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Journal

Analyst, 143(20)

ISSN

0003-2654

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Publication Date

2018-10-08

DOI

10.1039/c8an01343h

Peer reviewed

HHS Public Access

Author manuscript

Analyst. Author manuscript; available in PMC 2019 October 08.

Published in final edited form as:

Analyst. 2018 October 08; 143(20): 5008–5013. doi:10.1039/c8an01343h.

Microbiosensor Fabrication by Polydimethylsiloxane Stamping for Combined Sensing of Glucose and Choline

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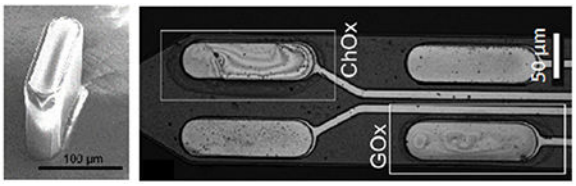
Abstract

High performance microprobes for combined sensing of glucose and choline were fabricated using microcontact printing (μCP) to transfer choline oxidase (ChOx) and glucose oxidase (GOx) onto targeted sites on microelectrode arrays (MEAs). Most electroenzymatic sensing sites on MEAs for neuroscience applications are created by manual enzyme deposition, which becomes problematic when the array feature size is less than or equal to ~100 μm. The μCP process used here relies on use of soft lithography to create features on a polydimethylsiloxane (PDMS) microstamp that correspond to the dimensions and array locations of targeted, microscale sites on a MEA. Precise alignment of the stamp with the MEA is also required to transfer enzyme only onto the specified microelectrode(s). The dual sensor fabrication process began with polyphenylenediamine (PPD) electrodeposition on all Pt microelectrodes to block common interferents (*e.g.*, ascorbic acid and dopamine) found in brain extracellular fluid. Next, a chitosan film was electrodeposited to serve as an adhesive layer. The two enzymes, ChOx and GOx, were transferred onto different microelectrodes of 2 × 2 arrays using two different PDMS stamps and a microscope for stamp alignment. Using constant potential amperometry, the combined sensing microprobe was confirmed to have high sensitivity for choline and glucose (286 and 117 μA/mM cm^2, respectively) accompanied by low detection limits (1 and 3 μM, respectively) and rapid response times (≤ 2 s). This work demonstrates the use of μCP for facile creation of multianalyte sensing microprobes by targeted deposition of enzymes onto preselected sites of a microelectrode array.

Graphical Abstract



A microprobe for sensing of glucose and choline is created by PDMS stamping of GOx and ChOx onto separate microelectrodes.

Conflicts of interest

There are no conflicts to declare.

Introduction

The ability to monitor neurotransmitter release and metabolite levels in the brains of freely moving animals is key to understanding neuronal processes underlying complex behaviors and disorders. Such processes are controlled by neuronal networks influenced by interactions among multiple neurotransmitters and metabolites such as glucose (Glu), dopamine (DA), glutamate (Glut) and acetylcholine (ACh).[1–4] Therefore, our understanding of the connection between neurochemistry and behaviors would be greatly facilitated by the capability to monitor *in vivo* multiple neurochemicals simultaneously and in near-real time. The combination of microelectrode array (MEA) microprobes and electroenzymatic sensing approaches potentially offers a means for combined monitoring of multiple neurochemicals *in vivo* with high spatiotemporal resolution. The existing microdialysis technique also provides for multiple analyte measurements, yet achievement of sufficient spatiotemporal resolution commonly is challenging.[5] Thus, there is impetus to develop implantable microprobes with an array of microsensors capable of combined monitoring of multiple neurochemicals with desirable sensitivity, selectivity and response time characteristics.

We have reported an implantable probe with arrayed microsensors for combined amperometric monitoring of Glut and DA.[6] However, the glutamate oxidase enzyme used in constructing the Glut sensing sites was manually applied in the usual manner to selected microelectrodes, which is very challenging to achieve consistently given the less than 100 μm spacing between sites. In contrast, DA is directly electrooxidizable, and DA sensing sites were constructed straightforwardly through selective electrodeposition processes.[6] Clearly, if multiple oxidases are to be deposited on selected microelectrodes on the same microprobe for combined sensing of nonelectroactive analytes such as Glut, ACh, and Glu, higher resolution, non-manual methods for enzyme transfer and immobilization must be developed.

Microcontact printing (μCP) based on polydimethylsiloxane (PDMS) stamping is an emerging method for transferring proteins to surfaces in high-resolution patterns with feature size down to 500 nm.[7,8] After mold and stamp fabrication, the stamping process begins by “inking” a protein solution onto the PDMS stamp. The protein pattern defined by the stamp is then transferred onto a target substrate upon contact of the protein-covered PDMS stamp with the surface for a few moments. The process can be designed to maintain activity of transferred proteins, and the PDMS stamp can be re-used after appropriate cleaning.[7–13]

Previously, we utilized PDMS stamping to transfer glucose oxidase (GOx) onto macroscopic, 1.6-mm-dia. platinum disk electrodes to demonstrate the feasibility of PDMS stamping for fabrication of high performance electroenzymatic biosensors.14 The glucose biosensors made using PDMS stamping showed excellent properties with a sensitivity of ~29 μA/mM cm^2, a detection limit of ~4 μM, and a response time of ~2 s. In this report, we demonstrate PDMS stamping with microscopic alignment to transfer choline oxidase (ChOx) and glucose oxidase (GOx) separately onto selected individual sites of an MEA. In so doing, we provide the first example of an oxidase-based dual sensing microprobe for nonelectroactive analytes (*i.e.*, glucose and choline) that is suitable for application *in vivo*.

Experimental

Reagents

Glucose oxidase (from Aspergillus niger, CAS NO. 9001-37-0), pyrrole (Py), choline oxidase (Alcaligenes, 9028-67-5), m-phenylenediamine (PD), choline chloride, glutaraldehyde solution (25%), bovine serum albumin (BSA) lyophilized powder, hydrogen peroxide solution (30%), chitosan (from crab shells, minimum 85% deacetylated), D-(+)-glucose, L-ascorbic acid, dopamine hydrochloride, potassium hexacyanoferrate (II) trihydrate, and potassium hexacyanoferrate (III) were purchased from Sigma-Aldrich (St. Louis, MO). Isopropyl alcohol and 1 M sulfuric acid solutions were obtained from Fisher Scientific (Pittsburgh, PA). Ag/AgCl glass-bodied reference electrodes with 3 M NaCl electrolyte and 0.5-mm-diameter Pt wire auxiliary electrodes were purchased from BASi (West Lafayette, IN). Sodium phosphate buffer (PBS, pH 7.4) was composed of 50 mM sodium phosphate (dibasic) and 100 mM sodium chloride. Ultrapure water was generated using a Millipore Milli-Q Water System and was used for preparation of all solutions. Four-inch Si wafers were purchased from Silicon Valley Microelectronics (Santa Clara, CA). SU-8 2075 and SU-8 developer were obtained from MicroChem (Westborough, MA). The Sylgard® 184 silicone elastomer kit was purchased from Dow Corning (Auburn, MI).

The microelectrodes used in this work were silicon-based multielectrode arrays manufactured in house using microelectro-mechanical-system (MEMS) technologies. The fabrication and array details are described in our previous work.[15] The MEA consists of four 6000 μm^2 (40 μm × 150 μm) Pt sites, situated in pairs at the tip of a 9-mm-long shank. The pair nearest the shank tip is 100 μm from the pair farthest from the shank tip, and the paired sites are 40 μm apart. Each site may be modified to act either as a working, control, reference, or counter electrode.

Instrumentation

Electrochemical experiments for sensor development, evaluation and calibration were performed using a Versatile Multichannel Potentiostat (model VMP3) equipped with the ‘p’ low current option and N’Stat box driven by EC-LAB software (Bio-Logic USA, LLC, Knoxville, TN) in a three electrode configuration consisting of the sensing electrode, a Pt wire auxiliary electrode, and a Ag/AgCl glass-bodied reference electrode. An FEI Nova Nano 230 was used for environmental SEM images.

Fabrication of mold and PDMS stamps

The fabrication process for a mold and a PDMS microstamp is illustrated in Fig. 1a. SU-8 2075 was spin-coated on a four-inch Si wafer at 2000 rpm for 30 s to give a ~100 μm thick layer. The layer was soft-baked at 65 °C for 5 min and then at 95 °C for 40 min followed by 27 sec of UV exposure (total 216 mJ/cm^2). Post exposure baking was done at 65 °C for 5 min and at 95 °C for 10 min. After the layer was patterned in SU-8 developer for 20 min, the mold was cleaned using isopropanol and then left to dry in air at room temperature. PDMS microstamps were fabricated using the Sylgard® 184 silicone elastomer kit. To cover a 4 inch mold, 6 g of monomer was mixed with 0.6 g of curing agent (1:10; monomer:curing agent) and then centrifuged at 15,000 rpm for 5 min to minimize air bubbles. After pouring

onto the SU-8 mold, the mixture was subsequently degassed under vacuum to remove air bubbles and then cured at 60 °C for 4 h. The PDMS microstamps were detached from the mold and cut into 1 cm × 1 cm pieces. A fabricated PDMS microstamp is shown in Fig. 1b. To ensure that the enzyme mixture is transferred to the entire microelectrode surface (40 μm × 150 μm), the size of a microstamp surface was designed to be slightly bigger than the size of the microelectrode (50 μm× 160 μm). The PDMS stamps were cleaned in 7.5 % hydrogen peroxide with sonication before re-use.

Sensor preparation

Microelectrodes on microprobes were rinsed with isopropyl alcohol followed by an electrochemical cleaning step with 0.5 M sulfuric acid and sonication in ultrapure water. Next, a polyphenylenediamine (PPD) film was electrodeposited (5 mM PD in stirred PBS, 0.85 V *vs.* Ag/AgCl, 10 min) onto the microelectrode surfaces.

A solution (0.04% m/v) for chitosan electrodeposition was adjusted to pH 3 using HCl (0.5 M) to dissolve the chitosan flakes. After filtering with a 0.2 μm syringe filter, the pH was adjusted to 5 using NaOH solution (0.5 M). A constant potential of −0.7 V *vs.* Ag/AgCl was applied at the PPD-coated Pt electrode surface for 2 min while immersed in the chitosan solution to electrodeposit a chitosan film.[16,17]

PDMS μCP with alignment

A droplet (~3 μL) of enzyme mixture was placed on a PDMS microstamp and allowed to rest at room temperature for ~60 min in a closed chamber to prevent evaporation. Enzyme mixtures consisted of ChOx (17.5 mg/mL) or GOx (10 mg/mL) mixed with bovine serum albumin (BSA) in a 1:1 mass ratio in phosphate-buffered saline (PBS). After this “inking” step, the excess enzyme solution was wicked away using a Kimwipe, and the microstamp was dried using a nitrogen gun for ~15 s. Since our previous work used a stamp matched with a single, 1.6-mm-dia. disk electrode, microscopic alignment was not required for stamping onto the electrode.14 However in this work, microscopic alignment was necessary to deposit “inked” enzyme on selected microelectrodes in a 2 2 array on a microprobe where the separation between microelectrodes was 40 μm and 100 μm in the lateral- and axial-directions, respectively. The alignment setup consisted of a microscope with an adjustable stage and a separate custom-built, fixed stage to secure the PDMS stamp, as shown in Fig. 2. The microprobe was attached to the microscope stage and was moved into focus with the surface of the stamp. Alignment of the PDMS microstamp and the target microelectrode was achieved by manipulation of the microscope stage. The microscope stage was then raised further to make contact with the PDMS microstamp. The ChOx mixture and the GOx mixture were stamped onto the upper right and bottom left sites of the microelectrode array. The remaining two microelectrodes, upper left and bottom right, were left as control sites. The contact time was ~1 min to transfer the enzymes from the microstamp to the microelectrode. Subsequently, the microprobe was exposed to vapor from a 5% glutaraldehyde (GAH) solution at room temperature for 1 min to crosslink the chitosan, enzyme and BSA on the microelectrode surfaces. This enzyme stamping and crosslinking process was repeated twice to achieve sufficient enzyme surface concentrations for high-

performance sensing of choline and glucose. The fabricated sensors were preserved at 4 °C under dry conditions when not in use.

Electrochemical measurements

Constant potential amperometric measurements were conducted in PBS buffer at 0.7 V *vs.* Ag/AgCl and at ambient laboratory temperature. More than 30 min of equilibrium time in PBS buffer was allowed to achieve a stable current before adding analytes. Sensitivity was calculated based on the first few points of sensor calibration curves.

Results and discussion

PDMS stamping of enzymes

An optical image of the microelectrodes before and after stamping are shown in Fig. 3. A clear deposit of the ChOx and GOx mixtures is evident in the boxed areas of the image (Fig. 3b) that extends slightly beyond the edges of the microelectrode as planned (see Methods). There are no evident surface abnormalities, which implies that alignment and deposition were successful. By increasing the “inking” time (BSA and enzyme mixture on top of stamp) from our previously reported 10 min to 60 min,[14] more consistent enzyme layers were formed resulting in more consistent microsensor performance (see below).

Glucose microbiosensor performance

Calibration data for the glucose biosensor is presented as Fig. 4. Glucose biosensors fabricated on the same day exhibited a repeatable, high sensitivity of 117 ± 14 μA mM^{-1} cm^{-2} (n = 9) and detection limit of 3 ± 0.5 μM (n = 9) at a signal-to-noise ratio of 3. The 4-fold improvement in sensitivity as compared to our previously reported work (117 vs. 29 μA mM^{-1} cm^{-2}) is due in large part to reduced exposure to glutaraldehyde vapor (1 min exposure to 5% solution *vs.* 45 s to 12.5% solution),14 which suggests that even brief exposure to glutaraldehyde vapor damages enzyme. However, different permselective coatings also were used previously, polypyrrole (PPy) and Nafion, which may have contributed to a difference in performance as well. The sensors described here typically displayed a linear detection range up to ~1.4 mM (R2 = 0.9997) and a response time (95% of the steady-state current) of <2 s when exposed to a step change in glucose concentration to 40 μM in a stirred beaker.

The performance of our glucose microbiosensor fabricated by μCP compares very well with those described in recently published reports (see Table 1 for examples). In our previous work,[14] we carefully reviewed the performance characteristics of recently reported glucose biosensors based on immobilized GOx and found our macroscale sensor fabricated by PDMS stamping to exhibit a rare, if not unique, combination of high sensitivity and selectivity, fast response time and low limit of detection. Here, we describe a stamped glucose microbiosensor with substantially improved sensitivity, lower detection limit and still fast response time and impressive selectivity.

Choline microbiosensor performance

A choline biosensor was fabricated here tp provide another example of the utility of PDMS microstamping. A typical calibration curve for the choline biosensor is presented in Fig. 5.

Choline biosensors fabricated on the same day exhibited a repeatable, high sensitivity of 286 ± 32 μA mM^{-1} cm^{-2} (n = 4) and detection limit of 1 ± 0.2 μM (n = 4) at a signal-to-noise ratio of 3. The biosensor also displayed a relatively fast response time (~2 s) to a choline step change of 10 μM in a stirred beaker.

Our choline biosensor created by μCP is also competitive with those recently described in the literature (see Table 2). Keihan et al. reported a very high sensitivity (345.4 μA mM^{-1} cm^{-2}) with a low detection limit (0.45 μM), but the biosensors were composed of a complex system consisting of a multi-walled carbon nanotube (MWCNT)/ionic liquid (IL)/Prussian blue (PB) nanocomposite-modified glassy carbon (GC) electrode.[22] Ricci *et al.* presented results describing a choline biosensor assembled on screen-printed electrodes (SPEs) with a low detection limit (0.5 μM), but with lower sensitivity than our device (Table 2).[23] Rahimi *et al.* described a choline biosensor based on an amine functionalized multi-walled carbon nanotubes (NH_2-MWCNT)/IL system with performance not as good as this report (sensitivity of 125.8 μA mM^{-1} cm^{-2}, detection limit of 3.85 μM), and the response time was not mentioned.[24] Zhang *et al.* reported a similar response time (2 s) with lower detection limit of 0.4 μM, but the sensitivity was significantly lower (75.2 μA mM^{-1} cm^{-2}). In addition, the biosensors were fabricated using a complex system consisting of poly(diallyldimethylammonium chloride (PDDA) deposited on PB-iron phosphate nanostructures (PB–$FePO_4$).[25] Burmeister *et al.* reported a ceramic probe-based choline biosensor with low detection limit of 0.4 μM and fast response time of 1.4 s, but a self-referencing recording mode was needed to remove interfering dopamine signals.[26] Thus, the choline biosensor presented in this work offers an appealing combination of simplicity of design, high performance, and excellent selectivity (see below).

Dual sensor and effect of interferents

Fig. 6 shows the combined sensing of glucose and choline by our microprobe created by PDMS microstamping. The glucose and choline microbiosensor selectivity was tested against ascorbic acid (AA) and dopamine (DA), common electrooxidizable interferents found in brain extracellular fluid (Fig. 6). Physiologically relevant concentrations of 5 μM dopamine and 250 and 500 μM ascorbic acid were used to test for the false positive signals that might arise in applications *in vivo*. A negligible biosensor response to AA and DA was observed at the constant operating potential of 0.7 V (*vs.* Ag/AgCl), while the appropriate biosensing sites exhibited the expected responses to hydrogen peroxide and to glucose or choline. These results show that the permselective polyphenylenediamine film effectively blocks access of these key electroactive interferents, which suggests that this microprobe may be a useful implantable tool for neuroscience research.

Conclusions

PDMS stamping has been employed successfully to deposit GOx and ChOx onto selected, distinct sites of a microelectrode array (MEA) to fabricate an implantable microbe for combined sensing of glucose and choline. The microbiosensor sites showed high sensitivity for choline and glucose (286 and 117 μA/mM cm2, respectively), fast response times (≤ 2 s), and low detection limits (1 and 3 μM, respectively). The sensors also were selective against

ascorbic acid and dopamine, two electroactive interferents common to brain extracellular fluid. The PDMS microstamping technique is expected to contribute to neuroscience research by making possible the controlled deposition of different enzymes on selected microelectrode sites on a microprobe thereby enabling the combined sensing of multiple neurochemicals in close proximity simultaneously. The high resolution and non-manual nature of this stamping approach for enzyme transfer also should enable a decrease in size of MEAs in order to minimize tissue damage and increase spatial resolution, as well as a higher throughput process to generate microprobes for combined electroenzymatic sensing of multiple analytes.

Acknowledgements

The research was supported by NIH (R01NS087494).

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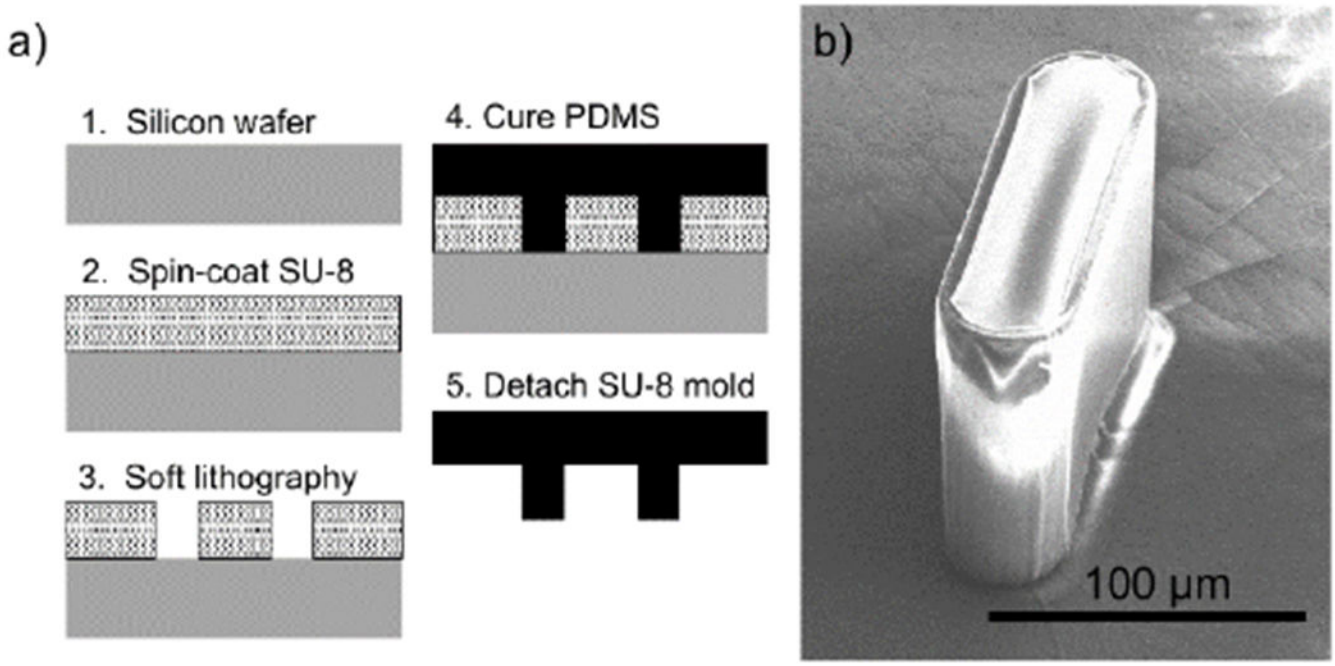

Fig. 1.

a) Fabrication process for a SU-8 mold and a PDMS microstamp. b) Scanning electron microscope (SEM) image of a PDMS microstamp.

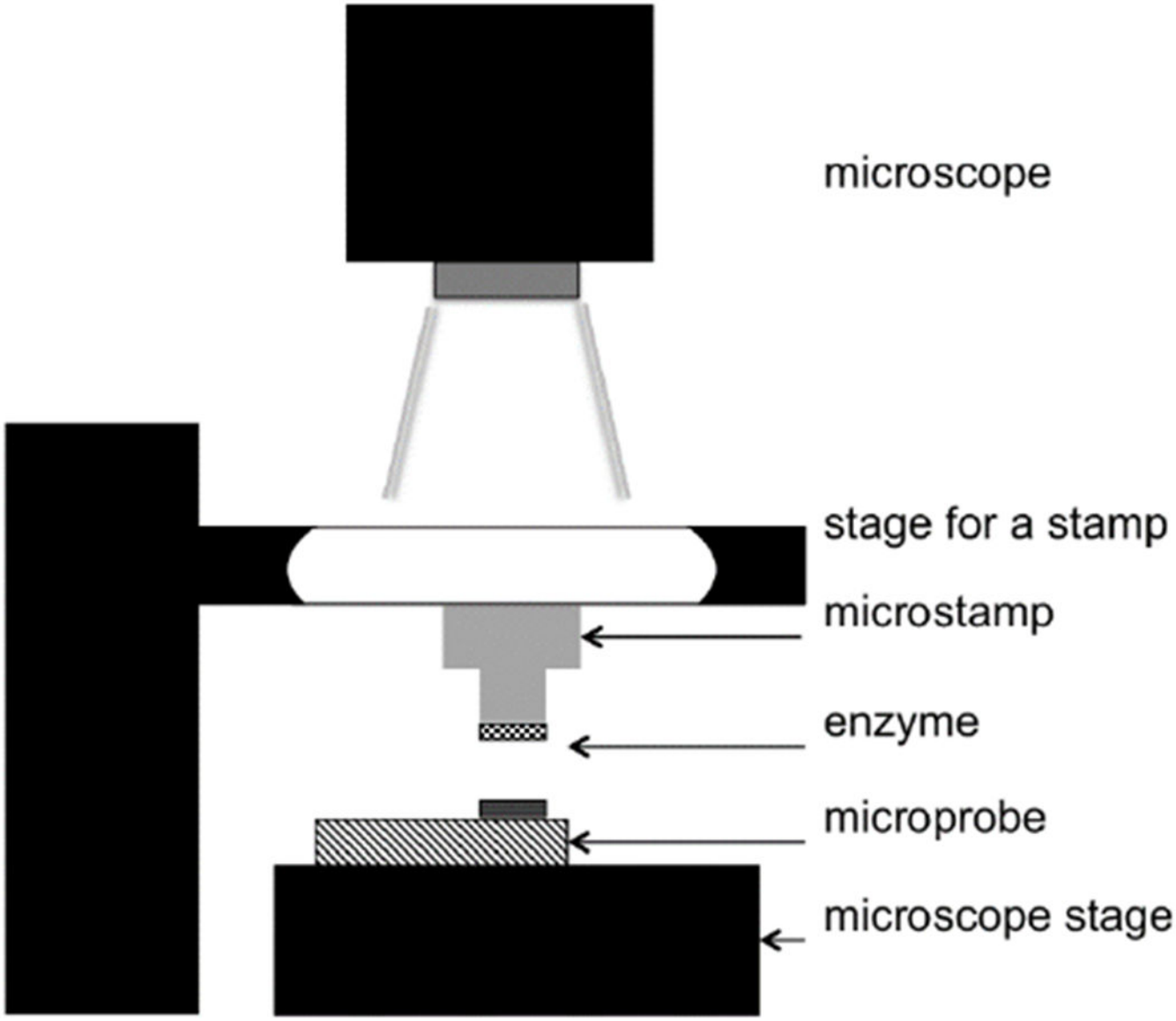

Fig. 2.
Alignment setup for a PDMS microstamp and a microelectrode array on a silicon-based microprobe.

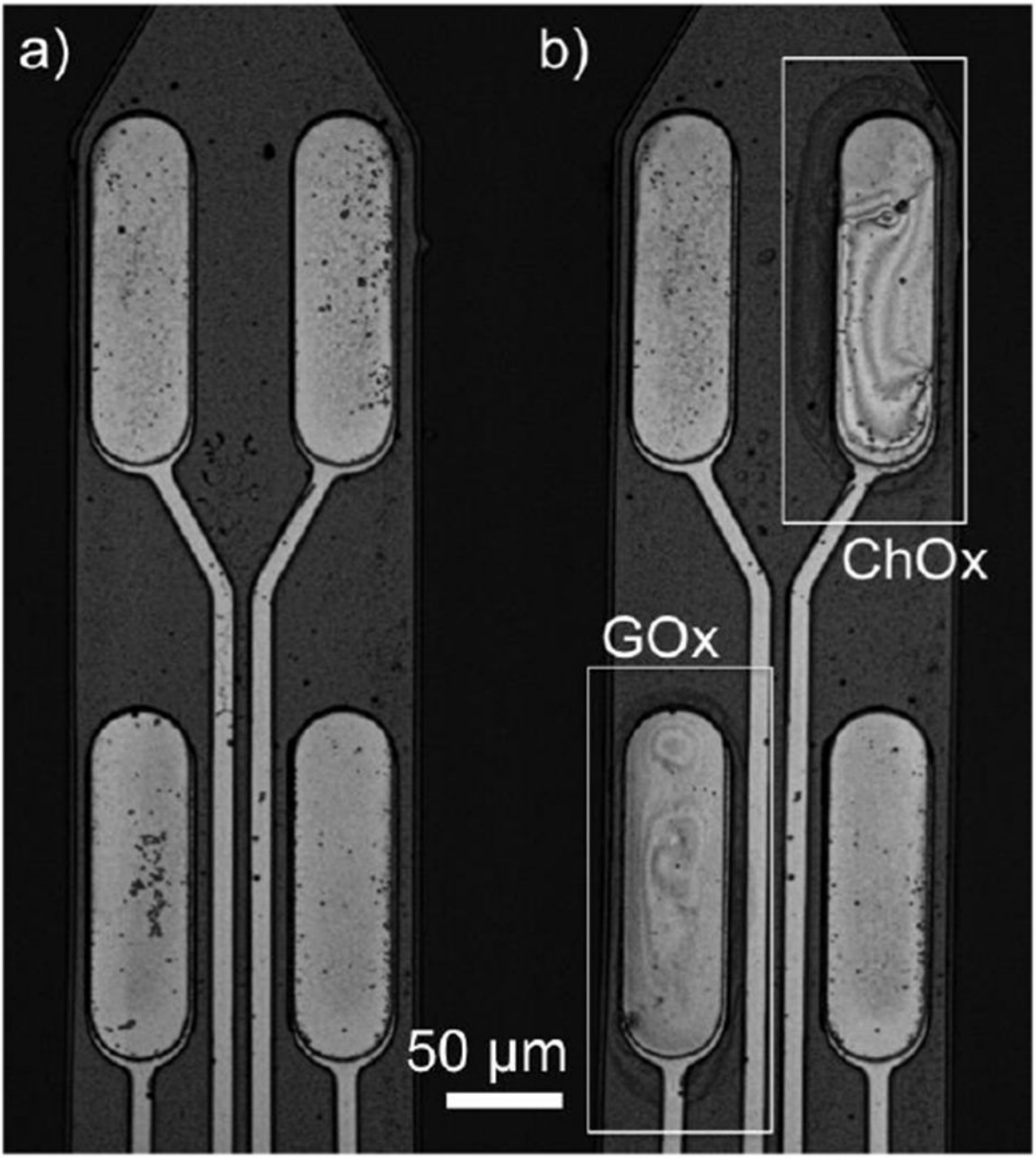

Fig. 3.
100× Optical microscope image of a microelectrode array on a microprobe a) before and b) after PDMS stamping of ChOx and GOx with alignment.

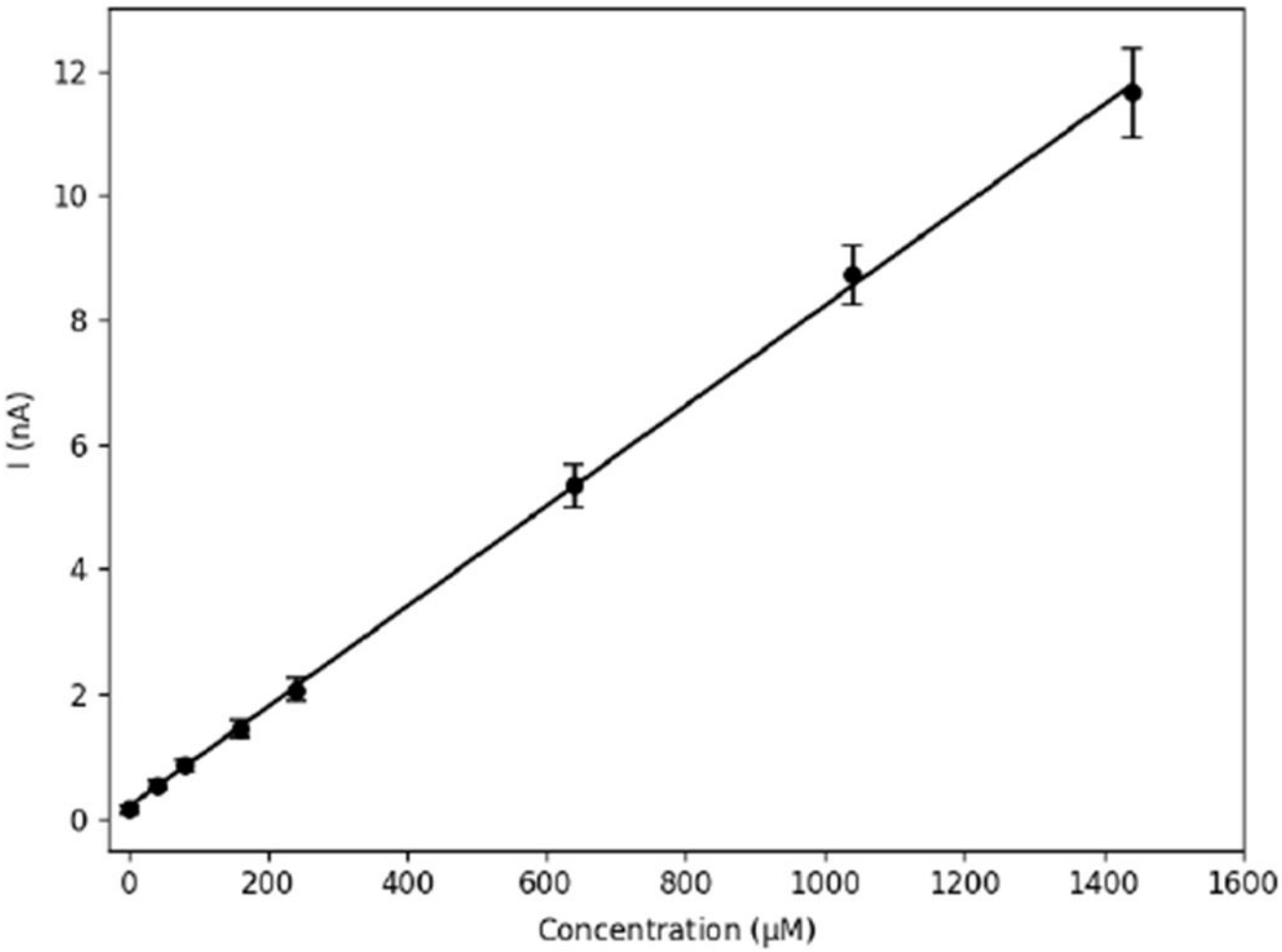

Fig. 4.

Calibration plot for the glucose biosensor. The biosensor response in stirred solution was recorded for sequential injections of glucose to give concentrations of 0, 40, 80, 160, 240, 440, 640, 840, 1040, 1240 and 1440 μM, at a constant potential of 0.7 V (*vs.* Ag/AgCl) in stirred PBS buffer (pH 7.4). Error bars: Standard error of the mean (n = 5).

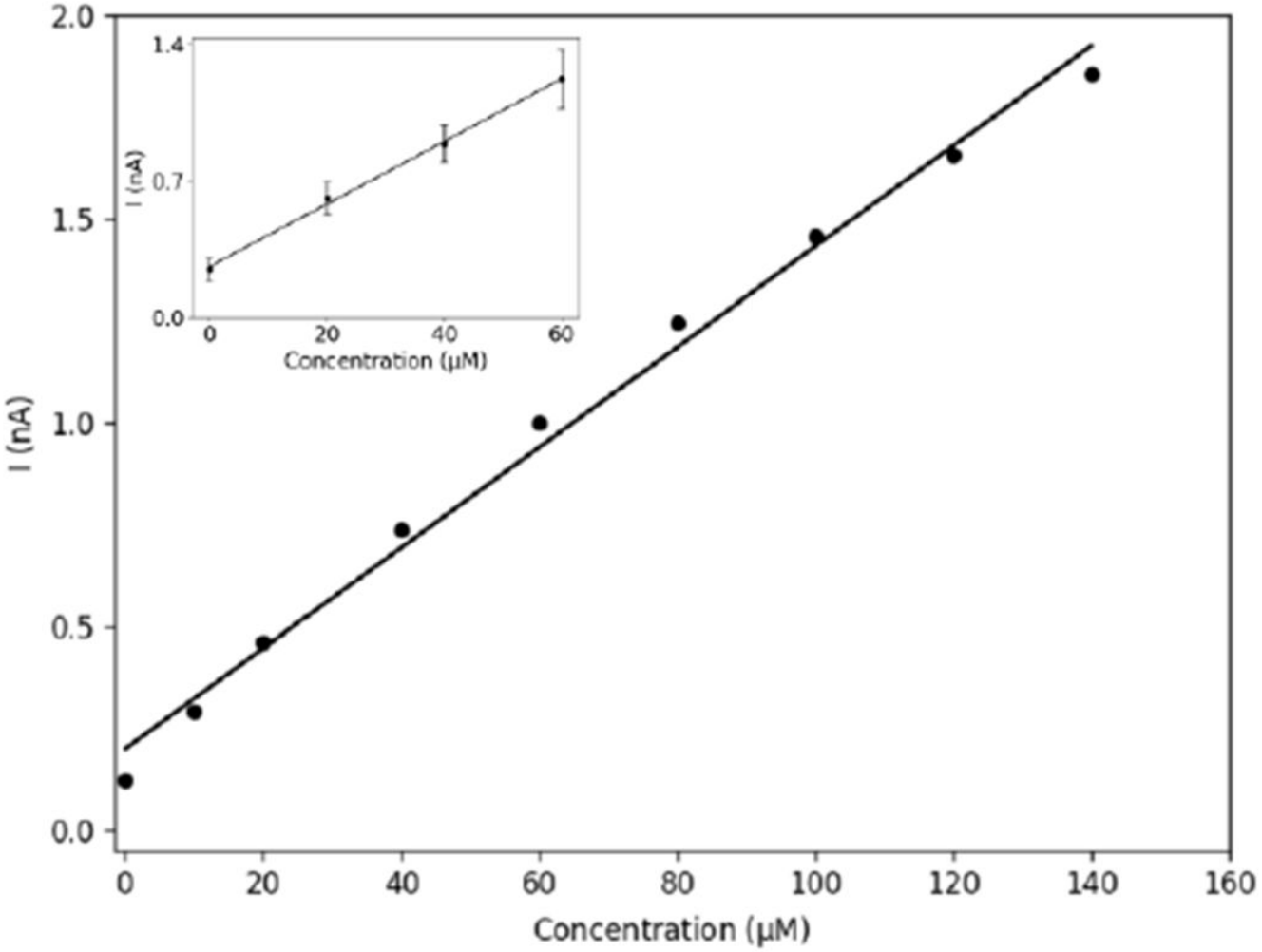

Fig. 5.

Representative calibration plot for the choline biosensor. The biosensor response in stirred solution was recorded for sequential injections of choline chloride to give concentrations of 0, 10, 20, 40, 60, 80, 100, 120, and 140 μM, at a constant potential of 0.7 V (*vs.* Ag/AgCl) in stirred PBS buffer (pH 7.4). The inset plot shows the lower analyte concentration range (0-60 μM). Error bars: standard error of the mean (n = 4).

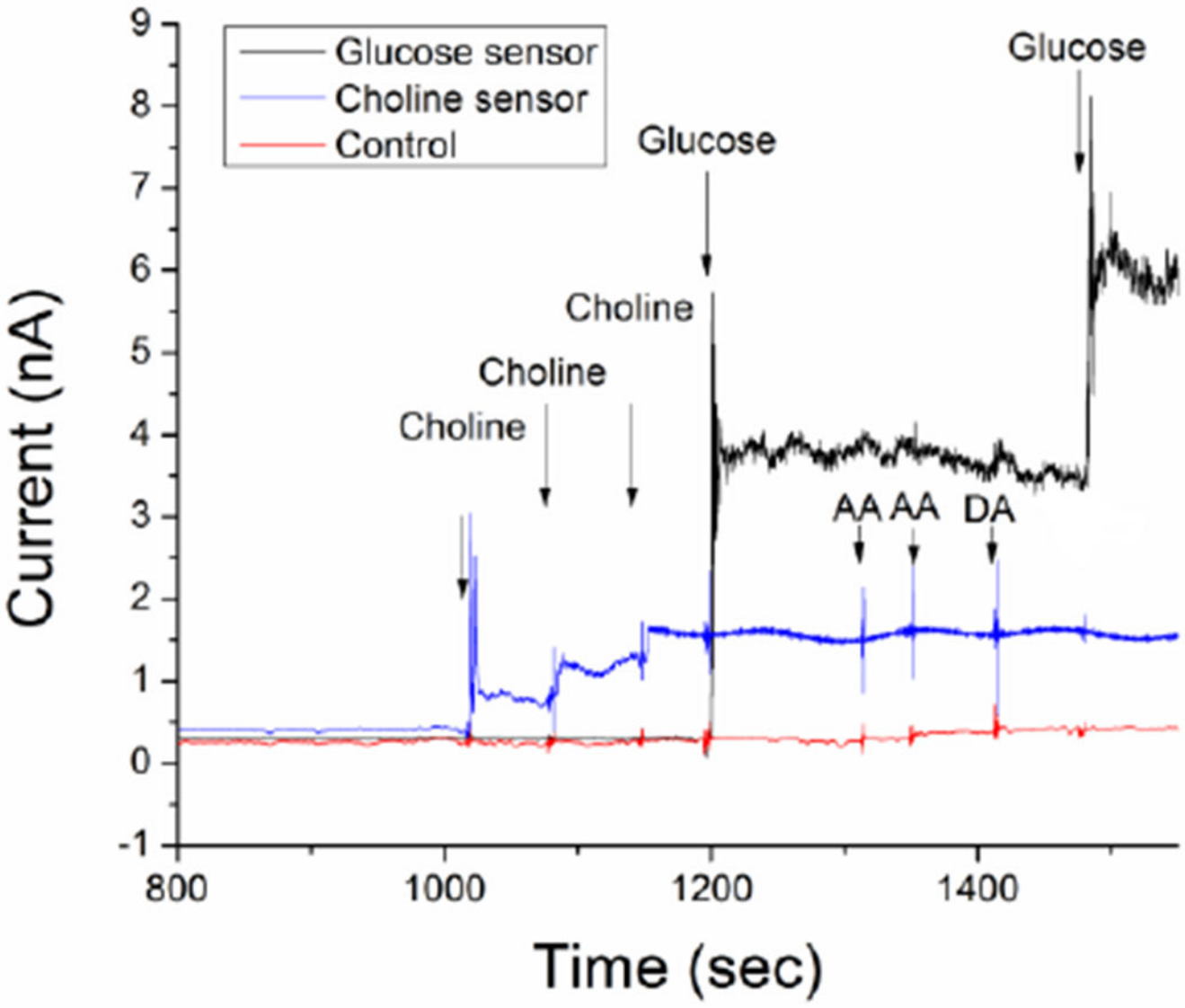

Fig. 6.

Combined sensing of glucose and choline at a constant potential of 0.7 V (*vs.* Ag/AgCl). The microprobe was tested in stirred PBS solution with sequential injections to give 20 μM, 40 μM and 60 μM of choline chloride, 0.6 mM of glucose, 250 μM and 500 μM of ascorbic acid (AA), 5 μM of dopamine (DA) and 1.2 mM of glucose. The Control site was coated with the same permselective PPD film as the sensing sites.

Table 1.

Comparison of the performance characteristics of the glucose microbiosensor of this work with other recently reported electroenzymatic glucose biosensors

Enzyme electrodes	Sensitivity (μA mM^{-1} cm^{-2})	LOD (μM)	Resp Time (s)	Refs.
GOx/Chitosan/PPD/Pt	117	3	~2	This work
GOx/Chitosan/Nafion/PPy/Pt	29.4	4.3	~2	14
GOx/Graphene-Polyaniline/Pt	22.1	2.77	~5	18
Chitosan/GOx/Cysteamine/Au	8.91	49.96	~9	19
GOx/Silver-Chitosan/Pt	67.17	1	NR*	20
Nafion/GOx/Carbon-ZnO nanowire/Ti	35.3	1	~5	21

*NR: not reported

Table 2.

Comparison of the performance characteristics of the choline microbiosensor of this work with other recently reported electroenzymatic choline biosensors

Enzyme electrodes	Sensitivity (μA $mM^{-1}cm^{-2}$)	LOD (μM)	Resp. Time (s)	Refs.
ChOx/Chitosan/PPD/Pt	286	1	~2	This work
ChOx/Nafion/PB/SPE	110	0.5	NR *	23
ChOx/IL/NH_2-MWCNT/GC	125.8	3.85	NR *	24
ChOx/PB/MWCNT/IL/GC	345.4	0.45	2	22
ChOx/PDDA/PB–$FePO_4$/GC	75.2	0.4	2	25
ChOx/Nafion/Pt	264	0.4	1.4	26

* NR: not reported