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# Amperometric Immunosensor for Detection of Celiac Disease Toxic Gliadin Based on Fab Fragments

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**Immunosensor sensitivity is strongly dependent on the density of free active epitopes per surface area, which could be achieved via well-oriented immobilization of antibody fragments as bioreceptor molecules. Here, we report on the development of an electrochemical gliadin immunosensor based on the spontaneous self-assembly of antigliadin Fab fragments (CDC5-Fab) on Au surfaces. The analytical performance of this immunosensor is compared with a similar containing whole CDC5 antibodies previously modified with thiol groups (CDC5-SH) as the recognition element. Fab fragments were generated by reduction of the disulfide bond of $F(ab)_2$ fragments obtained by bromelain digestion of CDC5 antibody. Surface plasmon resonance (SPR) was used to evaluate the degree of immobilization and recognition ability of immobilized CDC5-Fab and CDC5-SH on gold surfaces. The studied surface chemistries were evaluated in terms of time required for SAM formation, stability, susceptibility to nonspecific interactions, and sensitivity using surface plasmon resonance, electrochemical impedance spectroscopy (EIS), and amperometry. CDC5-Fab formed a stable monolayer on gold after 15 min and retained >90% of antigen recognition ability after 2 months of storage at 4 °C.Detection of gliadin of Fab modified electrodes was evaluated by impedance and amperometry. Labeless impedimetric detection achieved a LOD of 0.42 μg/mL while the amperometric immunosensor based on Fab fragments showed a highly sensitive response with an LOD of 3.29 ng/mL. The Fab based immunosensor offers the advantages of being highly sensitive, easy, and rapid to prepare, with a low assay time.**

Protein/peptide molecules strongly interact with a wide variety of solid surfaces through hydrophobic, electrostatic, and hydrogen bond interactions.[1] Many studies have compared methods of antibody attachment to biosensor surfaces including physicochemical adsorption and covalent attachment.[2,3] Covalent methods improve the uniformity and reproducibility of the bound proteins and have been applied for immobilizing proteins onto different solid substrate surfaces using defined linkages or strong Au−S bonds to form self-assembled monolayers (SAM),[4,5] generally addressing the lysine residues randomly present in the antibodies. This gives rise to a random orientation of the antigen binding site toward the sensor surface[5,6] and may also lead to loss of the biological activity of the antibody.[7]

An alternative to whole antibodies is the use of antibody fragments as recognition element. Recombinant Fab fragments, as well as the even smaller Fv and single-chain Fv fragments, can be readily generated using robust engineering approaches, such as phage display. While the generated fragments possess definitive advantages over their whole antibody counterpart, they are generally labeled with a histidine or biotin tail for linking to a functionalized surface rather than by direct chemisorption for subsequent immunoassay/immunosensing. In an alternative approach, the production of $F(ab')_2$ and Fab fragments using enzymatic proteolysis is a well established technique.[8]

Fab fragments can be directly generated using thiol proteases such as papain or ficin or, alternatively, $F(ab')_2$ fragments can be generated using bromelain, pepsin, or ficin, and the disulfide hinge is subsequently cleaved using a reducing agent, generating Fab fragments.[9,10] Pepsin is not applicable to all antibody types (e.g., it cannot be used for mouse IgG1 subclasses), and the low pH required for pepsin digestion can destroy antibodies.[11] Bromelain or ficin provide significantly higher yields than pepsin[12] and with more rapid and reproducible digestion.[13] The $F(ab')_2$ fragments are dimeric structures of two Fab units linked

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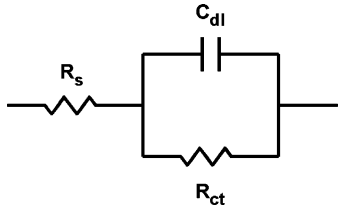
**Scheme 1. Equivalent Circuit Used to Model the Impedance Data[a]**




[a] $R_s$, solution resistance; $R_{ct}$, resistance to charge tranfer; $C_{dl}$, double layer capacitance.

together by a disulfide bridge that can be cleaved generating two Fab fragments with active thiol groups, which are located on the opposite side of the molecule with respect to the binding site.[14] These thiol groups can interact with gold surfaces leading to a monolayer of Fab fragments displaying a highly controlled orientation that is expected to maximize their antigen-binding efficiency with a concomitant increase in sensitivity and selectivity[15,16] when compared to randomly immobilized whole antibodies (Figure 1).[17] Lu et al. have demonstrated that the antigen binding activity of the Fab fragments immobilized in the oriented form on derivatized silica surfaces is 2.7 times higher than that of the random form.[15] Vikholm-Lundin[18] reported on a generic platform where the spaces in between chemisorbed Fab fragments are filled with the disulfide bearing polymer of *N*-[tris(hydroxyl-methyl)methyl]-acrylamide, resulting in a marked decrease in nonspecific binding. The same group went on to apply the developed platform to the detection of C-reactive protein, comparing $F(ab')_2$ and Fab immunocapture layers, with a 5-fold improvement in specific binding observed with the Fab monolayer.[19] The self-assembly of Fab onto a gold surface, followed by surface plasmon resonance transduction was applied to the detection of insulin,[20] with another report detailing the detection of differentiated leukocyte antigens for immunophenotyping of acute leukemia via the direct adsorption of Fab fragments onto gold nanoparticles with piezoelectric transduction.[21] In last two examples, the authors did not compare their approach with a full length antibody strategy.

In some cases, the use of $F(ab')_2$ fragments has resulted in lower detection limits as compared to whole antibodies.[22] There have also been reports of the exploitation of Fab fragments, but without taking direct advantage of the ordered monolayer that can be formed via the direct chemisorption of the thiolated Fab onto a gold surface, but rather focusing on antibody orientation as a means of increasing sensitivity. Examples of this are the immobilization of biotinylated antiatrazine Fab on neutravidin modified gold electrodes[23,24] for the detection of atrazine and monobiotinylated Fab against human chorionic gonadotropin and using surface plasmon fluorescence measurements, the biotin-Fab achieved a detection limit of $6 \times 10^{-13}$ M as compared to $4 \times 10^{-12}$ M when biotinylated whole antibody was used.[25] Additionally, exchange reactions between disulfide-terminated SAMs and thiolated Fab fragments have also been employed to generate sensor surfaces with well oriented Fab fragments.[15−17,26] However, the preferred method for Fab immobilization is the spontaneous adsorption of Fab motifs on gold, giving rise to surfaces of higher epitope density, high antigen-binding constants, and operational stability or adhesion.[27−29]

Celiac disease (CD) is a condition associated with the ingestion by susceptible individuals of gluten from wheat, barley, rye, and oats triggered causing histological changes in the small intestine mucosa and leading to a malabsorption syndrome.[30,31] CD affects possibly 1:100 people in Northern Europe and Northern America,[32,33] and symptoms revert when a strict gluten-free diet is established, being the only treatment available thus far. Therefore, accurate assays for detecting gluten in foodstuffs are mandatory. The new benchmark recently approved by Codex Alimentarius (July 2008) states that "gluten-free" foods may not exceed 20 ppm of gluten, while those with a gluten content to a level between 20 and 100 ppm may be referred to as "low gluten" or "reduced gluten".

Industries generating gluten-free foods have a definitive need for on site and easy to use gluten assays so that incoming raw materials and possible gluten contamination throughout the production process can be rapidly tested. Biosensors could provide a rapid and convenient alternative to conventional analytical methods for monitoring substances under interest in various application fields. Our group has recently reported an electrochemical immunosensor for the detection of gliadin with a detection limit of 5.5 ng/mL which exploits a gold electrode functionalized with a dithiol self-assembled monolayer. This sensor was based on an antibody, coined CDC5, which was raised against the putative immunodominant celiac disease toxic epitope of α-gliadin, 56−75[34] and applied to the analysis of gliadin in real food samples.[35]

In this paper, we exploit the spontaneous self-assembly of CDC5 Fab fragments on gold surfaces for the construction of a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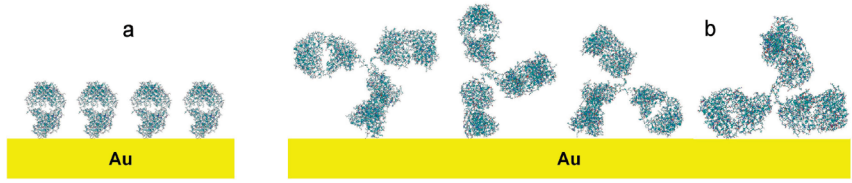


**Figure 1.** Comparison of oriented disposition of F(ab) fragments on the gold surface (left) with randomly oriented SATA-modified antibody (right).

sandwich electrochemical immunosensor for gliadin and compare its performance with the corresponding whole antibody in which thiol groups have been selectively introduced using the *N*-hydroxysuccinimide ester of *S*-acetylthioacetic acid (SATA). Surface modification and antigen affinities were studied by surface plasmon resonance (SPR), and the electrochemical assay was optimized in terms of deposition and incubation times. The immunosensor we report herein is highly sensitive, easy, and rapid to prepare, with a lower assay time and improved detection limit as compared to our previous reported method,[35] with minimal requirement of operator manipulation.

## EXPERIMENTAL SECTION

**Chemicals, Materials, and Instrumentation.** *Materials.* The Prolamin Working Group (PWG, martin.stern@med.uniuebingen.de) provided a gliadin preparation[36] to be used as a basis for standardizing the analysis and detection of gliadin. Monoclonal antigliadin CDC5 antibody was kindly provided by H. J. Ellis and P. J. Ciclitira.[37] Monoclonal antigliadin horseradish peroxidase conjugate was used as received. 1-(Mercaptoundec-11-yl)-tetra-(ethyleneglycol) (PEG) was purchased from Aldrich. Antibody $F(ab)_2$ and Fab fragments of CDC5 were prepared according to a method provided by Fujirebio Diagnostics AB. Gliadin stock solutions were freshly prepared in PBS-Tween containing 60% (v/v) ethanol and diluted in the appropriate buffer. All other reagents were of analytical grade and used without further purification.

*Electrochemical Instrumentation.* All the electrochemical measurements were performed using a PGSTAT12 potentiostat (Autolab, The Netherlands) controlled with the General Purpose Electrochemical System (GPES) software, with built-in frequency response analyzer FRA2 module. The immunosensor consisted in a three electrode cell composed of a modified Au (BAS model MF-2014, 1.6 mm diameter) as a working, Pt wire as a counter (BAS model MW-1032), and Ag/AgCl-3 M NaCl as a reference electrode (CH Instruments., model CHI111) immersed in 5 mL of the corresponding supporting electrolyte. The working surface was cleaned as reported previously.[35]

Faradaic impedimetric measurements were carried out in 0.1 M phosphate buffer saline (PBS) (pH 7.4) containing 1 mM $Fe(CN)_6^{3-}$ and 1 mM $Fe(CN)_6^{4-}$ within the frequency range of 0.1 Hz−100 kHz at a bias potential of +0.22 V and an ac amplitude of 5 mV. The nonfaradaic measurements were carried out in PBS (pH 7.4) at 0.0 V and an ac amplitude of 5 mV. The equivalent circuit used to model the impedance data is shown in Scheme 1. All amperometric measurements were carried out at a fixed potential (−0.2 V) under continuous stirring at 350 rpm.

*SPR Instrumentation.* A Biacore3000 SPR instrument was used for all the SPR studies. It was operated at a constant temperature of 20 °C. A gold (Au) sensor chip was used as a solid support for immobilization of CDC5-Fab and CDC5-SH. The data were evaluated with the Biacore3000 control software version 4.1.

**Preparation and Characterization of Antibody Fragments.** *Preparation of F(ab′)$_2$ Fragments.* CDC5 antibody was purified by YM 100 kDa microcon and washed four times with 0.15 M NaCl. To 500 μL of the purified CDC5 antibody (1 mg/mL), 50 μL of 0.5 M Tris buffer containing 50 mM EDTA (pH 7) was added. To the previous mixture, 50 μL (10 mg/mL) of bromelain solution in PBS (pH 7.2) was added. The mixture was incubated at 37 °C overnight. The produced $F(ab')_2$ fragments were separated with a Sephacryl S-100 column using 0.15 M NaCl as the eluting agent. The fractions with maximum absorbance at 280 nm were concentrated using a YM10 KDa microcon, washed several times with PBS (pH 7.4), and stored at −20 °C.

*Preparation and Characterization of Fab Fragments (CDC5-Fab).* Fab fragments were always freshly prepared before use, using 1500 μL of 10 mM cysteine in phosphate buffer (pH 10.3) treated with 60 μL (0.483 mg/mL) of $F(ab')_2$ added. The mixture was incubated for 2 h at room temperature under gentle shaking, and the excess of cysteine was removed using a YM 10 kDa microcon. The obtained Fab fragments were washed four times with PBS (pH 7.4) and spectrophotometrically quantified at 280 nm, and the number of free sulfhydryl groups was determined using Ellman's reagent using a molar absorptivity of 14 150 $M^{-1}$ $cm^{-1}$ at 412 nm.[38]

CDC5-Fab were characterized by nonreducing sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis (SDS-PAGE, 12% acrylamide in TRIS-glycine gel).[39] A precision protein standard marker (Bio-Rad, Nazareth Eke, Belgium) was used as a molecular weight reference. The protein fragments were stained with Coomassie brilliant blue R250 (Sigma, Spain).

*Modification of CDC5 with Thiol Groups (CDC5-SH).* To 100 μL of CDC5 (4 mg/mL), 5.4 μL of SATA (1 mg/0.9 mL in DMSO) was added and the mixture was incubated for 30 min at room temperature under gentle shaking. Then, 100 μL of deacetylation solution (0.5 M hydroxylamine hydrochloride containing 25 mM EDTA in PBS, pH 7.4) was added, and the mixture was incubated at room temperature for 2 h under gentle shaking. The produced SATA modified antibody (CDC5-SH) was dialyzed in PBS (pH 7.4) overnight and concentrated using a YM 30 KD microcon. The

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final concentration of CDC5-SH was determined spectrophotometrically at 280 nm and then stored at −20 °C.

*Enzyme Linked Immunosorbent Assay (ELISA) Characterization of the Prepared Fab Fragments and the SATA Modified Antibody.* The ELISA analysis was performed as reported previously[35] with the following modifications. The capture layer was formed using 100 μg/mL of CDC5-Fab or CDC5-SH in carbonate buffer, pH 9.6. In the immunorecognition step, 50 μL of 1 μg/mL PWG in PBS-Tween prepared from PWG stock solution prepared in 60% v/v ethanol in PBS buffer (pH 7.4, 0.1 M) was added for each well. Detection was carried out with 100 μg/mL of commercial HRP-labeled monoclonal antigliadin antibody. The analysis was carried out in triplicate.

**Surface Chemistry.** *Optimization of Time Required for SAM Formation.* Clean Au electrodes were modified by direct immersion in 100 μg/mL CDC5-Fab or CDC5-SH in PBS solution (pH 7.4), and the time dependence of impedance variations was recorded for selected immobilization times. CDC5-Fab immobilization was monitored between 0 and 60 min by nonfaradaic impedance while faradaic responses were recorded between 0 and 180 min for CDC5-SH. $R_{ct}$ values were calculated for different exposure times using the Autolab impedance analysis software.

*Immobilization of CDC5-Fab and CDC5-SH on SPR Au Chips.* A 100 μg/mL solution of CDC5-Fab and CDC5-SH in PBS (pH 7.4) was injected onto a clean Biacore SIA Au chip during the immobilization phase until a saturation level was obtained. CDC5-Fab or CDC5-SH (100 μg/mL) in PBS pH 7.4 were injected directly over the sensor chip for 20 min, at a flow rate of 5 μL/min. The free space of the modified gold chip was then backfilled with 25 μL of 1 mM PEG in PBS-Tween.

*Impedimetric Study of the Stability and Affinity of the Antibody Fragments with Time.* Clean electrodes were immersed in 100 μg/mL of CDC5-Fab or CDC5-SH in PBS (pH 7.4) for 15 min. The modified Au electrode was then stored in PBS (pH 7.4) at 4 °C. The nonfaradaic EIS was recorded weekly during 2 months in PBS for the modified electrodes with Fab fragments at a bias potential of 0.0 V (frequency range, 0.1 Hz−100 kHz; ac amplitude, 5 mV), and log $Z'$ was calculated at a fixed frequency (1.1 Hz).

*Nonspecific Binding Study.* First, the EIS response was recorded before and after incubation of a pure SAM of PEG, which is used as the backfiller with the Fab fragments, with 30 μg/mL PWG for 30 min.

Different conditions were used in the amperometric study at fixed potential (−0.2 V) in PBS (0.1 M, pH 7.4) solution with two consecutive injections of 2 mM $H_2O_2$ and 2 mM HQ, using 30 μg/mL PWG and 100 μg/mL antigliadin-HRP: (i) PEG-modified electrodes were first immersed in 0.05 M PBS-Tween for 30 min followed by incubation with antigliadin-HRP in the absence and in the presence of PWG and (ii) CDC5-Fab modified electrodes were first backfilled with PEG followed by immersion in 0.05 M PBS-Tween for 30 min followed by incubation with antigliadin-HRP.

**Cross-Reactivity Studies.** *Amperometric Detection.* Amperometric measurements were carried out using the same conditions explained in the previous section. The clean gold electrodes were modified with different antibody Fab fragments, by immersion in a 100 μg/mL solution of CDC5-Fab or CEA-Fab (as nonspecific antibody) in PBS pH 7.4 for 15 min. The remaining free space of the gold surfaces were then blocked with 1 mM PEG for 10 min, followed by incubation in 0.05 M PBS-Tween for 30 min. Subsequently, 30 ng/mL of the corresponding protein (CEA or PWG, respectively) were incubated for 30 min. After incubation with 100 μg/mL of antigliadin HRP for another 30 min, the amperometric response was recorded.

*Surface Plasmon Resonance.* Different nonspecific analytes (at 5 μg/mL) such as recombinant high molecular weight glutenin (r-HMW-Glu), CEA, and PSA (prostate specific antigen) were injected during 6 min to a CDC5-Fab and CDC5-SH modified Au chip, and the SPR responses were measured. Before each measurement, three regeneration steps were carried out by injecting 20 μL of glycine (10 mM, pH 2.2) for 1 min. Specific signals were recorded using 5 μg/mL of PWG or gliadin (from Sigma) dissolved in PBS pH 7.4.

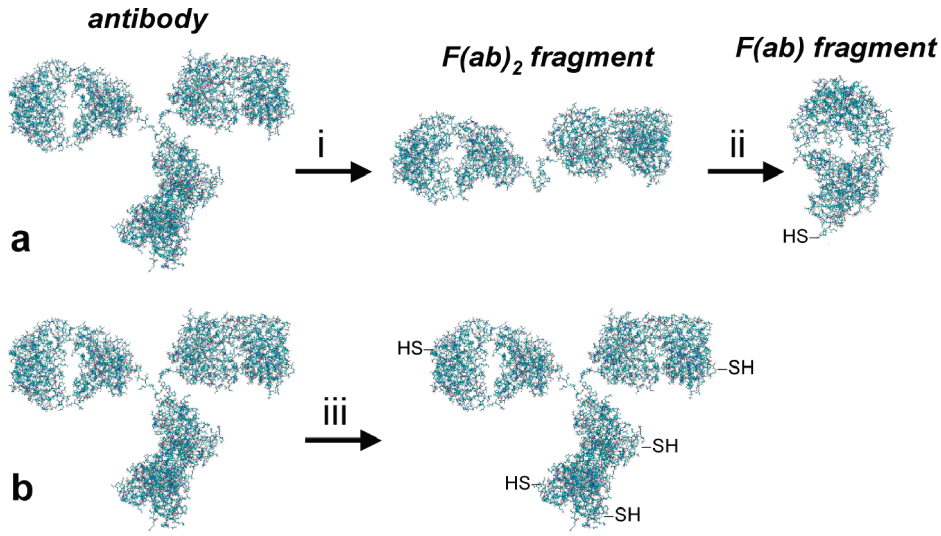
**Immunosensor Construction and Calibration.** *Formation and Characterization of the Sandwich Assay.* SAM formation of the Fab and CDC5-SH were carried out at their corresponding optimal times and following blocking with 1 mM PEG, and the modified surfaces were incubated in PBS-Tween (0.05 M, pH 7.4) solution for 30 min. The electrodes were then exposed to different concentrations of PWG (1−20 μg/mL) in PBS-Tween solution for 10 min. Each building step during the fabrication of the biosensor was characterized by CV and EIS using $Fe(CN)_6^{3-/4-}$ in PBS solution as an electroactive marker. In the case of the Fab modified surfaces, an additional amperometric measurement were carried out at different concentrations of PWG (5−30 ng/mL) after an additional layer of labeled antibody was incubated with 100 μg/mL antigliadin-HRP in PBS for 30 min at room temperature under stirring conditions.

*Optimization of Incubation Times.* The incubation time of the specific recognition between PWG and the immobilized Fab was optimized as follows. The modified electrodes with Fab were blocked with PEG and PBS-Tween as described in the previous section. The electrodes were then incubated with 30 ng/mL PWG in PBS-Tween solution at 12 different incubation times (between 0 and 45 min), and the nonfaradaic EIS was measured at each incubation time in PBS (pH 7.4) in the frequency range of 0.1 Hz−100 kHz at a bias potential of 0.0 V and 5 mV amplitude. The influence of the incubation time on the logarithm of impedance of the real part at a fixed frequency (0.0383 Hz) was investigated.

For the optimization of incubation with labeled antigliadin-HRP in the sandwich assay, the Fab modified gold surface was blocked with PEG and PBS-Tween, followed by incubation with 10 ng/mL PWG in PBS-Tween. The electrodes were then incubated with 100 μg/mL mAb-HRP solution at different incubation times (between 0 and 60 min). The amperometric responses were then recorded at a fixed potential (−0.2 V vs Ag/AgCl) in PBS (0.1 M, pH 7.4) in the absence and presence of 2 mM $H_2O_2$ and 2 mM HQ.

**Electrochemical Detection of PWG.** *Impedimetric Detection.* Detection of PWG on the modified electrodes was carried out using EIS and amperometry. EIS measurements were carried out in the presence of the redox probe 1 mM $Fe(CN)_6^{3-/4-}$ in PBS (0.1 M, pH 7.4) in the frequency range of 0.1 Hz−100 kHz at a bias potential of +0.22 V and 5 mV amplitude before and after binding of different concentrations of PWG (5, 10, 15, 20, 25, and 30 ng/mL). The obtained spectra were fitted to an

**Scheme 2. Preparation of (a) Fab Fragments (i, Bromelain; ii, Cysteine) and (b) SATA-Modified Antibody (iii, SATA, Hydrolysis)**




equivalent electrical circuit using the Autolab impedance analysis software.

*Amperometric Detection.* Clean gold electrodes were modified with Fab as previously explained. The free surfaces were then blocked with PEG and PBS-Tween, followed by incubation of the modified electrodes in different concentrations of PWG (5, 10, 15, 20, 25, and 30 ng/mL) in PBS-Tween for 10 min. Followed by a final incubation with 100 $\mu$g/mL of mAb-HRP for 30 min, the amperometric response of the antibody-modified surface was recorded in PBS before and after the two consecutive injections of 2 mM $H_2O_2$ and 2 mM HQ. The limit of detection was calculated by measuring the sensor response in triplicate at zero analyte concentration, calculating 3× the standard deviation of these measurements and interpolating this value in the linear calibration curve.

## RESULTS AND DISCUSSION

**Preparation of CDC5 Fragments and Characterization Using Nonreducing Electrophoresis (SDS-PAGE).** Scheme 2 shows the strategy employed for the preparation of Fab fragments and for the introduction of thiol groups in CDC5 using SATA. Whole CDC5 antibody was first digested with bromelain to obtain the corresponding $(Fab')_2$ after chromatographic purification. Purified $(Fab')_2$ fragments kept in PBS (0.1 M, pH 7.4) at −20 °C were stable for more than 6 months, which allowed the preparation of a large batch of $(Fab')_2$ from which Fab fragments were generated when needed.

The fragmentation process of CDC5 and the chemical reductions of the produced mouse mAb CDC5 $(Fab')_2$ to Fab fragments using cysteine as a reducing agent were characterized by nonreducing electrophoresis. The mouse mAb CDC5 $(Fab')_2$ were successfully reduced to Fab fragments. Figure 2 illustrates the SDS-PAGE (12%) analysis of the full length CDC5 antibody and their $(Fab')_2$ and Fab fragments under the nonreducing conditions. Lane 1 of Figure 2 clearly shows one major band around 40 kDa, indicating that a significant amount of Fab was produced. In lane 1, there were no significant bands less than 40 kDa, indicating that the produced Fab fragments were not further reduced into smaller inactive peptides. Lanes 2 and 3 present major bands around 120 and 150 kDa, respectively, which can be attributed to the prepared $(Fab')_2$ fragments and whole CDC5 antibody, respectively. In Lane 2, a few faint bands were visible around 40 kDa, indicating that some Fab was produced due to further reduction of $F(ab')_2$ during the enzymatic fragmentation process, which is expected. The successful cysteine-mediated reduction of CDC5 $F(ab')_2$ to Fab fragments was also demonstrated using Ellman's reagent. The concentration of free sulfhydryl groups after reduction was 1.697 $\mu$M, a value 2.35-fold higher than that of $F(ab')_2$ (0.722 $\mu$M). This indicates a successful reduction of the $F(ab')_2$ fragments. The biorecognition affinity of the prepared Fab and modified CDC5-SH was further studied by ELISA. The ELISA results indicated that the Fab and CDC5-SH are still active and easily recognize PWG, and the preparation processes for both biocomponents does not affect their activity.

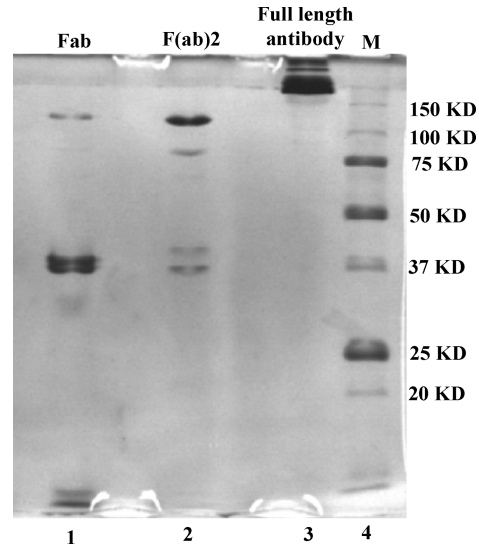



**Figure 2.** SDS-PAGE analysis (12% gel; nonreducing conditions) of the full length CDC5 antibody and their $F(ab)_2$ and Fab fragments: (a) lane 1, cysteine reduction of $F(ab)_2$ to F(ab) fragments; lane 2, $F(ab)_2$ fragments (obtained by enzymatic bromelain fragmentation); lane 3, full-length CDC5; and lane 4, molecular weight marker.

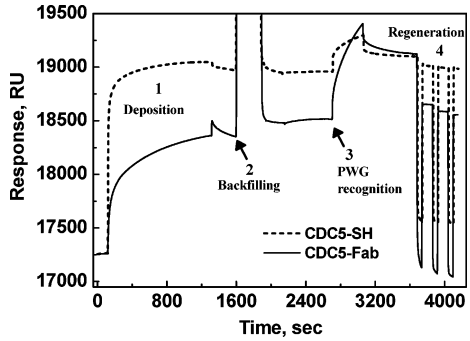



**Figure 3.** SPR sensorgrams obtained at CDC5-Fab (—) and CDC5-SH (- - -) modified Au chips: (1) SAM formation, (2) blocking step with 1 mM PEG and PBS-Tween, (3) injection of 5 $\mu$g/mL PWG for antigen binding, and (4) regeneration with three pulses of glycine (10 mM, pH 2.2).

**Table 1. SPR Responses (ΔRU) Values for the Immobilization of CDC5-Fab and CDC5-SH and the Corresponding Binding with 30 ng/mL PWG**

| | ΔRU | |
|---|---|---|
| surface | immobilization | PWG recognition |
| CDC5-Fab | 1091 | 606 |
| CDC5-SH | 1708 | 136 |

**Surface Chemistry.** *Surface Plasmon Resonance Study.* The deposition of CDC5-Fab and CDC5-SH onto Au surfaces was primarily studied using SPR. Figure 3 represents the SPR sensograms of consecutive immobilization, blocking, and recognition steps for both CDC5 forms, and Table 1 details the RU values obtained for the immobilization and recognition steps. The surface mass density obtained for CDC5-Fab and CDC5-SH after the immobilization step were 109 and 171 ng/cm$^2$, respectively. These values translate into molar concentrations of 2.72 and 0.85 pmol/cm$^2$, considering a molecular weight of 40 kDa for CDC5-Fab and 200 kDa for CDC5-SH as revealed by SDS-PAGE. Hence, an antigenic CDC5-Fab monolayer contains about 1.6 times more recognition epitopes per square centimeter than a monolayer of full antibodies (considering two binding sites per whole antibody molecule). It has been recently estimated that a full monolayer contains between 130 and 650 ng/cm$^2$ of whole antibodies of approximate size $15 \times 15 \times 3$ nm$^3$) and 260−700 ng/cm$^2$ of Fab fragments ($8.2 \times 5.0 \times 3.8$ nm$^3$).[17] Therefore the observed RU values indicate that an incomplete monolayer was obtained with the Fab fragments in contrast with the whole antibody.

SPR was also used also to assess antigen (PWG) binding to the immobilized full-length antibodies and fragments. Figure 3 shows the antigen binding responses on the covalently immobilized CDC5-Fab and CDC5-SH. The Fab fragment showed the highest antigen binding response (606 RU for 5 $\mu$g/mL of PWG) (Table1) in comparison to the 136 RU for immobilized CDC5-SH, i.e., a decrease of about 77% antigen recognition ability was observed for CDC5-SH. This is presumably due to the fact that the modification process of the full length antibody with SATA reagent converts the free amines distributed throughout the antibody structure to sulfhydryl groups, resulting in a randomly oriented immobilization of CDC5-SH which leads to a decrease

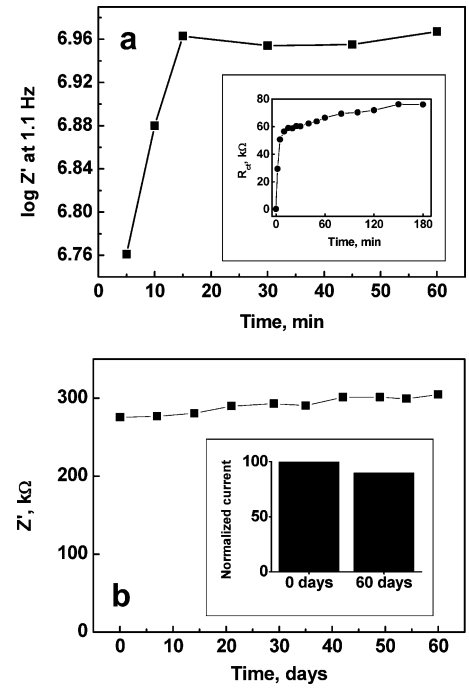



**Figure 4.** (a) Impedance variations obtained upon formation of SAMs of CDC5-Fab (■) and CDC5-SH (●). (b) Time dependence of the impedance response obtained for Au electrodes immobilized CDC5-Fab stored in PBS (0.1 M, pH 7.4) at 4 °C. Inset: amperometric responses obtained to test the recognition ability of stored electrodes.

in antigen accessibility to the recognition epitope (see Figure 1). An additional factor could be the steric hindrance of the whole antibody onto the sensor surface, since it has larger dimensions ($15 \times 15 \times 3$ nm$^3$) and consequently consumes a larger space on the sensor surface, which can therefore negatively influence antigen binding efficiency as compared to the smaller receptor molecules (Fab) well oriented on Au which resulted in a higher number of accessible active epitopes.[27]

*Immobilization of CDC5-Fab and CDC5-SH on Gold Electrodes.* The immobilization of CDC5-Fab and CDC5-SH on the Au electrode was optimized in terms of time of formation and minimization of nonspecific interactions using impedance spectroscopy. Faradic impedance was used to follow CDC5-SH deposition, while the nonfaradic technique was used for CDC5-Fab since it gave a more sensitive response variation than faradic impedance. In both cases, the impedance response increased steadily with time (Figure 4a), reaching a saturation after 15 min for CDC5-Fab and 3 h for CDC5-SH. In both cases, the impedance increase does not account for multilayer formation since each point represents the constant impedance value obtained after repeated washings to remove physically adsorbed molecules. This difference in deposition time highlights the advantage of using Fab fragments for biosensor construction, where SAM formation is notably faster than that achievable using the whole antibody or chemical cross-linking to alkanethiol SAMs.[35]

*Stability of CDC5-Fab and CDC5-SH Modified Electrodes.* The stability of Fab-modified Au was tested by recording the nonfaradaic impedance variations weekly for 2 months for electrodes stored in PBS buffer at 4 °C (Figure 4b). The electrodes modified with CDC5-Fab showed a slight impedance increase at fixed frequency (1.1 Hz) in the first 2 weeks, remaining constant during

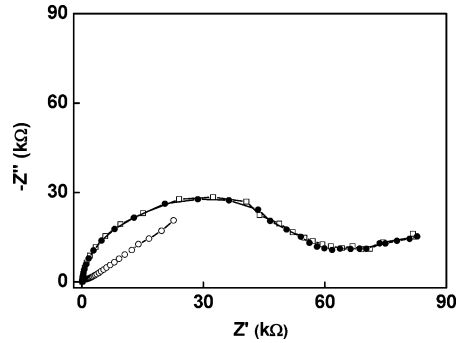



**Figure 5.** Complex impedance plots (in 1 mM $K_3Fe(CN)_6$ solution in PBS pH 7.4) recorded at bare Au (○), PEG modified Au covered with Tween 20 (●), and after incubation with 20 $\mu$g/mL PWG (□).

**Table 2. Amperometric Responses at Different Surfaces Corresponding to Nonspecific Binding and Cross-Reactivity**

| surface | $\Delta i$ (nA) | % NSB[1] |
|---|---|---|
| Au\|PEG&Tween\|30 ng/mL PWG | 0 | 0 |
| Au\|Fab (CDC5)\|30 ng/mL CEA | 7 | 4.4 |
| Au\|Fab (CEA)\|30 ng/mL PWG | 25 | 15.8 |
| Au\|Fab (CDC5)\|30 ng/mL PWG | 158 | |

[1] NSB = $100(\Delta i^{\text{nonanalyte}}/\Delta i^{\text{analyte}})$, where NSB is the degree of nonspecific binding and $\Delta i$ represents the change in the amperometric current responses before and after injection of hydroquinone.

the rest of the stability study. A similar behavior was obtained for the CDC5-SH modified electrodes (not shown) indicating a stable antibody layer was attached to the Au surface. After 2 months of storage, <10% loss of antigen recognition activity was observed for the Fab modified Au electrodes as revealed by amperometric measurements, indicating the high stability of the immobilized Fab fragments.

*Evaluation of Nonspecific Interactions.* In immunosensors, it is critical to avoid the interaction of the target analyte or labeled reporter antibody with the surface (nonspecific binding) while maximizing the recognition by the immobilized biocomponent (specific binding). In this study (1-mercaptoundec-11-yl)-tetra-(ethyleneglycol) and Tween 20 were used to prevent nonspecific adsorption. EIS and amperometry have been used to evaluate the occurrence of nonspecific interactions on SAMs of PEG further blocked with Tween (0.05% w/v), as well as gold surfaces modified with specific and nonspecific Fab fragments against gliadin, using CEA as a nonspecific analyte.

Impedance measurements (Figure 5) shows the occurrence of a very low degree (<3%) of nonspecific adsorption on a bare SAM of PEG when exposed to 20 $\mu$g/mL of PWG for 30 min. Similarly, amperometric measurements (Table 2) carried out using 30 ng/mL PWG are in agreement with the EIS results. In a situation more comparable to the final immunosensor format, where antibody Fab fragments are immobilized on gold and incubated with a nonspecific antigen (carcinoembryonic antigen, CEA), the nonspecific binding value was 4.4%.

In addition, the SPR responses corresponding to the binding of 5 $\mu$g/mL of the nonspecific analytes recombinant high-molecular weight peptic triptic gluten (r-HMW PTGlu), carcino-embryonic antigen (CEA), and prostate specific antigen (PSA) with the immobilized Fab molecules resulted in a very small

**Table 3. Observed RU Values for CDC5-Fab and CDC5-SH Modified Au Surface After Incubation with Different Nonspecific Analytes**

| | ΔRU | |
|---|---|---|
| sample | CDC5-Fab (% NSB)[a] | CDC5-SH (% NSB)[a] |
| Sigma gliadin | 376.8 (62.12) | 0 (0) |
| r-HMW PTGlu | 8.2 (1.35) | 6.7 (4.9) |
| CEA | 5.2 (0.86) | |
| PSA | 6.2 (1.02) | |

[a] NSB = $100(\text{RU}^{\text{nonanalyte}}/\text{RU}^{\text{analyte}})$, where NSB is the degree of nonspecific binding and ΔRU represents the change in RU values before and after incubation of the surface with 5 $\mu$g/mL of the selected analyte.

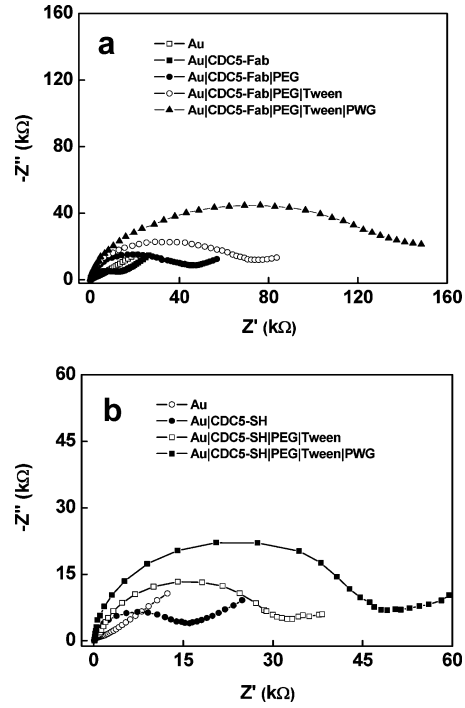



**Figure 6.** Complex impedance plots (in 1 mM $K_3Fe(CN)_6$ solution in PBS pH 7.4) recorded at a SAM of CDC5-Fab (a) and CDC5-SH (b) modified Au electrodes for the sequential immobilization steps of the immunosensors.

increase in SPR responses of 8.2, 5.2, and 6.2 RUs, respectively (Table 3), which represents 0.86−1.35% of the signals observed for the specific antigen. These results indicating that the Fab modified Au surfaces are highly specific to PWG antigen. Moreover, the presence of nonspecific proteins in the detection media does not interfere with PWG. Therefore, this immunosensor could have a wide range of applications for the detection of PWG in real samples.

**Electrochemical Detection of Gliadin.** *Immunosensor Construction.* Figure 6 represents the impedance spectra of the successive building steps leading to the construction of the gliadin immunosensors using CDC5-Fab and CDC5-SH. The impedance spectra of each building step of both biosensors consisted of a semicircle whose extrapolation to the *x* axis gives the charge-transfer resistance ($R_{ct}$) of each layer. This value is closely related with the hindering of the flux of the redox couple toward the electrode surface and, therefore, addition of suc-

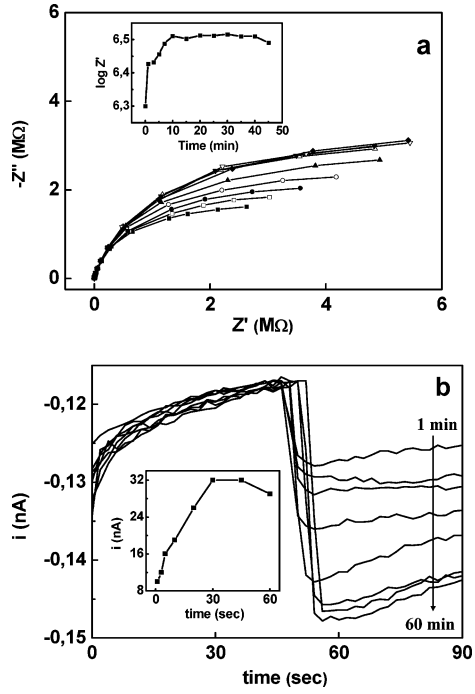



**Figure 7.** (a) Nyquist plots of the nonfaradaic impedance in PBS (pH 7.4) at CDC5-Fab modified electrodes after interaction with 30 ng/mL PWG at incubation times: 0(■), 1(□), 3(●), 5(○), 7(▲), 10(Δ), 15(▼), 20(▽), and 25(◆) minutes. Inset: the time dependence of log *Z*′ 0.0383 Hz. (b) Amperometric responses at CDC5-Fab modified immunosensor in PBS (pH 7.4) at different incubation times with 10 ng/mL R5-HRP (0, 1, 3, 5, 10, 20, 30, 45, and 60 min) Inset: time dependence of current responses.

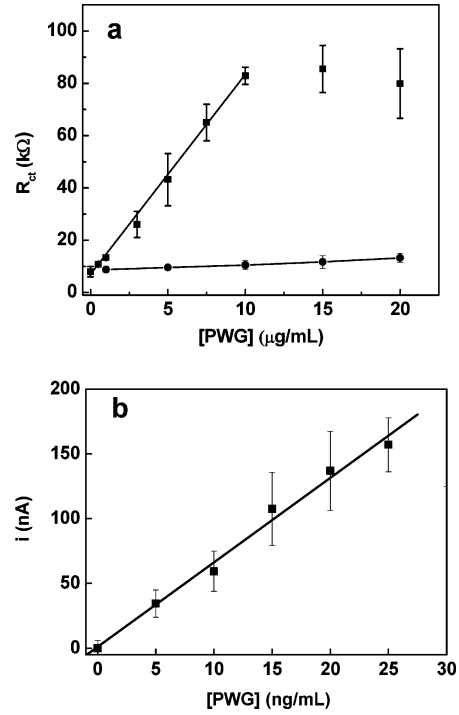



**Figure 8.** (a) Impedimetric calibration curves for CDC5-Fab (□) and CDC5-SH (●) and (b) amperometric calibration plot at the CDC5-Fab modified immunosensor.

cessive layers increases this value. As shown in Figure 6, the diameter of the semicircle was increased with the binding of each corresponding protein layer, providing evidence of the successful formation of the immunocomplex.

*Optimization of Incubation Times of Recognition and Detection Steps for a Fab-Based Biosensor.* Since the analytical performance of the immunosensor is directly related to its ability to recognize the target PWG, it is essential to optimize the time required for the complete reaction between the antigen and the immobilized biorecognition probe. Figure 7a shows the influence of incubation time (0−45 min) on the impedance response of the system in the presence of 30 ng/mL PWG. The log *Z*′ values improved with increasing incubation time and then maintained the maximum value after 10 min incubation. Therefore, this incubation time was used in further experiments.

The incubation time required for the complete interaction between the detecting conjugate (antigliadin/HRP) and the CDC5-Fab/PWG complex was studied using amperometry (Figure 7b). After modification of the Au surfaces with CDC5-Fab fragments and blocking and incubation with 30 ng/mL PWG for 10 min, the system was exposed to 100 $\mu$g/mL mAb-HRP at different incubation times (0−60 min). The amperometric responses were then recorded reaching saturation after 30 min incubation. Therefore, this incubation time was used for the amperometric detection of PWG.

*Electrochemical Detection of PWG.* The impedance technique allows a fast, direct, labeless detection of targets. We have investigated the possibility of using this technique for PWG detection using the constructed immunosensors. Resistance and capacitance values were obtained from simulation using a $R_s(R_{ct}C_{dl})$ equivalent circuit (Scheme 1), where $C_{dl}$ is the double layer capacitance, $R_s$ is the solution resistance, and $R_{ct}$ is the charge transfer resistance of the circuit. Figure 8a shows the calibration plots obtained using both CDC5-Fab and CDC5-SH modified electrodes. At higher concentration levels of PWG ($\mu$g/mL), $R_{ct}$ values were linearly correlatable with PWG concentrations (1−20 $\mu$g/mL) with an LOD of 0.42 $\mu$g/mL. Double layer capacitance values obtained from simulation decreased with increasing PWG concentration with a sensitivity of 15 nF/$\mu$g mL$^{-1}$ and a LOD of 8 $\mu$g/mL (data not shown); hence, in this case, the use of capacitance values gives a higher detection limit than the variations of $R_{ct}$.

At lower concentration levels of PWG (nanograms per milliliter), the impedance technique was not sensitive enough to detect the minimal changes in the biointerface structure and not suitable for gliadin detection with the CDC5-Fab fragments. However, with using CDC5-SH, the $R_{ct}$ variations were substantially less sensitive, which may be due to noncontrolled orientations of the modified antibody.

Detection of very low PWG concentrations levels (nanograms per milliliter) was achieved with the CDC5-Fab biosensor using amperometric detection. Figure 8b shows the amperometric calibration curve, in which the current response showed a linear relationship with the concentration of PWG over the range of 5−30 ng/mL with a limit of detection of 3.29 ng/mL. This value is 2 times lower than that previously obtained by our group using a CDC5 covalently immobilized on a bipodal alkanethiol based self-assembled monolayer[35] and highlights the advantage of using antibody fragments for the detection of lower levels of target.

## CONCLUSIONS

An electrochemical gliadin immunosensor based on the spontaneous adsorption of antigliadin Fab fragments on Au surfaces has been developed and compared with whole antibody modified electrodes. SPR results revealed that the antigenic CDC5-Fab monolayer contains about 3 times more epitopes per square centimeter than CDC5-SH. CDC5-Fab formed a stable monolayer on gold after 15 min and retained >90% of antigen recognition ability after 2 month of storage at 4 °C. Detection of gliadin of Fab modified electrodes was evaluated by impedance and amperometry. Labeless impedimetric detection achieved a LOD of 0.42 $\mu$g/mL while the amperometric immunosensor based on Fab fragments showed a highly sensitive response with an LOD of 3.29 ng/mL. The Fab based immunosensor offer the advantages of being highly sensitive, easy, and rapid to prepare, with a low assay time.

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