Detection of anti-gliadin autoantibodies in celiac patient samples using a cyclodextrin-based supramolecular biosensor

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ABSTRACT: Celiac disease is a condition affecting at least 1% of the population in North America and Europe and is associated with the ingestion of gluten by genetically susceptible individuals. Measurement of serum anti-gliadin antibodies is a diagnostic tool also used as a means of assessing continued inflammation of the small intestine and monitoring patient's compliance to a gluten-free diet. In this work we demonstrate the applicability of an electrochemical supramolecular platform based on cyclodextrin-modified gold surfaces to detect antigliadin antibodies in real serum samples. Several support layer-biorecognition element combinations were tested in order to maximize the electrochemical response and the assay was optimized in terms of incubation times and resistance to non-specific interactions. The developed supramolecular biosensor was then applied to the amperometric detection of antigliadin IgA and IgG autoantibodies in real samples of celiac disease patients under follow-up treatment and the results are compared with a commercial ELISA test observing an excellent correlation between both methods.

KEYWORDS Cyclodextrin, adamantane, supramolecular chemistry, biosensor, celiac disease

MANUSCRIPT TEXT

1. Introduction

Celiac disease is a condition associated with the ingestion by susceptible individuals of gluten from wheat, barley, rye and oats causing histological changes in the small intestine mucosa and leading to a malabsorption syndrome.[1] The condition is also strongly associated with other autoimmune conditions, such as diabetes mellitus (10% of celiacs affected), auto-immune thyroiditis and many others, and the likelihood of developing such conditions may increase with the length of time an individual is exposed to dietary gluten, in other words when the condition remains undiagnosed and untreated.[2] Celiac disease affects at least 1:100 people in Northern Europe and Northern America and screening studies have shown that celiac disease is severely under-diagnosed, with a prevalence of 0.5-1.0% among the European population. Celiac disease is frequently unrecognized by physicians, because of its variable clinical presentation and symptoms.[3] Assuming a conservative prevalence of 0.5% this corresponds to about 4 million celiac disease cases in Europe alone. Approximately 85% of cases are unrecognized and thus also untreated, and the health burden of celiac disease is thus considerable, reducing the quality of life both for the affected subjects and their families, with extensive negative economical consequences on a societal level. However, upon diagnosis and with subsequent withdrawal of gluten from the diet, the patient's symptoms are completely reversed and the earlier this diagnosis is made, the better the long term outlook for the patient, thus highlighting the need tool for the widespread screening of celiac disease.

While the gold standard for diagnosis for celiac disease remains small intestinal biopsy with assessment of the morphology, serological screening tests have become increasingly more specific and sensitive, and it is expected that the criteria for diagnosis will be based on a combination of serology and genetic tests. Serum IgA and IgG antibodies against gliadin, reticulin and endomysium (components of connective tissue) are detectable in individuals with celiac disease. Measurement of serum anti-gliadin antibodies was firstly used as a diagnostic tool, and secondly as a means of assessing continued inflammation of the small intestine, and thus of monitoring patients' compliance to a gluten-free diet, which is the only treatment available thus far.[4] Currently there is no cost-effective, easy to-use

technology, that can be used in situ, for example at the doctor's office, allowing for screening of celiac disease, either population based or within specific high-risk populations.

Among the many existing bioanalytical tools, electrochemical biosensors have been used in the past to detect auto-antibodies related to autoimmune diseases such as systemic lupus erythematosus[5], multiple sclerosis[6] and rheumatoid arthritis.[7] In the case of celiac disease related auto-antibodies, there are only a few reports on the application of electrochemical biosensors.[8] To the best of our knowledge, there is only one report on the amperometric detection of anti-gliadin autoantibodies.[9] This system was based on an automatic immuno-microfluidic device fabricated in a low-cost plastic support and allowed the detection of anti-gliadin IgG autoantibodies in real samples with a limit of detection of 0.52 U/mL and excellent correlation with a commercial ELISA assay.

Surface engineering relies on the modification of materials at the molecular scale with the aim of providing an interface of enhanced performance and improved physical and (bio)chemical properties. This is especially relevant for the construction of biosensors where the challenge is not only to engineer the surface of the device such that the biorecognition molecule tolerates the "foreign" object but also to correctly orientate the surface functional groups in order to control the communication between the device and its bio-environment,[10,11] whilst taking into account other important biosensor operational parameters such as sensitivity and stability. For example, considerable attention has been drawn during the last two decades to the functionalization of noble metals with self-assembled monolayers (SAM) of thiolated compounds as a route to modify the electrode surfaces with functional organic molecules which, depending on the terminal functionality, can control the microenvironment nature of the interface or the distance and/or orientation of the attached biomolecule.[12]

As an alternative to classical methods for biosensor construction, the fabrication of highly organized molecular systems with the aid of supramolecular interactions has opened up new prospects for the control of molecular interactions and the design of novel functional materials and devices.[13,14] In particular, the use of β–cyclodextrin (βCD)[15] coated surfaces offers an unconventional route for the reversible immobilization of different biomolecules based on the interfacial inclusion of adamantane (ADA) containing polymers, dendrimers or biomolecules.[16-18] In this arrangement, two well-defined

types of supramolecular interactions can be identified: the CD/ADA host-guest complex serves to dock the biological element to the surface, which is also responsible for the specific biorecognition event used to detect the target analyte (Scheme 1). The strength of the inclusion process is in many cases dictated by the multivalency of the host-guest interaction[16] while also offering, if needed, the possibility of a stepwise surface regeneration using allowing the possibility to re-use the supramolecular platform.[19]

In this work we report a novel surface modification strategy based on the interfacial self-assembly of a bifunctionalized polymer bearing adamantane units and an antigenic fragment onto a βCD-containing support and its applicability to the detection of anti-gliadin antibodies in both immunoassay and immunosensor formats using real patient serum samples. To optimize the electrochemical response, several forms to arrange the βCD-ADA linkers over the surface were investigated (Scheme 2) and the assay was optimized in terms of incubation times and resistance to non-specific interactions. The developed supramolecular biosensor was then applied to the amperometric detection of anti-gliadin IgA and IgG auto-antibodies in real samples of celiac disease patients under follow-up treatment (i.e. following withdrawal of gluten from the diet) and the results are compared with a commercial ELISA test with an excellent correlation obtained.

2. Experimental

2.1. Reagents

All chemicals used were of analytical grade and used as received without any further purification. β-cyclodextrin was a generous gift from Roquette (France). Carboxymethyl cellulose sodium salt (CMC, M_W 90 kDa) potassium hexacyanoferrate, 1-ethyl-3-(3-dimethyl-aminopropyl) carbodiimide hydrochloride (EDC), phosphate-buffered saline (PBS) with 0.05% v/v Tween 20 (dry powder), hydroquinone (HQ), adamantane carboxylic acid, gliadin, anti-gliadin polyclonal-IgG-antibody (anti-rabbit), anti-rabbit IgG (whole molecule)–peroxidase antibody produced in goat (Ab-HRP), were purchased from Sigma-Aldrich. All solutions were prepared with Milli-Q water (Millipore Inc., Ω = 18 MΩ·cm). Maleimide-activated plates were purchased from Pierce.

The thiolated β-cyclodextrin polymer (CDPSH. Mw ~ 18 000 mol/L, degree of substitution: 13 mol SH per mol polymer)[17c] and hepta-6-thio-6-deoxy-β-cyclodextrin (CDSH7)[20] were prepared as previously described. The synthesis of adamantane conjugates of CMC and gliadin is described in the Supporting Information.

2.2 Enzyme-Linked "Self-Assembled" Assay.

Reacti-Bind™ 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 7.2). The strips were initially washed three times prior to CDPSH immobilization via addition of 150 μL of a 100 μg/mL solution in binding buffer (0.1 M sodium phosphate, 0.15 M sodium chloride, 10 mM EDTA; pH 7.2) and incubation for 3 hours at room temperature.

After washing and blocking of the unreacted maleimide groups with 200 μL of 20 μg/mL mercaptoethanol solution in water (prepared immediately before use), 150 μL of variable concentrations of ADA-CMC-GLI (0, 1.25, 2.5, 5, 10, 20, 40 μg/mL) in binding buffer were added to the wells and incubated overnight at 4°C. After washing, 100 μL of variable concentrations of anti-gliadin antibody (0, 0.2, 0.8, 1.6, 3.3, 6.5, 13 μg/mL) in binding buffer were incubated for 1 h at room temperature followed by washing. Detection was carried out by addition of 100 μL of a 1:15000 dilution of anti-mouse/ IgG-HRP conjugate, again for 1 hour and at room temperature. After a final washing step, 50 μL of TMB solution was added and 15 minutes later, the absorbance was recorded at 450 nm after addition of 50 μL of 1 M H_2SO_4 using a multiplate reader Wallac Victor2 1420 Multilabel counter from Perkin Elmer.

2.3. Surface plasmon resonance (SPR) studies.

SPR studies were carried out in a Biacore® 3000 instrument operating at 20°C. Gold chips from a Biacore SIA kit were first cleaned by treatment with ozone using a PSD-UVT cleaning instrument (from Novascan, USA) for 10 min followed by rinsing with ethanol and drying under a filtered Ar stream. The chip was modified with 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 20 μL/min flow of running buffer (10 mM PBS pH 7.0) was established. After baseline stabilization (~ 3 hours) layers of ADA-CMC-GLI or ADA-GLI were formed by injecting 100 μL of a 1 and 0.1 mg/mL solution in PBS, respectively, until signal saturation. Glycine buffer (10 mM, pH 2) was used for antigen surface regeneration. To calculate the concentration changes on the sensor surface an equivalence of 1 RU = 1 pg/mm^2 was used. To test the specificity of the surface anti-CEA as non-specific analyte was used.

2.4 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. Electrochemical impedance experiments were performed at a formal potential of 0.20 V vs Ag/AgCl with an amplitude of 5 mV at the frequency range from 100 kHz to 10 mHz. A Nyquist plot (Z' vs $-Z''$) was drawn to analyze the impedance results.

Electrode arrays were fabricated as previously described, consisting of 16 gold working electrodes (dimensions: 1 × 1 mm^2) arranged in a 4×4 disposition.[21] Each working electrode is positioned between a silver pseudo reference 0.2 × 1 mm^2 and a gold counter electrode of the same size. The working electrodes were electrocleaned by applying a series of 25 potential cycles in 1 M H_2SO_4 in the range 0-1.6 V vs. Ag/AgCl. The quality of the cleaning step was checked using cyclic voltammetry with 1 mM $K_3[Fe(CN)_6]$ in 0.1 M KCl. Samples were spotted on the working electrodes using a spotting device fabricated as previously reported.[22]

2.5 Modification of gold electrodes and antibody detection.

The support layer was formed by spotting the electrodes with 5 μL of a 10 mg/mL solution of CDPSH in water or CDSH7 in DMSO and incubated overnight to form a SAM containing cyclodextrin hosts. After rinsing with water or DMSO, 5 μL of the gliadin conjugated adamantane polymer (1 mg/mL of ADA-CMC-GLI or 0.1 mg/mL of ADA-GLI in PBS) were spotted onto the functionalised electrode and allowed to incubate overnight. The next incubation steps were carried out immediately prior to the amperometric measurements. For this purpose, 5 μL of polyclonal anti-gliadin IgG antibody of the appropriate concentration in PBS was incubated for 30 minutes. After rinsing with water, a solution of anti-IgG peroxidase conjugate (IgG-HRP) conjugate was then added and incubated for 10 minutes. To study the effect of serum on the electrochemical response, mixtures of PBS pH 7 and fetal calf serum of composition 0:100, 50:50 and 25:75 (v/v) were spiked with 1 μg/mL of polyclonal anti-gliadin antibody.

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 a mixture of 1 mM hydroquinone/1 mM H_2O_2 in 0.1 M PBS pH 6 containing 0.15 M KCl.

2.6 Detection of anti-gliadin antibodies in real samples

Real serum samples were kindly provided by Dr. H. J. Ellis (King's College London) from two anonymous celiac patients undergoing a follow-up treatment for compliance to a gluten-free diet. The samples were divided in four portions, two were used for the detection of anti-gliadin IgG and IgA autoantibodies using the supramolecular platform with amperometric tmeasurement and the other two using a commercial ELISA kit (α-Gliatest S IgG Chromo and (α-Gliatest S IgA Chromo, kindly supplied by Eurospital SpA, Trieste, Italy).

Respective calibration curves using both methods were obtained using the calibrators included in each kit (0, 10, 20, 50, 100 AU/mL for both IgA and IgG) and initially checked against the negative and positive controls also included in the test. These controls should lay in the ranges of AU/mL of 0-10 (negative IgG), 50-90 (positive IgG), 0-5 (negative, IgA), 35-75 (positive, IgA). The autoantibody concentrations in each real sample were obtained in duplicate by interpolation on the calibration plots.

3. Results and discussion

3.1 Enzyme Linked "Self-Assembled" Assay

Commercially available maleimide-precoated plates are useful for binding thiol-containing molecules and have been applied for the colorimetric or fluorescent detection of biomolecules in a similar way to hydrophobic microtitre plates.[23] Unlike immunosorbent plates, where the capture molecules are passively adsorbed on a hydrophobic polystyrene surface, maleimides react with thiol groups at slightly acidic pH forming stable thioether linkages[24] offering an alternative platform to gold surfaces to characterize the supramolecular detection system which, at the same time, would be close to a real sensing scenario in terms of the building blocks used to construct the biorecognition surface. The assay is thus based in the use of an anti-mouse IgG linked HRP conjugate as reporter antibody to detect the target antibodies using a cyclodextrin surface (CDPSH) supporting a self-assembled adamantane-containing gliadin carrier (ADA-CMC-GLI) in what we term an *Enzyme Linked "Self-Assembled" Assay (ELSAA).*

Figure 1a shows a representation of the ELSAA for the detection of anti-gliadin antibody. The plate is initially incubated with CDPSH at pH 6 in order to form the cyclodextrin support layer. Unreacted maleimide groups are then blocked with mercaptoethanol followed by the incubation of different concentrations of ADA-CMC-GLI (from 0-40 μg/mL) and anti-gliadin antibody (from 0-13 μg/mL) to form a matrix of 7 × 7 points. As can be seen from Figure 1b, the absorbance signal increases proportionally with both ADA-CMC-GLI and antibody concentrations, indicating a good performance of the assay. At ADA-CMC-GLI concentrations above 20 μg/mL the signal remains constant with variations in antibody concentration, indicating a saturation of the antigen binding sites immobilized on the surface. At 40 μg/mL of ADA-CMC-GLI, the limit of detection of was 0.21 ± 0.02 μg/mL (n = 7). It is also noteworthy that in the absence of ADA-CMC-GLI the background signal is very low, corresponding to 6% of the maximum signal obtained under saturation conditions, demonstrating the highly effective anti-fouling ability of the cyclodextrin support. Furthermore, the optical response is also

very low (<10%) in the absence of target, indicating negligible affinity of the tracer conjugate for the ADA-CMC-GLI layer.

3.2 Electrochemical characterization of CDPSH/ADA-CMC-GLI deposition

Qualitative information on surface modification can be obtained from cyclic voltammetry by studying the blocking effect caused by substance deposition on the electrode surface that inhibits electron transfer from the electroactive probe. Chemisorption of CDPSH and deposition of ADA-CMC-GLI at the gold surface provoked a 180 and 270 mV increase in ΔE_p of $Fe(CN)_6^{3-}$ anion, respectively, with respect to the bare surface (ΔE_p = 70 mV, see Supporting Information). Simultaneously, the current intensities decreased by 20 and 55%, although the current response is not totally suppressed demonstrating that the surface is permeable to electron transfer. Reductive stripping of CDPSH in alkaline solution also confirmed the chemisorption of CDPSH by observing a cathodic peak at -1.02 V (Figure S2, inset), the integration of which indicated a surface concentration of 2×10^{-12} mol/cm^2, which translates into 2.4×10^{-11} mol/cm^2 of cyclodextrin units taking into consideration that each CDPSH 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.[20]

The immobilization of CDPSH at a bare electrode was also evaluated using impedance spectroscopy. A 175 kΩ increase in the charge transfer resistance (R_{ct}) of the electroactive marker after interaction of CDPSH with the bare electrode ($R_{ct\ bare}$ = 2 kΩ) was observed indicating the successful deposition of the CD support layer. A further increase in R_{ct} was obtained after deposition of ADA-CMC-GLI with a R_{ct} = 295 kΩ (Figure 2). In a control experiment, interaction of ADA-CMC-GLI with a surface modified with a αCD polymer caused a negligible impedance variation, which can be explained considering that the adamantane moiety does not fit in the αCD cavity.[25] This indicates that specific host-guest interactions between the βCD cavities of CDPSH and adamantane are responsible for the interfacial complexation of ADA-CMC-GLI .

3.3 Surface chemistry for electrochemical detection

Four different supramolecular platforms were explored as alternatives for detecting anti-gliadin antibodies combining different formats of both the support layer and antigen (Figure 3). These supramolecular platforms are composed of thiolated β-cyclodextrin polymer (CDPSH, Figure 3a and 3c) or hepta-thiolated β-cyclodextrin (CDSH7, Figure 3b and 3d) deposited on the gold surface acting as a support layer able to immobilize the gliadin auto-antigen. Two different presentations of the antigen were tested: a doubly-modified carboxymethylcellulose polymer carrying gliadin and adamantane units (ADA-CMC-GLI, Figure 3a and 3b) and gliadin modified on the protein surface with adamantane(ADA-GLI, Figure 3c and 3d).

The four supramolecular platforms were evaluated in terms of the amperometric signal response obtained for the detection of 1 μg/mL of anti-gliadin antibody and compared with a blank solution. As can be seen from Figure 4, the highest response and the lowest background signal were obtained with the system CDPSH/ADA-CMC-GLI. If the polymeric antigen is replaced by an easier-to-prepare conjugate of adamantane and gliadin the signal drops by about 40%. When ADA-CMC-GLI was immobilized on a heptathiolated cyclodextrin monolayer there is also a signal drop (~15%) with respect to CDPSH/ADA-CMC-GLI. This indicates that a multivalent presentation of both, the support layer and the gliadin recognition layer favors and improves the performance of the sensing platform.

The marked signal differences observed between systems CDPSH/ADA-CMC-GLI and CDPSH/ADA-GLI can also be attributed to the number of immobilized gliadin residues on the sensor surface. For this reason, both systems were studied using surface plasmon resonance of a CDPSH-modified Biacore® chip (Figure 5). The responses obtained after the injection of 1 mg/mL of ADA-CMC-GLI and 0.1 mg/mL of ADA-GLI were 610 and 145 RU, respectively (Figures 5a and 5b). The signal increase observed for ADA-CMC-GLI corresponds to a surface coverage of 2.8×10^{-15} mol/cm^2, assuming a molecular weight of 217 kDa, which corresponds to a CMC polymer with 80% of COOH groups and a degree of substitution of 0.9 adamantane and 0.07 gliadin residues per glucose unit, respectively. This translates into 2×10^{-16} mol/cm^2 of gliadin residues while in the case of ADA-GLI the

amount of gliadin attached to the sensor surface is equivalent to 7×10^{-16} mol/cm^2. The responses of both platforms to the injection of 1 μg/mL of anti-gliadin antibody were 450 and 120 RU, respectively (Figure 5c). This difference could be explained considering that a more densely packed layer of gliadin antigens on the surface of ADA-GLI exerts some steric hindrance to the recognition of the target antibody. Regeneration of the CDPSH/ADA-CMC-GLI surface was possible using an acidic glycine solution (Figure 5d) and, furthermore, this system showed negligible affinity for a non-specific antibody (anti-carcinoembryonic antigen, Figure 5e).

3.4 Effect of serum on anti-gliadin antibody detection

The presence of high concentrations of other globulins, enzymes, hormones and nutrients in serum are potential sources of non-specific responses and false positives. The possible effect of the serum matrix on antibody detection using the CDPSH/ADA-CMC-GLI and CDPSH/ADA-GLI systems was studied by spiking foetal calf serum with 1 μg/mL of anti-gliadin antibody at different serum dilutions (Table 1). As can be seen, the CDPSH/ADA-CMC-GLI system is able to selectively detect the target antibody in this complex matrix, demonstrating that the sensor performance is not affected and highlighting the applicability of the system to real sample analysis. In contrast, the signal recovery obtained with the CDPSH/ADA-GLI was considerably lower. A plausible reason for this behavior could be the markedly hydrophilic structure of the CMC carrier arising from the glucose units, which prevents non-specific interactions of matrix components.

3.5 Optimization of incubation times for amperometric detection of antigliadin autoantibody.

The system CDPSH/ADA-CMC-GLI was thus selected for the construction of an amperometric biosensor for the detection of anti-gliadin antibodies. In order to optimize the conditions, studies at different incubation times of target antibody and the reporter anti-IgG-HRP conjugate were carried out. The optimum incubation times for target antibody and IgG-HRP were 30 and 10 minutes, respectively (Figure 5) at 25 ºC. In addition, pre-incubation conditions for the detection of anti-gliadin autoantibody

were studied by mixing anti-gliadin autoantibody and tracer antibody for a given time followed by incubation of this mixture in the electrodes. The highest response was obtained after 15 minutes of off-chip mixing and 15 minutes of incubation on the electrode, but the signal was markedly lower than that observed using step-by-step incubation.

3.6 Amperometric detection of antigliadin antibodies.

Figure 6 shows the amperometric calibration curve for the detection of anti-gliadin antibody using the optimum incubation conditions. A linear relationship was observed from 0 to 0.75 μg/mL, while above this value the signal tends to saturation. The sensitivity obtained was 1.48 $\mu A \cdot mL \cdot ng^{-1}$, with a limit of detection of 20 ng/mL, with an RSD of lower than 6%.

For comparison, a standard ELISA was carried on Nunc plates coated with native gliadin (see Supporting Information). In this case, the limit of detection was 260 ng/mL, which is more than 10 times higher than that obtained using amperometry, highlighting the advantage of the developed supramolecular immunosensor, in terms of sensitivity, detection limit and assay time, (~40 min as compared to > 3 hours required for ELISA).

3.7 Detection of antigliadin antibodies in real samples

The supramolecular platform was then used for the detection of anti-gliadin antibodies in real samples. Figure 7 shows the calibration curves obtained for both anti-gliadin IgA and IgG calibrators as determined by ELISA (using a commercial ELISA kit provided by Eurospital Spa (Trieste, Italy) and with the amperometric supramolecular immunosensor. As can be seen the antibody concentrations obtained for these calibrators with the supramolecular immunosensor showed a good correlation with those determined by ELISA.

Using these calibration curves, real samples obtained from patients immediately after detection of celiac disease by biopsy and during different steps of treatment following withdrawal of gluten from the diet (named *follow up samples)* were analyzed. As can be seen from Table 2, the anti-gliadin IgA and

IgG levels of both patients decreased with time, indicative of a successful implementation of a gluten free diet. In addition, there is an excellent correlation between the results obtained by ELISA and with the amperometric immunosensor and the measurements showed a good reproducibility, indicative of the good performance of the immunosensor and demonstrating the effectiveness and applicability of the developed supramolecular to a real clinical scenario.

Conclusions

In this work, we demonstrate the applicability of a simple and reversible surface modification tool based on the supramolecular self-assembly of adamantane-gliadin conjugates and adamantane-carboxymethylcellulose-gliadin polymers on cyclodextrin surfaces for the detection of anti-gliadin autoantibodies in complex matrices such as real serum samples. The surface modification strategy only involves two steps and the immunosensor parameters were optimized in terms of incubation times and matrix effect. The developed supramolecular biosensor was successfully applied to the amperometric detection of anti-gliadin IgA and IgG autoantibodies in real samples of celiac disease patients following removal of gliadin from the patient's diet, with results demonstrating excellent correlation with a commercial ELISA test.

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Supporting Information Available: Synthesis of CMC and gliadin conjugates and cyclic voltammetry of electrode modification steps. This material is available free of charge via the Internet at http://pubs.acs.org.

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FIGURE CAPTIONS

Scheme 1. General principle for the construction of a biosensor based on cyclodextrin-adamantane host-guest interactions.

Scheme 2: Structures of cyclodextrin hosts and gliadin conjugates used in this work.

Figure 1. a) Enzyme-Linked "Self-Assembled" Assay (ELSAA): i) CDPSH, ii) mercaptoethanol, iii) ADA-CMC-GLI, iv) anti-gliadin antibody, v) anti-mouse HRP, vi) color development with TMB. b) 3D plot of the variation of optical response with ADA-CMC-GLI and anti-gliadin antibody concentrations.

Figure 2. Complex impedance plots (in 1 mM $K_3Fe(CN)_6$ in 0.1 M KCl) obtained at bare gold electrode (♦), CDPSH monolayer (●) and deposition of ADA-CMC-GLI (■). Inset: Complex impedance plots after the deposition of αCD polymer (○) and ADA-CMC-GLI (□).

Figure 3. Supramolecular platforms studied for the electrochemical detection of anti-gliadin autoantibodies: a) CDPSH/ADA-CMC-GLI, b) CDSH7/ADA-CMC-GLI, c) CDPSH/ADA-GLI, d) CDSH7/ADA-GLI. GLI: gliadin fragment.

Figure 4. Relative amperometric signals obtained with surface chemistries **a**-**d** of Figure 2 in the detection of 1 μg/mL of polyclonal anti-gliadin anti-IgG antibody (target).

Figure 5. SPR sensorgram for the interaction of ADA-CMC-GLI and ADA-GLI with a CDPSH-modified surface: a) injection of 1 mg/mL of ADA-CMC-GLI, b) a) injection of 0.1 mg/mL of ADA-GLI, c) injections of 1 μg/mL of polyclonal anti-gliadin anti-IgG antibody, d) regeneration of antigen surface with 10 mM glycine buffer pH 2, e) injection of 1 μg/mL of anti-CEA antibody. Conditions: temperature: 20ºC; running buffer: 10 mM PBS pH 7.0; flow rate: 5 μL/min.

Figure 6. Dependence of the amperometric signal for the detection of 1 μg/mL of polyclonal antigliadin anti-IgG antibody with incubation times of target antibody (a) and tracer conjugate (b) using the CDPSH/ADA-CMC-GLI system.

Figure 7. Calibration curve for the detection of polyclonal anti-gliadin IgG antibodies using the CDPSH/ADA-CMC-GLI system with the developed amperometric immunosensor.

Figure 8. Calibration plots employed for the detection of anti-gliadin IgG (●) and IgA (■) autoantibodies in real samples. a) with α-Gliatest Chromo ELISA kit, b) with the CDPSH/ADA-CMC-GLI system and amperometric detection. The open symbols (□,○) indicate the negative (-) and positive (+) controls.

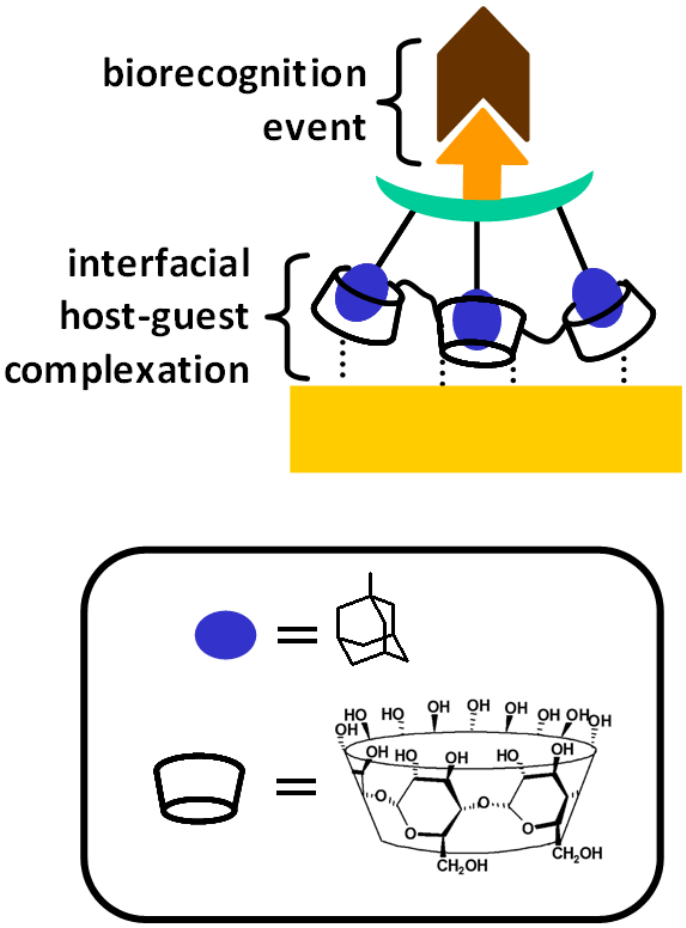
Table 1. Amperometric signal recovery (%) for the detection of 1 μg/mL of anti-giadin antibody in the presence of variable amounts of serum.

Surface chemistry	Serum content (%)			
	0	**25**	**50**	**100**
CDPSH/ADA-CMC-GLI	100	105	101	97
CDPSH/ADA-GLI	100	93	85	82

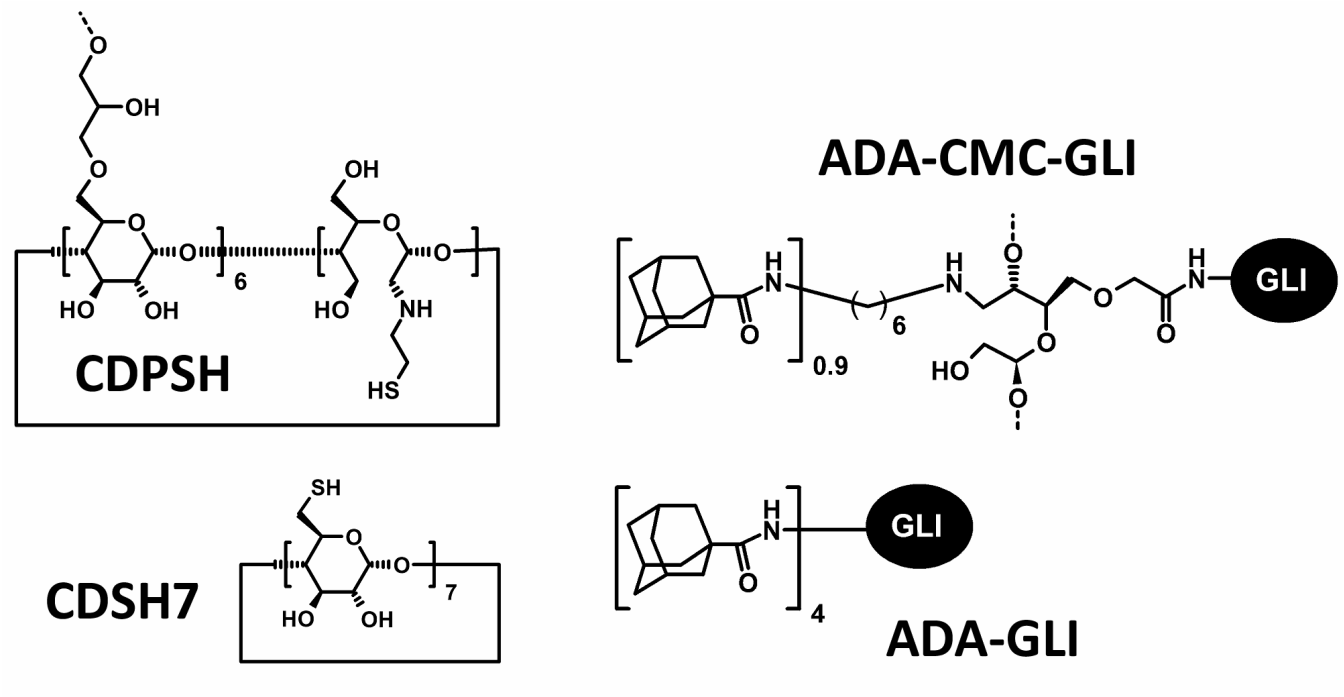
Table 2. Comparison of the anti-gliadin IgA and IgG autoantibody levels (Arbitrary Units/mL (AU/mL)) obtained by ELISA and with the supramolecular platform in two patients before and after introduction of a gluten-free diet.

Patient	Treatment status[a]	Months on GFD[b]	IgA (AU/mL)		IgG (AU/mL)	
			ELISA	**Sensor**	**ELISA**	**Sensor**
A	I	0	16.5 ± 0.4	14 ± 5	81.1 ± 0.9	85 ± 10
	II	4	4.7 ± 0.2	7 ± 4	60.5 ± 0.4	53 ± 3
	III	5	-	-	22.4 ± 0.6	25 ± 4
B	I	0	55.3 ± 0.4	46 ± 8	78.3 ± 1.1	71 ± 7
	II	4	14.7 ± 0.3	12 ± 5	70.1 ± 0.3	60 ± 8
	III	5	-	-	46.1 ± 0.6	42 ± 6

a) I: immediately after detection of celiac disease by biopsy; II and III: during different steps of treatment (compliance to a gluten free diet). b) Gluten Free Diet.


biorecognition
event
interfacial
host-guest
complexation

Scheme 1



Scheme 2

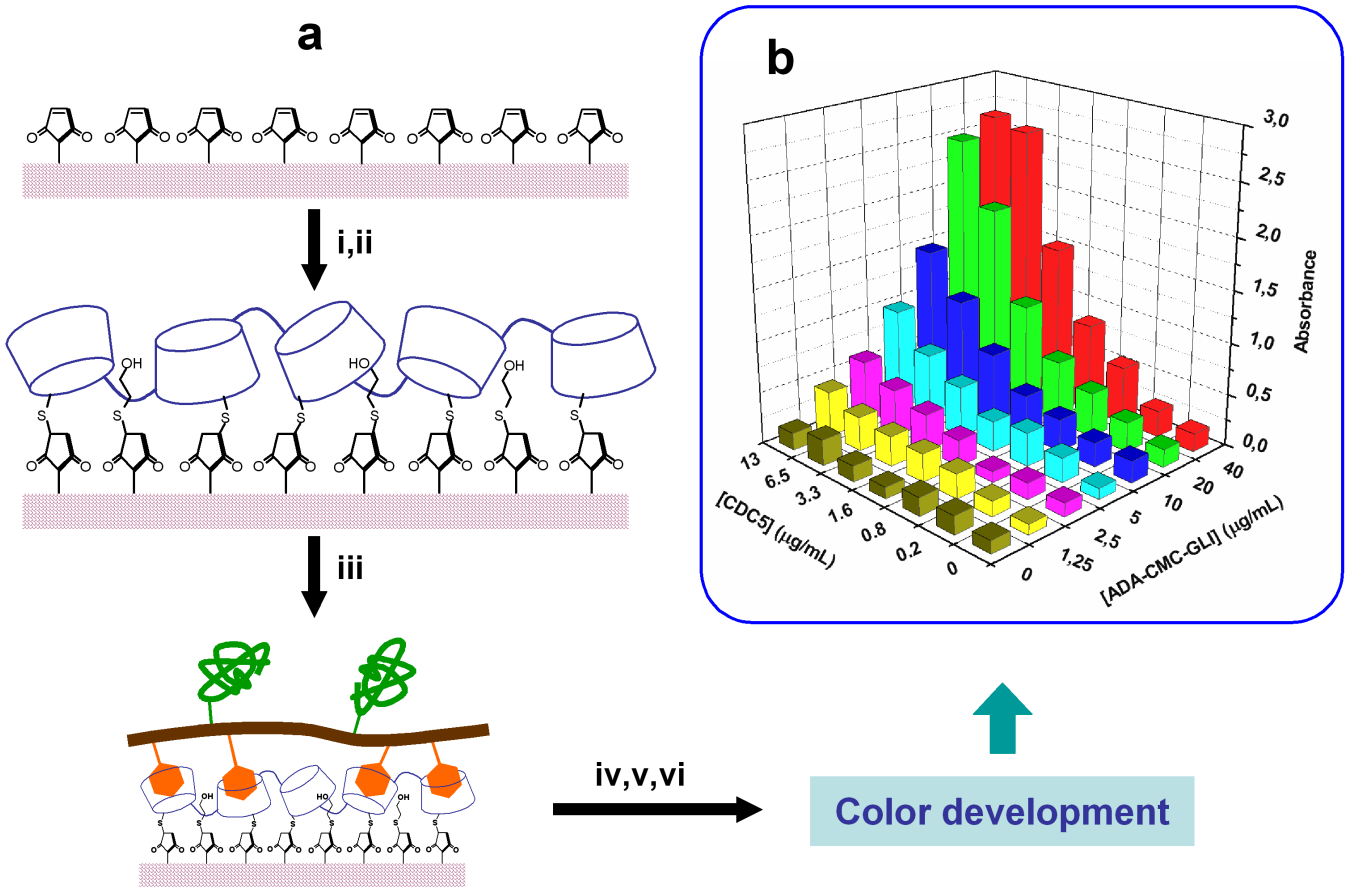

a
i,ii
iii
iv,v,vi
b
Absorbance
3,0
2,5
2,0
1,5
1,0
0,5
0,0
[CDC5] (µg/mL)
13
6.5
3.3
1.6
0.8
0.2
0
[ADA-CMC-GLI] (µg/mL)
0
1,25
2,5
5
10
20
40
Color development

Figure 1

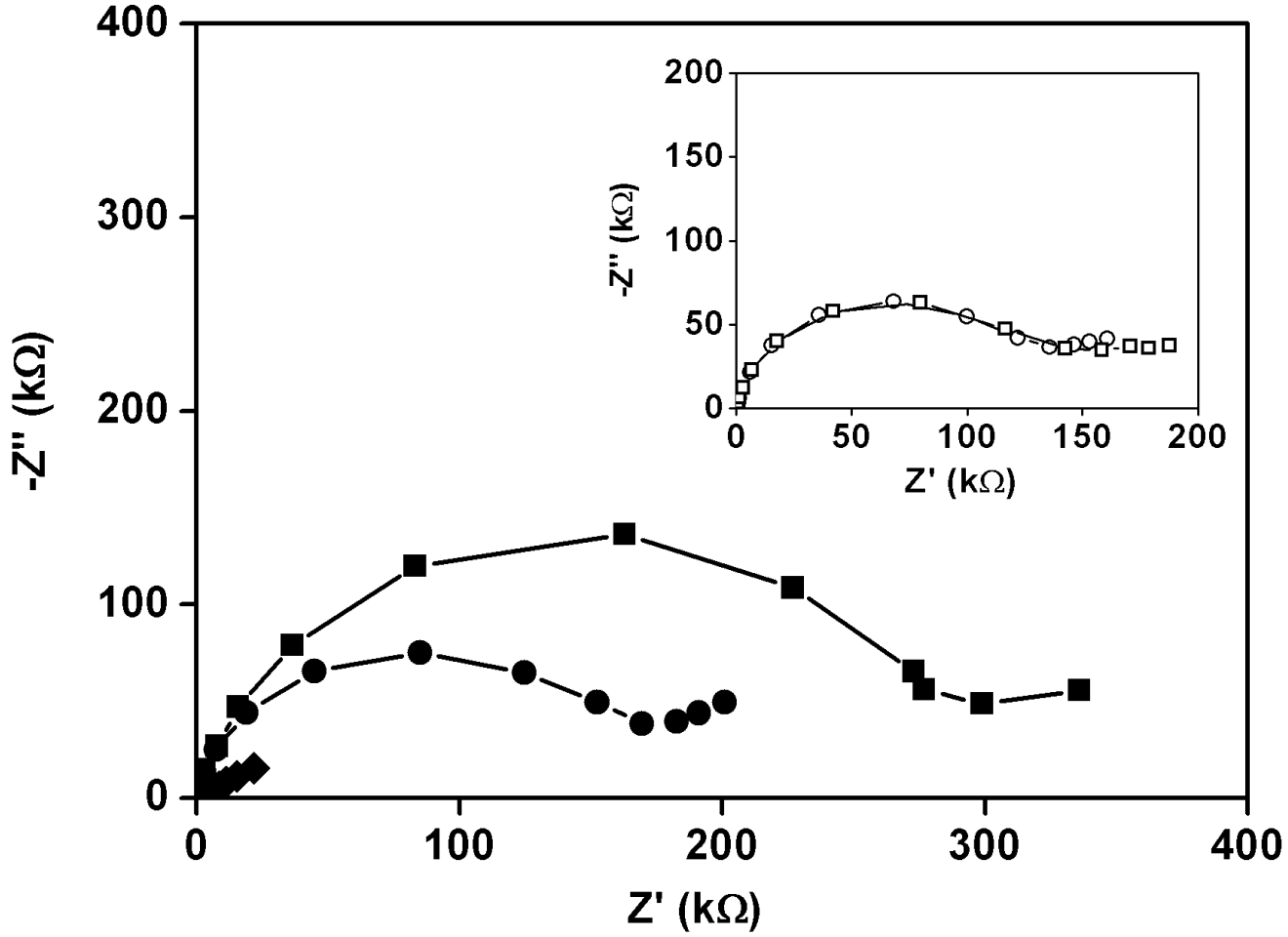

400
300
200
100
0
-Z'' (kΩ)
0
100
200
300
400
Z' (kΩ)
200
150
100
50
0
-Z'' (kΩ)
0
50
100
150
200
Z' (kΩ)

Figure 2

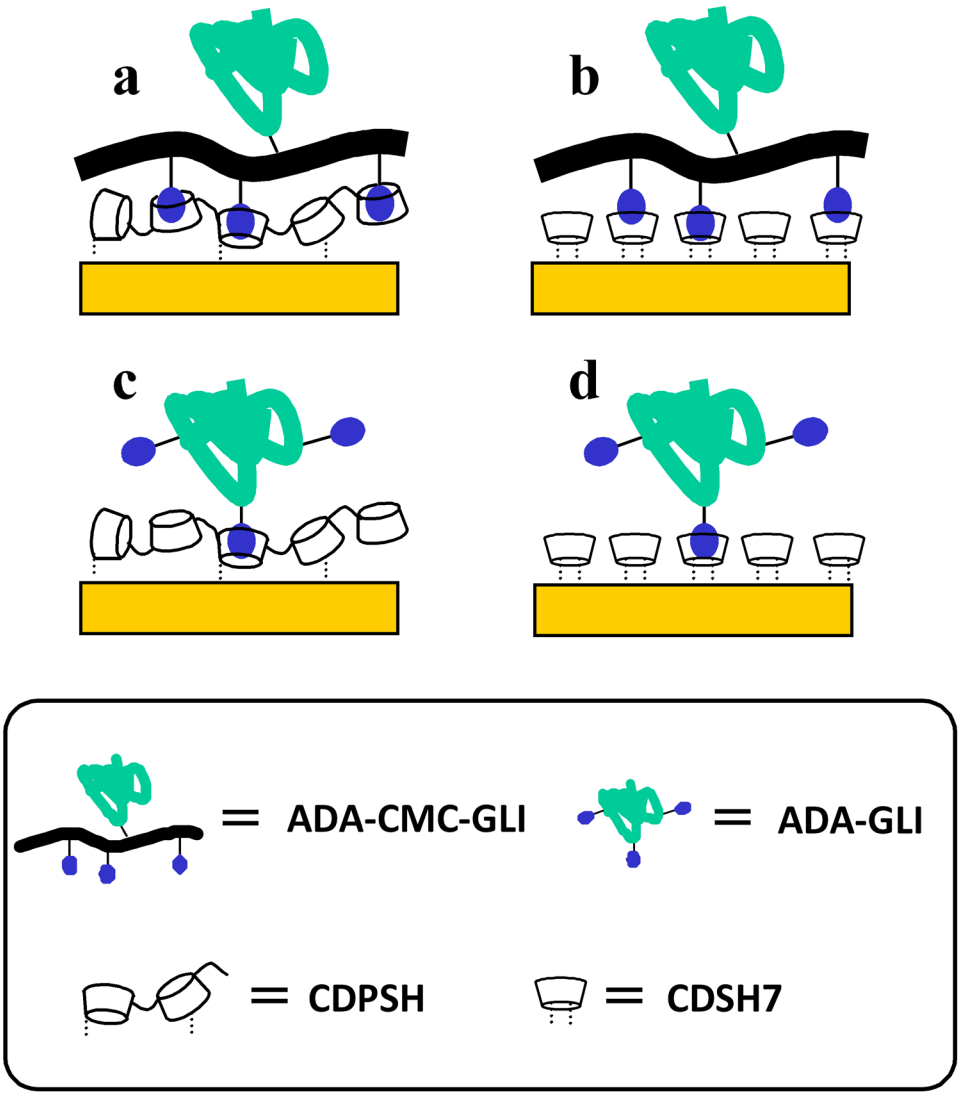


Figure 3

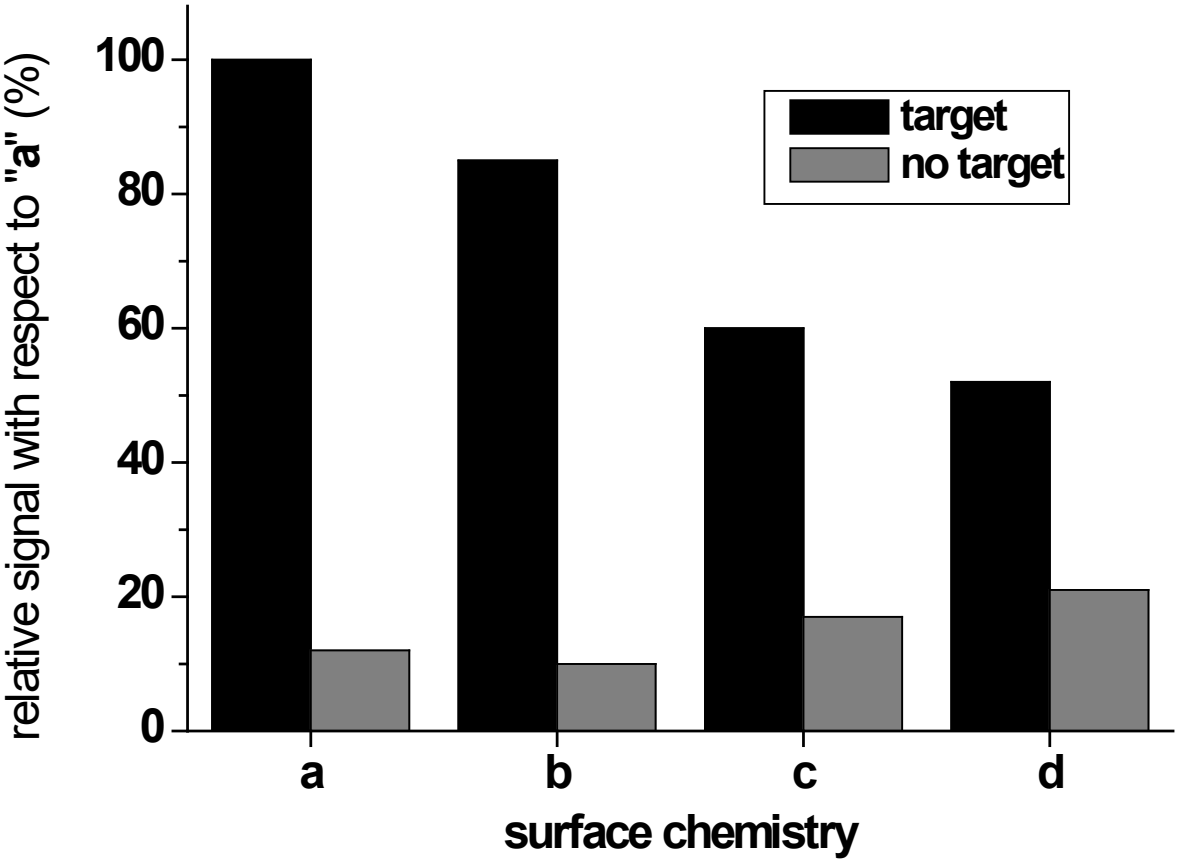

relative signal with respect to "a" (%)
100
80
60
40
20
0
target
no target
a
b
c
d
surface chemistry

Figure 4

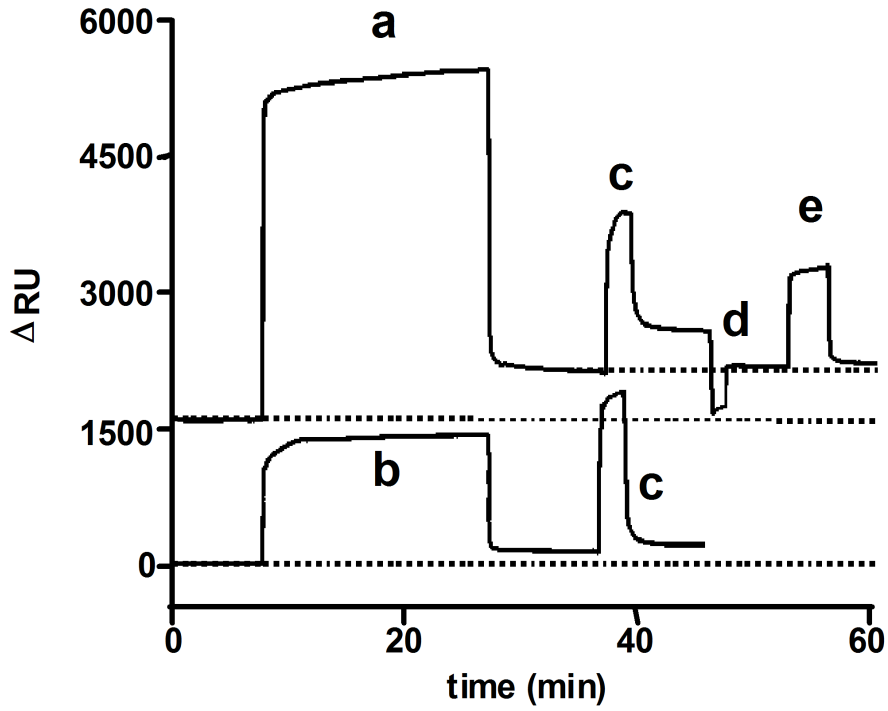


Figure 5

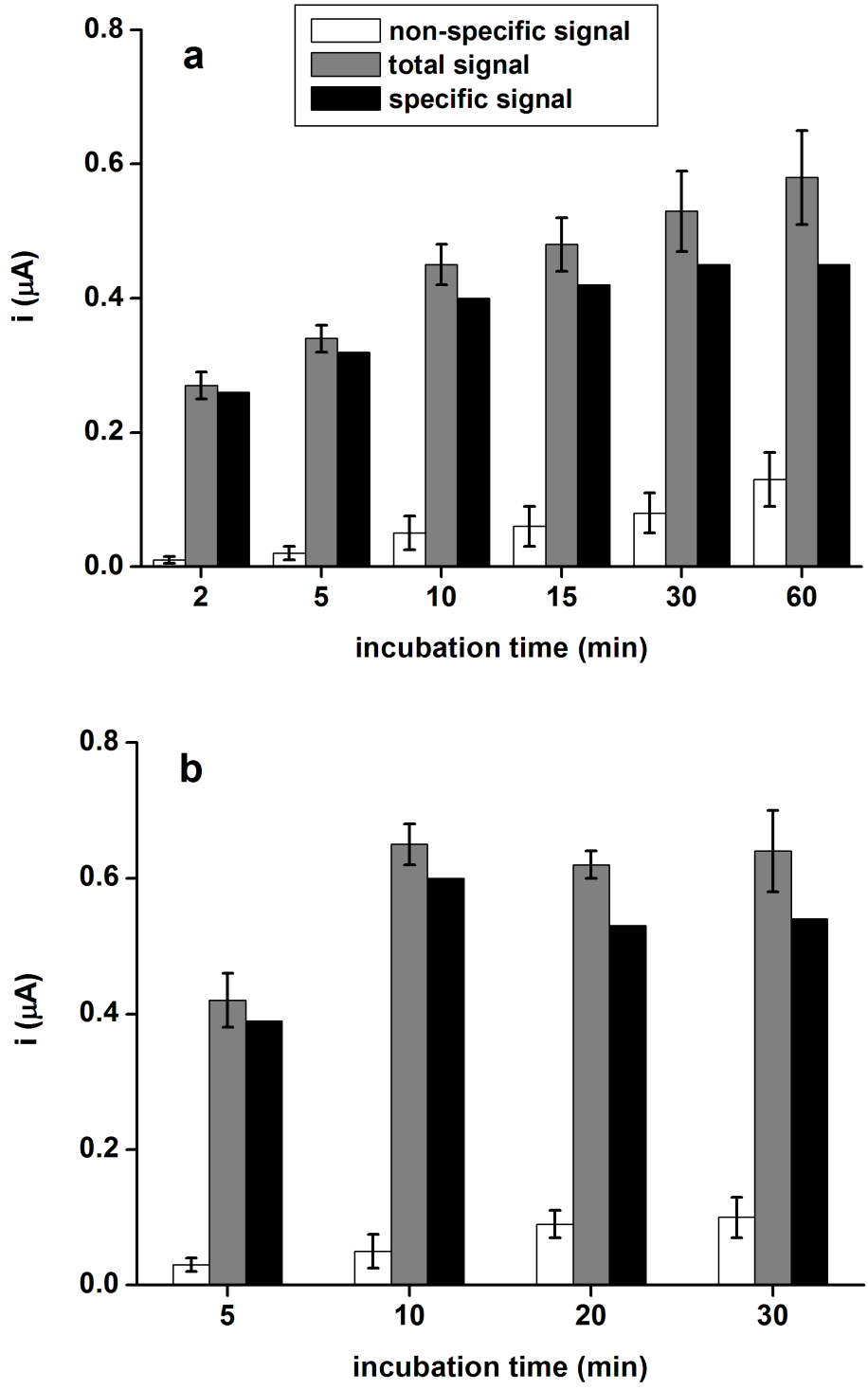

a
non-specific signal
total signal
specific signal
0.8
0.6
0.4
0.2
0.0
i (μA)
2
5
10
15
30
60
incubation time (min)
b
0.8
0.6
0.4
0.2
0.0
i (μA)
5
10
20
30
incubation time (min)

Figure 6

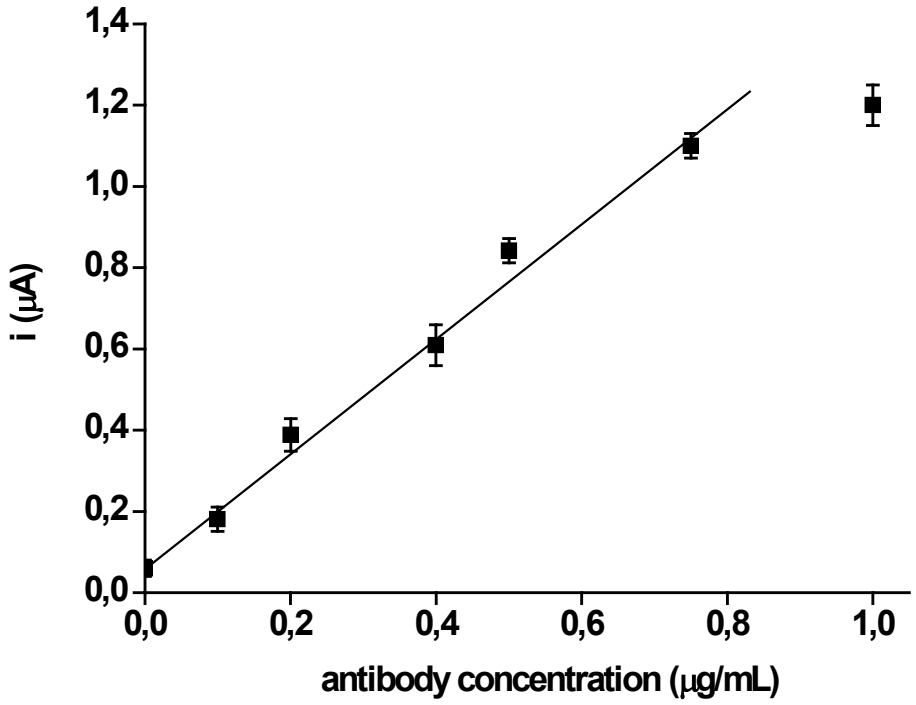

1,4
1,2
1,0
0,8
0,6
0,4
0,2
0,0
i (μA)
0,0
0,2
0,4
0,6
0,8
1,0
antibody concentration (μg/mL)

Figure 7

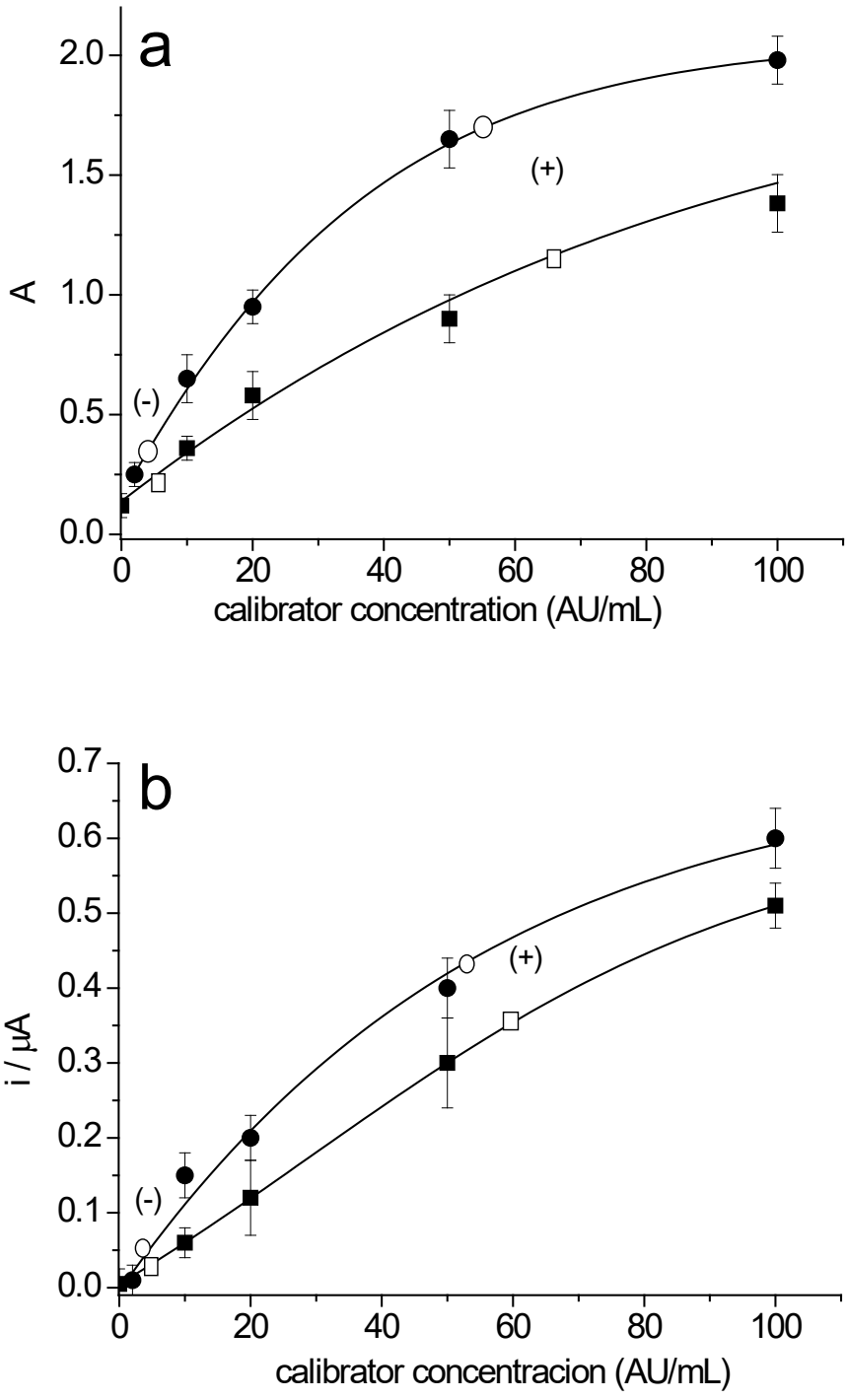

a
2.0
1.5
1.0
0.5
0.0
A
(+)
(-)
0
20
40
60
80
100
calibrator concentration (AU/mL)
b
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
i / µA
(+)
(-)
0
20
40
60
80
100
calibrator concentracion (AU/mL)

Figure 8