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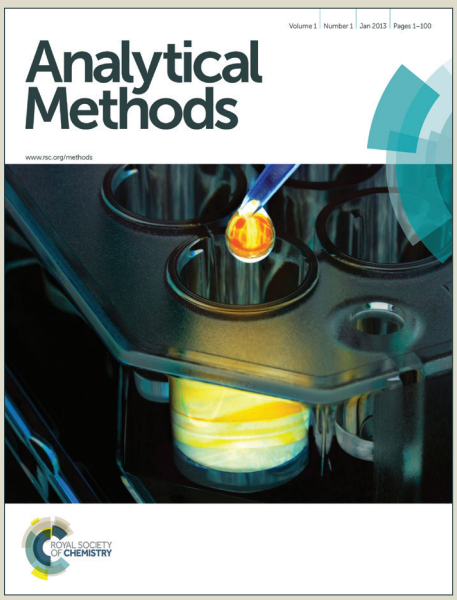

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Toll-Like Receptor 3 modified Au electrodes: An investigation into the interaction of TLR3 immobilized on Au surfaces with poly(I:C)

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Abstract

The rapid detection of viruses is crucial for medical diagnosis, environmental monitoring, public health, and homeland security. In this report, the development of a novel electrochemical impedance spectroscopy-based method utilizing Toll-Like Receptor 3 (TLR3)-modified Au electrodes for studying the interaction of TLR3 on a surface with polyinosinic-polycytidylic acid (poly (I:C)) as a dsRNA mimic (a molecular signature of viruses)[1] has been described. The modified Au electrodes were prepared by covalent immobilization of TLR3 protein using lipoic acid N-hydroxysuccinimide ester (Lip-NHS) linkers. The electrochemical behavior of the modified electrodes and their applicability for sensing poly(I:C) was examined.

Introduction

Detection of viruses in a rapid and precise manner is of crucial importance to medical diagnosis, bio-defense[2] and environmental monitoring[3] and is seriously considered as a public health, homeland security, and armed forces issue.[4] Traditional virus detection methods including cell culturing, enzyme linked immunosorbent assays (ELISA), and polymerase chain reaction (PCR) require complicated instrumentations and are not suitable for *in situ* analysis.[5-6] Therefore development of highly sensitive, fast, low-cost, and easy-to-miniaturize methods for virus detection is a necessity. To date, a number of different novel sensing methods have been introduced for this purpose, the majority of which rely on utilization of the signature proteins[7-11] or DNA[11-15] of viruses to characterize them. These virus biosensors consist of bio-receptors immobilized on a transducer and with high specificity towards the target analyte; the detection methods employed include surface plasmon resonance (SPR), fiber optics, acoustic wave technologies, quartz crystal microbalance (QCM) and electrochemical techniques.[11, 16-17] Electrochemical impedance spectroscopy (EIS) has been widely employed to study kinase-based biological processes[18], protein-DNA interactions[19], detection of hepatitis C viral NS3-4A protease[20] and Tau protein phosphorylations.[21] Detection of pathogens such as bacteria and viruses also comprises a significant portion of the EIS applications reported to date[22-24]. The innate immune system in higher organisms plays the key role of prevention of pathogen dissemination employing receptors encoded in the genome to sense pathogen-associated molecular patterns (PAMPs).[25-28] Pattern recognition receptors (PRRs) target the membrane of bacterial cells or different components of the cell wall such as lipopolysaccharide, lipoteichoic acid, and peptidoglycan.[29-30] Double-stranded RNA (dsRNA), a molecular signature of viral infections, is distinguished from nucleic acids of higher organisms by particular immutable

features, and it is recognized by several PRRs of the innate system, including Toll-Like Receptor 3 (TLR3).[30-32] Although in solution TLR3 is monomeric, recent studies have shown that it interacts with 45-bp segments of dsRNA as dimers and multiple TLR3 pairs can bind to longer dsRNA strands. In addition, it has already been shown that the minimum dsRNA length required for binding to TLR3 is ~40 bp and the binding affinity increases with the dsRNA size.[33] Leonard *et al* have indicated that the binding of dsRNA to TLR3 is also pH-dependent so that no binding of 540-bp dsRNA to TLR3 at pH 7.0 has been observed however; dramatic pH-dependent differences in affinity have been reported.[33] At pH 7.5, the TLR3-dsRNA complex completely and immediately dissociates.[33] Previous studies indicate that the minimal TLR3 signaling complex is a receptor dimer.[33-34] Each TLR3 dimer requires ~40–50 bp of ligand. Although double stranded DNA (dsDNA), single stranded DNA (ssDNA) and single stranded RNA (ssRNA) have been reported not to activate TLR3,[33-35] these oligonucleotides have been shown to inhibit the binding of TLR3 to immobilized dsRNA to some extent, which is an indicator of interaction and binding of these molecules to TLR3.[33] However, previous findings show that 100- to 1000-fold higher concentrations of these oligonucleotides are required to bind TLR3 as efficiently as dsRNA.[33] In the present study we report the construction and employment of the TLR3-modified Au electrode to investigate the interaction of immobilized TLR3 with poly (I:C) using the EIS method. To the best of our knowledge this is the first time that TLR3 has been employed for the detection of poly (I:C), a dsRNA mimicking molecule (0.2-1.0 kbp), as a molecular signature of viruses using EIS. Detection and sensing of viruses using different methods such as SPR[36], target-triggered cycled polymerization[37] and electrochemical techniques[38-39] has been already reported however, the majority of these methods include complicated procedures in preparation of a surface with multiple layers or require a large

quantities of sample for detection and analysis. This study reflects the results of investigation of the interaction of TLR3 on the Au surfaces and poly (I:C) at two different pH conditions; it opens a new avenue for virus detection by employing an immunoprotein and EIS as an easy-to-use, easy-to-miniaturize and low-cost method.

Experimental section

Reagents

TLR3 protein was purchased from Abcam (Cambridge, MA). Potassium ferrocyanide ($K_4[Fe(CN)_6]$), Potassium ferricyanide ($K_3[Fe(CN)_6]$), H_2SO_4, HCl, KOH and Phosphate Buffer Saline (PBS) were purchased from Sigma Aldrich (St. Louis, MO). Tris was obtained from United Biochemicals (Sanborn, NY). Boric acid was from BioShop (Burlington, ON). All buffers were prepared using ultrapure water (18.3 MΩ cm) from a Milli-Q system (Millipore, MA). Polyinosinic-polycytidylic acid (poly I:C) was obtained from Tocris Bioscience (Bristol, UK). Lip-NHS was synthesized as described before.[40]

X-ray photoelectron spectroscopy (XPS)

X-ray photoelectron spectroscopy (XPS) measurements (ThermoFisher, E. Greanstead) were carried out at the Surface Interface Ontario Center, University of Toronto, using Al Kα (15 μm, 200 eV) as the photo source. XPS was employed to characterize the TLR3 modified gold on silicon surfaces. Briefly, silicon chips coated with sputtered gold (Ti 6 nm, Au 140 nm, 0.2 cm2 surface area, fabricated at Nanofabrication Facility, University of Western Ontario) were cleaned for 20 s with piranha solution (H_2SO_4/H_2O_2 3:1 (v/v)) and rinsed with Millipore water and sonicated in ethanol for 10 min. After drying under N_2 flow, the chips were incubated with an ethanolic solution of 2 mM Lip-NHS for 48 h at 4 °C. Then the gold coated silicon chips were rinsed with ethanol and dried and incubated with 10 μg mL^{-1} TLR3 solution in 50 mM Tris

buffer pH 7.4 for 48 h at 4°C and then blocked in 1 M ethanolamine in 50 mM Tris buffer pH 8.5 (2 h). The chips were incubated in 0.5 and 5 μg mL^{-1} poly (I:C) in phosphate buffer saline (PBS) pH 7.00 for 5min, and prior to analysis the chip surfaces were thoroughly washed with Millipore water several times to remove any physically adsorbed poly (I:C) and dried under N_2 flow. The control chip was incubated only in phosphate buffer saline (PBS) pH 7.00 for 5 min and then rinsed thoroughly with Millipore water several times.

Electrochemistry

A CHI 660C potentiostat (Austin, TX) and microcrystalline gold disk electrodes (0.2 cm diameter) (CH Instruments, Austin, Texas) were employed for all electrochemical experiments. A three electrode setup consisting of an Ag/AgCl 3 M KCl as the reference, Pt wire as the auxiliary and TLR3-modified gold electrode as the working electrode was used. The reference electrode was connected via a salt bridge. All measurements were performed in $K_4[Fe(CN)_6]$ and $K_3[Fe(CN)_6]$ (5 mM each) in 1 M $NaClO_4$ (pH 7.0) or 100 mM Borate buffer (pH 6.5) solutions. ZSimWin 2.0 (EChem Software) was employed to analyze and fit the EIS experimental data to the appropriate equivalent circuit.

Electrode cleaning

Gold electrodes were incubated for 20 s in piranha solution (H_2SO_4/H_2O_2 3:1(v/v)), rinsed with H_2O and then polished with alumina slurry (0.1 mm and 0.05 mm, respectively) for 2 min. After rinsing, the electrodes with H_2O, electrodes were further cleaned electrochemically by cycling in the range of -2 to 0 V vs. Ag/AgCl in 0.5 M KOH and then cycling in the range of 0 to +1.5 V in 0.5 M H_2SO_4.

Modification of electrodes with TLR3 protein

Fig. 1A illustrates the different steps of modification of gold surfaces with TLR3 protein. Cleaned electrodes were incubated in 2 mM ethanolic solution of Lip-NHS for 48 h at 4 °C. The electrodes were then rinsed with ethanol and dried under N_2 flow and incubated in 10 μg mL^{-1} TLR3 protein solution in 50 mM Tris buffer pH 7.4 or 100 mM Borate buffer pH 7.4 for 48 h at 4°C. After rinsing with H_2O, 1 M ethanolamine in 50 mM Tris buffer pH 8.5 was used to block the electrodes (2 h), and then the electrodes were rinsed with H_2O.

Immunoassay and detection of poly (I:C)

The whole immunoassay process has been schematically illustrated in Fig. 2. The TLR3 modified electrodes were incubated in poly (I:C) solution at different concentrations of 0.2, 0.3, 0.5, 1, 2, 5, 10 and 20 μg mL^{-1} in PBS pH 7.0 or Borate buffer pH 6.5 for 5 min at room temperature (23°C) while shaking at 400 rpm using a VWR incubating shaker. The K_d for TLR3-immobilized dsRNA (540 bp) has been reported to be the lowest at pH 5.5-6.0 (5-28 nM respectively) and a considerable amount of binding has still been observed at pH 6.5, however the affinity of TLR3 to dsRNA at pH 7.0 is very low[35]. We performed the immunoassays at pH 6.5 and pH 7.0 to study the electrochemical behaviour of the TLR3-modified Au electrodes arising from the interactions of TLR3 and poly (I:C) under these two conditions. Before electrochemical measurements (Electrochemical Impedance Spectroscopy (EIS), Square-Wave Voltammetry (SWV) and Cyclic Voltammetry (CV)) the electrodes were rinsed thoroughly with H_2O to remove any physically adsorbed poly (I:C).

Construction of calibration curves

To assess the applicability of TLR3-modified Au electrodes for the determination of poly (I:C), calibration curves (R_{ct} vs poly (I:C) concentration) were evaluated at pH 6.5 and 7. The characteristics of the calibration curves for determination of poly (I:C) under these two pH conditions are summarized in Table 2. The lower limit of detection (LOD) was calculated using the expression: $LOD=3SD_b/m$, where SD_b is the standard deviation of the blank and m is the slope of the calibration curve.[41-42]

Results and discussion

Characterization of TLR3 modified gold surfaces

XPS was used to characterize the surface after each chemical modification step, including the immobilization of Lip-NHS onto gold, followed by the attachment of TLR3. In addition, XPS was used to investigate the interaction of immobilized TLR3 with poly(I:C). A representative XPS spectrum of the Au surface after modification with TLR3 is shown in Fig. 3A. The presence of S, C, O and N atoms in all samples is revealed by XPS survey scans; Table 1 presents their respective values. A significant S coverage was observed in the case of all films, which is due to Lip-NHS and the TLR3 protein. The deconvoluted S 2p spectrum showed a doublet indicating the S $2p_{3/2}$ peak at the binding energy of 162.7 eV. This peak represents a thiolate species which is S atoms bound to Au surfaces.[18] Furthermore, after the incubation of the TLR3 modified Au surfaces with 5 μg mL^{-1} poly (I:C), the results of XPS analysis of the P $2p_{3/2}$ region showed the modification of these surfaces by the binding of phosphate at 133.0 eV, associated with the presence of poly (I:C) (P $2p_{3/2}$). The atom percentage of phosphorus after the incubation with poly (I:C) increased from 0.1% to 1.4%, representing the interaction of poly (I:C) with immobilized TLR3 and its presence on the surface. No phosphate binding was observed after

incubation of the TLR3 modified Au surfaces at 0.5 μg mL^{-1} poly (I:C). These results indicate that a very weak interaction between poly (I:C) and immobilized TLR3 takes place at pH 7.00. In addition, when rinsing the surfaces incubated in 0.5 and 5 μg mL^{-1} poly (I:C) with equal amounts of Millipore water, the poly (I:C) was easily washed off the surface when incubated at a low concentration (0.5 μg mL^{-1}) of it. However, when incubated at high concentrations, (5 μg mL^{-1}), some poly (I:C) remains on the surface (Fig. 3 B).

Electrochemical characteristics of the modified electrodes

The modification process of the gold electrodes by TLR3 was characterized step by step by CV (Fig. 1B), SWV (Fig. 1C) and EIS (Fig. 1D) in 1 M of $NaClO_4$ solution containing 5.0 mM of $[Fe(CN)_6]^{3-/4-}$. Upon modification of the electrode, a significant increase in the charge-transfer resistance (R_{ct}) in each step was observed (Fig. 1D). A straight line (curve a) was observed for the bare Au electrode, which indicated a fast charge-transfer process. Immobilization of insulating Lip-NHS (curve b) and in particular TLR3 layer (curve c) and blocking of the surface with 1 M ethanolamine in 50 mM Tris buffer pH 8.5 (curve d) subsequently gave rise to a dramatically enhanced R_{ct}. The CVs of $[Fe(CN)_6]^{3-/4-}$ at different stages of modification of the electrode are shown in Fig. 1B. A well-defined redox peak pair was observed for the bare Au electrode (curve a). After each step of modification including immobilization of Lip-NHS, TLR3 and blocking with ethanolamine, the current decreased and the peak-to-peak separation was increased (curves b-d). Fig. 1C illustrates the SWVs of $[Fe(CN)_6]^{3-/4-}$ at different stages of the electrode modification and subsequent decreases in current due to immobilization of insulating Lip-NHS, TLR3 protein layers and surface blocking (curves b-d). The CV and SWV results were well in agreement with those of EIS experiments, confirming the success of each step of the modification.

Comparison of the modification of Au surfaces with TLR3 in Tris and Borate buffers

Fig. 4 illustrates the EIS obtained for the TLR3-immobilized Au surfaces modified in Tris and borate buffers. Modification of the Au surface in 10 μg mL^{-1} TLR3 in borate buffer gave rise to the R_{ct} twice greater than that in 10 μg mL^{-1} TLR3 in Tris buffer. This result could be explained by the interaction of the amine group of Tris buffer and its competition with TLR3 to be immobilized on Lip-NHS linkers.

Investigation of the interaction of poly (I:C) with immobilized Lip-NHS

The Au surfaces modified with only Lip-NHS were deactivated by 1 M ethanolamine in 50 mM Tris buffer and were incubated in different concentrations of poly (I:C) to investigate the electrochemical behaviour arising from the interaction of poly (I:C) with immobilized Lip-NHS. Fig. S1 shows that no significant change was observed in R_{ct} before and after incubation in poly (I:C).

Interaction of immobilized TLR3 with poly (I:C)

The interaction of TLR3 immobilized on the Au electrode surface with poly (I:C) was studied, and the binding of TLR3-poly (I:C) was monitored electrochemically in the presence of $[Fe(CN)_6]^{3-/4-}$ at pH 7.0 and pH 6.5. At pH 7.0 and at concentrations < 1 μg mL^{-1}; by increasing concentrations of poly (I:C) an increase in the R_{ct} was observed. 1 μg mL^{-1} showed an R_{ct} comparable to 0.1 μg mL^{-1} poly (I:C). Higher concentrations of poly (I:C) (> 1 μg mL^{-1}), however, caused a dramatic decrease in the R_{ct} (Fig. 5A). At pH 6.5 by increasing concentrations of poly (I:C) the R_{ct} increased up to 2 μg mL^{-1} (Fig. 6). The R_{ct} values were obtained by fitting the EIS data to the appropriate equivalent circuit (Fig. S2, S3, scheme 1 and Table S1). When performing electrochemical studies at pH 7.0, at poly (I:C) concentrations below 1 μg mL^{-1}, R_{ct} was concentration-dependant; however at concentrations above 2 μg mL^{-1} the R_{ct} value remained almost constant (Fig. 5B). The decrease in the R_{ct} could be due to the aggregation and auto-

oxidation of the linker molecule[22], however observing no significant change in the electrochemical signal after the incubation of the Au electrodes modified only with Lip-NHS with different concentrations of poly (I:C) (Fig. S1) indicates that this cannot be the case in this research work. It has also been reported by Sethuraman and Belfort that immobilization of proteins on well-defined homogeneous solid surfaces can lead to their structural changes and aggregation.[43] In this work the decrease in R_{ct} was attributed to the aggregation of immobilized TLR3 at such high poly (I:C) concentrations and the steric hindrance effect of the immobilized TLR3 protein at pH 7.0. At pH 6.5 the R_{ct} value increased by increasing concentrations of poly (I:C), and the dramatic decrease in R_{ct} at high concentrations of poly (I:C) was not observed (Fig. 6). The increase in the R_{ct} is attributable to an electrostatic repulsion between the anionic marker ions ($[Fe(CN)_6]^{4-/3-}$) and polyanionic poly (I:C) bound to the TLR3 proteins on the electrode surface, as shown in Fig. 2. The EIS data obtained were in good agreement with the CVs and SWVs (Fig. S4). Table 2 compares the analytical figures of merit for the calibration curves obtained for quantification of poly (I:C) at two pHs of 7.0 and 6.5. The calibration curve obtained by the data collected for measurements at pH 7.0 showed smaller dynamic linear range and poor linearity, however better limit of detection in comparison to the calibration curve on the basis of the data obtained at pH 6.5. At pH 7.0 although an electrochemical signal indicating an interaction between poly (I:C) and TLR3 was observed, according to previous reports[33] at this pH no specific interaction happens between immobilized dsRNA and TLR3. Also according to the XPS data, the bound poly (I:C) is easily washed off with Millipore water pH 7.0; it can be concluded that the interaction at pH 7.0 is weak and there is a significant contribution of non-specific interaction.

Conclusion

The research work presented here discusses taking advantage of capabilities of the immune system of higher organisms for detection of viruses. Utilization of TLR3, a component of the immune system, as the bio-recognition element on an Au electrode and using EIS as the detection method provides a novel and simple technique for sensing viruses. The Au surfaces were successfully modified by TLR3 protein through Lip-NHS linkers. Modification in borate buffer gave a better result than the modification in Tris buffer, which was attributed to the interactions of the amine group of the Tris buffer with Lip-NHS linkers and its competition with TLR3. The electrochemical behaviour of TLR3-modified Au electrodes in addition to XPS studies showed that at pH 7.0 an interaction between poly(I:C) and the immobilized TLR3 could be detected. However due to the low affinity between TLR3 and poly(I:C) at pH 7.00,[33] the observed signal was presumably the result of non-specific interactions. At pH 7.0 and at high concentrations of poly (I:C), a dramatic decrease in the R_{ct} was observed which was due to the aggregation and structural changes of the immobilized TLR3 protein on the Au surface. The calibration curve obtained on the basis of the data collected at pH 7.0 showed poor linearity and smaller dynamic linear range; however at pH 6.5 where mainly specific interaction between TLR3 and poly (I:C) happens, the calibration curve obtained had better linearity and larger dynamic linear range.

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Table 1. XPS analysis of films with (A) NHS-ester and (B) TLR 3 and (C) after incubation with 5 μgmL^{-1} poly (I:C). The binding energies (eV) and atom percentages were obtained using XPS survey scans.

XPS binding energies (atom percentage) in films			
Films			
Core level	**A**	**B**	**C**
O 1s	532.2 (6.5)	532.7 (16.7)	532.2 (22.2)
N 1s	399.9 (1.8)	399.8 (3.65)	399.8 (3.81)
C 1s	285.1 (36.3)	285.8 (54.2)	285.7 (50.2)
S 2p	162.1 (1.5)	162.6 (2.4)	162.7 (2.9)
P 2p	132.3 (0.1)	132.0 (0.1)	133.6 (1.4)

Table 2. Analytical figures of merit for quantification of poly(I:C) using calibration curves obtained from the R_{ct} data at pH 7.0 and pH 6.5.

Parameters	R_{ct} at pH 7.0	R_{ct} at pH 6.5
Dynamic linear range (μgmL^{-1})	0.2 – 0.5	0.2 – 1.0
Correlation coefficient	0.9301	0.9910
Limit of detection (μgmL^{-1}), n=5	0.06	0.18
Equation of calibration curve (R_{ct} versus μgmL^{-1} of analyte)	y = 99.61x + 249.77	y = 301.61x + 363.27

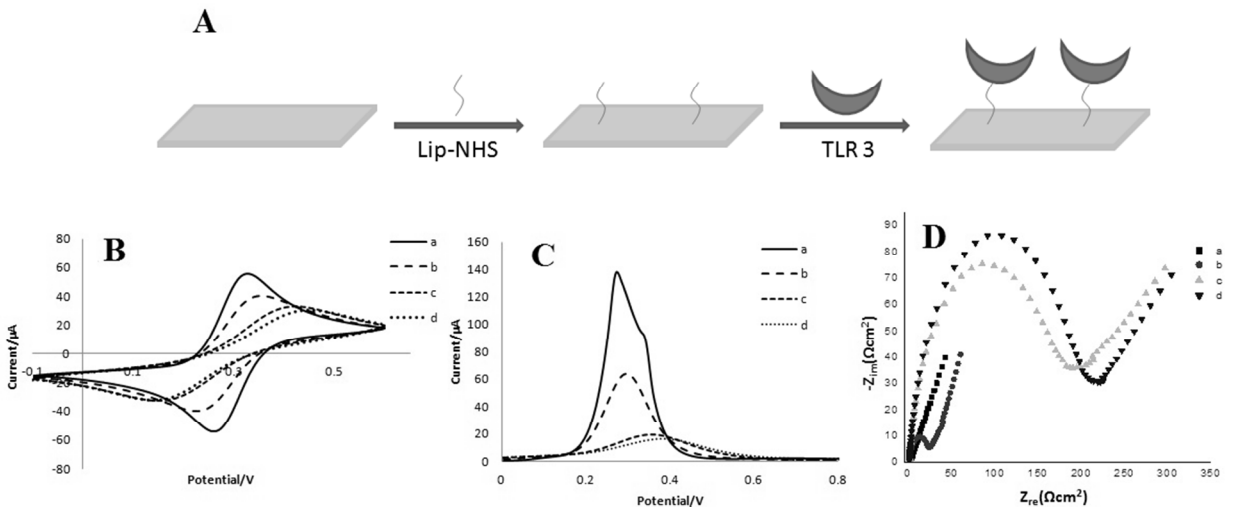

Fig.1 Constructing a TLR3-modified gold surface. (A) Schematic illustration of modification of gold surfaces with TLR3 protein (B) cyclic voltammogram (CV) (C) square wave voltammetry (SWV) and (D) electrochemical impedance spectroscopy (EIS) for (a) bare Au electrode, (b) Au electrode after modification with Lip-NHS, (c)TLR3 and (d) blocking with ethanolamine.

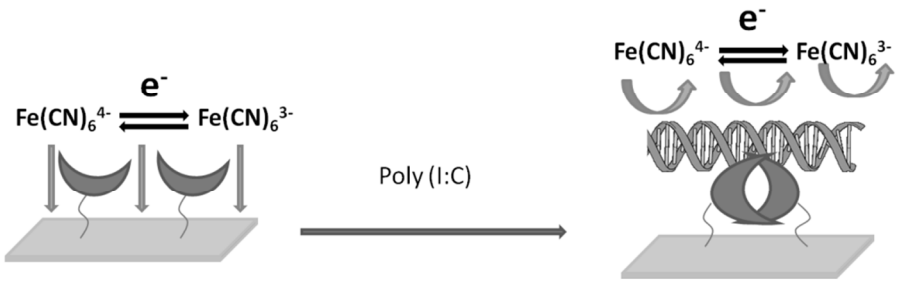

Fig. 2 Schematic illustration of the electrochemical immunoassay using a TLR3 immobilized Au electrode for poly (I:C).

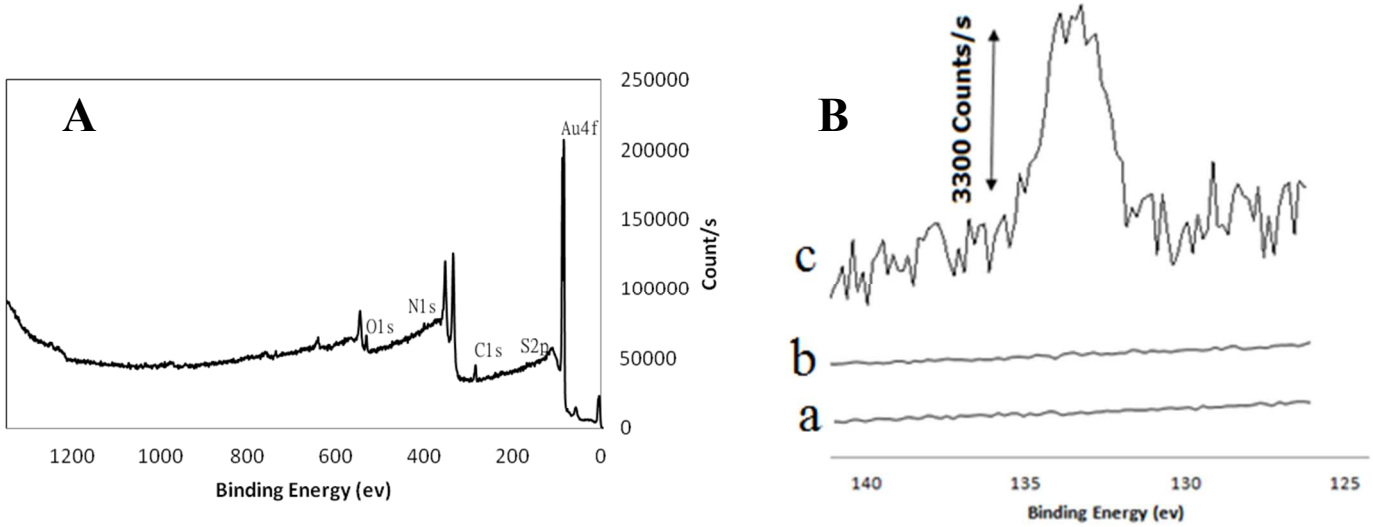

Fig. 3 (A) Representative XPS spectrum of the TLR3-modified gold on a silicon surface. (B) XPS data for the phosphorus 2p region for gold on silicon surface after (a) incubation in PBS buffer pH 7.00, (b) incubation in 0.5 μg mL^{-1} poly (I:C) and rinsing thoroughly with Millipore water and (c) incubation in 5 μg mL^{-1} poly (I:C) and rinsing thoroughly with Millipore water.

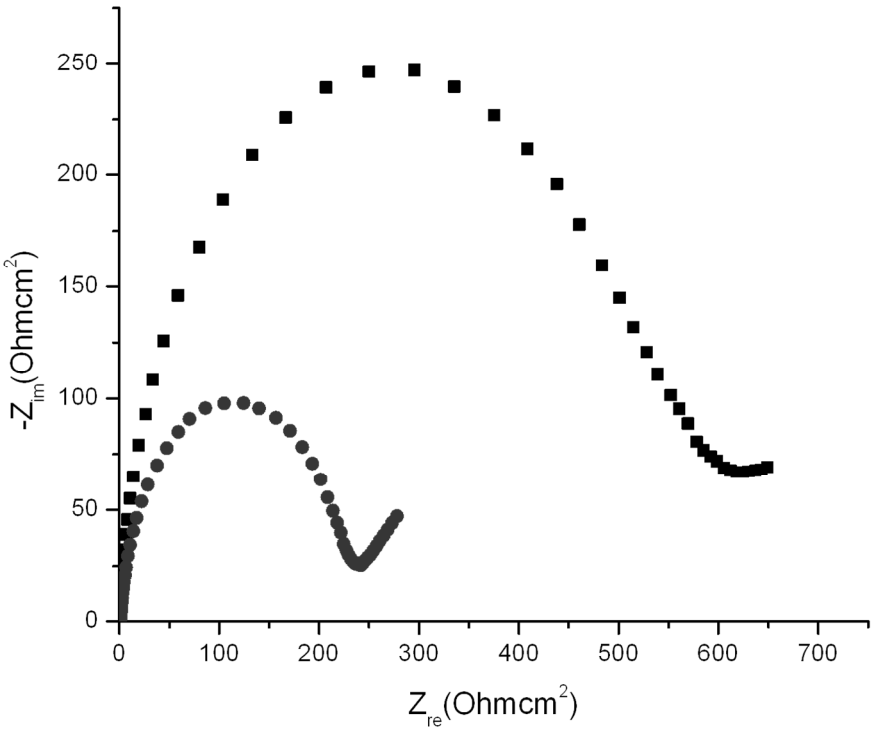

Fig 4 EIS obtained for the Au surfaces modified in (●) Tris buffer pH 7.4 and (■) borate buffer pH 7.4.

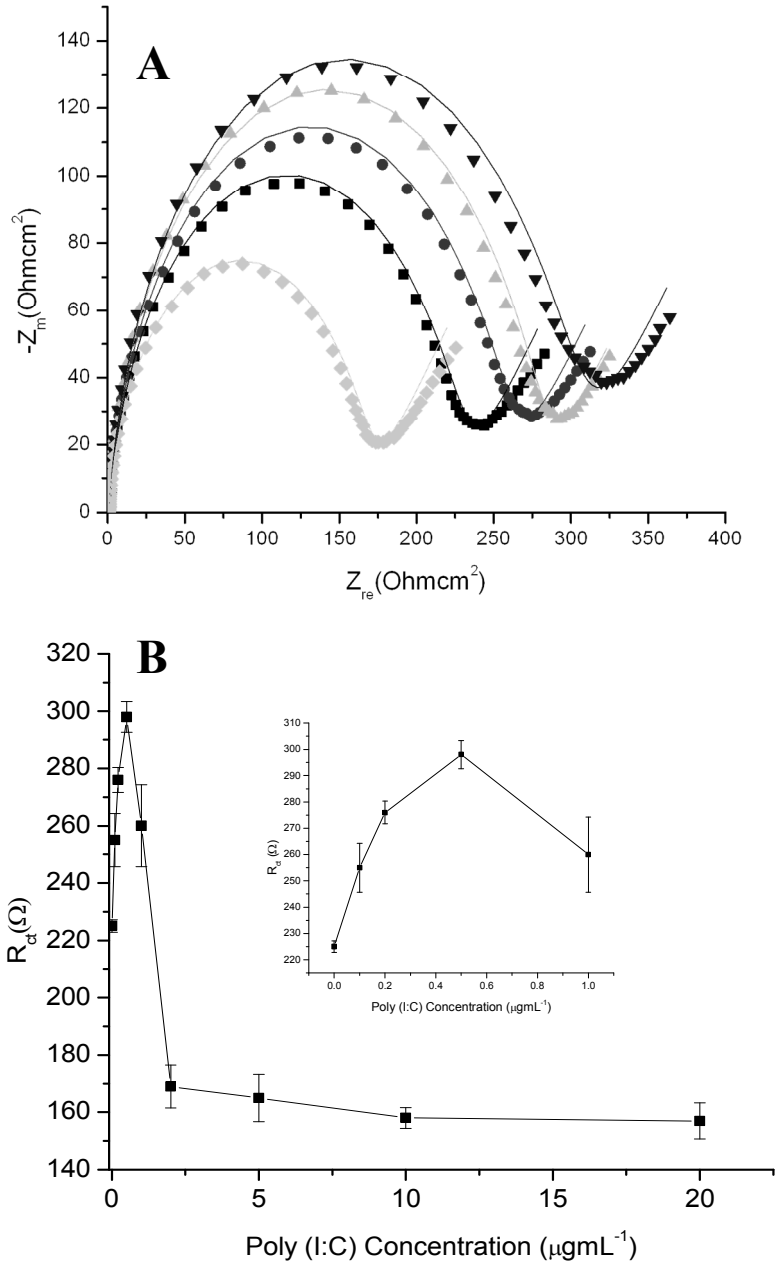

Fig. 5 (A) EIS obtained for the incubation of TLR3-modified Au electrodes with (■) 0.0, (●) 0.2, (▲) 0.3, (▼) 0.5, and (◆) 5, μg mL^{-1} poly (I:C) in the presence of 5 mM $[Fe(CN)_6]^{3-/4-}$ in 1 M $NaClO_4$, pH 7.0 (for the plot illustrating all EISs please refer to Fig. S2). Data points show experimental results while solid lines represent the spectra calculated for the equivalent circuit shown in

scheme 1. (B) R_{ct} values obtained by fitting the EIS data obtained after incubation of the TLR3-modified Au electrodes with different concentrations of poly (I:C).

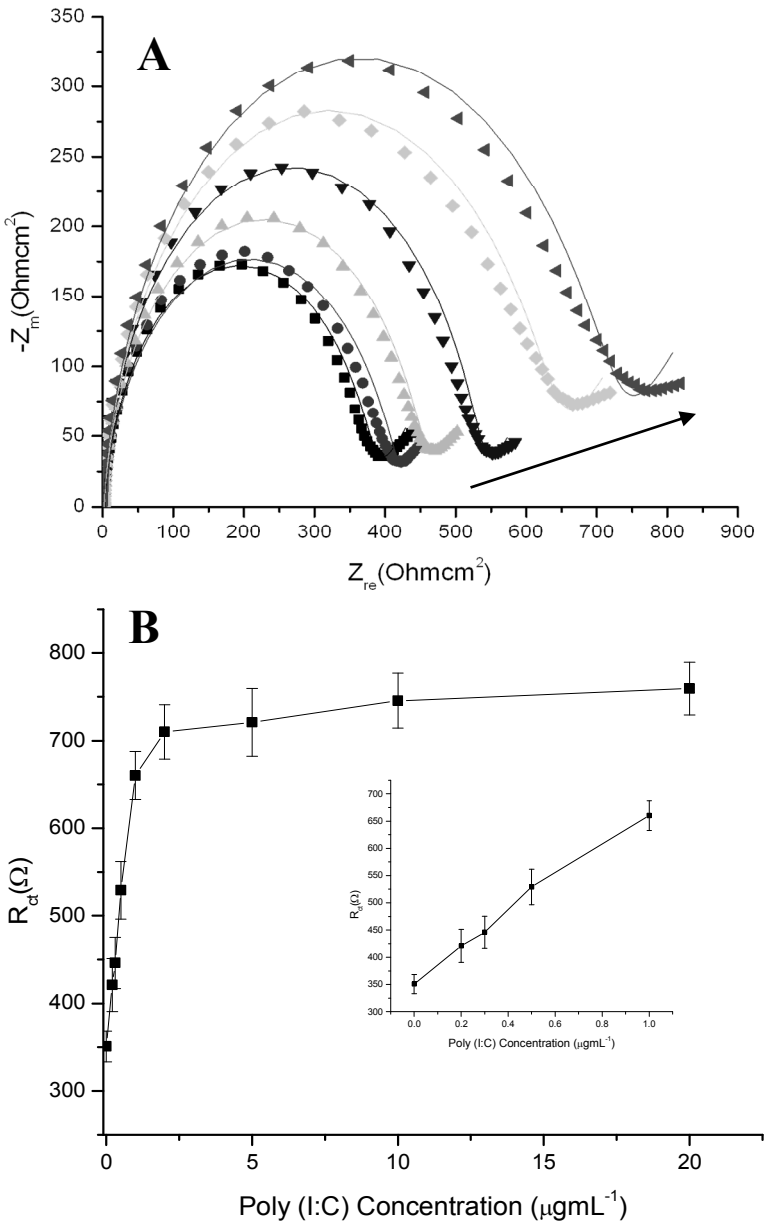

Fig 6. (A) EIS obtained for the incubation of TLR3-modified Au electrodes with (■) 0.0, (●) 0.2, (▲) 0.3, (▼) 0.5, (◆) 1 and (◀) 2 μg mL^{-1} poly (I:C) in the presence of 5 mM $[Fe(CN)_6]^{3-/4-}$ in 100 mM Borate buffer, pH 6.5 (for the plot illustrating all EISs please refer to Fig. S3). Impedance response increases with increasing poly (I:C) concentration. Data points show experimental

results while solid lines represent the spectra calculated for the equivalent circuit shown in scheme 1. (B) R_{ct} values obtained by fitting the EIS data obtained after incubation of the TLR3-modified Au electrodes with different concentrations of poly (I:C).