



HAL
open science

Rapid and label-free electrochemical DNA biosensor for detecting hepatitis A virus

Marisa Manzano, Sara Viezzi, Sandra Mazerat, Robert S. Marks, Jasmina Vidic

► **To cite this version:**

Marisa Manzano, Sara Viezzi, Sandra Mazerat, Robert S. Marks, Jasmina Vidic. Rapid and label-free electrochemical DNA biosensor for detecting hepatitis A virus. *Biosensors and Bioelectronics*, 2018, 100, pp.89-95. 10.1016/j.bios.2017.08.043 . hal-02621615

HAL Id: hal-02621615

<https://hal.inrae.fr/hal-02621615v1>

Submitted on 18 Sep 2024

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



UNIVERSITÀ
DEGLI STUDI
DI UDINE

Università degli studi di Udine

Rapid and label-free electrochemical DNA biosensor for detecting hepatitis A virus

Original

Availability:

This version is available <http://hdl.handle.net/11390/1118001> since 2020-03-05T13:40:10Z

Publisher:

Published

DOI:10.1016/j.bios.2017.08.043

Terms of use:

The institutional repository of the University of Udine (<http://air.uniud.it>) is provided by ARIC services. The aim is to enable open access to all the world.

Publisher copyright

(Article begins on next page)

and Bioelectronics

Elsevier Editorial System(tm) for Biosensors

Manuscript Draft

Manuscript Number:

Title: Rapid and label-free electrochemical DNA biosensor for detecting Hepatitis A virus

Article Type: Full Length Article

Section/Category: The others

Keywords: Electrochemical biosensor; Hepatitis A virus detection; virus quantification.

Corresponding Author: Dr. jasmina vidic, PhD, HDR

Corresponding Author's Institution: INRA

First Author: Marisa Manzano

Order of Authors: Marisa Manzano; Sara Viezzi; Sandra Mazerat; Robert Marks; jasmina vidic, PhD, HDR

**Institut de la Recherche Agronomique
Unité Virologie et Immunologie
Domaine de Vilvert
Bât. Biotechnologies
78350 JOUY EN JOSAS**

Dr. Jasmina Vidic
Tel.: +33 (0)134652623
fax: +33 (0)134652621
e-mail: jasmina.vidic@inra.jouy.fr

Prof. Marisa Manzano
Tel.: +39 (0) 432558127
fax: +39 (0) 432558130
e-mail: marisa.manzano@uniud.it

**Editorial Office,
Talanta**

Jouy en Josas, Mai 16th, 2017

Subject: Submission of the original research paper: "RAPID AND LABEL-FREE ELECTROCHEMICAL DNA BIOSENSOR FOR DETECTING HEPATITIS A VIRUS "

Dear Editor,

We are happy to submit our manuscript entitled "Rapid and label-free electrochemical DNA biosensor for detecting hepatitis A virus" by Manzano et al. for publication reviewing in the Biosensor&Bioelectronics journal.

Hepatitis A virus (HAV) is a significant human pathogen which causes acute liver disease hepatitis A. The World Health Organization has estimated that about ten millions of new HAV infections occur globally every year giving about 1.5 million of HAV clinical cases. HAV is transmitted via fecal-oral route through a person-to-person contact or from ingestion of food or water that has been contaminated with the virus. It is generally difficult to absolutely confirm a food source of HAV transmission taking into account the complexity of international traceback of food and the difficulties to detect the viral RNA in food and water. We have developed a quantitative DNA based electrochemical sensor for label free detection of specific nucleic acid sequence characteristic for the HAV. For this disposable screen-printed electrode was functionalized with the selective thiolated-ssDNA probe. DNA hybridization on the electrode surface was detected by cyclic voltammetry directly measuring changes in the peak potential of the redox probe triggered by the recognition event. All steps of sensor elaboration were characterized by AFM and SPR. The selectivity of the sensor was demonstrated using a non-complementary sequence. The limit of detection (LOD) of this electrochemical DNA biosensor was determined to fall in the picomolar range.

We suggest the following scientists as referees of our manuscript:

Prof Chung-Ming Chang, Chang Gung University, Taiwan.
chungming.chang@gmail.com,

Dr Emanuela Torelli, New Castle University, UK.
emanuela.torelli@ncl.ac.uk

Dr Reza Saber, Tehran University of Medical Sciences, Iran
rsaber@sina.tums.ac.ir

Dr Richard Crooks, University of Texas at Austin, USA
crooks@cm.utexas.edu

Dr Ronnie Eriksson, Science Division, Biology Department, National Food Agency, Uppsala, Sweden.
Ronnie.Eriksson@slv.se

Thanking you in advance for your consideration.

With kind regards,
Jasmina Vidic & Marisa Manzano

Rapid and label-free electrochemical DNA biosensor for detecting Hepatitis A virus

Marisa Manzano^{1,2,*}, Sara Viezzi¹, Sandra Mazerat³, Robert S Marks^{2,4}, Jasmina Vidic^{2,5,*}

¹Dipartimento di Scienze Agroalimentari, Ambientali e Animali, University of Udine, Udine, Italy.

²Nanyang Technological University-Hebrew University of Jerusalem-Ben Gurion University (NEW-CREATE) Programme, Singapore.

³Paris Saclay University, Institut de Chimie Moléculaire et des Matériaux d'Orsay Chimie Inorganique, Orsay, France.

⁴Ben-Gurion University of the Negev, Beer-Sheva, Israel.

⁵Paris Saclay University, Virologie et Immunologie Moléculaires, INRA, Jouy en Josas, France.

Corresponding authors :

Marisa Manzano: marisa.manzano@uniud.it, Tel.: +39(0)432558127 ; fax: +39(0)432558130

Jasmina Vidic : jasmina.vidic@inra.fr , Tel: +33(0)134652627, fax: +33(0)134652621

Abstract

The hepatitis A virus (HAV) presents one of the most important foodborne pathogens causing a worldwide health problem each year. The control of HAV outbreaks remains difficult as conventional PCR-based methods fail to detect low levels of the virus in water and foods. We developed a highly sensitive and specific analytical method for detection of HAV. The device comprises a thiol-terminated DNA probe, complementary to the specific HAV sequence, embedded onto a gold electrode where the DNA hybridization is a sensing mechanism. The electrochemical measurements demonstrated that this device detected HAV DNA template over a wide concentration range from 10 fg/ μ L to 1 ng/ μ L with the calculated limit of detection of 0.398 fg/ μ L for the complementary ssDNA sequence and 3.2 fg/ μ L for viral cDNA obtained by PCR, respectively. The DNA-sensor developed can be potentially adopted as an easy-to-use and low cost method for screening HAV in contaminated food samples.

Keywords: Electrochemical biosensor; Hepatitis A virus detection; virus quantification.

1. Introduction

Hepatitis A virus (HAV) is a waterborne and foodborne human pathogen which causes acute liver disease hepatitis A. HAV presents a major health problem as causing considerable morbidity and economic loss (Deinhardt 1992; Gossner and Severi 2014; Hollinger and Emerson 2007; Nainan et al. 2006; Pettrignani et al. 2014) that were estimated based on epidemiological studies to be 500 times higher than those of cholera (Lenfant 1994). The World Health Organization has evaluated that about ten millions of new HAV infections occur globally every year giving about 1.5 million of HAV clinical cases (WorldHealthOrganization 2000). HAV is transmitted via fecal-oral route through a person-to-person contact or from ingestion of food or water that has been contaminated with the virus. HAV outbreaks have been associated with contaminated shellfish, raw oysters, mussels, clams, lettuce, green onions, frozen strawberry, blueberries and raspberries (Nainan et al. 2006).

HAV prevalence is tightly related to the economic development. The incidence of hepatitis A in developed countries has decreased due to improved access to safe drinking water and hygienic standards recommended for food preparation as well as due to the vaccination programs (Pérez-Sautu et al. 2011). However, even in developed countries outbreaks occur with foods contaminated during traveling, harvesting and processing or with foods contaminated by an infected food-service worker at the point of sale, as well as with foods imported from endemic areas. For instance, in 2003 a large foodborne outbreak of hepatitis A in Pennsylvania implicated green onions that were apparently contaminated with virus during packing into shipping boxes on farms in Mexico (Wheeler et al. 2005). Raw shellfish and frozen mixed berries were identified as potential vehicles of infection that caused an hepatitis A outbreak in Italy in 2013(Rizzo et al. 2013). HAV was reported to survive and remain infectious on biological surfaces for at least one month (McCaustland et

al. 1982). However, it is generally difficult to absolutely confirm a food source of HAV transmission taking into account the complexity of international food traceback and the difficulties to detect the viral RNA in contaminated samples. The detection of HAV in food is additionally challenging comparing to other viruses due to its long period of incubation (Sánchez et al. 2007) and the inefficient replication of isolates of HAV in cell culture (Cristina and Costa-Mattioli 2007). It was suggested that only one (Grabow 1997) or 10-100 hepatitis A virions (Venter et al. 2007; Yezli and Otter 2011) were sufficient to cause disease. Moreover, clinical manifestations of an acute hepatitis A infection cannot be distinguished from infections caused by other hepatitis virus strains (Martin and Lemon 2006). Therefore, both food-security programs and diagnosis of acute HAV infection need the specific and high sensitive virus detection. Such an efficient analytical method able to detect HAV in food and water will help us to understand the virus transmission routes and to improve security measures.

Currently various molecular techniques are applied for the detection of HAV due to their sensitivity and selectivity and because they can be directly applied on isolates from contaminated samples (Carroll et al. 2000; Oberste and Pallansch 2005; Sano et al. 2016; Vidic et al. 2017). Restriction fragment length polymorphism (Goswami et al. 1997), single strand conformation polymorphism (Fujiwara et al. 2000), real-time reverse transcription-PCR (RT-PCR) (Coudray-Meunier et al. 2015; Cromeans et al. 1997), multiple RT-PCR (Fuentes et al. 2014; Jothikumar et al. 2000), Nested-PCR (n-PCR) (Hu and Arsov 2009; Hu and Arsov 2014), nucleic acid hybridization (Zhou et al. 1991) and Southern blotting (Buti et al. 2001) are reported as efficient molecular methods for the detection of HAV. However, many difficulties are related to nucleic acid extraction from food matrices. Usually, food and water present low level of viral particles as HAV cannot replicate in food or environment. It makes necessary to concentrate virus particles prior to extraction of their nucleic acids. The minimal

amount needed to perform test was estimated between 10 and 25 g of contaminated fruit and vegetables in many of published or methods under development (Crocì et al. 2000). In addition, complex food matrices contain ions and molecules that may act as inhibitors for PCR-based methods, impeding their efficiency. Thus, still a robust, sensitive and specific method to quantify HAV in various food and environmental samples has to be developed.

Here we report on the quantitative DNA based electrochemical sensor for label free detection of specific nucleic acid sequence characteristic for HAV. DNA hybridization on the electrode surface was detected by cyclic voltammetry (CV) directly measuring changes in the peak potential (ΔE_p) of the redox indicator tripropylamine (TPA) triggered by the recognition event. The selectivity of the sensor was demonstrated using a non-complementary sequence. The limit of detection (LOD) of this electrochemical DNA biosensor was determined to fall in the picomolar range.

2. Experimental section

2.1. Material and reagents

Phosphate buffer saline (PBS) containing 150 mM NaCl, 8.1 mM Na₂HPO₄, 1.9 mM NaH₂PO₄, tripropylamine (TPA) and bovine serum albumin (BSA) were purchased from Sigma-Aldrich (Singapore). Single strand DNA probes (ssDNA) were purchased from IDT Integrated Technology (Singapore). The capture probe was modified at 5' end (5ThioMC6-D). For better recognition of the DNA probe, a spacer of adenines was inserted between the thiol-tag and the nucleic acid sequence to prevent steric hindrance. A ssDNA of 50 bp, complementary to the capture probe sequence was used as a positive control. cDNA sequences characteristic for HAV target were obtained by Reverse Transcription (RT) on pure viral RNA. All nucleic acids sequences used in this study are given in Tables 1 and 2.

2.2 PCR and nRT-PCR

Reverse Transcription from RNA of HAV was performed using the iScript™ cDNA Synthesis Kit (Biorad, Mi, Italy) according to the manufacturer instructions. The obtained cDNA was used in the amplification protocols. The DNA of the reference bacterial strains was extracted and purified from 1 mL of overnight broth culture using the Wizards Genomic DNA Purification Kit (Promega, Milan, Italy) as previously described (Manzano et al. 2003). DNA concentrations were evaluated by the spectrophotometer Nanodrop 2000c (Thermo Fisher Scientific, Wilmington, DE, U.S.A.). Before performing control measurements DNA concentrations in all samples were adjusted to the same concentration using ultrapure sterile distilled water.

A couple of primers of 21 bp (forward, HAV_{fw}) and 20 bp (reverse, HAV_{rv}) (Table 1) were designed for the first amplification step by conventional PCR in a final volume of 50 µL containing: 10 µL Colorless GoTaq® Reaction Buffer 5X (Promega, Padua, Italy), 1 µL dNTPs mix (10 mM)(Promega), 1 µL each Primer at 10 µM, 0.25 µL GoTaq® G2 DNA polymerase (5u/µL), 1 µL DNA template obtained from RT and nuclease free water. Thermal cycler RT BIORAD (Milan, Italy) conditions consisted of 95°C for 2 min, 95°C for 45 sec, 56°C for 45 sec, 72°C for 45 sec and final extension at 72°C for 7 min to test their specificity. Amplicons obtained by conventional PCR were electrophoretically resolved in a 2% agarose gel in 0.5 M TBE, 2 mM EDTA, 80 mM Tris-acetate buffer, pH 8.0, and visualized under UV in a GeneGenius BioImaging System (SynGene, UK).

A second amplification by Nested Reverse Transcription PCR (nRT-PCR) was performed in a Rotor-Gene Q (Venlo, Limburg, Netherlands) using the primers previously designed (Hu and Arsov 2009) (Table 1). The amplification was carried out in a total volume of 50 µL containing 25 µL GoTaq® qPCR Master Mix (2x), 1 µL of each primer from Table 1 at 10 µM, nuclease-free water 13 µL and 10 µL of the HAV amplicon from the first

amplification step and subjected to purification using the AppliChem (Darmstadt, Germany) kit. Amplification conditions comprised of 1 cycle for hot-start activation at 95°C for 2 min, followed by 40 cycles denaturation at 95°C for 15 sec, annealing/extension 60°C per 60 sec, followed by the melt curve at 60-95°C. The standard curve for absolute quantitation was obtained using decimal dilutions of HAV cDNA from 1.0 ng/μL to 0.1 fg/μL.

2.2. Probe and sample preparation and analysis by dot blot tests

Prior to the electrochemical biosensor elaboration the specificities of all designed probes reported in Table 1 were verified first by *in silico* analyses using the software Blast (Altschul et al. 1990) and the OligoAnalyzer 3.1 (<http://eu.idtdna.com/calc/analyzer>). Subsequently probes were tested by dot blot according to the protocol previously described (Cecchini et al. 2012). For this ssDNA and cDNA (double strand) obtained by amplification were diluted in PBS at the following concentrations: 1 fg /μL, 10 fg/μL, 50 fg /μL, 100 fg/μL, 500 fg/μL, 1 pg /μL, 10 pg/μL, 100 pg/μL. The capture and template DNA were heated in a thermal cycler at 95° C for 5 min and preserved on ice prior to test.

2.3. Electrode modification

All electrochemical measurements were performed using a 1470E potentiostat (Solartron). Disposable screen-printed gold electrodes DropSens 220 BT (Au–Au–Ag/AgCl; 4 mm-diameter working electrode, Metrohm, Singapore) were used for detecting DNA templates. Before utilization electrodes were cleaned upon 10 cyclic voltammetry (CV) cycles from –0.1 V to –0.9 V at a scan rate of 100 mV s⁻¹ in 50 μL of 0.5 M H₂SO₄ aqueous solution. Thereafter, electrodes were rinsed first with water, then ethanol, after which they were incubated with 20 μL of 50 ng/mL thiolated-DNA probe in PBS, pH 7.4 for 1 h at room temperature. After multiple rinses with deionized water, the resulting DNA-functionalized

electrode surfaces were blocked with 50 μL of 3 % bovine serum albumin in PBS, pH 7.4 for 15 min at room temperature. The electrode surface was then rinsed with PBS and used for detection. All the experiments were carried out at room temperature.

2.4. AFM

High resolution atomic force microscopy (AFM) was used to visualize the gold surface modifications upon functionalization by the DNA probe and hybridization. Before observation gold surface was cleaned by immersion in a piranha solution (1:1 v/v, $\text{H}_2\text{SO}_4:\text{H}_2\text{O}_2$) for 10 min in order to get rid of inorganic and organic contaminants on the substrate surface. Atomic force microscopy measurements were performed using di Innova AFM Bruker with Nanodrive v8.02 software. Tapping mode images were acquired using silicon tips from Nanosensors (PPP-NCSTR) with a resonance frequency ranging between 76 and 236 kHz. Images were processed using WsXM software, freely available on internet.

2.5. Electrochemical measurements.

The hybridization on the biosensor surface was enabled by depositing 20 μL of the DNA template diluted in PBS at various dilutions onto the functionalized gold surface at room temperature for 30 min. Electrodes were then extensively washed with PBS and incubated with 100 mM TPA in PBS to allow CV measurements. The DNA-target sequence in a concentration range from 10 $\text{fg}/\mu\text{L}$ to 1 $\text{ng}/\mu\text{L}$ was used to evaluate the analytical performance of the DNA sensor. Non-complementary sequence of *Listeria monocytogenes* was used to demonstrate the selectivity of the biosensor assay. After hybridization, electrodes were washed with PBS in order to remove non-hybridized DNA molecules. CV measurements were conducted between - 0.2 V and + 1.2 V (vs Ag/AgCl reference electrode)

at 100 mV/s in PBS containing 100 mM TPA as a redox indicator. Three independent electrodes per DNA concentration tested were used to evaluate the reproducibility of results. A schematic representation summarizing different steps of the detection is shown in Figure 1.

3. Results and Discussion

3.1. DNA probes selectivity

The selectivity of the designed DNA primers were tested before its deployment as a HAV sensing element. For this, the subsequently conventional PCR and nRT-PCR were carried out on 100 ng/ μ L of DNA of the reference strains listed in Table 2. The cDNA obtained from HAV samples gave the expected amplicon of about 500 bp in the first amplification step by PCR with forward_{Hu-Arson} and reverse_{Hu-Arson} primers. In contrast, all non-related cDNAs from Norovirus and bacteria tested failed to produce the expected amplicon. The second expected amplicon of about 200 bp was obtained by nRT-PCR protocol with HAV_{fw} and HAV_{rv} using the first amplicon as a template. Figure 2A shows the resulting calibration curve obtained by nRT-PCR using decimal dilution of cDNA from 1.0 ng/ μ L to 0.1 fg/ μ L. The lowest amount of HAV cDNA detected was 0.1 fg/ μ L, obtained as the intersection between an amplification curve and a threshold line after 28.73 cycles as reported by the Ct value (Figure 2A). The efficiency was of 108 % proving the good result obtained as an optimal reaction should have an efficiency between 90 and 110%,

Figure 2A shows the calibration curve obtained by nRT-PCR using decimal dilution of cDNA. The lowest amount of HAV cDNA detected was 0.1 fg/ μ L, obtained as the intersection between an amplification curve and a threshold line after 28.73 cycles as reported by the Ct value.

Additionally, the specificity of the designed capture DNA probe (Table 1) was assessed by dot blot using DNA extracts from 13 reference strains listed in Table 2 at concentration of 100 ng/ μ L. Negative results were obtained for all bacteria strains and Norovirus used in the test, while positive blue spots were visible for both HAV ssDNA strand complementary to the probe and the HAV cDNA obtained by PCR (data not shown). Both nRT-PCR and dot blot demonstrated that no hybridization was obtained with control strains and, thus, validated designed capture DNA probe as a HAV sensing element.

3.2. Preparation and characterization of DNA-electrode

To obtain a clean gold surface prior to DNA immobilization the disposable electrode was cleaned and checked by CV measuring in a sulfuric acid solution. The cyclic voltammogram showed the typical wave of gold in a sulfuric solution suggesting no impurities (Figure 2B). Subsequently, the HAV specific DNA probe was grafted to the electrode via thiol-gold chemistry. Cyclic voltammograms recorded in PBS containing a redox indicator TPA showed the characteristic oxidation peak at 0.68V vs. Ag/AgCl reference electrode (Figure 2B). The electrochemical oxidation and reduction of TPA was previously characterized (Miao et al. 2002). Figure 2C shows the AFM images of the gold surface modifications after immobilization of the thiolated-DNA probe and upon the hybridization of the complementary DNA template (ssDNA). Before DNA probe grafting the gold surface was relatively smooth, with a root-mean-squared (RMS) roughness relatively constant at an average of ~ 2.7 nm. After DNA immobilization the gold surface is occupied by a large quantity of DNA which has self-assembled in a random organizing giving RMS roughness increase to an average of ~ 40 nm. Finally, the hybridization with the complementary template (ssDNA) increased the density of immobilized molecules on the gold surface, and

additionally increase RMS roughness to ~60 nm.

The peak potential (E_p) of TPA oxidation was used to evaluate hybridization of the DNA on the modified electrode. The hybridization of 500 fg/ μ L of the complementary DNA template shifted the E_p of TPA reduction towards high potentials (Figure 2D, right panel). This could be related to the blocking effects of the large sized of the immobilized DNA for the charge transfer and diffusion of the TPA and its radicals between the solution and the Au electrode surface. When the electrode was incubated with the same concentration of non-complementary DNA no E_p shift was observed (Figure 2D, left panel) suggesting the specificity of detection.

3.3. Analytical performance of the sensor

First, hybridization experiments were performed with HAV specific ssDNA in a concentration range from 10 fg/ μ L to 0.1 ng/ μ L. The E_p corresponding to the TPA reduction shifted towards more positive potentials with increasing HAV ssDNA concentrations as shown in Figure 3A (left panel). This shift could be related to the changes of the sensor surface properties caused by DNA hybridization and also due to a decrease of the permeability of the sensing layer after the formation of the large complex to the surface. Indeed, hydrogen bonds formed upon hybridization represent potential barriers that slow down the diffusion of ions and the electron transfer from the electrode to the redox indicator TPA. Figure 3A (right panel) shows the calibration curve obtained from the quantification of ΔE_p for each ssDNA concentration, taken from at least three independent electrodes per concentration. The sensor reproducibility was estimated to 5 % according to the signal obtained after addition of the same concentration of ssDNA. ΔE_p plotted against the logarithm of target concentrations was a linear with a correlation coefficient (R^2) of 99 %. The

regression equation obtained of the plot was $y(x) = 0.044x + 0.01595$, where x was \log (target DNA concentration), $\text{fg}/\mu\text{L}$ and y was ΔE_p , V. The limit of detection (LOD) of $0.398 \text{ fg}/\mu\text{L}$ was calculated using $3\sigma/d$ formula, where σ is a standard deviation of the blank solution and d is a slope of the obtained calibration line. Taking into account the molecular weight of the probe of 155.51, the calculated LOD was 2.56 pM.

Second, hybridization experiments were performed with HAV specific cDNA (double strand) obtained by nRT-PCR of the HAV RNA. Prior to test, target cDNA was denatured for 5 min at 95°C in PBS to allow double strand opening. Figure 3B shows CV curves obtained with cDNA in a concentration range from $10 \text{ fg}/\mu\text{L}$ to $10 \text{ pg}/\mu\text{L}$ and the corresponding calibration curve. For comparison, the sensor was probed with the same range of concentrations of DNA from *Listeria monocytogenes* used as a negative control (Figure 3B). Insignificant variation of ΔE_p was obtained with *L. monocytogenes* DNA template confirming the high selectivity of constructed DNA sensor for detecting complementary target. The regression equation obtained for HAV cDNA target was $y(x) = 3.21149x + 8.043$ and R^2 was 91%. The estimated LOD for HAV cDNA detection was $3.2 \text{ fg}/\mu\text{L}$.

Our goal was to develop an analytical method for specific and highly sensitive detection of HAV. The fabricated DNA electrochemical sensor specifically detected HAV target sequence in a simple and swift way within a few minutes. Besides holding a great potential for cost and time reduction in HAV detection, the DNA electrochemical biosensor showed the sensitivity comparable to that of nRT-PCR assay. The viral load of HAV in contaminated food samples is usually quite low. For instance, 0.2-224 infection particles/100 g were found in contaminated selfish meat (Williams and Fout 1992) and 10^3 - 10^5 HAV particles/g in contaminated clams (Costafreda et al. 2006). Our novel electrochemical sensor that specifically detects DNA hybridization in a concentration-dependent manner can be adapted to portable format for routine endpoint analyzes for the presence of pathogenic

enteric viruses in food and water. Additionally, it shows advantages over molecular methods as PCR-based methods require at least hours to provide a quantitative result and may be inhibited by ions and molecules present in biological and environmental matrices. Finally, our novel electrochemical biosensor does not require sequencing or nucleic acid pre-amplification step. Thus, the developed analytical method seems to be more adapted for screening pathogens in food and water samples than conventional PCR assays.

4. Conclusions

We presented the functionality of user-friendly electrochemical biosensor for detection of specific HAV nucleic acid sequences. Hybridization event on the functionalized electrode was detected by CV measurements evaluating the variation in redox potential of the redox indicator TPA from the solution. Conventional and molecular methods for HAV diagnosis are not only costly but also do not provide an instant detection and cannot be used onsite. The sensor described here could be adapted for rapid (a few minutes) and sensitive detection (a few fg/ μ L) of viral nucleic acids in food and environmental. The presented electrochemical DNA-based biosensor is a promising analytical tool that can be adapted for water and food safety and quality control for the presence of HAV which represents an important medical and economic pathogen.

Acknowledgements

The University of Udine (Italy), the Paris Saclay University (France) and the NEW-CREATE (Singapore) are acknowledged for experimental facilities. The Campus France is gratefully acknowledged for travel support through the BioAsia grant 35976PH. We thank the OIE laboratory ISZVe (Padua, Italy) for providing samples of pathogens used and Dr Nicole Jaffrezic-Renault (CNRS, France) for gold electrode gift. We are also thankful to Dr Sachin

Kumar Srivastava (CSIO, India), Prima Dewi Sinawang and Dr Kamal Elouarzaki (NTU, Singapore) for their expert technical assistance.

Figure captures

Figure 1. Construction of disposable DNA-based biosensor was performed through thiol-gold coupling of the thiolated-ssDNA probe on a screen-printed gold electrode. Complementary target DNA sequences hybridized on the biosensor surface giving changes in CV peak potential (ΔE_p) of the redox indicator TPA in PBS.

Figure 2. Elaboration of the DNA-based sensor for HAV detection. (A) Calibration curve obtained for the nRT-PCR amplification with forwardHu-Arson and reverseHu-Arson primers using HAV cDNA from conventional PCR as a template at various dilutions. Baseline value of 7.62928, Correlation coefficient (R) of 0.99895, R² value of 0.9979, and efficiency of 108 % were obtained. Ct, threshold cycle values. (B) CV curves obtained for the clean gold electrode surface in sulphinic acid solution and for the gold electrode bearing DNA probes is solution containing 100 μ M TPA. (C) AFM images showing the bare Au-electrode and DNA-modified electrode by thiol-gold chemistry before and after hybridization with the complementary ssDNA target sequence. (D) Potential of the TPA cathodic peak shifted only when complementary DNA template was deposited onto the sensor surface. DNA complementary template and non-complementary sequences were at 500 fg/ μ L.

Figure 3. CV curves obtained for detection of ssDNA (A) and cDNA (B) templates using DNA electrochemical sensor. Measurements were performed in PBS containing 100 mM TPA as a redox indicator. Corresponding calibration curves were obtained by plotting the shift of TPA peak potential in a function of DNA template concentrations.

Table 1: Sequences of the primers used in conventional PCR (HAV_{fw}-HAV_{rv}), and in nRT-PCR (forward_{Hu-Arson} and reverse_{Hu-Arsov}) methods and DNA probes used for sensor surface functionalization and characterization of its specificity and sensitivity.

PCR and nRT-PCR sequences

HAV _{fw}	5' - ACTTGATACCTCACCGCCGTT - 3'
HAV _{rv}	5' - AGTCCTCCGGCGTTGAATGG - 3'
forward _{Hu-Arson}	5' - CGG GGT CAACTC CAT GAT TA - 3'
reverse _{Hu-Arsov}	5' - CGC CGC TGT TAC CCT ATC C - 3'

Sequences of DNA probes used for hybridization purposes

Thiolated-DNA probe	5' - ThiC6-AAAAATCTTAACAACCTCACCAATATCCGC-3'
Positive control (ssDNA)	5'- GGTAACAGCGGCGGATATTGGTGAGTTGTTAAGACAAAAACCATT CAACG-3'

Table 2: List of viral cDNA and bacterial DNA used as references.

Virus	Bacteria
HAV cDNA ^a	<i>Bacillus subtilis</i> DSM 1029 ^f
Norovirus GI cDNA ^b	<i>Campylobacter jejuni</i> subsp. <i>jejuni</i> ATCC 49943 ^d
Norovirus GII cDNA ^c	<i>Escherichia coli</i> DISTAM ^f
	<i>Listeria monocytogenes</i> ATCC 7644 ^d
	<i>Pseudomonas fluorescens</i> DISTAM ^f
	<i>Salmonella enteritidis</i> DSM 4883 ^f
	<i>Vibrio</i> spp. DSM 14379 ^f
	<i>Aeromonas sobria</i> DSM 19176 ^f
	<i>Proteus vulgaris</i> DISTAM ^e
	<i>Staphylococcus aureus</i> DISTAM ^b

^a provided from the OIE reference laboratory ISZVe (Padua, Italy)

^b obtained by RT from Synthetic Norovirus G1 (I) RNA ATCC VR-3234SD

^c obtained by RT from Synthetic Norovirus G2 (II) RNA (ATCC VR-3200SD)

^d American Type Culture Collection (Manassas, VA, USA)

^e Deutsche Sammlung von Mikroorganism und Zellkulturen GmbH (Braunschweig, Germany).

^f Colección Española de Cultivos Tipo (University of Valencia, Spain)

References

- Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J., 1990. Basic local alignment search tool. *Journal of molecular biology* 215(3), 403-410.
- Buti, M., Jardi, R., Bosch, A., Rodriguez, F., Sanchez, G., Pinto, R., Costa, X., Sanchez-Avila, J., Cotrina, M., Esteban, R., 2001. Assessment of the PCR-Southern blot technique for the analysis of viremia in patients with acute hepatitis A. *Gastroenterologia y hepatologia* 24(1), 1-4.
- Carroll, K.C., Taggart, B., Robison, J., Byington, C., Hillyard, D., 2000. Evaluation of the Roche AMPLICOR enterovirus PCR assay in the diagnosis of enteroviral central nervous system infections. *Journal of clinical virology* 19(3), 149-156.
- Cecchini, F., Manzano, M., Mandabi, Y., Perelman, E., Marks, R.S., 2012. Chemiluminescent DNA optical fibre sensor for *Brettanomyces bruxellensis* detection. *Journal of biotechnology* 157(1), 25-30.
- Costafreda, M.I., Bosch, A., Pintó, R.M., 2006. Development, evaluation, and standardization of a real-time TaqMan reverse transcription-PCR assay for quantification of hepatitis A virus in clinical and shellfish samples. *Applied and Environmental Microbiology* 72(6), 3846-3855.
- Coudray-Meunier, C., Fraise, A., Martin-Latil, S., Guillier, L., Delannoy, S., Fach, P., Perelle, S., 2015. A comparative study of digital RT-PCR and RT-qPCR for quantification of Hepatitis A virus and Norovirus in lettuce and water samples. *International journal of food microbiology* 201, 17-26.
- Cristina, J., Costa-Mattioli, M., 2007. Genetic variability and molecular evolution of hepatitis A virus. *Virus research* 127(2), 151-157.
- Croci, L., De Medici, D., Scalfaro, C., Fiore, A., Divizia, M., Donia, D., Cosentino, A., Moretti, P., Costantini, G., 2000. Determination of enteroviruses, hepatitis A virus, bacteriophages and *Escherichia coli* in Adriatic Sea mussels. *Journal of Applied Microbiology* 88(2), 293-298.
- Cromeans, T.L., Nainan, O.V., Margolis, H.S., 1997. Detection of hepatitis A virus RNA in oyster meat. *Applied and environmental microbiology* 63(6), 2460-2463.
- Deinhardt, F., 1992. Virus infections: strategies of prevention. *Behring Inst Mitt*(91), 28-37.
- Fuentes, C., Guix, S., Pérez-Rodríguez, F.J., Fuster, N., Carol, M., Pintó, R.M., Bosch, A., 2014. Standardized multiplex one-step qRT-PCR for hepatitis A virus, norovirus GI and GII quantification in bivalve mollusks and water. *Food microbiology* 40, 55-63.
- Fujiwara, K., Yokosuka, O., Ehata, T., Imazeki, F., Saisho, H., 2000. PCR-SSCP Analysis of 5'-Nontranslated Region of Hepatitis A Viral RNA. *Digestive diseases and sciences* 45(12), 2422-2427.
- Gossner, C., Severi, E., 2014. Three simultaneous, food-borne, multi-country outbreaks of hepatitis A virus infection reported in EPIS-FWD in 2013: what does it mean for the European Union. *Euro Surveill* 19, 43.
- Goswami, B.B., Burkhardt, W., Cebula, T.A., 1997. Identification of genetic variants of hepatitis A virus. *Journal of virological methods* 65(1), 95-103.
- Grabow, W., 1997. Hepatitis viruses in water: Update on risk and control. *Water SA* 23(4), 379-386.
- Hollinger, F., Emerson, S., 2007. Hepatitis A virus, p 911–947. *Fields virology*. Lippincott Williams and Wilkins, Philadelphia, PA.
- <http://eu.idtdna.com/calc/analyzer>.
- Hu, Y., Arsov, I., 2009. Nested real-time PCR for hepatitis A detection. *Letters in applied microbiology* 49(5), 615-619.
- Hu, Y., Arsov, I., 2014. A rapid single-tube protocol for HAV detection by nested real-time PCR. *Food and environmental virology* 6(3), 189-195.
- Jothikumar, N., Paulmurugan, R., Padmanabhan, P., Sundari, R.B., Kamatchiammal, S., Rao, K.S., 2000. Duplex RT-PCR for simultaneous detection of hepatitis A and hepatitis E virus isolated from drinking water samples. *Journal of Environmental Monitoring* 2(6), 587-590.
- Lenfant, C., 1994. From the National Institutes of Health. *JAMA* 271(10), 738.
- Manzano, M., Giusto, C., Iacumin, L., Cantoni, C., Comi, G., 2003. A molecular method to detect *Bacillus cereus* from a coffee concentrate sample used in industrial preparations. *Journal of applied microbiology* 95(6), 1361-1366.

Martin, A., Lemon, S.M., 2006. Hepatitis A virus: from discovery to vaccines. *Hepatology* 43(S1).

McCaustland, K., Bond, W., Bradley, D., Ebert, J., Maynard, J., 1982. Survival of hepatitis A virus in feces after drying and storage for 1 month. *Journal of clinical microbiology* 16(5), 957-958.

Miao, W., Choi, J.P., Bard, A.J., 2002. Electrogenenerated chemiluminescence 69: the tris(2,2'-bipyridine)ruthenium(II), (Ru(bpy)₃(2+))/tri-n-propylamine (TPrA) system revisited—a new route involving TPrA*+ cation radicals. *Journal of the American Chemical Society* 124(48), 14478-14485.

Nainan, O.V., Xia, G., Vaughan, G., Margolis, H.S., 2006. Diagnosis of hepatitis a virus infection: a molecular approach. *Clin Microbiol Rev* 19(1), 63-79.

Oberste, M.S., Pallansch, M.A., 2005. Enterovirus molecular detection and typing. *Reviews in Medical Microbiology* 16(4), 163-171.

Pérez-Sautu, U., Costafreda, M.I., Lite, J., Sala, R., Barrabeig, I., Bosch, A., Pintó, R.M., 2011. Molecular epidemiology of hepatitis A virus infections in Catalonia, Spain, 2005–2009: circulation of newly emerging strains. *Journal of Clinical Virology* 52(2), 98-102.

Petrignani, M., Verhoef, L., Vennema, H., van Hunen, R., Baas, D., van Steenberghe, J.E., Koopmans, M.P., 2014. Underdiagnosis of foodborne hepatitis A, The Netherlands, 2008–2010. *Emerging infectious diseases* 20(4), 596.

Rizzo, C., Alfonsi, V., Bruni, R., Busani, L., Ciccaglione, A., De Medici, D., Di Pasquale, S., Equestre, M., Escher, M., Montano-Remacha, M., 2013. Ongoing outbreak of hepatitis A in Italy: preliminary report as of 31 May 2013. *Euro Surveill* 18(27), 20518.

Sánchez, G., Bosch, A., Pintó, R., 2007. Hepatitis A virus detection in food: current and future prospects. *Letters in applied microbiology* 45(1), 1-5.

Sano, D., Amarasiri, M., Hata, A., Watanabe, T., Katayama, H., 2016. Risk management of viral infectious diseases in wastewater reclamation and reuse: Review. *Environment international* 91, 220-229.

Venter, J., Van Heerden, J., Vivier, J., Grabow, W., Taylor, M., 2007. Hepatitis A virus in surface water in South Africa: what are the risks? *Journal of water and health* 5(2), 229-240.

Vidic, J., Manzano, M., Chang, C.-M., Jaffrezic-Renault, N., 2017. Advanced biosensors for detection of pathogens related to livestock and poultry. *Veterinary Research* 48(1), 11.

Wheeler, C., Vogt, T.M., Armstrong, G.L., Vaughan, G., Weltman, A., Nainan, O.V., Dato, V., Xia, G., Waller, K., Amon, J., 2005. An outbreak of hepatitis A associated with green onions. *New England Journal of Medicine* 353(9), 890-897.

Williams, J., Fred, Fout, G., 1992. Contamination of shellfish by stool-shed viruses: methods of detection. *Environmental science & technology* 26(4), 689-696.

WorldHealthOrganization, 2000. Hepatitis A vaccines 75, 38-44.

Yezli, S., Otter, J.A., 2011. Minimum infective dose of the major human respiratory and enteric viruses transmitted through food and the environment. *Food and Environmental Virology* 3(1), 1-30.

Zhou, Y.-J., Estes, M.K., Jiang, X., Metcalf, T.G., 1991. Concentration and detection of hepatitis A virus and rotavirus from shellfish by hybridization tests. *Applied and environmental microbiology* 57(10), 2963-2968.

Figure1

[Click here to download high resolution image](#)

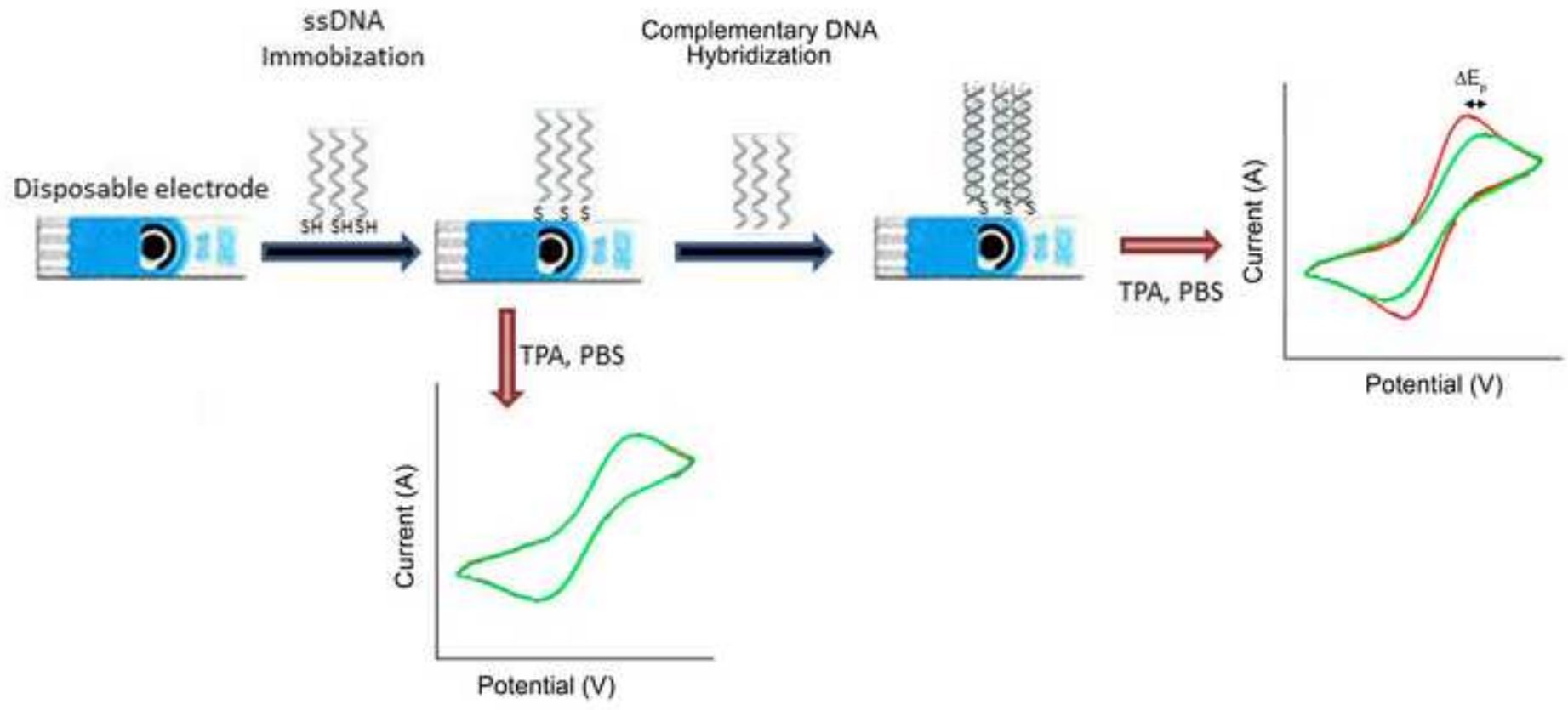


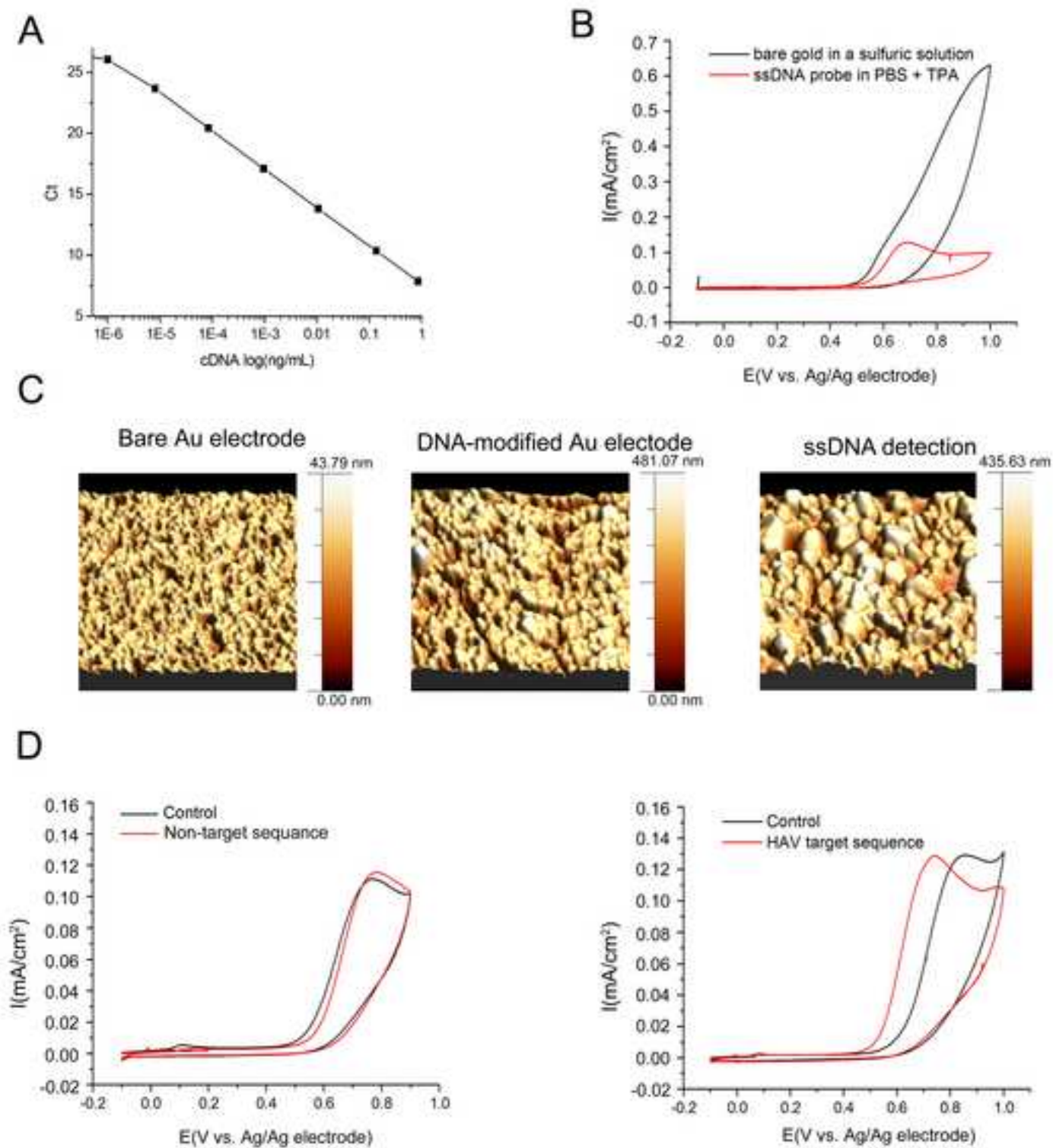
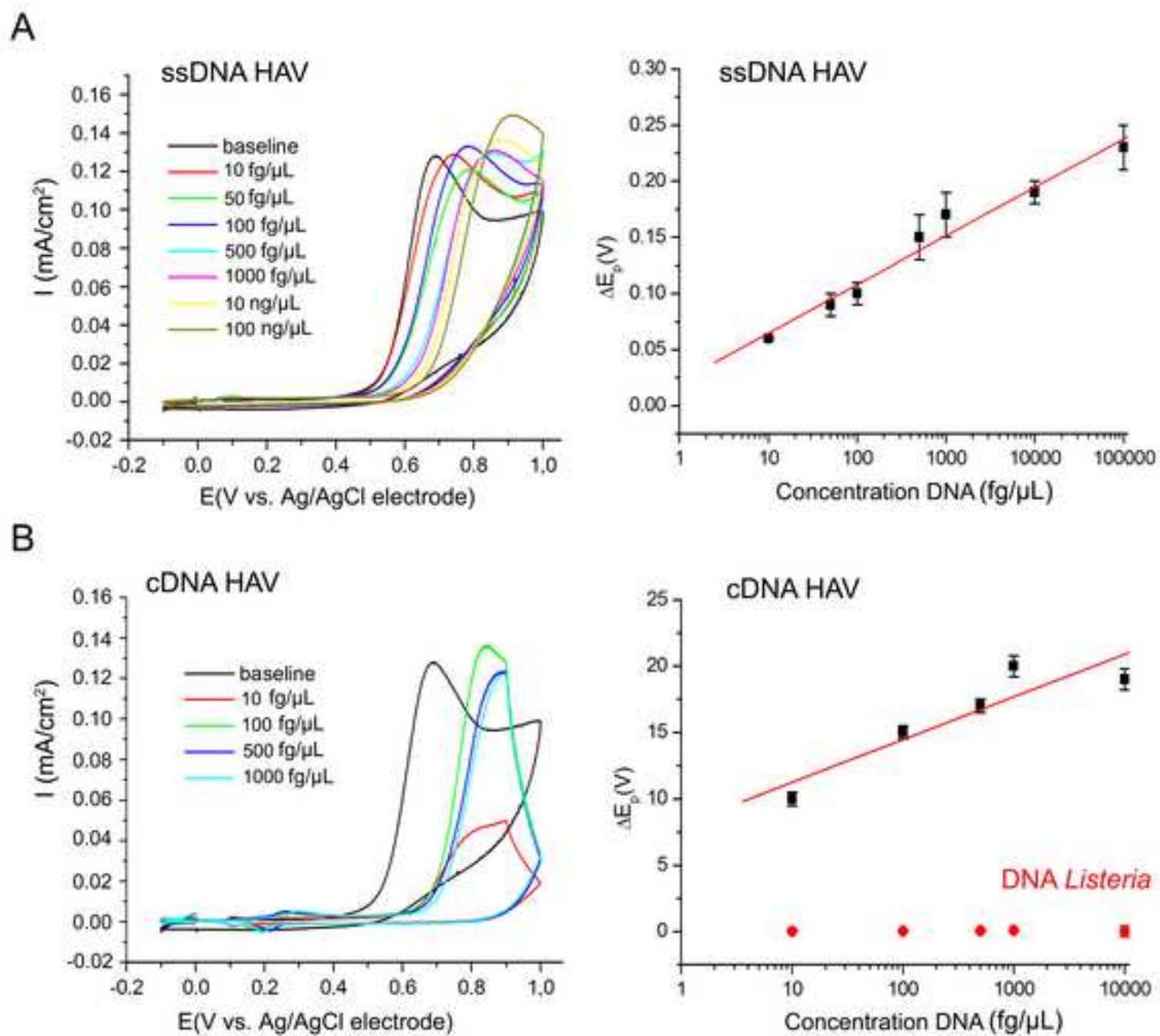
Figure2[Click here to download high resolution image](#)

Figure3

[Click here to download high resolution image](#)



Highlights

- An electrochemical method for quantitative detection of hepatitis A virus.
- Specific DNA probe designed and optimized for sensitive detection of hepatitis A virus
- Optimized modifications of disposable electrode for a stable and reproducible virus detection