Amperometric biosensors in flow injection analysis: silver amalgam-based transducers coupled to replaceable and reusable enzymatic mini-reactors

Sofiia TVORYNSKA¹,², Jiří BAREK², Bohdan JOSYPČUK¹

¹ J. Heyrovský Institute of Physical Chemistry of the Czech Academy of Sciences, Dolejskova 3, 182 23 Prague 8, Czech Republic
² Charles University, Faculty of Science, Department of Analytical Chemistry, UNESCO Laboratory of Environmental Electrochemistry, Hlavova 2030/8, 128 43 Prague 2, Czech Republic
AMPEROMETRIC ENZYME-BASED BIOSENSOR:

Bioresognition part → enzyme - oxidoreductase

substrate + enzyme_{ox} → product + enzyme_{red}

enzyme_{red} + O_2 → enzyme_{ox} + H_2O_2

detection part → working electrode

- monitoring of the enzymatically consumed O_2 via its reduction (e.g., Clark electrode)
- monitoring of the enzymatically produced H_2O_2 via its oxidation (e.g., GCE)

Literature overview

Biosensor of classic pen-type design in batch configuration

Two challenges:

- low stability and short lifetime
- possible interference effect

Biosensing platform in flow injection analysis - conceptualization

- biorecognition (1 enzymatic mini-reactor) and detection (2 silver amalgam-based transducer) parts are spaciously separated
- use of a silver amalgam-based transducer for amperometric monitoring of oxygen consumption by its four-electron reduction at a highly negative detection potential

**Uric acid biosensor**
1. LOx-based mini-reactor
2. Tubular detector of silver solid amalgam (TD-AgSA)

**Choline biosensor**
1. ChOx-based mini-reactor
2. Silver solid amalgam electrode covered by mercury film (MF-AgSAE)

**Acetylcholine biosensor**
1. ChOx-based mini-reactor
2. AChE-based mini-reactor

**Lactic acid biosensor**
1. Silver amalgam-based SPE (AgA-SPE)
2. LOx-based mini-reactor
Biosensing platform in flow injection analysis - conceptualization

- biorecognition (1 enzymatic mini-reactor) and detection (2 silver amalgam-based transducer) parts are spaciously separated
- use of a silver amalgam-based transducer for amperometric monitoring of oxygen consumption by its four-electron reduction at a highly negative detection potential

**Detection part:**

2 Electrochemical reaction at silver solid amalgam transducer (TD-AgSA/MF-AgSAE/AgA-SPE):

\[
O_2 + 2H_2O + 4e^- \rightarrow 4OH^- 
\]

**Biorecognition part**

1 Enzymatic reaction in oxidoreductase enzyme-based mini-reactor:

substrate + enzymeox \rightarrow product + enzymered

enzyme_{red} + O_2 \rightarrow enzyme_{ox} + H_2O_2

**Uricase-based mini-reactor:**

uric acid + O_2 + H_2O \rightarrow allantoin + CO_2 + H_2O_2

**Lactic acid oxidase-based mini-reactor:**

lactic acid + O_2 + H_2O \rightarrow pyruvic acid + H_2O_2

**Acetylcholinesterase-based mini-reactor:**

acetylcholine + H_2O \rightarrow acetic acid + choline

**Choline oxidase-based mini-reactor:**

choline + 2O_2 + H_2O \rightarrow betaine + 2H_2O_2
### Importance of determination

<table>
<thead>
<tr>
<th>Clinical analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Uric acid (UA)</strong></td>
</tr>
<tr>
<td>UA &gt; 4.4 mM in urine or &gt; 0.52 mM in blood:</td>
</tr>
<tr>
<td>goat kidney disease</td>
</tr>
<tr>
<td><strong>Choline (Ch)</strong></td>
</tr>
<tr>
<td>Ch &lt; 7.0 μM in blood:</td>
</tr>
<tr>
<td>neurological diseases liver disorders complications during pregnancy</td>
</tr>
<tr>
<td><strong>Acetylcholine (ACh)</strong></td>
</tr>
<tr>
<td>ACh (neurotransmitter) &lt; 6.0 μM in serum:</td>
</tr>
<tr>
<td>a biomarker of Parkinson’s and Alzheimer’s disease</td>
</tr>
<tr>
<td><strong>L-Lactic acid (LA)</strong></td>
</tr>
<tr>
<td>LA &gt; 0.2 mM in saliva or &gt; 2.0 mM in serum:</td>
</tr>
<tr>
<td>sepsis liver disorders cardiovascular diseases</td>
</tr>
</tbody>
</table>

**Pharmaceutical industry**

**Sport medicine**

**Winemaking**

**Food industry**
Advantages of the AgSAEs:

- a wide potential window
  \(-2.07 \ldots -0.06\) V in 0.1 M NaOH at MF-AgSAE
- mechanically stable and suitable for flow systems
- simple, low-cost preparation and easy miniaturization
- easy renewability of their surface (electrochemically / polishing)
- environmentally friendly

Oxygen reduction at liquid mercury electrodes and amalgam electrodes (neutral or alkaline medium):

I. \(\text{O}_2 + 2\text{H}_2\text{O} + 2\text{e}^- \rightarrow \text{H}_2\text{O}_2 + 2\text{OH}^-\) \((-100\) mV vs. SCE\)

II. \(\text{H}_2\text{O}_2 + 2\text{e}^- \rightarrow 2\text{OH}^-\) \((-900\) mV vs. SCE\)

\(\text{O}_2 + 2\text{H}_2\text{O} + 4\text{e}^- \rightarrow 4\text{OH}^-\)
Detection part: silver amalgam-based transducers

(1) Tubular detector of silver solid amalgam (TD-AgSA)
- simple, robust, inexpensive construction
- providing repeatable measurements
- lower sensitivity (compared to MF-AgSAE)

The laboratory-made 3-electrode flow-through cell
- simple design
- air bubbles easily go through the TD-AgSA with the flow of the CS

➢ use as a transducer for the UA biosensor

(2) Silver solid amalgam electrode covered by mercury film (MF-AgSAE)
- simple, robust, inexpensive construction
- providing repeatable measurements
- better sensitivity (compared to TD-AgSA)

The laboratory-made 3-electrode wall-jet cell
- the inlet PTFE capillary was cut off at the optimized angle of 30°
- more challenging to get rid of the air bubbles

➢ use as a transducer for the Ch biosensor as well as the ACh biosensor
Detection part: silver amalgam-based transducers

(3) Silver amalgam-based screen-printed electrode (AgA-SPE)

Preparation:
- fully automated and computer-controlled electrochemical deposition of mercury ions on the commercially available Ag-SPE (ø 1.6 mm, m(Ag) = 56.3 μg)

\[ E_{2H^+_{(aq)}/H_2(g)} = -1592 \text{ mV} \]

\[ E_{2H^+_{(aq)}/H_2(g)} = -1979 \text{ mV} \]

Detection of the hydrogen evolution potential at AgA-SPE:
(w(Hg) = 50%) appeared to be the most favorable

Electrochemical characterization (using [Ru(NH₃)₆]³⁺²⁺):

<table>
<thead>
<tr>
<th>Electrode</th>
<th>ΔE_p / mV</th>
<th>I_p/I_p⁺</th>
<th>A_geometric / mm²</th>
<th>A_eff / mm²</th>
</tr>
</thead>
<tbody>
<tr>
<td>AgA-SPE</td>
<td>63.3 ± 0.2</td>
<td>0.97 ± 0.01</td>
<td>2.0</td>
<td>1.61 ± 0.01</td>
</tr>
<tr>
<td>Ag-SPE</td>
<td>63.9 ± 0.6</td>
<td>0.90 ± 0.02</td>
<td>2.0</td>
<td>1.68 ± 0.04</td>
</tr>
</tbody>
</table>

- (i) cathode: Ag-SPE
- (ii) anode: silver paste amalgam (12 % (w/w) Ag)
- (iii) electrolyte: [0.05 mol L⁻¹ HgO, 2 mol L⁻¹ KI]
- (iv) \( E_{dep} = -200 \text{ mV} \)

fast electron transfer kinetics
(3) Silver amalgam-based screen-printed electrode (AgA-SPE)

- use as a transducer for the LA biosensor
  - FIA ($v_{\text{flow}} = 0.2 \text{ mL min}^{-1}, V_{\text{LA}} = 60 \mu\text{L}$)
  - the commercially available wall-jet cell
  - biorecognition part: Lox-based mini-reactor
  - amperometric monitoring of oxygen consumption ($E_{\text{det}} = -1.1 \text{ V vs. Ag pseudo-RE}$)
  - supporting electrolyte: 0.1 M PB, 1.0 mM Na$_2$EDTA, pH 7.5
**Immobilization method:**

**Support:**

**Coupling agent:**

**Covalent attachment**

**Mesoporous silica powders**
MCM-41 (surface area $\approx 1000 \text{ m}^2\text{g}^{-1}$, pore size $\approx 2.1 – 2.7$ nm)
SBA-15 (surface area $\approx 600 \text{ m}^2\text{g}^{-1}$, particle size $2 – 6$ $\mu$m, pore size $\approx 7$ nm)

**Glutaraldehyde (technique A) vs. EDC/NHS (techniques B and C)**

**technique A**

\[ \text{NH}_2 \rightarrow \overset{+}{\text{OHC - (CH}_2)_3 - \text{CHO}} \rightarrow \text{Glutaraldehyde} \rightarrow \text{NH}_2 \rightarrow \overset{-}{\text{NH}_2} \rightarrow \overset{-}{\text{NH}_2} \]

**technique B**

\[ \overset{-}{\text{COOH}} \rightarrow \overset{-}{\text{NH}_2} \rightarrow \overset{-}{\text{NH}_2} \rightarrow \overset{-}{\text{NH}_2} \]

**technique C**

\[ \overset{-}{\text{COOH}} \rightarrow \overset{-}{\text{NH}_2} \rightarrow \overset{-}{\text{NH}_2} \]
Silanization technique

Formation of the $\text{--NH}_2$ or $\text{--COOH}$ groups on the surface of the mesoporous silica powders (SBA-15, MCM-41)

Biorecognition part: enzymatic mini-reactor

APTES storage at 4°C for 2 years

CEST
Biorecognition part: enzymatic mini-reactor

- amount of $–\text{NH}_2$ groups introduced to the surface of 1 g SBA–15 is 2.0 times higher than $–\text{COOH}$ groups

- GA-activated support is stable for 24 h, while EDC/NHS is stable for around 30 min

- GA is a 5-atom spacer arm, contrary to zero-length EDC/NHS

GA-technique (technique A)
Determination of the immobilized enzyme quantity (Bradford method)

Biorecognition part: enzymatic mini-reactor

enzyme amount in solution before immobilization enzyme amount in solution after immobilization amount of the immobilized enzyme / 50 mg of silica powder-NH$_2$
Quantity of the immobilized enzyme:

- **UOx**-based mini-reactor: c.a. **955 μg (8.6 U)** of the **UOx**
- **ChOx**-based mini-reactor: c.a. **477 μg (6.6 U)** of the **ChOx**
- **LOx**-based mini-reactor: c.a. **270 μg (12 U)** of the **LOx**

### Biosensors of classic pen-type design

<table>
<thead>
<tr>
<th>WE (φ)</th>
<th>Immobilization technique</th>
<th>Enzyme (activity)</th>
<th>Immobilized amount</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pt (3 mm)</td>
<td>Avidin-biotin technique</td>
<td>ChOx (10 U/mg)</td>
<td>0.038 μg (0.4 mU)</td>
<td>[1]</td>
</tr>
<tr>
<td>Pt (3 mm)</td>
<td>Encapsulation</td>
<td>ChOx (10 U/mg)</td>
<td>5.6 μg (0.06 U)</td>
<td>[2]</td>
</tr>
<tr>
<td>GCE (3 mm)</td>
<td>Physical adsorption</td>
<td>ChOx (10 U/mg)</td>
<td>60.9 μg (0.7 U)</td>
<td>[3]</td>
</tr>
<tr>
<td>GCE (3 mm)</td>
<td>Physical adsorption</td>
<td>ChOx (11 U/mg)</td>
<td>9.26 μg (0.1 U)</td>
<td>[3]</td>
</tr>
<tr>
<td>GCE (3 mm)</td>
<td>Entrapment</td>
<td>HRP (318 U/mg)</td>
<td>0.61 μg (0.19 U)</td>
<td>[4]</td>
</tr>
<tr>
<td>GCE (3 mm)</td>
<td>Encapsulation</td>
<td>Lac (0.5 U/mg)</td>
<td>38.9 μg (0.019 U)</td>
<td>[5]</td>
</tr>
<tr>
<td>GCE (2 mm)</td>
<td>Covalent binding</td>
<td>GOx</td>
<td>2.57 μg</td>
<td>[6]</td>
</tr>
</tbody>
</table>

Optimization of the responses of the biosensors

<table>
<thead>
<tr>
<th>Biosensor</th>
<th>Analyte</th>
<th>pH and composition of the carrier solution (CS)</th>
<th>$E_{det}$ / mV</th>
<th>$v_{flow}$ / mL min$^{-1}$</th>
<th>$V_{inj}$ / μL</th>
</tr>
</thead>
<tbody>
<tr>
<td>TD-AgSA + <strong>UOx</strong>-based mini-reactor</td>
<td>uric acid (UA)</td>
<td>[0.1 M BB, pH 9.1]</td>
<td>-1100*</td>
<td>0.1</td>
<td>60</td>
</tr>
<tr>
<td>MF-AgSAE + <strong>ChOx</strong>-based mini-reactor</td>
<td>choline (Ch)</td>
<td>[0.1 M PB, pH 7.2, 1.0 mM Na$_2$EDTA]</td>
<td>-1400*</td>
<td>0.2</td>
<td>60</td>
</tr>
<tr>
<td>MF-AgSAE + <strong>AChE</strong>-based mini-reactor + <strong>ChOx</strong>-based mini-reactor</td>
<td>acetylcholine (ACh)</td>
<td>[0.1 M PB, pH 8.0, 1.0 mM Na$_2$EDTA]</td>
<td>-1400*</td>
<td>0.2</td>
<td>120</td>
</tr>
<tr>
<td>AgA-SPE + <strong>LOx</strong>-based mini-reactor</td>
<td>lactic acid (LA)</td>
<td>[0.1 M PB, pH 7.5, 1.0 mM Na$_2$EDTA]</td>
<td>-900**</td>
<td>0.2</td>
<td>60</td>
</tr>
</tbody>
</table>

*vs. SCE-AgA; **vs. Ag pseudo-RE
### Analytical performances

<table>
<thead>
<tr>
<th>Biosensor</th>
<th>Analyte</th>
<th>LDR / μmol L⁻¹</th>
<th>LOD / μmol L⁻¹</th>
<th>Repeatability (RSD&lt;sub&gt;n=11&lt;/sub&gt;) / %</th>
<th>Reusability</th>
</tr>
</thead>
<tbody>
<tr>
<td>TD-AgSA + UOx-based mini-reactor</td>
<td>uric acid (UA)</td>
<td>50 – 800</td>
<td>18.5</td>
<td>2.8</td>
<td>90.5 % / 365 days / 600 uses</td>
</tr>
<tr>
<td>MF-AgSAE + ChOx-based mini-reactor</td>
<td>choline (Ch)</td>
<td>40 – 500</td>
<td>13.0</td>
<td>2.4</td>
<td>83.0 % / 100 days / 500 uses</td>
</tr>
<tr>
<td>MF-AgSAE + AChE-based mini-reactor + ChOx-based mini-reactor</td>
<td>Acetylcholine (ACh)</td>
<td>30 – 400</td>
<td>13.6</td>
<td>2.3</td>
<td>89.8 % / 100 days / 400 uses</td>
</tr>
<tr>
<td>AgA-SPE + LOx-based mini-reactor</td>
<td>lactic acid (LA)</td>
<td>40 – 500</td>
<td>12.0</td>
<td>1.8</td>
<td>93.8 % / 95 days / 350 uses</td>
</tr>
</tbody>
</table>

### Calibration curve

![Calibration curve for LA biosensor](image1)

### Repeatability

1. **AChE-SBA15/ChOx-SBA15 mini-reactors #1**
   - 731±14 nA
   - RSD<sub>n=7</sub> = 2.22%

2. **AChE-SBA15/ChOx-SBA15 mini-reactors #2**
   - 746±13 nA
   - RSD<sub>n=7</sub> = 1.97%

- **recovery time for enzyme**
No interference caused changes in biosensor signals of more than 5.0%
<table>
<thead>
<tr>
<th>Biosensor</th>
<th>Analyte</th>
<th>Samples</th>
<th>Found</th>
<th>Declared</th>
</tr>
</thead>
<tbody>
<tr>
<td>TD-AgSA + UOx-based mini-reactor</td>
<td>uric acid (UA)</td>
<td>Human urine</td>
<td>2.98 ± 0.04 mM</td>
<td>&gt; 4.4 mM</td>
</tr>
<tr>
<td>MF-AgSAE + ChOx-based mini-reactor</td>
<td>choline (Ch)</td>
<td>Pharmaceutical: Otic solution®</td>
<td>0.143 ± 0.0023 g</td>
<td>0.140 g</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Pharmaceutical: Lipovitan® DUO</td>
<td>0.254 ± 0.0087 g</td>
<td>0.255 g</td>
</tr>
<tr>
<td>MF-AgSAE + AChE-based mini-reactor + ChOx-based mini-reactor</td>
<td>acetylcholine (ACh)</td>
<td>Human plasma (spiked)</td>
<td>102.3 μM</td>
<td>100.0 μM</td>
</tr>
<tr>
<td>AgA-SPE + LOx-based mini-reactor</td>
<td>lactic acid (LA)</td>
<td>Human saliva</td>
<td>87.4 ± 2.4 μM</td>
<td>&gt; 200 μM</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Red wine</td>
<td>1.36 ± 0.031 g L⁻¹</td>
<td>&gt; 3.0 g L⁻¹</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Yogurt</td>
<td>0.78 ± 0.007 (w/w)</td>
<td>~ 0.9 (w/w)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Kefir</td>
<td>1.27 ± 0.028 (w/w)</td>
<td>0.9 - 1.1 (w/w)</td>
</tr>
</tbody>
</table>

**Standard addition method**

**Recovery test**

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Added / mM</th>
<th>Expected / mM</th>
<th>Found / mM</th>
<th>Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>uric acid (UA)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>2.99</td>
<td>2.98 ± 0.04</td>
<td>103.0</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td>3.00</td>
<td>3.08 ± 0.03</td>
<td>105.6</td>
</tr>
<tr>
<td>lactic acid (LA)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>50.0</td>
<td>137.4</td>
<td>132.5 ± 3.9</td>
<td>96.7</td>
</tr>
<tr>
<td></td>
<td>100.0</td>
<td>187.4</td>
<td>190.4 ± 7.9</td>
<td>101.6</td>
</tr>
<tr>
<td></td>
<td>150.0</td>
<td>237.4</td>
<td>233.0 ± 8.5</td>
<td>98.3</td>
</tr>
<tr>
<td></td>
<td>200.0</td>
<td>287.4</td>
<td>297.5 ± 12.0</td>
<td>103.5</td>
</tr>
</tbody>
</table>
Conclusions

- the proposed platform, based on the preparation of the spatially segregated biorecognition part coupled with a principle of detecting oxygen consumption, solves the common biosensors’ limitations related to rapid enzyme deactivation and low selectivity of detection

- four biosensors have been successfully constructed

- it could be a promising new pathway for the development of electrochemical biosensors with other oxidoreductase enzymes

- the working electrodes based on AgSAs constructed in our laboratory can be successfully used for the monitoring of the reduction process in FIA

Acknowledgements

- the Grant Agency of Charles University in Prague (Project 1356120)
- the Grant Agency of the Czech Republic (Projects 20-01589S, 20-07350S)
- Specific Charles University Research (SVV 260560, 260690)

THANK YOU FOR YOUR ATTENTION!