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Rational Design of Matrix Metalloproteinase-13 (MMP-13) Activatable Probes for Enhanced Specificity

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Abstract

Due to the important roles of matrix metalloproteinases (MMPs) play in tumor invasion and metastasis, various activatable optical probes have been developed to visualize MMP activities *in vitro* and *in vivo*. Our recently developed MMP-13 activatable probe, L-MMP-P12, has been successfully applied to image the expression and inhibition of MMPs in a xenografted tumor model (Zhu L et al., *Theranostics*. 2011;1:18–27). In this study, to further optimize the *in vivo* behavior of the proteinase activatable probe, we tracked and profiled the metabolites by a high resolution LC/MS system. Two major metabolites that contributed to the fluorescence recovery were identified: One was specifically cleaved between Glycine (G⁴) and Valine (V⁵) by MMP, while the other one was generated by non-specific cleavage between Glycine (G⁷) and Lysine (K⁸). In order to visualize the MMP activity more accurately and specifically, a new probe D-MMP-P12 was designed by replacing the L-lysine with D-lysine in the MMP substrate sequence. The metabolic profile of the new probe, D-MMP-P12, was further characterized by *in vitro* enzymatic assay and no non-specific metabolite was found by LC/MS. Our *in vivo* optical imaging also demonstrated that D-MMP-12 had significantly higher tumor-to-background ratio (TBR, 5.55 ± 0.75) compared with L-MMP-P12 (3.73 ± 0.31) at 2 h post-injection. The improved MMP activatable probe may have the potential for drug screening, tumor diagnosis and therapy response monitoring. Moreover, our research strategy can be further extended to study other protease activatable probes.

Keywords

Liquid chromatography–mass spectrometry (LC-MS); activatable probe; matrix metalloproteinases (MMPs); metabolite; near-infrared fluorescence imaging

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Supporting Information

Experimental details and figures as noted in text. This material is available free of charge via the Internet at <http://pubs.acs.org>.

INTRODUCTION

As an art of ‘seeing and believing’, molecular imaging brings a better way to noninvasively elucidate the fundamental molecular pathways in living bodies^{1, 2}. Among various molecular imaging modalities, optical imaging has attracted considerable interest due to the non-ionization, cost-effectiveness, and convenience of this modality^{3, 4}. Especially with near-infrared (NIR) fluorophores to overcome the limitation of penetration depth and intrinsic autofluorescence, the applications of optical imaging are expanding in both preclinical and clinical settings.

Proteases are closely involved in most life-sustaining biochemical reactions, which run necessarily to keep cells functional, provide metabolic energy, and protect human bodies. Protease activity is finely tuned *in vivo* by complex mechanisms including the spatial and temporal expressions, small molecule binding, and posttranslational modifications⁵. Anomalous activity of proteases may cause diseases and also stimulate the disease development, such as inflammation^{6, 7}, cancer⁸, neurological disorders⁹, and cardiovascular diseases¹⁰. Considerable efforts have been made to identify the roles of certain proteases in biological processes and to screen specific molecules that can regulate protease expression^{11–15}.

With the development of hydrophilic NIR dyes and the corresponding quenchers, activatable probes have been developed as *in vivo* imaging agents for the detection of protease activity^{8, 16–18}. Composed of an enzyme specific peptide substrate, a NIR dye, and a fluorochrome quencher, these imaging agents are optically silent (quenched) in their native state and are activated in the presence of a specific protease, thereby boosting a strong NIR fluorescence signal. Imaging with these protease activatable optical probes has demonstrated its significance in the field of protease research^{11, 19–21} and protease-targeted drug development^{22, 23}. However, there have been only very few reports about the *in vivo* pharmacokinetics and metabolic profiling after systemic administration of the activatable probes.

To better understand the *in vivo* fate of the activatable probes, in this study, we investigated a matrix metalloproteinase 13 (MMP-13) activatable probe developed by us recently¹⁹. The probe was based on a well-studied MMP substrate peptide, GPLGVRGKGG, and was constructed by conjugating polyethylene glycol (PEG) molecules of various molecular weights. After *in vitro* and *in vivo* characterization, the PEGylated probe with a PEG-12 (P12, MW 545 Da) demonstrated faster activation, higher tumor/normal ratio, and prolonged *in vivo* half-life than the other analogs¹⁹. A high resolution LC/MS method was applied to analyze the *in vitro* and *in vivo* metabolic profiles of this probe. The metabolites that resulted from protease degradation were identified. Based on the LC/MS data, we redesigned a new activatable probe by replacing the L-lysine in the sequence into D-lysine to diminish non-specific degradation of the probe, which was denoted as D-MMP-P12. D-MMP-P12 demonstrated a longer *in vivo* half-life and better tumor-to-background contrast. This is believed to be the first reported metabolic study of an optical activatable probe. The approach to optimize an optical imaging probe could be effectively extended to study other probes.

RESULTS AND DISCUSSION

Investigation of the *in vitro* metabolism of MMP-P12 by LC/MS

The process of designing MMP-P12 was described as in Figure 1a. First of all, to profile the metabolites of the MMP-13 activatable probe L-MMP-P12, we incubated the probe with its specific enzyme, MMP-13 for 2 h. We then ran LC/MS and identified two major fragments

as shown in Figure 1b. Among them, metabolite 1 (Met1) was formed by cleavage between Gly⁴ and Val⁵ of the MMP-P12 peptide, which is consistent with the reported specific cleavage site of MMP enzyme²⁴. Metabolite 2 (Met2) was formed by cutting the probe between Gly⁷ and Lys⁸, and this non-specific metabolite has not been reported previously (Figure S1–S3). In fact, in the peptide sequence used for L-MMP-P12 (GPLGVRGKGG), GPLGVR is the MMP specific substrate sequence²⁵. The glycine was added to increase the flexibility of the probe and the lysine was added to facilitate dye or quencher conjugation with the ϵ -amine group on its side chain^{18, 24}.

Optimization of MMP-P12

Since both fragments restore fluorescence signals during optical imaging with one cleavage site being not enzyme specific, the imaging results will not truly reflect MMP activity. To eliminate the non-specific metabolite (Met2) and reflect MMP activity more accurately and specifically, we re-designed the substrate peptide by replacing the L-lysine with D-lysine. The newly designed probe was denoted as D-MMP-P12 probe to differentiate from the previous probe, L-MMP-P12. After 2 h incubation with MMP-13, only the MMP specific metabolite Met1 but not Met2 was found with D-MMP-P12 (Figure 2). This result indicates that the D-lysine modification could eliminate the non-specific cleavage between Gly⁷ and Lys⁸ by MMP enzyme. We also performed molecular modeling of the interaction of the two probes with the crystal structure of MMP-13. Both L-MMP-P12 and D-MMP-P12 docked with MMP-13 well with similar energy parameters (Figure S4). Moreover, D-MMP-P12 showed very similar enzyme specificity and selectivity with L-MMP-P12 over different MMPs, as shown in Figure 2a.

To compare the enzymatic kinetics of the two probes, we incubated MMP-13 with L-MMP-P12 and D-MMP-P12 respectively and the fluorescence signal was measured and recorded in real time (Figure 2b). D-MMP-P12 showed different enzymatic kinetics from L-MMP-P12. The Michaelis constant K_m calculated for D-MMP-P12 (44.47 μ M) is higher than that for L-MMP-P12 (19.33 μ M), indicating that the fluorescence signal from L-MMP-P12 increased at a faster rate than that from D-MMP-P12. We speculated that the additional cleavage site in L-MMP-P12 made the release of fluorescent dye faster from the quenchers, resulting in faster increase of the fluorescence signal as compared to the D-analog. To test the hypothesis, we applied LC/MS to evaluate the enzyme-cleaved fragments at different time points after incubation. As shown in Figure 2c, the amount of parent L-MMP-P12 decreased with time after incubation with the MMP-13 enzyme and it almost disappeared after 2 h, whereas the amount of enzyme degradation fragments, Met1 and Met2, increased gradually with time. At the beginning of the reaction, Met1 and Met2 showed a similar pattern of generation. After 45 min, the amount of Met1 increased faster than that of Met2. This phenomenon is at least partially due to the fact that Met2 can be further cut by MMP-13 to form Met1, especially when certain amount of Met2 was accumulated at late time points. For D-MMP-P12, only Met1 was found to increase with time by LC/MS. Met2 was not detectable at all (Figure 2d, Figure S6). Consistent with the enzymatic kinetics, the amount of intact D-MMP-P12 decreased at much slower rate than that of L-MMP-P12 over time after incubation with MMP-13, probably because only one enzyme recognition site is available in the D-MMP-P12 sequence.

Evaluation of L-MMP-P12 and D-MMP-P12 *in vivo*

After *in vitro* characterization, we performed imaging studies to evaluate the probe's *in vivo* behavior. It is expected that MMP imaging will be improved by removal of the second cleavage site as the *in vivo* MMP activity will be evaluated more accurately without the interference from non-specific cleavage and consequently reduced background signal. The serial images at various time points (15, 30, 60 and 120 min) after intravenous injection of

either L-MMP-P12 or D-MMP-P12 (Figure S5) into SCC-7 tumor-bearing mice were shown in Figure 3a. Both probes had similar signal intensity over the tumor region at 2 h time point. However, D-MMP-P12 demonstrated a much lower background and a higher tumor-to-background ratio than L-MMP-P12 (5.55 ± 0.75 vs. 3.73 ± 0.31 , $p < 0.05$). Although glycyl-lysine is a substrate of kidney enzyme, we did not observe much higher kidney signal from L-MMP-P12. This may be due to the urinary clearance of the metabolites. Moreover, effective blocking of tumor fluorescence signal by local administration of MMP inhibitors suggests MMP specificity of both D-MMP-P12 and L-MMP-P12 in the tumor area.

Biodistribution of L-MMP-P12 and D-MMP-P12

To validate the *in vivo* optical imaging results, the mice were sacrificed, tumor and other major organs were collected at 2 h after probe injection and imaged *ex vivo*. As shown in Figure 4, tumors had the highest fluorescence signal with either D-MMP-P12 or L-MMP-P12 as the imaging probe, indicating high MMP activity within the SCC-7 tumor. Kidneys showed relatively high signal for both the L- and D-analogs due to the non-specific degradation. Indeed, it has been reported that the glycyl-lysine sequence is a substrate for one of the brush border enzymes, carboxypeptidase M, expressed on the lumen of renal tubules²⁶. Only minimal signal was observed in other organs including liver and intestine. The tumor/muscle ratio for D-MMP-P12 is higher than that for L-MMP-P12 (7.92 ± 1.30 vs. 6.27 ± 1.46), again confirming the superiority of D-MMP-P12 to L-MMP-P12 for imaging MMP activity *in vivo*.

Characterization of the *in vivo* metabolism of L-MMP-P12 and D-MMP-P12 by LC/MS

In order to analyze the *in vivo* metabolism of the probes, plasma, tumor and major organs were collected for LC/MS analysis after intravenous injection. By plotting the amount of intact probes against time (Figure 5a, Figure S7, and Figure S8), D-MMP-P12 showed a much longer circulation half-life than L-MMP-P12 (29.82 ± 0.45 vs. 7.81 ± 0.25 min, $p < 0.01$). At 45 min after injection, there was almost no intact L-MMP-P12 can be detected in blood samples, while D-MMP-P12 showed a much longer circulation time. Moreover, the Met1/Met2 ratio of D-MMP-P12 is also much higher than that of L-MMP-P12 at all the time points examined (Figure 5b), indicating that D-MMP-P12 generated much less non-specifically cleaved Met2 fragment than L-MMP-P12 *in vivo*. Therefore, the relatively lower *in vivo* tumor signal of L-MMP-P12 than D-MMP-P12 can be partially explained by the much faster disappearance of the intact parent probe and more rapid clearance of Met2 from the circulation of the L-form (Figure 5).

To figure out the distribution of D-MMP-P12 and L-MMP-P12, the parent probe, Met1 and Met2 were identified by high sensitivity LC/MS at 60 min after probe injection (Figure S9). In the SCC-7 tumor, the parent D-MMP-P12 probe, Met1 and Met2 were all detectable. For L-MMP-P12, only Met2 was detected while no parent probe or Met1 was found. In plasma, parent form of D-MMP-P12 was still detectable after 1 h, while very little L-MMP-P12 was found. In the liver, only Met2 fragment could be detected for both L-MMP-P12 and D-MMP-P12.

CONCLUSION

A unique feature of activatable optical probes lies in that the fluorescence signal should be restored only under the condition of enzymatic cleavage²¹. Thus, specific fragmentation of the activatable probes is of critical importance for accurate evaluation of enzyme activities. However, there are very limited reports about the *in vivo* pharmacokinetics and metabolic profiling of enzyme activatable probes. To further improve the probe design and accuracy of the measurement of enzyme activity, it is necessary to perform a comprehensive analysis of

the enzymatic cleavage and *in vivo* fragmentation of the activatable probes. Herein, for the first time, we applied LC/MS to profile the metabolites of an MMP-13 activatable probe (MMP-P12), which was recently developed by our group. LC/MS has the unique combination of sensitivity and mass selectivity to provide sensitive detection and mass to charge ratio (m/z) data that can be used to predict parent and metabolite structures²⁷. Consequently, LC-MS has been applied intensively in pharmacokinetic studies, as well as metabolic profiling and metabolomics analysis^{28,29,30}. For example, we have applied LC/MS/MS system to quantitatively analyze the metabolism of gastrin-releasing peptide receptor binding peptidic agonist and antagonist. Based on the metabolite analysis, we found GRPR antagonist ligand is potentially more useful for at least receptor-targeted imaging purpose²⁸.

In this study, we used relatively small sized MMP activatable probes. Even with the same peptide sequence, it is not certain if the enzyme recognition and cleavage will be the same when incorporating the sequence into a polymer or nanoparticle based system^{18, 24}. However, we cannot exclude the possibility that the similar non-specific cleavage exists with those systems and we should be cautious about the data interpretation. The major components may be further degraded and thus complicating the situation. Based on the two major fragments, we are able to profile the metabolism of the MMP-P12 both *in vitro* and *in vivo*. The results provided valuable guidance on optimization of the probes.

In summary, we have presented a novel strategy to optimize an MMP activatable imaging probe guided by LC/MS. We are able to profile the metabolism of the MMP-P12 both *in vitro* and *in vivo* with the high sensitivity and high resolution power of mass spectrometry. The results allowed us to optimize the probe to be more specific to MMP enzymes and may have the potential for drug screening, tumor diagnosis and therapy response monitoring. Besides activatable probes, other peptide-based imaging probes could also be analyzed and potentially optimized by this approach.

METHODS

Chemicals

Cy5.5 monofunctional *N*-hydroxysuccinimide ester (Cy5.5-NHS) was purchased from GE Healthcare (Piscataway, NJ). MMP-13 catalytic domain was constructed, expressed and purified in our lab¹⁹. MMP inhibitor III was bought from EMD Millipore (Billerica, MA). Head and neck squamous cell carcinoma cell line SCC-7 was purchased from the American Type Culture Collection (Manassas, VA). 35-mm glass bottom dishes and 8-well chambers were purchased from MatTek Corporation (Ashland, MA). The amino acids and peptide synthesis related reagents were obtained from CS Bio. Co. (Menlo Park, CA). All other solvents and chemicals were purchased from Sigma-Aldrich.

Synthesis of L-MMP-P12, D-MMP-P12, Met1 and Met2

L-MMP-P12 was synthesized as previously reported¹⁹. Briefly, the side chain protected MMP-13 substrate, GPLGVRGKGG, was synthesized manually on 2-Cl-Trt resin at 0.1 mmol scale using the standard Fmoc (*N*-(9-fluorenyl)methoxycarbonyl) protocol and (benzotriazol-1-yloxy) tripyrrolidinophosphonium hexafluorophosphate (PyBOP) activation. The synthesized peptide was purified by reversed-phase high performance liquid chromatography (RP-HPLC, Dionex, CA) on a C18 semi-preparative column using a linear gradient of 10% to 55% acetonitrile/water (0.1% trifluoroacetic acid) for 30 min at a 10 mL/min flow rate, and lyophilized. Next, protected MMP-13 substrate was reacted with amine-PEG12-alcohol (BroadPharm, San Diego, CA) in the presence of dipyrrolidino (*N*-succinimidyl)oxycarbenium hexafluorophosphate (HSPyU) for 2 h. The Fmoc group on the

peptide was further removed and Cy5.5-NHS was applied onto the N-terminal. At last, all side chain protection groups were removed and a black hole quencher-3 (BHQ-3) dye was conjugated onto the lysine ϵ -amino group. D-MMP-P12 was conjugated in a similar procedure, with L-lysine in the sequence replaced by D-Lysine (Figure S1). Cy5.5-GPLG (Met1) and Cy5.5-GPLGVR (Met2) were also synthesized as controls (Figure S2 and S3). All the final products were confirmed by analytical RP-HPLC, using 5% to 65% acetonitrile containing 0.1% TFA versus distilled water containing 0.1% TFA over 30 minutes at a flow rate of 1 mL/min (C18 column, μ m, 120 Å, 250 $\times$ 4.6 mm) and LC-MS purity > 95% (Figure S1). The retention time for L-MMP-P12, D-MMP-P12, Met1 and Met 2 were 24.5, 24.5, 20.33 and 19.6 min, respectively.

***In vitro* enzymatic assay**

The fluorogenic property of L-MMP-P12 and D-MMP-P12 were examined by incubating 1 μ M of L-MMP-P12 or D-MMP-P12 in reaction buffer (50 mM Tris, 10 mM CaCl₂, 150 mM NaCl, 0.05% Brij35, pH 7.8) with 40 nM of catalytic domain of MMP-13. The NIR fluorescence emission signals of the samples were measured using a Hitachi F-7000 Fluorescence Spectrophotometer (Tokyo, Japan) every 10 min at 37 °C for 120 min. The excitation wavelength was set at 675 nm and the emission spectrum was recorded from 680 to 800 nm. Three independent experiments were conducted. During the incubation of L-MMP-P12 or D-MMP-P12 with MMP-13 enzyme, 100 μ L of reaction solution was taken out every 10 (from 0 to 60 min) or 20 (from 60 to 120 min) min. Additionally, 100 μ L of H₂O/acetonitrile (50/50, v/v) containing 10% HCOOH was added to stop the reaction. LC/MS analysis was then conducted.

Cell culture

SCC-7 squamous cell carcinoma cells were cultured in RPMI 1640 medium containing 10% (v/v) fetal bovine serum (Invitrogen) supplemented with penicillin (100 μ g/ml) and streptomycin (100 μ g/mL) at 37 °C with 5% CO₂. SCC-7 tumor model was developed by subcutaneous injection of 1 $\times$ 10⁶ cells into the right front flank of female athymic nude mice (Harlan Laboratories). The mice were used for optical imaging studies when the tumor volume reached about 300 mm³. All animal studies were conducted in accordance with the principles and procedures outlined in the Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of Clinical Center, NIH.

***In vivo* imaging and ex vivo biodistribution**

In vivo imaging was performed and analyzed using a Maestro 2.10 *in vivo* imaging system (Cambridge Research & Instrumentation, Woburn, MA; excitation = 675 nm, emission = 695 nm). L-MMP-P12 or D-MMP-P12 (10 nmol in 100 μ L PBS) was injected intravenously into SCC-7 tumor-bearing mice *via* tail vein, and imaging was performed at 10, 30, 60 and 120 min after the injection of the probe (n = 5/group). During the injection and image acquisition process, the mice were anesthetized with 2.0 % isoflurane in oxygen delivered at a flow rate of 1.0 L/min. To inhibit MMP expression, 1 μ mol of a broad spectrum MMP inhibitor (MMPI III) was injected intratumorally 30 min prior to intravenous injection of D-MMP-P12. All images were normalized and analyzed using Maestro software. For quantitative comparison, regions of interest (ROIs) were drawn over tumors and muscle, and the average signal ($\times 10^6$ photons $\times$ cm⁻² $\times$ s⁻¹) for each area was measured.

For *ex vivo* study, major organs were collected, rinsed with normal saline and placed on black paper followed by imaging using Maestro. For quantitative comparison, ROIs were drawn as described above. After imaging, the organs were weighed carefully and stored in liquid nitrogen. Before MS analysis, 1 mL of H₂O/acetonitrile (50/50, v/v) containing 10%

HCOOH was added into tube with each organ and subjected to homogenization. After removing the DNA and protein pellet, the solution containing L-MMP-P12 analogs were lyophilized and further dissolved in 1 mL of H₂O/acetonitrile (50/50, v/v) containing 10% HCOOH buffer for LC/MS analysis.

LC/MS

Qualitative LC/MS was conducted with a Waters Acquity UPLC system coupled to the Q-T of Premier high resolution mass spectrometer. An Acquity BEH Shield RP18 column (150 × 2.1 mm) was eluted with a two-solution gradient of solution A (2 mM ammonium formate, 0.1% formic acid, and 5% CH₃CN) and solution B (2 mM ammonium formate and 0.1% formic acid in CH₃CN). The elution profile, at 0.35 mL/min, was: 100% (v:v) A and 0% B initially; gradient 0 - 40% B over 5 min; isocratic elution at 40% B for an additional 5 min; washing with 100% B over 2 min; and re-equilibrium with A for an additional 4 min. The injection volume was 10 µL with entire column elution into the Q-T of mass spectrometer. Ion detection was achieved in ESI mode using a source capillary voltage of 3.5 kV, source temperature of 110 °C, desolvation temperature of 350 °C, cone gas flow of 50 L/h (N₂), and desolvation gas flow of 700 L/h (N₂). Quantitative LC/MS system consisted of an Agilent 1200 HPLC, and an AB/MDS Sciex 4000 Q TRAP. Separation was achieved on a Phenomenex Gemini column (5 µm, 110A, 50 × 4.6 mm) with 2 mM ammonium acetate and CH₃CN) with gradient system at flow rate of 1.0 mL/min. 100% (v:v) A and 0% B 1 min initially; gradient 0–46% B over 4 min; isocratic elution at 46% B for an additional 5 min; washing with 100% B over 1 min; and re-equilibrium with A for an additional 1 min. The different combinations of multiple-reaction monitoring (MRM) [MMP-P12 (951.8/660.1, 951.8/629.1), Met1 (815.2/660.1, 815.2/629.1), Met2 (659.1/629.1), and the full scan MS/MS experiments were performed. Standards were prepared for MMP-P12 covering the concentration range from 1 ng/mL to 160 ng/mL in 1:1 CH₃CN-water with 10% HCOOH. Three replicate injections (20 µL) were made for each concentration level. The specific comparisons made for quantitation used a single MRM transition per analysis.

Statistical analysis

Quantitative data were expressed as mean ± SD. Two-tailed paired and unpaired Student's t tests were used to test differences within groups and between groups, respectively. P values of less than 0.05 were considered statistically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

(a) Experimental design of metabolic profiling and optimization of an activatable probe (AP). (b) Structure of MMP-P12 and LC/MS analysis of the major fragments.

Figure 2.

In vitro characterization of L-MMP-P12 and D-MMP-P12 and LC/MS analysis. (a) Specificity and selectivity of L-MMP-P12 and D-MM-P-12 to MMP enzymes. (b) Fluorescence emission kinetic spectra of L-MMP-P12 and D-MMP-P12 in the presence of MMP-13. (c) Quantitative analysis of fragmentation of L-MMP-P12 incubated with MMP-13 at different time points. (d) Quantitative analysis of fragmentation of D-MMP-P12 incubated with MMP-13 at different time points.

Figure 3.

In vivo near-infrared (NIR) fluorescence imaging evaluation of D-MMP-P12 and L-MMP-P12 in SCC-7 tumor-bearing mice. (a) Representative sagittal images of SCC-7 tumor mice at different time points after intravenous injection of MMP-P12 analogs. Both L-MMP-P12 and D-MMP-P12 detected SCC-7 tumor, whereas D-MMP-P12 demonstrated lower background due to the less non-specific cleavage than that of L-MMP-P12. MMP inhibitor effectively prohibits the fluorescent recovery of both L-MMP-P12 and D-MMP-P12. White arrows indicate the tumors. (b) Region of interest (ROI) analysis of SCC-7 tumor at 2 h post injection (p.i.) of 10 nmol of L-MMP-P12 or D-MMP-P12.

Figure 4.

Ex vivo optical imaging with L-MMP-P12 and D-MMP-P12. (a) Representative images of dissected organs and tissues of tumor-bearing nude mice sacrificed at 2 hours after intravenous injection of L-MMP-P12 (left) and D-MMP-P12 (right) into SCC-7 tumor-bearing mice at a dose of 10 nmol per mouse. Organs were indicated as labeled. (b) Biodistribution of L-MMP-P12 (left) and D-MMP-P12 (right) in SCC-7 tumor-bearing nude mice at 2 h p.i. (mean $\pm$ SD, $n = 3$ /group). (c) Tumor and major organ signal ratios (muscle, M; liver, LI; Lung, LU; and Kidneys, K).

Figure 5. (a) LC-MS analysis of intact L-MMP-P12 and D-MMP-P12 in the blood over time. (b) Met1/Met2 ratio in the blood samples collected at 1, 5, 15, 30, 45, and 60 min postinjection (p.i.) of L-MMP-P12 and D-MMP-P12. *, $p < 0.05$; **, $p < 0.01$.