

MIT Open Access Articles

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

The MIT Faculty has made this article openly available. **Please share** how this access benefits you. Your story matters.

Citation: Warren, Andrew D., Gabriel A. Kwong, David K. Wood, Kevin Y. Lin, and Sangeeta N. Bhatia. "Point-of-Care Diagnostics for Noncommunicable Diseases Using Synthetic Urinary Biomarkers and Paper Microfluidics." *Proceedings of the National Academy of Sciences* 111, no. 10 (February 24, 2014): 3671–3676.

As Published: <http://dx.doi.org/10.1073/pnas.1314651111>

Publisher: National Academy of Sciences (U.S.)

Persistent URL: <http://hdl.handle.net/1721.1/91284>

Version: Final published version: final published article, as it appeared in a journal, conference proceedings, or other formally published context

Terms of Use: Article is made available in accordance with the publisher's policy and may be subject to US copyright law. Please refer to the publisher's site for terms of use.



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

Andrew D. Warren^{a,b,1}, Gabriel A. Kwong^{a,b,1}, David K. Wood^{c,1}, Kevin Y. Lin^{b,d}, and Sangeeta N. Bhatia^{a,b,e,f,g,h,2}

^aHarvard–Massachusetts Institute of Technology Division of Health Sciences and Technology, Institute for Medical Engineering and Science, Massachusetts Institute of Technology, Cambridge, MA 02139; ^bDavid H. Koch Institute for Integrative Cancer Research, Massachusetts Institute of Technology, Cambridge, MA 02139; ^cDepartment of Biomedical Engineering, University of Minnesota, Minneapolis, MN 55455; ^dChemical Engineering, Massachusetts Institute of Technology, Cambridge, MA 02139; ^eElectrical Engineering and Computer Science, Massachusetts Institute of Technology, Cambridge, MA 02139; ^fDepartment of Medicine, Brigham and Women's Hospital and Harvard Medical School, Boston, MA 02115; ^gBroad Institute of Massachusetts Institute of Technology and Harvard, Cambridge, MA 02139; and ^hHoward Hughes Medical Institute, Cambridge, MA 02139

Edited by Stephen R. Quake, Stanford University, Stanford, CA, and approved January 21, 2014 (received for review August 1, 2013)

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.

Author contributions: A.D.W., G.A.K., D.K.W., K.Y.L., and S.N.B. designed research; A.D.W., G.A.K., D.K.W., and K.Y.L. performed research; A.D.W., G.A.K., and D.K.W. contributed equally; A.D.W., G.A.K., D.K.W., and S.N.B. analyzed data; and A.D.W., G.A.K., D.K.W., and S.N.B. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

¹A.D.W., G.A.K., and D.K.W. contributed equally to this work.

²To whom correspondence should be addressed. E-mail: sbhatia@mit.edu.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1314651111/-DCSupplemental.

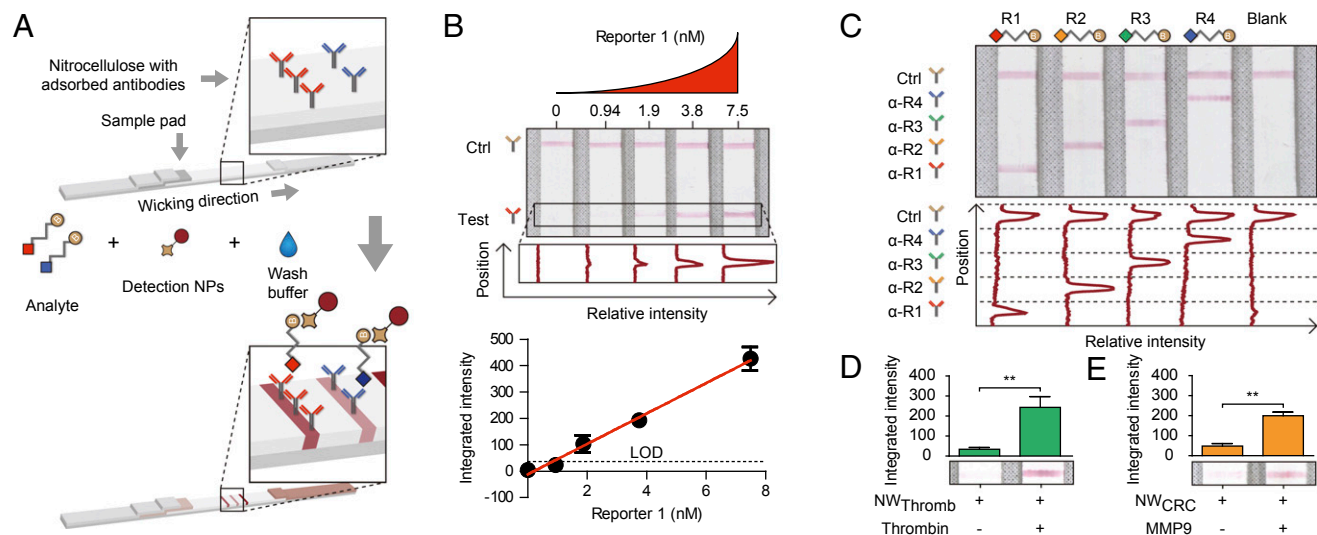


Fig. 4. Paper assay development and detection of protease activity. (A) Capture antibodies were adsorbed in spatially multiplexed lines on nitrocellulose membrane. The LFAs were developed by application of analyte, wash buffer, and streptavidin-gold detection NPs that wick past the capture antibodies 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.

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, $P = 0.0022$). Similar results were obtained when filtrate collected after incubation of R2-encoded MMP-sensitive NWs with MMP9 was analyzed by LFA (Fig. 4E, $P = 0.0022$). Together, these results demonstrated that the activity of distinct proteases can be detected by paper-based LFAs.

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. S4 C 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 diagnostic 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

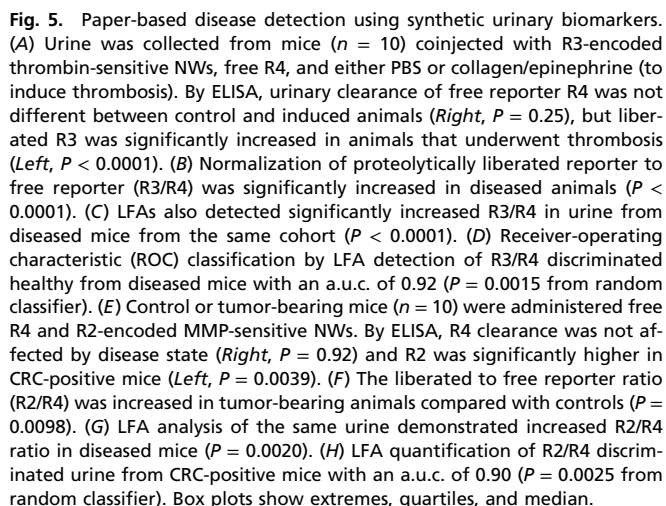
control mice accurately, with an area under the curve (a.u.c.) of 0.92 (Fig. 5D, $P = 0.0015$).

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



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

by plate reader at 37 °C. Inhibitors Argatroban or Marimastat were incubated with the protease-NW mixture at 100 μ M. To quantify reporter release by LFA, reporter-functionalized NWs were incubated with cognate proteases, passed through a 30-kDa M_r 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 and blocked with 1% (wt/vol) bovine serum albumin (BSA) in 1 \times 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 \times 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 specificity of each antibody to all reporters and normalizing signal to a cognate reporter ladder.

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. 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 \times 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-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 1:10² to 10⁴ 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).

ACKNOWLEDGMENTS. We thank Prof. B. Engelward for use of the robotic liquid handler, Dr. H. Fleming for helpful guidance, and J. Gómez-Márquez for LFA development insights. A.D.W. thanks the National Science Foundation Graduate Research Fellowship Program for support. D.K.W. acknowledges support by the Mazumdar-Shaw International Oncology Fellowship. G.A.K. acknowledges support from National Research Service Award F32CA159496-02 and holds a Career Award at the Scientific Interface from the Burroughs Wellcome Fund. K.Y.L. acknowledges support from Center of Cancer Nanotechnology Excellence Grant 5 U54 CA151884-03. We thank the Koch Institute Swanson Biotechnology Center for technical support, specifically Richard Cook and the Biopolymers and Proteomics Core. This work was supported in part by the Koch Institute Support (core) Grant P30-CA14051 from the National Cancer Institute. S.N.B. is a Howard Hughes Medical Institute investigator.

- Mathers CD, Loncar D (2006) Projections of global mortality and burden of disease from 2002 to 2030. *PLoS Med* 3(11):e442.
- Kanavos P (2006) The rising burden of cancer in the developing world. *Ann Oncol* 17(Suppl 8):i15, i23.
- World Health Organization (2011) *The Global Burden of Disease* (World Health Organization, Geneva).
- Yach D, Hawkes C, Gould CL, Hofman KJ (2004) The global burden of chronic diseases: Overcoming impediments to prevention and control. *JAMA* 291(21):2616–2622.
- Gutman S, Kessler LG (2006) The US Food and Drug Administration perspective on cancer biomarker development. *Nat Rev Cancer* 6(7):565–571.
- Hanash SM, Pitteri SJ, Faca VM (2008) Mining the plasma proteome for cancer biomarkers. *Nature* 452(7187):571–579.
- Brooks JD (2012) Translational genomics: The challenge of developing cancer biomarkers. *Genome Res* 22(2):183–187.
- Kwong GA, et al. (2013) Mass-encoded synthetic biomarkers for multiplexed urinary monitoring of disease. *Nat Biotechnol* 31(1):63–70.
- Lin KY, Kwong GA, Warren AD, Wood DK, Bhatia SN (2013) Nanoparticles that sense thrombin activity as synthetic urinary biomarkers of thrombosis. *ACS Nano* 7(10):9001–9009.
- Chin CD, Linder V, Sia SK (2007) Lab-on-a-chip devices for global health: Past studies and future opportunities. *Lab Chip* 7(1):41–57.
- Martinez AW, Phillips ST, Whitesides GM, Carrilho E (2010) Diagnostics for the developing world: Microfluidic paper-based analytical devices. *Anal Chem* 82(1):3–10.
- Yager P, et al. (2006) Microfluidic diagnostic technologies for global public health. *Nature* 442(7101):412–418.
- Pollock NR, et al. (2012) A paper-based multiplexed transaminase test for low-cost, point-of-care liver function testing. *Sci Transl Med* 4(152):ra129.
- Mudanyali O, et al. (2012) Integrated rapid-diagnostic-test reader platform on a cellphone. *Lab Chip* 12(15):2678–2686.
- Kim H-S, Ko H, Kang M-J, Pyun J-C (2010) Highly sensitive rapid test with chemiluminescent signal bands. *BioChip J* 4(2):155–160.
- Ellerbee AK, et al. (2009) Quantifying colorimetric assays in paper-based microfluidic devices by measuring the transmission of light through paper. *Anal Chem* 81(20):8447–8452.
- Posthuma-Trumpie GA, Korf J, van Amerongen A (2009) Lateral flow (immuno)assay: Its strengths, weaknesses, opportunities and threats. A literature survey. *Anal Bioanal Chem* 393(2):569–582.
- Zucker S, Vacirca J (2004) Role of matrix metalloproteinases (MMPs) in colorectal cancer. *Cancer Metastasis Rev* 23(1–2):101–117.
- Park J-H, et al. (2008) Magnetic iron oxide nanoworms for tumor targeting and imaging. *Adv Mater* 20(9):1630–1635.
- Park J-H, et al. (2009) Systematic surface engineering of magnetic nanoworms for in vivo tumor targeting. *Small* 5(6):694–700.
- Morris TA, et al. (2003) Urine and plasma levels of fibrinopeptide B in patients with deep vein thrombosis and pulmonary embolism. *Thromb Res* 110(2–3):159–165.
- Smyth SS, Reis ED, Väänänen H, Zhang W, Collier BS (2001) Variable protection of beta 3-integrin-deficient mice from thrombosis initiated by different mechanisms. *Blood* 98(4):1055–1062.
- Brand K, et al. (2000) Treatment of colorectal liver metastases by adenoviral transfer of tissue inhibitor of metalloproteinases-2 into the liver tissue. *Cancer Res* 60(20):5723–5730.
- Giljohann DA, Mirkin CA (2009) Drivers of biodiagnostic development. *Nature* 462(7272):461–464.
- Hermanson GT (2008) *Bioconjugate Techniques* (Academic, New York), 2nd Ed.
- Berenson A, Abelson R (June 29, 2008) The evidence gap: Weighing the costs of a CT scan's look inside the heart. *NY Times*, Section A, p 1.
- Rissin DM, et al. (2010) Single-molecule enzyme-linked immunosorbent assay detects serum proteins at subfemtomolar concentrations. *Nat Biotechnol* 28(6):595–599.
- Stern E, et al. (2010) A nanoelectronic enzyme-linked immunosorbent assay for detection of proteins in physiological solutions. *Small* 6(2):232–238.
- de la Rica R, Stevens MM (2012) Plasmonic ELISA for the ultrasensitive detection of disease biomarkers with the naked eye. *Nat Nanotechnol* 7(12):821–824.
- Harisinghani MG, et al. (2003) Noninvasive detection of clinically occult lymph-node metastases in prostate cancer. *N Engl J Med* 348(25):2491–2499.
- Heesakkers RAM, et al. (2009) Prostate cancer: Detection of lymph node metastases outside the routine surgical area with ferumoxtran-10-enhanced MR imaging. *Radiology* 251(2):408–414.
- Danielson BG (2004) Structure, chemistry, and pharmacokinetics of intravenous iron agents. *J Am Soc Nephrol* 15(Suppl 2):S93–S98.
- Choi HS, et al. (2007) Renal clearance of quantum dots. *Nat Biotechnol* 25(10):1165–1170.
- Mitragotri S (2013) Devices for overcoming biological barriers: The use of physical forces to disrupt the barriers. *Adv Drug Deliv Rev* 65(1):100–103.
- Yang W, Peters JL, Williams RO, 3rd (2008) Inhaled nanoparticles—a current review. *Int J Pharm* 356(1–2):239–247.
- Hori SS, Gambhir SS (2011) Mathematical model identifies blood biomarker-based early cancer detection strategies and limitations. *Sci Transl Med* 3(109):ra116.
- Lutz AM, Willmann JK, Cochran FV, Ray P, Gambhir SS (2008) Cancer screening: A mathematical model relating secreted blood biomarker levels to tumor sizes. *PLoS Med* 5(8):e170.
- Branson BM (2003) Point-of-care rapid tests for HIV antibodies. *Laboratoriums Medizin* 27(7–8):288–295.
- Chin CD, Linder V, Sia SK (2012) Commercialization of microfluidic point-of-care diagnostic devices. *Lab Chip* 12(12):2118–2134.
- Lee L, Nordman E, Johnson M, Oldham M (2013) A low-cost, high-performance system for fluorescence lateral flow assays. *Biosensors* 3(4):360–373.
- Thompson IM, et al. (2005) Operating characteristics of prostate-specific antigen in men with an initial PSA level of 3.0 ng/ml or lower. *JAMA* 294(1):66–70.