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Ultrasensitive PCR-Free detection of whole virus genome by electrochemiluminescence

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A B S T R A C T
Detection of nucleic acids is crucial in many medical applications, and in particular for monitoring infectious diseases, as it has become perfectly clear after the pandemic infection of COVID-19. In this context, the development of innovative detection methods based on signal-amplification rather than analyte-amplification represents a significant breakthrough compared to existing PCR-based methodologies, allowing the development of new nucleic acid detection technologies suitable to be integrated in portable and low-cost sensor devices while keeping high sensitivities, thus enabling massive diagnostic screening. In this work, we present a novel molecular sensor for the ultrasensitive PCR-free detection of Hepatitis B Virus (HBV) based on electrochemiluminescence (ECL). Thanks to the combination of surface cooperative hybridization scheme with ECL detection strategy, our sensor for the ultrasensitive PCR-free detection of Hepatitis B Virus (HBV) based on electrochemiluminescence represents a significant breakthrough compared to existing PCR-based methodologies, allowing the development of new nucleic acid detection technologies suitable to be integrated in portable and low-cost sensor devices while keeping high sensitivities, thus enabling massive diagnostic screening. In this work, we present a novel molecular sensor for the ultrasensitive PCR-free detection of Hepatitis B Virus (HBV) based on electrochemiluminescence (ECL).

1. Introduction

The detection of nucleic acids is nowadays of extreme importance in many medical fields for early and accurate diagnosis, personalized therapy, and preventive screening. In this context, DNA identification and quantification through molecular methods provides relevant clinical advantages with respect to the traditional laboratory methods, such as bacterial cultures or antibody detection, being much faster (hours versus days for bacterial cultures), specific (allowing the detection of genotypes), sensitive (few copies of pathogens in a sample) and accurate (able to detect different microorganisms through the specific molecular markers, but also their vitality through the mRNA monitoring).

However, molecular methods typically need an amplification step achieved through the well-known Polymerase Chain Reaction (PCR) that is intrinsically quite laborious because it involves a multi-step and expensive process (about $15–80 per sample) thus limiting its utilization to specialized laboratories (Espy et al., 2006). Therefore, although current PCR-based methods are well-established and consolidated, they are not suitable to be used by unskilled personnel near the patient at competitive costs. This represents a strong limitation for its massive use, limiting the potential of genome analysis for human health. This is proved by the diffused difficulty to perform massive and real time diagnosis of coronavirus disease 2019 (COVID-19) for prompt infection management and prevention which is stimulating new approaches and...
methodologies for molecular detection (Cheong et al., 2020; Fan et al., 2021; Nunez-bajo et al., 2020). In addition, it has become evident that COVID-19 antigen rapid diagnostic tests, although being fast and relatively cheap, yield a high percentage of false negative results. Therefore, the development of new molecular methods allowing rapid, sensitive, and simple detection of pathogens would be a significant breakthrough in molecular diagnostics opening new technological frontiers in the genome detection, as represented by the Genetic Point of Care (G-PoC) testing (Petraila and Conoci, 2017; Wang et al., 2021). G-PoC sensors indeed would present some unique advantages, such as a simple operation (performable by untrained personnel), rapid results, low power supply and use of reagents, and convenience of use specifically in areas where resources are limited (Arduini et al., 2016; Chan et al., 2017).

In this scenario, PCR-free approaches are extremely appealing because they do not require any analyte-amplification reactions significantly simplifying the instrumentation needed and thus lowering costs. However, these approaches are extremely challenging since detection of whole-genome sequences has to be achieved at lower concentrations. This is particularly true in the case of infectious diseases where the concentration of bacterial or viral genome can be few copies in few microliters of sample. For these reasons, only very few examples of PCR-free methods have been reported in the literature so far and, consequently, a limited number of devices has been proposed (Kerr et al., 2021). Furthermore, most of them use indirect strategies that target analytes different from the chased viral or bacterial genomes (Wen et al., 2021) and, in case of pathogens, they do not fulfill the Limit of Detection (LoD) requirements (10 copies of target-genome/reaction) (Petralia et al., 2021). Additionally, all the reported methods need pre-treatment or labelling of the sample, adding a further degree of complexity (M. Chen et al., 2020). Therefore, a fundamental prerequisite to achieve an effective PCR-free detection of the nucleic acid of pathogens is the design of a radically new biochemical strategy for molecular recognition combined with a highly efficient transduction method.

Among infections, Hepatitis B Virus (HBV) is one of the major worldwide infective agents (Schweitzer et al., 2015). The World Health Organization (WHO) estimates that in 2015 257 million people were living with HBV infection and that 887,000 deaths yearly (World Health Organization (WHO), 2016) are caused by the subsequent occurrence of hepatocellular carcinoma (HCC). From a genetic standpoint, HBV is featured by an incomplete circular partially double-stranded (ds)-DNA genome that can be present in a wide range of concentrations (from few copies to 10^6 copies µL^{-1}) in several biological fluids such as serum and dried blood spots (Brecho et al., 1981; Gupta et al., 1992) The molecular diagnosis of HBV is carried out using standard real-time PCR methods. To overcome the above reported limitations of this methodology, several types of alternative biosensors have been proposed including immunobiosensors (Chen et al., 2014; Kanan et al., 2019), paper-based electrochemical sensors (Srisomwat et al., 2020) and label-free sensors based on electrochemiluminescence (ECL) transduction (Babamiri et al., 2018).

ECL can in fact provide a very high sensitivity with the use of very simple instrumentation (Kanan et al., 2019; Pang et al., 2017; Xiang et al., 2018) being very appealing for the development of cheap G-PoC (Delaney et al., 2011). ECL is based on a luminescent phenomenon induced by electrochemical stimulus on a specific molecular system (Qi and Jiang, 2020). Thanks to the combination between electrochemical and spectroscopic methods, ECL possesses several advantages over chemiluminescence and photoluminescence, such as (i) superior temporal and spatial control on light emission, (ii) intrinsically very low background and high sensitivity (pmol L^{-1}) due to the absence of excitation light, and (iii) broad dynamic range (i.e., more than six orders of magnitude) and rapid measurement (i.e., few seconds) with low volumes (<1 mL). Finally, the electrical trigger allows both simplified optical architectures (light detection without any interference of light excitation) and the integration in miniaturized microelectronic systems. In particular, in the ECL luminescence/correactant mechanism, used in most of analytical applications and instruments (Ma et al., 2019; Zanut et al., 2020a), the excited state of the luminesphore is generated through its reaction with reactive radicals produced by the electro-chemical oxidation or reduction of the correactant (Fantacci et al., 2005; Hu et al., 2009; Yuan et al., 2012). Tris(2,2'-bipyridine) ruthenium(II) ([Ru(bpy)_3]^{2+}/[Ru(bpy)_3]^{3+})/tri-n-propylamine (TPPA) (A. Fiorani et al., 2018) is the most efficient ECL-correactant system widely exploited both in commercial assays (more than 2 billion tests per year) and in innovative biochemical sensors (Sciuto et al., 2015, 2019; Zanut et al., 2019, 2020a, 2020b). An alternative approach is represented by the so called “reductive oxidation” correactant mechanism using peroxodisulfate (S_{2}O_{8}^{2−}) as correactant, with negative potentials (Bolletta, 1982; Fiorani et al., 2018; White and Bard, 1982; Zhang et al., 2020). This has been proved to enhance the ECL intensity of about 450-fold compared with other alternative cathodic correactants (H_{2}O_{2}, Na_{2}C_{2}O_{4}) (Russell et al., 2016).

In this work, a novel molecular biosensor for ultrasensitive detection and quantification of HBV whole genome without target amplification is proposed. The method is based on the combination of surface cooperative hybridization of HBV genome and ECL transduction using the [Ru(phen)_{2}dppz][O_8^{2−}] complex (phen = 1,10-phenanthroline; dppz = quinoxalino[2,3-f][1,10]phenanthroline) as ECL-active dye. ("Patent pending – Italian submission N. 1020210000184222.” 2021) The analytical performances of the PCR-free biosensor using both synthetic HBV genome (SG ds-HBV) and samples extracted from real samples (EG ds-HBV) are presented and discussed.

2. Materials and methods

2.1. Chemicals and materials

Analytical grade reagents were used in this work. Phosphate buffered Saline (PBS) at different concentrations (0.1 M and 0.01 M) and pH (5.5, 6.5, 7.4 and 8.5), potassium persulfate (K_{2}S_{2}O_{8}) > 99%, sulfuric acid (H_{2}SO_{4}) 98%, Potassium hexacyanoferrate (III) (K_{3}[Fe(CN)]_{6}) and Ethanol (C_{2}H_{5}O) 100% were purchased from Sigma-Aldrich, Fetal Bovine Serum (FBS) produced by Gibco® 100% was purchased from ThermoFisher Scientific. [Ru(phen)_{2}dppz][O_8^{2−}] and [Os(bpy)_{2}dppz]Cl_{2} were prepared as previously described (Li et al., 2016) (Defever et al., 2011). 6-Mercapto-1-hexanol (C_{6}H_{12}O_{5}S) > 97% was purchased from Fluka.

Hepatitis B virus (HBV) clone complete genome (analytical genome - SG ds-HBV) was purchased from Clonit (ref 05960467) and consists of the HBV ds-DNA genome (3.2 kbps) inserted into plasmid PBR322 vector (3.8 kbps). It is provided in a TE (Tris 10 mM, EDTA 1 mM, pH = 8) solution. Extracted HBV real genome (EG ds-HBV) were provided by Clonit (extraction were carried out from human blood using Qiagen QIAamp DNA Mini Kit 'ref. 51306', following the instructions for use) at concentration of 12 × 10^6 IU/mL (quantified by real time PCR).

Mycobacterium Tuberculosis clone complete genome (MTB) was obtained from Clonit (ref 05960564) provided in a TE (Tris 10 mM, EDTA 1 mM, pH = 8) solution to test the selectivity of the system.

Complementary P1 probe (HS-(CH_{2})_{9}-GGTGAAGTGAAGGGTTT) and P2 Probe (HS-(CH_{2})_{9}-CACATCGAAGTCCCCAGGTT) were purchased from MWG (Germany).

2.2. Apparatus and measurements

All Electrochemiluminescent (ECL) and electrochemo (EC) measurements were conducted in static environment with a potentiostat (PGSTAT302N, Metrohm) using a single-compartment three-electrode Teflon cell in static condition with Au electrode (CHI101) as the working electrode (WE), a Pt wire as the counter electrode (CE), and an Ag/AgCl
(saturated KCl) electrode as the reference electrode (RE). The ECL signal was measured with a photomultiplier tube (PMT, Hamamatsu R928) placed at a fixed height from the electrochemical cell, inside a dark box. A high-voltage power supply socket assembly with a transimpedance amplifier (Hamamatsu C6271) was used to supply 750 V to the PMT, using an external trigger connection to the potentiostatic DAC module. Light/current/voltage curves were recorded by collecting the amplified PMT output signal with the ADC module of the potentiostat. ECL spectra were collected by a SEC2000 Spectra system UV–visible spectrophotometer (ALS Co. Ltd., Japan). As EC technique it was used the cyclic voltammetry (CV) applying negative potential from 0 to −2 V, with a scan rate of 0.3 V s⁻¹, in a buffer solution PBS 0.1M pH 5.5; using a transparent electrochemical cell. For the Electrochemical Impedance Spectroscopy (EIS) a buffer solution of PBS 0.1M, pH 5.5 containing [Fe(phen)₃]SO₄·H₂O 5.0 mM was used. All EIS measurements have been done in the range of frequency 0.1 MHz⁻¹ Hz, thus the current range has been kept equivalent at 1 mA. Finally, the value of open circuit potential (Eop) was fixed at 0.228V for the entire measurement.

2.3. Electrode preparation

Gold electrodes were polished with 0.3 and 0.05 μm alumina slurry in order to obtain a mirror surface and then washed by sonication in a 50:50 ethanol and deionized water for 5 min. Furthermore, electrodes were cleaned electrochemically with cyclic voltammetry in 0.5 M H₂SO₄ scanning from −1 to 1 V (10 scans and 0.1 V s⁻¹). Finally, they were dried with Ar gas. Next, P1 and P2 thiol 5'-terminated probes were dissolved in PBS 0.01M at pH 5.5 and incubated for 2 h until the solution containing the thiol-terminated probes. Afterwards, they were gently washed with PBS 0.01 M (pH 7.4) followed by an overnight blocking procedure with 10 μM of C₆H₄OS dissolved in PBS 0.01 M (pH 7.4) to stabilize the assembled layer of P1 and P2 specific probes (Fig. 1a).

2.4. Analytical test procedure

Synthetic HBV genome (SG ds-HBV) samples (1, 5, 10, 100, 1000, 10000 cps μL⁻¹) were prepared by diluting the starting clone solution (10⁶ cps μL⁻¹) in PBS 0.01 M at pH 5.5. P1/P2 modified electrodes were immersed in 1 mL of SG ds-HBV solutions at 50 °C in a chamber for 3 h. To intercalate [Ru(phen)₂dppz]²⁺ in the anchored HBV genome, electrodes were immersed in a solution containing 14 μM of [Ru(phen)₂dppz]²⁺ in PBS 0.01M at pH 5.5 and incubated for 2 h until the intercalation was completed. The Au electrode was then ready for the ECL procedure. The electrochemical cell was fixed in front of a PMT tube in order to always keep a 0.5 cm space between the cell and the PMT input window. The transparent nature of the cell allows the photocurrent emission, produced by [Ru(phen)₂dppz]²⁺ complex, to be detected and amplified by the PMT tube. For real sample analysis, 0–0.75 copies μL⁻¹ of EG ds-HBV (extracted from real samples) diluted in PBS pH 5.5 from the starting solution (12 × 10⁷ IU/mL) were used. For the biological fluid simulation testing, SG ds-HBV (10⁶ cps μL⁻¹) has been diluted into 1 mL Fetal Bovine Serum (FBS) to a final concentration of 100, 1000 and 10000 cps μL⁻¹, then the modified P1/P2 electrodes were immersed in these solutions and we repeated the same procedure as it is described above. Standard deviations were obtained by repeating the experiment with 5 independent samples. For details on the EC apparatus see the paragraph 2.2.

3. Results and discussion

To detect the whole double-stranded DNA genome of HBV (ds-HBV DNA) we developed an innovative strategy combining the surface cooperative hybridization of the ds-HBV whole genome with ECL transduction mechanism, as depicted in Fig. 1.

More specifically, the typical surface hybridization implies specific interactions, leading to a thermodynamic equilibrium between hybridized and unhybridized DNA, of immobilized single strand DNA probe with the target DNA. In the case of cooperative hybridization, instead, two NA probes (P1 and P2) – designed to recognize specific and different
sequences on both parallel and anti-parallel strands of the ds-HBV – were immobilized on a gold electrode surface (Fig. 1a). The ds-HBV DNA is recognized by the two surface probes that independently hybridize two complementary filaments of the ds-HBV DNA and anchor it on the electrode surface (Fig. 1c and fully characterized by EIS see Fig. S1). This leads to an effective DNA recognition reaction endowed with higher affinity, as confirmed by the comparison of the two individual probes performances (Fig. S2) and by our previous molecular dynamic investigation showing higher binding enthalpy for ds-HBV DNA – P1/P2 interaction (−67.3 kJ mol⁻¹) than the sum of the individual single probes interactions (−47.0 kJ mol⁻¹) (Petralia et al., 2020). A sequence gap in the target of 138 bps between the two regions recognized by the two probes (Fig. 1d) was proved to be effective in minimizing the steric hindrance (Petralia et al., 2020). To detect the whole genome recognition event occurring at the electrode surface, an ECL-active complex – [Ru(phen)₂dpzp]²⁺ – was used as efficient intercalating dye (‘Patent pending – Italian submission N. 102021000018422,” 2021) in the final step of our PCR-free detection strategy (Fig. 1d). Intercalation methods are appealing since they do not require any extra chemical labelling step of target or probes (He et al., 2015; Lin et al., 2016); in addition, since hundreds of complexes can intercalate in the whole viral genome, a strong signal amplification can be obtained. Moreover, [Ru(phen)₂dpzp]²⁺ shows an intense luminescence at 600–650 nm when intercalated with a double-strand DNA genome (Chambon and Sauvage, 1991; Friedman et al., 1990; Nair et al., 1997), while in aqueous solution the emission is dramatically quenched, increasing even more the signal-to-noise ratio. This behaviour results from a differential population of two close-lying dpzp-localized excited states, the non-emissive state being favoured in polar and H-bonding solvents (Hiort et al., 1993; Tuite et al., 1997). Therefore, once [Ru(phen)₂dpzp]²⁺ was intercalated to the HBV-SG anchored at the electrode surface, light emission at 600–650 nm was recorded at −0.8 V (Fig. S4) vs Ag/AgCl reference electrode, i.e. upon the reduction of the S₂O₈²⁻ coreactant which ignites the ECL process.

The effectiveness of the molecular recognition mechanism and the analytical performances of the above detection strategy were firstly tested at different pH values (from 5.5 to 7.4), using the SG ds-HBV concentration of 100 cps µL⁻¹ (Fig. S3). In fact, the use of an acid hybridization buffer (pH = 5.5) may lead to protonation of the -G and -C groups of DNA which promotes a stronger bonding with probes (Dai et al., 2015; Fiorani et al., 2020; Petralia et al., 2020). However, at pH < 7.4 unwanted reactions involving electrochemically generated SO₄²⁻ take place leading to a decreasing of the ECL signal (Huang et al., 2010). At pH = 7.4 we observed the highest ECL value intensity in agreement with previous results (Ismail et al., 2017). Finally, at pH > 7.4 the ECL intensity is strongly reduced due to the decreasing of the SG ds-HBV recognition efficiency by the P1 and P2 probes at the surface.

After the optimization of the ECL detection conditions, we tested our system with both the SG ds-HBV analytical synthetic genome and the EG ds-HBV extracted genome from real samples. Measurements on the synthetic genome have been performed in order to optimize the conditions and to investigate in details the mechanisms of the signal generation, while the analytical characterization has been performed on the genome extracted from real samples.

Fig. 2a reports the ECL intensities vs electrode potential measured in SG ds-HBV solutions at different concentrations (0–10000 cps µL⁻¹). Fig. 2a suggests a logarithmic dependence of ECL intensities on HBV genome concentration and a recordable signal is still detected with few copies of HBV genome (5 cps µL⁻¹), noteworthy without any analyte-amplification process. To assess the analytical performance of the method, ECL intensity vs the logarithm of the ds-HBV concentration was plotted. Fig. 2b displays a good linear behaviour in the 5 and 10000 cps µL⁻¹ range with a correlation coefficient of 0.987 with a good reproducibility; thus, the increasement of the cathodic current held at −0.8 V with and without the presence of [Ru(phen)₂dpzp]²⁺ (see Figs. S4 and S5). The calculated LoD (S/N = 3.3) was 2.4 cps µL⁻¹ (i.e. 4.5 nM) with a sensitivity (slope of the curve) of 1.35 a.u./decade.

At the heart of the transduction mechanism, the processes leading to light emission are described by equations (1)–(5) (Reshetnyak and Koval’chuk, 1997; Sojic, 2020): by applying 0.80 V, both the reduction of peroxydisulfate (Eq. (1)) and [Ru(phen)₂dpzp]²⁺ (Eq. (2)) see Fig. S6 (Liang et al., 2019; Wang et al., 2018; Zhou et al., 2020) take place, thus making the sequence of processes outlined by Eqs (3)–(5) possible. In particular, peroxydisulfate (S₂O₈²⁻) is reduced to generate a sulphate anion (SO₄²⁻) and a sulphate radical anion (SO₄*⁻) (Eq. (1));(Y. Chen et al., 2020) the latter also being indirectly obtained by the mediation of [Ru(phen)₂dpzp]⁺ (Eq. (3)). The reaction between [Ru(phen)₂dpzp]⁺ and the sulphate radical anion generates the [Ru(phen)₂dpzp]³⁺ excited state (Eq. (4)) which emits light (Eq. (5)).

\[ S_2O_8^{2-} + e^- + SO_4^{2-} + SO_4^{2-} \]  
\[ \text{[Ru(phen)₂dpzp]}^{2+} + e^- \rightarrow \text{[Ru(phen)₂dpzp]}^{3+} \]  
\[ \text{[Ru(phen)₂dpzp]}^{2+} + SO_4^{2-} \rightarrow \text{[Ru(phen)₂dpzp]}^{3+}, \text{SO}_4^{2-} \]  
\[ \text{[Ru(phen)₂dpzp]}^{2+} + SO_4^{*^-} \rightarrow \text{[Ru(phen)₂dpzp]}^{3+}, \text{SO}_4^{2-} \]
In order to evaluate its selectivity, the method was tested towards an
unspecific target. *Mycobacterium tuberculosis* (MTB) synthetic clone at
three different concentrations (10-100-1000 cps μL⁻¹) was tested
resulting in the absence of any ECL emission (Fig. S8 a-c), evidencing the
high selectivity of P1 and P2 probes for SG ds-HBV.

To further validate the high sensitivity of the method, EG ds-HBV
extracted from real samples was used. Noteworthy, much lower concen-
trations than those tested for SG ds-HBV could be detected. Fig. 3a
reports the ECL curves obtained for EG ds-HBV at concentrations below
0.75 cps μL⁻¹ and a signal was still detected at 0.06 cps μL⁻¹. The
 calibration curve shows a good linear correlation (Fig. 3b) with a LoD
(S/N = 3.3) of 0.06 cps μL⁻¹ (i.e. 0.1 aM).

The different sensitivity between the synthetic SG-dsHBV (1.35 a.u./
LogC) and the extracted real EG-ds HBV genomes (0.18 a.u./LogC) can
be imputed to the different structure of the two macromolecules.
Actually, the former consists of the HBV genome (3.2 kbps) inserted into
plasmid PBR322 vector of 3.8 kbps leading to a circular genome of about
7.0 kbps (Petralia et al., 2017). On the contrary, the latter is a real
extracted HBV genome consisting in an unusual genome partially dsDNA
of about 3.2 kbps. Such a difference may be responsible for a change in
the genome surface conformation and distribution of the ECL
luminophore which in turn would affect the efficiency of the ECL
generating mechanism (Zanut et al., 2021). The above data confirm the
higher sensitivity of our method compared to standard Real-time PCR
(RT-PCR) also for the real sample composed by extracted genome (10
cps μL⁻¹, slope 0.2 – See Fig. S7) (Alice et al., 2007; Petralia et al.,
2017).

An important factor in the electrically stimulated sensors is the
applicability in biological fluids that can interfere with the electrical
signal. The effect of biological matrixes on the modified gold electrodes
was tested according to the experimental conditions described in section
2. To simulate the biological matrix, PBS was replaced by Fetal Bovin
Serum (FBS) in the last step of triplex formation of our experimental
procedure, and three different SG ds-HBV concentrations (100, 1000,
and 10000 cps μL⁻¹) were examined. ECL intensities measured in FBS
are consistent with the one observed in PBS solution (Fig. S9). The
matrix effect was evaluated by calculating the Real Standard Deviation (RSD)
between PBS and FBS (Table S2), showing a satisfactory detection of SG
ds-HBV in the range of 83–104%, thus confirming the efficient
applicability of the proposed electrochemical DNA sensor in complex
fluids without detectable interferences. These results are very promising
for future application in real human complex matrices such as plasma or
blood.

Table 1 gathers the analytical performances of different methodol-
dgies for the detection of HBV DNA reported in the literature, compared
with the ones obtained with our proposed PCR-free sensors. It can be
noticed that our method represents an important step forward regarding
sensitivity with respect to those already described. Stimulated by these
results, we are currently applying this method to SARS-CoV2 detection
for the development of molecular point-of-care test for low-cost massive
screening.

4. Conclusions

In summary, we developed a novel molecular sensor for ultrasensi-
tive and PCR-free detection of HBV whole genome. Our method exploits
a surface cooperative hybridization at miniaturized gold electrode with
ECL transduction through the intercalation of [Ru(phen)₂dpdz]²⁺
complex, using potassium persulfate (K₂S₂O₈) as co-reactant. By
combining the superior analytical performances of ECL and the signal
amplification offered by the intercalation of a high number of ECL-active
Ru complexes, we were able to detect HBV genome from real sample
without any amplification step, reaching a LoD of 0.06 cps μL⁻¹ (i.e. 0.1
aM). This approach may be performed with extremely simple and
portable instrumentation and ongoing research in our laboratories is
dedicated to the detection of viral genomes using the same strategy.
The present results represent indeed an important step towards the devel-
opment of rapid, sensitive and amplification free molecular Point-of-
Care (PoC) test for monitoring infectious diseases.

CRediT authorship contribution statement

Pavlos Nikolaou: performed and analyzed the ECL experiments.
Emanuele Luigi Sciuto: optimized the protocol for the DNA recognition
and performed the RT-PCR analysis. Alessandra Zanut: performed and
analyzed the ECL experiments. Salvatore Petralia: optimized the pro-
tocol for the DNA recognition and performed the RT-PCR analysis.
Giovanni Valenti: planned and supervised the research and co-wrote
the paper with contributions from other authors. Francesco Paolucci:
planned and supervised the research and co-wrote the paper with
contributions from other authors. Luca Prodi: planned and supervised
the research and co-wrote the paper with contributions from other authors.
Sabrina Conoci: planned and supervised the research and co-wrote the
paper with contributions from other authors.

![Fig. 3.](image-url)
Table 1

| Strategies                  | Techniques                  | Linear range          | Sensitivity         | Reference          |
|----------------------------|-----------------------------|-----------------------|---------------------|--------------------|
| HBV DNA sandwich assay     | Anodic Stripping Voltammetry (ASV) | 0–500 × 10^3cps μL^−1 | 511 × 10^3cps μL^−1 | Li et al. (2015)   |
| Label-free HBV DNA         | Differential Pulse Voltammetry (DPV) | 50 × 10^3–10^3cps μL^−1 | 8.71 × 10^3cps μL^−1 | Srisontawatkul et al. (2020) |
| PCR-free HBV genome detection | Square Wave Voltammetry (SWV) | 1–100cps μL^−1 | 1.36 × 10^5cps μL^−1 | Peralia et al. (2017) |
| Multicolloids QDs based sensor | Electrochemiluminescence (ECL) | 0.0005–0.5 × 10^3cps μL^−1 | 0.49 × 10^3cps μL^−1 | Liu et al. (2016) |
| Carbon sphere/Polyaniline  | Differential Pulse Voltammetry (DPV) | 0.01 × 10^3–1 × 10^3cps μL^−1 | 3.62 × 10^3cps μL^−1 | Salimian et al. (2019) |
| Label-free platform using graphene QDs | Electrochemiluminescence (ECL) | 0.1–5 × 10^3cps μL^−1 | 1.07 × 10^3cps μL^−1 | Xiang et al. (2018) |
| PCR-free EG ds-HBV         | EG ds-HBV                   | 0.075–0.011–0.017cps μL^−1 | 0.06 × 0.011cps μL^−1 | This work         |

* This LoD it was obtained using SG ds-DNA.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

The authors have a potential competing interest having filed the following Patent Application: Italian submission N. 102021000018422, 2021

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Appendix A. Supplementary data

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References

Allice, T., Cerutti, F., Pittaluga, F., Varetto, S., Gabella, S., Marzano, A., Franchello, A., Colucci, G., Ghisetti, V., 2007. COBAS AmpliPrep-COBAS TaqMan hepatitis B virus (HBV) test: a novel automated real-time PCR assay for quantification of HBV DNA in plasma. J. Clin. Microbiol. 45, 828-834.

Ardini, F., Micheli, L., Moscone, D., Balleschi, S., Riccì, F., Volpe, G., 2016. Electrochemical biosensors based on nanomodified screen-printed electrodes: recent applications in clinical analysis. Trends Anal. Chem. 79, 114-126.

Babamiri, B., Hailali, H., Salimi, A. 2018. Ultraselective electrochemiluminescence immunosensor for determination of hepatitis B virus surface antigen using CdTe@CdS-PAMAM dendrimer as luminescent labels and Fe3O4 nanoparticles as magnetic beads. Sensor. Actuator. B Chem. 254, 551–560.

Bolletta, F. 1982. Polypryidine transition metal complexes as light emission sensitizers in the electrochemical reduction of the persulfate ion. Inorg. Chim. Acta. 62, 207-213.

Brechot, C., Scotto, J., Chamay, P., Hadchouel, M., Degos, F., Trepo, C., Tiollais, P., 1981. Detection of Hepatitis B virus DNA in liver and serum: a direct appraisal of the in the electrochemical reduction of the persulfate ion. Inorg. Chim. Acta. 62, 207–213.

Chambron, J., Sauvage, J., 1991. Ru (bipy)2(dpq)2 : a highly sensitive luminescent probe for micellar sodium dodecyl sulfate solutions. Chem. Phys. Lett. 182, 603–607.

Chen, H.N., Tan, M.J.A., Wu, H., 2017. Point-of-care testing: applications of 3D printing. Lab Chip 17, 2713–2739.

Chan, H.N., Tan, M.J.A., Wu, H., 2020. Fast detection of SARS-CoV-2 RNA via the integration of plasmonic electrochemiluminescent system in bioassay. J. Anal. Test. 4, 57–66.

Fan, Z., Yao, B., Ding, Y., Zhao, J., Xie, M., Zhang, K., 2021. Entropy-driven amplified electrochemiluminescence biosensor for RdRp gene of SARS-CoV-2 detection with self-assembled DNA…………

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bios.2022.114165.

References

Allice, T., Cerutti, F., Pittaluga, F., Varetto, S., Gabella, S., Marzano, A., Franchello, A., Colucci, G., Ghisetti, V., 2007. COBAS AmpliPrep-COBAS TaqMan hepatitis B virus (HBV) test: a novel automated real-time PCR assay for quantification of HBV DNA in plasma. J. Clin. Microbiol. 45, 828-834.

Ardini, F., Micheli, L., Moscone, D., Balleschi, S., Riccì, F., Volpe, G., 2016. Electrochemical biosensors based on nanomodified screen-printed electrodes: recent applications in clinical analysis. Trends Anal. Chem. 79, 114-126.

Babamiri, B., Hailali, H., Salimi, A. 2018. Ultraselective electrochemiluminescence immunosensor for determination of hepatitis B virus surface antigen using CdTe@CdS-PAMAM dendrimer as luminescent labels and Fe3O4 nanoparticles as magnetic beads. Sensor. Actuator. B Chem. 254, 551–560.

Bolletta, F. 1982. Polypryidine transition metal complexes as light emission sensitizers in the electrochemical reduction of the persulfate ion. Inorg. Chim. Acta. 62, 207–213.

Brechot, C., Scotto, J., Chamay, P., Hadchouel, M., Degos, F., Trepo, C., Tiollais, P., 1981. Detection of Hepatitis B virus DNA in liver and serum: a direct appraisal of the in the electrochemical reduction of the persulfate ion. Inorg. Chim. Acta. 62, 207–213.

Chambron, J., Sauvage, J., 1991. Ru (bipy)2(dpq)2 : a highly sensitive luminescent probe for micellar sodium dodecyl sulfate solutions. Chem. Phys. Lett. 182, 603–607.

Chen, H.N., Tan, M.J.A., Wu, H., 2017. Point-of-care testing: applications of 3D printing. Lab Chip 17, 2713–2739.

Chen, M., Ning, Z., Chen, K., Zhang, Y., Shen, Y., 2010. Recent advances of electrochemiluminescent system in biosensor. J. Anal. Test. 4, 57–75.

Chen, Y., Qian, C., Liu, C., Shen, H., Wang, Z., Jing, J., Wu, J., Chen, H., 2020. Biosensors and Bioelectronics Nucleic acid amplification free biosensors for pathogen detection. Biosens. Bioelectron. 153, 112049.

Chen, Y., Wang, J., Liu, Z., Wu, G., 2014. Determination of hepatitis B virus surface antigen in serum with a sandwich immunoassay and capillary electrophoresis-electrochemical detection. Anal. Methods 6, 2484–2489.

Cheong, J., Yu, H., Lee, C.Y., Lee, Jung-uk, Choi, H., Lee, Jae-lyn, Lee, H., Cheon, J., 2020. Fast detection of SARS-CoV-2 RNA via the integration of plasmonic thermocycling and fluorescence detection in a portable device. Nat. Biomed. Eng. 4, 1159–1167.

Dai, P., Yu, T., Shi, H., Xu, J., Chen, H., 2015. General strategy for enhancing electrochemiluminescence of semiconductor nanocrystals by hydrogen peroxide and potassium permisulfate as dual coreactants. Anal. Chem. 87, 12372-12379.

Deffever, T., Druet, M., Evrard, D., Marchal, D., Limoges, B., 2011. Real-time electrochemical PCR with a DNA intercalating redox probe. Anal. Chem. 83, 1815–1821.

Delaney, J.L., Hogan, C.F., Tian, J., Shen, W., 2011. Electrogenated chemiluminescence detection in paper-based microfluidic sensors. Anal. Chem. 83, 1300–1306.

Espy, M.J., Ubl, I.R., Sloan, L.M., Buckwalter, S.P., Jones, M.F., Vetter, E.A., Yao, J.D.C., Wengenack, N.L., Rosenblatt, J.E., Cockrell, F.R., Smith, T.F., 2006. Real-time PCR in clinical microbiology: applications for routine laboratory testing. Clin. Microbiol. Rev. 19, 165–256.

Fan, Z., Yao, B., Ding, Y., Zhao, J., Xie, M., Zhang, K., 2021. Entropy-driven amplified electrochemiluminescence biosensor for RdRp gene of SARS-CoV-2 detection with self-assembled DNA…………

This LoD it was obtained using SG ds-DNA.

Fan, Z., Yao, B., Ding, Y., Zhao, J., Xie, M., Zhang, K., 2021. Entropy-driven amplified electrochemiluminescence biosensor for RdRp gene of SARS-CoV-2 detection with self-assembled DNA…………

This LoD it was obtained using SG ds-DNA.
Ma, C., Cao, Y., Guo, X., Zhu, J.-J., 2019. Recent progress in electrochemiluminescence sensing and imaging. Anal. Chem. 92, 431–454.

Nair, R.B., Callum, B.M., Murphy, C.J., 1997. Optical properties of [Ru(phen)2dppz]2+ as a function of nonaqueous environment. Inorg. Chem. 36, 962–965.

Nunez-bajo, E., Silva, A., Collins, P., Kasimatis, M., Cotur, Y., Asfour, T., Tanriverdi, U., Grell, M., Kaiti, M., Sereni, G., Stevenson, K., Guidé, F., 2020. Disposable silicon-based all-in-one micro-pECL for rapid on-site detection of pathogens. Nat. Commun. 11, 6176.

Pang, X., Bian, H., Wang, W., Liu, C., Khan, M.S., Wang, Q., Qi, J., Wei, Q., Du, B., 2017. A bio-chemical application of N-GQDs and g-C3N4 QDs sensitized TiO2 nanopillars for the quantitative detection of pDNA3-HBV. Biosens. Bioelectron. 91, 456-464. Patent pending. 2021. Italian Submission N. 1020210000181422.

Petralia, S., Sciuto, E.L., Villaggio, G., Santangelo, M.F., Laudani, S., Federico, C., Saccone, S., Sciuto, E.L., Santangelo, M.F., Villaggio, G., Sinatra, F., Bongiorno, C., Nicotra, G., Salimian, R., Shahrokhian, S., Panahi, S., 2019. Enhanced electrochemical activity of a quantum dots. RSC Adv. 8, 1820–1825.

Qi, H., Zhang, C., 2020. Electrogenerated chemiluminescence biosensing. Anal. Chem. 92, 524–534.

Reshetnyak, O.V., Koval’chuk, E.P., 1997. A possible scheme of electrochemiluminescence generation on platinum cathodes in aqueous solutions of peroxydisulfate. Electrochim. Acta 43, 465–469.

Russell, R., Stewart, A.J., Denny, L., 2016. Optimising electrogenerated chemiluminescence of quantum dots via co-reactant selection. Anal. Bioanal. Chem. 408, 7129–7136.

Salmiyan, R., Shahrokhian, S., Panahi, S., 2019. Enhanced electrochemical activity of a hollow carbon sphere/polyaniline-based electrochemical biosensor for HBV DNA marker detection. ACS Biomater. Sci. Eng. 5 (5), 2587–2594. https://doi.org/10.1021/acsbiomaterials.8b01520.

Schweitzer, A., Horn, J., Nikolajczyk, R.T., Krause, G., Ott, J.J., 2015. Estimations of worldwide prevalence of chronic hepatitis B virus infection: a systematic review of data published between 1965 and 2013. Lancet 386, 1546–1555.

Sciuto, E.L., Santangelo, M.F., Villaggio, G., Sinatra, F., Bongiorno, C., Nicotra, G., Libertino, S., 2015. Photo-physical characterization of fluorophore Ru(bpy)32+. For optical biosensing applications. Sens. Bio-Sensing Res. 6, 67–71.

Sciuto, E.L., Villaggio, G., Santangelo, M.F., Laudani, S., Federico, C., Saccone, S., Sinatra, F., Libertino, S., 2019. Study of a miniaturizable system for optical sensing application to human cells. Appl. Sci. 9.

Sojic, N., 2020. Analytical Electrogenerated Chemiluminescence: from Fundamentals to Bioassays. Royal Society Of Chemistry (RSC).

Srisomwat, C., Teengam, P., Chaypen, N., Tangkijvanich, P., Vilaiivan, T., Chailapakul, O., 2020. Chemical Pop-up paper electrochemical device for label-free hepatitis B virus DNA detection. Sensor. Actuator. B Chem. 316, 128077–128082.

Tuite, E., Lincoln, P., Norden, B., 1997. Photophysical evidence that Δ- and Λ-[Ru(phen)]2(dpz)2+ intercalate DNA from the minor groove. J. Am. Chem. Soc. 119, 239–246.

Wang, C., Liu, M., Wang, Z., Li, S., Deng, Y., He, N., 2021. Point-of-care diagnostics for infectious diseases: from methods to devices. Nano Today 37, 101092.

Wang, X., Gao, H., Qi, H., Gao, Q., Zhang, C., 2018. Proximity hybridization-regulated immunosensor for cell surface protein and protein-overexpressing cancer cells via electrochemiluminescence. Anal. Chem. 90, 3013–3018.

Wen, Y., Pei, H., Shen, Y., Xi, J., Lin, M., Lu, N., Shen, X., Li, J., Fan, C., 2012. DNA Nanostructure-based Interfacial electrochemical analysis of microRNA. Sci. Rep. 2, 14–18.

White, H.S., Bard, A.J., 1982. Electrogenerated chemiluminescence. 41. Electrogenerated chemiluminescence and chemiluminescence of the Ru(2,2’-bipy)32+–S2O82− system in acetonitrile-water solutions. J. Am. Chem. Soc. 104, 6891–6895.

World Health Organization (WHO), 2016. Global Health Sector Strategy on Viral Hepatitis.

Xiang, Q., Huang, J., Huang, H., Mao, W., Ye, Z., 2018. A label-free electrochemical platform for the highly sensitive detection of hepatitis B virus DNA using graphene quantum dots. RSC Adv. 8, 1820–1825.

Yuan, Y., Han, S., Hu, L., Parv see, X., Gu, G., 2012. Coreactants of tris(2,2’-bipyridyl)ruthenium(II) electrogenerated chemiluminescence. Electrochim. Acta 82, 484–492.

Zanut, A., Fiorani, A., Rebec kani, S., Kesarkar, S., Valenti, G., 2019. Electrochemiluminescence as emerging microscopy techniques. Anal. Bioanal. Chem. 411, 4375–4382.

Zanut, A., Fiorani, A., Saito, T., Ziebert, N., Rapino, S., Rebec kani, S., Barbon, A., Irie, T., Josel, H., Negri, F., Marcacc io, M., Windfuhr, M., Imai, K., Valenti, G., Paolucci, F., 2020a. Insights into the mechanism of coreactant electrochemiluminescence facilitating enhanced bioanalytical performance. Nat. Commun. 11, 2668–2676.

Zanut, A., Palomba, F., Rossi Scota, M., Rebec kani, S., Marcacc io, M., Genoves e, D., Rampazzo, E., Valenti, G., Paolucci, F., Prodi, L., 2020b. Dye-doped silica nanoparticles for enhanced ECL-based immunoassay analytical performance. Angew. Chem. Int. Ed. 59, 21858–21863.

Zanut, A., Rossetti, M., Marcaccio, M., Ricci, F., Paolucci, F., Porchetta, A., Valenti, G., 2021. DNA-based nanowires: insights into electrochemiluminescence signal enhancement. Anal. Chem. 93, 10397–10402.

Zhang, J., Kerr, E., Usman, K.A.S., Doeven, E.H., Francis, P.S., Henderson, L.C., Razal, J.M., 2020. Cathodic electrogenerated chemiluminescence of tris(2,2-bipyridine)ruthenium(II) and peroxynitrite at pure Ti3C2Tx MXene electrodes. Chem. Commun. 56, 10022–10025.

Zhou, T., Huang, R., Huang, M., Shen, J., Shan, Y., Xing, D., 2020. CRISPR/Cas13a powered portable electrochemiluminescence chip for ultra-sensitive and specific miRNA detection. Adv. Sci. 7, 1–16.