IMPACTO CIENTÍFICO EDITORA

INTEGRATION OF ANTIBODIES AND METAL NANOPARTICLES IN MYCOTOXIN DIAGNOSTIC SYSTEMS: FIRST STEPS IN THE DEVELOPMENT OF BIOSENSORS FOR OCHRATOXIN A

INTEGRAÇÃO DE ANTICORPOS E NANOPARTÍCULAS METÁLICAS EM SISTEMAS DE DIAGNÓSTICO DE MICOTOXINAS: PRIMEIROS PASSOS NO DESENVOLVIMENTO DE BIOSSENSORES PARA OCRATOXINA A

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ABSTRACT

Ochratoxin A (OTA) is a mycotoxin produced by fungi of the genera Aspergillus and Penicillium, frequently detected in plant-derived products such as seeds, grains, cereals, and animal feed. This toxin has a recognized carcinogenic potential, in addition to neurotoxic, nephrotoxic, and immunotoxic effects, posing risks to human and animal health as well as economic losses to the production chain. Due to its occurrence at very low concentrations in food matrices, its detection requires highly sensitive and specific analytical methods. This study aimed to standardize the initial step of coupling monoclonal anti-OTA antibodies (mAbOTA) to gold nanoparticles (AuNPs), as a basis for the future development of biosensors for OTA detection in biological and food samples. AuNP concentrations of 1×10^{14}, 1×10^{13}, and 1×10^{12} particles/mL were tested under different agitation conditions. The coupling efficiency was evaluated by scanning spectrophotometry (400–1000 nm), comparing the absorption

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spectra of pure AuNPs, AuNPs functionalized with mAbOTA, and saline controls. The best performance was obtained using AuNPs at 1×10^{13} particles/mL and mAbOTA at 50 µg/mL, with magnetic stirring for 16 hours (overnight). The results indicate the feasibility of the initial antibody–nanoparticle conjugation step and provide essential parameters for optimizing functionalization and detection conditions in subsequent assay development stages.

Keywords: Gold Nanoparticles. Ochratoxin A. Monoclonal Antibodies. Biosensors. Functionalization. Spectrophotometry.

RESUMO

A ocratoxina A (OTA) é uma micotoxina produzida por fungos dos gêneros Aspergillus e Penicillium, frequentemente detectada em produtos de origem vegetal, como sementes, grãos, cereais e rações. Essa toxina apresenta reconhecido potencial carcinogênico, além de efeitos neurotóxicos, nefrotóxicos e imunotóxicos, representando risco à saúde humana e animal, bem como prejuízos econômicos à cadeia produtiva. Devido às baixas concentrações em que ocorre em matrizes alimentares, sua detecção requer métodos analíticos sensíveis e específicos. Este trabalho teve como objetivo padronizar a etapa inicial de acoplamento de anticorpos monoclonais anti-OTA (mAbOTA) a nanopartículas de ouro (AuNP), visando o desenvolvimento futuro de biossensores para detecção da toxina em amostras biológicas e alimentares. Foram testadas concentrações de 1×10^{14}, 1×10^{13} e 1×10^{12} partículas de AuNP/mL, sob diferentes condições de agitação. O acoplamento foi avaliado por espectrofotometria de varredura (400–1000 nm), comparando os espectros de absorção das AuNP puras, funcionalizadas com mAbOTA e controles em solução salina. A melhor condição obtida foi com AuNP a 1×10^{13} partículas/mL e mAbOTA a 50 µg/mL, utilizando agitação magnética por 16 horas (overnight). Os resultados indicam a viabilidade do acoplamento inicial e fornecem subsídios para ajustes nas condições de funcionalização e detecção, a serem explorados em etapas posteriores do desenvolvimento do ensaio.

Palavras-chave: Nanopartículas de Ouro. Ocratoxina A. Anticorpos Monoclonais. Biossensores. Funcionalização. Espectrofotometria.

RESUMEN

La ocratoxina A (OTA) es una micotoxina producida por hongos de los géneros Aspergillus y Penicillium, frecuentemente detectada en productos vegetales como semillas, granos, cereales y alimento para animales. Esta toxina tiene un reconocido potencial carcinogénico, así como efectos neurotóxicos, nefrotóxicos e inmunotóxicos, lo que representa un riesgo para la salud humana y animal, así como pérdidas económicas para la cadena de producción. Debido a las bajas concentraciones en las que se presenta en matrices alimentarias, su detección requiere métodos analíticos sensibles y específicos. Este trabajo tuvo como objetivo estandarizar el paso inicial del acoplamiento de anticuerpos monoclonales anti-OTA (mAbOTA) a nanopartículas de oro (AuNP), con miras al futuro desarrollo de biosensores para la detección de la toxina en muestras biológicas y alimentarias. Se analizaron concentraciones de 1×10^{14}, 1×10^{13} y 1×10^{12} partículas de AuNP/mL en diferentes condiciones de agitación. El acoplamiento se evaluó mediante espectrofotometría de barrido (400–1000 nm), comparando los espectros de absorción de AuNP puras, funcionalizadas con mAbOTA, y controles en solución salina. La mejor condición obtenida se obtuvo con AuNP a 1×10^{13} partículas/mL y mAbOTA a 50 µg/mL, utilizando agitación magnética durante 16 horas (durante la noche). Los resultados indican la viabilidad del acoplamiento inicial y respaldan la realización de ajustes en las condiciones de funcionalización y detección, que se explorarán en etapas posteriores del desarrollo del ensayo.

Palabras clave: Nanopartículas de Oro. Ocratoxina A. Anticuerpos Monoclonales. Biosensores. Funcionalización. Espectrofotometría.

1 INTRODUCTION

Mycotoxins are secondary metabolites produced by filamentous fungi of the genera Aspergillus, Penicillium and Fusarium, widely distributed in agricultural products and feed. Among them, ochratoxin A (OTA) stands out, produced mainly by species of Aspergillus spp. and Penicillium spp. The main characteristics of OTA include nephrotoxicity, neurotoxicity, immunotoxicity, and recognized carcinogenic potential (LIMONCIEL & JENNINGS, 2014; MALIR et al., 2016).

This toxin is often detected in products of plant origin, such as grains and cereals, and can contaminate food by-products such as flours and breads (MALIR et al., 2016; KROGH, 1997; DUARTE et al., 2010). From a sanitary, toxicological and economic point of view, OTA is of great relevance in animal production, as it interferes with feed conversion, reduces productivity and increases mortality (MATRELLA et al., 2006).

The detection of OTA in human serum or plasma is widely used as a biomonitoring strategy, as it directly reflects continuous exposure to the mycotoxin (GROSSO et al., 2003; DI GIUSEPPE et al., 2012; MALIR et al., 2013). Several studies demonstrate that OTA circulates in human blood for long periods due to its long half-life and strong binding to albumin, allowing its quantification to serve as a sensitive marker of systemic exposure (SCOTT, 2005; MALIR et al., 2012). Evidence also indicates that chronic exposure to low concentrations of OTA poses a greater risk for nephrotoxic and carcinogenic effects than acute episodes of high exposure (PFOHL-LESZKOWICZ; MANDERVILLE, 2007; PFOHL-LESZKOWICZ, 2009).

Several techniques have been developed for the detection of OTA, such as ELISA and high-performance chromatography (HPLC). However, these techniques require time, high cost, and specialized equipment (MEULENBERG, 2012). Nanotechnology emerges as a promising alternative, mainly due to the use of metallic nanoparticles in biosensors. Gold nanoparticles (AuNPs), usually spherical, have characteristic optical properties and are widely applied in diagnostics and therapies (BOISSELIER & ASTRUC, 2009).

In this context, the conjugation of monoclonal antibodies to AuNPs constitutes the basis of immune biosensors. Thus, the present work aimed to standardize ideal concentrations of nanoparticles and anti-OTA antibodies for the initial stage of functionalization, aiming at the development of optical biosensors for ochratoxin A detection.

2 MATERIALS AND METHODS

2.1 ANALYSIS OF GOLD NANOPARTICLE SUSPENSIONS (AUNPS) BY SCANNING SPECTROPHOTOMETRY

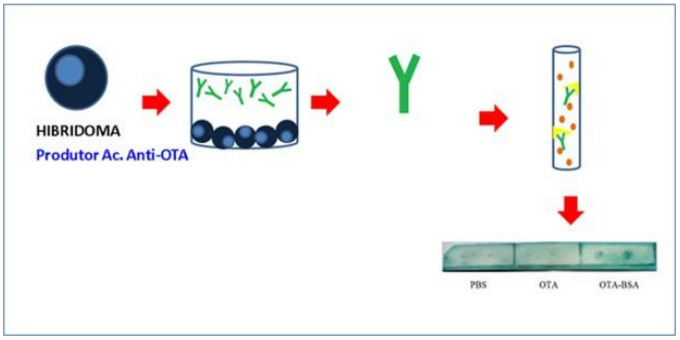
Initially, the reading of pure AuNP suspension at a concentration of 1×10^{14} NP/mL in saline solution was used to determine the optical absorption pattern and the peak of maximum absorbance, by scanning spectrophotometry in the range of 400 to 1000 nm. Lower concentrations of AuNPs (1×10^{13} and 1×10^{12} NP/mL) were also tested in saline suspension, under different stirring times, to evaluate stability and spectral behavior.

2.2 HYBRIDOMA EXPANSION AND ANTIBODY PREPARATION.

The hybridoma clone producing the anti OTA IgG antibody (mAbOTA), kindly provided by Prof. Dr. Osamu Kawamura (Kagawa University, Japan) was expanded in RPMI medium. The supernatant was concentrated by precipitation with ammonium sulfate and the purification of IgG was performed by affinity chromatography in a column of Sepharose conjugated with Protein G and the positive fractions detected with equipment nanodrop were gathered, conditioned, and stored for analysis. Antibody reactivity was evaluated by dot-blot: samples containing OTA (OTA or OTA-BSA) or PBS were applied to membrane, blocked, incubated with secondary anti-IgG antibody from mice peroxidase, and submitted to detection by MTT, as shown in Figure 1.

Figure 1

Anti-OTA monoclonal antibody procurement and analysis scheme



2.3 FUNCTIONALIZATION OF AUNPS WITH ANTI-OCHRATOXIN A MONOCLONAL ANTIBODY

Concentrations of 25 and 50 µg/mL of the monoclonal anti-ochratoxin A antibody (mAbOTA) were tested, with a final volume of 300 µL and addition of AuNPs at a concentration of 1×10^{13} NP/mL, in glass containers. The negative control was prepared by replacing the volume corresponding to the antibody with saline solution. The suspensions

were maintained under overnight magnetic agitation and analyzed in a scanning spectrophotometer (400–1000 nm).

2.4 ANALYSIS OF FUNCTIONALIZED AUNPS

The functionalized AuNPs samples were subjected to blockade to avoid nonspecific bonds. A 600 µL sample of the suspension (50 µg/mL) was centrifuged at 4000 × g for 10 minutes, with discarding of the supernatant. The pellet was resuspended in 600 µL of saline solution, centrifuged again, and the supernatant discarded. Then, 1 mL of albumin-containing saline solution (0.05 mg/mL) was added, incubating at 37°C for 40 minutes. After re-centrifugation and discarding of the supernatant, two washes were performed with buffer (0.5% BSA, 0.05% Tween 20 and solution saline), and the final volume adjusted to 600 µL with saline solution. For the detection of ochratoxin A (OTA) using functionalized AuNPs, a 96-well plate was prepared with OTA dilutions at concentrations of 20, 10, 5, 2.5 and 1.25 µg/mL, adjusting the final volume to 10 µL in each well. Then, 100 µL of the suspension of AuNPs functionalized with mAbOTA (50 µg/mL) were added to each sample. The plate was incubated for 1 hour at 37°C and analyzed by scanning spectrophotometry (400–1000 nm).

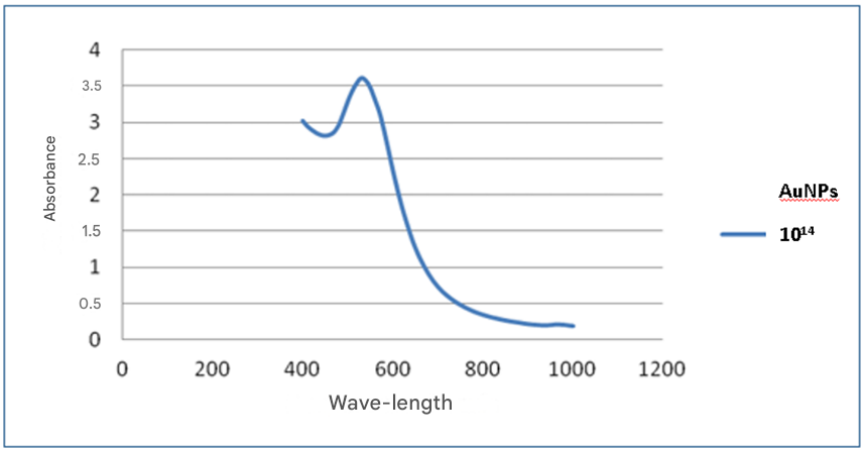
The data obtained were processed in Microsoft Excel 2010 software, with the construction of spectral absorption graphs for comparison between the samples and the controls.

3 RESULTS AND DISCUSSION

This work sought to functionalize AuNPs with anti-OTA monoclonal antibodies (mAbOTA), aiming to standardize a detection technique by testing and searching for the lowest concentrations of AuNPs and mAbOTA capable of recognizing OTA. Initially, the spectrophotometric reading was performed to establish the optical profile of the colloidal suspension of gold nanoparticles (AuNPs). The results obtained revealed a maximum absorption peak at approximately 530 nm with suspension of AuNPs at a concentration of 1×10^{14} NP/mL, (Figure 2), a typical value for spherical AuNPs in the range of 10–20 nm, as described by Boisselier & Astruc (2009). This result confirmed the colloidal stability of the suspension and its suitability for functionalization tests with antibodies.

Figure 2

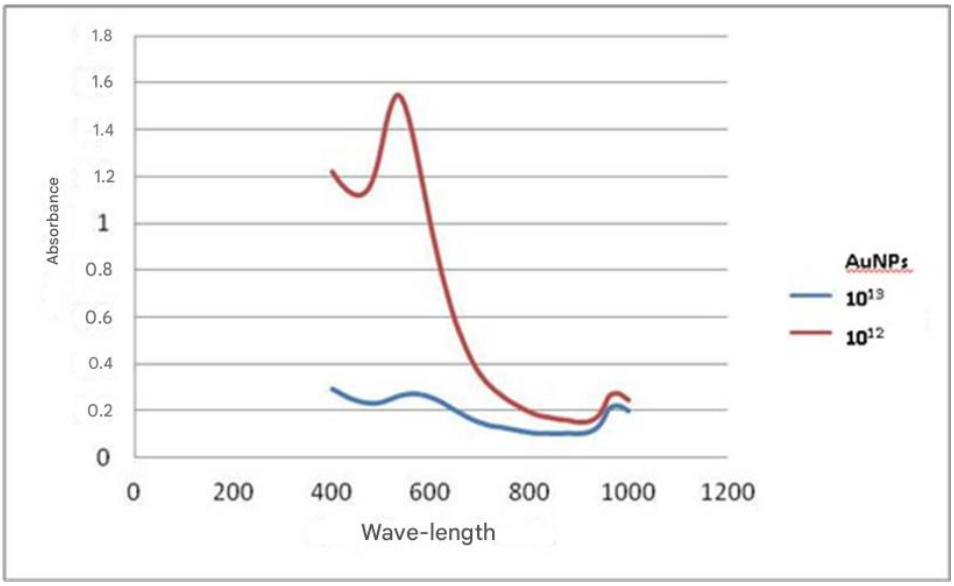
Reading profile of AuNP 1014 NP/mL by scanning spectrophotometry (400–1000 nm)



Successive dilutions of the nanoparticles were performed at concentrations of 1×10^{13} and 1×10^{12} NP/mL, a distinct spectral behavior was observed among the samples (Figure 3). The intermediate concentration of 1×10^{13} NP/mL showed an absorption profile very similar to that observed for the original suspension (1×10^{14} NP/mL), indicating maintenance of colloidal dispersion and less tendency to aggregation. On the other hand, the most dilute suspension (1×10^{12} NP/mL) showed slight instability, with variations in baseline and widening of the main peak (data not shown).

Figure 3

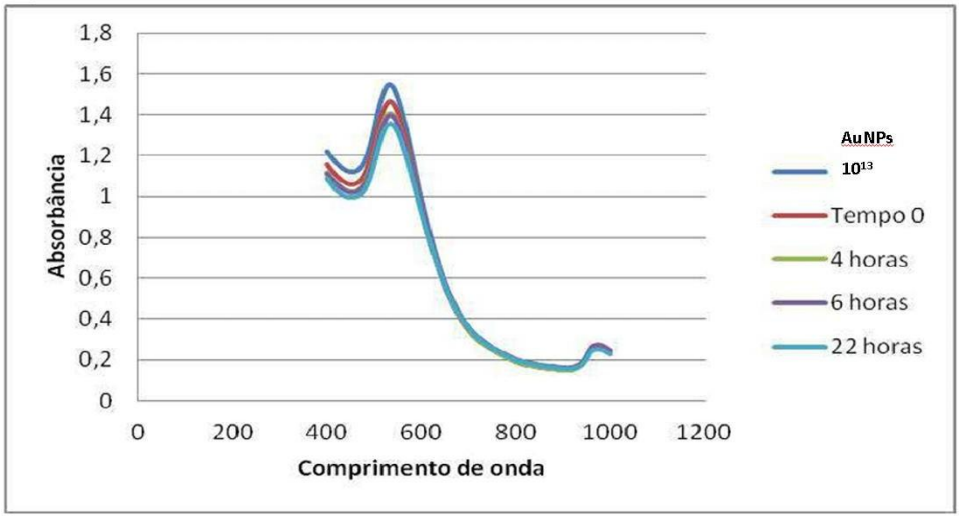
Spectrophotometric reading of AuNPs at concentrations of 1x1012 and 1x1013 NP/mL mL by scanning spectrophotometry (400–1000 nm)



Subsequent tests evaluated the effect of the addition of saline solution and different stirring times on the stability of the nanoparticles. Suspensions containing AuNPs at 1×10^{13} NP/mL maintained the characteristic spectral pattern, even after 22 hours of continuous agitation (Figure 4), demonstrating good colloidal stability under these conditions. This behavior was not reproduced at the lowest concentration (1×10^{12} NP/mL), suggesting that particle density directly influences the stability of dispersion, possibly due to the lower electrostatic repulsion between the particles.

Figure 4

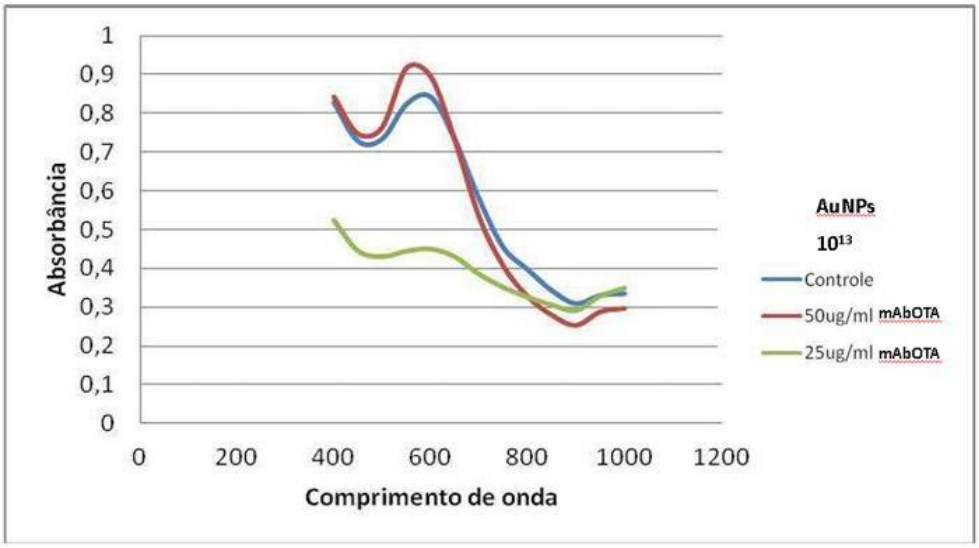
Spectrophotometric reading of AuNPs at a concentration of 1x1013 NP/mL with saline solution addition at different times (0, 4.6 and 22 hours) mL by scanning spectrophotometry (400–1000 nm)



In the functionalization step of AuNPs with the anti-ochratoxin A monoclonal antibody (mAbOTA), the spectrophotometric readings showed a peak absorption displacement of approximately 50 nm in samples containing 50 µg/mL of mAbOTA, when compared to the negative control (Figure 5). This displacement indicates the coating of the surface of the AuNPs by antibody molecules, which alters the local refractive index and modifies the surface plasmonic resonance — a phenomenon widely described in protein-nanoparticle conjugation systems (Boisselier & Astruc, 2009).

Figure 5

Spectrophotometric reading of AuNPs at a concentration of 1×10¹³ NP/mL and functionalized with 25 and 50 µg/mL of mAbOTA and saline solution control by scanning spectrophotometry (400–1000 nm)

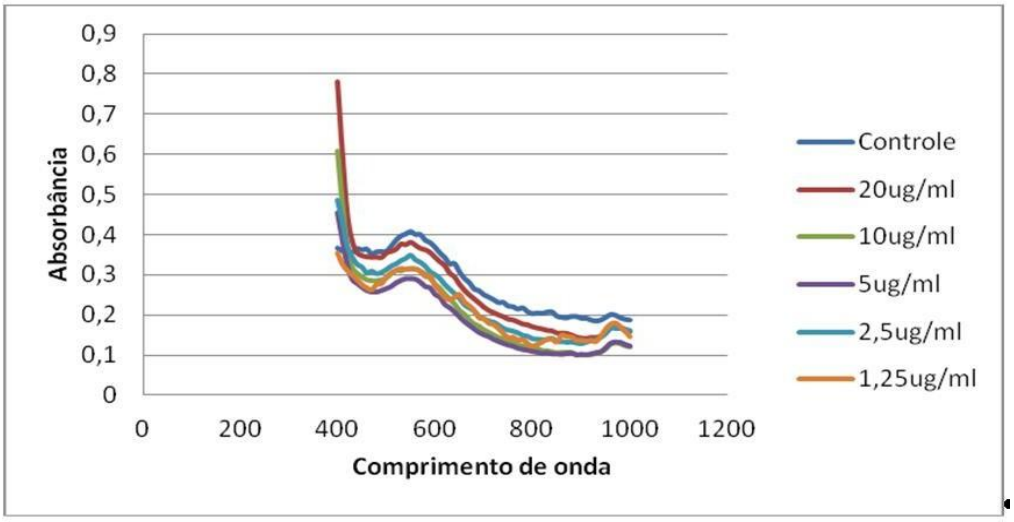


The 50 µg/mL concentration of mAbOTA was therefore selected as optimal for subsequent ochratoxin A (OTA) detection tests. However, during the assays with different OTA concentrations (20, 10, 5, 2.5 and 1.25 µg/mL), it was not possible to observe significant displacements in peak absorption in relation to the negative control. Even so, oscillations in the absorption curve were noted, possibly associated with the partial aggregation of functionalized AuNPs (Figure 6). This aggregation may have occurred due to prolonged exposure to saline solution or incomplete saturation of the surface of the nanoparticles,

factors that interfere with colloidal stability and impair the sensitivity of the detection system. According to WANG et al., 2020, small variations in physicochemical conditions such as the ionic concentration of the solution affect the aggregation and optical response of AuNPs.

Figure 6

Spectrophotometric reading of AuNP at a concentration of 1×10¹³ NP/mL functionalized with OTA mAb (50 µg/mL) and complexed with OTA at different concentrations and saline solution control by scanning spectrophotometry (400–1000 nm)



The results obtained so far represent an initial step in the standardization of the functionalization of AuNPs with specific monoclonal antibodies. Despite the absence of a clear spectral response in the presence of the toxin, the displacement observed during the conjugation step confirms the coupling of the antibody to the nanoparticles. This is a key step in the development of biosensors based on metallic nanoparticles for mycotoxin detection, such as OTA.

Recent studies reinforce the applicability of this approach. Ferreira (2017), for example, described the functionalization of gold nanorods with specific antibodies for the detection of circulating antigens of Paracoccidioides brasiliensis, demonstrating the diagnostic potential of these systems. Similarly, research focused on the detection of mycotoxins, such as those reviewed by Meulenberg (2012) and Malir et al. (2016), points to the need for fast, sensitive, and low-cost methods, requirements that nanotechnology can promisingly meet.

Thus, the present work contributes to the standardization of the physicochemical conditions of conjugation between antibodies and AuNPs, establishing the basis for the future development of a system for the detection of ochratoxin A, with potential application in several areas such as biological, animal/human health and food.

4 CONCLUSIONS

The standardization of conjugation conditions between anti-OTA monoclonal antibodies and gold nanoparticles proved feasible and provides an experimental basis for the development of immunological biosensors for mycotoxin detection. Future studies should focus on optimizing the colloidal stability and sensitivity of the system for application in food and biological samples.

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