Point-of-care diagnostics for noncommunicable diseases using synthetic urinary biomarkers and paper microfluidics

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With noncommunicable diseases (NCDs) now constituting the majority of global mortality, there is a growing need for low-cost, noninvasive methods to diagnose and treat this class of diseases, especially in resource-limited settings. Molecular biomarkers combined with low-cost point-of-care assays constitute a potential solution for diagnosing NCDs, but the dearth of naturally occurring, predictive markers limits this approach. Here, we describe the design of exogenous agents that serve as synthetic biomarkers for NCDs by producing urinary signals that can be quantified by a companion paper test. These synthetic biomarkers are composed of nanoparticles conjugated to ligand-encoded reporters via protease-sensitive peptide substrates. Upon delivery, the nanoparticles passively target diseased sites, such as solid tumors or blood clots, where up-regulated proteases cleave the peptide substrates and release reporters that are cleared into urine. The reporters are engineered for detection by sandwich immunoassays, and we demonstrate their quantification directly from unmodified urine; furthermore, capture antibody specificity allows the probes to be multiplexed in vivo and quantified simultaneously by ELISA or paper lateral flow assay (LFA). We tailor synthetic biomarkers specific to colorectal cancer, a representative solid tumor, and thrombosis, a common cardiovascular disorder, and demonstrate urinary detection of these diseases in mouse models by paper diagnostic. Together, the LFA and injectable synthetic biomarkers, which could be tailored for multiple diseases, form a generalized diagnostic platform for NCDs that can be applied in almost any setting without expensive equipment or trained medical personnel.

protease nanosensor | urine biomarker | image-free diagnostic | engineered disease reporter | global health

In the last several decades, global health challenges have dramatically shifted, with substantial reductions in the burden of infectious diseases (e.g., HIV, tuberculosis, and malaria) and simultaneous growth in the prevalence of noncommunicable diseases (NCDs) such as stroke, heart disease, and cancer, which constitute an increasing majority of global mortality (1, 2). Strikingly, NCDs disproportionately affect the developing world: low- and middle-income countries bear nearly 80% of the world’s NCD burden (3), and cardiovascular diseases and cancer have been the first and second, respectively, highest causes of mortality in the developing world since 2001 (4). Diagnosing NCDs in remote and/or poor settings is difficult without access to costly imaging modalities [e.g., computed tomography (CT)/MRI], well-equipped clinical laboratories [e.g., for histopathology], and trained medical personnel. Consequently, developing diagnostics for NCDs that are cost effective and can be easily implemented remains an important goal in global health. One promising approach is to detect disease biomarkers from readily accessible bodily fluids with point-of-care (POC) devices that are inexpensive, noninvasive, and do not require trained medical personnel. Despite widespread interest, the lack of predictive, validated biomarkers significantly limits the types of NCDs that can be detected at the POC (5–7).

Rather than searching for endogenous biomarkers, a promising strategy is to engineer exogenous agents that can specifically probe for the presence of diseased tissue. Radiolabeled glucose is an example of a common exogenous agent used with PET to reveal the location of metabolically active tumors. Inspired by engineered approaches, our group recently outlined a framework whereby protease-sensitive nanoparticles (NPs) called “synthetic biomarkers” are administered to detect NCDs including liver fibrosis, cancer, and thrombosis noninvasively (8, 9). These peptide-coated NPs probe diseased sites and, in response to cleavage by local dysregulated proteases, release mass-encoded reporters that then filter into the urine for analysis by mass spectrometry. In practice, administering NPs and collecting urine samples are both well-suited for the POC, but the need for a mass spectrometer to analyze the urinary cleavage fragments limits the utility of mass-encoded synthetic biomarkers for global health applications.

To address these difficulties, we reformulated our synthetic biomarkers to release ligand-encoded reporters designed for detection by a companion POC diagnostic comprised of paper test strips, a well-established technology used to screen and monitor diseases with readily available biomarkers (10, 11). The Significance

Noncommunicable diseases, including cardiovascular disease and cancer, are growing worldwide but are challenging to diagnose because biomarkers that can accurately detect them in patients are lacking. Here, we designed nanoscale agents that are administered to reveal the presence of diseased tissues by producing a biomarker in the urine that can be detected using paper strips similar to a home pregnancy test. Using mouse models, we show that we can detect diseases as diverse as solid cancer and blood clots using only a single injection of our diagnostic followed by urine analysis on paper. This platform does not require expensive instruments, invasive procedures, or trained medical personnel, and may allow low-cost diagnosis of diseases at the point of care in resource-limited settings.


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benefits of paper testing include low cost, rapid diagnosis, and no need for complex equipment or technical expertise (11, 12). Paper tests operate by wicking a biological specimen (commonly urine, saliva, or blood) containing a target analyte to regions where subsequent chemical or antibody-mediated analyses are detected by direct observation (13) or inexpensive and accessible quantitative imaging (14–16). Recently, paper diagnostics have been developed for quantitative assays like monitoring transaminases released by liver damage (13) or detecting infectious diseases like HIV-1, malaria, or trichinellis (17). Unfortunately, the scarcity of naturally occurring biomarkers has limited use of paper diagnostics for NCDs. Consequently, combining paper diagnostics with synthetic biomarkers that sensitively and specifically indicate disease from the urine may provide a simple and low-cost method to diagnose NCDs in resource-limited settings.

To pursue the goal of developing affordable POC tests for NCDs, we engineer synthetic biomarkers to detect thrombosis and colorectal cancer (CRC) from the urine by custom lateral flow assay (LFA), a variant of paper tests. Thrombosis, the formation of obstructive blood clots, occurs in many cardiovascular-associated disorders (e.g., stroke and heart attack) and is characterized by the activation of the plasma protease thrombin that mediates fibrin clot formation. In CRC and most solid cancers, tumors produce matrix metalloproteinases (MMPs) to facilitate growth, angiogenesis, and metastatic spread (18). To detect these diseases, we develop thrombin- and MMP-sensitive NPs by conjugating substrate–reporter tandem peptides to the surface of NPs (Fig. 1A). When administered, these NPs probe diseased tissues (blood clots or tumors) where local up-regulated proteases (thrombin or MMPs, respectively) cleave their surface coat of peptides, releasing reporters that are concentrated into the urine. The urinary reporters are functionalized with structurally distinct ligands for capture onto paper test strips adsorbed with ligand-binding antibodies (Fig. 1B). Beyond thrombosis and CRC, this approach may be amenable to many noncommunicable and infectious diseases in which aberrant protease activities are implicated.

**Results**

**Protease-Sensitive NPs for Urinary Monitoring of Disease.** To develop synthetic biomarkers for thrombosis and cancer, we first sought to design NPs for sensing the activity of the proteases thrombin and matrix metalloproteinase 9 (MMP9). We functionalized poly(ethylene glycol)-coated iron oxide nanoworms (NWs)—a long-circulating NP formulation previously characterized by collaborators and our laboratory (19, 20)—with fluorescein-labeled derivatives of thrombin- and MMP9-cleavable substrates [PLGLRSW and PLGVRGK, respectively (8)] at a surface valency of 20–30 peptides per NW to induce intermolecular quenching (Fig. 1A). To test the efficiency of peptidolysis, we incubated thrombin-sensitive NWs with thrombin and observed a rapid increase in sample fluorescence as cleaved peptide fragments released into solution fluoresced freely. By contrast, no increase in fluorescence was observed in the presence of Argatroban, a direct thrombin inhibitor, or when the substrate was synthesized with protease-resistant D-stereoisomers (Fig. 2A), indicating that thrombin activity was required to activate the NWs. We observed similar increases in sample fluorescence when MMP-sensitive NWs were incubated with MMP9 and no activity when the broad-spectrum MMP inhibitor Marimastat or D-stereoisomers were used (Fig. 2B). Together, these findings showed that peptides on the surface of NWs can be efficiently cleaved by thrombin or MMP9.

We previously showed that synthetic biomarkers composed of long-circulating NWs accumulate in diseased tissue by diffusing across fenestrated vessels such as in liver fibrosis and cancer (8), or when the sites of disease are intravascular as in thrombosis, sense protease activity while in systemic circulation (9). These studies also showed that free peptides administered i.v. are rapidly cleared by renal filtration, but their conjugation to NWs makes urinary clearance conditional upon cleavage by disease-specific proteases. To confirm that our synthetic biomarkers tailored for thrombin and MMP9 exhibit similar pharmacokinetics,
we synthesized NWs with substrates labeled with carrier peptide-linked near-infrared fluorophores to monitor peptide traffic and cleavage by in vivo fluorescence imaging. To promote renal clearance and to enable in vivo fluorescent visualization of the peptide-fluorophore reporter released by substrate proteolysis, we conjugated the near-infrared fluorophore VT750 (N-terminal) to the peptide glutamate-fibrinopeptide B (GluFib) (sequence eGvndneeGffsar), which we synthesized with 3-amino acids (lowercase) to confer stability against protease activity (8, 9, 21).

We chose a murine model of thrombosis in which the onset of clotting is controlled by the i.v. administration of collagen and epinephrine to activate platelets and thrombin, forming blood clots that embolize to the lungs (22). Consistent with our previous findings, coadministration of NWs to mice challenged with collagen and epinephrine resulted in a pronounced increase in their urinary and lung fluorescence relative to healthy controls (Fig. 2 C and D), indicating in vivo cleavage and renal clearance of peptides. To apply to CRC, we infused MMP9-sensitive NWs into nude mice bearing s.c. human colorectal tumors (LS174T), formed by a cell line that secretes MMP9 (23), and observed similar increases in fluorescence localized to the bladder (Fig. 2E). Immunofluorescent staining of tumor sections confirmed NW (green) extravasation localized to the tumor interstitium (Fig. 2F and Fig. S1). Collectively, these results verified the ability of our synthetic biomarkers to probe disease sites and release cleaved peptide fragments into the host urine.

Detecting Ligand-Encoded Reporters by Sandwich Complexes. Next sought to design a panel of ligand-encoded reporters that can be detected by protein-based sandwich complexes (Fig. 3A). The formation of a sandwich complex requires a target antigen to express two distinct epitopes that bind separately to a capture and a detection agent (Fig. 3A). Ligand-encoded reporters are designed based on an amino acid sequence specific for a particular target antigen (24). Here, we sought to determine whether ligand-encoded reporters could be detected in urine by sandwich ELISA.

Paper Assay Development and Detection of Protease Activity. First developed more than two decades ago to detect human choric gonadotropin as a home pregnancy test, paper-based LFAs have since been expanded for use in diverse settings to detect pathogens, drugs, hormones, and metabolites (17). LFAs detect antigens by a sandwich complex in which capture antibodies are adsorbed onto a highly porous test line. The nitrocellulose membrane, which serves to wick fluids and transport analytes from the sample pad to the capture regions (Fig. 4A). The immobilized analytes are then visualized by a detection agent coupled to NPs (typically gold or latex nanospheres) that create a colored line detectable by eye without enzymatic amplification.

Here, we sought to determine whether ligand-encoded reporters could be detected on paper. Using a low-volume robotic liquid handler, we deposited α-R1 antibody and α-streptavidin antibodies to capture test and control lines, respectively, on nitrocellulose paper strips. Unprocessed mouse urine samples spiked with R1 were then applied to the sample pads followed by a solution containing gold NP-conjugated streptavidin. Colored lines appeared where the test antibodies were printed, indicating R1 capture from urine and detection as a sandwich complex (Fig. 4B). Quantitative scans of LFAs used to analyze serial dilutions of R1 revealed a LOD of ∼1 nM and a working linear range of ∼1–7 nM (Fig. 4B and Fig. S5E). Similar performance metrics were observed for separate LFAs customized for the remaining reporters (Fig. S3 A–E).

To enable multiplex reporter detection, we printed capture antibodies into four parallel test lines relative to a control line and analyzed urine samples that contained one of the four reporters. Similar to the ELISA results, only the test lines printed with the cognate capture antibody developed a positive signal (Fig. 4C), highlighting the LFA’s specificity and the
capacity to detect distinct reporters with single spatially encoded paper strips.

To detect protease activity by LFAs, thrombin-sensitive substrates were conjugated in tandem with R3 onto NWs. Following in vitro substrate cleavage by thrombin, we collected the peptide fragments by size-exclusion filtration. Cleaved R3 was readily detected from the filtrate by LFA, developing into significantly darker test lines compared with control samples not exposed to thrombin (Fig. 4D, \( P = 0.0022 \)). Similarly, reporter concentration is proportional to band intensity. (B) Paper LFAs demonstrated a linear increase in band intensity with reporters diluted in urine by eye (Top) and automated image analysis (Middle), resulting in a linear detection region for R1 of \(-1-7\) nM (Bottom, linear fit \( R^2 = 0.99 \)). (C) Spatially multiplexed detection antibodies demonstrated specific detection of each of the four reporters and a test control line. (D) LFA detection of R3 released by incubation of thrombin-sensitive NWs with thrombin demonstrated darker bands visually (Lower) and by image analysis (Upper, \( P = 0.0022 \)) in the presence of enzyme than without enzyme. (E) Increased R2 was detected by LFA upon incubation of MMP-sensitive NWs with MMP9 than without enzyme visually (Lower) and by image analysis (Upper, \( P = 0.0022 \)). Error bars are SEM.

**Disease Detection on Paper with Synthetic Urinary Biomarkers.** Urine concentration is dependent on many host and environmental factors (e.g., diet, activity level, circadian rhythm, medical history); therefore, we sought to develop a normalization strategy for our test. We hypothesized that coadministered free reporters would pass into the urine independent of disease state and could be used to normalize the level of reporters released by protease activity. To investigate this approach, we infused a mixture of free R4 and thrombin-sensitive NWs (labeled with R3) into healthy or thrombotic cohorts of mice and collected all urine for 30 min postinjection. As anticipated, urinary concentrations of R4 were statistically equivalent between the two groups by ELISA, indicating unbiased clearance of the free reporter (Fig. 5A, Right, \( P = 0.25 \)). By contrast, urinary levels of R3, the reporter of thrombin activity, significantly increased in mice harboring thrombi when quantified independently (Fig. 5A, Left, \( P < 10^{-4} \)) or when normalized against R4 (Fig. 5B, \( P < 10^{-4} \)). Using a paper strip printed with multiple capture antibodies, we analyzed the urinary levels of R3 and R4 simultaneously (Fig. 4C and D) and similarly observed a statistically significant increase in the ratio of R3/R4 in diseased urine samples compared with healthy controls (Fig. 5C, \( P = 0.0015 \)). To determine the diagnostically accuracy of the assay, we analyzed the rate of true positives (sensitivity) and false positives (one-specificity) by receiver-operating characteristic (ROC) curves and found that the multiplexed paper test discriminated urine from thrombotic versus control mice accurately, with an area under the curve (a.u.c.) of 0.92 (Fig. 5D, \( P = 0.0015 \)).

To establish the ability to detect solid cancers, we adopted the normalization strategy developed for thrombosis by infusing a solution containing free R4 and R2-encoded MMP-sensitive NPs into nude mice bearing s.c. LS174T colorectal tumors and collecting all urine up to 1 h postinjection. As before, diseased mice cleared R4 with an efficiency statistically equivalent to healthy animals (Fig. 5E, Right, \( P = 0.92 \)), whereas the urinary concentrations of R2, the reporter of in vivo MMP activity, or its normalized intensity (R2/R4) were both significantly elevated in tumor-bearing mouse urine by ELISA (Fig. 5E, Left, \( P = 0.0039 \); Fig. 5F, \( P = 0.0098 \)). Analysis of the same urine samples by LFA demonstrated a significant increase in the ratio of R2/R4 in urine collected from tumor-bearing but not from control mice (Fig. 5G, \( P = 0.002 \)). By ROC analysis, this urine test was highly accurate and discriminated CRC with an a.u.c. of 0.90 (Fig. 5H, \( P = 0.0025 \)). Collectively, these results showed that LFAs can both detect synthetic biomarkers directly from the urine and discriminate NCDs with significant predictive power.

**Discussion.** In resource-limited environments, POC tests should be simple to operate, built from inexpensive components, and able to detect disease directly from biological fluids. Here, we outlined a strategy whereby NCDs are detected by a single infusion of synthetic biomarkers that release reporters into the urine in the presence of disease. Collected urine samples are then applied to custom LFAs that quantify reporter levels directly on paper without additional sample preparation or expensive instrumentation.

Building on our previous work on mass-encoded NPs, we developed NPs that sense protease activity by releasing rationally designed ligand-encoded reporters after substrate cleavage. We showed that these heterobifunctional reporters mediate the formation of sandwich complexes detectable by standard ELISA and LFA to allow POC testing as an alternative to expensive diagnostics platforms like CT scanners or mass spectrometers, which can cost over 100-fold more than a standard microplate reader (26). Because our ligand-encoded reporters are engineered to

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**Fig. 4.** Paper assay development and detection of protease activity. (A) Capture antibodies were adsorbed in spatially multiplexed lines on nitrocellulose membrane. The LFAs were developed by application of analyte, wash buffer, and streptavidin-gold detection NPs that wick past the capture antibodies and develop lines. LFAs may be scanned and reporter concentration is proportional to band intensity. (B) Paper LFAs demonstrated a linear increase in band intensity with reporters diluted in urine by eye (Top) and automated image analysis (Middle), resulting in a linear detection region for R1 of \(-1-7\) nM (Bottom, linear fit \( R^2 = 0.99 \)). (C) Spatially multiplexed detection antibodies demonstrated specific detection of each of the four reporters and a test control line. (D) LFA detection of R3 released by incubation of thrombin-sensitive NWs with thrombin demonstrated darker bands visually (Lower) and by image analysis (Upper, \( P = 0.0022 \)) in the presence of enzyme than without enzyme. (E) Increased R2 was detected by LFA upon incubation of MMP-sensitive NWs with MMP9 than without enzyme visually (Lower) and by image analysis (Upper, \( P = 0.0022 \)). Error bars are SEM.
Fig. 5. Paper-based disease detection using synthetic urinary biomarkers. (A) Urine was collected from mice (*n* = 10) coinjected with R3-encoded thrombin-sensitive NWs, free R4, and either PBS or collagen/epinephrine (to induce thrombosis). By ELISA, urinary clearance of free reporter R4 was not different between control and induced animals (*Right, P* = 0.25), but liberated R3 was significantly increased in animals that underwent thrombosis (*Left, P* < 0.0001). (B) Normalization of proteolytically liberated reporter to free reporter (R3/R4) was significantly increased in diseased animals (*P* = 0.0001). (C) LFAs also detected significantly increased R3/R4 in urine from diseased mice from the same cohort (*P* < 0.0001). (D) Receiver-operating characteristic (ROC) classification by LFA detection of R3/R4 discriminated healthy from diseased mice with an a.u.c. of 0.92 (*P* = 0.0015). Box plots show extremes, quartiles, and median. 

Methods

Expanded methods may be found in SI Methods.

Synthesis of Synthetic Biomarkers. Heterobifunctional ligand-encoded reporters R1–4 were synthesized by derivatizing GluFib with a capture ligand (fluorescein, DNP, TMR, or AF488) on the C terminus and a detection ligand (biotin) on the opposing terminus (Fig. S2). NWs (∼60 nm) were synthesized from the reaction of iron(III) chloride hexahydrate and iron(II) chloride tetrahydrate with dextran (15–25 kDa) as previously described (19, 20). Aminated NWs were derivatized with N-succinimidyl iodoacetate and reacted with sulfhydryl-terminated protease-sensitive reporters. Reporter valency (∼20–30) was quantified by absorbance or ELISA.

In Vitro Protease Activity Assays. Fluorescent reporter-bound thrombin- or MMP-sensitive NWs (substrates PLGRL5W or PLGVRGK, respectively) were introduced to recombinant thrombin or MMP9 (respectively). Release of homogenous fluorophores upon proteolysis was read as increased fluorescence intensity.

The ability to engineer synthetic biomarkers to produce a detection signal from urine has several advantages over POC tests that detect biomarkers from blood. Endogenous biomarkers are often limited by fundamental biological constraints, making detection challenging, especially at the earliest stages of disease when treatments are more likely curative (36, 37). By contrast, small inert analytes in plasma such as our ligand-encoded reporters are concentrated into the urine: in our animal models, reporters were enriched to levels that required the urine samples to be diluted (104-fold for LFA; 107- to 108-fold for ELISA) to prevent signal saturation when NPs were administered at a dose typical of nanomedicines (~1 mg/kg) (30, 31). In addition, detecting blood biomarkers depends on a blood draw that requires technical expertise unnecessary for urine collection.

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by plate reader at 37 °C. Inhibitors Argatroban or Marimastat were incubated with the protease-NW mixture at 100 μM. To quantify reporter release by LFA, reporter-functionalized NWs were incubated with cognate proteases, passed through a 30-kDa M cuto filter, quantified by LFA, and analyzed by Mann–Whitney test.

In Vivo Imaging. All animal studies were approved by Massachusetts Institute of Technology’s committee on animal care (protocol 0411–036–14). Thrombin- or MMP-sensitive NWs were functionalized with infrared fluorescent reporter VT750. Bladder and/or lung localization of proteolytically released fluorescent reporter was imaged in control and diseased mice. Thrombosis was induced by coinjection of collagen and epinephrine with synthetic biomarkers in female Swiss Webster mice; colorectal flank tumors were induced by s.c. injection of human cell line LS147T in female NCr nude mice.

ELISA Characterization. Ninety-six-well plates were adsorbed with capture antibodies LFD blocked with 1% (v/v) bovine serum albumin (BSA) in 1× PBS. Reporter standards were applied and detected by addition of Neutralizing antibodies and blocked with 1% (wt/vol) bovine serum albumin (BSA) in 1× PBS. Reporter concentrations were calculated and detected by addition of NeutraVidin horseradish peroxidase. Oxidation of chromogenic substrate TMB for 1–5 min allowed quantification of reporter concentration. All incubations were 1 hour and plates were washed with 1× PBS with 0.5% (v/v) Tween 20 or 20. Samples were assayed by inductive R1 in 1:100 control mouse urine. Assay specificity was measured by quantifying capture by human or control antibody to all reporters and normalizing signal to a cognate reporter control.

Paper LFA Characterization. Capture (same as for ELISA) or control (α-streptavidin) antibodies were printed in 2-mm-spaced lines with 50-nL droplets at 0.5-mm pitch onto cellulose ester membrane. Membranes were laminated to a plastic backing with glass fiber conjugate and absorbent pads.

Collection and Analysis of Urinary Peptides. Urine was collected from mice i.v. infused with synthetic biomarker mixtures (free R4 plus either R3-functionalized thrombin-sensitive NWs to detect thrombosis or R2-functionalized MMP-sensitive NWs to detect CRC) for 30 or 60 min postinjection (to detect thrombosis or CRC, respectively). Urine collection times were optimized from previous studies using these disease models (8, 9) and are dependent on site of disease and rate of enzymatic substrate cleavage. Reporter concentration in unprocessed urine was assayed by above protocols from urine diluted 1:10 to 1:100 for ELISA or 1:4–5 for LFA. Data were analyzed using ROC curves (both) and Wilcoxon signed-rank test (CRC) or Mann–Whitney test (thrombosis).

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Supporting Information

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**SI Methods**

**Synthesis of Ligand-Encoded Peptides and NWs.** Heterobifunctional ligand-encoded reporters R1–4 were synthesized by derivatizing the N terminus of glutamate-fibrinopeptide B-biotin (Glufib-biotin) (sequence, eGvndneeGffsar-biotin; lowercase, the N terminus of glutamate-fibrinopeptide B-biotin (GluFib-ligand-encoded reporters R1

S2 and Peptide). Peptide structure details may be found in Fig. S2F. Alexa Fluor 488-PEG-biotin (R4, PEG) was synthesized by reaction of AF488 maleimide (Invitrogen) with NHS-biotin (Pierce) and NH₂-PEG-thiol (5 kDa; Laysan) in a 10:10:1 dye:biotin:PEG ratio and purified using illus- trata NAP-25 columns (GE Healthcare). The resultant conjugates were purified by HPLC (Gilson). Reporter concentration was quantified by absorbance according to dye-specific extinction coefficients in a 96-well plate by plate reader (Molecular Devices SpectraMax Plus).

**In Vitro Protease Activity Assays.** Fluorescent-functionalized matrix metalloproteinase (MMP)- or thrombin-sensitive NWs (2.5 μM by peptide) were mixed in 1% (wt/vol) BSA (Sigma) with recombinant thrombin (15 nM; Haematologics Technology) or MMP9 (15 nM; Rockland Systems) in 100-μL final volume in a 384-well plate per manufacturer’s instructions, and fluorescent signal increase due to enzymatic release of homocoupled reporters was monitored at 37 °C (SpectroMax Gemini EM microplate reader). Argatroban (Sigma) or Marimastat (Tocris) were incubated with the NW–protease mixture at 100 μM final concentration. To test proteolytic reporter release by lateral flow assay (LFA), reporter-functionalized enzyme-sensitive NWs were incubated with MMP9 or thrombin as above at 37 °C for 4 h and passed through a 30-kDa Mₙ cutoff centrifugal filter. The filtered reporters were diluted to within LFA dynamic range and assayed by LFA as described below. Reporter stability experiments were performed using reporter 3 (1 μM) mixed in 1% (wt/vol) BSA with recombinant thrombin or MMP9 (both 15 nM) as above to 100-μL final volume and were incubated at 37 °C for 1 h. Following this, the reporters were passed through a 30-kDa Mₙ cutoff centrifugal filter as above and assayed by R3 ELISA.

**In Vivo Imaging.** All animal studies were approved by MIT’s committee on animal care (MIT protocol 0411-036-14). Synthetic biomarkers for in vivo imaging were prepared by reacting free amine groups on MMP- or thrombin-sensitive NWs (both on sub-strate N termini and on NWs) with VivoTag 750-NHS (Perkin-Elmer) and purified by FPLC.

Human LS174T colorectal cancer cells were grown in Dul-becco’s modified Eagle’s medium (ATCC) supplemented with 10% (vol/vol) FBS (Gibco) and 1% (vol/vol) penicillin-streptomycin (CellGro). Female NCr Nude mice (4–6 wk; Taconic) were inoculated s.c. with 5 × 10⁶ LS174T cells per flank and allowed to grow to ~0.5 cm³ total burden (volume = length×width×depth/2). Tumor-bearing and age-matched control mice were i.v. infused with 200 μL of VivoTag- and FAM-labeled MMP-sensitive NWs (1.67 μM by substrate), allowing visualization by an in vivo imaging system (IVIS) (Xenogen) 5–60 min postinfusion. For histology, mice were killed 1 h postinfusion. Tumors were removed, fixed in 4% paraformaldehyde, frozen in OCT (Tissue-Tek), sectioned, and stained with rat anti-CD3 (Santa Cruz), DAPI (Invitrogen), and goat anti-FAM (GeneTex) before imaging by fluorescence microscopy (Nikon Eclipse Ti).

To model thrombosis, female Swiss Webster (4–6 wk; Taconic) were coinfused with 200 μL of VivoTag- and FAM-labeled thrombin-sensitive NWs (0.84 μM by peptide), 10 μg/kg epinephrine (Sigma), and 280 μg/kg collagen (Chronolog). Fifteen minutes postinduction, mice were killed, and their lungs were inflated with PBS and excised. Infrared fluorescent imaging of lungs was taken using a LI-COR Odyssey infrared imager. Peptide substrates were PLGLRSW for thrombin and PLGVRGK for MMP (3).

**ELISA Characterization.** Mouse anti-fluorescein (GeneTex), rabbit anti-DNP and rabbit anti-AF488 (Invitrogen), and mouse anti-rodhamine (Rockland) antibodies were adsorbed to 96-well Bacti plates (Thermo) at concentrations of 0.4–0.8 μg/mL for 1 h in 1× PBS. Plates were then blocked for 1 h with 1× PBS with 1% (wt/vol) BSA (Sigma). Reporter standards were applied to blocked plates in twofold serial dilutions in 100-μL volume for 1 h to characterize assay linearity. To detect reporters, 100 μL of 0.4 μg/mL NeutrAvidin-HRP (Pierce) was applied for 1 h. Bound HRP was exposed with 50 μL of Ultra-TMB (Pierce) for 1–5 min followed by quenching with 50 μL of 1 M HCl. Between each step, plates were washed three times with 1× PBS with 0.5% (vol/vol) Tween 20 (Sigma). Absorbance at 450 nm was measured, plotted against known reporter concentration, and used to generate a linear fit over the assay’s linear absorbance region. Assay limit of detection (LOD) was calculated as 3 SDs above mean background signal.

To test interference due to urine, urine from untreated mice was added to R1 standard at a 1:100 dilution. To quantify assay specificity, reporter concentrations at the peak of each reporter’s linear region were applied to each of the four capture antibodies, and the ELISA was completed as normal. Signal for each of the four capture antibody types was quantified by comparison with a standard ladder and normalized to the maximal signal from reporters captured by their cognate antibody.

**Paper LFA Characterization.** Antibodies (same as above) were printed in lines spaced by 2 mm using 50-nL droplets at 0.5-mm pitch (Digilib MicroSysts) onto HiFlow Plus cellulose ester membrane (240 s/4 cm flow rate; Millipore). Control lines were anti-streptavidin antibody (Abcam) at 0.5 mg/mL, whereas reporter capture antibodies were the same as for ELISA and were applied at 1 mg/mL.
(α-R1, α-R3, α-R4) or 2 mg/mL (α-R2). Cellulose membrane (Millipore) was laminated to a plastic backing. Ten-millimeter glass fiber conjugate pad (Millipore) was laminated to the sample side of the cellulose membrane, and 20-mm cellulose fiber pads were laminated to both the sample side of the conjugate pad and the run-off end of the cellulose membrane. The resultant construct was cut into 4-mm strips that were stored at 4 °C.

Twofold dilutions of marker standards in 1× PBS with 1% (wt/vol) BSA with 1:1 control urine spiked in were applied to the conjugate pad and washed with 200 μL of wash buffer [1× PBS with 1% (wt/vol) Tween 80] on the sample pad. To detect the markers, 5 μL of streptavidin-conjugated gold nanoparticles (40 nm; BBI International) were applied to the conjugate pad and washed with an additional 200 μL of wash buffer. Test strips were allowed to dry and could be visualized by eye or applied to a scaling template and scanned (600 dpi; Epson V330 Photo) or imaged by cell phone (Fig. S3G only; Samsung Galaxy Nexus). Resultant images were loaded into MATLAB (MathWorks) and processed by a custom script that integrated signal over background across each antibody line. Marker orthogonality was characterized by comparing reporter capture by each antibody by applying a single reporter and quantifying signal over background noise across each antibody line. All strips were performed in at least triplicate.

**Collection and Analysis of Urinary Peptides.** Mice were i.v. infused with 200 μL of PBS with R4 [AF488-PEG-biotin; thrombosis model: 0.125 μM; colorectal cancer (CRC) model: 1 μM] as an injection control and either R2-functionalized MMP-sensitive NWs (1.67 μM by peptide; tumor volume, ∼0.5 cm³) or R3-functionalized thrombin-sensitive NWs (0.84 μM by peptide; thrombosis model). Immediately following infusion, mice were placed over 96-well plates enclosed by a cylindrical tube to collect urine for 30 min (thrombosis model) or 1 h (tumor model). Urine collection times were optimized from previous studies using these disease models (3, 4) and are dependent on site of disease and rate of enzymatic substrate cleavage. Urine was stored at −80 °C directly following collection.

Unprocessed urine was diluted (1:100–1:10,000) in 1× PBS with 1% (wt/vol) BSA, and reporters were quantified by ELISA (at least two replicates) using standards as described above. Urine was applied to lateral flow test strips in 5-μL volume, at 1:4 (thrombosis model) or 1:5 (CRC model) dilution. Lateral flow tests were performed in triplicate as described above and test strips were allowed to dry and were quantified by automated script as described above. ELISA and LFA data were analyzed using a Wilcoxon signed-rank test (CRC) and a Mann–Whitney test (thrombosis).

**Companion Diagnostic Cost Analyses.** Approximate costs for materials and labor costs to produce LFA were based on estimates from a technical document by LFA materials manufacturer Bangs Laboratories (5). The majority of costs are packaging and assembly, and the major variable costs are due to the specific antibodies used and region of manufacture, resulting in a raw material cost of roughly $0.60 and an assembled product cost of less than $2.

**Fig. S1.** Model system validation. Immunohistochemical staining of LS147T flank tumors 1 h postinjection of FAM-labeled NWs (Upper) or PBS (Lower). Staining of endothelial cells (CD31; red) indicates both colocalization of injected NWs (green) with blood vessels as well as extravasation of NWs into the tumor interstitium. Nuclei are counterstained blue with DAPI. (Scale bar: 100 μm.)
Fig. S2. Conventional sandwich ELISA validation. (A) Standard curve for reporter 1 (R1) diluted in 1:100 urine (black) or PBS (red). (B–D) Standard curves for R2–R4 diluted in PBS. (E) Analyses of linear regions of R1–4 standard curves. (F) Ligand-encoded reporter structures. All reporters were comprised of heterobifunctionalized peptides with the structure biotin-eGvndneeGffsarK(ligand) in which ligand$_2$ comprised one of four capture ligands (fluorescein, dinitrophenyl, tetramethylrhodamine, or Alexa Fluor 488). We chose the $\alpha$-stereoisomer peptide glutamate-fibrinopeptide B (eGvndneeGffsar) as a linker because it is biologically inert, stable, and efficiently filters into the urine (1, 2). Near-infrared reporters used for in vivo biodistribution studies (Fig. 2 C–E) made use of a modified reporter with VivoTag 750 substituted for biotin [VT750-eGvndneeGffsarK(FAM)]. (G) Stability of reporters to protease activity was assayed by exposing reporter 3 to MMP9 or thrombin for 1 h at 37 °C. ELISA quantification of recovered reporter demonstrated no significant differences between controls and enzyme-exposed reporters. Limit of detection (LOD) is defined as 3 SDs above mean background. Error bars are SEM. Means were compared using a nonparametric multiple-comparisons ANOVA (Kruskal–Wallis).


### Fig. S3. Paper LFA validation. (A–D) Standard curves for detection of R1–4 by paper LFA. Increasing dilutions of each reporter spiked 1:1 in urine were applied to each LFA, and test strips were imaged using a flatbed scanner and analyzed by an automated MATLAB script. (E) Statistics for linear regions of R1–4 standard curves. Limit of detection (LOD) is defined as 3 SDs above mean background signal. (F) Approximate costs for materials and labor costs to produce LFAs based on estimates from a technical document by LFA materials’ manufacturer Bangs Laboratories (1). The majority of costs are packaging and assembly, and the major variable costs are due to the specific antibodies used and region of manufacture. (G) The same automated analysis script was modified to quantify LFA band intensity using a 5-megapixel cellular phone camera (Samsung Galaxy Nexus). Analyses of decreasing concentrations of R1 on paper LFA performed using images from a scanner (black) or camera phone (red) produced indistinguishable results. Error bars are SEM.

Fig. S4. Conventional ELISA and paper-based detection of synthetic urinary biomarkers. (A) ROC curves for urinary clearance of synthetic biomarkers in our murine model of platelet-mediated thrombosis, as detected by ELISA. We coinjected mice \((n = 10)\) with R3-encoded thrombin-sensitive NWs, free R4, and either PBS or collagen/epinephrine and collected urine after 30 min. Both R3 (green, \(P = 0.00016\)) and the ratio R3/R4 (purple, \(P = 0.00016\)) demonstrated perfect discrimination \((\text{a.u.c.} = 1)\) of diseased from healthy animals by urine ELISA. (B) Receiver-operating characteristic (ROC) curves for urinary clearance of synthetic biomarkers in a flank tumor model of colorectal cancer. Urinary concentration of liberated R3 from MMP-sensitive NWs by ELISA discriminated disease with an a.u.c. of 0.96 \((P = 0.00051)\). Normalization of liberated R2 to free R4 resulted in an a.u.c. of 0.93 \((P = 0.0012)\). (C) Upon LFA interrogation of urine from mice with or without thrombosis, integrated intensity of R3 capture was higher in animals with thrombosis compared with controls \((P = 0.0089)\). (D) LFA band intensity of urinary free reporter R4 was not significantly different between control and thrombosis cohorts \((P = 0.12)\). (E) ROC curve analysis of discriminatory ability of our synthetic biomarkers detected in urine by LFA. Cleaved reporter R3 discriminated with an a.u.c. of 0.84 \((P = 0.010)\). Normalization of liberated R3 to free reporter R4 achieved an a.u.c. of 0.92 \((P = 0.0015)\). (F) LFA analysis of urine from the CRC model demonstrated increased urinary concentration of cleaved reporter R2 \((P = 0.027)\) in diseased animals versus controls, but no significant difference in urinary concentration of free reporter R4 \((P = 0.16)\) as expected from ELISA analysis of the same urine cohort. (G) ROC curve analysis of LFA quantification demonstrated an improved ability of urinary R2 \((\text{a.u.c.} = 0.82, P = 0.016)\) and the ratio R3/R4 \((\text{a.u.c.} = 0.90, P = 0.0025)\) to discriminate control from diseased animals compared with a random classifier. Box-and-whisker plots show extremes, quartiles, and median.