Fundamentals of Microfluidics with Applications in Biological Analysis and Discovery

Harvard Extension School
E155
Dr. Anas Chalah

04.19.16
E155 Final Project 2016
General Prospective
Design a lab-on-a-chip device for human breast milk sample analysis

Specific Goal
Integration of 5 different Human Breast Milk tests on one microfluidic chip/ Device

Rational
“Breast feed or not” kind of question
“If human breast milk came stamped with an ingredients label, it might read something like this: 4 percent fat, vitamins A, C, E and K, lactose, essential minerals, growth hormones, proteins, enzymes and antibodies”

Source: The New York Times
TOXIC BREAST MILK? By FLORENCE WILLIAMS  - Published: January 9, 2005
Quick Background

• Human Breast Milk has the perfect combination of **Proteins**, **Fats**, **Vitamins**, and **Carbohydrates**

• Human milk is ~ **85% water**

• Easy to digest

• Newborns feed 10 to 12 times in 24 hours

• Even if the mother’s diet is horrible, nutrients are “borrowed” from her body to make sure the breast milk is perfect for the baby
Quick Background

- Human Breast Milk has the perfect combination of **Proteins, Fats, Vitamins, and Carbohydrates**
- Mothers can breastfeed if they have a **cold** or **flu**
- Mothers should not breastfeed if they have **HIV**, active **untreated tuberculosis**
- Most **over the counter and prescription medications** are safe to take while breastfeeding
- Women who **abuse alcohol** (more than 2 drinks a day) should not breastfeed
The exact composition of human breast milk changes based on many factors including female diet and illness. This list presents some of the components found in human breast milk.
Project **SHOULD** Cover the Following Topics:

- **CONCEPT:** The choice of the analysis: Explain the rational behind each test/analysis you picked and any possible links between all tests – Explain what is already known about the tests you picked

- **CHIP DESIGN AND LAYOUT:** The integration of 5 different process on one chip and the order of the tests on the surface

- **FABRICATION:** The fabrication of the chip: Platform material, fabrication method, and needed components (micro valves, pumps, mixers, heaters, channels, incubation chambers, sensors … etc)

- **SIGNAL:** The detection methods: How would you combine the detection of different signals from 5 analytical process on one chip. Change in color, fluorescence, precipitation, change in viscosity, …etc

- **APPLICATION:** Possible applications and marketing: Describe the use of your designed device in real life - Targeted applications (disposable or reusable)
✓ **Short Introduction:**
It will be good if a mom knows when to …

✓ **Concept:**
Test A of breast milk is super important because it.. Test B is as important since..
Test C is linked to A and will offer so and so..

✓ **Chip Design and Layout:**
Test A is going to be put first because at this point the sample is…
The tested sample will be split into X different channels or it will flow in one channel and be treated sequentially

✓ **Chip Fabrication:**
The chip needs X reaction chambers for test B and C, X channels, X pumps to do so and so, and X heaters at location Z for test B… The chip itself is made of MMM since this material is..

✓ **Signal:**
Test A signal is going to be a rainbow color observed by naked eye, test B is going to be a change in so and so due to the following reaction..

✓ **Application and Market:**
Moms and kids will be happy 😊
Project Format: PDF file only!

Adhere to the following format:

- Short Introduction:
- Concept:
- Chip Design and Layout: (a drawing of the 5 test chambers)
- Chip Fabrication: (a drawing of the components on the surface)
- Signal:
- Application and Market:

Do not mix these sections together and write your project as a story.

The project should not exceed TEN pages, THREE of which need to be for the discussion of Chip design, fabrication and signal.
More Format Tips

Final project should be typed in **size 12 Arial** font

All pages should be **numbered**

Your **name** must appear on each and every page.

Again… Send your work ONLY in a .PDF format
Tuesday May xx\textsuperscript{th} 2016
Where to Send Your Final EMAIL

EMAIL your final project to:

achalah@seas.harvard.edu
pduane@seas.harvard.edu

You will receive a confirmation email no later than 24 hours after you sent your final

If you do not, please inquire by email
E155 CLASS GRADE FORMULA

Homework are worth 40% of the final grade

Midterm Exam is worth 30% of the final grade

Final Project is worth 30% of the final grade
Microfluidic for Biological Sensing
Any biochip fabrication method should take into account the following factors:

(1) Mild chemical fabrication conditions - Avoid denaturing of biomaterials

(2) Allow for large number of enzyme units to be immobilized on the surface

(3) Provide a favorable micro environment to maintain the enzymatic activity

(4) Provide a large surface area for the interaction between enzyme and substrate – Mass transport effect

(5) Signal should be detectable (strength, interference, background and noise)

(6) Storage consideration & reusability
What Do Biosensors Detect?

A Biosensor offers selective identification of ultra trace levels of variety of materials:

- **Biomedical** and **clinical** detection & diagnosis (blood, urine, tissue)
- **Environmental** and **agricultural pollution** (air, soil, and water)
- **Biological** microsystems (bacteria, virus) and **microbial contamination**
- **Industrial / mining gases and liquids** - analysis and detection
- **Food industry** - flavors and essences
- **Toxic chemicals**
- **Biodefense**
Building a Biosensor

To build a Biosensor, what kind of questions should you ask?

Question (1):
Components: What are the building blocks

Question (2):
Method of assembly: How to put components together

Question (3):
Detection mechanism: What is the outcome (generated signal)

Question (4):
Applications
A biosensor is an analytical device "natural or manmade"

Designed for the detection of an analyte by combining a biological active sensing component with a physical or chemical reporting component that could be translated into a quantifiable electrical signal

Biosensor = Receptor + Detector (transducer)

http://www.tms.org
The (bio)catalyst converts the substrate to product. This reaction is detected by the transducer which converts it to an electrical signal. The output signal from the transducer is amplified by the amplifier. The signal is received and analyzed by the processor. The signal is displayed on the device monitor.

http://www.lsbu.ac.uk/biology/enztech/biosensors.html
The Detector/Transducer of a Biosensor

The detector (transducer) is a nonselective component that translates the physical or chemical change produced by analyte recognition and converts it into an electrical signal.
Examples of detectable changes

- Heat release or absorption by the reaction (Calorimetric biosensors)
- Electrical potential production by the changes in the distribution of charges (Potentiometric biosensors)
- Light release (Emission) during the reaction
- Light absorbance difference between the reactants and products (Optical biosensors)
A biosensor owes its high selectivity to its receptor component.

The receptor component could be:

(a) Enzyme
(b) Chemoreceptor
(c) Antibody

All are proteins
Why Consider Proteins for Sensors

Proteins have properties ideal for biosensing engineering purposes:

- Sophisticated structure at nanoscale dimensions
- Very rich chemistry
- Versatile enzymatic activities
- Structure easy to genetically engineer, modify, extend or shorten
**Chemical Sensing:**

*E. coli* (as well as many other single-cell or multi-cellular organisms) direct their movements according to the detection of certain chemicals in their environment.

This phenomena is called **CHEMOTAXIS**

Chemotaxis is the process by which a migrating (or swimming) cell moves toward a higher concentration of an attractant gradient or lower concentration of a repellent gradient.
Bacteria such as *E. coli* can sense a variety of substances:

- Amino acids
- Sugars
- Dipeptides
- Temperature
- Oxygen
- pH

Several major chemoreceptors have been discovered:

- Aspartate receptor (Tar)
- Serine (Tsr)
- Dipeptide (Tap)
- Ribose and Galactose (Trg)
Chemoreceptors are dimers anchored in the inner membrane of the bacteria

~ 38 nm in length and 2.5 nm in diameter

They transmit signals from the periplasmic ligand-binding domain to the cytoplasmic part of the receptor

The end of the receptor is where two other proteins (CheW and CheA) bind to the receptor and form the signaling complex
In **Green** are components that induce **clockwise** motor rotation = tumbling

In **Red** are components that induce **counterclockwise** rotation = smooth swimming

J. S. Parkinson Journal of Bacteriology Mar 2003 1492-1494
Amperometric biosensors rely on an enzyme system that converts electrochemically non-active analytes into products that could be oxidized or reduced at the chip surface.

The electrode is maintained at a specific potential with respect to a reference electrode.

The produced current is linearly proportional to the concentration of the electroactive product.

The concentration of electroactive product is proportional to the non electroactive enzyme substrate which in turn is a clear indication of the activity of the enzyme.

Enzymes used in amperometric biosensors are typically oxidases that catalyze the following class of reactions:
Detection of Enzyme Inhibition

Substrate + O₂ ----(enzyme/catalyst)-----→ Product + H₂O₂
(non electroactive) (electroactive)

Substrate concentration can be determined by amperometric detection of O₂ ↓ or H₂O₂ ↑

Remember: This reaction is coupled to a specific enzyme and an electrode

Example: Acetylcholinesterase is coupled to an amperometric sensor used to detect hydrogen peroxide
Steps:
(1) AChE catalyzes the hydrolysis of acetylcholine into Acetate and Choline
(2) Choline is broken in the presence of oxygen to produce Hydrogen Peroxide
(3) A sensor platform would promote the electron transfer generated from the chemical reaction

Concept:
AChE inhibition is monitored by measuring current generated by product oxidation (Choline)

AChE = Acetylcholinesterase
Cho = Choline Oxidase

Question: How could we use such a biosensor?
When a functioning neuron is stimulated, it releases a neurotransmitter molecule (Acetylcholine) over a synapse to transmit the impulse to a muscle.

**Acetylcholine = Neurotransmitter**

Once the electric nerve impulse is sent, a subsequent hydrolysis (breaks down) of Acetylcholine is required.

Specific enzyme Acetylcholinesterase (AChE) breaks down Acetylcholine into Acetate and choline - both are non-reactive substances.

Therefore the muscle is set to relax.

In case of additional need of muscle tension, the neuron releases more acetylcholine.

http://www.anaesthesiakuk.com
Nerve agents disrupt the nervous system by inhibiting the enzyme \textit{AChE} (acetylcholinesterase).

Such agents form \textit{covalent bonds} with a particular \textit{serine} residue in the active site cavity of \textit{AChE} enzyme.

This specific serine site is where acetylcholine normally undergoes hydrolysis.

With the enzyme inhibited, acetylcholine builds up in the human synapse and continues to act so that any nerve impulses are continually transmitted.

Nerve agents stop the brain from sending nerve messages down the spinal cord within a very short time (~ 30 seconds).

Example: \textit{Sarin} and \textit{VX} work similarly through inhibiting the enzymatic function of Acetylcholinesterase.

Hermona Soreq & Shlomo Seidman \textit{Nature Reviews Neuroscience} 2, 294-302 (April 2001)
Sarin is an extremely toxic substance whose sole application is as a nerve agent.

\[ \text{C}_4\text{H}_{10}\text{FO}_2\text{P} = \text{O-isopropyl methyl phosphonofluoridate} \]

Colorless, Odorless, Highly toxic

Classified as a **weapon of mass destruction** by the United Nations in UN Resolution 687

Production of Sarin was outlawed by the Chemical Weapons Convention of 1993
Sarin disrupts the nervous system by inhibiting the Acetylcholinesterase enzyme.

Sarin forms a **covalent bond** with a particular **serine** residue in the enzyme.

This serine residue site is where acetylcholine normally undergoes **Hydrolysis**.

With the enzyme inhibited, **acetylcholine builds up** in the human synapse and continues to act so that any **nerve impulses are continually transmitted**.
Threat = Need for Detection

EARLY DETECTION = fast indication of terrorist activity + quick response for activating proper procedures

Samples and locations:
Environmental (water, air, soil,..) and biological samples (blood, urine,..) in public places, workplaces, and individual exposures

Current analysis methods:
Analytical techniques: Gas or Liquid Chromatography or Mass Spectrometry

Tests are performed at centralized laboratories

Requires technicians, analytical resources, sample preparation, and time

L.O.C are designed for rapid analytical in-field analysis with a real-time output
Goal:
Detect the activity of AChE enzyme

Nanobiosensor device:
A sandwich-like structure on the surface of CNTs formed by self-assembling of layer-by-layer (PDDA/AChE/PDDA)

Components:
Enzyme: Acetylcholinesterase (AChE)
Support Material: PDDA (Poly Diallyl Dimethyl Ammonium Chloride)
Substrate: Toxic agent – Inhibitor
Transducer: Carboxyl-functionalized Carbon nanotube

Assembly:
AChE is immobilized on the negatively charged CNT surface by assembling one AChE layer sandwiched in between two cationic PDDA layers
In general, The evaluation of sensor performance with respect to operating conditions includes:

(1) Inhibition time
(2) Detection range and limits
(3) Regeneration conditions and baseline analysis

**Example:** (Guodong Liu & Yuehe Lin - 2006)

Under the operation conditions:
(1) Described nanobiosensor had ~ 6 min inhibition time
(2) Measure as low as 0.4 pM of paraoxon (toxic agent)
(3) Had an operational stability with no decrease in the activity of enzymes for more than 20 repeated measurements over a 1 week period
Nanostructures in Biochips

SEM images of MWCNT nano electrode arrays:

(a) 3x3 electrode array
(b) array of MWCNT bundles on one of the nine pads
(c) And (d) arrays of MWCNTs at UV lithography and e-beam patterned Ni spots, respectively

Scale bars are 200, 50, 2 and 5 µm, respectively

In this nanobiosensor, CNTs play a dual role as:

(1) **Support platform** for enzyme (AChE) immobilization

(2) **Signal transducer** which amplifies the electrochemical signal generated by the product of the enzymatic reaction

**Fabrication Steps:** (Sandwich format)
(a) Assembly of positively charged PDDA on negatively charged CNT
(b) Assembly of negatively charged AChE
(c) Assembly of the second PDDA layer on top

Thickness of one PDDA/AChE/PDDA layer is ~9 nm

Guodong Liu and Yuehe Lin
Anal. Chem. 2006, 78, 835-843
Rational for the Sandwich Design

Among the sited drawbacks of physical adsorption, direct immobilization and entrapment:

(1) **Non-uniform coating**: Difficulties to achieve uniform enzyme distribution on the surface
(2) **Stability**: covalent binding reactions tends to partially denature the activity of the AChE

Tested acetylcholinesterase inhibitor: **Paraoxon**
Paraoxon = It is an organophosphate that is used as a pesticide
Example: 0.1 mg/kg paraoxon significantly inhibited AChE activity

Moslem Mohammadi *et al.*, 2008
*(Toxicology Vol. 244, Issue 1, 3 February 2008)*
CNT Biosensor Preparation

Step (1) Functionalization:
(a) Multiwall CNTs are functionalized by sonication in a mixture of HNO₃ and H₂SO₄ (v/v, 1:3) for 6 hours followed by washing in water until the filtrate is neutral
(b) The pH is adjusted to 8.0 to achieve net negatively charged –COO⁻ residues
(c) The negatively charged CNT is centrifuged for 30 min to eliminate any reagent leftovers

Step (2) Immobilization and Sandwich Formation:
The positively charged PDDA are adsorbed by dipping the negatively charged CNT in an aqueous solution of PDDA. Then, the PDDA/CNT structure are rinsed with distilled water and dried in nitrogen. A layer of negatively charged AChE is adsorbed to the surface. Another PDDA layer is adsorbed on the top of the AChE layer using the same procedure

TEM images of PDDA/AChE/PDDA/CNT hybrid material at low (A) and high magnification (B)
**Detection Chemistry**

\[
\text{Acetylcholine} + O_2 + H_2O \rightarrow \text{Choline} + \text{Acetate}
\]

\[
\text{Choline} + O_2 + H_2O \rightarrow \text{Betaine aldehyde} + H_2O_2
\]

**AChe** = Acetylcholinesterase  
**Cho** = Choline Oxidase

\[
\text{Acetylthiocholine} + H_2O \rightarrow \text{thiocholine} + \text{acetate acid}
\]

Acetylthiocholine (ATCh) is the substrate  
Acetylcholinesterase (AChe) is the Enzyme  
Thiocholine (TCh) is the product

**HYDROLYSIS**
TCh Oxidation Reaction = Electric signal :

(1) Acetylthiocholine is enzymatically hydrolyzed by AChE to TCh
(2) Thiocholine is oxidized (at a constant potential) at the electrochemical transducer CNT, producing the initial biosensor electric response

Paraoxon is used as the toxic agent: An organophosphate cholinesterase inhibitor that is used as a pesticide

**Question:** What would happen to the electric signal when Paraoxon is present, increase or decrease and why?
The electrochemical response of the CNT/PDDA/AChE/PDDA biosensor to the substrate Acetylthiocholine (ATCh) under the flow injection system appears to be sensitive and stable making it suitable for toxic agent monitoring (Paraoxon).
Signal Generation (before and after exposure)

The biosensor response before and after exposure of the AChE to paraoxon

(1) Two injections of 10 µL of 2 mM ATCh substrate produce the initial enzyme activity (peaks 1 and 2)

(2) Then 10 µL of 10⁻⁸ M paraoxon was injected into the cell, and the flow was stopped for 6 min (incubation)

(3) Reinjection of the 10 µL of 2 mM ATCh shows a significant decrease in enzyme activity (peaks 3 and 4)

(4) A strong wash with 0.1 mM PAM and 10 mM ATCh for 2 min, respectively, resulted in recovery of enzyme activity (peaks 5 and 6)

(5) Reincubation of the biosensor with 10 µL of 10⁻¹⁰ M paraoxon caused a decrease again in the activity (peaks 7 and 8)

(6) The relative decrease in the activity at the second inhibition is less than that obtained in the first inhibition (Why)

(7) After the second regeneration step, the activity of AChE was recovered again (peaks 9 and 10)

REMEMBER:
The enzyme Acetylcholinesterase (AChE) is immobilized
The substrate Acetylthiocholine (ATCh) is injected
Thiocholine oxidation = signal

Sarin Detection Concept

The detection concept is based on the **inhibition reaction** between the nerve agent and an enzyme.

**First:** The enzyme activity is tested by hydrolysis of a known substrate (**Chromophore**) (the substrate is broken down by reaction with water) and the extent of the hydrolysis is measured with an attached chromophore.

**Second:** The extent of Sarin binding is measured by the change in the hydrolysis signal (drop or increase?)

Example of LOC device made by a team from **Singapore**

The device allows early detection of Sarin exposure by first responders to a terrorist strike.

(Example: 1995 Tokyo subway Sarin attack)

Hsih Yin Tan et al., 2008
Nanyang Technological University & DSO National Laboratories, Singapore
Schematic Approach for Blood Sample Analysis (no Sarin yet !!)

First: blood sample is collected and blood cells are burst open

Second: blood proteins and other particulates are filtered out

Third: The purified mixture is mixed with a substrate and a chromophore, and passed over an immobilized enzyme

Third: If the sample contains no sarin, the enzyme reacts with the substrate, the chromophore is activated and detected by an external UV-visible spectrometer

Hsih Yin Tan et al., 2008
Sarin Detection by L.O.C.

- Stage (A): nerve agent is regenerated from whole blood. An aliquot of whole blood enters the device at inlet 1 and is mixed with a nerve gas regeneration agent entering at inlet 2. The regeneration reaction is performed by a micromixer.

- Stage (B): Cell lysing and introduction of a protein precipitation chemical into the sample followed by filtration of precipitated blood proteins.

- Stage (C): Chamber with packed beads for further particle trapping.

- Stage (D): The tested sample is mixed with a substrate for the enzyme-based hydrolysis reaction and a chromophore to indicate the enzyme activity.

- Stage (E) The detection chamber.

Hsih Yin Tan et al., 2008
Color or no color: If nerve agent exists in the blood sample, the enzyme is inhibited, hence hydrolysis of substrate is prevented and chromophore remained unconverted.

As a result, the sample entering stage (E) is almost colorless.

**Sarin = Color disappearance**

Baseline: Before testing the blood sample, a baseline measurement is carried out with the substrate, the chromophore and the enzyme only. The color is determined by an absorbance measurement at the wavelength of 412 nm.

Enzyme Protection: During chip storage, the immobilized enzyme (in chamber D) is protected by a coating layer for a longer shelf-life.

The coating layer is removed with an extra washing step before the measurement.
Schematic vs. Real

(a) Nerve gas regeneration reactor
(b) Cell lysing and filtering
(c) Removal of cellular debris
(d) Inhibition reactor (immobilized Acetylcholinesterase)
(e) Optical detection

This LOC device was fabricated in PMMA (polymethylmethacrylate) using CO$_2$-laser micromachining.

Hsih Yin Tan et al., 2008