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

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AMPEROMETRIC ENZYME-BASED BIOSENSOR:

biorecognition part → enzyme - oxidoreductase

$$\text{substrate} + \text{enzyme}_{ox} \rightarrow \text{product} + \text{enzyme}_{red}$$

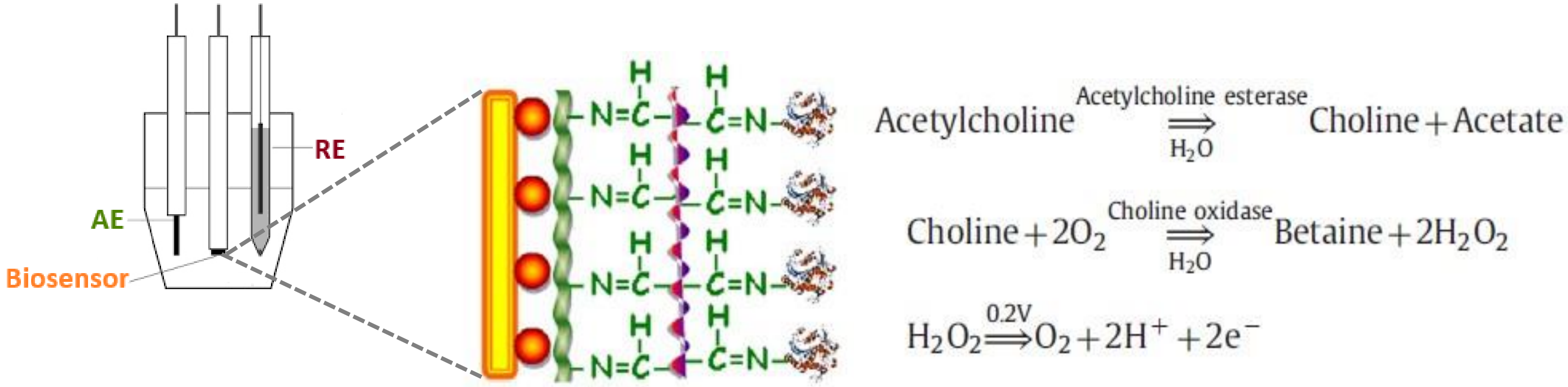
$$\text{enzyme}_{red} + O_2 \rightarrow \text{enzyme}_{ox} + H_2O_2$$

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



Au/Fe@AuNPs/Chit/GA/ChOx-AChE

N. Chauhan, C.S. Pundir, *Biosens. Bioelectron.* **2014**, *61*, 1-8.

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 UOx-based mini-reactor

② Tubular detector of silver solid amalgam (TD-AgSA)

Choline biosensor

① ChOx-based mini-reactor

Acetylcholine biosensor

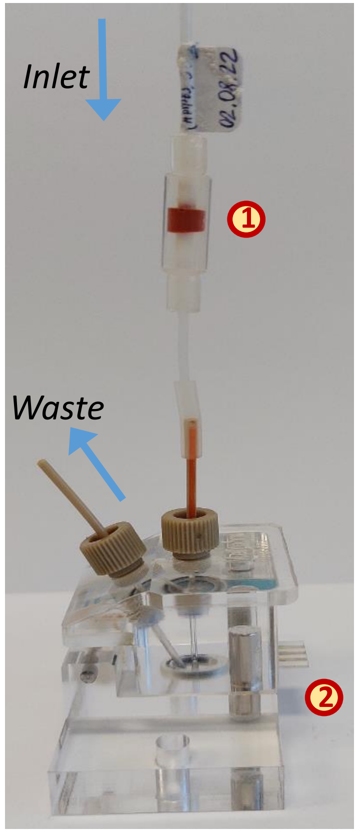
① ChOx-based mini-reactor
AChE-based mini-reactor

② Silver solid amalgam electrode covered by mercury film (MF-AgSAE)

Lactic acid biosensor

① LOx-based mini-reactor

② Silver amalgam-based SPE (AgA-SPE)



Biosensing platform in flow injection analysis - conceptualization

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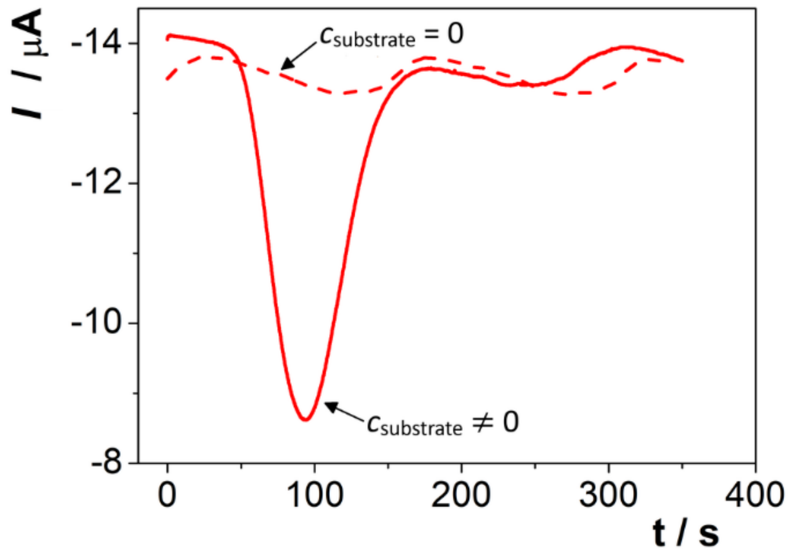
Detection part:

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

$$O_2 + 2H_2O + 4e^- \xrightarrow{-0.9\ V\ldots-1.4\ V} 4OH^-$$



Biorecognition part

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

$$\text{substrate} + \text{enzymeox} \rightarrow \text{product} + \text{enzymered}$$
$$\text{enzyme}_{red} + O_2 \rightarrow \text{enzyme}_{ox} + H_2O_2$$

Uricase-based mini-reactor:

$$\text{uric acid} + O_2 + H_2O \xrightarrow{UOx} \text{allantoin} + CO_2 + H_2O_2$$

Lactic acid oxidase-based mini-reactor:

$$\text{lactic acid} + O_2 + H_2O \xrightarrow{LOx} \text{pyruvic acid} + H_2O_2$$

Acetylcholinesterase-based mini-reactor:

$$\text{acetylcholine} + H_2O \xrightarrow{AChE} \text{acetic acid} + \text{choline}$$

Choline oxidase-based mini-reactor:

$$\text{choline} + 2O_2 + H_2O \xrightarrow{ChOx} \text{betaine} + 2H_2O_2$$

Importance of determination

Clinical analysis

- ❑ Uric acid (UA)

UA > 4.4 mM in urine or > 0.52 mM in blood:

goat **kidney disease**

- ❑ Choline (Ch)

Ch < 7.0 µM in blood:

neurological diseases **liver disorders** **complications during pregnancy**

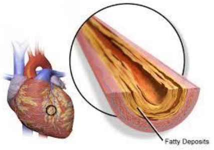
- ❑ Acetylcholine (ACh)

ACh (neurotransmitter) < 6.0 µM in serum:

a biomarker of Parkinson's and Alzheimer's disease

- ❑ L-Lactic acid (LA)

LA > 0.2 mM in saliva or > 2.0 mM in serum:



sepsis **liver disorders** **cardiovascular diseases**

Pharmaceutical industry

Sport medicine

Winemaking

Food industry

Detection part: silver amalgam-based transducers

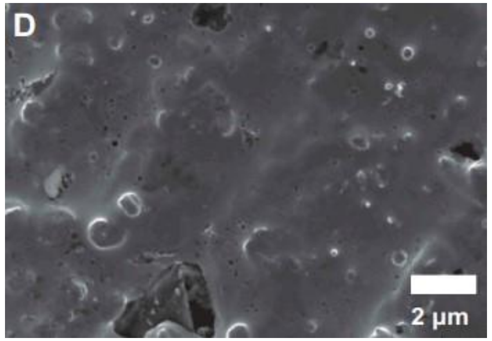
- *Silver solid amalgam electrodes were introduced in 2000 by Yosypchuk and Novotný*

Advantages of the AgSAEs:

- a wide potential window (−2.07 … −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

- a crystalline structure $\mathbf{Ag_2Hg_3}$
- no other phases
- no pores
- no liquid mercury

SEM image of the surface of AgSAE



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

I. $O_2 + 2H_2O + 2e^- \rightarrow H_2O_2 + 2OH^-$ (−100 mV vs. SCE)

II. $H_2O_2 + 2e^- \rightarrow 2OH^-$ (−900 mV vs. SCE)

$$O_2 + 2H_2O + 4e^- \rightarrow 4OH^-$$

Detection part: silver amalgam-based transducers

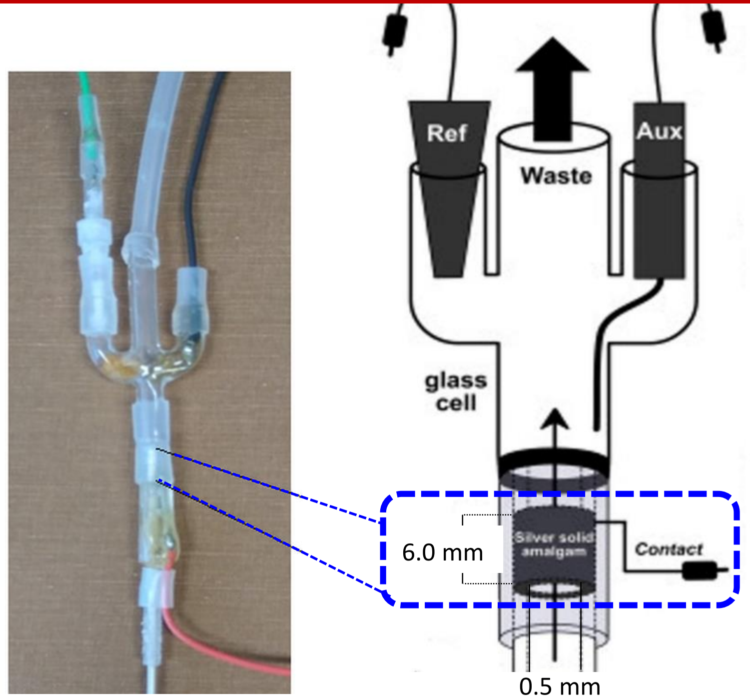
(1) Tubular detector of silver solid amalgam (TD-AgSA)

- 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***



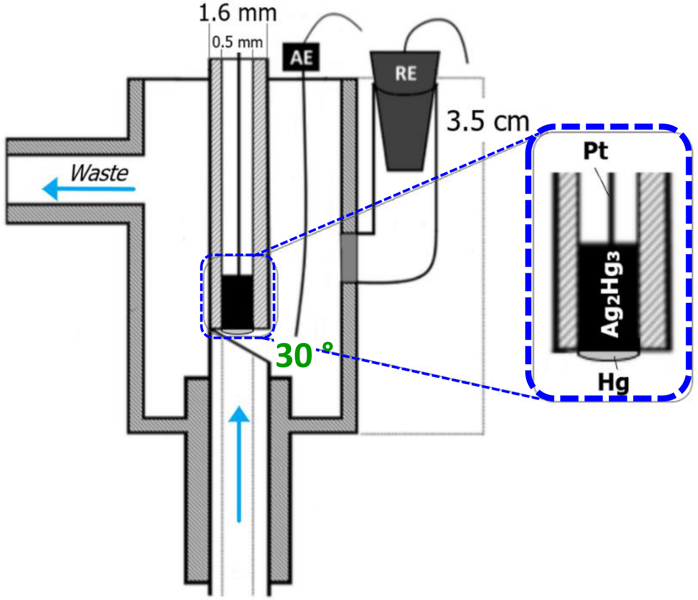
(2) Silver solid amalgam electrode covered by mercury film (MF-AgSAE)

- 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

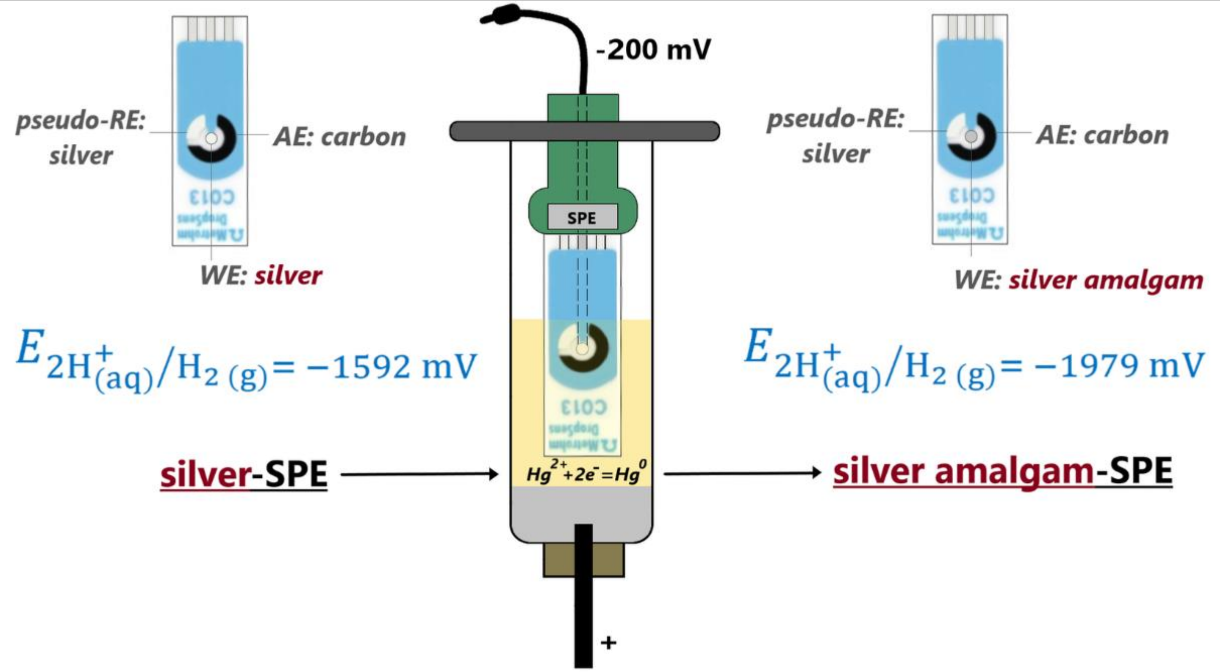
➢ *use as a transducer for the **Ch biosensor** as well as the **ACh biosensor***



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

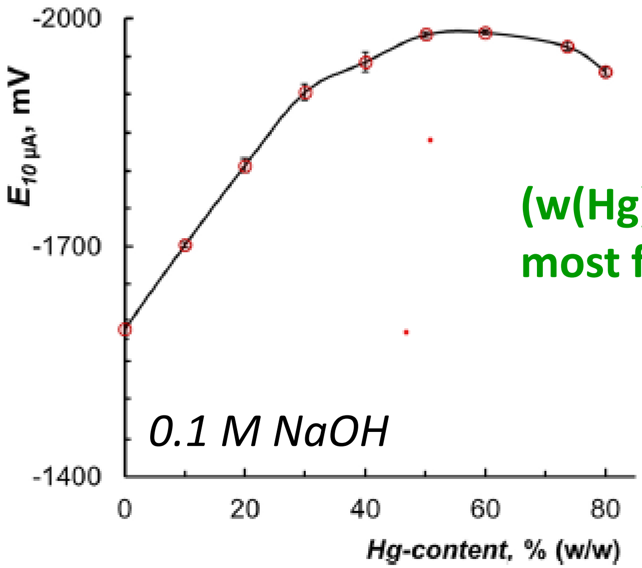
Preparation:

- 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)



(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 mV

Determination of the hydrogen evolution potential at AgA-SPE:



(w(Hg) = 50%) appeared to be the most favorable

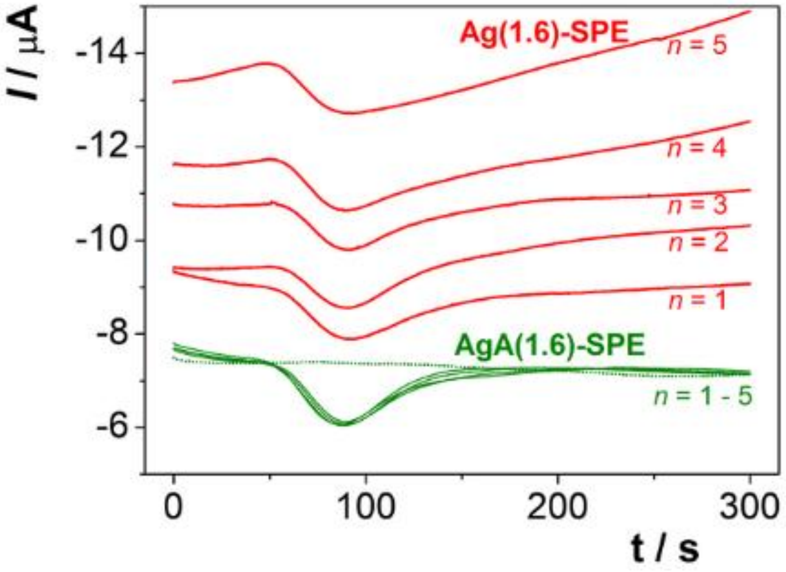
Electrochemical characterization (using $[Ru(NH_3)_6]^{3+/2+}$):

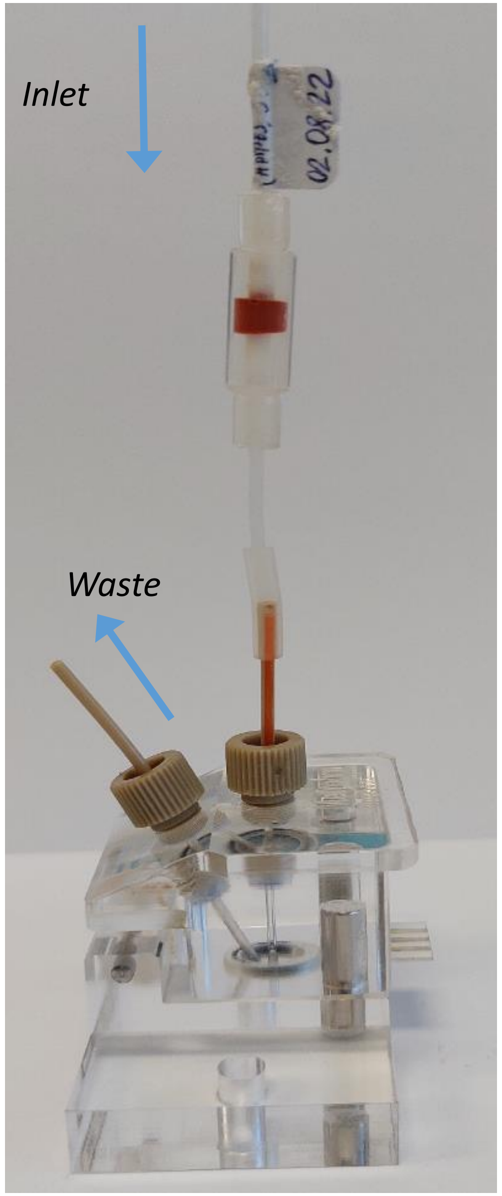
Electrode	ΔE_p / mV	I_p^c/I_p^a	A_{geom} / mm²	A_{eff} / mm²
AgA-SPE	63.3 ± 0.2	0.97 ± 0.01	2.0	1.61 ± 0.01
Ag-SPE	63.9 ± 0.6	0.90 ± 0.02	2.0	1.68 ± 0.04

fast electron transfer kinetics

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

- *use as a transducer for the **LA** biosensor*
  - *FIA (v_{flow} = 0.2 mL min^{-1}, V_{LA} = 60 µL)*
  - *the commercially available wall-jet cell*
  - *biorecognition part: **LOx**-based mini-reactor*
  - *amperometric monitoring of oxygen consumption (E_{det} = -1.1 V vs. Ag pseudo-RE)*
  - *supporting electrolyte: 0.1 M PB, 1.0 mM Na_2EDTA, pH 7.5*





Biorecognition part: enzymatic mini-reactor

Immobilization method: ***Covalent attachment***

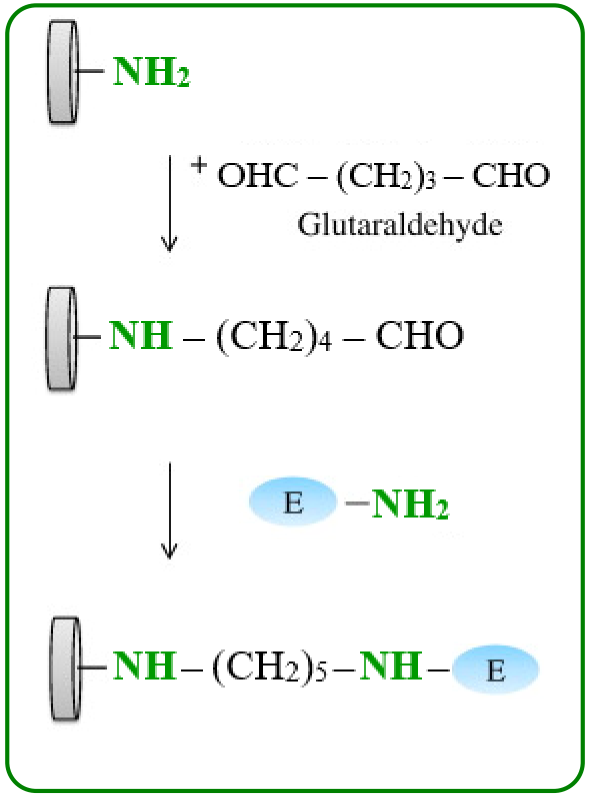
Support: ***Mesoporous silica powders***

MCM-41 (surface area ≈ 1000 m^2g^{-1}, pore size ≈ 2.1 – 2.7 nm)

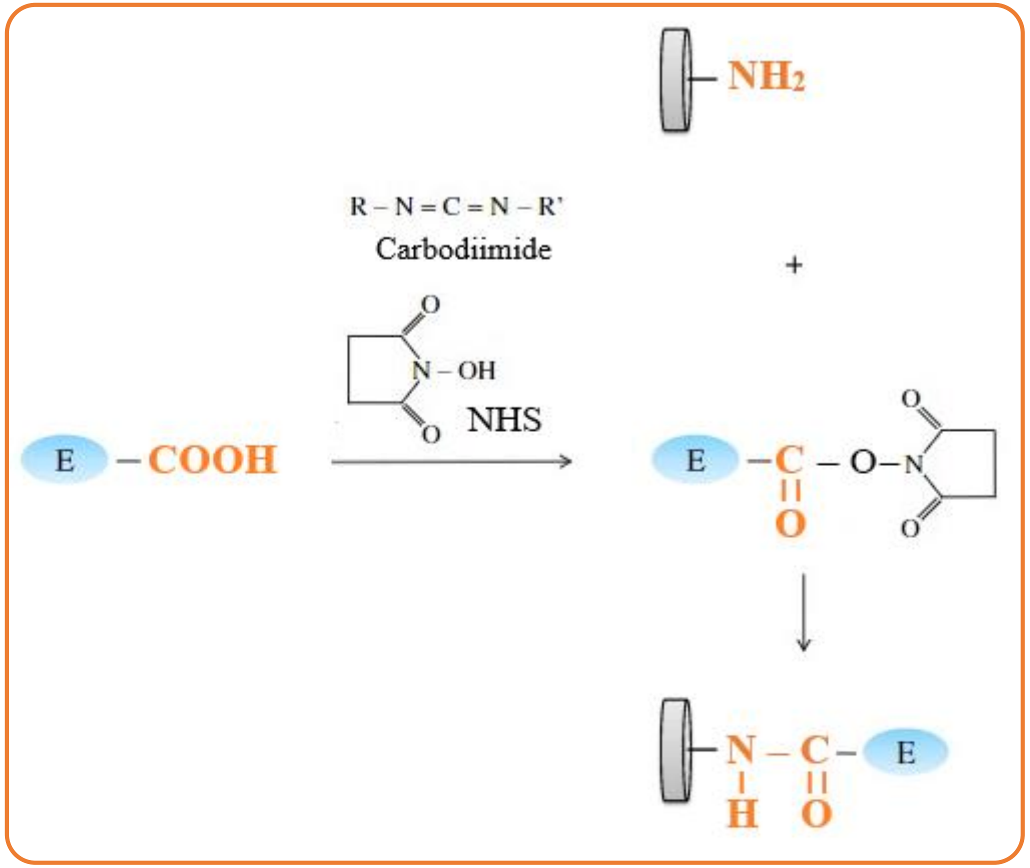
SBA-15 (surface area ≈ 600 m^2g^{-1}, particle size 2 – 6 µm, pore size ≈ 7 nm)

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

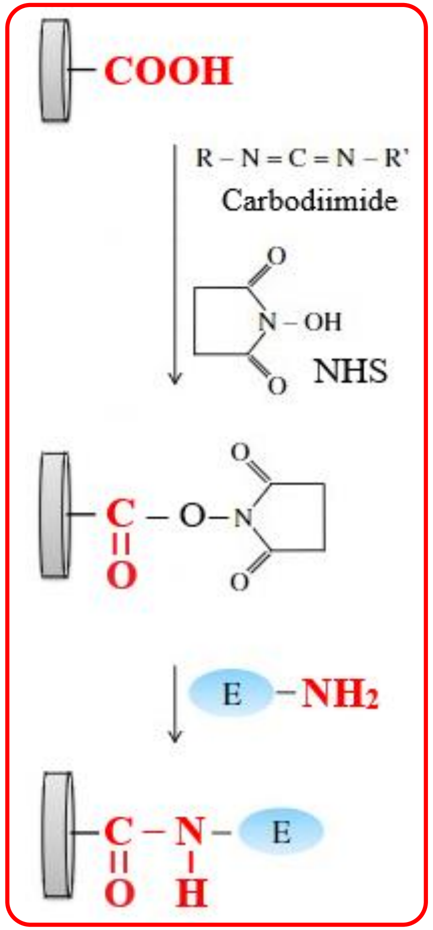
technique A



technique B

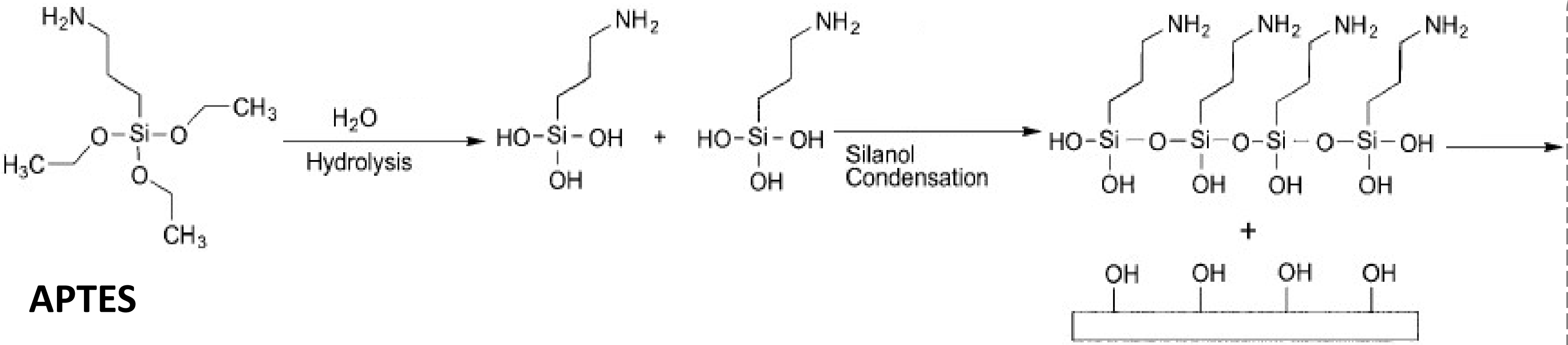


technique C



Formation of the –NH_2 or –COOH groups on the surface of the mesoporous silica powders (SBA-15, MCM-41)

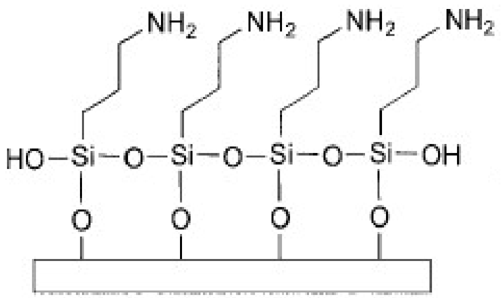
Silanization technique



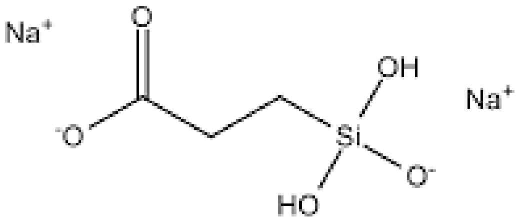
APTES

MCM-41 / SBA15

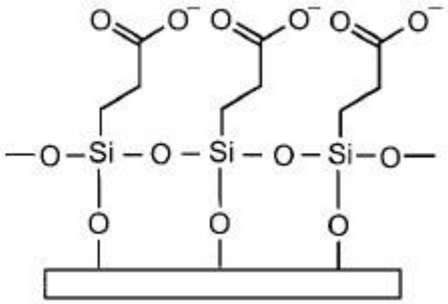
storage at 4°C for 2 years



MCM41–NH_2 / SBA15–NH_2

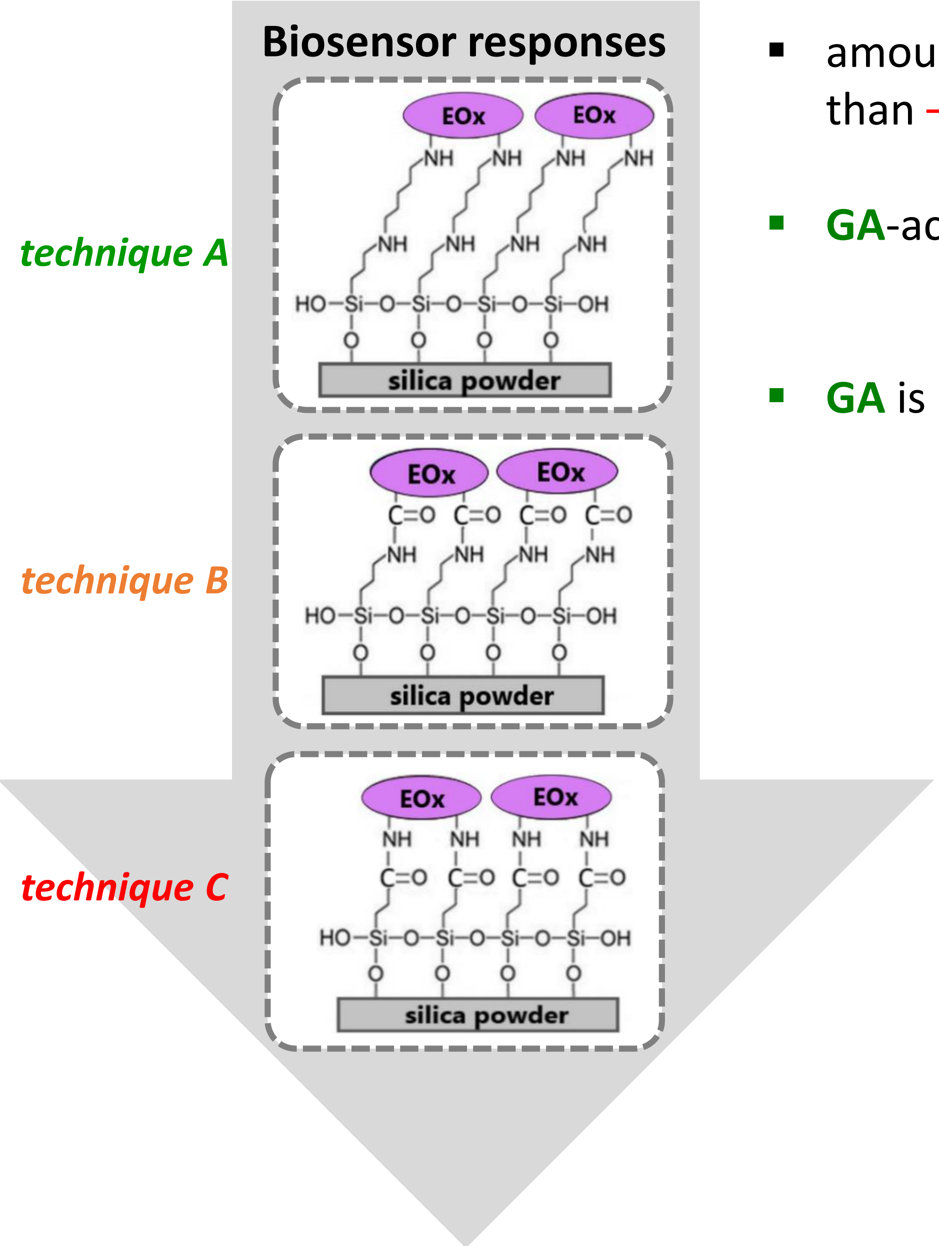


CEST



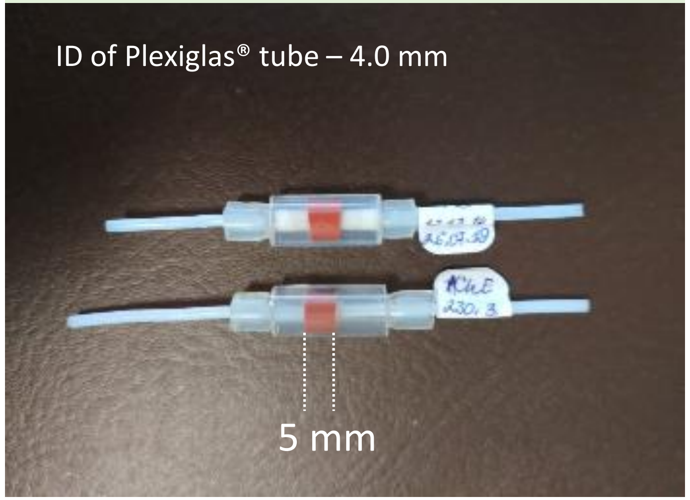
MCM41–COOH / SBA15–COOH

Biorecognition part: enzymatic mini-reactor

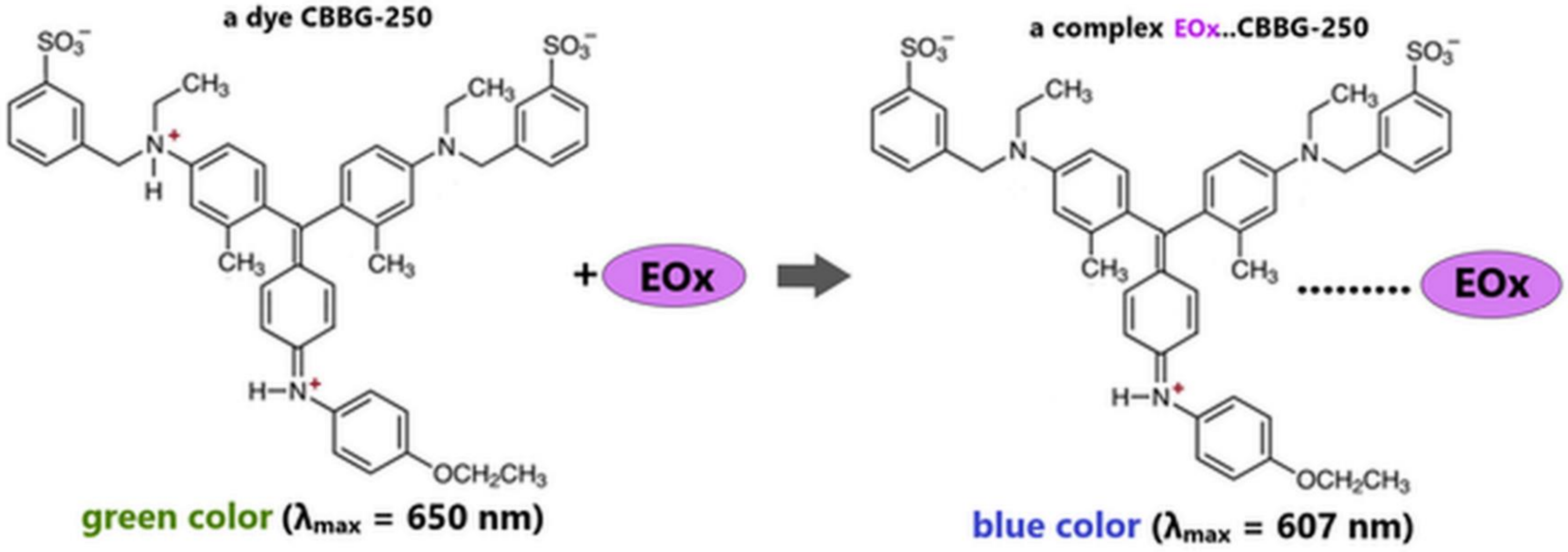


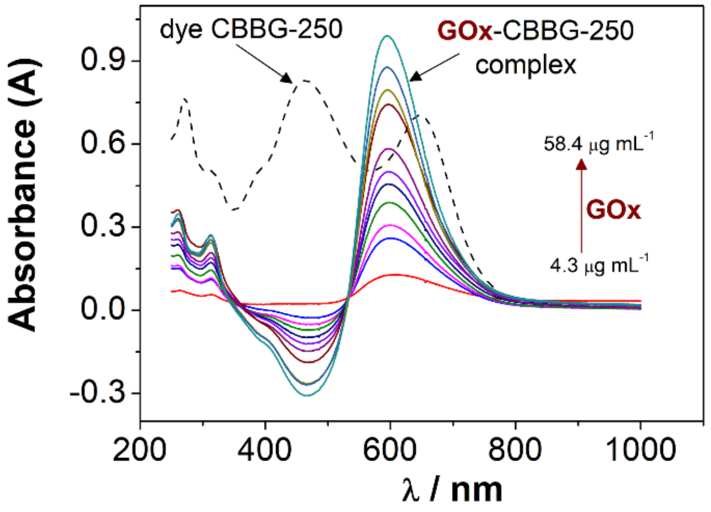
- amount of **$-NH_2$ groups** introduced to the surface of 1 g SBA–15 is 2.0 times higher than –COOH groups
- **GA**-activated support is stable for 24 h, while **EDC/NHS** is stable for around 30 min
- **GA** is a 5-atom spacer arm, contrary to zero-length **EDC/NHS**

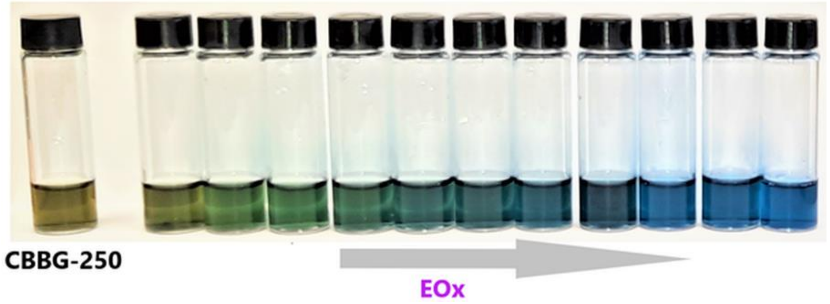
GA-technique (technique A)



Determination of the immobilized enzyme quantity (Bradford method)







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

vs.

Biosensors of classic pen-type design

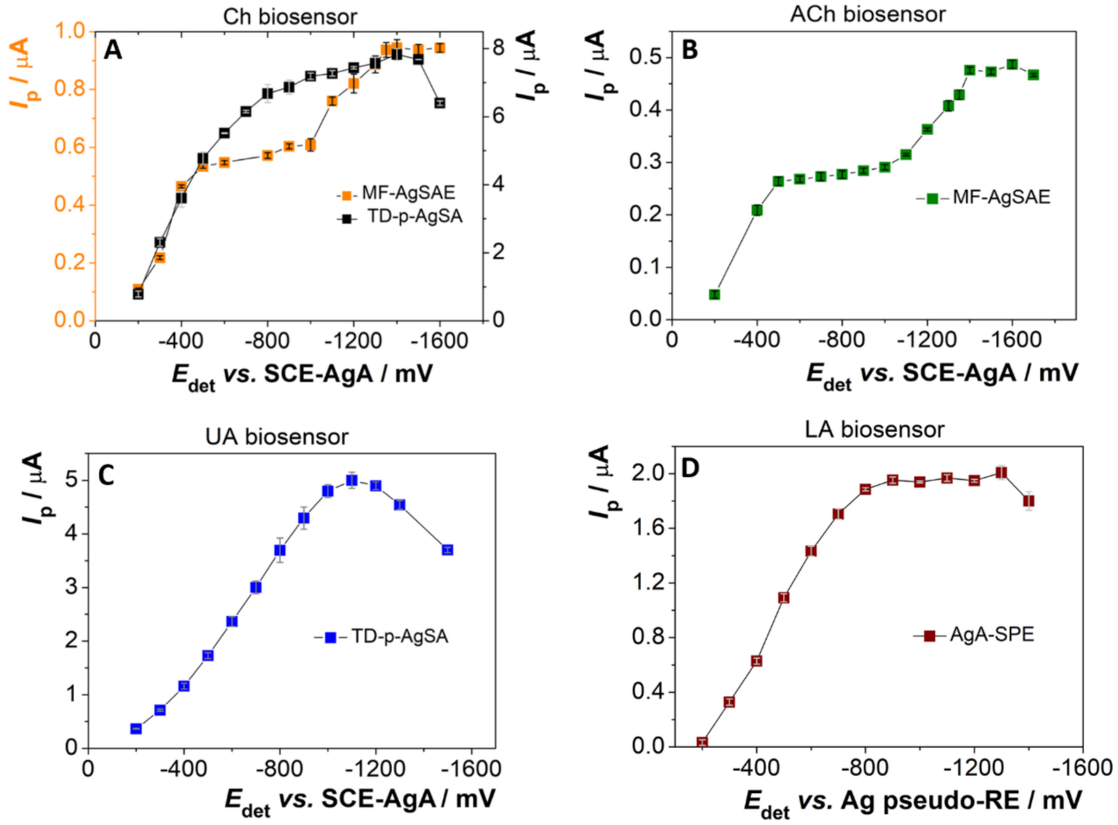
WE (ø)	Immobilization technique	Enzyme (activity)	Immobilized amount	Ref.
Pt (3 mm)	Avidin-biotin technique	ChOx (10 U/mg)	0.038 µg (0.4 mU)	[1]
Pt (3 mm)	Encapsulation	ChOx (10 U/mg)	5.6 µg (0.06 U)	[2]
GCE (3 mm)	Physical adsorption	ChOx (10 U/mg)	60.9 µg (0.7 U)	[3]
GCE (3 mm)	Physical adsorption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

[1] Chen et al., Electroanalysis 1998, 10 (2), 94-97.
[2] Shimomura et al., Talanta 2008, 78, 217-220.
[3] Sajjadi et al., Electrochim. Acta 2011, 56, 9542-9548.
[4] Jiang et al., J Solid State Electrochem. 2009, 13, 791-798.
[5] Shimomura et al., Sens. Actuators B: Chem. 2011, 153, 361-368.
[6] Abasiyanik et al., J Electroanal. Chem. 2010, 639, 21-26.

Optimization of the responses of the biosensors

Biosensor	Analyte	pH and composition of the carrier solution (CS)	E_{det} / mV	v_{flow} / mL min^{-1}	V_{inj} / µL
TD-AgSA + **UOx**-based mini-reactor	uric acid (UA)	[0.1 M BB, pH 9.1]	-1100*	0.1	60
MF-AgSAE + **ChOx**-based mini-reactor	choline (Ch)	[0.1 M PB, pH 7.2, 1.0 mM Na_2EDTA]	-1400*	0.2	60
MF-AgSAE + **AChE**-based mini-reactor + **ChOx**-based mini-reactor	acetylcholine (ACh)	[0.1 M PB, pH 8.0, 1.0 mM Na_2EDTA]	-1400*	0.2	120
AgA-SPE + **LOx**-based mini-reactor	lactic acid (LA)	[0.1 M PB, pH 7.5, 1.0 mM Na_2EDTA]	-900**	0.2	60

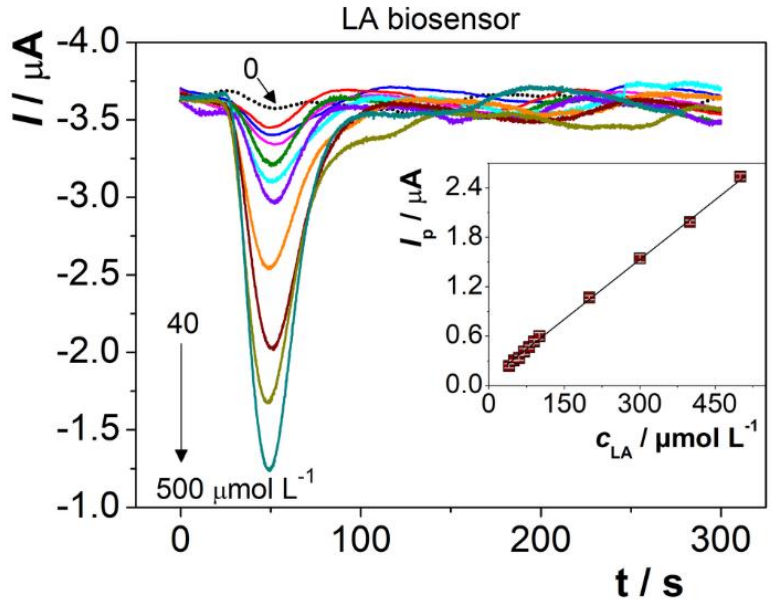
*vs. SCE-AgA; **vs. Ag pseudo-RE



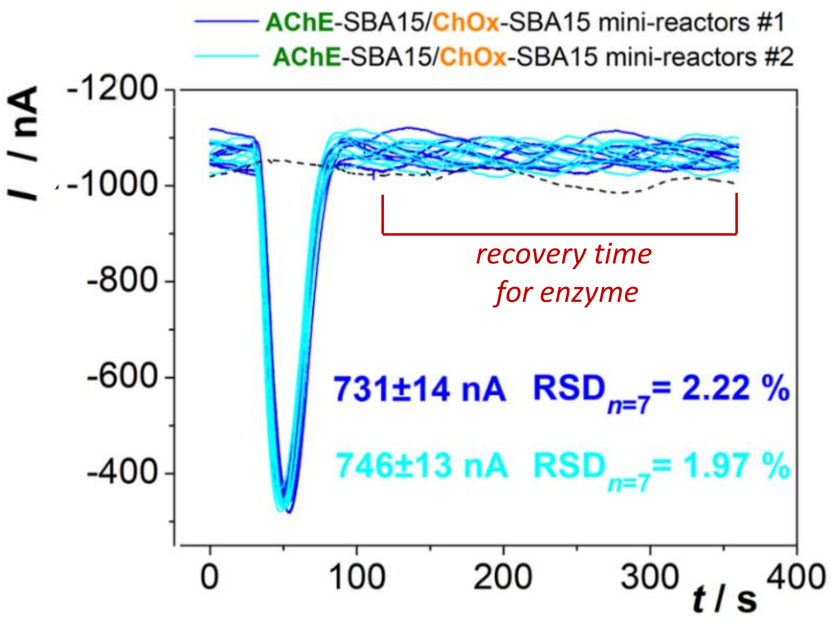
Analytical performances

Biosensor	Analyte	LDR / µmol L^{-1}	LOD / µmol L^{-1}	Repeatability ($RSD_{n=11}$) / %	Reusability
TD-AgSA + **UOx**-based mini-reactor	uric acid (UA)	50 – 800	18.5	2.8	90.5 % / 365 days / 600 uses
MF-AgSAE + **ChOx**-based mini-reactor	choline (Ch)	40 – 500	13.0	2.4	83.0 % / 100 days / 500 uses
MF-AgSAE + **AChE**-based mini-reactor + **ChOx**-based mini-reactor	Acetylcholine (ACh)	30 – 400	13.6	2.3	89.8 % / 100 days / 400 uses
AgA-SPE + **LOx**-based mini-reactor	lactic acid (LA)	40 – 500	12.0	1.8	93.8 % / 95 days / 350 uses

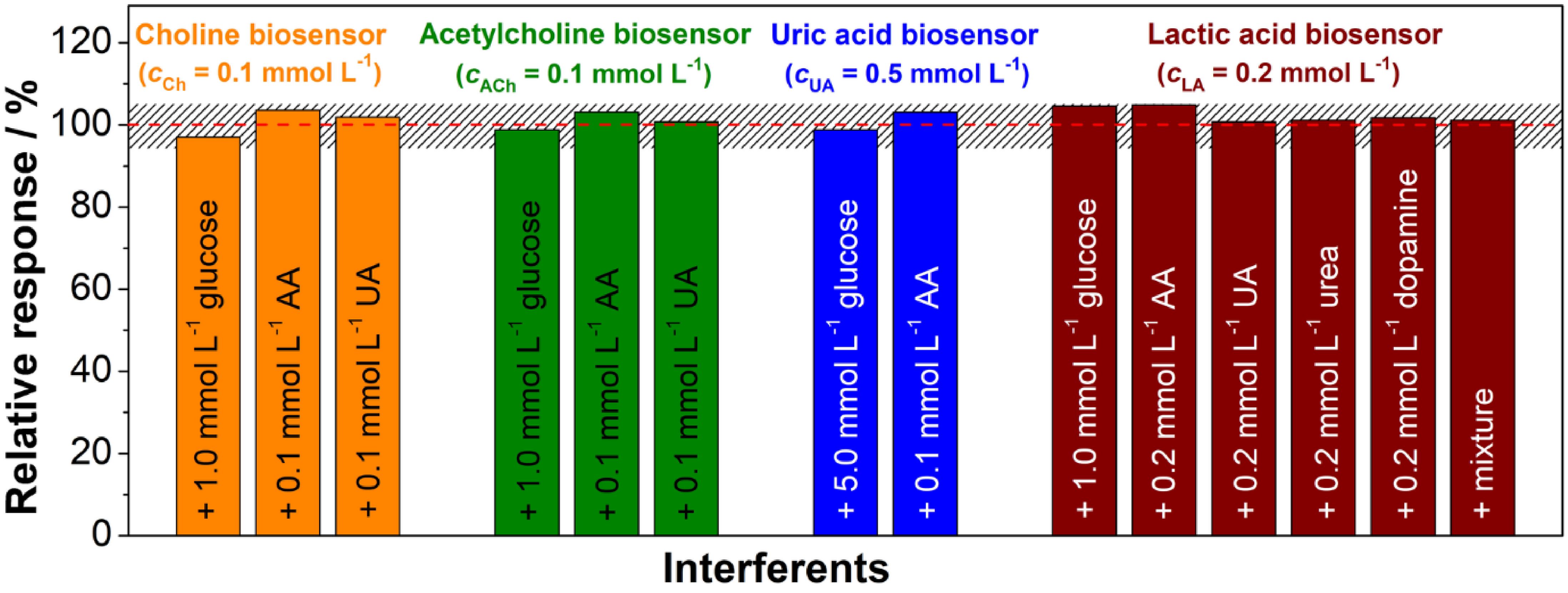
Calibration curve



Repeatability



Selectivity



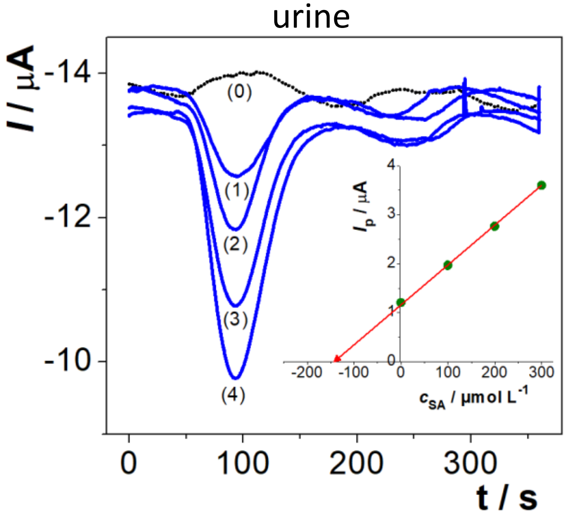
- No interference caused changes in biosensor signals of more than 5.0%

Practical application

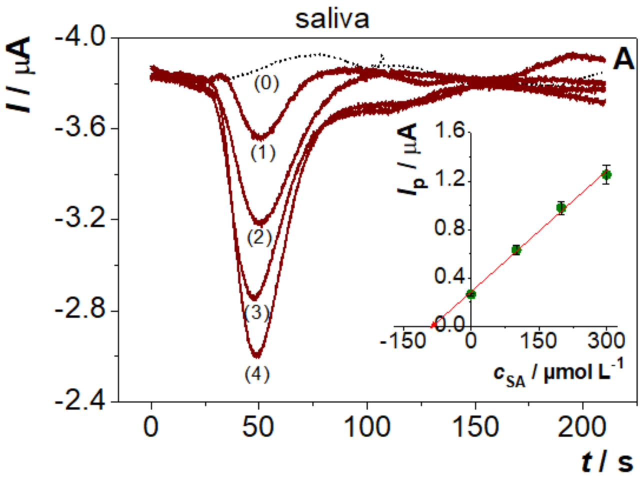
Biosensor	Analyte	Samples	*Found*	*Declared*
TD-AgSA + **UOx**-based mini-reactor	uric acid (UA)	Human urine	2.98 ± 0.04 mM	> 4.4 mM
MF-AgSAE + **ChOx**-based mini-reactor	choline (Ch)	Pharmaceutical: Otic solution® Pharmaceutical: Lipovitan® DUO	0.143 ± 0.0023 g 0.254 ± 0.0087 g	0.140 g 0.255 g
MF-AgSAE + **AChE**-based mini-reactor + **ChOx**-based mini-reactor	acetylcholine (ACh)	Human plasma (spiked)	102.3 µM	100.0 µM
AgA-SPE + **LOx**-based mini-reactor	lactic acid (LA)	Human saliva Red wine Yogurt Kefir	87.4 ± 2.4 µM 1.36 ± 0.031 g L^{-1} 0.78 ± 0.007 (*w/w*) 1.27 ± 0.028 (*w/w*)	> 200 µM > 3.0 g L^{-1} ~ 0.9 (*w/w*) 0.9 - 1.1 (*w/w*)

Standard addition method

UA biosensor



LA biosensor



Recovery test

Analyte	Added / mM	Expected / mM	*Found / mM*	*Recovery*
uric acid (UA)	-		2.98 ± 0.04	-
	0.1	2.99	3.08 ± 0.03	103.0
	0.2	3.00	3.17 ± 0.03	105.6
Analyte	**Added / µM**	**Expected / µM**	***Found / µM***	***Recovery***
lactic acid (LA)	-		87.4 ± 2.4	-
	50.0	137.4	132.5 ± 3.9	96.7
	100.0	187.4	190.4 ± 7.9	101.6
	150.0	237.4	233.0 ± 8.5	98.3
	200.0	287.4	297.5 ± 12.0	103.5

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

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