HIGHLY SENSITIVE COLORIMETRIC ENZYME LINKED OLIGONUCLEOTIDE ASSAY BASED ON CYCLODEXTRIN-MODIFIED POLYMERIC SURFACES

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1 ABSTRACT

In this chapter, the development of an enzyme-linked oligonucleotide assay for the detection of a human leukocyte antigen allele associated with celiac disease based on cyclodextrin-modified polymeric surfaces is described. The surface of maleimide-pre-coated plates was modified with a layer of thiolated cyclodextrin polymer and used for the supramolecular capture of adamantane or ferrocene-modified carboxy-methylcellulose polymers bearing DNA probes. The assay was optimised in terms of incubation time, temperature, and surface chemistry and applied to the highly sensitive and selective detection of HLA sequences with a limit of detection of 0.7 nM. A real sample analysed using this platform showed an excellent correlation with maleimide-activated plates using thiolated DNA probes.

2 INTRODUCTION

Genetic tests are diagnostic tools to detect genes associated with inherited disorders[1,2] and the enzyme-linked-oligo- nucleotide assay (ELONAs) has emerged as an attractive technique for clinical applications.[3] The necessity for a sensitive, rapid, reliable, and inexpensive alternative for DNA analysis has led to the development of this simple, sensitive, robust, and versatile bioanalytical technique that has been used for the

colorimetric detection of DNA.[3–5] In this technique, a probe is immobilised on the surface of a microtitre plate[4] and the target DNA is detected in a sandwich-type assay, exploiting a secondary-labelled reporter DNA probe,[3] for example via the use of a fluorescein-labelled oligonucleotide probe and an enzyme-labelled anti- fluorescein antibody.[5]

To immobilise the DNA probe on the surface of microtitre plates, the most extended strategy is the interaction of biotiny- lated probes with streptavidin (or avidin)-coated surfaces.[3, 5–7] This type of surface preserves the biological activity of the immobilised molecule better than direct passive adsorption and the high biotin–streptavidin affinity constant (Kd ~10^{-14} M) provides a robust system as compared with direct passive adsorption.[8]

However, in spite of the general acceptance of streptavidin-coated microtitre plates, some protein leaching and inter-plate variation between provider sources have been observed.[8] Another alternative for probe immobilisation is via covalent binding using maleimide-activated plates. These are useful for binding thiol-containing molecules[9] and have been used for the colorimetric or fluorescent detection of biomolecules.

The use of supramolecular interactions for the immobilisation of biomolecules on surfaces is garnering great interest.[10,11] Cyclodextrin-modified gold surfaces have been used to capture biomolecules using biotinylated bifunctional linkers terminating in hydrophobic moieties such as adamantane,[12, 13] or, more recently, using polymeric carriers.[14, 15] In this strategy, host–guest interactions act as linkers to promote immobilisation under mild conditions and have been demonstrated to be a useful platform for the detection of auto-antibodies in serum,[16] or DNA from PCR products.[17] In spite of the robustness of these platforms, the application of host– guest interactions as an immobilisation strategy on microtitre plates has not been reported to date. These plates are fabricated with cheap polymeric materials such as polystyrene allow high throughput genomic and proteomic analysis for many biomedical research and clinical diagnostics applications. In this paper we describe the development of an ELONA-type assay for the detection of a human leukocyte antigen (HLA) allele associated with celiac disease[18] an autoimmune condition of known genetic predisposition[19] The surface of maleimide-precoated microtitre plates was modified with a layer of thiolated cyclodextrin polymer and used for the supramolecular capture of adamantane (ADA) or ferrocene (Fc)-

modified carboxymethylcellulose (CMC) polymers bearing DNA probes (**Scheme 1.1**). The assay was optimised in terms of incubation time, temperature and surface chemistry and applied to the highly sensitive and selective detection of HLA-DQ2-associated sequences.

3 EXPERIMENTAL SECTION

Materials

All reagents were used as received. CMC (MW 90 kDa), 1- ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride, (EDC), phosphate buffered saline (PBS; dry powder), and tetrame- thylbenzidine (TMB) liquid substrate system were purchased from Sigma-Aldrich. SYBR Green I was purchased from Invitrogen and prepared at a 1:10,000 dilution in PBS (pH 0 7.4 containing 0.8 M NaCl and 50 mM KCl). All solutions were prepared with Milli-Q water (Millipore Inc., Ω 0 18 MΩ·cm). Maleimide-activated microtitre plates were purchased from Pierce. Thiolated α, β and γ-cyclodextrin polymers (CDPSH)[20] and bifunctionalised CMC polymers modified with ADA or Fc residues and DNA probes (ADA- CMC-DNA and Fc-CMC-DNA)[17] were prepared as previously reported. Synthetic oligonucleotides were purchased from Biomers.net (Ulm, Germany).

Sequences specific for HLA-DQA1*0201 are shown below:

HLA-DQA1*0201 aminated capture probe linked to ADA-CMC and Fc-CMC polymers: H_2N-C_6-5’-CAA ATC TAA GTC TGT GGA -3’.

HLA-DQA1*0201 thiolated capture probe: HS-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’.

HLA-DQA1*01* (interference 1): 5’-GAG AGG AAG GAG ACT GCC TGG CGG TGG CCT GAG TTC AGC AAA TTT GGA GGT TTT GAC CCG CAG GGT GCA CTG AGA AAC ATG GCT GTG GCA AAA CAC A-3’.

HLA-DQA1*03* (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’.

Instrumentation and methods

UV–vis spectra were recorded in a temperature controlled Cary 100 Bio spectrophotometer (Varian) in 1 cm quartz cells. Plate optical densities were recorded in a Wallac Victor2 1420 Multilabel counter from Perkin Elmer.

The fluorescence experiments were performed at 20 °C in a Cary Eclipse spectrofluorimeter equipped with a Peltier temperature control and plate reader. The excitation wavelength was set at 495 nm, which corresponds to the SYBR Green absorption maximum in aqueous solution. The fluo- rescence spectra were recorded in the wavelength interval of 510–570 nm with excitation and emission slits of 10 nm and a scan rate of 240 nm/min. All measurements were carried out in triplicate and the average value of the fluorescence changes was used.

Enzyme Linked Oligonucleotide Assay (ELONA)

Reacti-BindTM Maleimide Activated Plates (8-well strips) were used as received. All washing steps were carried out with 0.1 M sodium phosphate, 0.15 M sodium chloride (pH 7.4). After washing the strips three times, CDPSH was immobilised via the addition of 150 µL of a 10-mg/mL solution in binding buffer (0.1 M sodium phosphate, 0.15 M sodium chloride, 10 mM EDTA; pH 7.4) to each well and incubated for 3 h at room temperature. Unreacted maleimide groups were blocked for 1 h with 200 µL of a 10-µg/mL aqueous solution of mercaptoethanol (prepared immediately before use). After washing with PBS, 150 µL of ADA-CMC- DNA or Fc-CMC-DNA polymers was added to the wells and incubated overnight at 4 °C, and subsequent to another washing, 100 µL of 10 nM of HLA-DQA1*0201 target in PBS (pH 0 7.4 with 0.8 M NaCl) was added and incubated for different times (30, 60, 90, and 120 min) and temperatures (4 °C, 25 °C, and 37 °C) in order to elucidate optimum assay conditions. Detection was facilitated by

addition of 100 µL of 10 nM of HLA-DQA1*0201 HRP-labelled probe in PBS (pH 7.4 with 0.8 M NaCl), again for 1 h and at room temperature. After a final washing step, 50 µL of TMB solution was added and 30 min later, the absorbance was recorded at 450 nm following addition of 50 µL of 1 M H_2SO_4. In the case of the surface modified with Fc units, the absorbance was referenced to a CDPSH/Fc-CMC-DNA surface to subtract the possible contribution of the Fc chromo- phore to the absorbance at this wavelength.

To prepare the calibration curve, the target was incubated at the optimum conditions (37 °C and 1 h) with a range of concentrations of the HLA-DQA1*0201 target (from 0 to 300 nM) in PBS (pH=7.4 with 0.8 M NaCl) and the detection was carried out as described above. For comparison purposes, a calibration curve was obtained using a thiolated probe instead of the supramolecular system. In this case, the plate was initially modified with 150 µL of a 10 nM solution of HLA-DQA1*0201 thiolated capture probe in binding buffer (0.1 M sodium phosphate, 0.15 M sodium chloride, 10 mM EDTA; pH 7.4), incubated for 3 h at room temperature and blocked with mercaptoethanol. All other steps (washes, target incubation, detection) were carried out under the same conditions as described above.

Determination of amount of DNA attached to the supramolecular surface by polymer desorption

In order to determine the amount of DNA probe immobilised using the supramolecular system, CDPSH/ADA-CMC-DNA was prepared on maleimide-activated plates as previously described, and then the plate was rinsed with PBS and subsequently incubated with 200 µL of 1 M solution of sodium adamantanecarboxylate for 1 h at 37 °C to dissociate the ADA-CMC-DNA polymer from the cyclodextrin surface. The released ADA-CMC-DNA polymer was incubated with 100 nM of target for 1 h, followed by a 15 min incubation with SYBR Green I (1:10,000 dilution) for 15 min.

In order to construct a calibration curve for quantification of the number of DNA probes on the released ADA-CMC- DNA polymer, HLA-DQA1*0201 capture probe (0 to 100 nM) in PBS, pH 7.4 containing 0.8 M NaCl and 50 mM KCl, was incubated with a constant amount of target (100 nM) for 1 h followed by 15 min of incubation with SYBR Green (1:10,000 dilution). The fluorescence intensity at 525 nm was recorded and the

amount of DNA probe present in the released ADA-CMC-DNA was calculated by interpolation in the calibration curve obtained.

Determination of intraplate probe distribution homogeneity and stability

A maleimide-activated black microtitre plate for fluorescence measurement was modified with the supramolecular surface as previously described. The wells were rinsed with PBS and incubated with target (100 nM) and SYBR Green (1:10,000) in the presence of KCl (50 mM) before reading the fluorescence at 525 nm using a plate reader coupled to the spectrofluorimeter.

The stability of the supramolecular surface chemistry was carried out during 4 weeks at 4 °C. Each week the amount of coating DNA was measured in 8 wells of the plate, as previously described.

Real sample analysis

A PCR product obtained by amplification of a real HLA typed sample was diluted 1:10 and added as target following the procedure described above for the detection of HLA-DQA1*0201 target on CDPSH/ADA-CMC-DNA and CDPSH/Fc-CMC-DNA-modified plates. The values were interpolated in the respective calibration curves and compared with the results obtained using a maleimide plate modified with thiolated DNA.

4 RESULTS AND DISCUSSION

Two supramolecular platforms were evaluated using a modification of the Enzyme Linked Oligonucleotide Assay (ELONA). The plate was initially incubated with βCDPSH at pH 7.4 in order to form the cyclodextrin support layer by addition of the thiol groups to the double bond of the maleimide group thus forming a stable thioether linkage. Unreacted maleimide groups were then blocked with mercaptoethanol followed by the incubation of the polymeric DNA carrier (ADA-CMC-DNA or Fc-CMC-DNA). In these polymers, the presence of adamantane and ferrocene units has the function of docking the polymer within the cyclodextrin layer via host-guest interactions. After hybridisation with the target DNA, a secondary probe labelled with HRP was used as a reporter probe and the detection was carried out colorimetrically by addition of tetramethylbenzidine (TMB) as substrate for HRP (**Figure 1**).

The supramolecular nature of the CDPSH/CMC interface was tested by comparing the response obtained using similar thiolated polymers derived from αCD and γCD, which have smaller and larger cavity diameters as compared to βCD. Both, αCDPSH and γCDPSH, were immobilised on the maleimide- activated surface in the same way as βCDPSH, and ADA or Fc-CMC-DNA polymers were allowed to with the host-modified surface. Since the amount of both hydrophobic moiety (0.86–0.90 mol/mol glucose) and DNA probe (0.012– 0.014 mol/mol of glucose) attached to both polymers is very similar, the differences in the optical response can be attributed to the amount of DNA probe immobilised on the surface. As can be seen from **Figure 2a**, the response showed the trend βCDPSH > γCDPSH > αCDPSH with the βCDPSH/ADA- CMC-DNA system showing the highest absolute response. **Figure 2b** shows the dependence of the optical responses obtained in **Figure 2a** with the reported stability constants for cyclodextrin/ferrocene[19] and cyclodextrin/adamantanecar- boxylic acid[20] systems. As can be seen, there is a direct relationship between the stability of the inclusion complex and the optical response obtained, clearly demonstrating that the immobilisation of the polymeric carrier on the surface takes place through inclusion complexation. The strength of the inclusion complex thus modulates the amount of DNA probe immobilised on the surface, and consequently, assay sensitivity. The role of the inclusion complexation in the immobilisation of the CMC carrier is also demonstrated by the very low signal observed

after the interaction of a CMC-DNA polymer (i.e. not carrying the hydrophobic moiety) with the βCDPSH- modified surface (see **Figure 2a**, inset).

To test the homogeneity of probe distribution in a plate (i.e. well–well reproducibility), the probes were incubated with the target sequence and the fluorescence of the intercalating agent SYBR Green was measured. As can be seen from **Figure 3a**, the resulting fluorescence readings were reproducible, with a standard deviation of 8 %, indicating a homogeneous distribution of the polymer on the wells of the plate. The supramolecular architecture was observed to be completely stable over a period of 4 weeks a 4 °C (**Figure 3b**).

Since the assay performance largely depends not only on the amount of capture probe attached to the CMC carrier but also on the amount of polymer immobilised on the surface, the ADA-CMC-DNA polymer was desorbed from the plate surface using a competitive displacement in the presence of a large excess of sodium adamantanecarboxylate in order to truly quantify the amount of probes at the surface. The detached DNA-carrying polymer was initially analyzed by UV–vis spectroscopy at 260 nm but the absorbance values were low and difficult to quantify. For this reason, the solution was incubated with a target sequence to form dsDNA, which was then quantified by fluorescence using SYBR Green as intercalating agent (**Figure 4**). Normalisation of the measured concentration considering the well area gave a surface probe density of $(8.6 \pm 0.6) \times 10^{-10}$ mol/cm^2, a value very close to the binding capacity of a thiolated peptide indicated by the manufacturer (1.5×10^{-10} mol/well, equivalent to ~5.8×10^{-10} mol/cm^2) and indicative of the formation of an almost complete monolayer at the surface.

The optical response for the detection of 10 nM of the target (specific response) at different hybridisation times and temperatures in PBS (with 0.8 M NaCl) was studied in order to optimise assay conditions and a control measurement was carried out in the absence of target (non-specific response). As can be seen in **Figure 5**, the optimum hybridisation conditions are 60 min at 37 °C, where the highest $A_{specific}/A_{non\text{-}specific}$ ratio was observed representing a 77 % and 51 % signal increase as compared to the highest response obtained at 4 °C and 25 °C, respectively. The decrease of the signal at 37 °C after 60 min in the case of βCDPSH/Fc-CMC-DNA is due to the stability of the βCD/Fc

inclusion complex, whilst in the case of CDPSH/ADA-CMC-DNA, this effect is less pronounced due to the higher stability of the βCD/ADA ($K_{\beta CD/ADA}$~10^5) inclusion complex, which is two orders of magnitude higher than the βCD/Fc system.

Using the optimum conditions to immobilise the target for both systems the corresponding calibration curves were obtained in the concentration range 0–300 nM (**Figure 6**). As can be seen, the optical response increased with target concentration and tends to saturation above 100 nM. At low concentrations (0–10 nM), the response showed a linear dependence with the concentration with a sensitivity (taken as the slope of the A vs c curve) of 0.034 and 0.056 AU/nM for the Fc and ADA systems, respectively and a limit of detection of 0.7 and 0.8 nM, respectively. These limits of detection are similar to the value obtained using a thiolated capture probe (0.5 nM), whilst the sensitivity of the supramolecular assay is 4 and 7 times higher compared with the thiolated probe (0.0086 AU/nM). This highlights the role of the CMC backbone in spatially orienting the capture probes, avoiding possible steric hindrance to target recognition. The use of an increased number of DNA probes attached to CMC or shorter CMC backbones that could be better accommodated on the underlying CDPSH layer may help to extend the linear range and sensitivity if necessary by increasing the density of probes on the plate and this is currently being explored. This probe density is more difficult to control in the case of the direct reaction of a thiolated probe with the maleimide surface, often requiring lengthy optimisation steps of probe/backfiller ratios as well as incubation times and temperatures. In addition, the hydrophilic nature of the CMC backbone effectively suppresses non-specific interactions, as is evident from the very low response observed in the absence of target, indicating that the HRP-labelled probe has a negligible tendency to interact with the CDPSH/CMC-modified surface.

For DNA detection, selectivity is critical, and **Figure 7** shows the optical response obtained with the βCDPSH surface in the presence of two potential interfering alleles, which only differ by base pairs, and no cross-reactivity was observed, and the low signal obtained is attributed to a minor amount of non- specific binding of the reporter probe.

5 CONCLUSIONS

In conclusion, we have demonstrated the applicability of supramolecular host–guest interactions in the development of colorimetric DNA tests based on the self-assembly of bifunctionalised CMC polymers on cyclodextrin-modified microtitre plates. Adamantane and ferrocene-appended polymers were demonstrated to have a preference to interact with βCD-modified surfaces over αCD and γCD, demonstrating the supramolecular nature of the immobilisation process. The CMC polymer facilitated a spatial orientation of the DNA probes on the surface giving better access to target DNA, References resulting in a markedly more sensitive colorimetric assay than a similar assay based on the immobilisation of thiolated probes, with subnanomolar limits of detection. In addition, the presence of the hydrophilic cyclodextrin and CMC polymers min- imise non-specific interactions, as demonstrated by the very low response obtained in the absence of target. A real PCR- amplified sample of a celiac patient, demonstrated to carry the HLA-DQA1*0201 allele using Luminex-based HLA typing, was tested using the supramolecularly coated plates and compared with the thiolated probe-coated plates. The results obtained using supramolecular plates were 0.78 ± 0.07 nM for ADA-CMC-DNA/CDPSH and 0.96±0.09 nM for Fc-CMC- DNA/CDPSH, showing an excellent correlation with thiolated DNA capture probe (0.86±0.05 nM), clearly demonstrating that the supramolecular chemistry is not affected by the sample matrix and does not need to be pre-treated. The multiplexing ability of this platform in the detection of several HLA alleles is currently under investigation.

6 ACKNOWLEDGEMENTS

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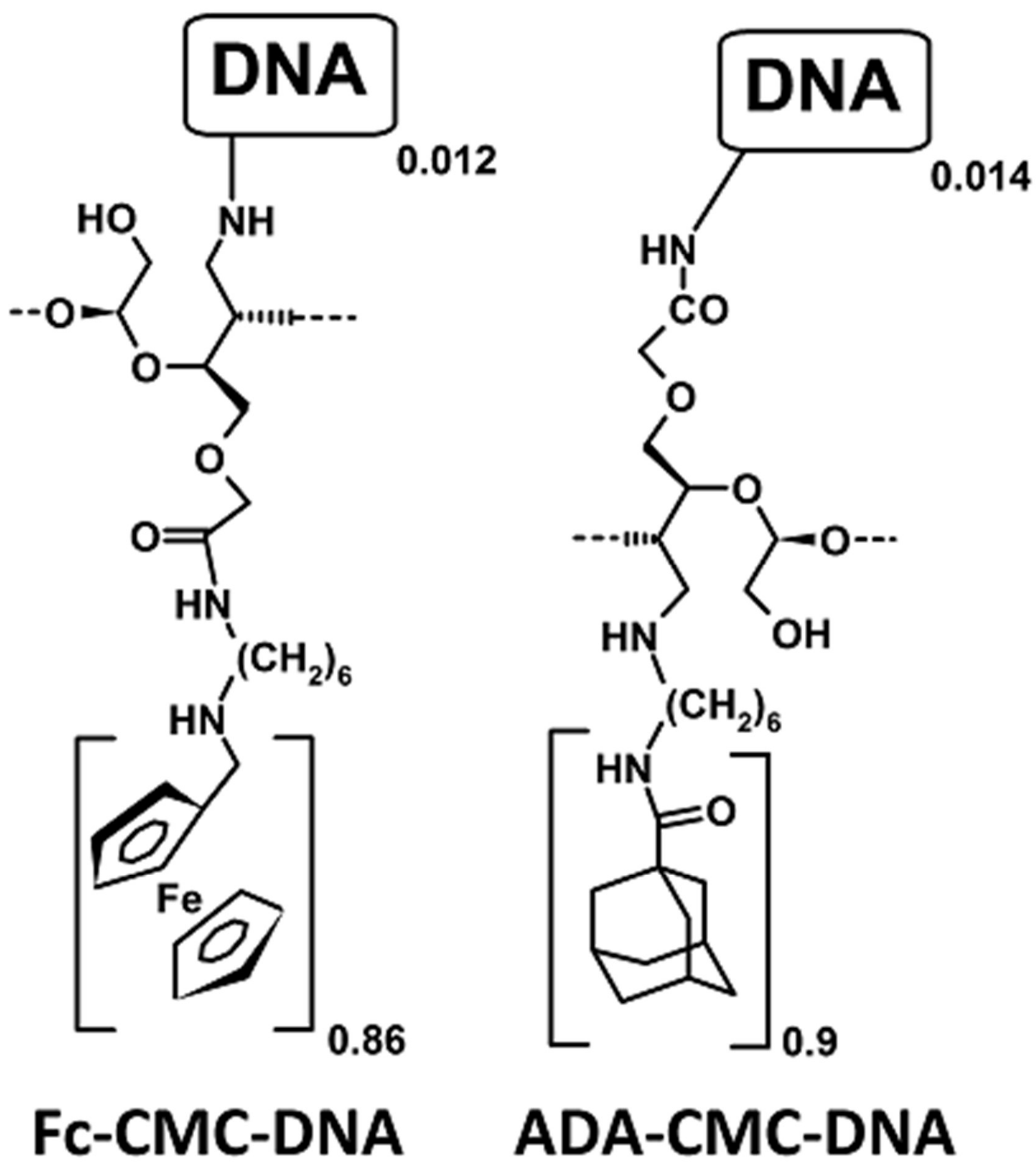

Scheme 1. Structure of polymeric carriers

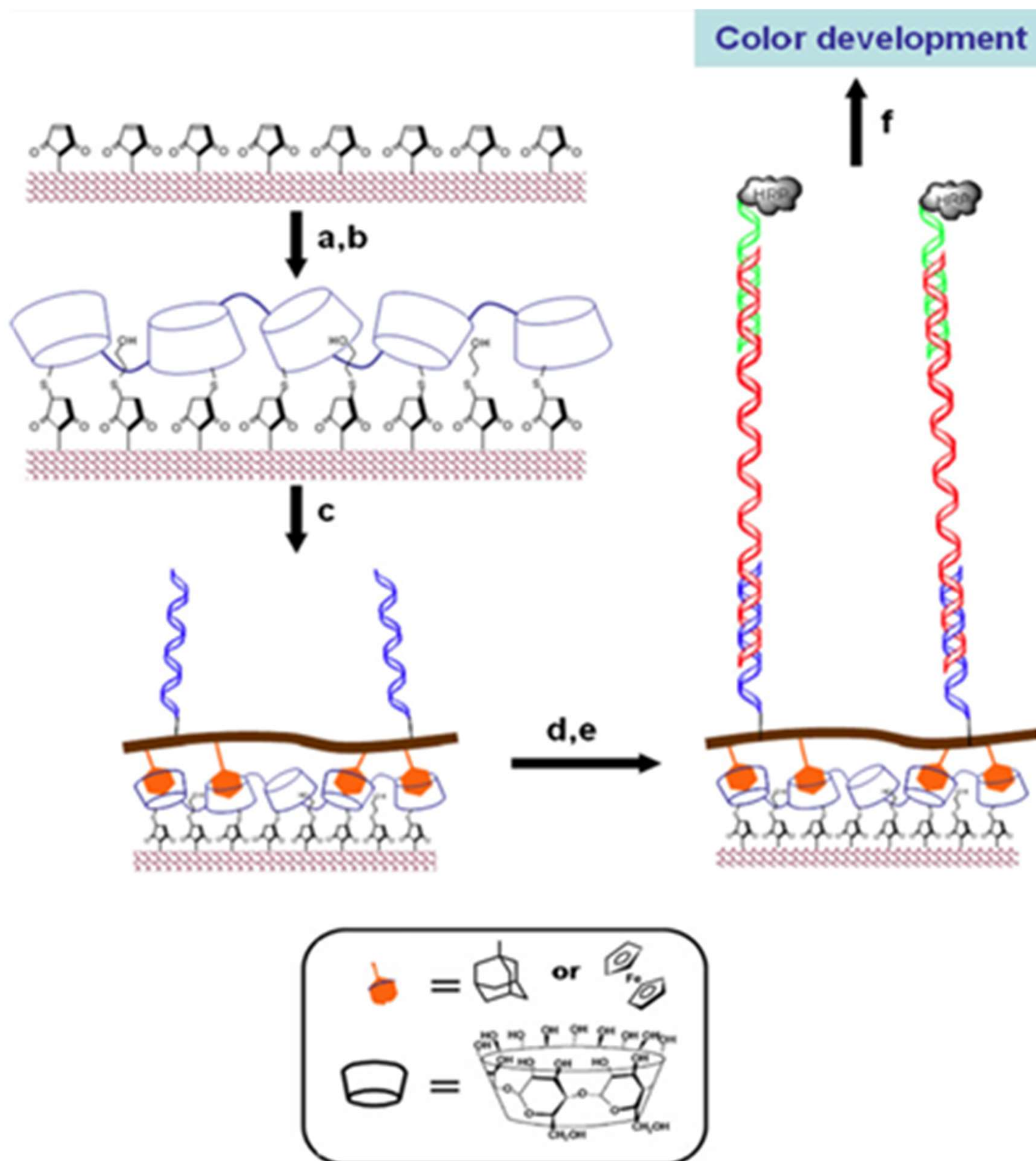

Figure 1. Principle of modified ELONA platform based on the self-assembly of CMC-polymers bearing DNA probes on cyclodextrin modified plates. a) CDPSH, b) mercaptoethanol, c) ADA-CMC-DNA or Fc-CMC-DNA, d) target DNA, e) HRP-labeled secondary DNA probe, f) colour development with TMB.

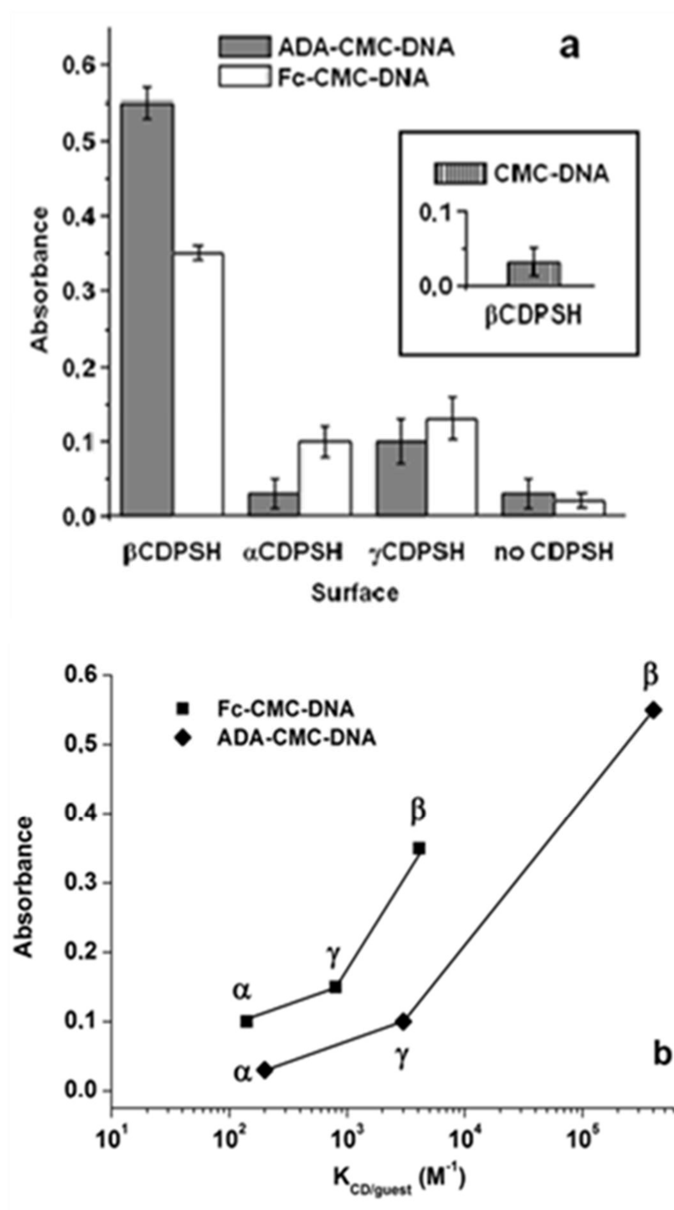

Figure 2. a) Optical response obtained in the detection of 10 nM HLA-DQA1*0201 target sequences on α, β, and γ-CDPSH modified surfaces using ADA-CMC-DNA and Fc-CMC-DNA polymers. Inset: response obtained using a CMC-DNA polymer (without hydrophobic moiety) on the βCDPSH surface. b) Dependence of the optical responses obtained in Figure 2a with the stability constants for the cyclodextrin/ferrocene and cyclodextrin/adamantanecarboxylic acid system.

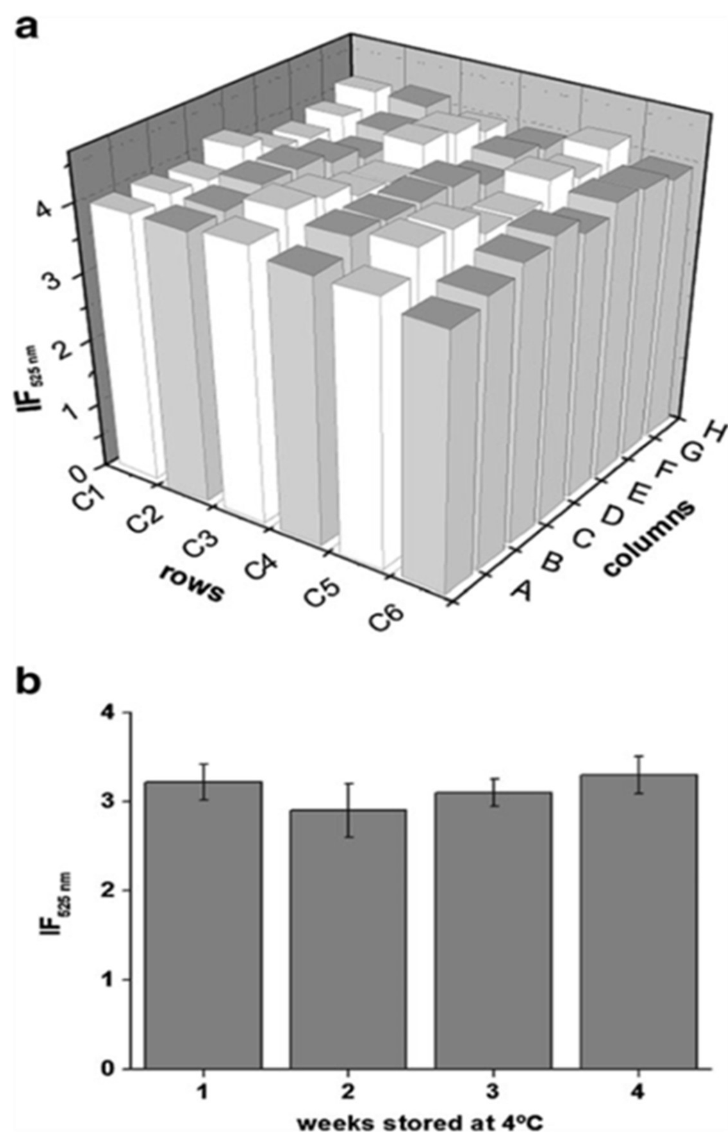

Figure 3. a) 3D plot showing the fluorescence values at 525 nm obtained on a 6×8 well section of a plate after incubation of the supramolecular surface with 100 nM of target and SYBR green (λ_{exc} = 495 nm). b) Stability of supramolecular surface coated plates with time at 4ºC.

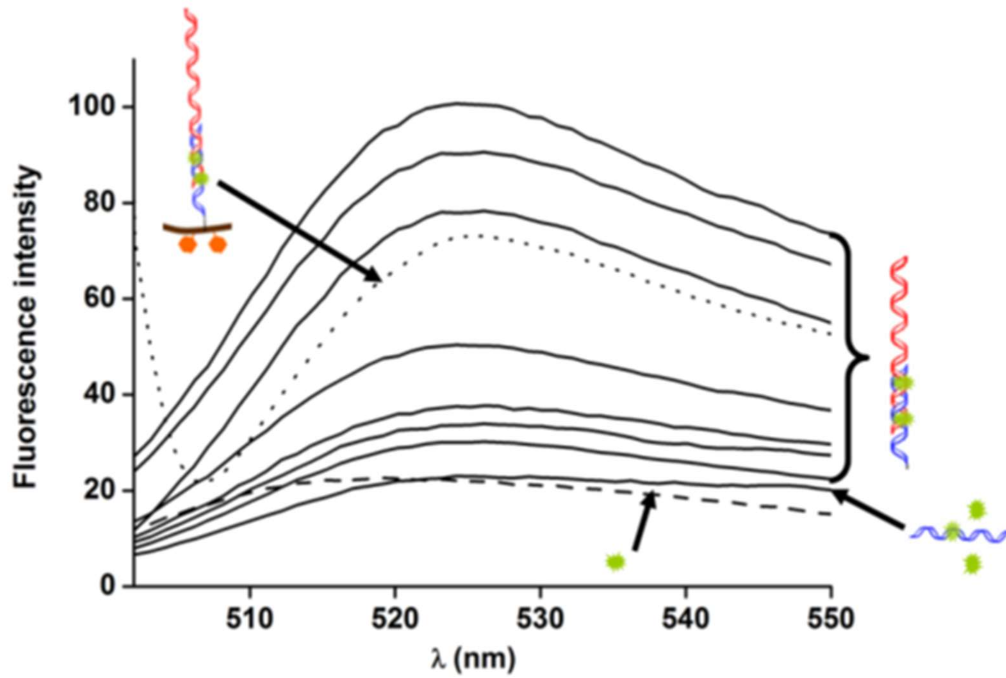

Figure 4. Fluorescence spectra of: SYBR Green intercalated in dsDNA formed by hybridisation of 100 nM target with different concentrations of HLA-DQA1*0201 probe in solution (———), ADA-CMC-DNA desorbed from supramolecular surface (……………) and SYBR green in the absence of any DNA (-------). Excitation wavelength: 495 nm.

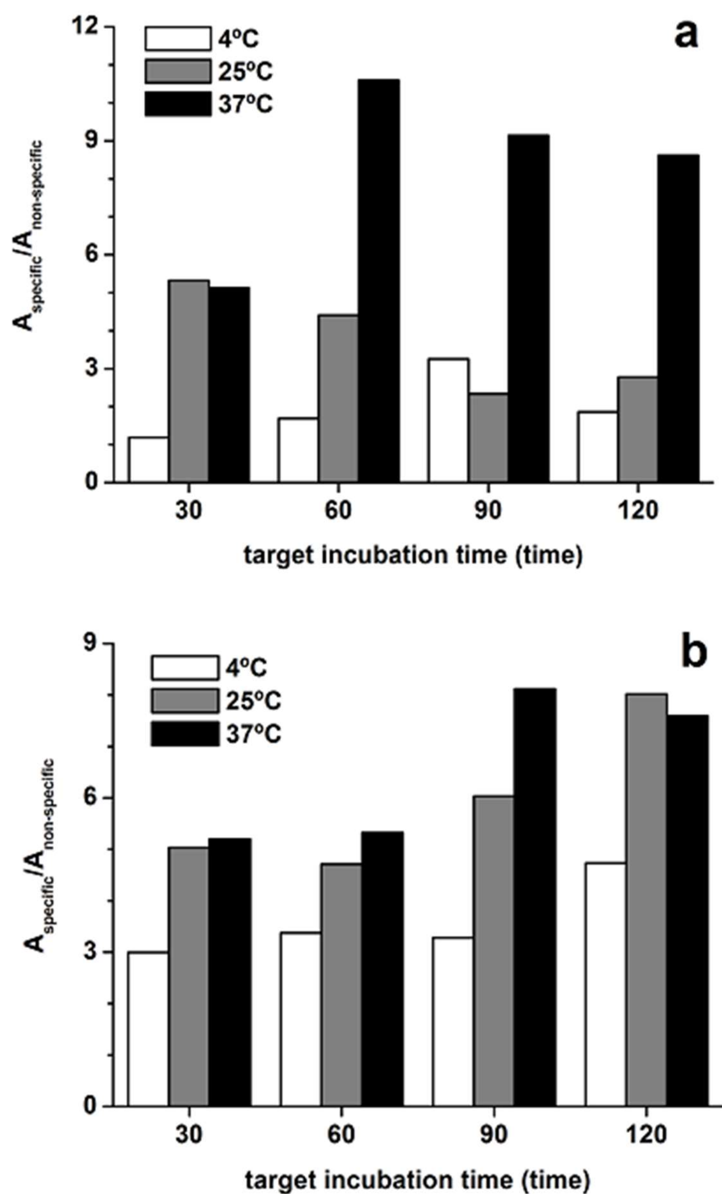

Figure 5. Dependence of the optical response for the detection of 10 nM HLA-DQA1*0201 target with target incubation times and temperatures for (a) βCDPSH/Fc-CMC-DNA system, (b) βCDPSH/ADA-CMC-DNA system. Conditions: Target concentration: 10 nM in PBS (with 1 M NaCl); Target incubation times: 30, 60, 90, 120 min; Incubation temperatures: 4, 25 and 37°C.

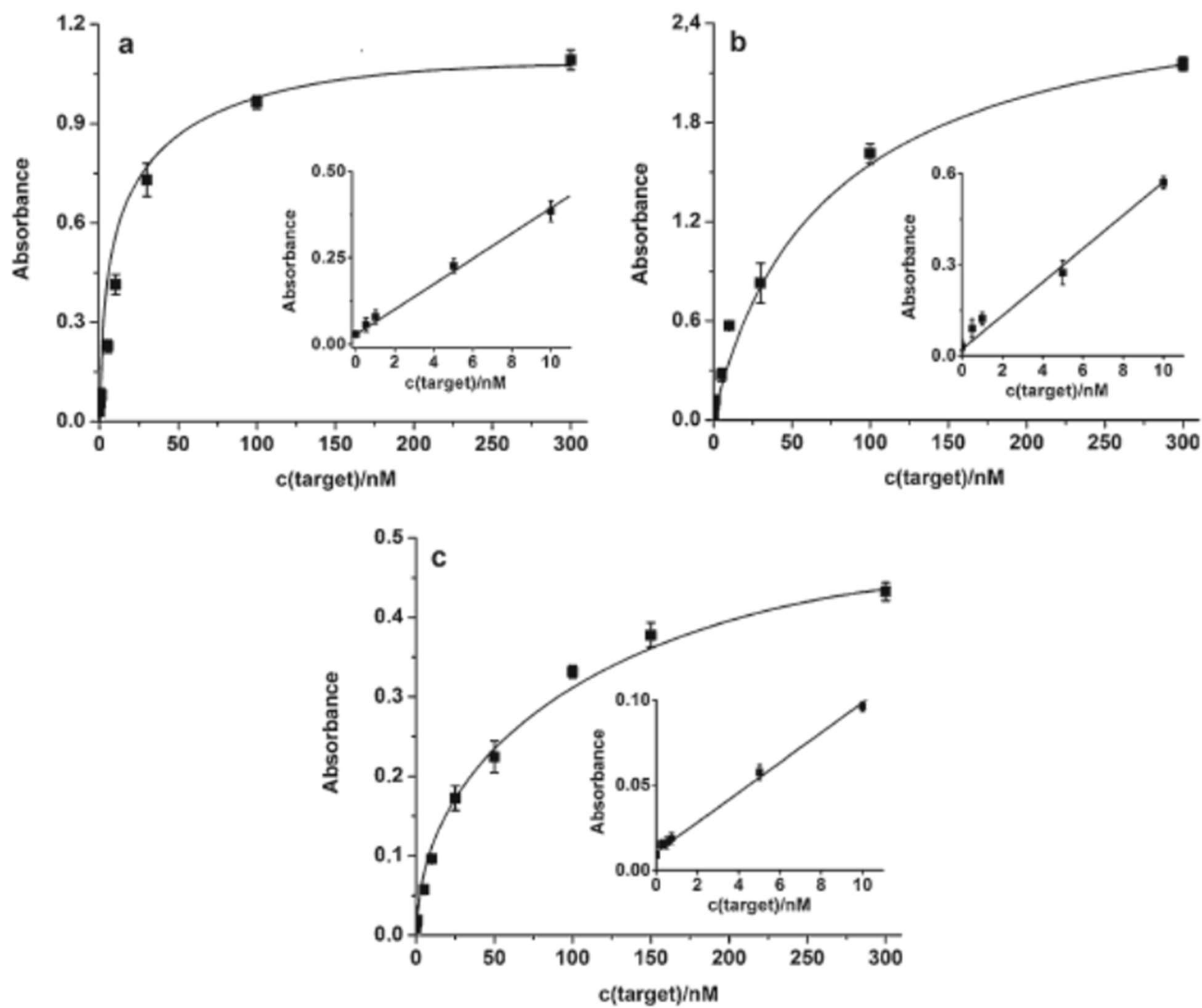

Figure 6. Calibration curves for the detection of HLA-DQA1*0201 target sequence using (a) βCDPSH/Fc-CMC-DNA system, (b) βCDPSH/ADA-CMC-DNA system, c) HLA-DQA1*0201 thiolated capture probe.

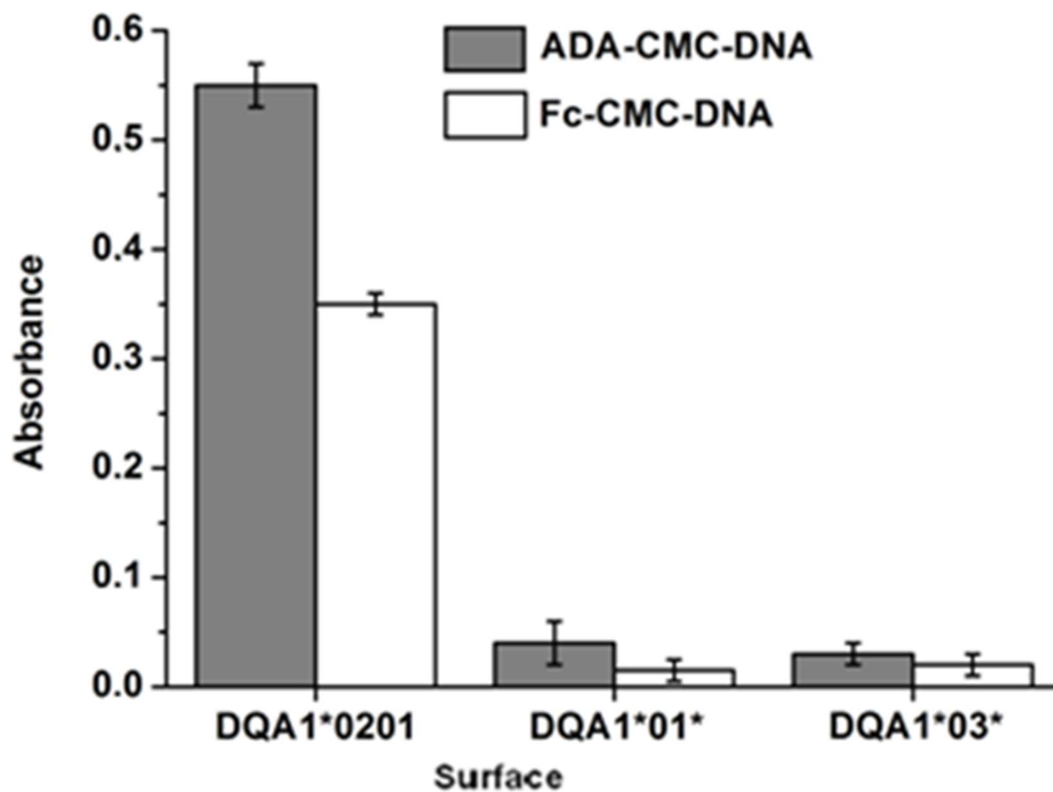

Figure 7. Optical responses obtained in the detection of 10 nM HLA-DQA1*0201 target and two HLA-related interferences.