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ZnO nanorods-gold nanoparticle-based biosensor for detecting hepatitis C

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Abstract
ZnO nanorods comprised of biosensors with Au nanoparticles are described in this study for detecting Hepatitis C virus (HCV). The biosensor device was constructed on a glass substrate with silver and gold electrodes. The ZnO nanorods were grown by microwave hydrothermal synthesis, and the Au nanoparticles were deposited by the sputtering method. The Au nanoparticles were deposited because of the right efficient of anti-HCV antibodies with cystamine and glutaraldehyde. The ZnO nanorods were evaluated using SEM images. The electrochemical biosensor shows a sensitive response to HCV with a detection limit of 0.25 µg/µL. The proposed sensor characteristics of high specificity, good reproducibility and remarkable stability will provide a sensitive, selective, and convenient approach for the detection of HCV.

1 Introduction

Recent advances in interdisciplinary research in molecular diagnostics have led to the rapid development of different classes of biosensors with increasingly better sensory characteristics. In addition, the development in the areas of engineering and nanotechnology has been fundamentally important for miniaturization and technological multifunctionality of biosensors.

Biosensors have emerged as a cheap, easy-to-handle, portable solution without sacrificing the sensitivity and specificity of other biosensors used currently. The term biosensor, or biological sensor, is a detection device composed of a transducer and a biological element, where the biological element interacts with the analyte being tested. This biological response is converted into an electrical signal. In a biosensor [1], the sensor biomolecules need to be integrated into a system with solid support. The type of solid support responsible for the detection of biomolecules (receptor) is known as a matrix. A suitable matrix increases the signal of the transducer and assists in the immobilization of the biomolecules while maintaining the activity. The physical–chemical properties of the matrix, dictate the immobilization method and the stability of the biosensor. In addition, the biomolecule resistance can be altered based on pH level, temperature, and change in the chemical composition of the matrix. The thin films and nanostructures of ZnO have been extensively studied as a potential matrix for several types of biosensors due to physicochemical characteristics and biocompatibility.

The chemical receptor acts as the biologically active element that will interact with the analyte. This reaction is very specific, reversible, and alters the physicochemical parameters associated with the above-mentioned interactions; this results in the production of ions, electrons, light or heat. The receptors used in the sensors can be any substance capable of detecting the biochemical signal; however, the receptor must be a living element for the biosensors such as a cell, enzyme, antibody, organelle, among others [2].

In recent years ZnO nanostructures have attracted a lot of interest for use in biosensor applications. This is because of the small increase in the surface area of ZnO nanostructures, which can achieve the detection of a single molecule. ZnO can be synthesized in highly anisotropic nanostructures and grown on various types of substrates; these include sapphire, glass, silicon, and conductive surfaces such as indium tin-oxide (ITO) with different morphologies [3]. ZnO nanomaterials can also act as excellent fluorescent materials for the detection of biomolecules. Research has been carried out with ZnO nanostructures for biosensor applications synthesized by various physical

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and chemical methods. These nanostructures include wires (ZnONW), rods, fibers, beads, particles, tubes, belts, flowers, among others [4]. Nanostructured ZnO films have been widely used in biosensor applications because of their ease of manufacturing that employs low-cost processes; therefore a variety of nanostructures can be manufactured. The characterization of these nanostructured materials is essential for the advancement in the field of biosensor electrochemistry, and to obtain more sensitive biosensors that can be incorporated in fast reading devices for cheap diagnosis.

Recently, different kinds of nanomaterials have been investigated for the process of analyte detection with a high efficiency. One type of graphene has attracted special attention because of its low production cost and unique physical properties. These properties include high specific surface area, high carrier mobility, high electrical conductivity, flexibility, and optical transparency. Graphene and its oxygenated derivatives, including graphene oxide (GO) and reduced graphene oxide (rGO), are becoming an important class of nanomaterials in the field of biosensors [5].

Graphene is a two-dimensional nanomaterial with a thick carbon atom. These carbon atoms are sp^2 hybridized and packed in a crystalline hull-like lattice. Recently graphene has received increased attention as an emerging material with a range of potential applications due to its electrical, mechanical, optical, and biological properties. The oxidized form of graphene or graphene oxide has been extensively explored in the biomedical area because of its decent compatibility. Studies have shown that the surface of graphene oxide can interact through π–π stacking and hydrogen bonding with various biomolecules such as proteins. These interactions are fundamental for biological applications of graphene oxide [6].

It is estimated that at least 2.5% of the Brazilian population and about 200 million people worldwide carry the Hepatitis C virus, the largest human epidemic today. It is five times greater than AIDS and HIV. The number of people with Hepatitis C is greater in the urban centers of the south and southeast regions of Brazil. Another problem is that the people that carry the virus do not know they are infected. Hepatitis C is the leading cause of liver transplantation worldwide according to the Ministry of Health around 405 cases and can cause cirrhosis, liver cancer and death.

Hepatitis is a disease characterized by inflammation of the liver and can be caused by an infection by viruses or bacteria, or excessive consumption of toxic products such as alcohol and medicines. This can trigger cirrhosis and liver cancer leading to death. Viral hepatitis can be caused by six different forms of cirrhosis that can be transmitted through water and food contaminated with feces (Hepatitis A and E) or by contaminated blood contact and sexual intercourse (Hepatitis B, C, D, and G).

Hepatitis C is one of the most common diagnosis, characterized by the inflamation of the liver due to viral infection by the RNA–HCV virus of the Flaviviridae family. This virus was first isolated in 1989, and is an enveloped type virus with about 50–70 nm in diameter. It results in a positive RNA tape and belongs to the genus of Hepacivirus [7]. In 1992, the first test was developed to identify the antibody against HCV (anti-HCV), providing greater safety in blood transfusions. Its genome consists of a single positive RNA strand of approximately 9500 nucleotides, and exhibits significant genetic variability due to spontaneous mutations that occur during viral replication [8]. The treatment of Hepatitis C aims to halt the progression of liver disease by inhibiting viral replication. The combination of two drugs, conventional interferon or pegylated interferon plus ribavirin, is now the standard treatment for patients with chronic Hepatitis C. Interferon is a protein produced by the body in response to an infectious agent, inhibiting or interfering with the replication of viruses and protecting healthy cells. Because this interfered with the virus by helping the body's defense mechanisms, the protein was called interferon. Interferon for therapeutic use is produced synthetically and was the first treatment for chronic Hepatitis C, approved by the FDA (Food and Drug Administration). The use of interferon causes a stimulus in the immune system that starts to act against HCV, thus obtaining a natural response [9]. In 1998 there was an advance in the treatment of Hepatitis C with the combination of ribavirin. This provided a significant improvement in the responses. Ribavirin is a drug that exhibits in vitro activity against a range of viruses, and when used in conjunction with interferon or pegylated interferon, improves the patient's response to therapy [10].

The laboratorial diagnosis is performed by serological tests for detection of anti-HCV antibodies (Enzyme Linked Immuno Sorbent Assay—ELISA), and molecular tests (HCV-RNA) which detect and quantify the RNA of the Hepatitis C virus. The Enzyme Linked Immuno Sorbent Assay (ELISA) tests are enzyme-linked immune Sorbent assays that allow the detection of specific antibodies in blood plasma. The method consists in adsorbing the antigen to a polystyrene plate, followed by the addition of test serum to the plate, allowing the specific antibodies to bind to the antigens. After the incubation period, the plate is washed to remove the unbound antibodies. A conjugate (anti-immunoglobulin antibody bound chemically to an enzyme) is used to detect antigen-bound antibodies. A chromogenic substrate is then added to detect the presence of antigen–antibody binding. The resultant color intensity developed by the substrate is proportional to the amount of antibodies (antigen-specific) present in the serum. This color intensity can be analyzed by a spectrophotometer, allowing a quantitative analysis [11].

The first generation ELISA test for HCV targeted only one antigen, the c100-3 polypeptide, from the non-structural

region S4. Three generations of anti-HCV tests have been developed to increase sensitivity and specificity. The second generation of the ELISA test (ELISA II) appeared in 1992 in the United States, which incorporated two recombinant HCV proteins: c22-3 (derived from the structural region, or core) and c33-c (derived from the NS3 non-structural region). The c33-c protein was fused with the c100-3 antigen to form the c200 protein. The ELISA I and ELISA II tests increased both sensitivity and specificity; further, these reduced the false-positive rate, identified about 95% of patients infected with HCV, and decreased the mean time of seroconversion from 16 to 10 weeks. This can also be referred to the time elapsed between the infection and the onset of the antibody [12]. The third generation ELISA test (ELISA III) included recombinant antigens or synthetic peptides to capture antibodies and added an antigen from the NS5 region. The advantage of this new generation test was the reduction in the mean time of seroconversion, which increased to 7–8 weeks, in addition to an increase in sensitivity of up to 97% [13, 14]. Despite the great improvement in sensitivity and the reduction in the rate of false-positives compared to the first generation tests, ELISA tests do not confirm all people infected with HCV. Further tests to confirm the disease must be performed, for example, liver biopsy and enzymatic dosing. However, these tests are invasive and can cause risks to patients and are costly [15].

The core protein is being used as an important antigenic target in several commercial diagnostic tests for the detection of anti-HCV antibodies due to its high sensitivity [16]. However, considering the limitations of ELISA tests and the importance of the development of a rapid, direct and specific test for the determination of HCV infection, the proposal is to develop a state of the art device to include the entire test process. This process must have a low cost, high specificity, and increased sensitivity for the successful diagnosis of the disease in Brazil.

2 Experimental details

Synthesized ZnO nanorods with Au nanoparticles were produced by preparing the seed layers by the Pechini method, then the ZnO nanorods were grown by the assisted microwave hydrothermal process followed by the Au functionalization utilizing the sputering process.

2.1 Synthesis of ZnO seeds layers

The ZnO solution was prepared by the Pechini method [17]. In this method the zinc acetate dehydrate ($((CH_3CO_2)2Zn{\cdot}2H_2O)$) was dissolved in a water solution of citric acidand ethylene glycol in the ratio 1:4:16, under stirring and heating at 90 °C to form the polymeric solution. The spin coater was used to deposit the ZnO solution in the substrate, the thin film formed was treated at 400 °C for 1 h.

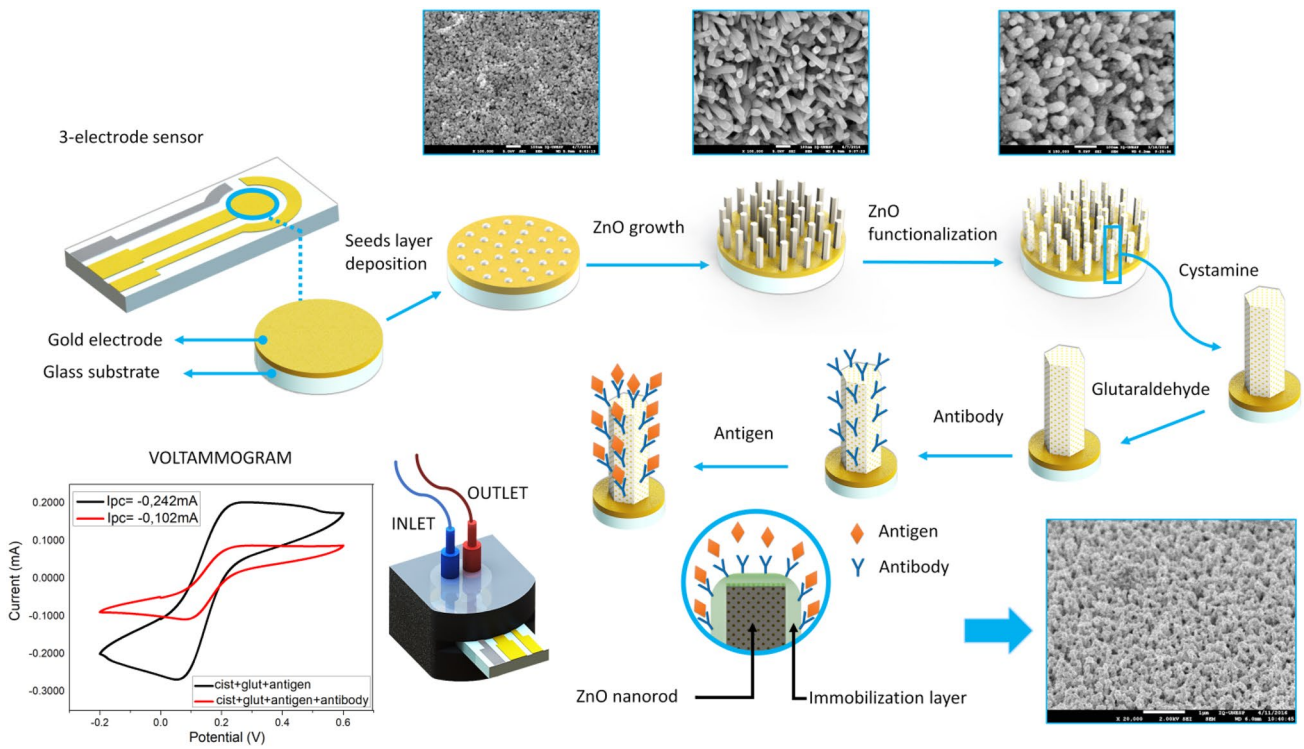

Fig. 1 Schematic construction of ZnO nanorods with Au nanoparticles for HCV detection

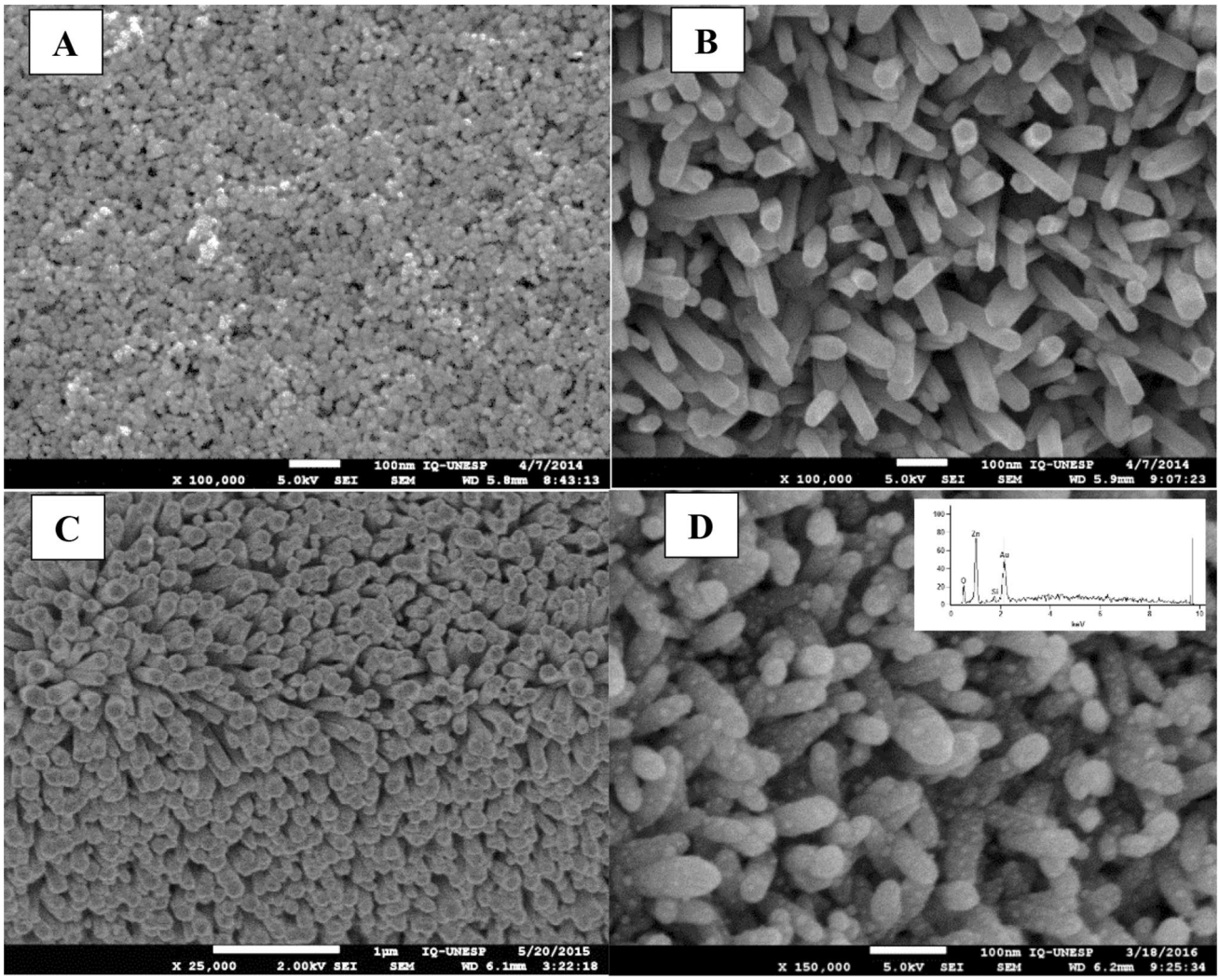

Fig. 2 SEM surface micrographs of seeds (**a**); ZnO nanorods top view (**b**); ZnO nanorods–Au NPs top view (**c**); and ZnO nanorods–Au NPs top view with EDS analysis (**d**)

2.2 Growth of ZnO nanorods

The substrates with ZnO seed layers were used in the assisted microwave hydrothermal synthesis to grow ZnO nanorods. In this method, zinc nitrate hexahydrate ($Zn(NO_3)_2{\cdot}6H_2O$, 99.0%) and hexamethylenetetramine (HMTA) ($C_6H_{12}N_4$, 99.0%) were mixed in equimolar aqueous solutions at 90 °C for 1 h.

2.3 ZnO nanorods funcionalization with Au nanoparticles

The substrates with ZnO nanorods were functionalizated with Au nanoparticles and the Au NPs were sputtered with a Bal-Tec model SCD 050 sputter coater for 4 s and then the material was treated at 450 °C for 1 h. The material was characterized by Scaning Electron Microscopy and energy dispersive X-ray spectroscopy (EDX) by using a JEOL-JSM 7500F.

2.4 ZnO–Au NPs electrochemical biosensor

The biosensor device used in this work comprised of a glass substrate with a working and counter electrode produced from gold (Au), and silver (Ag) as the reference electrode. The product electrodes were sputtered by sputtering Denton Vacuum, DV-502A model. The ZnO nanorods–Au nanoparticles were grown on the gold working electrode. The resulting gold working electrode was coated with 20 µL of cystamine solution, 20 µL of glutaraldehyde solution 5%, washed with deionized water and dried. Once that process was completed, 20 µL of HCV antibody solution diluted in PBS was immobilized onto the ZnO–Au NPs. The electrode was washed with deionized water and stored at 4 °C

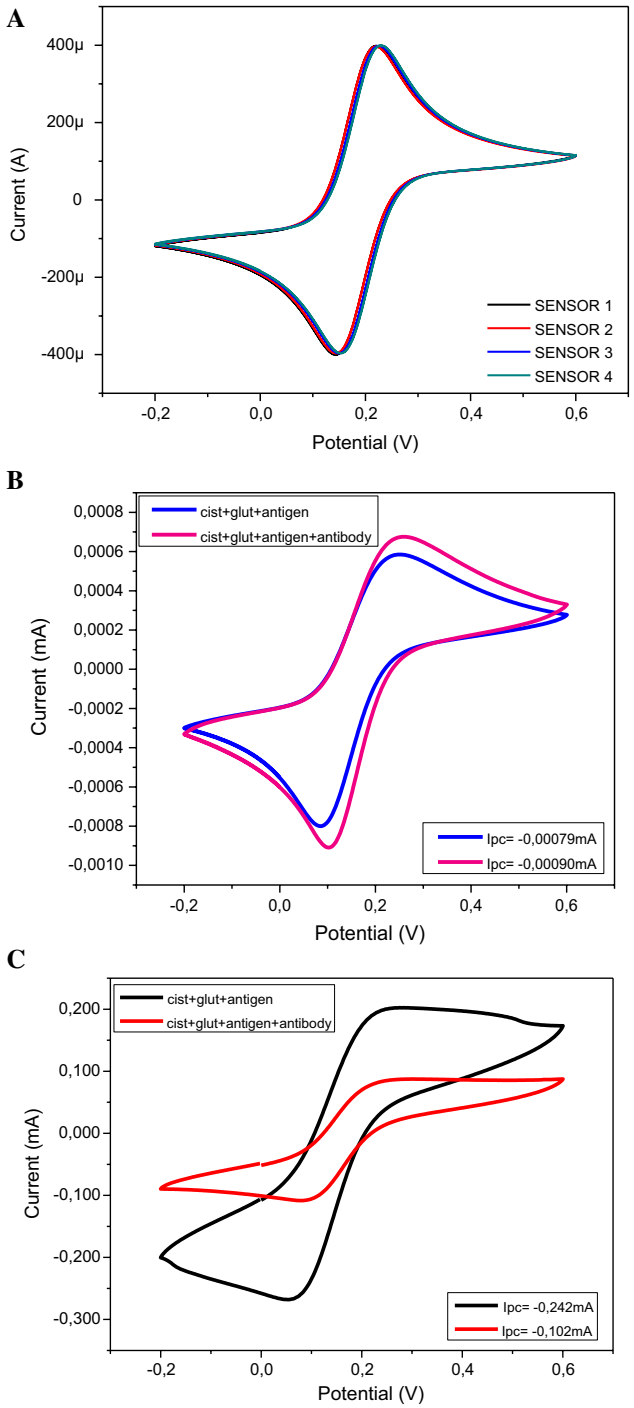

Fig. 3 Cyclic voltammograms of (**a**) calibration curves of the blank sensor; (**b**) blank sensor immobilized with cystamine (Cyst), glutaraldehyde (Glut) and antigen and antibody without ZnO nanorods; (**c**) ZnO nanorods–Au NPs immobilized with cystamine (Cyst), glutaraldehyde (Glut) and antigen and antibody. The curves were recorded in 0.1 M PBS solution containing 5 mM [Fe(CN)6]3−/4− as a redox mediator

overnight. Finally, HCV was added to the bioelectrode and after that the bioelectrode was immersed in PBS. The cyclic voltammetry (CV) measurements were carried out on a Potentiostat Metrohm Autolab PGSTAT 128 N in 0.1 M PBS solution containing 5 mM potassium ferri/ferrocyanide [Fe(CN)6]3–/4 as the mediator and with the scan rate of 1000 mV/s. The schematic process to obtain the ZnO nanorods–Au nanoparticles biosensor. The schematic illustration of the ZnO nanorods with Au nanoparticles biosensor construction is show in Fig. 1.

3 Results and discussions

The ZnO Pechini solution was deposited by spinning followed by a thermal treatment which consisted of heating the substrate to 400 °C for 1 h for the formation of the seed layer. Figure 2a shows the seed layer deposited on the working electrode, with small grains, homogeneously distributed along the substrate with porous microstructure. These layers are very important because the morphology and size will be the main determinant for the ZnO nanorods growth alignment in the *c* axis (Fig. 2b, c). The subsequent growth of ZnO nanorods by microwave-assisted hydrothermal synthesis at 90 °C for 1 h, starting from a solution of 0.03 M zinc nitrate and 0.3 M HMTA, is observed in Fig. 2b. The nanorods grow this uniform in length around 260 nm. The SEM images of the seed layer, ZnO nanorods, and ZnO nanorods fuctionalized with Au NPs are shown in Fig. 2. Figure 2d shows the Au nanoparticles in the ZnO nanorods. The diameter of the Au NPs is around 10 nm, which is confirmed by the EDS analysis as well as sample composition. This kind of morphology results in increased surface area. This is one of the most important characteristic for biosensor applications. After obtaining the nanorods functionalized with Au NPs on the working electrode, the samples were prepared, with cystamine, glutaraldehyde, antigen and antibody, and the sensor device was submitted to measurement cyclic voltammetry using a potentiostat.

Figure 3 show the cyclic voltammograms (CV) obtained for the different electrodes in the potential window from −0.2 to + 0.6 V and the scan rate of 100 mV/s recorded in 0.1 M PBS solution containing 5 mM $[Fe(CN)_6]^{3-/4-}$ as the redox species. The cyclic voltammograms show good defined oxidation and reduction peaks in the presence of $[Fe(CN)_6]^{3-/4-}$ which provides a lower electron potential.

To guarantee the quality, stability and reproducibility of the sensor developed in our laboratory, measurements of cyclic voltammetry were performed on four sensors as a blank sample. Figure 3a shows the curve with the cyclic voltammetry obtained for the sensor, where a high stability to the system is observed. In order to compare the behavior of the direct immobilized biological material in the sensor

Table 1 HCV detection by electrochemical immunosensors developments

Electrode	Antigen	Technique	Performance	Ref
3-Mercaptopropionic, acid was assembled on gold electrode to form a self-assembled monolayer	Thionine HCV horseradish peroxidase (HRP) antibody	Cyclic voltammetry and linear scan voltammetry	Hepatitis C range 3.2–16 mg/L	[19]
Glassy carbon electrode with nanocomposite	HCV core	Cyclic voltammetry and electrochemical impedance spectroscopy	Detection limit: 0.17 ng/mL and 128 ng/mL HCV core antigen	[20]
GMCs-MB nanocomposite	HCV core	Cyclic voltammetry and electrochemical impedance spectroscopy	Detection limit: 0.01 pg/mL	[21]
Glassy carbon modified with Au-MoO_3/ Chitosan	HCV non-structural 5Aprotein	Cyclic voltammetry and electrochemical impedance spectroscopy	Detection range: 1.0 ng/mL–50 µg/mL	[22]
Silk fibroin nanostructured films on printed carbon electrode	HCV non-structural 5Aprotein	Cyclic voltammetry	Detection range: 0–0.2 µg/mL	[23]

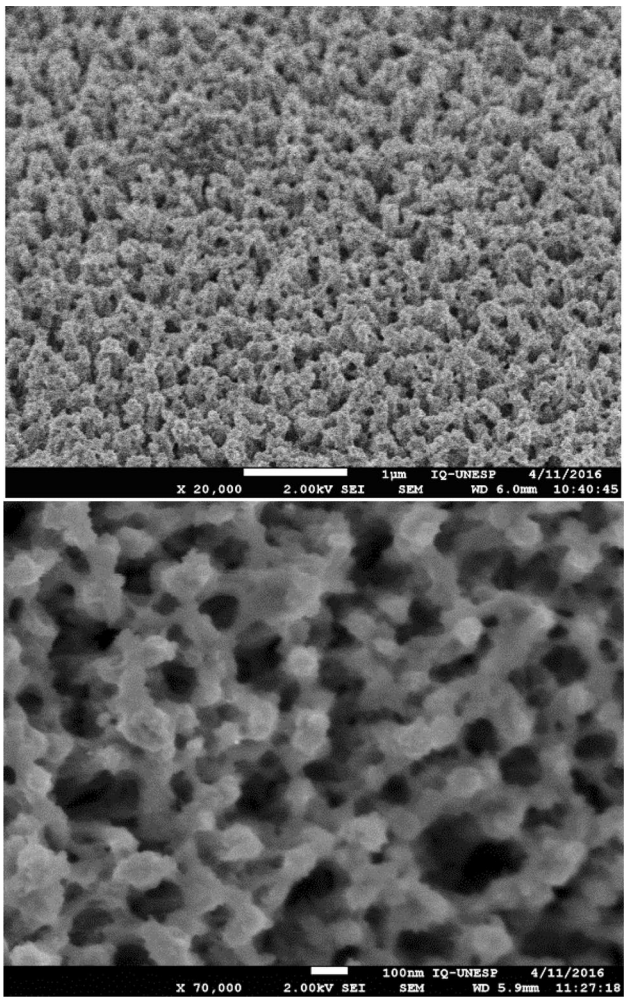

Fig. 4 SEM images of immunosensor ZnO NRs–Au NPs immobilized with Cys + Gly + antigen + antibody

without ZnO nanorods functionalized with Au nanoparticles, voltammetry measurements were performed under these conditions. In Fig. 3b the voltammogram measurements show the behavior of the biological material immobilized directly in the sensor. Without the nanorods of ZnO with the analysis of the curve, it is not possible to observe a significant variation in the current when we add the antibody. The cathode peak current Ipc is − 0.079 µA for the material without the antibody and − 0.090 µA with the antibody.

In Fig. 3c we can observe the cyclic voltammetry curve indicating the oxidation and reduction peaks for the ZnO nanorods functionalized with gold nanoparticles grown on the gold work electrode. There is a difference between the curves obtained in the presence and absence of the antibody. In both curves,there is the presence of the antigen. For the antibody curve, the cathodic peak current Ipc was − 0.102 mA and in the absence of antibody the Ipc was − 0.242 mA; these curves were obtained in the presence of the antigen at the concentration of 0.25 µg/µL. The difference observed in the current value is nearly 3 times less with the addition of the antibody. This difference is extremely significant. When the current values obtained are compared in the voltammogram of Fig. 3b and c, the importance of the ZnO nanorods is verified because of the current difference being in the order of 100 times.

The electrochemical immunosensors based on the molecular recognition of an antigen and antibodies have attracted popularity in clinical diagnostics and will provide highly sensitive detection of HCV antigen. There are few reports concerning theses immunosensors [18]. Table 1 indicates developments in HCV detection using electrochemical analysis.

In Fig. 4 we have the surface of ZnO nanorods with Au nanoparticles with antigen and antibody immobilization was observed the high surface area. With this, it is possible to

detect the Hepatitis C virus, with high biosensor efficacy developed using ZnO nanobastones functionalized with gold nanoparticles.

4 Conclusion

The methodologies used for the growth of nanorods by microwave assisted hydrothermal synthesis and the surface functionalization with Au nanoparticles proved to be efficient and extremely important for the development of the biosensor. In addition, the developed biosensor showed excellent repeatability and sensitivity, guaranteeing reliability for cyclic voltammetry measurements of the immobilized material (cystamine + glutaraldehyde + antigen core protein + anti-HCV antibody), which could detect the Hepatitis C virus. The developed biosensor showed good stability and excellent sensitivity for the detection of Hepatitis C. The development of the biosensor was performed at the Research Laboratory (LIEC) in partnership with the Laboratory of Clinical and Biomolecular Immunology (LICBM), since the development of the sensor performed in the work of Costa, growth, functionalization and measurement of the biosensor present in this work, as well as, obtaining the core protein performed in the work of Kenfe and showed to be very stable sensor.

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