analytical chemistry

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Detection of Antigliadin Autoantibodies in Celiac Patient Samples Using a Cyclodextrin-Based Supramolecular Biosensor

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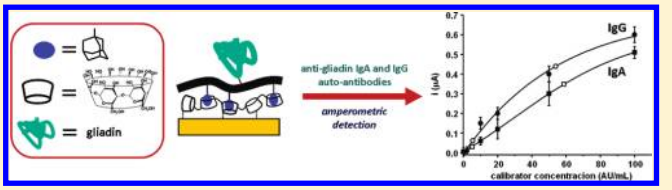
Supporting Information

ABSTRACT: Celiac disease is a condition associated with the ingestion of gluten by genetically susceptible individuals. Measurement of serum antigliadin antibodies is a diagnostic tool also used as a means of monitoring a 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 nonspecific 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; the results were compared with a commercial enzyme linked immunosorbent assay (ELISA) test, and an excellent correlation was observed between both methods.



Celiac disease is a condition associated with the ingestion of gluten by susceptible individuals causing histological changes in the small intestine mucosa and leading to a malabsorption.[1,2] Screening studies have shown that celiac disease is severely underdiagnosed, with seven undiagnosed cases for each diagnosed case. Approximately 85% of cases are unrecognized because of its variable clinical presentation and symptoms,[3] and thus, it is also untreated, 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 is the long-term outlook for the patient, thus highlighting the need of tools for the widespread screening of celiac disease.

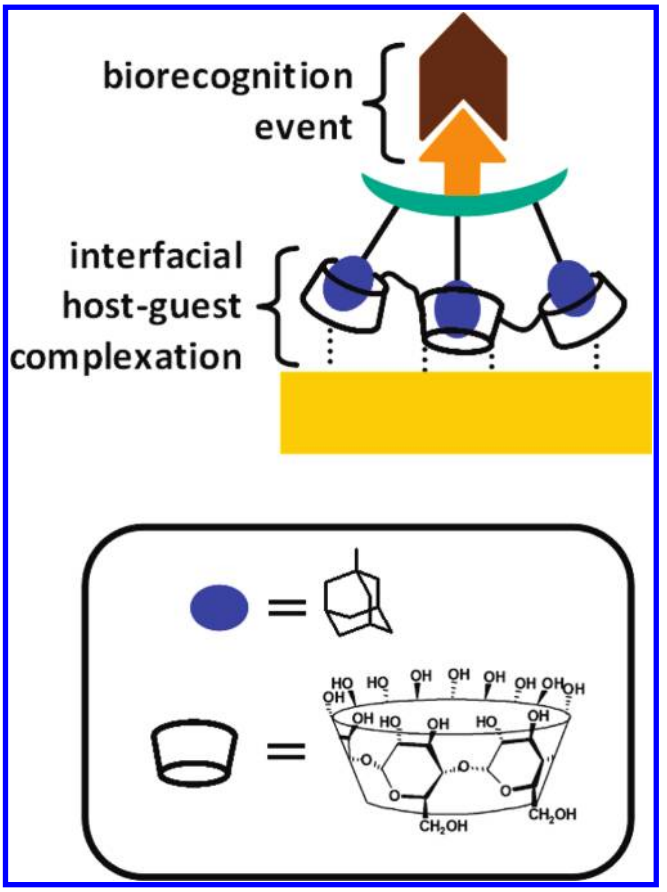
While the gold standard for diagnosis for celiac disease is currently based on a small intestinal biopsy, serological screening tests have become increasingly more specific and sensitive. Serum IgA and IgG antibodies against gliadin,[4] tissue transglutaminase,[5] reticulin,[6] and endomysium[7] (components of connective tissue) are detectable in individuals with celiac disease. Measurement of serum antigliadin antibodies was first used as a diagnostic tool and, second, 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.[8] 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 autoantibodies related to other autoimmune diseases such as systemic lupus erythematosus,[9] multiple sclerosis,[10] and rheumatoid arthritis.[11] In the case of celiac disease related autoantibodies, there are only a few reports on the application of electrochemical biosensors.[12] To the best of our knowledge, there is only one report on the amperometric detection of antigliadin autoantibodies.[13] This system was based on an automatic immuno-microfluidic device fabricated in a low-cost plastic support containing amino-terminated porous glass modified with gliadin molecules. Detection of antigliadin IgG autoantibodies was carried out in real samples with a limit of detection of 0.52 arbitrary units/mL.

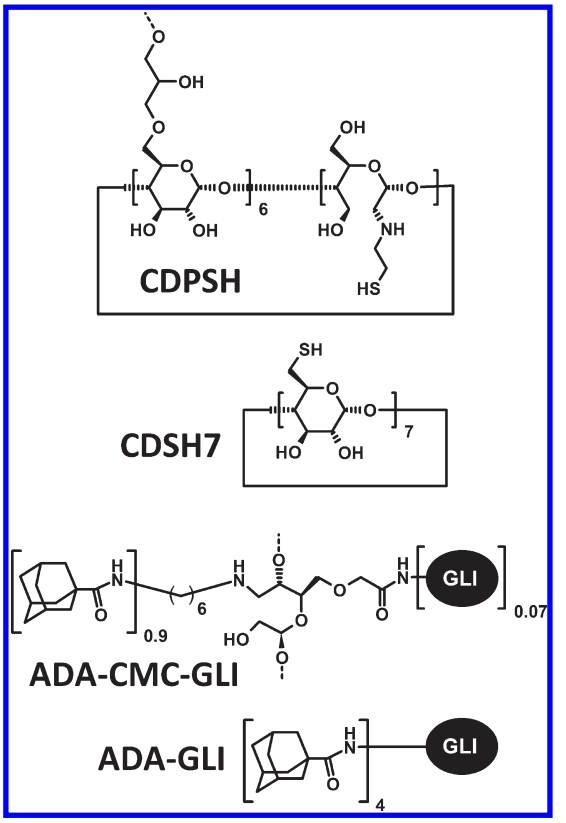
Surface engineering relies on the modification of materials at the molecular scale with the aim of providing an interface of enhanced performance and improved physical and (bio)chemical properties. This is especially relevant for the construction of biosensors where the challenge is not only to engineer the surface of the device such that the biorecognition molecule tolerates the "foreign" object but also to correctly orientate the surface functional groups.[14,15] 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 electrode surfaces with functional molecules able to control the microenvironment

Received: November 12, 2010
Accepted: March 18, 2011
Published: March 28, 2011

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



of the interface or the distance and/or orientation of an attached biomolecule.[16]

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.[17,18] In particular, the use of β−cyclodextrin (CD)[19] coated surfaces (i.e., with a thiolated CD on gold) offers an unconventional route for the reversible immobilization of different biomolecules based on the interfacial inclusion of adamantane (ADA) containing polymers, dendrimers, or biomolecules.[20−22] 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[20] Since the electrode surface is initially modified with a highly hydrophilic layer, nonspecific interactions are greatly reduced, which is particularly critical in immunosensor development. This advantage is not present in the case of modifying the electrode surface with a single polymer containing thiol groups and the biomolecule, in which the latter will also have some tendency to interact with the bare surface decreasing the performance of the device. In addition, the reversibility of the host-guest interactions allows the possibility of surface regeneration to reuse the supramolecular platform, useful in developmental work.[23]

In this work, we report a novel surface modification strategy based on the interfacial self-assembly of a bifunctionalized carboxymethylcellulose (CMC) bearing adamantane units and an antigenic fragment onto a CD-containing support and its applicability to the detection of antigliadin antibodies in both immunoassay and immunosensor formats. To optimize the electrochemical response, several forms to arrange the CD-ADA linkers over the surface were investigated (Scheme 2), and the assay was optimized in terms of incubation times and resistance to nonspecific 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 (i.e., following withdrawal of gluten from the diet), and the results are compared with a commercial enzyme linked immunosorbent assay (ELISA).

EXPERIMENTAL SECTION

Materials. Thiolated β-cyclodextrin polymer (CDPSH; $M_w \sim$ 18 000 mol/L; degree of substitution: 13 mol SH per mol polymer)[21c] and hepta-6-thio-6-deoxy-β-cyclodextrin (CDSH7)[24] were prepared as previously described.

Synthesis of Adamantane-Containing Carboxymethyl Cellulose Polymer (ADA-CMC). Aminated CMC (0.2 g; see Supporting Information) was dissolved in 20 mL of 0.1 M acetate buffer, pH 5, and treated with a previously prepared solution of activated

dx.doi.org/10.1021/ac102956p |Anal. Chem. 2011, 83, 2931–2938

adamantane carboxylate. The reaction mixture was stirred at 4 °C for 1 h and then at room temperature overnight. The mixture was concentrated to about half the initial volume, left to dialyze for 24 h to remove nonreacted material, was then concentrated to dryness to give ADA-CMC, and stored under vacuum (Yield: 0.21 g). ^{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).

Conjugation of Gliadin to ADA-CMC. ADA-CMC (0.06 g) was dissolved in 5 mL of 0.1 M acetate buffer at pH 5. To this solution, EDC (0.2 g) was added, and the mixture was stirred for 1 h at 4 °C. Gliadin was conjugated by adding 0.01 g of digested gliadin (see Supporting Information) to the activated polymer, and the solution was stirred overnight. The ADA-CMC-GLI polymer was purified using a Microcon centrifugal filter device (M_w cutoff 10 kDa) and freeze-dried. IR (KBr), ν_{max} (cm^{-1}): 1641, 1529 (s, C═O amide), 1601 (s, COO^-), 1446 (m, C−N). UV−vis λ_{max} (H_2O): 279 nm. The amount of gliadin in the polymer was estimated by UV spectroscopy at 279 nm using a digested gliadin calibration curve, and the measured absorbances were referenced to an ADA-CMC solution. This analysis gave an average gliadin content of 0.07 mol/mol of glucose units.

Introduction of Adamantane Units in Gliadin. Gliadin was conjugated to adamantane by stirring a mixture of 0.01 g of digested gliadin with 0.01 g of EDC-activated adamantane carboxylic acid overnight. The ADA-GLI conjugate was purified by dialysis. The number of introduced ADA units was calculated by measuring the number of free lysine residues on the gliadin molecule before and after modification using trinitrobenzenesulfonic acid (TNBSA).[26] This gave an average of 4 mol of adamantane per mol of gliadin.

Enzyme-Linked Immunoassay (EIA) on Maleimide-Precoated Plates. Reacti-bind maleimide activated plates (8-well strips) were used as received. All washing steps were carried out with 0.1 M sodium phosphate and 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 ethylenediaminetetraacetic acid (EDTA); pH 7.2) and incubation for 3 h 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 various concentrations of ADA-CMC-GLI (0−40 μg/mL) in binding buffer were added to the wells and incubated overnight at 4 °C. After washing, 100 μL of a range of concentrations of antigliadin antibody (0−13 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 1:15 000 dilution of mouse anti-IgG-HRP conjugate for 1 h at room temperature. After a final washing step, 50 μL of TMB solution was added, and 15 min later, after addition of 50 μL of 1 M H_2SO_4, the absorbance was recorded at 450 nm using a multiplate reader Wallac Victor2 1420 Multilabel counter from Perkin-Elmer.

Modification of Gold Electrodes and Antibody Detection. The amperometric detection system used in this work consists of the following main steps: (1) self-assembly of thiolated cyclodextrin and ADA-CMC-GLI on gold electrodes, (2) immunocomplex formation with the target autoantibody followed by mouse anti-IgG-HRP conjugate incubation, (3) amperometric signal generation using a hydroquinone (mediator)−hydrogen peroxide (substrate) mixture at fixed potential.

Electrode arrays consisting of 16 gold working electrodes (dimensions: 1 × 1 mm^2) in a 4 × 4 arrangement placed between a silver pseudoreference (0.2 × 1 mm^2) and a gold counter electrode of the same size were fabricated using a photolithographic process as previously described.[25] Briefly, a circular borofloat glass wafer (ϕ = 12 cm) was fully covered with a UV patterned lift-off resist, and 10 nm of titanium adhesion promoter was sputtered followed by a 150 nm silver layer. The patterned substrate was fully coated a second time with the same lift-off resist and UV patterned to reveal working and counter electrode areas and their corresponding connections, and finally, it was coated with a 10 nm thick titanium layer followed by a 150 nm thick gold layer.

The support layer was formed by spotting the electrodes with 5 μL of a 10 mg/mL solution of CDPSH in water or CDSH7[24] in dimethylsulfoxide (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) was spotted onto the functionalized 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 antigliadin IgG antibody of the appropriate concentration in PBS was incubated for 30 min. After rinsing with water, a solution of anti-IgG peroxidase conjugate (IgG-HRP) (1:15 000) was then added and incubated for 10 min. The electrochemical measurements were carried out by first recording the amperometric background response at −0.2 V vs Ag/AgCl in PBS buffer, pH 6, for 60 s followed by injection of a mixture of 1 mM hydroquinone (mediator) and 1 mM H_2O_2 (substrate) in 0.1 M PBS, pH 6, containing 0.15 M KCl.

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 antigliadin antibody and detected as previously described.

Detection of Antigliadin 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 follow-up monitoring for compliance to a gluten-free diet. The samples were collected after informed consent and only used after the Ethics Committee approval of King's College London. The samples were divided in four portions: two were used for the detection of antigliadin IgG and IgA autoantibodies using the supramolecular platform, and the other two used commercial ELISA kits (α-Gliatest S IgG Chromo and α-Gliatest S IgA Chromo, kindly supplied by Eurospital SpA, Trieste, Italy).

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), and 35−75 (positive IgA). The autoantibody concentrations in each real sample were obtained in duplicate by interpolation on the calibration plots.

RESULTS AND DISCUSSION

Enzyme-Linked Immunoassay on Maleimide-Precoated Plates. 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 microtiter plates.[27] Unlike

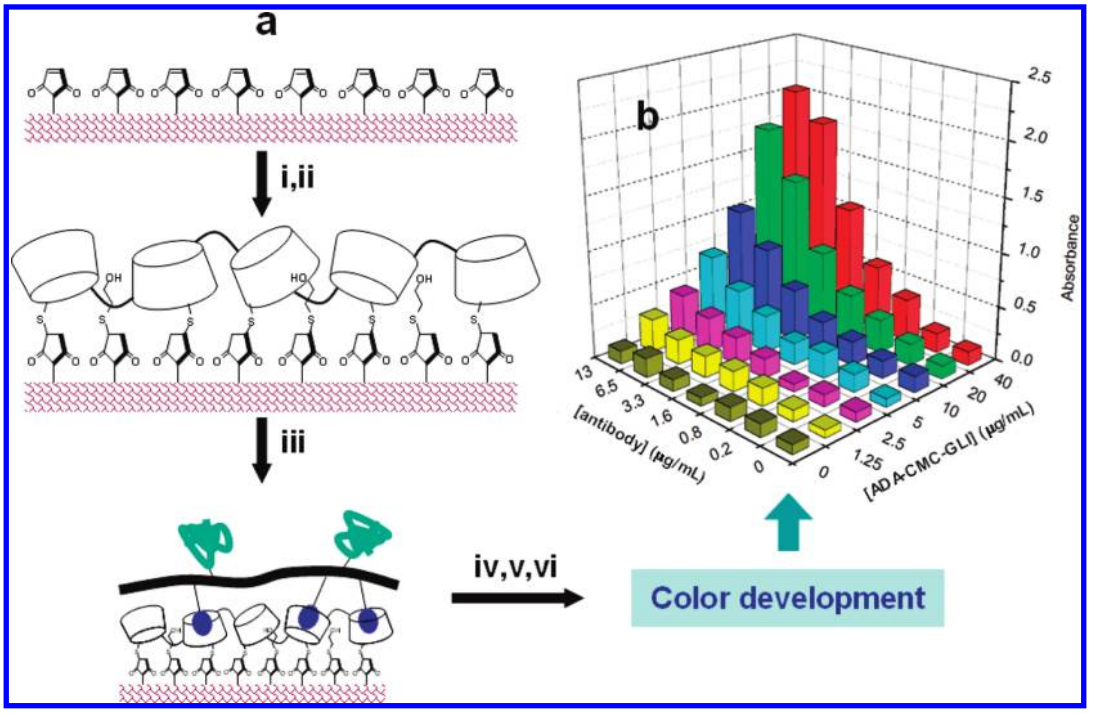


Figure 1. (a) Enzyme-linked immunoassay on maleimide-precoated plates: (i) CDPSH, (ii) mercaptoethanol, (iii) ADA-CMC-GLI, (iv) antigliadin antibody, (v) antirabbit IgG labeled with HRP, and (vi) color development with TMB. (b) 3D plot of the variation of optical response with ADA-CMC-GLI and antigliadin antibody concentrations.

hydrophobic immunosorbent plates, maleimides react with thiol groups forming stable thioether linkages,[28] thus 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 antimouse 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).

Figure 1a shows a representation of the immunoassay for the detection of antigliadin antibody. The plate is initially incubated with CDPSH in order to form the cyclodextrin support layer. After blocking the unreacted maleimide groups with mercaptoethanol, different concentrations of ADA-CMC-GLI and antigliadin antibody are sequentially added 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 40 μg/mL of ADA-CMC-GLI, the limit of detection was $0.21 \pm 0.02\,\mu$g/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 antifouling ability of the cyclodextrin support. Furthermore, the optical response is also very low in the absence of target, indicating negligible affinity of the tracer conjugate for the ADA-CMC-GLI layer.

Electrochemical Characterization of CDPSH/ADA-CMC-GLI Deposition. Chemisorption of CDPSH and deposition of ADA-CMC-GLI at the gold surface provoked, respectively, a 180 and 270 mV increase in the peak-to-peak response of $Fe(CN)_6^{3-}$ in cyclic voltammetry (Figure 2) with respect to the bare surface (ΔE_p = 70 mV). Simultaneously, the current intensities decreased by 20

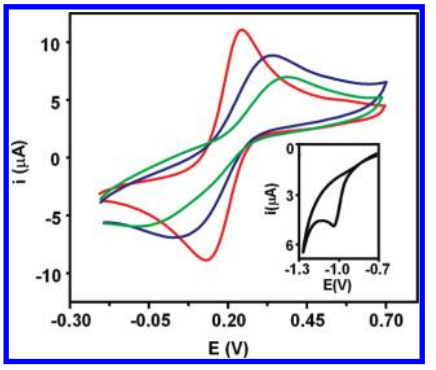


Figure 2. Cyclic voltammograms (in 1 mM $K_3Fe(CN)_6$ in 0.1 M KCl; scan rate: 100 mV/s) obtained at bare gold electrode (red), deposition of CDPSH monolayer (blue), and deposition of ADA-CMC-GLI (green). Inset: cyclic voltammogram (in 0.5 M KOH; scan rate: 10 mV/s) for the reductive desorption of CDPSH from a gold electrode.

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 2, 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.[21c]

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

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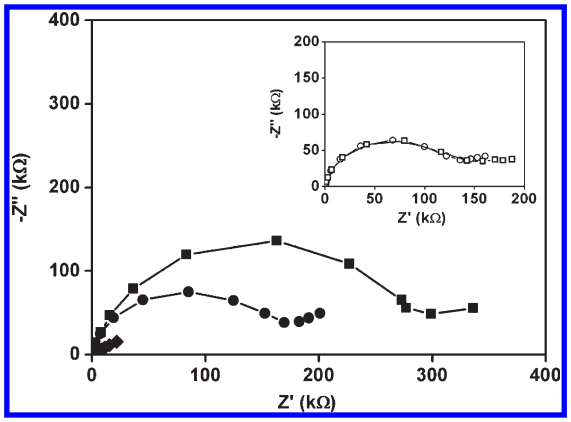

Figure 3. Complex impedance plots (in 1 mM $K_3Fe(CN)_6$ in 0.1 M KCl) obtained at bare gold electrode (♦), CDPSH monolayer (●), and deposition of ADA-CMC-GLI (■). Inset: Complex impedance plots after the deposition of αCD polymer (○) and ADA-CMC-GLI (□).

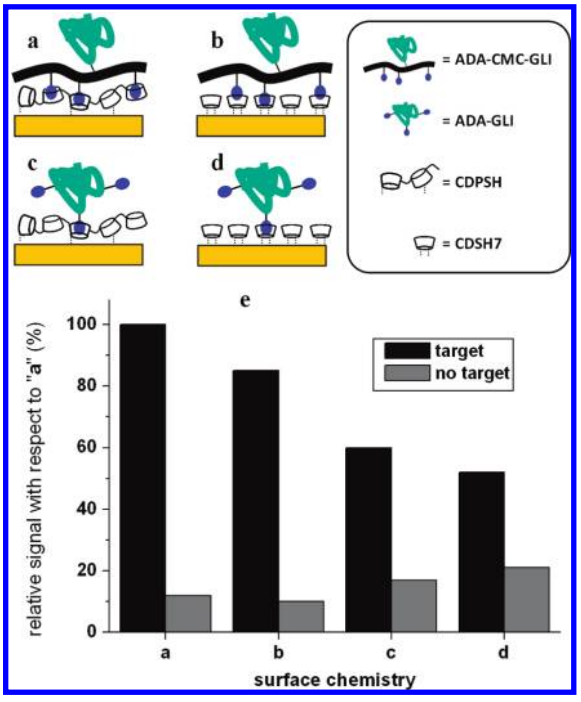

Figure 4. (a−d) Supramolecular platforms studied for the electrochemical detection of antigliadin autoantibodies; GLI: gliadin fragment. (e) Relative amperometric signals obtained with surface chemistries a−d in the detection of 1 μg/mL of polyclonal antigliadin anti-IgG antibody (target).

CD support layer. A further increase in R_{ct} was obtained after deposition of ADA-CMC-GLI with a R_{ct} = 295 kΩ (Figure 3). In a control experiment, interaction of ADA-CMC-GLI with a surface modified with a αCD polymer caused a negligible impedance variation, which is expected considering that the adamantane moiety does not fit in the αCD cavity (Figure 3, inset).[29] 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 .

Surface Chemistry for Electrochemical Detection. Four different supramolecular platforms were explored as alternatives for detecting antigliadin antibodies combining different formats

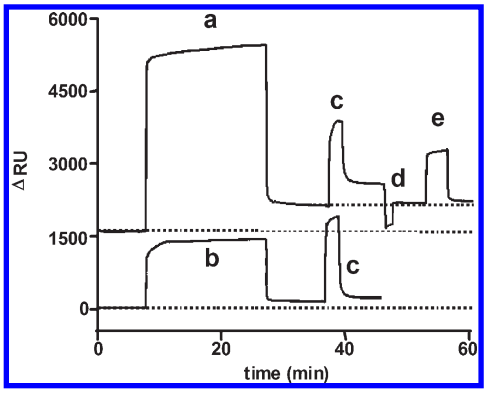

Figure 5. SPR sensorgrams for (a) injection of ADA-CMC-GLI, (b) injection of ADA-GLI, (c) injections of polyclonal antigliadin antibody, (d) regeneration of antigen surface with 10 mM glycine buffer, pH 2, and (e) injection of anti-CEA antibody. Conditions: temperature, 20 °C; running buffer, 10 mM PBS, pH 7.0; flow rate, 5 μL/min.

of both the support layer and antigen (Figure 4). These supramolecular platforms are composed of CDPSH (Figure 4a, c) or hepta-thiolated β-cyclodextrin (CDSH7, Figure 4b,d) deposited on the gold surface acting as support for the adamantane-modified polymers. Two different presentations of the antigen were tested: a doubly modified carboxymethylcellulose polymer carrying gliadin and adamantane units (ADA-CMC-GLI, Figure 4a,b) and gliadin directly modified with adamantane (ADA-GLI, Figure 4c,d).

The supramolecular platforms were evaluated in terms of the amperometric signal response obtained for the detection of 1 μg/mL of antigliadin antibody and compared with a blank solution. As can be seen from Figure 4e, 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 the heptathiolated cyclodextrin monolayer, there is also a markedly lower signal observed with respect to CDPSH/ADA-CMC-GLI, indicating that a multivalent presentation of both the support layer and the gliadin recognition layer favors and improves the performance of the sensing platform.

The notable 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. To further explore this, both systems were studied using surface plasmon resonance on 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,b). 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% COOH groups and a degree of substitution of 0.9 adamantane and 0.07 gliadin residues per glucose unit, respectively, representing 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 antigliadin 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

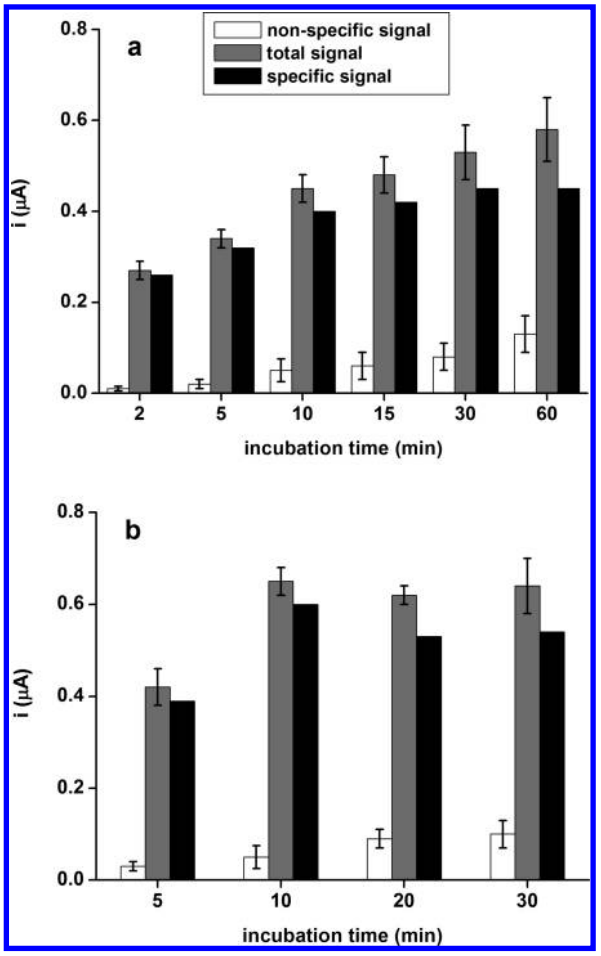

Figure 6. Dependence of the amperometric signal for the detection of 1 μg/mL of polyclonal antigliadin antibody with incubation times of target antibody (a) and tracer conjugate (b) using the CDPSH/ADA-CMC-GLI system.

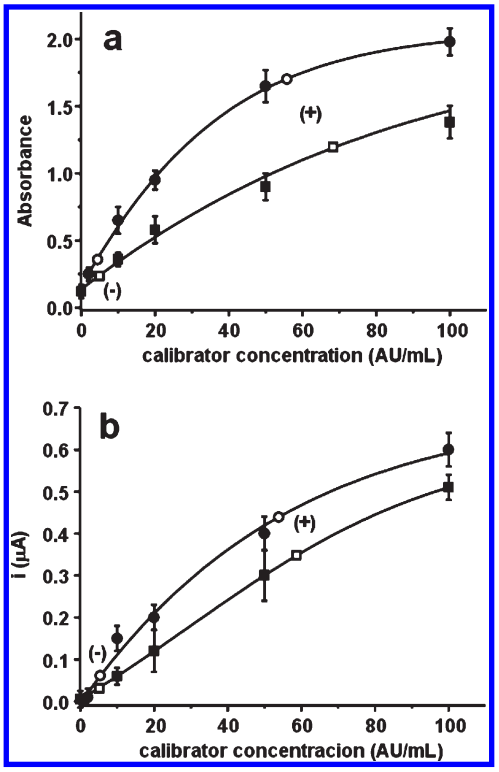

Figure 7. Calibration plots employed for the detection of antigliadin IgG (●) and IgA (■) autoantibodies in real samples: (a) with α-Gliatest Chromo ELISA kit and (b) with the CDPSH/ADA-CMC-GLI system and amperometric detection. The open symbols (□, ○) indicate negative (−) and positive (+) controls, respectively, included in the kits.

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 nonspecific antibody (anticarcinoembryonic antigen, Figure 5e).

Effect of Serum on Antigliadin Antibody Detection. The presence of high concentrations of other globulins, enzymes, hormones, and nutrients in serum are potential sources of nonspecific 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 fetal calf serum with 1 μg/mL of antigliadin antibody at different serum dilutions (Table S1, Supporting Information). The CDPSH/ADA-CMC-GLI system is able to selectively detect the target antibody in this complex matrix with signal recoveries near 100%, 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 (82% for pure serum). A plausible explanation for this behavior could be the markedly hydrophilic structure of the CMC carrier arising from the glucose units, which prevents nonspecific interactions of matrix components.

Amperometric Assay Optimization and Detection of Antigliadin Autoantibody. The system CDPSH/ADA-CMC-GLI was thus selected for the construction of an amperometric biosensor for the detection of antigliadin 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 min, respectively (Figure 6) at 25 °C. In addition, preincubation conditions for the detection of antigliadin autoantibody were studied by mixing antigliadin autoantibody and tracer antibody for a given time followed by incubation of this mixture on the electrodes. The highest response was obtained after 15 min of off-chip mixing and 15 min of incubation on the electrode, but the signal was markedly lower than that observed using step-by-step incubation.

Using the optimum incubation conditions, a calibration plot was linear from 0 to 0.75 μg/mL with a linear regression equation of $i = 1.48[\text{antibody}] + 0.05$ ($r = 0.991$). Above 0.75 μg/mL, the signal tends to saturation. The sensitivity obtained was 1.48 μA·mL·ng^{-1}, with a limit of detection of 20 ng/mL and a RSD lower than 6% ($n = 6$, see Supporting Information). For comparison, a standard ELISA was carried on Nunc plates coated with native gliadin (see Supporting Information). In this case, the absorbance variations followed the equation $A = 1.16[\text{antibody}] + 0.02$ ($r = 0.989$) with a limit of detection of 260 ng/mL, which is more than 10 times higher than that obtained using amperometry and highlighting the advantage of the developed supramolecular immunosensor, in terms of sensitivity, detection limit, and assay time (~40 min as compared to >3 h required for ELISA).

To test the reusability of the surface, an experiment was carried our in which the supramolecular CDPSH/ADA-CMC-GLI

Table 1. Comparison of the Antigliadin 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

			IgA (AU/mL)		IgG (AU/mL)	
patient	treatment status[a]	months on GFD[b]	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.

complex is formed on the gold surface, disrupted with 0.1% w/v SDS, and formed again, and detection of 1 μg/mL of antigliadin antibody is carried out before and after the regeneration step, obtaining that more than 95% of the initial signal is maintained.

Detection of Antigliadin Antibodies in Real Samples. The supramolecular platform was then used for the detection of antigliadin antibodies in real samples. Figure 7 shows the calibration curves obtained for both antigliadin 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 1, the antigliadin 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 were highly reproducible, demonstrating the successful performance of the immunosensor and highlighting the effectiveness and applicability of the developed supramolecular biosensor to a real clinical scenario. Work is ongoing to house the developed supramolecular immunosensor within a microsystem, which can be expected to facilitate completion of the assay in less than 5 min, based on previous experience of the authors.[30]

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 antigliadin autoantibodies in complex matrixes 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 antigliadin 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.

ASSOCIATED CONTENT

Supporting Information. Synthesis of CMC and gliadin conjugates, effect of serum, SPR details, calibration curve, and ELISA results. This material is available free of charge via the Internet at http://pubs.acs.org.

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ACKNOWLEDGMENT

This work has been carried out with financial support from the Commission of the European Communities specific RTD programme, FP7-2008-ICT-216031, CD-MEDICS. A.F. is grateful to the Ministerio de Ciencia e Innovacion, Spain, for a Ramon y Cajal Research Professorship and financial support under the grant BIO2008-02841. We also thank Dr. H. J. Ellis (King's College London) and Dr. Enzo Bravi (Eurospital SpA) for providing the samples and ELISA kits used in this work.

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