



Affinity of aptamers binding 33-mer gliadin peptide and gluten proteins: Influence of immobilization and labeling tags



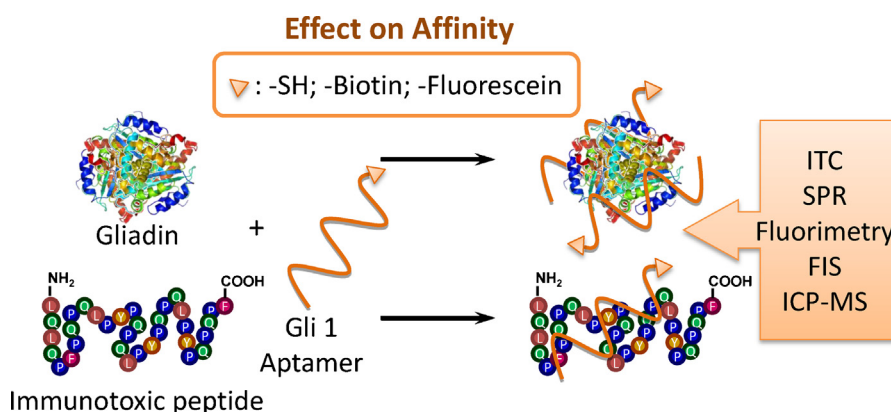
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HIGHLIGHTS

- Affinity of anti 33-mer peptide aptamer was evaluated by various techniques.
- Unlike other antipeptide aptamers, it recognizes the whole protein with minimal loss in affinity.
- Any modification at 5' end led to a decrease in affinity but at different extent.
- 6-FAM was the most deleterious marker molecule.
- Biotin modification seems to be less harmful than thiol modification, so recommended for analytical purposes.

GRAPHICAL ABSTRACT



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ABSTRACT

Aptamers are starting to increase the reagents tool box to develop more sensitive and reliable methods for food allergens. In most of these assays, aptamers have to be modified for detection and/or immobilization purposes. To take full advantage of their affinity, which decisively influence the detectability, these modifications must be faced rationally. In this work, a recently developed aptamer for an immunotoxic peptide of gliadin associated to celiac disease is used in different configurations and modified with various markers and anchored groups to evaluate the influence of such modifications on the real affinity. The interaction in solution with the peptide is strong for a relatively small molecule ($K_d = 45 \pm 10$ nM, 17 °C) and slightly stronger than that for the immobilized intact protein due to a cooperative binding effect. Comparatively, while only minor differences were found when the peptide or the aptamer were immobilized, labeling with a biotin resulted preferable over fluorescein ($K_d = 102 \pm 11$ vs 208 ± 54 nM, 25 °C). These findings are of prime importance for the design of an aptamer-based analytical method for gluten quantification.

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1. Introduction

The high affinity of aptamers for their cognate ligand is frequently invoked as one of the most prominent features that support their use in theranostic [1,2] or sensing applications [3,4]. Measurement of the

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affinity between aptamers and their ligands is usually carried out after SELEX evolution, to select the best binder among the enriched DNA pool obtained, and to characterize the winning sequence.

In order to select an aptamer among several described for a given target, K_d values are usually the primary criteria for comparison. Tables of K_d values, though useful, are reported irrespective of the method employed for their estimation [5,6]. True dissociation constants in solution are not always measured for several reasons. On one hand, some techniques such as isothermal titration calorimetry (ITC) [7] or NMR [8] need partner concentrations in the μM range, which is expensive and not always achievable because of the low solubility of some ligands in aqueous media. On the other hand, among the variety of techniques available to measure the constant binding [8–10], some of the most powerful ones (surface plasmon resonance (SPR), fluorescence intensity) require partner immobilization.

Interactions between the immobilized partner and the surface or steric hindrance, related to the proximity of the surface, are plausible and well-known explanations for the decrease in affinity commonly observed in heterogeneous binding events. Striking and not well-explained loss in affinity as large as three orders of magnitude have been found with aptamers directed against small molecules immobilized on SPR sensors [11,12]. Nonetheless, negligible [13] or even the opposite effects have been also reported and tentatively attributed to a more active presentation of the immobilized aptamer [14], cooperativity at the matrix or loss of anionic nature of the immobilized ligand [15].

From a practical point of view, not only the true affinity of an aptamer in solution but also the actual affinity after post-SELEX modifications is interesting. For sensing applications, labeling the aptamer with small molecules is the most wide-spread strategy that allows easy and inexpensive immobilization and/or detection. All these modifications, however, can exert a non-trivial impact on the affinity of the synthetic receptor towards the ligand. In general, this aspect is set aside assuming that modification of oligonucleotides at their ends keeps enough affinity for most analytical applications. However, selection of the proper end for labeling as well as the length and nature of spacers has been proved to improve the affinity [16,17]. Rational selection would be straightforward if the aptamer–ligand complex structure is fully solved. Unfortunately, this is unknown for most aptamers, so they are modified at any end irrespective of the effect.

To take advantage of the full binding potential of the winning aptamer evolved by SELEX, detailed comparative studies on binding ability of aptamers modified for immobilization or detection purposes are desirable but scarce. We have recently developed by SELEX a pool of aptamers against the immunodominant peptide of gliadin, known as 33-mer, that triggers the celiac disease [18]. Taking as a model the most abundant in the final pool, we present here a systematic study of the extent to which different tags to facilitate the immobilization and detection of aptamer alters its affinity towards both the peptide and the whole protein. This is crucial in order not to impact further the solution-phase affinity and selectivity in the development of analytical approaches aimed at detecting gluten in food. Availability of more sensitive methods of gluten detection are essential to protect patients from inadvertent content of gluten in food below the current threshold (20 ppm) [19] that it is still high for a relevant proportion of the sensitized population.

2. Materials and methods

2.1. Reagents

Native (unmodified) and 5'-tagged (biotin and 6-FAM) gli1 aptamers were obtained from Integrated DNA Technologies (IDT,

Leuven, Belgium). 5'-thiolated gli1-aptamer and 5'-thiolated control DNA were obtained from Sigma-Life Science. Gli1 aptamer and control DNA sequences are the following:

Gli 1: 5' CTA GGC GAA ATA TAG CTA CAA CTG TCT GAA GGC ACC CAA T 3'

Control DNA: 5' AGT CAA CCA GTA TTA TCT GAC CGT CCC A 3'

Peptide 33-mer was obtained from Biomedal (Sevilla, Spain). The sequences of the unmodified and the biotinylated 33-mer are as follows:

33-mer: LQLQPFQPQLPYQPQLPYQPQLPYQPQLPYQPQPF

Biotin-33-mer: LQLQPFQPQLPYQPQLPYQPQLPYQPQLPYQPQPFH-HHHHH-Biotin

Gliadin standard from Prolamin Working Group (PWG) was acquired to R-Biopharm AG (Germany). Phosphorus and beryllium standards were purchased from High Purity Standards (Charleston, USA): $1000 \mu\text{g mL}^{-1}$ P in 0.05% HNO_3 and $1000 \mu\text{g mL}^{-1}$ Be in 2% HCl.

All aptamer interactions with peptide or PWG were carried out in the binding solution (BS) composed of 50 mM Tris-HCl pH 7.4 + 0.25 M NaCl + 5 mM MgCl_2 . This buffer was the one used for aptamer evolution by SELEX, so optimized for the best interaction [18]. All buffer components were obtained from Sigma-Aldrich. Milli-Q purified water was used.

2.2. ITC experiments

The isothermal titration calorimetric (ITC) experiments were performed at two temperatures (17 and 37 °C) in a VP-ITC instrument (MicroCal Inc., USA). The aptamer, loaded into the calorimetric cell (1.4189 mL) at a concentration of 33.4 μM , was titrated by the sequential addition of 15 μL -aliquots of the 33-mer gliadin peptide from the stirring syringe at a concentration of 338 μM (the volume of first injection was 4 μL and was not considered in the fit). The amount of power required to maintain the reaction cell at constant temperature after each injection was monitored as a function of time. The exchanged heat due to the recognition event between 33-mer and gli1 aptamer was obtained as the difference between the heat of reaction and the corresponding heat of dilution.

2.3. Faradaic impedance spectroscopy (FIS) experiments

All electrochemical measurements were performed with a standard three electrode cell controlled by an AutoLab 12 potentiostat equipped with GPES and FRA 4.9 software (EcoChemie, The Netherlands). A platinum wire acted as an auxiliary electrode and a 1.6 mm diameter gold electrode as a working electrode (Bioanalytical Systems, Inc., USA). All potentials are referred to the Ag|AgCl|KCl saturated reference electrode.

The Au electrode was cleaned and conditioned as indicated elsewhere [20,21]. The gli1 aptamer was immobilized onto the freshly cleaned surface under potential control due to accelerated self-assembly [22]. This way, the electrode was immersed in 20 mM sodium phosphate + 0.3 M NaCl + 2 mM EDTA for 5 min at +0.3 V. The reduced gold surface was then immersed in 200 nM thiolated gli1 (or thiolated DNA control for specificity studies) in identical buffer for 5 min at +0.4 V. After washing with water, the electrode was incubated with 1 mM mercaptohexanol for 50 min to fill the bare regions and minimize unspecific adsorption. The modified electrodes were stored in BS at 4 °C until their use.

The interaction was carried out by incubating the modified electrodes with increasing concentrations of 33-mer peptide in BS (or the control peptide for unspecific assessment). Each 5 min the electrode was transferred to the impedance measurement solution (10 mM Tris-HCl pH 7.4 + 100 mM KCl + 5 mM $[\text{Fe}(\text{CN})_6]^{3-/4-}$) where the corresponding spectra were recorded at a bias potential

of +0.223 V using a frequency range from 10 kHz to 100 mHz and an alternating current amplitude of 5 mV.

2.4. SPR measurements

A double-channel cuvette ESPRIT instrument (EcoChemie) was operated in automatic mode using the autosampler for dispensing the solutions and mixing. All SPR experiments were carried out using commercial BK7 disks coated with a 50 nm gold film supplied by Autolab (Metrohm, The Netherlands) under controlled temperature conditions of $25 \pm 1^\circ\text{C}$ obtained with a thermostat HaakeD1 (Germany).

The Au sensor was cleaned as indicated elsewhere [23]. Then, a mixed SAM monolayer of mercaptoundecanoic and mercaptopropionic acids was prepared overnight from a 10 mM ethanolic mixture of each acid in a 1:10 ratio [24]. After washing with water, streptavidin was covalently attached to the carboxylic acids by activating these groups with 4 injections of 50 μL of 200 mM EDC and 50 mM NHS in water for 5 min and subsequent reaction with the 50 $\mu\text{g mL}^{-1}$ protein in 50 mM acetate buffer pH 4.5 for 30 min. The unreacted carboxylic groups were blocked with 1 M ethanolamine for 30 min. The streptavidin-modified sensor was primed with 50 mM HCl. Finally, one channel was modified with 25 nM biotinylated-33-mer and the reference channel with 500 nM biotin in BS buffer.

Each independent interaction cycle carried out in BS includes baseline, association, dissociation, regeneration and back to baseline steps as follows: after obtaining a stable signal by acquiring several baselines, varying concentrations of aptamer prepared in BS (35 μL) were simultaneously injected in both channels and the association phase was recorded for 10 min. After draining the cuvettes, 50 μL of BS were injected and the dissociation phase was monitored for 5 min resulting in a decrease of the resonance angle until a stable value was achieved. Finally regeneration step was accomplished by several injections of 50 μL of 0.05 M HCl (1 min) followed by baseline registration until the initial value was reached. All steps were recorded under needle-based agitation to avoid mass transfer control.

2.5. Aptamer–PWG interaction assays on microtiter plates

Microtiter plates (Corning High Bind, NY) were modified overnight at 25°C with 100 μL of 5 $\mu\text{g mL}^{-1}$ PWG in 50 mM sodium carbonate/hydrogen carbonate buffer (pH 9.6). Then, the wells were washed with 10 mM Tris–HCl pH 7.4 three times. For each binding interaction assay, 100 μL of increasing concentrations (60–500 nM) of the corresponding native or 6-FAM-tagged gli1 aptamer was dispensed on the wells. After 30 min at 25°C under mild microtiter plate stirring, the aptamer solutions (free fraction) were transferred to a vial to be measured by ICP-MS (native aptamer) or to a microtiter plate for fluorescence measurements (6-FAM aptamer).

For the ICP-MS measurements, the free aptamer fraction was diluted 1:10 with 1% v/v HNO_3 to reduce the salt concentration. An Element 2 ICP-MS (Thermo Elemental, Bremen, Germany) operated in medium resolution mode ($R = 3000$) for detection of $^{31}\text{P}^+$ was used. This kind of double focusing instrument improves the detection of phosphorus, which is obscured by the low ionization efficiency and the spectra interferences [25]. ^9Be was used as an internal standard. The operating conditions were the following: 1280 W RF power, 14.2 L min^{-1} coolant gas, 0.91 L min^{-1} auxiliary gas, 0.965 L min^{-1} nebulizer gas, sample flow 0.4 mL min^{-1} . Inorganic phosphorus in 1% v/v HNO_3 was used as standard for calibration from 5 to 1000 ppb in the presence of 10 ppb of Be.

For the fluorescence measurements, a Flx800 microplate reader (BioTek®, VT, USA) was used. The excitation and emission

wavelengths were fixed at 480 and 528 nm, respectively. A calibration plot for each labeled aptamer in the range of 50–500 nM was needed. Differences in fluorescent response at identical total ssDNA concentration as estimated by UV–vis measurements at 260 nm were found probably due to discrepancies in the yield of the commercial labeling process.

As in the case of ICP-MS, the excellent correlation between the analytical response and the DNA concentration allowed the calculation of the unbound aptamer concentration after interaction with PWG.

3. Results

3.1. Homogeneous aptamer–peptide interaction

First, the evaluation of the binding affinity between gli1 aptamer to immunodominant peptide 33-mer was addressed. Among the available techniques, ITC provides not only direct measurement of all thermodynamic parameters from a single experiment but also valuable information about binding stoichiometry. It is also the method of choice to thermodynamically characterize binding reactions with affinity range from mM down to high nM as expected for most aptamer–protein interactions. This restriction derives from the need for performing the experiments at μM concentration of both reagents to generate enough binding heat change [7]. ITC measures the heat involved during macromolecular interactions. Usually small aliquots of the ligand are stepwise injected into a solution of the macromolecule (typically a protein) at a constant temperature. In our case the aptamer is larger (12 kDa) than the peptide (5 kDa), so the aptamer was titrated and the heat absorbed/released measured as a function of time. In Fig. 1 representative data of ITC experiments at 17 and 37°C are shown. In Table 1, the thermodynamic parameters obtained by ITC determined by fitting the experimental data to a single-site binding isotherm.

According to the estimated K_d values (45 ± 10 and 17 ± 6 nM at 17 and 37°C , respectively), a strong affinity between gli1 aptamer and peptide exists, especially at high temperatures, where the value approaches the limit of detection of the technique. The binding stoichiometry calculated from the titration equivalence point is 1:1, which is reasonable for a small peptide.

The aptamer binding is entropically driven since a large positive entropy at both temperatures is measured, while the enthalpic contribution is small but positive, that is, unfavorable to the binding. This thermodynamic signature is usually associated to the so called hydrophobic effect mainly arising from solvent reorganization. The entropy variation related to the formation of a ligand–macromolecule complex originates from two contributions: reduction of the conformational degree of freedom of both molecules, which is usually unfavorable ($\Delta S < 0$), and solvation effects [26]. When a hydrophobic molecule is involved, hydration is entropically unfavorable because of disruption of the stable hydrogen-bonding network of the bulk water and the formation of a shell of strongly bound water molecules to compensate [27]. This leads to ordering with a penalty in entropy. Binding regarded as a withdrawal of non-polar groups from water (desolvation process) can account for a favorable entropy change [26,28]. Then, a positive entropy change is a strong evidence of removal of water molecules from the complex interface [29]. The negligible heat capacity minimizes the role of hydrophobic interactions in the DNA–peptide binding.

A binding to DNA entropically driven is not unusual even for intercalating agents where stacking interactions are expected to give negative enthalpy changes as major contribution to the binding free energy [30]. The interaction of aptamers with small molecules such as antibiotics [31,32], adenosine [33] or

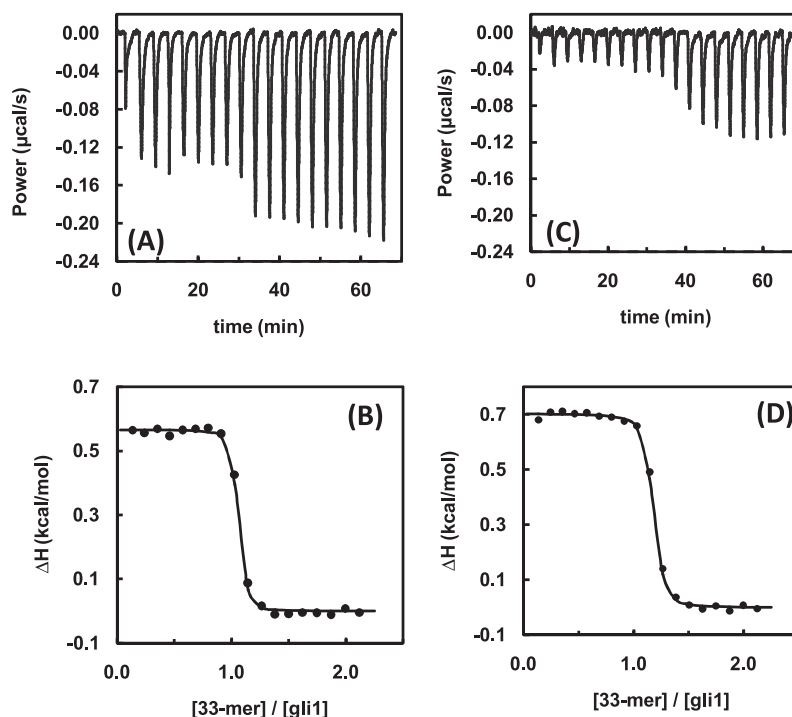


Fig. 1. ITC titration of 33.4 μM gli1 aptamer with 338 μM 33-mer in BS at 37 $^{\circ}\text{C}$ (A and B) and 17 $^{\circ}\text{C}$ (C and D). Top panels: power as a function of time. Bottom panels: integrated heat change per injection with the fitted curve to a single-site binding model.

L-tyrosinamide [34] was found to be controlled by enthalpy negative changes. With proteins, both thermodynamic signatures were found even for a single binding site, the exosite I of thrombin, with two different ligands [35]. The binding of thrombin [35], lysozyme [36] and peptide prion [37] aptamers is also entropically driven. In the lysozyme case, this behavior is accompanied by an increase in K_d with temperature, which was explained by progressive disruption of a weak secondary aptamer structure. In the case of gli1 aptamer, the secondary structure is very weak at room temperature [18] but exactly the contrary trend is observed.

3.2. Heterogeneous aptamer–peptide interaction

In heterogeneous aptassays and aptasensors, the aptamer is usually anchored to a solid surface assuming a minimal influence on affinity, but the ligand can be also immobilized in competitive or displacement assays. Since the immobilization of each partner can induce different loss in affinity both approaches were examined.

Thiolated gli1 aptamer was assembled on Au surfaces at a positive potential to accelerate the monolayer formation [22] and then, the surface was blocked with mercaptohexanol. Successive additions of increasing concentrations of 33-mer peptide were followed by faradaic impedance spectroscopy (FIS). An increase in electron transfer resistance (R_{et}) with time was observed for each addition due to the progressive blocking of the electrode surface

(Fig. 2(A) inset). The equilibrium R_{et} values at each concentration were expressed as percentage of R_{et} increase through the equation

$$S\% = (R_{eq} - R_0) / R_0 \times 100 \quad (1)$$

where R_{eq} denotes the R_{et} at equilibrium and R_0 the initial R_{et} . The plot of $S\%$ vs the peptide concentration takes the general form of a rectangular hyperbola. By non-linear fitting (Fig. 2(A)), a value of $K_d = 98 \pm 48 \text{ nM}$ was obtained. This calculation implies that the bound peptide concentration is negligible relative to the total peptide concentration in solution. Surface coverages in the range of 10^{12} aptamers cm^{-2} are typically achieved that implies an amount of about 30 fmol of aptamers on the surface. Since the lowest amount of 33-mer tested was 100 pmol it is apparent that this assumption is reasonable.

The sensing phase prepared did not exhibit significant unspecific binding. A set of blank experiments was carried out on bare Au and with sensing phases prepared with mercaptohexanol alone, or a mixture of a scrambled DNA sequence and MCH. These surfaces were incubated with 100 nM of 33-mer. Additionally, a mixed SAM prepared with gli1 was incubated with a 57-mer peptide control previously used for counter selection steps in the SELEX process [18]. In all cases, the increase in R_{et} was negligible with respect to that obtained in the specific interaction between 33-mer and gli1 aptamer (Fig. 2(B)).

SPR is a better suited technique for biomolecular interaction characterization. Like FIS is a label-free technique but kinetics is continuously monitored. Since peptide is a small molecule the direct observation of binding on self-assembled monolayers of aptamers is only detectable at high concentrations. So, this technique was used to evaluate the binding reaction with 33-mer peptide immobilized on the surface. To do this, a mixed monolayer of mercaptopropionic acid and mercaptoundecanoic acid was prepared and streptavidin was covalently linked through carbodiimide chemistry. About 200 m^2 of streptavidin were immobilized that is equivalent to 1.6 ng mm^{-2} assuming 120 m^2 corresponds to 1 ng mm^{-2} according to instrument specifications.

Table 1
Thermodynamic parameters and stoichiometry obtained by ITC for the gli1 aptamer–33-mer binding.

	17 $^{\circ}\text{C}$	37 $^{\circ}\text{C}$
ΔG (kcal mol^{-1})	-9.75 ± 0.14	-11.1 ± 0.3
ΔH (kcal mol^{-1})	0.70 ± 0.01	0.57 ± 0.01
ΔS (cal $\text{K}^{-1} \text{mol}^{-1}$)	36.0 ± 0.5	37.4 ± 0.7
n	1.12 ± 0.01	1.07 ± 0.01

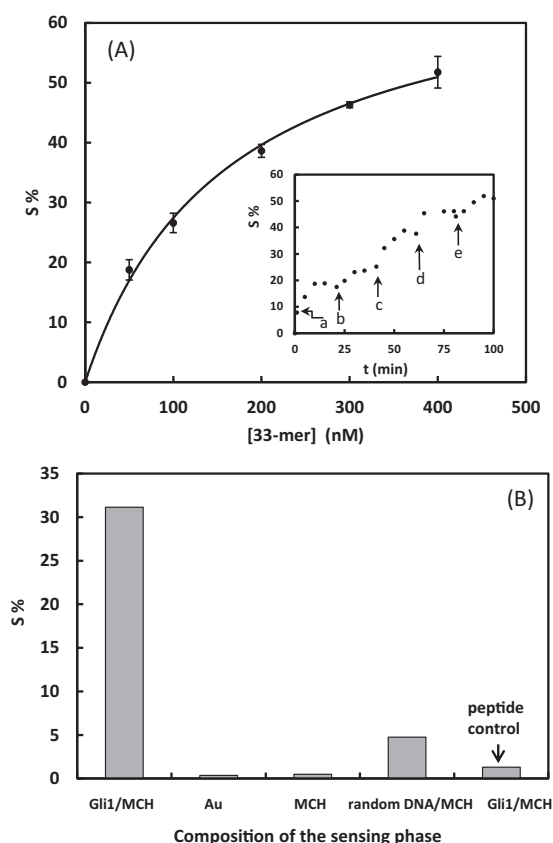


Fig. 2. (A) Binding isotherm obtained from FIS experiments fitted to the single-site binding model. Inset: representative data to estimate the K_d between thiolated gli1 aptamer and 33-mer peptide. Normalized R_{et} (S%) with time after successive incubation of a Au electrode modified with a mixed SAM of gli1 aptamer and mercaptohexanol with (a) 50 nM; (b) 100 nM; (c) 200 nM; (d) 300 nM; (e) 400 nM of 33-mer. (B) Variation of the R_{et} (S%) of different sensing phases to 100 nM of 33-mer and peptide control (last column).

An averaged angle shift of 37° was measured when adding 25 nM biotinylated 33-mer peptide, indicating that there was more than one binding site available for peptide binding. In the reference channel 500 nM of biotin was used to fill the streptavidin binding sites. Both channels were then challenged with concentrations of aptamer ranging from 50 nM to 5 μ M. After each interaction, a regeneration step with 0.05 M HCl was carried out until the initial baseline was recovered. The angle shifts at equilibrium were plotted against the aptamer free concentration calculated as the difference between the total aptamer in solution and the surface concentration of complex estimated from the angle shift. At all concentrations the amount of complex formed was negligible with respect to the total aptamer concentration in solution. After fitting to Langmuir isotherm, K_d was estimated to be 51 ± 16 nM. Although the mean value is slightly lower than the one estimated by FIS suggesting a larger influence on affinity when the aptamer is immobilized, both K_d values are identical within the experimental error. This indicates that there are subtle differences when immobilizing the aptamer through a thiol or the peptide through a biotin–streptavidin affinity interaction. The K_d value in solution at 25°C was estimated to be about 29 nM. Considering this value, in both cases the K_d values for the heterogeneous reaction are substantially higher than that of the homogeneous one calculated by ITC. It is worth noting that the peptide has a spacer of six histidine residues adjacent to the biotin and aptamer has a C6 spacer, which can contribute to somehow minimize the steric hindrance of the surface.

We have previously estimated the K_d of gli1 aptamer from a binding curve obtained with the peptide immobilized onto magnetic beads and chronoamperometrically (CRamp) measuring the aptamer bound using an streptavidin–enzyme conjugate [18]. In that experiment, the aptamer was biotinylated, so the influence of the labeling can be determined. A K_d value of 102 ± 11 nM indicates that the biotin tag also decreases the affinity. However, it is not significantly different from the K_d value obtained by FIS when the aptamer is immobilized. This points out that the influence of aptamer modification is larger than that of the peptide. This is reasonable although the peptide is half the size of aptamer. The binding site is probably constituted by a few amino acids while the entire aptamer might fold upon ligand binding. The separate influence of the tagging and the surface steric hindrance is difficult to interpret. K_d almost doubles from non-labelled (SPR) to biotin-tagged aptamer (CRamp). This suggests that the tagging effect dominates over the steric hindrance effect. However, the thiol group is much smaller than biotin, so its chemical interaction with the aptamer is expected to be minimal. Its strong impact on the affinity might be determined, in this case, by the proximity to the surface.

3.3. Heterogeneous gliadin–aptamer interaction

In real samples, the receptor has to recognize the 33-mer peptide within the native protein. Previous studies suggest that the aptamer developed binds to the complete protein [18] but the extent of the affinity in comparison with the peptide has not been accomplished yet. The K_d for the homogeneous reaction between the gliadin standard, PWG, and gli1 aptamer is difficult to estimate because of the limited solubility of the protein in aqueous solution. So only the constant for the heterogeneous reaction was estimated immobilizing the protein on microtiter plates. After interaction with gli1 aptamer in the concentration range from 60 to 500 nM, the unbound aptamer fraction (F), was determined by ICP-MS after tenfold dilution with 1% v/v HNO_3 to reduce the salt concentration. In spite of its high first ionization potential and the spectral interferences, the use of a double focusing instrument allowed the construction of calibration plots with inorganic P from 5 to 1000 ppb with excellent correlation ($r > 0.9997$). The bound fraction (B) was estimated as the difference between the total and free aptamer. Plot of B vs F did not fit to the one-binding site model indicating that gliadin has several binding sites for the aptamer. This is not unusual because gliadin is a mixture of peptide chains containing a high percentage of prolines and glutamines in repetitive motifs. It is known that the epitope for antigliadin antibodies G12 and A1 raised against 33-mer are hexameric and heptameric regions, respectively [38]. Since aptamer is smaller than antibody a small fraction of the 33-mer is also expected to be the epitope, so the chances for finding homologue sequences throughout the overall sequence of gliadin is high. Therefore, the saturation curve (Fig. 3(A)) was non-linearly fitted to the Hill equation (Eq. (2)), a simplification of the general Adair equation that accounts for macromolecules with interacting binding sites, that is, cooperativity.

$$B = \frac{F^n}{K_d + F^n} \quad (2)$$

where B is the bound aptamer concentration, F is the free aptamer concentration and n is a measure of the cooperativity.

Blank experiments on bare microtiter plates gave rise to undetectable changes in the concentration of the aptamer in solution, ruling out the unspecific binding to microtiter plates.

Using this approach, the K_d value is 58 ± 2 nM, with a cooperativity index of 3.4 ± 0.2 . This represents a value slightly

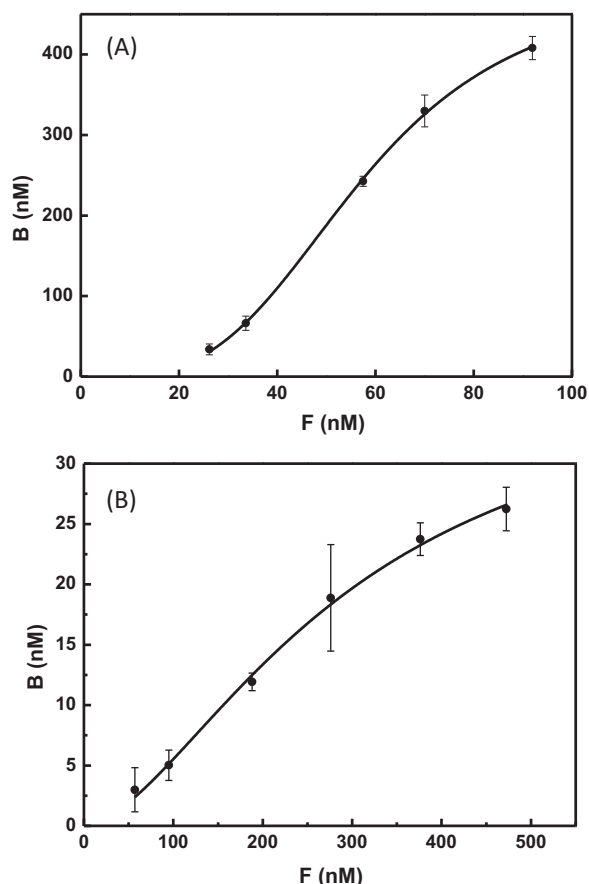


Fig. 3. Binding isotherms obtained from ICP-MS (A) and fluorescence (B) experiments at 25 °C between PWG and gli1 aptamer.

higher than that of the peptide, which correlates with previous studies with magnetic particles and electrochemical detection that suggested a lower affinity for the native protein. However, those experiments were carried out with a labeled aptamer, so the influence of a small tag was further evaluated using 6-carboxy-fluorescein (6-FAM) as fluorophore at 5' end to be able to directly monitor the free aptamer fraction by fluorescence. Performing analogue experiments on microtiter plates and fitting the binding curves to the Hill equation (Fig. 3(B)), the K_d value (208 ± 54 nM) clearly increases relative to the untagged aptamer. It is worth noting that in all cases, n values were always higher than 1 indicating a positive cooperativity.

4. Discussion

The immobilization and labeling tag effects we have observed for gli1 aptamer with regard to 33-mer and gliadin binding are summarized in Table 2. Gli1 was obtained using a short peptide (the 33-mer immunotoxic peptide) as a target, and it binds two

related ligands, 33-mer peptide and PWG-protein, with similar affinities. The possibility of generating aptamers from specific short amino acid sequences to bind that epitope in the protein was previously shown with a peptide from Rev protein but this strategy was never generalized [39] and led to up to 16 times decrease in affinity when challenged against the whole protein [40]. In this work we have shown that it is possible to obtain aptamers with similar affinity for the full-length protein. Although the highest affinity is obtained for the homogeneous interaction between gli1 and 33-mer peptide (ITC measurements), the immobilization of ligand produces only a slight increase in K_d (SPR measurements). Even when the whole protein, PWG, was adsorbed on the surface, only 1.7 fold less affinity than for 33-mer in solution was observed. This is attributed to a positive allosteric effect not previously reported to the best of our knowledge for other aptamers.

Remarkably, the aptamer evolved against the 33-mer presents a binding affinity larger than most of the few aptamers raised against food allergens. Currently aptamers for food allergen sensing are being developed due to steady increase of allergenic individuals to a great variety of food ingredients. The discovery of novel receptors with enhanced sensing properties fuels this trend. Although in all cases allergenic proteins were used as targets, a wide range of K_d values were reported. Lupine and peanut aptamers showed affinity larger than 300 nM when measured by SPR. In the former, the protein was covalently immobilized [41] and in the latter a biotinylated aptamer was bound to streptavidin coated surfaces [42]. The affinity of the peanut aptamer was also estimated by fluorescence anisotropy (FA) and nonequilibrium capillary electrophoresis of equilibrium mixtures (NECEEM) obtaining higher values than with SPR where immobilization of a biotinylated aptamer is needed. This increase was attributed to the unpurified aptamer used in FA obtained from the SELEX and to the high voltage applied in NECEEM that somehow disrupt the complex.

More interesting is the behavior of lysozyme aptamers. First an RNA aptamer against a biotinylated lysozyme was evolved using automated SELEX that showed slightly increased affinity for the unlabeled protein [43]. Since the aptamer sequence was published as the DNA template used to generate it, other researchers erroneously employed that DNA sequence in various successful analytical approaches [4]. Subsequent investigations revealed that the DNA-lysozyme interaction was governed by electrostatic interactions rather than sequence specificity [36]. In that detailed study, ITC experiments showed a binding driven by entropy, with a small positive enthalpy change and a high positive entropy change, similarly to that found for gli1 aptamer. Authors assigned this energetic pattern to electrostatic interactions by comparison with another adsorption process. However, in the case of gli1-33-mer interaction, the absence of charged amino acids in the sequence along with the much higher ionic strength used during interaction rules out this possibility. K_d values around 30 nM for the best clone against lysozyme were reported by filter binding assays [44], an order of magnitude lower than those for lupine and peanut. Gli1 aptamer raised against a much smaller molecule showed, nonetheless, similar affinity for the peptide and slightly lower

Table 2

Mean values of the binding constants and cooperativity degree (mean values $\pm$ SD) for the interaction between aptamer gli1 and 33-mer peptide or PWG.

Technique	Immobilized species	Species in solution	T (°C)	K_d (nM)	n
ITC	None	33-mer, gli1	17	45 ± 10	1.01 ± 0.01
			37	17 ± 6	1.12 ± 0.01
FIS	SH-gli1	33-mer	RT	98 ± 48	
SPR	Biotin-33-mer	Gli1	25	51 ± 16	
CRamp	Biotin-33-mer	Biotin-gli1	25	102 ± 11 [18]	
ICP-MS	PWG	Gli1	25	58 ± 2	3.4 ± 0.2
Fluorescence	PWG	6-FAM-gli1	25	208 ± 54	1.7 ± 0.5

RT: room temperature.

for the protein, which is remarkable taking into account the apolar nature of the target. Newly developed DNA aptamers against egg white lysozyme shown the lowest K_d values (6 nM by SPR) [45] for food allergens.

The group/molecule attached at 5' end of gli1 aptamer has a negative but diverse influence on the affinity. In our case, immobilizing the peptide resulted somehow less deleterious than immobilizing the aptamer with a thiol group. Biotinylation of aptamer, however, could have a milder influence on the affinity because the average K_d obtained when challenged to biotinylated 33-mer on magnetic beads matches the one measured with the thiolated aptamer and native peptide. A larger adverse effect of thiol groups with respect to biotin was observed for the thrombin aptamer [17].

Labeling aptamer at its 3' or 5' end with small molecules for detection has usually a limited influence on affinity [13,16,46]. As an example, labeling the protein with a fluorophore sometimes resulted more damaging than biotinylating the aptamer and this effect was dependent on the aptamer clone [44]. In our case, when the aptamer was labeled with 6-FAM, a clear decrease in binding ability for PWG was found even when a cooperative mechanism is operating. Although a direct comparison must be taken with care, 6-FAM seems to be more damaging for the affinity than biotin.

5. Conclusions

In this work, evaluation of the affinity of the recently developed aptamer for gliadin was carried out. Modification of the aptamer at 5' with thiol, biotin and 6-FAM allowed us to gain insight into the recognition ability. The solution phase binding constant between gli1 aptamer and 33-mer peptide was significantly low for such a small molecule. Interestingly, the binding to the whole protein was only slightly affected, which is unusual for aptamer developed against a peptide fragment. This encourages the implementation of that strategy for the generation of future aptamers.

Immobilization of peptide through biotin–streptavidin interaction resulted beneficial when compared with the immobilization of aptamer through thiol groups. This finding points to the potential advantages of developing competitive assays, which are desirable in the food allergen field because of hydrolysis of proteins during processing. The modification of gli1 aptamer with a fluorophore showed an unexpectedly substantial negative effect, probably larger than that of biotin. As a consequence, for future applications, the use of biotinylated gli1 aptamer is strongly recommended.

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