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Enzyme-Directed Assembly of a Nanoparticle Probe in Tumor Tissue

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The goal of targeted therapeutics and molecular diagnostics is to accumulate drugs or probes at the site of disease in higher quantities relative to other locations in the body. To achieve this, there is tremendous interest in the development of nanomaterials capable of acting as carriers or reservoirs of therapeutics and diagnostics in vivo.^[1] Generally, nanoscale particles are favored for this task^[2] as they can be large enough to function as carriers of multiple copies of a given small molecule, can display multiple targeting functionalities, and can be small enough to be safely injected into the blood stream.^[3] The general goal is that particles will either target passively via the enhanced permeability and retention (EPR) effect, actively by incorporation of targeting groups, or by a combination of both.^[3b,4] Nanoparticle targeting strategies have largely relied on the use of surface conjugated ligands designed to bind overexpressed cell-membrane receptors associated with a given cell-type.^[5] We envisioned an alternative targeting strategy that would lead to an active

accumulation of nanoparticles by virtue of a supramolecular assembly event specific to tumor tissue, occurring in response to a specific signal (Figure 1). The most desirable approach to stimuli-induced targeting would be to utilize an *endogenous* signal, specific to the diseased tissue itself, capable of actively targeting materials introduced via intravenous (IV) injection. Such an approach is in contrast to efforts to develop systems capable of targeting and release *via* the local application of *external* stimuli such as light^[6] or magnetic fields.^[7] With respect to viable endogenous signals, one could reasonably consider materials that accumulate in response to stimuli including pH changes,^[8] temperature variation,^[9] or redox reactions.^[10] However, we aim to develop nanoparticles capable of assembling *in vivo* in response to selective, endogenous, biomolecular signals.^[11] For this purpose, we aim to utilize enzymes as stimuli, rather than other recognition events, because they are uniquely capable of propagating a signal via catalytic amplification *in vivo* as in enzyme-prodrug therapy strategies.^[12]

We hypothesized that an enzyme-directed, nanoparticle accumulation and retention process would be possible if a specific enzymatic signal could be used to chemically alter nanoparticles and induce them to form a new, slowly clearing morphology within tumors.^[13] We reasoned that the best signal for exploring this concept would come from the catalytic activity specific to matrix metalloproteinases, MMP-2 and MMP-9, known to be overexpressed in certain tumor types and proven as viable biomarkers capable of activating peptide-based fluorogenic probes *in vivo*.^[13c,14] To achieve this, we designed a set of MMP-responsive spherical nanoparticles for IV injection into HT-1080 xenograft mice, known to have elevated levels of MMP-2 and MMP-9 within the tumor tissue.^[15] We hypothesized that nanoparticles would circulate throughout the organism then collect by virtue of a MMP-driven accumulation event occurring within the tumor tissue (Figure 1). Furthermore, the particles were labeled to generate specific fluorescent signals associated with the enzyme-induced structures that arise in conjunction with the tissue-specific accumulation events. Therefore, enzyme-driven accumulation and retention would give rise to a probe for tumor tissue.

To generate enzyme-responsive particles capable of generating a FRET (Förster resonance energy transfer) probe in tumor tissue, we designed a set of novel peptide-polymer amphiphiles (PPA) that consist of a peptide substrate for cancer-associated enzymes MMP-2 and -9 conjugated to form a brush copolymer, labeled only at the termini with donor or acceptor dyes (Figure 2).^[16] The PPAs were designed to assemble into fluorescent micellar nanoparticles when dialyzed from DMSO into buffered water, with the peptide substrate present as a hydrophilic shell at the outer surface of stable, spherical micellar nanoparticles. This spherical morphology could then be altered via MMP-directed cleavage of the peptide substrate because cleavage reactions facilitate changes in the steric bulk, and electrostatic properties of the polymeric amphiphiles resulting in a dramatic change in packing behavior through the establishment of new equilibria for surfactant aggregation.^[17] This type of enzyme-response can lead to the assembly of nanoparticles into micrometer scale network aggregates observed *in vitro*.^[16a] Therefore, we hypothesized that tissue selective transformations of this type, within tumors that overexpress MMPs, could possibly lead to accumulation and retention of the materials within those tissues as they transition from small

spherical particles in the blood stream, to micrometer scale aggregates in the tissue (Figure 1).

PPAs were prepared from block copolymers containing a hydrophobic phenyl-moieity and a conjugatable *N*-hydroxysuccinimide-ester prepared via ring-opening metathesis polymerization (Figure 2: Synthetic steps (i) and (ii). See also, Supporting Information, Figure 1S).^[18] The living polymer was modified either with novel rhodamine (**3**) or fluorescein (**4**) termination agents by splitting the reaction into two pots (Figure 2, (iii)), to generate two similar polymers consisting of either the donor or acceptor of a FRET pair. These polymers were further modified with peptide sequences (Figure 2, (iv)) consisting of either L-amino acids as cleavable substrates, or D-amino acids as non-cleavable controls. Therefore, four PPAs were generated, two consisting of the fluorescein-terminated polymer (PPA-F), and two as rhodamine-terminated polymers (PPA-R). From this set of four PPAs, two types of fluorescein-labeled micelles (**M1**, containing the L-amino acid substrate, and **M1_D**, the D-amino acid peptide control) and two rhodamine-labeled micelles (**M2** and **M2_D**) were prepared by dissolving fluorescein-terminated polymers (PPA-F), or rhodamine-terminated polymers (PPA-R) respectively in DMSO and dialyzing against buffered water over 24 h (Figure 2, (v)). In addition, PPA-F and PPA-R were mixed prior to dialysis to generate FRET-labeled micelles containing both donor and acceptor within the same particle (**M3**). Furthermore, a FRET-labeled micelle containing the D-amino acid sequence was generated (**M3_D**). Characterization of the resulting six micelles was performed by fluorescence spectroscopy, dynamic light scattering (DLS) and transmission electron microscopy (TEM). The diameters of these spherical particles was confirmed by TEM and by DLS as between 15-20 nm (Supporting Information). In addition, stabilities in the low nM range were measured from dilution studies of **M3**, whereby the critical aggregation concentration was determined as a loss in intensity of the FRET signal associated with dilution of the intact micelle (Supporting Information, Figure 2S).

Prior to in vivo studies, initial tests of enzymatic responses were conducted in buffered solutions in vitro and resulting micellar morphology changes from sphere to micrometer-scale assemblies were examined via TEM, DLS and fluorescence spectroscopy (Supporting Information, Figure 3S and 4S). The key experiment involved mixing **M1** and **M2** together and treating with activated MMP-9. The resulting reaction is observed over time indicating the formation of a new FRET-active, micrometer-scale assembly as PPA-R and PPA-F are cleaved and rearrange into new structures containing both dyes (Supporting Information, Figure 3S). By contrast, control experiments utilizing the **M1_D/M2_D** pair of particles showed they maintained their original structures as observed by TEM, DLS and via their unchanged spectroscopic properties in the presence of activated enzyme, as they are not cleaved by MMP-9. The cleaved peptide fragment was quantified by HPLC (44% cleavage efficiency after 24 h) and characterized by MALDI-TOF (Supporting Information, Figure 5S). We note that **M1** and **M2** can be stored together in buffer for at least 50 days without aggregating or exchanging PPAs, in the absence of MMPs (Supporting Information, Figure 6S).

With these six particles in hand (**M1-M3** and **M1_D-M3_D**), we aimed to determine if enzymatic signals within tumor tissue could be used to accumulate nanoparticles. For this

purpose we utilized a human cancer model known to overexpress MMPs (HT-1080 xenograft) within nude mice (Figure 3). Three groups of HT-1080 xenograft mice were treated via tailvein injection (40 nmoles injected with respect to PPAs). The first group was treated with a mixture of **M1** and **M2** (i.e. a **M1/M2** co-injection, Figure 3A), the second with a mixture of **M1_D** and **M2_D** (i.e. a **M1_D/M2_D** co-injection, Figure 3B) and the third with **M3** (Figure 3C—serving as a benchmark for the maximum signal possible from an initially FRET-active nanoparticle). The mice were then examined live at given time points via a scan of the tumor as the region of interest. The generation of new aggregates of particles within tumors was monitored via FRET signal development ($\lambda_{\text{ex}} = 470 \text{ nm}$, $\lambda_{\text{em}} = 590 \text{ nm}$). All animal procedures were approved by University of California, San Diego's institutional animal care and use committee. Unique FRET signals were observed to increase in intensity over time beginning at 1 day following administration of a mixture of **M1** and **M2** (Figure 3A). The observed signal comes from micelles forming a new FRET-active assembly induced by MMPs, observable because donor and acceptor carrying PPAs collect within the observable Förster distance with respect to one another. This conclusion is supported by the observation that this FRET signal is not seen in **M1_D/M2_D** co-injected mice (Figure 3B). This negative control result is consistent with in vitro studies confirming the D-amino acid based particles do not react with MMPs (Supporting Information, Figures 3S and 4S). Finally, mice injected with **M3** showed a similar pattern of accumulation as observed for the co-injected donor and acceptor particles (Figure 3C). This final experiment serves as a positive control for the observed FRET signal because **M3** carries both donor and acceptor dyes in its initial spherical form in addition to the accumulated morphology. Furthermore, having observed accumulation over 2 days, we conducted longer-term studies with two more groups of mice (Supporting Information, Figure 7S). In these studies, a FRET signal can be observed for **M1/M2** co-injected mice out to a 7-day time point compared to unobservable fluorescence in the case of **M1_D/M2_D** co-injected animals.

To confirm the observations made through the skin of live mice in our focussed imaging of the tumor only, and to examine off-target accumulation events, tumors and selected organs (liver, spleen, heart, lung and kidney) were excised and FRET signals were measured (Figure 3D-F). Ex vivo analysis reveals that **M1/M2** co-injected mice show accumulation of materials within the tumor with significantly less *detectable* accumulation in other organs. Indeed, we note that this does not rule out particles being present in these organs, simply that they are not detectable in these studies. Furthermore, in the case of **M1_D/M2_D** injected mice some fluorescence can be observed in the ex vivo tumor sample, albeit at significantly reduced levels indicating the possibility of some low level of enzymatic action on these substrates; an observation consistent with other studies of D-amino acid peptide sequences in vivo.^[9]

Live animal imaging data showing nanoparticle accumulation via rearrangement of PPAs were further confirmed by analysis of tissue slices collected from tumors (Figure 3G-H, and Supporting Information, Figure 8S-9S). The accumulated structures within the tumor, resulting from **M1/M2** co-injections, were clearly observed via FRET microscopy (excitation of fluorescein, with rhodamine emission observed, Figure 3G). This observation was further confirmed by traditional channel merged images showing colocalization of

fluorescein and rhodamine dyes (Figure 3H). Here, we observe widespread and homogeneous colocalization of rhodamine and fluorescein dyes, matching data for **M3** injected mice (Supporting Information, Figure 10S A-D). This serves as evidence for enzymatic cleavage occurring concomitantly with aggregation events causing a distribution of rearranged PPAs within the tissue as the mechanism of accumulation. Furthermore, only fluorescein fluorescence is observed in merged channel images from **M1** injected mice (that is, treated in the absence of **M2**), with no observable fluorescence over background from **M1_D/M2_D** co-injected mice.

The *in vivo* studies described above analyse the ability of particles to target by virtue of an enzyme-directed switch and *accumulation* mechanism. Our next efforts sought to determine if this targeting mechanism also resulted in relatively slow *clearance* rates of the materials from the tumor as a proof-of-concept study. To examine this, we required an intratumoral injection of FRET-active micellar nanoparticles, **M3** and **M3_D**. Intratumoral injections were required because we desired a starting reservoir of cleavable (**M3**) or non-cleavable particles (**M3_D**) present in the tumor from the starting time point to visualize clearance. Therefore, two groups of HT-1080 xenograft nude mice were subjected to intratumoral-injection of **M3** or **M3_D** (Figure 4, Supporting Information, Figure 11S). The mice were scanned at various time points; immediately post-injection, 1 h, 2 h, 1 day, 4 days, 7 days, and 8 days (Figure 4 shows selected time points, see Figure 11S for full range). These scans were performed via excitation at 470 nm and monitored for emission at 590 nm to detect the FRET signal inherent to **M3** and **M3_D**. The FRET signal is clearly observed immediately post-injection for both groups of mice (Figure 4A-2, 4B-2). However, after 1 h the D-amino acid containing control particles (**M3_D**) clear from the tissue and are only detectable as a signal in the liver via *ex vivo* analysis after 8 days (Figure 4B-5). This is in contrast to **M3**, with observable fluorescence over the entire 8 day period. These data are consistent with enzyme-driven accumulation and retention of the materials within the tumor tissue, together with undetectable levels in excised organs, as observed for tailvein injections (Figure 3). We note that tumors injected with **M3** showed no visible signs of change relative to any of the control animals.

We have demonstrated the general principle of utilizing endogenous, enzymatic signals to guide and control the accumulation properties of nanoparticles *in vivo*. Critically, the mechanism of accumulation is supported by **M1/M2** co-injection data showing a tissue specific FRET signal. Furthermore, D-amino acid based peptide labeled micelles show exceptionally limited accumulation, implying that the EPR effect is not sufficient to provide passive targeting of these particles. We note that the particles show no observable toxicity with respect to the tumor tissue as evidenced by no change in tumor growth over the 7-8 day observed time periods for any of the tumor bearing animals (Figure 3 and Figure 4). Furthermore, liver and kidney appear normal after 8 days following injection of both control (D-amino acid) and enzyme-responsive nanoparticle probes (Figure 12S). The key to implementing such an enzyme-directed strategy is the ability to program particles to respond to given patterns of enzyme expression associated with the tissue of interest. This concept has been demonstrated here in the context of enzyme-driven changes resulting in accumulation of micellar nanoparticles. These types of systems constitute autonomous,

injectable materials capable of pre-programmed responses to selective signals in complex biological milieu. Work is underway in our laboratories to determine the efficacy of this approach in the context of the delivery of therapeutics and other diagnostic probes. For example, next generation materials would include ones labeled with near infrared dyes to enable deep tissue, whole-animal imaging of live, orthotopic mouse models.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Hubbell JA, Chilkoti A. *Science*. 2012; 337:303–305. [PubMed: 22822138]
2. Merkel TJ, Jones SW, Herlihy KP, Kersey FR, Shields AR, Napier M, Luft JC, Wu H, Zamboni WC, Wang AZ, Bear JE, DeSimone JM. *Proc Natl Acad Sci USA*. 2011; 108:586–591. [PubMed: 21220299]
3. a) Nayak S, Lyon LA. *Angew Chem, Int Ed*. 2005; 44:7686–7708. b) Peer D, Karp JM, Hong S, Farokhzad OC, Margalit R, Langer R. *Nat Nanotechnol*. 2007; 2:751–760. [PubMed: 18654426] c) Vasir JK, Reddy MK, Labhasetwar VD. *Current Nanosci*. 2005; 1:47–64. d) Geng Y, Dalhaimer P, Cai S, Tsai R, Tewari M, Minko T, Discher DE. *Nat Nanotechnol*. 2007; 2:249–255. [PubMed: 18654271] e) Mailaender V, Landfester K. *Biomacromolecules*. 2009; 10:2379–2400. [PubMed: 19637907] f) Champion JA, Mitragotri S. *Proc Natl Acad Sci USA*. 2006; 103:4930–4934. [PubMed: 16549762]
4. a) Matsumura Y, Maeda H. *Cancer Res*. 1986; 46:6387–6392. [PubMed: 2946403] b) Torchilin VP. *Eur J Pharm Sci*. 2000; 11:S81–S91. [PubMed: 11033430]
5. a) Arap W, Pasqualini R, Ruoslahti E. *Science*. 1998; 279:377–380. [PubMed: 9430587] b) Pan D, Turner JL, Wooley KL. *Chem Commun*. 2003:2400–2401. c) Hilgenbrink AR, Low PS. *J Pharm Sci*. 2005; 94:2135–2146. [PubMed: 16136558] d) Liu J, Zhang Q, Remsen EE, Wooley KL. *Biomacromolecules*. 2001; 2:362–368. [PubMed: 11749193] e) Farokhzad OC, Cheng J, Teply BA, Sherifi I, Jon S, Kantoff PW, Richie JP, Langer R. *Proc Natl Acad Sci USA*. 2006; 103:6315–6320. [PubMed: 16606824]
6. a) Lee, Hi; Wu, W.; Oh, JK.; Mueller, L.; Sherwood, G.; Peteanu, L.; Kowalewski, T.; Matyjaszewski, K. *Angew Chem Int Ed*. 2007; 46:2453–2457. b) Fomina N, McFearn C, Sermsakdi M, Edigin O, Almutairi A. *J Am Chem Soc*. 2010; 132:9540–9542. [PubMed: 20568765]
7. a) Hasan W, Stender CL, Lee MH, Nehl CL, Lee J, Odom TW. *Nano Lett*. 2009; 9:1555–1558. [PubMed: 19271715] b) Singh N, Karambelkar A, Gu L, Lin K, Miller JS, Chen CS, Sailor MJ, Bhatia SN. *J Am Chem Soc*. 2011; 133:19582–19585. [PubMed: 21981330]
8. a) Du J, Tang Y, Lewis AL, Armes SP. *J Am Chem Soc*. 2005; 127:17982–17983. [PubMed: 16366531] b) Gillies ER, Frechet MJ. *Bioconjugate Chem*. 2005; 16:361–368. c) Li Y, Du W, Sun G, Wooley KL. *Macromolecules*. 2008; 41:6605–6607.
9. a) Dreher MR, Simnick AJ, Fischer K, Smith RJ, Patel A, Schmidt M, Chilkoti A. *J Am Chem Soc*. 2008; 130:687–694. [PubMed: 18085778] b) Moughton AO, O'Reilly RK. *Chem Commun*. 2010;

- 46:1091–1093.c) De P, Gondi SR, Sumerlin BS. *Biomacromolecules*. 2008; 9:1064–1070. [PubMed: 18288803] d) Roy D, Cambre JN, Sumerlin BS. *Chem Commun*. 2009:2106–2108.
10. Napoli A, Valentini M, Tirelli N, Muller M, Hubbell JA. *Nat Mater*. 2004; 3:183–189. [PubMed: 14991021]
11. a) Azagarsamy MA, Sokkalingam P, Thayumanavan S. *J Am Chem Soc*. 2009; 131:14184–14185. [PubMed: 19757790] b) Laromaine A, Koh L, Murugesan M, Ulijn RV, Stevens MM. *J Am Chem Soc*. 2007; 129:4156–4157. [PubMed: 17358069] c) Savariar EN, Ghosh S, González DC, Thayumanavan S. *J Am Chem Soc*. 2008; 130:5416–5417. [PubMed: 18384200] d) Yang Z, Liang G, Wang L, Xu B. *J Am Chem Soc*. 2006; 128:3038–3043. [PubMed: 16506785]
12. a) Bagshawe, KD.; Napier, M. *Enzyme-Prodrug Strategies for Cancer Therapy*. Melton, R.; Knox, R., editors. Springer; USA: 1999. p. 199–207.b) Campbell DA, Gong B, Kochersperger LM, Yonkovich S, Gallop MA, Schultz PG. *J Am Chem Soc*. 1994; 116:2165–2166.c) Carl PL, Chakravarty PK, Katzenellenbogen JA, Weber MJ. *Proc Natl Acad Sci USA*. 1980; 77:2224–2228. [PubMed: 6246527] d) Dubowchik GM, Walker MA. *Pharmacol Ther*. 1999; 83:67–123. [PubMed: 10511457] e) Duncan R, Gac-Breton S, Keane R, Musila R, Sat YN, Satchi R, Searle F. *J Controlled Release*. 2001; 74:135–146.f) Amir RJ, Zhong S, Pochan DJ, Hawker CJ. *J Am Chem Soc*. 2009; 131:13949–13951. [PubMed: 19739628]
13. a) Scherer RL, McIntyre JO, Matrisian LM. *Cancer Metastasis Rev*. 2008; 27:679–690. [PubMed: 18465089] b) Galande AK, Hilderbrand SA, Weissleder R, Tung CH. *J Med Chem*. 2006; 49:4715–4720. [PubMed: 16854078] c) Jiang T, Olson ES, Nguyen QT, Roy M, Jennings PA, Tsien RY. *Proc Natl Acad Sci USA*. 2004; 101:17867–17872. [PubMed: 15601762] d) Simberg D, Duza T, Park H, Ji M, Essler M, Pilch J, Zhang L, Derfus Austin M, Yang M, Hoffman Robert M, Bhatia S, Sailor Michael J, Ruoslahti E. *Proc Natl Acad Sci USA*. 2007; 104:932–936. [PubMed: 17215365] e) Von Maltzahn G, Harris TJ, Park JH, Min DH, Schmidt AJ, Sailor MJ, Bhatia SN. *J Am Chem Soc*. 2007; 129:6064–6065. [PubMed: 17447766] f) Tung CH, Mahmood U, Bredow S, Weissleder R. *Cancer Res*. 2000; 60:4953–4958. [PubMed: 10987312] g) Querol M, Chen JW, Bogdanov AA Jr. *Org Biomol Chem*. 2006; 4:1887–1895. [PubMed: 16688334]
14. a) Tarin D. *Cancer Microenviron*. 2012; 5:95–112. [PubMed: 22782446] b) Kessenbrock K, Plaks V, Werb Z. *Cell*. 2010; 141:52–67. [PubMed: 20371345] c) Tauro JR, Gemeinhart RA. *Bioconjugate Chem*. 2005; 16:1133–1139.d) Vartak DG, Gemeinhart RA. *J Drug Targeting*. 2007; 15:1–20.e) Olson ES, Jiang T, Aguilera TA, Nguyen QT, Ellies LG, Scadeng M, Tsien RY. *Proc Natl Acad Sci USA*. 2010; 107:4311–4316. [PubMed: 20160077]
15. Bremer C, Bredow S, Mahmood U, Weissleder R, Tung CH. *Radiology*. 2001; 221:523–529. [PubMed: 11687699]
16. a) Ku TH, Chien MP, Thompson MP, Sinkovits RS, Olson NH, Baker TS, Gianneschi NC. *J Am Chem Soc*. 2011; 133:8392–8395. [PubMed: 21462979] b) Chien MP, Thompson MP, Lin EC, Gianneschi NC. *Chem Sci*. 2012; 3:2690–2694. [PubMed: 23585924]
17. a) Wang Y, Xu H, Zhang X. *Adv Mater*. 2009; 21:2849–2864.b) Jain S, Bates FS. *Science*. 2003; 300:460–464. [PubMed: 12702869]
18. a) Smith D, Pentzer EB, Nguyen ST. *Polym Rev*. 2007; 47:419–459.b) Sanford MS, Love JA, Grubbs RH. *J Am Chem Soc*. 2001; 123:6543–6554. [PubMed: 11439041]

Figure 1.

General scheme for enzyme-directed accumulation and retention of nanoparticle probes based on dye-labeled peptide-polymer amphiphiles (PPAs) within tumor tissue. TOP: PPAs depicted as a hydrophilic brush of peptide conjugated to a polymer backbone. After enzyme cleavage, the peptide is truncated causing reequilibration to a new assembly. BOTTOM: [a] Intravenous injection with a mixture of acceptor- and donor-labelled micellar nanoparticles. [b] Particles circulate and passively diffuse into tumor tissue. [c] Accumulation occurs upon reaction of the particles within tumor tissue in response to the action of overexpressed proteases (MMP-2, MMP-9). [d] The accumulation event is detectable via a FRET signal unique to the newly formed assemblies (orange), which are larger in size and slower to clear than the original nanoparticles. [e] Particles continue to circulate and accumulate over time.

Figure 2.

Preparation of MMP-responsive fluorescent micellar nanoparticles. Peptide-polymer amphiphiles (PPAs) with fluorescent tags were generated utilizing ring-opening metathesis polymerization. Block sizes were determined by SEC-MALS and spectroscopy. Synthesis: i) **1** was mixed with Grubbs' modified initiator for 45 min and an aliquot analyzed by SEC-MALS to confirm degree of polymerization. ii) **2** was added, for 45 min and an aliquot analyzed for confirmation of the 21:8 block copolymer ratio by SEC-MALS. iii) The resulting polymer was split into two pots and mixed with novel fluorescent termination agents **3** and **4**, for 2 h followed by addition of ethyl vinyl ether to quench the catalyst. This procedure generates the fluorescently labeled polymers as shown (green circle – fluorescein; red circle – rhodamine). iv) Peptides were added to the block copolymers to form PPAs via amide-bond formation through the amino terminus of the peptide and the polymeric NHS-ester, with degree of incorporation determined by SEC-MALS. v) Dialysis of PPAs from DMSO to PBS (pH 7.4) buffered water over 24 h to generate micellar nanoparticles. **M1** contains PPA-F, **M2** contains PPA-R, and **M3** contains PPA-F and PPA-R mixed in a 1:1 ratio. Peptide sequences: L-amino acid sequence: GPLGLAGGWGERDGS. D-amino acid sequence: gplglagGWGERDGS (lower case indicates portion prepared from D-amino acids). See Supporting Information for synthetic details (Figure 1S).

Figure 3.

Intravenously injected mice imaged to show the time course of accumulation of enzyme-responsive particles in the region of interest (tumor). A) **M1 + M2** co-injected. B) **M1_D + M2_D** co-injected. C) **M3** injected. 1) Background prior to injection. 2) $t = 1$ day. 3) $t = 2$ days. D–F) Ex vivo organ analyses: D) Background; no injection. E) **M1 + M2** co-injected, removed after 2 days. F) **M1_D + M2_D** co-injected, removed after 2 days. General conditions: Micellar nanoparticles were injected via tail vein at 40 nmoles (100 μ L volume) as a solution in PBS buffer. HT-1080 xenograft nude mice with tumor sizes approximately 150 mm³ were utilized for this study. Red circles indicate tumor location identified visually and scanned. Fluorescence images are overlaid on the bright field images of the animals for point of reference as to tumor location.

Figure 4.

Intratumoral injection to determine relative levels of retention of enzyme-responsive nanoparticles vs. control particles with HT-1080 tumors. A) **M3** injected. B) **M3_D** injected. 1) Background prior to injection. 2) Immediately postinjection. 3) 1 h post-injection. 4) 8 days post-injection. 5) Ex vivo organ analysis; 8 days post-injection. General conditions: Micellar nanoparticles were intratumoral injection at 0.0284 nmol/mm³ tumor size. PBS buffer. HT-1080 xenograft nude mice with tumor size around 150 mm³ (~4.26 nmoles of nanoparticles injected) were utilized for this study. Red circles indicate tumor location identified visually and scanned as the region of interest. Fluorescence images are overlaid on the bright field images of the animals for point of reference as to tumor location.