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Current trends and challenges in point-of-care urinalysis of biomarkers in trace amounts

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ABSTRACT

Urinalysis enables non-invasive point-of-care (POC) testing of numerous biomarkers at their physiological and elevated levels, obviating the need for sophisticated equipment or trained personnel. POC urinalysis is used to identify biomarkers that are rich in urine (greater than 1 μM), such as lactate, uric acid, glucose, ions, and adenosine. Urine also contains biomarkers such as small molecules, nucleic acids, neurotransmitters, and drugs in trace amounts (less than 1 μM). These biomarkers are of significant importance for health care monitoring, diagnosis of various disorders (cancer, metabolic diseases, etc.) and illicit drug control (cocaine, steroids, etc.). While POC detection of urinary biomarkers at higher concentration (μM to mM) levels is feasible, direct assaying of biomarkers in nM to fM levels is challenging, as assay responses are typically masked by interferences from the urine sample matrix. This report is a consolidated review of emerging trends and challenges in the POC urinalysis for detecting biomarkers that are less abundant in urine. The sensing mechanisms, analytical device fabrication, discrete and integrated sample pre-treatment procedures for POC assaying of urinary markers in trace amounts are elaborated. Subsequently, the utilization of smart data analytics for facilitating personalized urinalysis is presented. A comprehensive outlook on associated challenges in POC urinalysis of biomarkers in trace amounts is further provided, which would facilitate the advancement of POC urinalysis for a wide range of healthcare applications.

1. Introduction

Urine analysis or urinalysis has great potential for healthcare monitoring and disease diagnosis, as urine contains a wide range of ions, proteins, and other biomolecules [1,2]. These molecules have been reported as biomarkers since variations in their levels could be correlated to specific diseases or health conditions. Urinalysis has been reported for numerous diagnoses, including diabetes, kidney malfunction, dehydration, and urinary tract infection (UTI) [3–6]. Besides being rich in biomarkers, urine can be collected effortlessly without the need for any special equipment, unlike blood, and can easily be utilized as a sample matrix for continuous monitoring of biomarkers. In addition, as urine is neither contagious nor biohazardous, it is convenient for handling and disposal. Therefore, urinalysis is advantageous and promising for advanced healthcare and clinical diagnosis in point of care (POC) settings [7–10].

An ideal POC assay can provide rapid response, facile operation, cost-effectiveness, and minimal use of instrumentation or trained personnel and thus possesses significance in personalized healthcare, disease outbreaks, or rapid testing in resource limited settings [11–13]. POC urinalysis possesses the potential to be ubiquitous for health monitoring and diagnosis, especially where clinical laboratory test facilities are not readily available. Therefore, research efforts have been devoted to develop POC urinalysis for various applications. Urinalysis includes physical analysis of urine by observing color, odor, specific gravity, etc. Chemical analysis is performed in urine by examining urine pH, protein concentration, blood traces, etc. Microscopic analysis is also performed for screening the existence of bacteria, casts, crystals, cells, etc. [7,14]. Rapid test kits for qualitative and semi-quantitative POC urinalysis

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are commercially available in the formats such as dipsticks, strips, and lateral flow assays (LFAs) [15]. For instance, dipsticks are often used to monitor ions, glucose, lactate, proteins, ketones, blood components, creatinine, etc. [4,16,17]. They are widely accepted in clinical settings due to their rapid processing time and reliability [18–21]. Besides dipsticks, LFAs are commonly used in pregnancy tests for hormone human chorionic gonadotropin (hCG) detection [22,23]. However, these commercial assays typically detect biomarkers that are in very high concentrations ranges (μM to mM), for example, glucose in 0.1–0.8 mM, lactate in 5.5–22 mM, uric acid in 1.5–4.4 mM [20], and K^+ ion in 25–125 mM [24] concentration ranges. Besides health care and disease diagnosis, urinalysis is also applicable for monitoring illicit drug usage. For instance, drug screening test cards are used to detect morphine, cocaine, and methamphetamine in urine in μM concentration levels [14,25,26]. Since the physiological as well as elevated concentration of the above-mentioned urinary markers are high in urine samples, their detection is comparatively easier and requires minimal or no sample preparation and increases affordability, user-friendliness, and rapid diagnosis. Apart from these markers, urine also contains nucleic acids, small molecules, protein, and drugs as important biomarkers in extremely low physiological concentrations, e.g., well below 1 μM, as illustrated in Fig. 1. These biomarkers provide valuable insight in the identification and therapy of various diseases and disorders, including chronic hepatitis, metabolic disorders, cardiovascular diseases, and cancer [5,27]. The detection of these trace amounts of biomarkers in urine in POC settings could pave the way for early screening of various health conditions. Therefore, various POC urinalysis platforms such as dipstick, LFA, paper-based, and microfluidic assays, and analytical devices are continuously being developed to detect urinary markers that are in trace amounts.

In contrast to urinary markers that are rich in urine, the detection of urinary markers in trace amounts is challenging for several reasons [27,28]. For instance, the presence of highly concentrated markers in urine could interfere with the responses associated with substantially less abundant markers [29]. Detecting trace amounts of small molecules in urine often suffer from sensitivity. Urine's inherent fluorescence might obscure colorimetric and fluorometric assay responses. In order to achieve sensitive and selective detection of trace amount markers without the interferences of urine matrix components, several urine sample pre-treatment techniques have been investigated, including centrifugation, filtration, ultrafiltration, dilution, dialysis, sample preconcentration, and solid-phase extraction [29–32]. However, the additional sample preparation steps reduce the feasibility of POC testing because of the associated tedious, expensive, or instrument-dependent pre-treatment processes. Besides interferences from the urine sample matrices, detection of structurally similar biomarkers often suffers from cross-reactivity and false positive responses [33]. Furthermore, some biomarkers, such as steroid hormone, cortisol, and DHEA require continuous and/or frequent measurements to obtain precise information on the circadian rhythm [34–36]. Therefore, rapid, sensitive, selective, and onsite/POC applicable urinary assays for tracking biomarkers in trace amounts are of significance for POC urinalysis.

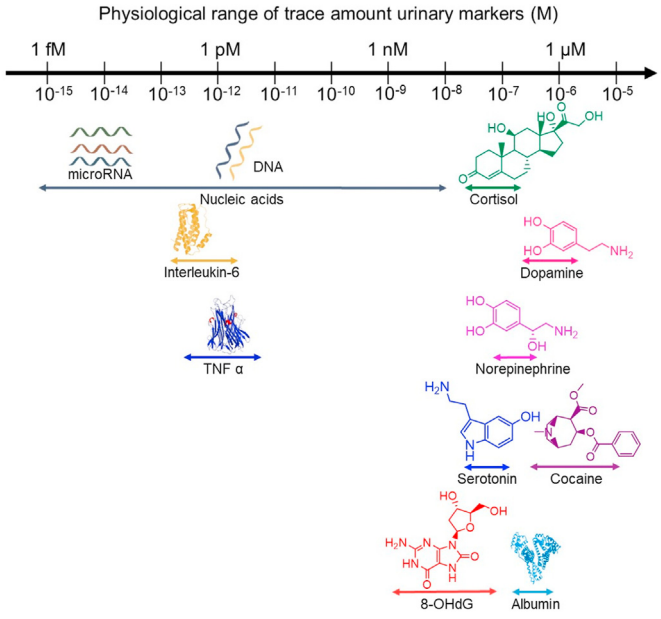

Fig. 1. Illustration of physiological ranges of typical urine biomarkers in trace levels.

Utilization of POC platforms for the detection of urinary biomarkers in trace amounts is still nascent, and research efforts are required to develop rapid, reliable, and accurate sensing platforms. In this perspective, we have attempted to highlight the current trends and challenges in POC urinalysis of biomarkers in trace amounts. First, we will summarize the significance of numerous urinary biomarkers in trace amounts for healthcare monitoring, disease diagnosis, and drug abuse, followed by a discussion on the sensing mechanisms and assay platforms. We will then elaborate on the existing methodologies for urine sample preparation in order to minimize interferences from the sample matrices. Efforts on utilization of smart data analytics for facilitating personalized urinalysis will then be reviewed. Subsequently, a comprehensive outlook on associated challenges in POC urinalysis of biomarkers in trace amounts will be provided, which would potentially broaden the scope of POC urinalysis.

2. Urinary markers in trace amount

Urinalysis paves the way for the future of POC diagnosis owing to the presence of a wide range of biomarkers and to the facile sample collection process. Commercially available urine dipstick/strip assays have been applied successfully for assaying highly concentrated urinary markers, while progress has been made for discovering and tracking urinary markers such as nucleic acids, reactive oxygen species, steroids, neurotransmitters, drugs and their metabolites, and proteins that are in trace amounts. Assaying these biomarkers is important for the early diagnosis of diseases and health complications. Important urinary markers that appear in trace amounts and their normal/elevated levels are consolidated and compiled (Table 1).

2.1. Nucleic acids-based markers

Novel nucleic acid biomarkers have been realized as possible diagnostic indicators, for instance, single stranded microRNAs (miRNAs). miRNAs are non-coding RNAs that can regulate gene expression via repressing or degrading the messenger RNA (mRNA) transcripts. miRNAs have a significant role in cell proliferation, development, differentiation, and other biological activities. Various diseases, such as cancer, develops through a down-regulated expression of miRNAs. The expression profile of miRNA varies significantly depending on the source of the RNAs, i.e., whether it is from malignant or benign tissue. As an example, tumor cells can release a particular kind of miRNAs in the bodily fluids, detection of which can indicate tumorigenesis. Therefore, urinalysis for detecting the presence of miRNAs can serve as a non-invasive diagnostic tool [64]. The circulating miRNAs are highly

Table 1
Summary of important urinary biomarkers in trace amounts.

Biomarker	Associated health condition	Concentration level
Nucleic acids		
MicroRNA	Prostate cancer [37,38], bladder cancer [39,40], kidney disease [41]	Healthy: Sub 10 nM or lower [27,42]
HBV-DNA	Chronic hepatitis and hepatocellular carcinoma [43], liver cirrhosis [44]	Infected: 4.3 log copies/mL [45]
Reactive oxygen species		
8-OHdG	Type 2 diabetes [46,47], neurodegenerative diseases [29]	Healthy: ~70 nM Infected: ~280 nM [48,49]
Steroid hormones		
Cortisol	Acute stress, Cushing's disease, chronic fatigue [35,50]	Healthy: 30–300 nM [51]
Testosterone	Prostate cancer [52], suicidal behaviour [53], drug abuse [54]	Healthy: 20–150 ng/mL [55]
DHEA	Bipolar disorder, obesity, diabetes, and drug abuse [56]	Cut off: 100 ng/mL [57]
Neurotransmitters		
Serotonin	Depression, dysbiosis, anxiety [58,59]	Healthy: 5–13 ng/24 h [60]
Dopamine	Anxiety, paroxysmal hypertension, PTSD [61,62]	Healthy: 52–480 ng/mL [60]
Epinephrine	Attention-deficit/hyperactivity disorder (ADHD), bipolar disorder, PTSD	Healthy: 0–20 ng/mL [60]
Norepinephrine	ADHD, bipolar disorder, PTSD	Healthy: 15–80 ng/mL [60]
Illicit Drugs		
Cocaine	Addictive, heart disease, heart attack, stroke, lung damage	Cut off: 150 ng/mL [63]
Marijuana	Addictive, respiratory infections, pneumonia, chronic bronchitis, reduced immune response	Cut off: 15 ng/mL [63]
Phencyclidine	Addictive, hallucinations, mental disorders, seizures, coma	Cut off: 25 ng/mL [63]
Proteins		
Tumor Necrosis Factor- α	Immune response, cell regulation, arthritis, Crohn's disease, cancer cachexia	Healthy: 0.03–0.4 ng/mL [51]
Interleukin-6	Inflammatory associated diseases, Alzheimer's disease	Healthy: 20–30 pg/mL [60]

stable biomarkers that can be used to diagnose diseases on a routine basis. For example, urine-circulating miRNAs, miR-148a, and miR-375 are reported as potential biomarkers for prostate cancer [37]. Moreover, urinary miR-99a, miR-204, miR-30b, miR-532−3p and miR-125b are identified in cancer patients [44]. miRNAs concentration in urine are typically in nM or lower levels [27,42]. Besides miRNA, DNA sequences in urine are also specific and sensitive biomarkers for numerous diseases, not limited to bladder, prostate, renal, and upper urinary tract cancers [70]. For instance, the hepatitis B virus (HBV) DNA sequence is an important biomarker for chronic hepatitis, hepatocellular carcinoma [48], and liver cirrhosis [49]. Mean HBV-DNA concentrations of 4.3 log copies/mL was recorded for patients chronically infected with HBV [45].

2.2. Reactive oxygen species

Oxidative stress (OS) can arise during the accelerated formation of reactive oxygen species (ROS). ROS are very reactive and can damage biomolecules and their functions and in adults, OS has been related to aging, atherosclerosis, high blood pressure, immune system failure, neurodegeneration, diabetes, carcinogenesis, acute respiratory distress syndrome, and a variety of other disorders. Recent findings indicate that OS may be involved in a variety of children's disorders or ailments, albeit it is not disease specific. As a result, accurately measuring OS status in children is important. As measuring ROS directly is challenging owing to their short half-lives, therefore, OS is typically quantified with stable metabolic markers. For instance, 8-hydroxy-2′-deoxyguanosine (8-OHdG) is a result of an oxidatively modified guanine base produced in urine and is now utilized as one of the most important biomarkers for oxidative damage associated to type 2 diabetes [46,47]. In healthy and diseased conditions, urine 8-OHdG concentrations are ~70 nM (20 ng/mL) and ~280 nM (80 ng/mL), respectively [48,49].

2.3. Steroids

Steroid hormones participate in various biological activities, and their lower/elevated levels are used to determine stress response, sexual maturity, and steroid abuse [65]. Steroids are small hydrophobic molecules regulated by adrenocorticotropic hormone and luteinizing hormone (LH) [66]. Steroids are released into the blood and then diffused into different body fluids, including urine. Some steroid hormones, such as cortisol and dehydroepiandrosterone (DHEA), maintain a diurnal rhythm (reaching the peak in the early morning and the minimum at night) and require frequent and continuous measurement for accurate assessment. For instance, the stress hormone cortisol is measured over a 24 h period in urine. The physiological range of cortisol in urine is 30 nM−300 nM [51]. The 24 h urinary cortisol test determines the levels of cortisol generated in the urine over the course of a day. In adults, levels of 50−100 μg/day is indicative of Cushing's syndrome [67]. Other steroids, such as anabolic-androgenic steroid hormones (e.g., DHEA, androstenedione (adione)), can increase muscle mass and strength and are often used as prohibited substances (doping) for boosting the performance of athletes. World Anti-Doping Agency (WADA) also fixed the cut-off threshold for steroid abuse in trace amounts (e.g., DHEA, 100 ng/mL and adione, 40 ng/mL) [57].

2.4. Neurotransmitters

Neurotransmitters are chemical messengers in the nervous system that are critical for physiological and physical health. Physical, psychotic, and neurological disorders, including dementia, Parkinson disease and Alzheimer disease, and schizophrenia are linked to abnormal neurotransmitter levels [68]. A high level of urinary serotonin is found during depression, anxiety, and dysbiosis [58,59], while a low level is linked with autism spectrum disorder [75]. Patients with elevated dopamine levels display anxiety, stress, paroxysmal hypertension, and post-traumatic stress disorder (PTSD) [61,62]. Alzheimer's disease, anorexia nervosa, hypertension, and periodic limb movement disorder conditions are associated with low dopamine levels [69,70]. The "fight or flight" response is regulated by epinephrine, and norepinephrine and elevated levels of these biomarkers are found in poor health condition, such as PTSD. The physiological concentration of neurotransmitters in urine is sub-μM or lower levels [51].

2.5. Drug and drug metabolite

It has been of great concern that the over-usage of drugs and the over-prescription of drugs, such as codeine, ephedrine, morphine,

amphetamines, methamphetamines, ketamine, and therapeutic drugs, may result in adverse physiological consequences. Excessive nervous system stimulation, stomach acid production, breathing restriction owing to relaxed bronchial muscle, along with several other toxic and dangerous symptoms, are among them. Furthermore, drug abuse and overuse can cause social problems. Determination of drug abuse is of significant importance in medical diagnosis, forensics, and pharmaceutical science, and doping control. The marijuana drug test is performed by analyzing tetrahydrocannabinol analyte, which is an active ingredient in marijuana. Federal regulated cut-off concentration for marijuana is 15 ng/mL. Similar to marijuana, cut-off concentration for other drugs is very low (e.g., cocaine as 150 ng/mL and phencyclidine as 25 ng/mL) [63].

2.6. Proteins

Urine contains numerous proteins that can act as valuable biomarkers in the diagnosis and monitoring of a variety of diseases. Urinary protein biomarker database contains biomarkers for cancer, renal diseases, pancreatitis, diabetic complications, and other diseases. For instance, advanced glycation end-products (AGEs) are modified proteins or lipids that are glycated after exposure to sugar. Micro and macrovascular diabetes problems, aging, cardiovascular illnesses, and neurodegenerative disorders are linked to AGEs [71,72]. An inflammatory cytokine (TNF alpha) generated by macrophages/monocytes during acute inflammation is in charge of a variety of cell signaling activities, including necrosis and apoptosis. This protein is also necessary for infection resistance and cancer resistance. Another important protein marker is cytokine storm, particularly interleukin-6 (IL-6), which is monitored for the identification of inflammatory associated diseases. In addition, IL-6 causes varying effects, from adverse to mild, on COVID-19 patients [73]. Therefore, early detection of IL-6 could facilitate treatment procedures. These important protein markers are typically found in trace amounts in urine samples.

3. POC urinalysis methods

An analytical device is constructed to selectively detect the analyte (target) through chemical or biological reactions or specific interactions (binding). As illustrated in Fig. 2, the sensing methodology involves three major steps: capturing and/or processing of the target analyte using a biorecognition element/catalyst, signal generation, and transduction, and signal processing and display. In this section, we will elaborate on each of the steps for a comprehensive understanding of POC urinalysis.

3.1. Biorecognition element

The molecular or biorecognition element is the crucial element for achieving sensitivity and selectivity. Biorecognition elements with high affinity are required for sensitive and efficient urinalysis [83,84]. Some of them combine recognition with catalytic action. In this section, we will discuss natural recognition elements as enzymes and antibodies utilized for urinalysis, followed by the progress in the development of synthetic recognition elements. Table 2 summarizes commonly used biorecognition elements/catalysts for POC urinary assays.

3.1.1. Natural recognition element

Currently, POC urinary assays depend on natural recognition elements, either based on combined recognition and catalytic action of an enzyme or a binding event to an antibody or aptamer. The enzyme (natural or synthetic) can catalyze biomolecules and produce a change in the concentration of protons, digested species, and gases that ultimately results in a change of emission or absorption of a probe [85]. Glucose oxidase is a well-known enzyme for glucose detection. Other enzymes, for instance, hydroxysteroid dehydrogenases (HSDs), have been reported for the catalytic reaction of steroid hormones [86,87]. However, achieving the long-term stability of these assays could be challenging due to the instability of enzymes [81]. Analyte-antibody reaction mechanism is established and successfully used in pregnancy test strips and other commercial immunoassays [1]. The sandwich format (antibody-analyte- antibody) is applicable to larger target molecules due to the presence of multiple epitopes. On the contrary, competitive assay formats are more suitable for the sensitive detection of small target molecules [51]. However, the additional signal labelling processes result in a longer reaction time. In addition, antibodies are not cost-effective, and they are unstable in the ambient environment over extended periods. They typically require facilities for controlled storage and transportation “cold chain” and are therefore not ideal for rapid POC urinalysis [88].

3.1.2. Synthetic recognition element

Although enzymes and antibodies have been widely used, their physiological state is influenced by ambient conditions, which significantly affects their affinity and catalytic activity. For overcoming these disadvantages, synthetic recognition elements have been developed. The most common synthetic recognition element is an aptamer, which is a short, single-stranded oligonucleotide. When aptamers attach to a target analyte, they can generate three-dimensional conformations. Aptamers are synthesized chemically [75,89]. The synthesis process has minimal batch-to-batch variations. It also enables chemical or functional alterations throughout the synthesis process [90,91]. Aptamers possess several advantages, including small size (10−15 KDa), lower immunogenicity, high specificity, reliability, and cost-effectiveness as compared to antibodies [34,76,77]. Over the years, aptamer-based POC urinary sensors have been established to detect diverse urinary markers at low concentrations, including 8-OHdG [28,29,92,93], cocaine [94,95], lysozyme [96,97], AGE [98], dopamine [99,100]. However, screening for individual target-specific aptamer is time-consuming, and target binding can be non-specific [78,101].

Alternatively, the molecularly imprinted polymer (MIP), which acts as a synthetic antibody, has drawn significant attention. It has numerous advantages such as simplicity of preparation, good stability, cost effectiveness, longer lifespan compared to antibodies and enzymes, high selectivity, and sensitivity [79,80,102]. MIPs work as synthetic recognition sites in polymer for target-specific binding. The formation of MIPs starts with the polymerization of monomers with a target molecule as a template. Upon removal of the templates, complementary cavities are left behind within the polymer, which serve as recognition sites for the target molecule. Recently, researchers are also mimicking the catalytic activities of enzymes by synthesizing the active site (prosthetic group) of enzymes [103,104]. Synthetic enzymes catalyze the electro-oxidation of different molecules such as ions, nitrate, H_2O_2, cortisol, dopamine, L-cysteine, etc. [105]. Iron single atom catalyst based LFA is reported to detect the presence of herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) in urine [106]. Copper phthalocyanine (CuPc) acts as a synthetic catalyst for the detection of 17β-estradiol hormone in urine sample [107]. Synthetic enzymes hold the potential for high catalytic activity, signal amplification, and high stability in ambient conditions [81,108].

3.2. Assay formats for urinalysis

This section will elaborate colorimetric, fluorescence,

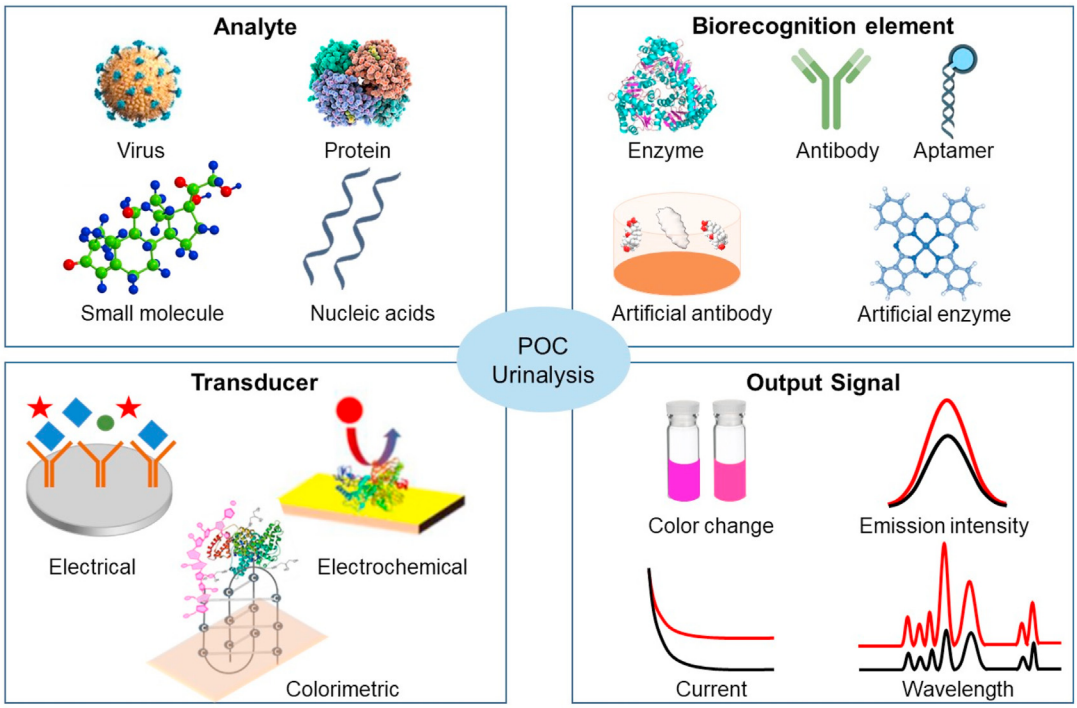

Fig. 2. Molecular requirements and sensing strategies for POC urinary assays.

Table 2
Summary of biorecognition elements/catalysts used in POC urinary assays.

Biorecognition elements	Pros	Cons
Natural enzyme	Sensitive and selective	Costly, short shelf-life and limited stability [74]
Antibody	Sensitive, and selective	Costly, short shelf-life and limited stability [75]. Difficult to generate antibodies specific to small molecules [76]
Aptamer	Small size, minimal batch to batch variation, long shelf life, and comparatively cheaper than antibodies [76,77]	Screening for individual analyte-specific aptamer is time-consuming, not cost-effective, and analyte binding affinities can be non-specific [78]
Synthetic antibody	Sensitive, selective, low cost, excellent stability and long shelf-life [79,80]	Unsatisfied sensitivity and require complex, time-consuming fabrication processes
Synthetic enzyme	Sensitive, high stability and long shelf-life [81]	Suffer from low selectivity and requires specific pH conditions for catalytic redox reaction [82]

electrochemical, and optical (intensity/spectral) POC assays for detecting trace amount biomarkers in urine. Fig. 3 represents different sensing mechanism based urinary assays. The highlights and challenges of different sensing mechanisms are summarized in Table 3.

3.2.1. Colorimetric biosensors

Colorimetric biosensors are widely utilized and commercially available as POC diagnostic devices. The sensing mechanism in colorimetric assays depends on the alteration in the color of a colorimetric probe during its biochemical interaction with the target biomarker [27]. There are numerous advantages of colorimetric biosensors, such as low cost and ease of usage. Even in some cases, they can be designed to yield easily observable naked eye responses. However, colorimetric sensors sometimes lack sensitivity and reproducibility because of non-uniform color distribution issues across the sample spot (region of interest, ROI). This also influences the dynamic/linear range making quantitative measurements challenging.

The main types of probes used in colorimetric assays are chromophores, noble metals, fluoroprobes, quantum dots, and luminescent polymers. Nanoparticles such as gold/silver nanoparticles (AuNPs/AgNPs) have gained significant research interest for the usage of colorimetric sensing. Out of these nanoparticles, AuNPs are the most frequently utilized due to their good stability, less toxicity, and excellent biocompatibility. For example, colorimetric and visual detection of urinary 8-OHdG was reported using unmodified AuNPs [29], as shown in Fig. 3a. Here, as a recognition element, an aptamer was utilized, and AuNPs were used as a probe. The presence of 8-OHdG facilitates a change in aptamer conformation, which is validated through the color alteration of AuNPs probe suspension. A limit of detection (LOD) of 1.7 nM is achieved by the proposed methodology. Although AuNP-based assay offers sufficient sensitivity, it has several limitations. It is extremely difficult to maintain a uniform size and shape of the AuNPs. This non-homogeneity can influence the colloidal stability and produce varying colorimetric assay responses, affecting the reproducibility of the assay [111–114].

3.2.2. Fluorescence biosensors

In past decades, fluorescence biosensors have gained interest due to their high sensitivity and the capability to yield quantitative

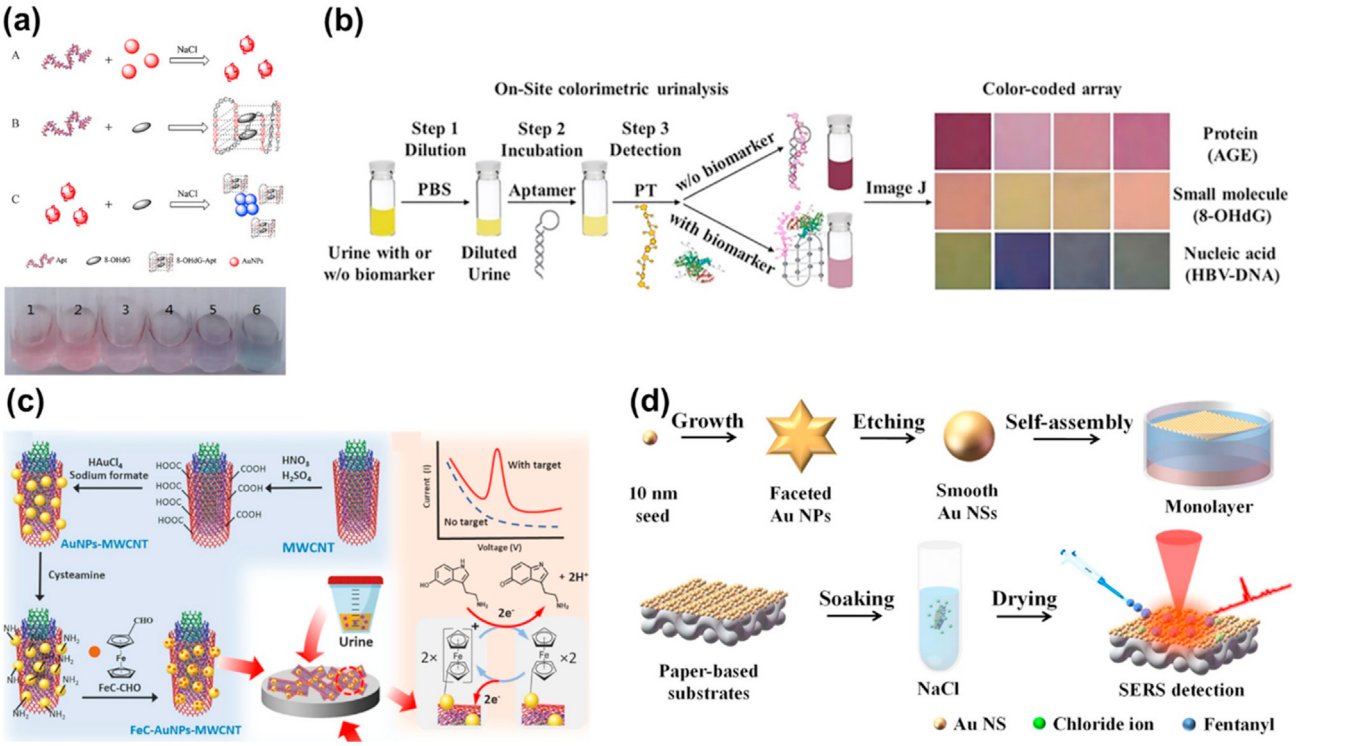

Fig. 3. Different sensing mechanisms for urinalysis. (a) Schematic diagram for colorimetric sensing of 8-OHdG with label-free aptamer and unmodified gold nanoparticles in solution. The optical image (bottom) present colorimetric responses of different concentration 8-OHdG [29]. (b) Schematic illustration of fluorescence assay for AGE and 8-OHdG detection with cationic polythiophene (PT) and target-specific aptamer. A color-coded array represents concentration-dependent responses for three makers (AGE, 8-OHdG, and HBV-DNA) on a paper substrate [27]. (c) Schematics of electrochemical detection of serotonin with ferrocene-gold nanoparticles-carbon nanotube nanocomposite based screen-printed electrodes [109]. (d) Schematic for SERS detection of fentanyl citrate onto paper-based SERS substrate [110]. All figures have been reprinted with permission.

Table 3
Summary of sensing strategies in POC urinalysis.

Sensing mechanism	Highlights	Challenges
Colorimetric	Facile, rapid, visual, equipment-free and cost-effective [115]	Limited dynamic range, some-times insufficient sensitivity, non-uniform color distribution over ROI that affects reproducibility and reliability [139,140]
Fluorescence	Rapid and sensitive [34]	Sensitive to interfering fluorescent species and suffers from photobleaching (depending on fluoroprobe) [27]
Electrochemical	High sensitivity, rapid response, low cost, and portability [109]	Interference vulnerability, poor stability, suffer from baseline drift [13]
SERS	Rapid, high sensitivity and low background noise [141]	SERS substrates degrade with time and cause decrease in signal, lack of homogeneity and reproducibility [142]
Chemiluminescence	Highly sensitive, wide dynamic range and use of simple instrumentation [143]	Require enzyme labeled antibodies, high cost and time-consuming labeling steps [144]
Surface plasmon resonance	Label-free, sensitive and real-time [145]	High excitation power, toxicity, and costly fabrication [146]

responses. The sensing mechanism is similar to that of colorimetric assays, except it requires additional instrumentation for fluorescence signal recording. An external optical source is also required to excite the fluorescent molecules, and subsequently, its fluorescence response is measured. The fluorescence intensity is subsequently correlated with the target analyte's concentration level for quantitative analysis [115]. In addition to fluorophores and dyes, various optical reporters such as quantum dots, carbon dots, AuNPs, and conjugated polymers have been utilized as probes. Recently, a cocaine fluorescence assay was developed with target-induced light-switching excimer signaling [116]. In this work, a single-strand aptamer was split into two portions with pyrene labeled terminals. The addition of a target biomarker enabled the self-assembly of two aptamer fragments and the development of an aptamer–target complex. As a result, two pyrene molecules formed an excimer and yielded a redshift in the fluorescent signal. The sensor was able to detect 1 μM of cocaine. In one of our previous works, we developed a luminescent cationic polythiophene polymer for fluorescent assaying of three different biomarkers, including a nucleic acid (HBV-DNA), a protein (AGE), and a small molecule (8-OHdG), shown in Fig. 3b [27]. Here, the presence of respective target biomarkers was detected with the alteration in optical properties of the luminescent polymer and aptamer, which is specific to the target. Three markers, HBV-DNA, 8-OHdG, and AGE were detected in urine at clinically significant levels, with a LOD of ~ 1 nM, ~ 650 pM, ~ 850 pM, respectively.

3.2.3. *Electrochemical biosensors*

Electrochemical biosensors are low cost, sensitive, and portable, thus a suitable platform for POC testing. One of the most popular

examples of the electrochemical assay is the blood glucose meter. Recently, electrochemical sensors have been evaluated for POC urinalysis of trace amount markers [117]. The sensing mechanism primarily relies on monitoring the change of electrochemical signals associated with oxidation/reduction of electroactive species participating in the recognition process. Therefore, if the target is electroactive, it can be monitored directly. On the contrary, if the target is not electroactive, then a standard redox probe is added into the analyte solution. For instance, electroactive molecule serotonin detection is performed with an electrocatalytic nanocomposite based portable screen printed sensor [109]. In this work, ferrocene-gold nanoparticle-decorated carbon nanotube nanocomposite is utilized as a high-density catalyst for an enhanced response toward oxidation of serotonin, and a LOD of 17 nM was obtained (Fig. 3c).

3.2.4. Surface-enhanced Raman scattering (SERS)-based biosensors

Surface-enhanced Raman scattering (SERS) uses enhanced light scattering upon absorbing biomolecules onto corrugated metal surfaces or nanoparticles. SERS may be able to overcome the typical disadvantages of colorimetric and fluorometric biosensors owing to its strong multiplexing capabilities, lack of photobleaching, high sensitivity, and low background noise [118–120]. Furthermore, SERS-based biosensors can enhance signals over three orders and possess the potential to detect single molecules [121,122]. Hence, SERS has been extensively utilized for screening cancer [123,124], liver cirrhosis [125], bladder tumors [126], illicit drug detection [127], and many other applications. A multiplexed SERS detection platform was developed for detecting urine metabolites, 20α-diol-3α-glucuronide, 5β-pregnane-3α, and tetrahydrocortisone at sub-nM concentration levels in 30 min [128]. For the diagnosis of prostate cancer, detection of two microRNA sequences (miR-10a and miR-21) was reported with a 3D plasmonic nanostructure-based SERS platform [129]. The formation of a 3D plasmonic hotspot accelerates significant amplification of the SERS signal, yielding a LOD of ~10 nM. A portable kit including reagents tubes, SERS substrate, and handheld Raman spectrometer was demonstrated for the detection of drugs in urine [130]. A LOD of 0.1 ppm of amphetamines in urine was reported. Multiplexed detection of 3,4-methylenedioxymethamphetamine and methcathinone in 30 urine samples was also demonstrated.

Besides the above-mentioned assays, other assays such as reverse transcription polymerase chain reaction (RT-PCR) [131], chemiluminescence [132,133], and surface plasmon resonance (SPR) [134,135] have been reported for POC urinalysis. Table 4 summarizes important transducers for urinary biomarker detection. Statistical methods such as principal component analysis (PCA), linear discriminant analysis (LDA), and hierarchical cluster analysis (HCA) were extensively used with sensing methods for identifying and discriminating the patterns obtained from sensor arrays [136]. A colorimetric array for assaying three catechol structure biomarkers (dopamine, epinephrine, and nephrine) has been reported. In this work, HCA, LDA, and PCA were employed for analyzing individual biomarkers from their mixture in urine sample [137]. In another work, the sensing performance of a colorimetric array for structurally similar seven carboxylic acids in urine samples was evaluated using HCA, LDA, and PCA recognition method [138].

4. POC urinalysis platforms

The attractiveness of urinalysis is due to the successful transformation of established clinical urinalysis techniques into low-cost, facile, yet accurate, and portable assay platforms. The recent approaches in miniaturization and design in portable diagnostic tools for urinalysis are summarized in this section. Miniaturized set-ups and portable diagnostic tools for urinalysis are briefly summarized in Table 5, based on their analytical methodology, fabrication technique, LOD, limit of quantification (LOQ), and assay format. The major advantages and concerns of the POC urinalysis platforms are also discussed in the following sections.

4.1. Homogeneous solution-based assays

Comprehensive urinalysis in a urine sample has been subjected to physical, chemical, and microscopic evaluation. In a physical inspection, the color and odor of the urine are assessed visually, while a chemical investigation evaluates the sample's pH and protein/chemical composition, and a microscopic examination examines the morphology particles in urine. Various assays have been developed to distinguish these features and to assist in the diagnosis of patients. As an example, casts and blood cells are indicatives of kidney conditions and may be utilized to diagnose acute renal damage and the progression of kidney disease over time. Sedimentary urinary markers might be classified in two categories; first category contains cells, fungi, casts, parasites, bacteria, and sperm and the second category includes crystals of various kinds. Owing to the distinctive morphology of each of these sediments, they can be easily identified by a microscopic investigation. Therefore, routine microscopy analysis is commonly used for casts, cells, crystals, parasites, and bacterial detection. Microscopy of urinalysis is typically performed using brightfield microscopy, while other microscopy techniques (e.g., phase contrast and dark field) also utilized. However, microscopy set-ups are bulky and are not cost effective, which mitigates POC urinalysis. Therefore, lensless microscopy is emerging as a viable alternative to traditional microscopy, which possesses advantages such as a vast field of view, three-dimensional reconstruction, cost effectiveness, and compactness. This method captures the specimen's image on a detector without requiring lenses. As illustrated in Fig. 4 (from a to

Table 4
Selected urinary biomarkers, sensing strategies, their LOD, and assay time.

Biomarker	Sensing Strategy	LOD	Assay time (min)
8-OHDG [29]	Colorimetric	1.7 nM	>20
Adenosine [30]	Colorimetric	130 pM	120
8-OHDG [27]	Fluorescence	650 pM	30
AGE [27]	Fluorescence	850 pM	30
HBV-DNA [27]	Fluorescence	1 nM	30
Cocaine [116]	Fluorescence	1 μM	40
Serotonin [109]	Electrochemical	17 nM	2.5
Ascorbic acid [147]	Electrochemical	200 nM	–
Dopamine [147]	Electrochemical	30 nM	–
Lysozyme [148]	Surface plasmon resonance	1 nM	20
Zika virus [131]	Polymerase chain reaction	5.0 PFU/mL	15

Table 5
Summary of POC urinalysis platforms.

Assay	Analytical technique	Fabrication technique	LOD/LOQ	Assay format
8-OHdG, 3-nitrotyrosine (3-NT) [149]	Electrochemical	Filter paper with hydrophobic barrier layers to construct 3D circular reservoirs and electrodes	LOD of 14 nM for 8-OHdG and 2.7 nM for 3-NT	Paper-based analytical device
Creatinine [150]	Colorimetric	The necessary form and hydrophobic barriers were printed in bulk paper using a solid ink printer	LOD - 0.35 mM and LOQ - 1.05 mM (Jaffe-based sensors) and LOD - 0.27 mM and LOQ- 0.82 mM (DNBA-based sensors)	Paper-based format
Lead ions (Pb(II)) [151]	Electrochemical	Patterned paper was made directly by printing microchannels on filter paper	LOD of 9 μg/L	Paper-based microfluidic chip
Nitrate [152]	Colorimetric detection	A laminator is used to generate a hydrophobic area	LOD - 0.04 mM and LOQ - 0.14 mM	Microfluidic paper-based analytical device
Fentanyl citrate [110]	Raman spectroscopy	To create a paper-based substrate, an efficient seed-mediated growth approach was paired with etching method, followed by a liquid/liquid self-assembly method	LOD- 0.59 g/mL	Paper-based SERS substrate
UTI (E. coli) and STD (Neisseria gonorrhoeae) [153]	Colorimetric smartphone camera as a detector	Using a conventional lithography approach with UV exposure and development, microfluidic channels were printed on paper. The multichannel paper chip was made from cellulose chromatography paper	LOD of 10 CFU/mL	Microfluidic paper analytical device
Pathogen [154]	Fluorescence immunoassay	Standard soft-lithography technique	Ten-million-fold enrichment for 1×10^3 cells/mL bacteria	The agarose-based micro-fluidic chip
Immunoglobulins [155]	Fluorescence	Photochemical immobilization technique	LOD of ~8 μg/L	Metal enhanced fluorescence
Zn [156]	Electrochemical	The layer-by-layer (LbL) technique	LOD of 46 nM	ElectroSens Platform
Exosomes [157]	Visual detection	CuS NPs and filter paper to enrich the exosomes at low concentrations	2×10^3 particles/μL in a complex matrix by the naked eye	Filter membrane
Drugs of abuse [158]	Volumetric-bar-chart	Standard photolithography process	Clinical sensitivity and specificity of 0.94 and 1.00 (recovery rate) respectively	Volumetric-bar-chart chip
Tuberculosis Lipoarabinomannan [159]	Mach−Zehnder Interferometer (MZI) transducer combined with an on-chip spectral filter	Six MZI sensors and a reference sensor are combined into the photonic sensor chip, which is fabricated in silicon nitride at wafer level	27 pM	Photonic sensor chip
N-acetyl-tyramine-O-glucuronide (NATOG) for onchocerciasis [160]	Lateral flow immunoassay	A sample pad, membrane, wick pad, and backing card were used to construct the LFA strip	NATOG concentration cutoff for onchocerciasis (25 μM)	Lateral Flow immunoassay dipstick
Bacterial detection [161]	Immunoluminescence	Magnetic and fluorescent nanoparticles were both produced and functionalized with antibodies	*Escherichia coli, Staphylococcus aureus* 10^3 CFU/mL	Photonic crystal-based biochip
Urinary protein [162]	Colorimetric	Chips and elements were designed with AutoCAD software and printed via 3D printer	LOD of 8.5 μg/mL	3D printed microfluidic chip
Nitrite [163]	Colorimetric	The colorimeter is made up of 80% off-the-shelf components and has a 3D-printable casing	LOD of 0.8 μM in buffer and 1.6 μM in synthetic urine	Transmission-based colorimeter

c), a typical lensless imaging device comprises of a LED light source with a pin-hole apparatus and a CMOS detector. These systems minimize the turnaround times for samples, health-care expenses, and workload in labs. Prescreening procedures obviate the need for an overnight urine culture, thereby minimizing the turnaround time. By removing negative samples from the screening process at an early stage, redundant culturing and administration of antibiotics are prevented. In an ideal case, this instrument could be used as a POC device in hospitals and health centers to minimize the amount of samples sent to the laboratories, while also allowing for tailored diagnosis due to the rapid recognition of the clinical status of the patient. The current studies utilizing lensless imaging analyze blood counts, pap smear, and sperm motility and facilitate health care monitoring. The basic theory, capabilities, limits, and the background of lensless imaging were extensively discussed by Ozcan et al. [164]. Lensless imaging has been successfully combined with the pixel super resolution (PSR) method to significantly improve image resolution. PSR is a method of combining numerous low-resolution photos into one high-resolution image, for instance, a lensless optofluidic system with shadow imaging and PSR demonstrated by Sobieranski et al. [165]. Fig. 4d and e, corresponding to images of objects prior to and after PSR application, respectively, illustrate the improvement in image resolution. Another demonstration of PSR for improving image resolution is illustrated by the ability to resolve 338 nm grating, as shown in Fig. 4f (before PSR) and 4g (after PSR).

The PSR application for a sperm motility test is shown in Fig. 5, which illustrates the potential of lensless imaging for POC urinalysis. Holograms were used to obtain images of sperm samples. The first three rows in Fig. 5 indicate the amplitude, phase, and multi-dimensional RGB arrangement, respectively. The bottom panel in Fig. 5 illustrates the normalized high resolution (HR) phase in radians, with a minimum of 1.43 and a maximum of 3.1416 [165].

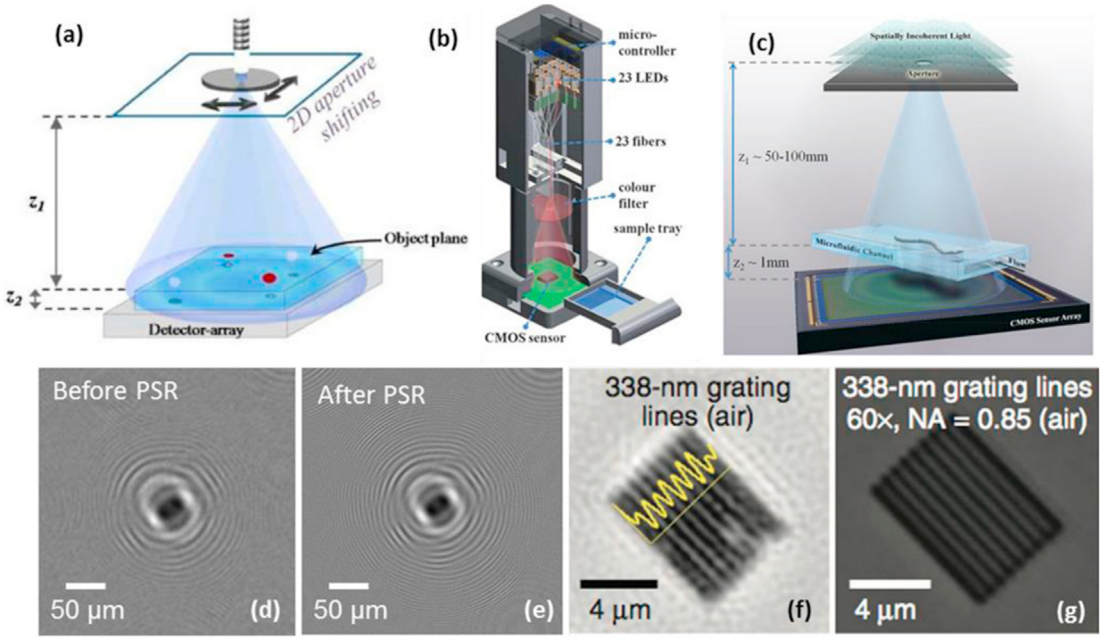

Fig. 4. Pixel super-resolution (PSR) for on-chip digitized hologram imaging. Subpixel shifts can be created in a digital hologram via (a) source-switching, (b) individual programmable fiber-coupled LEDs, sensor switching, or (c) sample switching utilizing a microfluidic flow. (d) Prior to PSR, an in-line holographic. (e) The same object's hologram in a pixel-resolved form. (f) PSR was used to restructure gratings with a line width as small as 338 nm. (g) Comparative microscopy of the same area of interest demonstrates the improvement in image resolution [164]. All figures have been reprinted with permission.

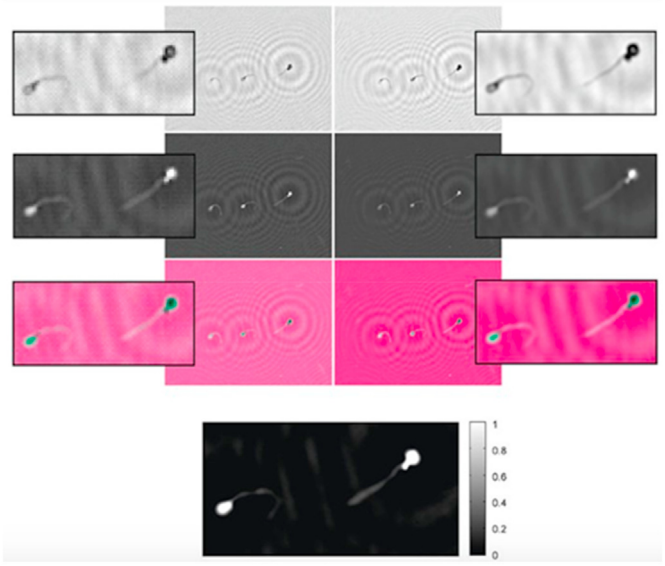

Fig. 5. Demonstration of PSR application for sperm motility test [165]. All figures have been reprinted with permission.

4.2. Heterogenous paper-based assays

The heterogenous POC based assay has certain advantages over a homogenous microscope and lensless imaging. In this section, major developments on heterogenous paper-based sensors will be discussed. As summarized in Table 5, the current heterogenous urinalysis assays exhibit promising potential due to precise fabrication techniques as well as the incorporation of nanotechnology detection methodologies. Jalal et al. demonstrated a device featured paper−plastic hybrid microfluidics for colorimetric urinalysis via smartphone [166]. The described platform was obtained by a cost-effective hot-embossing technique for patterning. The proposed device is capable of detecting pH (5.25−7.5), red blood cells (0−280 cells/µL), protein (0−2000 mg/dL), and glucose (0−350 mg/dL) [166]. Nontawong et al. has described a smart sensor for evaluating stress biomarkers that used an electrochemical paper-based electrochemical device with dual imprint [149]. The proposed device showed good analytical performance for the detection of 3-nitrotyrosine (3-NT) and 8-OHdG with a LOD of 2.7 nM and 13.8 µM, respectively. A colorimetric POC paper-based assay for the detection of urinary creatinine via a smartphone was described by Lewińska et al. [150]. The authors showed the utilization of a solid ink printer to print a specified form and generate hydrophobic obstacles in the paper. This heterogenous paper-based assay exhibit a LOD of ~0.35 mM and LOQ of 1.05 mM. As another example, an electrochemical paper-based microfluidic system was developed to perform protein separation and onsite detection of lead in urine (with a linear range of 10−500 µg/L and LOD of 9 µg/L). This work demonstrates the benefit of printing microchannel designs directly on filter paper using a printer [149]. In a recent study, a relatively new methodology for creating a hydrophobic barrier on micropads has been presented by Ferreira et al. to detect nitrate in urine [152]. This enzymatic assay shows good analytical performance and a LOD of 0.04 mM, a LOQ of 0.14 mM with a linear concentration range of 0.14−1.0 mM.

The high sensitivity of surface-enhanced techniques was combined in a paper-based platform by Han et al. Herein, chloride ion-treated paper platform was utilized for fentanyl citrate detection in serum and urine. The authors used a liquid/liquid self-assembly approach to build a paper-based substrate after introducing an efficient seed-mediated growth method [110]. Li et al. has used the advantage of agarose-based microfluidics for concentrating and detecting a pathogen in POC settings. The agarose-based microfluidic chip was prepared using a standard soft-lithography technique, and the authors utilized fluorescence immunoassay to detect bacteria in the range of 1×10^3 cells/mL [154]. Ventura et al. demonstrated POC assaying of immunoglobulins in urine, via

manipulation of biological solutions on a superhydrophobic surface (Fig. 6a) followed by concentration and delivery of target analytes using superhydrophobic pillars (Fig, 6b). Gold nanoparticle clusters that provide metal-enhanced fluorescence are utilized for the assay, as shown in Fig. 6c and d, respectively. Fig. 6e to 6i shows the Si micropillar sensor platform for providing control delivery of analyte, and this assay yielded concentration dependent responses from 10 μg/L to 100 μg/L with a LOD of ~8 μg/L (Fig. 6j). A rapid and ultrasensitive visual detection of exosomes, a class of emerging biomarkers, was proposed for POC disease diagnosis by Chen et al. [157]. The proposed assay utilizes CuS nanoparticles and a membrane for enriching exosome concentrations and providing visual detection of 2×10^3 particles/μL in a complex matrix.

Ying et al. has reported an assay based on a microfluidic platform for the detection of drug abuse. Analysis of cocaine spiked samples was demonstrated using ELISA, as shown in Fig. 7 (a) and (b). Drug-BSA-PtNPs are bounded on an antibody coated glass surface. The target drugs subsequently compete with the drug-BSA-PtNPs for binding with the antibodies immobilized on the glass surface. The sample is placed at the bottom position, while the control with the target drug's cutoff value is placed at the top position. A negative sample and a PBS spiked with the drug as positive samples (Fig. 7c) and (Fig. 7d) were tested for the proof of concept. Responses for a series of positive (Fig. 7e) and three positive/negative sample (f) were then recorded. Methadone (MTD) determination in patient serum samples has been further demonstrated by the proposed methodology (Fig. 7g). Negative (top) and positive (bottom) sample calibration curves were then obtained, as shown in Fig. 7h. Bar graphs representing MTD concentrations determined by the device and LC-MS/MS method in patient serum samples were then plotted (Fig. 7i). Correlation determination using Bland-Altman analysis between the proposed methodology and LC-MS/MS data with a confidence interval of 95% average value is shown in Fig. 7j [158].

The detection of N-acetyl-tyramine-O-glucuronide (NATOG) for onchocerciasis by LFA was analyzed by Shirey et al. where an LFA test strip was constructed using a sample pad, wick pad, and backing card. This diagnostic tool was able to detect NATOG concentration cut off for onchocerciasis (25 μM) [160]. The importance of urinary protein detection was discussed by Chan et al. where the authors conceptualized a facile 3D printed microfluidic platform for POC colorimetric detection of urinary protein. The obtained LOD was 8.5 μg/mL. The microfluidic platform was designed with AutoCAD software and printed via a 3D printer [162]. Ghaderinezhad et al. developed a miniaturized paper-based device for the detection of electrolytes in urine utilizing various fluorescent probes such as sodium green and fluozin calcium [167]. Yang et al. demonstrated a microfluidic immunoassay cassette comprised of a lateral flow strip and an optical handheld reader for the sensing of HIV in urine samples [168,169].

4.3. Miniaturized devices

Miniaturized platforms with numerous advantages, such as reduced sample volume, reduced processing time, low-cost analysis, and minimal reagent consumption, are increasingly in demand for biosensors coupled with microfluidics. As shown in Fig. 8, microfluidics integrated biosensors offer several advantages, including laminar flow, minimal handling of hazardous materials, parallel detection of many samples, mobility, and design flexibility. Microfluidic chips, when combined with biosensors, are predicted to improve analytical capability and broaden the scope for POC urinalysis. Developments in microfluidics have made it easier for researchers in business and academia to use these platforms for POC diagnosis. The commercial nitrite kits (Fig. 8a) have been demonstrated as cost-effective transmission-based POC colorimeter for detecting nitrite in buffer, synthetic, and human urine samples, respectively (Fig. 8b–d). The described device is capable of

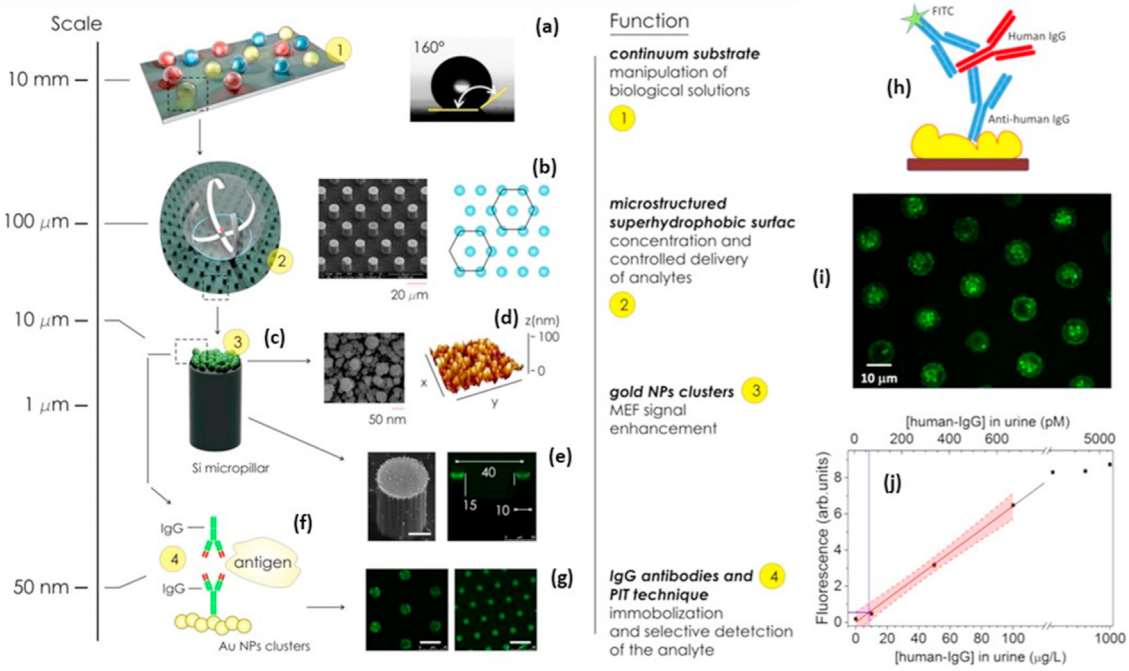

Fig. 6. Schematic illustration of POC assaying of immunoglobulins in urine showing (a) manipulation, (b) concentration and delivery of analytes, (c) gold nanoparticles clusters that provide (d) metal enhanced fluorescence. (e) Selective capturing of immunoglobulins using functionalized gold nanoparticles, (f) detection scheme, and (g) detection in extremely low concentration levels. (h) Schematic of fluorescein isothiocyanate (FITC)-tagged secondary antibodies that bind to human IgG in a sandwich arrangement, (i) fluorescence image obtained for 200 g/L IgG, (j) fluorescence intensity vs concentration of IgG (95% confidence bands region is indicated in red) [155]. All figures have been reprinted with permission.

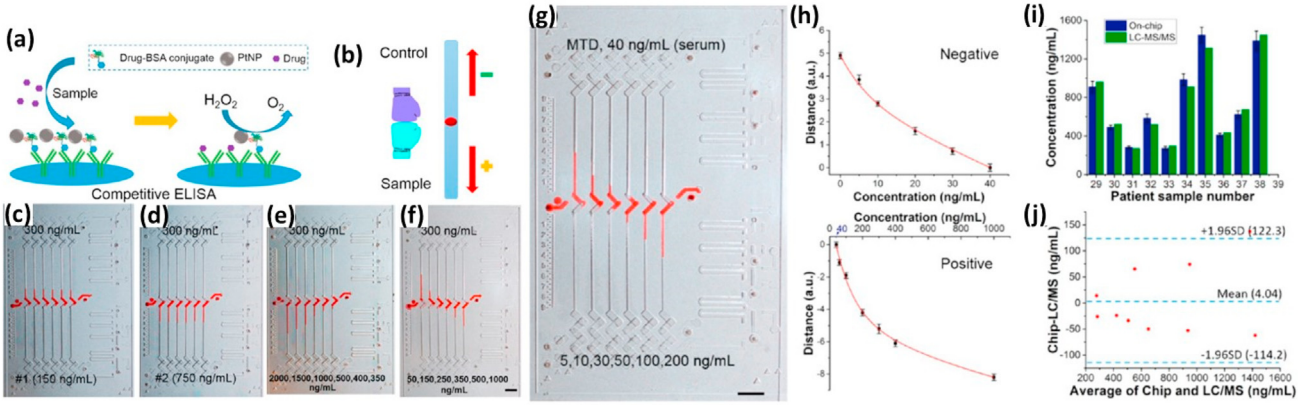

Fig. 7. Demonstration of (a) and (b) an ELISA approach for detection of cocaine showing responses for (c) negative, (d) positive, (e) series of positive, and (f) three positive/negative samples. (g) MTD determination showing responses, and (h) analysis for negative (top) and positive (bottom) samples, (i) benchmarking of responses against LC-MS/MS method, and (j) their correlation efficiency [158].

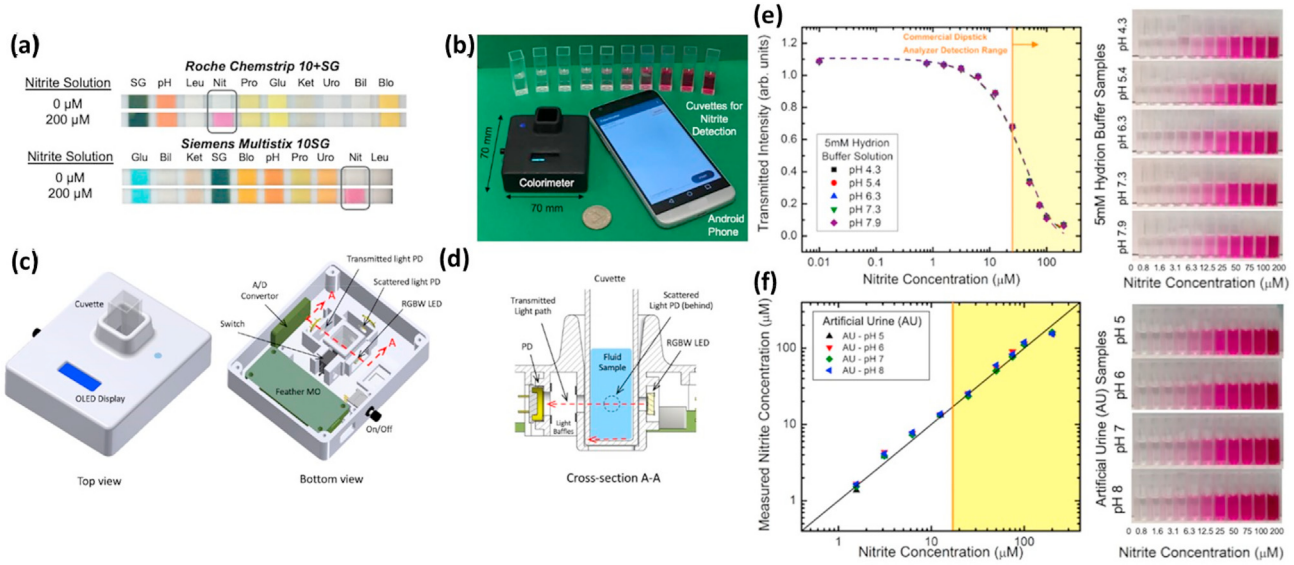

Fig. 8. Detection of nitrite: (a) commercially available urine dipsticks, (b) quantitative colorimeter based on transmission, smartphone, and cuvettes, including reagents. (c) Colorimeter's exterior and interior components. (d) Cross sectional view of two photodetectors for the transmitted and scattered light paths. (e) Transmitted intensity and (f) colorimetric detection of different concentrations of nitrite spiked synthetic urine samples and their corresponding optical images [163]. All figures have been reprinted with permission.

detecting nitrite in 1–100 µM concentration in urine, as illustrated in Fig. 8e and f. The authors also offered a smartphone-based mobile application, which can display the nitrite concentration. This portable analyzer may be used to identify various biomarkers, improving the sensitivity and specificity of the required experiment [163]. A miniaturized electrochemical platform to detect Zn ions in urine has been proposed by Nikolaev et al. [156]. Protein molecules are prevented from adsorbing on the carbon fiber surface by the ElectroSens platform, which is a polyelectrolyte-based sensor interface, increasing the sensitivity of Zn detection to 2.2 µA/M. ElectroSens has been designed with a wireless portable potentiostat linked to an android-based smartphone. The sensing performance of ElectroSens is promising for the detection of Zn in blood and urine.

Recently, a variety of microfluidic technologies have been reported for the detection of nucleic acids and proteins. Amplification methods such as PCR, loop-mediated isothermal amplification (LAMP), and recombinase polymerase amplification (RPA) are typically required for molecular diagnostics as the target RNA/DNA are typically found in low concentration levels [170–172]. Recent trend indicates that efforts are devoted to integrating these amplification methodologies with microfluidic devices. Ahrberg et al. developed a PMMA microwell array as a miniaturized portable digital PCR based on plasmonic heating for the detection of DNA [173]. Cao et al. demonstrated a digital LAMP platform utilizing a microscale hydrogel array and heater for sample partition and DNA amplification with a detection limit of 1 copy/µL [174]. Schoepp et al. also developed an antibiotic susceptibility test of E. coli from clinical urine samples using a digital LAMP SlipChip microfluidic device for nucleic acid quantification [175]. Moreover, Kalsi et al. developed a programmable digital microfluidic platform for sample preconcentration and RPA to detect antimicrobial resistant genes from urine samples [176]. Yang et al. reported an integrated microfluidic device for the isolation and detection of exosomes from urine samples. PMMA was utilized to fabricate the microfluidic device and gold nanorod probe with antibody for the

detection of lung-cancer derived exosomes [177]. The continuous development of microfluidic technologies and detection methodologies could help in overcoming the current challenges in urinalysis. POC diagnostic techniques could be utilized for the monitoring of urine samples and various sensing modalities can be integrated into a device for the simultaneous detection of multiple analytes [14].

5. Sample handling and pre-treatment for POC testing

As previously stated, due to obstacles from the urine sample interferences, direct assaying of urinary biomarkers in trace levels is difficult. For instance, the inherent variations in color, varying pH, high ionic strength, and the auto-fluorescence of urine can affect the output of colorimetric, fluorometric, and electrochemical sensing [27]. To overcome these background interferences, it is necessary to adopt pre-treatment protocols for enrichment and/or purification of samples prior assaying as well as for facilitating sample storage [4,5]. Sample pre-treatment facilitates obtaining accurate and consistent results, better sensitivity, and robustness (less interference or masking of analytes). Numerous methodologies like urine sample dilution, centrifugation, and solid phase extraction (SPE) are utilized to eliminate the interferences. Centrifugation is used to exclude solid parts that could be there in the urine. (e.g., blood cells or microorganisms). Most of the existing assays depend on centrifugation for detecting urinary biomarkers in trace amounts. Dialysis and ultrafiltration are two other pre-treatment options for removing peptide fragments and proteins from urine. The exact type of pre-treatment technique and combinations thereof depends on the analytical procedure and the concentration levels of the target analyte. The majority of these sample pre-treatment techniques are labor-intensive, making them less feasible for point of care setting [178]. In the following sections, different methods of sample handling and sample pre-treatment technologies for urinalysis involving conventional or benchtop instrumentation, miniaturized devices, and paper-based devices are discussed.

5.1. Sample collection and storage

Urinalysis could be affected by improper sample storage conditions and extended storage between collection and testing. The stability of the urine sample is in between 2 and 6 h at room temperature and 24 h for refrigerated samples without the addition of chemical preservatives [179–181]. Recently, paper cards have become an interesting alternative for sample collection and storage due to their low cost. Paper cards are chemically modified with appropriate surface treatments such as nitrification, chemical reagents and/or by adding suitable surfactants for sample collection and storage [182,183]. Various paper cards are commercially available with chemical modifications, as shown in Table 6. As an example, fast technology analysis (FTA) cards could be utilized for urine collection and storage. They contain chemicals to prevent bacterial growth, protein denaturation, and cell lysis. The chemicals also protect nucleic acids from nucleases. Most of the current commercial cards allow collection and storage of urine samples without the requirement of refrigeration and enables transportation of the sample for downstream analysis [5].

5.2. Sample preparation

Sample preparation methodologies could be classified broadly into two types: matrix scavenging and targeted extraction. Matrix scavenging methods are rapid and facile, however, does not yield satisfactory analyte recovery and/or detection limits. On the other hand, targeted extraction methodologies yield higher recoveries and sensitivities [188]. The composition of urine is schematically shown in Fig. 9. Since the urine components, such as urea, salts, proteins, etc., vary significantly with factors such as age, race, gender, etc., it is important to adopt appropriate sample preparation protocols. The various discrete sample preparation techniques are discussed in the following section.

5.2.1. Dilution

One of the most common and simplest ways of sample preparation is dilution, where urine samples are diluted with water or buffer and surfactant. This method is cheap, fast, and simple. As an example, a urine sample is diluted × 32 with 0.1 M PBS for electrochemical detection of 8-OHdG with polythiophene (PT) modified glass electrode [190]. Candace et al. developed a lateral flow immunoassay for detecting amatoxins in the urine where two-fold dilutions of the sample were made [191]. Urinary biomarkers such as N-acetyl-β-D-glucosaminidase (NAG), neutrophil gelatinase associated lipocalin (NGAL), and kidney injury molecule-1 (KIM-1) are utilized for detection of kidney injury using ELISA with 1:50 and 1:5000 dilutions of urine sample [192]. However, with extensive dilution, the sensitivity and achievable LOD may be jeopardized, and the presence of matrix components may also influence the assay responses.

5.2.2. Filtration

In this facile and cost-effective method, urine samples are filtered through a 0.2 or 0.45 μm filter to remove particulates, followed by dilution. For instance, urine was passed through a 0.2 μm membrane and diluted using phosphate buffer (pH 7.4) for detection of adenosine using aptamer-AuNP [193]. Dulal et al. developed a filtration method for detecting clenbuterol by utilizing SPR immunosensor. In this, filtration of urine samples was conducted using either a 0.2 μm syringe filter or monospin filter and 2-fold dilution with PBS for pH adjustment to 7.4 [194]. However, the

Table 6
Urine collection and storage using commercially available paper cards.

Types of paper	Modification	Sample volume	Advantages	Disadvantages	Applications/methodology
Chromato-graphy paper [184]	Impregnated with butylated hydroxytoluene ((BHT)	2 mL	Offer stability of target analyte	Require elution via centrifugation	Detection of metabolic disorders/ NMR Spectroscopy
Whatman™ 903 filter paper [185]	Impregnated with chemical reagent (proprietary)	10 mL	Wide range of samples	Takes time for drying up of urine (4 h)	Glycosaminoglycans/colorimetric
Whatman FTA Card [186]	Impregnated with chemical reagent (proprietary)	50 μL	Storage at room temperature Wide range of samples	Require centrifugation and heating	DNA Recovery/PCR
Whatman Body Fluid Collection Paper [187]	Impregnated with chemical reagent (proprietary)	2 mL	Wide range of samples	Takes time for drying	Hormones and metabolites/mass spectrometry

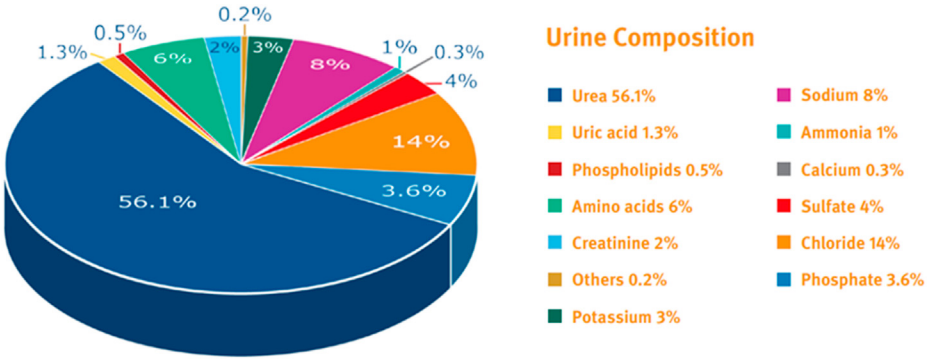

Fig. 9. General composition of urine excluding water (% values could vary) [189].

dissolved matrix components may still interfere with the assay responses.

5.2.3. Centrifugation

Centrifugation is another common method to remove the sample interferences in urinalysis, where the particulates are separated from the urine according to their size and density by adjusting the centrifugation speed. According to the European Confederation of Laboratory Medicine (ECLM) standards, urine samples should be pre-treated at a centrifugal speed of 400 g, in 4 °C, for 10 min, but other centrifugation speeds are also used in practice [195,196]. For example, cocaine in urine is detected by centrifuging the sample at 4000 g at RT for 15 min to remove the precipitates followed by dilution to 12.5% [94]. Jia-Cheng Wang et al. made an AuNP based biosensor utilizing aptamer for 8-OHdG detection, where the urine samples went through centrifugation at 3500 rpm for 10 min followed by dialysis using 100 Da molecular weight cut off dialysis bag [197]. Another paper depicts the electrochemical detection of toxins with low molar mass, where urine samples were treated with centrifugation (4500 g for 5 min) and dilution with assay buffer [198]. Centrifugation of urine for a longer time could cause artifacts such as the rupture of cells.

5.2.4. Ultrafiltration

In this method, molecular filters are used in combination with centrifugation and allow low molecular weight molecules to pass through the semipermeable membrane depending upon molecular weight cut-off, while high molecular weight molecules are retained on the membrane [199]. The process is performed at an extremely high centrifugal speed and varying temperatures according to the sample requirement. Baños et al. used a 5 kDa cut off filter for the detection of low molecular mass aldehydes along with centrifugation and 20 times dilution with DI water [200]. Another example is where a urine sample is incubated with proteinase K and $CaCl_2$ at 50 °C for 10 min followed by passing through a10 kDa spin filter at 12,300 rpm for 20 min at 20 °C for the electrochemical measurement of miRNA [201]. This is not a cost-effective method and requires multiple process steps for effective pre-treatment of urine.

5.2.5. Evaporation

Target analyte at low concentration in urine also may be preconcentrated by heating the sample. As an illustration, lipoarabinomannan (LAM), a tuberculosis urinary biomarker is concentrated by localized heating of a paper strip and thus evaporating the solvent to achieve a 20-fold increase in concentration [202]. This technique requires an external heating device, and most importantly, the target analyte should withstand denaturation at the heating temperature [178].

5.2.6. Protein precipitation (PPT)

This is a matrix scavenging method, where proteins in a urine sample are precipitated by adding acetonitrile or other organic solvents followed by either filtration or centrifugation of the precipitated proteins. For example, acetone and trichloroacetic acid (TCA), along with high-speed centrifugation are utilized for the separation of urinary proteins followed by SDS-PAGE (sodium dodecyl sulfate–polyacrylamide gel electrophoresis) detection [203]. This method is used to precipitate and isolate urinary proteins, but requires a high concentration of organic solvent, buffers with high salt strength, or very low pH. Although organic solvents provide high yields, some of them are toxic. In this method, proteins are only removed (not phospholipids), and it is not analyte specific. However, PPT is a facile and cost-effective method.

5.2.7. Dual mode extraction (DME)

This matrix scavenging technique is used for the detection of metabolites or drugs. In this method, urine is first hydrolyzed to deconjugate the analytes from glucuronide or sulfate using an enzyme, followed by acetonitrile treatment to remove matrix components such as urea, salts, and pigments and elution through two different sorbents [204]. Neeranuch et al. demonstrated the neonicotinoid (NEO) compounds and their metabolites detection using ISOLUTE® HYDRO DME + Dual mode extraction Biotage plates for extraction and then analyzed using liquid chromatography-tandem mass spectrometry (LC-MS) [205].

5.2.8. Liquid liquid extraction (LLE)

LLE is a targeted extraction technique where the target analyte is extracted based on its relative solubility in two separate immiscible liquids, such as water and an organic solvent. An LLE microchip for the detection of amphetamine-type stimulants in urine was developed by Hajime et al. [206]. A pretreated urine sample (alkalized) and an organic solvent (1- chlorobutane) were then pumped into a deep and shallow microchannel. The extracted sample was analyzed using gas chromatography. This method could only be utilized for the pretreatment of target analyte that are soluble in the organic solvent. LLE is a time-consuming process which usually requires a high amount of toxic solvents.

5.2.9. Solid phase extraction (SPE)

SPE is a powerful extraction technique where a solid phase or a dry packing material (usually made up of silica or polymer) is utilized to capture the target analyte based on the defined binding conditions. The silica or polymer is packed in a column and pretreated with the buffer followed by sample loading and target binding, washing of unbound molecules, and elution. For illustration, a microchip SPE is utilized for the extraction and enrichment

of ephedrine in urine. The extraction channels made up of fused silica capillaries containing UV-polymerized octadecyl silica beads provided 40-fold enhancement. The extracted ephedrine was quantified using capillary electrophoresis (CE)-laser induced fluorescence (LIF) technique [207]. Another example is SPE microchip coupled with mass spectrometry for preconcentrating and detecting imipramine. Here, an acrylate-based monolith was utilized as an extraction channel [208,209]. It is worthwhile mentioning that SPE is a multistep procedure that requires fine tuning of flow rate, pressure, and volume for desired target analyte extraction.

The above sample preparation techniques are discrete, where sample pre-treatment is performed separately followed by analysis or detection (summarized in Table 7), thus making some of them impractical for POC urinalysis in resource limited settings.

5.3. Integrated sample pre-treatment

Integrated sample pre-treatment implies that the sample collection/pre-treatment and signal transduction are integrated in a single device, as shown in Fig. 10 [172]. Discrete sample pre-treatment techniques require multiple steps, such as sample collection, incubation, and/or transfer steps for detection. These techniques may need multiple things such as pipettes, syringes as well as external equipment for sample pre-treatment, whereas the integrated sample pre-treatment method has all the procedures such as sample pre-treatment, extraction, amplification, and detection all done in the same chip as shown in Fig. 10. The integrated chip-based devices are typically fabricated using polymers, silica, glass, or paper. As an example, a paper-plastic hybrid device is developed by integration of finger actuating plastic pump (for sample collection) with a conventional reagent test strip for colorimetric sensing of urinary markers such as glucose, protein, and pH [166]. Table 8 provides a summary of integrated sample pre-treatment devices (μPADs) for urinalysis. Currently, integrated sample-pretreatment methodologies are developed for either other biofluids such as blood and saliva or for urine with high concentration target biomarkers. There is still a wide gap in integrated sample pretreatment for urinalysis, especially for low concentration analytes.

6. Smart data analytics

The integration of smartphone technology with POC diagnosis is an emerging area because of various factors such as economic benefits, advanced communication, and data processing protocols that offer on-site monitoring and rapid testing in resource limiting settings [221]. Smartphones can function as portable computer that is equipped with built-in sensors such as camera, GPS, and accelerometer, and thus they are capable of displaying and processing data and images rapidly [222]. Moreover, smartphones would allow the automation of analysis of the acquired data and also enable electronic storage of the data, which is essential for long-term monitoring and tracking for diagnosis purposes [223].

As for urinalysis, smartphones are usually incorporated with phone applications that replace expensive analyzers, for instance, spectrophotometers for analyzing colorimetric and fluorescence responses. Additionally, smartphone yields a digital signal readout, which is easily interpretable, thus facilitating POC diagnosis [224]. As for colorimetric assays, smartphones could provide a more accurate analysis of the colors of the reagent pads as compared to the visual interpretation by the naked eye [225]. In this section, we will be focusing on the use of smartphones for the detection of trace amount targets (nM to fM) in urine. As an illustration, as shown in Fig. 11, Taron et al. have designed a portable smartphone-based fluorometer to detect urinary Opisthorchis viverrine antigen (OvAg) by fluorescence ELISA [226]. The achieved LOD was 33 ng/mL.

Although Table 9 illustrates the potential of smartphone based POC urinalysis, there are still some drawbacks such as camera spectral sensitivity, camera settings, and the non-uniformity of the red, green, and blue color space that influence the assay response. It also can be difficult to standardize color space parameters for precise quantitative analysis of images captured using a smartphone, owing to the differences in smartphone models. The development of machine learning algorithms may circumvent the concerns mentioned above. The machine learning models can precisely identify colorimetric values [234]. As an example, as shown in Fig. 12, Rahman et al. designed an autonomous fluidic device consisting of a disposable plastic cup that is secured on an imaging box, and a smartphone is placed beneath the box for

Table 7
Discrete sample pre-treatment techniques and detection methodologies.

Technique	Analyte	Detection method
32 times dilution with 0.1 M PBS [190]	8-OHdG	Electrochemical detection using PT modified glassy carbon electrode
1000 times dilution with PBS [210]	8-OHdG	Electrochemical impedance spectroscopy
Phosphate buffer and tween (PBST) [211]	Morphine	Colorimetric
10 times dilution with PBST [27]	8-OHdG, AGEs and DNA	Colorimetric and fluorometric
Centrifugation followed by 20 times dilution [212]	8-OHdG	Electrochemical using DNA functionalized graphene coated glassy carbon electrode
Whatman filter paper, centrifugation, diluted 10-fold with PBS [213]	8-OHdG	Electrochemical and colorimetric
Centrifugation and dialysis [29]	8-OHdG	Colorimetric
Ultrafiltration [200]	Low molecular mass aldehydes	Capillary electrophoresis with fluorescence
Evaporation [202]	Tuberculosis-specific Glycolipid, lipoarbinomannan (LAM)	Immunodot blotting
Precipitation with high-speed centrifugation [203]	Proteins (cancer biomarkers)	Gel electrophoresis
Deproteinized sample is linked with an antibody and then trapped by molecular weight cut-off filtration [214]	OS biomarker (F2 isoprostanes)	Enzyme immunoassay
LLE [215]	Weakly ionizable analytes	Electrophoresis
Microchip LLE [206]	Amphetamine-type stimulants	Gas Chromatography
SPE [216]	Morphine and codeine	Electrochemical
Microchip SPE [208]	Imipramine	Mass spectrometry
MicroRNA extraction kit [217]	miRNA (prostate cancer)	Quantitative reverse transcription PCR (RT-qPCR)

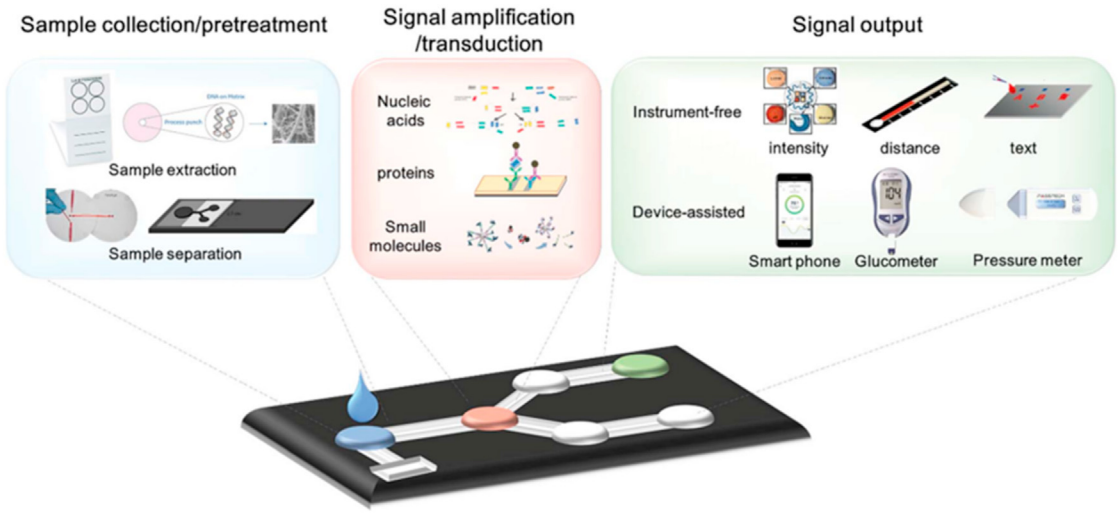

Fig. 10. Overview on an integrated paper-based μPAD [172]. All figures have been reprinted with permission.

Table 8
Summary of integrated sample pre-treatment devices.

Analyte	Sample Pre-treatment/material	Amplification	Detection
Albumin [218]	3D-μPAD	N.A.	Colorimetric (counting)
Cocaine [219]	Origami paper analytical device (invertase DNA conjugate)	Enzymatic reaction	Colorimetric (distance based)
Chlamydia Trachomatis (DNA) [220]	Paper based (filtration)	Thermophilic Helicase-Dependent Amplification (tHDA)	Colorimetric (intensity based)

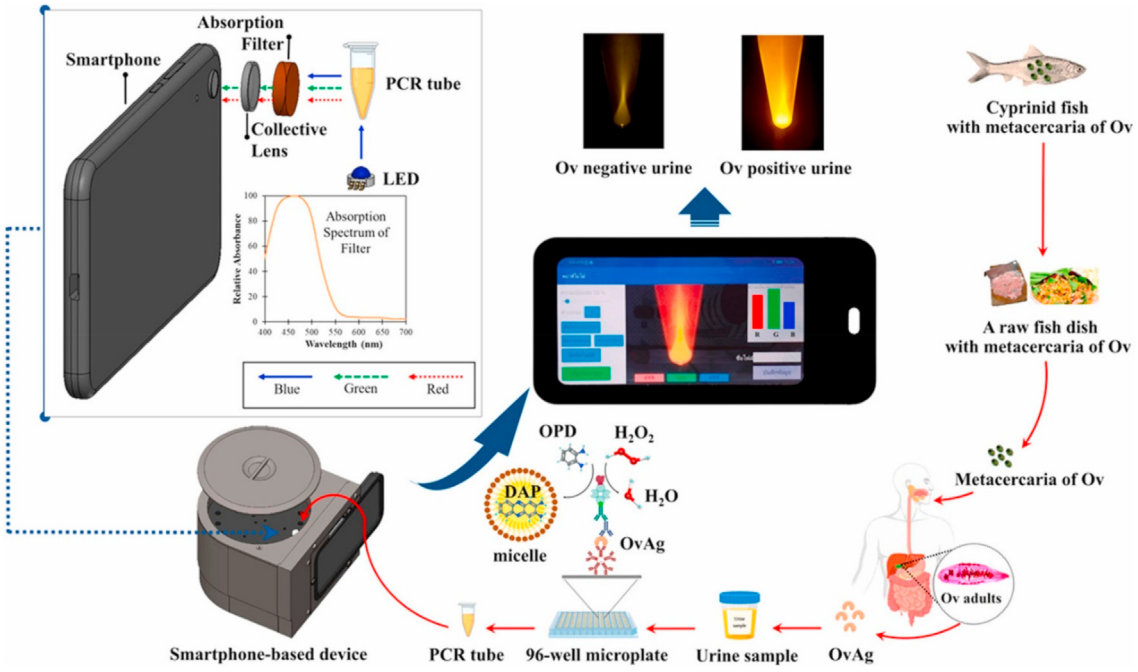

Fig. 11. Schematic Illustration of the portable fluorometer to detect OvAg in urine via fluorescence ELISA [226]. All figures have been reprinted with permission.

analysis. As for the urine sample analysis, it can be automated using a smartphone application with an image recognition algorithm [235].

7. Associated challenges in POC urinalysis of biomarkers in trace amounts

The necessity for POC detection of biomarkers in trace amounts stimulates the continuous development of novel assays. Although

Table 9
Smartphone-based POC detection of urinary markers in trace amounts.

Analyte	Method of detection	LOD	Health condition	Technique
HE4 [227]	Colorimetric	19.5 ng/mL (8.48 nM)	Ovarian cancer	Integration of a cell phone with microchip ELISA through a mobile application
Neutrophil gelatinase-associated lipocalin (NGAL)	SPR	0.19 ng/ml	Acute kidney injury	Smartphone's flashlight is employed as the light source which is coupled to a plastic optical fiber. The light reflected from the sensor chip is collected by the camera of the smartphone and the captured images are saved in the smartphone
Interleukin-18 (IL-18)		0.51 ng/ml		
Retinol-binding protein (RBP) [228]		0.7 ng/ml		
Bacteria [223]	Colorimetric	10^5 bacteria/mL	UTI	A platform consisting of a 3D printed chassis with a TLC plate opening, battery-powered LEDs for consistent plate illumination, and a port into which a standard smartphone might be placed. When the smartphone camera captures the plate, the device can upload it to a server automatically
Albumin [229]	Fluorescence	5–10 μg/mL	Chronic kidney disease	The fluorescent tubes are photographed with a cellphone camera and processed with Android application within 1 s
Pathogen-specific DNA [230]	Colorimetric	$<10^3$ copies/mL	General clinical diagnosis	A platform that consists of 2 parts: an integrate chip and a foldable detection box The smartphone serves as a heater, incubator, detector, and reader
Nitrofurazone [231]	Electro-chemiluminescence	0.09 nM	General diagnosis of cancer and other diseases	The smartphone is used to capture a luminescent image, which will be processed by a neural network and a software to analyze the color.
Opisthorchis viverrini antigen [226]	Fluorescence	33 ng/mL	Human opisthorch-iasis	A smartphone-based portable fluorometer, consisting of 3 parts: light source, auto sampler and detector.
Cocaine [232]	Colorimetric	2.5 ng/mL	Drugs	The smartphone is used to capture images of the test lines for a signal readout.
Phenyl-glyoxylic acid [233]	Fluorescence	10.8 ppb	Pollutant	The smartphone is combined with the fluorescence probe for on-site quantitative analysis via a color scanning application.

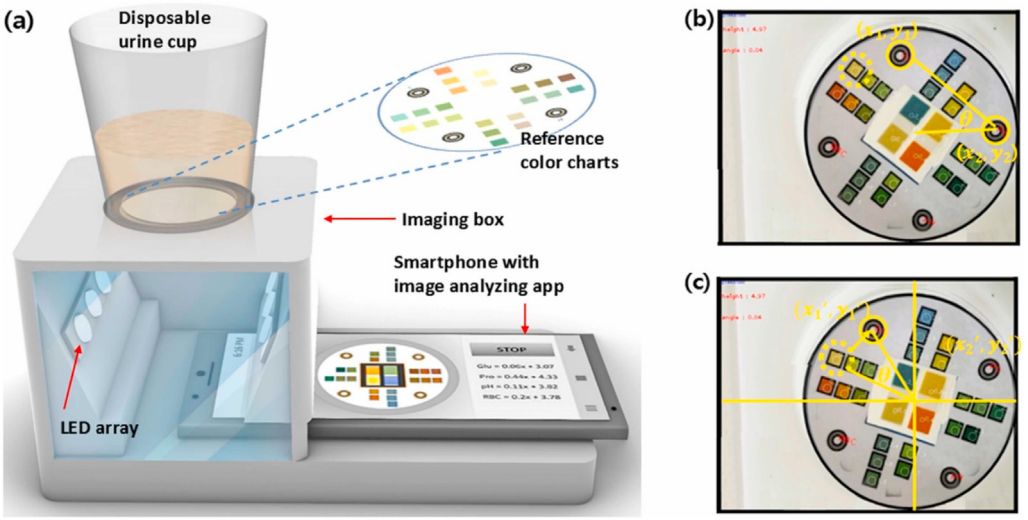

Fig. 12. (a) Illustration of HCG detection via smartphone and (b, c) 3D printed cassette for quantitative colorimetric measurement [235]. All figures have been reprinted with permission.

the reported POC assays are promising, there are numerous challenges that must be resolved for technology to be acceptable and reliable for clinical diagnosis. This section will discuss major challenges such as real-time and progressive monitoring of analytes, multiplexed detection of biomarkers, sensitivity, accuracy, specificity, sample pretreatment integration, and environmental and sample variations.

7.1. Sensitive, accurate and specific detection of biomarkers

Sensitive measurement is critical for the development of biosensors. The most popular POC LFA often suffers from sensitivity and cannot compete with laboratory-based assays [139]. Several advancements in recent years have improved LOD, including nanomaterial amalgamations, novel material synthesis, and robust device fabrications. For instance, fluorescent nano-spheres, quantum dots (QDs), and up-converting phosphors are utilized as probes in LFA for signal amplification [236]. The use of a microfluidic system in conjunction with nanomaterials and microarray technologies is extremely beneficial in reaching the objective of miniaturized, automated, and portable assays [237]. Accurate measurement of assay response is another critical factor. For instance, in colorimetric assays, colors may be seen differently due to varied ambient lighting, causing diagnostic inaccuracies that may impact the medical decisions [238]. In this regard, a new urine test strip is developed in which reference array color codes are arranged in a “donut-shape” adjacent to the reagent pad on the test strip [239]. The assay responses are interpreted by referring to the

reference color code, and there is no need for an additional reference sheet. Smartphone-based colorimetric detection system is a promising approach to convert colorimetric responses to a digital format to obtain precise diagnosis conclusions. This methodology is a potential research direction to develop a reliable POC assay for the detection of urinary biomarkers in trace amounts. Specificity is challenging for detecting target analytes of low molecular weight without the inference from structurally similar compounds and interferents. For instance, aptamers cannot bind specially to smaller target molecules with high affinity [240]. In this regard, research efforts are devoted to develop MIPs for specific binding. As an example, MIP-based quartz crystal microbalance sensor yielded a high selectivity for a number of regularly encountered cannabinoids in spiked urine with concentrations as low as sub pM range [241]. Thus, MIP based assays could enable reliable and sensitive POC urinalysis of biomarkers in trace amounts.

7.2. Real-time, frequent, and simultaneous monitoring of biomarkers

Real-time assessment of certain biomarkers is required to track disease fluctuations and for therapeutic control. For instance, infectious disease outbreaks (such as COVID-19, Ebola, and Zika) have highlighted concerns for accelerated diagnosis in POC settings [237]. In this context, the nanophotonic-based label-free and real-time urinary tuberculosis detection method is reported with a LOD of 27.14 pM [159]. For real-time detection, the SPR method is highly sensitive and label-free [145]. Thin-film synthesis, surface modification, robust detection, and other advances in SPR sensor technology have permitted the detection of urinary biomarkers in trace amounts. As an example, a competitive immunoassay was developed for urinary testosterone detection using a SPR methodology with a LOD of 0.1 ng/mL [242]. Besides infectious disease, some markers vary with circadian rhythm, for instance, steroid cortisol and DHEA [243]. An appropriate steroid test requires charting a patient's steroid levels frequently over 24 h in order to search for patterns that may suggest the occurrence of psychological disease. Simultaneous detection and multiplexing of biomarkers are also essential for POC urinalysis. As an illustration, the DHEA/Cortisol ratio detection is more reliable for assessing an individual's adrenocortex stress profile (ASP) rather than individual hormones [244].

7.3. Influence of sample status on correlation of assay responses to diseases

The fluctuations of urine pH, salt concentration, and color influence POC urinalysis. In addition, nonspecific binding is a major issue in urinary assays. The adsorption of urine components onto the container wall often causes loss of the target molecule. Nonspecific binding can be prevented by adding an appropriate anti-adsorptive agent, for instance, bovine serum albumin (BSA) and detergents, to the urine collection containers [245]. Another major concern is the inherent background fluorescence of urine. Several approaches have been reported to minimize the interference from fluorescently active urine components. In our previous work, we developed a urinary assay, where non-ionic surfactant and dilution were utilized to minimize the background fluorescence [27]. However, POC detection of urinary markers in trace amounts is still challenging in the presence of highly concentrated inorganic ions [246]. Therefore, as described in section 5, more research efforts are required on integrated sample preparation to develop a reliable POC assay for urinalysis. In addition to the urine components, the target concentration in urine also varies by age, race, body mass index, and sex. Thus, it would be mandatory to establish databases of normal physiological urine considering age and demographic conditions [247]. It may also be necessary to obtain individual baselines at normal physiological conditions by implementing personalized health monitoring.

7.4. Influence of environmental and logistic conditions on assay responses

The performance of POC urinary assays is affected by environmental conditions, especially for low concentration analytes. The natural receptor (antibody and enzyme) makes the assay temperature-sensitive and requires a cold chain for transportation and storage. The reported POC assays demonstrated high sensitivity in controlled circumstances. On-site reliability and accuracy remain a challenge for the detection of urinary markers in trace amounts. The POC platforms should include features to overcome the limitations of on-site validation. The capacity to process samples and analyze data on-site would minimize the demand for laboratory tests and significantly facilitate POC urinalysis [14].

8. Conclusion

POC detection of biomarkers in trace amounts is a potential alternative to centralized laboratory-based tests in emergency and low resource settings. Although detection of urinary markers in high concentrations regime is well-established, clinical validation for urinalysis of markers in trace amounts is still nascent. Besides conventional colorimetric LFAs, electrochemical and SPR-based assays have been reported for monitoring urinary markers in trace amounts. However, the reliability and accuracy of POC detection of urinary markers in trace amounts should be improved significantly for clinical validation. Novel assay formats for POC urinalysis should be rapid and involve environmentally stable biorecognition elements. On-site urine sample pre-treatment or integrated sample preparation within the device is a potential research direction for POC urinalysis. Frequent, real-time, and continuous monitoring of urinary markers is also crucial for the implementation of personalized healthcare monitoring, which enable early diagnosis of diseases and health conditions. Multiplexed detection could pave the way for a comprehensive POC urinalysis. The inclusion of smart data analytics offered POC urinary assays more flexibility, accuracy, and user-friendliness. Recent developments in smart data analytics, machine learning, and mobile apps could be utilized to realize POC detection of biomarkers in trace amounts. A stable regulatory code is also vital to ensure that POC performance, usability, and quality control. In conclusion, the current trends in POC detection of urinary biomarkers in trace amounts indicate the potential of advanced POC urinalysis for clinical and healthcare applications.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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