Nanoparticle-based probes to enable noninvasive imaging of proteolytic activity for cancer diagnosis

Proteases play a key role in tumor biology, with high expression levels often correlating with poor prognosis for cancer patients – making them excellent disease markers for tumor diagnosis. Despite their significance, quantifying proteolytic activity *in vivo* remains a challenge. Nanoparticles, with their ability to serve as scaffolds having unique chemical, optical and magnetic properties, offer the promise of merging diagnostic medicine with material engineering. Such nanoparticles can interact preferentially with proteases enriched in tumors, providing the ability to assess disease state in a noninvasive and spatiotemporal manner. We review recent advances in the development of nanoparticles for imaging and quantification of proteolytic activity in tumor models, and prognosticate future advancements.

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It is predicted that by 2030 there will be more than 22 million new cancer cases each year worldwide [1]. Successful management of cancer hinges on its early and accurate detection and subsequent treatment [2]. In many instances, both diagnosis and treatment planning rely on data gathered through biomedical imaging techniques. Conventional anatomical imaging modalities, which include magnetic resonance imaging (MRI), ultrasound and computed tomography, provide morphological information on the location and size of tumor lesion. Anatomical imaging, however, lacks specificity and sensitivity [3], and provides no information about abnormalities at the cellular and molecular level [4]. As a result, clinically utilized anatomical imaging techniques only detect tumors when the lesion diameter exceeds approximately 1 cm, at which point the tumor comprises more than a billion cells [5]. Molecular imaging, on the other hand, offers the potential to visualize and quantify the

molecular and cellular processes associated with early stages of disease progression and its remission [6]. For example, differential physiological characteristics, which include the overexpression of several cell receptors such as EGFRs [7], low pH, hypoxia and increased proteolytic activity, between tumor and normal tissues can be exploited in the development of tools for diagnosis and monitoring of disease state [6,8,9].

Imaging of tumor proteolytic activity is attractive for several reasons: protease expression can be elevated at even the early stages of tumor progression [10]; catalytic amplification provides for enhanced sensitivity [11]; the potential for improved signal-to-noise ratio through ON/OFF switching of probes by proteolytic activity; and the presence of proteases at the invasive front of tumors and at sites of angiogenesis – regions readily accessible to imaging probes [12]. Tumor progression and invasion makes significant use of proteases at the primary and meta-

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static sites [2]. In addition to degrading the extracellular matrix, proteases release growth factors and chemokines, which directly or indirectly affect tumor invasion, and they also activate latent proteins on the cell surface [13]. Matrix metalloproteinases (MMP), a family of extracellular, zinc-dependent enzymes involved in extracellular matrix remodeling, are of special interest as their increased activity is associated with the aggressiveness of many cancers [14,15]. Other proteases, including the lysosomal cysteine protease cathepsin B and the serine protease urokinase-type plasminogen activator have also been implicated in tumor progression [16].

Numerous molecular probes, including those based on peptides [17,18], polymers [19,20], polymeric nanoparticles [21,22], protein nanoparticles [14] or inorganic nanoparticles [23–25], have been developed to image proteolytic activity. Recent interest in nanoparticle-based probes to quantify *in vivo* proteolytic activity is driven by attempts to overcome the limitations of small molecule probes, which tend to have poor pharmacokinetics, high background noise due to nonspecific uptake by tissues and relatively poor detection limits [26]. Nanoparticles are able to overcome some of these limitations due to their size and vast surface area, which is readily functionalized with imaging agents for molecular imaging, cell targeting ligands for active targeting of the tumor site, and biocompatible surface coatings that can modulate pharmacokinetics and biodistribution [27]. Each nanoparticle has the potential to deliver numerous imaging agents, which enhances sensitivity for detection of the targeted molecular event [28]. Proper sizing of nanoparticles can also lead to preferential accumulation at tumor sites due to the enhanced permeability and retention effect [10,29]. Nanoparticles can also be designed to change size, charge and/or surface coating in response to environmental cues in order to optimize transport across the many physiological barriers encountered *in vivo* [9].

The various types of probes discussed in this review are summarized in **Table 1**. This discussion is focused on nanoprobe that provide the potential for noninvasive, spatial and temporal imaging of *in vivo* biological activity in tumor microenvironments [30].

Magnetic resonance imaging

MRI is a noninvasive imaging modality that provides high spatial resolution and deep-tissue imaging [32,48]. Hydrogen protons, which are most frequently imaged in MRI because of its abundance, are normally found in the body with their spin axes randomly aligned. When exposed to a strong magnetic field, the spin axes of protons align with the applied field, which is the low energy state for the system. This alignment is then displaced by application of a radiofrequency pulse,

which excites the protons to a higher energy level. Subsequent relaxation of the excited nuclei back into alignment with the applied magnetic field is characterized by a relaxation time, which varies based on tissue properties and is the means for discrimination between healthy and malignant tissues [50,51]. Contrast agents are used to improve the sensitivity and contrast of MR images by shortening either the T_1 (longitudinal) or T_2 (transverse) relaxation times. Standard MRI contrast agents used in the clinic are gadolinium chelates (T_1) and magnetic nanoparticles (T_2) [48]. Some clinical success has been achieved with magnetic nanoparticles as targetable MRI contrast agents [29]. The high magnetic susceptibility of iron oxide cores leads to noticeable enhancement of transverse (T_2 and T_2^*) relaxivity, which is observed as a darkening of T_2 -weighted MR images (hypointensity) [52].

While MRI allows for whole-body imaging [32], it generally suffers from poor sensitivity for measuring molecular events [53]. Since magnetic susceptibility and r_2 -relaxivity depend on particle size, the growth of single iron oxide crystals into larger aggregate structures is one method that has been explored to enhance the sensitivity of T_2 -weighted MRI. Growth of particle size, however, can also negatively affect the pharmacokinetic properties and biodistribution of the particles. A potential strategy to overcome this problem is to design particles that are initially stable in the blood but which then form aggregates in response to proteolytic activity. One approach utilizes two sets of complementary, colloidally stable superparamagnetic iron oxide nanoparticles (SPIONs) that only self-assemble in response to proteolytic cleavage, as depicted in **Figure 1**. To accomplish this, Gallo *et al.* designed two families of SPIONs that undergo a bi-orthogonal, copper-free click conjugation following MMP cleavage [25]. The nanoparticles were tagged with cyclopentapeptide, a C-X-C chemokine receptor type 4 (CXCR4) targeting ligand, for enhanced targeting of metastatic tumors, an MMP 2/9 cleavable peptide (PLGMWSR), PEG for enhanced *in vivo* stability, and either azide or alkyne moieties. The particles were colloidally stable in the absence of MMP 2/9, but proteolytic cleavage exposed the alkyne and azide moieties, which led to a [3+2] cycloaddition reaction that crosslinked the particles and altered magnetic relaxivity. Simultaneous administration of the two SPIONs in tumor-bearing mice displayed T_2 signal enhancement, which dropped significantly with inhibition of MMP.

The ability to simultaneously monitor the activity of multiple molecular targets associated with a given disease has tremendous diagnostic value. Von Maltzahn and colleagues developed a nanoprobe capable of simultaneously monitoring the activity of two

Table 1. Summary of nanoparticle-based systems for imaging *in vivo* proteolytic activity.

Imaging modality	Target proteases	Nanoparticle type	Ref.
MRI			
¹ H MRI	MMP-2, -7, -9 and -14, and legumain	SPION	[25,31–34]
¹⁹ F MRI	Legumain	¹⁹ F nanoparticles	[35]
Optical imaging			
Prequenched fluorophores	MMP, cathepsin B, caspase-3, -8 and -9, matriptase, trypsin and uPA	Ferritin protein, glycol chitosan, AuNP and Au-Fe ₃ O ₄ nanocomposites	[14,21,36–41]
Bioluminescence	Trypsin, MMP-2 and caspase-3	Poly (phenylene ethynylene) nanoparticles, AuNP and UCNPs	[42–44]
Theranostic nanoparticles	MMP	AuNP and AuNR	[45,46]
Photoacoustic	MMP	Copper sulfide	[47]
Secondary Cerenkov-induced fluorescent imaging	MMP-2	AuNP	[23]
Multimodal imaging			
MRI/optical	MMP	SPIONs and Gadolinium-labeled dendrimeric nanoparticles	[12,23,48]
PET/optical	MMP	⁶⁴ Cu-radiolabeled glycol chitosan	[49]
CT/optical	MMP	AuNP	[24]

AuNP: Gold nanoparticle; AuNR: Gold nanorod; MMP: Matrix metalloproteinase; SPION: Superparamagnetic iron oxide nanoparticle; UCNPs: Lanthanide-doped upconversion nanoparticle; uPA: Urokinase-type plasminogen activator.

matrix-metalloproteinases, MMP-2 and MMP-7 [31]. The SPIONs were designed to aggregate in response to logical ‘AND’ or ‘OR’ functions, causing an amplification of the T_2 relaxation rate and enabling MRI-based detection. In the AND case, two sets of nanoparticles, one with an MMP-2 specific substrate (GPLGVRG) and biotin and the other with an MMP-7 substrate (VPLSLTM) and neutravidin, self-assembled by biotin-neutravidin interactions only if both enzymes were present. In the OR case, two sets of nanoparticles, one having both MMP substrates in series and tethered with biotin while the other nanoparticle was tethered with only neutravidin, would self-assemble in the presence of either one or both proteases.

A significant limitation of the enzyme-activated MRI-nanoprobes discussed so far is that they require separate sets of SPIONs, which can exhibit different circulation times and biodistribution patterns *in vivo*. The co-delivery issue can be eliminated if a single nanoparticle is used. Schellenberger *et al.* designed protease-specific iron oxide nanoparticles (PSOP), which upon activation by MMP-9, switch from an electrostatically stabilized, low-relaxivity stealth state to aggregating, high- T_2^* relaxivity particles [32]. Initially, peptides comprising an arginine-rich coupling domain with an MMP-9-cleavable domain linked by a glycine bridge (NH₂-GGPRQITAG-K(FITC)-GGGG-RRRRR-G-RRRRR-amide) were reacted with amine-reactive *N*-hydroxysuccinimide-methyl-

PEG (NHS-mPEG). The resulting positively charged mPEG-peptide was electrostatically adsorbed onto the surface of negatively charged citrate iron oxide particles to yield PSOP. Cleavage of the peptide by MMP-9 results in the release of PEG and a loss of steric stabilization, causing aggregation due to both magnetic attraction as well as the electrostatic attraction between the positively charged arginine-rich coupling domain and the negatively charged citrate coat, as shown in **Figure 2**. While the probes discussed so far perform well in an *in vitro* setting, their *in vivo* application is limited by our inability to determine whether the increase in MRI contrast is due to proteolytic cleavage and subsequent aggregation of particles or if it is simply due to increased accumulation of particles at the target site, thus complicating quantitative analysis.

In one application, activity of the protease legumain was utilized to tag tumor-associated macrophages (TAM) with magnetic nanoparticles. Legumain is a lysosomal/vacuolar cysteine protease that cleaves substrates at the C-terminal of asparagine, and is overexpressed in prostate, breast and colon cancers [54,55]. TAMs overexpress legumain on their surface and play an important role in the progression of certain cancers [56,57]. Yan *et al.* designed a Y-shaped legumain-targeting peptide (Y-Leg) with the sequence AANLHK(HK)₂ and grafted it onto oxidized carbon nanotubes (OCNTs) loaded with Fe₃O₄ nanoparticles for *in vivo* targeting and MRI of TAMs [33]. T_2^* -

Figure 1. Two sets of complimentary, sterically stabilized iron oxide nanoparticles aggregate (e.g., through neutravidin/biotin interactions) following proteolytic cleavage of peptide substrate and removal of polyethylene glycol (PEG). Cluster formation enhances magnetic susceptibility and r_2 relaxivity to allow MRI detection of proteolytic activity.

weighted MRI images following intravenous administration of Y-Leg-OCNT/ Fe_3O_4 to 4T1 tumor-bearing mice revealed targeting of the nanotubes toward the TAM-infiltrated tumor microenvironment. On the other hand, control peptide-conjugated nanotubes showed no significant change in the MRI signal.

Chemotherapeutics are hindered by undesired systemic toxicity to healthy tissues. Ansari and colleagues developed an MMP-14 activatable theranostic probe (TNP) based on US FDA approved ferumoxytol nanoparticles conjugated to an MMP-14 cleavable peptide conjugate of azademethylcolchicine, a tumor vasculature-disrupting agent, for tumor-selective therapeutic delivery [34]. The probe allowed for simultaneous MRI imaging of TNP tumor accumulation and protease-specific drug activation. MRI scans following intravenous injection to MMP-14 positive, MMTV-

PyMT mammary tumor-bearing mice indicated significant accumulation of the theranostic probe at the tumor site, and a significant antitumor effect and tumor necrosis, with no detectable toxicity and MRI signal in healthy tissues. It is important to note, however, that the MRI images indicated TNP delivery but not drug activation.

Due to the abundance of hydrogen protons and the scarcity of intrinsic ^{19}F atoms in the body, ^{19}F MRI provides better contrast-to-noise ratio than ^1H MRI. Yuan *et al.* designed a ^{19}F nanoparticle contrast agent which self-assembles intracellularly in the presence of glutathione (GSH) and disassembles in the presence of legumain [35]. The 'off' and 'on' ^{19}F NMR/MRI signal was used to detect legumain activity in HEK 293T tumor-bearing zebrafish, which showed a strong signal compared with healthy zebrafish.

Optical imaging

Optical imaging detects photons emitted by fluorescent or bioluminescent contrast agents, and can simultaneously image multiple contrast agents depending on their excitation/emission spectra. Near infrared fluorescence (NIRF) imaging has become increasingly important for visualizing important *in vivo* processes occurring at the cellular and molecular levels [58]. NIRF offers several advantages over visible-range optical imaging, including lower light scattering and lower absorption by endogenous biomolecules that strongly absorb visible (e.g., hemoglobin) and infrared light (e.g., water and lipids), leading to deeper tissue penetration [59,60]. The specificity of NIRF imaging can be substantially improved by utilizing fluorescently quenched, peptide-based activatable probes. These probes emit little fluorescence in their native state, but become highly fluorescent in the presence of a target proteases [58]. The quality of an NIRF probe for imaging of *in vivo* proteolytic activity is determined by the efficiency of its quenching, its quantum yield, the target-to-background ratio, probe targeting and pharmacokinetics, protease specificity and the probe's photostability in physiological conditions [3,41]. The following section examines the various strategies to detect proteolytic activity *in vivo* utilizing optically active nanoprobe.

Prequenched fluorophores

The majority of probes developed for optical imaging of *in vivo* proteolytic activity utilize one or more fluorescence quenching effects that include self-quenching, fluorescence resonance energy transfer (FRET), or energy transfer between dye and nanoparticle surface to reduce the preactivation fluorescence signal. Fluorescence is recovered following release of the quencher or

dye by proteolytic activity. High quenching efficiency is essential for an effective probe.

FRET

FRET is a process in which an excited fluorophore (donor) transfers its excitation energy to a nearby chromophore (acceptor). The efficiency of FRET is strongly affected by the spectral overlap, dipole orientations and distance between the donor and acceptor. Separation of the two dyes causes a detectable change in signal, which is utilized to measure proteolytic activity [61]. Protease-activatable probes that employ FRET comprise an NIRF dye and quencher at both ends of a protease-specific peptide. Lin *et al.* developed two sets of ferritin protein cages, one with a fluorescently labeled MMP-specific peptide sequence (Cy5.5-GPLGVRGC) and another with black hole quencher (BHQ-3), that self-assemble to bring the energy donor and receptor into close proximity [14]. The ratio of donor and receptor that gave the highest fold increase in fluorescence following incubation with MMP was optimized and the probe validated following intratumoral injection *in vivo* against two MMP-positive cell lines, UM-SCC-22B and SCC-7.

Cathepsin B (CB), which normally remains in the intracellular lysosomal compartments, is secreted into the pericellular region in high amounts as cells gain metastatic potential [62]. Ryu *et al.* developed CB-activatable fluorogenic nanoprobe for early detection of metastases [36]. The nanoprobe was synthesized by conjugating a CB-responsive fluorogenic peptide substrate (GRRGKGG), which included a donor (Cy5.5) and a quencher (BHQ-3), onto the surface of tumor-targeting glycol chitosan nanoparticles. The probe displayed strong specificity to CB, compared with cathepsin L, cathepsin D and CB plus inhibitor, and was also able to discriminate metastases in liver, lung and peritoneal metastatic mouse models.

Simultaneous imaging of multiple proteolytic activities has the potential to provide a more complete map of disease progression. Park *et al.* developed various caspase substrate-linked fluorescent proteins immobilized on gold nanoparticles (AuNP-FPs) for real-time simultaneous detection of multiple caspase activities in cancer cells during apoptosis [37]. AuNP served as a broad-spectrum fluorescence quencher. As shown in Figure 3, AuNP-FPs were designed to release blue-, red- and yellow-fluorescent protein following activation by caspase-8 (IETD), caspase-9 (LEHD) and caspase-3 (DEVD), respectively, where the peptide sequence in brackets indicates the caspase cleavage site. While this is a promising approach to quantify multiple proteases, only *in vitro* results have been presented to date.

Figure 2. Sterically stabilized protease-specific iron oxide nanoparticles release peptide-mPEG in the presence of MMP-9, resulting in a loss of steric stabilization and enabling aggregation due to both magnetic attraction as well as electrostatic attraction between the positively charged arginine-rich coupling domain and the negatively charged citrate coat.

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Quenching by gold nanoparticles

Strong electronic interactions with chromophores near the surface make gold nanoparticles (AuNPs) very efficient quenchers of molecular excitation energy [63], with larger quenching distances (~20 nm) compared with FRET [39]. Deng *et al.* developed spherical and rod-shaped AuNPs for detection of *in vivo* matrilysin, a type II transmembrane epithelial serine protease which activates several proenzymes including urokinase plasminogen activator (uPA) and MMPs associated with several cancers [38]. The expression of matrilysin is known to correlate with tumor staging, making it an excellent target for molecular imaging. The molecular beacon comprised a fluorescent dye attached to AuNPs through a matrilysin cleavable peptide substrate linker (GRQSRAGC). NIRF images of mice bearing matrilysin-expressing HT-29 tumor xenografts showed enhanced fluorescence recovery following intratumoral injection of the molecular beacon.

The success of AuNPs that rely on the thiol-gold chemistry is hampered in an *in vivo* setting, due to off-target site activation in the thiol-rich blood. To overcome this limitation, flower-like Au-Fe₃O₄ nanocomposites with a single Au core and several Fe₃O₄ petals, were developed as an MMP activatable fluorescence imaging probe [39]. The nanocomposite sensor, which can be tuned for shape, size and composition, combines the robust surface chemistry of Fe₃O₄ for conjugation of an MMP-specific peptide-dye (Cy5.5-GPLGVRG), with the excellent quenching properties of nearby Au. The flower-like shape of Au-Fe₃O₄ creates an architecture whereby the fluorophores on the Fe₃O₄ petals are in close proximity to the Au core for efficient quenching. Separate groups of SCC-7 xenograft tumor-bearing mice received injections of flower-like activatable nanoparticles (FANPs), with and without preinjection of MMP inhibitor, or gold-based activatable nanoparticles (GANPs). NIRF images for the FANP group showed high signal at the tumor site after 30 min, which increased gradually up to 4 h postinjection. Preinjection of an MMP inhibitor significantly reduced the observed signal. Meanwhile, the GANP control group showed a weak optical signal in the tumor after 4 h (Figure 4).

Combination of different quenching mechanisms

The efficiency of quenching is enhanced by combining different quenching mechanisms into a single probe. In one example, self-quenching and FRET were combined in an MMP-responsive nanosensor for NIRF imaging of MMP [21]. The nanosensor comprised a self-assembled chitosan nanoparticle and an activatable MMP-specific peptide sequence with the NIRF dye Cy5.5 and dark

quencher BHQ3 (Cy5.5-GPLGVRGK(BHQ3)-GG) to create a FRET pair. NIRF of Cy5.5 was quenched by both the interaction of the dye with BHQ-3 and dye-dye self-quenching mechanism. High recovery of Cy5.5 fluorescence signal was observed *in vivo*, as evidenced by fluorescence tomography following intravenous administration of the nanosensor in an MMP-positive SCC-7 xenograft tumor and correlated with levels of active MMPs. Meanwhile, the signal was attenuated when animals were pretreated with an MMP-inhibitor. To determine response to tumor size, the nanosensor was intravenously injected into mice carrying SCC-7 tumors of varying sizes, between 3.5 and 381.5 mg. After 2 h, the animals were euthanized and the excised tumors analyzed by measuring fluorescence intensity, which was found to increase proportionally with tumor size (Figure 5A). MMP-2/9 activities were also quantified using gelatin zymography, and correlated strongly with the NIRF signal intensity (Figure 5B).

Lee and colleagues developed an MMP protease-sensitive probe with AuNPs and Cy5.5 linked together through an MMP-cleavable peptide substrate (Cy5.5-GPLGVRGC-amide) [40]. The combination of AuNP surface and close proximity of Cy5.5 induced a strong multi-quenching effect on the fluorescence of Cy5.5. Upon exposure to MMPs, the peptide was cleaved, releasing free Cy5.5 and recovering the NIRF signal, as shown in Figure 6.

In another example, self-assembled heterogeneous monolayers of fluorophore (Quasar 670) and dark quencher (BHQ-2)-labeled peptide were adsorbed onto 20 nm AuNPs as an activatable probe for the detection of trypsin and uPA [41]. Fluorescence, attenuated due to self-quenching and FRET between Quasar 670 and BHQ-2, is restored following proteolytic cleavage. The probe displayed high fluorescence image contrast in a subcutaneous tumor phantom model in athymic nude mice.

Bioluminescence

A luminescence 'turn ON'-turn OFF' system based on protease-responsive organic nanoparticles was developed and comprised a tightly packed, semiconducting poly (phenylene ethynylene) (PPE) core bearing perylene units and randomly inserted far red emissive dye (perylene) [42]. The probe also had an external, hydrophilic hydrogel coating with reactive succinimide groups. Aggregation induced quenching by tuning of the π -associations in the PPE core was accomplished by reacting the succinimide groups with a protease-sensitive peptide (KCRPLALWRSK), which leads to cross-linking of the shells of the nanoparticles (strained OFF state). Quenched luminescence was recovered upon exposure to the protease trypsin, which leads to highly

fluorescent noncrosslinked nanoparticles (ON state) with a 15-fold increase in luminescence.

Bioluminescence resonance energy transfer (BRET) offers several advantages over FRET-based systems, including larger differences in the emission spectra between the BRET donor and acceptor, high sensitivity and low background emissions. Kim *et al.* utilized the strong quenching properties of AuNPs (BRET acceptor) to silence the bioluminescence emission of *Renilla* luciferase [43]. Bioluminescence was recovered *in vitro* following 1-h incubation with MMP-2 and subsequent cleavage of an MMP-2 peptide linker substrate (IPVSLRSG).

Lanthanide-doped upconversion nanoparticles (UCNPs) can sequentially absorb multiple low-energy excitation photons to generate higher energy anti-Stokes luminescence [64]. UCNPs offer strong photostability, large anti-Stokes shifts and sharp emission bandwidths. Zeng *et al.* developed biostable luminescence resonance energy transfer (LRET) based UCNPs through facile peptide-mediated phase transfer to image proteolytic activity [44]. Oleic acid on the surface of UCNPs was displaced by chimeric peptides containing a polyhistidine-tag and a caspase-3 cleavage domain (DEVD). (H)₆-GDEVDAK-TAMRA-coated, caspase-3 responsive UCNPs were used to monitor the therapeutic efficacy of doxorubicin in a tumor mouse model. The upconversion luminescence (UCL) signal, which was collected between 450 and 600 nm (under 980 nm excitation), gradually increased the expression of light over a period of 12 h in DOX-treated tumors, as opposed to saline-treated tumors.

Theranostic nanoparticles in optical imaging

Multifunctional nanoplateforms that integrate *in vivo* imaging and drug delivery into a single theranostic nanoparticle (TNP) enable simultaneous visualization of probe biodistribution, therapy and response to treatment [65]. Prodrugs, which exploit the unique characteristics of the tumor microenvironment such as overexpressed proteolytic activity or pH, could be delivered for selective treatment of cancer cells while minimizing off-target toxicity [66,67]. The addition of imaging capability enables direct monitoring of the delivery and activation of the prodrug in the tumor microenvironment [68]. In one example, doxorubicin (Dox) was conjugated to AuNPs via an MMP-2 cleavable peptide substrate (CPLGLAGG) [45]. Fluorescence of Dox was quenched by AuNPs, and recovered following exposure to MMP-2. This switchable fluorescence property allowed for imaging of the activity of MMP-2 in tumor sites *in vivo*.

One disadvantage of incorporating both drugs and imaging agents on a nanoparticle is the competition

for space, which can lead to a less than optimal surface density and reduced imaging and therapeutic efficacy. Moreover, drug incorporation adds to the cost and difficulty of synthesis and purification [65]. One alternative is to take advantage of the intrinsic therapeutic ability of several types of nanoparticles [46]. The efficient absorption of light by gold nanorods (AuNR) and the subsequent conversion of that energy to heat make these particles good candidates for photothermal therapy [69]. Yi *et al.* developed a theranostic probe based on MMP-sensitive gold nanorods (MMP-AuNR) for simultaneous cancer imaging and photothermal therapy, as shown in Figure 7A [46]. A Cy5.5-labeled MMP substrate (Cy5.5-GPLGVRGC) was conjugated onto the surface of AuNR, which quenched NIRF. NIRF tomographic images of SCC-7 tumor-bearing mice after intratumoral injection of MMP-AuNR with and without inhibitor were taken and significantly greater NIRF signal was observed in the case when no inhibitor was added (Figure 7B). Additionally, following laser irradiation, the temperature of MMP-AuNR injected tumors increased up to 45° after 4 min (Figure 7C), which is sufficient to damage cancer cells, and coincided with *in vitro* results.

Photoacoustic imaging

Photoacoustic imaging (PAI) is an emerging technology that has the ability to provide structural, functional and molecular information of target tissues. PAI was developed as a means to overcome the scatter of signal from a source within an animal [70], a problem that

Figure 3. Fluorescent protein-conjugated gold nanoquenchers (AuNP-FPs) for simultaneous imaging of multiple caspase activities involved in apoptosis. AuNP-FPs were designed to release blue-, red- and yellow-fluorescent protein following activation by caspase-8, caspase-9 and caspase-3, respectively. Reproduced with permission from [37].

Figure 4. NIRF images following injection of flower-like activatable nanoparticles (FANPs), FANPs plus MMP inhibitor, or gold-based activatable nanoparticles (GANPs). The additional iron oxide phase incorporated into composite FANPs allowed higher loading of MMP-specific peptide-dye and better quenching properties, which resulted in higher NIRF signal in the tumor site. Reproduced with permission from [39].

dramatically reduces the resolution of optical imaging modalities. In general, PAI uses a focused excitation pulse to provide energy to target probes and cause them to enter the excited state. In doing so, the probes or endogenous molecules in tissue (e.g., hemoglobin) release a heat signature in the form of thermal expansion that results in an acoustic wave, which is detected by transducers and assembled by image reconstruction analysis [71].

Recently Yang and colleagues developed an activatable photoacoustic (PA) nanoprobe which comprised BHQ3 conjugated to 20 nm copper sulfide (CuS) nanoparticles via an MMP-cleavable peptide substrate (GPLGVRGKGG) [47]. The resulting CuS-peptide-BHQ3 (CPQ) probe displayed strong PA signals at 680 nm and 930 nm due to the strong optical absorbance of BHQ3 and CuS nanoparticles, respectively, which was used to distinguish the two components. It was hypothesized that MMP activity would disassociate BHQ3 from the CuS nanoparticles and lead to their rapid clearance from the tumor, due to their small size, while the larger CuS nanoparticles are retained. CPQ was intratumorally injected into mice bearing SCC7 tumors with and without MMP inhibitor. Ratiometric analysis of the PA signals (680/930 nm) showed MMP activity in the tumor; decreasing after 2 h in the case with no inhibitor, but remaining almost uniform when an MMP inhibitor was preinjected.

Other

NIRF signals obtained from *in vivo* lesions are a function of the intensity of incident light and the size and depth, from the surface, of the lesion. The observed signal will also depend on the proteolytic activity present in the lesion and the concentration of nanoprobe delivered, which complicates any attempt to quantify proteolytic activity *in vivo* [72]. A solution proposed by Scherer utilized a dual fluorochrome probe, in which polyamidoamine PAMAM-Generation 4 dendrimers were coupled to a Cy5.5 fluorophore-labeled, MMP7-cleavable peptide (RPLALWRS), to detect proteolysis (S, sensor) and AF750 as a noncleavable internal reference fluorophore, to monitor the total concentration (cleaved and uncleaved) of the reagent (R, reference), hence facilitating quantitative analysis [22]. The dye AF750 was also used as a quencher and its signal used to evaluate pharmacokinetics. The sensitivity of the probe was evaluated with two subcutaneous xenografted tumors on either flank of athymic nude mice that only differed in the expression of MMP7. Effective cleavage of Cy5.5 fluorophore in the tumor was calculated by dividing the sensor signal by that of the internal reference (S/R), a ratio that corrects for differences in the lesion size and depth. S/R increased over time and was consistently greater in MMP7 expressing tumors compared with control tumor.

Many of the probes discussed so far require post-synthesis modification, which causes variability in surface charge, payload and other characteristics. A controllable on-chip preparation of nanoprobe involved cadmium selenide quantum dot (CdSe QD) payload embedded into the hydrogel-like interior of MMP-responsive supramolecular gelatin nanoparticles (SGNs) to produce CdSe QDs encapsulated SGNs [73]. Self-assembly was done on a microfluidic device with hydrodynamic flow focusing, and the physiochemical properties were precisely controlled by changing the flow rates of the fluids. Degradation of the gelatin corona, upon exposure to MMP in the tumor, releases the QDs which can be subsequently internalized by cancer cells. *In vitro* cellular uptake by MMP secreting HT1080 cells was observed while addition of an MMP inhibitor decreased the fluorescence signal.

Thorek *et al.* utilized energy transfer between Cerenkov luminescence-emitting radionuclide and an activatable fluorescent AuNP probe for low background, secondary Cerenkov-induced fluorescent imaging (SCIFI) of MMP-2 activity [23]. The platform comprised FAM-labeled, MMP-2 cleavable peptide (IPVSLRSG) conjugated to AuNP, which quenched FAM fluorescence. Mice bearing SCC-7 xenografts were co-injected with [^{18}F]-FDG radionuclides and activatable AuNP. Cleavage of the peptide by MMP-2

at the tumor site releases the fluorophore, which is then excited by nearby [^{18}F]-FDG, leading to secondary Cerenkov-induced fluorescent conversion and detection by SCIFI.

Multimodal imaging

A growing trend in disease diagnosis is to synergistically combine several complimentary imaging modalities into a single platform, thus overcoming their individual limitations [24]. This has the potential to provide more complete information on disease pathology. Much research effort has been dedicated toward combining nanotechnology and molecular imaging to obtain a new generation of multimodal imaging nanoparticles. This section will look at the various multimodal, nanoparticle-based imaging probes developed thus far for imaging proteolytic activity *in vivo*.

MRI/optical

MRI provides excellent anatomical information and great tissue penetration but it has limited sensitivity, which hampers imaging of molecular events [53]. Optical imaging, on the other hand, provides excellent molecular imaging but weak anatomical information and limited tissue penetration [48,74]. Nanoprobes that can be imaged by both of these modalities could potentially provide excellent anatomical and molecular information. Iron oxide nanoparticles, which quench fluorescence, were fabricated with a thin silica coating (PCM-CS) that contained a Cy5.5-MMP substrate (Cy5.5-GPLGVRG) for MRI/NIRF dual imaging, with molecular (MMP) activity determined through optical imaging and anatomical information through MRI [53]. PCM-CS successfully visualized the tumor

regions in SC77 tumor-bearing xenografted mice using both imaging modalities. No NIRF signal was detectable in normal mice, whereas signal increased gradually in tumor-bearing mice up to 12 h, with NIRF intensity three- to four-times higher than in normal tissue. Administration of MMP-2 inhibitor 30 min before injection of PCM-CS significantly reduced NIRF signal. Noticeable darkening in T_2 -weighted MRI images appeared at 6-h postinjection in the tumor region as compared with the healthy muscle regions in the mice. While the decrease in MRI signal hit a maximum at 12 h, similar to the trend exhibited in the NIRF images, it should be noted that MRI and optical imaging were conducted on different mice.

Harris *et al.* developed a strategy for reversibly veiling a cell internalization domain on magnetofluorescent, dextran-coated iron oxide nanoparticles by shielding it with sterically protective, MMP-2 cleavable PEG (PEG-GK(TAMRA)GPLGVRGC) [12]. FITC was used to label the cell internalizing domain and thus track cellular internalization, while TAMRA-labeled peptide-PEG was used to measure MMP activity. Following administration to mice via tail-vein injection, unveiled controls cleared eight-times faster than veiled particles from the blood, and fluorescence molecular tomography and MRI showed that veiled particles accumulated to a greater extent in tumor xenografts.

A different shielding strategy was developed by Olson *et al.*, who instead of using PEG chains, used MMP cleavable polyanionic peptides to electrostatically neutralize short, cell-penetrating polycationic domains on the surface of dendrimeric nanoparticles [48]. Following cleavage of the cleavable linker (PLGCAG) by MMP, the polyanionic domain departs

Figure 5. To distinguish different stages of tumors *in vivo*, a fluorescently quenched, peptide-based activatable nanosensor was intravenously injected into mice carrying MMP secreting SCC-7 tumors of varying sizes. (A) NIRF images of differently sized excised tumor tissues (3.5, 66.8, 172.0 and 381.5 mg), with overall signal increasing with tumor size. (B) Total fluorescent intensity (tumor grade) increased proportionally to MMP 2/9 activity, as measured by gelatin zymography.

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Figure 6. (A) A series of NIRF tomographic images of normal and SCC-7 tumor-bearing athymic nude mice after intratumoral injection of an MMP-sensitive gold nanoprobe (AuNP), with and without inhibitor. Clear visualization of MMP-2 activity was evident in tumor bearing mice. Signal diminished significantly in normal mice and in MMP-2 inhibitor-treated tumor-bearing mice. (B) *Ex vivo* validation: NIRF signals of excised tumors were significantly higher (upper image) compared with inhibitor-treated tumors (lower image). Reproduced with permission from [40].

from the polycation and associated nanoparticle, which are then able to penetrate cells in the immediate vicinity of the protease. Tracking of nanoparticle uptake in tumor-bearing mice by optical imaging and T_1 -weighted MRI was possible by labeling the cell-penetrating peptide with Cy5, gadolinium (Gd) or both. Residual tumor and metastasis as small as 200 μm was detected with optical imaging, while Gd-labeled nanoparticles deposited 30–50 μM of Gd in tumor parenchyma, which resulted in significant T_1 contrast that persisted for 2–3 days after injection.

PET/optical

PET provides tomographic images with excellent sensitivity and information on metabolic activities [49]. PET, unlike optical imaging, is limited in its ability to provide molecular information on proteolytic activity. As a result, combining PET imaging with optical imaging can provide vital information on tumor-targeting efficacy and proteolytic activity. Lee and co-workers prepared a PET/optical imaging probe by conjugating ^{64}Cu radiolabeled DOTA complex and activatable MMP-sensitive probe onto azide-functionalized glycol chitosan nanoparticles via copper-free click chemistry [49]. The ^{64}Cu -radiolabeled DOTA was used as

an ‘always on’ PET imaging agent, while the MMP-sensitive probe (Cy5.5-GPLGVRGK(BHQ-3)GG) was used as an ‘activatable’ optical imaging agent. The activity of MMP and the biodistribution of the probe following intravenous injection were successfully measured in tumor bearing mice by both NIRF and PET. NIRF signal could be detected in the tumor region 1 h after injection and kept increasing until it reached a maximum value after 6 h. Meanwhile, administration of an MMP inhibitor significantly reduced the NIRF signal. PET imaging enabled *in vivo* real-time visualization of tumor accumulation and biodistribution of the probe. Tumor accumulation continued to increase until it reached a plateau 24-h postinjection.

CT/optical

Combining both CT and NIRF functionalities onto the same probe enables simultaneous gathering of CT anatomical images with high spatial resolution and optical images with high sensitivity [75]. It is also a cheaper alternative to MRI fluorescence multimodal imaging [58]. Sun *et al.* developed a CT/optical imaging agent based on X-ray absorption and optical quenching properties of AuNPs [24]. To increase the physiological stability of AuNPs, they modified the

surface with biocompatible glycol chitosan (GC) polymers (GC-AuNPs). For fluorescence optical imaging of MMP activity, an MMP-specific activatable peptide probe (Cy5.5-GPLGVAGL-BHQ3) was conjugated to GC-AuNPs (MMP-GC-AuNPs), which resulted in combinatorial quenching effect of NIRF of Cy5.5 by the black hole quencher (BHQ-3) and AuNP surface. *In vivo* dual CT/optical imaging studies were performed with HT-29 tumor bearing mice to confirm the specific accumulation and MMP-responsive behavior of MMP-GC-AuNPs. CT images provided anatomical information of the tumor (Figure 8A), while the NIRF signal was detected in the tumor region 1 h after injection, and increased gradually until reaching a maximum value after 4 h. Meanwhile, administration of an MMP inhibitor intratumorally 30 min before injection significantly reduced the NIRF signal (Figure 8B).

Conclusion & future perspective

Genomics and proteomics have provided tremendous insight into the genetic, biochemical and cellular abnormalities that occur during cancer pathogenesis [4]. With this greater understanding, imaging of

in vivo proteolytic activity provides the potential for early detection and staging of cancer. It is especially advantageous due to the catalytic nature of proteases, which allows for signal amplification and for the design of activatable probes with minimal background noise and enhanced contrast. To be effective, the imaging probe must display excellent pharmacokinetic properties, minimal toxicity, specific tumor targeting capability, specificity and selectivity to a target protease, and high signal-to-noise ratio.

Nanoparticles possess unique physical and chemical properties that can be varied for optimal tumor targeting and recognition of proteolytic activity. While small molecule probes may be optimum for some applications, nanoprobe do offer some advantages over small molecule based systems. Small molecule-based probes tend to be unstable and distribute nonspecifically into tissue, yielding a high background noise. On the other hand, nanoparticles, with a high surface area to volume ratio, enable the incorporation of large peptide-dye payloads, which leads to enhanced target selectivity, increased sensitivity through signal amplification, and the potential for multimodal imaging of proteo-

Figure 7. (A) Schematic of a theranostic probe based on MMP-sensitive gold nanorods (MMP-AuNR) for simultaneous cancer imaging and photothermal therapy. (B) NIRF tomographic images of SCC-7 tumor-bearing mice following intratumoral injection of MMP-AuNR without (1) and with (2) inhibitor were taken, showing a significantly enhanced NIRF signal in the tumor with no inhibitor added, compared with that treated with an inhibitor. (C) The hyperthermal therapeutic potential of MMP-AuNR was visualized through infrared thermal images of tumor-bearing mice and measured with a hypodermic thermocouple. Laser irradiation at various times was done after intratumoral injection of MMP-AuNR. The temperature increased up to 45° after 4 min of laser irradiation.

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Figure 8. The potential of MMP-GC-AuNP as an *in vivo* CT/optical dual imaging agent was evaluated following intravenous injection of the probe into MMP-2 positive, HT-29 tumor-bearing mice. CT images confirmed the tumor-targeting efficacy of the probe, while the NIRF images confirmed its MMP-2-responsive.

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lytic activity, not possible with small molecule systems. Moreover, nanoparticles can also offer multifunctionality through their inherent unique optical, magnetic and therapeutic properties. However, while incorporating surface coatings, targeting ligands, imaging agents and therapeutics can yield desirable properties for diagnosis and therapy, the additional complexity could introduce challenges to large-scale production and batch-to-batch reproducibility – potentially hampering clinical translation and commercialization. This is a significant problem that hinders the production of nanoprobe, as opposed to small molecule imaging systems. Also, since even small changes in any one of the physiochemical properties (e.g., size, surface chemistry, etc.) tend to affect the pharmacokinetics, biodistribution and toxicity of nanoprobe, these systems are inherently difficult to optimize due to the large parameter space. However, given their enormous potential in both diagnosis and therapy, significant effort in their development continues.

Several biological, physical and regulatory barriers must be overcome prior to clinical translation and commercialization of nanoparticle-based proteolytic imaging probes. Unfortunately, only a small number of probes are able to provide quantitative rather than semi-quantitative information about proteolytic activity at the target site. Semi-quantitative information, where the only signal obtained is from the activated product, can be misleading since the probe does not accumulate uniformly throughout the body or even in the tumor volume [76]. Moreover, following activation by proteases, it is difficult to pinpoint the exact location of the probe. To overcome this issue, the con-

centration of both the probe and the activated product should be co-localized simultaneously and the signal ratio used to correct for lesion heterogeneity. While the dual fluorochrome probes and some of the multimodality probes discussed in this review are capable of providing quantitative information about proteolytic activity, much work remains to be done [77].

Another challenge in measurement of *in vivo* proteolytic activity is the shielding of the peptide sequence from preactivation prior to reaching the target site. This is a difficult challenge due to the variety of proteases present in serum, which can increase the background noise. Shielding of the peptide with PEG instills a stealth property that can reduce preactivation, but can also inhibit the action of the target proteases. Advancement in nanoprobe design requires fine balancing between the nanoparticle physiochemical properties and safety features with imaging sensitivity. The choice of protease is also very important. Proteases that are present in the tumor interstitium require extravasation and penetration of nanoparticles through the various barriers to reach the desired regions in the tumor interstitium, a slow and complex process that requires extended plasma half-life, which in turn could lead to high background noise. Moreover, fluorescent molecules tend to photobleach and destabilize over time. Proteases present in the angiogenic tumor vasculature may be an easier target as those probes do not require prolonged circulation or tissue penetration properties. Otherwise, further effort should be placed toward discovering more selective and sensitive shielding strategies and into the discovery of specific peptide substrates as these in large part determine the signal-to-noise ratio of the contrast agent.

There is also a limited understanding of the potential adverse effects of these nanoparticle-based systems, which could have drastically different toxicity profiles from their constituent components [58]. Many of the studies discussed in this review, for example, focus on enhanced tumor targeting without assessing the impact on other organs such as the liver. Obtaining a full toxicity profile, short-term and long-term, as well as nanoparticle biodistribution to critical organs and their clearance is essential.

Quantification of *in vivo* proteolytic activity promises to tremendously increase our knowledge of disease progression, potentially also leading to improved therapies. For this to become reality, however, the specificity, selectivity and sensitivity of probes need to be further developed, along with the computational methods necessary for data analysis. Such an effort will require a multidisciplinary effort with collaboration between engineers, chemists, biologists, physicists and clinicians.

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Executive summary**Molecular imaging of proteolytic activity in tumor models *in vivo***

- Identifying and quantifying molecular-level changes associated with tumorigenesis helps in early detection and estimation of malignant potential; necessary for effective cancer treatment.
- Several proteases, including matrix metalloproteinases, cathepsins and caspases, have been implicated in tumor invasiveness and metastasis and their expression is an indicator of tumor aggressiveness.
- Proteases can propagate signals through their catalytic activity; specifically cleave certain peptide sequences; and are present at the invasive front of tumors, regions that are more easily accessed by probes.
- Imaging probes have been developed with nanoparticle technology to provide a noninvasive measurement of proteolytic activity in tumor models at different stages of disease progression. Nanoparticles have unique physical and chemical properties; excellent *in vivo* characteristics; and a vast surface area to deliver a large number of imaging agents and tumor-targeting ligands.

Nanoparticles for MRI of proteolytic activity *in vivo*

- Magnetic nanoparticles used to image proteolytic activity rely on signal amplification due to cluster formation following proteolytic cleavage, which enhances magnetic susceptibility and r_2 relaxivity.
- The most commonly used design strategy utilizes two sets of complimentary, polyethylene glycol (PEG)-conjugated iron oxide nanoparticles that self-assemble (e.g., through neutravidin/biotin interactions) following proteolytic cleavage and release of PEG.
- A significant drawback of utilizing separate sets of nanoparticles is they face co-delivery issues *in vivo*.

Nanoparticles for optical imaging of proteolytic activity *in vivo*

- Optical imaging remains the most commonly used modality for imaging proteolytic activity *in vivo*. The majority of probes utilize one or more fluorescence quenching effects to reduce the preactivation fluorescence signal. Fluorescence is recovered following release of quencher by proteolytic activity.
- Several theranostic probes have been developed for simultaneous therapy and imaging of proteolytic activity.
- Other examples of optical imaging include bioluminescence resonance energy transfer, photoacoustic imaging and secondary Cerenkov-induced fluorescent imaging.

Nanoparticles for multimodal imaging of proteolytic activity *in vivo*

- Multimodality combines several complimentary imaging modalities, each with its own strengths and weaknesses, into a single platform, thus overcoming their individual limitations.
- MRI/optical dual imaging has been the most thoroughly investigated combination
- PET/optical and CT/optical dual probes have been developed as well.

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