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Chemical Biology for Understanding Matrix Metalloproteinase Function

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Abstract

The matrix metalloproteinase (MMP) family has long been associated with normal physiological processes such as embryonic implantation, tissue remodeling, organ development, and wound healing, as well as multiple aspects of cancer initiation and progression, osteoarthritis, inflammatory and vascular diseases, and neurodegenerative diseases. The development of chemically designed MMP probes has advanced our understanding of the roles of MMPs in disease in addition to shedding considerable light on the mechanisms of MMP action. The first generation of protease-activated agents has demonstrated proof of principle as well as providing impetus for in vivo applications. One common problem has been a lack of agent stability at nontargeted tissues and organs due to activation by multiple proteases. The present review considers how chemical biology has impacted the progress made in understanding the roles of MMPs in disease and the basic mechanisms of MMP action.

Keywords

activity-based protein profiling; cell-penetrating peptides; FRET; matrix metalloproteinases; optical imaging; triple-helical peptides

Introduction

The matrix metalloproteinases (MMPs) are a family of 23 human zinc endopeptidases that play significant roles in embryonic implantation, tissue remodeling, organ development, and wound healing.^[1] The MMPs have also been implicated in numerous diseases, including multiple aspects of cancer initiation and progression,^[2] osteoarthritis,^[3] inflammatory and vascular diseases,^[4] and neurodegenerative diseases.^[5] The MMPs have long been recognized for their ability to catalyze the hydrolysis of extracellular matrix (ECM) molecules, including collagen, elastin, laminin, and fibronectin.^[6] More recently, attention has turned to the processing of cell surface and intracellular biomolecules by MMPs.^[7]

Unraveling the roles of specific MMPs in disease progression is a daunting task, made more so by the recent identification of certain MMPs as protective agents.^[8] Knowledge about MMP substrates is limited, due to the fact that most experiments are conducted in vitro and that substrates are often derived from late disease stages.^[9] However, the activity of a purified enzyme may not correspond to the in vivo function, as substrates may be post-translationally modified or bound to other proteins capable of modulating activity.^[9] Also,

the sought after substrates from late stage malignancies shed little light on the acquisition, early onset, and maintenance of the phenotype.^[9]

Due to the large number of proteases in the human genome (>500), better detection technologies are required to study the intricate network of proteases and their interactions with substrates, inhibitors, activators, receptors, and other binding proteins. Discovering the redundancy of the enzymes, as well as detecting the functional diversity, is also crucial for understanding the complete disease picture.^[9] All of the above knowledge will contribute to the development of selective probes and inhibitors for disease treatment. Imaging of pathological protease activity in vivo has already contributed to the discovery of novel substrates that lead to disease states.^[9–10]

In order to unravel the roles of MMPs in various pathologies, a great variety of chemical probes have been developed. Probes are believed to accumulate in tumors due to leaky and constricted vasculature.^[11] The use of chemical approaches to better define MMP function has greatly aided target identification. Current proteolytic profiling can be organized into two categories: 1) target-based profiling with targets based either on a given substrate or a given proteinase in a complex sample; and 2) activity-based protein profiling (ABPP; also known as mechanism-based profiling) with an enzyme active site-directed chemical probe to assess the proteolytic activity of a class of enzymes in a complex sample.^[12]

Target-based profiling of MMPs

Target-based profiling takes advantage of substrate sequences selected for specific proteases. Sequences may be derived from natural protein targets of the proteases or from combinatorial libraries (phage display or chemical). Substrate-based probes minimally consist of a peptide sequence and a monitoring group, such as a fluorophore. Upon peptide cleavage, either a fluorescent or a bioluminescent signal is measured. There are four basic applications of this technique (Figure 1).^[10] The first utilizes a fluorophore that changes its fluorescent spectrum upon cleavage from the peptide (i.e., 7-amino-4-trifluoromethyl coumarin). The second, frequently utilized in MMP studies, is based on FRET, where a peptide possessing donor fluorescent and acceptor molecules is either chemically synthesized or expressed (Table 1). Upon peptide cleavage, the FRET is lost, and the increase in donor fluorescence is measured.^[13] One example is an MMP probe with Cy5.5 as the fluorophore and near-infrared quencher NIRQ820 (Table 1).^[14] The third utilizes autoquenching fluorophores, such as Cy5.5 and Cy7, attached proximally within a substrate. Substrate hydrolysis liberates the fluorophore. The fourth exploits a change of fluorescence upon a change in cellular localization of the probe. Fluorescence microscopy is often used to detect such changes (for example, cleavage of a substrate bound to a membrane anchor).^[10]

Targeted-based profiling by FRET

There have been several reports on the use of MMP FRET substrates for screening biological fluids. Cellular metalloproteinase activity was analyzed by using FRET substrates, incorporating (7-methoxycoumarin-4-yl)acetyl (Mca) as the fluorophore and 2,4-dinitrophenyl (Dnp) as the quencher, wherein the substrate was added to the cells and then transferred to 96-well plates for fluorescence readings.^[15] In similar fashion, an Edans/Dabcyl FRET substrate was used for general MMP screening in cell culture medium and synovial fluid.^[16] This assay requires the use of protease inhibitors to minimize non-metalloproteinase activity. An MMP-3-selective sequence identified in our laboratory, Arg-Pro-Lys-Pro-Val-Glu-Nva-Trp-Arg,^[17] was modified to incorporate Edans/Dabcyl and was used for MMP-3 activity screening in rheumatoid synovial fluid.^[18] MMP-3 activity was found to be significantly higher in synovial fluid from patients suffering from rheumatoid arthritis (RA) compared with control subjects. Our laboratory recently described an in situ

MMP cell-based assay with FRET peptide substrates in which no transfer of reagents is needed.^[19] MMP activity assays with three different FRET substrates were utilized to determine whether protein production correlated with function for melanoma WM-266-4 and fibroblast BJ cell lines. Metalloproteinase activity was observed for both cell lines with a general MMP FRET substrate [Knight SSP; Mca-Lys-Pro-Leu-Gly~Leu-Lys-(Dnp)-Ala-Arg-NH₂], consistent with protein production data. However, although both cell lines promoted hydrolysis of a more selective MMP FRET substrate [NFF-3; Mca-Arg-Pro-Lys-Pro-Val-Glu~Nva-Trp-Arg-Lys-Lys(Dnp)-NH₂], metalloproteinase activity was only confirmed in the BJ cell line. The use of inhibitors to confirm metalloproteinase activities pointed to the strengths and weaknesses of in situ FRET substrate assays.

Significant MMP-9 overexpression in tumor tissue, serum, plasma, and urine has been observed in patients with breast cancer. ADAM12, a member of the disintegrin metalloproteinase family, has also been detected in the urine of breast and bladder cancer patients and its level correlated with disease status, progression, and risk. A secreted isoform of ADAM8 has been reported to correlate with head and neck cancers. In order to monitor MMP-9 and ADAM12 activity in the urine of breast cancer patients, FRET-based probes were designed and screened against other MMP and ADAM family members.^[20] Probes Flsub8 [Dabcyl-Pro-Cha-Gly-Cys(S-methyl)-His-Ala-Lys-(FAM)-NH₂] and Flsub11 [Dabcyl-Ser-Asn-Leu-Ala-Tyr-Tyr-Thr-Ala-Lys(FAM)-Lys-NH₂] were shown to be preferentially cleaved by MMP-9, whereas Flsub21 [Dabcyl-Leu-Ala-Gln-Ala-homoPhe-Arg-Ser-Lys(FAM)-NH₂] was used to detect ADAM12 activity, although it was shown to be cleaved by ADAM17 and ADAM10 as well. Another probe, Flsub13 [Dabcyl-His-Gly-Asp-Gln-Met-Ala-Gln-Lys-Ser-Lys(FAM)-NH₂], was tested against ADAM8 activity. MMP-9-specific substrates Flsub8 and Flsub11 only showed partial selectivity in urine samples from normal and metastatic groups of patients. When using Flsub21, only 19% of urine samples from normal controls displayed cleavage activity, whereas 34, 68, and 89% of samples from ductal carcinoma in situ (DCIS), inflammatory breast cancer (IBC), and metastatic patients, respectively, displayed Flsub21 cleavage activity. Similar trends were observed for Flsub13, with 28% of normal samples displaying activity and 37, 77, and 89% of samples from DCIS, IBC, and metastatic patients, respectively, exhibiting positive Flsub13 activity.

These studies were further developed in an effort to design a diagnostic tool for prediction of protease activity utilizing a mixture of substrates, with the ultimate goal of one-shot determination of which MMPs are predominantly active in a given sample.^[21] In order to accomplish this goal, the group created a mixture of soluble FRET-based substrates and analyzed proteolytic signatures given by MMPs studied in isolation and in combination. Elaborate analytic tools, referred to as PrAMA (proteolytic activity matrix analysis), were created in order to deconvolute the results. Analysis revealed that although some MMPs can be accurately and simultaneously studied in a given sample, better substrates are needed to distinguish between other MMPs. Although these observations were made in purified-enzyme samples, the group applied the method to cell-based experiments. Conditioned media from MDA-MB-231 cells stimulated with EGF and TNF α was "spiked" with MMP-7 and subjected to analysis. MMP-7 activity was detected with 100 % specificity but no other MMP activity was detected. In addition, PrAMA was applied to live cell analysis in ADAM10^{-/-} and ADAM17^{-/-} mouse embryo fibroblast (MEF) cells. PrAMA analysis applied with respect to five chosen enzymes (MMP-2, MMP-9, MMP-14, ADAM10, and ADAM17) indicated that 1) treatment of the cells with phorbol ester PMA stimulated ADAM17 activity; 2) substrate cleavage was sensitive to treatment of cells with metalloprotease inhibitor GM6001; 3) ADAM17^{-/-} cells contained 90% less ADAM17, and ADAM10^{-/-} cells showed 90% less ADAM10 activity than WT MEFs; and 4) ionomycin treatment induced an increase in cleavage by all five proteases in all three cell lines, but upregulation was the most striking with respect to ADAM10 and MMP-9. Upregulation of

ADAM17 with PMA stimulation and of ADAM10 with ionomycin stimulation had previously been shown, thus validating PrAMA as an additional tool which can be used in a real time manner without additional sample manipulations.

Our laboratory previously described a number of FRET triple-helical peptide (fTHP) substrates (Figure 2) that are either suitable for most collagenolytic MMPs or selective for different collagenolytic MMPs.^[13a,22] fTHP substrates were initially utilized for mechanistic studies of MMP family members (see later discussion). Subsequently, some of these substrates were applied to monitor cellular proteolytic potential. A solid-phase MMP/fTHP assay was developed to determine the collagenolytic capabilities of melanoma cells compared with fibroblasts by conditioned-media assays.^[23] Analysis of several melanoma cell lines correlated higher constitutive MMP-13 activity with higher levels of production of cell surface receptor CD44.

MMP/fTHP assays were further expanded to evaluate the effect of the tumor microenvironment on melanoma proteolytic potential. The microenvironment's influence has been suggested to be more important in initiating and affecting the development of cancer than the mutations found in the cancer cells.^[24] Tumor cells have altered expression patterns of cell surface receptors and intracellular signaling factors that respond differently to their microenvironment than their normal counterparts. Cancer cells are well known for their adaptability, yet their ability to proliferate and invade relies on signaling pathways, some of which are initiated by the ECM. Our laboratory found that ligands possessing either $\alpha 2\beta 1$ integrin or CD44 binding sites from type IV collagen promote expression of MMPs in a ligand-dependent and temporal manner.^[25] Gene and protein expression, as well as protease activity of MMP-1, MMP-2, MMP-3, MMP-13, and MT1-MMP, were modulated with an $\alpha 2\beta 1$ -specific ligand, whereas an CD44-specific ligand yielded significant stimulation of MMP-8 and lower levels of modulation of MMP-1, MMP-2, MMP-13, and MT1-MMP.^[25] These experiments demonstrated that cellular interaction with the ECM at different receptors induces different signaling outputs, that is, proteolytic profiles.

Four genetically encoded fluorescence indicators have been designed for MMPs. The first encodes a yellow fluorescent protein (YFP, the quencher) and a cyan fluorescent protein (CFP, the fluorophore) linked by the peptide Leu-Glu-Gly-Gly-Ile-Pro-Val-Ser-Leu-Arg-Pro-Val, which contains the MMP substrate site (mss). This sensor, designated YFP-mss-CFP^{display}, was used to detect secreted MMP-2 activity of MCF-7 breast cancer cells.^[26]

The same research group later designed a probe designated DMC (DsRed2-mss-CFP, expressed from pDisplay vector) and measured the decrease in the DsRed2/CFP ratio in MDA-MB435 breast carcinoma cells as an indicator of MMP activity.^[13b] The red-shifted spectrum of the DsRed fluorophore provided a brighter signal and less fluorescent cross-talk with the CFP molecule than did YFP-mss-CFP^{display}. This probe was also tested in shell-less cultures of chick embryo chorioallantoic membrane (CAM) as an in vivo tumor model for MMP detection and was proven to be readily detectable by fluorescence stereomicroscopy. However, MDA-MB435 cells express high levels of MMP-2 but also MMP-1, MMP-3, MMP-9, MT1-MMP, MT2-MMP, and MT3-MMP, so follow-up selectivity studies are needed.

The third indicator encodes a YFP variant (YPet, the quencher) and an enhanced cyan fluorescent protein (ECFP, the fluorophore) linked by the peptide Cys-Pro-Lys-Glu-Ser-Cys-Asn-Leu-Phe-Val-Leu-Lys-Asp, and was used to detect MT1-MMP activity in HeLa cells.^[27] This indicator was anchored to the cell membrane by platelet-derived growth factor receptor transmembrane domain (PDGF_TM). The indicator was further modified to incorporate mOrange2 as the donor and mCherry as the acceptor linked by Cys-Arg-Pro-

Ala-His-Leu-Arg-Asp-Ser-Gly.^[28] MT1-MMP activity was detected increasing slowly in HeLa cells and at the cell periphery upon EGF stimulation, in contrast to Src activity.^[28] Slow MT1-MMP activity was due to slow activation. The MT1-MMP cleavage sequence was not a substrate for MMP-2 or MMP-9.^[28]

An MT1-MMP indicator containing CyPet as the fluorophore and YPet as the quencher has been described.^[29] To improve selectivity compared with prior MT1-MMP probes, a pentapeptide library was screened, and the substrate C-10.1-Y (Ser-Leu-Ala-Pro-Leu-Gly-Leu-Gln-Arg-Arg) was found to be more selective for MT1-MMP compared with other MMPs, with the exception of MMP-9.^[29] MT1-MMP-deficient breast carcinoma MCF-7 and MT1-MMP-producing fibrosarcoma HT-1080 cells were screened with CyPet-[C-10.1-Y]-YPet and compared with other MT1-MMP sequences. Although all indicators had low backgrounds for MCF-7 cells, CyPet-[C-10.1-Y]-YPet showed the greatest levels of MT1-MMP activity for HT-1080 cells.

Other probes were designed to measure activity of MMP-12 in activated macrophages in a mouse pulmonary inflammation model.^[30] The FRET reporter LaRee1 was based on the MMP-12 preferential cleavage site sequence Pro-Leu-Gly-Leu-Glu-Glu-Ala. The cleavage sequence was flanked by two di(ethylene glycol) units and two Lys residues. The N-terminal Lys had the fluorophore coumarin 343 attached to the α -amino group and palmitic acid attached to the ϵ -amino group, whereas the C-terminal Lys had the fluorophore TAMRA attached to the ϵ -amino group. Upon cleavage by MMP-12, the two fluorophores separated, causing a large shift in the emission ratio, and the lipidated moiety linked to the donor fluorophore entered and accumulated within the cells. In addition, soluble forms of this same reporter with varying fluorophores were tested against the pericellular activity of MMP-12 not anchored to the cell membrane. The probes allowed for the identification of macrophages isolated from particulate-treated mice versus untreated or treated MMP-12^{-/-} mice with a three-fold signal increase.

Protease-activated imaging has been achieved by use of a fluorogenic proteolytic beacon for in vivo detection of tumor-associated MMP-7 activity.^[31] The MMP-7 substrate was fluorescein-Ahx-Arg-Pro-Leu-Ala~Leu-Trp-Arg-Ser-Ahx-Cys (Fl-M7). Fl-M7 was then attached to a polyamido amino (PAMAM) dendrimer core, followed by tetramethylrhodamine (TMR), to generate (Fl-M7)_m-PAMAM-(TMR)_n. Fl-M7 served as the optical sensor of protease activity, with TMR effectively quenching fluorescein (Fl) fluorescence in the intact conjugate. In addition, TMR provided an internal reference for the intact conjugate, as TMR could be monitored by using its own fluorescent properties. Thus, the cleaved and uncleaved conjugates could be differentiated, based on the ratio of green/red (Fl/TMR) fluorescence. The M7 sequence was hydrolyzed much more rapidly by MMP-7 compared with MMP-2, MMP-3, trypsin, and proteinase K. Imaging with Fl-M7_m-PAMAM-(TMR)_n was utilized to discriminate between MMP-7-positive and MMP-7-negative mouse xenograft tumors originating from SW480 human colon cancer cells.^[31]

A FRET-based sensor incorporating graphene oxide (GO) as a quencher was designed to monitor and quantify the activity of MMP-2.^[32] GO is single-atom thick, two-dimensional material that has unique electronic, optical, and thermal properties. The material is biocompatible, has good water dispersibility, and provides facile surface modification at low manufacturing costs, making it attractive for biomedical and biosensor applications. In addition to these qualities, GO is also an effective quencher because of its long-range nanoscale energy transfer property. The addition of FITC-Gly-Pro-Leu~Gly-Val-Arg-Gly-NH₂ peptide solution to GO led to the formation of a GO-peptide probe that exhibited 90% quenching of the fluorophore. The probe was incubated with increasing concentrations of MMP-2 and exhibited a linear activity range with excellent sensitivity (as low as 50 pm of

enzyme) and a five- to tenfold signal increase. The probe was tested against MMP-1 and exhibited a weak fluorescence increase. Conditioned cell super-natant from HeLa cells (which are known for production and secretion of MMP-2) was incubated with the GO-peptide, and MMP-2 levels were quantified against the linear curve obtained during validation with purified enzyme. It was determined that MMP-2 levels increased with increasing concentration of HeLa cells. This was the first example of quantitative measurement of MMP-2 enzyme in HeLa cells. Although the substrate used here may not be selective to MMP-2 alone, improvement in sequence selectivity can make this an attractive probe for future biosensor development.

In order to determine protease activity during cell migration and invasion, a fluorogenic probe anchored to a crosslinked collagen matrix was designed.^[33] The substrate peptide Lys-Asp-Pro-Ahx-Gly-Pro-Leu-Gly-Ile-Ala-Gly-Ile-Gly-Ahx-Pro-Lys-Gly-Tyr (where Ahx is 6-aminohexanoic acid), which was derived from a known collagen cleavage site, was doubly labeled on the Lys residues with rhodamine 6G. The self-quenched fluorescence probe was then immobilized on a solid support such as triple-helical collagen or gelatin by chemical crosslinking. Breast cancer MDA-MD-231 cells were incubated on the prepared 2D or 3D surface, and fluorescence was recorded. Contact with cell-associated protease resulted in cleavage of the probe and a red fluorescence pattern showing cellular migratory paths as they traversed the matrix. The location of MT1-MMP activity was confirmed by expressing GFP-labeled MT1-MMP, and its fluorescence correlated with cleavage of the substrate probe. Protease activity was localized to the leading edge in MDA-MD-231 cells, as well as BT549 and PC-3 cells. Interestingly, cell-derived protease activity was required for tumor migration in a crosslinked collagen matrix, but not in pepsin-degraded collagen. The data observed in these experiments not only provided valuable information on the spatial resolution of proteolytic activity in the cellular context but also allowed for quantification of tumor cell migration.

Targeted-based profiling by using FRET with near-infrared (NIR) optical imaging

Near-infrared (NIR) encompasses light in the $\lambda=650\text{--}900\text{ nm}$ range. The advantage of NIR compared with visible light for imaging is the potential for tissue penetration. It has been estimated that, in the NIR window, 7–14 cm of tissue penetration can be achieved.^[34] An additional advantage is that autofluorescence artifacts in the NIR range are minimal, providing a good signal-to-background ratio. A great variety of fluorochromes and quenchers have been developed for NIR optical imaging of protease activities.^[34] Agents developed primarily for optical-based molecular imaging have potential medical applications at the forefront.^[34–35]

The MMP-7 proteolytic beacon described previously was subsequently modified to contain Cy5.5, which monitors protease activity, with AF750 as the internal reference.^[11] The NIR probe (PB-M7NIR) showed a fivefold increase in fluorescence upon treatment with MMP-7. The probe was subsequently used for imaging of MMP-7-positive and -negative human colon cancer cell lines subcutaneously injected into mice. Tumors from the MMP-7-positive cell line produced fluorescence that increased for 3 h; tumors from the MMP-7-negative cell line did not. Ex vivo imaging of MMP-7 activity was achieved for intestinal adenomatous polyps in APC^{Min} mice and was subsequently utilized for histological imaging of adenomas.^[11]

NIR optical imaging of MMP-2 was developed by attaching Cy5.5-Gly-Pro-Leu-Gly-Val-Arg-Gly-Lys(FITC)-Cys-NH₂ to a synthetic graft copolymer composed of poly-L-Lys (PL) and methoxyPEG (MPEG): PL-MPEG₉₂.^[36] A control probe was generated by attaching Cy5.5-Gly-Val-Arg-Leu-Gly-Pro-Gly-Lys(FITC)-Cys-NH₂. MMP-2 and several other MMPs activated the designed probe, but the relative signal generated by other MMPs was

less than 30% of that for MMP-2 under equimolar conditions.^[36b] The control probe was not activated by MMP-2. The MMP-2-targeted probe was able to image HT-1080 human fibrosarcoma tumors in mice, whereas a much lower signal was obtained from low MMP-2-producing BT20 breast adenocarcinoma tumors.^[36] Inhibition of MMP-2 activity by intraperitoneal administration of prinomastat was also able to be imaged in mice and correlated with MMP-2 activity in tumors.^[36b]

An NIR probe for MMP-7 was designed with Cy5.5 as the fluorescence emitter and NIRQ820 as the fluorescence absorber.^[14] The substrate sequence was Gly-Val-Pro-Leu-Ser~Leu-Thr-Met-Gly-Cys, and the final probe was NIRQ820-Gly-Val-Pro-Leu-Ser~Leu-Thr-Met-Gly-Cys(Cy5.5)-NH₂. The probe was highly active against MMP-7 but not against MMP-9. Tumor extracts from human fibrosarcoma HT-1080 cells injected subcutaneously into mice were found to activate the probe, a presumptive sign of MMP-7 activity.^[14]

An additional MMP-7-activated molecular beacon was created by incorporating the sequence Gly-Pro-Leu-Gly~Leu-Ala-Arg-Lys between pyropheophorbide (Pyro) and black hole quencher 3 (BHQ3). Pyro served as both a photosensitizer (to produce ¹O₂) and a NIR fluorescent probe, whereas BHQ3 was both an ¹O₂ quencher and fluorescence quencher.^[37] The conjugate, referred to as PP_{MMP7}B, was activated upon MMP-7 treatment but not MMP-2 treatment. The hydrolyzed, Pyro-containing portion of the beacon was internalized by human nasopharyngeal epidermoid carcinoma KB (MMP-7⁺) cells. Internalization allowed for fluorescent imaging and induction of apoptosis by photodynamic generation of ¹O₂. Neither effect was observed following PP_{MMP7}B treatment of human breast cancer BT20 (MMP-7⁻) cells. In vivo application of PP_{MMP7}B in a KB mouse model resulted in imaging of tumors and reduction in tumor size.^[37]

Another NIR-based activatable probe was designed for MMP-13 for use in in vivo imaging of osteoarthritis (OA). MMP-13 is overexpressed in OA articulate cartilage, although occurring at very low levels in normal tissue.^[38] The probe was based on a previously determined MMP-13 substrate, Gly-Pro-Leu-Gly~Met-Arg-Gly-Leu-Gly-Lys, linked to Cy5.5 and BHQ3 (Probe 1).^[38] BHQ3 has no native fluorescence and minimal absorption, and can efficiently quench fluorophores that emit between λ =620–730 nm. Other dyes typically have their own fluorescent properties and do not provide complete quenching, contributing to limited image resolution attributed to a low signal-to-background ratio. Probe 1 was tested in vitro against MMP-13 and MMP-7.^[38] Although Probe 1 displayed a 32-fold increase in fluorescent signal when treated with MMP-13, it was also significantly cleaved by MMP-7. Three other probes with modified sequences were designed, and all probes were tested against MMP-13, MMP-2, MMP-9, and MMP-7.^[39] Only Probe 2, with the sequence Gly-Val-Pro-Lys-Ser~Leu-Thr-Met-Gly-Lys-Gly-Gly, displayed an improved signal-to-background ratio in vitro compared with Probe 1 (36-fold) and was not cleaved by other enzymes tested. Probe 2 was then tested in vivo in an OA rat model, in which the OA was induced in one knee, allowing for the other joint to serve as a control within the same animal. Probe 2 clearly identified the OA versus the normal knee joint in the acquired image, with a 3.8-fold increase in fluorescence emission. Although this probe proved successful in imaging of OA-associated changes (i.e., increase in MMP-13 activity), the limitation was that NIR could only penetrate the tissue up to a few centimeters, which may not provide enough depth when applied in humans.

Our laboratory described a selective MMP-2/MMP-9/MMP-12 FRET THP substrate, α 1(V)436–447 fTHP [(Gly-Pro-Hyp)₅-Gly-Pro-Lys(Mca)-Gly-Pro-Pro-Gly~Val-Val-Gly-Glu-Lys(Dnp)-Gly-Glu-Gln-(Gly-Pro-Hyp)₅-NH₂].^[22b, 40] This MMP-2/MMP-9/MMP-12 triple-helical substrate was utilized to develop a potentially selective NIR imaging agent by replacing the Mca and Dnp groups with LS276—a highly fluorescent, mono-functional,

water-soluble, heptamethine cyanine dye.^[41] Upon self-assembly of the triple-helical structure, the three peptide chains intertwine, bringing the fluorophores within close proximity and reducing fluorescence by self-quenching. Upon enzymatic cleavage of the THP, six labeled peptide chains were released, resulting in an amplified fluorescence signal. The fluorescent yield of the probe increased 3.8-fold upon activation. Kinetic analysis showed a rate of LS276-THP hydrolysis by MMP-2 ($k_{\text{cat}}/K_M=30000 \text{ s}^{-1}\text{M}^{-1}$) similar to that of MMP-2 catalysis of $\alpha 1(\text{V})436\text{--}447$ fTHP. Administration of LS276-THP to mice bearing a human fibrosarcoma xenografted tumor resulted in a tumor fluorescence signal more than five times greater than in muscle. These results were the first to demonstrate that THPs are suitable for highly specific in vivo detection of tumor-related MMP-2 and MMP-9 activity.

NIR FRET substrates have been successfully used for in vivo visualization of upregulated MMP-2/MMP-9 localization to atherosclerotic aorta. An MMPsense probe (with the sequence Gly-Gly-Pro-Arg-Gln-Ile-Thr-Ala-Gly) was administered to ApoE^{-/-} mice fed high-fat diets and compared to ApoE^{+/+} mice fed equal diets. The upregulation of MMP-2/MMP-9 in atherosclerotic plaques was detected by visualizing probe accumulation by fluorescent molecular tomography.^[42] The probe was also utilized to analyze distribution of MMPs within an excised human carotid plaque from a 74-year old woman with symptomatic carotid stenosis.^[43] The areas of highest NIR intensity were then excised and further analyzed by in-situ zymography and RT-PCR. Both methods confirmed hyperactivity of MMP-2 and MMP-9 at those locations, and PCR displayed 14-fold overexpression of MMP-2 and eightfold overexpression of MMP-9.

The MMPsense probe has been utilized for imaging of MMP hyperactivity in adenomas, considered an early tumor biomarker.^[44] Overexpression of MMP-7 has been found in 85% of colorectal adenocarcinomas and is associated with poor prognosis, whereas elevated levels of MMP-9 have been associated with severely dysplastic polyps, colorectal adenomas, and colorectal cancer metastases. In this study, adenomatous polyposis coli (Apc)^{+/Min-FCCC} mice that develop spontaneous colorectal adenomas were injected with MMPsense 680, and colons were imaged ex vivo. A strong correlation was observed between a positive signal and the presence of pathologically confirmed colonic adenomas. 92.9% of the 350 areas of interest examined were classified correctly, based on a comparison of MMPsense results to immunohistochemical staining of MMP-7 and MMP-9.

An MT1-MMP NIR probe was designed based on a peptide sequence identified in a phage display substrate library. A non-substrate peptide (His-Trp-Lys-His-Leu-His-Asn-Thr-Lys-Thr-Phe-Leu, denoted as MT1-AF7p) displayed high binding affinity to the MT-loop region of MT1-MMP and was shown to interact with the enzyme through hydrogen bonding and hydrophobic interactions.^[45] The loop is located within the catalytic domain and contains an eight amino acid insertion unique to MT-MMPs (MT1-, 2-, 3-, and 5-MMP). This insert is absent from all other MMPs. The assay design was distinct, as the phage display library was screened against a unique sequence from MT1-MMP (¹⁶⁰Arg-Glu-Val-Pro-Tyr-Ala-Tyr-Ile-Arg-Glu-Gly-His-Glu-Lys-Gln¹⁷⁴, designated MT1-160p) and not the entire enzyme or catalytic domain. Only peptides which bound specifically to this sequence (which is located within the loop structure as mentioned above) were evaluated. MT1-AF7 displayed the highest affinity towards MT1-160p ($K_d=0.075 \text{ nM}$), was labeled with Cy5.5 (Cy5.5-MT1-AF7p), and was chosen for further validation in vivo.^[45] Evaluation was performed in mice carrying MDA-MB-435 breast cancer xenografts (expressing high levels of MT1-MMP) and A549 xenografts (low MT1-MMP levels). MDA-MB-435 xenografts had significantly higher signal accumulation and better tumor contrast than A549 xenografts. However, more precise quantitative data on tumor uptake and pharmacokinetics will be needed to determine the further utility of this probe.

Another MT1-MMP fluorogenic probe was designed based on the substrate sequence Gly-Arg-Ile-Gly-Phe~Leu-Arg-Thr-Ala-Lys-Gly-Gly and labeled with Cy5.5 and BHQ3 quencher (MT-P)^[46] in an effort to target the membrane-bound form of MT1-MMP. MT1-MMP is found in both bound and soluble forms, and targeting the membrane-bound form could prove useful in localization of MT1-MMP-overexpressing tumor environments. MT-P was tested against MMP-2 and MMP-9 as well as against MT1-, MT2-, and MT3-MMPs. The probe displayed moderate selectivity. In vivo evaluation of probe activity in mice bearing MDA-MB-435 xenografts indicated strong NIR activation in the MT1-MMP-positive tumor region. Although MT-P displayed good specificity in the tumors, nonspecific activation and accumulation was also observed in the liver. This result suggests further optimization of the probe is needed.

It has been hypothesized that the delivery of chemically labeled activatable probes would be more selective in vivo if the probe was tethered to the surface of a nanoparticle. Effective accumulation of nanoparticles at the disease site would result in high probe specificity and sensitivity (due to sustained enzyme secretion and significant quenching of densely conjugated peptide probes prior to hydrolysis). To examine this hypothesis, nanosensors (NS) were developed that consisted of a self-assembled chitosan nanoparticle (CNP) and an activatable Cy5.5-peptide substrate, quenched by the NIR dark quencher BHQ3 (Figure 3).^[47]

The peptide sequence used in a proof-of-principle study was Cy5.5-Gly-Pro-Leu-Gly-Val-Arg-Gly-Lys(BHQ3)-Gly-Gly, which was shown to be cleaved by MMP-2, MMP-3, MMP-7, MMP-9, and MMP-13. In vivo imaging of pathologies of xenograft SCC-7 tumor and colon cancer in mouse models was performed. Intravenous administration of the NS resulted in observation of MMP-associated disease regions with high specificity and minimal background signals. For example, the in vivo SCC-7 tumor-to-normal tissue (T/N) ratio was 15.4, whereas inhibitor-pre-treated mice had a T/N ratio of 6.4. In azoxymethane-induced colon cancer mice, ex vivo imaging of all tumors produced high fluorescence signals compared with adjacent mucosa. Tumor-associated levels of MMPs were corroborated by ex vivo imaging and immunohistochemistry.

Based on the success of the above NS, an MMP-3-specific probe was developed to detect onset and progression of RA. MMP-3 is thought to be important in the pathology of RA (see earlier discussion). The NIR dye FPR-675 and BHQ3 were linked to the substrate Gly-Val-Pro-Leu-Ser~Leu-Thr-Met-Gly-Lys-Gly-Gly and attached to self-assembled chitosan nanoparticles.^[47-48] The NS were tested against MMP-3 and MMP-7 and displayed activity towards both enzymes. To determine whether the MMP-3 NS could be used for early diagnosis of RA development, mice were injected with the NS over several weeks, and fluorescent images were recorded. At 14 days post-RA induction, a significantly higher NIR signal was detected in arthritic joints compared to normal joints. The signal was detected before any swelling or erythema could be detected with the naked eye or any erosion was detected by histologic or micro-CT analysis. The NIR signal from the arthritic joints increased over the course of arthritis development and was maximal at five weeks post immunization; this was not observed in normal mice. Western blotting and immunohistochemistry confirmed upregulation of MMP-3 activity in joint tissues.

The above NS were developed as a high-throughput MMP-13 diagnostic kit (MMP-d-KIT).^[49] The MMP-13-specific peptide Gly-Pro-Leu-Gly-Val-Arg-Gly-Lys-Gly-Gly (described earlier) with Cy5.5 as a fluorophore and BHQ3 as a quencher was immobilized on the biocompatible glycol chitosan (GC) polymer and anchored to a 96-well plate. MMP peptide probes in the flexible polymer conjugates had free access to the MMP catalytic site. Upon the addition of MMPs, the increase in NIR fluorescence was measured over time. The

kit displayed 25-fold and 22-fold increases in NIR fluorescent signals over reaction time with MMP-2 and MMP-13, respectively. Other MMPs tested (MMP-3 and MMP-7) did not show any probe cleavage. The specificity of the kit was not ideal, and activity of other MMPs (such as MMP-1, MMP-8, and MT1-MMP) need to be tested. The MMP-D-KIT did show overall specificity towards MMPs, as other protease families tested (caspase-3 and 20 s proteasome) did not process the probe. The kit was further evaluated for a clinical application by using 100 μ L of synovial fluid from OA patients with progressive disease. The synovial fluid from the early-stage patients (grade 1) displayed the strongest (50-fold) NIR fluorescence signal as compared to later stage patients (grades 2–4). This indicated the early involvement of MMP-13, and possibly MMP-2, at the onset of OA. Even though the kit is not perfectly selective towards MMP-13, it might be useful as supportive evidence for early detection of OA onset in patients.

Targeted-based profiling by using FRET with quantum dots

In addition to the probes described above, targeting of MMPs has been used to create cell-imaging quantum dots (QDs).^[50] The QDs have typically been coated with streptavidin and conjugated with biotinylated peptide ligands. In one study, the peptide ligand contained an MMP-susceptible sequence sandwiched between a transporting sequence (to allow intracellular delivery of the QD) and a blocking group (which inhibited cellular uptake of the QD). Initial studies showed that (Arg)_n, where $n=4, 5, 7$, or 9 , allows for delivery of QDs into HT-1080 cells following protease processing. For MMP-2-activated delivery, the sequence biotin-(Arg)₄-Ahx-Pro-Leu-Gly~Val-Arg-Gly-(Glu)₄ was designed. This sequence was conjugated to QDs, and the resulting probe was found to exhibit MMP-2-dependent imaging of HT-1080 cells.^[50] MMP-7-dependent imaging was achieved with a probe that conjugated QDs to biotin-(Arg)₃-Gly-Arg-Pro-Leu-Ala~Leu-Trp-Arg-Ser-Gly-(Glu)₅.^[50] These probes were not applied to any MMP disease-related studies.

QDs have also been modified with gold nanoparticles (AuNPs), where the AuNPs suppress QD luminescence.^[51] A collagenase-susceptible sequence (Gly-Gly-Leu-Gly-Pro-Ala-Gly-Gly-Cys-Gly) was incorporated between the QD and the AuNP, allowing for protease-activated imaging, and a PEG₇₅₀ coating was applied to the QD to enhance enzyme activity. The probe was activated by using *Clostridium histolyticum* collagenase type XI, with a 52% increase in luminescence.^[51] However, it has been noted that this change in signal may not be sufficient for practical applications.^[52] In an analogous application, QD fluorescence was modulated by the attachment of rhodamine $\times$ via a collagenase-susceptible Arg-Gly-Asp-Cys sequence.^[53] This QD probe was utilized to monitor proteolytic activity in the ECM of HTB 125 normal breast cells and HTB 126 breast carcinoma cells. After 12 min, fluorescence from the HTB126 cells was ~1.7 times that of the fluorescence of the HTB 125 cells, indicating greater proteolytic activity in the carcinoma ECM.^[53] Control experiments (such as determining MMP levels in the respective cell lines or utilizing an MMP inhibitor) were not performed, so it is not clear which proteases were responsible for the activity.

The QD/AuNP approach was further tested by using a solid surface (*N*-hydroxysuccinimide-derivatized hydrogel glass slide). In this case, AuNP-Cys-Arg-Pro-Leu-Ala~Leu-Trp-Arg-Ser-Lys-biotin was prepared as an MMP-7 substrate.^[54] Fluorescence from QD605 was found to increase as a function of MMP-7 concentration and was detectable in a range of secretions from malignant tissues and in cancer patient sera.^[54] Other colors could be achieved by using QD525 and QD655, thus allowing for a multiplexed assay. This approach was not tested in cellular samples.

QDs for detecting MMP-7 activity were prepared by conjugating CdSe dots coated with ZnS to a PEGylated MMP-7 substrate [PEG₃₄₀₀-Ahx-Arg-Pro-Leu-Ala~Leu-Trp-Arg-Ser-Ahx-PEG₅₀₀₀-Lys(5-FAM)].^[55] Monitoring was performed at $\lambda_{em}=519$ nm, with a decrease in

5-FAM fluorescence corresponding to peptide hydrolysis by MMP-7. The assay was sensitive to MMP-7 concentrations attributed to tumor environments (50–100 nM).^[55]

A bioluminescence resonance energy transfer (BRET) assay was developed by using QDs attached to a bioluminescent protein (luciferase, Luc8) via an MMP-7 (Val-Pro-Leu-Ser~Leu-Thr-Met-Gly) or MMP-2 (Ile-Pro-Val-Ser-Leu-Arg-Ser-Gly) sequence.^[56] It was proposed that BRET-based assays would offer greater sensitivity than FRET-based assays due to lower background in biological environments. The QD/Luc8 construct was able to discriminate between MMP-2 activity in HT-1080 cells versus HT29 cells.^[56]

Targeted-based profiling by using FRET with nanoparticles

Silica nanoparticles (SiNPs) represent a class of nanomaterials possessing excellent biocompatibility, a lack of toxicity, and reactive surfaces for functionalization.^[57] Even though SiNPs are not naturally fluorescent, they can be rendered fluorescent by dye incorporation (doping) or surface modification. In one example, MMP-2 activity was measured by incubation of the enzyme with fluorescent core-shell SiNPs bearing a fluorescently labeled MMP-2 substrate.^[57] The fluorescent labels were chosen such that the emission band of label 1 (contained within the silica shell, emission maximum $\lambda \sim 470$ nm) overlapped with the absorption of label 2 attached to the substrate peptide (maximum at $\lambda = 505$ nm), allowing for FRET emission at $\lambda = 630$ nm. The SiNP described above may have good potential for early detection of dysregulated MMP activity in various cellular environments. The limit of detection of the SiNPs is as good as that for QDs and gold particles, although SiNPs are brighter due to the numerous single fluorophores contained within the shell. Additionally, the fluorophores are well shielded from the adverse effects often caused by cell culture media or fluids in cell homogenates.

Another probe was designed by using an iron oxide nanoparticle, a cleavable peptide linker (Gly-Arg-Arg-Arg-Arg-Gly-Cys), and spectrally matched dual far-red fluorophores ATTO647 and ATTO647N.^[58] The far-red fluorophores were used because of their superiority in *in vivo* applications. The iron oxide was used as a quencher and a particle platform,^[58] ATTO647 was used as a constant fluorophore, and ATTO647N was attached to the peptide linker to provide signal correlating to proteolysis. The ratio of the two fluorescent signals was used to calculate the percentage of activation. Although this approach was tested only with trypsin, it should also prove useful for the quantification of MMP activity with interchangeable linker sequences. An additional benefit of this type of probe is its small size, providing potential ease of *in vivo* usage.

Activatable cell-penetrating peptides

The Pro-Leu-Gly~Leu-Ala-Gly sequence has been used for construction of MMP activatable cell-penetrating peptides (ACPPs).^[59] A series of peptides was created in which an MMP-2-susceptible sequence was inserted between a cell-penetrating peptide (poly-Arg) and a polyanionic sequence that effectively masked the action of the cell-penetrating peptide by associating with it. A key aspect of the construct is that the MMP-2 cleavage site is located within a hairpin structure; this allows for favorable interactions between the poly-Arg and polyanionic sequences. The polyanion masks the adhesiveness of the polycation until the protease cuts the linker, releasing the polycation and associated cargo to adhere to and be taken up by cells in the immediate vicinity. The best activation and uptake of the cell-penetrating peptide by Jurkat and HT1030 cells was observed for succinoyl-(D-Glu)₈-Ahx-Pro-Leu-Gly~Leu-Ala-Gly-(D-Arg)₉-Ahx-Lys(FAM) and PEG₁₁₀₀₀-Ahx-(D-Glu)₉-Ahx-Pro-Leu-Gly~Leu-Ala-Gly-(D-Arg)₉-Ahx-Lys(Cy5). The latter construct was effective for imaging of HT-1080 tumor xenografts and, with replacement of PEG₁₁₀₀₀ by PEG₅₀₀₀, human squamous cell carcinoma tissue sections.^[59] One problem noted was the potential

leakage of soluble MMPs away from the tumor, leading to higher background values. A further modified form of the ACPP [succinoyl-(D-Glu)₈-Ahx-Pro-Leu-Gly~Leu-Ala-Gly-(D-Arg)₉-D-Cys(Cy5)] was found to be processed primarily by MMP-2, MMP-9, and MT1-MMP, with some MMP-8 hydrolysis.^[60] This ACPP was found to have better pharmacokinetics than conventional cell-penetrating peptides. Fluorescent imaging of MDA-MB-231 human adenocarcinoma cells and HT-1080 human fibrosarcoma cells suspended in Matrigel was achieved by using the ACPP.^[60] Confocal imaging of HT-1080 xenografted mice tissues found that the ACPP localized to punctate organelles, presumably endosomes, but not nuclei.^[60] This study noted that improvements in selectivity would increase tumor contrast and decrease systemic toxicity.^[60] A follow-up study utilizing HT-1080 xenografts in mice found that hydrolysis of the ACPP was 65–75% inhibited by a broad-spectrum MMP inhibitor (prinomastat) and the gelatinase inhibitor SB-3CT.^[61] The lack of complete inhibition was attributed to clearance of the inhibitor and/or hydrolysis of the ACPP by another protease. In transgenic tumor models, ACPPs were found to accumulate in regions of MMP activity, such as the tumor–stroma boundary. More accumulation was seen in the cartilage, liver, and kidney than in tumors. Attachment of ACPPs to carriers [such as a poly(amidoamine) dendrimer, albumin, or 70 kDa dextran] resulted in greater tumor accumulation and virtually eliminated uptake in cartilage and kidneys.^[61] The greater tumor accumulation was attributed to increased circulation time, and thus increased time for MMP cleavage, as well as to the enhanced permeability and retention (EPR) effect due to the leaky vasculature of tumors.^[61]

To test the biodistribution of the ACPPs, acetyl-(D-Glu)₉-Ahx-Pro-Leu-Gly~Leu-Ala-Gly-(D-Arg)₉-Ahx-Lys(DOTA)-NH₂ and acetyl-D-Tyr-(D-Glu)₉-Ahx-Pro-Leu-Gly~Leu-Ala-Gly-(D-Arg)₉-Ahx-Lys(DOTA)-NH₂ were labeled with ¹⁷⁷Lu and ¹²⁵I/¹⁷⁷Lu, respectively.^[62] Biodistribution studies were performed in HT-1080 tumor-bearing mice. Retention of Leu177-ACPP was higher than ¹⁷⁷Lu-non-ACPP in heart and muscle, even though no MMP-2/MMP-9 activity was detected in these tissues. As it was the activated form of the ACPP which accumulated, the conclusion of the study was that activation occurred in the vasculature, and accumulation in the tumor was due to the EPR effect.^[62] It was suggested that ACPP sequences be chosen carefully to avoid nonspecific cleavage, and that cell membrane-bound proteases be targeted to avoid ACPP activation in the blood.^[62]

Other fluorescence-based probes

Degradation of fluorescently labeled substrates has long been used for analysis of protease activity. As one specific example of functional live-cell imaging by using self-quenching fluorophores, degradation products generated from cleavage of fluorescently labeled type IV collagen (DQ collagen) were used for imaging prostate and breast cancer cell line functions.^[63] Self-quenching of the fluorophore (FAM) occurred in the intact substrate, and an increase in fluorescence was observed following type IV collagen hydrolysis. Although this approach provided information on β 1-integrin modulation of the type IV collagenolytic potential for each cell line,^[63] specific information on the presence and activity of a specific MMP was not conveyed. Type IV collagen is known to be a substrate for several classes of proteases, such as MMPs, the serine protease plasmin, and the cysteine protease cathepsin B.^[63]

Activated macrophages have been implicated in initiation and propagation of atherosclerosis. The hallmark of this disease is overexpression and secretion of active MMP-9 as well as scavenger receptor AI (SR-AI). To distinguish between resting versus activated macrophages, Suzuki et al. constructed a probe incorporating an MMP-9-cleavable linker with the sequence Val-Pro-Leu-Ser-Leu-Tyr-Ser-Gly.^[64] The probe contains trigger factor (TF) at the N terminus and a GFP- or rLuc-labeled apolipoprotein (ApoB₅₄₇₋₇₃₅). ApoB₅₄₇₋₇₃₅ binds to SR-AI and becomes internalized by activated macrophages upon

MMP-9 cleavage and release of TF.^[64] Both protein constructs were exclusively incorporated into active macrophages without incorporation into neighboring endothelial cells. rLuc and red-shifted rLuc technology can be a useful reporter in animal models, as the reporter does not suffer from autofluorescent artifacts and is widely used for in vivo imaging.

The mechanisms of MMP intracellular trafficking and exposure to the membrane surface are just beginning to be unraveled. MT1-MMP is highly enriched in primary macrophage “invadosomes,” cellular invasion structures that are preferred sites of ECM degradation. In order to visualize trafficking of this protein, an MT1-MMP-mCherry fluorescent construct was transfected and monitored with confocal time-lapse microscopy.^[65] Interestingly, MT1-MMP-mCherry was shown to co-localize to vesicles within microtubules, and coexpression of GFP- α -tubulin showed that the vesicles travel in bidirectional fashion.

Another probe developed for MT1-MMP trafficking was a rhodamine X-conjugated anti-MT1-MMP antibody (anti-MT1-MMP mAb-ROX).^[66] Anti-MT1-MMP mAb-ROX was an activatable probe, with a quenched form containing approximately three ROX molecules per antibody. The fluorescence of the probe increased 14-fold upon the addition of detergent. It was hypothesized that MT1-MMP internalization and delivery to lysosomes for degradation would activate the fluorescent probe. The probe was validated in C6 glioma cells and MCF-7 breast cancer cells as MT1-MMP-positive and -negative models, respectively. Intake of anti-MT1-MMP mAb-ROX bound to MT1-MMP was measured and compared to cells treated with nonspecific ROX-labeled antibodies (NC ab-ROX). The fluorescence intensity was significantly higher in C6 cells compared to MCF-7 cells, and was higher when anti-MT1-MMP mAb-ROX was used compared with NC ab-ROX. The effects of anti-MT1-MMP mAb-ROX were negated when endocytosis was blocked. The probe was successfully tested in other cell lines as well (MDA-MB-231 and HT-1080). The probe was tested in vivo in C6 and MCF-7 xenografted mice and displayed highly differential tumor-to-muscle (T/M) and tumor-to-basal (T/B) signals in C6-implanted mice but not in MCF-7 mice. The T/M ratios increased approximately tenfold within 24 h of injection, and 15-fold within 48 h, as compared to 2.6-fold and 4.6-fold, respectively, for NC ab-ROX. The advantage of this system is that the anti-MT1-MMP antibody has high specificity and affinity for its target, thus providing strict imaging of MT1-MMP. However, the fluorescence of the probe was too short for use in in vivo imaging, and the system may be improved by replacing ROX with a tissue-penetrating NIR group in the future.

Activity-based protein profiling (ABPP)

ABPP-based tools have been designed to measure the activity of enzymes in a temporal and spatial manner.^[10,67] ABPP probes have three characteristic elements: a group that binds the active site of enzymes within a given enzyme class, a reactive group for labeling ABPP-bound enzymes, and a reporter group that allows for observation and isolation of the ABPP-bound enzyme (Figure 4).^[67–68] Metalloproteinases do not form a covalent intermediate with their substrates during catalysis. Thus, in order for ABPP technology to be effective with metalloproteinases, the reactive group is required so that the probe remains bound to the enzyme. Crosslinking is often achieved by utilizing photo-reactive groups, such as benzophenone.

A strength of ABPP probes is that they can discriminate between active enzymes and their inactive counterparts (e.g., zymogens, inhibitor-bound forms, or non-post-translationally modified forms). Conversely, ABPP technology does require a large proteomic sample to ultimately purify the target enzyme. An additional potential weakness is that the efficiency of probe crosslinking is dependent upon individual metalloproteinase interaction with the

reactive group, that is, some metalloproteinases may have surface groups that react with the probe more readily than other metalloproteinases.

ABPP probes have been used to document metalloproteinases upregulated in invasive cancer cells.^[69] Probe design was based on MMP inhibitor GM6001, a hydroxamic acid that chelates zinc and was thought to selectively bind MMPs. Enzymes which were affinity-purified, and thus upregulated in invasive melanoma, included neprilysin (an integral membrane metalloproteinase), leucine aminopeptidase (LAP), and dipeptidylpeptidase III (DPPIII). These results suggested that nonspecific inhibition of metalloproteinases (not limited to the MMP family) may explain the toxicity observed with MMP inhibitors in clinical trials.^[70] A variety of hydroxamic acid-based ABPP probes have been developed for metalloproteinases,^[68b, 71] and comparisons between labeling agents (diazirine-based versus benzophenone) have been performed, but ultimately, none of these probes have yet yielded information about MMP activity in disease states.

Fluorescent labeling of hydroxamate derivatives CGS27023A and CGS25966 was undertaken, involving a PEGylated spacer to minimize the interactions between the activated MMP and the dye of the conjugate with an azide as a protected amino functionality.^[72] After reducing the azide and labeling with Cy5.5, the CGS-based MMP inhibitor was obtained with a fluorescent tag. The photoprobe was tested in three human cell lines (A-673, HT-1080, and BT-20) with respect to differential MMP-2 and MMP-9 production. The PEGylated CGS inhibitors caused complete inhibition of purified MMP-2 and MMP-9. The cryostat tumor sections found to be positive for gelatinase activity also displayed strong binding of CGS-Cy5.5.

Pyrimidine-2,4,6-triones (barbiturates) were recently shown to possess potent MMP inhibitory activities by binding to the zinc(II) active site in a different manner than radiofluorinated hydroxamate analogues.^[73] C-5-disubstituted pyrimidine-2,4,6-triones were exploited as a new class of potential ABPP radio-tracers with improved binding potency (Figure 5). Fluoro-chrome-based tracers have the advantage of being free from ionizing radiation and isotope decay and are applicable to tomographic imaging (such as fluorescence mediated tomography, FMT).^[74] The barbiturate was modified with Cy5.5 by using a short PEG linker to space the dye from the MMP binding site and to preserve gelatinase binding affinity. In vitro kinetic studies confirmed the inhibition of MMP-2 and MMP-9 in the nanomolar range. The probe was also characterized in three different cell lines: rhabdomyosarcoma (A-673), producing both MMP-2 and MMP-9; fibrosarcoma cell line HT-1080, only expressing MMP-2; and MCF-7, which produced neither enzyme. Incubation of each of the cell lines with the Cy5.5-labeled barbiturate caused strong fluorescence in A-673 cells, less fluorescence in HT-1080 cells, and no fluorescence in MCF-7 cells, corresponding to the expression and activity levels of MMP-2 and MMP-9 in each of the cell lines.

Another ABPP photoaffinity probe consisting of a phosphinic peptide core was created by grafting an azido photolabile group onto the inhibitor P₁' side chain (Figure 6).^[75] Initially, phosphinic peptide libraries having the general formula *p*-Br-Ph-(PO₂-CH₂)-Xaa'-Zaa'-NH₂ were screened for activity of ten MMPs.^[76] Two phosphinic peptides were identified to selectively inhibit MMP-12, with *K_i* values of 0.19 and 4.4 nM. A photolabile phosphinic peptide was then designed by incorporating an isoxazoline side chain into the P₁' position.^[75a] The isoxazoline side chain was expected to place the azido group within the conserved S₁' subsite pocket of the MMPs, thereby covalently modifying it. The probe was then radiolabeled with tritium, which was preferred over a fluorescent tag because of the bulkiness and hydrophobic properties of the latter. High sensitivity of detection and quantification of covalently modified crosslinked peptide-enzyme complex were additional

advantages of the radiolabel. UV crosslinking of the probe resulted in $42 \pm 5\%$ labeling of MMP-12, with sensitivity as low as 50 pg of enzyme. However, probe crosslinking was also observed with other MMPs at various levels (MMP-2=24%; MMP-3=5%; MMP-8=1%; MMP-9=15%; MMP-11=11%; MMP-13=35%; and MT1-MMP=19%), thus displaying nonspecific binding. Characterization of a human MMP-12 mutant indicated that modification of the enzyme predominantly involved the ϵ -amino group of Lys241, which is located within the S_1' loop.^[75b] Because the residue in this position is not conserved between different MMPs, the observed variability of cross linking of MMPs other than MMP-12 may be ascribed to structural differences within the S_1' loop, as well as the nucleophilicity of the residue in position 241.^[75b,c]

To further optimize the probe for in vivo applications, the Dive group evaluated an affinity approach versus photoaffinity.^[77] For this purpose, two probes were prepared by attaching a biotin moiety through a PEG spacer (Figure 7). This modification allowed capture of MMPs with magnetic beads when incubated with a streptavidin linker. Although the photoaffinity probe described above displayed affinity in the nanomolar range, the affinity probe constructed here displayed picomolar affinity towards MMP-2, MMP-8, and MMP-12, thus offering superior performance with regard to the quantity of captured enzyme. The efficiency of the capture was corroborated by the performance of affinity pulldown experiments from tumor extracts, leading the way to possible diagnostic tools.

Nuclear imaging (SPECT and PET) of MMP activity

Several groups have recently developed inhibitor-based radiotracers targeting MMPs in their activated forms for noninvasive imaging of MMP-associated disease by means of single photon emission computed tomography (SPECT) or positron emission tomography (PET).^[73] The ^{18}F -labeled N-sulfonyl amino acid hydroxamates CGS25966 and CGS27023A were explored as promising lead structures for the development of potential radiolabeled tracers (Figure 8).^[78] These compounds displayed nanomolar or subnanomolar inhibition of MMP-2, MMP-8, MMP-9, and MMP-13 and were proposed for use in diagnostic applications in animals. No adverse accumulation of the tracers was found in healthy animals.

Radioactive second generation barbiturates were developed and shown to effectively accumulate in areas of high MMP-2, MMP-9, MMP-8, and MT1-MMP activity (Figure 5).^[73] One compound, the 2- ^{18}F fluoroethylazide-conjugated pyrimidine-2,4,6-trione, was synthesized with improved hydrophilic properties which, in turn, exhibited accelerated in vivo excretion and clearance.

Radiotracers have also been developed to monitor MMP activation in a murine model of postinfarction remodeling.^[79] Initial studies were performed with an ^{111}In -labeled MMP-targeted radiotracer (^{111}In -RP782) and an enantiomeric compound (^{111}In -RP788) as a negative control. The experiments were performed in control mice as well as in mice one week post-surgically induced myocardial infarction (MI). Localization and a $334 \pm 44\%$ increase in retention of ^{111}In -RP782 was shown by micro-auto-radiography and gamma well counting. Subsequent in vivo imaging studies utilizing micro-SPECT/CT imaging studies with an analogous $^{99\text{m}}\text{Tc}$ -labeled MMP-targeted radiotracer ($^{99\text{m}}\text{Tc}$ -RP805) and ^{201}Tl demonstrated good biodistribution and clearance kinetics. Myocardial uptake in the MI region was found to be increased fivefold, and a significant twofold increase in myocardial activity in remote regions was also detected. This finding suggested activation of MMPs in regions remote from the MI. This approach has potential clinical utility as a diagnostic tool for in vivo localization of MMP activation and tracking of MMP-mediated post-MI remodeling.

Another radiotracer was designed to detect gelatinase activity of MMP-2 and MMP-9, as these enzymes are known to be overexpressed in various tumors. The cyclic decapeptide cyclo[Cys-Thr-Thr-His-Trp-Gly-Phe-Thr-Leu-Cys] (CTT) was designed based on the considerations that 1) the His-Trp-Gly-Phe motif of the peptide displays inhibition of gelatinase activity, 2) hydrophobic radiolabeled peptides have shown low-level accumulation in liver, and 3) negatively charged radiolabeled peptides previously exhibited good renal clearance.^[80] A hydrophilic and negatively charged radiolabel, indium-111-diethyle netriaminepentaacetic acid (¹¹¹In-DTPA), was attached N-terminally to the His-Trp-Gly-Phe motif (¹¹¹In-DTPA-CTT). This probe exhibited significant inhibition of MMP-2 activity in vitro, and when injected into normal mice, displayed fast clearance from liver and kidneys. In mice bearing MDA-MB-231 breast tumors, the probe displayed increased accumulation within the tumor as compared to MDA-MB-435S tumor-bearing mice. The gelatinase activity was compared between the two cell lines and was shown to be approximately three times higher in MDA-MB-231 tumor, thus indicating the potential usefulness of ¹¹¹In-DTPA-CTT in detecting gelatinase activity. However, the relatively low tumor contrast found in this study warrants further exploration of the molecular interactions between the inhibitor and the enzymes.

Expression of MT1-MMP is associated with poor prognosis in patients with advanced neuroblastoma, small cell lung cancer, tongue squamous cell carcinoma, head and neck carcinoma, bladder cancer, and ovarian cancer.^[81] In order to evaluate MT1-MMP activity in MDA-MB-231 cells, a tripartite probe was designed containing the following components: 1) a positively charged D-Arg octamer (r8) cell-penetrating peptide (CPP) attached with single amino acid chelate (SAAC) for technetium-99 m; 2) an MT1-MMP-specific substrate (Ser-Gly-Arg-Ile-Gly-Phe-Leu-Arg-Thr-Ala); and 3) a negatively charged D-Glu attenuation sequence (Figure 9).^[81] Several attenuation sequences were evaluated in order to achieve linear conformation of the cleavable sequence that would be available for MT1-MMP docking. A sequence comprising four D-Glu-Gly-Gly repeats (4egg) was chosen for cell-based studies. Probe activation was determined by treatment of transfected MDA-MB-231 cells with and without a broad spectrum MMP inhibitor (GM1489). The average uptake of the 4egg probe was two times greater in untreated cells, indicating successful cleavage and increased uptake of the activated probe into MT1-MMP-expressing cells. Negative results from cells treated with free ^{99m}Tc-tricarbonyl complex (^{99m}Tc(CO)₃)⁺ suggested that there was no nonspecific uptake of the radiolabel and that ^{99m}Tc accumulation in cells treated with the intact probe was the result of cleavage rather than leakage of free radiolabel into these cells. Further in vivo imaging analysis is needed to determine the clinical potential of this probe.

A radiotracer to monitor the development and calcification of aortic plaques has been designed, as MMP activity in atherosclerotic lesions is associated with plaque instability.^[82] A noninvasive tracer would allow for assessment of MMP activity in developing lesions and potentially the prevention of acute coronary events. The broad spectrum MMP inhibitor RP805 was labeled with ^{99m}Tc (MPI). Micro-SPECT/micro-CT images were performed in mice deficient in apolipoprotein E (ApoE^{-/-}), mice deficient in low-density-lipoprotein receptor (LDLR^{-/-}), and C57/BL6 mice as controls. Half of the mice were exposed to a high-cholesterol diet, and MPI uptake was visualized as well as examined ex vivo. The extent of pathological lesions, plaque infiltration by macrophages, and MPI uptake was shown to be highest in the most advanced lesions and correlated well with quantitative expression of MMP-2 and MMP-9. The amount of MPI uptake was also proportional to the extent of the plaques, making this a good method for future clinical noninvasive assessment of the extent of expression of various MMPs.

Antibody radiolabeling has also been explored as a potential novel probe for MT1-MMP activity.^[83] ^{99m}Tc-anti-MT1-MMP monoclonal antibody (mAb) was evaluated in breast tumor-bearing rodents. MT1-MMP was highly expressed in all malignant cells tested. Tumor radioactivity increased over time and displayed a three- to fivefold increase at 24 h as compared to 1 h after antibody injection. The radioantibody cleared other organs and blood rapidly, suggesting promising in vivo application of this probe.

Magnetic resonance imaging (MRI) of MMP activity

MRI is one of the most important diagnostic tools utilized to visualize soft tissue malignancies and tumors. Because it does not rely on ionizing radiation, it is a well-suited technology for monitoring disease progression with minimal risk to the patient. The advantage of MRI over fluorescence-based detection modalities is the ability to visualize all tissues without depth limitations.^[84]

Magnetic relaxation switches have been introduced to sense enzymatic activity based on superparamagnetic high-relaxivity probes for in vitro MRI. These crosslinked iron oxide particles (CLIO) are sterically stabilized and switch from large clustered high-relaxivity aggregates to smaller low-relaxivity single particles. However, the drawback of this technique is that it cannot be applied in vivo because of the large size of nonactivated particles, and reduction in relaxivity corresponds to lower contrast within the tissue.^[85]

Initial studies have been presented for MMP-activated nanoparticle self-assembly imaging of tumor cells.^[86] Superparamagnetic Fe₃O₄ nanoparticles were coated with neutravidin, followed by attachment of an MMP-2-susceptible sequence (Lys-Gly-Pro-Leu-Gly~Val-Arg-Gly-Cys) to Lys ε-amino groups on the neutravidin. Finally, MPEG-succinidyl α-methylbutanoate (for which PEG₁₀₀₀₀ was ultimately shown to be optimal) was linked to the peptide Lys ε-amino groups. Treatment with MMP-2 resulted in nanoparticle self-assembly, which caused shortening of T₂ relaxation times in MRI. Nanoparticles were incubated with HT-1080 cell cultures, and self-assembly led to a substantial shortening of T₂ relaxation times, maps of which were generated with MRI.^[86]

Protease-specific NS for MRI on the basis of very small iron oxide particles (VSOP) were also developed by Schellenberger et al. (Figure 10).^[87] This research group synthesized MMP-9-activatable protease-specific iron oxide particles (PSOP) based on electrostatically stabilized, citrate-coated VSOP. The same group previously developed another variant of these superparamagnetic particles (VSOP-C184) for angiography, which were tested in phase II clinical trials. In the present probe, the cleavage motif was based on a previously published activatable fluorescent probe (Gly-Gly-Pro-Arg-Gln-Ile-Thr-Ala-Gly-Lys(FITC)-(Gly)₄-(Arg)₅-Gly-(Arg)₅-amide) and was reacted with an mPEG copolymer (Figure 10A). A ratio of six peptide-mPEGs per VSOP was utilized and yielded MMP-9-PSOP with a small hydrodynamic diameter of 24.9±7.0 nm. When the sterically stabilized MMP-9-PSOP was cleaved by MMP-9 at the peptide recognition site, the stabilizing mPEG shell was lost. Due to the superparamagnetic properties of iron oxide cores, the particles aggregated (Figure 10B). Clustering of the superparamagnetic nanoparticles caused a substantial increase in R₂ relaxativity (the magnetic switch). The particles were also converted from mPEG-protected particles into highly aggregative particles with strongly charged surfaces. The results of size measurements and in vitro MRI experiments showed that, upon incubation with MMP-9, the NS began clustering after a few minutes. This activity was countered in a dose-dependent manner by preincubation with an MMP-2/-9 inhibitor. The above methods are valuable for in vivo imaging of protease activity. However, precipitation of large aggregates seems to be a potential problem.

Jastrzebska et al. described development of a protease-activatable contrast agent (PCA) containing paramagnetic gadolinium chelate (Gd-DOTA) with an MMP-2-cleavable sequence Ser-Pro-Ala-Tyr-Tyr-Thr-Ala-Ala attached via a hydrophobic alkyl chain (PCA2-switch).^[84] The PEG chain was added to the C terminus of the peptide to increase solubility. Upon MMP-2 cleavage, the PEG chain was liberated, and PCA2-switch became less water soluble, remaining at the tumor site. After injection of PCA2-switch, the relative ΔR_1 relaxation value reached a temporary plateau in MC7L1-WT tumors within 5–10 min of injection. A maximum followed at 40 min after injection, whereas only a continuous decrease was observed in the MMP-2 knockdown tumor line, MC7L1-KD. PCA2-scrambled displayed an early maximum, immediately followed by a continuous decrease.

Another PCA switch (PCA7) was designed to measure in vivo MMP-7 activity in tumor-bearing mice.^[88] Several PCA7 variants were generated: PCA7-switch, PCA7-scrambled, and PCA7-B. To generate an MMP-7-selective switch, a peptide sequence was used that had previously been reported to be MMP-7 specific (Pro-Leu-Ala-Leu-Lys-Arg-Asp-Arg). PCA7-scrambled was generated to retain the same physical characteristics as PCA7-switch but contain a noncleavable sequence (Arg-Ala-Lys-Asp-Arg-Leu-Leu-Pro). PCA7-B was another control used that had greater hydrophobic properties than PCA7-switch. The contrast agent Gd-DOTA was made hydrophobic by attaching insoluble moieties such as alkyl chains or aromatic rings. The probes were utilized in vivo in a bilateral xenograft mouse model of SW480 cells overexpressing MMP-7 and in an MMP-7-negative colorectal cancer cell line. The differential response of PCA-7 switch in the MMP-7-positive versus MMP-7-negative tumors was shown to be consistent with those measured by optical proteolytic beacons. Although the peptide sequence displayed MMP-7 selectivity against proteolysis by three other MMPs (MMP-2, -3, and -9), it is probable that this sequence is cleaved by other cellular proteases as well. However, it is still possible to utilize these types of switches to some extent for monitoring of general tumor-associated protease or inhibitor activity.

In vivo MMP activity was monitored by MRI and fluorescence of dendrimeric nanoparticles coated with ACPPs labeled with Cy5, gadolinium, or both.^[89] The core PAMAM dendrimer carried the attached contrast agent with the site of macromolecular ACPP attachment on its polycationic domain. The peptide used in this study was succinoyl-(D-Glu)₈-(Aop)-Pro-Leu-Gly~Cys(S-methyl)-Ala-Gly-(D-Arg)₉-D-Cys-NH₂, where Aop is 5-amino-3-oxapentanoyl and Pro-Leu-Gly~Cys(S-methyl)-Ala-Gly is the activatable linker preferentially cleaved by MMP-2/-9. Each dendrimer was labeled with 15–30 Gd-DOTA moieties. Uptake of these particles (ACPPDs) was four to 15 times higher than for unconjugated ACPPs, and tumors as small as 200 μ m were detected by using fluorescence. Uptake of these sensors was shown to be comparable to other NIR probes, with the added advantage of monitoring by MRI. Upon activation, the Gd-labeled nanoparticles deposited high levels of Gd in tumor parenchyma and higher levels within the infiltrative tumor. The deposition resulted in good T₁ contrast lasting several days post injection. These findings correlated the most intense ACPPD uptake with high MMP activity at the invasive edge of breast cancer tumors.

The activity of the ACPPD-Cy5 probe was compared to existing cleavable probes, ProSense (cathepsin-specific) and MMPsense (MMP-specific). ACPPD-Cy5 displayed equal or greater in vivo sensitivity and a better tumor-to-background ratio in MMTV-PyMT transgenic mice (carrying multiple breast cancer tumors and metastases), MMTV-PyMT mice with homozygous deletion of MMP-2 and MMP-9, and mice carrying HT-1080 xenografts, as compared to the other probes (Figure 11).

Specificity of targeting sequences

One overriding concern with most MMP-targeting sequences is a lack of stability at nontargeted tissues and organs due to nonspecific hydrolysis.^[90] For example, the Pro-Leu-Gly~Val-Arg-Gly sequence, used for NIR imaging of MMP-2-positive tumors and MMP-2-mediated cellular uptake of quantum dots, is additionally hydrolyzed by MMP-1, MMP-7, MMP-8, and MMP-9.^[36b,50] This NIR MMP-2 probe was not selective when tested in MMP-2-deficient mice.^[9] The Pro-Leu-Gly~Leu-Ala-Gly sequence used for MMP-2 activation of cell-penetrating peptides^[59] is hydrolyzed by numerous MMPs.^[91] The linker contained within MMPsense (Gly-Gly-Pro-Arg-Gln~Ile-Thr-Ala-Gly) is based on a motif that has not been tested against multiple MMPs.^[92] A few relatively selective sequences have been developed, such as Pro-Cit-Gly~Hof-Tyr-Leu, which is proposed to be favored by MMP-2, MMP-9, and MT1-MMP over neprilysin,^[93] and Arg-Pro-Leu-Ala~Leu-Trp-Arg, which is favored by MMP-7.^[31]

Several phage display and combinatorial chemical library studies have been reported with the ultimate goal of uncovering motifs that are unique for individual MMPs.^[92,94] However, more often than not, MMPs show overlapping sequence specificities.^[91,94f] As described earlier, a collagen model THP has been used to develop a selective MMP-2/MMP-9 imaging agent.^[41] The use of targets that combine sequence and three-dimensional structural preferences may provide MMP probes of desired selectivities.

Mechanism of fibril hydrolysis by MMPs

Catalysis of collagen hydrolysis (collagenolysis) is a well-known activity of several MMP family members. The precise mechanism of collagenolysis has been the subject of speculation for several decades, and recent use of MMP probes has allowed for atomic level analysis of this process.

In vivo collagenolysis initially involves MMP interaction with fibrils. MMPs bind to multiple sites in collagen, but hydrolysis ultimately occurs only at a single site.^[95] MMP-1 was fluorescently labeled with Alexa Fluor 488, and two-photon excitation fluorescence correlation spectroscopy and fluorescence photo-bleaching recovery was used to monitor MMP-1 interaction with collagen gels containing oriented fibrils. MMP-1 was found to be a diffusion-based “burnt bridge” Brownian Ratchet capable of biased diffusion on the surface of collagen fibrils, where the bias was driven by proteolysis.^[96] When using the same experimental approach, surface-bound MT1-MMP movement was found to occur through a similar mechanism as MMP-1, whereas MMP-2 and MMP-9 exhibited Brownian (unbiased) diffusion on the collagen fibrils.^[97] NMR spectroscopic analysis of ²H-, ¹³C-, and ¹⁵N-enriched THPs and MMPs have helped to reveal the initial stages of collagenolysis (Figure 12).^[98] MMP-1 is in equilibrium between open/extended and closed structures (Figure 12A). The HPX domain then binds chains 1T and 2T of the THP and, due to the flexibility of the linker, the catalytic domain is guided towards the Gly16–Ile17 portion of chain 1T (Figure 12B). This structure would thus correspond to the first event of collagen recognition by MMP-1. Back-rotation of the catalytic and hemopexin-like domains occurs to achieve the closed FL-MMP-1 conformation, with THP unwound as a result (Figure 12C). The domain movement drives chain 1T into the active site, allowing the polypeptide to establish a number of hydrogen bonding interactions and allowing the carbonyl oxygen of the cleavage site amide bond to coordinate the metal ion. Chain 1T is then cleaved, and the fragments begin dissociation from the active site (Figure 12D and E).

fTHP substrates (Figure 2) have been utilized to evaluate MMP collagen specificities and subsite preferences. A THP model of the MMP-9 cleavage site in type V collagen, α1(V)436–447 fTHP, was hydrolyzed by MMP-2 and MMP-9 with $k_{\text{cat}}/K_{\text{M}}$, k_{cat} , and K_{M}

values comparable to collagen hydrolysis by these enzymes, whereas it was not hydrolyzed by MMP-1, MMP-3, MMP-13, or MT1-MMP.^[22b] Melanoma cellular assays with this substrate revealed that binding of $\alpha_2\beta_1$ integrin resulted in the production of substantial triple-helical peptidase activity, the majority (>95%) of which was non-MMP-2/MMP-9.^[22b]

The triple-helical peptidase activities of MMP-1 and MT1-MMP were quantified by using fTHPs that differed in their thermal stabilities.^[22c] One series of substrates was modeled after a collagenolytic MMP consensus cleavage site from collagen types I–III; the other series had a single substitution in the P₁' subsite of the consensus sequence. The substitution of Cys(4-methoxybenzyl) for Leu in the P₁' subsite was greatly favored by MT1-MMP but disfavored by MMP-1. An increase in substrate triple-helical thermal stability led to a decreased ability of the enzyme to cleave such substrates but with a much more pronounced effect for MMP-1. Overall, MMP-1 was found to be less efficient at processing triple-helical structures than MT1-MMP.^[22c]

Subsequently, 12 different triple-helical substrates in combination with ten MMPs were used to better define the contributions of substrate sequence and thermal stability towards triple-helical activity and collagen specificity.^[22d] In general, MMP-13 was found to be distinct from MMP-8 and MT1-MMP, in that enhanced substrate thermal stability has only a modest effect on activity, regardless of sequence. This result correlates to the unique collagen specificity of MMP-13 compared to MMP-8 and MT1-MMP, in that MMP-13 hydrolyzes type II collagen efficiently, whereas MMP-8 and MT1-MMP are similar in their preference for type I collagen. In turn, MMP-1 was the least efficient of the collagenolytic MMPs at processing triple-helices with increasing thermal stability and thus favors type III collagen, which has a relatively flexible cleavage site. Gelatinases (MMP-2 and MMP-9) appear incapable of processing more stable helices and are thus mechanistically distinct from collagenolytic MMPs. The collagen specificity of MMPs appears to be based on a combination of substrate sequence and thermal stability.^[22d]

Two fTHP sequences were then used to compare the cleavage site specificities of ten MMPs.^[22e] One substrate featured a continuous Gly-Xxx-Yyy sequence (Pro-Leu-Gly~Met-Arg-Gly); the other incorporated an interruption in the Gly-Xxx-Yyy repeat (Pro-Val-Asn~Phe-Arg-Gly). Both sequences were selectively cleaved by MMP-13 while in linear form, but neither proved to be selective within a triple helix. This suggested that the conformational presentation of substrate sequences to an MMP active site is critical for enzyme specificity, in that activities differ when sequences are presented from an unwound triple-helix versus an independent single strand. Interruption of the triple-helix had different effects on secreted MMPs and MT-MMPs, as MT-MMPs could not hydrolyze the Asn-Phe bond but instead cleaved the triple-helix nearer the C terminus at a Gly-Gln bond. It is possible that MT-MMPs have a requirement for Gly in the P₁ subsite in order to be able to efficiently process a triple-helical molecule. Analysis of individual kinetic parameters and activation energies indicated different substrate preferences within secreted MMPs; MMP-13 preferred the interrupted sequence, whereas MMP-8 showed little discrimination between uninterrupted and interrupted triple helices. Unique triple-helical peptidase behaviors could be assigned to the collagenolytic MMPs.^[22e]

fTHPs and triple-helical transition state analogue inhibitors were utilized to dissect the roles of potential secondary binding sites (exosites) in MMP-9 collagenolytic behavior.^[99] Substrate and inhibitor sequences were based on either the $\alpha 1(V)$ 436–450 collagen region, which is selectively hydrolyzed at the Gly~Val bond by MMP-2 and MMP-9, or the Gly~Leu cleavage site within the consensus interstitial collagen sequence $\alpha 1(I-III)$ 769–783, which is hydrolyzed by MMP-1, MMP-2, MMP-8, MMP-9, MMP-13, and MT1-MMP.

Exosites within the MMP-9 fibronectin II inserts were found to be critical for interactions with type V collagen model substrates and inhibitors and to participate in interactions with an interstitial (types I–III) collagen model inhibitor. A triple-helical peptide incorporating a fibronectin II insert-binding sequence was constructed and found to selectively inhibit MMP-9 type V collagen-based activities compared with interstitial collagen-based activities. This represented the first example of differential inhibition of collagenolytic activities and was achieved through an exosite-binding THP.^[99]

A variety of THP substrates and inhibitors were used to dissect MMP-1 collagenolytic behavior.^[22f] Analysis of MMP-1/THP interactions by hydrogen/deuterium exchange mass spectrometry, followed by evaluation of wild type and mutant MMP-1 kinetics, led to the identification of three noncatalytic regions in MMP-1 (residues 285–295, 302–316, and 437–457) and two specific residues (Ile290 and Arg291) that participated in collagenolysis. Ile290 and Arg291 contributed to recognition of triple-helix structure, and facilitated both the binding and catalysis of the triple helix. The MMP-1 catalytic and hemopexin-like domains were proposed to collaborate in collagen catabolism by properly aligning the triple-helix and coupling conformational states to facilitate hydrolysis. This study was the first to document the roles of specific residues within the MMP-1 hemopexin-like domain in substrate binding and turnover.^[22f]

Three fTHP were constructed to evaluate if type I–III collagen sequences located N- or C-terminal to the physiological cleavage sites mediate substrate selectivity among MMP-1, MMP-2, MMP-8, MMP-13, and MT1-MMP.^[100] The first fTHP contained consensus residues 769–783 from collagen types I–III, the second had $\alpha 1(\text{II})$ collagen residues 763–768 inserted N-terminal to the consensus sequence, whereas the third had $\alpha 1(\text{II})$ collagen residues 784–792 inserted C-terminal to the consensus sequence. Insertion of C-terminal residues significantly increased the $k_{\text{cat}}/K_{\text{M}}$ and k_{cat} for MMP-1. MMP-13 showed the opposite behavior, with a decrease in $k_{\text{cat}}/K_{\text{M}}$ and k_{cat} and a greatly improved K_{M} in response to the C-terminal residues. Insertion of N-terminal residues enhanced $k_{\text{cat}}/K_{\text{M}}$ and k_{cat} for MMP-8 and MT1-MMP. For MMP-2, the C-terminal residues enhanced K_{M} and dramatically decreased k_{cat} , resulting in a decrease in overall activity. These changes in activities and kinetic parameters were good representations of the collagen preferences of MMP-8, MMP-13, and MT1-MMP. Thus, interactions with secondary binding sites (exosites) helped direct the specificity of these enzymes. However, MMP-1 collagen preferences were not recapitulated by the fTHP studies. The preference of MMP-1 for type III collagen appears to be primarily based on the flexibility of the hydrolysis site of type III collagen compared with types I and II.^[100]

Clinical trials with small-molecule MMP inhibitors have been largely unsuccessful, with a lack of selectivity that is considered particularly problematic. Enhanced selectivity could be achieved by taking advantage of differences in substrate secondary binding sites (exosites) within the MMP family. The noncatalytic sites identified in the fTHP studies described above could ultimately be utilized to create THP inhibitors that target MMPs implicated in disease progression while sparing proteases with functions beneficial to the host.

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Biographies

Anna M. Knapinska received her PhD in molecular pharmacology from a joint graduate program at the University of Medicine and Dentistry/Rutgers University in 2009. Until 2011, she was a Postdoctoral Fellow at Scripps Institute, Florida, where she was a part of the High Throughput Screening and Lead Identification Department. Dr. Knapinska recently joined Torrey Pines Institute for Molecular Studies where she manages Dr. Fields' laboratory. Dr. Knapinska's research interests are in the development of diagnostic tools and disease biomarkers, utilizing chemical methods for the determination of MMP activity.

Gregg B. Fields received his Ph.D. from Florida State University and was a Postdoctoral Scholar at UCSF. He was an Assistant, then Associate Professor at the University of Minnesota (1991–1997), a Full Professor at Florida Atlantic University (1997–2008), and a Welch Foundation Distinguished University Chair at UTHSCSA. He joined the Torrey Pines Institute in 2011, and is the Vice President of Scientific Affairs, and Distinguished Chair of Metalloproteinase and Multiple Sclerosis Research. G.B.F.'s research interests are in the use of chemical approaches to better understand how three-dimensional protein structures influence cellular and enzymatic behaviors.

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Figure 1. Mechanisms of target-based profiling for detection of protease activity.^[10] A) Cleavage between a fluorophore and a peptide results in detected fluorescence of the fluorophore. B) Removing a quencher group by cleavage of a peptide sequence between a fluorophore and quencher results in increased fluorescence. C) The close proximity of the fluorophores causes auto-quenching. Cleavage of the peptide results in separation of the fluorophores and leads to elevated fluorescence. D) Upon cleavage of a fluorescent substrate anchored to the cell membrane, the fluorophore localizes to the cytoplasm.

Figure 2.

Models of fTHP sequences. Left, fTHP-15 [(Gly-Pro-Hyp)₅-Gly-Pro-Lys(Mca)-Gly-Pro-Gln-Gly~Leu-Arg-Gly-Gln-Lys(Dnp)-Gly-Val-Arg-(Gly-Pro-Hyp)₅-NH₂]; center, fTHP-16 [(Gly-Pro-Hyp)₅-Gly-Pro-Ser-Gly-Ala-Glu-Gly-Pro-Lys(Mca)-Gly-Pro-Gln-Gly~Leu-Arg-Gly-Gln-Lys(Dnp)-Gly-Val-Arg-(Gly-Pro-Hyp)₅-NH₂]; right, fTHP-17 [(Gly-Pro-Hyp)₄-Gly-Pro-Lys(Mca)-Gly-Pro-Gln-Gly~Leu-Arg-Gly-Gln-Lys(Dnp)-Gly-Val-Arg-Gly-Leu-Hyp-Gly-Gln-Arg-Gly-Glu-Arg-(Gly-Pro-Hyp)₄-NH₂]. Pro and Hyp residues are shown in white, Gly residues in light blue, Lys(Mca) and Lys(Dnp) in yellow, polar residues (Gln, Ser) in green, nonpolar residues (Ala, Leu, Val) in red, Glu residues in orange, and Arg residues in blue. Peptides are arranged with their N termini oriented toward the top.^[100]

Figure 3.

A) Schematic diagram of MMP-sensitive NS. Nanoparticles can deliver a multiquenched fluorogenic peptide efficiently to the target site by the EPR effect. When MMPs interact with the NS, cleavage of the fluorogenic peptide occurs, resulting in a pronounced NIR fluorescence signal recovery due to dequenching of the dye. B) MMP-sensitive NS. Because of the efficient NIR fluorescence quenching ability of the quencher (BHQ3) and self-quenching of the Cy5.5 dye itself, fluorogenic peptides that are chemically conjugated on the surface of nanoparticles are in the strongly multiquenched state. After cleavage of the substrate by MMPs, NIR fluorescence dyes are released from nanoparticles and fluoresce brightly. C) Chemical structures of polymeric nanoparticles and MMP-sensitive fluorogenic peptide. Reprinted from ref. [47] with permission; copyright: American Chemical Society, 2009.

Figure 4.

Activity-based protein profiling (ABPP). All ABPP probes have a group that binds the active site of enzymes within a given enzyme class (arrow) and a reporter group that allows observation and isolation of the ABPP-bound enzyme (star). If active site binding results in a noncovalent interaction (panel B), then a reactive group for labeling ABPP-bound enzymes (hook) is needed.

Figure 5.

A) Biodistribution of radioactivity in adult C57/BL6 mouse after intravenous injection of $[^{18}\text{F}]\text{-30}$. Reaction conditions: a) $[^{18}\text{F}]\text{fluoride}$ (potassium 222 cryptate), K_2CO_3 , CH_3CN , 84°C , 15 min; b) $\text{CuSO}_4\cdot\text{H}_2\text{O}$, sodium ascorbate, DMF, RT, 8 min. B) Visualization by small-animal PET. Images represent maximum intensity projections as selected time frames. Labeled structures are liver (lv), gallbladder (gbl), bladder (bl), kidneys (kd), small bowel (sb), and lung (lu). Reprinted from ref. [73] with permission; copyright: American Chemical Society, 2012.

Figure 6.

MMP probe consisting of a phosphinic peptide core (to inhibit the enzyme) with an azido photolabile group (to covalently modify the enzyme) grafted onto the P₁' side chain.^[75b]

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Figure 7.
Chemical structures of phosphinic photoaffinity probe **1** and phosphinic affinity probe **2**.
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CGS 27023A,	X: N,	R ₁ : OCH ₃ ,	R ₂ :H
HO-[¹²³ I]-CGS 27023A,	X: N,	R ₁ : OH,	R ₂ : ¹²³ I
[¹¹ C]CGS 25966,	X: CH,	R ₁ : O ¹¹ CH ₃ ,	R ₂ :H
[¹⁸ F]1c,	X: N,	R ₁ : OCH ₂ CH ₂ ¹⁸ F,	R ₂ :H
[¹⁸ F]1d,	X: CH,	R ₁ : OCH ₂ CH ₂ ¹⁸ F,	R ₂ :H

Figure 8.

Lead structure CGS 27023A and a selection of radiolabelled derivatives. Reprinted from ref. [78] with permission; copyright: Elsevier, 2009.

Figure 9.

Outline of the probe structure and mechanism. The quenched probe (i) is able to freely circulate in vivo until it encounters its protease target. Cleavage of the probe at a defined point by MT1-MMP (ii) releases a cell-penetrating peptide, which can then translocate its radionuclide cargo across the target cell membrane (iii). After uncleaved quenched probe is washed away, internalized radioactivity can be imaged by SPECT. Reprinted from ref. [81] with permission; copyright: Elsevier, 2009.

Figure 10.

Synthesis and function of MMP-9-PSOP. A) The 25-residue peptides consisting of an Arg-rich coupling domain and a cleavage domain with the recognition site for MMP-9 linked by a Gly bridge are reacted with NHS-methyl-poly(ethylene glycol) (NHS-mPEG). The resulting peptide-mPEG copolymers are purified by gel filtration to remove unreacted mPEG and NHS. Peptide-mPEG is mixed with VSOPs, yielding MMP-9-PSOP. B) When sterically stabilized PSOP with an intact mPEG shell are exposed to a protease specific to the cleavage motif, the peptide-mPEGs are cut at the cleavage domain, resulting in a loss of sterical stabilization. C) The remaining particles aggregate due to the magnetic attraction of the iron oxide cores and the electrostatic attraction of the positively (Arg-rich coupling domain) and negatively charged surface areas (acid shell). Reprinted from ref. [87] with permission; copyright: American Chemical Society, 2008.

Figure 11.

ACPPD-Cy5 gives equal or better tumor-to-background contrast than existing dequenching probes. A) Fluorescence images through intact skin of MMTV-PyMT transgenic and HT1080 xenografted mice 48 h after injection with 1 nmol cleavable ACPPD-Cy5 or 24 h after injection with 2 pmol ProSense or MMPySense. Yellow arrows indicate tumor and red arrows indicate liver. Dim images have been brightened by the factors indicated to enable comparisons. B) Tumor-to-background ratios for ACPPD-Cy5 and ProSense in the MMTV-PyMT model. Black bars show ratios with skin attached, and gray bars show ratios after skin removal. *P* values for MMPySense and ProSense relative to ACPPD-Cy5s are shown.^[89] Reprinted from ref. [89] with permission; copyright: National Academy of Sciences, 2010.

Figure 12.

The initial steps of collagenolysis. A) Closed (left) and open/extended (right) forms of FL-MMP-1 in equilibrium. B) The extended protein binds THP chains 1T–2T at Val23–Leu26 with the HPX domain and the residues around the cleavage site with the CAT domain. The THP is still in a compact conformation. C) Closed FL-MMP-1 interacting with the released 1T chain (magenta). D) After hydrolysis, both peptide fragments (C- and N-terminal) are initially bound to the active site. E) The C-terminal region of the N-terminal peptide fragment is released. Reprinted from ref. [98] with permission; copyright: American Chemical Society, 2012.

Table 1

Fluorophores and quenchers used for MMP targeting.

Fluorophore	λ_{ex} [nm]	λ_{ex} [nm]	Quencher
(7-methoxycoumarin-4-yl)acetyl (Mca)	325	392	<i>N</i> -2,4-dinitrophenyl (Dnp)
5-[(2-aminoethyl)amino]naphthalene-1-sulfonic acid (Edans)	340	490	4-[4-(dimethylamino)phenylazo]-benzoic acid (Dabcyl)
CFP	433	476	YFP
CyPet	433	477	YPet
ECFP	434	485	YPet
Coumarin 343	450	490	TAMRA
5-carboxyfluorescein (5-FAM)	490	520	TMR, Dabcyl
mOrange2	549	565	mCherry
rhodamine X (ROX)	570	576	
ATTO647N	644	669	
ATTO647	645	669	
Pyro	660	674	BHQ3
Cy5.5	675	695	AF750, fluorescein isothiocyanate (FITC), NIRQ820
FPR-675	675	695	black hole quencher 3 (BHQ3)
LS276	780	810	