



Research review paper

Recent advances in micro/nanotechnologies for global control of hepatitis B infection



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ABSTRACT

The control of hepatitis B virus (HBV) infection is a challenging task, specifically in developing countries there is limited access to diagnostics and antiviral treatment mainly due to high costs and insufficient healthcare infrastructure. Although the current diagnostic technologies can reliably detect HBV, they are relatively laborious, impractical and require expensive resources that are not suitable for resource-limited settings. Advances in micro/nanotechnology are pioneering the development of new generation methodologies in diagnosis and screening of HBV. Owing to combination of nanomaterials (metal/inorganic nanoparticles, carbon nanotubes, etc.) with microfabrication technologies, utilization of miniaturized sensors detecting HBV and other viruses from ultra-low volume of blood, serum and plasma is realized. The state-of-the-art microfluidic devices with integrated nanotechnologies potentially allow for inexpensive HBV screening at low cost. This review aims to highlight recent advances in nanotechnology and microfabrication processes that are employed for developing point-of-care (POC) HBV assays.

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Introduction

Hepatitis B virus (HBV), the etiological agent of hepatitis B, can cause chronic hepatitis and lead to liver failure, cirrhosis and hepatocellular carcinoma (HCC), which accounts for 1 million deaths annually worldwide (Dienstag, 2008). The chronic HBV infection prevalence varies greatly in ethnicity and region in East and Southeast Asia, Western Pacific, and Sub Saharan Africa, it exceeds 8%, whereas in North America and Western Europe (Lavanchy, 2004) lesser than 1% of the population is infected. Although vaccination reduces occurrence rate of chronic HBV infection (Zanetti et al., 2008), an antiviral therapy emerges as a sole option for control and prevention of progression of disease (Hoofnagle, 2006). Suppression of HBV replication by antiviral drugs may reduce the incidence of cirrhosis to less than 1% year, and decrease the rate of HCC in patients with advanced fibrosis or cirrhosis by 50% (Liaw et al., 2004). In the United States, for example, only 20–30% of CHB carriers know their status, and only 12.5% of CHB carriers who are eligible for treatment under the guidelines receive it (Cohen et al., 2011). An important problem of prolonged therapy with the first generation nucleos(t)ide analogs is the occurrence of drug resistance, which may negate the therapeutic benefit. This problem can now be addressed by using 3rd generation drugs with minimal resistance in treatment-naïve patients (Chien and Liaw, 2008). The well-established serological and nucleic acid screening assays have been successfully implemented for diagnosis and screening, where the infrastructure is available. Despite the availability of HBV testing and potent drugs with minimal resistance in developed countries, access to diagnostics, treatment, and monitoring remains limited in developing countries due to resource constraints and lack of infrastructure. The primary motive of emerging diagnostic technologies remains to eliminate such limitations and facilitate HBV testing that leverages portable, inexpensive, but sensitive and specific diagnostic technologies.

Advances in nanotechnology and emergence of nanomaterials with exceptional electrical, optical and mechanical properties provide invaluable opportunities in developing new generation methodologies for HBV testing. Due to response capability of nanomaterials to minor stimulus, signal transduction at the molecular level is enabled that improves limit of detection and sensitivity by several orders of magnitudes with respect to traditional sensors. The combination of nanomaterials (metal/inorganic nanoparticles, carbon nanotubes) with microfabrication technologies also renders miniaturized sensors for rapid sensing of HBV and other viruses from ultra-low volume of biological samples. HBV nanobiosensors often rely on generalized approach “utilization of biomarker HBsAg” and “interaction with HBV” and subsequent transduction of the binding event to a detectable signal. In contrast to traditional serological methodologies used in virus (or nucleic acid) detection, nanomaterials such as gold nanoparticles provide an ultra-high surface area enabling immobilization of a number of biomarkers and inducing substantial change in plasmonic properties even in the presence of few target molecules. By the advancement of bioconjugation techniques, application of nanomaterials in complex bioassays such as HBV and HIV detection, are profoundly simplified, which lowers the assaying time and cost. The demand to develop fast, easy, preferably inexpensive detection strategies makes nanomaterials an attractive and indispensable component of HBV diagnostics for POC testing that enables quantification, ultra-sensitivity, and on-board-signal-amplification. Such an ideal detection platform can be potentially realized by leveraging several different cutting edge technologies and high performance nanomaterials. Fulfilling this highly demanding task requires, i) understanding the origin and nature of HBV infection, ii) resolving multistep biological mechanisms of infection and transfection, and iii) exploring sensing capability of nanomaterials such as gold nanoparticles, carbon nanotubes or graphene.

The purpose of this review is to highlight recent advances and future perspectives in nanotechnology and microfabrication processes

that are employed for developing HBV detection platforms and assay methodologies. First, we present insights into the natural history, epidemiology and treatment of HBV. Next, we discuss monitoring of therapy, cancer relationship and co-infections as well as the public health aspects such as screening, diagnostics, cost-effective tools and assays for HBV. Last advances in nanotechnological tools for diagnosis of HBV and HBV co-infections are discussed in detail highlighting the impact of nanotechnology and nanomaterials on developing novel HBV detection platforms and testing methodologies.

General description of hepatitis B virus and natural history

Hepatitis B virus is a DNA virus in the family of Hepadnaviridae (Tacke et al., 2004). The whole genome of HBV is a complex consisting of four partially protruding reading frames that encode entire viral structure (Tacke et al., 2004). HBV enters into the hepatocyte upon endocytosis process, followed by uncoating, and formation of covalently closed circular DNA (ccc DNA) in nucleus that serves as a viral replication template (Doo and Liang, 2001; Tacke et al., 2004). The replication of HBV is asymmetric by transcription of RNA. Due to lack of proofreading activity of its transcriptase (Pol/Rt), estimated mutations are at rate of one error/ 10^4 – 10^5 nucleotides daily (Locarnini and Mason, 2006). It is considered that random mutations may overlap with the antiviral-induced mutations and result in drug-resistant strains. Permanent elimination of HBV is extremely low or not achievable since virus capable of establishing persistent reservoir in the form of ccc DNA in hepatocytes (Delaney, 2013).

Epidemiology

Human body is a sole reservoir for HBV. However, the high replication activity of HBV leads to production of high concentration of viral particles circulating/present in blood and body fluids of infected person, and therefore, makes hepatitis B virus extremely transmissible. Most people become chronically infected at childbirth when the mother is a hepatitis B carrier (vertical transmission), while others become infected by close personal contact (infancy, unprotected sex) or by injections (medical and dental instruments or intravenous drug use) (horizontal transmission). The horizontal transmission risk of chronic of HBV infection incidence is 30% to 50% for the age group between birth and 5 years of age, decreases dramatically to 7–10% by aging. (McMahon, 2009a).

There are eight genotypes of HBV and their distribution varies in different regions of the world. Genotype A is found most frequently in North America, but it is also present globally. In Asia, genotypes B and C are dominant. Genotype D is prevalent in South Asia, Middle East and Southern Europe. Genotype E is frequent in sub-Sahara Africa while Genotype F is mostly found among Native Americans. Genotypes G and H are relatively uncommon (Fig. 1), (Magnius and Norder, 1995). It is generally accepted that HBV genotype and subgenotype are the certain variables to predict outcome of chronic HBV infection (McMahon, 2009a). The cross-sectional studies conducting the examination of relation of genotypes C and B to HCC incidence reveal that genotype C has a potential impact on increasing risk of HCC (Lee et al., 2003) (Fig. 1).

Chronic hepatitis B

Chronic hepatitis B (CHB) is described as necroinflammation in liver originating from prolonged presence of HBV, which is diagnosed by the persistent serum hepatitis B surface antigen (HBsAg) for 6 months or longer (Hollinger and Lau, 2006). The natural stages of CHB are classified as four major clinical phases based on levels of serum aminotransferase (ALT) and hepatitis B virus DNA (HBV DNA), presence of hepatitis Be antigen (HBeAg), and suspected immune status (McMahon, 2009b). These phases are: 1) Immunotolerant phase, 2) immunoeelimination or

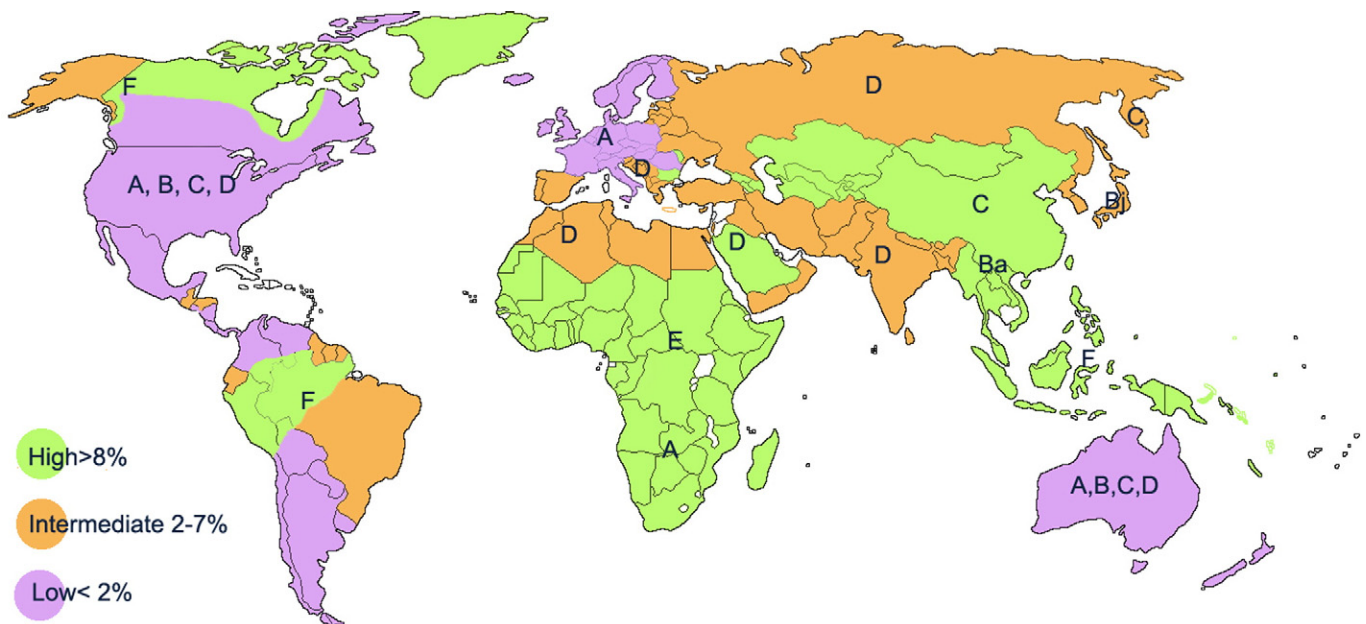


Fig. 1. Global distribution of HBV infection according to the WHO. (WHO vaccine-preventable diseases: monitoring system, in 2013 global summary. Geneva)

HBeAg-positive CHB phase, 3) non-replicative or inactive carrier phase, and 4) reactivation or HBeAg-negative CHB phase (Gish, 2008).

- 1) Immunotolerant phase: The individuals having HBeAg positive and high level of virus in blood (HBV DNA greater than 20,000 IU/mL) but normal liver enzyme level, are in the immunotolerant phase. These patients have potential to develop high level of liver enzymes that elevate the risk of cirrhosis.
- 2) Immunoelimination phase: Individuals who, are in the immunoelimination phase, are HBeAg positive and have a high level of HBV DNA in serum and elevated ALT. In liver biopsy moderate or severe necroinflammation is observed.
- 3) Non-replicative (inactive carrier) phase: Individuals exhibit HBsAg positive, HBeAg negative (or non-detectable), low level of HBV DNA (less than 2000–20,000 IU/mL) and normal ALT level. Since these patients have a risk to develop HCC in later stages of their life, monitoring of their status by performing HBV replication, serology, liver enzymes and synthetic tests every six months are required.
- 4) Reactivation phase: Individuals in this phase are HBeAg negative and anti-HBe positive. In patients who are in the non-replicative phase develop high level of HBV DNA and significantly elevated ALT, reactivation occurs.

The progression of HBV infection, are possible to vary, and patients might not proceed through all phases of the disease during the infection period (Okamoto et al., 1990). The pre-core and basal core promoter (BCP) mutations lead to HBeAg-negative CHB (Hunt et al., 2000; Okamoto et al., 1990, 1994) and moderate HBV replication sustains in these patients together with active liver disease.

Antiviral therapy for chronic hepatitis B

The aims of therapy for CHB are to improve the quality of life and increase the rate of survival by the prevention of progress of the disease to cirrhosis, liver failures, and HCC (European Association For The Study Of The, 2012). In the last 20 years, seven drugs were approved for curing of CHB: interferon alfa (IFN-alfa) and polyethylene glycol (PEG)-ylated interferon (PEG-IFN), as immune modulators, and lamivudine (LAM), adefovir (ADV), entecavir (ETV), telbivudine (LdT) and tenofovir (TDF) as nucleos(t)ide analogs. The first-line therapy currently consists

of PEG-IFN and the 2 NAs, ETV and TDF, which are potent HBV inhibitors and are known for their high resistance profile (Chang et al., 2006, 2010; Heathcote et al., 2011; Lai et al., 2006; Marcellin et al., 2008; Snow-Lampart et al., 2011). The eligibility of antiviral therapy has been recommended by several international guidelines (Table 1) (Liaw et al., 2012; Liver EAFTSOT EASL clinical guidelines, 2012; Lok and McMahon, 2009). LAM, the first nucleos(t)ide analogs, initiate a fast evolution in treatment of CHB, however it had a crucial downside (Yuen and Lai, 2011). Although about 16% of patients developed resistance after one year of treatment compared to placebo, LAM was associated with a good advantage of achieving HBV DNA suppression, normalization of ALT and seroconversion of HBeAg (Lai et al., 1998). Follow-up studies showed that LAM treated patients continued to develop resistance at a rate of 76% after 5 years (Lai et al., 2006; Moskovitz et al., 2005). The lowest resistance rates are observed in ETV (1.2%) and TDF (0%) (Snow-Lampart et al., 2011; Tenney et al., 2009). Recently, a study by Marcellin et al. (2013) on the long lasting treatment of patients including patients with advanced fibrosis or cirrhosis with tenofovir, confirmed that liver cirrhosis was reversible, with sustained HBV suppression and diminished activity of hepatitis. The endpoints of antiviral treatment were correlated by diminishing HBeAg and HBsAg and reaching the undetectable level of HBV DNA (Weinbaum et al., 2008).

Antiviral resistance is described as failure of primary or secondary treatments due to reduced susceptibility of a certain HBV mutant to the target drug. Although resistance predominantly causes failure of secondary treatment, it can result to primary treatment failure due to resistant mutant transmission or because of cross-resistance as a result of previous therapies (Pawlotsky et al., 2008). On the course of therapy there is risk of emergence of drug resistant mutants, in HBV DNA polymerase/reverse transcriptase increases by prolonged therapy (Kim et al., 2007; Lau et al., 2000). The resistance can be connected to acute hepatitis flare with decompensation of liver disease in particular individuals with advanced fibrosis (Liaw et al., 2004).

The rates of drug associated resistance differ among the nucleos(t)ide analogs based on their antiviral effectiveness and genetic barrier to evolve mutations. LAM and LdT have low genetic barrier and resistance can emerge with single nucleotide mutation. The resistance rate with ADV increased to 29% by year 5 (Hadziyannis et al., 2005;

Table 1
Treatment eligibility under international guidelines for the treatment of chronic hepatitis B.

Guideline	HBeAg-positive	HBeAg-negative	Cirrhosis
<i>AASLD</i>			
HBV DNA	>20,000 IU/mL	>2,000 IU/mL	Detectable
ALT	>2× ULN (3–6 months of observation with absence of spontaneous HBeAg loss)	>2× ULN	Any
Liver biopsy or non-invasive fibrosis assessment	If HBV DNA >20,000 IU/mL and ALT ≤2× ULN, and age >40 or family history of HCC	HBV DNA >2000 IU/mL and ALT > ULN	None
<i>APASL</i>			
HBV DNA	≥20,000 IU/mL	≥2000 IU/mL	Compensated: HBV DNA <2000 IU/mL monitor ALT, HBeAg or HBV DNA for 3 months, decompensated: treat
ALT	>5× ULN, if ALT 2–5 ULN (persistent for 3–6 months or concerns for hepatic decompensation)	>2× ULN (if persistent for 3–6 months) or has hepatic decompensation concerns	
Liver biopsy or non-invasive fibrosis assessment	If age >40 and HBV DNA ≥20,000 IU/mL and ALT <2× ULN	If age ≥40 and HBV DNA ≥2000 IU/mL and ALT <2× ULN	
<i>EASL</i>			
HBV DNA	>2000 IU/mL	>2000 IU/mL	Detectable
ALT	>ULN	>ULN	Any

Abbreviations: AASLD, American Association for the Study of Liver Diseases; APASL, The Asian Pacific Association for the Study of the Liver; EASL, European Association for the Study of the Liver; HBeAg, hepatitis B e antigen; ULN, upper limit of normal.

Papatheodoridis et al., 2005) since it has relatively low antiviral potency. The development of ETV resistance requires pre-existing LAM resistance, mutations (Colonno et al., 2006). The relatively low resistance rate of ETV at <1% in 5 years among previous treatment naïve patients is explained by the requirement of multiple mutations and its antiviral potency (Tenney et al., 2009). In contrast, for patients with pre-existing LAM resistance who were subsequently switched to ETV, resistance rate increased to 43% after 5 years of continuous therapy (Chang et al., 2010). This illustrates the important concept of the emergence of drug resistance in the setting of reduced genetic barrier. To date, there has no clinically relevant drug associated mutations with TDF or interferon therapy (Marcellin et al., 2013).

Monitoring on therapy

The initial evaluation of patient relying on HBV DNA quantification has high impact to monitor the treatment response and to perform early detection of virological failure. The ideal quantitative HBV assay has to exhibit broad dynamic range around five orders of magnitude (Pawlotsky et al., 2008). Since the real-time PCR quantification assays possess several advantages, they are recommended for monitoring during therapy (Lok and McMahon, 2009). According to International treatment guidelines (European Association For The Study Of The Liver, 2012), all patients should be tested for serum HBV DNA, ALT, liver function tests, and HBeAg/anti-HBe prior to initiating treatment. While on therapy, serum HBV DNA is monitored to determine antiviral effect, efficacy and end-points.

Antiviral effect is an early indication whether the patient is responding to therapy. An antiviral effect is defined as a $\geq 1 \log_{10}$ IU/mL reduction of serum HBV DNA from baseline within 3 months of starting therapy. Primary antiviral treatment failure is the failure to achieve more than $1 \log_{10}$ decrease from baseline within 3 months of starting therapy. Secondary antiviral treatment failure is defined by a rebound of serum HBV DNA $\geq 1 \log_{10}$ IU/mL from in patients with an initial antiviral treatment effect as confirmed by two consecutive determinations at a 1-month interval. The main causes of primary and secondary antiviral treatment failure are poor adherence to therapy and selection of drug-resistant HBV mutants.

Antiviral efficacy can be defined as the ability of a given therapy to achieve an undetectable HBV DNA applying a given HBV DNA assay. In clinical trials, treatment efficacy can be assessed by measuring both the mean or median \log_{10} reduction of HBV DNA level and the proportion of patients with undetectable HBV DNA (below the threshold of the assay) at various treatment time intervals (Pawlotsky et al., 2008).

The aim is to achieve optimal HBV DNA suppression to as a low level as possible to avoid drug-associated resistance, and to ensure adequate virological suppression that will then lead to histological improvement.

Endpoint of therapy can be defined as sustained response off treatment while the ultimate goal of therapy is HBeAg seroconversion (Lok and McMahon, 2009). For HBeAg positive CHB, sustained response off treatment can be defined as sustained HBe seroconversion, HBV DNA suppression and ALT normalization. In patients with HBeAg-negative CHB, the sustained response off treatment is defined as sustained HBV DNA suppression and ALT normalization. Since virological relapse with increased HBV DNA levels is common with nucleos(t)ide analogs, treatment duration with these agents tends to be extended with the goal to achieve continuous inhibition of HBV replication in both HBeAg-positive and -negative patients.

For standardization of comparison of different therapeutic agents, assessments of HBV DNA, ALT and HBeAg/anti-HBe should be performed at baseline and then every 1–3 months to evaluate viral kinetics/biochemical response. In clinical practice, 3 to 6-month assessments are adequate. In both clinical trials and clinical practice, if a serum HBV DNA measurement indicates that the patient may have primary or secondary treatment failure, but there is no elevation in serum ALT, a second serum HBV DNA sample should be assayed for confirmation one month later (Lok and McMahon, 2009).

Cancer relationship and co-infections

According to the WHO (2013) CHB is a leading risk factor for cancer in low-and middle income countries, and HCC, the most common type of liver cancer, with an estimated 746,000 deaths in 2012, is the 2nd most common cause of cancer death worldwide (Marcellin et al., 2013). There is high ecologic correlation between areas of HBV prevalence and HCC incidence and mortality (El-Serag, 2012). Hepatitis B infection is generally acquired by mother-child transmission in areas with high HCC incidence, mainly in Asia. Prospective cohort studies showed a range between 5 to 100-fold increase in the risk of developing HCC in CHB infected individuals (Chen et al., 2006, 2007; Iloeje et al., 2007). Hepatocellular carcinoma carries a poor prognosis with a global mortality to incidence ratio of 0.95 and the one-year survival rate remains less than 50%. According to recent meta-analysis results (Shen et al., 2012), antiviral therapy reduced 5-year cumulative incidence of HCC by 7.1% in patients with CHB. According to another study (Lai and Yuen, 2013), treatment with nucleoside analog therapy appears to be more effective in lowering HCC development compared to IFN- α , probably through more potent and persistent suppression of viral

replication, though according to the authors, the effect may be blunted with the occurrence of resistance. Hepatocellular carcinoma prevention by long term monitoring of those with inactive diseases and oral antiviral suppressive treatment for those, who meet the treatment criteria has the potential to be an innovative, simple and effective cancer prevention strategy that many countries can adopt (Toy, 2013).

Co-infection of HBV with HCV and/or HIV causes a rapid progression to end-stage liver disease and death (Tuma et al., 2010). HBV infection is prevalent in 25% of HIV infected people (Hoffmann and Thio, 2007). There are globally 15 million people living with HBV–HCV co-infection, 3 million with HBV–HIV and 0.5 million with HBV–HCV–HIV co-infection (Fernandez-Montero and Soriano, 2012). Cooper et al. (2009) suggests that chronic viral hepatitis may diminish the gains of HIV anti-retroviral therapy in sub-Saharan Africa. Successful treatment of HBV in HIV is dependent on implementation of pre-highly active antiretroviral therapy screening programs for hepatitis B surface antigen.

Public health: screening, diagnostics, cost-effective tools and assays

Diagnosis is an initial strategy to HBV-care and treatment. The one-time HBV screening may identify most individuals and giving the opportunity to vaccinate those who are susceptible to effective antiviral therapy before the development of advanced liver disease. Hepatitis B vaccination as well as cost-effective antiviral treatment is an advantage in the case of screening and justifies detecting people in high endemic areas. Reduction in screening assay costs could potentially mean that additional funding would be available for programmatic resources (treatment related or vaccination policies). In many high risk areas, particularly those in Asia, HBV is transmitted from mother to newborn (vertical transmission); as many as 90% of infected babies develop chronic infection (El-Serag, 2012). Hepatitis B screening during pregnancy and postpartum immunoglobulin and HBV vaccination in neonates born to HBV-infected mothers is far from being commonly accepted. In the United States, individuals are from high-prevalence countries, HIV-positive, injection drug users, close contacts with HBV infected person carries high risk for HBV infection (Weinbaum et al., 2008). Testing for CHB meets established public health screening criteria as formulated originally by Wilson and Jungner (1968): (1) it is a serious health disorder that can be diagnosed before symptoms develop; (2) it can be detected by reliable, inexpensive, and minimally invasive screening tests; (3) chronically infected patients have years of life to gain if medical evaluation, monitoring, or treatment is initiated early; and (4) the cost of screening are reasonable in relation to the anticipated benefits.

At individual level the association of disease progression with increased cost of disease management suggests that measures to prevent or delay progression by e.g. antiviral treatment of CHB related liver diseases will be economically beneficial (Toy, 2013). At the population level, however, the impact of therapy on the overall number of people with CHB will remain limited as long as the majority of infected patients will not receive treatment due to lack of resources for optimal treatment.

The United States Preventive Task Force (USPSTF) found convincing evidence that antiviral treatment in patients with chronic HBV infection is effective in improving intermediate and clinical outcomes (Chou et al., 2014). Given the accuracy of the screening test and the effectiveness of antiviral treatment, the USPSTF recommends that screening is of moderate benefit for populations at high risk for HBV infection, and this recommendation applies to screening for HBV infection in asymptomatic, non-pregnant adolescents and adults who have not been vaccinated and other individuals at high risk for HBV infection (Chou et al., 2014).

Serologic assays

Serologic assays have been widely used to diagnose HBV infection throughout the disease course. According to serum biomarkers, HBV

serologic assays can be designed to detect HBsAg and HBeAg, as well as anti-HBs, anti-HBc and anti-HBe antibodies. As shown in Fig. 2, acute HBV infection is diagnosed with HBsAg and anti-HBc IgM positive. By contrast, chronic HBV infection is characterized by HBsAg-positive for at least 6 months and the presence of anti-HBc IgG.

If the infection is resolved, anti-HBs and anti-HBe antibodies will be positive, which in turn suppresses the level of HBsAg and HBeAg to be below detectable. If the infection becomes active during the chronic stage, over production of HBeAg will negate anti-HBe antibody. It should also be noted that anti-HBc IgM is the only serum biomarker during the window period. According to the reaction mechanisms, HBV serologic assays can be divided into well or particle based enzyme immunoassay (EIA), chemiluminescent immunoassays (CIA) or electrochemiluminescent immunoassay (ECIA). For example, the ARCHITECT HBsAg Qualitative assay (Abbott) is an immunoassay that qualitatively detects HBsAg using a microparticle-based CIA technology (Lou et al., 2011). In this method, paramagnetic microparticles coated with anti-HBsAg monoclonal antibodies are used to capture HBsAg in serum or plasma. The captured HBsAg is then recognized by another type of acridinium-labeled monoclonal antibodies. The resultant chemiluminescence due to addition of hydrogen-peroxide and sodium hydroxide in the mixture is measured using the ARCHITECT System optics. It has been reported that this assay has an improved sensitivity in detection of HBV variants (Lou et al., 2011).

To prevent under diagnosis of HBV, serologic assays targeting various biomarkers should be used in combination. The reasons have two-folds; one is to increase the capability to detect HBV variants and the other is to include multiple biomarkers that vary at different clinical stages. One recent study showed that the combination of the ARCHITECT Anti-HBc II and HBsAg assays had a sensitivity of 100% and a specificity of 90% for detection of HBV among solid organ donors compared to the COBAS Ampliprep/TaqMan test for HBV DNA (Ba Alawi et al., 2013). Further, this study showed that the HBcAb assay alone missed one acute HBV infection, highlighting the importance of detecting multiple HBV serum biomarkers for optimal diagnosis. However, combination strategy will inevitably increase the cost of clinical diagnosis and blood screening. In resource-rich settings, NATs are widely used to minimize blood-borne transmission (Stramer et al., 2011), which will be discussed in detail in the next section. In contrast, rapid lateral flow tests are commonly used for blood screening because of affordability and simplicity. Although the sensitivity and subtype coverage have been significantly improved over years, one comprehensive study

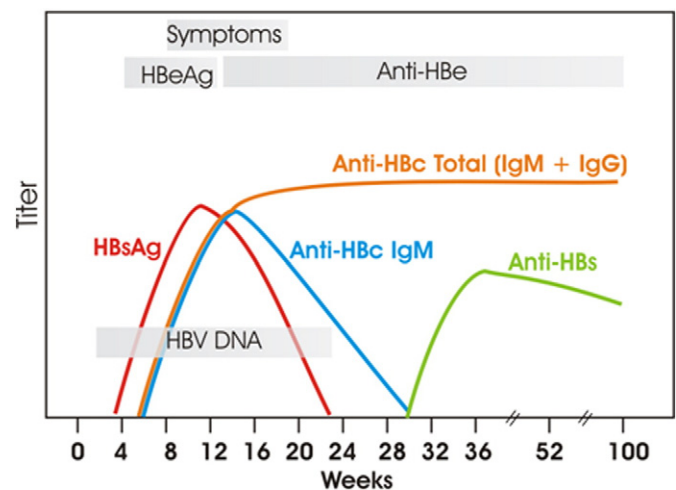


Fig. 2. Serum biomarkers for acute HBV infection. During acute HBV infection, HBsAg is the first antigen that appears. It clears out prior to the emergence of anti-HBs antibody. The period in between is called “window period”, in which anti-HBc IgM may be the only serological indicator of HBV infection. Therefore, HBsAg and the total anti-HBc antibodies (including IgM and IgG) are employed for HBV diagnosis.

showed that 70 HBsAg tests had substantially different performances and that 17 of these rapid assays showed low sensitivity in detecting various HBV genotypes (Scheiblaue et al., 2010). Thus, serologic assays need to be further improved in terms of sensitivity, genotype coverage, and multiplexing capability.

Nucleic acid test

In diagnosis of viral hepatitis, nucleic acid tests (NATs) play key a role from several aspects such as in reassuring diagnosis, defining virus genotype, assessing the risk of infection/transmission and guiding antiviral therapy. Clinically, NATs are commonly used to detect/quantify HBV DNA in patients. As stated above, the serum biomarkers vary during the course of disease, and HBsAg assays can under-detect acute HBV infection due to the absence of this antigen during the 'window period' or mutations in this antigen. Thus, NATs are an important supplemental methods to confirm the infection of HBV, particularly during the window period when specific antibodies are not yet generated. Since the level of HBV DNA is an indicator of HBV replication in the host, it can be used to assess treatment efficacy. HBV NATs also play an important role in identifying a subgroup of HBV infection, i.e., occult HBV infection (Allain, 2004). This subgroup of patients are characterized by HBsAg negative but HBV DNA positive (~200 copies/mL). In addition to its role in the clinical management of infected individuals including diagnosis and treatment monitoring, NAT has been widely implemented in blood banks to improve transfusion safety (Stramer et al., 2011). Because of the prohibitive cost of screening individual units of blood by NAT, a "minipool" strategy (16 plasma samples) is used to increase cost-effectiveness. However, this strategy demands sensitive and well-quality controlled assays for blood screening.

To date, two main amplification technologies are established to detect HBV DNA; one is polymerase chain reaction (PCR), and the other is isothermal amplification such as transcription-mediated amplification (TMA). In PCR, the most conserved region in HBV genome is used as the template for amplification by two flanking primers. The cyclic process can be divided into three phases i.e., denaturation, annealing and amplification, and the template of HBV DNA is doubled by Taq polymerase at the completion of each cycle. The use of fluorescent TaqMan probe improves the detection limit and expands the dynamic range of quantification in real-time PCR compared to end-point PCR. The PCR based technology has been utilized in commercial assays developed by Roche and Abbott. TMA generally starts the amplification with RNA templates. For HBV DNA detection, an additional RNA transcription is needed (Kamisango et al., 1999). Briefly, after initial denaturation, a promoter-primer is used to initiate RNA transcription by RNA polymerase. Once an RNA transcript is generated, a second primer is used to generate DNA-RNA intermediates by reverse transcriptase. RNase H then cleaves the RNA in the DNA-RNA intermediates, and reverse transcriptase completes the formation of double-stranded DNA (dsDNA) with addition of a T7 promoter. The RNA polymerase subsequently utilizes the synthesized dsDNA templates to rapidly generate a substantial amount of RNA amplicons for chemiluminescence detection. This TMA approach has been developed by Gen-Probe as an integrative part in a triplex assay together with HIV and HCV for blood screening. The performance of the FDA-approved or CF-marked HBV NAT assays is summarized in Table 2 (Chevaliez et al., 2012).

Miniaturized NAT devices

There have been some efforts to miniaturize HBV NAT in a portable medical device to increase the accessibility of HBV NAT in resource-limited settings. For example, a rapid real-time microchip-based PCR system was developed for rapid detection of HBV DNA (Cho et al., 2006). The microchip completed 40 cycles of HBV DNA amplification by real-time PCR in less than 20 min with the aid of a portable

amplification instrument containing a silicon-based heater plate, a cooling fan, an optic unit and a microprocessor. Three independent clinical evaluation studies (n = 563) showed sensitivity of 94% and specificity of 93%. In another study, an isothermal amplification technology referred to as loop-mediated isothermal amplification (LAMP) was developed to detect HBV DNA in a medical device without using fluorescence reagents (Lee et al., 2007). Since the temperature for LAMP can be controlled using a simple external heating source and expensive fluorescence detection can be replaced by turbidity-based detection, LAMP has shown promising applications for POC testing (Mori and Notomi, 2009; Notomi et al., 2000). Based the LAMP approach, Lee et al. (2007) were able to detect HBV DNA using the LAMP-based approach in 60 min with a detection limit of 60 copies/reaction.

Recent advances in nanotechnological tools for diagnosis of HBV and HBV co-infections

Advances in nanotechnology and microfluidic platforms rapidly enable significant developments of biosensing technologies and medical diagnostics (Wang et al., 2012a, 2012b). Particularly, advances in micro-nanofabrication technologies allow for developing rapid, user-friendly, accurate and specific diagnostic tools/methods, which achieve low detection limits of the target analytes/cells. Basically, a biosensing platform comprises of three main components (i.e., bio-recognition element, transduction mechanism and read-out system) that provide response for the physicochemical changes on the sensor surface. This response is translated to a read-out platform in terms of electrochemical, electrical, optical and mechanical strategies as discussed in the following sections. Fig. 3 summarizes the milestones of development of several sensor platforms that were utilized by micro/nanotechnologies. The various nanomaterials and advanced fabrication technologies were engaged cooperatively to produce new generation diagnostic tools.

Electrochemical biosensing strategies and technologies based on carbon nanostructures and metal/inorganic nanoparticles

Electrochemical-based sensing technologies have been one of the most studied platforms in the detection of HBV biomarkers such as nucleic acids, capsid proteins or other components. In these detection platforms, specific recognition elements are immobilized on a sensor surface or freely present in the sensing solution, conductivity/resistivity are monitored upon the recognition and capture of target molecules. A recent study demonstrated that simultaneous detection of 5-type HBV antigens (i.e., hepatitis A, B, C, D, E) is facilitated by an electrochemical immunosensor array (Tang et al., 2010). The described assay is based on following simple methodology; 5-type HBV antibodies were immobilized onto a self-made electrochemical sensor array using gold nanoparticles and protein A as matrices, and the immunosensor array is then used to capture their corresponding antigens from sample solution with a 1-step capture format (Fig. 4a–b). It is shown that described electrochemical assay has produced similar results with respect to ELISA method. The advances in the discovery of new chemical and biological indicators, attention towards platforms, have been increasingly attracting due to the low power requirement and simple design (Palek and Fojta, 2001; Ye et al., 2003). For instance, electrochemical DNA biosensors hold a great promise to develop biosensors for HBV diagnosis. These biosensors have demonstrated a broad range of application by utilizing DNA hybridization, mismatch analysis, direct DNA analysis-based on guanine signal, and PCR-based approaches (Ye et al., 2003). In the perspective of DNA hybridization approaches, sensor surface is chemically modified with single stranded DNA molecules, which are complementary to HBV specific DNA sequences. In one of the latest study, electrochemically deposited Au nanoparticles on single walled carbon nanotube (SWCNT) arrays were utilized, to self-assemble single-stranded probe DNA on the SWCNT/Au platform for HBV specific DNA sequences detection (Fig. 4c–d) (Wang et al., 2013a).

Table 2
Specifications of FDA-approved or CF-marked HBV NAT assays (Chevaliez et al., 2012).

Assay	Manufacturer	Method	Sample volume (μL)	Limit of detection (IU/mL)	Dynamic range (IU/mL)
COBAS TaqMan HBV test	Roche Molecular Systems	Real-time PCR	650	6–10	$29-1 \times 10^8$
COBAS Ampliprep-COBAS TaqMan (CAP/CTM) test, v2.0	Roche Molecular Systems	Real-time PCR	650	20	$20-1.7 \times 10^8$
Real-time HBV	Abbott Molecular	Real-time PCR	200 or 500	10 (for 500 μL) 15 (for 200 μL)	$10-1.0 \times 10^9$
Artus HBV QS-RGQ assay	QiaGen	Real-time PCR	1000	10.2	$31.6-2 \times 10^7$
APTIMA HBV quantitative assay	Gen-Probe, San Diego, CA	Real-time TMA			

Electrochemical-based sensing technologies have effectively employed single carbon nanotubes (CNTs) (Heller et al., 2005) or CNT network (Star et al., 2006) as electrode materials since they provide adequate electrocatalytic activity (Fig. 4e–j). Upon recent development in micro-nanofabrication and integration technologies, graphene (Jung et al., 2010a; Kwon et al., 2013; Liu et al., 2013) based devices have

been accessible for several of applications in biological as well as clinical detection. An interesting example was reported the use of a multilayered free standing graphene film as an electrochemical biosensor for rotavirus detection (Liu et al., 2011). This study showed that 105 pfu/mL of input cells were detected with ca. 30.7% sensitivity. Another study reports the successful modification of graphene by bifunctional

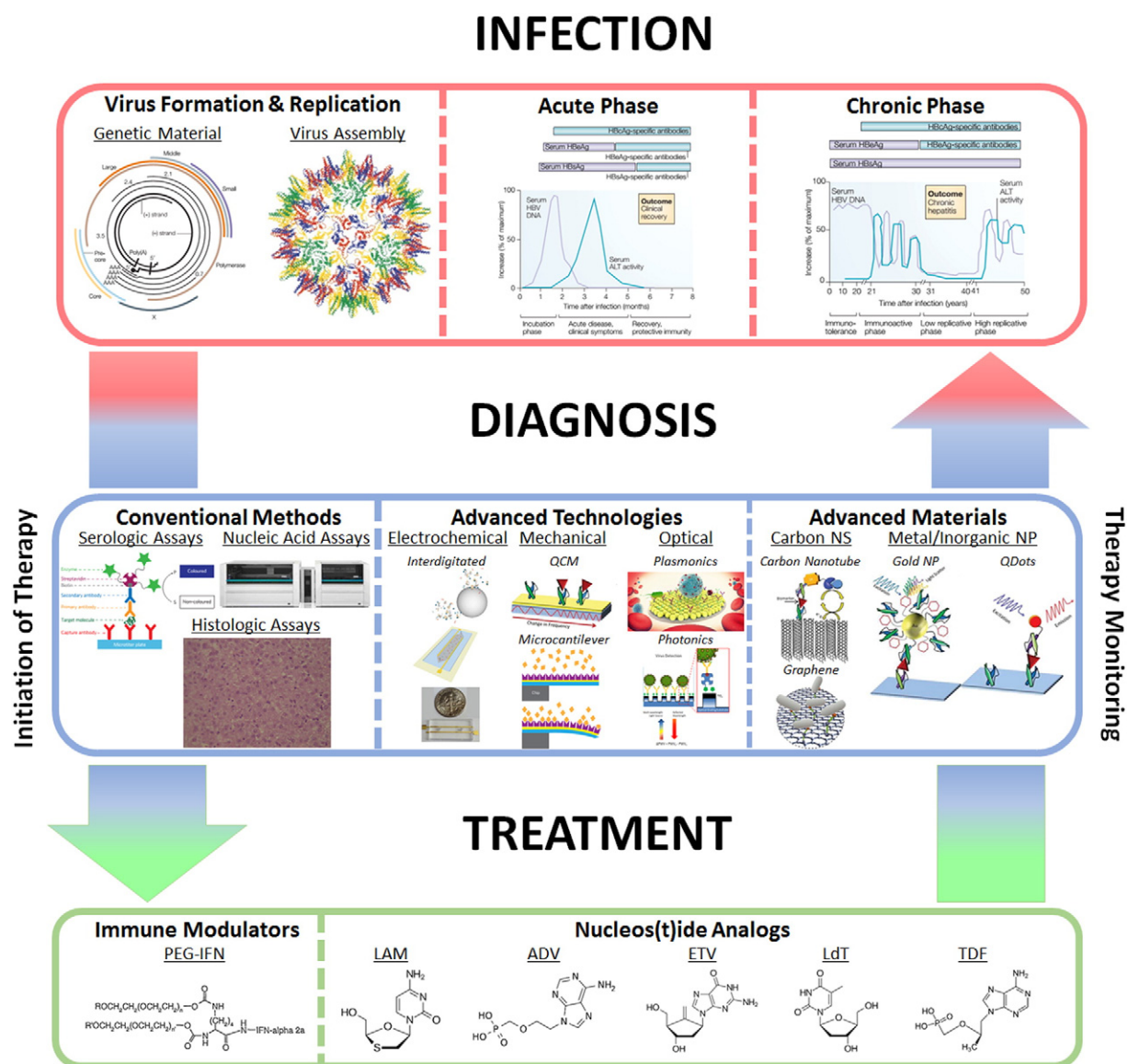


Fig. 3. HBV infection, diagnosis and treatment diagram. Upper panel illustrates the viral genetic material, and hepatitis B virus. Serum level of HBV DNA and ALT activity in acute and chronic phase. Middle panel shows the *conventional methods*: serologic assays and nucleic acid assays, *advanced technologies*: electrochemical sensor (interdigitated electrodes made of graphene, carbon nanotubes), mechanical sensors (quartz crystal microbalance and microcantilevers) and optical sensors (plasmonic platforms made of gold nanoparticles and photonic crystal based platforms). Lower panel shows chemical structure of immune modulator PEG-IFN and nucleos(t)ide analogs: LAM, ADV, ETV, LdT, TDF.

Reproduced from: Arlett et al., 2011; Mannoor et al., 2012; Kierny et al., 2012; de la Rica et al., 2012; Shafiee et al., 2013, 2014; Ferreira et al., 2012; Rehmann and Nascimbeni, 2005; Inci et al., 2013; Wynne et al., 1999.

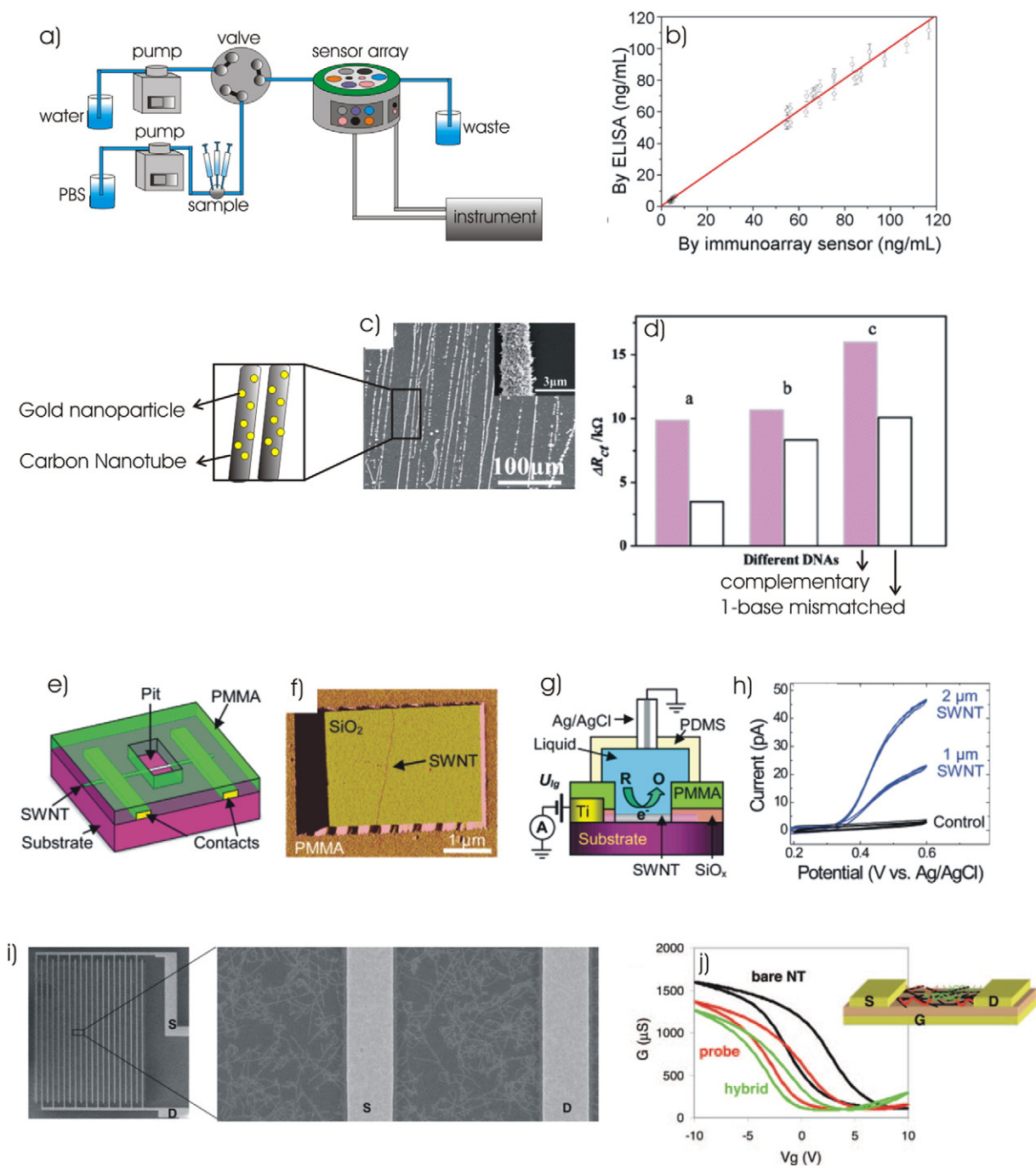


Fig. 4. An electrochemical immunosensor array enabling simultaneous detection of hepatitis virus antigens (hepatitis A, B, C, D, E) were utilized to develop a facile assay methodology. Five-type HBV virus antibodies functionalized electrochemical sensor that were made of gold nanoparticles and protein A. The prototype immunosensor array used for a single-step antigen capture. The detection relies on the potential change between priori and post antigen-antibody interaction. a) Illustration shows the immunosensor array integrated into fluidic system. Graphs (b) shows comparison of the results obtained by using the developed immunosensor array and the standard ELISA for HBV samples from 43 serum specimens (Tang et al., 2010). c) DNA biosensors proposed by Wang et al. (2013a), FE-SEM images of aligned SWCNTs-gold nanoparticle hybrid sensors for the detection of hepatitis B DNAs. d) Electrochemical response of the proposed sensor that compares 1-base mismatched and complementary DNAs. e) The use of individual single-walled carbon nanotubes as nanoelectrodes for electrochemistry (Heller et al., 2005), micro-fabricated device schematic. CNTs are grown on Si wafers with 500 nm thermal SiO₂ and contacted by Ti leads. A layer of SiO_x and PMMA is used as insulating layer, in which windows are opened to selectively expose the SWNTs. (f) AFM-amplitude image of an exposed section of a CNT crossing the bottom of a pit through the PMMA and SiO_x layers. (g) Low current measurement setup. The SWNT is exposed to a solution containing a redox-active species. (h) Sampled current voltammograms measured from two metallic SWNT devices (1 μm and 2 μm exposed). i) CNT network field-effect transistors (NTNFETs) that function as selective detectors of DNA immobilization and hybridization (Star et al., 2006). Scanning electron microscopy image of the random network NTNFET device. The distance between source (S) and drain (D) interdigitated metal electrodes is 10 μm. j) The response of the micro-fabricated device before and after incubation with 12-meroligonucleotide capture probes (5-CCT AAT AAC AAT-3), as well as after incubation with the complementary DNA targets.

perylene-tetracarboxylic acid di-imide (PDI) acting as a bridge to graphene and gold nanoparticles (AuNPs) (Zhang et al., 2014). By the fabrication of simple label-free impedance DNA biosensor, detection of human immunodeficiency virus 1 (HIV-1) pol gene sequence was achieved with a limit of detection $1.2 \cdot 10^{-15}$ M. More complex

microfabrication strategies were used to fabricate of liquid-ion gated field-effect-transistor (FET) by using large graphene micropattern (GM) hybrids with self-assembled polypyrrole nanoparticle (CPPyNP) (Kwon et al., 2013). It was hypothesized that the introduction of CPPyNP array enlarge surface areas and provide stable

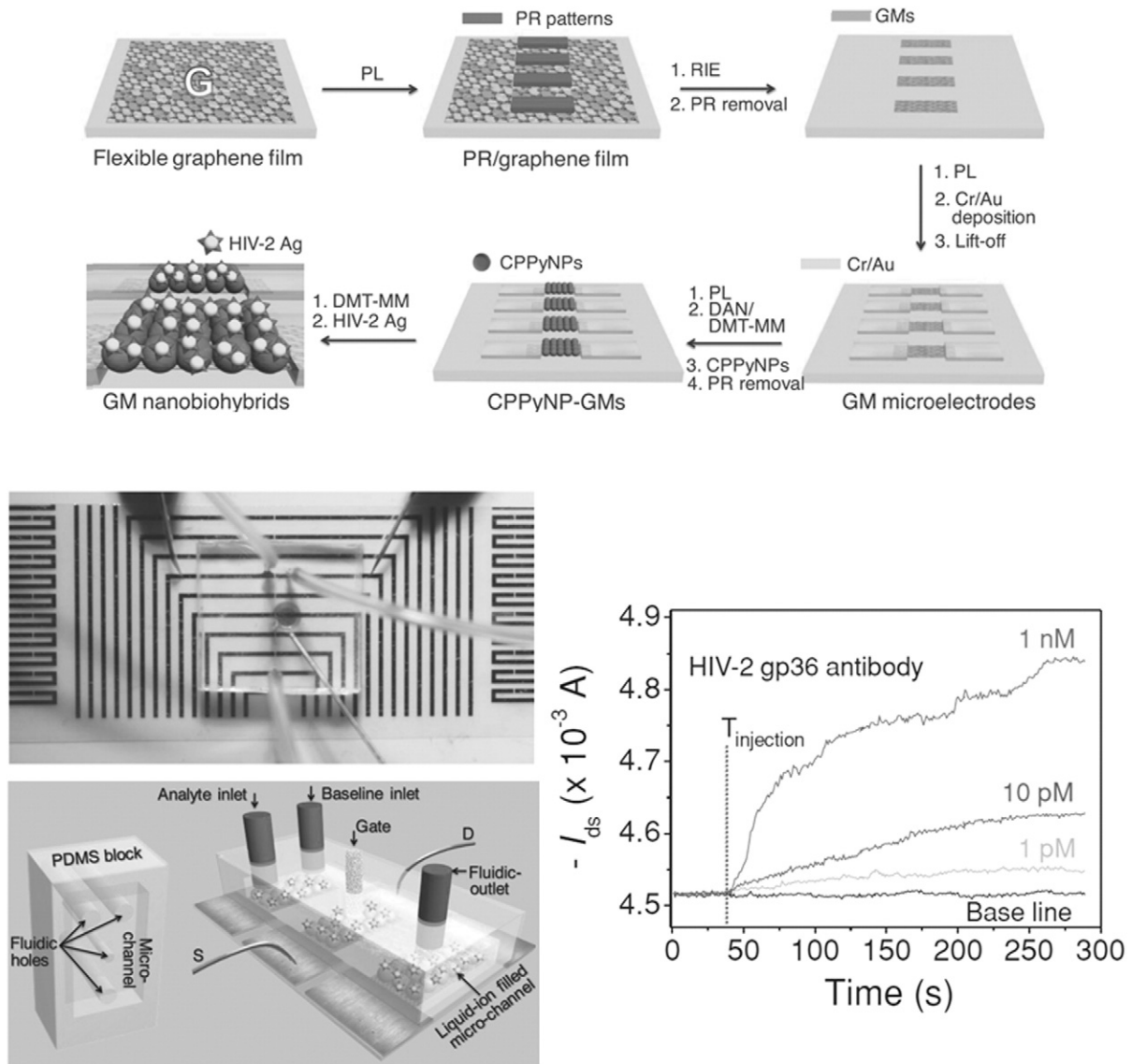


Fig. 5. The illustration of the FET-type micropatterned graphene nano-biohybrid immunosensor (GMNS) relies on GMs with close-packed carboxylated polypyrrole nanoparticle (CPPyNPs) on the flexible substrate (photolithography, PR: photoresist, RIE: reactive-ion etching, GM: graphene micropattern). Images (at middle left) shows flexi-FET coupled with microfluidic channel and illustration (low left) of the GMNS integrated microchannels. The utilized system was tested against leakage that exhibits no leakage. Graph at lower right shows I_{ds} responses of the device for different HIV-2gp 36 Ab concentrations (1 to 10 pM, and 1 nM) at $V_{ds} = -10$ mV ($V_g = 0$ V), a gradual increase in current was recorded upon Ab injection into the microchannel. The greater Ab concentrations the higher current values and saturation occurs rapidly (Kwon et al., 2013).

sensing in liquid, resulting that allow recognition of HIV biomarker at a very low concentration ~ 1 pM (Fig. 5).

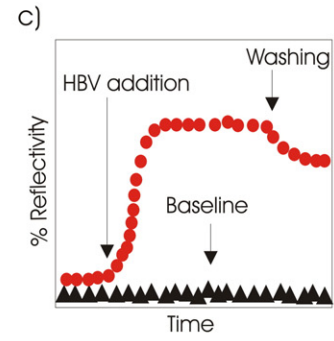
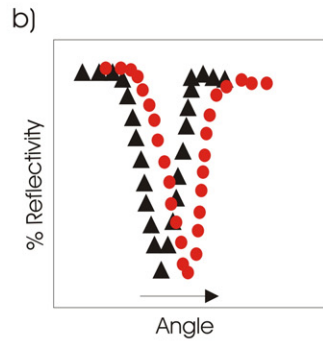
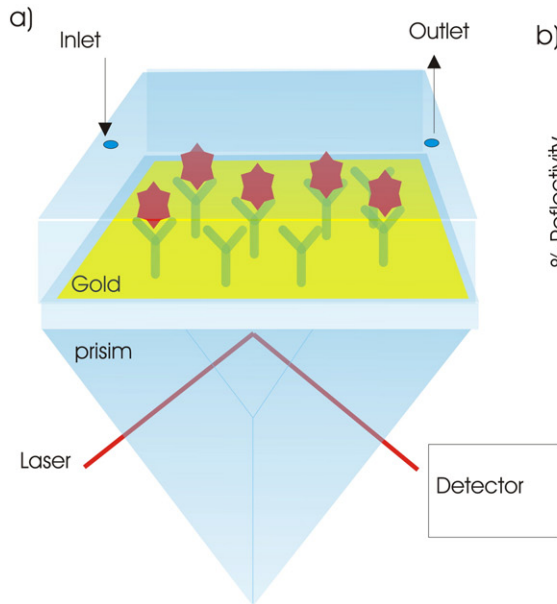
Beside utilization of their electrical properties, graphene and graphene oxide (GO) nanomaterials have been used as energy acceptors with their π -systems for viral molecular recognition based on fluorescence resonance energy transfer (FRET) (Zhang et al., 2012). For instance, GO-based immuno-biosensor was used for detecting a rotavirus (Jung et al., 2010b). In this sensor, sample containing viruses was incubated on GO micropatterns functionalized with specific

capture antibodies and then gold nanoparticles (AuNPs) labeled with detection antibodies were introduced on top. Rotaviruses could be detected down to 10^5 pfu/mL using FRET between GO sheets and AuNPs. This limit of detection (LOD) value was comparable with conventional ELISA tests. GO was also used as a quencher of quantum dots (QDs) fluorescence for multiplex detection of human Enterovirus 71 (EV71) and Coxsackievirus B3 (CVB3) (Chen et al., 2012). Two colored QDs were labeled with different virus capture antibodies and mixed with GO. Without the presence of target virus, QDs with antibodies were quenched by

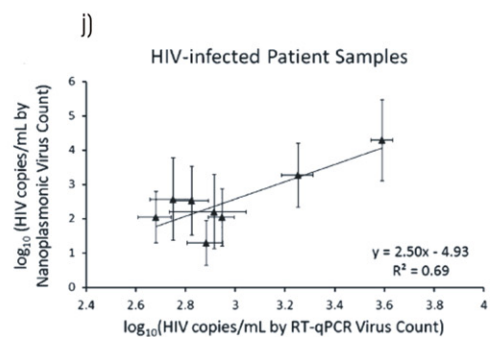
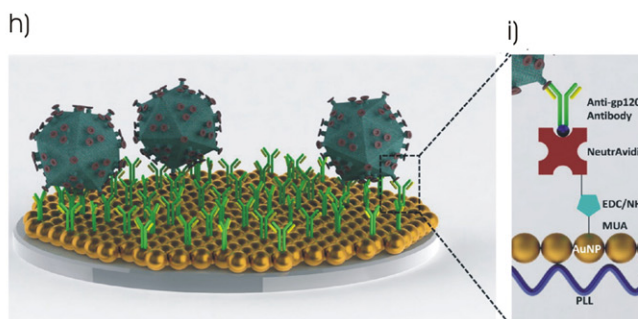
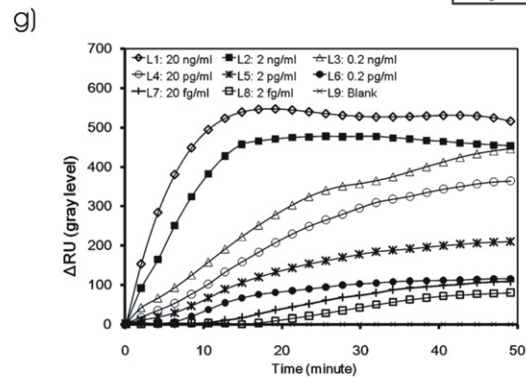
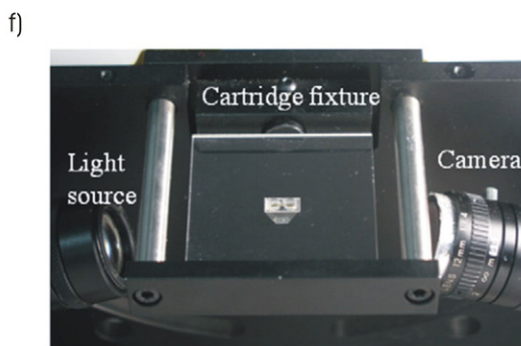
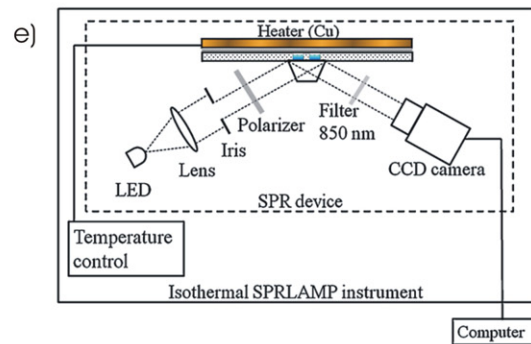
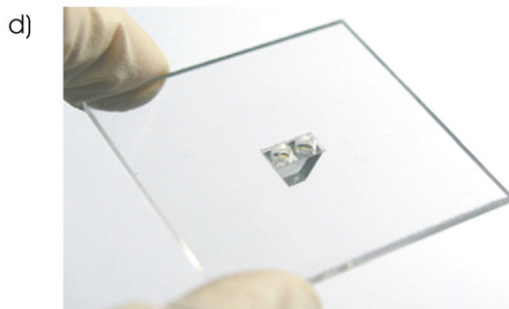
Fig. 6. a) Schematic illustration of SPR microfluidic device that is used for the development of SPR based HBV detection technology. The described assay is designed to detect recombinant HBV surface antigens (HBsAg) by immobilizing anti-HBsAg polyclonal antibodies to a dextran layer of N-hydroxysuccinimide activated CM5 sensor chips. b) Typical angular shift upon binding event c) real time monitoring of binding of HBV on to modified sensor surface (Tung et al., 1998) d) SPR-LAMP platform. Cartridge is constructed by integrating a polymethyl methacrylate (PMMA) with two micro-wells and a 50 nm Au film coated polycarbonate (PC) prism. e) Block diagram of SPR-LAMP microfluidic device. f) Top view of the fluidic compartment. g) Data collected by the SPR-LAMP set up. The LAMP reaction is performed in the micro-wells, and the changes in the refractive index of reaction mixture is recorded in real-time (Chuang et al., 2012). h) Nanoplasmonic viral load detection platform, HIV was captured on the antibody immobilized biosensing surface. i) Schematic representation of surface modification strategy to capture HIV on the biosensing surface. j) Viral load ranging $1.3 \pm 0.7 \log_{10}$ copies/mL to $4.3 \pm 1.2 \log_{10}$ copies/mL (Inci et al., 2013).

GO. With the target viruses, QDs were released from GO and they emitted strong fluorescent signal. This homogenous assay offers fast detection of EV71 and CVB3 down to 0.42 and 0.39 ng mL⁻¹, respectively.

A similar approach was conducted for the detection of HBV surface-antigen gene down to 10.4 nM using graphene and probe DNA labeled with QDs (Zhang et al., 2012). HBV, HIV and syphilis (*Treponema*



HBV
 Anti-HBsAg



pallidum) genes were also able to quantify using GO and probe DNA functionalized silver nanoparticles (AgNPs) (Liu et al., 2013).

The current demand induces quick advancement of one/two dimensional carbon-based sensors and enables validation of electrochemical detection methods by using clinical samples. The much broader applications including use of electrocatalytic activities of graphene, promise enhanced selectivity and sensitivity for these sensors. However, the extreme hydrophobic nature of the carbon interface, stands as main drawback limiting applicability since it causes severe aggregation, nonspecific binding, denaturation problems for delicate biological samples. The major future challenges to develop of CNT and graphene based biosensors for clinical applications requires miniaturization, easy-fabrication and with minimal non-specific binding as well as high sensitivity and rapid response.

Optical biosensing strategies: surface sensitive and nanoparticle assisted sensing technologies

Optical biosensors are powerful tools and alternative strategies to conventional analytical detection methods/techniques by improving sensitivity and specificity, and allowing us to develop cost effective tools for clinical diagnostics (Dey and Goswami, 2011). Optical biosensing technologies also provide significant advantages for real-time, label-free and reliable detection of targets of interest e.g., nucleic acids, peptides, and cells from biologically relevant media (Shafiee et al., 2013), (Dey and Goswami, 2011; Soldatkin et al., 2003). Surface Plasmon Resonance (SPR)-based sensors, one of the most common optical biosensors, employ conventional surface plasmons propagating along planar structures, and monitor binding/capture events on biosensing surface by real-time recording the changes in refractive index of metal-dielectric interface (Homola, 2008; Sevimli et al., 2012; Tokel et al., 2014). By utilizing surface plasmon strategy, a user-friendly, inexpensive SPR biosensing cartridge-based loop-mediated isothermal amplification (LAMP) method was developed for the on-site detection of the hepatitis B virus (HBV) (Liaw et al., 2012). In nucleic acid amplification assays, LAMP-based nucleic acid detection has become a preferred method for in-field screening by providing high amplification efficiency and specificity, and enabling higher DNA yield compared to conventional PCR methods (Harper et al., 2010; Liaw et al., 2012). However, this detection strategy requires a labeled indicator to measure and monitor DNA polymerization (Tomita et al., 2008). Thus, SPR-integrated LAMP platform provides a label-free, highly sensitive and versatile detection method and addresses the current challenges in LAMP amplification strategies (Chuanga et al., 2012). In this platform, SPRLAMP cartridge was first constructed by integrating a polymethyl methacrylate (PMMA) with two micro-wells and a 50 nm Au film coated polycarbonate (PC) prism (Fig. 6). The LAMP reaction was completed in micro-wells, and changes in the refractive index of reaction mixture were recorded in real-time. In this method, highly conserved fragments of HBV DNA templates (323 bps) were detected with a sensitivity of 2 fg/mL in 17 min. Thus, SPRLAMP platform provided a novel flexible, inexpensive, rapid nucleic acid detection system with high sensitivity and specificity, and in future, this technology would be adapted to detect other viruses in complex biological samples for resource-constrained countries. Another SPR-based HBV detection technology was reported to detect recombinant HBV surface antigens (HBsAg) by immobilizing anti-HBsAg polyclonal antibodies to a dextran layer of N-hydroxysuccinimide activated CM5 sensor chips (Hwang et al., 2005). HBsAg is reported as the most important biomarker for diagnosis HBV, and its serum levels exhibit an acute and chronic HBV infection and probable infectivity (El-Ghitany and Farghaly, 2013; Wang et al., 2010b). This microfluidic-integrated SPR platform is utilized to examine the binding characteristics between recombinant HBsAg and polyclonal antibody, and this binding analysis demonstrated that approximately six antibody molecules captured one HBsAg molecule at saturation level. The highest level of the linearity range was reported

as ~40 µg/mL, which is much higher than conventional ELISA platforms (~100 ng/mL) (Hwang et al., 2005).

The localized surface plasmon resonance (LSPR) that utilizes collective oscillations of nanoparticles' conduction band electrons and monitors association and/or dissociation of bioagents/molecules onto metal nanoparticles leading to changes in their individual wavelength (Sherry et al., 2005). Optical biosensing platforms based on LSPR technology have been used to monitor HBsAg levels for rapid and highly sensitive HBV detection (Wang et al., 2010b). In this study, anti-HBsAg monoclonal antibodies interacted with Chloroauric acid and cetyltrimethylammonium bromide (CTAB)-modified gold nanorods through nonspecific physical adsorption to detect HBsAg biomarkers from complex substances such as serum and plasma. This detection strategy was validated with HBsAg positive and negative serum and plasma clinical samples, and the limit of detection was reported as 0.01 IU/mL in Tris buffer. Although homogeneous and label-free biosensor has been successfully developed using anisotropic gold nanorods, the detection strategy has many obstacles in the signal distortion due to nonspecific adsorption of other proteins in complex media. Recently, a quantitative nanoplasmonic viral load platform was reported by generating "hot-spots" on gold nanoparticle-assembled surface for ultrasensitive virus capture, detection and isolation from unprocessed whole blood (Inci et al., 2013). Nanoplasmonic platform was evaluated with multiple HIV subtypes (A, B, C, D, E, G, and panel) spiked in whole blood and HIV-infected patient samples, and the detection limit was reported as ~100 copies/mL (Fig. 6). The presented platform technology can also be broadly applicable to capture and detect other pathogens causing HBV co-infections by adapting the surface chemistry approaches. Thus, this widely applicable platform technology would play a key role to translate laboratory-based investigations into multiple settings including POC diagnostics, hospital, POC, and primary care settings. The similar other optical concepts were expanded for screening and early detection of other infectious diseases for example, successful adoption of point-of-care detection platform for TB and HIV diagnosis, reaching the sensitivity and specificity required clinically, were also realized (Wang et al., 2010a, 2012c 2013b, 2014; Kim et al., 2009). Regarding the social and economic impact of major infectious diseases such as HBV, tuberculosis or HIV, demand to develop portable, affordable diagnostic platforms early detection and monitoring one of the priorities in developing countries. In this review, novel methodologies that facilitate the diagnosis and treatment of HBV are discussed, therefore, other methods for different viruses and proteins have not been comprehensively included. New methods such as biosensors for HIV and TB provide promising applications that can potentially be adapted for diagnosis of HBV. Although there are multiple challenges for accessible and inexpensive healthcare solutions for infectious diseases, rapid advances in microfluidics and nanotechnology has potential to address many of the challenges.

Conclusion

In this review, we summarized the recent progress in HBV detection methodologies. We highlighted the potential, capabilities and promising applications of nanomaterials together with microfabricated and nano-assembled technologies that serve as a sensory system for detection of viral infections. To achieve effective viral detection, the detection systems need to have high sensitivity and selectivity, and label-free detection capabilities. Moreover, the ideal sensor must be cost-effective and user-friendly. The recent advances in nanomaterial synthesis facilitate microfabrication technologies to generate promising microfluidic lab-on-a-chip viral detection platforms. The primary driving force of nanomaterials is the physical and chemical properties that provide capabilities to create ideal sensors or powerful transducers. The new generation of sensing platforms promises

sensitivity down to a single virus level. Some of the advances in other diagnostic areas such as recombinant antibody coupled nanomaterials (Kierny et al., 2012), graphene based wireless sensor (Mannoor et al., 2012) microcantilever resonator (Lissandrello et al., 2014), and surface plasmon resonance aptasensor (Chuang et al., 2014) can be adapted to develop diagnostic tools for HBV. These new generation platforms and nanomaterials provide significant benefits. However, minimizing the cost of whole detection process is still a challenging problem, since current methodologies in HBV screening are labor intensive and require expensive instruments. The concept “instrument free detection” or “naked eye detection” for viral infection detection emerges as a possible future perspective. The recent cutting-edge technology facilitates sample handling and allows detection of low level of HBV in blood. The current strategies in instrument-free viral detection rely on unique optical properties of metal (and inorganic) nanoparticles. These achievements enable ultra sensitive viral detection and pave the way to utilize inexpensive detection methodologies. The new generation technologies in this field have to revolutionize classical testing methodologies together with understanding the clinical needs for current screening and prevention.

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