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Probing of microgel–enzyme films on graphite substrates by means of atomic force microscopy and amperometry

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Experimental Section

Materials: Glucose oxidase (GOx) from *Aspergillus niger*, E.C. 1.1.3.4, activity 168100 U/g solid was purchased from Sigma-Aldrich (Steinheim, Germany), β-D-glucose was received from ICN Biomedicals, Inc. (Aurora, OH, USA). Tris(hydroxymethyl)aminomethane (TRIS) and its hydrochloride (TRIS-HCl) were obtained from Sigma-Aldrich (Steinheim, Germany). The microgel was synthesized via precipitation polymerization of N-isopropylacrylamide (NIPAM) and a cationic comonomer N-(3-aminopropyl)methacrylamide (APMA) in the presence of a cross-linker N,N'-methylene bisacrylamide according to procedure described in [S1]. The content of NIPAM, APMA, and the cross-linker in the reaction mixture were about 92%, 3% and 5% (mol.), respectively. All other chemicals were of analytical grade and used without further purification. Deionized water (18.2 MΩ cm) purified with a Milli-Q purification system from Millipore (Burlington, MA, USA) was used as a solvent for preparation of all solutions.

Electrochemical Microgel-Enzyme Constructs: The screen-printed electrodes (SPEs) were fabricated on poly(vinyl chloride) substrates of 0.2 mm thickness by means of conductive graphite paste from Gwent Group Co. (Pontypool, UK) screen-printed by a semi-automated Winon machine (model WSC-160B, Winon Industrial Co., Ltd., Hong Kong, China) with a 200 mesh screen stencil. Each SPE consisted of a round-shaped working area (2.5 mm diameter), a conductive track (30 mm × 1.5 mm), and a square extremity (3 mm × 7 mm) for electrical contact. The SPEs were pre-modified with a peroxide-sensitive layer of manganese dioxide nanoparticles according to procedure described elsewhere [S2]. The SPE/MnO_2 electrodes were stored dry at an ambient temperature until further use. Microgel particles were adsorbed onto SPE/MnO_2 at a temperature of 50 °C via the dip coating method by dipping the electrodes into a preheated 1 g/L dispersion of P(NIPAM-*co*-APMA) microgel in 10 mM TRIS of pH 9.5 for 60 min adsorption. After that time, the electrode surface was rinsed with Milli-Q water and was very shortly (for 1−2 s) blown by a stream of air. Directly after this, GOx was adsorbed in a similar way from 9×10^{-5} M solution in 10 mM TRIS of pH 7 at 20 °C for 40 min, followed by rinsing with Milli-Q water and shortly drying with a stream of air. To prevent the loss of enzymatic activity, the SPE covered by microgel-enzyme films were stored at +4 °C until further use.

Electrochemical Assay: Electrochemical experiments were performed in a water-jacket one-compartment electrochemical cell with stirring (volume of 1 mL), using a three-electrode configuration. The SPE-MnO_2/P(NIPAM-*co*-APMA)/GOx constructs were used as the working electrode, while an Ag/AgCl and a platinum wire served as a reference and a counter electrode, respectively. A potentiostat IPC Compact (Kronas Ltd., Moscow, Russia) used for electrochemical measurements was interfaced with a PC and electrochemical parameters were controlled by the potentiostat software. The necessary temperature of

the water-jacket electrochemical cell was maintained by a thermostat Huber CC-K6 (Huber, Offenburg, Germany). Electrochemical responses were assayed in a 50 mM HEPES/30 mM KCl buffer of pH 7.5 by recording the current arising after the addition of a solution of β-D-glucose with a standard concentration (2 mM in the cell). The oxidative current is generated in response to the addition of a substrate (β-D-glucose) solution at an applied potential of +450 mV *vs* Ag/AgCl. Each electrochemical response was determined as a value of steady-state baseline current change (the difference between an average value of steady-state baseline current before and after the analyte addition).

Atomic Force Microscopy (AFM): Freshly cleaved highly oriented pyrolytic graphite (HOPG) was used for visualization of the microgel and microgel-enzyme coatings by means of AFM. The P(NIPAM-*co*-APMA) microgel was adsorbed onto HOPG (slices with the size of 5 mm × 10 mm) at ambient temperature by covering the substrate with a 20-40 μL drop of the microgel solution (1 g/L), followed by adsorption for 1 hour. After that time, the substrate was gently rinsed with Milli-Q water and shortly blown by a stream of air. The enzyme was adsorbed similarly by depositing a drop of the GOx solution (1×10^{-5} M) for 40 min. After that the substrate was again gently rinsed with Milli-Q water and shortly blown by a stream of air. AFM images were taken with a commercial atomic force microscope Asylum MFP-3D-SA (Asylum Research, Santa Barbara, CA, USA) operating in a semicontact mode in air. The cantilevers (fpN 10S (Super), F.V. Lukin State Research Institute for Problems in Physics, Russia) with a tip curvature radius ≤10 nm, a tip cone angle ≤22°, and a resonance frequency 190-325 kHz were used. The obtained results were presented as typical images chosen on the basis of at least five 5 μm × 5 μm uniform-sized images obtained from different places of each AFM sample. They were initially flattened using the MFP3D software version 120804 + 2702 (Asylum Research, Santa Barbara, CA, USA) run in Igor Pro 6.36 environment and then further analyzed with the Gwiddion 2.62 program. Lateral diameters and heights of the adsorbed microgel or microgel-enzyme complex particles were calculated for the separated ones.

Spectrophotometric assay: The GOx activity was assayed in a 96-well microplate using a microtiter plate reader xMark Bio-Rad (USA), according to a protocol described in the literature [S3] with slight modifications. Each well was filled with 290 μL of the reagent mixture containing 131 mM of β-D-glucose; 0.2 mM of 4-aminoantipurine; 0. 875 mM of phenol, and 5mU of horse radish peroxidase diluted in 50 mM HEPES/30 mM KCl buffer (pH 7.5). The microplate was preincubated at specified temperature in a range of 25-45°C. The enzymatic reaction was started by the addition of a 10mL aliquot of GO from a stock solution preincubated for 10 min in 50 mM HEPES/30 mM KCl buffer (pH 7.5) at the same temperature. The appearance of the colored product was measured at λ=500 nm in five cycles with 20 s duration. The GO activity was assumed to be proportional to the increase in the absorbance of the samples with time and it was expressed as mOD/min.

References

S1. L. V. Sigolaeva, D. V. Pergushov, M. Oelmann, S. Schwarz, M. Brugnoni, I. N. Kurochkin, F. A. Plamper, A. Fery and W. Richtering, *Polymers*, 2018, **10**, 791.

S2. E. A. Dontsova, Y. S. Zeifman, I. A. Budashov, A. V. Eremenko, S. L. Kalnov, I. N. Kurochkin, *Sens. Actuators, B,* 2011, **159**, 261.

S3. https://www.toyobo-global.com/seihin/xr/enzyme/enzyme_list/index.html

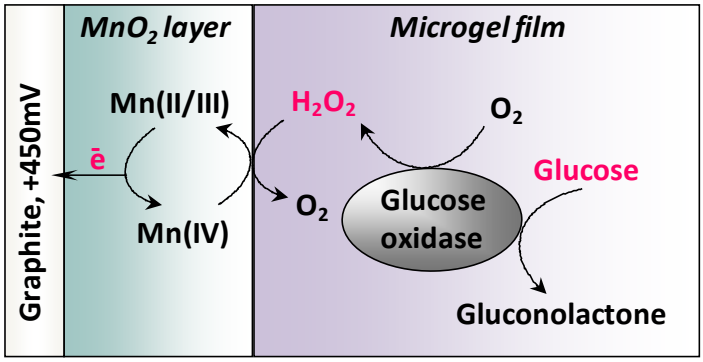

Figure S1. A principal scheme of electrochemical analysis of glucose by microgel-based biosensor.

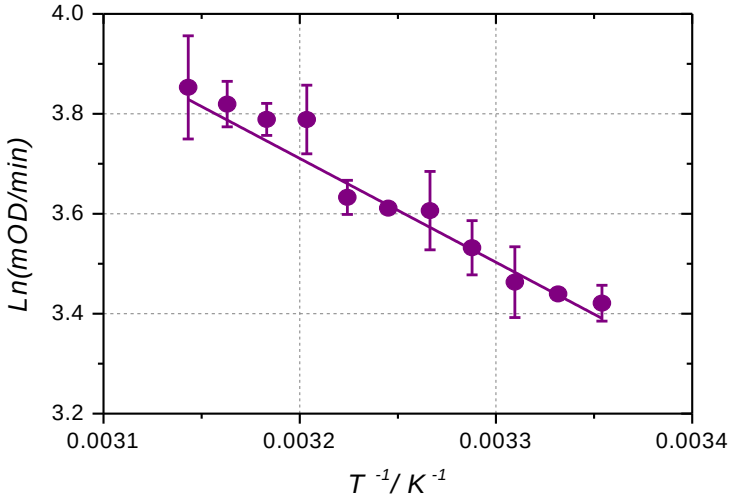

Figure S2. The temperature dependence (in the Arrhenius' coordinates) of the GO activity measured spectrophotometrically in 50 mM HEPES/30 mM KCl (pH 7.5).