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Enzyme-triggered gelation: targeting proteases with internal cleavage sites†

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A generalizable method for detecting protease activity *via* gelation is described. A recognition sequence is used to target the protease of interest while a second protease is used to remove the residual residues from the gelator scaffold. Using this approach, selective assays for both MMP-9 and PSA are demonstrated.

Because both overexpressed and overactive enzymes correlate with disease, simple visual assays that respond to enzymatic activity are in high demand. Colorimetric and fluorimetric assays dominate the field, but the analysis can be challenging in the complex medium of biological fluids. An alternative approach is to use an enzyme to trigger a phase transition (e.g., solution-to-gel) by cleaving a covalent bond. Such assays can be performed in media as complex as blood or cells, and the results are unambiguously detected without any instrumentation. In 2004, Xu and coworkers reported the first enzyme-mediated gelation of small molecules. Since then, several different enzymes have been used to trigger gelation.

Though promising, only a handful of reported enzymetriggered gelations are selective for a single enzyme. Most of these selective systems use peptide-based recognition sequences. Enzymatic cleavage at the N-terminal side of the recognition sequence serves to completely separate this sequence from the gelator and trigger gel formation. In 2012 we reported a highly selective and generalizable method for detecting protease activity based on this approach. While this method is suitable for most serine and cysteine proteases, it is not suitable for aspartyl proteases and metalloproteinases because these enzymes require specific residues on both sides of the scissile bond. Targeting these enzymes is more challenging because the leftover peptide residues attached to

Scheme 1 General design strategy for detecting proteases *via* gelation using recognition sequences and aminopeptidase M.

the gelator scaffold can disrupt the gelation process,⁷ and as a consequence, a new gelator may need to be discovered for each enzyme.

We reasoned that an aminopeptidase, which nondiscriminately cleaves amino acids off the N-terminus of a peptide, ⁸ could be used to remove the leftover prime-side recognition sequence residues from the gelator scaffold (Scheme 1). As a consequence, the same gelator can be used for detecting any protease by simply varying the recognition sequence. Using this approach, we describe herein the development of selective assays for matrix metalloprotease-9 (MMP-9) and prostate specific antigen (PSA) using a single gelator scaffold and aminopeptidase M (AP-M). Aminopeptidase M (AP-M) was selected because it will remove residues from nearly every sequence of natural amino acids. ⁸

The sensing scheme is comprised of three key components: a solubility factor (SF), recognition sequence, and gelator. The solubility factor increases the solubility and ensures that the recognition sequence is accessible to the target enzyme in solution. The solubility factor also prevents premature cleavage of the substrate by AP-M because it contains a non-natural oligoethylene glycol unit at the N-terminus that is not recognized by AP-M. The recognition sequence contains a short peptide segment that is easily interchangeable and optimized for each target enzyme. Once this sequence is cleaved by the protease,

enzyme-specific cleavage site

solubility factor — recognition sequence — gelator

dependence — gelator

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the AP-M will non-discriminately cleave the remaining residues. To prevent the AP-M from cleaving the peptide-based gelator, a non-natural p-aminobenzamide (PABA) unit was included at the N-terminus. Gelator $\mathbf{1}^{6a}$ was selected for these studies because it forms stable gels in buffer (50 mM HEPES, 200 mM NaCl, 10 mM CaCl₂, 7.5% DMSO, pH = 7.5) with a low critical gel concentration (cgc, 1.7 mM).

We first targeted MMP-9¹⁰ because it has stringent requirements about which residues are present on both sides of the cleavage site, ¹¹ and it has been implicated in several pathological conditions, including cancer metastasis ¹² and arthritis. ¹³ A scaffold containing an optimized recognition sequence for MMP-9 (GPKG-LKGA)¹⁴ was treated with both MMP-9 and AP-M in buffer. Gratifyingly, a stable gel was observed within 7 h (Fig. 1A and Fig. S10, ESI†). The importance of prime side residues was demonstrated when a recognition sequence lacking these residues showed no conversion over the same time (Fig. 1B and Fig. S10, ESI†).

Because, in principle, the assay depends on the concentration and activity of both enzymes, identifying conditions wherein the target enzyme (MMP-9) is rate-limiting is desired, ensuring a low detection limit for the target enzyme. LC-MS studies were used to monitor conversion of LKGA-1 to 1 mediated by AP-M over time. These studies revealed that approximately 45% conversion is reached within 160 min, at which point gelation occurs (ESI,† Fig. S17). This rate is significantly faster than the 7 h needed for conversion of parent scaffold: SF-GPKG-LKGA-1 (ESI,† Fig. S10). Thus, the rate-limiting step must be the initial MMP-9 triggered cleavage. Overall, the detection limit is 50 nM under these optimized conditions, which is within the levels present in cancer patients with elevated levels of circulating MMP-9 (71 \pm 60 nM for non-small cell lung cancer patients, 36 \pm 13 nM for healthy subjects). 15

MMP-9 cleavage site

SF - GPKG-LKGA - 1

MMP-9

SF - GPKG + LKGA - 1

AP-M

1

AP-M

no reaction

B

Fig. 1 (A) An opaque gel was observed within 7 h of adding MMP-9 (50 nM) to a buffer solution containing AP-M (1.2 μ M) and the gelator scaffold with the optimized recognition sequence. (B) No gel was observed when the prime side residues were not included under otherwise identical conditions. Pictures were taken after 24 h.

This suggests that our MMP-9 sensing gelator could readily discriminate between healthy patients and patients with aggressive cancers.

To demonstrate the generality of this approach, we targeted a different protease: prostate-specific antigen (PSA). PSA is a serine protease, however, it was selected because the prime side residues have a dramatic influence over the reactivity. 16 In addition, PSA is an important biomarker for prostate cancer. 17 Although overexpression of active PSA correlates with cancer prognosis, current screening methods measure total PSA concentration, not the fraction that is active. 18 While total PSA levels as a prostate cancer diagnostic is controversial, 18a recent studies have demonstrated that active PSA enzyme in the blood is highly correlative to aggressive prostate cancer. 19 Hence, an assay based on activity is highly desirable. A substrate containing an optimized PSA recognition sequence (HSAKFY-SG)16a was synthesized and treated with PSA and AP-M in buffer. Within 24 h, a stable gel was formed (ESI,† Fig. S14). A control experiment lacking PSA showed no gelation over the same time (ESI,† Fig. S14). Similarly, if the prime side residues were missing (HSAKFY) from the substrate, no reaction was observed by LC-MS even after 24 h (ESI,† Fig. S13).

Because PSA activity is rate-limiting, we hypothesized that the response time could be shortened by adding the cleaved substrate (SG-1) to the reaction mixture. Although SG-1 was largely insoluble, adding a lysine (i.e., SGK-1) significantly improved its solubility. This substrate is rapidly converted to 1 in situ using AP-M. Gratifyingly, including SGK-1 (1 mM) reduced the time-togelation from 24 h to 7.5 h (Fig. 2). The detection limit of this PSA sensor is 32 nM of active enzyme, which is above the physiological levels of active PSA found in the blood of prostate cancer patients $(\sim 0.1 \text{ nM} \text{ active enzyme})^{20}$ However, high levels of active PSA are found in the extracellular fluid of prostate cancers (1.8 μM). ^{18b} On this basis, our PSA sensing gelator could be used as an aid in biopsy diagnosis. Our detection limit must be lowered considerably to be useful as a diagnostic for measuring active PSA levels in blood.20 In principle, a lower detection limit can be achieved if a gelator with a lower cgc is used. Alternatively, a different recognition sequence with higher activity towards PSA would lead to lower detection limits. Additionally, patients with prostate cancer have high levels

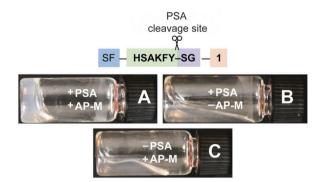


Fig. 2 (A) An opaque gel was observed within 7.5 h when PSA (1.6 μ M) was added to a buffer solution containing AP-M (1.2 μ M), SGK-**1** (1 mM) and the gelator scaffold with the optimized recognition sequence. No gel was observed when either (B) AP-M or (C) PSA was excluded, under otherwise identical conditions.

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of proPSA, an inactive precursor of PSA, that could be converted into active PSA in situ (\sim 30 nM proPSA, compared to less than 1 nM for healthy individuals)²⁰ using an additional enzyme.²¹

In summary, a generalizable method for detecting protease activity using gelation is described. We demonstrated that aminopeptidase M, which nondiscriminately cleaves amino acids off the N-terminus of a peptide, can be used to remove the residual components of a recognition sequence from the gel scaffold. As a consequence, the same gelator can be used to detect different proteases by simply switching the recognition sequences. Using this approach, we were able to detect both MMP-9 and PSA at physiological concentrations. Given that these proteases are validated biomarkers for cancer, we believe that with further development this simple visual assay may be useful for early detection. Our future efforts will focus on accelerating the time for gelation by employing additives (i.e., growth promoters),22 designing alternative gelators with lower critical gel concentrations, and improving the activity of the recognition sequences. Overall, the system described herein is significantly more versatile than other enzyme-triggered gelations in the literature. As a result, we anticipate that this method will be utilized for the detection and diagnosis of other diseaserelevant proteases.

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