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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 immunooassays, 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.

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 trichinellosis (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.

Fig. 1. Protease-sensitive NPs for POC urinary monitoring of disease. (A) Synthetic biomarkers were synthesized by conjugating substrate–reporter tandem peptides to carrier iron oxide NWs. Proteolytic cleavage of the linking peptide substrate liberates ligand-encoded reporters that filter into urine. (B) (I) A patient suspected of harboring a disease receives a disease-tuned diagnostic nanoworm (NW) mixture. (II) NWs infiltrate the disease site and release reporters upon proteolytic cleavage of peptide substrates. Although intact NWs are too large to pass the glomerular basement membrane, liberated reporters passively filter through the kidney. (III) The patient collects a urine sample. (IV) Application of unprocessed urine to a low-cost POC paper lateral flow assay (LFA) enables diagnosis.

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 sites of disease are intravascular as in thrombosis, or when the broad-spectrum MMP inhibitor Marimastat (A) or MMP inhibitor Marimastat (B) abrogated fluorescent increase, as did use of d-isomer substrates. (C and D) Induction of thrombosis increased bladder (C) and lung (D) localization of near-infrared fluorophore-labeled thrombin-sensitive NWs over controls. (Scale bar: 5 mm.) (E) Intravenous injection of near-infrared fluorophore-labeled MMP-sensitive NWs resulted in increased bladder localization of reporters in tumor-bearing mice compared with controls. (F) NW mixture administration showed colocalization of blood vessels (CD31; red) and NW/reporters (fluorescein; green) and significant NW/reporter tumor infiltration. Nuclei were DAPI counterstained (blue). (Scale bar: 50 μm.)
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 d-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 from the vasculature (red) into 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. We 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 antibody to specifically bind only its cognate reporter. (Upper) Reported R1–R4 consist of a nondegradable spacer, GluFib, conjugated to a specific capture ligand on one terminus and a common detection ligand, biotin, on the other. Each reporter is captured by a paired antibody (α-R1–α-R4), and all are detected by NA-HRP. (B) Signal dilutions of R1 spiked in urine were captured on an α-R1-coated plate. Oxidation of chromogenic substrate by HRP (Lower) resulted in a linear detection range of ∼0.1–6 pM (upper, linear fit R² = 0.95). (C) A 96-well plate coated with four different capture antibodies visually demonstrated the ability of each capture antibody to specifically bind only its cognate reporter. (D) Quantification of marker specificity demonstrated that noise generated by other probes was below the LOD of the correct signal. Error bars are SEM.

Fig. 3. Detecting ligand-encoded reporters by sandwich complexes. (A) Reporters R1–R4 consist of a nondegradable spacer, GluFib, conjugated to a specific capture ligand on one terminus and a common detection ligand, biotin, on the other. Each reporter is captured by a paired antibody (α-R1–α-R4), and all are detected by NA-HRP. (B) Signal dilutions of R1 spiked in urine were captured on an α-R1-coated plate. Oxidation of chromogenic substrate by HRP (Lower) resulted in a linear detection range of ∼0.1–6 pM (upper, linear fit R² = 0.95). (C) A 96-well plate coated with four different capture antibodies visually demonstrated the ability of each capture antibody to specifically bind only its cognate reporter. (D) Quantification of marker specificity demonstrated that noise generated by other probes was below the LOD of the correct signal. Error bars are SEM.

Detection and detection agent; thus to construct the synthetic hetero-

Paper Assay Development and Detection of Protease Activity. First developed more than two decades ago to detect human chorionic 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 strip that is fabricated from a 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 and α-streptavidin antibodies to create 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. S3A–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
To determine the di-
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 from random classifier). (E) Control or tumor-bearing mice (n = 10) were administered free R4 and R2-encoded MMP-sensitive NWs. By ELISA, R4 clearance was not affected by disease state (Right, P = 0.92) and R2 was significantly higher in CRC-positive mice (Left, P = 0.0039). (F) The liberated to free reporter ratio (R2/R4) was increased in tumor-bearing animals compared with controls (P = 0.0098). (G) LFA analysis of the same urine demonstrated increased R2/R4 ratio in diseased mice (P = 0.0020). (H) LFA quantification of R2/R4 discriminated urine from CRC-positive mice with an a.u.c. of 0.90 (P = 0.0025 from random classifier). Box plots show extremes, quartiles, and median. ** P < 0.01, *** P < 0.001.
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, cutoff 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 LfA blocked with 1% (wt/vol) bovine serum albumin (BSA) in 1× PBS. Reporter standards were applied 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 h and plates were washed with 1× PBS with 0.5% (wt/vol) Tween 20 between steps. Urine interference was assayed by spiking R1 in 1:100 control mouse urine. Assay specificity was measured by quantifying capture antibodies on cognate antibody to all reporters and normalizing signal to a cognate reporter ladder.

Paper LFA Characterization. Capture (same as for ELISA) or control (α-strep-tavidin) antibodies were printed in 2-mm-spaced lines with 50-μl droplets at 0.5-mm pitch onto cellulose ester membrane. Membranes were laminated with a 0.5-mm pitch onto cellulose ester membrane. Membranes were laminated to a plastic backing with glass fiber conjugate and absorbent pads. The resultant construct was cut into 4-mm strips and stored at 4 °C. Reporters diluted 1:1 in urine were applied to the conjugate pad and flushed with wash buffer [1× PBS with 1% (wt/vol) Tween 80]. Reporters were detected using 40-nm streptavidin-gold nanoparticles. Dried strips were scanned and processed by a custom script that integrated and quantified band intensity.

Collection and Analysis of Urinary Peptides. Urine was collected from mice i.v. infused with synthetic biomarker mixtures (free R4 plus either R3-functionalyzed thrombin-sensitive NWs to detect thrombosis or R2-functionalyzed 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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