Amperometric supramolecular genosensor self-assembled on cyclodextrin-modified surfaces

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Abstract:

A novel genosensor platform based on supramolecular interactions has been developed based on the self-assembly of bifunctionalised polymer bearing adamantane and DNA onto cyclodextrin surfaces. The surface chemistry can undergo a controlled regeneration, as revealed by SPR and impedance spectroscopy, has an excellent detection limit of 0.08 nM and demonstrated high selectivity, clearly differentiating between complementary and non-complementary DNA sequences. The performance of the developed genosensor was validated by applying it to the detection of DNA in a real patient sample that had been previously genotyped.

1. Introduction

Supramolecular architectures [1] are an attractive strategy for the construction of biosensor platforms as they facilitate the fabrication of highly organised molecular systems on surfaces and the design of novel functional materials and devices [2]. Recently, the host-guest interactions of cyclodextrins (CDs) [3] with size-compatible hydrophobic molecules have been used for the reversible immobilisation of different biomolecules based on the inclusion of adamantane (ADA) containing polymers, dendrimers or enzymes [4]. In this strategy, the CD/ADA host-guest complex docks the biological element to the surface of the transducer whilst also offering the possibility of a stepwise surface regeneration to re-use the supramolecular platform [5]. This method has been employed to immobilise proteins such as cytochrome c [6] and xanthine oxidase [7]. More recently, our group has reported the construction of a tri-dimensional catalytic biosensor surface based on the layer-by-layer technique, [8] in which successive layers of enzyme-adamantane conjugates are deposited on a CD-modified surface using CD-coated gold nanoparticles as the gluing element.

To the best of our knowledge, this type of supramolecular architecture has not been applied to the construction of DNA biosensors. Here we report a novel strategy for the construction of genosensors exploiting the interfacial self-assembly of ADA-modified carboxymethylcellulose (CMC) polymers on CD-modified surfaces. We exemplify this approach in the construction of a genosensor, where the self-assembled polymer is modified with an oligonucleotide capture probe, which acts as the biorecognition element. The target DNA is detected by hybridisation in a sandwich format, between the capture probe and an enzyme labelled reporter probe (Figure 1). The surface was characterised using surface plasmon resonance (SPR) and electrochemical impedance

spectroscopy (EIS). The genosensor platform was applied to the detection of a human leukocyte antigen allele associated with celiac disease [9], an autoimmune condition of known genetic predisposition [10].

2. Experimental

2.1. Reagents

Cyclodextrins were a gift from Wacker Chemie (Germany). Thiolated α-, β-, and γ–cyclodextrin polymers (CDPSH) [8] and aminated CMC (CMC-NH_2) [11] were prepared as reported. Synthetic oligonucleotides were purchased from Biomers.net (Ulm, Germany) with the following sequences:

HLA-DQA1*0201 aminated capture probe: NH_2-$(CH_2)_6$-5'-caaatctaagtctgtgga-3'

HLA-DQA1*0201 target: 5'-gagaggaaggagactgtctggaagttgcctctgttccacagacttagatttgac ccgcaatttgcactgacaaacatggctgtgctaaaacata-3'

HLA-DQA1*0201 HRP-labeled probe: 5'- gacagtctccttcctctc-HRP-3'

2.2 Synthesis of modified CMC carrying adamantane and DNA (ADA-CMC-DNA)

0.5 g of adamantane carboxylate sodium salt dissolved in 0.1 M acetate buffer pH 5 were treated at 4°C with 0.5 g of EDC for 1 hour and added dropwise over a solution of CMC-NH_2 [11] (0.5 g) in 2 mL carbonate buffer pH 9 under stirring conditions overnight. The mixture was dialysed against water and concentrated to dryness to give ADA-CMC (Yield: 0.8 g). IR (ATR): 3310 (ν_{O-H}), 2931 (ν_{C-H}), 1106 (ν_{C-O}), 1579 ($\nu_{C=O}$). ^{1}H-NMR (300 MHz, D_2O, 300 K) δ (ppm): 0.5-1.2 (m, adamantane protons); 2.0-3.2 (m, $N(CH_2)_6N$); 3.2-4.6 (m, glucose skeletal protons). 4.9-5.3 (anomeric protons). Integration of the protons in the 0.5-1.2 ppm region with respect to the

anomeric protons (δ = 4.9-5.3 ppm) indicate an average of 0.9 adamantane residues per glucose unit.

To attach the DNA probe, 0.5 g of ADA-CMC in 5 mL of 0.1 M acetate buffer pH 5 was activated with 0.5 g of EDC and the mixture stirred for 1 hour at 4ºC. The DNA probe was conjugated by adding 500 μL of 0.5 mM HLA-DQA1*0201 aminated capture probe and the solution stirred overnight. The ADA-CMC-DNA conjugate was purified using a Microcon® centrifugal filter device (MW cut-off 10 kDa) and absence of DNA in the residual water was tested using UV spectroscopy. The obtained stock solution was stored at -20 ºC. The amount of DNA attached to ADA-CMC (0.012 mol of DNA per mol of glucose unit) was estimated using UV-Vis spectroscopy at 260 nm.

2.3 Surface plasmon resonance

SPR studies were carried out using a Biacore® 3000 instrument at 20°C. Clean gold chips were modified overnight with a 10 mg/mL solution of thiolated cyclodextrin polymers, mounted in the Biacore support and a 5 μL/min flow of running buffer (10 mM PBS pH 7.4) was established. After baseline stabilisation a layer of ADA-CMC-DNA polymer was created by injecting a 1 mg/mL solution in PBS. Target hybridisations (0.5 and 1 nM) were followed by a denaturation step in 10 mM NaOH. The CDPSH surface was regenerated with 0.1% v/v sodium dodecylsulfate (SDS).

2.4 Measurements

Electrochemical measurements were performed using a standard three-electrode configuration; working electrode: gold disk (ϕ = 1.6 mm), reference electrode: Ag/AgCl(sat), counter electrode: Pt wire. The gold electrodes were polished three times

with alumina slurry (1, 0.5, 0.03 μm) followed by cleaning in hot *Piranha's* solution (30% H_2O_2/H_2SO_4 (conc) = 3/1 (v/v)) for 5 minutes (*Warning: Piranha's solution is very corrosive)*.

To modify the electrodes, 100 μL of a 10 mg/mL βCDPSH solution were incubated on the electrodes overnight. After rinsing with water, 100 μL of ADA-CMC-DNA (0.1 mg/mL) were incubated overnight. The next incubation steps were carried out immediately prior to the amperometric measurements. For this purpose, 100 μL of HLA-DQA1*0201 target (previously heated at 70ºC to disrupt any self-folding) at different concentrations (0, 0.1, 0.2, 0.5, 0.75, 1, 1.5, 2, 5 nM) in PBS pH 7.4 containing NaCl 0.8 M were incubated with the surface modified genosensor for 1 hour at 37ºC. Following another rinse with water, a 100 nM solution of HLA-DQA1*0201 HRP-labelled probe was added and the genosensor was incubated for 1 hour at 25ºC. The amperometric measurements were carried out by first recording the background response at 0.2 V in PBS buffer pH 6 followed by injection of 1 mM TMB/H_2O_2 in 0.1 M PBS pH 6 + 0.15 M KCl.

3. Results and Discussion

The formation of the interfacial supramolecular platform involves a two-step process: i) deposition of βCDPSH on bare gold electrodes, ii) supramolecular capture of ADA-CMC-DNA by ADA/CD host-guest interactions. The deposition of a SAM of βCDPSH was confirmed via observation of a cathodic desorption peak of the thiolated polymer at -1.02 V in alkaline solution (0.5 M KOH), the integration of which indicated a surface concentration of 2×10^{-12} mol/cm^2. This translates into 2.4×10^{-11} mol/cm^2 of cyclodextrin units taking into consideration that each βCDPSH molecule has ~12 mol of

cyclodextrin per mol of polymer, which is in the same order of magnitude of the surface coverage of a monolayer of hepta-6-thio-6-deoxy-β-cyclodextrin [12].

Electrical impedance spectroscopy is a powerful tool to study bimolecular interactions at interfaces [13]. The formation of the interfacial supramolecular platform was confirmed using EIS by observing the variations of the charge transfer resistance (R_{ct}) exerted by the deposition of the successive layers on an electroactive ferricyanide probe (Figure 2). Deposition of a SAM of βCDPSH caused a 175 kΩ increase in R_{ct} with respect to the bare electrode (R_{ct} = 2 kΩ). The R_{ct} values further increased to 348 kΩ after interaction of the βCDPSH-modified surface with ADA-CMC-DNA and then to 530 kΩ after capture of the HLA-DQA1*0201 target sequence by the Au/βCDPSH/ADA-CMC-DNA surface. In contrast, the interaction of ADA-CMC-DNA with a αCDPSH-modified surface provoked only a 6% variation in R_{ct}, indicating that the immobilised αCD hosts cannot recognise the ADA polymer, in agreement with the SPR results (see below).

The interaction of ADA-CMC-DNA carrier with βCDPSH was also studied by surface plasmon resonance (SPR) (Figure 3). The individual channels of an SPR Au chip were modified with α-, β- and γCDPSH polymers. When ADA-CMC-DNA was injected to the βCDPSH-modified channel, a significant response of 400 RU was observed (Figure 3a). Assuming the equivalence 1 RU = 1 pg/mm^2 [14], this response affords a surface coverage of ~2 × 10^{-13} mol/cm^2 assuming a molecular weight for ADA-CMC-DNA of 205 kDa, corresponding to a CMC polymer of molecular weight 90 kDa and a degree of substitution of 0.9 adamantane and 0.012 oligonucleotide probes per glucose unit, respectively. This represents a probe density of ~1.2 × 10^{11} molecules/cm^2, which is in

the typical range of 10^{11}-10^{13} molecules/cm^2 considered to be the optimal to avoid inter-probe electrostatic repulsions on the surface [15]. The SPR response obtained with βCDPSH markedly differs from those observed with αCDPSH (19 RU, Figure 3b) and γCDPSH (25 RU) modified surfaces. These differences are explained by the lower association constants (about two orders of magnitude) observed for ADA derivatives with αCD (smaller cavity) and γCD (larger cavity) compared with βCD ($K_{ass} = 4 \times 10^5$ M^{-1}), due to an optimum geometric matching of cavity size and ligand size in the ADA/βCD system [16]. In addition, when a CMC-DNA polymer (i.e. not carrying ADA units) was injected on the βCDPSH-modified surface, a very small response was obtained (11 RU). Finally, no interaction with the βCDPSH modified surface was observed by SPR using a CMC polymer prepared in a similar manner to ADA-CMC but modified with a bulkier residue (3,5,7-trimethyladamantane-1-carboxylic acid). The selectivity observed in the interaction of the ADA-CMC-DNA polymer with the different surfaces and the lack of response in the absence of ADA units or presence of bulkier residues clearly indicate that the ADA-CMC-DNA polymer is immobilised on the βCDPSH surface via specific ADA-βCD host-guest interactions.

Injection of 0.5 nM of target to a channel modified with the Au/βCDPSH/ADA-CMC-DNA supramolecular platform gave a response of 44 RU (Figure 3c). The biosensor surface could be regenerated by applying two pulses of 10 mM NaOH to denature the formed DNA duplex. This restored the SPR signal at ~400 RU corresponding to the Au/βCDPSH/ADA-CMC-DNA surface, which indicates that the ADA-βCD assembly is essentially unaltered (Figure 3d). Subsequently 1 nM of target was injected, obtaining 90 RU, a value that is in good correspondence with the value obtained with 0.5 nM target (Figure 3e). Finally, the DNA probe surface was again regenerated in alkaline

conditions (Figure 3f), demonstrating the reusability of the biosensor surface. Whilst it is not envisaged that the genosensor would be re-used when applied to clinical diagnostics, re-usability of the sensor surface is very useful during developmental work. Injection of a 0.1% v/v solution of SDS caused desorption of ADA-CMC-DNA from the cyclodextrin support, rendering it available to capture a new probe layer (Figure 3g). The developed βCDPSH/ADA-CMC-GLI surface was then applied to the amperometric detection of the HLA-DQA1*0201 target sequence using a peroxidase-labelled secondary probe as reporter. Figure 4a shows a comparison of the specific (presence of target) and non-specific (absence of target) signals obtained. The non-specific signal represented only 4% of the specific signal obtained at 1 nM concentration, indicating a very low tendency of the reporter probe to interact with the surface in the absence of target. In addition, when the βCDPSH support layer was not present, a signal about 10% was observed demonstrating the importance of the cyclodextrin layer in assisting the immobilisation of the probe. Three possible interference sequences were added in place of the target and only ~5% of the signal was observed, demonstrating the high selectivity of this biosensor.

Figure 4b shows the calibration curve obtained with the βCDPSH/ADA-CMC-GLI modified surface, which was linear in the range 0-2 nM with a sensitivity of 0.35 nM/μA and a limit of detection of 80 pM. A preliminary study was carried out using a real PCR-amplified sample of a coeliac patient, who had been previously genotyped and shown to carry the HLA-DQA1*0201 allele using Luminex based HLA typing. Single stranded DNA was generated via exonuclease digestion and directly applied to the electrode surface and the quantitative result obtained compared to enzyme linked oligonucleotide assay, showing a high degree of correlation with values of 0.75 ± 0.09

nM) and 0.86 ± 0.05 nM, obtained respectively, demonstrating the genosensor to have detection limits that easily allow it to be applied to the direct analysis of real PCR products. Negligible performance changes (<5%) were observed for the supramolecular biosensor in the detection of 1 nM of target sequence after one week of storage at 4ºC in a commercial stabilising buffer (StabilCoat® Plus Microarray Stabilizer), indicating an excellent stability of the self-assembled structure.

Conclusions

A novel biosensor platform based on supramolecular interactions has been developed for genosensor construction based on the self-assembly of bi-functionalised polymer bearing adamantane and DNA onto cyclodextrin surface. The developed amperometric genosensor has an excellent LOD of 0.08 nM as well as high selectivity and was applied to the detection of DNA in a real patient's sample. The combination of a hydrophilic support layer (βCDPSH) with the controlled attachment of the capture probe to a polymeric backbone minimises non-specific interactions and provides an optimal probe separation to avoid electrostatic repulsions, which is essential in the development of DNA biosensors. The CMC backbone allows an optimal spacing of the DNA probes to avoid steric hindrance for target binding due to an excessively dense layer of probe DNA, with the negative charge of the CMC vertically orienting the probe, and the combination of the CD and the CMC facilitate maximal binding of the target DNA. The work reported here highlights the feasibility of using cyclodextrin based supramolecular surface chemistries for the detection of DNA and the work is being extended to the multiplexed, microsystem packaged, genosensor array with a focus on reducing hybridisation time and the number of PCR cycles required.

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Figures:

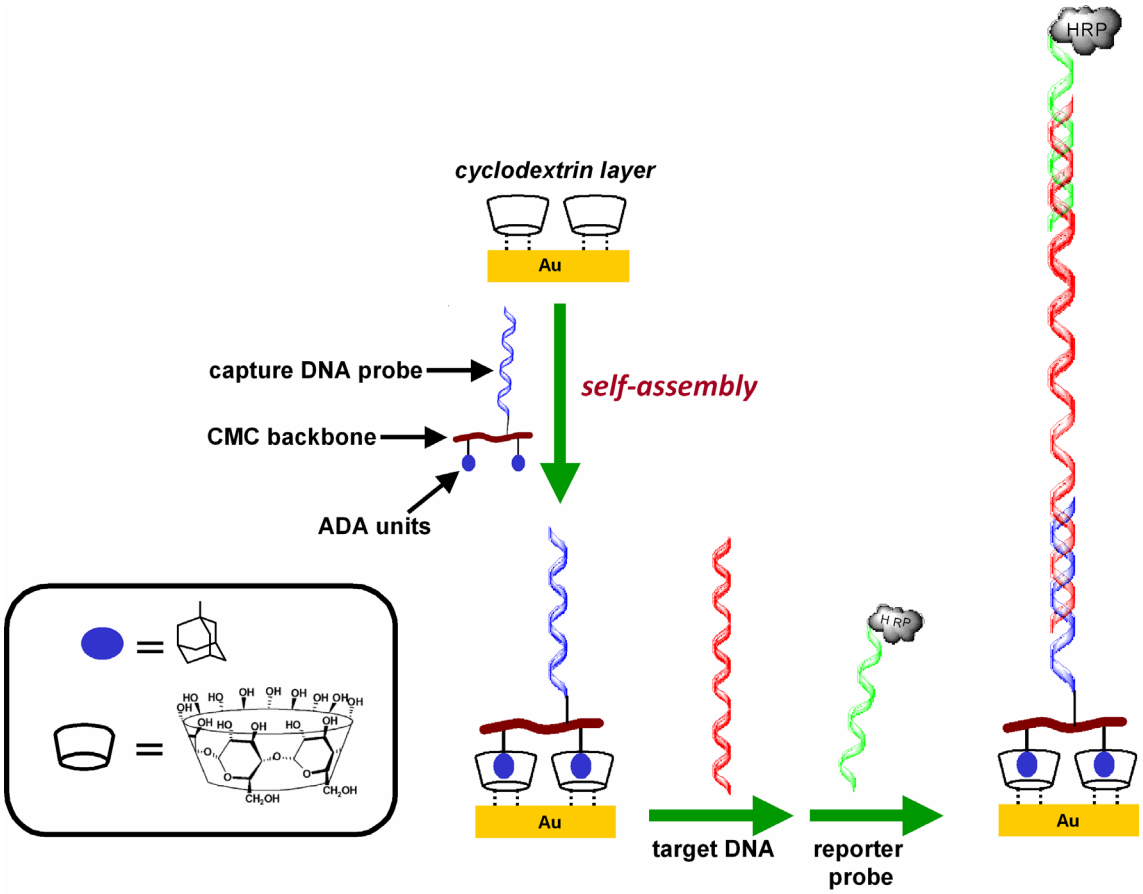

Figure 1. Strategy employed for the construction of the supramolecular genosensor.

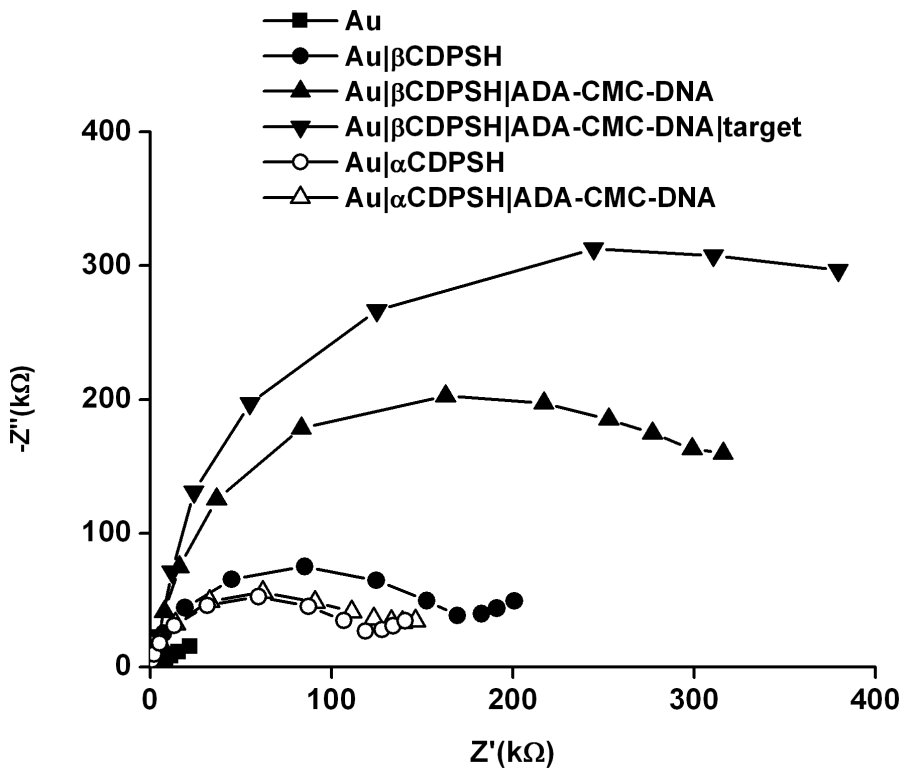

Figure 2. Complex impedance plots (in 1 mM $K_3Fe(CN)_6$ in 0.1 M KCl) obtained alter successive electrode modifications.

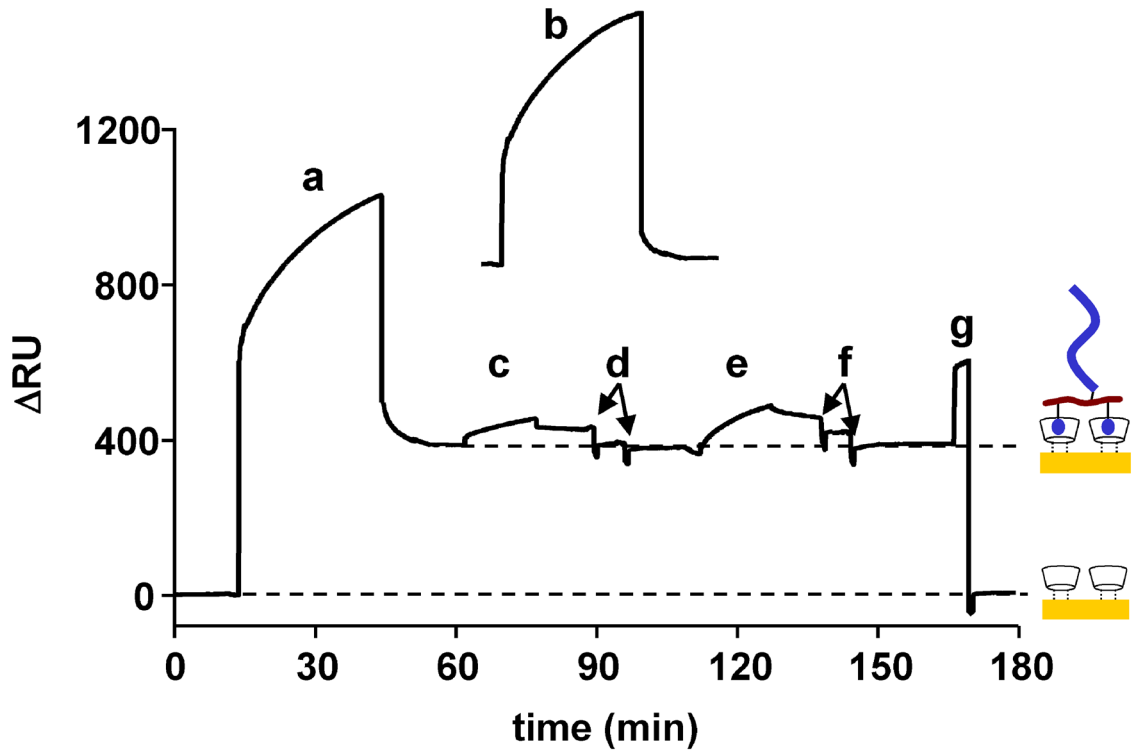

Figure 3. SPR sensorgram of injections of (a) 1 mg/mL βCDPSH, (b) 1 mg/mL αCDPSH, c) 0.5 nM HLA-DQA1*0201 target, (d) 10 mM NaOH pulses, (e) 1 nM HLA-DQA1*0201 target, (f) 10 mM NaOH pulses, (g) 0.1% SDS. Conditions: running buffer: 0.1 M PBS pH 7.4, flow rate 5 µL/min.

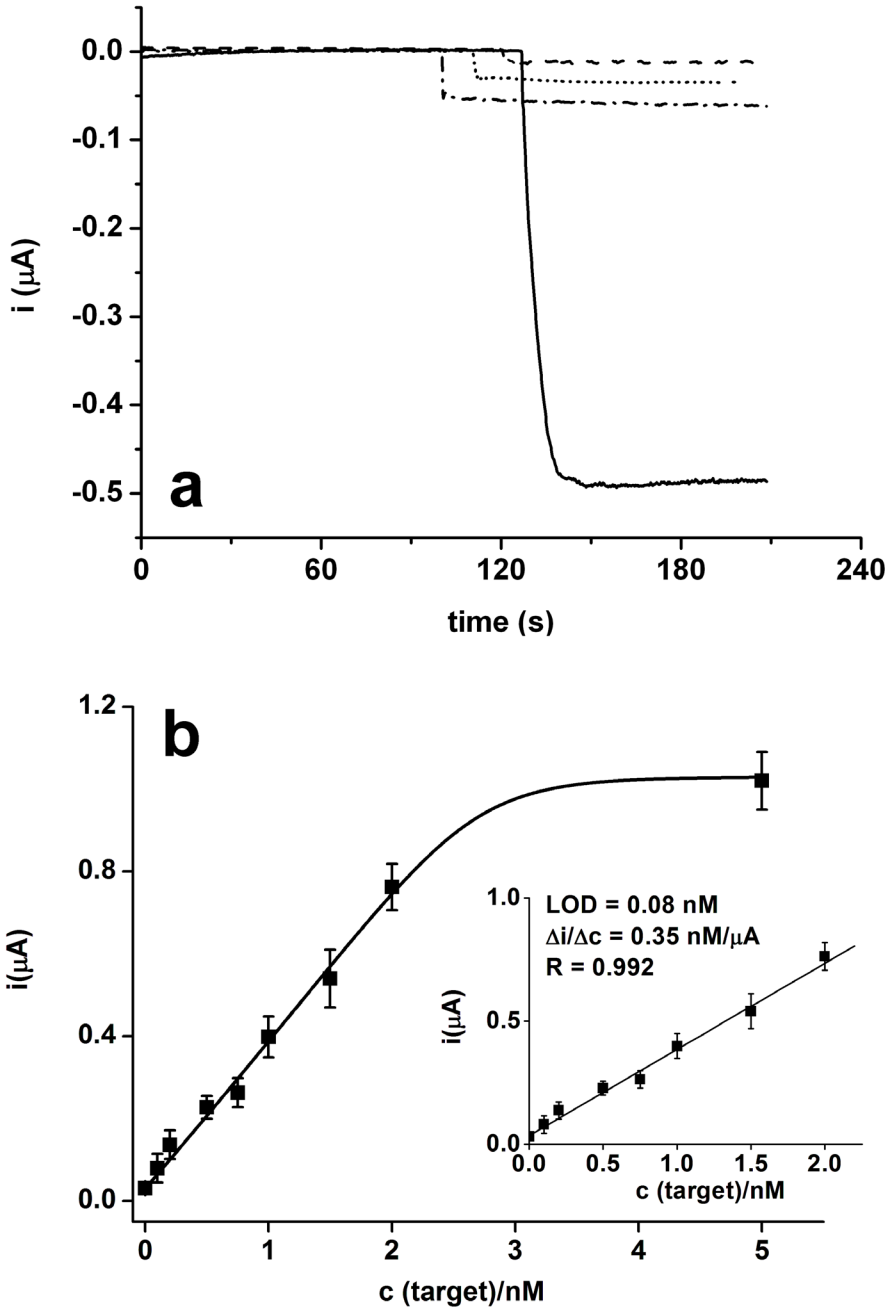

Figure 4. a) Amperometric responses for the detection of HLA-DQA1*0201 sequence in the presence (——) and absence of target (-------), absence of cyclodextrin support (·-·-·-·-·-·) and in the presence of a non-complementary target (·····). b) Calibration curve for the amperometric detection of HLA-DQA1*0201 target sequence.