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OPTICAL NANOFIBER AS A SMART DETECTOR OF MYCOTOXINS IN BLOOD

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ABSTRACT

The objective of this study is to use the optical fiber, an emerging device due to their biocompatibility, photo-sensitivity, as a biosensor which can detect the mycotoxin produced by the *Aspergillus sp*. found in food and feed product of animals. First, we have treated the Red Blood Corpuscles (RBCs) of human blood with different concentration of Aflatoxin B1 and Aflatoxin G1 (AflB1 and AflG1respectively) and after certain time slot, we have studied the changes of the RBC and its protein by Circular Dichorism spectroscopy (CD)and, Fluorescent Spectroscopy. From these Spectroscopic data, it has been found that there was a change in the structure of the Haemoglobin (Hb), supported by theReactive Oxygen Species (ROS), which gives the apoptotic dimension of the RBC. These experiments followed by the preparation of the nanoprobe by etching optical nanofibre with Hydrofluoric Acid (HF) upto certain extent. The prepared nanoprobe was then used to detect theAflB1and AflG1treated RBC.The outcome of the experiments it has been found that the optical nanofiber can detect a small variation of pH of the solution.

Keywords: Aflatoxin B1, Red Blood Corpuscles, Optical Nanofiber, Fluorescent Spectrophotometer, Fourier-transform infrared spectroscopy, Reactive Oxygen Species, Circular Dichorism

INTRODUCTION

Biosensors are those which can be capable of sensing the biological (cells, Blood, serum etc.), biochemical changes in the cells, Deoxy-ribo Nucleic Acid (DNA),and proteins. These biosensors may be a chemical device (like different solution) **(Bing et al., 2013; Bing et al., 2011; Kamrul et al., 2011; Peveler and Algar, 2018)** an optical device (like different light wave and fibers) **(Wang, T. et.al)** or a mechanical device **(Kausal et al., 2013; Nayak et al., 2013).** Optical nanofiber has found to be the most emerging biosensor **(Zhang et al., 2011)** which has found to be cost-effective and also a good sensor for the biological species such as protein marker of cells, DNA **(Pollock et al., 1995)**. The development and improvement of optical biosensors are driven by the continuous demand for simple, rapid, sensitive, and in-situ monitoring techniques compared to the costly immunological assay **(Leunga et al., 2007)**. These processes of detection are to be used in a broad range of areas including medical, pharmaceutical, environmental, defense, bio processing, and food industries which include the food and the feed products **(Leunga et al., 2007; Wolfbeis et al., 2008)**. Optical fiber biosensors use the fibers as the transduction element, and rely completely on optical transduction mechanisms for detecting targeted bio molecules **(Khijwania et al., 1999; Littlejohn et al., 1999; Bures et al., 1999**) by the parameter of changes in pH of the solution or maybe the antigen binding receptor molecules. One reliable and sensitive optical method is evanescent sensing. These fibers are mostly the single mode fiber **(Tong et al., 2004; Lou et al., 2005)** which are then tapered to get the ultimate width of the necessity. The tapered fiber follows the wave guideline and this particular phenomenon has led them to have the highest sensitivity and selectivity, fast response, low detection limits **(Tong et al., 2003)** with a very smaller and thinner in size. As per our review is concerned the optical fiber has got the selective size of the range between 100µm to 1mm **(Bhatia 1996; Kersey et al., 1997; Bhatia And Vengsarkar 1999; Stephen And Ralph, 2003).**
Biologically the detection of the fungi was made visually but the mycotoxins produced by the fungi changes the chemical and the physical composition of the food and the feed product and that has found to be the fatal cause of death these days **(Abdulkadar et al., 2004).** As per our review is concerned Aflatoxins, produced by the toxigenicstrains of *A. Flavus*and, *A. parasiticus* has found to be the most dangerous mycotoxins due to their range of toxicity. On the basis of their toxicity, AflB1 and AflG1 are categorized as most hazardous and fatal among the pool of other Aflatoxins such as B2, G2, M1, M2 **(Cast, 1989; Smith et al., 1991)**. A fluorescent property has also been noticed in these mycotoxins. The AflB1 produce blue fluorescence whiles the AflG1 produce green fluorescence and moreover they are more reactive as they all have two difuran rings with oxygen **(Acar, 1998)**. They all may have a selective effect on various cell membranes or target organs where they interfere with the synthesis of themacromolecule and the functions of the organs. This can influence the immunological functions directly or indirectly. Some of these mycotoxins have found to be aneurotoxic or causeotherorgan pathology, and these compounds alsomay activate the endocrine mechanisms, such asthe stress-induced release of corticosteroids inhibits immune function **(Sharma et al., 1985; Sharma et al., 1993)**. Aflatoxins are found as the potent liver toxins, and the effects in animals vary with theamount of the dose, length of exposure of the mycotoxin, species,breed, and diet or nutritional status. In animals, acute aflatoxicosis has been found and the signs and symptoms consist of reduced feed consumption, drastic drops in milkproduction, abnormal weight loss, and acute liver damage **(Bodine et al., 1983; Charoenpornsook et al., 2006)**.
Because mycotoxins are resistant to high temperatures and chemicals, they can be accumulated in grains and heavily contaminate grain-based food and feed that may result in the ill detection of these toxins **(Gendloff et al., 1986)**. There have been radical developments, incremental developments, and techniques for the detection of mycotoxins nowadays **(Maragos, 2004)**. More recently, the changes occurred by the mycotoxins are detected by the image analysis, machine vision systems, and infrared spectroscopy **(Pearson 1996; Hirano et al., 1998; Ruan et al., 1998; Dowell et al., 1999; Pearson et al., 2001; Garden et al., 2001; Dowell et al., 2002; Lohninger et al., 2002)**.Testing for mycotoxins is conducted under many different circumstances and for a variety of reasons and selection of the appropriate method depends upon the usefulness of the method. Chromatography, HPLC, ELISA were the most common method used in past days and were not very user-friendly and cost-effective **(Sydenham et al., 1997; Bhattacharya et al., 1999; Shephard et al., 2001; Trucksess et al., 2001; Koe et al., 2004)**. Deciding factors of a method selection may vary between the accuracy andspeed of the method, the skill level required to perform the assay,

and the cost-effectiveness **(Maragos et al., 2004)**. The opticalfiber has already been used in case of sensing the mycotoxins **(Maragos, 1997; Maragos and Thomson, 1999; Jeon et al., 2013)** but those have been used by the antibody coating which is a very costly affair to detect the same in all cases and in a huge quantity.
Our aim is to use the Optical fiber in the range of nano (10^{-9} cm) where it is capable of sensing a very nominal change in the pH of a solution due to its dangle bonds. Using the properties of light and the pH, the detection of the AflB1 and AflG1 has been done in our work.

MATERIAL AND METHODS

Materials

The chemicals PBS buffer, NaCl, 1% Methanol as a solvent,AflB1,and AflG1 from Sigma –Aldrich. Distilled water and isolated the RBC with the standard Protocol **(Hanson et al., 2008)**.

Red blood corpuscles isolation

The 5 ml peripheral venous blood sampled on Na_2EDTA (Sodium Ethylenediaminetetraacetic acid) as anticoagulant and centrifuge (Eppendorf) at 500xg for 10 min at 4 degrees. Aspirate supernatant (plasma) and add Phosphate Buffer Solution to erythrocyte pellet cells from the top layer containing White Blood Corpuscleswas removed. Centrifuge erythrocytes at 500xg for 10 min at 4 degrees C. this has repeated for two more times for a total of 3 washes of the blood and collect the RBC pellet. In all of the experiments, we have used RBC preparations with less than 1% other blood cells.

AFLB1 AND AFLG1 solution preparation

A stock solution of 20 µM as shown in Table 1, concentrations was prepared by dissolving 5mg of AflB1 and AflG1 in 8 ml 1% Methanol **(Sonmez et al., 2013)**.

Table 1 Making of different concentration of the working solution

Concentrations	The volume of the 20µM stock	Volume of Methanol
10 µM	2.5 ml	2.5 ml
5µM	1.25 ml	3.75 ml
2.5µM	0.625 ml	4.375 ml

Preparation of optical nano fibre

Length of 1m of single mode optical fiber is taken and etched with the HF for 57 mins to obtain the nano range of the fiber.

Methodology

Treatment of RBC with AFLB1 AND AFLG1

Isolated RBCs are treated with different concentration of AflB1 and AflG1 and incubated at 37^0 C for different time frame like 3 hours, 6hours, and 12 hours in a CO_2 incubator.

Scanning Electron Microscopy

A field emission scanning electron microscopy (FE-SEM) study was performed to characterize and determine the morphology of the treated RBCs and to find the dimension of theOptical nanofiber. A thin layered RBC sample was coated with a carbon under high vacuum and examined by FE-SEM (Carl Zeiss Supra SEM instrument). Detection of the size of the etched optical fiber has also determined by SEM.

Fluorescence Anisotropy

The fluorescence anisotropy of PBMC was assessed by the determination of TMA-DPH steady-state fluorescence polarization after the cell membrane exterior phospholipid layer permeation of the probe **(Katona et al., 2004; Lakowicz et al., 2004; Shrivastava et al., 2007)**.
For the measurement of the changes in the TMA-DPH fluorescent properties following the membrane permeation, we added 2.5µMTMA-DPHto a 2 ml of RBC suspension an aliquot of TMA-DPH stock solution in DMSO to get 2.5 µM TMA-DPH in the measuring cuvette.The cell suspension with the fluorescent probe was incubated for 30 minutes at 37°CThe measurement has been done between excitation and emission state, 360 nm and 430 nm respectively (on an average of three).

Fourier-Transform Infrared Spectroscopy

The Fourier-transform infrared spectroscopy has done (triplicate) to analyze the bond between the Hb and the mycotoxins. FTIR (Jasco)of the RBC has done with the preparation of the KBr (Potassium Bromide) palate on which the RBC solution was added. KBr (Potassium Bromide) has been used because of its absorption of excess water from the solution.

Circular Dichorism Spectroscopy

CD spectra (Jasco-J814) of hemoglobin and hemoglobin treated with different concentrations of AflB1 and AflG1 were taken after incubation for adifferent hour and centrifuged at4032 g (6000 rpm) for 5 mins. Scan speed 50 nm/min; bandwidth 1 nm; spectral response 0.2 nm; (taken on an average of three).

Reactive Oxygen Species Analysis

Membrane fluidity of blood cells was shown to have a decisive role in the direct cell to cell contact and the modulation of the activity of membrane enzymes and to be affected by the increased release of ROS **(Hollan, 1996)**.
For the measurement of the changes in the reactive oxygen of cells, DCF-DA was added to a 2 ml of RBC suspensions. The cell suspension with DCF-DA was incubated for 30 minutes at 37^0 C. The measurement was done at 540 nm on an average of three.

RESULTS AND DISCUSSION

Scanning Electron Microscopy

After etching by the HF for 55 minutes, the dimension of the fiberhas been measured by the SEM and shown in the Fig:1. Dimension of the fiber has found to be the order of 50nm.We have performed the experiment by using the property of propagation of light through the fiber as the etching process is going on. We found that the intensity of the light at the power meter connected in the receiving end of the fiber falls to zero suddenly. This phenomenon is being observed as the dimension of fiber has etched to the dimension of the wavelength of the light and due to this the area of the nano dimension of the fiber creates an evanescent field. The plot shows in Fig 2the power received by the power meter gradually decreased with the time of etching and after 55-57 minutes the power has falls to zero as there were no light passed though the nano dimensioned fiber.
In Fig 3 (a), (b), (c), (d) the SEM of normal RBC and the treated RBC has shown. The results have taken after 6 hrs of incubation with the drug as the changes have found in the other experiments mostly in that time frame and the changes of the structure of AflB1 to the AflG1 occurs durion that period of time. These results of SEM have shown that the drugs have broken down the membrane of RBC and entered into it. This leads us to do the fluorescence anisotropy and the CD spectroscopy.

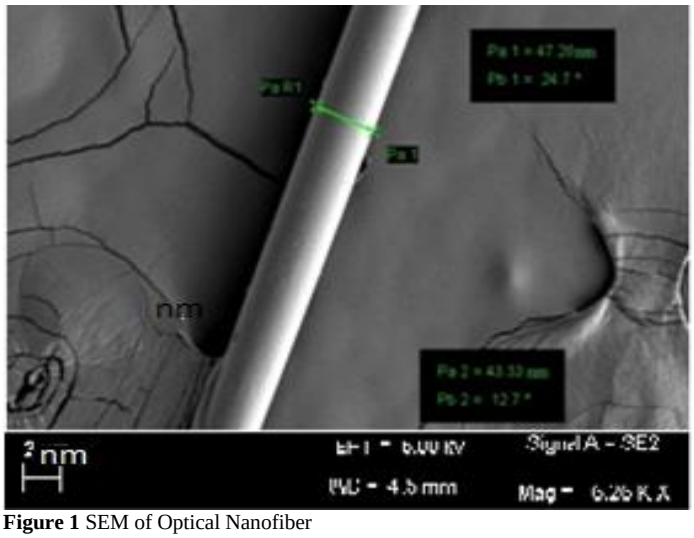


Figure 1 SEM of Optical Nanofiber

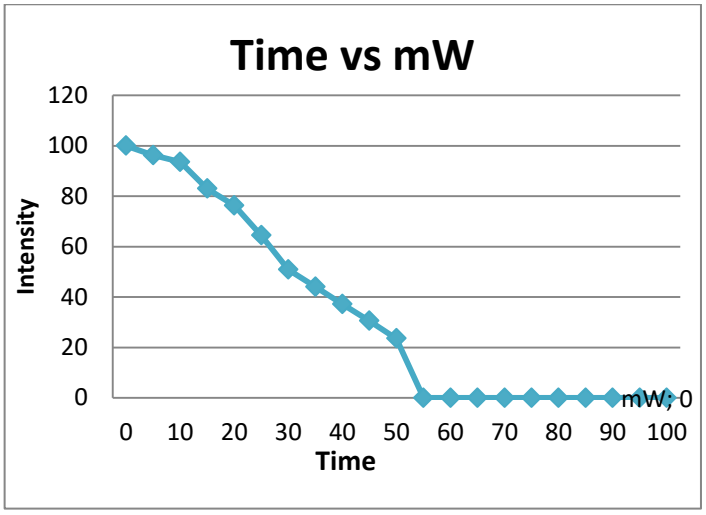
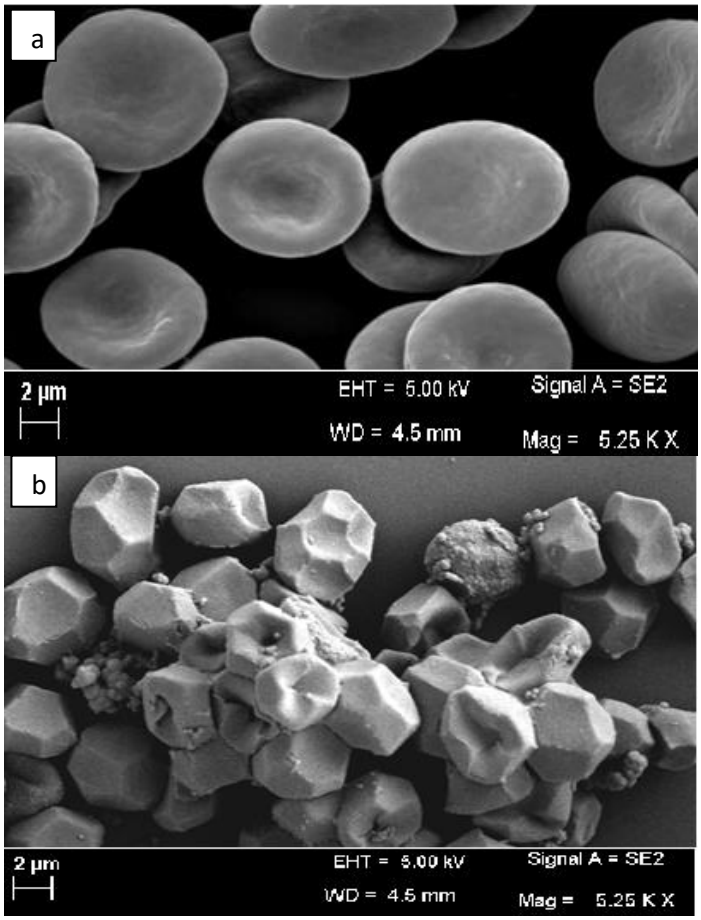


Figure 2 Time vs mW plot



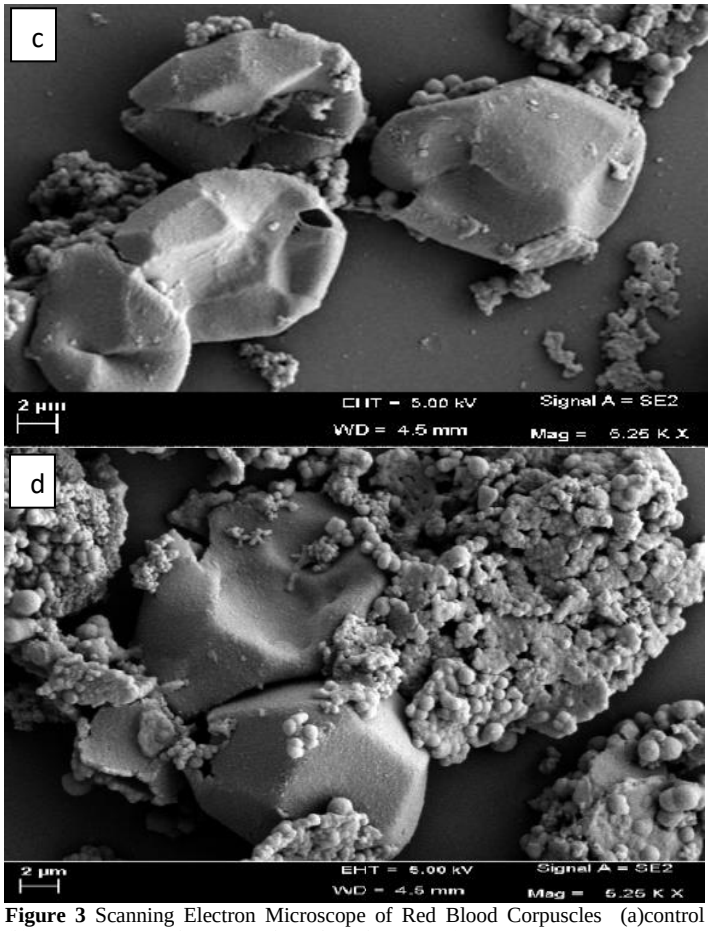


Figure 3 Scanning Electron Microscope of Red Blood Corpuscles (a)control (b)2.5µM (c)5µM (d) 10µM (after 6 hrs of treatment)

Fluorescent Anisotropy

The fluorescence spectra have been studied for all the samples (triplicate) and shown in the Fig:4 The decreasing value of the fluorescence anisotropy leads to the conclusion about the interaction between the Hb and the AflG1 got minimal due to the lower concentration and not any significant changes are noticed with the increment of the exposure time of this mycotoxin. But on the other hand, the changes in structure of Hb due to the addition of AflB1 is similar as in the samples treated with AflG1 in a particular time frame which implies that the conformational change ofAflB1 to AflG1 occurs and revives. This is also a confirmatory test that the AflB1 and the AflG1 have shown significant changes in the structure of Hb and as the values are decreased, the changes in the structural configuration of AflB1 and AflG1 changes to the minimal energy state. The changes of the structure of Hb have studied further by the CD spectroscopy. This fluorescent anisotropy is also a confirmatory test that the drug interacts only with the Hb and not with the membrane proteins present on the surface of the RBC as the intensity

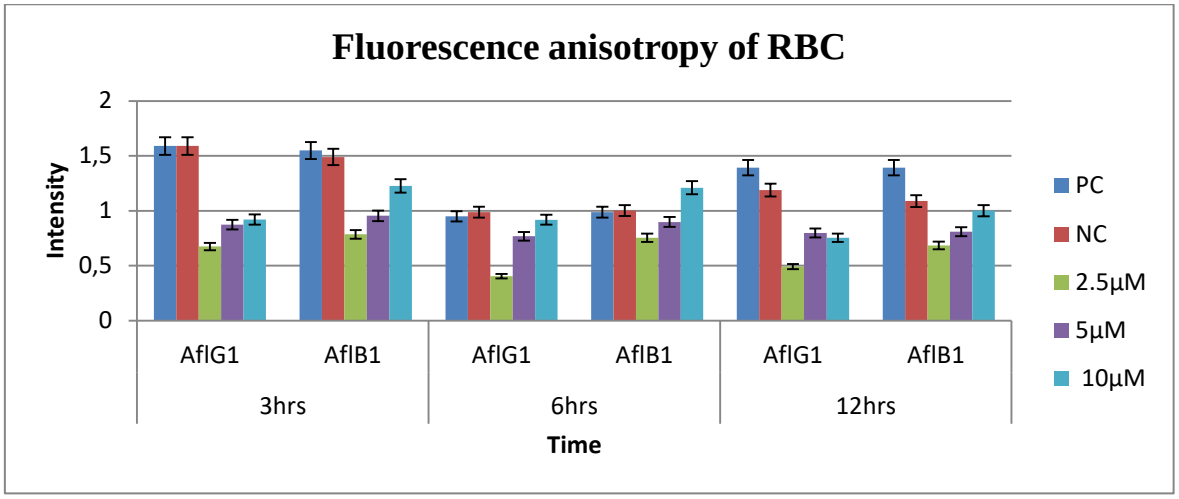


Figure 4 Fluorescence anisotropy of Red Blood Corpuscles with the treatment with AflG1 and AflB1

Reactive Oxygen Species Analysis

The ROS analysis isthe cell viability test based on the presence of mitochondria. The ROS binds to the mitochondria of a viable cell and changes the density accordingly. The triplicate analysis report of ROS in Fig:4 has clearly shown that the changes of number of RBCs are due to the changes of the structural deformation of Hb with the interaction of the mycotoxins and it has gradually decreased by the increment of the time of interaction and also with the increment of the concentration of the mycotoxins.It has also be found that there is a similarity with the results after 6 hrs of incubation with the mycotoxins which indicates the structural changes of AflB1 to AflG1.

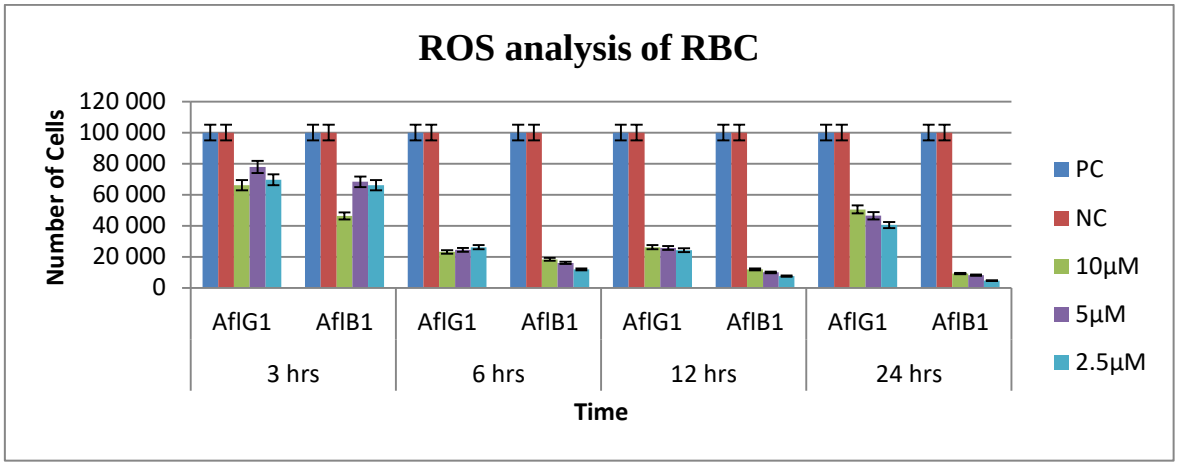


Figure 5 Reactive Oxygen Species analysis of Red Blood Corpuscles with the treatment with AflG1 and AflB1 (cell number (+/-) 1000)

Fourier-Transform Infrared Spectroscopy Analysis

The unconventional peak has found along with the suppression of characteristics peaks of AflB1 in those samples which are incubated for 6 hours for the concentrations of 10µM and 5µM both and these peaks are surprisingly disappeared at 24 hours of incubation. But in the case of 2.5µM, the coexistence of unconventional peaks and characteristics peaks of AflB1 occur for different times of incubation of the study. From these results, it can be concluded that the free radical of the Hb has changed the structure of the AflB1 and again after acertain time the structure revived. So far the molecular structure of different forms of Aflatoxins, the conclusions have been made that the unconventional peaks are due to the formation of AflG1 from AflB1 and as the review is concerned **(Mirghania et al., 2001)** the scale of toxicity of AflB1 is more than that of AflG1 and this is a good achievement of this work so far reported. One more important findings of this study is that the change in internal energy (preferably known as collision energy) of the structure of AflB1 to AflG1 calculated based on the peaks found in our FTIR data isfairly in agreement with the data reported by ref 47 who are estimated the collision energy value from another experiment like mass spectrographs etc.

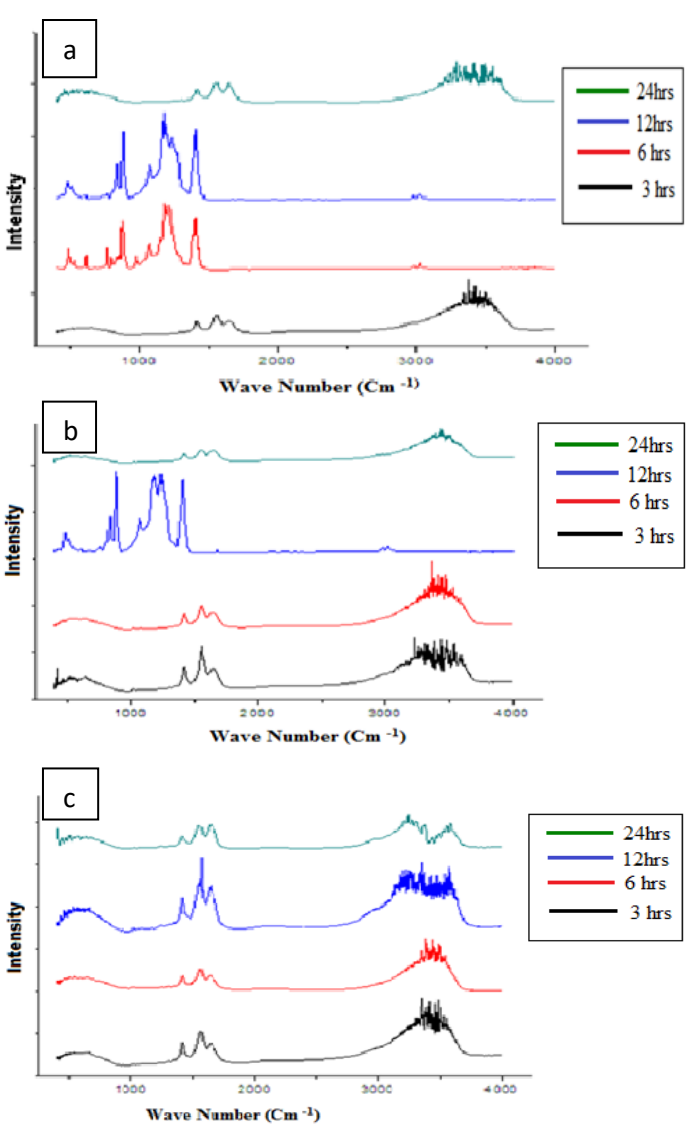
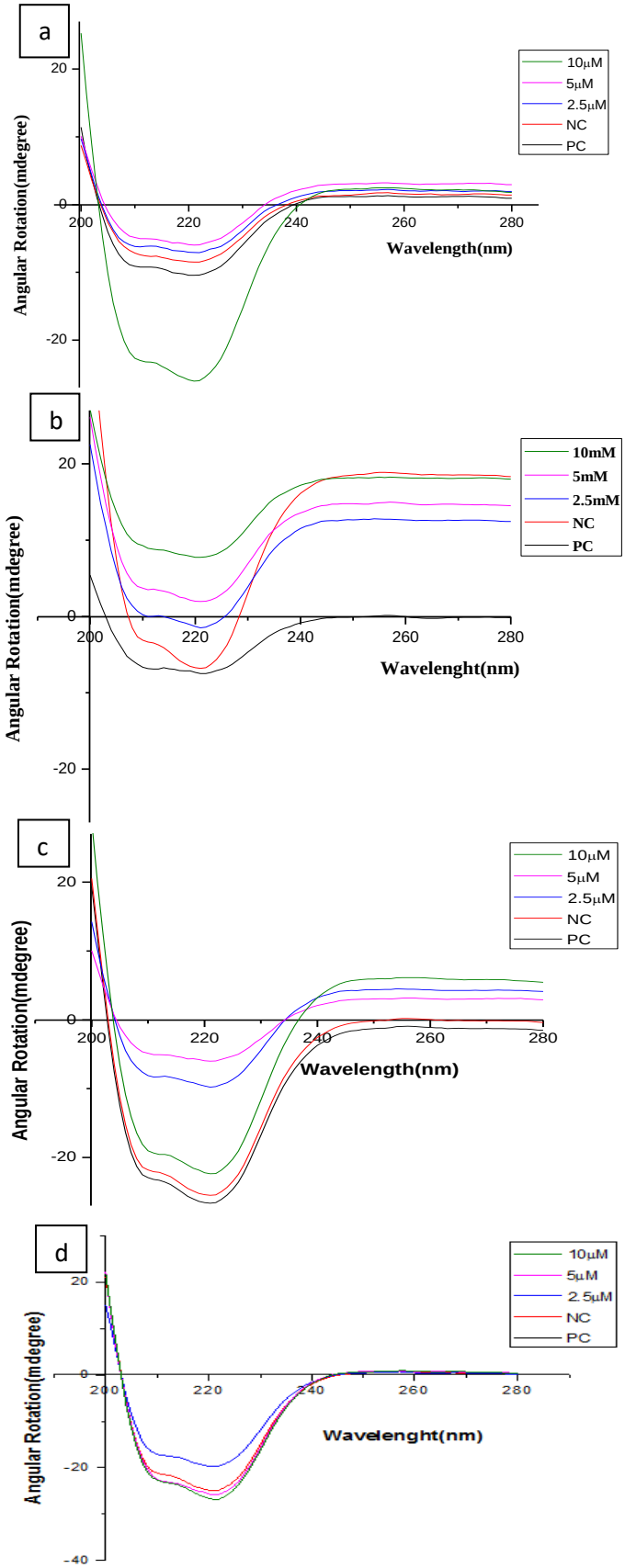


Fig 6 FTIR results of Treated RBC with different concentration of AflB1 (a) 10µM (b)5µM (c) 2.5µM (The graphs is showing the changes in the structure of RBC due to the treatment of AflB1 and AflG1)

Circular Dichorism Spectrophotometer

The interaction of Hb and the mycotoxins have studied by the CD spectroscopy. The peak of hemoglobinhas shifted with the change in time of interaction and the concentration of mycotoxins. From our previous finding **(Bhadra et al., 2017)**, from the FTIR analysis, we foundthat AflB1 has changed its conformation to AflG1 and after acertain period of time it regains its previous structure. The presence of Fe ion in the haemoglobin made easier for a molecule to change its molecular configuration as the Fe molecule has both the state of +3 and +2 state and as all the molecule wants to stay in its nominal energy state, the AflB1 undergoes the oxidation as it has a CH-N in its arm and changes its configuration to AflG1. After acertain period of time, it again releases its energy and comes back to the normal state of AflB1.CD Spectroscopic data havealso shown (Fig:6 (a,b,c,d,e)) that there is a significant changes in the structure of Hb due to the interaction with AflB1 which have a similarity to the changes due to AflG1 after 6 hour of incubation, which implies that the mycotoxins interact with the Hb only, not the other membrane proteins present in the RBCs as the normal CD spectra of Hb has maintained a regular structure throughout the reaction and also shown that the effect of toxicity of the AflB1 is higher than the AflG1 as we get the change in spectroscopy of the Hb with AflB1 and the AflG1. The shifting of the peaks from the negative to the positive region signifies that the interaction between the Hb and the AflB1 and AflG1 configure a new protein structure and that leads to a change in the structure and as well as in the morphology of RBCs.



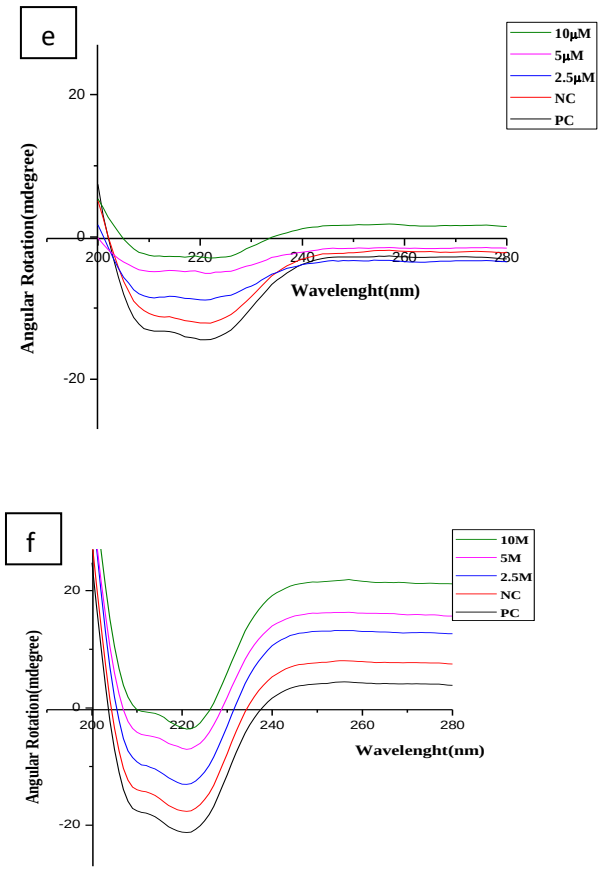


Figure 7
a, b, c: Confirmation of Protein change in RBC after treatment with AflB1 after 3, 6 and 12 hours respectively with CD spectroscopy
d, e, f: Confirmation of Protein change in RBC after treatment with AflG1 after 3, 6 and 12 hours respectively with CD spectroscopy

Detection by Power Meter

Due to the nano dimensions, the fiber has many dangle bonds and it can react with the OH^- (hydroxyl ion) and the H^+(hydrogen ion) present in the solution.As the interaction of two different molecules leads to the changes of pH of the solution, thus we took the solution of the treated Hbwith different concentration of AflB1 and AflG1 and used the etched optical nanofiber to detect the changes in pH of the solutions. By studying the power received from the optical nanofibre we first made a calibration curve by using the solution of the known pH. Now as per the schematic diagram shown in Fig 8, we have measured the pH of our solutions (containing the cell suspension of RBCs alone and the treated one) and compared their results with the calibrated curve, we can estimate the pH of the solution of the RBCstreated by the mycotoxins. All the results have shown in table 2. We have calculated the changes of ph of the treated cells with the standard curve shown in the Fig: 9.

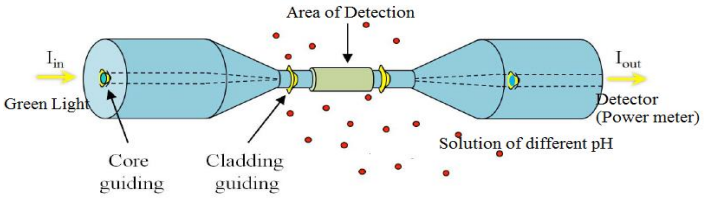


Figure 8 Schematic diagram showing the light detection pathway using optical nano fiber

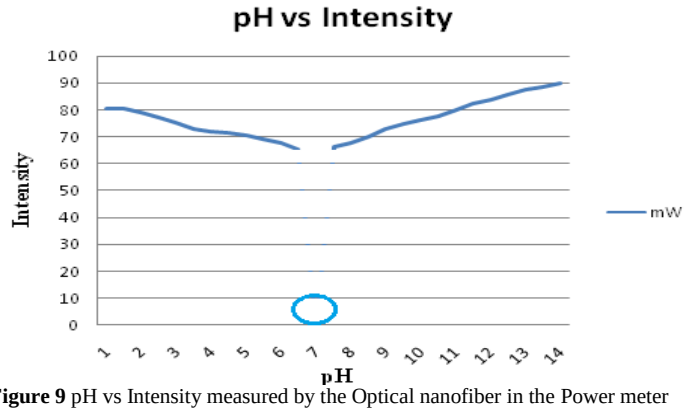


Figure 9 pH vs Intensity measured by the Optical nanofiber in the Power meter

Table 2 Intensity measured by the Optical nanofiber with a Power meter (accuracy: (+)(-) 0.786) (square mean value of triplicate study)

TIME (HRS)	CONCENTRATION	AFLB1		AFLG1	
		Intensity With Blood Cells(mW)	Intensity Without Blood Cells(mW)	Intensity With Blood Cells(mW)	Intensity Without Blood Cells(mW)
3	10µM	75.126	85.630	70.934	79.630
	5µM	70.126	76.231	65.103	75.967
	2.5µM	61.310	70.985	60.843	71.008
6	10µM	71.250	82.560	70.605	79.560
	5µM	63.872	79.945	62.498	72.879
	2.5µM	51.058	77.987	51.052	67.586
12	10µM	58.379	80.637	55.484	70.637
	5µM	50.351	73.237	47.579	61.289
	2.5µM	48.829	62.037	41.558	54.289

CONCLUSION

RBC has shown the morphological changes because of the changes in thestructure of the Haemoglobin due to the mycotoxin treatments. The changes of the oxidation state of the AflB1 were seen on a particular time variation. Thus from the experiments, we can conclude that the AflB1 and the AflG1 react with the Hb and not with the other proteins present in the Hb and changes of state occurs due to the chemical nature of the Iron present in the Hb. This signifies that the hazards occurred only in Hb of the RBCs which is more harmful to the living beings. Another serious conclution can be made from the FTIR analysis that, determination of the bonding between the drugs and the proteins can be determined by this analysis by calculating the collision energy of an electron which can be derived from the intensity found in the FTIR graph.Though there are many sensors present in the market, the important finding of our workis that the use of optical fiber in nano dimensions is quite fruitful and those are easily available in the market in lower price. These fibers are capable of detecting the minor changes in the pH of a solution due to the presence of dangle bonds in it and may improve the detection procedure of the affected cell by the changes in pH of a solution and also the marker proteins present in the cells. The other market procedure for detecting the biochemical changes are the lengthy, costly and time-consuming. Thus this sensor made of the optical fiber is very useful as the light does not interact with the medium and thus it can be used in different medium also.In near future we are planning to work in vivo model and standerize the protocol for the making of optical bio sensor and using this as a powerful sourse to detect abnormalities in the cells and others.

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