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Nanobiotechnology enabled approaches for wastewater based epidemiology

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ABSTRACT

The impacts of the ongoing coronavirus pandemic highlight the importance of environmental monitoring to inform public health safety. Wastewater based epidemiology (WBE) has drawn interest as a tool for analysis of biomarkers in wastewater networks. Wide scale implementation of WBE requires a variety of field deployable analytical tools for real-time monitoring. Nanobiotechnology enabled sensing platforms offer potential as biosensors capable of highly efficient and sensitive detection of target analytes. This review provides an overview of the design and working principles of nanobiotechnology enabled biosensors and recent progress on the use of biosensors in detection of biomarkers. In addition, applications of biosensors for analysis of the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) virus are highlighted as they relate to the potential expanded use of biosensors for WBE-based monitoring. Finally, we discuss the opportunities and challenges in future applications of biosensors in WBE for effective monitoring and investigation of public health threats.

1. Introduction

Early detection and assessment of the threat of pollutants in drinking water and wastewater systems are immensely important from the standpoint of public health and safety. The application of environmental sensing for real-time monitoring of changes in biomarkers (e.g., chemicals, pathogens, metabolites, etc.) can help in the implementation of countermeasures and mitigate the risk of public health outbreaks. Wastewater has been examined as a potential discharge source of illicit drugs to elucidate collective drug usage levels within a community since the early 2000s [1]. The idea of obtaining population information from biomarkers curated from concentrations found in wastewater has grown into the field of wastewater-based epidemiology (WBE). WBE has expanded from primarily looking at drug use in a community to many other aspects surrounding community health, including heavy metal exposure, infectious diseases, and the prevalence of antibiotic resistance genes (ARGs) [2]. Most recently, WBE has been used by research groups across the world to track patterns and outbreaks of COVID-19 as a tool against the pandemic [3].

The use of appropriate analytical tools is necessary for the precise quantification of biomarkers in wastewater at environmentally relevant concentrations. As WBE continues to develop as a field, so does the challenge of detecting biomarkers with both high sensitivity and low detection limits. Nanobiotechnology enabled biosensors are sensing platforms that can be modified with target specific recognition elements (e.g., antibodies, proteins, enzymes, etc.) that have biochemical affinity towards target analytes (e.g., chemicals, pathogens, DNA/RNA, etc.) [4]. These interactions between the target and the probe molecules can modify the unique optical, electrical, magnetic, and other properties of the system which can be used for analyte detection and quantification [4]. Advantages, such as low-cost, straightforward application and rapid detection of nanobiotechnology enabled sensing platforms can potentially be used to develop point-of-use sensors for real-time field monitoring of analytes in water and wastewater.

This paper provides an overview of the existing and emerging nanobiotechnology enabled sensing platforms. Initially, we summarize the types of biomarkers present in wastewater as potential WBE targets and introduce biosensor technologies for potential applications in WBE. Then, we review the current state-of-the-

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science of biosensing technologies involving indirect biosensing platforms (polymerase chain reaction (PCR), loop-mediated isothermal amplification (LAMP), genome sequencing, and clustered regularly interspaced short palindromic repeats (CRISPR)) as well as surface enhanced Raman scattering (SERS) based approaches and electrical biosensors. In addition, recent progress in the application of these biosensors in water and wastewater analysis, including applications related to COVID-19 are highlighted. Finally, we discuss potential avenues for future research and development of nanobiotechnology enabled sensing platforms for expanded use in WBE.

2. Wastewater-based epidemiology targets

Analysis of different biomarkers present in wastewater collection networks can inform policy making decisions and emergency responses to public health crises, such as the propagation of infectious agents and the prevalence of drug use in a community. WBE has been used as a powerful tool for real-time monitoring and analysis of a variety of biomarkers in wastewater. For example, the presence of viral (e.g., SARS-CoV-2) genomes in wastewater provides promise for better understanding of the spread of infectious disease within a population [5]. The monitoring of phthalate metabolites in wastewater can be used as an economic alternative for estimating human exposure to phthalates [6]. The target classes of biomarkers in wastewater consisting of inorganic and organic chemicals, microbes, and other pollutants are summarized in Table 1.

3. Nanobiotechnology enabled sensors

Nanobiotechnology merges nanotechnology and biotechnology for applications in life sciences. Research in nanobiotechnology has evolved from molecular imaging techniques and drug delivery into the rapidly evolving area of biosensing applications. Fig. 1 illustrates the basic methodology involved in biosensor development. Biosensors are usually designed and implemented after considering potential biomarkers as target analytes for detection and quantification. The design of sensors, at the basic level, involves (1) the use of a material or combinations of materials with unique properties to make nanocomposites, or nanobiocomposites; (2) the use of recognition elements for target specific binding; and (3) a signal transduction method (Fig. 1). For nanobiotechnology enabled sensors, indirect sensing platforms using nucleic acid based diagnostic tools (i.e., PCR, LAMP, genome sequencing, CRISPR) are sometimes miniaturized in microfluidic or paper-based chips for analyte detection. For example, Wang et al. detected methicillin-resistant *Staphylococcus aureus* (MRSA) at 10 fg μL^{-1} with a magnetic bead based microfluidic system with integrated LAMP technology for amplification of target MRSA DNA [18]. The target analytes interact with recognition elements (e.g., proteins, aptamers, antibodies, etc.) and generate a detectable signal via a signal transduction method (e.g., optical, electrical, magnetic, etc.). The implementation of biosensors involves one or a combination of different physical, chemical, and biological techniques (Fig. 1). The following sections discuss in detail the detection mechanisms and the latest progress in biosensing applications of sensing platforms using nucleic acid based diagnostic tools, SERS based sensing, and electrical/electrochemical based approaches. Key information on the sensors discussed herein is summarized in Table 2.

4. 'Indirect' sensor platforms using nucleic acid based diagnostic tools

The robust applicability of biomolecular analyses is appealing for WBE. Nucleic acids extracted from wastewater can provide information on biological identity and function, which can then be used to investigate the prevalence, the spread, and the scale of infectious agents in the sewer catchment. This information can be used as an early warning system for recurrent large-scale epidemics. In addition, monitoring the prevalence of ARGs and mobile gene elements (MGEs) in wastewater plays a significant role in keeping track of the spread of antimicrobial resistance (AMR) [19].

4.1. Polymerase chain reaction (PCR)

PCR-based techniques are the most commonly used and reliable biomolecular analytical tools to detect nucleic acids. In brief, PCR uses Taq polymerase to amplify a target DNA strand through replication using multiple thermal cycles. For the detection of RNA, an additional step of reverse transcription (RT) is required. Quantitative PCR (qPCR) has become the gold standard PCR approach as it enables real-time monitoring of gene amplification using an intercalating fluorescence dye that binds to double-stranded DNA. The recent development of droplet digital PCR (ddPCR) that relies upon the partitioning of several PCR reactions into reaction droplets increases the scalability and sensitivity of the PCR platform. It has been reported that ddPCR has better sensitivity and lower

Table 1
Main classes and representatives of WBE targets.

WBE targets	Representative contaminants	References
Inorganic ions		[7,8]
Heavy metals ions	Cd, Cr, Cu, Hg, Ni, Pb, Zn	
Nonmetallic ions	sulfate, phosphate, chloride, perchlorate, nitrate, nitrite, fluoride, arsenate	
Organic chemicals		[9–11]
Pesticides	atrazine, carbendazim, diazinon, diuron, glyphosate, isoproturon	
Pharmaceuticals and personal care products (PPCPs)	ibuprofen, caffeine, ciprofloxacin, metronidazole, musk ketone, triclosan, octocrylene	
Endocrine disruptors compounds (EDCs)	estrone, bisphenol A, progesterone, estriol, 17-β-estradiol	
Polycyclic aromatic hydrocarbons (PAHs)	anthracene, acenaphthene, fluoranthene, fluorene, naphthalene	
Surfactants	linear alkylbenzene, secondary alkane sulfonate, alkyl sulfate, perfluorooctanoic acid	
Industry emitted synthetic dyes	acridine orange, Sudan I, neutral red, methylene blue, rhodamine B, malachite green	
Pathogens and biomolecules		[5,12–15]
Microorganisms	*Escherichia coli*, fecal coliforms, *Legionella* spp., antibiotic resistant bacteria	
Viruses	coronavirus, adenovirus, noroviruses, hepatitis A virus, sapovirus	
Pathogenic genetic material	pathogenic DNA/RNA	
Antibiotic resistance genes	*blaKPC, blaSHV, ermB, mefAE, sul1, vanA, intI1*	
Other chemicals		[16,17]
Disinfection by products (DBPs)	trihalomethanes, haloacetic acids, haloacetonitriles, haloacetamides	
Microplastics		

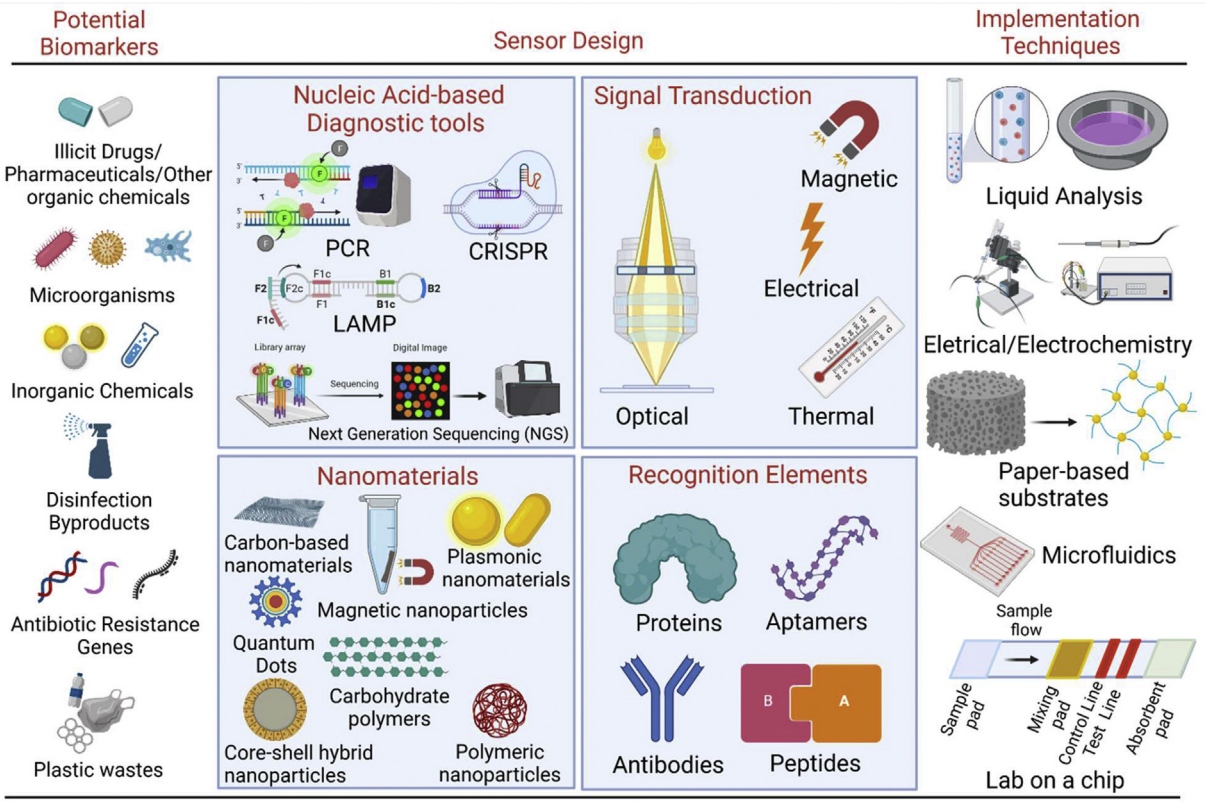

Fig. 1. Schematic illustration of the components involved when designing nanobiotechnology-enabled sensors. At first, the potential biomarker of interest is selected for detection. Next comes the sensor design step. The design of biosensor involves the selection of core materials, target specific recognition elements and one or more signal transduction methods. The nucleic acid based diagnostic tools can be applied for both indirect sensing using a separate instrument (e.g., amplification of target genes for subsequent detection), or direct sensing by incorporating the tools into the sensor platform. Finally, sensor is deployed using an implementation technique (image created with https://biorender.com).

probability of false negatives for SARS-CoV-2 detection in clinical samples than qPCR [20].

The PCR platform has been successfully used for wastewater surveillance of SARS-CoV-2 [21–23]. Curtis et al. compared the concentrations of SARS-CoV-2 RNA in wastewater from grab and 24-h composite samples using RT-qPCR [23]. The result showed the low variability of SARS-CoV-2 RNA concentrations in wastewater via these two sampling approaches. Pecson et al. found that 80% of recovery-corrected concentrations of SARS-CoV-2 RNA in wastewater across a total of eight sample concentration methods fell within the error of 1.15 $\log_{10}$ copies/L [21]. This result suggests that with recovery-correction that there was no significant impact of a solid removal step and selection of a concentration method on the measurement. Another study conducted using RT-ddPCR from wastewater treatment plants (WWTPs) in Southeastern Virginia determined that wastewater loading changes arising from the Virginia phase reopening and rainfall events could increase the uncertainty in SARS-CoV-2 surveillance [22].

To monitor the spread of AMR, a variety of ARGs and MGEs in wastewater have been detected using qPCR. For example, five ARGs: *tet*A, *tet*W, *sul*I, *sul*II, *bla*TEM were detected in wastestreams from six WWTPs in different swine farms [24]. Caucci et al. investigated the seasonality of ARG concentrations in wastewater and found higher levels in autumn and winter coincide with higher rates of overall antibiotic prescriptions [25].

4.2. Loop-mediated isothermal amplification (LAMP)

LAMP is a simple, rapid, and sensitive biomolecular platform for the detection of nucleic acids. LAMP uses four (or six) different primers that bind to six (or eight) distinct regions of a target DNA fragment for subsequent gene replication using *Bst* polymerase. LAMP has been shown to have a simpler and higher efficiency of amplification than PCR [26]. Compared to *Taq* polymerase, *Bst* polymerase is active under various inhibitory conditions. In addition, LAMP can amplify the gene within 30–60 min at a constant temperature in the 60–70°C range. Owing to such advantages, LAMP is not constrained by the availability of thermocyclers and is more field-deployable than PCR with higher rapidity. Huang et al. reported a colorimetric RT-LAMP approach that was effective for the detection of SARS-CoV-2 RNA in clinical samples, with a detection sensitivity of 80 copies of viral RNA/mL of sample [27]. LAMP was successfully applied for the detection of human specific-mitochondrial DNA (mtDNA) from untreated wastewater in the field (Fig. 2A) [28]. mtDNA is a model population biomarker reflecting the presence of carcinogenesis. The detection limit of LAMP in this study was 40 copies per reaction volume. Recently, direct detection of SARS-CoV-2 RNA in wastewater was achieved using RT-qLAMP [29]. The results showed that even in a region with a low number of confirmed cases (e.g., 1–10 per 100,000 people), positive detection was confirmed using RT-qLAMP. This result demonstrates that LAMP-based detection can directly detect SARS-CoV-2 in wastewater while avoiding viral concentration and RNA extraction steps.

4.3. Genome sequencing

Next generation sequencing (NGS) enables rapid and large-scale whole-genome sequencing that can be applied to sequence WBE targets. Several NGS based platforms have been applied for WBE.

Table 2
Summary of previous studies on the application of biosensors.

Type of biomarker	Recognition element	Output Signal	Sample Matrix	Limit of detection (LOD)	References
Bacterial (MRSA) DNA	Aptamer	Optical/magnetic	Clinical sample	10 fg/μL	[18]
Viral RNA (SARS-CoV-2)	Aptamer	Optical	Clinical sample	–	[20,31]
Viral RNA (SARS-CoV-2)	Aptamer	Optical	Wastewater	14.6, 2, and 2.18 copies/20 μL for SARS-CoV-2 N1, N2, and N3	[22]
Viral RNA (SARS-CoV-2)	Aptamer	Optical	Wastewater	58 copies/100 mL	[23]
DNA (ARGs)	Aptamer	Optical	Wastewater	–	[24,25]
Viral RNA (SARS-CoV-2)	Aptamer	Optical	Clinical sample	80 copies/mL	[27]
DNA (mtDNA)	Aptamer	Optical	Wastewater	40 copies/20 μL	[28]
Viral RNA (SARS-CoV-2)	Aptamer	Optical	Wastewater	–	[29,30]
Viral RNA (SARS-CoV-2)	Aptamer	Electrical	Clinical sample	–	[32]
Bacteria (*P. aeruginosa*)	Aptamer	Optical	Cell medium extracts	1 CFU/mL	[35]
Viral RNA (SARS-CoV-2)	Aptamer	Optical	Clinical sample	10 copies/10 μL	[36]
Antibiotic Resistant Bacteria	Antibody, protein	Optical/magnetic	DI water	10^1 CFU/mL	[37]
Bacteria	Nanomaterial (Au nanorods)	Optical	DI water	–	[38]
Virus (adenovirus, rhinovirus, and HIV)	Nanomaterial (Ag nanorod arrays)	Optical	DI water	100 PFU/mL	[39]
Viral RNA (SARS-CoV-2)	Aptamer	Optical	DI water	5.5×10^4 $TCID_{50}$/mL	[40]
Viral protein (SARS-CoV-2)	Aptamer	Optical	DI water	250 nM	[43]
Virus (H1N1)	Aptamer	Optical	DI water	97 PFU/mL	[44]
Protein biomarker	Antibody	Optical	Blood plasma	0.86 ng/mL	[45]
Virus (H1N1, adenovirus)	Antibody	Optical/magnetic	PBS, blood, serum, and sputum	50 PFU/mL (H1N1), 10 PFU/mL (adenovirus)	[46]
Virus (H5N2, HPIV 3)	Aligned carbon nanotube	Optical	Clinical sample	10^2 EID_{50}/mL (50% egg infective dose per microliter)	[47]
Human prostate cells	Wheat germ agglutinin	Optical	Cell medium	–	[49]
Virus (Hep B)	Antibody	Optical	Human blood plasma	0.01 IU/mL	[50]
Virus (SARS-CoV-2)	Antibody	FET	Culture medium and clinical samples	1.6×10^1 PFU/mL in culture medium, 2.42×10^2 copies/ml in clinical samples	[54]
Viral RNA (SARS-CoV-2)	DNA probe	Electrochemical	Clinical sample	6.9 copies/μL	[56]
Viral RNA (Hep C)	Peptide	SEC	10 mM PBS	264.5 IU/mL	[59]
Viral protein (H5N1)	Primary and secondary antibodies	SEC	Clinical samples	4 ng/mL, or 77 pM	[60]

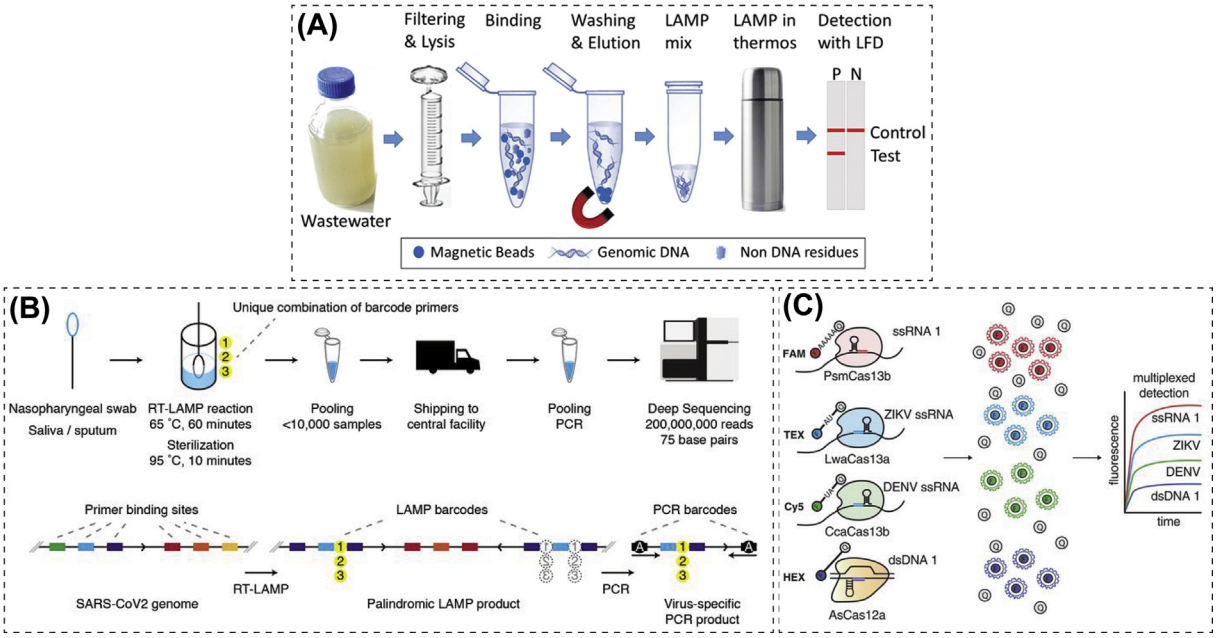

Fig. 2. (A) The workflow of extraction and detection of the genomic population biomarker, mtDNA, in wastewater using LAMP and lateral flow device (Reprinted with permission from Ref. [28]); (B) The illustration of the highly scalable detection of SARS-CoV-2 in the swab samples using Illumina sequencing of combinatorial RT-LAMP-PCR barcoded amplicons (Reprinted with permission from Ref. [31]); (C) Four-channel multiplexed CRISPR-Cas system for detection of nucleic acids with orthogonal CRISPR enzymes: PsmCas13b, LwaCas13a, CcaCas13b, and AsCas12a for dsDNA target (Reprinted with permission from Ref. [34]).

Illumina MiSeq provides short read (typically 100–150 base pairs in length) DNA sequencing and data analysis and has enabled metatranscriptomic sequencing of wastewater to investigate SARS-CoV-2 variants [30]. First, the targeted region of SARS-CoV-2 RNA was amplified using RT-PCR and the amplicon was sequenced using Illumina MiSeq with single-nucleotide sensitivity. The result illustrates that viral genotypes from wastewater sequencing can provide information about how transmission is occurring in advance of that detected by clinical sequencing.

To increase the scalability of NGS, a short DNA fragment (barcode) is attached to the amplified target region of the gene during PCR or other amplification processes. The process, called DNA barcoding, allows for easy identification using the barcode library after DNA sequencing. A highly scalable SARS-CoV-2 detection method was introduced using barcoded RT-LAMP products, which were sequenced using Illumina MiSeq (Fig. 2B) [31]. Nanopore sequencing is an emerging NGS platform that enables real-time analysis of extremely long-reads of DNA fragments exceeding 20 kilobases (kb) in length. Nanopore sequencing uses multiple-nanopore channels in a membrane that is immersed into electrolyte solution where the magnitude of the electric current can be measured. The duration of ion current blockage events induced by passing DNA differs depending upon base identity and can be used in their identification. Recently, a multiplexed highly scalable platform combining LAMP and nanopore sequencing (LAMPore) was developed for detection of SARS-CoV-2 RNA in clinical samples [32]. This platform succeeded in rapid testing of 96 clinical samples in under 2 h. With the advantage of high scalability and single base-resolution, DNA sequencing techniques have great potential for WBE.

4.4. Detection using clusters of regularly interspaced short palindromic repeats (CRISPR)

The CRISPR-associated (CRISPR-Cas) system has adaptive immunity against invading nucleic acids. CRISPR-Cas system enzymes (e.g., Cas9, Cas12, Cas13) have been used as nucleases for detection of nucleic acid. Such enzymes are activated upon recognition of target RNA/DNA and engage in collateral cleavage (i.e., indiscriminate cutting) of non-target nucleic acid. A CRISPR-Cas based detection platform, termed Specific High-sensitivity Enzymatic Reporter un-LOCKing (SHERLOCK), was introduced for nucleic acid detection combined with isothermal pre-amplification with Cas13 [33]. The collateral cleavage of reporter RNA (quenched fluorescence linked by sequence of RNA) by activated Cas13 allowed real-time detection of Zika and Dengue viruses. CRISPR-Cas systems have also shown multiplexed detection with orthogonal CRISPR enzymes: PsmCas13b, LwaCas13a, CcaCas13b for ssRNA targets and AsCas12a for dsDNA target (Fig. 2C) [34]. The CRISPR-Cas platforms show high sensitivity for point-care-use detection of *Pseudomonas aeruginosa* [35] and SARS-CoV-2 [36] using a lateral flow biosensor, implying great potential for WBE targets.

5. SERS based sensing

SERS is a rapidly evolving technique for biosensing applications. In SERS, the inelastic light scattering of a target molecule is greatly enhanced by a factor of up to 10^{12} or higher, thereby making single molecule detection a possibility [37]. This phenomenon occurs when target molecules are adsorbed onto plasmonic metal nanoparticles such as gold (Au) or silver (Ag) and enhanced Raman scattering occurs due to the localized surface plasmon resonance (LSPR) of the particles. SERS has gained wide interest due to its ultrasensitive detection limits and relatively simple implementation. Continuous progress in the development of nanocomposite materials and nanolithography have driven forward the development of a wide range of SERS substrates. As a result, SERS based approaches have proven to be robust and reliable for biosensing and environmental sensing applications.

5.1. Liquid SERS techniques

Dried droplets of analytes are still widely used for SERS given their ease of preparation and signal acquisition. However, the drying process can sometimes be detrimental to cells and poses challenges for dynamic studies of particle interactions. SERS of biomolecules in controlled liquid environments, or liquid SERS, is often desired due to greater control over experimental conditions, cell viability, and the study of physical, chemical, and plasmonic interactions between target molecules and SERS probes. Previous studies have demonstrated high SERS signal intensities for liquid SERS platforms with low Raman background. Liquid SERS has been quite effective for detection of both Gram negative (*Escherichia coli* and *Serratia marcescens*) and Gram positive (*Staphylococcus aureus* and *Staphylococcus epidermidis*) bacteria using Au nanorod probes (Fig. 3A) [38]. The use of SERS reporter molecules, such as malachite green isothiocyanate (MGITC) or 4-(1H-pyrazol-4-yl)-pyridine (PPY), is often done to tag target molecules with a unique label [37]. SERS spectra of adenovirus, rhinovirus, and human immunodeficiency virus (HIV) were collected previously by dropping small volumes (0.5–1 μL) of these viruses on a substrate consisting of Ag nanorod arrays [39]. A SERS-based aptasensor was developed by functionalizing colloidal AgNPs with olegonucleotides for detection of SARS-CoV-2 in water at 5.5×10^4 TCID50/mL level [40]. A portable handheld Raman system was used to detect influenza A virus using 10 μL of sample in water applied to Ag nanorod substrates [41].

5.2. Paper-based SERS sensors

Cellulose paper-based nanomaterials are often used as SERS substrates. The flexible and porous structure of paper-based substrates enables fabrication of plasmonic nanostructures and induces interaction with a wide range of analytes. Properties such as high tensile strength, biocompatibility, and the low cost of paper substrates allow for development of cost-effective and widely applicable biosensors.

Paper based SERS sensors can be differentiated based on direct contact and flow-based measurements. Direct contact-based SERS sensors have nanostructures that are either synthesized within the paper or post-decorated onto the paper surfaces [42]. For a deposited droplet on the substrate or a substrate submerged into sample solution, target molecules interact with the nanostructures and SERS signals are generated. However, for wastewater matrices where different types of contaminants (e.g., metals, organics, microbes, etc.) are present, paper sensors can be functionalized with specific recognition elements (e.g., proteins, antibodies, aptamers) for specific binding and detection [4]. Recently, SARS-CoV-2 spike proteins were detected at the ~250 nM level by applying 10 μL of sample to oligonucleotide aptamers and Ag colloids immobilized onto polytetrafluoroethylene (PTFE) membrane filters [43]. In addition, Au coated polyethylene naphthalate (PEN) polymer substrate have been modified with aptamer DNA for detection of influenza A H1N1 virus at a 97 PFU/mL detection limit [44].

Lateral flow and vertical flow assays are commonly used in paper-based SERS sensors. Typically, samples are loaded onto a sample pad and flow, via capillary force, towards the conjugation pad, where the target molecules interact with SERS probes (Fig. 3B) [45]. The target molecule-SERS probe complex is captured by recognition elements on the test line and the acquired SERS signals

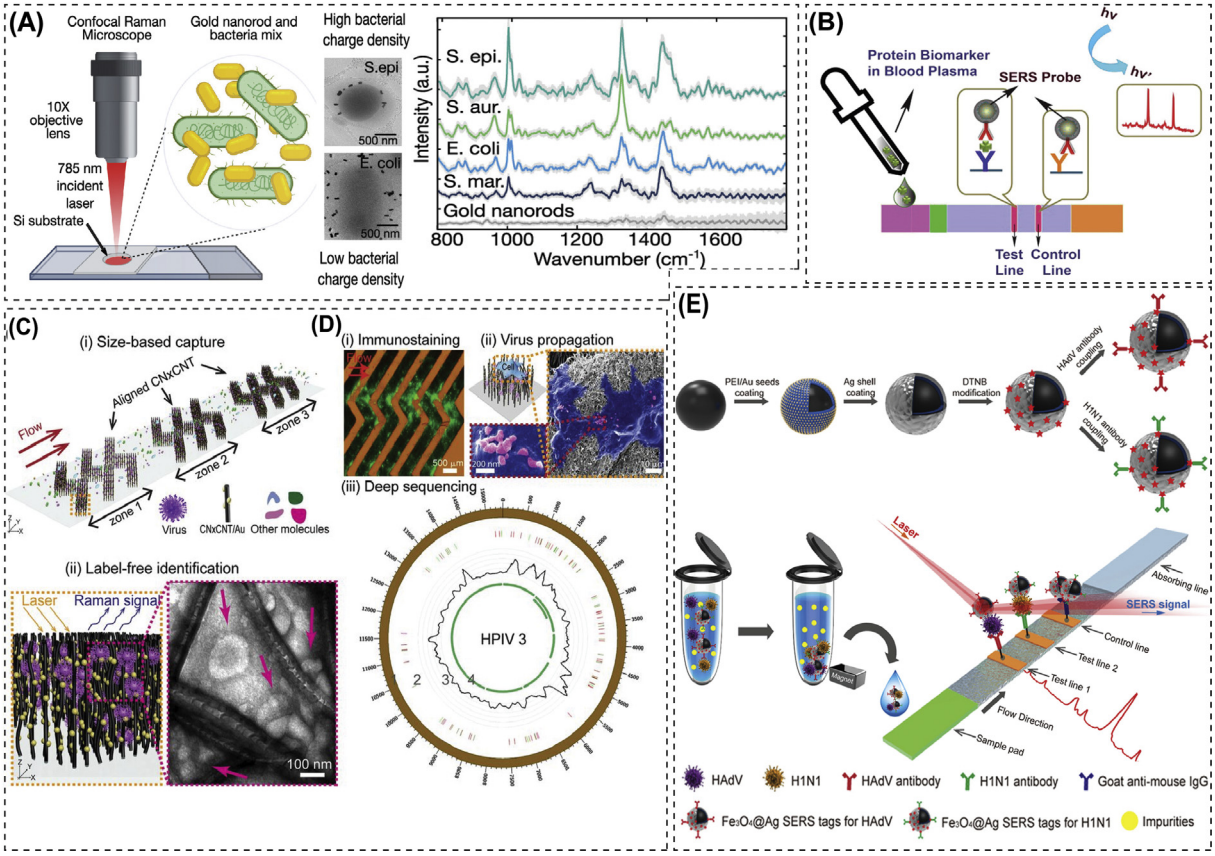

Fig. 3. (A) Detection of bacteria using a liquid SERS platform (Reprinted with permission from Ref. [38]); (B) Illustration showing the detection of the protein biomarker, neuron specific enolase (NSE) in blood plasma using a paper based lateral flow strip (PLFS) immunoassay (Reprinted with permission from Ref. [45]); (C) a microfluidic platform for the capture of avian influenza A viruses from clinical samples and rapid label-free SERS identification (Reprinted with permission from Ref. [47]); (D) The captured viruses on the chip are (i) immunostained, then (ii) propagated via cell culture and are finally (iii) genome sequenced for identification of subtypes (Reprinted with permission from Ref. [47]); (E) Application of a SERS based lateral flow immunoassay (LFIA) for detection of Influenza A H1N1 virus and human adenovirus (Reprinted with permission from Ref. [46]).

can be used for quantification. Unlike direct contact mode, flow-based SERS devices do not embed nanostructures on the surface of the paper devices. Instead, the nanoparticles are initially prepared and modified with a recognition element for specific binding to the analytes and then labelled with a reporter molecule for readout. The obtained SERS signals arise from the Raman reporter rather than the analytes. The Raman reporter and the recognition element enable high sensitivity and specificity, respectively. In addition, multiple analytes can be detected in one analysis run by immobilizing different recognition elements and Raman reporters [46].

5.3. SERS microfluidic sensors

Microfluidics, which integrates all analytical procedures on a chip, offers numerous advantages, such as low sample consumption, precise control, fast response, and high efficiency. Continuous flow platforms and segmented flow platforms are the two most common categories of SERS microfluidic sensors. One type of continuous flow platform is a built-in nanostructured microfluidic device, which consists of an inlet, an outlet, and pre-created nanoarrays within the microchannels. After the analytes are injected into the channels, the highly-designed plasmonic nanostructures specifically bind to the target analytes for SERS detection. This setup has been applied successfully as an effective disease-monitoring system (Fig. 3C,D) [47]. Another commonly used technique is colloidal nanoparticle-based microfluidics, where mixing between the analytes and nanoparticles is the greatest challenge. Passive and active mixers are usually introduced to enhance the mixing process. The design of micromixers has been described in detail previously [48]. In a segmented flow platform, the flow of the mixed sample and nanoparticles is separated by an immiscible fluid or gas phase. Segmented flow in microfluidics has multiple advantages, such as increased interfacial area, enhanced mixing, and minimal sample dosage. The microchannel in segmented flow microfluidics can be made hydrophobic to minimize sample retention and effectively decrease cross-contamination. By encapsulating single prostate cancer cells and SERS nanoprobes in water-in-oil droplets, we previously identified cell-to-cell and intracellular variability in the expression of glycans on the cell membrane [49]. A Au-Ag coated GaN substrate in a microfluidic device was modified with antibodies for SERS detection of hepatitis B virus antigen at 0.01 IU/mL [50].

5.4. Magnetic separation and SERS detection

Magnetically assisted SERS employs magnetic nanomaterials to capture, isolate, and enrich target molecules that can be interrogated using SERS nanoprobes. The surface of magnetic nanoparticles can be functionalized using inorganic materials (e.g., Au, Ag, etc.) or analyte specific biomolecules (e.g., antibodies, proteins, DNA, etc.), which enables the design of magnetic SERS tags of a wide range of properties. Iron-based nanoparticles (e.g., Fe^0, Fe_3O_4, γ-Fe_2O_3) are widely used as magnetic nanomaterials for biosensing

applications due to their ease of synthesis and biocompatibility. Recently, Wang et al. used Ag coated Fe_3O_4 (Ag@Fe_3O_4) nanoparticles as magnetic SERS tags in a SERS based lateral flow immunoassay (LFIA) for ultrasensitive detection of influenza A H1N1 virus (up to 50 PFU/mL) and human adenovirus (up to 10 PFU/mL) (Fig. 3E) [46] Functionalized magnetic nanoparticles are often used to specifically bind to the target (i.e., bacteria, viruses, ARGs) in solution and the target-NP conjugate can be isolated via a magnetic field. Furthermore, Au or Ag nanoparticles can be combined with magnetic particles to form a sandwich-type SERS assay for biosensing [37].

6. Electrical/combined approaches to sensing

Electronic biochemical sensors are devices that transduce signals arising from target molecules in the biochemical system into electrical signals [51]. Compared with spectroscopic sensing techniques, electrical biosensing can be performed with simple and portable instrumentation that requires only low power and are easy to operate, thus enabling on-site sensing capability. Electrical measurements are unaffected by factors such as sample turbidity or interference from fluorescing compounds, which can significantly impact spectroscopic data quality. In the last two decades, the use of nanoscale electronic transducers such as noble metal nanoparticles, silicon nanowires, and carbonaceous nanomaterials (graphene, carbon nanotubes) have enabled ultrasensitive and selective detection of target molecules due to the unique intrinsic properties of the nanomaterials employed [51]. These properties include 1) a high surface to volume ratio enabling superior physical and electronic properties, 2) size compatibility with biomolecules, and 3) easy and stable surface functionalization of the nanomaterial surface for biochemical sensing [52,53]. Here we cover two prominent electrical biosensing techniques: field effect transistors (FETs) and electrochemical sensors and we will discuss the possibility of combining electrochemical and spectroscopic modalities in a single platform for the detection of target analytes using WBE.

6.1. Field effect transistor (FET) sensing

FET nanosensors rely upon measurement of the change in conductance that occurs upon binding of a target analyte to a nanoscale transducer [52]. FET nanosensors are functionalized with a recognition element (antibodies, aptamers) that selectively bind to the target molecules in the biochemical system. Due to the electrostatic charge possessed by the trapped target molecule, the charge at the FET surface is tuned which leads to a change in carrier density. Accordingly, molecular binding events tune the electrical conductivity, which can be monitored in real time enabling ultrasensitive and selective detection capability [52]. The applicability of FET nanosensors for biomarker detection has been described previously. For example, Seo et al. demonstrated a FET nanobiosensor using graphene transducers modified with an antibody specific for the SARS-CoV-2 spike protein. SARS-CoV-2 in clinical samples was detected with a detection limit of 2.42×10^2 copies/mL (Fig. 4A) [54]. Despite the success of FET nanosensors for ultrasensitive and selective detection of target analytes, their potential remains underexplored for WBE due to potential limitations such as the Debye screening effect in physiological environments.

6.2. Electrochemical sensing

Electrochemical sensors measure voltage or current changes that occur due to an electron transfer reaction between the electrode surface and a target analyte or intermediate. The emergence of nanostructured electrode surfaces has enabled ultrasensitive detection of target analytes with long−term operational stability [53]. Different electrochemical analytical methods can be used for the transduction of target analytes including: 1) Voltammetric or amperometric methods that measure the change of current by techniques (e.g., cyclic voltammetry (CV) and differential pulse voltammetry (DPV)), and 2) impedimetric methods that measure the change in impedance by electrochemical impedance spectroscopy (EIS). Several electrochemical sensors with nanostructured electrode surfaces functionalized with recognition elements have already been developed for the detection of population and health biomarkers via WBE [4].

As noted previously, paper based electrochemical devices have recently gained attention because of the attractive properties of paper [55]. Paper based electrochemical sensors have been demonstrated in the literature for the detection of health biomarkers (e.g., dopamine), inorganic toxic contaminants (e.g., Pd and Cd in sea water) and organic toxic contaminants (e.g., nerve agents in wastewater) [55]. Recently, a paper based electrochemical sensor chip made of graphene and gold nanoparticles conjugated with antisense oligonucleotides was developed for the rapid detection of SARS-CoV-2 viral RNA with a detection limit of 6.9 copies/μL (Fig. 4B) [56]. These portable, disposable, and low-cost paper based electrochemical sensing platforms with 1) nanoscale electronic transducers for ultrasensitive and selective sensing and 2) integrated microfluidics for sample processing have huge potential for on-site detection of target molecules via WBE.

6.3. Spectroelectrochemical (SEC) sensing

Both electrochemical and spectroscopic sensing approaches have demonstrated highly sensitive and selective detection of target analytes. However, combining the two methods in a single platform, SEC sensing, can enable unique advantages [56]. First, access to complementary and uncoupled information is provided from the two sensing modalities, which neither of the respective techniques provides in isolation, thus leading to a richer set of data [57]. Second, the interaction between the target molecules and the metallic transducers can be regulated via changing the electrochemical potential to improve the performance of the spectroscopic sensing modality. For example, electrochemical SERS (EC-SERS) devices, where electrochemical potentials are applied on the metallic surface of the SERS substrates, have demonstrated improved sensing performance relative to conventional SERS substrates due to electrode potential dependent changes at the metal-molecule interface, including: 1) electrostatic adsorption of low-affinity target molecules, 2) potential dependent orientation of adsorbed molecules for the alignment of the vibration modes and local plasmonic fields, and 3) the photon-driven charge transfer enhancement between the metal structure and adsorbed molecule [58]. Various spectroscopic techniques such as SERS and surface enhanced infrared absorption spectroscopy (SEIRAS) have been combined with electrochemistry for the detection of DNA, proteins, bacteria, and health biomarkers (e.g., uric acid, 6-thiouric acid) [57]. For example, Au nanodot modified indium tin oxide (ITO) substrates were used for SEC detection of hepatitis C virus-RNA at 264.5 IU/mL [59]. A SEC immunoassay was developed using primary antibodies to capture the hemagglutinin (HA) protein from the H5N1 avian influenza A virus [60]. Then methylene blue-labeled secondary H5N1 antibodies were adsorbed to the target for sub picomolar detection using a single-mode, electro-active, integrated optical waveguide (SM-EA-IOW) device [60].

SEC sensing remains an evolving field and improved understanding of the SEC mechanisms and further exploration of the various SEC techniques for sensing applications is required. With further development, SEC sensing techniques such as EC-SERS, that

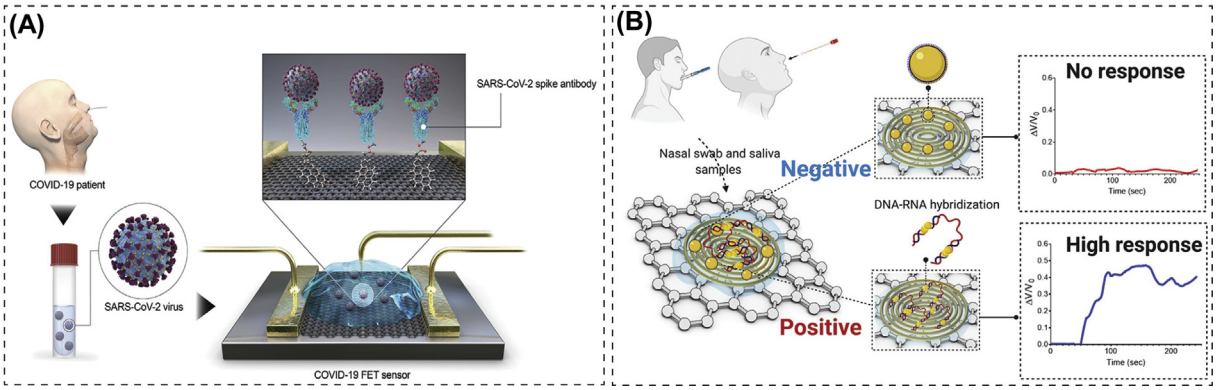

Fig. 4. (A) The illustration of the detection of SARS-CoV-2 via FET nanobiosensors with graphene transducers modified with an antibody specific for the SARS-CoV-2 spike protein (Reprinted with permission from Ref. [54]); (B) The illustration of the rapid detection of SARS-CoV-2 viral RNA using an electrochemical sensor made of graphene and gold nanoparticles modified with antisense oligonucleotides (Reprinted with permission from Ref. [56]).

Table 3
Summarized key information on the applicability of different sensing platforms.

Technique	Advantages	Disadvantages	Potential for WBE Applications	Challenges in implementation	References
Indirect sensing (PCR, LAMP, genome sequencing and CRISPR)	Most commonly used for detecting nucleic acids; Precise and sensitive detection; Established protocols and standards.	Require centralized facilities, specialized equipment, and trained personnel; High cost; Time consuming.	Established methods for nucleic acid detection; Detection of SARS-CoV-2 RNA; Analysis of complex matrices (e.g., wastewater, biofluids).	False negatives; Interpretation of findings in terms of disease propagation and human health risks; Variability of strains in samples vs reference strains.	[20,26,36,61]
SERS based sensing (liquid SERS, paper-based SERS, microfluidic SERS, magnetic SERS)	Rapid, highly sensitive and low-cost detection; Wide range of SERS nanotags are already available; Great potential for field deployment.	Requires plasmonic substrates; Nanomaterial and SERS tag orientation induce large variability in scattering response.	Single molecule detection capability; Detection at environmentally relevant concentrations; Low-cost SERS active substrates for wastewater monitoring; Field diagnosis using handheld Raman systems.	Heterogeneity of SERS substrates; Weak SERS signals and similarity of SERS profiles of biomolecules require additional data analysis; Reproducibility; Detection at sub nanomolar concentrations in complex media (e.g., wastewater, biofluids).	[37,42,62]
Electrical approaches (FET sensing, electrochemical sensing)	Rapid, highly sensitive, low cost and real-time detection; Simple and portable instrumentation; Electrical signals unaffected by factors such as sample turbidity or interference from fluorescing compounds.	Low stability and reproducibility in physiological environments; Reduced sensitivity and specificity due to non-specific adsorption of interfering species.	Detection at environmentally relevant concentrations; Easy lab on a chip integration due to low power requirements; Portable instrumentation and compatibility with microfabrication technology for on-site analysis; Real-time detection with simple operation.	Operation in complex media (e.g., wastewater, biofluids) has several challenges including non-specific adsorption of interfering molecules, Debye screening effect in FET nanosensors, and stability of electrochemical signals under changing physiological conditions.	[51–53,63]
Combined approaches (SEC sensing)	Highly sensitive and selective due to simultaneous acquisition of complementary electrochemical and spectroscopic data; Improved spectroscopic modality (e.g., SERS).	Requires advanced understanding of SEC mechanisms for accurate data interpretation; Incident light beam can affect the electrochemical results.	Single molecule detection capability; Overlapping signals of interfering molecules can be resolved using complementary data allowing detection in complex media (e.g., wastewater, biofluids).	Reproducibility of devices (e.g., EC-SERS substrates); Complex data interpretation and analysis; Improvement and miniaturization of instrumentation for on-site analysis	[57,58,64]

provide synergistic electrochemical and spectroscopic information with high detection sensitivity, can be successfully implemented for the monitoring of target analytes via WBE.

7. Conclusions and future directions

Nanobiotechnology enabled sensors offer great advantages, such as miniaturization of the detection assay, multiplex detection, and device portability. This review highlighted the rapidly expanding research on indirect sensing methods using nucleic acid based diagnostic tools, and methods based on signal transduction, such as optical and electrochemical signals. Key information on the various sensing platforms is presented in Table 3, which summarizes their applicability for WBE applications. For efficient operation in inhibitory conditions presented in complex sample matrices (e.g., wastewater, biofluids, etc.), target specific recognition elements are often used to modify biosensors (Table 2). Furthermore, deployment of biosensors based on a specific detection technique or combining multiple techniques can be used for reliable detection and monitoring of biomarkers in the complex environments of water and wastewater systems. The simplicity and reliability of these methods offer great potential for future application in WBE.

The disruption to public health and health care systems around the world caused by the COVID-19 pandemic has shown the importance of early detection and diagnosis of public health

outbreaks. Improved monitoring of biomarkers in wastewater networks is necessary for maximizing the benefits of WBE. Nano-biotechnology enabled sensing platforms have great potential for the development of field deployable point-of-use (POU) sensor networks for real-time monitoring of biomarkers in wastewater. However, there remains challenges for implementation. Biosensors need further development to operate with increased efficiency, multiplex-functionality and flexibility in the complex matrix of wastewater where there are different types of biomarkers present. The nano and biomaterials required for sensor design need to be stable in all operating and storage conditions to ensure proper functioning of the biosensors. There needs to be standardized and established analytical procedures for detection of analytes to endure reproducibility and reliability of methods. Further research and development to overcome these challenges are necessary to ensure wide implementation of biosensors in real-world environments.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Peter Vikesland reports financial support was provided by National Science Foundation.

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