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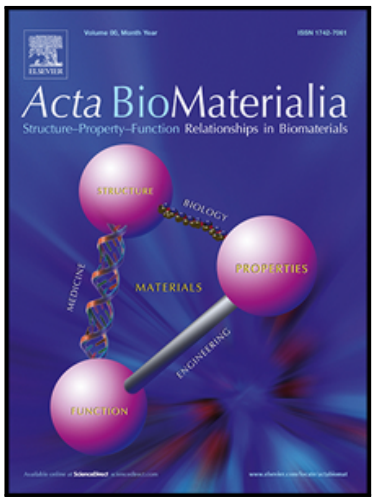

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Past, Present, and Future of Affinity-based Cell Separation Technologies

Kaitlyn Bacon[1,†], Ashton Lavoie[1,†], Balaji M. Rao[1,3], Michael Daniele[2], Stefano Menegatti[1,3,*]

[1] *Department of Chemical and Biomolecular Engineering, North Carolina State University, Raleigh, NC 27695-7905, USA*

[2] *Joint Department of Biomedical Engineering, North Carolina State University – University of North Carolina Chapel Hill, North Carolina, United States.*

[3] *Biomanufacturing Training and Education Center (BTEC), North Carolina State University, Raleigh, NC 27695-7928, USA*

† denotes equal contribution.

** Author to whom correspondence should be addressed; email: smenega@ncsu.edu; phone: (919) 515 6398.*

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Abstract

Progress in cell purification technology is critical to increase the availability of viable cells for therapeutic, diagnostic, and research applications. A variety of techniques are now available for cell separation, ranging from non-affinity methods such as density gradient centrifugation, dielectrophoresis, and filtration, to affinity methods such as chromatography, two-phase partitioning, and magnetic-/fluorescence-assisted cell sorting. For clinical and analytical procedures that require highly purified cells, the choice of cell purification method is crucial, since every method offers a different balance between yield, purity, and bioactivity of the cell product. For most applications, the requisite purity is only achievable through affinity methods, owing to the high target specificity that they grant. In this review, we discuss past and current methods for developing cell-targeting affinity ligands and their application in cell purification, along with the benefits and challenges associated with

different purification formats. We further present new technologies, like stimuli-responsive ligands and parallelized microfluidic devices, towards improving the viability and throughput of cell products for tissue engineering and regenerative medicine. Our comparative analysis provides guidance in the multifarious landscape of cell separation techniques and highlights new technologies that are poised to play a key role in the future of cell purification in clinical settings and the biotech industry.

Keywords: cell purification, immunoaffinity, MACS, FACS, microfluidics.

1. Introduction

The ability to sort cells into distinct, mono-disperse populations is crucial to advance our knowledge of specific phenotypes, and explore their potential in tissue engineering and regenerative medicine [1, 2]. Efficient cell separation is therefore paramount in a multitude of fields, including personalized cell therapy [3-6], organ recellularization [7-11], diagnostics and disease monitoring [12-17], drug discovery [18-22], and basic cell biology [23-25]. To meet the growing demand for highly pure cell products, there has been considerable effort to develop efficient and high-throughput separation methods. As a result, a multitude of techniques have emerged, which are classified into separations by *(i)* physical characteristics (*i.e.*, cell volume and shape, density, and light scatter properties or fluorescence), *(ii)* surface properties (*i.e.*, electrical charges, hydrophobicity, etc.) and cell constituents (*i.e.*, such as nucleic acids, enzymes and other proteins), and *(iii)* adherence/affinity features [26-29] (**Figure 1**).

When supplying cells for therapeutic applications, separation technologies must meet analytical benchmarks and regulatory compliance [30-32]. Consistency in product quality, in terms of cell viability and phenotype purity, is highly controlled to ensure product efficacy

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and patient safety [33-35]. The presence of adventitious agents is also rigorously monitored, and all processing steps must be compatible with sterility requirements [33, 36, 37].

Affinity-based separations have emerged as the main technology for cell isolation, as they meet the demand for high yield and purity, together with scalability and sterility [27, 38]. After three decades of developments, however, a systematic review is needed to recapitulate the diversity and complexity of affinity-based cell separation technologies and guide new users through the selection of appropriate purification methods. To this end, we present a comprehensive survey of affinity-based methods for cell purification, including traditional chromatographic techniques to more recent, non-chromatographic or pseudo-chromatographic systems (**Figure 2**, **Table 1**). These methods employ a variety of biorecognition agents for capture, ranging from traditional protein ligands to ~~novel~~ synthetic ~~agents~~ binders. Through this comparison, we also aim to identify emerging opportunities for improving the manufacturing of cells for tissue engineering and regenerative medicine.

2. Cells of interest

A list of clinically relevant cell products is provided in **Figure 3**. The isolation of erythrocytes is a prerequisite for estimating erythrocyte aging [39] and diagnosing conditions such as anemia[40] as well as vascular [41] and neurodegenerative diseases (Alzheimer's and Parkinson's) [12, 42]. Similarly, the isolation of lymphocytes is needed when assessing immune activation [13, 14], and as such, these cells are valuable in diagnosing or studying HIV infections [43], autoimmune diseases [44], post-operative infections [45], transplant rejection [46], and graft-versus-host disease (GvHD) [47, 48]. Mast cells (MCs) also represent a relevant class of targets, especially for studying innate immune response, as their specific role *in vivo* is still unclear; while often associated with allergic response, specifically anaphylaxis, and hypersensitivity reactions [49, 50], MCs have also been found to have

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significant roles in a host's defense against infections [51-53], angiogenesis during pregnancy [54], wound healing [55, 56], and autoimmune diseases [57]. Obtaining pure mast cell isolates has the potential to greatly improve our knowledge of disease mechanisms through the study of mast cell activation and immune response stimulation [58, 59]. Stem and progenitor cells are key ingredients in regenerative medicine and developmental biology, where they are used to reconstruct decellularized organs or to seed scaffolds for tissue and organ engineering [7, 9, 60]. For these reasons, stem cells have shown promise to help relieve the shortage of transplant organs [61, 62], to treat a number of conditions including macular degeneration [63] and Parkinson's disease [64, 65], or as a therapy to repopulate heart tissue after myocardial infarction [66, 67]. The isolation of stem cells involves an additional challenge compared to common cell purification, as undifferentiated cells must be removed prior to implantation to reduce the risk of teratoma formation [68, 69]; with an average of 10^7 - 10^9 cells being required for a transplant [70, 71], even a 0.1% impurity level can result in a load of 10^6 undifferentiated cells and teratoma formation [72, 73]. Several technologies have been developed for stem cell purification based on cell phenotype, including density-gradient separation [74, 75], fluorescence-activated cell sorting (FACS) [73, 76, 77], and metabolic selection [78]. Improved cell purification techniques would also be beneficial to detect and monitor circulating tumor cells [79, 80] and pathogen infections [81]. It is in fact particularly difficult to isolate circulating tumor cells due to their rarity (~ 1 circulating tumor cell per 10^8 red blood cells [82]). Additionally, cell separation techniques have been used to remove virus-infected cells from a patient to reduce a their overall viral load, as shown with malaria and hepatitis C [83-87]. Improved pathogen infection detection is not only beneficial for human related infections like those caused by HIV [88, 89], but also for the monitoring of food-related pathogens [90-92].

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3. Non-affinity methods

Outside of affinity-based methods, cell separation is typically based on the physical properties of cells [93, 94]. These methods include density gradient centrifugation [95-100], dielectrophoresis [101-105], field-flow fractionation [102, 106-111], filtration [112-118], and elutriation centrifugation [119-123]. While useful for primary enrichment, these methods lack the specificity and resolution to achieve the levels of purity required for therapeutic and analytical applications [93, 94, 124, 125], and typically afford low yield for rare cell types [107, 126]. To overcome these limitations, affinity-based methods have been implemented to improve recovery and purity [29, 127-131]. These rely on the specific recognition and binding of a cell surface target by a complementary molecule, called *ligand*, immobilized on a suitable carrier or surface [132, 133]. Protein ligands, especially antibodies, are currently the major workhorse in affinity-based cell purification, owing to their high capture strength and selectivity [134-137]. Biological ligands, however, are expensive and often suffer from low biochemical stability. Furthermore, their strong binding often makes the elution of cells challenging [133, 138, 139]. Thus, improvements in affinity methods are needed to enable therapeutic and analytical approaches that rely on consistent and cost-effective cell purification. The main channels of innovation are *(i)* the identification of ~~novel and~~ cost-effective synthetic affinity or pseudo-affinity ligands for replacing biological ligands, *(ii)* the development of purification formats that improve upon classic chromatography originally designed for protein purification, and *(iii)* the determination of unique surface receptors on the target cells that are appropriate for use as affinity targets to ensure high phenotypical purity of the cell product.

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4. Conventional Affinity Ligand Formats and Selection for Cell Separation

In cell separations, ligands bind proteins that are ideally unique or overexpressed on the cell membrane of the population of interest. Three ligand families are currently the most employed in cell separation: antibodies, proteins, and lectins. More recently, however, ~~novel~~

synthetic ligands have emerged as promising and cost-effective alternatives. In this section, conventional ligands used for cell separation are described as well as the desired ligand properties to achieve successful cell purification.

4.1. Cell properties that determine the outcome of affinity-based cell purification. Cell sorting relies on the identification of a target receptor on either the cell phenotype of interest (positive cell enrichment) or the background cells (negative cell enrichment) [13, 14, 140, 141], based on the target's abundance on the cell surface, the heterogeneity of cell population, and the requirements for the final cell product [142-144]. Typically, each receptor forms only ~ 0.01% of the total membrane protein content [145, 146], although proteins considered of "low-abundance" can be considerably less, as occurs on T or B lymphocytes [147]. The difficulty in identifying unique biomarkers for a target cell phenotype complicates the separation process and renders the assessment of cell product purity challenging [140, 148-150]. Cell surface receptors may vary among donors and different tissues isolated from an individual donor [151-153]. This heterogeneity complicates the selection of target cell markers for affinity purification and has slowed considerably the study of certain cell classes. This has particularly been the case for mast-cells [51, 53, 56], whose purification by affinity is predominantly based on CD117 (c-Kit) targeting, although this receptor is not specific to mast cells and is present in many stem cell phenotypes [154]. For target cells featuring a particularly low surface density of unique receptors, negative enrichment is the preferred strategy [13, 155-157]. When low expression level is combined with low target cell abundance, microfluidic devices integrating negative selection strategies and physical separation methods (*e.g.*, fluid, electric, or magnetic field) represent the technology of choice [156, 158-160].

Additional considerations when selecting the target receptor come from the biochemical effects that occur upon receptor binding. External cell receptors are inherently

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connected to cell metabolism, and ligand/receptor interactions can trigger undesired events such as internalization of the receptor, metabolic alteration, and even differentiation, in the case of stem cells [151, 152, 161]. Metabolic changes caused by affinity binding have been observed on mast cells enriched by targeting c-kit and FcεRI; while utilized for the positive selection of mast cells, these markers are crucial in IgE activation and are likely to impact cellular metabolism [58, 162].

Ligand selection must also take into account both kinetic (k_{on} and k_{off}) and thermodynamic (K_D) binding parameters [163-166]. Binding strength (K_D) is crucial to ensure product purity, and, in the case of positive selection, to ensure that the target cells can be eluted from the affinity adsorbent. High-affinity ligands (low K_D), while binding target cells specifically, make cell elution difficult, whereas low-affinity ligands (high K_D), while allowing for easier elution, may not provide sufficient throughput. Thus, an ideal affinity ligand offers a balance between specific binding and effective elution [133]. Furthermore, quantifying cell adsorption in terms of K_D only is not accurate, due to multi-point interactions between a target cell and multiple immobilized ligands known as avidity. Cell size, aspect ratio, and receptor density can be used to estimate the number of interactions per cell, and select an appropriate ligand density for a given value of K_D [167, 168]. Finally, cell elution conditions are also crucial, as they strongly affect the viability of the recovered cells [138]. Elution can be achieved *(i)* non-specifically, by manipulating the salt concentration, pH, or temperature, or *(ii)* specifically, by using eluents that inhibit the ligand-cell interactions [139, 140]. Non-specific methods can damage the cells, while specific methods tend to be expensive. To overcome these issues, specific elution methods using multivalent competitive inhibitors have been presented, which have shown increased cell recovery compared to monovalent inhibitors [139, 169].

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4.2. Antibodies. "Immunoaffinity", *i.e.* the use of antibodies as affinity ligands (**Figure 4**), has been widely applied for cell purification, owing to the antibodies' binding selectivity and ability to operate effectively under physiological conditions [93, 150, 170-175]. Following the seminal work by Peterson [176] and Wigzell [177, 178], immunoaffinity has been employed to purify a wide variety of cells, including pathogenic bacteria [179], lymphocytes [180-185], mast and inflammatory cells [186, 187], neural cells [188, 189], and stem cells [190-192]. Recently, antibody fragments, such as Fabs and scFv, have been utilized as ligands in lieu of whole antibodies, as they possess the same binding activity while being produced more affordably [193, 194]. The strength of the interaction between the antibody and target protein, however, requires harsh elution conditions that may impact the cells' viability. To address this issue, elution strategies have included competitive elution [181, 195, 196] and cleavable linkers [197, 198].

4.3. Protein A/G. Another antibody-based method for cell isolation relies on Protein A and Protein G, two antibody-binding proteins expressed respectively by *Staphylococcus aureus* and group C and G Streptococcal bacteria [199]. In Protein A/G-based methods, a cell mixture is incubated with a receptor-specific antibody and passed through a Protein A/G-linked adsorbent [200-205], where the antibody-labeled cells are selectively retained (**Figure 5**). As the binding to Protein A/G is less impacted by steric hindrance than binding to immobilized antibodies directly, this variant of immunoaffinity cell chromatography is more efficient, and has been demonstrated in different formats, such as rosetting [206, 207] and solid-phase chromatography [201, 208].

4.4. Protein and synthetic antigens. Antigens represent a broad class of ligands ranging from proteins to small synthetic molecules [93, 171, 209-211]. The use of antigenic ligands for purifying white cells has been pioneered by Wigzell *et al.*, who isolated immunized mouse lymph node cells using glass and plastic beads functionalized with human serum albumin,

bovine serum albumin, and ovalbumin with yields between 60-95%, but poor enrichment (2.5-fold) [212]. Later work on lymphocytes has utilized enzyme-substrate interactions to isolate lymphocytes raised against enzyme antigens [213]. To purify enzyme-binding lymphocytes, Deluca *et al*. contacted white cells with the antigen enzyme and then exposed the solution to beads decorated with the enzyme's substrate to specifically capture enzyme-bound lymphocytes [210].

Synthetic antigens represent the first use of synthetic ligands for cell purification [214-217]. Truffa-Bachi *et al.* utilized haptens as antigens to stimulate an immune response, and subsequently as immobilized ligands to isolate white cells with anti-hapten activity [214, 215]. This method addresses two main difficulties encountered in affinity-based capture, namely *(i)* non-specific binding of non-target cells and *(ii)* detaching cells from the adsorbent without impacting their viability. In this context, Haas *et al*. utilized a gelatin matrix containing dinitrophenyl as a ligand for adsorbing mouse spleen cells, demonstrating that 30-fold enrichment and high viability could be achieved by melting the gelatin, providing for a gentle elution strategy [217].

4.5. Lectins. Lectins recognize specific carbohydrate sequences on glycoprotein cell surface markers and have been widely utilized for cell fractionation (**Figure 6**) [218]. Herz *et al.* have used soybean agglutinin as a ligand to isolate T lymphocytes from peripheral blood for use in the prevention of graft *vs.* host disease in bone marrow transplants [219]. Hellström *et al*. have shown how helix pomatia A hemagglutinin can bind T cells treated with neuraminidase by targeting surface carbohydrates [220]; because only a small fraction of B cells interact with helix pomatia hemagglutinin, this method represents an efficient strategy to separate T cells from B cells [221]. This work shows how lectins enable highly specific cell fractionation as they target post-translational modifications; helix pomatia hemagglutinin, in fact, is selective for human T cells over many B cells since T cells express proteins with

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unique post-translational modifications [222]. Another major advantage of lectins is that cell elution can be triggered by mono- and disaccharides, which are harmless to cells [171]. In one instance, though, the elution of mouse thymocytes from concanavalin A was accomplished by cleaving the mercury-sulfur bond conjugating the lectin ligands from the chromatographic substrate using a short thiol, affording quantitative recovery and high cell viability [223].

5. Formats of affinity-based cell separation

The principles of affinity purification have been applied in different ways for cell capture, depending on the source fluids and the required throughput [26, 100, 140, 150]. Cell-binding ligands have been immobilized onto solid substrates (chromatographic-like methods) [128] or polymer carriers (pseudo-/non-chromatographic method) [224] as well as magnetic particles (MACS) [225] and fluorescent markers (FACS) [226] that enable separation by an electromagnetic field. More recently, affinity ligands have been displayed on the channels of microfluidic devices. This latest frontier of cell separation offers higher resolution and holds great promise to expedite the clinical implementation of cellular therapies relying on rare cell types.

5.1. Rosetting. Rosetting was the first isolation method to combine affinity with traditional density-gradient separation methods [150, 227-229]. In this technique, antigen-specific cells are incubated with antigen-coated erythrocytes, with which they form aggregates, called "rosettes", that are separated from non-rosetted cells by gradient centrifugation (**Figure 7**) [230]. Rosetting was first utilized to separate two mouse immune cell populations using sheep red blood cells [231]. Further work demonstrated that greater quantities and purities of rosette-forming antigen-specific cells could be obtained through avidin-biotin affinity [207], gradient density centrifugation [18], and in combination with magnetic fields [232]. Rosetting

is now routinely employed for purifying B and T lymphocytes and stem cells [233], with commercial products such as the RosetteSep^{TM} kit from StemCell, which offer good recovery and purity.

5.2. Chromatography. Besides recovery and purity, other parameters, such as scalability and capacity, are critical to extend cell separation processes to clinical and commercial applications [128, 234]. In this context, cell affinity chromatography (CAC) shows great promise as a scalable technology [235], given its successful use in industrial protein purification [236, 237]. In CAC, cells are injected into a column packed with a porous material functionalized with affinity ligands. Target cells are retained by affinity on the chromatographic medium, while other components flow through (**Figure 4**). While similar to traditional protein chromatography, CAC faces unique challenges, due to the major differences between cells and proteins: cells are large, sensitive to shear stress [204], and possess a low diffusivity, which results in the need for convective transport to achieve sufficient interaction with the affinity surface [238-240]. Most importantly, high binding avidity requires harsh elution conditions to elute the cells from the chromatographic substrate [204, 241-243]. These challenges have highlighted the need for ~~novel~~ matrices that are tailored for chromatographic cell separations.

Computational modeling has been utilized to simulate cell interactions with affinity surfaces and guide the design of CAC substrates. Hammer *et al.* modeled the receptor-mediated adhesion of cells to ligand-decorated surfaces [238] and found that adhesion mainly depends on *(i)* the cell receptor-ligand interaction, such as the bond formation rate (k_{on}) and strength (K_D), and *(ii)* the fluid mechanical force, receptor mobility, and contact area [244-250]. The model predicts two regimes governing CAC, *i.e.* a rate-controlled high-affinity regime and a low-affinity regime. Additional studies have expanded on CAC modeling [251] by implementing ~~novel~~ advanced analytical [252-254] and numerical [244, 255-257]

approaches, understanding the effect of contact time and presence of inhibitors on cell adhesion [258], evaluating the effect of cell deformability on adhesion to surfaces [259, 260], and observing cell binding in microfluidic channels [261, 262]. A model based on "cell rolling" behavior, inspired by leukocytes rolling against blood vessel walls [263, 264], was designed to increase the likelihood of ligand-receptor interactions [143, 145, 256, 265-268], reduce residence times, and secure the binding of cells with low surface marker density.

In place of traditional chromatographic substrates, alternatives such as fluidized bed CAC, cryogel CAC, and microfluidic CAC, have been proposed [234].

5.3. Fluidized Beds. Fluidized bed, or expanded-bed, affinity adsorption is frequently used to harvest from crude feedstocks [269]. A fluidized bed is comprised of porous particles coated with cell-binding affinity ligands that are agitated by an upward flow of fluid containing the target cells (**Figure 8**). The advantages of this technique over traditional CAC are *(i)* improved mass transfer and *(ii)* large inter-particle volume, and *(iii)* high surface area [221, 270-272]. In one study, perfluorocarbon-based beads functionalized with lectin Concanavalin A were utilized to capture *Saccaromyces cervisiae* cells [271, 273]. The rapid adsorption kinetics enabled the capture of up to $6.8 \cdot 10^9$ cells/mL, although elution was hindered by the "avidity" effect; to facilitate elution, ion-exchange groups were used in lieu of Concanavalin A [270, 271]. Fluidized bed separation was also utilized to isolate monocytes labeled with biotinylated antibodies from human peripheral blood using streptavidin beads [272]; cells were eluted using mechanical shear to a purity of 90%, yield of 77%, and viability of greater than 65%. While promising, fluidized beds suffer from limitations such as shear stress on cells, the need for large columns, long equilibration times, non-specific capture by the adsorbent base material, limited flow velocities, disengagement of absorbed cells from ligands [270], and fouling of the beads [274].

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5.4. Cryogels. Another alternative to traditional CAC is represented by monolithic cryogels [128, 234, 275, 276]. Cryogel matrices are prepared by gelation or polymerization at sub-zero temperatures to create a continuous macroporous structure that enables cell suspensions to flow through [275] (**Figure 9**). While initially designed for the separation of proteins [277], oligonucleotides [278], and plasmids [279], cryogels have been shown to be ideal for the purification of viruses [280], cell organelles [281], and whole cells [234] owing to their *(i)* uniform and highly interconnected pores [128, 234], *(ii)* high channel width (>30 μm) that provides for efficient transport of cells between 2 and 15 μm [128, 282], *(iii)* efficient ligand conjugation [204, 205, 283], and *(iv)* high elasticity and hydrophilicity, which is particularly suited for mammalian cells [234, 284]. Finally, cryogels are attractive for large scale manufacturing as they exhibit high storage stability and have an extended life cycle [205, 285]. The two main approaches for cell capture using cryogels are mechanical entrapment in the cryogel matrix and ligand-mediated binding [140, 275, 286, 287]. Ligands are conjugated to the cryogel either during or after cryogenic pore formation [281, 288]. A wide array of ligand formats [234, 285, 288] including antibodies [204, 205, 247, 289, 290], proteins [128, 204, 205, 286, 291], lectins, and synthetic ligands [290, 292-294] have been incorporated into cryogels for the separation of lymphocyte cells [128, 204, 285], myeloid cells [276, 295], microbial cells like *Staphylococcus aureus* [290], *Escherichia coli* [286, 291, 296], *Bacillus halodurans* [297], and yeast cells [286, 291]. Elution from cryogels can be achieved by traditional methods, as well as by elastic deformation and thermally-induced shrinkage of the matrix to ensure viability of the recovered cell product [291].

6. Pseudo-chromatographic systems

6.1. Gel Affinity Separation. Among polymer-based media, gels are particularly attractive as single-use adsorbents for cell purification, as they can be disintegrated thermally or

enzymatically to release viable cells [298-305]. Haas and Layton developed antigen-coated gelatin layers to separate spleen cells with a 30-fold enrichment [217]; the bound lymphocytes were recovered by melting the gelatin. Because cell recovery could only be performed below gelatin's melting temperature, Maoz *et al*. modified this process by including matrix-specific enzymes (*i.e.*, collagenase) [303]; less than 5% of non-specific cells bound the gel, and non-adherent cells had significantly lower cytotoxicity than the bound cells, indicating that this method can specifically isolate functional T cells. Bröcker *et al*. developed antigen-functionalized gelatin for purifying T cells with up to 100-fold enrichment and purity of 80-90% [304]. Similarly, Webb *et al.* used an anti-mouse IgG for the selective capture of B cells [302]; on average 250 cells/mm^2 attached to the immobilized antibody and the B cells had a minimum viability of 60%.

6.2. Fiber-based affinity separations. Arrays of parallel hollow fibers have gained popularity as substrates for affinity purification of cells [306-313]. Fibers introduce a new component in cell adhesion, represented by the fiber's cross-section geometry and flexibility [309, 312]. Fiber-based adsorbents are also attractive as they can be regenerated by washing at high shear [308, 312] and can be manufactured affordably at large-scale. The first use of fibers for cell isolation was published by Edelman *et al.* [306], who described the isolation of spleen cells from mice, immunized against Dnp38-bovine IgG, using nylon fibers coated with Dnp38-BSA, tosyl$_{30}$-BSA, and BSA antigens. The cells were detached mechanically, chemically, or competitively by incubation with inhibitors. While the eluted cells were up to 90% viable, significant non-specific binding occurred, which limited purity to 63-88%. To increase specificity, several authors have coupled antibodies and antigens to the luminal surface of cellulose hollow fiber modules. Pope *et al*. covalently attached goat anti-mouse antibodies to cellulose fibers to capture CD4$^+$ lymphocytes, resulting in 63-99.9% depletion of the CD4$^+$ cells from the starting population [314]. Similarly, Nordon *et al.* covalently coupled an anti-

CD34 antibody directly to the luminal surface of their system's fibers to enrich $CD34^+$ cells from mononuclear cells at 94% purity and 61% yield [307]. Other groups have developed fusion proteins comprising an antibody-binding domain and a fiber-binding domain for mediating the adhesion of antibody-labeled cells onto the fibers. Specifically, Craig *et al*. developed a fusion protein ("protein LG") that captured more than 90% of the antibody-labeled $CD34^+$ cells onto a cellulose fiber module [311]. The use of these chimeric proteins helps overcome problems associated with ligand conjugation to hollow fibers such as low yield, random orientation, and structural alterations or degradation caused by the conjugation chemistry [311]. Hollow fiber systems enable the implementation of ~~novel~~ unconventional elution techniques. For example, bound cells can be fractionated into populations with varying binding strength by adjusting the flow rate (shear elution) [312]. Bound cells can also be eluted if labile links (*e.g.*, a disulfide bond) are included between the ligands and fibers.

6.3. Affinity Membranes. Membranes are suitable substrates for affinity cell separations, as the balance between trans-membrane flux and fluid velocity parallel to the surface can be easily controlled to optimize adsorption and elution [138, 315]. Additionally, the pore size of membranes and surface shear can be varied to minimize concentration polarization and fouling, which is advantageous when processing high-density cell suspensions. In an early example, Mandrusov *et al*. used a cellophane dialysis membrane functionalized with goat anti-mouse immunoglobulin to purify mouse B-lymphocytes [316]: cells were eluted with a low pH buffer by trans-membrane diffusion, while a shear-producing flow was applied to promote detachment of the cells from the membrane and neutralization of the acidic environment. Feeding the elution buffer on the membrane side opposite to the bound cells afforded a 100% yield and 60% viability, indicating that a trans-membrane pH gradient is needed to elute cells effectively without decreasing cell viability.

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Affinity membranes enable cell separation processes that employ bubble-induced cell detachment [317]. This technique is attractive as cells can be removed from adsorption surfaces without excessive dilution. Wang *et al*. utilized this method with tubular capillaries coated with antibodies to purify specific blood cell populations [127], obtaining 85.7% yield, 97.6% purity, and 85.8% viability of $CD4^+$ cells isolated from blood samples. Specifically, > 90% of cells detached by bubble-induced elution, whereas compression and flow-induced elution resulted in 40-80% and 10-40% of cell detachment, respectively [317].

Thermo-responsive polymers have also been integrated in membranes to improve elution. Specifically, a poly(N-isopropylacrylamide)-grafted polypropylene (PNIPAAm-g-PP) membrane functionalized with monoclonal antibody ligands was developed for purifying $CD80^+$ cells [318]. PNIPAAm displays a thermo-responsive phase transition at 32°C, where it switches from a hydrophilic to a hydrophobic state. At 37°C, antibody ligands adhere to the PNIPAAm-g-PP membranes by hydrophobic interaction, enabling the affinity capture of $CD80^+$ cells; at 4°C, the IgG ligands detach from the PNIPAAm coating, thereby releasing the cells. The recovered cells were enriched from a 1:1 cell suspension to 72%, proving to be the first case of affinity-based capture of cells where temperature is used for cell elution. In a similar work, anti-CD34 antibodies were adsorbed onto a PNIPAAm-g-PP membrane and utilized to enrich $CD34^+$ cells. The $CD34^+$ cell concentration was increased from 50% in the feedstock to 85% in the eluate, and 95% of the recovered cells were viable [319].

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7. Non-chromatographic affinity purification methods

A variety of non-chromatographic techniques have been developed, such as two-phase separations, magnetic-activated cell sorting (MACS) and fluorescence-activated cell sorting (FACS) [148]. Two-phase separations employ polymeric materials often labeled with affinity ligands to drive the selective migration of cells into an aqueous phase (**Figure 10**). In MACS

and FACS, the target cells are tagged with labeled affinity ligands that enable separation; magnetic labels are used in MACS (**Figure 11**), while fluorescent labels are used in FACS (**Figure 12**). While MACS and two-phase separations isolate cells into bulk groups, FACS is unique in its ability to analyze and sort single cells, allowing for more precise cell separation.

7.1. Affinity two-phase partitioning. Affinity two-phase partitioning is a powerful preparative method for cells, cell membranes and organelles, and viruses [140, 320-329]. Aqueous two-phase systems (ATPS) form when two polymers added to a water solution produce two non-miscible liquid layers, across which other components in solution migrate based on their differential affinity towards the polymers (**Figure 10**). To improve the selectivity of cell migration, affinity ligands such as lectins, antibodies, and receptor-specific molecules have been conjugated to the phase-forming polymers [330, 331]. Polyethylene glycol (PEG) and dextran are the most commonly utilized polymers for ATPS, with PEG being used as the ligand carrier and the dextran-rich phase acting as the receptacle for the bulk contaminants [325, 332-334]. Monoclonal antibodies coupled to PEG have been utilized for separating human red blood cells from sheep and rabbit blood cells, resulting in up to 92% partitioning of the human red blood cells to the top phase [321, 335, 336]. Antibody-PEG conjugates have also been used to purify hybridoma 16-3F cells from their parental NS-1 cell line, resulting in 24% recovery and 80% purity [332].

Two-phase affinity partitioning has flourished with the introduction of stimuli-responsive polymers. Kumar *et al.* have utilized PNIPAM decorated with antibody ligands to separate $CD34^+$ human acute myeloid leukemia KG-1 cells from Jurkat cells (immortalized human T lymphocytes) [337]. While more than 80% of the KG-1 cells were partitioned to the top phase, a small contamination of Jurkat cells was observed; however, incomplete recovery of the conjugates lowered the yield of KG-1 cells to 75% during subsequent use. In addition to antibodies, cell separation by two-phase partitioning has also been demonstrated with other

ligands, such as transferrin [323], synthetic dyes [338-342], and immobilized metals [333, 343].

Owing to its biocompatibility, mild operating conditions, and scalability, ATPS is regarded as a high-potential technology for the recovery of cell targets for which the minimization of mechanical stimuli is critical (*e.g.*, stem cells and neurons) [327, 328]. Sousa *et al.* used PEG800-dextran functionalized with anti-CD34 antibodies to separate and recover $CD34^+$ stem cells from umbilical cord blood [325]. The $CD34^+$ cells were enriched from a starting population of ~ 0.2% $CD34^+$ cells to ~ 42% in the final population, and recovered with 81-95% yield; in contrast, with PEG alone, a cell enrichment of 13% and 2.3% recovery was achieved. Using a three-polymer (PEG, ficoll, dextran) system and an anti-CD133 antibody, González-González *et al.* recovered $CD133^+$ stem cells from umbilical cord blood with a final recovery of 62% and 98% viability [328].

A significant limitation of two-phase separations is the recurring presence of impurities in the top (product) phase. Accordingly, separations using ATPS generally result in lower purity than what is achieved using chromatographic technologies. Further work to improve ATPS separations, especially by increasing the partition preference of antibodies to a top clean phase, is considered a worthwhile effort to achieve a truly scalable process for cell separation.

7.2. Magnetic-activated cell sorting (MACS). MACS is a relatively recent cell separation technology that employs affinity ligands conjugated to magnetic particles comprising an iron core coated by a hydrophilic shell to reduce non-specific binding (**Figure 11**) [344]. Upon incubating ligand-coated particles with a cell mixture, a magnetic field is applied to separate the target cells bound to the magnetic particles from the unbound cells [345]. Pioneered by Zborowski and co-workers [142, 346-350], MACS is now recognized for its speed of separation, with rates in the range of ~10^{11} cells/hour [100]. Recent developments enable

simultaneous separation of multiple cell types using magnetic field gradients [348, 351] or by combination with microfluidic devices [352, 353]. The predominant MACS format is antibody-based, where target cells are either directly bound onto antibody-coated beads [225, 347], or are labeled in solution with a primary antibody and subsequently captured onto beads coated with a secondary antibody [354, 355].

Ligand immobilization techniques are highly dependent on the nature of the ligand. Methods for antibody immobilization to magnetic particles include covalent binding [356, 357], streptavidin-mediated immobilization (specific for biotinylated antibodies) [358, 359], Protein A-/G-mediated immobilization [360], conjugation to boronic acid or hydrazinyl groups [360, 361], and oligo-dT coating [362]. Besides antibodies [225, 345, 363-365], other affinity ligands have been successfully utilized in MACS [366-375]. Herr *et al*. utilized DNA aptamers to capture acute leukemia cells from complex mixtures with a 40% recovery [371]. Magnetic nanoparticles coated with bis-Zn-DPA, a synthetic ligand that binds Gram-positive and Gram-negative bacteria, have been utilized for separating *Escherichia coli* from blood with complete bacterial clearance in two separation cycles [375]. This work has also demonstrated that nanoparticles outperform micrometer-scale particles in terms of binding capacity and kinetics, and separation output.

The increasing relevance of immunomagnetic separation technology is demonstrated by the recent FDA approval of the CellSearch system, which can isolate circulating tumor cells using a epithelial cell-adhesion molecule (EpCAM) antibody [376-378].

7.3. Fluorescent-Activated Cell Sorting (FACS). In FACS, fluorescently-tagged ligands are utilized to individually sort cells using fluorescence and light scattering [379-381]. When injected into the sorter, the stream of cells tagged with fluorescent ligands is broken into droplets that contain a single cell; each droplet passes through an illumination detection zone, and a charge is placed onto any cell that meets the separation criteria. As the charged droplets

fall through electrostatic deflecting plates, they are sorted into different containers based on their charge (**Figure 12**). FACS has been extensively utilized for sorting therapeutic cell products, especially stem cells [29, 31, 382-384] and blood cells [385, 386]. FACS has gained popularity as it provides highly pure (>95%) cell populations and can sort at the single cell level due to the high sensitivity of fluorescence detection [100, 387]. FACS also allows population-averaged single cell data, as it can be used to efficiently perform high throughput cell sorting and counting [387]. Recent advances in fluorescent dyes and laser detectors allow researchers to simultaneously track multiple cell parameters [388, 389]. On the other hand, FACS requires the use of expensive equipment and suffers from limited throughput (~ 10^7 cells/hour) and long processing times (3-6 hours), which prevents its use in large scale manufacturing of therapeutic cells [380].

Elements of MACS and FACS sorting can be combined in a method known as "ratcheting cytometry" to perform multicomponent purifications of specific subpopulations [390]. This method is frequently used for continuous and quantitative purification of T cell subsets for cell therapy manufacturing. Specifically, T cells from apheresis or peripheral blood mononuclear cell samples are magnetically labeled using magnetic particles featuring different iron oxide content and size, and antibody functionalization. As magnetic particles travel differently within the sorting cartridge based on their magnetization and size, cells specifically bound to a magnetic particle population can be isolated from other cells in the mixture [390]. Ratcheting cytometry also enables sorting cells based on differential levels of antigen, as this determines the number of magnetic particles bound to a cell. This method has been used to simultaneously isolate CD4 and CD8 T cells from a sample via labeling with antigen-specific magnetic particles [391].

8. Emerging Trends

Cell purification technology is rapidly evolving, owing to the introduction of ~~novel~~ target-specific biorecognition moieties for capture (*i.e.*, biological and synthetic ligands) and isolation formats (*e.g.*, microfluidic devices).

8.1. Microfluidic devices for cell separation. The latest frontier of CAC is represented by microfluidic devices that comprise sub-millimeter channels coated with affinity ligands (M-CAC) [129, 131, 159, 392-396]. The high surface-area-to-volume ratio of microfluidic channels, enhanced by micro-fabricated structures with complex geometry, has enabled the capture of cells at extremely low concentrations by M-CACs [261, 392, 397-399]. M-CAC systems have been utilized to separate T- and B-lymphocytes at high purity (> 97%) from mixed suspensions [127, 175, 400]. To ensure binding specificity, the channels are often grafted with hydrophilic polymer brushes (*e.g.*, PEG) or coated with hydrogels (*e.g.*, alginate) functionalized with antibody ligands [401, 402]. Chang *et al.* have developed a M-CAC system coated with E-selectin IgG molecules to separate HL-60 and U-937 myeloid cells with purity greater than 70% and 200-fold enrichment [262]. M-CAC also enables the sorting and capture of multiple cell types from a complex mixture. Li *et al.* incorporated a pneumatic-actuated control layer into an affinity separation layer to create different antibody-coated regions within the same channel [129]. Ramos cells were flown through anti-CD19- and anti-CD71- coated regions, with the anti-CD19 region having a capture density 2.44-fold higher than the anti-CD71 region. The authors also coated a second channel with two different antibodies targeting either Ramos or HuT 78 cells, allowing specific retention of the cells in their complementary region at greater than 90% purity. Lastly, a four-region antibody-coated device was developed for the simultaneous capture of three different cell lines in a single channel, thereby enabling multiple cell sorting.

Microfluidic devices coated with antibodies against specific cell markers have gathered considerable interest as tools for detecting rare tumor circulating cells (CTCs) [397, 403-405].

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Isolating CTCs from the bloodstream enables the detection, characterization, and monitoring of non-hematological cancers [406], but is made extremely challenging by their low concentration (1 -100 CTCs per mL of blood [82, 376-378, 407, 408]). Researchers have shown that microfluidic devices (CTC-Chip) containing an array of microposts functionalized with epithelial cell adhesion molecules (EpCAM) can capture CTCs [397]. Many attributes of the device have been explored to enhance CTC enrichment. Gleghorn *et al.* described how the geometry of the microposts can enhance CTC enrichment [398]. CTC-ligand binding has also been improved by introducing a high-throughput microfluidic device called "HB-Chip", which mixes the blood cells by generating micro-vortices that increase the interactions between the target CTCs and the antibody-coated channels [406]. To increase binding sensitivity, Myung *et al.* developed high-avidity ligands by conjugating multiple EpCAM ligands to dendimers [409]. The combination of multivalent binding and cell rolling in the channels mediated by E-selectin granted high sensitivity and specificity towards CTCs. This work has led to a device, commercialized by Biocept, which employs streptavidin-coated microposts to capture CTCs tagged with biotinylated antibodies, followed by fluorescent microscopy-based detection and *in situ* cytogenic interrogation [399, 410]. Another approach for the isolation of CTCs is represented by negative enrichment (or negative selection) using microfluidic technologies [411], which takes advantage of the physical and biochemical properties of cells [412]. Unlike hematopoietic cells, which display the cell surface markers CD15 (granulocytes), CD66b (granulocytes) and CD45 (leukocytes), CTCs are CD15/45-negative. Accordingly, negative enrichment technologies feature microfluidic channels, nanoparticles, and micro-scale adsorbents functionalized with anti-CD15, anti-CD66b and, most commonly, anti-CD45 antibodies [155-157]. The affinity-based selection alone, however, is often not sufficient to achieve the desired enrichment factor, and must be complemented by size exclusion-based or fluid dynamic-based separation techniques [157, 158, 160, 413, 414].

Lastly, emerging technologies for droplet-based single cell analyses are flooding the contemporary literature landscape. While there is significant focus on droplet barcoding for

single cell sequencing and transcriptomics [415-418], some efforts are aimed at employing droplet-based technologies for human cell isolation, sorting, and studying biomolecular interactions [419-425]. An in-depth review of droplet-based cell analyses was recently provided by Huck *et al.* [426]. Briefly, the formation of water-in-oil plug flow in microfluidics can generate picoliter-sized droplets for carrying cells or other biomolecular residents, and these droplets can be generated in a highly repetitive and chemically-defined manner [427-430]. When employed for cell isolation and sorting, the physico-chemical properties of the droplet can be tuned to promote interaction with specific surface features of the microfluidic device, resulting in droplet isolation and sorting [431-435]; for example, the interfacial tension of the droplet can be made sensitive to pH causing the droplets to interact with the microfluidic channel's surface [422]. The physico-chemical properties of the resident cell can also be made responsive, so that the droplet can be sorted via imaging and fluorescence-activated techniques [436-440].

In regard to using these techniques for studying affinity-based chemistries, droplet microfluidics have been employed to screen drug and antibody binding by generating sub-nanoliter reactors [441-444]. In one example, hybridoma cells secreting antibodies were individually co-encapsulated with a target cell in nanodroplets to select hybridoma clones expressing antibodies featuring affinity for the target cell [440]. While there have been limited studies of strictly affinity-based sorting via droplet microfluidics, based on the aforementioned examples there is an emerging lane of study for using droplet microfluidics for therapeutic antibody discovery, especially since the single-cell droplet approach is amenable to use with primary human plasma cells, which secrete antibodies.

8.2. ~~Novel~~ Synthetic Ligands. A significant barrier to improving the affordability of cell products is represented by the cost of biological ligands [133]. While highly selective, proteins and antibodies are biochemically labile [445], and a complex engineering process is

required to discover viable ligands [446]. Further, they are generally characterized by high binding strength, which can trigger undesired intracellular signaling cascades upon binding and even cell death [447-449]. To overcome these limitations, synthetic ligands have been proposed to maintain targeted affinity while lowering binding strength to facilitate cell elution. In addition, synthetic ligands are biochemically stable and can be synthesized affordably at large scale.

Hormones are the first small molecules to ever be utilized as affinity ligands [450]. In particular, histamine [451, 452], catecholamines [453], and prostaglandins [454, 455] coupled to Sepharose beads have been used to separate 19S and 7S plaque-forming cells from the total spleen leukocyte population. The hormone-based adsorbents were able to capture 56% and 84% of the 19S and 7S plaque-forming cells respectively. Similarly, glycans (*e.g.*, mannose) immobilized on Dowex resins have been utilized to separate *E. coli* K12 and *Campylobacter jejuni* NCTC 11168 cells with high yield (94–96%) and selectivity [456].

Recent advances in selection technology have spurred the use of synthetic ligands with engineered affinity and selectivity for any target cell [133, 457-459]. Aptamers and peptides represent the main classes of synthetic ligands [449, 460-470]. Aptamers consist of single-stranded DNA or RNA molecules and have seen rising popularity in cell purification [469]. The development of these ligands is supported by a high-throughput screening method known as "systematic evolution of ligands by exponential enrichment" (SELEX) [464, 471-474]. Xu *et al.* have selectively captured three leukemia cell lines (CCL-119, Ramos cells, and Toledo cells) using a microfluidic device coated with cell line-specific aptamers, with up to 136-fold enrichment [475]. Aptamers have also been demonstrated as cell capture ligands in more traditional pseudo-chromatography applications, as described by Zhang *et al.*, where aptamers coupled to a hydrogel bound and eluted target cells with a resulting viability of ~ 99% [476]. Aptamers have also been successfully used in MACS applications [371, 372, 475,

477-480], microfluidic devices, and hydrogels, with reported capture efficiencies and cell purities at > 80% [131, 403, 481-483]. These case studies showcase the value of aptamers as cell capturing ligands. Nonetheless, some improvements are still needed, such as increasing binding selectivity to improve capture [484], tuning the binding strength to facilitate cell elution [485], and addressing safety concerns by implementing rigorous tests of biocompatibility [486].

Peptides have also emerged as robust and cost-effective alternatives to protein ligands [133]. Over the past two decades a number of selection techniques have been developed, ranging from the screening of biological and synthetic libraries in liquid or solid phase to *in silico* approaches, such as computational design and machine learning. This has resulted in a myriad of peptides targeting analytical and medically relevant target cells. Veleva *et al.* identified an angiogenic tumor-binding peptide via *in vitro* enrichment of a peptide library against peripheral blood outgrown endothelial cells followed by *in vivo* screening of the enriched library to identify tumor-binding peptides [487]. Similarly, Oyama and coworkers identified peptides from a phage library to bind human lung cancer cell lines and noted that the selected peptides were specific towards the target cancer cells without negative selection [461, 466]. In 2008, Choi *et al.* identified a Raji cell-targeting peptide as a model for Burkitt lymphoma cells with seemingly high specificity for these cells as determined by lack of binding to normal, non-cancerous B cells, peripheral blood cells, or other leukemia cells [488]. Wang *et al.* also utilized phage display to identify affinity peptides for imaging detection of human colorectal cancer cells (Caco-2); the specificity of the peptide was confirmed using negative-control cell lines HEK293, SGC-7901, and SMMC-7721 [489]. Peptide ligands have also been employed in a number of cell adhesion applications, which are of primary interest for cell separations. De *et al.* demonstrated the use of peptides in pathogen removal applications by isolating pancreatic beta-cells infected by *Mycoplasma arginii* from

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healthy cells, showcasing a 10-fold reduction in the number of infected cells [490]. Success in separating different phenotypes of primary cells has also been shown in multiple cases by microcontact printing of tetrameric peptides in microfluidic devices. These have been used for the separation of osteoblasts from fibroblasts by Hasenbein and coworkers [491], or the fractionation and characterization of different human cell phenotypes by Murthy and coworkers [492-494].Peptide ligands have been discovered for a number of cell surface markers that identify analytically or therapeutically relevant cells, including CD-34 [495], CD-133 [496, 497], CD-38 [498], VCAM-1 [499-502], and Flt-3 [503, 504].

Large peptides, developed from non-antibody scaffolds, also represent a viable alternative for cell separation purposes. Our group has identified the first non-antibody binders for CD-117 by screening a yeast-display scaffold library against magnetized yeast cells expressing the extracellular domain of CD-117 [505]. Two nanobody mutants were identified with good affinity (*i.e.*, 131 and 204 nM) for CD-117. While binding of these mutants for CD-117 was only confirmed for yeast displayed CD-117, a combination of these ligands would likely enable the purification of phenotypically pure cells such as endothelial stem and progenitor cells (ESCs, HSC), and hematopoietic stem and progenitor cells (EPCs, HPCs) [506-517]. Additionally, the mid-nanomolar affinities of the nanobody ligands promote gentler compared to antibody ligands.

Peptide ligands can also be engineered to enable the control of cell binding and release upon exposure of biocompatible stimuli. To this end, stimuli-responsive monomers can be incorporated into the amino acid sequence, allowing the peptide to reversibly switch between a binding and a non-binding mode upon cooling, or exposure to light or a magnetic field (**Figure 13**). Our group, for example, has developed VCAM1-binding azobenzene-cyclized peptides for the light-controlled labeling of endothelial progenitor cells [518]. Upon exposure to light, the ligands undergo a remarkable ~ 1300-fold variation in binding strength, which enables selective and stable light-controlled labeling of cells. Notably, modified

azobenzene linkers have been engineered to photo-switch in different wavelength windows, namely red, green, and blue (RGB) light [519-524]. Therefore, a combination of peptide ligands targeting different cell markers, whose binding/release is triggered at different wavelengths, could be used to produce and dynamically modify patterns of cells on solid substrates by exposure to sequences of red, green, or blue photo-patterns, for example using liquid crystal display light-emitting diodes (LCD-LED) arrays.

9. Conclusions. Cell separation technologies have progressed steadily to meet the demands for basic research, diagnostic, and therapeutic applications, resulting in cell isolation methods that are more efficient, scalable, and dependable. Affinity-based approaches are now the most utilized, owing to their ability to achieve high purity. A wide variety of affinity-based approaches are available, ranging from traditional chromatographic to pseudo- and non-chromatographic systems. Each system has advantages and disadvantages, as outlined in this work, which must be carefully considered when choosing a cell separation method. Microfluidic technologies represent the next frontier of cell manufacturing as they offer the capacity to perform multiple functions (mixing, counting, lysis, single cell analysis, etc.) in a single device. Advances in parallelization and scale-up hold great promise to overcome the low throughput of current devices and enable processing of large sample volumes. Further, the ability to integrate post-sorting molecular, cellular, and functional characterization furthers the appeal of using microfluidic devices for cell separation.

On the biorecognition front, affinity-based separations are shifting from protein and antibody ligands towards synthetic ligands. Biological ligands, in fact, while highly specific, are limited by their high cost and exceedingly strong binding. Synthetic ligands, on the other hand, can be synthesized affordably, at large scale, and with no batch-to-batch variability. The need to develop gentle cell elution conditions has stimulated the development of stimuli-

Journal Pre-proof

responsive ligands, such as photo-switchable peptides, whose binding activity can be controlled by exposure to biocompatible stimuli. In this regard, further progress in the fields of *in vitro* and *in silico* selection methods is needed to expand the portfolio of peptides and aptamers with tailored affinity and binding mechanism for cellsurface markers. Further studies – both experimental and modeling – are also needed to understand the balance between affinity, multivalent binding due to expression level of surface markers, in order to optimize the balance between efficient cell capture and elution, ultimately enabling high recovery and bioactivity.

Currently, the major challenge for cell therapies and related clinical applications resides in achieving rapid, efficient, and affordable separation while minimizing costs and attaining the required purity, yield, and functionality of the cellular product. Membrane-based separations show exceptional potential in large-scale production, particularly in combination with ~~novel~~ cell-specific biorecognition moieties that ensure high recovery, purity, and bioactivity of the cell product. Owing to their high pore diameter and porosity, in fact, membranes enable processing high volumes of cell suspensions at high flow rates, thereby increasing throughput and minimizing processing time, which aids in maintaining the viability of the cell product. On the front of basic cellular research and personalized medicine, the continued identification of highly specific markers defining cell populations [525], combined with the advancements in integrating physical and affinity-based strategies in miniaturized devices, will be critical for the fruition of patient-specific cellular therapies.

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Conflict of interest. The authors have no conflict of interest to acknowledge.

List of figure captions

Figure 1. *Cell properties and corresponding purification techniques.*

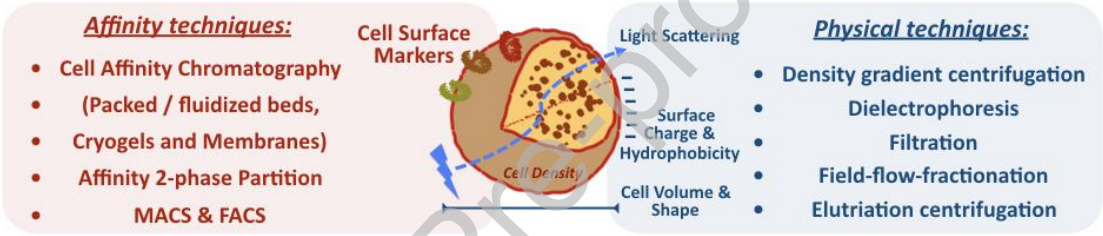

Figure 2. *Cell purification technologies.*

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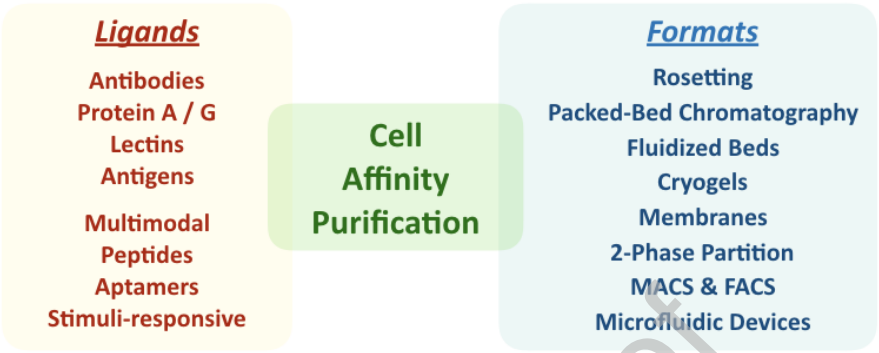

Figure 3. *Cell targets and their diagnostic or therapeutic applications.*

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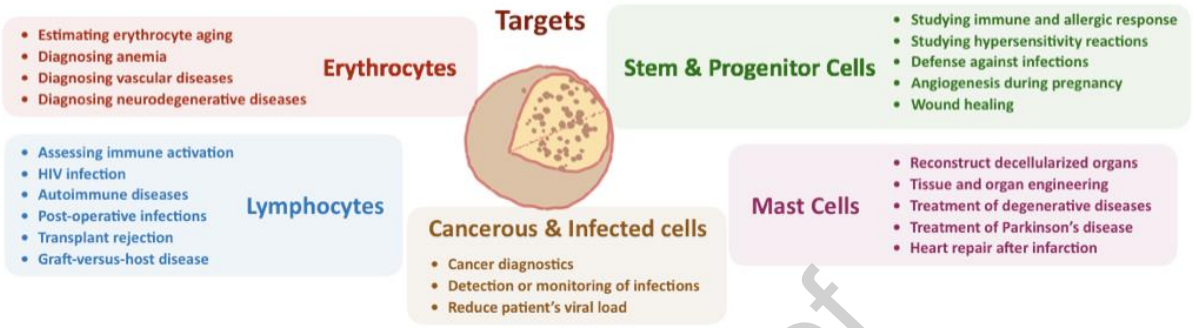


Figure 4. *Cell immunoaffinity chromatography. (A) Contacting a mixture of cells with the affinity substrate (e.g., an immunoaffinity adsorbent); (B) Removing the unbound cells by washing; (C) Eluting the target cell.*

Journal Pre-proof

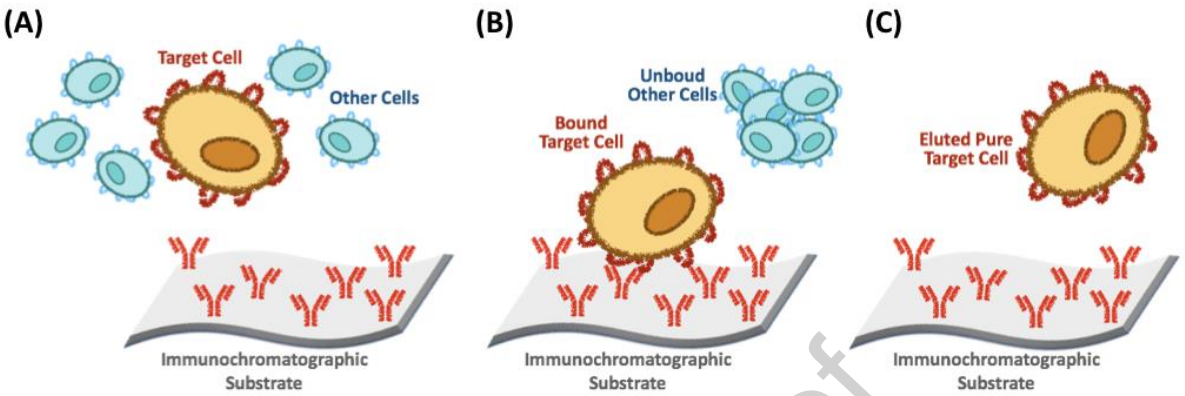


Figure 5. *Protein A-based cell affinity chromatography. (A) Mixing the target cell and other cells with target-specific antibodies (e.g., an immunoaffinity adsorbent); (B) Removing the unbound cells by washing; (C) Eluting the target cell.*

Journal Pre-proof

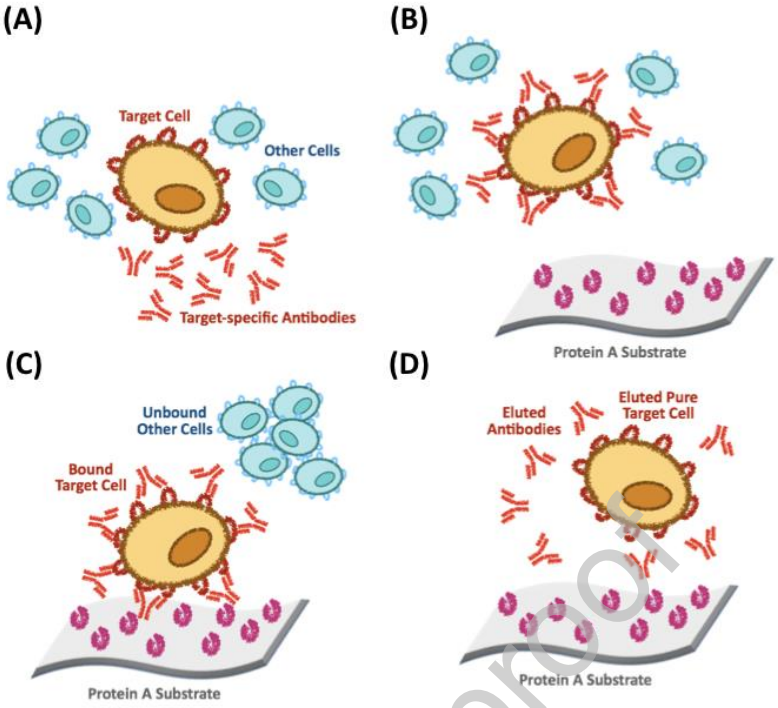


Figure 6. *Lectin-based cell affinity chromatography. (A) Contacting a mixture of cells with the lectin substrate; (B) Removing the unbound cells by washing; (C) Eluting the target cells using a mixture of sugars.*

Journal Pre-proof

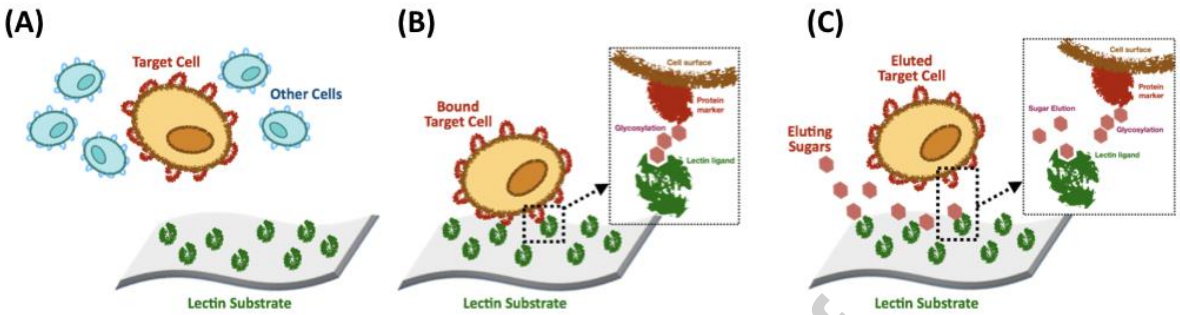


Figure 7. *Cell rosetting technique. (A) Contacting the target cell and other cells with bi-specific (target cell and red blood cells) ligands resulting in (B) the formation of a complex; (C) Incubating the tagged target cells with red blood cells; (C) Procedure of cell purification by rosetting.*

Journal Pre-proof

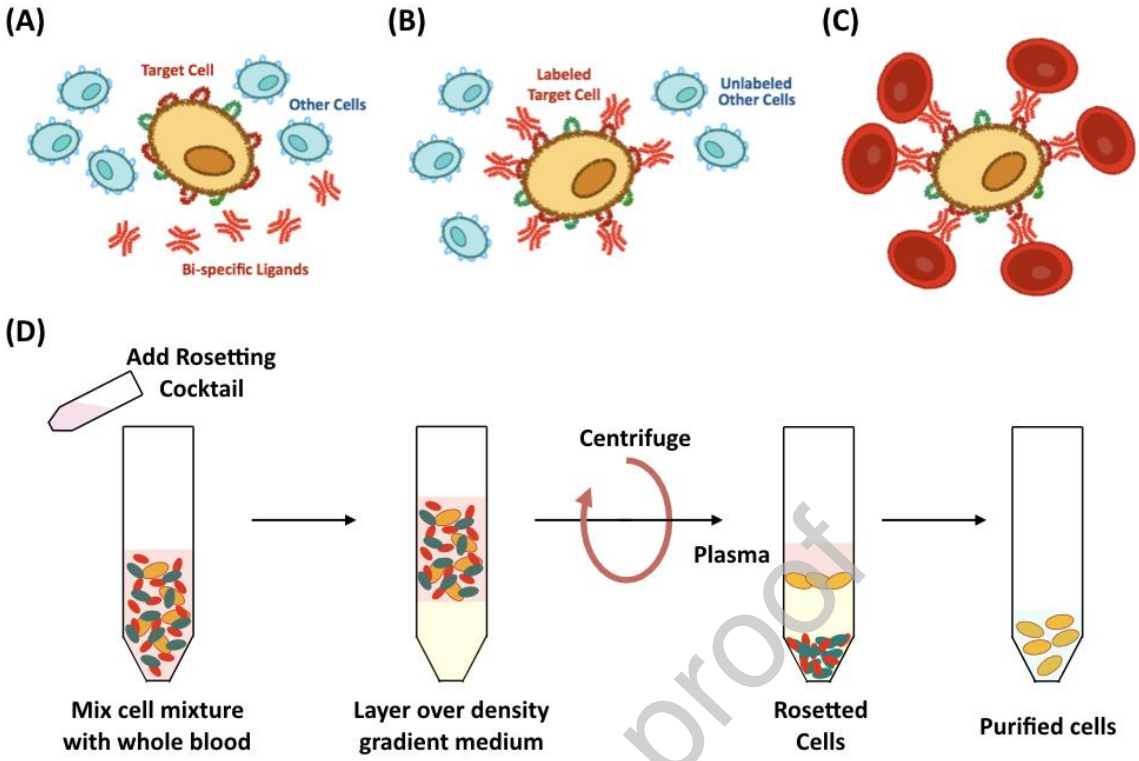


Figure 8. *Cell purification by expanded bed chromatography. (A) Filling the column with beads; (B) Expanding the beads; (C) Loading the cell mixture; (D) Compacting and washing the beads; (E) Eluting the target cells*

Journal Pre-proof

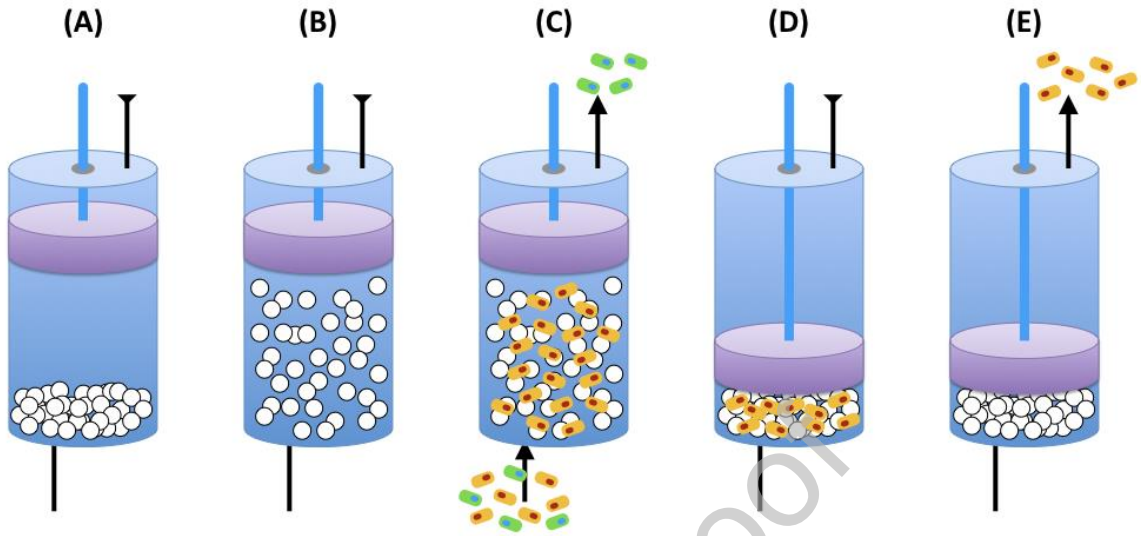


Figure 9. *Process of cryogel production.*

Journal Pre-proof

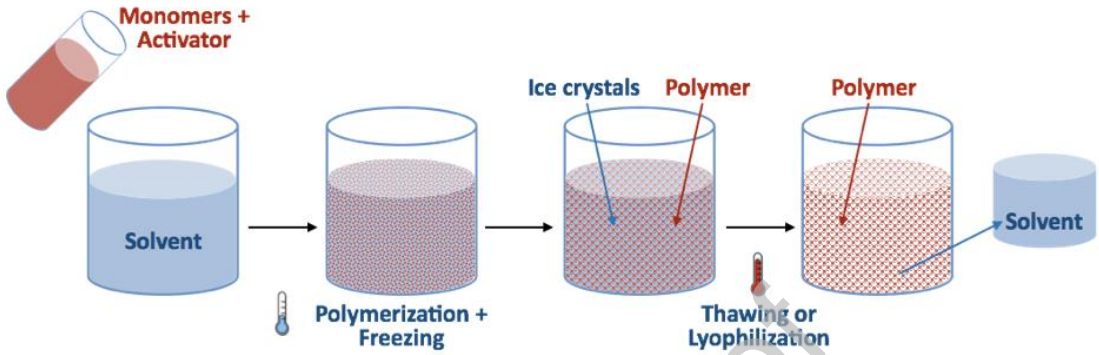


Figure 10. *Cell purification by affinity-based aqueous two-phase partition. (A) Suspending the cell mixture; (B) Adding the affinity-polymer forming the second phase; (C) Mixing the two phases; (D) Allowing the two phases to separate and recovering the target cells in the top affinity phase.*

Journal Pre-proof

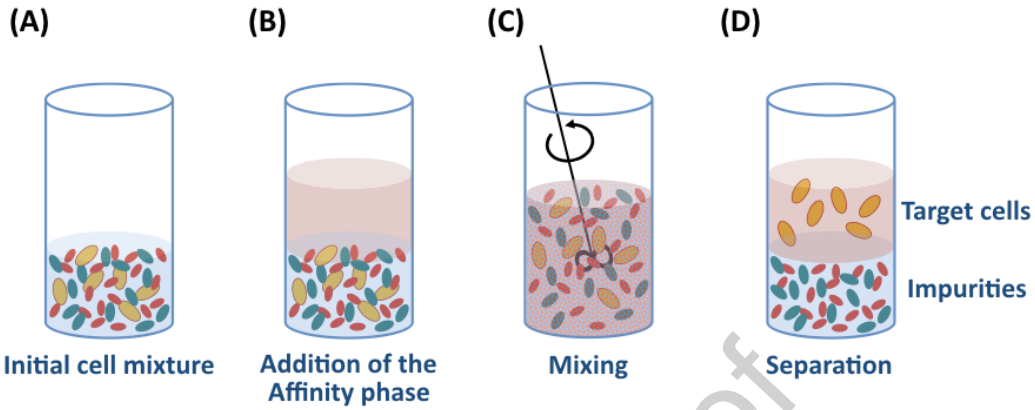


Figure 11. *Cell purification by MACS. (A) Contacting the cell mixture with ligand-functionalized magnetic beads; (B) suspending the magnetic beads in the cell mixture; (C) Applying a magnetic field to isolate the magnetized target cells and remove all unbound cells; (D) Resuspend and wash the magnetized target cells; (E) Elute the target cells from the magnetic beads.*

Journal Pre-proof

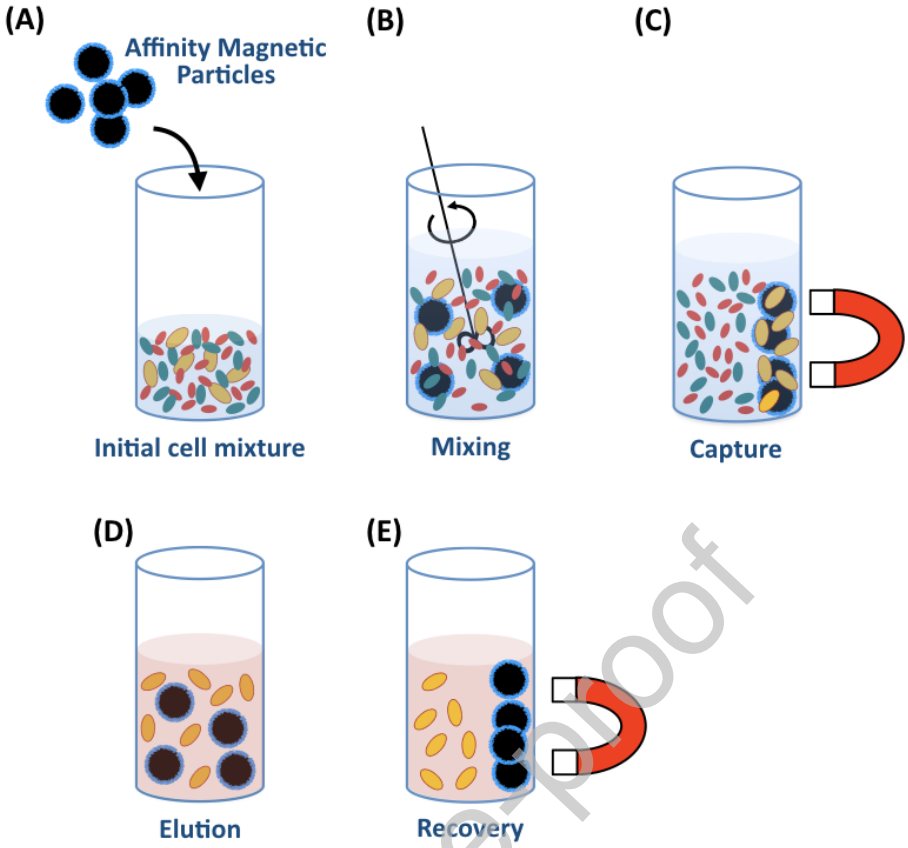


Figure 12. *Cell purification by FACS.*

Journal Pre-proof

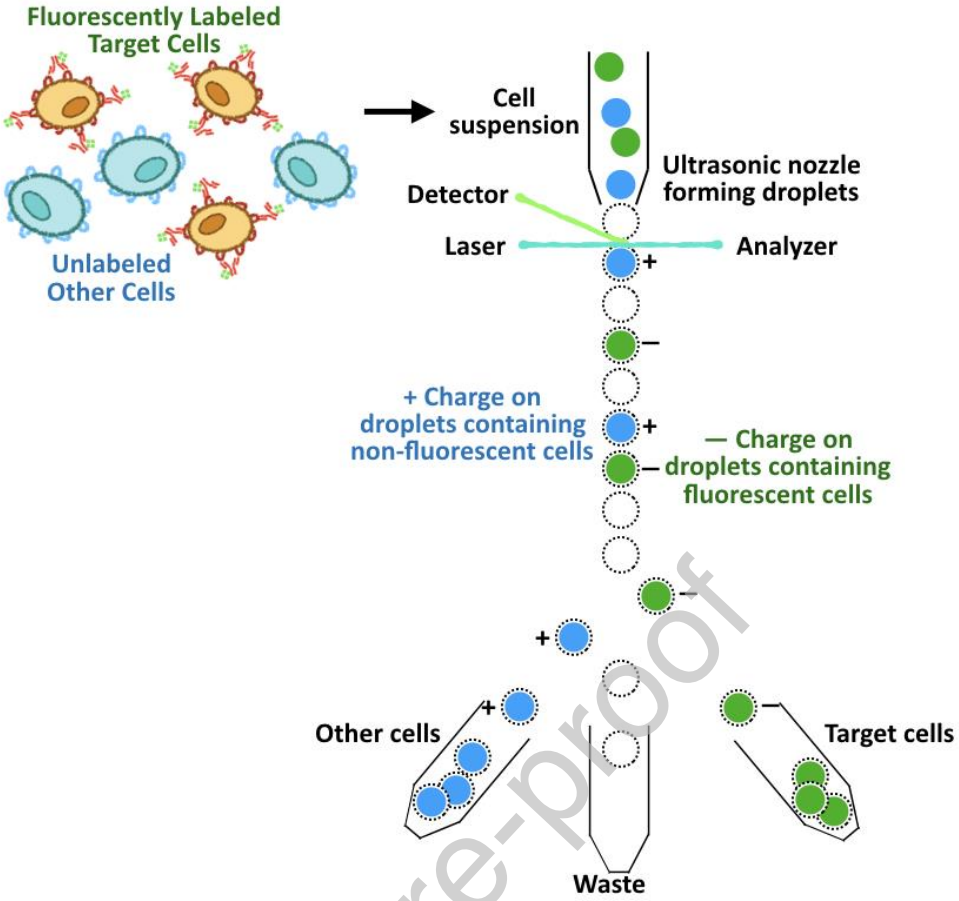
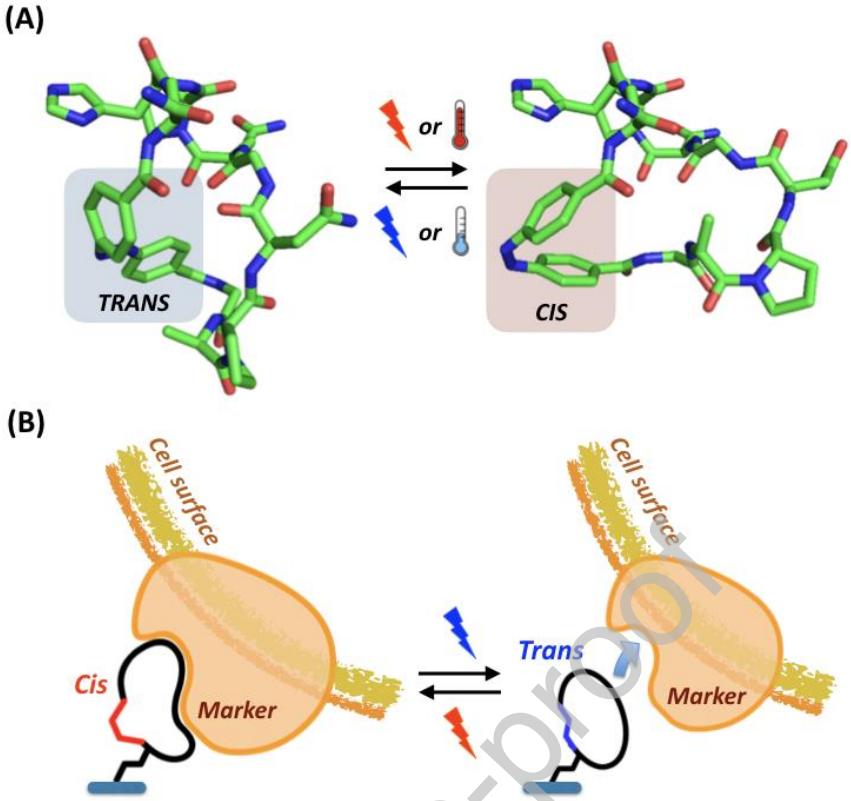


Figure 13. *Cell purification using stimuli-responsive peptide ligands. (A) Reversible photo- or thermo-switching of an azobenzene-cyclized peptide; (B) Principle of cell capture and release using stimuli-controlled cyclic peptides.*

Journal Pre-proof



List of table captions

Table 1. *Comparison of physical (non-affinity) and affinity-based cell separation techniques.*

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Method	Mechanism	Target cells	Advantages
		Physical (non-affinity) methods	
Density gradient Centrifugation	Cells migrate through a vertical density gradient (aqueous solutions of biopolymers) during centrifugation and collect in the region where the local density corresponds to their own.	Human mesenchymal stem cells, hematopoietic stem and progenitor cells, blood cells (erythrocyte, platelets, granulocytes, lymphocytes, monocytes), circulating tumor cells, sperm cells, and neurons. Rat pancreatic islets.	Label-free technology, which enables processing large volumes in short process times and concentrating the cell product; high viability of the cell product; reproducible results; facile scale up; commercially available equipment.
Dielectrophoresis	Cells placed in a gradient electric field act as induced dipoles and migrate at different rates based on their size and dielectric properties as well as the dielectric properties of the medium.	Hematopoietic stem and progenitor cells, leukocytes (B and T-lymphocytes, monocytes, and granulocytes), circulating tumor cells, astrocyte and neuron-biased cells, neural stem and progenitor cells. Isolation of pathogenic bacteria from blood. Fractionation of viable *vs.* non-viable cells (yeast and mammalian cells).	Label-free, continuous technology that enables sorting cells based on viability without dilution, thereby reducing sample volumes; short processing time and high sensitivity; can be integrated with microfluidic devices.
Field flow fractionation	A cell suspension is flown through a channel where a field (*e.g.*, crossflow, sedimentation, and electrical) is applied perpendicular to the direction of flow enabling separation based on mobility differences.	Pathogenic bacteria, yeast cell subpopulations. Human blood cells (erythrocyte, platelets, leukocytes), cancer cells, neurons from cerebral cortices, embryonic stem cells, mesenchymal stem cells, hematopoietic stem cells, electroporated vs. non-electroporated cells, and cells undergoing apoptosis.	Label-free, continuous technology that grants high viability and bioactivity of the cell product, under short process time; reduced sample volumes; high reproducibility.
Filtration	Cells are captured non-specifically on the surface of a material with controlled porosity based on physical properties such as cell diameter (volume) and aspect ratio. Generally used as preparative tool for further purification.	Blood cells (leukocytes, erythrocytes), hematopoietic stem and progenitor cells, adipose-derived stem cells, mesenchymal stem cells, circulating tumor cells. Bacterial and mammalian (e.g., Chinese Hamster Ovary (CHO) cells) cells for metabolomics preparation.	High-throughput, simple, and scalable technology, which can be integrated into a microfluid device platform.
Elutriation Centrifugation	Cells are separated based on their sedimentation velocity.	Blood cells (granulocytes, lymphocytes, monocytes, platelets), macrophages, Kupffer cells from liver, mast cells, hepatocytes, sperm cells (rats and human), separation into age-related fractions (yeast, erythrocytes), prostate and ovarian cancer cells from tumors, and hematopoietic stem and progenitor cells.	Rapid processing of large volumes of cells featuring a wide range of sizes; applicability at low temperatures to impede cell activation; high recovery and viability of the cell product.

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Declaration of Interest Statement For:

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Past, Present, and Future of Affinity-based Cell Separation Technologies

Kaitlyn Bacon, Ashton Lavoie, Balaji M. Rao, Michael Daniele, Stefano Menegatti

No personal or financial interests exist.

Statement of significance. Technologies for cell purification have served science, medicine, and industrial biotechnology and biomanufacturing for decades. This review presents a comprehensive survey of this field by highlighting the scope and relevance of all known methods for cell isolation, old and new alike. The first section covers the main classes of target cells and compares traditional non-affinity and affinity-based purification techniques, focusing on established ligands and chromatographic formats. The second section presents an excursus of affinity-based pseudo-chromatographic and non-chromatographic technologies, especially focusing on magnetic-activated cell sorting (MACS) and fluorescence-activated cell sorting (FACS). Finally, the third section presents an overview of new technologies and emerging trends, highlighting how the progress in chemical, material, and microfluidic sciences has opened new exciting avenues towards high-throughput and high-purity cell isolation processes. This review is designed to guide scientists and engineers in their choice of suitable cell purification techniques for research or bioprocessing needs.

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