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NON-ENZYMATIC ELECTROCHEMICAL DETECTION OF GLUTAMATE USING TEMPLATED POLYMER-BASED TARGET RECEPTORS

Habib M. N. Ahmad¹, Bo Si¹, Gaurab Dutta¹, John R. Csoros², William Rudolf Seitz², Edward Song¹

¹Department of Electrical and Computer Engineering, University of New Hampshire, USA

²Department of Chemistry, University of New Hampshire, USA

Abstract

We present a novel electrochemical biosensing platform for the detection of neurotransmitter glutamate using templated polymer-based target receptors. Our sensing approach demonstrates, for the first time, a non-enzymatic approach without the need of glutamate oxidase, leading to a more specific and rapid response. The proposed detection principle is based on the following two claims: (1) our templated polymer-based receptor results in specific molecular recognition of the target glutamate and, (2) upon target binding, the polymer undergoes a conformation change which can then be measured via electrochemical techniques. This sensing platform has the potential to provide direct monitoring of a variety of non-electroactive species and to eliminate the incorporation of enzymes thereby providing a simpler and more robust alternative to enzyme-based sensors.

Keywords

Non-enzymatic; Biosensing; Templated Polymer; Electrochemical Detection; Neurotransmitter; Glutamate

INTRODUCTION

Electrochemical sensing techniques generally rely on the analyte's redox activities. However, the presence of other common interferents that have similar redox activities as the analyte often makes such direct electrochemical detection with reasonable target selectivity difficult. Moreover, electrochemical detection of redox-inactive species is even more troublesome due to the absence of a measurable signal. Hence, enzymes are often incorporated into the biosensors for detecting the non-electroactive analyte. The enzyme-based sensing performances usually depend on many factors including the permeability of the enzyme layer as well as the level of enzyme activity which is often influenced by the external environment. Cross-reactivity of the interferents also affects the stability and the reaction kinetics of the enzyme layer, resulting in inaccurate sensor readout. Therefore, a

*CONTACT E. Song, tel: +1-603-862-5498; Edward.Song@unh.edu.

novel non-enzymatic electrochemical sensing platform that allows direct quantification of such non-electroactive species is highly desirable. Electrochemical sensing in general offers many advantages in biosensing because it is less expensive, can be easily miniaturized, and easier to operate compared to other advanced detection approaches, such as spectrophotometry and chromatography [1].

In achieving an enzymeless detection of non-electroactive species, one practical approach is to use a molecular templating method, also commonly referred to as molecular imprinting [2]. Generally, molecularly imprinted polymers are based on a highly crosslinked polymer matrix with the templated molecular cavities that exhibit preferential binding to the analyte. However, such polymer layer suffers from poor permeability resulting in slow response time. Moreover, under this platform, the templated polymer matrix often requires a label to transduce a molecular recognition event into a measurable electrochemical signal.

In this paper, we propose a new approach to molecular templating based on a linear, single-chain stimuli-responsive templated polymer. In this platform, the covalent interchain crosslinks were eliminated, and the molecular recognition was achieved primarily using non-covalent, weak forces including hydrophobic interactions and ionic bonding. Our hypotheses are two-fold; first, the proposed flexible single-chain templated polymer binds with the target analyte showing fast response and high affinity; secondly, the templated polymer chain exhibits conformation change from the extended random coil to a collapsed globular form upon specific binding of the analyte.

A single-chain poly-N-isopropylacrylamide (PNIPAM) was used as the backbone into which functional monomers were incorporated to form a templated polymer. The intrachain interactions include hydrophobic crosslinks between the N-isopropyl groups PNIPAM as well as the acid-base crosslinks between the acidic and basic functional monomers. Such monomers were incorporated during the polymerization to increase the binding affinity with the target analyte via intrachain interactions [3].

In this work, we have chosen glutamate as a model analyte to test the feasibility of the proposed sensing platform. Glutamate, a non-electroactive neurochemical, plays a key role in the central nervous system especially as it relates to certain brain disorders such as Parkinson's disease, Alzheimer's disease, and schizophrenia [4]. Abnormalities in glutamate signaling in the synaptic cleft are also implicated in cerebral hypoxia/ischemia, and other traumatic brain injuries [5].

TEMPLATED POLYMER SYNTHESIS

The glutamate (Glu) receptor was developed using a single chain PNIPAM copolymerized with functional monomers 4-vinylpyridine (4-VP) and methacrylic acid (MAA) [3]. The synthesized polymer exhibits a lower critical solution temperature (LCST) of 31–33°C [6]. Figure 1 shows the conceptual diagram of the synthetic Glu receptor before and after binding. The polymers were synthesized via reversible addition-fragmentation chain transfer (RAFT) method in the presence of N-methylglutamic acid (Methyl-Glu) as a template. The methylated glutamate analog was used instead of Glu to prevent cross-reaction between the

primary amine group of the Glu and the RAFT agent [6] resulting in much higher polymerization yield.

The synthesis of Methyl-Glu templated polymers were performed by mixing 8.7 mmol of NIPAM, 0.5 mmol of MAA, 0.8 mmol of 4-VP, 0.1 mmol of RAFT chain transfer agent (CTA) 2-(Dodecylthio-carbonothioylthio)-2-methyl-propanoic acid (DDMAT), 0.1 mmol of template molecule Methyl-Glu and 0.01 mmol of azobisisobutyronitrile (AIBN) in 35 mL dioxane solution. The freeze-pump-thaw process was performed three times for degassing. Finally, the polymerization was initiated at 70°C. After the polymerization is complete, the product is dialyzed against 50% THF to remove the template and the unreacted species. The polymer powder was then obtained via lyophilization after purification.

For the sensor development, the polymers were attached to the gold electrode in the presence of a reducing agent tris-(2-carboxyethyl) phosphine (TCEP) to promote thiol bonding [7]. The Glu rebinding to the templated polymer was performed via immersing the polymerized electrode in a Glu solution (at pH 7.0) prepared in deionized water for 5 minutes. Afterward, the sensor was washed in deionized water in order to minimize non-specific bindings of Glu to the receptors.

NMR ANALYSIS OF THE POLYMER

The composition and structure of the Methyl-Glu templated polymer were determined by ¹H-NMR spectroscopy. Figure 2 shows the theoretical structure of the polymer and the corresponding NMR spectrum [8]–[11]. The $\delta = 4.0$ ppm and $\delta = 1.1$ ppm are attributed to methine protons and methyl protons of NIPAM, respectively [11]. $\delta = 8.5$ and $\delta = 7.1$ ppm are attributed to aromatic protons of 4-VP [10]. The feature signals of DDMAT were observed at $\delta = 3.64$ ppm ($-\text{S}-\text{CH}_2(\text{CH}_2)_{10}\text{CH}_3$) and at $\delta = 0.9$ ppm ($-\text{S}-\text{CH}_2(\text{CH}_2)_{10}\text{CH}_3$) [10]. The molar ratio between functional monomer 4-VP and NIPAM can be calculated via equation (1) below:

$$\frac{\text{Mol}(4 - \text{VP})}{\text{Mol}(\text{NIPAM})} = \left[\frac{A_1/2_{(\text{protons})}}{A_2/1_{(\text{protons})}} \right] \quad (1)$$

where A_1 is the area of aromatic protons at 8.5 ppm, A_2 is the area of methine protons of isopropyl group at 4.0 ppm. The calculated ratio is 8:92.8, which is in close agreement with the initial composition (8:87) during synthesis.

EXPERIMENTAL SETUP

The electrochemical characterization and sensing were carried out in a 5 mM potassium ferro/ferricyanide $\text{K}_4/3\text{Fe}(\text{CN})_6$ dissolved in 1X PBS buffer. The pH level recorded was 7.2. The cyclic voltammetry (CV) was performed at a scan rate of 100 mV/s from 0 V to +0.8 V vs. Ag/AgCl reference. The electrochemical impedance spectroscopy (EIS) was performed with an amplitude of 10 mV with frequencies ranging from 1 Hz to 100 KHz. All circuit fitting was performed using EC-lab software to obtain the circuit parameters. The electrochemical experiments were carried out in a glass cell, and a platinum wire used as a

counter electrode. A Bio-Logic VSP dual channel potentiostat was used for all electrochemical studies.

RESULTS AND DISCUSSION

The CV curves in Figure 3(a) show that bare Au electrode before and after exposure to Glu have similar electrode kinetics suggesting that Glu is not readily adsorbed on the gold surface. Binding of Glu to the polymer receptor decreases the intensity of redox peaks which further decreases the rate constants. Also, the Nyquist plot obtained from EIS shows an increase in charge transfer resistance upon analyte binding. The circuit fitting in Figure 3 (b) shows that, upon binding of Glu, the charge transfer is more kinetically controlled, indicating more surface blockage upon Glu binding. The circuit fitting parameters summarized in Table 1 shows that, as more Glu are bound to the receptors, it not only increases the R_{ct} but also increases the Warburg coefficient (A_w) obtained from Warburg element (W) fitting [12]. The increasing value of A_w also suggests that the diffusion of electrolyte through the polymer is hindered upon gradual binding of Glu.

Figure 4(a) and 4(b) shows the Bode magnitude and phase plot, respectively. The bare Au electrode shows parasitic shunt capacitance at $Z < 100$ Hz due to the exposed metal surface resulting in enhanced charge transfer at a lower frequency range. The lower frequency domain at the Bode plot shows decreased shunt capacitance upon the polymer attachment and further binding of higher concentrations of Glu to the receptors. The conformation change of the polymers is confirmed by a decrease in the parasitic leakage pathway. This is observed by a rising impedance due to the passivation of the metal surface via the polymer deposition and subsequent Glu binding. The phase plot in Figure 4(c) shows that the peak phase response shifts toward the lower frequency upon binding of polymer and Glu [13]. At the lower frequency domain ($f < 100$ Hz), the phase differences among the responses is maximized at 25 Hz [14]. Figure 5(a) shows the change in rate kinetics before and after binding of Glu. The rate constant (k_0) values are obtained from CV and EIS by equations (2) and (3) by Kochi and Klinger, respectively [12], [15]:

$$K_0 = 2.18 \left[\frac{\alpha n F \vartheta}{RT} \right]^{-0.5} \times \exp \left[\frac{-\alpha^2 n F (\Delta E_p)}{RT} \right] \quad (2)$$

$$K_0 = \frac{R \cdot T}{n \cdot F^2 \cdot C \cdot A \cdot R_{ct}} \quad (3)$$

where F = Faraday's constant (96,485 C/mol); A = electrode area (0.07 cm²); T = Temperature (298K); n = number of electrons = 1; C = concentration (5 mM); ϑ = scan rate (0.1 V/s); α = transfer coefficient; R_{ct} = Charge transfer resistance from Nyquist plot. The K_0 value obtained from EIS (Figure 5(a) red curve) is significantly lower than that from CV (blue curve) because of the electrochemical irreversibility at the electrode-electrolyte interface [16]. The increase in Faradaic impedance after Glu binding shows an almost linear trend in Figure 5(b). A nine-fold increase in R_{ct} ratio indicates the specific binding of Glu [17]. The R_{ct} increment (blue curve) of approximately 90% was achieved at the highest

Glu concentration. The exponential increment in R_{ct} suggests an association of Glu molecule in polymer chain results in a globule formation of polymers [15].

The angular phase displacement (θ) obtained from EIS in Figure 6 depicts the impediment in diffusion through the porous network of the polymer due to the binding of Glu. The conformational change (from a random coil to a globule) of the single-chain templated polymer contributes towards change in phase. The maximum angular phase obtained from the phase plot at 25 Hz in the lower frequency domain (< 100 Hz) has proportionally increased upon Glu binding. The slope calculated from the phase graph shows the sensitivity of our polymer-based Glu sensor which is $0.86 \pm 0.12^\circ/\mu\text{M}$. The limit of detection (LOD) for Glu detection is calculated to be $1.7 \pm 0.2 \mu\text{M}$. The selectivity of the templated polymer is assessed by exposing the Glu-templated polymer to gamma-aminobutyric acid (GABA), another neurochemical. The comparison in the calibration curve between Glu and GABA in Figure 6 The phase response under GABA exposure yields a straight line with negligible slope. demonstrates the selective nature of the templated polymers.

CONCLUSION

The Methyl-Glu templated polymer was developed for the selective electrochemical detection of Glu. The synthesized target receptor has demonstrated a specific affinity towards Glu. Moreover, upon specific binding with Glu, the templated polymer undergoes conformation change from the extended to the collapsed state. The detection limit obtained for Glu was $\sim 1.7 \mu\text{M}$ with reasonable selectivity against another neurotransmitter GABA.

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Figure. 1:
Schematic representation of the sensor platform using templated polymers (a) before and (b) after binding with glutamate.

Figure 2:

The chemical structure of Methyl-Glu templated polymer after template removal and the corresponding ^1H -NMR spectrum.

Figure 3:

(a) Change in CV curves and (b) Change in R_{ct} after circuit fitting of Nyquist plot with circuit parameters R_s (solution resistance), Q (non-ideal capacitor due to frequency dispersion), R_{ct} (resistance for charge transfer) and W (Warburg element for diffusion). A modified Randles circuit is shown in the inset.

Figure 4:

(a) Corresponding change in the impedance for various concentrations of Glu binding derived from the Bode plot under impedance spectra. (b) The change in phase plot for different concentrations of Glu binding with respect to 0 μM Glu.

Figure 5:

(a) The changes in the rate constant under various concentrations of Glu binding obtained from CV (blue) and EIS (red) measurements; (b) The percentage change in R_{ct} (blue) and the ratio of R_{ct} (red) before and after Glu binding.

Figure 6:

The calibration curve showing angular phase vs. concentration. The changes in angular phase were measured before and after binding of various concentrations of Glu (blue) and GABA (red) at 25 Hz.

Table 1:

EIS circuit fitting parameters obtained before and after Glu exposure to the templated polymer-based sensor.

Circuit Parameters					
Polymer coated gold electrode with and without Glutamate binding					
Samples	Electrolyte Resistance	Electrode-Polymer Interface			
	$R_s (\Omega)$	$Q_1 (\mu F. s^{n-1})$	n_1	$R_{ct} (\Omega)$	$A_W (\Omega. s^{-0.5})$
Gold	84.3±0.2	10±3	0.87±0.5	40.6±1.2	440±1
0μM-Glu	97.9±0.2	10.4±0.3	0.84±0.5	351±0.9	456±3
5μM-Glu	95.2±0.2	8.5±0.1	0.88±0.5	661±0.5	502±3
10μM-Glu	97.4±0.2	9.5±0.1	0.85±0.5	824.3±0.8	598±2
20μM-Glu	95.9±0.2	7.2±0.5	0.85±0.5	1219±1	632±3
40μM-Glu	97.5±0.2	10.1±0.2	0.85±0.5	3162±6	1064±7