 Highly sensitive aptasensor based on ‘rose petal’ shaped iron nanoparticles decorated on 3D graphene for detection of zearalenone 

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Abstract. Zearalenone (ZEA), a mycotoxin mainly synthesized by Fusarium graminearum and F. culmorum is a widespread contaminant of several important crops such as wheat, maize, and paddy causing major plant diseases. Environmental factors such as rain and wind aids in the dispersal of ZEA in the soil and waters which affects aquatic lifes. ZEA causes detrimental health effects such as hyper-oestrogenism and premature abortions to human and animals when contaminated crops are ingested. Hence, it is vital to detect ZEA as early precautionary step in lowering the risks related with the health impairment to human and animals, as well as environmental contamination. Conventional methods are time-consuming and complex, thus, this study aimed on developing a highly sensitive biosensor using graphene-nickel decorated with ‘rose petal’ shaped iron nanoflowers (GNINF) as the transducer and aptamer as the bioreceptor. Low-pressure chemical vapour deposition is used to grown 3D-graphene followed by electrochemical deposition of iron (II) sulphate on its surface to form iron nanoflowers. Immobilisation of chemical and biomolecules were done using the layer-by-layer technique. Field-Emission Scanning Electron Microscopy showed prominent ‘rose petal’ shaped nanoflowers on the graphene surface. This unique assembly creates large surface area for immobilisation and better electric charge transfer on the material surface. The existence of hydroxyl group on the surface of GNINF also plays a role as linker to the surface. Besides, the sensitivity of the aptasensor was characterised using electrochemical impedance spectroscopy. The limit of detection achieved in this study is 1 fg ml⁻¹ and the linear range is 1 fg ml⁻¹ to 1 ng ml⁻¹, which is highly sensitive than most reported biosensors. Overall, this highly sensitive aptasensor is a straightforward and cheap alternative for detecting ZEA in crops and the environment.

1. Introduction
Zearalenone (ZEA) is one of the most commonly found pathogenic mycotoxin from the Fusarium species in the world. ZEA is a nonsteroidal, resorcylic acid lactone which allows the mycotoxin to combine to estrogen receptors and prompt estrogenic effects. ZEA generally contaminates cereals but
its presence is also detected in processed products of food and feed due to the stability of the mycotoxin during storage under exposure to high temperature, pressure, ultraviolet light, and mechanical processes such as grinding [1,2]. Accidental ingestion of ZEA contaminated food and feed causes reproductive disorders in human and animals. In humans, the disruption of the endocrine system could lead to cancer development in later stages. Besides, clinical studies of ZEA reported DNA damage, chromosomal abnormalities, peroxidation of lipid biofilms, and immunosuppression in rats and pigs. Apart from that, ZEA leached from the infected crops, excretion of human and livestock, food industry wastewater, or wastewater treatment plants can enter surrounding water systems to contaminate the soil and groundwater [3]. As a result, a variety of freshwater organisms such as planktonic rotifers, insects and larvae, crustaceans, cnidarians, molluscs, and protozoa were affected by toxicity of ZEA [4].

Conventional techniques used to detect ZEA include chromatographic methods like high-performance liquid chromatography (HPLC) [5], HPLC coupled with tandem mass spectrometry (LC-MS/MS) [6], and gas chromatography tandem mass spectrometry (GC-MS) [7]. Albeit their high sensitivity and specificity towards ZEA, these techniques are tedious, slow, expensive and unsuitable for on-site application. Another major problem associated with ZEA detection is its determination at minute level concentrations in complex matrices which needs sensitive and selective detection techniques which is not achievable via conventional methods [8]. To overcome the disadvantages of these conventional techniques, biosensing techniques have been considered a promising alternative due to its miniaturization and better performance in terms of sensitivity of detection.

Recently, an optical fiber-based localized surface plasmon resonance (LSPR) biosensor was developed for detection of ZEA [9]. Besides, a ratiometric fluorescence aptasensor using ZEA aptamer-modified nitrogen doped graphene quantum dots (NGQDs-apt) and silica sphere-encapsulated cadmium telluride quantum dots (CdTe QDs@SiO$_2$) could determine ZEA as low as 0.32 pg ml$^{-1}$ [10]. However, these methods incorporate expensive noble metals and complex material preparation for biosensor. On the other hand, electrochemical biosensor using chitosan functionalized acetylene black and multi-walled carbon nanotubes (CS@AB-MWCNTs) nanocomposite and carboxylated graphene oxide-labeled ZEA binding aptamer (CGO-ZBA) was designed for ZEA detection [11]. In addition, a study on electrospinning technique used to fabricate pencil-graphite electrode to determine presence of ZEA in food simulants was reported [12]. Although successful, these studies involve long material preparation time and complex procedures to prepare the nanocomposites [8]. As the field of biosensing is rapidly evolving, an easier and cost-effective approach, as well as a more sensitive and selective biosensor is needed for ZEA detection.

Therefore, in this study, a highly conductive graphene-nickel decorated with ‘rose petal’ shaped iron nanoflowers (GNINF) was developed for sensitive detection of ZEA. This study aims to overcome the long preparation time and complex procedures as well as exclude the use of expensive noble metals in the developed biosensor. The three dimensional graphene was grown via chemical vapour deposition method which is straightforward and easy to be used. Besides, the decoration of iron oxide on the 3D graphene surface only takes 4 minutes. Thus, the GNINF biosensor is easy to prepare in a short duration and possess great electrical conductivity and large surface area for biosensing application.

2. Methodology
In this study, surface morphology and chemical component characterization has been done on GNINF to determine the suitability of GNINF as biosensor material. Then, the GNINF biosensor was analyzed for its sensitivity and selectivity performance on detection of ZEA.

2.1. Materials and reagents
The nickel foam was purchased from Shanghai Winfay Industry Ltd., China. (3-Aminopropyl)triethoxysilane (APTES), glutaraldehyde, acetone (99.5%), ethanol (70%), iron (II) sulphate, ZEA, deoxynivalenol (DON) and ochratoxin A (OTA) mycotoxins, phosphate buffer saline (PBS), and ethanolamine were obtained from Sigma Aldrich, USA. ZEA aptamer with sequence 5'-
NH2-C6-TCA TCT ATC TAT GGT ACA TTA TCT GTA ATG TAT G-3' was synthesized commercially from Apical Scientific Sdn. Bhd., Malaysia [13]. All chemicals were stored in supplier recommended condition and used without further purifications.

2.2. Synthesis of GNINF
The 2×2 cm nickel foams were cleaned with acetone and propanol to remove contaminants. Then, the dried nickel foams were placed in the chemical vapor deposition equipment (planarTECH CVD System) at a pressure of 3 Torr with constant argon and hydrogen flow. Once the furnace is stabilized for 10 minutes at 1000°C, methane gas was purged into the furnace for the growth of 3D graphene.

Then, iron nanoflowers were deposited on the 3D graphene surface using electrochemical deposition for 4 minutes using pure iron (99.99%) as anode, 3D graphene as cathode and 0.1 M iron (II) sulphate as the electrolyte [14]. The GNINF was then washed with deionized water to remove excess iron and dried.

2.3. Surface morphology and chemical composition characterization of GNINF
Field emission scanning electron microscopy (FESEM, Zeiss Supra55 VP) with energy-dispersive X-ray (EDX) analyses were used to determine the surface morphology of 3D graphene decorated with iron. X-ray photoelectron spectroscopy (XPS, Thermo Scientific, K-alpha with a monochromatic Al Kα source with step size of 1000 eV) was used to verify the chemical composition of GNINF.

2.4. Immobilization of GNINF and electrochemical measurement of ZEA detection
Layer-by-layer immobilization on GNINF surface was initiated by washing the surface of GNINF with 70 % ethanol to remove any impurities. Then, 2.5 % APTES was incubated on GNINF surface for an hour. The unbound APTES is then rinsed with 10 times volume of 10 mM PBS (pH 7.4). APTES was followed by adding 3 % glutaraldehyde for an hour, pre-heated ZEA aptamer for 30 minutes and ethanolamine for 15 minutes. Then, 10 μL of diluted ZEA mycotoxin was incubated with the surface for 5 minutes. ZEA was diluted with PBS in a range of 1 fg ml-1 to 1 ng ml-1 via serial dilution. Each layer-by-layer deposition was followed by washing steps with 10-times volume of PBS and electrochemical impedance spectroscopy (EIS) measurements were conducted after addition of each layer using the Autolab PGSTAT302N. Besides, the selectivity of the GNINF biosensor towards ZEA was tested in a cross-reactivity study with DON and OTA. All electrochemical measurements were analyzed with the Nova 2.1 software.

3. Results and discussion
Surface morphology characterization of GNINF confirms the formation of iron nanoflowers on the 3D graphene surface. Besides, chemical component characterization of GNINF reports the presence of biocompatible chemical components on it which makes it suitable for biosensing application. As a result, the developed biosensor also showed high sensitivity and selectivity towards ZEA detection.

3.1. FESEM and EDX studies of GNINF
A comparison of FESEM micrograph of 3D graphene (Figure 1a) and GNINF (Figure 1b) showed prominent formation of ‘rose petal’ shaped iron nanoflower on the surface of GNINF. The formation of iron nanoflower provides a large surface area on its surface which deems it suitable for the effective immobilization of the biomolecules. The shape and pattern of the iron nanoflower could be attributed to the presence of the wrinkles on the 3D graphene layer. These wrinkles are the results of variation in thermal expansion coefficients between the nickel foam and graphene where the nickel foam retracts faster than graphene. Thus, the wrinkles act as accumulators of electrons during the electrochemical deposition process which attracts the iron ions to be deposited on it forming ‘rose petal’ shaped iron nanoflowers on the 3D graphene surface [15].

The EDX and elemental mapping analyses of GNINF showed that the distribution of iron was more prominent in the area with the abundant of wrinkles on the 3D graphene (Figure 2 a-b). This confirms
that the irons are attracted towards the wrinkled parts of the 3D graphene due to its high current density [16].

![Figure 1. FESEM micrograph of a) 3D graphene b) GNINF.](image)

**Figure 1.** FESEM micrograph of a) 3D graphene b) GNINF.

![Figure 2. a) EDX and b) elemental mapping of GNINF.](image)

**Figure 2.** a) EDX and b) elemental mapping of GNINF.

### 3.2. XPS analysis of GNINF

Deconvoluted narrow scans for Fe2p and O1s regions for GNINF were demonstrated in Figure 3 a-b and Table 1. In the Fe2p region, peaks at 711.98, 719.91, 725.27, and 733.69 eV were recorded. Broad peaks at 711.98 and 725.27 eV for Fe2p$_{3/2}$ and Fe2p$_{1/2}$, respectively, are caused by electrostatic interaction between photoionized Fe2p core hole and unpaired Fe3d electrons, spin–orbit coupling and crystal field interactions. Furthermore, minor satellite peaks found could be due to the charge transfer [16].

Meanwhile, in the O1s region, the peaks at 530.4 and 531.9 eV were attributed for Fe–O and Fe–OH, respectively. The presence of these components could be due to the surface oxidation. Besides, the presence of the hydroxyl groups provides biocompatible surface for the binding of APTES on the biosensor during immobilization [16].
Figure 3. XPS analysis of GNINF.

Table 1. XPS analysis of GNINF.

| Element | Binding energy (eV) | Chemical component          |
|---------|---------------------|-----------------------------|
| Fe2p    | 711.98              | Fe2p_{3/2}                  |
|         | 725.27              | Fe2p_{1/2}                  |
|         | 719.9               | Satellite                   |
|         | 733.7               | Satellite                   |
| O1s     | 530.4               | Fe-O                        |
|         | 531.9               | Fe-OH Lattice               |

3.3. Sensitivity of detection of ZEA with GNINF biosensor

Based on the surface morphology and chemical component characterizations, it was determined that GNINF is a suitable biosensor material. Therefore, the GNINF biosensor was tested with ZEA of concentration ranging from 1 fg ml\(^{-1}\) to 1 ng ml\(^{-1}\) and its sensitivity was measured with the EIS method. It was observed that the resistance increased as the concentration of ZEA was increased (Figure 4 a). As the ZEA concentration was increased, more molecules will bind to the aptamer which stimulates electron transfer, decrease conductivity and enhancing the charge transfer resistance. The limit of detection (LOD) of ZEA using the GNINF biosensor was 1 fg ml\(^{-1}\) determined through observation.

3.4. Selectivity of GNINF biosensor towards ZEA

The selectivity of GNINF biosensor towards ZEA was determined by a cross reactivity study with DON and OTA. It was found that ZEA showed higher resistance value compared to DON and OTA suggesting that it bonded with the ZEA aptamer thoroughly (Figure 4 b). This is due to the specificity of the tailor-made ZEA aptamer towards ZEA.
4. Conclusion and recommendation

In summary, the GNINF aptamer based impedimetric biosensor has been developed for sensitive and selective sensing of ZEA. The surface morphology characterization shows high surface area for biomolecule attachment, whereas X-ray spectroscopy found biocompatible chemical components on GNINF surface. These properties make the GNINF a suitable biosensor material. Based on the EIS measurements, it can be concluded that GNINF has high electrical conductivity and this aids in successful electron transfer of the biosensor which increases the sensitivity of the biosensor. Besides, the tailor-made aptamer results in highly selective detection of ZEA. Therefore, the GNINF biosensor is an easy to prepare and use as well as cost effective biosensor for detection of ZEA in crops and the environment. This biosensor also opens the revenue the detection of other mycotoxins presents in the environment.

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