Electrochemical evaluation of a protein-imprinted polymer receptor

Najmeh Karimian, Anthony P.F. Turner and Ashutosh Tiwari

Original Publication:

Najmeh Karimian, Anthony P.F. Turner and Ashutosh Tiwari, Electrochemical evaluation of a protein-imprinted polymer receptor, 2014, Biosensors & bioelectronics, (59), , 160-165.
http://dx.doi.org/10.1016/j.bios.2014.03.013

Electrochemical evaluation of troponin T imprinted polymer receptor

Najmeh Karimian[a,b], Anthony P.F. Turner[a] and Ashutosh Tiwari[a*]

[a]*Biosensors and Bioelectronics Centre, Department of Physics, Chemistry and Biology (IFM), Linköping University, S-58183 Linköping, Sweden*

[b]*Department of Chemistry, Faculty of Sciences, Ferdowsi University of Mashhad, Mashhad, Iran*

[*]Corresponding author. Tel: (+46) 1328 2395; Fax: (+46) 1313 7568; E-mail: ashutosh.tiwari@liu.se

Abstract

The selective detection and quantification of macromolecular targets is a fundamental biological mechanism in nature. Molecularly imprinted polymers (MIPs) have been identified as one of the most promising synthetic alternatives to bioreceptors. However, expanding this methodology towards selective recognition of bulky templates such as proteins appears to be extremely challenging due to problems associated with removal of the template from the polymeric network. In this study, polymer imprinted with troponin T (TnT) was assessed using electrochemical methods and the influence of various extraction methods, including conventional immersion extraction, thermal annealing and ultrasonic-assisted extraction, on the binding characteristics of the troponin-to-imprinted polymer receptor was elucidated. Cyclic voltammetric deposition of *o*-phenylenediamine (*o*-PD) film in the presence of TnT as a template was performed in acetate buffer (0.5 M, pH 5.2) on a gold substrate. Solvent extraction of the target molecule was optimised and followed by subsequent washing with water. The electrochemistry of a ferro/ferricyanide probe was used to characterise the TnT MIP receptor film. The incubation of the TnT MIP receptor-modified electrode with respect to TnT concentration resulted in a suppression of the ferro/ferricyanide redox current. The dissociation constant (K_D) was calculated using a two-site model of template affinity for the TnT MIP receptor. The synthetic TnT MIP receptor had high affinity for TnT with a K_D, of 2.3×10^{-13} M.

Keywords: Synthetic receptor, imprinted polymer, Troponin T, template affinity, electrochemical study.

Introduction

There is a rapidly expanding demand for suitable diagnostics to measure and monitor an increasingly wide range of expressed proteins elucidated in proteomic studies of various pathophysiologies. Molecular recognition is a fundamental property of biological processes and has proved a powerful analytical tool in the form of, for example, antibody/antigen recognition and enzymatic catalysis (Kryscio and Peppas, 2012). Despite the successes of systems based on natural recognition elements, however, there is a steadily growing interest in mimicking natural recognition systems using synthetic analogs, which offer improved stability, cost-effectiveness and means of rapid fabrication (Haupt and Mosbach, 2000; Sapsford *et al.*, 2008).

The design and synthesis of artificial receptors, i.e. molecularly-imprinted receptors that are able to recognise and bind different target molecules with high affinity and specificity comparable to their biological counterparts, offers a number of advantages; notably greater long-term storage stability, potential re-usability, resistance to microbial spoilage and custom synthesis of selective receptors without the need to inoculate laboratory animals as well as facile integration with transducers (Whitcombe *et al.*, 2011). Among the different formats for the preparation of MIPs, bulk polymerisation is most frequently used (Karimian *et al.*, 2013). This technique produces monolithic structures, which are then grounded and sieved the final product. These steps are time consuming and show slow template-to-MIP binding kinetics. To overcome these drawbacks, electropolymerisation is a smart way to prepare MIPs directly on the surface of a transducer by simply controlling the thickness of MIP receptor layer by the amount of charge passed. This approach is particularly attractive for making small devices for clinical diagnostics, environmental control and the pharmaceutical industries. Moreover, determination and monitoring of a wide range of analytes based on electrochemical techniques has made

electrochemical transducers very popular due to the ease of measurement and the ready availability of instrumentation. They also offer good limits of detection at low cost with the possibility of easy miniaturisation and automation (Suryanarayanan *et al.,* 2010; Piletsky and Turner, 2002; Merkoci and Alegret, 2002).

There are many descriptions of using MIP films based on electropolymerisation of *o*-phenylenediamine (*o*-PD) for determination of different kinds of templates, such as glucose (Cheng *et al.,* 2001), sorbitol (Feng *et al.,* 2004), DL-phenylalanine (Peng *et al.,* 2001), theophylline (Kan *et al.,* 2010), dopamine (Song *et al.,* 2010), triclosan (Liu *et al.,* 2009) single-stranded oligodeoxyribonucleotide (Tiwari *et al.,* 2012), TnT (Karimian *et al.,* 2013) and folic acid (Karimian *et al.,* 2013). Although, there are many reports of MIPs for small templates however, substantially less articles are published on MIPs for large templates, e.g. proteins (Kan et al., 2012; Ramanaviciene and Ramanavicius, 2004; Ratautaite et al., 2013). Furthermore, removal of proteins from imprinted polymers is rather difficult due to their high molecular weights, which retard diffusion through the dense polymer network. Several studies have shown that a small portion of the template remains even after extensive washing using different extractive solvents containing acid or base additives (Wulff, 1995; Ellwanger *et al.,* 2001). The residual template can constitute a problem as it might leach out from the polymer during the measurement and may limit the application of the protein MIP receptor in quantitative analysis. Thus, it is of interest to investigate the effect of extraction and to optimise it (Sauerbrey, 1959). In order to remove the entrapped template, different strategies are possible. First and most straightforwardly, the use of a solvent that will strongly interact with the network polymer may lead to the required swelling of the backbone necessary for template release. The preferred solvents may then be combined with acid or base additives in order to disrupt the electrostatic

interactions between protein and polymer. Moreover, the extraction may be performed at elevated temperature or ultrasonically assisted to increase the rate of diffusion.

Unfortunately, rather few reports discuss the effect of the extraction methods on the removal of macromolecules and the regeneration of the imprint sites. In the current work, the influence of various extraction processes for creation macro imprints have been investigated and their performances have been compared. Troponin T (TnT) is a specific biomarker for myocardial tissue that it is used as an inexpensive and fast cardiac biomarker test for early cardiac disease diagnosis (Kemp, 2004). A wide variety of immunoassay configurations have been developed for its determination (Klein *et al.*, 1998; Asano *et al.*, 2012; Dengler *et al.*, 1998; Katus *et al.*, 1989). We sought to replace antibodies with smaller, more stable counterparts and have also been searching for alternative ways to obtain antibody like receptors. The design and synthesis of an artificial macromolecular receptor of TnT based on molecularly imprinting was achieved by electropolymerisation of the functional monomer, *o*-phenylenediamine, in the presence of TnT as target molecule (Scheme 1). Electrochemical methods showed that TnT receptor possesses remarkable recognition properties with affinities and specificities comparable with the natural receptor.

2. Experimental

2.1. Materials

o-Phenylenediamine (*o*-PD, ≥98%, Sigma-Aldrich, USA), potassium ferricyanide ($K_3[Fe(CN)_6]$, 99%, E. Merck, Germany), potassium ferrocyanide ($K_4[Fe(CN)_6]$, 99%, E. Merck, Germany) and troponin T (TnT, *M*w = 37 kDa, from human heart, Sigma, USA) were used as received. Standard stock solution of TnT (50 µg/mL) was prepared in phosphate buffer solution (PBS, 1x at pH 7.4) and stored at -20 °C if not in use. $K_3[Fe(CN)_6]$ (10 mM) and $K_3[Fe(CN)_6]$)/

$K_4[Fe(CN)_6]$ (1: 1, 0.5 mM) were prepared in 0.1 M KCl. All other reagents were of analytical grade and solutions were prepared using Milli-Q water (18.2 MΩ/cm^2).

2.2. Instrumentation

Electrochemical measurements were performed using an Iviumstat potentiostat (Ivium, The Netherlands) controlled by software supplied by the manufacturer. A standard three-electrode configuration was used. A gold disk (2.0 mm diameter), a platinum wire and an Ag|AgCl|KCl (3 M KCl) electrode were used as working electrodes, counter and reference electrodes respectively. A digital instrument Nanoscope IIIa (New York, USA) with a Si tip (NT-MDT NSG01) in a taping mode at ≈300 kHz resonance frequency was opted for atomic force microscopy (AFM). All measurements were carried out at 22 ^{o}C.

2.3. Preparation of TnT MIP receptor

The surface of the gold electrode was conditioned before modification by polishing with 1.0 and 0.05 μm wet alumina slurry followed by 1 min cleaning in distilled water. Then, the electrode was subjected to cyclic potential sweeps between 0.2 and 1.5 V in 0.5 M H_2SO_4 until a stable cyclic voltammogram was obtained. Electropolymerisation of *o*-PD film was carried out by cyclic voltammetry (CV, 20 scans) in the potential range 0 to 1.1 V at a scan rate 50 mV/s in a solution containing 7.5 mM *o*-PD in 0.5 M acetate buffer solution (pH 5.2). TnT was added in a solution as a template molecule before polymerisation at concentration of 1 μg/mL. A control electrode modified with non-imprinted polymer (NIP) was obtained in the same way, but without TnT being added as a template. Modified surfaces were dried under a nitrogen flow and stored at room temperature. The conditions for preparation of the synthetic receptor for TnT and its corresponding NIP are summarised in Table 1.

2.4. Optimisation of template removal

Various treatment conditions were explored to remove the template from the polymer. Pure or mixed extraction solvents were added to stoppered vessels containing imprinted electrodes and were then placed in an ultrasonic bath (SB5200, Shanghai, Branson) with controllable temperature. For comparison, conventional immersion extraction was performed without the ultrasonication and heating. The template molecule was successfully washed out with ethanol-water (2:1, v/v) solution containing 0.25 M NaOH within 15 min at 50°C, followed by subsequent washing with water (Table 1). In order to characterise the imprinted electrodes, electrochemical measurements were carried out in the presence of 0.5 mM $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ (1:1) solution containing 0.1 M KCl at room temperature (22 °C). CVs of the imprinted receptor electrode were recorded in the potential range of 0.0 to 0.6V vs. Ag/AgCl with a scan rate of 50 mV/s. Differential pulse voltammetry (DPV) runs were quantified over a potential range of 0.0 to 0.4 V at a scan rate of 50 mV/s and pulse amplitude of 25 mV.

2.5. Binding affinity of TnT MIP receptor

The binding studies were performed by incubating the appropriate concentration of TnT with the synthetic receptor electrode in buffer solution at pH 7.4 for 10 min with stirring @250 RPM, followed by washing with water to remove any loosely bound materials that may have been absorbed on the surface. A control measurement was also performed with NIP receptor without TnT. The electrochemical measurements to characterise the receptors were carried out in the presence of 0.5 mM $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ (1:1) solution containing 0.1 M KCl at 22 °C.

3. Results and discussion

3.1. Formation of TnT MIP receptor

Fabrication of the MIP-film was achieved by cyclic voltammetric deposition of *o*-PD in the presence of TnT on a gold substrate. The voltammograms recorded during electrodeposition of *o*-phenylenediamine are shown in Figure 1a. The CV depicts a decreasing current during the oxidation process (~0.5 V) from the 1^{st} cycle to the 20^{th} cycle, indicating the deposition of the non-conducting polymer receptor film. This is also confirmed by the disappearance of the redox peaks of ferro/ferricyanide probe (Fig. 1b). The same oxidation peak was observed with the electropolymerisation of the monomer alone (NIP), which means that the template was electrochemically stable over the scanned potential window and only the monomer underwent electropolymerisation. Formation of ultrathin polymer films on the transducer surface was preferred to improve the sensitivity of the devices. The thickness of the polymer could easily be adjusted by controlling the scan rate and the number of cycles during electropolymerisation.

3.2. Electrochemical evaluation of TnT MIP receptor

Solvent extraction is one of the most important elements in the fabrication of an efficient MIP-based receptor. An optimal extractive solvent should swell the polymer and suppress interactions between the functional groups of the template and the polymer, but the procedure should also allow fast diffusion of the template molecules from the polymer. For characterisation of the polymerised films before and after template removal, CV with ferro/ferricyanide redox probe was utilised. The treatment of the modified surface in presence of TnT MIP led to the appearance of a sluggish response to the redox probe. It should be noted that to study the effects of the various post-treatments on the backbone of polymer and the success of washing out the template from the polymer network, each of the following post polymerisation treatments were performed

on the NIP electrode as a control. In fact, the washing process can deliver some of the binding properties attributed to imprinting. So for interpreting specific binding interactions with MIPs, careful use of controls is needed.

Initially, in order to remove troponin from the imprinted polymers deposited on the surface of gold electrodes, a washing solution of PBS (pH 7.2) tried for 2 h. Also, the solutions of electrolyte and surfactant, i.e., 1M potassium chloride and sodium dodecyl sulphate (SDS), respectively were tested. This was found to be ineffective in quantitatively removing the template. These solutions were poor solvents for P*o*PD. The use of strong acid, $HNO_{3,}$ was also evaluated, but was not successful in removing the template (Fig. 2a). Acidic and alkaline solutions of organic solvents were also explored. Methanol brought about partial release, but this was not reproducible (Fig. 2b). Among the organic solvents tested, alkaline ethanol was found to be the best (Fig. 2c). The effect of ultrasonic-assisted extraction with heating was also investigated for template removal. The highest difference in voltammetric signal between imprinted and non-imprinted polymers was observed after treatment in 0.25 M NaOH solution in ethanol-water (2:1 v/v) with 15 min heating (50°C). Here, a new Au electrode was taken as a reference for these studies Fig. S1. An alkaline medium was chosen to avoid degradation of polymerised *o*-PD films (Kennedy and Cunnane, 2008) and denaturation of template protein molecules at extreme pH. The resulting MIP TnT receptor film was further characterised using AFM, while NIP was taken as reference. The images shown in Fig. 2d, illustrate the surface topography of (i) MIP and (ii) NIP after washing with alkali ethanol solution, show active deposition of the polymer film on the electrode surface, and reveal a noticeable change in the roughness of the receptor surfaces. The root-mean-square (RMS) value of roughness of MIP and NIP TnT receptor films was calculated to be 3.69 and 2.61 nm, respectively.

3.3. Affinity of TnT MIP receptor

In order to investigate the binding performance of the troponin-imprinted polymer, imprinted film was dipped into the binding buffer solutions containing various concentrations of TnT for 10 min with stirring, followed by washing. Cyclic voltammetry and DPV were used to monitor the ferro/ferricyanide probe response as affected by TnT binding on the MIP-receptor. Fig. 3a represents the decrease of redox peak currents and increase of peak-to-peak separation of the ferro/ferricyanide couple for an increase in TnT concentration in the binding buffer. The investigation of the binding performance was also performed using NIP-electrodes. The polymer film deposited on the electrode in the absence of TnT, but treated the same way as MIP, did not give a detectable response to TnT (Fig. 3b). With increasing concentration of TnT more imprinted sites were rebound. When the electrode reaction of the ferro/ferricyanide probe blocked by the binding of protein with MIP an analytical signal was observed (Fig. 3c).

Recognition in the MIP system was evaluated by estimation of the equilibrium dissociation constant (K_D) with the corresponding maximum number of binding sites present in the film (B_{MAX}). The binding isotherm of the synthetic receptor of troponin T was fitted using a model for two types of simultaneous binding (Fig. 3d): on the sites of specific recognition inside polymer film and on the surface of electrode due to non-specific adsorption:

$$\frac{i - i_0}{i_0} = \frac{B_{MAX}\, c}{K_D + c} + N_S c$$

where c is bulk concentration of the target and N_S – binding constant for nonspecific adsorption. Table 1 summarises the binding properties (K_D and B_{MAX}) of the interaction sites. It can be observed that there is a considerable difference between K_D values of MIP and NIP systems; the

K_D value for troponin synthetic receptor was 2.3×10^{-13} M compared to 1.4×10^{-9} M for the NIP electrode. The significant difference in the interaction between template and binding sites present in the two polymer matrices demonstrates the effectiveness of this approach in creating a high affinity synthetic receptor for the target protein molecules.

Conclusions

The design and synthesis of artificial receptors, i.e. molecularly-imprinted receptors that are able to recognise and bind different target molecules with high affinity and specificity comparable to their biological counterparts, offers a number of advantages; notably greater long-term storage stability, potential re-usability, resistance to microbial spoilage and custom synthesis of selective receptors without the need to inoculate laboratory animals as well as facile integration with transducers. In this study, evaluation of a synthetic receptor for TnT was performed by observing the effect of protein binding on the electrochemistry of a well-characterised redox probe. Successful rebinding of the template proteins by the TnT synthetic receptor suggested that, indeed, the mild washing procedure adopted succeeded in freeing specific binding sites with reduced damage. This resulted in improved rebinding properties as demonstrated by the K_D values, which indicated excellent affinity for the imprinted protein. This approach is particularly attractive for making protein receptors for clinical diagnostics, environmental control and the pharmaceutical industries. In a more general context, this work shows that additional attention should be paid to the post-imprint treatment of polymers in respect of interpreting their binding properties and potential usefulness in biosensors.

Acknowledgments

The authors wish to acknowledge the European Commission (PIIFGA- 2009-254955), LIST, Ferdowsi University of Mashhad, Iran (code: 15616/3, dated: 2010) for generous financial

support to carry out this research at Linköping University. We also thanks to Mikhail Vagin for his fruitful discussion.

References

Asano, S., Suzuki, A., Ishii, J., Sekiguchi-Ueda, S., Shibata, M., Yoshino, Y., Nakamura, K., Akiyama, Y., Kitagawa, F., Sakuishi, T., Fujita, T., Itoh, M., 2012. Diabetology International 3, 29–36.

Cheng, Z., Wang, E., Yang, X., 2001. Biosensors and Bioelectronics 16, 179–185.

Dengler, T.J., Zimmermann, R., Braun, K., Muller-Bardorff, M., Zehelein, J., Sack, F.U., Schnabel, P.A., Kubler, W., Katus, H.A., 1998. Journal of the American College of Cardiology 32, 405–412.

Ellwanger, A., Berggren, C., Bayoudh, S., Crecenzi, C., Karlsson, L., Owens, P.K., Ensing, K., Cormack, P., Sherringtonc, D., Sellergren, B., 2001. Analyst 126, 784–792.

Feng, L., Liu, Y., Tan, Y., Hu, J., 2004. Biosensors and Bioelectronics 19, 1513–1519.

Haupt, K., Mosbach, K., 2000. Chemical Reviews 100, 2495–2504.

Jozefowicz, M., Jozefonvicz, J., 1997. Biomaterials 18, 1633–1644.

Kan, X., Xing, Z., Zhu, A., Zhao, Z., Xu, G., Li, C., Zhou, H., 2012. Sensors and Actuators B 168, 395– 401.

Kan, X., Liu, T., Zhou, H., Li, C., Fang, B., 2010. Microchimica Acta 171, 423–429.

Karimian, N., Vagin, M., Arbab Zavar, M. H., Chamsaz, M., Turner, A.P.F., Tiwari, A., 2013. Biosensors and Bioelectronics 50, 492–498.

Karimian, N., Arbab Zavar, M.H., Chamsaz, M., Turner, A.P.F., Tiwari, A., 2013. Electrochemistry Communications 36, 92-95.

Katus, H.A., Remppis, A., Looser, S., Hallermeier, K., Scheffold, T., Kubler, W., 1989. Journal of Molecular and Cellular Cardiology 21, 1349–1353.

Kemp, M., Donovan, J., Higham, H., Hooper, J., 2004. British Journal of Anaesthesia 93, 63–73.

Kennedy, B.M., Cunnane, V.J., 2008. Journal of Electroanalytical Chemistry, 615, 197-204.

Klein, G., Kampmann, M., Baum, H., Rauscher, T., Vukovic, T., Hallermayer, K., Rehner, H., Muller-Bardorff, M., Katus, H.A., 1998. Wiener Klinische Wochens chrift 110, 40–51.

Liu, Y., Song, Q.J., Wang, L., 2009. Microchemical Journal 91, 222–226.

Merkoci, A, Alegret, S., 2002. Trends in Analytical Chemistry 21, 717-725.

Peng, H., Zhang, Y., Zhang, J., Xie, Q., Niea, L., Yao, S., 2001. Analyst 126, 189–194.

Piletsky, S. A., Turner, A.P.F., 2002. Electroanalysis 14, 317-323.

Ramanaviciene, A., Ramanavicius, A., 2004. Biosensors and Bioelectronics 20, 1076–1082.

Ratautaite, V., Topkaya, S.N., Mikoliunaite, L., Ozsoz, M., Oztekin, Y., Ramanaviciene, A., Ramanavicius, A., 2013. Electroanalysis 25 (5), 1169–1177.

Sapsford, K.E., Bradburne, C., Detehanty, J.B., Medintz, I.L., 2008. Mater Today 11, 38–49.

Sauerbrey, G., 1959. *Zeitschrift für Physik* 155, 206-222.

Song, W., Chen, Y., Xu, J., Yang, X.R., Tian, D.B., 2010. Journal of Solid State Electrochemistry 14,1909–1914.

Suryanarayanan, V., Wu, C.T., Ho, K.C., 2010. Electroanalysis 22, 1795–1811.

Tiwari, A., Deshpandea, S. R., Kobayashi, H., Turner, A.P.F , 2012. Biosensors and Bioelectronics 35, 224–229.

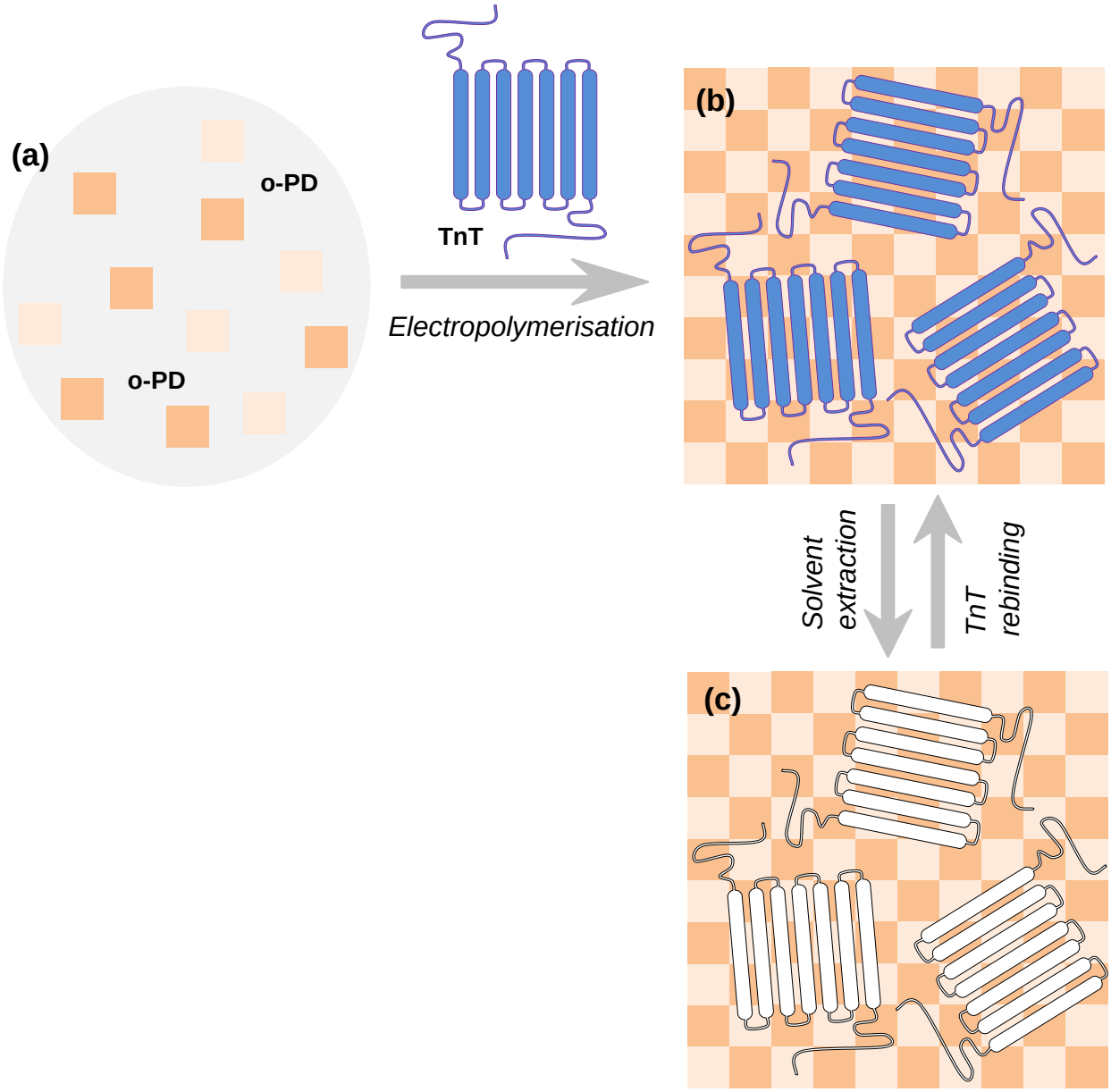
Whitcombe, M.J., Chianella, I., Larcombe, L., Piletsky, S. A., Noble, J., Porter, R., Horgan, A., 2011. Chemical Society reviews 40, 1547-1571.

Wulff, G., 1995. Angew. Chem. Int. Ed. Engl. 34, 1812.

Table 1. Summary of preparation steps of TnT MIP and NIP receptors and their binding properties.

Receptor films	Electrosynthesis conditions			Washing conditions			Binding parameters	
	Functional monomer, mM	*Template, µg/mL*	*Electrocycles, No.*	*Solvent extraction*	*Temperature, °C*	*Time, min*	K_D[b], *M*	B_{MAX}[c], *pmol*
TnT MIP	*o*-PD, 7.5	TnT, 1	20	Ethanol-water/ NaOH	50	15	2.3×10^{-13}	1.35
TnT NIP	*o*-PD, 7.5	-	20	Ethanol-water/ NaOH	50	15	1.4×10^{-9}	0.014

[a] *Refer to (2:1, v/v) ethanol-water solution containing NaOH (0.25 M).*
[b] *K_D; equilibrium dissociation constant*
[c] *B_{MAX}; maximum number of binding sites*



Scheme 1. Schematic representation of TnT MIP receptor. (a) Functional monomer (*o*-PD), (b) formation the recognition site around a bulky template, TnT and (c) specific TnT binding sites on the PoPD matrix.

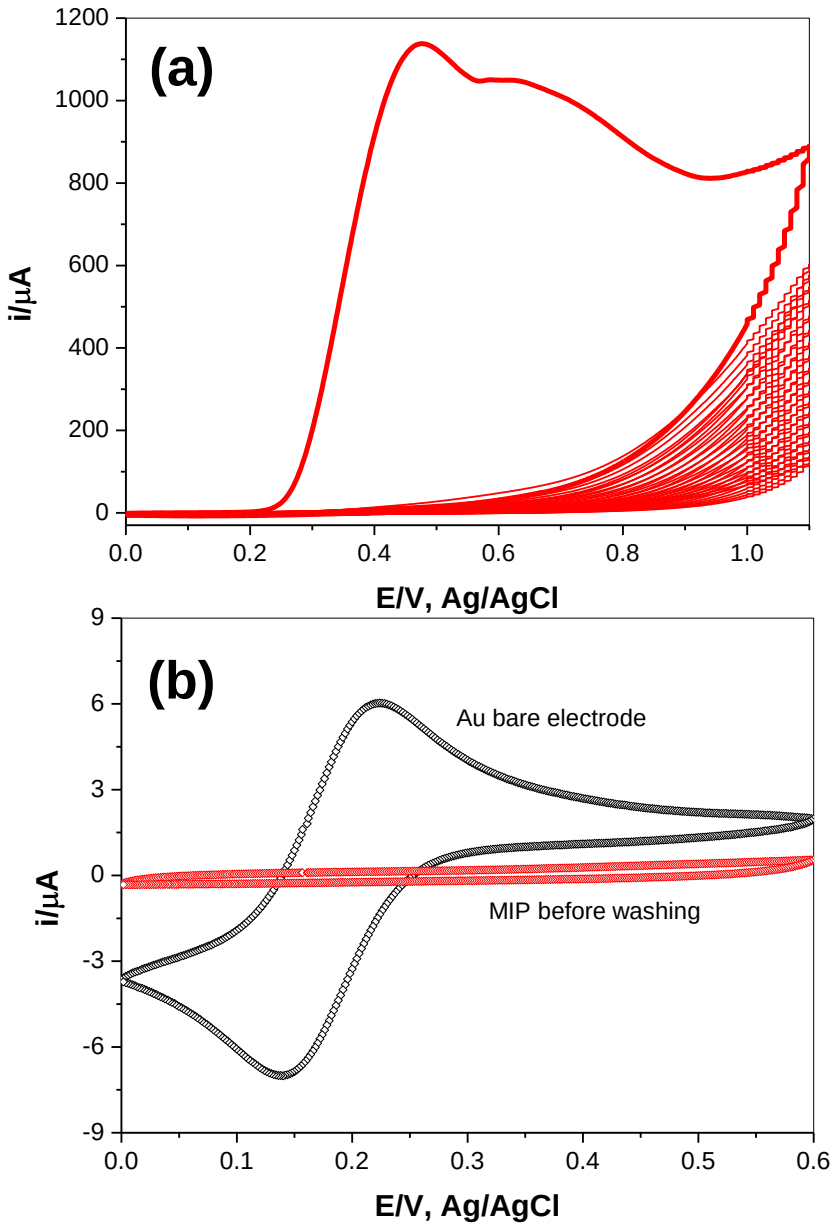


Figure 1. TnT MIP receptor formation using *in situ* electrochemical polymerisation. (a) Cyclic voltammetric deposition of 7.5 mM *o*-PD in the presence of 1 µg/mL TnT in acetate buffer (pH 5.2) on the gold substrate. Scan rate: 50 mV/s, Number of scans: 20. (b) Insulating of synthetic receptor of troponin T before washing the template. Cyclic voltammograms recorded with bare gold electrode and MIP-receptor; monitoring by 0.1 M KCl, 1 mM $K_3Fe(CN)_6$, 1 mM $K_4Fe(CN)_6$).

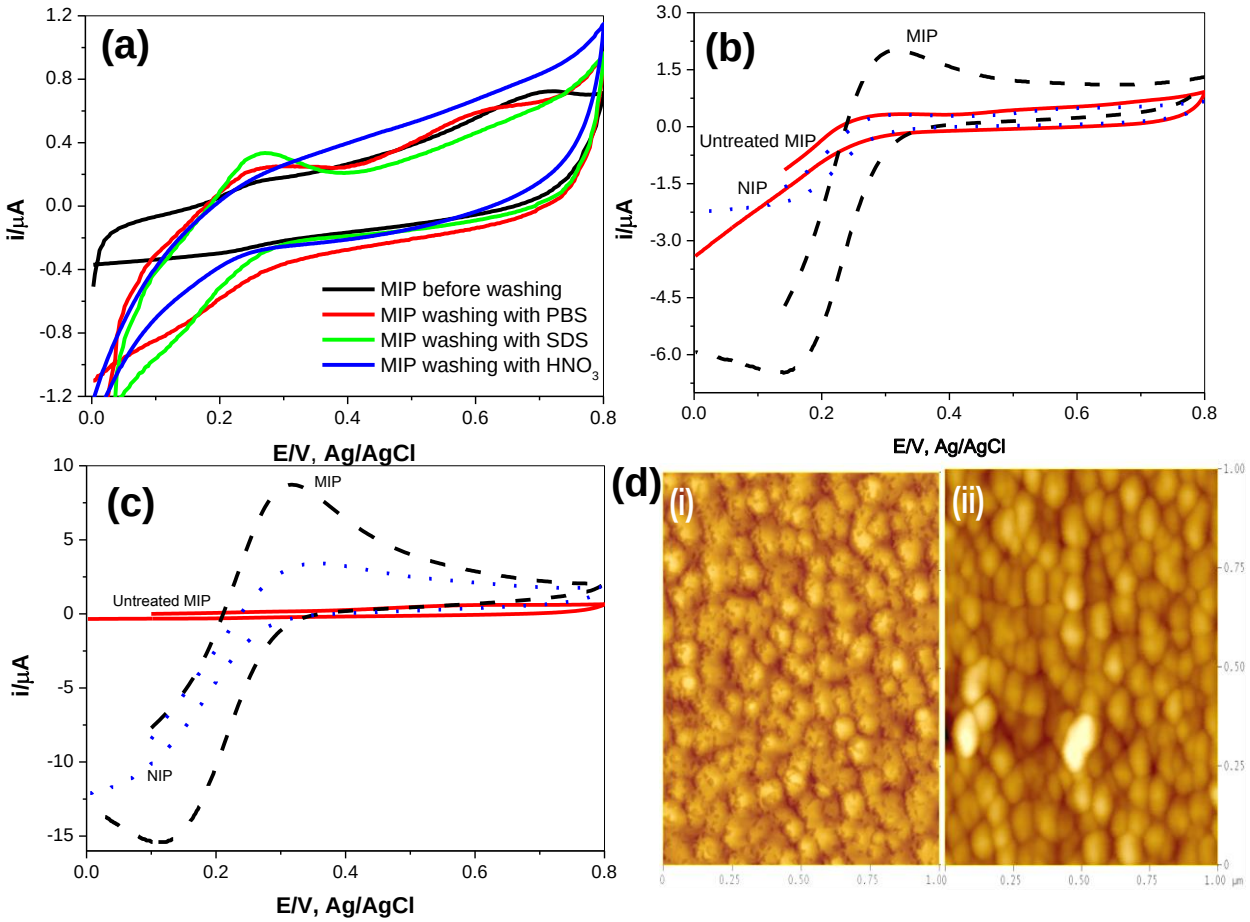


Figure 2. Electrochemical evaluation of TnT MIP receptor via solvent extraction process. (a) Black curve- MIP without exposure to extractive solvent, red curve- MIP after exposed to PBS (pH:7.2) for 2 h, green curve- MIP after exposed to surfactant, sodium dodecyl sulfate (SDS) for 3.5 h, blue curve- MIP after exposed to 1M HNO_3 for 1 h. (b) MIP and NIP receptor films after exposed to methanol at 50°C for 2 h, (c) MIP and NIP receptor films after exposed to 0.25 M NaOH solution in ethanol-water (2:1 v/v) at 50°C for 15 min. Conditions: concentration of TnT: 1μg/mL, concentration of *o*-PD: 7.5 mM, number of scan: 20, scan rate: 50mV/sec. The monitoring was done by using of the ferri/ferro cyanide redox couple, 0.1 M KCl, 1 mM $K_3Fe(CN)_6$, 1 mM $K_4Fe(CN)_6$. (d) AFM micrographs of (A) MIP and NIP receptor films after exposed to methanol at 50°C for 2 h, (d) i-MIP and ii-NIP receptor films after exposed to 0.25 M NaOH solution in ethanol-water (2:1 v/v) at 50°C for 15 min.

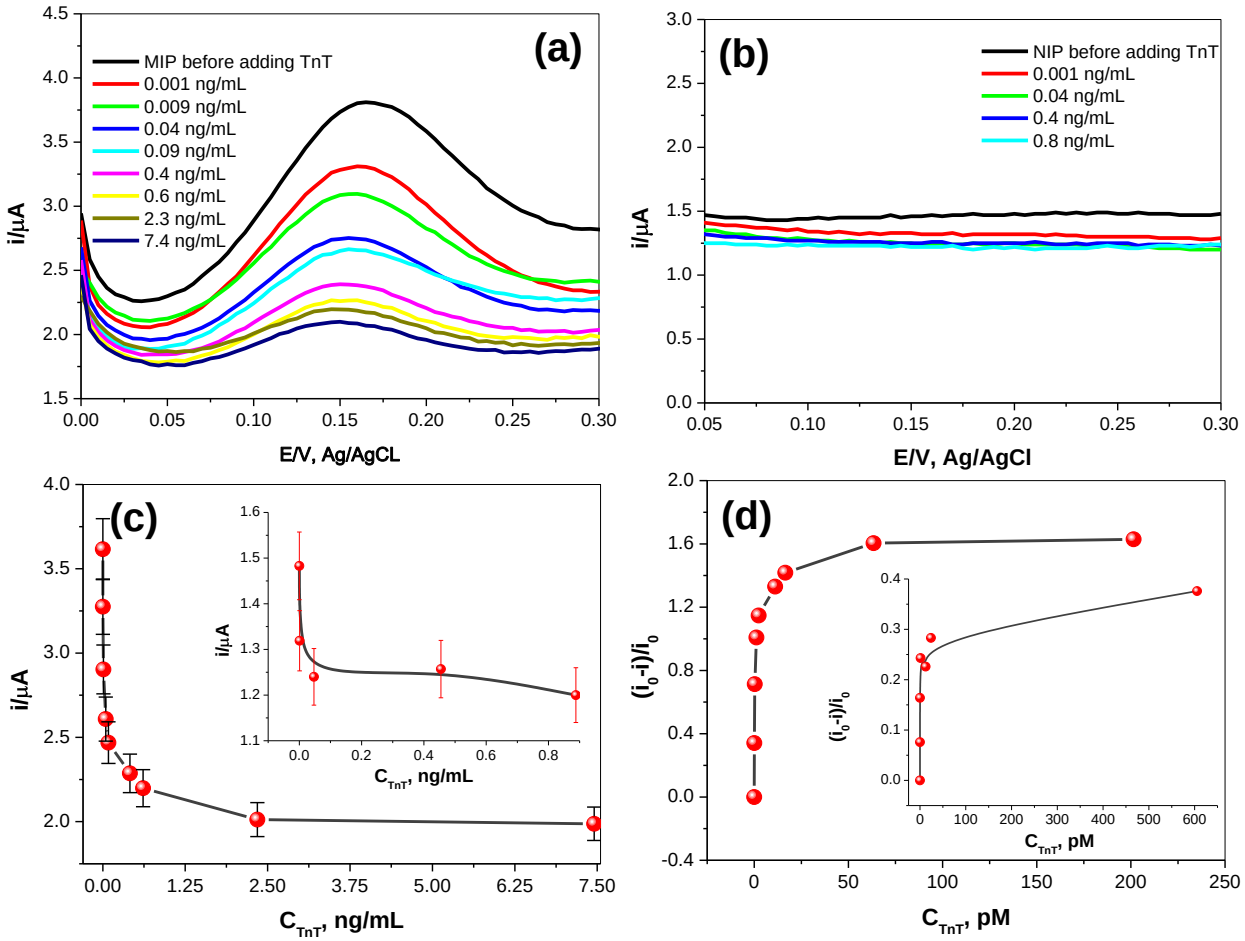


Figure 3. Electrochemical evaluation of template rebinding and affinity of synthetic receptor to template with (a) TnT MIP receptor film, (b) TnT NIP receptor film; after incubation in different concentrations of TnT for 10 min. (c) The plots of peak current versus TnT concentration on MIP and NIP receptor films (inset). (d) Binding isotherm of synthetic receptor of TnT and NIP receptor (inset) at different concentration of TnT. Solid line – fitting curve for specific binding accompanied with non-specific adsorption. The monitoring was done by using of the ferri/ferro cyanide redox couple, 0.1 M KCl, 1 mM $K_3Fe(CN)_6$, 1 mM $K_4Fe(CN)_6$.