Engineering of Biosystems for Detection of *Listeria monocytogenes* in Foods

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Co-founders of Biovitesse include Michael Ladisch and Rashid Bashir;
Arun Bhunia is a consultant for Biovitesse

Outline

Introduction and Background: Goals
Rapid Cell Concentration and Recovery (CCR)
Membrane Systems: Fouling and bacterial capture
Mammalian cell receptor for capture of pathogens on biochip/biosensor surface
Microfluidics device design for pathogen detection: systems Integration of biochip functions
Conclusions/Next steps

Biochip Detection Process

100-250 ml fluid → 100 μl fluid (w. cells) → Biochip sensor → Readout

Sample 100 – 250 ml

Biochip Detection Process

Off-chip Concentration

Detection/ID

Data Analysis/Results

Electrical Detection of cell Growth

Automated Off-Chip Cell Concentration and Recovery - 1000X

~ 40 min

~ 30 min

~ 1-3 hr

Goal: Total Time less than < 4 hours

Bashir et al, 2004

Goals

Detect low levels of foodborne pathogens in complex and various foods and in quick and precise way
Achieve rapid sample preparation Scale-down of bioseparations couple to specific and rapid detection
Biochip: Buffers, Receptors, Devices Amplify, detect, identify pathogens Sample volumes of 100 μL at 10 cell level

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Benchmarks (Metrics)

Concentrate sample containing bacteria
Final concentration of $10^3$ to $10^4$ cells / mL
Final viable cell count on chip > 10 cells
Concentrate cells in 30 min
Process samples in 60 min
Maintain cell viability
Introduce samples on chip, detect cells in 3 hr

Membrane Concentration
Recognizing Role of Liquid Film

![Diagram]

-700 cells / ml * 50 ml
Syringe holder
Membrane filter
Liquid film
Assumption: 1 mg = 1 μl
Membrane retains 15 μl of liquid

Liquid film concentrates $10^5$ cells into a volume of 15 μl of liquid.
Concentration factor equivalent to $6.7 \times 10^6$ cells / mL.
Challenges: membrane fouling, recovering viable cells after concentration

Chen et al, 2005

Flat Membrane CCR: 100 mL sample volume

![Diagram]

Liu et al, 2005; Banada et al, 2007

Minimum Number of Cells in Sample for Recovery by CCR

![Graph]

To recover an initial cell number of (Log_{10} cfu/mL)

Banada et al, 2007

Larger Volumes = Higher Sensitivity

Flat membranes have limit of 120 mL before flow stops
Moderating loss of permeation rate (flux) and increasing throughput

1. Lipases and proteases may hydrolyze macromolecules
   believed to cause pore occlusion; improvement in permeability is small
2. High cross membrane fluid velocity
3. Low trans-membrane pressure drop

Cross flow membrane configurations: hollow fiber, flat membrane
Dead end filtration has limit of 120 mL. Cross flow an alternative option.

Cross Flow HF Microfiltration

- Liquid solution passes through the HF membrane. Particles retained on the inner HF membrane surface and module surface.
- Permeate flux decreases rapidly.
- A fouling layer build-up causes the system to plug up
Particle Transport

Φ = Particle volume fraction

\( u \) = axial velocity

\( v \) = transverse velocity

\( x \) = axial position

\( y \) = transverse position

\( D \) = hydrodynamic diffusion coefficient

\( \frac{\partial (u \Phi)}{\partial x} + \frac{\partial (v \Phi)}{\partial y} + \frac{\partial (D \frac{\partial \Phi}{\partial y})}{\partial y} = 0 \)

Boundary Conditions

- Boundary Condition at Membrane Wall:
  - Zero Particle Transport
  - Transverse Fluid velocity is a function of trans-membrane pressure and the cake layer
    \( \vec{v} = f(TMP, \delta) \mid_{\text{membrane}} \)
  - This function is dependant on many system parameters and is not well characterized for many systems

Sheer Induced Diffusion

Use the Navier-Stokes Equations to solve for the flow and pressure field.

Balance particle transport to solve for particle concentration and cake layer.

Particulates transport occurs via convection and shear induced diffusion.

Equations coupled because flux is function of trans-membrane pressure and cake layer.

Hollow Fiber Membranes

Same flowrates, much smaller cross-sectional area

Cross Flow System

Bacterial Recovery

Initial test was done with E. coli GFP

Use of Hollow Fiber System (HST)

cross-flow, 0.45 µm pore size

Done with hot dog massage

250 ml starting volume

2 filtration steps prior to concentration

50 to 100% recovery

Test milk, vegetables
Processing of Stomached Hot Dog

Homogenized Hot Dog Permeate Concentrate

Liu et al., 2007

Pressure, Permeate Volume vs. Time
220 mL Stomached Hot Dog Processed

<table>
<thead>
<tr>
<th>Initial Cell Concentration (cells/mL)</th>
<th>Pressure (PSI)</th>
<th>Leakage</th>
<th>Recovery viable cells</th>
<th>Total Captured Cells</th>
<th>% recovered E. Coli</th>
</tr>
</thead>
<tbody>
<tr>
<td>190</td>
<td>25-28</td>
<td>0.0</td>
<td>810</td>
<td>53 x 10^3</td>
<td>114</td>
</tr>
</tbody>
</table>

Cross flow hollow fiber

Able to process 250 mL or more
Homogenized hot dog
Dry milk
Vegetables (leafy matter)
Mechanisms being studied
Testing being carried out

McKinnis, Rodriguez et al., 2007

SEM Photos of Membrane Fouling:
with Baby Formula (contains fat)

Stainless Steel Membrane

- Stainless Steel Construction
- Smooth Inner Surface
- Low Adhesion
- High Chemical Resistance
- Small Pore Size 0.1 µm

Summary on Cell Concentration

Cell Concentration and recovery should result in 1000 To 10,000 Cells / mL

CCR for a single large volume is preferred over replicates of smaller proportions of the same volume

Cross flow membranes are able to process homogenized or stomached samples that block flat membranes
Mammalian cell receptor for capture of pathogens on sensor surface

Goal: to investigate if mammalian cell receptor can be used as a capture molecule for biosensor application

- **LAP** (~94 kDa), a membrane bound alcohol acetaldehyde dehydrogenase enzyme responsible for adhesion to mammalian cells
- Bi-functional protein: (i) Enzyme (ii) Adhesion
- N-terminal part is ALDH (acetaldehyde dehydrogenase)
- C-terminal end is ADH (alcohol dehydrogenase)
- Interacts with eukaryotic Hsp60 (chaperone protein)
- Hsp60 is present on mammalian cell surface

**Hsp60 (receptor) immobilization**

Bacteria

LAP

Bioreceptor

Biotin

Biotinylated Hsp60

Strepavidin

Sensor surface

**Silicon dioxide chips with microfluidic set up**

- Bacteria added: 5x10^7 cfu/well
- Incubate for 2 h at RT
- Wash and stain with propidium iodide
- Count under microscope (area: 130 x 98 μm)

**Comparison of MAb C11E9 and Hsp60-mediated capture of L. monocytogenes cells on silicon dioxide surface**

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Net binding (cell count/μm²)</th>
<th>Estimated total cell number/well</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hsp60</td>
<td>65.56 ± 25.26</td>
<td>1.11E+05 ± 6.96E+04</td>
</tr>
<tr>
<td>MAb C11E9</td>
<td>0.69 ± 13.18</td>
<td>1.03E+03 ± 2.38E+04</td>
</tr>
</tbody>
</table>

Koo et al (unpublished)
E-cadherin (InlA)-mediated capture of *L. monocytogenes* cells in microtiter plate

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>NET Binding (counts/image)</th>
<th>Initial CFU/well</th>
<th>Estimated total cells/well</th>
<th>% Capture</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>L. monocytogenes</em></td>
<td>110.93 ± 26.29</td>
<td>5.80E+07</td>
<td>2.01E+05 ± 4.75E+04</td>
<td>0.401 ± 0.095</td>
</tr>
<tr>
<td><em>L. ivanovii</em></td>
<td>79.46 ± 60.11</td>
<td>4.10E+07</td>
<td>1.44E+05 ± 1.09E+05</td>
<td>0.287 ± 0.217</td>
</tr>
<tr>
<td><em>L. innocua</em></td>
<td>4.92 ± 2.34</td>
<td>2.20E+07</td>
<td>8.40E+03 ± 6.22E+03</td>
<td>0.018 ± 0.017</td>
</tr>
<tr>
<td><em>L. welshimeri</em></td>
<td>1.92 ± 4.27</td>
<td>2.20E+07</td>
<td>4.40E+03 ± 7.75E+03</td>
<td>0.007 ± 0.015</td>
</tr>
<tr>
<td><em>L. seeligeri</em></td>
<td>4.72 ± 2.06</td>
<td>3.30E+07</td>
<td>8.52E+03 ± 5.35E+03</td>
<td>0.017 ± 0.011</td>
</tr>
<tr>
<td><em>L. grayi</em></td>
<td>2.52 ± 3.12</td>
<td>2.50E+07</td>
<td>4.55E+03 ± 5.65E+03</td>
<td>0.009 ± 0.011</td>
</tr>
</tbody>
</table>

Hsp60 mediated capture profile of *Listeria* cells on SiO2 surface (Selectivity)

**Surface LAP expression**

**Hsp60 binding assay**

Capture profile of various food-associated microorganisms on Hsp60-coated SiO2 surface (Selectivity)

(Inoculation concentration was ~10^7 CFU/well)

**EM10 MAb**

Secondary only

**Anti-LAP MAb**

**Anti-Hsp60 MAb**

**LAP expression assay**

**Flow cytometry**

**Microtiter plate assay**

*Koo et al. (unpublished)*

*Schubert et al. 2002. Cell 111:825*

*Burrkholder et al. unpublished*

*Burkholder et al. unpublished*

- The area for each image was 130 x 98 μm.
Application of Hsp60 for capture of Listeria on optical waveguide (Fiber Optic)

Next steps with Hsp60

- Determine capture efficiency on microfluidic chip with or without DEP
- Determine capture efficiency on SPR
- Determine capture efficiency with magnetic beads
- AFM to examine the binding strength/patterns
- Determine the interaction domains for LAP and Hsp60

Microfluidics device design for pathogen detection: systems integration of biochip functions
Overview of Biochip-based Detection Process

- Sample Off-chip
- Concentration
- Detection/ID
- Data Analysis/Results

- Water
- Food
- Air
- Body Fluids

- 100-250ml fluid
- 100ul fluid (w. cells)
- Biochip sensor
- Readout

~ 30 min
~ 1-3 hr
~ 1-3 hr

On-Chip vs. Automated Off-Chip

- Electrical Detection of cell Growth
- On-Chip Concentration: 1000X
- ~ 15 - 30 min
- Automated Off-Chip Cell Concentration And Recovery - 1000X
- ~ 1-3 hr
- On-Chip Concentration: 1000X

Outline

1. Brief review of prior work
2. PCR in the “Petri Dish on a Chip”
   a. Optical Detection
   b. Label-Free Electrical Detection
3. Future Work

Integrated BioChips for Study of Microorganisms and Cells

Lab on a Chip with microfluidics and micro/nanosensors

Outline of “PCR in Petri Dish”

Current Work

- PCR reaction with a 508 bp Listeria monocytogenes prfA gene.
- Calibration of the thin film temperature sensor on chip using LabView data acquisition.
- Design and realization of automatic thermal cycling on chip at low average power values.
- Development of a real time PCR protocol for Listeria monocytogenes on chip
- Direct electrical detection of PCR products

System and PCR Details

- Integration of a PMT (photomultiplier tube) module for fluorescence detection.
- 0.5µl/min flow rate at a 20Vpp DEP voltage with frequency 100 KHz in LCGB
- PCR reagents introduced, cells lysed at 95°C, and PCR cycling initiated
  - SYBR Green based PCR Assay
  - prfA 508 bp segment target for specific detection of LM
    - 5'CGGGATAAAACCAAAACAATTT3' and R-
      - 5'TGAGCTATGTGCGATGCCACTT3'

- Bhattacharya et al. 2007, Submitted
**PCR Thermal Cycling**

- Average power 2.5 W
- Total time 112 min for active cooling
- Times at each temperature can be reduced

**End Point PCR Detection Sensitivity**

**Specificity Trials Without DEP**

**Towards Label Free Electrical Detection of PCR Products**

- Polymerase Chain Reaction
  - Target molecule doubles every cycle

<table>
<thead>
<tr>
<th>Cycle #</th>
<th># of Molecules</th>
<th>Conc. (pM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>5 x 10^7</td>
</tr>
<tr>
<td>3</td>
<td>2</td>
<td>1 x 10^7</td>
</tr>
<tr>
<td>5</td>
<td>4</td>
<td>5 x 10^6</td>
</tr>
<tr>
<td>7</td>
<td>8</td>
<td>1 x 10^6</td>
</tr>
<tr>
<td>9</td>
<td>16</td>
<td>5 x 10^5</td>
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<tr>
<td>11</td>
<td>32</td>
<td>1 x 10^5</td>
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<td>13</td>
<td>64</td>
<td>5 x 10^4</td>
</tr>
<tr>
<td>15</td>
<td>128</td>
<td>1 x 10^4</td>
</tr>
</tbody>
</table>

- What is the minimum concentration of dsDNA molecules (e.g. 500bp) that can be directly detected in solution using impedance measurements ???

**Electrical Nature of DNA Molecules**

- DNA polarization (dipole effect)
  - Dielectric relaxation (Debye relaxation):
  - Counter ion movement: Rest | Z |
Dielectric Relaxation of DNA in Solution

Local changes of counter-ions around the phosphate ions
Reorientation of water molecules around the DNA

Counter-ions around the phosphate ions

Log Frequency
-4 -3 -2 -1 0 1 2 3 4 5 6 7 8 9 10

ε'
α relaxation
δ relaxation
γ relaxation

Dielectric Relaxation of DNA in Solution

Label free DNA detection in DI water

500 bp dsDNA
10⁹ #/µl dsDNA

ε increases with # or L, C increases, Z decreases
Detection limit in DI Water for 500bp DNA = 1e9 #/µl (1.33 nM)

DNA prepared by QIAquick Gel Extraction Kit, QIAGEN, Valencia, CA.

Label free DNA detection in PCR Solution

Extracted Solution Conductance
Extracted Solution Capacitance

Sample (PCR mix plus DNA template)
Control (PCR mix only)
1 µg starting concentration (3e8 bacterial cells)

Selectivity Measurements

Sample Control
Sample Control
1/Rsol (µmho)
Cdi (pF)

1.10E+03 1.15E+03 1.20E+03 1.25E+03 1.30E+03 1.35E+03
Cdi (pF)
0 cycle 30 cycles

Listeria monocytogenes
E. coli

Sample Control
Sample Control

Selectivity Measurements

1 µg starting concentration (3e8 bacterial cells)
Selectivity Experiment Summary (L. m. & E. coli):
- 10 - 12% increase in Cdi with L. m. template and L. m. prfA gene primer.
- 0.5% increase in Cdi with E. coli template and L. m. prfA gene primer.
- If primer-dimers formed, the increase in Cdi is about 9% in Cdi.
- We have to avoid primer-dimers and unspecific amplifications!
- The detection is real, i.e., Cdi will change whenever there are significant DNA molecules.

Detection Limits
- We used 1 μg of initial genomic DNA → 3e8 bacterial cells (we used 25µl solution) → ~1e7 #/µl in PCR mix
- 1e7 #/µl → after 30 cycle PCR → 1e10 #/µl
- 1e7 #/µl → 1e4 #/nl before 30 cycle PCR
- Use 1000 cells in 0.1nl
- Mechanical Filter
- On-Chip PCR and direct electrical detection

Next Steps
- Optimize real time PCR with fluorescence detection for reduced time
  - Also demonstrate for Escherichia coli (in progress) and Salmonella
- On-chip label-free electrical detection of PCR product in microfluidic device
  - Move to RNA detection so as to
    - lower the limit of detection
    - have the possibility of live/dead information about Bacterium.
- Isothermal RNA (TMA) fluorescence detection off and on chip
- Isothermal RNA amplification (TMA) electrical detection on chip
  - To obviate the need for thermal cycling requirement
  - To eliminate time loss in ramp up/down

Isothermal RNA Amplification
- Promoter primer binds to rRNA target
- Reverse transcriptase creates a DNA copy of the rRNA target
- The RNA - DNA duplex
- RNase H activity of the reverse transcriptase degrades the rRNA
- Primer 2 binds to the DNA and reverse transcriptase creates a new DNA copy
- Double stranded DNA template with a promoter sequence

Isothermal RNA Amplification
- RNA polymerase initiates transcription of RNA from the DNA template.
  - 100 to 1000 copies of RNA amplicon are produced.
- Promoter primer binds to each RNA amplicon and reverse transcriptase creates a DNA copy.
- RNA - DNA duplex
- RNase H activity of the reverse transcriptase degrades the rRNA.

Final Conclusions
- Cell concentration and recovery using large volumes demonstrated
- Processing of complex samples (baby milk, vegetables) begun
- Very promising receptor (hsp-60) identified for capture of Listeria monocytogenes
- Capture demonstrated on biochips and fiber optic biosensor
- PCR on a Chip with fluorescence detection of 60 cells
- Label-free electrical detection of PCR product on chip initiated