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REVIEW ARTICLE

Miniaturized technology for protein and nucleic acid point-of-care testing

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The field of point-of-care (POC) testing technology is developing quickly and producing instruments that are increasingly reliable, while their size is being gradually reduced. Proteins are a common target for POC analyses and the detection of protein markers typically involves immunoassays aimed at detecting different groups of proteins such as tumor markers, inflammation proteins, and cardiac markers; but other techniques can also be used to analyze plasma proteins. In the case of nucleic acids, hybridization and amplification strategies can be used to record electromagnetic or electric signals. These techniques allow for the identification of specific viral or bacterial infections as well as specific cancers. In this review, we consider some of the latest advances in the analysis of specific nucleic acid and protein biomarkers, taking into account their trend toward miniaturization and paying special attention to the technology that can be implemented in future applications, such as lab-on-a-chip instruments. (Translational Research 2012;160:332–345)

Abbreviations: POC = point-of-care; LFI = lateral flow immunochromatography; PSA = prostate-specific antigen; hCG = human chorionic gonadotropin; TSH = thyroid-stimulating hormone; SEB = staphylococcal enterotixin B; FRET = Förster resonance energy transfer; MMP = matrix metalloproteinase 9; BNP = B-type natriuretic peptide; CRP = C-reactive protein; PDMS = polydimethylsiloxane; Ig = immunoglobulin; Hb A_{1c} = hemoglobin A1c; Ag = antigen; Ab = antibody; TNFα = tumor necrosis factor α; PCT = procalcitonin; IL = interleukin; PCR = polymerase chain reaction; CA = cancer antigen; CEA = carcinoembryonic antigen; NMP = nuclear matrix protein; S100β = S100 calcium binding protein beta; ELISA = enzyme-linked immunosorbent assay; VEGF = vascular endothelial growth factor; PMMA = methyl methacrylate; cTnI = cardiac troponin I; EGF = epidermal growth factor; IP = interferon-inducible; MCP = monocyte chemoattractant protein; Timp-1 = tissue inhibitor of matrix metalloproteinase-1; RANTES = regulated upon activation, normal T cell expressed and secreted; MIP-1 β = macrophage inflammatory protein-beta; cTnT = cardiac troponin T; HRP = horseradish peroxidase; Si-FET = silicon field-effect-transistor; AFP = alpha fetoprotein; ACT = antichymotrypsin; MIA = magnetic immunoassay; APC = allophycocyanin; HE4 = human epididymis protein 4; TMB = 3,3′,5,5′-tetramethylbenzidine; hp = hairpin; LAMP = loop-mediated isothermal amplification; MRSA = methicillin resistant *Staphylococcus aureus*; FMDV = foot-and-mouth disease virus; mμLAMP = multiplex microfluidic LAMP; HAD = helicase-dependent amplification; NASBA = nucleic acid sequence based amplification; LFM = lateral flow chromatography microarrays; HSP = heat shock proteins; SPR = surface plasmon resonance; MEMS = micro-electro-mechanical systems; MIMED = magnetic integrated microfluidic electrochemical detectors

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The field of point-of-care (POC) testing technology is developing quickly and producing instruments that are increasingly reliable, while their size is being gradually reduced. In this review, we consider some of the most recent advances in the analysis of specific nucleic acid and protein biomarkers, paying special attention to the analytical techniques that can be accomplished by current and future miniaturized devices.

ANALYSIS OF PROTEINS

Proteins are a common target for many POC analysis methods (see Table I). They can be detected and quantified in a number of ways, but lateral flow immunochromatography (LFI) is the most common detection method due to its specificity. The following are some examples of this and other techniques currently under development for POC applications.

Lateral flow immunochromatography. Currently, most commercially available devices are based on LFI. The assays are conducted on paper, nitrocellulose or plastic supports. The analyte migrates up the support by capillarity, along with the appropriately-conjugated antibodies, up to a test zone where another antibody immobilizes the complex. A second antibody binds to the analyte, which is then sandwiched between the 2 antibodies. The conjugated antibody that did not initially bind to the analyte continues migrating up to a third antibody that binds it within a control zone. To detect the complex that is formed with the analytes, the first antibody is usually bound to markers such as colloidal gold particles, colored latex, or selenium, depending on the kind of detector used. As a result, qualitative or quantitative measures are typically obtained within 15 min.

Many of these detectors are based on reflectometry and measure visible light. The analytical devices can be designed for a single-strip test or for multiple analytes. Blood is the most common source of samples for analysis, and instruments often include a wicking pad to separate plasma from whole blood.

The portable LFI can also be used for urine samples. Some examples include the detection of anti- *Helicobacter pylori* IgG,[1] as well as the bladder cancer tumor marker NMP22 (nuclear matrix protein).[2]

There is a wide spectrum of commercial LFI POC devices designed for the detection of antigens related to infectious diseases,[3] inflammation and sepsis proteins (such as C-reactive protein [CRP] or procalcitonin [PCT]),[4] cardiac markers (creatine kinase isoenzyme MB, troponins, B-type natriuretic peptide, or myoglobin),[5] tumor markers (prostate specific antigen [PSA]),[6] glycosilated hemoglobin, and some hormones (such as human chorionic gonadotropin [hCG] or thyroid-stimulating hormone [TSH]).[7] These devices use different markers for the detection of the first binding antibody. Among them, fluorescent markers are most popular because they provide good sensitivity for analysis.

An example of this strategy is the work of Ahn et al,[8] who used a fluorescence scanner to measure CRP. These authors obtained a limit of detection of 0.133 μg/mL with good precision (CV $<$ 10%) and good correlation with the reference method based on immunoturbidimetry. A similar approach was used by Meagher et al[9] who designed a portable device for the detection of biotoxins. Their quick (less than 20 min) microfluidic chip-based immunoassay was tested in ricin, Shiga toxin I, and Staphylococcal enterotixin B (SEB). The limit of detection for SEB was 300 pM, which was reduced to 10 pM when adding a pre-concentration step on the chip. Herr et al[10] designed an even faster assay to identify periodontitis populations in less than 20 microliters. They could detect collagen-cleaving enzyme matrix metaloproteinase-8 (MMP-8) in less than 2 min.

The last 2 examples illustrate that many of the instruments developed for POC applications must account for the need to use small sample amounts. As a result, research or commercial[11] applications often include complex microfluidic systems. These systems contain microcapillaries that allow the flow of very small liquid volumes. Plasma can separate from the blood sample through a filter and reach the reaction chamber, where it meets fluorescent antibodies. The analytes are detected as antigen-antibody complexes, which move toward an antibody array where they are distributed according to different capture antibodies. The concentration of each analyte is measured from the Förster resonance energy transfer (FRET) fluorescence, in which fluorescence results from a distance-dependent interaction between an electron donor and an acceptor, with excitation wavelengths close to infra-red, as appropriate for plasma and serum samples.[12,13]

A clinical evaluation of this type of system was carried out with a commercial device, which incorporated 4 biomarkers related to brain ischemia (matrix metalloproteinase 9, D-dimer, S100β, and B-type natriuretic peptide) to evaluate the existence of a stroke in patients who showed related neurological symptoms.[14] Authors concluded that although the diagnosis was not perfect with these tests (the area under the receiver-operating-characteristic curve was 0.76 for hemorrhagic stroke and 0.69 for all strokes): they do aid in forming a diagnostic picture when they are applied together with other tests.

Microfluidic schemes can also be used, for instance, for the analysis of CRP in human serum.[15] This method compares well with the reference enzyme-linked

Table I. Summary of the latest works integrating different POC instruments to measure protein concentrations

Tested target	Relevance of sample*	Detection strategy	Sensitivity/Limit of detection (LOD)†	Timeframe	Ref.
Lateral flow immunoassays					
Thyroid-stimulating hormone (TSH) and human chorionic gonadotropin (hCG)	Whole blood and plasma	Piezofilm sensor	LOD: 18 pg/mL for TSH	5–10 min	(7)
C reactive protein (CRP)	Whole blood	Fluorescence (in plastic disposable cartridge)	LOD: 133 ng/mL	10 min	(8)
Biotoxins ricin, Shiga toxin I and Staphylococcal enterotoxin B	Biological toxins	Fluorescence (Microfluidic with polyacrylamide electrophoresis)	LOD: 20 nM for ricin; 500 pM for Shiga toxin I; 300 pM for Staphylococcal enterotoxin B	Incubation Ag-Ab: 30 min Electrophoresis: 3 min	(9)
Matrix metalloproteinase-8 (MMP-8)	Saliva	Fluorescence (Microfluidic with polyacrylamide electrophoresis)	LOD: 130 ng/mL	2 min	(10)
CRP	Serum	Chemiluminescence (Microfluidic channels)	Linear range: 2–15 μg/mL	26 min	(15)
CRP	Serum and cerebrospinal fluid	Fluorescence (Microfluidic capillary system for immunoaffinity chromatography)	Calibration ranges: 0.05–3.0 μg/mL for 1:10 diluted serum 0.01–30 for undiluted cerebrospinal fluid	10 min	(16)
CRP	Serum	Fluorescence (Microfluidic on PDMS)	LOD: 10 ng/mL LOD: ng/mL	<3 min 14 min	(17)
CRP	CRP-enriched human serum	Fluorescence (Microfluidic on PDMS)	LOD: 0.42 nM	20 min	(18)
Vascular endothelial growth factor (VEGF)	Human plasma	Fluorescence (Elastomeric microfluidic chip)	LOD: 4 pM	~10 min	(19)
CRP and ferritin	Serum	Fluorescence (Elastomeric microfluidic chip)	LOD: 12 μg/mL for CRP Linear range: 30–300 ng/mL for ferritin		(20)
CRP, tumor necrosis factor α (TNFα), and procalcitonin (PCT)	Serum	Fluorescence (Sensor chip of PMMA) Multiplexed	LOD: 1.265 μg/mL for CRP LOD: 10 ng/mL for TNFα LOD: 50 ng/mL for PCT	25 min approximately	(21)
Prostate specific antigen (PSA)	Serum	Voltammetry (Electrochemical inmunosensor in plastic disposable cartridge)	LOD: 20 pg/mL	20 min	(22, 23)
Cardiac troponin I (cTnI)	Serum	Magnetic signal (plastic disposable cartridge)	LOD: 10 pg/mL	15 min	(24)

Microarrays (multiplex)					
CRP, interleukin 6 (IL-6) and PCT	Plasma	Microfluidic fluorescence	LOD: 0.35 ng/mL for CRP LOD: 0.08 ng/mL for IL-6 LOD: 1.01 ng/mL for PCT	35 min	(25)
12 tumor markers (PSA and 11 cytokines)	Plasma	Fluorescence	LOD: ~30pM	10 min	(26)
PSA, TNFα, IL-6, and IL-23	Protein solutions	Bright white "light-emitting diode" (LED) Multiplexed	3.4 ng/mL for PSA 0.1 ng/mL for TNFα, IL-6, and IL-23		(27)
Tumor markers (cancer antigens CA 153, CA 125, CA199, and carcinoembryonic antigen: CEA)	Serum	Voltammetry (Electrochemical inmunosensor)	Detectable linear ranges: 0.42–80 U/mL for CA 153 0.5–65 U/mL for CA 125 0.8–75 U/mL for CA 199 0.8–46 ng/mL for CEA	<5 min	(28)
Anti-hepatitis C virus (anti-HCV) antibodies (HCV core and nonstructural 4 antigen [NS4])	Artificial targets and human serum	Fluorescence	LOD: 10 ng/mL for anti-CHV core LOD: 50 ng /mL for NS4	14 min	(29)
CRP	Saliva (1000-fold dilution)	Fluorescence	LOD: 10 pg/mL	10 min	(30)
10 cytokines	Saliva	Fluorescence	LOD: From 8 pM for IL-8 to 469 pM for (VEGF)	Incubation: 2 h Assay: 30 min approximately	(31)
Other methods					
Cardiac troponin T (cTnT)	Serum	Amperometric (Electrochemical inmunosensor)	LOD: 0.2 ng/mL Linear range: 0.1–10 ng/mL	2 h	(38)
Tumor markers (PSA, CEA, and AFP)	Serum	Multiplexed silicon field-transistor (Si-FET)	Detection quantitative: from 0.2 to 114 ng/mL	20 min	(39)
PSA and PSA-α 1-antichymotrypsin	Serum	Quartz crystal microbalance e sensor	LOD: 0.39 ng/mL Detection range: 0.39–200 ng/mL	8 min	(40)
CRP	Whole blood	Magnetic permeability inmunoassay	LOD: 3 μg/mL Linear range: 0–260 μg/mL	5.5 min	(41)
CRP	Whole Blood	Noncompetitive immunoassay Fluorescence	LOD: 160 ng/mL	2 min	(42)
PSA	Serum	Homogeneous fluoroimmunoassay	LOD: 56 pg/mL	30 min	(43)
No antibodies					
Thrombin	Plasma	GoldBio strip reader after aptamers binding	LOD: 2.5 nM Linear range: 5–10 nM	10 min	(36)

(*Continued*)

Table I. (*Continued*)

Tested target	Relevance of sample*	Detection strategy	Sensitivity/Limit of detection (LOD)†	Timeframe	Ref.
Generation of thrombin and other coagulation activators	Whole blood and plasma	Amperometric (Substrate cleaved for coagulation activators)	LOD: 0.4 U/mL thrombin	15–20 min	(44)
Albumin	Urine	Visual (Spot test with erythrosin B)	LOD: 0.5 mg/mL	5 min	(45)
Albumin	Urine	Reflectance (Test strip)	LOD: 10 μg/mL	1 min	(46)
α-amilase	Saliva	Reflectance with LED device (Test strip)	Linear range: 10–132 kU/L at 37°C	0.5 min	(47)

*We indicate in this column what kind of samples (actual patient samples, synthetic test oligos, etc.) were used to compare them with what could be found in an actual patient sample.
†Some studies focus on LOD and others on sensitivity. In either case, the values may depend on specific experimental conditions, and these values should be taken as merely indicative. Samples are usually applied in volumes of a few microliters.

immunosorbent assay (ELISA), and it could be carried out in a shorter time (25 min). In addition to raw serum, microcapillary systems allow for the use of diluted serum and cerebrospinal fluid in the detection of CRP within 10 min.[16]

The use of microcapillary arrangements has driven some researchers to use polydimethylsiloxane (PDMS) as an elastomeric support. PDMS is a soft organic silicon polymer that is widely used for cheap microfluidic devices, and some of these supports contain multiple channels. Gervais et al[17] designed a capillary-driven microfluidic chip built on PDMS, which was capable of analyzing CRP from human serum using a fluorescence microscope. This method allowed the detection of 10 ng/mL of CRP in serum in a single step within less than 3 min. For smaller sample amounts, this device had a capillary pump that permitted slower sample speed. In this way, a 1 ng/mL concentration was detected by slowing the detection process to 14 min.

In a similar approach, Hosokawa et al[18] produced a PDMS microchip with no pump. Instead, channels were filled with liquid as a consequence of the pressure difference produced by degassing. They tested the method for the detection of human CRP and rabbit IgG (they also tried a competitive method for the latter) and achieved limits of detection of 0.21 nM (0.21 fmole) and 0.42 nM, respectively. A number of analytes can be measured using these microfluidic systems, such as vascular endothelial growth factor (VEGF),[19] ferritine,[20] or CRP.[20] An alternative method used PDMS microchips with affinity microcolumns to isolate and asses Hb A_{1c}. However, for this method, the Ag-Ab (antigen-antibody) mix had to be incubated for 1 h and 37°C prior to detection.

Although PDMS provides a good support for miniaturized POC applications, some authors prefer to use more rigid supports such as poly(methyl methacrylate) (PMMA). This is the case of the work carried out by Kramer et al,[21] who achieved simultaneous determination of 3 inflammation and sepsis parameters: TNF α, PCT, and CRP from human serum. This method involved an incubation step that took 20 min.

In addition to fluorescence detection, there are other approaches to improving the efficiency of LFI biosensors. One of these alternatives includes the use of quantum dots (a particular kind of semiconductors that can produce signals with good sensitivity). An example of this strategy can be found in the work by Liu et al.[22] They used the classic LFI technique with electrochemical immunosensors, where the conjugated antibody was marked with (CdS/ZnS) quantum dots. They obtained electrochemical measurements of tumor marker PSA. A similar method was also used by Lin et al[23] who achieved a limit of detection of 0.02 ng/mL and

good reproducibility (with a relative standard deviation of 6.4%) using a serum sample. An alternative to the quantum dots could be the use of markers with magnetic properties. Xu et al[24] explored this option for the detection of very small amounts (0.01 ng/dL) of cardiac marker cTnI (cardiac troponin I) in serum. They used superparamagnetic nanobeds as markers for the conjugated antibody and they could measure the magnetic signal produced by sandwich type superparamagnetic complexes.

Microarrays. Microarrays consist of multiple microscopic detection spots placed on a chip, and they are suitable for POC applications due to their small dimensions and capacity for multiplexing. The work by Kemmler et al[25] is an example of an application using this set-up. These authors used a compact microarray system for the determination of inflammation and sepsis markers CRP, IL-6, and PCT in plasma. The system contained a fluidic apparatus work with small volume samples. Fan et al[26] used this principle to carry out a simultaneous analysis on 12 plasma proteins (PSA and 11 cytokines). The detection was performed using an integrated barcode designed with polydimethylsiloxane (PDMS) on glass and containing a multichannel matrix capable of measuring plasma proteins *in situ*. These channels contained DNA oligomers that could bind specific sequences from DNA-tagged antibodies and plasma proteins that could be captured between 2 antibodies. Prior to this step, plasma had to be separated from blood cells right on the chip. Results were obtained within 10 min from a single blood drop.

Schroeder et al[27] developed a similar technique for PSA, TNFα, IL-6, and IL-23; and Wu et al[28] also developed an electrochemical immunosensor to simultaneously determine tumor markers (cancer antigens) CA153, CA 125, CA 199, and CEA (carcinoembryonic antigen). In these microarrays, immobilized gold nanoparticles had peroxidase-tagged antibodies, which provided a direct electrochemical response. When the antigen bonded to the antibodies and immunocomplexes formed, a signal reduction was observed in relation to the steric hindrance of the peroxidase. Ember et al[29] also described the preparation of a platform that contained a microarray for the detection of hepatitis C antibodies by fluorescence. They obtained analytical results within 30 min.

In saliva, Christodoulides et al[30] could detect 10 pg/mL CRP in 1000-fold diluted samples. Additionally, Blicharz et al[31] could detect 10 proteins (VEGF, epidermal growth factor [EGF], interferon-inducible [IP-10], IL-8, monocyte chemoattractant protein-1 [MCP-1], tissue inhibitor of matrix metalloproteinase-1 [Timp-1], regulated upon activation, normal T cell expressed and secreted [RANTES], macrophage inflammatory protein-beta [MIP-1 β], eotaxin-2, and IL-6) related to asthma attacks, and Floriano et al[32] detected 21 proteins related to myocardial stroke, showing the multiplexing potential of microarrays.

Aptamers. Aptamers are *in vitro*-evolved nucleic acid molecules that are capable of binding specific target molecules or cells,[33-35] and it is possible to use them for the analysis of protein samples. This case is demonstrated in a recent article where they used aptamers in a dry-reagent strip application for the detection of thrombin from human plasma.[36] Aptamers substituted for antibodies in the test and control zones. A set of aptamers was labeled with gold nanoparticles, which was shown to have equal specificity and greater sensitivity to bench top equivalent methods. The authors obtained a limit of detection of 2.5 nM and good linearity in the 5–100 nM range for thrombin. Similarly, Liu et al 2009[37] detected whole Ramos cancer cells with aptamers, showing that this molecular tool is very versatile for a wide range of analytes because they can be used even in the absence of specific structural knowledge about the target.

Other methods. Silva et al[38] used anti-cTnT (cardiac Troponin T) Ab-containing streptavidin-conjugated microspheres, which they incubated with serum cTnT samples for 1 h and followed with another hour with Ab-conjugated HRP (horseradish peroxidase). After a wash and addition of H_2O_2, they could measure an amperometric signal. Kim et al[39] also measured an electric signal to analyze tumor markers PSA, CEA, and alpha fetoprotein (AFP) simultaneously. They used silicon field-effect-transistor ([Si-FET]; a kind of transistor that uses the electric field itself to alter the conductivity of the material) sensors that contained antibodies for each of the tumor markers from serum. A similar method was used by Uludag et al[40] with quartz crystal microbalance sensors to analyze PSA and PSA-α1-antichymotrypsin (ACT) complexes. The method described by Ross et al,[7] using piezoelectric sensors to measure concentrations of hCG and TSH in blood, plasma, and serum, falls in a similar category, and they achieved a low limit of detection (28 pg/mL), with a good linearity in the reference range (80–800 pg/mL).

Some applications use magnetic particles as a convenient way of separating target proteins such as CRP from the medium, as well as a way to detect proteins based on their magnetic properties Ibraimi et al.[41] This analysis could be done with 4 μL of whole blood samples within 5 min and with a good correlation to reference

clinical immunoassays (magnetic immunoassay [MIA] and turbidimetric). Similarly, Tarkkinen et al[42] developed a single-step noncompetitive assay for the same protein, measuring the fluorescence from an organic complex of Europium conjugated to the detection Ab. Kupstat et al[43] also used a similar complex as a donor for FRET, and allophycocyanin (APC) as the acceptor. These authors adapted a reference homogeneous fluoroimmunoassay into a commercial plate-reader system (KRYPTOR) to achieve miniaturization and to reduce assay costs. They achieved better sensitivity and limit of detection than the standard plate-reader method.

It is also possible to carry out specific analyses without using antibodies. Thuerlemann et al[44] monitored the formation of thrombin as it cleaved a specific substrate that released an amperogenic moiety. Kaneko et al[45] developed a visual method for the detection of urine proteins with a visual limit of detection for albumin at 0.5 μg/mL. Similarly, Kouri et al[46] evaluated a new method to measure albumin and creatinine by reflectance. The limit of detection was established at 10 μg/mL. Additionally, hand-held monitoring of the sympathetic nervous system could also be carried out using a reactive strip to measure α-amilase in saliva Yamaguchi et al.[47]

Wang et al[48] used a sandwich-type ELISA on a microchip for the detection of ovary cancer marker human epididymis protein 4 (HE4). The detection antibody was labeled with horseradish peroxidase so that when TMB (3,3',5,5'-tetramethylbenzidine, a chromogenic substance) was added, the resulting blue color could be recorded in a system coupled to a cell phone. When diagnostic specificity and sensitivity of this device were compared with microplate conventional immunoassays, they observed that specificity is 90% in both cases and sensitivity was lower than for the microplate assay (94.7% for the microplate and 84.2% for the chip).

ANALYSIS OF NUCLEIC ACIDS

Although proteins are a common target for the POC analyses, specific nucleic acid analyses can also contribute to the characterization of biological samples (see Table II). One of the main challenges in the analysis of nucleic acids for clinical, forensic, and other applications is the small amount of sample. To read a sufficient signal in the corresponding instrument, the amount of sample, the signal intensity (but not the noise), or both need to be increased. Some interesting methods that can be used for sample and signal amplification are described below as an exploration of current research direction.

Signal amplification. Some authors focus on the amplification of the detection signal instead of the sample. This is the approach of Wei et al,[49] who used a hairpin (hp) RNA molecule coupled with horseradish peroxidase to obtain specific and very sensitive detection of endogenous IL-8 mRNA, a proposed salivary biomarker for oral cancer. The hp molecule contained a sequence complementary to the target mRNA sequence. Absent the target mRNA, the hp was self-annealed and the horseradish peroxidase was too close to the solid support of the hp probe to interact with its substrate. However, in the presence of the target mRNA, the hp hybridized with it, changing its conformation and moving the peroxidase away from the solid support. As a result, the peroxidase, free from steric hindrance, could interact with its substrate and produce a measurable electrochemical signal.

Isothermal amplification. In spite of attempts aimed at increasing detection signal, given the availability of the polymerase chain reaction (PCR), amplification of DNA is still a useful strategy. However, even with the high sensitivity and specificity of PCR-based methods, they have drawbacks such as the dependence on a thermocycler, which can increase the cost and complexity of miniaturized portable devices. As a result, different strategies have been proposed to amplify samples without using a thermocycler. One of these strategies is the loop-mediated isothermal amplification (LAMP) of DNA.[50] This technique relies on strand displacement by a DNA polymerase and on the appropriate design of primers (4 primers that can bind in 6 distinct sequences) to amplify target sequences at a constant temperature ($\sim$60°C) with high selectivity. Based on this principle, Bearinger et al[51] developed a prototype of a small device capable of analyzing swab-collected samples in $\sim$90 min. They could detect $\sim$17 copies of methicillin resistant *Staphylococcus aureus* (MRSA) genomic DNA and foot-and-mouth disease virus (FMDV) from an epithelial homogenate in100 μL of reaction volume. They used a simple colorimetric detection system based on the detection of free Mg^{+2}. The method is based on the principle that the concentration of this ion diminishes as it binds to the pyrophosphate that is released in the amplification reaction. The versatility of this method also permits applications that use reverse transcription.[52]

Fang et al[53] also used a LAMP system, but in this case, they developed a multiplex gene assay using a microfluidic set-up with 10 microchambers connected to 1 microchannel per chamber. The microchannels had low mass-transfer coefficients to prevent cross contamination among different microchambers and, in addition to the direct visualization by the white precipitate

Table II. Summary of the latest works integrating different POC instruments to analyze nucleic acids[‡]

Tested target	Relevance of sample*	Detection strategy	Sensitivity /Limit of detection (LOD)†	Timeframe	Ref.
IL-8 mRNA (proposed salivary marker for oral cancer)	Clinical saliva samples	Signal amplification taking advantage of steric self-hindrance of a hairpin probe that prevents horseradish peroxidase to reach its substrate and produce and electrochemical signal	LOD: 0.4 fM	1 min	(49)
Methicillin resistant *Staphylococcus aureus* (MRSA) genomic DNA and foot-and-mouth disease virus (FMDV)	Swab collected sample	Loop-mediated isothermal amplification (LAMP) and colorimetry	LOD: 17 copies in 100 μL of reaction volume	~90 min	(51)
Nervous necrosis virus RNA	Infected biological samples	Magnetic purification, reverse-transcript LAMP (RT-LAMP) and slab gel electrophoresis	LOD: 100 fg cDNA	60 min	(52)
Flu viruses including influenza A H1N1	Clinical samples	Visual and fluorimetric detection on multiplex microfluidic LAMP (mμ-LAMP) with 10 microchambers	LOD: 10 copies/ μL in 2 μL	0.5 h	(53)
Genomic DNA of *N. gonorrhoeae* and *S. aureus (MRSA)*	Laboratory preparations of *N. gonorrhoeae* and *S. aureus*	Fluorescence after helicase dependent amplification (HAD) at 65°C	LOD: 250 pg genomic MRSA-DNA (5×10^4 *S. aureus* cells) LOD : 1 ng of piv_{NG} gene (1.32×10^5) *N. gonorrhoeae* cells	~145 min	(54)
RNA from *Bacillus anthracis*	RNA purified from a laboratory strain	Lateral flow microarray (LFM) after nucleic acid sequence based amplification (NASBA)	LOD: 250 fg (2–3 *B. anthracis* cells)	~60 min	(56)
Cryptosporidium parvum oocysts	Purified total mRNA from *C. parvum*	Oligonucleotide-gold nanoparticles after amplification of heat shock proteins (HSP)	LOD: 5000 *C. parvum* oocysts	~2–3 h	(57)
pUC18	pUC18 amplicon	Sanger sequencing	LOD: 1 fmole of DNA template	~30–35 min	(58)
Single nucleotide variations/ interaction of DNA with polymerase	Synthetic oligonucleotides	Nanopore analysis	LOD: 100 fmole	~5 min	(60-63)

*We indicate in this column what kind of samples (actual patient samples, synthetic test oligos, etc.) were used to compare them with what could be found in an actual patient sample.
†Some studies focus on LOD and others on sensitivity. In either case, the values may depend on specific experimental conditions and these values should be taken as merely indicative. Samples are usually applied in volumes of a few microliters.
‡See Table III for PCR applications.

Table III. Summary of the latest works integrating different steps in the sample preparation, PCR-amplification, and detection of biological samples on solid supports as a step toward the fabrication of versatile POC instruments

Tested target	Relevance of sample*	Sensor	Device features	Sensitivity/Limit of detection (LOD)†	Timeframe	Ref.
Cyanobacterial 16S rRNA gene (2060-bp) and human anti-α-1-chymotrypsin gene (330-bp)	Test oligos	Off the device	PCR only	LOD: 0.5 ng/μL genomic DNA	22.5 min (for amplification only)	(72)
H1N1 virus	Swab saliva sample	E-DNA	MIMED	LOD:10 $TCID_{50}$	3.5 h (PCR$\sim$150min)	(68)
Influenza A, influenza B, corona virus OC43, and human metapneumovirus	Cloned target viral sequences in plasmid standards	Fluorescence labeling of forward PCR primers	Integrated microdevice for concentration, amplification and multiplex analysis	LOD: 10 copies/reactor	within 2 h	(73)
STR‡	Cells from a buccal swab sample	optical excitation/ detection module	DNA analysis compatible with CODIS	Sensitivity: 1.15bp	3.5 h	(74)
STR (Y STR ie, STR from the Y chromosome)	Buccal swab sample	Fluorescence from fluorescence-labeled primers	Just for stacking capillary electrophoresis (no PCR)	LOD: 25 pg (1:1000 male:female genomic DNA + 15 min. DNase I digestion)	6 min just the electrophoresis	(75)
STR	Biological stains from a mock crime scene	Fluorescence	Real-time PCR in 100mm wafers	LOD: 100 copies for complete DNA profile; sensitivity: 0.8 bp allele typing accuracy	2.5 h	(69)
Determination of monozygous twinning	Bucal swab sample	Fluorescence	microfabricated capillary array electrophoresis (μCAE)	Sensitivity: single base resolution	$<$30 min	(76)
Escherichia coli stx1	Lab culture	Fluorescence reporter probes	Miniature real-time PCR	Minimum amount used: 1.25 ng/μL	Regular real-time PCR	(70)
Synthetic test oligo	Synthetic test oligo	Fluorescence of Cy3-labeled probe	Microstructured optical fiber (MOF)	10 μM (in 10 nL)	$<$30 min (hybridize and detect)	(77)
Methicillin-resistant *Staphylococcus aureus* (MRSA)	DNA extracted from clinical MRSA isolates	FRET from probes labeled with AlexaFluor 647 reporter dye and BHQ3 quencher dye	PDMS microfluidic system for PCR and fluorescence detection	Used sample containing 11.2 pg of DNA	Regular real-time PCR	(78)

λDNA	In test buffer	FRET from fluorescein and MGB-NFQ maker-containing primers	Microfluidic device to characterize PCR in aqueous-in-oil droplets and FRET	Minimum used concentration: 3.5×10^{-4}ng/μL	Regular real-time PCR	(67)
Escherichia coli and Bacillus	Lab culture	Electrochemical detection on gold nanoparticles	Thermal lysis, magnetic isolation, asymmetric PCR (one primer more abundant than the other), and detection using silver-enhanced gold nanoparticles	LOD: 0.5 nM genomic DNA; linear between 10^2–10^5 cells/sample	About 3 h	(79)
Genetic breast cancer markers: estrogen receptor-α, plasminogen activator urokinase receptor, epidermal growth factor receptor, and erythroblastic leukemia viral oncogene homolog 2	Test samples	Surface Plasmon Resonance	Multiplex device	LOD: ~3 nM	90 min (excluding PCR)	(66)
Hepatitis C virus RNA	Total RNA extracted from clinical samples	Cy3-conjugated oligonucleotide detection probes	Microarray combining protein and nucleic acid detection	LOD: 10 pM	16 min after PCR	(29)

*We indicate in this column what kind of samples (actual patient samples, synthetic test oligos, etc.) were used to compare them with what could be found in an actual patient sample.
†Some studies focus on LOD and others on Sensitivity. In either case, the values may depend on specific experimental conditions and these values should be taken as merely indicative. Samples are usually applied in volumes of a few microliters.
‡Short tandem repeats (STR) can be used for identification in forensic science, a field that could also benefit from new POC applications.

formed by Mg^{+2} and pyrophosphate, they added a fluorescent dye to obtain the positive signal. They called this system mμLAMP (multiplex microfluidic LAMP), and it allowed them to detect as few as 10 copies/μL of the target sequence in 2 μL quantities of sample within 30 min. In this study, they used flu viruses to test the device, and they were able to detect the seasonal influenza A H1N1 virus from clinical samples.

Another example for the amplification of target nucleic acid sequences without thermocyclers was developed by Andresen et al[54] based on helicase dependent amplification (HDA), where a helicase carried out strand separation. They achieved specificity by carrying out the amplification reaction at 65°C with a combination of thermophilic helicase and polymerase enzymes. These authors used labeled primers to detect amplified sequences from genomic DNA of laboratory preparations of *N. gonorrhoeae* and *S. aureus*.

Nucleic acid sequence based amplification (NASBA)[55] is a similar strategy that can be useful for point-of-care analytics. This technique relies on the T7 RNA polymerase activity, which can catalyze the synthesis of RNA from dsDNA, performing a role similar to what the helicase does in HDA. Using this principle in miniaturized lateral flow chromatography devices, Carter et al[56] detected RNA from pathogens such as *Bacillus anthracis* in 10 μL volumes, achieving subfemtomole limit of detection. They could detect RNA from as few as 2 *B. anthracis* cells within 120 s after sample preparation and amplification. In this case, they carried out the detection of laboratory preparations of the bacillus by hybridizing them to oligonucleotides attached to dyed microspheres. The separation was carried out in lateral flow chromatography microarrays (thus named LFM), which allow the determination of analytes without the use of fluorescence detection systems and provide the ground for multiplexing.

Alternative methods. There are some specific methods for the detection of nucleic acids that deserve a separate mention due to their originality. One of these methods involves the use of heat shock proteins (HSP). More specifically, Javier et al[57] developed this strategy to detect mRNA targets derived from *Cryptosporidium parvum* oocysts using oligonucleotide-gold nanoparticles. By incubating the oocysts at 42°C for 20 min, they induced the synthesis of the mRNA for HSPs and they purified them with magnetic beads that were coupled with oligo-dT. They detected the mRNA for HSP70 using gold nanoparticles coupled with oligonucleotides specific to their target mRNA. These gold nanoparticles facilitate the formation of aggregates in the presence of the target mRNA, and they can be detected by simple colorimetric changes in to the surface plasmon resonance (SPR). This detection method is based on the distortion that adsorbed analytes produce on measurable electron oscillations on the surface of the gold particle, and it is common among lab-on-a-chip microdetection devices. The use of HSP mRNA is a clever way of achieving nucleic acid amplification with low material requirements. However, oocysts need to be alive so that they can respond to the heat shock, a factor that must be taken into account for future applications and extensions of this method.

The specific interactions of nucleic acids are based on their base sequence. Hybridization with appropriate probes is an indication of this specificity. However, the most accurate method for the identification of a given nucleic acid is sequencing. In this sense, it is remarkable that the traditional Sanger sequencing method was implemented by Blazej et al,[58] who were able to complete Sanger sequencing from only 1 fmole of DNA template. These authors could sequence up to 556 continuous bases with 99% accuracy, demonstrating read lengths required for *de novo* sequencing of human and other complex genomes.

In their work, Blazej et al[58] implemented a classical sequencing scheme that included dye-terminator sequencing and laser-induced fluorescence detection, and they were able to obtain sequence data within 35 min with a small circular bioprocessor that was 100 mm in diameter. However, as described in the next section, faster sequencing technology may become available in the future.

Nanopore analysis. As stated in the previous section, sequencing nucleic acids from analytical samples is one of the most direct ways of characterizing this molecule. Since its introduction in 1996 as a method for the analysis of nucleic acids,[59] nanopore technology has experienced substantial progress to the point where it is currently possible to obtain sequence information on single nucleic acid molecules[60] as well as on complexes formed by nucleic acids and proteins.[60-63] These analyses are based on the principle that nucleic acids go through nanoscopic pores by electrophoresis. The dimensions of these pores are such that when nucleic acids go through, they cover most of the pore area. The aqueous buffer in which the pore is immersed contains ions that produce a measurable electric current. When the nucleic acids traverse the pore, a blockade in the current can be detected and, on average, this blockade has different properties depending on the specific characteristics (including base sequence) of the analyte that is going through. Nucleic acids are driven to the pore by the electric voltage as soon as they are added to the detection solution and the measurements can be carried out

within 5 min for a single nucleic acid sample. Given these properties, it is likely that this technology can be used for sequencing applications in the near future.

Currently, the most sensitive nanopores are formed with α-hemolysin toxins inserted in lipid membranes. However, there are technical difficulties associated with the formation and maintenance of such pores and this factor is encouraging research on solid state nanopores, for which sensitivity is gradually improving.[64] Solid state nanopores are more durable than biological ones, but their sensitivity is smaller. Thus, some researchers are working to combine the best features of both types of nanopores by coating the metallic surface of solid nanopores with lipids.[65] This sequencing approach has great potential to develop POC sequencing instruments due to the small dimensions of the pore and the small analyte amounts required.

PCR. In spite of the multiple alternatives presented so far, PCR still remains an important method for target nucleic acid sequence amplification, a technique that helps in the acquisition of a good signal and in the efficient and specific detection of analytic targets. Table III summarizes some of the latest works dealing with different steps in the sample preparation, PCR-amplification, and detection of biological samples on solid supports as a step toward the fabrication of miniaturized POC instruments. In this area, technological improvements may involve variations in electrode preparation[66] or control software.[67]

Many of these works involve the fabrication of solid supports with micro-electro-mechanical systems (MEMS) or magnetic integrated microfluidic electrochemical detectors (MIMED),[68] to achieve temperature control or to perform all steps of the analysis, respectively.

Instead of the reverse-transcriptase-PCR used by some of the devices mentioned before,[52,68] some of the POC instruments capable of PCR perform real-time PCR.[69,70] In this sense, it is worth mentioning the work by Lee et al[71] who compared conventional real-time PCR machines with real-time PCR chips. They concluded that PCR chips, which are mostly made using MEMS, have the advantage of their compact size, low sample volume (in the nanoliter range), and their short analysis time (which can be as short as 10 s per PCR cycle and 370 s for the whole quantification process). However, it seems that these devices still have room to improve in terms of detection limit, quantification uncertainties, and melting analysis ability.

CONCLUSION

In conclusion, there are many devices that are currently being designed to carry out POC analyses. Their aim is to detect and quantify proteins and nucleic acid markers for a variety of health conditions: from infectious agents such as the influenza virus, to conditions such as breast, ovary, or bladder cancers. Many of these devices still need some work to become functional parts of a complete POC analysis device. However, given the fast progress in the field and the large number of research groups involved, we believe that remaining issues will be soon overcome, and portable and economical POC devices that include the features described in this article will become commercially available in the near future.

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