

Circulating tumor cell isolation for cancer diagnosis and prognosis



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Summary

Circulating tumor cells (CTCs) are tumor cells that shed from the primary tumor and intravasate into the peripheral blood circulation system responsible for metastasis. Sensitive detection of CTCs from clinical samples can serve as an effective tool in cancer diagnosis and prognosis through liquid biopsy. Current CTC detection technologies mainly rely on the biomarker-mediated platforms including magnetic beads, microfluidic chips or size-sensitive microfiltration which can compromise detection sensitivity due to tumor heterogeneity. A more sensitive, biomarker independent CTCs isolation technique has been recently developed with the surface-charged superparamagnetic nanoprobe capable of different EMT subpopulation CTC capture from 1 mL clinical blood. In this review, this new strategy is compared with the conventional techniques on biomarker specificity, impact of protein corona, effect of glycolysis on cell surface charge, and accurate CTC identification. Correlations between CTC enumeration and molecular profiling in clinical blood and cancer prognosis are provided for clinical cancer management.

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Introduction

Cancer is one of the terminal diseases that threaten human lives. According to the statistics from International Agency for Research on Cancer (IARC), there were 19.29 million new cancer cases and 9.96 million cancer deaths worldwide in 2020.¹ Clinical reports have revealed that cancer metastasis is the primary cause of mortality and accounts for more than 90% cancer-related deaths.^{2,3} Since vast majority of cancer cases are found at an advanced stage, with metastasis at their first diagnosis, the current cancer managements face serious challenges despite of the advanced medical diagnosis and therapeutics, including surgery, chemotherapy, radiotherapy, immunotherapy, cellular therapy, proton-heavy ion therapy and other physical methods. If cancer can be detected at early stage, combined with appropriate therapeutics, the chance of cure will be significantly increased. In cancer management, the evaluation of treatment effect, post-treatment monitoring, and early

warning of the risk of tumor recurrence and metastasis are critically important in cancer prevention, detection, and treatment. Therefore, different techniques on early detection of tumor biomarkers from various clinical samples have been developed and extensively researched. However, due to the complex nature of tumorigenesis and heterogeneity of the tumors, there have not been exclusive biomarkers with acceptable specificity for early cancer diagnosis. Although some serum markers, such as CEA, PSA, AFP, CA-199, etc. are currently applied as tumor screening markers in routine physical examination, none is guaranteed with absolute tumor specificity.^{4,5} To address this critical issue, the alternative methods for tumor liquid biopsy are emerging with great successes and promises.⁶

Circulating tumor cells (CTCs) are tumor cells that shed from the primary tumor or metastasis loci and intravasate into the peripheral blood circulation system spontaneously or even possibly resulting from surgical operations. Since its first discovery in 1869, critical and versatile roles of CTCs have been increasingly recognized in cancer biology, molecular profiling, and precision medicine via tumor liquid biopsy.^{7,8} In 1993,

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Professor Klaus Pantel from University of Hamburg, Germany, proposed a new concept of prototype which is equivalent to the current definition of “tumor liquid biopsy.”⁹ This concept was demonstrated with a toolbox refilled with circulating tumor cells, circulating tumor DNA, exosomes, and other markers in patients’ blood based on which the cancer patients were distinguished from healthy individuals, and classified in the different disease stages.¹⁰ However, due to significant variations among different tumor types, there has not been a universal tumor liquid biopsy technique that is valid for all cancer diagnosis and prognosis. Nonetheless, compared to ctDNA and exosomes, cell is a more complete biological entity that can provide dynamic information of proteins, DNA, RNA, etc. They can also be cultured *in vitro* to build CTC lines for further studies on metastasis mechanisms, prevention, and intervention.¹¹ To this end, an ideal technology must be sufficiently versatile to target a heterogeneous subpopulation of CTCs and enable cell-friendly release for further *in-vitro* culture and analysis. More important, targeting CTCs via different interactions between nanoprobe and tumor cells must curb the influence of serum protein in the physiological environment. CTC detection and identification are also complicated by its rarity (one CTC out of a billion cells), cancer heterogeneity, and dynamic changes in cell biological properties during the metastasis process. Clinically, molecular profiling based on CTC studies provides critical information for developing treatment strategies and monitoring metastasis and recurrence.

This review summarizes the most recent CTC research outcomes in both fundamental cancer biology and CTC detection technology. In particular, a new CTC isolation strategy based on the Warburg effect and

cancer cell surface charge is introduced and compared with the conventional techniques that rely on immunoaffinity reaction and size dependent recognition. According to the Warburg effect, cancer cells do not produce energy via oxidative phosphorylation in the mitochondria, but through the process of aerobic glycolysis characterized by high level of glucose uptake and secretion of lactic acid. The cross-membrane movement of lactate results in removal of the labile inorganic cations, leaving a net of negative charges on cancer cell surfaces which distinguishes them from the normal blood cells except for red blood cells. The level of the negative charge is dynamically regulated by glycolytic capacity. Taking advantage of this bio-electrical manifestation of the Warburg effect, various magnetic nanoprobe can be designed with surface positive charge enabling them to electrostatically bind onto the cancer cell surface for detection and magnetic separation. Compared to the conventional CTC isolation strategies, the charge-based approach does not require any biomarkers such as commonly used epithelial cell adhesion molecule (EpCAM), but much more sensitive due to strong electrostatic binding of the probes on cancer cells. Moreover, the glycolytic regulated surface charge is a hallmark characteristic of all cancer cells regardless of any phenotypic and genotypic differences. The biomarker independent cell targeting paves a new way to isolate a wide spectrum of CTC subpopulations for more accurate and sensitive cancer prognosis and feasible *in vitro* culture. This review places considerable emphasis on introduction to the charge-based CTC isolation strategy while giving historical assessment of previous achievements by technologies that rely on surface biomarkers and cellular morphologies (Figure 1).

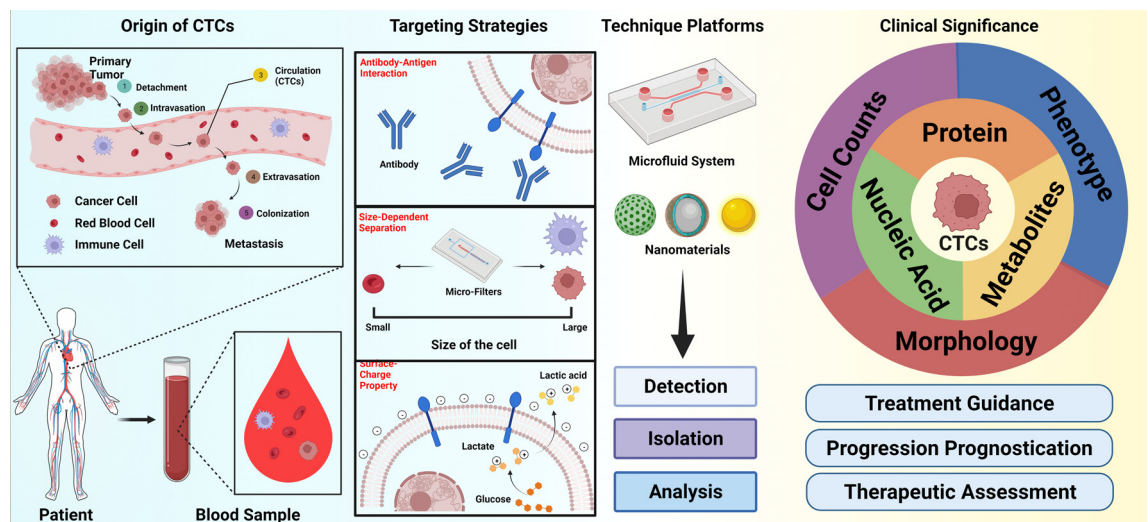


Figure 1. Schematic illustration of CTCs enrichment, analysis and clinical significance. Created with BioRender.com

Cancer biology fundamentals

Hanahan and Weinberg described the biological hallmarks of cancer that play the predominant roles in the processes of tumorigenesis, progression, and metastasis individually or synergistically.^{12,13} They showed that the biomarkers can be used to distinguish tumor cells from normal cells and attributed the origin of the biomarkers to carcinoma cells. Later, some of these biomarker characteristics were gradually recognized and utilized for identification of CTCs in blood samples. Numerous studies have shown that tumor cells are evolved from somatic cells, resulting from the synergistic effects of the accumulation of genetic damage, variations of genetic characteristics at the chromosomal level, specific environmental stimuli, and other factors. Among them, the classified proto-oncogenes and tumor suppressor genes play critical roles not only in regulating signal transduction pathways but also in reprogramming cellular metabolism.¹⁴ Moreover, human genes are complicated by variations of the physiological environments, thus typically characterized with heterogeneity, which is an intrinsic feature of tumors. As is well-known, differences at the genetic level can lead to differentiated expression of certain proteins in cells, and most solid tumors are of epithelial origin.¹⁵ During cancer development, cellular progression and metastasis are inherently linked to cellular communications and interactions. If the specific cancer cell surface molecules, such as protein and sugar molecules, are different from those on the normal cells, they can be used as biomarkers to identify CTCs.¹⁶

For detection of CTCs from the epithelial-derived tumors, cancer cell targeting through the differentiated EpCAM expression as the biomarkers has been extensively reported in the literature.^{16,17} The CTC-based tumor metastasis is clinically defined with five important stages: 1) tumor cells detach from primary tumor tissue; 2) dissemination in extracellular microenvironment (ECM); 3) intravasation into the circulatory system, mostly referring to the blood circulation system; 4) extravasation from blood circulation system into the microenvironment of an organ, and 5) the cancer cells carry the genetic or molecular information of the primary tumor, regulate the environment by interacting with immune cells and extracellular matrix cells in the microenvironment to facilitate the colonization, invasion and proliferation for metastasis.^{18,19}

Based on the breast cancer animal model, a proposed concept of “self-seeding tumor metastasis” that CTC metastasis *in vivo* is multidirectional and may move to the primary site.²⁰ CTCs have been found to accelerate the growth, angiogenesis, and stromal recruitment of tumor of origin through tumor-derived cytokines such as IL6, IL8, and CXCL2. CTC enrichment is mainly executed in stages 3 of the metastasis. Studies have shown that Epithelial-Mesenchymal Transition (EMT) takes place during CTC detachment from primary or

metastatic loci, which can promote tumor cell migration in the circulatory system, downregulate the expression of cell surface adhesive proteins, upregulate some mesenchymal markers, and provide CTCs with stem cell like biological features.²¹ Accordingly, a quantifiable, dual-colorimetric RNA–in situ hybridization (ISH) assay was established for a phenotype analysis of CTCs isolated from lung cancer patients’ clinical blood samples, in which three sub-phenotypes of CTCs were identified, exclusively epithelial, intermediate, and mesenchymal.²² However, EpCAM dependent CTC detection is usually found to have missed the partial CTC stem-cell like phenotypes, which compromises capture and recognition sensitivity.²³ In *CellSearch* system, 7.5 mL blood is needed and the cut-off value for cancer prognosis is 2 CTCs per 7.5 mL sample. Besides the epithelial cell markers, there are some other cell membrane protein biomarkers for the specific tumors, but their clinical applicability still needs to be evaluated through clinical trials. Considering the limitations of protein biomarker-based strategy, researchers started looking for new CTCs detection strategy with higher sensitivity.²⁴ Biophysical characteristics of tumor cell are independent of the surface protein markers during tumor development. The most direct biophysical feature is cell morphology, including cell size, nucleus-cytoplasmic ratio, cytoskeleton, and cell membrane plasticity.

Dr. Otto Heinrich Warburg first discovered that solid tumor tissue was a relatively hypoxic environment, in which tumor cells preferred to metabolize glucose through an anaerobic glycolysis consisting of high level of glucose uptake followed by secretion of lactic acid, even under the hypoxic circumstance.²⁵ This tumor cell abnormal metabolic behavior is distinctively different from that of the normal cells which mainly undergo aerobic respiration. According to the “Warburg Effect,” tumor cells produce large amounts of lactate through aerobic glycolysis, which pass through the cellular membrane via the mono-carboxylate transporter protein family (MCT-1 to -4) on the membrane in a perpetual fashion.^{26,27} The continuous secretion of lactate through cellular membrane disrupts the equilibrium of membrane potential by taking away cations such as H⁺, Na⁺, K⁺ resulting in a net of negative surface charge. As the charge is a direct consequential effect of lactic secretion, its level is proportional to the rate of glycolysis. This relation was established in our previous work where the cancer cell surface charge was correlated to the glucose level and monitored by a positively charged magnetic nanoprobe.²⁸ Due to electrostatic force, the positively-charged nanoprobe were found to strongly bind onto the negatively-charged cancer cells of all twenty-two different types.²⁸ These nanoprobe-captured cancer cells were then separated magnetically and counted as cell capture efficiency.²⁸ In addition, the cell capture efficiency was found to depend on the glucose

level provided, reflected on the one to one relationship between glycolysis rate and cell surface charge. The lactate secreted from the cells was varied by reducing the supply of glucose in the culture media of the cancer cells for 48 h and well correlated to the negative surface charge via cell capture efficiency. For instance, significant drop (17%) in the K562 cancer cell capture efficiency was found when decreasing the glucose concentration from 10 to 0 mM. This correlation provides the experimental evidence that the cancer cell surface negative charge is a bio-electrical manifestation of the Warburg effect.

More profoundly, this glycolysis-driven negative change on cell surfaces was observed on twenty-two different cancer cell lines. Chen et al. reported the percentage of cancer cells captured by applying both positively and negatively charged nanoprobles (NPs) respectively.²⁸ The cancer cells are mixed with the fluorescence labelled positively- or negatively- charged magnetic nanoprobles. In that case, HeLa cells were bound with numerous positive NPs due to the charge sign difference, while no significant binding was observed for the negative NPs, clearly indicating the cancer cell surfaces are negatively charged. It was found that although the binding efficiencies vary, all twenty-two different cancer cells were electrostatically bound to the positive NPs, and none is captured by the negative NPs, while 4 normal cells were left intact by either positive or negative NPs. Furthermore, positive NP cancer cell capture can also be reduced by inhibiting aerobic glycolysis and lactate production by 3-bromopyruvate (3BP) or Dichloroacetate acid (DCA), both are inhibitors of glycolysis. Those experimental results are consistent with the glycolytic-driven negative change on cancer cell surfaces.

Method of CTC recognition and impact of protein corona

There are several major approaches for CTC enrichment, which can be classified as, namely, magnetic beads platform, microfluidic chips systems, and hybrid systems which are designed and developed based on the different CTC capturing strategies. They can also be classified into positive, negative, and combined separation strategy according to the targeting cell types. [Table 1](#) provides the main characteristics of different cell targeting strategies and representative CTC isolation methods from the spiked or clinical blood samples of cancer patients. Compared to the enumeration results of CTCs from the clinical blood samples, the isolation efficiency of the cancer cell line spiked into the donor's blood sample is more reasonably determined. However, reliability of the cell line as surrogate of the CTC still needs to be verified by further investigations. The advantages and shortages of various platforms are also listed in [Table 1](#). Besides capture efficiency, purity, sample volume, and identification of the epithelial-to-mesenchymal

transition (EMT) are key factors for evaluating cancer metastasis and recurrence.

As shown in [Figure 2A](#), CTC recognition can be divided into two categories of immuno-affinity strategy and biophysical factors which respectively underlie the magnetic beads and microfluidic chip methods.⁵⁰ When in contact with the clinical samples, the magnetic nanoparticles with either conjugated ligands or electrically-charged are inevitably modified to exhibit new surface morphologies and bio entities due to formation of protein corona. In this fashion, the newly grafted surface corona structures will significantly render the biophysicochemical properties of the original nanoparticles at the nano-bio interfaces and in turn affect cell targeting. It has been reported by our and other groups that the protein corona on the surface of functionalized nanoparticles plays a dual-effects on the targeting ability of the original nanoparticles, which is determined by the physicochemical features of the nanoparticles to large extent. The dual-effects of electrostatic interaction and immuno-affinity on the CTC targeting ability by the protein corona decorated nanoparticles are shown in [Figure 2B](#). The characteristics of these targeting methodologies and the influence of protein corona on CTC targeting are described in the following sections.

Receptor-ligand interactions

The CTCs antigen-antibody recognition analysis system employs EpCAM as the hallmark. To overcome the limited sensitivity for CTC capture due to EMT, several cell membrane protein markers have been developed that are independent of EMT. A new biomarker VAR2CSA malaria protein was found to be effective for capturing tumor cells which otherwise would have been missed by the EpCAM dependent methods.³³ CD44 exon6 has also been used to recognize the phenotypes of cells that do not express EpCAM antigens.⁵³ The other candidate, aptamer, with specific design produced with help of SELEX technology, can be used separately to target specific CTC phenotype or combined for targeting different phenotypes of CTCs with improved specificity.^{54,55} In some cases, peptide was developed to identify CTCs as well.³¹ Both the magnetic bead and microfluidic chip platforms can be effectively integrated into the aforementioned receptor-ligand recognition approach.⁵⁶ In the magnetic bead system, the ligand can be directly conjugated onto the nanoparticle surface by chemical coupling, enabling cell binding for magnetic enrichment.

It should be noted that positive enrichment strategy aims at targeting tumor cells via surface-conjugated magnetic nanoparticles, while the negative enrichment removes other interference cells that are attached by the conjugated ligand.³⁴ For example, anti-CD45 antibody-coupled magnetic beads are used to bind and remove leukocytes from the red blood cell in the lysed blood samples, recovering the tumor cells in the supernatant.

Targeting Strategy	Isolation System Descriptions	Sample Type and Volume	CTCs capture number or Efficiency	Patients diagnosis accuracy	Capture Purity	EMT Identification	Ref.
Immuno-affinity strategy	CTC-chip, a microfluidic platform with antibody (EPCAM)-coated microposts under precisely controlled laminar flow conditions	Whole blood from patients; Average 2.7 ml per sample (range: 0.9 to 5.1 ml)	A range of 5–1281 CTCs per mL	≥ 5 CTCs/mL were identified in 115 of 116 (>99%) patient samples	Approximately 50%	No	29
	¹⁸ F-TO chip, a graphene oxide sheets functionalized with high-density anti-EPCAM antibodies and herringbone structures	Cancer cell spiked PBS buffer solution (50–200 cells/mL); 2 × 10 ⁷ MCF-7 cells injected canine models; 5 mL	≥ 90% at a flow rate of 1 mL/h in Cancer cell spiked PBS buffer solution; 212 MCF7 cells were isolated in ex vivo capture; 762 MCF7 cells were isolated in vivo capture;	-	-	No	30
	Peptide H13 (HER2-positive CTCs targeting)-functionalized nanomaterials	Cancer cell spiked Whole blood Sample (50 cells/mL); 1 mL	68.56% for SKBR3 cell; 20.04% for MCF7 cell; 79.26% for SKOV3 cell	-	-	No	31
	A controlled protein pre-modified immunomagnetic beads (IMBs)	MCF-7 cancer cell (2.5 × 10 ⁴ cells) and J774A.1 mouse monocyte macrophage cells (2.5 × 10 ⁶ cells) spiked 100% FBS; 0.5 mL	94.00% in cell spiked FBS at 200 μg/mL; 84.0–90.0% MCF-7 cells from mimic blood samples contained 25–55 MCF-7 cells	-	85.4% in cell spiked FBS at 200 μg/mL; 99.996% leukocytes were depleted	No	32
	The rVAR2 protein conjugated streptavidin coated magnetic CELLlection™, Biotin Binder Dynabeads®	Cancer cell spiked blood (Red blood cell lysed before isolation); 3–500 cells in 5 mL; Blood sample from patients (Red blood cell lysed before isolation); 5 mL	Average recovery was 60.0%–91.9% depends on the number of PDAC cells spiked in spiked samples; 12–23 cells were captured from 4 prostate patient samples	CTCs detected from 100% (4/4) of patients	Contaminating PBMCs was 82% less compared to the EPCAM-based capture strategy	Yes	33
Negative selection	Human CD45 Depletion Kit (immunomagnetic nanobeads)	SW620 cell spiked healthy blood samples (100 cells in 5 mL); Red blood cell lysis blood samples from patients 5mL for spiked blood samples; 20 mL for patients' blood samples	Average 58% (range 50–66%) for spiked blood samples;	CTCs detected from 56% (47/84) of peripheral samples	Total number of leukocytes: Before enrichment: 3 × 10 ⁷ ; After enrichment: 6.0 × 10 ³ .	Yes	34

Table 1 (Continued)

Targeting Strategy	Isolation System Descriptions	Sample Type and Volume	CTCs capture number or Efficiency	Patients diagnosis accuracy	Capture Purity	EMT Identification	Ref.
Biophysical feature strategy	Size dependent separation	HN5CC cells spiked whole blood samples (9–129 cells/mL); whole blood samples from HN5CC patients; 9 mL	Cell capture rate of 53.5% for unfixed cells; Cell capture rate of 65.3%, 73%, 73.1% at time points 0h, 24h, 72h using Transfix-collected samples; Cell capture rate of 51%, 32.3%, 6.0% at time points 0h, 24h, 72h using EDTA samples	CTCs detected from 50% (2/4) of patients	-	Yes	³⁵
	Optimized the Parsortix system	Cancer cell spiked whole blood samples (25, 50, 100 cells/mL); whole blood from prostate patients 7.5 mL	Capture rate of 25, 50, 100 PC3 cells spiked blood sample was 44.0% ± 4.0%, 48.0% ± 6.9%, 60.8% ± 5.2%; Capture rate of 25, 50 DU145 cells spiked blood sample was 57.3% ± 8.3%, 56.0% ± 8.7%; Capture rate of 25, 50 MCF-7 cells spiked blood sample was 54.7% ± 6.1%, 58.7% ± 13.3%	More than five CTCs in 90% (9/10)	3.1% ± 2.7%	Yes	³⁶
	Microfluidic devices with multiple arrays of crescent-shaped isolation wells in the microchannel	MCF-7, MDA-MB-231, HT-29 spiked PBS (1-3 cells/mL); 1 mL	80%	-	-	Yes	³⁷
	Isolation by size of Tumor cells (ISET) filtration	Diagnostic leukapheresis (DLA) samples contain CTCs and mononuclear cell populations; 10 mL	3.8 CTC/mL (IQR = 1.3–4.0)	CTCs detected from 88% (14/16) of patients	-	Yes	³⁸
Biophysical feature strategy	Surface charge based targeting	Red blood cell lysis blood samples from patients; 1 mL	2-8 CTCs cells/mL	CTCs detected from 100% (25/25) of patients	-	Yes	³⁹
	Others	Cancer cell and WBC spiked PBS (each 1000 cells/mL); Whole blood samples from patients; 5 mL	82% ± 5% of H1650 cell cancer cells from spiked PBS; Average of 417 CTC/mL (10.2–5068) from patient	CTCs detected from 100% (25/25) of patients	78% ± 1.8% of WBCs were removed from spiked PBS	Yes	⁴⁰
	ApoStream, a dielectrophoretic device separate cells by dielectric property of the cells	SKOV3, MDA-MB-231 spiked peripheral blood mononuclear cells (PBMCs); 1 mL PBMCs (isolated from 7.5 mL whole blood samples)	75.4 ± 63.1% SKOV3 cells (5000 cell spiked); 71.2 ± 61.6% MDA-MB-231 cells (500 cell spiked); Average 68.3% at low number of cancer cell spiked sample (4-23 cells spiked)	-	99.33% ± 0.56% of PBMCs were removed	Yes	⁴¹

Table 1 (Continued)

Targeting Strategy	Isolation System Descriptions	Sample Type and Volume	CTCs capture number or Efficiency	Patients diagnosis accuracy	Capture Purity	EMT Identification	Ref.
Combined Strategy	CD45+ Jurkat Clone E61 cell lines spiked PBS (7 × 10 ⁶ cells/mL); Cancer cells spiked blood samples (3 × 10 ³ , 4 × 10 ⁷ /mL); whole blood samples from patients; 10 mL for patient blood samples	~90% tumor cell recovery rate for Cancer cells spiked blood samples	-	-	-	Yes	42
	Microfluidic chips with ISET filtration combined aptamer modified microbeads and Anti-CD45 modified nanoparticle	91% for cancer cells spiked whole blood samples; 0–35 CTCs can be detected from patient blood samples from patients.	CTCs detected from 83% (5/6) of patients	Less than 0.01% of WBCs are detained	Yes	Yes	43
Combined Strategy	CTC-iChip, an integrated platform combined deterministic lateral displacement, inertial focusing and Magnetophoresis, for EpCAM positive cell line, anti-EpCAM antibody was functionalized on the magnetic beads, for negative depletion, anti-CD45 and anti-CD15 functionalized beads were used	98.6 ± 4.3% for SKBR3 human breast cancer cells; 89.7 ± 4.5% for PC3-9 human prostate cancer cells; 77.8 ± 7.8% for MDA-MB-231 "triple-negative" mesenchymal breast cancer cell line	≥0.5 CTC/mL in 37 of 41 (90%) prostate cancer patients	Removed 96.7 ± 1.9% for MCF10A cell lines; 97.0 ± 1.7% for the MCF10A-LBX1 derivatives	Yes	Yes	44
	A biomimetic microfluidic system combined with leukocyte membrane and SYL3C aptamer coated biomimetic nanoparticles	Above 91% MCF-7 cells could be detected in PBS and blood sample; 1:107/cancer cell spiked blood sample from mice (104 cells); 20 μ L	-	Almost no leukocyte non-specific adsorption	No for SYL3C aptamer	-	45
	EpCAM-targeted nanoparticles combined with microfluidic devices contains X-shaped velocity valleys	Near 100% when using 200 target VCap cells in spiked sample	CTCs detected from 100% (21/21) of patients	No more than 200 white blood cells were typically observed from patient samples	Report on the status of EMT	-	46
	Microchip-based immunomagnetic separation combined microchip and magnetic nanoparticles functionalized EpCAM antibody	Cancer cell capture rates are 90% for COLO205; 86% for SKBR3 2000 cells/mL, prepared in CellSave™ tubes; 2.5 mL	-	-	-	No	47

Table 1 (Continued)

Targeting Strategy	Isolation System Descriptions	Sample Type and Volume	CTCs capture number or Efficiency	Patients diagnosis accuracy	Capture Purity	EMT Identification	Ref.
Combined Strategy	<p>¹⁹CTC-iChip combined inertial separation array devices (RBCs and platelets removal) and magnetic beads (WBCs depletion)</p> <p>SDI-Chip with immunocoated micropillar surfaces combined EpCAM antibody selection and size dependent selection</p>	<p>Recovered 89.2 ± 5.7% CTCs from spiked samples</p> <p>99.2 ± 3.5% in spiked PBS buffer 92.2 ± 6.4% in spiked blood CTCs detected in a range of 8–161 CTCs/mL</p>	<p>CTCs detected from 100% (15/15) of patients</p> <p>CTCs detected from 100% (15/15) of patients</p>	<p>Removed 99.96% WBCs, 99.998%RBCs, and 99.998% platelets from spiked samples</p> <p>92.4 ± 4.6% in spiked PBS buffer 82.3 ± 3.8% in spiked blood</p>	<p>Yes</p> <p>No</p>	<p>Yes</p> <p>No</p>	<p>48</p> <p>49</p>

Table 1: Comparison of different CTC isolation strategies.

The advantages of the magnetic bead systems include facile scaled-up synthesis, easy surface modification, available characterization, and acceptable cost. The main challenge is the difficulty in purifying the tumor cell from leukocytes, resulting in a lower purity of the isolated CTCs, except for the magnetic nanoprobe with pre-treatment of surface property by serum protein or cellular membrane.^{32,51} In that study, a biomimetic immuno-magnetsome (IMS) is prepared by coating a leukocyte membrane (decorated with anti-epithelial cell-adhesion molecule antibody) on a magnetic nanocluster, enable superior CTC recognition efficiency and undetectable leukocyte background (Figure 2C). However, multi-step preparation pathway may increase the processing complexity and compromise the advantage of the low cost of magnetic beads.

Besides magnetic beads, microfluidic chips have recently attracted extensive interests in association with the concept of “Lab on a chip”, which has been elucidated in some review articles.^{57,58} With this system, the whole blood sample flows through the channels of the microfluidic chip at a moderate speed controlled by microvalves and micropumps. In this process, only CTCs are captured by binding onto the ligands-grafted surface of the protruding side with other background cells rinsing away. One of the well-known examples of microfluidic chips for CTC isolation based on the EpCAM biomarker strategy is the CTC-chip developed by Haber’s group at Harvard University in 2007. They reported a broad range of enumerated single CTCs isolated from 1 mL blood samples by the anti-EpCAM antibody-grafted micropillars of the chip from cancer patients’ peripheral blood sample, indicating good sensitivity of the method and reflection of heterogeneity of cancer progression.²⁹ To overcome the shortcoming of limited accessible blood sample volume during the *in vitro* CTC detection, a functional graphene oxide sheets decorated microfluidic system has been designed to continuously collect intravascular CTCs directly from a peripheral vein also based on the anti-EpCAM antibody targeting moiety (as shown in Figure 2D).³⁰ Section shape and pattern of the micropillar of the chip influence the flow dynamics and biomolecules reaction dynamics. How to balance between the processing efficiency and ligand-receptor reacting efficiency is one of the key issues to be addressed. To this end, Chaoyang Yang’s group at Xiamen University developed a tilted triangular micropillars array, which can change the flow pathway of both CTCs and normal blood cells to increase the collisions of the CTCs with ligands and reduce blocking of blood cells. As a result, capture efficiency, sensitivity, and selectivity of CTCs were considerably improved.^{49,59} Based on the foregoing, one can see various merits from the magnetic beads and microfluidic chips in the CTC isolation applications. The advantages of the microfluidic chip over the magnetic bead system lie on the enhanced purity of the captured

CTCs, automated operation process, and the ability of dealing with whole blood sample directly.⁴⁵ Despite of these advancements, overall efficiency of the microfluidic chip in processing liquid samples is still low, which remains to be improved for commercialized applications.⁶⁰ Furthermore, although microfluidic chips can be designed with numerous of micro patterns for cell interaction, the interfacial efficacy is not as good as nanomaterials. Therefore, the combination of magnetic beads and microfluidic chips may provide a better performance by taking advantage of the higher surface-to-volume ratio of the nanoparticles and improving purity of the microfluidic system.⁴⁷

Size dependent separation

Microfiltration membrane is a representative technology, such as the *CelSee* System, that sets a certain pore size to allow normal blood cells, which are generally smaller in size, to pass through the membrane, while tumor cells, larger in size, are trapped and separated.⁶¹ CTC enrichment using size and cell deformability as a marker has been extensively studied for the microfluidic chips. For example, the *Parsortix* system (as shown in Figure 2E) is a microfluidic chip with specific micron-sized microfluidic channels, which allows the cells with small size pass but block the larger-sized cells like CTCs.³⁶ Another effective platform to separate CTCs is the inertial flow microfluidic devices with a spiral micro-channel. The cancer cells are collected at the outlet of the channel according to different inertial forces on the cells due to size differences (Figure 2F).^{44,52,62–64} In addition, Prof. Chwee Teck Lim's group at the National University of Singapore designed a microfluidic chip with well-arranged micropillars inside the channel, with three to four micropillars as a group, allowing normal cells to leak through the gaps between the pillars while efficiently trapping tumor cells of larger sizes by a group of adjacent micropillars.³⁷ Except the microfluidic chips, it may be difficult for the filtering membrane to release the trapped CTCs due to cell deformation and entrapment in the membrane pore. Some commercial systems, such as the *CanPatrol* system, using immunonegative selection and size principle for CTC isolation have been validated for several different cancerous species for metastasis recurrence prediction. These systems can separate leukocytes from blood samples by CD45 antibody followed by removal of smaller size cells via nano-filtration membrane, and further classification of the isolated CTCs with different EMT phenotypes using immune-fluorescence staining.⁶⁵

CTC isolation via cancer cell surface negative charges – a non-biomarker approach

Due to tumor cell heterogeneity, there have been well-known limitations in the CTC capture strategies using

either biomolecular markers or size-dependence. A cell surface electrical charge-based approach has been recently developed for highly sensitive and efficient broad-spectrum CTCs isolation from clinical blood (requires only 1 mL clinical blood sample). Cancer cells exhibit the so-called “Warburg Effect” characterized by high glycolysis capacity. High levels of glucose uptake and lactate secretion are the two most distinguishable metabolic behaviors commonly observed from all cancer cells. The continuous secretion of lactate through cellular membrane disrupts the equilibrium of membrane potential by removal of the cell surface cations such as H⁺, Na⁺, K⁺ resulting in a net of negative surface charge. Metabolically, the cancer cell surface negative charge is dynamically regulated by glycolysis capacity and can be well controlled by glucose uptake. Sufficient glucose facilitates high level of glycolysis that results in greater negative surface charge. Chen et al showed a close correlation between the cell surface charge and the lactate secreted from the cells into the culture media by varying the glucose concentration.²⁸ Based on this unique metabolic behavior of cancer cells, unique magnetic nanoprobles (MNPs) were developed for cancer cell detection, capture, magnetic separation, and isolation. These MNPs were designed to be charge variable, label-free, fluorescent, and superparamagnetic for electrostatically binding onto the negative cancer cells.^{28,39,66–68} Recent works have shown that CTCs can be readily captured by positive NPs from clinical blood samples with high sensitivity without any protein biomarkers.^{39,66,67}

Charge-mediated CTC isolation (CMCTCI) has been widely applied in cell specific targeting.^{69–72} CMCTCI from the pretreated blood sample by red blood cell lysing has been proven to be an efficient strategy through EpCAM-independent tumor cells recognition.^{39,73} The charged NPs provide new bio-electricity-based cell targeting and capturing that is not relying on any protein-based biomarkers whose specificity has been a universal issue. Since the glycolytic-regulated surface negative charge is the hallmark characteristic of all cancer cells regardless of genotypic differences, the charge-based targeting will be unique, exclusive, and highly specific only to those that exhibit significant glycolysis. Therefore, this unique approach will address the universal non-specificity issue of cancer biomarkers and allow for development of new assay methods for early detection of cancer cells in clinical blood.

The captured CTCs can be magnetically separated and enumerated by immuno-fluorescence aided identification. These identified CTCs can be used for cancer diagnosis or screening, real-time long-term disease monitoring and even therapy guidance. Moreover, molecular analyses of CTCs can provide new insights into the biology of metastasis with important implications for the clinical management of cancers.

CMCTCI is known for several unique characteristics and advantages: 1) The cancer cell surface negative

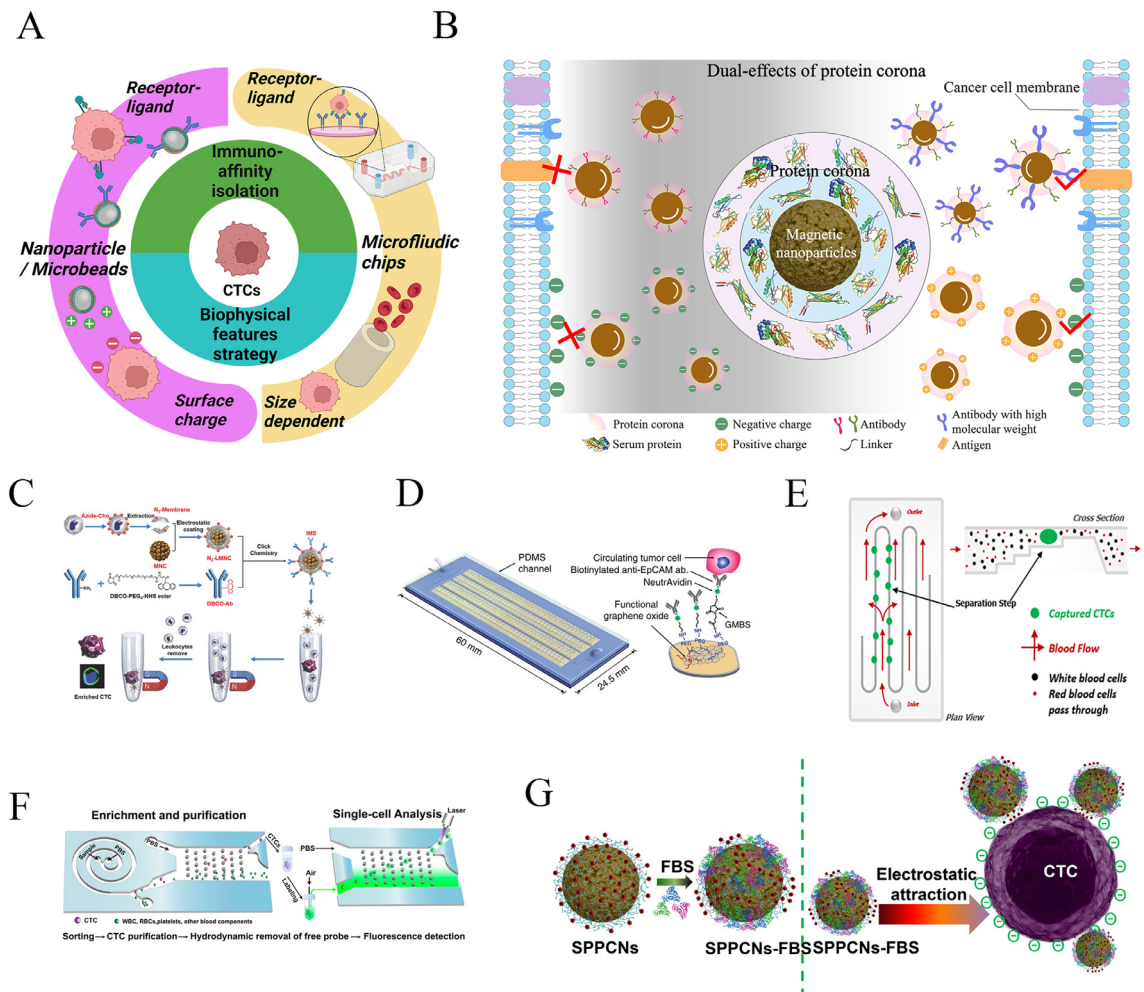


Figure 2. (A) Schematic diagram showing the main targeting strategies of CTCs isolation based on either nanoparticles or microfluidic chip platforms. Created with BioRender.com (B) Scheme showing the dual-effects of the formed protein corona on the binding ability of cancer cells by both immune-affinity and electrostatic interaction mediated magnetic nanoparticles, which can be tuned by the physicochemical feature of the functionalized nanoparticles. (C) Schematic illustration of construction of IMS and the procedure of CTC enrichment. Reprinted with permission from Ref.⁵¹ (D) Schematic of the ¹⁸BGO chip and the conjugation chemistry between functional graphene oxide and anti-EpCAM antibody. Reprinted with permission from Ref.³⁰ (E) Scheme of isolation principle inside the cassette, in which blood is forced along a series of channels and to flow through a 10 μm patented step which separates particles on the basis of size and compressibility. Reprinted with permission from Ref.³⁶ (F) Schematic of CTC sorting microchip with rapid CTC separation relying on inherent centrifugal force. Reprinted with permission from Ref.⁵² (G) Schematic of electrostatic interaction mediated cancer cell binding by the FBS corona decorated positively-charged magnetic nanoparticles. Reprinted with permission from Ref.³⁹

charge is directly regulated by high glycolytic metabolism which is the bio-electrical manifestation of the Warburg effect; 2) The cancer surface charge, as a bio-electrical analyte, can be quantitatively utilized for detection and capture of cancer cells without need of expensive monoclonal antibody which is required in the magnetic bead system and microfluidic chip platform; 3) CTC capture via glycolytic-regulated surface charge can address the problems of subpopulations with different EMT phenotypes, sizes, and chromosome ploidy with much

greater sensitivity in a clinical setting, and 4) Only 0.5~1 mL of blood sample is needed to complete the CTC capture and enumeration. Preliminary experimental results have shown the feasibility of the charge-based CTC detection in clinical blood of colorectal cancer (CRC) patients. Moreover, the sensitivity of the charge-based CTC capture has been compared with a commercialized inertial flow instrument using the same clinical blood samples from patients with lung, gastric, breast cancers, and melanoma.³⁹

Impact of protein corona

As has been reported, the nanoparticles are inevitably decorated with biological macromolecules when they are dispersed in the physiological media (for instance, human serum) and subsequently developing the so-called “protein corona”.⁷⁴ The biological decoration of the nanoparticles renders their bio-chemical-physical properties, ultimately altering the functionalities and behaviors in cell targeting, systemic circulation, fibrillation, cellular uptake, and biocompatibility. The protein coronas structurally and morphologically transform nanoparticles beyond recognition as compared to the original as-synthesized counterpart. Consequently, the effects of bio-decoration on a variety of nanoparticle properties have been extensively studied in many biomedical applications, such as bio-detection, nano-therapeutics, tissue engineering, and regenerative medicine.^{75–79} For cancer cell targeting, the protein corona on the surface of nanoprobe poses a dual-effects (Figure 2B). However, the level of corona influence during CTC isolation can be adjusted by modifying the physiochemical properties of the nanoprobe. Professor Caruso's group at the University of Melbourne demonstrated the impact of serum proteins on the targeting efficacy of the antibody-grafted inorganic microspheres.⁸⁰ It was found that in the presence of human serum albumin (HSA) alone, protein coronas exhibited a favorable effect on ligand-receptor interaction, while in the condition of many types of serum proteins, the targeting ability was eliminated due to alteration of the nanoprobe surface properties. In contrary, in the case of small molecular weight targeting ligands like transferrin or nanobody, the negative influence of the protein corona on the targeting ability is amplified to some degree (Figure 2B).^{81,82}

Since most of the serum protein molecules display a negative potential, they tend to bind onto the positively-charged nanoparticles by forming a protein corona. This is a critical issue in charge-based CTC detection and isolation as the protein corona can significantly alter the overall nanoprobe charge conditions. We investigated the effects of hard and soft protein corona formed on the positively-charged (MNCs \oplus) and the negatively-charged magnetic (MNCs \ominus) nanoprobe in the PBS solution with different FBS concentrations and its influence on the capture efficiency of HeLa cells.⁶⁸ At a moderate FBS concentration, Zeta potential of MNCs \oplus was rapidly reversed from +32 mV to -14 mV, and further changed to -15 mV after incubating in pure FBS solution. Interestingly, the FBS-MNCs \oplus complex with the reversed Zeta potential still kept nearly 78% HeLa cell capture efficacy, while both FBS-MNCs \ominus and MNCs \ominus showed negligible cancer cell binding efficiency (Figure 2B). With LC-MS analysis, different types of proteins were found to be absorbed on the positively- and negatively-charged magnetic nanoparticles with equal amounts of adsorptions.

However, when the Zeta potential of the FBS-MNCs \oplus complex was further dropped to -49.3 mV by introduction of polystyrene sulfonic (PSS), the HeLa cell capture efficiency dropped to the same level by the original negatively-charged counterpart.³⁹ Those experimental results indicate the strong electrostatic interactions between the tumor cells and the positively charged particles (MNCs \oplus) even with the particle surface-decorated corona. It is believed that the corona structure may not completely cover the surface of a positively-charged nanoparticle. Therefore, regions on the particle surface may locally appear positive resulting in considerable cell binding as shown in Figure 2G. Structurally, the conformation of the protein corona on the surface of MNCs \oplus may form islands rather than continuous layer.⁶⁸ Commonly, in the aqueous dispersion of charged nanoparticles, there are the stern layer and the diffusion layer according to the “Stern double layer” theory. The Zeta potential of nanoparticles measured by Dynamic Light Scattering (DLS) is calculated from the potential on the surface of shear. Therefore, the Zeta potential value is not equivalent to the available charge on the nanoparticle surface after protein adsorption, which is consistent with the conclusion in literature.⁸³

Identification of the isolated CTCs

Analysis of CTCs in clinical blood samples includes two essential steps, isolation and identification. Most of the CTC nanotechnologies focused on developing methods for efficient isolation of single CTCs and CTC clusters (e.g. CTC-Neutrophil clusters). Immunofluorescence staining has been widely applied to determine the types of the isolated cells using multichannel fluorescence staining of cytokeratin CK, nuclear DAPI, and leukocyte CD45 markers. Usually, the captured cells with the CK and DAPI positive and CD45 negative fluorescence signals are recognized as CTCs.²⁹ This method is quite repeatable, but the cells have to be fixed and mounted, a process that reduces the cell activity. Meanwhile, specificity of CK as a positive marker for CTC identification cannot be fully guaranteed.⁸⁴ Therefore, staining for EMT-related protein markers has been added to improve the accuracy and reliability in CTCs identification, such as EpCAM, Vimentin, etc. Typically, normal somatic cells are diploid, and more triploid karyotypes are present in all malignant tumor types.⁸⁵ In fluorescent photography, the cells with no less than three bright spots in the cell nucleus are considered as the cancer cells. This approach has been developed as an important tool known as RNA fluorescence in situ hybridization (FISH). Combined with immunofluorescence staining, iFISH is an effective method to increase the accuracy of CTC identification.⁸⁶

For clinical applications, there are still some shortcomings with the traditional immunofluorescence staining and iFISH: 1) staining has certain

requirements for the negative and positive control samples and staining techniques, 2) significant effort and time is required in searching for the positive cells via microscopy, but the results could be influenced by human errors, and 3) staining reagents for multiple protein or RNA marker are relatively expensive. It is, therefore, important to develop novel strategies to combine CTCs isolation and identification with high specificity and sensitivity. To this end, some techniques have been emerging^{87–89}: 1) developing the integrated system for CTC capture, separation, *in situ* fluorescence detection, and *ex-vivo* expansion based on microfluidic chips, 2) using fluorescence loaded MOF materials for fluorescent molecule release detection and fluorescence imaging of captured CTCs, and 3) entrapping a single cell within micron-sized droplets and labelling the unique uptake or metabolic substance with fluorescent signals. In addition, based on the traditional immunofluorescence staining process, replacing manual reading with automated reading equipment has been shown to have practical advantages.

Several novel systems for CTC isolation, capture and identification have been developed and technically available.^{7,90,91} The ultimate goal is to develop a CTC-based liquid biopsy in clinical setting for prognosis. Despite the rapid progress in this field, however, there is still a gap between nanoscience and clinical practice due to the interdisciplinary boundaries.⁹² More collaborations are therefore needed between physical and medical sciences in jointly developing the tumor liquid biopsy. Precise information on enumeration and molecular profiling of the captured CTCs will be critical for determining a specific clinical stage of the cancer patient. The CTC numbers must be well correlated with the clinicopathological results for assessment of cancer recurrence and metastasis. The CTC data must also be acquired from a large number of clinical samples in predicting the overall survival and progression-free survival of patients. As approved by Food and Drug Administration (FDA), *CellSearch* has been the globally applied CTC detection system for prognostic monitoring of metastatic breast, colorectal and prostate cancers.^{93,94}

On the other hand, molecular profiling of the CTCs can provide more significant insights into the tumor progression and metastasis.⁹⁵ Correlation of CTCs biology with clinical implications is needed on the basis of whole subpopulation CTC isolation and appropriate analyzing packages. Although single cell RNA sequencing technique has been proved to be effective, its accessibility and accuracy will have to be resolved.⁴⁴ Upon isolation, rapid CTC proliferation *in vitro* will enable more accurate sequencing, drug screening, and construction of CTC-based PDX model for drug resistance study.^{96,97} Currently, the CTC-friendly release is another important issue for the downstream molecular analysis and *in-vitro* proliferation.⁹⁸

Cellular viability of isolated CTCs

Effect of geometry of nanoprobes on the cellular viability

In our previous work, we studied the electrostatic interactions between cells and the nanoprobes of different geometries. Cytotoxicity of the nanoprobes was investigated on cell viability, collapse of cell membrane, and destruction of the cell pseudopod. Considering the spherical geometry of the nanoparticles (~100 nm) and the size of tumor cells (~20–30 microns), hundreds of nanoparticles were found to bind onto the surfaces of the tumor cells either through ligand-receptor or electrostatic interaction strategy. Theoretically, a micron-scale magnetic graphene sheet may trap the cells with much less membrane surface area therefore reducing cellular internalization and cytotoxicity. The 2D graphene oxide nano-sheets were decorated with the charged nanoprobes for HeLa cell capture.⁹⁸ It was found that the 2D graphene composites resulted in higher cells capture efficiency, but caused severe damages on cell membrane and pseudopods, leading to increased cytotoxicity compared with spherical nanoparticles. The cell damage was attributed to the sharp edges of the 2D thin films.⁹⁹

Effect of serum protein on the cellular viability

The roles of serum protein were investigated for the charge-mediated CTC isolation in our previous work.³⁹ This study addressed the critical issue of protein decoration on the nanoparticles in the physiological environment for clinical application of CTC detection. In charge-mediated CTC detection, the nanoparticles are rendered positively charged via PEI surface functionalization. However, PEI (Mw=25 kd) as a representative polycationic polymer is well-known for its toxicity to tumor cells. It is widely-recognized that nanoparticles tend to aggregate at cost of its colloidal stability after administration into the physiological environment.¹⁰⁰ Interestingly, the protein corona on the surface of MNCs⁺ can reverse the nanoprobes' Zeta potential from considerably positive to moderate negative, thus improving colloidal stability of the MNCs. In addition, FBS was found to provide certain protection for the rare tumor cells in the spiked blood samples (Figure 3A).³⁹ Thus, the capture efficiencies of spiked cells at different concentrations in healthy donors' blood samples when incubated in FBS and PBS after red blood cell lysing is apparently different. The well maintained cell viability contributes to the enhanced capture efficiency of HeLa cells in FBS.³⁹

Furthermore, it was found that the FBS-MNCs⁺ complexes efficiently captured 2–8 CTCs per mL clinical blood samples from 25 CRC patients, identified by immune-staining and iFISH staining. Using the same procedure, an average of 0.4 abnormal cells was captured and identified from 10 healthy donor's samples.

More importantly, from 1 mL of clinical sample of CRC patient, the CTCs with different sizes, chromosome ploidy, and EMT phenotype were detected successfully. Comparison of CTC isolation sensitivity between the label-free capture method and a commercialized inertial microfluidic device indicated better performance of the former, both were evaluated by using CD45/iFISH staining.³⁹

As for the negatively-charged magnetic nanocomposites, the capture efficiencies were much lower as compared to their positively-charged counterpart either in the medium of PBS or FBS. The bamboo-leaf like colloidal aggregates were formed after cell capturing due to protein corona decoration on the nanoparticles (Figure 3B). The formation mechanism of the colloidal nanoparticle assembly was identified involving multitude of parameters like protein types, protein concentration, magnetic force, and ions in medium. Using fibrinogen (Fg) and bovine serum albumin (BSA), two kinds of typical components of plasma, as models, the facile and reproducible colloidal assembly were investigated.¹⁰¹ It was found that the MNCs[⊖]-protein complex clusters acted as the seeds for the formation of the subsequent anisotropic self-assembly with the bamboo-leaf like morphology. This process was triggered by the temporary magnetic force and intermolecular electrostatic interaction of proteins on the nanocomposite surfaces. The one-dimensional fiber-shaped magnetic nanoparticle assembly was decorated by a variety of protein types. The formation mechanism provides the possibilities of constructing smart, dynamic, responsive, and biocompatible scaffolds for applications in tissue engineering and CTCs *in-vitro* culture.¹⁰²

As shown in Figure 3, the surface protein corona plays major roles in the dynamic interactions between the tumor cells and the surface-charged magnetic nanoprobe, which not only maintain the CTC viability in the blood sample but also enhance the colloidal stability of positively-charged nanoprobe (Figure 3B, 3C). These are critical functions to ensure highly efficient and whole subpopulation CTC isolation. On other hand, protein corona decorated nanoparticles may have important potential in biomedical applications that require both the protein structures and super-paramagnetic properties.

Cell release for molecular profiling and *in-vitro* proliferation

High cell viability can be well-achieved through efficient responsive release of the captured cells by the spherical magnetic nanoprobe. There have been extensive studies on the efficient release of the captured tumor cells using either the microfluidic chips or the nanoparticles platform through different release triggers, like light, sound, electricity, magnetism, heat, enzyme cleavage, pH value, hydrophobic force, polymer conformational

changes, ligand substitution, etc.^{7,55,98,103–105} Among these approaches, three concerns remain to be addressed: 1) the influence of the trigger on the cellular viability, 2) the impact of the protein corona formed on the surface of nanoparticle, and 3) the substrate effect on releasing efficacy.

We have developed an inorganic core-shell calcium oxide magnetic nanocomposite with strong positive potential.⁶⁶ The nanocomposite exhibits mild acidic circumstance responsive release property and high viable release efficiency without interference from the serum protein in the medium. It is worth noting that the pH value of the medium can be easily adjusted by changing the components' ratio of PBS for the *in vitro* cell culture medium without introducing any new acidic substances. This system has been initially validated in breast cancer patients' blood and ascites samples with peritoneal metastases.⁶⁷

With the highly efficient CTCs release techniques, powerful single-cell-omics technologies can be developed. However, as a cell population, CTCs are inherently heterogeneous. Therefore, for drug screening and treatment assessment, the captured CTCs must typically represent the tumor characteristics of the cancer patients.¹⁰⁶ Yu et al established brain metastasis mouse model based on *ex-vivo* cultured CTC lines isolated from blood samples of brain metastatic breast cancer patients. These data provided the direct experimental evidence on the promising role of CTCs as a prognostic factor for site-specific metastasis.¹⁰⁷

Clinical implications and significance of the CTC analysis

Since the early clinical applications of CTC enumeration from the patients' peripheral blood samples, tremendous progresses have been made in three areas: 1) prognostic evaluation and risk estimation for metastatic relapse,^{108,109} 2) therapeutic outcome assessment and medication guidance for oncology precision medicine,^{110,111} and 3) dynamic marker monitoring during the treatment.¹¹² *Cellsearch* has been approved by FDA for clinical prognostic monitoring of patients with breast, colorectal and prostate cancers. As this is a mature and commercial analytical system that can provide stable results in different laboratories, it has been widely and clinically applied and validated consistently for many cancer types. With a large number of clinical studies based on this system, the value of CTC count in the prognostication of metastasis has been confirmed.^{113–115} In addition, a study based on 5 independent randomized clinical trials has proved that the changes in the number of CTCs in peripheral blood at a specific stage demonstrate better accuracy than the conventional method of using Prostate-Specific Antigen (PSA) protein for predicting drug efficacy in the metastatic castration-resistant prostate cancer (mCRPC).¹¹⁶

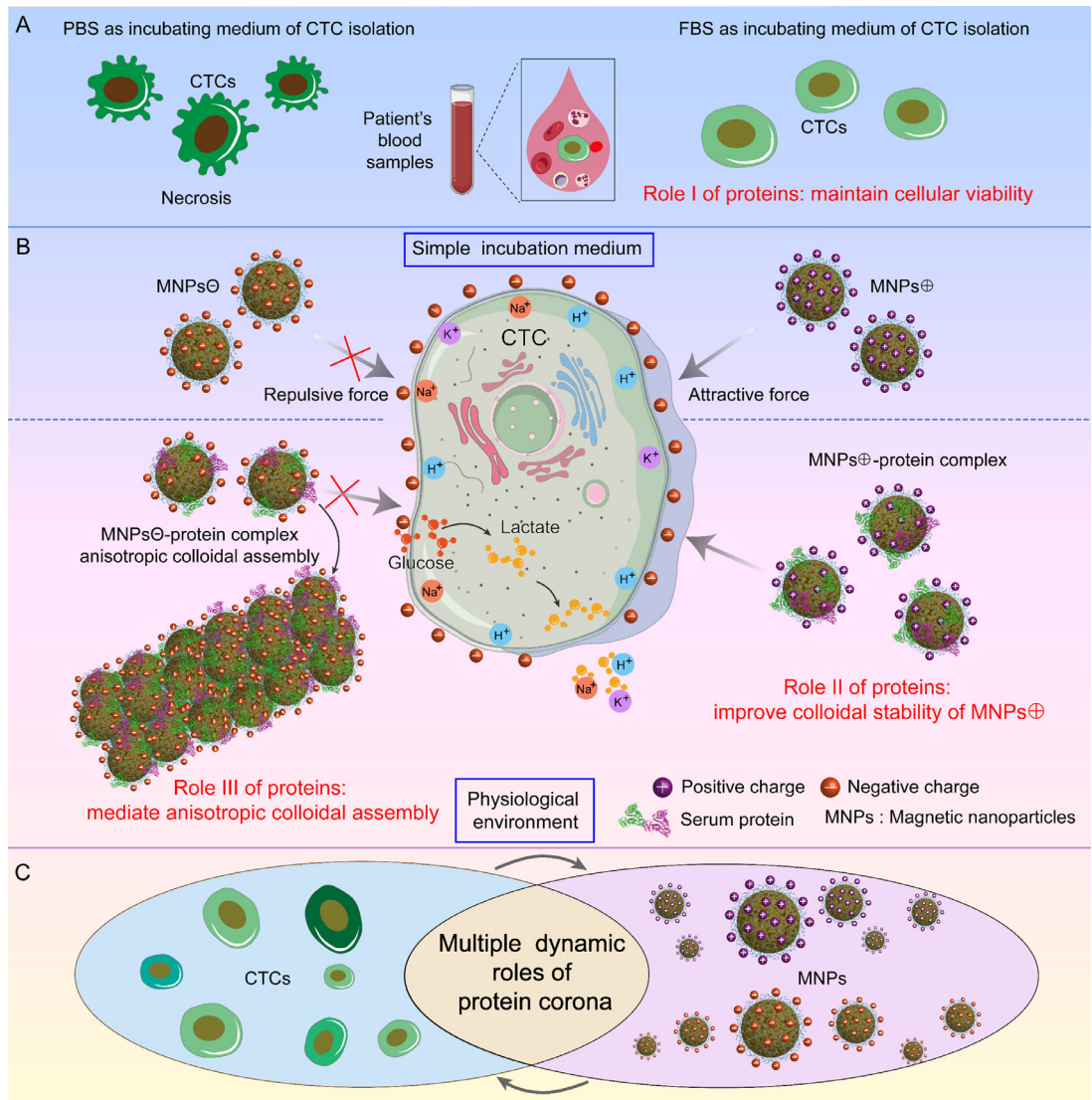


Figure 3. Schematic diagrams showing multitude of dynamic roles of protein corona in the interaction of cancer cells with the surface-charged magnetic nanocomposites. (A) Role I: maintain cellular viability of rare cancer cells including CTCs. (B) Role II: improve colloidal stability of positively-charged magnetic nanocomposites, Role III: mediate anisotropic colloidal assembly based on negatively-charged magnetic nanocomposite with the help of temporary magnetic force. (C) The multiple “bridging roles” of the protein corona in cancer cells targeting and isolation.

In addition to enumeration of CTC in the blood sample of cancer patients, correlation of cellular heterogeneity of CTC with clinical sequence via single CTCs molecular profiling and bioinformatics tools will be essential for translation of the fundamental understandings to clinical practice as illustrated by the scheme in Figure 4A.^{117–119}

Studies have also shown that CTCs with different EMT phenotypes play different roles in the clinical cytopathology and prognostic monitoring of tumors. Based on animal models of breast cancer and clinical samples, Wang et al investigated the heterogeneity of four EMT-

related CTC phenotypes and their relevance to migration, metastatic outgrowth and proliferation of CTCs or disseminated tumor cells (DTCs). These studies were carried out from the perspectives of morphological, molecular and phenotypic analyses, demonstrating the clinical value of EpCAM-positive CTC phenotypes for early detection and evaluation for the risk of metastasis in breast cancer.¹²² Another study employed the *CanPatrol* system for CTCs isolation and iFISH staining. Apart from CTC counts, the mesenchymal-CTC (M-CTC) percentage showed a clinical significance where the ovarian cancer patients with M-CTCs percentage ≥ 0.3 were

associated with a 2.1-fold increase of recurrence rate when compared to those with M-CTC < 0.1.¹²³ Besides, CTCs of ovarian cancer in liquid biopsy have been clinically applied for prognosis prediction and precision diagnosis.¹²⁴

As one of the markers in tumor liquid biopsy, CTCs can provide a variety of biological information such as protein, RNA, DNA and metabolites as compared with ctDNA markers,¹²⁵ which can be realized based on a microfluidic chip (Figure 4B).¹²⁰ Therefore, in addition to CTC count for tumor development monitoring, clinicopathologists and oncologists are increasingly interested in obtaining more information on cell biology and molecular biology from CTCs for clinical efficacy assessment in recent years.^{126–128} For example, Nagrath and colleagues reported a new way to allow real-time monitoring of immune activation of tumor by measuring PD-L1 expression in isolated CTCs during the radiation therapy (Figure 4C). In this approach, gene expression analysis based on the CTCs revealed that higher levels of PD-L1 were associated with poor prognosis, which implied the critical role of monitoring the dynamic changes of PD-L1 as one of potentially prognostic markers for responsive treatment.¹¹² There are two basic strategies to perform dynamic monitoring: 1) single cell technique based on the isolated CTCs, including mass spectrometry (MS) analysis of protein, DNA, RNA and metabolites from a trace of CTCs,¹²⁹ 2) CTCs *in vitro* culture to obtain a large number of CTC clusters for analyzing their various biological characteristics.¹³⁰ The former has the advantage of preserving the condition and biological properties of the original tumor cells. However, the rarity and weak signal intensity make the analysis difficult. The latter has a much higher number of cells with the increased signal intensity, but the changes at molecular level of the proliferated cells, as compared to the original CTCs, can create certain uncertainties. The microfluidic technology provides an excellent platform that integrates multiple functions including CTC isolation, enrichment, identification, and molecular analysis, for obtaining protein, gene, and metabolomics related information directly from blood samples. To improve the accuracy of CTC single cell sequencing by considering heterogeneity, it is necessary to effectively connect the different micro-regions with various functions in the microfluidic chip for suitable sequencing technology.

Yoon et al attempted a novel approach to address cellular heterogeneity in terms of gene expression and pathway regulation analysis using a massively parallel single-cell RNA sequencing (scRNA-seq) by using a microfluidic chip platform.¹²¹ They engineered the Hydro-Seq chips composed of 800 chambers per chip (can be expanded to an array of 12,800 chambers) with 16 branch channels. This Hydro-Seq chip utilizes size-based separation to isolate the CTCs from other blood cells. Using the Hydro-Seq chip, the captured CTCs can

be lysed in the chambers with the mRNA released from CTCs that are uniquely labelled by a barcode bead and identified using single-cell whole-transcriptome sequencing (Figure 4D). With multiple processes and valve valves controlled by pneumatics, the design is capable of various functions, such as protein expression level analysis, which provides a wealth of information that can be used in breast cancer gene sequencing.

Meanwhile, microdroplet and microfluidic platforms for metabolism, fluorescence analysis and sequencing of single cells are the emerging methods in the CTC studies. Combined with hydrogel microdroplets, there is a potential to integrate molecular analysis of single cells with *in-vitro* cell cultures, enabling CTC-based precision diagnosis and drug evaluation.¹³¹ However, this system still needs to be validated in more clinical samples. Although these platforms are rapidly updated, it is critical to further explore the clinical potential of single-cell sequencing. Three major types of systems are currently available for single cell analysis: (1) microdroplet systems, (2) microfluidic chips, and (3) microchips. The single-cell analysis focuses on transcriptome and genome analysis, where the controlling of the side products from CTCs specific amplification will be the key in obtaining the accurate genetic information due to rarity of CTCs. Furthermore, the analysis of protein expression from a few CTCs is also critical for cancer diagnosis and prognostic monitoring. A microfluidic western blotting technique was developed capable of reporting protein expression on a per CTC basis and two statistically distinct GAPDH subpopulations within the patient-derived CTCs from estrogen receptor-positive (ER+) breast cancer patients (Figure 4E). This targeted single-CTC proteomics has shown a capacity for multiplexed protein analysis that offers a unique, complementary taxonomy for understanding CTC biology and ascertaining clinical impact.¹¹⁹ After microfluidic isolation, the CTC protein can also be recognized by using an aptamer probe based on inductively coupled plasma mass spectrometry (ICP-MS), which exhibits a 73% measurement efficiency for single CTCs.¹³²

Conclusion and perspectives

As one of the important markers of tumor liquid biopsy, CTCs have been recognized to provide valuable information in both fundamental cancer biology studies and clinical diagnosis and prognosis. The single CTCs or CTC clusters isolated from peripheral blood can be used for prognostic monitoring and efficacy evaluation of many cancers. The molecular analysis of different CTC phenotypes associated with EMT can establish biological profiles of the metastatic tumors accurately. Longitudinal sampling and monitoring of tumor progression by CTC isolation before and after a specific treatment will provide dynamic molecular information for evaluating the driving cues for treatment resistance.

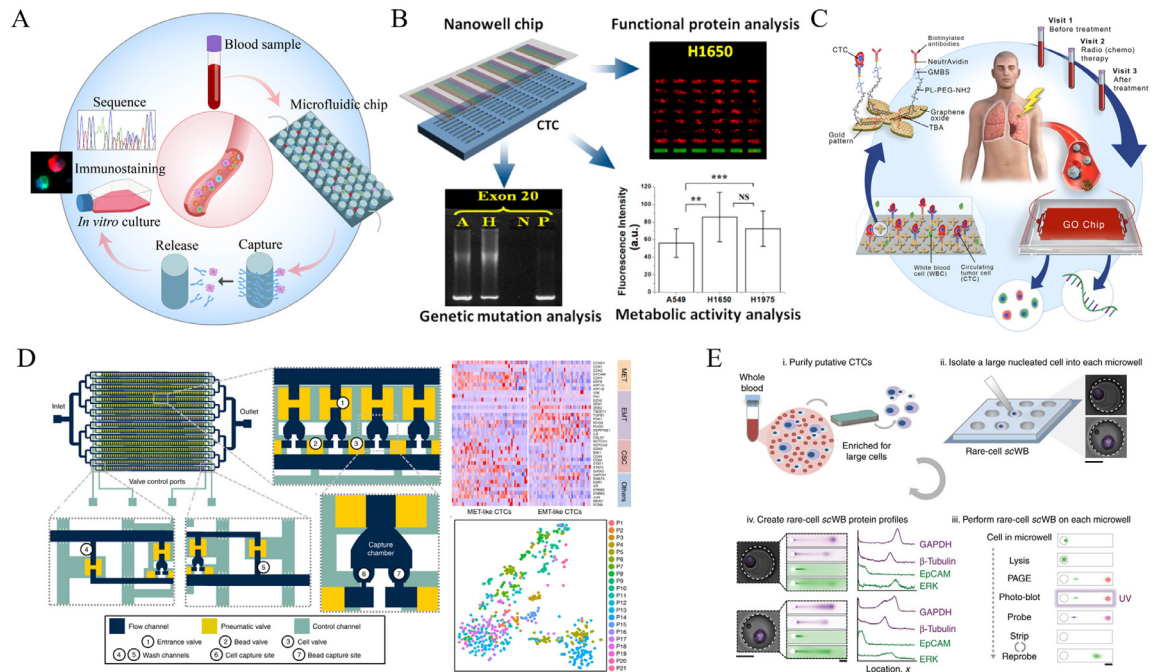


Figure 4. (A) Schematic illustration of molecular profiling of the captured rare CTCs for some representative microfluidic platforms. (B) Multiplexed biological information provided by isolated CTCs. Reprinted with permission from Ref.¹²⁰; (C) Real-time monitoring of immune activation of tumor microenvironment from isolated CTCs. Reprinted with permission from Ref.¹¹²; (D) Single-cell whole-transcriptome sequencing from isolated CTCs by a well-designed microfluidic chip. Reprinted with permission from Ref.¹²¹; (E) Multiplexed protein profiling of isolated CTCs through microfluidic western blot. Reprinted with permission from Ref.¹¹⁹

Additionally, cancer heterogeneity, including different patients, stages, and cells, presents both challenges and opportunities for precision diagnosis and treatment. The current CTC studies call for development of stable, reproducible, and clinically viable CTC enrichment technologies for analyzing tumor cells at genomic, phenotypic, and cellular levels. Integrated with proteins, nucleic acids and metabolites analysis at the single cell level, CTC studies will deepen the understanding of the underlying mechanisms of tumor metastasis and recurrence. Nanoparticles are inevitably decorated with protein coronas when dispersed in the physiological solutions. The interfacial behaviors between the nanoparticles and the corona through the biological environmental dictate the outcomes of CTC detection, capture, and isolation. *Ex-vivo* culture of the captured CTCs can be used for establishing the mouse metastasis model and *in-vitro* drug screening. Therefore, there is an urgent need to achieve stable and efficient capture of CTCs for phenotypic analysis, single cell biological characterization, and nano-bio interface studies that are extremely valuable for both fundamental cancer biology research and downstream applications. To this end, some critical issues need to be addressed in future research for clinical practice: (1) develop clinically viable CTC isolation strategies against specific requirements of liquid biopsy. (2) Target those that have not been well

reported such as head and neck cancer etc. (3) need more powerful and facile methods to identify CTCs without reducing its cellular viability. (4) With more advanced techniques for CTC ex vivo culture, calibrate different CTC isolation techniques by using cancer patients derived CTC-line as the standard base for evaluating the efficacy of CTC isolation.

Outstanding questions

Although CTC isolating technologies have been advanced in many areas, critical issues remain in detection sensitivity, technical and commercial viability, and CTC identification accuracy. The potential drawback of the CTC capture and detection system is the limitations in treating a small quantity of sample with multiple washes to increase sensitivity and the fractured identification of captured cells. The nano-probe based capturing may be tailored to simultaneous, small, multi-sample screening. For clinical settings, the key question is the possibility of developing a device that is capable of high throughput and large volume of blood sample filtering with synergy of CTC isolation, release, and molecular profiling. If CTCs can be efficiently captured from clinical blood, is it possible to develop a technique to remove CTCs from the blood in a continuous fashion, a process like *Dialysis*, for people with kidney failure.

Search strategy and selection criteria

The search strategy of this review relies on the most current research papers published in major medical and nanoscience journals focusing on circulating tumor cells (CTCs) and related cancer studies. We have applied explicit methods to perform a comprehensive literature search & critical appraisal of individual study searches of Web of Science, MEDLINE, Current Contents, PubMed, and references from relevant articles using the search terms such as “circulating tumor cells”, “Warburg Effect”, “protein corona” and “metastasis.” The selection criteria are based on research works published by well-known experts in the field of CTC studies, cancer biology, oncology, and pathology. Our own research on this topic is also included in this review.

Contributors

Writing - original draft preparation, Z.D., Y.W.; writing - review and editing, Y.W., Z.D., S.W., and D.S.; Figure - original draw preparation, Z.D., S.W.; Figure - review, Y.W. D.S.; All authors have read and agreed to the submission of the manuscript.

Declaration of interests

The authors declare no conflict of interest.

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