Determination of Asymmetric Dimethylarginine by Using Organic Semiconductor-Based Molecularly Imprinted Polymer Film

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A simple and sensitive chemosensor for the determination of asymmetric dimethylarginine (ADMA) was fabricated and tested. In this chemosensor, an electrochemically deposited molecularly imprinted polymer film of thiophene-based organic semiconductor served as the recognition unit. Analytical parameters of the chemosensor were tested with use of voltammetric and impedimetric signal transduction. The devised chemosensor responded linearly from 0.3 to 2.2 μM , with LOD of 0.3 or 0.4 μM for DPV and EIS transduction, respectively. Moreover, the chemosensor operated in the presence of excess of interferences. The determined analytical parameters indicated applicability of the chemosensor for determination of ADMA in body fluids.

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Manuscript submitted February 21, 2018; revised manuscript received April 13, 2018. Published May 17, 2018. *This paper is part of the JSS Focus Issue on Semiconductor-Based Sensors for Application to Vapors, Chemicals, Biological Species, and Medical Diagnosis.*

Asymmetric dimethylarginine (ADMA), is a naturally occurring analogue of L-arginine. Presumably, it is an endogenous inhibitor of nitric oxide synthase (NOS) enzyme.¹ It competes with the binding of its natural substrate, L-arginine.² In living organisms, ADMA is generated by enzymes, protein methyltransferases, from methylated arginine residues in proteins.³ It is then partially metabolized by the enzyme *N*^G,*N*^G-dimethylarginine dimethylaminohydrolase 1 and 2 (DDAH) to citrulline and dimethylamine.⁴ Partially, it is eliminated from the body via renal excretion.

Generally, high ADMA levels in the body are associated with decreased nitric oxide (NO) biosynthesis. Importantly, ADMA concentration in plasma is increased almost two-fold in patients with hypercholesterolemia,⁵ however it is unaffected by L-arginine concentration. ADMA accumulates in the plasma of patients with atherosclerosis,⁶ hypertension,⁷ diabetes,⁸ renal failure,² and chronic heart failure.⁹ Moreover, ADMA concentration is elevated during pregnancy.¹⁰ Furthermore, ADMA might be an independent predictor of future vascular events.¹¹ Interestingly, concentration of the ADMA is independent of the patient age or gender.¹ Therefore, development of fast, simple, and reliable procedure of ADMA determination in blood plasma would be of great interest. Until now, analytical methods used for ADMA determination include mainly HPLC.^{12–14} Application of a few other methods have been also reported including capillary electrophoresis (CE),¹⁵ liquid chromatography-mass spectrometry (LC-MS)¹⁶ and gas chromatography coupled to either mass spectrometry (GC-MS)¹⁷ or tandem mass spectrometry (GC-MS-MS).¹⁸ Another method used for determination of ADMA is enzyme-linked immunosorbent assay (ELISA).^{14,19} Unfortunately, chromatographic methods typically require expensive equipment and highly-trained personnel to operate, which make them costly. Quite often derivatization of the ADMA is required to achieve high sensitivity of the analysis.¹⁴ Besides, ELISA tests are also costly and suffer from insufficient stability and reproducibility.

Development of a simple, reliable, and inexpensive procedure of ADMA determination may be achieved with chemo- or biosensors using artificial or natural receptors, respectively. Surprisingly, to the best of our knowledge, there is no literature report on such a sensor. The only work available related to this issue was fabrication of

a nanosensor for arginine.²⁰ This sensor used a genetically encoded Förster resonance energy transfer and employed the arginine repressor/activator *ahrC* gene from *Bacillus subtilis*. In this work, ADMA was used only as the interference to the main analyte.

In our present work we describe simple, fast and cheap procedure of ADMA determination using a new chemosensor with an artificial recognition unit. To this end, we deposited by electropolymerization a molecularly imprinted polymer (MIP) based on a thiophene organic semiconductor as the recognition unit of the devised chemosensor. MIPs form an interesting class of synthetic molecular receptors, which can be used as recognition elements in chemosensors for small organic molecules,^{21–23} macromolecules such as proteins, RNA or DNA,^{24,25} or even whole bacteria.^{24,25} They offer numerous advantages over biological receptors as they are easy to design, robust, and show high resistance to organic solvents, high temperatures and high or low pH. Briefly, the idea of MIPs preparation is based on the formation of a pre-polymerization complex of the analyte template with suitable functional monomers in solution. Then, this complex is polymerized and, subsequently, the analyte is extracted. This leads to the formation of molecular cavities in the polymer with the size, shape, and orientation of recognizing sites preferentially fitting the analyte. MIPs have found applications in separation, catalysis, drug delivery, and chemosensor development.²² For the purpose of sensor devising, formation of a MIP thin film is often required. To this end, deposition of conducting polymer films by electropolymerization has successfully been applied.^{26,27} These polymers belong to the class of organic semiconductors that has been applied in chemosensing as both recognition and transduction elements.^{26,28–30} Electropolymerization allows for facile preparation of conducting polymer films as the recognition element in the form of a thin film. Moreover, inherent electrical and optical properties of the conducting polymers can be used as a means to transduce the chemical binding event into a convenient analytical signal of the chemosensor.

In the present work, we used a thin film of an organic semiconductor based on thiophene derivatives for fabrication of a MIP recognition unit of the chemosensor for the ADMA determination. For this purpose, DFT quantum calculations have been first used to select most suitable functional monomers. Then, potentiodynamic deposition by electropolymerization has been applied for integration of the resulting MIP films with selected transduction element. The devised chemosensor showed the linear dynamic concentration range, selectivity, and

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the limit of detection suitable for ADMA determination in body fluids.

Experimental

Asymmetric dimethylarginine (ADMA), arginine, glucose, acetonitrile, and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich and used without further purification. Tetra-butylammonium perchlorate was purchased from Fluka. Sodium hydroxide and potassium nitrate were from POCh (Poland). Potassium hexacyanoferrates (II and III), and sulfuric acid were from Chempur (Poland). All chemicals were of analytical grade and were used without further purification. Functional monomers, *bis*(2,2'-bithien-5-yl)-methylbenzo-18-crown-6 (F.Crown) and *p-bis*(2,2'-bithien-5-yl)methylbenzoic acid (F.Carboxy) and crosslinking monomer 4,4'-Bisthiophene-3-yl-5,5'-bisthiophene-2-yl-3,3'-(2,2'-bithiophene) (CL), were synthesized according to the procedures described in literature.^{31–33}

The differential pulse voltammetry (DPV), cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS) experiments were performed with the SP-300 potentiostat (Bio-Logic Science Instrument, France) controlled by EC Lab software version 11.01 of the same manufacturer.

All electrochemical measurements were performed using a three-electrode single-compartment V-shape glass cell. A 0.44 mm² Pt disk glass sealed electrode, Pt coiled wire, and Ag wire were used as the working, counter, and pseudo-reference electrode, respectively. Before experiments, the working electrode was polished with 0.05 μm alumina, and treated with the "Piranha" solution (H₂SO₄: H₂O₂ = 3: 1 v/v).

For atomic force microscopy (AFM) and surface-enhanced Raman spectroscopy (SERS) measurements polymer films were deposited on Au-coated glass slides, with 100-nm thick Au layer and 10-nm Ti underlayer.

The AFM imagings were performed with Multimode 8 microscope equipped with Nanoscope V controller and PeakForce QNM module, and driven by MultiMode 8.15 software of Bruker (Germany). Silicon cantilevers with silicon tips having force constant of 16 N/m were used in all measurements.

The SERS measurements were carried out using in Via Raman system of Renishaw (United Kingdom) equipped with a 785-nm solid-state laser. The spectra were recorded after introducing 60 nm Ag nanoparticles on the sample surface. The recorded spectra were processed with Wire 3.4 software from the same manufacturer.

Polarization-modulated infrared reflection-absorption spectra (PM-IRRAS) were recorded on films deposited on Au-coated glass slides by using Vertex 80v spectrophotometer and PMA50 accessory equipped nitrogen-cooled MCT detector of Bruker (Germany). For each measurement, 1024 spectra were recorded with spectral resolution of 2 cm⁻¹.

The quantum-chemical modeling of the pre-polymerization complex structure were performed in vacuum at the DFT level of theory by using the B3LYP/6-31G functional and the basis set implemented in Gaussian 09 rev. E software. This basis set and functional has been selected as allowing for obtaining reasonably precise calculations at reasonable calculation costs.

The films of MIP and control non-imprinted polymer (NIP) were deposited by potentiodynamic potential cycling between 0 and 1.30 V vs. Ag pseudo-reference electrode with the scan rate 50 mV/s. The MIP film was deposited by electropolymerization from 0.1 mM ADMA, 0.2 mM F.Crown, 0.1 mM F.Carboxy, 0.5 mM CL, 0.1 M (TBA)ClO₄ in the ACN : DMSO (95: 5 v/v) solution. Composition of the solution for NIP deposition was the same except of ADMA absence. After film deposition, electrodes were rinsed with ACN to remove not polymerized monomers and the supporting electrolyte. The ADMA template was extracted from the polymer in 0.1 M NaOH solution at room temperature for 1 h. NIP film was also treated in identical way before further experiments.

The DPV measurements for confirmation of template extraction and then analyte determination were accomplished under batch conditions using 1 mM K₄[Fe(CN)₆] in 0.1 M KNO₃. The DPV measurement conditions were as follows: the step potential was 5 mV the pulse amplitude was 25 mV and the pulse width was 50 ms. Potential was scanned in the range of 0 – 0.50 V vs. Ag pseudo-reference electrode. Aliquots of the stock solution of ADMA in 1 mM K₄[Fe(CN)₆] in 0.1 M KNO₃ were added stepwise to achieve the necessary concentration of ADMA in the test solution. After each ADMA aliquot addition, the solution was stirred for 30 min to equilibrate. The EIS measurements were performed using a mixed solution of 0.1 M K₄[Fe(CN)₆] and 0.1 M K₃[Fe(CN)₆] at open circuit potential (OCP). We used this approach to avoid possible problems with potential shifts between measured samples. In our studies electrode was coated with relatively thick and weakly conductive polymer film with substantial resistance. This could lead to ohmic losses and shifts of the observed potential of the studied redox process leading to substantial errors if a constant potential would be used. Sinusoidal wave potential with 100 mV amplitude has been superimposed on the OCP. The range of scanned frequencies was from 500 kHz to 0.1 Hz.

Results and Discussion

Quantum chemical modeling of pre-polymerization complexes.—For rational selection of functional monomers, DFT calculations employing the B3LYP/6-31G functional and basis set were used for estimation of the ADMA binding by various functional monomers (Table S1 in Supporting Information). For that purpose, structures of the template and functional monomers were optimized separately. Subsequently, structures of the complexes of the template with selected functional monomers were optimized. Imaginary frequency analysis was subsequently performed in order to ascertain that the calculated structure indeed corresponds to the energy minimum. The calculated Δ*G* values of the complex formation are shown in Table S1. The lowest Δ*G* value (−499 kJ/mol) indicate formation of a very stable complex composed of the ADMA molecule with two molecules of F.Crown and one molecule of F.Carboxy. The resulting optimized structure of the pre-polymerization complex, together with the complex structural formula, is presented in Scheme 1.

Deposition of the recognition polymer layer.—In Figure 1, potentiodynamic curves recorded during deposition of both MIP (Fig. 1a) and NIP (Fig. 1b) are presented. Peak at ~1.20 V, characteristic for irreversible oxidation of *bis*(bithienyl)methane moieties, appears in curves recorded during deposition of both MIP and NIP. Noticeably, peak current decreases during consecutive cycles in both cases, which is indicative of poorly conductive films formation. There are relatively small differences between the CV curves recorded during MIP and NIP deposition. Notably, there is a small shift in peak potential of monomers oxidation of ca. 80 mV toward more positive potential values for the CV curves recorded during MIP deposition (Fig. 1a) when compared to those observed during NIP deposition (Fig. 1b). This would be consistent with formation of the pre-polymerization complex of monomers and ADMA leading to small changes in oxidation potential of the thiophene monomers. Furthermore, there is slower decrease of peak current in the consecutive cycles in case of the CV curve recorded during MIP deposition than it was observed during NIP deposition. Moreover, more pronounced oxidation peak at around 1.05 V is visible after few consecutive cycles during formation of the MIP film. This peak is less pronounced during the NIP deposition. This seems to indicate that NIP film is still less conducting and/or more compact than MIP and it is more efficiently blocking the electrode.

Still, in both cases observed differences are relatively small indicating formation of films with quite similar properties and morphology, as corroborated by AFM imaging (Fig. 3).

Scheme 1. (a) Structural formulas of the asymmetric dimethylarginine, functional monomers *bis*(2,2'-bithien-5-yl)-methylbenzo-18-crown-6 (F.Crown) and *p-bis*(2,2'-bithien-5-yl)methylbenzoic acid (F.Carboxy) as well as cross-linking monomer 4,4'-bisthiophene-3-yl-5,5'-bisthiophene-2-yl-3,3'-(2,2'-bithiophene) (CL). (b) DFT calculated structure of the ADMA-F.Crown-F.Carboxy complex with the ratio 1: 2: 1.

Template extraction.—The ADMA template extraction step, allowing for emptying the cavities formed in the MIP film, is very important for sensor operation. To prove efficient extraction, DPV and SERS experiments were performed. DPV studies allowed proving the extraction indirectly using, so called, “gating effect”.³⁴ This concept is based on differences in diffusion of redox probe through a polymer film before and after the analyte removal from the imprinting cavities. In the simple case, diffusion of the redox probe to the electrode surface shall be easier if the imprinted cavities are unoccupied. This would lead to increase of the redox probe peak current after successful MIP extraction. For that, the DPV measurement were performed for MIP before and after extraction, as well as for NIP-coated electrodes in 1 mM solution of $K_4[Fe(CN)_6]$ as red-ox probe in 0.1 M KNO_3 . The results are shown in Figure 2a. Before polymer deposition, there was a well-pronounced DPV peak of the red-ox probe oxidation at 0.17 V. On the other hand, this peak almost completely disappeared after deposition of the MIP or NIP films indicating the blocking effect of both films. That is, these films did not allow for the red-ox probe diffusion to the electrode surface. After template extraction, the red-ox probe peak reappeared, however, its current was much smaller than that recorded at the bare electrode. This is indicative of the red-ox probe facilitated diffusion through the polymer film because of emp-

tying imprinted cavities. For the NIP, both before and after treatment with 0.1 M NaOH, the red-ox probe peak was not observed.

To further confirm the extraction, a direct method was employed. To this end, surface-enhanced Raman spectra (SERS) of the MIP film before and after extraction as well as the NIP film (Fig. 2b) were recorded after deposition of Ag nanoparticles on the film via drop-casting method. In the MIP film after extraction peaks at 1118 and 795 cm^{-1} ascribed to ADMA (Fig. 2b) disappeared from the spectrum. Moreover, they were also not seen in the spectrum of the NIP film.

MIP and NIP films characterization.—To confirm deposition of the polymer film with the required composition, as well as its preservation after template extraction, PM-IRRAS spectra of MIP and NIP after extraction were recorded (Fig. S1). Bands which can be attributed to vibrations of thiophene moieties at 1430, 873 and 728 cm^{-1} characteristic for symmetric C=C stretching, asymmetric C-S stretching, and C-S in-plane deformation,³⁵ respectively, can be discerned in the spectra of both films. Moreover, bands between 1710 and 1730 cm^{-1} characteristic for C=O stretching of the carboxyl group of F.Carboxy monomer and the band at 1103 cm^{-1} characteristic for C-O-C stretching of the crown ether moiety of the F.Crown monomer are seen in the spectra of both MIP and NIP.³⁶

Figure 1. Current-potential curves recorded during potentiodynamic electrodeposition of (a) molecularly imprinted polymer and (b) non-imprinted polymer on Pt electrodes. Polymerization was performed in 0.1 mM ADMA, 0.2 mM F.Crown, 0.1 mM F.Carboxy, and 0.5 mM CL in 0.1 M (TBA)ClO₄, ACN: DMSO (95: 5, v: v) at 50 mV/s scan rate during 10 potential cycles. NIP film was deposited from the solution of the same composition but without ADMA.

Figure 2. (a) Differential pulse voltammetry curves recorded at MIP and NIP-coated Pt electrode in 1 mM $K_4[Fe(CN)_6]$, 0.1 M KNO_3 solution. Pulse duration and pulse amplitude were 50 ms and 25 mV, respectively. (b) Surface enhanced Raman spectra of Au-coated glass slides coated with the MIP or NIP film. The Raman spectrum of the ADMA powder is given as reference. Excitation wavelength was 785 nm.

The AFM topography images as well as maps of adhesion, deformation and dissipation have been recorded for the MIP film before and after template extraction, and for the NIP film. Topography, adhesion and deformation maps are shown in Fig. 3, while calculated nanomechanical parameters of the studied films are presented in Table S2. All films were composed of non-uniform ill-defined grains with diameter in the range of 50 to 100 nm. Images indicate also the presence of

spheroidal depressions in the deposited films. The thickness is quite similar for all deposited films. The thickness values for the MIP before and after extracted, as well as for the NIP were 158, 162 and 153 nm, respectively. Roughness of the MIP film slightly increased after ADMA template extraction, but it was still lower than that of the NIP film. Mapping of the film's nanomechanical properties indicated that the MIP film after template removal became much softer,

Figure 3. Atomic force microscopy topographic as well as adhesion and deformation images of MIP before and MIP after ADMA extraction with 0.1 M NaOH, and NIP films. Films were deposited during 10 potential cycles with 50 mV/s scan rate on Au-coated glass slides from 0.1 mM ADMA, 0.2 mM F.Crown, 0.1 mM F.Carboxy, and 0.5 mM CL in 0.1 M (TBA)ClO₄ solution of ACN: DMSO (95: 5, v: v). The NIP film was deposited from the solution of the same composition but without ADMA.

Figure 4. (a) Differential pulse voltammetry curves recorded at MIP film-coated Pt electrode in 1 mM $K_4[Fe(CN)_6]$, 0.1 M KNO_3 solution after addition of (1) 0.0, (2) 0.2, (3) 0.8, (4) 1.0, (5) 1.4, and (6) 2.2 μM ADMA. Pulse duration and pulse amplitude were 50 ms and 25 mV, respectively. Inset shows magnified region around the DPV peak maximum. (b) DPV calibration plots for MIP and NIP-coated Pt electrodes in 1 mM $K_4[Fe(CN)_6]$, 0.1 M KNO_3 solution.

as pointed out by the increased deformation and energy dissipation of that film.

Analyte determination.—Analytical performance of the devised recognition unit was tested under batch conditions. The DPV and EIS measurements with the use of $K_4[Fe(CN)_6]$ as the red-ox probe were performed as transduction techniques. To this end, a sample of the analyte solution was added to the test solution in the cell. Then the solution was stirred for 30 min, and DPV and EIS curves were recorded. For the DPV experiment, a consecutive decrease of the DPV peak current was observed after each ADMA addition (Fig. 4a). The DPV peak current for solution without the analyte was subtracted from each peak current for ADMA addition to bring calibration plot for MIP and NIP to a common scale. On the basis of the normalized peak current, the calibration plot was constructed (Fig. 4b). This plot was linear at least in the range of 0.3 to 2.2 μM obeying the linear regression equation $i_{DPV}(\mu A) = -0.052 c_{ADMA}(\mu M) - 0.014$ ($R^2 = 0.9354$). The analytical parameters determined are summarized in Table I. Identical experiments were performed using the electrode coated with the NIP film. The apparent imprinting factor (AIF) for the DPV transduction was extremely high as the NIP film-coated electrode practically did not respond to changes in the ADMA concentration. This result indicates effective formation of imprinted cavities and small non-specific binding of the ADMA in the MIP.

The impedance spectra recorded for MIP- and NIP-coated electrodes after addition of the ADMA aliquots are shown in Fig. 5a. All the spectra have been recorded at OCP in the equimolar solution of $K_4[Fe(CN)_6]/K_3[Fe(CN)_6]$. The obtained EIS results can readily be

fitted with the Randles-Ershler model with use of a constant phase element instead of a pure capacitor,^{37–39} allowing for calculation of various parameters of the equivalent electric circuit, such as charge transfer resistance (R_{ct}), constant phase element (CPE) and Warburg impedance. By the way of example, the fitting of the experimental data to the model are shown in Fig S2 for solution without ADMA as well as for solution of 2.2 μM ADMA. Resulting parameters of the model circuit are presented in Table S3. For all fits the χ^2 parameters were at the level of 2×10^{-3} which indicates a reasonably good fit. The only visible deviation was observed at frequencies below few Hz in the region governed by the diffusion processes.³⁷ This is most probably due to diffusion alterations introduced by the presence of relatively thick polymer film on the electrode. As expected, the calculated charge transfer resistance increased after each addition of the analyte indicating blocking of electron transfer to the electrode surface after the analyte binding in the film.^{38,39} This increase was linear at least in the range of 0.4 to 2.2 μM ADMA (Fig. 5b) described by the equation of $R_{ct}(\Omega) = 86.1 c_{ADMA}(\mu M) + 21.8$ ($R^2 = 0.9495$). The analytical parameters determined are given in Table I.

In order to determine selectivity of the MIP toward selected interferences, the DPV curves of the red-ox probe were recorded on the MIP film-coated electrode for a given ADMA concentration at a large excess of either arginine or glucose interference. Results of these experiments are shown in Fig. 6. The recorded curves show that there is a smaller DPV peak current decrease after addition of ADMA of a given concentration in the presence of a large excess of interferences (curves 2, 3 and 2', 3' in Figs. 6a and 6b) than after addition of pure ADMA only (curves 1 and 1' in Figs. 6a and 6b). The decrease of the DPV peak current is proportional to available binding sites in the MIP film. As expected, arginine interferes more strongly with the sensor response because its structure is relatively similar to that of the ADMA. Still, a substantial peak current drop was observed after ADMA addition even in this case.

The obtained results indicate respectable selectivity and sensitivity of the devised MIP recognition layer in combination with DPV and EIS transduction methods. The LOD reached indicate possibility of determination of ADMA at the concentration level of 0.2 to 1 μM , expected in body fluids.

Summary

Herein, we describe development of an electrochemical chemosensor with recognition unit based on molecularly imprinted polymer film

Table I. Analytical parameters of the DPV and EIS chemosensors with ADMA-imprinted polymer recognition film.

Analytical parameter	DPV transduction	EIS transduction
LOD (μM)	0.3	0.4
Sensitivity ($nA \mu M^{-1}$)/($\Omega \mu M^{-1}$)	52	86.1
Linear dynamic concentration range (μM)	0.3–2.2	0.4–2.2
Apparent imprinting factor (AIF)	Very high ^a	10

^aIt was not possible to calculate AIF as the NIP-coated electrode practically did not respond to the ADMA presence.

Figure 5. (a) Electrochemical impedance spectra recorded in solutions with (1) 0.0, (2) 0.4, (3) 1.0, and (4) 2.2 μM ADMA in the 500 kHz to 0.1 Hz range at the MIP film-coated Pt electrode in 0.1 M $\text{K}_4[\text{Fe}(\text{CN})_6]$ and 0.1 M $\text{K}_3[\text{Fe}(\text{CN})_6]$ at open circuit potential. (b) Plot of normalized R_{ct} calculated from fitting of EIS curves vs. ADMA concentration for the MIP and NIP film-coated Pt electrodes.

Figure 6. DPV peak current changes in 1 mM $\text{K}_4[\text{Fe}(\text{CN})_6]$, 0.1 M KNO_3 solution recorded for MIP-coated Pt electrode in (1, 1') ADMA-containing solution, (2, 2') ADMA-containing solution in the presence of 40 μM arginine, (3, 3') ADMA-containing solution in the presence of 40 μM glucose, and (4, 4') NIP-coated electrode in solution of ADMA. Concentration of ADMA was (a) 0.4 μM and (b) 2.0 μM .

of organic semiconductor for asymmetric dimethylarginine determination. Quantum-chemical calculations allowed for selection of appropriate *bis*(2,2'-bithienyl)methane functional monomers capable of stable complex formation with ADMA. These monomers were used for MIP film deposition via potentiodynamic electrodeposition. The PM-IRRAS spectroscopy confirmed formation of thiophene-containing films with the desired functional groups. The AFM imaging of the nanomechanical properties of the films showed that the MIP film after template removal became rougher and softer. The MIP film deposited on the electrode was tested as the recognition unit of the electrochemical sensor for ADMA determination. The chemosensor showed high sensitivity and appreciable selectivity toward ADMA. Its LOD was at the level of 0.3 or 0.4 μM ADMA depending on the transduction methods used. That indicates that the devised chemosensor is suitable for determination of ADMA in body fluids.

Acknowledgments

The Authors gratefully acknowledge financial support of the Polish National Science Center (NCN) through grant No. 2014/15/B/ST4/04642 to K. N. and V. M.

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