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Fluorometric Aptasensor: Evaluation of Stability and Comparison to Standard ELISA Assay

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Abstract—Celiac disease (CD) is an immune system disorder that is related to eating gluten. As gluten-free (GF) diet has become a concern of many people for health reasons, a gold standard had to be nominated. Enzyme-linked immunosorbent assay (ELISA) has taken the seat of this role. However, multiple limitations were discovered, and with that, the desire for an alternative method now exists. Nucleic acid based aptamers have become of great interest due to their selectivity, specificity, simplicity, and rapid-testing advantages. However, fluorescence-based aptasensors have been tagged as unstable, but lifespan details are rarely stated. In this work, the lifespan stability of a fluorescence-based aptasensor is shown over a 8-week long study displaying the accuracy of the sensor and false negatives. This study follows 22 different samples, including GF and gluten-rich (GR) and soy sauce products, off-the-shelf products, and reference material from laboratories; giving a total of 836 tests. The analysis shows an accuracy of correctly classifying GF and GR products of 96.30% and 100%, respectively, when the protocol is augmented with molecular sieves. The overall accuracy remains around 94% within the first 4 weeks and then decays to 63%.

Keywords—Aptasensor, PEG, rGO, FAM, RM, ELISA.

I. Introduction

CELIAC disease (CD) is an immune system disorder triggered by ingesting gluten [1]. CD has gathered the attention of many researchers due to its growing incidence of 7.5% every year [2]. The repercussions of eating gluten as a CD patient lead to unfavorable outcomes like diarrhea, vomiting, and even worse outcomes if not treated promptly [1]. As of now, the most reliable treatment is adopting a gluten-free diet [2]. This increasing interest in gluten-free food relates to CD patients and the popularity of a healthy diet among non-CD patients [3]. Therefore, the gluten-free label is granted only for food that contains no more than 20 mg of gluten per kg (or 20 ppm) as standardized by Codex Alimentarius in 2008 [4]. Nevertheless, the demand for gluten-free labeled food employed the use of a gluten quantification standard: ELISA, now baptized as "The gold standard" for foodborne allergen quantification [5], [6]. ELISA has served to ensure compliance with the gluten-free regulation to date. In 2010, two Brazilian researchers found that among 185 different gluten-free labeled products 13% still contained more than 20 ppm [7], [8]. And 5 years later, in 2015, Thompson and Simpson did the same experiment with 158 gluten-free labeled products, and 5.1% of them were also mislabeled [9]. Additionally, the implementation of ELISA in the gluten-free food industry, the required specialized staff, and the complexity of handling GF products along the chain of production have contributed to the cause of the increasing the prices of such products reaching up to 450% (242% on average) more expensive than its regular version [10]. [11]

Subsequently, alternative gluten quantification methods have been sought. Scherf and Poms studied various analytical sensors for tracing gluten: immunological, proteomics-based, and genomics-based. Aptamer-based methods outstood as novel methods due to their high sensitivity and lower cross-reactivity [12]. Shaban and Kim reviewed the various aptamer-based methods: colorimetric, fluorometric, and electrochemical. Aptamer-based fluorometric sensors were also considered very sensitive and suitable. However, they concluded that photobleaching and instability was considered an obstacle to their development [13]. Röthlisberger and Hollenstein agree with the limiting stability of the aptamer [14]. Despite the shortcomings of the aptamer's stability, researchers have demonstrated great interest [15], [16] due to its portability, cost-effectiveness, lower time consumption, and reliability [17].

Therefore, this study focuses on exploring this uncertainty aiming to provide quantitatively a graphical representation of the stability of the fluorometric aptamer-based sensor for gluten in terms of accuracy over time. The accuracy is assessed utilizing gluten-free (GF) and regular food samples, where GF samples are expected to contain no more than 20 ppm of gluten, and regular samples contain grater than 20 ppm of gluten. The assessment finds the ratio of correctly classifying the samples based on their respective gluten concentration. The graphs represent the accuracy over time of reference material (RM) and off-the-shelf samples separately. The RM is utilized because it is believed that the current assessments lack of reliable RM [12], [17]. Hence, this study uses the same RM and ELISA kit for which the RM was quantified.

II. Materials and Methods

A. Materials

The gli4 DNA aptamer sequence is CCAGTCTCCCGTT TACCGCGCCTACACATGTCTGAATGCC. The 6FAM aptamer is labeled in the 3' prime end with a fluoroscence derivative and was obtained from Integrated DNA Technologies (IDT www.idtdna.com [18]) as a lyophilized product. The FAM-aptamer is diluted in Tris-EDTA buffer (100 μM) and stored at 253 K. Tris-EDTA (TE) buffer, are from IDT.

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The R5 antibody sandwich ELISA kit is obtained from R-Biofarm (QQPFP epitope - RIDASCREEN Gliadin R7001 [19]).

The RM is obtained from Trilogy Labs, measured with R-Biopharm RIDASCREEN Gliadin R7001. This study refers the RM with a mean of 6.2 $\pm$ 1.5 ppm gluten and a coefficient of variation (CV) of 24.8% as "6ppm" (Lot N°121110), the RM with a mean of 15.7 $\pm$ 3.0 ppm gluten and a CV of 19.1% as "15ppm" (Lot N°121109), the RM with a mean of 25.0 $\pm$ 3.1 ppm gluten and a CV of 12.2% as "25ppm" (Lot N°121103), the RM with a mean of 43.6 $\pm$ 5.7 ppm gluten and a CV of 13.1% as "43ppm" (Lot N°121111). All RM were obtained from Trilogy Labs [20], and the GF samples and regular samples were collected from a local grocery store.

The Phosphate Saline Buffer (or PBS buffer), graphene oxide, gliadin, molecular sieves, 4-dimethylaminopropyridine (DMAP), sodium chloride (NaCl), Dimethyl sulfoxide (DMSO), 1-etyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), hydrazin (N_2H_4), sodium phosphate dibasic (Na_2HPO_4), sodium phosphate monobasic (NaH_2PO_4) and ethanol 95% are from Sigma Aldrich [21].

B. Methods

1) Aptamers: Aptamers are single short strands of oligonucleotides (either DNA or RNA) tailored to bind to a specific target through a complex selection process called SELEX [22]. In this case, our group sought an aptamer that targets and binds the triggering factor of celiac disease patients: gliadin; a component of gluten. Amaya et al. found few DNA aptamer sequences that bind to the 33-mer peptide, which 33-mer peptide is the immunodominant section of gliadin and the triggering factor of celiac disease [23].

2) Fluorescence Aptasensor: 'turn on' method: Based on the previous work of Nikhil [24]; the graphene oxide (GO) is utilized for its quenching properties caused by the Fluorescence resonance energy transfer (FRET) phenomenon [25]. The FRET quenches the FAM aptamer fluorescence until the aptamer binds to its target (also called the 'turn-on' strategy) [16], [26], [11]. The more bindings the aptamer makes, the more fluorescence in the assay. This fluorescence is measured by a well-plate reader and compared to a standard curve to quantify the gluten concentration in the sample through interpolation.

3) rGO-PEG FAM aptamer preparation: 50 ml of DMSO and 15 ml of GO are sonicated for 15 minutes. After sonication, GO and DMSO are mixed in a round bottom flask with a magnetic stirrer. The flask is sealed using septum and parafilm. The oxygen is removed out of the flask using a vacuum pump. Nitrogen gas is injected into the sealed flask. This was archived using a nitrogen-filled balloon with a syringe attached and sealed with parafilm. After 5 minutes, the nitrogen gas is left attached and the stirrer is turned on for 10 minutes. In the meantime, one prepares 150 mg of PEG, 300 mg of DMAP, and 195 mg of EDC. The PEG is dissolved in 2.5 ml of distilled water (DI water) and the EDC in 2.5 ml of DI water in different containers. The diluted PEG and DMAP are added to the flask using a syringe. The Nitrogen balloon is removed with vacuum for 5 minutes, then the vacuum is removed with the nitrogen balloon for another 5 minutes. The previous step is repeated 2 more times. The flask is unsealed and EDC is added. The flask is sealed back with septum and parafilm. The oxygen is removed out of the flask using vacuum for 5 minutes and a nitrogen balloon is injected overnight. The flask is unsealed and 21 μl of hydrazine is added followed by 1 hour of stirring between 343 K to 373 K The flask is set at room temperature to cool down for 10 minutes. The content of the flask is added in 2 falcon tubes of 50 ml in equal quantities. The falcon tubes are centrifuged for 10 minutes at 5000 rpm, and after the removal of the supernatant; 7.5 ml of DI water is added to each falcon tube and vortex for 30 sec. The previous step is repeated 2 more times. The falcon tubes are combined in a single falcon tube and labeled "rGO-PEG". At this point, the rGO-PEG is stored at 253 K. This is the initial day of the preparation of the stock of rGO-PEG.

The day prior to the testing day, rGO-PEG is taken out of the freezer and sonicated for 20 minutes. The FAM-aptamer that should be stored at 1000 μl of rGO-PEG is added to a 1.5ml Eppendorf tube along with 100 μl of FAM-aptamer. The tube is gently mixed up and down overnight. The tube is centrifuged for 10 minutes at 2000 rpm, and after removing the supernatant; 1000 μl of TE buffer is added and vortex. The previous step is repeated 2 more times. The tube is labeled "rGO-PEG FAM aptamer".

4) Gluten extraction from sample:

1) The work area and tools are thoroughly cleaned with 70% ethanol
2) The food sample is ground with a pill pulverizer machine
3) 250 mg of powdered food sample or 250 μl of the liquid sample is added to a 15 ml falcon tube
4) 100 mg of activated charcoal/molecular sieves or 250 mg of skimmed milk powder is added in the same tube
5) 2.5 ml of ethanol 70% is added and vortex for 30 seconds (dilution factor of 11)
6) The falcon tube is sealed and incubated in a water bath for 10 minutes at 323 K
7) The falcon tube is set outside the water bath for 5 minutes at room temperature
8) The falcon tube is unsealed and 7.5 ml of TE buffer is added and vortex for 30 seconds (dilution factor of 3.7272)
9) The falcon tube is sealed and centrifuged for 10 minutes at 5000 rpm
10) The supernatant is collected
11) 80 μl of the supernatant is diluted in 920 μl of TE buffer for an additional 12.5 dilution factor (for a total dilution factor of 512.5)

5) Sample quantification: 940 μl of TE buffer are added to a 1.5 ml Eppendorf tube. The extracted gluten is briefly vortex and 60 μl of it is added to the tube. After a quick vortex, 100 μl of the tube is added to a new 1.5 ml Eppendorf tube for dilution. 1187 μl of PBS and 13 μl of rGO-PEG FAM aptamer are added to the new tube. After a quick vortex, 200 μl of the new tube is added to a well-plate. All samples follow

TABLE I
GLUTEN QUANTIFICATION OF REFERENCE MATERIAL, GF AND REGULAR FOOD SAMPLES USING APTAMER-BASED SENSOR AND ELISA

	Samples	Aptasensor		ELISA
		Without Sieves	With Sieves	Without Skim milk
		Gluten (ppm)	*Gluten (ppm)*	*Gluten (ppm)*
RM	6ppm	18 ± 6	19 ± 6	6 ± 1
	15ppm	24 ± 12	17 ± 12	19 ± 6
	25ppm	23 ± 7	27 ± 9	25 ± 1
	43ppm	38 ± 10	41 ± 8	53 ± 12
Gluten Free Samples	Crackers	4 ± 2	6 ± 1	5 ± 4
	Buns	5 ± 3	4 ± 1	7 ± 3
	Blueberry Waffle	3 ± 4	4 ± 5	25 ± 27
	Waffle	3 ± 3	1 ± 2	3 ± 2
	Bagel	5 ± 2	5 ± 3	1 ± 1
	Chocochip cookie	22 ± 15	13 ± 1	4 ± 3
	Choco doughnut	14 ± 11	1 ± 2	9 ± 5
	Aero	14 ± 11	18 ± 3	11 ± 10
	Soy sauce	111 ± 129	16 ± 2	5 ± 1
Regular Samples	Crackers	36 ± 7	12 ± 1	313 ± 121
	Annas	35 ± 6	37 ± 2	192 ± 30
	Hotdog bun	28 ± 10	34 ± 7	131 ± 23
	White bread	27 ± 9	36 ± 5	121 ± 14
	Beagle	35 ± 16	49 ± 6	135 ± 19
	Chocobar	44 ± 9	55 ± 8	2 ± 2
	Kitkat	29 ± 9	28 ± 3	196 ± 25
	Soy sauce	8240 ± 6093	15261 ± 640	3 ± 3
	Malt Vinegar	27 ± 15	32 ± 1	3 ± 1

the protocol in parallel. The well-plate is measured with a fluorescence well-plate reader (490/520).

III. RESULTS

A. Overcoming Aptamer Limitations

This study contains the results of gluten quantification of 22 different samples giving a total of 836 tests. The tested samples were classified by their content or purpose: RM, GF, and regular samples. The RM served to contrast the reliability of each method.

Gonzalez et al. have previously stated that the aptamer gli4 is unable to quantify gluten concentration from chocolate-rich samples even with addition of skim milk powder or gelatin [27]. ELISA has overcome the limitation of testing gluten concentration in chocolate-rich samples by adding Skim milk powder as suggested by the ELISA kit provider [28]. However, ELISA still suffers from limitations on fermented and hydrolyzed food samples. The current solution is testing the ingredients of GF products before they go through fermentation or breakdown [29].

The gli4 aptamer limitation on chocolate samples is overcome by adding 100 μg of molecular sieves. Adding molecular sieves also addresses the measurement of gluten in soy sauce and vinegar. As shown in Table I, the addition of molecular sieves improved the results in most of the samples; including chocolate samples, soy sauce, and malt vinegar. An exception to this rule is the regular cracker; where addition of molecular sieves causes a problem.

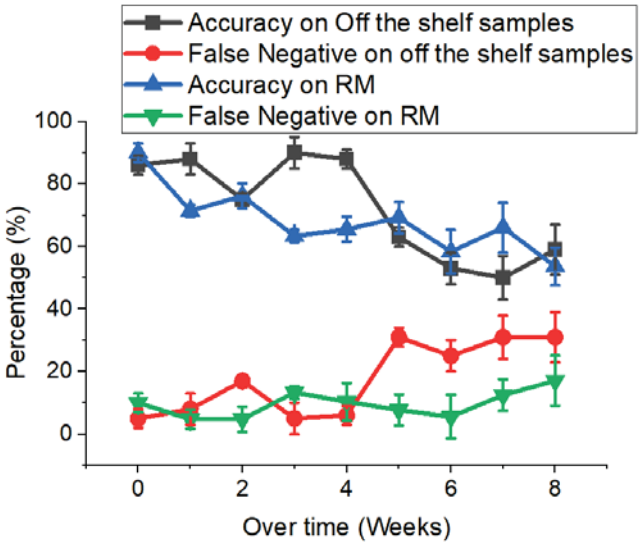

Fig. 1 Aptasensor's accuracy over time (in weeks) on different samples: Reference material (RM), Off the shelf samples, and the false negatives of each one. Starting from the first 3 days since the synthesis of the rGO-PEG as week 0

In the case of the aptasensor, the addition of molecular sieves in the assay gives classification accuracy of GF samples to 96.3%, and 88.9% on regular samples. On the other hand, not implementing the molecular sieves, the accuracy decreases to 71.1% and 73.3% respectively.

As shown in Table I, adding molecular sieves might worsen the measurements on certain types of food; like regular crackers. Therefore, if the addition of molecular sieves is cleverly done on each type of sample the accuracy of this application improves to 96.30% on GF samples and 100% on regular samples. Nevertheless, after a few days of using the synthesizing rGO-PEG, the accuracy of the aptasensor was observed to drop with no changes in the method.

B. Accuracy over Time

As shown in Fig. 1, the accuracy of the aptasensor decreases over time, the x-axis represents the weeks passed since the day of preparation of r-GO PEG. The accuracy is calculated by averaging the accuracy of correctly classifying the food samples to their correct category using both methods: with and without molecular sieves. The average is taking into consideration of 4 runs of experiments over 8 weeks. In this case, week 0 means the average of the first 3 days from the day of preparation of r-GO PEG, week 1; day 1 to 7, week 2; day 8 to 14, and so on.

It is perceived in Fig. 1 that in the first 4 weeks the aptasensor decreases drastically down to 63% of accuracy. However, within the same range, there is little to no false negatives. Meaning most of the inaccuracies come from false positives on GF samples.

In the case of a celiac disease patient, the most critical result is the false negatives, as a false positive means the patient misses out on that food with no harm. After the fourth week, most of the inaccuracies come from false negatives, meaning

regular samples are being classified as GF samples. This implies a lack of fluorescence or recognition of the aptamer's target.

IV. Future Work

In order to understand the behavior of this rGO-PEG aptasensor application, different extraction reagents or stabilizing agents have to be implemented and studied. Furthermore, this study has not explored colored, heated nor hydrolyzed food samples. So, in order to have a better comparison with ELISA, all ELISA limitations have to be contrasted with the aptasensor.

Finally, in order to avoid false negatives, all types of food samples have to be analyzed with and without molecular sieves. This study would indicate what samples should be measured with molecular sieves when using this aptasensor.

V. Conclusion

In this work, a 'turn on' fluorescence aptamer-based sensor (aptasensor for short) has been studied. The aptasensor accuracy of correctly classifying the food samples as GF or regular based on their gluten concentration has shown to be up to 96.30% on GF and 100% when molecular sieves are implemented for specific samples. However, the accuracy of the aptasensor decreases over time, where it drastically changes in the 4th week down to 63%. The false-negative remain around 6% within the first 4 weeks from the date of preparation of rGO-PEG. As these false negatives are the most critical parameter for celiac disease patients, this value remains concerning. Although the cause of this downtrend in the accuracy is uncertain, it is thought it could be due to the decomposition of the rGO-PEG conjugate. Further studies based on exploring different long-term stable replacements are expected for augmented reliability of the overall biosensor.

It should be important to have the reliability of the sensor over time for those bio-molecules that denature, change their performance over time or through storing storage conditions. This parameter should not be ignored in order to facilitate further technological advances.

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