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RAPID DETECTION OF PATHOGENIC MICROORGANISMS IN WATER, BY BIOSENSORS

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ABSTRACT

The integration of nanotechnology in combination with molecular biology and electrochemistry, have created a great expectation due to the development of new nucleic acid detection strategies based on nanomaterials and which are important tools in genomics, medical diagnosis, pharmacogenetics, pathology, criminology, food security, environmental monitoring, among others; these devices are known as biosensors.In this work, the DNA detection method to 4 microorganisms was performed: *Escherichia coli*, *Aspergillus niger*. *Salmonella typhi* and *Achlya sp*. by electrochemical detection, it provides simple, accurate and inexpensive platform for DNA detection. *E. coli* and *S. typhi* were donated, *Aspergillus niger*. and *Achlya sp*. were obtained from environmental samples; *Aspergillus niger*., was isolated from compost samples with used automotive oil, *Achlya sp*. was obtained from samples from the Xochimilco channels in Mexico City. For its identification, the techniques of PCR, sequencing and bioinformatic comparison of the sequence were used with the Genebank database.The specific probes were designed with the Primer-Blast program, they were synthesized and used in nanostructured genosensors, with gold nanoparticles (AuNPs) as a brand; the probes were functionalized with the AuNPs by the streptavidin-biotin system and hybridization was performed, which was detected electrochemically and it was observed that the signal decreases when there is less complementarity in the bases of the probe, so that the developed genosensors are an excellent tool for the rapid detection of microorganisms for purposes of monitoring, prevention and environmental control as well as gastrointestinal diseases in low-income populations and currently Covid 19; furthermore, if genosensors are produced on a large scale, they can be efficient, inexpensive, portable and simple to use.

Key words. Fast detection, Pathogenic microorganisms, water, biosensors, genosensors ,Covid 19

INTRODUCTION

The innovative research that includes disciplines such as molecular biology, electrochemistry and nanotechnology, has allowed the development of nucleic acid detection strategies that are important in genomics, medical diagnosis, pharmacogenetics, pathology, criminology, food safety, environmental monitoring, among others. (Wang, 2003; Erdem, 2007).

The nanoparticles (NPs) of noble metals have been studied during the last 20 years, highlighting an important characteristic, its optical resonance, which is in the visible spectrum, as well as its great sensitivity to environmental changes, however for its correct use the protocols specify the importance of their size and shape, which can be sphere, bar, cube, triangle; among others. (Turkevich et al., 1951; Sun and Xia, 2002; Kimling et al., 2006; Castañeda, et al., 2007; De la Escosura et al., 2011; Chavez-Sandoval et al., 2016), The nano particles can also be made functional with a wide range of ligands such as: antibodies, polymers, diagnostic probes, drugs, genetic material, including for the detection of chemical and biological threats (Upadhyayula, 2012). Therefore, gold nanoparticles (AuNPs) have aroused great interest in various fields of scientific knowledge, especially in studies of biomedical and environmental areas (Castro-Ortíz, 2007; Wei et al., 2010; Díaz et al., 2011; Chavez-Sandoval et al., 2020; Chávez-Sandoval et al., 2021).

Specifically, for the environmental area, the diagnosis and subsequent recovery of different environments, should be done in addition, considering the implications in ecological and health terms represents the degradation of natural resources. In this sense, microorganisms are good indicators of the trophic status of ecosystems and respond to the disturbances that occurred by modifying their structure in terms of composition and abundance. The detection of changes in aquatic ecosystems, specifically from the communities of fungi, bacteria, and algae, is currently widely used and constitutes a reliable and low-cost mechanism, because it does not require very sophisticated equipment to evaluate the alterations in the systems, since these microorganisms reflect the changes that have occurred in a water resource, unlike the physicochemical parameters that only show the specific situations of the moment of the sample. The indicator microorganisms are those that have a behavior like pathogens, in terms of concentration and reaction to environmental factors, but are easier, faster, and cheaper to identify (Arcos et al., 2005).

The conventional techniques of analysis of a specific genetic sequence are based on methodologies of sequencing and hybridization of nucleic acids (DNA and / or RNA). In techniques based on the hybridization of DNA, the sequence of interest or analyte (target) is identified by a probe or oligonucleotide (small DNA sequence of no more than 50 bases), whose sequence is complementary to the analyte; this hybridization reaction occurs with great affinity and specificity. The DNA probes must be marked since the "marks"

are responsible for giving the analytical signal to quantify the hybridization event (Yamanaka et al., 2009). The use of DNA associated with different transducers is a new field of research since DNA detection is of interest in genetics, pathology, criminology, environmental monitoring, among others (Wang, 2003, Erdem, 2007).

Electrochemical Genosensors are devices that convert the hybridization event into an electrochemical signal. Advances in the synthesis and characterization of nanostructured materials have produced dramatic changes in the design and capabilities of sensors since devices with electrical, optical and mechanical properties have been developed; These devices are used to determine the possible interaction of drugs and DNA, as well as in the early and precise diagnosis of infectious agents in different environments. For this, different electrochemical techniques are used: differential pulse voltammetry (DPV), linear voltammetry (LV), square linear voltammetry (LSV), cyclic voltammetry (VC), among others combined with different electrochemical transducers (Erdem, 2007).

The nanoparticles are used in a wide range of applications, in the biosensors, they are specifically linked to the recognition biomolecule that can be a protein or DNA, among others. The interaction of streptavidin with biotin is of high affinity and allows that once the complex is formed it is not destabilized, neither by changes in pH nor by multiple washes when immobilized. (Diamandis and Christopoulos, 1991). In this way the recent developments in the design and fabrication of efficient sensor platforms based on nanostructures, such as metal carbon or polymeric nanoparticles, make the highly sensitive sensors which a very low detection limit to the level of few molecules, a genuine possibility (Rasheed and Sandhyarani 2017).

In this work we carried out the molecular identification of the microorganisms *Escherichia coli*, *Aspergillus niger*. *Salmonella typhi* and *Achlya sp*., for the design of specific probes, with gold nanoparticles as a mark to be used in Genosensors.

MATERIALS AND METHODS

All reagents used were analytical grade and all solutions were prepared using double deionized water (Milli-Q, 18MW cm) from a Millipore purification system.

OBTENTION OF MICROORGANISMS

E. Coli and *S. Tiphy*, were donated from the microbiology laboratory of the Professional Biotechnology Unit (UPIBI-IPN), in Mexico City; while *Achlya sp*. it was isolated from water samples from the Xochimilco canals in Mexico City. 1 mL of the water sample was taken and inoculated in a Petri dish with Sabouraud agar, incubated at 28 ° C for 48 hours. Subsequently, microcultures were performed for identification. *Aspergillus niger*. it was obtained from compost samples with automotive oil used in the UAM-Azcapotzalco as follows: 10 gr. of compost was dissolved in 90 mL. of dilution water, 1 mL was taken and inoculated in a Petri dish with Sabouraud

agar and incubated at 28 ° C for 48 hours. Microcultures were performed.

DNA EXTRACTION

Once the cultures were pure, the DNA extraction was performed with the MoBioUltraCleanTMsoil DNA Kit, as well as using the extraction protocol described in Dellaporta, et al., 1983. The obtained DNA was visualized on an Alpha Imager 2000, in 0.8% agarose gels stained with Ethidium bromide.

AMPLIFICATION OF DNA BY PCR

The DNA obtained from the microorganism was amplified using a thermocycler MultiGeneOptiMax. The PCR conditions were 95 ° C for denaturation, 57 ° C for alignment, 72 ° C for extension, for 30 cycles using the ITS 4 primers, sequence: 5 'TCCTCCGCTTATTGATATGC 3' and ITS 5, sequence: 5 'GGAAGTAAAAGTCGTAACAAGG 3' (White *et al.* 1990; Faria et al., 2012).

SEQUENCING OF PCR PRODUCTS

The PCR products were sent to the sequencing service of the Molecular Biochemistry Laboratory of the Facultad de Estudios Superiores Iztacala (FES-I) UNAM. With the obtained sequences we proceeded to design the specific probes to each microorganism. Finally, the synthesis of the designed probes, as well as their modification with biotin: probe, probe with 1 error, probe with 3 errors and non-complementary probe, to the commercial house Alpha DNA in Otawa, Canada.

SYNTHESIS OF GOLD NANOPARTICLES (AuNPs)

To carry out the synthesis of the AuNPs, the method described in Turkevich et al. (1951), with some modifications. Glass material was used, washed with regia water and Milli-Q water to remove any traces of gold that could interfere during the synthesis process. In a 250 mL Erlenmeyer flask, 500 µL of 1% hydrogen tetrachloroaurate (25 mM) was placed in 50 mL of double deionized water (Milli-Q), it was placed on a heating grate with stirring, until it reached boiling point; subsequently, 1% sodium citrate was added, which acted as a reducing and stabilizing agent. They were kept under stirring and heating for no more than 15 minutes. Finally, they allowed to cool to room temperature, while maintaining the agitation and kept in a sterile bottle at 4 ° C for further characterization.

CHARACTERIZATION OF AuNPs

The UV-Vis spectroscopic measurements were made using a PerkinElmer Lambda 25 Spectrophotometer, with a wavelength range of 190-700 nm. The images of Atomic Force Microscopy (AFM) were obtained using a SPM (Scanning Probe Microscope) brand Digital Instruments. Transmission Electron Microscopy (TEM) images were obtained with a JEOL JEM-100CX II Electron Microscope. The electrochemical characterization of the AuNPs to evaluate the oxidation signal typical of gold reduction, was performed by differential pulse voltammetry (DPV) under the following conditions: a sweep of +1.25 V at 0.0 V, potential step 10 mV, amplitude 50 mV, scanning speed 33.5 mVs-1 and oxidation time 120 s, in 0.1 M HCl and 0.2 M HCl. The AuNPs were deposited on the surface of the M-GECE electrode, which were left for 5 min. After this time, the

electrode was placed in the three-electrode system for analysis by DPV.

FUNCTIONALIZATION OF THE PROBES

For the designed probes to be functional, the streptavidin-biotin system was used. Streptavidin was resuspended in sterile Milli Q water to obtain a concentration of 0.1 mg / mL.

The AuNPs synthesized together with the streptavidin solution were placed in 1.5 mL Eppendorf tubes in a ratio of 1: 1 (v / v). Subsequently, they were incubated in a thermoshaker (Thermo-Shaker MS-100), for 30 min at 650 rpm and at 25 ° C.

Immediately after the incubation was finished, they were centrifuged for 20 min at 14000 rpm and at a temperature of 4 ° C. The supernatant was discarded and 1 mL of phosphate buffer solution (PBS) was added.

HYBRIDIZATION AND ELECTROCHEMICAL DETECTION

The DNA-Target was added to the solution containing the DNA-probe and incubated at 42° C for 15 minutes at 800 rpm in a Thermo-Shaker TS-100, then magnetically separated and the supernatant was decanted. The resulting conjugate was washed twice with 100 µL of TT-buffer and resuspended in 20 µL of TTL-buffer, being ready to add the streptavidin-activated gold nanoparticles (AuNPs / Str.) That were used as a label.

The Target used were: Complementary probe, non-complementary probe, probe 1 error, probe 3 errors.

The electrochemical detection was carried out by means of cyclic voltammetry (VC) analyzing the current-potential response of a polarizable electrode GECE and M-GECE. The electrochemical cell was assembled and filled with 0.2 M hydrochloric acid, the electrodes were connected, a graphite electrode corresponding to the working electrode, one of platinum as against electrode and one of Ag / AgCl as a reference electrode. The sample was placed on the working electrode and a sweep potential of 1.25 mV / s was applied. on a Palm Sens computer.

M-GECE Electrodes Characterization

The electrodes were prepared according to Céspedes et al. (1993) and Santandreu et al. (1997), following the same procedure for the construction of the M-GECE but incorporating a magneto of neodimium of 3 mm in diameter and 1.5 mm in height. To analyze the surface morphology of the electrodes to know the structure and distribution of the graphite in the paste of the electrode, as well as its roughness, the electrodes were characterized by SEM and FCM. Finally, its electrochemical behavior was evaluated by cyclic voltammetry (CV).

RESULTS AND DISCUSSION

Isolation of microorganisms

In Figure 1.A, the micrograph of *Achlya sp* is observed, while in Figure 1.B, *Achlya sp* obtained from the Xochimilco canals in Mexico City is observed. 1.C shows the microorganism Aspergillus niger, isolated from compost samples.

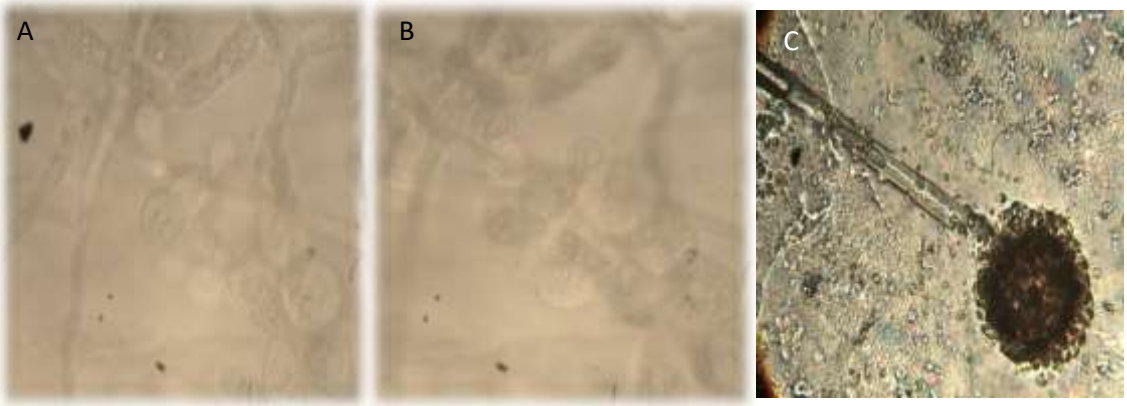


Figure 1. 40X micrographs. 1.A Micrography of Achlya sp. 1.B Microculture of *Achlya sp.* collected in the Xochimilco canals in Mexico City; 1.C. Aspergillus niger from compost samples.

DESIGN OF SPECIFIC PROBES

With the sequences obtained and through bioinformatic analysis it was confirmed that the microorganisms of interest (Achlya sp.) And Aspergillus niger were present, the design of the probes was carried out using the Primer-Blast program. In Table I the result is observed.

Table I. Probes designed by each microorganism.

Microorganism	NCBI/ GeneBank	Probe Designed
Achlya sp.	JQ974991.1	5' GATCAATACGCCGGTCTCCG 3'
Aspergillus niger.	HQ850370.1	5' CATACGCTCGAGGATCGGAC 3'
E.Coli	NZ_AERR00000000.1	5' GCACCGGAAGTACAGACCAA 3'
S. Tiphy	FJ460240.1	5' CGGTCGGCTTGAACGAATTG 3'

The designed probes contain high percentage of GC (60%) in order that the complementation in the hybridization was specific and stable.

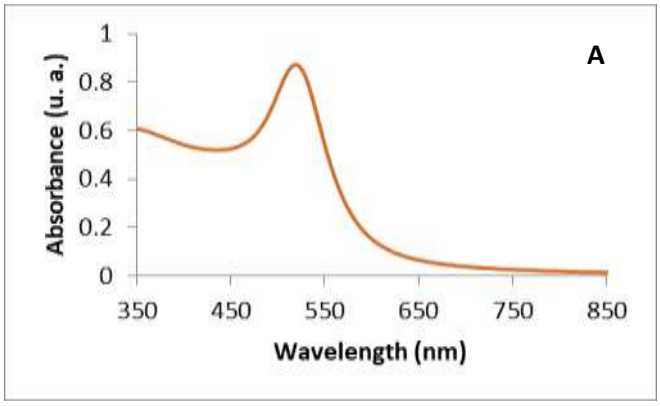
SYNTHESIS AND CHARACTERIZATION OF GOLD NANOPARTICLES (AuNPs)

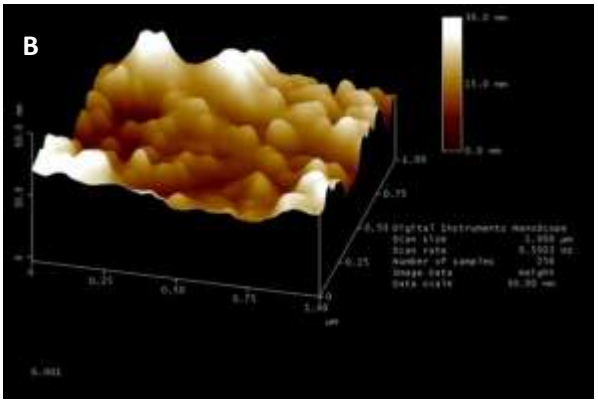
The synthesized AuNPs were characterized using UV-VIS spectroscopy, atomic force microscopy (AFM) and transmission electron microscopy (TEM), the results of each of them are presented in figure 2 A-C.

For the synthesized AuNPs a maximum wavelength of absorption of 519.5 nm was obtained (Fig. 2 A), this indicates that they are approximately 20 nm, Carralero, 2009 reported a spectrum of 520 nm for these AuNPs.

In the AFM images obtained, AuNPs with a diameter of approximately 20 nm and spherical morphology are observed (Fig. 2 B).

The TEM image of the synthesized AuNPs (Fig. 2C) corroborated the size and shape of the synthesized AuNPs, which are observed in spherical morphology and approximately 20 nm





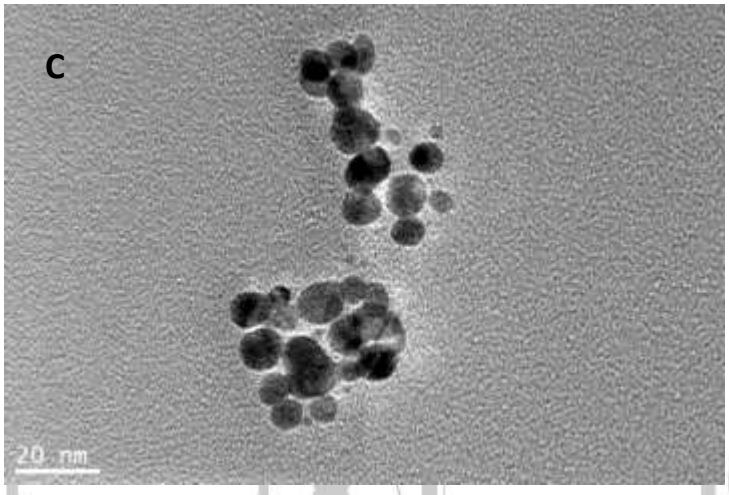


Figure 2. A. UV-Vis characterization, 2B. AFM characterization, 2C. TEM characterization.

In the characterization of the AuNPs by differential pulse voltammetry (DPV), a greater response was obtained with the 0.2 M HCl solution (Fig. 3).

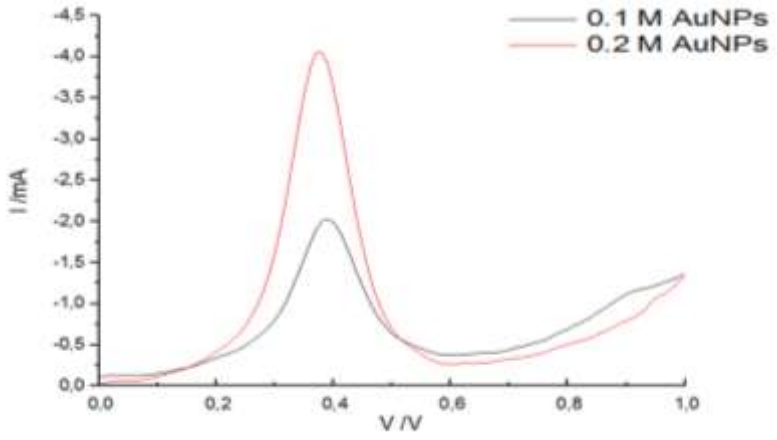


Figure 3. Differential pulse voltammetry (DPV). AuNPs at the electrode with 0.1 and 0.2 M HCl.

Characterization of the electrodes by scanning electron microscopy (SEM)

The SEM image in the figure 4 show the surface of the electrodes, observing that they have an adequate distribution of the epoxy resin and graphite.

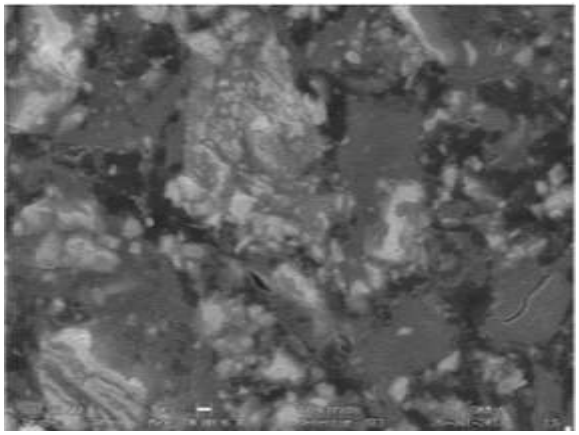


Figure 4. SEM micrographs of the electrode, taken with a resolution of 1 µm and an acceleration potential of 20 KV.

Characterization of the electrodes by fluorescence confocal microscopy (FCM)

The electrode surface obtained by FCM is observed uniform in terms of the distribution of the epoxy resin and the graphite. (Fig. 5).

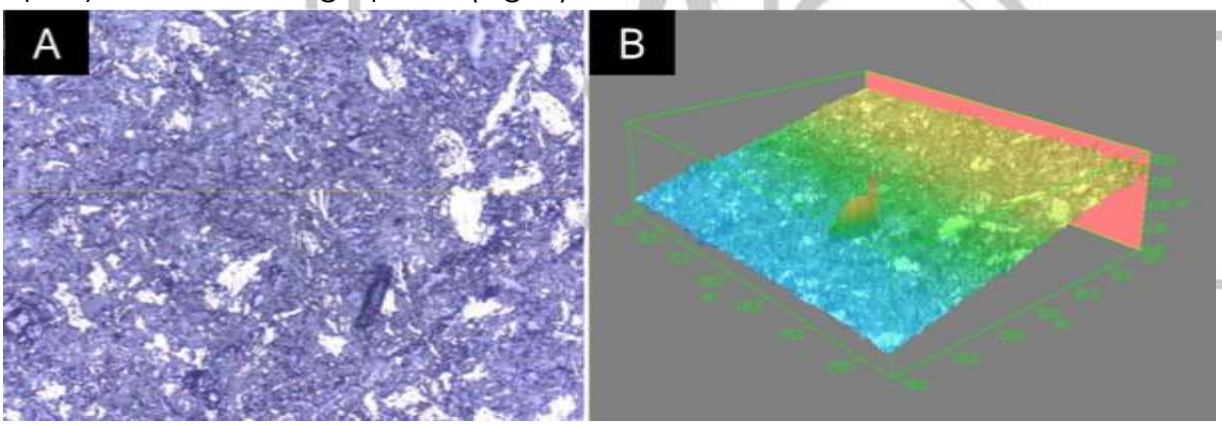


Figure 5. A. surface of the electrode by FCM; B. 3D image where a good roughness of the electrode surface is observed.

In the figure 6 the cyclic voltammogram (CV) obtained with the electrode is shown.

This electrode was chosen because it has the fastest oxidation response and gives the best signal.

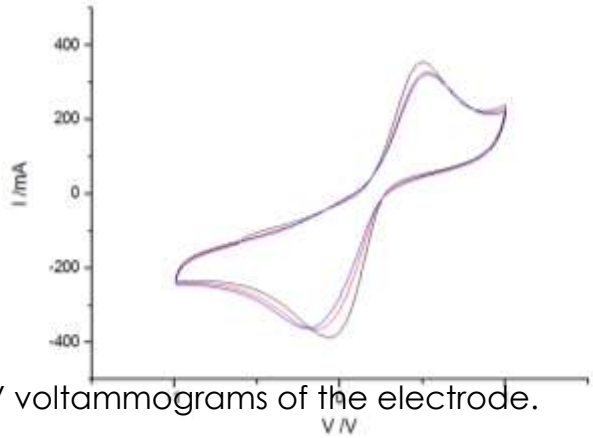


Figure 6. CV voltammograms of the electrode.

HYBRIDIZATION AND ELECTROCHEMICAL DETECTION

Hybridization (Fig. 7) and electrochemical detection (Fig. 4) were performed, characteristic signals were obtained for each probe: complementary, 1 error, 3 errors and not complementary, it was observed that the signal decreases when there is less complementarity, however it is advisable to perform some optimizations to minimize the signal from the probe not complementary.

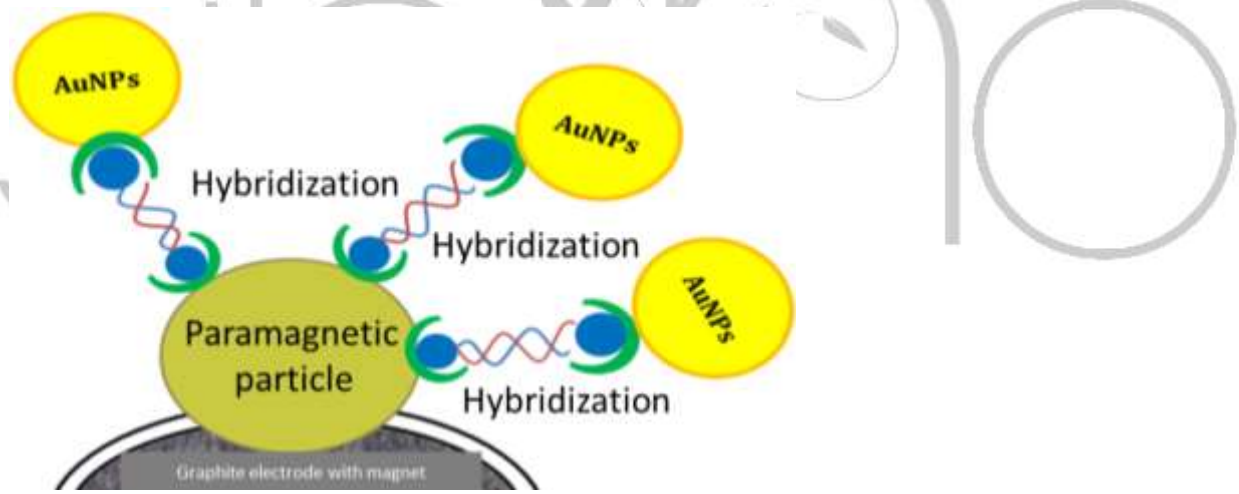


Figure 7. Hybridization event with DNA of the specific probes designed for each microorganism, using the AuNPs brand, for electrochemical detection.

It is observed in Figure 8, that the event of 3 errors is greater than 1 error, this may be due to non-controllable or unidentified parameters However, the main interest is to know whether the microorganism of interest is found in the sample and this objective is achieved with this methodology.

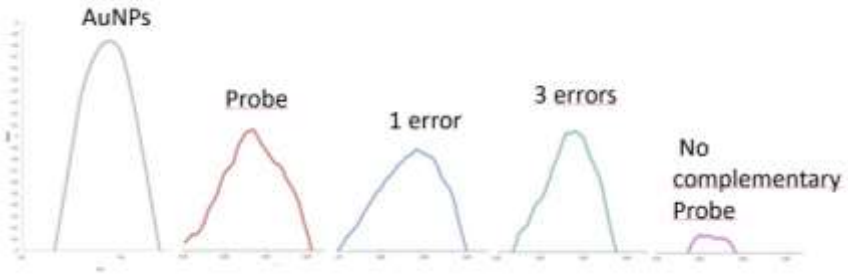


Figura 8. Voltamperograms DPV of the genosensor with the complementary probe, an error, 3 errors and not complementary for S. Typhi

As seen, electrochemical detection methods make available simple, accurate and inexpensive platform for DNA detection. In addition, the electrochemical genosensors developed in this work, provide direct electronic signal without the use of expensive signal transduction equipment and facilitates the immobilization of single stranded DNA (ssDNA) probe sequences on an electrode.

CONCLUSIONS

Spherical AuNPs of about 20 nm were obtained, which became functional with the specific DNA probes. The molecular characterization of the microorganisms *Aspergillus sp*. and *Achlya sp*. It allowed to identify at the species level the first, for the second it is necessary to continue sequencing another fragment until obtaining the species. However, the sequence obtained was enough for the design of the specific probes. Electrochemical detection was achieved for *E. Coli*, *S. Typhi*, *A. niger and Achlya sp*. using the specific designed probes and characteristic electrochemical signals were obtained for each probe.

The genosensor developed for each microorganism offers portability, sensitivity, ease of handling, rapid response, small sample volume, low cost and application in real samples, environmental samples, medical samples among others.

PERSPECTIVES

Highly sensitive and stable miniaturized amperometric sensors have been developed by integrating the nanomaterials and biocatalyst with the transducers and there are apply in a lot of fields like medicine, food security and environment security among others, like Covid 19, and all their variants.

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