ABSTRACT: Rapid detection of single nucleotide variations (SNVs) is of critical importance to early diagnosis of several diseases and the prediction of diverse responses to a specific treatment. Based on the information published in the literature, discrimination of SNVs is a developing area of study with great research enthusiasm and is also an area that can benefit from microfluidics-integrated designs. This review provides a brief overview of different microfluidics-based strategies for rapid detection of SNVs and mismatched bases. Sensors based on various microfluidic formats, such as paper-based microfluidic biosensors, droplet-based microfluidic systems, and magnetic bead-based microfluidic biosensors, have been discussed with respect to their specific pros and cons for SNV detection. These systems have shown promise for distributed on-site diagnostics in personalized medicine.

1. INTRODUCTION

It is becoming widely recognized that rapid detection and management of diseases is of critical importance to improve healthcare and the survival rate of patients worldwide. Point-of-care (POC) tests can quantify disease biomarkers and estimate physiological parameters within minutes of the test being administered, without the need for sophisticated laboratory equipment and technicians. POC diagnostics will enable convenient and on-time decisions for patient care and increases the testing capacity. A wide range of diseases, such as neurodegenerative diseases, genetic diseases, cancer, and cardiovascular diseases, are associated with single nucleotide variations in DNA sequences, that is, single nucleotide polymorphisms (SNPs) and single nucleotide mutation. Various POC devices are being developed by genotyping SNVs to detect liver cancer, hepatitis B, cryptosporidiosis, and genetic predispositions to diseases and gene mutation. Clinical evaluation of responsiveness of patients to drugs, such as antiepileptic drugs, based on their unique genetic profile, has also been conducted by genotyping of SNVs using POC devices.

Thus, the capability of POC diagnosis of SNVs is increasingly expanding for the realization of personalized medicine and the prediction of patient responses to a specific treatment, which consequently leads to a personalized treatment. Different techniques have been adopted to discriminate SNVs, such as DNA hybridization, mass spectrometry, molecular beacons, and high-resolution DNA melting analysis. However, these methods often require time-consuming processes, such as amplification, enzymatic
digestion or extension, stringency washing steps, and postisolation manipulation. These disadvantages limit the practical application of these techniques and motivate researchers to develop new SNV genotyping devices that are rapid, cost-effective, and sufficiently sensitive and do not require complicated operation steps.10

As such, microfluidics-based detection techniques have attracted great research interest for potential easy-to-do detection or multiplexed SNV genotyping. This review discusses different microfluidics-integrated devices developed for rapid SNV genotyping, with the focus on the integration of microfluidics and sensors. Information on the properties of microfluidic systems and biosensors can be found in other published reviews, in which different aspects of SNV genotyping methods and devices have also been discussed.1,10,11

2. MICROFLUIDICS-INTEGRATED BIOSENSORS FOR GENOTYPING

Microfluidic strategies have gained popularity for a wide range of applications from chemical processes to biomedicine and biosensing. A current trend of biosensor development is to incorporate the detectors with microfluidics to produce portable diagnostic devices.12 Multiple functions can be compactly integrated in a single device. Owing to their dimensions and designs, such devices can handle and facilitate sample analysis with reduced manual intervention, leading to lowered assay cost and minimized handling errors, and, in many cases, in a high-throughput fashion. Other advantages include high mixing efficiency of reagents, some degree of automation, disposability, and miniaturized dimensions. Thus, microfluidics-integrated biosensors can potentially realize real-time biosensing of multiple analytes with high precision and accuracy.11 In the following, different kinds of microfluidics-integrated systems for SNV genotyping are discussed.

2.1. Paper-Based Microfluidic Devices for SNV Detection. Among diverse categories of microfluidic chips, paper-based lateral-flow assays (LFAs) have attracted great research interest to meet POC demands. In these systems, microfluidic channels made of paper strips are integrated with biosensing mechanisms at the end. Commonly, a lateral flow biosensor comprises four parts: a sample pad for loading the sample, a conjugation pad for deposition of a receptor/probe conjugate, a nitrocellulose membrane for dispensing detection and control receptors, and an absorbent pad. Lateral-flow biosensors have been demonstrated for the discrimination of SNVs.5,13 In most cases, gold nanoparticles (AuNPs) are used on the paper-based LFAs as labeled probes for visual detection of SNVs. These nanoparticles are loaded on the conjugate pad, which forms colloidal lines in the presence of target biomarkers. AuNPs can both amplify the signals and also convert the produced signals to color for nucleic acids.14 They are a good alternative to labels such as radioisotopes and fluorescent dyes in a POC setting, by allowing for visual detection with the naked eye. These paper-based LFAs can be found in both single test and multiplexed detection strips, with the latter commonly used for high-throughput analysis. The multiplexed detection means simultaneous detection of two or more SNVs in a single device.

Various nucleic acid probes have been incorporated with paper-based LFAs. Lin et al. used an enzymatic genotyping system based on LFA combined with a primer extension (Figure 1).5 A portable strip reader could be used for semiquantitative analysis. These systems did not use sophisticated devices, which would be advantageous for POC analyses. In another report, AuNPs modified with hairpin oligonucleotides (HO) were used for SNP detection.15 Target DNAs hybridizing with HO would induce conformational changes of HO and break free the biotin on the AuNP surface. The freed biotins then led to the accumulation of AuNPs, hence changing the color at the LFA reading zone. The signal ratio between SNP and wild-type DNA could reach 28. Xiao et al. used a paper-based lateral-flow biosensor downstream of a circular strand displacement reaction. This scheme allowed for visual inspection of a single base mismatch even at a detection limit of 0.01 fM. The assay time was 2.5 h for the circular strand displacement reaction to amplify the matching sequences.16

Previously, colloidal gold-strip-based biosensors have been designed for biodiagnosis of up to two gene loci. Recent advancements in paper-based biosensors, for example, the design of two- and three-dimensional flows,16 made these devices promising for the detection of multiple analytes simultaneously.2 For example, a biosensor designed by Ma et al.3 could qualitatively detect the SNP locus of the cancer susceptibility gene CYP1A1 and semiquantitatively detect AFP, a tumor protein marker of liver cancer using two double-labeled gold probes. Litos et al. also developed a biosensor comprising four test zones in a single strip-based biosensor functionalized with gold nanoparticles for simultaneous detection of four alleles and genotyping of two SNPs.17

Paper-based sensors benefit from the advantages of low cost, straightforward fabrication, and disposability. These POC devices, however, also have some critical disadvantages. The most important problem is the fouling of channels, which reduces the quantities of targets that reach the sensing region. Paper-based microfluidic biosensors also cannot handle multistep sensing processes and are best suited for single step diagnostic devices, such as pregnancy test strips.1

2.2. Lab-on-a-Chip (LOC) Devices for SNV Detection. In chip-based microfluidics, fluid can flow in a continuous or intermittent manner through a system with functions such as valves, pumps, as well as mixers. These functions prepare the samples for detection, which can be realized with various sensing mechanisms and often integrated at the back end of the chip. Such chips are known as laboratories-on-a-chip or micrototal analysis systems (μ-TAS), commonly used for analyzing bioparticles such as cells, proteins, and DNA.1,12 For POC purposes, it is highly important to design a fully integrated and all-in-one device.
In some of the previously reported LOC-based genotyping devices, fluorescence detection was carried out using a charge-coupled device, which was inherently of relatively large dimensions and needed complicated signal analysis. Some alternative solutions include using a photomultiplier module, which is highly sensitive, or photodiodes, which can be readily integrated into microchannels. Both schemes can be realized for low-power, battery-operated, and standalone biosensing devices. For multiple SNV detection and high-throughput analyses, microarray chips are preferred choices. Ren et al. recently used a microarray chip based on competitive allele-specific polymerase chain reaction (PCR) for studying ancestry inference of East Asian populations by detection of 72 autosomal SNPs. Instead of optical detection, electrochemical sensors can also be integrated into microfluidic devices to detect mutations in DNA sequences. For example, Liu et al. combined PCR amplification with electrochemical detection of mutations. Another example is a 3D electrochemical biosensor system developed by Ilkhani et al. The device comprised of two substrate layers, one deposited with gold dots and another deposited with a gold electrochemical cell. The detection limit and sensitivity, as well as the specificity for detection of a single base mismatch, were found to be much higher than those of commercial screen-printed electrodes.

2.3. Droplet-Based Microfluidic Chips for SNV Detection. Droplet-based microfluidic chips are a well-known class of microfluidic systems which can be potentially used for high-throughput diagnostic tests. They are flexible for use in several types of diagnoses with different subtypes of transducers. The formation of droplets is accomplished by introducing immiscible fluids into the system and using external electric fields and injectors. For SNV detection, they can be advantageous because on-chip dilution and rapid mixing can be achieved. Individual droplet forms in and moves through the microchannels, with each droplet acting as a separate reactor.

The major limitation of this method is the short residence time of the sample in the system, which ranges from minutes to hours, so this method cannot be used for long-term observation. Some solutions have been suggested for overcoming this limitation. Cui et al. developed a system for long-term observation of SNVs (10 s to 150 min) and a high capability of discrimination between matched and single base mismatched targets, with discrimination factor ranging from 100 to 550 (Figure 2). Using serpentine and long paths, stopping the flow through the channels, and continuous monitoring of the captured samples, they broadened the monitoring time of SNVs. Moreover, unlike other droplet-based microfluidic systems, which used end-point analysis, they used kinetic measurement in their system. This system demonstrated high discrimination capability and specificity of detection, which could be used for low abundance mutation in the genome. The high specificity of their method was attributed to the longer residence time of the samples in the system.

2.4. Bead-Based Biosensor Chips for SNV Detection. Incorporation of magnetic beads into microfluidic chips can be very beneficial for bioanalytic and diagnostic applications. Integrating magnetic beads into microfluidic devices can provide a substrate for the separation of bioanalytes and possible downstream amplification of nucleic acids by the PCR process.

Surface-modified magnetic beads not only can increase the surface-to-volume ratio and accessibility but also help purification and enrichment of samples by binding with the target nucleic acid segments, which promotes hybridization and leads to a high degree of sensitivity and specificity. They are mostly labeled with ligands to selectively bind with target cells and extract DNA or with DNA probes to bind target DNAs. In bead-based biosensors, rapid thermal response and high signal/noise ratio are the additional advantages when integrating beads into microfluidic chips. For example, the number of thermal cycles for bead-based detection of SNP from a sample of gDNA related to Tiwanian chicken was found to be just 5 cycles, whereas this number is about 30 cycles for traditional methods. Lien et al. used magnetic beads to extract leukocytes from blood. Their work showed that the incubation time was shorter than lab-scale counterparts, and interestingly, the binding ratio of bioanalyte to magnetic beads was also considerably higher. Chang et al. developed a bead-based biosensor chip for SNP genotyping, which employed an allele-specific primer that can be extended, and DNA can be amplified only in the presence of perfectly matched sequences. The discrimination specificity between the perfectly matched and mismatched samples was significant at a considerably short turnaround time, approximately 95 s, while such efficiency would take about 1 h using the traditional PCR method.

3. CONCLUDING REMARKS

This paper provides a brief overview of different microfluidics-based strategies for rapid detection of SNVs, with focuses on the novel methods reported in the most recent years. Each of these approaches has specific advantages and weaknesses for SNV detection. A comparative summary of these methods is given in Table 1.

Lateral-flow test strips are simple and affordable with much potential in bioanalysis. They only need a small amount of a sample for a test and can realize multiplexed analyses at low cost. Currently, paper-based SNV tests are still being developed, with a handful reports at the proof of concept stage for SNV genotyping. They have exhibited functionality.
| Type of Sensor                      | Sample Type and Sample Processing                                                                 | Specific Characteristics and Outcomes                                                                 | Ref |
|-----------------------------------|-----------------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------------|-----|
| Lateral-flow biosensor            | Genomic DNA isolated from the blood of Tsvia ducks; PCR amplification was performed                  | Easy spotting the sample                                                                          | 5   |
|                                   | DNA synthesized by Sangon Biotechnology, China                                                      | Detection time less than 2 h                                                                        |     |
|                                   | DNA isolated from clinical blood samples by DNA extraction kits, then amplified by circular strand displacement reaction | Visual detection by naked-eye observation                                                            |     |
|                                   | Hairpin oligonucleotides and wild-type DNA obtained from Integrated DNA Technologies, Inc. (Coralville, IA) | Cost-effective                                                                                        |     |
|                                   | DNA extracted from blood sample of patients with genomic DNA extraction kit, then PCR amplification of target fragments | Limit of detection: 0.01 fM                                                                         | 14  |
|                                   | DNA extracted from blood sample of patients with genomic DNA extraction kit, then PCR amplification of target fragments | Processing time: 2.5 h                                                                             |     |
| Microfabricated multielectrode array sensor | DNA extracted from blood samples of epilepsy patients; PCR amplification of targets, and Förster resonance energy transfer based dual hybridization probes assay for genotyping | High sensitivity for α-fetoprotein, combined detection of tumor marker and susceptibility gene        | 2   |
| Channeled (capillary) microfluidic chip | DNA extracted from blood samples of epilepsy patients; PCR amplification of targets, and Förster resonance energy transfer based dual hybridization probes assay for genotyping | Combined detection of tumor marker and susceptibility gene                                          |     |
| 3D μTAS electrochemical DNA biosensor | Thiolated 34 nucleotides, single-base mismatched, 3-base mismatched, and 5-base mismatched, from Eurofins MWG Operon (Huntsville, USA). | Detection limit of 1.8 ng/mL                                                                       | 4   |
| Microarray chip                   | DNA extracted by DNA extraction kit from 160 blood samples, followed by PCR amplification           | Detection limit was 6 times higher than that of the commercial one                                  | 18  |
| Droplet-based microfluidic system | Template DNA-labeled with Cy3 fluorophore (33 nucleotides) and blocker DNA-labeled with BHQ-2, from Sangon Biotech, on-chip PCR amplification | Possible to observe droplets over a wide time range from subseconds to hundreds of minutes            | 20  |

Table 1. Comprehensive Overview on Published Articles for Rapid Detection of SNVs
and promising potential in a laboratory setting. Further optimization is required to be used for practical POC applications.

LOC devices possess several advantages over traditional assays and biosensors, especially in terms of speed, portability, and cost. Some LOC devices demonstrated comparable and even, in some cases, better sensitivity and accuracy than traditional methods. However, SNV detection has stringent requirements for detection sensitivity and specificity. Therefore, many of them need a series of sample preparation processes off-chip for POC SNV genotyping. One developmental direction is to integrate DNA amplification and SNV detection onto a microfluidic chip.

Droplet-based microfluidic chips benefit from rapid mixing of samples, programmable droplet makeup, high-throughput analysis, and ready multiplexing. However, the short residency of samples is a major challenge, as the binding reactions take time to happen. There are some solutions to this challenge. Using longer microchannels, trapping the droplets, or stopping the flow to monitor the droplets could help to overcome this problem with droplet-based microfluidic biosensors.

In bead-based biosensor chips, faster hybridization, higher signal-to-noise ratio (sensitivity), and better separation of bioanalytes can be achieved. However, the specificity of SNP detection in bead-based biosensors is one of the issues that needs to be investigated further. Primer extension is a promising method that can be employed in bead-based biosensors for SNV genotyping, which can result in quick response, while eliminating the need for complex and expensive modification enzyme procedures. Discrimination of SNV can be achieved by labeling the beads with predesigned primers that are extendable in the presence of perfectly matched SNP sites of the sample, while the mismatched ones cannot be amplified.

Most of the reported techniques require sample processing and initial amplification of genomic DNA by methods such as PCR. However, these sample processing steps can be potentially incorporated into microfluidic chips (on-chip processing) to eliminate the need for supporting equipment and to attain a fully integrated and all-in-one POC device.

In conclusion, due to the advantages of microfluidics-integrated systems, including high efficiency, rapid detection, low cost, and minimized reagent consumption and sample volume, the research community around the world remains highly interested in the development of these devices. Nevertheless, there is still much to be desired with their analytical specificity and sensitivity. For clinical applications, high specificity is one of the critical characteristics of POC devices especially for low abundance variants. Thus, exploring new designs to improve sensitivity and specificity of these devices is an important topic to be pursued in the era of personalized medicine.

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ACKNOWLEDGMENTS
The authors acknowledge the USDA National Institute of Food and Agriculture for their support (Grant No. 2017-67007-26150), and the support from the Institute for a Secure & Sustainable Environment at the University of Tennessee.

ABBREVIATIONS
SNVs, single nucleotide variations; SNPs, single nucleotide polymorphisms; POC, point-of-care; ssDNA, single-stranded DNA; gDNA, genomic DNA; μ-TAS, microtial analysis system; LIFs, lateral-flow assays; LOC, lab-on-a-chip

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