Towards Ultrasensitive, High-speed Diagnostics: Nanoscience Meets Health Care

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Nanomedicine Short Course
University of Minnesota
2019.06.06

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Acknowledgements

NIH:    R01AI111495    UG3CA211551    U01CA151650
        R21-EB016925
Kerith Foundation
Center on Aging (UofU)
Disease Diagnostics: Team Sport

- Goals and Challenges in Diagnostic Tests
- Nanodiagnosics - Surface-enhanced Raman Scattering (SERS)
- Sample Pretreatment
- Solid Phase MicroExtractions (SPME)
- What Are We Measuring Anyway?
Dave is a blue-collar worker in early stages of pancreatic cancer (PC), but is not aware of his affliction.

PC is the fourth most common cause of cancer deaths in men and women.

PC has a 4% five-year survival rate, and its onset is asymptomatic.

Dave has yet to exhibit clinically suspect signs and will not be diagnosed until there is obvious need to seek medical care.

Dave’s cancer is unfortunately at an advanced stage, and he is no longer eligible for tumor resection. He then receives palliative care, succumbing to his cancer within six months.
Early diagnosis is paramount

Stage-specific survival: 440 pancreatic ductal adenocarcinoma (PDA or simply PC) patients

Detection when tumor is small and confined to pancreas (IA+IB) improves survival rates.

Current staging does not optimally stratify patients.

Novel paradigms (multiplexing) needed for early diagnosis and for better treatment options.

Kaplan-Meier Survival Curve: University of Utah SOM and HCI
Relative distributions for PC diagnostic biomarkers

Large overlap of control (CON), chronic pancreatitis (ChPT) and pancreatic ductal adenocarcinoma (PDAC - PC) in patients.

Green lines indicate 95% specificity threshold.

Definitions and Binary Classifiers

**Analytical sensitivity:** slope of calibration plot

**Limit of detection (LOD):** analyte concentration with response equal to the blank signal plus $3 \times$ the standard deviation of the blank signal

**Clinical sensitivity:** fraction of infected people identified being infected
  It equals the number of TPs vs. number of TPs and false negatives (FNs).

  \[ \text{Sensitivity} = \frac{TP}{TP + FN} \]

**Clinical specificity:** fraction of uninfected people correctly identified uninfected
  It equals the number of True Negatives vs. number of TNs and false positives (FPs).

  \[ \text{Specificity} = \frac{TN}{TN + FP} \]
PC’s clinical sensitivity/specificity problem

Asymptomic - The Need to Test Everyone

- Population of 100M individuals -50 years or older tested for PDA
- Prevalence: ~4 in 10,000 or 40,000 in a population of 100M
- Assume 90% test sensitivity and specificity

<table>
<thead>
<tr>
<th>Binary Classifier: Condition</th>
<th>Positive Patients</th>
<th>Negative Patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive test outcomes</td>
<td>True positive (TP = 36,000)</td>
<td>False positive (FP = 9,996,000)</td>
</tr>
<tr>
<td>Negative test outcomes</td>
<td>False negative (FN = 4,000)</td>
<td>True negative (TN = 89,964,000)</td>
</tr>
<tr>
<td>Total</td>
<td>40,000</td>
<td>99,960,000</td>
</tr>
</tbody>
</table>

Sensitivity = TP/(TP+FN) = 90%
Specificity = TN/(FP+TN) = 90%
### The false positive problem

<table>
<thead>
<tr>
<th>100,000,000 Subjects Tested</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>4 in 10,000 Prevalence</strong></td>
</tr>
<tr>
<td>40,000 with PDA</td>
</tr>
<tr>
<td>99,960,000 without Disease</td>
</tr>
</tbody>
</table>

**Test Sensitivity & Specificity**

<table>
<thead>
<tr>
<th></th>
<th>TP</th>
<th>FN</th>
<th>Sensitivity</th>
<th>TN</th>
<th>FP</th>
</tr>
</thead>
<tbody>
<tr>
<td>*</td>
<td>36,000</td>
<td>4,000</td>
<td>90%</td>
<td>89,964,000</td>
<td>†9,996,000</td>
</tr>
<tr>
<td>†</td>
<td>39,600</td>
<td>400</td>
<td>99%</td>
<td>98,960,400</td>
<td>999,600</td>
</tr>
<tr>
<td>†</td>
<td>39,960</td>
<td>40</td>
<td>99.9%</td>
<td>99,860,040</td>
<td>99,960</td>
</tr>
</tbody>
</table>

* The false negative problem: patients fail to receive treatment

† The false positive problem: (1) severe patient stress

(2) $5.5$ billion annual cost for a single CT follow-up screen based on 2011 Medicare reimbursement rate of $554/CT
Target Attributes for Advanced Diagnostics

- Multiplexing
  - Everyone is different
  - Signal overlap at multiple addresses: cross reactivity and/or non-specific binding
  - One marker may be sufficient for some diseases (tuberculosis)
  - Multiple marker signature may be needed for other diseases (pancreas cancer)
  - Low limits of detection (LOD)
  - Sample volume: sample scarcity

- Instrumentation
  - Simple, cost-effective
  - Short time to results

- Complex sample matrices (more later)

The Gold Standard: ELISA

- Enzyme-linked Immunosorbent Assay (ELISA)
  - Antibody: specific capture of target antigen
  - Solid phase (sorbent): wash off of material not specifically captured
  - Enzymatic amplification: transforms capture into quantifiable visible color

Adapted from C. Roth, Rutgers University
SERS: Robust Hardware Platform

**Sensitivity**
Pico to femtomolar detection

**Multiplexing**
Raman bands 10-100 times narrower than fluorescence

**Hardware Simplicity**
Excitation wavelength is substrate-dependent

**Electromagnetic Enhancement** (dominant)

- Incident radiation is resonant with surface plasmon of conductors
  - plasma resonance: oscillation of conduction electrons
  - nanoparticles smaller than excitation wavelength

- Raman signal increased by $10^6$-$10^7$
- Enhancement decays by $d^{-10}$

SERS: Extensible Assay Platform

1. 20-µL sample
2. Rinse
3. 20-µL suspension of ERLs
4. Rinse
5. Measure Raman spectrum
Extrinsic Raman Label (ERL) Preparation

Self-assembled monolayer on Au
Scatterer close to Au surface
4.0 ± 0.2 × 10^4 RRM per particle
Red excitation for Au NPs

Intrinsically strong Raman scatterer: \( \nu_s(\text{NO}_2) \)
Small particle size dispersity
190 ± 20 mAbs per particle
SERS: Single Binding Event Detection

AFM: DSNB-coated ERLs tethered to aminoethanethiol adlayer on smooth Au

Cancer Diagnostics: Pancreatic Cancer (PC) Markers$^{1,2}$

LODS [ng/mL (pM)] for PC cancer markers in human serum

<table>
<thead>
<tr>
<th>MARKER</th>
<th>SERS</th>
<th>ELISA</th>
<th>SERS/ELISA</th>
</tr>
</thead>
<tbody>
<tr>
<td>CA19-9</td>
<td>0.040</td>
<td>1.10</td>
<td>~30</td>
</tr>
<tr>
<td>MMP-7</td>
<td>0.003</td>
<td>0.041</td>
<td>~15</td>
</tr>
</tbody>
</table>

Data for MMP-7 and CA 19-9 in patient samples.$^1$

<table>
<thead>
<tr>
<th>Specimen</th>
<th>Antigen</th>
<th>SERS</th>
<th>ELISA</th>
<th>SERS</th>
<th>ELISA</th>
</tr>
</thead>
<tbody>
<tr>
<td>DIDM199</td>
<td>MMP-7</td>
<td>3.53 ± 0.12 (3.42%)$^4$</td>
<td>3.91 ± 0.16 (4.24%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DIDM30</td>
<td></td>
<td>4.77 ± 0.12 (2.60%)</td>
<td>4.36 ± 0.26 (5.91%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DIDM17</td>
<td></td>
<td>1.92 ± 0.08 (3.97%)</td>
<td>2.79 ± 0.16 (5.90%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DIDM199</td>
<td>CA 19-9</td>
<td>50.4 ± 2.9 (5.7%)</td>
<td>30.7 ± 2.0 (6.40%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DIDM30</td>
<td></td>
<td>ND$^5$</td>
<td>ND</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DIDM17</td>
<td></td>
<td>ND</td>
<td>ND</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

MUC4 not detected with ELISA and Radioassays in sera, but is with SERS.$^2$

Sanura lives in a rural Kenya village, with her husband and 3 young children.

She has been complaining of general malaise and cough, finally traveling to the closest clinic 4 h away by bus to visit a care giver.

At the clinic, she provided a sputum sample for tuberculosis (TB) testing by acid fast bacteria (AFB) smear and culture.

Sputum microscopy was negative for AFB, and she was treated empirically with antibiotics for suspected pneumonia and returned home.

At two weeks, the culture grew Mycobacterium tuberculosis, but Sanura could not be located to begin proper treatment.

What can be done to give every Sanura a fighting chance?
Tuberculosis (TB)

- TB has killed more people than any other pathogen in history

- TB is the world’s deadliest infectious disease
  - 1.6 million deaths/year\(^1\)
  - 30% of people globally are infected with latent TB
  - Economic burden estimated at $1-3T in next 10 years\(^2\)

- WHO urges ban on TB blood tests (July 20, 2011)

- Diagnosis in epidemic regions relies on symptoms and sputum (phlegm) microscopy

- Today’s methods cannot detect components of the TB organism for reliable early diagnosis of active nad/or latent infection

- 15-20% of TB infections are extrapulmonary only

Today’s TB Diagnostics Landscape

**Sputum (Phlegm) Smear Microscopy**
- Fast and low cost, but poor sensitivity (20-80%)

**Culture**
- Gold standard, but causative agent of TB is slow grower

**Nucleic Acid (DNA) Amplification Tests (GeneXpert Mtb/RIF)**
- High sensitivity in sputum; ~120 min
- Ineffective for specimens from children and HIV patients due to extrapulmonary infection
- Cost: Instrument: $17,000 USD (actual: ~$65k USD)  
  Cartridge: $9.98 USD per test (actual: ~$100-200 USD)

**Bottom Line:** No all encompassing test for all forms of TB (pulmonary, extrapulmonary and latent) or for response to treatment

**Our Approach:** Markers of TB microorganism in blood serum and urine

## Ideal Attributes: Point-of-Need (PON) Diagnostics

### Assay
- Simple/no sample preparation (matrix agnostic)
- Short sample-to-answer (<5 min)
- Accessible Cost (1-2 days of economic income – goal of $2-3 USD per test)
- No refrigeration needed to store reagents - break cold chain
- Multiplexed detection (disease signature)

### Readout
- Lightweight: < 2 lbs (0.9 Kg)
- Battery or solar powered
- Ethernet or WiFi connectivity (telemedicine)
- Embedded software and database for Yes/No answer (easy-to-use)

### High Clinical Accuracy
TB Diagnostics Consortium

Colorado State University

The University of Utah

B&W Tek, Inc.

University of Utah School of Medicine

UGA College of Veterinary Medicine

London School of Hygiene & Tropical Medicine

National Jewish Health

CDC

UMDNJ New Jersey Medical School

Denver Health

University of Medicine & Dentistry of New Jersey

Level One Care for ALL
Biomarker: Mannose-capped Lipoarabinomannan (LAM)

- 17.5 kDa structure\(^1\)
- Phosphatidylinositol anchor with mannosyl backbone and arabinosyl side chains
- Unique to mycobacteria
- \(~40\%\) of cell wall composition (1.5\% total mass)
- Diagnostic potential
  - ELISAs (96-well microplate) & LFAs (DipStick)
    - Limit of Detection (LOD): \(~1\) ng/mL
    - LAM detection in urine\(^2\)
      » SN 13-93\%, SP 87-99\%

1. Chatterjee D. et al. JBC 1992, 267, 6228

LAM Image: Achim Treumann and Steve Homans
LAM spiked into Saline (PBS) Solution

Saline calibration run: 0.0 to 10.0 ng/mL LAM  LOD: 0.2 ng/mL

y = 1458x + 85  
R² = 0.9796
LAM Spiked into Blood Serum of Healthy Individuals

Serum calibration run: 0.0 to 10.0 µg/mL LAM  
LOD: 800 ng/mL
Pretreatment Method

pretreatment

centrifuge

supernatant to platform

pelleted agglutinate

: TB antigen (LAM)

: complexing agent

: other proteins

: denatured complexer

: denatured proteins
LAM Spiked into Blood Serum and Pretreated

Serum calibration run: 0.0 to 100.0 ng/mL LAM

LOD: 2 ng/mL
LAM Detection in TB Patient Serum

24 TB-positive subjects: adult, HIV-negative: 21/24
10 TB-negative subjects: adult, healthy: 10/10
Reducing the global burden of tuberculosis: the contribution of improved diagnostics

Box 1 | Key messages

- A rapid and widely available diagnostic for tuberculosis (TB) with \(\geq 85\%\) sensitivity for smear-positive and smear-negative cases, and 97% specificity, could save \(\sim 400,000\) lives annually.

- Ideally, new diagnostics for TB should require no electricity, refrigeration or access to clean water, and should be easy to use with minimal or no training. Test results should be available within 1 h.

- All the parameters examined in this study (test performance, speed and access) are important for achieving gains, and improvements can be realized in any of these areas. The best health outcomes result from implementing multiple solutions.
LAM detection in serum before/after serum pretreatment

- Signal plateaus at similar SERS intensities
- Spiked serum has a ~10 x dynamic range
- Spiked serum (our approach) has >100x dynamic range
- LOD after pretreatment is 250x lower than without pretreatment
What Next?

Ongoing/planned work:
A. Unravel complexation mechanism - LOD
B. Test Development and Automation
C. Test Validation (3200 specimens)
   • HIV positive/TB positive
   • HIV negative/TB positive
   • Serum/urine longitudinal studies
   • Patients treated with antibiotics
   • Pulmonary vs. Extrapulmonary
   • BCG vaccinated
C. Innovations for field deployment

Goal: Improved TB diagnostics could help save ~400,000 lives/yr

Distribution Challenge: Estimated PON distribution at ~80M tests/yr

Overall Goal: Add malaria and dengue fever [$36B (USD) in 2010]
Motivation for Point-of-Need Diagnostic Tests

- Liver cancer is the world’s 3rd most prevalent cancer.
- Patient survival compounded in Mongolia by limited access to preventative care.
- Nearly all HCC cases in Mongolia are referred to the National Cancer Center of Mongolia (NCCM) in Ulaanbaatar.
- ~90% of patients seeking HCC treatment at NCCM are late stage (i.e., tumors >2 cm) and not eligible for resection or other curative treatments.
- ~40,000 new cases in USA in 2017

### Proposed Panel Screen for PON Screening of Liver Cancer

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>Abbreviation</th>
<th>Function</th>
<th>Target Levels for Risk Assessment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alphafetoprotein</td>
<td>AFP</td>
<td>fetal glycoprotein that is increased in HCC</td>
<td>≥10 ng/mL</td>
</tr>
<tr>
<td>Lens-culinaris agglutinin binding Alphafetoprotein</td>
<td>AFP-L3</td>
<td>fucosylated variant of AFP that has a high affinity to Lens culinaris produced by malignant hepatocytes</td>
<td>&gt;10% of total AFP (1 ng/mL)</td>
</tr>
<tr>
<td>Des-Gamma-Carboxy Prothrombin</td>
<td>DCP</td>
<td>arises from an acquired defect in post-translational carboxylation of the prothrombin precursor in malignant hepatocytes</td>
<td>≥10 ng/mL</td>
</tr>
<tr>
<td>Core Antibodies to Hepatitis B Virus</td>
<td>HBV</td>
<td>small enveloped DNA virus that can lead to HCC</td>
<td>5 ng/mL</td>
</tr>
<tr>
<td>Core Antibodies to Hepatitis C Virus</td>
<td>HCV</td>
<td>small enveloped DNA virus that can lead to HCC</td>
<td>5 ng/mL</td>
</tr>
</tbody>
</table>
C-SPE: Technology to Monitor ISS Crew Health

Prior to analysis: membrane impregnation with colorimetric reagent and load in filter holder

Step 1: draw sample into syringe

Step 2: pass sample through disk

Step 3: acquire reflectance spectrum with portable spectrometer

Low LoDs for biocides in drinking water on ISS (2 ppb of iodine) in ~2 min from concentration strengths of SPE and high rates for reactions in confined domains.
### SERS and SPME

**STEP 1:** Sample drawn through membrane address by wicking action of absorbent pad, <10 s).

**STEP 2:** Biolyte captured by immobilized antibodies as sample flows through membrane.

**STEP 3:** Buffer rinse, followed by antibody-labeled ERLs to tag captured biolyte at an enhancement factor of $\sim 10^6$.

**STEP 4:** SERS signal measured with handheld Raman spectrometer at high accuracy and low limit of detection.

Total assay time with a little practice: $\sim 2$ min
R-SPE: AFP Directly from Human Serum (10 µL)

Bovine Serum Albumin as a blocking agent

LOD ~3 pg/mL (40 fM)

Why?
The Nuts and Bolts of R-SPE (SERS Version)

1. Binding affinity ($K_d = 1.67 \times 10^{-10}$ M)

2. SERS enhancement ($\sim 10^6$)

3. Concentration factor ($>100\times$)

4. “Blank” blank (immeasurable level of nonspecific adsorption)

5. Fast on (capture) rate coupled with reactions in confined submicron channels

6. Very slow off rate ($k_{\text{off}}: 2 \times 10^{-4}$ s$^{-1}$ or a $t_{\frac{1}{2}}$ of $\sim 60$ min)
Reactions in “Nanodomains” - Large Surface Area to Volume Ratio

Reaction rates enhanced up to 1000 times and more
