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Electrochemical Immunosensor for Detection of Celiac Disease Toxic Gliadin in Foodstuff

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Celiac disease is a gluten-sensitive enteropathy that affects as much as 1% of the population. Patients with celiac disease should maintain a lifelong gluten-free diet, in order to avoid serious complications and consequences. It is essential to have methods of analysis to reliably control the contents of gluten-free foods, and there is a definitive need for an assay that is easy to use, and can be used on site, to facilitate the rapid testing of incoming raw materials or monitoring for gluten contamination, by industries generating gluten-free foods. Here, we report on the development of an electrochemical immunosensor exploiting an antibody raised against the putative immunodominant celiac disease epitope, for the measurement of gliadin content and potential celiac toxicity of a foodstuff. To develop the gliadin immunosensor, we explored the use of two surface chemistries, based on the use of dithiols, 22-(3,5-bis((6-mercaptohexyl)oxy)phenyl)-3,6,9,12,15,18,21-heptaoxadocosanoic acid (1) and 1,2-dithiolane-3-pentanoic acid (thioctic acid) (2), for anchoring of the capture antibody. The different surface chemistries were evaluated in terms of time required for formation of self-assembled monolayers, stability, susceptibility to nonspecific binding, reproducibility, and sensitivity. The thioctic acid self-assembled monolayer took more than 100 h to attain a stable surface and rapidly destabilized following functionalization with capture antibody, while the heptaoxadocosanoic acid surface rapidly formed (less than 3 h) and was stable for at least 5 days, stored at room temperature, following antibody immobilization. Both surface chemistries gave rise to highly sensitive immunosensors, with detection limits of 5.5 and 11.6 ng/mL being obtained for 1 and 2, respectively, with nonspecific binding of just 2.7% of the specific signal attained. The immunosensors were extremely reproducible, with RSD of 5.2 and 6.75% obtained for 1 and 2 (n = 5, 30 ng/mL), respectively. Finally, the immunosensor was applied to the analysis of commercial gluten-free and gluten-containing raw and processed foodstuffs, and excellent correlation achieved when its performance compared to that of an ELISA.

Gluten sensitivity, manifesting as celiac disease (CD), affects possibly 1:100 people in Northern Europe and North America.[1,2] Celiac disease is an inflammatory disease of the upper small intestine, results from gluten ingestion in genetically susceptible individuals, and is the only lifelong nutrient-induced enteropathy.[3,4] The small bowel abnormalities are reversed on withdrawal of gluten from the diet. CD is a familial condition with ~10−15% of first degree relatives being similarly affected.[5]

Celiac disease, when untreated or poorly treated, that is with continued ingestion of gluten, leads to a large number of complications, [6,7] which can result in considerable morbidity and repeated hospital visits. In children, symptoms such as growth retardation among others are observed[8,9] and patients with osteoporosis have a higher prevelance of CD,[10] with bone mineral density in CD patients improving with a gluten-free diet.[11] Up to 50% of women with untreated celiac disease experience miscarriage, and long-term, silent undiagnosed celiac disease can lead to infertility.[12] The condition is also strongly associated with other autoimmune conditions.[13,14] Celiac disease sufferers who do not adhere to a strict gluten-free diet have an increased incidence of a fatal small intestinal lymphoma,[15,16] and the standardized mortality rate is twice that of the general population, non-Hodgkin lymphoma being the main cause of death.[17]

Dieterich and colleagues identified tissue transglutaminase as the autoantigen of celiac disease,[18] which was supported by a publication by Arentz-Hanson et al.,[19] the sequence identified was further backed up by Anderson et al., and it was later shown that

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a 19-mer peptide formed by the two overlapping peptides of Arentz-Hansen is indeed disease activating to the small intestines of celiac disease patients in vivo, causing the classic parameters of the condition.[21]

The development of legislation on levels of gluten permissible in foods labeled as gluten-free has been hampered by lack of a suitable assay system. The present standard has stood since 1981 (Codex Stan 118−1981) and is very nonspecific.[22,23] Codex Alimentarius have recently approved new standards for gluten-free foods (July 2008). The new benchmark states that foods labeled "gluten-free" may not exceed a gluten content of 20 ppm, while those with a gluten content to a level between 20 and 100 ppm may be called "low gluten" or "reduced gluten". A range of methods for the detection of gliadin have been reported, such as the use of gluten-specific PCR,[24,25] and SDS-PAGE in combination with immunoblotting, counter-immunoelectrophoresis, or mass spectroscopy and MALDI-TOF analysis.[26] High-performance liquid chromatography and capillary electrophoresis have been widely used to analyze prolamins in food,[27,28] and gel permeation high-performance liquid chromatography has been described for the quantitative determination of gliadin. More recent reports have looked at the use of flow cytometry detecting as low as 10 pg/mL levels of gliadin,[29] as well as the first report of a biosensor for gliadin detection, which exploited the use of a recombinant glutamine-binding protein.[30] The same group have gone on to report a fluorescence correlation spectroscopy assay, reporting a detection limit of 0.006 ppm, which would require huge dilution of extracts from gluten-free samples for detection.[31]

The most common method of measurement of gliadin is that of the enzyme-linked immunosorbent assay (ELISA). Early assays used whole gliadin as the immunogen to produce polyclonal antisera and gave rise to antibodies that were insufficiently specific, for example, giving spurious cross-reactivities with nontoxic maize.[32] Later, advantage was taken of monoclonal antibody technology to produce more precisely targeted reagents.[33] The first toxic sequence to be identified in vivo was A-gliadin 31−49,[34] and a monoclonal antibody has been raised against the celiac-toxic 19-mer A-gliadin peptide (LGQQQPFP-PQQPYPQPQPF) and used for detection of gliadin and gluten hydrolysates.[35,36]

Two commercial immunoassays are currently available. The first is based on a monoclonal antibody to the heat-stable ω-gliadin fraction;[37] however, measurement of this subfraction with extrapolation to total gliadin has theoretical errors of −44 to +80%.[38] The R5 method[39] has been proposed as the standard method of gluten analysis to back up the Draft Codex Standard (2003) on gluten-free foods, but this method weakly recognizes the immunodominant gliadin T cell stimulating epitope,[19,20] and measurement of this important epitope is highly pertinent. It can be argued that measurement of the immunodominant T cell epitope is the method more likely to give a true indication of the potential celiac toxicity of a foodstuff. Moreover, there is a definitive need for an assay that is easy to use and can be used on site, so that industries generating gluten-free foods can rapidly test incoming raw materials as well as checking for gluten contamination throughout the food production process.

Here, we report the use of an electrochemical immunosensor exploiting an antibody, coined CDC5, which was raised against the putative immunodominant celiac disease toxic epitope of α-gliadin, 56−75. Immunosensors offer the advantages of being easy to use, inexpensive, rapid, and in this particular case, importantly they can be used in situ allowing rapid assay turnaround time in labeled or labeless formats.[40,41] This facilitates punctual measurement of gliadin levels throughout a food production lifetime as well as control of raw materials. The immunosensor we report herein is highly sensitive, detecting low ppb levels of gliadin, and a reliable quantitative detection of gliadin content can be attained within 90 min, with minimal requirement of operator manipulation. Moreover, the immunosensor exploits a monoclonal antibody that can be correlated with celiac disease toxic gliadin, giving a correct indication of toxicity, rather than total gliadin content. Furthermore, the reported immunosensor is extremely reproducible, compatible with solvents required for extraction of gliadin from foodstuffs, and demonstrates excellent correlation with ELISA when applied to the detection of real samples.

EXPERIMENTAL SECTION

Chemicals and Materials. 1. Materials. Dithiol **1** (22-(3,5-bis((6-mercaptohexyl)oxy)phenyl)-3,6,9,12,15,18,21-heptaoxadocosanoic acid) was purchased from SensoPath Technologies (Bozeman, MT) and used as received. One mM stock solution

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was prepared in absolute ethanol, purged with argon, and kept at −20 °C when stored. The Prolamin Working Group (PWG, martin.stern@med.unituebingen.de) provided a gliadin preparation[42] to be used as a basis for standardizing the analysis and detection of gliadin. Monoclonal antigliadin CDC5 antibody[43] and rabbit polyclonal antigliadin[44] (PAb) were developed by Ellis and Ciclitira as previously reported. Goat antimouse IgG (Fc specific) alkaline phosphatase conjugate, (±)-1,2-dithiolane-3-pentanoic acid (lipoic acid, **2**), N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide (EDC), *N*-hydroxysuccinimide (NHS), bovine serum albumin (BSA), and phosphate-buffered saline (PBS) with 0.05% Tween 20 (dry powder) were purchased from Sigma-Aldrich. *p*-Aminophenylphosphate monosodium salt (*p*-APP) was purchased from LKT laboratories Inc. and was used as received. All aqueous solutions were prepared with Milli-Q water (Millipore Inc., Ω = 18 MΩ·cm). Gliadin stock solutions were freshly prepared in PBS−Tween containing 60% (v/v) ethanol and diluted in the appropriate buffer. Prostate-specific antigen (PSA) and anti-PSA monoclonal antibody (α-PSA) were provided by Fujirebio Diagnostics AB, Sweden.

2. Electrochemical Instrumentation. All the electrochemical measurements were performed using a PGSTAT12 potentiostat (Autolab) controlled with the General Purpose Electrochemical System software, with built-in frequency response analyzer FRA2 module. A three-electrode configuration of Ag/AgCl−3 M NaCl as a reference (CH Instruments., model CHI111), Pt wire as a counter (BAS model MW-1032), and bare or modified Au (BAS model MF-2014, 1.6-mm diameter) as working electrode was used. All the impedance measurements were carried out in 0.1 M 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 ac amplitude of 5 mV.

Surface Chemistry. 1. Optimization of Time Required for Self-Assembled Monolayer (SAM) Formation. Prior to the electrode modification, the gold surfaces were extensively cleaned by polishing in a slurry of alumina powder of 0.3 μm to a mirror finish and then sonicated in Milli-Q water and in ethanol for 5 min to remove any alumina remnants. The electrodes were placed in hot (∼70 °C) Piranha's solution (1:3 v/v, 30% H_2O_2, in concentrated H_2SO_4) for 5 min, thoroughly washed with Milli-Q water, and ethanol, and then dried with dry nitrogen. (*Warning:* Piranha solution is highly corrosive and violently reactive with organic materials; this solution is potentially explosive and must be used with extreme caution.) The bare electrodes were characterized using cyclic voltammetry (CV) and faradic impedance spectroscopy in $Fe(CN)_6^{3-/4-}$ in PBS (0.1 mM, pH 7.4). The formation of self-assembled monolayers was facilitated by immersion of the cleaned Au electrodes in 1 mM ethanolic solutions **1** or **2**, and the time dependence of EIS variations was recorded between 0 and 180 min for **1** and 0.5 and 312 h for **2**. R_{ct} values were calculated for different exposure times using the Autolab impedance analysis software.

2. Stability of Antibody-Modified SAMs of 1 and 2. Clean electrodes were immersed in an ethanolic solution of **1** and **2** for 3 and 120 h, respectively. The carboxylic acid groups of the SAM of both **1** and **2** modified electrodes were activated by stirring in 0.1 M MES buffer (pH 5) containing 0.2 M EDC and 50 mM NHS for 15 min, followed by incubation of the electrode with 100 μg/mL PAb in PBS (pH 7.4) for 1 h at 4 °C. The residual activated carboxylic groups were blocked with 1 M ethanolamine solution (pH 8.5) for 15 min. The EIS was recorded in $Fe(CN)_6^{3-/4-}$ in PBS (0.1 mM, pH 7.4) every day for seven days.

3. Nonspecific Binding and Cross-Reactivity Studies of the SAMs. The nonspecific binding and cross-reactivity studies were carried out using EIS in $Fe(CN)_6^{3-/4-}$ in PBS (0.1 M, pH 7.4) solution. Different conditions were used to study the nonspecific binding, using 20 μg/mL PWG: (i) EIS was recorded before and after incubation of the corresponding SAM with PWG for 30 min; (ii) the SAM-modified electrodes were first immersed in 0.05 M PBS−Tween (in the absence and in the presence of 0.1% w/v BSA) for 30 min followed by incubation with PWG; (iii) the modified gold electrodes were EDC/NHS activated for 15 min followed by immersion in a 100 μg/mL solution of PAb or anti-PSA (as nonspecific antibody) in PBS pH 7.4 for 1 h at 4 °C. The remaining carboxyl groups for both SAMs **1** and **2** were then blocked with 1 M ethanolamine hydrochloride (pH 8.5) for 15 min, followed by a second blocking step by incubation in 0.05 M PBS−Tween and 0.1% w/v BSA (in PBS−Tween), respectively, for 30 min. EIS of the antibody-modified surface was recorded in $Fe(CN)_6^{3-/4-}$ before and after incubation with 20 μg/mL of the corresponding protein (PSA or PWG, respectively) for 30 min.

Biosensor Fabrication and Calibration. 1. Formation and Characterization of the Sandwich Assay. SAM formation was carried out at their corresponding optimal times, followed by activation of the carboxylic groups and immobilization of PAb as previously described. Following blocking in ethanolamine hydrochloride, the modified surfaces of **1** or **2** were incubated in PBS−Tween (0.05 M, pH 7.4) and in 0.1% w/v BSA (in PBS−Tween) solutions, respectively, for 30 min. The electrodes were then exposed to different concentrations of PWG (10−60 ng/mL) in PBS−Tween solution for 30 min, followed by incubation with 100 μg/mL CDC5 in PBS for 30 min and 100 μg/mL α-mouse-ALP in PBS for 15 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 electroactive marker.

2. Optimization of Incubation Time of PWG. The incubation time of the specific recognition between PWG and the immobilized PAb was optimized as follows. The SAM-modified electrodes were biofunctionalized with PAb and blocked as described in the previous section. The electrodes were then incubated with 20 μg/mL PWG in PBS−Tween solution at different incubation times (2, 10, 15, 20, 30, and 45 min), followed by sequential interaction with 100 μg/mL CDC5 for 30 min and 100 μg/mL α-mouse-ALP for 15 min. The immunosensors were subsequently immersed in Tris buffer solution (0.1 M, pH 9) containing 0.1 mM $MgCl_2$ and 0.1 mM $ZnSO_4$ in the presence of 10 mM *p*-APP. The differential pulse voltammetry (DPV) response was then recorded after 10 min of stirring in *p*-APP solution in

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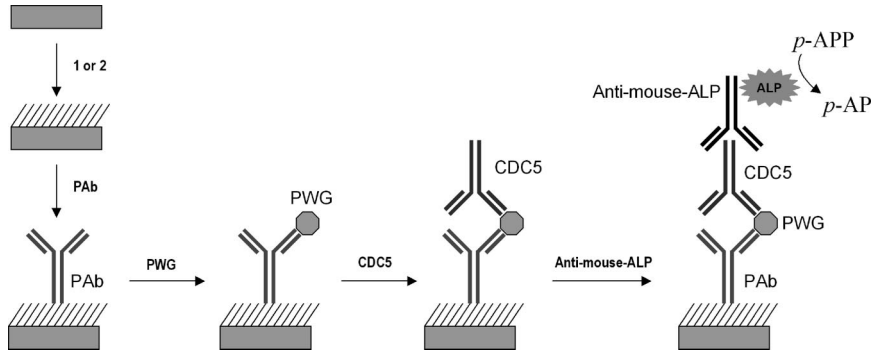


Figure 1. Stepwise construction of the gliadin immunosensor.

the potential range −0.2 to +0.6 V versus Ag/AgCl using a modulation amplitude of 25 mV), a step potential of 5 mV, and a scan rate of 50 mV·s^{-1}).

3. Electrochemical Detection of PWG. Detection of PWG on the modified electrodes (see the section Formation and Characterization of the Sandwich Assay) was carried out using EIS and DPV. EIS measurements were carried out in 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 (10, 20, 30, 40, 50, and 60 ng/mL). The obtained spectra were fitted to an equivalent electrical circuit using the Autolab impedance analysis software. DPV responses were recorded in Tris buffer solution (0.1 M, pH 9) containing 0.1 mM $MgCl_2$ and 0.1 mM $ZnSO_4$, in absence and in presence of 10 mM *p*-APP (after 10 min stirring) using the same parameters described in the section Optimization of Incubation Time of PWG.

Real Sample Analysis. 1. Extraction. Four commercial gluten-free samples were chosen: (i) raw materials, "mix B" bread mix (from Dr. Schär Srl, Italy) and "Damhert Nutrition" cake mix (from Damhert NV, Belgium); (ii) processed foods, "Glutafin" Sweet Biscuits (from Nutricia Dietary Care, UK) and "Harisín" dietetic pasta (from Sanavi, S.A., Spain); as well as (iii) a gluten-containing sample, "Tostagrill" toasted bread (from Diatosta España, S.L.). To extract the gliadin from the commercial gluten-free samples, an extraction buffer consisting of 60% v/v ethanol in the presence of reducing agent in Tris-HCl 50 mM pH 7.4 was prepared. The sample was blended to homogenity, and five portions of this (combined weight 100 mg) was added to 1 mL of the prepared extraction solution. The mixture was then vortexed, before heating under stirring conditions at 60 °C for 10 min, and finally centrifuged for 5 min at room temperature. The supernatant was recovered and divided into two aliquots—one aliquot for analysis using ELISA and the other for analysis using the developed immunosensor.

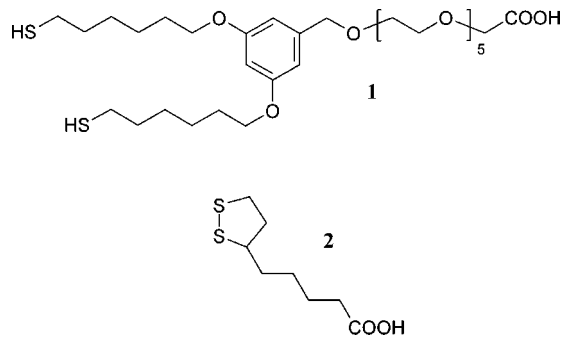
2. Enzyme-Linked Immunosorbent Assay Analysis of Commercial Gluten Free Samples. Rabbit antigliadin polyclonal antibody (1 μg/mL in carbonate buffer, pH 9.5) was added to each well of a Nunc Immunosorp microtiter plate and incubated for 1 h at 37 °C. Following thorough washing with PBS−Tween (pH 7.4, 50 mM 0.05% v/v Tween), the plate was then blocked by addition of 200 μL of PBS−Tween with 1% w/v BSA and incubated for 1 h at 37 °C, before thorough washing of the plate. In the immunorecognition step, 50 μL of a 1 in 10 or 1 in 25 dilution as well as 1 in 50 000 or 1 in 70 000 dilution (prepared by a 1 in 2500 or 1 in 3500 dilution in 60% v/v ethanol in PBS−Tween and a subsequent 1 in 20 dilution in PBS−Tween) of a range of concentrations of gliadin stock solutions prepared in 60% v/v ethanol in PBS buffer, pH 7.4, 50 mM, was used to prepare a calibration curve with a dynamic linear range between 1 and 150 ng/mL. The extracts from the four gluten-free samples were diluted 1 in 10 and 1 in 25 and the gluten-containing sample diluted 1 in 50 000 and 1 in 70 000 (again prepared by a 1 in 2500 or 1 in 3500 dilution in 60% v/v ethanol in PBS−Tween and a subsequent 1 in 20 dilution in PBS−Tween) to avoid precipitation of the gliadin. Analysis of both standards and samples was carried out in triplicate. The plate was again incubated, under stirring conditions for 1 h at 37 °C, and subsequently thoroughly washed with PBS−Tween, prior to exposure to 50 μL of 1 μg/mL CDC 5 antibody, and again left to incubate under stirring conditions for 1 h at 37 °C. Following a further thorough washing, a 1 in 10 000 dilution of commercial alkaline phosphatase-labeled antimouse antibody was added to each well of the microtiter plate, and the plate incubated under stirring conditions for 1 h at 37 °C. After a final washing, 50 μL of the enzyme substrate *p*-nitrophenylphosphate was added to each well and product formation allowed to proceed for 2 h at 37 °C, the reaction was finally stopped by addition of 1 M H_2SO_4, and the absorbance read at 450 nm, the complete indirect sandwich assay requiring more than 8 h to be completed.

3. Immunosensor Detection. The detection of gliadin in real samples was carried out using gold electrodes modified with a SAM of **1**, as previously described in the section Formation and Characterization of the Sandwich Assay. The extracts (see the section Extraction) from the four gluten-free samples were diluted 1/25 v/v in PBS−Tween buffer (0.1 M, pH 7.4), while the gluten-containing sample was diluted 1/3500 v/v in PBS−Tween buffer (0.1 M, pH 7.4) containing 60% (v/v) ethanol, followed by a subsequent 1/20 v/v dilution in PBS−Tween (0.1 M, pH 7.4). The modified electrodes were then incubated with the diluted extracts for 30 min at room temperature. The rest of the sandwich assay biocomponents were built up (see the section Formation and Characterization of the Sandwich Assay), and the DPV responses were recorded as previously described in the section Optimization of Incubation Time of PWG.

RESULTS AND DISCUSSION

Surface Chemistry. Figure 1 shows the strategy employed for the construction of the gliadin immunosensor. SAMs of dithiol **1** or **2** (Scheme 1) were used as supports for the capture PAb, which was covalently linked to the COOH groups of the SAM via carbodiimide chemistry.

Scheme 1



1. Formation of Dithiol Self-Assembled Monolayers. Prior to polyclonal antibody immobilization, the SAMs were optimized in terms of time of formation and minimization of nonspecific interactions. Figure 2 shows the variation of charge-transfer resistance obtained at a SAM of **2** in the presence of 1 mM $Fe(CN)_6^{3-/4-}$ at different lengths of exposure time to the thiol solution. Impedance values increased steadily with time before reaching a constant value after 100 h, in stark contrast with the 3 h needed to form a SAM of **1** (Figure 2, inset). This impedance increase does not account for multilayer formation since each point represents the constant impedance value obtained after repeated washings with ethanol in order to remove physically adsorbed molecules and indicates that the formation of a complete lipoic acid monolayer on gold is an extremely slow process. This apparent disadvantage is compensated in part by the immobilization of a higher number of biorecognition units in comparison to **1** (see below), which could be the result of the presence of a higher number of carboxylate groups in the surface due to its smaller molecular footprint (28 $Å^2$ for **2**, 49 $Å^2$ for **1**).

2. Immobilization of Capture Antibody and Stability of Biofunctionalized SAM. Immobilization of the capture antibody was achieved via activation of the COOH groups of the SAMs as labile NHS esters followed by covalent coupling of the PAb through its amino groups. The stability of antibody-modified SAMs was tested by recording the impedance variations daily for one week for electrodes kept in PBS buffer at room temperature (Figure 3). An impedance decrease for the SAM of **2** in the first three days of interaction was observed, which could be related with the partial loss of some physisorbed antibody material or destabilizing effect of the antibody immobilization on the thioctic acid SAM. In contrast, electrodes modified with **1** showed a slight impedance increase in the first day, remaining constant during the rest of the stability study.

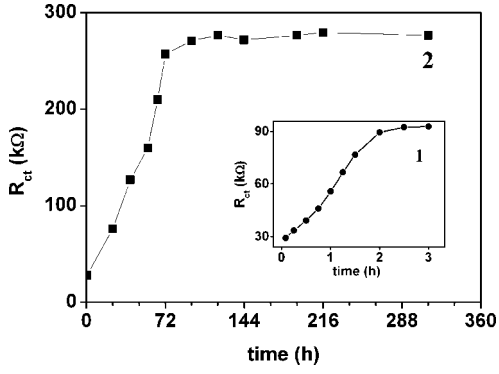


Figure 2. Time dependence of R_{ct} values (obtained from simulation) for the formation of SAMs of dithiols **1** (●) and **2** (□).

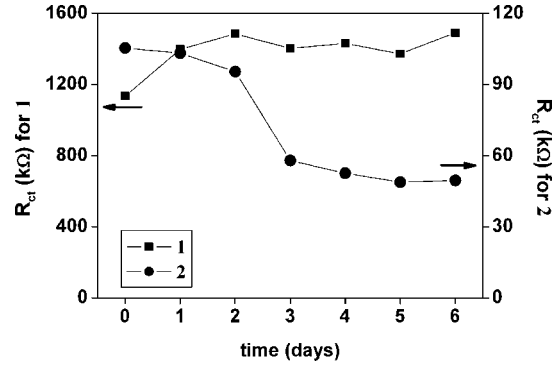


Figure 3. Time dependence of the charge-transfer resistance of SAMs of **1** and **2** modified with PAb.

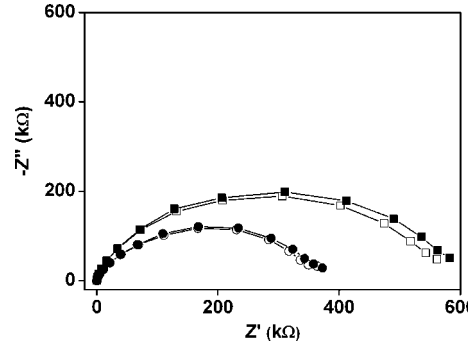


Figure 4. Complex impedance plots (in 1 mM $K_3Fe(CN)_6$ solution in PBS pH 7.4) recorded at a SAM of **2** modified with the following: PAb (□), PAb in the presence of 2 μg/mL PSA (■), anti-PSA (○), and anti-PSA in the presence of 20 μg/mL PWG (●).

3. Evaluation of Nonspecific Interactions. Using EIS we evaluated nonspecific interactions on SAMs of **1** and **2** as well as modified SAMs carrying specific and nonspecific antibodies against gliadin or using PSA as a nonspecific analyte. Impedance measurements showed the occurrence of a relatively high degree (>50%) of nonspecific adsorption on a bare SAM of **2** as compared with **1** (15%) when they are exposed to 20 μg/mL PWG. The degree of nonspecific interactions of both surfaces toward PWG was 2-fold reduced when this SAM was further incubated with 0.1% BSA in PBS−Tween. In a situation more akin to the true final immunosensor format, where antibodies are immobilized on the SAMs of **1** or **2** and incubated with a nonspecific antigen, nonspecific binding values were markedly decreased to below 10%, with values of 2.7 and 6.6% observed when PWG was allowed to interact with a SAM of **1** or **2**, respectively, modified with a monoclonal nonspecific PSA antibody (Figure 4).

Electrochemical Detection of Gliadin. 1. Labeless Impedance Detection. Figure 5 represents the impedance spectra of the successive building steps leading to the construction of the gliadin immunosensors using **1** and **2**. As shown in Figure 5, the charge-transfer resistance was increased with the binding of each corresponding protein layer, providing evidence of the successful formation of the immunocomplex. Interestingly, in spite of the relative complexity of the formed sandwich structure, the surface is capable of detecting a hydrophobic redox probe such as *p*-aminophenol even at submicromolar levels (LOD = 15 nM) as evidenced by DPV titration experiments in the concentration range

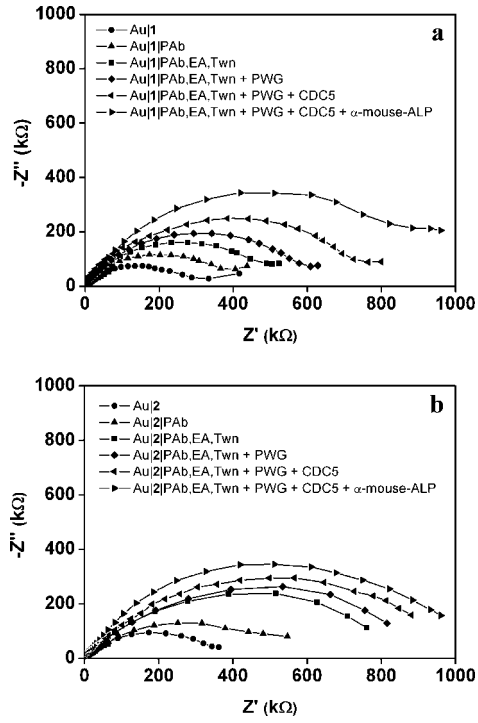


Figure 5. Complex impedance plots (in 1 mM $K_3Fe(CN)_6$ solution in PBS pH 7.4) recorded at a SAM of **1** (a) and **2** (b) for the sequential immobilization of biocomponents in the sandwich assay.

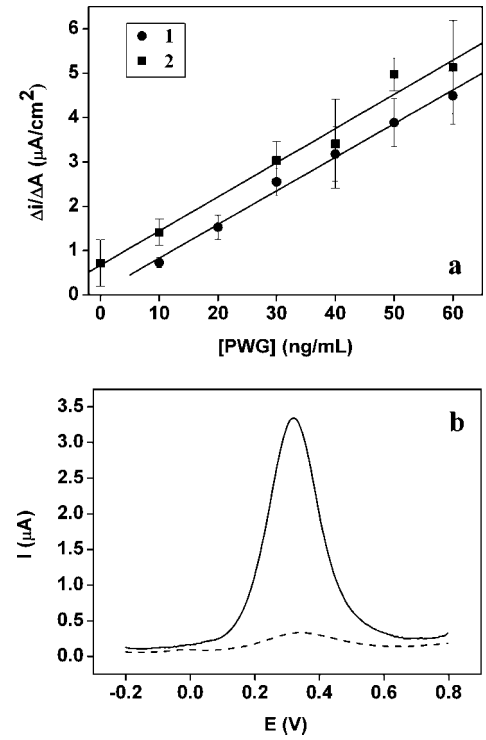


Figure 6. (a) Calibration plots for the detection of PWG on surfaces modified with **1** and **2**. (b) Differential pulse voltammograms of surfaces modified with **1** (—) and **2** (- - -) after 3-h SAM formation, followed by antibody immobilization and formation of sandwich immunocomplex and final addition of 10 mM *p*-aminophenylphosphate substrate in 100 mM Tris buffer, pH 7.4.

of 0−10 μM. However, the R_{ct} values showed no linear relationship with increasing PWG concentrations in the range of 10−60 ng/mL, indicating that the impedance technique is not suitable for gliadin detection under the studied conditions. This might be provoked by a dampening effect of the physically adsorbed BSA blocking agent, which makes impedance measurements irreproducible as it is very sensitive, not only to analyte binding but also to the occurrence of minimal changes in the biointerface structure.[45]

2. Optimization of Incubation Time. The current response of the immunosensor at different incubation times (2, 10, 15, 20, 30, and 45 min) of 50 ng/mL PWG was increased with increasing reaction time and then maintained the maximum value after 30-min incubation. Therefore, this incubation time was used in further experiments.

3. Differential Pulse Voltammetry Detection. Figure 6a represents the calibration plots obtained for PWG detection for immunosensors based on both SAMs **1** and **2**. The current peak height showed a linear relationship with the concentration of PWG over the range of 10−60 ng/mL for both **1** and **2** modified biosensors. As further proof of the requirement to allow the SAM of **2** to form for 100 h prior to biofunctionalization, the SAMs of both **1** and **2** were allowed to form for 3 h before immobilization of the capture antibody, and exposure to a saturating concentration of gliadin and subsequent formation of the complete sandwich immunocomplex and detection, as previously described. As can be seen in Figure 6b, a markedly lower signal is obtained at 0.32 V for the immunosensor based on SAM **2** when the SAM is only allowed to form for 3 h, similar measurements were made for various formation times of SAM **2** at periodic times between 0 and 100 h, and it was clearly observed that only after 100 h were comparable responses obtained (data not shown). Table 1 shows the analytical parameters obtained for the immunosensors based on SAM **1** formed after 3 h and SAM **2** formed after 100 h. As can be seen, the sensitivity of each immunosensor was similar for both surfaces, although electrodes modified with a SAM of **1** showed a lower limit of detection (LOD) when compared with **2** and the value of the LOD obtained for both is applicable to detection of gluten in gluten-free food samples.

Table 1. Analytical Parameters for Gliadin Detection Using 1 and 2

surface	sensitivity (μA ng^{-1} mL^{-1})	LOD (ng/mL)	r
Au|**1**	75 ± 3	5.5	0.996
Au|**2**	77 ± 6	11.6	0.989

4. Real Sample Analysis. The developed immunosensor was then applied to the detection of commercial gluten-free food products, as well as to a commercial gluten-containing food product. Extracts from gluten-free samples were diluted 1 in 25 and from the gluten-containing sample 1 in 70 000. As can be seen in Table 2, an excellent correlation was obtained between the immunosensor and the ELISA, but with the former representing a dramatic reduction in assay time from more than 8 h to less than 90 min, demonstrating the applicability of the immunosensor to in situ use, and work is ongoing to further reduce the required assay time.

(45) Pänke, O.; Balkenhohl, T.; Kafka, J.; Schäfer, D.; Lisdat, F. *Adv. Biochem. Eng. Biotechnol.* **2008**, *109*, 195.

Table 2. Comparison of Gliadin Detection in Real Samples Using Two Methods

sample	immunosensor (ppm)	ELISA (ppm)
"Glutafin" Sweet Biscuits (from Nutricia Dietary Care, UK)	0.56 ± 0.09	0.24 ± 0.08
"Harisín" dietetic pasta (from SANAVI, S.A., Spain)	12.4 ± 1.5	11.2 ± 1.3
"mix B" bread mix (from Dr. Schär Srl, Italy)	2.26 ± 0.04	1.60 ± 0.40
"Damhert Nutrition" cake mix (from Damhert NV, Belgium)	0.8 ± 0.1	1.65 ± 0.04
"Tostagrill" toasted bread (from Diatosta España, S. L.)	29000 ± 8000	33000 ± 9000

CONCLUSIONS

An electrochemical immunosensor for the measurement of potential celiac toxicity in raw and processed foodstuffs has been developed. The use of two different surface chemistries for the anchoring of the capture antibody has been compared in terms of time required for formation of self-assembled monolayers, stability, susceptibility to nonspecific binding, reproducibility, and sensitivity. The thioctic acid self-assembled monolayer took more than 100 h to attain a stable surface, and rapidly destabilized following functionalization with capture antibody, while the heptaoxadocosanoic acid surface rapidly formed (less than 3 h) and was stable for at least 5 days, stored at room temperature, following antibody immobilization. Both surface chemistries gave rise to highly sensitive immunosensors, with detection limits of 5.5 and 11.6 ng/mL being obtained for **1** and **2**, respectively, and nonspecific binding was significantly lower for the heptaoxadocosanoic acid based immunosensor, at just 2.7% of the specific signal. Better reproducibility was attained with **1** with RSD of 5.2% as compared with an RSD of 6.75% obtained for **2** ($n = 5$). Finally, the immunosensor was applied to the analysis of commercial gluten-free and gluten-containing raw and processed foodstuffs, its performance compared to that of an ELISA, and excellent correlation achieved. Ongoing work is looking at the use of antibody fragments as compared to whole antibodies as a means of improving sensitivity, as well as exploring formats to reduce assay time.

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