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Tutorial

Paper-Based Analytical Devices for Cancer Liquid Biopsy

Liquid biopsies have caused a significant revolution in cancer diagnosis, and the use of point of care (PoC) platforms has the potential to bring liquid biopsy-based cancer detection closer to patients. These platforms provide rapid and on-site analysis by reducing the time between sample collection and results output. The aim of this tutorial content is to provide readers an in-depth understanding regarding the choice of the ideal sensing platform suitable for specific cancer-related biomarkers.

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INTRODUCTION

Cancer is a significant global health issue, with the number of new cases at 1.9 million and a mortality of 609,820 each year.[1] Late detection of cancer that metastasizes lowers the efficiency of surgery and medicine, and it may cause fatalities.[2] In low- and middle-income countries (LMICs), access to early detection is critical in reducing the economic, cultural, and political impacts of treating cancer.[3] Standardized biomarkers play a vital role in the diagnostics of cancer for early control, because a traditional biopsy has some disadvantages, such as pain, side effects, and cost, and therapy can lack effective monitoring of cancer cell growth.[4] Liquid biopsy, a noninvasive technique that offers a global perspective, distinguishes itself from traditional biopsies by offering more complete genome analysis as technologies advance in sequencing.[5−7] In cancer research, researchers are working on blood-based tests toward solid tumors that involve biorecognition components for identifying and binding cancer-associated molecules, thus developing an effective diagnostic modality in oncology.[8] Figure 1 demonstrates that noninvasive health assessment is achievable via the use of a liquid biopsy technique through circulating tumor cells (CTCs) and cell-free tumor DNA (ctDNA), proteins, exosomes, and microRNA (miRNA).[9,10]

The liquid biopsy has the ability to transform cancer treatment because it uses target identification and understanding of resistance mechanisms. In addition, fluids can be analyzed in real-time point of care (PoC) systems, which could eventually lead to easier and more accessible diagnostics. Furthermore, liquid biopsy can also diagnose other disorders.[11,12] The paper-based method for cancer diagnosis through liquid biopsy is both cost-efficient and straightforward. This allows healthcare workers in remote areas to conduct tests with minimal equipment, making it especially advantageous in economically limited regions. This portable approach enables bedside testing of multiple cancer biomarkers on readily available paper substrates, ensuring quick, accurate diagnostics and optimal resource use. The tutorial highlights design adaptations in paper-based analytical devices (PADs) for cancer detection, focusing on optimizing development and examining colorimetric and electrochemical detection mechanisms within liquid biopsy contexts. This overview of paper-based sensing technology concludes by underscoring future research areas and novel developments aimed at boosting the efficacy and applicability of these methods, particularly in simplifying and reducing the cost of cancer diagnostics via liquid biopsy techniques. Figure 2 shows a paper-based diagnostic system that can be used to detect circulating biomarkers, such as miRNA, exosomes, CTCs, ctDNA, and cfDNA.

OVERVIEW OF FABRICATION METHODS

PADs are developed through the utilization of paper as a substrate, offering a straightforward and economical approach to analytical tool development. The selection of manufacturing methods for PADs is contingent on the intended objectives of the device. Factors such as the necessary infrastructure, cost considerations, production scale, and the resolution of patterned designs play pivotal roles in determining the most suitable patterning technique.[13]

The fabrication process typically begins with the selection of a suitable paper material, considering factors such as porosity, thickness, and wettability. The fabrication process primarily revolves around establishing hydrophobic zones within hydrophilic paper or shaping physical boundaries through paper cutting.[14] Whatman paper No. 1, with high absorbency, 180

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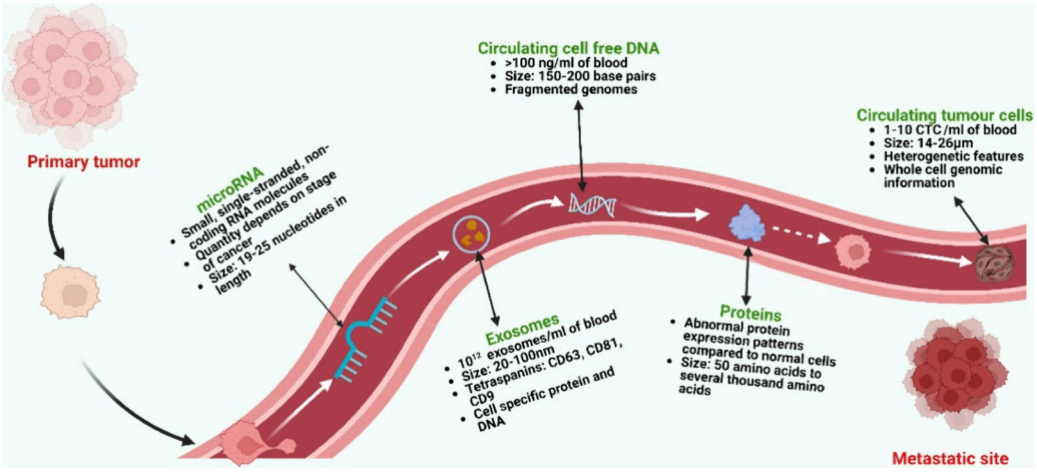

Figure 1. Biomarkers in circulation among cancer patients. Created with BioRender.com.

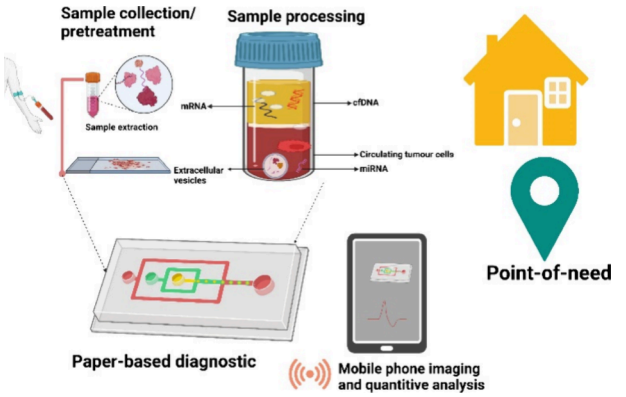

Figure 2. Paper-based diagnostic system for the detection of circulating biomarkers. Created with BioRender.com

μM thickness, and 11 μM pore size, is a popular choice. For variants like Whatman 4 (thickness: 205 μm; pore size: 25 μm), the thickness is smaller but the pore size is larger than those of Whatman 3 (thickness: 390 μm; pore size: 6 μm).[15] Filter paper, with uniform thickness and good wicking properties, is also common.[16] Nitrocellulose membranes, known for their smooth surface and pore size, are widely used in lateral flow assays.[17] Bioactive paper, nylon membranes, conventional printing paper, and bacterial cellulose nanopaper are also employed, each offering unique features like biomolecule modification, flexibility, and transparency.[18] The choice is determined by the nature of the intended use and other required attributes, including absorption, wetting characteristics, and chemical nature of the surfaces.

The design and patterning of microfluidic devices involve the use of computer-aided design (CAD) software to create a virtual blueprint. CAD allows for the meticulous planning of channels, reaction zones, and other components.[19] Patterning techniques, such as wax printing, inkjet printing or screen-printing, and photolithography, are then employed to transfer the design onto a paper substrate.[20] The precision achieved in both design and patterning is essential for accurate functionality of the final device, showcasing the synergy between digital planning and physical fabrication in microfluidics. The next important step is reagent deposition, a crucial step in assays that determines the accuracy of results. Techniques like inkjet printing, spray coating, and manual spotting offer versatility.[21] The paper-based device is created by laminating and assembling paper layers, forming a sealed reservoir for reagents. Methods like heat sealing, adhesive bonding, or pressure sealing are employed.[22] This assembly ensures controlled fluid flow in designated channels, preventing cross-contamination. After assembly, the device is ready for sample introduction and analysis. Samples such as blood or urine are introduced through a sample inlet, and capillary action directs the fluid across channels toward reagents, enabling chemical or biological reactions. Results can be observed visually or measured externally with devices such as smartphones or portable readers.

Technological Advancements in Liquid Biopsy Diagnostics. PAD provides a noninvasive diagnostic technique, offering major improvements in biomarker detection with enhanced specificity, sensitivity, affordability, portability, and convenience. The development of paper with color has been a significant milestone. Capillary motion and other benefits of the paper substrate make these assays simple to perform on any surface. Colorimetric indicators provide a visualization of the sample without the need for expensive equipment. Diagnostic possibilities and PoC testing are feasible in limited resource settings. Biomarkers can easily produce color changes, enabling earlier disease identification and self-monitoring for cancers.[23]

Liquid biopsy diagnostics now use parallel electrochemical assays, which have increased sensitivity and specificity for detecting biomarkers in low concentrations. Portable electrochemical units enable point-of-care testing and disease progress monitoring over time. Nanomaterials enable signal amplification, pushing detection limits to unprecedented levels. This integration has facilitated the identification of mutations and low-abundance biomarkers, crucial for early cancer detection and monitoring treatment responses.[24]

The development of paper-based colorimetric and electrochemical tests for liquid biopsy has experienced ongoing advancements, progressing from basic color alteration reactions to more complex multiplexed examination and signal enhancement approaches. These tests have emerged as effective resources for the diagnosis and tracking of cancers, providing an opportunity for early identification and personalized treatment strategies. The continuous progress in

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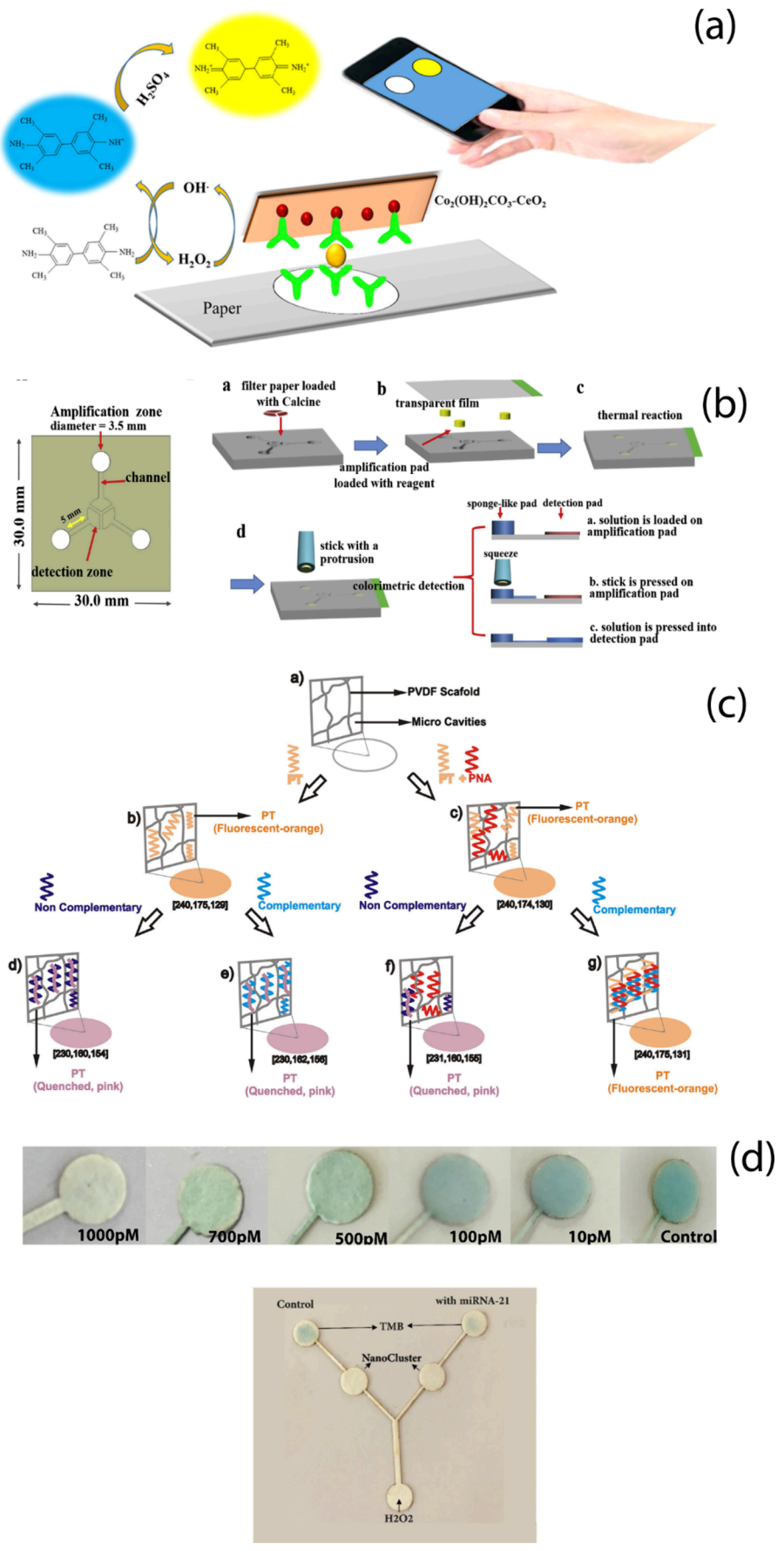

Figure 3. (a) $Co_2(OH)_2CO_3$−CeO_2 nanocomposite for smartphone-based detection of tumor marker using paper-based microfluidic immunodevice; (b) PCA 3 test apparatus with 3D printed RT-LAMP chip; (c) paper-based platform for mir21 detection;[38] (d) paper based

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Figure 3. continued

colorimetric detection of miRNA-21. Reprinted with permission from ref 36, Copyright 2018 Elsevier. Reprinted with permission from ref 37, Copyright 2020 Elsevier. Reprinted with permission from ref 39, Copyright 2020 Elsevier.

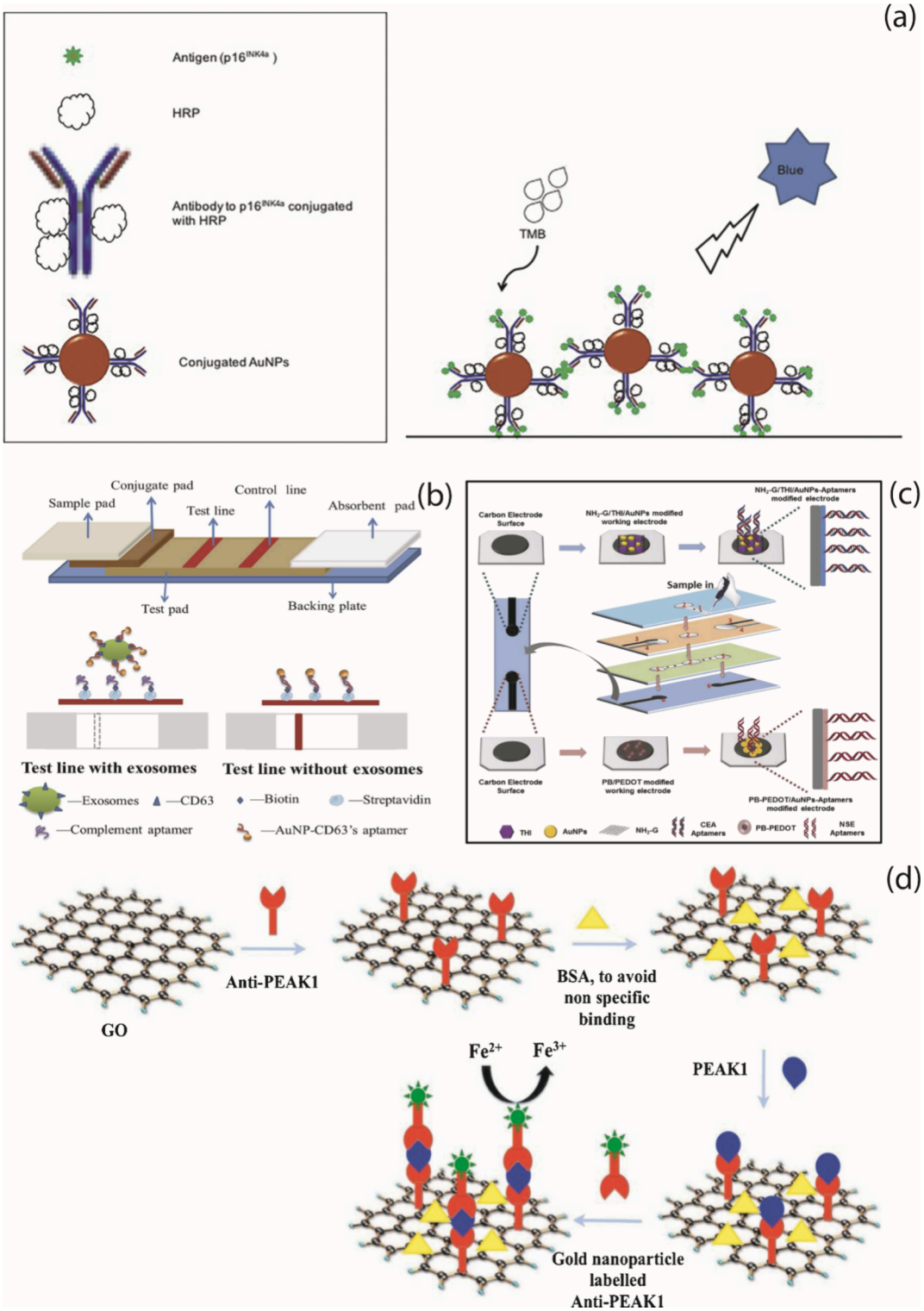

Figure 4. (a) Components of an immunosensor and immune reaction; (b) configuration and principle of LFAA test strips for exosome detection; (c) creating and enhancing a multiparameter electrochemical paper-based aptasensor; (d) operating principle of the PEAK1 immunosensor. Reprinted with permission from ref 47, Copyright 2018 Elsevier. Reprinted with permission from ref 48, Copyright 2020 Elsevier. Reprinted with permission from ref 49, Copyright 2019 Elsevier. Reprinted with permission from ref 50, Copyright 2020 Elsevier.

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these methods presents the potential for incorporation into standard clinical procedures to enhance patient results.

Colorimetric Biosensors. Colorimetric assays employ color-changing reagents to quantify the magnitude of the target molecules. These have been crucial because they aid fast detection of cancers and fit well with the latest technologies. In addition, colorimetric assays offer affordability, user-friendliness, and applicability in LMICs.[25] Qualitative measures can be turned into quantifiable data using smart devices, making them essential for improved disease detection, especially for cancer.[26] These assays can be customized to target selected cancer biomarkers for personalized diagnostics. Paper-based assays are economical, durable, and convenient to carry around even in harsh and remote situations. This approach can reveal a number of markers that improve cancer diagnostic access in impoverished areas. With these advanced technologies, cancer diagnosis is following a more personal and easier path.

Proteins as Biological Recognition Elements in Colorimetry Paper-Based Detection. Protein detection in bodily fluids is important for the diagnosis and management of diseases, including cancer. Some proteins from tumor cells can be detected in the blood. Besides the diagnosis of the diseases, this procedure can be used to forecast the disease stages.[27] Proteins serve as receptor agents binding certain analytes in paper-based substances. They bind with proteins, which are usually attached to the surfaces of substances such as filter paper or membranes.[28,29] Once the analyte, the substance of interest, binds to the immobilized protein, a noticeable alteration in color occurs. This transformation is a result of various processes, including catalysis or the dispersion of nanoparticles.[30,31] Proteins' advantages include specificity, versatility, and easy immobilization, making them valuable for cancer diagnostics.[32] It is important to use proteins in the design of cheap and portable paper-based detection devices for use in resource-limited parts of the world. The biomarker PSA in one specific application, the colorimetric paper detection technique, improves cancer detection methods of prostate studies.[33,34] Its primary purpose revolves around detecting prostate cancer and monitoring its progression.[35] Alizadeh et al. developed a simple paper sensor to detect CEA using a $Co_2(OH)_2CO_3$−CeO_2 nanocomposite with peroxidase-like abilities and functionalized antibodies in an ionic liquid/chitosan solution. The detection method involved changing the color by oxidizing tetramethylbenzidine with H_2O_2 using the nanocomposite. Color-based sensing took place using paper while a smart phone captured and analyzed colors with linearity of 0.002−75.0 ng/mL $^{-1}$ and a LOD at 0.51 pg/mL (Figure 3a).[36] PCA3, an important tumor biomarker, was detected by Wang et al. using a 3D printed RT-LAMP setup with colorimetric changes documented on dry paper pieces. The device has a LOD of 0.34 fg/μL and operates using a 12 V battery, making it suitable for PoCT and easily viewable with the naked eye or a smartphone camera (Figure 3b).[37]

Nucleic Acids as Biological Recognition Elements in Colorimetry Paper-Based Detection. Using DNA and RNA in paper-based colorimetry for cancer detection is a promising and innovative approach.[37,40,41] This approach utilizes the attraction between nucleic acids and cancer biomarkers, with probes immobilized on paper substrates containing color markers for easy detection without requiring complex equipment.[42,43] An innovative biosensing platform developed by Yildiz et al. can accurately determine a single DNA strand without complex laboratory instruments. It detects miR-21 of lung cancer using special paper with two substances emitting specific light signals upon binding with different sets of miRNAs. This breakthrough could reshape medical diagnostic testing and provide a cheaper approach for identifying miRNA across different applications to improve health care (Figure 3c).[38] Fakhri et al. carried out research using a novel paper-based biosensor in order to identify the miRNA-21 expression. A nanosensor showed that it was possible to effectively monitor the peroxidase-like activity of miRNA21 leading to accurate estimation of its concentration. There was great linearity found in an assay with LOD of 4.1 pM at 10−1000 pM (Figure 3d).[39]

Cells and Exosomes as Biological Recognition Elements in Colorimetry Paper-Based Detection. Using cells and exosomes for colorimetric cancer detection on paper has the potential to revolutionize disease diagnosis and monitoring by facilitating easy identification of specific molecules through observable color alterations.[44] On the other hand, exosomes refer to tiny vesicles that cells release.[45] Exosomes containing distinct biomarkers enable precise identification of diseases and targets through colorimetry.[46] Cells and exosomes are used in a colorimetric-based detection system for accurately locating the target. A paper-based immunosensor using multifunctional AuNPs to enhance the signal by binding antibodies to the p16-INK4a cancer marker on AuNPs was developed by Yokchom et al. The peroxidase enzyme's oxidation of the TMB substrate resulted in a positive response within 30 min, demonstrating 85.2% accuracy and aiming to create an efficient paper-based sensor for visual interpretation without requiring specialized imaging systems (Figure 4a).[47]

Yu et al. Have recently developed a strip-based technology for detecting A549 exosomes from NSCLC using a lateral flow assay. They employed a CD63 aptamer-based exosome assay to visualize and isolate exosomes for a more affordable approach (Figure 4b).[48]

■ ELECTROCHEMICAL BIOSENSORS

Electrochemical biosensors offer high sensitivity, quick response, and the ability to detect multiple cancer biomarkers concurrently.[33,51,52] Functionalizing electrode surfaces with specific bioreceptors enables the detection of cancer biomarkers by quantifying the resulting alterations in electrical characteristics.[53−55] Various electrochemical techniques can be used to detect and measure cancer biomarkers.[56−59] The use of nanotechnology has improved the sensitivity and selectivity of electrochemical biosensors, allowing for accurate detection in complex biological samples such as blood or urine.[60,61] These nanostructures provide extensive surface area for biomarker capture and facilitate electron transfer, ultimately improving detection performance.[62−64]

These biosensors are portable and sensitive and are primarily used in cancer detection and personalized medicine. They use paper-based substrates with bioreceptors to capture cancer biomarkers in liquid biopsies for electrochemical detection.[65−68] Cost-effective, portable, paper-based electrochemical biosensors are convenient for applications in LMICs. These sensors employ electrodes developed from conductive inks, carbon components, or metal nanoparticles to detect and quantify cancer biomarkers.[69−72] Smartphone integration improves the functionality of paper-based electrochemical sensors for real-world use.[73]

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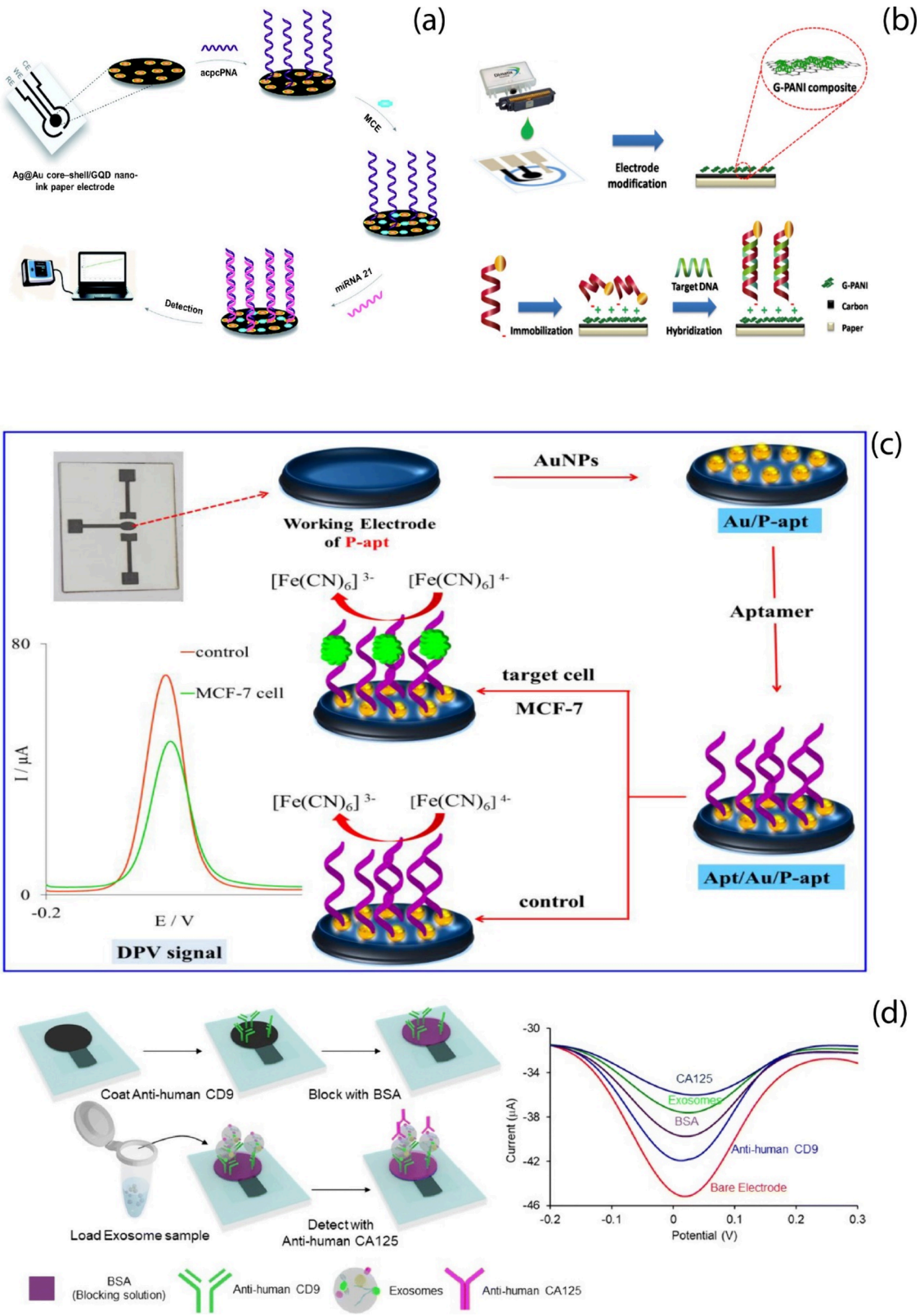

Figure 5. (a) PNA-based electrochemical biosensor on photographic paper to detect miRNA-21; (b) modifying and immobilizing electrodes in paper-based DNA biosensors; (c) development of Apt/Au/P-apt; (d) illustrating the cancer cell-derived exosome detection process. Reprinted with permission ref 80, Copyright 2021, RSC. Reprinted with permission from ref 81, Copyright 2017, Elsevier. Reprinted with permission from ref 82, Copyright 2022 Elsevier. Reprinted with permission from ref 83, Copyright 2022 RSC.

Proteins as Biological Recognition Elements in Electrochemical Paper-Based Detection. Proteins, with their varied structures and functions, have a significant impact on improving electrochemical paper-based detection devices for cancer biomarkers.[74] Proteins improve the performance of electrochemical paper-based detection devices for cancer

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biomarkers, resulting in systems that are more sensitive and adaptable.[26] Incorporating proteins into paper-based devices results in a bioactive interface that interacts with particular target proteins, initiating electrochemical reactions on the surface of the paper and generating identifiable signals.[68] This innovative method integrates paper-based systems with electrochemical detection to streamline device structure, enabling cost-effective and widespread accessibility for measuring cancer-related proteins.[75] Wang et al. have developed paper-based sensors to identify cancer biomarkers CEA and NSE in samples from patients. This method provides a label-free approach for analyzing properties, successfully detecting CEA at an incredibly low concentration of 2 pg/mL and NSE at 10 pg/mL. The results obtained from serum tests were comparable to those achieved using larger-scale equipment, which is particularly beneficial in areas with limited resources, as shown in Figure 4c.[49] Prasad et al. developed a practical immunosensor using paper-based electrodes and graphene oxide for early pancreatic cancer detection in resource-limited areas, achieving precise detection of PEAK1 substances at low levels (10 pg mL^{-1}). This user-friendly design supports early intervention and optimized treatments in resource-constrained settings (Figure 4d).[50]

Nucleic Acids as Biological Recognition Elements in Electrochemical Paper-Based Detection. Recently, nucleic acids such as DNA and RNA have emerged as promising biomolecular recognition elements within the realm of cancer detection using electrochemical paper-based platforms. The distinct attributes of nucleic acids, encompassing their capacity for discerning binding to complementary sequences, have paved the way for precise and sensitive identification of cancer biomarkers.[76] Nucleic acids were attached to paper to form a versatile electrochemical detection system. This can aid in personalized medicine by analyzing biomarkers. MiRNAs have roles in cancer therapy, gene regulation, and diagnostics.[77−79] Farshchi et al. developed a novel paper-based electrochemical sensor for the sensitive detection of miRNA-21 by utilizing Ag@Au core−shell nanoparticles on graphene quantum dot nanoink, created using pen-on-paper technology. This cost-effective biosensor exhibited high stability and sensitivity, with a linear detection range of 5 pM to 5 μM. The bioassay's potential for early cancer diagnosis was supported by its ability to inhibit miRNA-21 expression, activating caspase enzymes and inducing apoptosis in tumor cells, as shown in Figure 5a.[80]

Teengam et al. developed a paper-based electrochemical biosensor for detecting HPV, utilizing an anthraquinone-labeled pyrrolidinyl peptide nucleic acid probe and a graphene-polyaniline electrode. This biosensor, confirmed via EIS, effectively identifies synthetic HPV type 16 DNA, with LOD of 2.3 nM and linearity of 10−200 nM. Its efficacy in screening HPV-DNA type 16, crucial in early cervical cancer detection, was demonstrated with DNA from the SiHa cell line, as illustrated in Figure 5b.[81]

Cells and Exosomes as Biological Recognition Elements in Electrochemical Paper-Based Detection. Using cells and exosomes in paper-based biosensors offers a cost-effective and readily available approach to detect and monitor cancer. Khoshroo reported a straightforward technique for detecting circulating tumor cells in MCF-7 cells by using a paper-based sensing platform. A patterned sticker was used to establish a three-electrode system, enabling label-free detection of the target cells. The working electrode of a P-apt was enhanced by attaching aptamers. These aptamers were designed to specifically bind to mucin 1 proteins found on the surfaces of MCF-7 cells. P-apt's performance was evaluated using CV, EIS, and DPV. The sensor exhibited a linearity of 20 to 1×10^6 cells/mL and LOD of 7 cells/mL. Significantly, P-apt was capable of detecting MCF-7 cells in serum samples. The sensor shown in Figure 5c is an efficient and long-lasting device that allows the detection of circulating tumor cells. This improvement increases its potential for application in monitoring the spread of breast cancer and evaluating the effectiveness of treatments.[82] Another work by Kasetsirikul et al. developed a cost-effective electrochemical paper-based tool for measuring the overall amount of exosomes from both bulk and cancer cell sources in cell culture media. They utilized a sandwich immune assay approach involving common (CD9) antibodies as well as cancer-specific CA125 antibodies to capture and identify exosomes on electrodes. This innovative device holds promise for accurately quantifying exosomes in a more accessible manner. LOD was achieved to be 9.3×10^7 exosomes/mL for total bulk and 7.1×10^8 exosomes/mL for cancer cell-derived exosomes, with a <10% relative standard deviation (n = 3) (Figure 5d).[83]

OUTLOOK AND FUTURE PROSPECTIVE

Biosensor technology is poised to revolutionize cancer diagnosis and detection of biomarkers rapidly and accurately. Specifically, POC diagnosis, particularly liquid biopsy using colorimetric and electrochemical approaches, has the potential to identify cancer. This technology can be used to make early diagnoses, monitor patients under treatment, and enable personalized medicine for cancer.

Specific biomarkers are used for colorimetric biosensors that offer simplicity and affordability by presenting visible color changes. The paper substrate application of this technique yields qualitative or semiquantitative results, demonstrating its potential for use in cancer biomarkers. On the other hand, their sensitivity may be limited. However, the use of electrochemical techniques in liquid biopsy, such as impedance and amperometry, results in more sensitive and accurate detection of biomarkers. Nonetheless, such approaches may need sophisticated tools that are only useful with specific skills.

PADs used for liquid biopsy in the future require addressing fundamental hurdles. They include improvements in the sensitivity and specificity of PADs for the accurate detection of cancer biomarkers in liquid biopsy samples with minimal false-positive and false-negative results. This can be attained by improving the limit of detection via design optimization, signal amplification, and a multibiomarker panel. Furthermore, there is an urgent requirement to improve multiplexing capabilities to enable detection of several cancer biomarkers in order to collect vital data for effective disease classification and personalized treatments. New multiplexing approaches that use a different detection technique or an integrated microfluidic channel are being researched continually. Effective sample preparation continues to be an important issue, particularly in view of complex matrices and low target biomarker concentrations in liquid biopsies. Adding filtration, concentration, and purification sample preparation techniques into paper-based devices is seen as a way to enhance sensitivity and reliability. However, clinical validation becomes an important requirement in making PADs clinically useful. This entails thorough studies involving substantial cases to pit the PADs against conventional approaches for measuring gold standards. It is therefore crucial to determine the clinical

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efficacy, effectiveness, and usability of PADs under operating circumstances in order to enable their smooth integration into a health system.

Going forward, there is always room for improvement. Integrating PADs into smartphones would lead to greater utility. It would aid in remote monitoring, real-time information provision, and inclusion in electronic health records, which play an important role in personalized cancer care and remote patient management. Nevertheless, challenges remain, such as improving performance, tackling multiplexing complexities, reviewing sample preparation, and performing clinical validation. The development of low-cost, rapid, and widely available analytical tools could revolutionize cancer detection, monitoring, and treatment if successful in overcoming these challenges.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.analchem.3c04478.

Table S1: comprehensive overview of various technologies used in cancer detection, including details on biomarkers, types of samples, platforms, detection methods, fabrication techniques, recognition elements, linear ranges, and LOD; these technologies encompass both colorimetric and electrochemical approaches, each offering distinct characteristics and advantages (PDF)

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Notes

The authors declare no competing financial interest.

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Sima Singh is a Postdoctoral Fellow under Fondazione Umberto Veronesi. Currently, she is developing nanomodified electrochemical systems to be applied in prostate cancer liquid biopsy. She has been awarded an MSCA fellowship to be started in 2024.

Ada Raucci is a PhD student in the Department of Pharmacy, University of Naples Federico II. Currently, she is working on the development of portable electrochemical devices for user-friendly application in clinical and environmental fields.

Wanda Cimmino is a PhD student in the Department of Pharmacy, University of Naples Federico II. Currently, she is working on merging electrochemical sensors and biosensors with multivariate analysis tools, with a main application towards cancer detection.

Stefano Cinti is an Associate Professor in the Department of Pharmacy, University of Naples Federico II. He leads the uninanobiosensors Lab (uninanobiosensors.com) at University of Naples "Federico II", and his research interests include the development of electrochemical sensors, portable diagnostics, paper-based devices, and nanomaterials. He has published more than 80 papers in peer-reviewed journals, with an H-index of 33 and >4000 citations.

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