

Formulation of detection bioconjugates of gold coated iron oxide nanoparticles for aflatoxin M1 in milk

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ABSTRACT

Mycotoxins particularly aflatoxins are gaining increasing importance due to their deleterious effects on human and animal health and also due to ubiquitous presence of aflatoxigenic fungi in all the agricultural products. Corn, groundnuts and other plants were infected by *Aspergillus flavus* & *Aspergillus parasiticus* and secrete the mycotoxins. Aflatoxin B1 transmitted to cow by feeding these infected plants and aflatoxin B1 transformed into its hydroxylated product such as aflatoxin M1 and M2 and such aflatoxin secreted in cow milk. In the present study, we explored the nanobiotechnology approach to prepare detection bioconjugates of gold coated iron oxide nanoparticle for the detection of aflatoxin M1 in milk. Gold coated iron oxide nanoparticles (Au-Fe₃O₄ NPs) ranging between 10-20 nm were synthesized by co-precipitation method and functionalized by EDC-NHS then labeled with FITC-labeled streptavidin (FITC-STV). Biotinylated aflatoxin M1 (biAFM1) specific to monoclonal anti-aflatoxin M1 antibodies (mAFM1) were prepared, separately. The fluorophore FITC-STV-Au-Fe₃O₄ NPs and bimAFM1 antibodies were allowed to interact to obtain Au-Fe₃O₄ NPs-FITC-STV-bimAFM1 antibody and AFM1 bioconjugates. The bioconjugates were characterized by Transmission Electron Microscope (TEM), Scanning Electron Microscope with Energy Dispersive X-ray (SEM-EDAX), Fourier Transform Infrared (FTIR), Particle Size Analyzer with zeta potential (PSA) and Fluorescence Microscopy. The bioconjugation formation was confirmed by pinkish red color in bright field microscopy same field observed by fluorescence microscopy shows the green fluorescence which confirm the aggregation between Au-Fe₃O₄ NPs-FITC labeled STV-bimAFM1 antibody and AFM1.

KEY WORDS: AFLATOXIN M1, BIOCONJUGATES, GOLD COATED IRON NANOPARTICLE, MONOCLONAL ANTIBODY AFM1

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INTRODUCTION

From the last decade iron oxide nanoparticles (Fe_3O_4 NPs) and their combinations with gold (core/shell) has become more attractive because of their vast applications in different fields such as biosensor, medical field and drug delivery etc. The surface binding of various chemical and biological molecules onto gold particle was due to thiol chemistry of gold surface for attachment of functionalized compounds (Kouassi *et al.*, 2007). Gold coated iron oxide nanoparticle (Au- Fe_3O_4 NPs) were synthesized by reduction of metal with sodium borohydride through sonication method (Baniukeyevic *et al.*, 2013). Using hydroxylamine as a reducing agent gold (shell) coated iron oxide (core) nanoparticle also synthesized by iterative reduction method and it analyze by transmission electron microscope (TEM) and superconducting quantum interference device (SQUID) magnetometer reported (Lyon *et al.*, 2004). The measurement of fluorescent and optical properties of Au- Fe_3O_4 NPs has been reported by (Baniukeyevic *et al.*, 2013 Carrasco *et al.*, 2018).

Corn, groundnuts and other plants were infected by *Aspergillus flavus* and *Aspergillus parasiticus* and secrete the mycotoxins which is responsible for the food born disease. Corn, groundnuts and other plants were infected by *Aspergillus flavus* and *Aspergillus parasiticus* and secrete the mycotoxins. Aflatoxin B1 transmitted to cow by feeding these infected plants and aflatoxin B1 transformed into its hydroxylated product such as aflatoxin M1 and M2 and such aflatoxin secreted in cow milk which quite stable during storage, pasteurization and milk product preparation (Stroka & Anklam, (2002). The aflatoxin is high temperature resistant and is not inactivated after milk treatment processes such as pasteurization, sterilization and others. Mycotoxins particularly aflatoxins are gaining increasing importance due to their deleterious effects on human and animal health and also because of ubiquitous presence of aflatoxigenic fungi in all the agricultural products. Animals contact to mycotoxins by consumption of infected food, it may be harmful to their health as well as humans, who are consumers of the animal products such as milk (Gacem and Hadj-Khelil 2016, Ketney *et al.*, 2017).

Aflatoxin is a kind of mycotoxin that was discovered from the mass poisoning of turkeys in Britain in 1960, and has strong carcinogenicity. Aflatoxin M1 (AFM1) is hydroxylated product of aflatoxin B1. Numerous diagnostic methodology like, chromatographic method includes, TLC, HPLC and OPLC, FT-NIR and enzyme-linked immunosorbent assay (ELISA) were available for the detection of aflatoxins but it is time consuming and requiring sophisticated instruments as well as trained manpower (Espinosa *et al.*, 2011). Diagnostic immu-

noassay have been use with specific antigen antibody interaction for the detection of many molecules such as AFM1 because of their sensitivity, easy to handle and quantitatively measurements. In fluorescence microscopic technique antibody labeled with so many fluorescent dyes such as Fluorescein isothiocyanate (FITC), Rhodamine, Alexa fluor and other are used as an indicator which provide optical contrast for better analysis (Adarsha *et al.*, 2015).

Till now, the use of gold coated iron oxide nanoparticle was comparatively less because it require more time to synthesis, require high temperature for the synthesis of nanoparticle and very difficult to prevent aggregation without chemical modification or use of surfactant. Here in this study, we have used simple and rapid method for the synthesis of gold coated iron oxide nanoparticle at room temperature by chemical co-precipitation, reduction of sodium borohydride and sonication method. In first step, synthesis of iron oxide nanoparticle as a core by chemical co-precipitation and then it coated with gold as shell by reduction of sodium borohydride with sonication to enhanced particle monodispersity. Then prepared particles were characterized by Transmission Electron Microscope (TEM), Scanning Electron Microscope with Energy dispersive X-ray (SEM-EDAX), Fourier Transform Infrared (FTIR), Particle Size Analyzer with zeta potential (PSA) and Fluorescence Microscopy. Surface charges and further modifications with mercaptopropionic acid and EDC-NHS also characterization by the same.

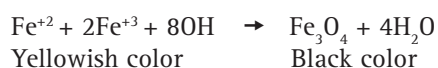
Our research effort is in direction of the detection of AFM1 with biotin-streptavidin binding approach by labeling of fluorescence dye with gold coated iron oxide nanoparticle. The bioconjugation formation between streptavidin-AFM1 and biotinylated monoclonal antibody of AFM1 was confirmed by fluorescence microscopy shows the fluorescence compounds which confirm the interaction. Mycotoxins particularly aflatoxins are gaining increasing importance due to their deleterious effects on human and animal health and also due to ubiquitous presence of aflatoxigenic fungi in all the agricultural commodities under field and storage conditions. Common established methodologies for aflatoxin detection include thin-layer chromatography (TLC), (Flores and Gonzalez 2017) and high performance liquid chromatography (HPLC) (Carrasco *et al.*, 2018).

These techniques have excellent sensitivities but they require skilled operators, extensive sample pre-treatment and expensive equipments. The present investigation was an attempt to develop basic mechanism of new nanotechnology based detection system with a minimal size, weight and real low cost and rapid detection will have significantly impact the practice of monitoring program for aflatoxin.

MATERIAL AND METHODS

All chemicals were obtained from commercial source and used as received. $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, FeCl_3 , HAuCl_4 , FITC-streptavidin, biotin and NHS-biotin, aflatoxin M1 (AFM1) were purchased from Sigma Aldrich (USA). Sodium hydroxide, perchloric acid, sodium borohydrate, cetyltrimethyl-ammonium bromide (CTAB), ethylene diamine tetra-acetic acid (EDTA), 3-mercaptopropionic acid, N-hydroxysuccinimide (NHS), N-Ethyl-N-(3-dimethyl-aminopropyl)-carbodiimide, (EDC), streptavidin (STV), Dimethyl Sulfoxide (DMSO), phosphate buffer solution (PBS) at pH 7.4 HPLC grade pure water, were purchased from HiMedia (India). Aflatoxin M1 monoclonal antibody (mAFM1-Ab) were purchased from MyBiosource (USA). All chemicals materials were used as received.

The synthesis of Fe_3O_4 NPs were carried out by modified Massart's co-precipitation method. The ratio of iron salt (Fe(III)/Fe(II)) was kept as 2:1 in an alkaline solution. Briefly, 0.64M FeCl_3 and 0.32M $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ were dissolved in 40ml of deionized water and the solution was stirred (TARSON SPINOT DIGITAL) until the iron salt completely dissolved. Subsequently add 40ml of 1M NaOH solution drop wise into stirred mixture and stirred it for 20minutes. The formation of Fe_3O_4 (magnetite) nanoparticle by indicating the color change from yellowish brown to black. The chemical reaction of magnetite precipitation may be written as follows (Tamer *et al.*, 2010).



The black color precipitate was separated by permanent magnet and washed with deionized water. To obtain oxidized magnetite nanoparticles, separated black precipitate was first washed with 2M perchloric acid and waited for 2-3 hours to oxidized iron salt to magnetite until the color was change from black to brown under inert condition. Then particle was centrifuged (REMI CM-12plus) at 10,000 rpm for 20 minutes. After centrifugation, discard the supernatant and washed with deionized water. Repeat the washing procedure for 2-3 times or until pH was reduced to neutral pH. The gold coating procedure was carried out in the presence of CTAB to encapsulate the synthesized Fe_3O_4 NPs by gold as a shell. Briefly, 10mg Fe_3O_4 NPs were add into 5ml 0.27M EDTA solution which prepared in 1M NaOH. The mixture was stirred in sonicator (LABMAN) at 30 amplitude for 5 minutes. Then resulting solution was centrifuge at 10,000 rpm for 10 minutes. Supernatant was discarded and pellet was washed with deionized water. Washing procedure was repeated for 3 times. Then add 7ml of 0.1M CTAB, 3ml 0.01M HAuCl_4 and 300 μl 1M NaOH into resulting

precipitation and stirred vigorously, subsequently add 150mg sodium borohydrate into stirred mixture and stirred vigorously for 3minutes. The color was change from yellow to dark red indicated the gold was coated into core (iron oxide nanoparticles (Fe_3O_4 NPs) as a shell (Tamer *et al.*, 2010).

SYNTHESIS OF GOLD COATED IRON OXIDE NANOPARTICLES WITH FITC LABELED STREPTAVIDIN AS A CARRIER MOLECULE

10mg gold coated iron oxide nanoparticles were added in 1ml of 20 mM 3-mercaptopropionic acid and sonicated for 4 hours. After sonication particles were washed with pure water. This procedure created the carboxyl group onto surface of gold coated iron oxide nanoparticles with EDC and NHS linked and it was used as a carrier material for streptavidin binding. For cross link of carboxylated nanoparticles to EDC-NHS, first 5mg of nanoparticle was added into 2ml EDC (0.05mg/ml) solution containing 0.2% NHS prepare in cold water then it sonicate for 5 minutes at 4°C, after sonication particles were separated out by using permanent magnet. After this process, add 1ml of 2mM streptavidin prepared in PBS (pH-7.4) in separated nanoparticle, mix it properly then incubate it for 2 hours.

Streptavidin fluorescently labelled with Fluorescein isothiocyanate (FITC) at a stock concentration of 1 mg/mL. This dye has an absorption peak at 495 nm and an emission peak of 525 nm. The resultant FITC labeled streptavidin binded gold coated iron oxide nanoparticles were separated by permanent magnet from reaction mixture, then air dried for 12 h and resuspended in water. The procedure was repeated for three times to remove impurities (Eivari and Rahdar (2013). Anti-AFM1 monoclonal antibody having sulfosuccinimidyl group was prepared separately by covalently binding primary amines of antibody (Gretch (1987). 10mg (10mM) NHS-biotin solution in 1ml DMSO was added and dissolved in it and was prepared prior to use. Then was added monoclonal AFM1 antibody with 80 μg of NHS-biotin/mg ratio, mixed immediately and was incubated for 3 hours in shaking condition. After incubation period dialysis procedure of NHS-biotin binded antibody was carried out at 4°C for overnight to remove unbinded NHS-biotin molecules. Subsequently, store the biotin labeled AFM1 antibody at 2-8°C.

The procedure was started with 50 μl biotinylated monoclonal AFM1 antibody and 20 μl streptavidin conjugated gold coated iron oxide nanoparticles were mix together and incubated at 4 °C for 1 h in dark condition. The biotinylated antibody had bound non-covalently to streptavidin and had formed bridge for direct sensing of aflatoxin M1. After the formation of nanoparticle bio-

conjugate add 10 μ l AFM1, it was incubated at 4 $^{\circ}$ C for 30 minutes in dark condition. Characterization of bioconjugates aggregation was done by different techniques (Adarsha *et al.*, 2015).

RESULTS AND DISCUSSION

TRANSMISSION ELECTRON MICROSCOPE

The morphological characterization iron oxide nanoparticle was done by transmission electron microscopy (TEM). A small drop of formulated iron oxide nanoparticle was placed on the copper grid surface and dried it at room temperature. TEM analysis was carried out by JEOL-JEM2100 transmission electron microscope.

The shape of nanoparticles was spherical having average dimension of 10.8 ± 4.6 nm. The TEM image of Fe_3O_4 NPs shows in Figure 1. The morphology and magnetic properties can be controlled by varying in pH solution, ionic strength, temperature, reaction time, type of salts and stirring speed. Eivari and Rahdar (2010) has reported that Fe_3O_4 NPs was almost spherical and their mean size was 10 nm. Our TEM result of Fe_3O_4 NPs morphology is at par to this.

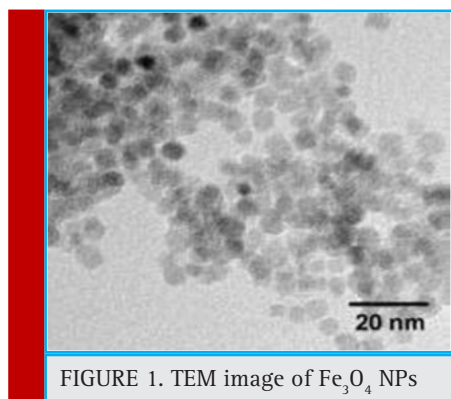


FIGURE 1. TEM image of Fe_3O_4 NPs

Figure 2 shows that the $\text{Au-Fe}_3\text{O}_4$ NPs was darker than Fe_3O_4 NPs. TEM images shows that the average particles size was increased from 10nm to 14nm after gold coating. The reduction of gold onto spherical surface of Fe_3O_4 NPs and it has average size of $\text{Au-Fe}_3\text{O}_4$ NPs was $14\text{nm} \pm 3\text{nm}$. Eivari and Rahdar (2010) has reported that the after coating of gold onto the Fe_3O_4 NPs it appear more darker than Fe_3O_4 NPs because of gold having more electron density than iron. Our TEM result of $\text{Au-Fe}_3\text{O}_4$ NPs is at par with them.

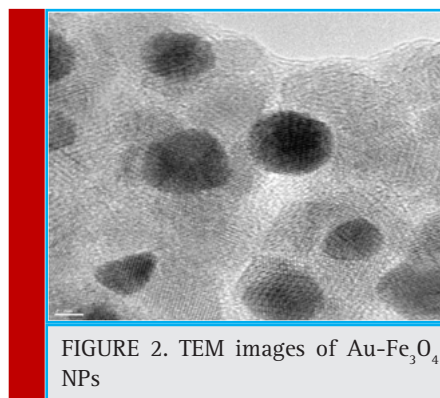


FIGURE 2. TEM images of $\text{Au-Fe}_3\text{O}_4$ NPs

SCANNING ELECTRON MICROSCOPE (SEM) WITH ENERGY DISPERSIVE X-RAY (EDAX)

The formulated $\text{Au-Fe}_3\text{O}_4$ NPs were further examine using a Zeiss EVO-18 Scanning Electron Microscope with Energy Dispersive X-Ray facility (SEM with EDAX), operating at 20 kV in vacuum and at 20KX magnification.

Confirmation of the morphology of the formulated Fe_3O_4 NPs and $\text{Au-Fe}_3\text{O}_4$ NPs analyze by SEM with EDAX which show in Figure 3.

After the synthesis of Fe_3O_4 NPs it was coated by gold with reduction of sodium borohydride in the sonication

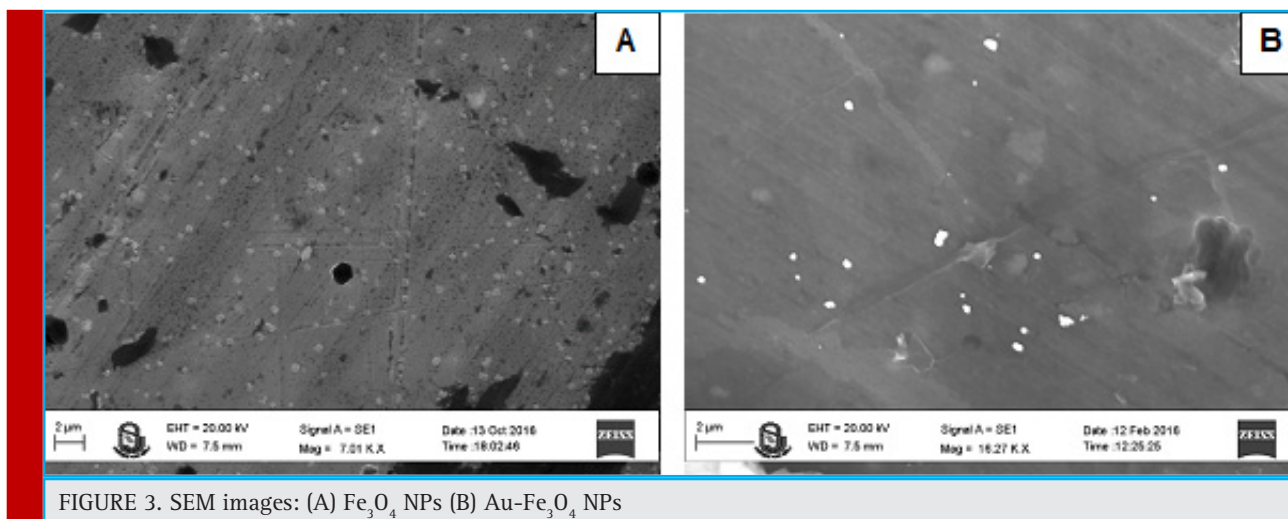
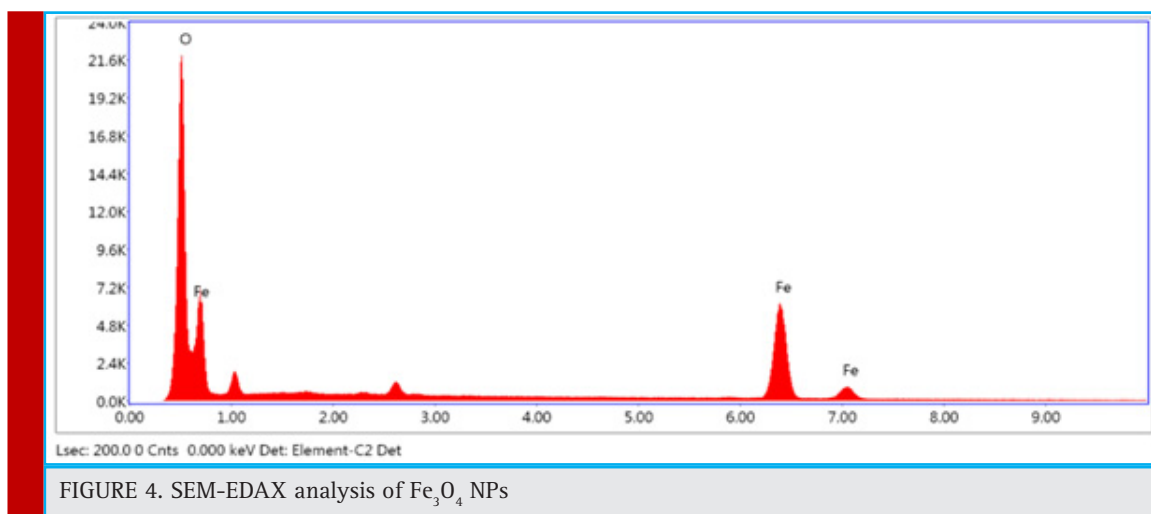


FIGURE 3. SEM images: (A) Fe_3O_4 NPs (B) $\text{Au-Fe}_3\text{O}_4$ NPs



chamber which shows in Figure 4. The difference in the size of Fe₃O₄ NPs shows that the coating of gold onto Fe₃O₄ NPs may be successful.

The analysis of Au-Fe₃O₄ NPs by EDAX was done to investigate the presence of Au and Fe in the synthesized nanoparticle. EDAX spectrum of the Au-Fe₃O₄ NPs was shown in Figure 5, which confirms the existence of Au and Fe in the synthesized nanoparticle. Hoskins *et al.*, (2012) has reported the presence of Au-Fe₃O₄ NPs by EDAX analysis. Our EDAX analysis result of Au-Fe₃O₄ NPs is affirmation to this.

PARTICLE SIZE ANALYZER AND ZETA POTENTIAL

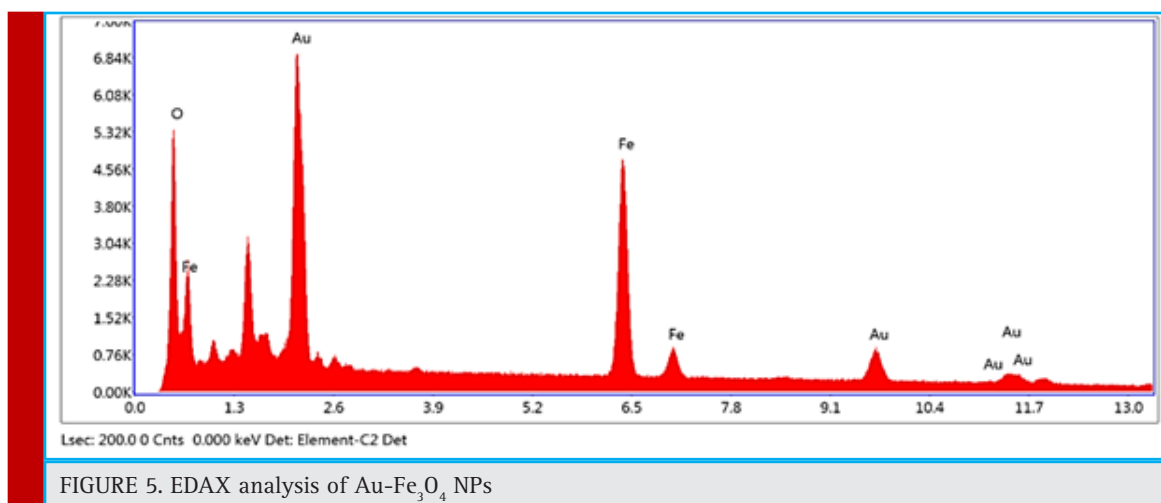
In the formulation of Fe₃O₄ NPs experiments, known volumes (normally 500 µL) of Fe₃O₄ NPs suspension were taken for particle size analyzed by a Malvern Mastersizer 2000. The particle size analyzer utilizes laser technology based on the Mie light-scattering theories. Fe₃O₄ NPs

was characterized to examine the particle mean size and understand the properties under different physiological conditions and also determine the surface charges of nanoparticle.

The average size of Fe₃O₄ NPs was 52.04nm ± 5nm shown in Figure 6. The Zeta Potential of synthesized Fe₃O₄ NPs was -31.0 mv and polarity was negative, so it has good particle stability reflected by this result. Our result is affirmation to study of Behera *et al.*, (2012).

VIBRATING SAMPLE MAGNETOMETER

Iron oxide (magnetic) nanoparticle may give different magnetic properties depending on condition of formulation Gupta and Gupta (2005). At room temperature iron oxide nanoparticle has superpara-magnetic property where at 60 emu/g reported for saturation. The vibrating sample magnetometer measure the iron oxide nanoparticle and gold coated iron oxide nanoparticle characteristic at 300K.



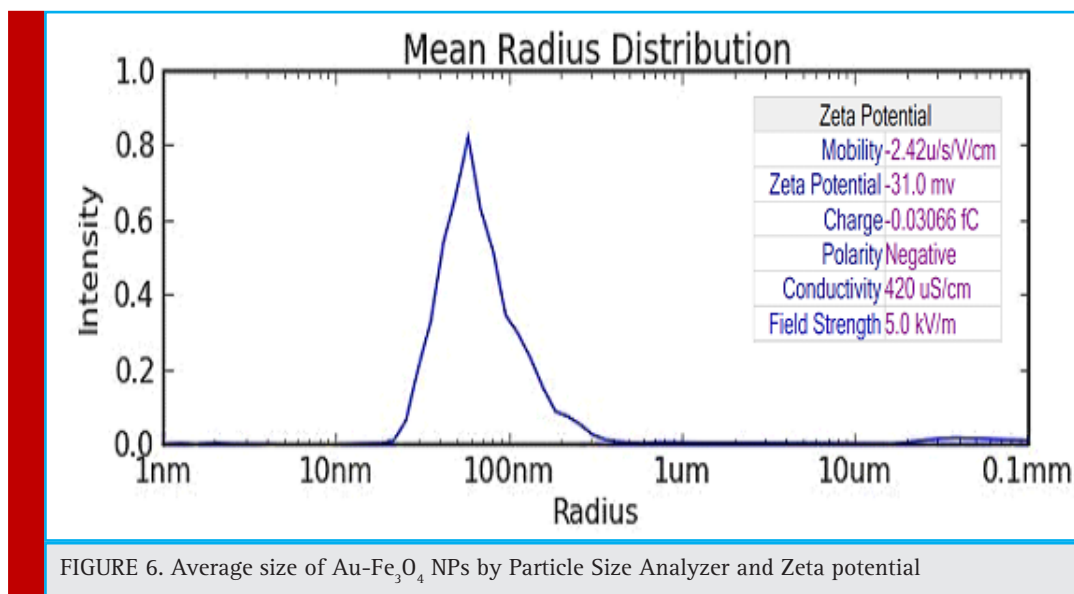


FIGURE 6. Average size of Au-Fe₃O₄ NPs by Particle Size Analyzer and Zeta potential

Figure 7 shows hysteresis loop of uncoated & gold coated iron oxide nanoparticle. The superpara-magnetism was observed in both synthesized nanoparticles. The magnetism form saturation to superpara-magnetism curve the value of iron oxide nanoparticle and gold coated iron oxide nanoparticle was 30 and 4.5 emu/g respectively at 300K. Reduction in magnetism was indicated that the magnetism was indicated that the formation of gold shell into iron oxide nanoparticle core.

FOURIER TRANSFORM INFRARED (FTIR) SPECTROSCOPIC ANALYSIS

The carboxylic group attached on the surface of Au-Fe₃O₄ NPs by treatment of ethanolic solution having

3-mercaptopropionic acid. The activation of carboxylic group for the bonding between amide and carboxylic group, the activation of carboxylic group was catalyzed by carbodiimide (EDC) in presence of N-hydroxysuccinimide (NHS). The addition of NHS catalyzed the formation of the intermediate active esters that further react with the amine function of the streptavidin (STV) to yield finally the amide bond between the monoclonal antibody aflatoxin M1 (mAFM1) and the carboxyl group on the nanoparticles.

Characteristic of C=O and N-H stretch analysis was done with various peaks in the FTIR spectrum region shown in Figure 8. However the sharp FTIR absorbance peaks 3426 cm⁻¹ specific to amide indicate the presence of proteins.

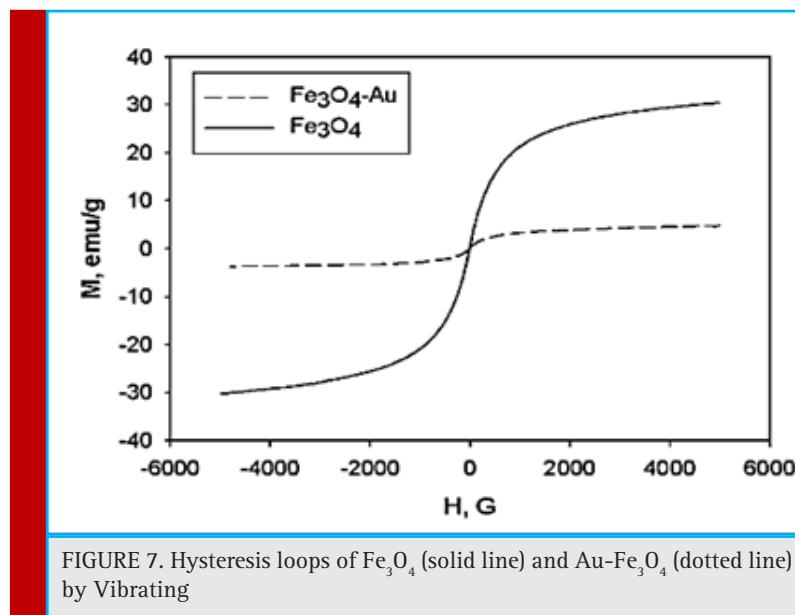


FIGURE 7. Hysteresis loops of Fe₃O₄ (solid line) and Au-Fe₃O₄ (dotted line) by Vibrating

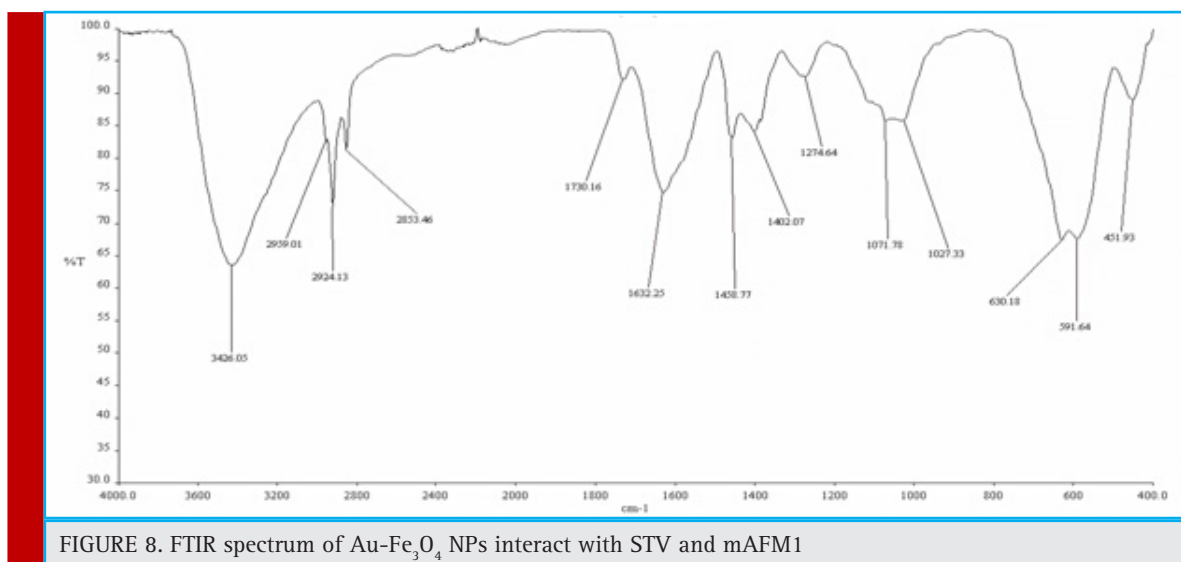


FIGURE 8. FTIR spectrum of Au-Fe₃O₄ NPs interact with STV and mAFM1

The FTIR spectrum in Figure 8 shows peaks at various ranges corresponding to different stretching and bending modes of the amine and carboxylic group. Peaks at 3426 cm⁻¹ are assigned to N-H stretching of the amide group. The peak at 1632 cm⁻¹ has been assigned to C=O stretching of the carboxylic group. The peak at 3426 cm⁻¹ is due to O-H stretching. This type of study has been reported previously to find functional properties, binding and to be used as detection system Adarsha *et al.*, (2015).

DETECTION OF BIOCONJUGATES BY FLUORESCENCE MICROSCOPY

Fluorescence is a process where a fluorophore (FITC) absorb light (492nm) and turns in to an excitation form with apple green color which result in emitted light (515nm). The Au-Fe₃O₄ NPs and its interaction with FITC labeled STV and biotinylated monoclonal antibody of AFM1 (bimAFM1) was measured under fluorescence microscope (Nikon Eclipse Ni) under fluorescence mode at constant flow of 50 a.u with FITC filter (EX 465-495nm, DM 505 & BA 512-558nm). The background of image became brighter making the particles more difficult to visualize so, it track with the Nikon D-element software.

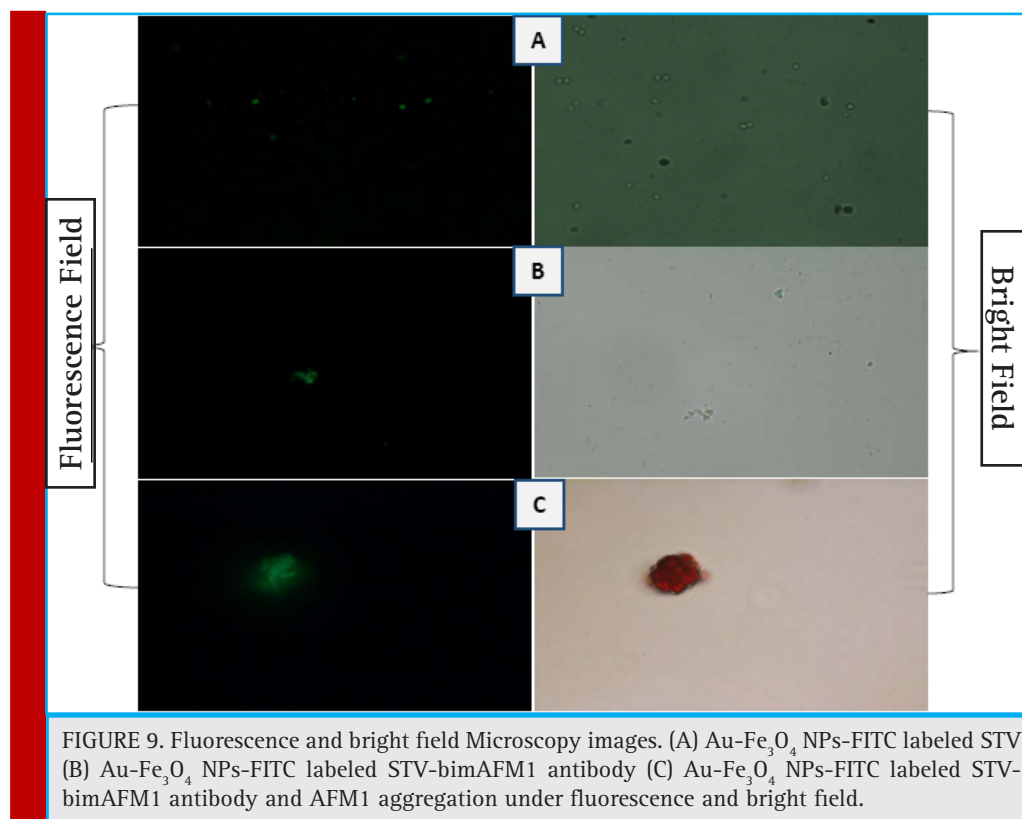
The fluorescence microscopic image of Figure 9 (A) Au-Fe₃O₄ NPs-FITC labeled STV (B) Au-Fe₃O₄ NPs-FITC labeled STV-bimAFM1 antibody and (C) Au-Fe₃O₄ NPs-FITC labeled STV-bimAFM1 antibody-AFM1 bioconjugate was examined under fluorescence and bright field shown in Figure 9. There was no any aggregation found in case of Figure 9 (A) and (B) while in case of (C) shows the aggregation between Au-Fe₃O₄ NPs-FITC labeled STV-bimAFM1 antibody and AFM1 which indicating the successful bioconjugation. Streptavidin are used ubiquitously

due to its remarkable specific binding affinity with biotin which ultimately leads to the aggregation. This aggregation could be exploited to examine at nano level due to ultra-small structure of aflatoxin M1 molecule.

The binding of bimAFM1 antibody and Au-Fe₃O₄ NPs-FITC labeled STV was shown in fluorescence microscopic images. Figure 9 (A), (B) and (C) shows the green fluorescence which indicating the FITC labeled STV binding with Au-Fe₃O₄ NPs, bimAFM1 antibody binding with Au-Fe₃O₄ NPs-FITC labeled STV and aggregation between Au-Fe₃O₄ NPs-FITC labeled STV and bimAFM1 antibody with AFM1. By antigen-antibody reaction anti-AFM1 and aflatoxin M1 developed color by the aggregation of gold nanoparticle conjugate Hoskins *et al.*, (2012). Figure 9 (C) shows the pinkish red color in bright field microscopy which confirm the aggregation Au-Fe₃O₄ NPs-FITC labeled STV-bimAFM1 antibody and AFM1.M. Adarsha *et al.*, (2015) has reported the detection of aflatoxin B1 by Fe₃O₄ NPs bioconjugation. Our study is at par to this with slight difference i.e. aflatoxin M1 detection instead of aflatoxin B1.

CONCLUSION

Iron oxide nanoparticles of 10±2 nm has been prepared by the co-precipitation method and in a next step, the nanoparticles have been coated with gold shell of gold it confirmed by super paramagnetic properties of Au-Fe₃O₄ shows a difference of the magnetization for the coated magnetite nanoparticles in comparison with the uncoated ones. The Fluorescence microscopic characterization shows the successful bioconjugation between Au-Fe₃O₄ NPs-FITC labeled STV-bimAFM1 antibody and AFM1.



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