SUPRAMOLECULAR CONFINEMENT OF POLYMERIC ELECTRON TRANSFER MEDIATOR ON GOLD SURFACE FOR PICOMOLAR DETECTION OF DNA

Mayreli Ortiz [a], Mabel Torréns [a], Nuria Canela,
Alex Fragoso *[a] and Ciara K. O'Sullivan *[ab]

* Corresponding authors

[a] Nanobiotechnology & Bioanalysis Group, Departament d'Enginyeria Química, Universitat Rovira i Virgili, Avinguda Països Catalans 26, Tarragona, Spain
E-mail: alex.fragoso@urv.cat, ciara.osullivan@urv.cat

[b] Unitat de Biologia Molecular, Servei de Recursos Científics i Técnics, Universitat Rovira i Virgili, Avinguda Països Catalans 26, Tarragona, Spain

[c] Institució Catalana de Recerca i Estudis Avançats, Passeig Lluis Campanys, 23, Barcelona, Spain

1 ABSTRACT

In this work a novel route for the immobilisation of an electron transfer mediator on electrode surfaces based on the interfacial complexation of a bifunctionalised carboxymethylcellulose (CMC) polymer backbone bearing ferrocene units and a DNA probe on a cyclodextrin-functionalised surface is described. The interfacial self-assembly has been studied using surface plasmon resonance and electrochemical techniques and the applicability of the modified surface for the construction of an amperometric genosensor was explored for the detection of a celiac disease associated allele. The supramolecular strategy simplifies the operation of the biosensor, only requiring the addition of enzyme substrate and the proximity of the mediator to the electrode surface greatly improves the detection limits attained (10 pM) with respect to a similar supramolecular system based on electrochemically inactive adamantane/CD inclusion complexes (80 pM) and requiring addition of the mediator in solution. In addition, the use of the hydrophilic CMC backbone contributes to the elimination of non-specific interactions and to an optimal spacing of the immobilised DNA probes.

2 INTRODUCTION

Supramolecular strategies based on self-assembly and host-guest interactions[1] have recently attracted great interest as surface modification tools to achieve pre-organization and improved functional properties in a vast array of molecular assemblies.[2] An interesting approach to construct organized structures on surfaces exploits the formation of host-guest pairs using molecular receptors (cyclodextrins, calixarenes, cyclotriveratrylenes, etc.), allowing the self-assembly of two-dimensional[3-8] and three-dimensional nanoarchitectures.[9-12] These systems have been used for the immobilisation of different molecules, rendering functional structures with high specificity and affinity. In the case of cyclodextrins (CD),[13] supramolecular immobilisation is in most cases achieved by the interaction of adamantane-appended bifunctional linkers or biomolecule conjugates with a surface modified with a monolayer of thiolated CD.[14] This method has been employed to immobilise proteins such as cytochrome c[15] and streptavidin[10] or for the construction of catalytic biosensors.[16,18] Recently, we reported a supramolecular genosensor for the detection of a human leukocyte antigen allele associated with celiac disease based on the self-assembly over a cyclodextrin surface of a bifunctionalised polymer bearing adamantane units and a DNA probe.[19] Using amperometric detection, a very low limit of detection (LOD) of 80 pM was obtained and the genosensor was validated using a previously genotyped patient sample.

Ferrocene (Fc) is a redox active metallocene commonly used as an electron transfer mediator in oxidase-based biosensors as it undergoes reversible one-electron oxidation at a low potential to give a ferrocenium cation. Fc moieties have been incorporated on electrode surfaces for the construction of reagentless biosensors via anion-exchange of ferrocenecarboxylate on polypyrrole films,[20] layer-by-layer deposition of Fc-terminated dendrimers,[21] casting of Fc-polysaccharide derivatives,[22] sol-gel,[23] and carbon nanotube[24] composites, among others. The supramolecular deposition of linear Fc-functionalized polymers derived from chitosan and poly(allylamine)[25] and Fc-appended biotin terminated linkers[26] on gold surfaces modified with b-cyclodextrin has recently been reported, where a quartz crystal microbalance coupled with cyclic voltammetry was used to monitor the deposition of the Fc-polymers and their subsequent desorption, triggered by the in situ oxidation of the Fc moieties that destabilize the inclusion complex. In another report, layer-by-layer polymer films based on host-guest interactions were formed by the stepwise adsorption of poly(allylamine) and poly(*N*-

hydroxypropylmethacrylamide) derivatives bearing ferrocene or β-cyclodextrin moieties.[27]

Here we report the interfacial complexation of a Fc-containing polymer backbone on a cyclodextrin surface and explore its applicability in the construction of an amperometric genosensor for the detection of DNA (**Figure 1**). A bifunctionalised carboxymethylcellulose (CMC) polymer tethers ferrocene units on one side and a short linear DNA probe on the other. The target DNA sequence is then detected via hybridization to the immobilised probe using a reporter probe labelled with horseradish peroxidase (HRP) in a sandwich type format. Thus, the Fc residues present in the structure of the self-assembled platform not only serve to dock the polymer structure on the CD-surface by via inclusion complexation but also act as an electron transfer mediator for the peroxidise label and the

interfacial association constants are measured by surface plasmon resonance (SPR). As a model detection system, we selected a target oligonucleotide sequence belonging to the human leukocyte antigen HLA-DQA1 allele family, which is associated to celiac disease,[28] an autoimmune condition showing almost 100% genetic predisposition.[29,30] As a consequence of the proximity and confinement of the Fc mediator at the electrode surface, a significant signal enhancement and a markedly lower detection limit are observed as compared to the use of a solution-based mediator.

3 EXPERIMENTAL SECTION

Materials

All reagents used were commercially available and used as received. Carboxymethylcellulose (CMC, MW 90 kDa), N-Ethyl-N′-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC), Phosphate Buffered Saline (PBS) dry powder and ferrocenecarboxaldehyde were purchased from Sigma-Aldrich. All solutions were prepared with Milli-Q water (Millipore Inc., Ω = 18 MΩ·cm). Thiolated α, β and γ cyclodextrin polymers were prepared as previously reported.[17] The synthesis of Fc-appended CMC conjugate bearing DNA probes is depicted in **Scheme 1.**

Synthetic oligonucleotides were purchased from Biomers.net (Ulm, Germany) and their sequences are shown below:

HLA-DQA1*0201 aminated capture probe: NH_2 C_6-5’-CAA ATC TAA GTC TGT GGA-3’.

HLA-DQA1*0201 target: 5’- GAG AGG AAG GAG ACT GTC TGG AAG TTG CCT CTG TTC CAC AGA CTT AGA TTT GAC CCG CAA TTT GCA CTG ACA AAC ATG GCT GTG CTA AAA CAT A-3’.

HLA-DQA1*0201 HRP-labeled probe: 5’- GAC AGT CTC CTT CCT CTC-HRP-3’.

Interference 1: 5’-GAG AGG AAG GAG ACT G**C**C TGG **CG**G T**G**G CCT **GA**G TTC **AG**C A**A**A **T**TT GGA GGT TTT GAC CCG CAG GGT GCA CTG AGA AAC ATG GCT GTG GCA AAA CAC A-3’.

Interference 2: 5’-GAG AGG AAG GAG ACT GTC TGG CAG TTG CCT CTG TTC CGC AGA TTT AGA AGA TTT GAC CCG CAA TTT GCA CTG ACA AAC ATC GCT GTG CTA AAA CAT A-3’.

Interference 3: GGG AGG AAG GAG ACT GTC TGG TGT TTG CCT GTT CTC AGACAA TTT AGA TTT GAC CGG CAA TTT GCA CTG ACA AAC ATC GCT GTC CTA AAA CAC A-3’.

Instrumentation and methods

Synthesis of Fc-CMC (2)

0.5 g of aminated CMC[31] (1.5 mmol of aminohexane groups) were dissolved in 25 mL of Milli-Q water. A solution of 0.32 g (1.5 mmol) of ferrocenecarboxaldehyde in 2 mL of DMSO was added dropwise with continuous magnetic stirring. After 3 hours an excess (60 mg, 15 mmol) of sodium borohydride was added and the solution was stirred overnight at room temperature. The mixture was concentrated to about half the initial volume by roto-evaporation and dialysed for 24 hours to remove impurities and was then dried *in vacuum* to give Fc-CMC (Yield: 0.31 g). ^{1}H-NMR (300 MHz, D_2O, 300 K) δ (ppm): 1.9-3.2 (m, Fc-CH_2-N, N(CH_2)$_6$N); 3.2-4.6 (m, overlapped Fc and glucose skeletal protons). 4.9-5.3 (m, anomeric protons). ^{13}C-NMR (75 MHz, D_2O, 300 K); 169 (bs, N*C*=O), 181 (bs, O*C*=O). UV-Vis: λ_{max} 430 nm (e = 1700 cm·M^{-1}, Fc M→L charge transfer). The amount of Fc units in **2** (0.86 mol Fc/mol glucose) was estimated by UV-Vis spectroscopy at 400 nm by interpolation of absorbance values of a polymer solution in a calibration curve prepared using aminoferrocene.

Synthesis of Fc-CMC-DNA conjugate (3)

Sodium meta-periodate (20 mg) was added to **2** (20 mg) in water (5 mL) and stirred for 3 hours at room temperature, followed by overnight dialysis to remove non-reacted material. The dialysed solution (containing aldehyde-activated Fc-CMC) was used in the next step. The presence of aldehyde groups was qualitatively confirmed using 2,4-dinitrophenylhydrazine. The dialyzed solution was treated with 2.5 µmol of HLA-DQA1*0201 aminated capture probe under stirring for 3 hours, after which 10 mg of sodium cyanoborohydride were added and the solution was stirred overnight. The Fc-CMC-DNA conjugate was purified using a Microcon® centrifugal filter device (Mw cut-off 10 kDa) for 3 minutes at 10000 rpm and washed twice with water. The absence of DNA in the filtrate was confirmed using UV spectroscopy at 260 nm and the obtained stock solution was stored at -20 ºC. The amount of DNA in Fc-CMC-DNA (0.014 mol of DNA per mol of glucose unit) was estimated using UV-Vis spectroscopy at 260 nm by interpolation of Fc-corrected absorbance values of a solution of Fc-CMC-DNA in a calibration curve prepared using HLA-DQA1*0201 aminated capture probe and considering that the molecular weight of the probe is 5700 Da. To correct the absorbance

at 260 nm from the contribution of Fc, solutions of Fc-CMC-DNA and Fc-CMC having identical absorbance at 430 nm (where only Fc absorbs) were prepared and the absorbance at 260 nm of the Fc-CMC solution was subtracted from Fc-CMC-DNA to calculate the contribution of DNA to the overall absorbance at this wavelength.

Synthesis of CMC-DNA conjugate (4)

CMC (10 mg) dissolved in 5 mL of water were treated with 20 mg of sodium meta-periodate with stirring at room temperature. DNA probe was attached in a similar way as described for the synthesis of **3**. The CMC-DNA conjugate contained 0.012 mol of DNA per mol of glucose unit and was purified as described above.

Surface plasmon resonance (SPR) studies.

SPR studies were carried out using a Biacore® 3000 instrument operating at 25°C. Gold chips from a Biacore SIA kit were cleaned with Piranha's solution (*Warning: Piranha's solution is very corrosive)* for 3 minutes, washing with water, followed by thorough washing with water and finally treated with ozone using a PSD-UVT cleaning instrument (from Novascan, USA) for 9 min, rinsed with ethanol and dried under a filtered Ar stream. The chip was modified with thiolated cyclodextrin polymer (CDPSH) by overnight immersion in a 10 mg/mL solution followed by extensive rinsing with water, after which the chip was 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 (~ 3 hours) a layer of Fc-CMC-DNA polymer was created by injecting 50 µL of a 1 mg/mL solution in PBS followed by HLA-DQA1*0201 target injection (1 nM in PBS pH 7.4 containing NaCl 0.8 M). Surface regeneration was carried out using 10 mM NaOH to dehybridise the target-probe complex or 1 mM adamantanecarboxylate to remove the Fc-containing polymer from the surface.

Electrochemical Instrumentation

Electrochemical measurements were performed on a PC controlled PGSTAT12 Autolab potentiostat (EcoChemie, The Netherlands) with a built-in frequency response analyzer FRA2 module using a standard three-electrode configuration (working electrode: gold disk, reference electrode: Ag/AgCl(sat), counter electrode: Pt wire).

The gold disk electrodes (f = 1.6 mm, from Bioanalytical Systems) were first polished three times with alumina slurry (1, 0.5, 0.03 μm) until a mirror finish was obtained. After sonication in water for one minute, the electrodes were cleaning in hot *Piranha* solution (30% H_2O_2/H_2SO_4(conc) = 3/1 (v/v)) for 5 minutes (*Warning: Piranha solution is very corrosive)*. The electrodes were then electrocleaned by applying a series of 40 potential cycles in 1 M H_2SO_4 in the range 0-1.7 V vs Ag/AgCl at 0.2 V/s. The quality of the cleaning step was checked using cyclic voltammetry in 1 mM $K_3[Fe(CN)_6]$ in 0.1 M KCl.

Modification of gold electrodes and DNA detection

In a first step, 100 μL of a 10 mg/mL CDPSH solution was incubated on the electrodes overnight to form a self assembled monolayer (SAM) containing cyclodextrin hosts. After rinsing with water, 100 μL of Fc-CMC-DNA (1 μg/mL) was added and incubated overnight. The next incubation steps were carried out immediately prior to the amperometric measurements. One hundred microlitres of HLA-DQA1*0201 target at different concentrations (0 - 5 nM) in PBS pH 7.4 containing NaCl 0.8 M were incubated for 1 hour at 37 ºC. Before each incubation, the target solution was briefly heated to 70 ºC (above the melting temperature measured spectrophotometrically) in order to disrupt any self-folding. After rinsing with PBS, 100 μL of a 100 nM solution of HLA-DQA1*0201 HRP-labeled probe was further incubated for 1 hour at 25 ºC.

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 H_2O_2 in 0.1 M PBS pH 6 in a 2 cm^3 cell.

4 RESULTS AND DISCUSSION

Preparation of ferrocene-appended polymeric DNA carrier

The synthesis of the Fc-CMC-DNA conjugate is shown in **Scheme 1**. CMC was chosen as a carrier polymer as it provides two individually addressable modification points for the attachment of the DNA probe and Fc residues: the COOH group in the C6 position and the vicinal diol group formed between C2 and C3. The COOH groups of the polysaccharide were activated with EDC followed by amidation with 1,6-

diaminohexane, which acts as a spacer. The amino-terminated precursor was reacted with ferrocenecarboxaldehyde under reductive alkylation conditions resulting in the Fc-modified CMC polymer. This polymer was activated with sodium periodate to oxidise the diol groups yielding a reactive aldehyde intermediate that was further reacted with an amino-terminated DNA probe followed by reduction of the formed imino-bond with sodium cyanoborohydride to give the desired Fc-CMC-DNA conjugate. The amounts of Fc and DNA were estimated using UV-Vis spectroscopy, indicating 0.86 mol of Fc and 0.014 mol of DNA per mol of glucose unit. For comparison purposes, a CMC-DNA conjugate was prepared from CMC by oxidation with $NaIO_4$ followed by coupling with amino-terminated DNA in the same reductive alkylation conditions as used for the preparation of the Fc-CMC-DNA.

Electrochemical characterisation of Fc-CMC-DNA deposition

The cyclic voltammogram of the CDPSH/Fc-CMC-DNA platform in PBS buffer pH 7.4 showed a reversible signal at $E_{1/2}$ = 0.19 V with a peak-to-peak separation ΔE_{ac} of 24 mV (**Figure 2**). ΔE_{ac} is essentially scan rate independent up to 0.3 V/s and the peak currents depend linearly on scan rate, indicative of the presence of a surface confined species. The ΔE_{ac} value is slightly higher than the ideal value of 0 mV for a surface-confined reversible redox couple, which may be by considering the polydispersion of the Fc-CMC-DNA polymer that causes the existence of multiple formal potentials for the Fc/Fc^+ couple in the monolayer film. Integration of the cathodic peak (to calculate the charge associated with the process and thus the number of moles of Fc from the Faraday's Law) and normalisation to the electrode surface indicated a surface coverage for the Fc units of 4×10^{-15} mol/cm^2.

Surface plasmon resonance characterisation and measurement of interfacial association constants

The construction of the CDPSH/Fc-CMC-DNA platform was studied using surface plasmon resonance (**Figure 3**). Fc-CMC-DNA was injected into the chip previously modified with βCDPSH giving a SPR response of 433 RU (**Figure 3a**). This response affords a surface coverage of ~2×10^{-15} mol/cm^2 assuming a molecular weight for Fc-CMC-DNA of 213 kDa, which corresponds to a CMC polymer with 80% of COOH

groups and a degree of substitution of 0.86 Fc and 0.014 DNA residues per glucose unit, respectively.

The role of specific Fc/βCD interactions in the immobilisation of the Fc-CMC-DNA was confirmed by the low SPR signals observed due to its interaction with γCD (51 RU) or αCD (37 RU) modified surfaces (**Figure 3 b, c**). γCD and αCD have, respectively, larger and smaller cavity sizes as compared with βCD and, therefore, form weaker inclusion complexes.[32] This explains the marked difference in SPR response observed in the interaction of Fc-CMC-DNA with the three CD-modified surfaces and indicates a major contribution of specific Fc/βCD interactions in the immobilisation process. Due to the relative structural complexity of the DNA-modified polymer it can be expected that other types of interactions might occur between the Fc-CMC-DNA and the βCD-modified surface, such as hydrogen bonding to the amine, amide, and hydroxyl groups as well as non-specific hydrophobic interactions that do not involve inclusion of the Fc groups into the βCD cavities. To assess the contribution of these interactions, a polymer that carries the DNA probe but lacks the Fc units (CMC-DNA), was prepared and allowed to interact with the βCD-surface. In this case, the SPR response was 23 RU (**Figure 3d**), which represents about 6 % of the signal obtained with the ferrocene functionalised polymer, indicative of a minor contribution of these interactions to the immobilisation process.

The target sequence (1 nM) was hybridised to the βCDPSH/Fc-CMC-DNA surface (**Figure 3e**), and surface regeneration via rupture of the DNA target-probe interaction was studied using a range of NaOH concentrations (1-50 mM) and number of pulses (1-3). Regeneration was successfully achieved by applying three pulses of 20 μL of 10 mM NaOH (**Figure 3f**). This restored the SPR signal at ~430 RU corresponding to the Au/βCDPSH/Fc-CMC-DNA surface, which indicates that the Fc/βCD assembly is essentially unaltered. DNA target at the same concentration was injected for a second time (**Figure 3g**) obtaining a response of 63 RU, which was very similar to the first hybridization value of 67 RU. The βCDPSH/Fc-CMC-DNA surface was easily regenerated again with NaOH following the second target injection (**Figure 3h**), demonstrating the reusability of the supramolecular platform for the detection of DNA. Injection of a large excess of adamantanecarboxylate (1 mM) displaced Fc-CMC-DNA from the βCDPSH support indicating the reversibility of the interfacial

Fc/βCD complexation and further confirming the host-guest nature of the immobilisation process (**Figure 3i**).

The interfacial association constants for the complexation of Fc-CMC-DNA with the three different CD surfaces was determined by SPR using the Langmuir equation: $c/\Gamma = c/\Gamma_{max} + 1/K\Gamma_{max}$, where c and Γ are the bulk concentration and surface coverage of the polymers, K is the interfacial association constant and Γ_{max} is the maximum surface coverage of the polymers (**Figure 4**). Linear regression analysis of the data afforded $K_{\beta CD} = 4.4 \times 10^{10}$ M^{-1}, $K_{\gamma CD} = 2.4 \times 10^{7}$ M^{-1} and $K_{\alpha CD} = 7.2 \times 10^{6}$ M^{-1}. These values are several orders of magnitude higher than those observed for individual Fc/CD complexes in solution[32,33] as expected for a multivalent interaction and indicate a higher affinity of the Fc units for the βCD hosts although Fc-CMC-DNA is also able to form interfacial complexes with αCD and γCD modified surfaces. Interestingly, the obtained $K_{\beta CD}$ is very similar to the value recently obtained in our group for the complexation of an adamantane-appended CMC polymer.[34]

Electrochemical detection

The possibility to develop an electrochemical genosensor based on the self-assembled CDPSH/Fc-CMC-DNA platform was evaluated using amperometry, where the presence of the ferrocene units in the platform serves, not only as a docking molecule of the probe to the CDPSH-modified surface, but also acts as an electron transfer mediator by shuttling electrons between the enzyme and the electrode. **Figure 5a-c** shows a comparison of the specific (trace a) and non-specific (trace b) amperometric signals obtained for the CDPSH/Fc-CMC-DNA system and the non-specific signal corresponding to the absence of target represented ~5 % of the specific signal in these conditions, highlighting the multifunctionality of the CMC polymer to not only hold the Fc docking molecules and DNA probes but also prevents non-specific interactions due to its hydrophilic nature. The role of the βCD support in assisting the immobilisation of the Fc-modified polymer is also evident by comparing the response obtained in the presence (trace a) and in the absence of the βCDPSH support (trace c). In the latter case, only 12 % or the original signal is observed, which can be attributed to some physical adsorption of the polymer on the gold surface.

Figure 5d shows the variation of the amperometric signal with target concentration in the range 0-5 nM. The excellent analytical performance observed, with a limit of detection of 10 pM, can be attributed to the presence of the mediator in the same structure of the recognition layer, which avoids a possible slow diffusion of the mediator toward the surface. This explains the 8-fold improvement in the LOD found in this case with respect to a similar supramolecular platform but using adamantane as docking molecule and thus requiring the addition of the mediator in solution.[19] Furthermore, the supramolecular incorporation of ferrocene as mediator into the platform simplifies its operation as it only requires the addition of hydrogen peroxide. Finally, sequence selectivity was studied in the presence of three possible interfering sequences also associated to the HLA DQ system. These sequences gave less than 5 % amperometric signal with respect to the HLA-DQA1*0201 target sequence, indicating an excellent selectivity of the system.

5 CONCLUSIONS

In this work we describe a novel route for the immobilisation of electron transfer mediators on electrode surfaces based on the interfacial complexation of a polymer backbone bearing ferrocene units on a cyclodextrin surface and explore the applicability of the modified surface in the construction of an amperometric genosensor using a sandwich detection system involving a peroxidase labeled secondary probe. This strategy simplifies the operation of the biosensor, only requiring the addition of enzyme substrate and could be an attractive alternative to the development of packaged genosensors as it avoids the need to use and store unstable redox mediators such as TMB or hydroquinone. The proximity of the mediator to the electrode surface greatly improves the detection limits attained with respect to a similar supramolecular system based on electrochemically inactive adamantane/CD inclusion complexes and requiring addition of the mediator in solution. In addition, the use of the hydrophilic CMC backbone contributes to the elimination non-specific interactions and to an optimal spacing of the immobilised DNA probes. Therefore, the excellent performance of this type of self-assembled structure opens new perspectives in the development of highly sensitive biosensors. Studies in this direction are currently underway.

6 ACKOWLEDGMENTS

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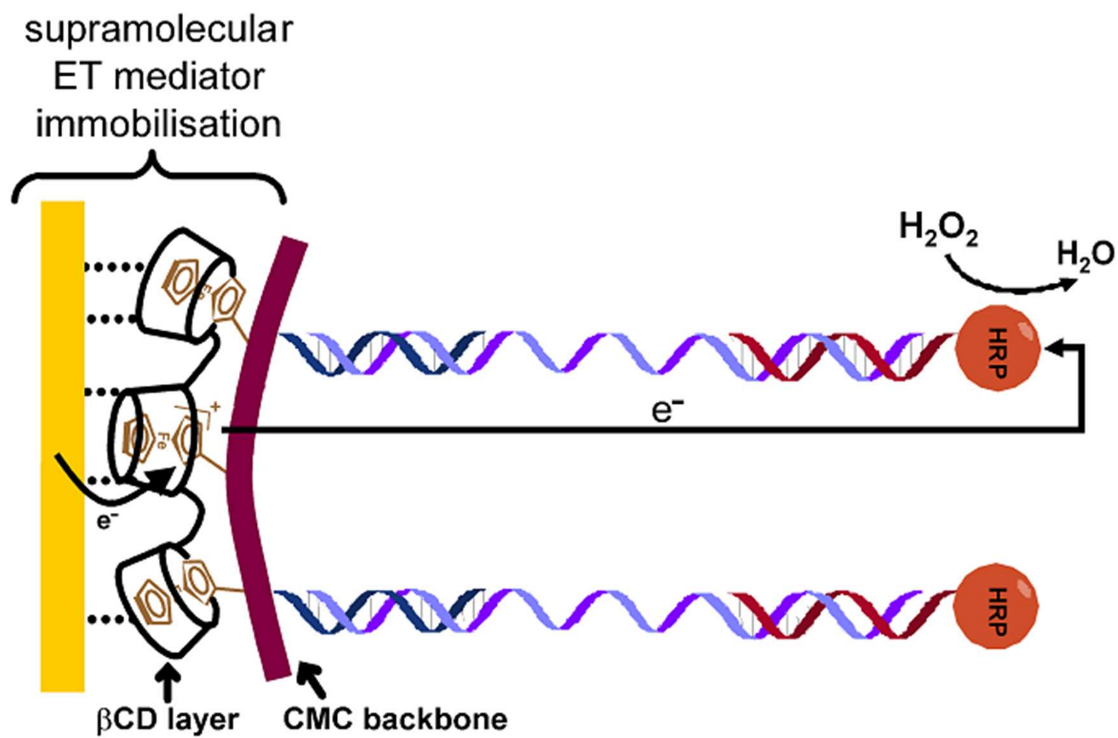
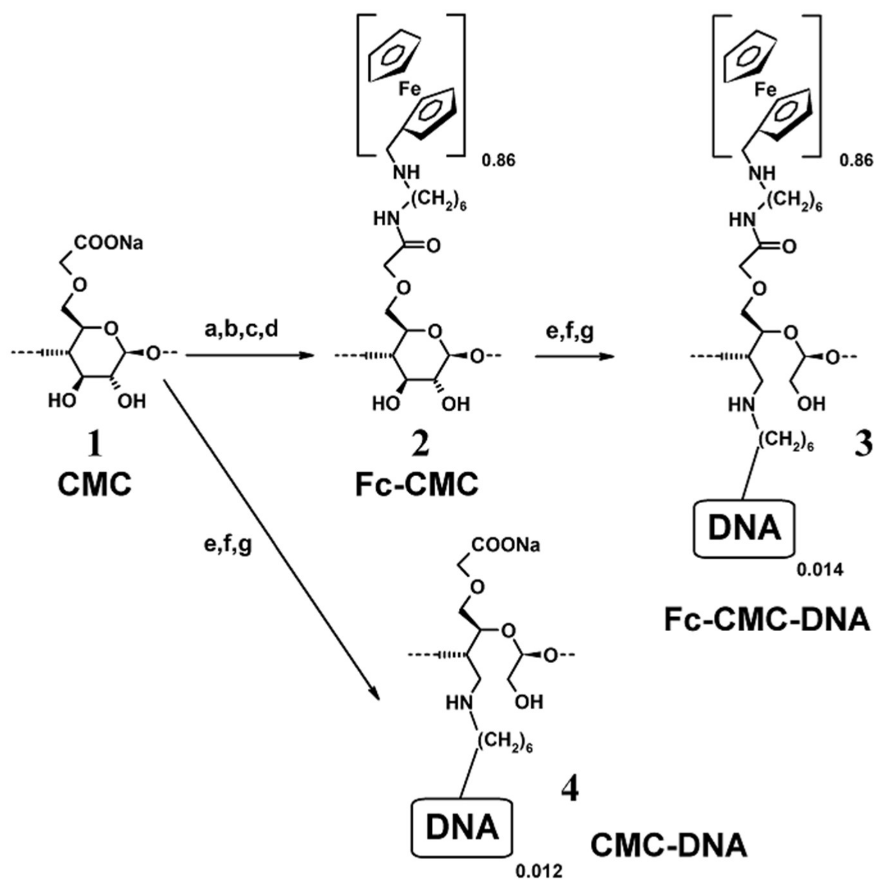


Figure 1. Strategy employed for the supramolecular immobilisation of electron transfer mediator.



Scheme 1. Synthesis of Fc-modified polymers: a) EDC, b) 1,6-diaminohexane, c) Fc-CHO, d) $NaBH_4$, e) $NaIO_4$, f) aminated DNA, g) $NaCNBH_3$.

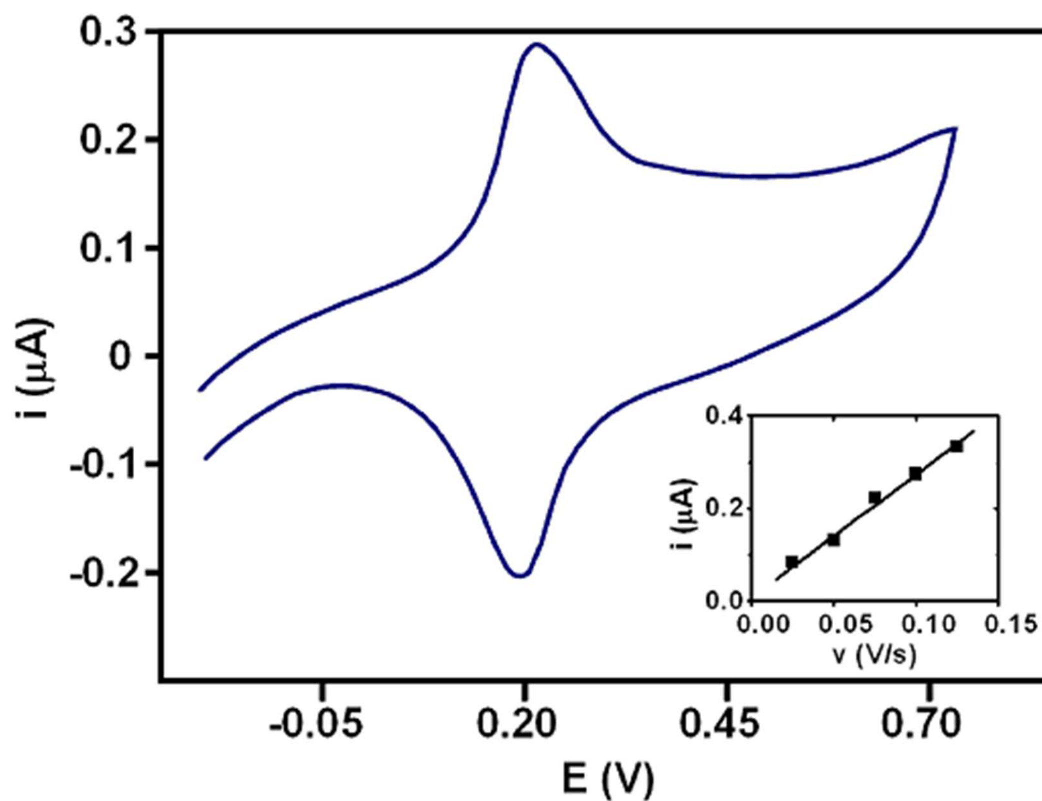


Figure. 2. Cyclic voltammogram (in 0.1 M PBS buffer pH 7.4, scan rate: 100 mV/s) obtained after the deposition of Fc-CMC-DNA on CDPSH-modified gold electrode. Inset: Dependence of peak currents with scan rate.

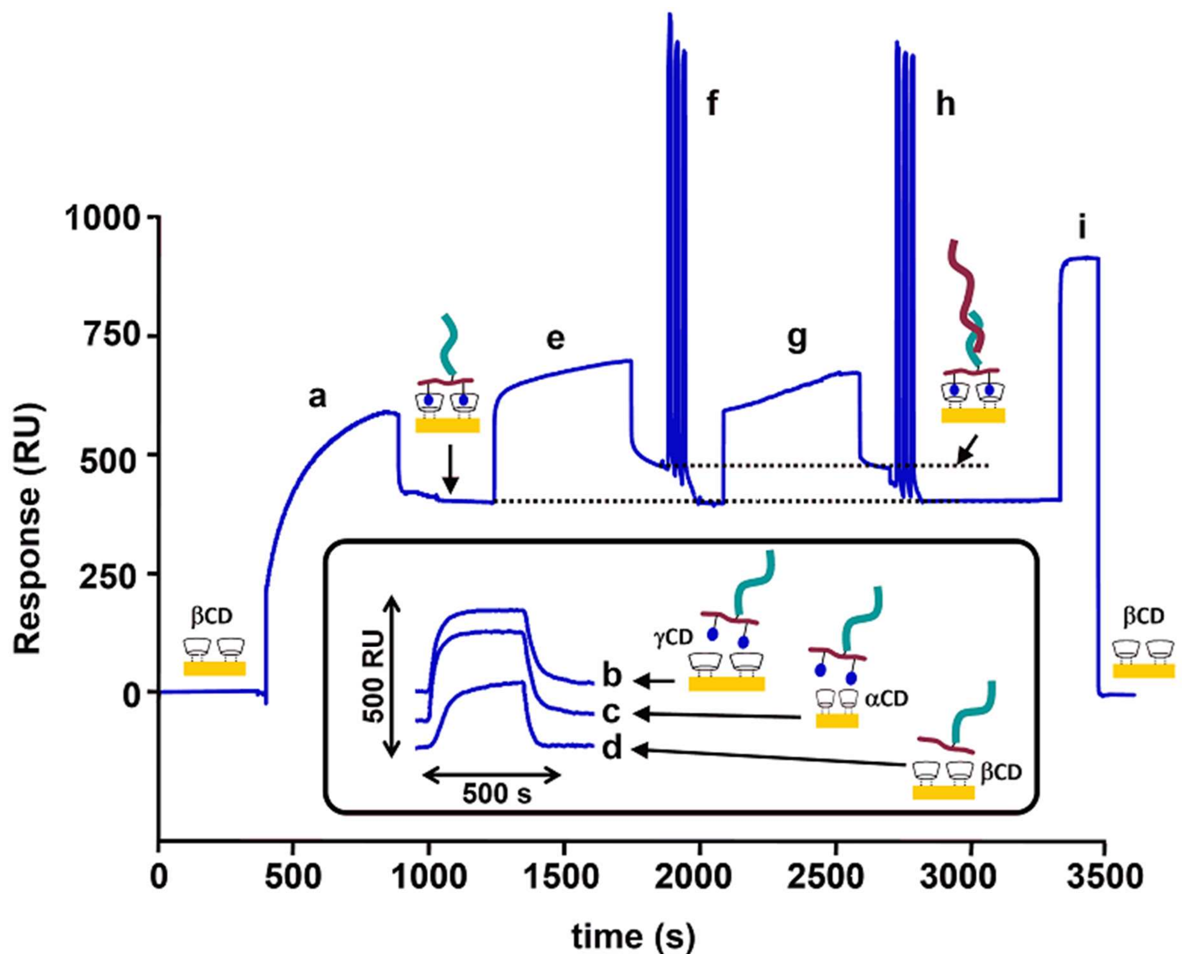


Figure. 3. SPR sensorgram for the immobilisation of Fc-CMC-DNA on bCDPSH surface (a), interaction of Fc-CMC-DNA with γCDPSH (b), and αCDPSH (c) modified surfaces, interaction of CMC-DNA with βCDPSH surface (d), injection of 1 nM HLA-DQA1*0201 target (e), regeneration of βCDPSH/Fc-CMC-DNA surface with three pulses of 10 mM NaOH (f), second 1 nM target injection (g), second NaOH regeneration (h) regeneration of βCDPSH surface with 1 mM adamantanecarboxylate (i). Conditions: running buffer: 0.1 M PBS pH 7.4, flow rate 5 mL/min.

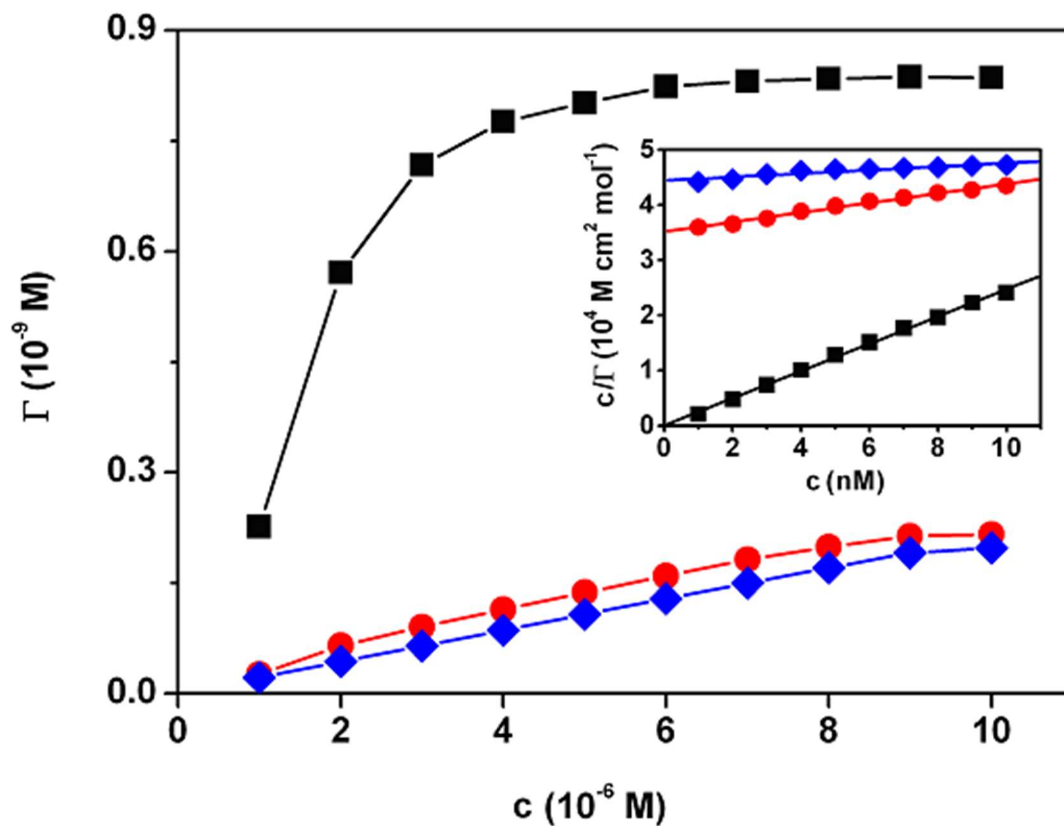


Figure 4. Variations of the surface coverage (Γ) with bulk concentration (c) for the interfacial complexation of Fc-CMC-DNA with αCD(♦), βCD(■) and γCD(●) modified surfaces. Inset: Langmuir plots.

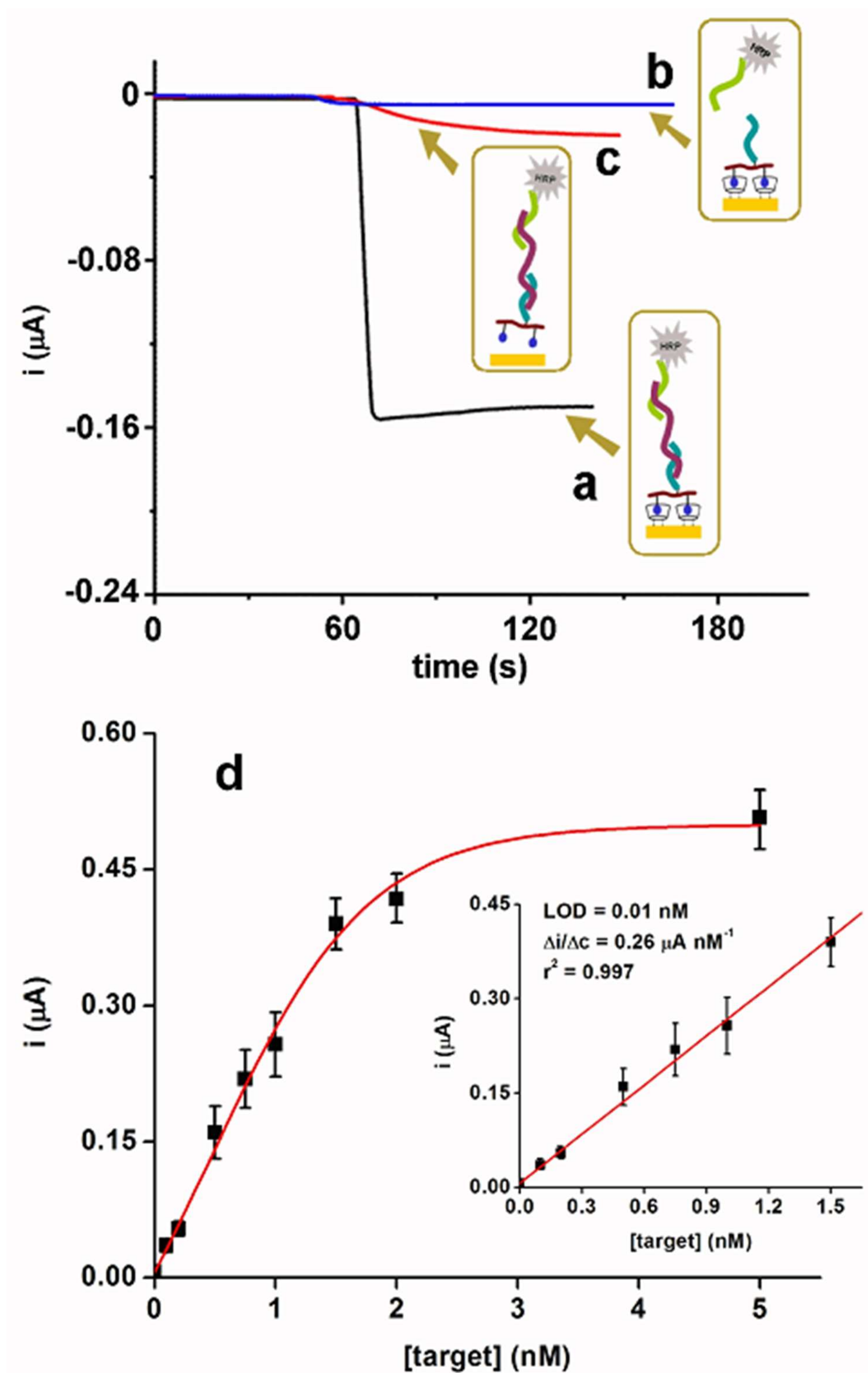


Figure. 5. Top: Amperometric responses obtained for different systems: a) full detection system, b) absence of target DNA, c) absence of CD layer. Conditions: E = 0.2 V, supporting electrolyte: 1 mM H_2O_2 in 0.1 M PBS + 0.15 M KCl (pH 6), Bottom: Amperometric calibration curve for the detection of HLA-DQA1*0201 target sequence (d).

SUPPLEMENTARY INFORMATION

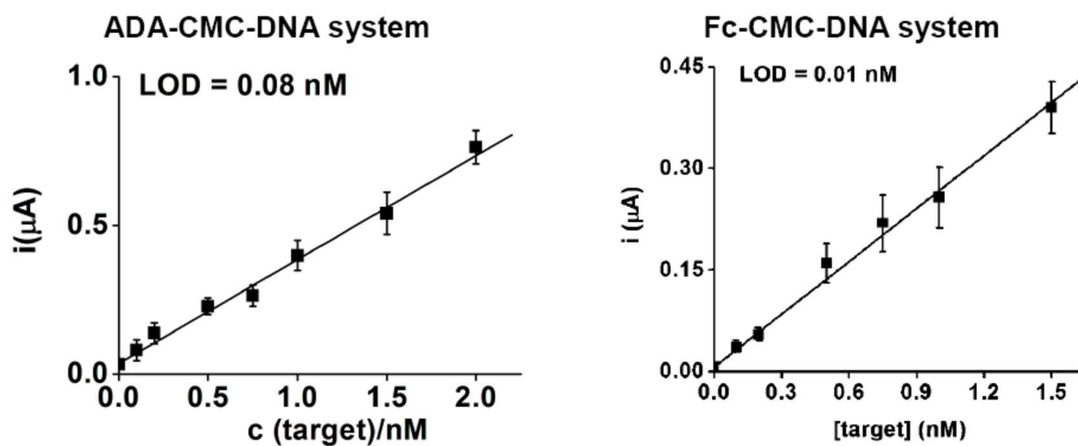


Figure. SI 1. Comparison of calibration plots obtained for ADA-CMC-DNA and Fc-DNA-DNA systems.

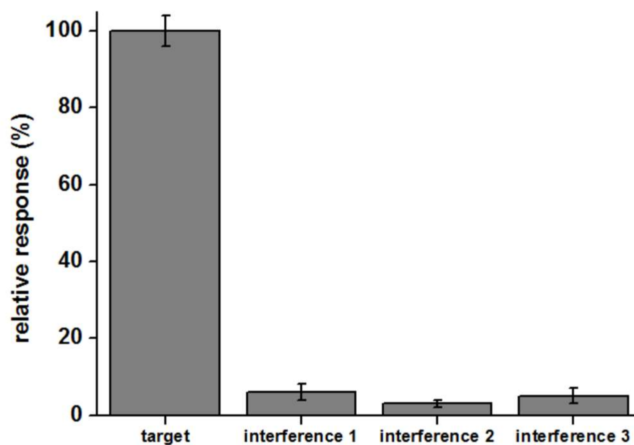


Figure. SI 2. Sequence selectivity. Comparison of amperometric responses obtained with the HLA DQA102 target sequence and with interfering probes 1-3 (see Experimental section for oligonucleotide sequences)