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Research review paper

Advances in nanomaterials and their applications in point of care (POC) devices for the diagnosis of infectious diseases

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ABSTRACT

Nanotechnology has gained much attention over the last decades, as it offers unique opportunities for the advancement of the next generation of sensing tools. Point-of-care (POC) devices for the selective detection of biomolecules using engineered nanoparticles have become a main research thrust in the diagnostic field. This review presents an overview on how the POC-associated nanotechnology, currently applied for the identification of nucleic acids, proteins and antibodies, might be further exploited for the detection of infectious pathogens: although still premature, future integrations of nanoparticles with biological markers that target specific microorganisms will enable timely therapeutic intervention against life-threatening infectious diseases.

Contents

Abbreviations: POC, point-of-care; SARS, severe acute respiratory syndrome; HIV, human immunodeficiency virus; ELISA, enzyme-linked immunosorbent assay; NAT, nucleic acid test; LOD, limit of detection; RT-PCR, real-time polymerase chain reaction; HBV, hepatitis B virus; HCV, hepatitis C virus; ECM, electrochemical; AuNPs, gold nanoparticles; QDs, quantum dots; MWCNTs, multi-walled carbon nanotubes; HEV, hepatitis E virus; RT-LAMP, reverse transcription loop mediated isothermal purification; NMOF, nano metal-organic framework; SPR, Surface Plasmon Resonance; HbsAg, hepatitis B surface antigens; MNPs, Magnetic NanoParticles; MAP, *Mycobacterium avium* spp. *Paratuberculosis*; BSA, bovine serum albumin.

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1. The threat of infectious diseases

According to the World Health Organization (WHO), in 2012 infectious diseases claimed 15 million lives worldwide (World Health, 2013). Among them, Human Immunodeficiency Virus (HIV) and tuberculosis were the leading causes of death at all age groups. In 2011, HIV claimed 1.3 million lives in sub-Saharan Africa alone (Tarantola et al., 1993). The extent of damage exerted by a particular infectious disease could reach well beyond the people directly plagued by the germs. A recent Ebola outbreak caused so much trouble for the healthcare system in West Africa that there were insufficient resources available for measles vaccination programs, thereby further adding to the death toll (Takahashi et al., 2015). An even more recent outbreak is represented by the Zika virus, currently spreading in the Americas and the Pacific region. This has resulted in increased infections during pregnancy and microcephaly, as well as Guillain-Barré syndrome in adults.

The transmission of pathogens is not limited to just humans. A number of transmissible microbes originated from animal vectors (e.g. birds, bats, ticks, etc.) could subsequently switch host to humans. Severe acute respiratory syndrome (SARS) virus, hantavirus, Nipah virus and human immunodeficiency virus (HIV) are just a few of such examples (Morse et al., 2012).

In the past few decades, the spread of once dreaded maladies such as smallpox and poliomyelitis have generally been kept under control, but these rigorous vaccination programs are far from being equally practiced across the globe (Fonkwo, 2008). In developing countries, a lack of proper sanitation, technologies, equipment, and human resources has been hampering efforts to provide timely treatments (Batt, 2007).

2. Current diagnostic tools

Identification of microorganisms by observing characteristic features of cultures has been in practice for decades. However, several limitations render this classical technique impractical for on-site diagnosis of infectious diseases, especially in resource-poor regions (Kaittanis et al., 2010).

Being time-consuming is one of the principal flaws of current diagnostic approaches. For preliminary results, each analysis takes 2–3 days. For more definite results, it might take up to 7–10 days. Detection of *Salmonella typhimurium* consumes 3–5 days before yielding results (He et al., 2013), whereas diagnosis of tuberculosis *via* microbiological means may take weeks (Dinnes et al., 2007).

An additional complication derives from the fact that, in order to procure meaningful observations, the initial serum samples must contain pathogen loads above a certain threshold level. This prerequisite might not be met if the patients are in early stages of infection. To worsen the situation, the life cycle of some bacterial strains includes a dormancy state, whereby organisms do not grow significantly in number when cultured. This could culminate in false negative results that critically undermine diagnoses.

Interferon gamma (INF-γ) release assay detects INF-γ produced by T-cells when the patient is exposed to *Mycobacterium tuberculosis* antigen. However, a tuberculosis patient is usually affected by HIV at the same time. Concurrent presence of HIV could readily impair the patient's immune systems. The resulting low T-cell count could mask a clinically relevant quantity of *Mycobacterium tuberculosis*, hence leaving tuberculosis undetected (Diel et al., 2011).

In the case of microbes more diminutive than bacteria (e.g. viruses, with average size of only about one-hundredth that of the average bacterium), an electron microscope is required for detailed visualization of the viral particles (i.e. virions). The growth of viral particles also necessitates a more sophisticated protocol than the one adopted for bacterial cultures (Shinde et al., 2012).

Technological advances have empowered medical professionals with a wide range of diagnostic tools. However, even state-of-the-art techniques are still far from being suitable for application in resource-poor contexts, wherein infectious diseases have proven to be the most widespread.

As of 2007, the gold standard for HIV diagnosis is an enzyme immunoassay which detects IgM antibodies in the patient's serum, followed by Western blot (Branson, 2007). Two popular methods are enzyme-linked immunosorbent assay (ELISA) and nucleic acid test (NAT).

In order to credibly detect a few virions in 100 μl of plasma sample, most commercially available methods require nucleic acid amplification (Calmy et al., 2007, Fiscus et al., 2006, Rouet and Rouzioux, 2007). Fourth-generation ELISA, a combination assay capable of detecting both HIV IgG/IgM and the capsid protein p24, has a limit of detection (LOD) of 4 pg/ml (Speers et al., 2005), thereby removing the need for nucleic acid amplification. The main downside is its high cost.

Amidst the outbreak of Severe Acute Respiratory Syndrome coronavirus (SARS-CoV) in 2003, real-time polymerase chain reaction (RT-PCR) (Chan et al., 2004) was widely employed. However, sensitivity of the assay represented the main limitation. In specifics, it would appear below clinically established standards, were the patients infected fewer than six days before the sample extraction date (Vasoo et al., 2009). While a refinement of specimen extraction process does improve the sensitivity level, it leaves the cost issue unaddressed. Another pathogen whose diagnosis utilizes RT-PCR as the standard test is the avian flu H1N1. Commercially available immunochromatography-based strip for the diagnosis of H1N1 (Welch and Ginocchio, 2010) is not as costly, but low sensitivity and specificity limit its clinical utility (Lee-Lewandrowski and Lewandrowski, 2001, Posthuma-Trumpie et al., 2009).

Other than diagnosis, NAT sees extensive use in screening of blood supply for common pathogens such as HIV, Hepatitis B virus (HBV), and Hepatitis C virus (HCV) (Fiscus, Cheng, 2006). It is also employed to monitor patient progress throughout treatment courses. GeneXpert is the first fully integrated NAT system. It could produce test outcomes in 2 h. Despite the relatively shorter assay time, the problems of cost and energy consumption remain (Meyer-Rath et al., 2012).

3. Point-of-care (POC) tests

3.1. Background information

According to the College of American Pathologists, POC testing could be considered as on-site diagnostic tests carried out using mobile devices readily accessible to the patients and the in-charge physicians (Lamb et al., 1995). Another more concise definition is 'testing done in the proximity of patient care' (Kiechle et al., 1990). The portable devices employed can be either hand-held or transported on a cart (Urdea et al., 2006). The acronym "ASSURED" was coined by WHO to denote the fundamental criteria of POC testing: affordable, sensitive, specific, user-friendly, rapid and robust, equipment-free, and deliverable to end user (Sista et al., 2008).

As mentioned above, there is an increasing demand for diagnosis of infectious diseases in resource-poor regions. A paucity of laboratory technicians with necessary know-hows is, among others, a major

concern under such circumstances. Hence, POC devices need to be user-friendly with easy-to-follow instructions. As such, routine tests may be performed by family members, or even the patients themselves.

Apart from mobility, short assaying time is crucial to POC testing. In cases where such time reduction does not hold much clinical significance, cutting down on the waiting time helps alleviate patients' discomfort (Holland and Kiechle, 2005).

Conventionally, POC tests are classified into 4 main categories (Hu et al., 2013):

1) Those whose speediness is the most valuable attribute, to reinforce decision on treatment regimen of lethal conditions (e.g. meningitis);
2) Those whose short assaying time is also a crucial element for prompt measures to restrain an outbreak (e.g. MRSA in hospitals);
3) Those simply for verification of the disease-causing microbes;
4) Those for self-monitoring by patients who do not attend follow-ups (e.g. in the case of patients with sexually transmitted diseases).

Despite this classification, POC devices are still at their infant stage. By 2005, several POC systems had been investigated in clinical trials. However, none had been released for commercial uses (Liao and Huang, 2005). In general terms, a POC device is designed to detect, either qualitatively or quantitatively, the presence of a specific biomarker characteristic of the malady at stake. At the moment, the analytes of interest range from nucleic acids of the microbes or proteins released by them during their time residing in the host, to antigens located on the surface of the microbes themselves. Therefore, the review of the current POCs has been organized into 1) nucleic acids, 2) whole pathogens and 3) proteins & antibody detection systems.

3.2. Nucleic acids

3.2.1. Electrochemical sensing

Many diagnostic techniques have been revamped and adopted for detection of microorganisms' genetic materials: electrophoresis, spectrophotometry, RT-PCR, etc. (Cagnin et al., 2009). However, adopting them not only for laboratory experiments, but also clinical applications at patient sites has proven to be a challenge.

Electrochemical (ECM) sensing was originally contrived for applications in laboratories, but recent advancements in technology have refined its suitability for the development of POC devices (Liepold et al., 2005).

On its own, ECM already has desirable properties (Lucarelli et al., 2009, Pohlmann et al., 2009, Wakai et al., 2004, Wei et al., 2010). Firstly, ECM sensors do not require much expertise to maneuver. The steps entailed in sample manipulation process are straightforward as well. In terms of resources, these systems can work with sample size smaller than usual, which ranges from a couple of microliters all the way to nanoliters, and do not consume much energy. All these fortes render ECM sensors suitable for POC assays.

A typical ECM sensor consists of an electrode, a capture probe, and a reporter probe (Fig. S1 (Siangproh et al., 2011) and S2). Capture probe is essentially an oligonucleotide whose sequence is complementary to that of the target nucleic acid. In most cases, the probe is conjugated to a surface, such as an electrode. After sample introduction, binding of target DNA/RNA to the capture probe generates a series of changes that eventually trigger the release of ECM signals by the reporter probe. Even though a number of disposable electrodes such as glassy carbon (Rivas et al., 2007) and pyrolytic carbon (Stoner et al., 2014) have been in use for quite some time, non-disposable alternatives (e.g. indium tin oxide, pencil graphite, screen-printed carbon) are slowly but steadily taking over. After all, the latter are more economical and easier to produce (Yeung et al., 2006). For instance, indium tin oxide electrodes were exploited in a silicon- and glass-based microchamber for simultaneous diagnosis of *Escherichia coli* and *Bacillus subtillis* (Xu et al., 2009). Capture probes were attached to the electrode surface by electrochemical copolymerization.

Nanoparticles (NPs) have lately been used to complement electrodes in immobilizing probe (Fig. 1). Sun et al. employed gold nanoparticles (AuNPs) and multi-walled carbon nanotubes (MWCNTs) to conjugate single-stranded DNA probes for the detection of *Staphylococcus aureus* DNA (Sun et al., 2015). Gold electrodes were also included in the set-up. Rather than the common purpose of immobilizing capture probes, the electrode was instead used to concentrate the nano-sized anchors. In another example, each AuNP served as the core for co-conjugating a hairpin sequence of DNA and a reporter DNA. Interestingly, the reporter DNA only carried a sequence complementary to half of the target *Helicobacter pylori* sequence. The other half was recognized by a capture probe anchored to a gold electrode (Cui et al., 2015). With a great surface-to-volume ratio, NPs could potentially bind a greater density of capture probes. This indirectly magnifies the ECM signals ultimately generated (Cui et al., 2015; Sun et al., 2015). At the same time, signal-to-noise (SNR) ratio is improved.

Out of various kinds of NPs, AuNPs are arguably the most extensively investigated as far as ECM sensing is concerned (Table 1). Like most other nano-sized materials, AuNPs have inherently large surface area and surface free energy. These properties facilitate the adsorption of nucleic acid strands. Nonetheless, there exist some challenges in terms of reliability, reproducibility, scalability in manufacturing AuNP-based biosensing assays and long-term stability; these aspects have hampered the successful translation into clinical trial research. Indeed, nanoparticles with different size ranges might show variable surface area, reactivity and orientation towards biosensing molecules. Quite recently, AuNPs have been produced with very narrow size distribution (e.g. 12.7 ± 1 nm (Lu et al. 2008) or even smaller, 1.6 ± 0.3 nm (Kim et al., 2004)) and further research efforts have resulted in samples with desirable polydispersity indices (<0.3). This process could be further enhanced *via* functional groups such as thiols and disulfides (Galow et al., 1999, Niemeyer and Ceyhan, 2001). Since AuNPs are capable of forming

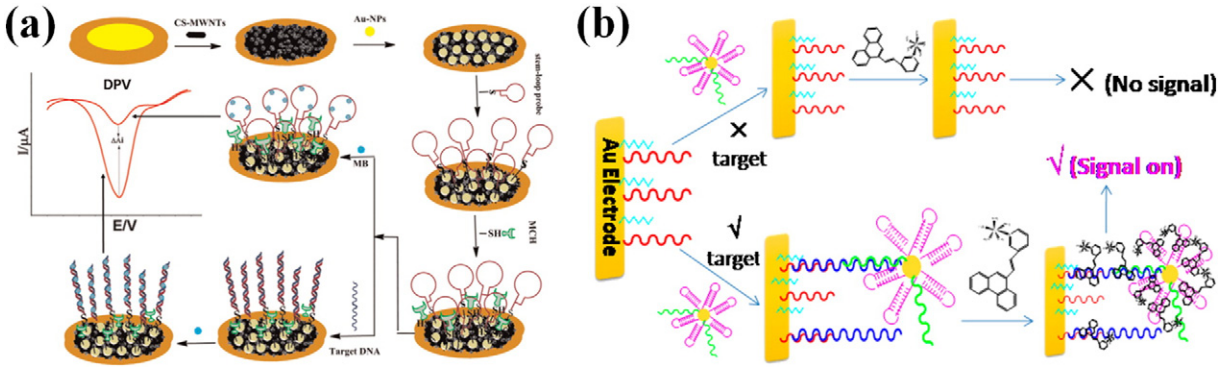

Fig. 1. (a) A highly selective and sensitive electrochemical CS–MWCNTs/Au-NPs composite DNA biosensor for *Staphylococcus aureus* gene sequence detection (Sun et al., 2015). (b) Hairpin DNA as a Biobarcode Modified on Gold Nanoparticles for Electrochemical DNA Detection (Cui et al., 2015).

Table 1
Examples of diagnostic POC systems whose target analytes are nucleic acids.

Technique	Authors	Key Features
ECM	(Yeung et al., 2006)	Analyte: *Escherichia coli* DNA or *Bacillus subtillis* DNA Sample size: 1 μL Sample pretreatment: • genome isolation using avidin-coated magnetic particles • amplification using PCR Detection range: 10^2–10^5 cells Clinical/real-world samples tested? No Performance: • capture probes were thermally stable throughout thermal cycling process • insignificant interference with quantification due to non-specific adsorption onto the electrodes • use of magnetic particles for DNA isolation was compatible with PCR process
	(Baeumner et al., 2002)	Analyte: Dengue virus RNA Sample pretreatment: • amplification using isothermal nucleic acid sequence-based technique • mixing with liposomes Assay time: 15 min (excluding RNA amplification process) Clinical/real-world samples tested? Yes (human serum) Performance: • sensitivity and specificity comparable to that of lab-based techniques • accurately detect Dengue serotypes 1, 2 and 4 in clinical samples • minimal cross-reactivity with Dengue serotype 3
	(Authier et al., 2001)	Analyte: 406-base pair HCMV DNA Sample pretreatment: • DNA extraction from cell culture • amplification using PCR • denaturation in alkaline media at room temperature • 12-fold dilution with coating solution Reproducibility: • ensured by slicing off a small segment at the end of the electrodes in between trials • enhanced by maintaining the screen-printed microband electrodes in a solution of (ferrocenylmethyl)trimethylammonium hexafluorophosphate • undermined with the use of manual screen-printer Detection range: 5–500 pM Clinical/real-world samples tested? No Performance: • AuNPs label employed for hybridization assay proved to be more stable than radioisotopic or enzymatic labels • non-specific binding present in low level • selectivity demonstrated by testing against non--complementary human ETS2 gene • LOD better than that reported in an electrochemiluminescent HCMV DNA method tested on 578-base pair HCMV DNA (Boom et al., 1999)
	(Sun et al., 2015)	Analyte: *Staphylococcus aureus* nuc gene Sample size: 1 μl (after purification) Sample pretreatment: • filtration of tap water samples through 22 μL membrane • inoculation with different amount of *Staphylococcus aureus* • centrifugation at 10,000 rpm for 5 min • DNA extraction using rapid boiling method • amplification using PCR • dilution with TE buffer solution, followed by denaturation in boiling water bath Detection range: • 1 fM–10 nM of *nuc* gene • 10–10^6 CFU ml^{-1} for real-world samples Clinical/real-world samples tested? Yes (tap water) Performance: • achieved a lower LOD (for tap water samples) than other reported methods such as AuNP-based immunosensors (Hejazi et al., 2008) or fluorescence-based assay using CdSe quantum dots(Yang and Lai, 2011)
Optical	(Chen et al., 2014)	Analyte: detection of HEV RNA Sample pretreatment: • reverse transcription of HEV RNA, followed by denaturation at 95 °C • amplification using RT-LAMP Assay time: <3 min (excluding amplification step) Detection range: >10 HEV RNA copies Clinical/real-world samples tested? Yes (human serum) Performance: • selectivity demonstrated by testing against three other hepatitis strains HAV, HBV, and HCV • results validated using agarose gel electrophoresis
	(Phillips et al., 2008)	Analyte: *Escherichia coli* DNA Sample size: 10 μl Reproducibility: • reproducible fluorescence signals • extent varied depending on bacterial strains and species Assay time: within min Performance: • selectivity demonstrated by testing against twelve other species of bacteria
	(Griffin et al., 2009)	Analyte: HCV RNA Detection range: 60–250 pM Clinical/real-world samples tested? No Performance: • no tagging is required • about two orders of magnitude more sensitive than some common colorimetric techniques • selectivity down to the level of single-base mismatch • quantitative signal intensity varied with the length of target RNA sequence
	(Nam et al., 2004)	Analyte: nucleotide sequence indicative of anthrax lethal factor Sample size: 30 μl Assay time: 3–4 h Detection range: 500 zM–5 fM Performance: • sensitivity on par with that of PCR-based methods, but did not require enzymatic amplification process • selectivity down to the level of single-base mismatch
	(Inci et al., 2013)	Analyte: HIV subtypes (A, B, C, D, E, G, and panel) Sample pretreatment: • system capable of effectively sequestrating separating viruses without the need for pretreatment Reproducibility:

Table 1 (*continued*)

Technique	Authors	Key Features
		• surface chemistry of NPs demonstrated to be reproducible to a considerable extent • analysis results reproducible for several HIV subtypes Assay time: 1 h of capturing and 10 min of detection and analysis Detection range: • varied between different subtypes • ranging from 98 ± 39 copies/ml (subtype D) to 120,159 ± 15,368 copies/ml (subtype E) Clinical/real-world samples tested? Yes (unprocessed whole blood)

strong covalent bonds with sulfhydryl groups, thiolation reaction could be readily achieved (Daniel and Astruc, 2004). In contrast, nucleic acid strands with adenosyl phosphothiolate tails could be conjugated in a more direct manner (Patolsky et al., 2006). Ease of functionalization and excellent biocompatibility (Liu and Ju, 2003) render AuNPs a great asset in both optical (Cao et al., 2002) and electronic (Park et al., 2002) DNA detection methods.

To incorporate AuNPs into ECM biosensors for POC devices, researchers have devised a handful of approaches for fully exploiting their potential (Castaneda et al., 2007). AuNPs, while being immobilized on genosensors, could be directly detected. In one experiment, target nucleic acid strands were first anchored onto Au_{67} quantum dots (QDs) (Pumera et al., 2005). Binding between target sequence and the capture probe, by then already conjugated to paramagnetic beads, led to the formation of a complex that enabled voltammetric detection of the gold QDs.

Alternatively, it is possible to quantify Au^{3+} ions generated after AuNPs are exposed to a mixture of hydrogen bromide and bromine (i.e. acid dissolving step). Such a strategy was employed in the detection of human cytomegalovirus (HCMV) DNA sequence (Authier et al., 2001). Since many Au^{3+} ions are released as each AuNP is suspended in a medium, the ECM signal is enhanced. As a result, a limit of detection (LOD) of 5 pM could be achieved. However, the mixture used for dissolving AuNPs is extremely harmful (hydrogen bromide is highly corrosive and irritating by inhalation). As such, this could limit the practicality of the technique (Lucarelli et al., 2004).

For signal amplification, silver enhancement could be employed. Cai et al. conjugated DNA capture probe to AuNPs, and targeted DNA to a glassy electrode (Cai et al., 2002). A silver enhancer solution was introduced to allow metallic silver to coat itself onto AuNPs. Such coating process helped boost the voltammetric signals by more than 80 times. Alternatively, signal amplification may be achieved by letting AuNPs act as carriers of electroactive labels. The incorporation of 6-ferrocenylhexanethiol decreased the LOD level to 2 pM for a sample size of 5 μl (Wang et al., 2003).

AuNPs could be combined with other nano-size materials for application in ECM sensors. Watanabe *et al.* recently combined the use of AuNPs and magnetic NPs (MNPs) in the detection of *mec*A gene, a popular biomarker for MRSA (Watanabe et al., 2015). Two DNA probes were employed. One was anchored to MNPs, whereas the other to AuNPs alongside ferrocene. The sequences of these probes were designed to be complementary to nearby regions located on *mec*A gene. The co-binding of MNPs permitted the isolation and enrichment of analyte complex prior to ECM measurement. Even without the help of nucleic acid amplification *via* PCR, this system managed to detect as low as 10 pM of target DNA.

Disposable biosensors are slowly but steadily assuming a larger role in POC technology, given the troubles commonly associated with non-disposable counterparts. A POC device that could be reused requires thorough cleaning after every assay to ensure no cross-assay contamination occurs, hence preserving the reliability of the assay. With that prerequisite, there is still the issue of how to do the washing without inadvertently damaging the integrity of the test reagents. This renders non-disposable POC devices unpractical in resource-poor settings. However, calibration and sterility problems have been reported for example in disposable clinical sensors, where the sensor-imbedded device required sensor calibration and/or validation by the clinician immediately prior to each use. Some recent advances have enabled the production of pre-calibrated and pre-validated sensors (e.g. US 7857506 B2 patent), but they still require optimization in lowering the costs and increase performance before becoming suitable for a single-use sensor application.

AuNPs have also been integrated into the design of disposable biosensors. They were used together with screen-printed electrodes for the diagnosis of respiratory pathogens such as *Mycoplasma pneumonia, Streptococcus pneumonia,* and *Chlamydophila pneumonia* (Bessede et al., 2010).

In another example of disposable biosensors, liposomes (another type of NPs) were employed as carriers of dyes (Ho et al., 2008). This strategy was examined for the detection of serotype-specific RNA fragments of Dengue virus (Baeumner et al., 2002). A portable reflectometer was utilized for quantification of the nucleic acid materials. Taking into account its many fortes (i.e. portable, inexpensive, user-friendly with easy-to-follow instructions, etc.), this system seems very promising. Following isothermal nucleic acid sequence-based amplification, which requires only basic tools such as water baths, it only took another 15 min to produce results. The same research group has adopted a similar system for detection of viable *Escherichia coli* in drinking water (Baeumner et al., 2003).

Thus far, we have discussed systems in which ECM labels play a major role in the quantification process. However, label-free sensors have also been investigated by numerous researchers. There are two main methods *via* which ECM sensing works without having to rely on electroactive labels (Siangproh et al., 2011). The first one is rather straightforward. Nucleotide bases do have intrinsic redox properties. Thus, they can generate ECM signals, which are indicative of the amount bound to the electrode. In the second approach, molecules which stably orient themselves into the groove of target DNA duplex (e.g. methylene blue, daunomycin, aromatic amines, $Co(2,2'\text{-bipyridyl})_3^{3+}$) are employed. After the two DNA strands get detached, these duplex-intercalating entities are freed, hence generating ECM signals. The latter strategy has been adopted in the investigation of infectious pathogens such as *Escherichia Coli*, *Mycobacterium tuberculosis*, HIV (Haddache et al., 2014), and HBV (Meric et al., 2002). However, a grave downside of this technique is the interaction between the chemicals and DNA duplex, which makes them potentially mutagenic (Watanabe et al., 2015).

Depending on the nature of the pathogens (e.g. DNA or RNA viruses, according to the Baltimore classification (Baltimore, 1971)), RNA detection is another viable strategy that could benefit from the advent of nanotechnology into the development of POC systems. For instance, AuNPs were incorporated into a lateral flow test strip for the diagnosis of HIV through the quantification of viral RNA in plasma samples (Rohrman et al., 2012). Lateral flow nucleic acid test strips derived from the well-established immunochromatographic strips (Mao et al., 2009). At the moment, this kind of device has several limitations (Carter and Cary, 2007, Corstjens et al., 2001). Before letting the sample flow through the devices, nucleic acid hybridization process is normally carried out in advance. This results in the addition of 10–30 min to the total assaying time. In general, quantification done with lateral flow assays necessitates the use of expensive equipment. The high cost, however, is not necessarily translated into great sensitivity of the assays (He et al., 2011). There have been several attempts to augment the sensitivity of lateral flow assays. Most of these ended up further complicating the protocol without really addressing the other issue (i.e. high cost) (Rohrman et al., 2012). An example of such attempts to improve sensitivity exploited antigens and antibodies to detect HBV, HCV, and HIV viruses (Dineva et al., 2005).

Indeed, the specific detection HIV-1 RNA is particularly challenging (Rohrman et al., 2012). It is known that the level of HIV genetic material present in the patients' bloodstream is naturally not very high. To be more precise, it is only a few copies per milliliter of blood. Therefore, duplication of the nucleic acids prior to the assay is a vital prerequisite for an adequately sensitive test. To this end, Rohrman et al. opted for isothermal nucleic acid sequence-based amplification, a popular technique mentioned above (Baeumner et al., 2002). This particular system has many laudable qualities (Rohrman et al., 2012), including a low cost (each strip costs no more than one US dollar) and simple production steps that involve commercially available reagents. From the very beginning to the completion of the assay, the user only needs to perform three steps. It takes in total only about 20 min, which is much shorter than the duration of any assay discussed thus far. In addition, simple-to-operate and relatively inexpensive instruments (e.g. heat block, scanner, camera, and pipette) are sufficient to perform the assay. In addition, when tested under different temperature conditions, the results produced by the assay remained essentially consistent. Such a commendable level of robustness testifies to its suitability for use in Africa and other places where a high temperature is a normal occurrence. Consistent performance was also observed when the system was tested against varying storage periods. However, this technology is still far from ideal, as the use of heat blocks does consume a considerable amount of energy (LaBarre et al., 2011; Liu et al., 2011). It could be replaced by heater equipment that runs on battery, hence cutting down on costs. Instead of imaging instruments, a color scale could be exploited to qualitatively simplify data interpretation process.

3.2.2. Optical sensing

Up to this point, it should be fairly apparent that ECM sensing is one of the most extensively studied methods for the detection of pathogenic nucleic acid. Another method that has also attracted much attention in the field of POC technology is optical sensing. In essence, presence of the biomarker of interest in the sample will trigger a chain of biochemical reactions. The end result is a change in optical properties of the system. Such variation is designed to be proportional to the amount of the nucleic acid sequence to be analyzed (Fig. 2). One key advantage of optical sensing is that electroactive labels are not indispensable (Shafiee et al., 2013).

Once again, nanotechnology plays a huge role. Metal NPs especially show tremendous promises. Among them, gold and silver NPs are the better options, since they are less susceptible to oxidation than their copper counterparts (Jain et al., 2008). Reportedly, nanorods (Bi et al., 2015) and AuNPs were employed in a colorimetric assay for the diagnosis of Hepatitis E virus (HEV) (Chen et al., 2014). To ensure that the concentration of the biomarker fell within detectable range, real-time loop-mediated isothermal amplification (RT-LAMP) was employed as part of sample preparation process. Streptavidin molecules were conjugated to AuNPs, which were then added to the sample that already underwent RT-LAMP. If the sample was HEV-positive, AuNPs would clump together as a response. This would in turn trigger a color change from red to purplish blue, hence permitting visual readout by naked eyes without any need for sophisticated instruments. On the contrary, had there been no HEV genetic material in the test sample, the biotin added during RT-LAMP process would help stabilize the AuNPs. As a result, the solution would remain red. A notable advantage demonstrated by this system was a remarkably short assay duration. Not including RNA amplification process, the test consumed only 3 min in total.

Surface plasmon resonance (SPR)-based sensors have rapidly emerged as a popular type of optical biosensors (Homola, 2008, Tokel et al., 2014). They work by measuring changes in refractive index of metal-dielectric interface, which could occur following binding events between the analyte molecules and capture probes. Surface plasmon bands absorbed by metal NPs are closely related to the size of their

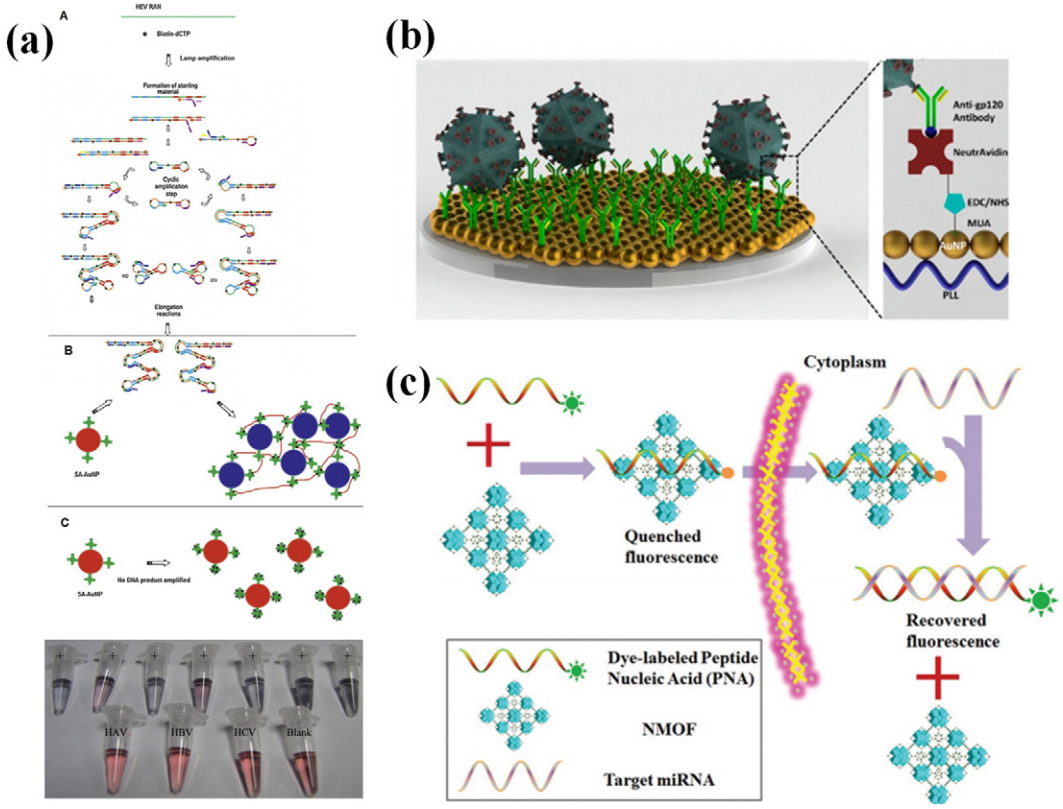

Fig. 2. (a) Colorimetric detection of hepatitis E virus based on reverse transcription loop mediated isothermal amplification (RT-LAMP) assay (Chen et al., 2014); (b) (Tang et al., 2010) (Figure 2 was under DNA section, but this system detects whole pathogens); (c) Nano metal–organic framework (NMOF)-based strategies for multiplexed microRNA detection in solution and living cancer cells (Lou et al., 2011).

aggregates, which come about as a consequence of nucleic acid hybridization (Hazarika et al., 2004). The more NP aggregates there are, the greater the resulting red-shift becomes. An example of SPR-based biosensor was used for the detection of HBV (Chuang et al., 2012). AuNPs were also employed in the said assay, which was economical and exhibited a LOD of 2 fg/ml with just 17 min of assay time.

NAT has always been a valuable diagnostic tool of HBV infection. Its utility is even more conspicuous during the 'window period' where other common techniques (e.g. immunoassays) are not practically reliable. The reason for this setback of immunoassays is the lack of antibodies against HBV in the body throughout the 'window period' (Yildiz et al., 2015). NAT is also particularly useful in the diagnosis of occult HBV infection (Ozsoz et al., 2003). While DNA of occult HBV are present in the bloodstream, there is no trace of their surface antigens. Given its unique capability, NAT is the preferred technique when it comes to scanning of blood transfusion sources (Stramer et al., 2011).

Fluorescence-based assays are categorized under optical sensing as well. Storhoff investigated the use of this type of assay for the detection of *mec*A gene of MRSA (Storhoff et al., 2004). The integration of nanosized materials (i.e. AuNPs) helped augment the sensitivity of the assay relative to that of similar methods. Conveniently, nucleic acid amplification prior to quantification process was not necessary.

A separate study adopted fluorescence-based sensors for the detection of *Escherichia Coli* DNA (Esteban-Fernandez de Avila et al., 2015). In details, anionic molecules of poly(para- phenylenethynylene) (PPE) were immobilized onto AuNPs, which were already functionalized with ammonium groups. The resulting complex efficiently subdued fluorescent property of PPE. After sample introduction, if bacteria were present, there would be electrostatic interaction between their surface and the various positive charges lining along the surface of AuNPs. This triggered the release of PPE from the complex. Once freed, PPE molecules regained their fluorescence, hence allowing quantitative measurements of the bacteria. As additional advantage, the assay is capable of identifying three distinct bacterial strains within min.

Fluorescence-based assays can also offer a final readout by naked eyes. Zhang et al. reported a POC device that made use of microcapillaries for detection of two RNA biomarkers that belong to different HIV strains (Zhang et al., 2014). A simple UV-flashlight was sufficient to generate visible readouts of fluorescence signals from the indicator, calcein. In addition, the system is self-sufficient in the sense that it did not utilize any external source of electricity. More precisely, a pocket warmer was all it needed. The use of capillaries to introduce and hold samples permitted concurrent analysis of several samples. In brief, this system did manage to tackle some of the most troubling issues associated with diagnostic tools in resource-poor settings, namely time and energy consumption.

3.3. Whole pathogens

These assays are generally based on immunoreactions between antibodies and antigens, which are characteristics of individual strains. Relative to NAT, immunological tests are generally capable of producing more robust results within a shorter span of time (Shinde et al., 2012). However, their level of specificity and sensitivity often pales in comparison.

Over the time, different kinds of antibodies (e.g. conventional, heavy chain, monoclonal, polyclonal, and recombinant antibodies) have been investigated in immunological tests. None of them have proven to be perfectly suitable for the role (O'Kennedy et al., 2005, Shinde et al., 2012). Polyclonal antibodies could be produced in a more rapid and economical manner than monoclonal antibodies, but their intrinsically poor specificity represents a valid concern. The latter have their fair share of shortcomings though. The production of monoclonal antibodies requires more well-trained personnel, and more sophisticated machineries, whose cost is a setback. Moreover, recombinant antibodies do not promise an acceptable level of sensitivity and affinity. To worsen the matter, they are rather vulnerable to interference from contaminants.

Monoclonal antibodies were utilized in the commercially available ARCHITECT Qualitative Assay by Abbott (Lou et al., 2011). This system was designed to detect hepatitis B surface antigens (HbsAg) by conjugating anti-HBsAg monoclonal antibodies to paramagnetic NPs. Recognition of the HbsAg in plasma samples, now bound to paramagnetic NPs, was handled by another set of acridinium-functionalized antibodies. Upon the introduction of hydrogen peroxide and sodium hydroxide into the system, chemiluminescence signals indicative of the amount of HbsAg were emitted. They were exploited by ARCHITECT System optics for quantification purposes.

3.3.1. Electrochemical sensing

In immunological tests, ECM sensing plays a significant role (Table 2). AuNPs served as the carriers for five different antibodies in an ECM immunosensor array that concurrently detects five separate strains of HBV (Tang et al., 2010). While its performance was comparable to that of conventional ELISA, it was demonstrated to be more energy-efficient and able to produce results within 5 min (Ye et al., 2003).

If AuNPs attract all the limelight in NAT, a wide range of NPs have found utility in the detection of pathogenic antigens. One such nanosized material is graphene. By virtue of its distinguished electron transfer quality, graphene film was used to construct electrodes for detecting rotavirus (Liu et al., 2012) (Fig. S3(a)). Antibodies specific to the viral particles were anchored onto the surface of the graphene-based electrodes. A wide range of graphene-based nanomaterials have been investigated for application in the field of nanomedicine. That alone is proof of the utility of graphene NPs. However, development of graphene-based biosensors has been to some extent impeded by a lack of reproducibility and scalability of the manufacturing processes.

Graphene NPs have also been exploited for other functions. For instance, graphene oxide NPs were employed by Chen et al. as fluorescence quenchers in an assay capable of simultaneously detecting both human Enterovirus 71 (LODs: 0.42 ng/ml) and Coxsackievirus B3 (LOD: 0.39 ng/ml) (Chen et al., 2012). This assay also made use of QDs, another type of NPs. For the detection of two unrelated species of viruses, two kinds of QDs, which possessed distinct optical behaviors for selective quantification, were required to conjugate the two kinds of antibodies.

Both kinds of QDs were in turn functionalized with graphene oxide, which efficiently suppressed fluorescent signals from QDs. When either human Enterovirus 71 or Coxsackievirus B3 was present in the samples, the corresponding QDs detached themselves from graphene oxide NPs and emitted fluorescence. The signals could be picked up and quantified. In another study, graphene oxide NPs were used together with silver NPs (AgNPs) for simultaneous diagnosis of HBV, HIV and *Treponema pallidum* (Liu et al., 2013). One major defect of graphene-based nanomaterials is their high hydrophobicity, which is responsible for formation of clumps in solution. These bulky aggregates indiscriminately bind biomolecules other than the desired targets, and could cause denaturation of the sample (Yildiz et al., 2015).

MNPs were employed in a sandwich-type immunoassay for the detection of *Salmonella typhimurium* (Gehring et al., 1996). Anti-*S. typhimurium* antibodies were conjugated onto superparamagnetic beads while being functionalized with alkaline phosphatase. Phosphatase was the key element of ECM sensing mechanism in that investigation. After sample introduction, the inherent magnetism of MNPs allowed to attract the complex towards disposable graphite ink electrodes. This step was included in the protocol to enhance the efficiency of ECM detection. A drawback of this particular assay was that it took in total 80 min, which made it much more time-consuming than many other POC systems. Given the fact that Gehring et al. reported this immunoassay more than a decade ago, the lack of efficiency in assay time was understandable.

Table 2
Examples of diagnostic POC systems whose target analytes are whole pathogens.

Technique	Author	Key features
ECM	(Lou et al., 2011)	ARCHITECT ® Prototype Assay (Abbott) Analyte: HBV surface antigen Sample size: 75 μl Detection range: 0.016–0.500 IU/ml Clinical/real-world samples tested? Yes (human serum/plasma) Performance: • excellent specificity (99.94%) when tested on 6482 specimens • performed better than other HBsAg assays in terms of accurate detection • capable of detecting more substitution mutants than an earlier versions
		Analyte: multiple types of hepatitis virus antigens (HAV, HBV, HCV, HDV, HEV) Reproducibility: inter-assay imprecision level at 8.1% Assay time: 5 min Detection range: • LOD slightly varied between antigen types, ranging from 0.8 ng/ml for HBV to 1.5 ng/ml for HCV and HEV • same upper limit of linear range (350 ng/ml) Clinical/real-world samples tested? Yes (human serum) Performance:
	(Tang et al., 2010)	• quality of results comparable with that of conventional ELISA • some cross-reactivity between adjacent sites (≤7.5%) Purpose: *Enterovirus 71* and *Coxsackievirus B3* Assay time: shorter than equivalent methods (e.g. RT-PCR) Detection range: • *Enterovirus 71*: 1–14 ng/ml • *Coxsackievirus B3*: 1–19 ng/ml Clinical/real-world samples tested? Yes (human throat swabs) Performance:
Magnetism	(Chen et al., 2012) (Perez et al., 2003)	• selectivity demonstrated by testing against *Coxsakievirus* A9, Enteric Cytopathic Human Orphan virus, mumps virus, and pseudorabies virus • good recovery from clinical samples (98.7% to 101.8% respectively) • insignificant cross-reactivity between the two virus antibodies employed Analyte: *Herpes simplex* virus or *Adenovirus* Sample size: 10 μl Sample pretreatment: minimal Detection range: • 5 viral particles in 10 μl samples • 100 viral particles in 100 μl samples Clinical/real-world samples tested? No Performance:
	(Lien et al., 2007)	• superior than common PCR-based techniques • capable of analyzing complex turbid samples • more sensitive than ELISA assays Analyte: Dengue virus serotype 2 Sample size: 25 μl Sample pretreatment:
	(Setterington and Alocilja, 2011)	• mixing with antibody-conjugated magnetic beads and a rotary micromixer • enrichment using magnetic field from planar microcoils Detection range: LOD of 100 cfu/ml Clinical/real-world samples tested? No Performance: • 87% viral particles separation efficiency Analyte: *Escherichia coli* O157:H7 Sample pretreatment: • dilution with PBS • magnetic separation using commercial antibody-coated immunomagnetic beads • labeling with immune-modified polyaniline nanostructures Reproducibility: validated for qualitative analysis Assay time: approximately 75 min (including sampling time) Detection range: 70–7 × 10^5 cfu/ml Clinical/real-world samples tested? No Performance: • LOD of 70 cfu/ml meets standard set by the FDA • efficiency of magnetic separation process could be impaired when applied to real-world samples, potentially affecting sensitivity level of the assay • suspected to detect non-viable bacteria alongside viable ones (not verified)
Optical	(Ho et al., 2008)	Analyte: heat-killed *Salmonella typhimurium* Sample size: 40 μl Assay time: 30 min Detection range: LOD of 1680 cells/ml Clinical/real-world samples tested? No Performance: • the concentration of anchored antibody does influence performance of the assay (reducing the concentration from 4 mg/ml to 2 mg/ml lengthens the detection range) • selectivity demonstrated when tested against *Escherichia coli* O157:H7 and *Liseria* genus Analyte: *Escherichia coli* O157:H7 Sample pretreatment: • no amplification or enrichment required Assay time: 20 min Clinical/real-world samples tested? Yes (spiked ground beef) Performance:
	(Zhao et al., 2004)	• could even detect a single bacterium in the samples (verified using two distinct quantitative techniques)

In an enzyme-free ECM immunosensor, silicon nanowires were functionalized with antibodies for the detection of influenza A virus (Patolsky et al., 2004) (Figure S3(b)). A change in conductance was recorded once the complex was exposed to viral particles. Such quantifiable change was observed when the sample contained paramyxovirus and adenovirus, but not influenza A virus. This helped establish that the assay possessed a clinically relevant level of selectivity. Remarkably, samples containing single viruses could be detected without compromising the selectivity. This level of performance would compare favorably against the mainstream PCR-based techniques. Moreover, the system was demonstrated to be capable of multiplexing.

3.3.2. Magnetic resonance sensing

As mentioned in the previous section, MNPs have been integrated into ECM immunosensors before. Nonetheless, they are more extensively applied in POC devices, which utilize magnetic resonance detection method (Fig. 3(a)). A phage-based magnetoelastic biosensor was adopted to analyze *Salmonella typhimurium* on fresh tomato surfaces (Li et al., 2010). Use of filamentous E2 phages facilitated the binding of the analytes. The resonance frequency generated by the wireless biosensors could be quantified *via* magnetic fields.

Traditional magnetic beads typically used in biological separation have a diameter of about 1 − 5 μm. In contrast, MNPs are much smaller (<10 nm in diameter). As such, they have a substantially larger surface-to-volume ratio (Josephson et al., 2002).

Superparamagnetic iron oxide NPs were used to anchor antibodies for the detection of herpesvirus or adenovirus in 10 μl of sample volume (Perez et al., 2003). The iron oxide NPs were coated with dextran. This layer could be further functionalized with amino groups, thereby facilitating antibody conjugation (Josephson et al., 1999). Existence of viral particles in the samples triggered self-aggregation of the MNPs to form a complex with augmented magnetic properties. This change in structure then allowed for quantitative detection. It was observed that the percentage of serum of the samples did have an impact on the sensitivity of the assay. In 100% serum samples, the LOD was 10 virions, but it dropped to as low as 5 virions when 25% serum samples were investigated.

An immunosensor with that impressive LOD is undoubtedly promising. While it can efficiently scan serum samples for viral infections, its application in the detection of bacteria seems far from being ideal (Kaittanis et al., 2007). Upon being exposed to a low bacteria count, the MNPs would clutter together on the surface of the pathogens. However, if the count was above a certain threshold value, the MNPs would revert back to a dispersed state just like how they would behave under pathogen-free circumstances. Therefore, the assay could potentially produce false negatives. With this serious flaw left unaddressed, applications of this system are confined to analysis of infectious diseases generally known to display a low serum pathogen count (e.g. *Mycobacterium avium* spp. *Paratuberculosis* (MAP)) (Kaittanis et al., 2007). A separate study investigated the use of dextran-coated iron oxide NPs, this time in the form of nano-sized rods, also for the diagnosis of MAP (Liao et al., 2009). An LOD of 6cfu could be achieved after just 5 min.

One principal shortcoming of magnetic resonance detection method is the requirement of machineries such as magnetic relaxometers or other instruments specialized to perform nuclear magnetic resonance (NMR). Aside from being too costly, their operation demands a certain level of technical skill of the personnel. This ultimately undermines the suitability of this detection method for application in POC devices.

3.3.3. Magnetic separation of pathogenic particles

Magnetic properties of MNPs have not only been exploited for magnetic resonance sensing, but also for deliberate isolation of the pathogens of interest from the sample (Fig. 3(b)(c)(d)). Such maneuver allows for sample enrichment. Lien et al. immobilized antibodies onto MNPs to trap Dengue virus from the sample (Lien et al., 2007). Using a

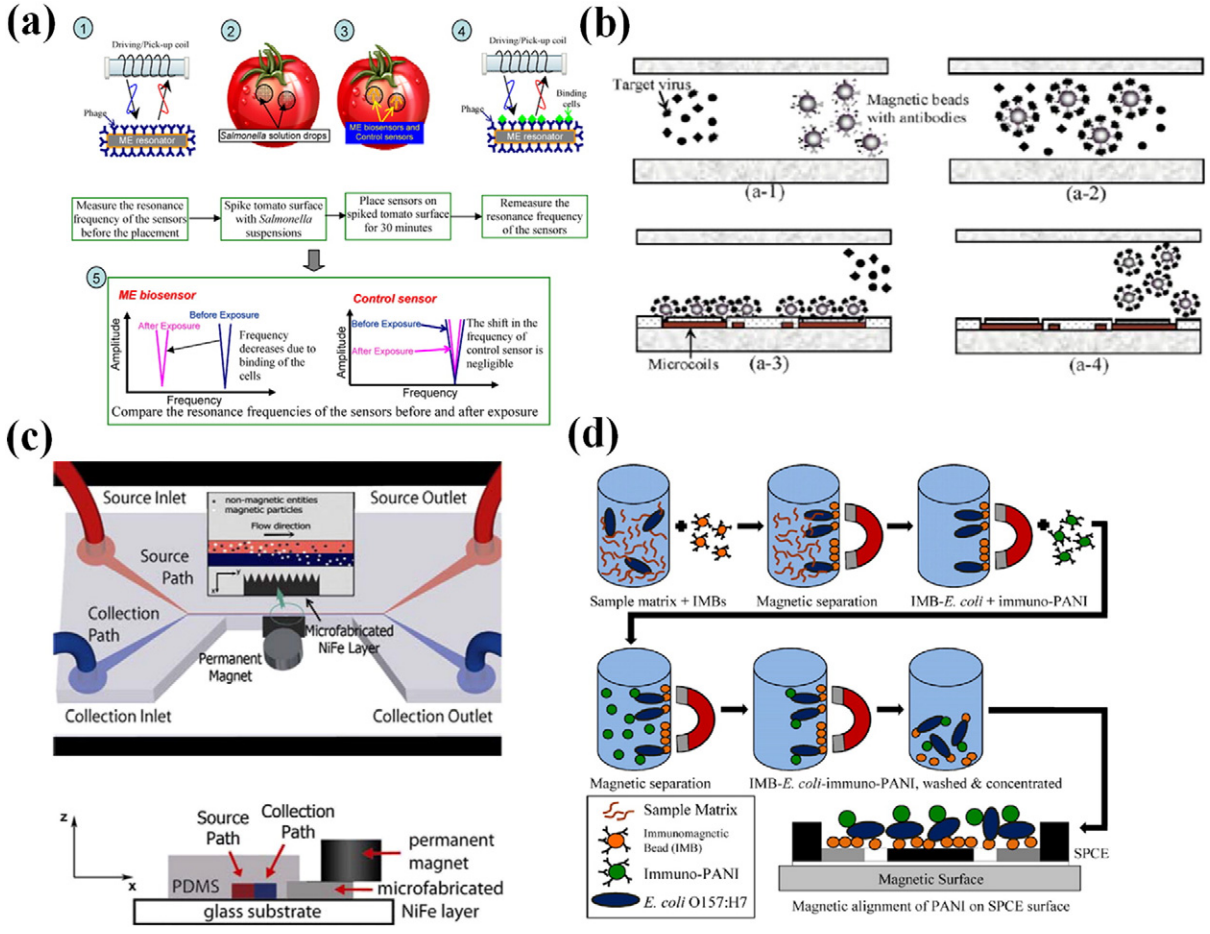

Fig. 3. (a) Direct detection of *Salmonella typhimurium* on fresh produce using phage-based magnetoelastic biosensors (Li et al., 2010). (b) Purification and enrichment of virus samples utilizing magnetic beads on a microfluidic system (Magnetic separation of pathogenic particles) (Lien et al., 2007). (c) Combined microfluidic-micromagnetic separation of living cells in continuous flow (Magnetic separation of pathogenic particles) (Xia et al., 2006). (d) Rapid electrochemical detection of polyaniline-labeled Escherichia coli O157:H7 (Magnetic separation of pathogenic particles) (Setterington and Alocilja, 2011).

planar micro-sized coil, a magnetic field gradient was set up to attract the viral particles captured on MNPs. The separation efficiency achieved was fairly high at 87%. As a result, pathogenic quantification could then be executed with a greater level of sensitivity. To be specific, the LOD was brought down to 100 fu/ml.

In another study, Xia et al. employed MNPs to bind and extract *Escherichia coli* virions from the flow of a solution that simulated human blood samples (Xia et al., 2006). In place of a planar microcoil, a high-gradient magnetic field concentrator was used to generate the necessary magnetic field gradient. It was noticed that the separation efficiency of the system did not deteriorate with time. At flow rates from 25 to 40 μl/h, separation efficiency of MNP-bound bacterial cells ranged from 78% to over 90%. Raising the cell density of input flow brought about a great increase in throughput rate.

One common setback faced by both systems was a poor capacity for concentrating samples. Nevertheless, it is not universally observed among experiments that perform magnetic separation using MNPs. In a microfluidic chamber device, which employed a close-packed columns of polydispersed iron NPs and a NdFeB permanent magnet, 0.5 ml of HIV-inflicted plasma samples was concentrated by 44 times. This remarkable concentrating power would significantly raise the sensitivity level of any quantification method subsequently employed for the diagnosis of HIV (Chen et al., 2010a).

ECM sensing was one of the methods which have been coupled with magnetic separation to enhance sensitivity. *Escherichia coli* O157:H7, an enterohemorrhagic serotype of *Escherichia coli*, was subjected to diagnosis using this combination of techniques (Gu et al., 2003). Setterington et al. took the modification a step further and bioconjugated the bacterial cells with polyaniline after they were magnetically separated (Setterington and Alocilja, 2011). The electroactive label allowed quantification using cyclic voltammetry. This played a huge role in strengthening the ECM signals emitted. Consequently, a LOD of 70 cfu/ml was attained. Notwithstanding the relatively lengthy sampling time of 70 min, this experimental POC system can be carried out using compact and mobile devices, hence confirming its suitability for a broad range of relevant applications.

It has been demonstrated that magnetic separation of the pathogenic particles also allows for microscopic identification of the microorganisms. In one study, vancomycin was conjugated to MNPs in order to trap vancomycin-resistant enterococci (Gu et al., 2003). With the help of an external magnetic field, the MNPs-bound bacteria were accumulated into an area around 1 mm^2 large. Biological separation was followed by observation using optical microscope, and finally verification with the help of electron micrograph. A LOD as low as 10 cfu/ml was achieved.

3.4. Proteins & antibodies

This kind of assays has several advantages (Ghindilis et al., 1998, Lin and Ju, 2005, Warsinke et al., 2000). It has inherently excellent sensitivity relative to other kinds of diagnostic tests, could easily be adopted as POC technology, and is, above all else, cost-effective. Nano-sized materials have been widely studied as add-ons to accompany transducers so as to facilitate electron transfer process, magnify the SNR of the system, or to heighten the efficiency of antibody conjugation(Liu and Lin, 2007). In some cases, NPs complex have also been explored as ECM labels, and anchor points for antibodies. It has been demonstrated that antibodies do retain their biological binding activity after being conjugated to NPs (e.g. AuNPs) (Liao et al., 2009) (Table 3).

3.4.1. Electrochemical and optical sensing

A range of NPs have been investigated for application in protein-sensing ECM immunosensors (Fig. S4(a))(Hansen et al., 2006, Liu et al., 2004, Yuan et al., 2015). Among them, QDs are fairly popular in multiplexed assays for parallel detection of several biomarkers at the same time (Fig. S4(b)) (Liu et al., 2004). To this end, one set of antibodies is immobilized onto magnetic beadsEach type of antibody belonging to the other set is conjugated to a distinct type of metal sulfide semiconductor. The examined QDs included CdS, ZnS, PbS, and CuS, which all have comparable levels of sensitivity. The hydroxyl terminals of these nano-sized colloidal tracers enabled the antibody functionalization process *via* carbamate bonds. The use of 2 sets of antibodies served as the central elements of an ECM sandwich immunoassay. The antigens of interest, be it proteins or a human antibodies, were captured between magnetic beads and QDs with the corresponding antibodies. Each individual antibody-antigen binding event would produce a characteristic voltammetric peak. The position and magnitude of the peaks provide detailed information on how much of each

Table 3
Examples of diagnostic POC systems whose target analytes are proteins and antibodies.

Technique	Author	Key features
Electrochemical	(Ambrosi et al., 2007)	Analyte: human IgG Sample size: 150 μl Sample pretreatment: • mixing with antibody-coated magnetic beads • labeling with double codified AuNPs Detection range: varied between the two quantification methods employed • spectrophotometry: LOD of 52 pg/ml • ECM: LOD of 260 pg/ml Clinical/real-world samples tested? No Performance: • more sensitive than conventional ELISA techniques • selectivity demonstrated by testing against goat IgG
	(Yuan et al., 2015)	Analyte: C-reactive protein (hsCRP) and soluble CD40 ligand (sCD40L) Sample size: 6 μl Sample pretreatment: minimal Reproducibility: • acceptable level of reproducibility • inter-assay standard derivations were 5.04% and 4.08% for hsCRP and sCD40L respectively Detection range: 0.05–100 ng/ml • LOD of hsCRP: 16.7 pg/ml • LOD of sCD40L: 13.1 pg/ml Clinical/real-world samples tested? Yes (human serum) Performance: • stability of system demonstrated by storing the immunosensor at 4 °C for 30 days in between assays (91.63% and 90.02% of initial response achieved for hsCRP and sCD40L respectively)
Optical	(Zhu and Yang, 2015)	Analyte: anti-rabbit human IgG Sample size: 18 μl Sample pretreatment: minimal Assay time: 25 min Detection range: 1–10 μg/ml Clinical/real-world samples tested? No Performance: • reducing sample size to 6 μl helps reduce assay time to around 15 min, but at the same time compromises the sensitivity (higher LOD of 5 μg/ml) • selectivity demonstrated by testing against bovine serum albumin (at much higher concentration than the target analyte)

biomarker (e.g. β_2-microglobulin, IgG, bovine serum albumin, and C-reactive protein) was present in the sample.

QDs were accompanied by AuNPs and thiolated aptamers in an ECM immunesensor system, which reportedly yielded an LOD of 20 ng/L (Fig. S4(c))(Hansen et al., 2006). Aptamers are synthetic nucleic acid ligands which have been studied as substitutes to antibodies. They possess several desirable qualities, out of which resistance to denaturation is perhaps the most laudable. In general, aptamer-based biosensors could achieve excellent sensitivity. This forte was apparent in this particular investigation, whereby trace amounts of proteins could be detected. Being energy-efficient, easily miniaturized, and economical, the assay satisfied a handful of ASSURED criteria.

In another study, cadmium tellurite QDs were conjugated to silica NPs for magnification of ECM signals. The system was designed for detecting Epstein-Barr virus-derived latent membrane protein 1 (LMP-1) (Chen et al., 2010b). The especially large surface area of nano-sized carriers allowed for the immobilization of numerous QDs. This served as the basis for the augmentation of ECM signals detected by square wave voltammetry. As a result, an LOD of 1 pg/ml was achieved. The invariable efficiency of QDs immobilization process also worked in our favor by ensuring excellent reproducibility of the assay.

The flexibility of QDs is apparent when we take into account the properties that make them excellent fluorescence emitters. By adjusting the size of these nano-sized semiconductors, it is possible to manipulate their emission wavelength range(Pinaud et al., 2006). Ergo, a single absorption wavelength could be used to trigger a range of emission wavelength, given that QDs of varying size are employed. This can be tapped on for potential development of fluorescence-based multiplexed assays (Hare et al., 2015).

Multiplexed assays can also be achieved with MNPs. In one study, hybrid NPs, which consisted of a $NiFe_2O_4$ core enclosed within a SiO_2 shell, were used to anchor different kinds of antibodies for concurrent detection of four distinct biomarkers (Tang et al., 2007). The extent of ECM signal interference between adjacent electrodes (each electrode designed to detect one biomarker) was minimal.

Ambrosi et al. reported an immunoassay compatible with two separate detection methods (ECM and optical) for quantifying human IgG (Ambrosi et al., 2007). AuNPs were conjugated with antibodies specific to human IgG. These antibodies were then bonded to horseradish peroxidase. Detection step could be done spectrophotometrically by measuring the intensity of the solution's color emanated from AuNPs. Alternatively, innate ECM behaviors of the AuNPs could be quantified with stripping voltammetry. The use of paramagnetic beads enabled magnetic separation of the labeled antibody complex. As a consequence, the sensitivity of the assay outperformed conventional ELISA tests. The LOD of optical and ECM detection methods was 52 pg/ml and 260 pg/ml respectively. In addition, the use of MNPs helped curtail incubation and washing time, which then contributed to a more desirable total assay time.

Just considering optical detection method alone, europium (III) NPs (EuNPs) have been contemplated as an excellent substitute for AuNPs. They were meant to help reduce the sophistication of the assays without compromising their sensitivity (Tang et al., 2009). The optical properties of these nano-sized fluorophores render them suitable for immunosensors. After all, EuNPs are capable of producing robust and lasting fluorescence (Hemmila et al., 1984). In one investigation, they were encapsulated inside polystyrene NPs for the detection of anthrax protective antigen (Tang et al., 2009). The system was considerably reliable, since no false negatives were observed. Meanwhile, the assay attained a level of sensitivity 100 times greater than that of conventional ELISA, whose LOD was known to be around 1 ng/ml (Moayeri et al., 2007).

3.4.2. Systems with visible readouts of assay results

POC devices which permit visible readouts are generally associated with a more affordable cost, since the need for advanced instruments for quantitative detection is eliminated. It is therefore a welcomed addition to POC technology, considering how it helps realize one key element of the ASSURED criteria.

One recent example microfluidic immunoassay with naked-eye readouts leveraged on the changing appearance of liquid crystals (Zhu and Yang, 2015) (Fig. S5(a)). Binding events between antigens and immobilized antibodies triggered a shift of the LqC appearance from dark to bright. This phenomenon could be visualized without the need for sophisticated devices. The technique exhibited good robustness, a LOD of 1 μg/ml. In addition, good specificity was demonstrated using bovine serum albumin as the non-target interference (10-fold concentration compared to the target analyte).

Another example is the volumetric bar-chart chip reported by Song et al. for the detection of disease-specific proteins (Song et al., 2012) (Fig. S5(b)). In this study, catalase and antibody molecules were both conjugated onto the surface of silica NPs. If the target analyte was present in the sample, the enzyme would catalyze the decomposition of hydrogen peroxide in the solution to give out oxygen. The extent of pressure build-up inside the enclosed columns was proportional to the amount of oxygen gas produced. The rise in column pressure elevated the ink columns upwards. In a nutshell, the extent of elevation was designed to be indicative of the amount of the protein biomarkers in the sample. The presentation of quantitative results in the form of bar charts, as suggested by the name of the device, could be readily interpreted with just naked eyes. Moreover, the oxygen-producing reaction facilitated by catalase occurred very rapidly, within seconds (George, 1947). This was arguably the key feature of the system, which helped shorten the total assay time. Duration was further decreased by virtue of this POC system's impressive multiplexing power, which permitted up to 50 concurrent tests.

Apart from the aforementioned strengths of this system, it did exhibit certain limitations (Zhu et al., 2014). The biocatalytic capability of the enzyme could possibly be impaired during the conjugation step. The fact that catalase enzyme itself is highly susceptible to hydrolysis further cast doubt on the reliability of the assay.

More recently, Zhu et al. developed an immunoassay based on similar concepts, but with certain alterations to expunge the existing drawbacks (Zhu et al., 2014). To tackle the problem at its root, the easily degraded catalase was replaced by hybrid NPs comprised of a gold shell and a platinum core (Au@PtNPs). These Au@PtNPs were encapsulated inside aptamer-modified hydrogels. When the biomarker of interest was added, its interaction with the aptamers found on the exterior of the hydrogels would trigger their disintegration. Au@PtNPs would then come into contact with hydrogen peroxide molecules already present in the test solution. In this manner, the hybrid NPs assumed the role of catalase. This led to the formation of visible bar-chart displays, as explained above in the study of Zhu et al. (Zhu and Yang, 2015). This novel system could be conveniently adopted for detecting numerous protein and antibody biomarkers. After all, a diverse selection of aptamers could be procured through different means (Ellington and Szostak, 1990, Tuerk and Gold, 1990).

Subramaniam et al. demonstrated another system with visible readouts, referred to as metal-amplified density assay(Subramaniam et al., 2015). Levitation of diamagnetic polystyrene beads was employed as the parameter for interpretation by naked eyes. In essence, antibodies specific to the biomarkers were conjugated onto these nano-sized beads. After sample introduction, successful immunoreactions would bring about a change in the density of the polystyrene beads. At first glance, such physical alteration proved to be too minute to be reliably detected. To address this issue, the authors revised the protocol by incorporating AuNPs into the POC system to amplify the change in density. Visible readouts using floating height of small particles are much more attractive than those dependent on colorimetric interpretation(Martinez, 2008). The former have shown promises in parallel testing capacity by virtue of several kinds of colored beads. For instance, it was explored for simultaneous diagnosis of syphilis and

hepatitis C (Subramaniam et al., 2015). However, its protocol required a handful of steps which could appear confusing to on-site test performers.

4. Future outlooks

By producing diagnostic test results within a short period of time, at low cost, and without the need for advanced instruments or well-trained technicians, POC devices are undoubtedly dream companions for medical professionals in resource-poor regions. Sometimes, the availability of POC devices could very well make the difference between life and death, given their ability to produce timely test outcomes. For those mobile devices whose operational instructions have been sufficiently simplified, the task of carrying out the tests could be entrusted to the caretaker, or even the patients themselves if the need arises.

Notwithstanding its far-reaching medical applications, POC technology in general, and nano-sized materials in particular, are still in early phases of development. Throughout this review, we have discussed several POC devices currently in experimental stage. They all excel in certain aspects, but at the same time fail to satisfy every single ASSURED criterion. This makes a case for further improvements.

One direction for improvement is to develop a platform which is able to deal with samples without the requirement of preprocessing (e.g. HIV in whole blood (Inci et al., 2013) or pathogen on fresh tomato surface (Li et al., 2010)). This can be truly helpful to users for a one-step diagnosis. Two feasible methods may be adopted for this purpose. One is further enhancement of sensitivity and another is having integration with microfluidic components for sorting and purification functions (Bi et al. 2015).

Advancements in the field have rendered the synthesis of inorganic NPs (e.g. AuNPs (Craig et al., 2012), SiO_2 NPs (Li and Zhao, 2013)) largely reproducible, with respect to physical parameters such as size distribution. Attaining uniformity of organic NPs used to be a real challenge, but progresses have been made in that aspect. Recently reported syntheses achieved acceptable levels of reproducibility and desirable polydispersity indices (<0.3). Since NPs are primarily employed as carriers onto which biomolecules are conjugated, their size distribution is one of the key factors predisposing the performance of the POC systems. Therefore, future researches into fine-tuning reproducibility of NPs will further enhance the reliability of POC systems.

It also helps that the developments in POC technology are easily adopted horizontally, as long as the biomarkers belong to the same class (e.g. nucleic acids). With the current amount of time and effort invested into researches on POC technologies, it is not much of an exaggeration to state that breakthroughs are bound to come in the near future.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.biotechadv.2016.09.003.

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