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In Vivo Tumor Cell Targeting with “Click” Nanoparticles

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Abstract

The *in vivo* fate of nanomaterials strongly determines their biomedical efficacy. Accordingly, much effort has been invested into the development of library screening methods to select targeting ligands for a diversity of sites *in vivo*. Still, broad application of chemical and biological screens to the *in vivo* targeting of nanomaterials requires ligand attachment chemistries that are generalizable, efficient, covalent, orthogonal to diverse biochemical libraries, applicable under aqueous conditions, and stable in *in vivo* environments. To date, the copper(I)-catalyzed Huisgen 1,3-dipolar cycloaddition or “click” reaction has shown considerable promise as a method for developing targeted nanomaterials *in vitro*. Here, we investigate the utility of “click” chemistry for the *in vivo* targeting of inorganic nanoparticles to tumors. We find that “click” chemistry allows cyclic LyP-1 targeting peptides to be specifically linked to azido-nanoparticles and to direct their binding to p32-expressing tumor cells *in vitro*. Moreover, “click” nanoparticles are able to stably circulate for hours *in vivo* following intravenous administration (>5h circulation time), extravasate into tumors, and penetrate the tumor interstitium to specifically bind p32-expressing cells in tumors. In the future, *in vivo* use of “click” nanomaterials should expedite the progression from ligand discovery to *in vivo* evaluation and diversify approaches toward multifunctional nanoparticle development.

INTRODUCTION

The ability to target nanomaterials to precise biological locations would have wide-ranging impact in biology and medicine. In living systems, highly controlled transportation networks continually shuttle payloads to and from specific molecular addresses. The efficiency of these systems provides strong motivation for the advancement of targeted nanoparticle technologies, particularly for the diagnosis and treatment of human diseases. Towards this goal, high throughput strategies for ligand discovery have generated a multitude of chemical and biological motifs with the potential to direct nanomaterials to specific biomolecular targets. However, translation of these ligands towards *in vivo* nanoparticle targeting has been limited by the number of nanoparticle attachment methods that are efficient, generalizable, aqueous-

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compatible, chemically orthogonal to broad ranges of functional groups, and suitable for *in vivo* applications.

Previous work has demonstrated that *in vivo* bacteriophage display may be used to select for peptide sequences that mimic the ability of endogenous shuttles to target vascular and parenchymal tissue addresses (1-5). Already, linear peptide candidates of phage screens, as well as small molecule targeting candidates, have been translated towards nanomaterial targeting (6-9), primarily via use of exogenous or non-essential thiols, carboxylic acids, or amines. Still, some of the most powerful targeting motifs developed to date are those that contain essential thiol, amine, and carboxyl groups, thereby prohibiting their specific attachment via traditional methods. In particular, conformationally-constrained, disulfide-cyclized targeting peptides are desirable for their enhanced affinity to biological receptors (10,11), and resistance to proteolytic degradation *in vivo* relative to their linear counterparts (12,13). However, specific intramolecular cyclization makes it difficult to add exogenous cysteine residues, while essential amines and carboxyl groups prohibit selective conjugation via exogenous lysine, aspartic acid, or glutamic acid residues. Additionally, non-covalent methods of ligand attachment relying on hydrophobic or electrostatic effects, although widely used *in vitro* (14-16), are unlikely to remain stable in blood or to resist rapid clearance *in vivo*. Recently, copper(I)-catalyzed “click” chemistry has emerged as an extraordinarily selective chemistry and an attractive solution in applications where commonly used thiol-reactive (maleimide, 2-pyridyldithio, iodoacetyl) or amine-reactive (NHS, epoxy, aldehyde, EDC) chemistries are not suitable (17). *In vitro*, “click” chemistry has been utilized to generate functionalized polymers (18-20), surfaces(21-23), and nanoparticles (24-32), and meets the previously mentioned criteria for broad utility in nanoparticle functionalization (chemical orthogonality, aqueous efficiency, applicability for diverse substrates). However the use of “click” nanoparticles for *in vivo* applications has not been investigated. Particularly, as opposed to small molecule reagents with circulation times on the order of minutes (8), ligand attachments on long circulating nanomaterials must remain stable against *in vivo* degradation for hours while nanoparticles circulate systemically and identify molecular targets.

Here, we find that alkyne-azide “click” chemistry provides a facile, single-step method for specifically linking the cyclic tumor-targeting peptide LyP-1 (CGNKRTRGC; (1,33)), which contains essential thiol and amine groups, to polymer-coated magneto-fluorescent nanoparticles. LyP-1 binds to p32, a mitochondrial proteins that is both overexpressed and aberrantly localized at the cell surface of tumor cells, macrophages and lymphatic endothelial cells in certain experimental tumors and in human cancers (34,35). We find that “click” LyP-1 nanoparticles are able stably traverse the systemic circulation, extravasate into tumors, and penetrate the tumor interstitium to specifically bind to receptors on p32-expressing cells in the tumors. Together, these results provide strong motivation for future use of “click” functionalization as a strategy for developing nanoparticles for *in vivo* biomedical applications.

EXPERIMENTAL SECTION

Unless otherwise stated all reagents were purchased from Sigma-Aldrich and all reactions were performed at room temperature.

Iron Oxide Nanoparticle Synthesis

Cross-linked, aminated, fluorescent, superparamagnetic iron oxide nanoparticles were synthesized according to the published protocol (36,37). Briefly, dextran-coated iron oxide nanoparticles were synthesized, purified, and subsequently cross-linked using epichlorohydrin. After exhaustive dialysis, particles were aminated by adding 1:10 v/v ammonium hydroxide (30%) and incubated on a shaker overnight. Aminated-nanoparticles were subsequently purified from excess ammonia using a Sephadex G-50 column and concentrated using a high-

gradient magnetic-field filtration column (Miltenyi Biotec). The near infrared-fluorochrome VivoTag 680 was added to remotely detect particle accumulation *in vitro* and *in vivo* by reacting in 0.1M HEPES buffer with 0.15M NaCl at pH 7.2. DMSO-solubilized fluorochromes were added into particle solutions at 4°C under mixing and allowed to warm to room temperature to react for 2 hours. The yield was approximately 10 fluorochromes per nanoparticle for all experiments.

Succinimidyl 4-azidobutyrate synthesis (Schematic 2A)

Ethyl 4-azidobutyrate (1)—To a solution of ethyl 4-bromobutyrate (5.85 g, 30 mmol) in dimethyl sulfoxide (DMSO, 20 ml), sodium azide (2.925 g, 45 mmol) was added with stirring. The reaction mixture was stirred for 22 hrs at 55 °C, and cooled to room temperature. Water was added to the reaction mixture and extracted with ethyl ether (3 × 30 ml). Combined organic layer was washed with water and brine, and reduced *in vacuo* to afford 3.80 g of the azido compound **1**.

4-Azidobutyric acid (2)—Ethyl 4-azidobutyrate **1** (3.14 g, 20 mmol) was dissolved in aqueous sodium hydroxide solution (1 N, 24 ml) with minimum amount of methanol to make the solution homogeneous. The reaction mixture was stirred for 3 hrs at room temperature. After removal of methanol *in vacuo*, the aqueous solution was acidified to pH 0 with HCl and extracted with ethyl ether (3 × 50 ml). The ether layer was then dried over sodium sulfate and filtered. Removal of solvent gave the acid **2** (2.25 g).

Succinimidyl 4-azidobutyrate (3)—To a solution of N-hydroxyl succinimide (1.65 g, 14.3 mmol) in methylene chloride (100 ml) was added acid **2** (1.68 g, 13 mmol) followed by 1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDAC, 2.74 g, 14.3 mmol). After stirring for 4 hrs at room temperature, the reaction mixture was washed with water and brine, dried over sodium sulfate, and filtered. Solvent was removed under reduced pressure to yield 1.15 g of the succinimidyl 4-azidobutyrate **3**.

Peptide Synthesis—Peptides were synthesized in the MIT Biopolymers Lab and their composition was confirmed via HPLC and mass spectrometry. The LyP-1 and LyP-1CTL peptides were synthesized with either heptynoic acid or propargylglycine at the N-terminus for conjugation. Each peptide is also labeled with a TAMRA fluorophore (Anaspec) via a lysine residue separated by an aminohexanoic acid (Ahx) spacer. (final sequence for LyP-1: Heptynoic acid or propargylglycine - K(Tamra)[Ahx] - CGNKRTRGC; for LyP-1CTL: Heptynoic acid or propargylglycine - K(Tamra)[Ahx] - CRVRTRSGC). Peptides were cyclized by bubbling air into 10 μM aqueous peptide solutions overnight. Finally, peptide solutions were lyophilized for subsequent use.

“Click” attachment of peptides to nanoparticles—Succinimidyl 4-azidobutyrate was linked to 5kDa thiol-PEG-amine polymers in 0.1M HEPES 0.15M NaCl pH 7.2 for 1 hour at a 2:1 molar ratio between linker and polymer. Simultaneously, amino-modified, fluorochrome-labeled nanoparticles were activated with N-[γ-maleimidobutyryloxy] succinimide ester (GMBS) (dissolved in DMSO) cross-linker under similar conditions at a 200:1 molar ratio between cross-linker and nanoparticles. To remove excess GMBS, nanoparticle samples were filtered on a G50 column into 50mM Na Phosphate buffer at pH 7.2 supplemented with 10mM EDTA. Purified nanoparticles were then combined with the polymer reaction mixture and allowed to react at room temperature overnight. Azido-PEG-nanoparticles were then purified from excess polymer and succinimidyl 4-azidobutyrate on a size exclusion column (ACA-44 media: Pall) into 0.1M HEPES pH 7.2 buffer. Finally, the azide-PEG-bearing particles were concentrated using Amicon Ultra-4 (Millipore) filters and stored at 4°C.

To optimize catalyst concentrations for the “click” reaction by HPLC, a 10-fold excess of azido-PEG-NH₂ or O-(2-Aminoethyl)-O’-(2-azidoethyl)pentaethylene glycol (Polypure) was added to a 100 μ M peptide solution. CuSO₄ and Na ascorbate were dissolved in H₂O and added to the reaction mixture to final concentrations of 1 mM/5 mM and 10 mM/50 mM CuSO₄/Na Ascorbate. This mixture was shaken at 37°C for various times (1 day to 3 days), after which it was characterized via HPLC.

Alkyne-bearing peptides (35:1 peptide:nanoparticle molar ratio), CuSO₄ (1 mM), and sodium ascorbate (5 mM) in H₂O were added to a solution of particles and the mixture was shaken at 37°C for 48 h. Following the reaction, nanoparticles were purified from copper catalyst and excess peptides by filtration in ACA-44 size exclusion media into 0.1M HEPES 0.15M NaCl pH 7.2 buffer.

Cell Culture—Cell uptake experiments were performed using a human MDA-MB-435 cancer cell line. Grow media was minimum essential medium eagle (Invitrogen) with fetal bovine serum (10%; Invitrogen). Cells were passaged into 96-well plates and used at 60-80% confluency.

For peptide uptake experiments (Figure 1), LyP-1 or LyP-1CTL peptides were added to cell monolayers in serum-containing media at a final concentration of 10 μ M. After 45 minutes of incubation at 37 °C, cells were washed with media, PBS, treated with trypsin (0.25%) and EDTA, and resuspended in 1% BSA (in PBS) for flow cytometry (BD LSRII). Fluorescence data on 10,000 cells was collected for each sample.

For nanoparticle uptake experiments (Figure 2), particles bearing an LyP-1, LyP-1CTL, or terminal azides were added to the cells in serum media at a final concentration of 100 nM. After 2 h of incubation, cells were trypsinized and assayed for particle fluorescence by flow cytometry. For peptide inhibition experiments, free LyP-1 peptides (10 μ M to 100 μ M) were first incubated with the cells for 1 h. The cells were then washed with media and 100 nM of LyP-1-coated nanoparticles were added to the cell culture. For imaging, cells were washed with PBS and observed using a 20x objective. Images were captured with a CCD camera mounted on a Nikon TE200 inverted epifluorescence microscope.

In Vivo Studies of Nanoparticle Targeting—Nude athymic mice were inoculated subcutaneously with human cancer cells (MDA-MB-435). After tumors had reached ~.5 cm³ in size, LyP-1- and azide-bearing nanoparticles were injected intravenously in the tail vein (1mgFe/kg). Twenty-four hours after the injection, tumor tissues were excised, snap frozen, and cut into 15 μ m histological sections. Rat anti-mouse CD-31 (1:50, BD Pharmingen) and polyclonal anti-p32 antibody (1:200; (34)) were used for immunohistochemical staining of frozen tissue sections. The corresponding secondary antibodies were added and incubated for 1 hour at room temperature: Alexa Fluor 594 goat anti-rat IgG (1:500, Invitrogen) for CD-31 and Alexa Fluor 594 goat anti-rabbit IgG (1:500, Invitrogen) for p32 antibody. The slides were washed three times with PBS and mounted in Vectashield Mounting Medium with DAPI (Vector Labs). The stained tumor sections were observed with a fluorescence microscope (Nikon, Tokyo, Japan). Histological quantitation of nanoparticles localization was done using ImageJ software. Stacks of CD31-stained sections and nanoparticle fluorescence images were utilized for intra- and extravascular particle distribution quantitation. Regions with CD31-staining were selected to denote intravascular accumulation of nanoparticles and surrounding areas were classified as extravascular. The net nanoparticle fluorescence signal above background was quantified for each of these regions to determine the approximate percent of nanoparticle fluorescence localized to the vasculature vs the extravascular space. Three sections from each set of mice were randomly chose for analysis.

RESULTS AND DISCUSSION

In order for “click” chemistry to be applied to the development of peptide-targeted nanomaterials, peptides must be able to harbor pendant alkyne or azide moieties without abating peptide activity. To investigate the efficacy of targeting peptides harboring pendant alkyne moieties, LyP-1 peptide (CGNKRTRGC) and untargeted cyclic control peptide, LyP-1CTL (CRVRTRSGC) in which the essential NKRTTR motif is replaced with RVRTR to maintain net charge but abate p32 targeting(33), were synthesized to incorporate either of two alkyne moieties (the unnatural amino acid propargylglycine or 6-heptynoic acid) and a 5,6-carboxytetramethylrhodamine fluorophore (TAMRA) (Figure 1A; spectra: Supplemental Figure 1). Because the alkyne moieties provide molecularly small chemical handles that may be incorporated in Fmoc synthesis to either the N- or C-terminal of essential peptide sequences, we hypothesized their presence could be tailored to allow chemical attachment to azido-nanomaterials without interfering with LyP-1 peptide activity. In previous investigations, we found that N-terminal addition of visible and near-infrared fluorophores do not disrupt peptide binding to its receptors (1,33). Accordingly, we reasoned alkyne moieties located near the N-terminus would be well-tolerated by the peptide (Figure 1A). To verify the specificity and efficacy of alkyne-LyP-1 targeting, 10 μ M of LyP-1, bearing either propargylglycine, heptynoic, or no alkyne group were incubated for 45 minutes on monolayers of MDA-MB-435 human tumor cells, which have been shown to bind and internalize LyP-1 and express p32 at the cell surface (33,34), LyP-1CTL peptides were included as a control sequence to verify targeted enhancement of uptake over non-specific cyclic peptide structures. Cellular uptake of LyP-1 peptide was quantified via flow cytometry (Figure 1B) and plotted as the populational fluorescent intensity, relative to cells incubated with vehicle alone. *In vitro* targeting of LyP-1 peptides bearing either propargylglycine or 6-heptynoic acid was similar to that of native LyP-1 and control peptides (Figure 1C), indicating that alkyne modifications N-terminal to targeting sequences were innocuously chaperoned by peptides and did not affect cell binding.

We next probed the effect of three variables on “click” reaction conditions between our peptides and an azido-PEG-amine (catalyst, catalyst concentration, and reaction time). Azido-PEG-amine was chosen to emulate the azido-PEG surface of the nanoparticles to be used subsequently and to provide a model reaction amenable to HPLC quantitation of product formation. Copper(I) catalyst was added either directly as an iodinated salt (Cu(I)I), or indirectly as soluble copper sulfate (Cu(II)SO₄) and reduced by sodium ascorbate *in situ*. The degree of product formation was measured via HPLC with mass spectrometric verification of product identity. The addition of the azido-PEO-amine rendered peptides more hydrophilic and decreased retention times compared to unconjugated peptides. As shown in Supplemental Table S1, product formation proceeded more completely in the tested reaction conditions for the heptynoic acid-LyP-1, likely due to reduced steric hindrance provided by the extended hydrocarbon chain. Optimal reaction conditions were found to be either: 1 mM CuSO₄ / 5 mM Na ascorbate or 1 mM -100 mM CuI for 72 h. Notably, the reaction yields with 10 mM CuSO₄ levels were dramatically lower than 1mM, likely due to global precipitation of reduced Cu(I) in solution. Nevertheless, we found 1mM CuSO₄ reactions to yield more reliable conjugations than 1 to 100 mM CuI reactions, possibly because the insolubility of CuI in aqueous solutions produced variations in the amount of available catalyst delivered to the reactions. Therefore, the optimal conditions for subsequent nanoparticle modification were determined to be 1 mM CuSO₄ and 5 mM Na ascorbate. Under these conditions we did not observe any reduction of peptide disulfide bonds due to copper catalyst or Na ascorbate reduction as determined by MALDI mass spectrometry and HPLC analysis (data not shown).

Having verified that alkyne-bearing LyP-1 peptides could effectively target p32-expressing MDA-MB-435 cancer cells and become linked to azido-bearing PEG polymers, we next

developed a protocol for linking these peptides onto azido-PEG bearing, near-infrared fluorochrome-labeled (VivoTag 680) iron oxide nanoparticles. Dextran-caged iron oxide nanoparticles were used as the parent formulation to provide a highly stable, relatively non-cytotoxic, and *in vivo*-tested nanoparticle scaffold. Briefly, a heterobifunctional linker bearing an azide on one end and an N-hydroxysuccinimide leaving group on the other was synthesized and attached to an amine-PEG-thiol polymer (MW 5000 Da) via its terminal amine (Schematic 2A, 2B). Azido-PEG-thiol polymers were subsequently linked to surface of cross-linked, aminated, and fluorochrome-labeled dextran-coated iron oxide nanoparticles via the linker N-[γ -maleimidobutyryloxy] succinimide ester (GMBS) (Schematic 2C). Long PEG polymers were utilized to carry pendant azide groups in order to enhance particle circulation time *in vivo* and to provide a generalizable nanoparticle surface, whereby optimized “click” attachment conditions might be applicable to other PEG-coated organic and inorganic nanomaterials in the future. Azido-PEG particles were purified from excess polymer and linked to alkyne-bearing peptides in 1 mM CuSO₄, 5 mM Na ascorbate. Finally, the conjugated nanoparticles were purified and sterile filtered for *in vitro* and *in vivo* applications. Peptide valency on nanoparticles was assessed spectrophotometrically by quantifying the number of TAMRA dyes added onto nanoparticles following “click” reaction (Figure 2A). In the presence of catalyst, approximately 30 peptides were added per nanoparticle for both LyP-1 and LyP-1CTL peptides, whereas no addition was observed in the absence of catalyst (Figure 2A). LyP-1 nanoparticles, LyP-1CTL nanoparticles, or azide nanoparticles were incubated over MDA-MB-435 tumor cells for 2 hours and nanoparticle accumulation was quantified using flow cytometry (Figure 2B). LyP-1-nanoparticles showed significant tumor cell accumulation, while LyP-1CTL-nanoparticles or azide-nanoparticles displayed minimal cell uptake (Figure 2B). The effect of serum on nanoparticle uptake was also studied, as low serum levels enhance the stress-induced expression of the p32 receptor (33). The slight increase in LyP-1-nanoparticle targeting in lower serum levels provided further validation of receptor-specific targeting, as decreased serum protein concentrations lowers the likelihood of non-specific serum protein mediated uptake. To further confirm the uptake specificity of LyP-1-nanoparticles, free LyP-1 peptide was added to cells along with LyP-1-particles (Figure 2C). Dose-dependent inhibition of uptake was observed with LyP-1 peptide concentrations from 10 to 100 μ M, suggesting the free LyP-1 and LyP-1-labeled particles share common cellular receptors. We attribute the large excess of free peptide required for inhibition compared to the concentration of nanoparticles used (100nM) to the presence of multiple copies of the LyP-1 peptide on each nanoparticle, thus improving nanoparticle avidity to receptors through polyvalent binding (38). In order to visualize LyP-1 peptide-mediated cell uptake, nanoparticles bearing pendant LyP-1 peptides, control LyP-1CTL peptides, or azides were incubated over MDA-MB-435 cells for 30 minutes, washed, incubated with a nuclear stain, and imaged via epifluorescence microscopy (Figure 3). LyP-1-nanoparticles were seen associated with cells, while markedly less binding of azide-bearing or control peptide-bearing nanoparticles was not observed. If the same staining procedure was instead performed at 24 hrs post incubation, LyP-1-nanoparticles were seen in punctate locations consistent with sequestration in endosome-like compartments (Supplemental Figure 2). To assess the cytotoxicity of “click” nanoparticles, NH₂-PEG-, azido-PEG- and peptide-conjugated nanoparticles were incubated for 24hrs of incubation above HeLa cell cultures (Supplemental Figure 3). In all three formulations, the TC₅₀ is >7mM Fe, or over 16 times higher than maximal blood concentrations during *in vivo* experiments performed here and 32 times higher than concentrations used *in vitro* here.

Having found that “click” attachment of homing peptides could mediate the targeting nanoparticles *in vitro*, we evaluated the ability of “click” chemistry to direct nanoparticle targeting to specific tumor cells *in vivo*. Again, near-infrared fluorochrome-labeled (VivoTag 680) nanoparticles were “clicked” to LyP-1 peptides, resulting on average of ~30 LyP-1 peptides per particle, while the parent azido-PEG nanoparticles were used as a negative control.

Each population of nanoparticles was injected intravenously into mice bearing human MDA-MB-435 cancer xenografts. Nanoparticles were allowed to circulate and distribute in mice for 24hrs, after which the mice were sacrificed and organs collected for immunohistochemical or whole organ fluorescence analysis. Vascular staining with anti-CD31 antibodies showed that azide nanoparticles in tumors remained localized within the immediate periphery of blood vessels (Figure 4B). This perivascular distribution of untargeted nanomaterials is in agreement with previous histological and intravital observations of passive liposomal accumulation in tumors(39, 40). By contrast, LyP-1 “click” nanoparticles appeared to have extravasated from the tumor vasculature, penetrated into the interstitial space of the tumor, and bound to p32-expressing cells (Figure 4B, 4C). As a result, the fraction of LyP-1 nanoparticles that get sequestered beyond the perivascular space was significantly higher than that of azido-nanoparticles (Figure 4D) ($P<0.005$). This pattern was observed in all injected mice and is characteristic of LyP-1 peptide and phage homing observed previously(1). Interestingly, previous LyP-1 bacteriophage experiments showed that the LyP-1-expressing phage concentrate in non-vascularized sites of tumors within minutes after injection while insertless phage do not reach these regions(1). Thus, there may be unique transportation pathways within tumors that are exploited by this ligand after extravasation that are not available to untargeted materials. In the future, the localization of LyP-1 nanoparticles in avascular tumor regions may be of use for directing therapeutics into hypoxic regions of tumors, where most nanoparticle therapies do not reach. Whole organ assessment of near-infrared tumor fluorescence demonstrated that, despite the distinct microscopic behavior of LyP-1 nanoparticles, the macroscopic tumor accumulation of LyP-1 nanoparticles and PEG-azide nanoparticles was similar (Supplemental Figure 4), indicating that the targeted accumulation of LyP-1-nanoparticles was on par with passive delivery, whereby long-circulating materials accumulate in tumors via their hyper-porous vasculature over time(41, 42). These results are in accordance with data showing that the development of targeted nanoparticle formulations that amplify the macroscopic accumulation in tumors requires systematic *in vivo* optimization of multiple material parameters, including target avidity, circulation time, and particle size(43, 44). Experiments of this kind are ongoing in order to probe the power of the LyP-1 targeting ligand for amplifying the accumulation and efficacy of nanoparticle-based imaging and therapeutic agents.

CONCLUSION

In this work, we have demonstrated that “click” chemistry may be used to develop nanoparticles that seek out specific cells *in vivo* based on their surface expression of protein markers. Ultimately, these findings suggest that “click” chemistry meets the criteria of being applicable under aqueous conditions, efficient, orthogonal to thiol- and amine-containing targeting motifs, and stable in the complex *in vivo* environments of the blood and tumor milieu. In the future, this work may empower the development of “click” nanomaterials that seek out specific tumor cell types, including tumor stem cells and angiogenic endothelial cells, or amplify the macroscopic accumulation of imaging agents or therapeutics in tumors. Further, the modularity of this “click” attachment strategy should allow it to be adapted to a diversity of *in vivo* nanoparticle platforms and both biological and synthetic ligands, potentially empowering novel on-nanoparticle screen approaches to targeted nanomaterial development.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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REFERENCES

- (1). Laakkonen P, Porkka K, Hoffman JA, Ruoslahti E. A tumor-homing peptide with a targeting specificity related to lymphatic vessels. *Nat Med* 2002;8:751–5. [PubMed: 12053175]
- (2). Lee SM, Lee EJ, Hong HY, Kwon MK, Kwon TH, Choi JY, Park RW, Kwon TG, Yoo ES, Yoon GS, Kim IS, Ruoslahti E, Lee BH. Targeting bladder tumor cells in vivo and in the urine with a peptide identified by phage display. *Mol Cancer Res* 2007;5:11–9. [PubMed: 17259343]
- (3). Rajotte D, Ruoslahti E. Membrane dipeptidase is the receptor for a lung-targeting peptide identified by in vivo phage display. *J Biol Chem* 1999;274:11593–8. [PubMed: 10206967]
- (4). Porkka K, Laakkonen P, Hoffman JA, Bernasconi M, Ruoslahti E. A fragment of the HMGN2 protein homes to the nuclei of tumor cells and tumor endothelial cells in vivo. *Proc Natl Acad Sci U S A* 2002;99:7444–9. [PubMed: 12032302]
- (5). Ruoslahti E. Vascular zip codes in angiogenesis and metastasis. *Biochem Soc Trans* 2004;32:397–402. [PubMed: 15157146]
- (6). Simberg D, Duza T, Park JH, Essler M, Pilch J, Zhang L, Derfus AM, Yang M, Hoffman RM, Bhatia S, Sailor MJ, Ruoslahti E. Biomimetic amplification of nanoparticle homing to tumors. *Proc Natl Acad Sci U S A* 2007;104:932–6. [PubMed: 17215365]
- (7). Reddy GR, Bhojani MS, McConville P, Moody J, Moffat BA, Hall DE, Kim G, Koo YE, Woolliscroft MJ, Sugai JV, Johnson TD, Philbert MA, Kopelman R, Rehemtulla A, Ross BD. Vascular targeted nanoparticles for imaging and treatment of brain tumors. *Clin Cancer Res* 2006;12:6677–86. [PubMed: 17121886]
- (8). Zhang C, Jugold M, Woenne EC, Lammers T, Morgenstern B, Mueller MM, Zentgraf H, Bock M, Eisenhut M, Semmler W, Kiessling F. Specific targeting of tumor angiogenesis by RGD-conjugated ultrasmall superparamagnetic iron oxide particles using a clinical 1.5-T magnetic resonance scanner. *Cancer Res* 2007;67:1555–62. [PubMed: 17308094]
- (9). Weissleder R, Kelly K, Sun EY, Shtatland T, Josephson L. Cell-specific targeting of nanoparticles by multivalent attachment of small molecules. *Nat Biotechnol* 2005;23:1418–23. [PubMed: 16244656]
- (10). Giordano RJ, Anobom CD, Cardo-Vila M, Kalil J, Valente AP, Pasqualini R, Almeida FC, Arap W. Structural basis for the interaction of a vascular endothelial growth factor mimic peptide motif and its corresponding receptors. *Chem Biol* 2005;12:1075–83. [PubMed: 16242650]
- (11). Colombo G, Curnis F, De Mori GM, Gasparri A, Longoni C, Sacchi A, Longhi R, Corti A. Structure-activity relationships of linear and cyclic peptides containing the NGR tumor-homing motif. *J Biol Chem* 2002;277:47891–7. [PubMed: 12372830]
- (12). Rozek A, Powers JP, Friedrich CL, Hancock RE. Structure-based design of an indolicidin peptide analogue with increased protease stability. *Biochemistry* 2003;42:14130–8. [PubMed: 14640680]
- (13). Ruoslahti E, Pierschbacher MD. New perspectives in cell adhesion: RGD and integrins. *Science* 1987;238:491–7. [PubMed: 2821619]
- (14). Green JJ, Chiu E, Leshchiner ES, Shi J, Langer R, Anderson DG. Electrostatic ligand coatings of nanoparticles enable ligand-specific gene delivery to human primary cells. *Nano Lett* 2007;7:874–9. [PubMed: 17362046]
- (15). Sokolov K, Follen M, Aaron J, Pavlova I, Malpica A, Lotan R, Richards-Kortum R. Real-time vital optical imaging of precancer using anti-epidermal growth factor receptor antibodies conjugated to gold nanoparticles. *Cancer Res* 2003;63:1999–2004. [PubMed: 12727808]
- (16). Huang X, El-Sayed IH, Qian W, El-Sayed MA. Cancer cell imaging and photothermal therapy in the near-infrared region by using gold nanorods. *J Am Chem Soc* 2006;128:2115–20. [PubMed: 16464114]

- (17). Kolb HC, Finn MG, Sharpless KB. Click Chemistry: Diverse Chemical Function from a Few Good Reactions. *Angew Chem Int Ed Engl* 2001;40:2004–2021. [PubMed: 11433435]
- (18). Lee JW, Kim JH, Kim HJ, Han SC, Kim JH, Shin WS, Jin SH. Synthesis of symmetrical and unsymmetrical PAMAM dendrimers by fusion between azide- and alkyne-functionalized PAMAM dendrons. *Bioconjug Chem* 2007;18:579–84. [PubMed: 17335177]
- (19). Wu P, Malkoch M, Hunt JN, Vestberg R, Kaltgrad E, Finn MG, Fokin VV, Sharpless KB, Hawker CJ. Multivalent, bifunctional dendrimers prepared by click chemistry. *Chem Commun (Camb)* 2005:5775–7. [PubMed: 16307142]
- (20). Shi Q, Chen X, Lu T, Jing X. The immobilization of proteins on biodegradable polymer fibers via click chemistry. *Biomaterials* 2008;29:1118–26. [PubMed: 18035410]
- (21). Rozkiewicz DI, Gierlich J, Burley GA, Gutmiedl K, Carell T, Ravoo BJ, Reinhoudt DN. Transfer printing of DNA by “click” chemistry. *Chembiochem* 2007;8:1997–2002. [PubMed: 17854021]
- (22). Ciampi S, Bocking T, Kilian KA, James M, Harper JB, Gooding JJ. Functionalization of acetylene-terminated monolayers on Si(100) surfaces: a click chemistry approach. *Langmuir* 2007;23:9320–9. [PubMed: 17655337]
- (23). Such GK, Tjpto E, Postma A, Johnston AP, Caruso F. Ultrathin, responsive polymer click capsules. *Nano Lett* 2007;7:1706–10. [PubMed: 17530811]
- (24). Li H, Cheng F, Duft AM, Adronov A. Functionalization of single-walled carbon nanotubes with well-defined polystyrene by “click” coupling. *J Am Chem Soc* 2005;127:14518–24. [PubMed: 16218649]
- (25). Cavalli S, Tipton AR, Overhand M, Kros A. The chemical modification of liposome surfaces via a copper-mediated [3 + 2] azide-alkyne cycloaddition monitored by a colorimetric assay. *Chem Commun (Camb)* 2006:3193–5. [PubMed: 17028740]
- (26). Fischler M, Sologubenko A, Mayer J, Clever G, Burley G, Gierlich J, Carell T, Simon U. Chain-like assembly of gold nanoparticles on artificial DNA templates via ‘click chemistry’. *Chem Commun (Camb)* 2008:169–71. [PubMed: 18092076]
- (27). Opsteen JA, Brinkhuis RP, Teeuwen RL, Lowik DW, van Hest JC. “Clickable” polymersomes. *Chem Commun (Camb)* 2007:3136–8. [PubMed: 17653366]
- (28). Antoni P, Nystrom D, Hawker CJ, Hult A, Malkoch M. A chemoselective approach for the accelerated synthesis of well-defined dendritic architectures. *Chem Commun (Camb)* 2007:2249–51. [PubMed: 17534506]
- (29). Zeng Q, Li T, Cash B, Li S, Xie F, Wang Q. Chemoselective derivatization of a bionanoparticle by click reaction and ATRP reaction. *Chem Commun (Camb)* 2007:1453–5. [PubMed: 17389990]
- (30). Brennan JL, Hatzakis NS, Tshikhudo TR, Dirvianskyte N, Razumas V, Patkar S, Vind J, Svendsen A, Nolte RJ, Rowan AE, Brust M. Bionanoconjugation via click chemistry: The creation of functional hybrids of lipases and gold nanoparticles. *Bioconjug Chem* 2006;17:1373–5. [PubMed: 17105213]
- (31). Sun EY, Josephson L, Weissleder R. “Clickable” nanoparticles for targeted imaging. *Mol Imaging* 2006;5:122–8. [PubMed: 16954026]
- (32). Said Hassane F, Frisch B, Schuber F. Targeted liposomes: convenient coupling of ligands to preformed vesicles using “click chemistry”. *Bioconjug Chem* 2006;17:849–54. [PubMed: 16704226]
- (33). Laakkonen P, Akerman ME, Biliran H, Yang M, Ferrer F, Karpanen T, Hoffman RM, Ruoslahti E. Antitumor activity of a homing peptide that targets tumor lymphatics and tumor cells. *Proc Natl Acad Sci U S A* 2004;101:9381–6. [PubMed: 15197262]
- (34). Fogal V, Zhang L, Ruoslahti E. Mitochondrial/cell surface protein p32/gC1qR as a molecular target in tumor cells and tumor stroma. *Cancer Research* (Submitted).
- (35). Rubinstein DB, Stortchevoi A, Boosalis M, Ashfaq R, Ghebrehiwet B, Peerschke EI, Calvo F, Guillaume T. Receptor for the globular heads of C1q (gC1q-R, p33, hyaluronan-binding protein) is preferentially expressed by adenocarcinoma cells. *Int J Cancer* 2004;110:741–50. [PubMed: 15146564]
- (36). Shen T, Weissleder R, Papisov M, Bogdanov A Jr, Brady TJ. Monocrystalline iron oxide nanocompounds (MION): physicochemical properties. *Magn Reson Med* 1993;29:599–604. [PubMed: 8505895]

- (37). Josephson L, Tung CH, Moore A, Weissleder R. High-efficiency intracellular magnetic labeling with novel superparamagnetic-Tat peptide conjugates. *Bioconjug Chem* 1999;10:186–91. [PubMed: 10077466]
- (38). Montet X, Funovics M, Montet-Abou K, Weissleder R, Josephson L. Multivalent effects of RGD peptides obtained by nanoparticle display. *J Med Chem* 2006;49:6087–93. [PubMed: 17004722]
- (39). Unezaki S, Maruyama K, Hosoda J, Nagae I, Koyanagi Y, Nakata M, Ishida O, Iwatsuru M, Tsuchiya S. Direct measurement of the extravasation of polyethyleneglycol-coated liposomes into solid tumor tissue by in vivo fluorescence microscopy. *International Journal of Pharmaceutics* 1996;144:11–17.
- (40). Son DH, Park SA, Lee SW, Cho YW, Roh JL, Kim SY. In vivo tumor targeting and radionuclide imaging with self-assembled nanoparticles: Mechanisms, key factors, and their implications. *Ejc Supplements* 2006;4:47–47.
- (41). Moghimi SM, Szebeni J. Stealth liposomes and long circulating nanoparticles: critical issues in pharmacokinetics, opsonization and protein-binding properties. *Prog Lipid Res* 2003;42:463–78. [PubMed: 14559067]
- (42). Moghimi SM, Hunter AC, Murray JC. Long-circulating and target-specific nanoparticles: theory to practice. *Pharmacol Rev* 2001;53:283–318. [PubMed: 11356986]
- (43). Bartlett DW, Su H, Hildebrandt II, Weber WA, Davis ME. Impact of tumor-specific targeting on the biodistribution and efficacy of siRNA nanoparticles measured by multimodality in vivo imaging. *Proc Natl Acad Sci U S A* 2007;104:15549–54. [PubMed: 17875985]
- (44). Gu F, Zhang L, Teply BA, Mann N, Wang A, Radovic-Moreno AF, Langer R, Farokhzad OC. Precise engineering of targeted nanoparticles by using self-assembled biointegrated block copolymers. *Proceedings of the National Academy of Sciences of the United States of America* 2008;105:2586–2591. [PubMed: 18272481]

Figure 1.

Native and alkyne-bearing LyP-1 peptides target p32-expressing MDA-MB-435 cells *in vitro*. **(A)** Structures of LyP-1, propargylglycine-LyP-1, and Heptynoic acid-LyP-1, all labeled with a TAMRA fluorophore (red). The cyclic nonapeptide is in blue. The pendant alkyne moieties were conjugated to the N-terminus of the peptide during standard Fmoc peptide synthesis. **(B)** Flow cytometry shows that peptides bearing different alkyne groups target MDA-MB-435 cancer cells similarly, while a scrambled control (LyP-1CTL) do not target (P=propargylglycine, H=6-heptynoic acid, $*p < 0.01$, unpaired Student's *t*-test). **(C)** Flow cytometry histogram shows LyP-1, P-LyP-1, and H-LyP-1 peptides (in different shades of blue) target MDA-MB-435 cells *in vitro*, while LyP-1CTL peptide (green) did not show targeting relative to peptide-free control cells (red).

Figure 2.

LyP-1-conjugated nanoparticles target p32-expressing MDA-MB-435 cells *in vitro*, while nanoparticles conjugated to control cyclic peptides (LyP-1CTL) do not target. **(A)** The amount of peptide bound per particle was quantified spectrophotometrically by measuring the absorbance of the TAMRA dyes added following the click reaction. With the addition of catalyst $\text{CuSO}_4/\text{Na ascorbate}$ (dark circles), the TAMRA absorbance at 555nm was quantified to equal approximately 30 peptides per particle, whereas no TAMRA signal was observed without catalyst (light circles). **(B)** LyP-1-nanoparticles or control LyP-1CTL-nanoparticles (both at ~30 peptides per particle), or parent azido-bearing particles (N3), were added to MDA-435-MB cancer cells in normal 10% serum (dark green) and 0.1% serum-starved (light green) conditions. Flow cytometry histogram shows marked increase in uptake of LyP-1-nanoparticles (blue) vs. LyP-1CTL-nanoparticles (green) and particle-free control cells (red). Each error bar represents 6 parallel experiments. **(C)** Addition of free LyP-1 peptides at concentrations from 10 to 100 μM inhibited cellular uptake of LyP-1-coated nanoparticles, suggesting that the LyP-1 peptide and LyP-1-labeled particles target the same receptor.

Figure 3.

LyP-1-nanoparticles target MDA-MB-435 cancer cells (right). Fluorescence imaging of cells incubated with LyP-1-nanoparticles showed increased near-infrared fluorescence (red). Uptake of azido-bearing or scrambled control peptide (LyP-1CTL)-bearing nanoparticles are not visible or show minor background (left and middle).

Figure 4.

“Click” LyP-1-nanoparticle targeting to tumor cells *in vivo*. Nanoparticles bearing only azide groups (top) or labeled with LyP-1 peptides (bottom) that are matched in circulation time were injected intravenously via the tail vein into mice bearing human MDA-MB-435 cancer xenografts. Histological sections were obtained 24 hours post injection. **(A)** Light reflectance images of mice bearing the tumor xenografts. **(B)** Fluorescent LyP-1-nanoparticles (VT680 fluorescence pseudocolored as green) did not colocalize with CD31, a blood vessel marker (red) while untargeted azide-PEG nanoparticles remained localized to the blood vessels or their immediate periphery. **(C)** LyP-1-nanoparticles (green) accumulated in regions of high p32 expression (red), whereas untargeted, azido-bearing nanoparticles did not accumulate in these areas. **(D)** Histological quantitation using CD31 stain to assess nanoparticle localization to immediate periphery of blood vessels. The fraction of LyP-1 nanoparticles outside of the perivascular space of CD31-stained blood vessels is significantly higher than azido-nanoparticles ($P < 0.005$) as assessed from 3 randomly chosen views in each set of mice ($n = 3$). Together, LyP-1-coated nanoparticles penetrate into the tumor interstitium to target p32-expressing cells.

Scheme 1.

Design of a “click” nanoparticle that targets tumor cells *in vitro* and *in vivo*. Cross-linked, fluorescent, superparamagnetic iron oxide nanoparticles are modified to display azido-PEG groups. Conjugation of cyclic targeting peptides (purple circles) bearing pendant alkynes to azido-PEG nanoparticles via the copper(I)-catalyzed Huisgen 1,3-dipolar cycloaddition (“click” reaction) allows specific targeting of the nanoparticles to cells expressing the receptor (red).

Scheme 2.

Synthesis of LyP-1-coated nanoparticles using “click” chemistry. **(A)** Synthesis of Succinimidyl 4-azidobutyrate. **(B)** Synthesis of azide-PEG-thiol by linking Succinimidyl 4-azidobutyrate to a 5kDa thiol-PEG-amine. **(C)** Aminated, cross-linked, fluorochrome-labeled superparamagnetic iron-oxide nanoparticles are activated with GMBS, filtered, and then reacted with the thiol-PEG-azide from (B) to yield azido-PEG bearing nanoparticles. After purification, the particle solutions were reacted with alkyne-bearing LyP-1 peptides with CuSO₄ / Na Ascorbate as catalysts to yield LyP-1-coated nanoparticles for *in vitro* and *in vivo* use.