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Truncated aptamers as selective receptors in a gluten sensor supporting direct measurement in a deep eutectic solvent

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Abstract

Enzyme-linked immunosorbent assays are currently the most popular methods to quantify gluten in foods. Unfortunately, the antibodies used as specific receptors in such methods are not compatible with the usual solvents for the extraction of gluten proteins. In consequence, commercial tests require a high dilution of the sample after the extraction, increasing the limit of quantification and decreasing convenience. In this work, we have rationally truncated an aptamer capable of recognizing gliadin in a deep eutectic solvent (DES). The truncated aptamer is a 19-nucleotides-long DNA that minimizes self-hybridization, allowing the development of an electrochemical sandwich-based sensor for the quantification of gluten in the DES ethaline. The sensor incorporates two identical biotin-labeled truncated aptamers, one of which is immobilized on a carbon screen-printed electrode and the other reports the binding of gliadin after incubation in streptavidin-peroxidase. This sensor can detect gliadin in DES, with a dynamic range between 1-100 µg/L and an intra-assay coefficient of variation of 11%. This analytical performance allows the quantification of 20 µg of gluten/kg of food when 1 g of food is extracted with 10 mL of ethaline. We demonstrate the ability of this method to achieve the measurement of gluten in food samples, after the extraction with pure ethaline. The assay is useful for the analysis of residual gluten levels in foods, thus facilitating the evaluation of any potential health risk associated with the consumption of such food by people with celiac disease or other gluten-related disorders.

Keywords: aptamer; deep eutectic solvent; gluten; sandwich assay

1. INTRODUCTION

Celiac disease (CD) is an autoimmune pathology triggered by gluten proteins found in food containing wheat, rye, barley, or some oat varieties (Wieser et al., 2008). Consumption of gluten containing food by celiac people causes the damage of the mucosal villi and can lead to a flattened mucosa, resulting in malabsorption of nutrients and deficiency-related illnesses (Heyman et al., 2012). In consequence, CD patients, as well as people with other gluten intolerances, must follow a lifelong gluten-free diet. In order to guarantee safety of foodstuff for celiac people, including the so called "sensitive celiac population", the development of inexpensive and rapid methods for gluten determination at low concentration is critical (Diaz-Amigo et al., 2012, 2013; Slot et al. 2016).

The term gluten actually describes a complex mixture of water-insoluble storage proteins, for which an effective and universal extraction method is not available. This constitutes the first challenge to achieve a reliable and accurate determination of gluten in foods. There are various gluten extraction cocktails that were shown to be efficient for the extraction of gluten from different matrices (Fallahbaghery et al., 2017; Satsuki-Murakami et al., 2018; Mena et al., 2012), but their compatibility with the subsequent gluten detection method is not always guaranteed (Scherf and Poms, 2016). Consequently, large dilutions of the extracts are necessary before carrying out the determination, which compromises the final sensitivity of the analytical method, and introduces opportunities for errors and cross-contamination leading to inappropriate food labelling.

At present, enzyme-linked immunosorbent assay (ELISA) is the most commonly used method for gluten analysis not only for its selectivity and sensitivity but also for the lack of other reference methods (García-García et al., 2020; Lacorn et al., 2019; Morón et al.,

2008; Sajic et al., 2020; Sherf, 2017; Skerrit and Hill, 1990; Valdés et al., 2003; Zhanga et al., 2019). Besides ELISA, other analytical methodologies are currently being developed like mass spectrometry combined with proteomic technologies (Colgrave et al., 2015; Fiedler et al., 2018), methods based on DNA amplification (García-García et al., 2019; Garrido-Maestu et al., 2018; Martín-Fernández et al., 2015, 2016) and electrochemical sensors using as recognition elements molecular imprinted polymers (Iskierko et al., 2019) and aptamers (López-López et al., 2017; Malbano et al., 2017; White et al., 2018). Among electrochemical sensors, aptasensors are emerging as alternatives to reference methods. Aptamers are short single-stranded oligonucleotide sequences selected *in vitro* through a process called Systematic Evolution of Ligands by EXponential enrichment (SELEX) to bind a specific target (Ellington and Szostak, 1990; Tuerk and Gold, 1990; Wang et al., 2019). Their selection is usually carried out in aqueous buffers and only in few cases in mixtures of aqueous buffers and organic solvents for the recognition of poorly water-soluble chemical species. Recently, we selected aptamers targeting gluten proteins in a deep eutectic solvent (DES) named ethaline (Svigelj et al., 2018). Ethaline is a mixture of choline chloride and ethylene glycol (in a molar ratio 1:2), which has proved to be a very efficient extraction medium for gluten from both unprocessed and processed food (Svigelj et al., 2017). DESs are a class of ionic liquids displaying high solubilization power with improved biodegradability and low toxicity (Liu et al., 2015; Zhang et al., 2012). First reported in 2004 by Abbott (Abbott et al., 2004), they have been applied in many different fields such as organic synthesis, electrochemistry and bio-catalysis. Specifically, the use of DES in the preparation of sensing layers in electrochemical sensors has been shown to improve their analytical performance (Brett, 2018; Shahamirifard et al., 2018).

Moreover, DESs based on the choline ion ensure long-term stability of biomolecules like DNA and proteins (Gállego et al., 2015).

Motivated by the potential advantages of using aptamers for the recognition of gluten proteins directly in a DES extraction medium, without the need for additional dilutions, we present here the development of a sandwich-based aptasensor. The practical application of this kind of assay may be jeopardized by the partial hybridization between the capture and signaling aptamers, especially when using the relatively long aptamers generated from SELEX. Therefore, we seek to reduce the probability of hybridization by eliminating (truncation) the portion of the aptamers that is not essential for gluten binding. Indeed, it is widely accepted that a way to enhance aptamers affinity for targets is the truncation of non-essential nucleotides, thus improving the accessibility of targets to the aptamer resulting in stronger aptamer-target complexes (Sharma et al., 2017; Wu et al., 2019). This post-SELEX optimization process will also decrease the production cost of the synthetic receptors, whereas improving their use in DESs by facilitating their diffusion in these high viscosity solvents (Zhang et al., 2012).

In response to the above arguments, we focus on two aptamers, Gli1 and Gli4, previously selected against the 33-mer immunotoxic peptide of gluten by SELEX, performed in aqueous buffer (Amaya-González et al., 2014) and in ethaline (Svigelj et al., 2018). These two aptamers have been subjected to a truncation study to improve their analytical performances. The results presented here highlight that a truncated anti-gluten aptamer (Gli4-T) may be successfully employed for the selective recognition of gluten in DES (ethaline), thus allowing the development of a sandwich electrochemical sensor for the detection of residual gluten levels that can be harmful to people affected by CD or gluten sensitivity.

2. EXPERIMENTAL SECTION

2.1 Chemicals

5'-tagged (biotin) aptamers were obtained HPLC-purified from Metabion International through Laboratorios CONDA (Madrid, Spain). All sequences are summarized in Table 1. Gliadin standard from Prolamin Working Group (PWG) was acquired to R-Biopharm AG (Germany). Ethaline was supplied by Scionix Ltd. (London, UK) and employed as received. Potassium ferricyanide and ferrocyanide were purchased from Fluka. All aqueous solutions were prepared with water purified with a MilliQ system (Millipore, Spain). Salts for buffer solutions, Tween-20, bovine serum albumin (BSA), 1 M Tris/HCl pH 7.4, phosphate buffered saline (PBS) 10×, 3,3′,5,5′-tetramethylbenzidine (TMB) liquid substrate system for ELISA solution, streptavidin and sorbitol were obtained from Sigma-Aldrich (Spain). Dynabeads™ MyOne™ streptavidin C1 (strep-MPs), Dynabeads® M-280 tosylactivated, DNA, Protein Lo-bind Eppendorf tubes and streptavidin-peroxidase conjugate (strep-HRP) were obtained from Thermo Fisher Scientific. The magnet (DynaMag-2) for magnetic separation was purchased from Life Technologies (Madrid, Spain). Thermomixer for temperature control and mixing the solutions was from Eppendorf Ibérica (Spain)

Table 1. Nucleotide sequence of aptamers. The highlighted bases represent the repeating motifs. 1st motif (bold); 2nd motif (underlined); central part of the 2nd motif (italics). Gli1-T and Gli4-T also include 5 thymines at the 5´-end.

Name	Sequence	Length	ΔG (kcal/mol)
Gli1	CTA GGC GAA ATA TAG *CTA CAA* C**TG TCT GAA** GGC ACC CAA T	40 bases	-1.24
Gli1-T	AG *CTA CAA* C**TG TCT GAA**	17 bases	-1.59
Gli4	CCA GTC TCC CGT TTA CCG CGC *CTA CA*C A**TG TCT GAA T**GC C	40 bases	-1.72
Gli4-T	*CTA CA*C A**TG TCT GAA T**GC C	19 bases	-2.86

2.2 Electrochemical measurements

Chronoamperometry for evaluating the sensor response and cyclic voltammetry (CV) to follow its fabrication were performed with a μ-AutoLab type II potentiostat controlled by Nova 2.1 software (EcoChemie, The Netherlands). Disposable screen-printed carbon electrochemical cells (SPCE) were purchased from Metrohm-Dropsens (Spain).

Electrochemical impedance spectroscopy (EIS) was performed using an Autolab Pgstat-12 controlled by Nova 2.1 software (EcoChemie, The Netherlands). Impedance spectra were recorded after each of the modification steps for the construction of the sensor, with a DC bias potential of 0.115 V (the equilibrium potential of the $[Fe(CN)_6]^{3-/4-}$ redox system) and an AC amplitude of 0.01 V, in the frequency range 0.1 Hz – 100 kHz.

2.3 Modification of tosylactivated-magnetic particles with PWG

165 μL of Dynabeads® M-280 tosylactivated magnetic beads were washed with 1 mL 0.1 M phosphate buffer pH 7.4 and then coated with gliadin by incubating them in 150 μL of 0.66 mg/mL PWG under continuous shaking at 37 °C for 18 h. Then, the supernatant was removed and a 0.5 % solution of BSA, prepared in PBS, was added and let under continuous shaking at 37°C for 1 h. After two washing steps using PBS with 0.1% BSA, the beads were resuspended in 250 μL of ethaline.

2.4 Binding assays

A volume of 10 μL of the tosylactivated-MPs with PWG was equilibrated with 490 μL of increasing concentrations of each biotinylated aptamer in ethaline for 1 h at 40 °C. Subsequently, the beads were subjected to two washing steps with 50 mM Tris, 250 mM NaCl, 5 mM $MgCl_2$ containing 0.01% Tween-20, and then incubated with 500 μL of 2.5 μg/mL strep-HRP conjugate for 30 min under shaking at 30 °C. After two washing steps with 50 mM Tris, 250 mM NaCl, 5 mM $MgCl_2$ containing 0.01% Tween-20 and one with the same buffer without Tween-20, the MPs were resuspended in 10 μL

of Tris buffer. Lastly, the amount of bound aptamer was evaluated electrochemically. Magnetic beads were placed on the working electrode of a disposable screen-printed carbon electrochemical cell and collected with a magnet (diameter 4 mm) placed under it. After 1 min, 40 μL of TMB solution were added. The reduction current of the product generated during 1 min of enzymatic reaction was chronoamperometrically recorded at 0 V.

2.5 Sandwich assay on carbon screen-printed electrodes

10 μL of streptavidin (1 mg/mL) were immobilized on the bare carbon electrode by adsorption at 4 °C overnight, then the surface was blocked for 30 min with a solution of 1% BSA and 6% sorbitol prepared in PBS. Subsequently, the Gli4-T aptamer modified in 5' end with a biotin was immobilized exploiting the streptavidin-biotin interaction by incubation with a 1 μM solution in PBS for 30 min. Afterwards, the remaining sites were blocked with a biotin solution (2 μM) in PBS for 30 min. At this point, solutions of increasing concentration (between 0.1 and 1000 μg/L) of PWG gliadin in buffer (Tris 50 mM, NaCl 250 mM, $MgCl_2$ 5 mM) and in parallel in ethaline were incubated for 30 min. Subsequently the biotin-Gli4-T aptamer at a concentration of 1 μM was incubated in the Tris buffer or in ethaline for 30 min. Finally, the streptavidin-HRP enzyme conjugate (0.75 μg/mL) was incubated for 10 min in the Tris buffer. The electrochemical transduction was performed by adding on the electrode 40 μL of TMB solution and measuring by chronomaperometry at 0 V the reduction current of the product generated after 1 min of the enzymatic reaction.

2.6 Sample preparation and extraction procedure

Sample preparation was carried out in a laboratory separated from that where analyses were performed, to avoid contamination. Gluten was extracted with ethaline. 0.1 g of the obtained powdered sample was extracted in vials with 1 mL of pure DES. These

vials were shaken in a vortex for 2 min, and then they were left in a water bath at 55 °C for 45 min. After this time, they were shaken again for 2 min and centrifuged for 10 min at 5000 rpm. Depending on the level of gluten, the supernatant is directly applied on the electrode surface or properly diluted in 100% DES before the analysis.

3. RESULTS AND DISCUSSION

3.1 Design of truncated aptamers

Our previous studies described two anti-gliadin aptamers, called Gli4 and Gli1, with a length of 40 nucleotides. These aptamers were identified by a SELEX process, using as the target molecule the 33-amino acid immunotoxic peptide from α-gliadin known as 33-mer. These two aptamers showed good affinity for both the immunotoxic peptide and the entire protein. However, not all the nucleotides of these aptamers necessarily play an essential role in their binding to the target. The region of the recognition site, which interacts with the target molecule, is usually no larger than 10-15 nucleotides (Wu et al., 2019). These nucleotides are usually also involved in secondary structures of loop type, guanine quartets or pseudoknots. A second region includes nucleotides that, although not directly in contact with the target molecule, play an important role in directing the affinity interaction. The rest of the nucleotides are not essential and could be eliminated, even improving the affinity with respect to the initial aptamers (Dhiman et al., 2018; Heilkenbrinker et al., 2015; Le et al. 2014; Macdonald et al., 2016).

The rational design of truncation studies was carried out taking as starting point the analysis of the secondary structures of the described aptamers and the two most repeated motifs previously identified in the output sequences of the SELEX process (Svigelj et al., 2018). With this objective the Mfold web server for nucleic acid folding was employed (Zuker, 2003). For this analysis, 25 °C and the saline concentration of the aqueous buffer employed for the selection, 250 mM NaCl and 5 mM $MgCl_2$, were considered. The two short consensus motifs are an 8-nucleotide motif TGTCTGAA, and a 9-nucleotide motif AGCTACAAC (see Figure 1).

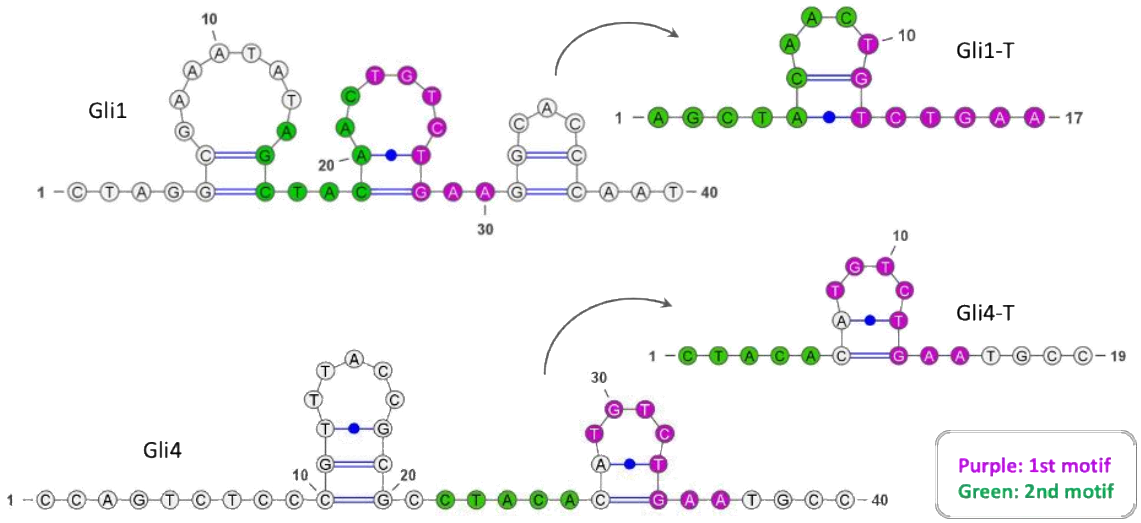

Figure 1. Secondary structures of Gli1 and Gli4, and the corresponding truncated aptamers. The repeating motifs are highlighted.

Both aptamers have a similar structure, with more than an internal loop, and the nucleotides involved in these hairpins correspond to conserved regions. The first motif is present in one of the loops of both Gli1 and Gli4 sequences. The second motif is present only in Gli1, whereas in Gli4 just its central part (CTACA) appears (Table 1). Since these motifs are believed to be involved in the recognition process, shorter aptamers were obtained by a rational truncation. Gli1 was truncated at both 5' and 3' ends to obtain a 17-nucleotide sequence (Gli1-T) containing the first and the second motifs. Gli4 was truncated only at the 5' end obtaining a 19-nucleotide sequence (Gli4-T) containing the 1st and the central part of the 2nd motif. It is worth noting that in Gli1-T the secondary structure is not maintained, while on the contrary it is maintained in Gli4-T. In addition, it was previously found that the affinity of the whole aptamers toward protein and peptide decreases when a marker molecule is incorporated at the 5' end (Amaya-González et al., 2015). For this reason, to evaluate the truncated aptamers it was decided to incorporate a sequence of 5 thymines between the aptamer sequence and biotin tag at the 5' end.

3.2 Evaluation of the affinity of the truncated aptamers against PWG Gliadin

To verify whether truncation of aptamers would benefit their recognition ability, we evaluate the affinity between them and the target protein by means of a titration experiment, where a fixed amount of protein interacts with increasing amounts of the aptamer, either in aqueous buffer or in DES. Once the recognition reaction reaches the equilibrium, the concentration of the bound form of the aptamer is measured, which allows the dissociation constant (K_d) for the aptamer-protein complex to be calculated. This is achieved by immobilizing a fixed amount of PWG gliadin onto tosylactivated magnetic beads, followed by incubation with increasing concentrations of the biotinylated-aptamers. Upon magnetic separation and binding to streptavidin-HRP conjugate, the chronoamperometric response generated by the enzymatically oxidized TMB gives a measure of the bound aptamers.

We performed the binding curves both in aqueous buffer and in ethaline. Binding curves for Gli4 and Gli4-T are shown in Figure 2. Gli4-T conserves the functional site of the aptamers and the corresponding binding curves are fitted to the Hill equation, which provides a dissociation constant of 148 ± 8 nM (R^2 = 0.997) in aqueous buffer, and of 515 ± 144 nM (R^2 = 0.94) in ethaline. In both cases, Gli4-T reaches saturation more quickly than Gli4 and has a lower dissociation constant compared to Gli4 (K_d = 186 ± 55 nM, R^2 = 0.98 in aqueous buffer and K_d =1.3 ± 0.4 μM, R^2 = 0.97 in ethaline). This confirms that the sequence of Gli4-T includes the region that is responsible for binding to gliadin. Interestingly, the truncation leads to a change in the binding model resulting in an increase in the cooperativity index, *n*, from 0.7 ± 0.1 for Gli4 to 2.4 ± 0.2 for Gli4-T, when the interaction is performed in aqueous buffer. This increase is less apparent when the binding is performed in ethaline (*n* = 1.5 ± 0.4 for Gli4, and *n* = 2.0 ± 0.9 for Gli4-T). Overall, these results indicate that ethaline may affect the structure of

both the protein and the aptamer, and the structural changes may lead to a change in the binding model. We presume that the affinity of the truncated aptamer in ethaline improves more consistently compared with the improvement in aqueous buffer because ethaline could stabilize a different secondary structure of the aptamer, as already reported in literature (Zhao, 2015). On the contrary, Gli1-T affinity toward gliadin strongly decreases (data not shown), conceivably because of some regions necessary for the interaction have been ousted in the truncation process or because the completely loss of the original secondary structure after truncation. These results suggest that eliminating exclusively non-essential nucleotides of the complete aptamer while maintaining the secondary structure, steric hindrance is reduced thus improving the affinity.

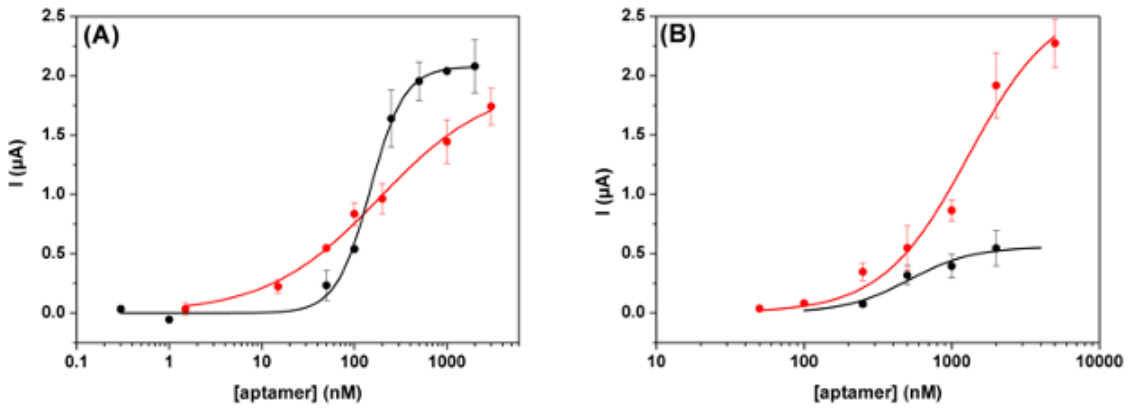

Figure 2. Binding curves of Gli4 (red) and Gli4-T (black) vs. PWG Gliadin in (A) buffer, and (B) ethaline.

3.3 PWG quantification by a sandwich assay

Encouraged by the results of the binding assays presented above, we developed an electrochemical method for gluten quantification using a sandwich format. The principle of the assay is illustrated in Figure 3. In this approach, Gli4-T was employed

as a capture as well as a reporting aptamer. In sandwich assays using aptamers, the hybridization between both aptamers should be minimized, and truncation is an effective way of achieving this. In this particular case, the free energy of the homo-hybridization between two molecules of Gli4 is -6.6 kcal/mol (at 25 °C, 250 mM Na^+ and 5 mM Mg^{2+}), while for Gli4-T is -5.7 kcal/mol (calculations performed with Mfold (Zuker, 2003)). As most of the hybridization region corresponds to the essential binding site, there is a slightly decrease in the probability of hybridization between the capture and reporting aptamer when using the truncated version, however, this may make a difference in the magnitude of blank signals. Hence, we used an electrode-bound Gli4-T aptamer, attached to the working electrode of a SPCE exploiting a biotin-streptavidin interaction. After that, the working electrode was covered with a solution of biotin to block the remaining free binding sites in streptavidin.

In order to control the fabrication of the sensor, we have performed EIS (Figure 3A) and CV (Figure 3C) measurements after each modification step. Successive electrochemical measurements can cause a rearrangement of the molecules immobilized on the electrode surface (Bogomolova et al., 2009), it is because that we employed different sensors to run in parallel each monitoring technique. The adsorption of streptavidin, followed by blocking with BSA and sorbitol lead to an increase in the charge transfer resistance, R_{ct}, of the SPCE from around 410 Ω to 5.4 ± 0.2 kΩ and 6.6 ± 0.5 kΩ, respectively. We observed a further 4% increase in R_{ct} upon the binding of the capture aptamer as a consequence of the electrostatic repulsion by the polyanionic phosphate backbone of the DNA. Finally, the blocking of the remaining surface streptavidin binding sites with biotin, in order to preclude the binding of the signaling aptamer during the operation with the sensor, produces a slight increase in R_{ct} to 7 ± 1 kΩ. These results are in good agreement with those obtained by CV monitoring. The successive modifications

provoke a progressive decrease in the oxidation and reduction peak currents for the electroactive probe, and a concomitant increase in the differences between the oxidation and reduction peak potentials (ΔE_p). The most important irreversibility occurs after the specific binding of the capture aptamer, with a ΔE_p of 340 mV and an 83% of suppression in the peak current with respect to the bare electrode. These results suggest that the fabrication of the aptasensor has occurred successfully and reproducibly.

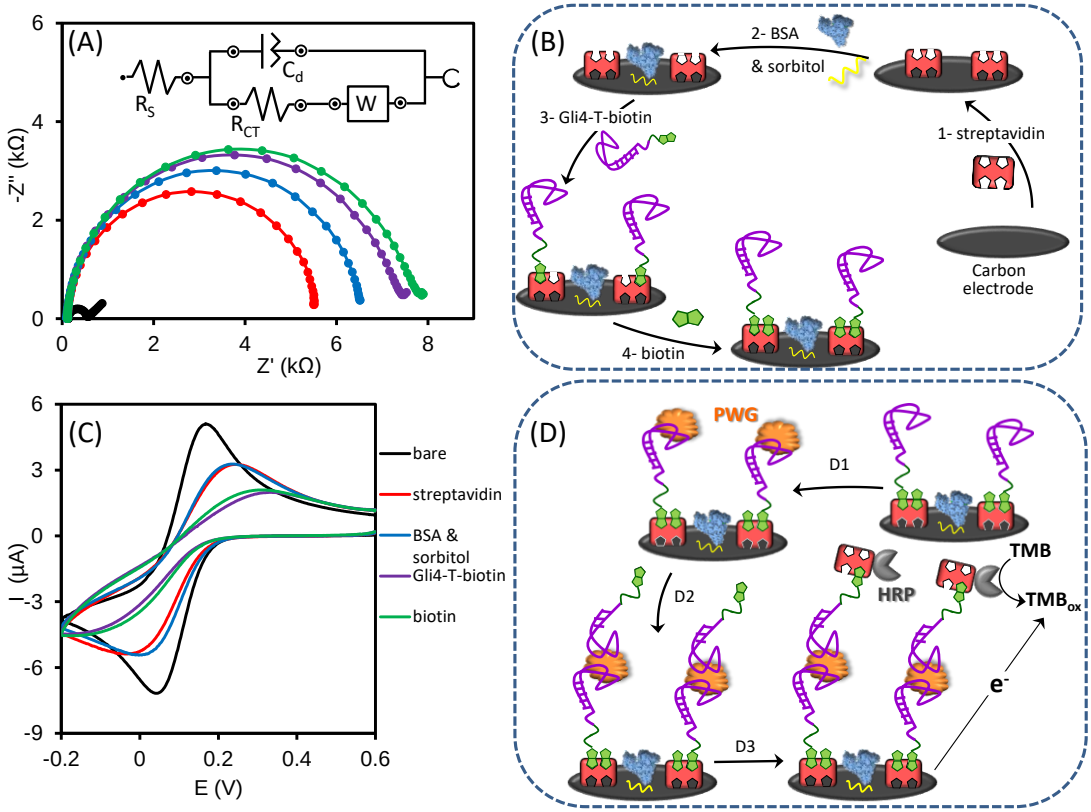

Figure 3. Construction of the aptasensor: (A) Nyquist plots recorded in a PBS solution containing 3 mM KCl and 0.5mM $[Fe(CN)_6]^{3-/4-}$ at SPCE after each of the modification steps involved in the preparation of the aptasensor, which are schematically represented in (B). The inset in A shows the Randles equivalent circuit used to fit the experimental data. (C) Cyclic voltammograms recorded in KCl 0.1 M with 0.5 mM $[Fe(CN)_6]^{3-}$ after each modification step. (D) Steps involved in the sandwich assay; D1: interaction with the sample containing (or not) PWG, D2: incubation with the detection aptamer (Gli4T-biotin), D3: labeling with streptavidin-HRP conjugate and chronoamperometric measurement of the oxidized tetramethylbenzidine enzymatically produced.

The operation with the sensor is represented in Figure 3D. As a consequence of the reduced homo-hybridization between the truncated aptamers and after the blocking of free-remaining streptavidin sites with biotin, it is possible to use a sandwich assay where, after the incubation with the sample, a biotinylated aptamer (biotin-Gli4-T) acts as the second receptor. Subsequently, the streptavidin-HRP conjugate is incorporated as the reporter molecule. The sensor is finally interrogated by adding hydrogen peroxide and 3,3',5,5'-tetramethylbenzidine (TMB), employing chronoamperometry to measure the amount of oxidized TMB that is enzymatically obtained. This strategy leads to a current signal, which increases with the amount of gliadin present in the sample.

In the absence of PWG gliadin, we observed a relatively high signal, both in aqueous buffer and ethaline. To clarify the origin of the background, several control tests were performed without gliadin, removing the secondary aptamer first, and both the secondary and the capture aptamer afterwards. Similar background signals were seen in the three situations, with currents around 2 μA when the incubations were performed in aqueous buffer, and even larger when ethaline solutions were used. These results suggest that the origin of the background is mainly the non-specific interaction of the enzyme conjugate with the electrode surface and not the self-hybridization between capture and detection aptamers. Consistent with this, the background decreased when the concentration of the enzymatic conjugate was decreased from 1.5 μg/mL to 0.75 μg/mL, obtaining under these conditions a net signal of 1.74 μA for a gliadin concentration of 100 μg/L. A further decrease in the concentration of the enzyme conjugate to 0.15 μg/mL led to a decrease in the specific signal, so a concentration of streptavidin-HRP 0.75 μg/mL showed the best performance and it was the selected one for sensor operation. Both, aqueous buffer and ethaline media, support efficient and sensitive gliadin sensing. However, the magnitude of the electrochemical signals is

decidedly greater when the test is conducted in ethaline, which can be explained by the possible enhancement of the enzymatic conjugate activity by the small amounts of DES that can reach the measurement medium. An increased peroxidase activity has been previously reported in choline chloride-based DES (Gotor-Fernández and Paul, 2019). This opens the possibility of developing the sandwich assay in DES, in order to directly measure the extracted food samples in the same medium. For this reason, we have tested the performance of the sensor when deployed in solutions with increasing concentrations of gliadin prepared in different media. Precisely, the calibration was carried out in an aqueous buffer and in 100% ethaline, see Figure 4. Under these conditions, the electrochemical measurement was conducted in buffer solution. Signals were normalized by expressing the percentage of the maximum net current avoiding variations between different days, and calibration curves were fitted to the Hill equation.

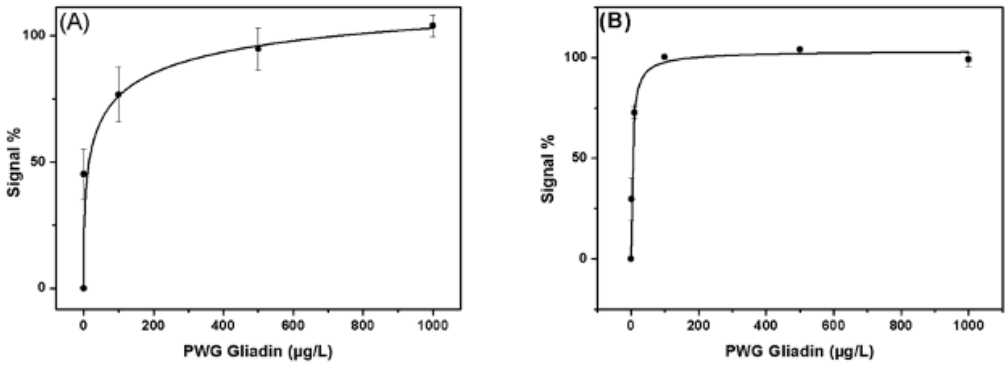

Figure 4. Calibration curve of the sandwich assay in aqueous buffer (A) and in 100% ethaline (B). Fitting equation for A: $\%S = \frac{(131\pm10)\,[PWG\ Gliadin]^{(0.43\pm0.05)}}{(46\pm18)^{(0.43\pm0.05)}+[PWG\ Gliadin]^{(0.43\pm0.05)}}$, R^2= 0.9991; Fitting equation for B: $\%S = \frac{(104\pm3)\,[PWG\ Gliadin]^{(0.81\pm0.11)}}{(3.3\pm0.6)^{(0.81\pm0.11)}+[PWG\ Gliadin]^{(0.81\pm0.11)}}$, R^2=0.98

As it can be seen, the calibration in DES proved to be more sensitive and affected by slightly less error. The intra-assay coefficient of variation (CV) was 13 % in buffer and

11% in 100% DES. The aptasensor dynamic range, however, expands up to lower PWG concentrations when the sample is incubated in DES (up to 100 μg/L) than when it is deployed in aqueous buffer, where it stretches up to 1000 μg/L. The LOQ, determined as the lowest gliadin concentration likely to be reliably detected from the blank to afford quantitation with a CV of at least 13%, was 10 μg/L in buffer, while it lowers to 1 μg/L in the calibration performed in pure DES. Since gliadin is used as reference, and its content in gluten is commonly assumed to be 50%, the LOQ in the food expressed as gluten is then calculated as the double of gliadin quantity. In consequence, for the analysis of food samples after the extraction with ethaline (1 g of food in 10 mL of ethaline), we obtained a LOQ of 20 μg/kg. This value successfully competes with that of the immunoassays present on the market, as it is evident from the data summarized in Table 2, which compiles a variety of commercial tests using different antibodies with the LOD in mg of gluten/kg of sample. It also outperforms the competitive aptasensor previously described (López-López et al., 2017), which showed an LOD of 380 μg/kg, 19-fold higher than with the sandwich aptasensor in DES.

Given that the formation of the sandwich complex during the sensor operation requires two simultaneous binding events with the target protein, the aptasensor achieves the improved detection without sacrificing selectivity. Specifically, we analyzed ethaline extracts of soya and rice flours, which are safe for celiac patients. We find no significant differences between the blank signals (1.7 ± 0.4 μA) and both soya (1.5 ± 0.2 μA) and rice (1.5 ± 0.4 μA) flours, even when the sensor is directly incubated in the extracts without any additional dilution.

Table 2. Comparison of the analytical performance of the sandwich assays for gluten detection. pAb means polyclonal antibody, whereas mAb is a monoclonal reagent.

Manufacturer	ELISA kit	Principle	Antibody	LOD (mg/kg)
Biomedal Diagnostics	GlutenTox ELISA Sandwich	Sandwich	A1/G12 mAb	0.6
Romer Labs	AgraQuant® ELISA Gluten G12	Sandwich	G12 mAb	2
Biocontrol	Transia Plate Prolamins	Sandwich	R5 mAb	3
R-Biopharm	Ridascreen® Gliadin	Sandwich	R5 mAb	1
Eurofins	Ingezim Gluten®	Sandwich	R5 mAb	3
BioCheck (UK)	Gluten-Check ELISA kit	Sandwich	R5 mAb	0.15
	AgraQuant® ELISA Gluten	Sandwich	pAb	0.6
Immunolab	Gliadin/Gluten	Sandwich	pAb	0.3

3.4. Application of the sandwich assay to food samples

The developed sandwich assay in DES could be suitable for the determination of gluten in food at very low levels, in order to ensure more information and safety to celiac people. Thus, we analyzed two dessert powders, panna cotta and vanilla cream, suitable to be labeled as gluten-free samples. The samples were extracted with ethaline, and the ethaline extracts were directly analyzed using the described aptamer-based sensor. The quantification was performed using PWG gliadin in ethaline as a standard. Both samples contain levels of gluten above the concentration range 1-100 μg of gliadin/L. After the suitable dilution of the extracts to match the dynamic range, we were able to estimate gluten concentrations of 3.4 and 1.6 mg/kg for panna cotta and vanilla cream, respectively. However, when the same samples were analyzed by a certified laboratory, the results were below the LOD of the ELISA test. These preliminary results prove that

the aptasensor in combination with ethaline extraction could be successfully used for the quantification of residual levels of gluten in foods, to improve their safety for celiac patients. It is important to note that the useful dynamic range of the assay in ethaline is relatively narrow and far below the threshold of 20 mg /kg stablished by EU for foodstuffs to bear a gluten-free claim (Commission of the European Communities, 2014). Consequently, dilution after the extraction with ethaline could be required to match the measurable range. However, the negative effects of gluten in people with celiac disease are accumulative, and patients with high gluten sensitivity may begin to have symptoms of intolerance with intakes as low as 0.015 mg of gluten per day (FDA, 2011). In consequence, the possibility of reliably quantifying so low residual amounts of gluten would be beneficial for such patients.

4. Conclusions

We have demonstrated the feasibility of a sandwich assay with truncated aptamers in DES. To the best of our knowledge, this is the first analytical method based on truncated aptamers in a non-aqueous solvent, which could significantly improve the capability of detecting gluten in foods. We have identified a truncated aptamer with good affinity toward gliadin, which allowed the development of a sandwich assay minimizing hybridization problems between capture and detection aptamers. Moreover, the use of shorter aptamers in a viscous medium, such as DES, represents a great advantage in terms of diffusion processes. Our sensor has proven to be suitable for the determination of very low concentrations of gluten, with a LOQ of 20 μg of gluten/kg of sample, which is much lower than the methods currently used. Additionally, the sandwich format with a truncated aptamer provides 19-fold and 25-fold lower than a competitive aptasensor and aptaassay on magnetic beads, respectively, using the whole

sequence of the same aptamer. Furthermore, the employment of a DES, such as ethaline in this case, allows an efficient extraction of gluten from foods, which combined with the use of aptamers selected in the same medium, represents an interesting alternative to traditional methods for gluten quantification.

CRediT authorship contribution statement

Rossella. Svigelj: Methodology, Investigation, Data curation, Writing - original draft, Writing - review & editing. **Nicolo Dossi:** Supervision, Writing - review & editing. **Stefania Pizzolato:** Investigation. **Rosanna Toniolo:** Conceptualization, Resources, Supervision, Writing - review & editing. **Rebeca Miranda-Castro:** Data curation, Methodology, Writing - review & editing. **Noemí de-los-Santos Álvarez:** Conceptualization, Data curation, Methodology, Writing - review & editing. **María Jesús Lobo-Castañón:** Conceptualization, Resources, Project administration, Supervision, Writing - review & editing.

Declaration of competing interest

The authors declare no competing financial interest.

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