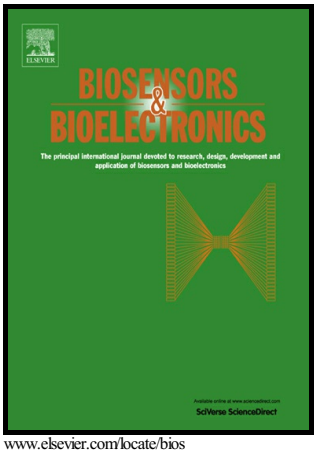
Rapid and label-free electrochemical DNA biosensor for detecting Hepatitis A virus

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



www.elsevier.com/locate/bios

PII: S0956-5663(17)30578-X
DOI: http://dx.doi.org/10.1016/j.bios.2017.08.043
Reference: BIOS9953

To appear in: *Biosensors and Bioelectronic*

Received date: 12 June 2017
Revised date: 16 August 2017
Accepted date: 18 August 2017

Cite this article as: Marisa Manzano, Sara Viezzi, Sandra Mazerat, Robert S. Marks and Jasmina Vidic, Rapid and label-free electrochemical DNA biosensor for detecting Hepatitis A virus, *Biosensors and Bioelectronic,* http://dx.doi.org/10.1016/j.bios.2017.08.043

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

Marisa Manzano[1,2,*], Sara Viezzi[1], Sandra Mazerat[3], Robert S Marks[2,4], Jasmina Vidic[2,5,*]

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

[2]Nanyang Technological University-Hebrew University of Jerusalem-Ben Gurion University (NEW-CREATE), Programme, 1 CREATE Way, Research Wing, #02-06/08, Singapore 138602, Singapore

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

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

[5]Paris 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

Diagnostic systems that can deliver highly specific and sensitive detection of hepatitis A virus (HAV) in food and water are of particular interest in many fields including food safety, biosecurity and control of outbreaks. Our aim was the development of an electrochemical method based on DNA hybridization to detect HAV. A ssDNA probe specific for HAV (capture probe) was designed and tested on DNAs from various viral and bacterial samples using Nested-Reverse Transcription Polymerase Chain Reaction (nRT-PCR). To develop the electrochemical device, a disposable gold electrode was functionalized with the specific capture probe and tested on complementary ssDNA and on HAV cDNA. The DNA hybridization on the electrode was measured through the monitoring of the oxidative peak potential of the indicator tripropylamine by cyclic voltammetry. To prevent non-specific binding the gold surface was treated with 3% BSA before detection. High resolution atomic force microscopy (AFM) confirmed the efficiency of electrode functionalization and on-electrode hybridization. The proposed device showed a limit of detection of 0.65 pM for the complementary ssDNA and 6.94 fg/µL for viral cDNA. For a comparison, nRT-PCR quantified the target HAV cDNA with a limit of detection of 6.4 fg/µL. The DNA-sensor developed can be adapted to a portable format to be adopted as an easy-to- use and low cost method for screening HAV in contaminated food and water. In addition, it can be useful for rapid control of HAV infections as it takes only a few minutes to provide the results.

Keywords: DNA-electrochemical biosensor; AFM; Hepatitis A virus detection; Tripropylamine oxidation; On-electrode hybridization.

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; Petrignani et al., 2014). The World Health Organization has evaluated that about 10 million of new HAV infections occur globally every year giving about 1.5 million of HAV clinical cases (WorldHealthOrganization, 2000).

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 imported from endemic areas. For instance, 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 trackback 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 contamined 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 (Croci 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.

Electrochemical biosensors for virus gene sequence detection are relatively new, but of rapidly growing interest due to their high sensitivity, high portability and rapidity to provide response. Various electrochemical biosensors have been developed for detection of enteric virus based on specific DNA sequences detection (Bouchet et al., 2007; Chen et al.,

2016; Galán et al., 2015; Hassen et al., 2008; Li et al., 2016; Liu et al., 2016; Zheng et al., 2014). The hybridization at the sensor surface results in a detectable change transduced into a quantitative amperometric, potentiomentric or impedimetric signal. The small dimension and simplicity of fabrication of electrochemical DNA sensor together with their good tolerance to the inhibitory components from environmental and clinical samples make them particularly attractive for development of inexpensive point-of-care platform for DNA analysis.

Here we report for the first time the development of an electrochemical DNA biosensor 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 (ΔEp) of oxidation of the 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 for complementary target sequences related to HAV, which is comparable with expensive quantitative PCR-based method that use extra labeling.

2. Experimental section

2.1. Material and reagents

Phosphate buffer saline (PBS) containing 150 mM NaCl, 8.1 mM Na_2HPO_4, 1.9 mM NaH_2PO_4, 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 andenines 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. The target ssDNA used as a template was 50 bp, and its molecular weight was 15,531.1 as calculated by the synthetizer.

2.2 PCR and nRT-PCR

Reverse Transcription from RNA of HAV was performed using the iScript™ cDNA Synthesis Kit (Biorad, Milan, 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 kit (Darmstadt, Germany). 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 1.2 V at a scan rate of 100 mV s^{-1} in 50 µL of 0.5 M H_2SO_4 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 than 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 after hybridization. Before observation gold surface was cleaned by it immersion in a piranha solution (1:1 v/v, H_2SO_4:H_2O_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 ips 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 60 min. Electrodes were then extensively washed with PBS and incubated with 100 mM TPA in PBS for 5 min to allow CV measurements. The DNA-target sequence in a concentration range from10 fg/µL to 1 ng/µL was used to evaluate the analytical performance of the DNA sensor. Non-complementary sequence of *Listeria*

monocytogenes (Table 2) 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 an 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

3.1. DNA probes selectivity

The selectivity of the designed DNA primers was tested before its deployment as a HAV sensing element. For this, the subsequently conventional PCR and nRT-PCR were carried out on 100 ng/µL 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 $forward_{Hu\text{-}Arson}$ and $reverse_{Hu\text{-}Arson}$ 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/µL to 0.1 fg/µL. The lowest amount of HAV cDNA detected was 1 fg/µL (ie., 6.4 pM, taking into account the molecular weight of the target cDNA of 155.51), 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 %.

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/µL. 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 the indicator TPA showed the characteristic oxidation peak at 0.68V vs. Ag/AgCl reference electrode (Figure 2B). The electrochemical behavior of TPA was previously characterized (Miao et al., 2002).

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/µL of the complementary DNA template shifted the E_p of TPA oxidation towards high potentials (Figure 2C, 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 2C, left panel) suggesting the specificity of detection.

Figure 3 shows the AFM images of the gold surface topologies 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 (Figure 3A). After DNA immobilization the gold surface is occupied by a large quantity of DNA which has self-assembled in a random organizing giving a RMS roughness increase to an average of ~ 40 nm (Figure 3B). Markedly, upon the addition of the thiolated-DNA to the surface, the surface became rougher and morphological heterogeneity increased. For comparison, when non-thiolated DNA probe was deposited onto the gold, the surface showed a high density of small features suggesting the physisorption of DNA onto the gold (Figure 3C). The height of features observed with non-thiolated DNA was inferior to those found with thiolated-DNA, with a RMS of around 35 nm. A maximum thickness observed was around 150 nm and 300 nm, for non-thiolated- and thiolated-DNA, respectively. Two morphologies observed suggested that when thiol-gold covenant bonds were formed the thiolated-DNA molecules rested rather vertically positioned over the surface. It seems that at given experimental conditions, the formation of covalent bonds occupied the surface and, thus, prevented nonspecific adsorption of DNA. Vertical orientation observed upon the covalent bond formation between gold and thiol at the 5' end of DNA allows higher density of DNA immobilization, which is expected as thermodynamically favorable. These features of vertically attached DNA molecules became larger in area upon addition of the complementary strand of DNA. Figure 3D shows that an increase in the density of immobilized molecules on the gold surface was observed upon the complementary ssDNA template hybridization giving an additional increase in RMS roughness to ~ 60 nm. Overall, AFM images verified that the

surface was becoming more complex at each stage of the modification process which is consistent with electrochemical finding in Figure 2. To avoid physisorption of DNA onto the gold surface all electrochemical measurements were done after surface saturation with 3 % BSA.

3.3. Analytical performance of the sensor

First, hybridization experiments were performed with HAV specific ssDNA in a concentration range from 10 fg/µL to 0.1 ng/µL. The E_p corresponding to the TPA oxidation shifted towards more positive potentials with increasing HAV ssDNA concentrations as shown in Figure 4A (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 and the electron transfer from TPA to the electrode. Figure 4A (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), fg/µL and y was $\Delta E_{p,}$ V.

The limit of detection (LOD) of 0.15 fg/µL was calculated using 3s/m formula, where ‘s’ is a standard deviation of the blank solution and ‘m’ is a slope of the linear calibration

graph of low concentration of the analyte. Taking into account the molecular weight of the ssDNA used as a target of 155.51, the calculated LOD was 0.65 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 strain opening. Figure 4B shows CV curves obtained with cDNA in a concentration range from 10 fg/µL to 10 pg/µL 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 4B). 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 95 %. The estimated LOD for HAV cDNA detection was 1.08 fg/µL, i.e. 6.94 pM (3s/m).

4. Discussion

Our goal was to develop an analytical method for specific and highly sensitive detection of HAV that can reduce the time of the analysis. 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.

Nowadays, mostly viral presence is tested only in shellfish, but vegetables such as salads and fruits can be responsible for viral hepatitis and gastroenteritis as well (Butot et al., 2007). The viral load of HAV in contaminated food samples can be quite low as reported by Williams and Fout (Williams and Fout, 1992) that found 0.2-224 infection particles/100 g selfish meat. Few information in official guidelines about food sampling methods for viral

detection are given, thus usually microbiological guidelines for the detection of bacteria are followed when food are screened for HAV presence. Right now there are no validated methods for detecting enteric virus, and the methods currently used are too expensive and time-consuming for the routine screening of food (Lopman et al., 2002). Croci et al., (Croci et al., 2000) reported the elution methods to collect viral particles from a fruit surface followed by centrifugation to concentration viral particles prior to nucleic acid extraction. These steps concentrate the particles in a reduced volume which may simplify the pretreatment of the sample to allow detection. Recently, analytical methods allowing increasing sensitivity for DNA detection are reported. For instance, use of nanoparticles enables increasing the dynamic range of detection. A quantitative electrochemical sensor with Ag nanoparticles was shown to detect DNA at concentrations as low as pM without any enzymatic amplification (Scida et al., 2014). However, the experimental protocol requires a delicate step of oxidation/precipitation of the Ag nanoparticles before detection. Isotachophoresis used in electrokinetic microarray to preconcentrate DNA target molecules amplified the hybridization rate and increased over 4 orders of magnitude dynamic range of DNA detection (Han et al., 2014). Microfluidic devices offer advantages compared to macroscopic equivalents in terms of rapid heat transfer and small volumes that reduce sample and reagent consumption, leading to inexpensive operation of the systems. The detection sensitivity in microfluidic channel may be adapted for detection of low-abundance biomolecules by electrokinetic concentration based on ion concentration polarization (Wei et al., 2016). Such device detected DNA target at concentration as low as 10 pM within 15 min.

The sensor developed here is based on direct DNA hybridization and thus avoids the problem connected to the inhibition due to the presence of compounds that can affect DNA polymerase activity. In fact, the presence of low amount of ions and chemicals from nucleic acid extraction seems not to prevent the DNA hybridization (Torelli et al., 2017). Moreover,

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 that require at least hours to provide a quantitative result. Finally, our novel electrochemical biosensor does not require sequencing or nucleic acid pre-amplification step, and can be used on DNA from different extraction methods as not sensitive to ions effect. 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 potential of the oxidation of 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/µL) 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 and Galilée grant 37184VG. 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 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 forward$_{Hu\text{-}Arson}$ and reverse$_{Hu\text{-}Arson}$ primers using HAV cDNA from conventional PCR as a template at various dilutions. Baseline value of 7.62928, Correlation coefficient (R) of 0.99895, R^2 value of 0.9979 and efficiency of 108 % were obtained. (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 µM TPA. (C) 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/µL.

Figure 3. AFM images taken in tapping mode of (A) gold surface, (B) gold surface functionalized with thiolated-single-stranded DNA probe, (C) gold surface with adsorbed non-thiolated-DNA probe, and (D) DNA-modified electrode by thiol-gold chemistry after hybridization with the complementary ssDNA target sequence. Scale bar, 600 nm.

Figure 4. 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 an indicator. Corresponding calibration curves were obtained by plotting the shift of TPA peak potential in a function of DNA template concentrations. Data points are the mean values obtained in 3 independent experiments ± SD.

Table 1: Sequences of the primers used in conventional PCR (HAV_{fw}-HAV_{rv}), and in nRT-PCR ($forward_{Hu\text{-}Arson}$ and $reverse_{Hu\text{-}Arsov}$) 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'
$forward_{Hu\text{-}Arson}$	5' - CGG GGT CAACTC CAT GAT TA - 3'
$reverse_{Hu\text{-}Arsov}$	5' - CGC CGC TGT TAC CCT ATC C - 3'

Sequences of DNA probes used for hybridization purposes

Thiolated-DNA probe	5'- ThiC6-AAAAATCTTAACAACTCACCAATATCCGC-3'
Positive control (ssDNA)	5'-GGTAACAGCGGCGGATATTGGTGAGTTGTTAAGACAAAAACCATTCAACG-3'

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

Virus	Bacteria
HAV cDNA[a]	*Bacillus subtilis* DSM 1029[f]
Norovirus GI cDNA[b]	*Campylobacter jejuni* subsp. *jejuni* ATCC 49943[d]
Norovirus GII cDNA[c]	*Escherichia coli* DISTAM[f]
	Listeria monocytogenes ATCC 7644[d]
	Pseudomonas fluorescens DISTAM[f]
	Salmonella enteritidis DSM 4883[f]
	Vibrio spp. DSM 14379[f]
	Aeromonas sobria DSM 19176[f]
	Proteus vulgaris DISTAM[e]
	Staphylococcus aureus 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 (Braunschweigh, Germany).
[f] Colección Española de Cultivos Tipo (University of Valencia, Spain)

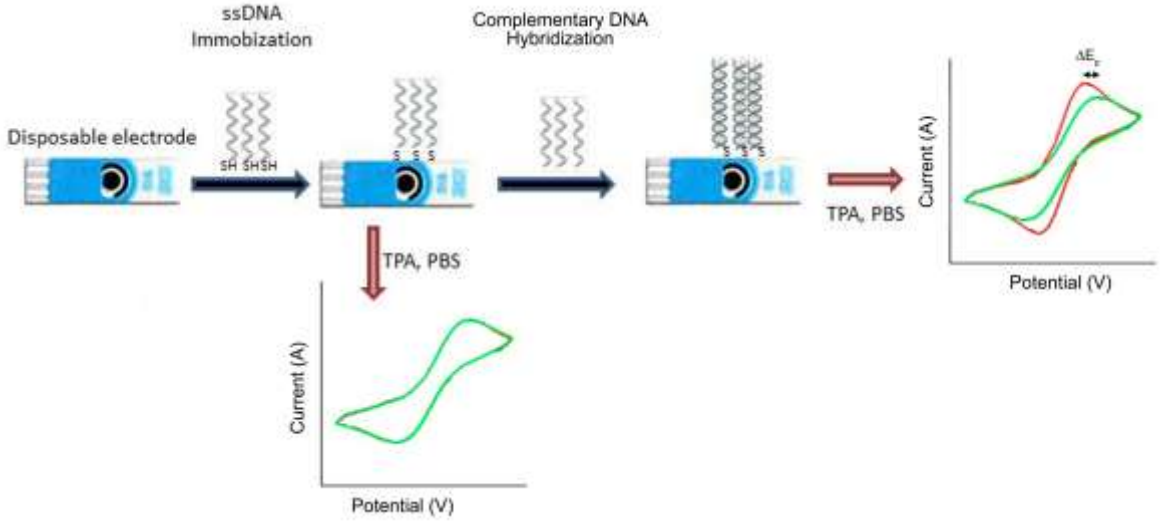
References

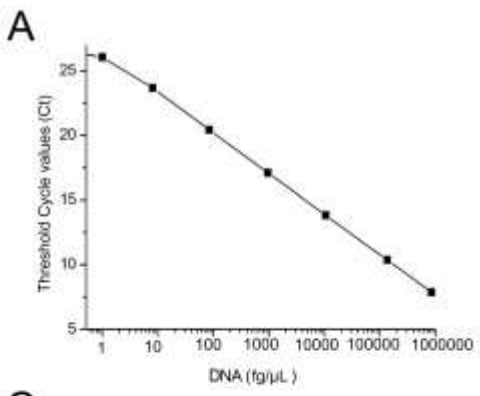
Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J., 1990. J. Mol. Biol. 215(3), 403-410.
Bouchet, A., Chaix, C., Marquette, C.A., Blum, L.J., Mandrand, B., 2007. Biosens. Bioelectron. 23(5), 735-740.
Buti, M., Jardi, R., Bosch, A., Rodriguez, F., Sanchez, G., Pinto, R., Costa, X., Sanchez-Avila, J., Cotrina, M., Esteban, R., 2001. Gastroenterologia y hepatologia 24(1), 1-4.
Butot, S., Putallaz, T., Sanchez, G., 2007. Appl. Environ. Microbiol. 73(1), 186-192.
Carroll, K.C., Taggart, B., Robison, J., Byington, C., Hillyard, D., 2000. J. Clin. Virol. 19(3), 149-156.
Cecchini, F., Manzano, M., Mandabi, Y., Perelman, E., Marks, R.S., 2012. J. Biotechnol. 157(1), 25-30.
Chen, C.-C., Lai, Z.-L., Wang, G.-J., Wu, C.-Y., 2016. Biosens. Bioelectron. 77, 603-608.
Coudray-Meunier, C., Fraisse, A., Martin-Latil, S., Guillier, L., Delannoy, S., Fach, P., Perelle, S., 2015. Inter. J. Food Microbiol. 201, 17-26.
Cristina, J., Costa-Mattioli, M., 2007. Virus Res. 127(2), 151-157.
Croci, L., De Medici, D., Scalfaro, C., Fiore, A., Divizia, M., Donia, D., Cosentino, A., Moretti, P., Costantini, G., 2000. J. Appl. Microbiol. 88(2), 293-298.
Cromeans, T.L., Nainan, O.V., Margolis, H.S., 1997. App. Environ. Microbiol. 63(6), 2460-2463.
Deinhardt, F., 1992. Behring Inst. Mitt (91), 28-37.
Fuentes, C., Guix, S., Pérez-Rodriguez, F.J., Fuster, N., Carol, M., Pintó, R.M., Bosch, A., 2014. Food Microbiol. 40, 55-63.
Fujiwara, K., Yokosuka, O., Ehata, T., Imazeki, F., Saisho, H., 2000. Digest. Diseases Sci. 45(12), 2422-2427.
Galán, T., Prieto-Simón, B., Alvira, M., Eritja, R., Götz, G., Bäuerle, P., Samitier, J., 2015. Biosens. Bioelectron. 74, 751-756.
Gossner, C., Severi, E., 2014. Euro Surveill. 19, 43.
Goswami, B.B., Burkhardt, W., Cebula, T.A., 1997. J. Virol. Meth. 65(1), 95-103.
Grabow, W., 1997. Water SA 23(4), 379-386.
Han, C.M., Katilius, E., Santiago, J.G., 2014. Lab Chip 14(16), 2958-2967.
Hassen, W.M., Chaix, C., Abdelghani, A., Bessueille, F., Leonard, D., Jaffrezic-Renault, N., 2008. Sens. Actuators B: Chem. 134(2), 755-760.
Hollinger, F., Emerson, S., 2007. p 911–947. Fields virology. Lippincott Williams and Wilkins, Philadelphia, PA.
Hu, Y., Arsov, I., 2009. Lett. Appl. Microbiol. 49(5), 615-619.
Hu, Y., Arsov, I., 2014. Food Environ. Virol. 6(3), 189-195.
Jothikumar, N., Paulmurugan, R., Padmanabhan, P., Sundari, R.B., Kamatchiammal, S., Rao, K.S., 2000. J. Environ. Monit. 2(6), 587-590.
Li, F., Xu, Y., Yu, X., Yu, Z., He, X., Ji, H., Dong, J., Song, Y., Yan, H., Zhang, G., 2016. Biosens. Bioelectron. 82, 212-216.
Liu, L., Wang, X., Ma, Q., Lin, Z., Chen, S., Li, Y., Lu, L., Qu, H., Su, X., 2016. Anal. Chim. Acta 916, 92-101.
Lopman, B., van Duynhoven, Y., Hanon, F., Reacher, M., Koopmans, M., Brown, D., Europe, C.o.F.V.i., 2002. Euro Surveill. 7(4), 61-65.
Manzano, M., Giusto, C., Iacumin, L., Cantoni, C., Comi, G., 2003. J. Appl. Microbiol. 95(6), 1361-1366.
Martin, A., Lemon, S.M., 2006. Hepatology 43(S1).

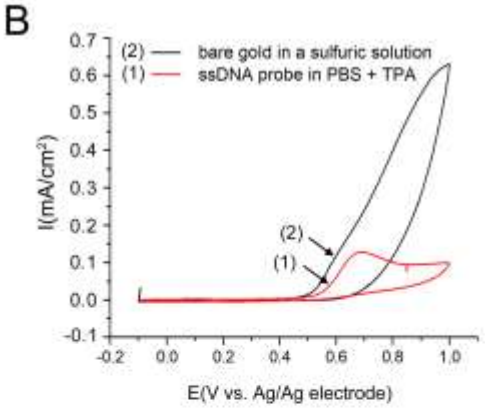
McCaustland, K., Bond, W., Bradley, D., Ebert, J., Maynard, J., 1982. J. Clin. Microbiol. 16(5), 957-958.
Miao, W., Choi, J.P., Bard, A.J., 2002. J. Am. Chem. Soc. 124(48), 14478-14485.
Nainan, O.V., Xia, G., Vaughan, G., Margolis, H.S., 2006. Clin. Microbiol. Rev. 19(1), 63-79.
Oberste, M.S., Pallansch, M.A., 2005. Rev. Med. Microbiol. 16(4), 163-171.
Pérez-Sautu, U., Costafreda, M.I., Lite, J., Sala, R., Barrabeig, I., Bosch, A., Pintó, R.M., 2011. J. Clin. Virol. 52(2), 98-102.
Petrignani, M., Verhoef, L., Vennema, H., van Hunen, R., Baas, D., van Steenbergen, J.E., Koopmans, M.P., 2014. Em. Infect. 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. Euro. Surveill. 18(27), 20518.
Sánchez, G., Bosch, A., Pintó, R., 2007. Lett. Appl. Microbiol. 45(1), 1-5.
Sano, D., Amarasiri, M., Hata, A., Watanabe, T., Katayama, H., 2016. Environ. Int. 91, 220-229.
Scida, K., Cunningham, J.C., Renault, C., Richards, I., Crooks, R.M., 2014. Anal. Chem. 86(13), 6501-6507.
Torelli, E., Manzano, M., Marks, R.S., 2017. Sens. Actuators B: Chem. 247, 868-874.
Venter, J., Van Heerden, J., Vivier, J., Grabow, W., Taylor, M., 2007. J. Water Health 5(2), 229-240.
Vidic, J., Manzano, M., Chang, C.-M., Jaffrezic-Renault, N., 2017. Vet. Res. 48(1), 11.
Wei, X., Panindre, P., Zhang, Q., Song, Y.-A., 2016. ACS Sensors 1(7), 862-865.
Williams, J., Fred, Fout, G., 1992. Environ. Science Technol. 26(4), 689-696.
WorldHealthOrganization, 2000. 75, 38-44.
Yezli, S., Otter, J.A., 2011. Food Environ. Virol. 3(1), 1-30.
Zheng, J., Chen, C., Wang, X., Zhang, F., He, P., 2014. Sens. Actuators B: Chem. 199, 168-174.
Zhou, Y.-J., Estes, M.K., Jiang, X., Metcalf, T.G., 1991. Appl. Environ. Microbiol. 57(10), 2963-2968.

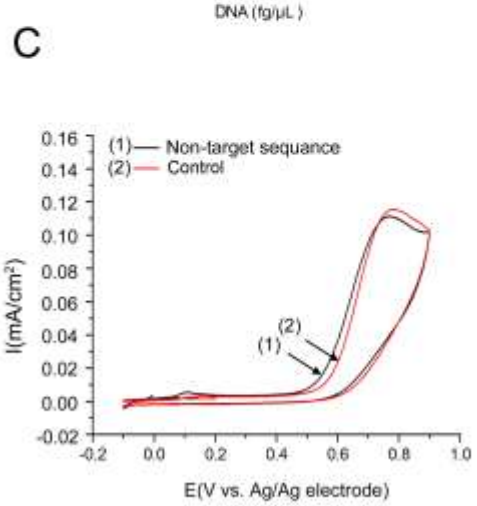
Highlights

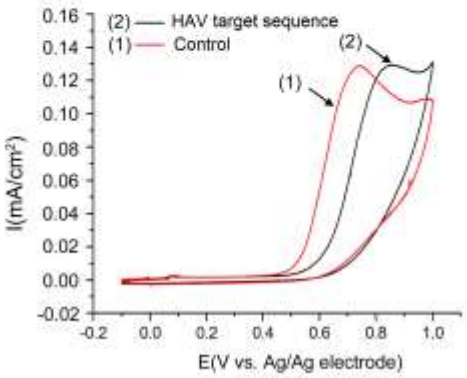
- An electrochemical method for quantitative detection of hepatitis A virus.
- Specific DNA probe designed and optimized for sensitive detection of hepatitis A virus
- Optimized modifications of disposable electrode for a stable and reproducible virus detection

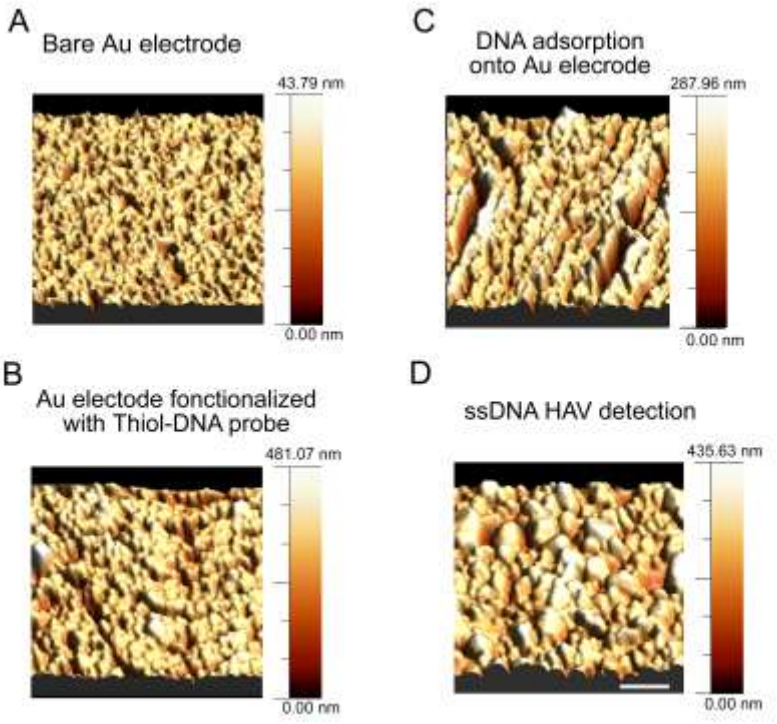


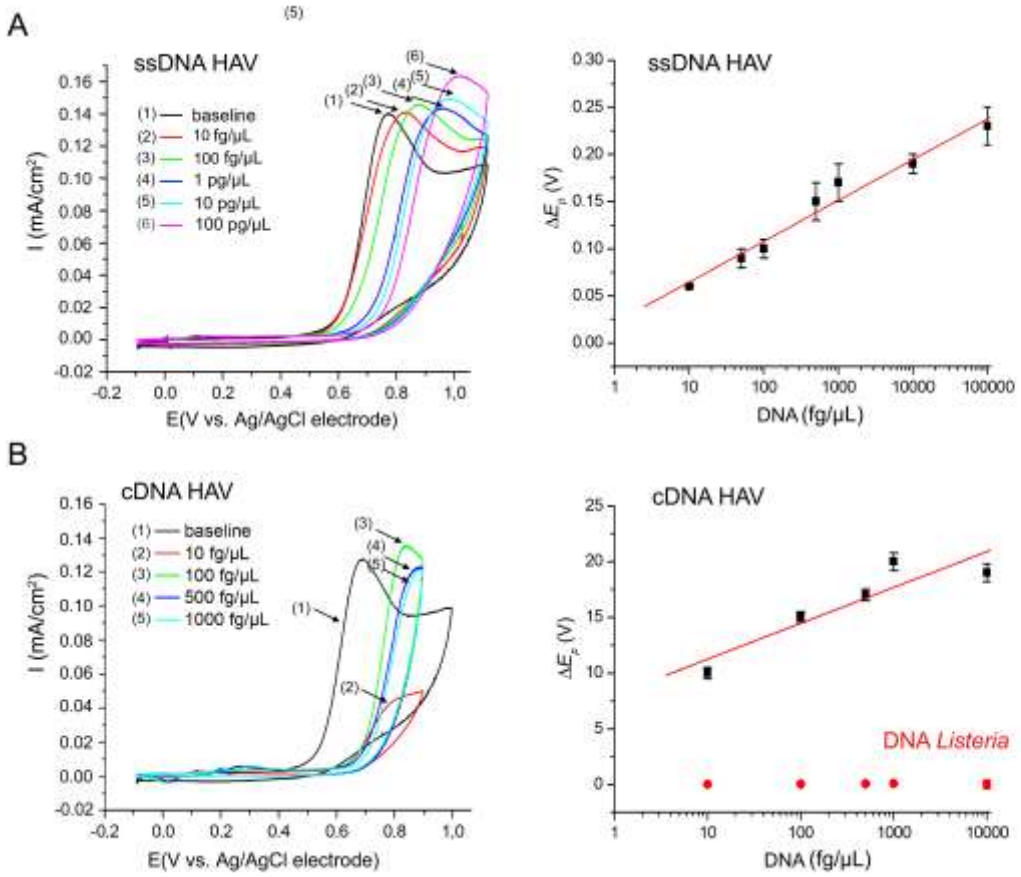

A
Threshold Cycle values (Ct)
DNA (fg/μL)


B
(2) bare gold in a sulfuric solution
(1) ssDNA probe in PBS + TPA
I(mA/cm²)
E(V vs. Ag/Ag electrode)


C
(1) Non-target sequance
(2) Control
I(mA/cm²)
E(V vs. Ag/Ag electrode)


(2) HAV target sequence
(1) Control
I(mA/cm²)
E(V vs. Ag/Ag electrode)




A
ssDNA HAV
(1) baseline
(2) 10 fg/µL
(3) 100 fg/µL
(4) 1 pg/µL
(5) 10 pg/µL
(6) 100 pg/µL
I (mA/cm²)
E(V vs. Ag/AgCl electrode)
ssDNA HAV
ΔEp (V)
DNA (fg/µL)
B
cDNA HAV
(1) baseline
(2) 10 fg/µL
(3) 100 fg/µL
(4) 500 fg/µL
(5) 1000 fg/µL
I (mA/cm²)
E(V vs. Ag/AgCl electrode)
cDNA HAV
ΔEp (V)
DNA Listeria
DNA (fg/µL)