

Nanosensing of Hepatitis E Virus in Swine Using Graphene

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Abstract— Sensing of the hepatitis E virus is crucial for effective porcine health management and prevention of spread to humans. This study presents the development of a nanosensor using graphene nanosheets to detect hepatitis E antigen within a minute. The graphene layer not only increases the loading of antibodies specific to the hepatitis E virus but also enhances sensitivity and selectivity. This sensor is sensitive to 10 fM of hepatitis E antigen. This nanosensor holds significant potential for the rapid and early detection and monitoring of hepatitis E, thereby contributing to enhanced public health outcomes and the safety of pork products.

Keywords—hepatitis E, antibody, nanosensor, graphene

I. INTRODUCTION

Hepatitis E virus (HEV) is a significant yet under-researched zoonotic pathogen responsible for both acute and chronic hepatitis, as well as various extrahepatic diseases in humans.[1-3] In the US, HEV is present in 11% of pig livers sold, highlighting significant food safety concerns. Further, agricultural biosecurity plays a crucial role in controlling zoonotic diseases like HEV infection in swine. Annually, HEV infects approximately 20 million people worldwide, resulting in 3.3 million cases and 44,000 deaths, with a mortality rate of about 3.3%. HEV ranks 6th among over 800 viruses for spillover infection risk and is recognized as one of the top three foodborne viral pathogens by a joint WHO/FAO expert panel. Pigs serve as the primary reservoir for HEV, [4] which can be transmitted to humans through contaminated food or water. Among the known genotypes, HEV genotypes 3 and 4 (HEV-3 and HEV-4) are zoonotic, infecting humans as well as various animals, including pigs, deer, rabbits, and mongooses. [1-3] In the US, HEV RNA is commonly found in growing pigs aged 2-4 months,[5] but was also detected in 6% of slaughtered pigs,[6] and in 11% of pig livers sold in grocery stores.[7] So far, the US Food and Drug Administration (FDA) has not yet approved a

diagnostic test, vaccine, antiviral, or immune globulin for preventing and treating HEV infection. Rapid and accurate HEV detection in swine on farms or pork in slaughterhouses is vital for reducing HEV transmission within animals, pork contamination, and improving health and productivity, yielding economic benefits.

Three main approaches, serology, qRT-PCR, and viral capsid antigen in blood or stool are commonly used to detect HEV infection,[8] yet none is suitable for real-time on-site testing. While the ELISA test does not indicate active virus infection, may yield false results and require lengthy processing, qRT-PCR offers sensitivity and selectivity[8] but lacks on-site screening capabilities. Current tests for anti-HEV (antibodies specific to hepatitis) sometimes have limited specificity and accuracy,[9] leading to inconsistent results. Graphene-based nanosensors are highly effective for the detection of virus surface biomarkers[10] due to their large surface area, high conductivity, and ability to functionalize with specific antibodies, enhancing sensitivity and selectivity.[11] Additionally, the functional properties of 2D graphene, including its functional groups such as -COOH and -OH, improve the loading of antibodies or antigens, allowing for the detection of targets even at low concentrations.[11] These properties enable rapid, accurate detection of HEV antigens at very low concentrations, making them ideal for early diagnosis and monitoring.

This report presents the development of a low-cost graphene-based nanosensor for the detection of genotype-3 HEV (HEV-3) through measurements of antigen concentrations. We used a prototype nanosensor made of -COOH functionalized graphene and HEV-3 antibodies on a screen-printed electrode, employing electrochemical modalities to sense dose-dependent HEV-3 concentrations (0.01 pM to 0.1 μ M). With 1 minute of incubation time, this

nanosensor provides sensing results within 30 seconds. It also demonstrates excellent selectivity for HEV-3, even in the presence of a similar antigen from the hepatitis B virus (HEB).

II. FABRICATION OF NANOSENSOR

The nanosensor utilizes a thin layer of graphene nanosheets on a carbon screen-printed electrode, which is connected to a portable reader. We chose this sensor configuration due to its ability to perform on-site screening of biological samples (such as blood, urine, or feces) from pigs. The nanosensor (**Fig. 1**) has three electrodes: the working electrode (WE), reference electrode (RE), and counter electrode (CE). The WE of the sensor is circular and has a diameter of 4 mm, which was modified to detect HEV-3 antigen.

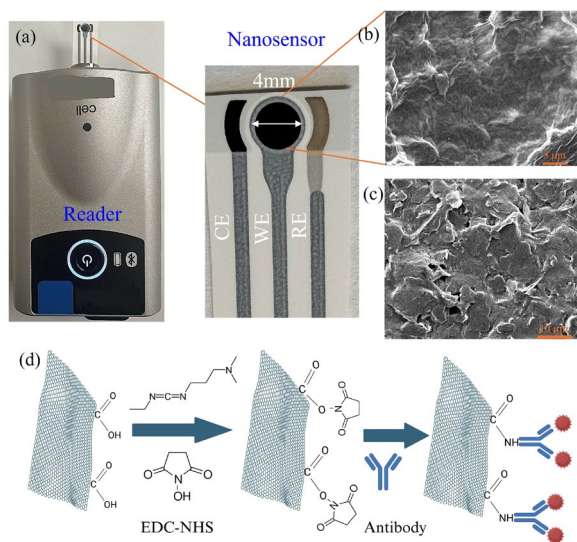


Figure 1. Nanosensor for the Detection of HEV-3 Antigen. (a) Photograph of the screen-printed sensor using carbon, along with the corresponding readout. The diameter of the working electrode (WE) is 4 mm. (b) The carbon electrode (as shown in the SEM image in part c) is modified with graphene nanosheets. (d) Schematic illustrating the functionalization process of the WE. EDC-NHS chemistry is used to create binding sites for the antibody. The red circles represent HEV antigens attaching to the blue antibodies, which are specific to the HEV antigen.

To functionalize the nanosensor, the graphene nanosheets were conjugated with HEV-3 antibodies using a well-known chemistry, such as EDC-NHS. To create layers of graphene nanosheets, 20 μL of a 0.5 mg/mL graphene suspension was drop-cast onto the working electrode (WE) of the nanosensor and dried on a hot plate for one hour at 80°C. This process was repeated once to form two layers of graphene nanosheets on the WE. Due to π - π interactions

between the graphene sheets and the carbon electrode, the graphene adhered strongly to the carbon-printed electrode. After drying, 20 μL of a 1:1 ratio solution of 0.2 M EDC and 0.05 M NHS were applied to the WE, and the sensor was incubated in a humid chamber for 4 hours at room temperature. This allowed the formation of an amide bond (C-N) between the antibodies and graphene via an amidation reaction (**Fig. 1d**). The nanosensor was then washed with phosphate-buffered saline (PBS), after which a 1 mg/mL solution of HEV-3 antibody was applied to the WE. The sensor was incubated in a humid chamber for at least 12 hours at room temperature. After incubation, the sensor was washed with PBS and stored in a 4°C refrigerator until use. To block non-specific binding sites on the nanosensor surface, a layer of bovine serum albumin (2 mg/mL) was applied.

Scanning electron microscopy (SEM) images reveal the morphology of the carbon electrode before and after the graphene layer is applied. The surface texture of the bare carbon film is not smooth, exhibiting large patches and irregularities (see image c in Fig. 1). After graphene modification, a uniform coating is observed, with the presence of wrinkles on the surface (**Fig. 1b**). This is due to the interaction between graphene and the carbon surface, including van der Waals forces and functional groups. Additionally, the quality of graphene dispersion contributes to the smooth coating, enhancing sensor performance.

III. NANOSENSOR CHARACTERIZATION

We first tested our fabricated nanosensor in PBS electrolyte, both with and without an electroactive mediator such as ferro/ferricyanide, using cyclic voltammetry (CV) and chronoamperometry (CA) modalities (**Fig. 2**). In the CV studies, no peak was observed when the nanosensor was tested with only the PBS solution (**Fig. 2a**). However, a strong peak appeared when the mediator was used, due to the oxidation and reduction of ferro/ferricyanide. Therefore, we used the mediator for evaluating the nanosensing measurements. A similar phenomenon was observed when using the CA method (**Fig. 2b**). The current increased by 327-fold when the ferro/ferricyanide mediator was used. This significant increase can be attributed to the mediator's ability to facilitate electron transfer between the electrode and the analyte. Ferro/ferricyanide, being an electroactive species, effectively shuttles electrons, thereby

enhancing the overall conductivity and electrochemical response. Therefore, we used this mediator to evaluate the nanosensing performance.

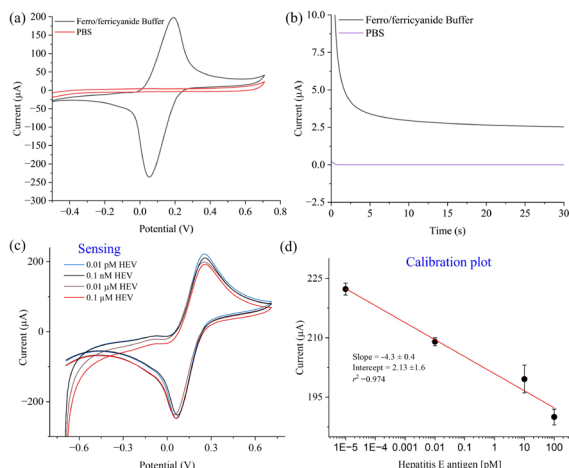


Figure 2. Nanosensing of HEV-3 antigen using CV. (a) CV of the nanosensor with and without the mediator in PBS electrolyte. (b) Chronoamperometry comparing the performance of the nanosensor with and without the mediator. (c) Nanosensing using CV by varying the HEV-3 antigen concentration from 0.01 pM to 1 μM. (d) Nanosensor calibration plot showing the difference in peak current at a potential of 0.25 V for different concentrations of HEV-3 antigen.

IV. HEV-3 ANTIGEN SENSING

The nanosensor was tested using a series of dilutions of the HEV-3 antigen, the target biomarker for identifying HEV-3 infection. In this detection principle, the surface of the nanosensor captures the HEV-3 antigen through interactions between antibodies on the nanosensor surface and the target antigen, blocking electron transfer and resulting in a decrease in current.

For our CV sensing experiments (Fig. 2c), we selected a dose-dependent range of HEV-3 antigen concentrations, from 0.01 pM to 1 μM. We tested the nanosensor across a range of concentrations, from low to high, and found that the resulting current is inversely proportional to the target concentration. Between concentrations, the sensor surface was cleaned by adding 1 M formic acid to the WE, allowing it to sit for 1 minute, and then washing with PBS before adding the next antigen concentration. Each concentration was tested three times, and the average results from 0.01 pM to 1 μM are presented in Fig. 2d to generate a nanosensor calibration plot. Figure 2d shows the near linear relationship between the decrease in current versus the increase in concentration of the analyte. This nanosensor showed

a slope value of 2.13 (*i.e.* sensitivity) while tested with CV.

Figure 3 presents the CA nanosensing results for various concentrations of HEV-3 antigen. The sensing principle of this CA-based method is similar, but we chose a sensing potential of 2.5 V and recorded the current for 30 seconds. First a buffer solution with no HEV present was tested to create the baseline. Then, we tested our titrate dose-dependent concentration of HEV-2 antigen (Fig. 3a). Figures 3b-c show the

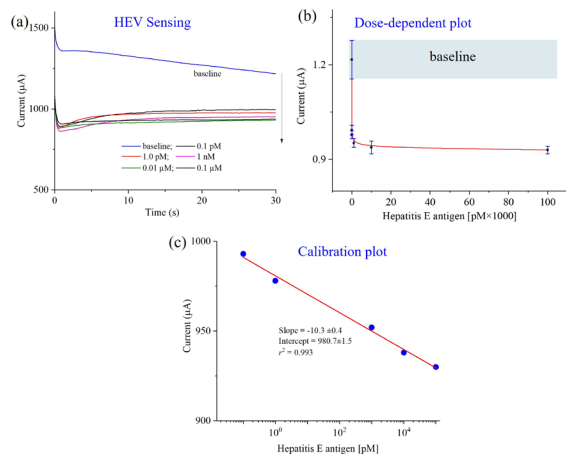


Figure 3. Nanosensing of HEV-3 antigen using CA. (a) CA graph showing the difference in current over time between different concentrations of HEV. (b) Dose-dependent plot showing the relationship between HEV antigen concentrations and current. (c) Calibration plot created from the HEV antigen concentrations versus current.

relationship between the increase in concentration of HEV-3 antigen and the decrease in current caused by the insulation of the WE. This indicates that the antigen is binding to the antibodies on the surface of the WE, which creates an insulating effect. Similar to CV, the resulting current is inversely proportional to the HEV-3 concentration. The slope value of this CA-based testing is 10.3, which is 4.9 times higher than that of the CV-based sensing. However, both sensing methods can detect a 10 fM concentration of HEV-3.

Selectivity testing was performed in the presence of hepatitis B (HEB) antigen. We mixed both HEV and HEB antigens at a 1:1 ratio, each with a concentration of 4 ng/mL, and conducted the CA test (Fig. 4a). The sensor showed a significant change in the signal when tested with only the HEB solution. However, when HEB was added to the HEV-3 solution, the sensor signal showed an insignificant change (Fig. 4b), indicating no cross-reactivity with the HEB antigen.

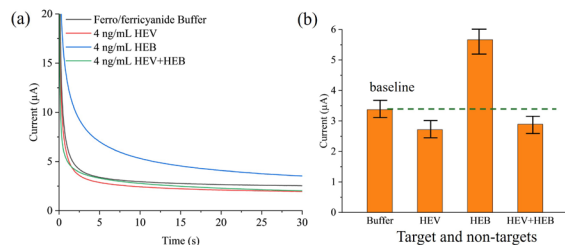


Figure 4. Selectivity testing with HEV-3 and HEB antigens. CA test showing a significant change in the sensor signal when tested with only the HEB antigen (4 ng/mL). Bar graphs show an insignificant change in the sensor signal when HEB antigen is mixed with HEV-3 antigen (4 ng/mL each), demonstrating that the sensor does not exhibit cross-reactivity with the HEB antigen.

V. CONCLUSION

This graphene-based, low-cost nanosensor shows promise for detecting hepatitis E in a short amount of time and is portable for use on swine farms. The nanosensor's sensitivity is high enough (~10 fM) for rapid testing to detect infections in swine and slaughterhouses. While it provides high selectivity, testing with different types of hepatitis (A and C) will be conducted to evaluate cross-reactivity. In the future, we will explore approaches to: a) introduce a 3D-printed sensing platform using a graphene layer, and b) analyze real samples obtained from a swine farm.

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