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Smart Material-Integrated Systems for Isolation and Profiling of Rare Cancer Cells and Emboli

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This review presents a broad aspect of smart material-integrated systems for isolating and profiling rare circulating tumor cells (CTCs) and circulating tumor microemboli (CTM) to provide physiological, biological, and mechanistic insights into cancer research. In particular, CTCs/CTM have emanated as essential pieces of evidence that can reveal clonal evolution, tumor heterogeneity, and disease progression within the metastatic cascade. Morphologies, cellular compositions, and rarity of CTCs/CTM make them difficult to track and isolate for profiling distinct molecular characteristics in the case of metastatic potential. Accordingly, with the advanced-characterization techniques, examining the aspects of the specific surface markers of CTCs/CTM, epithelial-to-mesenchymal (EMT), mesenchymal-to-epithelial (MET) transitions, and timing of tumor cell dissemination would assist us to understand cancer biology and metastatic characteristics. Existing clinical and research methods for the enrichment and isolation of these sporadic cells depend on mainly conventional methods with low-yield and expensive features. Owing to their specialized functions and analytical performances, smart material-based technologies hold an enormous impact not only on cell detection, but also on cell isolation for downstream analyses. Herein, the main reasons for cell isolation are discussed and the recent developments in CTCs/CTM approaches for identifying further methods and future perspectives are elaborated on.

1. Introduction

Personalized treatment for cancer patients depends on the identification of the molecular drivers of disease because millions of different cells join the circulation during carcinogenesis.[1,2] The current conventional methods to isolate cancer cells focus mostly on biomarkers, predicting therapy measured from biopsy samples.[3] Due to the broad area of cancer encompassing multiple disciplines, impractical invasive methods, cancer cell evaluation, and restricted repertoire of targeted therapies have been encouraging us to design new modalities and techniques in monitoring strategies that anticipate the future journey of cancer cells much ideally.[4] To overcome associated challenges, one of the new avenues is circulating tumor cells (CTCs), and approximately 2–5% of them comprised their clusters 3–100 cells—as known as circulating tumor microemboli (CTM).[5,6] CTCs/CTM can noninvasively snapshot genetic intratumor heterogeneity and provide real-time information better than any single-site biopsies whereby cells migrate between the primary tumor, marrow, and metastases.[7–9] As CTCs/CTM have been spanning the topic of tumor invasion and metastasis, they have been associated with pharmacodynamic,[10] prognostic,[11] biomarker utility,[12] and identification[13] for therapeutic selection.[14]

Historically, for the microscopic examination of metastatic cancer from blood, CTCs were explored by Récamier in 1829.[15] Later on, they were for the first time identified as cells of the original tumor by Langenback,[16] and this investigation was followed by Thomas Ashworth in 1869.[17] In the following century, many studies on tumor cells were reported; as a result, the scientists found out that the tumor emboli or the unusual cells/clusters in blood could be more malignant than the individual tumor cell.[18,19] Consequently, CTCs became widespread with the great certainty of cancer biomarkers.[20] Shortly after this identification, several studies had been launched about the role of CTCs within the metastasis.[21] Especially, the clusters or aggregates of tumor cells were spotted with higher metastatic potential.[22,23] In these studies, rare tumor cells have been differed in density, size, concentration, shape, and internal structure properties to evaluate metastatic risk.[24–26] With recent advanced techniques, DNA and RNA profiles of CTCs have been examined for determining the degree of heterogeneity through the aggregation or single-cell profiling methods, yet the primary obstacle in CTCs analysis is their low abundance (1–3000 CTCs mL^{-1}[27]) in the bloodstream (10^7 white blood cells [WBCs] mL^{-1}; 10^9 red blood cells [RBCs] mL^{-1}[28]).[29,30] To hurdle this challenge, CTCs were initially identified with epithelial cell adhesion molecule (EpCAM)—surface protein and cytokeratin (CK)—cytoskeletal proteins whereas they are negative for WBc-. So far, mesenchymal CTCs have been conversely identified because of the epithelial-to-mesenchymal transitions (EMTs) and downregulation of

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DOI: 10.1002/adem.202100857

CK or EpCAM markers.[31] Moreover, this took plenty of time to know-how the role of CTM that could be identified in human peripheral blood, and it is vital for a full appraisal of cancer metastasis.[32] In this review, a better understanding of cancer metastasis and point-of-care (POC) applications will be elaborated.[33–37]

1.1. Cancer Metastasis

Metastasis is the dissemination of the cells from the initial neoplasm to distant organs; the known primary site of metastasis can or cannot affect the prognosis. Therefore, this ambiguity creates the most fearsome aspect of cancer. To clarify the insight of this process, the biology of the primary site of cancer and metastatic circulation needs to be examined comprehensively. In this regard, tumor heterogeneousness prevails for virtually every phenotype measured, and it mostly consists of three types as positional, temporal, and genetic heterogeneities.[38] The main concern is the origin of heterogeneous tumors. Are they unicellular or multicellular? As suggested, tumors generally originate from a single cell, and they express maternal or paternal isoenzymes; hence, the generation of heterogeneity necessitates the divergence of single cells into multiple phenotypically distinct progeny that can also occur in normal physiology, such as pluripotent hematopoietic stem cells creating multicellular organism in fertilization.[39] Tumor progression is a unique, constant, and stepwise pattern described by Peyton Rous,[40] the first formalized conception framework for skin and breast carcinomas, and later on, Leslie Foulds[41] emphasized the irreversible qualitative changes of neoplasm characteristics.

To understand the reasons for neoplasm characteristics, the theory of mutative selection needs to be examined. This theory suggests that the genetic instability within a tumor contributes to the random generation of variants within the population.[42] Either highly or poorly metastatic clones conversely contained their metastatic characteristics in the experimental setup, consisting of changes in cell adherence to the extracellular matrix (ECM) and cells, exhibiting that clonal populations cannot be homogenous due to the absence of invasion.[43] The basic representation of metastatic determinants is summarized in **Figure 1**a. Furthermore, benign tumors refer to the site that has failed to invade, whereas the one which has the strength of escaping through a basement membrane is called malignant. For metastasis, the migration of tumor from the primary location to elsewhere is required, and rather than individual cells, the mass of tumor in the penetration stroma is measured to clarify the progression. In addition, the epithelial cell-derived carcinomas represent 90% precursors of human cancers involving drastic changes in cell shape.[39]

1.2. Epithelial-to-Mesenchymal Transition and Mesenchymal-to-Epithelial Transition

On the course of wound healing and embryonic development, epithelial–mesenchymal transition (EMT) occurs, but if the tumor microenvironment disturbs this process in the paucity

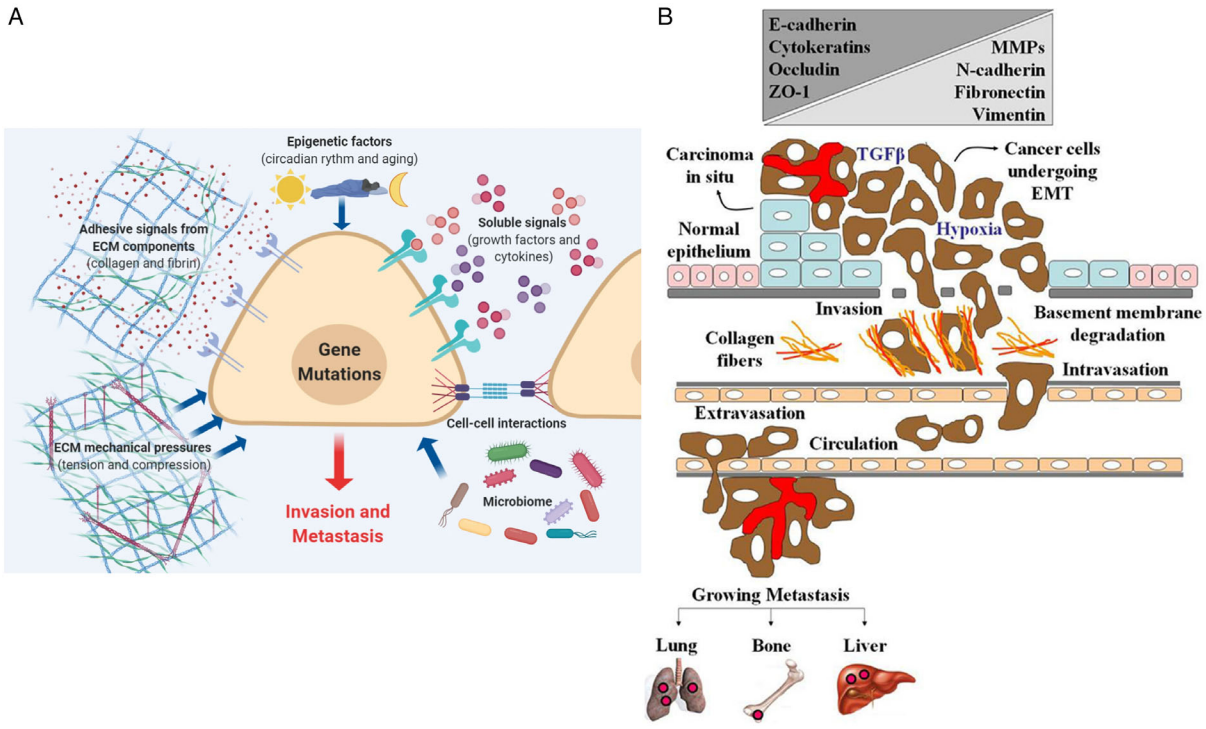

Figure 1. a) The schematic represents metastasis and invasion, which are related to cell–cell and cell–ECM adhesive signals; ECM mechanical pressures; soluble signals in the ECM; intratumoral microbiota; and epigenetic factors induced by living conditions. Reproduced with permission.[56] Copyright 2021, Springer Nature. b) EMT is mainly characterized by the loss of epithelial markers and regulated by versatile effectors, such as growth factors (TGFβ). Reproduced with permission.[192] Copyright 2021, Frontiers Research Foundation.

of EMT-inducing signals with influencing regulators of EMT, which are hepatocyte growth factor/scatter factor and growth factor-β, the progression may reverse to mesenchymal–epithelial transition (MET). This points out the loss of epithelial-specific cadherin, E-cadherin, and transmembrane glycoproteins that operate as a metastasis suppressor and a tumor suppressor.[44] For instance, with the degeneracy of transcriptional repressors Snail and Slug, β-catenin, and p120 catenin, regulators of cadherins and all the listed conditions affect the regulation of EMT. Apart from that, these regulators function in different signaling pathways relevant to cell–cell adhesions (Figure 1b).[39] Tumor cell adherence to the ECM is basically mediated by integrins, heterodimers of 1 of 18 α and 1 of 8 β transmembrane proteins, and each of them binds to specific proteins and transmits the signals between cells and ECM.[45] Furthermore, CD44 is another type of cell receptor in ECM, and it is an excessively polymorphic receptor for hyaluronan, surface proteoglycans, and immunoglobulin superfamily.[46]

1.3. Malignancy and Motility of Cancer Cells

Proteolytic degradation of the surrounding is a hallmark of malignancy. Proteolytic enzymes are classified as serine proteinases plasmin, seprase, hepsin, plasmin activator, and metal-dependent proteinases of the matrix metalloproteinase (MMP).[39] Especially, elevated MMP,[47] the plasminogen activator/plasmin, plasminogen activator inhibitor-1 levels, and urokinase plasminogen activator have been associated with cancer progression.[48]

Cell motility in the direction of favorable environments is a conserved fundamental ability. The motility of tumor cells is associated with metastasis since cell migration affects cell survival.[49] Indeed, age-related physical conditions and epigenetic factors mainly affect tumor cell motility. For instance, tumor cell-produced lysophospholipase D (autotaxin), correlated with the chemokinetic activity of epithelial cells, stimulates the motility. The modulation of motility converts the coordination of cancer invasion. Merely the ability of invasion is not enough for metastasis; some of the tumor cells, such as carcinoma of lung melanoma, is capable of forming secondary lesions that is the mainstay performing every steps of the metastatic cascade.[39]

1.4. Timing of Tumor Cell Dissemination

Dissemination of tumor cells is likely to be an early stage in tumor progression. For instance, colorectal cancer initially presenting with resectable tumors subsequently leads to metastatic disease in ≈30–50% of patients. In these cases, neoplastic cells can be disseminated either before or during surgical operation of primary cancer.[50] Although the intravasation of tumor cells is ambiguous, perivascular macrophages in mammary tumors are associated with this progression even if in the absence of local angiogenesis.[51]

In addition, tumor cells can move actively through motility or passively by fluid flow. While they are moving, natural killer (NK) cells or monocytes can execute them as well.[52] The ones that could escape from NK cells/monocytes are continually killed by hemostatic shear forces, which depend on tumor type and biophysical parameters, such as cytoskeletal organization, membrane fluidity, the existing number of tumor cells, and cellular elasticity.[39,53,54]

After that, the attachment of cancer cells is the next process accompanied by the engagement of integrins, and it happens preferentially at endothelial cell junctions like leukocyte extravasation.[55] The microvascular rupture or extravasation involving ligand–receptor interactions, chemokines, and circulating nontumor cells occurs when CTCs become entrapped.[56] Moreover, host microenvironment and adaptive process play a critical role in extravasation. For instance, breast cancer has a high metastatic potential, especially for bone tissues because they activate bone cells by providing osteoclasts in which cancer cells can grow.[57] The extravasated cells have harsh physiological conditions in the stroma, and thereby, only a few of them can persist in such an environment. The organ selectivity and colonization of metastasis depend on specific tumor-derived factors. Therefore, there are anatomic or mechanical considerations for each type of cancer, such as liver, lung, kidney, and breast.[39]

2. Biological Origin and Properties of CTCs/CTM

For the isolation of CTCs, the expressions of cell surface markers, i.e., EpCAM(+) and CD45(–), are ubiquitous biological assets that guide technologies to develop and utilize recognition elements (aptamer and antibodies), such as anti-EpCAM antibodies, anti-CD5 antibodies, anti-HER2/neu antibodies, anti-EGFR antibodies to capture and detect cancer cells with high specificity.[58] Besides, other biomarkers including N-cadherin, O-cadherin, ICAM-1, CEA, EphB4, hMUC1, CD44, CD133, CD146, PSMA, VCAM-1, TROP-2, and FAPα have been investigated for CTCs selection.[31,59–62] The unique functional and phenotypic characteristics of CTM, which can be defined with the minimum of three CTCs, are pivotal for the development of metastases since they have the ability to avoid immune surveillance,[7] avoidance of anoikis,[63] and traveling niche[64] under the favor of the presence of stromal cells. Apart from that, CTM is defined as a split from primary tumor mass clusters.[6]

2.1. Morphology, Cellular Composition, and Rarity of CTCs/CTM

CTCs have similar sizes (15–25 μm in diameter[65]) like surrounding leukocytes, yet the technologies distinguishing the size of cells would be potentially hindered by similarity in size, limiting their specificity.[5] Therefore, other characteristics, which are dielectrophoretic property[66] and deformability,[67] can be used for the physical-based separations. CTM displays a high level of heterogeneity, and it has specific physical properties and morphologic appearances like clusters, rings, elongated strands, and different geometries.[32] Furthermore, they can be a cell–cluster that includes the groups of tumor cells alone or tumor cells associated with platelets,[68] fibroblasts,[64] endothelial cells,[64] leukocytes,[69] and pericytes.[70] CTM is related to the poor prognosis of patients and the increased metastatic potential, but they are rarer than single CTC and account for 1–5 microemboli mL^{-1} blood[71] that have a shorter lifespan in bloodstream.[6] For instance, in the patients with melanoma, CTM displays more aggressive

characteristics than CTCs, and also, they constitute CD10, SOX10, and TRF2 expressions after sentinel lymph node extirpation.[72]

3. Molecular Characterization Techniques of CTCs/CTM

Because of the high heterogeneity and extreme rarity of CTCs and CTM, sophisticated characterization techniques are required to detect these cells reliably. The technologies for this manner could be listed, but not limited to, physical property-based methods, antibody-based methods, and additional detection strategies.[73] The cellular origins of rare tumor cells can be identified from metastatic and primary tumor deposits, so that physical property differences in cell size, dielectric properties, density, and mechanical plasticity can be employed to isolate both CTC and CTM, holding distinct physical characteristics compared to blood cells. In a study, cancer cell lines, for instance, were drained through silicon nitride microsieves, polymer track-etched filters, and metal TEM grids.[74] The size-based filtration system was designed according to pore dimension, the number of pores, spacing between pores, filter surface material, and filter thickness. Antibody-based methods are mostly employed as the cell capture techniques, and among them, EpCAM is the most commonly used marker for this regard. Protein-based immunofluorescence (CellSearch) or flow cytometry; nucleic acid-based fluorescence in situ hybridization (FISH), real time-polymerase chain reaction (RT-PCR), real time quantitative-PCR (RT-qPCR), microarrays, or sequencing are the other assay-based technologies.[75] For CTM identification, the most widely used batch purification approaches are likely to disturb cellular aggregates, so that some extra approaches have been launched by taking the advantages of biological and physical clues of epithelial cells as aforementioned. Likewise, CK(+) and CD45(–) markers can be utilized with enrichment methods to isolate microemboli.[76] It is worth mentioning that micropost array-based herringbone microfluidic chip (HB-Chip) is an impactful tool to preserve multicellular aggregates owing to its size-based intriguing design.[77]

4. Clinical/Research Methods for CTCs/CTM Enrichment and Isolation

Various enrichment methods focusing on determining biological and physical differences between CTCs/CTM and blood cells have been examined in the literature.[8,78] The specification of CTCs/CTM markers is complicated due to intra/interpatient heterogeneity in tumor biology. On the other hand, these properties can be employed to isolate and distinguish CTCs/CTM against around billions of white and red blood cells in circulation. Briefly, smart materials, including aerogel-based polymers, bioconjugates, nanofibers, metal foams, piezoelectric materials, and shape-memory materials, have been utilized in diagnostic devices for this manner.[79–81] For example, graphene—a conductive and 2D smart material with a very long periodic carbon honeycomb chain in the horizontal plane[82]—was integrated into a platform to identify and enrich CTCs/CTM as high as 98.15% of efficiency thanks to its high conductance ability.[80] The most widely used physical approaches for the isolation and enrichment of these tumor cells involve size and deformability-based filtration,[71,83] density-gradient centrifugation,[84] and electrical property-based dielectrophoresis (DEP) separation.[26] The biological feature-dependent enrichment methodologies are either a positive selection targeting surface markers, especially for the stages of metastasis or a negative selection strategy derived from the depletion of blood cells.[31] Apart from the conventional methods, microfluidic devices and batch purification methods are currently in use for CTCs/CTM enrichment.[61,85] This enrichment strategy also impedes with notable challenges that include low enrichment, recovery, and purity rate. Underlining again, the enrichment of tumor clusters is more struggling than the way of capturing single tumor cells because of rarity and limited lifespan. Furthermore, a significant amount of challenges including cost, energy efficiency, and manufacturing defects are encountered when smart materials moved from lab-bench to industry, whereas there is a huge demand for advanced technologies to meet out affordable, easy-to-produce, consistent, and controllable manufacturing. Prior to reaching clinical applications, these aforementioned obstacles need to be considered and fulfilled properly. In addition, more sophisticated designs, fabrication, and characterization techniques hold crucial potential to significantly improve the current bar for producing smart materials with high quality.

4.1. Performance Metrics for CTCs/CTM Technologies

To have a comparative picture among all the methods, we here benchmark the performance of CTCs/CTM enrichment platforms with the following parameters: 1) capture sensitivity and efficiency, 2) specificity and purity, 3) enrichment rate, 4) throughput, 5) viability, and 6) clinical yield. Sensitivity of detection/isolation relies on the smallest number of CTCs detected/isolated in the input sample, which is crucial for prediagnostic cases (Equation (1)). Purity rate is the ratio of isolated or detected CTCs compared to all captured cells from a sample (Equation (2)). Enrichment rate is the ratio of CTCs to blood cells before and after the enrichment process (Equation (3)). Throughput is the number of tumor cells processed per unit of time. Indeed, the throughput is the speed of the sensor, and it also can be calculated from the volumetric flow rate in microfluidics (Equation (4)). Viability is the percentage of the viable population of CTCs (Equation (5)). Clinical yield is the ratio of CTCs isolated from patients with an established cancer stage and stage by considering the total volume of the sample (Equation (6)).[8,62,86,87] All these parameters are also formalized as follows

$$\text{Capture Efficiency}_{\text{Tumor Cells}} = \frac{\text{Tumor Cells}_{\text{output}}}{\text{Tumor Cells}_{\text{input}}} \tag{1}$$

$$\text{Purity}_{\text{Tumor Cells}} = \frac{\text{Tumor Cells}_{\text{recovered}}}{\text{Tumor Cells}_{\text{recovered}} + \text{Background Cells}_{\text{input}}} \tag{2}$$

$$\text{Enrichment Rate}_{\text{Tumor Cells}} = \frac{(\text{Tumor Cells/Background cells})_{\text{recovered}}}{(\text{Tumor Cells/Background cells})_{\text{sample}}} \quad (3)$$

$$\text{Throughput} = \frac{\text{Volumetric Flow Rate}}{\text{Time}} \quad (4)$$

$$\text{Viability}_{\text{Tumor Cells}} = \frac{\text{Viable Tumor Cells}_{\text{recovered}}}{\text{Tumor Cells}_{\text{recovered}}} \quad (5)$$

$$\text{Clinical Yield Percentage}_{\text{Tumor Cells}} = \frac{\text{Tumor Cells}_{\text{recovered}}}{\text{Total Volume}} \times 100 \quad (6)$$

4.2. Batch Purification Methods

Multiple batch approaches—one of the earliest methods of isolating single CTCs—have been employed to separate cells according to their density gradient and immunomagnetic characteristics.[24] Microfluidic devices over batch purification contribute to noteworthy advantages, such as enabling excessively efficient processing of extracting complex cellular fluids with minimum damages due to low-scale shear forces.[88] For instance, a study conducted on demonstrating the effectiveness of a microfluidic mixing model has increased the interactions between the immunofluorescence-conjugated antibody-coated chip surface and CTCs with a periodically staggered herringbone grooves-based low shear design of the chip. Compared to the other micropost-based microfluidics, HB-Chip captured PC3 prostate cancer cells more efficiently, especially at below 3 mL h^{-1}, and almost all of the patients with metastatic disease (14 out of 15 patients: ≈93%) were detected as depicted in **Figure 2**a.[89]

4.3. Conventional Laboratory Methods

Magnetic affinity selection is a frequently employed method to isolate CTCs from patient samples. The CellSearch is considered as a gold standard, and it is so far the first and only CTCs capture assay validated by the FDA as a prognostic tool for patients, who have metastatic prostate, breast, or colorectal cancer. It is designed for enumerating epithelial-originated CTCs and utilizes anti-EpCAM antibody-coated magnetic beads. After the capture process, magnetically labeled CTCs are extracted by applying a magnetic field with a nuclear stain DAPI(+). Furthermore, fluorescent-tagged antibodies can be used for differentiating CTCs according to their surface markers such as EpCAM(+), CK(+), and CD45(–) from white blood cells. However, this method has some limitations, such as 1) the low recovery of CTCs,[90] 2) difficulties to monitor the subpopulation of CTCs undergoing the EMT process,[91] and 3) high background signals due to sensor contaminations caused by WBCs.[5]

4.4. Physical Principles for Selecting CTCs/CTM

Physical assets of CTCs/CTM for enrichment and isolation technologies rely on differences in physical parameters, including density, size, deformability, electrical polarizability, or the distinguishable phenotypes between leukocytes and CTCs. For instance, the epithelial cell-originated CTCs are assumed that they are larger than leukocytes and through a porous membrane, CTCs can be isolated from media via using size difference-based microfiltration devices.[74]

The selection of CTCs through the size of epithelial tumor cells (ISET[92] and ScreenCell[93]) is a method that has been used since the 1960s.[94] By ISET, the subpopulation of EMT can be captured by filtration thanks to their larger sizes than that of peripheral blood leukocytes. Briefly, the filtered blood is first subjected to red blood cell lysis and fixation in a module that has 12 wells with pores of 8 μm diameter. ISET eliminates CTCs from blood in a deformability and size manner, thereby improving cell recovery.[92] In spite of these valuable features, the challenges with filtration include low CTC recoveries (≈50%) and high background signal due to WBCs.[95] Furthermore, a combination of multiorifice flow fractionation (MOFF) and dielectrophoresis (DEP) hydrodynamic size-based separation technique for human breast cancer cells has high efficiency (the removal of >99% for RBCs and >94% for WBCs) without labeling process.[96]

Advanced filter membranes have combined lithographic methods patterned with pores into polymers that are capable of isolating viable CTCs from blood. For instance, a 3D microfilter device, which consists of two layers of 5–2.5 μm-thick parylene-C photolithographic membrane with pores and gap, results in high cell viability of the captured cells.[97] As another study, a flexible microspring array (FMSA) device has enabled to minimize cell damage for increasing the viability of CTCs/CTM by altering the chip design parameters in order to reduce shear stress. The FMSA device has 90% capture efficiency along with 80% viability and higher CTCs capture yield for breast, lung, and colorectal cancer patients compared to the CellSearch method.[98] Lastly, a microfiltration system (CellSieve), made with high porous patterns working under low pressure, is designed to isolate CTCs through size exclusion and their subcategories while sustaining intracellular content. CellSieve showed high isolation throughput for EpCAM/CD45/CK biomarkers compared to CellSearch thanks to its sophisticatedly arranged and distinguishable design.[99]

Considering the physical methods, analyzing cellular phenotypes of CTCs/CTM would be an effective way to gain insight into the isolation methods because the capacity of cell invasion, cell microenvironment interactions, and metastatic cascade of tumor cells are considered to have a strong relationship with biophysical properties. Comprehensive analysis of tumor cell motility, adhesion, and drug response might be key strategies to design and provide deeper insights into the enrichment methods.[100] In the aspect of invasive phenotype, collagen adhesion matrix (CAM) assay, which is a functional cell separation method, is employed to explore invade of tumor cells in the circulation. For example, the CAM-coated system has recovered tumor cells with a $54 \pm 9\%$ of recovery rate, 0.5–35% of purity, and detected invasive tumor cells with 100% of yield. In this study, the researchers have also correlated stage I–III breast cancer (28/54 ratio) to lymph node status and survival of patients in the early stage.[101]

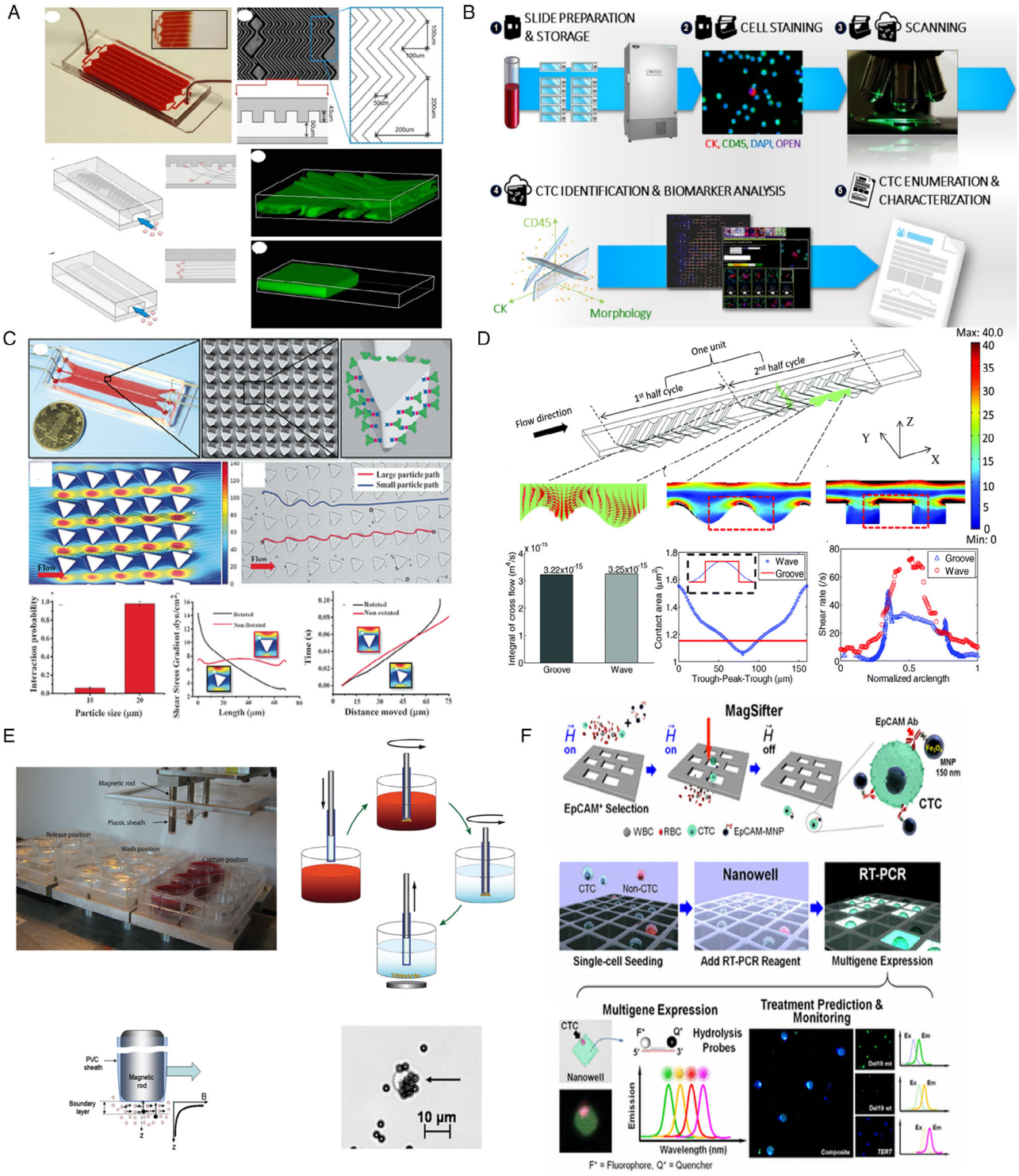

Figure 2. a) The herringbone chip consists of a microfluidic array of channels with a single inlet and outlet. The schematic of these herringbone grooves shows the periodicity and asymmetry of the surface, and the schematic representation compares the cell–surface interactions and the chaotic microvortices in the traditional microfluidic device (no grooves) and herringbone chip. Reproduced with permission.[89] Copyright 2021, PNAS. b) A schematic demonstrates the biomarker expression and enumeration of CTCs. Reproduced with permission.[104] Copyright 2021, Journal of Circulating Biomarkers. c) The schematic of SDI-Chip exhibits size-based separation. Reproduced with permission.[107] Copyright 2021, Angew Chemie International Edition. d) The principle of herringbone microfluidic chip is represented. Reproduced with permission.[115] Copyright 2021, Royal Society of Chemistry. e) A schematic of MagSweeper isolation protocol is exhibited. Reproduced with permission.[124] Copyright 2021, PLoS One. f) The workflow of MagSifter system is demonstrated. Reproduced with permission.[113] Copyright 2021, PNAS.

4.5. Immunocapture of CTCs/CTM

Immunocapture of CTCs/CTM usually accomplishes high-throughput via surface markers with highly specific interactions. For instance, immunostaining-FISH (iFISH) was developed for both CTCs and CTM enrichment with >69% capture yield for all the cultured tumor cell types.[102] Moreover, combining immunofluorescence with DNA fluorescent in situ hybridization (DNA FISH) method analyzes cell capture because a functionalized medical wire permits in vivo isolation of CTCs.[103] In addition to FISH, a clinically feasible Epic CTC Platform is designed to assess analytic assay performance using immunofluorescence and genetic biomarkers in the samples collected from a liquid biopsy of healthy donors and prostate cancer patients. CTM and CTCs were found as 89% and 100% of patient samples, respectively (Figure 2b).[104]

4.6. Microfluidics Isolation Methods

Microfluidics typically manipulates fluids at a micrometer scale with high throughput, specificity, sensitivity, and biocompatibility fashions.[105,106] The basic elements of microfluidics consist of microchannels, chambers, and valves. This technology requires three main actions, such as sampling, processing, and validation. The capability of isolating rare cells comes from different passive methods that tailor the microfilters of varying pore sizes,[107] flow chamber geometries,[108] microstructures,[109] and flow density[110] with precision and active methods, which rely on compressibility,[111] polarizability,[112] and magnetic susceptibility as shown in **Table 1**.[113] For the CTCs/CTM enumeration and further analysis for downstream processes, the surface chemistry of microfluidic platforms needs to be designed to control the capture and release of tumor cells. For instance, a surface-coated microfluidic chip (CMx platform) has been performed under a biomimetic supported lipid bilayer conjugated with anti-EpCAM antibodies to detect CTCs/CTM, which are abundant for patients with pancreatic ductal adenocarcinoma (PDAC).[114] Another example is reliant on a size-dictated immunocapture chip (SDI-Chip) with hydrodynamically optimized, two-mirrored anti-EpCAM antibody-coated micropillar surfaces that can capture different antigen levels with more than 92% efficiency, as illustrated in Figure 2c.[107] Moreover, a passive mixing within a wavy-herringbone microfluidic chip (HB-Chip), which was functionalized with anti-EpCAM antibodies, has achieved 85% of capture efficiency along with 39.4% purity of HCT-116 colorectal cancer cells as exhibited in Figure 2d.[115]

On the other hand, in the literature, several label-free microfluidic devices have been used to isolate CTCs/CTM from the

Table 1. Various approaches for isolating CTCs/CTM..

CTCs/CTM enrichment and isolation methods[a)]	Technology	Capture efficiency [%]	LOD [CTCs and CTM mL^{-1}]	Sample (media) volume and/or flow rate	Detection range [CTCs and CTM mL^{-1}]	Average detection time	Number of cells in the media [CTCs and CTM mL^{-1}]	References
Batch purification methods	Herringbone (HB)-Chip	93	12	1.2 mL h^{-1}	386 ± 238	1–405.8 s	12–3167	[89]
Conventional laboratory methods	FISH	60–70	≦1	7.5 mL	8 ± 92.4	7–14 days	3149	[27]
Physical principles for isolating CTCs/CTM	MOFF and DEP	94–99	–	126 µL min^{-1}	–	300 s	10^6	[96]
	3D microfiltration	86	–	3.75 mL min^{-1}	342 ± 58	180–300 s	$4.5–11 \times 10^6$	[97]
	FMSA	90	≦7	7.5 mL	–	<600 s	1000	[98]
	CellSieve	68–100	28.0	7.5 mL	46.6 ± 37.0	–	1000	[99]
	Adhesion Matrix (CAM) assay	52	18	1 mL	126 ± 25	1–2 months	18–256	[101]
Immunocapture of CTCs/CTM	FISH and NGS	89	≦1	10 mL	–	–	1–28	[104]
Microfluidics isolation methods	Surface-Coated Microfluidic Chip (CMx platform)	81	≦15	2 mL	–	1 h	600	[114]
	HB-Chip	85	≦10	1 mL	10–1000	≈9 h	$10^3–10^5$	[115]
	Parsortix	42–70	–	2 mL	0–6.5	≈3 h	10^6	[117]
	CTC-iChip	≥90	–	8 mL h^{-1}	$1,200 \pm 900$	2 h	10^3	[118]
Magnetic affinity-based selection	MagSweeper	70	–	10 mL	–	≈2–3 h	–	[124]
	CTC-Chip (Ephesia)	≥90	≦13	10 mL	13–1000	<4 h	2.5	[125]
	CTC-Chip	≥90	≦1	1–3 mL	31–96	≈4 h	4–470	[126]
	µNMR	≥60	≦3		3–27	–	140–6300	[127]

a)Abbreviations: (HB)-Chip, herringbone-chip; FISH, fluorescence in situ hybridization; MOFF, multiorifice flow fractionation; DEP, dielectrophoresis; FMSA, flexible microspring array; NGS, next-generation sequencing; CMx, coated microfluidic; iChip, antigen-independent microfluidic; µNMR, micronuclear magnetic resonance. Note: Some of the parameters were not reported in the original research, and hence, they were not stated with a value on this table.

microenvironment. For instance, a microchip technology, i.e., the Cluster-Chip, isolates CTM through independently tumor-specific markers and bifurcating traps under low shear stress from blood.[116] Additionally, the prostate adenocarcinomas and human breast cancer cells were captured with artificial clusters.[71] Another perspective is the Parsotrix cell separation system, which provides marker independent capture of CTCs according to their size and deformability with a 42–70% of efficiency range and 99% of viability.[117] Last but not least, an antigen-independent microfluidic CTC-iChip technology employs a passive method, which is a deterministic lateral displacement system reliant on the size separation of WBCs and CTCs from whole blood. The system is composed of two separate parts that initially perform inertial focusing for precise positioning, magnetophoresis to separate up to 10^7 cells s^{-1}, and then the depletion of antibodies against leukocytes with a 97% yield of rare cancer cells.[118]

4.7. Magnetic Affinity-Based Selection

Enrichment reliant on magnetic affinity is another method, in which both immunomagnetic assays and microfluidics are employed to distinguish rare cells and cellular entities.[119–122] As previously mentioned, the CellSearch platform is the first validated CTCs enrichment assay using magnetic fields.[27,99] On the other hand, immunomagnetic cell separation and density gradient are some of the first recorded studies of CTM isolation from whole human blood.[123] Most of the immunomagnetic systems utilize antibody functionalized magnetic nanobeads to isolate CTCs.[95,124] Presenting functional, reliable, and reproducible fashions are the main strengths of these systems, yet they still have some impediments regarding imaging, optical analysis, and poor detection range due to excessive saturation of the surfaces of the beads.[125] From an application perspective, MagSweeper technology—a robotic liquid biopsy device—isolates and purifies viable CTCs efficiently through magnetic rods covered in plastic sleeves. The MagSweeper especially addresses leukocyte contamination or limited methodological sensitivity, thereby overcoming this challenge (Figure 2e).[5,124] Moreover, an inexpensive and sophisticated method called magnetic sifter or MagSifter employs an electromagnetic device that pulls the nanoparticle-labeled CTCs into a flat array of tiny wells, and each of them accommodates only one cancer cell (Figure 2f).[113,126] Many other innovative platforms, such as micronuclear magnetic resonance (μNMR),[127] MagDense,[128] and Maglev,[129] can be also listed under the umbrella of separation methods for CTCs/CTM.

5. Why Do We Need to Release Tumor Cells?

So far, we have elaborated fundamental aspects in CTC/CTM biology through surface markers and expanded this view to employ cell separation and detection technologies. Once cancer cells are captured specifically, we need to understand their origin and heterogeneity to introduce the most efficient therapy. The release of CTCs/CTM from a surface is the mainstay in this regard. Elaborating this aspect, the mode of CTCs/CTM release enables culture expansion, phenotype identification, and molecular analysis of captured cancer cells. Here, surface chemistry is one of the most pivotal aspects that allow the controlling of cancer cell release with viable manners. In the course of this action, tumor cells might have some structural damages or hold some contaminations that could potentially hinder the downstream analyses. While keeping performance metrics at the highest levels possible, this process needs to be very specific and very gentle for accurate analysis of CTCs/CTM genome, transcriptome, and proteome. For instance, in the case of low intact viability, the impurity of CTCs/CTM disturbs true positive signals and obfuscates the downstream molecular profiling.[61] To hurdle these obstacles, CTCs/CTM release techniques can occur via mechanosensitive or thermal modes, enzymatic, chemical, and self-assembly-based interactions with the favor of smart materials.

6. Smart Materials for the Release of CTCs/CTM

Intelligent materials can actively or passively change their original properties against external stimuli.[130] Not only altering conformation, but also transferring and converting an energy type to another energy type is the most important capability of advanced materials. Therefore, they are mainly integrated into sensing devices and actuators as piezoelectric, electrostrictive, magnetostrictive, self-actuated, self-healing, and self-diagnostic components. Especially for releasing the captured CTCs/CTM, smart material-based emerging platforms have exhibited a notable impact on the isolation of cells. Contemporary applications are mostly utilized on microfluidic devices for light-sensitive, thermal, magnetic, electrochemical, aptamer mediated, affinity-based, ligand competitive, and enzyme degradative affect-based hybrid technologies (**Table 2**).[131] On the other hand, there are still challenges about smart material fabrication including 1) inaccurate manufacturing properties; 2) limitations on construction platform dimensions and aspect ratio; 3) limited repeatability; 4) the lack of adaptation to different industrial fields; 5) interlayer imperfectness; 6) impeded massive production; 7) the paucity of handiness; 8) nonsustainability; and lastly; 9) poor data management for executing practical functions while altering, transferring, and converting stimuli types.[132] In this aspect, well-designed, fabricated, and characterized methods and also the integration of new strategies such as AI-based techniques would be key solutions to hurdle these challenges. Smart biomaterials such as hydrogels, bionanoparticles, bioconjugates, bionanofibers, and shape-memory biomaterials are widely used in the field of rare tumor cells isolation,[79,133] and herein, we denote the developmental trends, challenges, and next-generation methods of nanotechnology approaches from a CTC/CTM release perspective.

6.1. External Stimuli-Based Strategies

In this section, we elaborate on the feature and applications of photosensitive, thermoresponsive, and hybrid (combining at least two stimuli) systems. Mentioning the photoresponsive systems, light is the major external factor that triggers the molecular structure of a material, including changes in size,

Table 2. Versatile smart materials for isolating CTCs/CTM.

Type of smart materials	Advantages	Disadvantages	Applications	References
Magnetostrictive	Energy density is excessive	The complexity of the system due to the composite shape and internal structure	Aptamer-assisted tumor cells isolation	[153,154]
	Robustness		Applying an external magnetic field to the nanoparticles for the CTC capture	
Shape-memory materials	Corrosion-resistive	High cost	Immunomagnetic enrichment via shape-memory polymer films	[193,194]
	Excessive fatigue failure life	Excessive cycle fatigue	Inkjet-Print Micromagnets-assisted polymer films	
	Extrinsic and intrinsic robustness	Temperature sensitivity may be challenging		
	High damping ability	Complicated designs		
		Heavy metal feature of the material diminishes portability aspects		
Magnetorheological fluid	Excessive permeability	High fidelity fluids are expensive to produce	Microfluidic device for CTCs separation and isolation	[195,196]
	Excessive saturation of magnetization	In ferroscale, the particle stabilizing is limited	Mechanical Degradation of tumor cells via iron particles consisting of magneto-rheological fluids	
	A minute amount of remnant magnetization			
Electrorheological fluid	Highly stable system	Density may be high	Electro-rheological fluid fabrication for tumor cells induction	[197]
	Simple design	After an extended application time, the radius of fluid flow becomes larger		
	The advantage of power amplifier	The need for liquid refreshment in the setup		
Optical fibers	High bandwidth substructure	Not convenient for higher optical powers	Fiber optic arrays scanning technology (FAST) for high-speed detection of CTCs	[96,198]
	Highly resistive to the electromagnetic force interferences	Design and fabrication are not affordable	Optical fiber integrated fluids for the fluorescence quantification of cells	
	Highly flexibility	The electrical power operation to terminal devices may not be possible		
	Resistive to the corrosive environment			
	Not bulky, convenient for portable system design and integration with the other modalities			
Piezoelectric	Highly responsive to frequency changes	Heat and wear generation	Acoustic separation of CTCs via piezoelectric substrates	[199-201]
	Converting electrical signals to mechanical forces	The nanoscalability is limited	Piezoelectric pumps for the alignment of the flow in order to transport CTCs into the detection region of the system	
		Limited manufacturing	Microdispenser focusing on impedance and actuation differences	
		Complex structure and designs		

diameter, or surface charge.[130] For instance, to elucidate the interfacial nature of platforms, light stimuli can be performed conceivably due to its maneuverability of being controlled precisely.[134–136] Considering optical sensors, such as surface-enhanced Raman scattering (SERS), surface plasmon resonance (SPR), luminescence, and fluorescence aptasensors,[135,137–144] light stimuli-integrated systems would have controlled the harvesting of rare tumor cells from isolation platforms. For instance, a near-infrared (NIR) light-responsive substrate has been designed for immunocapture and site-release of individual CTCs through the utilization of plasmonic signals derived from gold nanorods (GNRs), which was conjugated with a thermoresponsive hydrogel.[135] Briefly, target tumor cells were initially imprinted on GNR-pre-embedded gelatin hydrogel substrate. Immunoaffinity interactions and nanostructures created by an artificial cell stamp have improved cell recognition efficiency. The hydrogel substrate

dissolved at physiologic temperature (37 °C) has altered surface characteristics, and this has enabled the bulk release of the captured cells. Applying a cell-size NIR laser spot has also achieved for the site-release of cells owing to the photothermal effect of GNRs at a small region. By employing anti-EpCAM- antibody-coated gelatin hydrogels, the capture efficiency of MCF-7 cells was observed around $92 \pm 6\%$, and also, the efficiencies of cell release from the bulk and a small region were found as $95 \pm 4\%$ and $92 \pm 6\%$, respectively.

Another CTC analysis platform has combined triangular silver nanoprisms (AgNPRs) and superparamagnetic iron oxide nanoparticles (SPIONs).[138] Briefly, AgNPRs were treated with 4-mercaptobenzoic acid (MBA), and this step was followed with the modifications of reductive bovine serum albumin (rBSA) and folic acid (FA), respectively. Likewise, SPIONs were modified with same reagents to present FA molecules. All these particles were designed to capture HeLa cells through the interactions between FA and folate receptor alpha (FRα). By simply applying a magnet, the captured cells through the modified AgNPRs and SPIONs were collected, and this step was further processed with the addition of free FA in order to release cells in the tube. In addition, the researchers were able to monitor all these processes through the changes in SERS signals. As a result, with only the SERS strategy, the method achieved to detect as low as 5 cells per mL, whereas this was further improved with the combination of SPIONs and SERS strategy and resulted in detecting only a single cell per mL (**Figure 3**a).

Thermoresponsive systems also can be used in terms of the release of captured CTCs from the surface of such capturing device. Either polymers or carbon-based materials can be utilized as composites to improve the viability of the isolated CTCs/CTM.[145] Thereinto, graphene-based polymer composites, poly(*N*-isopropyl acrylamide, PIPAAm), or hydrogel grafted polymer brushes/surfaces have been mostly used platforms, which are subjected to surface hydrophobic-to-hydrophilic transitions at the lower critical solution temperature (LCST).[146–148] One of the examples is a tunable thermoresponsive graphene oxide (GO)-based poly(*N*-acryloyl piperidine-*co*-*N*, *N*-diethyl acrylamide) copolymer composite chip that can efficiently capture and reversibly release the CTCs, which are isolated on a microfluidic platform.[146] To achieve downstream investigation, molecular analysis, FISH, and single-cell analysis with an LCST of 13 °C were performed. The device was functionalized by immobilizing anti-EpCAM antibodies and the capture efficiency of 95.21% was obtained at a $1\ mL\ h^{-1}$ of the flow rate for EpCAM-positive cancer cells. For the release study, the efficiency of 95.21% in buffer and 91.56% in blood was observed along with a 91.68% of viability of the released cells.

In addition, a thermoresponsive gelatin hydrogel-coated 3D gelatin self-assembled (polydimethylsiloxane) PDMS scaffold chip has been developed through the layer-by-layer hydrogel for capture and release of both single tumor cell and cluster by compelling cells undergoing vortex or chaotic migration.[148] Gelatin is basically dissolved at 37 °C and below this temperature; the characteristic of the platform has a transition from a hydrophobic collapsed state to a hydrophilic swollen state, thereby allowing the release of viable cells. The modification of sulfo-NHS-biotin, streptavidin, and anti-EpCAM antibodies enables the capture of MCF-7 cells. The experiments demonstrated that a high capture efficiency, and an 80% of recovery yield of CTCs/CTM has been obtained along with more than 90% of viability at 2, 1, 0.5, and $0.2\ mL\ min^{-1}$ of flow rates as shown in Figure 3b.

6.2. Aptamer-Mediated Release

The aptamers are single-stranded oligonucleotides or peptides that fold into distinct secondary and tertiary structures to recognize target molecules or cells. The isolation of aptamers is mostly performed via the cell-systematic evolution of ligands by exponential enrichment (SELEX),[149] and the aptamers have unique characteristics such as high affinity, rapid response, reproducible synthesis, ease of modification, small size, and nontoxicity as a biorecognition molecule, bringing them prominent predominance in the studies for CTCs/CTM release.[150] By changing their conformation, aptamers would lose specificity and affinity, which results in allowing a myriad of alternatives, especially to antibodies, to release viable CTCs/CTM. For instance, smart cyclic signal ampliative DNAzyme probes as ion sensing elements were designed to capture and release of CTCs.[151] In this context, the Sgc8c (Cu^{2+}-DNAzyme-sgc8c) and TD05 (Mg^{2+}-DNAzyme-TD05) aptamer modifications were utilized for capturing CCRF-CEM and Ramos cells. The addition of Cu^{2+} and Mg^{2+} (cofactors in the reaction) catalyzed the cleavages of the substrate strands, thereby enabling the release of the captured cells. In conclusion, this strategy was capable of capturing CTCs with approximately 90% of efficiency in the buffer and with 80% of efficiency in the blood samples, as well as the platform was able to release around 70% of the captured cells from two different cell lines. Another confirmation strategy was the enzyme degradation of aptamers, which was established through a tetrahedral DNA nanostructure with a pendant aptamer grafted onto a deterministic lateral displacement (DLD)-patterned microfluidic chip (ApTDN-Chip).[152] The microfluidic chip was homogenously oriented in order to capture and release of CTCs at the top vertex of the structure. The rigid tetrahedral DNA scaffold helped to control the arrangement of aptamers for enhancing target interaction, and also, reduced the local overcrowding effect in order to make aptamers more accessible to DNA nuclease. Furthermore, the triangular micropillar array-based DLD enabled a high number of collisions between CTCs and micropillars. Compared with the other aptamer-based microfluidic interfaces, the capture efficiency has been enhanced to nearly 60% by using the ApTDN-Chip, and an 83% of release efficiency along with a 91% of cell viability (Figure 3c).

6.3. Magnetic Particle-Based Release

The magnetic particle-based strategies mostly depend on the isolation of magnetically labeled cells or clusters. These nanoparticles are mostly functionalized with different antibodies by CTCs (positive enrichment) or blood cells (negative enrichment) as previously stated in the literature.[90,91] The core idea about the extraction of tumor cells is to apply a proper magnetic field that can pull both the labeled CTCs and free magnetic nanoparticles onto the platform surface.[153] The crucial parameter for this strategy is the recovery rate of the immunoaffinity coupled with

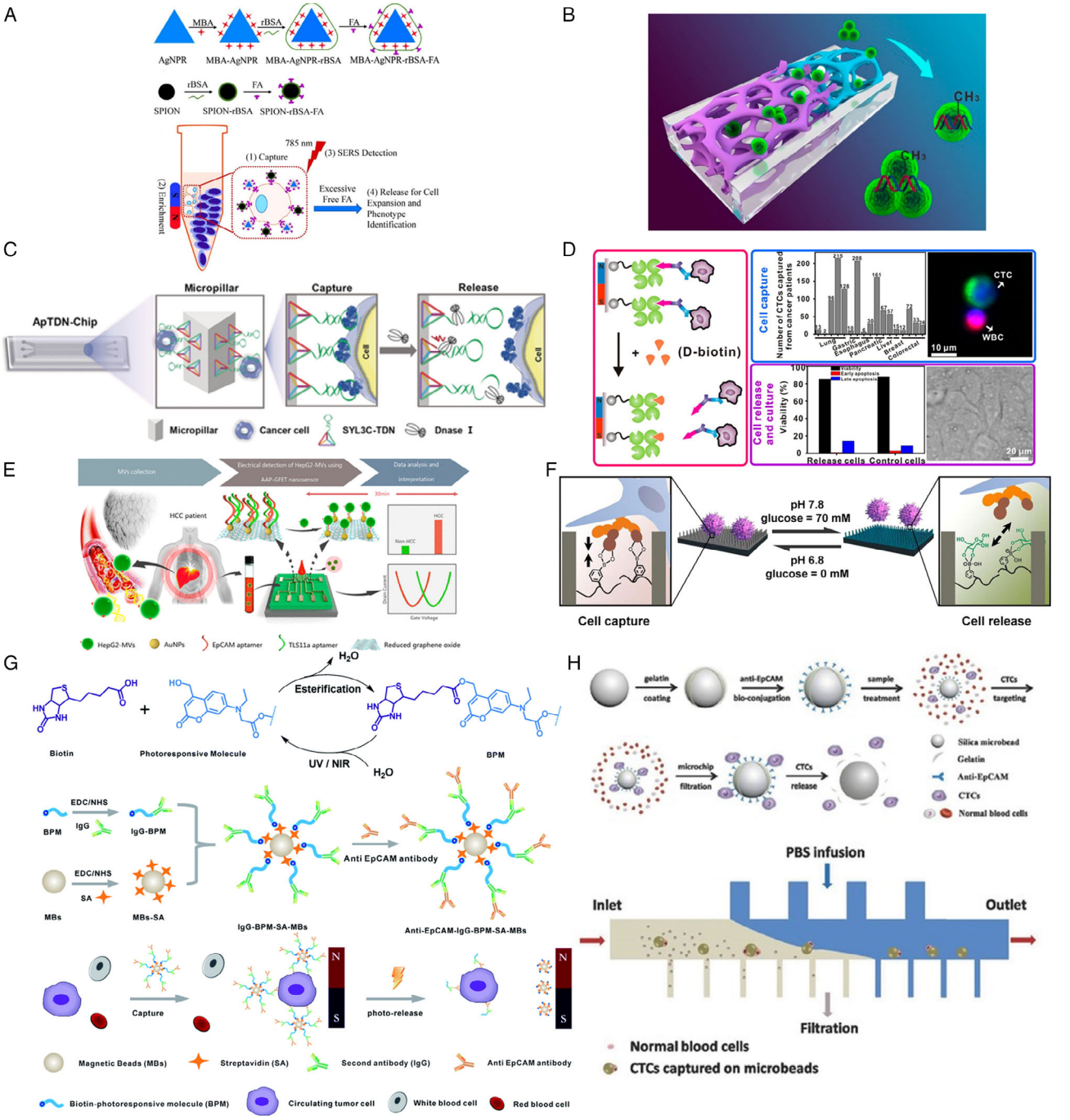

Figure 3. a) The schematic represents CTCs analysis system reliant on AgNPR and SPION. Reproduced with permission.[138] Copyright 2021, ACS Biomaterials Science & Engineering. b) Thermoresponsive 3D scaffold chip is represented. Reproduced with permission.[148] Copyright 2021, Analytical Chemistry. c) The workflow of ApTDN-Chip is presented. Reproduced with permission.[152] Copyright 2021, Angew Chemie International Edition. d) A schematic of biotin-triggered decomposable immunomagnetic beads along with the results from the capture and release studies. Reproduced with permission.[155] Copyright 2021, ACS Applied Materials & Interfaces. e) The scheme shows the electrical detection on a nanosensor. Reproduced with permission.[162] Copyright 2021, Analytical Chemistry. f) A schematic presents the glucose and pH dual-responsive surface for the capture and release of CTCs. Reproduced with permission.[168] Copyright 2021, Journal of the American Chemical Society. g) The schematic exhibits the photosensitive immunomagnetic system for the capture and release of CTCs. Reproduced with permission.[171] Copyright 2021, Chem Science Journal. h) A schematic represents the isolation of CTCs through the microbead-mediated size amplification. Reproduced with permission.[177] Copyright 2021, Advanced Healthcare Materials.

magnetic particles, relying on the integrity and expression level of the antibody binding epitopes of the target antigen.[95,154] For instance, anti-HER2 antibody, anti-EpCAM antibody, and anti-EGFR antibody were employed for capturing CTCs in a nano-bio-probe immunomagnetic system.[155] Furthermore, in this study, after the release of cells, it was accomplished ex vivo culture of viable CTCs for providing a genotype of the primary tumor. Here, the capture of cells was created on the basis of the interactions of Strep-tag II (a short peptide sequence) with Strep-Tactin (a mutated streptavidin molecule with the biotin-binding site)-coated magnetic beads (STMBs). Strep-Tactin and Strep-tag II-derived antibody-STMBs were used as a capture agent, and by introducing biotin, the cancer cells were detached from STMBs. Quantitatively, approximately 70% of the captured cells were released, and around 85% of the released cells have remained viable as depicted in Figure 3d. From another perspective, a hybrid magnet-deformable CTC chip was established to enumerate CTCs bonded with magnetic immune beads.[156] Adjacent micropillars were developed by gradually decreasing gaps formed by microellipse arrays filter, enabling small or compliant cells transition through the constriction to capture CTCs. After turning off the magnetic field, CTCs were released from the microellipse microfluidic chip. Different types of cell samples were applied to this system as a clinical validation, and the platform was able to capture cells with more than 90% of efficiency at the flow rate of 3 mL h^{-1} and provided a 96% of viability at the flow rate of 1.0 mL h^{-1}.

6.4. Electrochemical-Based Release

Electrochemical sensing provides the quantitative analysis of CTCs/CTM release by mainly investigating the redox state of the systems. Electrochemical stimuli-responsive materials can be employed by applying a voltage to the electrode surface in order to result in an conformational transition and alter the adhesion of particles.[157,158] Briefly, these strategies focus on potential, current, scan rate, impedance, and conductance alterations thereby resulting in efficient and fast response. However, the sensing capabilities of electrochemical sensors could be improved more due to the rarity of CTCs/CTM in blood. Furthermore, the accuracy of cell recognition could be also an obstacle since the protein structure of cell membrane of tumor cells causes the complexity of specification. Hence, electrochemical biosensors have been mostly hybridized with different materials, such as immunoassays, nanoparticles, composites, magnetic beads, nanowires, transistors, and nanosheets, as illustrated in Figure 3e.[159–164] To exemplify, an electrochemical assay system based on PdIrBPMNS (palladium–iridium–boron–phosphorus alloy-modified mesoporous nanospheres) and KB (Ketjen black)/AuNPs was designed for the isolation of MCF-7 cells.[159] Capture antibodies and signal antibodies chosen in this work were a cocktail of anti-vimentin antibodies and anti-EpCAM antibodies in order to improve the poor clinical relevance of the detection resulted from EMT. Creating a signal probe to catalyze H_2O_2 and also, in order to amplify the signal current, PdIrBPMNS was modified with a carboxylated PEGylation of thiolated heterobifunctional polyethylene glycol and anti-vimentin in the platform. KB/AuNPs were employed as an electrode to enhance the conductivity and antibodies binding through the interactions of Au-NH_2. CTCs were quantitatively analyzed by a differential pulse voltammetry (DPV) assay. The response of the method increased gradually from 1×10^1 to 1×10^6 cells mL^{-1} of CTCs, and 2 cells mL^{-1} was observed as the limit of detection (LOD) of this platform. On the other hand, introducing glycine hydrochloride (Gly-HCl) buffer as eluent enabled to dissociate the interactions between the biomarkers and antibodies, thereby resulting in the release of target cells.

6.5. Ligand Competition-Based Release

To form more stable chemical bonds by inserting ligands with stronger affinities is a favorable way of ligand competition-based release. With this technique, not only the selection of an appropriate approach, but also available experimental conditions are quite possible.[165,166] For instance, a herringbone chip with a thiolated ligand–exchange reaction via *N*-hydroxysuccinimide ester (NHS)-functionalized gold nanoparticles (NP-^{HB}CTC-Chip) was designed to isolate and release breast cancer cells from whole blood.[167] The nanoroughened structures of the chip enhanced specific interactions between cancer cells and antibodies, whereas biocompatible thiol molecules exchanged ligands and antibodies via metal–thiol interactions. Furthermore, the release of captured cells was enabled under the favor of the increased surface area through irregular surfaces of the NP assemblies. Briefly, NHS-terminated AuNPs were bonded to NeutrAvidin, and then, the unmodified chip was bound to NeutrAvidin–NP assemblies and finally coated with antibodies via tetravalent biotin–NeutrAvidin binding. For the cell release purposes, the addition of free glutathione (GSH), the most plentiful thiol species in the cytoplasm, was selected as a cell-release reagent. In this study, cell viability, capture, and release efficiency were found higher than that of unmodified herringbone chip. In another example, a glucose/pH-sensitive sensor was developed with a modified surface via poly(acrylamidophenylboronic acid) (polyAAPBA) brushes from an aligned silicon nanowire (SiNW) array that was able to reversibly capture and release the targeted cancer cells through a precise control of glucose concentration and pH.[168] Varying from a cell-adhesive condition to a cell-repulsive state was enabled by altering pH from 6.8 to 7.8 in the presence of 70 mM glucose. At pH 6.8, polyAAPBA brushes grafted on the SiNW array created a specific binding with sialic acid, which is localized on the membrane of MCF-7 cells, and then, rare tumor cells were released by elevating the pH value from pH 6.8 to pH 7.8. About 6% of 3-AAPBA units in polyAAPBA with further addition of glucose replaces the polyAAPBA/sialic acid complex having the binding constant (K_a) (Figure 3f).

6.6. Digestion of the Affinity Agent or Bond Cleavage-Based Release

To enhance the affinity for CTCs capture, 3D nanostructured agents can be used owing to their local topographic interactions of cellular surface elements at the same scale. Digestion of these nanocomponents would be favorable models in terms of cell release.[169] For example, a multivalent dual-aptamer-tethered

rolling circle amplification (MA-RCA) system based on DNA assembly was designed for capturing CTCs, and this was accomplished by tailoring interval hybridized dual receptor-recognizing aptamers to a long DNA scaffold.[170] The elasticity of the hybridization was able to enable the extending of DNA strands into the cell suspension and the interactions with cancer cells. CTCs were reversibly captured and released by DNA-triggered toehold-mediated strand displacement in a noninvasive protocol, particularly for human acute lymphoblastic leukemia cell lines (CCRF-CEM). In another example, a photoresponsive immunomagnetic carrier was developed for CTCs capture and release with a photo-trigger 7-Aminocoumarin bridge in order to connect the magnetic beads with anti-EpCAM antibodies.[171] Under NIR light illumination and UV, the C—O bonds generated by the coumarin moieties between streptavidin (SA)-modified immunomagnetic beads and capture antibodies were dissociated, and thereby, CTCs were released with $52 \pm 6\%$ and $73 \pm 4\%$ of capture efficiencies on the course of NIR and UV light irradiation, respectively. Moreover, 97% and 90% of these cells were viable in these irradiation conditions, respectively (Figure 3g).

6.7. Enzyme Degradation-Based Release

Enzymes are very well-known catalyzers of biochemical reactions that basically transform substrates into products. In the manner of CTCs/CTM capture and release, enzymes have been also employed in smart reaction fashion.[172] For instance, a two-stage platform was designed in this context through utilizing on-chip purification and off-chip enzymatic exposure.[173] Herein, cells were first attached with a herringbone structured microfluidic channels by inducing transverse flows, treating like a chaotic mixer, and then, by applying exonuclease enzyme, these cells were released from immunomagnetic beads along with both purity and recovery rate of >60%. Alternatively, because of biological limitations as the enzyme disruption of surface markers, biopolymers could be another option to tackle such obstacles in the restrained downstream analysis. To exemplify, by treating benzonase nuclease enzyme solution, silicon nanowire substrates (SiNWS)-grafted DNA aptamer agent was able to not only capture cell lung cancer cells, but also they enabled to release from NanoVelcro Chip with a 78–83% of viability.[174] In addition, biodegradable nanolayered films or nanofilms, i.e., enzymatically degradable polymers, were employed to screen downstream analysis by applying enzyme solutions.[175,176] Lastly, by employing metalloproteinases-9 (MMP-9) enzyme to gelatin-coated silica microbeads (SiO_2@Gel MBs), tumor cells were able to be released, yet it might be harmful to cell microenvironment due to the MMP-9 activity (Figure 3h).[177]

7. Artificial Intelligence of Cell Isolation and Characterization

Today, the healthcare system has benefited greatly from a number of advantages of artificial intelligence (AI)-based strategies, such as the chance of storing, comparing, and classifying enormous data via high-speed computers, and all these fashions have been precisely implemented into the fields of drug delivery, medicine, and cancer research (treatment, imaging, and cell sorting).[178–183] In particular, machine learning (ML)—an application of AI—enables automatically learning of the trained datasets without any internal programming. The attribution of recognizing and analyzing patterns employed in biomedical and clinical areas creates an enormous level of heterogeneous data. To hurdle this complexity, predicting a smooth model for increasing homogeneity via ML algorithms could be a way, which can operate the decisions according to the individual separated models.[184,185] In particular, genomic analysis of CTCs is a quite struggling process because of the cell heterogeneities, unknown mutations, and limitations in the analysis methods. To address such challenges, deep learning recommends autoencoding convolutional neural networks.[186] For example, a predictive method was designed by random forest algorithm in order to enumerate CTCs and circulating tumor DNA (ctDNA) through examination sites of metastatic actions in breast cancer. In this study, it was observed that epigenetic and genetic alterations of the *ESR1* gene were the potential factors to activate mutations.[187] Moreover, the label-free or marker-based imaging methods could be a great subject in the manner of tumor cell identification.[184] Additionally, a strategy called ClearCell Polaris was developed for the size-dependent enrichment of CTCs by focusing on the negative selection of CD45 biomarker. To label these cells, single-cell representations of Peripheral Blood Mononuclear Cells (PBMCs) and CTCs were used as training tools for unique cancer types of integrative detections.[188]

As we have known that tumor cells in circulation do not constitute only a single cell, and they could form a cluster (CTMs),[73] the AI-based view would be more dominant than that of the conventional way of thinking in biological properties of tumor cells and the EMT/MET process (**Figure 4**a).[189,190] Talking more on the smart material design, an AI-based nanoarray sensor was designed through a heterogeneous collection of chemosensitive nanostructured films to diagnose lung cancer.[191] In order to detect a single tumor cell, the AI-assisted nanoarray was employed to form chemisensitive films by using volatile organic mixture emanating in the air trapped above blood specimens. The strategy of this study was based on the difference of volatile mixture's chemophysical properties that could be resulted either from a cancer cell or a blood cell. Overall, the system provided a 95% of specificity, >90% of accuracy, and >85% of sensitivity via ML-based discriminate factor analysis **(**Figure 4b**)**.

8. Conclusion and Future Perspective

CTCs/CTM are special tools to understand tumor biology, metastasis, and also, provide treatment options through conventional and emerging biomarkers. In practice, "biopsy" is the most widely utilized method for cancer diagnosis, and it majorly guides the associated therapy. However, the heterogeneity of tumor microenvironment causes background impurities for the systems detecting the subpopulations of cancer cells, and consequently, this might lead to low capture efficiency. In this manner, new technologies are required to deploy more sophisticated solutions to routine clinical tests. By taking advantages of discovered biomarkers such as EpCAM for specific CTC detection, CellSearch was announced as the first validated technology. In the course of cancer research, the need for CTC analysis was

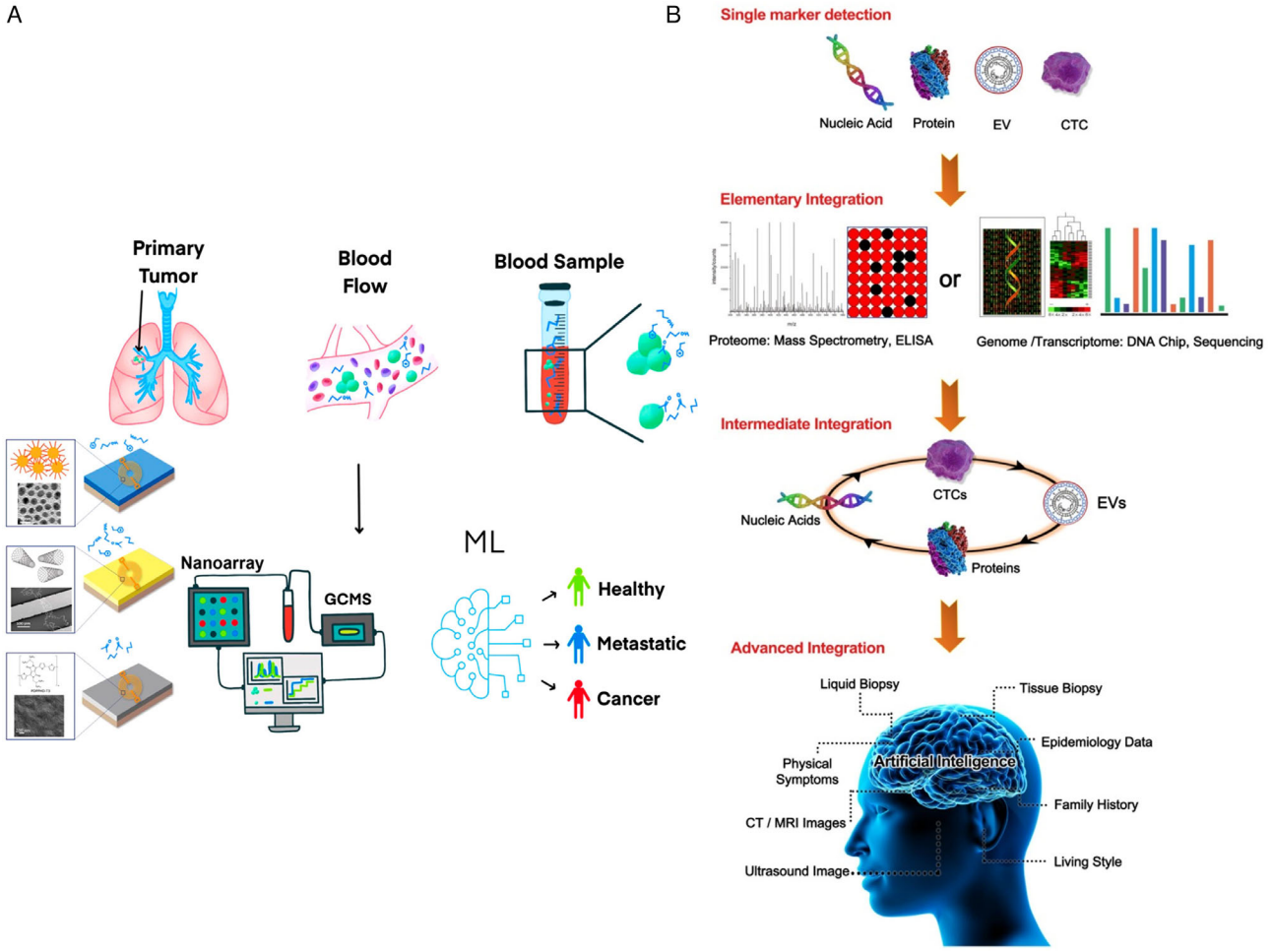

Figure 4. a) The working principle of artificial intelligence-integrated nanoarray sensors from primary tumor to metastatic prognosis period. Reproduced with permission.[191] Copyright 2021, ACS Nano. b) An overall schematic of liquid biopsy application and the integration of artificial intelligence-based strategy is presented. Reproduced with permission.[190] Copyright 2021, Ivyspring International Publisher.

not only limited to detect or capture cancer cells, but also isolate them through smart release mechanism with multiple external stimuli, such as thermal, optical, magnetic, electrochemical, aptamer mediated, affinity-based, ligand competitive, and enzyme degradative. On the other hand, a few of them could have received clinical validation from FDA. In addition to CTC studies, there is an urgent need for early diagnostic identification and enumeration techniques for CTM because of their crucial role in metastasis.[8] However, the effects of metastatic feature, size, and quantity on tumor cells within CTM have not been unearthed. In addition to new designs for material and/or detection systems, AI- and ML-integrated platforms would accelerate our understanding of the fundamentals of CTC/CTM research, and consequently, they would provide unprecedented solutions for extenuating metastatic cancer spread.

Acknowledgements

Dr. Fatih Inci gratefully acknowledges the support from TÜBITAK 2232—International Fellowship for Outstanding Researchers (Project No.: 118C254) and TÜBITAK 1001—The Scientific and Technological Research Projects Funding Program (Project No.: 120Z445). However, the entire responsibility of the publication/article belongs to the owner of the publication/article. The financial support received from TÜBITAK does not mean that the content of the publication is approved in a scientific sense by TÜBITAK. Dr. Fatih Inci and Kutay Sagdic also thank the support from TÜBITAK 3501—Career Development Program (CAREER) (Project No.: 120Z335). This work was supported by the BAGEP Award of the Science Academy.

Conflict of Interest

The authors declare no conflict of interest.

Keywords

circulating tumor cells, circulating tumor microemboli, diagnostics, microfluidics, smart materials

Received: July 8, 2021
Revised: October 26, 2021
Published online:

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