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Recent advances in nanotechnology and microfluidic-based approaches for isolation and detection of circulating tumor cells (CTCs)

Jyotish Kumar [a,1], Soumyadeep Basak [a,1], Ashish Kalkal [a], Gopinath Packirisamy [a,b,*]

[a] Nanobiotechnology Laboratory, Department of Biosciences and Bioengineering, Indian Institute of Technology Roorkee, Roorkee, Uttarakhand 247667, India
[b] Centre for Nanotechnology, Indian Institute of Technology Roorkee, Roorkee, Uttarakhand 247667, India

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ABSTRACT

The event of cancer metastasis, wherein tumor cells detach from the primary tumor site and travel to distant tissue or organs through the circulatory system of the body, is currently considered one of the most exciting areas of research throughout the world. The potent tumor cells, capable of invading blood vessels and transporting themselves towards the secondary spread site, are called circulating tumor cells or CTCs. The detection and isolation of such CTCs from a patient's bloodstream, followed by molecular biological investigations for determining a correct intervention plan, are of prime importance to curb the menace of cancer. In such a scenario, a multitude of research is going on to understand the biology of CTCs and develop highly sensitive and rapid detection strategies. Pertaining to this, a review has been proposed to outline the recent advancements in the development of various CTC isolation and detection strategies, as well as the commercially available ones. The role of microfluidic-based fluid dynamic systems related to such efficient isolation/detection devices is also highlighted. Nanotechnology, on the other hand, has opened a myriad of excellent possibilities for developing more clinically relevant and efficient platforms. Such nano-based strategies are also summarized in this review. Moreover, recent investigations on novel platforms combining nanotechnology and the microfluidics avenue for CTC isolation and detection are also discussed, along with recent challenges and future perspectives.

Contents

* Corresponding author at: Nanobiotechnology Laboratory, Department of Biosciences and Bioengineering, Indian Institute of Technology Roorkee, Roorkee, Uttarakhand 247667, India.
E-mail address: gopi@bt.iitr.ac.in (G. Packirisamy).

[1] The authors contributed equally to the manuscript.

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1. Introduction

As per the data from the Global Cancer Observatory in 2020, breast (11.7%) and lung (11.4%) cancer contributed most to the total of ∼19.3 million new cases reported worldwide, with colorectal cancer being the 3rd most common cancer in the world [1]. Reportedly, tumor metastasis has been the leading cause of mortality in patients who have cancer [2]. Metastasis occurs when the tumor cells detach from a primary tumor site and seeds to a distant secondary tumor site via the circulatory system. These highly specialized, heterogeneous, individual or group of cells detached from a tumor, invading into and traversing through the vasculature are called Circulating Tumor Cells or CTCs. CTCs have been reported to be one of the most reliable tools for early cancer diagnosis and treatment [3]. Therefore, the detection and isolation of CTCs from the patient's circulatory system have provided a comparatively non-invasive alternative to other cancer diagnostic approaches.

Traditionally detection strategies are based on either positive or negative selection. Positive selection relies upon interaction with a specific surface-active antigen (e.g., EpCAM expression by CTCs), and negative selection is where elimination based on a physical attribute that distinguishes a particular target from the rest of the population (e.g., the larger size of CTCs compared to other blood cells) takes place. Moreover, with the progress in molecular biology, -omics analysis has also provided much exciting information regarding CTC detection processes [4]. However, these traditional methodologies are limited by factors like a very low number of cells in patients in the early stages of cancer (low sample volume), high cost, long time required to acquire data, and CTC's inherent immune-escaping strategies making them not properly detectable by antibody-based methods. However, the last decade (2010–2020) has seen a steady increase in the number of publications related to circulating tumor cells. The keyword "Circulating tumor cells" yielded 16299 articles in total (almost 1482 articles per year) in PubMed [5]. The more insights we get into CTC biology and its molecular information, the more we can devise novel detection and isolation methodologies.

From its very inception in 1959, through the vision of Nobel Laureate physicist Richard P Feynman, nanotechnology has been considered by many the "seed of a new industrial revolution" [6,7]. Because of their unique physicochemical properties, nanomaterials have found their use in almost every scientific research sector [8–12]. For example, metal quantum dots, carbon dots, graphene, and the recent addition of MXene-like nanomaterials have revolutionized the field of rapid detection or sensing [13–19]. The role of nanotechnology in the fight against cancer was realized almost a couple of decades ago when superparamagnetic (SPM) nanoparticles (e.g., Fridex, an injectable SPM iron oxide(IO) solution) were employed as a contrast-enhancing agent in magnetic resonance imaging (MRI), depending upon the differential uptake behavior of such nanomaterials by cancerous and non-cancerous cells [20]. Apart from that, in cancer theranostics (therapy and diagnosis through a single platform), precise detection and targeted delivery of therapeutic molecules have been achieved using specialized nanomaterials like nanocages, core-shell nanoparticles, etc. [21–23]. Therefore, nanotechnology can provide a promising avenue for isolating and detecting CTCs. This review discusses such novel nano-based CTC isolation and detection strategies.

Another rapidly developing technology is Microfluidic-based devices, where microscale manipulation of fluid flow is employed for various applications, including biomedical, tissue engineering, and many others being explored [24,25]. Although the technology itself was introduced in the early 1950s, the true diversity of applications achievable is now tremendously being realized throughout the world due to a myriad of advantages, like low sample volume requirement, low-cost fabrication, the rapid response of sample volume, portability, enhanced sensitivity, the ability of single-cell manipulation, potential for multiplexing, etc. [24,26]. There are several thorough reviews regarding the effectiveness of microfluidic-based platforms in CTC isolation and detection [3,27,28]. However, literature discussing the potential of a careful amalgamation of nanotechnology and microfluidic-based platforms is of prime importance. This review focuses on applying nanotechnology and microfluidic-based approaches in the isolation and detection of CTCs, focusing on the commercially available strategies (see Fig. 1).

2. Circulating tumor cells

2.1. CTC biology

Events associated with the metastatic cascade involving CTCs, as illustrated in Fig. 2, are biologically dynamic and complex events, and variations exist in various cancer types. Broadly, metastasis through CTCs occurs by one of two possible mechanisms; active invasion or intravasation, supported by an epithelial-to-mesenchymal transition (EMT), or passive shedding of cells, where clumps of cells break off from the primary tumor [30]. The EMT of individual cells was reported to be triggered when tumor cells try to intravasate and get covered with platelets, while the reverse, i.e., the mesenchymal-to-epithelial transition, occurs during extravasation at a distant site to form the metastatic epithelial deposition [30,31]. During the event of passive shedding, clumps consisting of 2–50 CTCs, called the circulating tumor microemboli (CTMs), break off and travel through the blood vessels. The CTMs were also reported to possess significantly enhanced (23–50 times) metastatic potential than single CTCs because of certain inherent advantages: firstly, the absence of apoptotic cells, and secondly, the absence of proliferating cells. Moreover, an enhanced expression of plakoglobin, a constituent of adherence junction and desmosome, was reported to contribute to the formation of CTMs, which would also assist in its survival [30].

After the CTCs enter the circulation, they either try to home and form metastasis at a distant organ (secondary metastases) or may re-seed the primary tumor site under the influence of chemo-attractants cytokines IL-6 and IL-8 produced by the tumor or immune cells at the primary site (tumor self-seeding or cross seeding, generating more aggressive metastases). Such

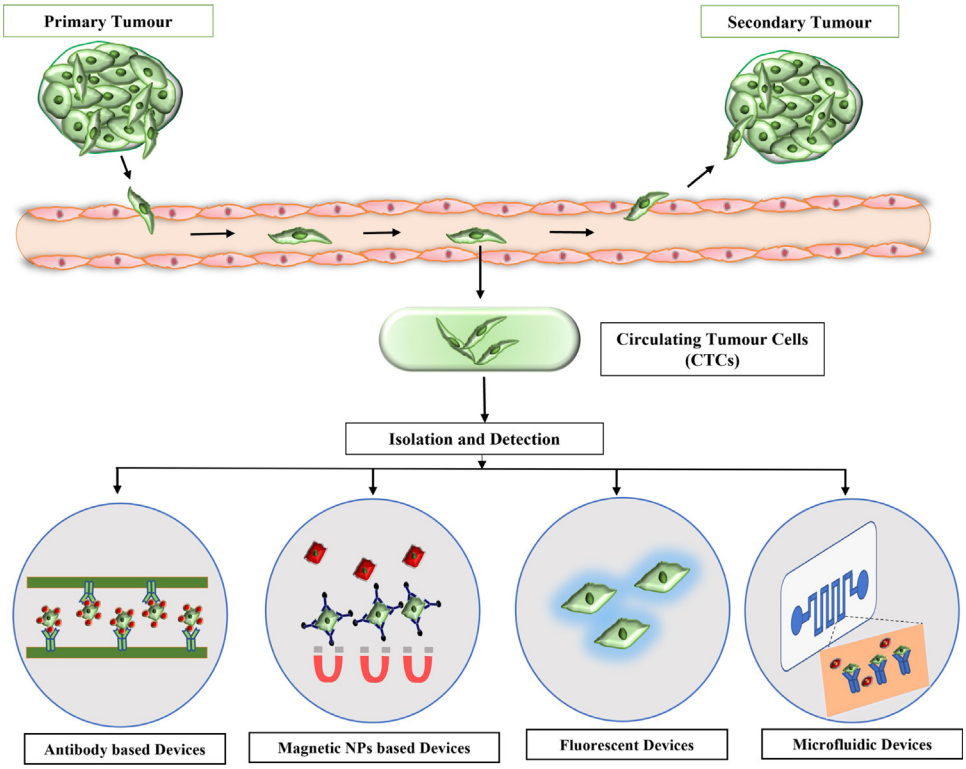

Fig. 1. Illustration of the metastatic events led by CTCs and their detection by various strategies.

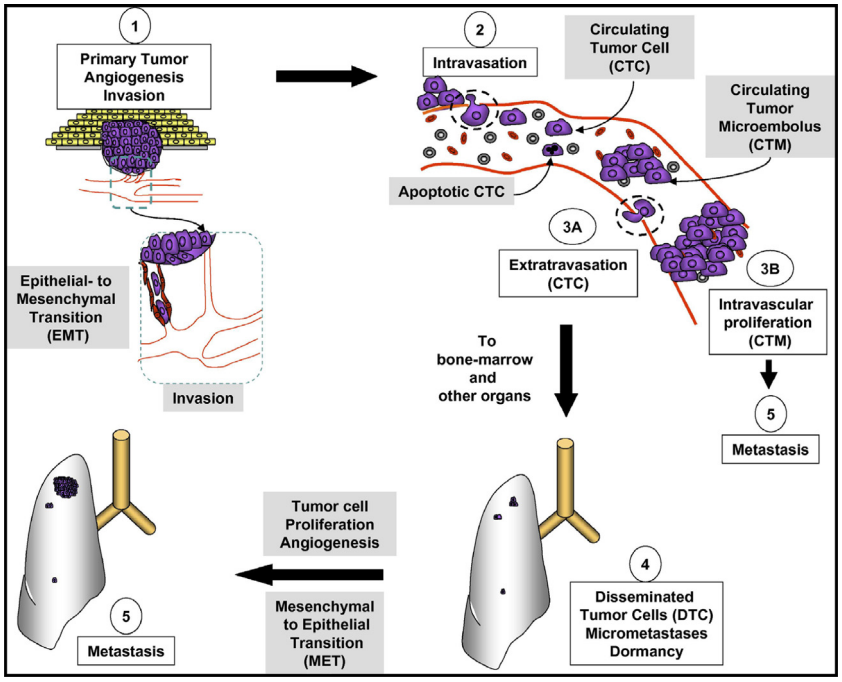

Fig. 2. The biology of circulating tumor cells (CTCs), illustrating different stages of metastasis events involving CTCs, Reproduced with permission [29].

CTCs, capable of remaining dormant for years followed by forming micro-metastases, are called Disseminated Tumor Cells or DTCs, and the event is named Minimal residual disease (MRD) [30,32]. Bone marrow (BM) has been reported to be the most common homing organ for CTCs, especially in the osteoblastic niche of the BM. It has also been noticed that DTCs isolated from the BM express specific stress-associated genes, enabling them to survive in the hypoxic condition of the BM niche. Another significant characteristic of the CTC is the "organ-mimetic phenotype", which explains the adaptation of osteomimetism and lung- or liver-mimetic adaptation of the CTCs as they arrive and interact with the acceptor niche [32]. Gene expression studies have also

supported that activation of the RAS pathway, thereby enhancing cell proliferation and activation of the hypoxia-induced transcription factor (HIF-1α), has considerably increased the homing of CTCs to the BM [33]. Therefore, a BM liquid biopsy has been considered a potentially reliable source of isolation and detection of CTCs.

On the other hand, the CTC's diameter is 3–4 times more than the vessel diameter at distant organs. Therefore, it can be expected that only the deformable and/or small CTCs can be detected in the circulation [34]. Recently, mechanical deformability has been investigated as a potential CTC detection strategy (cancer mechanobiology). However, such mechanical measurements can only be performed using sophisticated and specialized techniques, like single-cell/small tissue core liquid biopsy, fine needle aspiration, single-cell atomic force microscopy (AFM) techniques, etc. [35]. Additionally, the homing site also tends to differ depending on the kind of cancer being investigated. Thus, determining the variable homing site to collect clinically relevant CTCs for getting helpful information on the cancer progression is not minimally invasive [34].

2.2. Clinically important signatures of CTCs

Several protein-based markers expressed on the CTCs surface can be used for their detection using antibody-based approaches. Such selectable markers can be used for the isolation/enrichment of the CTCs for further analysis. The epithelial cell adhesion molecule (EpCAM) is one of the most used cell surface markers for the positive selection of CTCs. Along with that, cytokeratins, cytoskeletal proteins expressed exclusively by epithelial cells, e.g., CK8, CK18, CK19, etc., are also significantly used for positive enrichment of CTCs [36]. Enhanced expression of EMT markers has also been reported as an important phenomenon during CTC invasion and migration. Therefore, EMT markers like N-cadherin and vimentin can be valuable candidates for positive selection markers. Transcription factors like SNAI1, TWIST, ZEB1, etc., are also important EMT markers. However, reports also have suggested that CTCs that are "frozen" in the mesenchymal stage cannot form metastasis to distant organs. Thus, cancer cells with an intermediate phenotype, having the characteristic plasticity between epithelial and mesenchymal states, thereby representing the cancer stem cells (CSCs, e.g., low expression of EpCAM with high MET expression) are suggested to be suitable for a successful metastasis event and the detection of which is of paramount importance [37].

On the other hand, low EpCAM expressing CTCs have been reported using the CellSearch system [38]. CTCs expressing NOTCH1, HER2, EGFR and HPSE, while being EpCAM negative, have also been reported to be forming brain metastasis [39]. Thus, a wide range of phenotypes has been reported for CTCs, depending upon the type and stage of cancer. In general, tumor-specific markers like HER2 and EGFR (epidermal growth factor receptor) are widely overexpressed in CTCs compared to normal cells, contributing to the further selection of CTCs and other markers. A negative selection marker, like CD45, is not expressed in carcinomas but is expressed in leukocytes. CK^{-} $CD45^{+}$ cells are also signatures for tumor-associated macrophages [36]. Depleting such a background of cells expressing negative selection markers is also a strategy of choice for many commercial CTC isolation and detection devices, which will be discussed in more detail later in the review.

Due to such a high degree of heterogeneity, the detection of CTCs with prominent clinical relevance should rely upon more specific detection approaches like reverse transcription PCR (RT-PCR) or genome sequencing analysis, which can be further complemented by genomic interventions like fluorescent *in-situ* hybridization (FISH) or single-cell analysis [36]. Therefore, developing a reliable, clinically relevant CTC detection strategy demands a combinatorial approach rather than a single trait dependence.

3. Mechanism of CTC isolation and detection

The isolation and detection methods of CTCs have broadly been categorized as label-dependent and label-independent methods. Label-dependent methods for positive CTC isolation use specific CTC cell surface markers, such as EpCAM, EGFR, MUC1, HER2, etc., whereas negative selection markers include CD45 and CD66b. On the other hand, label-independent methods rely upon the physical attributes of the cell population. Several mechanisms are employed for such label-dependent and label-independent CTC isolation and detection, as represented in Fig. 3.

3.1. Label-dependent methods

3.1.1. Immunocapture

Among all the methods available for CTC isolation, immunocapture is considered one of the most traditional methods available for CTC isolation in the category of label-dependent methods. It is based on the interaction between CTCs and cancer-specific antibodies [38]. For example, when EpCAM-specific antibodies are used for CTC isolation, the method can be called Positive immunocapture [41]. Other alternative markers used are prostate-specific membrane antigen (PSMA), human epidermal growth factor receptor 2 (HER2), and epidermal growth factor receptor (EGFR) [28]. These markers are either used alone as an alternative or can be used in combination for CTC isolation. However, negative isolation focuses on depleting WBCs using anti-CD45 antibodies and hence CTC enrichment.

The immunocapture method includes chaotic mixing, nanostructure or nanomaterial-based microchannel, and micropost array. The antibody-functionalized micropost array is the most common method [42]. In this method, a turbulent flow is created within the microchannels and thus increases the chances of contact of CTCs with functionalized antibodies.

3.1.2. Immuno-magnetophoresis

Immuno-magnetophoresis was among the early methods developed for CTC isolation and still remains one of the most commonly used methods [43]. In this method, magnetic nanoparticles bind to CTCs using tumor-specific antibodies or aptamers. Due to its accuracy and ease of use, it was a widely adopted method of CTC isolation and the first FDA-approved CTC isolation product (CellSearch ™ system) [44]. The CellSearch ™ system remains the "gold standard" among all the methods available for CTC isolation or detection. While the immunocapture method isolates CTCs in a discontinuous fashion, immunomagnetophoresis collects CTCs discontinuously or continuously in the microchannel reservoir for downstream analysis. Mostly, the discontinuous approach is used because of the simplicity of the procedure. Positive isolation is preferred over negative selection due to its higher specificity and selectivity. Sometimes positive isolation methods face the problem of adsorption of captured CTCs, limiting the retrieval of target cells for downstream analysis [42].

3.1.3. Immunofluorescence

Due to the limitation of the large cell size and the need for trained personnel for CTC isolation using conventional fluorescence-activated cell sorters (FACS), microfluidic-based FACS have been developed for CTC isolation [45]. Such devices are simple and consist of a component with tumor-specific fluorescence-conjugated antibodies and a cell sorting component to isolate

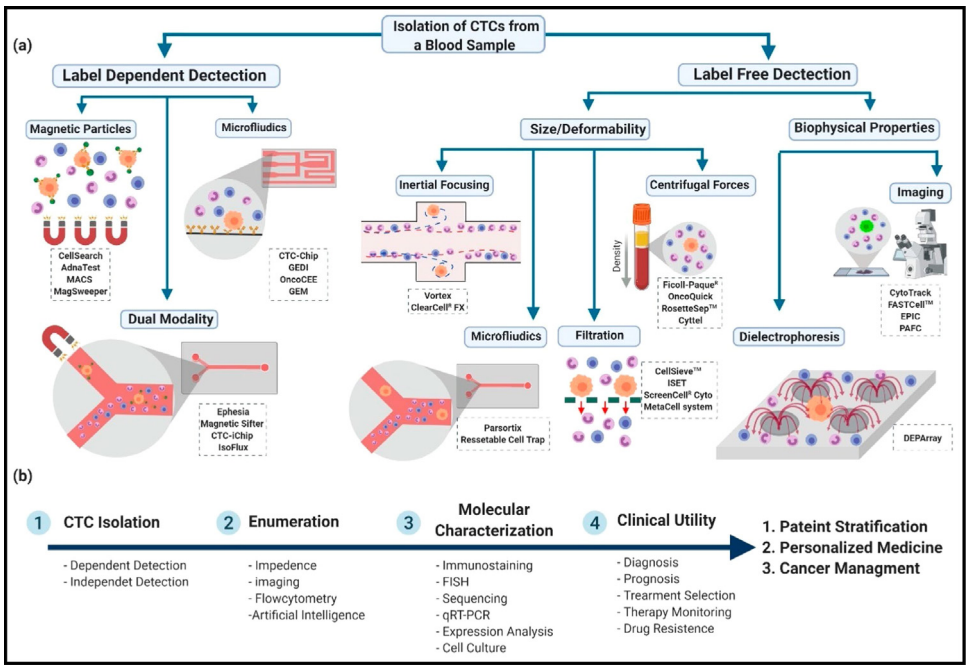

Fig. 3. Schematic representation of the CTC isolation and detection techniques, (a) various mechanisms for the isolation/detection; (b) overview of different steps and their associated techniques for CTC isolation/detection; [40].

the detected CTCs using integrated approaches. The immunofluorescence detector detects CTCs and then isolates them using hydrodynamic valve-based actuators, an optical switch, and piezoelectric modalities [28].

3.1.4. Deterministic cell rolling

Deterministic cell rolling is an exciting approach for CTC isolation/detection based upon the interactions of CTCs with ligand-tagged surfaces and their adhesion to them through lateral passive rolling [46]. Deterministic cell rolling is based on the interaction between the ligands present on the cell surface of the CTCs and the microchannel wall. Cells that show an interaction with the wall are separated by slanting ridges present on the microchannel floor, which changes the pathway of the flow of cells [47]. This method of CTC isolation has a low throughput because of the incomplete rolling of CTCs on the microchannel floor. Additionally, limited use of this technology has been achieved due to the inability to detect CTCs in a whole blood sample as the cell surface is blocked by other blood-derived cells.

3.2. Label-independent methods

While label-dependent methods depend on tumor cell-specific markers, the expression of such surface markers is not uniform in CTCs [48]. Because of this heterogeneity, label-independent technologies are more advantageous. Biophysical characteristics, like larger cell size or enhanced mechanical plasticity of CTCs compared to normal blood cells and so on, can be employed as a label-independent cell surface marker. Depending upon such markers, the following technologies were devised:

3.2.1. Mechanical filtration

Mechanical filtration represents a label-independent, size-based isolation/detection method, which is simple and has a high throughput. The advancements in nanomaterial synthesis have enabled us to fabricate highly uniform and precise nano/microstructures or fillers, which have ultimately increased the performance of this CTC isolation technique. In general, mechanical filtration includes microsieve, pillars, cross flow, and weir. For instance, a group of researchers (Na sun et al.) has developed a low-cost and small-sized microfluidic-based capture chip using a conventional polycarbonate membrane for CTC isolation from blood [49]. They have used the size enlargement method by using modified microbeads that can bind to the CTCs. Using this system, up to 91% of target cell isolation was achieved from a whole blood sample.

3.2.2. Hydrodynamics

This is a size-based isolation approach based on hydrodynamic inertial forces. Cell-size dependent during continuous flow, hydrodynamic inertial forces were found to operate during the interaction of CTCs and microscale obstacles [28]. One significant advantage of this technology is that it allows further manipulation of the isolated CTCs for downstream analysis, which overcomes the limitation of the mechanical filtration method. For example, studies performed by Kyung et al. have introduced a parallel multi-orifice flow fractionation (p-MOFF) device-on-chip for isolating CTCs continuously using hydrodynamic forces and cell-size differences [50]. These devices do not require any labeling processes; hence a heterogeneous population of CTCs can be isolated regardless of any biomarker expression.

3.2.3. Electrokinetics

Dielectrophoresis (DEP), an electrokinetic phenomenon caused by the asymmetrical displacement of electric fields, has been used to isolate CTCs [51]. This phenomenon creates a dielectrophoretic (DEP) force on cells under a non-uniform electric field, which varies with cell size and other dielectric properties. Tumor cells have different sizes and dielectric properties and are easily differentiated by DEP. For example, a discontinuous CTC cell isolation method developed by Gascoyne et al. reported using the DEP forces within a parabolic fluid flow profile, resulting in increased cell elution [51]. A 100% efficiency in purging viable MDA-MB-231 cells from blood was reported. However, DEP methods need complicated sample preparation processes where the cellular components are resuspended in an isotonic medium with low conductivity.

3.2.4. Acoustophoresis

With the advancement in microfluidic technologies for CTC isolation, acoustophoresis has been developed as a method having many advantages over the previously developed technologies, such as being fast, simple, biocompatible, label-independent, and a contact-free system. In this system, cells are forced towards the minimum acoustic pressure exposed to standing waves placed within the microchannel. Acoustophoresis enables manipulation of viable and non-viable cells, blood cell subpopulations, cancer cells, etc., because the cells are exposed to different magnitudes of acoustic radiation force depending upon several factors, like their size, compressibility, and density [28,52]. Although it is easier to separate cancer cells from WBCs, isolating CTCs in blood samples is not that easy as the acoustic properties between other blood cells and CTCs do not vary considerably [53].

4. CTCs isolation and detection strategies

Based on the mechanism discussed above, several strategies have been developed for CTC isolation and detection. Some recent investigations will be discussed in this section, focusing mainly on nanotechnology and microfluidic-based approaches.

4.1. Role of nanotechnology in CTC isolation and detection

Theoretically, a class of materials having one of their dimension in the range of 0–100 nm is usually considered nanomaterials. Due to their unique and exciting properties, nanomaterials have been exploited to develop highly sensitive and specific techniques for identifying and isolating heterogeneous CTCs [54,55]. Nowadays, the development of nanotechnology-based systems for CTC isolation is gaining interest among researchers around the globe. Such CTC-based methods can give unique opportunities for early diagnosis. Due to their scarce abundance in the bloodstream, effective cancer prognosis needs more sample volume, necessitating CTC enrichment strategies.

Moreover, isolation methods are either based on physical properties, such as size and density, or biological properties, such as the type of expression protein that serves as a cell surface marker. EpCAM is the most used marker for the recognition of CTCs [56]. In such a scenario, nanomaterials, offering the largest surface area among other classes of materials, can provide many useful platforms to tag/apply the identification moieties. Broadly, the available methods of CTC isolation include microchip-based capture platforms and magnetic separation. Here are some nano-based techniques for the isolation of CTCs.

4.2. Nanotechnology- and microfluidic-based methods for CTCs isolation

4.2.1. Magnetic separation

Magnetic nanoparticles (MNPs) containing elements like iron (Fe), cobalt (Co), nickel (Ni), their oxides, etc., can be separated easily from a resting solution with the help of a magnetic field [54,57]. The separation of magnetic nanoparticles is widely utilized as one of the commonly used methods for CTC isolation. This method can be easily manipulated and promises high specificity and capture [54]. There are two widely popular approaches to magnetic separation available, either using microbeads made up of polymers embedded with magnetic materials with a diameter less than 0.5 μm or polymer-coated magnetic NPs. Among these, polymer-coated magnetic NPs are preferred over microbeads due to their higher propensity to bind with the cells and more excellent stability in blood, therefore having the requirement of a lesser quantity and thus less cytotoxicity [58]. Moreover, they can adhere to cells without aggregation due to steric repulsion. Another benefit of using magnetic NPs is that they allow for multiplexed detection on labeling with different tags and with different-sized nanoparticles, thus increasing the efficacy of the separation and detection process. A related study using magnetic NPs for CTC capture has been done where iron oxide-based immunomagnetic separation of CTCs was investigated using a whole blood sample. In this study, polymer-based modified antibodies were used against HER2 (overexpressed in many cancer cells) as a targeting cell membrane protein, and nanosized magnetic nanoparticles have given excellent results with an enrichment factor of $1{:}10^6$ and promise a better results strategy for CTC capture in fresh whole blood [59]. Further, the magnetic separation-based approaches can be broadly grouped into two categories, namely bulk and microfluidic-based magnetic separation, as discussed in the following sections.

4.2.1.1 Bulk magnetic separation

External magnets separate magnetic nanoparticles bound CTCs in a bulk system. Since the magnetic force is directly proportional to the total number of bound NPs, under the same magnetic field, the isolation rate of NP-bounded cells is faster than that of the free NPs in the solution; hence, the former are selectively enriched [60]. The FDA-approved CellSearch ™ system uses a similar approach where Fe-NPs are linked with the anti-EpCAM antibodies used for the isolation and detection of CTCs in breast cancer patients using whole blood samples [61]. In combination with immunofluorescence, the CellSearch system has offered excellent efficiency (over 80% recovery rate in spiked breast cancer cells) [62]. However, one significant limitation of the system is that it can only detect EpCAM- positive cells, and the expression of EpCAM is not homogeneous in cancer cells, sometimes the downregulation of which can be a potential indication of CTCs in peripheral blood circulation [63]. To address this issue, a recently reported investigation by Ding et al., tannic acid (TA) tagged magnetic nanoparticles were fabricated for cancer cell detection from blood [64]. The EpCAM independent approach selectively detects and isolates the blood-derived cancer cells, and the platform can be utilized for broad-spectrum CTC detection.

Interestingly, although the positive selection is considered a gold standard for CTC isolation and detection, it cannot capture the CTCs with low or no expression markers. CTCs are also reported to be cloaked using blood-derived platelets [34]. Such a problem can be resolved using the negative depletion method, where immunomagnetic beads were used to capture cells other than CTCs, ultimately facilitating CTC enrichment [65]. In such negative selection strategies, RBCs are first removed from the blood samples by lysis, and then WBCs are removed using magnetic NPs coated with anti-CD45 by magnetic separation [66,67]. A similar approach was also reported by Bhuvanendran et al. A chip for isolation of CTCs from patients using whole blood samples without targeting any tumor-specific antigens was developed [68]. Strong magnetic field gradients with customized antibody complexes to capture WBCs followed by depletion of RBCs were used with a micro-slit membrane. The approach reported specificity of more than 80% tumor cell recovery from a 2 mL blood sample in just 50 mins.

4.2.1.2. Microfluidic-based magnetic separation

Microfluidic devices have recently received tremendous attention for developing CTC isolation and detection platforms. These devices offer portable, cost-effective, tunable, multiplex-capable platforms with the ability to effectively isolate/detect CTCs and single-cell analysis [27]. In an excellent approach developed by Weislsleder et al., magnetic nanoparticles are used for direct detection using a whole blood sample and measured specific biomarkers using a multiplexed detection technique [69]. The group developed a microfluidic chip-based micro-Hall detector

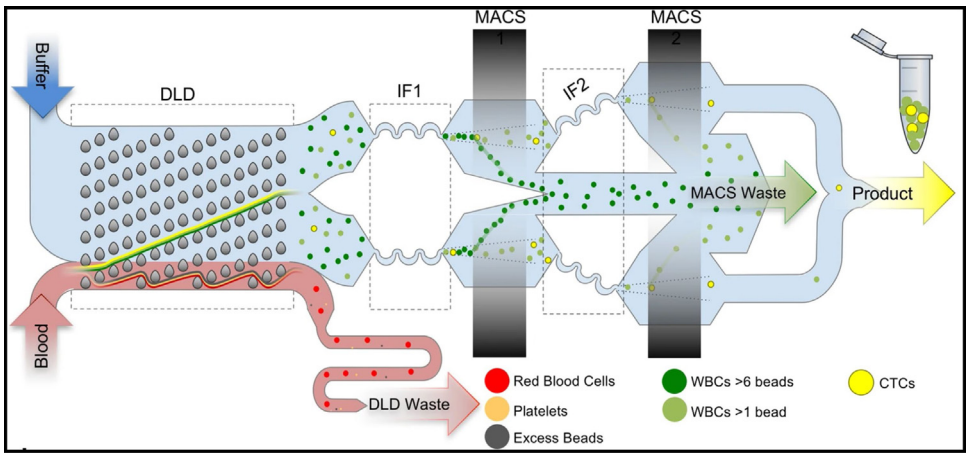

Fig. 4. Illustration of CTC isolation using a monolithic microfluidic device and magnetically activated cell sorting (MACS) [72].

system based on the Hall effect that can detect induced magnetic moments of magnetically labeled cells when exposed to an external magnetic field. It was observed that the signal intensity is directly proportional to the number of bound magnetic NPs and thus the level of biomarkers. Hence, the targeted biomarker can be detected and profiled on single cells in a pool of blood cells. MNPs of different sizes can be used for multiple markers. On testing with cell lines using different biomarkers, the results are satisfactory and agreed with flow cytometry. In a study with samples from 20 ovarian cancer patients, the developed method was found to be more efficient than the widely used commercial system and was able to detect CTCs in 100% of patients in comparison with the commercial system, which can detect CTCs in a much lower number of instances. The higher efficiency in detecting CTCs is due to the use of the multiplexed targeting strategy.

A recent investigation reported a microfluidic system that can be used for CTC isolation and immunomagnetic separation. In this proposed system, flow conditions and computational analysis have shown that the CTC capture efficiency from a non-Newtonian particulate blood flow can be controlled by manipulating the ratio of the magnetic force and drag force [70]. Similar conclusions were also drawn by Zhang et al., both theoretically and practically, by studying the effect of these parameters on the CTC capture efficiency using an immunomagnetic detection system [71]. These methods resulted in an excellent recovery of 83% of head and neck cancer cells. However, it is not easy to deplete all the background cells. Additionally, the RBC lysis process can cause damage and loss of CTCs, decreasing the platform's capture efficiency. A monolithic CTC-iChip was also reported by Fachin et al., an automated immuno-magnetophoretic cell sorting device, as illustrated in Fig. 4 [72]. An unbiased, highly efficient CTC detection could be achieved using ~1.5 million microstructures in the chip. In another recent study, Mishra et al. developed an ultrahigh-throughput microfluidic chip, LPCTC-iChip, capable of sorting over 6 billion nucleated cells, thereby providing increased CTC isolation capacity by two orders with 86% recovery and 10^5 enrichment [73]. Soft iron-filled channels were used to deplete massive numbers of magnetically labeled leukocytes within microfluidic channels. Additionally, a hybrid magnetic and deformability-based isolation approach was developed by Chen et al. for CTC isolation. A magnet-deformability hybrid integrated microfluidic chip to capture CTCs bonded with magnetic immune beads was designed. Their clinical test results have shown over 90% of capture efficiency and 96% viability at a high flow rate of 3 mL/h [74].

4.2.2. Nanostructured substrates

Nanostructure substrates provide a large surface area for grafting capture agents, improving cell capture efficiency and promising high purity, sensitivity, and high reproducibility [78]. Aided by the recent advances in nanostructure substrate fabrication, researchers have explored different parameters, including cell migration, alignment, and proliferation on nanostructured surfaces, to find novel methods for capturing CTCs [54]. As shown in Figs. 5A and B, the nanostructure substrates with immobilized targeting ligands for CTC capture, using ligand–antigen interactions, offer several benefits like increased surface area and hence increased integration and binding affinity between the substrate and targeting cell surface as compared to flat surfaces [75]. There are different types of nanostructures available for CTC isolation, such as nanosheet [79], nanofiber [80], nanoarray [81], nanotextured substrate with immobilized aptamers [82], nanoporous substrates, and nanoparticle deposited rough surfaces [83]. A very recent investigation by Wu et al. have reported the fabrication of a microfluidic device (illustrated in Fig. 5C) consisting of bovine serum albumin (BSA) modified poly(lactic-co-glycolic acid) (PLGA) nanofiber chips, glutaraldehyde(GA) linked with a dual aptamer system (EpCAM-5-1 and NC3S aptamers) for the efficient capture and release of various phenotypic variants of CTCs [76]. Excellent capture (89%–91%) and release (88%–95% efficiency) were reported for OVCAR-3 and A2780 cells. Another recent investigation by Zhou et al. reported a microfluidic device containing a degradable porous nanoflower substrate (PNFS) [77]. Micro/nanofabricated zinc oxide (ZnO) nanostraws were hydrothermally treated under low temperature to form the zinc phosphate composite structures upon a nanoporous membrane, as shown in Fig. 5D. The nanoflowers can be easily degraded using a 20 min treatment of sodium citrate solution, thereby making interactive CTC recovery possible. By incorporating the PNFS in a microfluidic device, excellent CTC capture efficiency ($93.9 \pm 1.7\%$) could be achieved.

4.2.3. Other nano-based methods for CTC isolation/detection

Broadly, the approaches available for CTC detection are based either on cytometric analysis or nucleic acid analysis. The cytometric assays examine cells based on protein expression, whereas nucleic acid-based methods detect genetic variation specific to cancer cells. Cytometric methods are more advantageous over nucleic acid-based approaches because there is no need for cell lysis, and cell morphology analysis can be performed using microscopic techniques. Spectroscopic detection, flow cytometry, and immunohistochemistry imaging are the methods of choice for cytometric detection. In contrast, nucleic acid-based approaches analyze the genetic information of a complete cell or extracted DNA

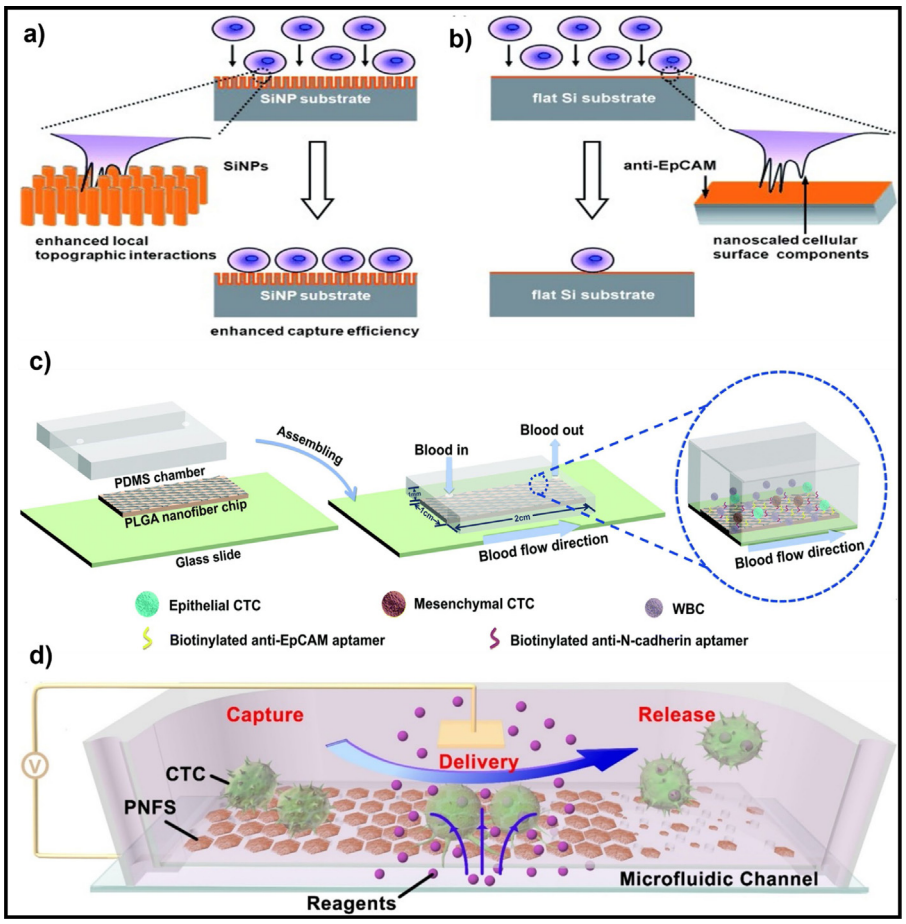

Fig. 5. Illustration of employing a nanostructured substrate for CTC detection; (a) enhanced cell capture efficiency due to the 3D nanostructured surface; (b) reduced topographical interaction on a flat surface; Reproduced with permission [75], Copyright 2009, WILEY-VCH Verlag GmbH & Co.; (c) aptamer tagged microfluidic device containing PLGA nanofibers, Reproduced with permission [76], Copyright 2013, Royal Society of Chemistry; d) degradable PNFS based microfluidic device for CTC capture; Reproduced with permission [77].

or RNA using PCR, quantitative RT-PCR, FISH, and whole genome amplification [84]. Nucleic acid-based methods are highly sensitive, but their specificity is not impressive due to interference from the level of expression of markers by normal cells and the low sample volume (a single diploid cell offering the amount of DNA as little as 6.6 pg) [85]. Nanomaterials are exploited in various CTC detection methods due to their unique properties in such a scenario. Depending upon the readout signal and the type of nanomaterial used, isolation/detection strategies can be broadly classified into three categories as optical, magnetic, and conductive nanomaterial-based systems.

4.2.3.1 Fluorescence-based and magnetic nanoparticles in CTC isolation/detection

Although fluorescence using organic dyes is one of the most commonly used techniques for CTC detection, several challenges limit their utility, like photobleaching, spectral overlapping, low signal intensity, and the requirement of multiple light sources while exciting multiple fluorophores in a multiplex detection [86]. To address these issues, quantum dots (QDs) are used as they have a narrow emission, high photostability, and superior brightness [87–89]. Another significant benefit of using QDs is their large absorption spectrum. The size and composition of nanoparticles play a key role in controlling the emission, resulting in multicolor NPs using a single excitation laser source. Such a wide range of tunability in emission behavior makes them an excellent candidate for biomedical imaging and has great potential in detecting CTCs by examining the extracted nucleic acids [90]. In line with this, Zhang et al. have developed a microfluidic-based sensor for nucleic acid detection using multienzyme-nanoparticle amplification and QDs [91]. Fluorescence intensity was measured using QDs, which provided a quantitative analysis of targeted DNA. Park et al. have also reported a CTC-capturing microfluidic device, called NP-^{HB}CTC-Chip, involving antibody interaction and ligand exchange, as illustrated in Figs. 6a and b [92]. Another CTCs detection strategy was reported by Tkaczyk et al. by using *in-vivo* fluorescence flow cytometry to detect two different colored QDs (Qdot585 and Qdot655) for monitoring breast cancer cell lines [93].

Due to the highly dynamic nature of cancer prognosis, it is disadvantageous to rely upon the detection and analysis of cancer cells at a single time point [94]. Therefore, *in vivo* imaging techniques are gaining tremendous popularity for real-time monitoring. Luckily, one major advantage of using quantum dots is their rapid permeability and capability to access various tissue or organs in the body due to their extremely small size. Kuo et al. recently reported an *in vivo* real-time CTC imaging strategy using streptavidin-conjugated QDs (Qdot525, Qdot705) tagged with CD41, CD24, and CD133 antibodies [95]. $CD24^{+}$ and $CD133^{+}$ CTCs could be identified *in vivo* in real-time.

Other than antibodies, aptamers have also been extensively studied as a potential biosensing platform. Many excellent reviews on the suitability of such apta-sensors for CTC detection/enrichment have been published [97,98]. Moreover, metal quantum dots are also reported to impart a certain level of cytotoxicity, limiting their applicability [99]. To address these issues,

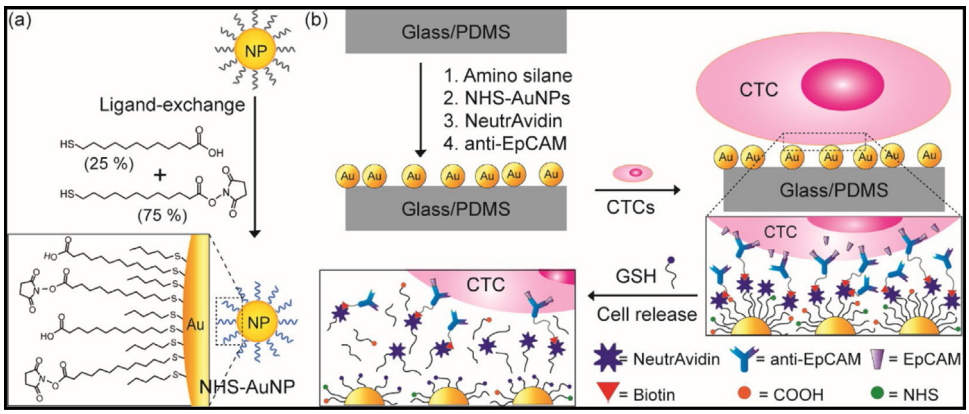

Fig. 6. Illustration of NP-[HB]CTC-Chip fabrication; (a) pentanethiol-functionalized NPs fabrication process; (b) CTC isolation by a functionalized NP based chip; Reproduced with permission [92].

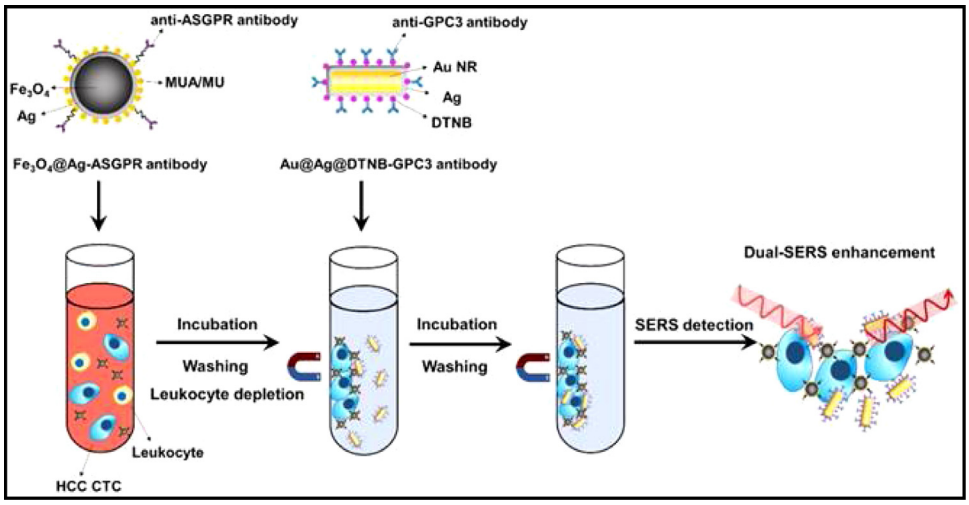

Fig. 7. Antibody-tagged Ag coated magnetic NP-based SERS CTC detection [96].

carbon-based materials, like graphene quantum dots (graphene QD), carbon dots, etc., have gained increasing popularity. In one such recent investigation by Cui et al. a "turn on" magnetic fluorescent nanocomposite (MFNs) was fabricated containing Ep-CAM Aptamer tagged Fe_3O_4, which was further associated with graphene QDs [100]. Due to the presence of graphene QDs, Ep-CAM expressing CTCs could be tagged and imaged in 15 mins, and the presence of iron oxide makes it possible to enrich the tagged cell population using an external magnetic field, serving the dual purpose of detection and sorting for a whole blood sample. Folic acid (FA) tagged carbon dots (FA-CDs) have also been studied for their potential to tag along with the folate receptor (FR) of FR+ cancer cells [101]. Such FA-CD-based nanomaterials have enhanced biocompatibility with high fluorescence emission, making such systems a potential candidate for CTC detection.

4.2.3.2. Surface-enhanced raman scattering-based approaches

Metal nanoparticles were also applied to adsorb highly delocalized π electrons of organic dyes and quench the fluorescence signal, resulting in strong Raman signal enhancement. Such enhancement increases the detection sensitivity and is helpful in the detection of a single molecule or even a single particle [102]. Such dye-adsorbed metal NPs are surface-enhanced Raman scattering nanoparticles (SERS NPs). SERS NPs based detection is a new era of biomedical diagnosis and imaging [103,104]. Other than providing ultrasensitive detection by giving sharp signals, there is no need for signal separation in the case of SERS. Additionally, it gives excellent results in multicolor probe detection and has good multiplex capability along with its need for only a single source for excitation, and also has minimal photobleaching [96,105]. The overall process outline is illustrated in Fig. 7. Therefore, metal nanoparticle-employed SERS-based CTC detection strategies are gathering attention worldwide. In this context, Lin et al. have discussed several SERS-based CTC detection strategies using various surface-modified metal nanoparticles [106]. Very recently, Jibin et al. have come up with a portable centrifugal prototype for CTC isolation detection [107]. Anti-EpCAM antibody-coated polycarbonate (PC) filters were used for centrifugal CTC isolation, and quantitative detection was performed based on an anti-ErbB2 antibody tagged gold–graphene nanohybrid (Au-rGO@anti-ErbB2) SERS nanotag. The prototype could detect up to 5 tumor cells/mL of whole blood. Such a selective enrichment platform combined with an efficient detection platform can provide a highly efficient and sensitive CTC quantification strategy for cancer diagnosis.

4.2.3.3. Semiconductor carbon nanotube-based approaches

Carbon nanotubes are one more amazing category of nanomaterials that are exploited for making excellent chemical and biological sensors. They have remarkable electrical properties, due to which they can behave as metal or semiconductors, based on their helicity and diameter [108]. The application of carbon nanotubes for cancer detection using their electrical properties

was first explained by Shao et al. in 2008. The authors used 20 pairs of electrodes, inserted a single pair of antibody functionalized CNTs between each pair of electrodes, and mounted the whole setup in a single wall [109]. This binding of CTCs decreased the conductivity by 60%, whereas in control experiments, the reduction was only 5%, which confirmed the presence of antibody-bound CTCs. The significant advantage of using this system was its ability to detect low protein expression as the sensing area is limited to only a few receptors in cells; therefore, it can be used to detect CTCs in whole blood samples directly. However, this system did not allow the quantification of CTCs as a signal is only determined if the cell is present in the space between the electrodes. Moreover, the volume was very small (<10 μl), and rare CTCs might not be present in such a small blood volume.

Very recently, an excellent colorimetric detection strategy was reported by Zhu et al. based on aptamers and single-wall carbon nanotubes (SWCNT) [110]. Fe_3O_4 immunomagnetic nanoparticles (INMs) containing the EpCAM aptamer P1 conjugated with mDNA sequences when interacting with CTCs; the mDNAs released from the aptamer-mDNA conjugate could be quantitatively detected. The SWCNTs associated with the INMs would provide visual identification of CTCs because of their peroxidase activity. Therefore, very high accuracy and selectivity were reported through the system, with a promising clinical potential.

Besides those above diverse nano-based technologies, microfluidic-based approaches are being investigated extensively, specifically approaches that carefully integrate nanomaterials and microfluidic systems. Some of the most promising strategies will now be discussed below.

5. Commercially available CTC isolation/detection devices

Technologies have been developed consistently for CTCs isolation/detection since the early 2000s. Moreover, the fact that, as of 2021, the market size value of CTC-based technologies is 9.3 billion USD and is expected to triplicate its value (to almost 18.3 Billion USD) by 2027 has significantly fueled the research and development sector. Some of those technologies are now available as products in the market. Almost all of them are highly reproducible, easy to use, and high-performance [42]. Both label-dependent and label-independent types of isolation/detection devices are available commercially. The available technologies provide only a moderate performance but are utilized because they are simple to use, readily available, and have a well-established procedure. More efforts are being made to increase the performance by developing advanced microfluidic technologies, integrating *in-silico* approaches, and much more. Some of the most recent as well as popular commercially available CTC isolation/detection technologies are discussed below and are given in Table 1.

- **ISoFluX**™ - This has been developed by Fluxion Biosciences. The IsoFluX system uses flow control and immunomagnetic capture, particularly Fluxion's patented microfluidic system, to increase the CTC isolation [111]. The significant advantage of using this system is that it has a high sensitivity to CTCs from many tumor types, and multiple markers can be used simultaneously [112]. Additionally, kits for lab usage are available for cell enrichment, isolation, and downstream analysis [111].
- **CellSearch**® - The Cell search system developed by MenariniSilicon Biosystems uses anti-EpiCAM targeting immunomagnetic beads for separation [113], followed by fluorescent image analysis of blood samples for identification [114]. It is the only FDA-approved blood test used for care diagnostics for different cancers, including breast, prostate, and colorectal cancer. It has a limitation as some CTCs have a low level of EpiCAM expression and are unlikely to be captured [115].
- **AdnaTest**- The AdnaTest system is based on a positive selection assay that uses immunomagnetic beads-coated antibodies to capture CTCs [116]. The captured CTCs are detected using a polymerase chain reaction (PCR) assay for tumor-associated transcripts [117]. A significant advantage of using this system is that a variety of antibodies help characterize cells for multiple cell surface markers, and antibody cocktails are used with a high specificity towards certain cancer types [112]. The limitation of this system is that sometimes it gives false-positive results due to the detection of selection markers on cells other than CTCs or nucleic acid contamination [118]. Additionally, AdnaTest allows the profiling of custom-selected mRNA targets and isolation of CTCs from whole blood. It is a nanotechnology-based immunomagnetic cell selection method coupled with multiple tumor-associated antibodies using magnetic beads.
- **Megsweeper** - This sweeping blood system developed by Stanford University consists of a magnetic rod covered with an ultrathin non-adherent polymeric film of a diameter of 25 μm [119]. Like other CTC isolation methods, centrifugation or lysis is not required in Megsweeper, and CTCs labeled magnetic beads are captured using a magnetic rod. After that, washing is performed to remove non-bound normal cells using a fresh buffer solution. CTCs isolated using this method can be studied by genetic profiling [120].
- **MACS**® - Miltenyi Biotec developed magnetically activated cell sorting (MACS). It uses immunomagnetic beads of 50 nm for CTC isolation from a blood sample. Anti-EpCAM magnetic beads are used for direct isolation, based on the positive isolation method. In the positive isolation method, an external magnetic field is removed to retrieve the captured CTCs in the column, whereas in the negative isolation method, normal cells are depleted in the process of CTC isolation in the column [39].
- **Dynabeads**™ - Dynabeads is a method developed by ThermoFisher Scientific based on immunomagnetic capture using magnetic microbeads of 4.5 μm in diameter. Dynabeads, for both positive and negative isolation methods, are available for CTCs, which use Anti-CAM and anti-CD45-coated Dynabeads, respectively [121,122].
- **CellMag**™ - CellMag is a newly developed system by silicon biosystems based on the same technology used by the gold standard Cellsearch ™ system, but with a cost-effective and straightforward protocol. It is based on ferrofluid technology using immunomagnetic separation and staining of CTCs, and isolated cells can be used for various downstream analyses using fluorescent microscopy, PCR, flow cytometry, and single-cell sorting. CellMag has been designed for research only and not for diagnostic purposes [123].
- **ADMONITRIX** - Admonitrix is another newly developed innovative medical device used for real-time monitoring of CTCs. The best part is that testing can be performed in just 40 min by a physician, and it can estimate the total number of CTCs with an accuracy of 95%. It is a combination product for diagnostic uses and is mainly used by a metastatic cancer patients with advanced-stage cancer for real-time detection of CTCs. It provides an idea to medical professionals about the treatment strategies used for the best medical outcomes. IV Diagnostics holds a provisional patent over Admonitrix [124].
- **StraightFrom** - StraightFrom Whole Blood CD326 (EpCAM) MicroBeads are a newly developed product by Miltenyi Biotec for CTC enrichment and detection. These Microbeads have been developed for rapid automated selection of CD326 positive cells using a whole blood sample. The main benefit of using these MicroBeads is that it does not require additional sample preparation steps, such as density centrifugation and erythrocytic lysis [125].

Table 1
Commercially available nano/micro/fluidic-based CTC isolation and detection devices.

Device	Technology used (*Nano/Micro/Fluidic*)	Manufacturer	Cancer/Cell type	Advantages	Disadvantages	Efficiency	Ref.
CellMag ™ CTC	*Nano-based;* Ferrofluid technology, immunomagnetic enrichment method	Menarini Silicon Biosystems	Breast, prostate	Cost-effective and straightforward protocol, semiautomated aspiration tool	Only for research, no clinical application reported	–	[123]
StraightFrom Whole Blood CD326	*Microbead based,* Fluorescent labeling and flow cytometry.	Miltenyi Biotec	Breast, lung, prostate	Fast and easy protocol for magnetic isolation directly from whole blood	A single selection marker is used, Costly and need special detection equipment.	–	[125]
CELLSEARCH®	*Nano-based;* Anti-EpCAM targeting immunomagnetic beads	Janssen Diagnostics	Prostate, breast, colorectal	Only FDA approved blood tests, easy to use	Low CTCs expression levels are not detected. Low purity of captured CTCs	71%–80%	[126,127]
AdnaTest	*Nano/Microbeads based;* Anti-EpiCAM and anti-MUC-1 targeting immunomagnetic beads	Adnagen	Ovarian, breast, colon, prostate	Use of multiple selection markers. Specific to particular cancer type	False-positive findings, CTCs undergoing EMT are EpCAM negative	73%	[116,128]
MagSweeper	*Microbeads-based;* Immunomagnetic isolation using anti-EpCAM	Stanford University, Miltenyi Biotech	Breast, colorectal, prostate.	CTCs isolation without leukocyte contamination	Unable to detect CTCs with low or no expression of Epi-CAM	62%–70%	[127,129]
MACS system	*Microbeads-based;* Immunomagnetic CTCs enrichment by anti-pan CK antibody	(Miltenyi Biotec)	Non-small-cell lung cancer (NSCLC), breast (HER2+)	Able to work with leucocyte depletion (negative enrichment by anti CD45)	Can identify Ep-CAM negative CTCs but not CK negative ones.	NA	[39,130]
Modular Sinusoidal Microsystems	*Microfluidic-based;* Chip-based microchannels for CTCs capture and detection	BioFluidica	Pancreatic	Use of electrical sensor for counting and determination		86%	[131,132]
LiquidBiopsy®	*Microfluidic-based;* Microfluidic chip with coated antibodies	Cynvenio	Lung, breast	High purity of detected cells	Non-specific binding and decrease in the sheath flow		[112,133]
GEDI chip	*Microfluidic-based;* Geometrically enhanced differential immunocapture using HER2 and PSMA antibodies	Cornell university	Breast, prostate	Correlation with primary tumor, treatment regimen	High purity and sensitivity	94%	[112,134]
Graphene oxide	*Nano-based;* Nanosheets are made up of graphene oxide functionalized cell surface markers	GO Chip	Lung, breast, pancreatic	High capture yield and low processing time	Minimum studies have been done; all of them are preclinical.	84%–90%	[79]
IsoFlux Rare cell Access systems	*Microfluidic-based;* Immunomagnetic capture system based on microfluidics	Fluxion	Breast, prostate	Ability to detect genetic alterations, high sensitivity for many tumors	Biomarker heterogeneity due to EMT; capture rate is 50%	50%–70%	[111]
ISET	*Nano-based;* Size exclusion approach for isolation of CTCs	Rarecells Diagnostics	Melanoma, breast, lung, pancreatic	Efficiency higher than CellSearch systems and label-free technique	It is a multistep process, so it damages CTCs.	76%–82%	[135]

6. Summary and future perspectives

CTCs are heterogeneous, individual or groups of cells that invade into and traverse through the vasculature after escaping from the original tumor. CTCs can be used as a promising cancer biomarker in early cancer diagnosis and therapy. The detection and isolation of CTCs from the circulatory system provides a non-invasive alternative to other cancer theragnostic approaches. Traditional CTC isolation and detection strategies are based on either positive or negative selection. However, these traditional methodologies are limited by multiple factors, such as low abundance in peripheral blood, high cost, leukocyte contamination, long time required to acquire data, low throughput, etc. The most challenging part of CTC isolation is that it is not that easy to differentiate between the CTCs and true cancer cell lines due to differential expression on markers. The nature of CTCs varies in

different cancers, among individuals and within the same patient. This further increases the complexity of the isolation and identification process. Cell heterogeneity of CTCs is why researchers are not confident enough to claim a single biomarker that can be used for CTC isolation. EpCAM has been widely used as a biomarker for CTC isolation; however, not all malignant tumors express EpCAM as a surface biomarker. Isolating CTCs and their early detection using effective techniques can be a reliable modality for cancer prediction and can be helpful in cancer diagnosis and therapy. Hence, it is the need of the hour to develop practical, low-cost, and reliable methods for CTC isolation and detection methods that can be useful for accurate identification of cancer.

Over the last decade, several efforts have been made to overcome the existing issues, utilizing microfluidics and nanotechnology-based approaches. This review discusses the recent advancements pertaining to these microfluidics and nanotechnology-based approaches. The incorporation of nanomaterials into microfluidic channels has provided a better strategy for developing new devices for CTC isolation and detection. Despite the advancements in CTC isolation and detection methods using nanotechnology and microfluidic-based techniques, many significant challenges are yet to be addressed. The available techniques need more improvement to enhance sensitivity, efficiency, and accuracy. Besides, these devices need clinical validation and market utility. Most of these methods remain in the laboratory and must be translated from lab to clinic/bench. An in-depth investigation of the existing challenges and incorporation of advanced tools, such as Artificial Intelligence machine learning coupled with bioinformatics, may aid the scientist and researchers in developing novel and more efficient CTC isolation and detection strategies in the near future.

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.

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