

The synergy of nucleic acid amplification and miniaturized systems in enhancing liquid biopsy applications

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“This commentary explores the synergistic combination of nucleic acid amplification techniques and miniaturized systems, advancing the field of liquid biopsy.”

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Chips-based bioanalytical devices for liquid biopsy applications

Lab on a chip and miniaturizing systems have been a part of the analytical chemistry and bioanalysis community since the 90s [1,2]. Recently, some reports [3,4] show significant contributions that recent advances in the field of miniaturized bioanalysis for biopsy liquid have furthered the area of clinical diagnosis. In our view, it is essential to connect the developments of these fields to their roots for a deeper understanding and further improvement. To do so is the purpose of this commentary. This commentary explores the synergistic combination of nucleic acid amplification techniques and miniaturized systems, advancing the field of liquid biopsy. We aim to clearly delineate the recent developments and their applications in clinical diagnostics. Our discussion traces the evolution from traditional methods to innovative, miniaturized approaches, highlighting the transformative impact on biomarker detection. The force that drives the miniaturizing and bioanalysis systems for liquid biopsy to the recent advances can originate primarily from three factors: the micro-, nano-technology advances, the material advances and the biotechnology advances. In the micro-, nano-technological aspect, the intensive development of micro-, nano-fabrication and fast prototyping such as 3D printing, micromachining, i.e., laser cutting, milling and injection molding has offered the ground tools for lab on a chip and microfluidics to grow in an unexpected and exciting direction, some of such examples have also been shown in the review of Tabata *et al.* [4]. In the material aspects, the appearance of new materials, for instance, graphene, carbon nanotubes, new types of polymers and copolymers such as cyclic olefin copolymer have given rise to sophisticated applications of microfluidics as well as the commercialized possibility of portable, cheap components for the outcome point-of-care devices. As for the biotechnology part, focusing on liquid biopsy, the nucleic acid amplification plays a vital role due to the blooming of the two critical technologies including PCR and isothermal amplification. In addition, in order to realize lab-on-a-chip technology for commercialized systems such as point-of-care devices, the roles of open-source hardware and software have become increasingly important for the electrical readout and controlling parts in the outcome devices [5–7]. The other authors [4] focus mainly on the electrochemical sensors together with isothermal amplification methods and applications of liquid biopsy on the lab scale. In this discussion, we shall broaden this further to the commercialized level, i.e., toward bioanalysis point-of-care devices. Discussion on other detection methods besides electrochemical sensors is also briefly treated. In addition, we provide some additional examples of possible applications of miniaturizing systems for nucleic acid amplification methods toward point-of-care devices for liquid biopsy and clinical diagnosis.

Micro-nano fabrication

In the realm of micro- and nano-technology, the intensive development of microfabrication and nanofabrication methodologies, alongside rapid prototyping technologies, has laid a foundational framework for the burgeoning field of lab-on-a-chip and microfluidic systems. These technological advancements have not only accelerated the pace of innovation but have also expanded the potential applications of these miniaturized devices in ways that were previously unanticipated.

Microfabrication & nanofabrication techniques

Microfabrication techniques, including photolithography, soft lithography and etching, have been the foundation of the microelectronics industry and have found new life in developing microfluidic devices [8,9]. Nanofabrication extends these capabilities to an even smaller scale, allowing for manipulating materials at the molecular or atomic level [10]. This has opened doors to unprecedented precision in creating fluidic channels and structures that are essential for manipulating and analysing biological and chemical samples in lab-on-a-chip devices.

Advances in photolithography, for instance, have enabled the creation of complex, layered structures with features on the order of nanometers. The ability to pattern materials with such precision is critical for developing microfluidic devices that can perform many functions on a single chip, such as sample preparation, mixing, reaction, separation and detection.

Rapid prototyping technologies

Rapid prototyping, including 3D printing and micromachining, has dramatically reduced the time and cost associated with the development of lab-on-a-chip devices. 3D printing, with its capacity for additive manufacturing, allows for the creation of 3D microfluidic devices with complex geometries that would be challenging or impossible to fabricate using traditional methods. Materials such as acrylonitrile butadiene styrene (ABS), polylactic acid (PLA) and photopolymers are commonly used in 3D printing and offer a range of mechanical and chemical properties that can be tailored to specific applications [11,12].

Micromachining techniques, including laser cutting and milling, provide another avenue for rapidly creating microfluidic devices. These subtractive manufacturing processes can be used to create intricate designs on various substrates such as glass, silicon and polymers. Laser cutting, in particular, is ideal for the rapid prototyping of microfluidic devices due to its precision and the ability to cut complex shapes without needing physical contact with the material.

Injection molding

Injection molding has been adapted from the plastics industry and is now utilized for the high-throughput production of microfluidic devices. This technique is particularly suited for the mass production of devices as it allows for the replication of high-precision microfluidic components at a low cost per unit. A wide range of thermoplastic polymers can be used in injection molding, providing a variety of mechanical and optical properties that can be selected based on the requirements of the microfluidic application [13,14].

The combination of these advanced fabrication techniques has catalyzed a paradigm shift in the field of lab-on-a-chip and microfluidics. The ability to rapidly prototype and mass-produce microfluidic devices has not only made these technologies more accessible but has also spurred innovation across a spectrum of scientific disciplines. From personalized medicine to environmental monitoring, the applications of these devices continue to grow, each benefiting from the tailored capabilities that these advanced fabrication techniques provide.

Furthermore, the convergence of micro- and nano-fabrication with rapid prototyping technologies is fostering a new era of interdisciplinary research. Engineers, chemists, biologists and medical professionals are collaborating to push the boundaries of what is possible with lab-on-a-chip devices. As these technologies continue to mature, they are expected to play an increasingly central role in advancing scientific research, diagnostics and therapeutics.

Advanced materials

The advancement of materials science has played a pivotal role in the evolution of lab-on-a-chip and microfluidic technologies. Progress in this domain has led to the creation of new materials with tailored properties and the innovative application of these materials in the micro- and nano-fabrication of devices.

Innovative materials in microfluidics

The selection of materials for microfluidic devices is driven by the need for specific chemical and physical properties such as optical transparency, chemical inertness, biocompatibility, mechanical flexibility and thermal stability. For example, glass and silicon have traditionally been used due to their chemical resistance and the ease with which they can be integrated with semiconductor devices. However, the fragility of glass and the high cost of silicon micromachining have led researchers to explore polymers as viable alternatives [15].

Polymers in microfluidic fabrication

With their diverse and tunable properties, polymers have emerged as materials of choice for many microfluidic applications. Polydimethylsiloxane (PDMS) is extensively used because of its transparency, gas permeability, and ease of fabrication. However, its hydrophobic nature can adsorb hydrophobic analytes and affect measurement accuracy. Researchers have developed surface modification techniques to overcome this or turned to other polymers such as PMMA, PC and COC. These materials offer distinct advantages in terms of cost, ease of mass production through injection molding, and lower analyte adsorption, making them suitable for a broad range of applications, including point-of-care diagnostics and high-throughput screening [16].

Emerging materials

Recent advancements have seen the emergence of novel materials such as hydrogels [11], which can respond to environmental stimuli such as pH, temperature, and ionic strength, making them suitable for creating dynamic microfluidic systems. Another area of significant interest is the development of conductive polymers, which can be used to integrate electrical functionalities directly into microfluidic devices, paving the way for new types of biosensors.

Biodegradable & sustainable materials

Sustainability in material science is also gaining attention, leading to research in biodegradable and eco-friendly materials (e.g., paper-based materials) for microfluidic applications [17,18]. This addresses environmental concerns and opens up the field to new applications in agriculture and environmental monitoring where disposable devices are particularly advantageous.

Future directions in material science for microfluidics

The future of materials for microfluidics is likely to see continued diversification, with the development of composites and hybrid materials that offer multifunctional capabilities. Additionally, the field may see increased use of additive manufacturing techniques, such as 3D printing with advanced materials, allowing for the rapid prototyping and fabrication of devices with complex architectures that were previously impossible [19].

In conclusion, the advancements in materials science have provided many opportunities for innovation in the design and functionality of lab-on-a-chip and microfluidic devices. As the material properties become increasingly tailored to specific applications, one can expect the emergence of new device architectures that will further expand the horizons of what is possible in diagnostics, research, and beyond.

Advanced biotechnologies

In the domain of liquid biopsy, biotechnological advancements have been largely propelled by innovations in nucleic acid amplification, a process central to the detection and analysis of genetic material from biological samples. Two pivotal technologies, PCR and isothermal amplification, have dominated this landscape, each contributing significantly to the field's growth and application in clinical diagnostics.

Recently, Tabata [4] described the importance of liquid biopsy in healthcare professionals and introduced the five crucial candidates as detection targets, namely, blood circulating tumor cells, cell-free DNA (cfDNA), circulating tumor DNA (ctDNA), exosomes and micro-RNA (miRNA). The authors focus the attention on cancer detection using circulating miRNA as a biomarker and introduce the two current bold techniques: real-time polymerase chain reaction (real-time PCR) and next-generation sequencing (NGS). The authors express the limitation of these two methods connected to the use of fluorescence-based detection, hence fluorescence labelling and external excitation and optical filters. Then, the authors introduce the key point of the paper, namely, the electrochemical sensor and isothermal nucleic acid amplification techniques and devices which aim to overcome the limitation of fluorescence-based and thermal cyclers as in PCR and NGS techniques. We would like to broaden those detection

techniques, which can also help to overcome the limitation of fluorescent detections. The bioluminescent use [20–22], for example, does not require an external excitation and fluorescent labelling. Bioluminescence is a natural phenomenon in which living organisms produce and emit light through a chemical reaction. This process involves a light-emitting molecule called luciferin and an enzyme called luciferase. The luciferase catalyzes the oxidation of luciferin, resulting in the emission of light. This biochemical emission of light is found in various organisms, including fireflies, certain species of fish, jellyfish and microorganisms. In a laboratory setting, bioluminescence has been harnessed for various applications, particularly in bioanalysis and diagnostics. The high sensitivity of bioluminescent assays allows for detecting small amounts of biological materials, such as nucleic acids, proteins and small molecules. The non-toxic and self-contained nature of the bioluminescent system means that it does not require external light sources and can operate in living cells, making it an indispensable tool for *in vivo* imaging and studies. On-chip quantitative nucleic acid amplification detection in real-time using bioluminescence has been reported [21]. The byproduct of the DNA isothermal amplification, i.e., inorganic pyrophosphate (PPi), is proportional to the amount of polynucleotide synthesized [20–24]. The detection of PPi can thus be used to quantify the amount of the original target DNA in the sample.

Another example we think we could also eliminate the use of fluorescence is phosphorescence.

Phosphorescence, a distinctive form of photoluminescence, diverges from its counterpart fluorescence, manifesting a temporally delayed re-emission of absorbed radiation. This delayed luminescence is a quantum mechanically “forbidden” transition from a metastable electronic excited state, typically a triplet state, to the ground state. The triplet state is energetically lower than the fluorescence-associated singlet state, resulting in a prolonged emission time as the transition does not proceed with the same quantum efficiency as fluorescence. Consequently, phosphorescent materials are characterized by their ability to sustain emission long after the initial excitation has ceased. Mn-doped ZnS quantum dots were reported [25] to be used for a long-lived phosphorescent on-off-on probe for the sensitive and selective detection of pyrophosphate ions (PPi). These materials can be tactically incorporated into isothermal amplification assays to enable the detection of nucleic acid sequences. Upon successful amplification, the phosphorescent reporters emit light in the presence of the target DNA or its amplification byproducts, eliminating the necessity for continuous external excitation. This innovative approach not only simplifies the detection apparatus but also augments assay sensitivity, as the phosphorescent signal persists and can be measured with greater temporal flexibility, thereby enhancing signal-to-noise ratios. The strategic implementation of phosphorescent probes in isothermal amplification techniques thus advances molecular diagnostics, offering a robust platform for developing next-generation point-of-care diagnostic assays. The use of phosphorescence could also eliminate the necessity for optical filters since phosphorescent emission lasts much longer than fluorescence [26].

There are different nucleic acid isothermal amplification techniques, namely, linear, exponential and loop-mediated isothermal amplification (LAMP). When compared with PCR, the advantages of isothermal amplification are less power consumption due to using a constant lower temperature than a thermal cycler, portable capacity and cost-efficient. The downside of LAMP or, in general, isothermal amplification techniques, which we think is the lack of multiplex detection capability due to the requirement of primers for each specific target of interest, compared with solid-phase (SP) PCR [13,16,27]. In the SP-PCR, the target biomarkers are synthesized and amplified on a solid substrate with one or both primers, immobilized on the surface beforehand [13,16,27]. The SP-PCR provides a much higher multiplexing capability since numerous primers can be arranged in an array.

Tabata [4] introduced different miniaturizing electrochemical sensors in combination with nucleic acid isothermal amplification. The critical sensing technique is the detection of redox reactions, and it stays on the detection of byproducts, either PPi or H^+ , at the chemical reactions on the electrodes. The SP RCA could not perform real-time detection but end point observation. Regarding the SP isothermal amplification, it is worth mentioning that SP LAMP has recently been invented [28], which might lead to multiplex detection in the near future with SP LAMP.

Accurate identification of cancers or pathogens directly from blood without proper sample preparation steps is highly challenging in miniaturized systems. Therefore, sample concentration or enrichment of microorganisms or biomarkers has been a crucial step in overcoming the inhibitory effects of biological matrices. Since bio-molecules are negatively charged, possible sample extraction and preconcentration techniques use electrokinetics such as electrophoresis [29] or dielectrophoresis [30].

The cfDNA can be used as the target biomarker for cancer detection. There should be difficulties in the extraction and preparation of samples toward the point of care for early diagnosis. Furthermore, data analysis with the help of artificial intelligence (AI) may be one of the solutions to tackle the issue of big data obtained from real-time monitoring using electrochemical sensors described in the work of Tabata [4]. We would like to note that

in addition to cancer, diseases characterized by blood-based biomarkers, such as sepsis, may also benefit from the integration of nucleic acid amplification and miniaturized systems. Working in such a big clinical project requires multidisciplinary partners and large consortium. It is hence essential to establish worldwide cooperation to push forward boundaries of current techniques and revolutionize the point of care testing for liquid biopsy.

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