



Recent Nano Technological Approaches on Capturing, Isolating, and Identifying Circulating Tumor Cells

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ABSTRACT: Nano technological approaches are the latest modality for early stage detection of cancer. The need of rapid, non-invasive, patient specific, and informative techniques in cancer diagnostics lead to the utilization of nanotechnology, microfluidics, and lab-on-a-chip platforms for liquid biopsy, and the developments through these technologies increased the knowledge also for case specific applications. In this review, nanotechnology-based methodologies that are developed in the last decade for cancer diagnostics are investigated and are discussed under four main categories for the purpose of simplification as; Nano chip based, Nano film based, magnetic nanomaterial-based methods, and combinational utilization of multiple methodologies. We suggest a combinational approach on device development with an aim of producing a compact, cost effective, rapid, sensitive, and non-invasive diagnostic device as a conclusion of literature review.

Keywords: Cancer, Circulating Tumor Cells (CTCs), Liquid Biopsy, Translational Oncology, Nanotechnology.



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1. Introduction

Cancer is one of the leading causes of death in the world; the Global Cancer Observatory (GCO) estimated during 2018. The inadequacy of current technologies; tissue biopsy and imaging, to discover the cancer in early

stages while it is more treatable leads the research focus to other alternatives that are more informative and are less invasive [1-2]. Liquid biopsy is a promising alternative to the current technologies due to its ability for rapid and multiple analyses of samples, and non-invasive features. The recent developments in nanotechnology, microfluidics, and lab-on-a-chip platforms enabled the liquid biopsy applicable; supplying the demand of non-invasive, rapid, and informative techniques [3]. The latest developments in the field of capturing and identification of CTCs suggested new possibilities to answer questions that were left unanswered previously; like effects of the cancer type on CTCs, dynamics between tumor type and CTC capturing technique, and prognostic significance of CTCs [4]. CTCs are cells that are detached from tumor side and entered blood or lymph circulation of the body, which is a crucial step in metastasis but also has great clinical significance for cancer diagnosis [5]. During their passage to blood or lymphatic vessels, CTCs are often challenged by immune cells causing them to be rare in the blood stream however they have been seen in patients with almost all types of cancer which makes the isolation of CTCs more significant and also more challenging [6-7]. Another factor that is causing challenge in isolation and detection of CTCs is that there is no single marker that can distinguish the cells; and for reliable diagnosis high-purity isolation of the CTCs should be carried out [8]. It has been previously stated that the CTC counts in blood correlate with the clinical stage of cancer, metastasis, and relapse; further improving the importance of detection of CTCs with lower occurrence in blood [9]. As the target of liquid biopsy platforms extracellular vesicles and various other cancer biomarkers such as; circulating tumor cells (CTCs)

had a lot of attention from the researchers to become the new golden standard for cancer diagnosis [5]. Today various new techniques had been developed to isolate or detect CTCs; microscopic biomechanical immunocytology-based and polymerase chain reaction (PCR) based techniques are included in detection methods. However, these methods are hindered by aforementioned challenges of low CTC count in blood, and also absence of a distinguished single marker [10-15]. The development in nanotechnology delivered the potential to identify the CTCs before any symptom is appeared, and well earlier than conventionally used methods [16-20]. Advanced nanotechnological developments and their applications; which are highlighted through this review, enabled CTC detection and cancer diagnosis at its early stages.

State of Art

There have been various studies on CTCs and the majority can be categorized based on the techniques that had been used as; Nano chip-based, nanofilm-based, magnetic nanomaterial-based methods, and combinational utilization of multiple methodologies, figure 1 demonstrates these concepts.

As summarized and compared in Table 1, each method provides an edge towards isolation, detection, or identification of CTCs; while nanochip-based techniques utilize microfluidic technologies with the aid of specific antibodies and physical isolation methods, magnetic nanomaterial-based methods exploit magnetic properties of various nanomaterials, Their interaction with CTCs and other cells, and electromagnetic potential of cells, and nanofilm-based

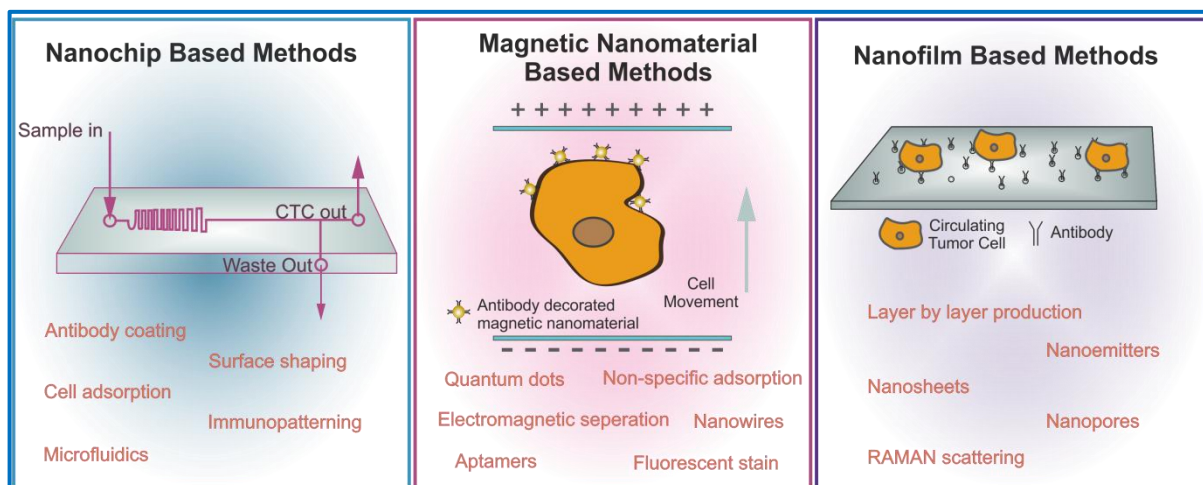


Figure 1. Nano-technologies that are utilized for capturing, isolation, and detection of CTCs

Table 1. Summary of various studies on Capturing, Isolating, and Identifying of CTCs.

	Function	Device principle	LOD* (cells/ml)	Response time	Sample type	Cost**	Reference
Nanochip based methods	Capturing and detection	Quantum dots functionalized with EpCAM	8	60 minutes	Cancer cell line	\$\$\$	Wu et al., 2018
	Capturing	Microfluidics	5	5 hours	Clinical samples	\$\$	Huang et al., 2019
	Capturing	Amide functionalized surface	NG	NG	Cell models	\$	Qin et al., 2017
	Capturing	Dual immunopatterned surface	NG	NG	Clinical samples	\$\$	Kang et al., 2018
	Detection	Electrical impedance spectra measurement	3	10 minutes	Clinical samples	\$	Nguyen et al., 2018
	Capturing	Microfluidics and antibody functionalized surface	100	Several minutes	Clinical samples	\$\$	Liu et al., 2013
	Capturing and detection	Microfluidics and antibody functionalized surface; DNA extraction	155	NG	Clinical samples	\$\$\$	Sequist et al., 2009
Magnetic Nanomaterial- based methods	Isolation	Peptide functionalized magnetic nanoparticles	50	30 minutes	CTC spiked blood samples	\$\$	Bai et al., 2014
	Capturing	Magnetic nanowires	0.14	NG	Clinical sample	\$\$	Hong et al., 2016
	Detection	Targeted magnetic fluorescent stain	50	30 minutes	CTC spiked cell line	\$\$	Sun et al., 2017
	Capturing and detection	Folic acid attached magnetic nanoparticles	20	2 hours	Clinical samples	\$	Li et al., 2018
	Capturing and detection	QD capped DNA aptamers with magnetic nanoparticles	25	NG	Clinical samples	\$\$	Li et al., 2019
Nanofilm based	Capturing and detection	Reflectometric interference spectroscopy measurements	1000	5 minutes	Cell line	\$\$	Kumeria et al., 2012

	Capturing and isolation	Enzymatic and degradable nanofilm layers	3.4	NG	Clinical samples	\$\$	Li et al., 2015
	Detection	RAMAN scattering	NG	NG	Clinical samples	\$\$\$	Krasnoslobodtsev et al., 2015
	Detection	Ratiometric electrochemoluminescence	2	3 hours	Cell line	\$\$	Feng et al., 2016
	Detection	Electrochemical sensor utilizing gold nanoparticle-grafted functionalized graphene	2	30 minutes	Cell line	\$\$	Salahandish et al., 2018
Combinational methods	Capturing and detection	Surface plasmon resonance measurements	13	Real time monitoring	CTC spiked blood samples	\$\$\$	Mousavi et al., 2015
	Isolation and detection	CTC enrichment via magnetic nanoparticles and microfluidics	NG	NG	CTC spiked blood samples	\$\$	Gourikutty et al., 2016
	Isolation and detection	Magnetic nanoparticles and size based discrimination with nanoporous system	1	NG	Clinical samples	\$\$	Ko et al., 2017
	Detection	Amperometric immunosensor with targeting aptamer	2	60 minutes	Cell line	\$\$	Zhou et al., 2019

*Limit of Detection

**In terms of need of antibody utilization and external device usage.

NG: Not given or calculated in the related article

Methods include usage of nanomaterial decorated or attached surfaces with thin layers, nanopores, or similar nanostructures. Most of the nanochip-based sensors are used for detection purposes which utilize fluorescent labelings and stainings. On the other hand magnetic nanomaterial-based methods provide highly specific isolation assays while nanofilm-based methods allow more versatile approaches due to the structure of the film.

Nanochip-Based Methods

One of the landmarks of nanochip-based CTC detection technologies was a distinguishable design of a nanochip that carried out by Sequist et al. [21]. The

nanochip was manufactured from a standard microscope slide that has been etched to have 78000, μm -sized posts on it. These posts were etched by a specific pattern and coated with antibodies (EpCAM), for CTC capturing. Later captured CTCs were used analyzed through applied anti-cytokeratin staining and DNA extraction. The chip was tested on 116 blood samples; which yielded ~ 155 cells/ml from all samples. In a later work of another research a fast and efficient way of CTC isolation and capture was suggested. Their developed microfluidic platform utilized lateral displacement array and affinity-based cell capture and the device consists of one inlet, twelve outlets with capture and enrichment chambers. Enrichment was

carried out via microposts of 25 μm radius, after enrichment step affinity-based cell capture was carried out by antibody coated chambers. The enrichment factor of 1500 fold was achieved during the study and more than 50% capture was observed at a cell concentration of 100 cells/ml [22]. Later a label-free detection method was proposed by Lee et al., suggesting that EpCAM based techniques may cause overlooking of CTCs without the specific antigen while a label free approach might isolate them too. The study proposed μ -LaFF chip which included one inlet and outlet with filter gaps of varied sizes, in total 1050 holes, with various widths were fabricated by a conventional soft lithography method. Fluorescent labeling was carried out to confirm the captured CTCs, 2 to 12 CTCs were captured from 1ml blood samples of cancer patients. Although label-free capturing of CTCs provides a higher sensitivity it also is susceptible to non-specific cell adsorption. Qin et al. utilized dendrimers which either negatively or positively functionalize the surfaces to overcome this effect or also prevent the fouling of the microfluidic devices. Surface functionalized PDMS microfluidic device was utilized where amino groups were used and APTMS, G4 and G4+G7 dendrimers were attached. APTMS and G4+G7 surface adsorbed much more live cells compared to G4 surfaces [23]. Later on dual-immunopatterned microfluidic device, utilizing two different antibodies (EpCAM and anti-63B3 antibody), was suggested which would supposedly get through limitations of the single anti-body systems. The device consisted of circular chambers aligned in two similar layers, both containing evenly spaced microposts arrayed in rings. The rings had alternating antibodies conjugated and spaced apart from the neighboring rings. The experiment results showed that the device captures CTCs more efficiently than only EpCAM based devices with a yield of $\sim 95\%$ [24]. Wu et al. utilized Nickel pillars and poly (lactic-co-glycolic acid) (PLGA) nanofibers with quantum dots to develop a 3D electrochemical cytosensor for CTC detection. The nanochip is fabricated via soft lithography on an ITO glass slide and electrospinning of PLGA finally gold nanoparticles were attached to nanochip. The researchers exploited electrical conductivity of Ni micropillars and its special structure via quantum dots functionalized EpCAM antibodies. The suggested nanochip showed a detection limit of 8 cells/ml [25]. A novel method of CTC detection was suggested by Nguyen and Jen utilizing dielectrophoretic (DEP) manipulation and impedance

measurement. The device was designed to enable the enrichment and detection of CTCs; all cells in the sample were captured between two circular electrodes then an inward stepping electric field is generated to separate normal cells from CTCs. Impedance measurements are used for the identification of cancerous cells afterwards; and limit of detection was calculated to be 3 cells/ml [26]. A nanochip that is comparable to this sensitivity was developed by another research group; geometrically enhanced mixing (GEM) chip with a dislocation herringbone etch was used to capture CTCs to a capture rate of 87% and capturing purity of 99.58%. Limit of detection of this immune affinity chip is calculated to be 5 cells/ml [27].

Magnetic Nano material-based Methods

Magnetic nanomaterial-based methods have been started to be used widely for CTC isolation purposes; either as a complex with CTC specific antibodies or other with other agents which have affinity towards CTCs. EpCAM antibodies are widely used for CTC detection, a novel peptide; Pep10 functionalized iron oxide magnetic nanoparticles (Pep10@MNPs) is proposed by Bai et al. as an alternative. The designed peptide has high binding affinity that is comparable to EpCAM. Moreover, the technique allows viable isolation of CTCs for further analysis and reached around 90% capture of CTCs in spiked samples, which in case of controls with EpCAM antibody was around 91% [28]. Another method that is able to detect CTCs even at the early stages of non-metastatic cancer is developed exploiting magnetic properties of alumina oxide and using five different types of antibodies coated on nanowires. CTC isolation was carried out with precision in 29 patients out of 29 breast cancer patients with small amounts of blood sample requirement (250 μl – 1 ml). As a result of developed system, the promise of early detection and detection of non-metastatic cancer offer great clinical significance [29]. Another study was carried out with the motivation of utilizing other molecules than antibodies and specifically EpCAM because of its detection restrictions, and relatively high cost of the assay. For this purpose, fluorescent staining and magnetic nanoparticles (Fe_3O_4) were utilized that targets tumor cells. The first step of the study was to synthesize IR780 preparation of IR780- Fe_3O_4

nanoparticles. It was claimed that the nanoparticles could target a variety of CTCs, and distinguish tumor cells from healthy cells in simulated blood [30]. Later Folic acid (FA) functionalized magnetic nanoparticles were developed by Li et al., with same motivation as previous study; using other molecules than antibodies for more affordable biosensors. It is concluded that FA nanoparticles provided efficient capture of CTCs from blood without any pretreatment. The CTC detection was possible at concentration as low as 20 cells/ml with minimal non-specific adsorption and the captured cells showed high viability [31]. A study suggested the utilization of DNA aptamers for high selectivity CTC detection with magnetic nanoparticles for isolation and quantum dots (QD) for enumeration. DNA-capped QDs and Fe₃O₄ functionalized nanoparticles were prepared for this purpose. The results revealed high capture efficiency of CTCs from human blood samples and with a high purity around 80%, with CTC concentrations as low as 25 cells/ml (Li et al., 2019) [2].

Nanofilm-based Methods

Nanofilm-based methods are considered to be one of the most suitable way to fully utilize the interactions between CTC and nanostructured surfaces. Kumeria et al. developed a nanoporous sensing platform that utilizes reflectometric interference spectroscopy; multiple functionalization steps were followed to reach desired attachment of EpCAM antibodies on anodic aluminum oxide (AAO) which is fabricated by electrochemical anodization of aluminum foil. Results showed that the proposed platform was able to measure cell concentrations down to 1000 cells/ml with a small amount of sample (<50µl) [32]. In another study a capture and release methodology was developed with the purpose of isolating CTCs without harm. Suggested platform consists of enzymatically degradable nanofilm layers, conjugated with specific cell marker antibodies. A layer-by-layer approach was followed for fabrication of the platform and achieved 80% capture and 95% release efficiency in spiked samples. In patient samples it was possible to achieve a lower detection limit of 3.4 cells/ml compared to control samples where it was as low as 0.5 cells/ml [33]. Another promising methodology is a Surface-Enhanced Raman Scattering (SERS) that carries a potential for detection of low levels of CTCs in cancer

patient blood samples, with this foresight Krasnoslobodtsev et al. Developed and optimized a SERS-based assay. The method briefly includes coating of mica surface with thiol-functionalized gold nanoparticle solution and 4-nitrobenzenethiol (NBT) where NBT is utilized as Raman reporter molecule. It is concluded that the developed platform successfully distinguishes healthy individuals from patients [34]. Later a dual-potential ratiometric electrochemoluminescence (ECL) based platform was developed to increase the detection sensitivity. For this purpose, carbon nitride nanosheets and luminol-reduced gold nanoparticles were used as nanoemitters and gold nanoparticles were used to conjugate aptamers for CTC capture. The sensor showed a lower detection limit of 2 cells/ml depending on glycan expression on cell surface [35]. Another electrochemical sensor was proposed recently utilizing gold nanoparticle functionalized graphene and nanostructured polyaniline (PANI) which reached a wide linear response between 10 – 5 x 10⁶ cells/ml with a lower detection limit of 2 cells/ml and a response time of 30 min. Furthermore, the gold nanoparticles were replaced with silver nanoparticles, decreasing the cost of the device providing a low cost, rapid, and sensitive detection of CTCs. The platform was composed of three layers on fluorine tin oxide (FTO); graphene layer, silver nanoparticles, and PANI. Onto the three-layer nanocomposite structure HER2⁺ antibody was attached, providing affordable, sensitive, and robust platform for detection of CTCs (Salahandish et al., 2018) [36].

Combinational Methods

Combining two or more of the previously mentioned techniques for isolation and detection CTCs is suggested to improve efficiency and precision of assays. A label-free Surface Plasmon Resonance (SPR) based technique was developed by Mousavi et al., utilizing magnetic nanoparticles for isolation of target cells and SPR technology to enhance the detection limit. Antibody functionalized (Ab1) magnetic nanoparticles were used to isolate CTCs in a magnetic field and a secondary antibody (Ab2) was then used to capture the antibody attached CTCs on a surface with gold nanoslits. Later on CTC captured nanoslits were used for SPR measurements and a lower detection limit of 13 cells/ml were achieved while also allowing real time monitoring of the capturing

process [37]. A combination of microfluidic technologies and magnetic properties were utilized to develop a CTC enriching chip by Gourikutty et al., the chip was designed to firstly deplete white blood cells, then red blood cells; retaining only CTCs. First diluted blood was mixed with antibody functionalized magnetic nanoparticles that target white blood cells, and the white blood cells are separated via magnetophoresis. Then the red blood cells were depleted by a micro-slip membrane. Developed microfluidic nanochip was successfully removed around 99.98% of white blood cells and achieved a CTC recovery of 80% [38]. Ko et al. took another approach to overcome detection challenges of rare CTCs and also low throughput isolation. CTC Fluorescence In-Situ Hybridization (CaTCh FISH) chip was designed for this purpose. First non-cancerous cells in large portions were removed via size exclusion in the chip; cells that are similar sized with CTCs were labeled with magnetic nanoparticle

s. Afterwards micropore size-based sorting structures were used to separate red blood cells and platelets. Lastly single cell RNA analysis was performed in this CTC enriched medium left, utilizing newly developed rapid FISH assay. The clinical trials showed high sensitivity; 11 out of 12 patient samples were positive with small number of CTCs (<1 cell/ml) and when higher volumes of blood sample used (>10 ml) extremely rare CTCs can also be detected [39]. Recently, an amperometric immunosensor was developed by Zhou et al.; nucleolin-targeting aptamer (AS1411) (CP) was attached to the gold coated surface to capture tumor cells; a nanoparticle that consists Pt, horseradish peroxidase, and CP was used as a catalytic probe, and infinite coordinate polymer of ferrocenedicarboxylic acid attached tyramine (ICP@Tyr) was utilized to reach a sandwich-like assay. Attachment of tumor cells to gold surface followed by addition of Pt nanoparticles, and H₂O₂ and ICP@Tyr H₂O₂ reduction peak in the current of ICPs generated was measured and used for detection of cells. The assay had a lower detection limit of 2 cells/ml and was able to distinguish CTCs from blood cells with great specificity [40].

Conclusion and Future Aspects

Through the last decade research on CTC and CTC isolation, detection, and identification techniques flourished. All the methodologies discussed above

reaching a lower detection limit around 2 cells/ml indicate that developed assays and technologies become to achieve great specificity and sensitivity. The papers that were discussed through this review are summarized in Table 1. Highest sensitivity was achieved by Hong et al., among the included articles, which utilized a magnetic nanomaterial-based method. Methods developed by Feng et al., Salahandish et al., Ko et al., and Zhou et al. also reached proximal sensitivities. It is clear that through the last decade sensitivity of devices for CTC capturing, isolation, and identification has improved extremely.

Although there have been great development in cancer diagnosis and therapeutics, the demand of medicinal industry have been shifting through a point-of-care approach where sensitivity alone is not a decisive parameter. Diagnostic devices are also ought to have lower costs, portability, and deliver rapid responses without need of expert handling. Studies are receding from use of multiple antibodies because of its cost-ineffectivity and single antibody systems such as EpCAM do not provide the required specificity for CTC detection and identification. Without multiple antibodies the best alternative appears to be the combination of different techniques to reach the same specificity. Methodologies suggesting alternatives to antibody utilization usually have lower costs and most of them are applicable without the need of highly equipped laboratory conditions. On the other hand, portability requirement is satisfied by microfluidic technologies such as; nanochip based methods, which also bring ease to utilization; via simple injection of sample. Overall nanochip-based systems with integrated magnetic nanomaterial-based assays, or nanofilm-based assays, or both are seemingly where the technology and demand will carry researches in the upcoming years. In addition, device development studies are expected to be more focused to gather more information of CTCs. We foresee that along with advances in diagnostic systems for CTCs, progress in known data and available CTC libraries promise development of sensitive, simple, and inexpensive technologies in near future.

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