

Recent Advances in Detection of Ochratoxin-A

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ABSTRACT

Ochratoxin-A [7-(L-β-phenylalanylcarbonyl)-carboxyl-5-chloro-8-hydroxy-3,4-dihydro-3R-methyl-isocoumarin, OTA] is a common food contaminant mycotoxin that enters the human body through the consumption of improperly stored food products. Upon ingestion, it leads to immuno-suppression and immuno-toxicity. OTA has been known to produce nephrotoxic, teratogenic, and carcinogenic activity (via oxidative DNA damage) in several species. This review introduces potentials of electrochemical biosensor to provide breakthroughs in OTA detection through improved selectivity and sensitivity and also the current approaches for detecting OTA in food products.

Keywords: Biopolymer; Mycotoxins; Ochratoxin-A; Chitosan; Organic-Inorganic Hybrid Nanocomposite; Biosensors

1. Introduction

Mycotoxins are secondary metabolites produced by microscopic (micromycetes) and macroscopic (macromycetes) fungi which can contaminate food crop, or entire food crops, throughout the food chain. The toxic effects of these metabolites in mammals (including humans) is known as mycotoxicosis [1,2]. **Figure 1** describes the various factors involved in mycotoxicosis. These factors lead to the production of fungal toxins *i.e.*, mycotoxin. Fungal toxins are produced by species within the genera *Fusarium*, *Aspergillus*, and *Penicillium*, which frequently occur in crops or during storage of food [1]. These mycotoxins have deleterious effect on agricultural productivity, human health (including carcinogenesis, immune suppression, teratogenicity), growth retardation, and animal toxicities including the possible carry-over of mycotoxins or their metabolites into the human food chain [1].

Mycotoxins can appear in the food chain from infected crop, either by direct human consumption or through the use of livestock feed (**Figure 2**).

The high incidence of human food contamination with mycotoxins has led a research effort in the areas of mycotoxicology to study its biochemical mode of action, implications on human health, and development of analytical tools for quantitative and qualitative analysis of these toxins in human food [3-5]. Various techniques used for the detection of mycotoxins are summarized in **Table 1** with their advantage and drawbacks.

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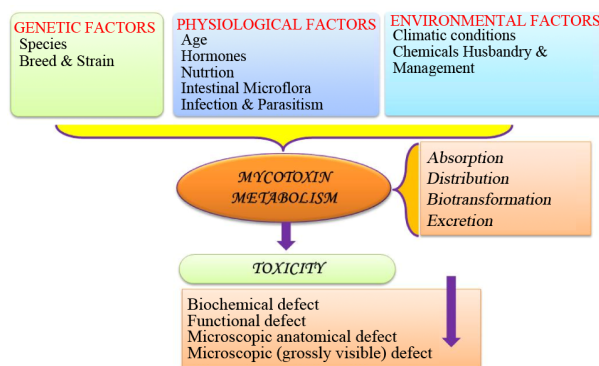


Figure 1. Representation of mycotoxicosis.

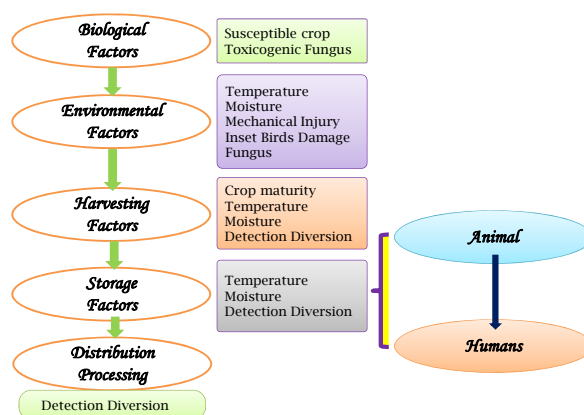


Figure 2. Factors affecting mycotoxin occurrence in the food and animal feed chains.

Table 1. Analytical methods used to detect mycotoxins.

Analytical Techniques	Advantages	Disadvantages
Thin Layer Chromatography (TLC)	Simple, inexpensive & rapid Can be used for screening Simultaneous analysis of multiple mycotoxins Suitable for ochratoxin and aflatoxin	Poor sensitivity (for some mycotoxins) Poor precision Adequate separation may require 2 dimensions analysis Quantitative when used with a densimeter
Gas Chromatography (GC)	Simultaneous analysis of multiple mycotoxins Good sensitivity May be automated (auto sampler) Provide confirmation (MS detection)	Expensive instrument Need expertise Derivatization required Matrix interface problem Non-linear calibration curve Drifting response Carry-over effect from previous sample Variation in reproducibility and repeatability
High Performance Liquid Chromatography (HPLC)	Good sensitivity Good selectivity May be automated Short analysis time Official method available	Expensive instrument Need expertise Derivatization may be required
LC/Mass Spectroscopy	Good sensitivity LC/MS/GC Simultaneous analysis of multiple mycotoxins Provide confirmation No derivation require	Expensive instrument Need expertise Matrix assisted calibration curve (for quantitative analysis)
Real Time polymerase chain reaction (PCR)	Good Sensitivity Accurate Suitable for high throughput analysis	Time taking Error during polymerase
Enzyme Linked Immunosorbant Assays (ELISA)	Simple sample preparation Inexpensive equipment Simultaneous analysis of multiple mycotoxins Suitable for screening Limited used of organic solvents Visual assessment	Cross reactivity with related myco toxins Matrix interface problem Possible false +ve/-ve results Critical quantization near regular limit Semi-quantitative (visual assessment)
Rapid tests Membrane based cards Antibody based tubes Immuno cup test	Simple and fast (5 - 10 min) No expensive equipment required Limited use of organic solvents Suitable for screening Can be used in-situ	Quantitative or semi quantitative (cut off level) Possible false +ve/-ve results Cross reactivity with related mycotoxins Matrix interface problem Lack of sensitivity near regular limit
Electrochemical Biosensor	Rapid and cost-effective Good selectivity and sensitivity Portable, no expertise required Used for multiple mycotoxins detection Suitable for screening Commercial issues are open	Selection of immobilizing matrix and techniques is crucial to optimize best electrical parameters to obtain enhanced sensing performance

Among mycotoxins, OTA, OT-B & C, aflatoxin B1, B2, B3 & M1, citroviridin, patulin, citrinin and zearalenon are widely studied due to their known adverse effect on human/animal health through food and beverage consumption. Among these OTA has gained much attention recently.

OTA, the toxic metabolite, produced by several fungal species such as genera *Aspergillus* and *Penicillium*, is present in food and food products due to the thermostability of its derivatives [6]. OTA has been widely detected in cereal-derived products, dried fruits, spices, beer, and wine [7]. Strains of OTA from *Aspergillus*

carbonarius and *Aspergillus niger*, that contaminate grapes, has been found in wines [8]. Additionally, OTA in large amounts has been found in animal feed [9,10]. OTA in cereals is mainly produced by *P. verrucosum*, while OTA in grapes, coffee and cocoa is formed by *A. Carbonarius*. As concerned with agricultural food safety, OTA has been found to be responsible for the contamination of several agricultural products.

The critical factors that affect fungal growth during farming, harvesting, and storage of commodities susceptible to OTA are temperature, moisture content, and exposure time. Additional factors that support fungal growth

are the presence of spores, the physical location (the internal part of a vegetable is more vulnerable to fungal invasion than the external area), insects (due to their metabolism, they increase the moisture and temperature of the plant and break its protective shield), storm and rain damage, moisture stress, the availability of mineral nutrient, pH (in general, mold tolerates acid media and is able to acidify the media themselves), oxygen and carbon dioxide levels, chemical and physical treatments, and, for some commodities, the product drying and re-wetting speed [11].

OTA is the most detected mycotoxin in human blood globally due to its binding with plasma protein, long elimination half-life (~35 days in serum), its enterohepatic circulation and its reabsorption from urine [12]. Human exposure to OTA has been clearly demonstrated through its detection in human blood, urine and food [13]. In animal species, OTA has shown many adverse effects such as teratogenicity [14], immunotoxicity [15], genotoxicity [16] and mutagenicity [17]. The most important toxic effect of this mycotoxin is its nephrotoxicity [2] leading to conditions such as “Balkan Endemic Nephropathy” [18]. In 1993, the International Agency for Research on Cancer (IARC) classified OTA in group 2B, a possible human carcinogen due to induction of oxidative DNA [18]. The proposed hypothesis of OTA causing cancer is shown in **Figure 3**.

Keeping these adverse effects in mind various health organizations have set OTA consumption limits. The World Health Organization has established the provisional tolerable weekly intake of OTA as $100 \text{ ng}\cdot\text{kg}^{-1}$ of body weight (equivalent to $14 \text{ ng}\cdot\text{kg}^{-1}$ body weight/day) [6,7]. The European commission has set the following limits of OTA contamination: $10.0 \text{ }\mu\text{g}\cdot\text{kg}^{-1}$ for dried fruits and soluble coffee, $5.0 \text{ }\mu\text{g}\cdot\text{kg}^{-1}$ for raw cereal grains and roasted coffee, $3.0 \text{ }\mu\text{g}\cdot\text{kg}^{-1}$ for cereals intended for human consumption, $2.0 \text{ }\mu\text{g}\cdot\text{kg}^{-1}$ for wine and

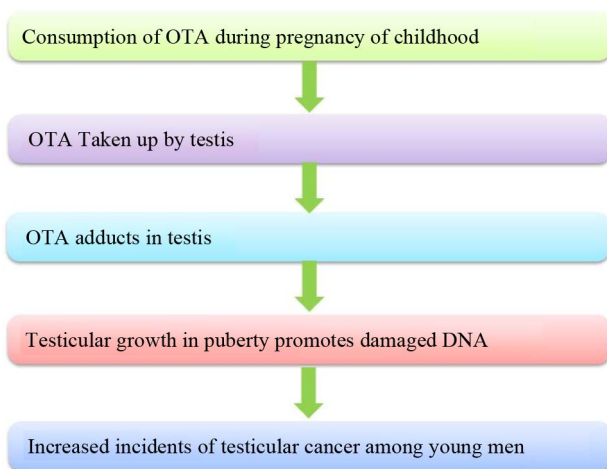


Figure 3. Hypothesis for OTA to cause testicular cancer.

grape juice and $0.5 \text{ }\mu\text{g}\cdot\text{kg}^{-1}$ for baby foods and cereal-based foods intended for young children (European Commission) [6,7]. Thus precise, rapid and selective detection of OTA is warranted for human health care and for diagnostics. The various methods used for the estimation of OTA are discussed in next section.

2. Current Approaches for Detection of OTA

OTA and other food borne contaminants come in a variety of sizes ranging from simple chemical compounds to entire bacterial cells. Due to public health concerns, there is an overwhelming need in the food industry for sensitive, specific, and rapid methods to monitor the presence of OTA [19,20]. This can be achieved by setting up hazard critical control point concepts, which “filter” out all those lots displaying certain levels of the toxin. According to the hazard critical control point philosophy, analytical concepts demand methods that fulfill the following needs a) analytical target must be a direct marker for the hazard that needs to be controlled, b) marker must be indicative for the future toxigenic potential of a sample as well as for its mycological history, c) sensitivity of the analysis must be high enough to detect hazardous samples, d) results must enable decisions to be taken on whether or not a given lot should pass or fail the control point, e) screening of vast numbers of samples must be possible, e.g. the procedure must have an automatable format for high-throughput applications, f) results must be available in a short time, g) tests must be usable by relatively unskilled personnel and h) Tests must be inexpensive and easy to use, as must the equipment used to perform them.

In this context, chromatographic analytical tools such as thin layer chromatography (TLC) and high performance liquid chromatography (HPLC) have been used for detection OTA using low volume samples as well as quicker sample clean-up. Pittet *et al.* reported a screening method for the detection of OTA (range 0.2 to $136.7 \text{ }\mu\text{g}\cdot\text{kg}^{-1}$) in green coffee at a control level of $10 \text{ }\mu\text{g}\cdot\text{kg}^{-1}$ using TLC [21]. Varelis *et al.* described the use of reverse-phase HPLC-fluorescence detection (FD) with post column ammoniation to improve the detection limit for OTA in wine and beer with the high precision and concentration range of 0.016 - $1.284 \text{ mg}\cdot\text{l}^{-1}$ [22]. Reinsch *et al.* combined the anion exchange/reversed phase clean-up and liquid chromatography (LC) with tandem mass spectrometry to detect OTA in beer [23]. The method exhibits a detection range from 1.17 to $5.56 \text{ }\mu\text{g}\cdot\text{kg}^{-1}$, and a detection limit of $0.4 \text{ }\mu\text{g}\cdot\text{kg}^{-1}$ and limit of quantification of $0.8 \text{ }\mu\text{g}/\text{kg}$. Juan *et al.* reported HPLC-FD to detect OTA in maize bread samples via extraction with PBS, methanol (50:50) and clean-up with immuno-affinity column [24]. The limit of quantification was obtained as 0.033 ng/g

and recoveries ranged from 87% to 102% for concentration range 2.0 and 0.5 ng·g⁻¹, respectively. Caputo *et al.* fabricated a hydrogenated amorphous silicon (a-Si:H) sensor for detection of OTA [25]. Selma *et al.* developed a new real-time (RTi) polymerase chain reaction (PCR) based procedure for the rapid and specific detection and quantification of OTA. RTi-PCR assay is a promising tool for the prediction of potential ochratoxicogenic risk, even in the case of low-level infections, and is suitable for rapid, automated, and high throughput analysis [26]. Flajs *et al.* reported enzyme linked immunosorbent assays (ELISA) and HPLC for OTA analysis on wine samples collected in Croatia [19]. The results of ELISA and HPLC analysis of OTA in naturally contaminated red wines correlated well at higher OTA concentrations. Ghali *et al.* studied the detection of 180 samples of OTA contaminated food using HPLC [27]. The experimental analysis indicated that 45% of monitored samples were contaminated with levels ranging from 0.11 - 33.9 ng·g⁻¹. Lai *et al.* prepared a rapid, inexpensive and user-friendly lateral flow strip assay ideally suited for onsite testing of OTA [28]. Mimotope peptide capable of mimicking OTA by panning from a M13 phage-displayed random seven-peptide was used instead of OTA-protein conjugate. 10 ppb of OTA was detected in 10 min using this new strip. These techniques provide precise results however they are very expensive, require expertise and are susceptible to false positive results. Conversely, ELISA is a rapid, simple, specific, and sensitive technique but may occasionally result in a systematic overestimation when compared to the chromatographic methods. Recently, immunosensors have attracted considerable attention for OTA and other mycotoxin detection because they lead to rapid analysis, selectivity, sensitivity, cost-effectiveness and are user friendly [20,29-31].

3. Recent Advances in OTA Detection Using Immunosensors

Estimation of OTA using immunosensors is getting main stream [32]. Tsai *et al.* fabricated a piezoelectric immunosensor based on self-assembled monolayer of 16-mercaptohexadecanoic acid (16-MHDA) to detect OTA using quartz crystal microbalance (QCM) [33]. The fabricated immunosensor exhibited detection range from 50 to 1000 ng·mL⁻¹, detection limit ~16.1 ng/mL with negligible effect of interference. Ngundi *et al.* reported the development of a rapid and highly sensitive competitive immunoassay for the detection and quantification of OTA [34]. OTA was quantified by measuring the formation of the fluorescent immuno-complex on the waveguide surface. The limit of detection for OTA in several cereals has been reported in a range of 3.8 - 100 ng·g⁻¹, while in coffee and wine, detection limits were 7

and 38 ng/g respectively. Adanyi *et al.* used the optical waveguide light mode spectroscopy (OWLS) techniques for the detection of aflatoxin and OTA in both competitive and direct immunoassays wherein a microchip utilized a flow-injection analyser (FIA) system [34].

Prieto-Simon *et al.* reported OTA detection [35] through immobilization of polyclonal (PAb) and monoclonal (MAb) antibodies. The sensor demonstrated values at least one-order of magnitude lower (IC₅₀) when working with MAb. Considering 80% of antibody binding, both HRP and alkaline phosphatase (ALP) labelled immunosensor showed detection limit of 0.7 and 0.3 ng/mL, respectively.

Ansari *et al.* have reported a zinc oxide (ZnO) based impedimetric immunosensor for OTA detection that exhibited a linearity of 0.006 - 0.01 nM·(dm³)⁻¹, detection limit of 0.006 nM·(dm³)⁻¹, response time of 25 s and sensitivity of 189 Ω·nM⁻¹·(dm³)⁻¹·cm⁻² with a regression coefficient of 0.997 [36]. Kaushik *et al.* have utilized sol-gel derived cerium oxide (CeO₂) nanostructured platform for the fabrication of electrochemical immunosensor to detect OTA [37]. This CeO₂ based immunoelectrode exhibited a linear range (0.5 - 6 ng·dL⁻¹), low detection limit (0.25 ng·dL⁻¹), fast response time of (30 s) and high sensitivity (1.27 μA·ng⁻¹·dL⁻¹·cm⁻²). The obtained high value of the association constant (K_a, 0.9 × 10¹¹ l·mol⁻¹) reveals the high affinity of immobilized antibody with OTA. Radi *et al.* prepared a novel, label-free, Electrochemical Impedance Spectroscopy (EIS) immunosensor to detect OTA [38] that exhibited linearity in the range 1 - 20 ng·mL⁻¹, with a detection limit of 0.5 ng·mL. Radi *et al.* also proposed a protocol to fabricate competitive immunosensor for OTA detection via immobilizing horseradish peroxidase (HRP) labelled OTA antibodies onto 4-nitrophenyl modified screen-printed gold (Au) electrode. The electrochemical response was observed using 3,3',5,5'-tetramethylbenzidine as substrate. This immunosensor exhibits detection limit of 12 ng·mL⁻¹ and a dynamic range up to 60 ng·mL⁻¹ of OTA [39]. Aptamer-DNA enzyme hairpin as a biorecognition element has been used to detect OTA, wherein self-assembly of the active HRP-mimicking DNA enzyme occurs, and exhibits detection up to 10 nM and detection limit of 2.5 nM [40]. DNA aptamers based electrochemical impedance biosensor was utilized for the detection of OTA which exhibited detection range of 0.1 - 100 nM with the detection limit of 0.44 nM [41,42].

OTA-bovine serum albumin (OTA-BSA) has been conjugated onto gold-coated quartz crystals (AT-cut/5 MHz) to fabricate flow-injection based piezoelectric immunosensor to detect OTA. 3D procedures for the direct adsorption and covalent attachment to two alkane thiol self-assembled monolayers were characterized using QCM in real time. The immunosensor was efficient

to detect OTA in concentration range from 10 to 128 $\text{ng}\cdot\text{mL}^{-1}$, and detection limit as 8 $\text{ng}\cdot\text{mL}^{-1}$. Moreover, the immunocomplex formed due to antigen-antibody binding can be released pepsin of 2 $\text{mg}\cdot\text{mL}^{-1}$ ($\text{pH} = 2.1$) solution to regenerate the bio recognition surface [43]. EIS coupled with QCM has been utilized for the detection of OTA via immobilizing antibodies onto mercaptoundecanol/mercaptoundecanoic acid which was mixed with self-assembled monolayer modified gold electrode. It was observed that the increase of the relative change of charge transfer resistance was proportional to OTA concentration and found linear between 100 and 1000 $\text{pg}\cdot\text{mL}^{-1}$ [44].

Liu *et al.* fabricated an electrochemical immunosensor based on self-assembled monolayer of 1,6-hexanedithiol fabricated onto Au colloid layer via immobilizing OTA-ovalbumin conjugate [45]. The fabricated immunosensor exhibited the detection ranging from 10 $\text{pg}\cdot\text{mL}^{-1}$ to 100 $\text{ng}\cdot\text{mL}^{-1}$ and detection limit of 8.2 $\text{pg}\cdot\text{mL}^{-1}$. Prabhakar *et al.* utilized a Langmuir-Blodgett film of polyaniline (PANI)-stearic acid (SA) to fabricate an aptamer based impedimetric immunosensor to detect OTA [46]. The electrochemical response studies suggested that Apt-DNA/PANI-SA/ITO exhibits linear range of 0.1 $\text{ng}\cdot\text{mL}^{-1}$ to 10 $\text{ng}\cdot\text{mL}^{-1}$ and 1 $\mu\text{g}\cdot\text{mL}^{-1}$ - 25 $\mu\text{g}\cdot\text{mL}^{-1}$ with detection limit of 0.1 $\text{ng}\cdot\text{mL}^{-1}$ in 15 min. Sensing electrode was found to be 13 times reusable with affinity constant, estimated using Langmuir adsorption isotherm, as $1.21 \times 10^7 \text{ M}^{-1}$. Zamfir *et al.* proposed magnetic nanoparticles based label-free immunosensor for the detection of OTA using EIS/surface plasmon resonance (SPR) techniques. The impedimetric parameters *i.e.*, R_{ct} variation was found to be antibody-OTA interaction dependent which exhibited linear range of 0.01 - 5 $\text{ng}\cdot\text{mL}^{-1}$ with a detection limit as 0.01 $\text{ng}\cdot\text{mL}^{-1}$. A larger response range from 1 to 50 $\text{ng}\cdot\text{mL}^{-1}$ with a detection limit of 0.94 $\text{ng}\cdot\text{mL}^{-1}$ was observed in case of OTA detection using SPR [47]. Solanki *et al.*, have immobilized anti-OTA antibody onto self-assembled monolayer of 11-Amino-1-undecanethanol for the selective detection of OTA in coffee sample using EIS technique [48]. The results of the studies revealed linearity from 0.5 $\text{ng}\cdot\text{dL}^{-1}$ - 6.0 $\text{ng}\cdot\text{dL}^{-1}$, detection limit of 0.08 $\text{ng}\cdot\text{dL}^{-1}$ and sensitivity of 36.83 $\Omega\cdot\text{ng}^{-1}\cdot\text{dL}^{-1}\cdot\text{cm}^{-2}$ with a regression coefficient of 0.999. Authors have validated fabricated immunosensor to estimate OTA in coffee samples.

Recently, nanostructured platform such as metals, metal oxides, quantum dots, self-assembled monolayers, conducting/biopolymers and hybrid nanocomposites have been employed in immunosensor fabrication [49]. Inorganic nanostructured materials such as metals or metal oxides are crystalline materials with precise chemical composition, surface terminations, and are dislocation-defect free [50,51]. Surface effects appear be-

cause of the magnification in the specific surface of nanostructures, which leads to an enhancement of the properties such as catalytic activity or surface adsorption. This enhancement allow these materials to be used in many applications such as catalysis, fuel cell, heavy metal detection, photonic band-gap materials, single electron transistors, non-linear optical devices, biotechnology etc. [52,53].

The unique electrical and optical properties of organic nanostructures such as conducting polymers [PANI, etc.] [54-57], biopolymers [chitosan (CH), etc.] [58-63], and self-assembled monolayers [64] helps to exhibit the ease in synthesis with desired functionality and therefore makes them promising candidates for a wide range of electronic, optoelectronic and molecular electronic applications. The changes in electrical and optical properties of conducting polymers combined with interaction of oxidizing or reducing agents make them suitable materials for desired applications. The conducting polymers which can make a transition from an initial insulating state to an electrically conducting state after the chemical treatment with redox active agents have been widely exploited for optical and bio/gas sensors [55,56]. However, the problem of stability and its interaction with moieties present in environment limits the application of most of the organic materials. Consequently, biopolymers such as CH, due to their excellent optical, mechanical, and electrical properties, have been used in industrial and medical applications such as bioplastics, medical devices, tissue engineering, and drug delivery [58]. Efforts are continuing towards the engineering of nanomaterials to obtain improved optical and electrical properties for desired applications. In this context, the possibility of combining properties of organic and inorganic components at the same platform on the basis of organic-inorganic hybrid materials at nanoscale is a new approach to design new functional materials with desired properties.

Organic-inorganic hybrid nanocomposites, such as conducting polymer-metal/metal oxides and biopolymer-metal/metal oxides, are the new class of materials wherein nanocomposites exhibit the properties of organic as well as inorganic counterparts, along with some new properties that are absent in counterpart molecules. These unique properties of organic-inorganic hybrid nanocomposite can be exploited for various applications such as mechanically reinforced lightweight components, non-linear optics, battery cathodes, nanowires, optics, electronics, ionics, mechanics, membranes, protective coatings, catalysis, sensors, biology etc. [65-67]. Hybrid nanocomposites systems can be used for biomimetic artificial systems (*i.e.* membranes, vesicles, liposomes, enzymes, etc.) that exhibit behaviour similar to those of living bodies and are also complex architectural assemblies [68-70]. Because of their exceptional biocompatibility, optical,

and electrical properties due to electron and phonon confinement organic-inorganic hybrid nanocomposites are receiving a great deal of attention as alternative matrices for biomolecule immobilization aiming to improve stability and sensitivity of desired biosensor [71,72]. These systems can be used to immobilize desired biomolecules such as enzymes, antibodies or micro-organisms with high activity and natural state to perform specific reactions that would not be possible with the usual chemical routes. The organization and properties that can be obtained for organic-inorganic hybrid nanocomposite materials certainly depend on the chemical natures of their parent components, but they also rely on the synergy between their counterparts. These hybrid nanocomposites can be prepared using sol-gel technique, assembly of nanobuilding blocks, self-assembly of nanomaterials, integrative, and electrochemical methods [66,73-79]. Recently, among hybrid nanocomposites, nanostructured CH and metal/metal oxide nanoparticle based hybrid nanocomposite systems are being studied for fabrication of biosensor fabrication [58,61-63,80] and have been utilised for OTA detection (discussed in next section).

4. CH Nanocomposites Based Immunosensor for OTA Detection

In recent years, CH has potentially been used as an immobilization matrix for cell carriers, living organisms, or enzymes/DNA/antibody [70,81-84]. The unique structural feature of CH is the presence of amine groups at the

C-2 position of glucosamine residue resulting in important functional properties that can be used for biosensor applications. CH has been reported as a rather important emerging immobilization matrix in various pharmaceutical, environmental and biotechnological applications due to its excellent film-forming ability, good adhesion, biocompatibility, high mechanical strength, and susceptibility to chemical modifications due to the presence of reactive hydroxyl and amino functional groups. Moreover, widely present amino and hydroxyl groups present in CH molecules facilitate immobilization of biomolecules via covalent binding, electrostatic interactions, etc. Therefore numerous processes using CH and enzyme immobilization are well-established in various fields [85-88] for example in contact lenses [89], as drug nano-carriers [90], as a matrix for cell [91] and as desired biomolecules immobilization [92] and artificial skin [93]. The derivatives of CH are being potentially used for biomedical applications [94].

Recently, nanocomposite of CH-metal/metal oxide has emerged as an appropriate, suitable, and micro-friendly immobilizing matrix and has been employed to fabricate electrochemical immunosensor for OTA detection with improved sensing characteristics. The proposed schematic for the fabrication of CH-metal/metal oxide based nanocomposite platform to fabricate electrochemical immunosensors for the rapid, selective, specific, and sensitive detection of OTA is shown in **Figure 4**.

Electrochemical impedimetric immunosensors have

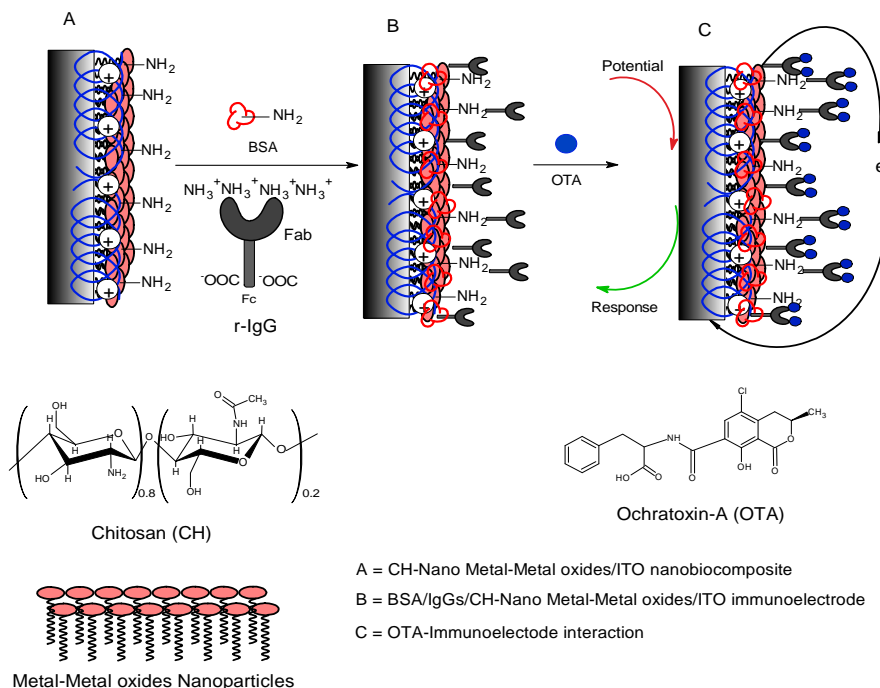


Figure 4. The proposed schematic for the stepwise fabrication of CH-Metal oxide nanocomposite based immunosensor for OTA detection.

been proposed by Khan and Dhayal using nanocomposite films based on CH and TiO₂ nanoparticles to immobilize Abs for OTA detection. The CH-TiO₂ nanocomposite based immunosensor exhibited linearity in 1 - 10 ng·dL⁻¹ range with the detection limit of 1 ng·dL⁻¹ [95]. Khan *et al.* also prepared an impedimetric immunosensor for OTA detection using hybrid film of PANI and CH to immobilize r-IgGs. OTA interaction with IgGs increases R_{CT} values and shows linear response up to 1 - 10 ng·mL⁻¹ with absolute sensitivity of 53 ± 8 mL·ng⁻¹ [96].

Studies by Kaushik *et al.* revealed that surface charged CH film can be used to detect OTA via immobilization of r-IgGs. Further, the presence of NH₂/OH groups in CH provides a favorable microenvironment for immobilization of IgGs leading to enhanced electron transfer to the electrode. They showed that the CH based immunosensor shows improved characteristics such as linearity (1 - 6 ng·dL⁻¹), low detection limit (1 ng·dL⁻¹), fast response time (25 s), high sensitivity (4.8 × 10⁻⁸ A·dL⁻¹), reproducibility (>10 times), and long term stability (30 days) in comparison to that of the CH/ITO based immunosensor [97]. In another study, nanoCeO₂ was incorporated into CH to fabricate CH-NanoCeO₂ nanobiocomposite film onto ITO substrate for the co-immobilization of r-IgGs and BSA to detect OTA [61]. The results of electrochemical studies revealed that the presence of NanoCeO₂ in CH-NanoCeO₂/ITO nanobiocomposite increases the effective surface area of CH resulting in improved loading of r-IgGs. Electrochemical response of BSA/r-IgGs/CH-NanoCeO₂/ITO immunoelectrode was obtained as a function of OTA concentration exhibits linearity in the range of 0.25 - 6.0 ng·dL⁻¹, detection limit of 0.25 ng·dL⁻¹, response time of 25 s, sensitivity of 18 μA·ng⁻¹·dL⁻¹·cm⁻² with 0.997 regression coefficient and standard deviation of 0.28 × 10⁻⁵ μA·ng⁻¹·dL⁻¹. BSA/r-IgGs/CH-NanoCeO₂/ITO immunosensor was found to exhibit improved sensing characteristics than that of the sol-gel derived NanoCeO₂ and CH based immunoelectrode for OTA detection [61].

Recently, Kaushik *et al.* fabricated NanoSiO₂ and CH based nanobiocomposite film onto an ITO substrate to co-immobilize r-IgGs and BSA for OTA detection [63]. The observed three-dimensional (3D) arrangement of NanoSiO₂ in CH matrix via hydrogen bonding, available NH₂/OH groups, and excellent film forming abilities of CH resulted in an increased effective surface area of CH-NanoSiO₂ nanobiocomposite for r-IgGs immobilization resulted in enhanced electron transport between r-IgGs and electrode. It has been shown that BSA/r-IgGs/CH-NanoSiO₂/ITO immunoelectrode can be used for OTA detection with improved sensing characteristics. Electrochemical studies suggest that the presence of NanoSiO₂ leads to enhanced electrochemical behavior of CH resulting in increased electron transport between the

medium and the electrode. BSA/r-IgGs/CH-NanoSiO₂/ITO immunoelectrode exhibited improved sensing characteristics such as linearity (0.5 - 6 ng·dL⁻¹), detection limit (0.3 ng·dL⁻¹), response time (25 s) and sensitivity (18 μA·ng⁻¹·dL⁻¹·cm⁻²) with a correlation coefficient of 0.98. CH-NanoSiO₂ nanobiocomposite based immunosensor platform can be used for detection of other mycotoxins. Moreover, authors have studied the co-immobilization of r-IgGs and BSA onto nanobiocomposite films of CH and superparamagnetic Fe₃O₄ nanoparticles for detection of OTA [95]. Herein, surface charged Fe₃O₄ nanoparticles were self-assembled in CH bio-polymeric matrix to prepare nanobiocomposites. It was observed that in the nanobiocomposites, NanoFe₃O₄ results in increased electro-active surface area wherein, affinity of surface charged NanoFe₃O₄ supported the immobilization of r-IgGs and led to enhanced electron transfer. Differential pulse voltammetry (DPV) studies indicate that Fe₃O₄ nanoparticles provide increased electro active surface area for loading of r-IgGs and improved electron transport between IgGs and electrode. BSA/r-IgGs/CH-NanoFe₃O₄/ITO immunoelectrode exhibited improved sensing characteristics such as low detection limit (0.5 ng·dL⁻¹), fast response time (18 s) and high sensitivity (36 μA·ng⁻¹·dL⁻¹·cm⁻²) with respect to other CH based nanocomposite immunoelectrode. Kaushik *et al.*, incorporated MWCNT/CWCNT into polymer backbone of CH to prepare hybrid nanocomposite film for the immobilization of r-IgG to detect OTA [62]. The results of electrochemical studies revealed that CH-SWCNT based biosensor exhibited better sensing characteristics than that of CH-MWNCTS based immunoelectrode due to more electro-active surface area and enhanced electron transfer kinetics. CH-SWCNT/ITO immunoelectrode exhibits improved linearity as 0.25 - 6 ng·dL⁻¹, detection limit as 0.25 ng·dL⁻¹, response time as 25 s, and sensitivity as 21 μA·ng⁻¹·dL⁻¹·cm⁻² with the regression coefficient as 0.998. The work presented by Kaushik *et al.*, was based on polyclonal antibody based OTA detection and may exhibit the problem of specificity. Besides this, the utilized CH based nanocomposites are found to be highly electroactive and are suitable for fabrication of efficient immunosensors for OTA detection. To overcome the problem of selectivity, polyclonal antibody can be replaced with monoclonal antibody or aptamers during immunosensor fabrication using CH-metal/metal oxide hybrid nanocomposite for the selective detection of OTA.

5. Conclusion

Among the various OTA detection techniques, electrochemical biosensors have proven potential for the detection of OTA. In the future, new types of organic-inorganic hybrid nanocomposites having capabilities to pro-

vide better matrices for immobilization of desired biomolecules in the fabrication of biosensors will provide better optical, electrical and molecular responses for better selectivity and sensitivity. As recent developments, polyclonal antibody has been replaced by monoclonal antibody and aptamers to detect OTA. Efforts are being made to optimize OTA immunosensors for real sample analysis. Studies suggest that there is market for the commercial development of these immunosensors for practical, on-site, selective and specific detection of OTA.

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