High selective spectroelectrochemical biosensor for HCV-RNA detection based on a specific peptide nucleic acid

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ABSTRACT

Hepatitis C virus (HCV) is a blood-borne virus that causes infectious chronic hepatitis. Egypt has the largest epidemic of HCV in the world, with about 14.7% of the Egyptian population. Thus, HCV, which could cause severe risks for public health including liver failure, becomes a public health concern for Egyptians. Development of highly selective and sensitive biosensors for accurate detection of HCV levels without extensive sample preparation has received great attention. The present work reported on developing a new rapid, highly selective and sensitive biosensor for early detection of HCV-RNA extracted from clinical samples. The HCV-based biosensor was constructed by immobilization of a specific peptide nucleic acid (as bio-receptors) terminated with thiol group onto gold nanodots/indium tin oxide substrate and followed by immobilization of a specific peptide nucleic acid (as bio-receptors) terminated with thiol group onto gold nanodots/indium tin oxide. The principle of the developed biosensor was based on the selective hybridization between the peptide nucleic acid and the HCV-RNA at the untranslated regions (5′-UTR). Raman spectroscopy and Square wave voltammetry techniques were used to monitor the interaction between the HCV-RNA and the immobilized peptide nucleic acid. The reported HCV-biosensor demonstrated a high capability to detect HCV-RNA.

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1. Introduction

Hepatitis C virus (HCV) is a frequent cause of infectious chronic hepatitis. Unlike hepatitis A and B, there is no vaccine to prevent hepatitis C infection yet. According to WHO, around 200 million people are infected with HCV worldwide [1], in addition to three to four million newly infected patients are detected each year. In 2009, Egyptian Demographic and Health Survey (EDHS) have reported an overall anti-HCV antibody prevalence of 14.7% of the Egyptian population (excluding the patients that are under 15 or above 60 years old) was positive for the anti-HCV antibody with overall 0.6% new patients getting infected each year. In addition, Egypt has the highest prevalence of HCV in the world [2]. Therefore, HCV becomes a public health concern for Egyptians. HCV could cause several risks for human health including chronic effects that damage the liver and lead to liver failure. An accurate and early diagnosis of active HCV infection is critical not only because of its associated morbidity and mortality but also because the early diagnosis is the most important factor for successful treatment [3]. Several techniques such as enzyme-linked immunoassays (ELISA), recombinant immune blot assays (RIBA), Surface Plasmon Resonance, electrochemiluminescence, piezoelectric genosensor, cell-based assays, a biosensor based on fluorescence detection, and electrochemical detection were used for detecting anti-HCV antibodies [4–7]. However, early detection of HCV using these methods is not feasible due to the absence of antibodies against HCV-antigens at early stages of the disease; in addition, the testing for anti-HCV antibodies couldn’t differentiate between the current or past infection [8,9]. Several commercial assays are available for the detection of anti-HCV antibodies [10–12]. Although, the anti–HCV test has significantly reduced the risk of HCV transmission, the time frame for detection of infection remains a concern. The anti–HCV antibodies can be detected 7–8 weeks after infection and usually persist for life. Also, false negative results may arise in immunocompromised patients, such as those with human immunodeficiency virus (HIV) infection or uremia. Therefore, a highly sensitive and reliable test is needed for the early detection of HCV infection. In addition, Reverse transcriptase polymerase chain reaction (RT-PCR) and branched DNA-based assays were used for quantitative detection of HCV with high sensitivity and specificity. However, they are time-consuming, labor intensive, expensive, and require specialized equipment. Nucleic acid testing (NAT) for HCV RNA was developed as a more accurate method for the disease diagnosis and monitoring, as well as a confirmatory diagnostic tool for anti-HCV assays [13,14]. The introduction of NAT has greatly reduced the risk of HCV transmission. But, it is costly, liable to environmental contamination, and laborious work has hampered the wide application of NAT in clinical settings. However, the detection of HCV RNA is too expensive and labor-intensive for routine...
use. In view of these limitations, there is a need to develop a low-tech assay for the direct detection of unamplified HCV-RNA with high sensitivity, selectivity, short turnaround time, and cost-effectiveness. Electrochemical HCV-biosensors have many advantages in comparing with other molecular detection methods; the advantages including the high sensitivity, its capability to analyze a complicated matrix in addition a compact device and a low power are needed [15–17]. Riccardi et al. have reported a label-free DNA hybridization electrochemical method for detection of HCV [18] by using a film of polypyrrole modified Pt micro-electrodes. In addition, Cai’s group have developed an HCV-biosensor based on immobilization of DNA-thiol (DNA-SH) onto Au electrode in the presence of thionine as an electrochemical indicator [19,20]. Furthermore, Pourmagni-Azar et al. (2009) has reported the immobilization of DNA on a pencil graphite electrode to detect HCV 1a genotype [21]. Recently, nanoparticles (NPs) have emerged as promising tools with potential applications in many fields such as biosensing and drug delivery. Due to the unique properties of NPs and their ability to interact with biomolecules, several NPs showed a great promise to meet the rigorous criteria of early disease diagnosis and treatment [10]. In this regard, researchers’ efforts have especially concentrated on developing nano-biosensors that provide rapid detection by responding to their target with high sensitivity and selectivity. Liu et al. have reported on the development of an electrochemical HCV RNA level for HCV 1b genotype based on the specific DNA modified gold nanoparticles (Au NPs) [22].

In the present work, we have successfully developed a highly sensitive and selective biosensor for early detection of very low concentrations of HCV-RNA as an in-vitro diagnostic tool for HCV. Here, we have prepared Au nanostructures modified indium tin oxide (ITO) substrate and then modified it with peptide nucleic acid (PNA) and used as a probe for the detection of the extracted HCV-RNA that have the ability to hybridize with the HCV-RNA at the untranslated regions (5'-UTR) of the HCV-RNA. Scanning electron microscope images were used to investigate the morphology of these nanostructures; in addition, the chemical structures and the purity of the PNA-SH were confirmed by using MS spectra and high-performance liquid chromatography (HPLC) techniques. The capability of the developed biosensor for detecting HCV-RNA was monitored by using Raman spectroscopy and two electrochemical techniques (cyclic voltammetry and square wave voltammograms).

### 2. Experimental

#### 2.1. Materials

Gold (III) chloride tetrahydrate (HAuCl4·4H2O) and ITO slides were purchased from Aldrich (3050 Spruce St, St. Louis, MO63103, USA). Ethanol and phosphate buffer saline (PBS, pH = 7.4) were obtained from Sigma. The aqueous solutions were prepared by using deionized water (DIW) that purified a Purite purification system.

#### 2.2. Instruments

The surface morphology of the Au/ITO substrate was analyzed using a scanning electron microscope (SEM) (JEOL JSM-5400 LV, Japan). All electrochemical measurements were performed by using micro-Autolab, potentiostat/galvanostat instrument (Metrohm Model 663VA stand) that controlled by Autolab Nova software. The electrochemical system consisted of a handmade three-electrode system consisted of a platinum wire as a counter electrode, Ag/AgCl as a reference electrode, and Au nanodots/ITO (10 mm × 20 mm) as the working electrode. The immobilization of the PNA molecules and their interaction with the HCV-RNA were monitoring by recording their Raman spectra with a Bruker Senterra Raman microscope (Bruker Optics Inc., Germany) with 785 nm excitation, 1200 rulings mm-1 holographic grating and a charge-coupled device (CCD) detector. The acquisition time was 3 s with a power of 50 mW.

#### 2.3. Preparation of Gold nanostructures/ITO substrate

Au nanostructures/ITO was fabricated by deposition of Au nanodots onto the surface of the ITO substrate based on electrochemical deposition according to our previous method [23] as follows. At the beginning, ITO substrates were cleaned by sequential sonication in 1% Triton X-100, DIW, and ethanol, respectively for 15 min for each one. Then, the substrates were immersed in a basic piranha solution that consists of a mixture of H2O2:N H3:H 2O ratio (1:1:5, v/v) for 90 min at room temperature (RT). The ITO substrates were rinsed with DIW and ethanol and dried under N2 gas. Finally, ITO substrates were immersed in 1 mM HAuCl4 aqueous solution and a negative potential of −0.9 V against Ag/AgCl (reference electrode) was applied for 30 s. The surface morphologies were analyzed with SEM.

#### 2.4. HCV RNA extraction

Several serum samples from healthy persons and chronic HCV patients were obtained from the microbiology unit at Assiut University. All the positive samples were evaluated by the RT-PCR technique. HCV-RNA was extracted from serum samples using two different kits: Stratagene total RNA Kit (Cat. No. 400800) according to standard manufacturer’s instruction, and Promega total RNA extraction kit (Cat. No. Z3100) according to the modified manufacturer’s protocol for HCV RNA isolation [24]. The viral load of the HCV was determined by RT-PCR and was used to investigate the selectivity and sensitivity of the developed sensor by using serial dilutions of the HCV RNA samples.

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**Fig. 1.** SEM images of Au nanodots modified ITO substrate.
2.5. Design, synthesis and characterization of HCV biosensor

The solution of PNA-SH molecules was self-assembly immobilized onto the Au nanostructures modified ITO substrate, in which PNA-SH was dissolved in ethanol and then the Au modified ITO substrates were immersed in the PNA-SH solution for about 6 h at 4 °C. The non-immobilized PNA molecules were removed by washing the substrate with DIW and dried under nitrogen stream. The immobilization of PNA was confirmed by using Raman spectra and square wave voltammetry (SWV) techniques. The Au/ITO with HS-PNA will be dipped into the complementary target RNA solution prepared in 10 mM PBS for 1 h. Then, the hybridization events between the PNA molecules and the complementary target HCV-RNA was monitored based on Raman spectra as well as the changes of its cyclic voltammetry (CV) and SWV response.

3. Results and discussion

3.1. Design, synthesis and characterization of the HCV biosensor

The principle of the developed HCV-RNA biosensor based on PNA with a specific sequence of nucleic bases related to the following facts. The HCV genome is composed of 9600 bases that contain two untranslated regions (5′-UTR and 3′-UTR) at both ends, the sequence of these UTRs are specific for each virus (5′-GGAGAUUUGGGCGUG-3′); the sequence of the UTR can differentiate mRNAs [13,14]. Moreover, in the 5′-UTR, there is an internal ribosome entry site (IRES), which is one of the most conservative regions in the whole HCV genome. In the current work, we have used a complementary PNA with a bases sequence 5′-CACGCCCAAATCTCC-3′ and terminal with thiol group to enable their direct immobilization onto Au nanostructures/ITO substrate. The PNA/Au nanostructures/ITO was used as receptors that could hybridize with the target HCV-RNA as shown in Scheme 1. The chemical structures and the purity of this peptide were confirmed by using mass spectra and HPLC techniques as shown in Figs. S1a and b, respectively. The mass spectrum of the peptide showed a single peak that corresponds to the molecular weight of the peptide plus one (Fig. S1a). Furthermore, the purity of the peptide was confirmed by using the HPLC technique that showed a single peak as shown in Fig. S1b, which confirmed that the peptide was obtained in high purity grade (more than 99%). Then, in order to develop the HCV-PNA based sensor, Au nanostructures modiﬁed ITO substrate was fabricated based on electrochemical deposition of Au onto the ITO surface. The morphology and the size of the Au nanostructures were investigated by using SEM image as shown in Fig. 1, which demonstrated the formation of Au nanodots with an average size of about 20 nm. The Au nanodots could be acting as hot spots to enhance the Raman effect [25,26] as well as enhancing the electrochemical conductivity, in addition to their role as a scaffold for direct immobilization of PNA.

To develop the HCV RNA-based biosensor, the PNA-SH peptide was immobilized into the Au modified ITO based on the self-assembly technique, in which the immobilization process was depended on the interaction between the thiol group from the PNA molecules and the Au nanostructures [27]. Fig. 2 (black curve) showed the Raman spectrum of PNA immobilized onto Au/ITO that confirmed the immobilization of the PNA peptide. Then, we used this probe to monitor the HCV-RNA, in which the PNA-S Au/ITO was dipped in the complementary target RNA solution prepared in 10 mM PBS for 1 h to allow hybridization between the extracted HCV-RNA and the PNA molecules, and washing the substrate with DIW and dried under nitrogen stream. Then, the hybridization events between the PNA molecules and the complementary target HCV-RNA was monitored based on Raman spectra as well as the changes of its SWV response. Fig. 2 (red curve) represented the Raman spectrum of HCV-RNA after hybridization with the PNA, which showed

![Scheme 1. Development of HCV-RNA biosensor, Step 1: Fabrication of Au nanostructures modified ITO substrate; Step 2: immobilization of PNA-SH onto Au nanodots modified ITO substrate; and Step 3: detection of complementary target HCV-RNA.](image1)

![Fig. 2. Raman spectra of PNA-SH immobilized onto Au nanostructures modified ITO substrate and HCV's RNA hybridized with PNA.](image2)
the characteristics peaks of HCV, the assignments of these peaks were summarized in Table 1. These results demonstrated the capability of the developed probe for sensing the HCV-RNA.

### 3.2. Evaluation of the sensitivity of the HCV biosensor based on Raman spectroscopy

Here, we have studied the sensitivity of the developed HCV biosensor based on measured the Raman spectra for different concentrations of HCV-RNA as shown in Fig. 3a. We have extracted the HCV-RNA from different samples and the contents of the HCV were measured by using the RT-PCR technique. Fig. 3a indicated the increase of the Raman intensity with increasing the HCV-RNA concentration. Fig. 3b showed the relationship between the HCV-RNA concentration and the Raman intensity at 1232 cm$^{-1}$, which showed a linear relationship with $R^2$ of 0.98. These results illustrated the capability of our sensor to detect different concentrations of the HCV-RNA. The limit of detection (LOD) of the developed HCV-sensor based on Raman spectroscopy was calculated by using the following equation LOD = 3.3*standard deviation/slope of the calibration curve, and the LOD was found to be 264.5 IU/mL.

### 3.3. HCV-RNA sensing based on electrochemical techniques

Electrochemistry techniques have widely applied for detecting many important biomolecules markers such as DNA bases dopamine, glucose and beta-amyloid [28–32]. Here, we reported the application of cyclic voltammetry (CV) and SWV techniques as simple, easy and cost-effective methods for detecting different concentrations of the extracted HCV-RNA. The principle for developing the electrical HCV-RNA sensor is the same used principle for Raman measurements, in which the presence of Au NPs on the surface of the ITO substrate would enhance the electrochemical conductivity and also for the direct immobilization of the PNA-SH onto the substrate. Fig. 4a showed the CV of PNA-SH immobilized on the surface of Au nanostructures modified ITO substrate, which demonstrated an irreversible response with an anodic peak at about 0.5 V and no cathodic peaks were observed. Furthermore, the CV behavior of HCV-RNA hybridized with the PNA showed two new peaks in addition to the shift of the anodic peak of PNA. These new peaks would use for monitoring the different concentrations of the HCV-RNA. The SWV was used to monitor the different concentrations of HCV-RNA as shown in Fig. 4b, which indicated that the increase of the anodic current peak with increasing the concentration of HCV-RNA. Fig. 4c showed the relationship between the anodic current peak and the concentration of the HCV-RNA within a range from $1 \times 10^3$ IU/mL to $2 \times 10^3$ IU/mL, which illustrated that the value of the oxidation current peak shows a linear increasing ($R^2 = 0.998$) with the concentration of the HCV-RNA. The LOD of the fabricated HCV-sensor based on SWV was found

<table>
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<tr>
<th>Raman shift (cm$^{-1}$)</th>
<th>Assignments</th>
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<tbody>
<tr>
<td>668</td>
<td>Riboguanosine</td>
</tr>
<tr>
<td>828</td>
<td>O-P-O</td>
</tr>
<tr>
<td>1170</td>
<td>CO-O</td>
</tr>
<tr>
<td>1233</td>
<td>Uricil</td>
</tr>
<tr>
<td>1550</td>
<td>Riboadenosine</td>
</tr>
<tr>
<td>1635</td>
<td>Riboadenosine</td>
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Fig. 3. (a) Raman spectra of different concentrations of HCV’s RNA hybridized with PNA and (b) relationship between the Raman intensity at 1232 cm$^{-1}$ and the concentrations of HCV’s RNA within concentrations range from $5 \times 10^3$ IU/mL to $20 \times 10^3$ IU/mL.
to be about 101.5 IU/mL, which lies in the normal range for investigation the HCV infection and lower than the level of HCV in serum after therapy (more than 600 IU/mL) [33], thus this LOD is sufficient for discerning the infection in serum clinical samples. However, the sensor performance needs to improve for early HCV infection detection by using other modified electrodes.

3.4. Selectivity of the developed HCV-based sensor

To investigate the selectivity of the reported sensor against the HCV-RNA, the HBV- RNA was used instead of HCV- RNA and allowed it to hybridize with the PNA molecules by incubating the extracted RNAs on the PNA/Au NPs modified ITO substrate for about 6 h at 4 °C and finally washing the substrate with DIW and dried under nitrogen stream. Fig. 5a showed the Raman spectra of different concentrations of HBV-RNA after their interaction with the PNA, which showed very weak Raman peaks within the range from 200 to 600 cm$^{-1}$ only with a strong broad peak centered at about 1500 cm$^{-1}$ that characteristic for glass. In addition, there isn't any effect of the HBV-RNA concentration on the Raman intensities. These results confirmed that there is no interaction between the PNA peptide molecules and the HBV-RNA; and hence, confirmed the high selectivity of our sensor towards HCV-RNA. Furthermore, the selectivity of the developed electrode as HCV-RNA sensor was investigated as shown in Fig. 5b, which demonstrated the SWV of a high concentration of HBV-RNA at PNA/Au NPs modified ITO. Although high concentration of the HBV-RNA (15 × 10^3 IU/mL) has been used, only shoulder peak could be observed at 244 mV on a counter to strong oxidation peak at 184 mV in case of HCV-RNA. Thus these results demonstrated the capability of the developed probe for selectively sensing the HCV- RNA.

4. Conclusions

This work represented the developing of highly selective HCV-RNA sensing strategy based on Raman and electrochemical techniques. A PNA-SH with a specific sequence of nucleic bases was used as a probe for monitoring the HCV-RNA. The immobilization of PNA-SH molecules onto Au nanostructures modified ITO substrate and their hybridization with HCV-RNA was confirmed by using Raman spectroscopy and electrochemical techniques. Also, the selectivity and specificity of this biosensor were verified by employing HBV-RNA instated of HCV-RNA, which showed high selectivity towards HCV-RNA. Thus, we have prepared a new system that could be used for developing a selective HCV sensor.

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References


