commonly used flexible (GGSG)_{*n*} ($n = 1, 3$) linkers and rigid A(EAAAK)_{*n*}A ($n = 2, 4$) linkers. Although both linkers have been reported to successfully separate protein domains,⁴⁴ they failed to display the AHNP ligand on the ELP_{BC} micelle surface in an orientation that led to increased cell uptake over that of the unfunctionalized parent micelle, indicating that simply length or rigidity of the linker is not sufficient to solve this problem.

We found that charged linkers that consisted of four or eight amino acids of K and/or D failed to separate AHNP ligands from hydrophobic ELP blocks during ELP_{BC} micelle formation, irrespective of whether the linker was anionic, cationic, or zwitterionic.

However, extending the length of the charged linker to 12 hydrophilic amino acids finally resulted in the identification of two linkers—K₈D₄ and D₄K₈—that enhanced the display of both hydrophobic peptide ligands tested herein—AHNP and EC-1—as seen by the increased cell uptake of ligand-functionalized ELP_{BC} micelles by SK-BR-3 cancer cells which overexpress the ErbB2 receptor. However, the precise sequence is not important, as the K₈D₄ linker and the D₄K₈ linker showed no significant difference in cell uptake. In contrast, when the charges were reversed, the K₄D₈ and D₈K₄ linkers failed to enhance the cell uptake of micelles, because they impart a greater negative charge to the micelle, as seen by their more negative zeta potential. The cell uptake of AHNP-functionalized ELP_{BC} micelles in terms of linker length and zeta potential of ELP_{BC} micelles are summarized in Figure S5. These results suggest a simple rule—a hydrophilic linker of at least 12 amino acids in length with a compositional bias in favor of cationic lysine (K) residues that imparts a zeta potential close to neutral—enables optimal presentation of these two anionic and hydrophobic peptide ligands at the micelle surface in an orientation that maximizes receptor binding.

To decouple the impact of linker length and overall charge, we distributed the K and D residues in separate ELP_{BC} and created two groups of mixed micelles that present the AHNP peptide ligand. One group was ELP_{BC}-K₄-AHNP/ELP_{BC}-D_{*n*} (*n* = 4, 8, 12) mixed micelles, and the other group was ELP_{BC}-D₄-AHNP/ELP_{BC}-K_{*n*} (*n* = 4, 8, 12) mixed micelles. In the first series, that has an overall anionic bias in its composition, ELP_{BC}-K₄-AHNP/ELP_{BC}-D_{*n*}, for all values of *n* (4, 8, 12), the mixed micelles did not exhibit enhanced internalization because the overall charge at the interface as seen by their zeta potential is far too negative, resulting in electrostatic repulsion from the cell. In contrast, for the second series that has an overall cationic bias in its composition, the ELP_{BC}-D₄-AHNP/ELP_{BC}-K₄ mixed micelles did not enhance the internalization of micelles by SK-BR-3 cancer cells. However, for *n* = 8, 12, the ELP_{BC}-D₄-AHNP/ELP_{BC}-K_{*n*} mixed micelles successfully showed enhanced AHNP-triggered ErbB2-mediated internalization of the mixed micelles by SK-BR-3 cancer cells. ELP_{BC}-D₄-AHNP/ELP_{BC}-K₈ micelles have an overall hydrophilicity and charge that is similar to ELP_{BC}-K₈D₄-AHNP micelles, and this is reflected in the similar cell uptake of these two AHNP-functionalized micelles by SK-BR-3 cells. We attempted to further increase receptor-mediated endocytosis by increasing the length of the linker but the ELP_{BC}-K₁₂D₄-AHNP construct proved difficult to express and purify. Fortunately, the ELP_{BC}-D₄-AHNP/ELP_{BC}-K₁₂ mixed micelles provided an alternative that showed a higher level of cell uptake than the best performing—ELP_{BC}-K₈D₄-AHNP—single-linker construct (Figure 2). These results were corroborated by the EC1 ligand and suggest that there is a minimum threshold of length of the linker for hydrophobic anionic ligands to engage the receptor.

These results clearly show that the optimal presentation of a hydrophobic ligand requires balancing both the length of the linker and its overall hydrophilicity to prevent the ligand from burying into the core of the micelles as well as the charge at the micelle-water interface to counteract the negative charge presented by the ligand and the C-terminal carboxyl

moiety presented on the corona of the micelle. Micelles with a negative charge above a certain threshold cannot overcome the repulsive electrostatic barrier imposed by the negatively charged cell membrane to enable engagement of the cell surface receptors by the ligand.⁴⁷ Hence, linkers that can overcome this initial energetic barrier by reducing the net negative charge on the corona of the micelle and that at the same time present the peptide ligand in a receptor accessible orientation is critical for optimal presentation of an anionic peptide ligand on the surface of a polymer micelle to engage their cognate cell surface receptor. However, the length of the linker and the charge at the interface can be decoupled, as long as an overall slight compositional bias toward cationic residues is maintained.

Clearly, there are two forces at play here. One parameter is that the hydrophobic ligand needs to be positioned away from the core of the micelle. The other parameter is a slight compositional bias toward cationic residues, which can be independently controlled in mixed micelles by the diluent linker, and whose length, by definition, must be longer than the oppositely charged linker that presents the ligand to bias the overall charges toward cationic residues. The use of mixed micelles with two different linkers also provides a modular, mix-and-match approach to identify a suitable linker for a new peptide ligand, as one only needs to synthesize two unique constructs—the ELP_{BC}-K₄-ligand and ELP_{BC}-D₄-ligand—and then mix each of these constructs with different ELP_{BC}-linker constructs that have a different length and charge to optimize ligand presentation.

Consistent with this picture, we found that the compositional bias at the interface between the peptide ligand and the ELP_{BC} toward cationic residues that is needed for optimal ligand presentation correlates with the zeta potential of the micelles. The zeta potential value of the parent ELP_{BC} micelles is -6.9 ± 0.4 mV, consistent with the fact that the C-terminal carboxyl residue in each polymer chain is exposed at the corona-water interface. By introducing K and D residues and adjusting the K-to-D ratio, we can easily tune the zeta potential of the micelle (Figure S6). By doing so, we found a set of linkers that successfully recovered the accessibility of the AHNP peptides for the ErbB2 receptor, and all of them had higher (less negative) zeta potential than the parent ELP_{BC} micelles (Table S1), including ELP_{BC}-K₈D₄-AHNP micelles (-2.5 ± 0.5 mV), ELP_{BC}-D₄-AHNP/ELP_{BC}-K₈ mixed micelles (-3.1 ± 0.5 mV) and ELP_{BC}-D₄-AHNP/ELP_{BC}-K₁₂ mixed micelles (-1.0 ± 0.4 mV). Similar results were obtained for the analogous EC1-functionalized micelles that showed enhanced receptor-mediated cell uptake, with zeta potentials that range from -1.2 to -2.8 mV (Table S1). The use of an R₈ linker in ELP_{BC}D₄/ELP_{BC}-R₈ mixed micelles, however, provides far too much cationic charge at the interface, as seen by its zeta potential of 2.2 ± 0.6 mV, which competes with ErbB2 receptor-mediated cell uptake.

We note that these studies would be difficult to carry out with any other nanoparticle system. The thermally triggered ELP_{BC} micelles are an enormously experimentally convenient model nanoparticle system to understand the impact of different elements of the design of nanoparticles for targeted delivery because of the following reasons: (1) the ease with which peptides can be conjugated to one end of an ELP at the gene level,⁴² (2) the ease with which ELP fusion proteins can be purified by ITC, a nonchromatographic method we have developed to purify ELPs and ELP fusion proteins,⁴³ and (3) the ease with which micelles can be prepared simply by temperature-triggered unimer-to-micelle transition of the ELP.

^{24–27} BC Finally, swapping one linker for another is easy as the design is completely modular and only requires that the DNA sequence that encodes the new linker be inserted into the vector that encodes the parent ELP_{BC} followed by the expression of the gene.

Although the major findings of this study were validated with two hydrophobic peptide ligands and provide simple design rules for the presentation of hydrophobic, anionic ligands—the need for a minimum linker length of 12 hydrophilic amino acid residues and an overall cationic compositional bias at the ligand-micelle interface to bring the zeta potential close to neutrality, an outstanding question that remains is the generality of the design rules uncovered here, across peptide ligand classes and nanoparticle types. In future studies, we will address this issue by expanding upon this study with a larger set of compositionally diverse peptide ligands and examine the cell uptake of each ligand-decorated ELP micelle with a set of hydrophilic peptides linkers with systematically varied sequence, length, and charge. We recognize, however, that these design rules may not necessarily translate to structurally different nanoscale delivery systems such as metal or silicon nanoparticles, and we hence pose a challenge to nanomedicine researchers to carry out similar studies across nanoparticle types—with diverse composition size, morphology, and architectures—to identify the key factors that control ligand presentation on the surface of nanoparticles. In conclusion, this study provides design rules for the presentation of hydrophobic and charged peptide ligands on polymer micelles and more generally establishes a rational framework for the systematic optimization of the presentation of peptide ligands by nanoparticles that can be applied across structurally diverse nanoparticle types.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

CMT	critical micelle temperature
CMC	critical micelle concentration
DLS	dynamic light scattering
ELP_{BC}	elastin-like polypeptide diblock copolymer
EPR	enhanced permeability and retention
ITC	inverse transition cycling
Pre-RDL	recursive directional ligation by plasmid reconstruction
SEC	size-exclusion chromatography

cryo-TEM	cryogenic transmission electron microscopy
HSPG	membrane-associated heparin sulfate proteoglycan

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Figure 1.

(A) Temperature-programmed turbidity and size of the parent ELP_{BC} and the ELP_{BC}-K₈D₄. The temperature at T_{i-1} corresponds to the collapse of the hydrophobic block in the ELP_{BC}, which drives the self-assembly of diblock copolymers into micelles and is the critical micelle temperature (CMT). The temperature at T_{i-2} corresponds to the temperature at which the hydrophilic block in the ELP_{BC} desolvates sufficiently to trigger the transition of micelles into micron-size aggregates. OD_{350nm}: optical density at 350 nm. (B) Cryo-TEM images of the parent ELP_{BC} micelles, ELP_{BC}-K₈D₄ micelles, and ELP_{BC}-K₈D₄-AHNP micelles. The cryo-TEM images show spherical micelles (as indicated by black arrows). The black scale bar represents 200 nm.

Figure 2.

Cell uptake of ELP_{BC}-AHNP micelles without linker, with (GGSG)_n or A(EAAAK)_nA linkers (gray set), with K_n, D_n or (KD)_n linkers (red set), with K_nD_m or D_mK_n linkers (green set), and AHNP-functionalized mixed micelles (blue set) compared with cell uptake of their corresponding control micelles without attaching AHNP ligand. Cell uptake study was performed by incubating 20 μ M of Cy5-labeled micelles with SK-BR-3 cancer cells at 37 °C for 1 h, and then cell fluorescence of Cy5 was quantified by flow cytometry. ** p < 0.01.

Figure 3.

(A) Confocal fluorescence microscopy images of the cellular distribution of ELP_{BC}-K₈D₄-AHNP micelles, ELP_{BC}-D₄-AHNP/ELP_{BC}-K₁₂ mixed micelles, and their negative control micelles in SKBR-3 cancer cells, and their colocalization with ErbB2 receptors. 20 μ M of Cy5-labeled micelles was incubated with SK-BR-3 cancer cells at 37 $^{\circ}$ C for 3 h, and then fixed, permeabilized, blocked, stained with PE-conjugated anti-ErbB2 antibody, and imaged by confocal fluorescence microscope. DIC: differential interference contrast. The black scale bar in the image indicates 12 μ m. (B) Free AHNP peptides inhibit the cell uptake of ELP_{BC}-K₈D₄-AHNP micelles and ELP_{BC}-D₄-AHNP/ELP_{BC}-K₁₂ mixed micelles by SK-BR-3 cancer cells at 37 $^{\circ}$ C. Cells were coincubated with 500 μ M of free peptides and 20 μ M of Cy5-labeled AHNP-functionalized micelles at 37 $^{\circ}$ C for 1 h, and then cell fluorescence of Cy5 was quantified by flow cytometry. (C) Cell uptake of ELP_{BC}-AHNP micelles, ELP_{BC}-

K₈D₄-AHNP micelles, ELP_{BC}-D₄-AHNP/ELP_{BC}-K₁₂ mixed micelles and their corresponding control without AHNP by ErbB2-negative MCF-7 cancer cells. 20 μ M of Cy5-labeled micelles was incubated with cells at 37 °C for 1 h. ** $p < 0.01$.

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Figure 4.

(A) Cell uptake by ErbB2-positive SK-BR-3 cancer cells of ELP_{Bc}-EC1 micelles, ELP_{Bc}-K₈D₄-EC1 micelles, ELP_{Bc}-D₄-EC1/ELP_{Bc}-K_n ($n = 4, 8, 12$) mixed micelles and their corresponding negative control micelles without EC1 peptide. (B) Confocal fluorescence microscopy images of the cellular distribution of ELP_{Bc}-K₈D₄-EC1 micelles and ELP_{Bc}-D₄-EC1/ELP_{Bc}-K₁₂ mixed micelles in SK-BR-3 cancer cells and their colocalization with ErbB2 receptors. The black scale bar in the image indicates 12 μ m. (C) Free EC1 peptides inhibit the uptake of ELP_{Bc}-K₈D₄-EC1 micelles and ELP_{Bc}-D₄-EC1/ELP_{Bc}-K₁₂ mixed micelles by SK-BR-3 cancer cells at 37 °C. (D) Uptake of ELP_{Bc}-EC1 micelles, ELP_{Bc}-K₈D₄-EC1 micelles, ELP_{Bc}-D₄-EC1/ELP_{Bc}-K₁₂ mixed micelles, and their corresponding negative control micelles without EC1 by ErbB2-negative MCF-7 cancer cells. ** $p < 0.01$.

Scheme 1.

(A) Scheme of the ELP_{BC}-Linker-Ligand Constructs and (B) Scheme of Self-Assembly of an ELP_{BC} Ligand Fusion without or with an Optimized Hydrophilic Linker by an ELP Micelle^a

^a(A) The parent ELP_{BC} consists of 40 repeats of a hydrophobic XGVPG repeat unit, where X = W:V = 1:4, at the N-terminus and 40 repeats of a hydrophilic SGVPG repeat unit at the C terminus. The peptide linker and the peptide ligand are incorporated at the C-terminus of the parent ELP_{BC}. (B) A hydrophobic ligand is buried in the micelle interior in the absence of a suitably optimized linker, whereas introducing an optimized linker enables the presentation of the ligand on the micelle surface in a solvent accessible orientation.