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A three-dimensional microfluidic flow cell and system integration for improved electrochemical substrate detection in HRP/TMB-based immunoassays

Alexander Ecke,[a,c,†] Jérémy Bell,[a] and Rudolf J. Schneider [a,b*]

[a]*Department of Analytical Chemistry; Reference Materials, Bundesanstalt für Materialforschung und -prüfung (BAM), 12205 Berlin, Germany.*

E-Mail: rudolf.schneider@bam.de

[c]*Department of Chemistry, Humboldt-Universität zu Berlin, 12489 Berlin, Germany*

[b]*Faculty III Process Sciences, Technische Universität Berlin, 10623 Berlin, Germany.*

[†]*Current address: Bundesgesellschaft für Endlagerung mbH; 38226 Salzgitter, Germany.*

I. Microfluidic setup

I.1 2D microfluidic chip manufacturing

For the 2D chip, the channel structure was designed using the software LayoutEditor (juspertor, Unterhaching, Germany) which was then used to produce a mould made of the epoxy-based negative photoresist SU-8 (Kayaku Advanced Materials, Westborough, MA, USA) on a 2-inch silicon wafer (Siegert, Aachen, Germany). The wafer was first rinsed with acetone and dried with compressed air before being activated in a plasma cleaner Zepto (Diener Electronic, Ebhausen, Germany) equipped with vacuum pump ScrollVac SC 5 D from Leybold (Cologne, Germany) at 0.6 mbar in air plasma for 2.5 min. The wafer was then heated to 200 °C for 2.5 min on a hot plate C-MAG HP 4 (IKA, Staufen, Germany) to remove any humidity from the wafer. Those steps ensured stability of the SU-8 resin.

The resin SU-8 2100 (3 mL) was spin-coated onto the wafer using a spin-coater POLOS SPIN150i (SPS, Putten, Netherlands). The spinning speed was first ramped up to 500 rpm at an acceleration of 100 rpm s^{-1} and held for 5 s to spread the photoresist. After-wards, to coat a layer of 100 µm thickness, the speed was ramped up to 3000 rpm at an acceleration of 300 rpm s^{-1} and held for 30 s.

The spin-coated photoresist was then soft-baked in a two-step process for 3 min at 65 min and subsequently 15 min at 95°C on a precision hot plate PZ 28-2 with temperature controller Type 2860 SR (Harry Gestigkeit, Düsseldorf, Germany). After that, the wafer was allowed to cool down to RT before it was placed in a maskless aligner MLA100 (Heidelberg Instruments, Heidelberg, Germany) where it was exposed to UV irradiation at a power of 330 mJ cm^{-2}. Post exposure baking was then performed on a hot plate for 2 min at 65 °C followed by 14 min at 95 °C, before the wafer was allowed to cool to RT.

To develop the cross-linked SU-8, the wafer was immersed in SU-8 photoresist developer (Kayaku Advanced Materials, Westborough, MA, USA) for 15 min with vigorous stirring and further rinsed with the developer. Afterwards, the wafer was dried with compressed air. Finally, the SU-8 mould was made permanent by hard baking in an oven (UN30, Memmert, Schwabach, Germany) for 2 h at 180 °C. A second mould with a slot for the SPE was produced by gluing an SPE on another silicon wafer with commercial cyanoacrylate glue.

For fabrication of the microfluidic chip, PDMS from Sylgard 184 Silicone Kit (Dow Corning, Midland, MI, USA) was mixed with the corresponding curing agent in a ratio of 10:1 and stirred in a beaker. The beaker was then sonicated in an ultrasonic bath Sonorex Super RK 52 (Bandelin, Berlin, Germany) for 5 min and degassed afterwards in a desiccator under vacuum for 20 min. In the meantime, the beforehand produced SU-8 mould and SPE slot moulds were coated with hexamethyldisilazane (Sigma-Aldrich, Taufkirchen, Germany) by chemical vapor deposition for 20 min (RT, 1 atm) to prevent removing the SU-8 when de-moulding the PDMS at a later stage. The degassed PDMS mixture was then poured slowly onto the moulds each in a Petri dish covered with aluminium foil (10 g of PDMS per mould).

The PDMS in the Petri dish was baked in an oven at 140 °C for 15 min and allowed to cool to RT afterwards. The Petri dishes and aluminium foils were removed and the PDMS was carefully peeled off the silicon wafers. Using knife and scalpel, the cured PDMS blocks were cut to chips and holes for channel inlet and outlet were made with a biopsy punch of 1.5 mm diameter.

The two chip parts were cleaned by applying and removing tape on the surface. Afterwards, they were placed in the plasma cleaner to activate the surface with air plasma at 0.6 mbar for 100 s. Subsequently, the two parts were pressed together to form the final chip.

I.2 3D microfluidic chip manufacturing

For the 3D chip, the sacrificial inner structure was modelled using the FreeCAD software and then printed piecewise by fuse deposition modelling of the copolymer ABS using the 3D printer Ultimaker 2 (Ultimaker, Utrecht, Netherlands) equipped with a 0.25 mm nozzle at 255 °C while the bed temperature was set to 70 °C, with 120 % material flow (25 mm s^{-1}), 50 % infill,

no brim. The single parts were combined with cyanoacrylate glue and three prints of the complete model were glued to a 2-inches silicon wafer.

The production of the PDMS chip was performed as described above with 15 mL of PDMS mixture and curing agent. Another chip with a slot to receive an SPE was produced as described above. After baking of the PDMS, the inner structure of the 3D chip was removed by placing the chip in acetone and sonicated for 1h.[27] The resulting PDMS block was dried with compressed air, cut, and bonded to the SPE slot part by plasma activation as described above to form the final 3D chip.

I.3 Chip holders

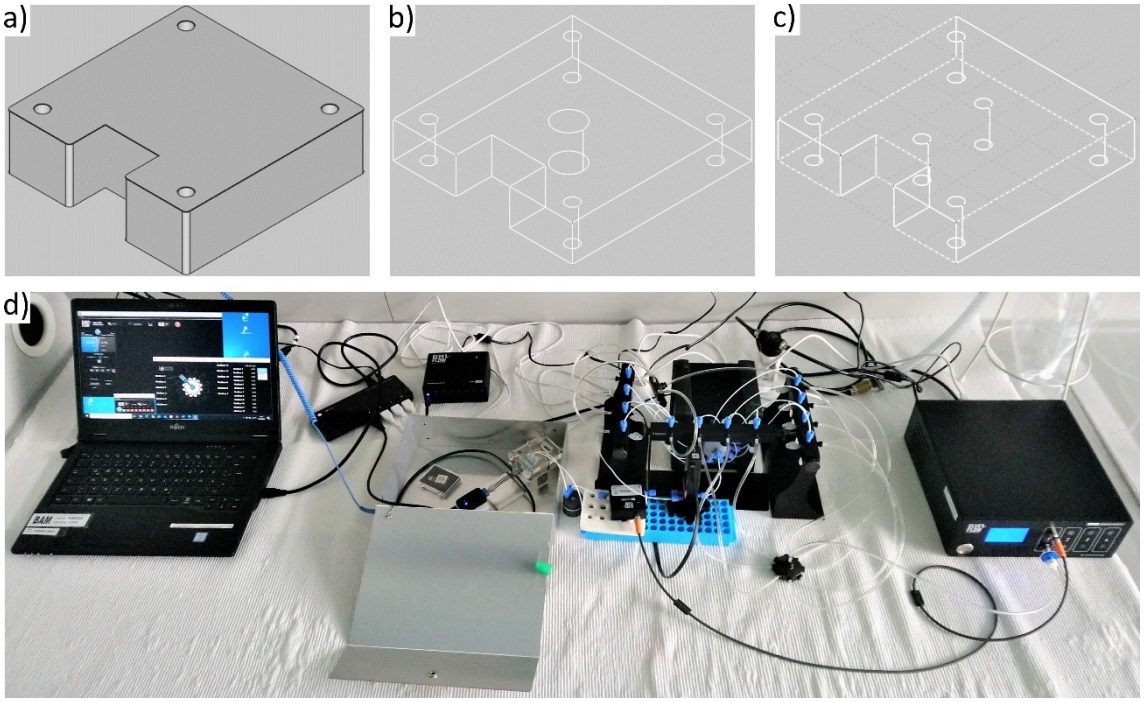

Figure S1. 3D models for chip holder parts: a) bottom plate for both chip designs; B) top plate for 2D chip design, c) top plate for 3D chip design; d) Picture of the setup described in Figure 1.

II. Electrochemical immunoassays

II.2 Immunosensor protocols

All measurements were carried out using the potentiostat Sensit Smart from PalmSens (Houten, Netherlands) controlled with the software PSTrace 5.9, connected to a DropSens SPE 250AT (Metrohm DropSens, Oviedo, Spain) in one of the above-described microfluidic chips. Potentiostat and chip were placed in a Faraday cage shield case from Ivium Technologies (Eindhoven, Netherlands). The electrode surface was activated prior to the measurements by performing cyclic voltammetry with 0.1 M sulfuric acid (0 – 1.6 V, 0.1 V s^{-1}, 5 scans). A magnetic bead-based immunoassay (MBBA) for the quantification of diclofenac (DCF) was conducted as described in a previous study[26] with the difference that potassium citrate monobasic (Sigma-Aldrich) was used instead of sodium citrate in the substrate solution, and 1 M sulfuric acid with 0.3 M potassium chloride (Alfa Aesar, Kandel, Germany) was used for stopping the reaction. The oxidized substrate solutions from the MBBA were transferred to 2 mL amber reaction tubes from Eppendorf (Hamburg, Germany) which were connected to the respective reservoir

holders of the microfluidic system. Additionally, three reaction tubes were filled with buffer solution consisting of 5.5 mL of 220 mM potassium citrate buffer (pH 4.0), 2.1 µL of 30 % hydrogen peroxide (Sigma-Aldrich), 2.82 mL of 1 M sulfuric acid with 0.3 M potassium chloride, and 138 µL of TMB stock solution containing 8 mM of tetrabutylammonium borohydride (Sigma-Aldrich) and 40 mM of 3,3',5,5'-tetramethylbenzidine (Serva) in dry *N,N*-dimethylacetamide (Sigma-Aldrich). Another reaction tube was filled with 25 % *i*PrOH (Th. Geyer, Renningen, Germany) in Milli-Q® water (MQ).

The microfluidic system was then flushed with 250 µL of 25 % *i*PrOH, followed by 500 µL of MQ and 300 µL of buffer at a pressure of 600 mbar. During the last step, the open-circuit potential (E_{OCP}) was measured by switching on the potentiostat without applying a potential. When the E_{OCP} reached a value of approximately 0.3 to 0.4 V and remained constant, a potential of 0.33 V was applied and the chronoamperometric measurement was started. The flow rate was adjusted to 500 µL min^{-1} (3D chip) or 50 µL min^{-1} (2D chip) and maintained by automatically adapting the pressure (1320 – 1340 mbar). The oxidized substrate solutions (250 µL) were then injected successively each followed by flushing with 200 µL buffer until every substrate solution was analysed three times. Afterwards, the microfluidic system was flushed with 400 µL of buffer, 1000 µL of MQ, 500 µL of 25 % *i*PrOH and dried by flowing compressed air at a pressure of 500 mbar through the system. The peak currents of the signals were plotted against the concentration of calibrators to obtain a calibration curve using the software Origin® 2019 (OriginLab, Northampton, MA, USA).

II.2 Electrochemical measurement optimisation

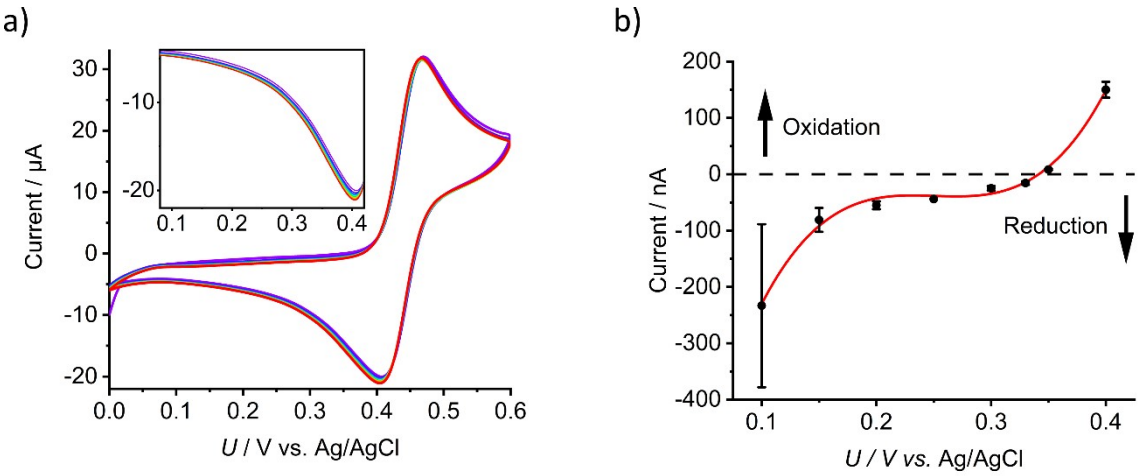

Figure S2. a) Cyclic voltammogram of TMB at pH 1 at a scan rate of 0.1 V s^{-1} on a gold SPE (10 scans). Peak potentials: E_{pa} = 469 mV; E_{pc} = 408 mV. b) Background current as a function of the applied potential in chronoamperometry with 650 µM TMB in 143 mM potassium citrate buffer containing 2.5 mM H_2O_2, 333 mM H_2SO_4, and 100 mM KCl.

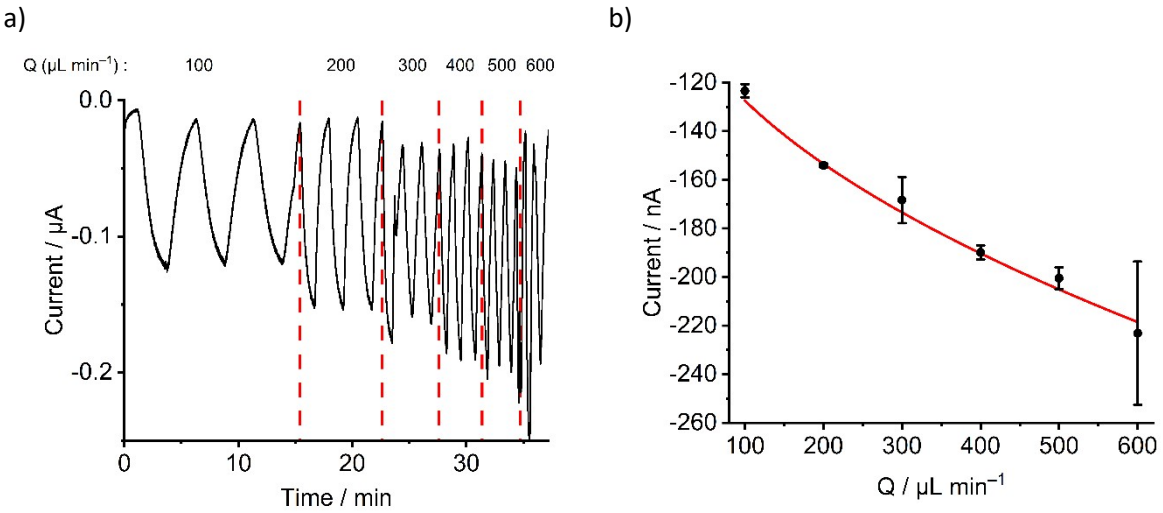

Figure S3. a) Time course of the current signal during reduction of oxidized TMB upon consecutive injection of 250 µL oxidized TMB solutions (with O.D. (450 nm – 620 nm) of approximately 1.0) and buffer in triplicate for each flow rate. b) Plot of the peak currents against the respective flow rate and curve fitting using a negative square root function.

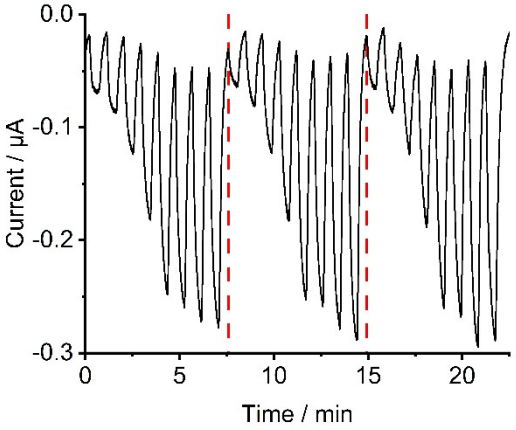

Figure S4. a) Time course of the current during three successive injection cycles of eight different substrate solutions from the DCF-MBBA with different DCF concentrations (U = 330 mV *vs.* Ag/AgCl, Q = 500 µL min^{-1}). b) Plot of the peak currents against the concentration of the DCF calibrators and comparison with the calibration curve obtained in optical detection showing high concordance.