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A fluorescent aptasensor for the detection of Aflatoxin B1 by graphene oxide mediated quenching and release of fluorescence

Sai Keerthana Setlem [a,*], Bhairab Mondal [b], Shylaja Ramlal [c]

[a] Senior Research Fellow (DRDO-SRF), Defence Food Research Laboratory (DFRL), Mysore 570011, India
[b] Senior Research Fellow, (Inspire fellow, DST), Defence Food Research Laboratory (DFRL), Mysore 570011, India
[c] Scientist 'E', Defence Food Research Laboratory (DFRL), Mysore 570011, India

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ABSTRACT

Aflatoxin B1 contamination in food and agro commodities has been major concern of global food safety and trade industry. There is an urgent need to develop sensitive and on-site detection methods for aflatoxins mainly, AFB1 monitoring. In the present study, a fluorophore (Alexa Fluor 488) based aptamer biosensor was devised in combination with graphene oxide (GO) for the detection of Aflatoxin B1 (AFB1). The optimized diagnostic procedure consisted of a fluorescent modified aptamer (Ax-AFLA5) as detection probe and GO mediated quenching of the same; to the quenched system AFB1 was added resulting in subsequent release of fluorescence. The principle of GO based adsorption of ssDNA and successive desorption in the presence of target mycotoxin was utilised in development of the bioassay. In presence of target mycotoxin, the GO adsorbed ssDNA attained a structural conformation resulting in desorption and subsequent release of fluorescence. Assay parameters such as concentration of fluorescent probe, GO and incubation time were evaluated and optimized. The optical signal thus generated could determine presence of AFB1 in the given sample. Selectivity of the method with other mycotoxins was evaluated. The linear range of AFB1 from 0.2–200 ppb was assessed. Visible green fluorescence release was observed at 20 ppb under UV transilluminator and the detection limit of the developed assay was interpreted as 20 ppb of AFB1. The suitability of the assay for AFB1 quantification in groundnut and natural samples was also evaluated. Thus, the developed assay can be a field deployable, reliable and rapid alternative tool for onsite screening method of aflatoxins and other mycotoxins at field level.

1. Introduction

Aflatoxins (AFs) are one of the major mycotoxins responsible for creating global food insecurities in the increasing population [Pankaj et al., 2018]. Produced as secondary metabolites by *Aspergillus* species, these non proteinaceous toxins contaminate wide variety of food such as cereals, nuts, fruits, oilseeds, and dried fruits both in field and storage. More than 4.5 billion people from developing countries are chronically exposed to myotoxins predominantly aflatoxins [Nazhand et al., 2020]. Among 20 different types of AFs identified, aflatoxin B1 (AFB1) is a highly toxic metabolite and is classified as Group I carcinogen by the IARC (International Agency for Research on Cancer). AFB1 is associated with wide range of biological activities, such as genotoxicity, teratogencity, hepatotoxicity, nephrotoxicity and immunosuppression [Ostry et al., 2017; Marchese et al., 2018]. Considering the hazardous effects, legislative limits have been set for AFB1 levels in food commodities by several countries. These include 15–30, 20 and 2 ppb of AFB1 by FSSAI (Food Safety and Standards Authority of India), FDA (Food and Drug Administration) and EU (European Union), respectively as permissible levels in crop and food products [Setlem et al., 2020].

Due to the stringent regulations, diagnosis of aflatoxins in food commodities are crucial for many applications, including import – export trade, food manufacturing, poultry and cattle industries. Modules with improved detection resolution and significantly shortened analysis time are of paramount importance for onsite analysis of mycotoxins. Many methods are being used in aflatoxin detection which include chromatographic methods, real – time PCR, immunological assays and advanced biosensors with combination of one or more modules. These methods demonstrate admirable applicability in aflatoxin detection. However, few limitations such as expensive equipment, extensive assay time, ethical concerns etc., for on – site analysis restrict their applicability [Mahfuz et al., 2018; Negash, 2018].

* Corresponding author.
E-mail address: keerthana.set@gmail.com (S.K. Setlem).

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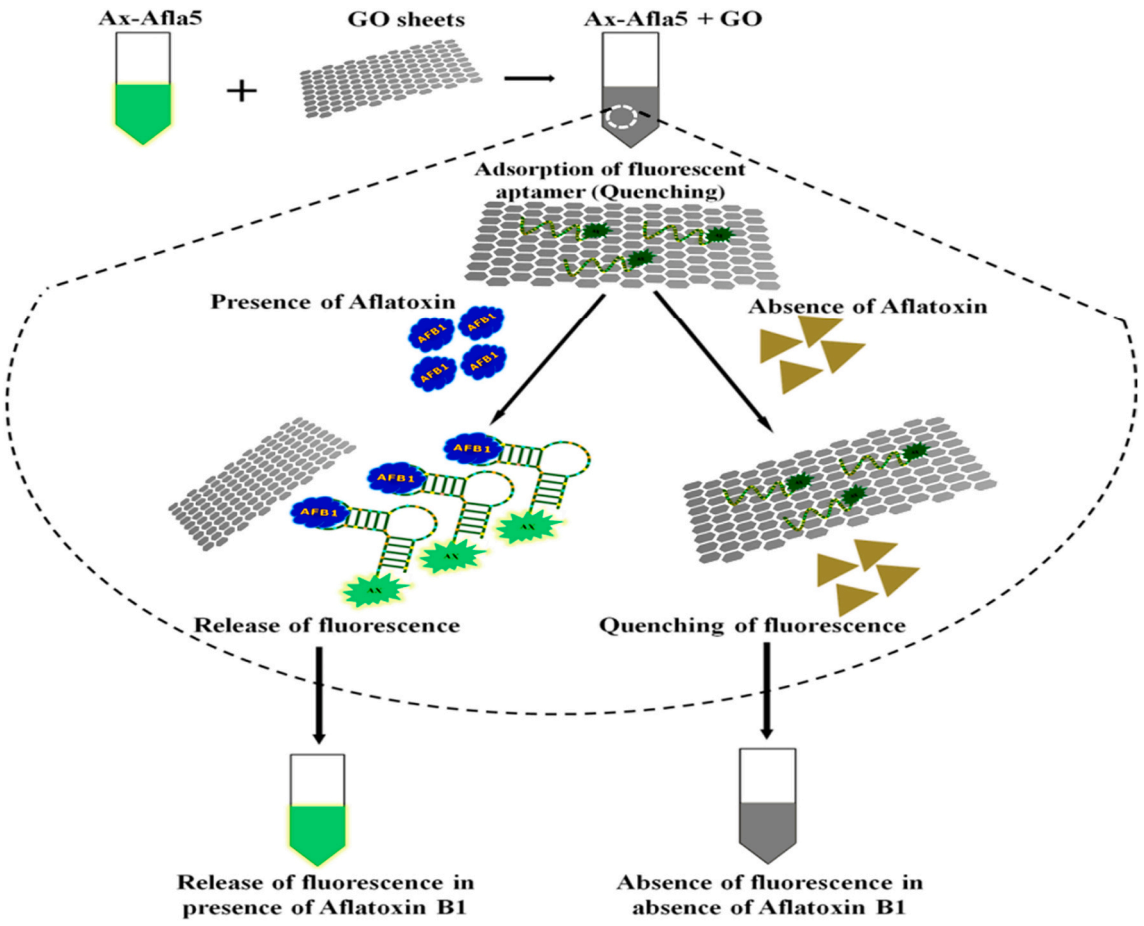

Fig. 1. Schematic illustration of AFB1 detection by graphene oxide - Alexa Fluor 488 aptamer bioassay.

With Significant advances in the scientific community, another molecule called aptamer have gained importance as recognition probes in the field of diagnostics and drug delivery for their sensitivity and specificity. Aptamers are short oligonucleotide sequences either ssDNA or RNA molecules selected by an in vitro procedure known as SELEX (Systematic Evolution of Ligands by EXponential enrichment) [Bunka and Stockley, 2006; Zhou and Rossi, 2017]. Merits such as cost effective production, stability under various physiological conditions and ease of modifications make aptamers attractive tools in diagnostics assays. These molecules interact with wide variety of targets by intra - molecular folding of oligonucleotides into three-dimensional structures. Due to their stem loop formations aptamers possess high specificity and affinity for targets. Many formats of bioassays employing aptamers have been developed for detection of mycotoxins. Recently, aptamers are assembled with nanomaterials as biomolecular recognition components in aptasensors for detection of various targets such as dyes, metal ions, toxins, and contaminants [Cai et al., 2018; Pehlivan et al., 2019; Guo et al., 2020].

Optical aptamer sensors are one of the most commonly used modules in target detection for their direct, real-time and label-free detection of many biological and chemical substances. In general an optical biosensor consists of a biorecognition sensing element integrated with an optical transducer system to produce a signal which is measured and found proportionate to the concentration of the target analyte. Optical signal generation by colorimetric, fluorescent or luminescent are different formats of label based optical biosensors [Damborský et al., 2016; Sharma et al., 2018; Ye et al., 2019; Setlem et al., 2020]. Different targets like metal ions, cells, proteins, pathogens, mycotoxins, nucleic acids and other small molecules are detected by optical aptasensors [Goud et al., 2017; Zhao et al., 2019; Wu et al., 2020].

Fluorescent dyes or fluorophores have been widely used as probes (for physical and structural parameters), indicators (for molecular concentrations) or labels (for visualization) in various bioassays [Valeur and Berberan-Santos, 2012]. With the development of new biotechnological tools and devices, fluorescent dyes have been widely used in biosensing platforms. One such, fluorophore is Alexa Fluor 488 (Ax488) synthesized through sulfonation of coumarin, rhodamine, xanthene and cyanine dyes, and have characteristics of greater photostability and brightness as well as lower pH sensitivity than common dyes with excitation/emission (green; $\lambda_{max}/\lambda_{em}$ = 495/519 nm) [Panchuk-Voloshina et al., 1999; Spence and Johnson, 2010]. Due to these interesting features, Ax488 has been recently used in fluorescence based bioassays such as flow cytometry, fluorescence microscopy, fluorescent reporter-gene assay and live-cell imaging [Danielli et al., 2010; Nishi et al., 2015].

On the other hand, graphene oxide (GO) has drawn much attention in various research fields due to its intrinsic properties. GO is the oxidized derivative of graphene and possess both the 2D honeycomb-like carbon sheet structure with various oxygen functional groups such as epoxy, carboxyl, carbonyl, hydroxyl groups [34]. This distinctive structure offer GO certain useful physicochemical and chemical properties like amphiphilicity, strong adsorption, superior mechanical strength, thermal/electrical conductivity and optical property [Allen et al., 2010; Huang et al., 2011]. Due to its favourable intrinsic properties, GO has been harnessed for the development of various types of biosensors including electrochemical, optical (fluorescence, colorimetric and Raman), and mass analysis [Kim et al., 2009; Raccichini et al., 2015; Lee et al., 2016]. In the recent years priority has been given to adapting the biochemical properties of other functional materials along with GO in the development of biosensors [Gao et al., 2014;

Bogue, 2014].

In the present study, fluorescence-based bio-sensing platform was developed for detection of aflatoxins. The main objective of the present study was developing an assay platform for onsite analysis and initial screening of aflatoxin contamination in agricultural produce by unskilled personnel prior to intensive laboratory testing. The aptamer (AFLA5) from our previous work has been used in the present assay. Aptamer, AFLA5 was selected and characterised by in - house developed imunoaffinity SELEX method [Setlem et al., 2016]. The bio-assay comprised of a fluorescent probe which was a fluorescent molecule modified aptamer (Alexa Fluor 488 – AFLA5) and graphene oxide (GO) as a quenching agent. The assay was designed by utilizing the unique properties of GO matrices for adsorption of ssDNA aptamers. This phenomenon facilitated in the quenching of fluorescence of reporter probe on to the GO sheets in the absence of AFB1 and release of fluorescence in the presence of AFB1, respectively. Standardization of various assay parameters was carried out. The effectiveness of the aptasenor was evaluated using spiked and naturally contaminated food samples. Altogether, this method is simple, rapid, and reliable for routine analysis of aflatoxins.

2. Material and methods

2.1. Chemicals and reagents

The fluorescent molecule (Alexa Fluor 488) modified anti-aflatoxin aptamer, AFLA5 (Alexa Fluor 488–**5'**TTCTTCTGGCTTGGTGGTT GGTGTGTCTGCTGATTTGGTA**3'; Ax-AFLA5)** was synthesized and procured from Sigma-Aldrich (India). All chemicals and mycotoxins were procured from Sigma-Aldrich (St. Louis, USA) unless mentioned otherwise. The aptamer probes were prepared in 20 mM Tris–HCl buffer (pH 7.4) and stored at 4 °C until use. Working standards of aflatoxins and other related mycotoxins (1 mg/ml) were prepared in absolute methanol (HPLC grade). The chemicals and solvents used in the study are of chemical grade and are listed in the supporting information 1.

2.2. Optimization of fluorescent probe concentration

Fluorescent molecule, Alexa Fluor 488 tagged AFLA5 aptamer was used as a fluorescent probe for the development of the assay. Different concentrations of Ax-AFLA5 ranging from 0 to 350 nM were investigated for optimal concentration with unbiased fluorescence signal. The reaction was carried out in black 96-well microwell plates (Nunc), wherein each well consisted of 150 µl of DPBS buffer and 50 µl of respective concentrations of Ax-AFLA5. The plates were then incubated for 10 min at RT in dark conditions. After incubation, the fluorescent intensity was measured (top read) with excitation at 493 nm and emission at 519 nm using the Infinite M1000 spectrophotometer (TECAN, India).

2.3. Optimization of the quenching system

The quenching system was optimized with different concentrations of GO ranging from 0 to 100 µg/ml. Each concentration was incubated with 200 nM of Ax-AFLA5 for 15 min at RT in dark with mild shaking. After incubation, the fluorescence spectra were measured as mentioned prior. Also, the incubation time from 0 to 30 min was tested for appropriate quenching of fluorescence with GO.

2.4. Determination of aflatoxin B1 by GO mediated fluorescent bioassay

Alexa Fluor – GO system was used for the detection of AFB1 as illustrated in Fig. 1. The assay was performed in black microtiter plates (Nunc, Sigma-Aldrich, India) and the fluorescence was top read by Infinite M1000 spectrophotometer (TECAN, India). Initially, 50 µl of 200 nM of Ax-AFLA5 was added to 10 µl of 20 µg/ml of GO and volume was made up to 150 µl with DBPS (pH 7.4) containing 3% methanol. The reaction was then incubated for 15 min at RT in dark conditions. After incubation, the fluorescence intensity was measured with excitation at 493 nm and emission at 519 nm. After complete quenching, the release of fluorescence was mediated by the addition of 200 ppb of AFB1 and the dequenching was monitored at regular intervals of time. After an incubation of 15 min at RT in dark conditions, the release of fluorescence was measured. Table. 1 contains the components of individual reactions used in the assay.

Table 1
The buffer components used in GO mediated fluorescence biosensor.

Components	Volume (µl)	Concentration
Alexa Fluor 488 – AFLA5	15	2.5 nM
GO (Graphene Oxide)	6.0	12 µg/ml
AFB1 (Aflatoxin B1)	50	150 ppb
DPBS	134	1×
Total Volume	**200**	–

2.5. Specificity analysis of GO mediated fluorescent bioassay

In order to check the selectivity of the developed assay, specificity analysis was carried out. Independent solutions containing 50 µl of 200 ppb of related and other mycotoxins namely AFB2, AFG1, AFG2, CIT (citrinin), OTA (ochratoxin A), DON (deoxynivalenol), PAT (patulin), and FB1 (fumonisin B1) were prepared and added to the previously quenched Ax-AFLA5 + GO complexes, respectively. These assemble were then incubated for 15 min at RT in dark conditions. The fluorescence intensity upon dequenching was measured and compared with that of fluorescence release on the addition of AFB1.

2.6. Sensitivity analysis of GO mediated fluorescent bioassay

The robustness of the developed GO – Ax-AFLA5 assay was evaluated with quantitative detection of various concentrations of AFB1 from 0.02–200 ppb. Individual wells of the microwell plate were loaded with 150 µl of pre - prepared quenched complex, containing 200 nM of Ax-AFLA5 and 20 µg/ml of GO and DPBS+3% methanol. The release of fluorescence upon addition of 50 µl different amounts of AFB1 (0.02–200 ppb) was observed and measured with excitation at 493 nm and emission at 519 nm.

2.7. Detection of aflatoxin from natural and food samples by employing GO – Ax-AFLA5 bioassay

The pertinence of the standardised Alexa Fluor - graphene oxide based system for effective detection of aflatoxin B1 was determined by conducting spiking studies. Groundnuts were collected from local vendors and surface sterilised [Setlem et al., 2016]. The samples were then ground by mechanical grinding and made to a sample size of 5 g each. The samples were tested for mycotoxin contamination by HPLC prior to perform spiking studies in order to avoid false positives (supporting information 04) . Known concentrations of 500 µl AFB1 ranging from 0.2 to 200 ppb in 2.5 ml of DPBS +3% methanol were added to each 5 g of powdered groundnut samples. The samples were then allowed to air dry for 24 h in dark conditions. After drying, toxin extraction was carried out (supporting information: 02); the extracted toxin of 50 µl was then added to the pre-quenched Ax-AFLA5 + GO complex and incubated for 15 min at RT in dark conditions. The release of fluorescence after toxin addition was measured.

2.8. Detection of aflatoxin from food samples and fungal isolates by GO mediated fluorescence bioassay

The applicability of the developed assay was tested with naturally contaminated food samples (corn, $n = 10$; groundnut, $n = 5$; wheat, $n =$

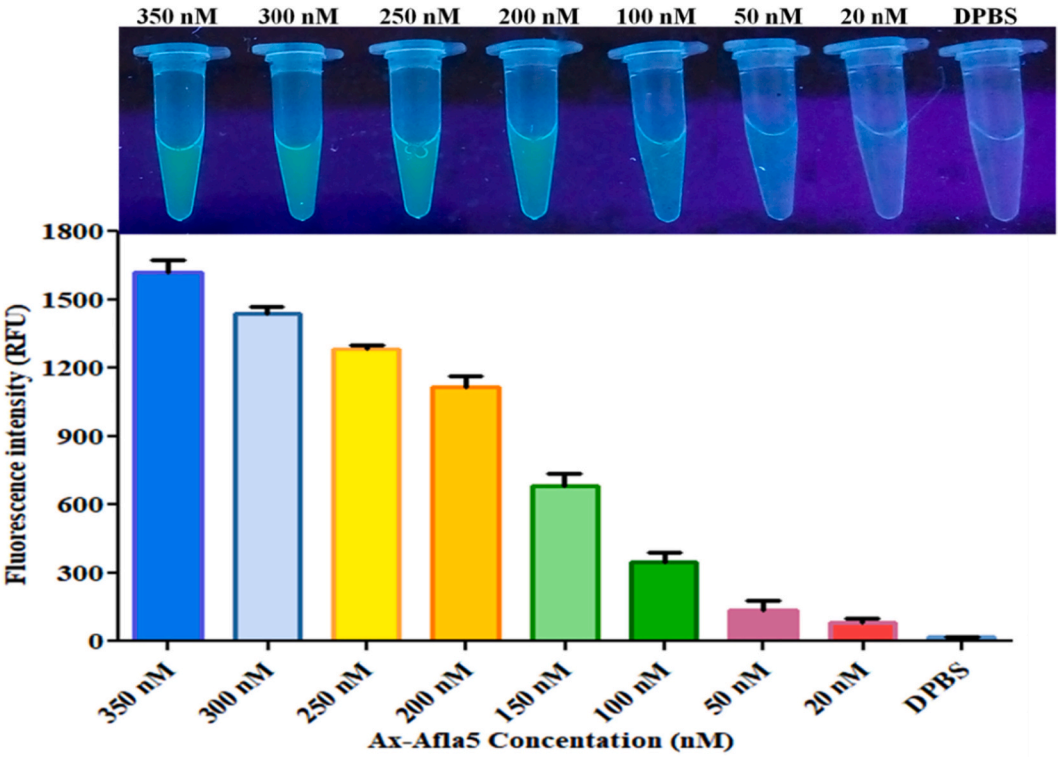

Fig. 2. Optimization of concentration of detection probe (Ax-AFLA5) for optimal fluorescence, different concentrations from 350 to 20 nM of Ax-AFLA5 are depicted in the figure. Visual interpretation of microfuge tubes for green fluorescence by UV transilluminator at wavelength 365 nm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

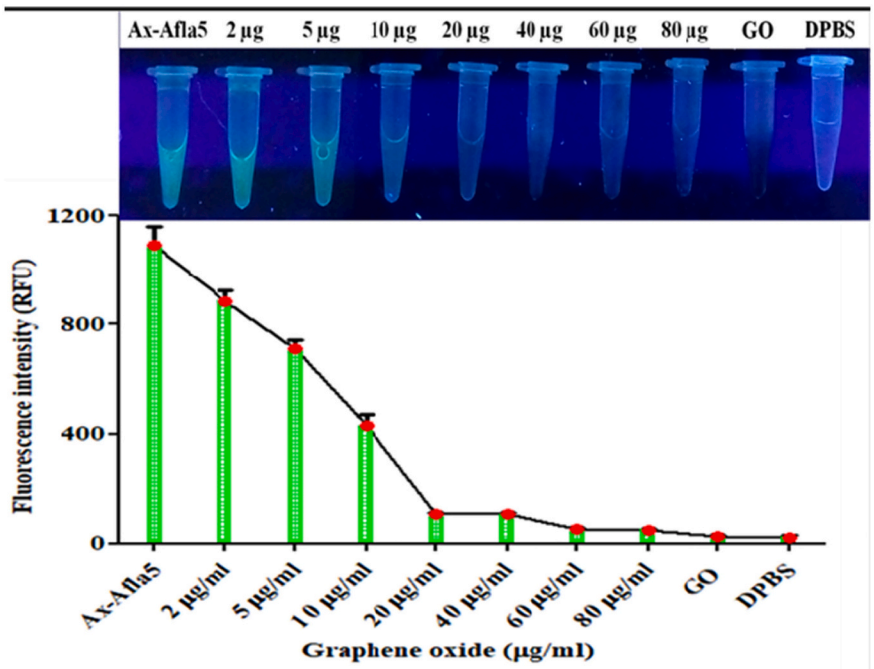

Fig. 3. Optimization of graphene oxide (GO) concentration for complete quenching of fluorescence, different concentration of GO from 2 to 80 µg are tested for complete quenching of Ax-AFLA5 by GO, naive Ax-Afla5, higher GO and blank DPBS are used as blank in the present study. Visual interpretation of microfuge tubes for green fluorescence by UV transilluminator at wavelength 365 nm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

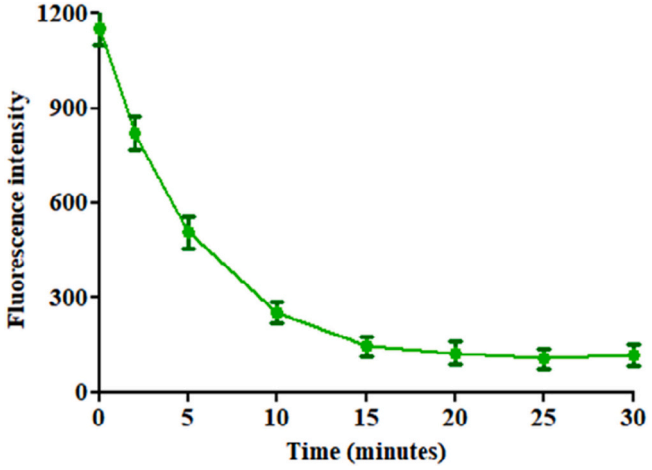

Fig. 4. Optimization of incubation time upon addition of GO for complete quenching of fluorescence, different time intervals ranging from 0 to 30 min are are analyzed for complete quenching of Ax-AFLA5 from GO.

4; and resins, $n = 3$) and fungal isolates from DFRL, Mysore repository. Toxin extraction from the samples was performed as described in supporting information 02; 03. The samples were tested with GO mediated fluorescence bioassay for the presence of aflatoxin.

2.9. Stability analysis of GO mediated fluorescent bioassay

The reliability of the developed assay was evaluated with the stability analysis of the fluorescence release for longer durations of time. Pre - prepared quenched complex, containing 200 nM of Ax-AFLA5 and 20 μg/ml of GO and DPBS+3% methanol was incubated with 20 ppb of AFB1. The fluorescence release was measured at different time intervals for 15 min to 48 h with excitation at 493 nm and emission at 519 nm under standardised assay parameters.

2.10. Statistical analysis

All the experiments were carried out in triplicates and data obtained were statistically interpreted with the help of graphpad prism version 5.3. Two way ANOVA and non linear regression analysis was performed. Standard deviation (SD) was obtained and graphs were constructed. The level of significance was set at $p < 0.05$ and the sensitivity was considered at values three times of the blank.

3. Result and discussion

3.1. Optimization of the detection probe

Anti Aflatoxin B1 aptamer, AFLA5 from our previous work was employed in the present study for fluorescent-based detection of AFB1 [Setlem et al., 2016]. Aptamer, AFLA5 was tagged with Alexa Fluor 488 (Ax-AFLA5) and used as fluorescent probe. An optimal concentration of the Ax-AFLA5 was investigated for the visual detection of AFB1. The results in Fig. 2 suggested a faintly visible green fluorescence at 100 nM and the intensity increased with the concentrations of the probe. Hence, 200 nM of Ax-AFLA5 was selected based on optimal green fluorescence that was visible with unaided eye under UV transilluminator with wavelength 365 nm (Fig. 2).

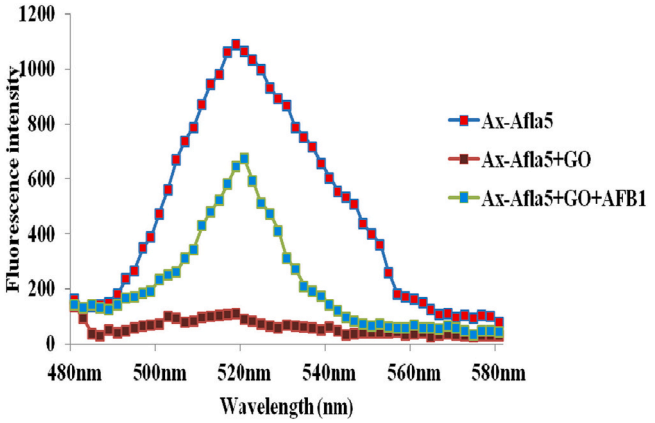

Fig. 5. Detection of AFB1 by GO mediated quenching and subsequent fluorescence release by Ax-AFLA5 aptamer.

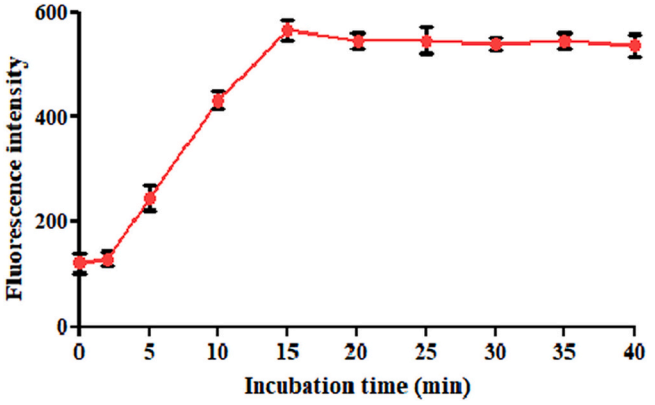

Fig. 6. Optimization of incubation time for optimal release of fluorescence upon addition of AFB1 to quenched GO - Ax-AFLA5 complex, different incubation time from 0 to 40 min for desorption of Ax- AFLA5 from GO is depicted in the figure.

3.2. Optimization of the quenching system

Since quenching of fluorescence plays an important role in subsequent detection of AFB1, the concentration of GO was optimized to achieve the maximum quenching efficiency. As shown in Fig. 3, complete quenching for fluorescence was observed at 20 μg/ml of GO with no further change in the fluorescence. When the assay was tested for dequenching with the toxin, higher concentrations of GO resulted in the hindrance of fluorescence. This may be attributed to nonspecific adsorption of the fluorophore to GO. Hence 20 μg/ml of GO was used for the standardization of the assay. Also, the time of incubation for efficient quenching was investigated simultaneously. As depicted in Fig. 4, 15 min of incubation of 20 μg/ml of GO resulted in complete quenching of 200 nM of Ax-Afla5 with no further change in intensity at longer intervals of time. The ssDNA absorption and desorption properties of GO plane through π- π stacking facilitated for on and off detection of AFB1 without altering the basic function of DNA aptamer [Wu et al., 2012; Kim et al., 2017].

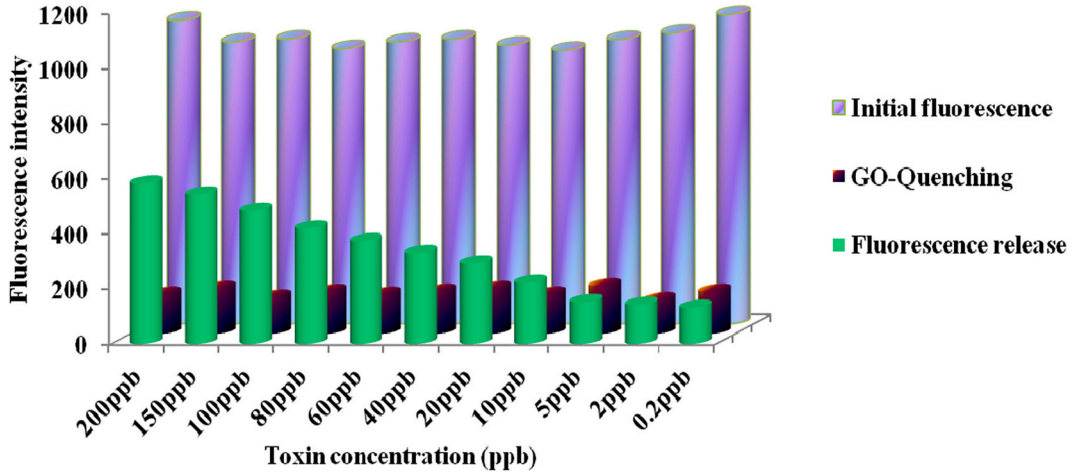

Fig. 7. Sensitivity analysis of GO mediated quenching and subsequent fluorescence release by Ax-AFLA5 aptamer upon addition of varying concentration of AFB1 (0.2 to 200 ppb).

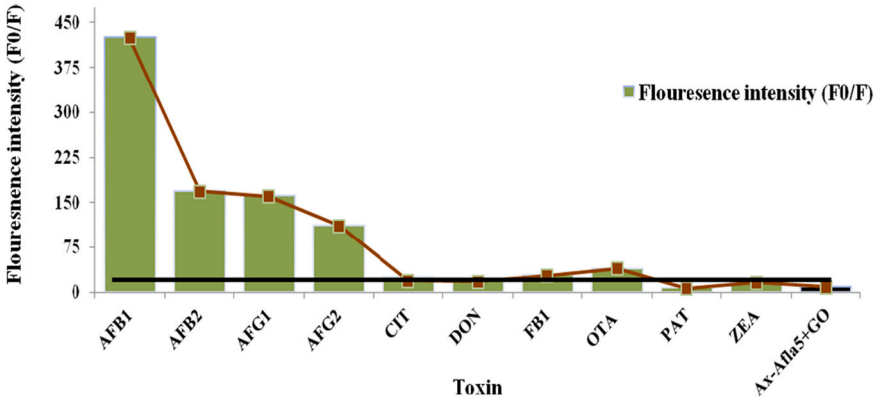

Fig. 8. Specificity analysis of GO mediated quenching and subsequent fluorescence release by Ax-AFLA5 aptamer using aflatoxins and other mycotoxins.

3.3. Detection of aflatoxin by GO mediated fluorescent biosensor

In the present assay, AFB1 was detected by graphene oxide-mediated quenching and subsequent fluorescence release by Alex Fluor 488 conjugated AFLA5 aptamer (Fig. 1). The use of fluorophore tagged aptamer facilitated in the exclusion of preparation step for detection probes as in other detection platforms. Additionally, use of DNA aptamers benefited the assay in terms of stability when compared to immunological detection platforms [Jayasena, 1999].

Fluorescence of the detector probe, Ax-AFLA5 was quenched due to the adsorption of single-stranded DNA aptamer (AFLA5) on to the GO sheets. This subsequently led to the loss of fluorescence of the fluorophore molecule, AX-488 tagged to the ssDNA aptamer, AFLA5. Upon addition of AFB1, ssDNA aptamer attained a secondary structural confirmation and bound to AFB1. These conformational changes in the ssDNA aptamer led to desorption from the GO matrices with subsequent dequenching and release of green fluorescence resulting in the detection of AFB1 (Fig. 5).

3.4. Optimization of incubation time of target toxin to quenched complex

The duration of incubation of AFB1 for efficient fluorescence release and detection of the target was evaluated with different time intervals. As shown in Fig. 6, the increase in the fluorescence intensity can be observed with the increase in the time. It can be observed that there was no further shift in fluorescence after 15 min of incubation of toxin (AFB1) with the previously quenched (Ax-AFLA5-GO) complex. This suggested the incubation time as 15 min for the present assay.

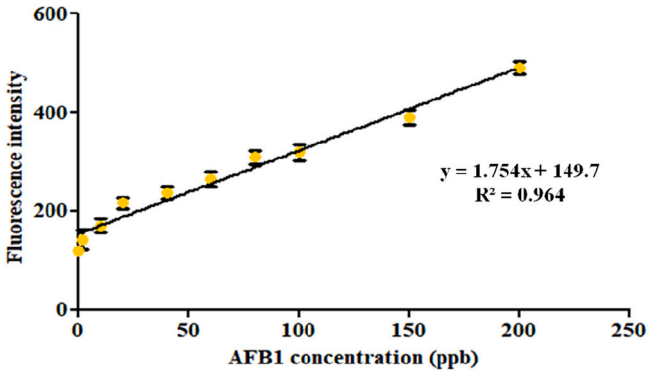

Fig. 9. Calibration curve of fluorescence intensity versus aflatoxin B1 concentration (ppb) employing GO mediated quenching and subsequent fluorescence release by Ax-AFLA5 assay.

3.5. Sensitivity analysis of GO mediated fluorescent bioassay

The efficiency of the developed assay was evaluated with various concentrations of AFB1 ranging from 0.02 to 200 ppb. As shown in Fig. 7, initial fluorescence release upon addition of AFB1 was visually observed from 20 ppb under UV transilluminator (λ, 365 nm) and

Table 2
List of food samples analyzed and found positive for aflatoxin by GO mediated quenching and subsequent fluorescence release by Ax-AFLA5 assay.

Sample name	No. of samples	No. of samples positive for aflatoxin
Corn	10	8
Wheat	4	2
Groundnut	5	5
Resins	3	1
Total	**22**	**16**

Table 3
Standard fungal cultures examined by the GO mediated fluorescence bioassay.

Fungal ID	ITS sequencing	HPLC	Alexa Fluor- assay
NCIM 152	*A. flavus*	+	+
MTCC 2797	*A. parasiticus*	+	+
DFRL, Mysore	*Ammophilus fumigatus*	–	–
DFRL,Mysore	*A. parasiticus*	+	+
DFRL, Mysore	*A. flavus*	–	–

Table 4
Isolated fungal cultures examined by for aflatoxin production in the present study.

S·No	Fungal ID	ITS sequencing	HPLC	GO - Ax-AFLA5 assay
1	AFC8	*A. flavus*	+	+
2	AFC13	*A. flavus*	+	+
3	AFC14	*A. flavus*	+	+
4	AFC20	*A. parasiticus*	+	+
5	AFC24	*A. parasiticus*	+	+
6	AFC27	*A. flavus*	+	+
7	AFC 28	*A. flavus*	+	+
8	AFC30	*A. flavus*	+	+
9	AFC31	*A. parasiticus*	+	+
10	AFC33	*A. flavus*	+	+
11	KFC7	*A. parasiticus*	+	+
12	KFC11	*A. parasiticus*	+	+
13	KFC14	*A. flavus*	+	+
14	KFC33	*A. flavus*	+	+
15	KFC32	*A. flavus*	+	+
16	KFC44	*A. flavus*	+	+
17	KFC48	*A. flavus*	+	+
18	KF66	*A. parasiticus*	+	+
19	KFC70	*A. parasiticus*	+	–
20	KFC71	*A. flavus*	+	+
21	TFC1	*A. parasiticus*	+	+
22	TFC4	*A. parasiticus*	+	+
23	TFC6	*A. flavus*	+	+
24	TFC7	*A. flavus*	+	+
25	TFC8	*A. flavus*	+	–
26	TFC13	*A. parasiticus*	+	+
27	TFC15	*A. flavus*	+	+
28	TFC42	*A. parasiticus*	+	+
29	TFC43	*A. parasiticus*	+	+
30	TFC44	*A. parasiticus*	+	+

gradually increased with higher concentrations with prominent signal at 40 ppb and above of ABF1 concentrations (supporting Fig. 1). Hence, considering the main objective of the present assay, visual fluorescence signal initiation at 20 ppb of AFB1 was interpreted as the detection limit (LOD) of the developed assay.

3.6. Specificity analysis of GO mediated fluorescent bioassay

The preciseness of the present assay was evaluated with related and other mycotoxins. The fluorescence release after addition of AFB1 was considered as 100% and the resulting fluorescence with respective mycotoxins was calculated. As shown in Fig. 8 there was a significant release of green fluorescence in the wells with AFB1, however certain percentage of fluorescence release of 32, 12, and 14% was observed in wells with AFB2, AFG1, and AFG2, respectively. With the other mycotoxins, no observable fluorescence was measured in comparison to AFB1. These observations substantiate the specificity of the developed fluorescence assay and also pave a possibility for simultaneous detection of one or more aflatoxins.

3.7. Detection of aflatoxin from spiked food sample

The applicability of the developed fluorescence biosensor in the detection of aflatoxin from food samples was evaluated by spiking studies. Powdered groundnut samples were artificially treated with known concentrations of AFB1 (0.2–200 ppb). The extracted toxin was then added to the quenched Ax-AFLA5 + GO complex and the fluorescence release was monitored. As depicted in Fig. 9, there was the release of fluorescence upon addition of toxin and showed linearity with the increase in the concentration of AFB1. The linear regression analysis was carried out and results were fitted into equation $y = mx + C$. The obtained results were in high correlation with an R^2 value of 0.964 and limit of detection (LOD) of 20 ppb. Hence, the developed aptasensor demonstrated applicability and futuristic approach in conversion to lab on chip biosensor for bio emergencies.

3.8. Detection of aflatoxin from fungal sample by GO mediated fluorescent bioassay

The robustness of the bioassay developed in the present study was evaluated from naturally contaminated food samples and fungal isolates. Toxins extracted from naturally contaminated food samples were tested. Table. 2 depict out of 22 food samples tested 16 samples were found contaminated with aflatoxins and the results were in synchronous with that of HPLC analysis. The developed assay was evaluated with toxins extracted from standard fungal cultures and isolates from DFRL, Mysore repository. As shown in Table. 3; Table 4 the results were in synchronous with that of HPLC analysis. The fungal isolates from DFRL, repository were identified by ITS sequencing and aflatoxin production was tested by the developed fluorescence bioassay. The results were then correlated with HPLC analysis which is an officially accepted toxin detection method. Out of 30 fungal isolates, 28 were tested positive for aflatoxin production; however two isolates namely KFC70 and TFC8 showed negative in fluorescence bioassay but were found positive in HPLC analysis. We assume this deviation may be the result of production aflatoxin in lower concentration than the detection limit of the developed GO mediated fluorescence bioassay. As HPLC being highly sophisticated method, these low concentration of aflatoxins can be negligible as per the legislative limits.

3.9. Stability analysis of GO mediated fluorescent bioassay

Reliability of the developed assay was evaluated with the stability studies of fluorescence release at different time intervals. As shown in the Fig. 10, the fluorescence release remained steadied at longer durations of time up until 10 h and at 24th hrs the decreased fluorescence was observed and it equalled to the GO quenched value. Hence this suggested that the developed method was found reliable at field level analysis for testing at longer duration of time under standardised assay parameters.

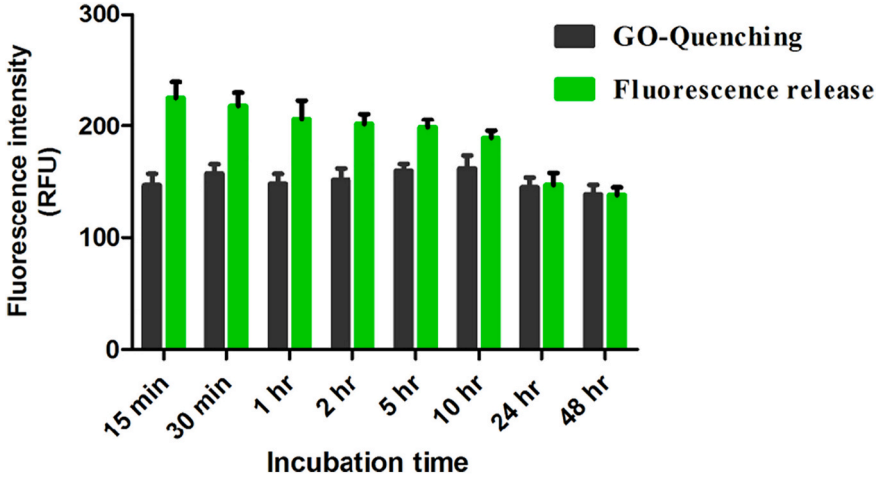

Fig. 10. Stability analysis of fluorescence release by GO quenched Ax-AFLA5 aptamer upon addition of 20 ppb concentration of AFB1. Fluorescence release at different tie intervals (min, hr) was measured.

4. Conclusion

In the present study, we have reported a GO mediated fluorescence based bioassay for visual detection of aflatoxins at field level. The main objective of the present study was developing an assay platform for onsite analysis and initial screening of aflatoxin contamination in agricultural produce by unskilled personnel prior to intensive laboratory testing. The bioassay was designed by utilizing the phenomenon of graphene oxide quenching and release of fluorescence in the absence and presence of target aflatoxin. Adsorption of property of GO to ssDNA resulted in the quenching of fluorescence of detection probe, Ax-APTA5. Addition of AFB1 resulted in the conformational changes of the aptamer on GO matrix hence the release of fluorescence and subsequent detection of target mycotoxin. The obtained results prove the stability and specificity of the developed bioassay. The effective detection of aflatoxin from natural food samples and fungal isolates substantiated the usability of the bioassay for simple and rapid detection of aflatoxins in routine food analysis by an UV light source. Also, the developed bioassay can be converted into more sophisticated sensing methods such as voltammetry and electrochemical techniques for use at biological emergencies.

Author contributions

Conceptualization, Sai Keerthana Setlem; **methodology**, Sai Keerthana Setlem and Shylaja Ramlal; **software**, Sai Keerthana Setlem and Bhairab Mondal; **validation**, Sai Keerthana Setlem and Shylaja Ramlal; **formal analysis**, Sai Keerthana Setlem; **investigation**, Sai Keerthana Setlem, Shylaja Ramlal; **resources**, Sai Keerthana Setlem; **data curation**, Sai Keerthana Setlem and Bhairab Mondal; **writing—original draft preparation**, Sai Keerthana Setlem; **writing—review and editing**, Sai Keerthana Setlem, Shylaja Ramlal and Bhairab Mondal; **visualization**, Sai Keerthana Setlem; **supervision**, Shylaja Ramlal; **project administration**, Shylaja Ramlal; **funding acquisition**, Shylaja Ramlal. All authors have read and agreed to the published version of the manuscript.

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Declaration of Competing Interest

The authors declare no financial or commercial conflict of interest.

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Appendix A. Supplementary data

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