A review of recent progress toward the efficient separation of circulating tumor cells via micro-/nanostructured microfluidic chips

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Abstract
Circulating tumor cells (CTCs) are closely correlated with cancer diagnoses, and their early detection can help patients receive accurate prognoses in a noninvasive manner. However, the rarity, fragility, and heterogeneity of CTCs introduce significant technical challenges to their separation from blood. Recent advances in micro-/nanofabrication techniques have made microfluidic chips better for CTC separation. In this review, we mainly focus on recent progress made in the development of microfluidic chips incorporating micro-/nanostructures for the CTC separation. Specifically, we reviewed two major CTC separation mechanisms, that is, physical and chemical approaches. Representative works on physical mechanism-based approaches (include pillar filtration, cross-flow filtration, deterministic lateral displacement, smart materials, and bionics); immunomagnetic beads, and surface modification; and chemical mechanism-based approaches include diversity of affinity ligand selection, are reviewed. Their advantages and disadvantages are compared and discussed, and a perspective on the future direction of CTC is provided.

KEYWORDS
cancer diagnosis, CTCs, microfluidics, microstructure, nanostructure

1 | INTRODUCTION

According to the latest data, cancer is the second leading cause of death worldwide, with the vast majority of deaths due to the metastasis of solid tumors.[¹] A critical set of mediators in this metastatic process are circulating tumor cells (CTCs), which are thought to be responsible for the hematogenous spread of cancer. CTCs are tumor cells...
that have escaped from the primary tumor. They can then invade blood vessels, spread into the peripheral blood, and colonize in different tissues distally in the body.\cite{2} More importantly, CTCs contain the genetic and phenotypic information of both the primary and metastatic tumors, so they can be used as markers for in vitro diagnostics, for prognosis, and for therapeutic evaluation.\cite{3} 

To monitor and characterize CTCs, researchers need to separate them from the blood of patients. However, their concentration is so small that, in patients whose tumors have metastasized, there are only about 10 CTCs per milliliter of whole blood, compared to 50 million red blood cells and 100 million white blood cells (WBCs). The scarcity, heterogeneity, and fragility of CTCs present a considerable challenge to the development of isolation techniques, and the effect of CTC sorting directly affects subsequent assays (such as counting, gene amplification, gene sequencing, etc.).\cite{4} Therefore, CTC sorting and enrichment methods with high purity, high sensitivity (i.e., no CTC loss), high throughput, and high cellular activity become the key to using CTCs in clinical applications.\cite{5} 

Since the discovery of CTCs, multiple separation strategies have been used, including both physical approaches based on cell size and density\cite{6} and chemical approaches like immunoaffinity reactions.\cite{7} Current commercial cell-sorting platforms suffer from limited sample throughput, slow processing speeds, cumbersome operation, and large footprints, making it difficult to process clinical samples (>500 million cells) in parallel in large batches.\cite{8} As a result, researchers have focused on turning microfluidic chips into a platform for the next generation of translatable cell sorters.\cite{9} The preparation of microfluidic chips is already a fairly mature technology, and the maturity enables a variety of functions to be performed on microfluidic chips such as sample preparation, mixing reactions, separation, detection, and the provision of precise spatial and temporal control over cell processing.\cite{10} It reduces the size of the necessary equipment, eliminates potentially biohazardous aerosols, and simplifies the complex protocols typically associated with cell sorting.\cite{11} Microfluidic chips have, therefore, given CTC prognostic techniques the potential of becoming truly ideal and effective tools for cancer patient prognosis.\cite{12} 

Currently, in vitro microfluidic chip-assisted separation techniques for CTCs can be broadly classified into physical approaches and chemical approaches, as shown in Figure 1. Physical approaches enhanced microfluidic chips are label-free, and are guided by physical mechanisms alone—relying on the fabrication of fine and effective micro-/nanostructures to capture CTCs efficiently.\cite{5b,13} On the other hand, chemical microfluidic chips require further enhancements to their CTC capture efficiency by modifying affinity ligands or including novel materials at particular interfaces, in addition to, the specific micro-/nanostructures being fabricated.\cite{14} Common affinity ligands here include antibodies and aptamers that specifically recognize surface marker proteins coming from cancer cells, with the diversity of these ligands and their combinations greatly improving the sensitivity of microfluidic chip methods. 

At the same time, a range of new materials, such as temperature-sensitive hydrogels, polymers that mimic cell footprints, ultra-long nanofibers, and graphene, have been developed, and these produce excellent performance properties when used in chemical approaches to microfluidic chips.\cite{15} Note that, temperature-sensitive hydrogels and polymers that mimic cellular footprints are known as smart bionanomaterials, whose action and function can be consciously adjusted and repaired in response to changes in external conditions. This makes them a particularly hot research topic for medical materials. 

In addition to natural antibodies and smart bionanomaterials, it is also possible to combine chemical modifications and topological structuring to increase
responsiveness at the reaction interface such as by coating nanospheres with responsive topological nanostructures.\[7c,16\] All of these approaches can significantly improve the performance of different reaction interfaces, accelerate the development of more precise enrichment methods, provide new prospects for future research in cell biology, and lead to possible clinical applications of these interactive interfaces.\[17\] In the remainder of this review, we will summarize the recent progress made toward the development of methods used to efficiently separate CTCs via the incorporation of micro-/nanostructures into microfluidic chips.

2 PHYSICAL APPROACHES FOR CTC SEPARATION VIA MICRO-/NANOSTRUCTURED MICROFLUIDIC CHIPS

Label-free microfluidic chips achieve sensitive cell sorting through physical mechanisms, as controlled by fine micro-/nanostructures. By physically manipulating fluids or cells, or by utilizing external fields, selection can be achieved on the basis of properties like size, shape, density, elasticity, polarizability, and magnetic susceptibility. Label-free sorting usually requires minimal preparation in most sorting assays, making it an attractive option for cell sorting.\[18\] The integration of label-free sorting concepts into microfluidic chips has yielded some satisfactory results, as we will illustrate with the following examples of pillar filtration, cross-flow filtration, vortex design, and deterministic lateral displacement (DLD) correlation. In this section, we reviewed representative micro-/nanostructured microfluidic devices for physical separation of CTCs.

2.1 Pillar filtration

Pillar filtration typically sorts cells based on the cell size using micropillar arrays situated in the fluid channels. In which, larger cells are retained while smaller cells pass through the gaps between the pillars. CTCs (15 to 40 μm in size), WBCs (∼10 μm in size), and other blood cells all have different sizes, Cui and co-workers designed a “shoulder” structure, as shown in Figure 2A,\[19\] and their results showed that the highest capture efficiency of about 95% was achieved with a gap of 8 μm between the two shoulders in the channel. They further used surface-enhanced Raman spectroscopy to perform in-situ isolation and analysis of breast cancer cell subtypes at a single-cell resolution. By achieving a more comprehensive phenotypic analysis and a more accurate subtype identification, this allowed for more sensitive subtype sorting of breast cancer cells from different individuals.

In another study, Sarioglu et al., developed a microchip technology for capturing cell clusters (Cluster-Chip) that can capture CTC clusters directly from untreated blood, and distinguish them from single migratory CTCs. Three micropillars form a basic structural unit.\[20\] The researchers used a flow rate well below the human blood flow rate, such that the resulting shear stress was low. This allowed CTC clusters to become immobilized at the edges of the bifurcated pillars while maintaining their integrity, thus, allowing for effective capture. Their chip method achieved more than 80% capture efficiency at a flow rate of 2.5 mL/h, and an 80% release efficiency at 4°C using a low reverse flow rate.

The above-mentioned methods of pillar filtration are used to sort CTCs by cell size, are relatively simple to operate, and can both guarantee high separation efficiencies. In particular, column structured arrays are popular and can be prepared with more technically mature methods. However, filtered sorting of cells also has its disadvantages; the gaps in the arrays, the micropores, and the porous films are all prone to blockage, causing intercellular extrusion and rupturing.\[8b,21\]

2.2 Cross-flow filtration

Cross-flow filtration typically employs the tangential flow of cell suspensions through a filter structure. This technique typically utilizes a membrane with special micropores or structures that can capture CTCs based on their size and deformability. Small cells pass through the pores, while larger cells are flushed away with the original sample, thus, eliminating the clogging problem often encountered in traditional membrane or pillar filters.\[21,22\] A triple separation strategy using membrane filtration combined with cross-flow filtration was prepared by Huang et al. to be used for CTC separation. Their microfluidic chip was fabricated based on the principles of cross-flow and dead-end filtration, and the entire device could be disassembled without affecting CTC separation, thereby enabling in-situ drug analysis. Blood samples from prostate cancer patients were used for the analysis, and the data showed that cancer cells were efficiently separated (>95%), had a high survival rate (78%), and that overall the chip had excellent performance.\[23\]

According to a similar principle, Li et al., achieved efficient separation of CTCs by controlling the fluid. As shown in Figure 2B,\[18b\] the chip demonstrates a label-free, size-dependent, and high-throughput isolation of rare tumor cells from untreated whole blood by interfacial viscoelastic microfluidics. By exploiting the competition between
FIGURE 2  (A) Pillar filtration. Data showed that the highest capture efficiency of about 95% was achieved with a gap of 8 μm between the two shoulders in the channel. Reproduced with permission. Reproduced with permission from Ref. [19]. Copyright 2018, Wiley-VCH. (B) Engineering the fluid flows in microchannels for CTC separation. Reproduced with permission from Ref. [18b]. Copyright 2018, Royal Society of Chemistry. (C) DLD-based approach for tumor cell separation. Reproduced with permission from Ref. [25]. Copyright 2017, Royal Society of Chemistry. (D) CTC separation based on interfacial viscoelastic microfluidics. Reproduced with permission from Ref. [26]. Copyright 2019, American Chemical Society.

The inertial lift forces and interfacial elastic lift forces, the sharp flow interfaces between the sample flow and viscoelastic flow (0.05% PEO solutions) in the straight microchannel allow for the penetration of large tumor cells while blocking small blood cells. The microfluidic paradigm does not involve external force fields or complicated fabrication procedures, and achieved 95.1% separation efficiency and 77.5% recovery rate for isolating as few as 50 tumor cells in 1 mL whole blood. The viability of tumor cells after separation is approximately 100%, and normal proliferation of separated tumor cells is observed.

2.3 Deterministic lateral displacement

Deterministic lateral displacement (DLD) is another method that sorts CTCs based on their size and deformability. Here, CTCs are sorted out from background cells by the guidance of microcolumn arrays, as they each follow different migrating flow trajectories as they move through.\textsuperscript{[24]} The reason for this difference in trajectories is that the combination of the critical radius ($R_c$) of the specially designed array and the diameter of the cells ($a$) determines a particular deviation angle experienced by the cells. Those cells larger than the critical radius ($a > R_c$) will gradually deviate laterally from the initial flow direction, while the flow direction of smaller cells ($a < R_c$) will not be sharply deviated by the microcolumns. As shown in Figure 2C, Tran et al. used this principle to successfully sort MCF-7 breast cancer cells using cylindrical arrays with a 95% recovery rate and a viability rate of over 90%.\textsuperscript{[25]} However, the DLD structure also has its disadvantages, such as easy clogging, excessively long-reaction channels, and the requirement of a complex structure to achieve significant lateral displacements.

When the liquid flows in a microfluidic channel, it would generate shear- and wall-induced inertial lift forces, which can cause cell migration across the laminar flow lines. Moreover, when cells are flowing in curved channels, they are not only subject to inertial lift forces but also to lateral Dean drag forces. To address this, Xiang et al. developed a two-stage sorting device combining helical inertial microfluidics with DLD sorting to achieve high precision separation of CTCs. Comparing with a cylindrical array, cells become less deformed in a triangular column array, which is preferable for CTC sorting. They applied such a
triangular microcolumn array in the device, as shown in Figure 2D, which resulted in a CTC capture rate of 91.34% and a blood cell depletion rate of 99.95%.[26]

To briefly summarize, label-free microfluidic chips based on physical properties enjoy the advantages of high throughput, low cost, and simple operation. However, there are still some disadvantages that need to be overcome, such as the loss of CTCs smaller than the screening size, which results in an incomplete separation of CTCs and, hence, false negatives. In other cases employing especially designed-enhanced microfluidic chips, some CTCs may pass quickly through the filter along with the buffer or accumulate between the micropillars, leaving them prone to rupture due to extrusion by strong mechanical forces.

3 CHEMICALLY APPROACHES FOR CTC SEPARATION VIA MICRO-/NANOSTRUCTURED MICROFLUIDIC CHIPS

We need a progressively deeper understanding of cancer mechanisms, and we will require a higher level of functionalization of microfluidic chips. It has been shown that some materials, including affinity ligands, new materials, and smart materials, can be used to capture CTCs by modifying the reaction interface to achieve specificity and selectivity, which can greatly reduce the possibility of disordered aggregation of liquid or rapid take away of target cells, and also avoid false negatives or false positives and improve the effective immobilization of captured CTCs.[8b,27] Therefore, in addition to the need for advanced micro-/nanoprocessing, efforts are needed in the selection of chemical modifications.[28] In this section, we will also present different combinations of approaches to improve CTC capture efficiency including surface chemical modifications, meaningful micro- and nanostructures, and physical mechanisms.

3.1 Diversity of affinity ligand selection

In one example shown in Figure 3A, Yang et al., applied an aptamer to modify the capture interface of a device. They combined the DLD mechanism with a triangular micropillar array, thus, forming a size-determined immunocapture chip (SDI-chip). The key point of the whole device was to increase the roughness of the capture interface by

FIGURE 3 (A) A size-determined immunocapture chip (SDI-chip), the data show that the highest capture efficiency exceeds 92% and the purity level reaches 82%. Reproduced with permission from Ref. [29]. Copyright 2019, Wiley-VCH. (B) A three-dimensional hierarchical spiky micro straw array (HS-MSA), the device achieved sufficient specificity and a high efficiency (~84%). Reproduced with permission from Ref. [48]. Copyright 2019, Wiley-VCH. (C) Chaotic herring bone mixer. The results showed a CTC separation efficiency in the buffer of over 90% and a purity of 84%. Reproduced with permission from Ref. [31c]. Copyright 2017, American Chemical Society. (D) A nanoparticle-herringbone CTC (NP-HBTC) chip. The capture efficiency of the device reached 98.15 ± 1.1% at an antibody concentration of 10 μg/mL. Reproduced with permission from Ref. [31b]. Copyright 2015, Wiley-VCH. (E) The device combined the herringbone with bioinspired intestinal nanovilli, efficient and reproducible capture of tumor-derived EVs from plasma samples. Reproduced with permission from Ref. [32]. Copyright 2019, American Chemical Society. (F) Silicon nanowire/microscale pyramid (Si-NW/MP). The CTC capture efficiency could reach 84.5 ± 1.19%, their specificity was 74 ± 2.68%, and their sensitivity was 78.25 ± 5.19%. Reproduced with permission from Ref. [33]. Copyright 2019, Elsevier. (G) Cell capture efficiency with multiple recognition ligand pattern reaching 80% for renal cell carcinomas (RCC-CTCs). Reproduced with permission from Ref. [14c]. Copyright 2020, Elsevier.
combining numerous SYL3C aptamer tentacles with gold nanoparticles to form a multivalent structure similar to octopus whiskers, which greatly improved the interface sensitivity. Their data show that the highest capture efficiency exceeds 92% and the purity level reaches 82%, as obtained by optimizing the distribution of the nanoparticle positions. This structure not only exploits the benefits of aptamer pairing diversity, but also improves CTC capture by increasing the physical contact between the cells and the functionalized capture structures through the designed micro-/nanostructure arrays.

Building multilayered micro-/nanostructures also has the effect of increasing the surface area of microfluidic substrates, thereby increasing the availability of binding sites for markers, and thus, further contributing to improvements in CTC capture efficiency. These structures can include hierarchical indium tin oxide nanowires, polystyrene nanotubes, silicon nanopillars, etc. Tseng’s group have done pioneering works based on this principle, that they designed a three-dimensional nanostructured substrate, as shown in Figure 3B, namely, a silicon-nanopillar array coated with epithelial-cell adhesion-molecule antibody (anti-EpCAM), shows enhanced local topographic interactions between nanoscale cell-surface components and the substrates surface, resulting in enhanced cell-capture efficiency when employed to isolate viable cancer cells from whole-blood samples. Cell viability with this platform is as high as 84–91%, which is conducive to subsequently releasing the cells, culturing them, and performing molecular biological diagnosis.

In addition to building multilayered structures, classic herringbone microfluidic channels can also be used in sensitive methods for capturing CTCs. Herringbone channels are microstructures prepared following the physical principles related to the Reynolds number (Re) and are used to shorten mixing distances. A side view of such a structure, as designed and optimized by Fan and his coworkers, is shown in Figure 3C. The lateral flow induction generated in this device greatly enhanced the interaction of CTCs with the antibody-coated surface, and the results showed a CTC separation efficiency in the buffer of over 90% and a purity of 84%. In an experiment using this device, CTCs were found in 17 out of 18 blood samples taken from patients with metastatic pancreatic cancer—an efficiency of over 94%. The experimental data show that chemical modification can improve the specificity, sensitivity, and separation efficiency.

In another example shown in Figure 3D, the Hammond’s group designed a nanoparticle-herringbone CTC (NP-HBCTC) chip along with a similar pattern, pairing it with gold nanoparticles modified with sulfhydryl ligands to improve the sensitivity of the capture interface. After surface modification with specific recognition antibodies, the capture efficiency of the device reached 98.15 ± 1.1% at an antibody concentration of 10 μg/mL, and the activity of CTCs after the capture of target cells was high due to the high release efficiency of the sulfhydryl exchange reaction. This NP-HBCTC chip design is easy to fabricate, flexible with regards to the functional group exchange of different ligands, and has an overall structure with its three-dimensional surface that ensures both cell binding and release.

Extracellular vesicles (EVs), also known as exosomes, can serve as a new and controllable tool for intercellular messaging including between tumor cells. Tseng’s group developed the herringbone chips with bio-inspired nanovilli, as shown in Figure 3E. Nanovilli arrays grafted by anti-EpCAM were designed to mimic intestinal microvilli, dramatically increasing surface area and enhancing tumor-derived EV capture. They studied the influence of anti-EpCAM concentrations and other factors to identify conditions that yield optimal EV-capture efficiency. The captured EVs are subsequently subjected to mRNA assays for quantitative detection and monitoring of alterations in targetable oncogenes. Therefore, chemical modification allows specific capture and serves as a source for detecting specific oncogenic alterations associated with treatment response and disease progression in clinically relevant applications for the clinical management of patients.

Ming-Ying Lan and his group developed a silicon nanowire/microscale pyramid (Si-NW/MP) layered substrate based on a modified epithelial cell adhesion molecule (EpCAM), as shown in Figure 3F. Their device was used for specific detection and monitoring of patients with nasopharyngeal carcinomas, and they achieved this by changing the roughness of the reaction interface to improve sensitivity. Si-NWs/MPs can not only effectively capture CTCs, but the rough surfaces of the Si-NWs can also allow cancer cells to adhere easily, as shown in the SEM inset to Figure 3F. The key reason for the increased sensitivity of Si-NWs/MPs here was that the cell filaments were even able to extend sufficiently into the gaps of the nanowires. As a result, the CTC capture efficiency of Si-NW/MP chips could reach 84.5 ± 1.19%, their specificity was 74 ± 2.68%, and their sensitivity was 78.25 ± 5.19%.

In another study, a cocktail antibody-modified capture interface was developed for the effective isolation of CTCs from renal cell carcinomas (RCC-CTCs). There had been a clinical gap due to the difficulty of isolating RCC-CTCs with a single antibody. This was discovered when it was shown that EpCAM expression in RCC-CTCs was down-regulated in 75% of samples, while the expression rate in primary tumors was less than 40%. The cocktail antibody used to address this gap consisted of a mixture of EpCAM, carbonic anhydrase IX (CA9), and surface receptors such
as epidermal growth factor receptor (EGFR) and hepatocyte growth factor receptor (c-Met). This multicapture method is shown in Figure 3G. Cell capture efficiency with this multiple recognition ligand pattern greatly improved, reaching 80% for RCC-CTCs. We can, therefore, say that the use of a diversity of affinity ligands (antibodies or aptamers) and of their combinations (antibody or aptamer cocktails) can enrich CTCs more precisely after grafting them onto microfluidic chips. This can then provide researchers with accurate and reliable information for CTC detection and subsequent downstream analysis. [14c]

The EpCAM used in the above-mentioned study is a traditional affinity ligand and is still widely used. However, it has some shortcomings. First, certain cancer cells have low or no EpCAM expression, which can easily cause false negatives or false positives. Second, the modification site of EpCAM is not specific, which reduces the applicability of the antibody. By using an aptamer as an affinity ligand, the usual advantages of EpCAM can be maintained while also improving the versatility and suitability of the method. [28b,34] New nucleic acid aptamers, for example, can be screened for and used to target tumor cell surface markers like EpCAM, EGFR, and prostate-specific membrane antigen. [36] In summary, the diversity and specificity of cancer mechanisms require that functionalized micro/nanostructures be combined with diverse affinity ligands if they are to be able to demonstrate superior and plausible results when it comes to CTC separation. If mass commercial production can be achieved, devices with these features could provide a wealth of information for the clinical treatment of patients in later stages.

### 3.2 New materials and bionics

As one example, electrospun nanofibers have demonstrated their potential for use in a wide range of applications since they emerged as a new material made with a simple process. [35] As shown in Figure 4A, Zhang et al. prepared a method of detecting CTCs based on the deposition of PLGA nanofibers by an electrospinning process, in combination with the introduction of TiO₂ nanostructures as supports. They were inspired by the presence of villi on cell surfaces and by the construction pattern of
extracellular matrix scaffolds, both of which can enhance local topological interactions and significantly improve capture efficiency. Their data showed that CTCs were captured in samples from two out of three colorectal cancer patients, with the number of CTCs per 0.5 mL blood sample ranging from 0 to 2. In another case of seven gastric cancer patients, the number of CTCs per 0.5 mL blood sample ranged from 3 to 19. This shows that their device exhibited a fairly high capture efficiency in human blood samples.[35b]

Yu et al. similarly used an electrospinning process to prepare PLGA nanofibers, producing a rough interface for efficient CTC capture. They were also able to induce high shear stresses with the help of air bubble technology, useful for nondestructive and effective release of the cells. As shown in Figure 4C, their device achieved a capture efficiency of up to 80% for spiked human blood samples, and was able to obtain a 90% release efficiency (~73% recovery) using continuous air foam injection from the microfluidic device.[35a]

As another interesting new material, carbon nanotubes (CNTs) are ideal as the basis for building future superstructures and carbon-based semiconductor devices. Assembling CNTs into macroscopic bodies (e.g., fibers, films, and foams) is one important way of them achieving macroscopic applications, and these bodies can be used not only alone but can also be woven together to form two-dimensional films or diverse three-dimensional structures.[36]

Indeed, CNTs were used by Loeian et al. to develop CTC detection chips, as shown in Figure 4B. This device combines a CNT surface with microarray bulk fabrication technology, and is used for the capture and isolation of tumor-derived epithelial cells. Their data show that this nanotube-CTC chip successfully captured CTCs in the peripheral blood of breast cancer patients (stages 1–4), with concentrations ranging from 4–238 CTCs per 8.5 mL of blood, or 0.5–28 CTCs per milliliters. CTCs were successfully identified in all seven breast cancer patients tested (based on CK8/18, HER2, and EGFR), and no CTCs were captured in the healthy controls (n = 2).[14a]

In recent years, graphene has occupied a lot of scientific niches among carbon-based materials, following on from the hot topic of CNTs. The high surface-area-to-volume ratio of graphene, and it having a similar size to many biomolecules, means it can improve the sensitivity of biomolecule detection, thereby making functionalized graphene oxide a promising nanomaterial. In one case, Hyeun Joong Yoon et al. applied functionalized graphene oxide modified with a flower-shaped gold pattern to a planar silicon substrate, and used this to sensitively capture CTCs (as shown in Figure 4F). These researchers collected blood samples from pancreatic, breast, and lung cancer patients, and achieved high sensitivity capture of CTCs at low target cell concentrations with this device (3–5 cells per mL of blood, 73 ± 32.4%).[37]

Smart hydrogels are another group of materials with a lot of promise. They are a class of hydrophilic polymers with three-dimensional network structures capable of responding significantly to small changes or stimuli present in the external environment (e.g., temperature, pH, electromagnetic fields, specific biomolecules, etc.).[38] Based on the three-dimensional network structure and environmental sensitivity of these smart hydrogels, they have become widely used in memory materials, drug retardation, wound dressings, tissue engineering, smart spinning, chemomechanical chips, substance separation, enzyme sequestration, and other applications.[39]

For example, Wang and coworkers prepared thin hydrogel coatings for use in CTC capture. They did so by utilizing the surface hydrophobicity and morphology of a particular hydrogel, produced when N-isopropylacrylamide (NIPAAm) and the cross-linker N,N’-methylenebisacrylamide form a gel through free-radical copolymerization. The critical solution temperature of these poly(N-isopropylacrylamide) (PNIPAAm) chains is about 32°C; below which it is hydrophilic and the interface is spreading, and above which the interface becomes hydrophobic and contracted. Even at 37°C, a PNIPAAm hydrogel will collapse, and the resulting surface will appear wrinkled due to uneven contraction (as shown in Figure 4D). These nanowrinkles thereby provide more contact sites for cell protrusions, and this enhances the adhesion of cells to the substrate. The experimental results from this study showed that the CTC capturing efficiency was as high as 98%, the survival rate of captured cells was 98.7%, and the survival rate of released cells was 96.8%.[15b]

Bionic interfaces have also been a hot topic in various fields including in the research on cell sorting. Molecular imprinting technology is one example of a desirable technique that uses molecularly imprinted polymers to mimic enzyme-substrate or antibody-antigen interactions for the exclusive recognition of imprinted molecules (also called template molecules). The predetermined recognition and practical nature of this technique has led to considerable progress in the imprinting of biomolecules, proteins, viruses, and even whole cells.[27c,38c,40]

Lu et al., just such a bionic interface has been applied to capture CTCs in vitro—with highly desirable results. By studying in detail the synergistic effects of molecular recognition and topographic guidance during CTC capture, a cell-imprinted substrate was prepared using soft lithography by replicating the morphology of target cancer cells on polydimethylsiloxane (PDMS), as shown in Figure 4E. In this case, the human breast cancer cell line MCF-7 was used to mimic CTC capture. The comparative experimental data from this study showed that the physical
structural complementarity and chemical signal matching of template cells play a special role in the capture of target cells, and that the anti-EpCAM-modified cell-blot substrate showed a significant increase in target cell capture and low nonspecific adsorption of control cells. Overall, the MCF-7 cells used in these capture experiments resulted in a capture efficiency of up to 80% and a high activity. [15a]

4 | COMBINED APPROACHES FOR CTC SEPARATION VIA A COMBINATION OF MAGNETIC BEADS AND MICRO-/NANOSTRUCTURED MICROFLUIDIC CHIPS

Microfluidic immunomagnetic beads technology is a CTCs capture method that uses either the biochemical properties of immunomagnetic beads alone, or uses them in combination with the physical properties of the beads. [41] A microfluidic chip fabricated by Hongwei Song et al., using this method obtained a high capture efficiency and could effectively control the capture and release of cells. They used inverted silicon nanowires (SiNWs) combined with multifunctional composite magnetic beads (Fe3O4@C6/Ce6@Silane; with coumarin 6 (C6) and chlorin E6 (Ce6)) for real-time monitoring and photodynamic treatment of CTCs by confocal laser scanning microscopy. This treatment proved to be efficient at hindering the proliferation and spread of cancer cells. As shown in Figure 5A, the SiNWs in the device were inverted, allowing CTCs and other unlabeled cells like leukocytes to be naturally separated by magnetic force and by gravity. As this avoided the adhesion of background cells at the interface that can occur in the case of vertical SiNWs, their technique was able to improve both CTC capture efficiency and purity—the former reaching 82%, and the latter exceeded 90%. [42]

Considering the rarity of CTCs in blood, it is crucial to explore methods for capturing them with high precision and purity from a complex background of other cells. Recent studies have shown that platelets (PLTs), whose homologous WBCs do not form clumps during blood
circulation, can recognize and interact with CTCs. To use platelets to effectively remove background cells, especially leukocytes, Xie et al., proposed the scheme shown in Figure 5B. Their Fe₃O₄ magnetic nanoclusters (NCs) were disguised as mimetic nanoparticles (M/NCs) using leukocyte membrane fragments. These M/NCs inhibited the nonspecific binding of leukocytes and promoted the binding of the DSPE-modified SYL3C aptamer (A-M/NCs) to the clusters. The whole capture process lasted about 20 min and captured more than 90% of the rare tumor cells from whole blood, with little nonspecific adsorption of leukocytes in the device.[43]

Due to the heterogeneity of CTCs, their low-expressing isoforms are sometimes missed in experiments, resulting in false negatives. To address this issue, Kwak et al., used immunomagnetic beads combined with a microfluidic technique based on the application of a magnetic field gradient and differential immunoreactive fluorescence. They were thereby able to isolate eight different CTCs isoforms from breast cancer based on three biomarkers (HER2, ER, and PR), further enabling in-situ measurements on microfluidic chips. As shown in Figure 5C, a magnetic field gradient from left to right was established by placing permanent magnets on the right side of a PDMS section. Their results showed that the magnetic nanoparticles bound to cancer cells with an extremely high capture efficiency of more than 99%, making up for all the difficult work they performed in applying immunofluorescent labels to the magnetic nanoparticles before cell separation.[44]

In another approach, Gwo-Bin Lee et al. designed a pneumatically controlled integrated microfluidic chip for cholangiocarcinoma (CCA) prognosis and monitoring of metastasis, as shown in Figure 5D. The first four layers of their device are made from PDMS, with the fifth being a double-sided adhesive tape layer (3 M, USA). The first layer collects cell particles from blood, while the second and third layers are the air-controlled and liquid channel layers, respectively. The fourth layer of the device is the thinnest and keeps the contents of the liquid channel layer intact. The PDMS layers are bonded together by oxygen plasma. The coupling of CCA-specific aptamer probes to magnetic beads was achieved in this study. Their experimental data showed that the efficiency of isolating CTCs from 35 advanced/metastatic patient samples was close to 100%, indicating that the application of this microfluidic platform for active diagnosis of CCA looks promising.[45]

5 | SUMMARY AND PROSPECTS

In summary, we reviewed the recent research progress on the CTC separation based on micro-/nanostructured microfluidic chips. These microfluidic chips can be turned into powerful tools for capturing CTCs by pairing specific affinity ligands with novel materials and surface modifications, thereby making the fast, accurate, low-consumption, and low-damage capture, and detection a reality. The intactness and activity of captured CTCs after being released are of vital importance to obtain a more complete picture of the tumor cells. However, captured CTCs tend to adhere to the substrate or be trapped in microstructures, which hinder the release process. To address the problem, many strategies have been reported on how to efficiently release the captured CTCs. For example, CTCs can be released using counter currents in column filtration microfluidic microarrays, or by using trypsin-hydrolyzed proteins in chips employing immunoaffinity reactions. Temperature-controlled, lamp-controlled, and hybridized complementary sequence methods have also been put forward.[46]

Although all the above-mentioned methods have achieved the release of CTCs to some extent, the complete biological properties of CTCs are still essential to develop an active, controlled release strategy with low CTCs self-damage.[39c,47] Furthermore, the current progress of chips is still far from the requirements of clinical practice, requesting further optimization and innovation. The development of multifunctional and efficient microfluidic analysis platforms requires a lot more effort in future research. Advanced detection systems that integrate the capture, release, and single-cell analysis of CTCs into a single miniaturized device will be developed to reduce cell loss and interference. We expect more advances in microfluidic chip-based CTC isolation methods to be made, thereby furnishing more accurate tools for cancer diagnostics, treatment and evaluation, and for use in drug sensitivity studies.

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CONFLICT OF INTEREST
The authors declare no conflict of interest.

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